ABSTRACT

"EXAMINATION OF GEOGRAPHIC VARIATION IN MALE MELANISTIC COLOR PATTERN IN THE LAKE MALAWI MAYLANDIA ZEBRA CICHLID SPECIES"

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Director: Patrick Danley, Ph.D.

The purpose of this thesis project is to examine geographic variation in male melanistic color pattern in the Lake Malawi cichlid Maylandia zebra. Maylandia zebra, like other members of Lake Malawi's rock-dwelling cichlid clade, are highly philopatric, meaning very few individuals migrate into or out of geographically isolated populations. The genetic isolation of these populations may facilitate their phenotypic differentiation and contribute to the extraordinary rate of speciation observed in this system. I will test the hypothesis that populations of M. zebra are phenotypically isolated by examining the within and between population variation in male melanistic pigmentation. Specifically I will quantify bar density and pigment intensity from photos of M. zebra from differing locations in order to quantify phenotypic variation. Next, I will test the hypothesis that geographic variation in pigmentation pattern is correlated with environmental conditions at each site. To do this, I will quantify the number of rocks for the location of each population. I predict that populations with larger than average melanistic patterning will live in habitats with many smaller rocks. This analysis will provide insight into the ecological forces influencing population differentiation of Lake Malawi cichlids and may inform on the speciation process in this system.

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EXAMINATION OF GEOGRAPHIC VARIATION IN MALE MELANISTIC COLOR PATTERN IN

THE LAKE MALAWI MAYLANDIA ZEBRA CICHLID SPECIES

A Thesis Submitted to the Faculty of Baylor University

In Partial Fulfillment of the Requirements of the Honors Program

By

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CHAPTER 1

Introduction

1.1 Motivation

In his 1973 essay denouncing anti-evolution creationism, Theodsius Dobzhansky

delivered the famous quote, "nothing in biology makes sense except in the light of

evolution" (Dobzhansky, 1973). This persists throughout modern biology. A deeper

understanding of evolution and its processes lends itself to a greater appreciation of all

of biology. The cichlid species of Lake Malawi are one of the best examples of adaptive

radiation. Adaptive radiation refers to the evolution and divergence of species from a

common ancestor and becoming adapted to available niches.

Lake Malawi alone contains over 500 endemic species of cichlids which have

evolved from a common ancestor within the last one million years. There are two major

clades of cichlids; the rock dwelling and the sand dwelling cichlids (Albertson et al.,

1999). The immense variability of color patterns, morphologies and mating behaviors of

the cichlids have been intensely studied. Morphological adaption and sexual selection

are recognized as the most probable mechanisms for the rapid diversification in both

clades. The particular species of cichlid covered in this study is *Maylandia zebra*. They

are named "zebra" as a result of their eight dark grey bars ranging from their head to

caudal peduncle, the location where the tail fin attaches. (Seehausen et al., 2001). *M.

zebra*, like other members of Lake Malawi's rock-dwelling cichlid clade, are highly

philopatric meaning very few individuals migrate into or out of geographically isolated

populations (Knight & Turner, 2004). After Dr. Patrick Danley collected photographs of *M. zebra* cichlids from different areas around Lake Malawi such as: Boadzulu Island, Harbour Island, and Otter point, it became apparent that the relative dark pigment density of individuals differed based on their location. Several important questions emerged from this observation. Is this evidence of adaptive divergence taking place and is it based on the particular environment? Is it possible to quantify these differences in order to make analyses and predictions? Does the type of terrain of these locations have a correlation with the phenotype of its inhabitants? Each of these questions will be addressed in my thesis.

Lack of gene flow between populations may be a major cause of the cichlid phenotypic differentiation, and may be a precursor to the speciation of cichlid species. There are hundreds of individual isolated populations throughout Lake Malawi allowing scientists to observe the relationship between geographic variance and morphological and genetic variances (Kocher, 2004). The study of a particular cichlid species may allow for the possibility to witness the process of adaptive divergence take place. Observing adaptive divergence in an extant species may result in the ability to test predictions for future speciation events and will further expand scientists' knowledge of adaptive divergence. Knowing the relationship between geographic variance and phenotypic variance of a specific species allows for a better understanding of the mechanisms of why and in what context adaptive divergence takes place.

1.2 State of Research

There has been a plethora of research conducted on numerous cichlid species across the world. Much of the research relating to *M. zebra* has been focused on female mate choice, male-male interaction, genomic analyses, and aggression studies. While there has been some research on nuptial coloration of M. *zebra*, the research topic I am examining, the geographic variation in male melanistic color patterns, has not been conducted. The following studies will help elucidate the results of my research as well as provide support for my conclusions.

Allender et al. (2003) recognized that nuptial coloration is a very important factor in cichlid mate choice. Their research went about testing whether speciation through sexual selection was observable in isolated ecologically isolated geographic regions. Nuptial coloration refers to the coloration of cichlids during their mating cycle. During their research on the genetic differences between individuals living in the same region versus those of separate regions they confirmed a positive correlation between geographic distance and genetic difference. They hypothesized that sexually selected color patterns relate to visual ecology, and also that visual conspicuousness may determine mating success. For example, depending on the rock environment, the depth, ambient light, etc. a lighter shade of blue may be more conspicuous than a darker shade and vice versa. They did not manage to test these predictions; however, those ideas relate heavily to the goals of my research. Quantifying the dark pigment percent area and intensity of cichlids of different locations, along with determining the variances in their rocky habitats, may lead to better understanding the link behind the phenotypic and geographical differences. The hypotheses of this study relates to the goals of this

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thesis. By determining how melanistic color patterns vary with differing geographic locations, it may be possible to also find a link between environment and phenotype. If a link could be found, it would help illuminate the mechanisms of speciation in *M. zebra*.

