

Sexual Dimorphism and Trophic Trait Evolution in the Carolina Bay *Fundulus* Complex

By

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ABSTRACT

Convergent evolution in similar environments is widely held to provide some of the strongest evidence for natural selection's role in evolution, and convergent or parallel evolution of reproductive isolation, best studied in some fish systems, has been interpreted as evidence of selection's role in speciation. Most such fish studies have emphasized the role of selection on trophic traits in explaining trait convergence and speciation, but lately the roles of sexual selection and sexual dimorphism have also been considered in the studies of speciation and adaptive radiation. In the present study I investigated a to date little studied system, the Carolina Bay *Fundulus* species complex, in which patterns of convergence and divergence of body shape (and even speciation) have been suggested to be driven largely by predation. I investigated this complex for the trophic trait evolution seen in other fish systems, and for evidence of sexual dimorphism in body shape that could be important to the interpretation of shape evolution. I found that gill raker length and density, key trophic traits in fish, exhibited similar patterns of divergence and convergence in *Fundulus sp.* from Carolina Bays and streams as are seen for body shape. I further found that body shape divergence between Lake Phelps (a Carolina Bay) and the adjacent Scuppernong River was uneven between the sexes, females diverged

significantly whereas males did not. In a second lake-river pair, however, Lake Waccamaw and the Waccamaw River, sexual dimorphism in body shape was not statistically detectable. These results suggest that selection on trophic traits may also be important to the divergence of Carolina Bay *Fundulus* from populations in other habitats, and that shape divergence between habitats may involve sexual dimorphism.

Sexual Dimorphism and Trophic Trait Evolution in the Carolina Bay *Fundulus* Complex

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INTRODUCTION

Fish systems have played a central role in the development of hypotheses of ecologically based diversification and speciation. Schluter (1996) used the Threespine Stickleback, *Gasterosteus spp.*, as a case study to articulate the theory of adaptive radiation caused by ecological selection, which may ultimately lead to speciation between populations inhabiting different ecological niches. Speciation is known to arise through divergent selection in differing habitats and has been seen, in sockeye salmon (*Oncorhynchus nerka*), to progress substantially within 13 generations (Hendry *et al.* 2000). Based on such examples, Schluter (2000) proposed what he called “the Ecological Speciation Hypothesis” which states that differences in phenotype, and ensuing speciation, are caused by competition among species and differences in environment that lead to divergent selection. McKinnon & Rundle (2002) reviewed speciation in the stickleback complexes, and concluded the process was driven by divergence in natural selection, as well as ecologically based sexual selection. Sexual selection is also considered in this model to be closely related to variation in a few, or even a single phenotypic trait (e.g. body size), that can contribute substantially to reproductive isolation among populations within this complex (McKinnon *et al.* 2004). Hendry (2009) later suggested that evidence for ecological speciation may be overstated and argued that populations thought to be exhibiting ecological speciation may exhibit differing levels of reproductive isolation anywhere between no isolation and being fully isolated.

One major factor in comparing evolutionary divergence is relative energy, or the speed and volume of an aquatic system, found in the aquatic habitats classified as different watershed types. Watershed type is an abiotic factor that can have a great effect on different populations of the same species in a number of different areas. Striped bass *Morone saxatilis* showed

sensitivity to relative energy in the water system causing changes in their spawning, egg characteristics, and offspring's characteristics after they have hatched (Rulifson & Manooch 1990). Striped bass have also adapted to the energy regime in different environments. Bergey *et al.* (2003) observed variation in egg size, weight, surface to volume to ratio, and even the proportion of fat types found in eggs in different watershed types. This selective pressure is not well studied or understood and could have further effects on populations of a single species.

One aspect of fishes that has a well-studied connection to relative energy in aquatic systems is body shape. Body shape in fishes has been shown to follow divergent patterns between lacustrine and riverine forms. European minnows, *Phoxinus phoxinus*, display deeper bodies and caudal peduncles in lakes than in streams (Collin & Fumagalli 2011). This is the expected trend between lake and river forms of a species complex. Webb (1978) described the most common occurrences of stream-lined body shapes, or those with shallower bodies and caudal peduncles, as an adaptation for sustained swimming speeds that increase predator evasion and swimming efficiency in moving water. Threespine stickleback from the Haida Gwaii archipelago in Canada do not support this hypothesis; anadromous populations exhibit more stream-lined body shapes compared to freshwater populations (Spoljaric & Reimchen 2011). Marine populations in the Vancouver Island (Canada and Washington State, US) as well as in Japan both showed more streamlined body shapes compared to stream-resident forms (Kitano 2012).

Morphological characteristics are also known to evolve with trophic context in fishes; gill raker size and count are key metrics in this regard. Within the Coregonid whitefish species model gill raker count was shown to have a close relationship with ecological factors including nutrient levels (Vonlanthen *et al.* 2012). The whitefish exhibited similar phenotypic divergence from

historical forms in terms of gill raker count in environments affected by eutrophication, showing the rapid evolutionary response of gill raker traits to prey availability and trophic niche differentiation. Because niche partitioning can play a major role in sexual isolation, differences in trophic traits may be involved in ecological speciation (McKinnon *et al.* 2004).

Another increasingly widely appreciated aspect of ecological variation is sexual dimorphism, or the phenotypic differentiation between the sexes. Sexual dimorphism has been suggested to be a product of both natural and sexual selective pressures that differ between the sexes (Lande 1980). Lande (1980) states that these pressures may drive change in each sex away from an ecological optimum. Sexual dimorphism of ontogenetic characters, which include allometric growth, are also suggested to reflect differing natural and sexual selective pressures within a population (Lande & Arnold 1983). Reimchen and Nosil (2004) also suggested that in the threespine stickleback trophic niche-partitioning of the sexes leads to different predation pressures, which has in turn driven sexual dimorphism. They further suggested that differences in ecology can drive selective differences between sexes through many different habitat or niche specific mechanisms (Reimchen & Nosil 2004). Cooper, Gilman, and Boughman (2011) conducted a theoretical analysis of the importance of sexual dimorphism in relation to niche structure and speciation. They found that sexual dimorphism can be a response to a selective pressure in place of speciation, and that a selective regime with strong selective pressures occurring on more than one axis can lead to sexual dimorphism and speciation sequentially (Cooper, Gilman, and Boughman 2011).

Carolina Bays are distinctive freshwater environments that in some cases are home to endemic fish faunas. These water bodies are similarly shaped, point in the north-west direction, and are characterized by their high density of bay trees including bald cypress *Taxodium*

distichum and many others. The origin of these environments is not known but highly debated. North Carolina is home to the two largest Carolina Bays, Lake Phelps and Lake Waccamaw, which are natural lakes. These Carolina Bays provide uniquely different habitats from the rivers and lakes in North Carolina, as well as other Carolina Bays. With 42 and 28 different species of fish in Lake Waccamaw and Lake Phelps respectively, as well as their size and proximity to other bodies of water they also provide different environments from one another.

Lake Waccamaw is found in the southeast of North Carolina near the South Carolina border where it is isolated from other bodies of water aside from its tributaries and distributaries. It is estimated by Stager & Cahoon (1987) to be between 15,000 and 32,000 years old, and is located on a limestone base, which raises its pH closer to neutral and increases the visibility in the water compared to other local Carolina Bays and North Carolina watersheds.

Lake Phelps is found in the northeast of North Carolina in between the Pamlico and Albemarle Sounds. It is located in close proximity to other lakes as well as rivers and the sounds. Lake Phelps is the largest Carolina Bay with an area of 16,600 acres and also rests on a limestone base causing the pH to be closer to neutral and the water being much clearer. The origin of this lake is not known but it is estimated to be around 38,000 years old or less (Holley 1989).

These habitats allow for possible evolutionary convergence and divergence from other local habitats as well as potential divergence from one another. The distributaries of the these lakes, Waccamaw River and Scuppernong River respectively, both exhibit common traits of North Carolina and South Carolina watersheds. They each have a low pH, low visibility due to sediment and plant leeching, low river gradients, and common anoxic areas. They also have traits that differ from one another: Waccamaw River is estimated to contain up to 62 fish species and is tidally influenced for the last 40 miles leading up to Winyah Bay (Shute *et al.* 1981), whereas

the Scuppernong River has a low species diversity and has relatively little energy, as a function of tides or river gradient, throughout the entire system leading up to the Albemarle Sound.

All four of these water systems are home to a form of *Fundulus* killifish. *Fundulus diaphanus* is split into two subspecies, *F. d. diaphanus* and *F. d. menona*, which are thought to have been glacial races that have partially speciated along a hybrid zone (April & Turgeon 2006). *Fundulus diaphanus diaphanus* is the Atlantic race and the subspecies that will be examined in this study (April & Turgeon 2006).

Lake Waccamaw has an endemic form of *Fundulus diaphanus* that was described by Hubbs & Raney (1946) as a distinct species, *Fundulus waccamensis*. This species differs from its presumed ancestral form through the presence of a more streamlined body shape, specifically a longer body that is less deep (Hubbs & Raney 1946). The description of *F. waccamensis* was later confirmed as being a different species from *F. diaphanus* (Frey 1951). The distribution of this species is limited to within Lake Waccamaw and as a result it has been considered a threatened species. *F. waccamensis* was added to the IUCN Red List as a State Endangered Species in 2010 (NatureServe 2013).

Sexual dimorphism displayed in *Fundulus diaphanus* was originally described by D.S. Jordan and B.W. Evermann as differences in the coloration of males and females, primarily in the number and coloration of the bands along the sides of the body (Newman 1907). Newman (1907) also posits that the sexual dimorphism exhibited in *F. diaphanus* is that of a primitive condition that is elaborated in its descendants. The sexual dimorphism of *F. waccamensis* is understood to be differences in coloration, genitalia and their contact structures, and differences in body shape (Shute *et al.* 1983).

The *F. diaphanus* and *F. waccamensis* species complex along with two other species pairs were shown to exhibit the opposite trend in body shape. Krabbenhoft *et al.* (2009a) showed that the Lake Waccamaw endemics, including *F. waccamensis*, possessed shallower body depths compared to their respective river forms, including riverine *F. diaphanus*. All three species analyzed in the Krabbenhoft study showed the same body shape divergence, though the patterns of divergence leading to shallower bodies were taxon specific. This phenomenon was hypothesized to be an adaptation for predator evasion in Lake Waccamaw, due to its higher water clarity than the surrounding tributaries (Krabbenhoft *et al.* 2009a).

