ONTOGENY OF THE CYPRINIFORM PHARYNGEAL JAW APPARATUS AND ITS ASSOCIATED MUSCULATURE

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Abstract of Thesis

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The pharyngeal jaw apparatus is an assemblage of bones and muscles found within the head of fishes. While the oral jaws are used for prey capture in fishes, the pharyngeal jaws are used for prey processing. There is a basic form of the pharyngeal jaw apparatus found amongst all fishes; however, some fish groups possess features of the pharyngeal jaw apparatus that are specialized for the demands of their diet (Vandewalle, 2000). One such group are the Cypriniformes.

Cypriniformes is one of the world's largest clades of freshwater fishes (Nelson, 2006). They are widely distributed in North America, Europe, Africa, and Asia, but are absent from Australia and South America. The clade is united by several characters, including the presence of a kinethmoid, a lack of teeth on the oral jaws, and an enlarged fifth ceratobranchial that opposes a pad on the basioccipital of the skull. Using developmental and histological methods, I investigated the development of the cypriniform pharyngeal jaw apparatus, a morphological novelty for this group of fishes, using the zebrafish model system.

Results indicate that the zebrafish's enlarged fifth ceratobranchial is the largest ceratobranchial very early on in development. Additionally, the levator posterior, which mediates movement of this bone, is also larger than the other levators through ontogeny, beginning very early on in development. Lastly, a unique pattern of bone and muscle development was observed, where the levators externi developed prior to the appearance of the bony elements on which they attached. In contrast, the levators interni developed at

approximately the same time as the bony elements on which they attach. This study is one of the first to provide insight into how the pharyngeal jaw apparatus as a whole develops.

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List of Symbols/Nomenclature

AC = anterior copula

AD5 = adductor 5

B = basibranchial

BH = basihyal

Boc = basioccipital

CB/Cb = ceratobranchial

CH = ceratohyal

Cl = cleithrum

CP = chewing pad

Cyprinid = Any member of the family Cyprinidae

Cypriniform = Any member of the order Cypriniformes

dpf = days post fertilization

EB/Eb = epibranchial

GH = geniohyoideus

H = hypobranchial

HH = hypohyal

Hy = hyoid

LE = levator externus

LI = levator internus

LP = levator posterior

LPJ = lower pharyngeal jaw

mm = millimeter

NCR = neurocranium

ON = overnight

PB/Pb = pharyngobranchial

PC = posterior copula

PCE = pharyngocleithrus externus

PCI = pharyngocleithrus internus

PCIa = pharyngocleithrus internus anterior

PCIp = pharyngocleithrus internus posterior

PJA = pharyngeal jaw apparatus

PO = palatal organ

RC = rectus communis

Re = retractor muscle

RP = retractor pharyngeus

SL = standard length

TP = tooth plate

TV4 = transversus ventralis 4

UPJ = upper pharyngeal jaw

X-section = cross section

Chapter 1: Introduction

Cypriniforms (minnows, loaches, algae-eaters, and catostomids) are a morphologically diverse clade of freshwater fishes that inhabit the continents of North America, Europe, Asia, and Africa. They are included in the Otophysi, which also includes Characiforms (tetras and piranhas), Siluriforms (catfish), and Gymnotiforms (American knifefish) (Nelson, 2006). While the Otophysi are united by the presence of the Weberian apparatus, Cypriniforms are set apart by several unique characters. These include the presence of the kinethmoid, a protrusible mouth, lack of teeth on the oral jaws, an enlarged fifth ceratobranchial, and an enlarged basioccipital that bears a keratinized pad (Nelson, 2006; Howes, 1991). The enlarged fifth ceratobranchial is one of the key components that form the unique structure that is the Cypriniform pharyngeal jaw apparatus.

The Cypriniform Pharyngeal Jaw Apparatus

All fishes possess some form of the pharyngeal jaw apparatus and its associated musculature, whose function is to process prey after it is captured. The pharyngeal jaw apparatus is usually composed of upper and lower pharyngeal jaws, which are constructed from modified parts of the pharyngeal skeleton (Lauder and Liem, 1992). The form most commonly seen in fishes is described briefly.

The bones comprising the pharyngeal skeleton include the basibranchials, hypobranchials, ceratobranchials, epibranchials, and pharyngobranchials. The first four ceratobranchials provide support for the gills, while the fifth ceratobranchial is often modified into lower jaw elements. In several fish, an upper pharyngeal jaw is formed from tooth plates on the ventral surface of the pharyngobranchials, and it is against these

that the lower pharyngeal jaw processes food. These bony elements are suspended from the neurocranium by a set of external levator muscles and a set of internal levator muscles. Most fish show little specialization in these muscles (Vandewalle et al., 2000).

However, this lack of specialization is not the case in cypriniform fishes (Vandewalle et al., 2000), where key derived features in the pharyngeal jaw apparatus give it a unique conformation not seen in most other fishes. First, cypriniforms have a hypertrophied fifth ceratobranchial, which bears teeth and forms the lower pharyngeal jaw. Secondly, cypriniforms lack upper pharyngeal jaws and instead process food against a keratinized pad on the basioccipital.

Cypriniforms also possess characteristic musculature that helps control movement of the unique lower pharyngeal jaws (Vandewalle et al., 2000). First, the levator posterior is larger in size and inserts on the fifth ceratobranchial instead of the fourth epibranchial, which is the case in most other fishes. This allows for greater movement and control of the lower pharyngeal jaws. Cypriniforms also possess a retractor muscle, known as the retractor pharyngeus, which runs from the pharyngeal jaws to the basioccipital and adducts the pharyngeal bones. Lastly, they possess a ventral transversus muscle that helps in positioning the teeth against the pharyngeal pad on the basioccipital during prey processing (Vandewalle et al., 2000; Sibbing, 1982; Springer and Johnson, 2004).

Goals of Study

The goals of this study, described in Chapter 2, are twofold. First, I determine and described the adult structure of the pharyngeal jaw apparatus and its associated musculature of the model cypriniform, the zebrafish *Danio rerio*. The timing of the appearance of the bone and muscular elements associated with the pharyngeal jaw

apparatus during development is then determined and compared with that of other cypriniforms to determine if there is a common developmental pattern associated with the presence of an enlarged fifth ceratobranchial. These findings add to those previously published that have described the adult structure of the pharyngeal jaw apparatus and its associated musculature in other Cypriniforms. Also, this study provides new information on how the pharyngeal jaw apparatus is constructed during the course of development, on which little work has been done.

Chapter 2: Ontogeny of the pharyngeal jaw apparatus and its associated musculature in *Danio rerio*

INTRODUCTION

Cypriniformes is a speciose group of freshwater fish with approximately 3,269 species represented within 5 families (Balitoridae, Cyprinidae, Catostomidae, Cobitidae, and Gyrinocheilidae) (Nelson, 2006). While notably absent in South America, these fishes inhabit a wide range of habitats in North America, Africa, and Eurasia, with their greatest diversity being found in southeastern Asia (Nelson, 2006). Cypriniforms are morphologically diverse and adapted to their unique feeding ecologies, from the small algae-scraping golden algae eaters, to the large herbivorous grass carp (Chilton and Muoneke, 1992; Berra, 2007). Moreover, Cypriniformes is united by several morphological novelties including the kinethmoid (a unique median ossification used to effect premaxillary protrusion), a lack of oral jaw teeth, and a unique conformation of the pharyngeal jaw apparatus (Nelson, 2006).

The pharyngeal jaw apparatus plays an integral part in mastication and/or food transport in a great many fish groups. While the oral jaws are used for prey capture, the pharyngeal jaws are used for prey processing and transport into the esophagus. The pharyngeal jaw apparatus, located in the pharynx, is made up of toothed bony elements and the musculature responsible for effecting their motion (Lauder and Wainwright, 1992). The pharyngeal skeleton is composed of many bony parts, and the clupeid pharyngeal skeleton represents the basal condition for teleostean pharyngeal structure (Fig. 2.1). Anteriorly are paired ceratohyals with their associated hypohyals, and the medial basihyal. The ceratohyals are followed posteriorly by five paired ceratobranchials.

