Spring 2013

The Zebrafish as a Model for the Evolution and Development of Breeding Tubercles in Fishes

Alexandra Rodriguez

University of Colorado Boulder

Follow this and additional works at: https://scholar.colorado.edu/honr_theses

Recommended Citation
https://scholar.colorado.edu/honr_theses/478

This Thesis is brought to you for free and open access by Honors Program at CU Scholar. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of CU Scholar. For more information, please contact cuscholaradmin@colorado.edu.
The Zebrafish as a Model for the Evolution and Development of Breeding Tubercles in Fishes

By Alexandra Rodriguez | Ecology and Evolutionary Biology

Thesis Advisor
Dr. David Stock | Ecology and Evolutionary Biology

Defense Committee
Dr. David Stock | Ecology and Evolutionary Biology
Dr. Barbara Demmig-Adams | Ecology and Evolutionary Biology
Dr. Pei-San Tsai | Integrative Physiology

University of Colorado - Boulder
April 5, 2013
Abstract

Breeding tubercles are multicellular, keratinized epidermal projections that are used in intraspecific competition and maintaining contact during spawning in fishes. The development of these novel structures has been little studied, despite their presence in the Zebrafish (*Danio rerio*) model system. The aim of the present study was to help establish the Zebrafish as a model for the development and evolution of breeding tubercles in fishes. The appearance of breeding tubercles during development was documented for this species with special focus on tubercles of a lateral flap of the lower jaw, which represents a synapomorphy (shared derived feature) of the genus *Danio*. As this fleshy flap is supported by a lateral extension of the dentary bone, the possibility of induction of tubercles by this structure was investigated by comparing tubercle and projection development in the Zebrafish. Breeding tubercles, the fleshy jaw flap, and dentary projection were also characterized in eight additional species of the subfamily Danioninae. This study revealed the coordinated appearance of the three structures as well as loss of tubercles in one clade. Finally, the Ectodysplasin signaling pathway was determined to be necessary and sufficient for tubercle development, establishing variation in this pathway as a candidate cause of variation in breeding tubercles of fishes.
**Introduction**

Some of the most diverse structures found in vertebrates are derivates of the skin, including hair, feathers, mammary glands, teeth, baleen, claws, nails, beaks, and horns (Kardong 1998). Entire vertebrate classes can be defined by the presence of particular skin derivatives, such as hair and feathers. Conversely, variation in these derivatives can differentiate closely related species, as with scale counts in fishes and feather coloration in birds (Peterson and Peterson 2008; Page 2011).

Skin is made up of three parts: an internal dermal layer, a basement membrane, and an external epidermal layer (Kardong 1998). Skin derivatives can be produced exclusively from the epidermis (hair, feathers, scales in reptiles and birds), uniquely from the dermis (bony plates in the skin of alligators), or from the epidermis and dermis in combination (scales and teeth in fishes). Despite these differences, all skin derivatives likely develop through interactions between an epithelium (e.g. epidermis) and mesenchyme (embryonic connective tissue, producing e.g. the dermis) (Krejsa 1979). In addition, it has recently been shown that skin derivatives as diverse as teeth, hair, and feathers develop using many of the same gene networks (Mikkola 2007).

Most studies of skin appendage development have focused on hair, feathers or teeth (Mikkola 2007). A type of skin appendage that has received almost no attention in developmental and genetic studies is the breeding tubercle of fishes. Such tubercles are multicellular projections of the epidermis with a keratinized (non-living) covering (Wiley and Collette 1970; Collette 1977). Proposed functions of these structures include defending resources such as nests and territories, or maintaining contact between spawning individuals.

Breeding tubercles are of interest for a number of reasons: 1) keratinized structures in fishes are rare, as compared to the skin of land vertebrates that has a keratinized outer layer (Krejsa 1979), 2) breeding tubercles are often sexually dimorphic and conjectured to be subject to sexual selection (Helfman *et al.* 2009), 3) breeding tubercles are likely to have arisen convergently, as they are found in
six distantly-related orders of fishes (Wiley and Collette 1970), 4) the pattern of breeding tubercles can be used to diagnose closely-related species (Page 2011).

The order Cypriniformes is one of the six groups that display tubercles. This order includes the most prominent fish model species for the study of the genetic control of development, the Zebrafish (*Danio rerio*). This species possesses a number of advantages compared to other vertebrate models including external fertilization, transparent embryos, rapid development, and large clutch sizes (Nüsslein-Volhard and Dahm 2002). For this reason, numerous methods have been developed for embryological and genetic manipulation, and an extensive collection of mutant and transgenic lines exists.

