Variation in morphology and physiology of introduced populations of the Virile Crayfish, *Faxonius virilis* Hagen 1870

By

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Abstract

Crayfish often show high levels of morphological variation between and within populations. Studies have shown that environmental factors can play a role in determining external morphology. Increased levels of phenotypic variation can be advantageous for individuals in a novel environment. I used geometric morphometrics to determine the differences in the external morphology of introduced populations of *Faxonius virilis* over a broad spatial scale. I compared carapace morphology in populations collected from lentic and lotic habitats using geometric morphometric techniques. I found that crayfish from lotic habitats had more fusiform body shapes with greater relatively wide areola and that crayfish from lentic habitats had broader body shape with less relatively wide areola. To determine the effects of this variation in shape on the physiology of this species, I performed a respirometry study on individuals collected from an introduced population in Alabama. Using curves derived from 26 closed respirometry trials, Regulator Index scores were calculated to quantify the degree of regulation of respiratory rate. Following the respirometry trials, the carapace shapes of each crayfish was quantified using GMM techniques. I found that crayfish with

conformatory strategies most often had a fusiform body shape, while crayfish with more regulatory strategies had a broader body shape. These body shapes correspond to the body shapes found in lotic and lentic crayfish, respectively.

Acknowledgements

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List of Abbreviations

PCA Principle Components Analysis

CVA Canonical Variate Analysis

CV Canonical Variate

DO Dissolved Oxygen

DO_{crit} Critical Concentration of Dissolved Oxygen

RI Regulator Index

TSA Total Surface Area

MO_2 Rate of oxygen consumption

AUC Area under the curve

GLM General Linear Modeling

Chapter 1

Variations in Morphology of Introduced Populations of the Northern Crayfish, *Faxonius virilis* (Hagen 1870) Across a Broad Spatial Scale

Introduction

Morphological variation within a species may be driven by a variety of evolutionary forces such as natural selection and spontaneous genetic mutations (White 1978). Given enough time and isolation, divergence leads to the rise of new species (White 1978). Examples include the explosive radiation of cichlid species found in the lakes of eastern Africa (Kocher 2004). Advances in molecular techniques and statistical analyses have allowed scientists to gain a better understanding of the causes of variation seen between and within different populations (Sint et al. 2007). For example, increases in bioinformatics techniques and genome sequencing have allowed for the full sequencing of the genomes of Darwin's finches across the Galapagos Islands, painting a more complete picture of the speciation process and driving forces behind one of the most well-known examples of adaptive radiation and speciation (Lamichhaney et al. 2015).

Freshwater ecosystems display a high level of spatial complexity and heterogeneity across the landscape. Factors such as pH, salinity, dissolved oxygen concentration, temperature, and water velocity vary between major habitat types such as rivers, lakes, and estuaries and may exert selection pressure on populations (Lampert and Sommer 2010). It is not surprising then that many freshwater species display variations in morphology across populations as the environment changes (Grandjean and Souty-Grosset 2000, Pakkasmaa 2001,

Hepp et al. 2012, Santos and Araujo 2015). Fish species often display differences in body shape based upon habitat, with species commonly found in slower moving water having deeper bodies, while fish in fast moving water had more fusiform shapes (Pakkasmaa 2001, Santos and Araujo 2015, Langerhans et al. 2003, Beachum et al. 2016). Crustaceans also follow a similar pattern (Hepp et al. 2012).

Freshwater crustaceans such as crayfish have been the focus of several studies concerning morphological variation. Studies have focused on indigenous populations of species that are threatened or endangered (). A better understanding of their morphology may help address issues that hinder the conservation process. The ability to quickly identify threatened species within an area allows for better detection rates of the species of interest and may increase the chances of protecting their habitats (Sint et al. 2007). Understanding morphological differences between isolated populations and the process of speciation within these populations may help also help with species identification and determining which populations may be more important to the overall conservation of the species (Duretanovic et al. 2017). Determining the effects of different habitats on external morphology and its effect on relocation success when moving threatened populations could significantly increase the success of relocation and reintroduction efforts (Haddaway et al. 2012).

Invasive crayfish, particularly species native to North America that have established populations well outside their native range, have caused serious damage to the areas they have invaded (Ahern et al. 2008). The introduction of

Procambarus clarkii and *Pacifastacus leniusculus* to European streams has had detrimental effects on the populations of native crayfish (Hänfling et al. 2011). *Procambarus clarkii* populations also have had negative effects on rice production in Europe and Asia, damaging the plants before they can be harvested (Hänfling et al. 2011).

Many introduced organisms tend to have high levels of variation in morphology, physiology, and behavior within a population (Ghalambor 2007). Being morphologically plastic may confer an advantage to invasive species, allowing them to quickly adapt to a new habitat and establish populations once they reach a new area, often within a generation (Baldwin 1896, Ghalambor 2007, Dlugosch et al. 2015). A recent study focused on the role of water velocity in determining the shape of chelae in *Faxonius rusticus,* a species of crayfish that is often a successful invader when introduced outside of its native habitat (Perry et al. 2011). Differences in the shape of the chelae, as well as in the overall body shape of the organism, were noted between habitats with high and low water velocity. Individuals from high velocity areas had broader chelae with a more fusiform body shape, while individuals from low velocity areas had narrower chelae and a stouter body shape (Perry and Jones 2018). This suggests that chelae play a role in the hydrodynamics of the crayfish, with broader chelae being better able to deflect water up and over the carapace than narrower chelae. However, other factors such as competition, mate attraction and defense against predators may also have an effect on chela size (Garvey and Stein, 1993 and Stein and Magnuson, 1976).

For my study, I wanted to determine if the patterns observed in *Faxonius rusticus* could also be detected in introduced populations of a closely related species, *F. virilis*, over a broader spatial area. *Faxonius virilis* (Virile or Northern Crayfish) is native to the northern and midwestern portions of the United States and southern Canada, but has been introduced throughout the continental United States and Europe (Adams et al. 2010). It prefers streams and lakes with rocky bottoms more than sandy-bottomed streams (Caldwell and Bovbjerg 1969). *Faxonius virilis* is an omnivorous species that tends to take advantage of a wide variety of available food sources, from plant material to the larvae of other aquatic organisms (Hanson et al. 1990, Love and Savino 1993). Introductions of this species outside of its native range have been attributed to recreational fishing activity in the US and the pet trade in Europe (Adams et al. 2010). The species often co-occurs with *F. rusticus*, so individuals may possess similar traits and environmental tolerances (Phillips et al. 2009).

Based upon the patterns observed in *F. rusticus*, *F. virilis* individuals taken from lotic environments should have longer, slender, more fusiform carapaces with a wider areola, while the individuals from lentic environments would have shorter, broader carapaces with a narrower areola (Perry et al. 2011, Perry and Jones 2018). The fusiform body shape is more advantageous for individuals living in faster moving waters because it reduces drag and decreases the chances of being carried off by the current (Perry et al. 2011). Without the need to reduce drag, lentic crayfish may exhibit broader frames that may have

other advantages, such as increased success in intraspecific and interspecific interactions (Hill and Lodge 1999).

Carapace shape does not seem to be influenced by intraspecific competition like the chelae or sexual dimorphism like the abdomen (Grandjean and Souty-Grosset 2000). However, the carapace does house a majority of the crayfish's organs, including the gills. The gills are the primary location of gas exchange and respiration. Because respiratory needs will most likely be different between habitat types, carapace shape may also change. The width of the areola has also been observed to change between species. The areola is indicative of gill attachment and gill volume. Some of the earliest surveys of crayfish noted that lotic crayfish had wider areolae than lentic and burrowing crayfish (Hobbs 1942, Hobbs 1981.) Thus, a plastic species may show variation in carapace shape, particularly in the region associated with the areola, between disparate environments due to different respiratory needs (Haddaway et al. 2012).

In order to determine if any shape variation exists in the carapace of crayfish from different habitats, I used geometric morphometrics to analyze shape variations in crayfish populations from different habitats. I also wanted to determine if any observed variation can be attributed to environmental factors, as opposed to differences between the sexes or factors unique to each site. Geometric morphometrics differs from traditional morphometrics in that it is a multivariate analysis of the overall shape of the organism as opposed to relying on a set of distances, ratios, and angles that may not be able to capture the exact spatial arrangement of the landmark (Slice 2007). It allows users to take into

account the overall shape of the object, whereas traditional morphometrics only takes into account the individual measurements of distance or ratios, Geometric morphometrics was developed to provide a more powerful and comprehensive analysis of morphology than the previous traditional morphometric approaches (Slice 2007).

Methods

Specimen Selection

Preserved specimens of *F. virilis* were obtained from the North Carolina Museum of Natural Science Non-Molluscan Invertebrate Collection in Raleigh, North Carolina, along with the dataset containing locality, introduction status, haplotype groupings, and body measurements. The specimens were originally collected across several provinces in Canada for a study of the range expansion of *F. virilis* (Williams et al. 2011). To quantify potential variation across a broad spatial scale, 76 individuals representing six different sites from Alberta Province in Canada were chosen from approximately 400 individuals representing 36 sites and digitized for morphological comparison. These individuals were chosen because they represented known introductions and shared a haplotype group (B. Williams, pers. comm.). Three sites, Beyette Lake, East Pit Lake, and McLeod Lake, represented the lentic habitat and three sites along the South Saskatchewan River, at Suffield Military Base, at Sandy Point Park at Route 41, and at the River Landing Provincial Park, represented the lotic habitat (Table 1). The lentic sites were somewhat isolated from the nearest waterways, ranging in distance from 60

km to 160 km to the nearest river. McLeod Lake is used heavily for recreational purposes, such as boating and fishing, and East Pit Lake is a reclaimed coal mine with no in-or out-flow connecting it to any other body of water (Williams et al. 2011). The lotic sites were all along the South Saskatchewan River. Sampling of all of the sites in 1967 did not yield any reports of *F. virilis* at the time, so the invasions of these areas have occurred since then (Williams et al. 2011). The lentic sites covered 6,374 km^2 and the lotic sites covered 2,804 km^2, for a combined total of 9,178 km^2 (Figures 1 and 2).

