THE DENTITION OF CYPRINIFORM FISHES AS A MODEL FOR THE NATURE OF DEVELOPMENTAL CONSTRAINTS ON EVOLUTION

by

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The dentition of cypriniform fishes as a model for the nature of developmental constraints on evolution

Thesis directed by Associate Professor David W. Stock

The extent to which constraints on adaptive evolution are imposed by the genetic and developmental architecture of organisms is a fundamental question in evolutionary biology. The evolution of dentition in cypriniform fish presents a unique opportunity to study such constraints. Teeth in ray-finned fishes are commonly found on the jaws as well as in the posterior pharynx (throat). In cypriniforms, they are restricted to a single pair of bones in the pharynx as a result of tooth loss in evolution. That the mechanisms of tooth loss or subsequent genetic changes represent constraints on the reappearance of lost teeth is suggested by the conservation of reduced dentition even in species with feeding modes that would likely benefit from additional teeth. The present study investigated a potential role of modification of Wnt signaling in the reduction of cypriniform dentition, a process that might contribute to a constraint on regaining lost teeth. The expression of the transcription factors *lef1* and *tcf7*, two downstream targets of Wnt signaling, was compared between a representative cypriniform, the zebrafish (*Danio rerio*), and a member of a related order with a more complete dentition, the Mexican cave tetra, *Astyanax mexicanus*. Both genes were found to be expressed in all tooth germs examined and to have lost their expression in regions from which teeth were lost in the zebrafish lineage. To determine whether such loss of expression was the cause of cypriniform dentition reduction, the necessity of Wnt signaling for tooth development in both species was examined. Injection of morpholino antisense oligonucleotides and application of pharmacological inhibitors revealed that Wnt signaling is necessary for the formation of tooth germs, as evidenced by the blocking of tooth germ molecular markers. However, some markers retained their expression, suggesting that the constraint on regaining teeth

lost in cypriniforms is likely the alteration of genetic pathways in addition to Wnt signaling.

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INTRODUCTION

A fundamental question in evolutionary biology is the extent to which the production of adaptive phenotypic traits by natural selection is limited by features of the developmental and genetic architecture of organisms (Gould and Lewontin, 1979; Futuyma, 2010). Constraints on adaptive evolution are illustrated most starkly by the increasing pace of anthropogenic extinction (Futuyma, 2010), but the nature of such constraints is poorly understood. An opportunity to study the developmental genetic features underlying constraint is provided by the evolution of dentition in fishes of the order Cypriniformes (Stock, 2007).

Cypriniforms are a diverse group of freshwater fish comprising over 3,000 species (Nelson, 2006) with more species being named regularly. Members of this group, which include minnows, carps, suckers and loaches, are extremely diverse in size and shape, ranging from deep-bodied goldfish to streamlined minnows and including some of the smallest (*Paedocypris progenetica* at 8 mm) and largest (*Catlocarpio siamensis* at 3 m) freshwater fishes (Helfman et al., 2009). A diversity of feeding modes are represented in the group, ranging from suction feeding in bottom deposits to filter feeding on zooplankton to biting pieces from aquatic macrophytes to capturing other fishes (Sibbing, 1991). Such diversity is reflected in the diversity of tooth size and shape in the pharyngeal dentition (Pasco-Viel et al., 2010). In contrast, tooth location is highly conserved, being restricted to the fifth ceratobranchial bones of the lower posterior pharynx. Teeth in the oral cavity and upper pharynx are thought to have been lost in the common ancestor of this group at least 50 million years ago (Stock, 2007).

The loss of cypriniform teeth has likely constrained re-expansion of dentition within the group. Several cypriniform lineages have evolved to feed on other fishes, a habit for which oral teeth would likely be advantageous, but none have regained such teeth (Stock, 2007). The most striking evidence for a constraint on cypriniform dental evolution comes from the species *Danionella dracula*. As the name implies, the species has large oral fangs and several tiny tooth-like structures on the upper and lower jaws (Britz et al., 2009). Despite their superficial resemblance to true teeth, however, all are bony protuberances lacking enamel, dentine and pulp cavities.

The utility of studies of cypriniform dentition reduction for identifying the genetic and developmental bases of constraint is strengthened by the fact that the zebrafish (*Danio rerio*), a biomedical model organism, is a cypriniform. Additionally, the Mexican blind cavefish (*Astyanax mexicanus*), an emerging model species for Evo-Devo (Jeffery, 2008), is a close relative of the zebrafish that has retained the ancestral locations of teeth (Stock et al., 2006). These species allow comparative studies of the developmental genetic processes that have been retained in cavefish, but lost in zebrafish, which are candidate features underlying constraint.

Tooth development has been studied most extensively in mammals (Catón and Tucker, 2009). The first morphological sign of tooth development is thickening of oral epithelium to form a dental placode. The epithelium invaginates into the underlying mesenchyme, which condenses to form the dental papilla. Folding of the epithelium prefigures the crown shape of the final tooth, and cytodifferentiation leads to formation of epithelial ameloblasts and mesenchymal odontoblasts, which produce the organic components of the enamel and dentine, respectively. Development of teeth in fishes is

similar to that of mammalian teeth, although some of the initial structures are less apparent due to their small size (Huysseune, 1983; Van der heyden and Huysseune, 2000). Studies of a variety of molecular markers of dental tissues in the zebrafish and *A. mexicanus* suggest that oral tooth development in the former species is arrested before the placode stage (Stock et al., 2006; Wise and Stock, 2006; Stock, 2007). This result suggests that the developmental genetic mechanisms underlying cypriniform tooth loss are likely to involve pathways functioning in the earliest stages of tooth development. One such signaling pathway is initiated by the Wnt family of extracellular ligands (Catón and Tucker, 2009).

Wnt signaling is essential for a variety of developmental processes in the early embryo, including axis specification and gastrulation, as well as for the maintenance of adult structures, such as the heart and limbs (Croce and McClay, 2008). The canonical Wnt pathway involves the binding of Wnt ligands to members of the Frizzled family of transmembrane proteins (Amerongen and Nusse, 2009). Such binding results in the interaction of Frizzled with lipoprotein receptor-related protein (LRP), which transduces the signal to the protein Disheveled and activates it. Disheveled inhibits the activity of *GSK-3β* (glycogen synthase kinase 3*β)* (Nusse, 2005). In the absence of Wnt signaling, GSK3-β forms a destruction complex comprised of Axin2, Ck1 and APC, which targets *β*-catenin for destruction (Willert and Nusse, 1998). When Wnt signaling is active, this destruction complex is inhibited, and β-catenin accumulates before translocating to the nucleus, where it associates with the Tcf/Lef family of transcription factors (Chen et al., 2009). It then regulates the expression of a variety of target genes (Liu et al, 2008; Nusse, 2005).

The Wnt pathway has been shown to be necessary for the initiation of teeth and other epithelial appendages such as hair and feathers (Andl et al., 2002). In transgenic mice overexpressing the Wnt inhibitor *Dickkopf-1* (*Dkk1*), hair and tooth placodes were absent or failed to undergo invagination (Mikkola, 2007). A similar tooth phenotype is induced by deletion of β-catenin in the dental epithelium (Liu et al, 2008). Gain of function experiments also support the role of Wnt signaling in tooth initiation in mice. Constitutively stabilizing β-catenin in oral epithelium leads to the formation of supernumerary teeth in the regions of pre-existing teeth (Jarvinen et al., 2006). The stabilization of β-catenin in mesenchyme results in ectopic invaginations of palatal epithelium characteristic of tooth development (Chen et al., 2009). Gene expression analysis has placed Wnt upstream of many other genes necessary for the development of skin appendages (Andl et al., 2002; Zhang et al., 2009).

