2020

AMPHIBIAN VOCALIZATION: IMPLICATIONS OF A NOVEL LARYNGEAL MUSCLE IN THE CALLING MECHANISMS OF THE TÚNGARA FROG ENGYSTOMOPS PUSTULOSUS

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Recommended Citation

Lagorio, Amy D.. (2020). *AMPHIBIAN VOCALIZATION: IMPLICATIONS OF A NOVEL LARYNGEAL MUSCLE IN THE CALLING MECHANISMS OF THE TÚNGARA FROG ENGYSTOMOPS PUSTULOSUS*. University of the Pacific, Thesis. https://scholarlycommons.pacific.edu/uop_etds/3683

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By

Amy D. Lagorio

A Thesis Submitted to the

Graduate School

In Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

College of the Pacific
Biological Sciences

University of the Pacific
Stockton, CA

2020

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DEDICATION

I dedicate this work to my mother, Nona Jo Dabney, who fiercely believed in me and my ability to succeed. Above all else it was your love and support that made this study possible. With unending love and admiration, thank you.

ACKNOWLEDGEMENTS

My sincere thanks to my committee members, Dr. Eric Thomas and Dr. Des Maxwell, who not only provided mentorship throughout the course of my thesis, but inspired and supported me for the duration of my time at Pacific. I am forever grateful for their encouragement, guidance, and wonderfully unique senses of humor which fueled my success throughout my graduate studies.

My gratitude to the wonderful faculty members of Pacific, whose combined efforts played a key role in my development into a better student, researcher, and person. Thank you to Dr. Jerry Tsai, for all your support during my undergraduate studies. I will always be thankful for the consistently entertaining coursework and novel research opportunities you allotted me. Thank you to Dr. Tara Thiemann and Carrie Kozina, who taught me the skills necessary to become a better educator. I am so grateful for all your patience and will continue to draw from your lessons as I move forward with my career in academia. Thank you to Dr. Vierra. You were an amazing advisor. Your advice was just as invaluable as the technical skills you shared, both of which helped see this study through to publication. Thank you to Dr. Lisa Wrischnik, whose confidence and grit were awe inspiring. It has truly been my pleasure to have studied under such a spirited scientist. Thank you to Dr. Stacie Hooper, I can’t even begin to express here how much you mean to me. The love and lessons you’ve shared will last a lifetime and I am so glad every day to have met such a wonderful person. Thank you to Debbie Walker, whom I admire greatly and could not imagine my time at Pacific without. You are the hardest working and most creative individual I know. Thank you so much for taking me under your wing.

A big thank you to Dr. Marcos Gridi-Papp and the previous members of the GPLab. To Dr. Gridi-Papp, thank you so very much for this incredible research opportunity and for being so supportive of my academic endeavors. To my lab mates and colleagues, thank you for not only going on this journey of discovery with me, but for helping to pave the way for the current study.

Love and wholehearted thanks to the amazing individuals I am lucky enough to call my friends and family. To my loving parents, thank you for being my number one support system and for giving your everything in the aid of my success. To my grandmother, thank you for always believing in me. I'm so grateful you were able to see me on this journey. Special thanks to Jenica LaRay Emerson, who was and will always be my favorite person. You continually inspire me to better myself and I’m so excited for the future adventures we both will share. To all my friends at Pacific, thank you for being there to remind me that happiness can be found, even in the darkest of times, if one only remembers to turn on the light.

Thank you to Professor Michael J. Ryan and to the High-Resolution X-Ray Computed Tomography Facility of The University of Texas at Austin for providing the CTscans used throughout this study. The data provided has been an invaluable basis of both learning and discovery with regards to the túngara frog larynx.

Lastly, I would like to extend thanks to the Pacific Fund and the Hunter Nahhas Summer Research Fellowship which helped to fund this study. Thank you for your support.

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Abstract

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The current functional model of the anuran larynx includes four pairs of laryngeal muscles. Their contractions do not account, however, for the behavioral control of call complexity observed in male túngara frogs (*Engystomops pustulosus*), which optionally add a secondary note with distinct harmonic structure to their advertisement call. Examination of the túngara frog's laryngeal morphology through dissection, microtomography, and resin histology has revealed that the m. dilatator laryngis is divided into two separate bundles (superficial and deep). The superficial bundle closely matches the typical description of the m. dilatator laryngis and is well positioned to open the glottis. The deep bundle is exclusively innervated by the short laryngeal nerve and has an attachment to the fibrous mass, an internal laryngeal structure necessary for complex call production. This attachment indicates a separate role for the deep bundle in controlling the complexity of the call. Based on physical separation, exclusive attachments, distinct fiber orientation, exclusive innervation, and potential action, this study recognizes the deep bundle of the m. dilatator laryngis as a separate muscle. It also revalidates the name m. arylabialis which had been previously used to describe it. The split of the m. dilatator laryngis into two muscles results in a laryngeal innervation pattern that closely matches that of mammals. This study identified a novel laryngeal muscle in túngara frogs, a potential

mechanism for the control of call complexity, and revealed new evidence of homologies between the laryngeal structures of amphibians and mammals.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
cm	centimeter
h	hour
Hz	hertz
g	gram
g/l	grams per liter
kHz	kilohertz
min	minute
ml	milliliter
mm	millimeter
ms	millisecond
µg	microgram
µm	micrometer
s	second

CHAPTER 1: INTRODUCTION

Evolution of Communication

Since Charles Darwin's 1859 publication of *On the Origin of Species by Natural Selection*, elucidating the evolutionary origin of complex characteristics has been the pursuit of countless scientific minds. Current studies on the evolution of complexity extend beyond the examination of morphological structures to include behavioral traits such as those involved in social interactions and communication (Ghazanfar, 2013). Animals communicate in various contexts, from alarm against predators to attracting mates, disputing territories or teaching offspring (Naguib and Price, 2013). Similarly, humans are capable of producing a wide range of complex sounds used in communication, the mechanisms behind which have been studied in great detail (Garcia, 1856; Kimppa, 2015). Human vocalization primarily involves the passive vibration of a pair of vocal folds within the larynx, the acoustic vibrations of which can be manipulated to produce a wide range of variable sounds (Fig. 1; Garcia, 1856; Berg, 2007). Attempting to understand a complex communication system, such as that of humans, can be very time-consuming and difficult. An alternative approach is to analyze a simpler system that shares the relevant characteristics with the complex system, allowing for extrapolation of the results.

The evolution of complexity in animal communication systems is perplexing because while some exhibit relatively complex signal repertoires, others retain a communication system based exclusively on simple signals (Prestwich, 1994; Mougeot and Bretagnolle, 2000). Communication complexity is not limited to a single mode of signal transmission either. Modes of communication most commonly include auditory, visual, olfactory, tactile, chemical, electric,

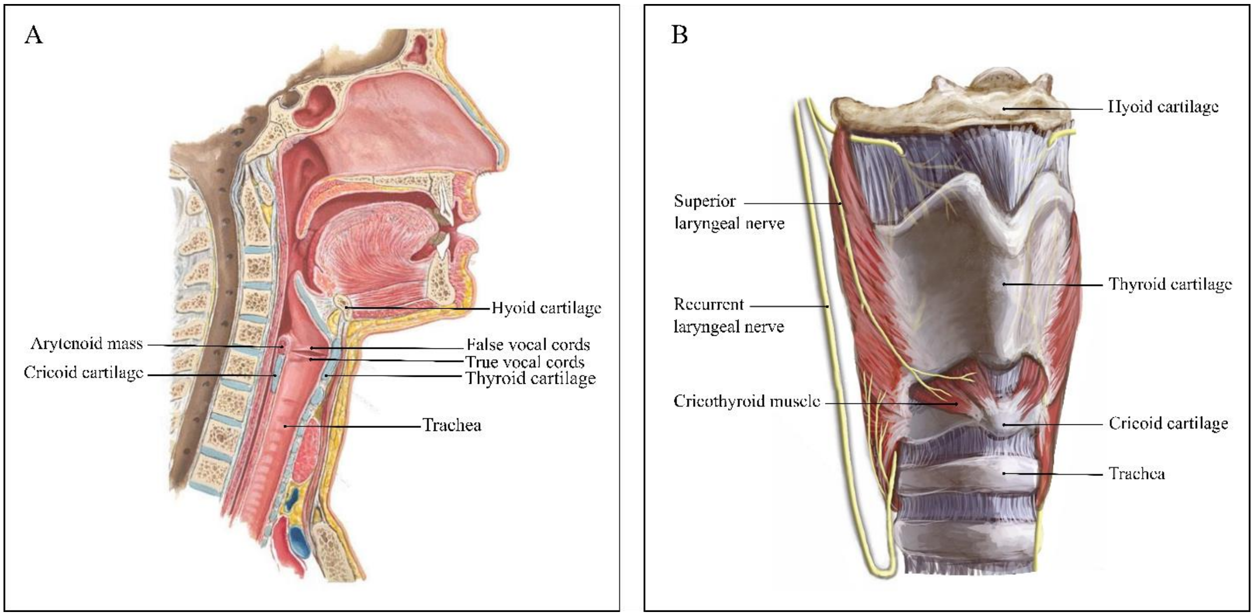

Figure 1. Organs involved in human vocalization. (A) Midsagittal section of the human upper respiratory tract. Sound is produced by vibration of the true vocal cords within the larynx. The larynx spans from the epiglottis superiorly to the cricoid cartilage inferiorly.[1] (B) Innervation of the human hyolaryngeal apparatus. The superior laryngeal nerve only innervates one laryngeal muscle, the cricothyroid, while the recurrent laryngeal nerve innervates all the remaining laryngeal muscles.[2]

or seismic signals (Bro-Jørgensen, 2010). Many behaviors involve combinations of signals emitted via multiple modes (Bro-Jørgensen, 2010). Acoustic communication is among the easiest of modes to study as the signals tend to be easily recorded, manipulated, and re-created for use in experiments on behavioral response (Ryan, 1985; Gerhardt and Huber, 2002; Marler and Slabbekoorn, 2004). In acoustic communication, organisms produce sounds that usually indicate their state of sexual readiness, dominance, alarm, hunger, or aggressiveness (Bradbury and Vehrencamp, 1998). These signals can be as simple as the rasp of a grasshopper to as complex as the arias of a songbird.

The lack of elaborate acoustic signaling within a given species does not imply a lack of complex calling capability. It can instead be an indication that complex calls do not provide sufficient benefit to be used as the default means of communication for that species. For instance, when intrasexual competition is low and the cost of complex call production is energetically high, males of a particular species may produce simple advertisement calls, even when complex calls are preferred by females (Wells, 1977). As such, it would be advantageous to males if they could choose when to elicit complex advertisement calls, such as when chorus density is high and competition over mates increases. The túngara frog (*Engystomops pustulosus*) is a prime example of a vertebrate whose males facultatively increase their call complexity in situations of increased competition for potential mates (Ryan, 1985). Additionally, the ability to regulate the complexity of acoustic signals can serve as a trade-off between the pressures of natural and sexual selection. This concept is also well demonstrated by the advertisement calls of the male túngara frog. The same acoustic complexity that increases the attractiveness of the call to females also facilitates localization by the fringe-lipped bat (*Trachops cirrhosus*) and makes the male túngara frog a target for predation (Ryan et al., 1982; Page and Ryan, 2008). Similarly, the added complexity increases the attraction of parasitic flies, which debilitate the male by sucking its blood while transmitting parasitic diseases (Bernal et al., 2006).

Mechanism of Call Production in Frogs

Understanding the evolution of facultative complex signals demands the identification of the anatomical structures contributing to sound complexity and an explanation of how they are controlled. Comprehension of what role the laryngeal anatomy plays in determining call complexity in frogs begins with a deeper understanding of the general morphology of anuran larynges. The amphibian order Anura includes frogs and toads, which are ideal organisms for studying acoustic communication due to the simplicity of their advertisement calls and the diversity of species in the group (Ryan, 2001). Male anurans produce distinctive, simple calls with most of the energy contained within one or two frequencies (Fig. 2). These calls are repeated at high intensities and in a simple timing pattern throughout the night (Wells, 1977). Females typically do not produce advertisement calls, but instead respond to the calls of males with phonotaxis (Tobias et al., 1997).

