

**Freshwater Mussel Growth in the Holston River: Water
Quality and Inferences from Sclerochronology,
Racemization and Low Precision C14 Dating**

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DEDICATION

This work is dedicated to Timothy Slater, the man who introduced me to the water and helped me learn to love it. Thank you, Dad.

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This work could not have been completed without the help of many. Ranging from all walks of life and those in Tennessee and outside our water driven state, I would like to thank everyone who contributed to this research project.

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ABSTRACT

The goal of this research was to compare modern shell material to relic material of the main branch of the Holston River to examine freshwater mussel growth and mortality to discover possible inferences from changes in water quality. Relic shell material collected from two sample sites were dated using low precision C¹⁴ dating (a relatively new method) and racemization (used to give an idea of the time between death and discovery). Relic shell material was then compared to modern shell material for the species *Actinonaias ligamentina* to illustrate changes in growth and rates of mortality using growth annuli analysis.

Relic specimens of the four species collected, *Actinonaias ligamentina*, *Elliptio crassidens*, *Amblema plicata* and *Pleurobema rubrum*, from the two sample sites along the Main Branch of the Holston River were geologically dated back to the Woodland and Early Mississippian Period in archaeological time using low precision C¹⁴ dating.

Racemization indicated that most amino acids (apart from alanine) were at a low ratio of conversion, meaning that these shells were in the early stages of decay. While the acidity of the water and colder temperatures could have blurred the results of this test, it is also possible that other aspects affecting preservation of the shell may have caused the low ratio conversion of amino acids.

Growth in the relic shells followed the pattern of more growth occurring in the warmer, summer months with a constant cessation of growth as shown by the halting of growth during the winter. For the modern shells (five recently dead *Actinonaias ligamentina*), individuals grew faster compared to some relic specimens of the same species and comparable age, with thicker growth annuli being observed in particular specimens. This was possibly due to an increase in nutrient content, as found through historical water quality data recorded by the Tennessee Department of Environment and Conservation (TDEC) and the Tennessee Valley Authority (TVA). A reduction of growth during the primary growth season for the modern specimens of *Actinonaias ligamentina* was

evident, perhaps caused by temperature-induced growth disruption resulting from the release of the Cherokee Dam.

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ABBREVIATIONS

AD: anno Domini
AFS: American Fisheries Society
AIC: Akaike information criterion
AL: *Actinonaias ligamentina*
Ala: Alanine
AMS: Accelerator Mass Spectrometry
ANOVA: Analysis of Variance
AP: *Amblema plicata*
Arg: Arginine
Asp: Aspartic Acid
BC: Before Christ
BP: Before Present
CFAMS: Continuous Flow Accelerator Mass Spectrometer
CFS: Cubic feet per second
C¹⁴: Carbon 14
C¹²: Carbon 12
D: *Dextro*
DPI: Dot per inch
EC: *Elliptio crassidens*
E. coli: Coliform bacteria
GC: Gas chromatography
Glu: Glutamic Acid
Gly: Achiral glycine
HPLC: High performance liquid chromatography
H₀₁: Null Hypothesis one
H₁: Hypothesis one
H₀₂: Null Hypothesis two
H₂: Hypothesis two
IBLC: N-isobutyl-L-cysteine
IEC: Ion exchange chromatography
I-hArg: Internal standard
I-Ile: Isoleucine
L: *Laevo*
Leu: Leucine
OPA: Ophthalaldehyde
OX-I: Oxalic Acid
Phe: Phenylalanine
PR: *Pleurobema rubrum*
RPC: Reverse phase chromatography
SE: Southeast
Ser: Serine
TDEC: Tennessee Department of Environment and Conservation

Thr: Threonine
TN: Tennessee
TVA: Tennessee Valley Authority
TWRA: Tennessee Wildlife Research Association
Tyr: Tyrosine
USGS: United States Geological Survey
Val: Valine
µm: Micrometers

CHAPTER 1: PROLOGUE

Implications of Research

Freshwater mussels can be used to assess changes in water quality through the analysis of yearly growth annuli, found in the internal structure of a mussel's shell. These growth annuli vary in thickness as growth of the shell and organism occurs, and can be subdivided between summer and winter through visual differences in color (Haag, 2012).

Attributes of water quality contribute to variation in growth annuli thickness or alter the thickness of growth annuli, such as nutrient and mineral content, water velocity, water pH, and sedimentation (Brown et al., 2005; Haag, 2012, Fritts et al., 2017). These attributes, coupled with habitat stability, influence the growth and longevity of living freshwater mussels and the preservation of relic freshwater mussel shell material. Water quality factors that contribute to changes in mussel growth are often brought on by human activities such as: agriculture, impoundment structures, and mining and industrial effluents, to name a few (Haag, 2012). This application of freshwater mussels to the interpretation of changes in water quality is important when trying to reconstruct how a water body has changed over time. Additionally, these changes in water quality, in part due to human intervention, may be visible through the rate or magnitude of change to the mortality and longevity of freshwater mussels (Popejoy et al., 2017).

Important questions to ask include: is it possible to determine differences in growth between modern and relic freshwater mussels? How can the time of death and geologic age of relic freshwater mussel material be determined to compare it to modern shells? Is there possible evidence of variation in growth brought on by anthropogenic changes to the water quality standards that most affect freshwater mussels' longevity and mortality? To answer these questions,

relic shell material should be dated and compared to modern mussels regarding growth as shown through the thickness of yearly growth annuli.

This study compares modern shell material to relic material from two study sites of the Holston River. Seasonal and yearly growth data is then used to possibly infer changes in water quality. In addition, changes in mussel species diversity and stability can be assessed, while also giving possible insight to the paleoclimate of the Woodland and Early Mississippian Period (Regional Archaeological timescale).

CHAPTER 2: INTRODUCTION AND BACKGROUND

Known by many names, the Holston River has been considered a major river system since the time of the Native Americans (TDEC, 2007). Renamed after Stephen Holston, who was an explorer on The Expedition of 1748 (TDEC, 2007), this stretch of water has long been used by humans for industrial, residential, and recreational purposes.

The Holston River

The Holston River contributes to the local water drainages and watersheds of Southeastern Virginia, Northeastern Tennessee, and Northwestern North Carolina. Originating from a spring in Bland County, Virginia, the North Fork of the Holston River flows through Bland County, Virginia continuing for 138 river miles. The South Fork flows for 112 river miles through Smyth County, Virginia before joining with the North Fork in Kingston, Tennessee. The Middle Fork stems near Smyth County, Virginia and flows for 56.5 river miles before coalescing with the South Fork in Washington County, Virginia. The Main Branch of the Holston River is marked by its confluence of all three forks in Kingsport, Tennessee and extends for another 136 river miles until its confluence with the French Broad River just outside of Knoxville, Tennessee to form the Tennessee River.

Ecology and Geology

The Holston River flows over a karst system. This karst system is dominated by water soluble rocks, including; dolomite bedrock, limestone, rock salt, and gypsum (TDEC, 2007). The diversity of these rocks contributes to the many landforms that typify this region: underground caverns, caves, sinkholes, natural springs, and vanishing smaller tributaries (TDEC, 2007) (Figure 1).

The Holston River is characterized as a level 3 ecoregion due to it being situated between a ridge and valley and can be further subdivided into 4 different 4th level sub-ecoregions (TDEC, 2007). These 4 sub-ecoregions are the

Southern Limestone/Dolomite Valleys and Low Rolling Hills, the Southern Shale Valleys, the Southern Sandstone Ridges and Southern Dissected Ridges and Knobs (Figure 2) (TDEC, 2007).

The Southern Limestone/Dolomite valley and hill region is recognized as being composed of limestone and cherty dolomite with white oak forests, bottomland oak forests, and riparian forests filled with sycamore, ash, and elm trees dominating most of the natural landscape of rolling hills and valleys (TDEC, 2007).

The Southern Shale Valleys consist of two main areas of shale: Ordovician-age calcareous shale in the north, with Cambrian-age shale mixed with bands of limestone in the south (TDEC, 2007). The shale beds in the north are surrounded by porous, well-drained soils that tend to be slightly acidic. The shale beds to the south are also surrounded by porous soils, but is recognized as being extremely acidic (TDEC, 2007). Steeper slopes, hills and valleys are common within this area and as a result, agriculture dominates the area.

The Southern Sandstone Ridges are mostly composed of sandstone bedrock, but do have some siltstone and shale throughout the area. Dense forests cover the steep ridges of the area with rocky and infertile soils being prominent (TDEC, 2007). This region is extremely diverse in local composition and is one of the only areas in the Ridge and Valley of Tennessee to have Pennsylvanian-age strata (TDEC, 2007).

Finally, the Southern Dissected Ridges and Knobs region is counted as one of the more varied sub-ecoregions of this drainage. Ridges on the eastern side of the Tennessee Ridge are formed from Ordovician strata including the Sevier Shale, Holston Limestone, Lenoir Limestone, and Athens Shale (TDEC, 2007). In the central and western portion of the area, the Cambrian-age Rome Formation is composed of shale and siltstone interspersed with sandstone (TDEC, 2007). Ecologically, chestnut oak and pine forests cover the higher elevations while white oak, mesophytic forests, and tulip poplars appear on the slopes and knobs of the lower elevations (TDEC, 2007).

The main branch of the Holston River, which is the area of focus for this study, can be divided into the Southern Shale Valley region and Southern Limestone/Dolomite Valley with Low Rolling Hills region. Due to the varying geology, the local waterway of this area has been impacted by many human uses, and subsequently, is labeled as an impaired waterway.

Impairments of the Main Branch of the Holston River

The Main Branch of the Holston River is recognized per the 2016 303 D list (TDEC, 2017) as being an impaired water system due to the presence of elevated levels of *E. coli*, excess levels of nitrates/nitrites, loss of natural habitat due to human-induced habitat alteration, siltation, presence of heavy metals such as excessive amounts of mercury, absence of vegetative buffering, and low dissolved oxygen. Along with these impacts, the construction of hydroelectric dams and coal-powered steam plants have had ecological impacts on the Holston River. Sixteen dams impact the three branches of the Holston River with most of the dams holding up to 30 acres of water (TDEC, 2007). In the headwaters of the Holston River, the Watauga and Wilbur Dam affects the contributing water body of the Watauga River, which is a tributary of the Holston River. The Boone Dam, South Holston Dam, and Fort Patrick Henry Dam were built in the 1950s and have caused ecological habitat alteration for the South Fork of the Holston River (Parmalee and Polhemus, 2004). In the main branch of the Holston River, the Cherokee Dam remains one of the major influences on flow and habitat stability along this reach.

Freshwater Mussel Fauna of the Main Branch of the Holston River

Historical

Prior to the construction of the Cherokee Dam in 1941, the Main Branch of the Holston River was home to a diverse range of mussel fauna (Lydeard et al., 2004; Williams et al., 2008). The extent of this mussel fauna was examined using archaeological specimen data excavated by the late James H. Polhemus and Richard R. Polhemus, from the archaeological Loy site and T-22 Cave found between river miles 36 and 26 of the Holston River (Parmalee and Faust, 2006). The first location, the Loy site, was identified as a Dallas phase Mississippian Village (AD 1400) located on the right ascending bank at river mile 24.7 (Parmalee and Faust, 2006). The second site (T-22 Cave) was located along river mile 35.6 on the right ascending bank in Grainger County and dates to Woodland Culture (AD 200 to 600). Between these two sites, 3,064 shells were found and 35 species were identified (Parmalee and Faust, 2006). Of the archaeological shell material excavated, the most numerous species found were: *Actinonaias ligamentina*, *Dromus dromas*, *Lemiox rimosus*, *Pleuronaia dolabelloides*, and *Quadrula intermedia*. A complete species list can be seen in Table 1 (all tables, graphs and figures will be featured as appendices). Importantly, these locations may have served as human created accumulations for discarded shell material (middens) over a lengthy period, unaffected by the distributing effects of the water channel. However, the proximity of these archaeological sites to the river reinforce the idea that the shells found within these locations originated from the Holston River and could be considered an approximate representation of the mussel fauna that was present at the time (Parmalee and Faust, 2006).

Modern

Many of the species identified in the excavation of the archaeological sites of Loy and T-22 Cave were extirpated from the main channel of the Holston River with the installation of the Cherokee Dam in 1941. Steve Ahlstedt completed a historical mussel survey of the main branch of the Holston River in 1981, in which it was noted that of the species originally found from Loy and T-22, only thirteen species were found below Cherokee Dam (Ahlstedt, 1992b). In another survey of the main branch below Cherokee Dam completed in 1993, of the archaeological species previously excavated, nine are now known to be extinct, twenty are listed as being endangered, four are currently threatened, nineteen species are listed as being of special concern, and eighteen are established as being currently stable (Williams et al., 1993). Another collection by Parmalee and Faust (2006), in which the archaeological species list taken from Loy and T-22 were compared to modern day species counts noted that 16 of the original 35 species remain in the channel, with *Lexingtonia dolabelloides* being completely extirpated from this branch of the river (Parmalee and Faust, 2006).

Mussels: Ecological Record Keepers

Freshwater mussels are natural record keepers of water quality through seasonal and yearly growth as shown through their shells. The shell of a mussel is secreted through the mantle, which is an expansion of the body wall found underneath the external portion of the shell nearer the actual mussel body (Haag, 2012). However, the three main layers of a mussel's shell (the periostracum, the prismatic layer, and the nacre) are secreted at different rates and at different points of a mussel's lifespan. The prismatic layer and nacre are the internal layers of a mussel's shell and are composed of thin sheets of calcium carbonate crystals mixed into an organic matrix made up of conchiolin protein (Haag, 2012). Meanwhile, the periostracum is the external delicate protein-rich layer that varies from species to species in color and texture. The prismatic layer and the periostracum are produced at the mantle's edge and are associated with the

developing shell margin. The nacre is the only part of the shell that is continuously secreted along the entire inner surface of the shell (Haag, 2012) and is the portion of the shell that contributes to the thickening and strengthening of the shell as the mussel ages.

Shell enlargement and growth occurs mostly during the warmer months of the year and stops or slows down during the colder months (Howard, 1922; Negus, 1966; Dettman et al., 1999). Consequently, in most bivalves, seasonal variation occurs within the shell by the ratio of organic matrix to calcium carbonate, causing the formation of yearly annuli (Day, 1984; Lutz and Clark, 1984). Shell secreted during the primary growth season will have a higher concentration of calcium carbonate (aragonite) when compared to the surrounding organic matrix. In contrast, once the prime growth season has ceased, the nacre will have a higher concentration of organic material to calcium carbonate (aragonite). The growth annuli produced during the primary growth season will appear lighter or whiter in color (due to the higher ratio of calcium carbonate) while the annuli produced during the non-growing season will appear darker in color (because of excess organic material). Provided there is not any large temperature fluctuation or other major disturbance affecting growth during the year, it is possible to measure annuli thickness and calculate yearly and seasonal growth rates within individuals of a mussel population. This method has been used to measure the human impacts on growth in conjunction with historical changes in water quality, the impacts of handling on the longevity of freshwater mussels, and growth patterns between freshwater mussel species (Haag and Commens-Carson, 2008; Brown and Daniel, 2014; Ehlo and Layzer, 2014).

Possible ecological effects of human development, including the building of hydroelectric dams and coal-powered steam plants and contamination caused by other water pollutants, are apparent in several different trophic levels of a given water body. As a result, as seen within the river continuum concept, the ecological effects on one trophic level can cascade to other trophic levels and

can disrupt the stability and function of each other (Vannote et al., 1980; Vaughn, 2008). Included in one of the “lower” trophic levels of freshwater systems are freshwater mussels, as these creatures are benthic or reside on/in the underlying channel bottom. Freshwater mussels feed via a siphoning action through one of the siphons located on the posterior end of their body. This method of feeding not only makes them more susceptible to changes in food availability and nutrient content, but also makes them highly sensitive to water quality (e.g., mineral and nutrient content, heavy metals, sedimentation) and composition (e.g., pH, dissolved oxygen) (Nichols et al., 2005). Due to this sensitivity, freshwater mussels are effective biological indicators of water quality and ecological health.

For these reasons, freshwater mussels may be considered good indicators of human impacts on aquatic habitats. Such impacts include: the building of dams that reconstructs the natural flow regime of a given water body, runoff into local waterways that may contain pesticides (as shown in Strayer, 2014), fertilizers or coliform bacteria, habitat alteration through sedimentation, channel alteration, and the removal of natural habitat structures. It should therefore be possible to use individual relic specimens to identify previous (historical) human impacts on the growth of these individuals.

