A Proposed Methodology for the Cardiac Analysis of Parasitically Infected Cuban Treefrogs

Thomas Horton

Florida Southern College

Abstract

Cuban treefrogs are a successful invasive species within the state of Florida, which have harmed Florida's native ecosystem (Johnson, 2017). The parasite *Apharyngostrigea pipientis* has been found using the Cuban treefrog tadpole as a secondary intermediate host during development. During this process, the parasite encysts around the heart and mesenteries. It is unknown how these parasites impact the survivability of the tadpoles and whether their presence impedes cardiac function. This paper proposes a methodology to examine both heart function, and myocardial thickness as a result of the presence of *A. pipientis.* Due to the spring semester not having enough rain to support tadpoles, as well as the COVID-19 pandemic, only parts of the protocol were able to be done. Pulse oximetry on tadpoles found that Cuban Treefrog tadpoles between Gosner stages 26 and 39 had an average oxygen saturation of 97.2%. Parameters for the successful use of pulse oximetry on tadpoles was generated, alongside a protocol for histological analysis of tadpole myocardium and endocardium.

Introduction:

Osteopilus septentrionalis, otherwise known as the Cuban treefrog, is an invasive species to Florida, which first appeared in Florida records in the 1920s (Johnson, 2017). Since this time, the Cuban treefrog has expanded to live in much of the state, and is currently expanding into the Panhandle (Johnson, 2017). Cuban Treefrogs are considered to be an invasive species (Johnson, 2017). Due to the presence of climate change, it is possible *O. septentrionalis* could inhabit much of the South Eastern U.S. in the coming decades (Johnson, 2017).

While Cuban treefrogs are mostly observed in urban environments, they are found everywhere and are causing a great deal of harm to Florida's native ecosystem (Johnson, 2017). *O. septentrionalis* are predators of Florida's native treefrog population, and have been linked to

population declines experienced amongst native species (Johnson, 2017). An example of one species being detrimentally affected by the presence of the Cuban treefrog is the squirrel treefrog, which has been shown to have a significantly decreased survivorship while in the presence of *O. septentrionalis* (Johnson, 2017).

Cuban treefrog tadpoles develop very quickly, only requiring 3-4 weeks in warmer weather to metamorphosize (Johnson, 2017). Since the mating season for *O. septentrionalis* stretches from March to October (and perhaps even year-round in South Florida) (Johnson, 2020), some females can lay around 15,000 eggs in a single season (Johnson, 2017). Typically, Cuban treefrogs are stimulated to mate by rainfall, such as the tropical rainfall which occurs during the Summer months (Johnson, 2017). These tadpoles are considered to be superior competitors when compared to tadpoles of the native southern toad and the green treefrog (Johnson, 2017).

Cuban Treefrogs have been observed to be targets for multiple parasites during development (Ortega et al., 2015). One such parasite is *Apharyngostrigea pipientis,* which uses the ghost rams-horn snail (*Biomphalaria havanensis*) as the primary intermediate host and the Cuban tree frog as a secondary intermediate host (G. Langford, personal communication). *A. pipientis* cercariae have not been observed to penetrate the skin of tadpoles, and it has been concluded that they penetrate through either the branchial or digestive epithelium (Oliver, 1940).

Post-infection, *A. pipientis* was observed to undergo metamorphosis in the body cavity before encysting around the heart and mesenteries (Oliver, 1940). This process of metamorphosis has been recorded down as taking anywhere between 18 and 23 days post-infection (Oliver,

1940). It is unclear how, if any way, *A. pipientis* impacts the survivability of Cuban treefrog tadpoles, specifically in relationship to functional impacts made to the heart.

In 1891, Haycraft discovered that external pressure applied to amphibian hearts resulted in impaired function. Specifically, during diastole, in which cardiac muscle is in a state of relaxation, external pressure altered the internal shape of the amphibian heart (Haycraft, 1891). During Diastole, the heart's chambers fill with blood, and a disruption to the shape of the heart could be detrimental in amphibians due to the structure of their three chambered heart.

While a single, stronger ventricle has the advantage of being able to force a greater amount of blood to the lungs for oxygenation, it has the downside of allowing oxygenated and deoxygenated blood to mix together (Molnar & Gairm, 2012). However, due to the internal structure of the heart, only about 17% of oxygenated and deoxygenated blood gets mixed at a heart rate of 50 beats per minute in *Bufo marinus* (Hedrick, Palioca, & Hillman, 1999). Hedrick, Palioca, & Hillman (1999) also recorded the mean arterial O_2 saturation for all conditions they tested, except for those conducted at 30 ºC, was 93% in *B. marinus*. External pressure to the heart caused by *A. pipientis* cysts may hinder the capability of the amphibian heart to maintain this high standard of arterial oxygen saturation.

