Insight from Frogs: Sonic Hedgehog Gene Expression and a Re-evaluation of the Vertebrate Odontogenic Band

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While the identification of conserved processes across multiple taxa leads to an understanding of fundamental developmental mechanisms, the ways in which different animals fail to conform to common developmental processes can elucidate how evolution modifies development to result in the vast array of morphologies seen today—the developmental mechanisms that lead to anatomical variation. Odontogenesis—how teeth are initiated and formed—is well suited to the examination of both developmental conservation and phenotypic diversity. We suggest here that the study of early tooth development, the period of odontogenic band development, reveals departures from conserved mechanisms that question the role of players in the developmental process. In the earliest stages of odontogenesis, *Sonic hedgehog* (*Shh*) gene expression is interpreted as critical evidence of tooth initiation prior to any histological indication. However, a detailed examination of studies of tooth development across a wide range of taxa reveals that several vertebrate species fail to conform to the expectations of the *Shh* Consensus Model, calling for a reconsideration of the assumed causality of epithelial *Shh* in tooth initiation. We present new *Shh* gene expression data for an amphibian, the frog *Silurana (Xenopus) tropicalis*. In these animals, craniofacial and odontogenic developmental processes are more disjunct, and thereby provide a natural test of the hypothesis that *Shh* is immediately required for subsequent tooth development. Our results suggest that *Shh* expression may actually be related to the formation of the mouth rather than a required precursor to subsequent tooth formation. Anat Rec, 00:000–000, 2016. © 2016 Wiley Periodicals, Inc.

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Existing models for organogenesis represent the aggregation of decades of work, experimental validation, and a reasonable assumption of conservation of developmental gene expression and function across vertebrates. Departures from this conservation allow us to understand how mechanisms change with different

morphological and physiological features across the vertebrate tree of life. Odontogenesis is well suited to a comparative approach that explores the mechanisms of development and ultimate phenotypic diversity. Although much of the foundational developmental genetic work on odontogenesis was done in laboratory mice, the last 15 years have seen a large number of studies in other vertebrates, including cartilaginous and bony fishes (e.g., Fraser et al., 2004, 2008; Smith et al., 2009a;), squamates (e.g., Vonk et al., 2008; Handrigan and Richman, 2010), crocodylians (e.g., Tokita et al., 2013; Weeks et al., 2013), carnivorans (Järvinen et al., 2009), and other rodents (e.g., Keränen et al. 1999; Yamanaka et al. 2007; Fig. 1a). Although some differences in odontogenesis have been identified, such as the probable uniqueness of enamel knots to mammals, and superficial placodes in mice instead of the dental lamina seen in many amniotes, these non-mouse studies have supported the broad-scale conservation of gene networks, signaling pathways, and morphogenesis in tooth development (Fraser et al., 2009; Jernvall and Thesleff, 2012). Among these conserved pathways is the early expression of *Sonic hedgehog* (*Shh*).

The *Shh* Consensus Model for Tooth Initiation

One of the earliest markers of tooth development is expression of *Shh*. Odontogenesis in the oral jaw of many vertebrates begins with the formation of an odontogenic band (OB). The OB is a region of oral epithelium competent to form teeth and marked by the gene expression of *Shh* and *Pitx2* (Fraser et al., 2004), and the more recently recognized *Sox2* expression (Juuri et al., 2013). The OB is presumed to be homologous to the primary epithelial band (Smith et al., 2009b) or to the dental lamina (Jernvall and Thesleff, 2012) when accompanied by epithelial thickening. This OB then gives rise to a dental lamina or to individual tooth placodes that are marked by *Shh* expression and presage the locations of individual teeth. Tooth morphogenesis begins at these individual locations with a proliferation of the epithelium into the mesenchyme, creating a tooth bud.

Shh is expressed in dental epithelium at least as early as E10.5 in mice (Sarkar et al., 2000), with a clear band of expression along upper and lower jaws at E11 (Keränen et al., 1999). When a similar pattern of expression was observed along the developing jaws of fish, specifically the trout, the OB was named (Fraser et al., 2004), and the discrete domain of early *Shh* expression began to be taken as the developmental genetic signature of tooth initiation in vertebrates (Fig. 1a). Broad conservation of this epithelial field suggests a developmental function for *Shh* at this time and location (Table 1).

A functional role for *Shh* during tooth initiation was first described in mice, where it is known to cause epithelial proliferation as well as mesenchymal condensation in combination with Msx1 (Chen et al., 1996; Hardcastle et al., 1998; Zhang et al., 2000). Furthermore, *Shh* is necessary for morphogenesis past epithelial thickening stages in the first generation teeth of several taxa, including mice (Hardcastle et al., 1998; Cobourne et al., 2001), cichlids (Fraser et al., 2008), zebrafish (Jackman et al., 2010), and pythons (Buchtová et al., 2008). Although the precise stage(s) of its earliest involvement remain to be clarified, the conservation of

the OB expression domain suggests that this particular pattern of *Shh* expression may be causally and immediately required for tooth initiation in vertebrates. For brevity's sake, we call this expectation the *Shh* Consensus Model for Tooth Initiation (*Shh*CMTI).

Discrepancies Between an *Shh*-Defined OB and Tooth Formation

While expression of *Shh* in an OB is widely assumed to be a precursor for odontogenesis, a nuanced look at published *Shh* expression across vertebrates suggests that while *Shh* expression may be correlated with odontogenesis, it may not be causal. For example, in some cases an OB correlates with rudimentary tooth formation or no teeth at all. In other instances, the OB persists until well after tooth morphogenesis is underway, suggesting that it has a permissive rather than an instructive role in positioning individual tooth sites. In other examples, teeth form despite the lack of a preceding *Shh*-defined OB. We review the evidence for these discrepancies below.

