Jaw Muscle Development and Metamorphosis in Tadpoles of Eastern Narrowmouth Toads (*Gastrophryne carolinensis*: Microhylidae)

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ABSTRACT

Most amphibians have two distinct periods of development; embryogenesis which results in the formation of the larval stage, and metamorphosis which transforms the larva into an adult. The most dramatic metamorphic transformations occur in frogs where virtually every tissue is remodeled. The current work uses myosin antibodies and whole-mount immunohistochemistry to describe jaw muscle development and metamorphosis in tadpoles of Eastern Narrowmouth Toads (*Gastrophryne carolinensis*) and American Toads (*Anaxyrus americanus*). In Narrowmouth Toads the primary differences in jaw muscles involve a more anterior origin of the levator mandibulae muscles from the palatoquadrate, reduced size and dorsal-most margin of the orbitohyoideus, and the increased size and more oblique fiber orientation of the interhyoideus. Similar differences have been reported in previous work comparing microhylid tadpoles with members of other frog families. Architectural changes in the orbitohyoideus and interhyoideus are associated with the transition from a generalized feeding strategy based on scraping food from the substrate using keratinized mouthparts to a strategy focused on filter feeding of small-suspended particles. Our descriptions of jaw muscle development in *G. carolinensis* are consistent with decreased developmental rate and early offset of jaw development hypothesized for closely related frogs with similar feeding strategies.

INTRODUCTION

Amphibians, particularly frogs, are commonly used models for vertebrate development. Of the close to 6,500 species of extant frogs, detailed morphological analyses of early development are confined to very few species chosen largely due to ease of collection, laboratory maintenance, or the ability to induce breeding (Elinson and del Pino, 2012). In contrast, larval and metamorphic stages have been examined in a much wider range of taxa (e.g., Haas et al., 2014, Roelants et al., 2011; Vera-Candiotti 2007; Ziermann and Diogo, 2014).

In frogs, the ancestral life history strategy is biphasic (Duellman and Trueb, 1986). Eggs are laid in water, where they hatch and develop into herbivorous larvae (tadpoles). The tadpole then grows for a period of time before metamorphosing into a terrestrial, carnivorous adult. Evolutionary changes to this ancestral life-history pattern range from species that develop highly specialized tadpoles adapted to a variety of habitats and diets, to species that reduce or eliminate the feeding larval stage (Elinson and del Pino, 2012; Ziermann et al., 2011; Kerney et al., 2007; Vera-Candiotti et al., 2011). Phylogenetic analysis suggests that most tadpole diversity was generated relatively early in the evolutionary history of frogs and that subsequent changes were relatively minor and often involved morphological convergence or reversals (homoplasy; Roelants et al., 2011). The one apparent exception to this is the Microhyloidea, a monophyletic group of frogs that has evolved distinct larval anatomies and exhibits relatively low levels of convergence in comparison to other anurans (Roelants et al., 2011; Haas, 2001).

Tadpole morphology and diversity are closely correlated with two main aspects of larval ecology: water flow regime and feeding strategy (Duellman and Trueb, 1986). While flow regimes are primarily associated with differences in body shape and tail morphology (Oberhummer et al., 2014), diversity in feeding strategies involves morphological changes in mouth and jaw orientation, jaw cartilages and muscles, and gut length (e.g., Vera-Candioti, 2007). Evolutionary changes in tadpole jaw anatomy are well documented and descriptions of jaw cartilages and muscles are available for tadpoles ranging from ancestral herbivores (Haas, 2001; Vera-Candioti 2007), to obligate carnivores (Haas et al., 2014; Fabrezi and Lobo, 2009, Ziermann et al., 2011), to species with non-feeding larvae or direct development (Vera-Candioti et al., 2011; Hanken et al., 1997; Kerney et al., 2007; Ziermann and Diogo, 2014). Microhylid frogs, one of the most speciose and morphologically diverse groups of amphibians (Trueb et al., 2011; Roelants et al., 2011), have received relatively little attention. Jaw cartilage and muscle anatomy have been described for a relatively narrow range of larval and postmetamorphic stages in several microhylid taxa (Haas, 2001; Vera-Candioti, 2007; Trueb et al., 2011). The most complete developmental analysis includes detailed descriptions of jaw cartilage remodeling, but does not examine the associated musculature (Fabrezi et al., 2012). The primary objective of the current work is to describe larval jaw muscle development and metamorphosis in a North American microhylid, the Eastern Narrowmouth toad (*Gastrophryne carolinensis*). Developmental patterns documented in Narrowmouth toad larvae are then compared with descriptions of jaw muscle development available for tadpoles with a range of feeding modes.