Digitization

Specimens were positioned dorsoventrally next to a scale bar and photographed using a Canon eOS Rebel t5i digital camera with an 8-48mm lens at 30 cm. The photographs were then digitized with tpsDig (Rohlf, 2017). Twenty-three landmarks were placed on each image, with 17 fixed landmarks and six sliding semilandmarks. The fixed points marked homologous structures on the dorsal side of the carapace and remain static during analysis, while the sliding points are used to capture variation that cannot be quantified by fixed points alone, such as the curvature of the posterior region of the carapace. The sliding points are placed between the fixed points that border the area of interest and marked as sliding points, which allow them to move between the fixed landmarks and capture any changes in the shape of the area. The 17 fixed landmarks were the tip of the rostrum, the marginal spines, the post-orbital processes, the insertion point of the scapes, the cervical spines, the insertion points of the abdomen, and

four points to capture the length and width of the areola (Figure 3). Two of the six sliding landmarks were placed between the insertion of the scape and the cervical spines to help estimate curvature of the anterior portion of the carapace. The other four were placed between the cervical spines and the insertion points of the abdomen, two on each side, to capture the curvature of the posterior portion of the carapace (Figure 3).

Once all of the landmarks had been digitized, a tps file was created containing the coordinates of each point using the tpsUtil program (Rohlf, 2013a). Landmark coordinates were aligned using Procrustes superimposition to account for differences in size and rotation of the specimens. Consensus, partial warps, and relative warp analysis were performed on the aligned coordinates to generate a set of relative warp scores using tpsrelw (Rohlf 2013a). The relative warp scores were used to perform multivariate analysis of variance (MANOVA) to detect significant shape change between habitat types and sex.

Analysis

The coordinates from the tps file were used to create a dataset within MorphoJ. The coordinates were modified to remove the semilandmarks, because MorphoJ considers all points to be homologous landmarks. Classifier data concerning site, habitat type, and sex were added to the dataset for further analysis of potential drivers of observed variation. A wireframe outlining the overall shape of the carapace was created in order to visualize the changes detected by the analyses. After aligning the coordinates, a covariance matrix was

generated and used to perform Canonical Variates Analysis (CVA) of the data using groupings based on the sex of the individuals, which site they were collected from, and which habitat type the site represented.

The scores generated by the relative warp analysis, along with classifier data concerning habitat, collection site, and sex to run a MANOVA to determine if any relationships existed between the observed variation in morphology and the habitat type, collection site, or sex of the individuals and any interactions between the variables (R Core Team, 2013, Adams et al. 2017).

Results

All 76 individuals from the selected populations were photographed and landmarked. There were 33 lentic individuals and 39 lotic individuals, 43 of the individuals were male and 29 were females, and all individuals were adults. The average carapace length of the individuals was 34.3 mm (21.4–48.4 mm) (Table 2).

CVA analysis suggests some minor differences in carapace shape between the sexes, but they were not statistically significant (MANOVA, $p=0.980$, $F=0.3394$, d.f.=1, CVA, $p=0.33$) (Figure 4, Table 3 and 4). The canonical variate scores displayed on the frequency graph show overlap between the sexes.

Comparison of shape among the six sites reported some pairwise shape differences between sites. However, the only significant differences were observed when comparing a lentic site and a lotic site (Table 5). The CVA also

supported this trend, with the sites grouping by habitat type (Figure 5). The first CV explained 45.17% of the variance and the second CV explained another 33.76% of the variance for a total explained variance of 78.93%. The third CV explained an additional 10.4%. The fourth and fifth CVs explained 6.4% and 4.5% of the remaining variance (Table 4).

Shape differences detected by the CVA between the habitat types were highly significant (MANOVA: p=>0.001, r^2=0.0699 F=5.2103, d.f.=1, CVA: p=0.001, d.f.=1) (Tables 4 and 5). Geometric morphometric analysis revealed a shorter rostrum and wider carapace in the lentic group, compared to the lotic group (Figure 6). Because only two group options were compared, CV1 explained 100% of the variance observed in the analysis (Table 4). The frequency graph displays less overlap between the habitat groups and a wider range of canonical variate scores, indicating a broader variety of morphologies with zero misclassified individuals (Figure 6).

Discussion

External morphology of crayfish varied over a broad spatial scale. The pattern of variation was consistent with the habitat of the individuals. I found that the crayfish living in the lotic habitats had longer, thinner carapaces and a sharper rostrum whereas crayfish living in lentic habitats had shorter, wider bodies and shorter rostrums. In general, the trends detected in this study are in agreement with previous studies in both crayfish and other aquatic invertebrates.

Some of the earliest studies of crayfish biology noted differences between different habitats, with crayfish from oxygen-poor habitats having adaptations that may increase their chances of survival, including increases branchial ventilation and a narrowing of areola (Hobbs 1942, Dejours and Beekenkamp 1977, Holditch and Reeves 1981). More recent studies have begun to tease apart the factors that may be influencing variation, from environmental factors such as variations in water velocity to interactions between different species (Perry and Jones 2018, Hayes et al. 2009). Similar patterns have been observed in fish and other aquatic macroinvertebrates (Langerhans et al. 2003, Orlofske and Baird 2014). Blacktail shiners (*Cyprinella venusta)* that had been collected from reservoirs had deeper and slightly wider bodies than individuals collected from rivers (Haas et al 2010). Fusiform bodies reduce the amount of drag experienced an animal moves through the water, which could prevent being swept away by a particularly strong current (Perry et al. 2011). It could also be easier for them to wedge into crevices and small holes to anchor themselves, instead of constantly having to swim to stay in place (Perry et al. 2011). The crayfish in lakes presumably do not experience as much water current, and therefore may not benefit from the fusiform body shape or may gain an advantage from having larger, wider bodies. Larger bodies may discourage predation or increase success in fighting off other crayfish and attracting mates.

Another difference between the habitat groupings was the relative width of the areola, with crayfish from lentic habitats having narrower areola than the crayfish from lotic habitats. Some of the earliest studies of crayfish have observed

differences between species that occupy different kinds of habitats, reporting narrower areola in burrowing species and wider areola in stream-dwelling species (Hobbs 1942). Researchers speculate that changes in the structure may have an effect on the internal morphology of the crayfish, but a direct study has yet to be completed (Haddaway et al. 2012). Relative areola width may reflect gill volume, with narrower areola providing more room in the gill chambers for the attachment of gill filaments, and thus more surface area available for gas exchange. A crayfish with more surface area available for gas exchange should be better suited for survival in low-oxygen conditions, like those typically found in lentic habitats due to higher water temperatures and decreased surface area for diffusion of atmospheric oxygen into the water (Lampert and Sommer 2010).

This observed variation in morphology might be beneficial to individuals who are introduced to new environments. *F. virilis* is a common bait item for fishermen, especially when targeting game species like largemouth bass, which has led to *F. virilis* being introduced to areas far outside its native range (Caldwell and Bovbjerg 1969). Being able to express a variety of phenotypes within a population without genetic recombination increases the odds of survival for any individuals released into a new area, which in turn increases the odds that a small number of individuals can survive long enough to reproduce and establish a population (Davis 2009). The odds of survival would increase even more if the morphological variation were accompanied by other changes in behavior or physiology, such as increased aggression towards other species or changes in dietary choices (Sargent and Lodge, 2014, Hanson et al. 1990). Further study

needs to be conducted on the potential interactions between morphology and other aspects of the crayfish biology, such as physiology or behavior, to determine how the crayfish might benefit from these changes, as well as what trade-offs may exist that might limit the amount of variation observed. Understanding these relationships will provide a better overall understanding of the crayfish themselves and how they interact with their environments. This knowledge, in turn, could prove vital for preventing damage, or even total lose, of populations to the looming threats of climate change and stymie the spread of invasive species across the globe.

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Figures

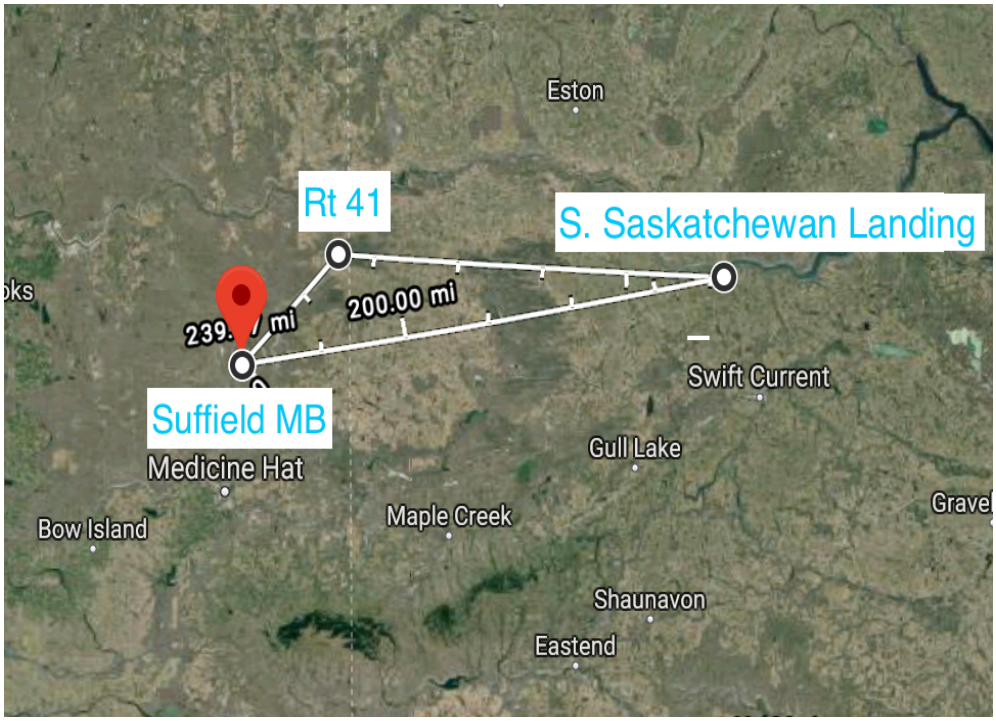

Figure 1: River sites. All sites along the South Saskatchewan River in Alberta, Canada.

Figure 2: Lake Sites. All sites in Alberta, Canada.

