5-1-2018

HISTOLOGICAL FEATURES OF THE LARYNX OF GONATODES ANTILLENSIS

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HISTOLOGICAL FEATURES OF THE LARYNX OF *GONATODES ANTILLENSIS*

by

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Presented to the Faculty of the Honors College of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

HONORS BACHELOR OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

May 2018

ACKNOWLEDGMENTS

This project is the result of the effort of many people who came together in the name of science. Thank you to all the extraordinary people who contributed in one way or another to make this project possible.

Special thanks to Dr. Schargel for allowing me to be a part of this beautiful and fascinating research and for trusting me with this project.

Thank you to my wonderful family, who has always been there to keep me going and to help me overcome every obstacle in the way. I could not be where I am now if it was not because of my parents, who never stop believing in me and encouraging me to never give up.

Special thanks to Dr. Rhonda Prisby, who selflessly allowed us to work in her lab and provided guidance during this project, as well her wonderful lab team: David Lee, Seungyong Lee, Darlene Ngwenya, Sunggi Noh, and Lizzette Partida.

Finally, I am extremely grateful for Ms. Hazel Dalton, who selflessly obtained and donated the reagents needed for the histological stains.

April 20 , 2018

ABSTRACT

HISTOLOGICAL FEATURES OF THE

LARYNX OF *GONATODES*

ANTILLENSIS

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The University of Texas at Arlington, 2018

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Within the *Gonatodes* genus, the diurnal state is the common state for all but one of its members: *Gonatodes antillensis*. *G. antillensis* is also the only member of this genus known to be capable of vocalization. The ability to vocalize is a feature thought to be lost for this genus, and now regained. To further understand the evolutionary process that allowed this species to regain the ability to vocalize, histological preparations were made from the larynx of a male *G. antillensis* and from three other males belonging to three different species from the same genus not known to vocalize. These were: *G. humeralis*, *G. ligiae*, and *G.vitattus*. The histology of the larynx of the four species was compared using routine and special stains. The observations revealed striking differences between the four species, with *G. antillensis* having a remarkably differentiated laryngeal structure which, may account for its ability to vocalize.

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CHAPTER 1

INTRODUCTION

Geckos are considered the only primarily nocturnal clade of lizards (Gamble et al., 2015). As of 2015, 72% of all known species in this clade (1552) were known to be active primarily at night. However, diurnality has independently evolved in many groups multiple times (Gamble et al. 2015). Geckos have also been recognized in literature as the only type of reptiles with true vocal abilities, given they are the only group where true vocal cords have been observed (Russell et al., 2000).

Given that the majority of geckos are nocturnal, they have developed a high number adaptations to such lifestyle. Some of the general adaptations they display include: olfactory specialization, enhanced sustained locomotion at low temperatures, absence of parietal foramen and pineal eyes, and the ability of vocalization and acoustic communication, among others. Diurnal geckos in turn had to developed their own set of adaptations like round pupils, smaller eyes, cone-like photoreceptor cells in the retina, as well as they returned to a higher energetic cost locomotion (Gamble et al., 2015).

Geckos are also known to utilize both visual and acoustic means for communication. There are several ways by which reptiles can produce sounds or calls. Some notable mechanisms include explosive expulsion or air, modulation of intermittent air through modified glottis or larynx, or even through rubbing or vibration of the integument (Moore et al. 1991). Gekkonoid lizzards are known to have a remarkable ability to vocalize and produce sounds that may have tonal and harmonic qualities (Russell et al.

2000). Geckos differ from other reptiles in their ability to produce sound by means of vibration of elastin rich vocal cords. (Russell, 2000). The different sounds that geckos are capable of producing range from barely perceptible squeaks and chirps to loud growling and barking noises (Marcellini, 1977).

Although geckos are known for their vocal abilities, little is known about the different structures that play a role in their sound-producing mechanisms. Furthermore, some literature describes some members of the Lacertid family as having true vocal cords (vocal cords that are highly elasticized), a feature that has only been observed in the Gekkonidae family (Russell, 2000). True vocal cords are defined as out-foldings into the laryngeal lumen of the mucosa and the submucosa, with the submucosa being densely packed with elastin fibers (Moore et al., 1991).

Interestingly enough, after the transition from nocturnality to diurnality, reversion to nocturnality has been observed within diurnal clades such as *Sphaerodactylus*, *Gonatodes*, *Phelsuma*, and *Pygopodidae* (Gamble et al., 2015).

Sphaerodactyl geckos (Gamble et al, 2011) comprise a rich family of Neotropical lizards, accounting for about 10% of all known gecko species; this family includes five genera: *Coleodactylus*, *Lepidoblepharis*, *Gonatodes*, *Pseudogonatodes*, and *Sphaerodactylu*. The majority of the species belonging to this group are diurnal. However, they are thought to be secondarily diurnal, having evolved from a nocturnal ancestor.

The genus *Gonatodes* constitutes a monophyletic group of mostly diurnal lizards with small bodies (Gamble et al., 2011). Currently, there are about 30 recognized different species of *Gonatodes*. This genus can be found in the region ranging from southern Mexico, passing by many Caribbean islands, and all the way to Brazil and Bolivia

(Schargel, 2008). The genus *Gonatodes* is a group composed mostly of diurnal geckos, except for one: *Gonatodes antillensis* (see fig. 1.1). *G. antillensis* is the only strictly nocturnal species of the group, and it is also the only one known to have the ability to vocalize and produce calls (Schargel, 2008). Little is known about the different roles that vocalization may play in the social interactions of *G. antillensis*. Only release calls have been noted at the moment of capture. The sounds that they produce can better be described as high pitch chirps (W. Schargel, personal communication with author, April 2018).

Figure 1.1: *Gonatodes antillensis*

Something that brings attention to the G. *antillensis* is the development of a nocturnal lifestyle, while retaining the chromatic sexual dimorphism of diurnal species, and resurrecting the once lost ability to vocalize. In an attempt to understand the adaptations that *G. antillensis* underwent in order to regain the ability to vocalize, the larynx of one male from this species, along with three other males from three non-vocalizing species from the same genus were extracted and processed for histological preparations. The species selected were: *Gonatodes humeralis*, *Gonatodes ligiae*, and

Gonatodes vitattus. Phylogenetically, *G. ligiae* is the closest species to G. antillensis within the species observed for this research. It is closely followed by G. humeralis. The most distant species in the group is G. vitattus (see figure 1.2).