Dating and Aging Methodology of Freshwater Mussels: Growth Annuli Analysis, Racemization and Low Precision C14

Growth Annuli Analysis

Growth line (annuli) counting is a common approach to age freshwater mussels and approximate growth. The major premise is to count the layers or growth annuli that are formed (and exposed within the nacre of the shell) as a mussel ages. It is possible to measure yearly and seasonal growth rates within individuals of a mussel population using these annuli. This method has been used to validate the human impacts on growth, the impacts of handling stress on longevity of freshwater mussels, and growth patterns for freshwater mussel

species (Haag and Commens-Carson, 2008; Brown and Daniel, 2014; Ehlo and Layzer, 2014).

Exposed inner portions of the shell can be analyzed using a compound light microscope or scanning images into a program, such as WinDendro that is traditionally used for tree-ring analysis (Guay, 2014; Arenas-Castro et al., 2015) to allow for the annuli to be counted and measured.

Racemization (AAR)

Amino acid racemization is a relative dating method that has been applied to numerous fossil taxa including: foraminifera, bone, eggshells, teeth, ostracods, and mollusks (Martin, 1999). This approach can give an estimate of time since death by measuring the ratio of the configuration of the α particle or isomer of an amino acid found in the organic matrix of the shell (Demarchi et al., 2011). This process is species specific, as the interpretation of the results of this method is dependent on average lifespan of the species and amino acid conversion rate and is calibrated using age calibration curves (Kosnik et al., 2008; Krause et al., 2010). The amino acids used for this analysis are found within the conchiolin protein in the organic matrix that make up the nacre and prismatic layer of a mussel shell. When an individual is alive, the central α particle of the amino acids that make up the conchiolin protein is in the left-hand image (*laevo*), and is denoted as having an L configuration. As that individual ages and eventually dies, the configuration of the central α particle of the amino acids within that conchiolin protein change to having a right-hand image or D (*dextro*) configuration. Previous work with freshwater bivalves using this method was conducted on *Lampsilis* to construct an advanced timeline of historical freshwater events (Brown et al., 2005; Huntley et al., 2012; Kaufman, 2006).

This method was incorporated into this project to estimate the time between death and discovery (collection for my analysis), therefore giving a more reliable estimation of the period in which these mussels lived in the Main Branch of the Holston River.

Low Precision C¹⁴ Dating

Otherwise referred to as low precision radiocarbon dating, this method measures the amount of the negative carbon ion concentration in reference to C¹⁴ relative to C¹². This method differs from traditional C¹⁴ dating by incorporating the use of an iron catalyst that is mixed directly with the calcium carbonate sample. This mixture is then placed into a targeted aluminum tray that allows for the direct measurement of the negative ion concentration, thus giving an estimate of geological time. This is a relatively new method (McIntyre et al., 2011; Bush et al., 2013; Longworth et al., 2013; Wood et al., 2015; Grothe et al., 2016). Also described as rapid screening, this method can circumvent the steps of leaching, hydrolysis, and graphitization and conduct C¹⁴ analysis within a much shorter time frame as opposed to the time it takes to complete traditional C¹⁴ dating analysis (Bush et al., 2013; Grothe et al., 2016). This analysis has been performed on deep-sea and shallow-sea corals (Bush et al., 2013; Grothe et al., 2016), but has not been used on freshwater invertebrates.

Modern and Relic Freshwater Mussel Shells: The Dating Game

Rationale and Goal

If it is possible to determine the approximate time of death, geologic age and approximate yearly and seasonal growth rates of a given mussel using the internal growth annuli, are the effects of changes in water quality and ecological habitat apparent in growth and mortality rates? Three different techniques, growth annuli analysis, racemization and low precision C¹⁴ dating, are used to age and date recently dead and relic shell specimens ontogenetically and chronologically. Using the methods of racemization, and low precision C¹⁴ dating, it may be possible to develop a technique to date relic and freshly dead shell material. Subsequently, changes in water quality, correlating ecological history, and growth trends can be compared to see how these changes affected the longevity and mortality rates of freshwater mussels.

Hypotheses

H₀₁: Freshwater mussels of relic and modern age cannot be dated using racemization and low precision radiocarbon dating.

H₁: Freshwater mussels of relic and modern age can be dated using racemization and low precision radiocarbon dating.

H₀₂: No distinct differences in growth are seen in yearly growth annuli of modern and relic freshwater mussel material.

H₂: Distinct differences in growth are seen in yearly growth annuli of modern and relic freshwater mussel material.

Approach

Collection locales were situated within the main branch of the Holston River, Tennessee, USA. The first collection site is below the Cherokee Dam along river mile 30 of the main branch. Relic and freshly dead mussels was collected from this area. The second collection area was located SE of Surgoinsville, TN between river miles 119 and 120 and only relic shell material was available from this site.

A total of 241 specimens ranging across four selected species were collected from these two areas of the main branch of the Holston River. The method for aging freshwater mussel material being tested was the analysis of growth annuli using the tree-ring measuring program WinDendro (Guay, 2014; Arenas-Castro et al., 2015). Racemization and low precision C¹⁴ dating were used to date (geologically) the relic and modern shell material to a point in time. I processed 178 of shells using WinDendro, racemization, and low precision C¹⁴ dating.

All 178 specimens were measured individually using growth annuli analysis and WinDendro. Subsequently, 18 relic shells from both sites were dated using amino acid and low precision C¹⁴ techniques to estimate absolute time since death. Additionally, 5 freshly dead shells were racemized to determine time of death as relevant to the species lifecycle and act as a control for racemization of relic material. Data from growth annuli analysis, racemization and

low precision C¹⁴ dating were combined to determine changes in growth and time at death.

CHAPTER 3: MATERIALS AND METHODS

Collection Site Locations

Along the Main Branch of the Holston River, two primary sites were chosen for collection of mussel specimens (Figure 3).

Site One: McBee Island

The first collection site was McBee Island, located on river mile 30 with exact coordinates of N 36.10368 W 83.63297 (Figure 4). This location is a site of possible historical archaeological significance due to evidence of aboriginal or Native American inhabitation near the local area (Polhemus and Parmalee, 2004). Covered with native and invasive plant species (most notably privet and river weed), the soil of the island is composed of mostly clay and fine-grained sediments (Figure 5). The soil is highly compacted with substrates ranging from a 5/4 (brown) to a 4/3 (brown) according to the 7.5YR Munsell Soil Color Chart. Relic shell material was collected along the northwest side of the island. Additionally, shell material recently eroded out of the lower strata of the island and has been observed to be an area of active shell erosion at throughout the year (H. Faust Per. Comm.). However, this portion of the island could be acting as a sand bar, or an area in which moving water slows down and there is a reduction in velocity casing the river to deposit shell material. Therefore, shell material from upstream could be deposited in this area on the banks of the island in addition to shell material eroding out of the lower strata of the island.

The Holston River that flows along McBee Island, which is covered with silty soil, contrasts with the local island in strata. Aquatic reeds and ferns are prominent during the spring and summer months. Small cobbles litter the bed of the main channel; however, rocks and boulders lie within the riffles of the channel. Riffles were found in numerous places along this portion of the river. Trees and other vegetation along the shoreline extended for 3 to 5 feet into the channel, lending shade to a small portion of the channel. No fallen logs or debris

were observed. However, vegetation (ferns and reeds) within the main channel either side of the island provided possible habitat for fish and other benthic invertebrates.

Site Two: SE Surgoinsville, TN

The second collection location was located southeast of Surgoinsville, TN with exact coordinates of N 36.47024 W 82.83567 (Figure 6). Located above the Cherokee Dam, this area was directly accessible via vehicle. The site of collection was surrounded by residential and agricultural land, with some riparian vegetation acting as a buffer.

In contrast to the sedimentology of the first sight, the underlying channel bed was mostly layers of limestone bedrock (Figure 7). Little to no gravel or cobble was present, and nearer the shoreline. Loamy and muddy quartz-rich and medium-grain soils extended about 4 to 6 feet from the bank. According to the 7.5YR Munsell Soil Color Chart, the soils of the bank of this site ranged from a 6/2 (pinkish grey) to a 5/2 (brown) with a hint of 5/3 (brown) in some places. The stretch of river was mostly runs with a few riffles peppering the central portion of the channel. Isolated areas nearer the bank had shallow pools with fallen trees. Little to no aquatic vegetation was found within the channel.

Shell material was collected along the northwest bank, as shells were scarce nearer the central portion of the channel. Unlike the McBee Island location, shell material was actively eroding out of the banks (Figure 8).

Collection Procedure and Events

Freshwater mussels of four species (*Actinonaias ligamentina*, *Amblema plicata*, *Elliptio crassidens*, and *Pleurobema rubrum*) were collected on five separate collection trips (Table 2). The first collection trip was on October 21st, 2016 at McBee Island, during which only relic shell material was collected. The collection origin point is documented as N 36.10925 W 83.63596.

The second collection trip occurred on February 19th, 2017 at McBee Island and only relic shell material was collected. The geographical coordinates for origin of collection are N 36.10925 W 83.63596.

The third collection event occurred on March 10th, 2017 at McBee Island. Only relic shell material was collected. The geographical coordinates for the origin of collection are N 36.10925 W 83.63596.

The fourth collection trip was on August 7th, 2017. This marks the only collection event in which live shell material was collected exclusively (*Actinonaias ligamentina*). The geographical origin coordinates for this location are N 36.10243 W 83.6695.

The fifth collection was on August 28th, 2017. This was the only collection event that took place southeast of Surgoinsville, Tennessee along the main branch of the Holston River. The geographical coordinates for this site are N 36.47024 W 82.83567.

Freshwater Mussel Species Biology and Ecology

The four species chosen for investigation in this study were selected due to adequate abundance, known ecology and distribution, and being known to be species substantially declining in abundance due to human impact, discussed below.

***Actinonaias ligamentina* (Lamarck, 1819)**

Known as the Common Mucket, *Actinonaias ligamentina* has a thick shell and is usually oval to elliptical in shape. The periostracum ranges from tan to light brown with notable dark bands running from the dorsal to the ventral side of the mussel. The nacre is commonly a creamy white color with heavy pseudocardinal teeth portrayed near the umbo or beak.

This species is ordinarily found within the shoals and runs of larger creeks and rivers in gravel and cobble substrates within the Tennessee River and other drainage (Williams et al., 2008). Males and females reach sexual maturity as early as four years old (Jirka and Neves, 1992). This species is classified as a

long-term brooder, as it becomes fertile or gravid around August, with glochidia reaching maturity typically in October, and brooded until July (Jirka and Neves, 1992). Known as a generalist, this species utilizes several different fish as possible hosts for glochidia (Williams et al., 2008). This species is reported as currently stable throughout its documented range, but may be endangered in particular drainage areas (Garner et al., 2004, Williams et al., 2008; Cordeiro, 2011; AFS, 2014).

Actinonaias ligamentina is also recognized as a species that is and has been encountered in the Holston River (main branch and forks) both currently and historically (Williams et al., 2008).

***Amblema plicata* (Say, 1817)**

Known as the Three-Ridge, *Amblema plicata* ranges in shell morphology depending on where the organism is found in the headwaters of larger streams or in smaller tributaries. Traditionally round to quadrate in shape, *Amblema plicata* is identified by the considerable oblique plications (usually three in total, even seen in younger individuals) that cover a great portion of the posterior side (additional indentations may be seen). Those found in larger streams and within the headwaters have a higher incidence of pustules and irregular concentric ridges running along the posterior side (Williams et al., 2008). The periostracum ranges from tawny to dark brown or black, depending on age of the individual (younger individuals will have a tawnier to light brown periostracum) and the habitat: those from smaller streams and tributaries often are found with a tawny nacre (Williams et al., 2008). *Amblema plicata* in smaller tributaries tend to have a more inflated shell shape, as opposed to the more compressed individuals found in larger streams and within the headwaters (Williams et al., 2008). The nacre color of this species is traditionally white, however, a purple or pinkish tint nearer the umbo is not uncommon (Williams et al., 2008). Notable pseudocardinal teeth with longer lateral teeth are common, as is the deep umbo cavity (Williams et al., 2008).

This species is very widespread, occurring in several different river drainages including the Mobile Basin, the Great Lakes Basin, the Mississippi Basin, the Hudson Bay Basin, the Cumberland River Drainage and the Tennessee River Drainage (Burch, 1975; Butler, 1990; Williams et al., 2008; Dawley, 1947; Cicerello et al., 1991; Parmalee and Bogan, 1998; Ahlstedt, 1992 and 1992a). Within the Mobile Basin and the Tennessee River drainages, this species is not usually found within the upper Tallapoosa River drainage and parts of the Coosa River drainage (Williams et al., 2008).

Preferring the shoals and pools of larger creeks and rivers, this species can also be found in impounded areas of larger rivers as well as natural lakes and man-made reservoirs (Williams et al., 2008). *Amblema plicata* reaches sexual maturity at around four years (Stein, 1969) and becomes gravid from May to August according to where it is found (Howells, 2000). As a generalist, this species has no specific preference for hosts of glochidia (Williams et al., 2008). Also, the rate of growth can vary according to habitat (Stansbery, 1970 and 1970a). Currently, the conservation status of this species is listed as stable as of 2012 by the IUCN Redlist for endangered species (Cordeiro and Bogan, 2012; AFS, 2014). However, strict harvesting regulations have been put forth for the benefit of this species as it is commonly used for producing cultured pearls and harvested for commercial industry (William et al., 1993; Waller et al., 1999; Williams et al., 2008). Its longer lifespan is the reason why this species was selected for this study.

***Elliptio crassidens* (Lamarck, 1819)**

Known as the Elephant Ear, *Elliptio crassidens* has a thick and moderately inflated shell that is normally rhomboid to subtriangular in shape (Williams et al., 2008). The periostracum is dark brown to near black with faint dark green rays being present in younger individuals. The color of the nacre widely varies from white to salmon or pink. Heavy and triangular pseudocardinal teeth are present near the umbo or beak.

Elliptio crassidens is widespread and commonly found in stable substrates such as cobble, sand, mud, and gravel of creeks and rivers found within the Tennessee River and Alabama river drainages (Williams et al., 2008). This species is known to be a short-term brooder, becoming gravid in April to August in water drainages from the Apalachicola Basin (Brim Box and Williams, 2000; O'Brien et al., 2003) and becoming fertile in areas of Alabama from April to June (Coker et al., 1921). As a generalist, this species has responded to several different fish as possible hosts for glochidia (Williams et al., 2008). This species is reported as currently stable throughout its documented range, but may be declining in areas or vulnerable (Williams et al., 1993; Cummings, 2011; AFS, 2014).

***Pleurobema rubrum* (Rafinesque, 1820)**

The common name for *Pleurobema rubrum* is the Pyramid Pigtoe. This species has a moderately inflated and thick shell that is predominately triangular in shape with a sharp twist of the umbo. The periostracum is greenish brown to reddish brown in color that can darken with age (Williams et al., 2008). This species can also have weak green rays that extend from the umbo towards the growth margin that fade with age. The color of the nacre varies from white to pink with thick, low, and triangular pseudocardinal teeth portrayed near the umbo (Williams et al., 2008).

Pleurobema rubrum prefers sandy and gravel substrates within shoal habitats of medium to large rivers (Williams et al., 2008). This species is found in drainages of the Mississippi Basin, the Cumberland River, the Ohio River headwaters, and the Kentucky and Tennessee drainages (Havlik and Stansbery, 1997; Ortmann, 1909; Cirerello et al., 1991; Parmalee and Bogan, 1998). This species is a short-term brooder, as it becomes gravid around late May to late July (Ortmann, 1919). Being a specialist, this species relies on particular fish host species for the survival of its glochidia. The fish that have been established as possible fish hosts are *Lythrurus fasciolaris* (Scarlet Shiner), *Erimystax dissimilis*

(Streamline Chub), *Cyprinella spiloptera* (Spotfin Shiner), and *Notropis photogenis* (Silver Shiner) (Culp et al., 2006). Due to this limited fish host list and impairments of the waterways within its documented range, this species is listed as threatened and endangered throughout its documented range (Stansbery, 1970; Bogan, 1996; AFS, 2014).

Shell Specimen Preparation

After collection, all relic and recent shells were transferred to the McClung Museum of Natural History and Culture located on the campus of the University of Tennessee, in Knoxville, Tennessee, USA. For the live specimens (*Actinonaias ligamentina*), all soft tissue was removed by hand prior to being moved from site. Following collection, all shells (recently dead and relic) were handled with gloved hands to minimize possible carbon contamination throughout the rest of the preparation procedure. Each shell was then cleaned using a soft bristled toothbrush and cool water to remove any excess algae and remnants of sediment from the river. After cleaning, each shell was allowed to air dry in a temperature and humidity controlled area of the archaeology processing and storage section of the McClung Museum and subsequently stored within a plastic storage container.