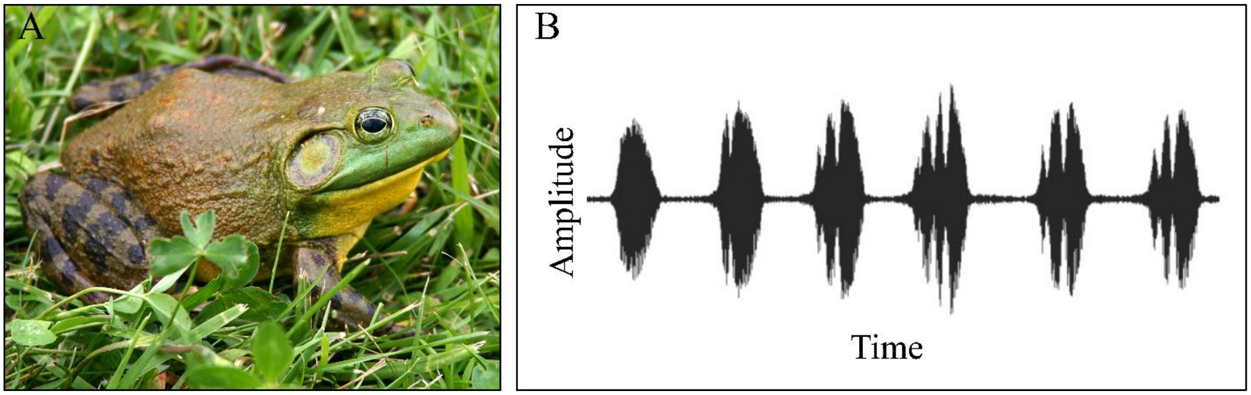

Figure 2. Structure of the simple anuran advertisement call. (A) The American bullfrog (*Lithobates catesbeianus*).[3] (B) Digitized oscillogram of a natural bullfrog call consisting of simple, repetitive vocalizations. Time is depicted along the horizontal axis and relative amplitude along the vertical axis.[4]

The anuran larynx is variable in the size and shape of its components, although the same basic structures are present in most species (Trewavas, 1933). Even species specialized in underwater communication exhibit a larynx modified from the same fundamental components (Yager, 1992). Typically a frog's larynx is composed of a cartilaginous framework, four pairs of muscles (m. dilatator laryngis, m. constrictor laryngis externus, m. constrictor laryngis anterior, and m. constrictor laryngis posterior), and a pair of vocal cords that can sometimes exhibit thickenings called fibrous masses (Fig. 3; Gaupp, 1904; Trewavas, 1933; Duellman and Trueb, 1986; Martin, 1971; Drewry et al., 1982; Walkowiak, 2007). Two arytenoid cartilages are

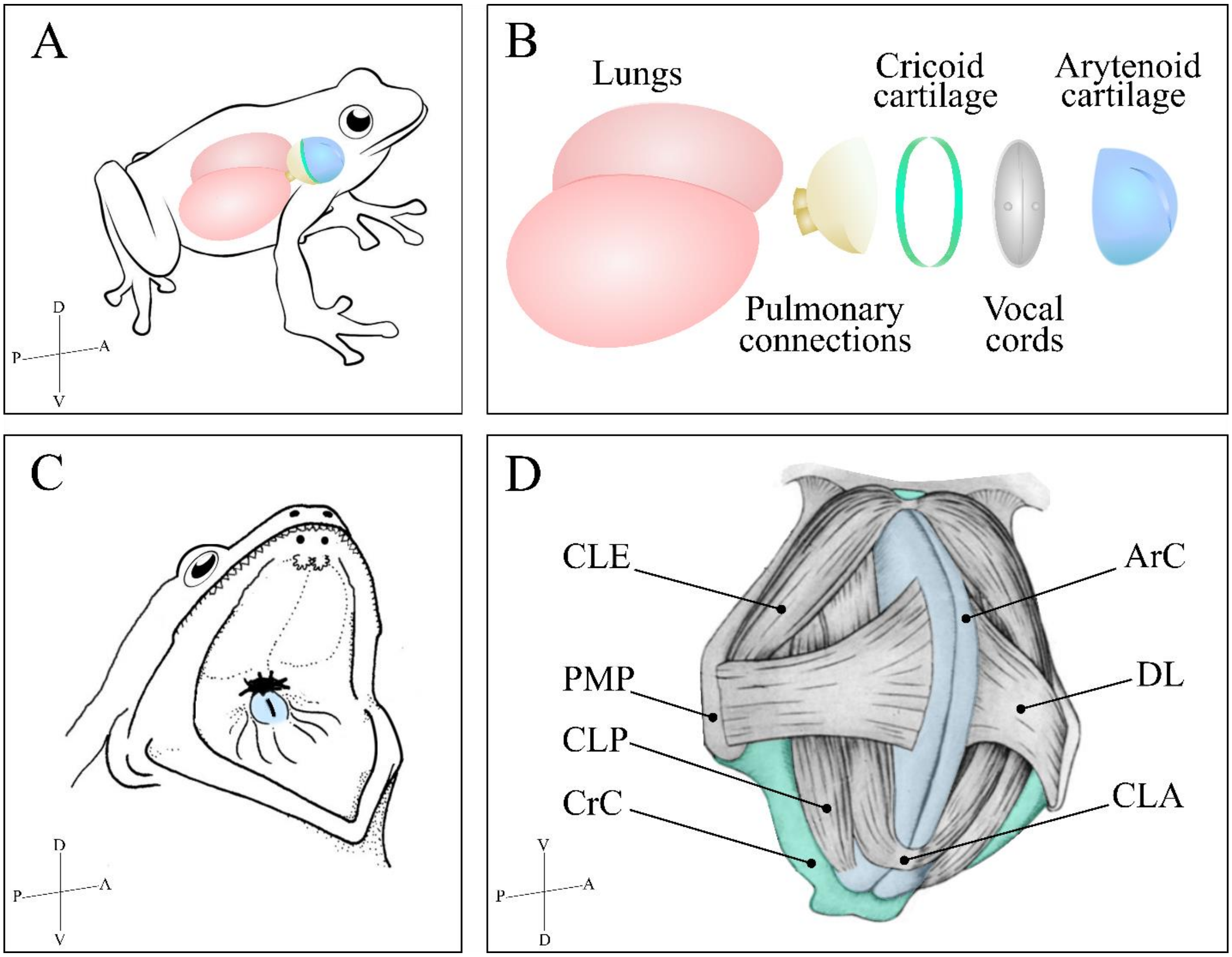

Figure 3. Anatomy of a typical anuran larynx. (A) Positioning of the laryngeal apparatus within the anuran body. Directional abbreviations: A, anterior; D, dorsal; P, posterior; V, ventral. These abbreviations apply to all subsequent panels. (B) Schematic representation of the laryngeal framework. Laryngeal elements have been distanced from one another to provide a clear view of each structure. The vocal cords are housed inside the combined arytenoid and cricoid cartilages which are joined to the lungs via the membranous pulmonary connections. (C) Positioning of the glottal opening and arytenoid cartilages (blue) within the oral cavity.[5] (D) Artistic representation of the laryngeal musculature in *Hyla a. aborea*.[6] Abbreviations: ArC, arytenoid cartilage (blue); CLA, m. constrictor laryngis anterior; CLE, m. constrictor laryngis externus; CLP, m. constrictor laryngis posterior; CrC, cricoid cartilage (green); DL, m. dilatator laryngis; PMP, posteromedial process of the hyoid cartilage. These abbreviations apply to all subsequent figures.

supported by a single ring of cricoid cartilage, and together they form the cartilaginous framework of the larynx. The arytenoid cartilages arch towards one another and can pivot over their attachments to the cricoid cartilage, thus creating a means of control for regulation of airflow through the glottis. This framework houses the vocal cords along with their fibrous masses and the entire laryngeal organ is supported by the posteromedial processes of the hyoid cartilage in the floor of the mouth (Fig. 3; Trewavas, 1933; Duellman and Trueb, 1986). Additional structural features within the anuran larynx have been documented in multiple species. These include cartilaginous structures whose presence and appearance are more varied than the rest of the laryngeal framework (Gaupp, 1904; Trewavas, 1933). The most common of these structures include the apical cartilage, membrane-embedded sesamoid cartilages, and the basal cartilage (Gaupp, 1904; Blume, 1930; Trewavas, 1933). The basal cartilage is a particularly well-established cartilage and it is present in multiple genera. It is located within the laryngeal cavity just medial to the inferior base of the arytenoids embedded in a portion of the vocal cords known as the frenulum labii vocalis (Fig. 4; sensu Gaupp, 1904; Blume, 1930; Trewavas, 1933).

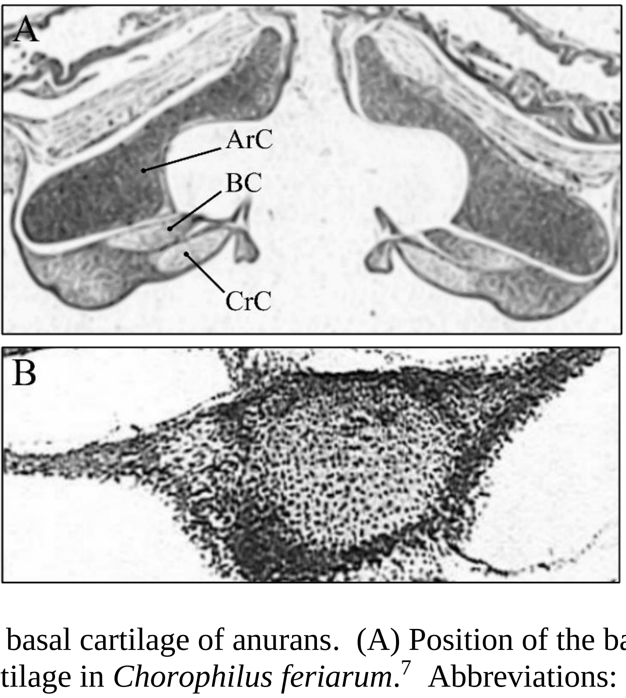

Figure 4. The laryngeal basal cartilage of anurans. (A) Position of the basal cartilage near the base of the arytenoid cartilage in *Chorophilus feriarum*.[7] Abbreviations: BC, basal cartilage. (B) Histological illustration of the basal cartilage embedded within the frenulum labii vocalis of *Phyllomedusa moschata*.[7]

Four pairs of muscles lie external to the cartilaginous framework of the anuran larynx. Each of these pairs carries out a specific function. The m. dilatator laryngis is the largest of these and is the most highly conserved across species (Trewavas, 1933). It has the role of opening the glottis to allow for air intake and expulsion. The glottis is opened laterally when the m. dilatator laryngis undergoes contraction. The m. dilatator laryngis originates on the posteromedial process of the hyoid cartilage and extends medially towards the glottal opening to insert onto the anterior edges of the arytenoid cartilages (Martin and Gans, 1972). The m. dilatator laryngis is opposed by the m. constrictor laryngis externus and m. constrictor laryngis anterior, both of which contract to close the glottis. The m. constrictor laryngis externus is the most ventral muscle of the anuran larynx. It originates from the posteromedial process of the hyoid cartilage and converges ventrally to attach to its other half near the ventral-most edge of the glottis (Trewavas, 1933). The two halves of the m. constrictor laryngis anterior originate ventrally from the hyoid cartilage and extend dorsally to meet each other at the dorsal-most end of the glottal opening (Trewavas, 1933). Control over the glottal opening is thus exerted by the m. dilatator laryngis which serves to open the glottis upon contraction, and the combined efforts of the m. constrictor laryngis externus and m. constrictor laryngis anterior which serve to close the glottis. The remaining laryngeal muscle, the m. constrictor laryngis posterior, generally originates on the cricoid cartilage and attaches to a fibrous cap on the apex of the arytenoid cartilages, adjacent to the vocal cords (Trewavas, 1933). Its most likely action is to bring the vocal cords into opposition (Schmidt, 1965; Martin and Gans, 1972).

Vocalization in frogs begins with inflation of the lungs through the process of buccal pumping (Walkowiak, 2007). Air is drawn in through the nares and allowed to accumulate within the lungs. Once sufficient volume has been achieved within the lungs, the frog will initiate vocalization by contracting the muscles within its body wall. Contraction of the body wall muscles forces air from the lungs, through the larynx, and into the buccal cavity. From there it passes through a pair of vocal slits located in the floor of the mouth and into the expanding vocal sac (Fig. 5). As air travels through the larynx, it causes the passive vibration of the vocal cords which are attached to the concave surface of the arytenoid cartilages (Walkowiak, 2007). The vibrations of the vocal cords are responsible for the production of sound. As airflow decreases, so does the amplitude of the vocal cord vibrations. This causes a decrease in the amplitude of the resulting sound.

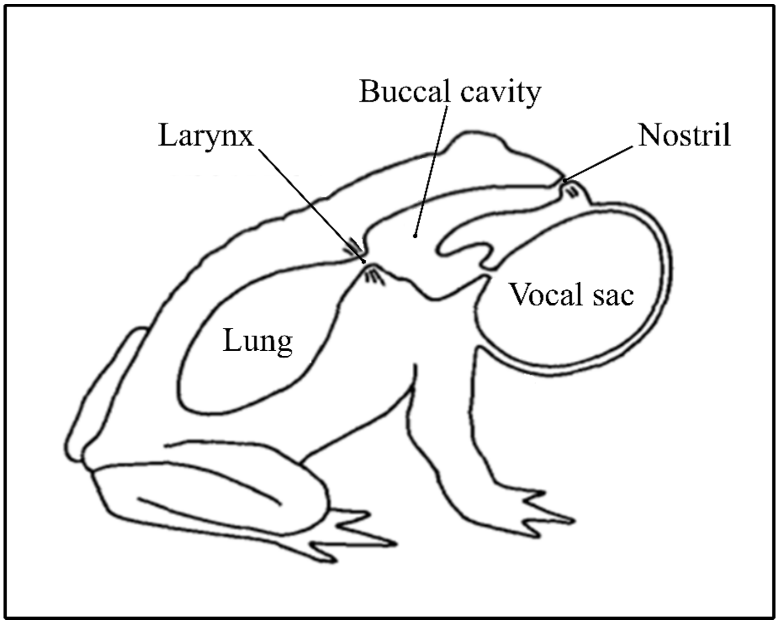

Figure 5. Schematic representation of an anuran showing the structures involved in vocalization. Air is expelled from the lungs, through the larynx and into the vocal sac, producing sound.[8]

Since frogs lack muscles inside their larynx, the laryngeal anatomy is believed to play a limiting role in frog call complexity. Positioning of the vocal cords is achieved by movement of the arytenoid cartilages (Martin, 1971; Martin and Gans, 1972), and potentially by the m.

constrictor laryngis posterior, which has several fibers that penetrate the laryngeal cavity (Gaupp, 1904; Trewavas, 1933; Schmidt, 1965). Phonation, for example, can be indirectly controlled by the movement of the arytenoid cartilages which can either be opened to facilitate airflow or closed to block sound. This mechanism has been observed in several frog species that produce pulsed calls characterized by a sharp onset of sound (Schmidt, 1965).