That altered Wnt signaling may have been involved in cypriniform tooth loss is suggested by the absence of expression of the ligand *wnt10a* and the Wnt target *axin2* from the oral cavity of zebrafish. (Alhajeri, 2010). Both genes are expressed in oral and pharyngeal tooth germs of *A*. *mexicanus* as well as in the pharyngeal tooth germs of zebrafish (Alhajeri, 2010). To further investigate the potential involvement of modification of Wnt signaling in cypriniform dentition reduction, I compared the expression of the transcription factors *lef1* and *tcf7*, two downstream targets of Wnt signaling, in the oral and pharyngeal cavities of *A*. *mexicanus* and *D. rerio*. Both genes are expressed in all tooth germs examined and appear to have lost their oral expression in association with cypriniform tooth loss. To determine whether such expression loss was a cause of cypriniform dentition reduction, I examined the necessity of Wnt signaling for

tooth development in the zebrafish and *A. mexicanus*. Injection of morpholino antisense oligonucleotides and application of pharmacological inhibitors revealed that Wnt signaling is necessary for the formation of tooth germs, as evidenced by blocking the expression of tooth germ molecular markers. However, since some tooth genes retain expression following such treatment, the constraint on regaining lost teeth in cypriniforms is likely to involve genetic changes in addition to those documented in the Wnt pathway.

MATERIALS AND METHODS

Animals

Zebrafish embryos were obtained from natural spawnings of the inbred Tübingen line (founders provided by the Zebrafish International Resource Center). Husbandry and staging of zebrafish followed Kimmel et al. (1995). *A. mexicanus* (hereafter referred to as cavefish) embryos and larvae were obtained from natural spawnings of a commercial line that originated from La Cueva Chica (Jeffery and Martasian, 1998).

Zebrafish embryos and larvae were raised in 100 mm tissue culture dishes in 30% Danieau's medium at 28.5°C. To inhibit pigmentation in embryos used for whole-mount *in situ* hybridization, embryos were transferred to Danieau's medium containing 1-phenyl-2-thiourea (PTU, 0.003% final concentration) at approximately 12 hours. Cavefish embryos and larvae were raised in Danieau's medium at 25°C (Jeffery et al., 2000).

Cloning and sequence analysis

Probes for zebrafish *lef1* and *tcf7* corresponded to nucleotide positions 640-1350 and 61-1128 in GenBank accessions NM_131426.1 and NM_001012389.1, respectively (D. Stock, unpublished). Cavefish *lef1* and *tcf7* were cloned to provide probes for *in situ* hybridization. Total cellular RNA was isolated from cavefish larvae using the TRIzol reagent (Life Technologies). cDNA was produced by reverse transcription with random hexamer primers and Superscript II reverse transcriptase (Life Technologies) and used as a template for PCR with the following degenerate primer (with added restriction sites underlined):

lef1: GCCGGGATCCGAYGARATGATHCCNTTYAA,

GCCGGAATTCCANCCNGGRTANARYTGCAT

tcf7: GCCGGATCCGGNGCNAAYGAYGARATGAT,

GCCGGAATTCTCRTARTAYTTNGCYTGYTC

PCR products were cloned into the pCR4-TOPO vector (Life Technologies) and subjected to automated sequencing. Sequences were conceptually translated and aligned with the program ClustalX (Larkin et al., 2007). Orthology to zebrafish genes was determined by phylogenetic analysis with the neighbor-joining method implemented in MEGA version 6.0. (Tamura et al., 2007). Settings used were the Jones-Taylor-Thornton (JTT) model of amino acid replacement, a gamma distribution of replacement rates among sites (gamma parameter = 1), a homogeneous pattern of evolution among lineages and complete deletion of sites with missing data. Support for the phylogeny obtained was determined by bootstrapping (1000 replicates).

Morpholino antisense oligonucleotides

Morpholino antisense oligonucleotides (MOs) used were a *lef1* splice-blocking MO (ACTGCCTGGATGAAACACTTACATG – Ishitani et al., 2005), a *tcf7* translation-blocking MO (AGCTGCGGCATGATCCAAACTTTCT – Bonner et al., 2008) and a *p53* translation-blocking MO (GCGCCATTGCTTTGCAAGAATG - Robu et al., 2007). MOs were prepared singly and in combination at concentrations ranging from 1.5 – 9.0 g/l in a solution containing 0.2M KCl and 0.2% phenol red. Approximately 1 nl of MO solution was injected into the yolk of 1-2 cell embryos. Concentrations used in the results reported were determined empirically to maximize effects on teeth while minimizing likely nonspecific defects such as necrosis. *p53* knockdown was included in the experiment to eliminate the non-specific apoptotic phenotype often associated with MO injection (Robu et al., 2007).

Drug treatments

Inhibition of Wnt signaling through stabilization of Axin (part of the destruction complex) was performed with the reagent XAV939 (3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3-d]pyrimidin-4-one; Calbiochem; Huang et al., 2011). This compound was dissolved in DMSO and added to dechorionated embryos in Danieau's medium to achieve a final concentration of 10-20 μM (determined through preliminary experiments as the lowest concentrations reliably producing effects on the dentition). Zebrafish embryos analyzed by *in situ* hybridization were maintained in XAV939 from 24, 30, 36, or 48 hpf (hours post-fertilization) through fixation at 56 hpf. Zebrafish larvae analyzed by clearing and staining for mineralized teeth were maintained in XAV939 from 24 hpf through fixation at 5 dpf. Cavefish analyzed by *in situ*

hybridization were maintained in XAV939 from 24, 36, 48, or 60 hpf through fixation at 84 hpf.

In situ hybridization and histology

Clearing and alizarin red staining of calcified teeth was conducted as described by Wise and Stock (2010). Whole mount *in situ* hybridization was performed as described in Jackman et al. (2004). Proteinase-K digestion treatments were carried out for 30 minutes at room temperature at concentrations of 2.5 μg/mL for examining the oral regions of each species, or 25 μg/mL for examining the pharyngeal region of zebrafish. Probes for zebrafish *bmp2b, dlx2b, eda, edar, fgf4, shha, pitx2* and cavefish *bmp2b, dlx2b*, *eda, edar, pitx2, shha* were as previously described (Jackman et al., 2004; Stock et al., 2006; Aigler et al., 2014).

Some animals used in *in situ* hybridization were cleared in 100% glycerol for whole mount observation, while others were dehydrated through a graded ethanol series and then embedded in glycol methacrylate (JB-4, Polysciences) for sectioning at a thickness of 4μm as described by Jackman et al. (2004). Images of the samples were taken using a Zeiss Axiocam digital camera mounted on a Zeiss Axiovert 135 inverted compound microscope. Image adjustments were conducted using the raster graphics editor *GNU Image Manipulation Program* (GIMP) and applied to the whole image.

RESULTS

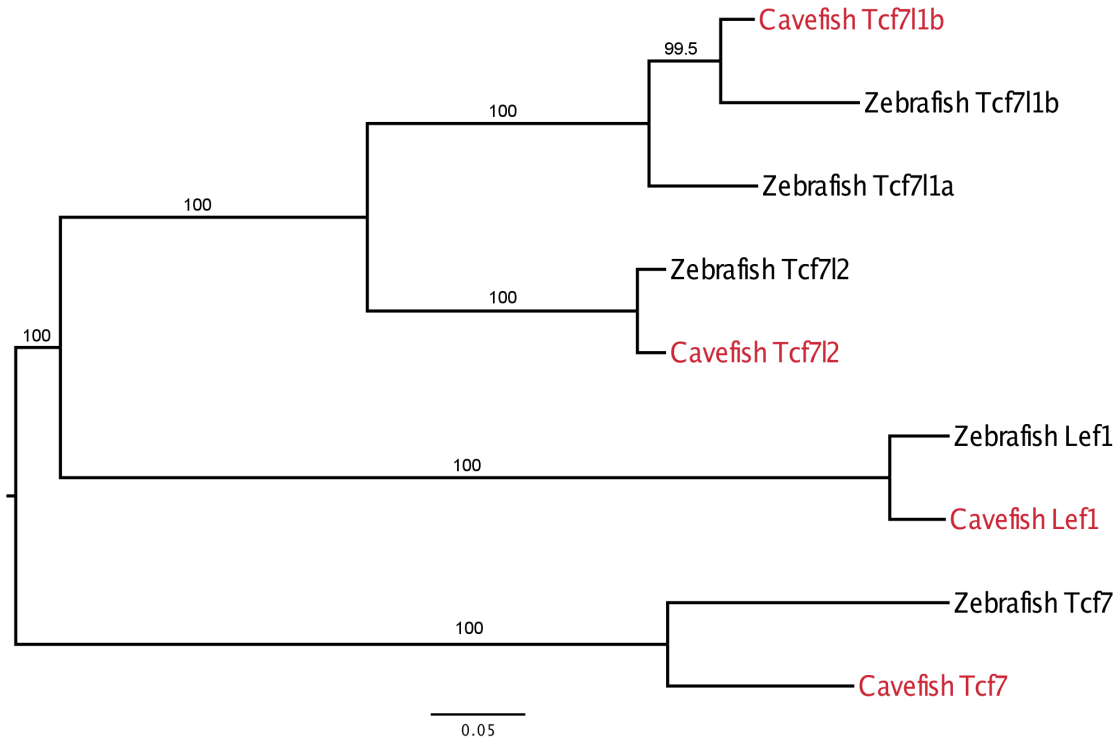

Figure 1. Neighbor-joining tree of *lef1* and *tcf7* amino acid sequences. Numbers above branches indicate bootstrap support, the genes cloned are indicated in red, and the scale bar indicates a sequence divergence of 5%.