Each mussel valve was sawed in half medially from the tip of the umbo to the ventral margin of the growth plate. This exposed the growth annuli as was required for the growth line counts. All recently dead shells of *Actinonaias ligamentina* were then separated from the relic shells of the other species and packaged to be sent for chemical analysis (racemization and rapid radio carbon dating). Each shell specimen sent for chemical dating was used for both procedures of racemization and low precision C¹⁴ dating. Following this procedure, the exposed inner surface of each relic shell valve was then polished using two different levels of grit, the first being 400 grit and the second being 900 grit, using a powered sander or by hand using glass plates. After each valve was polished, the exposed inner surface of each relic valve was dipped into 10%

hydrochloric acid solution to etch the growth annuli present within each of the valves. Note that each mussel has two valves, a right and left valve. Shell and valve are used interchangeably throughout this paper.

In total, 241 shells of the four species were collected between the two sample sites. Of the suitable shells (those able to be sawed, polished and dipped into the hydrochloric acid solution while remaining intact), 180 shells were chosen to be analyzed for growth annuli, racemized, and dated using low precision C¹⁴. Of the original 180 shells, 23 shells were racemized. Of the 23 that were racemized, 19 were subjected low precision C¹⁴ dating.

Of the 23 shells that were dated using racemization and low precision C¹⁴, ten represented the species *Actinonaias ligamentina*, three shells represented *Elliptio crassidens*, six represented the species *Amblema plicata* and the remaining four were *Pleurobema rubrum* (Table 3).

For the species *Actinonaias ligamentina*, five of the ten shells were from freshly dead individuals, and so only racemization was performed. These individuals served as a control group to determine if the methods of racemization and low precision C¹⁴ dating could properly date these specimens, as time of death was known.

Aging and Dating Methodology

Growth Line (Annuli) Counting

The growth line (annuli) counting method counts the layers or growth annuli that are formed as a mussel ages and are formed within the nacre of the shell. By counting these growth annuli, the age of the mussel upon death and amount of yearly/seasonal growth can be determined. The wider the growth annuli, the more growth occurred during that year/season (Haag, 2012). Upon cutting medially through either valve of a mussel nearer the umbo (Figure 9), growth annuli are visible within the nacre of the shell (Figure 10).

An important aspect of this analysis is how the growth annuli are exposed, as it affects the ability for them to be seen and counted. One of the most

common ways is to create a thin section of one of the valves medially through the umbo (the dorsal, mostly pointed end of the shell). This is done by slicing a thin section of the interior portion of the nacre and using a form of clear epoxy (specific type dependent on availability and shell density), and then gluing the section to a glass slide. The thin section can then be polished by sanding via a fine grit piece of sand paper to ensure that the annuli are not obstructed. For this study, each shell was first cut medially to expose the nacre's surface (Figure 1). Each shell was then etched using a 10% hydrochloric acid solution for 23.7 seconds, to allow for further visualization of the annuli. Following this, the internal surface polished using 600 and 400 level grit by hand. Allowed to air-dry, each shell was then directly scanned into the WinDendro program (Guay, 2014) without processing thin section slides or acetate peels.

Amino Acid Racemization (AAR)

Amino acid racemization estimates the relative time since death and its discovery (collection for study). To clarify, this method estimates the amino acid conversion rate and categorizes it as “young, middle or older” stage of decay following death and gives an estimate of time from death until collection and testing. This procedure is species dependent, as each mussel species varies in life expectancy and conversion rate due to differences in life history and physiology.

Amino acid racemization measures the ratio of the configuration of the central alpha particle or isomer of an amino acid (Demarchi et al., 2011). The amino acids primarily used for this analysis are found within the conchiolin protein that resides in the organic matrix that makes up the nacre and prismatic layer of a mussel shell. When the individual is alive, the central alpha particle of the amino acids that make up the conchiolin protein are in the shape of an L (*laevo*), and is denoted as having an L configuration. However, when the individual dies, the configuration of the central alpha particle of the amino acids within that conchiolin protein change to having a D (*dextro*) configuration. The ratio of D to L

configurations within individuals of many different species has been measured, and can be used to estimate the relative time of that individual after death until collection using the estimate of that species' lifespan (Kaufman and Manley, 1998).

Most geochronological laboratories use gas chromatography (GC) or high-performance liquid chromatography (HPLC) to separate the individual amino acids being measured (Kaufman and Manley, 1998). The most widely used of these two options is HPLC through ion-exchange chromatography (IEC) due to its minimal required preparation and straightforward automatization (Kaufman and Manley, 1998). The major disadvantage of IEC is that it can only be applied to specific amino acids, specifically alloisoleucine and isoleucine (Kaufman and Manley, 1998). The current method being employed by the Northern Arizona Geochronology Lab, led by Dr. Darrell Kaufman, uses a process that combines HPLC and reverse phase chromatography (RPC) to separate the DL amino acid pairs more efficiently and allows the use of more amino acid pairs (Kaufman and Manley, 1998). RPC is different from IEC because it can separate the DL amino acid pairs prior to being placed into a reverse phase column. This practice allows increased precision in separating the DL pairs and overall takes less time (approximately nine amino acid pairs per 75 minutes; Kaufman and Manley, 1998).

This procedure used a chromatograph (Hewlett-Packard HP1100 liquid chromatograph, Palo Alto, California) equipped with a quaternary pump and vacuum degasser, an auto-injector and auto sampler, and an HP1046A programmable fluorescence detector (Kaufman and Manley, 1998). The detector employs a xenon-arc flash lamp (frequency 55 hertz) and a 280 nanometers cut-off filter that functions at an excitation wavelength of 230 nanometers and an emission wavelength of 445 nanometers (Kaufman and Manley, 1998).

Pre-column derivatization of DL-amino acids with the use of o-phthaldialdehyde (OPA) in combination with chiral thiol, N-isobutyryl-L-cysteine (IBLC), to generate fluorescent diastereomeric byproducts of primary amino

acids are examples of the amino acids used (Kaufman and Manley, 1998). The derivatization of these amino acids is performed in sequence prior to each injection using the automated features of the HP1100 auto-injector. A derivatizing reagent (specifically for these amino acids) is collected by a syringe and injected consecutively onto an off-line sample loop both before and after the sample solution. The reagent and sample are then blended using a motorized plunger and then placed into the reverse phase chromatography column (Kaufman and Manley, 1998).

The amino acids are then separated using a linear gradient of aqueous sodium acetate, methanol, and acetonitrile (Kaufman and Manley, 1998). Computer software by HP Chemstation (Hewlett-Packard Development Company, L.P., California) performs the integration and calibration of the fluorescence signal and controls the eluent gradient and automated sample derivatization and injection program (Kaufman and Manley, 1998). Experimentation for ten pairs of racemic DL amino acids have been conducted at the Northern Arizona Amino Acid Geochronology Laboratory including, aspartic acid (Asp), glutamic acid (Glu), serine (Ser), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), phenylalanine (Phe), leucine (Leu), the diastereomer pair d-alle (alloisoleucine) and l-Ile (isoleucine), achiral glycine (Gly), and the internal standard l-hArg (Kaufman and Manley, 1998).

To calculate the DL Ratios, a macro (input as a user-defined value in the report layout of HP Chemstation; Kaufman and Manley, 1998) analyzes the peak wavelengths of these amino acids in a state of hydrolysis and electronically produces the ratios for each amino acid (Kaufman and Manley, 1998). The replicability of this method for nine DL Ratios averages about 7%. Asp and Glu DL ratios are considered the most reliable and consistent, with analytical variations of 2% and 3%, respectively (Kaufman and Manley, 1998). Subsequently, DL ratios in three fossil mollusk samples using this RPC method have measured as being within ± 1 standard deviation analytical error and ± 1 standard deviation inter-laboratory variation (Kaufman and Manley, 1998).

Racemization was conducted for 24 specimens from both collection sites. Experimentation for eight amino acids and subsequent pairs were done at the Northern Arizona Amino Acid Geochronology Lab including, aspartic acid (Asp), glutamic acid (Glu), serine (Ser), alanine (Ala), valine (Val), phenylalanine (Phe), leucine (Leu), and I-Ile (isoleucine). Total concentrations in picomoles per milligram (pMol/mg) were measured for each amino acid as well as phenylalanine (Phe).

Rapid Radiocarbon (Low Precision C¹⁴) Dating

Rapid radiocarbon dating, otherwise referred to as low precision C¹⁴ dating, uses an enriched iron (Fe) catalyst and specialized aluminum tray to conduct C¹⁴ dating. This method can circumvent the steps of leaching, hydrolysis, and graphitization and conduct C¹⁴ analysis within a much shorter time frame than materials processed using traditional C¹⁴ dating methods.

Sonication with deionized water is used to cleanse the shell material prior to treatment (Bush et al., 2013). Using a mortar and pestle, material (less than 1g in most instances) is ground to a fine powder. Opposed to high precision ¹⁴C AMS dating that requires carbonate samples to be prepared with leaching, hydrolysis, and graphitization prior to analysis, this newer method bypasses this process by combining powdered shell material (must be composed of calcium carbonate) with a powdered iron (Fe) catalyst (Sigma-Aldrich-400 mesh, 99.9% pure; Bush et al., 2013). This mixture is then placed within a small targeted aluminum tray to be placed into a calibrated accelerator mass spectrometer. To calibrate the spectrometer and to normalize the ¹⁴C/¹²C ratios that are present within marine material, three primary standards of oxalic acid (OX-I) >0.7 mg C graphite are used per wheel (Bush et al., 2013; Grothe et al., 2016; Santos et al., 2007). Following this calibration, samples are then placed within the spectrometer. This preparation and the powdered iron catalyst allows for the direct measurement of negative carbon ions allowing analysis to be completed within a relatively short time frame of approximately 72 hours (Bush et al., 2013;

McIntyre et al., 2011). Analytical accuracies ranging from $\pm 1.8\%$ (1 standard deviation) can be achieved on carbonate samples younger than 10 thousand years BP (before present) (Bush et al., 2013).

Radiocarbon concentrations were given as fractions of the modern standard, DC^{14} and conventional radiocarbon age according to the standards of Stuiver and Polach (Radiocarbon, v. 19, p.355, 1977). All results were modified or calibrated to account for isotopic fractionation (Stuiver and Polach, 1977), and DC^{13} values were determined using separately prepared graphite in conjunction with the use of the AMS (Accelerated Mass Spectrometry) spectrometer.

Analytical Equipment and Methods

Water Quality Testing: pH

pH testing was completed using a fisher scientific pH meter pen. Water samples were taken using a sterilized syringe, and placed into a sterile 10 ml graduated cylinder. The pen was calibrated before each use using 3 tablets and was left to sit in the water sample for 1.5 minutes. Three different measurements were taken during site observation visits at each collection site (which were done shortly before freshwater mussel collection events), with a total of six measurements being taken. Testing was completed on site.

Because only one mussel collection event occurred at SE Surgoinsville, it is important to note that one pH recording was done prior to the collection event while the other two pH readings occurred one week after the collection event at this site.

WinDendro

Each specimen or valve (only one from each specimen) was scanned directly after polishing and processing using an Epson 10000 XL scanner, that transferred the image to the WinDendro program (Guay, 2014). This program was originally used to observe the growth rings of trees and in doing so, estimate the growth of the tree. Each valve was scanned into this program using 48-bit

color, approximately 9,600 dpi and 0.001 μm resolution. This program automatically counted the annuli of each mussel specimen along with the space in-between each growth line, approximating seasonal growth and dividing it between early (winter) and late (summer) growth. The individual yearly growth for each specimen was calculated using the growth line nearest the ventral side of the mussel and measuring toward the inner cavity in micrometers per year, allowing for adjustment of any automatically detected annuli and calibration for precise measurement of summer and winter growth thickness.

R Statistical Programming and Data Analysis

To analyze patterns in data from the dating techniques and observed growth, multivariate correlation analysis was conducted using the R statistical program. Analysis of variance (ANOVA) models and Akaike information criterion (AIC) correlation analysis (Crawley, 2013) were used to detect patterns between growth, size, and longevity of the mussel specimens (both within species and across species) across yearly growth. Linear regression was used to look at species-specific site populations and yearly growth within and across the four species (Crawley, 2013).

To normalize the distribution of these growth measurements, each data set were log transformed and values of 0 (growth and correlating year) were removed.

CHAPTER 4: RESULTS

Water Quality: pH Testing

Six samples of pH were taken from the two collection sites (Tables 25 and 26). From McBee Island (Site 1), pH ranged from 7.89 to 8.37 beginning with the first collection event. For SE Surgoinsville (Site 2), pH ranged from 6.22 to 7.41.

Though the waters of the Holston River are noted for being slightly more acidic nearer the headwaters due to the acidic water-soluble rocks found within the karst region, it is possible that the quartz, limestone, and shale that dominate the Main Branch of the Holston River further downstream (TDEC, 2007) somewhat neutralize the acidity of the water flowing from upstream.

Low Precision C¹⁴ Dating

Low precision C¹⁴ dating was conducted on 19 relic valves, ranging across all four of the chosen mussel species from both collection sites.

McBee Island (Site 1)

This method was used to test 10 relic valves of the total 19 shells found from all four species (Table 4). For *Actinonaias ligamentina*, specimens ranged in geologic time from 1760 BP to 2320 BP with an error range of 60 to 70 years. Dates for *Elliptio crassidens* extended from 1830 BP to 2110 BP with an error range of 70 years. Specimens of *Amblema plicata* ranged from 2330 BP to 2210 BP with an error range of 70 years. Lastly, those of *Pleurobema rubrum* were estimated to date to 2120 BP with an error window of 70 years. The geologic ages of these relic shells correlate with the archaeological Woodland and Mississippian Period (Polhemus and Parmalee, 2004) habitation timeline of the Holston River in which shell middens were common, which may explain how relic shell found from this site can be eroding out of the banks in larger quantities, be relatively close together in geologic age, and be so well preserved taphonomically.

SE Surgoinville (Site 2)

Nine relic valves of the total 19 were dated using low precision C¹⁴ techniques (Table 5). Of these valves, 3 were from *Actinonaias ligamentina*, 3 were of *Amblema plicata* and 3 were *Pleurobema rubrum*. For *Actinonaias ligamentina*, specimens ranged in ages from 2120 BP to 2260 BP with an error interval of 70 to 100 years. Specimens of *Amblema plicata* ranged from 2130 BP to 2180 BP with an error interval of 70 to 100 years. Lastly, those of *Pleurobema rubrum* ranged from 2100 BP to 2230 BP with an error window of 70 to 90 years. As with the shell material found from site 1, geologic ages of the shells of site 2 also correlate with the Woodland and Mississippian Period of the regional archaeological timescale (Polhemus and Parmalee, 2004) habitation timeline of the Holston River. The dates for these shells show an even closer relationship, being closer individually in geologic age, also supporting an instance of rapid death and burial, similar to shell material of site 1.

Racemization

For shell material from both collection locations, the amino acid ratio was for alanine (Ala) exceeded the limits of detection by the spectrometry equipment. No individual concentrations of D and L configured amino acids were given for this series of experimentation.

Ratios of the amino acids are variable to interpretation and are species dependent due to differences in life expectancy (age calibration curves); therefore, methods for interpreting these results are still being revised when dealing with freshwater bivalves (Kosnik et al., 2008). Still, this method has been used with other archaeological biologic materials (such as bone), and is still considered a viable method for giving reliable results (Martin, 1999). Specimens from both sites had low ratios (closer to 0) for most of the amino acids being tested, with the highest values being observed for aspartic acid (Asp).

McBee Island (Site 1)

Ten relic and five recent valves from McBee Island were tested using the racemization method: eight of these valves were *Actinonaias ligamentina* (three being relic and five fresh dead), three as *Elliptio crassidens*, three for *Amblema plicata* and one for *Pleurobema rubrum*.

For the relic specimens of *Actinonaias ligamentina*, amino acid ratios spanned from 0.003 (DL Val ratio of specimen 2) to 0.175 (DL Ser ratio of specimen 1). This range suggests that these samples were also in the initial stages of degradation, thus, early stages of amino acid conversion. For the five recent shells of this species, amino acid ratios ranged from 0.005 (DL Val ratio of specimen 4) to 0.117 (DL Asp ratio of specimen five). For *Elliptio crassidens*, this analysis indicated that these specimens were in the very early phases of amino acid conversion, correlating with the known date of death for these specimens. Ratios for this species ranged from 0.003 (DL Val ratio of specimen one) to 0.169 (DL Asp ratio of specimen three). These ratios are relatively small, indicating that these individual specimens were in the early stages of amino acid conversion into the *dextro* configuration. For *Amblema plicata*, ratios ranged from 0.011 (DL Val ratio of specimen two) to 0.144 (DL Asp ratio of specimen one). The individuals tested were also in the preliminary stages of decomposition. Finally, for *Pleurobema rubrum*, the ratio range extended from 0.010 (DL Val ratio) to 0.132 (DL Asp ratio). In contrast to the other specimens of other species, this individual is estimated to be slightly older in ontogenetic age after death, meaning that it was further into the process of breaking down (Table 6). For specimens of this site, the most reliable amino acid ratios are for the amino acids glutamic acid and aspartic acid, as noted by Katherine Whitacre at the Northern Arizona Geochronology Laboratory (personal communication) who conducted the analysis.