Danley et al. (2011) similarly recognized the high degree of variability in male color pattern of rock dwelling cichlids. However, they took a different approach, focusing on the acoustic cues of cichlids in affecting mate choice instead of only visual communication. Of particular interest to my current research is their analysis of the *M. zebra* trill. They determined that *M. zebra* cichlids from Thumbi West produced a higher number of pulses per trill, showed a more prolonged pulse duration, and exhibited a shorter pulse period than *M. zebra* in Nkhata Bay. This finding strongly suggests that acoustic signals vary between geographically isolated populations and may contribute to mate selection and speciation of *M. zebra* and other cichlid species. For my research, it is important to acknowledge that other factors besides visual appearance, such as acoustic sounds, may be contributing to the genetic divergences of *M. zebra*.

Dalton et al. (2010) studied how coloration in cichlids is determined by both natural and sexual selective forces. They hypothesized and later demonstrated that male cichlids, such as in *M. zebra*, have darker and more conspicuous color patterns then females as a result of female mate choice and male to male competition. My current research implemented this knowledge by excluding female cichlids since their color patterns were unaffected by geographic location. Only dominant males had varying dark pigment density and intensity, suggesting sexual selection is a factor in the differences in phenotype from *M. zebra* of different locations. They also determined

that color pattern alone is not the only influence in mate choice. Another influence was the Euclidian distance in color space from the colors of the fish and that of the environment background. This suggests that isolated populations of *M. zebra* may exhibit different dark pigment intensity and density as a result of the environmental terrain.

Knight & Turner (2004) studied whether or not assortative mating could be measured. Assortative mating is when individuals with similar genotypes and/or phenotypes selectively mate with one another more frequently then seen in random mating. They did this by taking *M. zebra* in a lab and determining whether geographically isolated color forms would preferentially mate with similar patterned fish. The cichlids used were found from varying locations across Lake Malawi along with varying environmental terrains and depths. Their results showed that the cichlids of each location have developed partial reproductive isolation. There was a relatively high degree of assortative mating between cichlids of similar origin. In one instance, a group of cichlids from Nkhata Bay did not show assortative mating with those from Chisumulu. However, the researches attributed this to the high degree of similarity in male breeding colors, further suggesting a link between sexual selection and male color patterns. Because groups of *M. zebra* experience very low levels of gene flow due to their philopatric nature, the quantification of relative melanistic patterns both between individuals of the same population and of different populations may suggest sexual selection and the beginnings of reproductive isolation.

Assortative mating in cichlids was also studied by van Oppen at al. (2002). Their research focused on how male color patterns distinguished species as the speciation process was driven by sexual selection by female mate choice. Their results showed that many rock dwelling cichlids mate assortatively and there are most likely many species that have failed to receive species status. This research demonstrates how the beginning reproductive isolation seen in *M. zebra* from Knight et al. (date?) may be leading to, and in some cases already have led to, speciation in *M. zebra*.

Jordan et al. (2008) researched how nuptial coloration has resulted in reproductive isolation of rock dwelling cichlids as a result of female mate choice. The research set out to test the hypothesis that females, while generally mating with closer related species, would associate more with distantly related species if they had more similar color patterns. This study supported the hypothesis that female mate choice can sometimes fail to prevent hybridization of cichlid species. It also further demonstrates the importance of color patterns in regards to mate choice and speciation.

In a study by Kidd et al. (2006), *M. zebra* and *Maylandia benetos* were observed in the lab and mating trials were performed. The results provided support for the assumption that female cichlids utilize visual cues in order to identify and mate with conspecific males. This demonstrates sexual selection in the speciation between cichlid species. Conspecific refers to individuals of the same species. A goal of this thesis is to quantify and compare melanistic patterns of *M. zebra* cichlids, not only between populations, but also within populations. Comparing the degree of similarity between

closely related individuals may show further evidence of conspecific female mate choice in *M. zebra*.

A similar study by Couldridge et al. (2001) examined interspecific female mating choice between four related species of the Pseudotropheus zebra complex. The species chosen only differed in color pattern. The results of the study showed that female mate choice was positively correlated with conspecific males, suggesting color patterns are important in both female mating choice and species recognition. Quantifying color variations of one species would illuminate the process of speciation, rather than studying speciation after it has already occurred.

In a study by Seehausen (1995), Lake Victoria cichlids were studied for mate recognition among color morphs. They demonstrated positive assortative mating in these cichlids. Interestingly, he found evidence that the status of color morphs in species of cichlids relates to the environmental conditions which are considerably varied around the lake. He concludes that polymorphisms may precede species divergence and may be a key indicator of speciation in cichlids. Also in this paper, Seehausen (1995) determined three criteria in maintaining reproductive isolation: geographic isolation, behavioral isolation, and microhabitat isolation. This is important for my research since I am attempting to determine whether coloration can be attributed to one or all of these reproductive isolating mechanisms. For example, if sexual selection is at play, the female *M. zebra* may be selectively mating with males of a particular pigment brightness. The microhabitat of the cichlids may result in certain melanistic color patterns and hues being more conspicuous against the backdrop. A complex terrain with

numerous crevices may lead to brighter, more intensely pigmented, barred cichlids being selected by females. Contrarily, a location with larger smoother rocks may result in darker barred cichlids being sexually selected.

Another interesting question is whether or not predation impacts the variation in color pattern of *M. zebra*. It is possible that cichlids who stand out for females may also stand out for predators. Predation, therefore, may be slowing the speciation of cichlids by limiting the effect of sexual selection. In a study by Stuart-Fox et al. (2003), a link was found between conspicuous males and predation in Australian rock dragons. Coloration was categorized by brightness and dullness. The results showed that avian predators showed higher predation rates on lizards who were categorized as 'bright', supporting the assumption that differences in conspicuousness infers differential predation risks.

A study by Hurtado-Gonzales et al. (2014) dealt with the variation in the visual habitat of poeciliid fish and how it may mediate the maintenance of their color polymorphism. The data supported how conspicuousness of the fish was influenced by the contrast of their background. My research also focuses on the conspicuousness of cichlid fish as the melanistic color patterns relates with the examination of rocky terrain. Differences in both predation and environmental terrain of different locations may be responsible for observed phenotypic differences. Hurtado-Gonzales quantified male coloration through an absorbance spectrum. Then they assessed how conspecific females, in their natural habitats, perceived the males. They also assessed how *Aequidens*, a cichlid predator, perceived the males. The results showed that the most conspicuous morphs were more likely to mate with females yet were also more

susceptible to predation. These are both two hypothesis of my research with *M. zebra*. If conspicuousness of males resulted in sexual selection of the locations observed, then predation could be a factor hindering speciation and resulting in a larger variation of melanistic color patterns.

