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The Developmental Basis of Variation in Tooth and Jaw Patterning: Evolved Differences in the Silurana (Xenopus) tropicalis Dentition

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The Developmental Basis of Variation in Tooth and Jaw Patterning: Evolved Differences in the *Silurana* (*Xenopus*) *tropicalis* Dentition

By

Theresa Marie Grieco

A dissertation submitted in partial satisfaction of the

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in the

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University of California, Berkeley

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Abstract

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Perhaps the most evident conversion of genomic information into functional, morphological phenotypes in an animal occurs during organogenesis, and the study of vertebrate tooth development provides a phenotypically diverse system for which the mechanisms for patterning and morphogenesis have been extensively studied. An understanding of the developmental basis for evolved differences between teeth in different anatomical and phylogenetic contexts brings complementary information to our knowledge of odontogenic mechanisms. Examining difference, or variation, allows for the validation of hypothesized developmental mechanisms, identification of mechanistic flexibility that could be available to evolution or bioengineering, and the redefinition of phenotypes to better align with the natural biological variation available.

This dissertation examines the development of the dentition in the frog and emerging developmental model *Silurana* (*Xenopus*) *tropicalis*, including the first gene expression data for odontogenesis in any amphibian. Comparative data for the evolution of dental phenotypes are assembled from descriptions of tooth initiation, dentition patterning, and adult craniodental variation phenotypes, addressing developmental questions at population, subfamily, and phylum levels.

Using hematoxylin and eosin-stained histological sections and whole mount preparations of larval *S. tropicalis* jaws, I demonstrate that individual tooth initiation is broadly similar to that documented for phylogenetic relative *Xenopus laevis*, but that the process is temporally shifted relative to external developmental traits in the Nieuwkoop and Faber staging system. Furthermore, patterns of tooth initiation in *S. tropicalis* reveal a lack of synchrony in alternating tooth positions and dynamics that were previously undetected. The frequent presence of 'twinned' tooth germs in whole mount preparations argues against a robust model of local inhibition directing tooth initiation in this species. These findings rule out two hypothesized developmental mechanisms for tooth initiation in *S. tropicalis* that were derived from data in *X. laevis* and other homodont vertebrates.

In another investigation of first generation tooth development, I examine the expression of *Sonic hedgehog* (*Shh*), a marker for several phases of odontogenesis across vertebrates. I demonstrate

the utility of comparing ‘natural experiments’ in development with what is known from more anatomically conservative developmental models. In particular, I use the fact that *S. tropicalis* teeth do not initiate until just before metamorphosis as a case where tooth formation and mouth formation developmental programs are dissociated from one another to evaluate the current consensus odontogenic model for *Shh*. With *in situ* hybridization data from *S. tropicalis*, I fail to detect a *Shh*-expressing odontogenic band prior to tooth formation, counter to predictions from the consensus model. A review of published functional data and the correspondence between an odontogenic band domain and the presence of functional teeth in other vertebrates reveal several other taxa for which the consensus model appears insufficient to account for variation in the distribution of the marginal dentition.

Finally, I explore the ability to infer developmental processes from patterns of adult craniodental variation in three *S. tropicalis* genetic strains raised in captivity. Osteological measurements and tooth counts are analyzed for patterns of covariation at the functional phenotypic levels of the cranium, the jaw, and the tooth-bearing bone. I demonstrate that the bimodal sexual body length dimorphism does not carry over to any cranial metric trait measured; systemic factors affecting cranial length can explain much of the difference between male and female traits, which are distributed unimodally. Patterns of covariation with cranial size, size-adjusted patterns of pairwise phenotypic correlation, and significant differences between genetic strains all suggest a relative independence of variation in the premaxilla and maxilla in *S. tropicalis*, and I document evidence for a functional jaw module, in which the tooth row and jaw bones correlate when summed across the jaw, but in which tooth and jaw phenotypes lack integration at the level of individual tooth-bearing bones.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

For my parents, Paul and Julia,
for their love, encouragement, and curiosity.

And in loving memory of my grandfather,
John S. Grieco (1932 – 2013).

All members of monophyletic taxa share a common ancestor whose development pathway has been modified to produce descendant morphologies.

- David Lindberg, Heterochrony in Gastropods, 1988

Teeth happen.

- Paul Grieco

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INTRODUCTION

Organogenesis is perhaps the most overt demonstration of the conversion of genomic instructions into functional phenotypes that occurs during organismal development. Constructing an organ such as a limb, heart, or tooth from a specified group of cells requires patterning and morphogenesis, but these steps are not always distinct, and the same gene or set of genes often acts multiple times in a string of temporally contiguous phenotypes that lead to the final functional organ. We now know that it is possible to reuse the same signaling and regulatory genes in different spatiotemporal contexts due to differential and combinatorial cis-regulation of gene expression (Davidson 2006). From the perspectives of evolutionary change and the etiology of birth defects, understanding how temporally contiguous phenotypes that form an organ actually covary due to genetic architecture/common regulation (Schlosser & Wagner 2004, Stern 2000, Wagner 2001) helps us to understand biological cause and effect, and to identify the relevant action points for clinical intervention.

Because of its ability to create organismal form, organogenesis is also a system through which to understand the evolution and development of difference. Although the past 25 years of research in molecular genetics has shown us that gene expression patterns and developmental mechanisms are conserved among even the most divergent lineages, it is also plain that there is and has been a diversity of form in the animal tree of life. Animals have inherited common developmental mechanisms that must have been modified over the course of evolution in order to create the observable morphological diversity, the genetic and developmental basis for which has, in large part, yet to be identified. What follows from this are two principles: there is variation within developmental processes that is available to selection (e.g. Garstang 1922, Goldschmidt 1938, Haldane 1932, Huxley 1932, Gould 1988), and developmental mechanisms are channels through which genetic variants must be compatible in order for evolution to occur (e.g. Waddington 1957, Alberch 1982, Langille & Hall 1989). These principles are not new in evolutionary developmental biology, but the research and technology base has matured such that approaches to identifying and studying these natural variations are possible.

Identifying specific phenotype-genotype correlations has been accomplished through quantitative genetics, examining units of inheritance, and through developmental genetics, examining units of function. Identifying quantitative trait loci (QTL) responsible for phenotypic variants (e.g. Dunn et al. 2011, Klingenberg et al. 2000, Protas et al. 2008, Schoenebeck et al. 2012) and comparing patterns of correlation in genotype and phenotype matrices (e.g. Grieco et al. 2013, Hlusko & Mahaney 2009, Marroig & Cheverud 2001, Polly 2005) are complementary methods, with the former being a bottom-up approach and the latter a top-down approach to assess trait inheritance. Since the rise of molecular biology, however, the vast majority of developmental genetics studies has taken a bottom-up approach to identifying the functions of individual DNA sequences (Charles et al. 2011, Moskowitz et al. 2004, Garrity et al. 2002). Systems biology and transcriptomics have brought a more statistical approach to developmental genetics, and researchers are increasingly revisiting top-down approaches to gene function during organogenesis (Sharpe 2011, Young et al. 2010).

While in quantitative genetics it is not assumed that genetic architectures will carry across species, in developmental genetics a conserved developmental architecture is considered most parsimonious. Due to the difficulties of isolating functional signals in many but the most standard model organisms, much of the field has relied on the broad conservation of genes and developmental mechanisms across disparate taxa in order to understand genotype-phenotype relationships from a molecular perspective (e.g. Alexander et al. 2009, Juuri et al. 2013). In order to reconcile our understanding of gene function with a knowledge of the evolutionary change that has indeed occurred in these genes across these taxa, comparative analysis across developmental models is required (Woltering & Duboule 2010), as well as the use of variation to identify "integrated characters," those developmental and morphological phenotypes that covary spatially and/or temporally (Olson & Miller 1958). Such approaches are also essential to bring the information from developmental genetics more completely into our understanding of microevolution and macroevolution, and whether patterns of phenotypic variation among species can inform us about developmental and evolutionary mechanisms that work at population and individual levels (Wake et al. 2011).

A conserved developmental mechanism employed in different contexts

Vertebrate odontogenesis is a good model for taking both comparative and variational approaches to organogenesis. Much is known about the genetic basis of oral tooth development due to heavy study, particularly in the mouse. The system is characterized by iterative use of signaling pathways in patterning and morphogenesis (Jernvall & Thesleff 2000, 2012, Fraser et al. 2008) superimposed upon regional patterning instructions in the pharyngeal arch mesenchyme (anterior-posterior: Minoux & Rijli 2010, Graham 2008, Trainor & Krumlauf 2001, dorso-ventral: Depew et al. 2005, Medeiros & Crump 2012, oral-aboral: Tucker et al. 1999), and the frontonasal mass mesenchyme in the oral dentition of osteichthyans and tetrapods (Compagnucci et al. 2013). Serial homology within the dentition means that there are multiple instances within the same jaw to evaluate the function of odontogenic developmental mechanisms. Due to the iterative use of signaling pathways, *Sonic hedgehog* (*Shh*) mRNA expression can be used as a marker for most of the odontogenic time period across the vertebrates studied to date.

From an evolutionary perspective, odontogenesis is also labile so there are many natural phenotypic variants to work with for a deeper understanding of organogenesis. While individual teeth all possess the same basic construction of a mineralized structure formed from a single papilla and attached to the bone of the oral region (Ørvig 1967, 1977), dentitions vary in the number of tooth rows, the number, size, shape, and spacing of teeth. Because the dentition is a primary way of interacting with an organism's environment, selection for energetic constraints or sexual dimorphism can influence the form of the dentition based on the different feeding and behavioral functions it serves. Unlike the phenotypes produced in most experimental genetic manipulations, these natural modifications to a conserved developmental mechanism are viable and inherited because they exist in successful lineages of animals. The ability to form oral teeth has also been lost in several vertebrate lineages (reviewed in Davit-Béal et al. 2009).

Odontogenesis in the frog *Silurana* (*Xenopus*) *tropicalis*

Lissamphibians are notably understudied in terms of their dentitions; they are the only vertebrate class for which we have no developmental genetic data, although there are a number of descriptive and experimental studies of tooth morphological transitions at metamorphosis, and of tooth replacement (e.g. Gillette 1955, Wake 1980, Shaw 1986, Vassilieva 2005, Davit-Béal et al. 2006). The Lissamphibia (salamanders, frogs, and caecilians) diverged from amniotes ~360 Mya (Hedges et al. 2006), and have been evolving with their own natural experiments in dentition variation. Marginal dentitions range from edentulous in toads to up to 8 rows in caecilians (Wake 1980), tooth sexual dimorphism in plethodontid salamanders (e.g. Stewart 1958, Ehmcke & Clemen 2000) and the loss, and re-evolution, of mandibular dentitions in frogs (Wiens 2011).

Within the Lissamphibia, frogs possess a number of derived characteristics relative to other vertebrate groups that make them a key comparative lineage for the understanding of odontogenesis. Their single marginal tooth row is distinct from many of the teleosts' dentitions for which we have tooth development data, and their homodonty is genetically diverged from that of homodont squamates. Data from both of these homodont groups will help us understand the ancestral condition for the evolution of mammalian heterodont dentitions. In addition, several anuran families have lost teeth, including rhinophrynids, bufonids, brachycephalids, rhinodermatids, and most microhylids (Duellman & Trueb 1986). For those lineages that do possess teeth, having a prolonged, feeding, toothless larval stage means that unlike in other vertebrates, mouth development is decoupled from tooth development in frogs. In addition, the suprarostral and infralabial cartilages, which form the upper and lower "jaws" of tadpoles, are evolutionarily novel relative to anterior craniofacial development in other vertebrates (Schmidt et al. 2011, Svensson & Haas 2005) and provide a novel anatomical context in which teeth are developing.

This dissertation is an investigation of odontogenesis in the emerging developmental model *Silurana* (*Xenopus*) *tropicalis*, a pipid frog species that allows me to connect phenotype to mechanism, as well as provide key information for the evolution of odontogenesis in vertebrates. Because of the growing community of researchers using *S. tropicalis* for developmental genetics and embryology, there are established laboratory methods for embryonic and early larval development that could be extended into the later larval stage, and its recently sequenced, diploid genome (Hellsten et al. 2010) makes gene expression much easier to interpret and, in future studies, to manipulate through transgenesis.

Studying *S. tropicalis* allows for an immediate comparison with another pipid developmental model, *Xenopus laevis*, and together these provide a reference for subsequent comparison to other frog lineages. *S. tropicalis* and *X. laevis* are members of the subfamily Xenopodinae (Anura: Pipidae, Evans et al. 2004), and they diverged 57-76 Mya (Bewick et al. 2012). The Xenopodinae are ecologically and morphologically conservative, and have diversified primarily by allopolyploid speciation, resulting in an array of species with varying ploidy levels (Evans et al. 2008). Of these, *S. tropicalis* is the only diploid, and it has a differing basal chromosome number than *X. laevis* and its closer relatives (Figure 1). Although often presented in developmental genetics literature as *Xenopus tropicalis*, I will follow the convention of Cannatella and De Sá (1993) in applying the genus name *Silurana* on the basis of these fundamental genetic differences, although it was originally resurrected based on morphological

synapomorphies grouping pipids as (*Xenopus* (*Silurana* + *Hymenochirus*)) (Cannatella & Trueb 1988), a topology that is not supported by the genetic trees that have been published to date.

Although often viewed as interchangeable because of their similar early developmental biology and adult morphology, there are genetic and life history differences between the two models (Table 1). While much of the vast embryological literature on *Xenopus laevis* has been found applicable to studies in *S. tropicalis* (Khokha et al. 2002), some investigators have begun to compare the two models on a genomic level, using similarity to infer genes and regions most critical for developmental function (Yanai et al. 2011, Beer et al., 2012). Any phenotypic differences occurring between the models are also interesting from an evolutionary perspective, providing a way to empirically investigate the kinds of changes that occur on a subfamily level along with the ability to find their genetic basis, and whether these evolved differences can be associated with size or ploidy changes, with developmental plasticity, or with phenogenetic drift (Weiss & Fullerton 2000).

An understanding of odontogenesis in *S. tropicalis*, while yielding new insights into the flexibility and function of odontogenic mechanisms, is unlikely to be representative of a generalized frog strategy for tooth development. Within anurans, pipids have several derived oral morphologies associated with their secondarily aquatic lifestyle (Cannatella & De Sá 1993). They lack a tongue and adults feed in a manner very different from most other frogs (Carreño & Nishikawa 2010), and it has been questioned whether teeth even participate (Shaw 1979). *S. tropicalis* and other pipids also have simple conical teeth that lack the bony bases, or pedicels, common to most amphibian teeth (Parsons & Williams 1962, Katow 1979, Smirnov & Vasil'eva 1995).

As larvae, *S. tropicalis* and other pipids lack the keratinous beaks, denticles, and fleshy extraoral disc that are present in many other tadpoles (Altig 2006). The suprarostral and infralabial cartilages novel in frogs (Svensson & Haas 2005) are present but simple in pipids, largely because they are not acting as supports for the extraoral modifications. This relatively simple pipid tadpole (morphotype I, Orton 1957) is no longer thought to represent the primitive tadpole state for anurans (Sokol 1975, Cannatella 1999, Altig 2006), but it does make odontogenesis technically easier to observe. The absence of keratinous structures that must be shed may also be a reason why at least some pipids (*Xenopus laevis*, Shaw 1979) initiate their teeth relatively earlier than most frogs do, prior to metamorphosis (Davit-Béal et al. 2009, Altig & McDiarmid 1999). Despite these unique features, information about the patterning and individual morphogenesis of pipid dentitions, along with knowledge of these processes in other vertebrates will serve as reference points around which to frame hypotheses for tooth development in other frogs.

An integrative approach to *S. tropicalis* odontogenesis

This dissertation uses the dentition to try to understand the behavior of developmental mechanisms on evolutionary timescales, taking a comparative and variational approach to understand how the mechanisms of odontogenesis operate in the context of pipid oral anatomy. In describing tooth initiation, dentition patterning, and adult craniodental variation phenotypes in *S. tropicalis*, this work uses the lens of odontogenesis to provide key comparative data for

questions at multiple levels of phenotypic evolution. In each chapter, I focus on a different hierarchical level of comparison (population, subfamily, phylum), using each as an opportunity to examine the function of different developmental mechanisms and the influences they have on phenotypic variation.

Chapter 1 makes an explicit comparison between *S. tropicalis* and *X. laevis* tooth initiation dynamics, testing the hypothesis that the two developmental models have the same patterns of development despite their relative size, generation time, and ploidy differences. While their similarity has been widely acknowledged early in development, the development closer to the time of metamorphosis may differ in these two taxa. Morphological time series and histological data from *S. tropicalis* are used in conjunction with previous descriptions of *X. laevis* tooth initiation to assess whether initiation patterns are compatible with recent models of local inhibition and reaction-diffusion processes in setting up the dentition.

Chapter 2 begins by exploiting the natural differences between anuran craniofacial anatomy and that of other vertebrates to test our knowledge of the developmental relationships between tooth development and mouth development. Because the primary mouth forms in the earliest larval stages for feeding, but teeth are not initiated until just before or during metamorphosis, *S. tropicalis* and other frogs represent a natural experiment in decoupling tooth and mouth developmental programs. Using the signaling ligand-encoding gene *Shh* as a marker for odontogenesis-related phenotypes, whole mount *in situ* hybridization was performed on perimetamorphic tadpoles and these data are added to a detailed comparative analysis of the odontogenic band among vertebrates. These data, along with the functional data available for *Shh* in vertebrates, are used to discuss whether *Shh* specifies tooth-forming regions and tooth epithelia in *S. tropicalis*, and how expression patterns have evolved in vertebrates.

Chapter 3 takes an inferential approach to developmental processes, asking to what extent population-level variation can reveal developmental and physiological effects on adult craniodental phenotypes. Osteometric measurements and tooth counts are analyzed from a population of skeletonized *S. tropicalis* adults raised in captivity, from 3 different genetic strains. Phenotypic covariation and asymmetry are quantified with respect to sexual size dimorphism and variation in jaw morphology. By examining the dentition in its functional state, and in the context of more systemic effects on development, this chapter describes patterns of morphological integration in *S. tropicalis* and aims to identify potential constraints on the dentition as well as to highlight phenotypes that may be more free to vary in response to evolutionary pressures.

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Figure 1. Phylogeny of the Xenopodinae (Anura: Pipidae). *S. tropicalis* and *X. laevis*, model systems for developmental genetics, are highlighted in green. Phylogeny adapted from Evans (2007, 2008) based on autosomal *RAG1* and *RAG2* genes. Geographic range (from Evans et al. 2004), chromosome number, and species name are noted for each extant lineage in the tree. Reticulation marks hybridization events leading to allopolyploid speciation. †denotes a lineage predicted but not known to be represented by an extant lineage. W = West Africa, C = Central Africa, E = East Africa, S = South Africa.

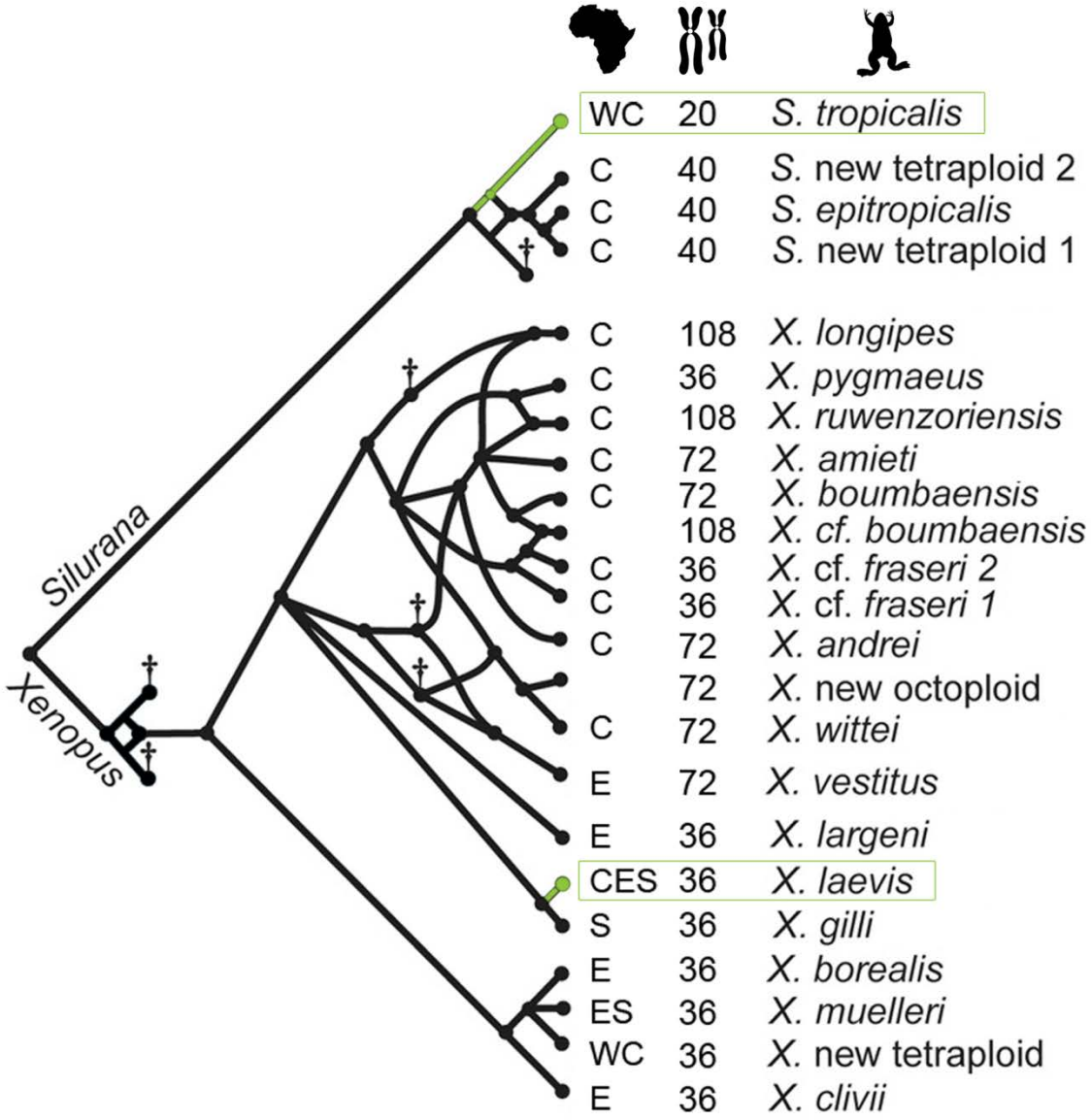

Table 1. *Silurana tropicalis* vs. *Xenopus laevis*. Adapted from http://www.xenbase.org, Hellsten et al. 2010, Evans 2008, Evans 2007, Evans et al. 2004.

	S. tropicalis	***X. laevis***
ploidy	diploid	allotetraploid
N	10 chromosomes	18 chromosomes
genome size	1.7×10^9 bp	3.1×10^9 bp
temperature optima	$25\text{-}30^o$ C	$16\text{-}22^o$ C
adult size	4-5 cm	10 cm
eggs/spawn	1000-3000	300-1000
generation time	4 months	1-2 years
geographic distribution	coastal West Africa	sub-Saharan Africa
preferred habitat	tropical forest	generalist

CHAPTER 1

Tooth initiation in *Silurana (Xenopus) tropicalis* reveals dynamic patterning of the first tooth generation

ABSTRACT

Much has been learned about early embryology and morphogenesis from the frog model systems *Xenopus laevis* and *Silurana (Xenopus) tropicalis*. As the genetic resources for *S. tropicalis* have grown, knowledge from the study of *X. laevis* has been successfully applied to *S. tropicalis* at early developmental stages, but less is known about how these species might differ from each other during later development, leading up to metamorphosis and adult morphologies. To further elaborate developmental comparisons that would provide data between these model species and across vertebrates, I examined odontogenesis in perimetamorphic *S. tropicalis* in histological sections and in whole mount preparations. Description of individual tooth developmental stages, as well as patterns of first generation tooth initiation, test the hypothesis that the same mechanisms of odontogenesis are working in *X. laevis* vs. *S. tropicalis* despite overall size and developmental rate timing differences. While the broad trajectory of tooth initiation stages can be aligned between the models, *S. tropicalis* develops its teeth relatively later and gains a dental lamina in the tadpole stages, and the findings on initiation patterns to date suggest that tooth initiation is a more dynamic process in *S. tropicalis*.

INTRODUCTION

Xenopus laevis and *Silurana (Xenopus) tropicalis* have become formidable as model systems for the understanding of early developmental biology. These African clawed frogs have a long history of study in embryology (reviewed in Grainger 2012, Gurdon & Hopwood 2000), and have made the transition to powerful developmental genetics models (e.g. *Genesis Xenopus* Special Issue 2012) due to the large number of techniques available for functional manipulation (Khokha et al. 2002, Hoppler & Vize 2012, Sive et al. 2000). Through the infrastructure and research communities created around these models, much has been learned about fundamental induction, patterning, and morphogenesis mechanisms (e.g. Harland & Gerhart 1997, Wardle & Sive 2003, Schlosser 2006, Jones 2005) that can be extrapolated to or compared to other vertebrate models. In addition, the recently sequenced genome for *S. tropicalis* (Hellsten et al. 2010), and soon to be completed genome for *X. laevis* (Beer et al. 2012) have facilitated larger scale genetic and genomic studies.

For the processes and unfolding of early development, *S. tropicalis* and *X. laevis* have been viewed as synonymous, albeit over slightly different absolute time scales (Khokha et al. 2002). Even between these closely taxonomically related models, however, some developmental differences have been reported, such as differing ploidy levels (*X. laevis* is allotetraploid, while *S. tropicalis* is diploid, Amaya et al. 1998) and hatching during different developmental stages (Showell & Conlon 2009, Carroll & Hedrick 1974, Yanai et al. 2011). Given the significantly

more rapid generation time in *S. tropicalis* (4-6 months compared to 1-2 years, Amaya et al. 1998), one might expect other differences to manifest as the absolute duration of development is compressed.

For the most part, researchers have yet to evaluate whether the similarities between *X. laevis* and *S. tropicalis* in early development carry over to later developmental phenotypes. Xenbase only covers the biology of these animals up to Nieuwkoop and Faber (NF, 1967) stage 46, which spans approximately 4 days into development (www.xenbase.org/anatomy/alldev.do). Up to this point, the development of most tadpole structures is completed but ossified skeletal and endocrine structures present only in the adult have yet to form. Later development in both species is less well characterized in comparison to early development, but has been described by Nieuwkoop and Faber (1967) and Weisz (1945) for *X. laevis*.

For two frogs that are morphologically similar but separated by 57-76 My of evolution (Bewick et al. 2012), evaluating developmental differences is critical to knowing if these powerful models will be useful for studying the same developmental processes, or whether their evolved differences will open a window into the modification of these processes on a subfamily-level phylogenetic scale. *S. tropicalis* and *X. laevis* are divergent lineages in a clade (the Xenopodinae) which has diversified primarily by allopolyploid speciation, creating at least 17 species with varying numbers of chromosomes (Evans 2008). If there are natural comparisons which are mechanistically tractable in both lineages, *Xenopus* and *Silurana* represent a powerful taxonomic system for the study of heterochrony, sub- and neofunctionalization, parallelism, and developmental constraint.

Mechanistic study of later development has centered around the control of more uniquely anuran phenotypes of metamorphosis and life cycle control (Buchholz et al. 2006, Furlow & Neff 2006, Das et al. 2009). *X. laevis* has been used as a model for intestinal remodeling and tissue regeneration (Ishizuya-Oka & Shi 2005, Shi et al. 2011) as well as craniofacial morphogenesis (Gross & Hanken 2008, Rose 2009, Kerney et al. 2012). The dramatic changes during metamorphosis and ease of exposure of these species to environmental chemicals have made them good indicators in ecotoxicology studies, where the developmental response of *S. tropicalis* and *X. laevis* has begun to be compared (e.g. Fort et al. 2004, Song et al. 2003, Mitsui et al. 2006, Wang et al. 2008).

Later phenotypes with connection to development in other vertebrates have not been evaluated extensively, although investigators have studied limb development in some detail as a model for regeneration (reviewed in Beck et al 2009, Yakushiji et al 2009; Newth 1948, Tschumi 1957, Brown et al. 2005, Foster et al. 2008, Carreño & Smith 2009). Researchers have also collected some data on the patterns and mechanisms of skeletogenesis (e.g. Trueb & Hanken 1992, Miura et al. 2008, Slater et al. 2009) in these models.

To further elaborate developmental comparisons that would provide data between these model species and across vertebrates, I examined odontogenesis in *S. tropicalis*, a phenotype that has been documented by histological studies in *X. laevis* (Shaw 1979, Cambray 1976) and has been preliminarily described in *S. tropicalis* (Bulckaen et al. 2007). Both species have a homodont, single-rowed dentition spanning the maxillae and premaxillae as adults, and which is initiated

during late larval development just prior to metamorphosis. The lower jaw, like that of most anurans, is edentulous.

