

Fall 12-7-2018

# Reproductive Life History and Shell Shape Variation of Three Species of *Elimia* (Pleuroceridae) in the Lower Etowah River Basin

Ellen Winant

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Reproductive life history and shell shape variation of three species of  
*Elimia* (Pleuroceridae) in the Lower Etowah River Basin.

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A Thesis Presented in Partial Fulfillment of Requirements for the Master of Science in  
Integrative Biology for the Department of Evolution, Ecology, and Organismal Biology

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December 2018

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## ABBREVIATIONS

CDS	Connesena Creek, downstream site
CTS	Connesena Creek, tributary site
CUS	Connesena Creek, upstream site
CVA	Canonical Variates Analysis
DS	Downstream site
ECC	<i>Elimia carinocostata</i>
ECF	<i>Elimia carinifera</i>
EMD	<i>Elimia modesta</i>
GM	Geometric Morphometrics
PCA	Principal Component Analysis
SDS	Stamp Creek, downstream site
STS	Stamp Creek, tributary site
SUS	Stamp Creek, upstream site
TDS	Two Run Creek, downstream site
TPS	Thin-Plate Spline
TS	Tributary site
TTS	Two Run Creek, tributary site
TUS	Two Run Creek, upstream site
US	Upstream site

## ABSTRACT

Pleurocerid snail diversity in the Southeastern United States is thought to be high, but estimates of overall diversity are compromised by uncertain taxonomy. This is particularly true for the genus *Elimia*, where reliance on conchological characters described by early naturalists for species designation failed to recognize significant variability in shell morphology. In this study we examine three putative species of *Elimia* to determine if quantifiable conchological characters are associated with differences in life history characteristics. Individuals of *E. modesta*, *E. carinocostata*, and *E. carinifera* were collected from nine sites in three streams in the lower Etowah River Basin. Females were separated by species and placed in mono-specific tanks where egg deposition could be monitored. Shell shape was assessed using Geometric Morphometrics. Although there was some overlap, shell shapes differ significantly among the three forms. Shell sculpture differences appear early in juvenile forms and the three species are distinguishable by the establishment of the fourth whorl. Differences in egg oviposition among species were observed in the lab and in substrate samples obtained from the field. Our results suggest that there are at least three distinct species of *Elimia* occurring in lower Etowah River Basin tributaries.

## CHAPTER 1: Literature Review of *Elimia* in the Lower Etowah River Basin.

Pleuroceridae (Cerithioidea) is a family of freshwater gastropods that currently includes 7 genera and 162 species. Species in this family are characterized as gill breathing, operculate freshwater snails with a spired shell. They are sexually dimorphic; males are aphyllid and the females have an egg ovipositor (sinus) on the right side of the foot (Burch 1989). Pleuroceridae diversity is highest in the Southeastern United States (Johnson *et al.* 2013), particularly in the Mobile River Drainage Basin. They are important components of nutrient cycling in freshwater ecosystems and are often the most abundant macro-invertebrates present in these systems (Newbold *et al.* 1983, Richardson *et al.* 1988). Localized densities can exceed 500 snails/m<sup>2</sup> (Dazo 1965, Stock *et al.* 1987, Richardson *et al.* 1988). *Elimia* (H. & A. Adams 1854) is the second most speciose genus of Pleuroceridae (Johnson *et al.* 2013) and has been described as having 86 species and an additional 41 “morphs or forms” (Burch 1989). *Elimia* are iteroparous, and some are estimated to have lifespans of 6 years or more (Huryn *et al.* 1994, Richardson and Scheiring 1994).

Much confusion has surrounded the taxonomy and nomenclature of Pleuroceridae. Inconsistent use of names, uses of junior synonyms, ambiguous descriptive terminology, poor or lacking illustrations, and vague type localities have all contributed to difficulties in identification (Burch 1989, Burch 2001, Perez and Minton 2008). Recent examples include the use of the junior synonym for *Elimia*, *Goniobasis*, in the Ecology of Freshwater Mollusks (Dillon 2000), among other works (Dillon and Frankis 2004, Dillon *et al.* 2009), and the suggestion that *Elimia* be subsumed under *Pleurocera* based on a limited study of 15 populations using allozymes (Dillon 2011). Observations of diverse shell morphologies by early naturalist (Lea 1834, 1845,

1853, 1862, Say 1858) prompted the descriptions of many new species. Attempts were made by others to synonymize the information (Tryon 1863, 1864, 1873, Hannibal 1912, Goodrich 1934, 1935, 1936, 1941, 1944, Burch and Tottenham 1980). Graf (2001) published an inventory of the almost 1000 nominal names associated with 152 species of Pleuroceridae in North America, North of Mexico.

Identification based on shell characteristics alone is particularly problematic because shells can vary depending on several factors including, but not limited to, interspecific phenotypic variation (Whelan *et al.* 2012a), river position (Minton *et al.* 2008), and predation intensity (Trussell 1996). *Elimia potosiensis* and *Elimia livescens* exhibited variation in shell length and aperture width in relation to river position (Minton *et al.* 2011, Dunithan *et al.* 2012), where shells in the areas upstream have less sculpturing on the posterior portion of the body whorl, a narrower body whorl, and a smaller aperture. *Lithasia* in the Duck River, TN, is another example of the variation seen in Pleuroceridae where general shape and sculpturing differed in an upstream to downstream orientation. In the upper reaches, shells are more smooth sided, more narrow, and have a smaller aperture, but in the lower reaches shells have a more robust and sculptured body whorl and larger apertures (Minton *et al.* 2008, Minton *et al.* 2011).

Shell characteristics can also differ between juvenile and adult forms. Some species of Pleuroceridae have similar adult shells but distinct juvenile shell characteristics which are often lost due to apical erosion (Thompson 2000). The reverse has also been seen where juveniles from two closely related species of *Leptoxis* were very similar but differentiation was apparent in the adult shells (Whelan *et al.* 2014). Similarities in egg laying behaviors, mitochondrial DNA, and soft body pigmentation resulted in the conclusion that the two forms are one species.



Life history studies of *Elimia* have shown similar patterns among species. They have similar growth periods, population age structures, and reproductive periods where oviposition occurs in spring through early summer (Huryn *et al.* 1994, Huryn *et al.* 1995). Growth and production are highest and consistent throughout the year in higher alkalinity limestone steams where the close ground water connection moderates winter temperatures (Huryn *et al.* 1995). Larger size adult snails show a higher preference for slower current velocities and sunny habitats. Juvenile have been found in greater densities in higher velocity habitats (Johnson and Brown 1997).

Unfortunately, taxonomic boundaries within Pleuroceridae and *Elimia* remain unresolved. Molecular analysis using COI, 16S rRNA, H3 nuclear genes, and ISSRs (Lydeard *et al.* 1998, Whelan and Strong 2016, Minton *et al.* 2017) have not been definitive in delineating species. Studies that include timing of reproduction events and type of oviposition may provide critical information for any future taxonomic efforts. Recent studies of *Leptoxis* (Pleuroceridae) have demonstrated species-specific differences in egg laying behaviors, juvenile shell development and information on density and flow regimes necessary for recruitment events (Whelan *et al.* 2014). In that study, an examination of life history traits for the 13 extant species of the genus *Leptoxis* genus found differences in egg laying behaviors (single, line, and clutch) and temperature cues for the onset of oviposition. Captive propagation showed juveniles developed the same shell characteristics as seen in the adult wild-caught forms, indicated shell morphology to be under genetic control, and showed that morphologic differences were not a result of eco-phenotypic plasticity.

Comprehensive studies which include morphology and life history can provide additional important details to clarify taxonomic delineations. Dazo (1965) compared the life histories of

*Elimia livenscens* and *Pleurocera acuta*, considered sister taxa within Pleuroceridae. He reported shell morphology was not always distinct between the species, and while internal anatomy was very similar, egg-laying characteristics did differ in number, arrangement, and length of time laid each season. Dazo concluded there were enough similarities in anatomy and life history that their separation into two separate genera may need to be reconsidered. In contrast, Strong (2005) described the same two species as in Dazo (1965) adding detailed anatomical comparisons of the reproductive structures and descriptions of the midgut and kidney structures. The findings revealed important structural differences at the genus level, supporting the current phylogeny which separates these two genera.

The objective of this study is to examine life history characteristics and shell morphology of three putative species of *Elimia* in three tributaries of the Lower Etowah River Basin. I will address three questions: (1) Are there differences in timing or oviposition method between species, (2) What are the juvenile shell characteristics and do they differ from the adults, and (3) Are intra- or interspecific differences in shell shape statistically significant. Timing and method of oviposition will be assessed by comparing field samples, temperature data and laboratory observations. Juvenile shell development will be assessed by examining field collections and lab reared young. Geometric Morphometrics will be used to analyze intra- and interspecific variation in shell shape.

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## CHAPTER 2: Life history of *Elimia* in the lower Etowah River Basin.

### INTRODUCTION

*Elimia* diversity is highest in the Southeastern United States, and the genus has experienced more extinctions than any other group in Pleuroceridae (Brown *et al.* 2008 and Johnson *et al.* 2013). Even though there are 86 currently recognized species of *Elimia* (Burch 1989 and Johnson *et al.* 2013), life history studies are only available for a few species (Dazo 1965, Huryn *et al.* 1994, Richardson and Scheiring 1994, Richardson *et al.* 1988, Huyrn 1997, Johnson and Brown 1997, Cross and Benke 2002, Dillon 2011, Dunithan *et al.* 2012). The majority of studies available for *Elimia spp.* focus on size, growth, production, competition, or habitat preferences, but few studies are available that include reproductive characteristics (Dazo 1965, Huryn *et al.* 1994, Morrison 1954, Winsor 1933). The taxonomy of *Elimia* remains uncertain, and reproductive life history may aid in distinguishing species where molecular markers have been unsuccessful. Basic identification of *Elimia* species are difficult in part because some species have adult shells that look very similar, and the distinctive, species-specific characteristics only visible on the juvenile whorls are often lost due to apical erosion.

Two recent studies provided important juvenile shell descriptions for 23 species of *Elimia* (Thompson 2000, Milhalcik and Thompson 2002), including *E.carinocostata* and *E.modesta*. Juvenile shells of *E.modesta* are described as having a strong peripheral carina, vertical ribs, and a concave outline while adult shells have an obsolete peripheral angle, no carina, and weakly concave or straight sided whorls. *E.carinocostata* juveniles also have a carinate periphery and strong vertical ribs, but the carinate periphery is lost on adult shells, and the vertical ribs are not as strong. Strong differences in these juvenile characters suggest *E.carinocostata* and *E.modesta*



are distinct species. In addition to juvenile characters, Thompson (2000) also provided associations between juvenile and adult forms.