In a similar study by Kusche & Meyer (2013), the relationship between the conspicuousness of Midas cichlids and predation was examined by introducing the species to largemouth bass. The cichlids were distinguished into two color morphs, normal and amelanic gold. Neither had noticeable behavioral differences. The result of the study showed the bass preyed on normal cichlids much more frequently than on the gold cichlids. While similar studies have not been performed on *M. zebra*, this study further shows the link between predation and conspicuousness and how such a model may be at play in other cichlid species such as *M. zebra*.

1.3 Goals and Outline

The main goal of this thesis project is to examine geographic variation in male melanistic color pattern in the Lake Malawi cichlid *M. zebra. Maylandia zebra*, like other members of Lake Malawi's rock-dwelling cichlid clade, are highly philopatric and very few individuals migrate into or out of geographically isolated populations. The genetic isolation of these populations may facilitate their phenotypic differentiation and contribute to the extraordinary rate of speciation observed in this system. I will test the hypothesis that populations of *M. zebra* are phenotypically distinct by examining the species within and between population variations in male melanistic pigmentation.

Specifically, I will quantify relative dark pigment percent area and pigment intensity from photos of *M. zebra* from differing locations in order to quantify phenotypic variation. Next, I will test the hypothesis that geographic variation in pigmentation pattern is correlated with environmental conditions at each site. To do this, I will quantify benthic habitat characteristics (number of rocks, size of rocks) for the location of each population. I predict that populations with larger than average melanistic patterning will live on habitats with many smaller rocks. This analysis will provide insight into the ecological forces influencing population differentiation of Lake Malawi cichlids and may inform on the speciation process in this system.

I plan to use the following outline in my thesis in order to accomplish my goals. Chapter two will discuss the methods in which I went about achieving the goals discussed in this chapter. It will provide a detailed description of how I gathered the data and methods I used to interpret the data.

Chapter three will include a detailed description of all my findings and will include graphs, tables, and diagrams that I have constructed based on raw data.

Chapter four will consist of an interpretation of the data presented in chapter three and evaluation of the results. I will compare my results to other studies introduced in chapter one. I will also ascertain whether my methodology was sound and I will discuss the significance of my findings.

Chapter five will serve as my conclusion and will consist of the consequences of

my research. I will discuss the overarching findings, suggest ideas for further studies,

and speculate on possible implications of my results.

CHAPTER 2

Methodology

2.1 Locations studied

In order to achieve the goals outlined in chapter one, *M.*zebra cichlids from

locations across Lake Malawi were photographed. These photographs were taken with

an underwater camera by Dr. Patrick Danley. The only photos used for analysis were

male cichlids whose entire bodies were unobstructed. Male *M. zebra* exhibit male-male

competition and thus exhibit variation in their color patterns unlike the female of the

species. There are three locations which were studied. The first location is Boadzulu,

which is a small rocky island approximately 5 km away from the western shore of the

most southern part of Lake Malawi. 33 quality cichlid photos were taken from this

location. The next location that was sampled was Harbour Island. Harbour Island is a

small island in the mouth of Monkey Bay on the eastern side of the Nankumba

peninsula. 41 quality cichlid photos were analyzed. The last location sampled from was

Otter Point. Otter Point is located on the eastern side of the Nankumba peninsula. 16

quality cichlid photos were analyzed from this location. Otter Point is approximately 42

km away from Boadzulu Island and approximately 17 km away from Harbour Island.

Harbour Island is approximately 30 km away from Boadzulu island. Geographical

distances could be useful in better understanding the role genetic drift may have in the

melanistic color pattern variations between these populations. Overall 90 quality

pictures were taken from around the lake. Photos of the same fish from different angles

and lighting conditions were also analyzed in order to test the validity and reliability of the data.

Each of the locations varied in their terrain which could be a factor in the melanistic variation seen in *M. zebra* cichlids across the lake. Overall Otter Point contains very large rocks making the terrain relatively flat and homogenous. Boadzulu contained numerous smaller rocks making for numerous crevices and holes creating a relatively heterogeneous terrain. Harbour island was more of an intermediate terrain containing larger rocks then Boadzulu but smaller then Otter Point. Initially, the number and size of rocks were attempted to be quantified based on the photographs taken at each location. However, because these photos were specifically taken of cichlids and not purposed for the surrounding environment, they differed drastically in zoom and angle. It became impossible to accurately measure size of rocks based on the limited data available, however, the relative number of rocks in a given area was able to be determined. This was done by selecting 10 representative samples from each location in which the rocky habitat was in full view and not angled upwards or zoomed in. The cichlids in each of the photos were used as a standard and each photo was adjusted so the relative area being studied was the same across each of the photos. Every rock was then counted in the standardized photos to determine the density of rocks and the results for each location were compared to each other. Further data should be collected to quantify differences in rock size or other useful topographical data. An ANOVA test was used on the topographical data to determine if there was significant differences between groups. A Tukey HSD test was then used to determine which specific groups

differed from one another. All statistical tests in the experiment were used through JMP.

2.2 Determining dark pigment density

In order to determine the melanistic variation, two different aspects of the cichlid color pattern was analyzed. The first was the dark melanistic pigment density and the second is relative pigment intensity. Dark melanistic pigment density refers to percent of the dark pigment relative to the cichlid's body, head to caudal peduncle. The caudal peduncle is the narrow part of a fish that attaches the tail to the body. Relative dark pigment intensity was determined by quantifying the dark melanistic color on each cichlid into an average grey value between 1 and 255. 1 represents pure black and 255 represents pure white.