The presence of similar habitats and selective pressures may lead to convergent evolution, a well-studied topic within evolutionary biology. The *Fundulus sp.* found in Lake Phelps, North Carolina, have been suggested to belong to *F. waccamensis* and to have originated in the lake through human introduction (Bailey 1977; Wiley & Mayden 1985). This argument was contradicted a few years later by Shute *et al.* (1981) who stated that there were morphological differences in the length of the head and caudal peduncle, as well as the interorbital width. It was also mentioned in Krabbenhoft *et al.* (2009b) that unpublished molecular genetic data by Quattro *et al.* indicate that the Phelps population is related more to *F. diaphanus* in nearby tributaries than *F. waccamensis*. This leads to the currently best accepted hypothesis that the Lake Waccamaw and Lake Phelps populations exhibit convergent evolution. Northern lineages of *F. diaphanus* have been observed to feed primarily on benthic prey such as cladoceran macroinvertebrates through the use of benthic adapted trophic structures (Phillips *et al.* 2007). Interspecific competition among sympatric species and different ecological pressures may lead to divergence of trophic traits between Carolina Bay forms and sister stream forms. Variation in the number of endemic species in the different habitats of these populations may

imply differences in levels of resource competition. Differing prey availabilities due to environmental characteristics in the lakes compared to the rivers may also lead to differing trophic adaptations and/or niche partitioning between the sexes.

In the present study I investigated the *Fundulus diaphanus/waccamensis* complex to answer the following questions: 1). do lake forms diverge from their stream relatives in trophic structures in a pattern similar to that for body shape; 2). do patterns of sexual dimorphism contribute to, confound, or otherwise relate to morphological divergence and convergence between lake-river pairs? Studies of other fish taxa lead to the hypothesis that the novel and comparatively similar lacustrine habitats will result in convergent evolution not only of body shape but also of trophic structures (Hendry & Taylor 2004; Vonlanthen *et al.* 2012). This would predict similar results in body shape and trophic structures in the Carolina Bay forms. Similarly, studies of sticklebacks and other fishes lead to the hypothesis that sexual dimorphism will be present in the body shape and trophic structures of the lacustrine populations (Spoljaric & Reimchen 2008; McPhail 1992). This dimorphism could be associated with, and either contribute to or confound, ecological divergence between stream and Bay forms. Support for these hypotheses could suggest the need to reanalyze previous interpretations of Carolina Bay evolution in this species.

MATERIALS AND METHODS

Collection

Wild *Fundulus diaphanus* and *Fundulus waccamensis* were collected from 3 locations (**Figure 1**): Lake Waccamaw, Lake Phelps, and Scuppernong River. The dates, GPS locations, and sexes can be found in **Table 1**. Collections were attempted at Waccamaw River, the main distributary of Lake Waccamaw, as it is hypothesized to be the most closely related river form of *Fundulus diaphanus* to *Fundulus waccamensis*, but were unsuccessful. Collections at Northwest River (VA) were conducted to attempt to collect a representative sample of the northern population but collections only totaled three females and so were excluded from all analyses. Fish were collected using minnow traps, beach seines, and casting nets, supplemented with dip nets.



Figure 1. Sample Sites in North Carolina and South Carolina (1) Scuppernon River, NC (2) Lake Phelps, NC (3) Lake Waccamaw, NC (4) Waccamaw River, SC.

Species	Population	Date	Number of Males	Number of Females	Preservation	GPS
<i>F. diaphanus</i>	Lake Phelps	12/12/2012	9	0	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	1/21/2013	0	2	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	3/3/2013	2	2	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	3/11/2013	0	1	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	3/13/2013	0	1	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	5/20/2013	1	4	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	5/21/2013	0	4	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	5/23/2013	1	1	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	5/27/2013	2	1	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	6/1/2013	2	0	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	6/3/2013	1	0	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	8/1/2013	1	0	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	8/5/2013	0	1	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	8/6/2013	0	2	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	8/24/2013	1	0	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	9/18/2013	2	10	Frozen	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	9/18/2013	9	15	10% Formalin	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	9/21/2013	0	1	10% Formalin	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	9/22/2013	0	1	10% Formalin	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	9/25/2013	2	2	10% Formalin	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	9/30/2013	0	1	10% Formalin	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	10/2/2013	1	3	10% Formalin	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	10/6/2013	1	0	10% Formalin	35.789830, -76.411377
<i>F. diaphanus</i>	Lake Phelps	4/15/2015	1	1	Fresh	35.804882, -76.444806
<i>F. diaphanus</i>	Lake Phelps	5/5/2015	9	11	Fresh	35.789830, -76.411377
<i>F. waccamensis</i>	Lake Waccamaw	9/20/2013	0	1	10% Formalin	34.256733, -78.502954
<i>F. waccamensis</i>	Lake Waccamaw	10/21/2013	5	9	10% Formalin	34.256733, -78.502954
<i>F. waccamensis</i>	Lake Waccamaw	5/18/2015	0	3	Fresh	34.256733, -78.502954
<i>F. waccamensis</i>	Lake Waccamaw	4/20/2016	1	2	Fresh	34.261473, -78.480083
<i>F. waccamensis</i>	Lake Waccamaw	5/4/2016	1	2	Fresh	34.261473, -78.480083
<i>F. diaphanus</i>	Northwest River	8/19/2015	0	2	Fresh	36.572393, -76.159735
<i>F. diaphanus</i>	Waccamaw River	3/25/2013	2	0	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	4/6/2013	0	2	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	4/25/2013	2	0	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	5/16/2013	6	7	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	5/21/2013	1	0	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	5/23/2013	1	0	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	9/20/2013	5	5	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	9/20/2013	24	11	10% Formalin	33.562064, -79.085685
<i>F. diaphanus</i>	Waccamaw River	9/21/2013	3	4	Frozen	33.562064, -79.085685
<i>F. diaphanus</i>	Scuppernong River	4/4/2016	4	5	Fresh	35.944957, -76.312494
<i>F. diaphanus</i>	Scuppernong River	4/26/2016	2	1	Fresh	35.944957, -76.312494
<i>F. diaphanus</i>	Scuppernong River	5/2/2016	5	13	Fresh	35.944957, -76.312494

Table 1. Collected Specimens Information

Lake Waccamaw’s waters are close to neutral pH and it has very high visibility. The lake has an average depth of 7.5 feet and the bottom of the lake can be seen on a sunny day.

Collections were attempted at sites around the entire lake but were only successful in two areas,

both found in the State Park. The park is found on the southern and southeastern end of the lake which is a stretch of almost entirely sandy bottom.

The first area of collection was the public pier, which is entirely sandy bottom with dense foliage covering the banks. The second area was a large beach area found 2 miles east along the State Park trail from the last campsite. These areas were intermittently covered with dense patches of maidencane *Panicum hemitomon* and sparse bald cypress *Taxodium distichum* saplings. Few *Fundulus waccamensis* were collected during any visit, but all successful collections from Lake Waccamaw were conducted on sunny days using beach seines.

Sampling at Lake Phelps was attempted around the entire lake but was only successful in two places within the Pettigrew State Park, which was also a large stretch of sandy bottom on the Northeast of the Lake. Lake Phelps is also a neutral pH lake with very high visibility in the sandy portion of the lake and an average depth of 4.5 feet with a maximum depth of 9 feet.

The first collection area was at the lake's entrance to Western Canal, one of the creeks that forms a small canal or ditch and eventually joins with others at the beginning of the Scuppernong River. The entrance of this creek has a sandy bottom and is densely populated with bald cypress. The second collection area was along the sides of the State Park boat ramp, which was all sandy bottom and had a very high visibility. The fish collected in this area were all found within 100 yards of the opening of the canal along the boat ramp road, but none were collected in attempts in the canal or the ditch that the canal feeds into. This area was also densely populated with maidencane. Like Lake Waccamaw, specimens were only collected on sunny days, and were mostly collected using beach seines, though some were collected with minnow traps.

Scuppernong River only had one successful sample area. The Scuppernong is a river typical of North Carolina in its low pH, low visibility and low gradient. The majority of the river

is a ditch that sees little to virtually no current, even as it broadens before connecting to the Albemarle Sound. The river is also known to possess low movement and high nutrients with anoxic regions. The Scuppernong River was sampled from the joining of the creeks and canals near Lake Phelps all the way until just before it connects to the sound, but collections were only successful just before reaching the sound. The collections were all made at the same area, which is at the end of Riverneck Road, just north of Columbia, NC. The area seems to be a retired and degraded boat ramp. Collections were made next to this ramp where the bottom is sandy and forms shallow beach areas. All collections were made using beach seines, and collections were only successful in this area on sunny days as well.

In all successful collection locations adults were generally collected alone or in small groups, whereas juveniles were more often collected in shoals of up to 10 individuals. Adults during spawning season of late spring and early summer, when females were observed to be gravid, were most often found in shallow beach areas near or in groupings of maidencane in all three of these locations. Some individuals were collected on these beach areas in less than 4 inches of water. Females were observed in the laboratory to produce a stringy mucous coating around the eggs which are expected to allow the eggs to attach to vegetation.

Due to the low collection counts and difficulty of collecting these fishes, previous collections fixed in 10% formalin from past lab members were also used in the analyses of body shape and trophic traits. Frozen specimens from this previous project were also used in the trophic structure analysis, but were omitted from the body shape analyses due to the confounding effect freezing would have on the body compare to alive and fixed specimens. These collections included specimens from Lake Waccamaw, Lake Phelps, and Waccamaw River. All specimens

were collected in the summer of 2013 and fixed in 10% formalin immediately after euthanization. Correction for possible fixation effects is discussed in each analysis section.

The Lake Waccamaw collections were made in the same beach area of the lake as the recent collections (Lake Waccamaw Collection Site 2). The Lake Phelps collections were also made in the same boat ramp area of the Lake as recent collections (Lake Phelps Collection Site 2).

