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What's that Hooting Sound? A Survey on Novel Sound Producing Mechanisms in Chameleons

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WHAT’S THAT HOOTING SOUND?
A SURVEY ON NOVEL SOUND PRODUCING MECHANISMS
IN CHAMELEONS

A Capstone Experience/Thesis Project
Presented in Partial Fulfillment of the Requirements for
the Degree of Bachelor of Science with
Honors College Graduate Distinction at Western Kentucky University

By
Keyana Boka

*****

Western Kentucky University
2014

CE/T Committee: Approved by
Professor Steve Huskey, Advisor
Professor Michael Smith
Professor Dennis Wilson

Advisor
Department of Biology
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2014
ABSTRACT

This research project seeks to study how chameleons generate low frequency vibrations, some audible and some not. The mechanism responsible for this 'hoot' is unknown. A modified tracheal appendage we termed “the resonator” has been hypothesized as the potential source of this sound. An anatomical survey was conducted on various chameleon species including, *Chameleo melleri* (Meller), *Chamaeleo pardalis* (Ambanja, Nosy Be, Panther, Sambava), *Furcifer rhinoceratus, Chamaeleo dilepis* (Flapneck), *Chamaeleo rudis* (Side-striped), *Chamaeleo calyptratus* (Veiled), *Chamaeleo jacksonii* (Jackson’s), *Chamaeleo quadricornicus* (4-horned), *Chamaeleo quilensis* (Flapneck), *Chamaeleo senegalensis* (Senegal), *Chamaeleo jacksonii xantholophus* (giant Jackson’s), and *Rhampholeon brevicaudatus* (Pygmy). Each chameleon was dissected in order to examine its trachea and associated appendages. Sagittal-sections of resonators provided for gross anatomical descriptions. From this, it has been determined that, of the species known to hoot, a resonator is always present and is the likely source for sound production/modification. Chameleon species that have never been heard to hoot follow a pattern of possessing smaller, possibly vestigial, resonators or none at all. Such results will be useful in future studies of chameleon behavior and morphology to better understand this novel vocal structure and its functional significance.

Keywords: chameleons, chameleon sound production, animal vocalization, chameleon hoot, anatomical survey, clearing and staining protocol.
Dedicated to my friends and family
ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Methodology</td>
<td>6</td>
</tr>
<tr>
<td>3. Results</td>
<td>10</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>16</td>
</tr>
<tr>
<td>5. Future Research</td>
<td>23</td>
</tr>
<tr>
<td>Bibliography</td>
<td>29</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Gecko Laryngeal Skeleton</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Resonator of <em>C. dilepis</em></td>
<td>4</td>
</tr>
<tr>
<td>2.1</td>
<td>Ventral Image of <em>C. dilepis</em></td>
<td>7</td>
</tr>
<tr>
<td>2.2</td>
<td>Lateral Image of <em>C. dilepis</em></td>
<td>8</td>
</tr>
<tr>
<td>3.1</td>
<td>Resonator and Sagittal Sections Data</td>
<td>10</td>
</tr>
<tr>
<td>3.2</td>
<td>Resonator and Snout-Vent Data Bar Graph</td>
<td>12</td>
</tr>
<tr>
<td>3.3</td>
<td><em>C. dilepis</em> Syncroscopy</td>
<td>13</td>
</tr>
<tr>
<td>3.4</td>
<td><em>C. melleri</em> Syncroscopy</td>
<td>13</td>
</tr>
<tr>
<td>3.5</td>
<td><em>C. dilepis</em> Syncroscopy</td>
<td>14</td>
</tr>
<tr>
<td>3.6</td>
<td><em>C. dilepis</em> Syncroscopy</td>
<td>14</td>
</tr>
<tr>
<td>3.7</td>
<td><em>C. pardalis</em> Syncroscopy</td>
<td>14</td>
</tr>
<tr>
<td>3.8</td>
<td><em>C. calyptratus</em> Syncroscopy</td>
<td>15</td>
</tr>
<tr>
<td>3.9</td>
<td><em>C. melleri</em> Syncroscopy</td>
<td>15</td>
</tr>
<tr>
<td>3.10</td>
<td><em>C. jacksonii</em> Syncroscopy</td>
<td>15</td>
</tr>
<tr>
<td>4.1</td>
<td>Skull of Australian Cassowary</td>
<td>18</td>
</tr>
<tr>
<td>4.2</td>
<td>Skull of Veiled Chameleon</td>
<td>19</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