Preliminary investigation of breeding tubercles in the laboratory of D. Stock (M. B. Hawkins and D. Stock, unpublished) has characterized their anatomy and the locations in which they are found in the Zebrafish. The current study aimed to build upon this research to help establish the Zebrafish as a model for understanding the evolution and development of breeding tubercles in fishes.

A prominent location of tubercles in the Zebrafish is on lateral projections (fleshy flaps) from the rostral tip of the lower jaw. The present study focused on the tubercles in this location, as the flap is a synapomorphy (shared derived character) of the genus *Danio* (Liao et al. 2011). The appearance of tubercles during development of the Zebrafish was first characterized using scanning electron microscopy (SEM). SEM and skeletal staining were used additionally to examine the relationship of tubercles of the fleshy flap to an underlying projection from the dentary bone known to support it. Variation in the tuberculate flap and dentary projection was also explored in eight species related to the Zebrafish. These data were used to construct a plausible scenario for the evolution of the tuberculate flap in the genus *Danio*. Finally, the role of a candidate gene for the regulation of tubercle development and evolutionary variation, the secreted signaling molecule Ectodysplasin (*eda*), was explored. To do so, tubercle number was characterized in loss of function mutations in the Ectodysplasin pathway, as well as a transgenic line over-expressing *eda*. 
Materials and Methods

Animals

Wild type Zebrafish of the inbred Tübingen (TU) line (Nüsslein-Volhard and Dahm 2002) were obtained from natural spawnings. Zebrafish homozygous for the nkt and fls<sup>370f</sup> alleles (representing loss of function mutations in Ectodysplasin and Ectodysplasin receptor, respectively) were obtained from crosses between heterozygotes and identified by their fin phenotypes (Harris et al. 2008). Ubiquitous and continuous expression of Ectodysplasin during development was obtained with the ef1 α:eda transgenic line (S. Aigler and D. Stock, unpublished). This line expresses the Zebrafish Ectodysplasin gene under the control of the cis-regulatory region of the *Xenopus laevis* (South African clawed frog) ef1α gene, which encodes a translation elongation factor. Although the expression of the transgene (a gene transferred into the genome of one organism from another) has not been directly assayed, it likely occurs throughout development and throughout the body, as would be expected from the widespread need for translation factors. Since the ef1 α:eda transgene could not be assayed directly, crosses involving this gene also included dlx2b:gfp (Jackman and Stock 2006) or NFκB:gfp (Kanther et al. 2011) reporter transgenes. Ectopic (displaced) expression of Ectodysplasin induces ectopic teeth in the upper pharynx, which express green fluorescent protein (GFP) in the presence of the reporter transgenes (S. Aigler and D. Stock, unpublished). Larvae were screened for ectopic GFP expression at 3-5 days post-fertilization (dpf) with a Leica MZFLIII fluorescent stereomicroscope (Leica Microsystems, Germany).

Adults of additional species within the subfamily Danioninae (*Rasbora trilineata*, *Sundadanio axelrodi*, *Danio choprae*, *Danio margaritatus*, *Danio erythromicron*, *Danio albolineatus*, *Danio roseus*, and *Danio nigrofasciatus*) were obtained from aquarium suppliers (The Wet Spot Tropical Fish, Portland OR; Arizona Aquatic Gardens, Oro Valley AZ; That Fish Place, Lancaster PA). At least two specimens of each of these species (one male and one female where possible) were used for SEM and an additional two specimens for clearing and skeletal staining.
Zebrafish males and females exhibit distinctive differences in color and shape (Nüsslein-Volhard and Dahm 2002), which were used to identify the sex of adults. These identifications were confirmed by examination of tubercles on pectoral fins, which are present in males but not females (M. B. Hawkins and D. Stock, unpublished). Pectoral fin tubercles were also used to identify the sex of the other species examined, with the exception of *D. margaritatus* and *D. erythromicron*, which lack such tubercles in either sex (Roberts 2007; this study). Distinctive color patterns allowed the identification of sex in *D. margaritatus* (Roberts 2007); the sex of *D. erythromicron* individuals was tentatively assigned using body shape.

**Fixation of specimens**

Fish were euthanized with tricaine methanesulfonate at a concentration of 500 mg/L and larval and juvenile specimens staged according to Parichy *et al.* (2009). Standard length (from the tip of the snout to the end of the vertebral column) was recorded to the nearest 0.01 mm with dial calipers and a preliminary assignment of sex was made where possible. In most specimens, this assignment was confirmed subsequently by SEM imaging of the left pectoral fin. Following staging, measurement and identification of sex, fish were fixed in 4% formaldehyde in phosphate buffered saline (PBS). Fixation was either overnight at 4 °C with rocking (larvae and juveniles) or 1-2 days at room temperature (adults). Specimens were then rinsed with phosphate buffered saline (PBS) and transferred to 70-100% ethanol for storage.