The Waccamaw River collections from this previous project were collected at the Wacca Wache Marina in Murrell's Inlet, South Carolina. This is well within the tidally controlled portion of the river near Winyah Bay. The collections were made on and along the boat ramp using beach seines.

During this project sampling was conducted on the Waccamaw River from close to its connection with Lake Waccamaw, down to the same tidal area of the river including the Wacca Wache Marina, but none were successful.

Crosses

Crosses of collected specimens were attempted in order to conduct a "Common Garden Test" but all crosses other than one collapsed shortly after fertilization. The one successful cross was a Lake Phelps pure cross and yielded 6 offspring, 2 males and 4 females. This cross was used to determine a standard length at which *F. diaphanus* can be considered an adult, as there is no defined adult standard length in the literature. The standard length at which the individuals displayed sexually dimorphic coloration as well as courtship and dominance behavior was measured and used to determine the cutoff for adult size (**Table 2**).

Sex	Standard Length
M	54
M	51
F	52
F	50
F	50
F	52
Average	51.5

Table 2. Lake Phelps Pure Cross Adult Size

Branchial Skeleton Extraction

Branchial skeletons were extracted from all collected and freshly euthanized adult specimens, all adult fixed specimens, and adult frozen specimens, following the branchial skeleton extraction protocols from the Miller Lab (Ellis & Miller 2016). This protocol shows the steps to dissect and mount the branchial skeleton of Threespine Stickleback *Gasterosteus aculeatus* onto microscope slides, but as stated in the protocol many fishes can be dissected in the same manner. In the protocol Alizarin Red is used to stain the branchial skeletons, but this was not used in this analysis. Due to the difficulty of seeing the gill rakers compared to the gill filaments in ethanol, all branchial skeletons were dried before being photographed which allowed for the gill rakers and the gill filaments to dry different colors and were more distinguishable. After drying no difference in the structure was present.

The right primary gill arch was used for every specimen and the structures that were measured were the gill raker count, longest gill raker length, and the distance between the largest and second largest gill raker. These were all photographed using an Olympus SZX-7 dissecting microscope and camera under the same magnification (8x Magnification), and using the same

lighting and background. Each photograph had the scale of 1 millimeter according to the magnification burned into the photo to allow for correct and precise measurement.

The photographs were then imported into tpsDig264 (Rohlf softwares, 2016) to measure the previous mentioned aspects. Each photo was corrected for the scale of number of pixels (283) in the photo that equaled the scale of 1 millimeter. The ruler function was then used to measure the longest gill raker, and the gap between the longest and second longest gill raker, and the number of gill rakers was counted.

Trophic Structure Analyses

The number of specimens used in the Trophic Structure Analysis grouped according to their population and preservation type can be seen in **Table 3**. The effect of Preservation Type was included in the analyses of Gill Raker Length and Gap Length.

Location	Formalin	Frozen	Fresh	Total
Lake Phelps	36	51	22	109
Lake Waccamaw	15	0	9	27
Waccamaw River	35	38	0	73
Scuppernong River	0	0	30	30

Table 3. Trophic Structure Analysis Specimens

The Gill Raker Length and Gap Length analyses were conducted using R-Packages (3.2.3). The Gill Raker Length analysis was conducted by creating a linear model to test all of the specimens with all factors that could be affecting the change in Gill Raker Length (standard length, location, preservation type, sex) and the significance of all interactions were tested. The model was tested using a Type III Sum of Squares Analysis of Variance using the Anova() function in the “car” package in R. The model was then stepped down in complexity to determine the model that showed only significant terms. This model tested Gill Raker Length as a function of standard length, location, sex, and preservation type sequentially and all of the two-

way interactions between these factors (higher order interactions excluded because of missing factor levels between populations). A Tukey test using the same format was then conducted to determine the significance of group comparisons.

The least squares means were computed for each location in order to compare the corrected population means. This process was conducted in order to determine the mean of each population after adjusting for the covariates of standard length and preservation type using the `lsmeans()` function in R Packages following the protocols of Lenth (2016).

The Gap Length analysis was conducted using the same method in R. The linear model tested the Gap Length as a function of standard length, location, sex, and preservation type as well as their interactions. A Tukey Test was again conducted with the same format to determine group significance. The least squares means were also computed for this data to correct the means of each population corrected for the covariate of location.

Heteroscedasticity tests were run on the gill raker measurements through plotting of residuals. Distribution of residuals was entirely random, and resulted in a horizontal line suggesting that no corrections needed to be made.

Morphometric Data Collection

All photographs used for morphometric analyses were taken using a Canon EOS 40D and were taken of the left side of every specimen. The same ruler was placed in the edge of every photograph to allow for scale. The photographs were taken in a light diffusing box on top of a grey card using the same tripod at the same height, the same flash, and all were shot at 100 millimeters focal length to standardize any effect of image distortion. The preserved specimens were all photographed using the same protocols as stated above. The number of specimens in the morphometric analysis broken down by location and preservation type can be seen in **Table 4**.

The Alive Group included Lake Waccamaw, Lake Phelps, and Scuppernong River. The Preserved Group included Lake Waccamaw, Lake Phelps, and Waccamaw River.

Location	Formalin	Fresh	Total
Lake Phelps	36	22	58
Lake Waccamaw	15	9	24
Waccamaw River	35	0	35
Scuppernong River	0	30	30

Table 4. Morphometrics Analysis Specimens

Morphometric Analyses

Photographs were imported into tpsDig264 and were set to the scale according to the ruler in each photo. The landmarks were placed on the photographs using tpsDig264 to convert these to x-y coordinate pairs. There were 15 total landmarks and they were placed in the following order and positions: **(1.) Anterior Tip of Pre-Maxilla (2.) Anterior End of Orbital (3.) Posterior End of Orbital (4.) Center of Orbital 5. Ventral Connection of Operculum (6.) Dorsal Insertion of Pectoral Fin (7.) Ventral Insertion of Pectoral Fin (8.) Posterior End of Skull (9.) Anterior Insertion of Dorsal Fin (10.) Posterior Insertion of Dorsal Fin (11.) Dorsal Caudal Peduncle (12.) Ventral Caudal Peduncle (13.) Posterior Insertion of Anal Fin (14.) Anterior Insertion of Anal Fin (15.) Anterior Insertion of Pelvic Fin.** These landmarks can be seen in **Figure 5.**

The landmark data in the form of x-y coordinate pairs for each landmark was then input into R- Packages to conduct the morphometric analyses. Because preservation in 10% Formalin has a taxon specific confounding effect the alive and preserved specimen images were analyzed separately.

The input landmark data was first superimposed using the ‘gpagen’ function. This function conducts a Generalized Procrustes Analysis (GPA), which controls the variables for

variation in placement and size while still factoring in those changes. This is conducted by aligning the landmark coordinates into linear tangent space. This process results in converting the landmarks into relative warps that can then be analyzed. The relative warps were then input into the 'prcomp' function to conduct a Principal Components Analysis (PCA) of the relative warps to determine the major changes in shape. The first two Principal Components (most significant) were then biplotted to show any separation between the populations. The first and second principal components were analyzed in a linear model to test the correlation between PC1 and PC2 scores and the location, sex, and interaction of those two factors.

A type III Analysis of Variance (ANOVA) was run on this model and a Tukey test was run to determine comparisons of groups; in the case of significant interactions between sex and location a least squares means Tukey test was conducted to contrast each sex between all populations. Three-way interactions between the factors were not tested due to locations not containing all preservation types. A thin-plate spline was created using the 'tpsgrid' function to visualize changes in landmarks between groups. The 5 highest loadings from the Principal Components Analysis, or the 5 landmarks that contained the most variance between groups were taken and shown on top of the original landmarks to also help visualize the population shape variation.

Two initial analyses were run following these protocols, the first using the alive specimens, and the second using the preserved specimens. Each time a new analysis was conducted the GPA was conducted with only the landmark data of those population as the GPA is dependent upon the number of samples. Lastly the watershed pairs were analyzed together, Lake Phelps and Scuppernong River from the alive data, and Lake Waccamaw and Waccamaw River from the preserved data.

RESULTS

Trophic Structure- Gill Raker Length

The Gill Raker Length analysis showed significant differences between populations. Standard length, location, and preservation type were all significant in analyses of gill raker length, but their two-way interactions were not, nor was sex ($p=0.3266$ when forced into a model including only the significant terms) (**Table 5**). Notably, the term for preservation type was relatively modest compared to location or body length. The Tukey Tests were conducted using the same model factors as in the linear model above.

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.0607	2.4508	0.1188450*
Standard Length	1	0.5656	22.8290	3.164e-06*
Location	3	5.3798	72.3752	<2.2e-16*
Preservation Type	2	0.4252	8.5801	0.0002551*
Residuals	229	5.6740		

Table 5. Gill Raker Length ANOVA Type III Results

Both Lake-River Pairs, Lake Waccamaw vs. Waccamaw River, and Lake Phelps vs. Scuppernong River, were significantly different from one another, with longer gill rakers in the river populations (Tukey tests, **Table 6; Figure 2**). In addition, the two rivers were significantly different from one another, and Waccamaw River was significantly different from Lake Phelps, but the two lakes did not differ.

