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Evolution of Developmental Control Mechanisms

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

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ABSTRACT

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.

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

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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 Hsp90α, 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 *shhA* and *shhB* (formerly *tiggy winkle 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*, *nkx2.1a* 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), *nkx2.1a* (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 digoxygenin-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′-CGGAATTCTTTTTTTTTTTTTTTTTTV-3′, Sigma Genosys, The Woodlands, TX). Blank cDNA was also created with total RNA as described, but with no reverse transcriptase, 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′-AGCGCTTCAAGGAGCTCATC-3′ and reverse primer, 5′-CGTGTTCTCCTCGTCCTTAAAGA-3′; *vax1* (AY661437) forward primer, 5′-TCTACAGGCTGGAGATGGAGTTC-3′ and reverse primer, 5′ TTGAGTTGGCGTGCAAGCT-3′; *pax2a* (AY661436) forward primer, 5′-GCACGACTTCTCCACCCGTAT-3′ and reverse primer, 5′-GATGCCGTTGATGGAGTAGGA-3′; *pax6* (AY651762) forward primer, 5′-TGGCTGCCAGCAATCAGATG-3′ and reverse primer, 5′-CTTCTGAGTCCTCCCCATTTGAG-3′; α-actin (Strickler and Jeffery, unpublished) forward primer, 5′-CACGGCATCATCACCAACTG-3′ and reverse primer, 5′-CCACACGGAGCTCGTTGTAGA-3′, and β-actin forward primer, 5′-CACACMGTGCCCATCTAYGA-3′ and reverse primer, 5′-CRGCARATCCAGACGCAGRAT-3′. 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 ΔCt 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 v.6.1 (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 inhibition

Shh activity was inhibited in two ways. First, Shh translation was inhibited by morpholinos. A *shh* MO (5′-GCCGTGGCGGAGCCGTGCGT-AAAA-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′-CCTCTTACCTCAGTTACAATT-TATA-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 *nkx2.1a* and *pax2a*. In rescue experiments, embryos were injected with 2 ng *shh* MO and 10 pg zebrafish *shh* mRNA (see below). Second, embryos were treated with 20 μM, 100 μM, or 200 μM cyclopamine (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 cyclopamine stock solution) for the same time interval.

Shh overexpression

Shh activity was increased in two ways. First, 20–800 pg *Astyanax* or zebrafish *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 zebrafish *shh*, the coding sequence of GFP (fused to the C-terminus of *shh*), and the SV40 polyadenylation signal, all flanked by ISceI meganuclease sites. An intermediate backbone for this vector was constructed by replacing the PstI–XbaI fragment from the vector described in Pyati et al. (2005) with a short sequence containing a BamHI restriction site. The zebrafish *shh* coding sequence was then amplified by PCR (Expand High Fidelity, Roche) from total zebrafish cDNA using the primers aGGATCCagccaccatgcggcttttgacgaga and aTCTAGAgcttgagtttactgacatccccaa 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 −20C. 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* over-expression 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 two 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 (1:500 dilution) and the Vectastrain 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 zebrafish (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–L). Taste buds can be distinguished from tooth germs by their positioning in single file along the lips, ring-like *shh* expression pattern (Figs. 1I, J), 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. 1O, 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.