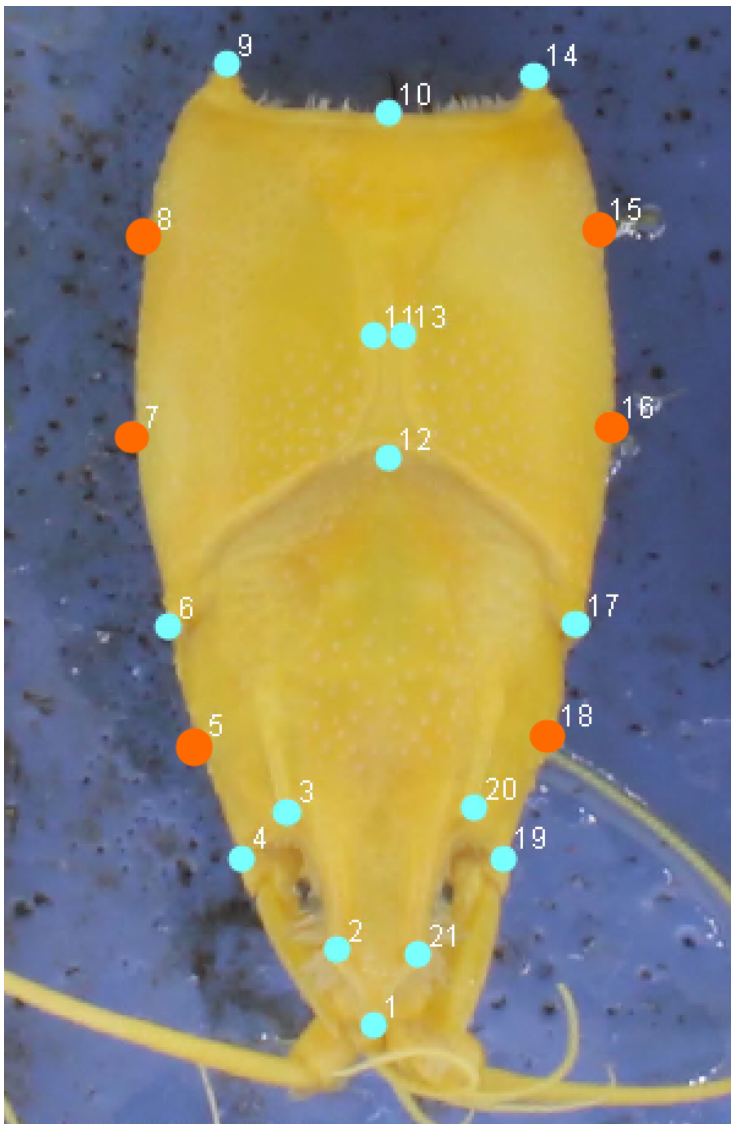

Figure 3: Digitized carapace with homologous (blue) and semilandmarks (orange). Homologous landmarks: 1-Rostrum, 2 and 21-Marginal Spines, 3 and 20-Post-orbital spines, 4 and 19-Scape insertion points, 6 and 17- Cervical spines, 9 and 14- End of carapace, 10 and 12- Areola length, 11 and 13- Areola width. Semilandmarks: 5, 7, 8, 15, 16, and 18.

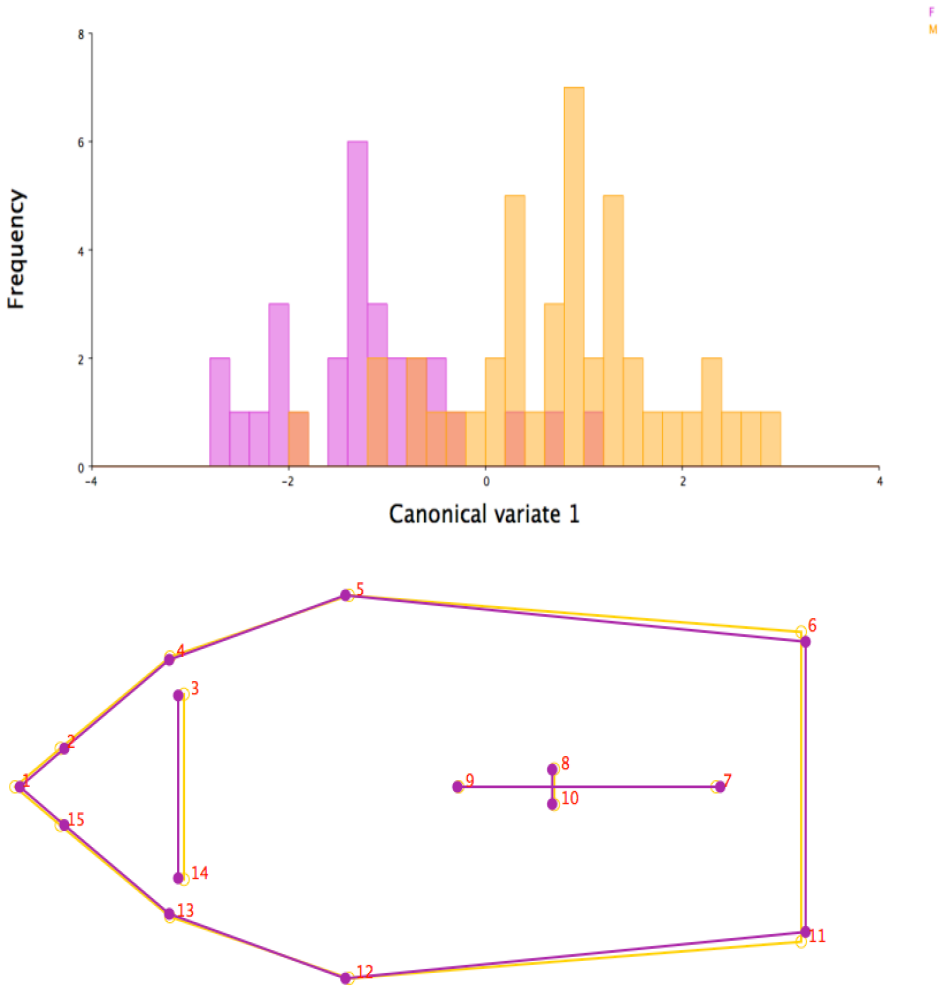

Figure 4: Canonical Variate Analysis of crayfish based on sex and wireframe of CV1 showing changes in carapace morphology. Male shape is displayed in gold, female shape in purple.

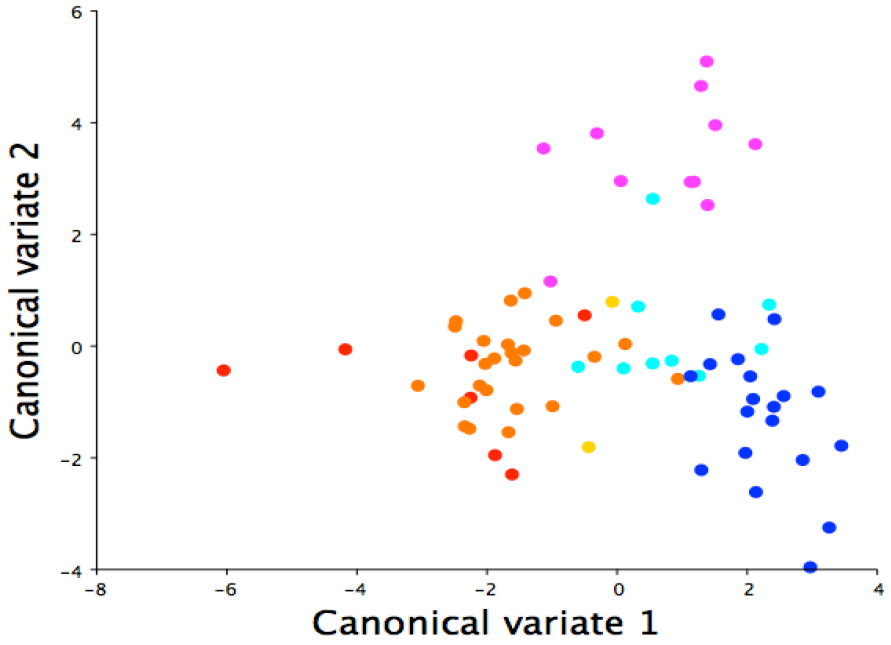

Figure 5: Canonical Variate Analysis of Sites. Blue and purple points represent lentic sites and red, orange, and gold points represent lotic sites

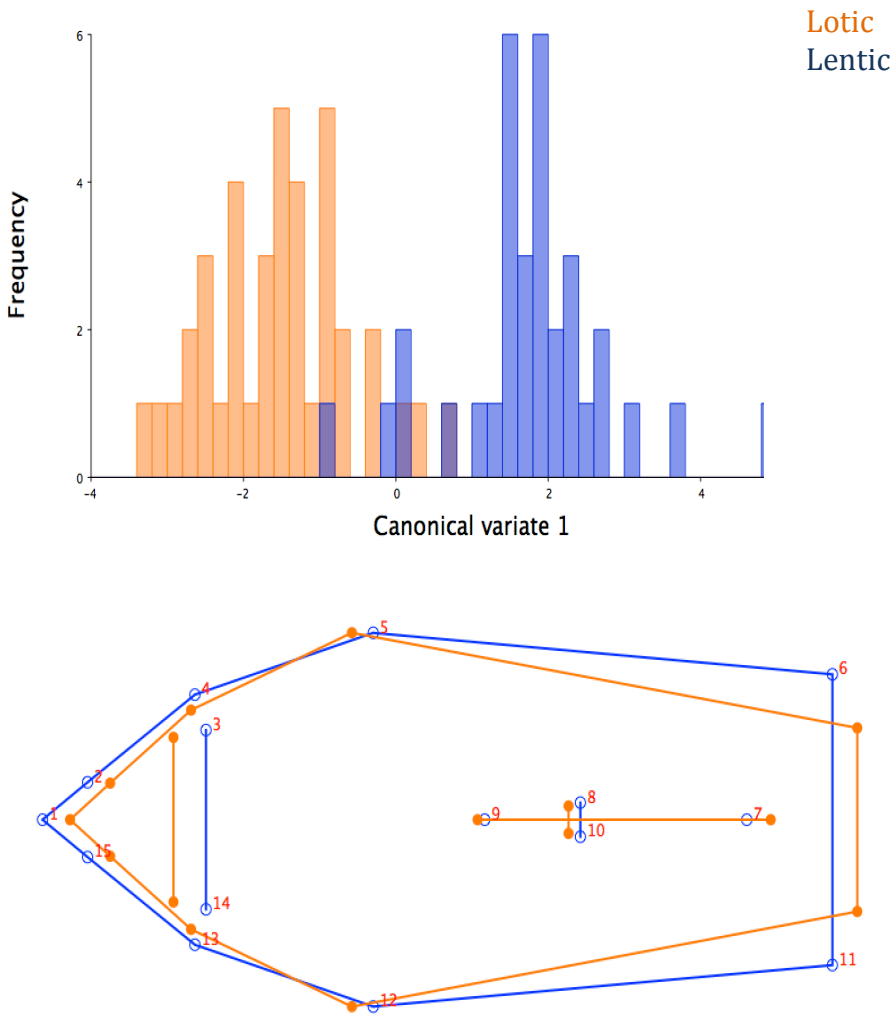

Figure 6: Canonical Variate Analysis of crayfish morphology based on habitat type and wireframe of coordinates showing changes in carapace morphology. The "River" group is displayed in orange, and the "Lake group" displayed in blue