There are not many studies related to the anatomy of the larynx of geckos. Some similar researches are the ones of Russell et al. (2000), and Moore et al.(1991). Russell et al.(2000), explored the variations seen in the morphology of the larynx of geckos of different species. Although it was found that some vocalizing species had structures more highly differentiated than others, there was no direct correlation between the level of specialization and the quality of the call.

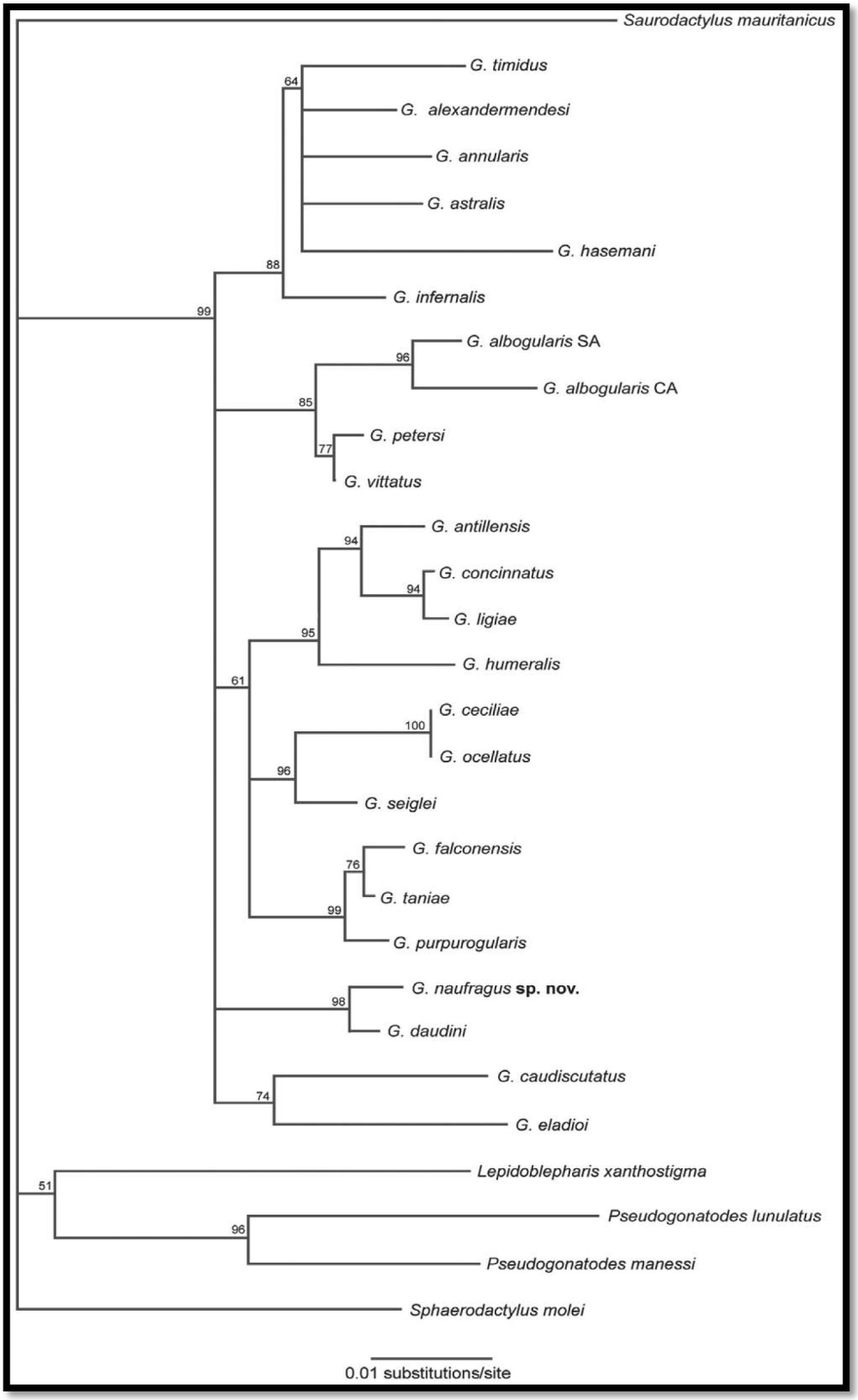

Figure 1.2: Consensus phylogenetic tree for the genus *Gonatodes* (Gilson et at., 2012)

CHAPTER 2

MATERIALS AND METHODS

The larynx of a male from four different species were excised. Three non-vocalizing gecko species were selected to serve as a comparison to *G. antillensis*. The species selected were: *G. ligiae*, *G. humeralis*, and *G. vitattus*. The larynxes were extracted with the assistance of a dissecting kit and a stereoscope.

The specimens selected had been previously fixed in 10% neutral buffered formalin for 48 hours and preserved in 70% reagent ethanol until processing. After processing, each specimen was embedded in a labeled cassette, and prepared for microtomy. Serial sections were cut until the block was depleted. The sections were cut at 3µm, given the small size of the specimens. Positively charged slides were used for this purpose.

A routine H&E was performed in all samples, as well as two special stains: Masson's Trichrome, and Verhoeff-van Gieson stain for elastic fibers (VVG). Masson's trichrome is particularly helpful to differentiate connective tissue, while the VVG stain is specialized to stain elastic fibers. All reagents used were manufactured by Poly-Scientific R&D Corp. The processing protocols were developed following the general guidelines provided in *Histotechnology: a Self Instructional Text* (Carson and Hladik, 2009), with some small variations in time.

2.1 Processing Protocol

Processing in histology refers to the procedure of dehydrating the tissue with a clearing agent and increasing concentrations of alcohol, to then infiltrate the tissue with paraffin

with the purpose of preparing the specimen for embedding (Carson and Hladik, 2009). All specimens were submitted for processing using an automated Benchtop TP1020 Leica processor, applying the following processing schedule:

1. 70% ethanol	60 minutes
2. 95% ethanol	45 minutes
3. 95% ethanol	45 minutes
4. 100% ethanol	30 minutes
5. 100% ethanol	30 minutes
6. Safeclear II (Xylene substitute)	45 minutes
7. Safeclear II (Xylene substitute)	45 minutes
8. Safeclear II (Xylene substitute)	45 minutes
9. Paraffin wax	30 minutes
10. Paraffin wax	30 minutes
11. Paraffin wax	30 minutes
12. Embed immediately	

2.2 Hematoxylin & Eosin Staining Protocol

Hematoxylin and Eosin staining is a routine stain performed in histology to demonstrate morphological features of tissue. Hematoxylin stains the nucleic acids purple-blue, while eosin stains the cytoplasm and extracellular matrix varying shades of pink (Carson & Hladik, 2009).