**Scanning electron microscopy (SEM)**

Specimens for SEM imaging were dehydrated by transfer to 100% ethanol. The portion of the specimen to be imaged was dissected, placed in a porous container and dried in a critical point dryer (Tousimis Autosamdri 815, USA). The dried sample was then mounted on an aluminum stub with clear nail polish. After drying overnight, the mounted specimens were sputter coated with gold for 120 seconds at 30 mA using a Cressington Model 108 Sputter Coater. The gold-coated specimens were then imaged.
with a JEOL JSM-6480 Scanning Electron Microscope (JEOL, Japan).

Clearing and staining for light microscopy

Dentary bones were visualized by clearing and staining with alizarin red (with or without alcian blue to stain cartilage) following the procedure of Walker and Kimmel (2007). Fixed fish in 70% ethanol were rinsed with 10 mM Tris HCl pH 7.5/25 mM MgCl₂ and stained overnight in 0.02% alcian blue/10mM MgCl₂/80% ethanol. The specimens were then rehydrated (with differentiation of the stain) in solutions of progressively lower concentrations of ethanol (80%, 50%, and 25%) and 0.5% KOH. Bleaching in 3% H₂O₂/0.1% KOH followed, along with rinsing in 35% saturated NaBO₄, clearing/digesting in 1% Trypsin in 35% saturated NaBO₄, washing in 10% glycerol/0.5% KOH, staining in 0.01% alizarin red overnight, and differentiating in 50% glycerol/0.1% KOH. Specimens were then transferred to 100% glycerol for storage.

A modified clearing and staining procedure was used for specimens targeted for subsequent examination by SEM. The 1% Trypsin solution was eliminated to prevent digestion of tubercles. The modified protocol therefore consisted of washing in a solution of Tris/MgCl₂, transferring to 0.5% KOH, bleaching in 3% H₂O₂/0.1% KOH, washing in 10% glycerol/0.5% KOH, staining in 0.01% alizarin red with a pH of 7.5, differentiating in 50% glycerol/0.1%, and storing in 100% glycerol. The lower jaws (dentary bones) of the fish were then dissected with fine forceps.

Clearing and staining followed by SEM

23 Zebrafish were imaged by SEM after being cleared and stained. In order to do this, cleared and stained specimens in glycerol were transferred (via 1X PBS) to 50%, 70%, and finally 100% ethanol where the SEM protocol was resumed (critical-point drying, sputter coating, and imaging).
Light microscopy and imaging

Cleared and stained specimens were imaged with a Zeiss AxioCam digital camera running AxioVision 4.6.3 software and mounted on a Zeiss Stemi SV11 stereomicroscope (Zeiss, Germany). Adobe Photoshop CS was used to adjust the contrast of entire images (Adobe, USA).

Results

Breeding tubercles in adult Zebrafish

The presence of breeding tubercles in the Zebrafish is indicated by a few published SEM images accompanied by no more than a brief text description (Roberts 1989; Sublette et al. 1990; Webb 2000); no comprehensive description of Zebrafish breeding tubercles has been published to date. The starting point of the present study was therefore an unpublished survey by M. B. Hawkins (Hawkins and Stock, unpublished). Hawkins’ survey found both sexes to possess tubercles on the lower jaw in two prominent locations: on fleshy flaps at the rostral tip of the lower jaw (Fig. 1A, arrowhead), and in a row several tubercles wide and caudal to each flap (Fig. 1A arrow). Additional tuberculation is sexually dimorphic. Males, but not females, possess tubercles on anterior pectoral fin rays (Fig. 1D) as well as scattered across the lower jaw, the dorsal surface of the head, and the operculum.

