Evolution of Developmental Control Mechanisms

Pleiotropic functions of embryonic sonic hedgehog expression link jaw and taste bud amplification with eye loss during cavefish evolution

Yoshiyuki Yamamoto a,b, Mardi S. Byerly c, William R. Jackman d, William R. Jeffery a,*

a Department of Biology, University of Maryland, College Park, MD 20742, USA
b Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK
c Program in Neuroscience and Cognitive Science, University of Maryland, College Park, MD 20742, USA
d Department of Biology, Bowdoin College, Brunswick, ME 04011, USA

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This study addresses the role of sonic hedgehog (shh) in increasing oral–pharyngeal constructive traits (jaws and taste buds) at the expense of eyes in the blind cavefish Astyanax mexicanus. In cavefish embryos, eye primordia degenerate under the influence of hyperactive Shh signaling. In concert, cavefish show amplified jaw size and taste bud numbers as part of a change in feeding behavior. To determine whether pleiotropic effects of hyperactive Shh signaling link these regressive and constructive traits, shh expression was compared during late development of the surface-dwelling (surface fish) and cave-dwelling (cavefish) forms of Astyanax. After an initial expansion along the midline of early embryos, shh was elevated in the oral–pharyngeal region in cavefish and later was confined to taste buds. The results of shh inhibition and overexpression experiments indicate that Shh signaling has an important role in oral and taste bud development. Conditional overexpression of an injected shh transgene at specific times in development showed that taste bud amplification and eye degeneration are sensitive to shh overexpression during the same early developmental period, although taste buds are not formed until much later. Genetic crosses between cavefish and surface fish revealed an inverse relationship between eye size and jaw size/taste bud number, supporting a link between oral–pharyngeal constructive traits and eye degeneration. The results suggest that hyperactive Shh signaling increases oral and taste bud amplification in cavefish at the expense of eyes. Therefore, selection for constructive oral–pharyngeal traits may be responsible for eye loss during cavefish evolution via pleiotropic function of the Shh signaling pathway.

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Introduction

Cave animals have evolved novel morphological, developmental, physiological, and behavioral phenotypes during the relatively short time since they diverged from surface-dwelling ancestors (Culver, 1982). The Mexican tetra Astyanax mexicanus, which consists of a sighted surface-dwelling form (surface fish) and a series of blind cave-dwelling forms (cavefish), is an emerging model system for studying development and evolution of cave-adapted phenotypes (Jeffery, 2008). Like many other cave-adapted animals, Astyanax cavefish have lost their eyes and pigmentation during evolution in perpetual darkness. In concert with regressive evolution, constructive traits have also evolved, including additional gustatory organs (taste buds) and changes in feeding behavior (Schemmel, 1967, 1980; Hüppop, 1987; Jeffery, 2001), which are probably adaptive and subject to enhancement by natural selection in the cave environment. It has been postulated that non-visual sensory systems were improved to compensate for loss of vision during cavefish evolution (Voneida and Fish, 1984; Teyke, 1990; Jeffery et al., 2000; Jeffery, 2001) but the responsible molecular causes have not been identified. Genetic studies have revealed overlapping quantitative trait loci (QTL) governing eye size and increased gustatory organs (taste buds), which could be explained by pleiotropic tradeoffs (Protas et al., 2008). Here we address the possible pleiotropic function of sonic hedgehog (shh) in linking the gain of oral and gustatory constructive traits to the loss of eyes in blind cavefish embryos.

Despite the absence of functional eyes in adults, small eye primordia with a lens and optic cup are initially formed in cavefish embryos but subsequently arrest in development, degenerate, and sink into the orbits, where they are covered by connective tissue and epidermis (Cahn, 1958; Langecker et al., 1993; Jeffery and Martasian, 1998). As a first step in eye degeneration, the cavefish lens undergoes apoptosis (Jeffery and Martasian, 1998; Yamamoto and Jeffery, 2000). Later in cavefish development, the dysfunctional lens fails to induce the anterior eye chamber, iris, and cornea, although a normally layered retina initially develops from the optic cup. Photoreceptor cells are formed in the layered retina but subsequently degenerate (Langecker...
et al., 1993; Yamamoto and Jeffery, 2000). The surface fish lens can restore eye development, including the cornea, iris, and retina with photoreceptor cells, after transplantation into the cavefish optic cup (Yamamoto and Jeffery, 2000), indicating that the lens has a fundamental role in sustaining eye development (Strickler et al., 2007a). Several factors have been discovered that may induce apoptosis in the cavefish lens. Two of these are the antiapoptotic factor αA-crystallin, which is downregulated in the cavefish lens (Strickler et al., 2007b) and maps near an Astyanax eye loss QTL (Gross et al., 2008), and the putative proapoptotic factor Hsp90x, which is upregulated during cavefish lens development (Hooven et al. 2005). A third is shh, which probably induces lens apoptosis indirectly following its overexpression in surface fish embryos (Yamamoto et al., 2004).