The father of modern physiology, Claude Bernard, once remarked that “the laboratory of a physiologist-physician must be the most complicated of all laboratories, because he has to experiment with phenomena of life which are the most complex of all natural phenomena,” and hundreds of years later his statement still holds true (Hill, 2008). Throughout history, what continues to fascinate human beings everywhere is the natural phenomenon of the animal world. The diversity of ways and reasons for which animals behave and interact is incredible. Yet, a full understanding and appreciation of all the marvels and phenomenon of the animal world depends on an analysis of how animals work. To learn how animals work, one must study the mechanisms by which they function.

Animal physiologists refer to “mechanism” as the components of actual, living animals and the interactions among those components that enable the animals to perform as they do (Hill, 2008). The structure of a mechanism and how the mechanism works maintains a very close, complex relationship. Often times it is difficult to distinguish which evolved first, the behavior or the mechanism by which the behavior functions, especially since the mechanisms of modern-day animals evolved in the past. The question of their origins is historical.
Therefore, by learning why evolution produced a certain mechanism, we will better understand what (if anything) animals gain by having the mechanism. Studies of animal mechanisms currently dominate physiological research, and the investigation all begins with the observation of a particular capability that sparks curiosity (Hill, 2008). My Honors Capstone project stems from a peculiar observation of a chameleon, in which spurned the question: what is that “hoot” sound?

Originating more than 60 million years ago, chameleons thrive as one of the most ancient lizard types (Necas, 2004). Popular for boasting distinct traits such as stereoscopic eyes and the ability to change skin color, family Chamaeleonidae is well known to human culture. People have devoted legends, laws, and libraries to these creatures. However, very little has been learned about the vocal communication and structure in chameleons. Vocal production is among the most principle forms of communication within the animal kingdom (Gans, 1973). It signifies mating calls, threats or attacks, foraging, and other messages relayed specifically within a certain species. Humans are raised to recognize various animal sounds –birds chirp, cows moo, and frogs croak, but what about chameleons?

Scientist Kenneth Barnett was the first to discover the chameleon “hoot”. One day he was handling Zappa, his pet veiled chameleon (Chamaeleo calyptratus) when he noticed that it was producing a buzzing sound from an area just in front of its front legs (Barnett, Cocroft, Fleishman, 1999).

Insects are known to communicate by producing vibrations which are transmitted along twigs and branches (Barnett, Cocroft, Fleishman, 1999). Although such communications have never been reported among reptiles, Barnett suspected that
something similar might occur with chameleons. This is what initiated my research project to learn more about this mysterious sound and how it is produced.

Reptiles produce sound in three ways: massive air expulsions, sporadic air movements through modified vocal folds, and rubbing or vibration of an exterior tissue (Gans & Maderson, 1973). In most reptiles, sound production is lacking or limited to hissing, except for the geckos. Figure 1.1 illustrates a gecko’s vocal cords, utilized for interspecies communication. Despite chameleons’ assumed silence, a very low frequency buzzing or hooting sound has been recorded in certain species, such as the veiled and meller chameleons. Yet, the behavior is baffling since chameleons do not possess vocal cords like the gecko.

**Figure 1.1.** A diagram of a gecko’s laryngeal skeleton.

It has been hypothesized that there must be potential pressure beneath the skin creating this sound. Dr. Huskey and I believe the biological component creating this
pressure and, potentially, sound is the “resonator”. An extension of the trachea, the resonator is an air sac-like mechanism whose movement may be responsible for the chameleon “hoot”.

The picture below shows the inflated resonator of an African flapneck chameleon, *Chamaeleo dilepis*.

