Same but Different: Ontogeny and Evolution of the *Musculus adductor mandibulae* in the Tetraodontiformes

P. KONSTANTINIDIS1* AND M.P. HARRIS2,3

1The Natural History Museum, Department of Zoology, Cromwell Road, London, United Kingdom
2Max Planck Institute for Developmental Biology, Tuebingen, Germany
3Orthopaedic Research Laboratories, Children’s Hospital, Department of Genetics, Harvard Medical School, Boston, Massachusetts

The morphological diversity of fishes provides a rich source to address questions regarding the evolution of complex and novel forms. The Tetraodontiformes represent an order of highly derived teleosts including fishes, such as the pelagic ocean sunfishes, triggerfishes, and pufferfishes. This makes the order attractive for comparative analyses to understand the role of development in generating new forms during evolution. The adductor mandibulae complex, the main muscle associated with jaw closure, represents an ideal model system within the Tetraodontiformes. The adductor mandibulae differs in terms of partitions and their attachment sites between members of the different tetraodontiform families. In order to understand the evolution of the jaws among the Tetraodontiformes, we investigate the development of the adductor mandibulae in pufferfishes and triggerfishes as representatives of two different suborders (Balistoidei and Tetraodontoidei) that follows two different adaptations to a durophagous feeding mode. We show that the varied patterns of the adductor mandibulae derive from similar developmental sequence of subdivision of the partitions. We propose a conserved developmental program for partitioning of the adductor mandibulae as a foundation for the evolution of different patterns of subdivisions in Tetraodontiformes. Furthermore, we argue that derived conditions in the higher taxa are realized by supplementary subdivisions and altered attachment sites. These findings support a reinterpretation of homology of different muscle partitions among the Tetraodontiformes, as muscle partitions previously thought to be disparate, are now clearly related. *J. Exp. Zool. (Mol. Dev. Evol.)* 316:10–20, 2011. © 2010 Wiley-Liss, Inc.


Although these new homologies may not change the phylogeny itself, they may radically affect any hypotheses based on the refuted homologies.

(Friel and Wainwright, ’97; p 460)

It is generally accepted that the evolution of the feeding apparatus is strongly linked with the great success of the ray-finned fishes (Actinopterygii), which represents, with more than 28,000 (Nelson, 2006) species, more than half of all living vertebrates. To understand the evolution of the actinopterygians, many studies have dealt with the morphological adaptations of the jaws in either a phylogenetic context (Schaeffer and Rosen, ’61; Lauder, ’79, ’80, ’82; Westneat, 2004) or with function in highly derived taxa, such as the flatfishes (Gibb, ’97; Gibb and Ferry-Graham, 2005), frogfishes (Pietsch and Grobecker, ’87), tube-eyes (Pietsch, ’78), seahorses (Roos et al., 2008, 2009), and members of the Tetraodontiformes (Turingan and Wainwright, 2007).

*Correspondence to: P. Konstantinidis, The Natural History Museum, Department of Zoology, Cromwell Road, London SW7 5BD, UK.
E-mail: p.konstantinidis@nhm.ac.uk
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The evolution of the free protrusible jaw was one of the key innovations of ray-finned fishes. In the bowfin *Amia calva*, as the sistergroup of all teleosts, the maxilla has become free from the skull, allowing the ventral end of the maxilla to swing forward (via the maxillomandibular ligament). The detachment of the maxilla from the skull was the key step that has led to the advent of a complex mechanism of upper jaw protrusion, evolved at least three times independently during teleost evolution (for details, see Schaeffer and Rosen, '61; Lauder, '82, '85; Johnson and Patterson, '93; Westneat, 2004).

Together with the alterations in the skeleton, muscles, and motor neurons associated with these skeletal elements show great diversity to enable a new function of the jaw. In non-teleost and basal teleost fishes, as well as in basal terrestrial vertebrates, the adductor mandibulae facilitates the closing of the lower jaw. With the advent of the protrusible upper jaw in higher teleosts, the arrangement of the *Musculus adductor mandibulae* has been altered, and acquired the retraction of the upper jaw as an additional novel function. For example, a general teleost fish has the adductor mandibulae subdivided into four portions, of which one is attached to the maxilla (portion A1). The other subdivisions remain attached to the lower jaw (A2 and A3) and one portion runs along the medial side of the dentary (A0).