The lower pharyngeal jaws are formed from modified ceratobranchial elements (the 4th ceratobranchial in *Polypterus*, the 5th ceratobranchial in all other fishes) (Lauder and Wainwright, 1992). The size and shape of the pharyngeal teeth found on the pharyngeal jaws are often specialized to fishes' feeding ecology (Streelman and Albertson, 2006). Articulating at the distal end of each of the first four paired ceratobranchials is an epibranchial. Each epibranchial in turn articulates with a more medially placed pharyngobranchial. The lower pharyngeal jaws oppose the upper pharyngeal jaws, which are formed from dermal tooth plates. In *Denticeps clupeoides* (Fig. 2.1) these tooth plates are located ventrally on the anterior tip of the fourth epibranchial, however, in other fishes the tooth plates are located on the ventral surface of the pharyngobranchial elements (Greenwood, 1968; Vandewalle et al., 2000). Paired hypobranchials are observed adjacent to the more proximal ends of the first three ceratobranchials. Three basibranchials occur along the midline in between the paired hypobranchials.

The pharyngeal skeleton of lower teleosts is suspended by several different cranial muscles (Fig. 2.2). Four levators externi originate from the ventral base of the neurocranium and insert upon the tip of their respective epibranchial. Additionally, there are 2 to 3 levators interni that originate medial to the levators externi and insert upon the dorsal surface of the pharyngobranchials. The internal and external levators function together in moving the upper pharyngeal jaws (Vandewalle et al., 2000). The levator posterior, if present, originates on the neurocranium posterior to the fourth levator externus and inserts upon the fourth epibranchial. The pharyngocleithralis (externus and internus) muscles insert on the lower pharyngeal jaw from their origin on the pectoral girdle, while the rectus communis connects the lower pharyngeal jaw to the third

hypobranchial. The adductor 5 muscle connects the fourth epibranchial to the fifth ceratobranchial. Lastly, the retractor dorsalis muscle, if present (as seen in *Amia calva*), extends from its origin on the vertebral column to the upper pharyngeal jaws (Vandewalle et al., 2000; Lauder and Wainwright, 1992; Springer and Johnson, 2004).

The cypriniform pharyngeal jaw apparatus has become highly specialized, and as previously mentioned, is characterized by several morphological novelties. Firstly, cypriniforms lack dermal tooth plates on the ventral surface of the pharyngobranchials, and have lost the upper pharyngeal jaws. Secondly, cypriniforms possess a keratinized pharyngeal pad on the basioccipital against which the teeth of a hypertrophied fifth ceratobranchial oppose and process food. Lastly, cypriniforms have a muscular sling formed by what some authors consider to be the levator posterior, that originates from the subtemporal fossa of the skull and inserts upon the enlarged fifth ceratobranchial (Sibbing, 1982; Nelson, 2006). This muscle rotates the pharyngeal jaws and move them towards the pharyngeal pad during prey processing (Sibbing, 1982). Internal levators, the pharyngocleithralis (externus and internus) muscles, and the rectus communis are also present in cypriniforms. A cypriniform retractor muscle, the retractor pharyngeus, originates on the basioccipital and inserts on the posterior surface of the lower pharyngeal jaws; however, this muscle is not homologous to the retractor muscle seen in *Amia calva* (Lauder and Wainwright, 1992). The transversus ventralis 4 connects the lower pharyngeal jaw to the fourth ceratobranchial, and adductor 5 connects the distal tip of the fifth ceratobranchial to the dorsoposterior margin of epibranchial 4 (Fig. 2.3) (Sibbing, 1982; Springer and Johnson, 2004).

The mechanisms that gave rise to the specialized morphology of the adult cypriniform pharyngeal jaw apparatus remain unknown. I hypothesize that differences in the developmental timing of the muscular and skeletal elements have led to the unique conformation seen in the cypriniform pharyngeal jaw apparatus. To investigate this hypothesis, I have examined and described the musculoskeletal ontogeny of these pharyngeal elements within the model cypriniform, *Danio rerio*. I first describe the development of musculoskeletal structures associated with the pharyngeal jaw apparatus in an ontogenetic series of larval to adult zebrafish. I chose ontogenetic stages that corresponded to key points in skeletal development in order to test the hypothesis that the levator externi develop before the epibranchials on which they insert. I then discuss how the structure and development of the pharyngeal jaw apparatus in zebrafish is comparable to that of the pharyngeal jaw apparatus in other cypriniforms. By doing this, I will determine if the zebrafish pharyngeal jaw apparatus has a structure that is common amongst the cypriniforms. I will also determine if there is a shared underlying developmental mechanism amongst the cypriniforms that leads to the unique conformation of their pharyngeal jaw apparatus.

MATERIALS AND METHODS

Maintenance of Zebrafish

Breeding stocks obtained from commercial breeders were maintained in 9.5 liter and 2.8 liter tanks on a stand-alone flow thru system at 28°C on a 12-hour light/12-hour dark cycle. Fish were feed a diet of brine shrimp. Embryos were collected through natural

breeding and were raised in embryo medium in a 31°C incubator. They were collected and staged according to Kimmel et al. (1995). Those being raised to later developmental stages were placed back in the flow thru system after one week of age.

Ontogenetic Stages

Ontogenetic stages studied included early larval stages of four to six days post fertilization (2.6-2.8 mm standard length (SL), late larval stages of fourteen days post fertilization (4.4-4.6 mm SL), juvenile stages (8.0-12.0 mm SL), and adult stages (~25.0-30.0 mm SL). These stages were chosen based on key thresholds of skeletal development described in the literature (Cubbage and Mabee, 1996; Schilling and Kimmel, 1997). Early larval stages were chosen for examining the presence of the ceratobranchials, late larval stages for the appearance of epi- and pharyngobranchials, juvenile stages for investigating the ossification sequence of the cerato-, epi-, and pharyngobranchials, and adult stages for documenting the fully formed pharyngeal skeleton.

Histology and Dissection

Fish specimens of all ontogenetic stages were fixed overnight in either 10% buffered formalin or 4% paraformaldehyde to be embedded in JB4 plastic or paraffin to be sectioned, or to be cleared and stained. Fish were embedded in plastic according to the instructions provided with the JB4 embedding kit (Electron Microscopy Sciences). Plastic blocks were sectioned at 3 μm on a microtome with a 6 mm glass knife and then stained with Lee's methylene blue and basic fuscin to differentiate tissues. Fish to be embedded in paraffin were first fully dehydrated in up to 100% ethanol, then quickly washed in xylene to clear the tissue. The length of the washes varied with size of the specimen, with larger fish needing longer washes. The fish were removed from the

xylene wash once the tissue was visibly clear. The container holding the specimen was then moved to a vacuum oven, where paraffin was added to the container, and the specimen was vacuum infiltrated. The length of the infiltration period varied with the size of the specimen, with larger fish being infiltrated for longer periods of time. Two more additions of paraffin and vacuum infiltration followed. The specimen was then transferred to a new container possessing clean paraffin where it was vacuum infiltrated one final time, and then embedded in a mold and allowed to cool. Once cooled, it was sectioned on a microtome at 12 μm and sections were stained with Hall's quadruple stain to differentiate tissues. Additional fish fixed in 10% buffered formalin or 4% paraformaldehyde were cleared and double-stained for cartilage and bone with alcian blue and alizarin red using a protocol presented by Dingerkus and Uhler (1977) with modifications by Potthoff (1984). These cleared and stained specimens, as well as fresh adult specimens, were then dissected under an Olympus SZX12 microscope. Adult specimens used for muscle dissection were dipped in Lugol's solution to stain all muscle fibers to facilitate fiber direction to determine their points of origin and insertion. Photographs of specimens were taken with an Olympus DP12 camera and drawings were produced using a Wacom Intuos 6x8-drawing tablet.

