INVESTIGATIONS INTO RAZORBACK SUCKER-FLANNELMOUTH SUCKER HYBRID VIABILITY AND IDENTIFICATION USING SHAPE

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ABSTRACT

INVESTIGATIONS INTO RAZORBACK SUCKER-FLANNELMOUTH SUCKER HYBRID VIABILITY AND IDENTIFICATION USING SHAPE

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Two catostomid species, Razorback Sucker (*Xyrauchen texanus*) and Flannelmouth Sucker (*Catostomus latipinnis*), live in sympatry in the Colorado River basin. Both species share similar spawning requirements and have overlapping spawning seasons. Although morphological intermediates have been described as early as 1889, hybrids were seemingly rare. Rarity of hybrids is likely attributed to the Razorback Suckers' ability to find conspecific mates within the entire basin. Several dams have segmented the Colorado River ecosystem and negatively affected native fish assemblages. As a result of interactions with nonnative fishes and a river segmented by dams, the Razorback Sucker was listed as endangered. The endangered Razorback Sucker is now restricted to spawning in reservoirs and sections of river between dams. The Flannelmouth Sucker dominates much of the riverine spawning habitats where they coexist with Razorback Suckers which makes it difficult for Razorback Sucker to find conspecific mates. With continued scarcity of the Razorback Sucker in riverine spawning habitats dominated by Flannelmouth Sucker, hybridization will continue and may even increase in the future. To understand the impacts of hybridization on the recovery of the Razorback Sucker, information on hybrid viability and identification is needed. We quantified hatch success and larval survival of artificially spawned Razorback Sucker, Flannelmouth Sucker, and their hybrids to determine viability at early life history stages.

We used the resulting progeny, wild, and hatchery reared fish to conduct geometric morphometric analyses and used conditional inference trees to determine at what length the progenies' shapes began to differentiate. Piecewise-linear models were used to determine how each progeny's shape changed with increasing total length. We found no difference in hatch success or larval survival between the parent species and hybrids and determined that differentiation between the three fish types is difficult when they are 136 mm total length or smaller. Understanding that hybrids are capable of hatching and surviving with similar success to that of the parent species, we have determined that there are little to no post-zygotic isolation mechanisms and F1 hybrids are likely to thrive in the wild. This finding demonstrates a need for identification tools of hybrids of all sizes. While shape can be used to classify Razorback Suckers, Flannelmouth Suckers, and their hybrids when they are larger than 136 mm total length with 91% accuracy, smaller fish were only classified with 73% accuracy. A more accurate method of field identification for small fish needs to be investigated to properly monitor the recruitment of Razorback Sucker in the wild.

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TABLE OF CONTENTS

CHAPTER 1: VIABILITY OF RAZORBACK-FLANNELMOUTH SUCKER HYBRIDS

CHAPTER 2: USING SHAPE TO IDENTIFY RAZORBACK SUCKER, FLANNELMOUTH SUCKER, AND THEIR HYBRIDS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

LIST OF FIGURES

CHAPTER 2

PREFACE

Each chapter of this thesis is formatted for journal submission. Therefore, each chapter is formatted differently. Chapter 1 is a note that will be submitted to the Southwestern Naturalist. Chapter 2 is a full manuscript that will be submitted to Transactions of the American Fisheries Society. Both chapters will be submitted by June 30, 2018.

Chapter 1

Viability of Razorback-Flannelmouth Sucker hybrids

Introduction

Hybridization occurs when two species successfully mate and is common to see in the plant kingdom, but not as common in animal taxa (Dowling et al. 2016). Reproductive isolating mechanisms restrict gene flow between species, generally making hybrids rare (Kocher 2004). Common reproductive isolating mechanisms include temporal and spatial separation of mating which reduces mating encounters between two species, zygotic failure, reduced fitness of hybrid offspring, and differences in breeding requirements (Freeman and Herron, 2007). Freshwater fishes have weakened reproductive isolating mechanisms because of similar spawning behaviors and external fertilization. Morphological intermediates between two catostomid species of the Colorado River, Razorback Sucker (*Xyrauchen texanus*) and Flannelmouth Sucker (*Catostomus latipinnis*), have been described as early as 1889 (Jordan 1891, pp. 26-27, pl. 5 fig. 12, cited from Hubbs and Miller 1953). However, hybrids were rarely reported possibly because the Razorback Sucker undertook long spawning migrations which allowed for large congregations of conspecific mates or even special and temporal isolation from Flannelmouth Sucker (Wick et al., 1982; Minckley, 1983).

Currently, dams block Razorback Sucker spawning migrations in much of the Colorado River (Holden and Stalnaker, 1975; Minckley, 1983; Douglas and Marsh 1998; Marsh et al., 2003; Albrecht et al., 2010). Both species are now confined to relatively small sections of the river compared to their historic ranges. Historic river conditions have been altered by the dams from warm and turbid to cold and clear. The altered habitat potentially resulted in an overlap in timing and location of spawning (Hubbs and Miller, 1953; Douglas and Marsh, 1998; Minckley et al., 2003; Dowling et al., 2012). The Razorback Sucker was designated as endangered in 1991 (United States Fish and Wildlife Service, 1991) largely because of habitat alteration and predation by nonnative species (Wick et al., 1982, Guttermuth et al., 1994; Weiss et al., 1997; Bestgen, 2008). Throughout the species' present-day ranges, the Flannelmouth Sucker remains relatively common despite these habitat alterations (Mueller and Wydoski, 2004), while the Razorback Sucker remains extremely rare. The rarity of the Razorback Sucker increases its risk of hybridization with Flannelmouth Sucker because of the limited number of conspecific mates (Hubbs, 1955; Tyus and Karp, 1990). Adult hybrids are captured in the wild (Hubbs, 1955; Wick et al., 1982; Guttermuth et al., 1994; Douglas and Marsh, 1998). However, the viability of hybrids at early life stages has not been evaluated (Tyus and Karp, 1990).

Genetic studies have already confirmed that hybrids can successfully reproduce in captivity (Buth et al., 1987) and in the wild (Douglas and Marsh, 1998; Dowling et al., 2012). Given this, information is needed on hybrid viability to understand how hybridization may affect recovery of existing Razorback Sucker populations. If the hybrids have inferior hatch success and larval survival compared to the parental species, then hybridization with the Flannelmouth Sucker would not be a risk to the recovery and conservation of genetically pure Razorback Sucker. However, if hybrids exhibit similar hatch success and larval survival to the parental species, then hybridization will likely impact pure Razorback Sucker genetics. In this study, our objective was to determine viability of hybrids verses parental sticks in early life stages by quantifying hatch success and larval survival of artificially spawned Razorback Sucker, Flannelmouth Sucker, and their hybrids under controlled laboratory conditions.

Methods and Results

We spawned hatchery-reared Razorback Sucker from Lake Mead Fish Hatchery, operated by the state of Nevada, and wild Flannelmouth Sucker collected from the Paria River, a tributary of the Colorado River, Arizona. Each fish was injected with 0.5 mL of Ovaprim® (Western Chemical, Ferndale, Washington) per kilogram of body weight to ripen gametes. Fish were checked for ripeness 24 hours post injection and all were given an additional half-dose of Ovaprim®. Gametes were collected in 50-mL centrifuge tubes 48 hours after the initial injection. Eggs from each female were divided approximately equally among multiple tubes, dependent on how many eggs were obtained from each individual female, and fertilized with the milt of a single male to make four progenies: pure Razorback Sucker, pure Flannelmouth Sucker, Razorback Sucker female x Flannelmouth Sucker male hybrid, and Flannelmouth Sucker female x Razorback Sucker male hybrids. The result was each tube only contained gametes from one female and one male. Tubes were labeled with unique codes to track parent combinations.

Even with hormone injections, we were unable to achieve all four progeny combinations within the same year because gametes did not ripen in some females. Therefore, pure Razorback Sucker and Razorback Sucker female x Flannelmouth Sucker male progenies were produced only in 2016 and pure Flannelmouth Sucker and Flannelmouth Sucker female x Razorback Sucker male progenies were produced only in 2017. In 2016, four replicates of fertilized eggs from each unique parent combination were placed into individual containers constructed of 6-inch diameter PVC pipes cut to

25.4 cm in length with four 7.62 cm holes drilled around the outside, openings covered with mesh. Each replicate container was placed into an 83 L round larval rearing tank filled with Flagstaff, Arizona city water and dechlorinated with sodium thiosulfate. In 2017, parent combinations were only replicated twice due to a reduced amount of eggs available.