To investigate the molecular basis of eye degeneration, we previously compared the expression of eye regulatory genes in cavefish and surface fish embryos (Strickler et al., 2001; Yamamoto et al., 2004; Jeffery, 2005). These studies pointed toward genes in the Shh midline–signaling system as regulators of cavefish eye regression. First, we observed that the bilateral eye domains of pax6 expression in the cavefish neural plate are reduced and separated by a larger gap along the dorsal anterior midline. Second, we showed that shh and shhb (formerly tiggy wrinkle hedgehog) expression is increased along the anterior midline (prechordal plate) in early cavefish embryos. Third, we found that expression of downstream genes in the Sonic Hedgehog (Shh) signaling pathway, such as the receptor patched, nlx2.1a in the neural plate, and pax2a and vax1 expression in the optic vesicles, is also amplified, implying Shh hyperactivity along the cavefish anterior midline. Vertebrate optic vesicles are patterned by reciprocal transcriptional repression between pax6 and pax2/vax1 (Schwarz et al., 2000; Take-uchi et al., 2003), and upregulation of the latter by Shh signals is partially responsible for the small cavefish eye. Together with effects on the lens, shh mediated changes in gene expression in the optic cup suggest that the Shh signaling pathway negatively controls cavefish eye development.

Because shh is a pleiotropic gene with both positive and negative roles in development (Ingham and McMahon, 2001), in addition to negative effects on eye development, Shh hyperactivity could be related to the evolution of constructive traits, such as taste buds. Taste buds are more numerous in adult cavefish than in surface fish (Schemmel, 1967; Boudriot, and Reutter, 2001; Schemmel, 1980), and this expanded gustatory sense may be beneficial for cave life. Overexpression of shh has been previously detected in Shh signaling domains in the developing cavefish brain (Menuet et al., 2007) but oral–pharyngeal structures have not been investigated. Here, we have followed shh expression during oral–pharyngeal development to identify features that may be under positive control of pleiotropic Hh signaling. We found that shh expression is expanded in the oral–pharyngeal region and is later expressed in taste buds. The results of functional experiments suggest that shh amplification is required for increasing taste bud number during the same developmental interval as it inhibits eye development. In addition, genetic crosses revealed an antagonistic relationship between eye size and taste bud number in Astyanax. The results support the possibility that increased oral and gustatory development may have occurred at the expense of eyes during cavefish evolution via pleiotropic effects of the Shh signaling pathway.

Materials and methods

Animals and embryos

Laboratory colonies of Astyanax mexicanus were derived from surface fish collected at Balmorhea Spring State Park, Texas and cavefish collected at Cueva de El Pachón, Tamaulipas, Mexico. Embryos were obtained by temperature-induced spawning and reared at 25 °C (Jeffery and Martasian, 1998; Jeffery et al., 2000).

In situ hybridization

RNA probes were generated from surface fish shh (AY661431), nlx2.1a (AY661435), and pax2a (AY661436) cDNA sequences as described previously (Yamamoto et al., 2004). Embryos or larvae were fixed in 4% paraformaldehyde–PBS (pH 7.2; PFA). In situ hybridization was done using digoxigenin-labeled RNA probes as described previously (Strickler et al., 2001; Yamamoto et al., 2004). Following in situ hybridization the specimens were post-fixed in PFA, dehydrated through an ethanol series, embedded in polyester wax, and sectioned at 10 μm. In situ hybridized specimens were viewed as whole mounts or sections and photographed.

Quantitative real time RT-PCR

Total RNA was extracted from 3-day post-fertilization (dpf) larvae with Ribopure kit (Ambion, Austin, TX) according to the manufacturer's protocol. Extracted RNA was quantified and its integrity verified using the UV absorbance (260/280) bioanalyzer (Agilent Technologies, Palo Alto, CA). Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) was used to create cDNA from 1 μg of RNA according to the Invitrogen protocol using an oligo (DT) primer (5'-CGAATTCCTTCCCCCATTTTGGTGGTGG-3', Sigma Genosys, The Woodlands, TX). Blank cDNA was also created to serve as a negative control for genomic contamination. mRNA levels were measured by quantitative real time RT-PCR (RT-qPCR) using 2 μl of diluted cDNA (1:100) in a 20 μl qPCR reaction with SYBR Green ER qPCR SuperMix using an iCycler (Invitrogen, Carlsbad, CA) and analyzed according to the manufacturer's protocol with the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

Primers were designed using Primer Express (v 2.0, Applied Biosystems) and either a known A. mexicanus sequence (see below) or the homologous region between zebrafish and Tetraodon nigroviridis cDNAs (for β-actin). The qPCR products were verified for the appropriate size by dissociation curve analysis and gel electrophoresis. Primers were 18–30 nucleotides in length with a melting temperature between 58–64 °C. The primer sequences were as follows: shh (AY661431) forward primer, 5'–AGCGCTTCAAGAGATCTAC3'– and reverse primer, 5'–CGTGTTCCTCCTCGTCTAAGAGA3'; vax1 (AY661437) forward primer, 5'TCCTACGCTGGAGATGCTTGC3'– and reverse primer, 5'TTGGATTGCGGCTGACAAGC3'; pax2a (AY661436) forward primer, 5'–GCACAGCTTCCACCGCTAT3'– and reverse primer, 5'–GATGCTGGTAGTGAGTAGATAGA3'; pax6 (AY651762) forward primer, 5'–GGCTGGCAACACTCAATGACTG3'– and reverse primer, 5'–CTCTGAGCTCCCTTATTG3'– β-actin (Strickler and Jeffery, unpublished) forward primer, 5'–CCGGCAATCTACACACTGCT3'– and reverse primer, 5'–CCACACGGACTGCTTGGTAGA3'– and β-actin forward primer, 5'–CACACMGTGCCCATCTAGA3'– and reverse primer, 5'–CRGCAATCTAGACGACG3'– The qPCR output provided a Ct value for the threshold cycle, which is representative of fluorescence derived from binding of SYBR green to the double-stranded PCR product. Data were transformed to a ΔΔCt value by subtracting the sample Ct value from the sample with the highest expression level in order to control for amplification efficiency. The ΔΔCt value was then calculated by normalizing gene expression to α- and β-actin using the geNorm software and methods (GeNorm v3.4, Vandesompele et al., 2002).