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

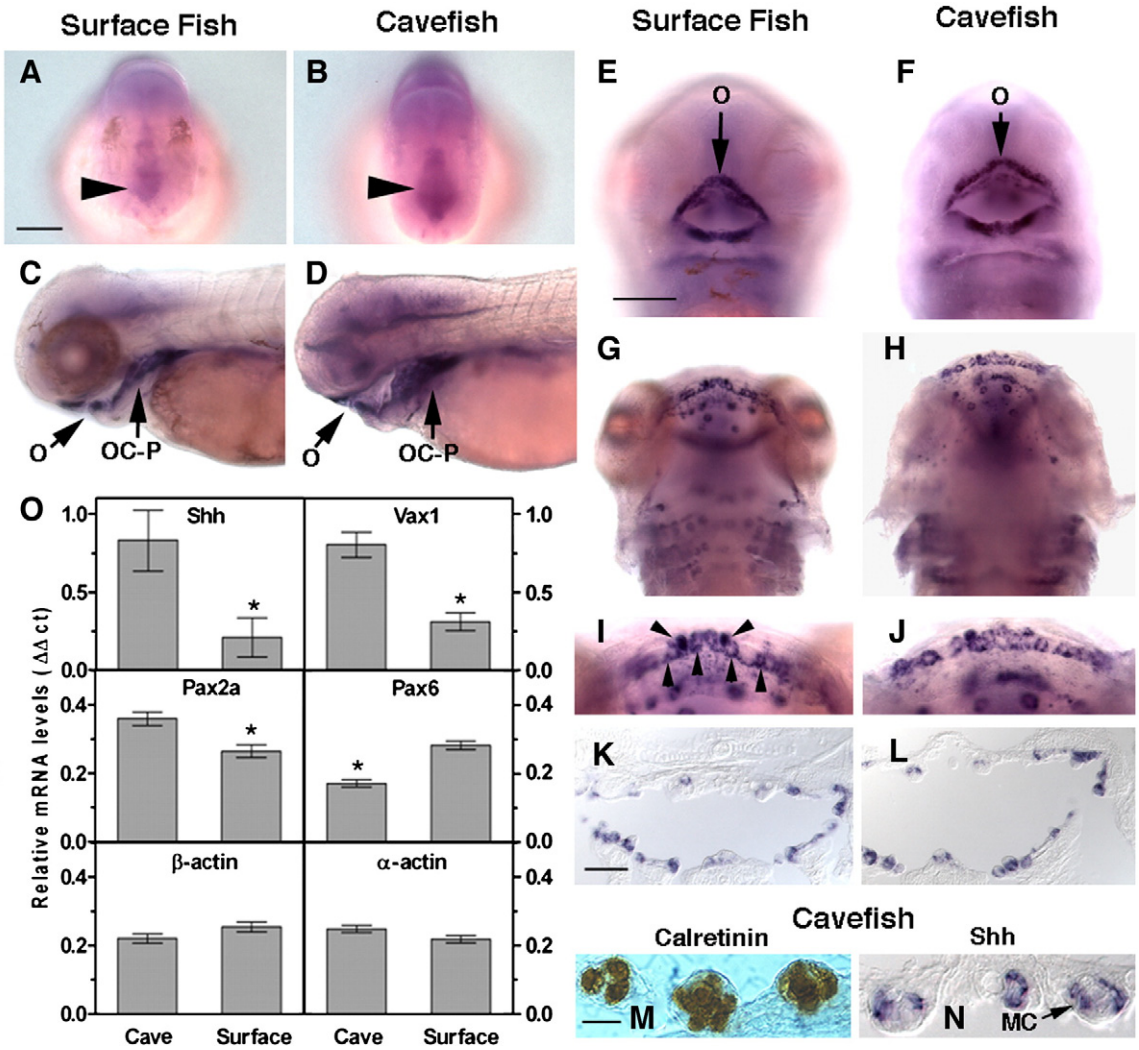

Fig. 1. Amplified *shh* expression in the cavefish oral–pharyngeal region. (A, B) Dorsal anterior views of 1 dpf surface fish and cavefish (B) embryos showing expanded *shh* expression in the cavefish anterior midline (A, B arrowheads). (C–F) Lateral (C, D) and rostral (E, F) views of 2 dpf surface fish (C, E) and cavefish (D, F) embryos showing expanded *shh* expression in the cavefish oral epithelium (o). O: oral area. OC-P: Oral–pharyngeal cavity. (G–J) Ventral views of 3 dpf surface fish (G, I) and cavefish (H, J) embryos showing *shh* expression in taste buds (upward pointing arrowheads in I) and primary tooth germs (oblique pointing arrowheads in I) on the lips. I, J are two-fold magnifications of G, H showing the ring-like *shh* expression pattern in taste buds. (K–N) Sections through 3 dpf surface fish (K) and cavefish (L–N) comparing the patterns of *shh* expression (K, L, N) and calretinin staining (M) in taste buds. MC: marginal cells. Scale bars: A (100 μm), E (50 μm), K (20 μm); M (4 μm); magnification is the same in A–D, E–H, I and J, K and L, M and N. O. Quantification by qRT-PCR showing increased levels of *shh*, *vax1*, and *pax2a* mRNA and decreased levels of *pax6* mRNA relative to β-actin and α-actin mRNA in 3 dpf cavefish larvae. Asterisks: $p<0.05$ in one-way ANOVAs comparing cave and surface fish mRNA levels ($n=4$).

manipulating *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 *nkx2.1a* and *pax2.1a* genes in the neural plate (Figs. 3A, B). We found that *shh* but not control MOs blocked *nkx2.1a* 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.

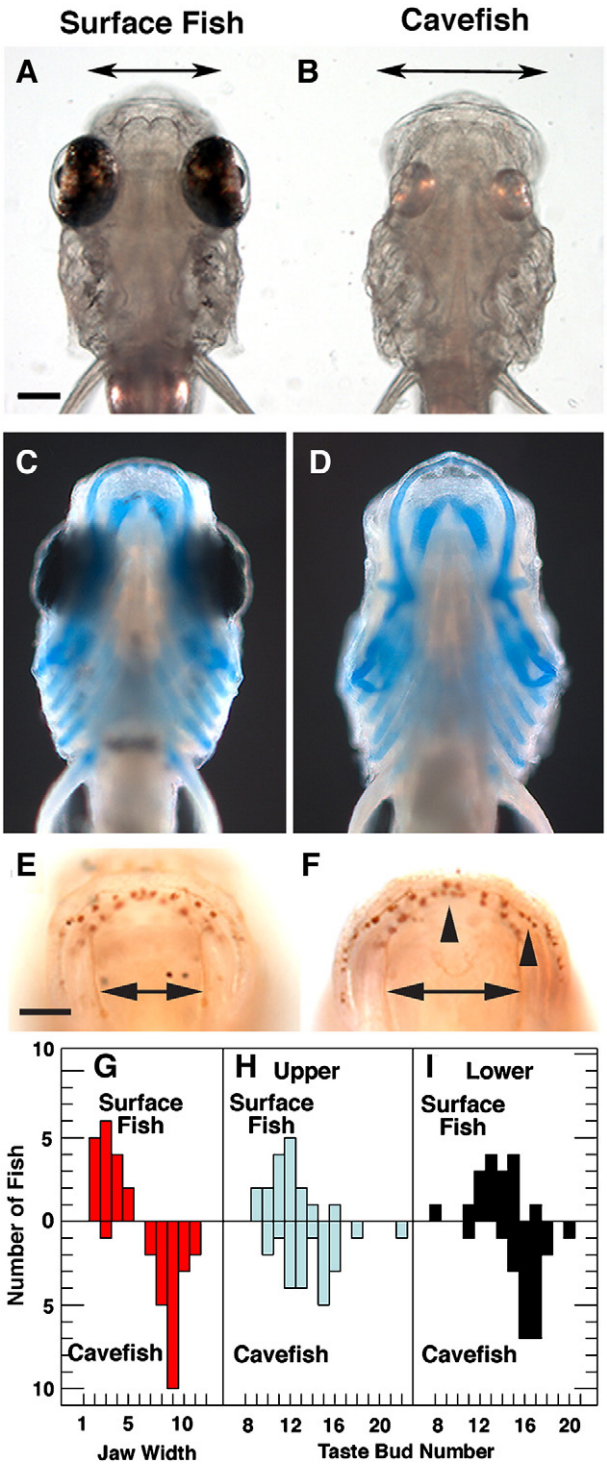

Fig. 2. Constructive oral–pharyngeal features in cavefish. (A–F) Dorsal (A, B) and ventral (C–F) views of 6 dpf surface fish (A, C, E) and cavefish (B, D, F) showing wider jaw span (A, B; double-headed arrows), larger Alcian Blue-stained mandibles (C, D), more calretinin-stained taste buds (E, F; upward 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.