Rearing tanks were equipped with one 7.62 x 3.81-cm air stone, a 380-L sump, and a biofilter containing 2 cubic feet of Kaldness® biofilter media. The outflow from the filter was designed to circulate through and under the containers, which provided oxygen to the eggs. Eggs were reared at 20 °C maintained by ambient air temperature. At 24 hours post fertilization, all eggs were treated for fungus in a dip solution of 10 drops of methylene blue per 3.78 L of water for five minutes each day for 2-3 days. Fungus treatments ceased when movement was noticeable within the eggs.

Photographs were taken of the eggs 24 hours post fertilization and of post-hatch larvae to facilitate accurate counts. Hatching occurred between three and eight days post fertilization. All photographs were taken using a Canon Powershot SD750 on macro setting with the flash off. Eggs and larvae were counted from the images using the multi-point tool in ImageJ (https://imagej.nih.gov/ij/) with the “Label points” option turned off. After counts had been completed, hatch success was calculated by dividing the number of larvae hatched by the number of eggs in each container. Confidence intervals and p-values were calculated by 10,000 bootstrap and permutation replicates using R statistical software (RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/).

There was no difference in hatch success between pure and hybrid progeny for either year (2016, *P* = 0.26; 2017, *P* = 0.11; Table 1). Survival was tracked for 36 days post fertilization by daily manual counts, image counts, and mortality collection. Survival was quantified to 36 days because that is when Razorback Sucker reach 25 mm length at 20 °C and chances of survival in the wild increases (Bestgen 2008). Percent survival was calculated by dividing the number surviving after 36 days by the number of larvae hatched. Permutation tests showed that there were no differences in percent survival between progenies for either year (2016, *P* = 0.57; 2017, *P* = 0.13; Table 2).

Discussion

Although genetic studies have shown that Razorback Sucker x Flannelmouth Sucker hybrids do produce viable offspring (Buth et al., 1987; Douglas and Marsh, 1998; Dowling et al., 2012), our study is the first to compare hatch success and larval survivability of Flannelmouth Sucker - Razorback Sucker hybrids to that of the parent species.

Our results indicate that Razorback Sucker female x Flannelmouth Sucker male and Flannelmouth Sucker female x Razorback Sucker male hybrids are capable of hatching and surviving at rates similar to that of the parental species. However, hatch success was generally low (3-8 %) despite all eggs being treated and reared under the same conditions. This suggests that our laboratory conditions were not ideal for egg rearing. Potential problems may have included the use of Flagstaff city water, fungal infections, and induced spawning. Marsh (1985) also reared Razorback Sucker eggs in the laboratory, but reported a relatively high (35%) hatch success at 20 °C. Marsh (1985) used well water to rear the eggs which may have contributed to the differences in hatch success between the two studies.

Considering the rarity of Razorback Sucker and the high abundance of Flannelmouth Sucker in the Colorado River in Grand Canyon, hybridization is likely to increase (Hubbs, 1955; Tyus and Karp, 1990) in the future, but is not frequently listed as a noteworthy threat to Razorback Sucker recovery. The results of this study, however, indicate that hybridization may pose a threat to Razorback Sucker genetic integrity. Populations of Razorback Sucker isolated between dams may be negatively affected by hybridization with Flannelmouth Suckers and may warrant management action. Determining the mechanism of natural hybridization, be it random mixing of gametes or interspecific mate selection, may inform the development of management options to reduce the potential for hybridization in the future.

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Table 1--Mean hatch success (%), lower and upper bounds of 95% confidence intervals (CI), and standard deviations (SD) for Razorback Sucker, Flannelmouth Sucker, and their hybrids from induced captive spawning.

Year	Progeny	*n*	Mean hatch success (%)	Lower CI	Upper CI	*SD*
2016	Razorback Sucker	29	2.92	1.33	4.80	4.82
2016	Razorback ♀ x Flannelmouth ♂	17	5.02	0.72	11.83	12.67
2017	Flannelmouth ♀ x Razorback ♂	8	7.88	1.91	14.97	10.25
2017	Flannelmouth Sucker	9	2.62	0.40	6.00	4.84

Table 2-- Mean survival (%), lower and upper bounds of 95% confidence intervals (CI), and standard deviations (SD) for Razorback Sucker, Flannelmouth Sucker, and their hybrids.

Year	Progeny	*n*	Mean survival (%)	Lower CI	Upper CI	*SD*
2016	Razorback Sucker	18	94.34	87.86	98.73	12.24
2016	Razorback ♀ x Flannelmouth ♂	8	94.80	90.85	98.25	5.74
2017	Flannelmouth ♀ x Razorback ♂	6	64.50	42.64	85.37	32.86
2017	Flannelmouth Sucker	5	40.03	14.140	64.79	32.52

Chapter 2

Using shape to identify Razorback Sucker, Flannelmouth Sucker, and their hybrids

Introduction

A hybrid is the offspring of two different species. Hybridization can lead to speciation, as it has for many plant species (Dowling et al. 2016) and a few animal species, but is a concern when trying to manage populations of endangered species (Allendorf et al. 2001, McDonald et al. 2008) because it leads to loss of genetic integrity (Quist et al. 2009). Loss of genetic integrity is of particular concern when hybrids are reproductively viable (Buth et al. 1987). When a first generation hybrid (F1) backcrosses with one of the parent species it leads to introgression, an amalgamation of the species' genomes (Buth et al. 1987) and can eventually lead to the endangerment and loss of species (McDonald et al. 2008) by loss of pure genetics.

Hybridization occurs naturally in many vertebrate taxa and can be facilitated by the introduction of exotic species, alteration of habitat, and limited conspecific mates (Hubbs 1955; Scribner et al. 2001). American black ducks *Anas rubripes*, and mallards *Anas platyrhynchos*, were allopatric before European settlement in the 20th century. Habitat alteration and the introduction of mallards as a game species has caused sympatry of these two species, which has led to introgressive hybridization between American black ducks and mallards. G_{ST} (a measure of genetic differentiation) was 0.146 before 1940 and was only 0.008 as of 1998. Molecular analyses have failed to distinguish them as two distinct taxa (Mank et al. 2004). The California tiger salamander *Ambystoma californiense* was declining in its native range due to habitat alterations before the introduction of eastern tiger salamanders *Ambystoma tigrinum*. Hybridization is now considered a threat to California tiger salamanders despite being genetically distinct from the invasive species (Riley et al. 2003). The Pecos Pupfish *Cyprinodon pecosensis* became endangered through introgressive hybridization with the Sheepshead Minnow *Cyprinodon variegatus* after it was introduced into the Pecos Pupfish's range (Scribner et al. 2001). These are all examples of how humans have facilitated hybridization in different vertebrate taxa.

Hybridization is relatively common in freshwater fishes (Hubbs 1955; Scribner et al. 2001). Fish hybrids often have intermediate morphology compared to the parental species. Green Sunfish X Bluegill *Lepomis cyanellus X Lepomis macrochirus*, hybrids have intermediate feeding mechanics to the parent species (McGee et al 2015). Hybrids between Arctic Char *Salvelinus alpinus,* and Brook Trout *Salvelinus fontinalis,* phenotypically resemble Brook Trout, but are intermediate in meristic counts (Argue and Dunham 1999). Hubbs (1955) reported hybrids in the sucker family, Catostomidae, are not only intermediates in morphology, but also meristics, external colors, body form, and size of scales. Morphological intermediates between two catostomid species, Razorback Sucker *Xyrauchen texanus*, and Flannelmouth Sucker *Catostomus latipinnis*, have been described as early as 1889 (Hubbs and Miller 1953; Holden and Stalnaker 1975). Both species are endemic to the Colorado River (Hubbs and Miller 1953; McCarthy and Minckley 1987; Douglas and Marsh 1998; Bezzerides and Bestgen 2002) and have evolved with pre-dam conditions which included seasonal flooding, warm summer water temperatures, and extremely turbid water.