SE Surgoinsville (Site 2)

Site 2 yielded 9 relic specimens ranging across all species that were dated by racemization. Three valves were from *Actinonaias ligamentina*, and amino acid ratios extended from 0.008 (DL Val ratio from all three of the relic shell specimens) to 0.113 (DL Asp ratio from specimen 8). These specimens were estimated to be in the preliminary phases of amino acid degradation. Three shell specimens were tested of the species *Amblema plicata*, and the amino acid ratios ranged from 0.010 (DL Val ratio from specimen 3) to 0.172 (DL Ser ratio from specimen 1). Again, specimens appeared to be in the beginning stages of amino acid conversion, according to the lifespan of this species. Finally, 3 specimens of *Pleurobema rubrum* were tested of which the ratios ranged from 0.007 (DL Val ratio from specimen 2) to 0.120 (DL Asp ratio from specimen 3). For this species, specimens were either in the very beginning or half-way through the process of L to D amino acid configuration, according to the shorter lifespan and amino acid conversion rates (Table 7). For this group of specimens, the most reliable amino acid ratios were for glutamic acid and aspartic acid. It should be noted that no specimens of *Elliptio crassidens* were dated, as no specimens of this species (recent or relic) were able to be located and/or properly identified at this collection location.

Growth Analysis

Statistics of Growth

The regressions show a negative relationship between age and growth (Figures 12 through 15). Most of the analysis resulted in positive AIC scores and mostly insignificant p-values when correlating the variables of year and growth. The growth models that were near significant or significant (p-value near or < 0.05) were for relic and recent *Actinonaias ligamentina* yearly and summer growth from site 2, *Amblema plicata* summer growth from sites 1 and summer and yearly growth from site 2, and *Pleurobema rubrum* summer growth from sites 1 and 2.

For AIC modeling selection, I found no evidence that the aspect of year when added to the dependent variable of growth improved the AIC scores for each model. While measuring for Cook's distance to see if any data point could be skewing the distribution and subsequent p-value, nine total data points were skewing the distributions and were deleted (Table 8).

Growth Patterns of Modern and Relic Shell Material

Actinonaias ligamentina was the only species in which recent (date of death known) and relic shells were collected. These recently dead specimens were located near site 1, McBee Island, and were compared to relic material found at McBee Island. The thickness of the growth annuli in the modern specimens were greater than that of the relic shells at the same ontogenetic stage regarding average yearly growth (Figure 16).

Growth Annuli Analysis

Relic specimens of site 1 show that more growth occurred during the summer months than the winter months, based on the thickness in micrometers given by the WinDendro program during annuli analysis (Table 10; all growth tables represent average growth). However, there was an observed dissimilarity for observed growth for the modern specimens of *Actinonaias ligamentina* (site 1), in that there was a noticeable reduction of growth during the warmer months in comparison to the consistently wider growth bands (formed by the cessation of growth) during the winter months. This reduction of growth, during what would be considered the primary growth season, could be attributed to changes in the nutrient levels, pH, or seasonal temperature of the water (TDEC, 2007; TDEC 2017; Bartsch et al., 2017).

To the support the reliability of these data, a pair t-test was conducted to compare specimens of *Actinonaias ligamentina* of the same chronologic age at the same location (modern specimen 1 and relic specimen 1 from site 1). Each test showed a p-value lower than 0 (1.445e-05), supporting that there was a

distinct large difference in means, supporting the alternate hypothesis or that there is an observed difference in growth between relic and modern specimens.

McBee Island (Site 1)

Mussel specimens (all species included) ranged in ontogenetic age from 0.5 years to 103 years old. At death, most of the individuals were relatively young, ranging from 13 to 26 years of age according to growth annuli analysis. Most growth occurred during the summer months for the non-modern specimens of McBee Island. Shell material of this site was relic (aside from the collected recent *Actinonaias ligamentina*) as shown by the results of the low precision C¹⁴ dating as presented above.

The range of growth for specimens of all four species during the winter months ranged from 0 to 2264 micrometers per year in thickness. During the summer, the range for growth varied from 0 to 4310 micrometers per year. The highest rate of growth for summer and winter occurred in the species *Amblema plicata*, which is one of the longest living species of the species collected (Williams et al., 2008).

Actinonaias ligamentina

As another of the longer lived species of mussels in this study, *Actinonaias ligamentina* showed the most variation in age range and ontogenetic growth rate due to the high number of relic shells that were able to be collected.

Relic

The observed ontogenetic range was from 0.5 years to 103 years of age, which was the longest observed ontogenetic range of all species studied. Many of these specimens were estimated to be 15 to 29 years of age. Growth rates for this species ranged from 2 to 2269 micrometers per year. This is quite young when compared to some of the older mussel species previously collected for this study (Table 9). Most growth occurred during the warmer months of the year. Growth for the winter months extended from 2 to 1208 micrometers per year and

the growth during the warmer or summer months reached from 22 to 1608 micrometers per year. Total yearly growth for recent specimens ranged from 21 to 1091 micrometers per year, with most growth occurring during the winter season (Table 10).

Recent

Opposed to the trend we see when looking at most freshwater mussel species, I found a noticeable reduction in growth during the primary growth season (summer or warmer months) with a consistently higher “growth” during the colder (winter) months. The span of growth for the summer months extended from 2 to 1208 micrometers per year. Conversely, the array of growth or laying of excess organic matrix during the colder or winter months extends between from 22 to 1608 micrometers per year (Table 11). Total average growth for the modern specimens ranged from 21 to 1091 micrometers per year (Table 12).

Amblema plicata

Many of the *Amblema plicata* individuals from this site were exceedingly young. The age range of this species was 1 year to 12 years. Yearly growth rates varied from 175 to 5694 micrometers per year (Table 13). Annuli thickness during the winter months ranged from 0 micrometers per year to 2264 micrometers per year. Summer growth rates ranged from 90 micrometers per year to 4310 micrometers per year. Mirroring the results from the relic *Actinonaias ligamentina*, most growth occurred during the summer months rather than the winter from this site (Table 14).

Elliptio crassidens

The ontogenetic range of *Elliptio crassidens* was 6 years old to 56 years old. Most of the specimens found averaged at 11 to 12 years of age according to WinDendro. Yearly growth rates fluctuated between 4 to 1240 micrometers per year (Table 15). Most growth occurred during the warmer months of the year.

The span of growth rates for the summer months extended from 1 to 988 micrometers per year. Conversely, the growth during winter months reached from 0 to 699 micrometers per year (Table 16).

Pleurobema rubrum

Of the four species in this study, *Pleurobema rubrum* is considered to have the shortest lifespan (about 30 years of age). The observed ontogenetic range was 6 to 18 years of age. Most specimens were around 10 years of age. Yearly growth rates for this species ranged from 95 to 2298 micrometers per year (Table 17). While I found little difference between seasons, more growth occurred during the warmer months of the year. The span of growth for the winter months extended from 1 micrometer to 1123 micrometers and the array of growth during the summer months extended from 63 to 1442 micrometers per year (Table 18).

SE Surgoinville (Site 2)

The ontogenetic age of the specimens (across all species) for site 2 ranged from 1 year to 13 years of age, which is generally younger than the ages of specimens from site 1. Growth across species ranged from 0 to 8196 micrometers per year, with the highest rate of growth in the species *Amblema plicata*. Most growth occurred during the summer months. Summer growth rates ranged from 1 micrometer per year to 8008 micrometers per year and winter growth rates varied from 11 micrometers per year to 6689 micrometers per year, with both being the highest in *Amblema plicata*. This group conforms to the natural pattern of more growth during the summer months, and conforms with the relic shell results for site 1.

Actinonaias ligamentina

As the most commonly found species within site 2 (as in site 1), *Actinonaias ligamentina* showed the most variation in age range and ontogenetic growth. The observed ontogenetic range was 1 year old to 8 years of age, which is exceedingly young considering the longer life expectancy. As these were relic shells that were dated to the Woodland and Early Mississippian Period of the archaeological timescale, it is again not possible that post-European settlement activities could have played a role in the premature death of these organisms. Most specimens were 2 to 3 years of age. General yearly growth ranges from 66 micrometers per year to 5588 micrometers per year (Table 19). Resembling the relic sample set from site 1, most growth occurred during the summer months of the year. The span of growth for the winter months extended from 42 to 1532 micrometers per year. Growth during the warmer or summer months reaches from 11 to 4963 micrometers per year (Table 20).

Amblema plicata

Many of the *Amblema plicata* individuals sampled from site 2 were very young. The age range of this species was observed to be from 1 to 9 years of age. Yearly growth rates varied from 0 to 8195 micrometers per year (Table 21). Most growth occurred during the summer months, with annuli thickness ranging from 4 micrometers per year to 8008 micrometers per year. Winter growth ranged from 27 micrometers per year to 6689 micrometers per year (Table 22).

Elliptio crassidens

Unfortunately, no live, recently dead, or relic *Elliptio crassidens* shell material was found in this area of the Holston River. While it has been documented historically that this species was found within this area, it was likely quite rare originally and is even more difficult to locate today (Gerry Dinkins and Hugh Faust, Pers. Comm.).

Pleurobema rubrum

The observed ontogenetic age range for *Pleurobema rubrum* at site 2 was 1 to 13 years at time of death. Most specimens were around 10 years of age. Yearly growth rates ranged from 269 micrometers per year to 5433 micrometers per year (Table 23). There was a statistically significant difference between seasonal growth, with more growth occurring during the warmer months of the year, rather than during the winter. Growth for the winter months spanned from 1 micrometer per year to 4612 micrometers per year. The growth during the summer months ranged from 38 to 2761 micrometers per year (Table 24). This is one of the few species for this group that shows such a drastic difference between summer and winter growth.

CHAPTER 5: DISCUSSION

Modern Specimen Growth and Human Impacts

Reduction of Growth in Modern Specimens of *Actinonaias ligamentina*

A main finding was the apparent reduction of growth during the primary (warm) growth season, as shown by wider growth annuli formed in the winter for recent *Actinonaias ligamentina* from McBee Island (site 1). Typically, freshwater mussel species experience greater growth during the warmer summer months than that of the colder winter months (Haag, 2012). Temperature of the water (seasonal and annual) functions to regulate the incorporation of calcium carbonate into the organic matrix present within the shell of a mussel, as higher temperatures promote a higher ratio of calcium carbonate within the matrix of the shell. In lower or colder temperatures, the amount of organic matrix will be higher than the amount of calcium carbonate. This ratio can either be disrupted or amplified with erratic or intensified changes in temperature (Ganser et al., 2015).

This reduction of growth during the primary season could be caused by colder temperatures within the water system of the Holston River downstream of the Cherokee and Fort Patrick Henry Dams during the primary growth season. Most reservoirs are thermally stratified, meaning that there is a hypolimnetic layer (colder layer usually with less dissolved oxygen and metal accumulation) below the thermocline or nearer the bottom of the reservoir while warmer water remains nearer the surface of the reservoir (Wetzel et al., 1991; Lerman et al., 1995; Beutel and Horne, 1999; Munger 2016). Most water released from the Cherokee and Fort Patrick Henry Dams comes from the bottom of the reservoir, thus colder water is released into the waterbody below. This water can be colder than natural waters below the dam depending on the season, thus disrupting the water temperature of the river downstream. If this cold water is released downstream more often during the spring and summer months, the primary growth season, freshwater mussel growth may be inhibited or stunted as freshwater mussel growth is partially temperature controlled (Haag, 2008; Ganser et al., 2015). This

is discharge variance between the summer and winter months is shown through monthly discharge data from 1941 till 1970 from collection unit 03490500 of the United States Geological Survey (USGS), located in the Holston River at Surgoinsville, TN (USGS, 2018). As the dams are less likely to be operated at high frequency during the winter months (due to less demand for electricity and recreational needs), the temperatures of the water downstream can remain more consistent and perhaps slightly warmer during the winter (possibly as a result of reservoir turnover, that would allow for the release of warmer water during the winter months), resulting in mussels having wider and more consistent “winter” annuli (TDEC, 2007; Haag, 2012; TDEC, 2017, USGS, 2018).

Rate of Growth: Modern vs. Relic

An observed trend of increased growth rate in the recent specimens that could have been caused by changes in water quality or ecological habitat brought on by human influence. For example, it might be possible that the increased number of nitrites, nitrates, and phosphates (fertilizers) listed as impairments for this section of the Holston River could be increasing the amount of nutrients within the water, thus possibly increasing freshwater mussel growth through an excess in food (Atkinson et al., 2013; Strayer, 2014; Bartsch et al., 2017).

Impoundment Structures: Influences on Shell Growth and Mortality

Currently, about 2 million dams exist within the waterways of the United States according to the United States, Army Corps of Engineers (Maclin and Sicchio, 1999). Hydroelectric dams and steam plants along freshwater waterways have been beneficial in providing energy and fulfilling the recreational needs of the people that reside in these areas. However, these structures have greatly altered natural habitats and flow patterns and, subsequently, affected the viability of organisms in these systems.

Several studies of different water drainages show that dams have had a negative impact on mussel diversity in waters once rich in mussels. For example, the number of freshwater mussels once supported in the Hannibal, McAlpine, and Smithland pools along the Ohio River has dropped from a range of 20 to 50 species to just 10 or less (Watters and Flaute, 2010). Within reaches of the Vermillion River Basin, mussel abundance and species diversity has decreased with the operation of three dams: Danville Dam, Ellsworth Park Dam and the Aqua Illinois Dam (or Waterworks Dam). In addition, the removal of another dam within the same river basin (Homer Park Dam) has contributed to the expansion of three mussel species since its removal in 1950 (Tiemann et al., 2016). Moreover, impoundment structures and other forms of river regulation can disrupt the growth of freshwater mussels by affecting natural flooding events that are otherwise beneficial to the species (Rypel et al., 2009).

Though some small impoundments can be beneficial to some freshwater species, distance gradients may control these benefits (Singer and Gangloff, 2011; Hornbach et al., 2014). In the Little River in Oklahoma, a linear correlation was found between increasing species richness and abundance with increasing distance downstream from the dam. (Vaughn and Taylor, 1999). In the Neosho River in Kansas, low-head dams cause habitat fragmentation and affect mean species richness and evenness, though not necessarily abundance (Dean et al., 2012).

Not only does evidence exist to support the negative effects that dams and other impoundment structures can have on freshwater mussels, but there is also evidence on how these barriers can inhibit the movement of fish species (without a fish ladder). Many fish serve as biological hosts for the glochidia of freshwater mussels, thus causing population fragmentation and reduced gene flow (Watters, 1996).

In the headwaters of the Holston River, the Watauga and Wilbur dams impact the contributing water body of the Watauga River. In the main branch of the Holston River, the Cherokee Dam remains one of the major influences on

flow and habitat stability. As many of reservoirs, created by dams that impact the Holston River, are thermally stratified and have a hypolimnetic layer, it is possible that varying released water temperatures can have impacts on the subsequent ecological systems below. Water release and its effect of the temperature of the water released are still being studied. Currently, the South Fork of the Holston River is completely impacted due to the presence of the Boone Dam, South Holston Dam, and Fort Patrick Henry Dam that were built in the 1950s by the Tennessee Valley Authority. Ecological habitat alteration for the South Fork of the Holston River remains one of the biggest impacts on mussels of several species (Parmalee and Polhemus, 2004). Additionally, discharge data from a USGS water quality recording station (Hydrologic Unit Code 06010104) shows annual discharge has remained relatively high, averaging at 4435 cfs since 1954 (USGS, 2018b). This indicates that the amount of discharge is highly controlled by the Cherokee Dam (built in 1941) in which the amount of water released into this system may influence the local ecology and habitat stability for freshwater mussels.

Low Precision C¹⁴ Dating

Examples of low precision C¹⁴ dating methods on freshwater mussel material are limited in the literature. Some have sought to aspects of this method through modification of the powdered catalyst by using titanium instead of iron (Longworth et al., 2013) or by used a continuous-flow accelerator mass spectrometer (CFAMS), instead of a regular accelerator mass spectrometer (McIntyre et al., 2011). Since most of these experiments have dealt with marine organisms, this method is limited in terms of application to freshwater shell material and information on the procedure. Therefore, exact location of the sampling of shell material from the fossil specimen is important due to how the shell of a freshwater mussel grows. To obtain an accurate account of time since death, the shell material obtained from each specimen for dating comes from the innermost portion of the shell, nearer the original mussel body. This is done to

get a time since the cessation of growth (the inner most layers of nacre are the “newest” in the process of mussel growth). This assures that the most accurate account of age is measured. However, until more work is completed with this method, it is difficult to assess the accuracy of this method for freshwater mussels.

