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Comparison of Behavior of Mangrove Mosquitofish Across Their Range and Identification of their Hybridization with Eastern Mosquitofish using Genital Morphology

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Undergraduate Honors Thesis of

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PREFACE

My research was inspired during my Environmental Science Laboratory class that was taught by Dr. J. Matt Hoch, an Everglades ecologist. This was my first class that was applicable to my newly added second major, environmental science, so I was nervous and excited for the course. One of our first assignments was analyzing fish lengths of a small, yet unique, looking fish. Even more unique was what we were studying: their gonopodiums! Learning about the history and background of why his lab studied the gonopodium, a reproductive structure seen in male mosquitofish, was fascinating to me as there was so much data and research that was conducted and it was all happening right in our beautiful yet remote backyard: Florida's Everglades. When Dr. Hoch mentioned he was looking for student researchers in his lab for the upcoming fall, I knew this was a project that I wanted to continue.

The next year of my studies included learning many new laboratory methods and procedures and also gave me first-hand experience in the field as well. We analyzed different conditions that could be affecting the Eastern Mosquitofish in different boat canals of the everglades while also balancing a project with the USDA invasive species lab on fish counts in relation to an invasive water hyacinth. During our research, we encountered a strange anomaly, a possible hybrid of our known eastern mosquitofish and a different, larger, mangrove mosquitofish. This led us to ask questions: Why were these hybrids present?

Over the next two years I developed and conducted my honors thesis work. Originally designed as a master's student thesis, Dr. Hoch and I worked together to learn everything we could about the mangrove mosquitofish and see where hybrids could be present, while also keeping the data and analysis manageable to be constructed in two years. Much of my junior year was spent collecting fish in canals or boat ramps from the northern tip of Broward County to South Miami. New methods were explored, and different experiments were conducted to best understand how these hybrids came to be. Of course, research never goes as planned. We had hoped to add a DNA component to our research but when our results came back that my mangrove mosquitofish were listed as "Friskies Cat Food: Salmon Flavor" we quickly realized the larger amount of error that could occur.

One of Dr. Hoch's favorite quotes is this: "Post Hoch, ergo propter Hoch". This exemplifies the logical fallacies we could make day to day (where A occurred, then B occurred. Therefore, A caused B), and is a good reminder of the wide range of what scientific research has to offer. While I can't say that "I was enrolled in a ENVS lab class and now just finished my honors thesis, therefore all students that enroll in the ENVS lab class will complete a thesis", I can say that every student who is lucky enough to have Dr. Hoch as an advisor, professor, or friend will emerge with skills that make them truly a one-of-a-kind scientist.

ABSTRACT

The Mangrove Mosquitofish is found in many brackish and freshwater ecosystems surrounding southeast Florida and Cuba. Historical range distribution in Florida has found these fish solely in the Florida Keys and parts of Miami. This research provides an update to the northernmost range that Mangrove Mosquitofish have been observed. As the name implies, Mangrove Mosquitofish reside in areas of critical habitat: Mangrove Forests. These forests are constantly battling habitat loss and reduction due to increased urbanization in native areas. Mangrove Mosquitofish are poecillid fish species with a modified anal fin called a "gonopodium" that allows for internal fertilization. This research found novel hybrids of the Mangrove and Eastern Mosquitofish as well as a new discovery into the mangrove mosquitofish range expansion. Geometric morphometric analysis of hybrid gonopodiums (Eastern Mosquitofish x Mangrove Mosquitofish) reveals an intermediate shape and shows the potential for genetic introgression between species. Hybridization may further threaten Mangrove Mosquitofish populations as they are more vulnerable than the Eastern Mosquitofish due to their use of threatened habitat and range limitation. The morphometric analysis between the two species and hybrids does confirm there is significant difference ($p < 0.05$) between the two species, and among sites. Boldness behavior trials of both male and female mangrove mosquitofish show slight differences in risk tolerance and exploration between the two sexes. The observations have important implications for the future of Mangrove Mosquitofish in the face of climate change and other anthropogenic habitat alterations.

ACKNOWLEDGEMENTS

To the Honors Thesis Program of the Farquar Honors College (NSU), as well as President's Faculty Research and Development Grant to Dr. Hoch and collaborators, June 2020-June 2022. To Dr. Hoch, I am forever grateful for your guidance and help as I learned to navigate all the processes behind scientific research. It was an honor to be a member of your lab for the last three years as it has carried some of my most memorable college memories. I have learned so much from you and hope to exemplify your teaching style and skills as I aid future scientists in their biological careers.

To Dean Nevins, thank you for giving me a voice in the Honors College, for funding my research, and supporting me. I am honored to share my experiences with my fellow classmates and hope I can bring inspiration to them just as you did to me.

To Dr. Feingold, thank you for your advice and friendship since the first day of my freshman year. Your experiences and stories motivated me to pursue my own research, and to always keep exploring and asking questions.

To my NOAA mentors, Dr. Seth Theuerkauf and Dr. Jefferson Hinke, thank you for helping me expand to be an interdisciplinary researcher. My summer internships with both of you helped me find my passion and gave me extraordinary experience as an undergraduate student and I will cherish my summer internships forever.

To my family, thank you for your undying love and support. My biggest support and team, I try to demonstrate the most amazing parts of each of you in my daily life. You are what helped me get here, and for that, I am forever grateful.

And lastly to my cat, Peanut. Thank you for coming into my life when I needed you most, and for keeping my lap warm as I wrote this thesis.

LAND ACKNOWLEDGEMENT

A land acknowledgement is a formal statement to recognize and respect Indigenous Peoples as stewards of the land and the traditions that exist between Indigenous Peoples and their territories.

I wish to acknowledge and pay respect to the Indigenous people past and present as the tradition owners of the land in which I conducted this research namely the sovereign homelands of the Seminole and Miccosukee tribes, whose ancestral lands Nova Southeastern University is built upon. I also wish to acknowledge the traditional homelands of the Native nations including the Tequesta and the Calusa and that many themes of this thesis including land use, geography, and population response have been studied and practiced by Indigenous Peoples on this land for thousands of years.

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I. Introduction

a. Mangrove Mosquitofish Historical Range

Mangrove Mosquitofish, *Gambusia rhizophorae* have historically been located in the Cuban archipelago and the South Florida region **(Rodriguez et al., 2016).** Mainly found in brackish waters and estuarine ecosystems, *G. rhizophorae* relies heavily on the protection and ecology that comes from mangrove forests **(Getter, 1982).** South Florida's waterways are lined with mangrove forest that help create a barrier against erosion, produce carbon, and foster habitat for many fishes and other vertebrates **(Rodriguez et al., 2016)**. In the northern border of the mangrove mosquitofish's habitat, many anthropogenic factors affect the surrounding waters where the fish reside. The natural conditions of mangrove habitats allow for a diverse variety of fish, including members of the Poecilid family, a live-bearing fish with internal fertilization **(Rivas, 1969)**. Poecilid fishes range from 20 to 200mm and are endemic to fresh, estuarine, and coastal waters, and never stray far from the coast **(Rivas, 1969)** . Mosquitofish, or Gambusia, are utilized as a mosquito control agent in wetland areas, whose diffusion into waterways has been linked to human interaction **(Polverino et al., 2013)**. Small in size, with colors of dark amber and brown, the mangrove mosquitofish blends in easily with its habitat and is well distinguished from its relative, the eastern mosquitofish, *Gambusia holbrooki.*

b. Environmental Factors Affecting Range

The Florida Power and Light (FPL) have a powerplant that resides in Port Everglades and has effluent runoff that can yield 10 to 15 °C warmer waters than the surrounding waters during the winter month **(Viragh, n.d.)**. As seen in charismatic megafauna, like manatees and species lower in the trophic levels, a change in water temperature ranges can drastically affect how populations reproduce and respond to change. The range of *G. rhozophorae* is restricted due to abiotic factors including water temperature, salinity, and habitat stability through the presence of mangroves. Different conditions have been found to alter the range pattern of *G. rhizophorae,* especially temperature tolerance **(Nordlie, 2006)**.

c. Range of Mangrove Mosquitofish in the Ecosystem

G. rhizophorae live in the unique and delicate mangrove ecosystem, where they feed on terrestrial insects and in turn are then preyed on by larger fish and birds. Besides their ecosystem role, *G. rhizophorae* are often used as pest control for household aquariums and are a good indicator of ecosystem health and are often studied for research purposes **(Reznick et al., 2017)**. While temperature changes are thought to be the main reason for change in range of *G. rhizophorae*, salinity varies heavily in South Florida, with a low of 13 ppt to a high of 53 ppt in a given season **(Getter, 1982)**.

d. Family Poecilliidae

Poeciliid fish are small laterally compressed fish that inhabit fresh and brackish waters of America and African continents. The subfamily of Poeciliinae has the following characteristics: 1) a unique gonopodium fin formed on males on anal-fin rays 3,4 and 5, 2) internal fertilization, and 3) viviparity **(Stockwell & Henkanaththegedara, n.d.)**. The live-bearing fish use internal fertilization to reproduce, allowing for different characteristics seen in reproductive ornamentation. These distinct characteristics allow for a wide range in fish behavior and morphology as many fish in the Poeciliinae family can have elaborate ornamentation for courtship and male to male aggression **(Reznick et

al., 2017). Because this subfamily has such a unique evolutionary history, understanding how species reproduce or hybridize can help understand future evolution advantages to the family.

e. Gonopodium Morphology

Male Mosquitofish have a modified anal fin called a Gonopodium that is used as an intromittent organ (delivering gametes for internal fertilization) and has been shown to be a target of sexual selection **(Kahn et al., 2010)**. In analysis done with mosquitofish native to The Bahamas, males exhibited a larger gonopodium in predator-free environments while males in the presence of predator fishes have reduced genital size **(Langerhans, 2011)**. Female mosquitofish prefer larger, longer males with larger, longer gonopodia **(Kahn et al., 2010)**. High genital diversity exists in Poecillid fish through both external display features and different courtship patterns. Male fish in the poecilid fish family often display courtship behaviors that can invoke sexual harassment towards female fish as the sexual activity can be as frequent as one sexual act per minute **(Dadda, 2015)**. In response, the female can store sperm for months and only require a few copulations to fertilize the eggs, so many behaviors including chasing and gonopodium movement is displayed in order to attract attention **(Dadda, 2015; Hoch et al., 2020)**. Hybridization indicators between *G. rhizophorae* and *G. holbrooki* can be seen through physical attributes on the gonopodium including bony structures referred to as the elbow, hook, amount of serrae and tip **(Getter, 1982)**. We identified established populations of Mangrove mosquitofish in Broward County Florida, north of their historic range. We also demonstrate that these populations occasionally form hybrids with Eastern Mosquitofish.