The Tetraodontiformes are well known for their highly specialized jaws to facilitate durophagy, or grasping and processing of hard-shelled prey (Tyler, '80; Turingan and Wainwright, '93; Turingan et al., '95). Although members of all families of the Tetraodontiformes are durophagous, the basal Triacanthodidae exhibit the least feeding specializations of the musculoskeletal jaw apparatus and resemble a general perciform however lacking the A0 (Winterbottom, ’74a). We consider this pattern as ancestral for the tetraodontiform order (Fig. 1). The most derived adaptations of the jaws are found in the suborder Tetraodontoidae, in which all members have parrot beak-like jaws without individual teeth. This is in contrast to the sister suborder, Balistoidei, which retain individual teeth as an oral dentition (Fig. 1).

Overall, the Tetraodontiformes show a trend toward morphological simplification, shown prominently in the loss or fusion of skeletal elements. However, in tandem, the more derived families...
harbor many novelties and specialized structures. For example, representatives of all families of the Tetraodontiformes, except the triacanthodids and triacanths, have secondarily lost the ability to protrude the upper jaw as seen in most percomorph fishes (Turingan and Wainwright, '93; Turingan, '94; Turingan et al., '95; Wainwright and Frien, 2000). In contrast, the jaw musculature has become extensively elaborated when compared with the common percomorph situation. The basal Triacantho-
didae show a similar configuration of the adductor mandibulae than a general percomorph (see above). The adductor mandibulae has evolved up to six portions in puffers and up to eight in some monacanthids (Winterbottom, '74a; Frien and Wainwright, '98). The complexity and variety (up to 350 species) within this group provides a good case study in which to address the changes in specification and patterning of the musculoskeletal system underlying the evolution of these novel structures.

Attempts to assign homology to the different subdivisions of the adductor mandibulae have led to contrasting hypotheses of the evolution of the musculoskeletal system associated with the morphological diversity of these fishes (Winterbottom, '74a; Frien and Wainwright, '97; Nakae and Sasaki, 2004). Winterbottom ('74a) tried to homologize muscle portions using the origin and insertion of the individual subdivisions. In addition, Frien and Wainwright ('97) analyzed the pattern of subdivision of the muscle in which the authors differentiated between orthologous subdivisions (homologous partitions among species, e.g. A1 in S. hepatus and Perca fluviatilis) and paralogous subdivisions (subdivisions of a common partition within a species, e.g. A1α and A1β in Microgadus tomcod). However, the results are incongruent with that of Winterbottom ('74a). Nakae and Sasaki (2004) used an alternative approach and tried to solve the homology of the adductor mandibulae subdivisions via the innervation pattern of the Ramus mandibularis trifurcatus of the trigeminal nerve, but failed to clarify the earlier difficulties of the homology of the partitions. All three cases show the obvious limits in the analysis of adult structures in classifying homology, especially in highly derived forms. However, determining homology of elements is essential to understand the evolution of anatomical structures.

We approached the question of the evolution of the adductor mandibulae in tetraodontiform fishes by analyzing the ontogeny of the adductor mandibulae complex of the freshwater pufferfish, Monotrete suvattii, a marine species of the genus Lagocephalus, and the triggerfish Balistapus undulatus. These genera represent different feeding specializations within the tetraodontiform clade. We looked at ontogeny of the partitions to identify (1) the homology of the different adductor mandibulae subdivisions between members of the different families (orthologs sensu, Frien and Wainwright, '97), (2) the developmental lineage of the individual muscle subdivisions within the balistids and the tetraodontids (paralogs sensu, Frien and Wainwright, '97), and (3) the developmental history that underlies the evolution of the subdivisions. Through tracing the development of the different partitions in the adductor mandibulae in larvae, we identified a conserved developmental sequence of muscle specification that is specifically altered within the different lineages leading to unique muscle arrangements in the “beaked” and “toothed” Tetraodontiformes.

MATERIAL AND METHODS

Specimens

Reared larvae of M. suvattii (size range between 3 mm notochord length (NL) and 8 mm standard length (SL)) were preserved in 4% paraformaldehyde (PFA) at 4°C between 24 and 72 hr and subsequently dehydrated to 100% methanol and stored at −20°C. Larvae larger than 4 mm were preserved in PFA for 4 days and then dehydrated. Lagocephalus sp. and B. undulatus larvae were obtained from unsorted material from the National Science Museum, Tokyo.