Terminology for the muscles associated with the zebrafish pharyngeal jaw apparatus follows Winterbottom (1974), with the exception of the pharyngocleithralis externus and internus, which are based on nomenclature modifications by Liem (1970).

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed on larvae from 3-6 days post fertilization (dpf). After being fixed overnight (ON) at room temperature in Carnoy's

solution (60% ethyl alcohol, 30% chloroform, 10% glacial acetic acid), fish were dehydrated in an ethanol series to 95% ethanol and then rehydrated back to distilled water. They were then treated with a proteinase K solution for 20 minutes and then rinsed and washed with PBT three times at 20 minutes per wash. Nonspecific binding was blocked with a blocking solution (PBT with 2% bovine serum albumin and 5% normal goat serum) for 30 minutes. Primary antibodies were diluted in blocking solution to obtain a 5 μg/ml concentration. Primary antibodies used were MF20, which stains all muscle fiber types, and II-II6B3, which stains collagen Type II and is thus a marker for cartilage, both were obtained from Developmental Studies Hybridoma Bank. After sitting ON at room temperature in primary antibodies, the larvae were rinsed several times with PBT and then washed 5 times at 15 minutes per wash. They were once again blocked and secondary antibodies (Jackson ImmunoResearch) were added at a 1:100 dilution in blocking solution. After sitting ON at room temperature in the secondary antibodies, the larvae were rinsed and then washed 5 times at 15 minutes per wash in PBT. They were further cleared in an 80% glycerol/PBT solution. They were stored at 4°C if they were not immediately viewed on a Zeiss Akioskop fluorescent microscope.

RESULTS

Adult Morphology

Adult zebrafish possess one paired ceratohyal and five paired ceratobranchials, the last pair of which is modified into hypertrophied lower pharyngeal jaws (Fig. 2.4a). Each of the first four paired ossified ceratobranchials articulates at their dorsal tip with an ossified epibranchial. These epibranchials in turn articulate with the pharyngobranchials,

of which position two and three are present as ossified elements (one and four remain cartilaginous throughout life). A fifth epibranchial also articulates with the fourth ceratobranchial; this small epibranchial remains cartilaginous into adulthood. Along the midline, one basihyal, three basibranchials, and three pairs of hypobranchials located at the proximal ends of the ceratobranchials are present.

The first three levators externi insert on the dorsal tip of their respective epibranchial, while the fourth inserts on the lateral margin of the fourth epibranchial (Fig. 2.4b). The large levator posterior (levator ceratobranchialis 5; Springer and Johnson, 2004) originates from the deep subtemporal fossa of the skull posterior to the fourth external levator and inserts on the distal tip of the greatly enlarged fifth ceratobranchial. Adductor 5 connects the dorsal tip of the fifth ceratobranchial to the fourth epibranchial. There are two levators interni, the first of which originates from the skull medial to the first external levator and inserts on pharyngobranchial 2. The second levator internus originates medial to the second levator externus, and then divides into an anterior and posterior head. Both heads insert on the third pharyngobranchial. The retractor pharyngeus originates on the ventral basioccipital and inserts on the entire posterolateral surface of ceratobranchial 5. The pharyngocleithralis externus is a large fan-shaped muscle that originates on the anteroventral margin of the cleithrum and extends dorsally to insert on then anteroventral region of ceratobranchial 5. The pharyngocleithralis internus posterior is a thin muscle that originates on the anterior surface of the cleithrum, medial to the pharyngocleithralis externus, and extends anterodorsally to insert via a tendon on the anteroventral margin of ceratobranchial 5 immediately anterior to the pharyngocleithralis externus insertion. The pharyngocleithralis internus anterior

originates on the cleithrum well anterior to the pharyngocleithralis externus, and runs dorsally to insert on the posterior copula, which extends caudally from basibranchial 3 to ceratobranchial 5. The rectus communis originates from the anterolateral tip of ceratobranchial 5 and extends anteriorly to insert on the posterior surface of hypobranchial 3. Transversus ventralis 4 originates on the anterolateral surface of ceratobranchial 5 and inserts on the posterolateral surface of ceratobranchial 4 (Fig. 2.4b) (Springer and Johnson, 2004; Sibbing, 1982).

2.6-2.8 mm SL (3-6 dpf)

At 6 dpf, the paired ceratohyals and all five paired ceratobranchial elements are present (Fig. 2.5a). Posterior to the basihyal, an undivided bar of cartilage, the anterior copula, delineates where the basibranchials will form. Posterior to the anterior copula is a round area of cartilage that will form the posterior copula. Between each of the first three paired ceratobranchials and the anterior copula is a small round area of cartilage representing the precursor to a hypobranchial. Ossification of skeletal elements is limited to the pharyngeal teeth on an already hypertrophied ceratobranchial 5 (Fig. 2.5a). The large levator posterior is present inserting on the dorsal arm of the fifth ceratobranchial in fish as early as 3 dpf (Figs. 5c, f). Other muscles including the retractor pharyngeus (originating on the basioccipital, inserting on ceratobranchial 5), pharyngocleithralis externus (originating on cleithrum, inserting on ceratobranchial 5), and the rectus communis (originating on ceratobranchial 5, inserting on hypobranchial 3), are also present (Figs. 2.5b, e, f). While the four levators externi are present from their origins on the neurocranium, the epibranchials upon whey they will eventually insert are not (Fig.

2.5a, b, c). These muscles instead appear to terminate in loose mesenchymal tissue adjacent to the area where these pharyngeal elements are fated to develop (Fig. 2.5d).

4.4-4.9 mm SL (12-14 dpf)

At 4.4 mm SL, approximately two weeks of age, the epibranchials begin to develop as cartilaginous elements articulating with the distal tips of each ceratobranchial. Epibranchials 3 and 4 appear first and all epibranchials are present by 4.6 mm (Fig. 2.6a, c, d). At this developmental stage, the cartilage cells of the anterior copula are condensed. Only ceratobranchial 5 is partially ossified, while other ceratobranchials are still cartilaginous (Fig. 4.6a, d). The pharyngobranchials develop between 4.7 and 4.9 mm, and appear at cartilaginous elements articulating with the epibranchials (Fig. 4.6e). It is not until 4.7 to 4.9 mm that the levator externi are observed clearly inserting on their appropriate epibranchial (Fig. 4.6b, e). Unlike the situation with the levators externi, which appear prior to the development of their epibranchial insertions, the levators interni arise at approximately the same time as the pharyngobranchials upon which they insert and are present by 4.7 to 4.9 mm (Fig. 4.6e).

7.0-12.0 mm SL (Juvenile)

By 8.6 mm, all ceratobranchials, epibranchials, and pharyngobranchials have developed. The fifth ceratobranchial is fully ossified, while ceratobranchials 1-4 have discrete ossification centers at their midpoint. The only epibranchial undergoing ossification at 8.6 mm is epibranchial 4, with ossification occurring in the center of the element. All other epibranchials remain cartilaginous at this stage (Fig. 2.7a). The levators externi are seen clearly inserting on the epibranchials (Fig. 2.7b, d). Cartilaginous pharyngobranchials are developed adjacent to the base of the neurocranium

and are separated from other pharyngeal elements by the palatal organ (Fig. 2.7b, c), a muscular organ on the roof of the pharyngeal cavity that is though to help process food (Sibbing and Uribe, 1985).

DISCUSSION

Development of the zebrafish PJA

Being a popular model system, zebrafish were utilized to investigate the development of an important fish feeding structure, the pharyngeal jaw apparatus. While the pharyngeal jaw apparatus is widely studied in terms of function and morphology, little work exists on the development of this structure (Aerts, 1982). Additionally, developmental studies addressing either the bones or muscles of this structure in zebrafish have failed to do so in the context of adult morphology. This is an unfortunate omission given that my study points to a unique developmental pattern of both the skeletal and muscular components associated with the pharyngeal jaw apparatus that has arisen in cypriniforms.