The first step in determining the dark pigment density on each cichlid was to outline the cichlid's body, head to caudal peduncle. This was done using a pen tool on a Lenovo tablet. The pen tool, while more time consuming, is a superior method to using an image processing program because the computer cannot correctly distinguish between the fins, tail, and body since their color shades are very similar. After body outlines were made for every cichlid, the pictures were converted into binary. This was accomplished by converting the image into an 8-bit, converting to binary, and removing all light and dark outliers. Converting the photos to 8-bit allows ImageJ to determine grey values for each pixel which is necessary to convert an image to binary. Binary

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converts the image to pure black pixels and pure white pixels. Removing outliers helped

eliminate parts of the image that were pure black or pure white since this data would

have skewed the results. Since the background of the cichlid outline is white this step

would eliminate the background from the data. The threshold converted pixels in the

photograph that were closer to 255 to white and converted pixels closer to 1 to black.

The threshold value remains constant for each of the pictures. After a binary image of a

cichlid was made, the area of the dark pixels was measured and divided over the area of

the whole body to determine the dark pigment percentage. A macro was created for

ImageJ and was applied to each cichlid body outline in order to calculate the dark

pigment percentage for each cichlid.

This data was grouped into their respective locations and converted to box and

whisker plots. Box and whisker plots break the data into quartiles showing the median

of the lower half of the data set, the median of the whole data set, and the median of

the upper half of the data set. They are useful for both visually seeing the shape of the

distribution, the central value, and the variability of the data. The plots for each of the

three locations were able to be compared using this method so that interpretations and

conclusions could be drawn. The data was also analyzed using a one-way ANOVA plot.

One-way ANOVA compares the means between the three locations to determine if

those means are statistically significantly different from one another. It specifically

attempts to see if the data rejects the null hypothesis that all means are equal. A Tukey

HSD test was also used to determine which specific groups differed between each other.

Lastly, a Pearson coefficient test was used to test the correlation between dark pigment percent area and habitat complexity.

2.3 Determining dark pigment intensity

The next part of the analysis was to determine the intensity of the dark pigmentation of the zebra cichlids. The first task was to standardize the brightness of the photographs using the light blue pigment of the cichlids as a reference. In dominant male *M. zebra* cichlids, the light blue pigment between individuals is a relatively constant intensity under normal conditions. Therefore, it can be used as a standard to eliminate variations in lighting conditions between photographs of cichlids taken at different areas of the lake. Lighting can be affected by time of day, camera angle, depth, and water clarity. All of these lighting factors, if not accounted for, could skew the data by giving incorrect reads on the dark pigment intensity of these fish.

In order to standardize the images, each of the body outlines was converted into 8-bit, and the mean grey values of the light pigment region and the mean grey values of the dark pigment region were calculated using ImageJ. The grey values range from 255-0 where 255 is pure white and 0 is pure black. The value of pure white, 255, was subtracted from the mean grey value of the light pigment for each individual cichlid. That value was then added to the mean grey values of the dark pigment. This reduces the light pigment areas to pure white, 255, and subtracts the difference from the dark areas. By setting every light segment to pure white and 'brightening' the dark segments by the respective amounts the data becomes more reliable. Along with mean grey

values, ImageJ was used to calculate the standard deviation when analyzing each segment.

The standardized body outlines were then separated into light and dark pigment sections. This is accomplished by manually setting a threshold in ImageJ that separates each section based on pigment values. The next step involves eliminating the white background and any white areas within the picture to avoid skewing the intensity values. The image is then converted to 16-bit in order to set it to a greyscale. An auto threshold is applied to the image which allows ImageJ to remove outliers such as 255 and 0. It is now possible to measure the intensity of both the light pigment section and the dark pigment section by measuring the mean grey value for each. The standard deviation was also measured. The mean grey value found for the light pigment sections was then subtracted from 255 and added to the mean grey values for the dark pigment sections. The standard deviations from both sections were combined for each fish image using the following formula:

$$\Delta k = \sqrt{(\Delta k_1)^2 + (\Delta k_2)^2}$$

The unstandardized intensity values for the dark pigment sections were recorded to compare with the standardized as a way to see the effectiveness of the standardization method. This was accomplished by analyzing images of the same fish from various angles and lighting conditions. If the standardization method was reliable the standardized dark pigment intensity values for the images of the same fish would have a very small percent difference then when compared to the unstandardized

images. The average intensities for all the cichlids in each of the three locations was then calculated along with the standard deviation for each. The results were converted into box and whisker plots and were compared amongst each other. A one-way ANOVA test was used for the mean intensity values for each of the three locations to determine if the differences between each population is statistically significant and rejects the null hypothesis. A Tukey HSD test was also used to determine which specific groups differed between each other. Lastly, a Pearson coefficient test was used to test the correlation between dark pigment intensity and habitat complexity.

2.4 Combining data

The average dark pigment area percentage for each individual cichlid was then plotted against each individual cichlid's standardized dark pigment intensity values in order to determine if there is a correlation between the two. The average dark pigment area percentage for each location was then plotted against the average standardized dark pigment intensity for each location to see if there is a correlation. The topographical data calculated from each location can be used to assess whether changes in environmental complexity has a correlation to differences in either pigment intensity or dark pigment density in each of the three locations. The distance between locations can also be compared with the data to check for any correlations.

CHAPTER 3

Results and Discussion

3.1 Results of dark pigment percent area study

Dark pigment percent area for each of the cichlids in the study and the results

were compared within and against varying locations around the lake. Validity of a study

is whether the study measures what it is intended to measure. The validity of the study

was tested in order to ensure the data is accurately measuring the dark pigment percent

area for these fish. This was done so by applying the method to two separate pictures at

different angles of the same fish to determine whether a significant difference was

measured in the results. Photos 1239 and 1243 from Boadzulu as well as photos 1385

and 1386 were used in this test. Each pair contained the same fish but taken at a

different point as the fish was traversing its environment. The dark pigment percent

areas of cichlids in photos 1239, 1243, 1385, and 1386 were 47%, 45%, 49%, and 50%

respectively. While only two examples were performed, the results show that the

method of determining the dark area provides a reliable indication of the true value.

While it would be preferable to meticulously measure cichlids in the lab, this is an

effective way to glean useful information from simple photos taken in the field without

disturbing the habitat.