As in most vertebrates, the morphogenetic stages of tooth formation in *X. laevis* comprise an epithelial thickening, bud, cap, and secretory stage prior to eruption (Shaw 1979). As is typical for the first generation teeth of teleosts and many other vertebrates (Donoghue & Aldridge 2001), the teeth of *X. laevis* arise quite superficially, possibly directly from the oral epithelium at NF stage 55 (Shaw 1979), although Cambray (1976) reports a late-developing dental lamina at the posterior ends of the metamorphosing jaw. He also reports tooth epithelial thickenings as early as NF 53 in *X. laevis*.

Patterns of first generation tooth initiation are far more variable across vertebrates, and less well-studied (alligator, Westergaard & Ferguson 1986, 1987, 1990, Kulesa et al. 1996; caecilians, Wake 1976; *Lacerta vivipara*, Osborn 1971). There are two published hypotheses regarding *X. laevis* tooth initiation: a lateral-to-medial pattern, and an alternating series pattern (Shaw 1979). In the first, individual teeth form in a wave from the lateral margin of the tadpole mouth and converge at the midline. In the second hypothesis, even- and odd-numbered tooth positions develop synchronously but with a delay in morphogenetic stage between the two series. Cambray's observations, which were not published outside of his doctoral dissertation (1976), describe a contrasting model for *X. laevis*, which includes 4 phases of initiation in the first tooth generation, with teeth initiating first in the larval maxillary region, second in the premaxillary region, and then third and fourth initiation stages expanding the dentition posteriorly and in interdental jaw segments during metamorphic climax.

The preliminary description of *S. tropicalis* identified even- and odd-numbered tooth series, "similar to what Shaw (1979) described in *X. laevis*" (Bulckaen et al. 2007), but this early report did not indicate the age of individual specimens that manifested this pattern nor how they were identifiable, both important criteria when multiple initiation patterns are reported in the literature. To bring additional data to clarify the early events of odontogenesis in *S. tropicalis*, this study examines individual tooth developmental stages, as well as patterns of first generation tooth initiation to test the hypothesis that the same mechanisms of odontogenesis are working in *laevis* vs. *tropicalis* despite overall size and developmental rate timing differences.

MATERIALS AND METHODS

Tadpole husbandry. Clutches were either F2 offspring of an outcross between inbred Nigerian and Golden strains from the Harland lab colony on the UC Berkeley campus. Tadpoles were reared in compliance with MAUP #R325-1010 at 23°C, on 12 hour light and dark cycles, daily water changes and feeding with a combination of Sera Micron suspension (Pondside Herp Supply), fish flake (TetraMin), and ground frog brittle (Nasco), and densities of approximately 30 tadpoles per 3L tank after 1 month of growth. Developmental series from Nieuwkoop and Faber (NF) stages 55-59 were sampled, based on limb morphology (Table S1, Figure S1). Tadpoles were sacrificed by immersion in 0.05% Benzocaine (Sigma), eviscerated, fixed in 10% buffered formalin for 2 days, and stored at 4°C in 100% ethanol. An additional NF stage 56

tadpole was derived from a wild type Nigerian breeding pair (Nasco) and raised and sacrificed as above. It was fixed in MEMFA and stored at -20°C in 100% methanol.

Additional specimens. Paraffin-embedded *X. laevis* tadpoles (NF stage 55, NF stage 56) were a gift from Xuan Luong (Hayes lab, UC Berkeley, USA).

Histological sectioning and staining. Timing of tooth development in *S. tropicalis* and the morphogenetic stages of odontogenesis were determined from examination of paraffin embedded and 8µm sectioned dehydrated tadpole crania in transverse, sagittal, and frontal planes. Descriptions are based on observation of at least 3 individuals per NF stage 55-58 in order to triangulate morphology and understand variation (with the exception of NF stage 57, which lacks a sagittally sectioned specimen). The *S. tropicalis* adult cranium was decalcified with 25% formic acid (Humason 1979) for 3 days prior to dehydration and paraffin embedding. Sections were stained with hematoxylin and eosin (after Humason 1979), and mounted with Permount (Fisher).

Histological imaging. Sections were photographed with transmitted light on a Zeiss Axiophot compound microscope using the SPOT flex image capture software and camera system (SPOT Imaging Solutions, Sterling Heights, MI). Composite images of entire transverse sections were created from a series of at least 2/3 overlapping images taken at the same exposure and focus in Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA), using Auto-Align to create a panorama, and then Auto-Blend Layers to create a seamless image.

Whole mount preparations. Additional visualization of the dental lamina and of tooth initiation patterns was based on whole mount preparations of dissected tadpole jaws between glass slides in glycerol. Alizarin Red staining was performed for 24hr on rehydrated fixed NF stage 59 specimens, followed by clearing in 1% KOH for 4 days and storage in 100% glycerol. All other whole mount specimens examined for tooth initiation patterns have been processed through a whole mount *in situ* hybridization protocol (see Chapter 2), so tissues have been subjected to chemical permeability, bleaching, and high temperatures prior to fixation and storage in glycerol at 4°C. Whole mount preparations were photographed with transmitted light on a Zeiss Axiophot compound microscope using the SPOT flex image capture software and camera system (SPOT Imaging Solutions, Sterling Heights, MI).

Determination of tooth initiation patterns. NF stage 56-58 tadpole upper jaws were examined in whole mounts at 20x magnification (N = 64), first tallying the numbers of teeth present to more finely stage dental initiation. In identifying a tooth-by-tooth sequence of initiation, jaw halves were considered independent from each other; given that the midline initiates teeth late in development (see below), the jaw halves were easily discerned at these stages. If teeth were present (N = 68 jaw halves from 37 individuals), tooth initiation patterns in jaw halves with the same numbers of teeth were compared to see if they were consistent with published tooth initiation models for *X. laevis*. Relative ages of tooth germs and comparisons between jaw halves with increasing numbers of teeth were used to infer the order of tooth initiation, with tooth germ age based on the morphogenetic stages defined in histological section and on overall size.

RESULTS AND DISCUSSION

Individual tooth morphogenesis

The histomorphogenetic stages during the development of a tooth in *S. tropicalis* are easily recognizable in transverse section and highly resemble those published for *X. laevis* (Shaw 1979, Cambray 1976). The first signs of tooth development appear in section approximately 100µm from the lateral edges of the suprarostral cartilage, as cells in the basal layer of the double-layered oral epithelium become more cuboid, causing a localized epithelial thickening. An aggregation of mesenchymal cells accompanies this early tooth placode (Figure 1a). A tooth bud forms as lateral and medial edges of the thickened epithelium proliferate basally while the mesenchyme condenses dramatically and protrudes apically at the center of the tooth primordium (Figure 1b).

During cap stage (Figure 1c), a more complex layering becomes visible in the tooth primordium as the epithelium begins to show a lateral-medial asymmetry. Medially, the most prominent epithelial layer is the differentiating inner dental epithelium (IDE), whereas the lateral edge of the tooth germ is flanked by a wedge of elongated epithelial cells and a set of round cells that is folding and/or proliferating to encircle the IDE as the IDE is encircling the condensed mesenchyme. In cap stage tooth germs, a no stellate reticulum in *S. tropicalis* was observed (Figure 2a), as reported by Bulckaen et al. (2007) and in contrast to cap stage teeth in *X. laevis*. By secretory stage, that the long axis of the tooth is oriented parallel to the body axis of the tadpole rather than apical-basally into the oral cavity (Figure 1d), and the cuboidal cells of the IDE and more elongate cells of the ODE completely encircle the dental papilla from this perspective. The tooth germ sits just tangential to the oral epithelium.

An intriguing compound structure was found in sections of a tooth germ in a more developed individual (NF stage 58) with secretory stage tooth germs nearby in the jaw (Figure 1e). The more lateral epithelial structure of the two is sectioned near the apex of a late cap stage tooth primordium, apparent from serial sections. Continuous with this cap stage tooth epithelium appears to be a budding and mesenchymal condensation for an additional tooth germ, although replacement teeth are observed to develop lingually in more mature tooth positions (Shaw 1979, Bulckaen et al. 2007). Another possible explanation for this structure is that it is a section through a ‘twinned’ tooth position, a phenomenon observed relatively commonly in whole mount preparations (see below) in which two cap or bell stage teeth appear to have been initiated in the same location where normally only one would develop (see Figure 5c,e for examples). More detailed reconstructions of serial sections could allow these two conditions to be distinguished.

First generation teeth

The first teeth to form in *S. tropicalis* are detected during NF stage 56 (Table S2), with no NF stage 55 individuals (0/11) displaying signs of tooth formation in section or whole mount. Tooth initiation occurs quite rapidly relative to the duration of NF stage 56, as evidenced by the fact that only 40% of individuals (17/42) had at least one developing tooth when sampled at this

stage. All of the specimens beyond NF stage 56 had at least one tooth at the time of sampling (22/22). The tooth row did not span the entire length of the oral cavity until late NF stage 57 or 58, with teeth continuing to initiate in all of the tadpoles examined.

As is seen in *X. laevis*, the first set of tooth germs to initiate in *S. tropicalis* develop superficially, and it is ambiguous as to whether they form an invagination or evagination of the epithelium (Figure 1a, b). By the time the epithelium begins to enclose the condensing mesenchyme, however, it is clear that these germs are developing close to the surface but in a position distinctly basal to the oral epithelial surface. Unlike superficial first generation teeth in some species which are considered rudimentary (e.g. in alligators, Westergaard & Ferguson 1990; bearded dragon, Handrigan & Richman 2010), *S. tropicalis* early teeth have fully developed outer dental epithelia (ODEs) at all stages of tooth morphogenesis (Figure 1, 2a). All teeth initiated in the first generation appear to reach a secretory stage with an enamel organ, based on a lack of arrested or resorbing structures in section or whole mount.

These superficially initiating first generation teeth do not appear to form from a laterally continuous dental lamina in the upper jaw, or at least not one which invaginates or thickens to any appreciable degree (NF stage 56, data not shown; NF stage 58, Figure 2a,b). As multiple teeth initiate across the jaw (Figure 2d,f), a prominent epithelial connection aside from the oral epithelium is not visible in whole mount.

At some point during NF stage 58, however, the same first generation teeth which have been accumulating across the jaw since NF stage 56 become laterally connected by a prominent dental lamina (Figure 2c,e,g, Chapter 2 Figure 3). This structure is not likely to be a blood vessel because it is unpigmented, whereas most cranial blood vessels in *S. tropicalis* are lined with melanocytes and appear black in whole mount preparations. Instead, this distinct squamous epithelial structure stains densely with eosin in section (Figure 2g). This structure, which I interpret to be the dental lamina, laterally joins tooth germs at differing morphogenetic stages of development, and appears to span the entire presumptive maxillary region (Figure 2e). Frontal sections of the anteriormost and medialmost (the presumptive premaxillary) oral region later in NF stage 58 clearly show a row of secretory stage germs connected lingually by a dental lamina which is separated from the lingual edge of the upper jaw tissue by intervening connective tissue (Figure 2g). It is unclear at this time whether the presumptive premaxillary and maxillary dental laminae are continuous with each other, in large part because there are no premaxillary and maxillary ossification centers detected in whole mount at this developmental stage to define these two tooth populations clearly (data not shown).

There is no report of a dental lamina developing in *X. laevis* during tadpole stages, although Cambray (1976) does identify paired dental laminae on either side at the posterior extent of the jaw during metamorphic climax. Although first generation teeth across vertebrates commonly develop without a laterally continuous dental lamina (Donoghue & Aldridge 2001), a laterally continuous dental lamina which forms after the first teeth are initiated remains a relatively unusual observation for vertebrate odontogenesis. A later-developing, laterally continuous dental lamina has been described for the alligator (see Chapter 2). Sectioning *S. tropicalis* tadpoles after NF stage 58 and into metamorphic climax may reveal additional roles of this later-developing structure.

Heterochrony in the onset of tooth initiation

The developmental timeline of tooth initiation between *S. tropicalis* and *X. laevis* reveals some clear differences between these species. The earliest indication of tooth development, laterally positioned epithelial thickenings inferior to the suprarostral cartilage, can be detected in both species in transverse section, but at different NF stages. In *S. tropicalis* this earliest morphological evidence is observed during NF 56, whereas Cambray (1976) reports an epithelial thickening stage far earlier, at NF stage 53 in *X. laevis* (Figure 3a-d).

The next morphologically equivalent stage for which data is available is when 1-3 secretory stage tooth germs are detectable on the lateral edges of the jaws, with enough space to fit another tooth in between each germ (Figure 3e-h). This phenotype is visible in transverse section for *S. tropicalis* around NF stage 58, whereas *X. laevis* continues its developmentally earlier timeline and displays this phenotype at NF stage 55. This heterochronic shift between species is not unprecedented (the two species hatch at different NF stages), but it is uncommon to see heterochronies at the level of gene expression, at least for pre-larval stages of development (Yanai et al. 2011).

There is considerable difference in the maturation of the hindlimb between NF stages 53-55 vs. between NF stages 56-58, suggesting that whatever mechanisms are controlling tooth initiation in *S. tropicalis* and *X. laevis* are not strictly coupled to the signals directing hindlimb morphogenesis. Indeed, within the *S. tropicalis* range of variation, the number of teeth present in a tadpole jaw during tooth initiation does not strictly correlate with absolute size, absolute age, or NF stage and its substages based on limb morphology (Grieco 2011, Kerney et al. 2009).

While the basic anatomical topology of where teeth are forming and the broad ontogenic trajectory in *S. tropicalis* and *X. laevis* match, the dynamics of tooth initiation may well be different. Shaw's (1979) study of first generation tooth development in *X. laevis* identified a nearly full tooth row with two sets of alternating germs in transverse section at NF stage 55. Adjusting for the heterochrony observed above, this predicts that an equivalent morphological stage would develop in *S. tropicalis* around the end of NF stage 58, about the time the dental lamina appears in this species. This later stage was outside the scope of the current histological survey, but no specimen sectioned for this study ever posessed anything resembling a full tooth row of germs that alternate as cleanly as that published for *X. laevis*. That Bulckaen et al. (2007) found alternation in *S. tropicalis* suggests that the two species are congruent in their broad trajectories of tooth initiation.

Change in the width of the jaw over NF stages 53-58 makes it very difficult to accurately infer or compare tooth initiation patterns from transverse sections. From the observations thus far it is difficult to say whether patterns of initiation in the two species are the same, excepting a heterochronic shift. The later transverse sections (Figure 3e-h) do show enough space between secretory stage tooth germs to allow for a second row to develop in between, but based on sections from both species capturing 1-3 germs per side and not a full tooth row, it is difficult to accept Shaw's series alternation hypothesis. The data presented here suggest that there are spatiotemporal dynamics that are being missed from these snapshots of anatomy. Regarding the

lateral-to-medial hypothesis (Shaw 1979), without invariant landmarks on the transverse sections as the jaws grow, one cannot say whether initiation is progressing medially, or that it just appears to be going that direction as the jaw expands laterally and adds teeth laterally.

Initiation patterns in the upper jaw

To better understand the dynamics of tooth initiation in *S. tropicalis* and to further evaluate the published hypotheses for tooth initiation patterns, I looked at whole mount preparations of tadpole upper jaws to characterize the variation within an NF stage and to more finely stage tooth initiation patterns. This process is not without its drawbacks, namely the difficulty of capturing all of the teeth in a focal plane and variation in staining from specimen-to-specimen. The most reliable markers of tooth initiation to trace are to compare teeth at the lamina vs. cap stage vs. secretory stage rather than to trace gradations within each morphological class, and so the method becomes harder to use as more teeth mature to secretory stages.

The process of populating the tadpole jaw with teeth in *S. tropicalis* is a gradual one, beginning at NF stage 56 and continuing through metamorphosis. The first tooth to initiate in the tadpole jaw does so between the lateral third and the lateral extent of the external nares, but whether this represents variation in jaw position or variation in the external nares is unclear. Figure 4 presents a sample of the earliest teeth to form in *S. tropicalis* tadpoles. Although the pattern at first looks stereotypical as the second and third germ are added laterally to the first, and the fourth is initiated medially to the group (Figure 4 a,b,c,d), the final example challenges this idea because the two cap stage germs are separated from each other by a younger tooth bud rather than adjacent to each other. In addition, the determination of which tooth germ was actually initiated first of the pair at nearly identical stages is based mostly on position relative to the bud stage germ in the first tadpole (Figure 4a), which may not be a reliable indicator without additional landmarks.

In tadpoles with more fully formed tooth rows (Figure 5), the patterns of tooth initiation become more complex. The first teeth to initiate have reached secretory stages at this point in development and it is nearly impossible to predict which tooth initiated first except by general location along the jawline. With these examples, it is possible to infer a general expansion of the tooth row at both ends, but sometimes also in intervening spaces between older tooth germs. There does not appear to be a spatial anchor to the initiation of first-generation teeth in *S. tropicalis*, or a reliable difference between right and left jaw halves in the pattern of tooth initiation. Additionally, it is unclear how the presence of 'twinned' tooth germs, seen in 10/37 specimens (e.g. Figure 5c,d,e), may be affecting the patterning of subsequent tooth germs in the jaw. Examining the distribution of these 'twinned' positions in more detail may eventually yield more information about tooth initiation mechanisms in *S. tropicalis*.

Based on the observations in 68 jaw halves which progressively add tooth germs to the tadpole tooth row, a few principles stand out:

- There is not a single stereotyped pattern to tooth initiation, nor is there commonly symmetry between jaw halves.

- Teeth may be initiated at lateral or medial ends of the tooth row, rejecting the hypothesis of a strict lateral-to-medial pattern of tooth initiation (Shaw 1979) in *S. tropicalis*. The effect of this mode of initiation is that the earliest tooth germs to initiate tend to be in the middle of the jaw half rather than at the ends.
- Newly developing teeth do not need to initiate in a position adjacent to other teeth; there may be gaps in the tooth row which are filled in later by younger tooth germs. This, combined with the observation that the first two teeth are often initiated adjacently, rejects the hypothesis of series alternation (Shaw 1979) as the dominant pattern of tooth initiation in *S. tropicalis*.

Overall, the pattern of tooth initiation in *S. tropicalis* appears to be much more dynamic than has been described with histological samples. Consistent with data from both histological sections and whole mounts, the midline is the last portion of the tadpole jaw to fill in, although no tadpole up to NF stage 58 has been observed to have more than 10 tooth germs on a jaw half, which is just over half of the average number of teeth present in adult *S. tropicalis* (17-18 tooth positions, see Chapter 3). This phenomenon of continuing to add tooth germs through metamorphosis and posteriorly as an adult has also been described for *X. laevis* (Shaw 1979, Cambray 1976).

The initiation patterns observed here in *S. tropicalis* align somewhat better with Cambray's 4 phase model (1976). Adjusting for the earlier onset of tooth initiation in *X. laevis*, all of the phenomena observed in *S. tropicalis* during this study would fall into his "first wave of teeth from epithelial odontogenic zones, medio-lateral, right and left and takes place stage 53 to 57" (Cambray 1976, trans.). The second phase described in Cambray's model begins at NF stage 58 and involves rapid initiation of teeth in the extreme anterior region, which is at least consistent with the patterns observed in *S. tropicalis* where it is rare to find a tadpole with mature tooth germs, or even any tooth germs, at the midline. In cases such as in figure 2g, where tooth germs were captured at the extreme anterior, they are all at the same mature stage, consistent with a more rapid initiation event. The temporal scope of this study prevents the evaluation of the two phases of Cambray's model which occur during metamorphic climax, in which the posterior dental laminae arise and additional intervening teeth are initiated in the jaw, although it does appear that in *S. tropicalis* new teeth are being added to the maxillary region during the entire leadup to metamorphosis.

In *S. tropicalis*, examination of tadpoles at late stages is required to understand whether the anteriormost, premaxillary region teeth form from the observed dental lamina originally or whether they form like the early-initiating maxilla teeth do, superficially in the oral epithelium. In either case, the observation of a dental lamina in *S. tropicalis* tadpoles contrasts with the Cambray model (1976) for tooth initiation. Cambray's 4 phase model does not invoke the presence of a dental lamina until the third phase, although he reports one continuous dental lamina in adult *X. laevis*.

Triggers for tooth development

Cambray (1976) ties his first two phases of tooth development in *X. laevis* to ossification patterns, observing in cleared and stained specimens that the maxilla ossifies around the time of maxillary tooth mineralization, followed by premaxilla ossification much later in development.

In fact, all tooth initiation in the tadpoles of both species begins long before the dermal ossifications of the jaw bones are detectable in whole mount. Trueb and Hanken's (1992) analysis of the order of skeletal ossification in *Xenopus laevis* using cleared and stained pre-metamorphic tadpoles reported that while the sequence of ossification was stereotypical, the timing did not correlate well with external morphology and NF stages. Looking more closely at the comparisons between ossification sequences documented by Trueb and Hanken (Table 2, 1992) and the studies of Bernasconi (1951), Sedra & Michael (1957), and Brown (1980), the sequence of jawbone ossification appears to be constant but either compressed or extended across multiple NF stages in each clutch and study. Teeth do not appear to be acting within this stereotypical sequence: when ossification is compressed, teeth tend to be mineralized at the same time as upper jaw bones, but when ossification is drawn out teeth are not observed until much later, during the metamorphic climax.

A more recent study of *Xenopus laevis* ossification showed that teeth are mineralized enough to stain with Alizarin Red only after maxillary ossification centers are apparent (NF 59), although in non-metamorphosing athyroid individuals, teeth are detectable prior to any maxillary ossification (Kerney et al. 2009). Thus, it appears that triggers for the ossification of various dermal bones may be distinct from the trigger causing mineralization in the tooth program. It has been suggested that ossification of the jaw bones may constrain the ability to add teeth in intervening spaces within the jaw (Wake 1976, Wake 1980), but it probably does not direct tooth initiation in the first place.

Another jaw structure that could potentially trigger tooth initiation is the suprarostral cartilage. Other than resorption during metamorphic climax, no changes are known to occur in this cartilage during development. Facial cartilages in tadpoles undergo isometric growth, meaning that there are no predictions for regionalization within the cartilage that would potentially correlate with tooth initiation in particular locations (Rose 2009).

Thyroid hormone (TH) is the trigger for most morphogenetic events studied in metamorphosing *X. laevis* or *S. tropicalis* to date (Wang et al. 2008, Das et al. 2010). TH is known to affect tooth morphologies in salamanders, where it is important in the transition to full pedicely and bicuspidity during the height of metamorphosis, but multiple generations of larval teeth are able to form when TH is blocked (*Salamandra keyserlingii, Triturus vulgaris*, Vassilieva 2005). Naturally occurring athyroid *X. laevis* tadpoles have varying numbers of monocuspid teeth as well as some cranial and postcranial bones associated with the adult skeleton (Kerney et al. 2009). The morphologies present in these tadpoles demonstrate that TH is not required for tooth formation, but it may serve to partially synchronize changes in the tadpole overall (Hanken et al. 1989, Kerney et al. 2009).

T3 treatment in *Bombina orientalis* causes precocious ossification dependent on which ossification centers are competent to respond at the stage of hormone exposure, but it remains unclear whether T3 can induce *de novo* ossification center differentiation in the cranium (Hanken & Hall 1988). Indeed, the role of TH as both morphological integrator and trigger for independent metamorphic events remains a fascinating area of investigation, demonstrated by the fact that TH regulates limb programs for musculature, nerves, and cartilage independently when targeted by dominant-negative transgenics (Brown et al. 2005).

Initiation patterns across vertebrates

A number of exquisitely detailed studies of tooth initiation have been performed in *Alligator mississippiensis* (Westergaard & Ferguson 1986, 1987, 1990). These authors and Osborn (1971), who looked in *Lacerta vivipara*, struggled to identify tooth positions reliably in the earliest phases of tooth development, in the first 5-7 teeth initiated, due to the changes in jaw size through development. Despite this, a reaction-diffusion computer model incorporating jaw growth was able to predict at least the sequence of initiation of the first 7 teeth in the lower jaw of the alligator (Kulesa et al. 1996). In the caecilian amphibian *Gymnopis multiplicata*, 10 early tooth germs were observed prior to jaw ossification in an embryo which had a morphological gradient getting younger more posteriorly, but limited sample material prevented a more detailed characterization of the initiation sequence (Wake 1976). A detailed ontogenetic study in another viviparous caecilian, *Dermophis mexicanus,* traced the addition of tooth positions from the earliest observation of 2 upper marginal tooth rows in the fetus; and, in contrast to other models of tooth initiation, observed a reduction in tooth row number and change in tooth morphology at birth (Wake 1980).

Alligator teeth initiate in both directions from tooth position 3 in the lower jaw, and the first tooth on upper jaw initiates in the middle of the presumptive premaxillary area (Westergaard & Ferguson 1986, 1990). Wake (1980) did not observe the initiation of the first teeth in *Dermophis mexicanus*, but reports an anteriorly-biased appearance of more mature tooth types. Observations in *S. tropicalis* contrast with this anterior onset of tooth development in that the first tooth to initiate is much closer to the lateral edge of the tadpole mouth. *Lacerta vivipara*'s first tooth develops at position 11, much more posteriorly in the jaw (Osborn 1971).

The initiation patterns reported for *Alligator mississippiensis* appear more regular than those in *S. tropicalis*; differential jaw growth creates spaces in between existing germs which are then filled in with new teeth. Consistent with a model of local inhibition by existing tooth germs (Osborn 1971, Kulesa et al. 1996), the intervening teeth first appear near the oldest teeth (Westergaard & Ferguson 1987). The high frequency of observation of twinned tooth germs in *S. tropicalis* tadpoles is a challenge to the model of local inhibition as the only guiding mechanism for the patterning of tooth initiation. A similar challenge to the model of local inhibition was seen in another amphibian; *Dermophis mexicanus* fetal tooth loci accumulate in multiple lingual rows that aggregate labially, creating up to 6 functional teeth per tooth position, or 6 marginal upper jaw tooth rows (Wake 1980). Based on this data, Wake (1980) suggests that tooth spacing is actually unregulated prior to birth and the change in tooth morphology in *Dermophis mexicanus*, which, in the absence of a sterotyped pattern of development, cannot be ruled out as a null hypothesis without experimental embryology manipulating the spatial extent of the jaw or positioning of individual teeth. In various caecilians, Wake (1976, 1980) observed that teeth are added interstitially with jaw growth as well as at the posterior limit of the jaw, an observation broadly consistent with the generalizations of tooth initiation in *S. tropicalis*.

CONCLUSIONS

Comparison of tooth initiation in *X. laevis* and *S. tropicalis* reveals that while the broad trajectory of tooth initiation stages can be aligned, *S. tropicalis* develops its teeth relatively later, by 2 NF stages, and the findings to date suggest that *S. tropicalis* teeth develop less synchronously. Alternatively, the current sampling scheme in *X. laevis* may fail to capture some of the dynamics in that species, and those dynamics may or may not be equivalent to those observed in *S. tropicalis*. Individual tooth morphogenesis stages in *S. tropicalis* align well with previous descriptions in *X. laevis* by Shaw (1979) and Cambray (1976), but the heterochrony in tooth development suggests that tooth development is not well-correlated to development in external morphology in these related species. It would be interesting to see the effects of rearing temperature on the dynamics of tooth initiation, given that a candidate mechanism guiding initiation patterns is a reaction-diffusion process.

One striking difference between *S. tropicalis* and *X. laevis* odontogenesis is the observation of a late-appearing dental lamina uniting tooth germs of various developmental stages in *S. tropicalis*. This laterally continuous dental lamina spans a large portion of the developing tadpole jaw at stages which have been observed in more detail for *X. laevis*, so it would be surprising if it were overlooked in this species.

Whether *X. laevis* and *S. tropicalis* employ the same mechanisms of tooth initiation despite their evolutionary separation remains an open question, but one that can be evaluated with denser sampling in both species: later in *S. tropicalis*, and earlier in *X. laevis*. Based on reconciling Cambray's multi-phase observations, Shaw's extreme anterior tooth series alternation, and latent midline tooth development in *S. tropicalis*, the intriguing possibility is presented that there may be separate maxillary and premaxillary phases to development in the tadpole dentition, and comparisons thus far are capturing different phases in detail. Further studies in these two species present the opportunity to observe whether relative size affects the patterning dynamics of the dentition, and a system in which to study the evolution of dental initiation and patterning mechanisms at the subfamily level.

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Figure 1. Transverse sections through individual *S. tropicalis* tooth primordia through histomorphogenesis, stained with hematoxylin and eosin. (a) lamina, (b) bud, (c) cap, and (d) secretory stages document the proliferation, folding, and differentiation of epithelial cells to form an enamel organ as mesenchymal cells condense and become encircled within the conical tooth. (e) possible replacement tooth formation initiated from a late cap stage tooth or anomalously coupled tooth germs. Scale bars = 0.1 mm.