The present study focused on tubercles of the fleshy flaps of the lower jaw rather than in any areas displaying sexual dimorphism for a number of reasons. It is difficult to follow sexually dimorphic features throughout development because both sexes of the Zebrafish undergo a stage in which the gonads exhibit an ovarian morphology (Anderson et al. 2012). In addition, the fleshy flaps are a synapomorphy (shared derived feature) of the genus Danio (Fang 2003; Fang et al. 2009; Liao et al. 2011). Finally, mutants in the Ectodysplasin pathway examined for phenotypic alterations of tubercles lack fins (Harris et al. 2008).
Breeding tubercles in fishes are characterized by a keratinized outer layer (Wiley and Collette 1970; Collette 1977), which has been confirmed through histological sections of the Zebrafish (D. Stock, personal communication). In SEM images (e.g. Fig. 1C), keratinization is manifest as a relatively smooth surface, which contrasts with unkeratinized surrounding cells that exhibit a pattern of microridges characteristic of the outer layer of fish epidermis (Elliot 2000). The spaces between tubercles on the fleshy flap also appear keratinized (Fig. 1B, C), with an irregular boundary separating the field of keratinized cells from unkeratinized ones (Fig. 1B).

**Appearance of breeding tubercles during Zebrafish development**

In order to determine the pattern in which breeding tubercles appear during Zebrafish development, larvae and juveniles were fixed weekly starting at approximately four weeks post-fertilization. The 75 fish examined were assembled into a developmental series on the basis of standard length. Heads and (at later stages) fins were imaged by SEM and the presence and/or number of tubercles noted. Tubercles first appear during development at 8-9 mm standard length, at an age of approximately one month post-fertilization (Fig. 2). The first tubercles to appear are those of the fleshy flap, followed by tubercles of the mandibular rows and finally the pectoral fins of males (Fig. 3; data not shown). The appearance of the first keratinized tubercle of the lower jaw is prefigured by a slight lateral projection in the region of the future fleshy flap (Fig. 3A-C). At the earliest stages in which the projection is present, its cells still exhibit microridges. In individuals with a single keratinized breeding tubercle on each side (Fig. 3D), the tubercle appears to project directly from the lateral edge of the jaw, suggesting that the early projection may be incorporated into the first breeding tubercle. A second tubercle appears next, either anterior or posterior to the first tubercle and in line with the first along the rostro-caudal axis (Fig. 3E). Following the appearance of a third tubercle in this line, a fourth tubercle appears at the base of the extending fleshy flap, forming a ring around the flap (Fig. 3H). The semi-circular shape of the fleshy pad begins to become apparent at this point, as the outer row of tubercles curves distally. The middle of the
fleshy flap is bare of tubercles at this stage, and becomes surrounded by an increasing number of tubercles (Fig. 3I-J) before tubercles appear on its surface (Fig. 3K-L). The flap continues to grow outward as the number of tubercles increases, eventually becoming completely covered by tubercles (Fig. 1B).

\begin{quote}
Developmental association between the tuberculate fleshy flap and a projection of the dentary bone in the Zebrafish
\end{quote}

The tuberculate fleshy flap in the Zebrafish is supported by a lateral projection of the dentary bone (Fang 2003). The above analysis of the appearance of tubercles during development of the Zebrafish revealed a close association between tubercle development and outgrowth of the fleshy flap. This association raises the possibility of inductive interactions between the supporting dentary bone projection and the first-forming tubercles. To investigate this possibility, an additional developmental series of 23 Zebrafish larvae and juveniles was first cleared and stained to visualize bone, and the same specimens were then processed further for SEM. Clearing and staining and SEM imaging of the same individuals revealed that tubercles and dentary bone projections were usually both absent (n= 4; Fig 4) or both present (n=15). If one structure appeared before the other, however, it was more commonly the dentary projection (n=3) rather than tubercles (n=1). These data are more consistent with induction of tubercles by the dentary projection than \textit{vice versa}.

\begin{quote}
Association between the fleshy flap, lateral projections of the dentary bone and breeding tubercles in the subfamily Danioninae
\end{quote}

The fleshy flap on the lower jaw in the genus \textit{Danio} is supported by a lateral projection of the dentary bone in all species from which both have been examined (Fang 2003). While this flap has been described as tuberculate in \textit{Danio} species beyond \textit{D. rerio}, \textit{e.g. D. kyathit} (Fang 1998), the association between the fleshy flap, dentary projections and tubercles has not been comprehensively addressed in a phylogenetic context. Such an analysis is expected to provide further insight into the inductive
interactions among these structures suggested by their order of appearance during development of the Zebrafish. The presence or absence of the fleshy flap, dentary projections, and breeding tubercles was determined for members of six genera in the cyprinid (minnow) subfamily Danioninae by a combination of literature review, clearing and staining for bone, and SEM (Fig. 5).