In the past, researchers have measured the oxygen saturation of *Xenopus laevis* frogs using pulse oximetry (Goulet et al., 2010). Goulet et al. (2010) were testing the change in oxygen saturation and heart rate over time relative to the *X. laevis* individuals' time in anesthesia, and how that impacted cardiovascular function. Pulse oximetry, therefore, is capable of being used effectively on small animal models.

Pulse oximetry is a non-invasive and painless test of oxygen saturation in the blood, which makes it a commonly used tool in medical practice (Johns Hopkins Medicine, 2020). Pulse oximeters use light absorption between oxygenated and deoxygenated blood to determine overall oxygen saturation (Gotter, 2017). Infrared and red light are used in pulse oximetry due to oxygen absorption between oxygenated and deoxygenated hemoglobin differing greatly (Hoffman, 2017). Such a difference in absorption allows the pulse oximeter to measure the saturation of oxygen within the bloodstream.

Due to their nature as an invasive species, Cuban treefrogs are excellent for research purposes. Not only are they available much of the year (Johnson, 2020), but they also reproduce in very large numbers as females lay between 100 and 1,000 eggs at a time (Johnson, 2020). In addition, Cuban treefrogs develop rapidly in warmer weather, which is vital in the use of pulse oximetry, as the tadpoles must be large enough to completely cover the sensor on the pulse oximeter in order to gain a consistent reading.

Another methodology for monitoring the cardiovascular health of small animal models has come through histology. In 2017, Marshall et al. monitored cardiac tissue recovery in adult anurans following injury to the heart. The purpose of the experiment was to determine approximately where in evolutionary phylogeny the ability to regenerate damaged cardiac tissue was lost (Marshall et al., 2017). The team performed immunohistochemical staining on the samples, and determined the regenerative capacity for anuran cardiac tissue was similar to that found in mammals (Marshall et al., 2017).

Prior research has shown that anurans are capable of being monitored through pulse oximetry, and histological analysis. This paper proposes a protocol for the analysis of Cuban

Treefrog tadpoles infected with *A. pipientis* through pulse oximetry, and histological analysis of cardiac tissue. Inadequate rainfall between January and early March led to constraints in progressing through this research. All stages of the methodology prior to "tissue embedding" were performed. However, due to the COVID-19 pandemic, later sections of the methodology were not completed.

Materials

Capillary Tubes

Collection tubes (with corks or cotton balls)

Dissection microscope

Ethanol (70%, 90%, and 100%)

Fish net

Forceps, straight

Forceps, curved

Formalin 10%

Hot plate

Kimwipes

Large net

Large plastic bucket

Large plastic container

Light Microscope

Medium sized plastic container

Microdissection scissors

Oven

Petri Dishes

Pulse Oximeter

Q-tips

Thermo Scientific HM 325 Rotary Microtome

Thermometer

Tissue-Tek VIP processing/embedding medium (paraffin)

Xylene

Methods

Collection of O. septentrionalis Tadpoles

Cuban treefrog tadpoles can be consistently caught close to campus within the mating season, as Common Ground Park has enough standing water to be able to sustain the frogs during that time. Tadpoles were found in a standing water runoff, or in the gentle stream that runs through the park. Cuban treefrog tadpoles can be found in other locations such as roadside ditches, streams, etc.

Figure 1: Collection of tadpoles at Common Gorunds Park.

Upon discovery of a population of tadpoles, a large plastic bucket was filled about halfway with water from the local area. A large net was used to cast through the water and collect the tadpoles. While in the net, tadpoles were gently picked up one-at-a-time using a non-abrasive tool or finger, and transferred into the pre-prepared bucket.

Collection of B. havanensis Snails

Snail collection was performed at the same time as tadpole collection. While making broad, but gentle sweeping motions with the large net, the tops of the vegetation below the

waterline were brushed to stir up the most snails for collection. Similarly to the tadpoles, a non-abrasive instrument or finger was used to transfer the snails into their respective container.

Figure 2: Image of ghost rams-horn snail taken under dissection microscope on 2/13/20

Ghost rams-horn snails can be identified by their discoidal shape, greyish-white colour and irregular growth striations (Benson et al., 2019). When freshly caught, these snails also have transparent shells (Benson et al., 2019). The aperture of the shell is relatively flush against the side of the whorl and does not protrude off to one side of the whorl. Only ghost rams-horn snails should be collected.

Ghost rams-horn snails were kept in a separate container to that of the Cuban treefrog tadpoles. The container was filled with local water, so as not to injure or kill the snails with water that does not match their native environment.