My observations on zebrafish pharyngeal skeleton development agree with observations made in previous studies (Schilling and Kimmel, 1997; Cubbage and Mabee, 1996). However, our study reveals some new aspects of the pharyngeal musculature development that are quite striking. First, the levator posterior in adult zebrafish is approximately two times larger in size when compared to the other branchial levators. In fact, this size differential is evident in zebrafish as young as 3 dpf. Additionally, the levators externi display a pattern of development in which they form

before the epibranchials on which they insert have chondrified. This result indicates that the levators externi are instead terminating in loose mesenchymal tissue. A similar situation was observed by Hall who noted that the levator mandibulae in larval amphibians terminated in loose mesenchyme due to the absence of the mandible (Scott, 1957). In contrast, it appears that the internal levators do not develop until the pharyngobranchials they insert upon have already appeared. Given this information, I propose that the zebrafish pharyngeal jaw apparatus and its associated musculature can be used as a model to better understand the interactions between muscle and bone during development, especially regarding the differences in developmental timing between the levators externi and interni and their respective skeletal structures.

Comparing pharyngeal skeleton development

The development of the pharyngeal skeleton of zebrafish, especially that of ceratobranchial 5, is markedly different when compared to groups basal to Cypriniformes. Previous work has shown that the fifth ceratobranchial in zebrafish develops earlier than the fourth ceratobranchial and also ossifies earlier than all other ceratobranchials (Schilling and Kimmel, 1997; Cubbage and Mabee, 1996). The fifth ceratobranchial is the last arch to develop in more basal taxa such as the anguilliform *Anguilla vulgaris* and the clupeiform *Sardinops japonicus*. The fifth ceratobranchial is also the last arch to form in *Chanos chanos*, a member of the Gonorynchiformes, the sister taxon to Ostariophysii (Norman, 1926; Matsuoka, 1997; Kohno et al., 1996).

In comparison to other cypriniforms, there are both similarities and differences between their skeletal development and that of zebrafish (Cyprinidae, Rasborinae). One of these differences observed is manifest in the skeletal development of *Barbus barbus*

(Cyprinidae, Barbinae) (Saitoh et al., 2006; Nelson, 2006). Unlike zebrafish, the 5th ceratobranchial of *B. barbus* is the last arch to appear (Vandewalle et al., 1992). Work on the appearance of the pharyngeal skeleton of *Catostomus commersonii* (Catostomidae) shows that the 5th ceratobranchial develops at approximately the same time as ceratobranchials three and four (Engeman et al. 2009). The sucker *Catostomus macrocheilus* (Catostomidae) shows a similar ossification pattern to zebrafish in that the 5th ceratobranchial is the first ceratobranchial to ossify (Weisel, 1967). More work is needed within Cypriniformes to understand how changes in skeletal development might have contributed to the unique conformation seen in the 5th ceratobranchial of cypriniforms.

Zebrafish PJA and its associated musculature compared to other Cypriniforms

Descriptions of both muscular and skeletal aspects associated with the pharyngeal jaw apparatus in some cypriniforms have been publishes (Matthes, 1963; Saxena, 1960; Sibbing, 1982; Springer and Johnson, 2004; Takahasi, 1925). The largest comparative study is that of Takahasi (1925), in which he examined the groups he calls cyprinoids and cobitoids, and notes several differences in the muscles that help to form the pharyngeal jaw apparatus. Below I discuss the major differences between zebrafish and those fish described by Takahasi.

Of the fish described by Takahasi, the zebrafish pharyngeal jaw apparatus seems most similar to that of *Cyprinus carpio* (Cyprinidae, Cyprininae). This said, there are some differences between that of the zebrafish pharyngeal jaw apparatus and its associated musculature and that of other cypriniforms described by Takahasi. First, zebrafish possess two levators interni. This is in contrast to *Pseudogobio esocinus* and the

five cobitoids studied by Takahasi, which have three internal levators. Another difference between zebrafish and cobitoids is in their retractor pharyngeus. In zebrafish and other cyprinoids, this muscle arises from the basioccipital and inserts on the 5th ceratobranchial. In cobitoids, this muscle arises partly from the basioccipital and partly from the pharyngeal wall and inserts on the last pharyngobranchial. Given the variation already described in the pharyngeal jaw apparatus by Takahasi, and the variation found in other cypriniform structures, I hypothesize that more variation exists in Cypriniformes that has yet to be recognized (Hernandez et al., 2007; Bird and Hernandez, 2007).

Conclusion

This study has provided a detailed look at how the pharyngeal jaw apparatus and its associated musculature develop during the course of ontogeny. The levators externi develop before their points of insertion (the epibranchials) while the levators interni develop at approximately the same time as their points of insertion (the pharyngobranchials). The levator posterior is large very early on in development. The development of these muscles needs further study in outgroup taxa to determine if this represents a specialized developmental pattern that might have contributed to the unique conformation of the pharyngeal jaw apparatus in cypriniforms.

More work is needed to investigate the development of the pharyngeal skeleton. As already discussed, the appearance and ossification sequence of the pharyngeal skeleton in zebrafish does not appear to be the pattern found throughout Cypriniformes, with the 5th ceratobranchial being the last arch to appear in some cases. This points to more work being done amongst all Cypriniformes to investigate how changes in the skeletal development might contribute to the conformation of the pharyngeal jaw

apparatus seen in this group. By investigating these developmental timings, one can hope to begin to understand why evolutionary mechanisms might have allowed for the unique conformation of the cypriniform pharyngeal jaw apparatus and its musculature to arise. This information would not only be helpful in the study of the Cypriniformes but will also help to gain a better understanding of how similar morphological novelties of the pharyngeal jaw apparatus might have arisen in other fish groups as well.

Figure 2.1. Dorsal view of the pharyngeal skeleton of *Denticeps clupeoides*, representative of the basal condition for the teleostean pharyngeal skeleton (modified from Greenwood 1968). The right side is reflected to clearly show the dorsal skeletal elements. The left side illustrates the skeleton as it is normally oriented in the head. Stippling indicates cartilaginous structures. B: basibranchial; BH: basihyal; CB: ceratobranchial; CH: ceratohyal; EB: epibranchial; H: hypobranchial; HH: hypohyal; PB: pharyngobranchial; TP: tooth plate.