Tables

Table 1: Collection Sites summary

Site Name	Abbreviation	Latitude/ Longitude	Habitat Type	Individuals per Site
Beyette Lake	BEYL	54.592414 -114.19854	Lentic	7
McLeod Lake, Carson-Pegasus Provincial Park	CPPP	54.293344 -115.65135	Lentic	24
East Pit Lake near Wabumum	EPL	53.584292 -114.46388	Lentic	2
S. Saskatchewan River at Rte 41, Sandy Point Park	SSR41	50.731667 -110.075	Lotic	9
S. Saskatchewan River at Saskatchewan Landing Provincial Park	SSRSL	50.655556 -107.97472	Lotic	19
S. Saskatchewan River on Suffield Military Base	SSRSUFF	50.399313 -110.58923	Lotic	11

Table 2: Data summary of individuals digitized for study.

Site Abbreviation	Number per Site	Number per Sex		Mean Carapace Length	Carapace Length Range		
		M	F		Low	High	Range
BEYL	7	4	3	37.158	32.25	41.65	9.4
CPPP	24	12	12	30.935	22.59	44.38	21.79
EPL	2	1	1	40.92	40.92	40.92	0
SSR41	9	7	2	40.43304	22.83	48.44	25.61
SSRSL	19	9	10	26.22	21.4	32.67	11.27
SSRSUFF	11	10	1	33.72181	25.1	43.99	18.89
Total	76	43	29	34.31083	21.4	48.44	27.04

Table 3: Summary of variance explained by Canonical Variates and associated eigenvalues.

Grouping	CV	Variance Explained	Eigenvalue
Sex	CV1	100%	0.997
Habitat	CV1	100%	2.78
Site	CV1	45.17%	3.359
	CV2	33.76%	2.51
	CV3	10.40%	0.77335
	CV4	6.40%	0.456
	CV5	4.50%	0.337

Table 4: MANOVA model analysis summary. Model used: shape~habitat+sex+habitat:sex where shape represents the relative warp scores of the specimens

Variable	df	Sum of Squares	MS	R^2	F	Z	p
habitat	1	0.01335	0.0133499	0.069953	5.21	3.8881	**0.001**
sex	1	0.00087	0.008696	0.004557	0.34	-2.1501	0.98
habitat**:**sex	1	0.002391	0.0023911	0.012529	0.93	0.108	0.474

Table 5: Table of shape differences between sites from CVA. Bolded numbers are significant p-values between sites.

Site Comparisons	BEYL	CPPP	EPL	SSR41	SSRSL
CPPP	0.1282				
EPL	**0.0017**	**0.03**			
SSR41	**0.0149**	**0.0001**	**0.05**		
SSRSL	**0.0139**	**0.001**	0.09	0.25	
SSRSUFF	**0.0001**	**0.0001**	0.15	0.43	0.1

Chapter 2

Effect of shape on respiratory strategy in an introduced population of *Faxonius virilis* (Hagen, 1870)

Introduction

Some of the most well known differences in the external morphology of crayfish species occur in the cephalothorax region of the crayfish body. The cephalothorax is covered by the carapace, a hardened portion of the exoskeleton that protects the internal structures found in this region (Holdich and Reeve 1988). Differences in the overall shape of the carapace, as well as structures such as the areola and rostrum, often appear as a diagnostic features in dichotomous keys for crayfish identification (Haddaway et al. 2012). Crayfish species living in lentic, or lake-like, habitats have been noted to have a wider carapace and a narrower areola compared to species living in lotic, or stream-like, habitats (Hobbs 1942, Hobbs 1988). It has been speculated that differences in the carapace width may have an impact on the branchial volume of the gill chambers, which are located on either side of the areola (Holdich and Reeve 1988). A wider carapace would imply that there was more room in the gill chambers for the gill filaments, improving a crayfish's chances of absorbing oxygen molecules (O_2) from water with low dissolved oxygen (DO) content (Haddaway et al. 2012). Studies have also noted that DO varies predictably between lentic and lotic habitats, with lentic habitats displaying more variable levels of DO compared to lotic habitats (Lampert and Sommer 2010).

In response to variation in oxygen availability, organisms can display various strategies for respiration. The strategies vary based upon the differences in their oxygen consumption rates (MO_2). A regulator maintains a constant rate of respiration, regardless of the amount of oxygen available. Regulation continues until the critical concentration of dissolved oxygen (DO_{crit}) is reached. The DO_{crit} is the concentration of DO below which an organism can no longer maintain its resting metabolic rate using aerobic respiration and switches to anaerobic metabolism and the metabolic rate slows (Prosser 1973). The regulation strategy allows the organism to maintain a constant respiratory rate despite fluctuations in DO levels.

A conformer is unable to maintain a constant respiratory rate as the DO levels decline and must alter the rate of respiration based on the amount of oxygen available, thus as O_2 levels drop, the organism slows its respiratory rates (Prosser 1973). It can take full advantage of increased levels of oxygen to increase its rate of respiration and produce more energy. However, as oxygen levels drop, it must slow its respiration rate down as well, and must make do with the lower amount of energy.

It is now generally recognized that most organisms do not utilize perfect conformation or perfect regulation strategies, but instead display strategies that fall somewhere between these two extremes. Recently, the Regulator Index (RI) was developed to quantify the varying patterns of respiration as oxygen availability changes (Mueller and Seymour 2011). The index is a relative measure of regulatory ability for an organism that assigns a score,

reflecting the degree to which an individual is regulating their respiration (Figure 7). As the score increases, respiration rate becomes more independent of the environmental O_2 levels. These scores range from 0 to 1, with 0 being a perfect regulator and 1 being a perfect conformer.

The RI is an improvement over calculating the point where an animal can no longer maintain a certain MO_2, because it displays the relationship between respiration rate and levels of dissolved oxygen both above and below the DO_{crit} .It does not assume that the respiration rate remains constant above the DO_{crit} (Mueller and Seymour 2011).

The degree of regulation seems to vary from species to species, and can vary from individual to individual depending on a variety of factors, including environmental conditions and physiological factors (Herreid 1980). An early study looked at the effects of sustained hypoxic environmental conditions on respiratory stress in *F. virilis* and suggested that *F. virilis* display a regulator strategy, increasing branchial irrigation to maintain a certain respiratory rate, until available oxygen dropped below a certain level (McMahon et al. 1973). Then, it becomes a conformer, dependent on surrounding oxygen levels to determine its respiratory rate.

Studies have also shown that physiological factors can affect the respiration of crayfish and other crustaceans, such as stage in the molt cycle (McWhinnie and Kirchenberg 1962, Huuskonen et al. 2014). The molting process involves major changes to physiology, biochemistry, morphology, and behavior of the crayfish and carries a significant energetic cost (Chang 1995, Penkoff and

Thurberg 1982). The crayfish must build an entirely new exoskeleton while breaking down the existing skeleton, which requires a significant input of energy and nutrients (Chang 1995). The minimum resting metabolic rate of crayfish (SMR) tends to remain consistently low during the intermolt stage, but increase significantly during the molting process (Huuskonen et al. 2014). Building a new exoskeleton increases the amount of energy needed, which in turn increases the demand for oxygen. While a regulator strategy would provide a stable, constant rate of respiration, and thus energy.

Given that no studies have examined potential relationships between variation in morphology and respiration in crayfish, I was interested in determining what, if any, relationships exist between morphology, particularly the carapace shape and gill surface area, and the respiratory strategy of individual crayfish. I also want to determine if there are any relationships between ability to regulate oxygen consumption and the molt stage of the individual.

I would expect to see crayfish with a wider carapace to have a higher RI score, while crayfish with a narrower carapace will have a lower RI score. I would also expect a higher total surface area (TSA) of gill filaments in the crayfish with a wider carapace compared to a crayfish with a narrower carapace. Because the molting process, from the construction of the new exoskeleton to ecdysis, is such an energetically demanding process for crayfish, I would expect to see them switch from a more regulating strategy to a more conforming strategy when the

crayfish enters the premolt stage of the molt cycle and begins to build its new exoskeleton.

Methods

Animals and Holding Conditions

A total of 36 *F. virilis* individuals were collected from the Cahaba River in Masonic Park, outside of Trussville, AL in September 2017. Individuals were collected using 16 minnow traps baited with canned cat food and placed in different areas of the river (Figure 8). The traps were collected after two days in the river. The crayfish were transported to the South Auburn Fisheries Unit and held individually in small tanks (1.89 L) (Figure 9). All of the tanks were connected to a common 40 L sump system that pumped artificial freshwater through the tanks and was changed once a week. Crayfish were provided with lengths of PVC piping for shelter and assigned a tank identification number and held for a one-month acclimation period. The first 2 weeks allowed them to acclimate to the holding tanks. For the last two weeks, the water in the system was gradually switched over to an artificial freshwater recipe to more closely mimic natural conditions from the previous recipe that was circulating in the system from a previous experiment.