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 *nkx2.1a* 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. 4 C–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

Table 1
Quantification of jaw width and taste bud number.

Manipulation type	Form	*N*	Stage	Mean JW (μm) +/− SD	Mean taste bud number +/− SD		Significance (*p*)		
					UJ	LJ	JW	UJ	LJ
None	SF	17	6 dpf	415 +/− 21	11.6 +/− 1.8	13.3 +/− 2.0			
None	CF	22	6 dpf	524 +/− 32	14.1 +/− 2.8	16.2 +/− 1.7	[a]0.004	0.000	0.000
Control MO injection	CF	29	6 dpf	512 +/− 52	11.4 +/− 2.9	13.4 +/− 3.3			
shh MO injection	CF	7	6 dpf	49 +/− 47	0.9 +/− 1.2	2.4 +/− 2.0	[b]0.000	0.000	0.000
shh MO + *shh* mRNA injection	CF	16	6 dpf	174 +/− 126	2.1 +/− 4.2	9.2 +/− 6.8	[c]0.400	0.850	0.018
Cyclopamine (control)	CF	11	5 dpf	Not measured	13.6 +/− 2.5	13.1 +/− 1.3			
Cyclopamine (20 μM)	CF	14	5 dpf	Not measured	11.9 +/− 2.3	14.2 +/− 1.6			
Cyclopamine (100 μM)	CF	6	5 dpf	Not measured	10.9 +/− 3.0	10.2 +/− 3.0			
Cyclopamine (200 μM)	CF	10	5 dpf	Not measured	0	0			
GFP mRNA injection	SF	38	6 dpf	411 +/− 54	11.1 +/− 2.7	13.2 +/− 2.9			
shh mRNA^{+} injection	SF	16	6 dpf	506 +/− 75	16.1 +/− 4.1	19.2 +/− 5.8	[d]0.000	0.000	0.000
shh mRNA^{++} injection	CF	27	6 dpf	Not measured	21.5 +/− 17.6	35.2 +/− 11.5			
shh heat shock	SF	28	TB	408 +/− 51	12.9 +/− 2.7	16.5 +/− 3.3	[e]0.369	0.010	0.019
shh heat shock	SF	23	1 somite	405 +/− 32	13.0 +/− 2.1	16.0 +/− 2.7	[e]0.399	0.007	0.039
shh heat shock	SF	27	1 dpf	391 +/− 75	11.1 +/− 2.5	14.6 +/− 3.0			
shh heat shock	SF	28	2 dpf	391 +/− 61	11.0 +/− 2.7	14.7 +/− 4.0			
shh heat shock	SF	36	2.5 dpf	376 +/− 90	10.2 +/− 2.4	13.4 +/− 3.5			
shh heat shock	SF	31	3 dpf	376 +/− 64	10.1 +/− 3.8	13.6 +/− 4.3			
Small eye	F3	30	6 dpf	465 +/− 73	15.5 +/− 2.4	14.4 +/− 1.6	[f]0.004	0.000	0.003
Large eye	F3	34	6 dpf	405 +/− 27	11.4 +/− 2.0	12.0 +/− 2.2			

N sample number. JW: jaw width. UJ: upper jaw. LJ: lower jaw. SD: Standard Deviation. SF: Surface fish. CF: Cavefish. F3: F3 hybrid progeny of SF × CF cross. $^{+}$20 pg *shh* mRNA injected. $^{++}$800 pg *shh* mRNA injected. Statistical comparisons:
[a] CF versus SF.
[b] *shh*MO versus control MO injection.
[c] *shh*MO versus *shh*MO + *shh* mRNA injection.
[d] *shh* mRNA versus GFP injection.
[e] Heat shock at the tailbud stage or 1-somite stage versus heat shock at 1 dpf.
[f] Small-eyed- versus large-eyed hybrids.

inspection indicated that many embryos showed a larger oral–pharyngeal region relative to controls (Fig. 5B). In contrast, conditional *shh* overexpression at 1, 2, 2.5, or 3 dpf resulted in taste bud numbers and levels of eye development resembling those of normal surface fish (Figs. 5C, D; Table 1). The differences observed between *shh* overexpression before and after 1 dpf were significant (Table 1). The results show that positive effects on taste bud development and negative effects on eye development 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 de-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 taste 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 (Menuet 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