All levels of gene expression were compared using a one-way ANOVA with cavefish and surface fish as the independent variables, and relative mRNA levels as the dependent variable. Values are reported as means±SE, and p<0.05 was required for significance. Statistica v6.1 (StatSoft, Inc., Tulsa, OK) was used for data analysis and Graphpad was used to construct graphs (Graphpad Prism Version 4.0, Graphpad Software, Inc.).
**Shh overexpression**

Shh activity was increased in two ways. First, Shh translation was inhibited by morpholinos. A shh MO (5′-GGCCGGGAGGCCTGCT-3′) was designed by Gene Tools Inc. (Summerton, OR) against part of the 5′ UTR of surface fish shh cDNA, a region in which cavefish and surface fish cDNAs do not differ in sequence. Embryos were injected with 1 or 2 ng shh or control (5′-CTTATCTAGCTAGTAAT-3′) MOs at the 2–4 cell stage. To test for Shh inhibition, MO injected embryos were subjected to in situ hybridization with probes for nkd2.1a and pax2a. In rescue experiments, embryos were injected with 2 ng shh MO and 10 pg zebrashf shh mRNA (see below). Second, embryos were treated with 20 μM, 100 μM, or 200 μM cycloamine (Sigma, St. Louis, MO) beginning at 10 and ending at 20 h post-fertilization (hpf) as described by Menuet et al., (2007), then washed into water and allowed to develop until 5 dpf. Controls were treated with 0.1% ethanol (which was used to prepare the cycloamine stock solution) for the same time interval.

**Shh overexpression**

Shh activity was increased in two ways. First, 20–800 pg Astyanax or zebrashf shh mRNA was injected into 2–4 cell embryos. Synthetic mRNAs were prepared and injected as described previously (Yamamoto et al., 2004). Control embryos were injected with Green Fluorescent Protein (GFP) mRNA. Second, to determine the effect of shh overexpression at different times in development, embryos were injected with the DNA expression construct hsp70:shh:GFP. This DNA construct consists of the hsp70 promoter, the coding sequence of zebrashf shh, the coding sequence of GFP (fused to the C-terminus of shh), and the SV40 polyadenylation signal, all flanked by IScel meganuclease sites. An intermediate backbone for this vector was constructed by replacing the PstI–XbaI fragment from the vector described by Yamamoto et al. (2004) with a short sequence containing a BamHI restriction site. The zebrashf shh coding sequence was then amplified by PCR (Expand High Fidelity, Roche) from total zebrashf cDNA using the primers aGGATCCagccaccatgcggcttttgacgaga and aTCTAGGctgttagttcagcatcctcaca and ligated into the BamHI/XbaI sites of the intermediate vector. The DNA construct was prepared for injection by digesting 600 ng of plasmid DNA with 10 U of I-Sce I meganuclease (New England Bio Labs) in the buffer supplied by the manufacturer for 1 h at room temperature and then stored at 20°C. We injected 30 pg of hsp70:shh:GFP DNA into 2–4 cell embryos and overexpressed shh by applying 36 C heat shocks for 1 h at various times in development. The effects of heat shocks on shh overexpression were monitored by following GFP expression.

**Cartilage staining and jaw width measurements**

Jaw cartilages were stained with Alcian Blue at 5–6 dpf as described by Yamamoto et al. (2003). Jaw width was measured across the hinge region in whole mounts of fixed larvae. Statistical analysis of jaw measurements was performed by Student’s unpaired (independent) t test.

**Taste bud detection and quantification**

For taste bud detection, embryos were raised to 6 dpf and fixed in 2 changes of fresh PFA for 2 h at room temperature. After washing in PBS, embryos were stained with calretinin antibody (1:1000 dilution; Swiss Antibodies, Bellinzona, Switzerland) and antigen-antibody complexes were detected using a biotinylated goat anti-rabbit secondary antibody (1:1500 dilution) and the Vecostain ABC Peroxidase kit (Vector Laboratories, Burlingame, CA), as described by Jeffery et al. (2000). The immunostained specimens were viewed as whole mounts and photographed.

Taste buds were counted on the upper and lower lips of calretinin-stained specimens viewed under a stereoscope or compound microscope. The rosette-like shape of taste buds distinguished them from much smaller solitary mechanoreceptor cells on the lips, which also stain positively for calretinin. Statistical analysis was carried out as described above for jaw width.

**Mating experiments to produce small- and large-eyed hybrids**

Cavefish were crossed with surface fish to produce an F1 generation. The F1 hybrids were interbred to produce an F2 generation, and the F2 hybrids were interbred to produce an F3 generation. The eye size of F3 hybrids was measured in living specimens at 6 dpf viewed under a compound microscope. Small-eyed F3 hybrids showed eye diameters lower than 268 μm (range = 192–268 μm). Large-eyed F3 hybrids showed eye diameters higher than 290 μm (range = 290–330 μm). After calretinin staining, jaw spans and taste bud numbers of small- and large-eyed F3 hybrids were determined and subjected to statistical analysis using unpaired Student’s t tests as described above.