The presence of an OB does not guarantee a full odontogenic program, as evidenced by the diastemal region of mice and venomous snakes. Keränen et al. (1999) found a continuous band of *Shh* in the mouse at E11 that then became restricted to budding teeth, including up to 7 diastema tooth germs that degenerate (Peterková et al., 2002). This lack of a dentition following OB expression is also observed in members of two distantly related venomous snake families (Elapidae and Viperidae). Both lineages have front fangs and a large anterior region of the developing maxilla that is edentate because the fangs arise posteriorly (Vonk et al., 2008). In both of these families the OB spans a region considerably anterior to the fang primordium, and is followed by a dental ridge—a dental lamina invagination that initiates transient tooth buds (Vonk et al., 2008).

Furthermore, the presence of an OB is not sufficient for tooth initiation, as in birds and zebrafish. Studies of early chick craniofacial development suggest that edentate birds also have an OB (Helms et al., 1997), with some chicken embryos displaying a thickened epithelial structure in the oral region (Chen et al., 2000). The expression of other genes suggests that the earliest epithelial patterning stages for odontogenesis occur but teeth fail to form for other reasons (Mitsiadis et al., 2003; Louchart and Viriot, 2011; Lainoff et al., 2015). The cyprinid zebrafish has similarly lost the ability to develop teeth in its oral cavity, although it does develop pharyngeal teeth. Despite no histological evidence for teeth in the oral region, *Shh* is co-expressed with *Pitx2* in the early mouth, coincident with the timing of pharyngeal tooth initiation (Stock et al., 2006).

Further complicating the *Shh*CMTI, a focal oral domain of *Shh* does not appear to be a required intermediate between the OB and individual tooth initiation and morphogenesis in all taxa. In non-venomous snakes, *Shh* expression in palatal and marginal tooth rows is reported to not become restricted to individual tooth-associated foci until the first teeth are at bell stage with apparent successional dental laminae (Buchtová et al., 2008). This further questions the downstream role of the OB.
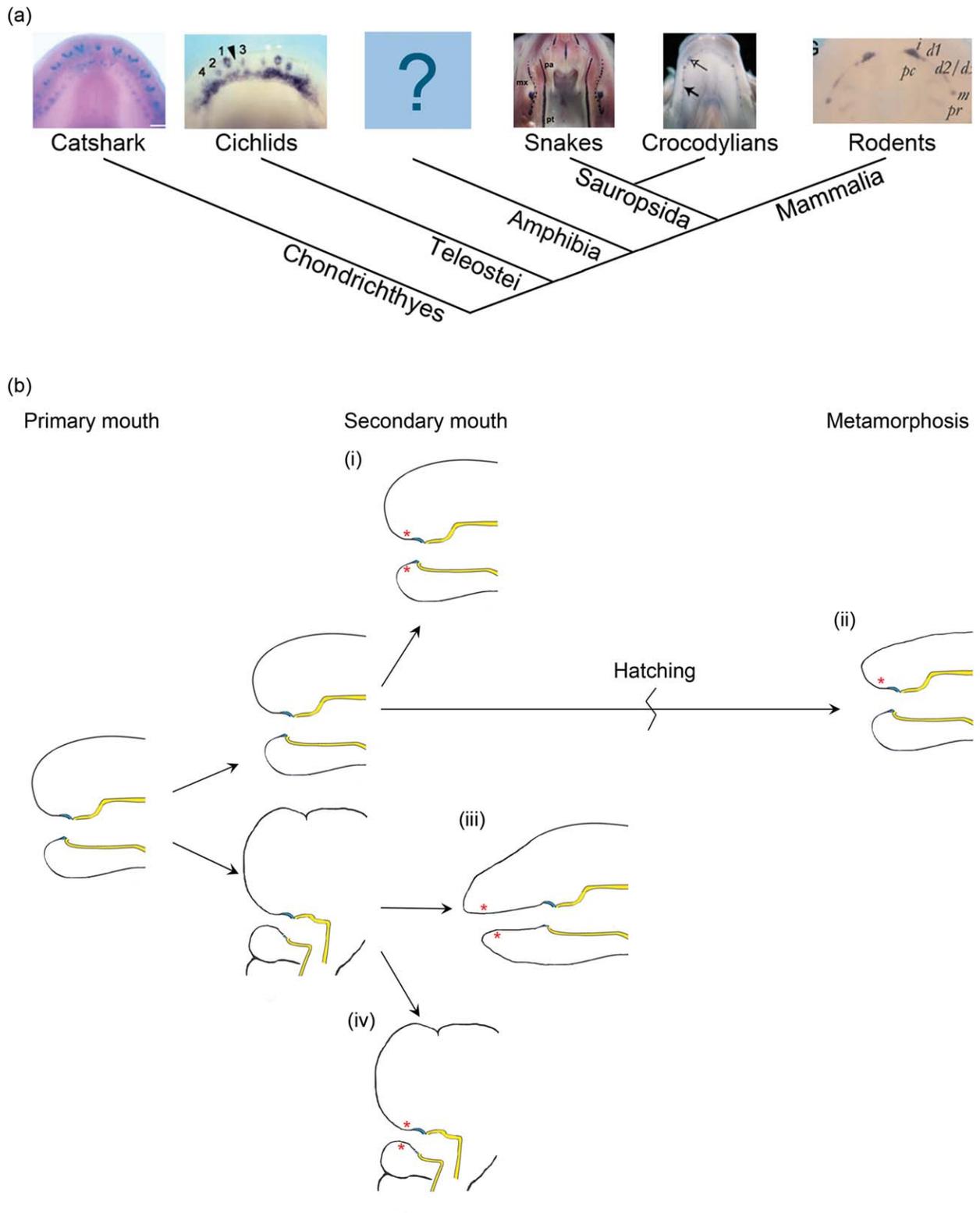
Fig. 1. Early stage tooth development across vertebrates. (**a**) Cladogram of whole mount *in situ* hybridizations for *Sonic hedgehog*. Individual tooth focus-like stages are present across the taxa studied thus far. Expression data compiled, from left to right: *Scyliorhinus canicula*, Smith et al., 2009a; *Metriaclima zebra*, Fraser et al., 2008; *Trimeresurus hageni*, Vonk et al., 2008; *Alligator mississippiensis*, Harris et al., 2006; *Mus musculus*, Keränen et al., 1999. See Table 1 for more detailed compilation of this trait. (**b**) Schematic representation of heterochrony in craniofacial development in vertebrate lineages. Odontogenesis occurs at differing phases and extents of craniofacial morphogenesis, with example trajectories presented for cichlids (i), *S. tropicalis* (ii), snakes (iii), and rodents (iv). Stomodeum boundary colored in blue, foregut cavity outlined in yellow. Red asterisks represent odontogenesis. Relative developmental time proceeds to the right.