This method was applied to each photo and the results showed that the

Boadzulu sample had a higher average bar area percentage, 62%, then Harbour, 56%,

which was higher than Otter Point, 50%. The standard deviation for each location were

0.124, 0.136, and 0.131 respectively. All statistical tests and graphs were made through

the JMP program. The specific steps involved in each test is outlined in the methods

section.

Location	Number	Mean	Std Dev
Boadzulu	33	0.617	0.123
Harbor Island	41	0.562	0.135
Otter Point	16	0.507	0.131

Figure 1: Means and standard deviation for percent area study

Before using statistical methods it is necessary to check to see if the data follows

a normal distribution since both ANOVA and Tukey HSD tests assume normality.

Therefore, a histogram was made of the data and it indeed followed a normal
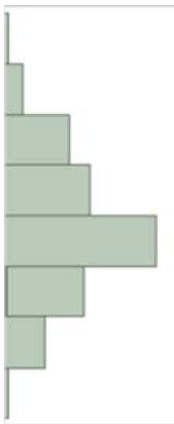
distribution as seen below.

Figure 2: Histogram of data for dark pigment percent area study

A box plot was made of this data comparing each of the three locations shown in Figure 3. A box plot is way of displaying data though quartiles. The whiskers of the boxes indicates variability outside the upper and lower quartiles.
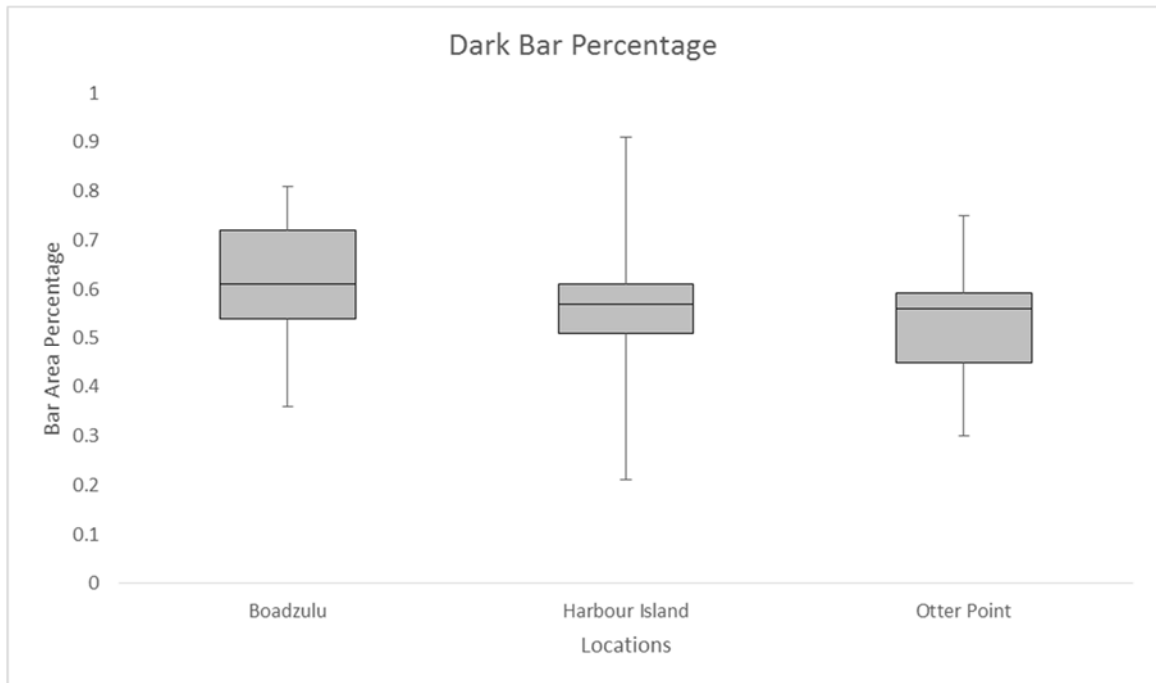

Figure 3: Box plot for dark pigment percent area study

A one way ANOVA test was used in order to determine whether the data is statistically significant. From the ANOVA test, the probability of this result, assuming the null hypothesis, is 0.0213. Therefore, there is sufficient evidence to reject the null hypothesis given if α=0.05.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Location	2	0.137	0.0687	4.024	0.0213
Error	87	1.486	0.0171		
C. Total	89	1.623			

Figure 4: ANOVA test results for dark pigment percent area study

A separate test was then conducted on this batch of data. A Tukey HSD test is used to determine which groups in the study actually differ. This test is typically performed after an ANOVA test. ANOVA describes whether or not groups in the sample differ; however it does not tell which groups differ. Since the ANOVA test determined there is a significant statistical difference in the data, the Tukey HSD test can determine which groups are significantly different. This test showed that Harbour Island was not significantly different from either location, but that there was a statistically significant difference between the Boadzulu cichlids and the Otter Point cichlids.

Location 1	Location 2	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Boadzulu	Otter Point	0.109	0.039	0.015	0.205	0.0192
Harbour Island	Otter Point	0.055	0.038	-0.036	0.147	0.323
Boadzulu	Harbour Island	0.054	0.030	-0.019	0.127	0.184

Figure 5: Tukey HSD test for dark pigment percent area study

The initial hypothesis of this study was that cichlids with lower bar area percentages would be found in locations that are less rocky, while cichlids with higher bar area percentages would be found in more rocky locations. The reasoning for this

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hypothesis was that areas with larger and fewer rocks create a homogenous coloration which may create a selection against cichlids with larger bars since they would stand out more for predators.

Another hypothesis made was that the further locations were from one another, the greater the difference in their relative dark pigment area percentages. The reasoning for this hypothesis was that the further populations are from one another, the less interactions there are between groups, and the greater the effect of genetic drift. The results seem to corroborate with both of these hypotheses.

In order to see whether there was a correlation between locations in the lake to dark pigment area percentage a Pearson product-moment correlation coefficient test was used in this study. A Pearson product-moment correlation coefficient is a measure of the linear association strength between two groups. It results in a line of best fit between the data of the two variables and the coefficient is indicative of how far away these points are from the line of best fit.