f. Hybridization in Fish

The presence of hybridization between species (Invasive alien species- IAS) has led to controversy on whether it is a benefit or not to the species as a whole. In order for an organism to respond to environmental changes, phenotypic plasticity allows the organism to respond to environmental variation without the present of genetic changes **(Zhou et al., 2022)**. This use of phenotypic plasticity can be observed in the early part of biological invasions including new range expansions. As the range expansion of fish further in the ecosystem and reproduces, the phenotypically plastic traits may lead to genetic evolution of the species as a whole **(Hewitt, 2011)**. This could affect processes like reproductive isolation, speciation, introgression and adaptive radiation, while it could also lead to more genetic diversity **(Canestrelli et al., 2016)**. The negative consequences of hybridization would disrupt the current species present and could alter which traits are favorable within an ecosystem as well as adding a competitive factor to those native and endangered species already present in the ecosystem.

g. Boldness

The brackish waterways of Broward and Miami- Dade County provide many sheltered habitats owing to the presence of mangrove forests and tree roots. Individuals from populations further away from their historical range are expected to experience stronger selection than those still in the historical range **(Phillips et al., 2010)**. Understanding the forces that pushed this selection can then be compared to those from the long-established population locals to understand adaptive response **(Lopez, 2011)**. This can further explain dynamics that contributed to the invader population settling in new ranges and allows us to study evolutionary change at a rapid rate **(Thomas et al.,**

2001). External conditions such as predators like wading birds, may influence the dense prey populations found in these waters as well **(Hoch et al., 2020)**. In terms of reproductive effort, invaders are likely to put forth more effort into reproductive success to further their population frontier to continue range expansion, which can lead to hybridization seen in adjoining fish populations **(Travis & Dytham, 2001)**. Sexual harassment from males has been documented in many members of poecilid fish and could be a driving force for range expansion of female fish to evade the stressors **(Dadda, 2015)**. Measuring how much the fish explores, based on location and species, will be a good indicator for the population's overall fitness and reproductive success.

II. Hypothesis

Morphometrics

Ho: There will be significant differences seen in both the body size and gonopodium morphometrics between the easter, mangrove, and hybrid species.

Ha: There will not be significant differences seen in both the body size and gonopodium morphometrics between the eastern, mangrove, and hybrid species.

Boldness:

Ho: Fish populations collected in the northern locations further from the historical range will exhibit more bold characteristics than the southern populations.

Ha: Fish populations of the north and south site locations will not exhibit any boldness behavior differences.

III. Materials and Methods

a. Site Locations

We collected fish in Broward and Miami-Dade County, Florida. The north sites were located in Broward County Florida USA at 26°01’N, 80°07’W, 26°02’N, 80°08’W, 26°06’N, 80°10’W, 26°07’N, 80°09’W, 26°08’N, 80°07’W, 26°09’N, 80°06’W and 26° 9'53.50"N, 80° 9'14.39"W. The south sites were located in Miami-Dade County Florida USA at 25°38’N, 80°17’W, 25°44’N, 80°09’W, and 25°55’N, 80°08’W (**Figure 1**).

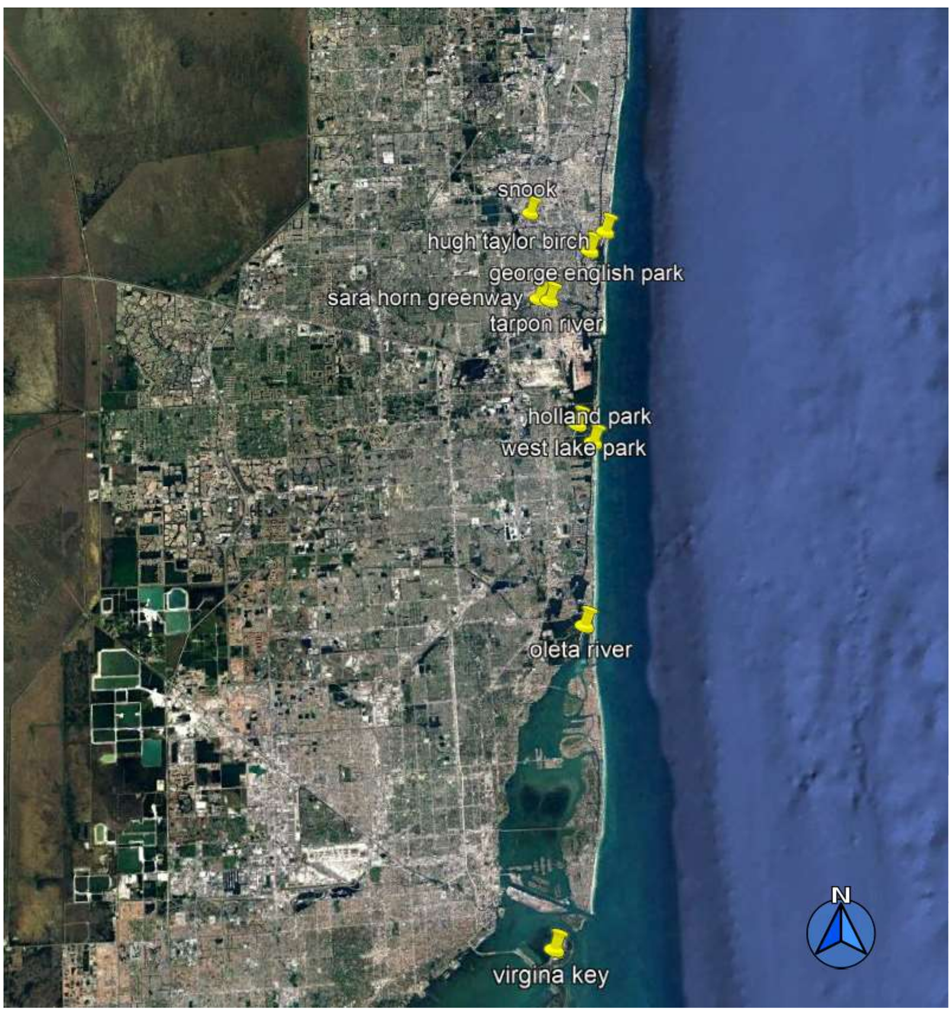

Figure 1: Map of the site locations where fish were collected in both Broward and Miami-Dade County.

b. Laboratory Methods

Both male mangrove and eastern mosquitofish were collected (if present). A dip net was used for fish collection along with 5mm minnow traps for deeper bodies of water. The deployed minnow traps were set out for a minimum of 1 hour but did not exceed 4 hours baited with a 14mL of Pedigree dog kibble. Once the fish were collected they were placed in a clear plastic aquarium so species identification could occur. Fifteen male fish were selected and any fish that were through to be hybrids were also taken. Samples were then placed in a cooler with water and a bubbler.

Fish were acclimated to the lab climate in a plastic 5-gallon plastic aquarium for two days. Hides, a bubbler, and food were also provided, and any deceased fish were removed. Boldness behavior trials were run (see data analysis for boldness behavior section) and then the fish was then painlessly euthanized individually with Tricaine MS-222, buffered with baking soda. Each fish is numbered and then photographed using a Canon digital camera for a full body portrait of the left side. A Hirox digital microscope was then used to photograph the gonopodium using 200x magnification.

c. Data Analysis for Morphometric Analysis

We selected 12 landmarks for the full body analysis and 18 landmarks for the gonopodium analysis that best represented visual differentiation between the species and hybrids (**Table 1 and 2)**. These landmarks outlined the basic shape and distinct anatomical features that different between species including the elongated fin rays seen on the posterior end of the gonopodium, commonly named “fingers” (Getter, 1976). The landmarks were conducted on the full body of both the eastern, mangrove, and hybrid mosquitofish (**Figure 2,3, and 4)** as well as the gonopodium of the three fish respectively

(Figure 5,6, and 7). Two dimensional coordinates were taken of each landmark, which were then digitized using TPSDIG version 2.17. Landmark data was then aligned using MorphoJ to perform a Procrustes superimposition that yields a canonical variate analysis and a principal component analysis for each species and location. A regression graph was conducted to further analyze results.

Standard length (SL) and gonopodium length (GL) were also measured using ImageJ, as differences seen between species of gonopodium length to body length have previously shown sign of sexual selection traits that could further yield hybridization between species. Standard length was taken from tip of the snout to the end of the caudal peduncle, and gonopodium length was measured from begging tissue of the gonopodium to the end of the tip.

Table 1: The description for the twelve different landmarks located for the male eastern, mangrove, and hybrid mosquitofish full body profile.

1	Tip of rostrum. (Sharp point of fish nose)
2	Cranial crease (Point where the head begins to warp and change shape into body)
3	Beginning of dorsal fin (intersection of fin to body)
4	End of dorsal fin (intersection of fin to body)
5	Top of caudal fin (where the muscle section meets the body)
6	Middle of caudal fin (where muscle section meets body)
7	End of caudal fin (where muscle section meets body)
8	Beginning of anal fin top (intersection of fin and body)
9	Beginning of anal fin bottom (intersection of fin and body, farthest left of gonopodium)
10	Bottom of operculum (underneath large flat of operculum flap)
11	Right of eyeball (middle section of eye)
12	Left of eyeball (middle section of eye)

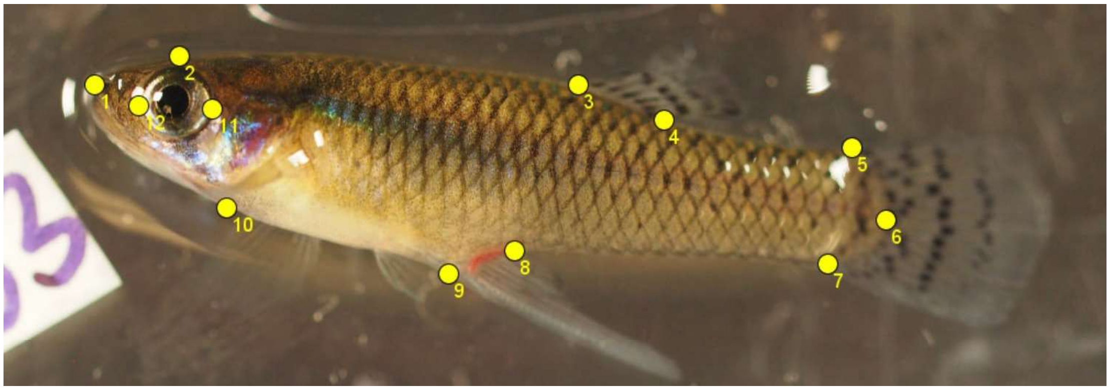

Figure 2: The twelve landmarks used for body morphometrics for the eastern male mosquitofish.