Immunolabeling

We analyzed the early development of the adductor mandibulae in larvae by antibody staining against myosin heavy chain (fast twitch). All larvae were carefully skinned and connective tissue was removed under a dissecting scope (ZEISS Stemi 2000, ZEISS DRC) before antibody labeling. The eyes were removed either before or after the staining. To specifically detect myofibrils, specimens were incubated with a primary antibody specific to fast twitch myosin (MF20, Developmental Studies Hybridoma Bank, 1:500). This was followed by secondary antibody detection conjugated with FITC (1:500, ALEXA 488, Invitrogen) or alkaline phosphatase antibody (1:5000, Dianova). The larvae were rehydrated in phosphate saline buffer (PBS) with several changes over the course of 2 hr. To make the cell membranes permeable, the larvae were treated for 1 hr with Proteinase K (10 mg/mL) and afterwards with Collagenase (10 mg/mL) for 30 min. Both steps were carried out at 37°C. Subsequently, the larvae were treated with Acetone for 1 hr at −20°C. The specimens were then washed several times with PBS at room temperature (RT). To block the unspecific antigens, a 10% normal goat serum in tris buffered saline with 0.1% Tween-20 (TBST) was applied for 3 hr at RT. Color reaction for alkaline phosphatase activity was done by washing the specimens in TBST + Levamisol (L; 0.5%) (TBST+L) o/n at 4°C, 5 hr with a basic buffer (pH 8) containing Levamisol and then placed in BCIP/NBT color substrate (Sigma FAST™) at 4°C until visible to eye.

Confocal Imaging

Formalin-fixed tissues exhibit autofluorescence. The high degree of organization of the muscle tissue causes less scattering and allows these structures to be differentially silhouetted against less organized tissue, such as connective tissue or skeletal elements. We used this advantage to trace the ontogeny of the adductor
mandibulae in museum specimens of *Lagocephalus* sp. and *B. undulatus*. The specimens were skinned only.

Specimens were mounted for observation in a small glass ring filled with 0.8% low melting agarose attached to a glass slide. Images of the whole mount specimens were taken with a ZEISS AxioCam HR digital camera attached to a ZEISS Discovery V20 dissecting scope.

Images of immunolabeled and formalin-fixed specimens were taken with a ZEISS LSM 510 confocal microscope controlled by the ZEISS ZEN software. Tile and z-stacks were aligned automatically.

**Materials Examined**

*Balistidae*. *B. undulatus* (Park), uncataloged from the collection of National Science Museum, Tokyo, five specimens, 2.6 mm NL–8.8 mm SL; Tetraodontidae: *Lagocephalus* sp. (Swainson), uncataloged from the collection of National Science Museum, Tokyo, two specimens, 3.1 mm NL and 4.0 mm NL; *M. suvattii* (Sontirat): uncataloged, three specimens, 4.5 mm NL–7.1 mm SL, Tokyo, two specimens, 3.1 mm NL and 4.0 mm NL; uncataloged from the collection of National Science Museum, *M. suvattii* (Sontirat): uncataloged, three specimens, 3.2 mm NL–4.1 mm NL, labeled with goat anti-ALEXA 488; *M. suvattii* (Park), uncataloged from the collection of National Science Museum, Tokyo, five specimens, 2.6 mm NL–8.8 mm SL; Tetraodontidae: *M. adductor mandibulae* by Vetter (1878) and labeled the different partitions of the *M. adductor mandibulae* (see Table 1), the result is a lack of clarity in the identification of muscle homology. Thus, we suggest a revised or complementary system of nomenclature that takes into consideration the developmental history as the primary measure of homology when available. It is likely that these systems of nomenclature will be similar in many cases, except in cases of diversification and novelty.

The base abbreviation for the adductor mandibulae subdivisions that we use here is AM. We introduce the term AMPRE for the initial stage of the adductor mandibulae. Separation of the AMPRE is denoted with a Greek symbol (e.g. AMx). Further separation of the partitions is labeled with hash marks (e.g. AMx\(\#\)y for the two partitions resulting from separation of the AMx). In discussing variation in placement or function of a partition, we follow Winterbottom (74b) who defined the origin of a muscle as the attachment closer to the center (e.g. suspensorium) and, consequently, the insertion as the point farther away from the center (e.g. maxilla).

Although Ao was described for trigger- and pufferfishes and the A1β of the triggerfishes, we were not able to detect them in the particular age specimens we examined.

**Anatomical Abbreviations**

Winterbottom (’74a,b) followed the nomenclature introduced by Vetter (1878) and labeled the different partitions of the *M. adductor mandibulae* based on relative position, origin, and insertion site in the adult; this classification also implies function. To demonstrate homology, portions of the adductor mandibulae were named as followed: A1, a portion that has its origin lateral on the suspensorium and its insertion point on the maxilla (either directly or dorsally on the primordial ligament that is associated with the maxilla) of the upper jaw; portions named A2 and A3 attach always on the lower jaw and A0 runs along the meckelian fossa. In puffers and other tetraodontiformes, more than one portion inserts on the upper jaw. Therefore, Winterbottom (’74a,b) extended the nomenclature and added a Greek letter after the abbreviation, e.g. A1α and A1β for the portions that insert on the maxilla of the upper jaw. This system was adopted by Friel and Wainwright (’97) and Nakae and Sasaki (2004); however, several additions were made to clarify different homology assessments including species/sub-order specific identifiers to "establish" functional identity in the adult.