MATERIALS AND METHODS

Animals: Larval Narrowmouth Toads (*Gastrophryne carolinensis*: Microhylidae) were purchased from a commercial supplier (Amphibians of North America, Charles Sullivan). Larval American Toads (*Anaxyrus americanus*: Bufonidae) were collected from temporary ponds in Madison County IL. All larvae were maintained in 10% Holtfreter solution in accordance with SIUE animal care regulations and fed ad libitum.

Staging: Tadpoles were anesthetized in 0.03% MS-222 and staged based on external morphology according to Gosner (Gos; 1960). Late larval stages (Gos 43-46) are

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based on changes in the position of the jaw joint, and the degree of tail resorption. Since jaw development was the focus of the current work, only tail resorption was used to differentiate among late stage larvae.

Muscle Immunohistochemistry: Larvae were fixed in Dent's fixative (20% DMSO / 80% Methanol; Dent et al., 1989). Subsequent steps follow the procedure of Klymkowsky and Hanken (1991). Tadpoles were immunostained using a monoclonal primary antibody to chicken myosin generated in mice (MF20; Developmental studies hybridoma bank; Bader et al., 1982). Primary antibody was diluted 1:250 in serum cocktail (5% newborn calf serum, 5% DMSO, 0.4% Triton X-100 in 0.1M PBS). After rinsing with saline cocktail (0.4% Triton X-100, 0.1M PBS), primary antibody localization was visualized using HRP-conjugate goat anti-mouse IgG (Bio-Rad) diluted 1:500 in serum cocktail. The individual muscle fibers were stained using 0.5 mg/ml diaminobenzidine (DAB) and 0.02% hydrogen peroxide in PBS for 1 hour. Specimens were dehydrated in ethanol, cleared through benzyl alcohol-benzyl benzoate/ethanol series and stored in benzyl alcohol:benzyl benzoate (1:1).

Image capture and analysis: For each specimen, images of musculature were captured using a Jenoptik ProgRes Capture Pro 2.7 system attached to a Leica S8APO dissecting scope. To insure consistency of orientation lateral photos were taken by aligning the two eyes, ventral photos were taken when both eyes were in the same focal plane. Muscle identification follows the nomenclature of Haas (2001), and muscle attachments follow chondrocranial descriptions of Trueb et al., (2011).

RESULTS

Gastrophryne carolinensis – **Lateral musculature:**

At the earliest stage examined (Gos 34), three distinct sets of jaw muscles are present (Fig. 1A). Two levator mandibulae muscles responsible for moving the larval lower jaw (m. levator mandibulae longus and m. levator mandibulae internus) originate from the subocular bar, ventral to the eye, and extend rostrally to insert on the jaw (Meckel's cartilage). The m. levator mandibulae longus inserts more medially on Meckel's cartilage than the m. levator mandibulae internus. In lateral view, the two muscles appear to overlap at their point of origin, but slightly diverge dorsoventrally at the point of insertion. Ventral to the levator mandibulae group, the primary muscles for opening the larval jaw (angularis group) are present. In lateral view, only one angularis muscle is visible. In ventral view, two distinct angularis muscles are apparent (Fig. 1E); the quadratoangularis (lateral) and suspensorioangularis (medial). Both of these muscles originate from the ventral surface of the palatoquadrate and insert on the retroarticular process of Meckel's cartilage The third muscle, located ventrally to the two levator mandibulae muscles and posterior to the angularis group, is the orbitohyoideus. At this stage, the orbitohyoideus is a broad muscle composed of short fibers oriented postero-ventrally from their origin on the muscular process of the palatoquadrate to their insertion on the lateral edge of the ceratohyal.