Figure 3: The twelve landmarks used for body morphometrics for the mangrove male mosquitofish.

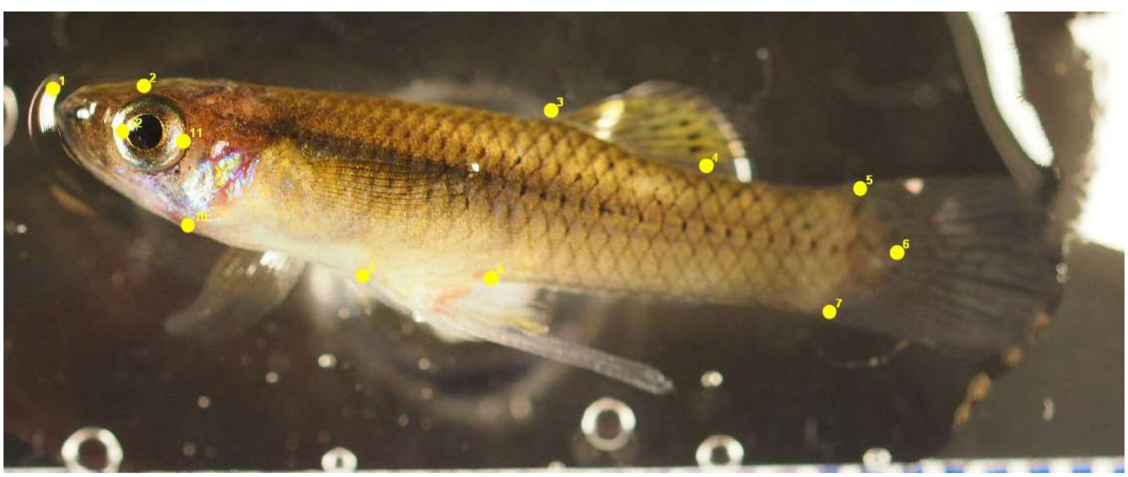

Figure 4: The twelve landmarks used for body morphometrics for the hybrid male mosquitofish.

Table 2: The description for the eighteen different landmarks located for the male eastern, mangrove, and hybrid mosquitofish gonopodium measurements.

1	Tip
2	Middle of bony elements at end of Ray 3
3	Ray 3, change of "feet" shape
4	Ray 3, middle of fingers top part
5	Ray 4A, right most point of elbow
6	Ray 4A, tip of elbow
7	Ray 4A, left most point of elbow
8	Ray 4A, bottom of intersection of Ray 4A and Ray 4P
9	Lowest dip of Ray 4A curve
10	Highest peak in dip on Ray 4P curve
11	Ray 4P beginning of tips (right side of said tip)
12	Intersection of Ray 4A and Ray 4P
13	3^{rd} tip on Ray 4P
14	Ray 5, above intersection of Ray 4A and Ray 4P
15	Ray 5, highest peak
16	Intersection of Ray 5 and Ray 4P
17	Ray 5 left hook point
18	Ray 5 right hook point

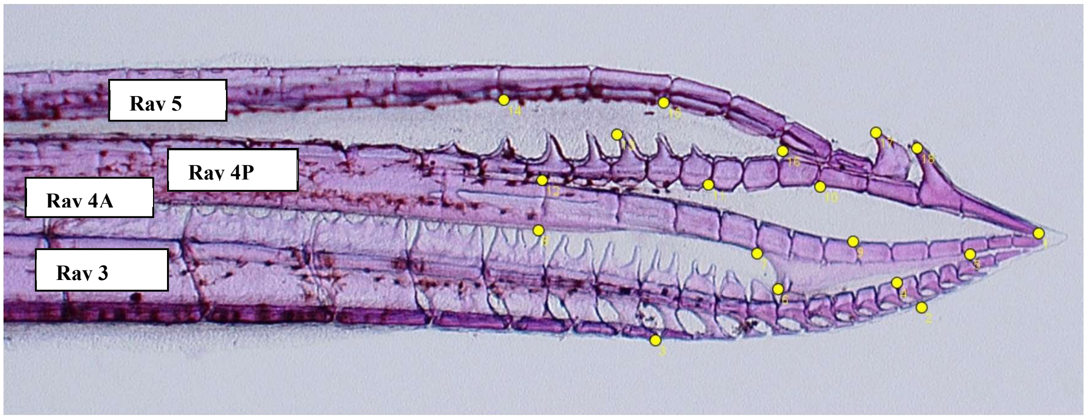

Figure 5: The eighteen landmarks used in the eastern male mosquitofish gonopodium for morphometric analysis.

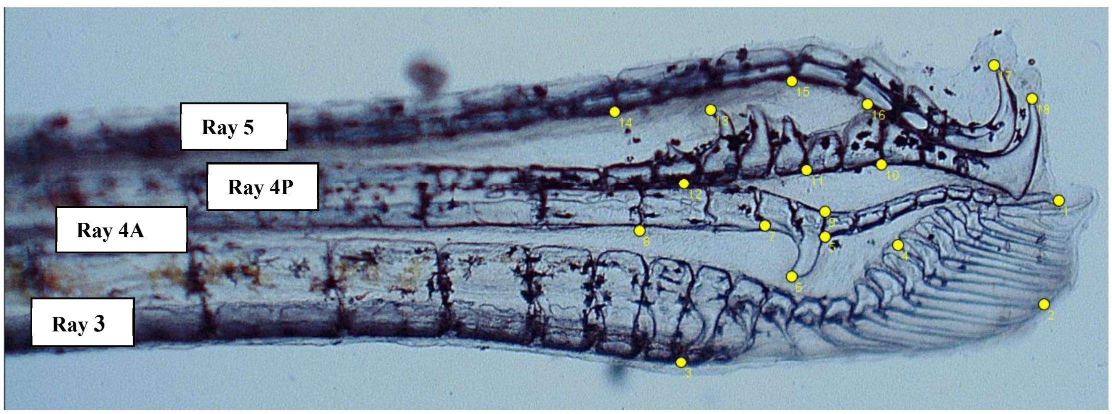

Figure 6: The eighteen landmarks used in the mangrove male mosquitofish gonopodium for morphometric analysis.

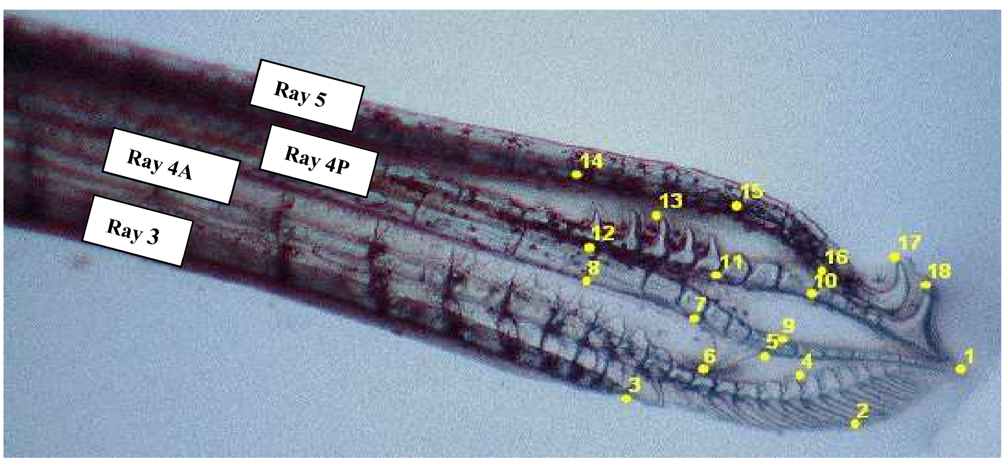

Figure 7: The eighteen landmarks used in the hybrid male mosquitofish gonopodium for morphometric analysis.

d. Data Analysis for Boldness Behavior

Once the fish were collected and acclimated in the lab, we began to run boldness behavior trials. These trials were set up in two laboratory aquaria in 200-micron filtered water collected from each site location with 10cm water depth and a total volume of water of 35 L **(Figure 8)**. The use of 50 cm x 35 cm polypropylene bins was enclosed with the use of several poster boards both on the sides and top so the fish could not see researchers or outside movement **(Figure 9).** Inside the tanks had a "hide" with a detachable door that was able to be removed after five minutes of acclimation time using a string tied to the top end. Around the door were five structures to mimic places for the fish to hide, while the other end of the set-up had a small tea strainer filled with Tetra: TetraMin Tropical Fish flakes. There was a computer webcam set up above each behavior trial **(Figure 10)**. The fish acclimated in the hide for five minutes and then five minutes of swimming time was recorded using iSPY (64bit) computer security camera software.

The iSPY computer software was set to take a picture frame every 1 second. Species and sex were also noted. Once downloaded, the latency time of the fish was determined based on how long it took for over 50% of the fish's body to leave the hide. From there, a stack of the image timelapse was put into ImageJ where it was converted to a 8-bit grayscale image and then had inverted colored correction. The "background" (a photo without the fish in it) was then subtracted from the stack using the image calculation plugin and then the threshold was adjusted for each stack to highlight only the fish in the entire image series. The polygon tool was used to erase any parts of the image that may have been colored but did now have the fish in it, and then the file was saved as a TIFF format.

Using the MTrack2 plugin in ImageJ, the path file was generated using the factors of a high velocity (>500) and a minimum track length of 5. The path length shows where the fish swam around the set-up and could be adjusted if they fish hid behind a barrier or if the image processing steps before lost coordinates of the fish. Once complete, the path length could be opened in excel where we converted the path length from pixels to centimeters (using the measure tool in ImageJ). The data was analyzed for total path length, coefficient of variation, efficiency, and time in open half of the tank (**Table 3)**. This was done for all fish collected starting January 2022 (sample size 144 fish) and was then analyzed based on species, sex, and location.