Identification of *Elimia* shells at the sites in this study are based on Thompson (2000) and are as follows. *E.carinifera* (Lamarck 1822) is characterized as having moderately indented sutures, a peripheral carina and nodules between the periphery and the suture. *E.carinocostata* (Lea 1854) is characterized as having moderately indented sutures, a peripheral carina, vertical costae, nodules on the upper portion of the costae, and where the costae meet the periphery it gives a scalloped appearance from the apical view. *E.modesta* (Lea 1845) has weakly indented sutures, straight sided or slightly rounded whorls, sometimes possessing a slightly shouldered whorl, but no costae, carina, or nodules. The types of *Elimia* present at each site in this study are listed in Table 2.3.

These descriptions are consistent with the forms found at the study sites listed in Table 2.3. Malacologists familiar with southeastern Pleuroceridae examined specimens collected in 2012 from tributary stream sites in the Lower Etowah basin. The identifications they suggested were not always consistent with the identifications I arrived at based on Thompson (2000) and Burch (1989). Additionally, a Bartow County survey (2001) of macro-invertebrates identified *E.c.f.gerhardti* as the only *Elimia* present in the three streams in this study. Novak (2004) identified *E.carinifera* and *E.modesta* in Two Run creek, *E.carinifera*, *E.carinocostata*, and *E.modesta* in Connesena Creek, and *E.modesta* and *E.murrayensis* in Stamp Creek. Given this uncertainty, the objective of the study described in this chapter is to determine if there are life history characteristics, particularly reproductive characteristics, that differ among the three forms described above.

## METHODS

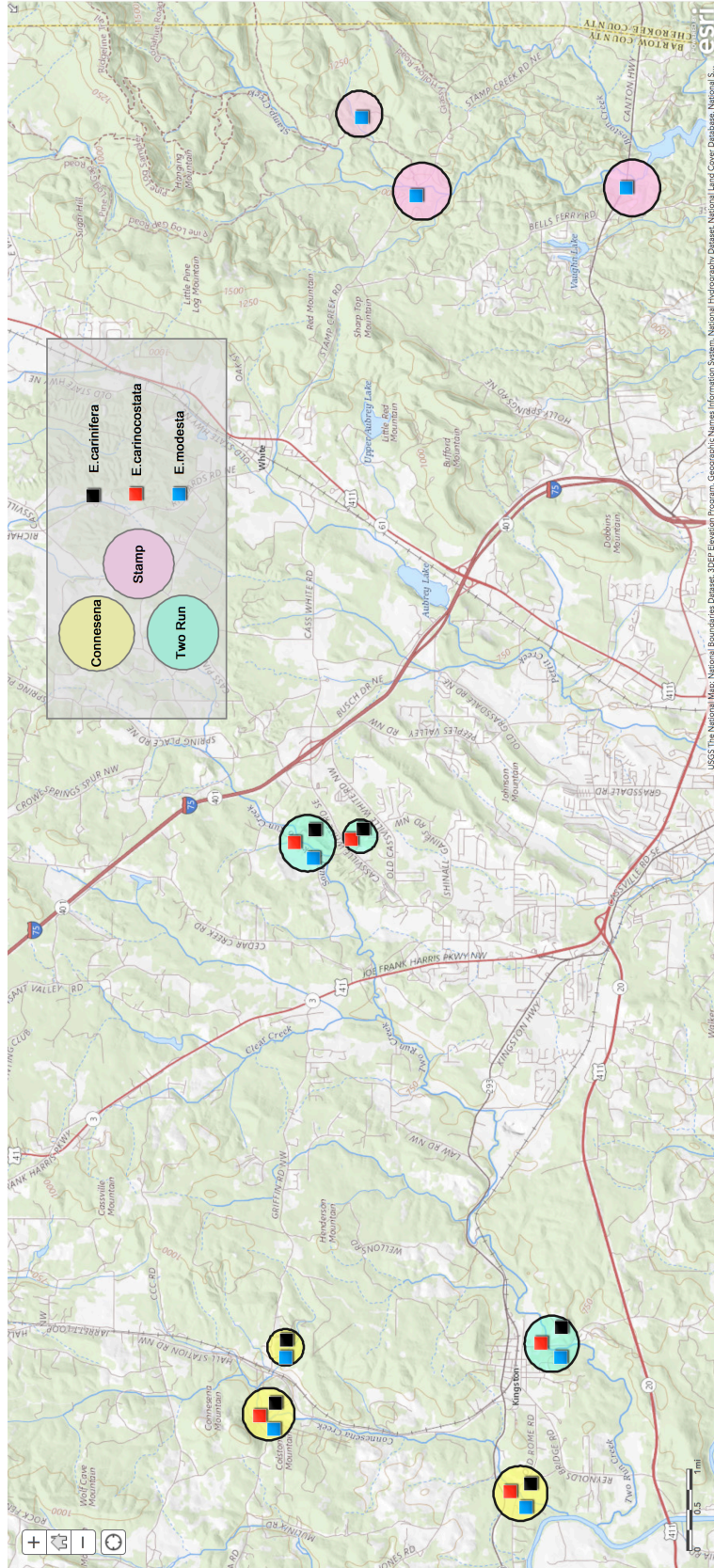
Three streams were selected for this study, and three sites within each were sampled- an upstream site (US), tributary (TS) and downstream site (DS), Figure 2.1. Two Run and Connesena Creeks are in the Valley and Ridge Ecoregion, with the later having a stronger spring influence. Stamp Creek is in the Piedmont Ecoregion. Abbreviations used throughout this thesis to identify the nine sites are as follows: Two Run upstream (TUS), Two Run downstream (TDS), Two Run tributary (TTS), Connesena upstream (CUS), Connesena downstream (CDS), Connesena tributary (CTS), Stamp upstream (SUS), Stamp downstream (SDS), Stamp tributary (STS). Watershed and water quality parameters are listed in Table 2.1 for the upstream and downstream sites in all three streams.

**Table 2.1.** Watershed and water chemistry for the upstream and downstream sites in Two Run, Connesena and Stamp Creeks. NA=not available. (Wikiwatershed and Dirnberger *et al.* 2001)

	TUS	TDS	CUS	CDS	SUS	SDS
Watershed area, km <sup>2</sup>	25	121	21	36	29	47
Stream order	3	4	3	3	3	3
Mean channel slope, %	0.25	.022	0.51	0.41	1.00	0.70
pH	7.9	8.3	NA	8.3	7.7	7.5
Alkalinity, mg/L	118	112	NA	115	48	38

Snails and sediment samples were collected from all nine sites. Three sites were sampled within each stream: an upstream site, a small tributary, and a downstream site. Samples were obtained monthly between March and July 2014, three times between October 2014 and January 2015, and in March and April, 2015. All nine sampling locations were used to study egg-laying behaviors in the field and to provide females for laboratory studies of those same behaviors.

**Figure 2.1.** Nine study sites in three streams in the Lower Etowah River Basin. An upstream, downstream and tributary site were sampled in each stream: Connesena Creek, Two Run Creek, and Stamp Creek.



During field collections, opportunistic sampling occurred with an attempt to obtain individuals and sediment samples from a variety of habitat types. Mineral substrates were inspected in the field for the presence of eggs, and sediment samples were collected using a 100 micron sieve and searched in the laboratory using a Leica Zoom 2000 stereomicroscope. All snail specimens were placed in Whirl-Pak containers with stream water, and the containers were placed in a cooler and transported back to the lab for further processing. Hobo data loggers were installed at each site to record stream temperatures every 4 hours.

Aquaria in the laboratory were used to study species-specific female egg-laying behaviors from TUS, TTS, and TDS. Two Run Creek was selected for the laboratory experiment because all three species were present, and the shells suffered little erosion making identifications of females straightforward.

A closed, flow-through aquaria system with 24 – 1 gallon tanks was established in the laboratory to house females from Two Run Creek sites (Ganser per comm., Kim *et al* 2009). Lights were controlled by a timer to simulate the normal day/night light cycle, and length of daylight was adjusted throughout the study to mimic the natural photoperiod. De-ionized water was used for aquaria water and was adjusted to pH 7.6, GH 9° and KH 11° (Funkhouser 2014) to be similar to the water chemistry of Two Run Creek. The flow rate was maintained at a level that exchanged water in each tank an average of 2.8x per hour. Water from each tank drained by gravity into the sump tank and was mechanically and UV filter sterilized before returning. An Aqua Euro 1 HP chiller was used to regulate water temperature. As with photoperiod, an attempt was made to maintain temperature at a level consistent with ambient temperatures in the streams from which the snails were obtained. Over the course of the study, temperatures in the aquaria ranged from 10°C to 22°C. A 2” square unglazed terracotta tile (Whelan *et al.* 2014) was leaned

against the front side of each tank, underneath the inflow, and a small amount of sand was added to the bottom of each tank.

Females from the Two Run Creek sites were separated by species and placed in mono-specific aquaria to observe egg-laying behaviors, temperature at onset of egg-laying, size of eggs, and length of time from oviposition to hatching. Processing time for each sample was approximately 3 hours using a Leica Zoom 2000 stereomicroscope. Approximately 20-30 snails at a time were placed in a container with stream water. As snails started to emerge from their shell or crawled on other snails, the presence/absence of an ovipositor on the right side of the foot was identified. All snails from all sites not selected for aquaria tanks were preserved in 85% Ethanol, using the method outlined in Fuduka *et al.* (2008).

Sediment samples from all sites were searched for presence/absence of eggs and juveniles for approximately 1 hour using a Leica Zoom 2000. Eggs collected from laboratory aquaria and field sediment samples were isolated in ~100mL flow-through ‘nursery’ isolation chambers or tissue culture dishes to make further observations about hatching and juvenile shell characteristics.

Female snails remained in laboratory aquaria for observations until the next field sampling was scheduled. At that time, females were preserved, eggs were moved to nursery isolation chambers or tissue culture dishes for further observation, and aquaria were cleaned out in preparation to house the next set of females collected.

Laboratory observations included temperature cues for egg laying, type of eggs laid, deposition method, size of eggs, days to hatching, and development of juvenile shell characteristics.

## RESULTS

All three species of snails were present and collected from the upstream and downstream sites of Two Run and Connesena Creeks. *E.carinifera* and *E.carinocostata* were the only two species present at the Two Run tributary site, and *E.carinifera* and *E.modesta* were the only two species present at the Connesena tributary site. *E.modesta* was the only species found at the upstream, tributary, and downstream sites of Stamp Creek.

Over the course of the laboratory egg-laying behavior study, a total of 701 females were isolated from field collections and placed in aquaria (*E.carinifera*=156, *E.carinocostata*=403, *E.modesta*=142) to study egg-laying behavior, Table 2.2.

**Table 2.2.** Total number of females isolated over the course of the study from three sites in Two Run Creek. Females collected from each site were separated by species and placed in mono-specific tanks to study egg-laying behavior.