From stages 34-40 (Fig. 1B), the levator mandibulae muscles shift so that they originate close to the anterior margin of the eye. A
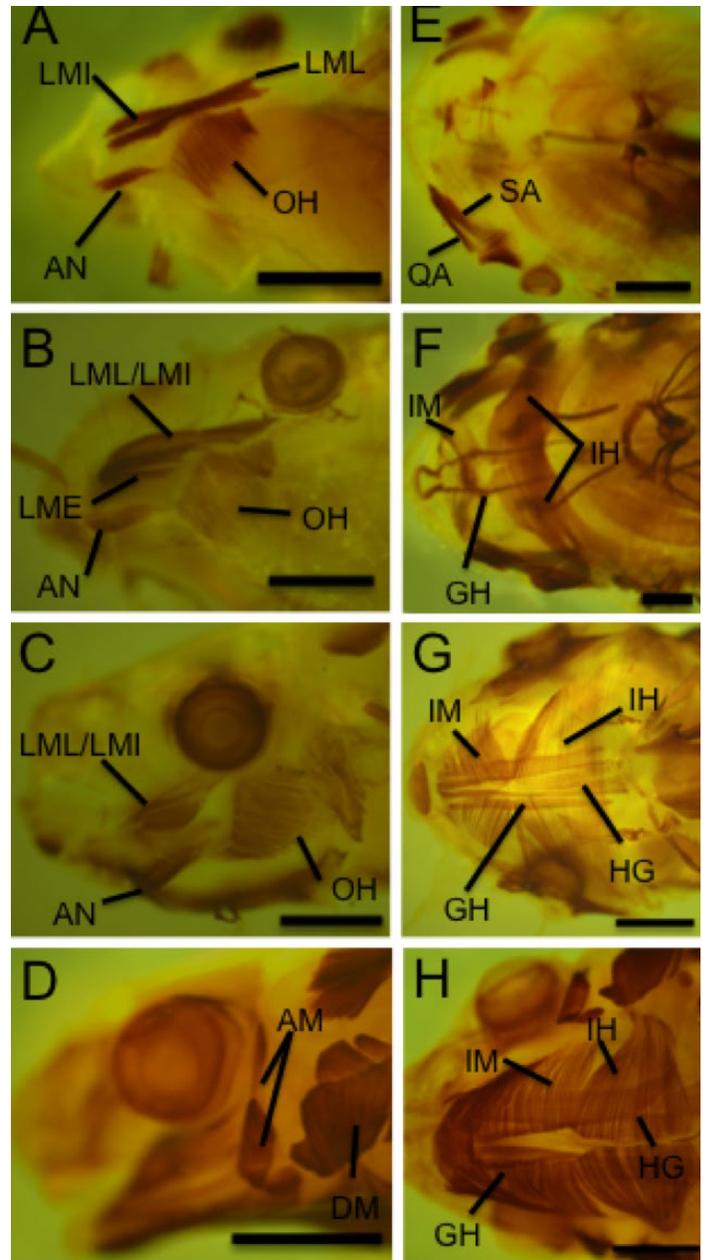

Fig. 1. *Gastrophryne carolinensis*, lateral (A-D) and ventral (E-H) views, jaw muscles stained with MF20 antibody. Anterior is to the left. A: Gos 34. B: Gos 36. C: Gos 42. D: Gos 46. E: Gos 34. F: Gos 39. G: Gos 43. H: Gos 46. Scale bar = 1mm. Abbreviations: AM – adductor mandibulae, AN – angularis group, DM – depressor mandibulae, GH – geniohyoideus, HG – hyoglossus, IH – interhyoideus, IM – intermandibularis, LME – levator mandibulae externus, LMI – levator mandibulae internus, LML – levator mandibulae longus, OH – orbitohyoideus, QA – quadratoangularis, SA - suspensorioangularis.

third levator muscle (m. levator mandibulae externus) composed of shorter fibers forms ventrally to the other levators. Muscle fibers in the angularis group elongate, nearly reaching the anterior border of the orbitohyoideus. The fibers of the orbitohyoideus also elongate, but the muscle is still broader than long.

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During subsequent stages (Gos 42-46), muscles shift in origin, insertion, and fiber orientation as the jaw begins to undergo metamorphic remodeling (Fig. 1C). The levator mandibulae muscles shift inferior to the eye at stage 42, and by stage 44 they originate from the anterior surface of the otic capsule. During this period, fiber orientation also changes from antero-posterior to more dorso-ventral as the origin of these muscles shift to the mandible as it forms and elongates posteriorly. Towards the end of metamorphosis (Fig. 1D), levator mandibulae muscles have begun to fuse and form the adductor mandibulae of the adult frog. Muscles of the angularis group also shift to the posterior margin of the eye during these stages, and fiber orientation shifts from horizontal to vertical. Migration of the orbitohyoideus follows a similar trajectory and retains its position as the most posterior muscle attached to the lower jaw. Changes in fiber orientation are less dramatic in the orbitohyoideus as they shift from an oblique orientation (antero-dorsal - caudoventral) to a dorso-ventral orientation. Coincident with this shift, the orbitohyoideus and angularis muscles begin to fuse to form the single adult depressor mandibulae.