1. Xylene	2 minutes
2. Xylene	2 minutes
3. Xylene	2 minutes
4. Xylene	2 minutes
5. 100% Alcohol	10 dips
6. 100% Alcohol	10 dips
7. 95% Alcohol	10 dips
8. 95% Alcohol	10 dips
9. Tap water	until water runs off evenly
10. 1 % Acetic acid	10 dips
11. Running tap water	was well
12. 0.25 % ammonia water	10 dips
13. Tap water	10 dips
14. Tap water	10 dips
15. 95% Alcohol	10 dips
16. Eosin	3 minutes
17. 95% Alcohol	10 dips

18. 100% Alcohol	15 dips
19. 100% Alcohol	15 dips
20. 100% Alcohol	15 dips
21. Xylene	15 dips
22. Xylene	15 dips
23. Xylene	15 dips
24. Mount and coverslip with synthetic resin	

2.3 Masson's Trichrome Staining Protocol

Masson's Trichrome is a special staining technique used to differentiate connective tissue. This technique stains the nuclei black, collagen and mucin blue, and the cytoplasm, keratin, and muscle fibers red (Carson and Hladik, 2009).

1. Xylene	2 minutes
2. Xylene	2 minutes
3. Xylene	2 minutes
4. Xylene	2 minutes
5. 100% Alcohol	10 dips
6. 100% Alcohol	10 dips
7. 95% Alcohol	10 dips
8. 95% Alcohol	10 dips
9. Distilled water	rinse until water runs evenly
10. 1 % Acetic acid	10 dips
11. Running tap water	was well
12. Bouin's solution	1 hour at 56 °C
13. Allow slides to cool	
14. Running tap water	Until yellow color disappears
15. Weigert Iron Hematoxylin	10 minutes
16. Running tap water	10 minutes
17. Biebrich scarlet-acid fuchsin	2 minutes
18. Distilled water	2 changes
19. Phosphomolybdic/phototunsgtic acid	15 minutes
20. Aniline blue	5 minutes
21. Distilled water	2 changes
22. 1% Acetic acid	5 minutes
23. 95% Alcohol	10 dips
24. 95% Alcohol	10 dips
25. 100% Alcohol	15 dips
26. 100% Alcohol	15 dips
27. 100% Alcohol	15 dips
28. Xylene	15 dips
29. Xylene	15 dips
30. Xylene	15 dips

31. Mount and coverslip with synthetic resin

2.4 Verhoeff-van Gieson Staining Protocol

Verhoeff-van Gieson stain is a special staining technique designed to differentiate elastic fibers. With this staining protocol elastic fibers are stained black, the nuclei are stained black-blue, collagen is stained pink-bright red, and other tissue elements yellow (Carson and Hladik, 2009).

1. Xylene	2 minutes
2. Xylene	2 minutes
3. Xylene	2 minutes
4. Xylene	2 minutes
5. 100% Alcohol	10 dips
6. 100% Alcohol	10 dips
7. 95% Alcohol	10 dips
8. 95% Alcohol	10 dips
9. Distilled water	2 changes
10. Verhoeff elastic tissue stain	1 hour
11. Distilled water	wash well
12. Differentiate microscopically in 2% ferric chloride	
13. Distilled water	wash well
14. 5 % Sodium Thiosulfate	1 minute
15. Running tap water	5 minutes
16. Van Gieson stain	1 minute
17. 95% Alcohol	10 dips
18. 95% Alcohol	10 dips
19. 100% Alcohol	15 dips
20. 100% Alcohol	15 dips
21. 100% Alcohol	15 dips
22. Xylene	15 dips
23. Xylene	15 dips
24. Xylene	15 dips
25. Mount and coverslip with synthetic resin	

CHAPTER 3

RESULTS

3.1 *Gonatodes antillensis*

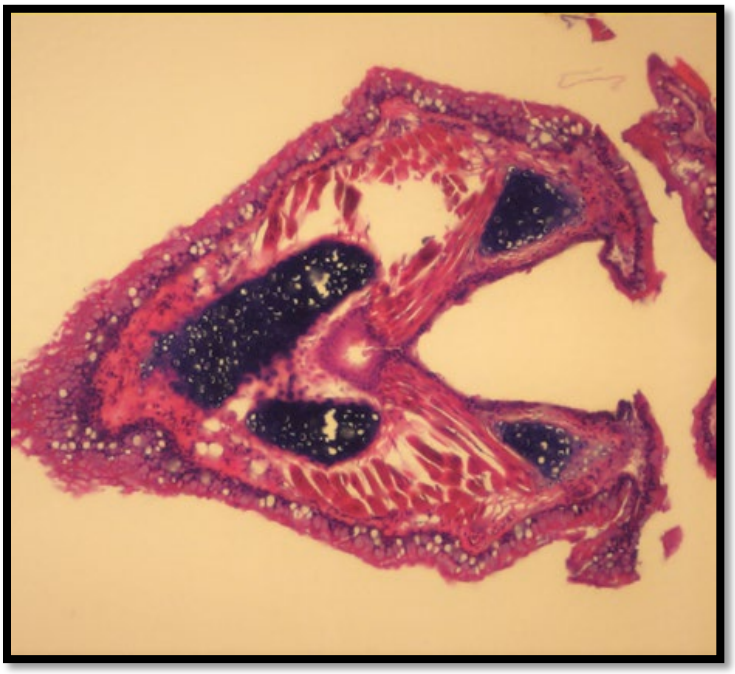

Figure 3.1: Frontal section of the larynx of *G. antillensis* H&E under 4X objective

Figure 3.1 shows the general structure of the superior part of the larynx of *G. antillensis.* The H&E staining of a frontal section of the larynx of *G. antillensis* revealed a highly specialized larynx. The vocal folds are lined by ciliated epithelium and mucus secreting goblet cells. Surrounding the cartilage and muscle fibers, there is non-keratinized stratified epithelium, as well as pseudostratified ciliated epithelium. Discontinued areas of Hyaline cartilage can also be observed. These likely correspond with the cricoid cartilage (left) and arytenoid cartilage (right) of the larynx.

Figure 3.2 shows a closer look at the muscle fibers. Skeletal muscle fibers can be seen running parallel to each in different orientations (elongated dark pink to red cells). Skeletal muscle tends to have peripherally located multiple nuclei, as well as striation. These are voluntarily controlled muscle cells. The collagen fibers in the matrix (light pink, string like fibers) can also be appreciated. Figure 3.3 shows the image as seen at 40X magnification. In this image, the pseudostratified ciliated epithelium can be better appreciated, as well as the goblet cells (pale pink cells), and the different levels of epithelium stratification.