**Results**

**Shh overexpression in the cavefish oral-pharyngeal region**

Previous studies showed that shh expression is expanded along the anterior midline during early cavefish development (Yamamoto et al., 2004) and later in Shh signaling centers in the forebrain (Menuet et al., 2007). During zebrash (Miller et al., 2000; Eberhart et al., 2006), chick (Marcucio et al., 2005; Haworth et al., 2007), and mouse (Yamagishi et al., 2006) development, shh expression is also prominent in the oral ectoderm and pharyngeal endoderm (oral-pharyngeal region). Accordingly, we asked if shh expression is also overexpressed in the cavefish oral-pharyngeal region at later stages of development.

In situ hybridization showed expanded shh expression along the cavefish anterior midline at 1 dpf (Figs. 1A, B). At 2 dpf a larger shh expression domain was observed in the oral-pharyngeal region (Figs. 1C–F), outlining a wider mouth in cavefish relative to surface fish (Figs. 1E–F). The expanded expression domain encompassed oral ectoderm and pharyngeal endoderm. By 3 dpf shh expression in the oral area was attenuated to tooth germs (Stock et al., 2006) and taste buds (Jeffery et al., 2000) (Figs. 1G–I). Taste buds can be distinguished from tooth germs by their positioning in single file along the lips, ring-like shh expression pattern (Figs. 1J, K), and staining by calretinin antibody (Fig. 1M; Jeffery et al., 2000). Sections through the oral area showed shh expression confined to the marginal (or basal) cells in each taste bud rosette (Fig. 1N). The marginal cells may be stem/precursor cells for taste receptor cells (Miura et al., 2006). No differences were apparent in the cellular organization of shh-expressing taste buds on the surface fish and cavefish lips. The results show that shh expression is amplified in the oral–pharyngeal region in cavefish relative to surface fish embryos, including both the oral ectoderm and pharyngeal endoderm, and later expressed in taste buds, one of the morphological features that is increased during cavefish evolution.

We quantified shh expression by qPCR at 3 dpf. As shown in Fig. 10, about 3 fold higher shh RNA levels were detected in cavefish relative to surface fish embryos, which would include the sum of expression in the oral–pharyngeal region, the brain, and possibility other embryonic regions. Furthermore, vax1 and pax2a mRNA levels, which are positively controlled in eyes by Shh signaling (Ekker et al., 1995; Take-uchi et al., 2003), are increased, whereas pax6 mRNA, which is negatively controlled in eyes by Shh signaling (Macdonald et al., 1995), is decreased in cavefish embryos (Fig. 1O). The results suggest a general elevation of Shh signaling in 3 dpf cavefish embryos.

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Oral–pharyngeal features are enhanced in cavefish

We next asked whether any differences in cavefish oral–pharyngeal and taste bud development correlate with increased shh expression. We observed that the expanded cavefish oral area, which is outlined by shh expression at 2 dpf (Figs. 1E, F), presages larger jaws later in development (Figs. 2A–D, G; Table 1). It has been reported that cavefish adults exhibit more taste buds than surface fish, particularly on the external surface of the lower jaw (Schemmel, 1967; Bensouilah and Denizot, 1991; Boudriot, and Reutter, 2001). Therefore, we also compared the number of taste buds in embryos of the two forms of Astyanax. Previous studies showed that Astyanax embryos begin to form calretinin–positive taste buds at 3–4 dpf (Jeffery et al., 2000). To determine whether surface fish and cavefish differ in the number of embryonic taste buds, we stained 5–6 dpf embryos with calretinin antibody. Calretinin-stained taste buds were seen throughout the oral–pharyngeal region, including the upper and lower lips (Figs. 2E, F). In contrast to adults, however, only a few taste buds were detected on the ventral surface of the lower jaw, and their number did not differ in cavefish and surface fish embryos. Calretinin antibody also stained solitary mechanoreceptor cells on the lips and head and cranial nerve fibers (Figs. 2C, D), as reported in another teleost (Diaz-Regueira et al., 2005), but calretinin-stained taste buds were clearly distinguishable by their large size and rosette-like morphology (see Fig. 1M). We focused on the lips, where taste buds are organized in single file. We observed significant elevations in taste bud number on the upper and lower lips in cavefish relative to surface fish (Figs. 2H, I; Table 1). The increased numbers of taste buds did not appear to be a consequence of higher density on cavefish lips. Instead, additional taste buds were present laterally in larger upper and lower jaws (Figs. 2E, F). Thus, cavefish appear to increase the size of lip epithelial surface devoted to taste bud formation rather than the foci of taste bud specification within the lip epithelium. We conclude that cavefish embryos have larger jaws with more taste buds than their surface fish counterparts.