Variable	By Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
Avg # of rocks per location	Body Percentage	0.2885	90	0.0866	0.4677	0.0058

Figure 6: Correlation tests for area percentage study

Both these correlation tests provide strong evidence that there is a correlation between body percentage and the average rockiness of each location. From data collected in the topographical study, discussed later, Boadzulu had the greatest number

of rocks, creating a relatively heterogeneous environment which correlated with the population having the greatest dark pigment percent area. Otter Point contained relatively few very large rocks, giving the location a homogeneous environment. This correlated to the relatively low dark pigment percent areas of this population. Lastly, Harbour Island contained an intermediate number of rocks which correlated to the relatively intermediate dark pigment percent area of this population.

Furthermore, the Boadzulu location is farthest away from Otter Point, with Harbour Island being an intermediate location between the other two. This correlates to the fact that only Boadzulu and Otter Point had significant statistical differences when compared.

3.2 Results and analysis of pigment intensity study

Raw pigment intensity data was standardized in order to mitigate the effects of variances of lighting in the photos. In order to test the validity of the standardization method, photos of the same cichlids taken from different angles and positions among the rocks were analyzed and compared. The photos tested were 1239 and 1243, which were taken of the same fish from Boadzulu. Photos 1385 and 1386 were also tested and were from the same cichlid taken at Harbour Island. Before standardization, the percent difference of the dark pigment intensity values between cichlid 1239, 1243, and between 1385 and 1386 was 11.6% and 6.0% respectively. After standardization the Boadzulu sample had a percent error decrease from 11.6% to 3.1%, while the Harbour

island sample decreased from 6% to 1.2%. These results show that this method of standardization greatly helps to eliminate the variable of lighting in the photos.

The raw intensity data for each of the cichlids were standardized and the results showed average dark pigment intensities of 201.9, 190.8, and 195.2 for Boadzulu, Harbour Island, and Otter Point, respectively. The standard deviations for each of these samples was 13.0, 15.3, and 25.1 respectively. These results can be seen below.

Location	Number	Mean	Std Dev
Boadzulu	33	201.997	13.048
Harbor Island	41	190.841	15.350
Otter Point	16	25.113	25.112

Figure 7: Means and standard deviation for pigment intensity study

Before using any statistical tests, a histogram was made of the data to check for normality. It indeed followed a normal distribution as seen below.
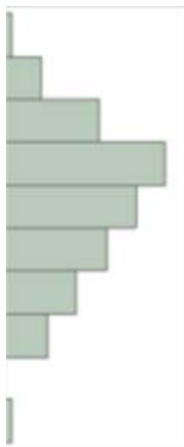

Figure 8: Histogram of data from dark pigment intensity study

A box plot was made in the same way as the dark pigment percent area study, comparing each of the three locations shown in figure 11.
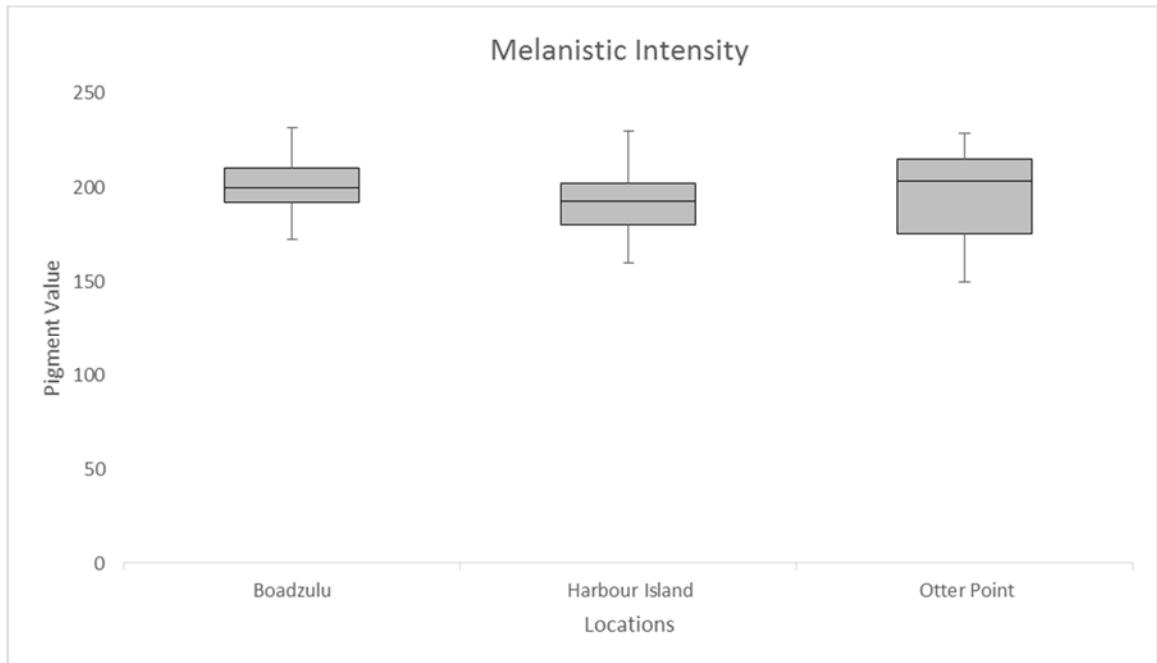

Figure 9: Box plot for dark pigment intensity study

A one way ANOVA test was used in order to determine whether the data is statistically significant and due to factors apart from random sampling fluctuations. The ANOVA test resulted in a p-value of 0.02, therefore providing strong evidence against the null hypothesis.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Location	2	2280.714	1140.36	4.0772	0.0203
Error	87	24333.303	279.69		
C. Total	89	26614.016			

Figure 10: ANOVA test for dark pigment intensity study

After performing a Tukey HSD test, it appears that the only two groups to differ by a significant statistical measure is between Boadzulu and Harbour Island. Therefore, although the pigment intensity values for all groups were statistically different, there is only a statistical significant difference between Boadzulu and Harbour Island.