The construction of dams has adversely affected the Razorback and Flannelmouth suckers by changing natural water conditions from warm and turbid with high seasonal fluctuations to relatively steady, cold, and clear (Minckley 1983). As a result of the altered habitat and predation and competition with nonnative species, young Razorback Suckers failed to recruit into the adult population leading to declining populations, and the Razorback Sucker was ultimately listed as endangered in 1991 (United States Fish and Wildlife Service 1991). Currently, the only natural known reproduction and recruitment of Razorback Suckers in the lower Colorado River Basin occurs in Lake Mead (Albrect et al. 2010, Kegerries et al. 2015). Natural spawning does occur in the Colorado River mainstem, but there is limited recruitment (Albrect et al. 2010; Kegerries et al. 2015). The Razorback Sucker and Flannelmouth Sucker are more likely to utilize similar spawning habitats where they co-exist between dams which may lead to increased hybridization between the two species, particularly where the Razorback Sucker is rare in relation to the Flannelmouth Sucker (Hubbs 1955; Tyus and Karp 1990).

Razorback Suckers are identified by a sharp protruding keel behind the head accompanied by a deep caudal peduncle, long head, and a thick, robust body. Flannelmouth Suckers lack a dorsal keel and are more slender in bodily dimensions, particularly in the caudal peduncle. Hybrids between these two species were originally described as a new species, *Xyrauchen uncompahgre,* in 1891 (Jordan 1891, pp. 26-27, pl. 5 fig. 12, cited from Hubbs and Miller 1953). However, Hubbs and Miller (1953) analyzed meristic and morphometric measurements of eight museum specimens of *X. uncompahgre* and determined that they were too variable to be considered a species, and dubbed them Razorback-Flannelmouth sucker hybrids. Morphological intermediates have been since been confirmed as Razorback-Flannelmouth sucker hybrids by genetics studies (Buth et al. 1987, Dowling and Minckley 1993)

To aid in the conservation and management of the endangered Razorback Sucker we wanted to understand the length at which we could accurately discriminate between Razorback Sucker, Flannelmouth Sucker, and their hybrids. Thus, we analyzed the shape of Razorback Sucker, Flannelmouth Sucker, and their hybrids using geometric morphometrics to define distinguishing shape characteristics. Conditional inference trees were used to determine at what length the characteristics begin to develop, e.g., keel development in Razorback Suckers, and used piecewise-linear models to visualize how shape changes with length. We were also interested in identifying any significant shape differences between the two different hybrid crosses, Razorback Sucker female *x* Flannelmouth Sucker male and Flannelmouth Sucker female *x* Razorback Sucker male.

Methods

Data acquisition. — Laboratory reared fish utilized in this study were obtained from the spawning that occurred in chapter 1. In chapter 1, hatchery-reared Razorback Sucker from Lake Mead Fish Hatchery, operated by the state of Nevada, and wild Flannelmouth Sucker collected from the Paria River, a tributary of the Colorado River, Arizona were spawned. Artificial spawning was facilitated by the use of Ovaprim® hormone injections (0.5 mL/kg body weight). Gametes were collected 48 hours post hormone injection and mixed in 50mL centrifuge tubes and labeled with each parent to monitor parent combinations. The spawning resulted in four progenies: pure Razorback Sucker, pure Flannelmouth Sucker, Razorback Sucker female *x* Flannelmouth Sucker male hybrids (herein referred to as “Razorback female hybrids”), and Flannelmouth Sucker female *x* Razorback Sucker male hybrids (herein referred to as “Flannelmouth female hybrids”). Progenies were reared at the Arizona Game and Fish Department Colorado River

Research office in 2.4 M diameter circular fiberglass tanks to be used for subsequent geometric morphometric analysis.

Fish were anesthetized with Aqui-S sedative and placed on a measuring board marked in millimeters. Pictures were taken of the left lateral side of each fish using a Canon Powershot SD750 camera on a macro setting. Pictures of 313 different laboratory-raised individuals were taken and grouped into ten different size strata: less than 60mm, 60-69mm, 70-79mm, 80-89mm, 90-99mm, 100-109mm, 110-119mm, 120-159mm, 160-199mm, and greater than or equal to 200mm. Pictures of Razorback Suckers from Bubbling Ponds Hatchery, Cornville, Arizona, and wild Flannelmouth Sucker from the Colorado River in Grand Canyon, Arizona supplemented images from the laboratory produced fish.

A modified-stratified random sampling approach was used to select specimens for analysis. Specimens were stratified by size class and progeny. Ten individuals were randomly selected from each stratum for analysis. All individuals were analyzed if less than ten existed within a given stratum. Random selection of specimens was modified for four size strata of laboratory reared fish to ensure all unique parent combinations were represented in the analysis.

After individuals were selected for analysis, pictures were blindly analyzed because all information on the images were censored (e.g., progeny, total length (TL), etc…, and each was given a unique identification code linked to a file with each individuals' specific information. Images were imported into ImageJ (https://imagej.nih.gov/ij/) where two-dimensional coordinate data from 13 landmarks were collected on each image. The landmarks used were: A) tip of snout; B) center of eye; C) back of operculum; D) top of operculum; E) top of the back of the head; F) top of the back; G) anterior dorsal fin insertion; H) dorsal and I) ventral caudal fin insertions; J) posterior and K) anterior anal fin insertion; L) anterior pelvic fin insertion; and M) pectoral fin insertion (Figure 1). Additional data recorded with landmarks were: unique fish id, image scale (pixels per mm), progeny, size class, total length, source of fish (laboratory, wild, or hatchery), and parental combination (only for laboratory reared fish).

Analyses. — Analyses were conducted in RStudio version 1.0.153 (http://www.rstudio.com/) using R version 3.4.1 “Single Candle”. Packages used were geomorph (Adams et al. 2017), Momocs (Bonhomme et al. 2014), MASS (Venables and Ripley 2002), Vegan (Oksanen et al. 2017), party (Hothorn et al. 2006), SiZer (Sonderreger 2012) and routines created by Julien Claude (2008). Coordinates for each image were divided by the scale to adjust for variable subject-to-camera distances between images. The scaled coordinates were put into a partial Procrustes superimposition which translated and rotated the original coordinates and allowed each individual’s shape to be compared to one another while preserving the individual's size. Partial Procrustes superimposition was performed in contrast to a full Procrustes superimposition because size information of an individual is lost in a full Procrustes superimposition.

All shape differences were tested using adonis models (Oksanen et al. 2017). The adonis model is a permutational Multivariate Analysis of Variation (MANOVA) that utilizes the Procrustes coordinates (shape variables) to partition sums of squares based on dissimilarities (Oksanen et al. 2017). Shape differences were tested between all progeny in a model where the response was the Euclidean distances of the principal components and predictor variables were progeny, TL, and source of fish. Hybrid's shapes were compared in a model where the response was the Euclidean distances of the principal components for the hybrid progeny and the predictor variables were progeny and total length. Source of fish was not included in this model because there was only one source of hybrids (laboratory). Laboratory reared and wild Flannelmouth Sucker shapes were compared in a model where the response was the Euclidean distances of the principal components of Flannelmouth Sucker that were smaller than 71 mm and the predictor variables were source of fish and TL.

A principal component analysis (PCA) was conducted on the Procrustes coordinates to evaluate allometric shape changes for each progeny, and a linear discriminate analysis (LDA) was performed on the Procrustes coordinates of each progeny to estimate how often the progenies were correctly classified independent of size. The LDA was repeated for fish less than or equal to 136mm TL and greater than 136mm TL to determine the effect of length on classification.

A piecewise-linear model (Sonderegger 2012) using total length and the first principal component was used to evaluate shape change with total length for each progeny in relation to the first principal component. This analysis showed the projected shape changes of a fish as it grows. The piecewise-linear model identifies a "change point" in the data, fits a line to the data before and after the change point, and used 1000 bootstrap replicates to estimate 95% confidence intervals for the change point and slopes of the lines.

Conditional inference trees (Hothorn et al. 2006) were used to determine the length that we can begin to differentiate between the three fish types and at which length

Razorback, Flannelmouth, and hybrid's shapes significantly change. A conditional inference tree is a multivariate regression analysis that uses recursive partitioning and permutations to determine associations between response variable and covariates (Hothorn et al. 2006). Default parameters were used for the conditional inference tree analysis, e.g., 9999 permutations, minimum split = 20, minimum criterion = 0.95, the only parameters specified were: test statistic was "max" and test type was "Test statistic." We first used this approach to split all fish into different branches using the first principal component and total length, and then we repeated the analysis for each progeny. The resulting branches showed the length at which each progeny's shape significantly changed.