Table 3: The description of the statistical tests and measurements used in the fish boldness behavior trials.

Term	Definition	Measurement
path length	Total length the fish swam during the 5-minute trial	Centimeters (cm)
coefficient of variation	Indicates how often the fish paused	Standard deviation/ population mean (no units)
efficiency	The percent of the habitat that the fish explored	Percentage (%)
time in open	The time the fish spent in the area without hides	Seconds (s)

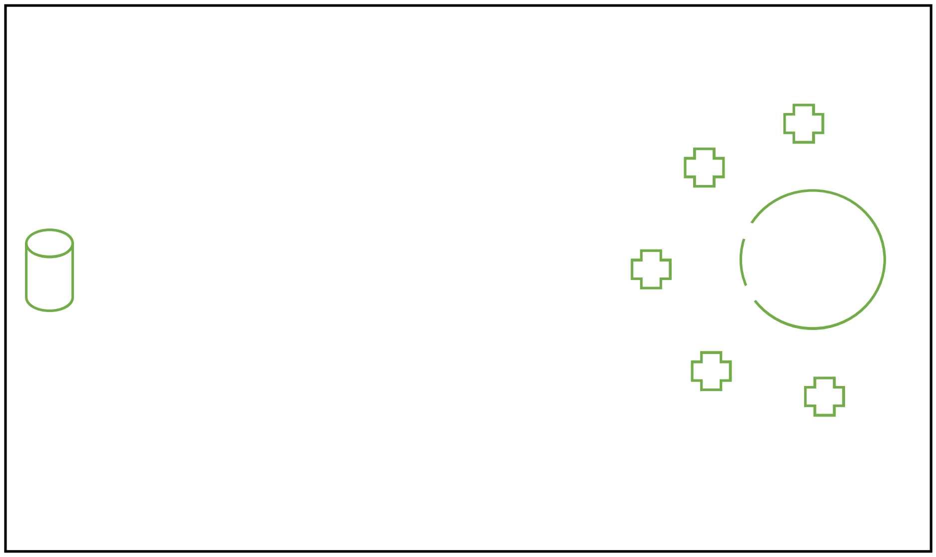

Figure 8: The top view of the boldness behavior trial tank, showing the position of the tea strainer with fish flakes, the obstacles, the hide, and removable door.

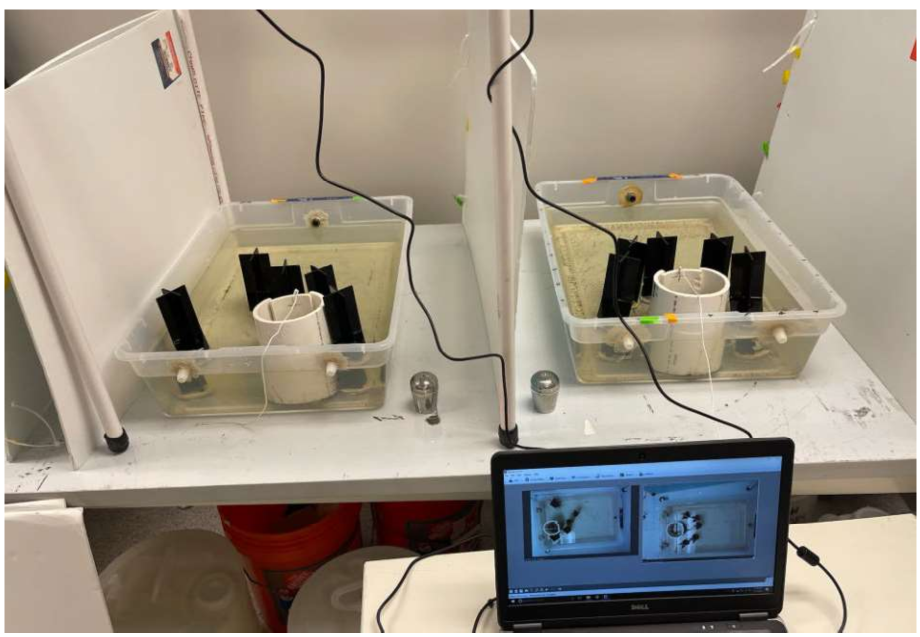

Figure 9: The side view of the boldness behavior trials with the two tanks set up side by side with poster board surrounding the perimeter and webcams directly above the tanks.

Figure 10: Rose Leeger positioning the webcams as Dr. Hoch checks for accuracy on the computer layout in the program iSPY.

IV. Results

a. Morphometric Analysis

The full body and gonopodium data of each fish was tested using a multivariate analysis of both Principal component Analysis (PCA) and Conical Variable Analysis (CVA). The PCA helped summarize variation in the original dataset in a new axis with PC1 resembling the major axis of body shape variation within the study. This can be resembled through different formats of graphs including a wireframe and lollipop graph. PC2 reflects the second most important axis of shape variation and the different PC's can be compared relative to each other using a classifier variable for each specific location category. This comparison helped see how the groups can be related to one another and include a 20% confidence ellipse. The use of PCA helps us explore differences among groups. CVA, identifies axes that differentiate between the groups and then computes new composite variables to then separate the found groups. It utilizes pairwise functions between the different groups. This process is best for generating hypothesis as it minimizes group variation and finds the maximum of variation to best find the axis of differences for shape variation. The Mahalanobis and Procrustes distances show different distance measures between groups and can help compare how groups differ between each other (for those most similar and those most divergent). P values are also calculated between he groups to look for significant differences in the groups as well. Similar graphs can be constructed to show the new CV scores (wireframe, lollipop, and transformation grid), and the CV scores can be compared to one another as well.

i. Full Body Data

PCA analysis for full body data showed that PC 1 had the highest percentage of variance with 79% (**Table 4**). This shows that PC 1 represented the new axis in relation to shape variation in the fish body as seen in the figure below **(Figure 11)**. This axis can also be shown in a lollipop graph which shows that landmarks 4, 8, and 9 have the highest variation **(Figure 12)**. PC 1 and PC 2 can be compared in contrast to one another where there is differentiation within the groups, using a 20% confidence ellipse **(Figure 13)**. The graph between PC 2 and PC 3 did not yield as clear of a result and was not considered in the analysis.

Table 4: The PCA statistical analysis of PC 1-4 with the corresponding variance for the full body morphometrics.

	Eigenvalues	% Variance	Cumulative %
1.	4.63325966	79.206	79.206
2.	0.52294964	8.940	88.146
3.	0.39766437	6.798	94.944
4.	0.29573261	5.056	100.000

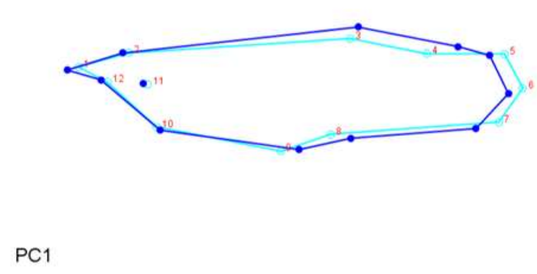

Figure 11: The PC1 wireframe graph that was used in MorphoJ to unite the landmarks to facilitate visualization of the shape changes in the full body morphology.

Figure 12: The PC1 lollipop graph that was used in MorphoJ to show the pattern of body shape variation with each PC axis where the landmarks mark the negative side of the PC axis and the lines indicate how the position changes as it gets closer to the positive PC side.

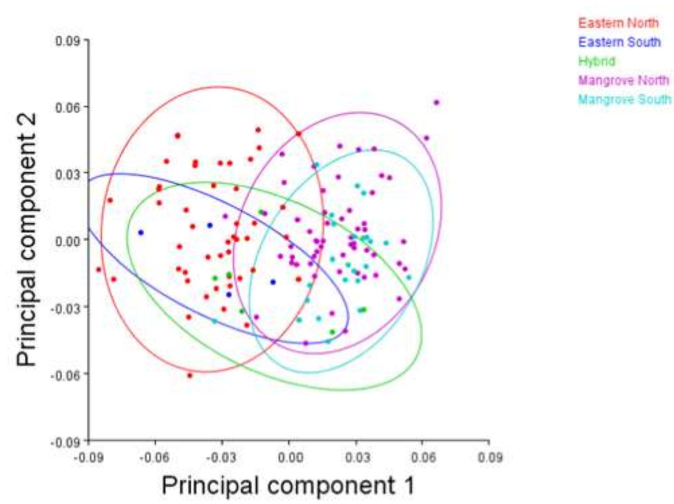

Figure 13: A comparison of the PC 1 to PC 2 with all specimens included to see how the groups are related, using a classifier file that separated the specimens into groups by color as well as 20% confidence ellipses around each group.

Using the CVA, there are noticeable differences in the CV 1 lollipop graph and the CV 2 lollipop graph **(Figure 14 and 15).** Both graphs show the change in variation in landmarks 4, 8, and 9, and population differences can be seen when comparing CV 1 to CV 2 **(Figure 16).**

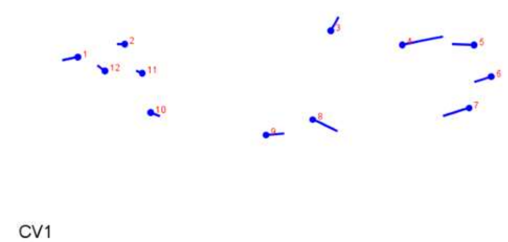

Figure 14: The CV1 lollipop graph that was used in MorphoJ to show the pattern of body shape variation with each PC axis where the landmarks mark the negative side of the CV axis and the lines indicate how the position changes as it gets closer to the positive CV side.

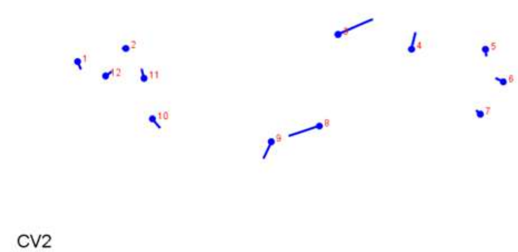

Figure 15: The CV2 lollipop graph that was used in MorphoJ to show the pattern of body shape variation with each PC axis where the landmarks mark the negative side of the CV axis and the lines indicate how the position changes as it gets closer to the positive CV side.