![Image of an inflated resonator of an African flapneck chameleon, *Chamaeleo dilepis*.](image)

**Figure 1.2.** Image of an inflated resonator of an African flapneck chameleon, *Chamaeleo dilepis*.

In collaboration with Dr. Huskey and Kenneth Barnett, I joined this research project during the spring semester of 2011. My objectives for this research project were to: conduct a survey on the trachea anatomy of various chameleon species, examine the morphological function of these resonator sound producing mechanisms, and propose how such vocalization signifies adaptively for chameleons. This novel research will not only provide advancement in the understanding of how chameleons communicate and
interact in their environments, but also contribute to the research of vocal communication evolution overall.
CHAPTER 2

METHODOLOGY

Dr. Huskey’s laboratory, the “Bone Room”, served as the site for the trachea dissection survey of various chameleon species. A total of 120 chameleon carcasses belonging to several different species were obtained by Dr. Huskey from Kenneth Barnett. 105 chameleons of the original 120 were utilized, since 15 were lost due to damage such as decomposition. The bodies were kept in Dr. Huskey’s lab freezer. The whole carcasses were well preserved and kept frozen when not used, and each chameleon was tagged with an identification number. The identification numbers were listed on a spreadsheet. The spreadsheet provided individual chameleons’ genus and species, common name, and whether or not the chameleon is a known “hooter” next to their identification numbers. Species include, *Chameleo melleri* (Meller’s), *Chamaeleo pardalis* (Ambanja, Nosy Be, Panther, Sambava), *Furcifer rhinoceratus*, *Chamaeleo dilepis* (Flapneck), *Chamaeleo rudis* (Side-striped), *Chamaeleo calytratus* (Veiled), *Chamaeleo jacksonii* (Jackson’s), *Chamaeleo quadricornicus* (4-horned), *Chamaeleo quilensis* (Flapneck), *Chamaeleo senegalensis* (Senegal), *Chamaeleo jacksonii xantholophus* (giant Jackson’s), and *Rhampholeon brevicaudatus* (Pygmy).

I worked my way through the list of chameleon species, cutting gross anatomy incisions with lab dissection tools. The gross dissection tools consisted of a scalpel, scissors, pins, and tweezers. Using a scalpel and scissors, I created incisions along the
ventral side of the chameleon from the tip of its chin to about a fourth of the way down its body, stopping just before the belly area. This opened to the trachea area where I cut through a thin layer of skin and muscle tissue to reach the resonator air sac beneath. From there I cut off any surrounding tissue and skin to better expose this region. Using metal pins to keep the desired resonator area propped open, I recorded digital images on Dr. Huskey’s camera. Both ventral and lateral snapshots of the resonator were recorded, and each of the pictures included a hand labeled tag of the specimen’s number for type identification. Afterward I transferred the images to my USB flash drive to be studied. A comparative analysis of the dissected chameleons was performed over the fully formed resonator versus the smaller, less noticeable or nonexistent resonator. The following two pictures are examples of ventral (left) and lateral (right) images of a *Chameleo dilepis*, flapneck chameleon’s resonator.

![Image of a dissected chameleon's resonator](image)

**Figure 2.1.** A ventral side image of a *Chamaeleo dilepis*, flapneck chameleon’s resonator.
Figure 2.2. A lateral side image of a Chamaeleo dilepis, flapneck chameleon’s resonator.

After ventral and lateral photos and length measurements of the resonator from each species were recorded, one resonator from each species underwent a sagittal section, while retaining parts of the attached trachea. Our goal was to preserve the whole resonator while in its fully extended/inflated state.