A. mexicanus possesses at least four members of the Tcf/Lef family

Twelve clones obtained from PCR amplification of cavefish cDNA with degenerate *lef1* and *tcf7* primers were sequenced. These clones represented four separate genes, which were found by phylogenetic analysis to be orthologous to zebrafish *lef1*, *tcf7*, *tcf7l2*, and *tcf7l1b* (Fig. 1). This result suggests that the cavefish possesses a similar complement of Lef/Tcf family members to the zebrafish. Because *lef1* and *tcf7* are considered to be the family members that function primarily as transcriptional activators (Veien et al., 2005), further analyses were restricted to these genes.

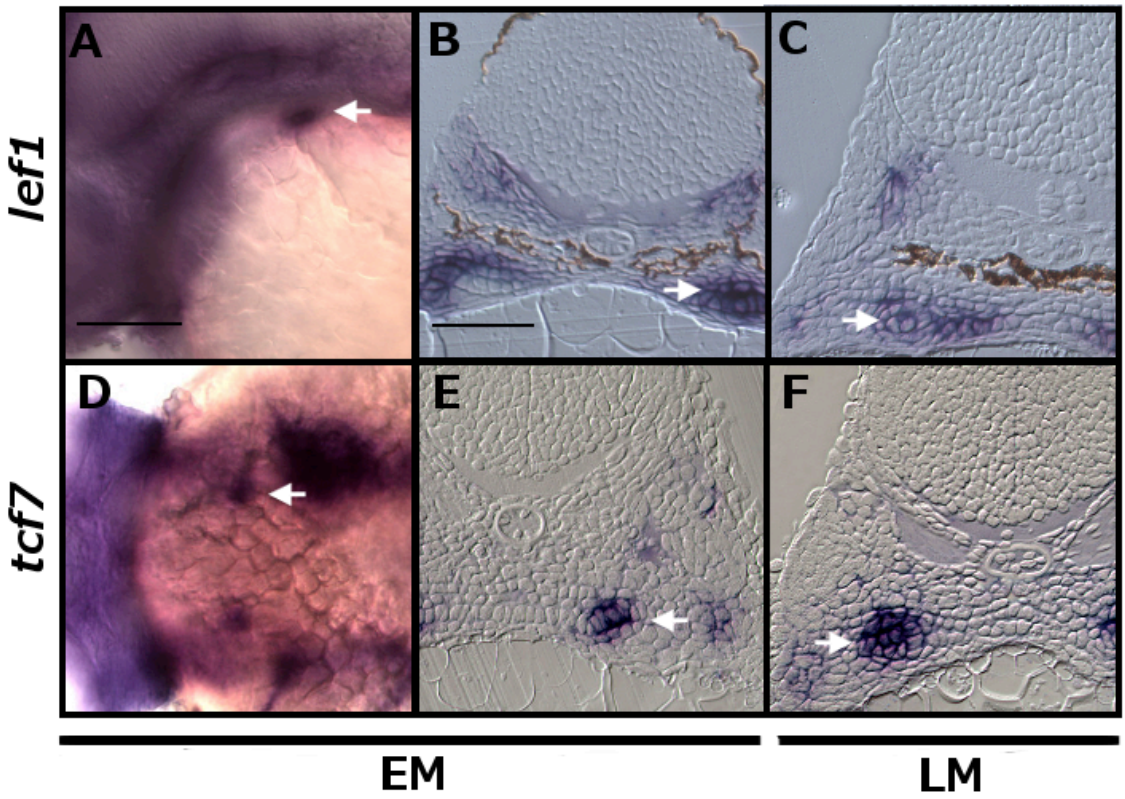

Figure 2. *lef1* and *tcf7* are expressed in zebrafish pharyngeal tooth germs. *In situ* hybridization analysis of gene expression in whole mounted (A, D) and sectioned (B-C, E-F) specimens. At early morphogenesis (EM) stages (Huysseune et al., 1998) of tooth development (A-B, D-E), both genes are expressed in dental epithelium (arrow). At late morphogenesis (LM) stages (C, F), both genes are expressed in epithelium and mesenchyme of tooth germs (arrows). Lateral view in (A), dorsal view in (D) and transverse sections in (B-C, E-F). Scale bar = 100 μm (A, D) or 50 μm (B-C, E-F).

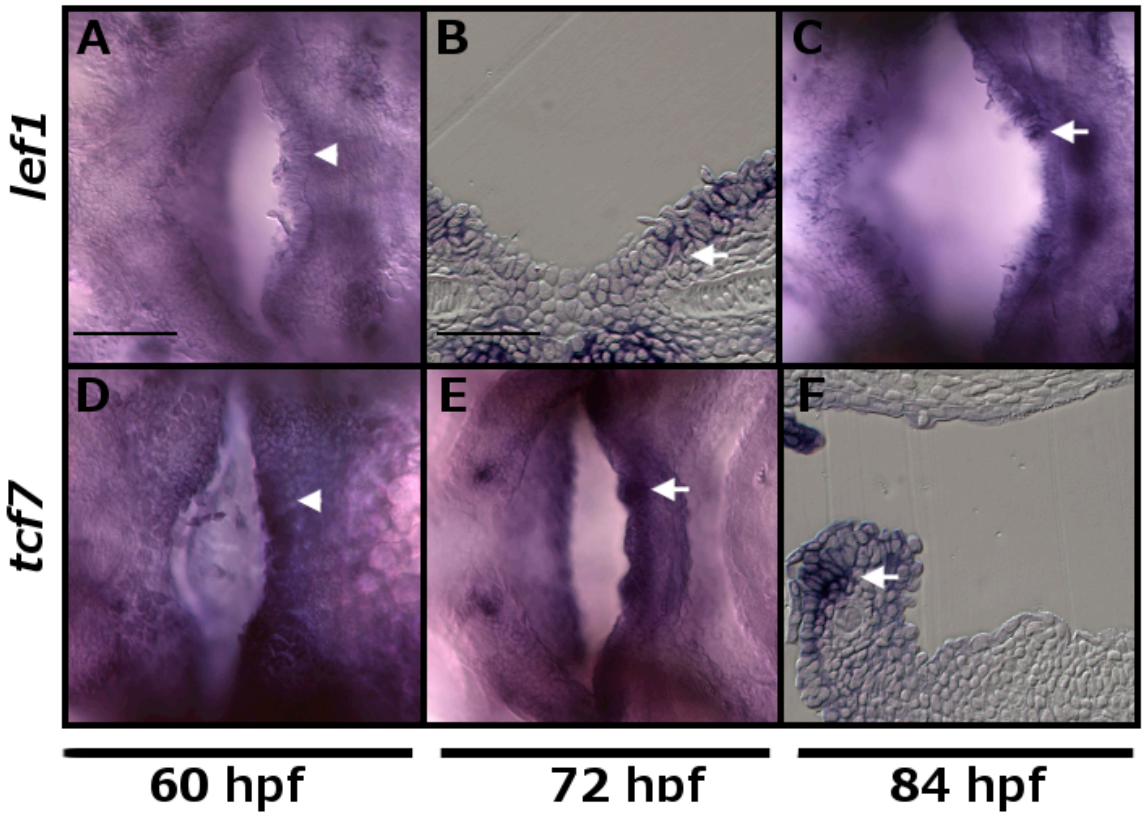

Figure 3. *lef1* and *tcf7* are expressed in cavefish oral tooth germs. *In situ* hybridization analysis of gene expression in whole mounted (A, C-E) and sectioned (B, F) specimens. Both *lef1* (A) and *tcf7* (D) are expressed broadly in the oral region (arrowheads) at 60 hpf, an age before tooth germs are morphologically visible (Stock et al., 2006). Later expression domains of both genes include oral tooth germs (arrowheads in B-C, E-F). Ventral views of mouth in (A, C, D-E), transverse view of symphyseal region of lower jaw in (B), and transverse section of lower (and a portion of the upper) jaw in (F). Scale bar = 100 µm (A, C-E) or 50 µm (B, F).