Gastrophryne carolinensis – Ventral Musculature

In contrast to lateral musculature, ventral muscles did not stain clearly at the earliest stages examined (Gos 34; Fig. 1E). At stage 39, the most prominent ventral muscle is the interhyoideus. The interhyoideus is a broad muscle, originating at the lateral margin of the ceratohyal extending medially to insert on the midline. Fibers of the interhyoideus are oriented both transversely and obliquely such that the medial portion extends well anterior to the point of origin (Fig. 1F). Anterior to the interhyoideus, the intermandibularis extends from its lateral origins on Meckel's cartilage to a median insertion. The most posterior fibers of the intermandibularis contact, but do not overlap the most anterior fibers of the interhyoideus. Paired geniohyoid muscles lie deep to both the interhyoideus and intermandibularis, extending rostral from their origin at the posterior margin of the interhyoideus to their midline insertion on the lower jaw.

During subsequent stages (Gos 40-46) ventral muscles do not change position markedly (Fig. 1G,H). Both the interhyoideus and intermandibularis increase in rostrocaudal width, but do not increase the degree of overlap. At the end of metamorphosis (Gos 46) fiber orientation of the two muscles is similar, and the border between them is less distinct (Fig. 1H). From stage 42 onward, the paired hyoglossus muscles are present medially to the paired geniohyoideus (Fig. 1G,H).

Anaxyrus americanus – Lateral Musculature

The two longest larval jaw levator muscles (m. levator mandibulae longus and internus) are present at the earliest stages examined (Gos 27; Fig. 2A). Both muscles originate from the ascending process of the palatoquadrate, ventral to the posterior margin of the eye, and insert on Meckel's cartilage of the lower jaw. The m. levator mandibulae longus inserts more medially on Meckel's cartilage than the m. levator mandibulae internus. The muscles are similar in site of origin, but diverge slightly at their insertions on Meckel's cartilage. At this stage, two angularis muscles (hyoangularis and suspensorioangularis) are also visible. The orbitohyoideus is a large
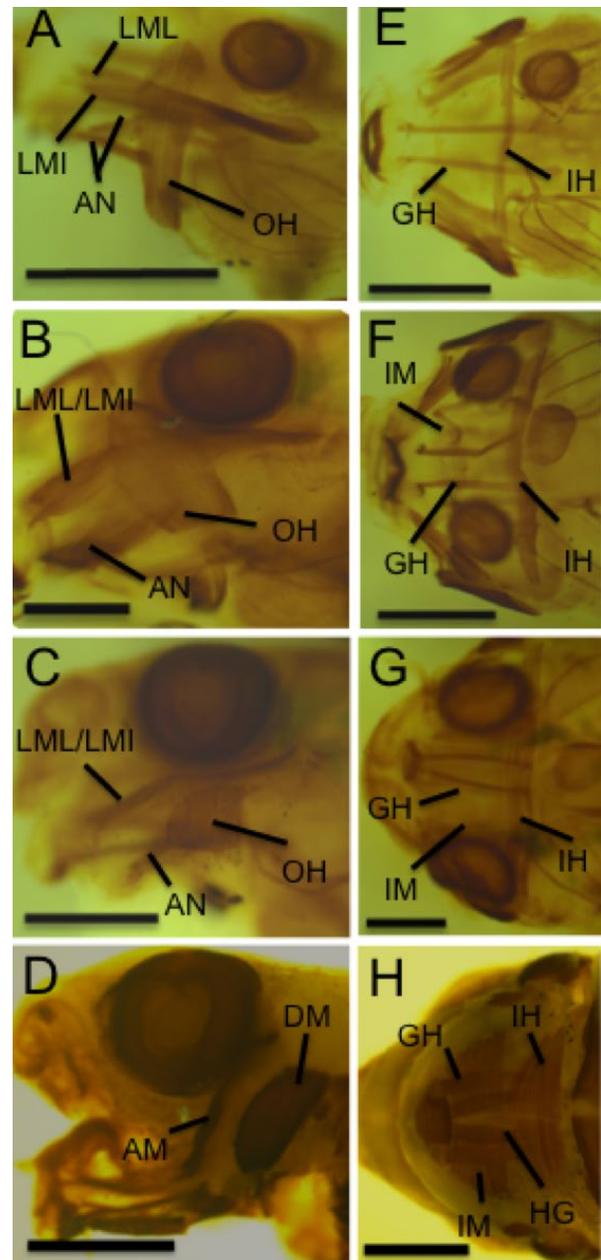