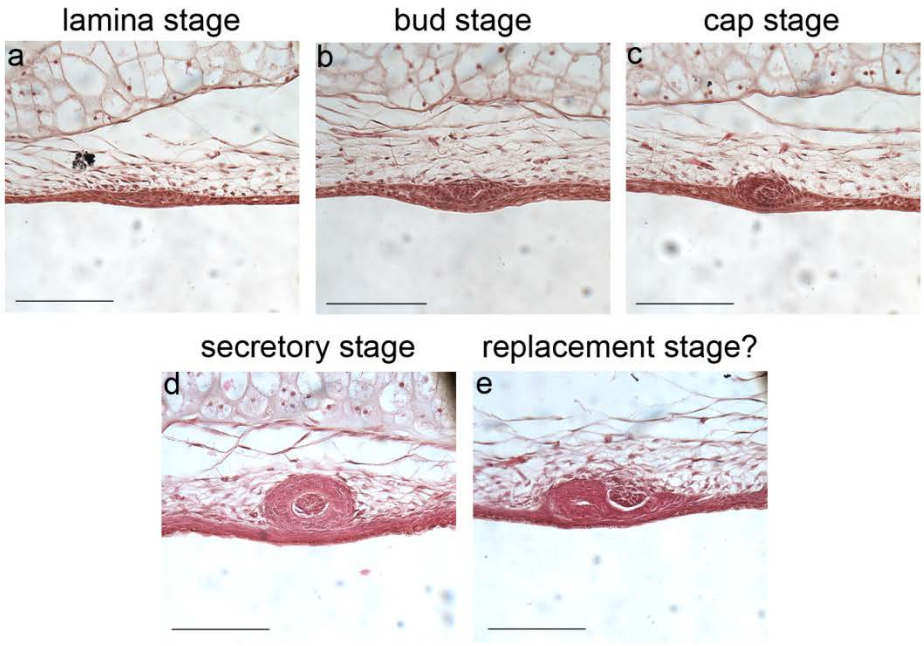


Figure 2. Development of the dental lamina in *S. tropicalis*. (a) and (b) Sagittal sections through a NF stage 58 tadpole jaw, stained with hematoxylin and eosin. (a) In the early phase of odontogenesis, a superficially developing cap stage tooth germ and (b) adjacent toothless region of the jaw showing no prominent dental lamina structures. (d) and (f) Whole mount preparation of an NF stage 58 upper jaw in the early phase of odontogenesis that has been processed through *in situ* hybridization, ventral view. (d) Tooth germs from the presumptive maxillary region and (f) tooth germs from the presumptive premaxillary region (the midline) which lack a prominent refractile epithelial connection. (c) and (e) Whole mount preparation of an NF stage 59 upper jaw cleared and stained with Alizarin red, ventral view. Box in (c) indicates region magnified in (e) to show a prominent refractile structure at the basal and lingual regions of the developing tooth row. This dental lamina is continuous for most of the upper left side tooth row present in (c). (e) A refractile structure (white arrow) spans laterally between tooth germs on the lingual side. The tooth with the red tip (actively mineralizing), 3rd from left, is in the position of the first tooth to initiate in the jaw, so the refractile structure, inferred to be a mature dental lamina, is joining tooth germs of varying ages and appearing after teeth are well on their way developing. (g) Frontal section through a later phase NF stage 58 tadpole jaw, stained with hematoxylin and eosin. An epithelial structure connecting midline tooth germs is clearly separated from the oral epithelium by intervening connective tissue. Scale bar = 0.1 mm.

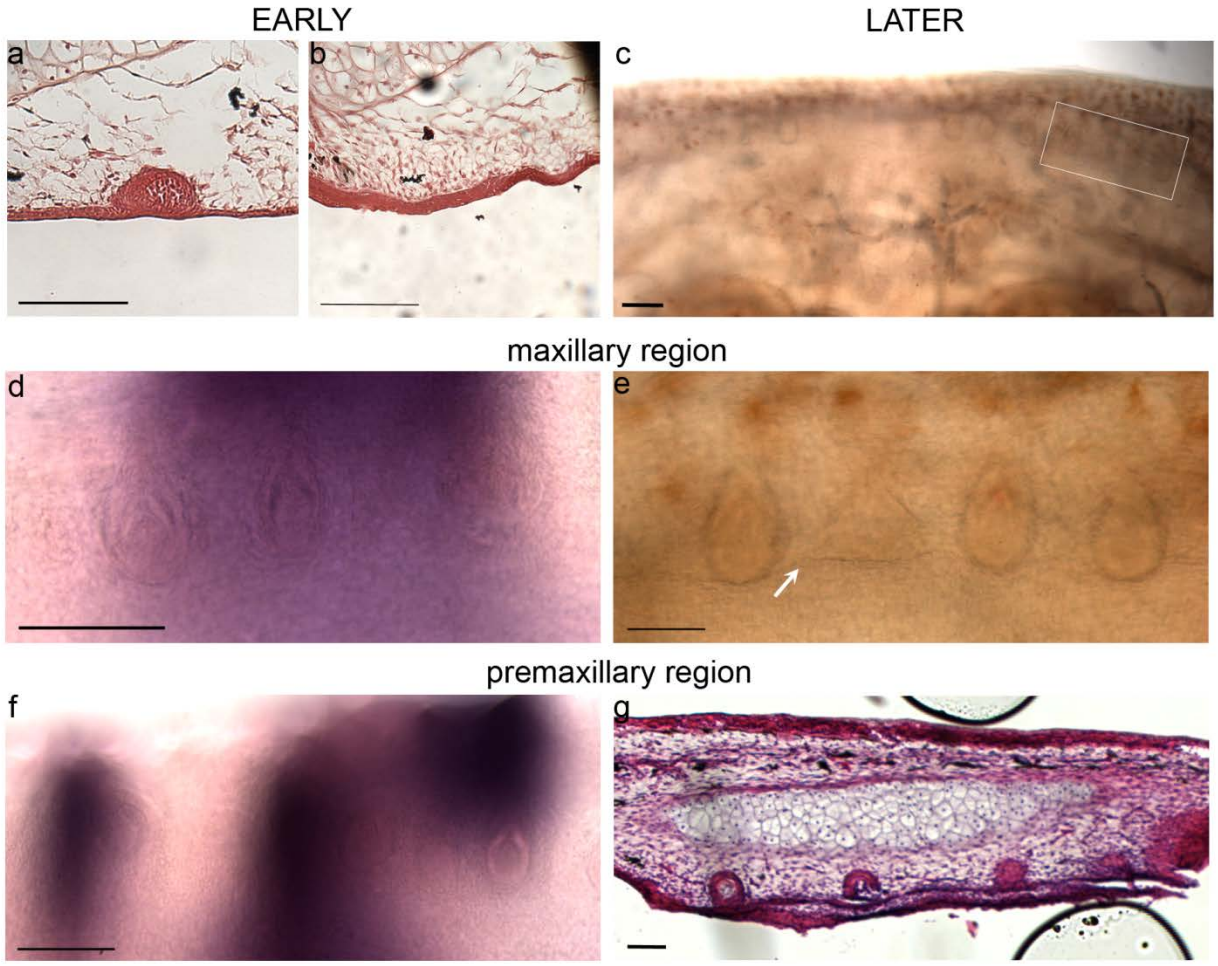


Figure 3. Comparison of the onset of tooth initiation in *S. tropicalis* and *X. laevis*. Composite transverse sections of tadpole jaws at the earliest (a-d) and later (e-h) phases of tooth initiation. (a,c) NF 56 *S. tropicalis* and (b,d) NF 53 *X. laevis* share the same tooth initiation morphology with thickened oral epithelia just medial and inferior to the ends of the suprarostral cartilages. (b,d) reproduced with modification from Cambray (1976). (e,g) NF 58 *S. tropicalis* and (f,h) NF 55 *X. laevis* both show the beginnings of adding to the tooth row, with secretory stage germs widely spaced and beginning to fill in medially (when controlled for size). Scale bars = 0.1 mm.

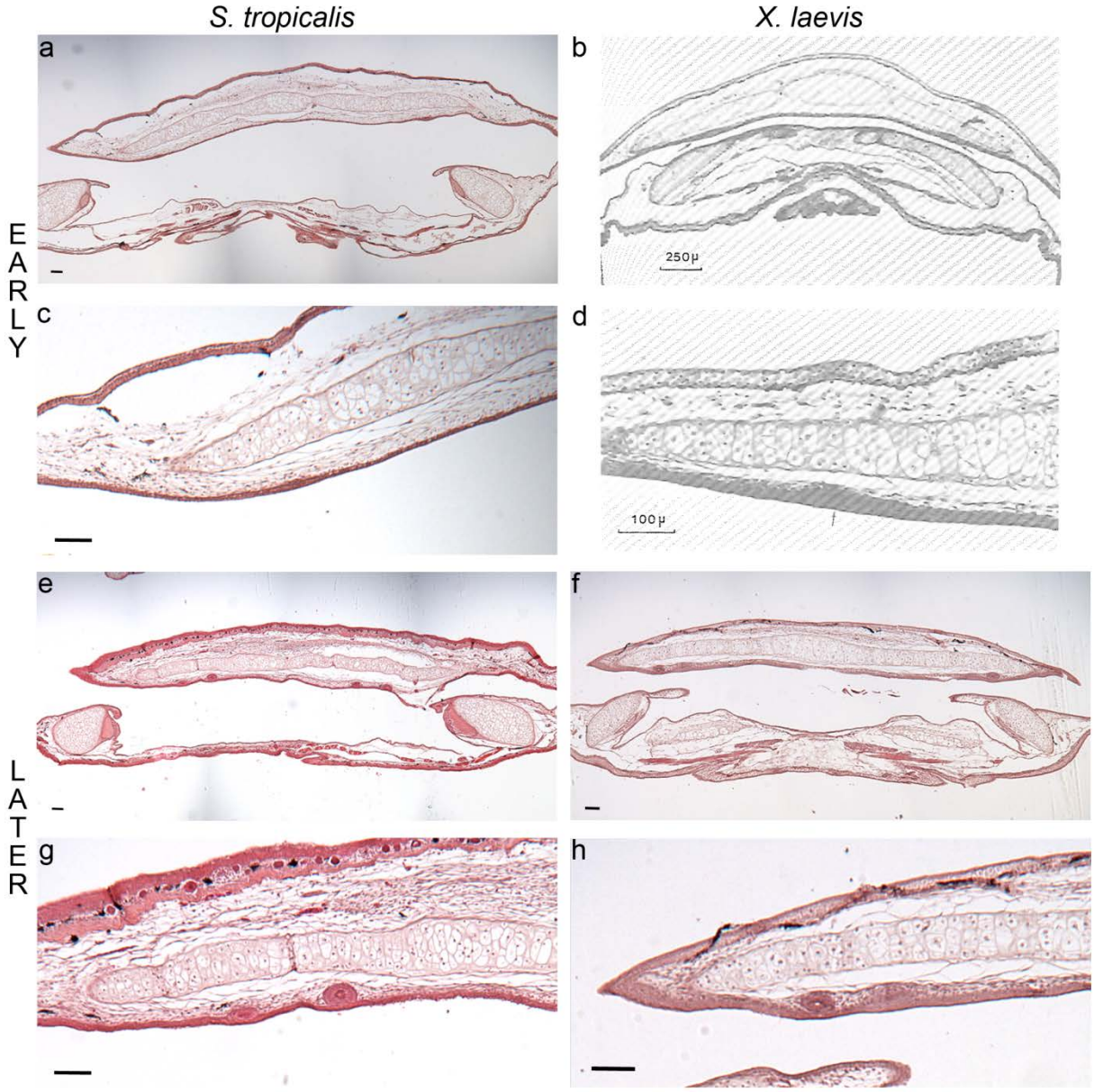


Figure 4. Partial tadpole tooth rows. Whole mount preparations after processing for *in situ* hybridization, ventral view. Jaw halves are all oriented such that medial is to the left and lateral is to the right. NF = Nieuwkoop and Faber stage, UL = anatomical upper left, UR = anatomical upper right. Scale bar = 0.1 mm

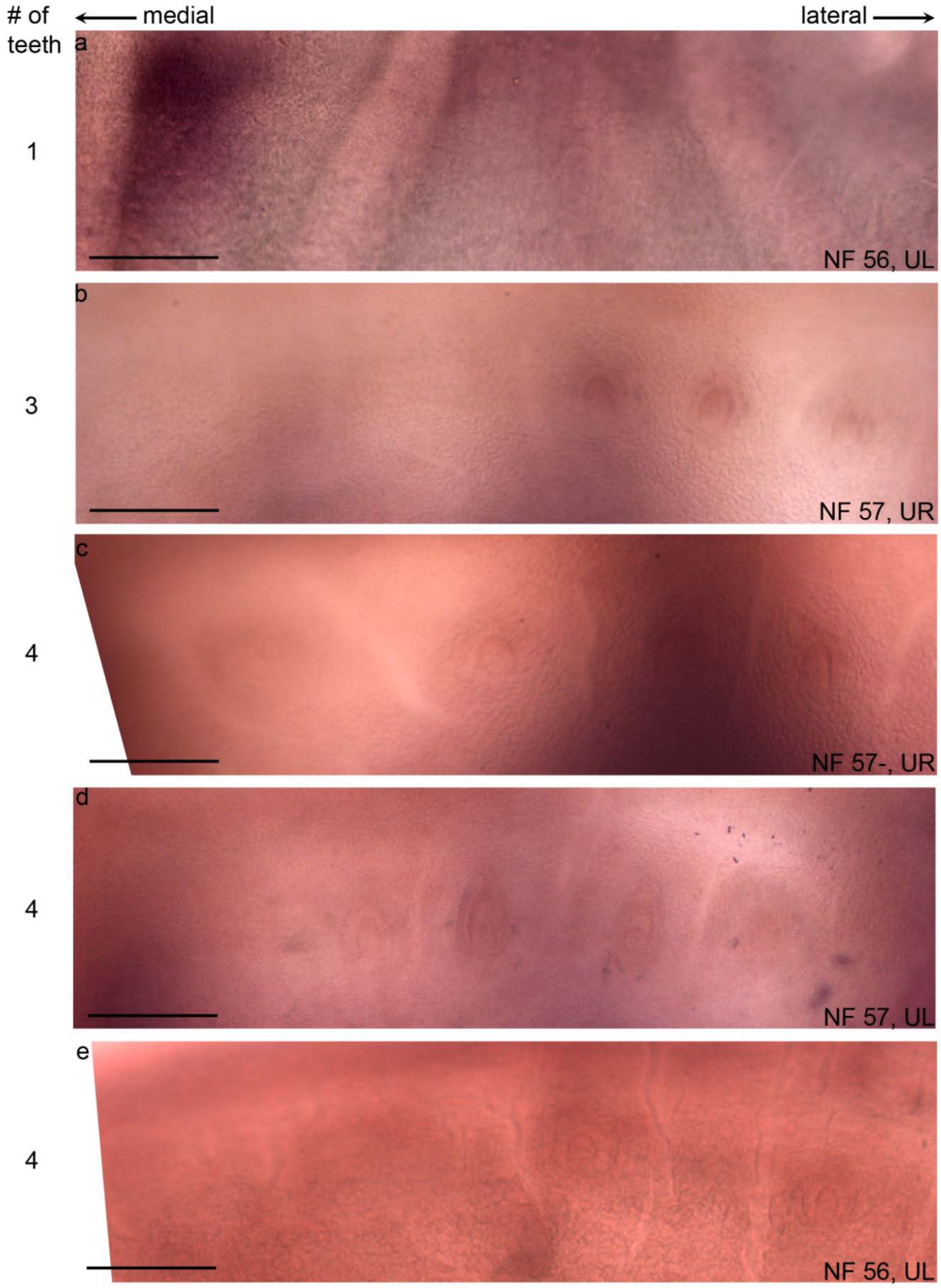
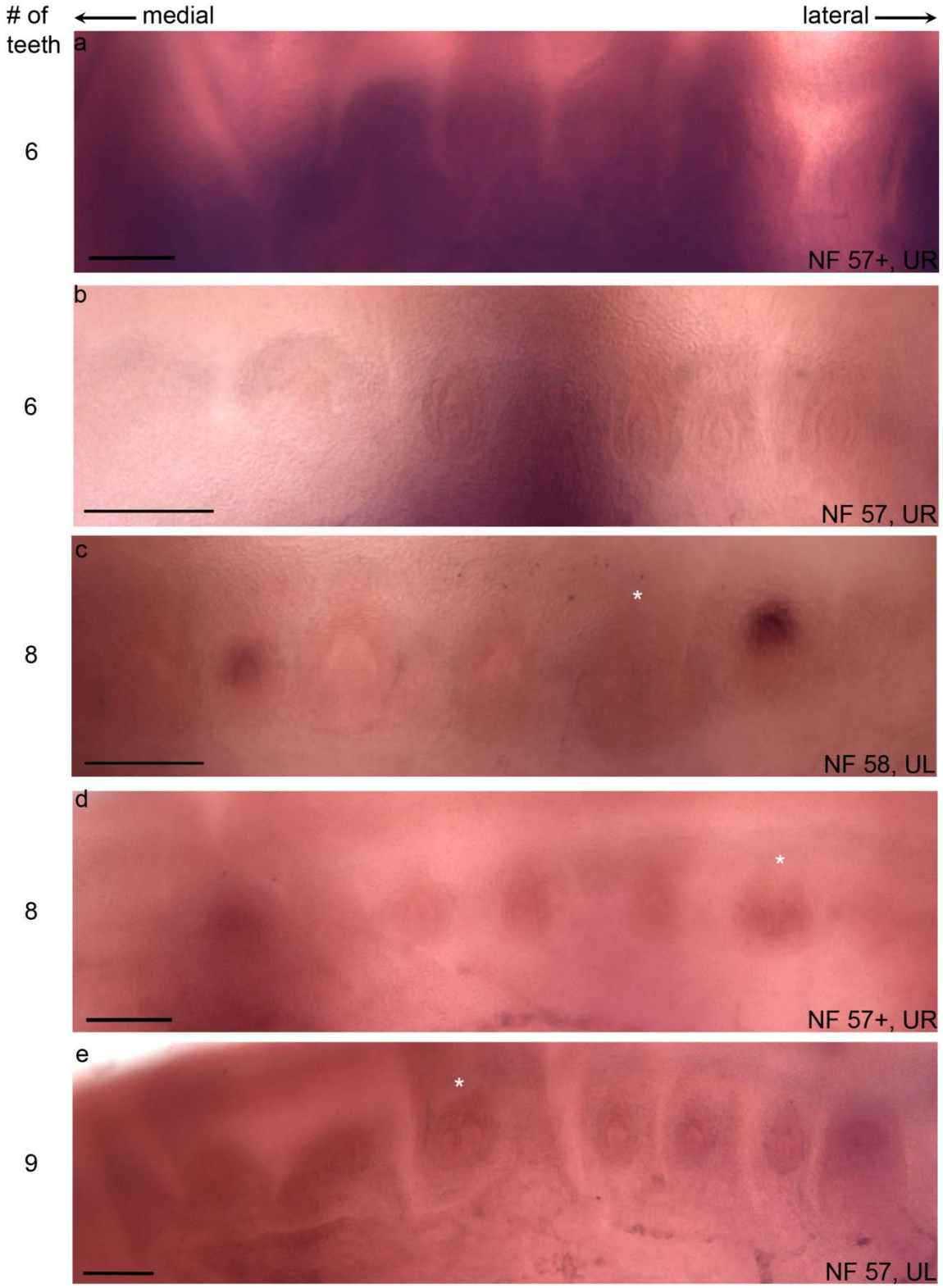


Figure 5. ‘Full’ tadpole tooth rows. Whole mount preparations after processing for *in situ* hybridization, ventral view. Jaw halves are all oriented such that medial is to the left and lateral is to the right. *denotes “twinned” tooth germs. NF = Nieuwkoop and Faber stage, UL = anatomical upper left, UR = anatomical upper right. Scale bar = 0.1 mm



Chapter 2

Shh gene expression in *S. tropicalis* teeth and a re-evaluation of the vertebrate odontogenic band

ABSTRACT

Developmental genetics and experimental embryology have successfully developed mechanistic models of organogenesis, aided by the broad conservation of gene expression and function in diverse species. It is this conservation of expression that makes it difficult to evaluate the adequacy of these mechanistic models to account for phenotypic variation in these organs without a comparative, more holistic anatomical approach. Odontogenesis is a system well-suited to the examination of both mechanistic conservation and phenotypic diversity, and the present study uses the odontogenic band as a case study in which to test the consensus model for odontogenesis as marked by *Shh*. *Shh* gene expression data are reported for an amphibian, the frog *Silurana (Xenopus) tropicalis*, and the correspondence between odontogenic band gene expression and the presence teeth is evaluated across vertebrates. When compared to the expectation for *Shh* expression in the consensus model, *S. tropicalis* gene expression appears unusual, but several other vertebrate species fail to conform to the expectations of the consensus model when odontogenic band phenotypes are examined in more detail. An evolutionary, comparative approach can play a crucial role in testing hypotheses put forward by developmental genetics, and can clarify the points of flexibility in developmental mechanisms by identifying phenotypes that may be decoupled.

INTRODUCTION

Existing mechanistic models for organogenesis represent the aggregation of decades of work, experimental validation, and what we now know to be a fairly reasonable assumption of conservation of developmental gene expression and function throughout phylogenetic diversity. This assumption of parsimony is often taken for granted in studies in developmental model organisms, and the ease of access or manipulation dictates which organism will be used to further elucidate organogenetic mechanisms. Testing this assumption, however, represents a powerful way to assess the adequacy of reductionist investigation at a phenotypic level where morphology and physiology vary significantly across the vertebrate tree of life. Consequently, researchers are increasingly using models, simulations, and network analyses to assess whether the known components of a developmental mechanism are sufficient to reconstruct the biological phenomena and phenotypes being observed. (e.g. Fisher et al. 2011, Marcon et al. 2011, Salazar-Ciudad & Jernvall 2010, Swalla et al. 2013, Langlois & Martyniuk 2013).

In order to understand whether genotype-phenotype associations persist above the species level and how phenotypic variation between species is able to be created by mechanisms with shared evolutionary history, comparative analysis across species is also required (Woltering & Duboule 2010). Assessing the correspondence between first-order gene expression and subsequent

morphological phenotypes in detail (e.g. Nemeschkal 1999, Jernvall et al. 2000, Reno et al. 2008, Shubin et al. 1997, Fish et al. 2011) is critical to test the adequacy of our bottom-up, reductionist models to explain the phenotypic outcomes of particular organogenetic mechanisms, and to help translational researchers understand how the results of experimental manipulation do and don't translate from one organism to another. Such comparisons are also essential to bring the information from developmental genetics more completely into our understanding of microevolution and macroevolution, and whether patterns of phenotypic variation among species can inform us about developmental and evolutionary mechanisms that work at population and individual levels (Wake et al. 2011).

Odontogenesis in vertebrates is an organogenetic system that is well-suited to a comparative approach to yield new insights into the mechanisms of development. Although much of the foundational genetic work on odontogenesis was done in laboratory mice, the last 15 years have seen a large number of tooth studies in other vertebrates, including scyliorhinids, salmonids, cichlids, squamates, crocodylians, carnivorans, and other rodents (Figure 1 for examples). These studies have supported the broad-scale conservation of tooth patterning and morphogenetic mechanisms, but have also pointed out key differences such as the probable uniqueness of enamel knots to mammals, and several phenotypic features that may be unique to the mouse's accelerated tooth development (placodes vs. dental lamina in many amniotes). In building consensus around odontogenic mechanisms, studies have focused largely on the idea of conserved gene networks and signaling pathways (e.g. Fraser et al. 2009, Jernvall & Thesleff 2012), while paying less attention to the craniofacial developmental and anatomical context in which these networks are expressed. The current consensus model for the morphological aspects of odontogenesis remains largely based on observations in laboratory mice and cichlid fish, despite the diversity of vertebrates for which odontogenesis has now been examined. In the vast majority of these studies in other vertebrates, *Shh* has been the marker gene of choice for its widespread expression through different stages of odontogenesis, as well as its functional role in many of them.

The consensus model for odontogenesis as marked by *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 more recently *Sox2* (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 & 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 tooth morphogenesis. At these periodic locations of individual tooth formation, epithelial-mesenchymal morphogenesis begins with a proliferation of the epithelium into the mesenchyme, creating a tooth bud. Cap stage begins as the proliferating basal edges of the epithelium begin to grow around a condensing mass of mesenchyme. At this point, *Shh* is expressed in the mammalian enamel knot, a cluster of cells at the apex of the folding epithelium (Vaahtokari et al. 1996, Keränen et al. 1998, Moustakas et al. 2011). In all other vertebrates examined to date, *Shh* is expressed at this stage in the inner dental epithelium (IDE), which has been considered homologous to the enamel knot at the level of its

expression of major signaling pathway genes and influence on tooth shape (Fraser et al. 2013, Handrigan & Richman 2010).

Bell stage is when the tooth crown shape is finalized, and cells of the entire inner dental epithelium and dental papilla differentiate into secretory cells. *Shh* is expressed in the differentiating IDE and is downregulated as ameloblasts begin to secrete matrix (Fraser et al. 2013, Buchtová et al. 2008, Handrigan & Richman 2010, Gritli-Linde et al. 2001). In mammals, tooth cusps are created by the positions of *Shh*-expressing enamel knots relative to basally-proliferating epithelia, and in most other vertebrates examined (salamanders, teleosts) with multicusped teeth, cusps are formed by localized proliferation of the IDE (Fraser et al. 2013), nonuniform matrix deposition (Davit-Béal et al. 2007), or concrescence of adjacent teeth (Jackman & Stock 2013). It is not until bell stages that interspecific differences in tooth shape become prominent (Handrigan & Richman 2010), though shape differences between cap and bell stages have not been quantitatively compared. Tooth shape is finalized during mineralization, when epithelial ameloblasts produce enamel (or enameloid) and odontoblasts at the margins of the dental papilla secrete dentin.

Reasons to question *Shh* function in the consensus model of tooth initiation

The function of the OB has been difficult to study with developmental and molecular genetics because OB expression of *Shh* is typically coincident with requirements for the pathway in midline and craniofacial morphogenesis, making it difficult to distinguish primary OB defects from indirect skeletal defects. In addition, the mouse model is difficult to access at this early stage *in utero*. Functional manipulations of Shh have now been done in more species along with comparative anatomical studies on epithelial appendage patterning.

Zebrafish knockout mutants for *Shha*, which normally have only pharyngeal teeth, do not appear to have mature teeth (Jackman et al. 2010). Early tooth marker *pitx2* is not expressed in the knockout, but *pitx2* expression does occur with cyclopamine inhibition just prior to tooth initiation at 30hpf, which shows that upstream Shh signals are involved in triggering early tooth-bearing area development in zebrafish (Jackman et al. 2010). Other tooth gene expression and morphogenesis is blocked with cyclopamine treatment. This study also examined zebrafish pharyngeal tooth development, but it is not clear whether a localized field of *shha* expression is necessary to initiate pharyngeal teeth or if these teeth are receiving *shha* from another source such as the broader pharyngeal endoderm.

Treating cichlid fish with cyclopamine during the development of the first oral tooth row disrupted patterning but did not eliminate the activation of later *Shh* expression in foci and the 2nd row OB (Fraser et al. 2008). This timing and level of inhibition was, however, enough to prevent all but the first-forming tooth from producing mineralized teeth, either directly or indirectly by removing midline tissue. Knocking down Shh once the second tooth row has started to form results in a normal dentition (Fraser et al. 2008), suggesting an indirect craniofacial mechanism for the first row patterning defect rather than a competence defect.

During mouse tooth initiation, *Shh* is in oral placodes at least by E10.5 (Sarkar et al. 2000). Shh is known to cause epithelial proliferation as well as mesenchymal condensation in combination

with Msx1 (Hardcastle et al. 1998, Chen et al. 1996, Zhang et al. 2000), but not basal layer epithelial thickening, since strong inhibition of Shh in cultured E10.5 mandibles causes tooth arrest after visible epithelial thickenings formed (Cobourne et al. 2001). A double knockout of Shh pathway targets *Gli2* and *Gli3* similarly causes an arrest of teeth at bud or placode stage, after morphogenesis has begun (Hardcastle et al. 1998). Similarly, injections of anti-Shh antibody at E10, as well as epithelium-specific *Shh* knockouts, produce M1-M2 fusion phenotypes, allowing a good deal of morphogenesis to take place without the action of Shh (Cho et al. 2011, Gritli-Linde et al. 2002). While many different strategies have been used to disrupt Shh signaling in mouse odontogenesis, the resulting morphogenetic phenotypes suggest that Shh has no role in mouse tooth initiation or that the window of functional manipulation is too early (in the case of *Shh* knockouts), or too late to test its role.

Tooth replacement is another process that gives insight into *Shh* function during tooth initiation, because a successional lamina must be induced to form a new functional tooth. In cichlids, Shh perturbation with cyclopamine does not interrupt tooth replacement, but it does affect replacement tooth shape (Fraser et al. 2013). Similar data for squamates show no effect of Shh on successional lamina induction or outgrowth in a gecko or a python (Handrigan & Richman 2010). In all of these taxa, in trout, and in alligator, *Shh* is not expressed in the successional lamina; it is only expressed in the IDE once replacement teeth begin morphogenesis (Fraser et al. 2013, Handrigan & Richman 2010, Buchtová et al. 2008, Fraser et al. 2006, Wu et al. 2013). These findings support the idea that Shh does not actively initiate tooth development but tooth-forming epithelial cells may need to have expressed *Shh* at some point in their past.