Location Pair	Difference	Lower	Upper	Adjusted P
Lake Waccamaw-Lake Phelps	0.06456281	-0.02688362	0.1560092	0.2629593
Scuppernong River-Lake Phelps	0.11983300	0.03621624	0.2034498	0.0014945*
Waccamaw River-Lake Phelps	0.29688611	0.23554935	0.3582229	0.0000000*
Scuppernong River-Lake Waccamaw	0.05527020	-0.05579818	0.1663386	0.5711937
Waccamaw River-Lake Waccamaw	0.23232331	0.13689469	0.3277519	0.0000000*
Waccamaw River-Scuppernong River	0.17705311	0.08909895	0.2650073	0.0000026*

Table 6. Gill Raker Length Tukey Results

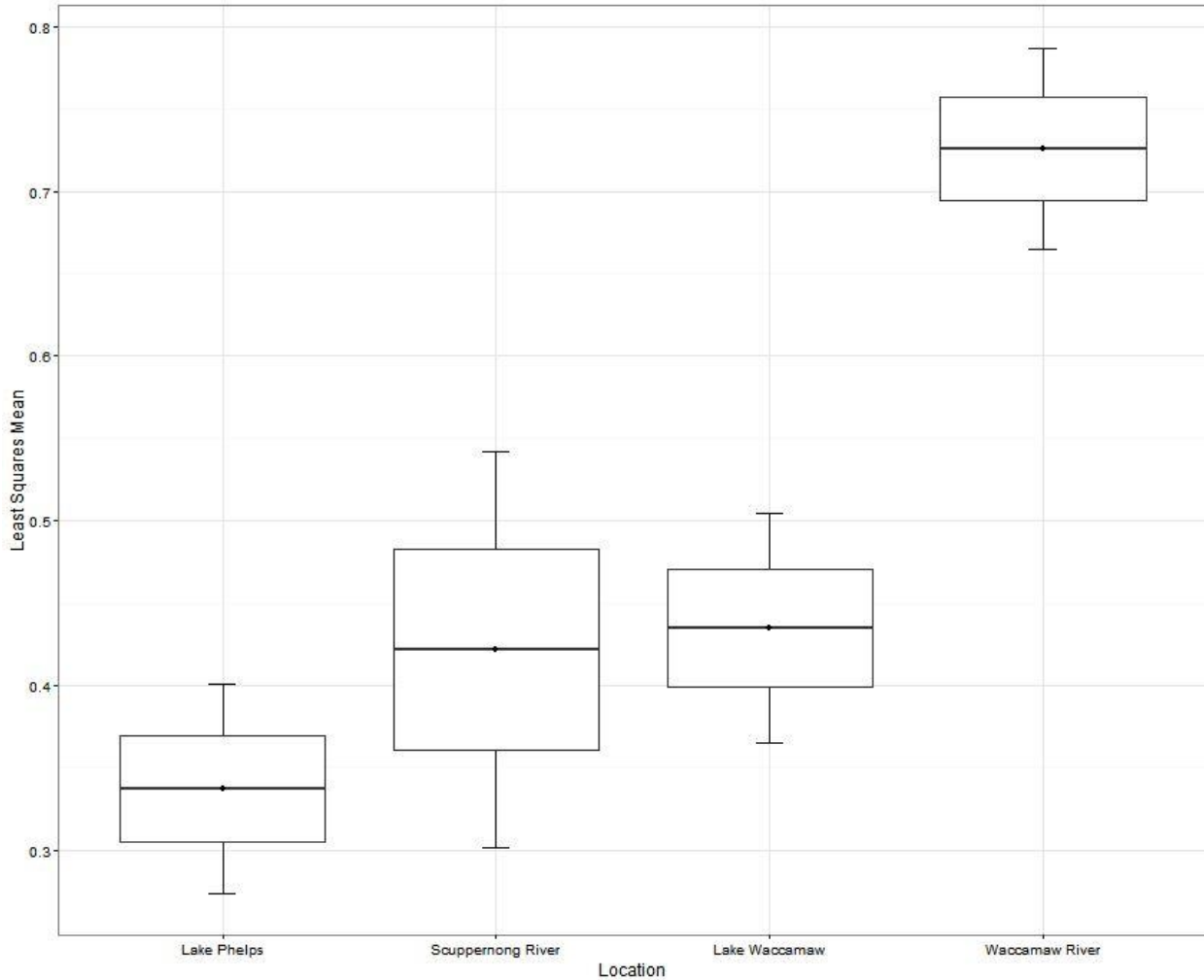


Figure 2. Gill Raker Length Plot of Least Squares Means. Results of the Least Squares Means are averaged over the levels of Location.

Trophic Structure- Gill Raker Gap

Standard length, location, and preservation type were all significant in the gill raker gap analyses, but their two-way interactions were not, nor the factor of sex ($p=0.1315$ when forced into a model including only the significant terms). The pattern in gill raker gaps was the reverse of that observed for gill raker length, with values for the rivers lower than values for the adjacent lakes; the linear model confirms significant effects of location as well as of standard length and preservation type (**Table 7**).

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.0258	0.9128	0.3404
Standard Length	1	1.2472	44.1488	2.195e-10*
Location	3	1.0996	12.9750	7.391e-08*
Preservation Type	2	0.5765	10.2046	5.684e-05*
Residuals	229	6.4690		

Table 7. Gill Raker Gap ANOVA Type III Results

Comparing pairs of locations, there were significant differences only between the two lakes and the Waccamaw River population (Tukey tests, **Table 8; Figure 3**).

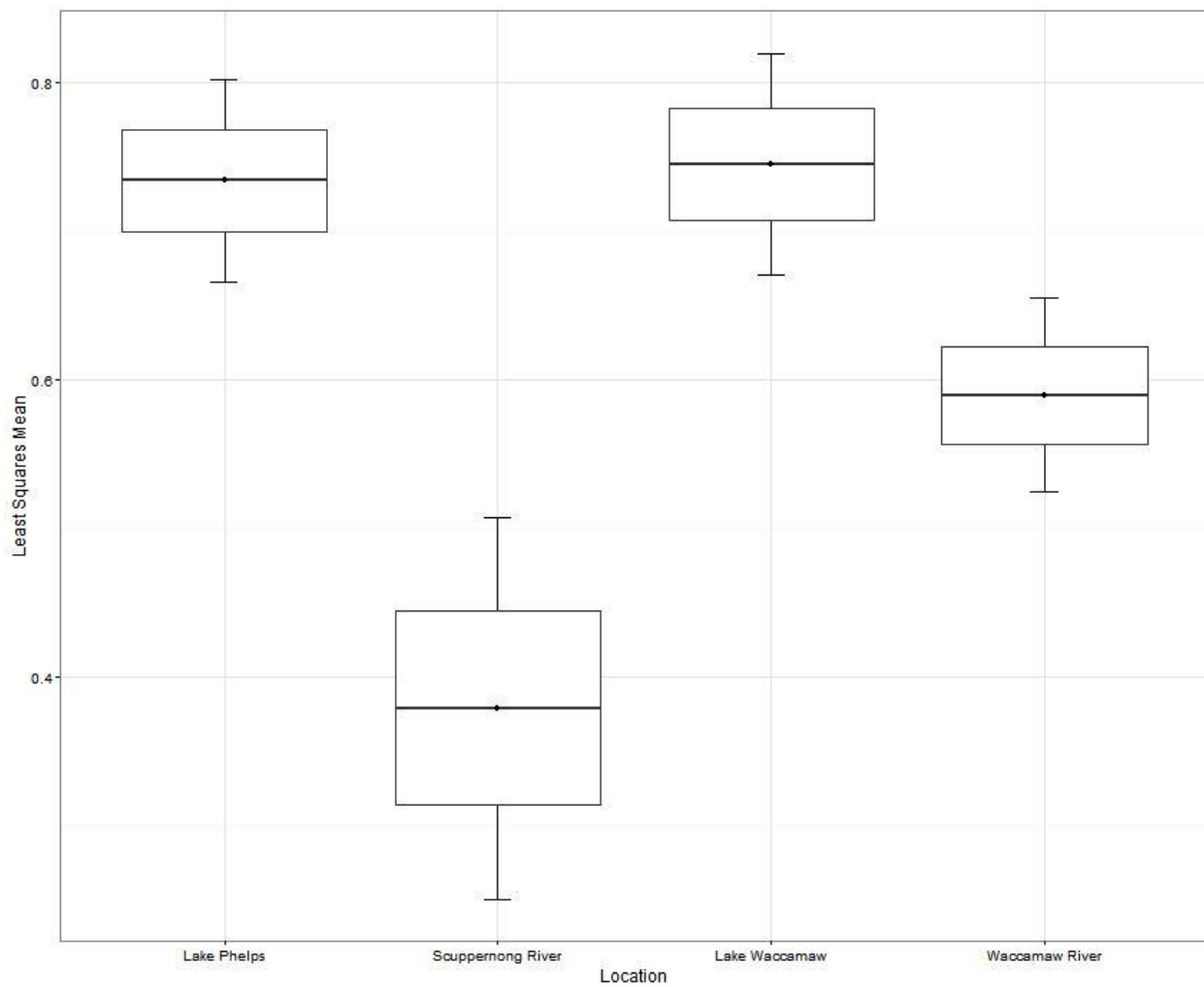


Figure 3. Gill Raker Gap Length Plot of Least Squares Means. Results of the Least Squares Means are averaged over the levels of Location.

Location Pair	Difference	Lower	Upper	Adjusted P
Lake Waccamaw-Lake Phelps	0.04440225	-0.0489688	0.137773291	0.6075818
Scuppernong River-Lake Phelps	-0.01629377	-0.1016704	0.069082822	0.9603148
Waccamaw River-Lake Phelps	-0.09894415	-0.1615718	-0.036316475	0.0003533*
Scuppernong River-Lake Waccamaw	-0.06069601	-0.1741020	0.052709954	0.5095477
Waccamaw River-Lake Waccamaw	-0.14334639	-0.2407834	-0.045909354	0.0010401*
Waccamaw River-Scuppernong River	-0.08265038	-0.1724557	0.007154887	0.0834261

Table 8. Gill Raker Gap Tukey Results

Both the Gill Raker Length Analysis and the Gill Raker Gap Analysis were repeated while excluding the 10% Formalin specimens to determine if the formalin fixed specimens had a major effect on these analyses. The same results were found in the formalin excluded dataset.

Morphometric Analysis- Fresh

The Principal Components Analysis (PCA) of the Fresh Specimens showed distinct separation between the lakes populations and Scuppernong River (**Figure 4**).