Injecting silicone failed to blow-up the air sac completely due to its high viscosity, and blasting air from an aerosol can into the resonator only worked temporarily before deflating. Finally, a 10% formalin solution was injected through a syringe into the resonator. Two small, natural openings near the top of the resonator allowed for the syringe to be relatively easily placed for injecting the solution. Once the resonator air sacs were fully extended from the 10% formalin inside, they were completely submerged in small jars of the 10 % formalin. The jars of soaking resonators were left untouched for 3-5 days in a cabinet. This was to insure that the resonators were able to completely fix as well as remain in the desired form. After the waiting period, the fully stiffened resonators were removed from the jars to undergo syncroscopy. Syncroscopy develops high resolution and magnitude 3-dimensional digital images. Next, with Dr. John Andersland’s direction, I used syncroscopy to photograph the morphological details of
the resonators’ physical form at a high resolution and magnitude. The syncroscopy photographs can be found in the following results chapter.
## CHAPTER 3

### RESULTS

<table>
<thead>
<tr>
<th>Chameleon Species</th>
<th>Snout-Vent Length (mm)</th>
<th>Resonator Length (mm)</th>
<th>Ratio of Res. Length (mm)</th>
<th>Hooter??</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Furcifer pardalis</em>, Ambanja</td>
<td>131</td>
<td>10</td>
<td>7.63</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Chamaeleo melleri</em>, Mellers</td>
<td>207.5</td>
<td>17</td>
<td>8.19</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Chamaeleo calyptratus</em>, Veiled</td>
<td>123</td>
<td>9</td>
<td>7.32</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Chamaeleo pardalis</em>, Panther</td>
<td>128.5</td>
<td>1</td>
<td>0.78</td>
<td>Yes</td>
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<tr>
<td><em>Furcifer pardalis</em>, Nosy Be</td>
<td>149</td>
<td>1</td>
<td>0.67</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Furcifer rhinoceratus</em>, Rhino</td>
<td>151</td>
<td>0</td>
<td>0.00</td>
<td>No prediction</td>
</tr>
<tr>
<td><em>Chamaeleo dilepis</em>, Flapneck</td>
<td>112.3</td>
<td>11.5</td>
<td>10.24</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Chamaeleo rudis</em>, Side striped</td>
<td>133</td>
<td>1</td>
<td>0.75</td>
<td>No</td>
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</tbody>
</table>
**Figure 3.1.** A table organizing the measured descriptions different chameleon species’ intact resonators and sagittal-sections.

<table>
<thead>
<tr>
<th>Species</th>
<th>Resonator Length</th>
<th>Snout-Vent Length</th>
<th>Correlation</th>
<th>Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chamaeleo jacksonii</em>, Jackson’s</td>
<td>106</td>
<td>1</td>
<td>0.94</td>
<td>No</td>
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<tr>
<td><em>Chamaeleo quadricornis</em>, 4 horned</td>
<td>120</td>
<td>0</td>
<td>0.00</td>
<td>No</td>
</tr>
<tr>
<td><em>Rhampholean brevicaudatus</em>, Pygmy</td>
<td>45</td>
<td>4</td>
<td>8.89</td>
<td>Yes</td>
</tr>
</tbody>
</table>

It can be inferred from the table’s data that all chameleon species known to hoot possess a resonator. Chameleon species recognized as a “hoot” with a resonator are the *Furcifer pardalis* (Ambanja), *Chamaeleo melleri* (Mellers), *Chamaeleo calyptratus* (Veiled), *Chamaeleo pardalis* (Panther), *Furcifer pardalis* (Nosy Be), *Chamaeleo dilepis* (Flapneck), and *Rhampholean brevicaudatus* (Pygmy).

The Meller’s chameleon possessed the longest resonator length of 17mm and the longest snout-vent length of 207.5mm. However, a lack of correlation exists between increased body length/size and increased resonator length, or being a hooter at all.

For example, the rhino chameleon (*Furcifer rhinoceratus*) has the second longest snout-vent body length of 151mm, yet it possesses no resonator, nor is it known to produce the hoot sound. Furthermore, the data confirms that chameleon species, like the rhino chameleon, unknown to hoot are found with significantly smaller seized or
nonexistent resonators (0-1mm on average). These non-hooter types consist of *Chamaeleo rudis* (Side striped), *Chamaeleo jacksonii* (Jackson’s), *Chamaeleo quadricornis* (4-horned), and *Furcifer rhinoceratus* (Rhino). The comparative analysis of the various dissected chameleons represented highlights the fully formed resonator versus the smaller, less noticeable or nonexistent resonator. Such results will aid in determining why some chameleons have the sound producing mechanism, some are partially there, and some not at all.