Fig. 2. *Anaxyrus americanus*, lateral (A-D) and ventral (E-H) views, jaw muscles stained with MF20 antibody. Anterior is to the left. A: Gos 27. B: Gos 38. C: Gos 43. D: Gos 46. E: Gos 29. Right eye was removed during specimen preparation. F: Gos 32. G: Gos 43. H: Gos 46. Scale bar = 1mm. Abbreviations: AN – angularis group, GH – geniohyoideus, HG – hyoglossus, IH – interhyoideus, IM – intermandibularis, LMI – levator mandibulae internus, LML – levator mandibulae longus, OH – orbitohyoideus.

muscle originating from the muscular process of the palatoquadrate anterior to the eye and inserting on the lateral border of the ceratohyal ventrally.

During subsequent stages examined (Gos 29-38, Fig. 2B), the only major change in jaw levators is in fiber orientation. In early stages levator muscles are oriented in an antero-posterior direction. By

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the late larval stages (Gos 38), fiber orientation in the anterior portion of these levator muscles is more ventral, while the posterior muscle fibers have retained their original orientation. The angularis group exhibits little change over the larval stages examined. Across the same set of stages, the orbitohyoideus shortens in length and begins to migrate posteriorly and inferior to the eye.

At mid-metamorphosis (Gos 43, Fig. 2C) the levator muscles still have different fiber orientations in the anterior and posterior regions, but have not started their posterior migration. In contrast, the angularis muscles are much less distinct, have begun to merge with the orbitohyoideus, and have the dorso-ventral fiber orientation typical of the adult depressor mandibulae. By the completion of metamorphosis (Gos 46, Fig. 2D) the levator muscles have shifted to originate on the otic capsule and insert on the mandible and have largely fused to form the adductor mandibulae of the adult frog.

Anaxyrus americanus – Ventral Musculature

Ventral musculature did not stain as darkly as lateral muscles in any of the larval stages examined. At early larval stages (Gos 27-30) the interhyoideus is visible as a thin, strap-like muscle that extends from the lateral margins of the ceratohyal to its median insertions (Fig. 2E). The muscle is equal in width along its entire length, and all fibers are oriented transversely. The geniohyoid is also visible from its posterior origin on the hypobranchial plate to its insertion on the infrarostral cartilages of the lower jaw. At these stages, the intermandibularis is not visible.

By stage 32, a few fibers of the intermandibularis are visible. The intermandibularis is fan-shaped, with narrow attachments to Meckel's cartilage at its lateral margins and much broader attachments at the midline (Fig. 2F). The interhyoideus and geniohyoideus are similar to earlier stages. The posterior margin of the intermandibularis and anterior margin of the interhyoideus are well separated. At mid-metamorphosis (Gos 43, Fig. 2G), fibers of the interhyoideus and geniohyoideus are clearly visible. The intermandibularis did not stain clearly at this stage. At the completion of metamorphosis (Gos 46, Fig. 2H), the interhyoideus and intermandibularis are clearly visible and the posterior margin of the intermandibularis approaches the anterior margin of the interhyoideus at the midline. The paired hyoglossus muscles are present medially to the paired geniohyoideus.

DISCUSSION

The current study uses myosin antibodies to document jaw muscle development in Eastern Narrowmouth Toads (*Gastrophryne carolinensis*: Microhylidae, Fig. 1) and American Toads (*Anaxyrus americanus*: Bufonidae, Fig. 2). The most apparent differences in jaw musculature between the two species include: (1) anterior position of the levator mandibulae muscles, (2) size and dorsal-most extent of the orbitohyoideus, (3) size and fiber orientation of the interhyoideus. Similar differences have been reported in previous work comparing microhylid tadpoles with members of other frog families (Haas, 2001; Vera-Candioti, 2007). Like most microhylids the levator mandibulae muscles of *G. carolinensis* originate further anterior on the palatoquadrate in comparison to other tadpoles, including *A. americanus*. The orbitohyoideus is also reduced in size in *G. carolinensis* along with its attachment site on the muscular process of the palatoquadrate (Trueb et al., 2011). Ventrally, the primary muscular difference among microhylid tadpoles and those of other frogs is the width of the interhyoideus. In most frogs this muscle is composed of a thin band of transverse fibers that originate from a central band of connective tissue and insert on each side of the larval ceratohyal. In microhylids this muscle is much broader, and is composed of both transverse and obliquely oriented fibers.