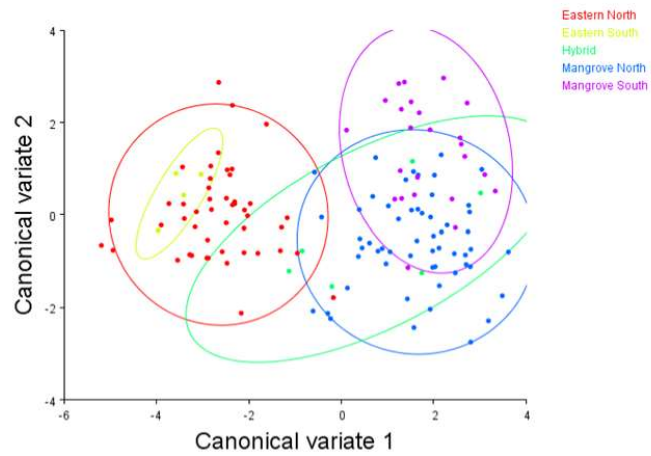

Figure 16: A comparison of the CV 1 to CV 2 with all specimens included to see how the groups are related, using a classifier file that separated the specimens into groups by color as well as 20% confidence ellipses around each group.

Lastly, the Mahalanobis distances and P-values were taken between groups **(Table 5)**. In the Mahalanobis test, there was most differences found between the Eastern South populations to the hybrids, mangrove north, and mangrove south populations (5.8, 5.98, and 6.22). The P-vales of significance were between eastern south and eastern north (0.0262), hybrid to eastern north (0.0440), hybrid to mangrove north (0.0411), hybrid to mangrove south (0.0421), and mangrove south to mangrove north (0.0216).

Table 5: P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups for the full body landmarks.

	Eastern North	Eastern South	Hybrid	Mangrove North
Eastern South	0.0262			
Hybrid	0.0440	0.0494		
Mangrove North	0.0571	0.0633	0.0411	
Mangrove South	0.0609	0.0635	0.0421	0.0216

A finalized graph comparing the full body morphology is seen below, with a visual representation of the differences in populations mentioned before.

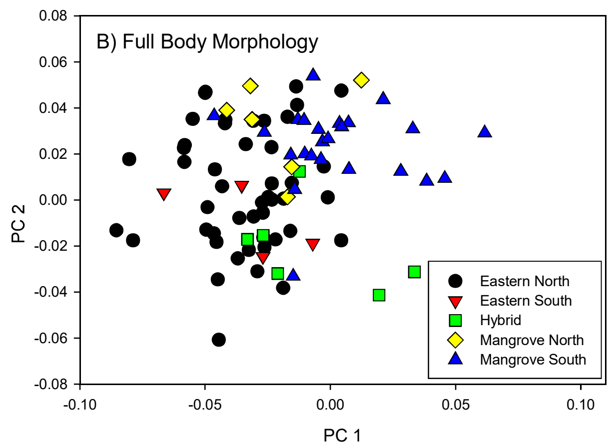

Figure 17: A comparison of the PC 1 to PC 2 with all specimens included to see how the groups are related, using a classifier file that separated the specimens into groups by color as well with no confidence ellipse.

ii.Gonopodium Data

PCA analysis for gonopodium data showed that PC 1 had the highest percentage of variance with 71.58% **(Table 6)**. This shows that PC 1 represented the new axis in relation to shape variation in the gonopodium as seen in the figure below **(Figure 19)**. This axis can also be shown in a lollipop graph which shows that landmarks 1, 3, and 5 have the highest variation (**Figure 18)**. PC 1 and PC 2 can be compared in contrast to one another where there is differentiation within the groups, using a 20% confidence ellipse **(Figure 20)**.

Table 6: The PCA statistical analysis of PC 1-4 with the corresponding variance for the gonopodium morphometrics.

	Eigenvalues	% Variance	Cumulative %
1.	0.04326609	71.583	71.583
2.	0.00497869	8.237	79.820
3.	0.00274273	4.538	84.358
4.	0.00157157	2.600	86.958

Figure 18: The PC1 lollipop graph that was used in MorphoJ to show the pattern of gonopodium variation with each PC axis where the landmarks mark the negative side of the PC axis and the lines indicate how the position changes as it gets closer to the positive PC side.

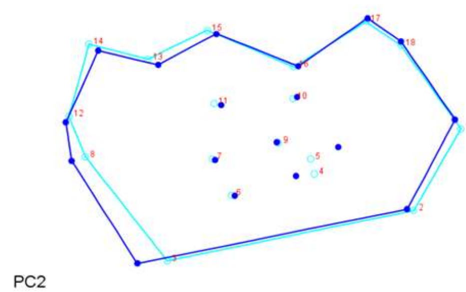

Figure 19: The PC1 wireframe graph that was used in MorphoJ to unite the landmarks to facilitate visualization of the shape changes in the gonopodium morphology.

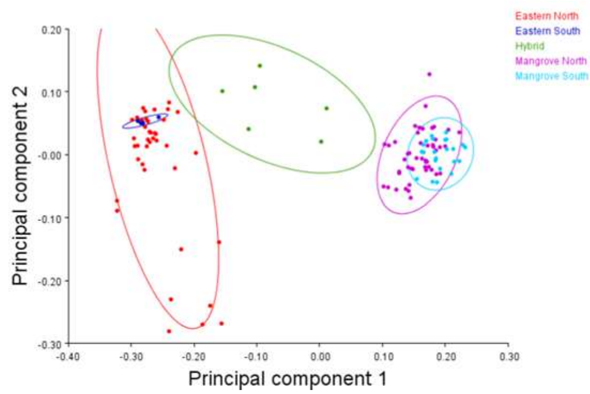

Figure 20: A comparison of the PC 1 to PC 2 with all specimens included to see how the groups are related, using a classifier file that separated the specimens into groups by color as well as 20% confidence ellipses around each group for the gonopodium analysis.

The CVA showed that CV 1had a 96.2% variance for the population analysis **(Table 7)**. Using the CVA, there is noticeable differences in the CV 1 lollipop graph **(Figure 21)** and the CV 2 transformational grid graph **(Figure 22)**. Both graphs show the change in variation in landmark 5 the most, and population differences can be seen when comparing CV 1 to CV 2 **(Figure 23).**

Table 7: The CVA statistical analysis of CV 1-4 with the corresponding variance for the gonopodium morphometrics.

	Eigenvalues	% Variance	Cumulative %
1.	90.22228714	96.277	96.277
2.	2.17791686	2.324	98.601
3.	0.89814370	0.958	99.559
4.	0.41325303	0.441	100.000

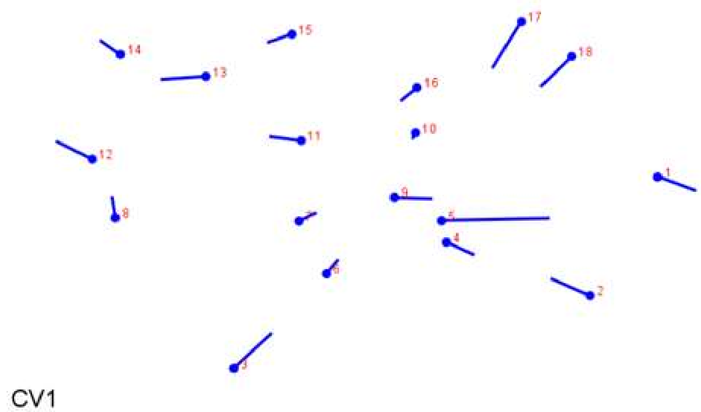

Figure 21: The CV1 lollipop graph that was used in MorphoJ to show the pattern of gonopodium variation with each CV axis where the landmarks mark the negative side of the CV axis and the lines indicate how the position changes as it gets closer to the positive CV side.

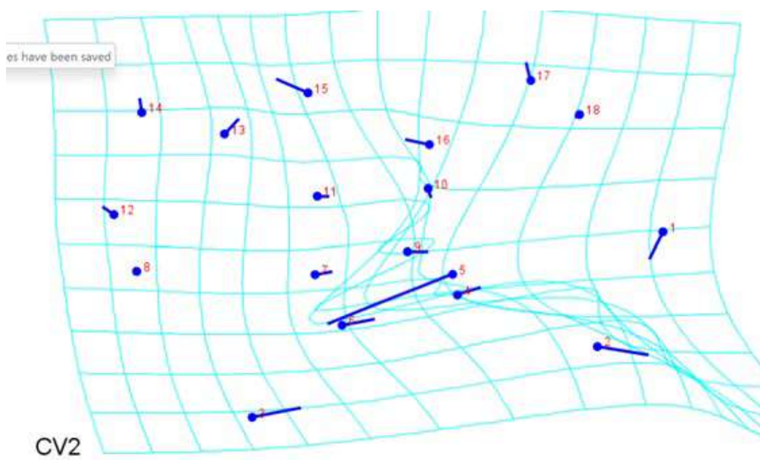

Figure 22: The CV2 transformation grid graph shows variance between the landmark points of variance for the gonopodium morphometrics.

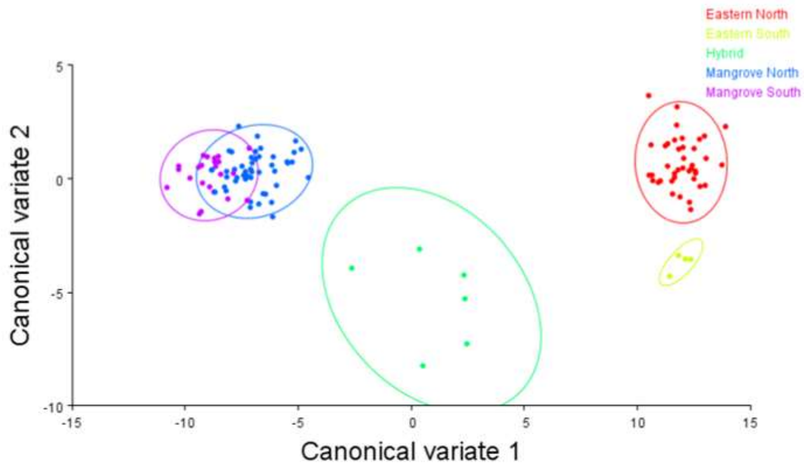

Figure 23: A comparison of the CV 1 to CV 2 with all specimens included to see how the groups are related, using a classifier file that separated the specimens into groups by color as well as 20% confidence ellipses around each group for the gonopodium analysis.