TABLE 1. Survey of taxa that have been examined for an OB[a]

Organism		Shh detected?	OB named?	Citations
Chondrichthyes	Scyliorhinus canicula	Y	Y	Smith et al. (2009a)
Teleostei	Oncorhynchus mykiss	Y	Y	Fraser et al. (2004)
	Astayanax mexicanum	Y	N	Stock et al. (2006)
	Danio rerio	Y[b]	N	Stock et al. (2006)
	Cynotilapia afra	Y	Y	Fraser et al. (2008)
	Metriaclima zebra	Y	Y	Fraser et al. (2008)
	Labeotropheus fuelleborni	Y	Y	Fraser et al. (2008)
	Monotrete abei	Y	Y	Fraser et al. (2012)
	Haplochromis piceatus	Y	Y	Cho et al. (2015)
	Polyodon spathula	Y	Y	Smith et al. (2015)
Amphibia	Silurana (Xenopus) tropicalis	N	Y	Present study
Sauropsida	Python sebae	Y	Y	Buchtová et al. (2008)
	Python regius	Y	Y	Buchtová et al. (2008); Handrigan and Richman (2010)
	Elaphe guttata	Y	Y	Buchtová et al. (2008)
	Trimeresurus hageni	Y	Y	Vonk et al. (2008)
	Causus rhombeatus	Y	Y	Vonk et al. (2008)
	Calloselasma rhodostoma	Y	Y	Vonk et al. (2008)
	Elaphe obsolete	Y	Y	Vonk et al. (2008)
	Natrix natrix	Y	Y	Vonk et al. (2008)
	Naja siamensis	Y	Y	Vonk et al. (2008)
	Aspidelaps lubricus infuscatus	Y	Y	Vonk et al. (2008)
	Liasis mackloti	Y	Y	Vonk et al. (2008)
	Pogona vitticeps	Y[c]	Y	Handrigan and Richman (2010)
	Eublepharis macularius	Y[c]	Y	Handrigan and Richman (2010)
	Trachemys scripta	N	Y	Lainoff et al. (2015)
	Alligator mississippiensis	Y[c], N	N	Harris et al. (2006); Wu et al. (2013)
	Crocodylus siamensis	N	N	Tokita et al. (2013)
	Gallus gallus	Y	N	Helms et al. (1997)
Mammalia	Mus musculus	Y	N	Keränen et al. (1999)
	Microtus rossiaemeridionalis	Y	N	Keränen et al. (1999)
	Suncus murinus	Y	N	Miyado et al. (2007); Yamanaka et al. (2007)

[a]Whether an OB is acknowledged within a publication partly reflects the history of study (the term OB was not defined until 2004, Fraser et al., 2004) and partly reflects author interpretations of trait homology relative to other vertebrates. Squamates and teleosts have domains of *Shh* expression early in tooth development that have been named OBs. Data from crocodylians are more ambiguous; in some cases early developmental stages were not examined, but no authors have acknowledged a model involving an OB in their publications. Several mammalian studies were published before the OB was defined, but in these and other papers not explicitly identifying an OB, published figures with *in situ* hybridization data for *Shh* in any vertebrate were examined for a laterally continuous epithelial signal at the oral margin that preceded any histological sign of tooth morphogenesis. Shrews have a continuous *Shh* band early in development that was called "dental lamina-like" prior to forming *Shh*-expressing epithelial thickenings (Miyado et al., 2007; Yamanaka et al., 2007).
[b]Oral *Shh* expression detected, but not overlapping *pitx2* as in other teleosts (Stock et al., 2006).
[c]Data not shown in publication.

Teeth are also able to form in the absence of an OB in several lineages. The first teeth to form in crocodylians are non-functional and develop quite superficially, evaginating, depositing dentine, and then submerging into the mesenchyme (Westergaard and Ferguson, 1990; Weeks et al., 2013). These species lack an OB (Tokita et al. 2013, A Lainoff, personal communication), indicating that an OB is certainly not necessary for creating individually spaced, functional tooth precursors with most of their histomorphogenetic properties (Westergaard and Ferguson, 1990; Tokita et al., 2013; Wu et al., 2013).

It is interesting to note that crocodiles express *Pitx2* but not *Shh* in a band prior to the formation of first-generation teeth (Tokita et al., 2013). Loss of *Pitx2* function in mice, however, causes tooth arrest after the first epithelial–mesenchymal signaling and mesenchymal condensation events of tooth initiation have occurred (Liu et al. 2003, Lin et al. 1999, Lu et al. 1999). While the role of *Pitx2* in craniofacial and odontogenic development should be further explored, comparative research on *Shh* expression and function is considerably more extensive than for *Pitx2*, and as such, we focus on evaluating the role of the best characterized and most frequently cited aspect of the OB.

A Test of the *Shh* Consensus Model for Tooth Initiation

To more definitively test the hypothesis that *Shh* expression is immediately essential for tooth initiation, we explored odontogenesis in the Lissamphibia. This

TABLE 2. Developmental series and *in situ*
hybridization sample sizes

NF stage	# Tadpole jaws sampled	% With teeth	Subset of jaws examined for *Shh* expression
55	11	0	0
56	42	40	5 toothless 3 toothed
57	17	100	7
58	5	100	3

lineage offers an interesting data point for models of odontogenesis because it diverged from Amniotes ~360 Ma and its members (salamanders, frogs, and caecilians) have adapted quite differently since their common Tetrapod ancestor with respect to their biphasic life histories and feeding requirements.