Information from Figure 3.1 is displayed in the bar graph below, Figure 3.2, depicting the correlation between the different chameleon species’ snout-vent length and resonator length.

**Figure 3.2.** A bar graph illustrating the comparison between resonator length (mm) and snout-vent length (mm) in various chameleon types.
My anticipated outcome is to contribute this new data and understanding to science’s knowledge of this animal’s communication in its environment, to supply another piece of support to science’s knowledge of chameleon vocalization. Furthermore, as the most primitive lizard, this could make head way on the evolutionary phylogenetic tree of when and how lizard sound production and communication evolved.

Figure 3.3. An image of a Chamaeleo dilepis, flapneck chameleon, resonator before (left) and after (right) syncroscopy.

Figure 3.4. An image of a Chamaeleo melleri, Meller’s chameleon, resonator before (left) and after (right) syncroscopy.
Figure 3.5. An image of a *Chamaeleo dilepis*, flapneck chameleon, resonator before (left) and after (right) syncroscopy.

Figure 3.6. An image of a *Chamaeleo dilepis*, flapneck chameleon, resonator before (left) and after (right) syncroscopy.

Figure 3.7. An image of a *Chamaeleo pardalis*, ambanja chameleon, resonator before (left) and after (right) syncroscopy.
Figure 3.8. An image of a *Chamaeleo calyptratus*, veiled chameleon, resonator before (left) and after (right) syncroscopy.

Figure 3.9. An image of a *Chamaeleo melleri*, Meller’s chameleon, resonator before (left) and after (right) syncroscopy.

Figure 3.10. An image of a *Chamaeleo jacksonii*, Jackson’s chameleon, resonator before (left) and after (right) syncroscopy.
CHAPTER 4

DISCUSSION

Based on the observations and data collected by my research results, it can be concluded that all chameleon species known to hoot possess a resonator. These species consist of *Furcifer pardalis* (Ambanja), *Chamaeleo melleri* (Mellers), *Chamaeleo calypttratus* (Veiled), *Chamaeleo pardalis* (Panther), *Furcifer pardalis* (Nosy Be), *Chamaeleo dilepsis* (Flapneck), and *Rhampholean Brevicaudatus* (Pygmy). This is a novel advancement in the understanding of the mechanism by which certain species of chameleons communicate. However, with this novel inference comes more questions – how exactly does the resonator work, what is its influence on a chameleon, and ultimately, why did the resonator evolve?

Kenneth Barnett’s research data suggests that chameleons respond to low frequency sound vibrations, no higher than “a middle C” in range (Barnett, Cocroft, Fleishman, 1999). This relates to the low frequency, infrasound wavelengths used by many animals, such as elephants, certain birds, and spiders, as a form of interspecies communication (Hill, 2008). Infrasound is sound that ranges from 20 hertz to 0.001 hertz, stretching beyond the lowest limits of human hearing. Animals benefit from recognizing or producing infrasound for its ability to travel long distances and through interferences (Mack & Jones, 2003).
With each movement of the air sac, a chameleon’s resonator could possibly be transferring infrasound wavelengths that send vibrations along a tree branch or other surface, to communicate with a potential mate, to warn other chameleons that a predator is approaching, to convey that food has been found, or for many other possible reasons.

The cassowary, a large, flightless bird native to the tropics of Australia and New Guinea, produces a unique, booming sound at very low frequencies. Recordings of species, *Casuarius casuarius* (Southern cassowary) and *Casuarius bennetti* (Dwarf cassowary) captured sounds as low as 32 and 23 hertz, barely making the audible range of humans (Mack & Jones, 2003). However, in groups the cassowaries’ “boom” has been described to feel like an earthquake, vibrating through the ground. These ancient birds are known to live solitary lives, and their low frequency vocalization is an ideal form of communication for a species spread out through a dense rainforest.

Scientists suspect the cassowaries’ booming sound is aided by the tall casques, or horn-like crests, that rise from the bird’s head. All cassowary species develop casques at a young age, and the crest-like structures are made up of a firm material covered in thick keratin. It has been proposed that a casque works to amplify sound by having a soft keratinous covering of one density and a fluid-filled center of a different density (Mack & Jones, 2003).