Results

Specimens

We analyzed pictures of 84 unique Flannelmouth Suckers, 14 of which were laboratory reared and 70 were pictures taken of wild fish. We used pictures of 95 unique Razorback Suckers, 60 of which were laboratory reared and 35 were hatchery raised. A total of 103 unique hybrids were analyzed, 73 of which were Razorback female hybrids and 30 were Flannelmouth female hybrids. Fish used in the analysis ranged from 37-305mm TL for all progeny in the study (Figure 2).

Analyses

The results of the adonis analysis of the principal components showed that progeny, total length, the source of an individual, as well as the two-way interactions between progeny: total length and source: total length were significant predictors of shape (Table 1). In a similar analysis using just hybrids, there was no detectable shape difference between the two hybrid progenies (adonis; F = 2.51; r^2 = 0.08; DF = 1,31; P-value = 0.121), therefore they were lumped together for further analyses. However, there was detectable shape differences between the laboratory reared and wild Flannelmouth Sucker (adonis; F = 7.04; r^2 = 0.29; DF = 1, 16; P-value = 0.02; Figure 3). The difference in shape was minimal to the eye (Figure 3), therefore laboratory reared and wild Flannelmouth Suckers were pooled for analysis.

Using the PCA for all fish in the study with the Procrustes coordinates, the first principal component accounted for 49% of the variation in shape between the three fish types (Figure 4). The PCA showed a separation of Razorback Sucker and Flannelmouth Sucker with hybrids occupying the space between (Adonis; F = 94.23; r^2 = 0.35; DF = 2, 272; P-value = 0.001; Figure 5). Considerable overlap between fish types can be attributed to smaller fish having similar scores for the first principal component (Figure 5).

In the LDA of the principal components of all the fish in the analysis, the first linear discriminant accounted for 91% of the variation in shape (Figure 6). A leave-one-out cross validation of all fish in the analysis correctly classified 59% of the fish based on shape; Razorback Suckers, Flannelmouth Suckers, and hybrids were commonly misclassified across all three fish types (Table 2). Based on a conditional inference tree analysis using the principal components of all fish with TL as the predictor variable, most overlaps in shape occurred at or below 136mm TL (Figure 7). As a result of the effect of size on shape, an LDA was done on specimens less than or equal to 136mm TL (small fish) and greater than 136mm TL (large fish). The LDA on small fish correctly classified 73% of the specimens based on shape. Small Razorback Suckers were misclassified 29%

of the time, 23% of small Flannelmouth Suckers were misclassified, and 28% of hybrids were misclassified as either Razorback or Flannelmouth suckers (Table 2). A LDA on large fish correctly classified 91% of the specimens based on shape. All misclassifications of Razorbacks (5%) and of Flannelmouths (7%) were misclassified as hybrids, and 21% of hybrids were misclassified as either Razorback or Flannelmouth suckers (Table 2).

A piecewise-linear model (Sonderegger 2012) for all Razorback Suckers using the first principal component and total length showed no significant change in shape with increasing total length (change point: 196 [80, 261]mm TL; slope 1 [-1.86e^{-4}, 8.58e^{-4}]; slope 2 [-1.58e-4, 6.91e-4]; Figure 8A). The total lengths of specimens in the study were not evenly distributed and were heavily weighted towards smaller fish (Figure 2). Consequently, a second piecewise-linear model was conducted using a random subsample (n = 25) of the small fish to ensure a more even total length distribution. This resulted in a significant change in Razorback Sucker shape with increasing total length at 183 [155, 258] mm TL (slope 1 [-4.02e^{-4}, -4.33e^{-5}]; slope 2 [5.61e^{-5}, 7.13e^{-4}]; Figure 8B). The piecewise-linear model for Flannelmouth Sucker showed no significant change in shape with increasing total length (change point: 119[58, 264] mm TL; slope 1 [1.34e^{-4}, 3.75e^{-3}]; slope 2 [-4.75e^{-4}, 2.50e^{-4}]; Figure 8C). Using a random subsample (n=25) of the small Flannelmouth Suckers failed to show a significant change in shape with total length (change point: 171 [58, 264]mm TL; slope 1 [-1.62e^{-4}, 2.95e^{-3}]; slope 2 [-5.44e^{-4}, 3.11e^{-4}]; Figure 8D). Hybrids showed a significant change in shape with increasing total length both when the full sample was analyzed and when a random subsample (n=25) was analyzed. The change in shape was at 148 [93, 165] mm TL (slope 1 [1.60e^{-4}, 4.55e^{-4}];

slope 2 [$-3.60e^{-4}$, $2.56e^{-5}$]; Figure 8E) for the full analysis and 148 [90, 203] mm TL (slope 1[$5.60e^{-5}$, $4.58e^{-4}$]; slope 2 [$-3.43e^{-4}$, $5.49e^{-5}$]; Figure 8F) for the subsampled analysis.

The conditional inference tree (Hothorn et al. 2006) analysis divided Razorback Suckers into two main groups, those that are greater than 100mm TL and those that are less than or equal to 100mm TL (p-value = <0.001). The group that was less than or equal to 100mm TL was subdivided further into two groups, less than or equal to 71mm TL and 72-100mm TL (P-value = <0.001; Figure 9A). Flannelmouth Suckers were divided into two main groups, those that are less than or equal to 145mm TL, those that are greater than 145mm TL (P-value = 0.0010). The two main groups were further divided into two sub-groups each, less than or equal to 55mm TL and 55-145mm TL, and 146-208mm TL and greater than 208mm TL (Figure 9B). Hybrids were split into two main groups, less than or equal to 81mm TL and greater than 81mm TL (Figure 9C). Each division within a tree represents shapes that are statistically different from one another.

Discussion

The results of our study show that Razorback Suckers, Flannelmouth Suckers, and their hybrids are similar in shape when they are less than or equal to 136mm TL, but their shapes begin to diverge when larger than 136 mm TL (Figure 7). Razorback Sucker and hybrid’s shapes change differently with length. As a Razorback Sucker grows, it develops a keel and a robust body (decreasing PC1 score) before its growth becomes more proportional with elongation (higher PC1 score) (Figure 8B). In contrast, hybrids tend to grow more elongated (increasing PC score) before developing a more robust body (decreasing PC1 score) (Figure 8E). Based on how Razorback Suckers’ shapes change with increasing length, we can use the results of the conditional inference tree analysis to interpret keel development in Razorback Sucker (Figure 9A). The keel and deep bodies in Razorback Suckers become distinctly visible around 100mm TL (Figure 9A). To interpret the length that the keel forms in the hybrids, we have to refer to the change point in the piecewise-linear model because this is where the hybrid's shape begins to converge with the Razorback Sucker's shape based on the first principal component. The hybrid's keel develops between 92 and 165mm TL in (Figure 8E).

Our findings are relatively consistent with Minckley and Gustafson (1982) who reported that keel development in Razorback Sucker was noticeable by touch at 90mm (about one-year old). We did not detect keel development until 100mm TL because the keel may not be visible until 100mm TL. Our results also agree with the traditional morphometric analyses by Hubbs and Miller (1953) which concluded that hybrids are intermediate to Razorback and Flannelmouth suckers in several morphological features including peduncle depth, body depth, and keel size. Our unique use of artificially spawned hybrids allowed for larger sample sizes and more definitive analyses of this pattern of intermediacy for hybrids. However, our study lacked hybrid specimens between 125 and 200mm TL. This data gap decreases our confidence in estimates of the specific length when hybrids' shapes begin to change, i.e. keel development.

The LDA of small fish showed small Flannelmouth Suckers were correctly classified at the highest rate (77%) over the small Razorback Sucker (71%) and hybrids (72%). The classification rates for fish smaller than or equal to 136 mm TL were higher than expected and were likely caused by our large sample size (n = 218). We expected the classification analysis of larger fish (>136 mm) to show improved classification, which it did for all fish types: Razorback (96%) and Flannelmouth Sucker (93%), and hybrids (79%) (Table 2). The classification of large hybrids did not increase as much as we were expecting which was likely an artifact of our small sample size of hybrids greater than 136 mm (n = 14). The variable length at which a hybrid develops its keel could lead to a misclassification as either Razorback or Flannelmouth sucker. A large hybrid that converges on Razorback shape earlier in life could result in a misidentification of a hybrid as a Razorback Sucker based on the presence of a keel. A large hybrid that lacks a keel could be misclassified as a Flannelmouth Sucker.