Innervation Patterns of the Anuran Larynx

The general morphology of the anuran larynx has been previously described in specific and comparative studies of the laryngeal skeleton and musculature (Trewavas, 1933). While the number of laryngeal muscles across organisms varies (e.g., there are six present in modern humans, while there are typically four in anura, and only three in monotremes), in general, all the muscles of the vertebrate larynx are innervated by two branches of the vagus nerve (Diogo et al., 2008).

Schneider's (1970) description of the laryngeal muscles in the anuran *Hyla arborea* summarized their innervation patterns. Two branches of the vagus nerve innervate the anuran larynx: the long and the short laryngeal nerves (Fig. 6). The long laryngeal nerve loops around the aorta and reaches the larynx, where it forms three branches. Schneider's illustrations further suggest that all the laryngeal muscles are innervated by the long nerve. Schmidt (1972) presented anatomical evidence that the short nerve strictly innervates the m. dilatator laryngis, although Schneider's 1970 illustration suggests that it also has a branch extending into the m. constrictor laryngis externus. In mammals, the superior laryngeal nerve (thought to be homologous to the anuran short nerve) only innervates one laryngeal muscle, the cricothyroid, while the recurrent laryngeal nerve loops around the aorta and innervates all the remaining laryngeal muscles in a manner similar to that of the long nerve.

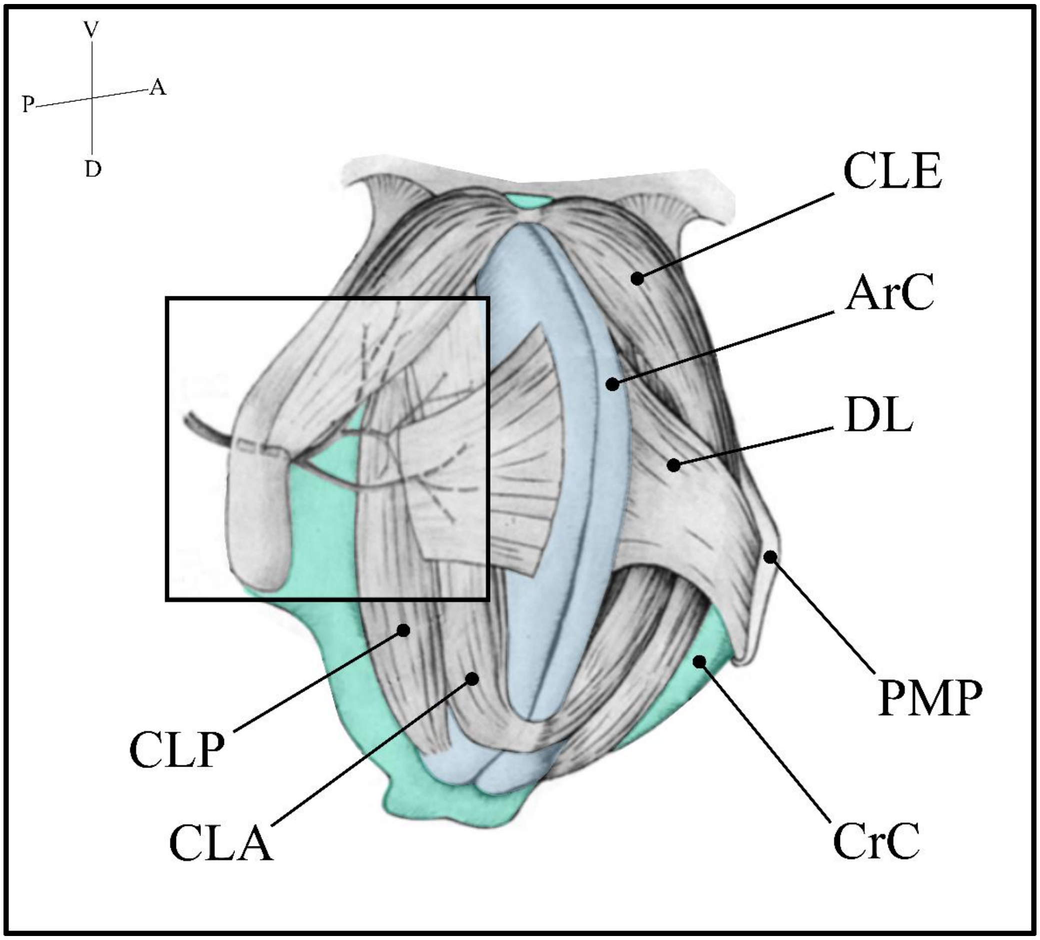

Figure 6. Representation of the anuran hyolaryngeal apparatus with depiction of the long laryngeal nerve (black box).[6] The long laryngeal nerve enters the m. dilatator laryngis, m. constrictor externus, m. constrictor anterior, and m. constrictor posterior. Directional abbreviations: A, anterior; D, dorsal; P, posterior; V, ventral.

The laryngeal muscles of mammals were not considered homologous with those of amphibians because the m. dilatator laryngis of frogs is innervated by both the long and short laryngeal nerves. It is important to determine how these differences in laryngeal innervation patterns arose between organisms with similar complex signaling abilities (i.e.; anurans and mammals). Such information could potentially clarify how the vocal control mechanisms of complex calling evolved in general.

Study Model: The Túngara Frog

The majority of anuran species utilize simple advertisement calls to attract mates for the purpose of reproduction, however, a number of species produce comparatively complex calls (Gerhardt and Huber, 2002; Wells, 2007). The túngara frog (*Engystomops pustulosus,* formerly known as *Physalaemus pustulosus*) from Central and Northern South America (Fig. 7) has complex signaling capabilities. Túngara frogs are small nocturnal amphibians, with a snout-to-vent length of 23.0-33.0 mm and an average body mass of around 2.0-3.0 g (Ryan, 1985). The mating season of the túngara frog is long compared to most, as they breed year-round with their most active months spanning the rainy season of April to December (Ryan, 1985). During this time males will come out at night to produce advertisement calls from small temporary pools of water. Females will listen to the calls while floating on the surface of the water approximately 15.0 cm away from the males (Ryan et al., 1983). The female's proximity and behavior towards the male at this range may cause increased excitement in the male, as it elicits increased call production (Ryan, 1985). Females will listen to several male vocalizations before choosing a mate. After making a decision, the female will directly approach the male within 5.0 cm and the male will initiate amplexus by grasping the female while still in the water using the thumb-like appendages on his front legs (Ryan et al., 1983). Sometime after a state of amplexus is achieved, both sexes release their gametes into the water. The male is then responsible for whipping this mixture into a floating foam nest through rhythmic mixing of the sperm and egg deposits with his hind legs (Dalgetty and Kennedy, 2010). This entire mating process lasts for approximately one hour, during which an average of 250 eggs become fertilized (Fig. 8; Ryan et al., 1983).

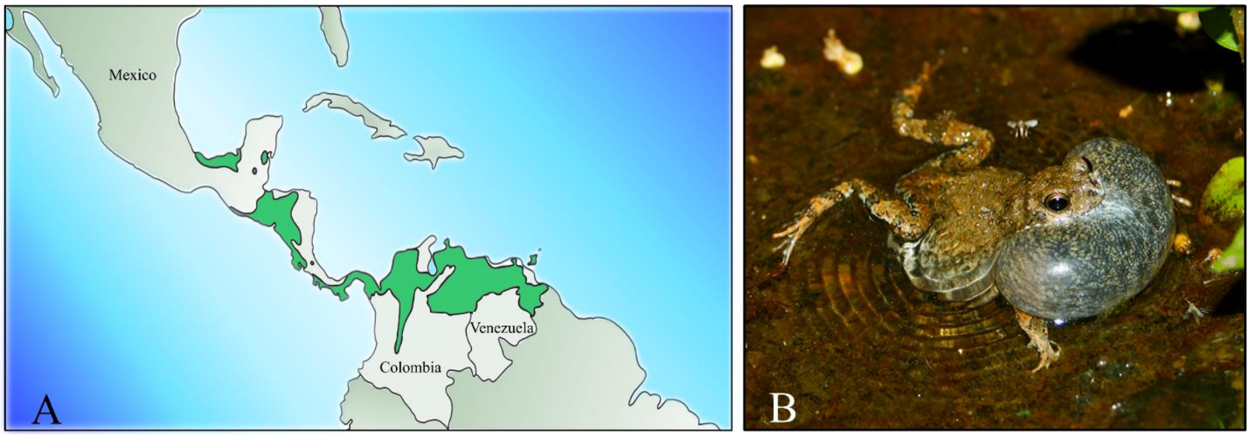

Figure 7. The neotropical túngara frog. (A) Geographic distribution highlighted in green.[9] (B) Floating male producing advertisement call.[10]

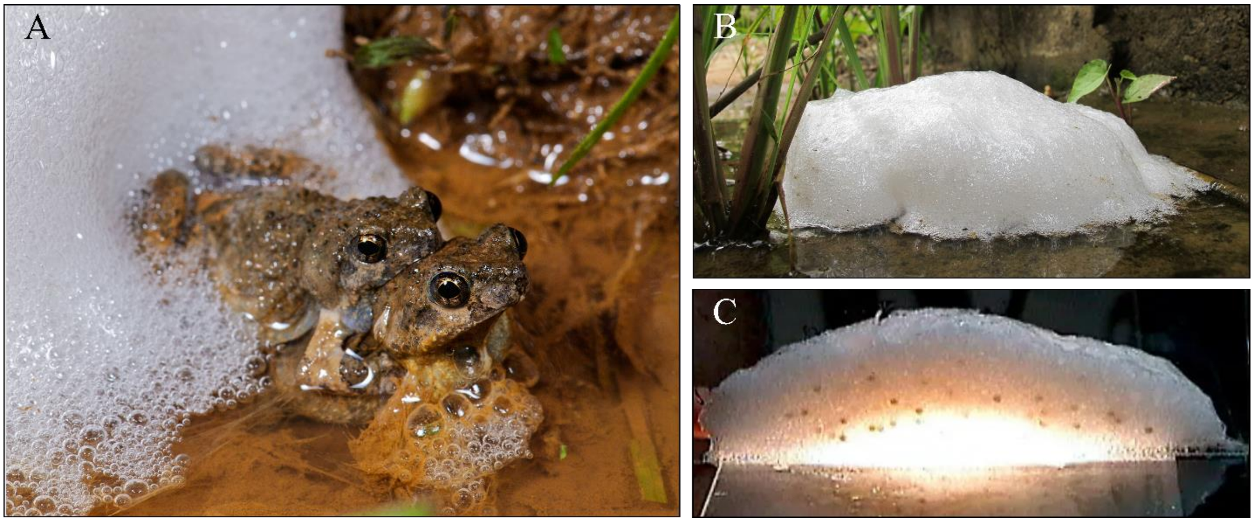

Figure 8. Reproduction in túngara frogs. (A) Male (top) and female (bottom) in amplexus. The female has released her eggs and the male uses his hindlegs to whip them into a foamy egg nest.[11] (B) A completed egg nest containing fertilized eggs.[12] (C) Light shown through an egg nest to highlight the presence of individual eggs.[13]

Túngara frogs are one of the best-known examples of complex calling in anurans. The male túngara frog produces a call composed of two components: an obligatory whine and a facultative chuck (Fig. 9; Ryan, 1985). The whine is formed by a frequency-modulated harmonic structure and mediates species recognition. The chuck is added as 0-7 short notes with half the fundamental frequency of the whine and it enhances the attractiveness of the call (Ryan, 1980, 1985). Most species in the genus *Engystomops* produce simple whine-like calls lacking chucks and exhibit only subtle differences in the size and shape of their laryngeal components (Ryan and Drewes, 1990). As such, examination of the túngara frog's vocal mechanism can assist in explaining the evolution of complexity in sexual communication.