Therefore, as sound passes through the resonating crest, they will vibrate differently to the incoming wavelength and the degree of difference in their response could tell the bird about the sound (Mack & Jones). Veiled chameleons, known to create the low frequency “hoot” sound, possess very similar skull crest structure and appearance to the cassowary’s casque. This could indicate a connection to chameleon
sound production. Figures 4.1 and 4.2 depict the skull and crest structure in cassowaries and a veiled chameleon.

**Figure 4.1.** The skull of an Australian cassowary.
The head crests of chameleons may hold similar functions and communicative significance to a cassowary’s casque, and future studies are needed to compare the two animal’s head crests further. Cassowary studies confirm that females tend to have larger casques than males (Mack & Jones, 2003). A future study would be to compare the crests of chameleon males and females of the “hooter” species. Another important study would be to place a male and female of the same “hooting” species together, in order to observe potential behaviors and reasons that would cause the chameleons to create low frequency vibrations.

Furthermore, dinosaur crests may have functioned similarly in receiving these low calls. The structures of fossilized ear bones in certain dinosaurs point to them hearing frequencies much lower than those detectable by humans (Bakker, 1998). Types of
"duck-billed" dinosaurs, such as the *Corythosaurus* and *Parasaurolophus*, which lived more than 65 million years ago, had similar crests. Many scientists think they used these for sound modification or amplification. One study in the 1990s detailed the acoustics of the *Parasaurolophus* crest. Despite having no vocal organs, the plant-eating dinosaur may have been able to produce deep, low-frequency sounds using resonating air cavities (Bakker, 1998). A Casper College study, *Channeling the Thunder in the Thunder Lizards: Cranial/Cervical Adaptations for Infra-Sound Control in Apatosaurine Dinosaurs* (1998) found that an aptosaurine specimen possesses a vibratory shell in its cheek and neck ribs. The research suggests that heavy soft tissue layers lying on the outer cranial surfaces are able to dampen the vibrations. This information provides greater direction in our research of possible physical and survival adaptations that shape how chameleons produce low frequency sound.

Charles Darwin repeatedly stressed that evolution is far from perfection, and the 1965 Noble Peace Prize winner, François Jacob, first explained these evolutionary imperfections as analogous to tinkering. Jacob’s revolutionary concept proposed that nature functions by integrations (Jacob, 1994). Instead of forming a mechanism from scratch, natural selection does what it can from the materials at its disposal, similar to a “tinkerer”.

It works on what already exists, either transforming a system to give it a new function or combining several systems to produce a more complex one. This process is not very different from what evolution performs when it turns a leg into a wing, or a part of a jaw into pieces of ear (Jacob, 1994). For example, nothing compares so closely to the human eye as the octopus eye, but neither evolved the same way. The photoreceptor cells
of the human eye’s retina point away from the light, while in mollusks they point toward the light (Hill, 2008). Although they are derived differently, both types of eyes are complex and equally solve the problem of photoreceptors. More signs to Jacob’s concept of evolution by tinkering are evident from the various organisms that are also found to produce the low frequency type vibrations made by “hootling chameleons”. Although these animals may have evolved differently, they each produce low frequency sound for similar survival purposes. A comparative analysis to the vocalization and sound producing mechanisms in other animals including birds, mammals, and other reptiles emphasizes the resonator’s significance to chameleons. Overall, this is a critical gateway. By studying and comparing the sound producing mechanisms of other animals, we can come closer to answering the ultimate question of how the chameleons’ resonator came to be.

Future research on the chameleon resonator is needed. The following chapter outlines my current and future research plans, including clearing and staining the chameleon tissue and nerves as well as scanning electron microscopy studies over certain skin samples.

I also plan to present my findings at future research conferences such as the Kentucky Academy of Science conference and Alltech Young Scientist conference. During the previous spring semester, I showcased my research project at the 42nd annual WKU Student Research conference.