Figure 3: Image taken during tadpole collection. This was at the end of a long sweep made through the water.

Storage of O. septentrionalis Tadpoles

Tadpole storage was kept primarily outside. Mesocosms worked well for the storage of

large numbers of individuals, as they typically contained enough water and nutrients for the tadpoles to develop while being free of predators. Tadpoles were kept in these containers up until Gosner stage 26, as at this point they are large enough to generate readings in the pulse oximeter.

Tadpoles of Gosner stage 26 or later were kept indoors to be used in pulse oximetry and later histology. During this time, tadpoles were left in a bucket containing water from the mesocosm and nutrient rich food comprised of decomposing vegetation and algae. No tadpoles stored in this manner should be developed past Gosner stage 42, due to complications involving the pulse oximeter.

Storage and Examination of B. havanensis Snails

Within the immediate hours following collection of ghost rams-horn snails, the snails were separated off into collection tubes. These tubes were prepared with water obtained at the site of snail collection and filtered through a coffee filter so as not to introduce unnecessary particles and lifeforms. The collection tubes were filled between half and two-thirds of the way full, and the container was sealed either with a cork fitted with an air hole or with a cotton ball. The collection tubes were stored upright overnight and examined in the morning.

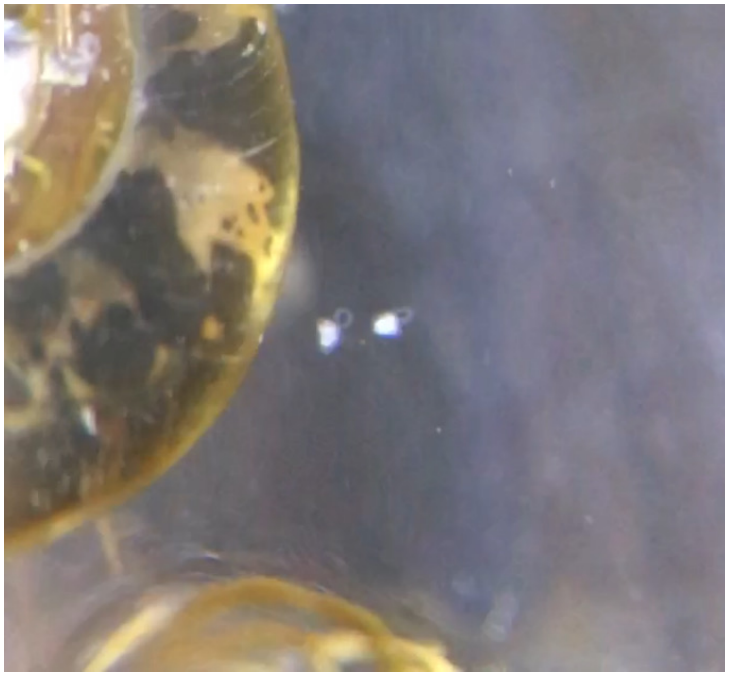

Figure 4: *A. pipientis* cercariae free swimming in a collection tube. Taken on 2/13/20

The following day, each tube was examined under a dissection microscope making sure to

plug the top of the container with a finger to prevent any water from leaking out. If the snails were infected with *A. pipientis*, then small swimming cercariae were visible in the water.

A. pipientis cercariae were identified by their distinctive swimming pattern. They are propelled by a vibratory motion through the water. The center of this motion comes from the node at the end of the tail stem (Oliver, 1940). This action causes the cercariae to primarily be moved tail first through the water (Oliver, 1940). Upon discovery of infected snails, quick action was taken to place the infected snail in the same mesocosm as the Cuban treefrog tadpoles. This gave the snail an opportunity to infect the tadpoles.

Pulse Oximetry of Tadpoles

For use in the pulse oximeter, only tadpoles between Gosner stages 26 and 39 were analyzed. The fish net was used to pick up a single tadpole, which was transferred using a non-abrasive tool or finger into a capillary tube. The tadpole was gently pushed down to the bottom of the tube, while the tube was held horizontally so as not to injure the tadpole.

The capillary tube was sealed off and the bottom of the tube was placed into the pulse oximeter and the device was started. Tadpole specimen were aligned so their body was over the sensor. The oxygen saturation and heart rate were recorded for each tadpole. Repeat this step 5 times with 30 second intervals between readings for each individual.

Euthanasian, measurement, and Dissection of Tadpoles

A batch of MS-222 solution buffered with sodium bicarbonate was prepared by mixing 30mg of MS-222 and 60 mg of sodium bicarbonate per 1L of D.I. water (Brown, 2020). Following pulse oximetry, tadpoles were immediately transferred over into the MS-222 solution.