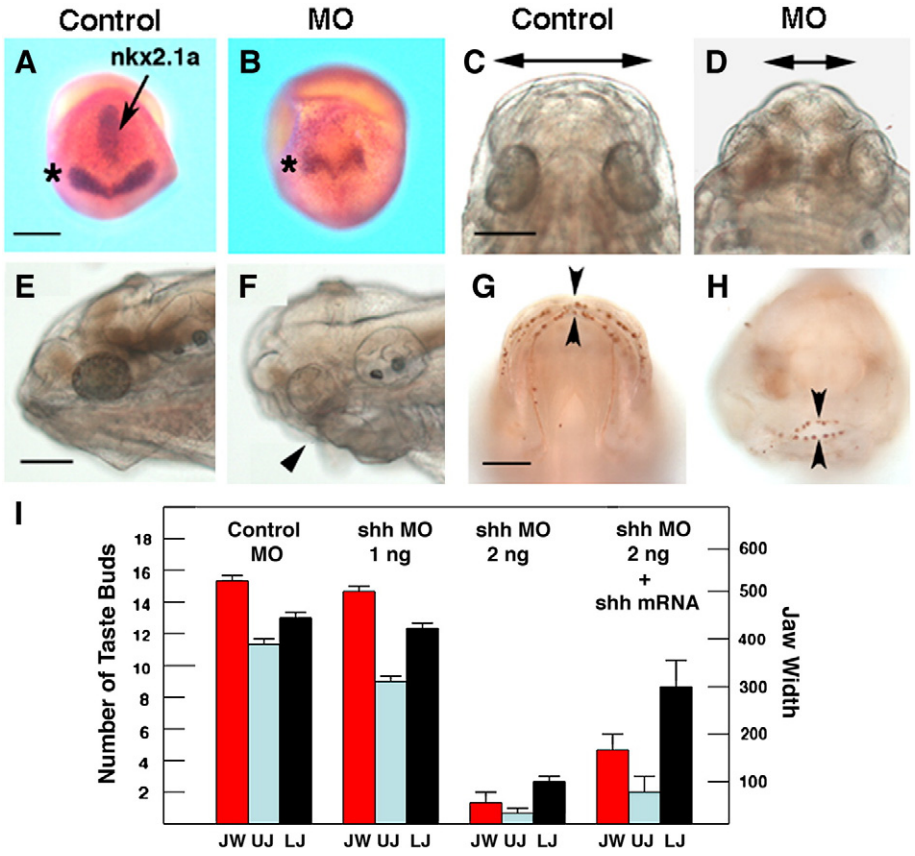

Fig. 3. Effect of MO-mediated *shh* inhibition on oral and taste bud development. (A–H) Cavefish were injected with control (A, C, E, G) or *shh* (B, D, F, H) MOs (2 ng) and analyzed at the tailbud stage (A, B) or 6 dpf (C–H). (A, B) *In situ* hybridization showing downregulation of *nkx2.1a* but not *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 cavefish larvae injected with *shh* MO. 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, 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 cavefish 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* expression in the cavefish prechordal 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), 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 latter form taste buds directly from the oral–pharyngeal epithelium.

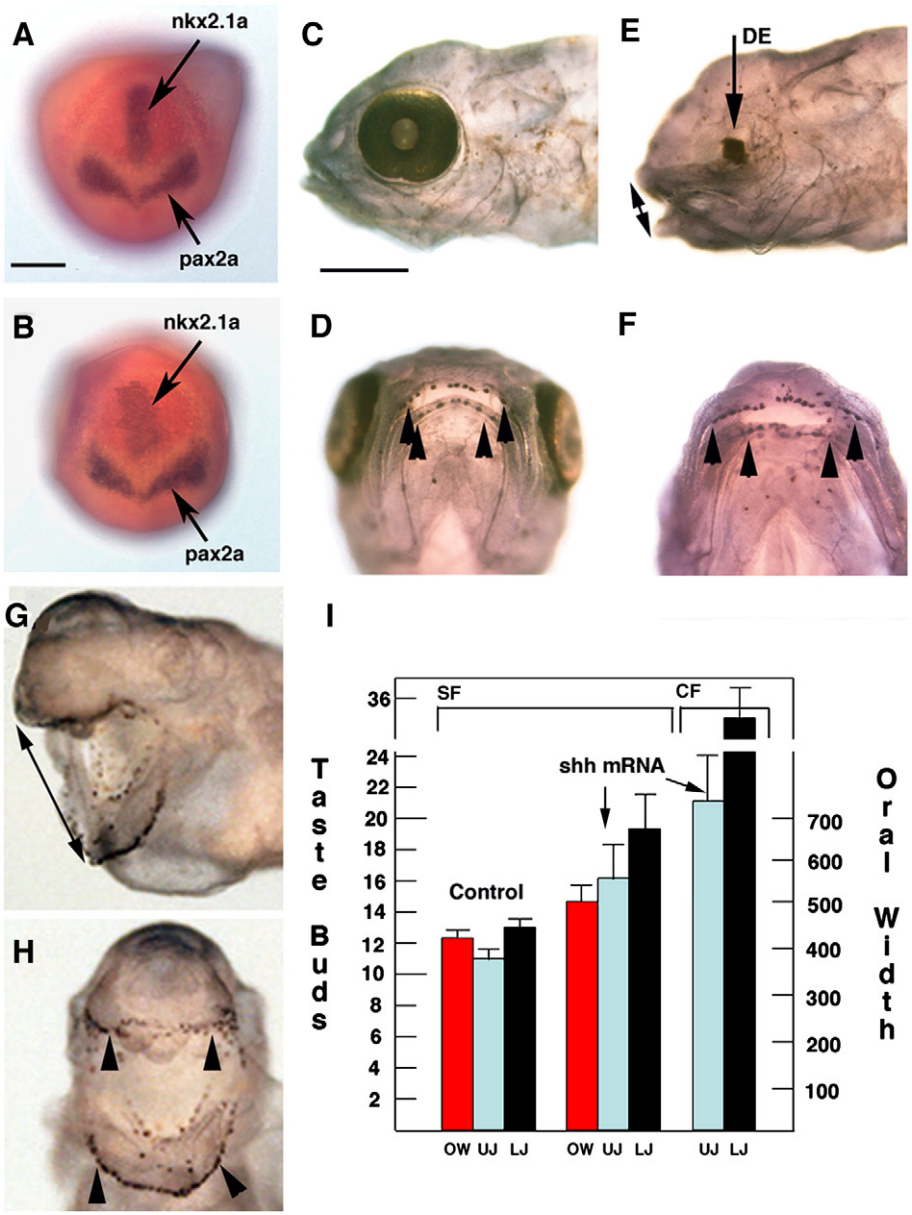

Fig. 4. Effect of *shh* overexpression on oral and taste bud development. (A–H) Surface fish (A–F) or cavefish (G, H) embryos were injected with *shh* (B, E, F–H) or GFP control (A, C, D) mRNAs and analyzed at the tailbud stage (A, B) or 6 dpf (C–H). (A, B) *In situ* hybridization showing expansion of *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.