Racemization

The reliability of this method in dating freshwater mussel specimens can be affected by amino acids and their ability to be altered by the chemical qualities of water. pH is a notable aspect of water quality that affects amino acids and the accurate determination of the D to L ratio. Freshwater mussels are protected from extreme pH fluctuations by the carbonate naturally found within the shell when alive (Smith et al., 1978). However, amino acids found between the carbonate crystals, not within them, comprise the nacre of the shell and are more likely to be affected by external pH fluctuations (Sykes et al., 1995; Penkman et al., 2008). Freshwater mussel shells found in waters with a higher average pH (more basic) show increased D to L number ratios (Orem and Kaufman, 2011). Though water quality records of pH for this area of the Holston River are limited, pH measurement done prior to mussel collection showed the area to be slightly more acidic, but not extremely so. The acidic nature of the waters of the Holston River may be caused in part by the karst system (consisting of quartz, shale, siltstone, and shall) that this portion of the river runs over and by the acidic soils that dominate the region (TDEC, 2007). However, the pH of the water may be slightly tempered by the presence of limestone found within this area and so somewhat slightly moderate the results of racemization.

Water temperature can also alter the effectiveness of amino acid racemization. In principle, for a fossil to be dated using racemization or AAR, an independently dated nearby site that correlates with the location site of the fossil must be used as a control. One of the major priorities when choosing a control site is similarity in temporal history (Kaufman, 2003). This is because the

reliability of results varies according to site temperature. Usually, warmer sites will have better clarity of results. Generally, sites with a mean annual temperature of 30 °C have a maximum range of 200 thousand years and resolution of about 10 years. Sites at 10 °C have a maximum age range of relatively 2 million years, and a resolution of approximately 20% of the age. Sites with temperatures of –10 °C have a maximum age of approximately 10 million years, and, consequently, imprecise resolution (Kaufman, 2003). In this study, it is conceivable that water temperature variation may have affected the results of the amino acid racemization, as both collection sites are affected by colder waters from upstream dams being released. This contributes to the cooler waters that dominate the Holston River year-round.

The pace of racemization greatly varies between amino acids (taking into consideration taxonomic disparity). In general, Glu and Val are among the most slowly racemizing amino acids (Kaufman and Manley, 1998). In contrast, Asp and Ala are among the fastest; while Ile and Phe racemize at an intermediate rate (Kaufman and Manley, 1998). Amino acids that exhibit low D to L ratios in young samples (Glu, Ala, Tyr, Val, Phe, Ile) tend to racemize at a more consistent rate over the course of the reaction (Kaufman and Manley, 1998). These varying reaction rates can result in differing D to L ratios, depending on the amino acids being measured (Kaufman and Manley, 1998). It must also be mentioned that results of these varying amino acids differ between various laboratories. This has been an issue directly related to consistency of results and remains an issue with amino acid racemization (Wehmiller, 1984). Moreover, this method has not been commonly used to measure the age of freshwater bivalves. Previous work with freshwater bivalves has dealt with the genus *Lampsilis* and has been used to construct advanced timeline of historical freshwater events (Huntley et al., 2012). Clearly, more research needs to be performed to confirm that this method can be used for freshwater mussel dating.

Taphonomy and Preservation

One of the major questions pertaining to any fossil or subfossil is what factors contribute to the preservation of the specimen after death (Martin, 1999). Researchers have considered the preservation of marine mussels to be a function of shell microstructure (thickness and durability), shell shape (spherical or not), size, and relative density (Martin, 1999). Water velocity and sediment type also play a role in shell transport and burial, which are key to the probability of fossilization and preservation of a specimen. Menard and Boucot (1951) found that shells are more likely to be transported on gravel rather than sandy strata, even more so than on muddy substrates. Another role in the preservation of shell material is the disarticulation of the mussel. Shells are more likely to remain coupled in low-velocity environments and if rapidly buried. Articulation is also more probable in lower temperature environments (slows down rate of decay of mussel body and the buildup of gases), anoxic environments (slows down the mineralization of the mussel body after death), epifaunal vs. infaunal habitats (infaunal inhabitants are more likely to remain articulated). The amount of adjoining tissue (more tissue leads to the greater chance of remaining articulated after death) and the size of the pseudocardinal and lateral teeth (greater adjoining and interlocking teeth) also increase the likelihood of the shell remaining articulated (Alexander 1990; Schäfer, 1972; Sheehan, 1977; Martin, 1999).

While most of these principles can be applied to freshwater mussel fauna, conflicting ideas abound as to how other morphological attributes of freshwater mussels contribute to preservation. Some researchers have claimed that shell size does not play a role in the taphonomic preservation of freshwater mussels, rather that sphericity, presence of predators and/or parasites, and lamellar microstructure of the shell itself affect fragmentation rates (Wolverton et al., 2010; Randklev et al., 2009). While more work needs to be done regarding freshwater mussel taphonomy, it is possible to apply these concepts to the four species being studied in these two locations.

Velocity

A major factor contributing to the preservation of shell material is water velocity. Bone, fossil, and other relic materials found within aquatic systems with higher water velocities are more prone to abrasion than those in low velocity water systems (Mernard and Boucot, 1951; Martin, 1999). Within the Holston River drainage, several different hydroelectric dams and steam plants disrupt the natural seasonal and daily flow of this water body (USGS, 2018). Flows and water velocity are higher when more generators are active, which varies daily and seasonally based on water usage and demand. This variation in flow, leading to varying velocities, can alter rates of abrasion as seen in the specimens of these two sample sites. This is especially true for species with a thinner shell and specimens found within coarse-grain sediment environments.

The specimens found at site 1 showed signs of low to moderate abrasion, with most of the periostracum still intact along the medial and dorsal portions of the shell. In the more severe cases, mostly with *Pleurobema rubrum*, much of the growth margin on the ventral side of the shell has been abraded away. In contrast, the specimens found at site 2 showed higher signs of abrasion with little to no periostracum remaining and occasional chipping of the inner nacre near the umbo. This was found for *Pleurobema rubrum* and *Actinonaias ligamentina*, of which some had little to no shell remaining nearer the growth margin.

Both sites are affected similarly by sometimes extreme fluctuations in discharge, with daily ranges of 100 to 1500 cfs within 24 hours (USGS National Water Information System Data Portal), so the cause of variation of abrasion of mussel shells found from both sites is unclear. The variation in abrasion, therefore, is perhaps due to the differences in the substrate.

Substrate

Together with velocity, substrate type and grain size play a key role in fossil and material preservation. Fossils and materials found in fine-grain and compacted sedimentary environments are more like to be preserved than those found in loosely compacted large-grained sediments (Martin, 1999). Additionally,

specimens that were rapidly buried have a greater chance at being preserved and remaining articulated than those that were slowly covered with sediment (Martin, 1999).

Substrate type may have been the key to the preservation of relic specimens, especially regarding those found at site 1 (McBee Island). Site 1 has a fine grained silty and clayey channel bed with McBee Island being composed of heavily compacted distinct layers of clay. Specimens eroding out of the strata of the island were difficult to remove by hand and portions of shell are still eroding out slowly. Because of the fine-grained sediments, even with the large fluctuations in velocity, shells could be more readily preserved with little possibility of abrasion.

In contrast, site 2 has a very different sedimentary environment. Along the main channel of this portion of the main branch, sheets of limestone bedrock dominate the area with shells being moved along the channel. Nearer the bank, where most shells were found, medium to large grained quartz soil and mud is prominent. While many articulated shells were eroding out of the bank, several shells (especially those that were thinner) were heavily abraded. Due to the highly fluctuating velocity and main channel composed of bedrock substrate, it is likely that the shell material found at this site is more prone to abrasion.

Water Quality

The preservation of freshwater mussel shells is susceptible to change in pH because much of the shell (nacre and inner portions of the shell) is composed of thin sheets of calcium carbonate crystals (aragonite) mixed into an organic matrix made up of conchiolin protein. Water bodies with lower, more acidic, pH levels can cause the degradation and erosion of shell material (recent or relic). Though it has been argued that shells composed of calcium carbonate and aragonite have the same relative reaction to changes in pH and stability in preservation (Best and Kidwell, 2000), pH is still as destructive to those organisms mostly composed of aragonite.

Measurements of pH in the portions of the Holston River of the two-sampling location (both taken consecutively by TDEC monitoring stations and through testing at the time of mussel collection), show that these two sites have water that spans from slightly acidic to mildly basic, ranging from 6.22 to 8.37 (Tables 25 and 26). The moderate pH can likely be attributed to the presence of limestone rock and material found at both sampling sites, buffering the otherwise acidic water flowing from the headwaters. Therefore, it can be assumed that pH does not greatly contribute to the degradation of the shell material found at these two sites.

Water temperature and dissolved oxygen rates also play a role in the preservation of freshwater mussel material. Lower water temperatures are known to slow down the rate of decay and buildup of gases as the putrefaction of the mussel body begins, and the muscles that once held the shell together disintegrate (Martin, 1999). Due to colder waters being released from the Cherokee and Fort Patrick Henry Dam, this likely contributes to the colder water temperatures common even during the summer months along the main branch of the Holston River and perhaps explain the relatively high rate of the preservation of the shell material found from both sites. Dissolved oxygen aids preservation of shell material by limiting or increasing the rate of mineralization of the mussel body after death, if the mussel was not subject to predation and died of natural causes (Martin, 1999). Higher levels of dissolved oxygen can decrease the mineralization rates of several compounds such as glucose, sucrose, tannic acid, and metal compounds found within the mussel body and instead increase the rate of disintegration by promoting bacterial and parasitic activity (Martin, 1999). Thus, mineralization of the mussel body is more common in more anoxic environments. According to the 2016 303 d list, portions of the Holston River, especially those of the South Fork, Middle Prong, and Main Branch are recognized as being impaired due to low levels of dissolved oxygen (TDEC, 2016). This can improve preservation of shell material after death and may

explain why shell material from both sites is still relatively well preserved despite other possible destructive aspects of water quality found near these sample sites.

Shell Morphology

Many aspects of shell morphology may have an effect on the preservation of shell material (Martin, 1999). However, it has been observed that thicker shelled specimens with larger pseudocardinal teeth and adductor mussels are more likely to be preserved than thin shelled species (Alexander, 1990).

Of the four species being studied, the smaller and thinner organism of the group, *Pleurobema rubrum*, was most prone to chipping and abrasion at both sites. The more robust two species, *Amblema plicata* and *Actinonaias ligamentina*, seem to show the least amount of damage to the growth margin along the ventral side of the shell, loss of the periostracum and abrasion nearer the umbo. This held true for those observed from both the sample sites.

Predator and Human Alteration

Aboriginal or Native American inhabitants dating back to the Woodland and Mississippian Period of the archaeological timescale have been historically recorded as residing within several areas near McBee Island and portions of the South Fork of the Holston River (Polhemus and Parmalee, 2004). It is not uncommon for freshwater mussel material to have been exploited as a food source and as adornments, due to the pearly nacre most specimens exhibit (Parmalee and Klippel, 1974; Robinson et al., 1977; Archaeology, 2011; Bamforth, 2007). It was documented during an archaeological excavation of a portion of the South Fork of the Holston River that along river mile 17.4 evidence of human tools was found within a shell midden: a straight stemmed Iddin (pointed cutting projectile). This raises the possibility that aboriginals could have gathered freshwater mussels, dating back to the late Archaic or early Woodland period (ca. 1600 BC to 100 AD) (Parmalee and Polhemus, 2004). Traditionally, if used as a food source, mussels are cut open with rudimentary stone or flint tools nearer the adductor mussels to expose the meat or mussel body. This results in

cut marks or hatches within the interior portion of the nacre nearer the mussel body and chipping on the posterior and anterior portions of the shell. No evidence of this modification was observed for any of the collected shell specimens from either site.

Aside from human modification and exploitation, it is not uncommon for local wildlife to prey on freshwater mussels. The most prominent predators in this local area would be the Muskrat (*Ondatra zibethicus*), the Raccoon (*Procyon lotor*), and the River Otter (*Lontra canadensis*). Each of these animals feed on freshwater mussels by pulling apart the valves by using their hands and feeding on the mussel meat inside. In the case of muskrat feeding, most shells may still be articulated after feeding and be buried, creating small pockets of shells. These practices can contribute to the preservation of shell material as this process of rapid burial leads to greater chances of preservation and the shell of the mussels are often not damaged aside from a few scratches nearer the ventral portion of the shell and removal of portions of the periostracum. The shells collected from both sites, however, were evenly distributed along the strata and bed of the banks and were not deposited in pockets.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

This research sought to answer two questions. First, is it possible to determine at what point in time did these freshwater mussel specimens live and die within the Holston River. Specifically, is it viable to determine a time of existence and death of freshwater mussels by using racemization and low precision C¹⁴ dating? Secondly, do changes in growth and rates of mortality exist between freshwater mussels of the past and present of this water system?

Relic specimens of the four species collected, *Actinonaias ligamentina*, *Elliptio crassidens*, *Amblema plicata* and *Pleurobema rubrum*, from two sample sites along the Main Branch of the Holston River were geologically dated back to the Woodland and Early Mississippian Period of the archaeological timescale, in which aboriginal or Native American inhabitation was documented near the study region (Parmalee and Polhemus, 2004). Additionally, when specimens of the same species from the two collection sites were compared with one another, they were found to be relatively close in geological age, supporting the idea that the shells were deposited into the strata around the same time.

Racemization provided insight into the rate of decay of the relic and recently dead shelled specimens and gave an estimate of time since death through the conversion of amino acids from an L to D configuration. Most amino acids (aside from alanine, which was outside of detection range) showed a low ratio, meaning that these shells were in the early stages of decay. This method is subject to changes in water quality, particularly dealing with validity and visibility. Racemization was shown to be a unreliable option for determining time since death. While this measurement could have been affected by preservation factors such as, pH, sediment type, grain size, water temperature, and dissolved oxygen, these shells were observed to be, taphonomically, well preserved.

Relic shells were of an overall younger age, especially those of *Pleurobema rubrum* and *Amblema plicata*. Additionally, growth in the relic shells followed the classic patterns of more growth occurring in the warmer, summer

months with a relatively lower amount of growth as shown by the halting of growth during the winter.

Key differences were observed between the relic shells and the modern shell specimens collected of *Actinonaias ligamentina*. For the modern shells (5 recently dead), individuals grew faster annually compared to relic specimens of the same species and age, with thicker growth annuli being commonly observed. However, the most important difference between the modern and relic shell material was an observed reduction of growth during the primary growth season for the modern shell specimens. Though the cause of this reduction is unknown, either through human activities in this watershed or perhaps due water temperature that is altered by the release of water from Cherokee Dam, many factors may be affecting the rate of growth and mortality of freshwater mussels within this waterway.

Hypotheses Results

To understand how changes in freshwater mussel growth and mortality are affected by changes in water quality, it is first important to be able to age and date mussel material to the appropriate point in time. For this reason, two dating methods were tested; racemization and low precision C^{14} dating. Dating results from low precision C^{14} dating were consistent, with an error range of 60 to 100 years across both testing sites. Results from racemization, however, proved to be complicated with low amino acid ratios from both recently dead to relic mussel specimens of considerable age. This could have been due to water temperature and deposition preservation; however, conclusive results were limited.

Therefore, considering the first set of hypotheses being tested, I cannot reject the original hypothesis, as results from low precision radiocarbon dating were found to be reliable for this study. However, more work needs to be done to determine the ability for racemization to be implemented for freshwater mussel material.

Regarding the second hypothesis of my research questioned the existence of possible differences in growth patterns between modern and relic freshwater mussels from this area. It was confirmed through growth annuli analysis that modern freshwater mussels of the species *Actinonaias ligamentina* from this area were growing at a faster rate per year as compared to relic mussels of the same age, species, and relative location. However, there was a notable difference in growth during the primary growth season for modern freshwater mussels, as to indicate a reduction in growth. This reduction may be due to the colder waters released from the Cherokee Dam during the warmer months of year, as shown through discharge rates recorded by TVA or the Tennessee Valley Authority. Given this information, I choose to accept the original hypothesis, confirming a difference in growth rates between modern and relic shell material of freshwater mussels from this area.

Are freshwater mussels' viable biological indicators?

This research was conducted on the premise that freshwater mussels are biological indicators that react to changes in water quality. Freshwater mussels react to changes in water quality through either growth fluctuations, changes in reproductivity, and longevity or rates of mortality. The Holston River was once a river system noted for its richness and diversity of freshwater mussels (Parmalee and Polhemus, 2004; Williams et al., 2008; Haag, 2012). However, in subsequent years, there has been a noticeable reduction in species richness and reproductive activity. This could be due to numerous factors, such as; impoundment structures, habitat alteration, extreme changes in water velocity, siltation, heavy metals, levels of nitrates and phosphates, levels of *E. coli*, and loss of appropriate fish hosts for glochidia.