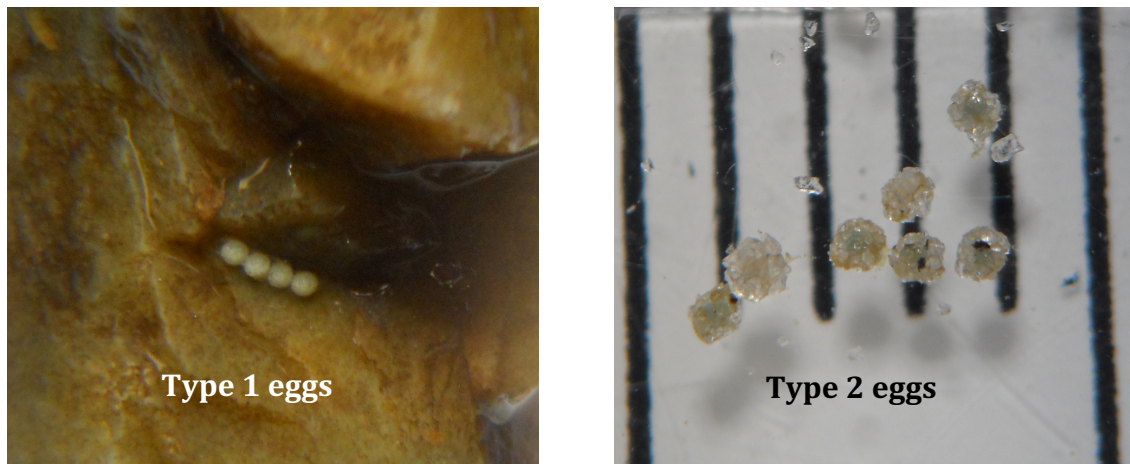
	TUS	TTS	TDS	Total
<i>E.carinifera</i>	45	56	55	156
<i>E.carinocostata</i>	159	109	135	403
<i>E.modesta</i>	92	0	50	142

Two types of egg deposition were observed in laboratory aquaria. *E.carinifera* laid eggs firmly attached to the sides of tanks or, less commonly, on the terracotta tiles (referred to hereafter as “Type 1” eggs). They were cream/white eggs laid singly, or in a line up to 6 eggs. *E.modesta* and *E.carinocostata* laid individual blue/green eggs loose in the sediment, unattached to any substrate, and each egg was surrounded by a case of sand grains (referred to hereafter as “Type 2” eggs). Type of eggs laid was consistent for each species of snail and for all observations. Loose eggs were never observed in *E.carinifera* tanks, and eggs attached to hard substrates were never observed in *E.carinocostata* or *E.modesta* tanks. Females of all species and

from all sites laid eggs in laboratory aquaria. A total of 498 eggs were collected from aquaria and isolated in nursery isolation chambers or tissue culture dishes (*E.carinifera*=219, *E.carinocostata*=144, *E.modesta*=135) for further observations.

As with the aquaria samples, two types of egg deposition were observed in field samples: (1) single eggs attached rocks in lines up to six eggs and (2) individual eggs, loose in the sediment, unattached to substrate, and surrounded by a case of sand grains, Figure 2.2.

**Figure 2.2.** Two types of eggs observed in laboratory and field samples for three *Elimia* in Two Run Creek, Bartow County, GA. As observed in the laboratory, Type 1 eggs were laid by *E.carinifera*, and Type 2 eggs were laid by *E.carinocostata* and *E.modesta*.



When field sediment samples were searched, individual unattached eggs surrounded by a case of sand grains were found (n=137) at all sites in Two Run, Connesena, and Stamp Creeks and isolated for observation. Eggs attached to rocks were observed in the field at all Two Run and Connesena Creek sites, but no attempt was made to quantify the number of eggs observed. No eggs were observed attached to rocks at the Stamp Creek sites. Egg coloration observed in laboratory for each type was consistent in field samples as well. Eggs from both the field and laboratory measured approximately 0.3mm in diameter.

**Table 2.3.** Species of *Elimia* present and type of eggs found at three streams in the Lower Etowah River Basin. Three sites were sampled for each stream: an upstream, tributary and downstream. Type 1 eggs = eggs firmly attached to rocks, Type 2 eggs = single eggs deposited loose in the sediment surrounded by a case of sand grains. Abbreviation for species are *E.carinifera*=ECF, *E.carinocostata*=ECC, *E.modesta*=EMD.

	Upstream		Tributary		Downstream	
	Species Present	Oviposition Type	Species Present	Oviposition Type	Species Present	Oviposition Type
<b>Two Run</b>	<i>ECF</i> <i>ECC</i> <i>EMD</i>	1 2	<i>ECF</i> <i>ECC</i>	1 2	<i>ECF</i> <i>ECC</i> <i>EMD</i>	1 2
<b>Connesena</b>	<i>ECF</i> <i>ECC</i> <i>EMD</i>	1 2	<i>ECF</i> <i>EMD</i>	1 2	<i>ECF</i> <i>ECC</i> <i>EMD</i>	1 2
<b>Stamp</b>	<i>EMD</i>	2	<i>EMD</i>	2	<i>EMD</i>	2

Eggs attached to rocks were observed in the field beginning in mid-January until late June. Average stream temperatures in mid-January were 9.1°C for the Two Run Creek sites and 10.4°C for the Connesena Creek sites. Average temperatures were calculated for the 14-day period prior to discovery, as egg gestation time observed in lab was an average of 14 days. Eggs attached to rocks were not observed for any of the Stamp Creek sites.

Eggs that were deposited loosely, unattached to substrate in the sediment, and encased in sand grains were first observed from field samples from mid-March in Stamp and Connesena and in mid-April in Two Run. The last observation of individually deposited eggs in sediment was in late June for all sites. Average stream temperatures at onset of observation was 16.7°C for Two Run sites, 14.0°C for Stamp Creek sites and 14.8°C for Connesena sites. Average temperature range, at final egg presence observation for all streams and types of egg deposition was 20.1 - 22.0°C, Table 2.4.



**Table 2.4.** Eggs observations from field samples for each stream. Temperatures (°C) are the average of 14 days prior to date of observation. \*Stamp Creek temperature data only includes the upper and lower sites. NA means egg type was not observed.

Stream	Eggs attached to rocks		Eggs loose in sediment	
	<i>First Obs.</i>	<i>Last Obs.</i>	<i>First Obs.</i>	<i>Last Obs.</i>
<b><i>Two Run</i></b>	Mid Jan	Late June	Mid Apr	Late June
	9.1	22.0	16.7	22.0
<b><i>Stamp*</i></b>	NA	NA	Mid March	Late June
			14.0	21.1
<b><i>Connesena</i></b>	Late Jan	Late June	Mid March	Late June
	10.4	20.1	14.8	20.1

The lowest temperature at which *E.carinifera* laid eggs in the laboratory was 10°C (lower limit of chiller). The lowest temperature *E.carinocostata* and *E.modesta* laid eggs in the laboratory was 12°C. The upper end of the temperature range where all species laid eggs in the laboratory was 22°C – ambient temperature in the laboratory, Table 2.5.

**Table 2.5.** Observed (n=51), temperature range of oviposition in Laboratory by type of deposition. The temperature range in the laboratory was 10°C – 22°C.

	<i>E.carinifera</i> Attached to hard substrate	<i>E.carinocostata</i> Loose in sediment	<i>E.modesta</i> Loose in sediment
Lowest	10	12	12
Highest	22	22	22

Stream water temperatures at the first observation of egg laying in the field followed a similar pattern as seen in laboratory aquaria, where eggs attached to rocks (*E.carinifera*) began at a lower temperature than individual eggs laid loosely in the sediment (*E.carinocostata* and *E.modesta*), Tables 2.4 and 2.5.

Eggs from field sediment samples and tanks were isolated in flow-through nursery isolation chambers or tissue culture dishes. The percentage of eggs that hatched after isolation was low likely due to two factors: free-swimming trematode predation and algae overgrowth. During the course of the laboratory study, two major algae blooms occurred. The algae choked the drain filters, covered the eggs, and many snails died. The entire system had to be dismantled and cleaned each time. When trematodes were present, many eggs and juveniles were missing. Free-swimming trematodes that had ingested both eggs and newly hatched juveniles were observed in tanks where no free living juvenile snails were observed.

Eggs that were left to hatch and develop in tanks where they were laid survived longer than eggs hatched in isolation. Hatched in isolation chambers, no juveniles developed beyond 2.5 whorls. *E.carinifera* juvenile snails reared in the original tank grew up to 5 whorls. *E.carinocostata* snails grew up to 3 whorls, and *E.modesta* juveniles developed up to 1.5 whorls. Carina become visible on the 2<sup>nd</sup> whorl for *E.carinifera* and *E.carinocostata*, and the nodules of *E.carinifera* start developing on the 4<sup>th</sup> whorl. None of the *E.modesta* juveniles developed enough to see any defining shell sculpture characteristics.

Juvenile shell characteristics for each species collected in the field were unique and could be clearly identified at the 5<sup>th</sup> whorl. Carina were visible starting on the 2<sup>nd</sup> whorl for *E.carinifera* and *E.carinocostata*, while the nodules of *E.carinifera* and the vertical ribs on *E.carinocostata* are clearly visible on the 5<sup>th</sup> whorl. *E.modesta* juveniles were smooth sided with no nodules but some did have weak vertical striae on the early whorls.

## DISCUSSION

Two types of egg laying behavior for three sympatric species of *Elimia* were observed in this study, (1) eggs firmly attached to hard surfaces and (2) single eggs deposited loose in the sediment, unattached to substrate and surrounded by a case of sand grains. *E.carinifera* laid Type 1 eggs in the laboratory, and we found the same type in the field samples when *E.carinifera* was present. *E.carinocostata* and *E.modesta* laid eggs of Type 2 in the laboratory, and the same type of eggs were found in sediment samples in the field where one or both were present. Only Type 2 eggs were observed in Stamp Creek sites where only *E.modesta* was present. Eggs of Type 1 that were gently dislodged from the sides of the tank had one side flattened, and this shape did not change for the length of time they were observed in isolation. Eggs of Type 2 were spherical and do not have a flattened side. Finding Type 2 eggs was unexpected, and we were unable to find any literature citations that mentioned Type 2 oviposition for *Elimia* or Pleuroceridae. No conclusions are offered as to why Type 2 oviposition may be beneficial in lotic environments. These findings illustrate the need for further research on the life history of *Elimia* and other Pleuroceridae.

Temperature cues for oviposition differed between the two types of egg laying behavior. *E.carinifera*, Type 1, began oviposition at lower temperatures in the field and in captivity. *E.carinocostata* and *E.modesta* (Type 2) began oviposition at warmer temperatures. Whelan *et al.* (2014) observed three types of oviposition in field and captive propagation of *Leptoxis*: clutch, single eggs, or eggs in a line; however, all of their specimens laid eggs firmly attached to hard substrates. They also found water temperatures for oviposition period and current velocity requirements were species-specific and generally agreed among populations with the same egg laying behavior. Dazo (1965) published egg laying for 12 species of Pleuroceridae, 5 of which

were *Goniobasis* (= *Elimia*). All the species were reported to lay eggs firmly attached to substrates as single eggs, lines of eggs, or masses of eggs.