Lastly, the Mahalanobis distances and P-values were taken between groups. In the Mahalanobis test, there was most differences found between the eastern north to mangrove north, eastern north to mangrove south, eastern south to mangrove north, and eastern south to mangrove south (18.90, 20.95, 19.53, 21.43). The P-vales of significance were found in all group comparisons except for eastern south to hybrid which had a p-value of 0.0018 (not significant) **(Table 8)**.

Table 8: P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups for the gonopodium landmarks.

	Eastern North	Eastern South	Hybrid	Mangrove North
Eastern South	<.0001			
Hybrid	<.0001	0.0018		
Mangrove North	<.0001	<.0001	<.0001	

A finalized graph comparing the gonopodium morphology is seen below, with a visual representation of the differences in populations mentioned before **(Figure 24)**.

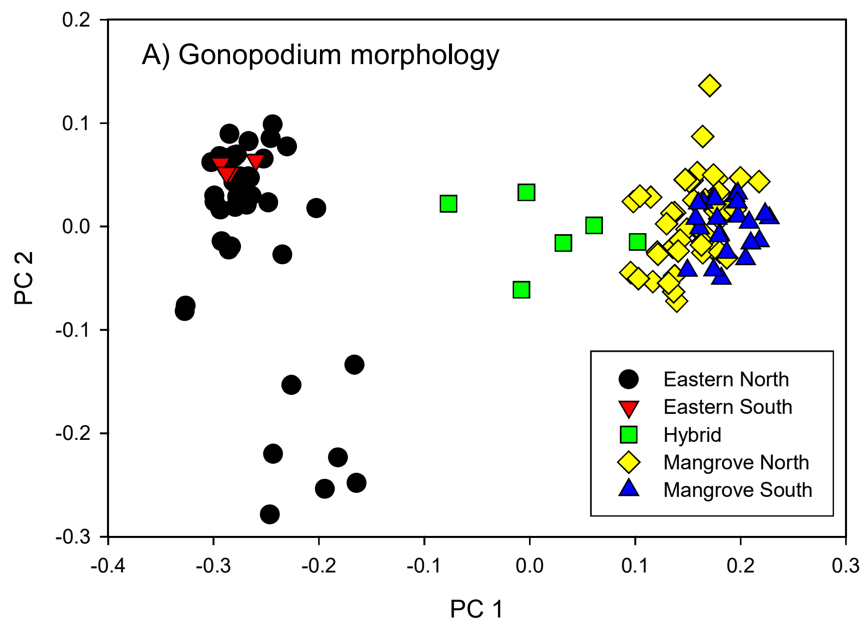

Figure 24: A comparison of the PC 1 to PC 2 with all specimens included to see how the groups are related based on gonopodium morphology, using a classifier file that separated the specimens into groups by color as well with no confidence ellipse.

A regression graph was created comparing the CV scores in relation to the centroid size. Here there are distinct groups within the population with the hybrid population falling directly in the middle of eastern and mangrove mosquitofish for the CV axis however there is no pattern seen in the centroid axis showing that body size is not a factor in comparison to CV values **(Figure 25).**

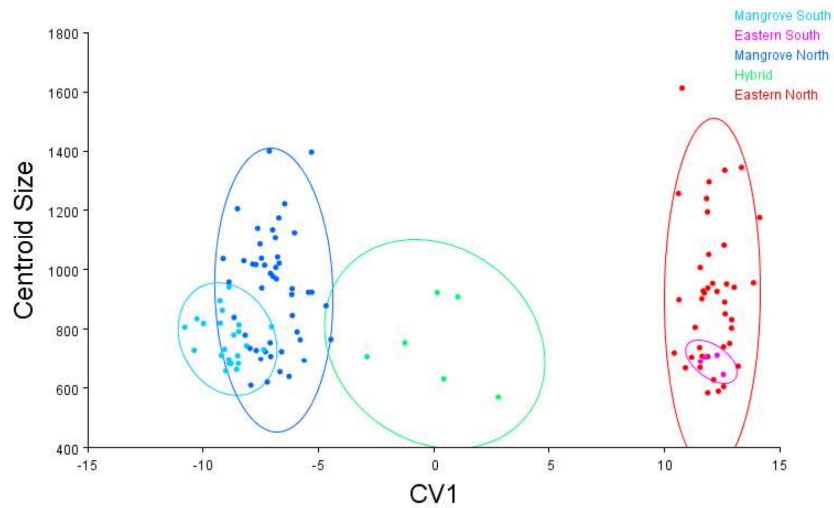

Figure 25: A regression graph comparing the centroid size (body length of fish) to the CV values, showing that there is no body size pattern in response to the CV change even as the fish grows in length.

b. Boldness Behavior

We tested the standard assumptions for ANOVA. For latency time and coefficient of variation of step length, log transformations were required to meet the assumptions of normality. We tested the effect of body size for each variable, and it was not significant for any of them; thus we did not use it as a covariate in our analyses. We tested for the effects of site of collection, sex and their interaction on all five dependent variables using Proc GLM (SAS).

We also measured fish size as a dependent variable but found that there was no significant difference for each of the variables, therefore it was eliminated from the analysis.

i. Latency

There is no significant difference between the latency times for fish populations between sex or location **(Table 9)**. Tarpon River male fish had the longest latency time (max: 185.2 seconds) and with the highest standard error (SE: +/- 113.08). West Lake Park had the shortest latency time with an average standard deviation of 9.5 seconds and a standard error of 3.77 **(Figure 26)**.

Table 9: Statistical test used for the latency time calculation in the boldness behavior trials.

Source	DF	Mean Square	F Value	Pr > F
sex	1	0.19288185	0.05	0.8250
Location	4	3.39592539	0.87	0.4887
sex*Location	3	3.11567044	0.80	0.5008

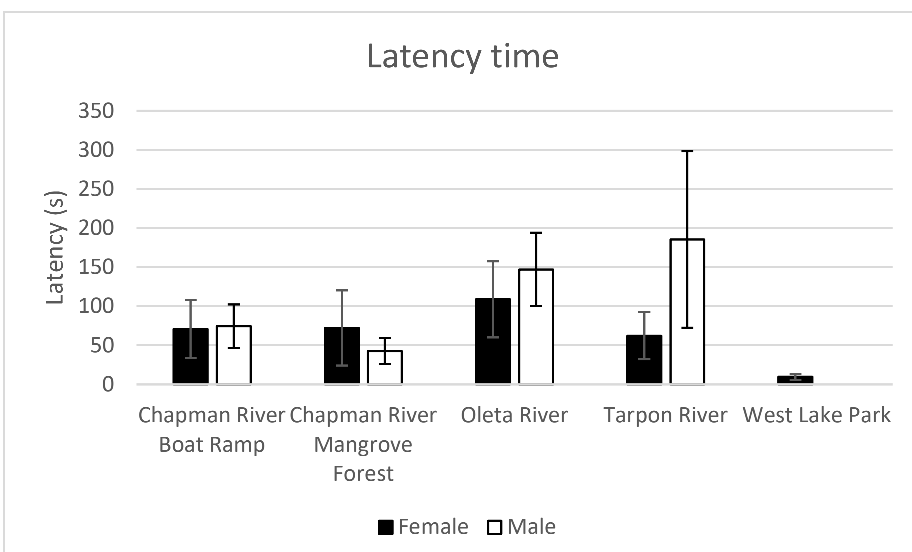

Figure 26: A graph representing the latency time in seconds between the different locations and sexes of fish along with the standard error bars.

ii. Path Length

The overall path length of each fish yielded some significant difference between the specific locations **(Table 10)**. Male mangrove mosquitofish of Chapman river Boat ramp had the longest path length and Female mangrove mosquitofish of Oleta River park had the shortest path length. There was significant difference between Chapman River and Oleta River for the total path length (Pr>F, <0.001) **(Figure 27)**.

Table 10: Statistical test used for the path length calculation in the boldness behavior trials.

Source	DF	Mean Square	F Value	Pr > F
sex	1	5840205.8	1.14	0.2917
Location	4	43790535.0	8.55	<.0001
sex*Location	3	8848655.5	1.73	0.1759

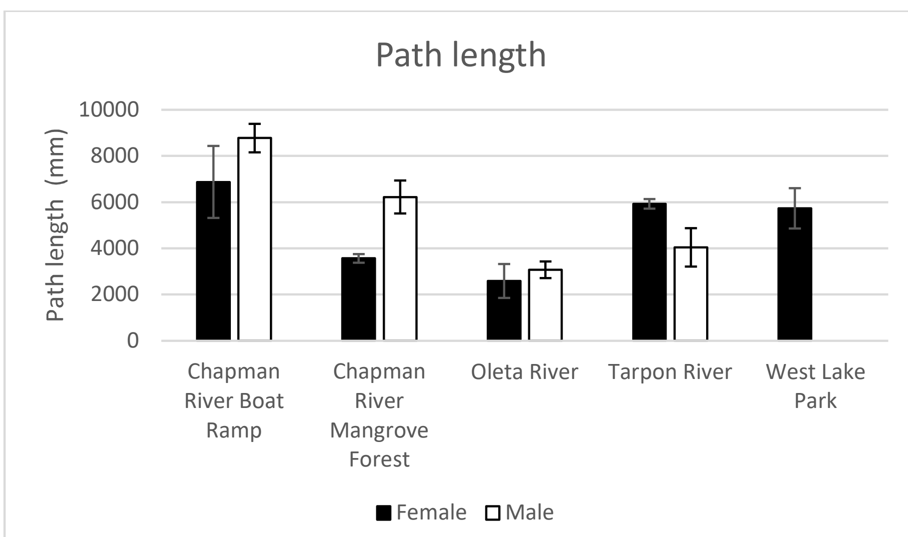

Figure 27: A graph representing the path length in mm between the different locations and sexes of fish along with the standard error bars.

iii. Coefficient of variation of step length

There is no significant difference between the coefficient of variation of step length for fish populations between sex or location **(Table 11)**. Chapman River mangrove forest females had the highest variation (max: 65.2) and with the highest standard error (SE: +/- 46.33). Tarpon River Park males had the shortest coefficient of variation with an average standard deviation of 11.1 and a standard error of 4.9 **(Figure 28)**.

Table 11: Statistical test used for the coefficient of variation of step length calculation in the boldness behavior trials.