Tadpoles were left in the MS-222 for 15 minutes before removal, as per standard protocol from Brown University (2020).

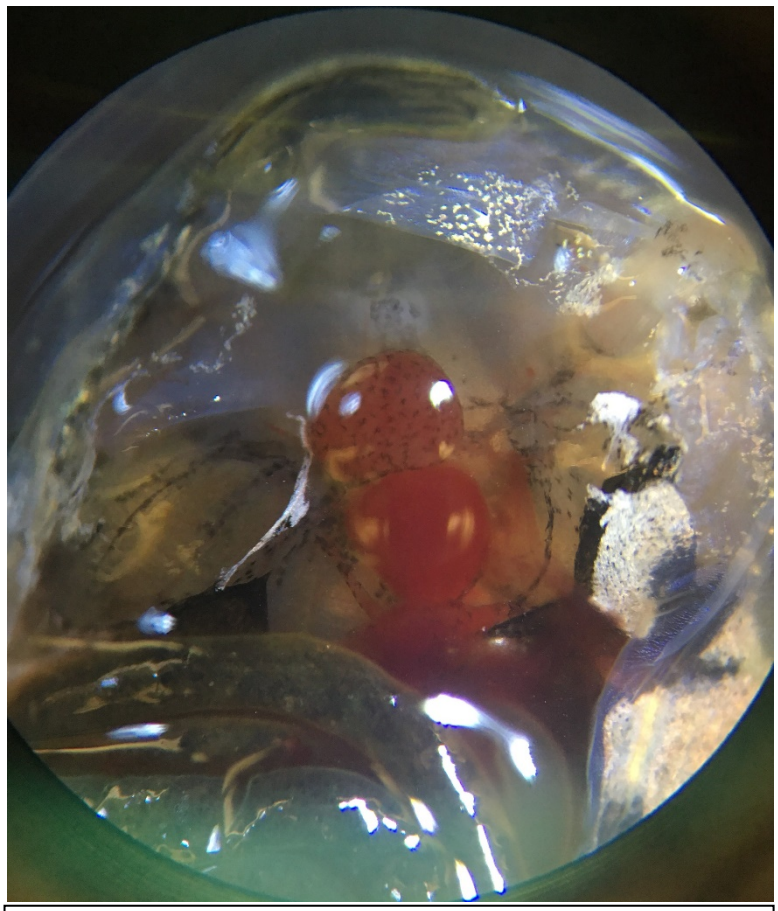

Figure 5: Picture of a Cuban treefrog thorax taken on 3/2/20. The silvery pericardium can be seen around the heart.

The euthanized tadpoles were then placed in a petri dish and examined to determine Gosner stage. Measurements of length (from head to tail) and width (from side to side while the tadpoles had their ventral side facing upwards) were taken using a standard ruler. Following this, the tadpoles were moved under the dissection microscope.

Before dissection, a slightly damp Kimwipe was placed between the petri dish and the tadpole, as Patmann et al. (2017) noticed this stabilized the sample during the dissection process. Using dissection scissors and forceps, the sample was rotated so the ventral side was up. A cut along the midline was made sagitally so as to expose the abdomen and thorax. Using the microscope, the abdomen and thorax were examined for signs of infection.

A. pipientis metacercariae undergo encystment around the heart and mesenteries (Oliver, 1940). Careful attention was given to the area surrounding the pericardium, as this was the most likely area for *A. pipientis* metacercariae to be found. These cysts are characterized by a thick,