Due to the readily declining longevity and rapidly growing rates of mortality of freshwater mussels from this system, I wanted to see if there was a noticeable difference in the growth rates of freshwater mussels when looking at modern and relic specimens to identify if these issues of water quality are impacting these

creatures as much as believed. Though noted that modern freshwater mussels were growing at high rates per year, there was a noticeable difference in growth during the primary growth season. Whilst this is probably a factor of water temperature, there has also been noted a noticeable difference in the ability to find live and reproducing freshwater mussels of most species that historically resided in this system (Ahlstedt, 1992a and 1992b; Parmalee and Polhemus, 2004). As freshwater mussels are one of the first to feel the effects of changes in water quality, due to their place in the ecological food web and their ability to “filter” water, I felt it important to look at these creatures to understand if the section of the Holston River in question was ecologically healthy.

The health of this ecological system does not just extend to the wildlife that inhabit these waters, but also the people that rely on this system. Recreational fishing (trout, bass, etc...), boating and general use is increasingly popular in the lower end of the Holston River. Additionally, as the Holston River does eventually flow into the Tennessee River, which serves a waterbody that supplies drinking water to the people of Knoxville, Tennessee, the cleanliness of this water system becomes an issue of public health.

Considering the importance of the Holston River and the rate in which freshwater mussels are decreasing within this system, this study serves not as only as a freshwater mussel and ecological survey, but a call to action. Freshwater mussels are viable biological indicators when combined with other testing methodology (regular water testing, fish surveys, riparian vegetation evaluations, etc...). The rapidly decreasing longevity and rising rates of mortality for freshwater mussels of this system may serve as a sign, that more work must be done for this system to become healthy once again. That aspects of water quality that may be affecting the modern freshwater mussels of this system are also affecting other aspects of this ecological system. This research serves to promote the use of freshwater mussels as biological indicators and to stress the idea that we, as humans living on this earth, need to begin to look at the health and longevity of the water systems and aquatic creatures that surround us.

The Complications of Aging and Dating: Freshwater Mussels

Through growth annuli analysis, racemization, and low precision C¹⁴ dating, I documented information regarding relic and recent freshwater mussel shell growth and mortality (geologically and ontogenetically) for the species *Actinonaias ligamentina*, *Amblema plicata*, *Elliptio crassidens* and *Pleurobema rubrum*, collected from the Holston River. However, perhaps what this research has accomplished more than anything else is the development of more questions. For example, are there water quality factors brought on by human influence that might affect the capability of racemization and low precision C¹⁴ dating in generating accurate information for freshwater mussels? Anthropogenic development of waterways through either the implementation of physical flow regulating structures (dams, reservoirs, etc.) and contamination brought on by human activity (fertilizers from agricultural activity, heavy metals leaching through the soil, siltation brought on by increased erosion, etc.) can greatly reduce or even negate the ability for these methods to provide reliable results. The major factors of water quality that most greatly contributed to the dependability of the results brought on by growth annuli analysis, racemization, and low precision C¹⁴ dating are pH and temperature (which are both greatly affected by the local karst system of the area and the release of cold water from the Cherokee Dam and Fort Patrick Henry Dam). Even more fundamental is the ability for freshwater mussel material to be preserved within a given water body. Water velocity, substrate type, and water pH can greatly affect the rates of preservation of shell material (living or fossilized) by contributing to abrasion and disarticulation rates.

For the four freshwater mussel species used for this experiment, if it is possible to determine age and time of death as well as monitor growth rate, would it be conceivable to be able to link changes in water quality and ecological habitat to the longevity and stability of populations? Could we develop better ways of conserving freshwater mussels as a species in other water systems if we

were able to answer these questions? The answer lies in more research and methodological progress.

Where do we go from here?

To allow for more statistically reliable results, more specimens should be collected and subjected to each form of dating (to correct for any possible outlying data points and corrupting of regression data). Additionally, supplementary testing sites and species should be incorporated to get a better understanding of the overall ecological health of the freshwater mussel fauna within the local area of the Holston River. Progress needs to be made on furthering the dating methods of racemization and low precision C¹⁴ to accommodate the needs of dating freshwater mussel material. Racemization, though applied to numerous fossil taxa including, foraminifera, bone, eggshells, teeth, ostracods, and marine and freshwater mollusks, needs to be calibrated for the study of freshwater mussels in different water bodies and on different species. Similarly, though low precision C14 dating is still an extremely new form of geological dating methodology, further research should be done to progress and validate this method for dating freshwater mussel material.

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APPENDIX

Table 1: Freshwater Mussel Species Archaeological Excavation (Parmalee and Faust, 2006)

Species	Loy	Cave T-22
<i>A. ligamentina</i>	42	326
<i>A. marginata</i>	0	5
<i>A. plicata</i>	11	85
<i>C. tuberculata</i>	72	94
<i>C. stegaria</i>	0	1
<i>D. dromas</i>	175	16
<i>E. crassidens</i>	1	2
<i>E. dilatata</i>	67	50
<i>E. arcaeformis</i>	2	7
<i>E. brevidens</i>	4	3
<i>E. capsaeformis</i>	0	51
<i>E. haysiana</i>	3	7
<i>E. lewisii</i>	0	2
<i>E. propinqua</i>	0	0
<i>E. stewardsonii</i>	4	3
<i>E. torulosa</i>	0	29
<i>E. triquetra</i>	0	0
<i>F. barnesiana</i>	0	17
<i>F. cor/F. cuneolus</i>	0	1
<i>F. subrotunda</i>	72	20
<i>L. abrupta</i>	0	0
<i>L. fasciola</i>	3	39
<i>L. ovata</i>	0	8
<i>L. costata</i>	0	6
<i>L. rimosus</i>	154	58
<i>L. dolabelloides</i>	923	50
<i>L. recta</i>	0	1
<i>M. conradicus</i>	0	2
<i>O. reflexa</i>	0	0
<i>O. subrotunda</i>	0	0
<i>P. cooperianus</i>	44	0
<i>P. cyhyus</i>	1	4
<i>P. cordatum</i>	82	2
<i>P. oviforme</i>	0	3
<i>P. plenum</i>	23	3
<i>P. rubrum</i>	11	1
<i>P. cf. sintoxia</i>	0	0
<i>P. alatus</i>	0	0
<i>P. fasciolaris</i>	37	65
<i>P. subtentum</i>	3	198
<i>Q. cylindrica</i>	16	0

List of species found from the archaeological excavation of T-22 Cave and Loy Site as reported from Parmalee and Faust, 2006.

Table 2: Results of Collection

Species	10/21/201 6	2/19/201 7	3/10/201 7	8/7/201 7	8/27/201 7	Total Across Species
Actinonaias ligamentina	7	49	8	5	56	125
Amblema plicata	1	8	0	0	23	32
Pluerobema rubrum	3	9	0	0	14	26
Elliptio crassidens	9	33	6	0	0	48
Total	30	99	14	5	93	241

This table shows the amount and species type of shell material that was collected from all the collection trips from both McBee Island and SE Surgoinville.

Table 3: Dating and Aging Shell Material Count

Species	Racemization	Low Precision C14	Growth Annuli Analysis
Actinonaias ligamentina	10	5	110
Amblema plicata	6	6	32
Pluerobema rubrum	4	4	26
Elliptio crassidens	3	3	10
Total	23	18	178

All 178 shells that were deemed stable for processing were measured using growth annuli analysis. Of those 178 shells, 23 shells were dated using racemization. Of those same 23 shells, 18 were also dated using low precision C¹⁴ dating.

Table 4: Low Precision C¹⁴ Dating Results (Site 1)

Sample name	Species	D14C	±	14C age	±
		(‰)		(BP)	
UAL15769	<i>Elliptio crassidens</i>	-231.34	6.087672	2110	70
UAL15770	<i>Elliptio crassidens</i>	-223.395	6.685549	2030	70
UAL15771	<i>Elliptio crassidens</i>	-203.347	5.995939	1830	70
UAL15772	<i>Actinonaias ligamentina</i>	-250.684	6.146601	2320	70
UAL15773	<i>Actinonaias ligamentina</i>	-197.14	6.579469	1760	70
UAL15774	<i>Actinonaias ligamentina</i>	-204.108	5.245745	1830	60
UAL16341	<i>Amblema plicata</i>	-240.1	6.2	2210	70
UAL16342	<i>Amblema plicata</i>	-240.6	5.9	2210	70
UAL16343	<i>Amblema plicata</i>	-252.2	6.4	2330	70
UAL16344	<i>Pluerobema rubrum</i>	-232.3	6.2	2120	70

Results were taken for all four species.

Table 5: Low Precision C¹⁴ Dating Results (Site 2)

Sample name	Other ID	D ¹⁴ C	±3	14C age	±4
		(‰)		(BP)	
UAL16332	<i>Actinonaias ligamentina</i>	-232.5	6.2	2130	70
UAL16333	<i>Actinonaias ligamentina</i>	-245.5	7.9	2260	90
UAL16334	<i>Actinonaias ligamentina</i>	-231.9	8.7	2120	100
UAL16335	<i>Amblyema plicata</i>	-237.8	6.0	2180	70
UAL16336	<i>Amblyema plicata</i>	-234.7	8.9	2150	100
UAL16337	<i>Amblyema plicata</i>	-232.8	8.3	2130	90
UAL16338	<i>Pluerobema rubrum</i>	-229.8	5.9	2100	70
UAL16339	<i>Pluerobema rubrum</i>	-242.7	8.2	2230	90
UAL16340	<i>Pluerobema rubrum</i>	-232.7	6.1	2130	70

Results were taken for only three species, as specimens of *Elliptio crassidens* were not found from this site.

Table 6: Racemization Results (Site 1)

Specimen No.	Species	Shell Status	DL Asp	DL Glu	DL Ser	DL Ala	DL Val	DL Phe	DL Ile	DL Leu	L-Ser/L-Asp
1	<i>Actinonaias ligamentina</i>	Recently Dead	0.09 33	0.02 18	0.04 43	0.01 09	0.0 050	0.01 43	0.0 110	0.01 30	1.0586
2	<i>Actinonaias ligamentina</i>	Recently Dead	0.08 91	0.02 47	0.07 38	0.01 65	0.0 081	0.03 42	0.0 163	0.01 67	0.6799
3	<i>Actinonaias ligamentina</i>	Recently Dead	0.09 88	0.02 28	0.06 39	0.01 10	0.0 130	0.02 47	0.0 143	0.01 47	1.0334
4	<i>Actinonaias ligamentina</i>	Recently Dead	0.09 52	0.02 35	0.05 37	0.01 24	0.0 055	0.01 61	0.0 071	0.01 21	0.9651
5	<i>Actinonaias ligamentina</i>	Recently Dead	0.11 74	0.03 74	0.18 61	0.01 51	0.0 061	0.02 29	0.0 075	0.01 43	0.8445
1	<i>Elliptio crassidens</i>	Relic	0.15 15	0.03 82	0.07 64	0.01 39	0.0 029	0.01 72	0.0 360	0.01 37	1.0130
2	<i>Elliptio crassidens</i>	Relic	0.14 56	0.03 32	0.07 38	0.01 76	0.0 150	0.01 94	0.0 297	0.01 35	0.9742
3	<i>Elliptio crassidens</i>	Relic	0.16 88	0.03 39	0.08 38	0.01 78	0.0 071	0.01 99	0.0 178	0.01 37	1.1222
1	<i>Actinonaias ligamentina</i>	Relic	0.11 66	0.04 07	0.17 52	0.03 87	0.0 096	0.02 36	0.0 052	0.01 34	0.4874
2	<i>Actinonaias ligamentina</i>	Relic	0.14 87	0.03 84	0.07 61	0.01 47	0.0 032	0.01 76	0.0 389	0.01 39	1.0152
3	<i>Actinonaias ligamentina</i>	Relic	0.12 25	0.03 13	0.04 93	0.01 35	0.0 114	0.01 45	0.0 262	0.01 09	1.1695
1	<i>Amblema plicata</i>	Relic	0.13 86	0.03 27	0.06 61	0.01 58	0.0 120	0.02 13	0.0 398	0.02 50	0.8768
2	<i>Amblema plicata</i>	Relic	0.14 38	0.02 79	0.07 35	0.01 41	0.0 107	0.02 26	0.0 356	0.02 64	0.9305
3	<i>Amblema plicata</i>	Relic	0.13 14	0.03 47	0.13 36	0.02 62	0.0 116	0.04 15	0.0 288	0.03 70	0.5826
2	<i>Pleurobema rubrum</i>	Relic	0.13 19	0.03 67	0.05 30	0.01 32	0.0 100	0.02 32	0.0 317	0.01 82	0.9552

Racemization results from those of site 1. Most of these were observed to be in the preliminary stages of amino acid conversion. This result, while accurate for the recently dead *Actinonaias ligamentina* is not as accurate for the relic shell material. The results for relic material may have been altered by water temperature of the channel and pH.

Table 7: Racemization Results (Site 2)

Specimen No.	Species	Shell Status	DL Asp	DL Glu	DL Ser	DL Ala	DL Val	DL Phe	DL Ile	DL Leu	L-Ser/L-Asp
6	<i>Actinonaias ligamentina</i>	Relic	0.10 49	0.02 85	0.05 16	0.0 115	0.0 085	0.01 88	0.0 226	0.01 46	0.9409
7	<i>Actinonaias ligamentina</i>	Relic	0.11 14	0.02 49	0.06 15	0.0 130	0.0 076	0.01 89	0.0 156	0.01 46	0.9486
8	<i>Actinonaias ligamentina</i>	Relic	0.11 30	0.02 65	0.06 02	0.0 131	0.0 076	0.01 90	0.0 159	0.01 48	0.9278
1	<i>Amblyma plicata</i>	Relic	0.16 22	0.04 18	0.17 21	0.0 239	0.0 139	0.04 77	0.0 407	0.04 72	0.5905
2	<i>Amblyma plicata</i>	Relic	0.15 43	0.03 17	0.10 79	0.0 162	0.0 105	0.02 80	0.0 205	0.02 82	0.8772
3	<i>Amblyma plicata</i>	Relic	0.12 60	0.02 93	0.09 25	0.0 167	0.0 083	0.02 63	0.0 184	0.02 69	0.7617
1	<i>Pluerobema rubrum</i>	Relic	0.10 60	0.02 06	0.03 97	0.0 109	0.0 129	0.02 32	0.0 214	0.01 33	1.0286
2	<i>Pluerobema rubrum</i>	Relic	0.11 77	0.02 70	0.07 60	0.0 153	0.0 066	0.02 38	0.0 177	0.01 73	0.7393
3	<i>Pluerobema rubrum</i>	Relic	0.11 96	0.02 62	0.07 68	0.0 148	0.0 085	0.02 42	0.0 212	0.01 77	0.7750

Racemization results from those of site 2. Most of these were observed to be in the preliminary stages of amino acid conversion. The results for relic material may have been altered by water temperature of the channel and pH.

Table 8: P-Values and AIC Scores for statistical models

Growth Model	P-Value	AIC with Variable	AIC without Variable
AL Relic and Recent Yearly (Site 1)	2.00E-16	1543.3	1482.2
AL Relic and Recent Summer (Site 1)	2.00E-16	1299.2	1257.7
AL Relic and Recent Winter (Site 1)	2.00E-16	1309.4	1247.1
AL Relic and Recent Yearly (Site 2)	0.00106	57.1	47.3
AL Relic and Recent Summer (Site 2)	0.0041	35.4	24.5
AL Relic and Recent Winter (Site 2)	2.00E-16	52.6	39.9
AP Relic Yearly (Site 1)	1.54E-05	145.7	136.8
AP Relic Summer (Site 1)	0.000677	114.5	111.2
AP Relic Winter (Site 1)	2.32E-05	134.9	127.2
AP Relic Yearly (Site 2)	0.000156	99.9	89.6
AP Relic Winter (Site 2)	0.00339	79.8	78.9
AP Relic Summer (Site 2)	2.32E-05	109.9	39.9
EC Relic Yearly (Site 1)	2.00E-16	576.8	554.8
EC Relic Summer (Site 1)	1.72E-14	528.7	502.6
EC Relic Winter (Site 1)	2.00E-16	478.3	468.7
PR Relic Yearly (Site 1)	1.51E-05	164.6	163.7
PR Relic Summer (Site 1)	0.000662	143.0	141.1
PR Relic Winter (Site 1)	7.83E-09	139.0	138.3
PR Relic Yearly (Site 2)	8.37E-07	128.5	120.4
PR Relic Winter (Site 2)	0.000759	101.0	100.3
PR Relic Summer (Site 2)	7.83E-09	111.5	109.4

Most p-values for these models were insignificant. However, there were some significance for models highlighted in yellow. This shows that particularly in these models, statistics were significant.