Juvenile shell characteristics are important for species identification for freshwater snails, as adult shell characteristics often differ from the juvenile. Erosion of the apex can further hinder identifications of adult shells if characteristics only seen on juvenile whorls are no longer visible. Adult shells of different species can look similar, and differences may only be distinguishable when looking at a size series where juvenile characteristics can be compared (Thompson 2000, Mihalcik and Thompson 2002). *E. modesta*, for example, has vertical striations on the first few whorls of the juvenile shells and raised spires, but this is not visible on adult shells which are smooth sided with weakly indented sutures. Adults of *E. carinocostata* and *E. carinifera* with highly eroded apex can look nearly identical. The species-specific sculpturing on the body whorl becomes less prominent and eventually obsolete with age, only remaining on the penultimate and upper whorls. Several studies of *Leptoxis* and *Elimia* have shown juvenile shells of some species have carina on the body whorl but become obsolete on adult shells (Whelan *et al.* 2012), while others develop shell characteristics such as costae and striae as they age (Whelan *et al.* 2014).

*E. carinifera* was the only species in our experiment that survived long enough to see both defining shell characteristics: carina and nodules. The carina on lab-reared *E. carinocostata* juveniles was visible beginning with the second whorl, but development of the vertical costae was not yet visible. *E. modesta* juveniles did not survive long enough for us to see any defining characteristics, so no conclusions could be made regarding the development of the shell morphology in these two species.

Several factors confounded attempts at captive propagation. Free-swimming trematodes (cercaria) were seen ingesting snail eggs and newly hatched juveniles. Trematodes are a common

parasite of *Elimia* (Dazo 1965, Tomba 2006, Richardson and Scheiring 1994). Trematodes were found in samples from every field site and were removed from tanks for all species and sites. Several snails, both male and female, were observed with smooth round trematode ‘cysts’ between the foot and operculum. These were isolated, and trematodes were observed hatching.

Algae overgrowth was another cause of egg mortality. Once the eggs became covered in algae, no hatching occurred. Fouling by dead snails also caused a cascade effect. If they were not removed quickly, many more snails would die soon after. Dazo (1965) had similar difficulties with high juvenile mortality and tank fouling from dead specimens in a life history and morphology study of *G.livescens* and *P.acuta*.

## CONCLUSION

Pleuroceridae have diverse methods of oviposition but all previous studies have indicated that eggs are firmly attached to a substrate. My observation of a novel type of oviposition where individual eggs were deposited loose in the sediment and surrounded in a case of sand grains is a first for *Elimia*. Evidence for this unique form of oviposition was observed both in laboratory aquaria and from sediment samples collected in the field. *E.carinifera* and *E.carinocostata* are most similar in juvenile and adult shell characteristics yet have different methods of egg deposition and temperature cues for the onset of oviposition. Species-specific juvenile shell characteristics are visible on the 5<sup>th</sup> whorl, and lab-reared *E.carinifera* exhibited the same shell characteristics as seen in wild-caught adults. Apical erosion of juvenile shell characteristics is common in freshwater streams and makes identification of similar looking adult shells difficult. In systems where sympatric species occur and where adult forms of different species look alike, the availability of datasets that include how the shell morphology changes from juvenile to adult

for each species will be important for conservation efforts, molecular analysis, and life history studies of *Elimia*.

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## **CHAPTER 3: Geometric Morphometric analysis of shell shape for sympatric species of *Elimia* in the Lower Etowah River Basin.**

### **INTRODUCTION**

Shell variation in gastropods is widespread and has been associated with many environmental factors. Predation pressure from decapods can influence aperture, ornamentation, and body whorl shape (Vermeij 1982, Krist 2002), fish predation can result in thicker shells (Palmer 1979), wave action is associated with aperture size (Conde-Padin *et al.* 2007), salinity levels can influence shell coloration and pattern (Minton and Gunderson 2001), river position may alter shape and ornamentation (Minton *et al.* 2008, Minton *et al.* 2011), and current velocity may influence body whorl width and spire length (Urabe 1998). Differences in conchological characters led many early naturalists to designate differing shell morphologies as separate species. In some instances, new species were named based on small samples sizes sometimes as small as one single shell from a specific location without the context of the variation seen across geographic regions (Lea 1853, 1862, 1864). It is clear that Pleuroceridae shells are variable, and this variability is a significant problem for species identification.

Geometric Morphometrics (GM) is a quantitative method used to analyze the variation observed in biological forms and is effective at discriminating between even subtle differences in shape. GM has an advantage over traditional morphometrics because it analyzes shape variability irrespective of size (Adams *et al.* 2004, Adams *et al.* 2013, Mitteroecker and Gunz 2009). The linear measurements of traditional morphometrics are highly correlated with the size of the organism being measured, and efforts to correct for size are not always successful, especially if shape doesn't change with size by a constant factor. GM considers not only the points

(landmarks) or lines (outlines) themselves but also the space between and preserves the geometry of the shape throughout the analysis. For example, Minton and Wang (2011) found statistically significant differences between male and female shell shape of *Viviparus subpurpureus* using landmark GM in contrast to a previous study that concluded there were no differences between the sexes based on traditional morphometric measurements (Falniowski *et al.* 1998). GM was also used to quantify clinal variation in *Lithasia geniculate fuliginosa* from the Duck River, TN. Minton *et al.* (2008) found intraspecific shell variation in a unidirectional pattern from the headwaters to the downstream reaches. In systems where sympatric species occur, GM has been used to visualize and statistically test for differences in shape variation among closely related and morphologically similar ecotypes of *Littorina saxatilis* in NW Spain (Carvajal-Rodriguez *et al.* 2005), NE England (Conde-Padin *et al.* 2007) and five morphologically similar species of *Conus*, a tropical reef snail (Cruz *et al.* 2012). Therefore, GM is an appropriate tool for investigating shape differences among the three sympatric species of *Elimia* in this study.

Shape, as defined by Kendall (1977), is “all the geometric information that remains when location, scale and rotational effects are filtered out of an object” and is the mathematical foundation for Geometric Morphometric Analysis. Landmark-based GM creates a Cartesian Coordinate system that represents the geometry of the form being studied. Landmarks are digital points placed on 2-D or 3-D images at homologous points or points that correspond to anatomically identical loci on each specimen. Placement of the landmarks must provide enough coverage of the form to convey changes in shape, should be reliably located on each specimen, and be in the same plane (Bookstein 1991). Partial Procrustes Superimposition (called Procrustes Superimposition from this point forward), creates a new set of shape coordinates which can be subjected to multivariate statistical analysis (Webster and Sheets 2010, Zelditch *et al.* 2012).

During Procrustes Superimposition, a reference form which is the average of all forms in the sample is created and centered at (0,0). All target configurations are translocated to the centroid (center) of the reference form, and all configurations are rescaled to a centroid size factor of 1. Target configurations are iteratively realigned and/or rotated to a position that minimizes the distances between corresponding landmarks for all configurations as compared to the reference form (Rohlf and Slice 1990). Procrustes Superimposition prepares the dataset for additional analyses including Thin-Plate Spline, Principal Component Analysis and Canonical Variates Analysis.

Thin-Plate Spline (Brookstein, 1989) provides a visual representation of shape change between sets of landmark coordinates and displays the change as a deformation grid. It is useful to see how shape is changing in particular regions of an organism relative to the reference form. Warp scores are produced which can be used in statistical analysis.

Principal component analysis (PCA) of warp scores, or relative warp analysis, is used to analyze shape differences among individuals and produces principal components which describe the variation among specimens relative to the reference form of all specimens in the sample (Webster and Sheets 2010).

Canonical variates analysis (CVA) produce canonical variates (CV) that explain between-group variation relative to within-group variation and statistical analysis of *a priori* group assignments. Cross-validation assignment rates indicate how well specimens would be assigned to the defined group by using a small portion of the sample as unknowns (Webster and Sheets 2010). Geometric Morphometrics will be used in this study to determine if the general shape of the three forms of *Elimia* present are statistically different, how the shapes differ, and if the individuals in the sample can be reliably separated based on species assignment.

## METHODS

Specimens used for shape analysis were randomly drawn from the field collections used in the life history analysis (Chapter 2), representing samples from the upstream and downstream sites in Two Run Creek, Bartow County, Georgia. Shells were soaked in a 10% bleach solution for approximately 10 minutes and gently scrubbed to clean away algae. Only specimens that included 3 intact whorls (10+mm) and a complete aperture were used for analysis. Gender was not determined for this analysis. A Leica 2000 stereomicroscope was used for a tentative initial assignment of individuals to a species group. Shells were photographed with a tripod-mounted Nikon D7000 camera. All shells were placed in the same orientation on soft polymer clay with the aperture in the same plane as the camera lens. Photo editing (brightness and contrast) took place in Adobe Photoshop (2017). GIMP 2.1 was used to rotate the images and draw a line through the central axis. Lines parallel and perpendicular to the central axis were drawn to aid in landmark placement (Figure 3.1). Open source software from Stony Brook University (<http://life.bio.sunysb.edu/morph/>) was used to perform Geometric Morphometric analysis. Landmarks were digitized using tpsDIG. A total of 77 specimens were digitized: Two Run Upper site included 12 individuals assigned to *E.carinifera*, 14 individuals assigned to *E.carinocostata*, and 12 individuals assigned to *E.modesta*; Two Run Lower site included 13 individuals assigned to *E.carinifera*, 13 individuals assigned to *E.carinocostata*, and 13 individuals assigned to *E.modesta*. Twelve landmarks were selected on all specimens for general shape analysis.

Landmark placements are indicated in Figure 3.1. They are: (1) intersection of columellar lip and central axis at the widest point of the aperture, (2) intersection of the central axis and the

lowest point on the aperture, (3) basal lip of aperture, (4) outer lip of aperture, (5) intersection of the aperture and the body whorl, (6-11) sutures, (12) outermost point on the body whorl, opposite of the outer lip of the aperture. All landmarks represent reproducible and comparable anatomical locations on all forms in the study (Zelditch *et al.* 2004).

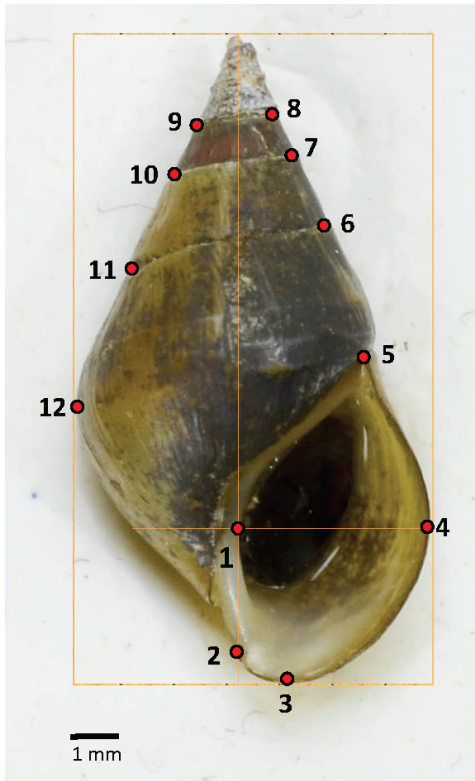


Figure 3.1. Location of landmarks used in Geometric Morphometric analysis of shell shape variation of *Elimia spp.* from Two Run Creek sites. Vertical and horizontal axes used to orient the landmarks are also indicated.