Male and female specimens of eight species representing the genera *Rasbora*, *Sundadanio*, and *Danio* were imaged by SEM and additional specimens of each of these species were cleared and stained for bone (Figs. 5, 6). Specimens of *Sundadanio axelrodi* imaged by SEM yielded poor quality results (not shown), but this species has previously been reported to have tuberculate fleshy flaps in males but not females (Roberts 1989; Conway *et al.* 2011). Tuberculate fleshy flaps were found in both sexes of *Danio choprae* (Fig. 5B), *D. albolineatus* (Fig. 5I), *D. roseus* (Fig. 5J), and *D. nigrofasciatus* (Fig. 5K), in addition to *D. rerio*. Neither tubercles nor fleshy flaps were found in either sex of *Rasbora trilineata* (Fig. 5A). *D. margaritatus* (Fig. 5C), and *D. erythromicron* (Fig. 5D) exhibited fleshy flaps that were larger in males than females, but lacked tubercles in either sex. In all species examined with fleshy flaps, projections of the dentary bone were found to support them (Fig. 5F-P). These projections varied extensively in size and shape, ranging from very small in *D. erythromicron* (Fig. 5H) to long and narrow in *D. choprae* (Fig. 5F) and to short and broad in *D. albolineatus* (Fig. 5M) and *D. roseus* (Fig. 5N). In the single species lacking fleshy flaps, *Rasbora trilineata*, dentary projections were also absent (Fig. 5E).

Mapping the presence of a fleshy flap supported by a projection of the dentary bone and covered with tubercles onto a phylogeny of the subfamily Danioninae revealed a pattern of parallel origins of this combination of characters in the common ancestor of the genera *Sundadanio* and *Paedocypris* and in the common ancestor of the genus *Danio* (Fig. 6). Interestingly, tubercles were lost from the common ancestor of *D. margaritatus* and *D. erythromicron*, that retained the fleshy flap supported by a dentary projection. This evolutionary dissociation suggests that tubercles are not necessary for the development of the dentary projection.
Candidate genes for the origin and diversification of breeding tubercles

Finally, the Ectodysplasin pathway was examined for involvement in the origin and diversification of breeding tubercles in the order Cypriniformes. Ectodysplasin (*eda*) is a secreted signaling molecule required for the development of a number of skin appendages, including hair, feathers, and teeth (Mikkola 2008). Loss of function mutations in *eda* and its receptor (*edar*) have been described to result in the absence of teeth, scales, gill rakers, and fin rays in the Zebrafish (Harris *et al.* 2008). A single specimen homozygous for each mutation was examined in this study. The specimen homozygous for the *finless* (*fls*) mutation in *edar* completely lacked lower jaw tubercles but retained prominent fleshy flaps (Fig. 7B). Fleshy flaps were reduced in the specimen homozygous for the *nkt* mutation in *eda* and a single tubercle was retained on the lower jaw of one of them (Fig. 7C). Both of these mutant phenotypes are reminiscent of the condition in *D. margaritatus* and *D. erythromicron*, suggesting that the evolutionary loss of Ectodysplasin signaling may have been the cause of the loss of tubercles.

Gain of function in the Ectodysplasin pathway was investigated with the *ef1α:eda* transgenic line of Zebrafish, that exhibits continuous and ubiquitous expression of Ectodysplasin (S. Aigler and D Stock, unpublished data). Comparison of specimens with the transgene to their wild type siblings revealed a statistically significant larger number of tubercles on the fleshy flaps in the former fish (means = 98, 37.9, standard errors of the mean = 10.8, 2.0, *p* = 4.1x10^{-5}, two sample t-test assuming unequal variances) (Fig. 8). The area of the fleshy flaps was not quantified, but the increased number of tubercles may have been the result of both larger flap area as well as a greater density of tubercles (compare Fig 7E, F with 7D). These results suggest that altered Ectodysplasin signaling is a potential mechanism for changes in the number of breeding tubercles in evolution.
Discussion

Developmental patterning of tubercles of the fleshy flap

At the time of first tubercle appearance during development there is only a slight indication of a precursor to the fleshy flap. The appearance of tubercles during flap outgrowth contrasts with the pectoral fin tubercles, which appear on already-formed fin rays. The most widely supported model for regulating the patterning of epidermal appendages suggests that appendage primordia secrete both activators and inhibitors of the formation of additional appendages (Hughes et al. 2011). The spacing of appendages therefore depends on the relative levels of activators and inhibitors. If a similar model applies to breeding tubercles, it is possible that later-forming tubercles along the base and in the center of the flap are able to form only when outgrowth removes the inhibitory effect of the initial tubercles on them.