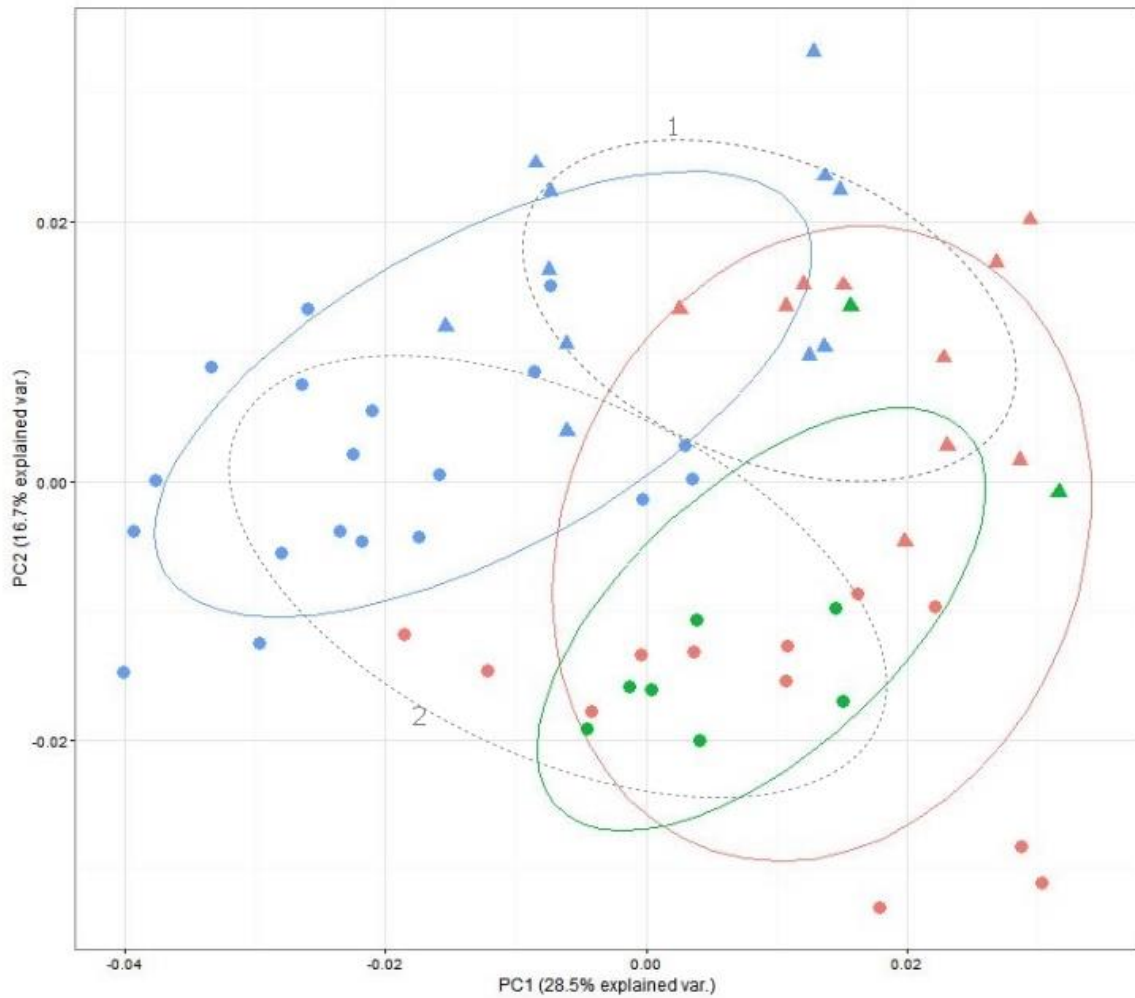


Figure 4. Biplot of Fresh PCA. Lake Phelps specimens denoted in red. Lake Waccamaw specimens denoted in green. Scuppernong River specimens denoted in blue. Males denoted by circles; females denoted by triangles. Dashed Ovals: (1) Grouping of males, (2) Grouping of females.

Principal Component (PC) 1 was significantly different among locations and between the sexes (**Table 9**), with significant differences between Scuppernong River and both lakes, but not between the lakes.

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.0002516	1.6018	0.2108
Location	2	0.0090031	28.6584	2.434e-09*
Sex	1	0.0041496	26.4175	3.497e-06*
Residuals	58	0.0089534		

Table 9. Fresh PC1 ANOVA Type III Results

PC2 was again significantly different among locations and between the sexes, and there was also a significant interaction between location and sex (**Table 10**). The males and females in each population were significantly different from one another; males from other populations were different from each other, and from females from different populations. The pairs of same sex in the two lakes were not significantly different, and it is notable that the males from each lake and the Scuppernong River were not significantly different. The most variation of shape was found in the body depth just anterior to the anal fin (**Figure 5**).

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.0036312	54.359	7.607e-10*
Location	2	0.0028671	21.4600	1.128e-07**
Sex	1	0.0064569	96.660	6.982e-14**
Residuals	57	0.0038076		

Table 10. Fresh PC2 ANOVA Type III Results

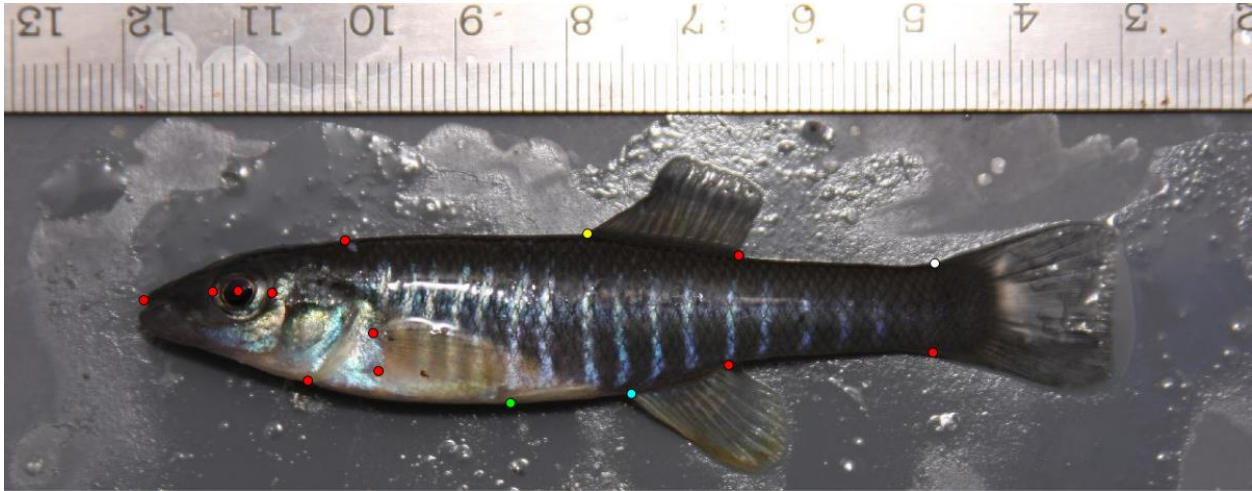


Figure 5. PC Loadings of Fresh PCA. PC Loadings Order: (1) Blue, (2) Green, (3) Purple, (4) Yellow, (5) White. Colors not shown are stacked on a landmark with a higher PC loading. Red dots are landmarks that were not found in the top 5 loadings.

The Lake Phelps vs. Scuppernong River subset PCA showed differentiation between the two populations (**Figure 6**).

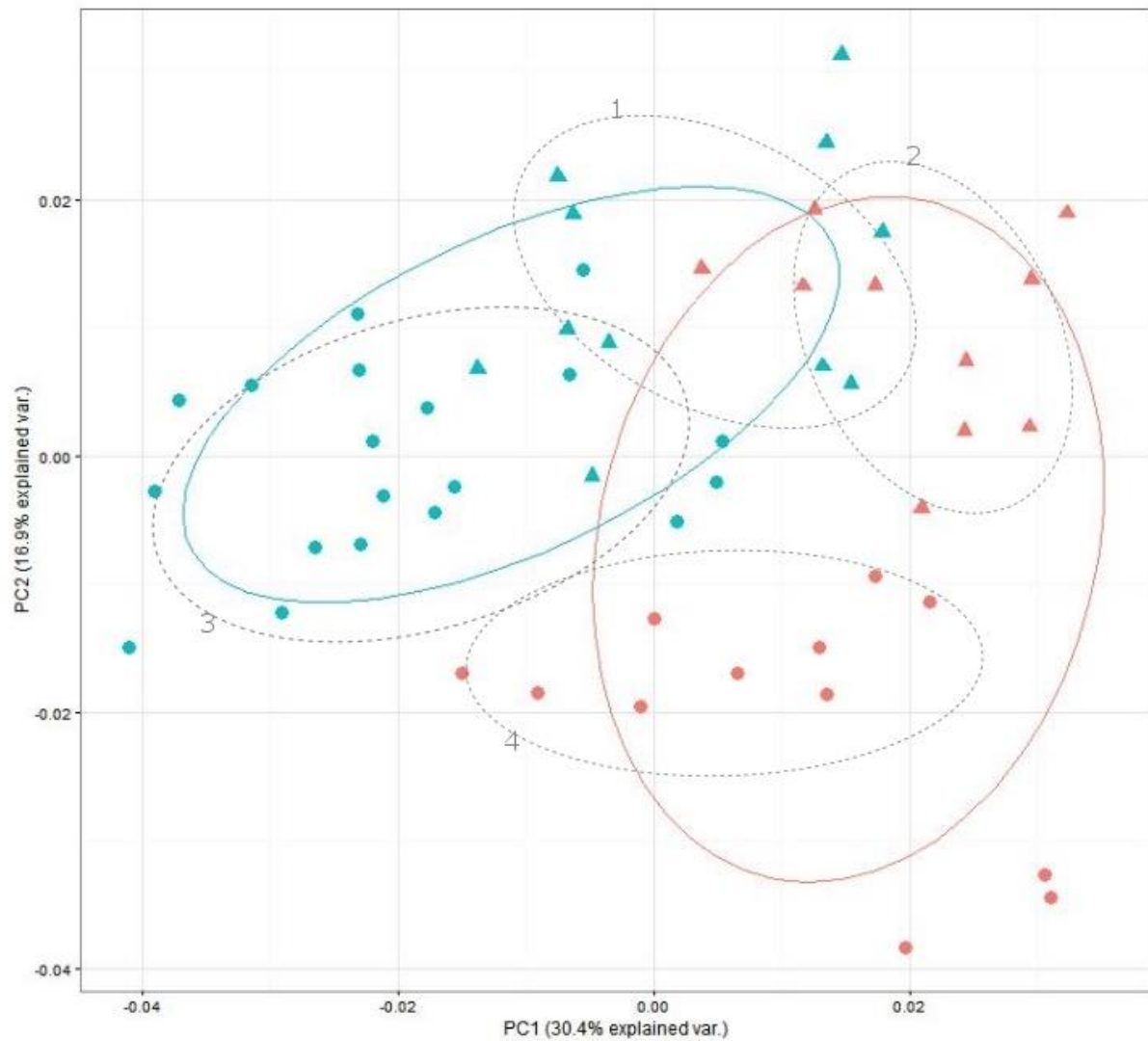


Figure 6. Biplot of Lake Phelps vs. Scuppernong River PCA. Lake Phelps specimens denoted in red. Scuppernong River specimens denoted in blue. Males denoted by circles; females denoted by triangles. Dashed ovals: (1) Scuppernong River females, (2) Lake Phelps females, (3) Scuppernong River males, (4) Lake Phelps males.