Frogs provide a particularly interesting case for testing the *Shh*CMTI because they do not develop teeth until the beginning of, or well into, metamorphosis. Their free-feeding tadpole forms a mouth well before odontogenesis in contrast to mice, for example, for whom odontogenesis and craniofacial development are temporally intertwined. Additionally, frogs lack the dramatic facial prominence outgrowth and reshaping characteristic of amniotes during oral cavity development (Fig. 1b, Kennedy and Dickinson, 2012). Studying frog tooth development, then, provides an opportunity to investigate what elements of tooth development are coupled to mouth development and which are not. The natural experiment in uncoupling the developmental timing for these phenotypes adds key comparative data for the evolution of odontogenesis across vertebrates. Based on the *Shh*CMTI, if *Shh* expression is conserved across all vertebrates, in frogs it should be visible broadly prior to tooth initiation, then condense into foci or a dental lamina and mark the oral epithelium through bell stage, when ameloblasts begin to differentiate.

MATERIALS AND METHODS

The S. tropicalis model. *S. tropicalis* is a genetically tractable representative of the phylogenetically basal Pipidae family within frogs. It is ecologically and morphologically conservative with respect to its closest relatives, members of the genera *Xenopus* and *Silurana*, which diverged from each other 57–76 Ma (Bewick et al., 2012) and have since diversified by allopolyploid speciation (Evans, 2008). Their morphological conservation allows for developmental comparison with the extensively studied *Xenopus laevis* and with the Nieuwkoop and Faber (1967) staging table, but future functional studies in the group will be facilitated by the diploid genome of *S. tropicalis*. In comparison with larval forms of less basal frogs, *S. tropicalis* has a simplified tadpole form allowing for a more direct investigation of odontogenesis, but due to their specialization for a fully aquatic lifestyle, additional frog taxa will need to be examined to determine the overall "frog" strategy for tooth development, if one exists.

Tadpole husbandry. Clutches were either F2 offspring of an outcross between inbred Nigerian and Golden strains from the R. Harland lab colony on the UC Berkeley campus or derived from wild type Nigerian breeding pairs (Nasco). Tadpoles were reared in compliance with MAUP #R325-1010 at 23°C, on 12-hr light and dark cycles, daily food and water changes, and densities of approximately 30 tadpoles per 3-L tank after 1 month of growth. Developmental series from Nieuwkoop and Faber (NF) stages 55–59 were sampled in each of three clutches, based on limb morphology. Tadpoles were sacrificed by immersion in 0.05% Benzocaine (Sigma), eviscerated, fixed in 4% paraformaldehyde or MEMFA at 4°C for 1–2 days, and stored at −20°C in 100% methanol.

Probe preparation. *xtShh* cDNA template was amplified from Xenopus Gene Collection library clone TNeu023n04 (Genbank accession #AL639263), a gift from R. Harland. *In vitro* reverse transcription with digoxigenin-labeled nucleotide mix (Roche) produced antisense and sense probes for *in situ* hybridization.

Whole-mount in situ hybridization. *Shh* mRNA detection was performed on *S. tropicalis* tadpole upper jaws and slices of posterior trunk spinal column dissected in cold 100% methanol. The whole mount procedure was designed after Sive et al. (2000) with modifications from Cleves et al. 2014. Additions to the protocol include 20-min Thisse Bleach treatment following rehydration, 50 mg/mL proteinase K treatment for 25 min, and an additional hybridization day. See Table 2 for sample sizes. Digoxigenin-labeled probe was detected with NBT/BCIP (Roche, Sigma). Tissues were mounted between slides in glycerol and photographed in bright field on a Zeiss Stemi dissecting microscope or in transmitted light on a Zeiss Axiophot compound microscope.

RESULTS
The earliest developing teeth in *S. tropicalis*

While specimens were studied beginning at NF stage 55, the earliest morphological signs of tooth development were not visible until NF stage 56, when 40% of individuals (17/42) had at least one developing tooth (Table 2). No specimen has been seen to have teeth prior to NF stage 56, and all specimens had at least one tooth by NF stage 57. The first teeth to form are very close to the oral surface, and it is difficult to find histological evidence for a dental lamina joining the earliest set of tooth positions laterally (Grieco, unpublished obs.). This situation is similar to what is observed in crocodylians (Wu et al., 2013), except that in *S. tropicalis* the first teeth invaginate into the oral mesenchyme whereas the superficial teeth of crocodylians evaginate into the oral cavity (Westergaard and Ferguson, 1990; Harris et al., 2006; Tokita et al., 2013; Weeks et al., 2013).