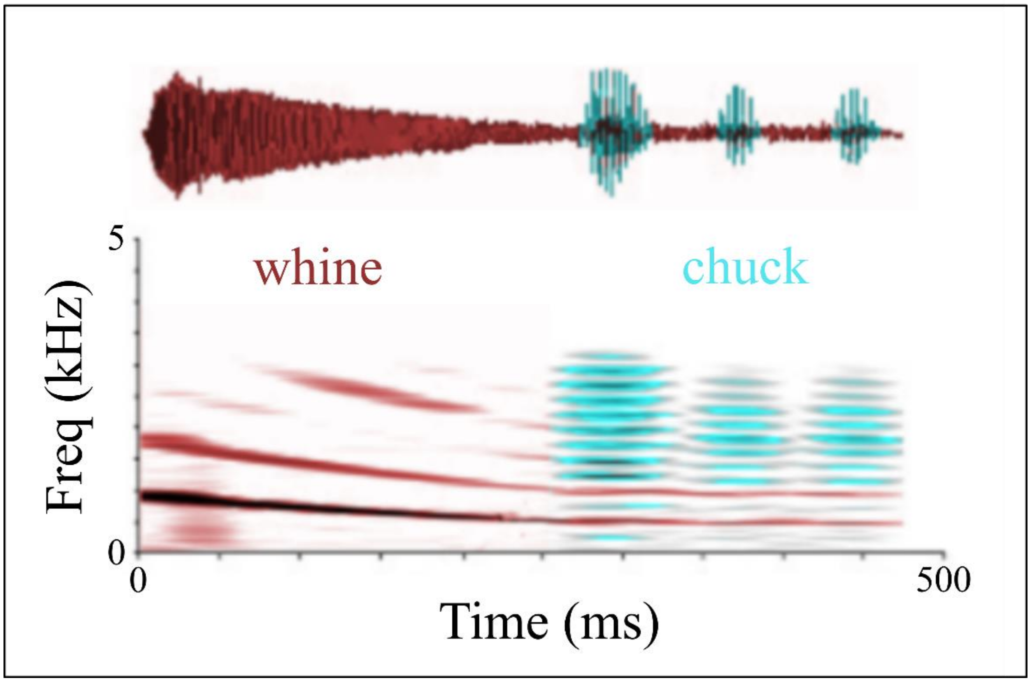

Figure 9. The complex call of the túngara frog.[14] Acoustic structure of the túngara frog call with a whine (in red) and three chucks (in blue). Oscillogram of the call showing the typical call's waveform (top) and spectrogram of the call with dark colors representing more energy at those frequencies (bottom).

A detailed morphological analysis can provide insight into the influence of laryngeal structure on call complexity. Túngara frogs have greatly expanded cricoid cartilages that give their larynges the appearance of a cartilaginous box. Two concave arytenoid cartilages articulate with the cricoid cartilage and close the glottis by touching each other medially. The vocal cords attach internally to the anteroventral and posterodorsal ends of the arytenoids. Halfway through its length, the medial edge of each vocal cord receives the attachment of a lateral extension of connective tissue known as the frenulum labii vocalis (senu Gaupp, 1904; Blume, 1930; Lagorio et al., 2019). The primary fibrous mass of the túngara frog is a midway swelling of this frenulum. The fibrous mass protrudes from the laryngeal cavity into the delicate pulmonary connections. A dorsal ligament also connects it to the wall of the larynx (Drewry et al., 1982; Gridi-Papp et al., 2006). The posterior end of each fibrous mass is round and free to vibrate over its attachments (Ryan and Drewes, 1990).

When a male túngara frog produces a chuck, its enlarged laryngeal fibrous masses vibrate passively in contact with the vocal cords. The size and attachments of the fibrous masses are determinant factors of both fibrous mass vibration and the ability of the species to produce chucks (Ryan and Drewes, 1990; Boul et al., 2007; Guerra et al., 2014). Removal of the fibrous masses abolishes the distinguishing spectral features of the chuck but has minor effects on the whine (Gridi-Papp et al., 2006; Baugh et al., 2017). The onset of the chuck has been suggested to be controlled directly through laryngeal muscle contractions (Drewry et al., 1982; Ryan and Drewes, 1990) or indirectly through manipulation of air pressure (Kime et al., 2018), but experimental assessments have not yet been conducted.

Throughout the course of this study the morphology of the túngara frog larynx was analyzed via photographic dissection, resin histology, microtomography, and three-dimensional modeling with the aim of generating precise hypotheses concerning the muscular control of call complexity. This was carried out, in part, by attempting to experimentally test previously proposed hypotheses of muscular involvement in the displacement of the fibrous masses, thus determining the onset of the chuck and regulating call complexity.

CHAPTER 2: METHODOLOGY

Animal Husbandry

The study subjects were raised in a colony consisting of 144 breeding pairs at the University of the Pacific. The colony is managed to maximize genetic diversity, having received genetic material from 32 wild individuals during the last four years. No signs of inbreeding depression have been detected since the colony's establishment. The male subjects were given the opportunity to call and breed for 2-3 nights per month throughout their lives. They matured naturally and developed normal larynges, producing both whines and chucks typical of their species. The tissues examined in this study were harvested from specimens euthanized for auditory or neurophysiological analyses under IACUC protocols 16R04 and 16R05 of the University of the Pacific.

Male Larynges

Tissue Harvesting

Larynges were excised from 10 sexually mature male túngara frogs. The specimens were euthanized in a bath of 2 g/l tricaine methanesulfonate at neutral pH, fixed in 4% formaldehyde solution for more than 24 h, and stored in 70% ethanol. The removal of each larynx was carefully conducted under a stereomicroscope to prevent damage to the laryngeal nerves which were exposed and photographed. Five of the ten larynges were further dissected and photographed whereas the other five were processed histologically.

Dissection

Dissections were conducted with the larynges submerged in deionized water following immersion in a 50% glycerol solution for approximately 1 min to prevent the tissues from floating. The origin and insertion of each laryngeal muscle was investigated with subsequent examination of the internal laryngeal structures. The dissections were performed under a Leica L2 stereo microscope and photographed using an Amscope MU500 digital camera (Fig. 10).

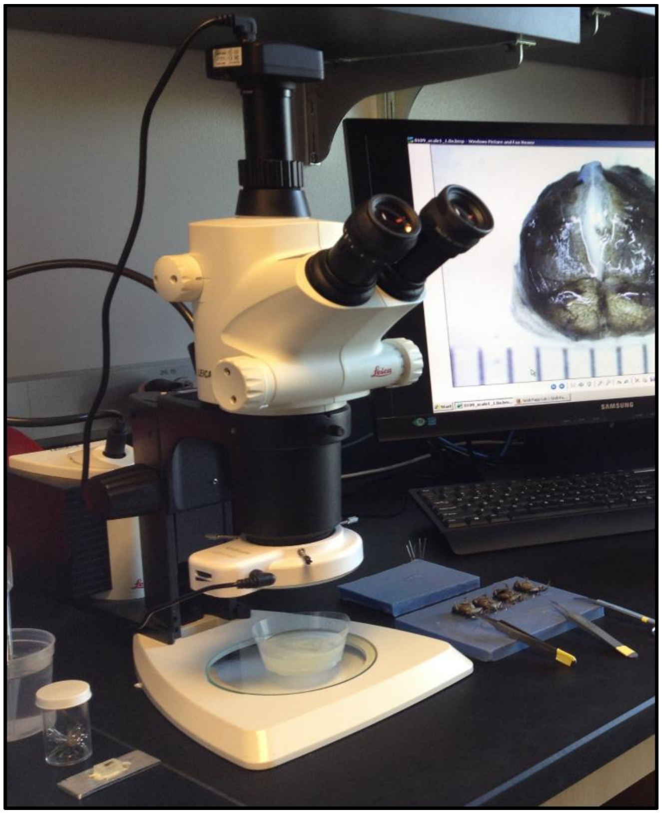

Figure 10. Dissection of adult male túngara frog larynges. The tissues were excised from 5 subjects using a set of fine dissection scissors and surgical tweezers. The larynges were dissected in a shallow cup of deionized water. A stereomicroscope equipped with a digital camera was used to monitor and document each dissection.

Histology

The remaining 5 specimens were simultaneously fixed and decalcified by immersion in a solution of 15% formaldehyde and 10% formic acid for 24 hours. The processed tissues were dehydrated in ethanol and embedded in glycol methacrylate (Technovit 7100, Kulzer). The embedded larynges were serial sectioned via rotary microtome into 5 µm-thick sections (Fig.

11). These sections were stained with a 1% toluidine blue, 1% sodium borate solution. The resulting slides were photographed with a resolution of 5867 dpi.

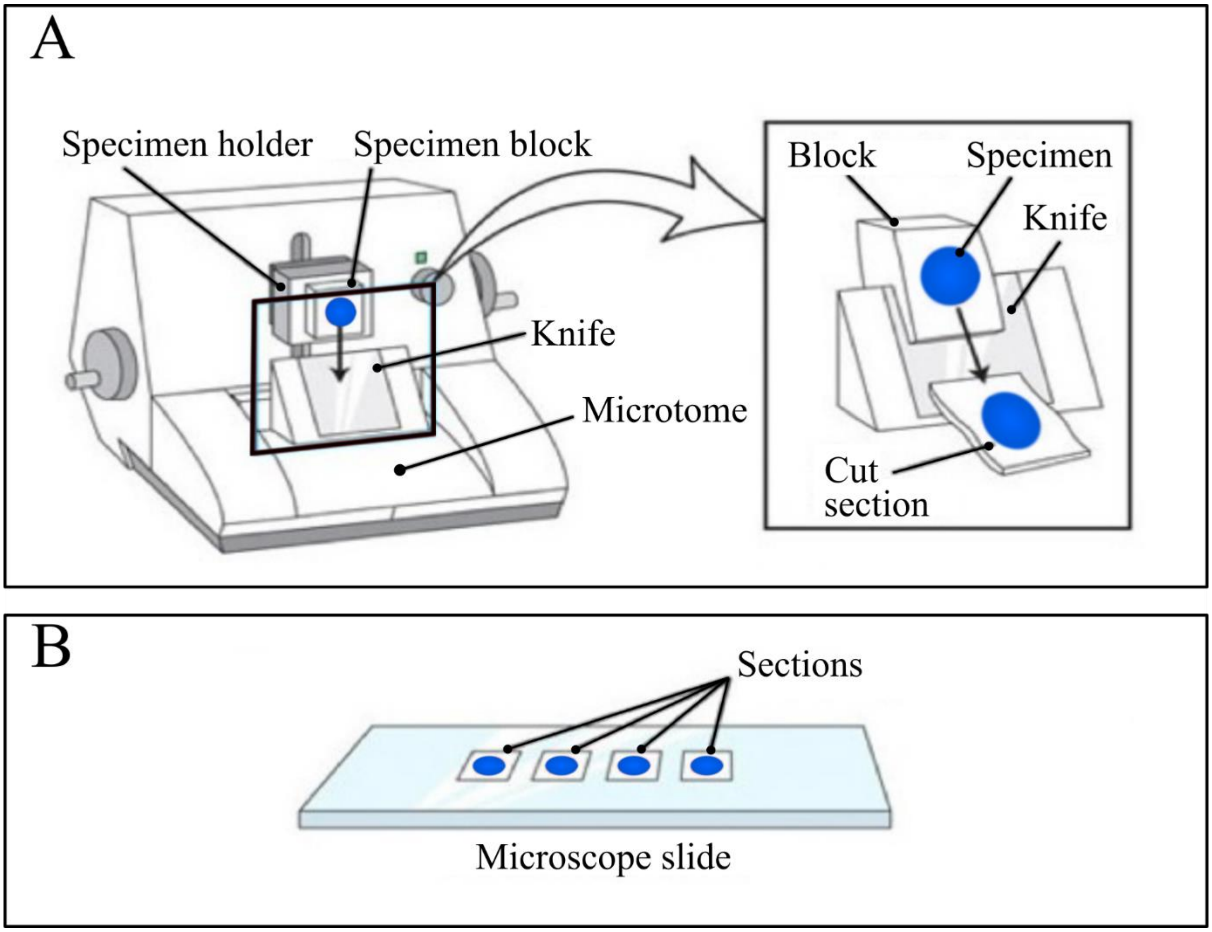

Figure 11. Artistic representation of histological methodology.[15] (A) Resin-embedded larynges were mounted in a rotary microtome for serial sectioning. As the blocked specimens moved across the knife, thin sections of tissue were produced. (B) The serial sections were slide-mounted so they could be viewed under a microscope.

3D Modeling

An image stack resulting from the microCT scan of a male túngara frog was provided by Professor Michael J. Ryan and the High-Resolution X-Ray Computed Tomography Facility of The University of Texas at Austin. The boundaries of each laryngeal structure were manually identified within each image and a digital mesh was created from the resulting outlines. This was used to generate accurate two- and three-dimensional representations of the laryngeal morphology (Fig. 12).