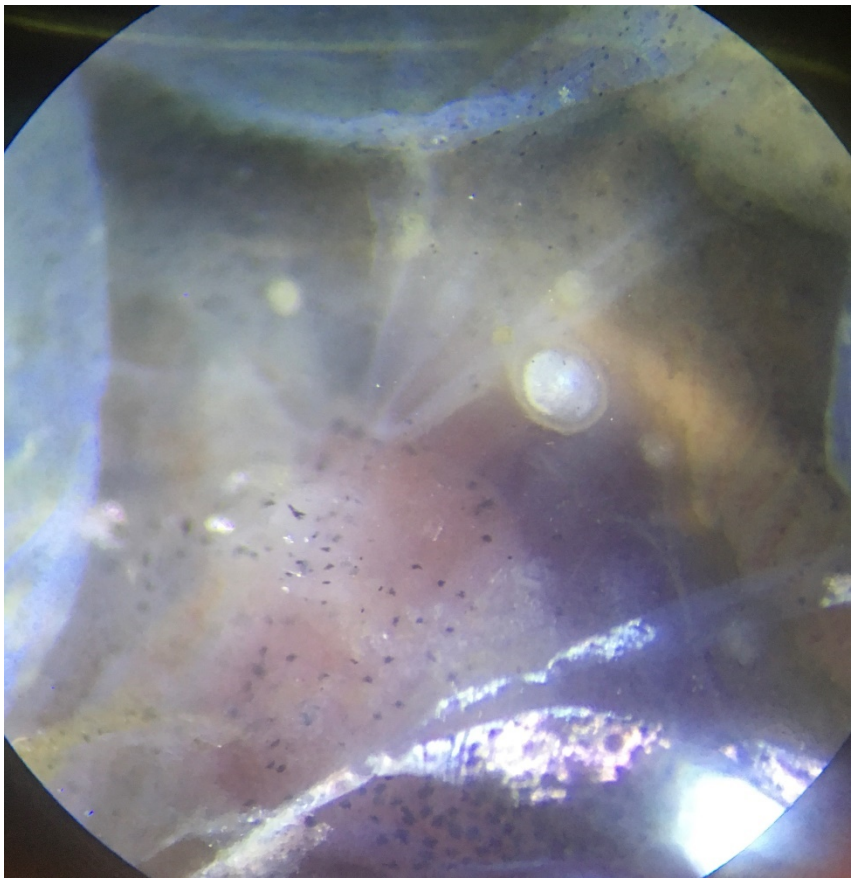

Figure 6: *A. pipientis* cyst found outside the pericardium. Picture taken on 2/5/20.

resistant wall, which protects the larval body from its environment (Oliver, 1940). Infection status was recorded down for each individual, including the number and location of *A. pipientis* metacercariae.

Preparation of Tadpole Samples for Histological analysis

Following dissection of the samples, tadpoles were placed in a 10% Formalin solution for 24 hours (St. Michael's Hospital, n.d.). Tadpoles were fixed immediately following dissection to prevent the tissue from being irreversibly damaged due to decay (Lisowski, 2019). The fixative volume was greater than 5 times the tissue volume so as to ensure proper fixation (St. Michael's Hospital, n.d.).

Following fixation, a progressive dehydration series was performed on the samples. By performing this series, it was possible to remove the water from the tissue while minimizing distortion to the tissue. The steps of the dehydration can be found in *Table 1.*

Xylene was employed to clear the alcohol present in the tissue and to remove fat which inhibited paraffin introduction (Lisowski, 2019). Due to the dilution of the xylene with alcohol

during this process, multiple charges were required (Lisowski, 2019). The protocol for clearing can be found in *Table 1.*

Stage of Preparation	Number of Charges	Solution	Time per charge
Dehydration	1	70% ethanol	15 minutes
	1	90% ethanol	15 minutes
	2	100% ethanol	15 minutes
	1	100% ethanol	30 minutes
	1	100% ethanol	45 minutes
Clearing	2	Xylene	20 minutes
	1	Xylene	45 minutes
Paraffin Infiltration	2	Liquid paraffin	30 minutes
	1	Liquid paraffin	45 minutes

Table 1: Detailed protocol for the dehydration, clearing, and paraffin infiltration of tissue sample. Protocol adapted from Rolls (n.d.).

Following the clearing, specimens were ready for paraffin infiltration. Paraffin infiltration grants the tissue rigidity and allows it to be sectioned on a microtome (Lisowski, 2019). Three charges of liquid paraffin were prepared at temperatures between 58 and 60 ºC (St. Michael's Hospital, n.d.). Samples were then placed in solution according to the protocol in *Table 1.*

Tissue Embedding

Liquid paraffin should be prepared in an oven overnight, alongside a flat surface of ice formed from freezing water in a dish. Appropriately sized molds should be placed on a hotplate to keep warm. Before picking up a tissue sample with forceps, the tips of the forceps should be warmed on the hot plate. Pour paraffin into the mold, and be sure not to overfill. The mold should be kept on the hot plate in order to prevent the paraffin from solidifying.

Place the specimen in the mold and orient the specimen with the ventral side facing upwards. Specimens should consistently be oriented in the same manner so as to allow for cutting of the sample in a coronal plane once placed on the microtome. Upon orienting the sample, place the mold on the ice plate so as to crystalize the paraffin at the bottom of the mold. Any final orientation adjustments should be made at this time. Place the corresponding cassette bottom on the mold and top off the mold with paraffin so that the wax barely connects the embedded sample to the cassette. Look through the cassette to confirm no air bubbles formed in the wax. Remove any such bubbles before proceeding. Place the mold back on ice to allow the wax to solidify and crystallize (Emge, 2013c).

Using the Microtome and Preparing Tissue Slides

Before calibrating the microtome, embedded sections along with a blank should be placed in an ice D.I. water bath. The bath will keep the paraffin wet and prevent tears to the block during the cutting process (Emge, 2013b). A 45 ºC water bath should also be prepared using D.I. water.