Group Membership lists for each data set were created to identify landmark configurations by species for PCA and CVA. Tps files were superimposed and converted to .cs files in CoordGen for analysis in PCAGen, CVAGen, and TwoGroup.

Principal Component Analysis (PCA) of partial warp scores was performed in PCAGen on the Two Run Creek landmark datasets. PCA reduces highly dimensional data (shape variables) and transforms them into a new linear dataset scaled to unit 1. Principal components (PC) are orthogonal eigenvectors that correspond to the direction of the most variance in the dataset. Each PC has an eigenvalue that expresses the magnitude of variance along the PC. PC1 describes the largest proportion of the variance in the dataset, and

PC2 describes the next largest not explained by PC1, and so on. The variance expressed is relative to the mean/consensus shape of the entire sample of individuals in the data set.

Specimens with the largest PC scores contribute a larger proportion of the overall variance and will be plotted farthest from zero, in the negative or positive direction. The number of significant

PCs was determined by performing an Anderson's test. The test analyzes consecutive PCs until no significance is found. The representation of variation of shape along the PCs was visualized by using Thin-plate spline deformation grids.

To determine if specimen groups can be statistically distinguished, a Canonical Variates Analysis (CVA) in CVAGen (IMP) was performed. A PCA is performed on landmark coordinate data sets and is rotated in a direction that maximizes the average within-group variances. The axes are then rescaled to the average within-group variances. A second PCA is performed to find the direction of maximum distance between the group averages. The direction of the first two CVs are orthogonal along the axes where group centroids are farthest apart. A MANOVA indicates how many statistically different CVs there are. Significant CVs indicate significant differences among groups. The Wilk's Lambda is computed as the within-group sum of squares/ total sum of squares (within-group + between group) and is analogous to the F-test in a traditional ANOVA. To test which groups are significantly different, pairwise tests are run in TwoGroup (see below).

A CVA re-substitution assignment test based on the Mahalanobis distance of each specimen to the closest mean was used to indicate how well the CV scores can distinguish species groups based on shape data. The results show how many individuals were assigned correctly or incorrectly to the pre-defined groups and the percentage of correct assignments. Jackknife cross-validation grouping tests- where one individual is left out at a time, the group means recalculated, and the remaining individuals are then classified according to distance from the new means- was also performed to indicate the percentage of correct assignments relative to the expected random rate of correct assignments, given the sample size of the groups.

To test for statistical differences between specific species or location, data-subsets were created in tpsUtil then loaded pairwise into TwoGroup (IMP). Landmark coordinate data sets were superimposed by Generalized Least Squares (GLS) Partial Procrustes, and a Goodall's Bootstrapped F-test was performed using 3600 iterations. Species groups were compared across and within stream sites.

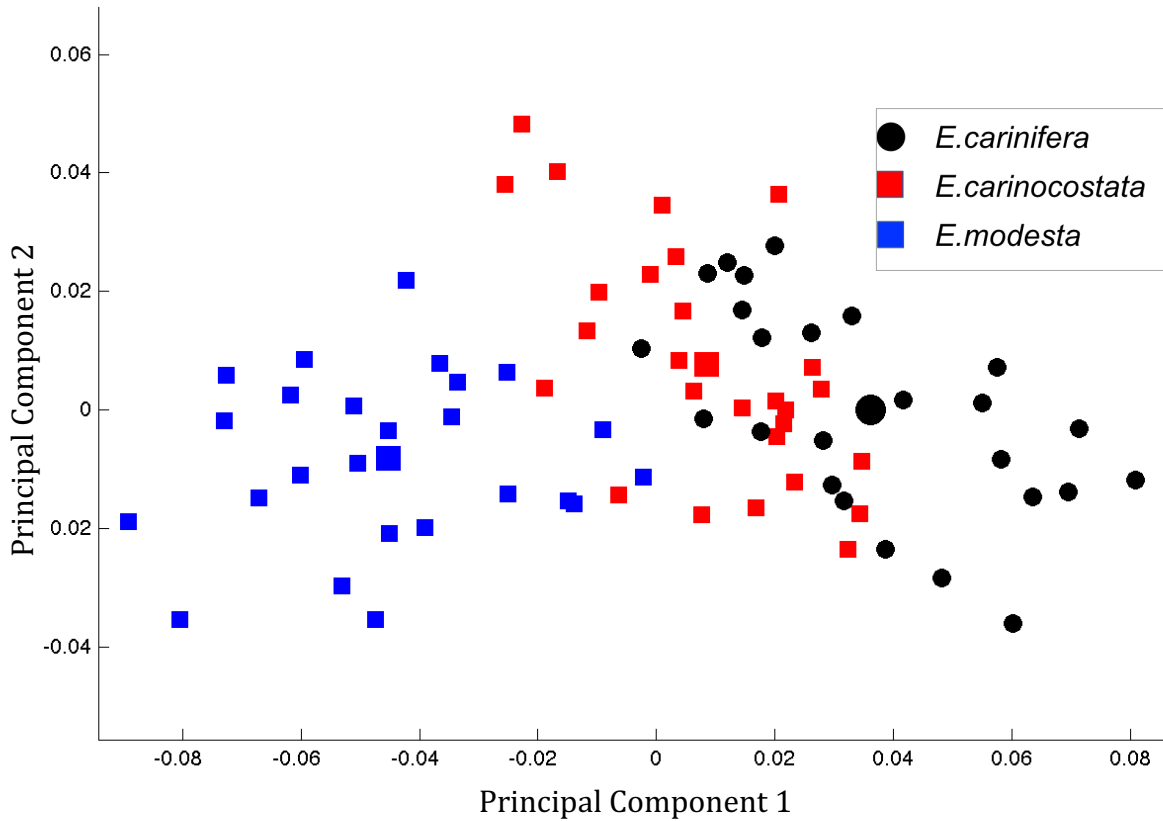
## **RESULTS**

### **PRINCIPAL COMPONENT ANALYSIS**

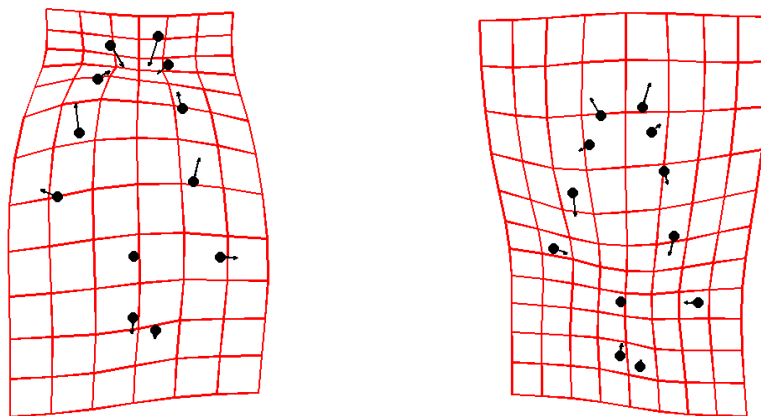
All specimens (n=77) at an upstream and downstream site in Two Run Creek were subjected to PCA (Figure 3.2). Two distinct eigenvalues were found ( $X^2=41.0237$  and  $19.9452$ ). PC1 (64.26% of variance) and PC2 (13.83% of variance) accounted for 78.09% of variance in the data set. Thin-Plate Spline deformation grids of PC1 in the positive and negative directions represent the variation in shape of the dataset from the mean or consensus form (Figure 3.3). PC1 in the negative direction shows the body whorl becomes more inflated (reflected in the movements of LM 6, 11 and 12 away from the centroid of the consensus form), the spire shortens, the angle is more obtuse (movements of LM 7-10 toward the centroid of the consensus form), and the aperture expands both in height and width (movement of LM 2,4 and 5 away from the centroid of the consensus form). PC1 in the positive direction represents compression/shrinking of the body whorl (movement of LM 6, 11 and 12 toward the centroid), an elongation of the spire with the angle becoming more acute (movements of LM 7-10 toward the centroid), and smaller apertures (movements of LM 2,4 and 5 toward the centroid).



**Figure 3.2.** Principal Component Analysis of 77 *Elimia* landmark configurations for three species collected from two sites of Two Run Creek, Bartow Co. GA. Two distinct eigenvalues were found ( $X^2=41.0237$  and  $19.9452$ ). PC1 (64.26%) and PC2 (13.83%) accounted for a total of 78.09% of the variance in the data set. Large symbols indicate the centroid of each species group.

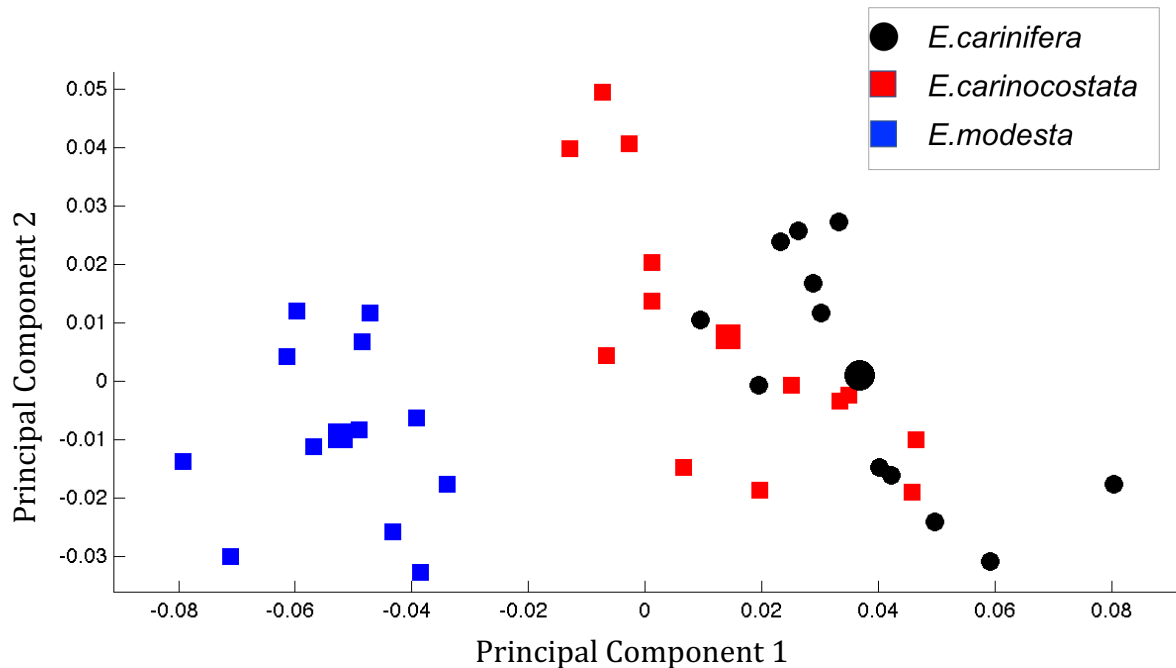


**Figure 3.3.** Thin-Plate spline deformation grid of PCA for two sites at Two Run Creek, Bartow Co. GA. Left grid represents PC1 in the negative position and the grid on the right represents PC1 in the positive position. Landmarks are shown as points and arrows indicate direction of shape change.