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 surface fish 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 transplantation or a smaller eye in surface fish 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.

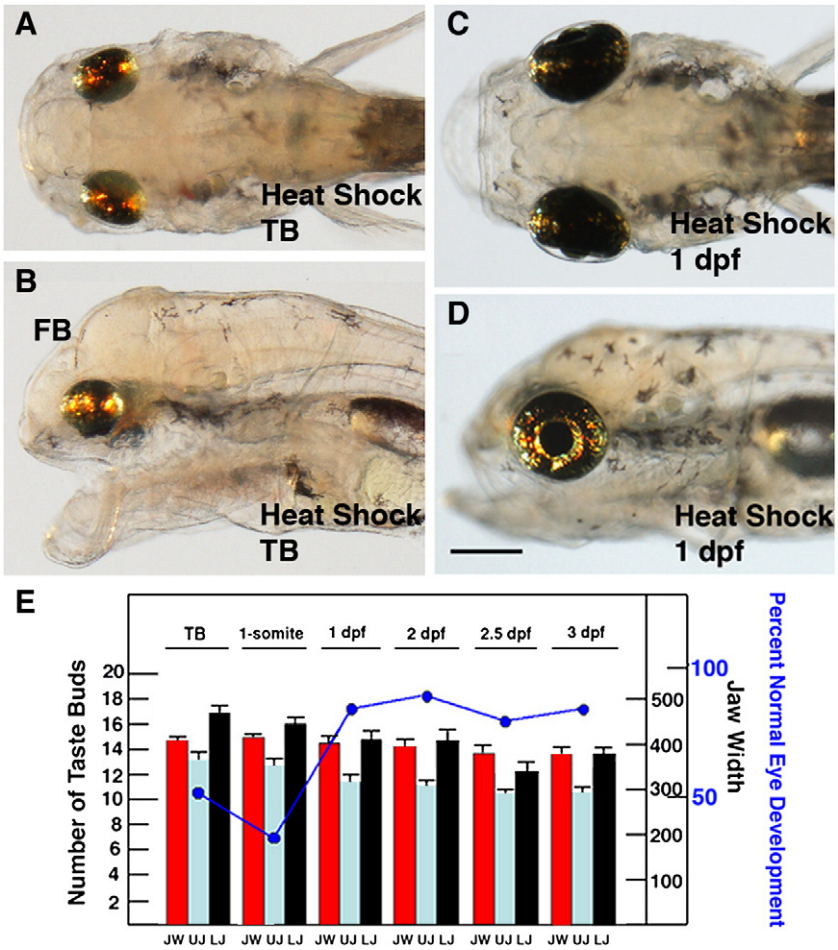

Fig. 5. The effects of conditional *shh* overexpression on oral–pharyngeal development and eye degeneration. Surface fish embryos were injected with the hsp70:shh:GFP transgene and heat shocked at various stages of development. (A, B) A transgene injected embryo (6 dpf) heat shocked at the tailbud (TB) stage showing a gaping mouth, enlarged forebrain (FB), and small degenerate eyes. (C, D) A transgene injected embryo (6 dpf) heat shocked at 1 dpf showing a normal mouth and eye. A and C: dorsal views B and D: lateral views. Scale bar in D is 200 μm; magnification is the same in A–D. (E) The *shh* sensitivity periods for increased taste bud development and eye degeneration in transgene injected surface fish embryos determined by heat shocking at different developmental stages. Red bars. oral width (μm). Blue bars: taste bud number on upper lips. Black bars: taste bud number on lower lips. Error bars indicate SE of the mean. Blue dots: percentage of embryos with normal eye development. 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.

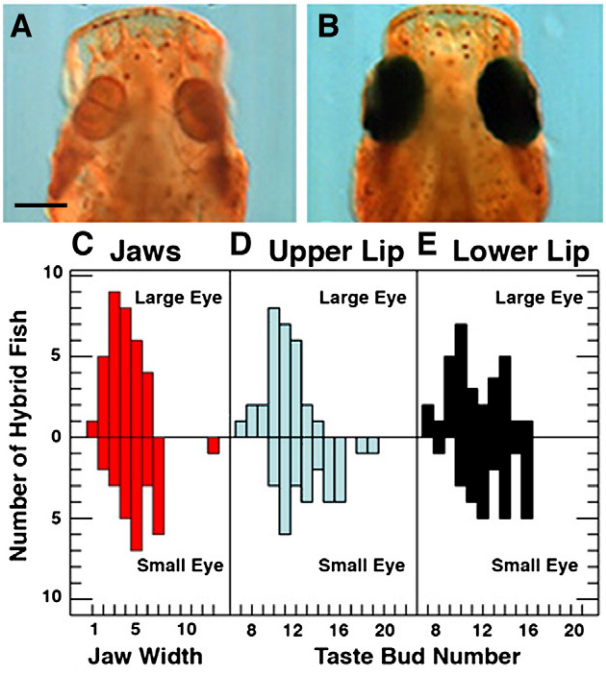

Fig. 6. The relationship between eye size and oral–pharyngeal development in F3 hybrid progeny of a surface fish × cavefish cross. (A) Examples of small- (A) and large- (B) eyed hybrids. The eye(s) of small-eyed hybrids are sunken into the orbit and sometimes de-pigmented, resembling those of cavefish, 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.