Figure 3.4 shows the larynx of *G. antillensis* stained with Masson trichrome. This protocol is good for the differentiation of connective tissue. Collagen and mucin are stained blue, while the cytoplasm, keratin, and muscle fibers are stained red. In this figure it is easy to appreciate the high number of mucin cells surrounding the structure.

Figure 3.5 shows the larynx of *G. antillensis* stained with Verhoeff-van Gieson protocol. This stain is useful to demonstrate elastic fibers. However, no elastic fibers are seen in this section. The black spots observed correspond with the nuclei of the cell. Collagen is stained pink, and muscle fibers are stained yellow-brown.

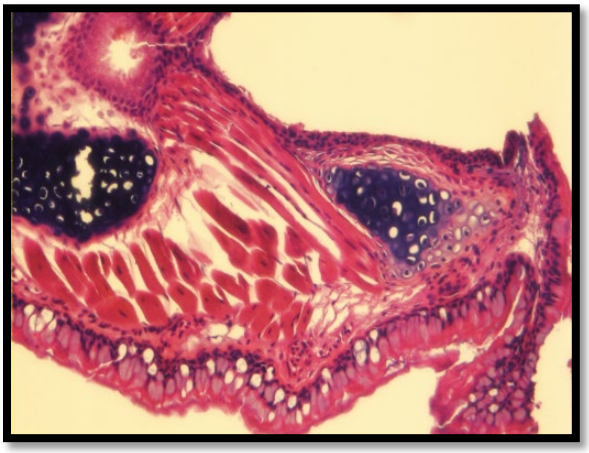

Figure 3.2: Frontal section of the larynx of G. *antillensis* H&E under 10X objective

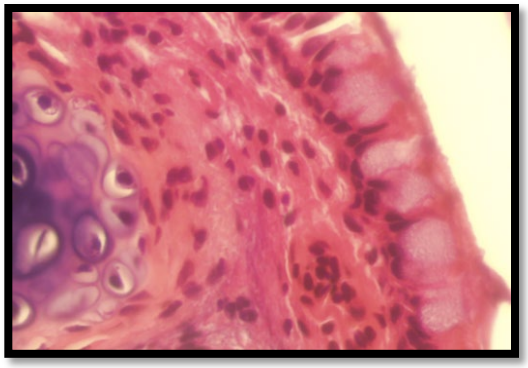

Figure 3.3: Frontal section of the larynx of G. antillensis H&E under 40X objective

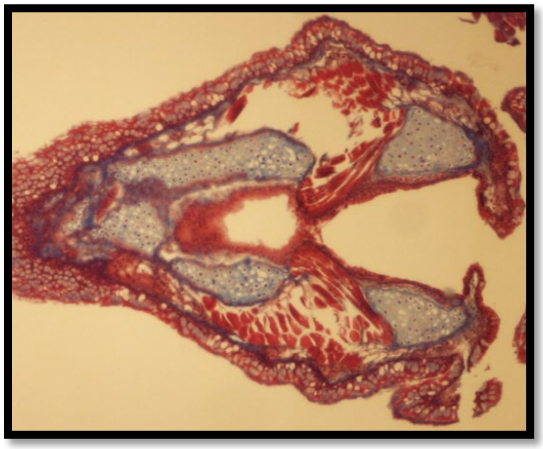

Figure 3.4: Larynx of *G. antillensis* stained with Masson's Trichrome under 4X objective

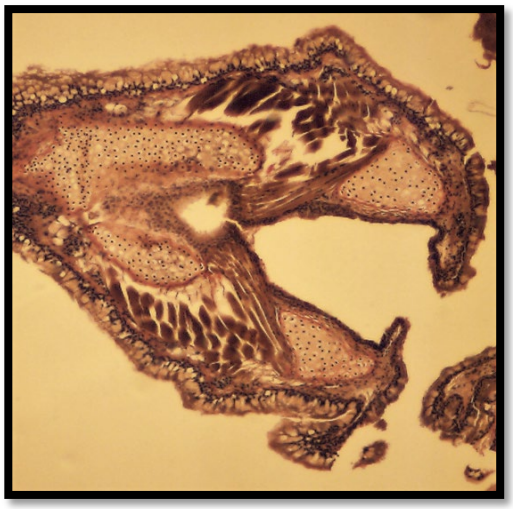

Figure 3.5: Larynx of *G. antillensis* stained with Verhoeff-van Gieson under 4X objective

3.2 *Gonatodes humeralis*

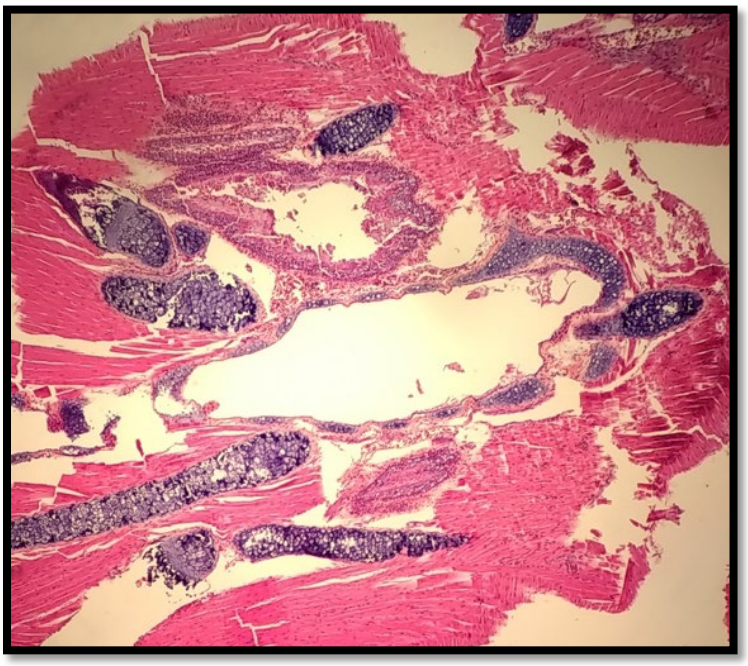

Figure 3.6: Cross section of the larynx of *G. humeralis* stained H&E under 4X objective

Figure 3.6 shows a cross section of the larynx of *G. humeralis.* The larynx is presented as a hollow ring of hyaline cartilage. The cartilage is lined internally with a single layer of epithelial cells. Goblet cells are not apparent. The epithelium appears to be ciliated. The hollow ring is flanked by solid rings of hyaline cartilage. These are likely to correspond to the cricoid (single ring of cartilage to the left) cartilage and arytenoid (pair of cartilages rings to the right of the image) cartilages. Dense fibers of striated muscle are observed surrounding the whole structure. Figure 3.7 shows the section under the 10X objective. In this image is possible to appreciate the different layers of collagen (light pink fibers), epithelial cells, and mucin producing cells surrounding the exterior aspect of the cartilage ring. Figure 3.8 presents the same section under the 40X objective. In this section it is easier to appreciate the single layer of epithelium lining the internal aspect of the cartilage ring.