PC1 was significantly different between both locations and between the sexes, though location was much more significant than sex (**Table 11**).

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.0007155	4.1043	0.04824*
Location	1	0.0078043	44.7652	2.000e-08*
Sex	1	0.0035156	20.1654	4.326e-05*
Residuals	49	0.0085426		

Table 11. Lake Phelps vs. Scuppernong River PC1 ANOVA Type III Results

PC2 was significantly different between both locations and between the sexes, and there was also a significant interaction between location and sex (**Table 12**).

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.0031544	42.770	3.736e-08*
Location	1	0.0029504	40.003	7.984e-08*
Sex	1	0.0060401	81.895	5.996e-12*
Location x Sex	1	0.0008259	11.198	0.001598*
Residuals	48	0.0035402		

Table 12. Lake Phelps vs. Scuppernong River PC2 ANOVA Type III Results

The only contrast that was not significant was between Lake Phelps and Scuppernong River males (Tukey tests, **Table 13**). The landmarks accounting for the most variation were landmarks 14 and 15 (**Figure 7**).

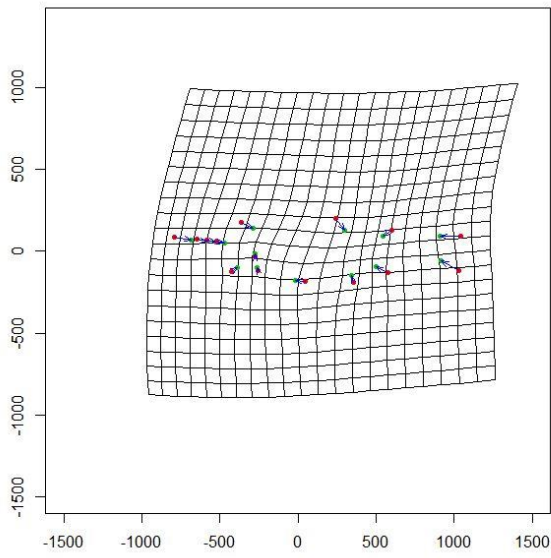
Contrast	Estimate	Standard Error	Degrees of Freedom	T Ratio	P Value
Lake Phelps,F - Scuppernong River,F	-0.020028608	0.003166686	48	-6.325	<.0001*
Lake Phelps,F - Lake Phelps,M	-0.030431869	0.003677158	48	-8.276	<.0001*
Lake Phelps,F - Scuppernong River,M	-0.034030247	0.003584830	48	-9.493	<.0001*
Scuppernong River,F - Lake Phelps,M	-0.010403261	0.003355164	48	-3.101	0.0165*
Scuppernong River,F - Scuppernong River,M	-0.014001639	0.003253712	48	-4.303	0.0005*
Lake Phelps,M - Scuppernong River,M	-0.003598378	0.003752363	48	-0.959	0.7732

Table 13. Lake Phelps vs. Scuppernong River PC2 Least Squares Means Tukey Results

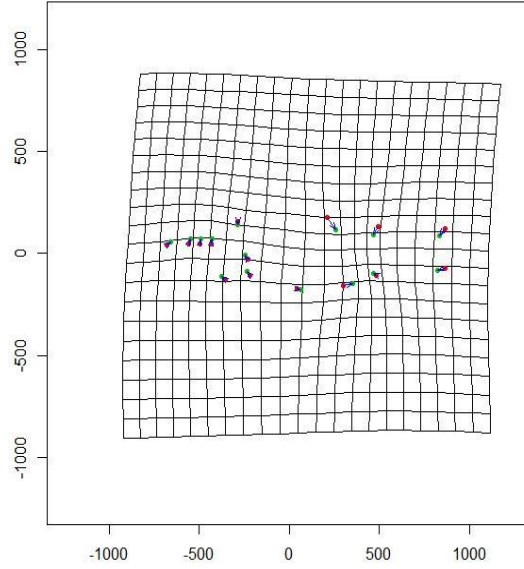


Figure 7. PC Loadings for Lake Phelps vs. Scuppernong River PCA. PC Loadings Order: (1) Blue, (2) Green, (3) Purple, (4) Yellow, (5) White. Colors not shown are stacked on a landmark with a higher PC loading. Red dots are landmarks that were not found in the top 5 loadings.

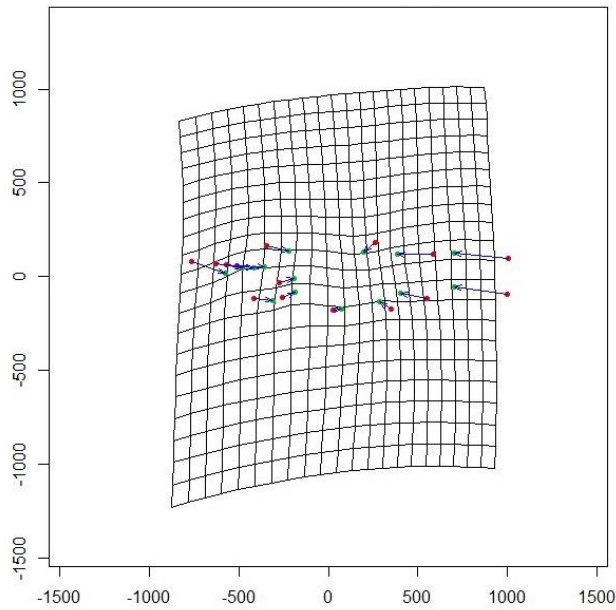
Lake Phelps males showed deeper bodies in between the dorsal and anal fins, and slightly greater lateral separation of landmarks compared to the females (**Figure 8A**). The Scuppernong River males also showed deeper bodies especially at the anterior insertion of the dorsal fin, as well as deeper caudal peduncles compared to the females (**Figure 8B**). The Lake Phelps specimens showed greater lateral separation of landmarks, especially the caudal peduncle, as well as shallower bodies (**Figure 8C**).



A



B



C

Figure 8. Lake Phelps and Scuppernong River Thin-Plate Splines (A) Thin-Plate Spline: Lake Phelps Male (Reference) to Lake Phelps Female (Target) 2x Amplification. (B) Thin-Plate Spline: Scuppernong River Male (Reference) to Scuppernong River Female (Target) 2x Amplification. (C) Thin-Plate Spline: Lake Phelps (Reference) to Scuppernong River (Target) 2x Amplification.

Morphometrics Analysis- Preserved

The PCA of the Preserved Specimens showed a significant effect of location with differentiation between the two lakes and Waccamaw River (**Figure 9**).

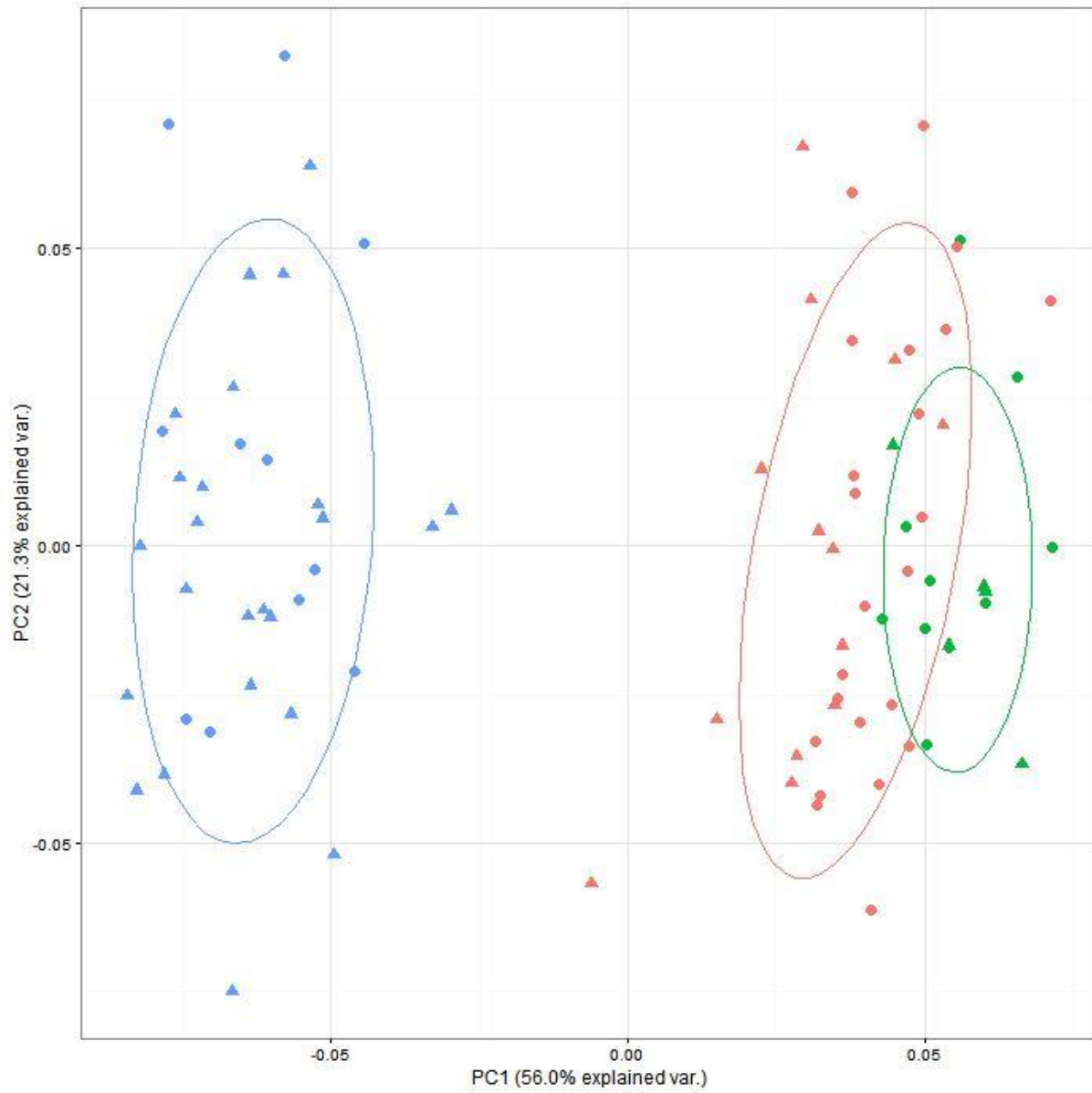


Figure 9. Biplot of Preserved PCA. Lake Phelps specimens denoted in red. Lake Waccamaw specimens denoted in green. Waccamaw River specimens denoted in blue. Males denoted by circles; females denoted by triangles.