Although the previous nomenclature (A1-A0) is sufficient for analysis of the functional changes of the adductor mandibulae complex, it is not suitable for discussions of homology—especially in cases of complex specialization. By looking at the observed homology of different partitions of the adductor mandibulae (see Table 1), the result is a lack of clarity in the identification of muscle homology. Thus, we suggest a revised or complementary system of nomenclature that takes into consideration the developmental history as the primary measure of homology when available. It is likely that these systems of nomenclature will be similar in many cases, except in cases of diversification and novelty.

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Although Ao was described for trigger- and pufferfishes and the A1β of the triggerfishes, we were not able to detect them in the particular age specimens we examined.

**Table 1. Comparative classification of adult adductor mandibulae partitions.**

<table>
<thead>
<tr>
<th>This study (lineage)</th>
<th>Winterbottom (function and lineage inferred)</th>
<th>Friel and Wainwright (function and lineage inferred)</th>
<th>Nakae and Sasaki (function and lineage inferred)</th>
</tr>
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<tbody>
<tr>
<td>AMx</td>
<td>A2α/A2β</td>
<td>A2β(#)t/A2β(#)t/A2β(#)b/A2β(#)α</td>
<td>A2α/A2β</td>
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<tr>
<td>AMx(#)y</td>
<td>A1α/A2γ</td>
<td>A1β(#)t/A2β(#)t/A2β(#)b/A2β(#)α</td>
<td>A1α/A2γ</td>
</tr>
<tr>
<td>AMx(#)z</td>
<td>A3/A3</td>
<td>A3/A3</td>
<td>A3/A3</td>
</tr>
<tr>
<td>AMβ(#)x</td>
<td>A2β(#)t/A2β(#)t/A2β(#)b/A2β(#)α</td>
<td>A2β(#)t/A1β(#)t/A2β(#)b/A2β(#)α</td>
<td>A2β(#)t/A1β(#)t/A2β(#)α/A2β(#)β/2γ</td>
</tr>
<tr>
<td>AMβ(#)y</td>
<td>A1β/A1α</td>
<td>A1β(#)t/A1β(#)t/A2β(#)b/A2β(#)α</td>
<td>A1β(#)t/A1β(#)t/A2β(#)b/A2β(#)α</td>
</tr>
<tr>
<td>N/A</td>
<td>absent/A1β(#)α</td>
<td>Absent/A1β(#)α</td>
<td>A3β(#)β/2γ</td>
</tr>
</tbody>
</table>

Partitions of the adductor mandibulae are classified using nomenclature according to the pattern of divisions (lineage) of the partitions or with previous classification scheme based on attachment site. For classification based on function, tetraodontids and balistids are shown (t/b); in classification based on ontogeny, the partitions are identical. N and S argue that the A1β of the balistids is a subdivision of A3; F and W did not discuss the unusual position of this muscle.
RESULTS
Development of the Adductor Mandibulae in Pufferfishes

Comparative studies of the development of Tetraodontiformes are hindered by the availability of larvae of different taxa of the group. When available, the method of fixation of the specimens often does not support immunological or in situ methods necessary for visualization of specific tissues in younger specimens. However, we were able to investigate the early formation of the adductor mandibulae using immunolabeling of muscles in a developmental series of larvae of the freshwater puffer M. suvattii bred in the lab.

Unlike the adults with several separate and distinct partitions of the adductor mandibulae, we find that the adductor mandibulae in early larvae of M. suvattii is represented by a single portion here referred to as AMPRE (3.2 mm NL; Fig. 2A, B). This portion has its origin on the hyomandibula, projecting anteriorly and bending slightly upward. The dentary is not yet ossified and AMPRE inserts at this stage on the posterior end of the meckelian cartilage (not shown). By 3.5 mm NL (Fig. 2C),