Closed Respirometry

Once the crayfish had acclimated to the laboratory conditions, closed respirometry was used to determine the respiratory rate of the crayfish. Eight 401 mL chambers were placed in a trough that was filled with 200 L of artificial freshwater held at 21°C. Each chamber was connected to two interchangeable pumps that circulated water through the chambers either as flow-through or a closed system. Each chamber had a fiber optic sensor to measure DO in water circulating through one of the closed-system tubes. This allowed for accurate DO measurements while conducting closed respirometry without risking the damage to the sensor from a curious crayfish. Each of the sensors is linked to one of two Witrox units that log the DO measurements from the O_2 sensors (Loligo ® Systems). The Witrox units then interface with the AutoResp software program. The AutoResp software also allows for the remote control of the pumps (Loligo ® Systems) (Figures 10 and 11).

Crayfish were run in batches of 7; the 8^{th} chamber was left empty and used as a control to determine the background microbial respiration occurring in the chambers. This background MO_2 was later used as a correction factor when determining the MO_2 of the crayfish. The crayfish were chosen for each run using a random number generator and were only run once.

Before a run, the chosen crayfish were removed from their tanks, weighed, and assigned to a chamber. Then, they were given a 24-hour acclimation period. For the first 12 hours, the crayfish were placed in mesh sided cups and allowed to acclimate to the trough water and release any stored gas bubbles trapped in their

gill chamber from when they were transported from their holding tanks to the respirometry trough. The gas release phase was especially important to prevent the crayfish from releasing air bubbles into the chambers. After 12 hours, the crayfish were transferred to their assigned respirometry chambers. The chambers were left open with water actively flowing through the chambers and the crayfish were allowed to acclimate to the chambers for the remaining 12 hours. The 24-hour system acclimation period also assured a 24-hour starvation period for the crayfish. After 24 hours, the chambers were closed and the measurement period began. All runs were performed at night. The crayfish were left in the closed chambers until the DO dropped below 0.5 mg/L. After the run, each crayfish was sacrificed. The chambers and tubing were bleached after each run and the trough water was changed after every 2 runs.

Respiratory Curve Calculations

To correct MO_2 estimates for background oxygen demand, the observed MO_2 was multiplied by the crayfish's mass in order to convert from mgO_2/grams Wet Weight/hour to mgO_2/chamber/hour. The background MO_2 (mg O_2/chamber/hr) taken from the control chamber with no crayfish, was then subtracted. Then, this number was divided by the crayfish's mass to convert back to a per gram basis.

$$\frac{((mgO_2/g/hr * mass)\text{-background } MO_2)}{mass}$$

After the curves were corrected for background oxygen demand, they were evaluated for their suitability to be used in RI score calculation. Individuals whose curves were similar to a hyperbolic or exponential curve with no significant spikes were deemed suitable for RI Score calculation. Individuals whose curves were irregular in shape or had significant spikes or drops in MO_2 did not have an RI score calculated.

RI Score Calculation

DO was plotted against MO_2 in SigmaPlot (Systat Software) and fitted with a model that best explained observed variation to generate an Observed curve. Typically this curve was an exponential or hyperbolic curve, but a few of the individuals were fitted with linear curves. Then, the area under the Observed curve (AUC) was calculated using the AUC function in SigmaPlot. To generate a Regulation curve, the first and last points of the observed curve were plotted and fitted with a linear model. The area under the Regulation curve was calculated using the same method as the AUC for the Observed Curve. Finally, the Conformation curve was plotted using the highest and lowest DO and MO_2 values from the Observed curve and fitted with a linear curve. The area below the Conformation curve was calculated using the same method as the AUCs for the Observed and Regulation curves.

The RI score was calculated using the following formula:

$$\frac{\text{(Observed AUC - Conformation AUC)}}{\text{(Regulation AUC - Conformation AUC)}}$$

Respiratory Strategy

After determining the RI score, each individual was assigned to a group based upon which strategy their RI score represented. Any individual with an RI score greater than 0.5 was placed in the Regulator (R) group. Any individual with an RI score of less than 0.5 was placed in the Conformer (C) group. The individuals that generated curves that could not be used for RI score calculation were denoted with a – and placed in the group NA.

DO_{crit} Calculation

DO_{crit} was calculated in Sigmaplot (Systat Software) using the method developed by Mueller and Syemour (2011). First, the conformation curve was plotted using the data from the RI score calculations and fitted with a linear curve with DO on the x-axis and MO_2 on the y-axis. Then, the intercept (y_o) and slope (a) from the linear curve equation were used to calculate a new curve using the formula $f=y_o+ax$. Then, the difference between the calculated f and the MO_2 from the linear curve was calculated using the formula $z=MO_2- f$. The DO associated with the highest value of z was the DO_{crit}.

Gill Surface Area Calculation

To calculate the total surface of the gill filaments, the carapace of each individual was removed to expose the gill chambers. The surface area of one filament was calculated using the formula for the surface area of a cylinder,

$$a=2\pi r^2+2\pi rh$$

where r is the radius of the filament and h is height of the filament. The surface area of the single filament was then multiplied by the total number of filaments in the gill chamber to calculate the total surface area (TSA) available for gas exchange.

Molt Stage Determination

The molt stage of each individual was determined from examination of a tail clipping taken just before the crayfish was sacrificed. Each clip was placed under a microscope and compared to images of individuals in the intermolt stage, premolt stage, molt stage, and postmolt stage from studies by Burton and Mitchell (1967) and Amer et al. (2015).

Geometric Morphometrics

Digitization

Specimens were positioned dorsoventrally next to a scale bar and photographed using a Canon eOS Rebel t5i digital camera with a 8-48mm lens at 30 cm. The photographs were then digitized with tpsDig (Rohlf, 2017). 23 landmarks were placed on each image, with 17 fixed landmarks and 6 sliding landmarks. The fixed points marked homologous structures on the dorsal side of the carapace and remain static during analysis, while the sliding points are used to capture the curvature of the carapace. The 17 fixed landmarks were the tip of the rostrum, the marginal spines, the post-orbital processes, the insertion point of the

scapes, the cervical spines, the insertion points of the abdomen, and four points to capture the length and width of the areola (Figure 12). Two of the six sliding landmarks were placed between the insertion of the scape and the cervical spines to help estimate curvature of the anterior portion of the carapace. The other four were placed between the cervical spines and the insertion points of the abdomen, two on each side, to capture the curvature of the posterior portion of the carapace (Figure 12).

Once all of the landmarks had been digitized a tps file was created containing the coordinates of each point using the tpsUtil program (Rohlf, 2013a). Landmark coordinates were aligned using Procrustes superimposition to account for differences in size and rotation of the specimens. Consensus, partial warps, and relative warp analysis were performed on the aligned coordinates to generate a set of Relative warp scores using tpsrelw (Rohlf 2013a). Shape data was analyzed using MorphoJ, a program created to perform multivariate analysis of landmark data and visualize any changes in shape (Klingenberg, 2011).

Analysis

The coordinates from the tps file were used to create a dataset within MorphoJ. Data concerning sex of the individual, mass of the individual, calculated RI score, respiratory strategy group based upon the RI score, length of the carapace, length of the areola, width of the areola, which stage of the molt cycle they were in during the tests, and the total surface area of the gill filaments were added to the dataset for further analysis. After aligning the coordinates, a

covariance matrix was generated and used to perform Canonical Variants Analysis (CVA) of the shape data. CVA allows for the detection of differences in shape between *a priori* groups. For my analyses, the groupings were based upon the sex of the individuals, male or female, the molt stage of the individual, intermolt or premolt, and the general RI strategy, regulator, conformer, or NA.

To determine if there were any significant relationships between the measured variables and the RI score, general linear models (GLM) were generated in R using the nlme statistical package (Pinhiero et al. 2018). A global model containing data on the sex, mass, RI Score, DO_{crit}, molt stage, and total surface area of the gills was run to determine if there were any significant relationships between the RI score and the other variables.

Results

The average mass of the crayfish was 22.4 g (SE= ±7.18 g). The average carapace length was 3.9 cm (±0.46 cm). The average total surface area of the gill filaments was 583.71 mm2 (±130.66 mm^2). The average RI score was 0.54, which falls slightly on the regulatory side, but ranged from0 to 0.94. Two molt stages were represented, with 13 individuals being in the intermolt phase during the experiment and 19 individuals being in the premolt stage. There were 19 females and 13 males (Table 6).

RI scores did not differ significantly between males and females (p=0.659, F=0.1999, r^2=0.008) (Table 7). There were also no shape differences between

sexes. The CVA of the sex groupings reported no significant differences in shape between males and females (p=0.96) (Figure 13).

Individuals in the intermolt stage had lower RI scores than individuals in the premolt stage (p=0.05, F=4.248, r^2=0.15) (Figure 14 and Table 7). The CVA of the molt stage groupings suggests significant differences between the groups (p=0.009) with all of the variation explained by the first canonical variate (Table 8). Visual analysis of the frequency graph showed little overlap between the groupings. The wireframe revealed that shape differences between the groups were found in a slight lengthening of the carapace and changes in the areola length and width (Figure 15).

Comparison of the shapes between the RI strategy groups revealed significant differences between the groups (Figure 16). CV1 explained 85.8% of the variation. CV2 explained the remaining 14.2% for a total of 100% of the variance explained (Table 8). CV 1 showed a slight lengthening of the carapace and areola while CV 2 displayed more extensive changes, including an overall narrowing and lengthening of the carapace, and a slight narrowing and lengthening of the areola and a forward shifting of the post-orbital spines (Figure 16). The R and C groups clustered close together and the NA group was separated from the other two groups on the CV 1 axis. On the CV 2 axis, the major separation was between the R and C groups.

Linear Modeling

Only the TSA of the gill filaments ($p=0.0032$, $F=10.73$, $r^2=0.309$, d.f.=24) and the mass of the crayfish ($p=0.00786$, $F=8.41$, $r^2=0.2595$, d.f.=24) were significantly correlated with the RI score (Table 9). As the TSA of the gill filaments increased, the RI score increased as well, indicating that individuals who utilized regulatory strategies also had more surface area available for gas exchange (Figure 14). The RI score also increased with the mass of the crayfish and the carapace length, indicating that the larger the crayfish, the stronger it's regulatory ability.