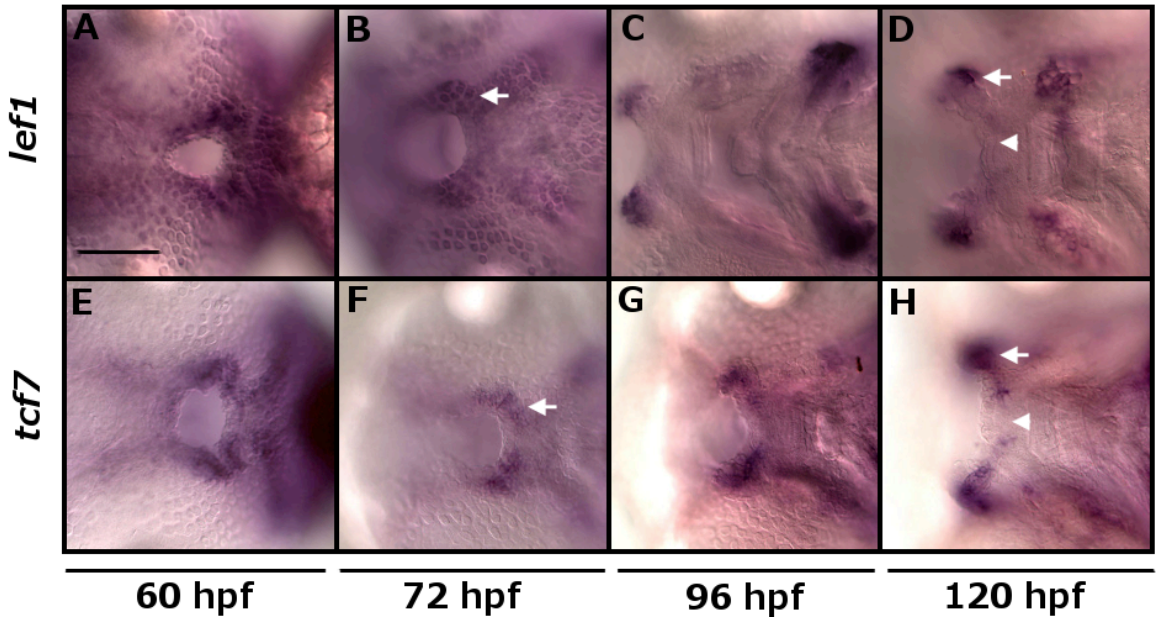

Figure 4. Tooth germ-associated expression of both *lef1* and *tcf7* is absent in the zebrafish mouth. Ventral views of the developing mouth. Expression of both genes at early ages (A-B, E-F) is strongest laterally (arrows). At ages comparable to those at which tooth germ appear in cavefish (C-D, G-H), expression is confined to the lateral margins of the jaw (arrows) and is not expressed medially at the jaw margin where tooth germs would be expected to develop (arrowheads; more caudal medial expression domains in H = neuromasts). Scale bar = 100 μm.

Expression of lef1 and tcf7 is present in teleost tooth germs but absent from formerly tooth-bearing regions of the zebrafish mouth

Expression of *lef1* and *tcf7* was first examined in the zebrafish pharynx. During early morphogenesis, expression of both genes is confined to the epithelium (Fig. 2 A-B, D-E), while during late morphogenesis, expression is found in both the epithelium and the mesenchyme (Fig. 2 C, F). In the cavefish mouth, *tcf7* is broadly expressed before the appearance of tooth germs (Fig. 3D). Expression then becomes restricted to tooth germs medially and the jaw hinge region laterally (Fig. 3E). Sectioning revealed that tooth germ expression is present in both epithelium and mesenchyme at the one stage examined (Fig.

3F). *lef1* expression was also detected in cavefish oral tooth germs but is less restricted to these structures than is *tcf7* (Fig. 3A-C).

The expression of both transcription factors was also examined in the oral region of zebrafish from 56-120 hpf. The mouths at these ages are morphologically similar to those of cavefish during the initiation and morphogenesis of oral teeth (Stock et al., 2006). As has been previously described with *wnt10a* and *axin2* (Alhajeri, 2010) expression of *lef1* and *tcf7* is exhibited in the lateral margins of the mouth (Fig. 4). This expression is also present in cavefish (Fig. 3). However, unlike in cavefish, expression of these two genes is not present in the medial epithelium from which tooth germs likely developed in cypriniform ancestors (Fig 4C-D, G-H). I conclude that evolutionary loss of oral teeth in cypriniforms is correlated with the loss of *lef1* and *tcf7* transcription factor expression in the oral epithelium.

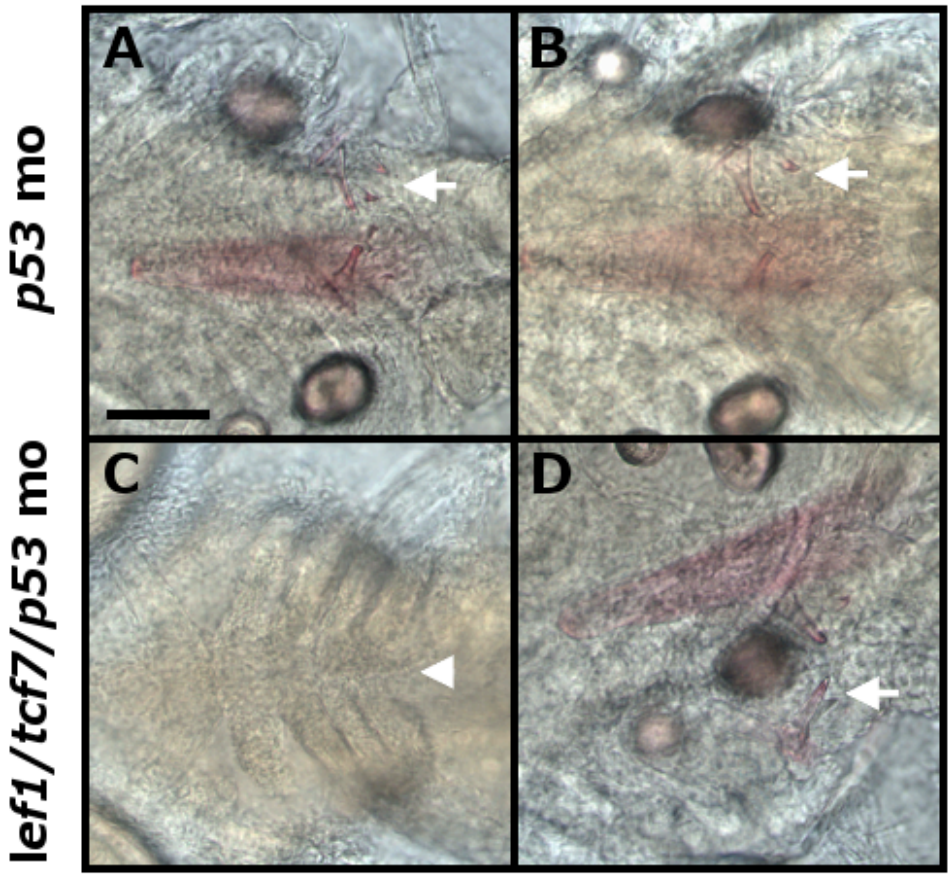

Figure 5. Co-injection of MOs targeting *lef1* and *tcf7* results in reduction of the number of mineralized teeth in the zebrafish . Ventral views of the gill arches of alizarin-stained 5 dpf larvae. Arrows and arrowheads indicate teeth and absence of teeth, respectively. *p53* MO injection (A-B) serves as a control. Three teeth per side (A) and two teeth per side (B) in the wild type pattern in control specimens. Absence of teeth (C) and two teeth per side (D) in specimens injected with *lef1*/*tcf7*. Scale bar = 100 µm.