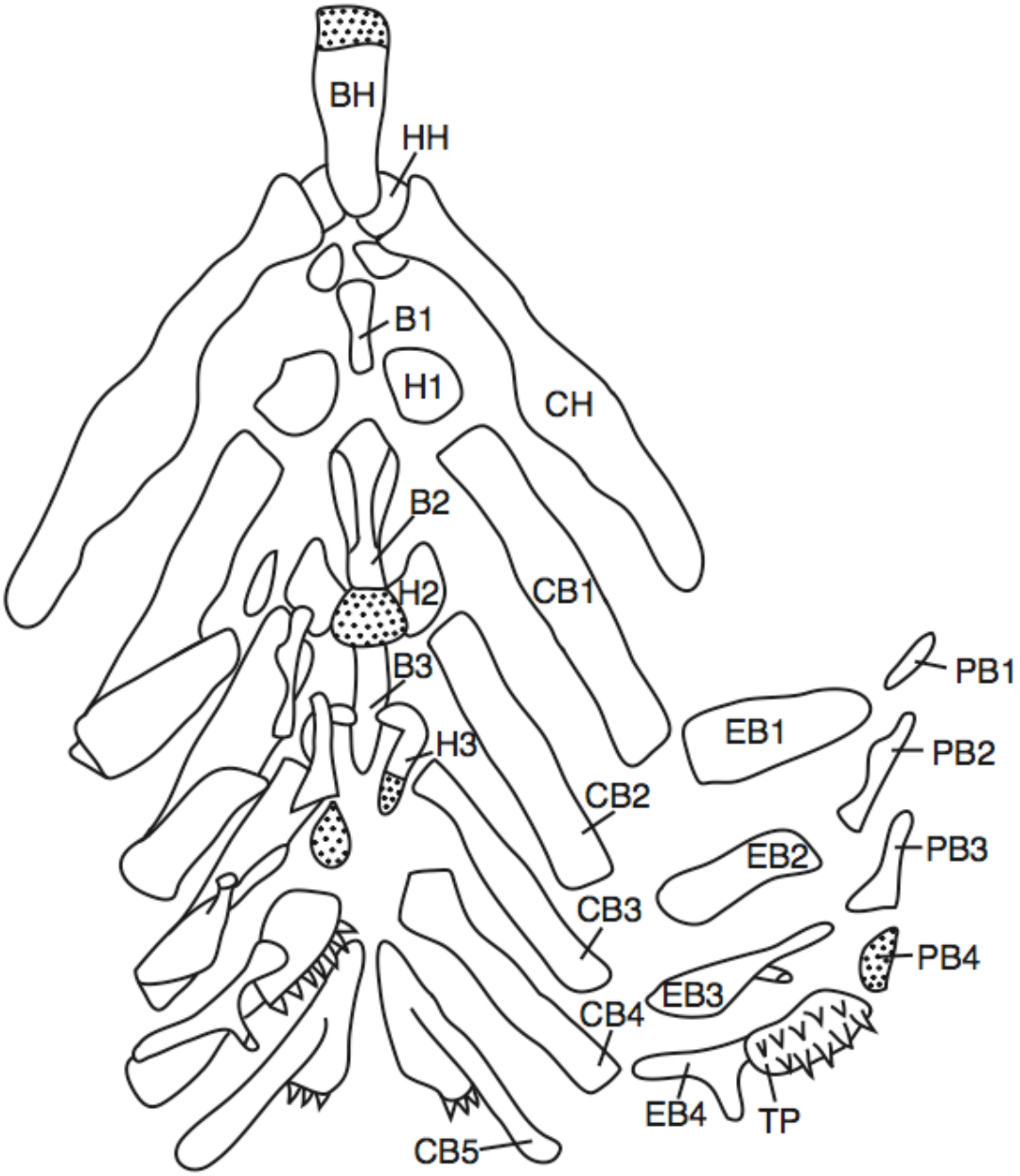

BH
HH
B1
H1
CH
B2
H2
CB1
B3
H3
CB2
CB3
CB4
CB5
PB1
EB1
PB2
EB2
PB3
EB3
PB4
EB4
TP

Figure 2.2. Schematic lateral view of a basal teleostean pharyngeal jaw apparatus. AD5: adductor 5; Cl: cleithrum; LE: levator externus; LI: levator internus; LP: levator posterior; LPJ: lower pharyngeal jaw; NCR: neurocranium; PCE: pharyngocleithralis externus; PCI: pharyngocleithralis internus; Re: retractor muscle; RC: rectus communis; UPJ: upper pharyngeal jaw.

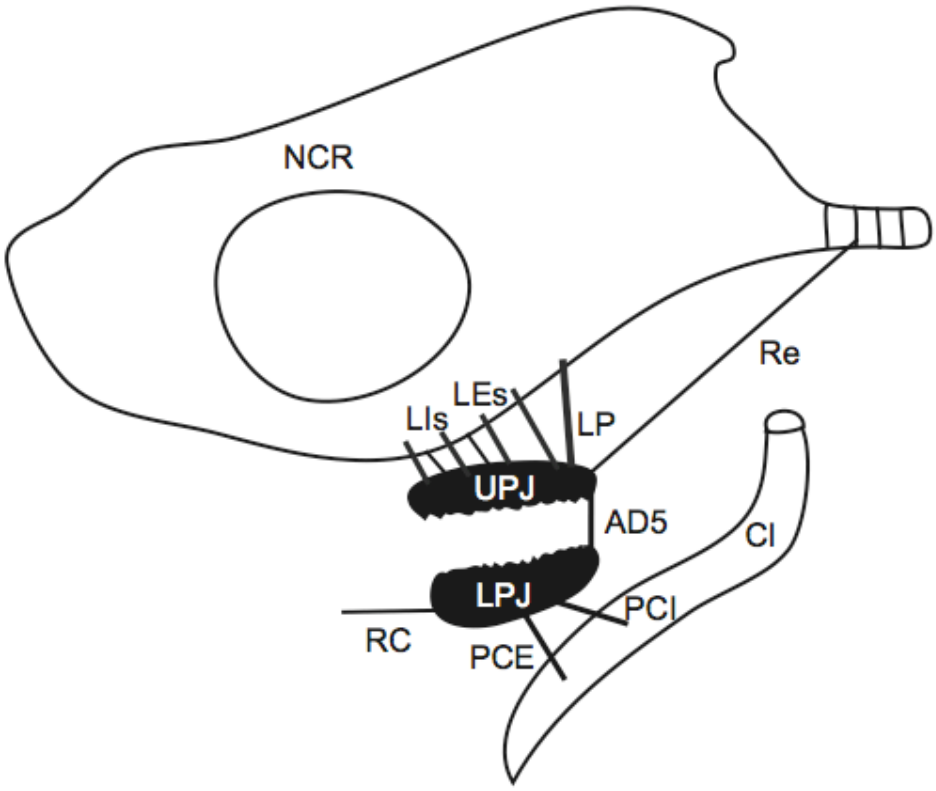

NCR
Re
LEs
LIs
LP
UPJ
AD5
Cl
LPJ
PCl
RC
PCE

Figure 2.3. Schematic lateral view of a generalized cypriniform pharyngeal jaw apparatus (modified from Springer and Johnson, 2004 and Vandewalle et al., 2000). AD5: adductor 5; Boc: basioccipital; Cb: ceratobranchial; Cl: cleithrum; CP: chewing pad; Eb: epibranchial; GH: geniohyoideus; Hy: hyoid; LE: levator externus; LI: levator internus; LP: levator posterior; NCR: neurocranium; Pb: pharyngobranchial; PCE: pharyngocleithralis externus; PCIa: pharyngocleithralis internus anterior; PCIp: pharyngocleithralis internus posterior; RC: rectus communis; RP: retractor pharyngeus; TV4: transversus ventralis 4.