Similar to the RI score, only the relationships between DO_{crit} and TSA of the gill filaments ($p=0.0117$, $F=7.86$, $r^2=0.3039$, d.f.=18) and DO_{crit} and mass ($p=0.0145$, $F=7.317$, $r^2=0.289$, d.f.=18) were significant (Table 9). As the TSA increased, the DO_{crit} decreased, indicating that having more gill surface area lowered the individual's DO_{crit} (Figure 17). Increasing the mass of the crayfish also decreased the DO_{crit}, meaning that larger crayfish tend to have lower DO_{crit} (Figure 17).

Discussion

I observed a significant difference in morphology of the carapace between respiratory strategies. Crayfish that displayed conformer respiratory strategies tended to have more fusiform body shapes. Conversely, crayfish with regulator strategies displayed the wider, shorter carapace. With this increase in width came an increase in gill filament TSA. Increases in the TSA of the gill filaments

lowered the DO_{crit} of the crayfish. The increase in the overall width of the carapace probably provided more space in the gill chambers, which provided room for more gill filaments. Increasing the number of gill filaments in turn increased the total surface area of the gills, meaning that there is more area available for gas exchange. Being able to absorb more oxygen, even at low levels of DO, would allow a crayfish to continue to utilize a regulator strategy by lowering the DO_{crit}. I did not see significant variation in the relative areola width in the CVA with differences in RI Score. This may be due to the fact that all of the individuals tested in this experiment came from a lotic habitat, or that I simply did not have enough individuals to detect a difference. Areola variation may be constrained by factors other than the DO levels, such as overall size of the crayfish (Haddaway et al. 2012).

Lentic habitats often have significant fluctuations in levels of DO, making the capture of as much oxygen while it is available very important. Interestingly, it has been noted that many crayfish invasions often begin in a lentic habitat, such as a lake or reservoir, and then spread into streams and river, but rarely do species move from lotic habitats into lentic habitats (Light 2003, Kerby et al. 2005). The crayfish with less regulatory ability may be less capable of coping with the fluctuations in DO levels in lakes and reservoirs may prevent them from establishing populations. On the other hand, the regulator strategies work well regardless of the ambient DO levels. I also saw an increase in RI score with an increase in mass and carapace length, meaning that larger crayfish had a stronger ability to regulate their respiration compared to smaller individuals. *F. virilis*

collected from lake sites are, on average, larger than individuals of the same species collected from streams (Perry et al. 2011).

I also saw a significant difference in both shape and RI scores between the two molt stages. The shape change was very slight, and may be attributed to the actual process of molting itself. The construction of the exoskeleton begins during the premolt stage, so the slight expansion of the carapace may be attributed to the new exoskeleton being formed (Burton and Mitchell 1987). The crayfish that had entered the premolt stage tended to display regulatory strategies, while crayfish in the intermolt stage displayed more conformer strategies. This observation is somewhat surprising as the entire molting process is energetically demanding, and an increase in energetic demands is often accompanied by an increased demand for oxygen (Chang 1995). However, switching to a regulatory strategy also has its advantages. It allows for consistent respiration in the face of fluctuating DO levels that could potentially hamper the molting process and leave the crayfish in a vulnerable state.

Conclusions and Implications

When considered together, the results from Chapter 1 and Chapter 2 reveal an interesting link between morphology and physiology. Crayfish that utilized regulatory strategies showed the same trends in external morphology as the individuals that were collected in lentic habitats, namely the wider, shorter bodies. The crayfish from the respirometry experiments that utilized the conformer strategies, on the other hand, displayed the same fusiform body shape as the

individuals from the morphology study that were collected from lotic habitat. Considering that lentic habitats generally have greater fluctuations in DO level compared to lotic habitats, it seems that the morphology of the crayfish allows it to best utilize the available DO. The wider body of the lentic habitat crayfish could allow for more room for gill filaments and increases the overall surface area available for gas exchange. This allows the crayfish that live in fluctuating DO environments to maintain a certain level of respiration. Lotic habitats tend to have more constant DO levels, so the crayfish do not need as much surface area in the gills to gather the oxygen they need. These crayfish may trade some of their regulatory ability for a more hydrodynamic body shape, allowing them to better navigate the higher water velocities found in lotic habitats, but further studies need to be done. It has also been noted that crayfish invading from lentic habitats into lotic habitats have greater success at establishing populations (Light 2003). The lower oxygen levels in the water of lentic habitats could hinder the crayfish with lower RI scores, as their gill chambers have less room for gill filaments and thus have more difficulty gathering enough oxygen to produce sufficient energy for survival. The crayfish from with higher RI scores do not seem to encounter these problems, as their gill chambers have enough surface area to absorb the oxygen they need to maintain their required level of respiration.

Being phenotypically plastic might also have implications for survival in novel habitats. Having multiple morphological strategies increases the odds that at least a few of the individuals that entered a new habitat or area could survive long enough to reproduce and being to establish a stable population. Plasticity in

respiratory strategy has been shown to be important in successful fish invaders, with air breathers and those tolerant of low DO being among the most successful invaders (Moyle and Marchetti 2006). Respiratory variation and its effects on physiology is likely also important in making *F. virilis* a successful invader, and I predict that similar plasticity will be found in other successful crayfish invaders. High levels of variation in morphology could predict the success of invasive crayfish species before they have a chance to spread into naïve ecosystems and cause significant damage.

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Figures

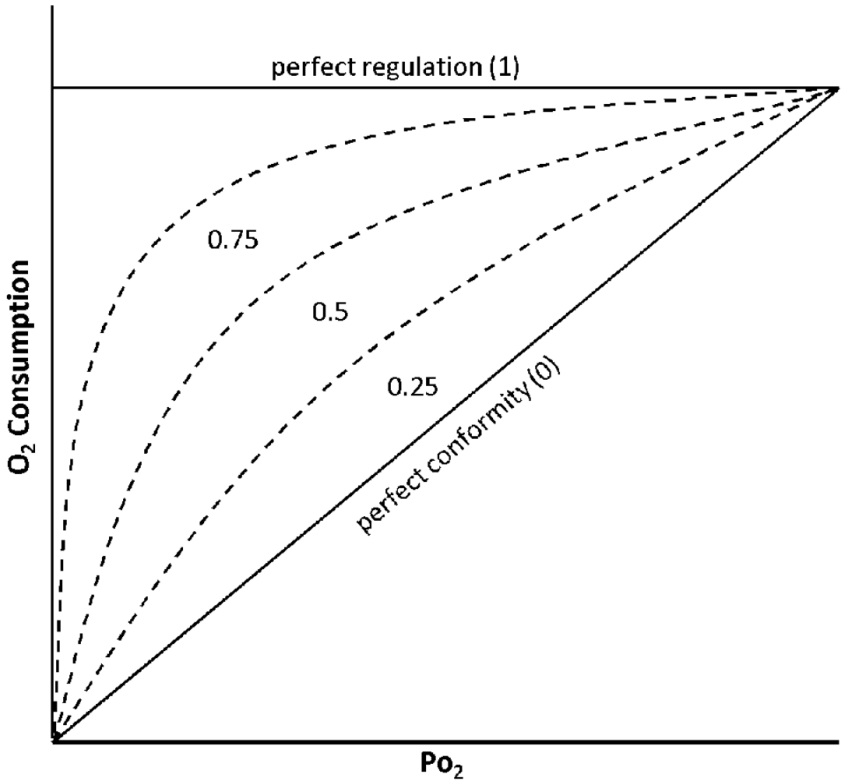

Figure 7: RI Score example graph. RI calculations assign a score ranging from 0 to 1, with 0 representing a curve of perfect confirmation and 1 representing a curve of perfect regulation. The higher the RI score, the more of a regulatory strategy is displayed by the organism. Adapted from Mueller and Seymour 2011.

Figure 8: Collection site along Cahaba River at Masonic Park, outside of Trussville AL (33o37’39” N, 86o36’9” W). All specimens were collected using minnow traps baited with canned cat food.

Figure 9: *Faxonius virilis* individuals in their tanks. Each individual was assigned a tank number and provided a length of PVC piping for shelter.

Figure 10: Crayfish reacting to flushing of chambers after the end of a closed run.

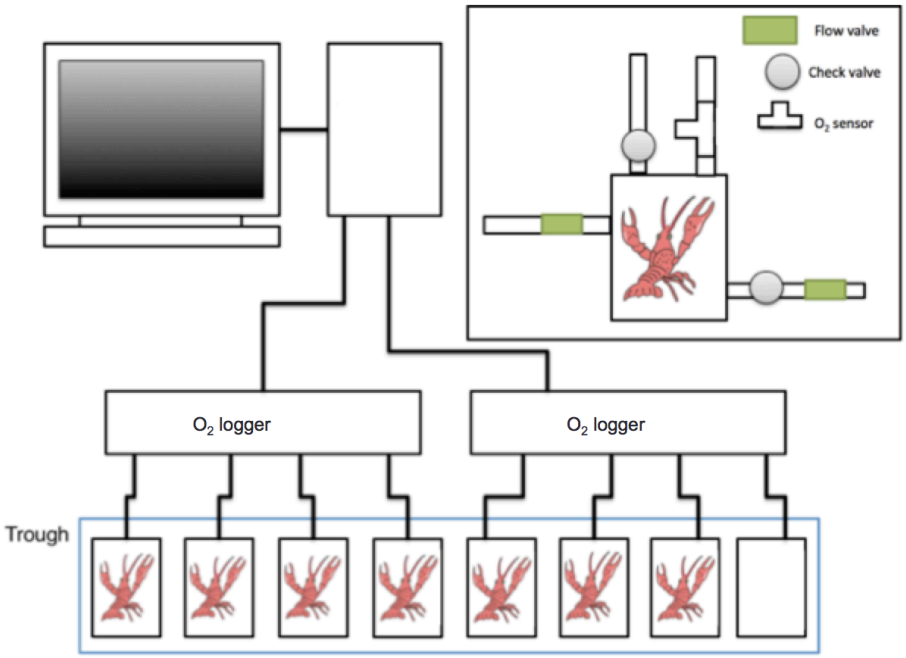

Figure 11: Diagram of respirometry equipment set-up. 8 chambers are set in a trough filled with ~200L of water. Each chamber is connected to two pumps, one that pumps water into the chamber and one that pumps water out. Each chamber has a fiber optic sensor in one of the tubes leading to the outtake pump. This allows for accurate DO measurements without risking the damage to the sensor from a curious crayfish. Each of the sensors is linked to one of two Witrox boxes that collect DO measures. The Witrox boxes then interface with the AutoResp software, which sends the readings back to the computer. The AutoResp software also allows for the remote control of the pumps.