Chuong et al. (2000) suggest, based on the growth of feather primordia, that *Shh* might control the size of initial placodes via lateral cell proliferation, but that it is not needed for induction of the primordia. EDA overexpression causes larger tooth placodes by promoting placodal cell fate (Mustonen et al. 2004) and is probably upstream of *Shh* (Pummila et al. 2007).

Snakes add a dental lamina morphogenesis stage between initiation and bud stage relative to mice and humans, and the downgrowth and polarization of the lamina at this stage seems Shh dependent (Buchtová et al. 2008). The more superficially forming premaxilla teeth do not go through the dental lamina stage and do not express *Shh* in the lamina at that time (only in the oral epithelium above the teeth). As in mice, however, epithelial thickenings still formed when Shh was blocked at initiation stages (Buchtová et al. 2008).

A comparative approach as a test of the consensus model

Among the vertebrate classes, the Lissamphibia are notably absent from more recent gene expression-based studies of odontogenesis. This lineage offers additional data for models of odontogenesis because it diverged from Amniotes ~360Ma and its members (salamanders, frogs, and caecilians) have adapted quite differently since their common Tetrapod ancestor with respect to their biphasic life history and feeding requirements.

To provide some information to fill this evolutionary and developmental gap, I have examined *Shh* gene expression and time course data for the pipid frog *Silurana (Xenopus) tropicalis*. Frogs provide a particularly interesting case for testing the sufficiency of odontogenic models

hypothesized from reductionist approaches because they do not develop teeth until the beginning of or well into metamorphosis. Despite this, their free-feeding tadpole stage formed a mouth well before odontogenesis. Additionally, frogs lack the dramatic facial prominence outgrowth and reshaping characteristic of amniotes forming their oral cavities. Studying their tooth development, then, provides an opportunity to investigate what elements of tooth development are coupled to mouth development and which are not, and the natural experiment in uncoupling these phenotypes adds key comparative data for the evolution of odontogenesis across vertebrates.

In this paper, I present the first tooth gene expression data in a frog, and use these results as a jumping off point for re-evaluating data from other vertebrates for their deviation from the consensus developmental genetics model of tooth initiation, in particular the role of the OB. I highlight cases of departure from this model based on a lack of expected phenotypic covariation, and put forth hypotheses for the evolvability and mechanistic decoupling of odontogenic phenotypes.

MATERIALS AND METHODS

The prevailing model for odontogenesis is based largely on laboratory mouse data, the study system for which the most is known. Subsequent studies of zebrafish pharyngeal teeth, and the oral teeth of salmonids and cichlid fish with similar gene expression patterns and functional responses, bolstered the idea that vertebrate odontogenic mechanisms were highly conserved at the level of gene expression across a diversity of developmental morphologies. An attempt to homologize gene expression patterns while accounting for histomorphological variation between the species previously studied made it difficult to know the expectations for odontogenesis in a frog. In addition to examining the *Shh* gene expression patterns in this developmentally unusual taxon, I undertook a more detailed survey of odontogenesis literature documenting *Shh* expression in early tooth development (Figure 1, Table 1).

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, other frog taxa will need to be examined to get a sense for 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 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 hour light and dark cycles, daily food and water changes, and densities of approximately 30 tadpoles per 3L tank after 1 month of growth. Developmental series from Nieuwkoop and Faber (NF) stages 54-59 were sampled in each of 3 clutches, based on limb morphology (Table S1, Figure S1). 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 Richard 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. Procedure after (Sive et al. 2000) with modifications from (C. Miller, unpublished protocols). Additions to the protocol include: 20 min Thisse Bleach following rehydration, 50 mg/ml proteinase K treatment for 25 minutes, and an additional hybridization day. See Table S2 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.

Literature survey/Phenotypic covariation analysis. Figures with *in situ* hybridization results were directly compared when available, along with verbal descriptions of the gene expression pattern of *Shh* during the pre-mineralization stages of odontogenesis, from initial patterning through cap stage morphogenesis.

Tooth initiation phenotypes were compiled across vertebrates for comparison to each other and to the consensus model. OB presence/absence was scored on *Shh* expression and on verbal descriptions consistent with or citing Fraser et al. (2004) (Table 1). OB presence/absence at a given embryological location was then compared to the presence of teeth at that same location in each taxon. Specific cases of taxa that do not fit the consensus model are discussed.

RESULTS

SHH GENE EXPRESSION IN *S. TROPICALIS*

In order to determine whether tooth development mechanisms are conserved across vertebrates even in those taxa which develop teeth much later in life, I examined *S. tropicalis* tadpoles for expression of the epithelial tooth marker *Shh*. Morphological signs of tooth development were not visible until NF stage 56 (see Chapter 1), where 40% of individuals (17/42) had at least one developing tooth (Table S2). 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.

Based on histological observations of tooth development, morphogenetic stages in *S. tropicalis* are broadly similar to those seen in teleosts and amniotes, although the first generation teeth

develop quite superficially (see Chapter 1). Following from this conservation, if *Shh* expression is conserved, it should be visible broadly prior to tooth initiation, then condensing into foci or a dental lamina and marking the oral epithelium through bell stage when ameloblasts begin to differentiate.

Shh is not detected in odontogenic band or placode stages

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 (Figure 2a). Specimens with a few teeth developed them laterally, and while these individual tooth positions expressed *Shh* (Figure 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 (Fraser et al. 2004).

The first teeth to form are very close to the oral surface, and it is difficult to find evidence for a dental lamina joining the earliest set of tooth positions laterally (see Chapter 1). 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 & Ferguson 1990, Harris et al. 2006, Tokita et al. 2013). Consistent with a lack of an early dental lamina, *Shh* is not detected in a ribbon at these stages.

No foci of *Shh* are detectable anywhere in the mouth when teeth are initiating, even adjacent to developing teeth where the next one would be predicted to form (Figure 2b, anatomical left). This is counter to what would be expected for a more teleost-like pattern of tooth initiation, in which many species do not have a laterally continuous dental lamina to express *Shh* in (Sire et al. 2002, Donoghue & Aldridge 2001), but in which those that have been examined for early tooth development reduce their *Shh* expression to individual foci, or tooth placodes, on the oral surface preceding morphogenesis of the first tooth generation (Fraser et al. 2004, Stock et al. 2006, Fraser et al. 2008).

Shh is expressed in the IDE of late-developing first generation teeth

The earliest morphogenetic stage at which *Shh* transcripts have been detected in *S. tropicalis* is the cap stage. *Shh* is expressed in the inner dental epithelium (IDE) of tooth germs (Figure 2d). 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 (Figure 3a).

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 (Figure S2). This is similar to the condition observed in squamates (Richman & 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 (Figure 3b). This dental lamina is not detectably expressing *Shh* 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 & Richman 2010, Wu et al. 2013, Fraser et al. 2013).

Without more detailed 3D reconstructions, it is not possible to verify whether the younger, *Shh*-expressing tooth germs are continuous with the dental lamina, whether they connect to adjacent germs via a successional lamina, or whether another, secretory-stage tooth occupies those positions as well. Observation of more developed tadpoles processed for clearing and staining in whole mount has shown a laterally continuous dental lamina at NF stage 59 joining teeth at varying stages of individual morphogenesis (see Chapter 1 Figure 2).

EVALUATING THE PHENOTYPIC DEFINITION OF THE ODONTOGENIC BAND

The limited patterns of *Shh* gene expression during *S. tropicalis* odontogenesis are surprising given the consensus model for tooth development via an OB, which is supported by broad conservation of expression across other vertebrates. One aspect of the model that has not received much attention is the conflation of a phenotype defined only by gene expression and its anatomical and functional contexts. To better delineate which of these factors covary and may contribute to the development of teeth, I have surveyed the existing tooth initiation literature to explicitly evaluate the correspondence between OB presence and tooth development. In addition, I review the variation in embryonic facial anatomy across vertebrates to evaluate the proposed function of the OB in marking competence to form teeth.

In surveying the published literature on tooth initiation in vertebrates, I have scored the presence or absence of an OB based on whole mount *in situ* data and/or verbal descriptions consistent with or citing Fraser et al. (2004) (Table 1). Whether an OB is acknowledged within a publication partly reflects the history of study (the term OB was not defined until 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 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). The present study does not contribute any evidence that amphibians have an OB, but there is also considerable variation in facial development within amphibians yet to be evaluated (see below).

Discrepancies between *Shh*-defined OB and tooth formation

One important question regarding the OB is whether a causal relationship can be inferred between it and later odontogenesis. To test whether the OB is necessary or sufficient for vertebrate tooth formation, the presence of a *Shh*-defined OB was mapped to the subsequent presence/absence of teeth in that jaw region. While the relationship holds true for oral teeth in the teleosts for which the OB was defined, there are numerous exceptions that question the role or identity of the OB in the consensus model.

<u>The OB is not sufficient to fully form teeth</u>

In some cases, 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 non-venomous snakes, oral *Shh* expression was reported to not become restricted to individual tooth-associated foci until those teeth are at bell stage with a successional dental lamina apparent (Buchtová et al. 2008). Thus, a focal oral domain of *Shh* does not appear to be required intermediate for individual tooth initiation and morphogenesis in this group. Consistent with the hypothesis that the OB marks dental-competent epithelium, the persistence of the snake OB later into development suggests that it is insufficient to trigger snake tooth morphogenesis on its own.

Venomous snakes, in particular *Naja siamensis*, *Trimeresurus hageni*, and *Calloselasma rhodostoma*, provide additional evidence that the OB's role in tooth initiation is permissive. These species, which belong to two distantly related snake families Elapidae and Viperidae, have fangs in the front of their mouths, lack a dentition anterior to the fangs, and a large anterior region of the developing maxilla is edentate because the fangs arise posteriorly (Vonk et al. 2008). During early development in both of these families, however, an OB is present that spans a jaw region considerably anterior to the fang primordium. What does develop in this region has been termed a dental ridge – an anterior dental lamina invagination that initiates transient tooth buds (Vonk et al. 2008). The lack of a dentition in the anterior maxillary region despite the expression of an OB means that additional signals are required to fully form teeth, and that the OB is not a reliable signal for where adult teeth will be found. That this phenomenon of *Shh* retention in the dental ridge appears to have evolved convergently within snakes indicates that *Shh* expression in this region of the maxilla may be serving a different, essential function to that hypothesized for the OB or that its regulation in that region is coupled to another expression domain. Data from the same study show an additional OB on the developing premaxilla, but the status of tooth development in this region remains to be evaluated.

Mice exhibit a condition similar to that seen in venomous snakes in their diastema region, where tooth buds form lateral to the incisor and anterior to the molars but then regress. At least a subtle inbudding of a dental lamina, as well as up to 7 transient tooth primordia have been detected in the mouse diastema (Peterková et al. 2002, but see also Keränen et al. 1999). If these observations are accurate, an OB delineating tooth competence is predicted to exist which spans the diastema. Consistent with this prediction, Keränen et al. (1999) found a continuous band of *Shh* in the mouse at E11 that then became restricted to budding teeth, including rudimentary diastema tooth germs, suggesting that an OB was present in a region that ultimately became toothless. By the time mandibular tissue transplant experiments have been conducted at E11.5, the diastema epithelium is not able to form teeth (Cobourne et al. 2004). Other mammals that have been examined at early developmental stages and do not have a diastema have an OB (Miyado et al. 2007, Yamanaka et al. 2007), although in both diastema and non-diastema possessing mammals, molars are initiated posteriorly, creating new tooth row tissue, after the OB stage has demarcated putative competent epithelium. It has been suggested that these develop from the primary dental lamina under a successional tooth-like mechanism (Juuri et al. 2013).

Studies of early chick craniofacial development suggest that birds have an OB (Helms et al. 1997) and that the earliest epithelial patterning stages for odontogenesis occur although they do not form teeth (Mitsiadis et al. 2003). In this case, a lack of response from the underlying

mesenchyme has been suggested as an explanation for the edentate phenotype (Mitsiadis et al. 2003, Louchart & Viriot 2011), reinforcing the insufficiency of the OB in the prediction of ultimate tooth phenotypes.

Observations of phenotypic correlations in these taxa are consistent with evidence from functional manipulations that there are additional epithelial factors involved in tooth initiation as well as reciprocal epithelial-mesenchymal interactions. Together, they suggest that a *Shh*-expressing OB is not sufficient to explain tooth presence/absence in comparative embryological studies (Figure 4a).

The OB is not necessary for most of the tooth developmental program to occur

Further examination of early tooth developmental events highlights several instances where no OB is present. Crocodiles express *Pitx2* but not *Shh* in a band prior to the formation of first-generation teeth (Tokita et al. 2013). Similarly, alligators lack an OB domain prior to tooth initiation (A. Lainoff et al., personal communication). The expression of *Shh* in the dental lamina of these taxa is distinct from any kind of *Shh* in a superficial epithelial field prior to tooth development. The dental lamina is marked by *Shh* in alligators when it originates within the jaw, after the first teeth have undergone morphogenesis (Westergaard & Ferguson 1990, Harris et al. 2006, Wu et al. 2013).

One aspect of the crocodylian teeth that initiate without an OB is their progressive histomorphogenesis relative to other tooth generations. The first teeth to form are non-functional and develop quite superficially, evaginating, depositing dentine, and then submerging into the mesenchyme (Westergaard & Ferguson 1990). First-generation teeth that are initiated as the *Shh*-expressing dental lamina is forming have a fully developed enamel organ and begin forming deeper in the jaw. The development of early, non-functional teeth in species that lack an OB might argue that oral *Shh* is necessary for the initiation of fully functional teeth, but an OB is certainly not necessary for creating individually spaced, functional tooth precursors with most of their histomorphogenetic properties (Tokita et al. 2013, Wu et al. 2013, Westergaard & Ferguson 1990).

The bearded dragon (*Pogona vitticeps*) has superficial teeth that form prior to dental lamina formation (Handrigan & Richman 2010), but the status of its OB is an open question, as the published description of the bearded dragon OB makes reference to oral epithelial *Shh* expression lingual to the dental lamina. Suggestively, inhibiting *Shh* during the development of bell stage teeth in the bearded dragon caused them to phenocopy the histology of its rudimentary teeth (Handrigan & Richman 2010). *Shh*, however, is expressed and functional during cap and bell stages of the rudimentary tooth epithelium, so *Shh* defects do not explain the mineralization defects seen in rudimentary teeth and suggest that the phenocopy in bell-stage functional bearded dragon teeth is a superficial comparison. Across vertebrates, first generation teeth are often rudimentary (Sire et al. 2002), including in cichlids, which have been shown to possess an OB (Fraser et al. 2008), so not every case of rudimentary tooth development can be attributed to the lack of an OB.

S. tropicalis gene expression data reflect similarities to the crocodylian condition where there is no OB (or dental lamina) before the first generation of teeth. In this frog, there is no current evidence whether its first generation teeth become functional (i.e. erupt), but they form submerged in the jaw, have a blood supply, and have more developed enamel organs than bearded dragon teeth although they lack a stellate reticulum (Handrigan & Richman 2010, see Chapter 1). As to the possibility that first generation *S. tropicalis* teeth are rudimentary, they do not histomorphologically resemble rudimentary first generation teeth in the amniotes for which morphological data are available. Developmentally delayed *S. tropicalis* tadpoles, which appear to have malformed first-generation teeth despite normal (though time-delayed) external morphology, exhibit tooth phenotypes which resemble bearded dragon rudimentary teeth, including a mineralized cap over a shallow condensation of mesenchyme (Figure S3). Bearded dragon rudimentary teeth lack cervical loops and an enveloping outer dental epithelium (ODE), both of which appear to develop in *S. tropicalis* first generation teeth (Figure 3b, see Chapter 1). When comparing first generation *S. tropicalis* teeth to the morphologies of crocodilian tooth generations displaying progressive histomorphogenesis, *S. tropicalis* teeth reach a secretory stage with an ODE that fully covers the other epithelial structures but remains close to the oral epithelial surface (see Chapter 1), much more closely resembling the later-forming 'submerged teeth' of alligators than the earliest 'surface teeth' of alligators which lack an ODE at the tooth apex (Wu et al. 2013, Westergaard & Ferguson 1990).

Although there is not currently a definitive case in which fully functional oral teeth are able to develop without the presence of an OB, the instances mentioned above suggest that most of the developmental program for patterning and morphogenesis of teeth can occur without the presence of an OB. In addition, even without an OB in alligators, functional teeth arise from successional lamina-like structures in association with rudimentary teeth (Wu et al. 2013), further arguing that an OB is not necessary to form teeth in the oral cavity (Figure 4b).

Based on comparative embryology and a lack of predicted covariation between OB and tooth phenotypes, *Shh*-expressing OB expression is insufficient to account for the patterns of oral tooth distribution in vertebrates.

TEMPORAL CONTEXT OF THE OB

The functional evidence in various vertebrates supports a role for *Shh* in the setup of the midline and of the very early oral domain, but it remains unclear what the source of this Shh signal is and it may well precede the existence of the OB phenotype. This possibility is further supported by comparison of the OB phenotype across vertebrates and its unreliable mapping to functional tooth distribution. In this case, a comparative approach to oral phenotypes occurring before the OB may further clarify the role of the OB in vertebrate tooth development.

Precursors of tooth development: The stomodeum

Development of the secondary mouth (morphogenetic modules involving neural crest cells that contribute to teeth and jaws) is superimposed upon the patterning and development of the primary mouth, or stomodeum (Dickinson & Sive 2006, reviewed in Soukup et al. 2013). The stomodeum is the endoderm-ectoderm boundary within the developing oral cavity (Dickinson &

Sive 2006, Helms et al. 1997), and later becomes the pharyngeal opening (Dickinson & Sive 2007). It has been suggested that the rupture of the buccopharyngeal membrane initiates secondary mouth development (Soukup et al. 2013). In some taxa, the stomodeum has been implicated in tooth induction (bichir, Kralovic et al. 2010; salamanders, Lumsden 1988; caecilians, Wake 1976).

There are also gene expression patterns that suggest a spatiotemporal link between stomodeum positioning and OB positioning. A recent review of stomodeal development does not mention *Shh* at all, although the authors concede that the research is too new to know the functional genes involved (Soukup et al. 2013). *Pitx1* and *Pitx2*, however, are implicated in marking the stomodeum from the earliest stages (Dickinson & Sive 2007, Schweickert et al. 2001, Lanctôt et al. 1997), and continue expression into the epithelial organ derivatives at least in the mouse (Lanctôt et al. 1997, St. Amand et al. 2000, Mucchielli et al. 1997). These data provide evidence of continuity between stomodeal gene expression and the eventual location of the OB. Although it was not reviewed, several species express *Shh* in their stomodeal epithelium after the rupture of the buccopharyngeal membrane (Sarkar et al. 2000, Eberhart et al. 2006, Buchtová et al. 2008, see below). In the zebrafish stomodeum *Shh* expression is required for NC survival and directs upper jaw cartilage condensation (Eberhart et al. 2006), and in amniotes there is a morphologically canalized stage of facial prominence fusion, after which time *Shh* dosage is important for species-specific outgrowth of facial prominences to create facial morphology (Liu et al 2010).

The current best developmental model for stomodeum formation, however, is *Xenopus laevis* (Dickinson & Sive 2006, 2007, 2009), which presents a conundrum for evaluating the present question of its relationship to the OB. Based on the results of this study in *S. tropicalis*, the *X. laevis* model is not likely to have an OB that forms teeth, although it may have a non-tooth-forming, *Shh*-expressing epithelial domain at these early stages (Figure 5). Overall, *X. laevis* has been assessed to have the plesiomorphic condition for stomodeum formation in gnathostomes (Soukup et al. 2013). Facial prominence fusion occurs subtly in *Xenopus*, but homologous prominences can all be identified ringing the developing stomodeum (Kennedy & Dickinson 2012).

Taken together, these comparative data suggest that *Shh* in the stomodeum is correlated with a conserved, likely plesiomorphic role in secondary mouth formation across vertebrates, if not always oral teeth. The role of the stomodeum in amniote tooth formation has not been tested as in amphibians, but the timing of tooth development does not rule it out as a factor (Lumsden 1988, Mina & Kollar 1987). The conserved OB phenotype identified for gnathostomes may well be a remnant/readout of gene expression that is maintained by contact between the endoderm and ectoderm during the course of oral development, but that does not necessarily have a role in tooth initiation.

Animals with a derived form of stomodeum formation provide testable cases for examination of the relationship between the stomodeum, the OB, and tooth formation. One (non-monophyletic) group of these animals includes salamanders, some teleosts and some frogs, which form a stomodeal collar in which the epithelium grows inward around an infilling of endoderm (Soukup et al. 2008, Soukup et al. 2013). In ray-finned fish (including zebrafish), a stomodeal wedge

forms in which no oropharyngeal tube separates upper and lower facial prominences (reviewed in Soukup et al. 2013). In both cases, the stomodeum then ruptures by cavitation, and the breakdown of a rostro-caudal series of epithelial bridges within the oral cavity (Soukup et al. 2013). Observations of development in the chondrostean fish *Polypterus senegalus* have shown that the tissue bridges span several invariant locations within the developing mouth, at the same locations where tooth buds first form, suggesting a series of buccopharyngeal membrane-like boundaries may contribute to the locally optimal conditions for tooth initiation (Kralovic et al. 2010).

If the OB is an endoderm-ectoderm marker, in these species one might predict a series of OB-like domains that mark where these teeth form, or a remnant domain of *Shh* unrelated to the locations of tooth formation because teeth are triggered by a mechanism other than *Shh* expression. The identification of oral teeth consisting of both ectodermally- and endodermally-derived enamel organs in *Ambystoma mexicanum* (Soukup et al. 2008) lends support to the idea that the endoderm/ectoderm boundary, where *Shh* is predicted to be expressed (Helms et al. 1997, see below), is not always the location of tooth formation.

Precursors of tooth development: *Shh* in craniofacial patterning

Shh expression in the stomodeal ectoderm has been studied intensively in the context of craniofacial patterning. All amniotes examined thus far express *Shh* expressed in a forebrain-induced signaling center called the frontonasal ectodermal zone (FEZ), located in the roof of the mouth at the equivalent of mouse E10/chick HH20 and then it is induced lateral to the FEZ in stripes along the maxillary processes (Hu & Helms 1999, Marcucio et al. 2011). This Shh signaling plays a role in facial prominence outgrowth and in determining facial width (Young et al. 2010). 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 (Jackman et al. 2010, Stock et al. 2006). These observations suggest a similar type of craniofacial patterning mechanism exists in non-amniote gnathostomes as well.

Other researchers have not made explicit connections between these *Shh* domains and the OB, most likely because craniofacial development studies have been dominated by observations in the edentate chicken. Helms et al. (1997), however, observed a common thread among the dynamic *Shh* expression domains in craniofacial primordia: that they were found at the locations of endodermal-ectodermal epithelial boundaries in the face and 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 (or lack thereof) of the OB.

SUMMARY AND DISCUSSION

As a test for the consensus model of early tooth development and to better understand how conserved developmental mechanisms can be reconciled with vertebrate dental diversity, I investigated *Shh* expression in *S. tropicalis*, a member of a phylogenetic group that is underrepresented in developmental genetic studies of odontogenesis and that has performed a natural experiment by delaying odontogenesis until well after primary and secondary mouth formation. Compared to *Shh* expression in the consensus odontogenic model, the reduction in *Shh* expression patterns, especially during initiation stages, in *S. tropicalis* seems unusual, but it is not outside the range of variation across vertebrates when phenotypic covariation is examined in more detail. To that end, I have also evaluated the OB in its anatomical context, and with the functional evidence available, found that *Shh* expression defining the OB can be understood in terms of its relationships to other anatomy and primary mouth development.

Across the range of developmental variation seen in vertebrates, there are several taxa that do not fit the 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. 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. Lumsden 1988, Mina & Kollar 1987, Chen et al. 1996). Given its anatomical and temporal context, it may be that a *Shh*-expressing OB is a readout of stomodeum rupture, or a marker of endoderm/ectoderm border areas (Helms et al. 1997, Huysseune et al. 2009).

Differences in frog odontogenesis provide insights into tooth initiation

In the case of frogs, there are two possible reasons for a lack of OB expression close to the time of tooth initiation: 1) the OB was not observed during the developmental time window under study, and 2) *Shh* does not function in tooth initiation. Regarding timing, frog teeth develop just before or during metamorphosis, a number of weeks after mouth formation has occurred. This evolutionary decoupling of phenotypes allows us to assess OB function more precisely. That no OB is detected just prior to tooth formation in *S. tropicalis* suggests that it is not necessary as an immediate trigger of tooth formation. The possibility that *Shh* expression is present at these stages, but that the whole mount method did not detect it seems unlikely, given the positive detection of *Shh* expression at both earlier and later stages. If *Shh* expression is a (functional or nonfunctional) readout of stomodeum development, one would expect to see an oral domain of *Shh* much earlier in *S. tropicalis* development. This possibility is suggested by early *in situ* data but should be tested further. It is also possible that there is an OB at an earlier tadpole stage that was missed by the study window, but the high amount of 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. The strong *in situ* 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 topographically superficial phenotype than an IDE is.

A lack of *Shh* function in tooth initiation could be manifest at several phylogenetic levels of investigation: it could be vertebrate-wide, frog-specific, or more restricted to the relatively simplified pipid tadpole condition. Based on the comparative anatomical and functional data presented here for vertebrates, other genes such as *Sox2* and *Pitx2* that are co-expressed in OB have a more significant function in tooth initiation than does *Shh*, given its lack of covariation with subsequent tooth phenotypes (see below). Another explanation that merits further investigation is that the expression domain called the OB might function to pattern other craniofacial tissues, and not the initiation of teeth.

The expression pattern of *Shh* in frogs may also differ from patterns in other vertebrates due to the evolution of novel tadpole craniofacial cartilages within the group. These suprarostral and infrarostral cartilages, which are located at the front of the mouth and support varied feeding modes in the tadpole phase of life, are resorbed or incorporated into Meckel's cartilage, respectively, during metamorphosis (Svensson & Haas 2005). In particular, the suprarostral cartilage is forming during mouth formation and present superior to the oral cavity during tooth development in *S. tropicalis* (see Chapter 1). A series of xenoplastic transplant experiments performed between salamander and frog tadpoles provide additional evidence for a frog-specific modification of odontogenesis. Frog neural crest gave rise to the mesenchymal components of teeth when transplanted into a salamander tadpole, in addition to giving rise to suprarostral and infrarostral cartilages (Wagner 1955). When the reciprocal experiment was performed, a salamander visceral skeleton but no teeth formed in the frog host, although larval salamanders typically bear teeth (Wagner 1949). A third experiment in which the presumptive stomodeum from a salamander was transplanted into a frog, the frog tadpole developed salamander-like teeth (Henzen 1957). This series of experiments suggests that the frog stomodeal epithelium lacks a signal for tooth initiation that salamander larvae possess during development.

Shh is unlikely to be that missing inductive signal in frogs based on the gene expression detected here, and it also suggests that there is a signal missing early on that comes on later or has a different identity in frog tadpoles. While neural crest competence is present in frogs during tadpole stages (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 with a novel mechanism during larval stages. If a *Shh*-expressing OB is present at early stages, it may still be the epithelial competence factor in frogs, but *Shh* does not appear to be acting as a competence signal during perimetamorphic stages. Later tooth *Shh* expression in *S. tropicalis* argues that *Shh* was not dispensed with entirely during tooth morphogenesis in frogs or in pipids. As is the case for at least some other pipid frogs (Shaw 1979, see Intro chapter), *S. tropicalis* initiates its teeth relatively earlier than most frogs do, which may be related to its simplified tadpole form (Cannatella 1999, see Intro chapter), or to yet another modification to the tooth induction and competence signals that involved displacement of *Shh* expression. While a single representative from a group with such disparate larval modifications (amphibians) is not sufficient for a complete picture of amphibian odontogenesis, these gene expression data in a model amphibian lay the groundwork for future comparison and experimentation.

Phenotypic correlates of the OB: What is functional in tooth induction/competence?

In species where more detailed tooth gene expression studies have been conducted, other genes have been found that are co-expressed in the region of the *Shh*-expressing OB, namely *Pitx2* and *Sox2*. *Pitx* gene expression precedes stomodeal invagination and rupture, being expressed from early on in the extreme anterior region of the embryo (Dickinson & Sive 2007, Soukup et al. 2013), whereas *Shh* expression is induced via the forebrain and neural crest after the stomodeum ruptures (Marcucio et al. 2005). *Pitx2* and *Shh* are co-expressed in each new tooth row for the cichlids examined during tooth initiation (Fraser et al. 2008), and interestingly, the successional lamina in cichlids expresses *Pitx2* but not *Shh* (Fraser et al. 2013). *Pitx2*'s expression pattern also tends to have sharper boundaries than does *Shh*'s during OB stages (e.g. Keränen et al. 1999, Stock et al. 2006) and its distribution responds to and regulates the levels of antagonistic FGF8 and BMP4 signaling in the early mouse jaw (St. Amand et al. 2000, Liu et al 2003). Together with recent evidence that Pitx2 is an upstream regulator of several other transcription factors in dental cell lineages (Venugopalan et al. 2011, Li et al. 2013, Zhang et al. 2013), this transcription factor is a good candidate for further analysis of a functional OB phenotype.