Understanding how the shape of these sucker species and their hybrids change with increasing total length is vital to field identification and proper monitoring of the endangered Razorback Sucker. Field scientists can become complacent when identifying closely related fish species, especially when processing large numbers of fish (Douglas and Marsh 1998). Complacency may easily lead to the misidentification of Razorback Suckers or hybrids that are less than 100mm TL as Flannelmouth Sucker. After keel development (approximately 100mm TL), it is likely that hybrids may be misidentified as Razorback Suckers until about 136mm TL.

The purpose of this study was to help managers differentiate between Razorback Suckers, Flannelmouth Suckers, and their hybrids. When these two species and their hybrids are small (less than or equal to 136mm TL), they are difficult to differentiate from one another using body shape alone. Mouth shape is commonly used as an identification method for suckers (Belk et al. 2016). We attempted to do shape analysis on lips of small fish, but insufficient contrast between the lips and body created difficulty in obtaining high quality pictures for analysis. Lip shape may also not serve as the best field identifier for small fish because the mouth parts are simply too small to see in any detail. It is possible that a combination of shape and meristic counts may serve as better field identifiers of small fish. However, obtaining meristic data in the field can be difficult and time consuming, particularly on small individuals. Based on our results, we suggest that field biologists take caution while measuring small suckers in the Colorado River basin and feel for presence of a keel in suckers that are greater than 90 mm TL. Genetics tests should be carried out on small suckers that are suspected of having a keel until a more accurate method of identification for these small fish is investigated.

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Tables

Table 1. Summary results of Adonis output displaying the significant predictors of shape for Razorback Suckers, Flannelmouth Suckers, and hybrids.

	Df	**Sum of Squares**	**Mean Squares**	**F. Model**	**R^2**	**P-value**
Progeny	2	0.153	0.076	94.231	0.347	0.001
Total Length	1	0.032	0.032	39.861	0.073	0.001
Source	2	0.021	0.011	13.181	0.049	0.001
Progeny: TL	2	0.005	0.003	3.346	0.012	0.007
TL: Source	2	0.008	0.004	4.745	0.017	0.001
Residuals	272	0.220	0.001		0.501	
Total	281	0.439			1.00000	

Table 2. Classification of all fish in the leave one out cross validation analysis, classification of fish less than or equal to 136mm TL (small fish), classification of fish larger than 136mm TL (large fish). Class correctness is the proportion of respective progeny that were correctly classified. Classified columns show how each fish in the respective progeny were classified.

		Classified		
	Proportion correctly classified	**Razorback**	**Flannelmouth**	**Hybrid**
All Fish				

Razorback Sucker	0.72	68	11	16
Flannelmouth Sucker	0.40	18	34	32
Hybrid	0.63	19	19	65
Small Fish (≤136 mm)				
Razorback Sucker	0.71	50	0	21
Flannelmouth Sucker	0.77	1	43	12
Hybrid	0.72	14	11	64
Large Fish (>136 mm)				
Razorback Sucker	0.96	21	0	1
Flannelmouth Sucker	0.93	0	26	2
Hybrid	0.79	1	2	11

Figures

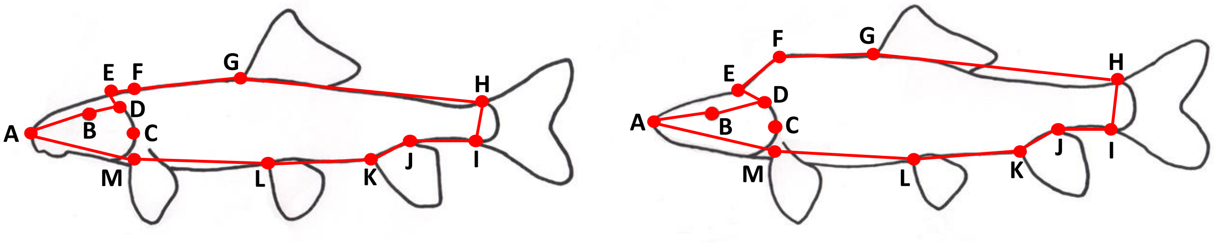

Figure 1. Visualization of landmarks used for geometric morphometrics: A) tip of snout; B) eye; C) back of operculum; D) top of operculum; E) top of the back of the head; F) top of the back; G) anterior dorsal fin insertion; H) dorsal and I) ventral caudal fin insertions; J) posterior and K) anterior anal fin insertion; L) anterior pelvic fin insertion; and M) pectoral fin insertion.

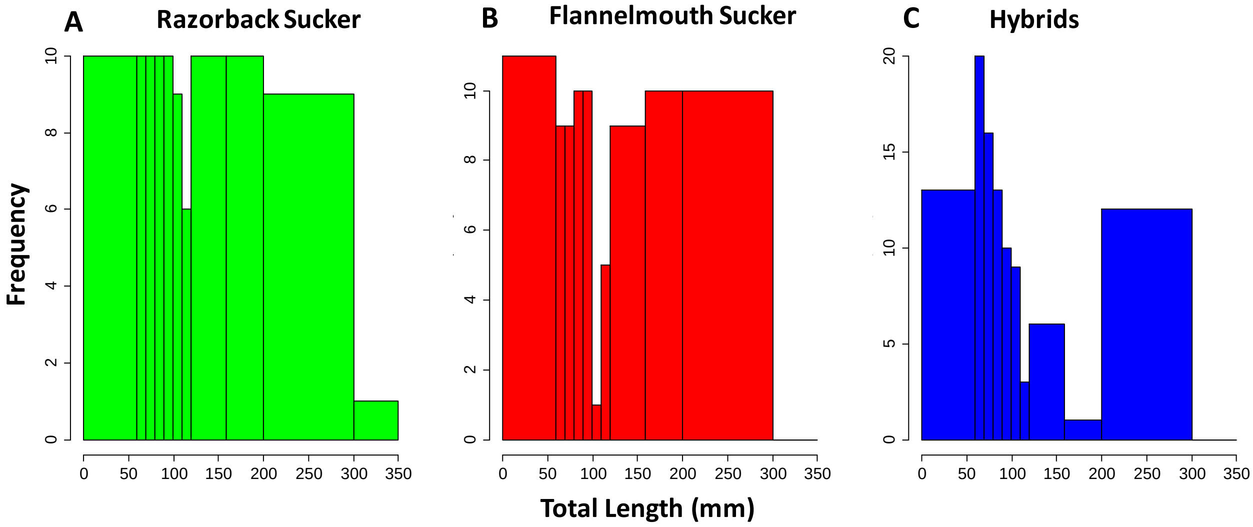

Figure 2. Length frequency histograms for A) Razorback Sucker, B) Flannelmouth Sucker, and C) hybrids used in the study. The width of the bars represents designated size classes: less than 60mm, 60-69mm, 70-79mm, 80-89mm, 90-99mm, 100-109mm, 110-119mm, 120-159mm, 160-199mm, and greater than or equal to 200mm.

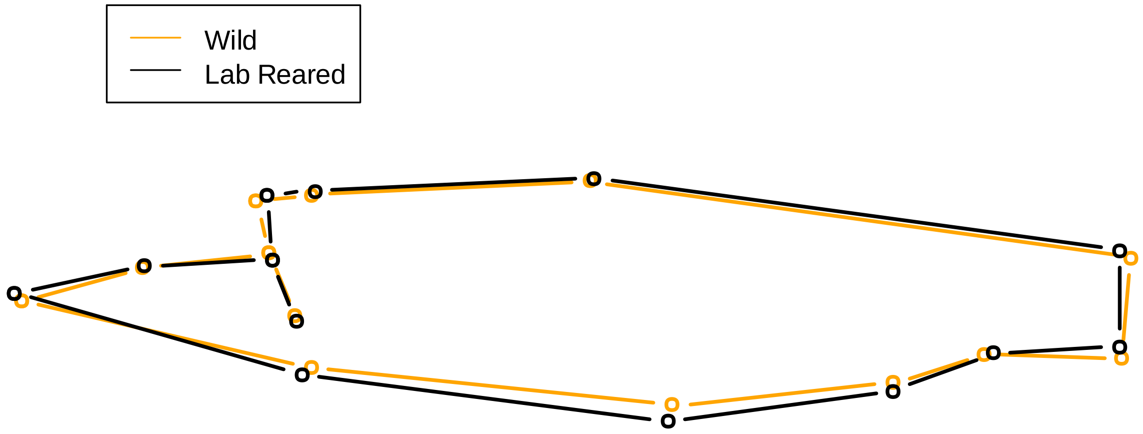

Figure 3. Laboratory Flannelmouth Suckers were in better condition than wild Flannelmouth Suckers. Average shape of wild (orange) and laboratory reared (black) Flannelmouth Suckers that were less than 71mm TL. P-value = 0.02