Figure 2. The formation and early partitioning of the adductor mandibulae of Monotrete suvattii. A. Lateral view of a 3.2 mm NL specimen. AMPRE is formed as a single primordia attaching to the lower jaw anteriorly and to the suspensorium posteriorly. B. Dorsal view of the same specimen as in A. Only the adductor mandibulae is marked. C. Lateral view of a 3.5 mm NL specimen. AMPRE has broadened mediodorsally. D. Schematic drawing of the suspensorium of a specimen of the size represented in C to visualize the attachment of AMPRE. E. Lateral view of a 4.1 mm NL specimen. AMPRE is now subdivided and represented by three portions. In lateral view only AMα0, AMα00 can be seen. AMα0 is attached to the maxilla F. Dorsal view of the same specimen as in E in which the third subdivision, AMβ, can be seen. The attachment of AMα0 to the maxilla is indicated by the red arrow G. Schematic drawing of the suspensorium of a specimen of the size represented in E and F to visualize the three different subdivisions. H. Lateral view of a 4.5 mm SL specimen. AMα0 has shifted its origin and AMβ now occupies most of the dorsal part of the suspensorium. AA, anguloarticular; AMα00, precursory portion of the adductor mandibulae; AMα0, AMα00, AMβ, subdivisions of the adductor mandibulae; D, dentary; DO, dilator operculi; HY, hyomandibula; LAP, levator arcus palatini; LO, levator operculi; MC, meckelian cartilage; MX, maxilla; NL, notochord length; PMX, premaxilla; PP, protractor pectoralis; PQ, palatoquadrate; SL, standard length.
AM\textsubscript{PRE} has broadened posteriomedially, but still remains associated with the meckelian cartilage (Fig. 2C and D). In a 4.1 mm NL specimen, AM\textsubscript{PRE} shows an initial subdivision (Fig. 2E and F). This separation of the AM\textsubscript{PRE} is denoted as AM\textsubscript{z} (AM\textsubscript{z}' and AM\textsubscript{z}''; see below) and AM\textsubscript{β}. The portion that is defined as AM\textsubscript{β} occupies the dorsal part of the palatoquadrate (Fig. 2F and G). Where the posterior end of the maxilla covers AM\textsubscript{z} laterally, a small bundle of muscle fibers encroached on the posteroventral end of the maxilla; this is the initial stage of AM\textsubscript{z}'' (Fig. 2E–G). The remaining portion of AM\textsubscript{z} is now termed AM\textsubscript{z}'. By 4.5 mm SL, AM\textsubscript{β} is now fan-shaped expanded and covers most of the suspensorium (Fig. 2H). In a slightly larger larva (5.2 mm SL), AM\textsubscript{z}' and AM\textsubscript{β} are fully separated (Fig. 3A).

At this juvenile stage, the anterior part of AM\textsubscript{β} has extended anterior and covers the entire dorsal and most anterior part of the suspensorium (Fig. 3A), and is attached to the parasphenoid dorsally. The anterior most part of AM\textsubscript{β} is larger and can be clearly distinguished from the more posterior portion (Fig. 3A). The subdivision AM\textsubscript{z}'' partially covers AM\textsubscript{z}' laterally (Fig. 3A). At this stage, a further division of AM\textsubscript{z} occurs medially and is termed AM\textsubscript{z}''' (not shown); this is the partition previously denoted as A3 by Winterbottom ('74a), Friel and Wainwright ('97), and Nakae and Sasaki (2004). This arrangement of the portions of the adductor mandibulae resembles the situation of adult puffers as described by the aforementioned authors, with the exception that the muscle portion that is attached to the maxilla, the ethmoid and the lateral ethmoid, has not developed. As development proceeds (7.1 mm SL; Fig. 3B), the more massive anterior part of AM\textsubscript{β} separates and forms a discrete entity, AM\textsubscript{β}'' (7.1 mm SL; Fig. 3B and C). Because of the separation, the posterior part of the former AM\textsubscript{β} is now renamed to AM\textsubscript{β}'. The new partition AM\textsubscript{β}'' is attached to the maxilla ventrally and to the ethmoid and lateral ethmoid dorsally. Because this portion is attached to the maxilla Winterbottom ('74a), Friel and Wainwright ('97), and Nakae and Sasaki (2004) believed that this portion is homologous to a subdivision that serves already the upper jaw (herein AM\textsubscript{z}''; see Table 1 for the nomenclature of the three aforementioned studies).

The development of the adductor mandibulae in \textit{M. suvattii} larvae suggests that (1) the major muscle partitions of the adductor derive from a common precursor and (2) that the diversity in the number of partitions is owing to successive divisions of this precursor. Furthermore, our data show that muscles with different

**Figure 3.** Comparative analysis of the development of the adductor mandibulae in puffers. A. Lateral close up of a 5.2 mm SL \textit{Monotrete suvattii}. AM\textsubscript{β} occupies the entire dorsal part of the suspensorium. B. Close up of C, the head of a 7.2 mm SL \textit{M. suvattii}. The most anterior part of AM\textsubscript{β} (now AM\textsubscript{β}') is separated and forms an individual portion. The arrow marks the gap between AM\textsubscript{β}' and AM\textsubscript{β}'' D. Lateral close up of a 3.1 mm SL \textit{Lagocephalus} sp. The adductor mandibulae arrangement at this stage is comparable to the \textit{M. suvattii} in Figure 2H. E. Lateral close up of a 4.0 mm SL \textit{Lagocephalus} sp. is comparable to the \textit{M. suvattii} in Figure 3A. AM\textsubscript{z}', AM\textsubscript{z}'', AM\textsubscript{β}, AM\textsubscript{β}', AM\textsubscript{β}'', subdivisions of the adductor mandibulae; DO, dilatator operculi; EM, eye muscles; LAP, levator arcus palatini; MX, maxilla; NL, notochord length; PHY, protractor hyoidei; SL, standard length.
attachment sites in the adult arise from similar antecedent partitions. This last fact has important implications for the analysis of homology of the adductor mandibulae complex (see below).