Comparative analyses of tadpole jaw development have documented shifts in muscle orientation, origins and insertions, and changes in muscle size that are primarily associated with changes in feeding strategy (Storz and Moerland, 2009, Ziermann et al., 2011, Haas et al., 2014). While some microhylids have non-feeding larvae, or lack a free-living larval stage, those with feeding tadpoles are primarily mid-water suspension feeders that pump high volumes of water through the oral cavity and filter out suspended particles (Vera-Candioti, 2007). Changes in the orbitohyoideus and interhy-oideus documented in the current study are associated with the transition from a generalized (ancestral) feeding strategy relying on grazing and suction feeding to a strategy focused on filtering of small suspended particles. Pumping mechanisms of generalized anuran larvae rely primarily on the lateral orbitohyoideus muscle, which is responsible for lowering the floor of the oral cavity resulting in water influx. Contraction of the ventral interhyoideus then elevates the floor of the oral cavity and, coupled with closure of the mouth, forces water to the posterior where respiratory and filtering structures are located (Larson and Reilly, 2003). Changes in the size of the orbitohyoideus in filter-feeding microhylids suggest that oral cavity expansion and generation of large negative pressure is reduced in these larvae. In contrast, increased width and addition of obliquely oriented fibers in the interhyoideus suggest that elevation of the oral cavity floor is enhanced in microhylid taxa that rely on suspension feeding. Similar jaw muscle anatomy is also characteristic of suspension feeding pipid tadpoles (e.g., *Xenopus*; Vera-Candioti, 2007; Ziermann and Olson, 2007) where feeding kinematics of the orbitohyoideus and interhyoideus have been examined (Wassersug and Yamashita, 2001; Ryerson and Deban, 2010). While general muscular arrangements appear similar among suspension feeding tadpoles, the extent of convergence is largely unknown as descriptions of jaw muscle anatomy and function in microhylids are only available for a small number of species, during a relatively narrow range of development (Haas, 2001; Vera-Candioti, 2007; Fabrezi et al., 2012).

In addition to changes in muscle architecture, jaw muscles also differ in fiber composition and the rate at which larval myofibers are replaced by adult myofibers. Fiber composition can alter how muscles function during feeding and respiration and may even alter in response to the types of food consumed (Larson and Reilly, 2003; Storz and Moerland, 2009). Future descriptions of jaw muscle development in tadpoles need to expand beyond morphological features and address how muscle physiology and kinematics differ among species (Venesky et al., 2011). The transition from larval to adult myofibers is also likely to impact the timing and rate of muscle remodeling

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during metamorphosis. Several adult muscles (e.g., depressor mandibulae) are derived from satellite cells embedded among larval muscle cells (Alley, 1989). Changes in the timing of adult myocyte differentiation potentially impact the onset and extent of muscle remodeling and may contribute to the early offset of jaw muscle development in *G. carolinensis*. In the direct-developing frog *Eleutherodactylus coqui*, the derived developmental pattern of jaw muscles is associated with the absence of larval myofibers and myofiber turnover (Hanken et al., 1997)

Comparative Developmental Patterns: Among amphibians, frogs are the most diverse group, and undergo the most substantial transformation between larval and adult stages (Elinson and del Pino, 2012). High levels of diversity in frogs have been attributed to two general mechanisms: (1) the evolution of a dramatic metamorphosis between larval and adult stages which potentially frees each stage to evolve relatively independently (Haas, 2001: Svensson and Haas, 2005), (2) heterochrony where features shift in their relative timing of development with respect to other features (Fabrezi and Quinzio, 2008; Fabrezi and Lobo, 2009; Vera-Candioti et al., 2011; Ziermann et al., 2011). These two mechanisms are not mutually exclusive as heterochrony can potentially result in features that evolved in one stage forming in a different stage (e.g., precocious development of adult features in tadpoles). To evaluate evolutionary changes in the extent of metamorphosis and the role of heterochrony in amphibian jaw development, a phylogenetic approach is essential. Detailed descriptions of larval and adult jaw anatomy are available for salamanders (Kleinteich and Haas, 2007; Ziermann and Diogo, 2013), caecilians (Kleinteich et al., 2014), and basal frogs (Johnston, 2011; Haas, 2001).