It should also be noted that as the first time such a project has been carried out, there have been challenges. All chameleons are protected by the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). This
international organization regulates human use of chameleons, which explains why it is so difficult to come across chameleon carcasses for lab work and why I do not have every species of chameleon in my survey.
CHAPTER 5

FUTURE WORK

Further research is necessary for an explanation of how the resonator in chameleons functions as well as its adaptive significance. A focus on the chameleons’ other anatomical components, such as the tissues, nerves, bone, and cartilage, is being directed, in order to better understand the targeted resonator area as a whole. By studying these characterizations, we can see exactly what makes up the structure of the resonator and related sound producing mechanisms. This could also show how the resonator connects to other parts of the body, which may aid in creating the hooting sound.

Clearing and staining of whole small vertebrates for displaying bone and cartilage are techniques used extensively in comparative vertebrate osteology (Song & Parenti, 1995). For example, the cleared and stained preparations of whole fish specimens provide osteological data that is used as a major resource in studies of fish phylogeny. One such clearing and staining method we are currently using and plan to continue in the future is through an innovative method for enzyme clearing and staining of whole animal specimens for the simultaneous demonstration of bone, cartilage, and nerves. Yet, a research method that reliably provides a cleared specimen with stained bone, cartilage, and peripheral nerves for study of the nerve-skeletal relationships has never been described for chameleons.
Such a method must use a formalin-fixed, alcohol-stored specimen that remains stained during long-term storage, and it must be straightforward enough to allow for preparation and results within a reasonable amount of time.

Past research shows comparative anatomical studies of nerves alone, or in combination with bone and cartilage, are rare due to a lack of reliable, repeatable protocol for preparing specimens that demonstrate all three characters. However, one study by Jiakun Song and Lynne R. Parenti (1995), published in the *American Society of Ichthyologists and Herpetologists*, does complete all three steps in their research of clearing and staining small species of fishes. Their method consists of using tissue-specific stains such as Sudan Black B, alcian blue, and alizarin red to provide evidence for the different tissue types in the specimen. Furthermore, it provides reliable, repeatable results. Yet, since Song and Parenti’s technique primarily applies to its tested specimens of small species of gobioid fishes, we must modify and adapt the method to apply to our chameleon specimens. All 20 fish specimens prepared by Song and Parenti were between the size range of 16.1-232mm, and our chameleon specimens’ range in size of 45-208mm. Although there is little size discrepancy, the fish specimens tend to have more visceral fat to account for in the timing of the experiments, whereas the chameleon specimens have little to no visceral fat to account for in the experiments.

It is critical to our experiment to adjust solution proportions and concentrations to compensate for the physical discrepancies between the specimen types, in order to obtain accurate and consistent results. Through trial and error, we are gaining progress in reaching a standard protocol for the clearing and staining of whole chameleon specimens.
By using Song and Parenti’s method as a model for the clearing and staining of various chameleon species, we will attempt to verify the exact kinds of muscle tissue and nerves that make up the chameleons’ vocal mechanisms, including the resonator, as well as its connections to other areas of the chameleon body. Such confirmation of the precise morphological composition will convey proof of how it works. The following paragraphs outline the adapted clearing and staining procedure.

The same eleven species types listed in Figure 3.1 were chosen to undergo the triple-staining procedure. Prior to fixation, the outer skin layers, including the skin fold covering the eyes, was completely removed from each chameleon’s body. The skinned chameleons were fixed in a 10% formalin solution for three to five days, depending on the size of the specimen. Afterwards, the specimen was washed in several changes of distilled water. The purpose of this step was to thoroughly remove the fixative solution from the chameleon.

For the cartilage staining, the specimen was placed in an alcian blue solution for about five to seven days or until the stain has been absorbed. The alcian blue solution was composed of 1120mL ethanol (80%), 280mL glacial acetic acid (20%), and 1.40g alcian blue dye (0.01%).