To further verify these results, we looked at the development of a marine puffer, *Lagocephalus* sp., as another example of the tetraodontid family. We were able to detect the structure of early developing muscles in batch-preserved museum specimens using simple autofluorescence of the muscle fibers (see Material and Methods). Although we were not able to obtain specimens of *Lagocephalus* sp. at small enough stages to detect the initial subdivisions of the adductor mandibulae, the smallest specimens we recovered showed partitioning of the *AMβ* and *AMα* as seen in *M. suvattii*. The earliest larval stages of *Lagocephalus* sp. (Fig. 3D) were comparable to the *M. suvattii* larva of Fig. 2E. *AMβ* covers the suspensorium and reaches far rostrally. In the older specimen, the most anterior part of *AMβ* is attached to the maxilla and to the ethmoid region of the skull, but not yet separated as an individual *AMβ'*. *AMβ'* is a fully separated portion in adult *Lagocephalus* sp. (Winterbottom, '74a); therefore, this partition likely separates in older juveniles.

The similarity of the development of the adductor mandibulae of the two puffer species, *M. suvattii* and *Lagocephalus* sp., suggests that the developmental trajectory of the adductor mandibulae, and in particular that the *AMβ''* binds to the maxilla, is conserved among members of this “beaked” family, the Tetraodontidae (Fig. 1).

**Development of the Adductor Mandibulae Complex in the Triggerfish, *Balistapus undulatus***

The jaws of members of the balistid suborder do not form a beak, such as the puffers, but rather retain a dentition. However, this suborder also exhibits a complicated, elaborate adductor mandibulae complex that functions to support derived feeding behaviors of the group (Turingan and Wainwright, '93; Turingan, '94; Ralston and Wainwright, '97). We, therefore, broadened our ontogenetic analysis to a member of this family to compare with the puffers. Such as *Lagocephalus* sp., we detected developing muscles using differential autofluorescence of formalin-fixed museum specimens using confocal microscopy.

Larval balistids are exceptionally rare in collections. Although we were able to get a series of developmental stages, the smallest balistid obtained (2.6 mm NL) showed the development of three adductor mandibulae portions (Fig. 4A). All teleosts analyzed to date show that the initial subdivisions derive from a single

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**Figure 4.** The development of the adductor mandibulae in the balistid, *Balistapus undulatus*. A. Close up of a 2.6 mm NL specimen, *B. undulatus*. *AMβ''* is still connected to *AMβ* anteriorly (red arrow). The attachment of *AMx* to the lateral crest of the preopercle is outlined to show the similarity to the puffers. B. Close up of a 3.4 mm NL specimen. C. Close up of a 4.9 mm SL specimen. D. Close up of a 5.9 mm SL specimen. E. Lateral view of the head of a 8.8 mm SL specimen. This stage closely resembles the adult. The attachment of *AMx* to the lateral crest of the preopercle is outlined. AAP, adductor arcus palatini; *AMz'', *AMx'', *AMβ'', *AMβ'', subdivisions of the adductor mandibulae; MX, maxilla; NL, notochord length; PHY, protractor hyoidei; SL, standard length.
progenitor; thus, we identified the subdivisions in this early larval stage of *B. undulatus* by the position and similar fiber direction as seen in the first division of AM fp in the puffers (Fig. 2A and C). We identified the two ventral portions as AMz, AMβ, and the dorsal portion as AMγ (Fig. 4A). AMβ is a narrow band that is connected to the preopercle and the dorsal part of the suspensorium. AMz is the largest muscle and, as in the puffers, it has its origin on the lateral crest of the preopercle (Fig. 4A). Importantly, these small larval AMβ is seen to derive from the most anterior part of AMβ (then becoming AMβ'); Fig. 4A)— identical to the case in the puffers. In a 3.4 mm SL larva, AMβ extends caudally (Fig. 4B) and occupies the dorsal part of the metapterygoid. In larger specimens, AMβ' is seen to develop in the rostrocaudal direction, shifts its position, and changes its attachment to the lateral ethmoid and ethmoid exclusively (Fig. 4C and D). AMz' appears last as an offshoot of AMβ originating on the preopercle ventral to the lateral crest (Fig. 4B–D).