Location 1	Location 2	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Boadzulu	Harbour Island	11.156	3.911	1.830	20.483	0.0148
Boadzulu	Otter Point	6.796	5.095	-5.352	18.944	0.3804
Otter Point	Harbour Island	4.360	4.360	-7.394	16.116	0.652

Figure 11: Tukey HSD test for dark pigment intensity study

Overall there was a significant difference in the pigment intensity of *M. zebra* between Boadzulu and Harbour Island but not between any of the other locations. In order to see whether there was a correlation between locations in the lake to pigment intensity, two tests were performed. A Pearson product-moment correlation coefficient test was used in this study.

Variable	By Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
Avg # of rocks per location	Body Percentage	0.2204	90	0.0139	0.4088	0.0369

Figure 12: Correlation tests for pigment intensity study

While the Pearson product-moment correlation coefficient test showed evidence for a correlation,

The large variance of pigment intensities observed may be related to factors outside of location in the lake, such as diet, age, etc. and therefore are outside the scope of this study. Possibly, a larger sample size may yield data that shows a stronger correlation. Lastly, a Pearson product-moment correlation coefficient and a Spearman's rank correlation coefficient was used to test for correlations between the pigment intensity data and the percent area data. These tests failed to show any correlation between body percentage and pigment intensity.

Variable	By Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
Avg # of rocks per location	Body Percentage	-0.149	90	-0.3453	0.0601	0.1613

Figure 13: Correlation tests between pigment intensity and percent area studies

3.3 Topographical analysis of locations

The analysis of the topography of each of the locations studied was performed. The rockiness of 10 standardized photos for each location was measured and converted into a box plot. Because only 10 samples were used for each location, as a result of limited available data, normality cannot be confirmed and thus was assumed. A histogram was made using this data.
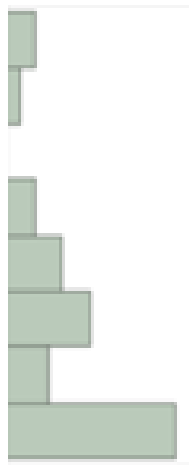

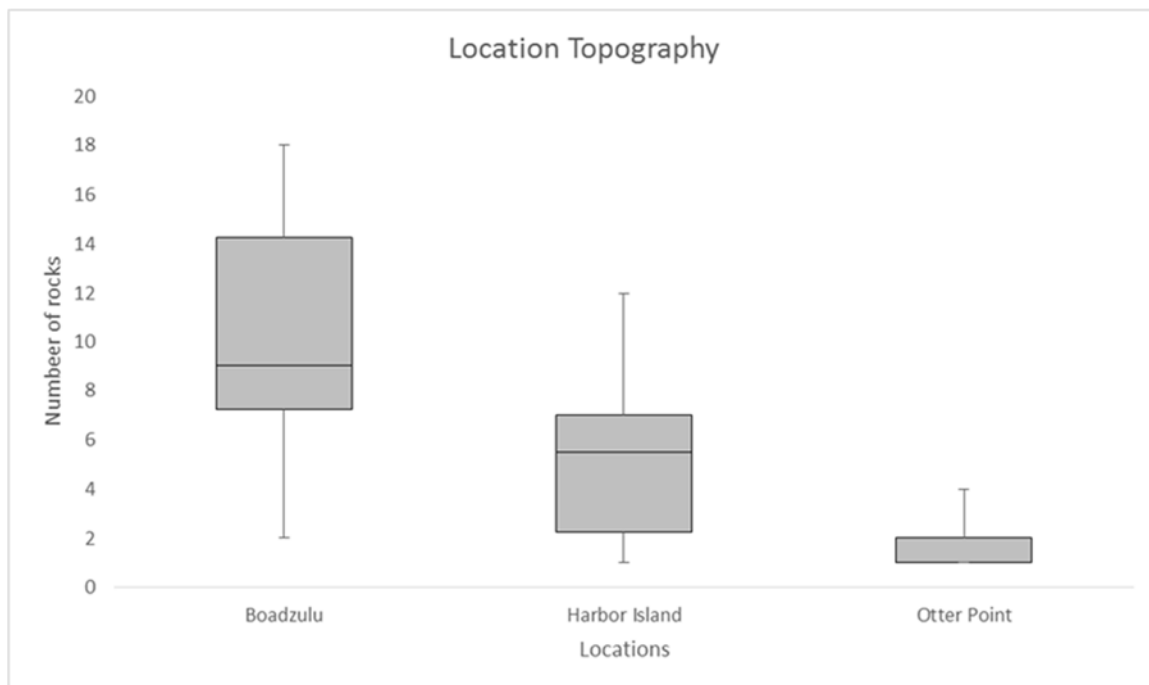*Figure 14: Histogram of topographical data*

Figure 15: Box Plot for topography study

The means and standard deviations for each location was also calculated.

Location	Number	Mean	Std Dev
Boadzulu	10	10.400	5.274
Harbor Island	10	5.300	3.401
Otter Point	10	1.800	1.229

Figure 16: Means and standard deviation for topographical study

An ANOVA Test was performed on the data to determine whether the results were statistically significant. The ANOVA test resulted in a p-value of <.0001, providing very strong evidence against the null hypothesis.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Location	2	374.0667	187.033	13.7188	<.0001
Error	27	368.100	13.633		
C. Total	29	742.167			

Figure 17: ANOVA test for topographical study

After performing a Tukey HSD test, the rocky topography of Boadzulu is

significantly statistically different from both Harbour Island and Otter Point. However,

Otter Point and Harbour Island were not found to be statistically significant. According

to this test there is very strong evidence of a statistically significant difference between

Boadzulu and Otter Point, $p<0.0001$, and between Boadzulu and Harbour island,

$p<0.0124$.

Location 1	Location 2	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
Boadzulu	Otter Point	8.600	1.651	4.506	12.694	<.0001
Boadzulu	Harbour Island	5.100	1.651	1.006	9.194	0.0124
Harbour Island	Otter Point	3.500	1.651	-0.594	7.594	0.1047

Figure 18: Tukey HSD test for topographical study

These results indicate that Boadzulu has numerous smaller rocks, Harbour Island

contains a smaller amount of intermediate sized rocks, and Otter point contains

relatively few very large rocks. The types of rocks at each of these locations reflect these

results. Otter Point contains large rocks, making the terrain relatively flat and

homogeneous. Boadzulu contains many small rocks, providing numerous crevices and

holes, forming a heterogeneous terrain. Harbour Island is an intermediate terrain,

containing larger rocks than Boadzulu but smaller rocks than Otter Point. A final

important distinction between each location is their approximate distance from one

another. Otter Point is 42 km away from Boadzulu and 18 km away from Harbour Island.