Shh downregulation reduces oral–pharyngeal development

The possibility that jaw width and taste bud number are controlled by Shh signaling was investigated by determining the effects of
selective "shh" expression levels. Shh activity was downregulated by "shh" morpholino injection and cyclopamine treatment (Fig. 3; Table 1). First, we injected translation-blocking "shh" MOs into early surface fish and cavefish embryos. The effects of "shh" inhibition in the MO injected embryos was evaluated by monitoring the expression of "nklx2.1a" and "pax2.1a" genes in the neural plate (Figs. 3A, B). We found that "shh" but not control MOs blocked "nklx2.1a" expression, which is positively regulated by Hh signaling (Pabst et al., 2000), but had less effect on "pax2.1a" expression, which is independent of "shh" at the midbrain–hindbrain boundary, suggesting that Shh activity was downregulated. The effects of "shh" MOs were dose dependent and inhibits oral–pharyngeal development in both surface fish (data not shown) and cavefish (Figs. 3C–I), shifting the mouth opening posteriorly along the longitudinal body axis (Figs. 3E, F). In surface fish, "shh" MOs also induced cyclopia at the highest concentration used in this investigation (data not shown), as described previously in zebrafish (Nasevicius and Ekker, 2000). However, cyclopia was not seen in cavefish injected with the same amount of "shh" MO (Figs. 3C, D), probably due to increased levels of "shh" expression (Fig. 1O). Morphants subsequently showed significant decreases in jaw width and taste bud number on their upper and lower lips (Figs. 3C, D, G, H, I; Table 1). Similar results were obtained with another MO directed against a splice site in the second "shh" intron (data not shown). Simultaneous injection of zebrafish "shh" mRNA with "shh" translation-blocking MOs partially alleviated the effects on lower jaws and taste buds (Fig. 3I; Table 1). Second, Shh activity was downregulated by cyclopamine treatment (Menuet et al., 2007). In these experiments, embryos were treated with 20, 100, or 200 μM cyclopamine beginning at 15 hpf, then at 1 dpf the treated embryos were washed into water lacking the inhibitor, and at 5 dpf the effects on oral–pharyngeal features were determined. Embryos treated with 20 μM cyclopamine showed similar taste bud numbers to controls, 100 μM treated embryos showed fewer taste buds, whereas embryos treated with 200 μM cyclopamine had very small mouths with no detectable taste buds (Table 1). The results show that Shh inhibition reduces the extent of oral and taste bud development.

**Shh upregulation amplifies oral–pharyngeal development**

The effects of Shh overexpression were determined by "shh" mRNA injection (Fig. 4). First, 20 pg of "shh" mRNA, a concentration known to promote eye degeneration (Yamamoto et al., 2004), was injected into surface fish embryos and the effects on jaw and taste bud development were determined. Embryos injected with "shh" mRNA showed lateral expansion of "nklx2.1a" in the neural plate (Figs. 4A, B), consistent with effective Shh overexpression, and eye degeneration at 6 dpf (Fig. 4E). The injected surface fish embryos showed significant increases in jaw width and taste bud numbers with respect to controls (Figs. 4C–F; Table 1). Second, a large excess of "shh" mRNA (800 pg) was injected into cavefish embryos. When the latter were examined at 6 dpf, most of them showed large mouths with increased lip surface containing 2–3 fold more taste buds than controls (Figs. 4G, H, I; Table 1). The results indicate that "shh" overexpression increases jaw size and taste bud number.

**Conditional "shh" overexpression positively affects taste buds and negatively affects eyes during the same early developmental period**

To determine the developmental interval in which taste buds and eyes are sensitive to "shh" upregulation, surface fish embryos were injected with the hsP70:shh:GFP transgene and subsequently heat shocked at various stages of development (Fig. 5). Similar to the results obtained when "shh" mRNA was injected into 2–4 cell embryos (Figs. 4C–F), heat shocks at the tailbud (8 hpf) or one-somite (10 hpf) stages increased taste bud numbers on the upper and lower lips to levels resembling cavefish (Table 1). Similarly, heat shocks at the tailbud and one-somite stages also induced eye degeneration (Figs. 5A, C). The increases in jaw width at these stages were not significantly different from normal surface fish (Table 1), but visual

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**Fig. 2.** Constructive oral–pharyngeal features in cavefish. (A–F) Dorsal (A, B) and ventral (C–F) views of 6 dpf surface fish (A, C, E) and cavefish (B, D, F) showing wider jaw span (A, B; double-headed arrows), larger Alcian Blue-stained mandibles (C, D), more calretinin-stained taste buds (E, F; upward pointing arrowheads), and wider oral palates (E, F; double-headed arrows) in cavefish. Scale bar in A is 100 μm; magnification is the same in A–D and E, F (G–I) Surface fish (top frames) and cavefish (bottom frames) show differences in jaw width (G, red bars) and taste bud numbers on the upper (H, blue bars) and lower (I, black bars) lips. Jaw width is indicated in units of 20 μm with unit 1 as 371–390 μm, unit 2 as 391–410 μm, and so forth.
The results showed that small-eyed hybrids have significant effects on eye development and negative effects can be induced in the same surface fish by conditional shh overexpression prior to 1 dpf, although taste buds are first apparent morphologically 2–3 days later (Jeffery et al., 2000).

**Inverse relationship between oral–pharyngeal traits and eye development**

The results described above opened the possibility that oral and taste bud development may be linked with eye development through the positive and negative effects of expanded Shh signaling. To test this hypothesis independently, we measured oral–pharyngeal traits in small- and large-eyed surface fish × cavefish hybrids. To create these hybrids, the F1 progeny of surface fish × cavefish crosses were mated to produce an F2 generation, and the latter were then interbred to produce an F3 generation. Cavefish eye regression is a multigenic trait (Wilkens, 1988). Accordingly, F3 hybrids showed a broad distribution of eye sizes, including large normal eyes resembling those of surface fish and small degenerating and sometimes deg-pigmented eyes, resembling those of cavefish (Figs. 6A, B). The small-eyed and large-eyed F3 progeny were fixed at 6 dpf, and jaw sizes and taste buds measured as described above. The results showed that small-eyed hybrids have significantly larger jaws and more jaw taste buds than large-eyed hybrids (Figs. 6C–E; Table 1). Thus, these experiments revealed an inverse relationship between eye size (e.g. extent of eye regression) and oral/taste bud development: hybrids with small degenerating eyes have the cavefish taste bud phenotype, whereas hybrids with large normal eyes show the surface fish bud phenotype.