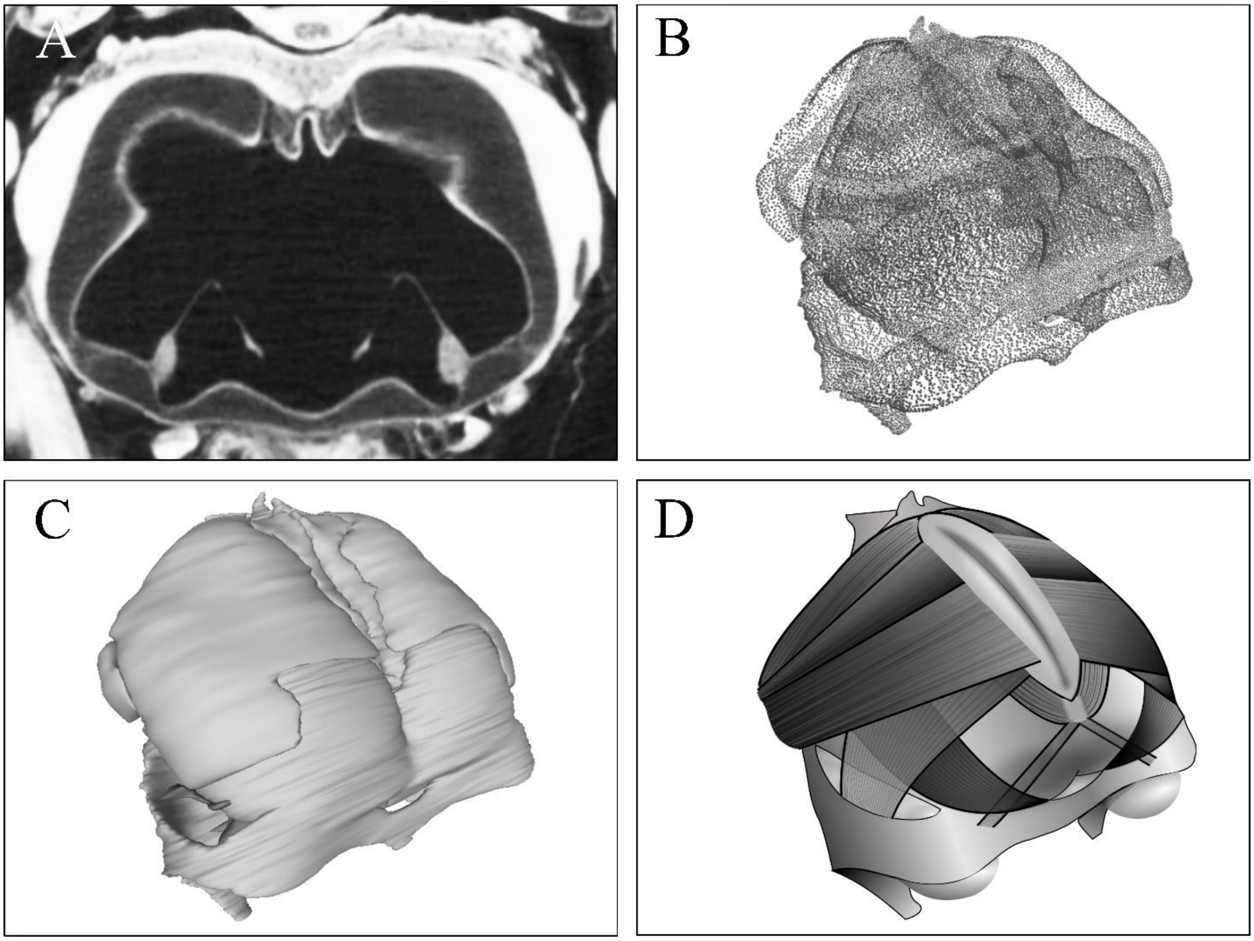

Figure 12. Production of 3D and 2D models of the túngara frog larynx. (A) Image from microCT scan of the laryngeal organ.[16] The cartilaginous framework of the larynx is shown in black with a distinct white outline. The softer muscular and membranous tissues are shades of white and gray. (B) Mesh view of the manual traces from the microCT scan that correspond to structures of interest within the larynx. (C) Skinned and smoothed mesh from B, which forms an accurate 3D representation of the túngara frog larynx. Muscles are not differentiated due to a lack of resolution in the microCT scan. (D) Artistic 2D representation of the túngara frog larynx using the 3D model as a base.

Female Larynges

Larynges from five adult females were also examined to assess sexual dimorphism in the laryngeal anatomy of túngara frogs. The tissues were excised and preserved using the same methods described previously for males. Dissections and photography supported the comparison of the position, size, and shape of the laryngeal elements with those of males.

Outgroup Larynges

The larynges of a mature male gray treefrog (*Hyla versicolor*) and a male American bullfrog (*Rana catesbeiana*) were carefully excised and photographed for outgroup comparisons of laryngeal morphology. These specimens had been previously euthanized for auditory analyses under IACUC protocol 13R08 of the University of the Pacific.

CHAPTER 3: RESULTS

Male Larynges

The m. dilatator laryngis of the male túngara frog is physically split into two major bundles of fibers: superficial and deep. These bundles differ in orientation of fibers, points of attachment, and innervation. They will be referred to as the superficial and deep m. dilatator laryngis (Fig. 13). Furthermore, the superficial m. dilatator laryngis exhibits two physically distinct bundles: anterior and posterior. These bundles, however, exhibit a convergent orientation of fibers, contiguous attachments, and shared innervation. They will be referred to as the anterior and posterior bundles of the superficial m. dilatator laryngis.

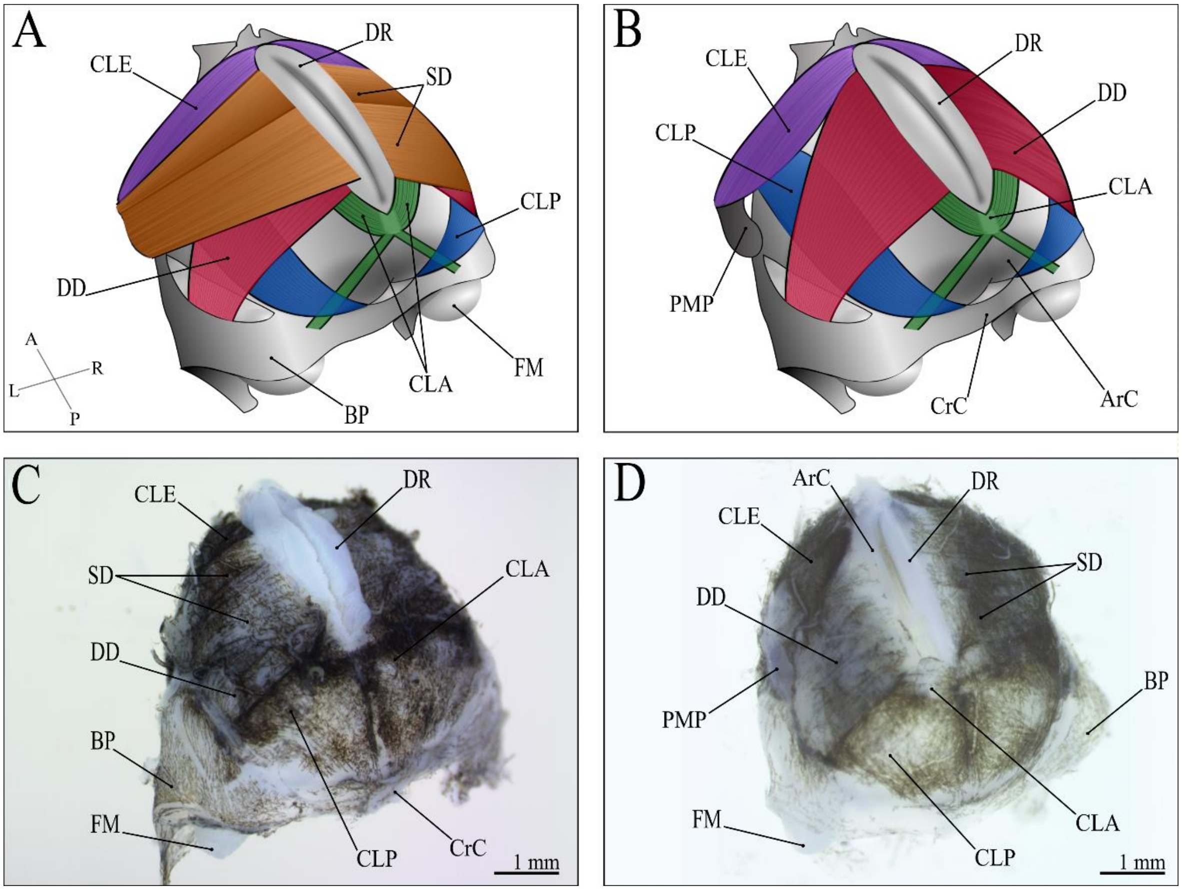

Figure 13. Laryngeal muscles in adult male túngara frogs. (A) Artistic representation of the intact laryngeal musculature. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. Orientation applies to all panels. (B) Artistic representation of the larynx with the superficial m. dilatator laryngis removed to reveal the deep m. dilatator laryngis. (C) Excised larynx with laryngeal musculature intact. (D) Excised larynx with the superficial m. dilatator laryngis removed on left side only, exposing the deep laryngeal musculature. Abbreviations: ArC, arytenoid cartilage; BP, bronchial process of the cricoid cartilage; CLA, m. constrictor laryngis anterior; CLE, m. constrictor laryngis externus; CLP, m. constrictor laryngis posterior; CrC, cricoid cartilage; DD, deep m. dilatator laryngis (m. arylabialis); DR, dense rod (covered in soft tissue); FM, fibrous mass; PMP, posteromedial process of the hyoid cartilage; SD, superficial m. dilatator laryngis (m. dilatator laryngis proper). These abbreviations apply to all subsequent figures.

The Superficial M. Dilatator Laryngis

The superficial m. dilatator laryngis is the most superficial of all the laryngeal muscles (Fig. 14). It originates from the distal end of the posteromedial process of the hyoid cartilage (Fig. 14D) and inserts along the length of a newly-discovered cartilaginous dense rod which runs adjacent to the medial edge of the arytenoid cartilage (Fig. 14C). The fibers of the anterior and posterior bundles are nearly parallel with each other with convergence toward their attachment site on the distal end of the posteromedial process of the hyoid cartilage.

The two bundles of the superficial m. dilatator laryngis differ from each other in size and insertion. The smaller anterior bundle inserts into the anterior portion of the medially located dense rod (Fig. 14B), whereas the larger posterior bundle inserts into the posterior portion of the dense rod (Fig. 14A). The insertions of the anterior and posterior bundles overlap by about 0.6 mm with the fibers of the posterior bundle being superficial to those of the anterior bundle. Both bundles of the superficial m. dilatator laryngis originate laterally from the distal end of the hyoid's posteromedial process.

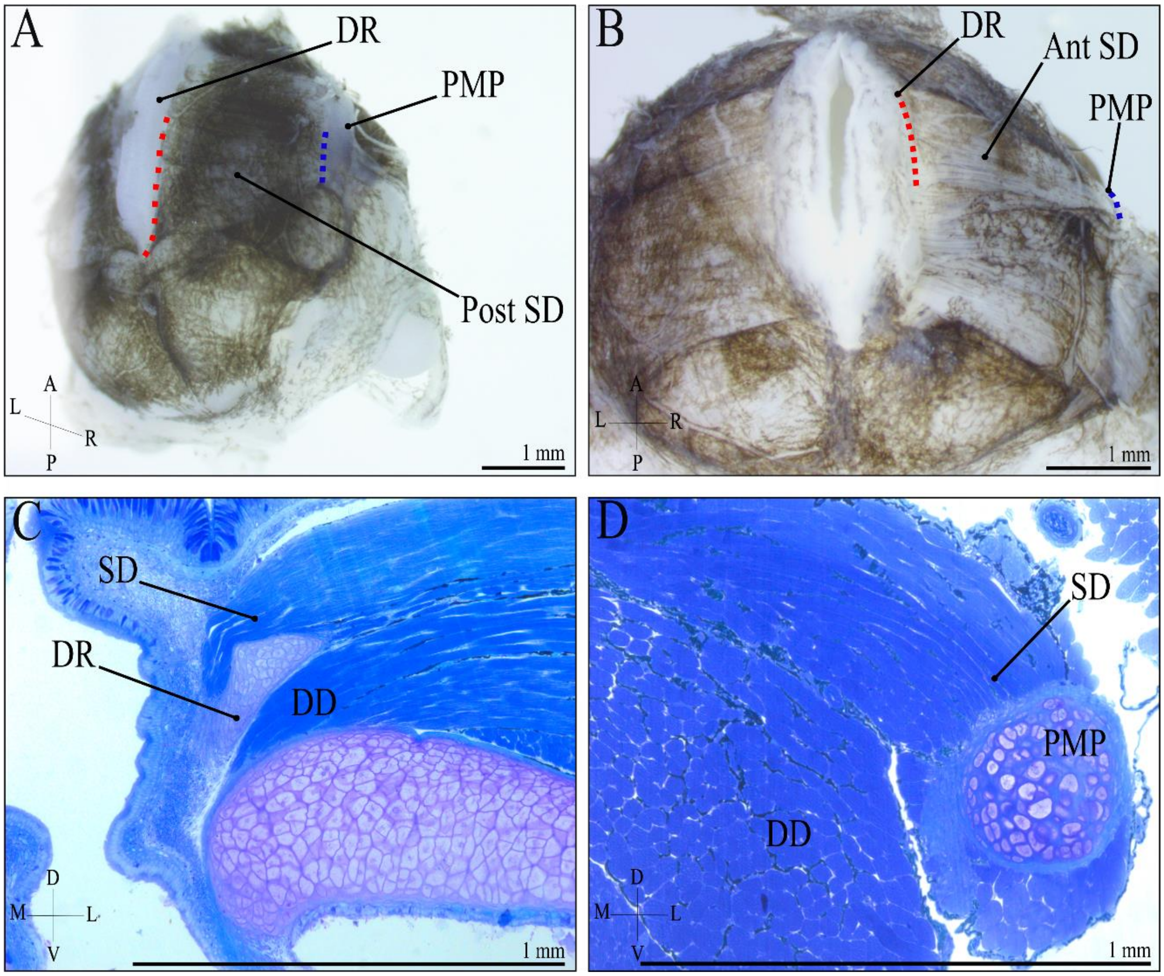

Figure 14. Structure of the adult male túngara frog's superficial m. dilatator laryngis (m. dilatator laryngis proper). (A) The posterior bundle of the superficial m. dilatator laryngis originates from the posteromedial process of the hyoid cartilage (blue dotted line) and inserts into the dense rod (red dotted line). Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (B) The anterior bundle of the superficial m. dilatator laryngis originates at the posteromedial process of the hyoid cartilage (blue dotted line) and inserts medially into the dense rod (red dotted line). The posterior bundle of the superficial m. dilatator laryngis has been retracted laterally. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (C) Transverse histological cross section of the larynx showing the insertion of the superficial m. dilatator laryngis into the dense rod. Directional abbreviations: D, dorsal; L, lateral; M, medial; V, ventral. Same orientation applies to the next panel. (D) Transverse histological cross section of the larynx showing the origin of the superficial m. dilatator laryngis on the posteromedial process of the hyoid cartilage.