Sox2, a stem cell marker, also marks the OB in mice, and then fades to foci just lingual to developing mouse placodes (Juuri et al. 2013). At later stages, Sox2 protein is found in the oral epithelium, dental lamina, and successional dental lamina of ferret, mouse, snake, alligator, and gecko teeth (Juuri et al. 2013) *Sox2* mRNA was also detected in the dental mesenchyme at E11 (Zhang et al. 2012), so in contrast to *Pitx2*, it is not an exclusively epithelial marker of the OB. However, the protein was only detected in the epithelium, suggesting control at the post-translational level (Zhang et al. 2012). Little is known about its functional role(s) in odontogenesis, but further experimentation may reveal a functional role during OB stages.

CONCLUSIONS

The re-evaluation of the OB consensus model presented here is a good example of the role that evolutionary biology and a comparative approach can play in developmental genetics and vice versa. In order to move the field forward, we need to move beyond the reasonable assumption of broad mechanistic conservation to understand at what phylogenetic nodes higher order covariations break down. It is these departures from conservation that indicates evolution: that something in the developmental system or the variation available has changed. For example, data from Old World monkeys suggest that developmental mechanisms during odontogenesis have the ability to constrain population-level dental variation (Grieco et al. 2013); getting a sense for the range of the possible given a generally conserved developmental mechanism will help us understand the flexibility of the genotype-phenotype map over time.

Careful attention to the timing of functional manipulations and precise sequence of ontogenetic events in different animals is critical to sorting out the OB's role, if any, in odontogenesis. *Shh* clearly has cascading functions in orofacial patterning, and looking for patterns of phenotypic covariation, whether in response to a specific functional hypothesis or at a larger scale, is another useful method to determine which portions of *Shh*'s dynamic gene expression phenotype are functional in what contexts. Comparative analyses can illuminate which functions and phenotypes can be decoupled, indicated by the *Shh* expression patterns for *S. tropicalis* tooth initiation vs. morphogenesis, and perhaps in craniofacial vs. dental patterning across vertebrates.

These hypotheses for the roles of *Shh* expression in different tissues during craniofacial and tooth development can be tested with tissue-specific removal of *Shh* expression, which now seems feasible given the development of transgenic methods in *S. tropicalis* that may allow for such tissue-specific gene inactivation.

At the genomic level, comparative analyses have begun to identify separate gene regulatory modules for *Shh* expression domains including an oral epithelial enhancer, MRCS1, with high conservation across amniotes and to some degree across gnathostomes (Sagai et al. 2009, Irimia et al. 2012). Studies providing phenotypic covariation data can yield predictions for gene interactions and binding sites, which may facilitate the identification of other enhancers and previously unknown cell- and tissue-level interactions. On the most translational level, identifying interactions between first-order gene expression and subsequent morphological phenotypes may well minimize the side effects of gene therapies and other efforts to treat birth defects by providing the ability to target discrete causative phases of the developmental process.

Can the consensus model for tooth initiation account for the variation of phenotypes observed in nature? The OB as currently defined by *Shh* expression is insufficient to explain how a conserved mechanism is able to accommodate patterns of heterochrony and toothlessness across vertebrates. Furthermore, the temporally preceding and following steps in oral development show a fair amount of variation across species, so the model based on gene networks in a limited anatomical context (i.e. epithelial-mesenchymal interaction), or perhaps any consensus model, becomes less useful as a guide for further research once it has been subjected to multiple tests and the results that validate the model are given the most attention. Perhaps the OB is better defined by the expression of additional genes, such as *Pitx2*, or reflects some other developmental process (e.g. a marker of endoderm-ectoderm boundaries), which would better correlate to the distribution of functional teeth in different species.

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Figure 1. Cladogram depicting early stage tooth development across vertebrates. Specimens are whole mount in situ hybridizations for *Sonic hedgehog*. Individual tooth focus-like stages are present across the taxa studied thus far. Image credits, 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.

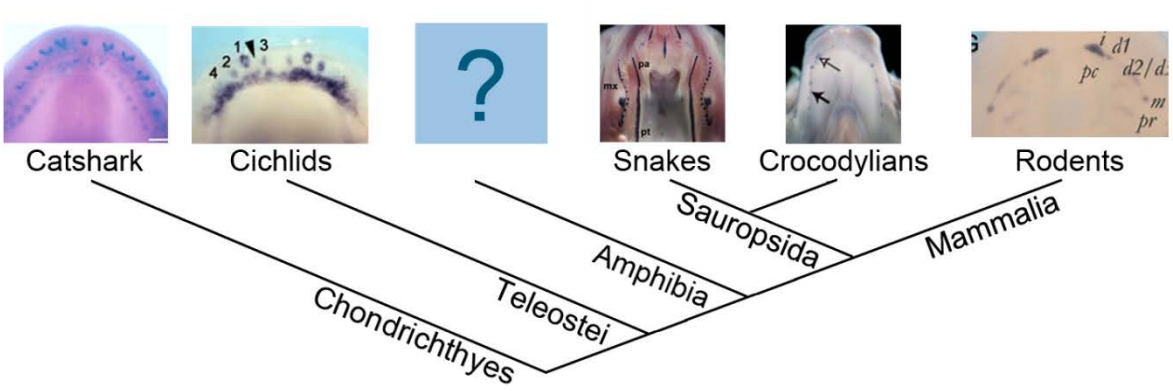

Figure 2. Whole mount *in situ* hybridization for *Shh* in *Silurana (Xenopus) tropicalis*. (a) Non-toothed and (b) 3-toothed upper jaw of NF stage 56 tadpoles. Ventral view. (a) No *Shh* expression is detected above background levels in the oral area prior to tooth formation. (b) Three developing tooth germs (arrowheads) express *Shh* transcripts with no gene expression detected in the intervening marginal jaw areas. White box marks area magnified in (d). (c) Schematic illustration of tadpole from dorsal view. Red box indicates tissue dissected for *in situ* hybridization. Compass points indicate orientation for ventral view images, with anatomical right at the left. (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 morphogenetic stage for which *Shh* transcripts have been detected.

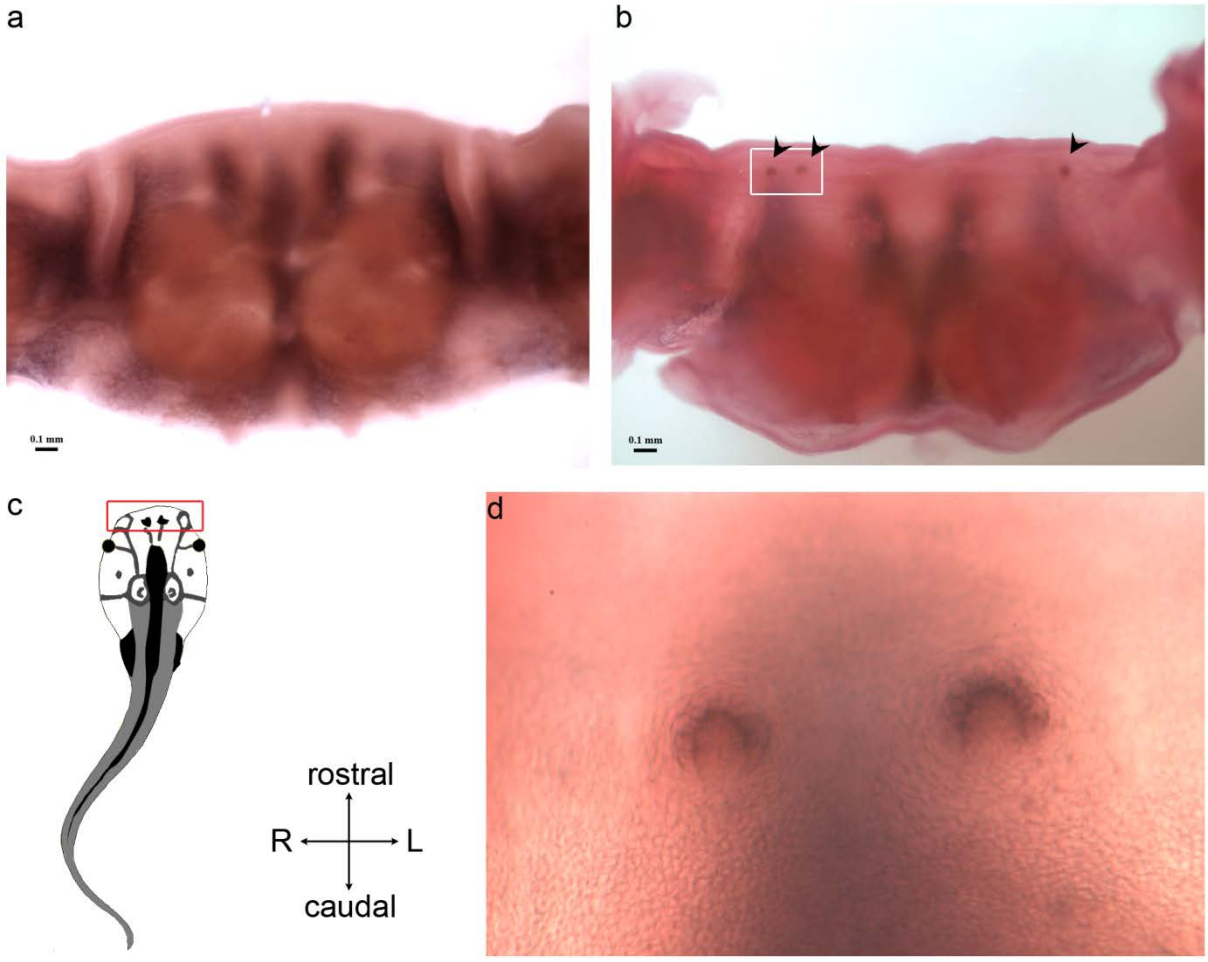

Figure 3. Whole mount *in situ* hybridization for *Shh* showing tooth expression. (a) A full tooth row of a NF stage 58 tadpole consisting of mostly secretory stage tooth germs. Four germs express *Shh* transcripts and are unevenly positioned across the jaw. White box marks area magnified in (b). (b) 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.

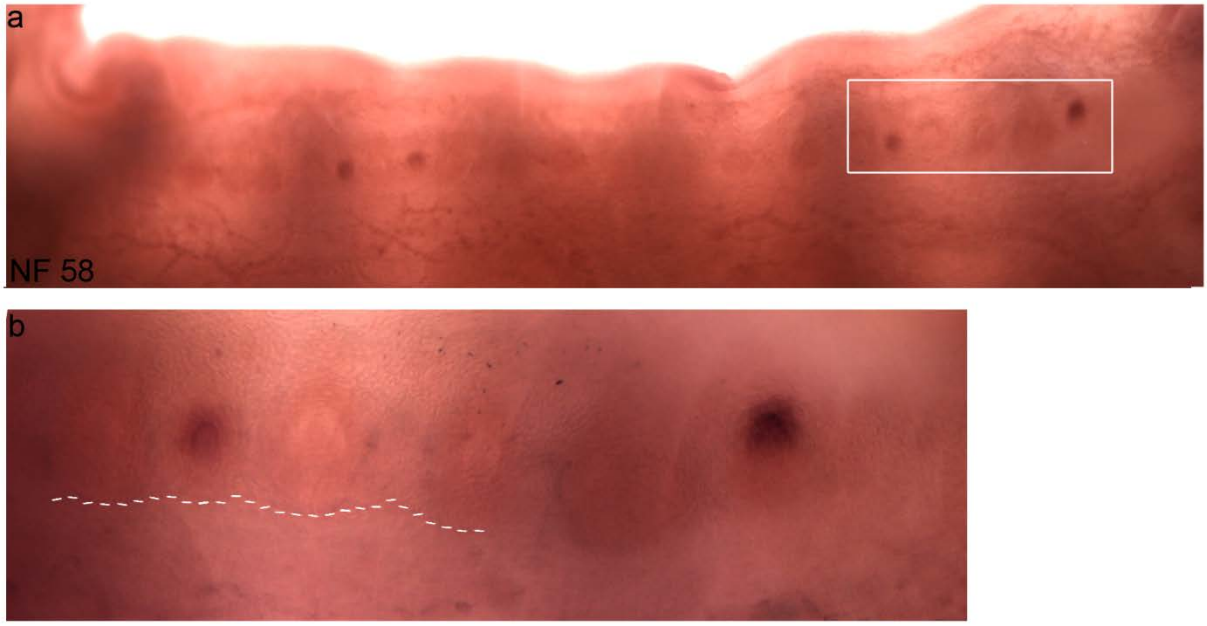

Figure 4. Scenarios in which the OB is not sufficient or necessary for fully formed teeth. Developmental transformations in time of an upper jaw with a *Shh*-expressing OB (purple arc) into a tooth-bearing upper jaw. Evolutionary "experiments" in which the OB is not sufficient to form teeth occur in (a) when at least one portion of the jaw expresses an OB but is ultimately toothless, such as in snakes, mice, and birds. 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*.

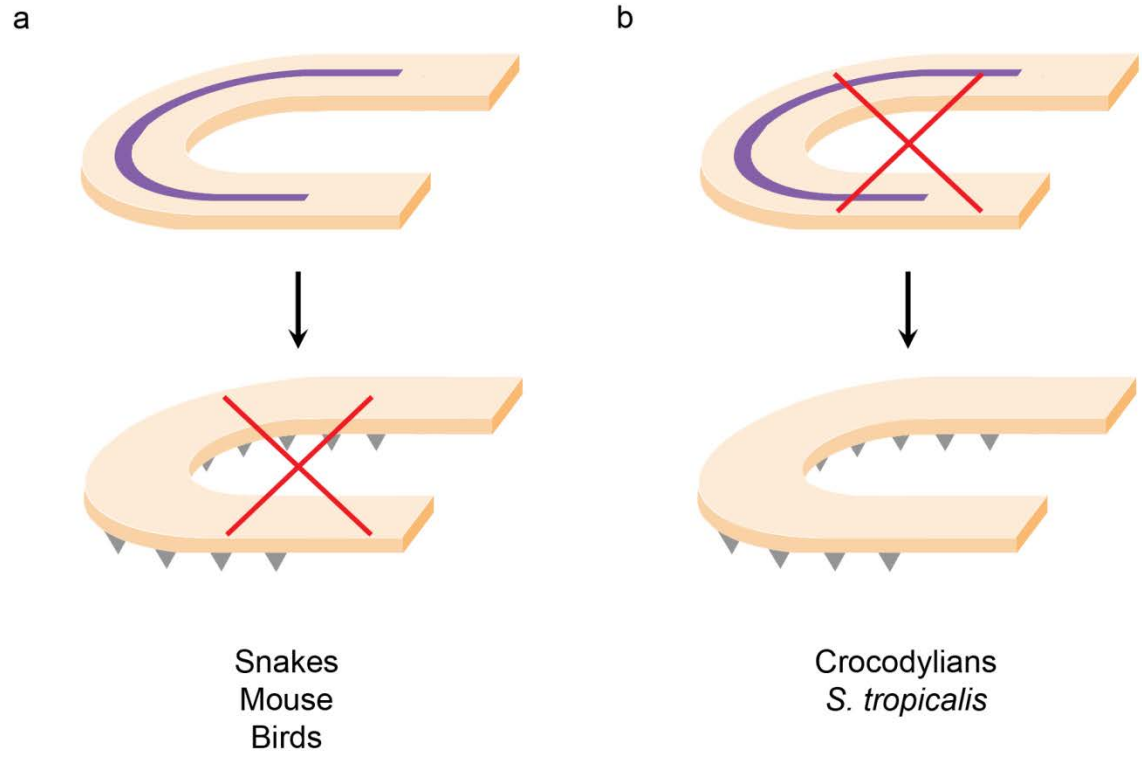

Figure 5. Whole-mount *Shh* expression in (a) lateral and (b) anterior views of the same NF stage 35 *S. tropicalis* early tadpole. (c) schematic of NF stages, midline sagittal sectioned, after Dickinson & Sive 2007 for *Xenopus laevis*. *S. tropicalis* stages have not been calibrated for stomodeum development, and stage determination in (a) and (b) was determined by the presence of eyes, tail ratio to body, and segregated somites extending into the tail (Nieuwkoop & Faber 1967). The *Shh* transcripts detected in the foregut endoderm and developing brain suggest conserved roles for Shh in craniofacial development prior to stomodeum rupture. In *X. laevis*, the stomodeum ruptures at NF stage 40 (Dickinson & Sive 2006).

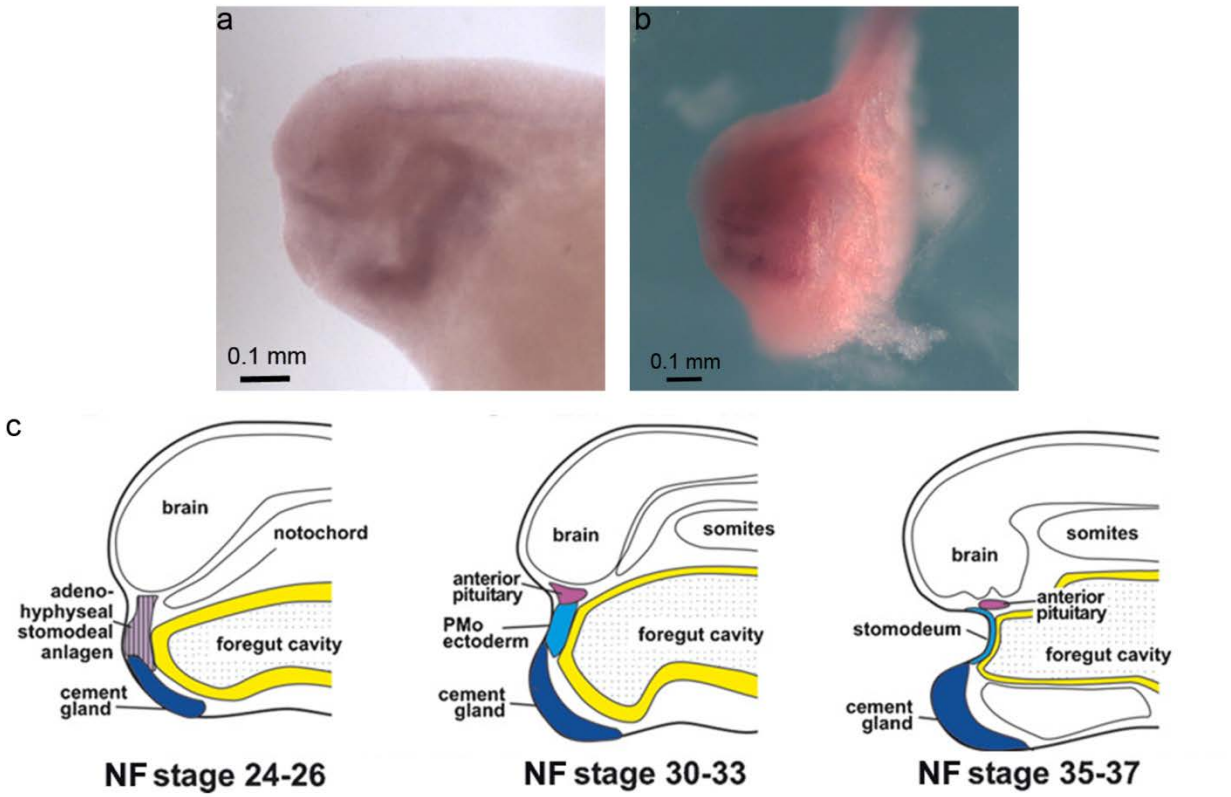

Table 1. Taxa that have been examined for an OB. OB presence/absence was scored based on published *Shh* gene expression images and on verbal descriptions consistent with or citing Fraser et al. (2004). *data not shown in publication. [1]Oral *Shh* expression detected, but not overlapping *pitx2* as in other teleosts (Stock et al. 2006)

	Organism	*Shh* detected?	OB named?	Citation
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[1]	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
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 & 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*	Y	Handrigan & Richman 2010
	Eublepharis macularius	Y*	Y	Handrigan & Richman 2010
	Alligator mississippiensis	Y*, N	N	Harris et al. 2006, Wu et al. 2013
	Crocodylus siamensis	N	N	Tokita et al. 2013
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

Chapter 3

Sex, size, and development: Narrowing down influences on adult craniodental variation in *Silurana* (*Xenopus*) *tropicalis*

ABSTRACT

Developmental and physiological processes have a large influence on the phenotypic variation that manifests in a population. Craniodental phenotypes are useful for studying the relative influences of these processes because the functional constraints of the oral apparatus are superimposed on the development of the entire cranium. Amphibians, with their largely homodont, marginal dentitions spanning multiple jaw bones, provide a good model in which to study these various influences, including body size, sexual dimorphism, embryological primordia, and localized remodeling. To examine these proximate influences on intraspecific variation, I assessed skeletal phenotypic variation in the sexually size-dimorphic frog *Silurana* (*Xenopus*) *tropicalis*. Craniodental linear measurement and tooth count data suggest that body length is less strongly correlated with the size of the jaw and dentition than cranial length, and overall cranial proportions may be operating under other constraints. Sexual dimorphism is apparent, but cranial size can explain most of the differences between males and females. Correlations at multiple hierarchical levels of investigation demonstrate a relative independence of the premaxilla or anterior midline from the rest of the jaw, and decoupling of tooth row and jaw bone phenotypes at more local developmental levels. At least some of this standing craniodental variation appears to have a genetic basis in *S. tropicalis*.

INTRODUCTION

The phenotypic variation expressed in a population is the outcome of developmental and physiological processes occurring during individual lifetimes. The expression of morphology, then, is the result of the superimposition of local onto systemic influences accumulating over ontogeny. Identifying the hierarchy of these local and global processes on a phenotype ─ the developmental architecture of a trait ─ is critical to both our understanding of the developmental processes at work in a population and to our interpretation of how morphology has evolved and may evolve (Hallgrímsson et al. 2009, Schlosser & Wagner 2004, Wagner 2001, Gould & Garwood 1969, Wright 1932). Each developmental process produces phenotypic integration, or the tendency for there to be spatial covariation in traits (Olson & Miller 1958) through its mechanism of action. Variational modules are subunits of the phenotype that tend to covary more with each other than other parts of the phenotype (Wagner 1996, Klingenberg 2008), and are commonly identified by examining patterns of phenotypic covariation and correlation in populations or species (e.g. Gingerich & Winkler 1979, Zelditch et al. 1992, Cheverud 1982, 1995, Goswami 2006, Klingenberg et al. 2004, Zelditch et al. 2008, Hlusko & Mahaney 2009, Drake & Klingenberg 2010). When observed in a set of adult organisms, any variational modules identified could well be the output of a series of inferred and identifiable developmental processes (Cheverud 1996, Willmore et al. 2007).

While it has been argued that examination of patterns of phenotypic covariation is unlikely to provide insight into developmental architecture due to the complexity of most morphological traits (Hallgrímsson et al. 2009, Grieco et al. 2013), approaching variation with *a priori* hypotheses about the effects of development at different hierarchical levels may allow us to understand developmental signatures in adult morphology (e.g., Nemeschkal 1999, Klingenberg & Zaklan 2000, Polly 2005, Willmore et al. 2006, Young & Hallgrímsson 2005, Reno et al. 2008, Zelditch et al. 2008, Grieco et al. 2013, Head & Polly 2013). The effects of body size, sexual dimorphism, embryological anlagen, and local physiological remodeling can all be traced back to developmental processes that predict patterns of regionalization in morphological variation. Evaluating the morphology affected (or not affected) by these different processes will allow us to better define phenotypes and trace their evolution (Lovejoy et al. 1999, Hlusko 2004).

Within the adult cranium, the dentition is a functionally important unit for mastication, prey capture, and social behavior. It develops in tandem with those developmental events responsible for its bony support structure and the hormonal influences on the developing body. Additionally, craniodental patterning and morphogenesis have been subjects of intensive study, providing information on localized developmental effects on adult phenotypes. When taken as a trait complex in which to identify the influence of superimposed developmental processes, craniodental phenotypes can be defined at the cranium, jaw, tooth row, and individual tooth levels. In the next section, I review some of the findings regarding developmental influences on the craniodental complex related to body size, sexual dimorphism, embryological anlagen, and local physiological remodeling as a prelude to their examination in intraspecific variation in the sexually size-dimorphic frog *Silurana* (*Xenopus*) *tropicalis* (Figure 1).

As an anuran amphibian, *S. tropicalis* exhibits protracted development via an extended larval stage and a dramatic metamorphosis. It also represents a unique case of frog tadpole development in which the dentition develops independently of early craniofacial development, but the tooth-bearing bones of the adult cranium develop during metamorphosis, close to the time of tooth initiation. *S. tropicalis* also has a homodont dentition that has had its development described (Bulckaen et al. 2007, see Chapter 1, Chapter 2) as well as compared to that of the often interchangeably studied phylogenetic relative *Xenopus laevis* (Shaw 1979, Cambray 1976, see Chapter 1). Due to the presence of a local breeding colony, I am able to report on intraspecific craniodental variation in *S. tropicalis* reared in a controlled environment, still a relatively rare dataset for an amphibian and one important for understanding the evolution of morphology.

SYSTEMIC INFLUENCES: BODY SIZE

Developmental factors serving to increase overall body length may also isometrically or allometrically increase the size of craniodental phenotypes. Functionally, it has been argued for mammal species, which replace their teeth only once or never, that occlusal surface area and therefore tooth size must scale allometrically with adult body size in order to meet the energetic needs of a species (Gould 1975, but see Copes & Schwartz 2010 questioning the pattern). Population variation often does not conform to this pattern due to the much longer influence of

environmental factors on body size than on mammalian tooth size before mineralization (Hlusko et al. 2006).

Tooth number in reptiles varies widely across species with no clear relationship to body size (e.g. Bauer & Russell 1990, Rasmussen 1996, Greer & Chong 2007). Developmental studies have identified a change in tooth size with growth from juvenile to adult with replacement (e.g. Poole 1960, Cooper et al. 1970, Kline & Cullum 1984, Bauer & Russell 1990, Mateo & Lopez-Jurado 1997, Torres-Carvajal 2002), and constant tooth replacement in most species creates heterogeneity in the composition of the tooth row because individual teeth develop at different body sizes. Many reptile species add tooth positions to the posterior ends of their jaws into adulthood (e.g. Kluge 1962, Edmund 1969, Cooper et al. 1970, Kline & Cullum 1984, Bauer & Russell 1990, Greer 1991, Ananjeva et al. 2003, Montanucci 1968, Osborn 1974), and some add to the lateral ends of the premaxilla as well (e.g. iguanids, scincids, Kline & Cullum 1984, Greer 1991).

The limited number of amphibian studies that report craniodental variation with body size have been conducted in divergent lineages, making generalizations about tooth and body size scaling in this diverse group difficult. Teeth in adult *Rana pipiens* frogs (Gillette 1955), adult *Desmognathus* as well as most other salamanders (Juterbock 1978, Wake 1963) and fetal teeth in viviparous caecilians (Wake 1980a), for example, are replaced with progressively larger ones as animal size increases. Several frogs, including *Xenopus laevis*, as well as salamander *Pleurodeles waltl* and several caecilians have been observed to add teeth posteriorly in the jaw (Shaw 1979, Cambray 1976, Gillette 1955, Davit-Béal et al. 2006, Wake 1976, Wake 1980a). Intraspecific variation in tooth number does not vary with body size or external head width in some salamanders such as *Plethodon cinereus* (Townsend 1998), but in many other salamanders, maxillary tooth number increases (e.g. Juterbock 1978, Ehmcke & Clemen 2000a, Wake 1963), and in others it decreases as tooth size increases (e.g. *Aeneides lugubris*, Wake et al. 1983). At the level of individual teeth, tooth height and width were correlated with snout-vent length in those measured for at least some species, such as *Plethodon cinereus* (Townsend 1998) and *Aeneides lugubris* (Wake et al. 1983).

SYSTEMIC INFLUENCES: SEXUAL DIMORPHISM

Craniodental phenotypes may also be affected by patterns of sexual dimorphism, tied at least indirectly to endocrine differences between the sexes. These effects can be a consequence of scaling from body size dimorphism or of more localized reception of systemic factors. Some of the most obvious examples of localized craniodental dimorphism come from mammalian dentitions, with dimorphic canines or incisors in many primates, walrus, elephants, suids, and hippos (Lincoln 1994, Martin et al. 1994). Male-biased and female-biased body size dimorphism are both common in reptiles, and the degree of sexual dimorphism can sometimes vary between populations of the same species (Cox et al. 2007). When this dimorphism does affect the cranium, it is largely through the jaw musculature for hypothesized functions in intrasexual competition or dietary partitioning (e.g. Camilleri & Shine 1990, Herrel et al. 1999, Vincent et al. 2004, Ljubisavljević et al. 2010). At the level of the dentition, there are some reports of a higher tooth number in males (iguanid *Sator grandaevus* (Etheridge 1962), *Lacerta* (Cooper 1963), *Ctenotus* (Greer 1991)) though perhaps not beyond what is predicted from body size

differences. The number of maxillary teeth is able to statistically discriminate between male and female snakes in *Achrochordus arafurae*, *Dendrelaphis punctulatus*, and *Dipsadoboa unicolor* (Camilleri & Shine 1990, Rasmussen 1996).