**Discussion**

The present investigation has revealed a link between constructive oral–pharyngeal development and eye regression via the pleiotropic Shh signaling pathway in the blind cavefish Astyanax mexicanus. The results support the following general conclusions. First, the expansion of shh expression, previously reported along the embryonic anterior midline (prechordal plate) in early cavefish embryos (Yamamoto et al., 2004), continues in the oral–pharyngeal region and taste buds later in cavefish development. Second, jaw size and oral taste bud numbers are increased in cavefish embryos and these constructive traits can be manipulated by Shh inhibition or overexpression. Third, eye degeneration and increased taste buds show similar shh sensitive periods during early development, although taste buds do not appear until much later. Finally, genetic crosses have revealed an inverse relationship between jaw size/taste bud number and eye size in F3 hybrid embryos. The results suggest that hyperactive Shh signaling is responsible for increased oral–pharyngeal traits in cavefish embryos, supporting an evolutionary model in which natural selection for larger jaws and more taste buds occurs at the expense of eyes via pleiotropic Shh signaling.

**shh expression in the oral–pharyngeal region and taste buds**

The domain of shh expression is wider along the embryonic midline in tailbud stage cavefish embryos compared to their surface fish counterparts (Yamamoto et al., 2004), as well as in classical Shh signaling centers in the forebrain later in development (Muenet et al., 2007). We have demonstrated here that shh expression is also expanded in the cavefish oral–pharyngeal region. Quantification by qRT-PCR showed an approximate 3-fold increase in shh transcripts in cavefish relative to surface fish at 3 dpf, which at least in part reflects the oral–pharyngeal increase.

The expanded shh expression domain in the oral–pharyngeal region consists of two parts: the pharyngeal region, which is probably a continuation of the original expanded shh expression domain.
Fig. 3. Effect of MO-mediated shh inhibition on oral and taste bud development. (A–H) Cavefish were injected with control (A, C, E, G) or shh (B, D, F, H) MOs (2 ng) and analyzed at the tailbud stage (A, B) or 6 dpf (C–H). (A, B) In situ hybridization showing downregulation of nkw2.1a but not pax2a (asterisks) expression in shh MO injected embryos at the neural plate stage. (C–F) Reduced jaw span (C, D; double-headed arrows) and oral–pharyngeal region (F, arrowhead) in shh MO injected larvae at 6 dpf. C, D: Ventral views. E, F: Lateral views. (G, H) Reduced numbers of calretinin-stained taste buds are formed in 6 dpf cave shh MO injected larvae. G: Ventral view. H. Anterior view. Downward and upward pointing arrowheads indicate upper and lower jaws respectively. Scale bars: A (250 μm), C, E, and G (200 μm); same magnification in A and B, C and D, E and F, and G and H. (I) Reduced jaw span (μm; red bars) and taste bud numbers on the upper (blue bars) and lower (black bars) lips in 6 dpf cave shh MO injected with 1 or 2 ng shh MO compared to control MO. Injection of a mixture of 10 pg zebrafish shh mRNA and 2 ng shh MO decreases the effects on jaw width and taste bud number. JW: jaw width. UJ: Upper jaw. LJ: Lower jaw. Error bars indicate SE of the mean.

present at earlier stages in the prechordal plate (Yamamoto et al., 2004), and the oral ectoderm, in which shh expression was not observed at earlier stages. Shh signaling has been implicated in regulating oral and pharyngeal development in other vertebrates (Moore-Scott and Manley, 2005). In zebrafish, shh is also expressed in the pharyngeal endoderm where it controls the condensation of skeletal elements in the developing pharyngeal arches and cranium, and in the oral ectoderm, where it promotes the formation of jaw cartilage (Miller et al., 2000; Wada et al., 2005; Eberhart et al., 2006). Furthermore, it has been proposed that shh expression in the oral ectoderm is induced by earlier Shh signals in the forebrain (Eberhart et al., 2006). Our data in Astyanax are consistent with what has been discovered in zebrafish. In the chick, shh is also expressed in pharyngeal endoderm, which regulates the formation of the first pharyngeal arch via fgf8 (Haworth et al., 2007). The early role for pharyngeal Shh in chick jaw development is mediated by its promotion of cranial neural crest cell survival (Brito et al., 2006). Accordingly, an additional set of lower jaws develop when an extra source of Shh is provided to the region around the first branchial arch, suggesting that the oral epithelium is an organizing center for the lower jaw (Brito et al., 2008). Finally, in the mouse, Shh emanating from the prechordal plate also functions through Fgf8 to promote development of the first pharyngeal arch and other craniofacial features (Yamagishi et al., 2006; Aoto et al., 2009). Thus, we propose that early expression of shh in the cavefish prechondal plate (Yamamoto et al., 2004) induces shh overexpression in the forebrain (Menuet et al., 2007; Rétaux et al., 2008), which in turn promotes overexpression in the oral epithelium, and this results in enhanced jaw and taste bud development. This possibility is also consistent with the changes in craniofacial development previously observed in cavefish relative to surface fish (Yamamoto et al., 2003).