Wnt signaling is necessary for teleost tooth development

To determine whether loss of *lef1* and *tcf7* expression from oral epithelium was a potential cause of the evolutionary loss of teeth in the zebrafish mouth, I first investigated whether these genes are necessary for tooth development in the zebrafish pharynx. Since *lef1* and *tcf7* are thought to function redundantly (McGraw et al., 2011), splice-blocking *lef1* (Ishitani et al, 2005) and translation-blocking *tcf7* (Bonner et al, 2008) MOs were co-

injected into zebrafish embryos. At 5 dpf, injected fish exhibited pectoral fin deformities, a phenotype previously reported for loss of *lef1* function (McGraw et al., 2011). In addition, many of these fish exhibited a reduction (one tooth per side in 5/16 individuals at 3 g/L and 12/15 at 1.5 g/L) or absence (6/16 at 3 g/L and 1/15 at 1.5 g/L) of dentition (Table 1, Fig. 5C). In contrast, 20/25 of the control fish injected with the *p53* morpholino exhibited two to three teeth per side (Fig. 5A, B) and none exhibited a complete lack of teeth (Table 1). A Fisher's Exact test of the number of individuals with reduced or absent teeth (0-1) versus wild type dentition (2-3) revealed that the difference was significant for both the 3g/l and 1.5 g/l injections ($p<0.0030$ and 0.0001, respectively). These results suggest that *lef1* and/or *tcf7* are necessary for pharyngeal tooth development in the zebrafish.

Table 1: Zebrafish pharyngeal teeth remaining at 5 dpf after morpholino injection

		Maximum number of teeth per side			
Injection	**Three**	**Two**	**One**	**None**	**Total Fish**
9 g/l *p53* MO	11	9	5	0	25
3 g/l *lef1*/*tcf7*/*p53* MO	3	2	5	6	16
1.5 g/l *tcf7*/*lef1*/*p53* MO	0	2	12	1	15

The effects of inhibiting Wnt signaling were additionally examined with the pharmacological compound XAV939. This drug functions by stabilizing Tankyrases1/2, which in turn stabilize the GSK-3 destruction complex, allowing the degradation of β-catenin (Baarsma et al., 2013). When treated with XAV939 at 20 uM beginning at 24 hpf, zebrafish cleared and stained with alizarin for dentition at 5 dpf exhibited reduced (one

tooth, 16/28) or absent (12/28) teeth while all fish treated with DMSO as a control exhibited two or three teeth per side (n=27). A Fisher's Exact test revealed that the difference between these treatments is significant ($P<0.0001$), suggesting, as do the results of the MO experiments, that Wnt signaling is necessary for tooth development in the zebrafish.

In order to identify the stage of arrest of tooth development in XAV939-treated zebrafish as well as to determine whether inhibition of Wnt signaling could produce a pharyngeal phenocopy of the gene expression profile of the zebrafish oral region, XAV939-treated larvae were examined by *in situ* hybridization. Treatment of zebrafish with XAV939 resulted in loss of expression of two dental placode markers, the transcription factor *dlx2b* and the signaling molecule *fgf4* (Fig. 6). In the case of both genes, treatment as late as 36 hpf was sufficient to block expression at 56 hpf (n=10/10 versus 0/10 in controls for *dlx2b*, n= 8/8 versus 0/6 for *fgf4*), but treatment at 48 hpf did not do so. Similar results were obtained for zebrafish *dlx2b* with an additional tankyrase inhibitor (IWR-1, data not shown). These results are consistent with the requirement of *lef1* function for *fgf4* expression in the mouse (Kratochwil et al., 2002).

In contrast to its effect on *dlx2b* and *fgf4* expression, continuous XAV939 treatment of zebrafish embryos from as early as 24 hpf did not reduce dental expression of the signaling molecules *eda* (n= 6/6), the receptor *edar* (n=8/8), or the transcription factor *pitx2* (n=9/9) (Fig. 7). In addition, although reliable tooth germ expression of the signaling molecule *shha* was not obtained in controls, the general expression of this gene in the pharynx was not affected by XAV939 treatment. These data are partially consistent with the effects of Wnt loss of function on the dentition of the mouse. Overexpression of

the Wnt inhibitor *Dkk1* or deletion of the Wnt effector *B-catenin* did not affect expression of *Pitx2* or *Eda* (Liu et al., 2008). In addition, *Edar* expression was not affected in the dental epithelium of *Lef1* knock-out mice (Laurikkala et al. 2001). However, dental *Shh* expression was lost in *Dkk1* overexpressing and *B-catenin* knockout mice (Liu et al., 2008). In addition, dental *Eda* expression was downregulated in *Lef1* knockout mice (Laurikkala et al., 2001). XAV939 treatment produces in the pharyngeal dentition of the zebrafish a partial phenocopy of the oral region of this species. *edar*, *pitx2*, and *shha* are expressed in the zebrafish mouth, while *dlx2b*, *eda* and *fgf4* are not (Stock et al., 2006; Aigler et al., 2014).

Differences in the effects of loss of Wnt function on the zebrafish pharyngeal dentition and the mouse oral dentition may be the result of differences between species or between regions of the oropharyngeal cavity. In addition, loss of Wnt function in the pharyngeal dentition may only partially phenocopy the zebrafish oral region because genetic changes in addition to loss of Wnt signaling were involved in cypriniform dentition reduction. In order to distinguish among these possibilities, I treated cavefish larvae with XAV939 and examined the effects on oral gene expression. Tooth germ expression of *dlx2b* was reduced relative to controls or completely absent in cavefish treated from 36 hpf with XAV939 (n=10/10) but not in individuals treated at 48 hpf (n=0/10, Fig. 8). In addition, while oral expression of *pitx2* and *shha* remained in cavefish treated from 36 hpf with XAV939, placode-like expression was lost (n=5/6 and 4/7, respectively for the genes). In contrast to the previous genes, XAV939 treatment had no effect on the oral tooth expression of *bmp2b* (a signaling molecule), *eda*, and *edar*. These results in the cavefish exhibit both differences and similarities with those

previously described for loss of Wnt signaling in the mouse and zebrafish. Reduction of *shha* and *pitx2* expression in the cavefish mouth contrasts with the effects of inhibition of Wnt signaling in both the mouse oral dentition and the zebrafish pharyngeal dentition. Retention of *eda* and *edar* expression in the cavefish mouth is consistent with the effects of inhibiting Wnt signaling on the zebrafish pharynx and those of at some of the methods of inhibiting Wnt signaling in the mouse. The effects of Wnt loss of function on *bmp2* expression in the mouse dentition have not ben reported, although Wnt signaling has been shown to be required for *bmp4* expression in this location (Liu et al., 2008), as well as for *bmp2* expression in the hair of mice (Andl et al., 2002). As in the case of the zebrafish pharynx, inhibition of Wnt signaling in the cavefish produces a partial phenocopy of the zebrafish mouth. Loss of *dlx2b* expression and placodal expression of *pitx2* and *shha* results in a pattern similar to that of zebrafish, while the retention of *eda* and *bmp2b* expression does not (Stock et al., 2006; Wise and Stock, 2006; Aigler et al., 2014). In addition, while *edar* expression is retained in the zebrafish mouth (Aigler et al., 2014), this gene is not expressed in the placode-like pattern seen in XAV939-treated cavefish (Fig. 10). Taken together, the results of loss of Wnt function on the zebrafish and cavefish dentition suggest that loss of Wnt signaling is unlikely to be the sole cause of dentition reduction in cypriniform fishes.