Harbour Island is 30 km away from Boadzulu.

CHAPTER 4

Conclusion

4.1 Chapter Outline

The results of this study shed light on many of the questions initially proposed in chapter one. These questions are important because of the role the species plays in understanding adaptive radiation and evolution has a whole. A better understanding of the role of divergence in *M. zebra* may lend itself to a better understanding of speciation in this incredibly complex and diverse group of fish. At the beginning of this study, the questions asked were the following: are the differences in male melanistic color pattern of zebra cichlids from different environments evidence of adaptive divergence? Are these morphological differences quantifiable, reliable, and useful for making predictions? Does the terrain of the individual populations have a noticeable correlation to its inhabitants' phenotype? This study addressed these questions and the conclusions for each will be evinced in the coming paragraphs. I will also address issues with this experiment, ideas for improvements in the method, and possible future studies that can help further answer these important questions.

4.2 Are these morphological differences sufficiently quantifiable, reliable, and testable?

The method used to quantify the pigment intensity and dark bar percent area of *M. zebra* photos is useful because it allows researchers to standardize and efficiently test these variables from photos. The goal to quantify these morphological differences into reliable and testable data was accomplished through a number of steps. ImageJ was used to differentiate light from dark pigment areas of the cichlids was useful twofold. First, by dividing the area of the dark pigment by the total area of the fish, the percent area of dark pigment was able to be determined. Second, by standardizing the dark pigment based on the grey value of the light pigment it was possible to find a standardized grey value of the dark areas which represented the intensity. The cichlid photos were standardized based on relative cichlid length in order to accurately measure number of rocks for photos in each location. The number of rocks, the pigment intensity values, and the dark pigment percent area values for each of the three studied locations were used to perform statistical tests in JMP.

4.3 Is there a correlation between population phenotype and environment?

A Pearson product-moment correlation coefficient test was used for correlation testing. A statistically significant, yet weak correlation, was found when comparing average number of rocks per location by body percentage. In regards to pigment intensity by average number of rocks per location, Pearson's coefficient test found there to be a weak yet statistically significant difference while Spearman's did not. There was no correlation found when testing dark pigment intensity vs dark pigment percent area.

These results allow us to suggest that there is a relationship between number of rocks

per location and the bar area percentage of these cichlids. However, correlation does

not suggest causation, so although these two variables may be related, the nature of

their relationship requires further study. An initial hypothesis of this experiment was

that a rockier environment would result in the less conspicuous, higher bar area

percentage cichlids having a selective advantage. Contrarily, an environment with large,

smooth rocks would result in cichlids with lower bar area percentage being selected for.

While the results of this study support this hypothesis it cannot not confirm it. While

conspicuousness of fish may be a major factor in the correlation between rockiness and

bar area percentage there are possibly many other factors not accounted for, such as

sexual selection.

4.4 Is this example evidence of adaptive divergence?

One of the results of this study was the finding that there was a significant

statistical difference in the dark pigment percent data between Boadzulu and Otter

Point. The results from the pigment intensity study were inconclusive. This was

significant for two reasons. First, these two populations have the greatest difference

with regards to topography. Otter Point has very few, large rocks while Boadzulu has

numerous small rocks. Second, these two locations are the furthest apart from the three

locations studied. These results support the idea that the differences in environment

and lack of gene flow between these two populations may be resulting in the beginning

of adaptive divergence. However, much more research needs to be undertaken to conclude that this is indeed the case.

4.5 Issues with experiment

There were several issues that limited this experiment. The main issue this study faced was the reliance on photographs. While photographs may be more convenient and less damaging to the ecosystem then bringing fish into the lab, it presents a number of problems. The first was the variances in lighting. Different times of days and different depths will change the lighting of the photos which will severely affect data such as pigment intensity. This however was accounted for with the standardization method used in the study. Studying the topography is where the use of photos fell short. Initially, I intended to not only quantify number of rocks, but also average length of rocks, and area of rocks. However, I was unable to do this because there was no way to account for how big objects were since I was looking at a 2d image of a 3d object. I was however, able to use the size of the cichlids in each photo as a reference from which to scale the size of the photos down in order to accurately assess the number of rocks. The photos were taken of cichlids, but were not intended to be taken of the topography. Therefore, many of the photos were taken from various angles and zooms which made it impossible to accurately quantify a great deal of info. To remedy this, only ten photos from each location that were similar in the angles taken and were not zoomed in were used. Another issue this study faced was the small sample size from which to measure, especially from Otter Point. Small sample sizes lower the chance of uncovering

significant differences, lower the confidence in results, and are less reliable. A larger sample size may show that Boadzulu and Harbour Island's percent area data was significantly different and may help clarify the pigment intensity results which are inconclusive.

4.6 Future Studies

Based on the results of this research there are several future studies which would help further answer the questions posed in chapter one. The first study would be a more expansive analysis of the standardization methods used in the pigment intensity study. In order to ensure reliability of the standardization method, several pairs of photos of the same fish were compared to see if the percent differences between standardized and unstandardized decreased. From the pairs studied the percent difference drastically decreased. Analyzing more pairs in the same way would greatly increase the reliability of this method which may be useful for researchers for studying pigment intensity of *M. zebra* as well as other specifies of cichlids. The second study would be a more expansive analysis of the topography of each of the three locations. Along with increasing sample size, more information on the environment would provide more context from which to test hypothesis and make conclusions. Due to restrictions, this study only accounted for number of rocks and doesn't take into account size of rocks, variability in rocks size, area of rocks, etc. Lastly, incorporating other locations into the study would be beneficial as it would provide more confidence in making correlations between factors such as rockiness and dark pigment percent area.

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