As development proceeds shh expression is downregulated in most of the oral–pharyngeal epithelium except for strong foci in the tooth germs and the marginal cells of taste buds. Taste buds are under continuous renewal in vertebrates, and the marginal cells may be stem/precursor cells involved in their replenishment (Miura et al., 2001, 2006). The precise role of Shh in taste bud development is unclear, however, and may differ among various vertebrate species. In axolotl, taste buds are specified and appear early in the pharyngeal epithelium (Barlow, 2001), in much the same way as they do in Astyanax (Jeffery et al., 2000) and zebrafish (Hansen et al., 2002). In contrast to our results in Astyanax, however, neither shh mRNA or Shh protein have been detected in the axolotl pharyngeal epithelium during taste bud formation (Parker et al., 2004). The situation is different in mammals, in which taste bud formation is preceded by the development of taste papillae on the emerging tongue. Expression of shh is initially uniform in the mammalian oral–pharyngeal and prelingual areas, then becomes progressively restricted to the tongue, the taste papillae, and finally to the taste buds (Hall et al., 1999; Jung et al., 1999; Miura et al., 2001, 2003; Liu et al., 2004). The mammalian situation is temporally similar to that in Astyanax embryos, although the former form taste buds directly from the oral–pharyngeal epithelium.
Oral and taste bud development in cavefish embryos

Taste buds begin to develop in Astyanax embryos between 3 and 4 dpf (Jeffery et al., 2000), and shh expression is detected in taste bud primordia as soon as they protrude above the oral and pharyngeal epithelia. The timing of taste bud development is similar in Astyanax and zebrafish embryos (Hansen et al., 2002). We have demonstrated that cavefish embryos have a larger number of taste buds on both their upper and lower lips than their surfacefish counterparts. The mouth, and later the jaws, are also increased in cavefish. The larger jaws are not related to increased head space created by degenerate eyes, however, as shown by experiments in which no changes in jaw size were observed after creating a larger eye in cavefish by embryonic lens extirpation (Yamamoto et al., 2003; unpublished). The constructive changes in oral development remodel the cavefish mouth into a shovel-like structure that is effective for sampling sediment from the bottom of cave ponds and therefore is likely to be under strong positive selection in the cave environment.

It is important to note that taste bud numbers increase in cavefish compared to surface fish without a detectable elevation in their density, at least along the lips, the only place in the oral–pharyngeal region that we can accurately determine their distribution. Thus, it is unlikely that enhanced numbers of taste buds are due to a change in the mechanisms that control taste bud specification within the oral–pharyngeal epithelium. It is probable that increased taste bud numbers reflect an enhancement in the global patterning mechanisms that are responsible for constructing a larger oral–pharyngeal area in cavefish.

Fig. 4. Effect of shh overexpression on oral and taste bud development. (A–H) Surface fish (A–F) or cavefish (G, H) embryos were injected with shh (B, E, F–H) or GFP control (A, C, D) mRNAs and analyzed at the tailbud stage (A, B) or 6 dpf (C–H). (A, B) In situ hybridization showing expansion of nkx2.1a but not pax2a expression in the neural plate of cavefish embryos injected with shh MO (B). (C–H) Increase in the oral–pharyngeal region and calretinin-stained oral taste bud numbers (arrowheads) in shh mRNA injected surface fish (E–F) and cavefish (G, H) embryos. Lateral (C, E, G), ventral (D, F), and anterior (H) views at 6 dpf. (A–F), 20 pg shh mRNA. (G, H), 800 pg shh mRNA (E, F). DE: pigmented remnant of degenerate eye. Arrowheads: calretinin-stained taste buds. Doubled headed arrows: mouth opening. Scale bars: A (250 μm), C (200 μm); magnification is the same in A and B, C–F. (I) Increased jaw span (red bars) and taste bud numbers on the upper (blue bars) and lower (black bars) lips in 6 dpf larvae that developed from embryos injected with 20 pg shh mRNA (middle) or 800 pg shh mRNA (right) relative to controls injected with 20 pg GFP mRNA (left). Error bars indicate SE of the mean. JW: jaw width. UJ: Upper jaw. LJ: Lower jaw.
The increase in taste buds observed in cavefish embryos is more modest than the 5- to 7-fold elevation reported in cavefish adults (Schemmel, 1967). Aside from the obvious reason of increased overall body size, there are several possible explanations for differences between our results in larvae and those that were obtained in adults. First, calretinin antibody could recognize only a sub-set of larval taste buds in Astyanax, as appears to be the case in amphibians (Barlow et al., 1996). We think that this explanation is unlikely, however, because all structures distinguishable as taste buds by their typical rosette-shaped morphology stained positively with calretinin antibody. Further, calretinin-stained taste buds are closely packed on the lips, leaving little or no room for additional taste buds between them. Second, some the structures originally described as taste buds by electron microscopy in adults (Schemmel, 1967) might actually be other types of sensory organs, such as solitary mechanosensory cells. If so, the difference in taste bud numbers between adult cavefish and surface fish would be inflated when assayed by electron microscopy. Third, external taste buds, which probably represent a large part of the difference between the two forms of Astyanax, may appear later during cavefish development and thus would not be detected in our analysis. We observed very few taste buds on the external surface fish or the jaws of surface fish or cavefish at 5–6 dpf. Furthermore, external taste buds appear much later in zebrafish and catfish development than larval oral–pharyngeal taste buds (Hansen et al., 2002; Northcutt, 2005). Thus, taste buds probably appear in two stages during Astyanax development. During early larval development, oral–pharyngeal taste buds are formed, and as shown here these are already more numerous in cavefish than in surface fish. Subsequently, taste buds may develop in the skin of the lower jaw, and these external taste buds are more prevalent in cavefish.