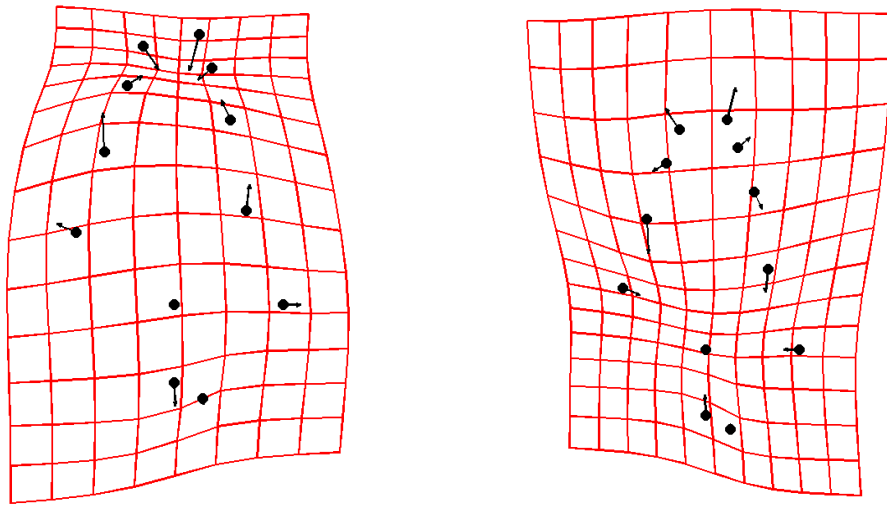


A PCA on Two Run Creek's upstream site (37 specimens / 444 landmarks) showed 2 distinct eigenvalues ( $X^2=15.9037$  and  $13.2621$ ) for PC1 and PC2. PC1 (64.92% of variance) and PC2 (16.35% of variance) accounted for a combined 81.27% of the variance in the dataset (Figure 3.4). The thin-plate spline deformation grid demonstrates PC1 in a positive direction represents compression of the aperture (LM 2-5) and body whorl (LM 6, 11 and 12) and elongation of the spire (LM 6-11). In a negative direction, PC1 represents expansion in the body whorl (LM 6, 7, 11 and 12) and aperture (LM 2-4), and shortening of the spire (LM 7-10), Figure 3.5.

**Figure 3.4.** Principal Component Analysis (PCA) of 37 *Elimia* from the Two Run Creek upstream site, Bartow Co. GA. Two significantly distinct eigenvalues were found ( $X^2= 15.9037$  and  $13.2621$ ). PC1 (64.92%) and PC2 (16.35%) account for 81.27% of the variance in the data set. Large symbols indicate the centroid of each species group.

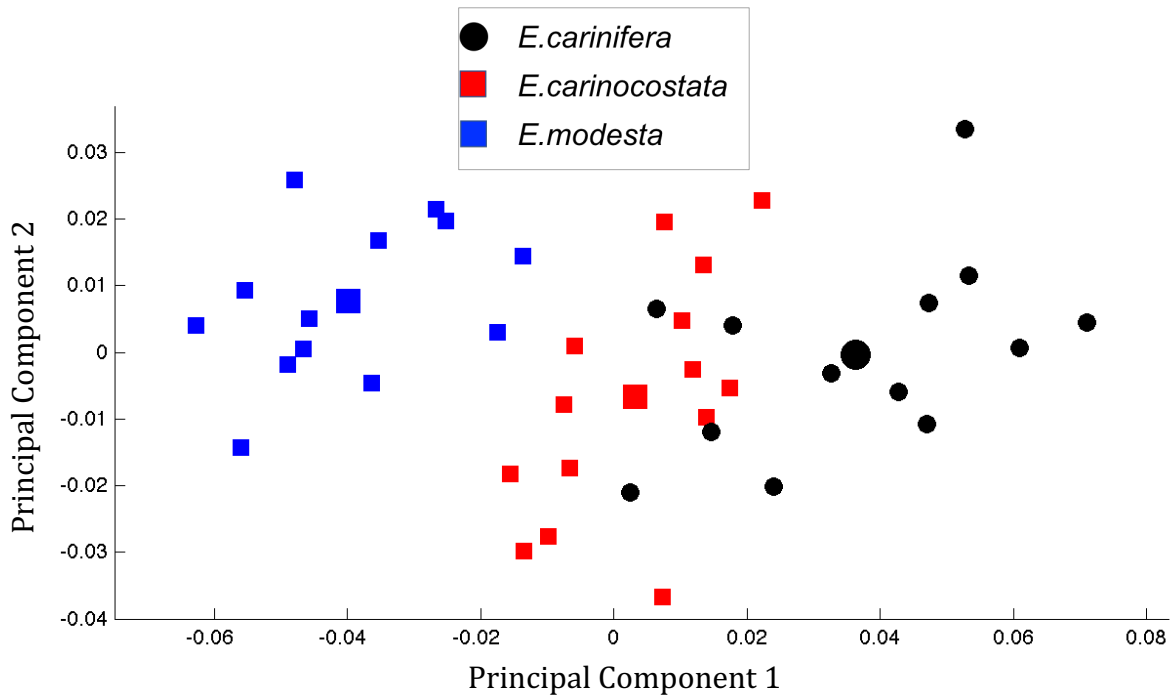


**Figure 3.5.** Thin-plate spline deformation grid of PCA for *Elimia* collected from the Two Run Creek upstream site. Grid on the left represents PC1 in the negative direction. Image on the right represents PC1 in the positive direction.

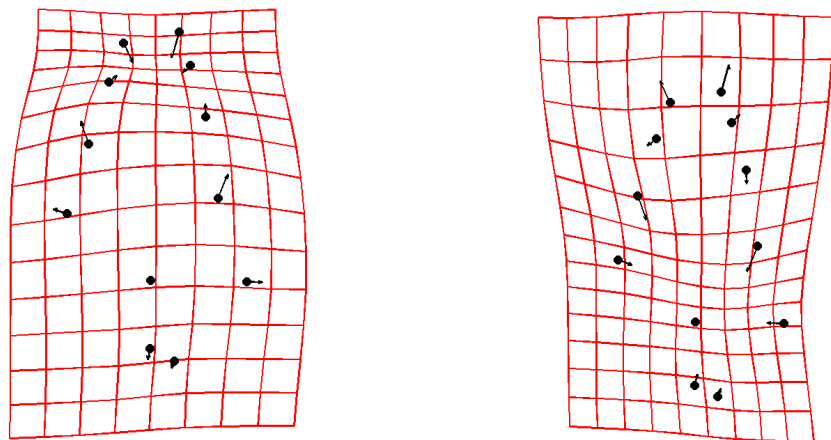


PCA on Two Run Creek's downstream site (40 specimens, 480 LM) indicated one distinct eigenvalue ( $X^2= 22.5734$ ), figure 5. PC 1 (63.89% of variance) and PC2 (12.95% of variance) accounted for 76.84% of the variance in the data set (Figure 3.6). Thin-plate spline deformation grids show the same patterns as for the Two Run Upper data set (Figure 3.7).

**Figure 3.6.** Principal Component Analysis (PCA) of 40 *Elimia* in Two Run Creek's downstream site, Bartow Co. GA. One significantly distinct eigenvalue was found ( $X^2= 22.5734$ ). PC1 (63.89%) and PC2 (12.95%) account for 76.84%% of the variance in the data set. Large symbols indicate the centroid of each species group.



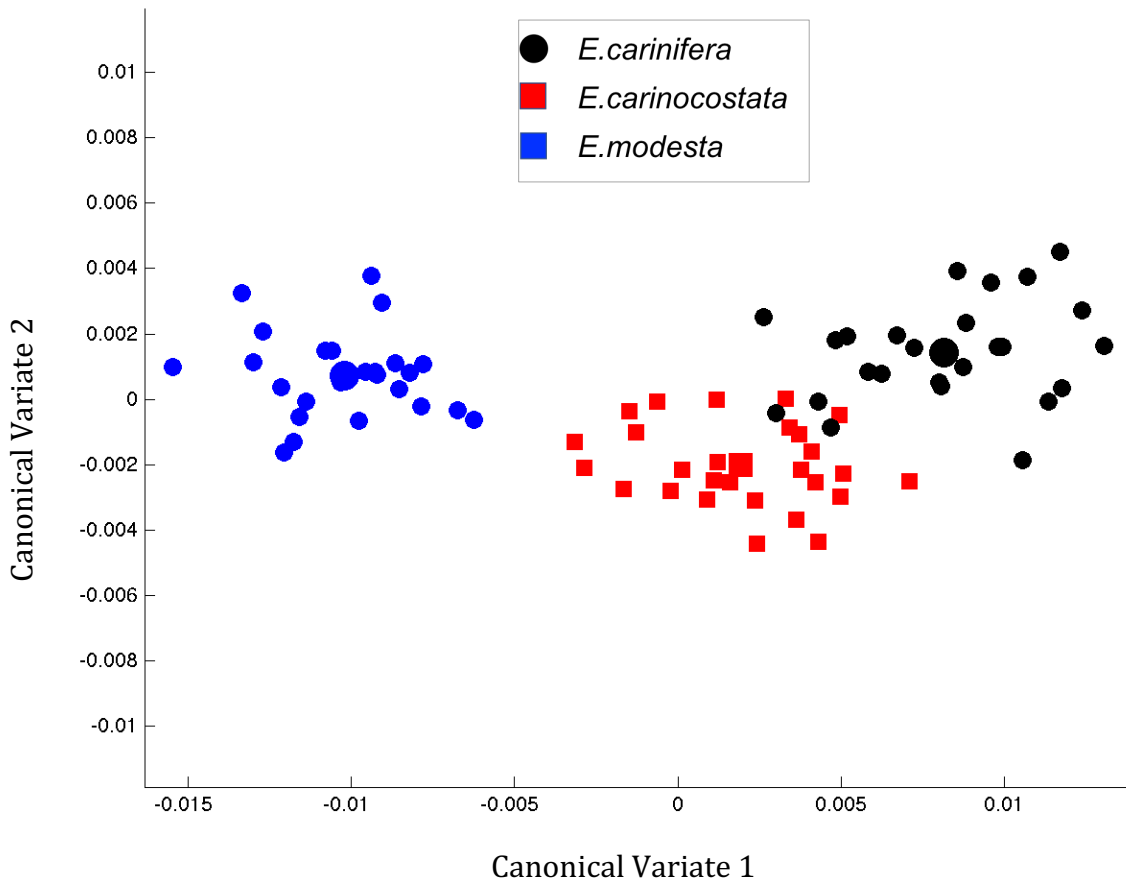
**Figure 3.7.** Thin-plate spline deformation grid of PCA for Two Run Creek's downstream site. Grid on the left represents shape variation associated with PC1 in the negative direction. Image on the right represents PC1 in the positive direction.