Calibrate the microtome ahead of time using blank slides. Make sure the microtome cuts evenly across the face of the wax, and the wax readily ribbons. Set the cutting angle of the microtome to 4º.

Transfer a cartridge onto the microtome in the required orientation to cut along a coronal plane moving cranially. Setting the microtome to 20 µm cuts, rough face the block until the block is at the depth of the heart (Emge, 2013a). During this time, make sure the specific block being worked on is ribboning well and does not produce any tears due to dehydration (Emge,

2013b). Smooth face the block by setting the microtome to 5µm cuts, and then proceed to cut samples maintaining the 5 µm cut setting (Emge, 2013b).

Upon generating a ribbon of between six and eight sections, use a pair of forceps and the handle of a long Q-tip to gently move the ribbon into the 45 ºC water bath. Maintain the orientation of the sections while they are in the water bath. This can be easily done by anchoring the ribbon to the side of the bath with excess wax (Emge, 2013b). The warm water will soften the paraffin and allow for easy transferring of the sample onto a slide.

Slides will be labelled using pencil only. With a prepared slide ready, gently separate two sections from the ribbon using forceps (Emge, 2013b). Place these samples on the glass slides and allow the samples to settle in place. Repeat this process with all sections involving cardiac tissue.

Sample Staining using Hematoxylin and Eosin (H&E)

Number of Charges	Solution	Time per charge
2	Xylene	2 minutes
2	100% Ethanol	2 minutes
1	95% Ethanol	2 Minutes
1	Water Wash	2 minutes
1	Hematoxylin	3 minutes
1	Water Wash	1 minute
1	Differentiator (mild acid)	1 minute
1	Water Wash	1 minute
1	Bluing (Using a weakly alkaline solution)	1 minute
1	water wash	1 minute
1	95% ethanol	1 minute
1	Eosin	45 seconds
1	95% Ethanol	1 minute
2	100% Ethanol	1 minute
2	Xylene	2 minutes

Table 2: Protocol for the rehydration and staining process using H & E. Adapted from Sampias & Rolls (n.d.).

The paraffinized slide should be prepared through progressive submersion of the sample in solutions according to the protocol in *table 2*. This protocol will rehydrate the sample and create the conditions for the stain H&E to be used. Following completion of the protocol, samples can be placed under a coverslip and viewed under the microscope.

Tissue Analysis of Specimen Slides

Under a light microscope, sample slides will be viewed in order to examine the cadiac tissue of the tadpole. The thickness of the myocardium will be recorded for each chamber of the heart. Analysis of the endocardium will also be performed to monitor for morphological changes occurring inside the heart.

Results

Data collection during the Spring semester was impeded by the weather. Due to the lack of rain, much of the standing water in Common Ground Park dried up. Numerous roadside ditches were examined all along Bartow Avenue and its cross streets between Florida Southern College and Winter Lake Road. No tadpoles were found in any of the nine bodies of standing water along this road. Four additional bodies of standing water were examined along Cleveland Heights Boulevard between Florida Southern College and Mikasuki Drive. None of these yielded any tadpoles or snails as well.

In an attempt to find tadpoles further South, Lakes Park in Fort Myers Florida was searched. The main lakes were all checked along with some of the smaller bodies of standing rainwater, but no Cuban treefrog tadpoles were found.

Common Grounds Park had both tadpoles and snails in January. Approximately 150 tadpoles were caught on January 7th, and a further 40 were collected on January 14th from the

same water source. Many of these tadpoles metamorphosized before they could be analyzed. A further 15 tadpoles were found in a separate and unused mesocosm outside of Polk Science on February 20th. Outside of these, no other tadpoles were found during the Spring semester.

A total of 369 snails were examined during the spring semester, and only one of them was infected with *A. pipientis.* After a few days delay due to a lack of tadpoles, this snail was added into a container with tadpoles, and left to infect them for one week. Unfortunately, none of the tadpoles exposed to the parasite in this manner developed an infection.

Over the course of the five readings separated by 30 second intervals of both heart rate and oxygen saturation, Pulse oximetry readings remained non-significantly altered between readings taken at 30 seconds and 150 seconds within the pulse oximeter (p=0.3262). The same is true for the heart rate of the tadpoles (p=0.7593). The average oxygen saturation of the Cuban treefrog tadpoles across 60 readings was 97.2 % and the average heart rate for these same readings was 75.14 bpm. Unfortunately, the sample size of individuals examined was small, only equaling 12 in total. Average tadpole oxygen saturation and heart rate may not be altered by being in the pulse oximeter between 30 seconds and 2 ½ minutes.