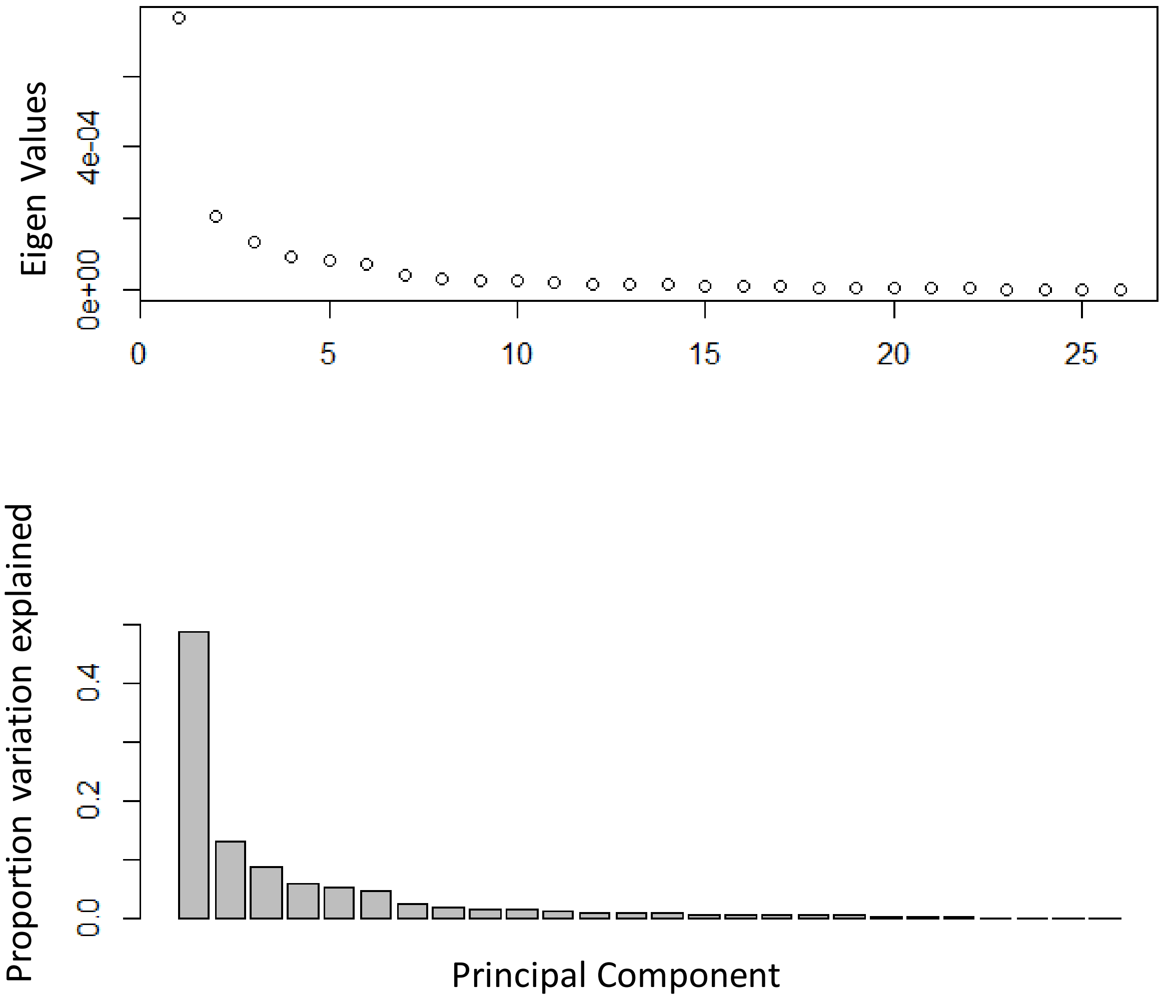

Figure 4. A principal component analysis was conducted using the shape variables of 95 Razorback Sucker, 84 Flannelmouth Sucker, and 103 hybrids. The resulting eigen values (top) and principal component loadings (bottom) are shown. The first component explains 49% of the variation in shape between the three fish types.

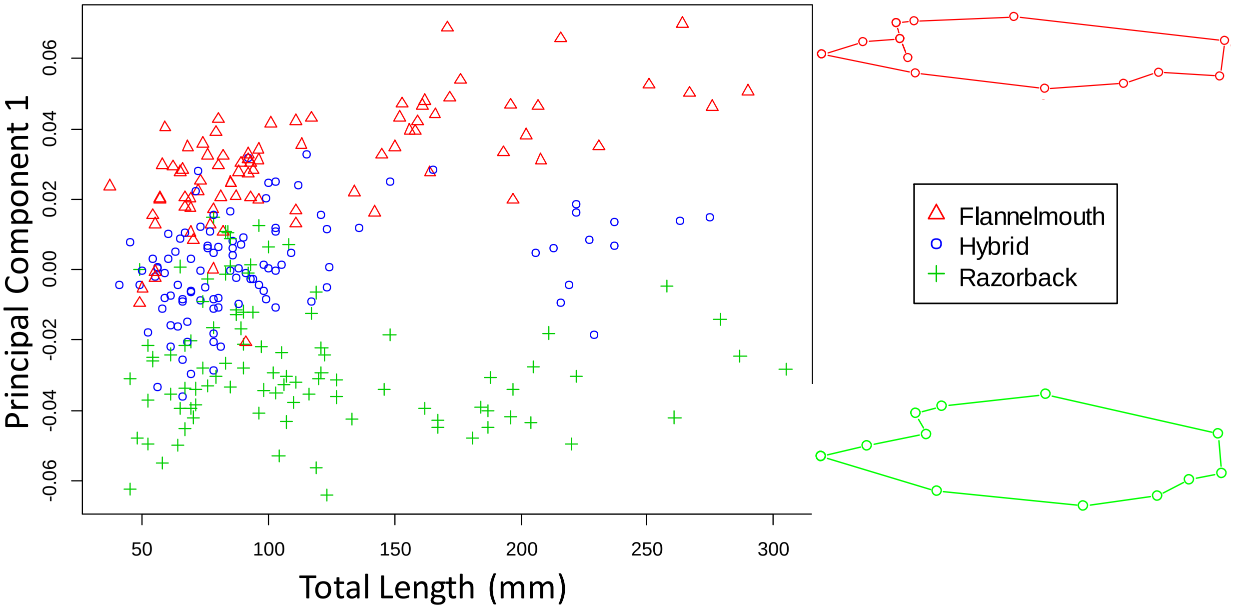

Figure 5. Left: Plot of the first principal component with total length. Flannelmouth Sucker in red, hybrids in blue, Razorback Sucker in green. The hybrids fall in intermediate shape-space between Flannelmouth and Razorback Suckers. Right: Red shape (top) represents the maximum principal component score, a Flannelmouth Sucker, and green shape (bottom) represents minimum principal component scores, a Razorback Sucker.

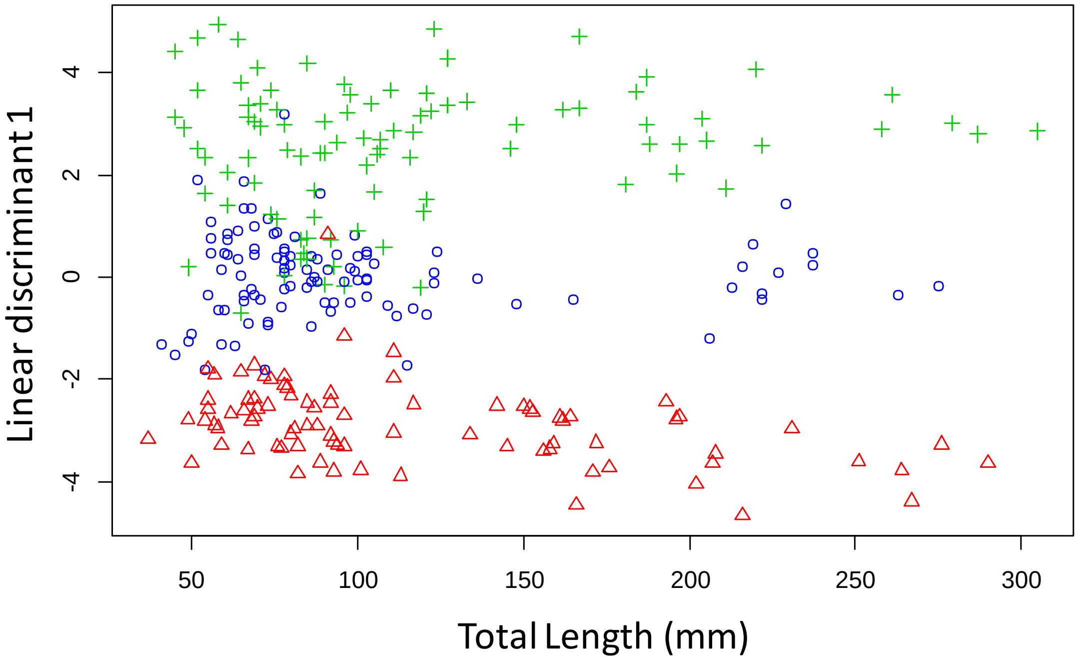

Figure 6. The first linear discriminant accounted for 91% of the variation in shape for the three fish types. Plot of first linear discriminant analysis that used Procrustes coordinates. Flannelmouth Sucker in red, hybrids in blue, and Razorback Suckers in green.