The solution was made fresh within the previous week. Next was the rehydration step. The specimen was transferred through two changes of 95% ethanol, two to three hours in each change, then through successive solutions of 75%, 50%, and 30% ethanol for two to three hours each, finalizing with two to three changes of distilled water for two to three hours.
The next step in the procedure was the muscle digestion phase. The specimen was placed in trypsin solution for several days to weeks depending on specimen size. The enzyme solution was made up of 30% sodium borate buffer - 20-25 grams of sodium borate saturated in 1000mL of distilled water- along with three pinches (three 1/16 teaspoon sized scoops) of trypsin enzyme. Every two to three days the enzyme solution was changed and the specimen container would be cleaned to prevent contamination until the bone and cartilage was visible.

Following the muscle digestion stage, the potassium hydroxide (KOH) environment was built in order to wash away the enzyme solution as well as to help the alizarin red S to penetrate the bone. The specimen was transferred to 0.5% aqueous potassium hydroxide for about one hour, but the length of time kept in solution was flexible. For the next bone staining step, the specimen was placed in alizarin red S solutions for about 24 hours or until the bones are distinctly red or reddish purple.

The alizarin red S solution was formed by slowly adding alizarin red S powder to 0.5% KOH while stirring until the solution turns deep purple. Song and Parenti advise to not overly stain during this step, due to undigested muscle possibly becoming reddish (Song & Parenti, 1995).

Destaining and bleaching made up the next steps of the procedure. The specimen are transferred to 0.5% KOH for 30 minutes, and then transferred to a bleaching solution for about one hour. The bleaching solution consisted of several drops of 3% H₂O₂ in 1000mL 0.5% KOH solution. Afterwards dehydration took place. Specimens went through solutions of 30%, 50%, and 70% changes of ethanol, and the specimen was left
in each solution for about 30 minutes to one hour. Now the specimen would be ready for nerve staining.

Nerve staining began by placing the specimen in 30-50% Sudan Black B solution for about five to ten hours depending on the size of the chameleon. Saturated Sudan Black B solution was made with 70% ethanol filtered and diluted with seven to five parts 70% ethanol to form a 30% or 50% Sudan Black B working solution. The concentration and length of time a specimen was kept in the Sudan B Black solution was flexible. However, in general, larger specimens needed to be placed in a more concentrated solution for a longer period of time than do smaller specimens. The solution was recommended to be freshly made and not more than two weeks old. Once nerve staining was complete differentiation follows. This was the critical step that determined the quality of the nerve staining.

For differentiation, the specimen would be destained by dipping it in 70% ethanol for two to five minutes to wash off excess Sudan Black B solution from the surface of the specimen, without destaining small, peripheral nerve branching. Specimens were left overnight in 50% ethanol to destain gradually. However, this step would be stopped once the remaining muscle fibers become clear and the solution is light blue to clear. The specimen was then placed in 0.5% KOH overnight. Lastly, it was time for storage of the specimen. This was conducted by first positioning the specimen in 70% glycerin and 30% 0.5% KOH, then storing it in 100% glycerin for long-term storage.

Aside from following the described clearing and staining protocol, another research method I plan to continue using for the project is scanning electron microscopy (SEM) work with WKU professor, Dr. John Andersland. I began SEM work on the
chameleon specimens during the summer of 2012. Dr. Andersland trained me on how to use the SEM microscope. My SEM work consists of closely examining pieces of skin cut from the front feet, back feet, and tail of the various chameleon species types. Pictures are taken of each anatomical area, and the visual results are compared and studied for signs of innervations of dome pressure receptors, superficial neuromasts, and nerve endings. Pressure receptors in these areas could indicate a possible pathway for vibration signals to be received/detected. Thus far, both microvillus looking feelers and smooth textures have been observed in different areas of the front feet, back feet, and tail. It is unclear if there is damage or not on the specimens.

Possible damage could be due to the dissection, handling, or frequent freezing and unfreezing of the specimens. Future SEM work is necessary for determining more information on whether there is a possible nerve connection through the chameleons’ front feet, back feet, or tail. Such research methods will highlight more in depth components that make up chameleon sound producing mechanisms and their connections to other areas of the chameleon body. This will aid in the ultimate goal of understanding the full function of a chameleon’s resonator.
REFERENCES