The results also suggest that *shh* expression is necessary as well as sufficient for jaw and taste bud development. Although Shh inhibition with MOs did not completely suppress taste bud development, and co-injection of *shh* mRNA did not entirely rescue the effects of *shh* MOs, complete inhibition of taste bud development did occur after cyclopamine treatment. Teleosts contain two paralogous *shh* genes, *shhA*, the gene we have focused on in these studies, and *shhB* (formerly *tiggy winkle hedgehog*) (Ekker et al., 1995). Both *shh* genes are expanded along the cavefish anterior midline (Yamamoto et al., 2004), and it is possible that they are functionally redundant, requiring a double knockdown to completely affect taste bud formation. However, cyclopamine can inhibit the function of both genes because it acts downstream of ShhA/B by binding to the Smo protein (Chen et al., 2002). There also may be functional redundancy between Shh and other signaling ligands and transcription factors involved in taste bud development. For example, Notch, Bmp, Fgf, Prox1, Mash1, Nkx2.2, and NeuroD (Jung et al., 1999; Seta et al., 2003; Jeffery et al., 2000, Suzuki et al., 2002; Miura et al., 2003) are expressed in vertebrate taste buds. Except for *shh*, which is required for taste bud development in the mouse (Mistretta et al., 2003; Liu et al., 2004), little is known about the roles of these molecules and how they may interact during taste bud development.

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 tradeoffs (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.

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* overexpression 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 OTL 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).

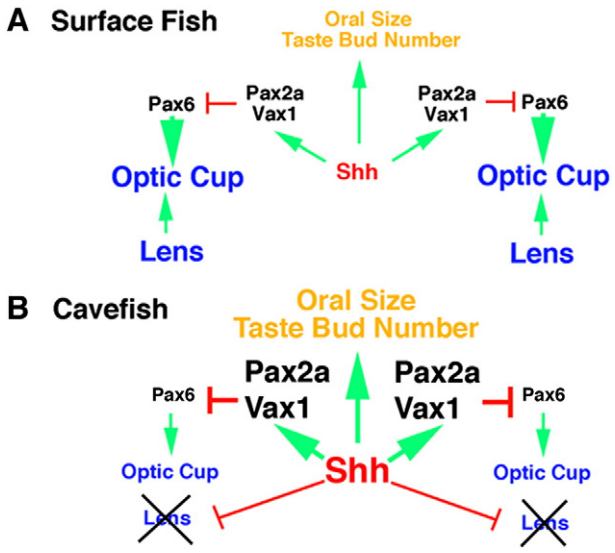

Fig. 7. The relationship between Shh signaling, oral–pharyngeal constructive traits, and eye degeneration in *Astyanax* surface fish (A) and cavefish (B) indicating the effects of Shh signaling on oral–pharyngeal, lens, and optic cup development. Letter size indicates relative increase or decrease in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

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 (Menuet 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.

Acknowledgments

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References

Aoto, K., Shikata, Y., Imai, H., Matsumaru, D., Tokunaga, T., Shioda, S., Yamamda, G., Motoyama, J., 2009. Mouse Shh is required for prechordal plate maintenance during brain and facial morphogenesis. Dev. Biol. 327, 106–120.

Barlow, L.A., 2001. Specification of pharyngeal endoderm is dependent on early signals from axial mesoderm. Development 128, 4573–4583.

Barlow, L.A., Chien, C.B., Northcutt, R.G., 1996. Embryonic taste buds develop in the absence of innervation. Development 122, 1103–1111.

Bensouilah, M., Denizot, J.-P., 1991. Taste buds and neuromasts of *Astyanax jordani*: distribution and immunochemical demonstration of co-localized substance P and enkephalins. Eur. J. Neurosci. 3, 407–414.

Boudriot, F., Reutter, K., 2001. Ultrastructure of the taste buds in the blind cave fish *Astyanax jordani* ("*Anoptichthys*") and the sighted river fish *Astyanax mexicanus* (Teleostei, Characidae). J. Comp. Neurol. 434, 428–444.

Brito, J.M., Teillet, M.-A., Le Douarin, N.M., 2006. An early role for Sonic hedgehog from foregut endoderm in jaw development: ensuring neural crest cell survival. Proc. Nat. Acad. Sci. U. S. A. 103, 11607–11612.

Brito, J.M., Teillet, M.A., Le Douarin, N.M., 2008. Induction of mirror-image lower jaws in chicken mandibular mesenchyme by Sonic Hedgehog-producing cells. Development 135, 2311–2319.

Cahn, P.H., 1958. Comparative optic development in *Astyanax mexicanus* and two of its blind cave derivatives. Bull. Am. Mus. Nat. Hist. 115, 75–112.

Chen, J.K., Taipale, J., Cooper, M.K., Beachy, P.A., 2002. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. 16, 2643–2748.

Culver, D.C., 1982. Cave Life: Evolution and Ecology. Harvard University Press, Cambridge.

Diaz-Regueira, S.M., Lamas, I., Anadon, R., 2005. Calretinin immunoreactivity in taste buds and afferent fibers of the grey mullet *Chelon glabrous*. Brain Res. 1031, 297–301.