Testing an activator-inhibitor model of tubercle patterning would be facilitated by knowledge of the specific location(s) at which new tubercles are being added. The data collected in the present study do not indicate whether initially adjacent tubercles remain so or are instead separated later in development by new tubercles appearing between them. The latter process would predict the presence of short tubercles between longer ones, a pattern that was not observed. However, small (and presumably newly-forming) tubercles were rarely observed in any location. A future direction of research would be to more precisely follow the fate of individual tubercles. This might be done by microinjecting tubercles in living fish with a vital stain, noting their initial position, and monitoring their subsequent position with fluorescence microscopy.

Several lines of evidence suggest that fleshy flap tubercles may be induced by outgrowth of a process from the dentary bone. Tubercles of the flap (and hence in the position of the dentary projection) appear well before other tubercles of the lower jaw, such as those forming a more medial strip on each jaw ramus. SEMs of some larval fish revealed a slight swelling of the tip of the lower jaw in the position of the fleshy flap before any tubercles were visible. Clearing and staining followed by SEM revealed more specimens without tubercles and with bony projections than with the reverse condition. No
Zebrafish relative examined exhibited a tuberculate fleshy pad without a bony support. While the absence of tubercles in the presence of a bony projection in *D. margaritatus* and *D. erythromicron* indicates that the projection is not sufficient for tubercle development, this situation does not rule out a requirement of the projection for tubercle formation in this location. The condition of these two species also suggests that an inductive interaction in the opposite direction (of the bony projection by tubercles) is unlikely, although in the absence of data on their development, it cannot be ruled out that tubercles may have been shed after development.

The hypothesis of induction of tubercles by the dentary projection might be further tested by mutagenic screens in the Zebrafish for fish lacking the dentary projection. In addition, it might be possible to insert a foil barrier between the future location of the dentary projection and the epidermis to block potential molecular signals, a classical technique in developmental biology (Gilbert 2011).

*The role of ectodysplasin signaling in the development of breeding tubercles*

Ectodysplasin signaling is generally considered to be an activator of epithelial appendage formation, with loss of function mutations in pathway components resulting in reduction of appendage number and gain of function mutations in additional appendages (Mikkola 2008). The results of the present study suggest that Ectodysplasin signaling plays a similar role in breeding tubercle development. An involvement of Ectodysplasin signaling for tubercle development is indicated by the virtual absence of these structures in homozygotes for the *fls* and *nkt* mutations (although additional specimens need to be examined to confirm this result). The same complete or near complete loss of epithelial appendages is observed for scales, gill rakers, and teeth in these Zebrafish mutants (Harris *et al.* 2008). In contrast, tooth numbers are only partially reduced in humans and mice with loss of function mutations in the Ectodysplasin pathway (Mikkola 2008).

The sufficiency of Ectodysplasin for tubercle development is suggested by the greater number of fleshy pad tubercles in Ectodysplasin over-expressing Zebrafish than in their wild type siblings. This
greater number appears to result from an increase in both the density of tubercles as well as the area that they occupy, a result that remains to be quantified. Interestingly, in feather tracts of the chick overexpressing a constitutively active form of Edar, increase in feather number is solely due to an increase in feather density (Drew et al. 2007). That the tuberculate area was increased in Zebrafish but the area of feather tracts was not increased in the chick may be due to the fact that tubercles appear on the fleshy flap during outgrowth, while initial feathers form on an already established area of skin. The effects of Ectodysplasin loss and gain of function on the dentary projection were not explored, although the size of the fleshy flap in the single nkt homozygote examined appears to be reduced. It will be interesting to determine whether the increased size of the fleshy flap in some eda-overexpressing Zebrafish is accompanied by an altered size or shape of the dentary projection.

Assembly of a synapomorphy in the genus Danio

The phylogenetic analysis presented in Figure 6 suggests that a fleshy flap supported by a projection of the dentary bone and covered with breeding tubercles is a synapomorphy of the genus Danio. Flap and projection are absent in the outgroup Rasbora trilineata and no breeding tubercles are found on the lower jaw, although they are found on the pectoral fin of males (data not shown). Members of the genus Devario, that is closer to Danio than is Rasbora (Tang et al. 2010) have tubercles in multiple rows likely corresponding to the medial row of tubercles present in the Zebrafish along the lower jaw (Fang 1997a,b), while fleshy flaps and dentary projections are absent in Devario (Fang 2003; Conway et al. 2008; Liao et al. 2011). A potential scenario for the origin of the complex, derived condition of a tuberculate flap in Danio is that mutation(s) leading to an outgrowth of the dentary bone induced development of breeding tubercles in the overlying epithelium. The capacity to produce breeding tubercles would have already existed, as evidenced by their presence along the lower jaw of Devario. Presence of the dentary projection may, however, have led to the heterochronic (earlier) induction of tubercles as suggested by the earlier appearance of fleshy flap tubercles in Danio relative to medial row
tubercles. A similar sequence of correlated morphological changes apparently happened independently in males of the common ancestor of Paedocypris and Sundadanio. Interestingly, Sundadanio males have two projections from the dentary bone, each covered by tubercles (Conway et al. 2011), further suggesting the ability of bony projections to induce breeding tubercles.