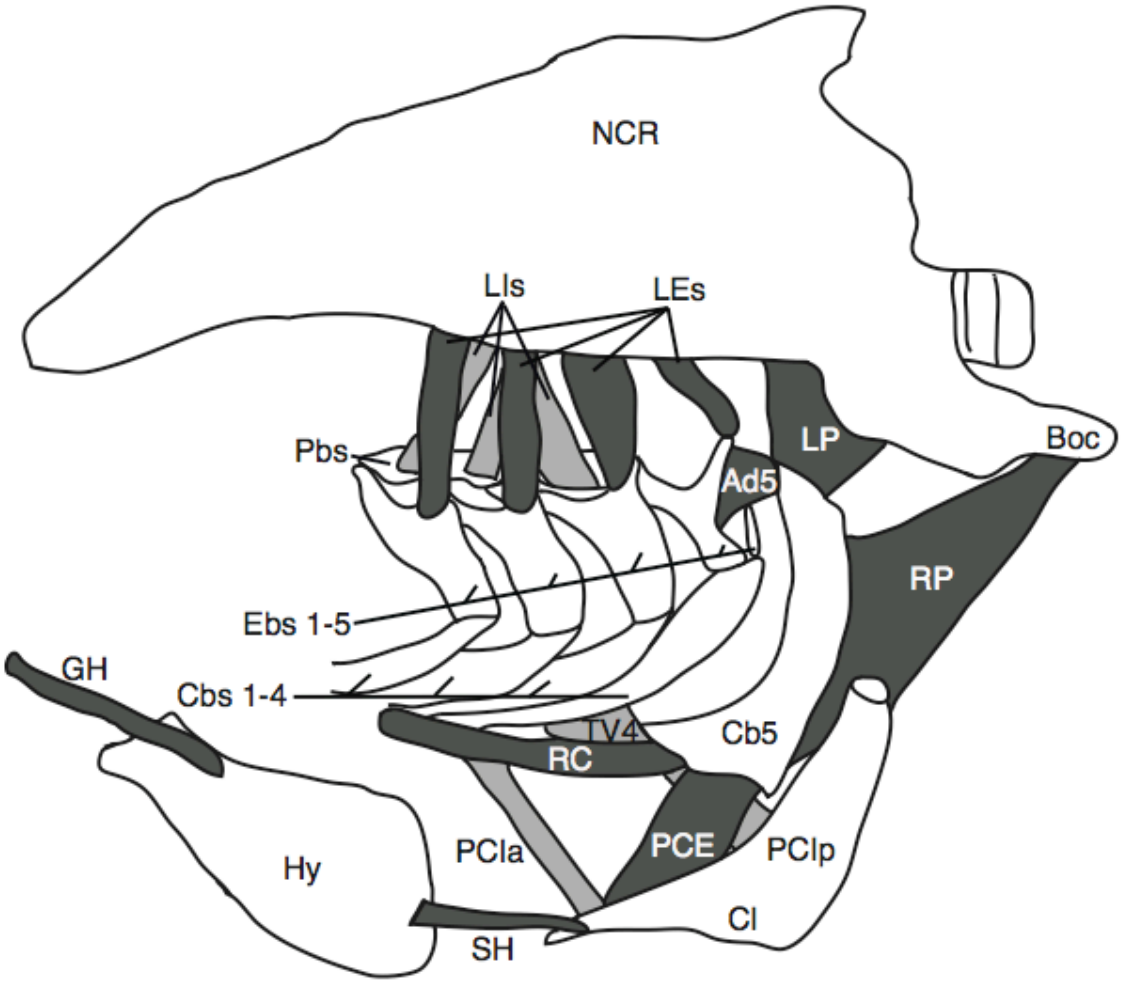
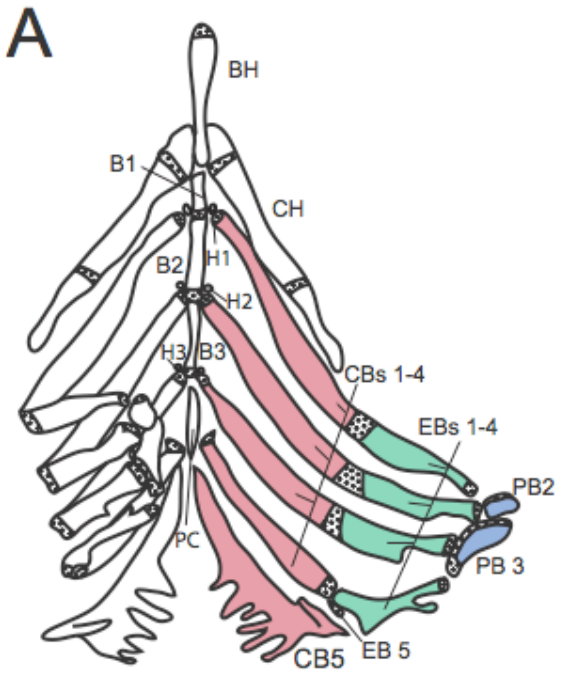

NCR
LIs
LEs
Pbs
LP
Boc
Ad5
RP
Ebs 1-5
GH
Cbs 1-4
TV4
Cb5
RC
PCE
PCIp
PCIa
Hy
Cl
SH

Figure 2.4. Adult zebrafish pharyngeal jaw apparatus. **A**: Diagram showing a dorsal view of the pharyngeal skeleton of an adult zebrafish. Stippling indicates cartilaginous elements. The right side is reflected to show the dorsal elements. Homologous elements are color coded, pink for the ceratobranchials, green for the epibranchials, and blue for the pharyngobranchials. **B**: Lateral view of the zebrafish head with a box outlining where in the head the pharyngeal structures are located. The picture on the right is a drawing of the musculoskeletal elements of the pharyngeal jaw apparatus. The small inset on the right picture illustrates the location of the internal levators. External levators on and two have been removed so the internal levators can be seen. Homologous elements are color coded, pink for the ceratobranchials, green for the epibranchials, and blue for the pharyngobranchials. AD5: adductor 5; B: basibranchial; BH: basihyal; CB: ceratobranchial; CH: ceratohyal; Cl: cleithrum; EB: epibranchial; H: hypobranchial; LE: levator externus; LI: levator internus; LP: levator posterior; NCR: neurocranium; PB: pharyngobranchial; PC: posterior copula; PCE: pharyngocleithralis externus; PCIa: pharyngocleithralis internus anterior; PCIp: pharyngocleithralis internus posterior; RC: rectus communis; RP: retractor pharyngeus; TV4: transversus ventralis 4.


A
BH
B1
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B2
H1
H2
H3
B3
CBs 1-4
EBs 1-4
PB2
PB 3
PC
CB5
EB 5

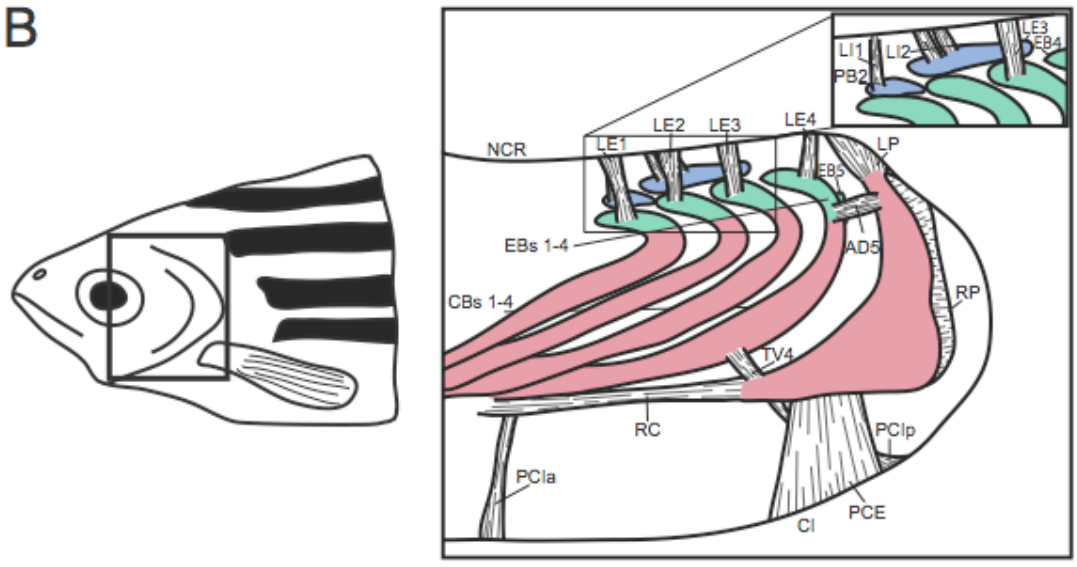

B
LE3
EB4
LI1
LI2
PB2
LE2
LE3
LE4
NCR
LE1
LP
EB5
EBs 1-4
AD5
CBs 1-4
RP
TV4
RC
PCIp
PCIa
PCE
CI

Figure 2.5. Early larval zebrafish pharyngeal jaw apparatus. **A**: Diagram showing a dorsal view of the pharyngeal skeleton of a 6 dpf fish. Stippling indicates areas of ossification. Homologous elements are color coded, pink for the ceratobranchials. **B**. Frontal section of a 6 dpf fish. **C**: Sagittal section of a 3 dpf fish. Note the presence of the LE and LP at this early developmental stage. **D**: Cross section of a 5 dpf fish. An LE is present while the epibranchial on which it inserts is not, as indicated by the asterisk. **E**: Sagittal section of a 3 dpf fish. Note the early presence of the PCE. All scale bars: 0.1 mm. AC: anterior copula; BH: basihyal; CB: ceratobranchial; CH: ceratohyal; H: hypobranchial; LE: levator externus; LP: levator posterior; PC: posterior copula; PCE: pharyngocleithralis externus; PO: palatal organ; RP: retractor pharyngeus; TE: teeth.