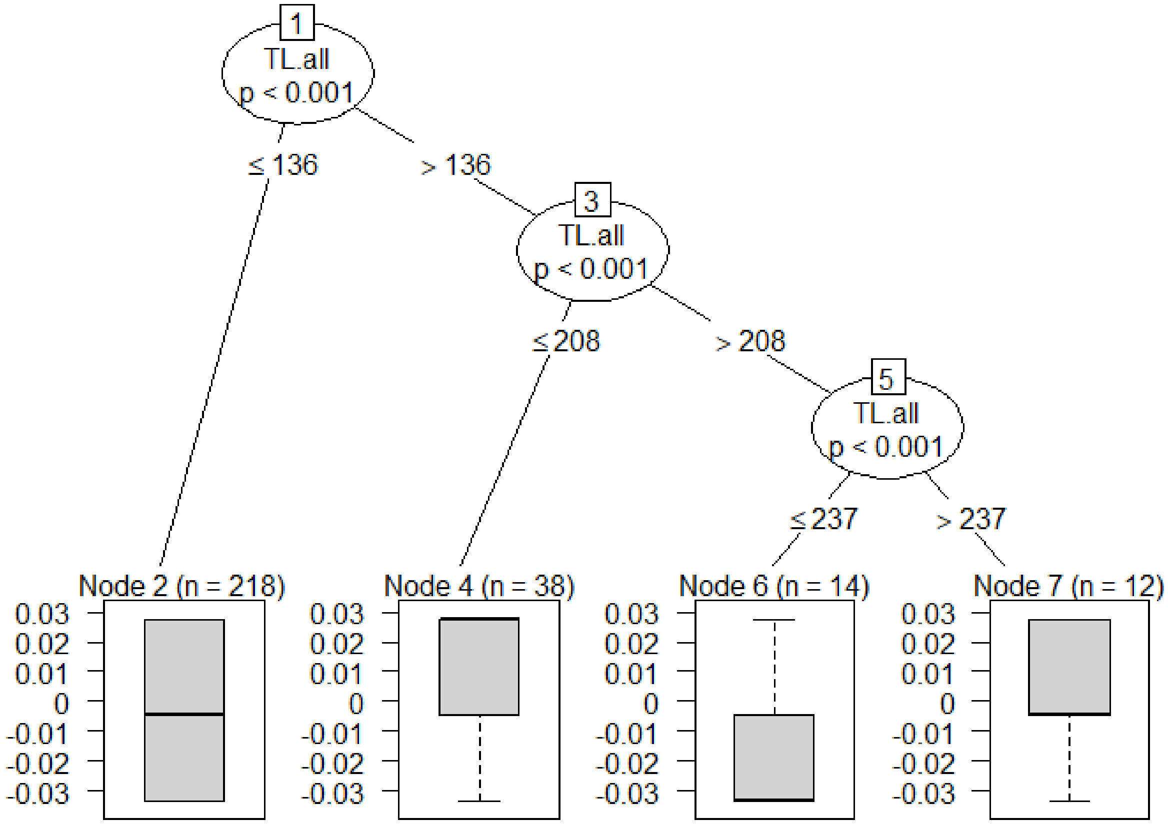

Figure 7. Conditional inference tree that used all shape variables as a function of total length that grouped all similar shaped fish into one group, less than or equal to 136mm TL, and fish with different shapes larger than 136 mm TL. The y-axis of the box plots represents the first principal component that resulted from the principal component analysis.

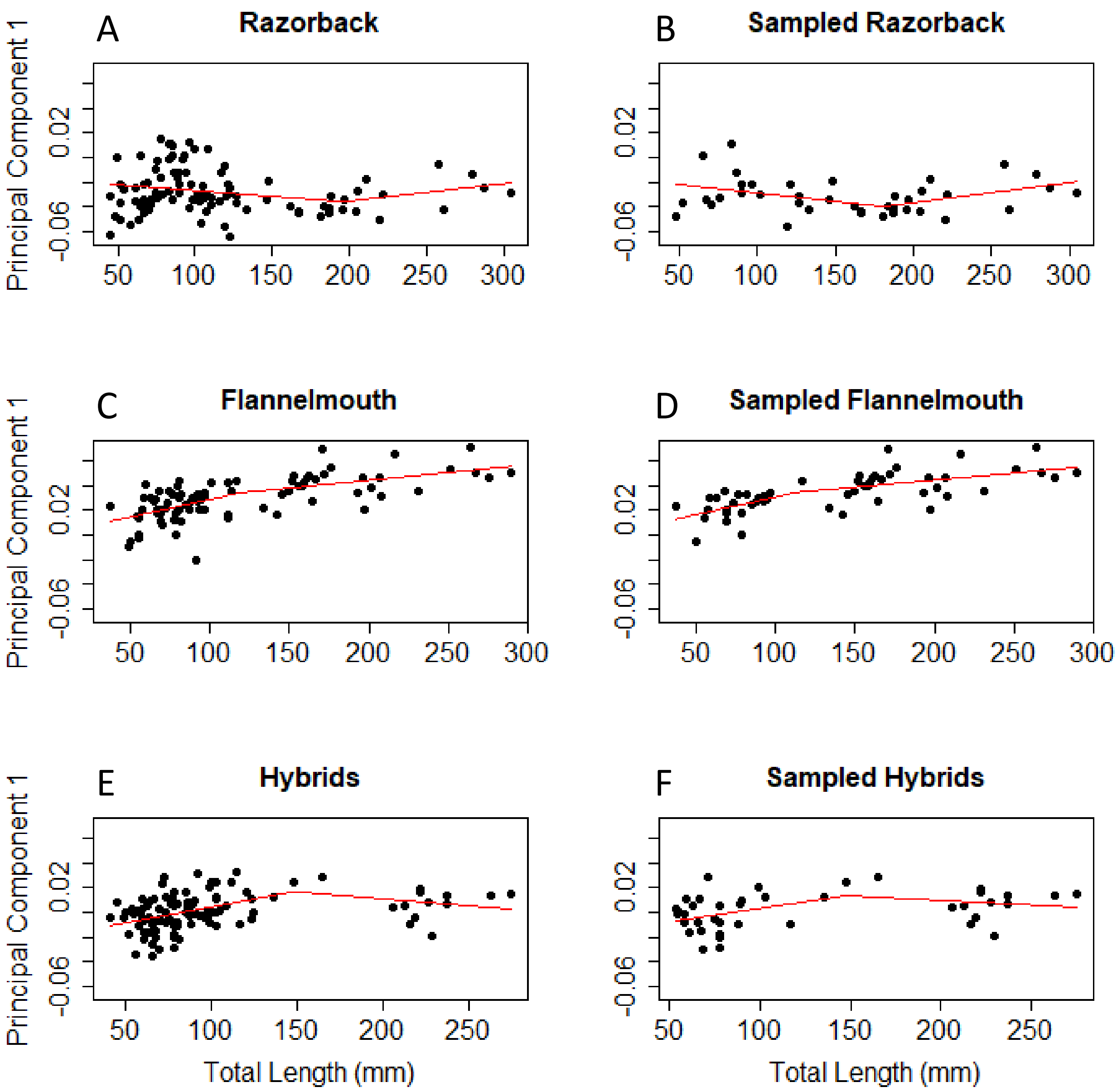

Figure 8. Each fish types shape changes differently with increasing length. The maximum principal component score represents a Flannelmouth Sucker shape and the minimum principal component score represents a Razorback Sucker shape. A) Razorback Sucker piecewise-linear model using all specimens. B) Razorback Sucker piecewise-linear model after using a random subset of small fish. C) Flannelmouth Sucker piecewise-linear model using all specimens. D) Flannelmouth Sucker piecewise-linear model after using a random subset of small fish. E) hybrid piecewise-linear model using all specimens. F) hybrid piecewise-linear model after using a random subset of small fish.