Shh Expression During the Time of Tooth Development

Our experiments do not detect *Shh* in tissues that immediately precede the histological manifestation of teeth. Whole mount *in situ* hybridization on dissected tadpole upper jaws did not detect *Shh* transcripts in the oral cavity prior to tooth formation in NF stage 56 individuals (Fig. 2a). Specimens with only a few teeth developed them laterally, and while these
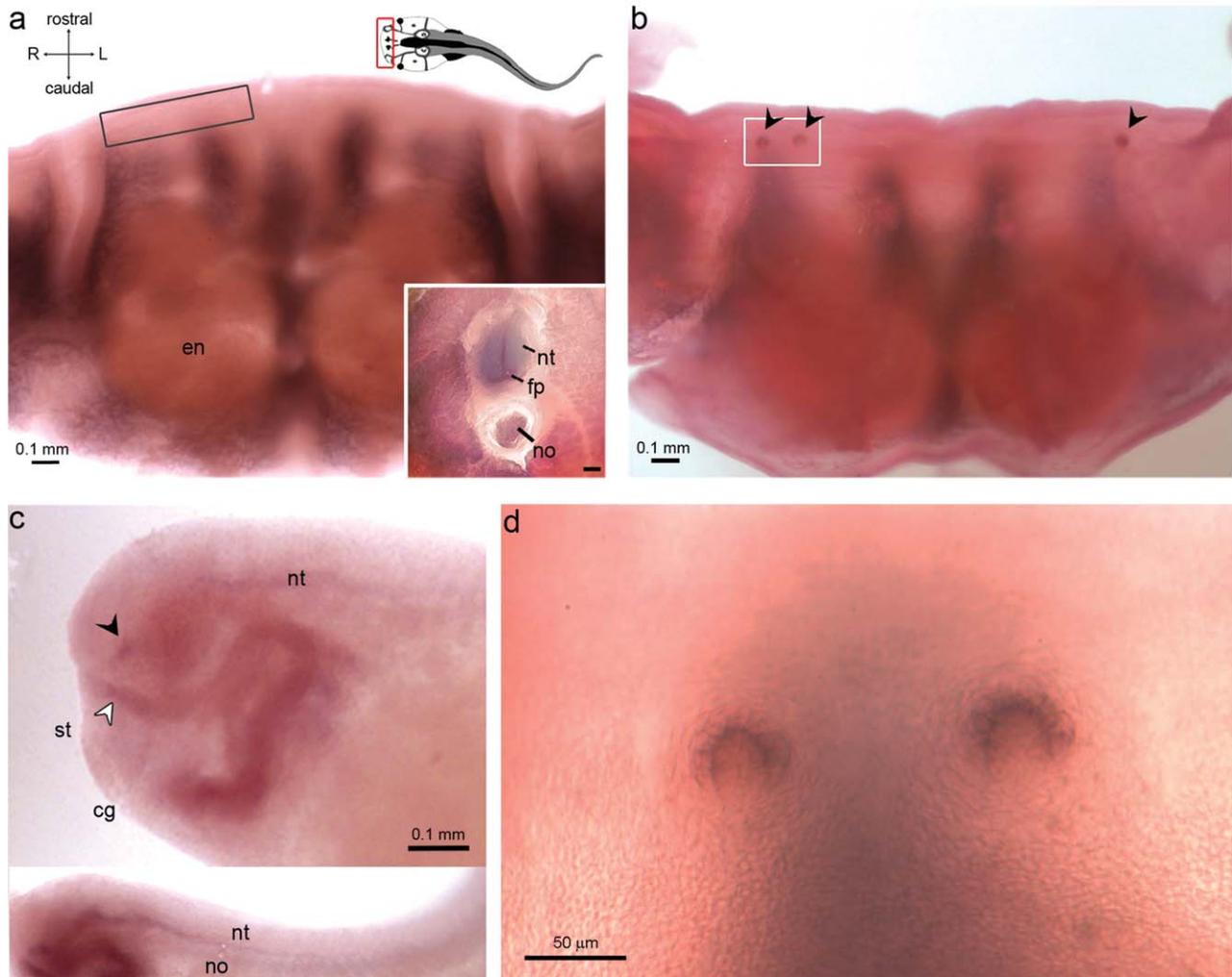
Fig. 2. Whole mount *in situ* hybridization for *Shh* in *Silurana (Xenopus) tropicalis*. (**a**) Non-toothed and (**b**) 3-toothed upper jaw of NF stage 56 tadpoles. Ventral view. (a) No *Shh* expression is detected above background levels in the oral area prior to tooth formation. Inset: red box indicates tissue dissected for *in situ* hybridization. Inset: cross section through a trunk fragment of an NF stage 56 tadpole with *Shh* expression in the notochord. (b) Three developing tooth germs (arrowheads) express *Shh* transcripts with no gene expression detected in the intervening marginal jaw areas. White box marks area magnified in (**d**). (**c**) NF stage 35 *S. tropicalis* early tadpole, prior to rupture of the buccopharyngeal membrane. Prechordal plate (black arrowhead) and foregut (white arrowhead) expression of *Shh* is detected. (d) Magnified view of two teeth in (b). *Shh* transcripts can be detected in the inner dental epithelium of cap stage tooth germs. This is the earliest odontogenic stage for which *Shh* transcripts have been detected. Abbreviations: cg, cement gland; en, external naris; L, left; no, notochord; nt, neural tube; R, right; st, stomodeum.

individual tooth positions expressed *Shh* (Fig. 2b), there was no broad field or ribbon of expression detected along the edge of the jaw marking where teeth would develop as would be expected for an OB (e.g., Fraser et al., 2004).

No foci of *Shh* are detectable anywhere in the upper jaw when teeth are initiating, even adjacent to developing teeth where the next ones would be predicted to form (Fig. 2b, anatomical left). This is counter to what would be expected for a more teleost-like pattern of tooth initiation, in which many species lack a laterally continuous dental lamina that could express *Shh* (Donoghue and Aldridge, 2001; Sire et al., 2002), but those teleost species that have been examined for early tooth development reduce *Shh* expression to individual foci, or tooth placodes, on the oral surface preceding morphogenesis of the first tooth generation (Fraser et al., 2004, 2008; Stock et al., 2006).