The Deep M. Dilatator Laryngis

The deep m. dilatator laryngis runs at an oblique angle of about 24.5 ± 3.4 degrees (n=5) and is deep to both bundles of the superficial m. dilatator laryngis (Fig. 15). Its broad medial attachment spans part of the arytenoid cartilage's medial border immediately deep to the dense rod (Fig. 15C). The lateral attachment is located more posteriorly. The deep m. dilatator laryngis extends from its medial attachment through a fissure between the arytenoid cartilage and the bronchial process of the cricoid cartilage (Fig. 15D). Inside the laryngeal cavity the tendon of the deep m. dilatator laryngis inserts into the frenulum labii vocalis, which extends from the lateral edge of the arytenoid cartilage to the medial free edge of the vocal cords (Fig. 16A). This frenulum contains a previously undiscovered basal cartilage and supports the fibrous mass, the latter of which is embedded approximately halfway through its length and attaches to both the cricoid cartilage and the vocal cords (Fig. 16B).

Innervation

The larynx of the male túngara frog is innervated by the short and long laryngeal branches of the vagus nerve (superior and recurrent laryngeal nerves in mammals). Dissections revealed that the long laryngeal nerve branches into all laryngeal muscles except the deep m. dilatator laryngis. This muscle is solely innervated by the short laryngeal nerve (Fig. 17). The long laryngeal nerve reaches the larynx posterolaterally and loops around the aorta before extending between the cricoid cartilage and the posteromedial process of the hyoid cartilage. It passes the cricoid cartilage at a position anterior to the distal end of the hyoid process. As it makes contact with the cricoid cartilage, the long laryngeal nerve forms branches that innervate both bundles of the superficial m. dilatator laryngis, the m. constrictor laryngis anterior, m. constrictor laryngis posterior, and the m. constrictor laryngis externus (Fig. 17B).

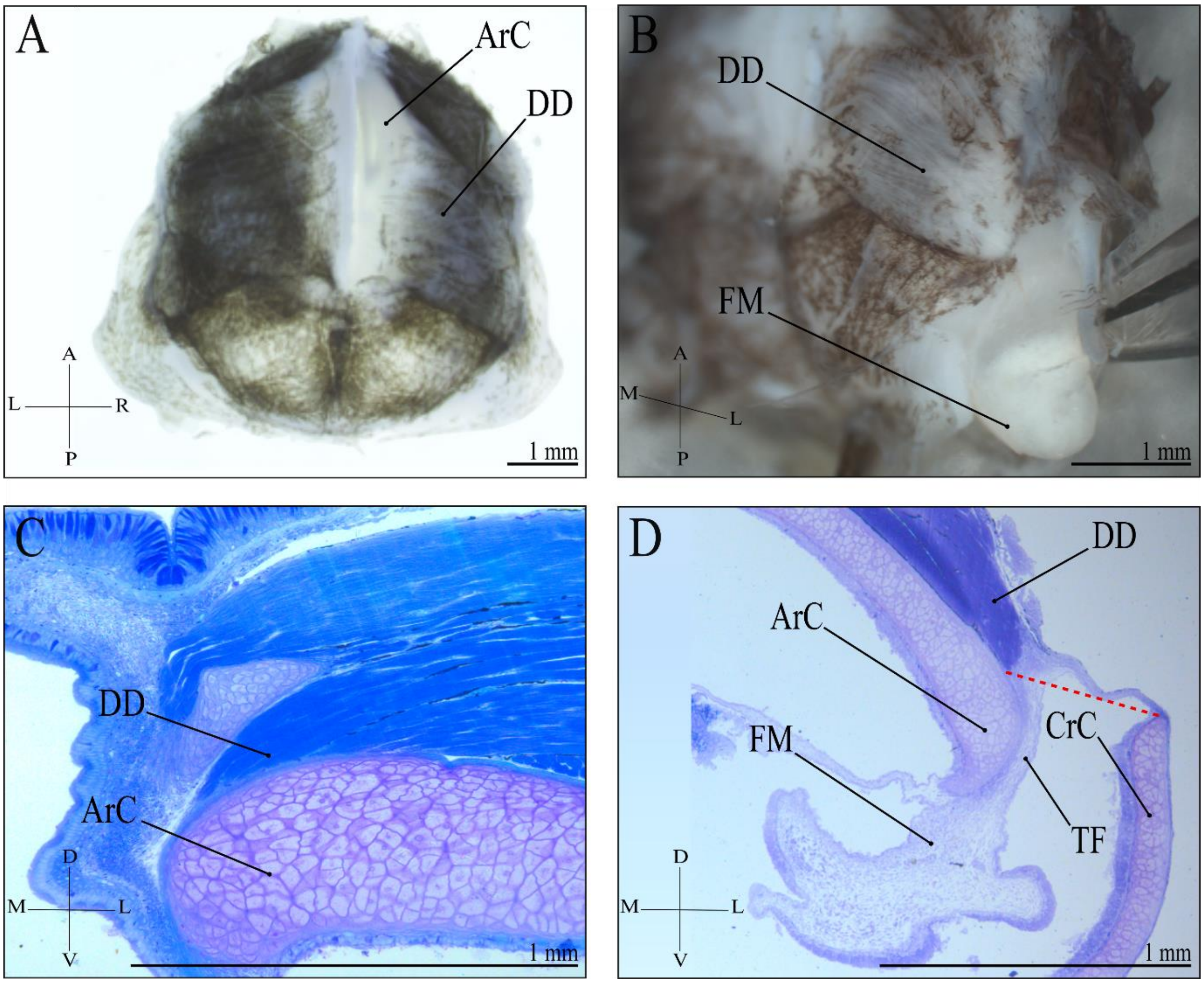

Figure 15. Structure of the male túngara frog's deep m. dilatator laryngis (m. arylabialis). The superficial m. dilatator laryngis was removed on the right side in panels A and B to expose the deep m. dilatator laryngis. (A) Medial attachment of the right deep m. dilatator laryngis to the arytenoid cartilage. Muscle fibers run deep to the dense rod (removed) to converge into the apex of the arytenoid cartilage. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (B) Attachment of the deep m. dilatator laryngis to the lateral attachment of the fibrous mass. Posterolateral view of the larynx with the bronchial process of the cricoid cartilage removed. Directional abbreviations: A, anterior; L, lateral; M, medial; P, posterior. (C) Transverse histological cross section of the deep m. dilatator laryngis' medial attachment to the apex of the arytenoid cartilage, deep to the dense rod. Directional abbreviations: D, dorsal; L, lateral; M, medial; V, ventral. Same orientation applies to the next panel. (D) Transverse histological cross section of tendon fibers (TF) from the deep m. dilatator laryngis passing through the crico-arytenoid fissure (red dotted line) in the laryngeal cartilaginous framework to converge within the lateral attachment of the fibrous mass.

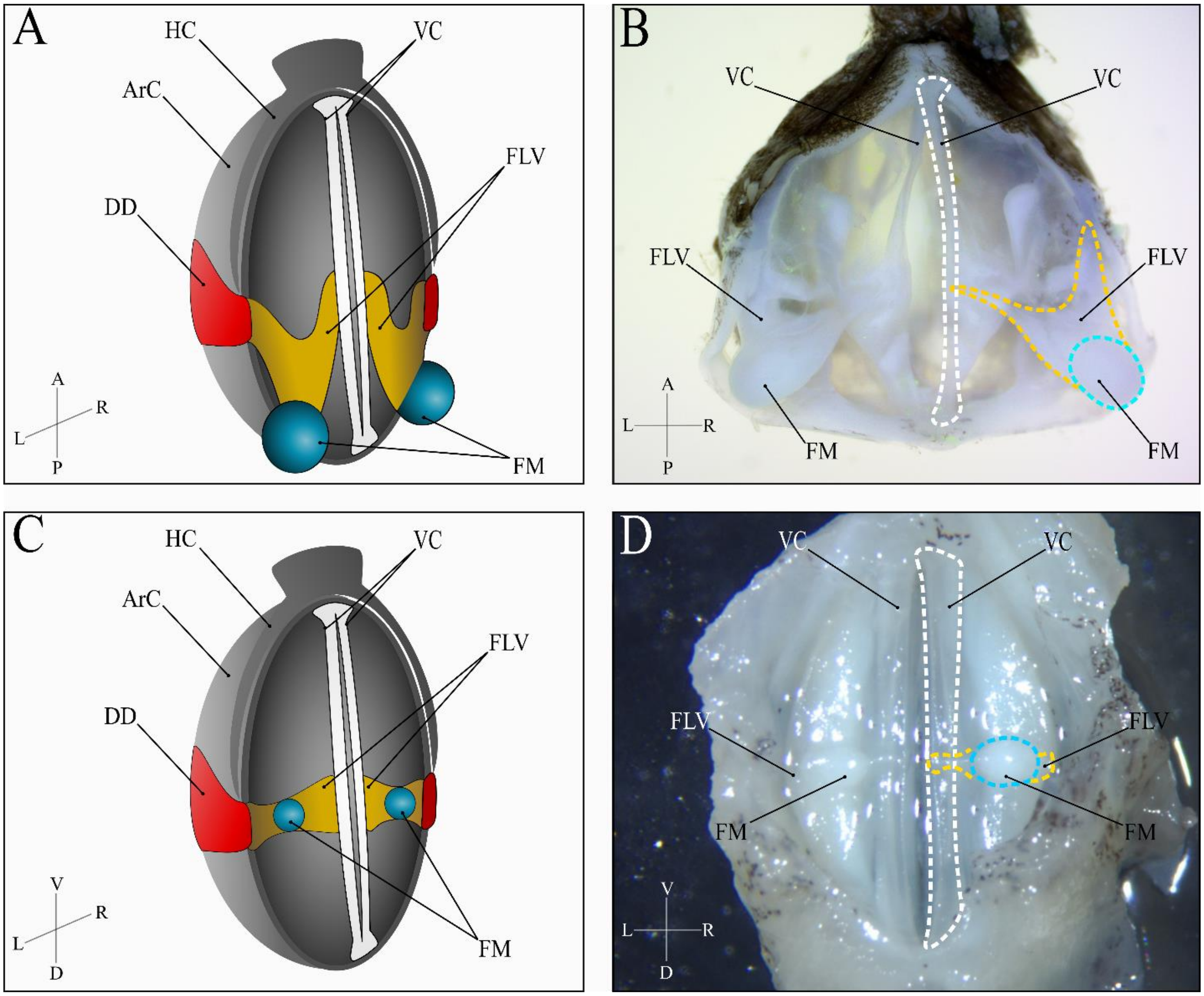

Figure 16. Comparison between the fibrous mass attachments of anuran larynges. The cricoid cartilage and pulmonary connections have been omitted for clarity. (A) Simplified artistic representation of a male *Engystomops pustulosus* larynx in the ventrolateral view. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (B) Excised larynx of *E. pustulosus* in the ventral view. Structures of interest have been highlighted on the right side for comparison. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (C) Artistic representation of a male *Hyla versicolor* larynx in posterolateral view. Directional abbreviations: D, dorsal; L, left; R, right; V, ventral. (D) Excised larynx of *H. versicolor* in posterior view. Structures of interest have been highlighted on the right side for comparison. Directional abbreviations: D, dorsal; L, left; R, right; V, ventral. Abbreviations: FLV, frenulum labii vocalis; HC, hyoid cartilage; VC, vocal cords. These abbreviations apply to all subsequent figures. Color coding: Blue, fibrous mass; gray, arytenoid and hyoid cartilages; red, deep m. dilatator laryngis; white, free edge of the vocal cords; yellow, frenulum labii vocalis.