Figure 3.9 shows the larynx of *G. humeralis* stained with the Masson trichrome protocol. In this is figure it possible to appreciate the one layer of epithelial cells (red) lining the inside of the hollow cartilage ring. The hyaline cartilage is stained blue, and the muscle fibers are stained dark red.

Figure 3.10 shows the larynx of *G. humeralis* stained with Verhoeff-van Gieson. The cartilage is stained pink. The muscle fibers are stained yellow-brown, and the nuclei of the cells are stained black. No elastic fibers are seen in this section.

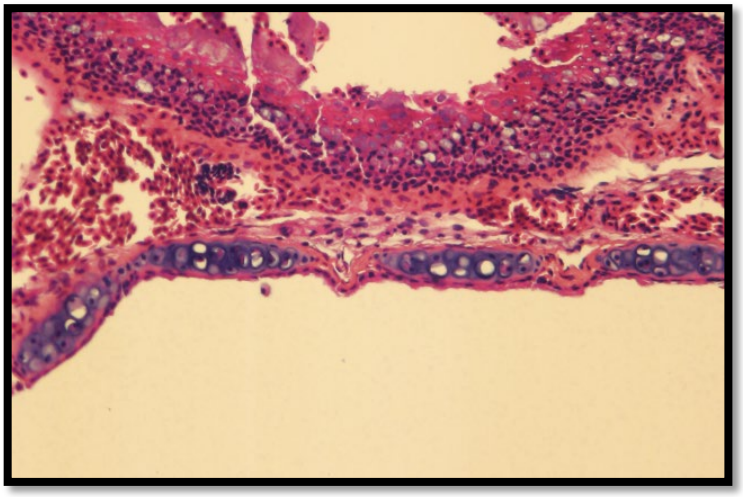

Figure 3.7: Cross section of larynx of *G. humeralis* stained H&E under 10X objective

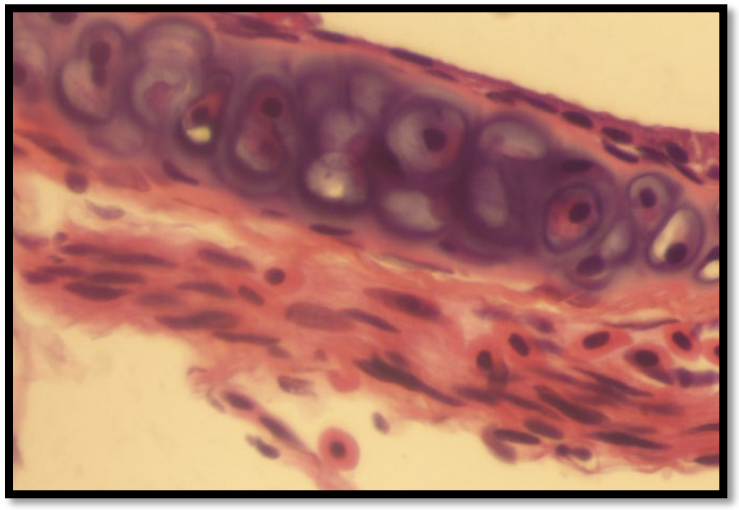

Figure 3.8: Cross section of the larynx of *G. humeralis* stained H&E under 40X objective

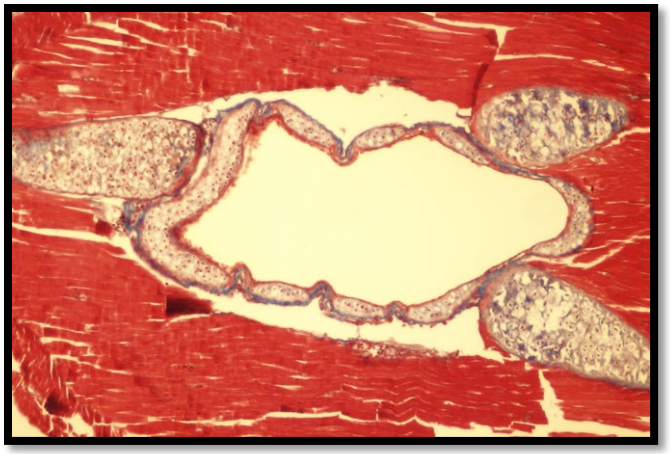

Figure 3.9: Larynx of *G. humeralis* stained with Masson's Trichrome under 4X objective

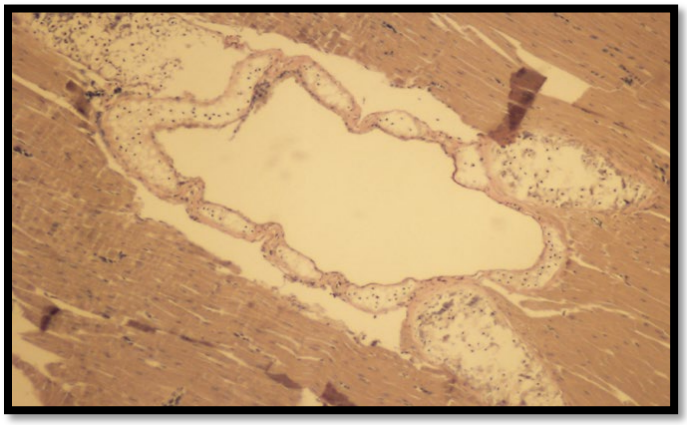

Figure 3.10: Larynx of *G. humeralis* stained with Verhoeff-van Gieson under 4X objective