**DISCUSSION**

The adductor mandibulae complex of the Tetraodontiformes presents an ideal model for the study of the evolution of morphological novelty. In particular, the homology and evolution of the highly derived adductor mandibulae has been the focus of previous studies [Winterbottom, '74a; Friel and Wainwright, '97; Nakae and Sasaki, 2004]. Given the complexity of the adductor complex of the Tetraodontiformes, the question of homology of the subdivisions within and among species becomes important in order to interpret the transformation of characters in a phylogenetic context. Does the ancestral muscle portion predetermine the pattern and functional attributes of the muscle or can descendant subdivisions adopt a new function? To allow an in-depth evaluation of the role of topological characters, such as origin, attachment, and innervation, as well as the function of specific muscle groups in the evolution of new feeding strategies, we compared the development of the adductor mandibulae of a balistid, as a representative of the suborder Balistoidae (toothed forms) with two tetraodontids, as representatives of the Tetraodontoidae (beaked forms; Fig. 1).

**Developmental Program and the Evolution of Complexity of the Adductor Mandibulae in Tetraodontiformes**

The multiple-partitioned adductor mandibulae seen in Tetraodontiformes derives from a single myogenic anlage and not from multiple muscle primordia that integrate into a complex. This property of the development of the adductor mandibulae complex is conserved throughout the teleosts and reported for a wide variety of taxa, e.g. *Danio rerio* (Hernandez et al., 2005; Diogo et al., 2008; Staab and Hernandez, 2010), Loricarioidei (Geerinckx et al., 2007; Huyseintro et al., 2007), *Kneria* sp. (personal observation), the Japanese flounder *Paralichthys olivaceus* (Uji et al., 2010), as well as for *Amia* (Jarvik, '80), as the sistergroup of the teleosts. Although pufferfishes and their relatives are highly derived, the initial development of the adductor mandibulae resembles the common ontogenetic trajectory among teleosts.

The initial anlage of the adductor mandibulae divides into two partitions, AMz and AMβ, of which AMz subsequently gives rise to three portions (Fig. 5B). This early state of complexity of the adductor resembles the adult pattern of the triacanthodids and the most plesiomorphic arrangement of the tetraodontiformes (Fig. 5C). This pattern of subdivision of the adductor can be applied to the triacanthids as well, although AMz' and AMz" are not completely separated in this group. This is either the result of an uncompleted separation of the two muscle subdivisions or a secondary fusion.

Although the balistids and the tetraodontids have very different adaptations of the skeletal elements of the jaws and arrangement of the associated musculature (e.g. AMγ'), the developmental history of the partitions are the same. Both groups share an additional subdivision of AMβ that differentiate them from the pattern in the basal triacanthodids (Fig. 5B). This model would also explain the pattern in representatives of the families, Diodontidae and Moliidae. In contrast to the balistids, the Monacanthidae show a tripartite AMβ, suggesting another duplication event of one of the AMβ partitions. Members of the family Ostraciidae are exceptional in having AMβ subdivided as the more derived Tetraodontiformes but have only an anteriorly bifurcated AMz.

**Homology of the Adductor Mandibulae Subdivisions in the Tetraodontiformes**

Winterbottom ('74a) and Friel and Wainwright ('97) used the pattern of duplicated partitions to understand the evolutionary trajectory of the adductor mandibulae of the tetraodontiformes. Winterbottom's ('74a) analysis does not present a hypothesis about character transformation between species. Friel and Wainwright ('97) were able to, through an analytical approach to provide a more parsimonious hypothesis than Winterbottom, explain the evolution of the adductor mandibulae complex.

When only adult specimens are used, as it is the case in Winterbottom ('74a) and Friel and Wainwright ('97), the only option to assess homology between an antecedent muscle and its decendent subdivisions is via anatomical similarity, e.g. relative position and anatomical “connectivity” (Remane, '52; Patterson, '82), which implies a correlation to a similar function of the related portion, e.g. A1 and A1β serve the maxilla and are the result of a duplication event of A1, which was already attached to the maxilla. The hypotheses of Winterbottom ('74a) and Friel and Wainwright ('97) are based on this correlation and state that all muscle portions that bind to the upper jaw (and, therefore, have also a certain function assigned) are offshoots from a single subdivision termed A1 by these authors. Additionally, all muscle portions that bind to the lower jaw derive from another subdivision (their A2). This, then, implies homology (Fig. 5A).
As the ontogeny of the adductor mandibulae in the puffer-fishes demonstrates, the contrary is the case: both subdivisions that bind to the upper jaw (AM$_a^0$ and AM$_b^0$) have their origin from different precursory muscle portions (Figs. 3A–C and 5B). The earlier view that the descended partitions of AM$_a$(A1) and AM$_b$(A2) were distinct separable units with a defined function is, therefore, no longer supported. In addition, a particular function of a partition does not indicate homology, e.g. AM$_a^0$ attached to the maxilla in the pufferfishes and to the lower jaw in the triggerfish has a similar developmental history and, therefore, represents in our view a homologous partition between the groups. The overlap between function and homology is common, but in cases of diversification the difference in these two properties become apparent.