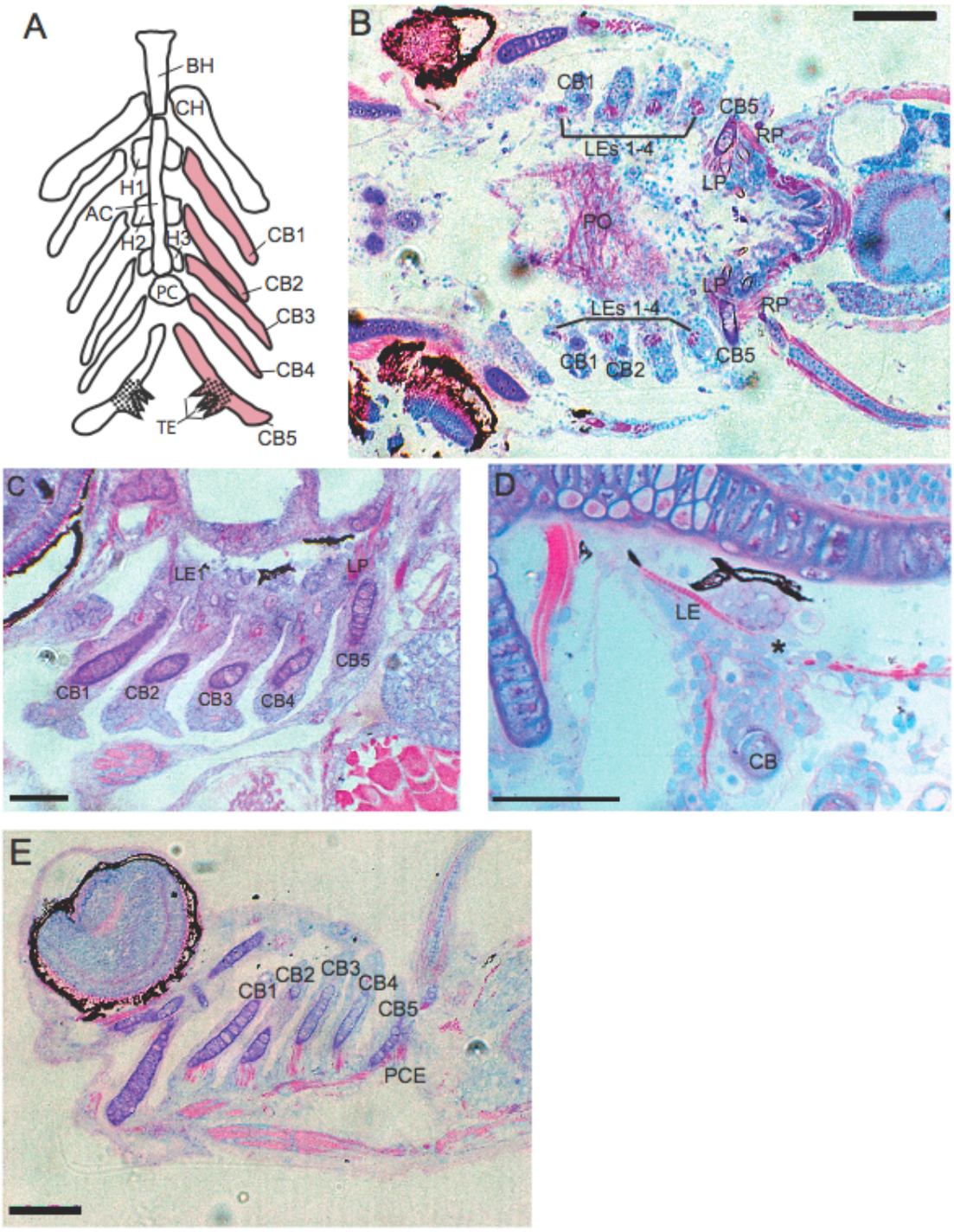
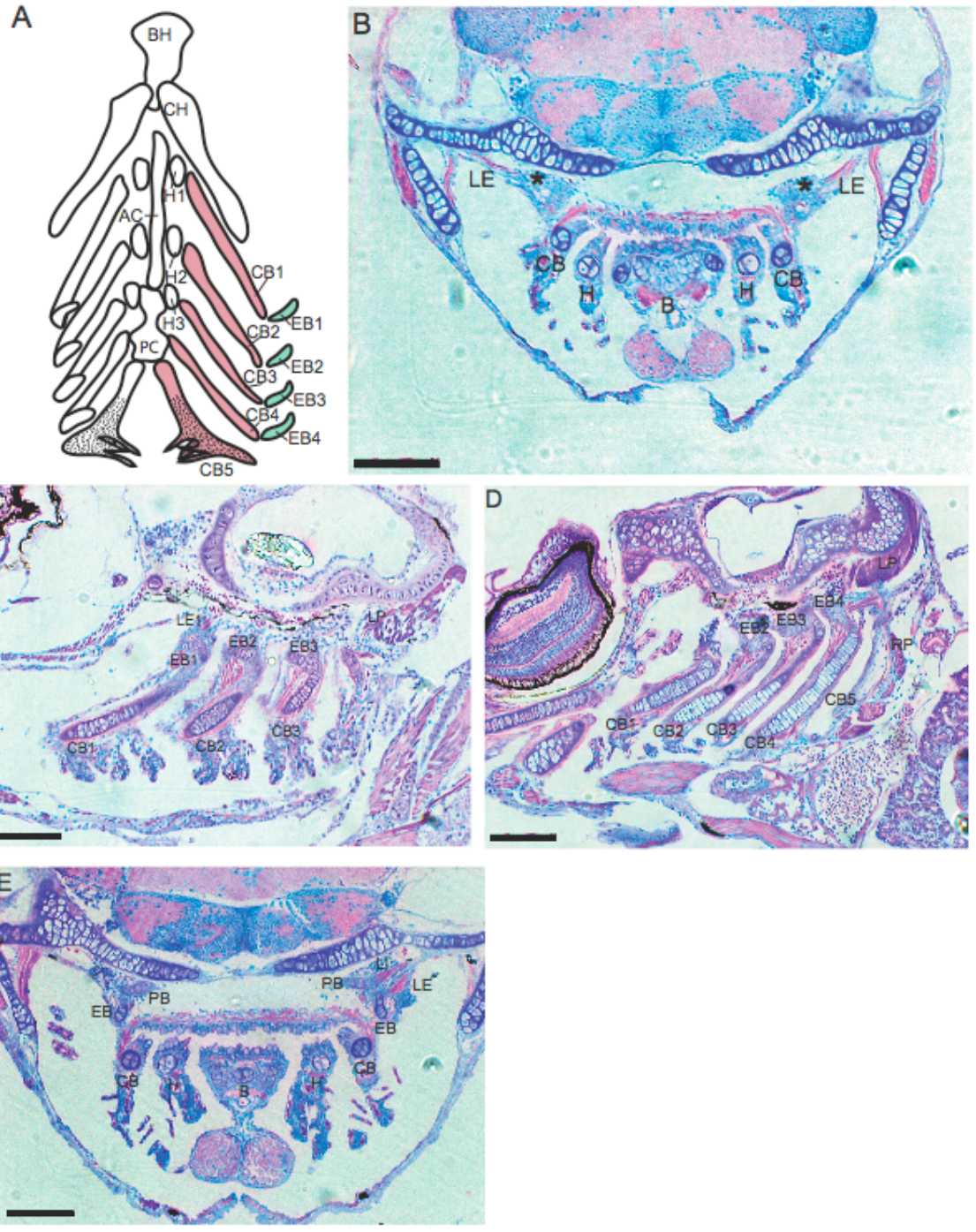

A
BH
CH
H1
AC
H2
H3
PC
CB1
CB2
CB3
CB4
TE
CB5
B
CB1
CB5
LEs 1-4
RP
LP
PO
LP
RP
LEs 1-4
CB1
CB2
CB5
C
LE1
LP
CB5
CB1
CB2
CB3
CB4
D
LE
*
CB
E
CB1
CB2
CB3
CB4
CB5
PCE

Figure 2.6. Late larval zebrafish pharyngeal jaw apparatus. **A**: Diagram showing a dorsal view of the pharyngeal skeleton of a 4.6 mm zebrafish. Stippling indicates ossification. The right side is reflected to show the dorsal elements. Homologous elements are color coded, pink for the ceratobranchials and green for the epibranchials. **B**: X-section of a 4.1 mm fish. Note the lack of the epibranchials that the levator externi insert upon, as indicated by the asterisks. **C**: Sagittal section of a 4.6 mm fish. Not the presence of the epibranchials. **D**: Sagittal section of a 4.5 mm fish. Note the epibranchials and the large size of the levator posterior. **E**: X-section of a 4.7 mm fish. Note the presence of the pharyngobranchial and its associated levator internus. All scale bars: 0.1 mm. AC: anterior copula; BH: basihyal; CB: ceratobranchial; CH: ceratohyal; H: hypobranchial; LE: levator externus; LI: levator internus; LP: levator posterior; PB: pharyngobranchial; PC: posterior copula; RP: retractor pharyngeus.