PC1 was significantly different among locations and there was a significant interaction between location and sex, though this interaction was marginally significant ($p=0.04998$), and the term for sex was not ($p=0.14193$, **Table 14**).

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.005516	38.7358	2.1e-08*
Location	2	0.098793	346.887	<2e-16*
Sex	1	0.000313	2.2001	0.14193
Location x Sex	2	0.000886	3.1112	0.04998*
Residuals	80	0.011392		

Table 14. Preserved PC1 ANOVA Type III Results

Most notably the contrasts of sexes in Lake Waccamaw, and in Waccamaw River were not significant (Tukey tests, **Table 15**). PC2 did not significantly vary with any factors or interactions. The most variation of shape was the shift of the caudal peduncle (**Figure 10**).

Contrast	Estimate	Standard Error	Degrees of Freedom	T Ratio	P Value
Lake Phelps,F - Lake Waccamaw, F	-0.011516641	0.004520100	80	-2.548	0.1228
Lake Phelps,F - Lake Phelps,M	0.013846235	0.004140670	80	3.344	0.0154*
Lake Waccamaw,F - Waccamaw River,F	0.116943772	0.005213967	80	22.429	<.0001*
Lake Waccamaw,F - Lake Waccamaw,M	-0.002214340	0.006536054	80	-0.339	0.9994
Waccamaw River,F - Waccamaw River,M	0.001530275	0.004344973	80	0.352	0.9993
Lake Phelps,M - Lake Waccamaw,M	-0.027577216	0.006279637	80	-4.392	0.0005*
Lake Phelps,M - Waccamaw River,M	0.093111171	0.004109401	80	22.658	<.0001*
Lake Waccamaw,M - Waccamaw River,M	0.120688388	0.005866288	80	20.573	<.0001*

Table 15. Preserved PC1 Least Squares Means Tukey Results

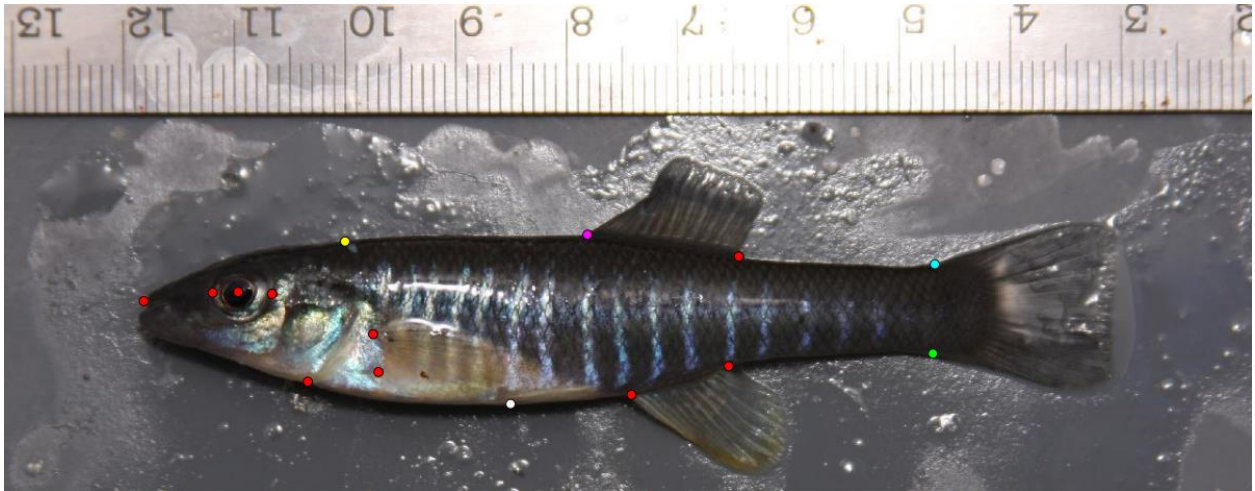


Figure 10. PC Loadings of Preserved PCA. PC Loadings Order: (1) Blue, (2) Green, (3) Purple, (4) Yellow, (5) White. Colors not shown are stacked on a landmark with a higher PC loading. Red dots are landmarks that were not found in the top 5 loadings.

The Lake Waccamaw vs. Waccamaw River subset PCA showed strong differentiation between the two populations (**Figure 11**).

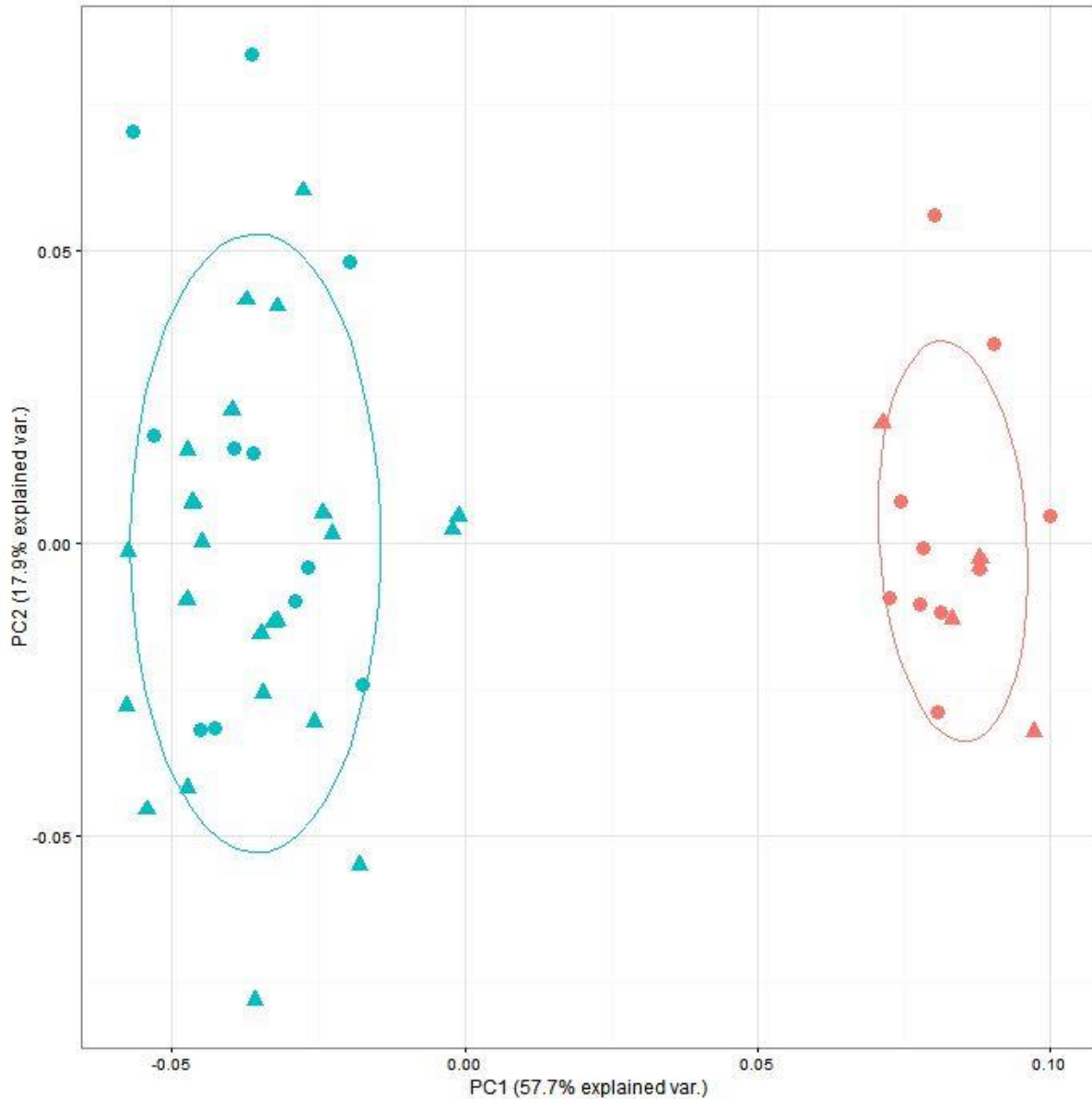


Figure 11. Biplot of Lake Waccamaw vs. Waccamaw River PCA. Lake Waccamaw specimens denoted in red. Waccamaw River specimens denoted in blue. Males denoted by circles; females denoted by triangles.

PC1 was significantly different between locations (**Table 16**). PC2 did not vary significantly different with any of the factors or their interactions. The landmarks with the most variation were landmarks 11 and 12 (**Figure 12**).

	Degrees of Freedom	Sum of Squares	F-Value	Pr(>F)
Intercept	1	0.023914	146.67	3.346e-16*
Location	1	0.149464	916.67	<2.2e-16*
Residuals	48	0.007826		

Table 16. Lake Waccamaw vs. Waccamaw River PC1 ANOVA Type III Results



Figure 12. PC Loadings of Lake Waccamaw vs. Waccamaw River PCA. PC Loadings Order: (1) Blue, (2) Green, (3) Purple, (4) Yellow, (5) White. Colors not shown are stacked on a landmark with a higher PC loading. Red dots are landmarks that were not found in the top 5 loadings.