## CANONICAL VARIATES ANALYSIS

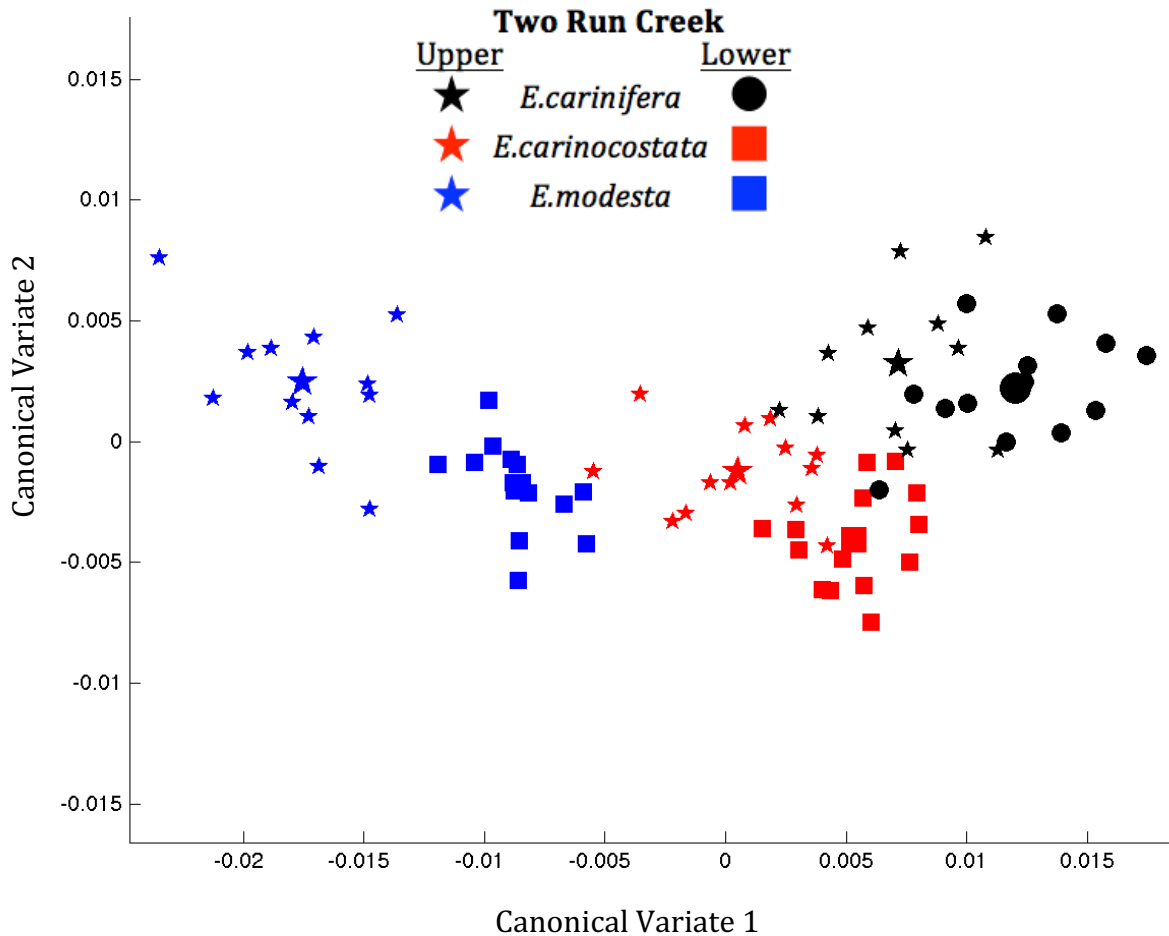
Canonical variates analysis was performed on 77 specimens of *Elimia* from Two Run Creek upstream and downstream sites, grouped by species (*E.carinifera*=25, *E.carinocostata*=27, *E.modesta*=25). The MANOVA indicated two statistically distinct canonical variates (CVA,  $p < 0.0001$  for both CVs), indicating that both CVs are factors in discrimination difference among species groups (Figure 3.8). A CVA re-substitution group assignment test was performed to indicate how well the CV scores could be used to assign individuals to species groups. Assignment of all *E.carinocostata* and *E.modesta* individuals were correct, but 3 individuals identified as *E.carinifera* were assigned to the *E.carinocostata* group. Jackknife cross-validation groupings test returned a correct assignment rate of 88.31% compared to an expected 33.34% random rate of correct assignments given the sample size of the groups.

**Figure 3.8.** Canonical Variates Analysis (CVA) for three species of *Elimia*: *E.carinifera* (n=25), *E.carinocostata* (n=27) and *E.modesta* (n=25) in Two Run Creek, taken from an upstream and downstream site. Two distinct CVs were found, both  $p < 0.0001$ .



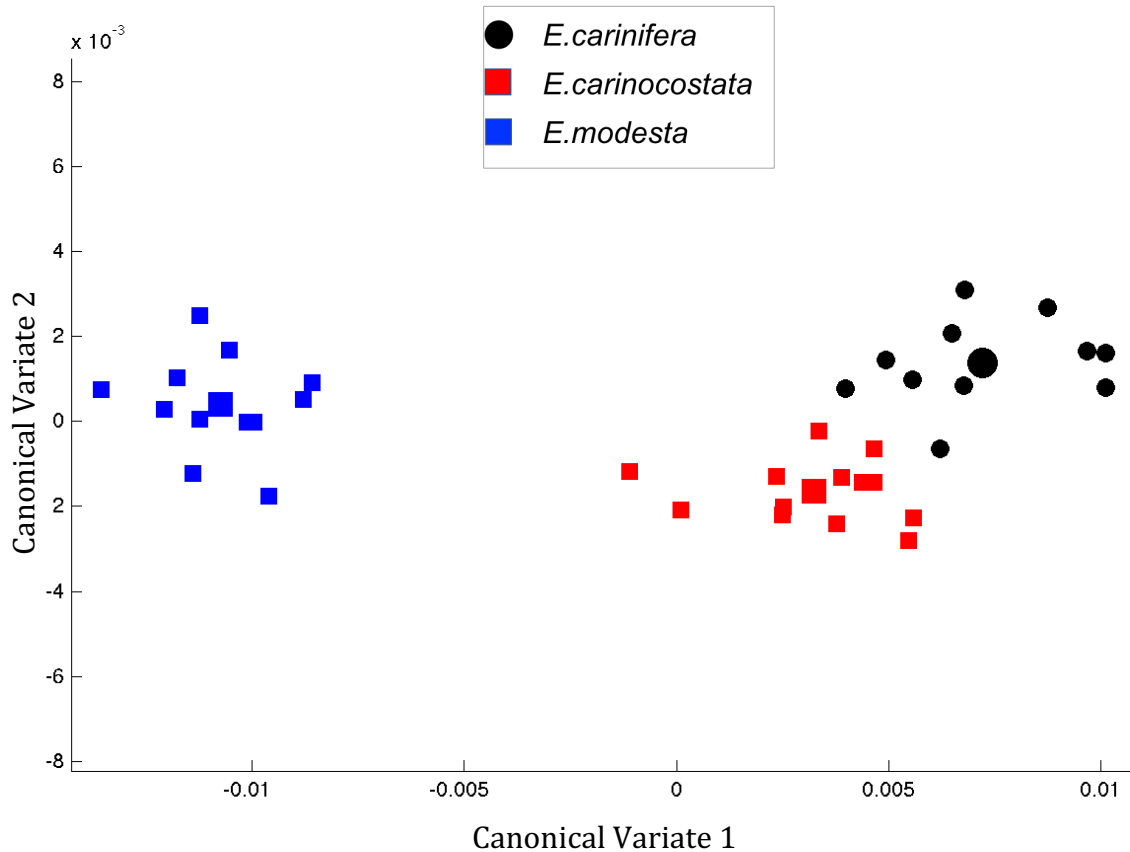
Canonical variates analysis was performed on 77 specimens from Two Run Creek, grouped by species across both upstream and downstream sites (6 groups). The MANOVA indicated 5 statistically distinct canonical variates: CVs 1-3= $p < 0.0001$ , CV4:  $p = 0.002$ , CV5:  $p = 0.0388$ . The CVA group assignment test results showed all specimen assignments were correct for *E.carinocostata* and *E.modesta* groups. One specimen of *E.carinifera* from each site was assigned to *E.carinocostata* (Figure 3.9). Jackknife cross-validation groupings test returned a correct assignment rate of 68.31% compared to an expected 16.88% random rate of correct assignments given the sample size of the groups.

**Figure 3.9.** Canonical Variates Analysis (CVA) of warp scores for three species of *Elimia* by stream location. Two Run Creek upstream site: *E.carinifera* (n=12), *E.carinocostata* (n=13) and *E.modesta* (n=12) and downstream site *E.carinifera* (n=13), *E.carinocostata* (n=14) and *E.modesta* (n=13): Five distinct CVs were found. CVs 1-3= $p<0.0001$ , CV4:  $p=0.0027$ , CV5:  $p=0.0388$ .



CVA of 27 *Elimia* from the Two Run Creek upstream site returned one distinct canonical variate,  $p<0.0001$ , Figure 9. The CVA group assignment test results showed all *E.modesta* were correctly assigned, two *E.carinocostata* were assigned to *E.carinifera*, and two *E.carinifera* were assigned to *E.carinocostata*. Jackknife cross-validation groupings test returned a correct assignment rate of 70.27%, compared to an expected 33.38% random rate of correct assignments given the sample size of the groups.