3.3 *Gonatodes ligiae*

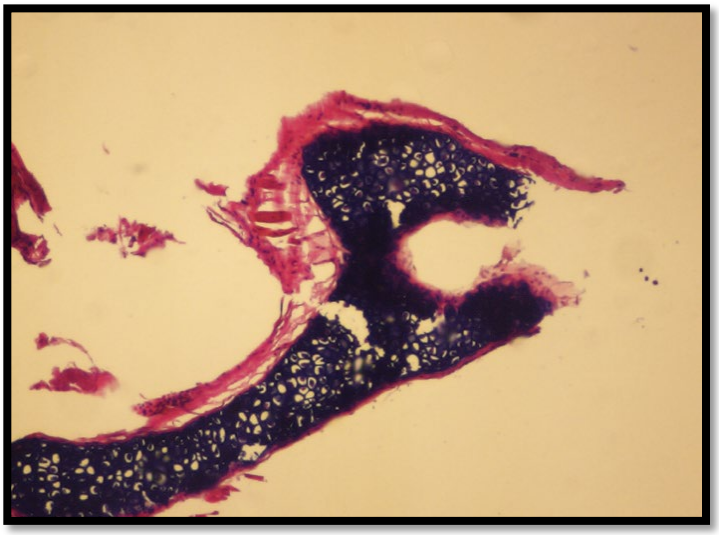

Figure 3.11: Larynx of *G. ligiae* stained H&E under 4X objective

A frontal section is used to represent the general structure of the larynx of *G. ligiae* as seen in figure 3.11. It shows prominent hyaline cartilage covered in stratified epithelium. This cartilage seems to correspond with the cricoid cartilage. Neither ciliated epithelium nor goblet cells are apparent in the section. Collagen and smooth muscle are both present. Figure 3.12 Shows the section under the 10X ocular. A flap of stratified epithelium can be seen extending to the right, and lining the hyaline cartilage. Some scattered smooth muscle cells can be seen to the left margin of the picture (dark pink to red cells). The inside ring of the cartilaginous structure is also lined by a single layer of epithelial cells. Figure 3.13 shows a closer look at the stratified epithelium lining the cartilage. The epithelial lining seems to be 2-3 layers thick.

Figure 3.14 shows a section from the larynx of *G. ligaea* stained with Masson trichrome under the 10X objective. The hyaline cartilage from the cricoid cartilage is stained blue. There are also some epithelial cells lining the outer surface of the cartilage. The blue staining reveals some collagen fibers in the matrix surrounding the cartilage. A few smooth muscle cells are also observed.

Figure 3.15 shows a section from the larynx of G. *ligiae* stained with Veroeff-van Gieson stain. The cartilage is stained light pink. Muscle fibers, cytoplasm and other tissue elements are stained yellow-brown. The nuclei are stained blue-black. This stain is good to demonstrated elastic fibers, however, there are no elastic fibers in this section.

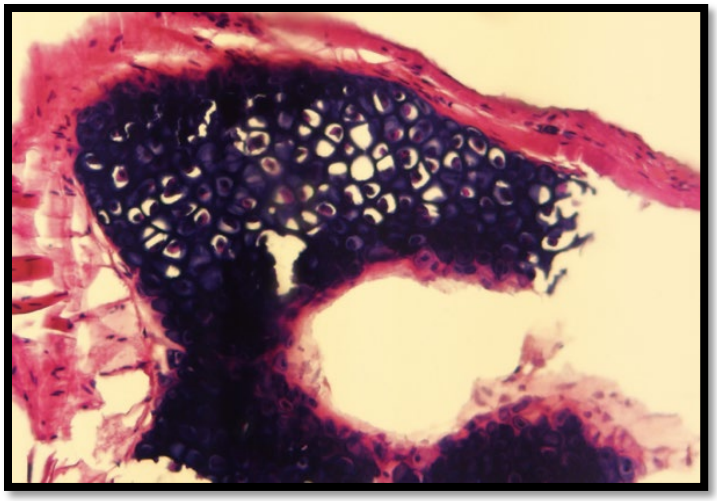

Figure 3.12: Larynx of *G. ligiae* stained H&E under 10X objective

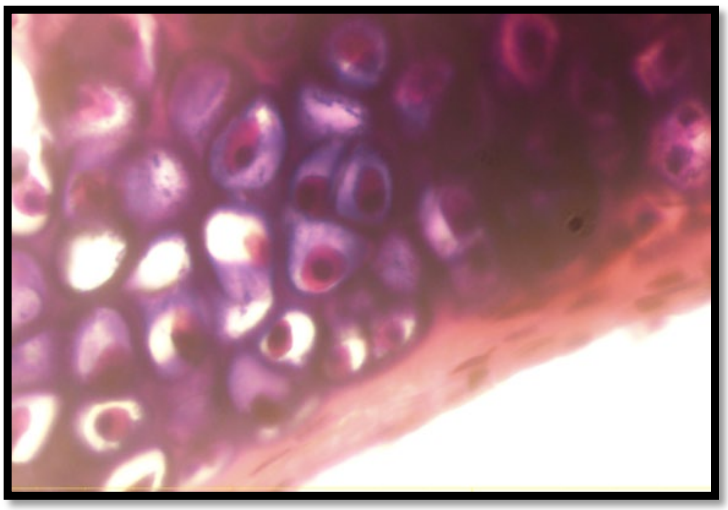

Figure 3.13: Larynx of *G. ligiae* stained H&E under 40X objective

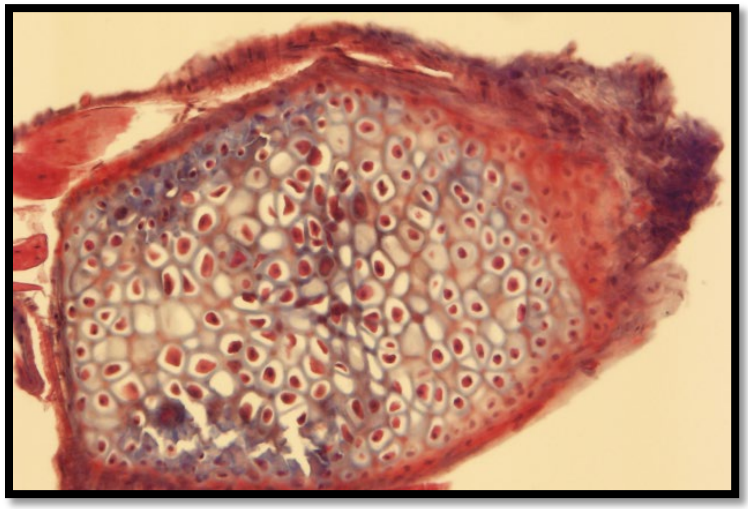

Figure 3.14: Larynx of *G. ligiae* stained with Masson's Trichrome under 4X objective