Table 9: Average Yearly Growth for relic *Actinonaias ligamentina* (Site 1)

Specimen	Average growth in micrometers per year	Specimen	Average growth in micrometers per year
1	92.6	29	450.6
4	400.5	32	686.3
5	234.1	33	593.8
6	674.9	34	690.3
7	298.6	35	690.7
8	570.4	36	1165
9	406.5	37	376
10	508.4	38	573.3
11	307	39	1464
12	250.8	40	492.4
13	277.5	41	839.6
14	504	42	840
15	470.7	43	617.4
16	501.9	44	1111
17	400.1	45	840.3
18	368.1	46	994
19	466.1	47	585
20	320.9	48	879.5
21	291.8	49	351.7
22	618	50	1019.3
23	658.2	51	643
24	545.1	52	738.6
25	320.8	53	898.7
26	468.6	54	677.8
27	763	55	806.8
28	456.9	56	1672

Average growth rates ranged from 111.0 micrometers per year to 1214.3 micrometers per year.

Table 10: Seasonal Growth for relic *Actinonaias ligamentina* (Site 1)

Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)	Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)
1	57.1	34.6	29	224.9	225.6
4	231.7	168.8	32	351.8	334.3
5	133.5	100.7	33	281.8	305.8
6	502.4	169.1	34	388.3	202
7	105.1	193.5	35	423.1	267.6
8	265.5	304.8	36	644.5	520.5
9	205.8	200.8	37	209.6	166.7
10	362.7	145.5	38	136.3	437
11	153.1	153.9	39	1162	302
12	82.7	168.1	40	294.5	197.9
13	101.7	175.8	41	421.5	418
14	261.2	243	42	453	387
15	199.9	270.5	43	227.9	389.4
16	263.8	238.1	44	654	457
17	173.2	226.7	45	507.3	333
18	197.5	170.6	46	734.3	260.3
19	282.3	183.8	47	304.3	280.5
20	152.5	167.7	48	663.8	216
21	104.1	187.6	49	234.4	117.6
22	213.8	404.5	50	602	417.5
23	405.2	253	51	425.2	217.6
24	287.8	257.7	52	494.6	244
25	121.2	199.7	53	511.7	386.7
26	186.3	282.3	54	485.6	192
27	508.2	255.2	55	582.8	224.2
28	211	245.8	56	589	1082.5

Average seasonal growth ranged from 78.6 micrometers per year to 734.3 micrometers per year during the summer, and ranged from 32.6 micrometers per year to 1082.5 micrometers per year during the winter.

Table 11: Yearly Growth for recent *Actinonaias ligamentina* (Site 1)

Specimen	Average Yearly Growth (µm)
1	93.1
2	148.2
3	198.2
4	188.6
5	84.0

Average yearly growth ranged from 84.0 micrometers per year to 198.2 micrometers.

Table 12: Seasonal Growth for recent *Actinonaias ligamentina* (Site 1)

Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)
1	32.6	78.5
2	56.6	91.5
3	70.6	127.7
4	81.2	107.3
5	31.4	52.6

Average seasonal growth ranged from 31.4 micrometers per year to 81.2 micrometers per year during the summer, and ranged from 52.6 micrometers per year to 127.7 micrometers per year during the winter. This represents the reduced amount of growth during the primary growth season, as compared to relic material of the same species.

Table 12: Yearly Growth for *Amblema plicata* (Site 1)

Specimen	Average Yearly Growth (µm)
1	1073.8
2	451.8
3	1349.7
4	560.7
5	871.1
6	1236.6
7	5694.0
8	1133.7
9	1735.5

Average yearly growth ranged from 560.7.0 micrometers per year to 5694.0 micrometers per year.

Table 14: Seasonal Growth for *Amblema plicata* (Site 1)

Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)
1	514.3	392.8
2	297.4	156.8
3	379.7	969.3
4	389.0	243.1
5	730.6	140.6
6	888.8	348.0
7	3881.0	1812.0
8	986.8	142.3
9	526.0	1210.0

Average seasonal growth ranged from 297.4 micrometers per year to 3881.0 micrometers per year during the summer, and ranged from 140.6 micrometers per year to 1812.0 micrometers per year during the winter.

Table 13: Yearly Growth for *Elliptio crassidens* (Site 1)

Specimen	Average Yearly Growth (µm)
1	190.5
2	422.6
3	571.6
4	435.0
5	626.1
6	460.6
7	737.4
8	622.5
9	596.2
10	378.2

Average yearly growth ranged from 190.5 micrometers per year to 737.4 micrometers per year.

Table 14: Seasonal Growth for *Elliptio crassidens* (Site 1)

Specimen	Average Summer Growth (μm)	Average Winter Growth (μm)
1	143.0	47.9
2	197.5	213.4
3	260.6	310.9
4	276.0	172.2
5	309.4	316.9
6	276.5	184.2
7	274.9	462.8
8	221.9	400.5
9	297.9	298.4
10	183.2	195.1

Average seasonal growth ranged from 143.0 micrometers per year to 309.4 micrometers per year during the summer, and ranged from 47.9 micrometers per year to 462.8 micrometers per year during the winter.

Table 15: Yearly Growth for *Pleurobema rubrum* (Site 1)

Specimen	Average Yearly Growth (μm)
1	968.2
2	581.2
3	1111.9
4	894.5
5	761.9
6	916.8
7	554.9
8	751.9
9	610.5
10	1055.0
11	494.2
12	1021.7

Average yearly growth ranged from 494.2 micrometers per year to 1111.9 micrometers per year.

Table 16: Seasonal Growth for *Pleurobema rubrum* (Site 1)

Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)
1	570.0	398.6
2	324.1	251.3
3	680.6	431.0
4	541.5	353.0
5	406.6	355.4
6	481.9	334.7
7	325.1	229.8
8	346.2	405.4
9	213.7	397.2
10	588.5	466.3
11	307.1	203.8
12	415.9	606.1

Average seasonal growth ranged from 213.7 micrometers per year to 680.6 micrometers per year during the summer, and ranged from 203.8 micrometers per year to 606.1 micrometers per year during the winter.

Table 19: Yearly Growth for *Actinonaias ligamentina* (Site 2)

Specimen	Average Yearly Growth (μm)	Specimen	Average Yearly Growth (μm)
1	694.5	27	1076
2	1314.3	28	909
3	721.7	29	1774.5
4	2161.5	30	577.3
5	603.5	31	575.8
6	420.5	32	1368.5
7	401.2	33	1602
8	1228.3	34	669.6
9	620.3	35	814
10	934	36	2169
11	862.3	37	657
12	487.2	38	2732
13	770.8	39	3210.5
14	1338	40	1215
15	724.8	41	552.3
16	584.3	42	729.3
17	503	43	618.5
18	784.7	44	2189
19	639.3	45	1519
20	1460.5	46	1249
21	998.3	47	764
22	942	48	1049
23	802.5	49	354.5
24	1492.5	50	1376
25	876.5	51	2214
26	679.7		

Average yearly growth ranged from 354.5 micrometers per year to 3210.5 micrometers per year.

Table 17: Seasonal Growth for *Actinonaias ligamentina* (Site 2)

Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)	Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)
1	307.8	262.5	27	424	652
2	495	819.3	28	408	501
3	521	201	29	1546.5	228
4	1537	625	30	369	207.8
5	296.3	307	31	241.3	334.5
6	195	225.5	32	390	978.5
7	300.8	100.6	33	421	1181
8	1138.3	90.3	34	393.6	275.8
9	95.7	524.7	35	343.5	471
10	358.6	575.8	36	1024.5	1144.5
11	750.2	112.3	37	472.7	184.3
12	267.3	219.8	38	2232	500
13	530	240.4	39	2820	390.5
14	218	1120	40	753	462
15	455.5	269.5	41	360	192.3
16	239	346	42	402.3	327.3
17	194.3	308.8	43	401.3	217.5
18	501.3	283.3	44	1170	1018
19	354.3	284.7	45	706	813
20	454.7	751	46	583	666.5
21	859.5	138.5	47	469	295
22	789	153	48	577	472
23	350.5	452.3	49	259	95
24	1270.5	222	50	406	970
25	721.2	155.5	51	708	1507
26	395	284.3			

Average seasonal growth ranged from 95.7 micrometers per year to 2820 micrometers per year during the summer, and ranged from 90.3 micrometers per year to 1507 micrometers per year during the winter.

Table 18: Yearly Growth for *Amblema plicata* (Site 2)

Specimen	Average Yearly Growth (μm)
1	3328.5
2	2161.0
3	2771.0
4	3696.0
5	8195.0
6	3062.0
7	1817.5
8	2034.7
9	2198.0
10	2088.3
11	1396.8
12	1143.8
13	2616.0
14	2342.0
15	777.4
16	2368.0
17	1610.0
18	722.1
19	815.2
20	1129.0
21	1664.0
22	963.5
23	825.8

Average yearly growth ranged from 722.1 micrometers per year to 8195.0 micrometers per year.

Table 19: Seasonal Growth for *Amblema plicata* (Site 2)

Specimen	Average Summer Growth (µm)	Average Winter Growth (µm)
1	459.5	2869.5
2	1186.7	974.0
3	1080.0	1691.0
4	2574.0	1121.5
5	8008.0	187.0
6	273.7	2721.3
7	901.5	916.0
8	218.3	1816.7
9	899.5	1298.0
10	172.0	1916.7
11	454.8	942.0
12	252.2	891.6
13	504.0	2111.5
14	1849.0	492.0
15	482.4	294.6
16	2260.0	108.0
17	1420.5	190.0
18	411.0	310.9
19	355.8	459.7
20	768.5	360.5
21	145.5	1518.5
22	402.8	560.5
23	350.0	475.8

Average seasonal growth ranged from 145.5 micrometers per year to 8008.0 micrometers per year during the summer, and ranged from 108.0 micrometers per year to 2869.5 micrometers per year during the winter.

Table 20: Yearly Growth for *Pleurobema rubrum* (Site 2)

Specimen	Average Yearly Growth (μm)
1	916.8
2	554.9
3	751.9
4	836.7
5	835.7
6	571.5
7	863.9
8	1199.6
9	1325.5
10	938.3
11	1623.0
12	5433.0
13	968.8
14	1148.0

Average yearly growth ranged from 554.9 micrometers per year to 1623.0 micrometers per year.

Table 21: Seasonal Growth for *Pleurobema rubrum* (Site 2)

Specimen	Average Summer Growth (μm)	Average Winter Growth (μm)
1	581.9	334.7
2	325.1	229.8
3	346.2	405.4
4	602.9	234.0
5	577.2	258.2
6	373.3	191.2
7	374.0	490.0
8	797.8	401.8
9	985.0	340.5
10	709.3	229.2
11	1221.0	401.8
12	821.0	4612.0
13	710.8	258.5
14	883.8	264.3

Average seasonal growth ranged from 191.2 micrometers per year to 4612.0 micrometers per year during the winter, and ranged from 325.1 micrometers per years to 1221.0 micrometers per year during the summer.

Table 22: pH Recordings for McBee Island (Site 1)

McBee Island (Site 1)	Nov-16	Feb-17	Aug-17
pH	8.37	7.89	8.02

Results from this site are slightly more basic than those from site 2. This might be due to the limestone found along the banks of McBee Island.

Table 23: pH Recordings for SE Surgoinsville (Site 2)

SE Surgoinsville, TN	Aug-17	Sep-17	Oct-17
pH	7.41	6.22	7.08

Results from this site are slightly more acidic than that from site 1. This may be due to the slightly more acidic waters flowing downstream from the headwaters.

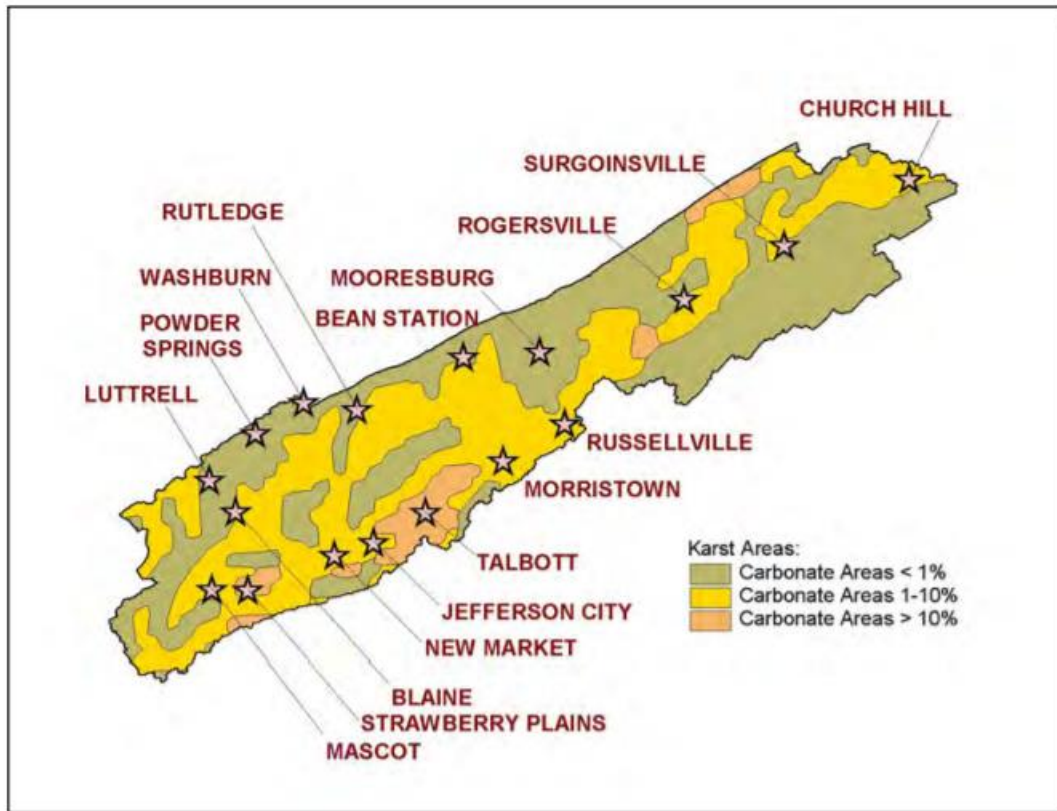


Figure 1: Holston River Watershed Karst Map (TDEC, 2007)

This map depicts the Holston River Watershed and the Karst Areas complete with Carbonate areas.

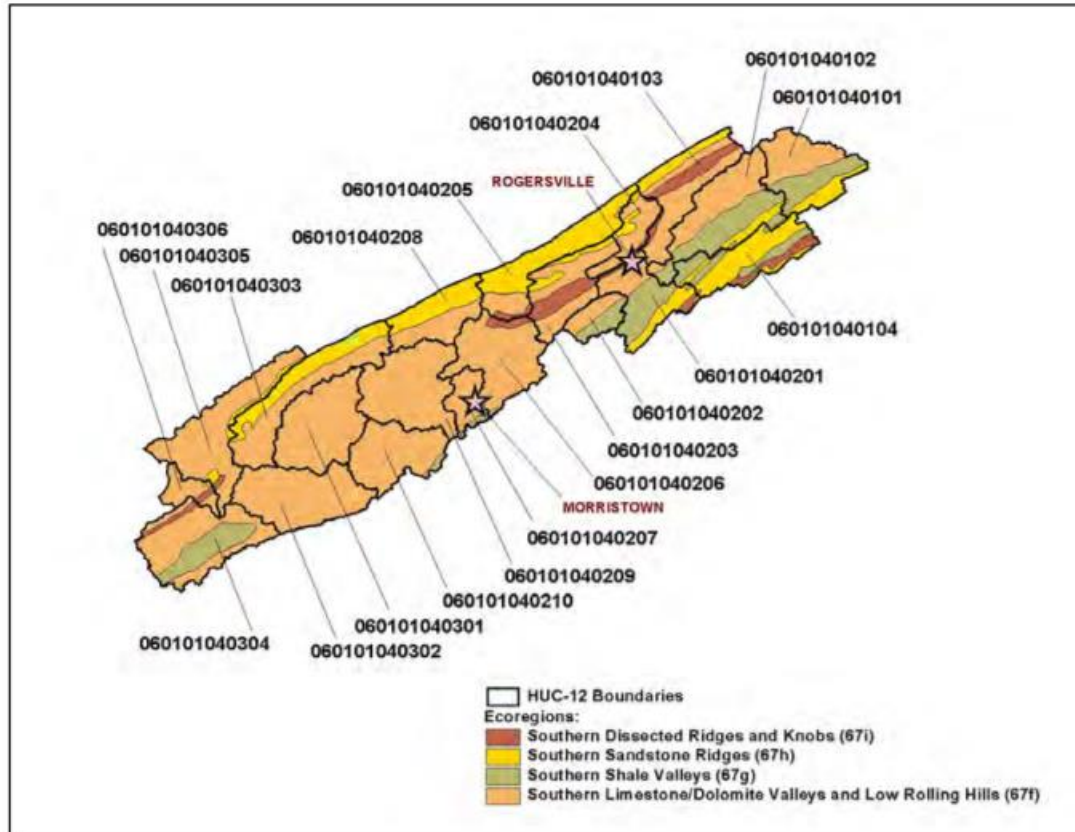


Figure 2: Holston River Watershed Eco-Region Map (TDEC, 2007)

This is a map of the ecoregions of the Holston River Watershed. The Holston River Watershed can be subdivided into 4th level 4 sub-ecoregions that are extremely diverse.