Table 2: Zebrafish pharyngeal teeth remaining at 5 dpf after XAV939 treatment

	Treatment	
Maximum number of teeth per side	**XAV939 [20 uM]**	**DMSO**
3	0	17
2	0	10
1	16	0
0	12	0

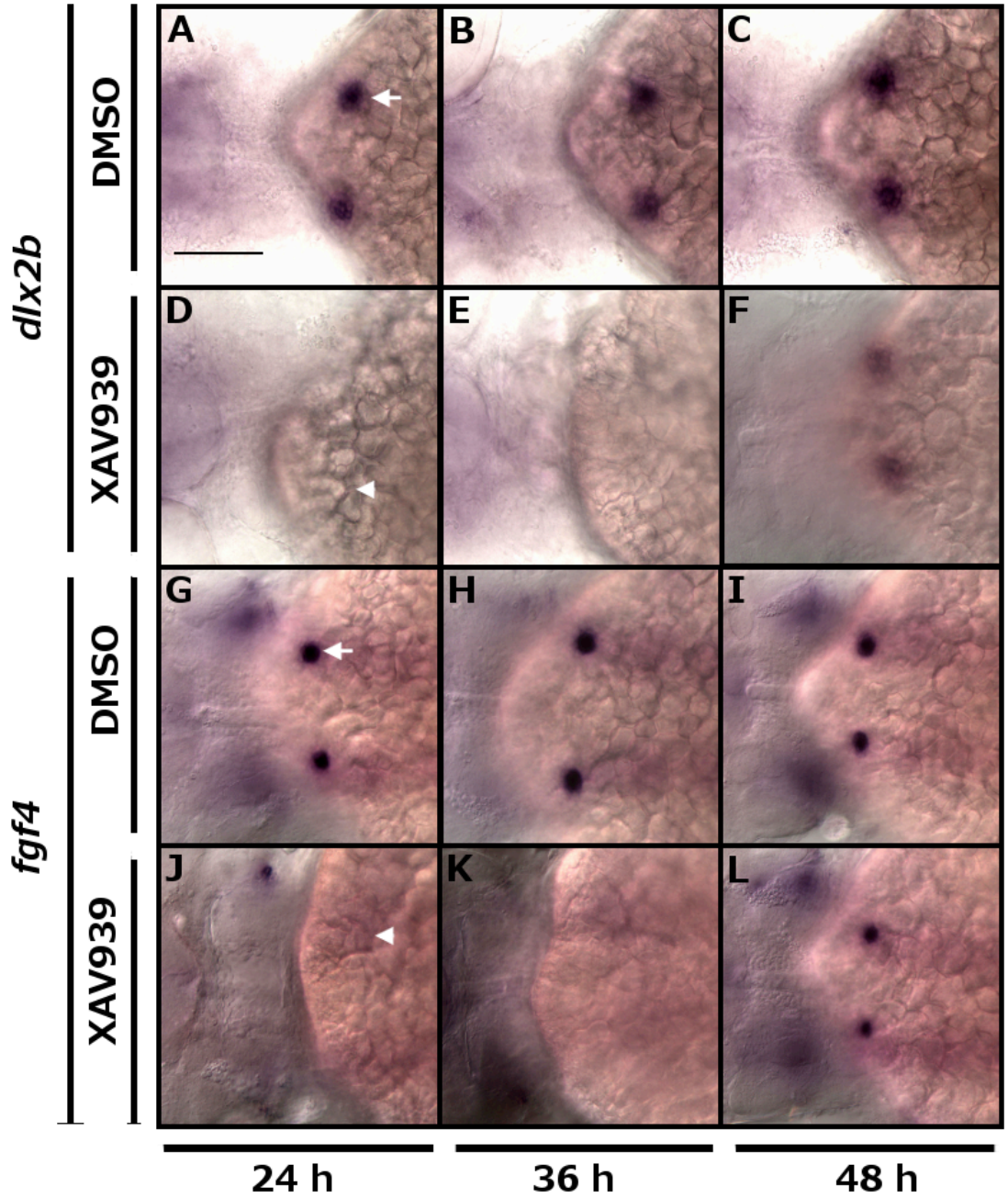

Figure 6. XAV939 treatment blocks the expression of the tooth germ markers *dlx2b* and *fgf4* in the zebrafish pharynx. Dorsal views of the pharynx of 56 hpf (late tooth morphogenesis stage) larvae treated continuously with XAV939 or DMSO from the indicated time point. Arrows indicate gene expression in tooth germs of DMSO-treated (control) and arrowheads indicate absence of such expression in XAV939-treated larvae. Scale bar = 100 µm.

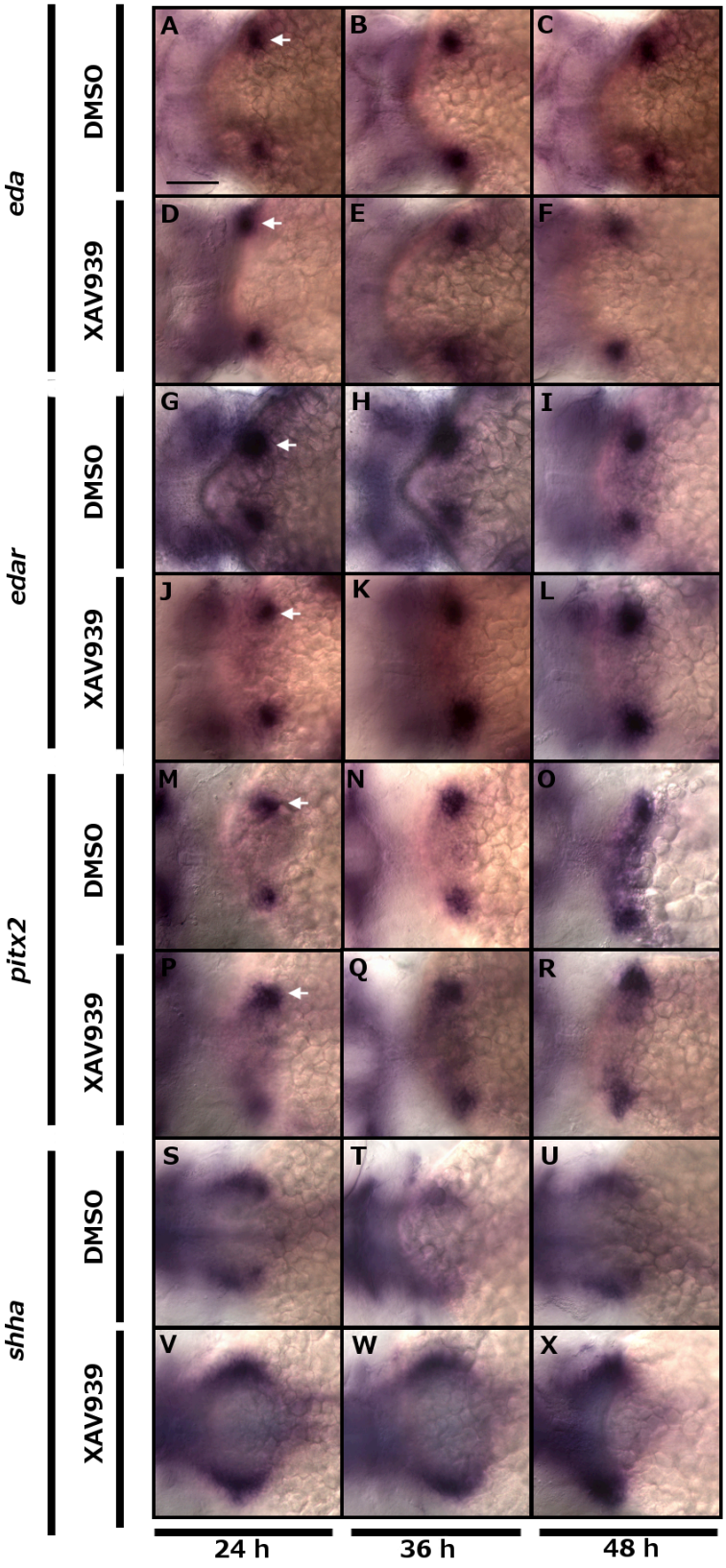

Figure 7. XAV939 treatment does not block expression of the tooth germ markers *eda, edar, pitx2* and *shha* in the zebrafish pharynx. Dorsal views of the pharynx of 56 hpf (late tooth morphogenesis stage) larvae treated continuously with XAV939 or DMSO from the indicated time point. Arrows indicate gene expression in tooth germs of DMSO-treated (control) and XAV939-treated larvae. Scale bar = 100 µm.