We are aware that for many taxa it is almost impossible to obtain larval material for ontogenetic studies and that in such cases topological characters, such as similarity and connectivity of structures (Remane, ’52; Patterson, ’82), are often the only way to assess homology. Analysis of ontogeny allows tracing complicated structures back to a more simple state and is independent of function and to a certain degree of connectivity and position. This, therefore, provides the most conclusive data for the identification of homologous structures.

Our findings show that topological characters, such as origin, attachment, and function of adductor mandibulae subdivisions, are not predetermined by the topology and the functional role of the antecedent precursor. This has a wider implication for evolutionary studies because the adductor mandibulae has often been used to elucidate the evolution of the highly kinetic jaws across teleosts. Gosline (’86) hypothesized that the complicated and highly derived subdivision pattern of the adductor mandibulae of higher teleosts (Acanthomorpha) are modifications of a basal pattern, already present in the ancestral sistergroup (Myctophidae). Our findings support this hypothesis.

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**Development and the Evolution of Feeding Specializations**

Members of the tetraodontiform order show unique evolutionary specializations of the jaw that are defining traits for the different suborders. The increase in the complexity of the adductor mandibulae is directly linked with the evolution of feeding specializations in the order. This functional articulation is in part owing to the increase in complexity of the number of partitions of the adductor mandibulae. By addressing the comparative development of the adductor mandibulae among groups with specialized feeding structures (e.g. beak or dentition), we show that the various patterns are due from differential partitioning of a single muscle primorida. Complexity arises in the derived tetraodontiform suborders owing to further subdivision of the AM$_b$ portion. It is noteworthy that the timing of specification of AM$_a^0$ is quite different between the puffer and triggerfish, as it develops early in _M. suvattii_ and it is the last subdivision that appears in _B. undulatus_.

It is not partitioning that differentiates the adductor mandibulae complex between the different suborders, rather the...
varied attachment of the AMz\(^{\prime}\). This defines a specific character change underlying these specializations. Although pufferfishes and triggerfishes have lost the ability to protrude their upper jaw, the AMz\(^{\prime}\) partition in pufferfishes retains the ancestral attachment site on the upper jaw as seen in higher teleosts (A1), whereas in triggerfishes AMz\(^{\prime}\) is attached to the lower jaw. Turingan (‘94) observed that the mass of the adductor mandibulae portions that serves the upper jaw (mass is linked with bit force) do not vary between different taxa within the tetraodontids. He inferred that these portions are not important for the different modes of processing prey (crushing vs. biting). Different articulation of the muscle to the skeletal system, however, might represent alternative evolutionary trends to increase the mobility (not protrusion) of the upper jaw. This is supported by the fact that tetraodontiformes process prey with their oral jaws exclusively (Turingan, ‘94; Wainwright and Friel, 2000), instead of with the pharyngeal jaws as is common for most of the teleosts.

**CONCLUSION**

A conserved pattern of subdivision of the adductor mandibulae is seen between the development of diverse tetraodontiform groups, as the balistids and tetraodontids–groups with varied feeding behaviors and jaw specializations. Thus, it is most likely that the common ancestor to both groups shared this rudimentary process of muscle patterning. Complexity in the adductor mandibulae in some of the tetraodontiformes apparently has arisen from added partitioning of a single portion (AMB) and subsequent alteration of positioning of attachments of AMz\(^{\prime}\) in the triggerfish.

These observations are in contrast to the previous hypotheses of Winterbottom (‘74a) and Friel and Wainwright (‘97) that the adductor mandibulae is comprised of two muscle partitions, A1 and A2, which are distinct in function and their evolutionary history. This last concept was used as the foundation to homologize different muscle partitions between species. However, by looking at the development of the complex, we clearly show that the different portions of the adductor mandibulae are not functionally related to their particular antecedent muscle anlagen. Although our work focuses on the diversity of the adductor mandibulae of a highly derived order within the teleost fishes, our results suggest that the condition in even generalized teleosts may not be as straightforward as implied by the nomenclature (e.g. A1–3) applied in previous studies to infer to homologous subdivisions.

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**LITERATURE CITED**