A
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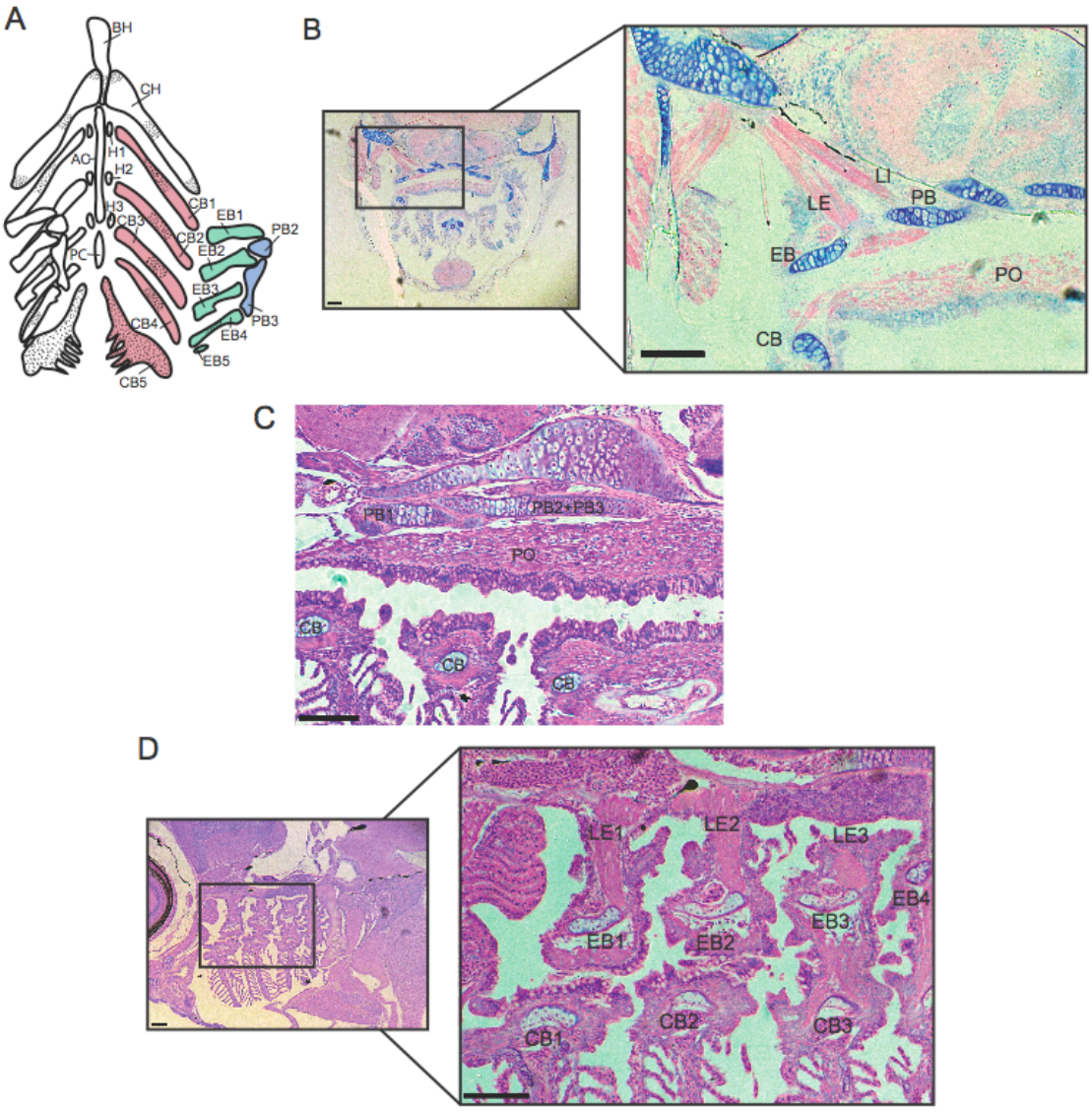
CB5
B
LE
LE
CB
H
B
H
CB
C
LP
LE
EB1
EB2
EB3
CB1
CB2
CB3
D
LP
EB4
EB2
EB3
RP
CB5
CB1
CB2
CB3
CB4
E
LP
PB
PB
LE
EB
EB
CB
CB
H
B
H

Figure 2.7. Juvenile zebrafish pharyngeal jaw apparatus. **A**: Diagram showing a dorsal view of the pharyngeal skeleton of an 8.6 mm zebrafish. The right side is reflected to show the dorsal elements. Homologous elements are color coded, pink for the ceratobranchials, green for the epibranchials, and blue for the pharyngobranchials. Stippling indicates ossification. Note the presence of the 5th epibranchial. **B**: X-section of an 8.0 mm fish. Note the levator externus and internus inserting on the epibranchial and pharyngobranchial, respectively. **C**: Sagittal section of an 8.8 mm fish. Note the pharyngobranchials' location dorsal to the palatal organ. **D**: Sagittal section of an 8.8 mm fish showing the external levators inserting upon their respective epibranchials. All scale bars: 0.1 mm. AC: anterior copula; BH: basihyal; CB: ceratobranchial; CH: ceratohyal; EB: epibranchial; H: hypobranchial; LE: levator externus; LI: levator internus; PB: pharyngobranchial; PC: posterior copula; PO: palatal organ.


A
BH
CH
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H1
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H3
CB1
EB1
PB2
CB3
CB2
EB2
PC
EB3
PB3
CB4
EB4
EB5
CB5
B
LI
LE
PB
EB
PO
CB
C
PB1
PB2+PB3
PO
CB
CB
CB
D
LE1
LE2
LE3
EB4
EB3
EB1
EB2
CB1
CB2
CB3

Chapter 3: Conclusion

The use of developmental and histological techniques has allowed us to examine how the pharyngeal jaw apparatus is constructed during the course of development in *Danio rerio*. As a result, we can start to draw conclusions about how the pharyngeal jaw apparatus is constructed and begin to apply this developmental data to generate hypotheses about the evolutionary origins of this structure.

This study has uncovered important aspects of the pharyngeal jaw apparatus in *Danio rerio* that were previously unknown. The structure of the adult pharyngeal jaw apparatus and its associated musculature in *Danio rerio* is very similar in structure to that of the pharyngeal jaw apparatus and its associated musculature in previously described cyprinids. The muscular sling exceeds the other levators in size from a very early point in development. I have uncovered differences in the developmental relationship that exists between the muscles and bones forming the apparatus. The levators externi develop at least a week before the epibranchials on which they attach. This is vastly different from the levators interni, which do not appear until approximately the same time as the pharyngobranchials on which they insert.

This study also lays the groundwork for further research not only for cypriniforms, but other groups of fish as well. Given the immense diversity of body plans in cypriniforms, additional work describing the pharyngeal jaw apparatus within all the families of this order is needed. Additionally, developmental studies similar to the one presented here would be useful in determining if the pharyngeal jaw apparatus is formed in the same manner in all cypriniforms. Lastly, this study is useful in beginning to

understand the formation of muscular slings that move hypertrophied 5th ceratobranchials not only in this group of fish, but in other fish groups as well.

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