The Lake Waccamaw specimens showed much shallower body depth at all landmarks, as well as greater lateral separation of landmarks compared to the Waccamaw River specimens (Figure 13).

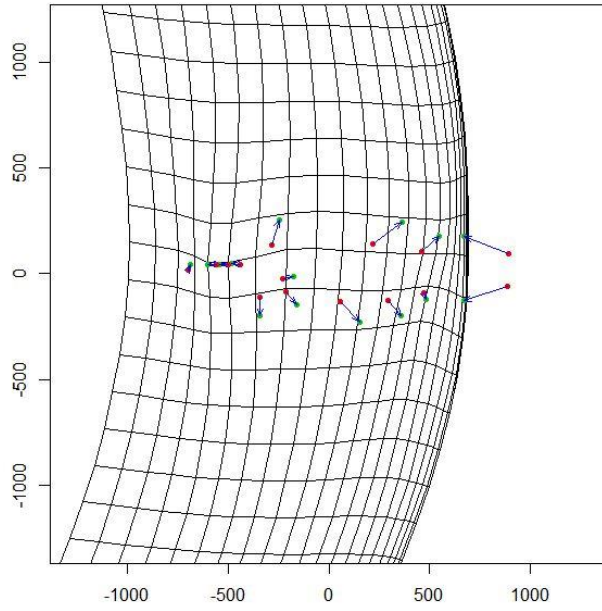


Figure 13. Lake Waccamaw to Waccamaw River Thin-Plate Spline: Lake Waccamaw (Reference) to Waccamaw River (Target) 2x Amplification.

DISCUSSION

In this thesis I conducted a novel test of whether trophic selection may have contributed to patterns of divergence and convergence in the North Carolina *F. diaphanus* complex. I also tested for the first time for evidence of sexual dimorphism in body shape divergence and convergence and whether incorporating sex into analyses of body shape divergence might lead to reconsideration of previous findings and interpretations.

My analyses of gill raker length indeed indicated a role for trophic adaptations in this system, with both Carolina Bay populations significantly different from their respective stream forms but lake populations not significantly different from one another. The divergence of the Carolina Bay populations away from their river counterparts supports the hypothesis that adaptation to these unique environments involves selection based on different feeding habits. Analyses of gaps between gill rakers provided further evidence of a role for trophic evolution in this miniature radiation. Comparison of the stream forms also provides support for the role of watershed type in trophic evolution. Results for sexual dimorphism supported the hypothesis that body shape evolution differed between the sexes although previous findings concerning the evolution of body morphology in this system were nevertheless supported.

These analyses provide new insight into the selective regime within this species complex. The diet of *F. diaphanus* is considered to be primarily benthic invertebrates, though diet variation with respect to environmental and ecological variation has not been tested. The diet of *F. waccamensis* is also considered to consist of invertebrates, primarily chironomid larvae and amphipods (Lindquist and Yarborough 1982). The driver behind selection in these Carolina Bays has been argued to be the unique water characteristics such as pH and temperature (Stager &

Cahoon 1987) as well as predation regime (Krabbenhoft 2009a). My results provide the first evidence of selective pressure on feeding habits in this complex.

Selection on trophic structures, especially gill rakers, has been observed in adaptation to different environments among disparate taxa, and parallel divergence has sometimes been observed across replicated environments. *Coregonid* whitefish have shown parallel adaptive divergence in gill rakers in environments affected by eutrophication (Vonlanthen *et al.* 2012). Whitefish gill raker adaptation has also been observed to occur in similar patterns as a result of trophic niche specialization in sympatric populations, providing evidence for the “Ecological Speciation Hypothesis” in this system (Lu & Bernatchez 1999). Populations (or arguably species) of threespine stickleback, *Gasterosteus aculeatus*, have also shown parallel divergence across replicated environments. Stickleback have been observed to diverge into sympatric benthic and limnetic pairs due in part to trophic niche adaptation (McPhail 1992; Schluter 1996; McKinnon & Rundle 2002). In sticklebacks, lake populations showed parallel divergence away from their respective stream pairs, with higher gill raker numbers and shallower bodies as an adaptation to zooplankton foraging in open lake waters (Hendry & Taylor 2004). My results also show a correlation between body depth and gill raker length/spacing, but in the opposite direction from the stickleback model. In the *F. diaphanus* complex the lake populations showed diminished gill rakers and slender bodies compared to the stream populations, which possess deeper bodies and elongated, more densely packed gill rakers. Body shape adaptation in this complex is likely resulting from similar selection as in the stickleback model but trophic traits show the opposite pattern, with the river forms having longer more closely spaced gill rakers and deeper bodies. This may be due to selection on body shape and feeding morphology being

correlated differently in killifishes and sticklebacks, causing different patterns in the evolution of these two traits.

Watershed type has also been observed to strongly correlate with evolutionary divergence of many phenotypic characteristics. Watershed type, classified in terms of differing relative aquatic energies, has been shown to affect the spawning, egg characteristics, and morphologies of offspring of striped bass, *Morone saxatilis* (Rulifson & Manooch 1990, Bergey *et al.* 2003). In the present study, the Waccamaw River population was sampled from the tidal region of the river while the Scuppernong River as a whole has little to no energy in the entire system. These two populations showed similar divergence in trophic structures from their corresponding lake populations but the Waccamaw River population shows much stronger divergence in both gill raker length and gap length. This supports the hypothesis that selective pressure on these structures is strongly correlated to aquatic system energy.

The analyses of gill raker characteristics were limited by the sample sizes. Collections of all locations were low, averaging 3-5 specimens per successful collection, and yielding few adult specimens from all locations and with varying sampling techniques. Consequently, I used previous collections frozen or fixed in 10% formalin in the analyses. All Waccamaw River specimens were from the fixed and frozen collections from the McKinnon Laboratory as no collections were successful from this location during this thesis. The 10% formalin fixation as well as the temporal variation of these specimens did introduce some error to the analyses that had to be corrected for.

To further test my findings, larger sample sizes for each location should be collected and evaluated, especially to more powerfully test sex's contribution to variation in the trophic structures. This would allow further elucidation of the sexual selection's (potential) direct or

indirect role in trophic evolution. Dietary analyses focusing on plankton consumption as well as stomach content analyses for invertebrates would further test the hypothesis of varying adaptation to prey availability and preference as well as explain feeding behavior within this species complex. Analyses of these diets, as well as genetic analyses and dietary rearing experiments would further elucidate the extent to which adaptation in gill raker traits is a result of plasticity or genetic divergence.

My analyses of body shape supported the hypothesis of uneven selection pressures acting upon the sexes, with the Lake Phelps males and females showing differing patterns of body shape divergence relative to the local stream form for the second, less statistically important PC, in addition to sexual dimorphism that was shared across that pair for both PC's. For the second PC, males from Lake Phelps were not significantly different in body shape from the Scuppernong River males, however the females of each population were significantly different from one another. Nevertheless, these analyses confirm the earlier findings of divergence of Lake Phelps fish from the local stream forms (Krabbenhoft *et al.* 2009a).

Comparing results of sexual dimorphism tests in Carolina bay and stream pairs shows substantial body shape sexual dimorphism for the Lake Phelps and Scuppernong River pair, but not for the Lake Waccamaw and Waccamaw River pair suggesting difference in natural or sexual selection between these systems. The Lake Waccamaw form in fact showed reduced sexual dimorphism, which may in part be a result of sampling bias (i.e. size of collected specimens) or an effect of fixation. I found no evidence that patterns of sexual dimorphism substantially confound or alter previous findings regarding convergence and divergence by habitat (Krabbenhoft *et al.* 2009a).

Body shape divergence and sexual selection have been well studied in the stickleback model. Sexual dimorphism in stickleback has been observed to vary due to selective pressures from predators and from their feeding ecology (Spoljaric & Reimchen 2008; Kitano *et al.* 2012). Sexual dimorphism has been shown to occur as an evolutionary response to a single axis of natural or sexual selection, taking the place of speciation, but the two may occur sequentially as a response to multiple axes of selective pressure (Cooper *et al.* 2011). Foraging evolution in stickleback has also been observed to be associated with body depth divergence, shallower bodies generally correlating with more and longer gill rakers (Hendry & Taylor 2004). My body shape analyses do not support the reduction of sexual dimorphism due to ecological factors in the fresh populations as no clear reduction in sexual dimorphism was observed in any of the populations. The results do however, support the hypothesis of multiple axes of selection acting upon this species complex as the divergence includes body shape, trophic traits, and, in the case of the Lake Phelps population, uneven body shape divergence between the sexes.

The body shape analyses were also limited by sample sizes. Due to the confounding effect of formalin fixation, which is taxon specific, the two groups were analyzed separately and frozen specimens were excluded. This limitation split the analyses into two three-way analyses and therefore a comparison could not be made of all populations together; analyses of larger samples collected and stored in the same manner would therefore be informative. In addition, further research utilizing genetic analyses as well as common garden crosses to determine possible body shape genetic control between the lake-stream pairs would help to elucidate the mechanisms of convergence in this complex. As the relatedness of the Carolina Bay forms is not well understood, double digest restriction enzyme DNA analyses are currently being conducted in the McKinnon and Brewer Laboratories in order to determine the lineage of this complex.

In conclusion, my work on the *F. diaphanus* species complex provides novel evidence of trophic selection contributing to divergence and convergence in this species complex. My results also provide the first evidence of sexually dimorphic body shape evolution in this complex but nevertheless confirm previous findings regard convergence in Carolina Bays and divergence from corresponding stream forms.

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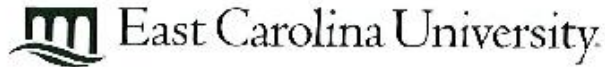
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APPENDIX



**Animal Care and
Use Committee**

241 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

April 14, 2015

252-744-2438 office
252-744-3488 fax

Jeffrey McKinnon, Ph.D.
Department of Biology
Howell Science Complex
East Carolina University

Dear Dr. McKinnon:

The Amendment to your Animal Use Protocol entitled, "Diversification and Speciation in the *Fundulus Diaphanus* Complex" (AUP #D264a) was reviewed by this institution's Animal Care and Use Committee on April 13, 2015. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

A handwritten signature in cursive script that reads "S. McRae".

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