A negative experimental result is not necessarily evidence of absence. While it is impossible to entirely rule out methodological reasons for our lack of detection of *Shh* preceding tooth initiation in frogs, we did extensive tests in an attempt to explain our results as a technical issue. Neurula stage embryos and thick sections through the trunk spinal cord and notochord showed the expected *Shh* expression in all experiments conducted, indicating a technically successful *in situ* protocol and probe specificity. *Shh* sense probe controls showed no signal in either the embryonic or larval spinal cord or notochord, or in stage 56 tadpole upper jaws. *Shh* in
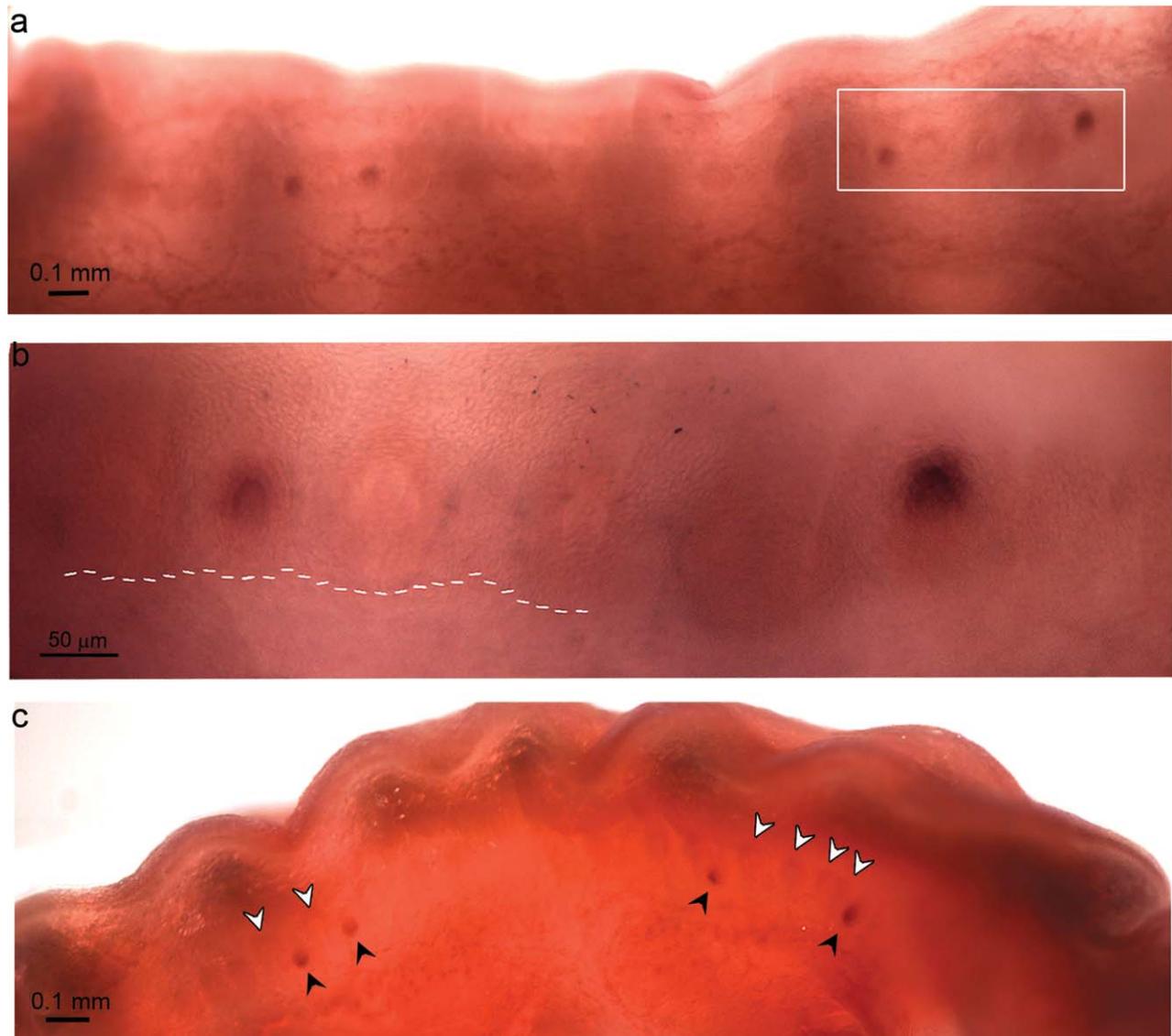
Fig. 3. Whole mount *in situ* hybridization for *Shh* showing tooth expression. (**a**) A full tooth row of a NF stage 58 tadpole consisting of mostly secretory stage tooth germs. Four germs express *Shh* transcripts and are unevenly positioned across the jaw. White box marks area magnified in (**b**). (b) Magnified view of tooth row boxed in (a). The two tooth germs where *Shh* signal is detected are at cap stage in morphogenesis, whereas the rest of the tooth row is made up of secretory stage germs. The *Shh*-expressing germs sit in a different, more ventral focal plane from the rest of the tooth row. Dashed white line marks a laterally continuous dental lamina within the image focal plane. (**c**) Oblique ventral view of the same specimen uncoverslipped. *Shh*-expressing teeth are ventro-lingually positioned relative to other germs in the tooth row.

tadpole upper jaws was detected in 3/8 hybridization experiments. This detection across independent experiments, along with expression patterns in the developing tooth consistent with those of other vertebrates, supports the interpretation of a true biological signal represented by at least one *S. tropicalis* specimen at NF stages 56, 57, and 58. Another possibility is that an OB was present at an earlier tadpole stage than those included in the current study, but the high variation in tooth number in NF stage 56 individuals sampled, including the large proportion (60%) that do not yet have teeth, suggests that if an OB were detectable just prior to tooth initiation this study would have captured

it. In the earliest tadpole stages, *Shh* transcripts are detected with our adapted protocol in the foregut endoderm and prechordal plate (Fig. 2c), expression domains known to play a role in craniofacial development prior to stomodeum rupture in *Xenopus laevis* (Li et al., 1997; Kazanskaya et al., 2000; Dickinson and Sive, 2006).

The earliest odontogenic stage at which *Shh* transcripts have been detected in *S. tropicalis* is the cap stage in NF stage 56 individuals. *Shh* is expressed in the inner dental epithelium (IDE) of tooth germs (Fig. 2d). The strong chromogenic signals widely distributed across the jaw in the form of IDE expression argue against technical issues preventing OB detection, as the

OB is a more anatomically superficial phenotype than an IDE is. In older tadpoles that have initiated most of a tooth row (NF stage 58), the newest tooth germs consistently express *Shh* in the IDE at cap stage, while the secretory stage germs do not have detectable levels of *Shh* transcripts (Figs. 3a–c).

When the tooth row is more fully established, the cap stage, *Shh*-expressing tooth germs sit more ventrally, in a more superficial location relative to the secretory-stage germs making up the rest of the tooth row (Fig. 3c). This is similar to the condition observed in squamates (Richman and Handrigan, 2011), mammals (Järvinen et al., 2009), and other lissamphibians (Davit-Béal et al., 2007) for replacement teeth, which are initiated lingually to functional teeth. In these older tadpoles, a laterally continuous dental lamina is visible in the maxilla and may be connecting germs of different ages (Fig. 3b). *Shh* expression is not detected in this dental lamina at NF stage 58. With the patterns of *Shh* expression examined in *S. tropicalis* to date, there is no evidence for an oral epithelium domain of *Shh* adjacent to developing tooth germs like that seen in reptiles and teleosts (Handrigan and Richman, 2010; Wu et al., 2013; Fraser et al., 2013).