Unexpectedly, when Cuban treefrog tadpoles were exposed to the light from the pulse oximeter, all individuals between Gosner Stages 26 and 39 became unconscious. The exact reason behind this is unknown, but it may have contributed to the steady blood oxygen saturation and heart rate observed during this stage. Upon placing the tadpoles back in water, they remained motionless for several seconds, not responding to touch, before eventually reawakening and swimming away.

Discussion

Much of this project was inhibited by the semester in which data was collected. While the Fall semester had plenty of rainfall, the Spring semester did not. Collection of Cuban treefrog tadpoles and ghost rams-horn snails would be most easily done from August through October, which aligns with the end of the Cuban treefrog mating season (Johnson, 2020).

Pulse oximetry of the tadpoles was largely successful, but too few infected individuals (one) were found to draw conclusions as to the affect *A. pipientis* cysts have on the heart. Pulse oximetry on tadpoles less developed than Gosner stage 26 was attempted, but the tadpoles were too small to cover the sensor, and so accurate readings could not be taken. Tadpoles between Gosner stage 26 and 39 were able to cover the sensor completely and generate a reading. Tadpoles developed past stage 42 used their legs to move around the capillary tube, which prevented the pulse oximeter from being able to give a consistent reading.

Similarly to Hedrick, Palioca, and Hillman (1999), the average Cuban treefrog tadpole had an oxygen saturation well above 90%, which displays the incredible nature of the amphibian heart in separating oxygenated blood from deoxygenated blood in their single ventricle. Hedrick, Palioca, and Hillman (1999) performed their research in adult frogs, so the values generated in this project suggest the tadpole heart is just as effective, if not more so than the adult anuran heart.

Very little research has been done into pulse oximetry in anurans, the vast majority of which were with adult frogs. This paper not only proves pulse oximetry can be performed on tadpoles, but the pulse oximeter used was meant for humans and was still able to generate readings providing the tadpoles were large enough to cover the sensor.

In terms of the introduction of the parasite to the tadpolesit likely failed due to the delay between the snails being placed in the bucket, and the tadpoles being introduced to them. During this time, the snails most likely stopped shedding, and thus spreading the *A. pipientis* cercariae (G. Langford, Personal communication). The tadpoles therefore never had an opportunity to be infected.

Further research could be done on the oxygen saturation of tadpoles as they switch from breathing through gills to breathing through lungs. In pre-metamorphic tadpoles, which were studied in this experiment, the gills are the primary source of oxygen, with air breaths being secondary (Perry et al., 2001). During metamorphosis, a pH change triggers the transition for tadpoles to begin using their lungs (Perry et al., 2001). Using pulse oximetry, it would be possible to ascertain whether there is any difference in oxygen saturation during this transition as well as whether a difference exists between the oxygen saturation provided by lungs and gills.

Conclusions

While this research did not end up generating much data, a couple of valuable conclusions can be drawn. Firstly, it is possible to perform pulse oximetry on tadpoles; however, the window for performing it is limited. Secondly, oxygen saturation in tadpoles appears to be similar to that recorded in adult anurans (97.2% in Cuban treefrog tadpoles vs. 93% in adult *Bufo marinus*).

Acknowledgements

I would like to thank Dr. Langford for persisting through this experiment. It has had a large number of set-backs from both external and internal factors, and his support has been greatly appreciated. I would also like to thank Vincent Colonna, who took many trips around

Lakeland with me searching for tadpoles, as well as Blake Gee for similar reasons. Lastly, I would like to thank Kianna Jackson, who not only travelled as far as Fort Myers in search of tadpoles and took some wonderful photos, but also was a great source of support.

References

Benson, A. J., Daniel, W. M., & Morningstar, C. R. (2019). *Biomphalaria havanensis.* USGS: Science for a Changing World. https://nas.er.usgs.gov/queries/FactSheet.aspx?SpeciesID=1030

Brown University. (2020). Guidelines for Preparation, Storage, Handling and Use of Tricane Methanesulfonate (Tricane-S, MS-222). *Brown University Institutional Animal Care and Use Committee.* https://www.brown.edu/research/sites/research/files/Tricaine-Guidelines-2-7-20.pdf

Emge, D. [Donna Emge, HT-ASCP]. (2013, March 16th). *Leica Jung 820 Histocut Rotary Microtome Part 1* [Video]. YouTube. https://www.youtube.com/watch?v=uxtuS1d1wH0

Emge, D. [Donna Emge, HT-ASCP]. (2013, March 17th). *Leica Jung 820 Histocut & RM 2025 Microtome Part 2* [Video]. YouTube. https://www.youtube.com/watch?v=1oyNbHMlkdo&t=1820s