The short laryngeal nerve reaches the larynx anteriorly, at a position medial to the distal end of the hyoid cartilage's posteromedial process. This nerve extends between the anterior and posterior bundles of the superficial m. dilatator laryngis (Fig. 17C) to innervate the deep m. dilatator laryngis exclusively (Fig. 17D).

The action of the deep m. dilatator laryngis is currently being examined as part of a study (unpublished) on the individual and combined actions of all the laryngeal muscles through stimulation of the laryngeal nerve branches using suction electrodes. Preliminary results appear to confirm the innervation patterns observed in this study: the short laryngeal nerve stimulates the deep m. dilatator laryngis exclusively, whereas the remaining laryngeal muscles are stimulated by the long laryngeal nerve.

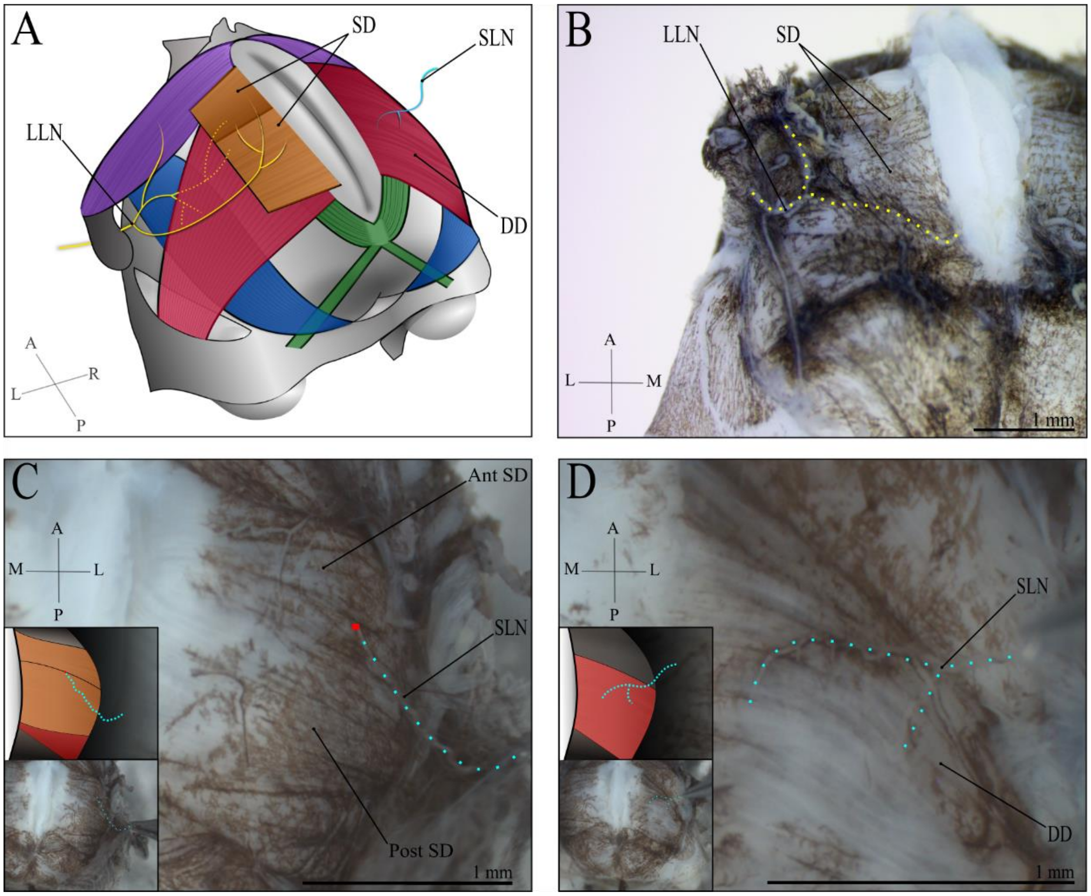

Figure 17. Laryngeal muscle innervation patterns of the male túngara frog. (A) Artistic representation of the long (LLN; yellow) and short (SLN; blue) laryngeal nerves. The portion of the long laryngeal nerve innervating the m. constrictors anterior and posterior (yellow dotted line) runs deep to the superficial and deep portions of the m. dilatator laryngis, a portion of which was removed. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (B) Long laryngeal nerve (yellow dotted line) shown innervating both the anterior and posterior bundles of the superficial m. dilatator laryngis. Directional abbreviations: A, anterior; L, lateral; M, medial; P, posterior. (C) Short laryngeal nerve (blue dotted line) as it approaches the larynx to pass between the anterior and posterior bundles of the superficial m. dilatator laryngis (red dot). Inserts: schematic representation of the short laryngeal nerve extending between the bundles of the superficial m. dilatator laryngis (top); and a less magnified view of the short laryngeal nerve as it reaches the larynx (bottom). Directional abbreviations: A, anterior; L, lateral; M, medial; P, posterior. Orientation applies to the remaining panel. (D) Short laryngeal nerve (blue dotted line) as it innervates the deep m. dilatator laryngis. Both bundles of the superficial m. dilatator laryngis have been removed. Inserts: schematic representation of the short laryngeal nerve innervating the deep m. dilatator laryngis (top); and a less magnified view of the short laryngeal nerve innervating the deep m. dilatator laryngis (bottom).

Female Larynges

The m. dilatator laryngis of the female túngara frog has a similar morphology to that of the male (Fig. 18A). It is divided into two distinct bundles: superficial and deep. As in males, the superficial portion of the m. dilatator laryngis flanks the glottal opening medially and has a lateral attachment on the distal end of the hyoid cartilage's posteromedial process (Fig. 18B). Unlike the male condition, however, the superficial portion of the muscle does not seem to be separated into anterior and posterior bundles. The deep bundle of the female m. dilatator laryngis lies immediately deep to the superficial portion. Its medial end attaches to the medial edge of the arytenoid cartilage near the glottal opening. Its lateral end is located more posteriorly on the fissure between the arytenoid cartilage and the bronchial process of the cricoid cartilage (Fig. 18C). As in males, the tendon of the deep m. dilatator laryngis appears to enter the laryngeal cavity through this fissure and attach to the frenulum labii vocalis but the small size of the structures prevented verification beyond doubt through dissection. The frenulum labii vocalis of female túngara frogs receives attachments from both the vocal cords and the fibrous mass (Fig. 18D).

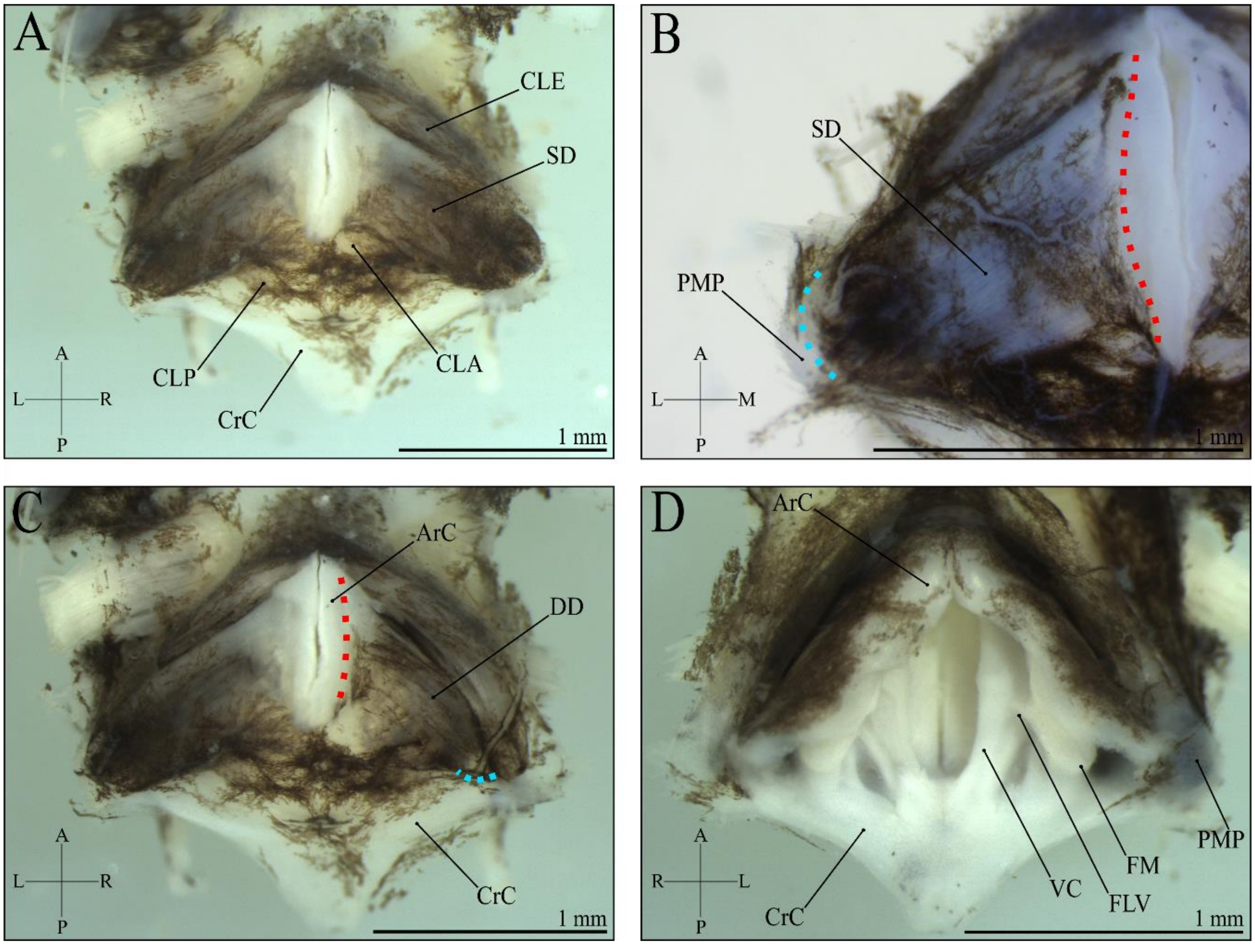

Figure 18. Overview of laryngeal structures in adult female túngara frogs. (A) Dorsal view of an excised larynx with laryngeal musculature intact. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (B) Medial (red dotted line) and lateral (blue dotted line) ends of the superficial m. dilatator laryngis near the glottal opening and the posteromedial process of the hyoid cartilage, respectively. Directional abbreviations: A, anterior; L, lateral; M, medial; P, posterior. (C) Medial (red dotted line) and lateral (blue dotted line) ends of the deep m. dilatator laryngis. The medial end is deep to the superficial m. dilatator laryngis (removed on right side) on the apex of the arytenoid cartilage. The lateral end of the muscle is on the fissure between the arytenoid cartilage and the bronchial process of the cricoid cartilage, possibly extending to the frenulum labii vocalis or to the cricoid cartilage. Directional abbreviations: A, anterior; L, left; P, posterior; R, right. (D) Ventral view of an excised larynx with the cricoid cartilage removed to expose the contents of the laryngeal cavity. Directional abbreviations: A, anterior; L, left; P, posterior; R, right.

Outgroup Larynges

Dissection of outgroup anuran species confirmed aspects of laryngeal morphology described in the literature. The m. dilatator laryngis of American bullfrogs exhibits two lateral origins, an anterior origin on the posteromedial process of the hyoid cartilage and a posterior origin on the lateral process of the cricoid cartilage. Within the laryngeal cavity of a male gray treefrog, the frenulum labii vocalis was observed attaching directly to the medial free edge of the vocal cord itself (Fig. 16D) and extending laterally to attach to the cricoid cartilage ring. Embedded halfway along its length was an enlarged fibrous mass (Fig. 16C).

CHAPTER 4: DISCUSSION

The Superficial and Deep Bundles of the M. Dilatator Laryngis

The morphology of the anuran larynx has been described in studies of the laryngeal skeleton (Gaupp, 1904; Blume, 1930; Trewavas, 1933), musculature (Henle, 1839; Wilder, 1896; Göppert, 1895; Gaupp, 1904; Krause, 1920; Trewavas, 1933), and innervation (Wahl, 1969; Schneider, 1970). In view of the scarcity of structural studies on other species within the genus and family of the túngara frog, their laryngeal structure will be discussed in comparison with the literature of other anurans. The m. dilatator laryngis is well conserved and is the least variable of the laryngeal muscles (Wilder, 1896). It is the largest laryngeal muscle and it has been described as originating from the posteromedial process of the hyoid cartilage and inserting into the medial border of the arytenoid cartilage. Features occasionally noted on the m. dilatator laryngis include: 1) A physical separation of muscle fibers into distinct bundles and slips of muscle (Gaupp, 1904); 2) A secondary lateral origin on the cricoid cartilage (Schmidt, 1972); 3) A medial insertion into an apical cartilage (sensu Wilder, 1896) as opposed to the arytenoid cartilage (Trewavas, 1933); and 4) A double innervation by the long and short laryngeal nerves (Schneider, 1970).

Physical Separation

The m. dilatator laryngis of amphibians has been divided into a variable number of muscle fiber bundles (Trewavas, 1933). Early descriptions of subdivisions within the m. dilatator laryngis included accessory slips of muscle with lateral attachments to the hyoid and cricoid cartilages (Göppert, 1895, 1898; Wilder, 1896). Gaupp (1904) later described in *Rana esculenta* (now *Pelophylax kl. esculentus*) both a superficial and a deep bundle of the m. dilatator laryngis, the latter of which was divided into crico-arytenoid and hyo-cricoid slips. These two bundles of the m. dilatator laryngis were commonly arranged in close proximity to one another but could have divergent orientations of muscle fibers (Gaupp, 1904; Wahl, 1969).