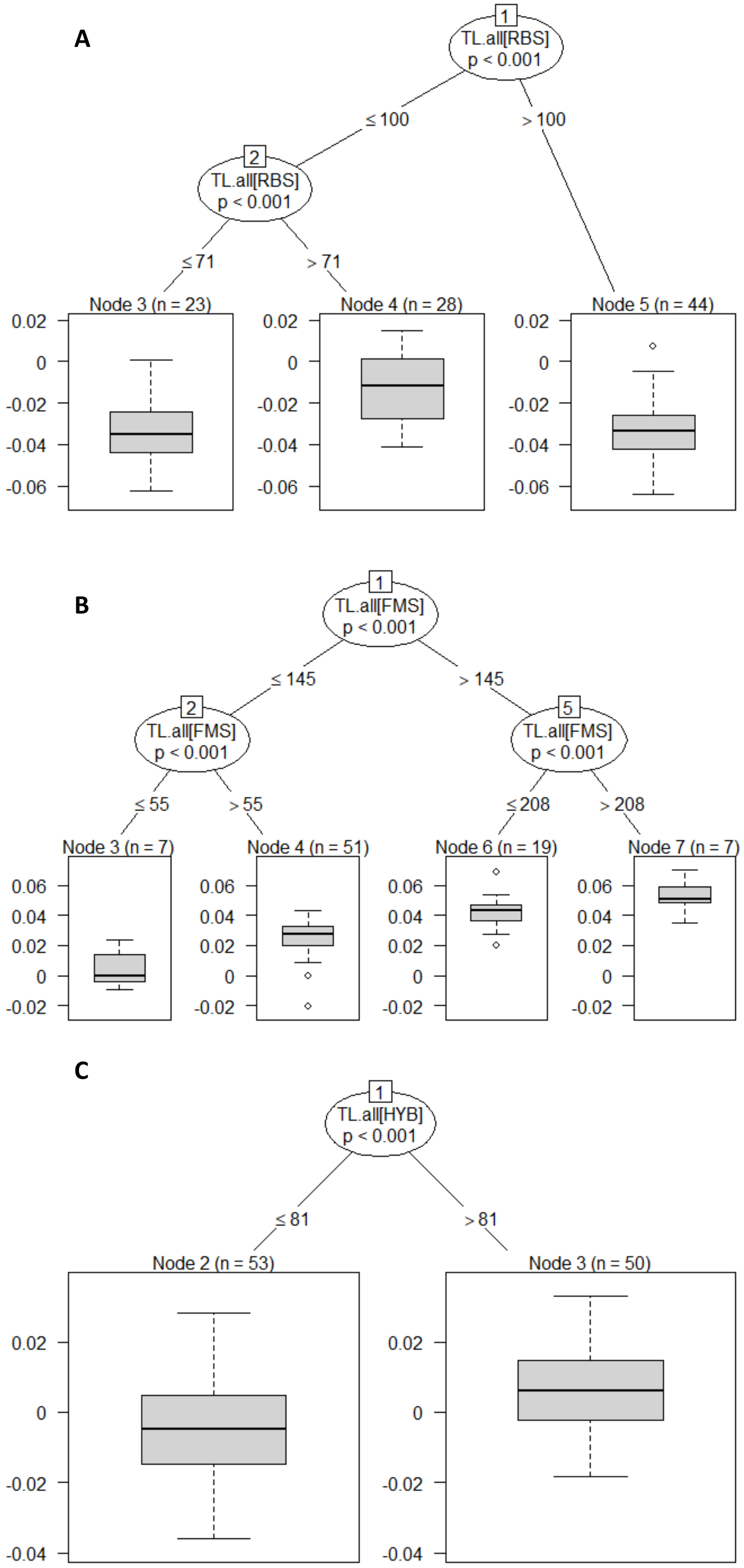
A
1
TL.all[RBS]
p < 0.001
≤ 100
> 100
2
TL.all[RBS]
p < 0.001
≤ 71
> 71
Node 3 (n = 23)
Node 4 (n = 28)
Node 5 (n = 44)
0.02
0
-0.02
-0.04
-0.06
B
1
TL.all[FMS]
p < 0.001
≤ 145
> 145
2
TL.all[FMS]
p < 0.001
5
TL.all[FMS]
p < 0.001
≤ 55
> 55
≤ 208
> 208
Node 3 (n = 7)
Node 4 (n = 51)
Node 6 (n = 19)
Node 7 (n = 7)
0.06
0.04
0.02
0
-0.02
C
1
TL.all[HYB]
p < 0.001
≤ 81
> 81
Node 2 (n = 53)
Node 3 (n = 50)
0.02
0
-0.02
-0.04

Figure 9. Conditional inference trees for A) Razorback Sucker, B) Flannelmouth Sucker, and C) hybrids. Each group is split by a total length. The y-axis for each boxplot in the trees are the PC1 scores. A) Razorback suckers that are less than or equal to 100mm TL have different shapes than Razorback Suckers that are greater than 100 mm TL. B) Flannelmouth Suckers that are less than or equal to 145 mm TL have different shapes than those that are greater than 145 mm TL. C) Hybrids that are less than or equal to 81 mm TL have different shapes than those that are greater than 81 mm TL.

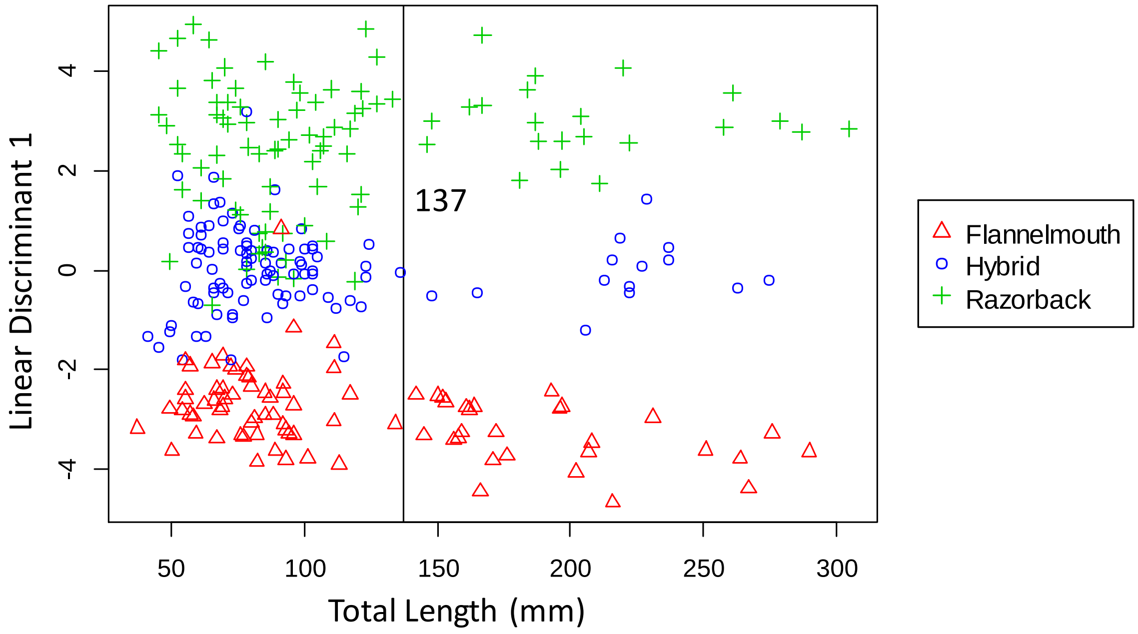

Figure 10. Plot of linear discriminant 1 with total length. The vertical line is the length threshold where to the left of the line there is not a statistical difference in shape between the three fish types, but to the right there is. Flannelmouth Sucker in red, hybrids in blue, Razorback Sucker in green.