Danio margaritatus and D. erythromicron retain the fleshy flap and dentary projection of their ancestors but the flap lacks tubercles. The common ancestor of these two species is likely to have experienced mutation(s) in gene(s) required for the development of all breeding tubercles. No breeding tubercles are found anywhere on the body of D. margaritatus (Roberts 2007) and the present study did not find any tubercles on the head or fins of D. erythromicron. The requirement of Ectodysplasin signaling for tubercle development in the Zebrafish identifies genes in this pathway as candidate causes of tubercle loss in the common ancestor of the former two species. This hypothesis can be tested by overexpression of Ectodysplasin in either D. margaritatus or D. erythromicron, which is predicted to result in the induction of tubercles.

Conclusion

Characterization of the timing and pattern of appearance of breeding tubercles reported in this study not only suggests mechanisms of their development, but provides a basis for evaluating the phenotypic effects of mutations on tubercle pattern. The pattern of tubercle variation in the subfamily Danioninae has been documented in a phylogenetic context, and a candidate cause of this variation (altered Ectodysplasin signaling) has been identified. Through these contributions, this study has helped to establish the Zebrafish as a model for investigating the development and evolution of breeding tubercles in fishes.
Acknowledgements

I would like to thank Dr. David Stock for his guidance, support, and feedback throughout this study. I would also like to thank Dr. Barbara Demmig-Adams for facilitating the Honors Thesis Seminar and for her encouragement. I would like to thank Brent Hawkins for access to his unpublished data and Rob Roscow for assistance with photography. I would like to thank Dr. Pei-San Tsai for agreeing to serve on my defense committee. Finally, I would like to thank the Undergraduate Research Opportunity Program (UROP) for providing me with two grants to perform my research and the Professional and Academic Conference Endowment (PACE), which allowed me to present some of this work at a professional meeting.

References


Table 1. Presence or absence of fleshy flap, dentary projection, and breeding tubercles in members of the subfamily Danioninae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Flap</th>
<th>Reference</th>
<th>Projection</th>
<th>Reference</th>
<th>Tubercles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rasbora trilineata</td>
<td>no</td>
<td>this study</td>
<td>no</td>
<td>this study</td>
<td>no</td>
<td>this study</td>
</tr>
<tr>
<td>Paedocypris sp.</td>
<td>?</td>
<td>yes</td>
<td>Reference</td>
<td>Conway et al. (2008); Britz and Conway (2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sundadanio axelrodi</td>
<td>yes</td>
<td>Roberts (1989); Conway et al. (2011); this study</td>
<td>yes</td>
<td>Fang (2003); Conway et al. (2008); this study</td>
<td>yes</td>
<td>Conway et al. (2008); Britz and Conway (2009); this study</td>
</tr>
<tr>
<td>Danio feegradei</td>
<td>yes</td>
<td>Hora (1937)</td>
<td>?</td>
<td></td>
<td>yes</td>
<td>Hora (1937)</td>
</tr>
<tr>
<td>Danio choprae</td>
<td>yes</td>
<td>Fang (2000); this study</td>
<td>yes</td>
<td>Liao et al. (2011); this study</td>
<td>yes</td>
<td>Kullander (2012); this study</td>
</tr>
</tbody>
</table>
Table 1. Presence or absence of fleshy flap, dentary projection, and breeding tubercles in members of the subfamily Danioninae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Presence</th>
<th>Reference 1</th>
<th>Reference 2</th>
<th>Reference 3</th>
<th>Reference 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danio margaritatus</td>
<td>yes</td>
<td>Roberts (2007); this study</td>
<td>Roberts (2007); Conway et al. (2008); Liao et al. (2011); this study</td>
<td>no</td>
<td>Roberts (2007); this study</td>
</tr>
<tr>
<td>Danio erythromicron</td>
<td>yes</td>
<td>Fang (2003); this study</td>
<td>Fang (2003); Roberts (2007); Conway et al. (2008); this study</td>
<td>n</td>
<td>this study</td>
</tr>
<tr>
<td>Danio albolineatus</td>
<td>yes</td>
<td>Fang (2003); this study</td>
<td>Fang (2003); this study</td>
<td>yes</td>
<td>this study</td>
</tr>
<tr>
<td>Danio roseus</td>
<td>yes</td>
<td>Fang and Kottelat (2000); this study</td>
<td>this study</td>
<td>yes</td>
<td>this study</td>
</tr>
<tr>
<td>Danio nigrofasciatus</td>
<td>yes</td>
<td>Fang (2003); Conway et al. (2008); this study</td>
<td>Fang (2003); Conway et al. (2008); this study</td>
<td>yes</td>
<td>this study</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>yes</td>
<td>Roberts (1989); Fang (2003); this study</td>
<td>Fang (2003); this study</td>
<td>yes</td>
<td>Roberts (1989); this study</td>
</tr>
</tbody>
</table>
**Figure Legends**