Females are larger than males in 90% of anuran species and 61% of urodele species (Shine 1979, Monnet & Cherry 2002), although some researchers caution that this high frequency of dimorphism may be attributable to differences in the age structure of male and female subpopulations (Howard 1981) or energetic constraints (Woolbright 1989) in the wild. In at least some anurans and urodeles the degree of sexual dimorphism varies between populations (e.g. Miaud et al. 1999, Ivanović et al. 2008, Angelini et al. 2008). The data available for gymnophionan species show that most species lack body size dimorphism (reviewed in Kupfer 2009), but many are dimorphic in head size, measured externally and including variation in jaw musculature (Nussbaum 1985, Jones et al. 2006, Malonza & Measey 2005, Wake 1980b, Presswell 2002, Nussbaum & Pfrender 1998, Teodecki et al. 1998, Delêtre & Measey 2004). Head width is male-biased after size corrections in some salamander species including brook salamanders *Euproctus platycephalus* (Bovero et al. 2003), the aquatic *Amphiuma tridactylum* (Fontenot Jr. et al. 2008) and *Salamandrella keyserlingii* (Hasumi 2010).

Localized hormonal influences have also been described in the craniodental complexes of amphibians. In salamanders, this has been most extensively studied in plethodontid salamanders, which exhibit sexually dimorphic dental variation that is regionalized between the maxillary and premaxillary teeth, either seasonally or year-round (*Eurycea bislineata,* Stewart 1958; *Bolitoglossa subpalmata*, *Oedipina uniformis*, *Nototriton abscondens*, Ehmcke & Clemen 2000a; *Bolitoglossa schizodactyla,* Ehmcke et al. 2003). Male salamanders in this group have monocuspid teeth localized to the premaxilla and occasionally the anterior maxilla (Ehmcke & Clemen 2000a), causing variations in the size and number of teeth between the sexes. The mechanism for the regionalization of these effects in the cranium has been attributed to differential expression of androgen receptors in the premaxillary and maxillary dental laminae (Ehmcke et al. 2003).

Some craniodental characteristics in anurans are also androgen-controlled (Lofts 1984); some are maintained by androgen levels in adults and may vary seasonally, but most osteological traits showing dimorphism are androgen-mediated during development (Emerson 1996, Kelley & Tobias 1989, Hayes & Licht 1992). One example is the fanged frogs of Southeast Asia and Australia, which exhibit male-biased jaw dimorphism in that males have larger heads, more developed jaw musculature, and larger dentary odontoid processes (not teeth) than their female counterparts (Emerson & Voris 1992, Emerson 1996, Katsikaros & Shine 1997, Emerson 1998).

Gonadal differentiation occurs around the same time as first generation tooth initiation in *X. laevis*, where sexual differentiation has been studied more extensively than in *S. tropicalis* (Kelley 1996), so hormonal influences are temporally positioned to play a role in craniodental development. Sexual dimorphism at a local scale has been studied in *X. laevis* larynx morphology (Kelley 1996). Within the dentition, Shaw (1979) reports differences in adult tooth number between the sexes, and Cambray (1976) reports that teeth in females are slightly larger. *X. laevis* has a low level of female bias compared to that of other anurans like *Pelobates* and *Alytes* (Monnet & Cherry 2002), so the effects of dimorphism in *S. tropicalis* may be relatively

subtle compared to those in other anurans. One advantage to sampling from a breeding colony as in the present study is that it reduces artifacts of longevity-related population structure and energetic constraints present on the dimorphism bias observed in the wild. In order to examine *S. tropicalis* for size effects and to identify more subtle local differences, it is necessary to test for those traits consistent with body size dimorphism and then for local, secondary sexual characteristics in the cranium.

LOCAL INFLUENCES

The appearance and structure of localized phenotypic variation depends on developmental particulars giving rise to that morphology, both anatomically and temporally. Changes in the presence, size, and timing of appearance of embryological anlagen may influence the way inter- and intraspecies variation are manifest. Similarities and differences in the development of the oral cavity and dentition between amphibians and other vertebrates have been described elsewhere (see Chapter 2, reviewed in Davit-Béal et al. 2007, Svensson & Haas 2005, Soukup et al. 2013, Jernvall & Thesleff 2012, Fish et al. 2011, Takechi et al. 2013), so the potential influences on local craniodental variation reviewed here are in the context of amphibian development and physiology.

In typically homodont salamanders (Greven & Clemen 1980), the formation of the marginal dental lamina and the development of a complete marginal tooth row are separated in space and time because maxillary bones do not exist until after metamorphosis. The premaxillary dentition is established during larval stages, several tooth replacement cycles before the establishment of the maxillary dentition, and the dental lamina from the premaxillary region appears to proliferate posteriorly near the beginning of metamorphosis to initiate the maxillary tooth row (Clemen & Greven 1979, Vassilieva & Smirnov 2001). In most of the adult salamanders examined (Greven & Clemen 1980), the premaxillary and maxillary tooth rows are joined by one continuous dental lamina; however, the examples of sexually dimorphic plethodontids reveal developmental interruption of the dental lamina at the premaxilla-maxilla boundary by connective tissue and severe constriction of the dental lamina in those species with monocuspid premaxillaries year-round (Ehmcke & Clemen 2000b). These data suggest that the number and extent of dental laminae can facilitate variation in the dentition.

A dental lamina has been described as an epithelium that laterally connects tooth germs of differing maturity in caecilians as early as fetal stages, but its lateral extent was not noted (Wake 1976). Within frogs, adult *Rana pipiens* have been described with 6 separate dental laminae, which are paired on the premaxillae, maxillae, and vomers (Gillette 1955). Although most *X. laevis* first-generation teeth develop in the absence of a dental lamina (Shaw 1979, Cambray 1976), adult *X. laevis* appear to have a continuous dental lamina across the maxilla and premaxilla, though its condition at the midline is not mentioned (Cambray 1976). Observations of a dental lamina in *S. tropicalis* at late larval stages indicate that one laterally connects tooth germs from just prior to metamorphosis, but the lateral extent across the jawbones is unclear (see Chapter 1, Chapter 2). Additionally, odontogenesis of the first tooth generation in *S. tropicalis* is hypothesized to occur in at least two phases corresponding to the presumptive premaxillary and maxillary regions, which may introduce fundamental developmental differences between teeth developing in those regions of the mouth (see Chapter 1, Cambray 1976).

The timing of dermal ossification in the metamorphosing cranium also affects craniodental variation. In addition to modification in the growth, differentiation, and matrix secretion processes contributing to variation in the shape of individual bones (e.g. Rücklin et al. 2012) and the fusion and course of sutures (Holmes 2012, Slater et al. 2009), dermal ossification is the primary reason why tooth rows elongate only posteriorly after a certain developmental time (Wake 1980a, Osborn 1974). In addition to being difficult to assess in adults directly, ossification sequences within *X. laevis* have been notoriously difficult to characterize due to their high variability and sensitivity to rearing conditions (e.g. Trueb & Hanken 1992, Brown 1980, Sedra & Michael 1957).

After embryological or metamorphic development is complete, variation in craniodental phenotypes may also come about through physiological processes and functional integration. The tooth row undergoes regular resorption by osteoclasts during the process of tooth replacement, in which the bony support and the majority of the dentine crown for each tooth are dissolved and bays are created through which replacement teeth erupt (Shaw 1986a). Remodelling of these bays was not reported in newly metamorphosed *X. laevis*, but reversal lines were detected in the bony tooth supports in adult specimens (Shaw 1986a). Very little is known about the prevalence and processes of bone remodeling in response to muscle attachment and loading in non-mammalian taxa, and it has only recently been described for zebrafish (Witten & Huysseune 2009).

To understand potential signatures of these various local and systemic influences on craniodental morphology, I have conducted a hierarchical morphometric study of *S. tropicalis* adult craniodental variation. Using three strains of sexually size-dimorphic *S. tropicalis* as a model with a homodont dentition (Figure 1), I assembled evidence of patterns of integration of the dentition with the jaw, cranium, and overall body size as well as quantified variation in the number of tooth positions on bones in the marginal jaw. Through these identified variational modules, this paper seeks to identify the main influences on the construction of the marginal tooth row within the craniodental complex.

MATERIALS AND METHODS

Specimens. We created a skeletonized population of *S. tropicalis* from natural deaths in an adult breeding colony (managed by the Harland lab, UC Berkeley, in compliance with their animal protocol), consisting of "wild-type" (Nasco, Inc., Modesto, CA) and inbred gamma crystalline-GFP Nigerian strains as well as inbred Ivory Coast strains. Individuals (N = 78, Table 1) were prepared in the lab after defrosting from storage at -20°C and skeletonized by dermestid beetles. Snout-vent length (SVL) and sex as diagnosed by dissection were recorded during specimen preparation by undergraduate research assistants. Strain associations for individual specimens were extracted from controlled breeding records (Harland lab, UC Berkeley).

Linear measurements. Crania (N = 67) were photographed from the ventral perspective with a Canon EOS-1D Mark II digital SLR camera with Canon Extender EF 2x II (Lake Success, NY) and HDF-2 Pro custom lens (Visionary Digital), with shutter speed 1/250 seconds, ISO 1000,

and BK Lab System (Visionary Digital). A series of images were captured on the 1/50 step setting of the Lift Controller software (Visionary Digital) and were Z-stacked to enhance depth-of-field (CombineZM, Hadley, 2008). Crania were oriented consistently with the occlusal margin of the upper jaw in the focal plane. Bilateral linear measurements (12) were defined to capture cranial, jaw, and dentition size and shape (Figure 2, Table S3) and were collected from photographs using ImageProPlus (version 5.1.0.20, Media Cybernetics, Inc., Rockville, MD). Two trials for each measurement were collected by a single observer who was blind to sex and strain categorizations and averaged. Measurement error between trials averaged 2.23%. Composite measurements were also generated for the tooth row by summation or ratios of the 12 measurements (total tooth row length, total jaw length, tooth row and jaw half lengths, cranial aspect ratio, proportion of maxilla length covered by teeth, tooth size, Table S3).

Suture scoring and tooth counts. Skeletonized crania (N = 77) were observed at 32x magnification under a dissecting microscope (Carl Zeiss, Inc., Oberkochen, Germany) under low-angle light in order to count the number of tooth positions on each of the premaxillae and maxillae and to assess the curvature of the ventral premaxillary-maxillary sutures. Five character states for this last phenotype were identified by their relationship to individual teeth on the premaxilla and maxilla, and were scored separately for each jaw half. Tooth positions were counted whether or not a functional tooth was currently present, and were distinguishable in the absence of a functional tooth by cylindrical resorption cavities in the bone of attachment. Any tooth position intersected by a suture was counted as 0.5 tooth on the premaxilla and 0.5 tooth on the maxilla. Tooth counts and suture character states were scored after at least 2 independent observations and subsequent conferral between the two observers.

Statistics. Sample sizes for regressions and correlations varied from measurement to measurement due to incomplete data. Histograms of raw data were constructed in JMP (2012). Two-tailed, Welch's two sample t-tests were conducted for SVL by sex, cranial length (CL) by sex, and cranial aspect ratio by sex to examine body size dimorphism in *S. tropicalis*. This test accommodates for unbalanced design and unequal variances between groups. Reduced major axis (RMA) regression was chosen to describe the relationship of CL on cranial width (CW) because the goal of the analysis was to identify the scaling relationship between these phenotypes (Smith 2009). To test for the effects of sexual dimorphism on *S. tropicalis* dental variation, a series of 4 MANOVA/MANCOVA were conducted on the specimens for which complete right side linear measurement data were available (N = 62). These tests used either dental linear measures alone or dental and jaw linear measures as the dependent variables, and sex as the independent variable. Size (cranial length, CL) was used as the covariate in the MANCOVA analyses. Multivariate regression coefficients were also calculated for the least squares regression of CL on the linear measurements. In cases where the sex factor was significant, Discriminant Function Analysis (DFA) was carried out to identify the morphological axis separating the sexes. Welch's two sample t-test was also conducted for whole jaw tooth number by sex and whole jaw tooth size by sex. Tests were implemented in R using the 'stats' and 'MASS' packages (R Development Core Team 2011, Venables and Ripley 2002) or in JMP (2012).

To compare tooth sizes between the premaxilla and the maxilla (see Table S3 for calculation), ANCOVA was carried out with CL as the covariate and bone as the grouping variable.

MANOVA/MANCOVA were also carried out with CL as the covariate and genetic strain as the grouping variable to identify a potential genetic basis for craniodental variation.

Pairwise Pearson correlations between right side collected and composite linear measurements and tooth counts were used to assess interrelationships (or lack thereof) within the cranium in response to particular questions. To examine patterns of morphological integration in the cranium more broadly, pairwise Pearson correlation matrices were assembled in JMP (2012) for 10 craniodental measurements (right premaxilla occlusal length, right maxilla occlusal length, right side occlusal length, total tooth row length, premaxilla breadth, maxilla length, maxilla depth, maxilla width, right side jaw bone length, total jaw length) across the entire *S. tropicalis* sample, in females only, and in males only. Additional matrices were generated for subsamples representing the 3 genetic strains, and for size-adjusted (for CL) linear measurements. CL adjustments to collected and composite linear measurements were implemented by bivariate least-squares regressions, saving the predicted value and the residuals for each individual specimen. For each phenotype, the predicted values were averaged and a new 'CL-adjusted' *S. tropicalis* population was created by adding the individual specimen residuals for each phenotype to the mean predicted phenotype values (after Zelditch et al. 2004). The size-adjusted matrix, then, contains the pairwise correlations between individual measurements as they would be in the absence of a cranial size scaling factor.

RESULTS

SYSTEMIC INFLUENCES

Body size dimorphism: convoluting size and sex

Male and female *S. tropicalis* differ most clearly and dramatically in body size. The female average SVL is significantly larger than the male's ($t = 8.48$, $p < 0.0001$), with a 9.58 mm difference between the sexes. Females have a larger variance in body size compared with males, but a bimodal distribution in SVL is apparent (Figure 3a). With this difference in overall size, the shape of the cranium could be expected to scale isometrically, allometrically, or vary with no real pattern in relation to SVL.

Cranial size and shape

Cranial length (CL) and cranial aspect ratio (CL/CW), a first approximation of cranial shape, are both distributed unimodally in contrast to SVL. The measure of CL used in this study does not capture variation in the shape or prominence of the occipital condyles, and as such represents a relatively simple measure of cranial size. While CL differs significantly by sex ($t = 2.93$, $p = 0.005$), the within-sex variances are much higher than those observed for SVL, with considerable overlap except at the largest cranial sizes (Figure 3b). Cranial aspect ratio does not significantly differ between the sexes ($t = -0.40$, $p = 0.691$), with male and female distributions nearly identical (Figure 3c).

Neither cranial size nor shape exhibits a strong pattern with respect to the dimorphic SVL. There is a significant but weak correlation between CL and SVL ($r = 0.451$, $p = 0.0002$, Figure 3d), and no correlation between cranial shape and SVL as a proxy for body size (Figure 3e). Taken together, the cranium is varying somewhat independently of overall body size, rather than scaling in relation to the body size dimorphism in *S. tropicalis*.

LOCALIZED EFFECTS OF SYSTEMIC INFLUENCES

Although not strongly coupled to variation in body size, systemic growth or hormonal factors may be influencing the size and shape of the cranium itself. To investigate possible relationships, I examined cranial proportions for evidence of constraint, and examined craniodental linear measurements and tooth counts for correlations with size and sex.

One possibility if cranial size and shape are not covarying strongly with body size is that cranial proportions themselves are constrained. I examined the bivariate relationship between CL and cranial width (CW), which was strongly correlated ($r = 0.766$). Most crania have an aspect ratio (CL/CW) between 0.85 and 1. No cranium in the sample is more than minimally longer than it is wide (maximum CL/CW = 1.01), and the RMA scaling relationship between CL and CW is 0.859 (Figure 3f).

Sex and size effects isolated to the cranium

To identify craniodental phenotypes that may be influenced by sexual dimorphism regardless of body size, a MANOVA was performed on right side dental and jaw size measurements (Table 2). Differences in cranial measurements between the sexes are apparent without a size correction, but DFA fails to produce significant axes separating the groups (data not shown). This means that either all measurements included in the analysis are varying in concert (would be an overall size difference), or the identity of measurements distinguishing the sexes is not consistent across the sample. Corresponding ANOVAs for sex were significant at an $\alpha = 0.05$ level for all of the maxillary measurements, and at $\alpha = 0.075$ for the premaxillary measurements, supporting the idea that all of the measurements vary in concert.

Including CL as a covariate with sex in the multivariate analysis eliminates the effect of sexual dimorphism in craniodental linear measurements, however a CLxSex interaction term is statistically significant (Table 2). This further suggests that all of the measurements vary in concert with cranial size, which was already demonstrated to be somewhat dimorphic, but also that different craniodental measures are contributing to differences between the sexes in different size classes within this sample. At the level of individual ANOVAs, the only phenotype carrying a significant CLxSex interaction term was maxillary depth ($F = 6.55$, $p = 0.0131$). This dimension has a bimodal distribution with the smaller mode dominated by male individuals and the rest of the distribution covered by both sexes. Sex explained additional variance after CL for maxillary length and width variables (length $F = 4.59$, $p = 0.0363$; width $F = 5.55$, $p = 0.0219$).

These multivariate data suggest that correcting for CL largely eliminates sex effects once covariances between the craniodental variables are taken into account, but that variables corresponding to gape size (maxillary depth, width, and length) may differ between the sexes at

the smallest size classes. In other words, *S. tropicalis* cranial dimensions scale with overall cranial size between the sexes, but in the smallest crania, perhaps the overall jaw morphology is affected by hormonal differences.

Size effects in the dentition

Counting tooth positions in the marginal tooth row reveals that the average number of teeth in *S. tropicalis* adults is 35, with a range of 28-44 (Table 3). This number is comparable to the number of teeth possessed by adult male and female *X. laevis* at 18 months (mean numbers of 28 and 34, respectively, Shaw 1979). In 3 year old or older *X. laevis*, however, the average total tooth count is 61, indicating a considerable increase in tooth number with age (Shaw 1979). Between these closely related species, average adult tooth number increases with increasing species size and ploidy, and the increase in tooth number with age in *X. laevis* indicates an increase with cranial size within that species. Although reliable age data were not available to test the former trend for the *S. tropicalis* sample, it is possible to look for an increase in tooth number with adult cranial size.

There is a positive and significant correlation between half jaw tooth count and CL (Figure 4a). The correlation is not much tighter for full jaw tooth count with total jaw length (Figure 4c), interpreted to mean that jaw size is not more influential than cranial size in determining the number of teeth an individual has. The highest tooth counts are rare at all size classes, but the range of cranial sizes for which the number of teeth is fewer than 20 is very broad. The estimated relationship of increasing tooth number with increasing size within *S. tropicalis* is driven largely by a few individuals with extreme high and low cranial sizes.

Tooth size for the right jaw half was estimated for each individual by dividing the occlusal length for the right jaw half by the tooth count for the right side. This measure of tooth size, which averages across the sizes of all teeth in the jaw and does not account for gaps, is positively correlated with CL (Figure 4b), suggesting that replaced and/or posteriorly added teeth are larger as the animal grows. Although not significantly different from 0, there is a slight negative relationship between tooth size and tooth count in the right half of the jaw ($r = -0.19$, $p = 0.127$). This indicates that within-species variation in tooth count may be explained by two different developmental strategies described for other species but not yet directly observed in *S. tropicalis*: one in adding fewer, larger teeth as the jaw grows, and one in adding additional teeth posteriorly to fill newly created jaw space.

Within the dentition, Shaw (1979) reports differences in adult tooth number between the sexes, and Cambray (1976) reports that teeth in females are slightly larger. In the *S. tropicalis* sample, there are no significant differences in tooth count between males and females ($t = 0.813$, $p = 0.420$), but female tooth sizes are slightly larger on average ($t = 2.46$, $p = 0.0169$).

Morphological integration in the cranium

To obtain insight into the relative responses of individual craniodental variables to size scaling, multivariate least squares regression coefficients were calculated for the variables on CL (Table 2, Table S4 for summary statistics). Maxillary width increases the most in response to an

increase in CL, with an incremental increase greater than 1. All other variables increase, but less so, beginning with maxillary depth and length. The least-responsive craniodental variables are those of the premaxilla, both in the breadth of the bone and the length of the occlusal area. This combination describes a change in cranial shape with increasing CL whereby the gape size, particularly the width, increases faster than the extreme anterior region, or anterior midline, of the cranium.

The basis for these differential scaling relationships in the cranium can be better understood at more intermediate morphological scales, such as in the context of the functional module of the jaw. To this end, linear measurements were coded into dental and jaw-specific phenotypes. A phenotypic correlation matrix for 4 dental and 6 jaw size measurements reveals nearly uniformly high positive correlations in *S. tropicalis* crania (Table 4a), indicating a highly integrated craniodental complex overall. The trait pairings with lower integration are premaxillary and maxillary tooth row lengths, and maxillary tooth row length with premaxilla breadth. That these phenotypes are more independent of each other indicates that the tooth row, in particular the maxillary tooth row, is not restricted by variation at the anterior midline.

Breaking this matrix down by males and females begins to show some evidence of developmental processes other than overall size scaling at work in the cranium. While the female correlation matrix is identical to the composite one (data not shown), the male correlation matrix has some additional areas of lower integration (Table 4b). In particular, there is reduced covariation with the premaxillary measurements relative to the phenotypes measuring maxillary size, but maintained covariation with those more indicative of overall jaw size such as total jaw length and maxilla width. These patterns of reduced integration between the premaxilla and the maxilla are based on a smaller sample size (N = 26), but they are consistent with the insights above that scaling relationships are differently structured at the smallest size classes or there are male-specific, perhaps hormonally influenced patterns of cranial shape development (these are not mutually exclusive).

LOCAL INFLUENCES

Submodules within the cranium

In an attempt to control for cranial size in the *S. tropicalis* skeletal population and reveal underlying patterns of developmental modularity in the jaw, I examined pairwise Pearson correlations after adjusting individual linear measurements for CL (Table 4c). Overall, the pairwise correlations are less strong after adjusting for CL, demonstrating that systemic factors are likely to contribute most to phenotypic correlation in *S. tropicalis* crania. In the absence of CL-based integration, maxilla depth fails to correlate with any other cranial measure, and it is one of the first measures to show lower correlations in males. Because it was also the only phenotype to demonstrate a ClxSex correlation, maxilla depth may have a unique developmental relationship that does not involve the jaw and tooth osteological measurements considered here and that manifests only at smaller size classes.

There are patterns of integration that remain after CL adjustments, predicted to reflect common developmental or functional constraints at a more local level in the cranium. One of the most

striking patterns is the strong integration of premaxillary tooth row and bone lengths, but their reduced correlation with all of the other dental and jaw phenotypes. The breadth of the premaxilla continues to correlate with overall maxilla width, but the anterior midline seems to covary independently of any other relationships with the overall tooth row, a pattern already suggested by its reduced correlation with CL compared to the rest of the tooth row. Functional demands are suggested by the continued integration of maxilla occlusal length and composite dental row phenotypes, but striking for their separation from integration within the jaw bone phenotypes themselves. That is, dental and jaw bone phenotypes only covary strongly when the phenotypes are chosen to span the entire tooth row or a half tooth row, suggesting a functional constraint defining a jaw module, but perhaps there is developmental modularity at the level of individual bones and between the tooth initiation and bone development programs.

Developmental remnants in adult variation

To explore the nature of developmental modularity, or decoupling, between the bones comprising the jaw and between the tooth row and jawbones further, I examined tooth counts and sizes as they relate to bone boundaries and potential developmental precursors in *S. tropicalis*.

The standard deviation for tooth number on the premaxilla bones is nearly 1 tooth (Table 3), demonstrating that there is no fixed tooth number on the premaxilla in *S. tropicalis* as reported for some reptile species (e.g. iguanid *Ctenosaura similis* Torres-Carvajal 2007). Although the average tooth numbers on right and left paired bones are indistinguishable (Table 3), asymmetry in tooth number is prominent at both the tooth row and individual bone levels (59/77 paired premaxillae, 63/77 paired maxillae, 55/77 jaw halves are asymmetrical by at least one tooth). The fact that tooth numbers are variable using jaw bone boundaries is consistent either with anterior and posterior dental laminae that add teeth posteriorly to both the premaxilla and maxilla prior to completed ossification of the jaw bones, with anterior and posterior laminae whose boundaries do not coincide with bone boundaries, or with a continuous dental lamina that is unaffected by bone boundaries.

Another phenotype that reveals developmental modularity in the jaw is tooth size, which can be separately estimated for the premaxilla and the maxilla (Table S3). Premaxillary and maxillary tooth sizes (defined in Table S3) are significantly different by ANCOVA (CL covariate, $F = 30.0$, $p < 0.0001$; bone after CL, $F = 6.57$, $p = 0.012$), with maxillary teeth larger on average. This heterogeneity within the tooth row could be due to developmental differences between premaxillary and maxillary teeth (and perhaps their dental laminae). Additional hypotheses consistent with this result, however, are that replacement patterns (with larger replacing teeth) are heterogeneous between the bones or that teeth added posteriorly to the maxilla are larger and drive the average maxillary tooth size up.

Decoupled maxillary tooth row and jaw bone phenotypes

One of the most straightforward observations of dental and jaw bone phenotypes in *S. tropicalis* is that the posterior extent of the tooth row does not coincide with the posterior extent of the maxillary bone, as is typical due to muscle attachment and jaw articulation sites (Figure 2). The

correlation between maxillary occlusal length and maxillary bone length is high but not 1 (r = 0.793, Table 4a), so to characterize variation on the maxillary bone, I examined the ratio of maxillary occlusal length to maxilla length, measuring the percentage of the bone covered by teeth in each individual. The maxilla varies fairly dramatically in the percent coverage by teeth, with a range between 50% and 80% (Figure 5a).

The maxillary tooth/bone ratio does not correlate with overall size as measured by SVL or CL, or with maxilla size (Figure 5b-d). Tooth size also does not correlate with the proportion of the maxilla covered by teeth, but tooth number does correlate (Figure 5e,f). Taken together, this means that of the phenotypes examined, more coverage on a maxillary bone is achieved largely by increasing the number of teeth initiated on the bone. In addition, there are both long maxillae with many teeth and short maxillae with many teeth, and vice-versa, indicating that dental features are following different spatial constraints besides overall bony element size in the posterior jaw.

Sutures and functional tooth positions

While the premaxillae are fused in many of the salamanders examined for dental development, the plesiomorphic condition is to have paired premaxillae (Wake 1963); most frogs have paired premaxillae, with a prominent midline symphysis in at least some frogs like *X. laevis* and *S. tropicalis*. This osteological feature allows for easy identification of teeth developing on the right or left sides of the midline suture. At the ventrolateral edges of the premaxillae, however, the articulations with the maxillae are more planar and the sutures intercalate with functional tooth positions in the marginal tooth row.

In counting the number of teeth present on each tooth-bearing bone, it became necessary to define several ways in which the suture relates to functional tooth positions, because often the suture transects a functional tooth position rather than dividing two positions neatly. This means that in many cases, a functional tooth and its replacement were situated on two different bones, supporting the independence of the tooth row and individual tooth positions from jaw bone developmental control. Five suture classes were defined based on the pattern of intersection with functional and replacement tooth positions (Figure 6).

Suture class diagnoses frequently were asymmetric across the midline, and while approximately 1/3 of premaxillary/maxillary sutures intercalated between discrete tooth positions, nearly 2/3 intersected either a functional or replacement tooth position. The most frequent intersection observed was through a functional tooth position but not through its replacement or its neighboring replacement tooth position (Class II, Figure 6). Shaw (1986a) classified the two *X. laevis* he found with intersecting sutures as abnormal, but this set of suture phenotypes does not appear to be abnormal, at least in captive populations of *S. tropicalis*.

That the sutures can be traced through empty functional tooth positions indicates that the resorption of the bone of attachment during functional tooth turnover is quite extensive and/or that deposition of the bone of attachment becomes discontinuous in the vicinity of the suture boundary. Consistent with a pleurodont mode of tooth attachment, the dental lamina is likely

situated ventrally and lingually to the articulation of the jaw bones, and the maturing teeth migrate towards the bone as they erupt and resorb the previous tooth.

GENETIC BASIS FOR CRANIODENTAL VARIATION

MANOVA for the original right side dental and jaw linear measurements between *S. tropicalis* strains did not return any significant differences ($F = 1.57$, $p = 0.175$). Individual ANOVAs detect significant differences only in premaxilla occlusal length between *S. tropicalis* strains ($F = 5.09$, $p = 0.0278$; $F = 4.03$, $p = 0.0493$ after size), providing an avenue for future work on variation in the premaxilla submodule of the dentition with the suggestion that some of it has a genetic basis.