Comparisons can be drawn between the patterns of muscle fiber subdivision within the m. dilatator laryngis of túngara frogs and those of ranids as described by Gaupp (1904). In both instances, the m. dilatator laryngis has a superficial and a deep portion which clearly diverge in orientation of muscle fibers. In contrast, it is the superficial m. dilatator laryngis in túngara frogs, as opposed to the deep portion in ranids, that is separated into distinct bundles. Additionally, there appears to be a much more pronounced physical separation between the superficial and deep m. dilatator laryngis of túngara frogs than observed between the superficial and deep portions of the m. dilatator laryngis in ranids.

Distinct Origins

Multiple points of origin at the lateral attachment of the m. dilatator laryngis have been identified in the literature. The crico-arytenoid and hyo-cricoid slips of the m. dilatator laryngis described by Gaupp (1904) on *P. kl. esculentus* were named for their distinct lateral origins on the cartilaginous framework of the larynx. This study’s dissection of a male American bullfrog (*Rana catesbeiana*) confirmed two lateral origins for the m. dilatator laryngis: the distal edge of the posteromedial process of the hyoid cartilage and the lateral process of the cricoid cartilage. An equivalent secondary attachment of the m. dilatator laryngis to a projection of the cricoid cartilage, illustrated as anterior to the main attachment of the muscle on the posteromedial process of the hyoid cartilage, has also been observed in North American leopard frogs (*Rana pipiens*, Schmidt, 1972).

In túngara frogs, the superficial and deep m. dilatator laryngis have distinct lateral attachments. The attachment of the deep m. dilatator laryngis to the frenulum surrounding the fibrous mass may correspond to the secondary attachment of the m. dilatator laryngis to the cricoid cartilage described by Schmidt (1972) in leopard frogs. The positions of these secondary lateral attachments are similar in these species; however, the secondary attachment is anterior to the primary attachment in leopard frogs whereas it is posterior in the túngara frog. This difference may be explained by the fact that the cricoid cartilage of túngara frogs and their close relatives is greatly expanded posteriorly, forming a cartilaginous box that is fused to the arytenoid cartilage (Trewavas, 1933). This expansion may have shifted the location of the secondary attachment posteriorly.

Distinct Insertions

Multiple attachments have also been described at the insertion of the m. dilatator laryngis along the midline of the larynx. In ranids, an apical cartilage is found along the medial edges of the arytenoid cartilages and it receives part of the insertion of the m. dilatator laryngis (Trewavas, 1933). In the leptodactylid *Physalaemus cuvieri* (closely related to the túngara frog), a rod of dense cellular connective tissue proper extends along the medial edge of the arytenoid cartilage and the two share the attachment of the m. dilatator laryngis (Trewavas, 1933).

The dense rod of *P. cuvieri* is also observed in túngara frogs, but in a chondrified form. This cartilaginous dense rod receives the entire attachment of the superficial m. dilatator laryngis. This completely separates the medial insertion of the superficial m. dilatator laryngis from that of the deep m. dilatator laryngis into the arytenoid cartilage.

Innervation

The laryngeal muscles of anurans have been described as having a shared innervation pattern involving the long and short branches of the vagus nerve (Walkowiak, 2007). Branches of the long nerve are thought to innervate all four pairs of laryngeal muscles (Schneider, 1970) while anatomical evidence suggests that the short nerve strictly innervates the m. dilatator laryngis (Gaupp, 1904; Wahl, 1969). This arrangement was confirmed by nerve stimulation experiments conducted by Schmidt (1972). This general innervation pattern changes, however, when the superficial and deep bundles of the m. dilatator laryngis are considered separately. In túngara frogs, the long nerve forms branches which innervate all laryngeal muscles except the deep m. dilatator laryngis. This suggests that the long nerve does not control the entire laryngeal musculature as previously suggested for other anurans. Instead, contraction of the deep m. dilatator laryngis is controlled by the short nerve exclusively.

The M. Arylabialis

This study proposes the recognition of the deep m. dilatator laryngis in túngara frogs as an independent muscle, separate from the superficial m. dilatator laryngis. This is based on 1) Complete physical separation with different orientations of muscle fibers; 2) Origins from distinct laryngeal structures without overlap; 3) Insertions into distinct cartilages without overlap; and 4) Mutually exclusive innervation. This muscle was first named by Gaupp (1904) as the m. arylabialis. This study therefore recognizes five laryngeal muscles in the túngara frog: the m. dilatator laryngis, m. constrictor laryngis externus, m. constrictor laryngis anterior, m. constrictor laryngis posterior, and m. arylabialis.

The m. arylabialis was first encountered by Gaupp (1904) in *P. kl. esculentus* as a deep crico-arytenoid slip of the m. dilatator laryngis. The muscle had distinct attachments from those of the m. dilatator laryngis and was physically separated from it. Gaupp (1904) described a tendon extending from the m. arylabialis into the frenulum labii vocalis. Krause (1920) also noted the deep-lying m. arylabialis in *P. kl. esculentus* and *Rana temporaria* (formerly *Rana fusca*), where a tendon from the muscle passed through a membranous wall between the arytenoid and cricoid cartilages near the hyoid-cricoid junction. Trewavas (1933) similarly found superficial and deep bundles of the m. dilatator laryngis in multiple species of *Rana*, noting that these bundles appear to be neither confined to nor universally present within this genus. She mentioned the m. arylabialis but considered it a portion of the m. dilatator laryngis due to a lack of evidence for functional separation. The recurring lack of a complete physical separation in combination with an approximate alignment of fibers between the m. dilatator laryngis and the m. arylabialis explains why previous studies have considered them a single muscle with multiple attachments and double innervation as opposed to distinct muscles in close proximity. The innervation of the m. arylabialis seems to not have been examined at the time of its initial description, which could have supported its separation from the m. dilatator laryngis in subsequent studies.

The abundance of evidence for a split in the structure of the m. dilatator laryngis in ranids indicates that the m. arylabialis is not a specialization restricted to túngara frogs or their family. There was no mention found of a split structure in treefrogs (Hylidae) or toads (Bufonidae) but the morphology of the m. dilatator laryngis has received little attention in these groups (Martin, 1971; Eichelberg and Schneider, 1973; McClelland et al., 1996). Broad comparative studies will be necessary to elucidate how ubiquitous this muscle is in anurans and what function it may serve.

A potential role for the m. arylabialis has been proposed based on morphological evidence (Gaupp, 1904; Blume, 1930; Wahl, 1969). Gaupp (1904) noticed that some tendon fibers originating from the crico-arytenoid slip of the deep portion of the m. dilatator laryngis extended through the space between the cricoid and arytenoid cartilages to enter the larynx. Inside the laryngeal cavity, the tendon of the m. dilatator laryngis was observed to extend medially until converging with the frenulum labii vocalis, with a portion of its fibers inserting into the vocal cords directly (Gaupp, 1904). This was observed again by Blume (1930), who noted that the deep portion of the m. dilatator laryngis penetrated into the larynx to radiate through the frenulum labii vocalis and into the vocal cord itself. Gaupp (1904) suggested that contraction of the m. dilatator laryngis could add tension within the vocal cords altering the frequency of the sound. The deep portion of the m. dilatator laryngis was therefore suggested to play a role in anuran vocalization. An equivalent arrangement in the túngara frog was observed in this study. The tendon of the m. arylabialis extends into the fibers that make up the fibrous mass' lateral attachment to the cartilaginous framework of the larynx. This lateral attachment stems off the base of the fibrous mass and is continuous with the frenulum labii vocalis. This configuration is consistent with the proposed role of vocal control. It indicates, however, that muscular action may adjust the position rather than the tension of the vocal cords. Pulling on the frenulum labii vocalis by the m. arylabialis should abduct the vocal cords and laterally displace the fibrous mass. This could provide a mechanism to oppose the action of the m. constrictor laryngis posterior as an adductor of the vocal cords (Schmidt, 1972). This study therefore speculates that the m. constrictor laryngis posterior and m. arylabialis of anurans may control the movement of the vocal cords, whereas the m. dilatator laryngis, m. constrictor laryngis externus and m. constrictor laryngis anterior likely control the movement of the arytenoid cartilages.

The non-vocal larynx of female túngara frogs is greatly reduced compared to those seen in males, with all the laryngeal structures appearing to be scaled down in size proportionally (Guerra et al., 2014). The m. arylabialis is present and does not appear to be more or less reduced in females than other laryngeal muscles or structures dedicated to calling, such as the vocal cords and the fibrous masses. Comparison of the larynges between male and female túngara frogs does not, therefore, confirm or dismiss the potential function of the m. arylabialis in the movement of the vocal cords in males.

The relationship described here between the vocal cords and the fibrous mass in túngara frogs qualitatively matches the structure observed in this study's dissection of a male gray treefrog (*Hyla versicolor*). The gray treefrog has its modest fibrous mass embedded near the free edge of the vocal cord, forming a thickening in the frenulum labii vocalis (Fig. 16C). This frenulum extends from the free edge of the vocal cord towards the vocal cord's attachment on the cricoid cartilage (Fig. 16D). This configuration produces a close match to what was observed in ranids by Gaupp (1904), in which the frenulum connects the tendon of the m. dilatator laryngis to the vocal cords.

Based on its ideal positioning, the m. arylabialis of túngara frogs may position the fibrous mass in addition to the vocal cords. Vibration of the fibrous mass is required for production of the facultative component of the male's advertisement call (Drewry et al., 1982; Ryan and Drewes, 1990; Gridi-Papp et al., 2006; Kime et al., 2013). Regulating the position of the fibrous mass in relation to the pulmonary airflow could allow the animal to control its passive vibration and the onset of the chuck. Stretching the fibrous mass across its attachments has been suggested to influence its passive vibration (Ryan and Drewes, 1990). Preliminary results of nerve stimulation in the Gridi-Papp Lab (unpublished) support this context by indicating that contraction of the m. arylabialis causes lateral displacement of the fibrous mass. Voluntary displacement of the fibrous mass through contraction of the m. arylabialis may therefore form the mechanism that controls the onset of the facultative portion of the túngara frog's complex call. Further experimentation is required, however, to confirm this action of the m. arylabialis in túngara frogs.

The current study sheds new light on the homology between anamniote and amniote larynges. The number of laryngeal muscles varies across organisms, but all the muscles of the vertebrate larynx are innervated by two branches of the vagus nerve, the superior (short) laryngeal nerve and the recurrent (long) laryngeal nerve (Diogo et al., 2008). Exceptions include the laryngeal innervation of reptiles (excluding Aves) which is accomplished by a single branch of the glossopharyngeal nerve (Smith, 1992; Diogo et al., 2008). In mammals, the superior laryngeal nerve only innervates one laryngeal muscle, the cricothyroid, while the recurrent laryngeal nerve loops around the aorta and innervates all the remaining laryngeal muscles. The relationship between the laryngeal muscles of mammals and amphibians was previously confused by the notion that the m. dilatator laryngis in amphibians was innervated by both the long and short laryngeal nerves. The m. dilatator laryngis of amphibians was thought to correspond exclusively to the posterior cricoarytenoid muscle of other tetrapods, including mammals, reptiles, and dipnoans (Diogo et al., 2008). The innervation pattern presently described for túngara frogs, however, matches the general arrangement known in mammals: the short laryngeal nerve exclusively innervates a single laryngeal muscle, the m. arylabialis, whereas the long laryngeal nerve loops around the aorta to innervate the remaining laryngeal muscles. This supports previous suggestions that the larynx of reptiles is not homologous to that of amphibians (Diogo et al., 2008).

CHAPTER 5: CONCLUSION

This study supports the recognition of the m. arylabialis as a novel muscle in the larynx of túngara frogs. Based on its attachments to the arytenoid cartilages and to the frenulum labii vocalis this muscle may play a role in either tensing the vocal cords, positioning the vocal cords, or positioning the fibrous masses. Tensing and positioning the vocal cords could potentially influence the whine portion of the male's advertisement call by altering its frequency. Alternatively, positioning the fibrous masses may allow the m. arylabialis to control the complexity of the call by determining the production of chucks.

The separation of the superficial and deep bundles of the m. dilatator laryngis into the m. dilatator laryngis and the m. arylabialis gives each of the muscles their own distinct innervation. This new innervation pattern for the túngara frog larynx directly matches the innervation pattern of the mammalian larynx. Based on this matching innervation, it is likely that the m. dilator laryngis is homologous to the mammalian posterior cricoarytenoid muscle and that the m. arylabialis is homologous to the mammalian cricothyroid muscle. These newfound similarities invite a reevaluation of the evolutionary relationships between amphibian and mammalian larynges.

The discovery of a novel laryngeal muscle in túngara frogs holds great significance. It not only has the potential to unveil the mechanism through which these animals add complexity to their calls but could help decipher the evolutionary path of complex call production in natural communication systems.

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APPENDIX A: IMAGE SOURCES

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