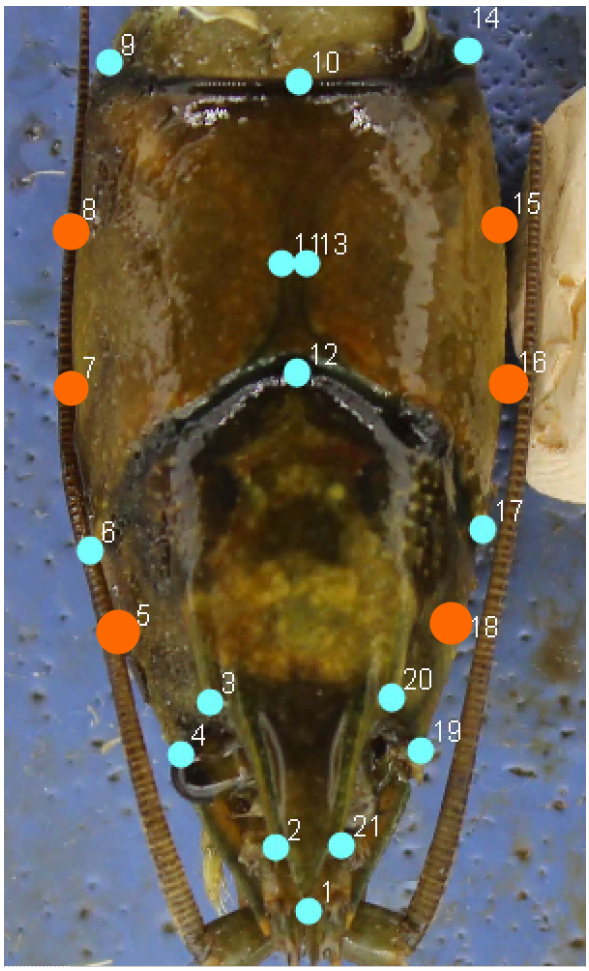

Figure 12: Digitized carapace with fixed and sliding landmarks. Landmarks in blue are fixed. Landmarks in orange are sliding points. Fixed landmarks: 1-Rostrum, 2 and 21-Marginal Spines, 3 and 20-Post-orbital spines, 4 and 19-Scape insertion points, 6 and 17- Cervical spines, 9 and 14- End of carapace, 10 and 12-Areola length, 11 and 13- Areola width. Sliding landmarks: 5, 7, 8, 15, 16, and 18.

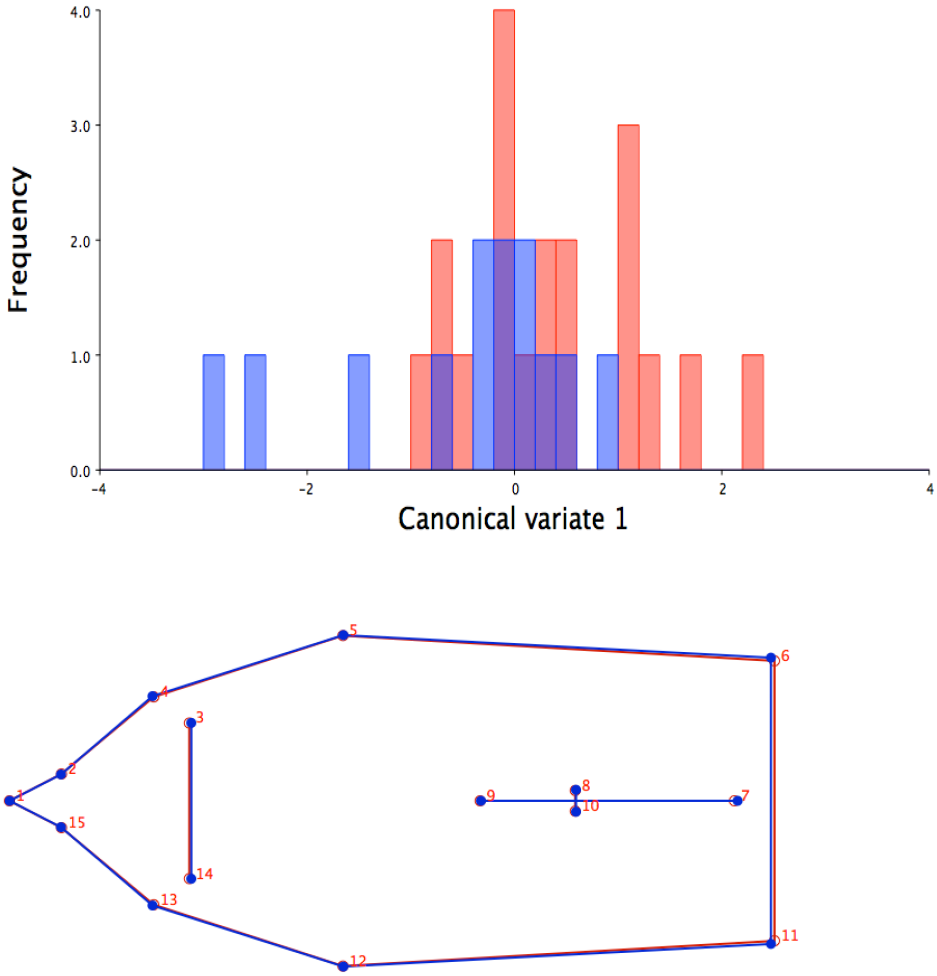

Figure 13: Canonical variate analysis of shape based on sex, with females in red and males in blue

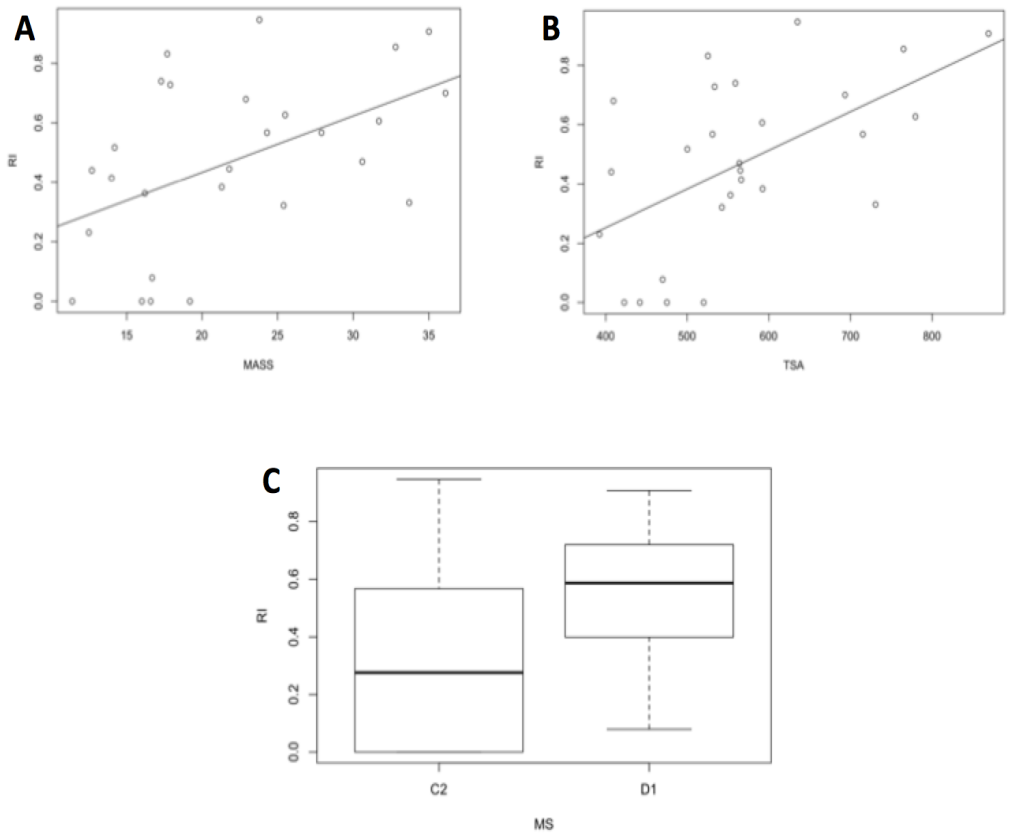

Figure 14: Graphs of significant linear relationships between RI score (y-axis for all graphs) and non-morphometric measures. Graph A displays the relationship between Mass (in grams) and RI score. Graph B is the relationship between Total Surface Area (mm^2) of the gill filaments and RI score. Graph C is the comparison of RI scores between the two molt groups, with C2 being the Intermolt stage and D1 being the Premolt stage.