Source	DF	Mean Square	F Value	Pr > F
sex	1	0.00187250	0.00	0.9653
Location	4	1.20904265	1.23	0.3107
sex*Location	3	0.51835476	0.53	0.6645

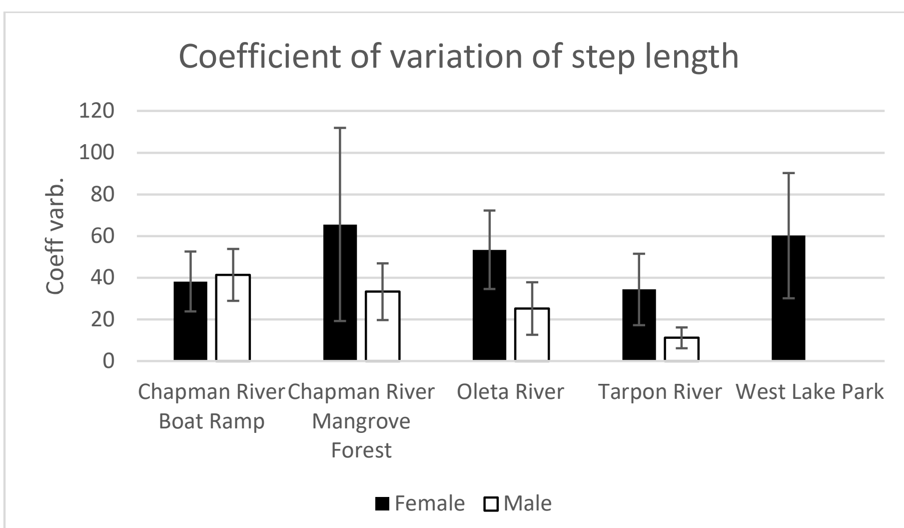

Figure 28: A graph representing the coefficient of variation between the different locations and sexes of fish along with the standard error bars.

iv. Efficency

There is no significant difference between the efficency for fish populations between sex or location **(Table 12)**. Oleta river females had the highest variation (max: 0.0389) and with the highest standard error (SE: +/- 0.025). Tarpon River Park females had the shortest coefficient of variation with an average standard deviation of 0.015 and a standard error of 0.0075 **(Figure 29)**.

Table 12: Statistical test used for the efficency calculation in the boldness behavior trials.

Source	DF	Mean Square	F Value	Pr > F
sex	1	0.00222209	3.26	0.0781
Location	4	0.00064090	0.94	0.4498
sex*Location	3	0.00122167	1.79	0.1631

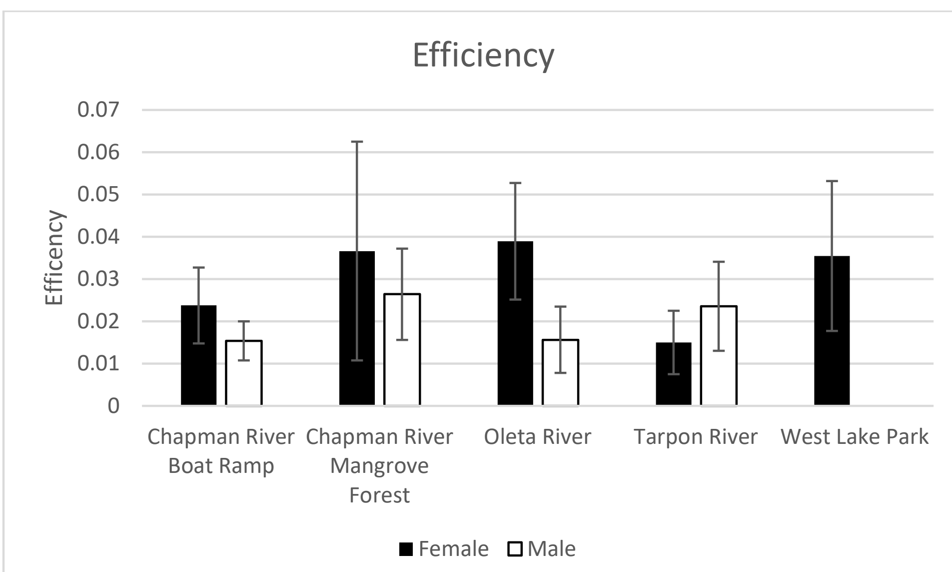

Figure 29: A graph representing the efficency between the different locations and sexes of fish along with the standard error bars.

v. Time in Open

The overall time in open of each fish yielded some significant difference between the different sexes **(Table 13)**. Female mangrove mosquitofish of Oleta river had the longest time in open (0.38) and Female mangrove mosquitofish of Chapman river mangrove park park had the shortest path length (0.003). There was significant difference between Chapman River and Oleta River for the total time in open (Pr>F, 0.0212) **(Figure 30)**.

Table 13: Statistical test used for the time in open calculation in the boldness behavior trials.

Source	DF	Mean Square	F Value	Pr > F
sex	1	0.20728298	5.73	0.0212
Location	4	0.02418578	0.67	0.6173
sex*Location	3	0.07927047	2.19	0.1032

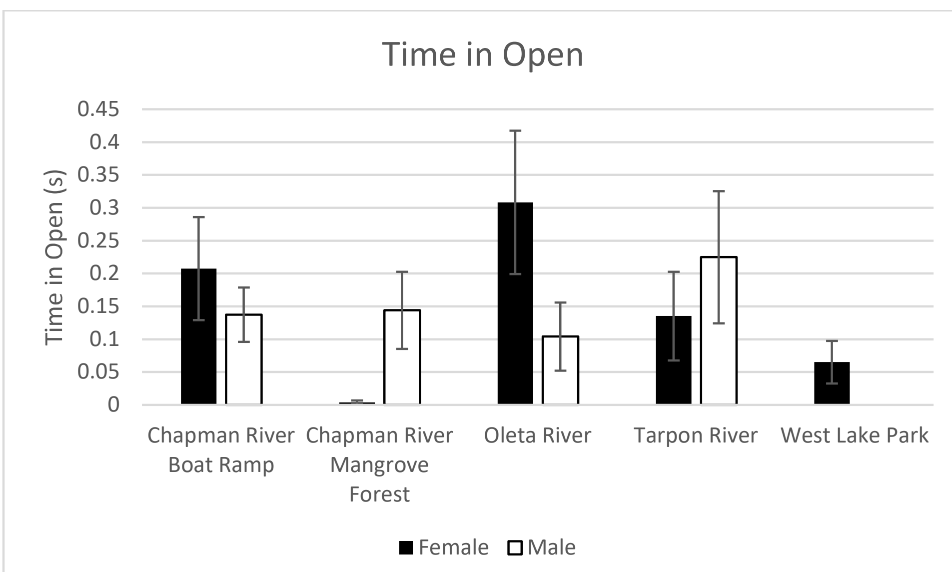

Figure 30: A graph representing the time in open in seconds between the different locations and sexes of fish along with the standard error bars.

V. Discussion and Conclusion

a. Morphometric Discussion for Full Body Analysis

The use of geomorphometric allows us to easily analyze and study variation in the organismal form. For both the full body morphometrics and the gonopodium morphometrics, the use of both the canonical variable analysis and principal component axis allow us to better understand the hybrid populations compared to populations of eastern and mangrove mosquitofish, along with their population location range.

For the full body data analysis, the eastern south population differed most between the mangrove south with a Mahalanobis distance of 6.22 **(Table 5)**. This aligns with our collection data as we expect two fish of two different species that are present in the same area (south- historical range of the mangrove mosquitofish) would have very different body size variations. We could also see this in the laboratory as the mangrove mosquitofish had a different body shape just by looking at it, and the landmarking and morphometrics confirmed our observations. Also, in the Mahalanobis distance, the mangrove north and mangrove south populations had the lowest value (2.02) which meant they were the most similar **(Table 5)**. This once again adds to our observations as we did not notice a different in looks or behavior during collection in the field.

When analyzing the p-values from permutation tests, we can see how the groups differed from one to another using a pairwise function. The eastern south to eastern north, eastern south to hybrid, hybrid to mangrove north, hybrid to mangrove south, and mangrove north to mangrove south, all have a p-value that was of significant value (< 0.05) **(Table 5)**. For the populations of the same species, this confirms that there is a slight difference in groups and sites of both the eastern north vs south populations and the mangrove north vs south populations as well. Furthermore, the significance in the hybrid populations compared to the mangrove north shows that there is a difference between the populations based on site. This is interesting as we found the hybrids in a northern part of Broward County that expanded past the historical range. We would expect that if they were true hybrids (50% of each species) then they would equally different from the associated parent species, which was confirmed as significant for the hybrids compared to the eastern north and eastern south populations. Hybrids and the south populations of both fish also were shown as significantly different, which further alludes to our original observation that the hybrid looked like a well-mixed version of both species of parent fish.

Lastly, these observations and statistical test can be seen clearly in the CV 1 vs CV 2 graph **(Figure 16)**. Here, the hybrids (green) fall directly in the middle of the eastern populations (red and yellow) and the mangrove populations (blue and purple), which can help prove that hybrids are morphologically in the center of both parent species based on the 12 body landmarks we analyzed.

b. Morphometric Discussion for Gonopodium Analysis

In the gonopodium analysis, the eastern south population differed most with the mangrove south population with a Mahalanobis distance of 21.43 **(Table 8)**. As we saw with the digital microscope, the gonopodiums of the two fish differed quite largely and was very clearly noticeable. This result furthers our justification of the differences present between the two species gonopodia, an idea that was drawn out and illustrated in Getter's original thesis, but is now confirmed using morphometrics. The gonopodium with the closest Mahalanobis distance was the mangrove north to mangrove south population with a distance of 3.23 **(Table 8)**. This low distance value helps affirm that we did catch the same species of fish during the various sites we collected at, as the gonopodiums of the two populations were very similar, despite being geographically far apart from one another.

When analyzing the p-values of each population compared to another, every single population differed greatly from one another (p value was listed as <0.001) **(Table 8)**. As seen in the PC scores graph and the CV graph, there is great variation with a few distinct landmarks on the gonopodium **(Figure 23 and 24)**. Landmark 5 had the greatest variation within the populations, which we referred to as the "elbow". The elbow had a distinct curve and hook like shape on the mangrove populations, while the eastern population had a much shorter and blunter elbow shape. Also, landmark 2 represents the fringes that hang off ray 3 of the gonopodium fin, which lengths varied greatly between the fish species. Eastern and hybrid populations had relatively short fringes, while the mangrove species had very long fringes that extended past ray 3. Lastly, ray 1 represented the initial hook and end of the gonopodium and there is variation seen there as mangrove gonopodiums seemed to be generally wider and block shaped compared to the longer slender shape of the eastern gonopodium.