DISCUSSION

As a test for the *Shh*CMTI, we investigated *Shh* expression in *S. tropicalis*, a member of a phylogenetic group that is underrepresented in developmental genetic studies of odontogenesis. As a frog with a biphasic life cycle, it is also interesting developmentally in that it has performed a natural experiment by delaying odontogenesis until well after primary mouth formation, temporally decoupling mouth formation and tooth formation.

We find no evidence of *Shh* expression in early odontogenesis of *S. tropicalis*. While this could be interpreted as a technical issue, our repeated tests and controls suggest strongly that our absence of evidence may be evidence of absence. While initially this result appeared in sharp contrast to expectations from the *Shh*CMTI, a detailed review of the published literature shows that the reduction in *Shh* expression patterns in *S. tropicalis* is not actually outside the range of variation across vertebrates, especially during initiation stages, when phenotypic and ontogenetic covariation is examined in more detail.

While neural crest is competent for odontogenesis during the very earliest tadpole stages in frogs (Wagner, 1955), it is unclear whether epithelial competence for odontogenesis is established early on and maintained until metamorphosis or whether there is a delay in establishing competence until larval stages. The apparent lack of an OB from NF stage 56 in *S. tropicalis* implies that *Shh* is not acting as an odontogenic competence signal or marker during perimetamorphic stages of development. While *Shh* expression is limited or nonexistent in early tooth development, our finding of later tooth *Shh* expression in *S. tropicalis* argues that *Shh* was not dispensed with entirely during tooth morphogenesis in frogs or in pipids.

In light of these results, we investigated the temporal context of the OB in an effort to better understand possible confounding factors. We hypothesize that the *Shh* expression that currently defines the OB may be better understood in terms of its relationships to primary and secondary mouth development in vertebrates.

Shh Expression in Primary and Secondary Mouth Formation

Development of the secondary mouth (morphogenetic modules involving neural crest cells that contribute to teeth and jaws) is superimposed upon and potentially directed by the patterning and development of the primary mouth, or stomodeum (Dickinson and Sive, 2006; reviewed in Soukup et al., 2013). The stomodeum is the endoderm–ectoderm boundary within the developing oral cavity (Helms et al., 1997; Dickinson and Sive, 2006; Rothova et al., 2012), and later becomes the pharyngeal opening (Dickinson and Sive, 2007). It has been suggested that the rupture of the buccopharyngeal membrane initiates secondary mouth development (Soukup et al., 2013). In some anamniotes, the stomodeum has been implicated in tooth induction (bichir, Kralovic et al., 2010; salamanders, Lumsden, 1988; caecilians, Wake, 1976).

In zebrafish, *pitx2*, *fgf8*, and s*hha* in the stomodeum are all at least indirectly activated by a Shh signal from the ventral brain (Eberhart et al., 2006). Later, *shha* is expressed in a band at the roof of the mouth and in a domain just lingual to a *pitx2* oral band (Stock et al., 2006; Jackman et al., 2010). *Pitx2*, another marker of the OB, is implicated in marking the stomodeum from the earliest stages (Lanctôt et al., 1997; Schweickert et al., 2001; Dickinson and Sive, 2007), and continues expression into the epithelial organ derivatives at least in the mouse (Lanctôt et al., 1997; Mucchielli et al., 1997; St. Amand et al., 2000). These data provide evidence of continuity between stomodeal gene expression and the eventual location of the OB. The role of the stomodeum in amniote tooth formation has not been tested as in amphibians, but the timing of tooth development in mice does not rule it out as a factor (Mina and Kollar, 1987; Lumsden, 1988). By examining an animal where stomodeum rupture and tooth developmental processes are quite disjunct, our work in *S. tropicalis* continues this line of investigation, but the consequences of heterochronic shifts between the events of stomodeum rupture, secondary mouth formation, and tooth formation in different vertebrate lineages should be further explored if the role of the OB and triggers for tooth formation are to be clarified.

Secondary mouth formation also involves *Shh* and may confound definition of the OB. In the zebrafish stomodeum *Shh* expression is required for neural crest survival and directs upper jaw cartilage condensation (Eberhart et al., 2006), and *Shh* expression in the prechordal endomesoderm is necessary for proper midline craniofacial patterning in *Xenopus* (Li et al., 1997; Kazanskaya et al., 2000). All amniotes examined thus far express *Shh* expressed in the frontonasal ectodermal zone (FEZ), a signaling center located in the roof of the mouth at the equivalent of mouse E10/chick HH 20, and then in stripe domains along the maxillary processes (Hu and Helms, 1999; Marcucio et al., 2011). This Shh signaling plays a role in facial prominence outgrowth and in determining facial width from the midline (Young et al., 2010), as well as in species-specific outgrowth of facial prominences to create facial morphology (Liu et al., 2010; Young et al., 2014).