Eberhart, J.K., Swartz, M.E., Crump, J.G., Kimmel, C.B., 2006. Early Hedgehog signaling from neural to oral epithelium organizes anterior craniofacial development. Development 133, 1069–1077.

Ekker, S.C., Ungar, A.R., von Greenstein, P., Porter, J., Moon, R.T., Beachy, P., 1995. Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. Curr. Biol. 5, 944–955.

Franz-Odendaal, T.A., Hall, B.K., 2006. Modularity and sense organs in the blind cavefish, *Astyanax mexicanus*. Evol. Dev. 8, 94–100.

Gross, J.B., Protas, M., Conrad, M., Scheid, P.E., Vidal, O., Jeffery, W.R., Borowsky, R., Tabin, C.J., 2008. Synteny and candidate gene prediction using an anchored linkage map of *Astyanax mexicanus*. Proc. Natl. Acad. Sci. U. S. A. 105, 20106–20111.

Hall, J.M., Hooper, J.E., Finger, T.E., 1999. Expression of *Sonic Hedgehog*, *Patched*, and *Gli1* in developing taste papillae of the mouse. J. Comp. Neurol. 406, 143–155.

Hansen, A., Reutter, K., Zeiske, E., 2002. Taste bud development in the zebrafish, *Danio rerio*. Dev. Dyn. 223, 483–496.

Haworth, K.E., Wilson, J.M., Grevellec, A., Cobourne, M.T., Healy, C., Helms, J.A., Sharpe, P.T., Tucker, A.S., 2007. Sonic hedgehog in the pharyngeal endoderm controls arch pattern via regulation of *Fgf8* in head endoderm. Dev. Biol. 303, 244–258.

Hooven, T.A., Yamamoto, Y., Jeffery, W.R., 2005. Blind cavefish and heat shock protein chaperones: a novel role for hsp90α in lens apoptosis. Int. J. Dev. Biol. 48, 731–738.

Hüppop, K., 1987. Food finding ability in cave fish (*Astyanax fasciatus*). Int. J. Speleol. 18, 59–66.

Ingham, P.W., McMahon, A.P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15, 3059–3087.

Jeffery, W.R., 2001. Cavefish as a model system in evolutionary developmental biology. Dev. Biol. 231, 1–12.

Jeffery, W.R., 2005. Adaptive evolution of eye degeneration in the Mexican blind cavefish. J. Hered. 96, 185–196.

Jeffery, W.R., 2008. Emerging systems in Evo–Devo: cavefish and mechanisms of microevolution. Evol. Dev. 10, 265–272.

Jeffery, W.R., Martasian, D.P., 1998. Evolution of eye degeneration in the cavefish *Astyanax*: apoptosis and the *pax6* gene. Am. Zool. 38, 685–696.

Jeffery, W.R., Strickler, A.G., Guiney, S., Heyser, D., Tomarev, S.I., 2000. Prox1 in eye degeneration and sensory organ compensation during development and evolution of the cavefish *Astyanax*. Dev. Genes Evol. 210, 223–230.

Jung, H., Oropeza, V., Thesleff, I., 1999. *Shh*, *Bmp-2*, *Bmp-4* and *Fgf-8* are associated with initiation and patterning of mouse tongue papillae. Mech. Dev. 81, 179–182.

Langecker, T.G., Schamle, H., Wilkens, H., 1993. Transcription of the opsin gene in degenerate genes of cave dwelling *Astyanax fasciatus* (Teleostei, Characidae) and its conspecific ancestor during early ontogeny. Cell Tissue Res. 273, 183–192.

Liu, H., MacAllum, D.K., Edwards, C., Gaffield, W., Mistretta, C.M., 2004. Sonic hedgehog exerts distinct, stage specific effects on tongue and taste papilla development. Dev. Biol. 276, 280–300.

Macdonald, R., Anukampa, Barth K., Xu, Q., Holder, N., Mikkola, I., Wilson, S., 1995. Midline signaling is required for *Pax6* gene regulation and patterning of the eyes. Development 121, 3267–3278.

Marcucio, R.S., Cordero, D.R., Hu, D., Helms, J.A., 2005. Molecular interactions coordinating the development of the forebrain and face. Dev. Biol. 284, 48–61.

Menuet, A., Alunni, A., Joly, J.-S., Jeffery, W.R., Rétaux, S., 2007. Shh overexpression in *Astyanax* cavefish: multiple consequences on forebrain development and evolution. Development 134, 845–855.

Miller, C.T., Schilling, T.F., Lee, K.-H., Parker, J., Kimmel, C.B., 2000. sucker encodes a zebrafish Endothelin-1 required for ventral pharyngeal arch development. Development 127, 3815–3828.

Mistretta, C.M., Liu, H.-X., Gaffield, W., MacCallum, D.K., 2003. Cyclopamine and jervine in embryonic rat tongue cultures demonstrate a role for Shh signaling in taste papilla development and patterning: fungiform papillae double in number and form in novel locations in dorsal lingual epithelium. Dev. Biol. 254, 1–18.

Miura, H., Kusakabe, Y., Sugiyama, C., Kawamatsu, M., Ninomiya, Y., Motoyama, J., Hino, A., 2001. *Shh* and *Ptc* are associated with taste bud maintenance in the mouse. Mech. Dev. 106, 143–145.

Miura, H., Kusakabe, Y., Kato, H., Jun, M., Tagami, M., Ninomiya, Y., Hino, A., 2003. Co-expression pattern of *Shh* with *Prox1* and that of *Nkx2.2* with *Mash1* in mouse taste bud. Gene Exp. Patterns 3, 427–430.

Miura, H., Kusakabe, Y., Shuitsu, H., 2006. Cell lineage and differentiation in taste buds. Arch. Histol. Cytol. 4, 209–225.