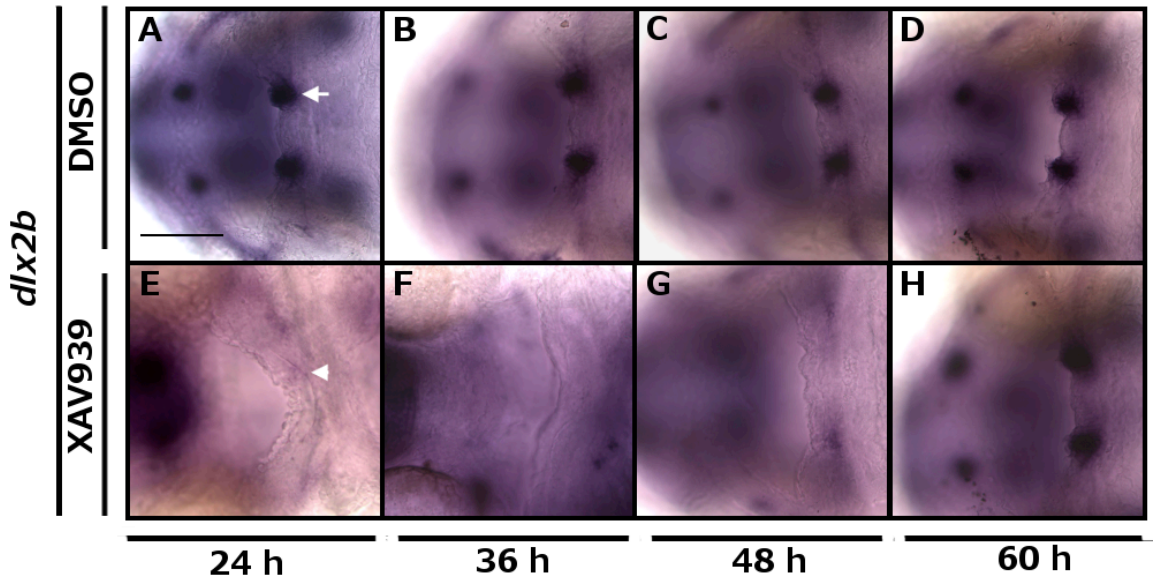

Figure 8. XAV939 treatment blocks the expression of the tooth germ marker *dlx2b* in the cavefish oral cavity. Ventral views of the developing mouth of 84 hpf larvae treated continuously with XAV939 or DMSO from the indicated time point. Arrow indicates gene expression in a tooth germ of a DMSO-treated (control) larva and an arrowhead indicates absence of such expression in an XAV939-treated larva. Scale bar = 100 μm.

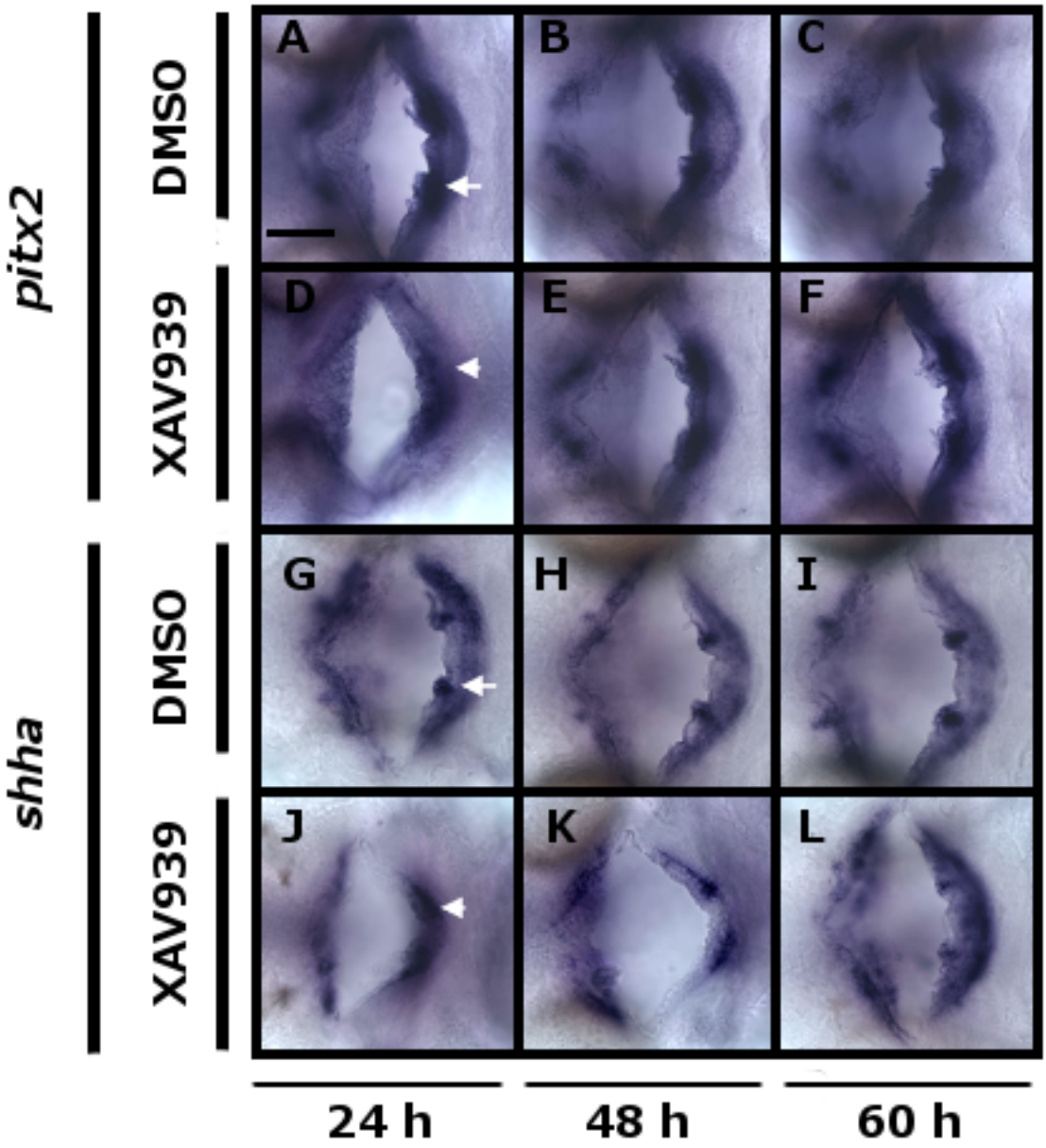

Figure 9. XAV939 treatment of the cavefish phenocopies zebrafish oral *pitx2* and *shha* expression. Ventral views of the developing mouth of 84 hpf larvae treated continuously with XAV939 or DMSO from the indicated time point. Arrows indicates gene expression in tooth germs of DMSO-treated (control) larvae and arrowheads indicate expression outside of tooth germs remaining in XAV939-treated larvae. Absence in tooth germs and presence outside of tooth germs characterizes the oral expression of *pitx2* and *shha* in the zebrafish (Stock et al., 2006) Scale bar = 100 µm.