These observations and statistical test can be seen clearly in the CV 1 vs CV 2 graph **(Figure 23)**. Here, the hybrids (green) fall directly in the middle of the eastern populations (red and yellow) and the mangrove populations (blue and purple), which can help prove that hybrid gonopodiums are morphologically in the center of both parent species based on the 18 gonopodium landmarks we analyzed.

As seen in the regression graphs, hybrids again fall directly in the middle of the parent species, now using centroid size as a dependent variable and the gonopodium data as a CV score on the independent axis **(Figure 25)**. In this graph there is no pattern of the centroid size to the CV scores, meaning that the size of the fish as it gets larger does not fall into a pattern based on location or landmark placement. The use of multiple statistical tests further our understanding of the newly discovered hybrids and how their gonopodium size and morphometrics align with the parent populations.

c. Boldness Discussion

The statistical analysis for each of the fish collected and ran in the boldness behavior trials looked at latency time, total path length, coefficient of variation of step length, time spent in open and exploration efficiency as the dependent variables **(Table 3).** The significant difference between the different locations the fish were collected for the total path length yield interesting questions for why fish in Chapman River swam longer than those fish in Oleta River. The Pr> F value of <0.001 demonstrates the differences in location for the path length having significant meaning to the overall data sampled **(Table 10)**. This result could be due to the fact that Oleta River is in the southern range of the sites visited and falls in the historical range of the Mangrove mosquitofish **(Getter, 1982)**. Furthermore, as Oleta River was in the historical range distribution, these fish would be less likely to need to swim as far since historically the fish have been in that same location for over the last forty years. Meanwhile, the fish located in Chapman River reside further north and fall slightly out of the historical range. As invader populations expand further from the historical range, the effects of behavior could be represented by the willingness to explore unknow areas, as well as the reproductive efforts into continuing the invasive front.

The significant difference between the males and females for time spent in the open is shown with a Pr> F value of 0.0212 **(Table 13)**. Here, females generally spend more time in the open than their male counterpart. This could be due to the general behavior of the fish when caught. Female mangrove mosquitofish are significantly larger visually than the males which makes it easier to spot them in the water. Many often were found to be pregnant or resembled a body shape that looked like the fish was at one time pregnant, which is drastically different to the slender male mangrove mosquitofish. Due to the sexual harassment characteristics from males, female fish could have an evolutionary reasoning to spending more time out in the open, with the possibility to do this in efforts to evade male presence. Also, as male mosquitofish are outnumbered to female mosquitofish, they could express more cautious behavior about the time they spend out in the open, unprotected area, for better reproductive success.

The remaining variables tested did not yield any significant results. This may be due to a variety of factors, but as our sample size was still relatively small (n=47), with more data and research collection, the other variables can be reevaluated. While our hypothesis cannot be confirmed using these results, the significance seen in both the location differences in path length and the sex differences in time in open can demonstrate the fitness and reproductive strategies different mangrove mosquitofish populations utilize around the historical and non-historical ranges.

d. Future Applications

These results help us better understand the different populations of mangrove mosquitofish found within the historical range and for the populations that have migrated north of the historical range. The geometric morphometric analysis of the full body data proved that there are differences in the body size between the hybrid species and both the eastern and mangrove species. This was the initial test used to notice a difference in the hybrids when first observed in the field, so the use of multiple statistical results further confirmed our initial observation. The analysis of the gonopodiums explained how diverse the hybrid gonopodium was in comparison to every other species and population sampled. This not only explained the uniqueness of the hybrid gonopodiums, but also represented the mix of both structural features that make up parts of the eastern and mangrove gonopodium. Further analysis with the boldness behavior trials shows the differences some populations and sexes have between each other, showing overlap with our main hypothesis that the new populations of mangrove mosquitofish found further north of the historic range will show different traits to better find a mate or reproduce.

Limits to this research include a relatively small sample size of 74 fish run for the boldness behavior trials and 138 specimens for the morphometric analysis. There is also a significant gap in data knowledge of the range of the mangrove mosquitofish from the late 1900s until now. There is the possibility that this invader front of mangrove mosquitofish could have began their invasion twenty or thirty years ago and we would have no way of knowing the effects during this time. We also did not measure the predator-prey risk exposure that would include the presence of wading birds or other larger fish. This would aid further in our boldness behavior trials to collect fish from environments with very little predator presence to environments with high predatory presence. Abiotic factors such as salinity and temperature were not recorded due to the vast changing dynamic of the brackish marshes of sample sites but could be a factor for further analysis.

Understanding the presence of hybridization in species can be vital to understanding the effects of climate change on ecosystems. As climate change threatens ecosystems including mangrove forests, colder climates are associated with limiting the range expansion of fish and other species that reside in this habitat. As the climate gets warmer, this could induce future continuation of range expansions for several species. Hybridization rates within vulnerable or endangered species poses a large risk for species survival and success. As seen between the endangered polar bear and grizzly bear, ice age climate change affects greatly increased the genome population of hybrid bears found during that temperature increase **(Cahill et al., 2018)**. This rate of hybridization can dilute the native population reproduction rates, further threating species survival rates. This novel discovery of a hybrid mosquitofish fish, as well as the northward range expansion can be used to understand the effects of climate change, hybridization effects, and behavior patterns that can be seen in a changing ecosystem.

VI. Literature Cited

Cahill, J. A., Heintzman, P. D., Harris, K., Teasdale, M. D., Kapp, J., Soares, A. E. R., Stirling, I., Bradley, D., Edwards, C. J., Graim, K., Kisleika, A. A., Malev, A. V., Monaghan, N., Green, R. E., & Shapiro, B. (2018). Genomic Evidence of Widespread Admixture from Polar Bears into Brown Bears during the Last Ice Age. *Molecular Biology and Evolution*, *35*(5), 1120–1129. https://doi.org/10.1093/molbev/msy018

Canestrelli, D., Porretta, D., Lowe, W. H., Bisconti, R., Carere, C., & Nascetti, G. (2016). The Tangled Evolutionary Legacies of Range Expansion and Hybridization. *Trends in Ecology & Evolution*, *31*(9), 677–688. https://doi.org/10.1016/j.tree.2016.06.010

Dadda, M. (2015). Female social response to male sexual harassment in poeciliid fish: A comparison of six species. *Frontiers in Psychology*, *6*. https://www.frontiersin.org/articles/10.3389/fpsyg.2015.01453

Getter, C. (1982). Temperature Limitations to the Distribution of Mangrove Mosquitofish in Florida. *Florida Scientist*, *45*(3), 196–200.

Hewitt, G. M. (2011). Quaternary phylogeography: The roots of hybrid zones. *Genetica*, *139*(5), 617–638. https://doi.org/10.1007/s10709-011-9547-3

Hoch, J. M., Spadafore, S., & Olesen, D. (2020). *Effects of predator cues on migration-related behaviors of Eastern Mosquitofish (Gambusia holbrooki) in temporary wetlands of the Everglades*. https://works.bepress.com/j-matthew-hoch/66/

Kahn, A. T., Mautz, B., & Jennions, M. D. (2010). Females prefer to associate with males with longer intromittent organs in mosquitofish. *Biology Letters*, *6*(1), 55–58. https://doi.org/10.1098/rsbl.2009.0637

Langerhans, R. B. (2011). *Ecology and Evolution of Poeciliid Fishes*.

Lopez, D. P. (2011). *Trait Variation in an Everglades Invasive Species: Life histories, Boldness, and Dispersal in the African Jewelfish* [Environmental Studies, Florida International University]. https://doi.org/10.25148/etd.FI11080804

Nordlie, F. G. (2006). Physicochemical Environments and Tolerances of Cyprindontoid Fishes Found in Estuaries and Salt Marshes of Eastern North America. *Reviews in Fish Biology and Fisheries*, *16*(1), 51–106.

Phillips, B. L., Brown, G. P., & Shine, R. (2010). Evolutionarily accelerated invasions: The rate of dispersal evolves upwards during the range advance of cane toads. *Journal of Evolutionary Biology*, *23*(12), 2595–2601. https://doi.org/10.1111/j.1420-9101.2010.02118.x

Polverino, G., Liao, J. C., & Porfiri, M. (2013). Mosquitofish (Gambusia affinis) Preference and Behavioral Response to Animated Images of Conspecifics Altered in Their Color, Aspect Ratio, and Swimming Depth. *PLOS ONE*, *8*(1), e54315. https://doi.org/10.1371/journal.pone.0054315

Reznick, D. N., Furness, A. I., Meredith, R. W., & Springer, M. S. (2017). The origin and biogeographic diversification of fishes in the family Poeciliidae. *PLOS ONE*, *12*(3), e0172546. https://doi.org/10.1371/journal.pone.0172546

Rivas, L. R. (1969). A Revision of the Poeciliid Fishes of the Gambusia punctata Species Group, with Descriptions of Two New Species. *Copeia*, *1969*(4), 778. https://doi.org/10.2307/1441802

Rodriguez, W., Feller, I. C., & Cavanaugh, K. C. (2016). Spatio-temporal changes of a mangrove–saltmarsh ecotone in the northeastern coast of Florida, USA. *Global Ecology and Conservation*, *7*, 245–261. https://doi.org/10.1016/j.gecco.2016.07.005

Stockwell, C. A., & Henkanaththegedara, S. M. (n.d.). *Chapter 12 Evolutionary conservation biology*.

Thomas, C. D., Bodsworth, E. J., Wilson, R. J., Simmons, A. D., Davies, Z. G., Musche, M., & Conradt, L. (2001). Ecological and evolutionary processes at expanding range margins. *Nature*, *411*(6837), 577–581. https://doi.org/10.1038/35079066

Travis, J., & Dytham, C. (2001). Dispersal evolution during invasions. *Evolutionary Ecology Research*, *4*.

Viragh, B. (n.d.). *The Effects of a New Bridge on Manatee (Trichechus manatus latirostris) Use of the FPL Discharge Canal at Port Everglades, Florida*.

Zhou, L., Ouyang, X., Zhao, Y., Gomes-Silva, G., Segura-Muñoz, S. I., Jourdan, J., Riesch, R., & Plath, M. (2022). Invasive fish retain plasticity of naturally selected, but diverge in sexually selected traits. *Science of The Total Environment*, *811*, 152386. https://doi.org/10.1016/j.scitotenv.2021.152386