Moore-Scott, B.A., Manley, N.R., 2005. Differential expression of Sonic hedgehog along the anterior–posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs. Dev. Biol. 278, 323–335.

Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene "knockdown" in zebrafish. Nat. Genet. 2, 216–220.

Northcutt, R.G., 2005. Taste bud development in the channel catfish. J. Comp. Neurol. 31, 1–16.

Pabst, O., Herband, H., Takuma, N., Arnold, H.H., 2000. NKX2 gene expression in neuroectoderm but not in mesendodermally derived structures depends on sonic hedgehog in mouse embryos. Dev. Genes Evol. 210, 47–50.

Parker, M.A., Bell, M.L., Barlow, L.A., 2004. Cell contact-dependent mechanisms specify taste bud pattern during a critical period early in embryonic development. Dev. Dyn. 230, 630–642.

Protas, M., Conrad, M., Gross, J.B., Tabin, C., Borowsky, R., 2007. Regressive evolution in the Mexican cave tetra, *Astyanax mexicanus*. Curr. Biol. 18, R27–R29.

Protas, M., Tabansky, I., Conrad, M., Gross, J.B., Vidal, O., Tabin, C.J., Borwosky, R., 2008. Multi-trait evolution in a cave fish, *Astyanax mexicanus*. Evol. Dev. 10, 196–209.

Pyati, U.J., Webb, A.E., Kimelman, D., 2005. Transgenic zebrafish reveal stage-specific roles for Bmp signaling in ventral and posterior mesoderm development. Development 132, 2333–2343.

Rétaux, S., Pottin, K., Alunni, A., 2008. Shh and forebrain evolution in the blind cavefish *Astyanax mexicanus*. Biol. Cell 100, 139–147.

Schemmel, C., 1967. Vergleichende Untersuchungen an den Hautsinnesorgagen over- and unterirdischlebender Astyanax-Formen. Z. Morphol. Tiere 61, 255–316.

Schemmel, C., 1980. Studies on the genetics of feeding behaviour in the cavefish *Astyanax mexicanus* f. *Anoptichthys*. An example of apparent monofactorial inheritance by polygenes. Z. Teirpsychol. 53, 9–22.

Schwarz, M., Cecconi, F., Bernier, G., Andrejewski, N., Kammandel, B., Wagner, M., Gruss, P., 2000. Spatial specification of mammalian eye territories by reciprocal transcriptional repression of Pax2 and Pax6. Development 127, 4325–4334.

Seta, Y., Seta, C., Barlow, L.A., 2003. Notch-associated gene expression in embryonic and adult taste papillae and taste buds suggests a role in taste cell lineage decisions. J. Comp. Neurol. 464, 49–61.

Stock, D.W., Jackman, W.R., Trapani, J., 2006. Developmental genetic mechanisms of evolutionary tooth loss in cypriniform fishes. Development 133, 3127–3137.

Strickler, A.G., Yamamoto, Y., Jeffery, W.R., 2001. Early and late changes in Pax6 expression accompany eye degeneration during cavefish development. Dev. Genes Evol. 211, 138–144.

Strickler, A.G., Yamamoto, Y., Jeffery, W.R., 2007a. The lens controls cell survival in the retina: evidence from the blind cavefish *Astyanax*. Dev. Biol. 311, 512–523.

Strickler, A.S., Byerly, M.S., Jeffery, W.R., 2007b. Lens gene expression analysis reveals downregulation of the anti-apoptotic chaperone αA-crystallin during cavefish eye degeneration. Dev. Genes Evol. 217, 771–782.

Suzuki, Y., Takeda, M., Obara, N., 2002. Expression of NeuroD in the mouse taste buds. Cell Tissue Res. 307, 423–428.

Take-uchi, M., Clarke, J.D.W., Wilson, S.W., 2003. Hedgehog signaling maintains the optic stalk–retinal interface through the regulation of Vax gene activity. Development 130, 955–968.

Teyke, T., 1990. Morphological differences in neuromasts of the blind cavefish *Astyanax hubbsi* and the sighted river fish *Astyanax mexicanus*. Brain Behav. Evol. 35, 23–30.

Wada, N., Javidan, Y., Nelson, S., Carney, T.J., Kelsh, R.N., Shilling, T.F., 2005. Hedgehog signaling is required for cranial neural crest morphogenesis and chrondrogenesis at the midline in the zebrafish skull. Development 132, 3977–3988.

Wilkens, H., 1988. Evolution and genetics of epigean and cave *Astyanax fasciatus* (Characidae, Pisces). Evol. Biol. 23, 271–367.

Voneida, T.J., Fish, S.E., 1984. Central nervous system changes related to the reduction of visual input in a natural blind fish (*Astyanax hubbsi*). Am. Zool. 24, 775–782.

Yamagishi, C., Yamamgishi, H., Maeda, J., Tsuchibashi, T., Ivey, K., Hu, T., Srivatava, D., 2006. Sonic hedgehog is essential for first pharyngeal arch development. Pediatr. Res. 59, 349–354.

Yamamoto, Y., Jeffery, W.R., 2000. Central role for the lens in cavefish eye degeneration. Science 289, 631–633.

Yamamoto, Y., Espinasa, L., Stock, D.W., Jeffery, W.R., 2003. Development and evolution of craniofacial patterning is mediated by eye-dependent and -independent processes in the cavefish *Astyanax*. Evol. Dev. 5, 435–446.

Yamamoto, Y., Stock, D.W., Jeffery, W.R., 2004. Hedgehog signaling controls eye degeneration in blind cavefish. Nature 431, 844–847.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by genomic averaging of multiple internal control genes. Genome Biol. 18 R34-1–R34-11.