Emge, D. [Donna Emge, HT-ASCP]. (2013, March 16th). *Tissue-Tek Embedding Center – Embedding Techniques* [Video]. YouTube. https://www.youtube.com/watch?v=Nab97hfUVPc&t=997s

Gotter, A. (2017, August 2nd). *Pulse Oximetry*. Healthline. https://www.healthline.com/health/pulse-oximetry

Goulet, F., Hélie, P., & Vachon, P. (2010). Eugenol Anesthesia in African Clawed Frogs (*Xenopus laevis*) of Different Body Weights. *Journal of the American Association for Laboratory Animal Science, 49* (4), 460-463. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2919187/

Haycraft, J. B. (1891). The Movements of the Heart Within the Chest Cavity and the Cardiogram. *Journal of Physiology.*

Hedrick, M. S., Palioca, W. B., & Hillman, S. S. (1999). Effects of Temperature and Physical Activity on Blood Flow Shunts and Intracardiac Mixing in the Toad *Bufo marinus. Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches, 72* (5). DOI:10.1086/316693

Hoffman, H. (2017). *Pulse Oximetry Basic Principles and Interpretation.* University of Iowa Health Care. https://medicine.uiowa.edu/iowaprotocols/pulse-oximetry-basic-principles-and-interpretation

Johns Hopkins Medicine. (2020). *Pulse Oximetry.* Johns Hopkins Medicine. https://www.hopkinsmedicine.org/health/treatment-tests-and-therapies/pulse-oximetry

Johnson, S. A. (2017). The Cuban Treefrog (*Osteopilus septentrionalis*) in Florida. *UF IFAS.* https://edis.ifas.ufl.edu/pdffiles/UW/UW25900.pdf

Johnson, S. A. (2020). Florida's Frogs & Toads: Treefrogs (Family Hylidae). *UF IFAS.* https://ufwildlife.ifas.ufl.edu/frogs/cubantreefrog.shtml

Lisowski, A. (2019). Science of Tissue Processing. *Leica Biosystems.* https://drp8p5tqcb2p5.cloudfront.net/fileadmin/downloads_lbs/190093_Rev_A_Science_of_Tissue_Processing.pdf

Marshall, L., Vivien, C., Girardot, F., Pericard, L., Demeneix, B. A., Coen, L., & Chai, N. (2017). Persistent Fibrosis, Hypertrophy and Sarcomere Disorganisation after Endoscopy-guided Heart Resection in Adult Xenopus. *PLoS ONE, 12* (3). https://doi.org/10.1371/journal.pone.0173418

Molnar, C., and Gair, J. (2012) *Concepts of Biology*. OpenStax.

Oliver, L. (1940). Life History Studies on Two Strigeid Trematodes of the Douglas Lake Region, Michigan. *The Journal of Parasitology, 26* (6), 447-477. https://www.jstor.org/stable/pdf/3272250.pdf?ab_segments=0%252Fbasic_SYC-5152%252Fcontrol&refreqid=excelsior%3A81731dad9c204450fd9e5ad6cd834482

Ortega, N., Price, W., Campbell, T., & Rohr, J. (2015). Acquired and Introduced Macroparasites of the Invasive Cuban Treefrog, *Osteopilus septentrionalis. International Journal for Parasitology: Parasites and Wildlife, 4* (3), 379-384. https://doi.org/10.1016/j.ijppaw.2015.10.002

Patmann, M. D., Shewade, L. H., Schneider, K. A., & Buchholz, D. R. (2017). *Xenopus* Tadpole Tissue Harvest, 870-879. *Cold Springs Harbor Protocols;* doi: 10.1101/pdb.prot097675

Perry, S. F., Wilson, R. J. A., Straus, C., Harris, M. B., & Remmers, J. E. (2001). Which came first, the lung or the breath? *Comparative Biochemistry and Physiology, 129,* 37-47. DOI:10.1016/s1095-6433(01)00304-x

Rolls, G. (n.d.). An Introduction to Specimen Processing. Retrieved from https://www.leicabiosystems.com/knowledge-pathway/an-introduction-to-specimen-processing/

Sampias, C. & Rolls, G. (2019). H & E Staining Overview: A Guide to Best Practices. Retrieved from https://www.leicabiosystems.com/knowledge-pathway/he-staining-overview-a-guide-to-best-practices/

St. Michael's Hospital. (n.d.). Standard Protocol for Formalin-Fixed Paraffin Embedded Tissue. Retrieved from http://stmichaelshospitalresearch.ca/wp-content/uploads/2017/05/Standard-Protocol-for-Formalin%E2%80%90Fixed-Paraffin-Embedded-Tissue.pdf