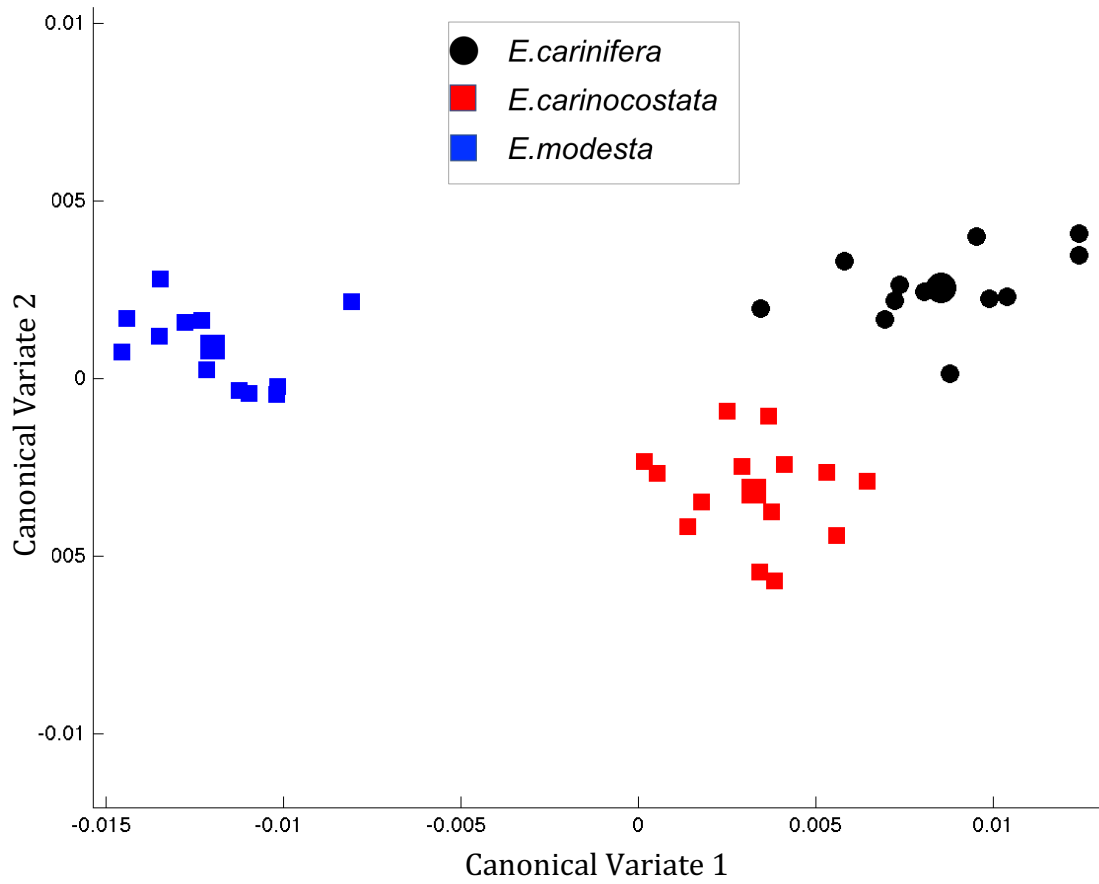
**Figure 3.10.** Canonical Variates Analysis (CVA) for three species of *Elimia* in Two Run Creek – upstream site. *E.carinifera* (n=12), *E.carinocostata* (n=13) and *E.modesta* (n=12). One distinct CVs was found,  $p < 0.0001$ .



Canonical Variate Analysis for three species of *Elimia* in Two Run Creek – downstream site returned two distinct CVs,  $p < 0.001$  (Figure 3.11). The CVA group assignment test results showed all specimens were correctly assigned. Jackknife cross-validation groupings test returned a correct assignment rate of 75.00%, compared to an expected 33.38% random rate of correct assignments given the sample size of the groups



**Figure 3.11.** Canonical Variate Analysis (CVA) for three species of *Elimia* in Two Run Creek-downstream site: *E.carinifera* (n=13), *E.carinocostata* (n=14) and *E.modesta* (n=13). Two distinct CVs were found, both  $p < 0.001$ .



## TWO GROUP ANALYSIS

To test for statistical differences between specific data sets, separate site and species data sub-sets were created in tpsUtil and then loaded pairwise into TwoGroup (IMP). The landmark coordinate data sets were superimposed by Generalized Least Squares (GLS) Partial Procrustes, and then a Goodall's Bootstrapped F-test was performed using 3600 iterations.

Differences between means for all species compared within each site location were statistically significant as determined by Goodall's Bootstrapped F-test. Pairwise tests for each site location were: *E.modesta* and *E.carinocosta* ( $p < 0.0003$ ), *E.modesta* and *E.carinifera* ( $p < 0.0003$ ), and *E.carinifera* and *E.carinocostata* (downstream  $p < 0.0003$ , upstream  $p = 0.0297$ ), Table 3.3.

**Table 3.3.** Goodall's Bootstrapped F-test (3600 iterations) for within site pairwise analysis of *Elimia* from an upstream and downstream location in Two Run Creek. ECF=*E.carinifera*, ECC=*E.carinocostata* and EMD=*E.modesta*.

Stream >	Two Run - Upstream			Two Run - Downstream		
	<i>F Score</i>	<i>P value</i>	<i>Distance between Means</i>	<i>F Score</i>	<i>P value</i>	<i>Distance between Means</i>
EMD & ECC	24.36	0.0003	0.0692	16.85	0.0003	0.0464
EMD & ECF	43.26	0.0003	0.0901	39.43	0.0003	0.0769
ECC & ECF	3.26	0.0297	0.0271	8.19	0.0003	0.0355

Samples were also grouped by species and compared across both site locations. There were statistically significant shape differences within species between sites for both *E.carinifera* ( $p = 0.0206$ ) and *E.modesta* ( $p = 0.0398$ ) but not for *E.carinocostata* ( $p = 0.0931$ ), Table 3.4.

**Table 3.4.** Goodall's Bootstrapped F-test (3600 iterations) for pairwise analysis of *Elimia* from an upstream and downstream location in Two Run Creek. Specimens were grouped by species and compared across sites. *E. carinifera* (n=25), *E. carinocostata* (n=27) and EMD=*E. modesta* (n=25).

	F-score	P value	Distance between Means
<i>E. carinifera</i>	3.62	0.0206	0.0268
<i>E. carinocostata</i>	2.06	0.0931	0.0191
<i>E. modesta</i>	11.52	0.0003	0.0398

## DISCUSSION

Principal Component Analysis and Canonical Variates Analysis found statistically significant differences in shell shape between three sympatric species of *Elimia* collected from Two Run Creek.

Landmarks chosen for this study were selected to specifically capture differences in overall shell shape in the absence of information on the sculptural characteristics (carina, nodules or costae) that have been used to distinguish species in Pleuroceridae. Landmarks were placed along the periphery at the sutures anterior to the penultimate and next upper whorl and none were placed between these sutures since two of the species (*E. carinifera* and *E. carinocostata*) possess peripheral carina and the third does not (*E. modesta*). Placement of landmarks between sutures on those whorls of *E. modesta* would have been arbitrary and not homologous to the peripheral carina on *E. carinifera* and *E. carinocostata*.

PCA of landmark data among all individuals in the sample and within each site location showed significant differences. The variance explained by PC1 for all analyses indicated the variation responsible for differences between species was related to the shape of the body whorl, spire and aperture. *E. carinifera* and *E. modesta* are most different in overall shell shape.

*E. carinifera* has a narrower body whorl, elongated spire, and smaller aperture, as seen on PC1 in

the positive direction. *E.modesta* has a more robust body whorl, compressed spire, and larger aperture, as seen on PC1 in the negative direction. *E.carinocostata* shell shape is intermediate, but is more similar to *E.carinifera* than *E.modesta* and is similar to the consensus form for all specimens in the data set. There was no overlap in scatterplot between *E.modesta* and *E.carinocostata*, which have the same type of oviposition (Chapter 2).

CVA indicated significant differences between species groups across and within sites for all species. *E.modesta* was well separated from both *E.carinocostata* and *E.carinifera*, in all analyses. *E.carinifera* and *E.carinocostata* clustered closer together for all analyses indicating their overall shape was more similar in individual analysis (PCA) and species group analysis (CVA).

Pairwise analysis between all species within each site location indicated statistically significant differences ( $p < 0.03$ ). Specimens of each species from the upstream site were compared to the downstream site. Significant differences were found for *E.modesta* ( $p = 0.0003$ ) and *E.carinifera* ( $p = 0.0206$ ), but not for *E.carinocostata* ( $p = 0.0931$ ). Differences in shell shape within a stream system for the same species has been reported in *Elimia* and Pleuroceridae. Geometric Morphometric analysis quantified intraspecific variation over small scale distances in *E.potosiensis* in Arkansas (Minton *et al*, 2011) and across large distances in *Lithasia* of the Duck River, TN (Minton *et al.*, 2008). Variation in shell shape followed a unidirectional pattern from headwaters to downstream reaches. Shells in the upper reaches generally had narrower body whorls, smaller apertures, and longer spires, compared to those downstream. Environmental factors correlated with variation in shell shape included predation pressures (Krist, 2002) and water chemistry (Minton *et al*, 2011). Further analysis would be needed to determine if intraspecific differences in shell shape seen between the upstream and downstream populations

of *E.carinifera* and *E.modesta* follow the same unidirectional pattern and which environmental factors may be influential.

*E.carinifera* and *E.carinocostata* are most similar in shell shape (and sculpturing) but had different egg-laying behaviors. *E.carinifera* began oviposition at lower temperatures and laid eggs of Type 1 (singly or in lines up to 6 eggs, firmly attached to substrates), and *E.carinocostata* began oviposition at higher temperatures and laid eggs of Type 2 (individual eggs, unattached to the substrate, deposited loose in the sediment and surrounded by grains of sand). *E.carinocostata* and *E.modesta* which had the same egg laying behaviors (Type 2) had much less overlap in the PCA, and CVA showed a greater distance between their group means.

This study has demonstrated that shell shape, regardless of differences or similarities in sculpture can effectively distinguish between species.

## CONCLUSION

This study has demonstrated quantifiable and significant differences in shell shape between three sympatric species of *Elimia* that exhibit two different methods of oviposition, as observed in laboratory experiments and in field collections. Similarities in shell morphology was not an indicator of similarities in reproductive life histories, as *E.carinifera* and *E.carininocostata* had the most similar shell shape but different methods of egg-laying. Between the two species that exhibit the same method of egg deposition (*E.modesta* and *E.carinocostata*) shell shape was significantly different and well separated in PCA and CVA scatterplots. This study should provide a baseline of evidence for further research for *Elimia* in the Lower Etowah River Basin.

## **INTEGRATION OF THESIS RESEARCH**

This study integrated high-powered morphometric analysis of multivariate shape data with a life history study of the reproductive behaviors between three sympatric species of *Elimia* in the Lower Etowah River Basin. Morphometric analysis statistically distinguished specimens into species groups based on shell shape and life history results indicated two distinct types of egg laying behaviors among three species of *Elimia*. Multiple geographic, temporal, and spatial scales were utilized to study egg laying behavior, seasonality of oviposition and morphological differences between species.

## **ACKNOWLEDGEMENTS**

I am immensely grateful to my thesis advisor and mentor, Dr. William Ensign, for his unwavering guidance, support, and patience. I'd like to thank my committee members Dr. Joseph Dirnberger and Dr. Joel McNeal for sharing their knowledge and providing feedback.

I could not have done this without the generosity of scientists in the field that were willing to share their time and knowledge: Dr. Paul Johnson, Dr. Terry Richardson, Dr. Ellen Strong, Dr. Nathan Whelan, and Dr. Riccardo Fiorillo. I am thankful to Thomas Tarpley, Dr. Kyle Gabriel, and Brandon Denney for technical/design assistance and Dr. Kerri Ackerly, Dr. Lori Trolley-Jordan, Dr. David Sheets, and Dr. Miriam Zelditch for assistance with resource materials.

I am indebted to David Haines and many others who braved the summer heat and cold winter water to help me collect data. The love, support, and encouragement of my family gave me strength throughout the entire process.

This project was made possible by funding from the College of Science and Mathematics and the Department of Ecology, Evolution and Organismal Biology.

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