If larval and adult stages are free to evolve independently, more recently derived species are predicted to undergo a more dramatic metamorphosis that are basal taxa (Wassersug and Hoff, 1982). In support of this prediction, larval and adult jaw orientations are generally more similar in basal than derived taxa (Wassersug and Hoff, 1982), and some of the most extreme differences between larval and adult jaw orientation are seen in derived frogs with carnivorous larvae (Fabrezi and Quinzio, 2008). However, recent morphological and phylogenetic analyses that include a wide range of larval characters conclude that diversity of tadpole morphology was largely generated during the basal radiations of frogs, and that subsequent evolutionary rates have been slower (Roelants et al., 2011). This pattern suggests that many tadpole features were established relatively early and that more recently derived taxa do not necessarily exhibit more dramatic metamorphic transitions. Convergent and parallel evolutionary changes (homoplasy) in tadpole morphology further obscure relationships among divergence time and degree of metamorphosis (Roelants et al., 2011).

A distinguishing feature of many microhylids is that as adults their jaw joint is located anteriorly in comparison to other frogs (Trueb et al., 2011). In these microhylid frogs the position of the jaw joint at the completion of metamorphosis resembles the condition seen in most frogs during mid-metamorphosis (Fabrezi et al., 2012). Mechanistically, the anterior location and jaw morphology of the microhylid frog *Dermatonotus muelleri* has been attributed to heterochronic slowing of developmental rate and early offset of jaw development (Fabrezi et al., 2012). Our descriptions of jaw muscle development in *G. carolinensis* are consistent with both of these possibilities. Development of the levator mandibulae and orbitohyoideus muscles in *G. carolinensis* occur at a slower rate relative to other features, and muscle organization of late larval stages resemble earlier stages of more generalized larvae. The location of the levator mandibulae muscles ventral to the anterior margin of the eye during larval development of *G. carolinensis* resembles the position of these muscles in Leopard frog tadpoles (*Lithobates pipiens*) shortly after their initial appearance (Fig. 1A; Ziermann and Diogo, 2014). During the entire larval period examined the orbitohyoideus of *G. carolinensis* is located ventral to the levator mandibulae complex, and fibers of the two muscles never overlap in lateral view (Fig. 1A-D). A similar arrangement of the orbitohyoideus and levator mandibulae occurs during early development in *L. pipiens*, but the orbitohyoideus lengthens shortly after hatching and by the onset of feeding fibers of the orbitohyoideus overlap fibers of the levator mandibulae muscles in lateral view (Ziermann and Diogo, 2014). Offset of jaw muscle development also seems to occur earlier in *G. carolinensis* than in other species. By mid-metamorphic stages, the origin of levator mandibulae muscles have shifted posterior to the eye and are beginning to establish the vertical orientation typical of the post-metamorphic adductor mandibulae muscle (Fig. 1C). The orbitohyoideus has also shifted and is now posterior to eye. At comparable stages, levator mandibulae muscles of *A. americanus* are still oriented obliquely, and the orbitohyoideus is ventral to the eye (Fig. 2C).

To fully document the role of heterochrony in jaw development in *G. carolinensis*, future work needs to examine muscular development in earlier stages. The earliest stages examined in the current work were all post-hatching, so it is unknown if the initial stages of muscle development are similar to what has been documented in other larval amphibians. Of particular interest are comparisons between early jaw development in *G. carolinensis* and *Xenopus laevis* as both species develop into microphagous filter-feeding tadpoles with similar jaw muscle architectures (Ziermann and Diogo, 2014; Vera-Candioti, 2007). Similar morphologies are not necessarily the result of similar developmental pathways and detailed descriptions of trait development are necessary to evaluate the evolution of developmental mechanisms (Kerney et al., 2011; Vera-Candioti et al., 2011). In addition, functional assays of jaw musculature are necessary to determine if changes in developmental timing are associated with changes in function (Ziermann and Olsson, 2007).

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