As might be expected from a decrease in heterozygosity if these craniodental phenotypes have a genetic basis, phenotypic correlations are much tighter in the inbred Nigerian strain than in the 'wild type' Nigerian strain, but the same overall pattern of high integration with decreased covariance between maxillary and premaxillary dental and jaw phenotypes holds (data not shown). The Ivory Coast sample is not large enough to comment on phenotypic correlations.

DISCUSSION

Through these developmental process-driven morphometric analyses, I have been able to describe the nature of female-biased dimorphism in *S. tropicalis* and its impact on craniodental morphological variation. Body dimorphism does not extend to crania, which have much more subtle patterns of dimorphism when they exist. Most craniodental variation between the sexes can be explained by variation in CL, although some phenotypes are differentially affected, either by scaling or by hormonal effects, to create small size class differences in shape. Maxillary depth may be integrated with a distinct cranial system than that of the rest of the jaw, and overall jaw size shows additional sexual dimorphism. Tooth number in the jaw is quite variable at a given size, but individuals with extreme cranial sizes tend to have a corresponding extreme number of teeth.

Between *X. laevis* and *S. tropicalis* species, there is evidence that sexual dimorphism manifests at least somewhat differently. The only comparable data reported for *X. laevis* are that both tooth number and size are greater in females compared to males (Shaw 1979, Cambray 1976). While *S. tropicalis* female teeth are larger on average, there are no significant differences in tooth count between males and females. Since *X. laevis* is a larger species overall, perhaps there are different scaling rules or demands for functional integration in larger individuals.

While systemic developmental influences are apparent, there is evidence for variational modularity within the craniodental complex as well. The anterior midline region varies distinctly from more posterior jaw segments, visible in differential scaling, possibly between the sexes, and in size-adjusted phenotypes. Functional tooth positions are not constrained by bone boundaries and suture positions are not constrained by the presence of teeth, which may or may not be a symptom of decoupled tooth row and bone length variation.

DEVELOPMENTAL AND FUNCTIONAL MODULARITY

The osteometric analyses in this study provide evidence for several different morphological influences based on the main variational modules identified in *S. tropicalis*. The homodont tooth program in *S. tropicalis* does not appear to have the strict bone-based constraints of some plethodontid salamanders, but hidden modularity within the odontogenic patterning program remains a possibility due to the identification of jaw and premaxillary modules. Androgen-based differences in the *S. tropicalis* premaxilla as in some specialized plethodontid salamanders (Ehmcke et al. 2003) are unlikely, but the possibility that dental laminae are compartmentalized cannot be rejected from the current data, particularly given observations in *S. tropicalis* and *X. laevis* tadpoles suggesting that there are different phases of anterior and posterior tooth row development (see Chapter 1), but they do rule out that the dental lamina compartments correspond directly to suture boundaries. The differential tooth size result between the premaxilla and maxilla underscores the challenges of superimposed developmental processes in testing for modularity based on hypothesized modular divisions. It is not possible to distinguish between the effects of uniformly compartmentalized size differences, heterogeneity of tooth replacement processes, and the posterior addition of larger teeth without additional data on individual tooth sizes. On the other hand, we would not have any knowledge that modularity between the premaxilla and maxilla is also detectable, and perhaps influenced, at the larger scales of cranial size scaling and sexual dimorphism had only the developmental processes of replacement been observed in the tooth row.

Modularity between tooth row development and jaw bone development can be explained by modularity on multiple levels. Evolutionarily, teeth and jaws did not develop at the same time, and co-option of developmental mechanisms has been hypothesized to place teeth on oral jaws (Smith 2003, Huysseune et al. 2009, Fraser et al. 2010) and pharyngeal jaws (Fraser et al. 2009). The expectation, then, would not be for the fundamental patterning and morphogenetic processes involved in these phenotypes to be completely integrated. During early development, however, the same genes and gene expression domains have been implicated in jaw and tooth patterning (Qiu et al. 1997, McCollum & Sharpe 2001, Fish et al. 2011, Nichols et al. 2013). Tight developmental and genetic integration between the mandible and the tooth row with respect to adult population and interspecific variation has been discounted in several mammalian species from similar kinds of osteometric data (Dayan et al. 2002, Boughner & Dean 2004, Zelditch et al. 2008, Boughner 2011).

Functionally, the jaw bone and tooth row need to be integrated, and aside from both being affected by scaling, investigators have suggested that jaw bone growth trajectories are under selection to accommodate the tooth row (Daegling 1996, Boughner 2011), and that jaw bone remodeling occurs in response to the forces of mastication (Zelditch et al. 2008). A partial explanation for the specific pattern of posterior jaw variation seen in adult *S. tropicalis* may be its derived mandibular morphology. Unlike most frogs, *Xenopus* and *Silurana* lack a quadratojugal and therefore lack a bony articulation between the posterior maxilla and the neurocranium (Shaw 1986b). One consequence of this is a shortened maxilla, and another is the presence of a substantial pterygomaxillary ligament attached to the posterior maxilla, both hypothesized to reduce torques that could damage the cranium upon feeding (Shaw 1986b).

INSIGHTS FOR AMPHIBIAN OSTEOLOGY

Most of the work on the craniodental complex in amphibians has focused on tooth morphological transitions from larval to adult stages, on the interpretation of particular characters for taxonomy, or on the ultimate causes for the evolution of sexual dimorphism. More recently, investigators have begun to use geometric morphometrics to compare ontogenetic trajectories in postmetamorphic crania (e.g. Yeh 2002, Ivanović et al. 2007, Ponssa & Candioti 2012, Vera & Ponssa 2013). The present study on adult variation in a controlled population adds to osteometric datasets available for comparative purposes, with a focus on the behavior of dental variation within the cranium. While age (or size as a proxy for age) was not explicitly considered, some of the variational modules identified in adult breeding colony variation align with those seen in anuran postmetamorphic trajectories. Maxilla growth and an orientation shift from latero-medial to antero-posterior is common for most postmetamorphic frogs, alhough the maxilla grows comparatively less in pipoids compared to its growth in other frogs (Yeh 2002). In *Leptodactylus* frogs, the maxilla grows to comprise 80% of the lateral margin from 40% (Vera & Ponssa 2013), as the main changes in shape (scaled to same size) occur in the nasal region and the otic region, where the squamosal and pterygoid are also growing laterally (Ponssa & Candioti 2012, Vera & Ponssa 2013). Thus, if the *S. tropicalis* ontogenetic trajectory is similar to that of most frogs including other pipoids (Yeh 2002), a change in posterior cranial width with size could explain decoupling of the premaxillary region from the maxillary length phenotypes.

In her study of *Hyla lanciformis* adult variation, Trueb (1977) found that among the least variable osteometric measurements in the cranium were the cranial widths at the pterygoids and at the temporals, and a relatively low amount of variation in pterygoid-maxillary length. She also detected sexual dimorphism more prominently in the premaxilla width, tooth count, and the more anterior region of the cranium. Trueb (1973) observed that interspecifically, there is dramatic variation in the position of the anterior pterygoid-maxilla articulation. Together, these osteological observations suggest that perhaps the maxilla represents a functional connection between anterior and posterior cranial evolutionary modules in adult anurans.

CONCLUSIONS

Approaching adult variation from a hierarchical developmental perspective allows for a more nuanced understanding of morphological evolution, useful for taxonomic and paleontological interpretation. The lack of a strong correspondence between cranial size and SVL, as well as the wide range of phenotypes observed at a given cranial size class in *S. tropicalis*, argue for caution in using size as a proxy for age, a common practice when examining museum specimens. As the present study demonstrates, examining scaling relationships and phenotypic correlation at each level reveals a little more insight into how the superimposition of developmental and physiological processes shape morphology. It may not allow us to immediately distinguish specific patterns of development, but it can rule some out, which is helpful for designing developmental genetics experiments and in thinking about candidate genes for the effects of natural selection. While it will be illuminating to compare phenotypic variation between lab-reared and wild-caught *S. tropicalis* populations, the differences in the magnitude of covariation

between inbred and ‘wild type’ strains with the same genetic background suggest that there is indeed a genetic component to the standing variation in cranial morphology.

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Figure 1. Female (left) and male (right) adult *S. tropicalis*. Mature individuals are sexually dimorphic in size, body shape, protuberance of the cloaca, and the presence/absence of nuptial pads on the forelimbs.

Figure 2. Linear measurements taken from a skeletonized *S. tropicalis* cranium, ventral view. Tooth row measurements are coded in blue, jaw measurements in green, and cranial measurements in tan. R = right, L = left, mx = maxilla, pmx = premaxilla. See Table S3 for measurement definitions.

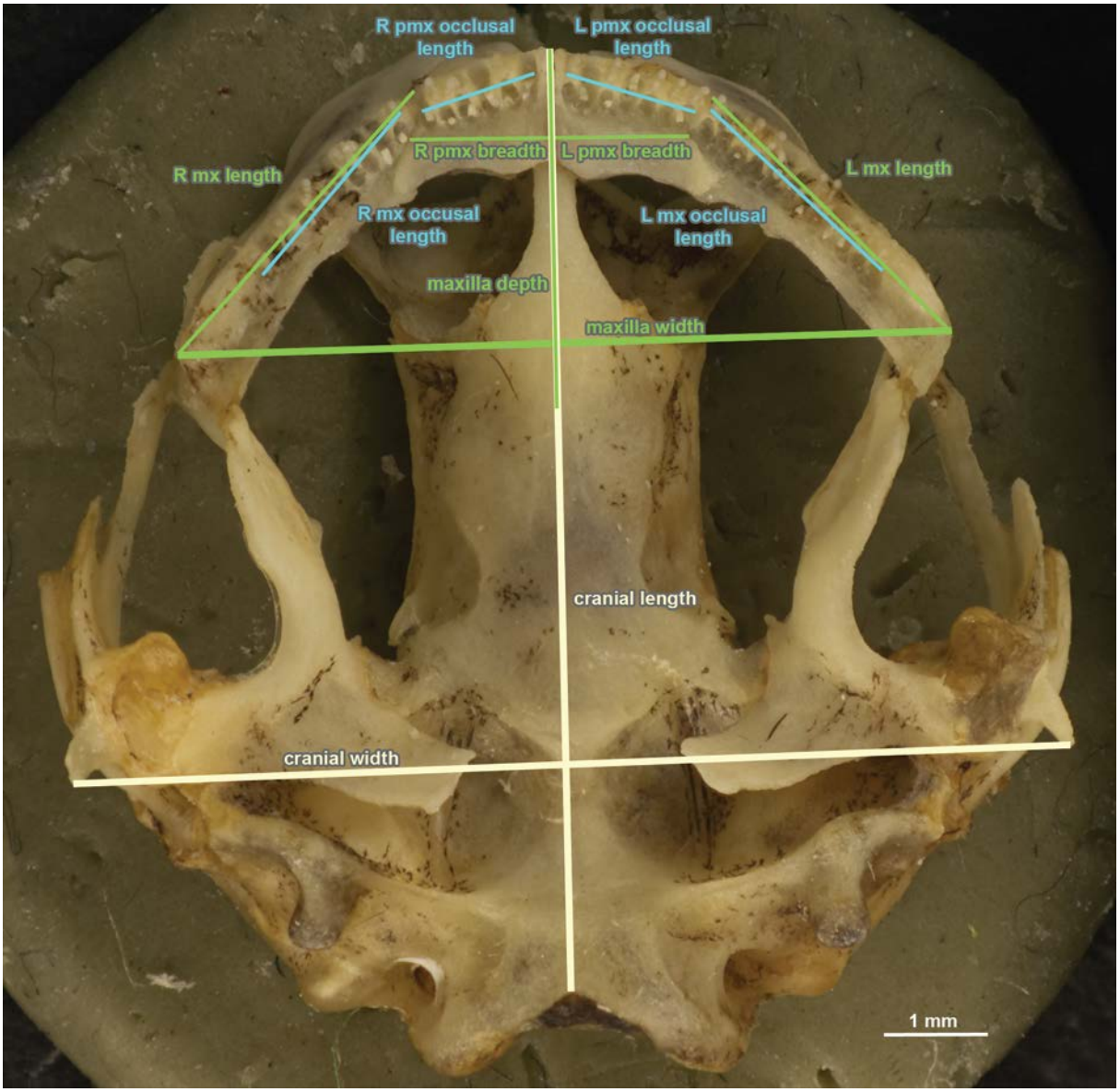

Figure 3. Sexual dimorphism and scaling relationships for *S. tropicalis* body and cranial size. (a-c) Histograms of linear or composite measurements from the skeletonized population, color-coded by sex. Bars illustrate distribution for entire population; female individuals in each bin are shaded dark green to illustrate dimorphism. Note the differing degrees of overlap between male and female trait distributions. (a) Distribution of snout-vent length (SVL). (b) Distribution of cranial length (CL). (c) Distribution of cranial aspect ratio, a first approximation of cranial shape. (d-f) Scatterplot comparing linear measurements of body and cranial size. (d) Correlation between CL and SVL. (e) Lack of correlation between cranial aspect ratio and SVL. (f) Scaling relationship between CL and cranial width (CW) as estimated by RMA regression (red trend line). The scaling factor for CL/CW is estimated to be 0.859. All linear measurements in mm. *denotes Pearson correlation r statistically significantly different from 0 ($p = 0.0002$).

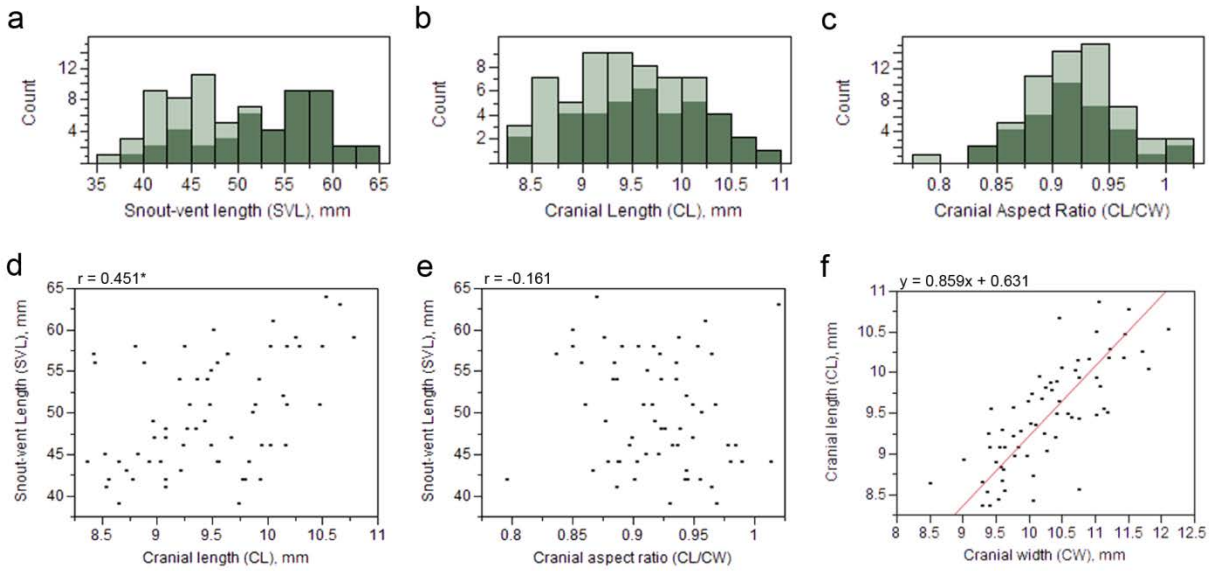

Figure 4: Size effects on the tooth row. Scatterplots of tooth measurements from skeletonized jaws, compared against cranial and jaw size proxies. (a) Correlation between CL and tooth count for the right half jaw, premaxilla + maxilla. (b) Correlation between CL and tooth size for the right half jaw. Tooth size was estimated as the premaxilla + maxilla occlusal length divided by the premaxilla + maxilla tooth count. (c) Correlation between total jaw length and whole jaw tooth count. Total jaw length was estimated by summing bone lengths for the premaxillae and maxillae, right and left. *denotes Pearson correlation r statistically significantly different from 0 ($p < 0.01$).

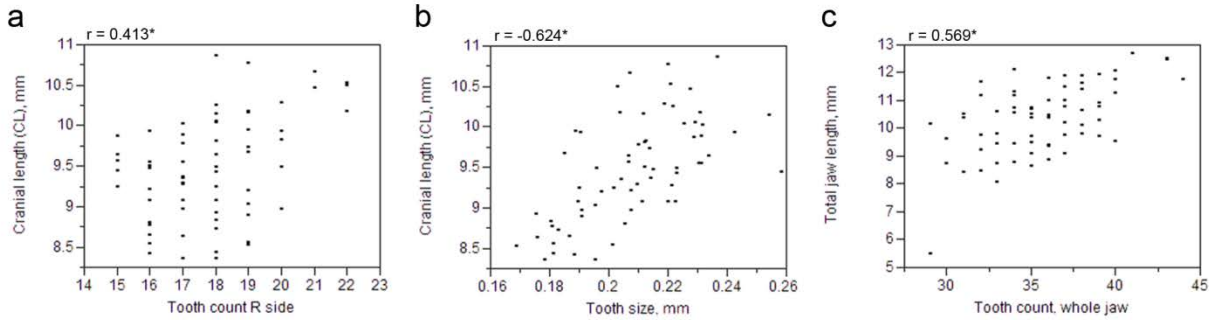

Figure 5. Maxillary tooth and bone variation. (a) Histogram of the ratio between maxillary tooth row length to maxillary jaw bone length. (b-f) Scatterplots of the maxillary tooth row to bone ratio compared against size proxies and dental parameters. (b) Lack of correlation between ratio and snout-vent length. (c) Lack of correlation between ratio and cranial length. (d) Lack of correlation between ratio and right maxilla length. (e) Correlation between ratio and number of teeth on the right maxilla. (f) Lack of correlation between ratio and maxillary tooth size. Tooth size was estimated as the maxilla occlusal length divided by the maxilla tooth count. *denotes Pearson correlation r statistically significantly different from 0 ($p < 0.0001$). R = right, mx = maxilla, occl/length = occlusal length to maxillary bone length.

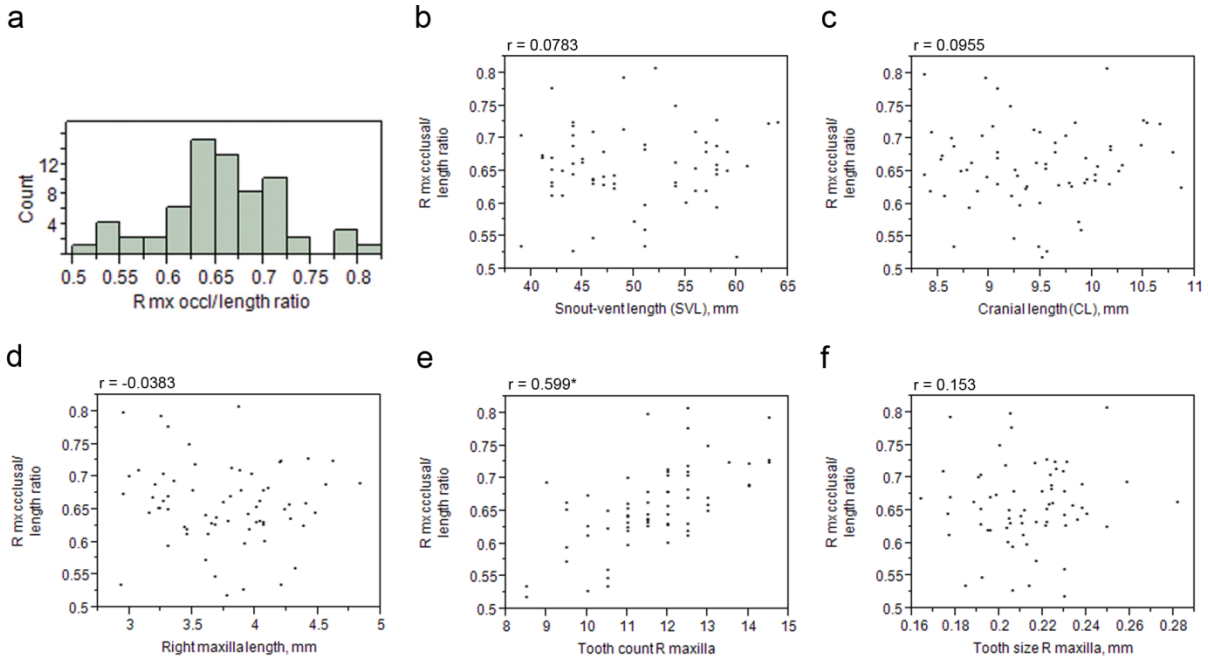

Figure 6. Suture classes reported by jaw half. Circles represent functional tooth positions, dots are replacement teeth lingual to them. Schematics represent the upper left premaxillary-maxillary suture as seen from an occlusal view of the cranium. The tooth position at left in this figure is located more on the premaxilla, while the right position is more on the maxilla. Position of the suture is diagrammed in green for each phenotypic class. Numbers below each class reflect the number of jaw halves observed for that class.

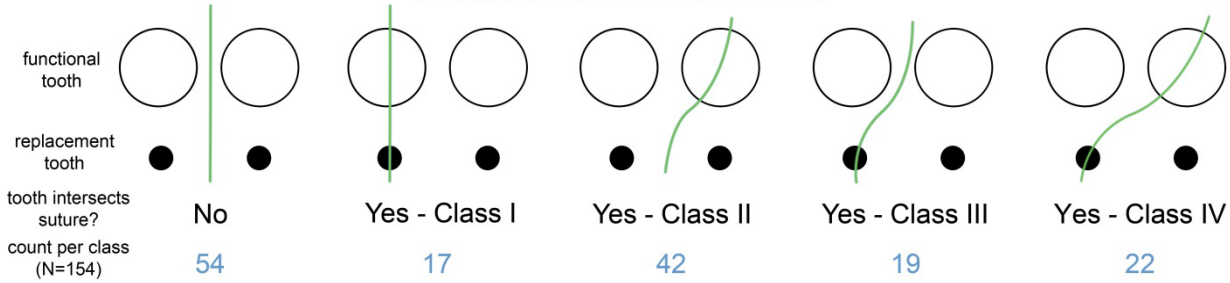

Table 1. *S. tropicalis* used in this study. Sample sizes for individual analyses varied slightly because it was not possible to take all measurements from all individuals. F = female, M = male, U = unknown.

Strain	**total**	***by sex***	
"wild type" Nigerian	36	*F*	*21*
		M	*13*
		U	*2*
inbred Nigerian	30	*F*	*18*
		M	*12*
		U	*0*
inbred Ivory Coast	9	*F*	*4*
		M	*3*
		U	*2*
unknown strain	3	*F*	*1*
		M	*2*
		U	
total	**78**		***78***

Table 2. MANCOVA for dental and jaw linear measurements. Linear measurements incorporated in the analysis were right premaxillary occlusal length, right maxillary occlusal length, maxilla depth, maxilla width, right premaxillary breadth, and right maxillary length (see Figure 2 and Table S3 for definitions). CL = cranial length, R = right, pmx = premaxilla, mx = maxilla, occl = occlusal length.

Sex only							
	Df	Hotelling-Lawley	approx F	num DF	den DF	Pr(>F)	
SEX	1	0.30196	2.7679	6	55	0.02008	*
Residuals	60						
Sex after size							
	Df	Hotelling-Lawley	approx F	num DF	den DF	Pr(>F)	
CL	1	0.3319	55.932	6	53	<2e-16	***
SEX	1	0.1975	1.745	6	53	0.1287	
CLxSEX	1	0.3236	2.859	6	53	0.01736	*
Residuals	58						
Regression coefficients for size							
		R pmx occl	R mx occl	Mx depth	Mx width	R pmx width	R mx length
Intercept		-0.154	-1.4982	-2.3722	-2.5388	-0.1195	-1.7907
CL		0.1484	0.419	0.6239	1.17	0.1654	0.5867

Table 3. Tooth count summary statistics (N = 77). Number of tooth positions was recorded for premaxillary and maxillary bones under magnification by dissecting microscope. The number of functional teeth could not be reliably documented due to processing and post-processing from dermestid beetles. For tooth positions that passed through a premaxillary/maxillary suture, 0.5 tooth was assigned to each bone. There are prominent medial ridges on the ventral paired premaxillae, so midline teeth clearly sit on the right or left jaw halves.

		Number of teeth						
	CL (mm)	R premaxilla	L premaxilla	R maxilla	L maxilla	R marginal jaw	L marginal jaw	marginal tooth row
mean	9.46	6.18	6.10	11.6	11.6	17.7	17.7	35.5
range	8.36 - 10.86	4 - 8	3.5 - 8.5	8.5 - 14.5	7.5 - 15	14 - 22	13 - 22	28 - 44
SD	0.644	0.895	0.870	1.41	1.52	1.82	1.85	3.42

(SD = standard deviation, CL = cranial length, R = right, L = left)

Table 4. Phenotypic correlation matrices for dental and jaw measurements. Pairwise Pearson correlations, shaded by strength of correlation. Tooth row measurements designated in blue, jaw measurements in green. (a) *S. tropicalis* males and females. (b) *S. tropicalis* males. (c) CL-adjusted measurements (see Methods for description), males and females. Dark grey, r = 0.5–1; medium grey, r = 0.3–0.5; light grey, r = 0–0.3. CL = cranial length, R = right, pmx = premaxilla, mx = maxilla.

a N = 72

	R pmx occlusal length	R mx occlusal length	Total R occlusal length	Total occlusal length	R pmx breadth	R mx length	Total R jaw length	Maxilla width	Maxilla depth	Total Jaw length
R pmx occlusal length	1									
R mx occlusal length	0.381	1								
Total R occlusal length	0.657	0.905	1							0.5-1
Total occlusal length	0.695	0.827	0.949	1						0.3-0.5
R pmx breadth	0.852	0.456	0.667	0.714	1					0-0.3
R mx length	0.506	0.793	0.809	0.790	0.582	1				
Total R jaw length	0.648	0.734	0.876	0.869	0.750	0.940	1			
Maxilla width	0.649	0.711	0.814	0.845	0.775	0.886	0.923	1		
Maxilla depth	0.525	0.689	0.704	0.710	0.550	0.836	0.779	0.759	1	
Total jaw length	0.695	0.692	0.942	0.920	0.759	0.876	0.942	0.936	0.808	1

b Males, N = 26

	R pmx occlusal length	R mx occlusal length	Total R occlusal length	Total occlusal length	R pmx breadth	R mx length	Total R jaw length	Maxilla width	Maxilla depth	Total Jaw length
R pmx occlusal length	1									
R mx occlusal length	0.139	1								
Total R occlusal length	0.487	0.797	1							
Total occlusal length	0.424	0.758	0.969	1						
R pmx breadth	0.816	0.035	0.328	0.310	1					
R mx length	0.375	0.754	0.735	0.727	0.319	1				
Total R jaw length	0.526	0.590	0.875	0.878	0.518	0.856	1			
Maxilla width	0.595	0.620	0.772	0.783	0.639	0.872	0.887	1		
Maxilla depth	0.422	0.636	0.565	0.562	0.418	0.847	0.670	0.779	1	
Total jaw length	0.516	0.616	0.871	0.901	0.534	0.847	0.974	0.900	0.711	1

c CL adj., N = 72

	R pmx occlusal length	R mx occlusal length	Total R occlusal length	Total occlusal length	R pmx breadth	R mx length	Total R jaw length	Maxilla width	Maxilla depth	Total Jaw length
R pmx occlusal length	1									
R mx occlusal length	-0.131	1								
Total R occlusal length	0.314	0.774	1							
Total occlusal length	0.404	0.592	0.864	1						
R pmx breadth	0.752	-0.086	0.246	0.369	1					
R mx length	0.012	0.480	0.434	0.412	0.067	1				
Total R jaw length	0.262	0.314	0.626	0.631	0.387	0.808	1			
Maxilla width	0.294	0.222	0.403	0.425	0.497	0.615	0.728	1		
Maxilla depth	0.002	0.163	0.054	0.048	-0.072	0.447	0.230	0.069	1	
Total jaw length	0.394	0.236	0.560	0.827	0.448	0.630	0.784	0.624	0.248	1

CONCLUSION

The studies performed here on *S. tropicalis* were designed to examine the developmental basis of phenotypic difference, a key component of understanding how organisms have diverged over time and how developmental mechanisms may be successfully modified. By exploiting natural phenotypic variation in odontogenesis and craniofacial development, this dissertation provides complementary data to that produced in more mainstream functional studies of development. This comparative approach has identified where conserved developmental mechanisms can be uncoupled or are already independent, revealing points of mechanistic flexibility that might otherwise look coupled in a single developmental model. Identifying these points of independence and change in phenotypes and the mechanisms that create them challenges the accepted notions of trait homology and helps to redefine phenotypes to better match the variation that can be acted on by selective forces and other genetic changes over the course of evolution.