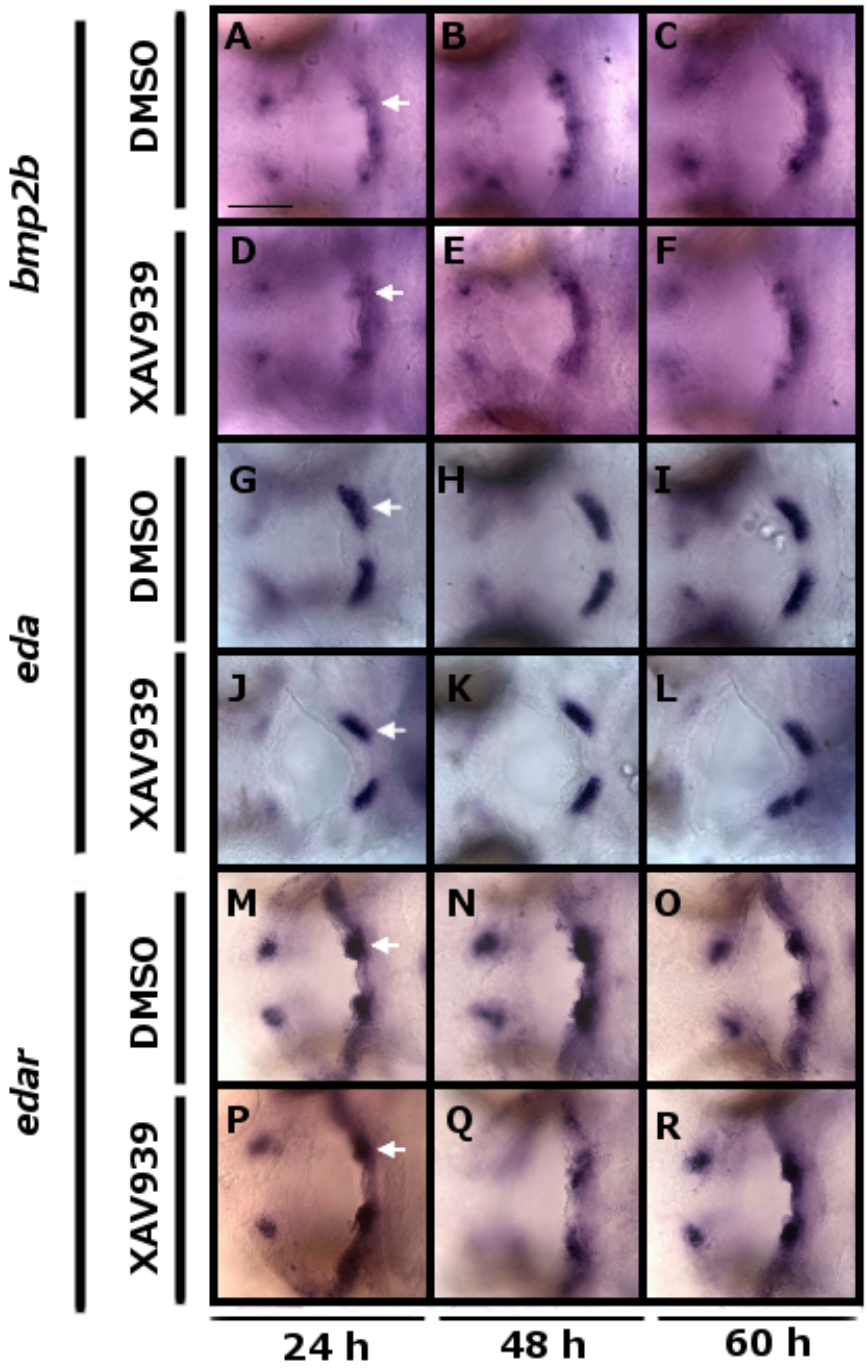

Figure 10. XAV939 treatment does not inhibit expression of the tooth germ markers *bmp2b, eda* and *edar* in the cavefish oral cavity. Ventral views of the developing mouth of 84 hpf larvae treated continuously with XAV939 or DMSO from the indicated time point. Arrows indicates gene expression in tooth germs of DMSO-treated (control) and XAV939-treated larvae. Scale bar = 100 µm.

DISCUSSION

The present study adds *lef1* and *tcf7* to a list of early tooth germ markers whose expression has been lost in the oral region of cypriniform fishes in association with loss of oral teeth. Such markers include the signaling molecules *bmp2a*, *bmp2b*, *bmp4*, *eda*, *fgf4*, *shha*, and *wnt10a*, the transcription factors *dlx2a*, *dlx2b*, and *pitx2*, and the intracellular component of the Wnt pathway *axin2* (Stock et al., 2006; Wise and Stock, 2006; Aigler et al., 2014; Alhajeri, 2010). Whether the loss of expression of these genes represents a constraint that has prevented cypriniforms from regaining teeth even in the presence of selection for their return (Stock 2007) depends on whether such losses represent multiple, independent genetic changes or the downstream consequences of one or a few genetic changes. The former situation would be most consistent with the hypothesis of constraint on the regain of oral teeth, as multiple genetic changes would be required to regain teeth (Fig. 11).

It is unlikely that all of the expression changes listed above are independent. Jackman and Stock (2006) found that loss of oral *dlx2b* expression was the result of changes in unidentified *trans*-acting factors rather than in the *cis*-regulatory region of the gene. Several candidates for such *trans*-acting factors among the genes whose expression was lost in association with cypriniform dentition reduction include the Bmps (Wise and Stock, 2006) because of the regulation of *dlx2* expression by *bmp4* in the mouse (Thomas et al., 2000), Fibroblast growth factors (Fgfs) because of the dependence of *dlx2b* expression in cavefish oral teeth on Fgf signaling (Stock et al., 2006), and *eda* because of the loss of dental *dlx2b* expression in zebrafish with mutations in genes of the Eda pathway (Aigler et al., 2014). This study adds *lef1* and *tcf7*, as well as Wnt signaling in general, as upstream regulators of dental *dlx2b* expression. Within the Wnt pathway

itself, loss of expression of *axin2*, *lef1*, and possibly *tcf7* could be the result of loss of *wnt10a* expression, as the former two genes are known targets of Wnt signaling (Kengaku et al., 1998; Jho et al., 2002) and the latter is related to *lef1* phylogenetically as well as in dental expression pattern.

Despite the likely function of many of the above genes in interacting networks, as suggested for example by the dependence of dental *dlx2b*, *fgf4*, *pitx2*, and *shha* on Wnt signaling in the cavefish, some of the results of the present study suggest the existence of multiple genetic changes associated with cypriniform tooth loss. Specifically, dental expression of *bmp2b*, *eda* and *edar* is not blocked by inhibition of Wnt signaling in the cavefish despite the necessity of Wnt signaling for zebrafish pharyngeal and cavefish oral tooth development. *eda* and *edar* are also necessary for tooth development (Harris et al., 2008) and Aigler et al. (2014) showed that while *eda* overexpression is sufficient to restore dentition to the upper pharynx of the zebrafish, it does not do so in the mouth. The latter authors found that the oral epithelium was responsive to Eda signaling, and argued that additional genetic changes outside of the Eda pathway contribute to the constraint on regaining cypriniform oral teeth. Such additional genetic changes could include those documented in the present study in the Wnt pathway. It is therefore important to determine whether Wnt signaling is downstream of Eda signaling in teleost tooth development through analysis of Wnt expression in zebrafish mutants in the Eda pathway (Harris et al., 2008). Studies in other epithelial appendages suggest that Wnt and Eda may act in parallel with feedback interactions (Fliniaux et al., 2008; Häärä et al., 2011); if such is the case in teleost teeth, mutations in these parallel pathways may be a component of the constraint on regaining oral teeth in cypriniforms. Further elucidation of such a

constraint would contribute significantly to our understanding of morphological conservatism in an important component of the freshwater ichthyofauna.

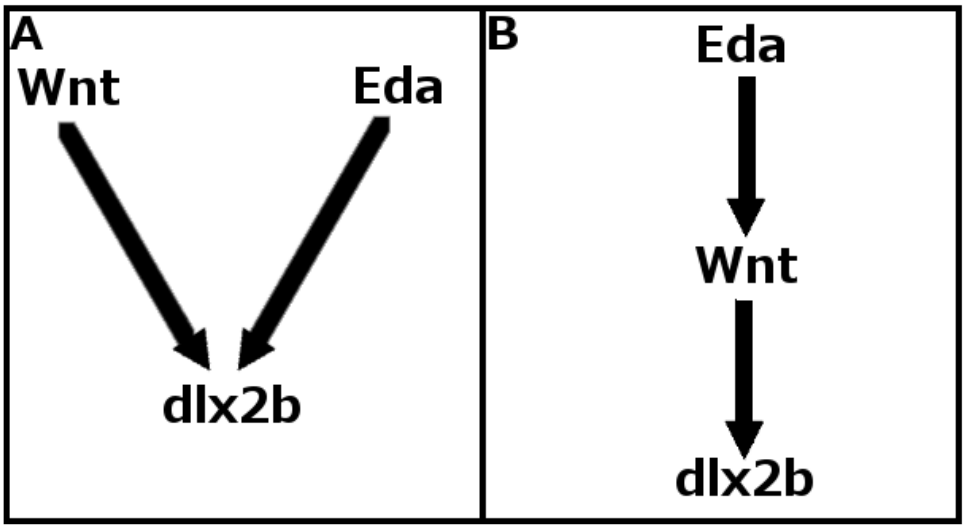

Figure 11. Alternative scenarios for gene networks governing dentition reduction in cypriniforms. (A) Wnt and Eda signaling act in parallel in early tooth development (represented by *dlx2b* expression). As components of both pathways have been altered in association with tooth loss, regain of lost teeth is likely constrained by the necessity of reversing at least two genetic changes. (B) Eda signaling acts upstream of Wnt signaling, so that reversal of Wnt signaling loss may only require restoration of Eda signaling. Regain of lost teeth is less constrained in this scenario than in (A).

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