**Figure 1.** Distribution of breeding tubercles in wild type adult Zebrafish. (A) Lower jaw of male, 25.9 mm standard length (SL). (B) Fleshy flap of the lower jaw of female, 30.6 mm SL. (C) Individual tubercle of the fleshy flap, same fish as in (B). (D) Pectoral fin of male, 23.5 mm SL showing breeding tubercles on the first five fin rays.

**Figure 2.** Number of tubercles on both lower jaw fleshy flaps as a function of standard length in larval and juvenile Zebrafish.

**Figure 3.** Development of fleshy flap tubercles. Ventral SEM views of anterior lower jaw halves are arranged by increasing tubercle number or development. Arrowheads indicate position or future position of fleshy flap. (A) No tubercles, 7.1 mm standard length (SL); (B) Unkeratinized swelling, 9.4 mm SL; (C) Unkeratinized swelling, 8.7 mm SL; (D) One tubercle, 9.0 mm SL; (E) Two tubercles, rostral tubercle less developed, 10.0 mm SL; (F) Two tubercles, 10.0 mm SL; (G) Three tubercles, 11.4 mm SL; (H) Four tubercles, 13.7 mm SL; (I) Five tubercles, 12.5 mm SL; (J) Seven tubercles, 13.1 mm SL; (K) Eleven tubercles, 16.7 mm SL; (L) Fifteen tubercles, 16.4 mm SL.

**Figure 4.** Number of tubercles on both lower jaw fleshy flaps as a function of standard length in larval and juvenile Zebrafish subjected to clearing and staining followed by SEM. Colors denote presence or absence of dentary bone projection in combination with absence or presence of tubercles: both absent (black), both present (purple), projections present but not tubercles (blue), tubercles present but not projections (red).
Figure 5. Lower jaws of additional species in the subfamily Danioninae. (A-D, I-L) SEM images showing presence or absence of fleshy flaps (arrowheads) and tubercles. (E-H, M-P) Cleared and stained dentary bones showing presence or absence of projections (arrowheads). (A, E) Rasbora trilineata, male, 28.1 mm standard length (SL); (B, F) Danio choprai, female, 22.5 mm SL; (C, G) Danio margaritatus, male, 19.3 mm SL; (D, H) Danio erythromicron, male, 16.7 mm SL; (I, M) Danio albolineatus, female, 27.2 mm SL; (J, N) Danio roseus, male, 33.1 mm SL; (K, O) Danio nigrofasciatus, unknown sex, 14.0 mm SL; (L) Danio rerio, female, 22.0 mm; (P) Danio rerio, unknown sex and SL.

Figure 6. Evolution of a tuberculate fleshy flap in selected genera and species of the subfamily Danioninae. Character states (black = presence, white = absence) were determined in this study (Fig. 5) or from the literature (Table 1) and mapped on a phylogenetic tree of species from Tang et al. (2010) using the program Mesquite version 2.75 (Maddison and Maddison 2011). Ancestral states were reconstructed using the parsimony option and treating lower jaw condition as a single unordered character.

Figure 7. Lower jaws of Zebrafish with altered ectodysplasin signaling compared with wild type. (A, D) Wild type, (B) Homozygous fls (loss of function mutation in ectodysplasin receptor) (C) Homozygous nkt (loss of function mutation in ectodysplasin), (E, F) ef1α:eda transgenic line (overexpressing ectodysplasin). Fish in D-F are siblings.

Figure 8. Comparison of number of tubercles between wild type and ef1α:eda zebrafish. Counts are of tubercles on both fleshy flaps of the lower jaw. P = 4.1 x 10^{-5}, t-test. Bars represent standard errors of the mean.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.

$p < 0.001$