Main findings

In chapter 1, the hypothesis of similarity between *S. tropicalis* and *X. laevis* was tested for the timing and dynamics of first generation tooth initiation. Despite their relative size, generation time, and ploidy differences, the broad trajectory of tooth initiation is conserved in these species. Tooth initiation does exhibit heterochrony, however, with *X. laevis* developing teeth earlier than *S. tropicalis* by two Nieuwkoop and Faber stages. In addition, initiation patterns in *S. tropicalis* reveal dynamics that were previously undetected, notably a lack of synchrony in developing maxillary teeth and an extreme anterior dentition that develops much later. Similar to the multi-phase model proposed by Cambray (1976) for *X. laevis*, the dynamics of tooth initiation *in S. tropicalis* suggest the possibility of two phases of initiation: first a slower and non-stereotypical sequence of development in the presumptive maxillary region, followed by a more rapid and synchronous development of the teeth in the presumptive premaxillary region. Although these teeth are initiated prior to the ossification of maxillary and premaxillary bones, a two-phase model would also be reminiscent of a 'fish' mode of tooth initiation in which a pioneer tooth is initiated on each tooth-bearing bone (Fraser et. al. 2004). Developmental mechanisms of strict tooth alternation and patterning by local inhibition were both unsupported in *S. tropicalis* due to the relative degrees of development between adjacent tooth germs and by the presence of twinned tooth germs in a high percentage of specimens.

Chapter 2 evaluated a consensus model for *Shh* in odontogenesis (e.g. Jernvall & Thesleff 2012, Fraser et al. 2009) in light of the natural experiment in *S. tropicalis* relative to non-anuran vertebrates in decoupling tooth and mouth development. While a conserved pattern of *Shh* expression was detected in the inner dental epithelium of cap stage *S. tropicalis* teeth, *Shh* expression corresponding to a tooth competence-determining odontogenic band was not detected in tadpoles. The expression patterns in *S. tropicalis* therefore suggest that odontogenic band and cap stage expression are semi-independent instances of *Shh* function, that *Shh* does not have the function hypothesized in the consensus model during odontogenic band stages, or that frogs may have evolved a novel mechanism to establish tooth competence. These data, along with a review

of the functional data available for the odontogenic band suggest that the odontogenic band *Shh* domain may regionalize the jaw for tooth development, but it does not determine individual tooth positions.

The apparent lack of a *Shh*-expressing odontogenic band in *S. tropicalis* seems unusual when compared to the consensus model for odontogenesis, but it is not outside the range of variation in vertebrates when the correspondence between the presence of an odontogenic band and the presence of teeth is examined in more detail. Several other taxa do not fit the consensus model for tooth initiation; in *S. tropicalis* and in crocodylians, teeth are able to form in the absence of recent *Shh* expression in the jaw, and in snakes, mice, and birds, an odontogenic band is present that does not lead to fully formed teeth. The odontogenic band as currently defined by *Shh* expression (Fraser et al. 2004) is insufficient to explain how a conserved developmental mechanism is able to accommodate patterns of heterochrony and toothlessness across vertebrates. Perhaps the odontogenic band is better defined by the expression of additional genes, such as *Pitx2*, or reflects some other developmental process (e.g. a marker of endoderm-ectoderm boundaries), which would better correlate to the distribution of functional teeth in different species. Another explanation that merits further investigation is that the expression domain called the odontogenic band might function to pattern craniofacial tissues other than the tooth competence domain.

In chapter 3, *S. tropicalis* osteological data were used to evaluate to what extent population-level variation are able to reveal developmental and physiological effects on adult craniodental phenotypes. By analyzing variation at the functional phenotypic levels of the cranium, the jaw, and the tooth-bearing bone, patterns of morphological integration were identifiable which correspond to potential constraints and points of flexibility in the dentition. For animals raised in captivity, cranial size is a main integrating factor, but while there is sexual dimorphism in body size, cranial size is not strongly dimorphic. The size of the jaw shows additional sexual dimorphism, but the maxillary depth phenotype used in this study may be integrated with a distinct cranial system than that of the rest of the jaw. Patterns of covariation with cranial size, size-adjusted patterns of pairwise phenotypic correlation, and significant differences between genetic strains all suggest a decoupling of variation in the premaxilla and maxilla in *S. tropicalis*, a trait shared with other vertebrate systems (Fraser et al. 2012, Stewart 1958, Hlusko et al. 2011, Grieco et al. 2013). There is evidence for a functional jaw module, in which the tooth row and jaw bones correlate when summed across the jaw, but at a local tooth-bearing bone level, asymmetry of tooth counts is common and the dentition is continuous across the premaxillary-maxillary sutures. Thus, any developmental influence of patterning teeth separately on each jaw bone, as in fish (Fraser et al. 2004), does not correspond to actual bone boundaries in adult *S. tropicalis*. Furthermore, at least some of this standing phenotypic variation in adult *S. tropicalis* has a genetic basis.

Maximizing model systems for EvoDevo

Following the investigation of odontogenesis conducted here, the *S. tropicalis* system presents additional opportunities to understand the evolution of odontogenesis. Functional knockdown of Shh with small molecules and the emerging transgenic technologies available for *S. tropicalis* should make it possible to further test the hypothesis that *Shh* does not function to initiate teeth

during perimetamorphosis or during early craniofacial development. Examination of other markers of the conserved tooth GRN (Fraser et al. 2009) will provide additional insight into the evolution of odontogenesis in frogs, as well as test the hypothesis of the GRN itself. The separation of tooth development from mouth development provides a unique context in which to identify the triggers for tooth initiation (in only the upper jaw, near the time of metamorphosis), for tooth loss (in the lower jaw), and for separating jaw patterning from dentition patterning.

Along with the advantages of investigation in an unusual model system for craniodental development, the potential of comparative studies between *S. tropicalis* and *X. laevis* is only beginning to be realized. Further enumeration of the larval differences between the two will create a suite of phenotypes in which questions of heterochrony, heterotopy, and heterometry can be addressed in two mechanistically tractable systems. Phenotypes in the developing dentition allow for the exploration of both pattern formation and morphogenesis, potentially in response to hormonal triggers surrounding metamorphosis. Examining the tooth initiation patterns and dental lamina formation of *X. laevis* may shed light on the effects of size and ploidy on what are presumed to be self-organizing tooth loci, and to see whether within-species size effects are distinct from between-species size effects. Similar questions can be investigated through the comparison of adult osteology, for which a skeletonized population of *X. laevis* has already been created (Grieco, unpublished).

The Inside-out, Outside-in debate

Inside-out vs. outside-in arguments about the evolutionary origins of oral teeth would benefit from clarification of the stomodeum *Shh* expression domain in vertebrates. The ouside-in hypothesis states that teeth evolved when skin denticles and ectoderm migrated into the oral cavity upon the evolution of jaws (e.g. Peyer 1968), whereas the inside-out hypothesis states that teeth evolved first within the pharyngeal cavity as patterned odontodes, and endoderm is responsible (e.g. Smith & Coates 1998, Smith 2003). This debate has seen heated arguments for which much of the argument stems from the paleontological diversity of tooth-like structures in fishes and placoderms. More recently, comparative embryology and gene expression have put forward modifications to these hypotheses, suggesting that contact between endoderm and ectoderm is critical for tooth formation (Huysseune et al. 2009) or that anywhere a core set of genes is deployed on an epithelium near neural crest, teeth will form (Fraser et al. 2010). The epithelium of the stomodeum and its function in tooth development sits at the juncture of these arguments.

Current data and further study based on *S. tropicalis* could play a critical role in this debate, as this frog embodies a natural experiment decoupling stomodeum formation from oral tooth formation. Xenoplastic transplants suggest that the frog stomodeal epithelium lacks a tooth inductive signal that salamander larvae possess (Wagner 1949, 1955, Henzen 1957), a condition that may not recapitulate the original edentulous vertebrate condition, but it will help us understand how tooth regulatory novelties are able to be established. In addition, accounting for the diversity of how teeth are patterned in development and getting a better understanding of the role of *Shh* in that process will help to understand what traits (phenotypes, gene regulatory modules, etc.) may have been co-opted to create teeth in the first place. Similarly, understanding the functional role of *Shh* in stomodeum development will help to define whether it marks

endoderm-ectoderm boundaries, is restricted to either the endoderm or ectoderm functionally, or is involved in craniofacial patterning more broadly. Future work along these lines has the potential to define tooth homology on multiple phenotypic and genetic levels, and presents a great opportunity for the successful integration of paleontology, embryology, and developmental genetics.

Implications for amphibian paleobiology

Knowledge of the developmental and physiological influences on the skeleton is also directly relevant to the study of morphology over time. Patterns of integration and variability from skeletal populations of *S. tropicalis*, in addition to a better understanding of the sequence of its tooth initiation, can directly bear on interpretations made of fossil pipid teeth and other bones, including those of *Xenopus* found at Olduvai Gorge in Tanzania (Leakey 1965, Leakey 1971), and other pipids, including larvae, in museums throughout North Africa and the Middle East (e.g. Roček & Van Dijk 2006, Henrici & Baez 2001). Although many of the known fossil anurans are fragmentary and their teeth are small, a study of this material in the context of known extant variation will allow us to understand the morphology of this specialized pipid lineage and to identify which phenotypes are relevant to trace backwards for their first appearances in the more enigmatic stem amphibian and stem frog fossil records.

The problem of stem amphibian relationships is not a simple one, with preservational gaps occurring at critical transitions in amphibian evolution (San Mauro et al. 2005), as well as the serious confounding factor of a biphasic life history on the interpretation of morphology. This latter issue, however, calls for a developmental approach to morphology in living amphibians to understand some of the potential constraints on adult phenotypes. Knowledge of ways larval developmental processes manifest in craniodental osteology may provide taxonomic links between larval and adult fossil remains. Furthermore, identifying skeletal traits that are reliably found as part of an ontogenetic series would allow us to better distinguish between instances of paedomorphosis and miniaturization, evolutionary scenarios that both use patterns of bone reduction and loss as primary data in amphibians (Wake 1986, Hanken & Wake 1993, Maddin et al. 2011).

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APPENDIX

Table S1. Collection strategy for tadpole clutches. Tadpoles were reared in 3L tanks in populations of ~30 individuals. Development (NF stage) was not strictly correlated with growth (size), and heterogeneity in both was apparent by 1 month of age, when they were split from a single tank into tanks of 30. At the time of splitting, attempts were made to make the size and development distributions the same across each tank. Heterogeneity in growth and development became more pronounced in the second month of age.

Clutch	Genetic Strain	Collection Strategy
Spring 2010	F2 offspring of Nigerian (F4) x Golden (F9) outcross	Tadpoles sampled as they developed, so the fastest developers were fixed as NF stage 58 specimens, and the slowest developers were fixed as NF stage 55 specimens. 44 days – 60 days.
Spring 2011	Wild type Nigerian	Two major rounds of sacrificing, one at 50-53 days (when the most developed tadpoles were at NF stage 59) and one at ~70 days (when tadpoles underdeveloped at ~50 days reached NF stages of interest).
Summer 2011	Wild type Nigerian	Entire tanks sacrificed at once, with mean NF stage 56, so that all had same age in absolute days ~55 days.

Figure S1. Growth chart for Spring 2010 and 2011 tadpole clutches. Sacrificing began on day 44 for the Spring 2010 clutch, so reported numbers are not meaningful as growth rates after that. I began reporting the oldest stage observed after that point; the tanks showed a bimodal distribution of development, with one mode around NF stage 53 and another mode around NF stage 56. Sacrificing began on day 50 for the Spring 2011 clutch.

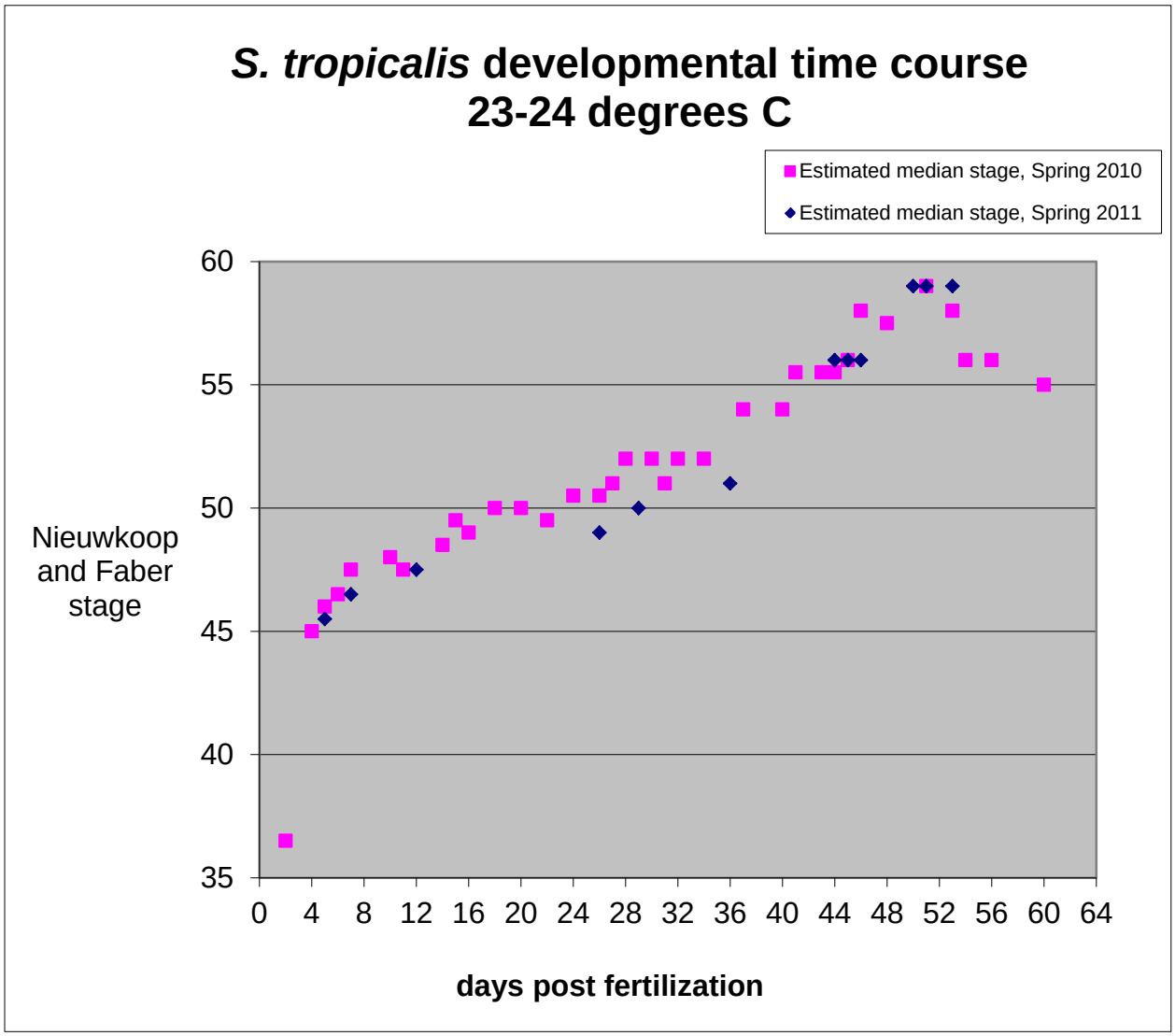

Table S2. *In situ* sample sizes and replicates. Positive controls in the form of neurula stage embryos and/or thick sections (dissections) through the trunk spinal cord and notochord showed positive *Shh* expression in all *in situ* experiments reported, suggesting a technically successful *in situ* protocol. *Shh* sense probe controls, when included, showed no signal in either the embryonic or larval spinal cord or notochord, or in tadpole upper jaws.

Positive results for *Shh* in tadpole upper jaws were retained from 3/8 *in situ* hybridization experiments. This detection across independent experiments, along with expression patterns in the IDE consistent with those of other vertebrates, support the interpretation of a true biological signal represented by at least one specimen at NF stages 56, 57, and 58. Biological replicates at NF stage 56 were confounded by the low frequency and abundance of tooth germs at this stage, despite the high sample sizes actually attempted. On the other hand, this high number of specimens without teeth also adds credibility to the idea of a true negative signal prior to IDE expression (i.e. a lack of an OB) because so many tadpoles were sampled just prior to tooth initiation.

The clutches used in 2013 experiments (Spring and Summer 2011) do appear to demonstrate a biological shift towards less developed tooth germs at a given NF stage relative to Spring 2010 specimens, so the lack of *Shh* signal in these experiments may also reflect biological reality in lacking *Shh* expression at bud stages more often observed in these clutches so far. Additional experiments with a wider range of stages may further clarify the situation. Alternatively, penetrance issues may remain. The thicker, more differentiated tissues of the jaw may be preventing diffusion of antibody and detection substrate, consistent with high background.

One other difference in specimen preservation between the successful experiments of 2012 as compared to more recent experiments is that specimens used in 2012 were stored at or above 25°C in 100% methanol for a period of 1-3 weeks, whereas more recent specimens have been maintained at -20°C since fixation. It remains to be tested whether a period of storage at room temperature enhances tissue permeability.

NF Stage	# tadpole jaws sampled	% with teeth	Subset of jaws for *Shh in situ*
55	11	0	0
56	42	40	21
57	17	100	8
58	5	100	4

Figure S2. Whole mount *in situ* hybridization for *Shh* in a tadpole with a full tooth row (NF stage 58). *Shh*-expressing teeth are ventrally positioned relative to other germs in the tooth row. Four tooth germs express *Shh* transcripts and are spaced unevenly through the jaw.

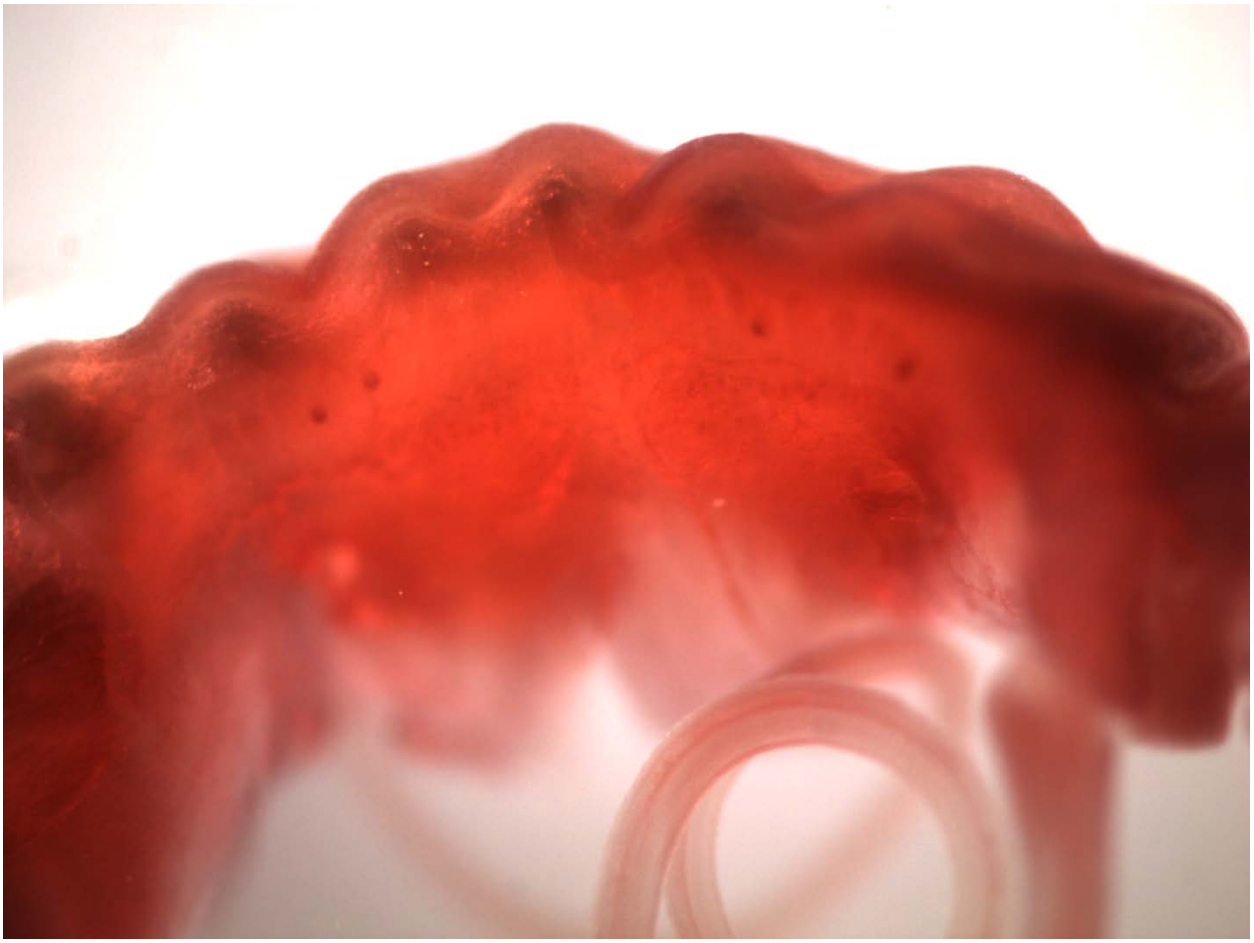

Figure S3. Comparison of bearded dragon (*Pogona vitticeps*) rudimentary tooth histology to *S. tropicalis* first generation tooth histology. Rudimentary teeth in the bearded dragon develop during the first generation and at least some of them form prior to dental lamina formation (Handrigan & Richman 2010). Some first generation *S. tropicalis* teeth also initiate prior to dental lamina formation, but it is unclear whether they are functional. (a) and (b) are coronal sections of the midline area of *S. tropicalis* tadpoles, stained with Hematoxylin and Eosin. (a) NF stage 58 tadpole based on hindlimb morphology, sacrificed after normal development of approximately 8 weeks. Adapted from Chapter 1. (b) NF stage 57 tadpole based on hindlimb morphology, sacrificed after >6 months with limited growth and development, likely attributable to high tank densities. Midline teeth in (a) are oriented dorsoventrally, connected to a dental lamina, and show ordered dentin mineralization. Midline teeth in (b) are oriented anteroposteriorly, have pockets of condensed, purple mesenchymal cells, and a highly mineralized cap (pink triangular shapes) without a dental lamina joining individual teeth. Teeth in (b) resemble the rudimentary germs in (c), a transverse section through a *P. vitticeps* jaw. Rudimentary germ in (c) boxed and inset. Image credit for (c): Handrigan & Richman 2010, Figure 1L. Picrosirius Red and Alcian Blue. dp – dental papilla, iee – inner enamel epithelium, oee – outer enamel epithelium, od - odontoblasts, de – dentin/predentin.

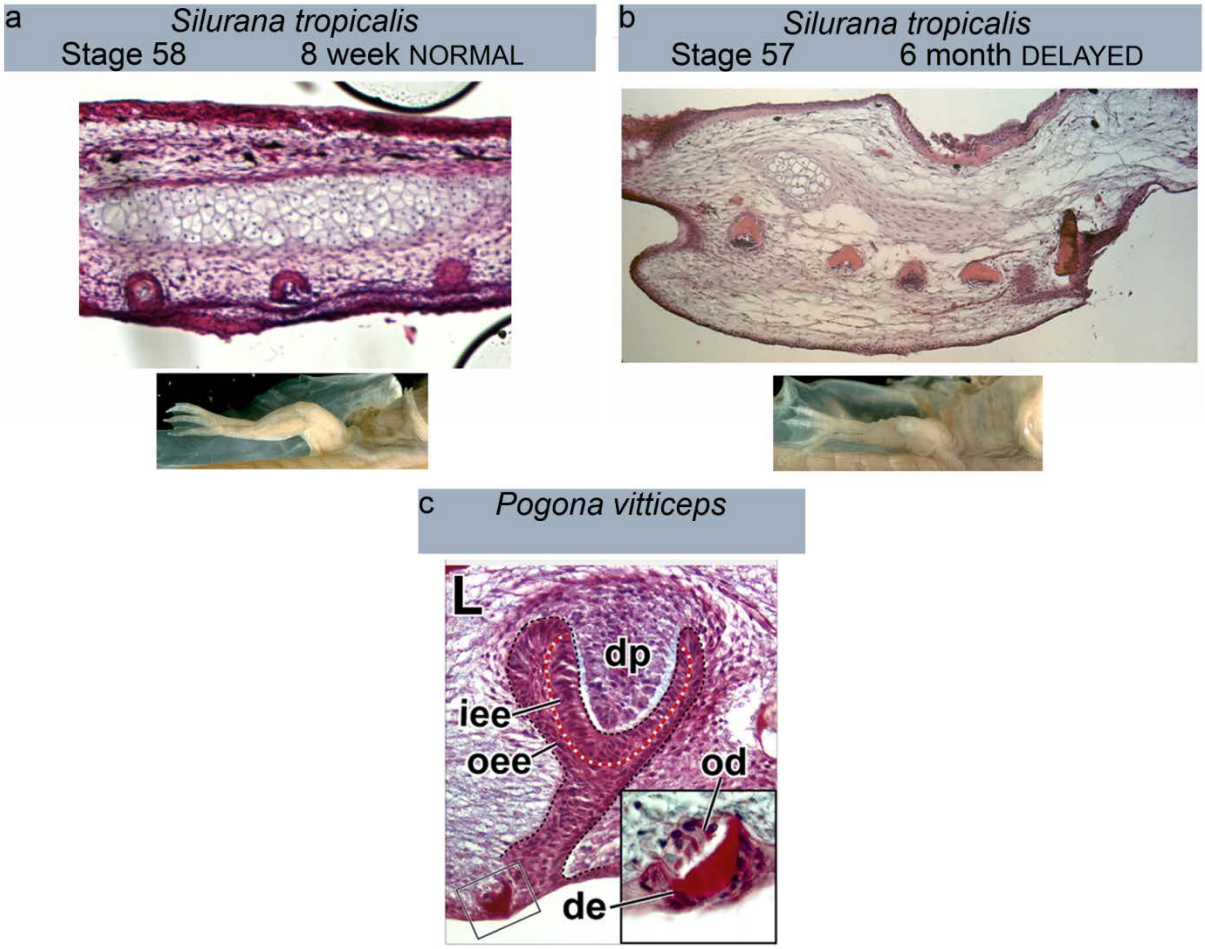

Table S3. Definitions of craniodental linear measurements.

Name	Definition
cranial width	posterior projection of squamosal on either side, outer edge to outer edge
cranial length	rostralmost point on the rostral cartilage to midline ventral presphenoid
maxilla depth	rostalmost point on the rostral cartilage along midline to posteriormost extent of maxilla
maxilla width	lateralmost edge to lateralmost edge of maxilla
right premaxilla breadth	breadth of premaxilla perpendicular to midline beginning at lateralmost point of replacement tooth row
left premaxilla breadth	breadth of premaxilla perpendicular to midline beginning at lateralmost point of replacement tooth row
right maxilla length	mesialmost point on maxilla at suture and labial tooth row to lateralmost point on the maxilla
left maxilla length	mesialmost point on maxilla at suture and labial tooth row to lateralmost point on the maxilla
right premaxilla occlusal length	a line lingual to functional teeth and labial to replacement row beginning at the mesialmost edge of the first functional tooth and ending at the distalmost edge of the tooth row on the premaxilla
left premaxilla occulsal length	a line lingual to functional teeth and labial to replacement row beginning at the mesialmost edge of the first functional tooth and ending at the distalmost edge of the tooth row on the premaxilla
right maxilla occlusal length	a line lingual to functional teeth and labial to replacement row beginning at the mesialmost point of the maxillary tooth row and ending at the distalmost edge of the tooth row
left maxilla occlusal length	a line lingual to functional teeth and labial to replacement row beginning at the mesialmost point of the maxillary tooth row and ending at the distalmost edge of the tooth row
cranial aspect ratio	cranial length divided by cranial width
total right occlusal length	right premaxilla occlusal length + right maxilla occlusal length
total occlusal length	right premaxilla occlusal length + left premaxilla occlusal length + right maxilla occlusal length + left maxilla occlusal length
total right jaw length	right premaxilla breadth + right maxilla length
total jaw length	right premaxilla breadth + left premaxilla breadth + right maxilla length + left maxilla length
tooth size, right jaw half	(right premaxilla occlusal length + right maxilla occlusal length) divided by right jaw half tooth count
tooth size, premaxilla	right premaxilla occlusal length divided by right premaxilla tooth count
tooth size, maxilla	right maxilla occlusal length divided by right maxilla tooth count

Table S4. Linear measurement summary statistics.

	snout-vent length (mm)	cranial width	cranial length	maxilla depth	maxilla width	R premaxilla breadth	L premaxilla breadth	R maxilla length	L maxilla length	R premaxilla occlusal length	L premaxilla occusal length	R maxilla occlusal length	L maxilla occlusal length
N	75	66	67	66	66	66	66	67	66	66	66	67	66
mean	49.32	10.28	9.46	3.55	8.50	1.44	1.46	3.76	3.74	1.24	1.29	2.47	2.49
min	37	8.51	8.36	2.57	6.67	1.07	1.10	2.93	2.72	0.790	0.927	1.57	1.75
max	64	12.1	10.86	4.40	10.32	1.78	1.98	4.83	4.50	1.59	1.87	3.35	3.50
SD	6.90	0.748	0.644	0.469	0.906	0.161	0.175	0.458	0.483	0.164	0.164	0.365	0.367

(min = minimum, max = maximum, SD = standard deviation, R = right, L = left)