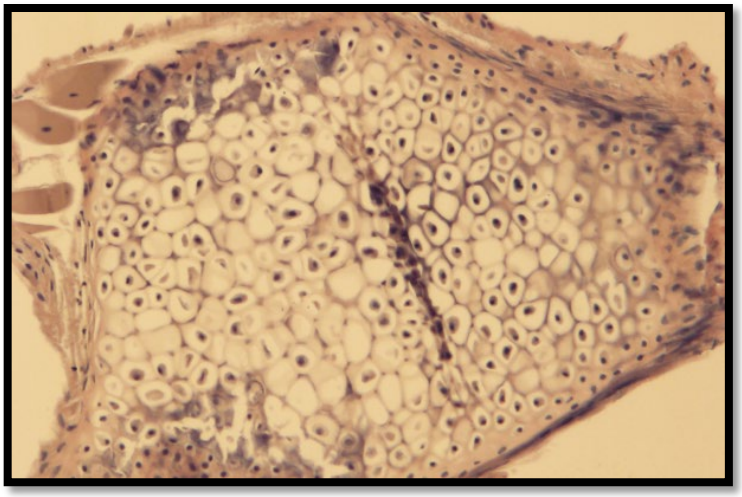

Figure 3.15: Larynx of *G. ligiae* stained with Verhoeff-van Gieson under 4X objective

3.4 *Gonatodes vitattus*

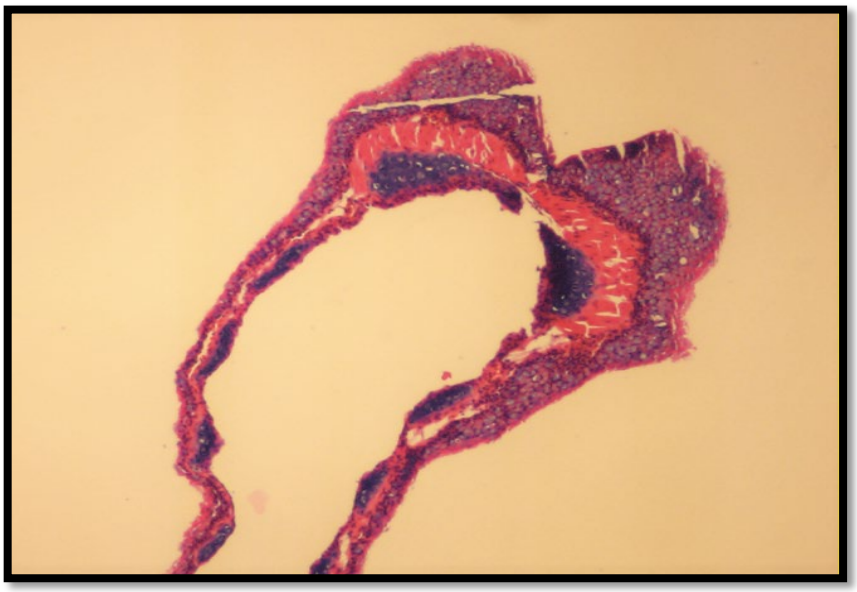

Figure 3.16: Larynx of *G. vitattus* stained H&E under 4X objective

The general structure of the larynx of *G. vitattus* can be observed in Figure 3.16. This figure shows a frontal section of the larynx. The outermost layer of tissue appears to be ciliated epithelium. There is a high number of mucin producing cells (mucin is stained light pink-purple). There also is a layer of smooth muscles cells between the epithelium and the hyaline cartilage. The hyaline cartilage is likely to correspond with the arytenoid

cartilage. Figure 3.17 shows the section under the 10X objective. In this section the pattern of the muscle cells can be better observed. Figure 3.18 shows the section under the 40X objective. In this image the ciliated cells, pseudostratified cells, stratified epithelium, and mucin producing cells can be clearly appreciated.

Figure 3.10 shows a section from the larynx of *G. vitattus* stained with Masson Trichrome. In this section collagen and mucin are stained blue, muscle fibers are stained dark red. Different bundles of muscle can be seen running in different orientation. Some of the muscle fibers seen re elongated and multi nucleated (skeletal muscle), and some others correspond with smooth muscle (non-striated, singly nucleated). Figure 3.20 shows a section from the larynx of G. vitattus stained with Verhoeff-van Gieson staining protocol. This stain is used to demonstrate elastic fibers. However, no elastic fibers are seen. The dark spots observed in this section correspond with the nuclei of the cells.