Helms et al. (1997) observed a common thread among the dynamic *Shh* expression domains in amniote craniofacial primordia: that they were found at the locations of endodermal–ectodermal epithelial boundaries in the face
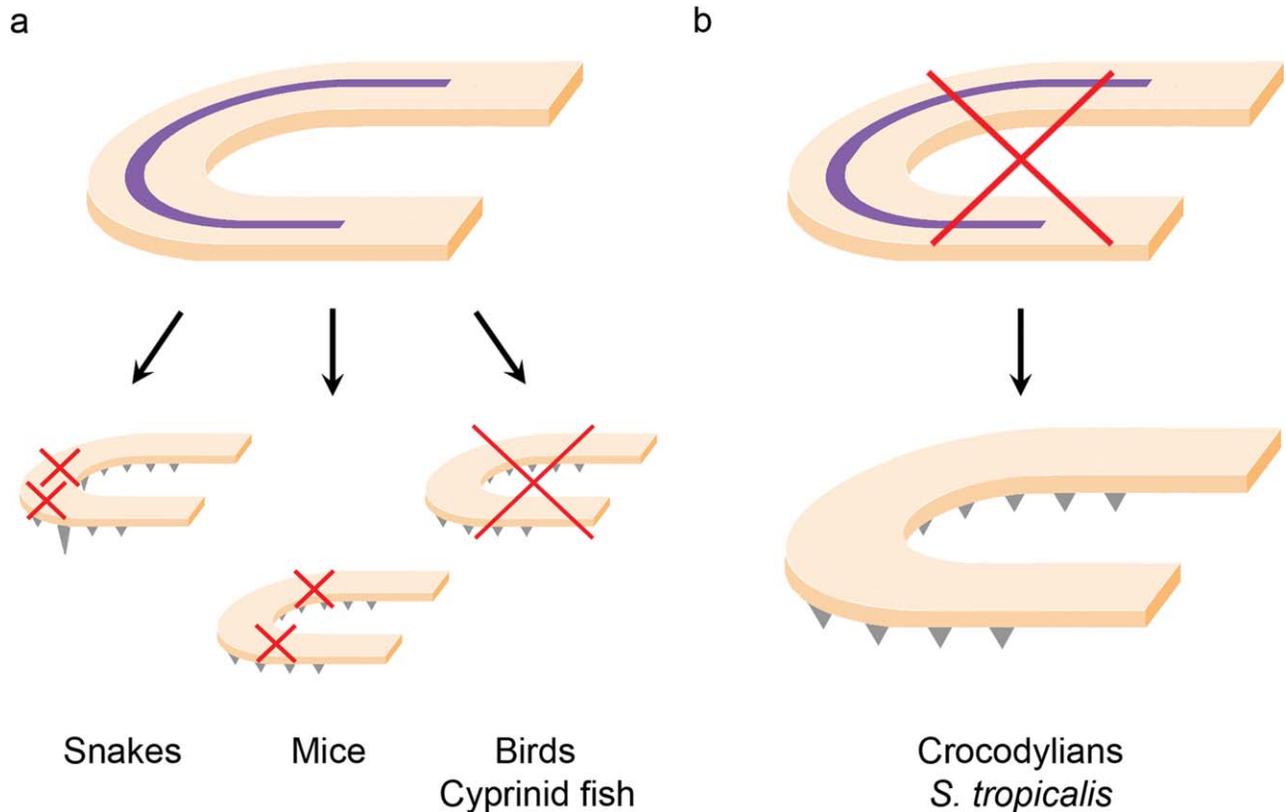
Fig. 4. Scenarios in which the OB is not sufficient or necessary for fully formed teeth. Developmental transformations in time of an upper jaw with a *Shh*-expressing OB (purple arc) into a tooth-bearing upper jaw. Evolutionary "experiments" in which the OB is not sufficient to form teeth occur in (**a**) when at least one portion of the jaw expresses an OB but is ultimately toothless, such as in snakes, mice, birds, and cyprinid fish. Evolutionary "experiments" in which the OB is not necessary to form teeth occur in (**b**) when teeth form in the absence of an OB, as in crocodylians and likely in *S. tropicalis*.

and in more posterior pharyngeal arches. Interestingly, after reviewing extensive tooth embryological literature, Huysseune et al. (2009) recently re-asserted the hypothesis that teeth are able to form only in areas where endoderm and ectoderm have had direct contact during development. Although *Shh* is not invoked by Huysseune et al. as a requirement for tooth development, perhaps this embryological connection provides an alternative phenotype to explore in regards to the function and phenotypic definition of the OB.

Re-evaluating the Vertebrate Odontogenic Band

Across the range of developmental variation seen in vertebrates, there are several taxa that do not fit the *Shh* Consensus Model for Tooth Initiation: in *S. tropicalis* and in crocodylians, teeth are able to form in the absence of an OB, and in snakes, mice, and birds, an OB is present that does not lead to fully formed teeth (Fig. 4). Based on the lack of covariation between the OB and tooth row phenotypes in several groups of vertebrates and a review of the functional genetic evidence, the OB *Shh* domain may regionalize the jaw for tooth development (or correlate to a gene that does) but it does not determine individual tooth positions. This insight into the function of the OB is consistent with embryological and functional data showing early epithelial direction for odontogenesis but that the mesenchyme must take over to fully form teeth (e.g., Mina and Kollar, 1987; Lumsden, 1988; Chen et al., 1996). Given its anatomical and temporal context, it may be that a *Shh*-expressing OB is a readout of stomodeum rupture, of secondary mouth development, or a marker of endoderm/ectoderm border areas (Helms et al., 1997; Huysseune et al., 2009). The retention of the OB phenotype across distantly related snakes, mice, and birds with convergent edentulous regions suggests that the OB is coupled to some function during development. Further research is required to determine whether the OB has a function in tooth development, is a by-product of these other anatomical phenotypes or of a different functional expression domain, or is a neutrally evolving phenotype. The variability of the OB phenotype with respect to tooth and craniofacial development across vertebrates also raises the question of what features are homologous and which may be modified in particular lineages.

CONCLUSIONS

The re-evaluation of the OB in the *Shh* Consensus Model for Tooth Initiation presented here is a good example of the role that evolutionary biology and a

comparative approach can play in developmental genetics and vice versa. It is these departures from conservation that indicate evolution: that something in the developmental system or the variation available has changed. As an increasing range of vertebrates are added to our understanding of odontogenesis, the OB as currently defined by *Shh* expression does not accommodate patterns of heterochrony and toothlessness across vertebrates. The temporally preceding and following steps in oral development show a fair amount of variation across species. We explored *Shh* expression in *S. tropicalis*, enabling us to explore tooth formation that was temporally delayed from mouth formation. Our results support the hypothesis that the *Shh* expression inferred as causally preceding tooth initiation may actually be related to the formation of the primary or secondary mouth. Perhaps the OB is better defined by the expression of additional genes, such as *Pitx2* or *Sox2* (Keränen et al., 1999; Stock et al., 2006; Fraser et al., 2008; Juuri et al., 2013). Alternatively, some other developmental process (e.g., a marker of endoderm–ectoderm boundaries) is a more precise determinant of the variation in vertebrate dentitions.

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