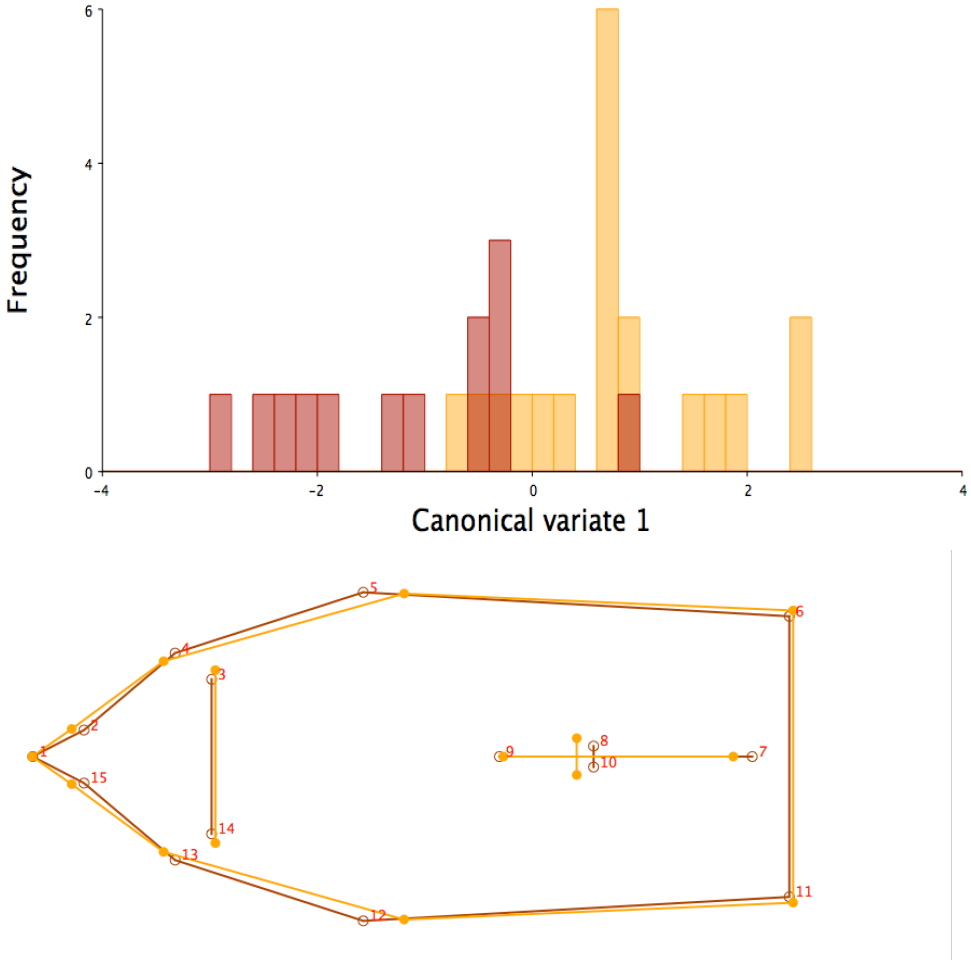

Figure 15: Canonical variate analysis of shape differences between molt stages, with Premolt (D1) in gold and Intermolt (C2) in red.

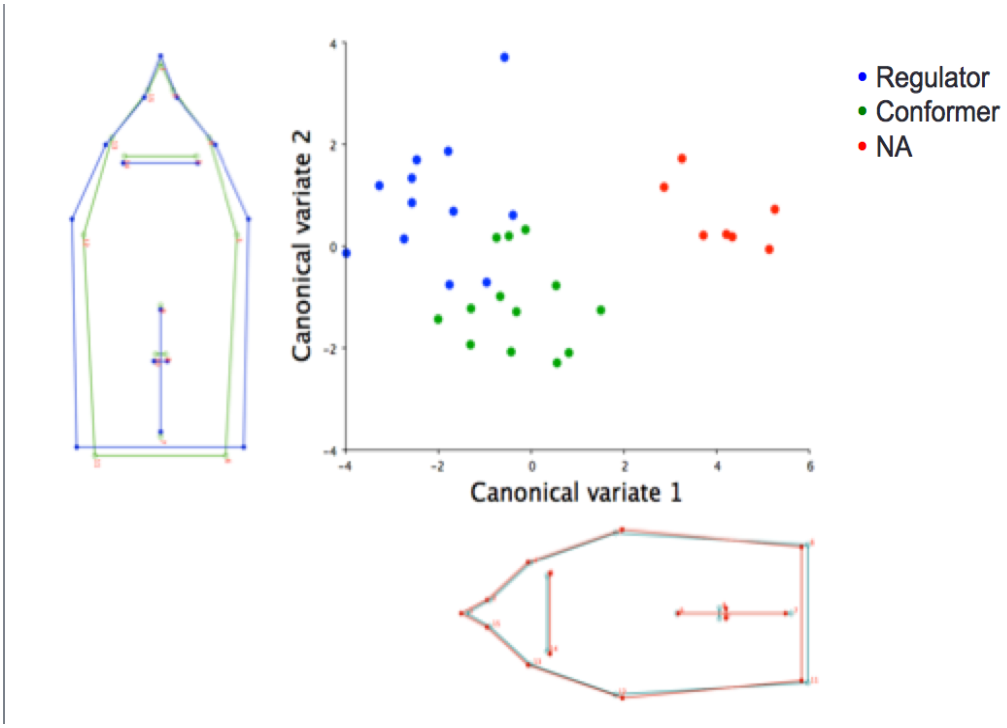

Figure 16: Canonical Variate Analysis using RI Strategy groupings. The blue points represent the Regulator group, the green points represent the Conformer group, and the red points represent the NA group.

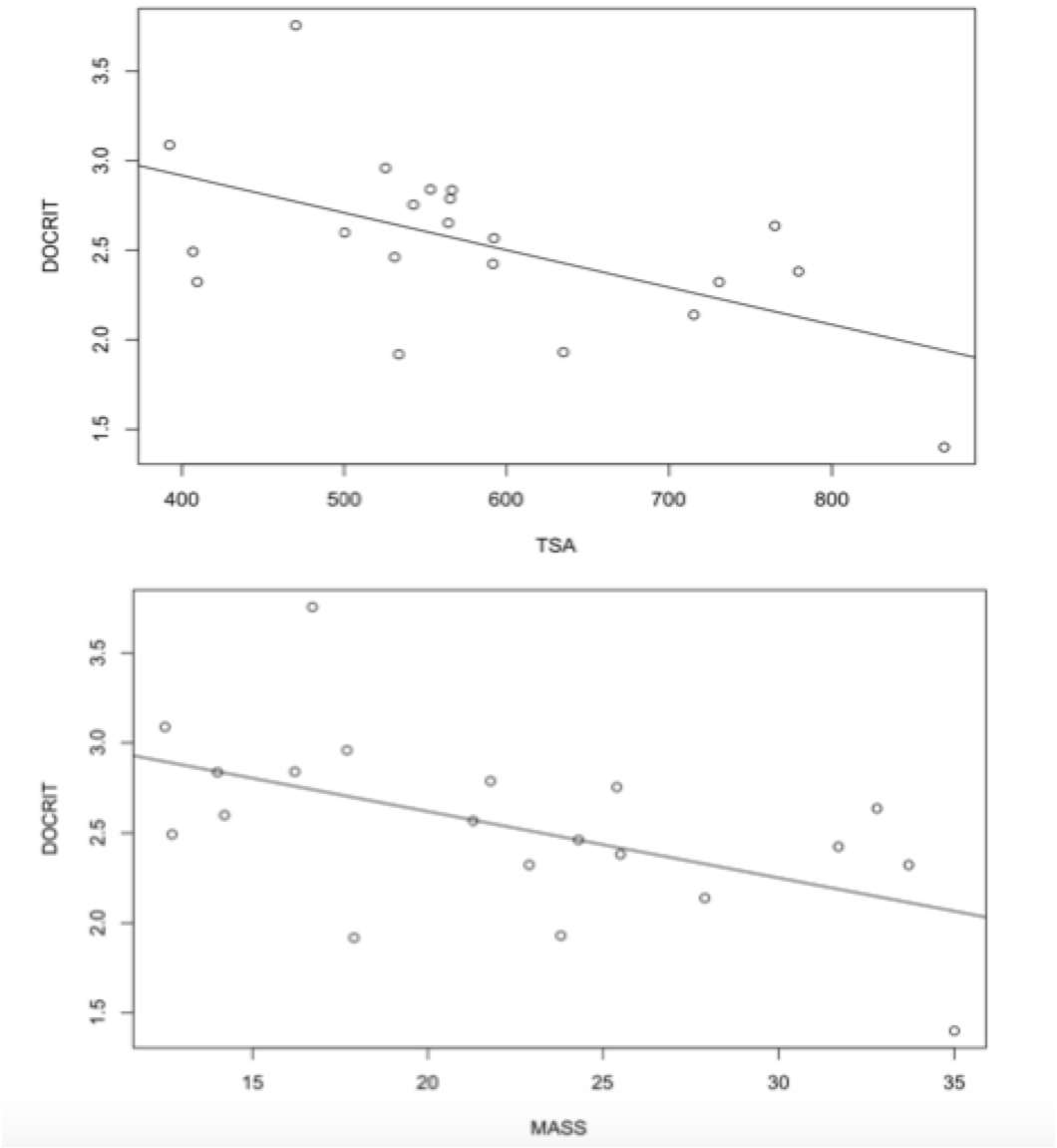

Figure 17: Graphs of significant linear relationships between DO_{crit}(y-axis for all graphs) and non-morphometric measures. Graph A displays the relationship between Total Surface Area (mm^2) of the gill filaments and the DOcrit. Graph B is the relationship between Mass (in grams) and DOcrit.

Tables

Table 6: Data summary

Number of Individuals	n=36
Number per sex	M=13, F=19
Usable Resp. Curves	n=26
Molt Stage	Intermolt=13, Premolt=19
Average Mass	22.4 g
Average Carapace Length	3.9 cm

Table 7: GLM models with RI as response variable for all characters. Full model was glm(RI ~ TSA + MASS + SEX + MS). Bold values are significant.

Variable	Std Err	t	p	R^2	F	df
TSA	0.0003975	3.276	**0.0032**	0.309	10.73	24
MS	0.11136	2.061	**0.05**	0.1504	4.248	24
SEX	0.12682	0.447	0.659	0.008258	0.1999	24
MASS	0.006923	2.9	**0.00786**	0.2595	8.41	24

Table 8: Summary of variance explained each canonical variate and associated eigenvalues for each grouping scheme

Grouping	CV	Variance Explained	Eigenvalue
Sex	CV1	100%	0.334
Molt Stage	CV1	100%	3.37
Strategy	CV1	85.831%	5.88
	CV2	14.169%	0.972

Table 9: GLM model with DOcrit as the response variable for all characters. Full model was glm(DOCRIT ~ TSA + MASS + SEX + MS). Bold values are significant.

Variable	Std Err	t	p	R^2	F	df
TSA	0.0007413	-2.804	**0.0117**	0.3039	7.86	18
MS	0.2432	0.739	0.469	0.02947	0.5467	18
SEX	0.2326	0.85	0.407	0.03857	0.7222	18
MASS	0.01363	-2.705	**0.0145**	0.289	7.317	18