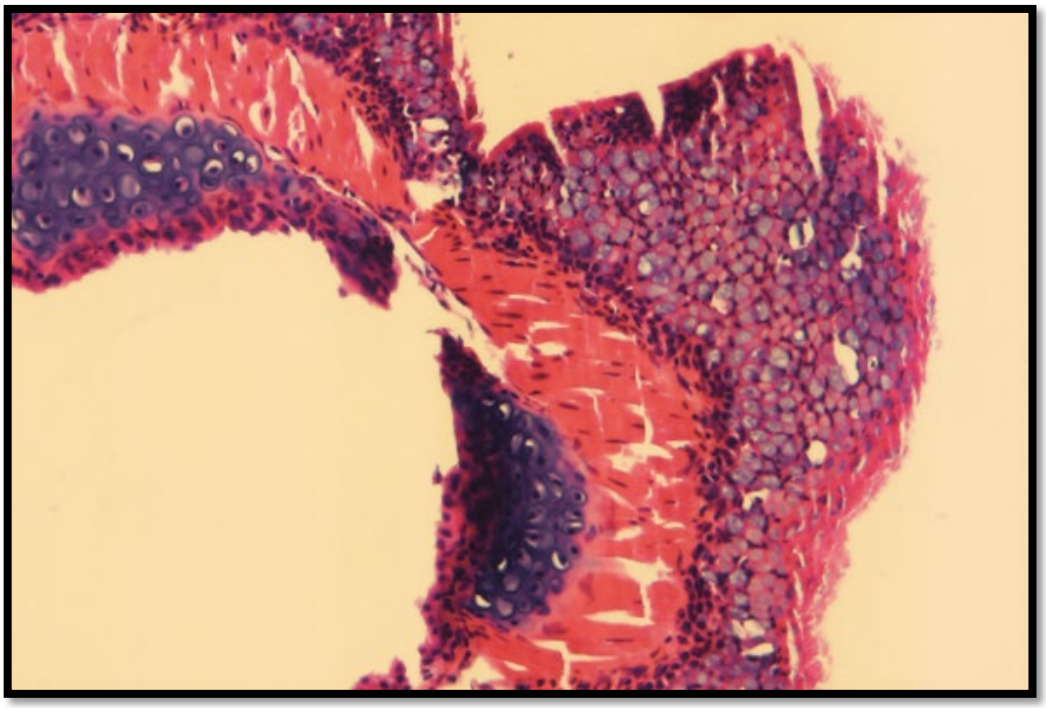

Figure 3.17: Larynx of *G. vittatus* stained H&E under 10X objective

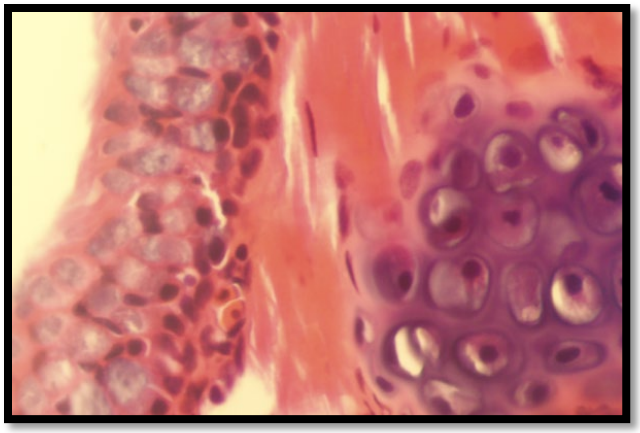

Figure 3.18: Larynx of *G. vitattus* stained H&E 40X under 40X objective

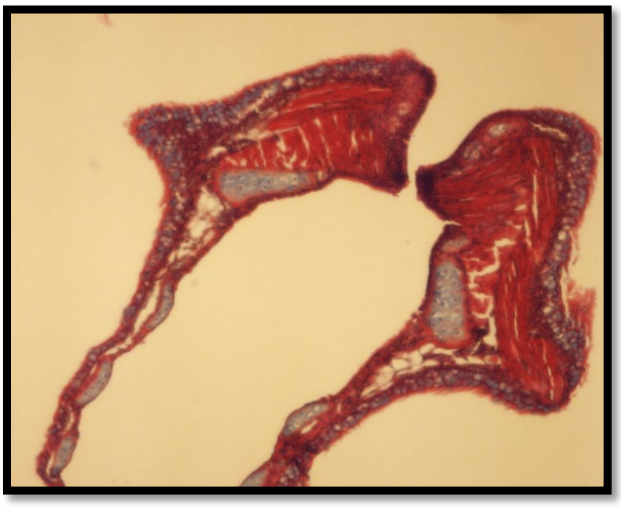

Figure 3.19: Larynx of *G. vitattus* stained with Masson's Trichrome under 4X objective

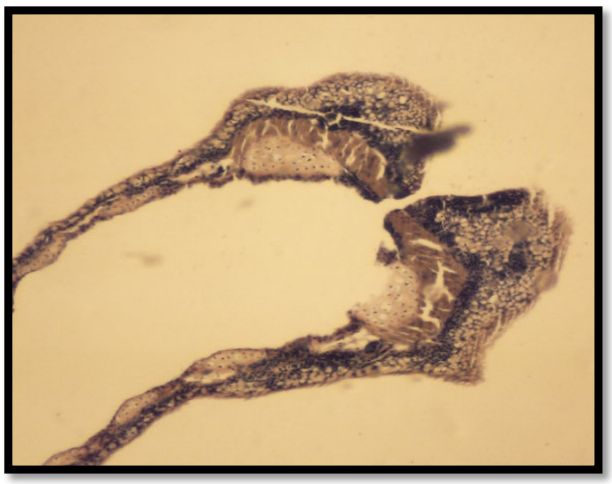

Figure 3.20: Larynx of *G. vitattus* stained with Verhoeff-van Gieson under 4X objective

CHAPTER 4

DISCUSSION

All four larynges showed common elements; there was a hyaline cartilage structure, lined by a mucosa membrane composed of stratified epithelium, pseudostratified epithelium and goblet cells. There was also a submucosa mostly composed of muscle fibers and collagen. However, the general morphology of the larynx differed from one specimen to the next.

Literature defines true vocal cords as foldings of mucosa and submucosa packed with elastic fibers (Moore et al., 1991). *G. antillensis* presented significant foldings of mucosa and submucosa into the larynx that were not observed in other species. Nontheless, the most important requirement to qualify the foldings as true vocal cords (elastic fibers) was not observed.

Although no elastic fibers were observed on the histological sections of the larynx *G. antillensis*, it became clear that the larynx of this species presents some adaptations and a level of development not seen in the other three species.

The larynx of the *Gecko gecko*, also known as the tokay gecko is described as having a pyramid-like elevation, with the main laryngeal skeleton being composed of cartilage: ring-shaped cricoid cartilage and a pair or arytenoid cartilage (Rittenhouse et al., 1998). Out of the four larynges observed, the larynx of *G. antillensis* was the only one to present characteristics similar to that of the *Gecko gecko*. Out of the four species used for this

comparison, the larynx of *G. vitattus* seems to be the most highly developed after that of *G. antillensis.*

The results obtained constitute preliminary information and further research is still necessary. Although elastic fibers were not observed in this specimen there are many factors that may have contributed to lack of elastic fibers, like the age of the specimen or the plane at which the specimen was sectioned. In order to reach final conclusion regarding whether or not *G. antillensis* has developed true vocal cords, a bigger cohort, including males and females need to be studied.

Besides basic histological techniques, scanning electron microscopy (SEM) and Computed Tomography (CT) scanning techniques may be employed to further the understanding of the internal anatomy. SEM provides details at the cellular level, allowing visualization of organelles, while CT scanning provides the opportunity to explore the anatomy of some of the preserved specimens, while eliminating the need of cutting all of them open to gain knowledge of the internal anatomy.

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BIOGRAPHICAL INFORMATION

María was born and raised in Venezuela where she graduated from a Catholic school with a high school diploma in Science. After moving to Texas, she attended Tarrant County College where she graduated with an Associate of Arts. While at TCC, María pursued her love for arts by serving as a drummer for TCC South's Jazz Band, making films with the film club, and serving as founder and president of the French Club. She then attended Tarleton State University where she graduated with an Associate of Applied Science in Histotechnology. Subsequently, María attended the University of Texas at Arlington, where she met Dr. Schargel and became interested in the phylogeny of Gonatodes. She graduated from UTA with an Honors Bachelor of Science in Biology. There were many things María loved about UTA, but the Honors College was her favorite.

María's ultimate goal is to become a College professor. She will take two years off before applying to a Graduate Program in Pathology. During this time, she will pursue her other three passions: music, jewelry making, and learning new languages.