Role of Shh signaling in jaw and taste bud development

The results of overexpression experiments suggest that shh is sufficient to promote the differences in oral and taste bud development we have seen between cavefish and surface fish embryos. Two key points are emphasized concerning these results. First, shh mRNA injection in surface fish embryos can increase the number of taste buds to levels typical of cavefish embryos while also inducing defective eye development. Previous results showed that shh overexpression in surface fish promotes eye degeneration by inducing lens apoptosis (Yamamoto et al., 2004), which occurs naturally in cavefish embryos (Jeffery and Martasian, 1998). Second, upregulation of shh at specific times during surface fish development by conditional activation of the hsp70:shh:GFP DNA construct showed that the sensitive periods for eye degeneration and increased taste bud number occur simultaneously prior to 1 dpf, although taste buds do not appear morphologically until 2–3 days later. The results suggest a tradeoff between eye and taste bud development that may be regulated by Shh signaling along the cavefish anterior midline.
The negative effects of Shh on eye development (Ekker et al., 1995; Yamamoto et al., 2004) and the corresponding positive effects on oral and taste bud development shown here suggest a developmental tradeoff between eyes and feeding organs. Three different lines of evidence support this possibility. First, the sensitive periods for eye degeneration and taste bud enhancement occur simultaneously during early development, prior to the appearance of taste buds. Second, independently of the shh results, genetic crosses show an inverse relationship between eye size and the extent of oral and taste bud development. An inverse relationship between these traits is consistent with offsetting positive and negative effects of shh over-expression in the concerted evolution of these two sensory modules.

Third, genetic linkage studies have revealed overlapping quantitative trait loci (QTL) governing eye size and increase in taste buds (Protas et al., 2008). One way of explaining this overlap would be to postulate a single pleiotropic gene within the QTL that controls both traits. Although shh appears to have role in eye degeneration and enhancement of constructive traits, it is not the gene that is mutated to give rise to these phenotypes in cavefish. Genetic analysis has shown that none of the multiple QTL underlying cavefish eye regression are located near a known hedgehog gene locus (Protas et al., 2007). Furthermore, the expression domains of upstream regulators of the Shh midline pathway, such as nodal and goosecoid, are also expanded in cavefish (Yamamoto unpublished). Thus, further progress in understanding the amplification of Shh-dependent phenotypes in cavefish will require identification of the upstream genes that have been mutated to cause hyperactivity of shh midline-signaling system.

Cavefish have evolved a specialized bottom feeding behavior that is more efficient than that of surface fish (Hüppop, 1987), which normally feed in the water column using visual cues (Schemmel, 1980). Efficient bottom feeding requires posture at an angle in which the mouth can sample substrate in cave pools. Thus, increase in jaw size and taste bud number could have evolved as an adaptation to the challenges of searching for and sampling the quality of food in the cave environment (Schemmel, 1967; Hüppop, 1987).

**Fig. 6.** The relationship between eye size and oral-pharyngeal development in F3 hybrid progeny of a surface fish × cavefish cross. (A) Examples of small- (A) and large- (B) eyed hybrids. The eye(s) of small-eyed hybrids are sunken into the orbit and sometimes de-pigmented, resembling those of cavefish, whereas the eyes of large-eyed hybrids are exposed and pigmented, resembling those of surface fish. (C–E) Differences in jaw width (red bars) and taste bud numbers on the upper (blue bars) and lower (gray bars) lips of small- and large-eyed F3 hybrids. Jaw width is indicated in units of 20 μm with unit 1 as 331–350 μm, unit 2 as 351–370 μm, and so forth.

**Fig. 7.** The relationship between Shh signaling, oral-pharyngeal constructive traits, and eye degeneration in Astyanax surface fish (A) and cavefish (B) indicating the effects of Shh signaling on oral-pharyngeal, lens, and optic cup development. Letter size indicates relative increase or decrease in cavefish compared to surface fish. See text for other details.

Modularity of sense organs, pleiotropic tradeoffs, and evolution of eye degeneration

It has been proposed that sensory organs are organized as developmental modules in Astyanax and that natural selection can affect developmental interactions between them, resulting in trade-offs (Franz-Odendaal and Hall, 2006). Further, regulatory genes could guide a sensory module into a specific type of differentiation, and if these genes are pleiotropic, there can be concerted negative consequences on development of other sensory modules. Accordingly, our results suggest that the Astyanax eye module may be linked to the oral taste bud module by pleiotropic effects of Shh signaling. A summary of the known pleiotropic activities of Hh signaling along the cavefish midline based on current knowledge of genes involved in oral/taste bud development and eye regression is shown in Fig. 7.
Whereas mechanical feeding efficiency may be one of the traits driving eye regression through shh overexpression, it is not the only example of a potentially adaptive phenotype produced by excess Shh signaling. In addition, cavefish also have an enlarged ventral forebrain controlled by an expanded Hh signaling center in the floor plate, which may lead to the production of more olfactory inter-neurons (Muenet et al., 2007). Together, dual Shh signals from the floor plate (Rétaux et al., 2008) and the prechordal plate (Yamamoto et al., 2004) may result in the development of multiple beneficial traits that synergistically drive rapid evolution of eye degeneration in blind cavefish.

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References