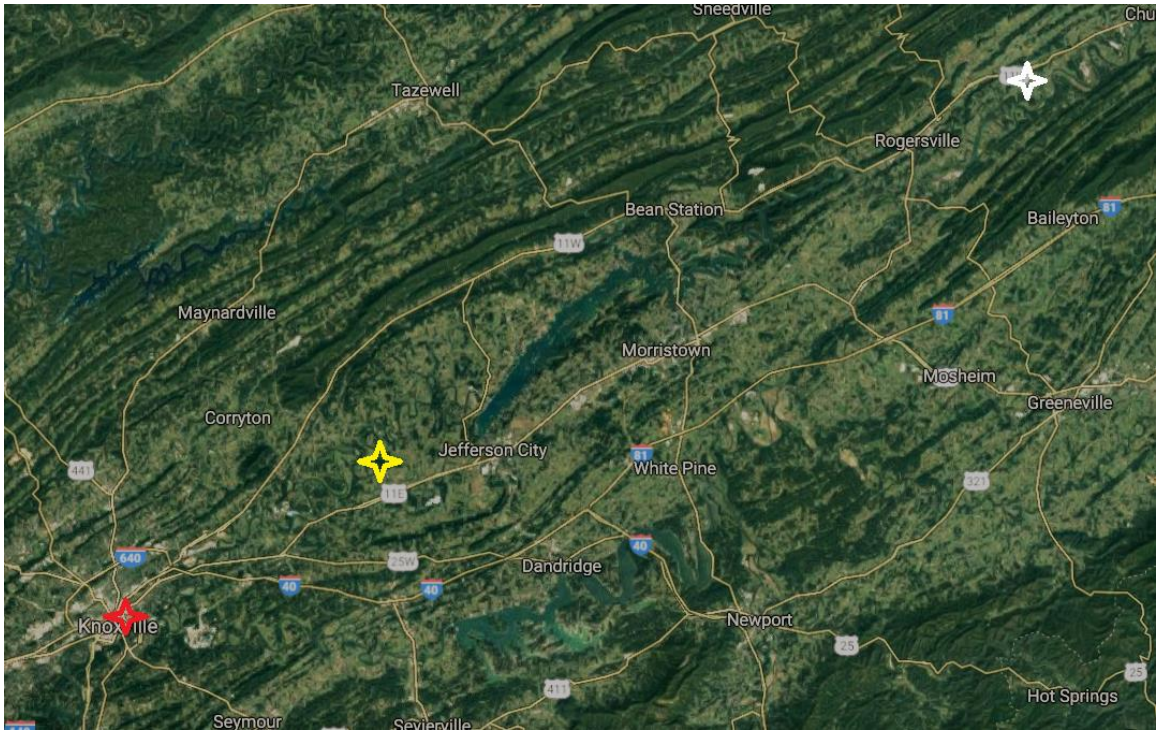


Figure 3: Collection Site Location Map

This map depicts the two sample sites, McBee Island and SE Surgoinsville, in relation to Knoxville, TN. The star in red represents Knoxville, TN. The yellow star is the location of McBee Island, located on river mile 30 of the Main Branch of the Holston River. The white star represents SE, Surgoinsville, right off of River Rd. on river miles 118 through 120, which was the main point of collection.



Figure 3: McBee Island (Site 1)

This is a representation of northwestern side of McBee Island in which relic shell material was collected.



Figure 4: Strata of McBee Island

Another photo of McBee Island and the silty strata that remain nearer the banks. Shells found around this area were mostly dislocated and very few were actively eroding out of the banks.



Figure 5: SE Surgoinsville (Site 2)

Taken from the entry point off of River Rd. at site 2 (SE Surgoinsville) on the Main Branch of the Holston River.



Figure 6: Limestone Bedrock Sheets of SE Surgoinville

Contrasting with McBee Island, SE Surgoinville (Site 2) is mostly composed of sheets of limestone bedrock littered with cobbles and slightly larger rocks.



Figure 7: Eroding Mussel Material from SE Surgoinville

Shells (mostly articulated) were found readily eroding out of the banks of site 2 (SE Surgoinville).

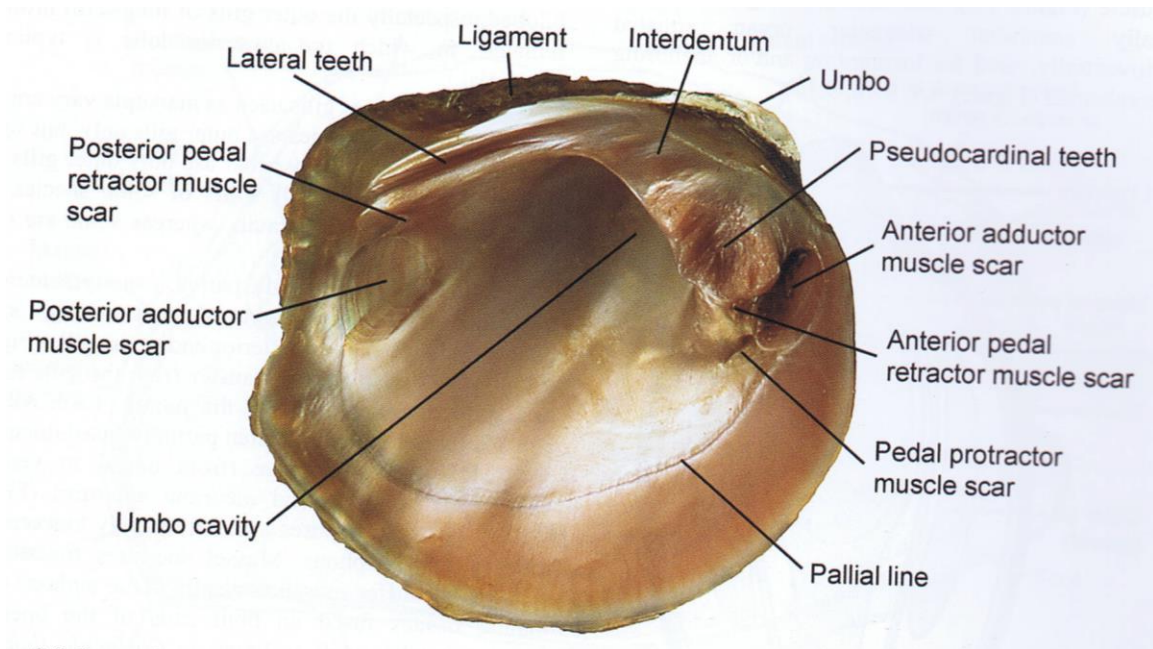


Figure 8: Freshwater Mussel Shell Anatomy (Williams et al., 2008)

This figure is an anatomical figure of a single valve representing the basic structure that most freshwater mussels possess. All specimens used for this project were cut medially through the umbo, exposing the inner nacre.



Figure 9: Exposed Inner Nacre of *Actinonaias ligamentina*

This picture shows a valve of *Actinonaias ligamentina* after it has been cut in half. This shows the exposed nacre that contain the growth annuli used for growth analysis.

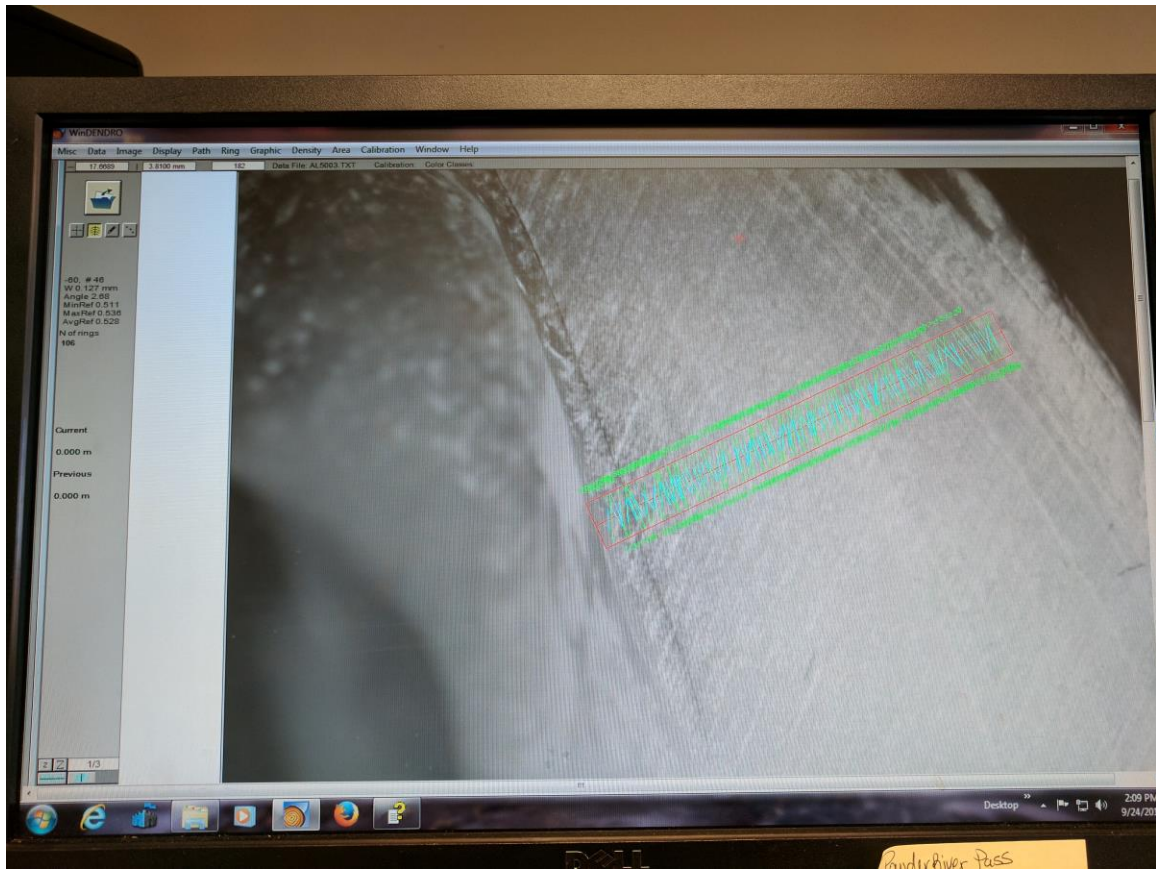


Figure 10: WinDendro Analysis of Growth Annuli

This is a representation of Growth Annuli Analysis using WinDendro. The program operates by determining establishing the inner-most (youngest) and outer-most (oldest) boundaries of the shell. From there, an algorithm can identify annual growth rings using light green lines (each year is composed of one lighter and one darker) and divide them using a light blue line into seasonal growth whilst also giving thickness in micrometers. Each lighter band represents summer growth, or that with more calcium carbonate. The darker bands represent winter or the cessation of growth. Every line (annual and divisional) can be manually moved if necessary.

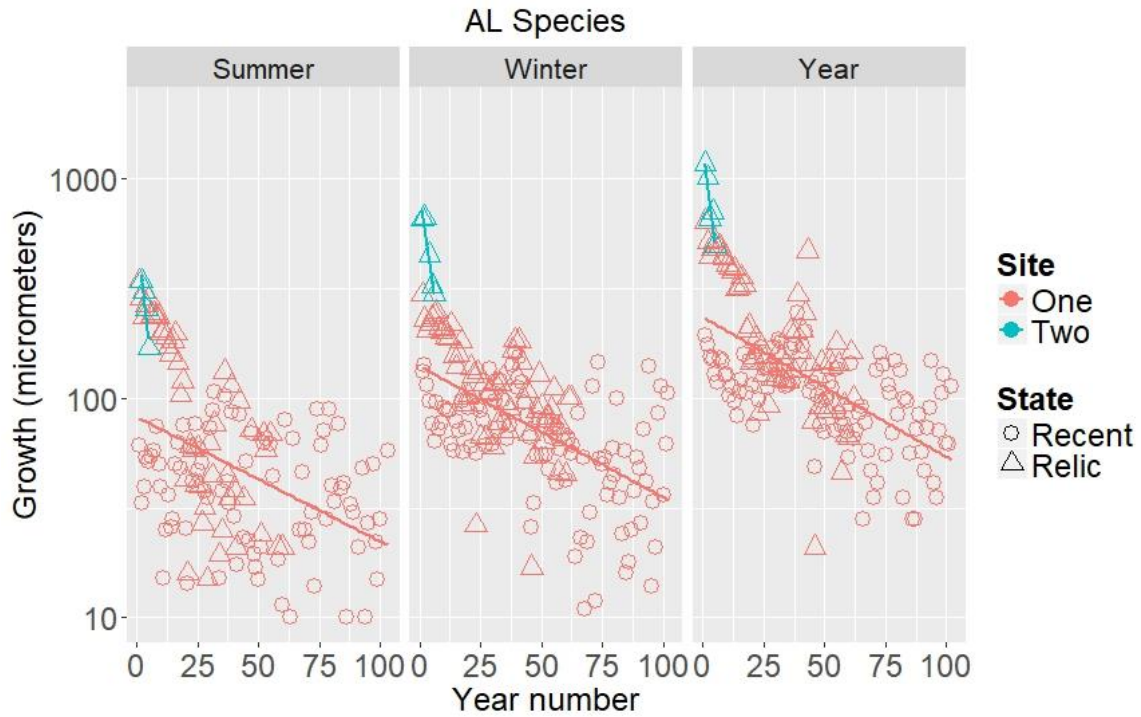


Figure 11: Regression of Growth: Relic and Recent *Actinonaias ligamentina*

All data were log averaged. There is an obvious negative correlation between growth and age. As the mussel ages, there is a noticeable decrease in growth noticeable between the relic and modern shell specimens.

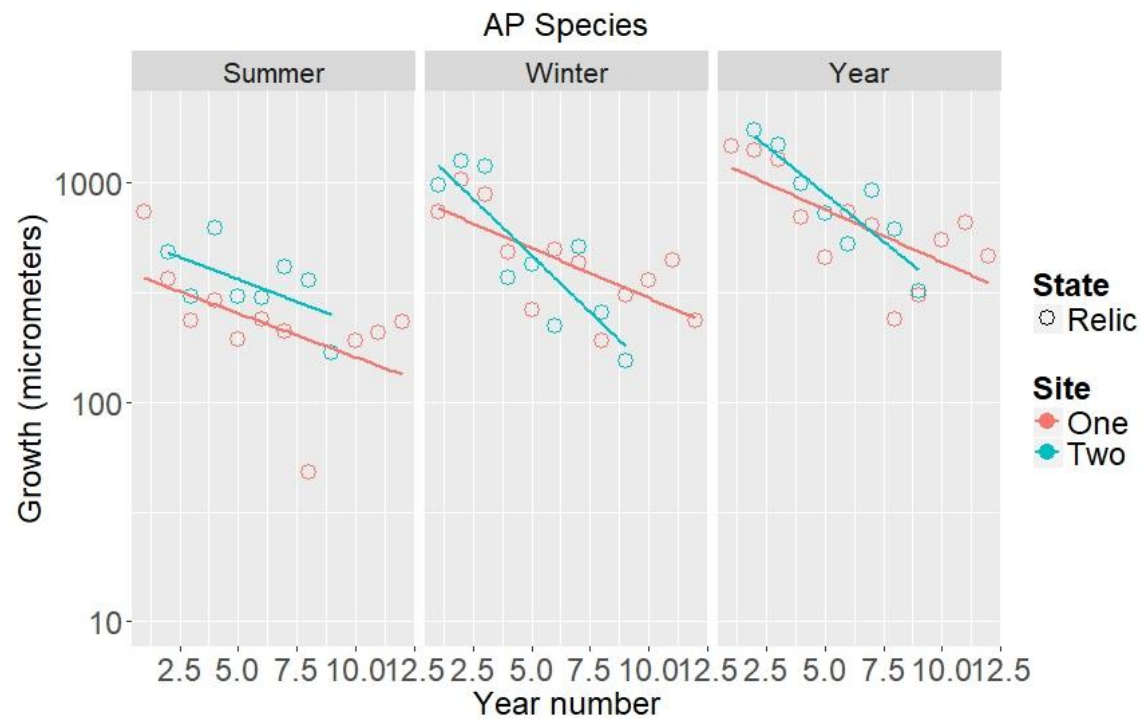


Figure 12: Regression of Growth: *Amblema plicata*

All data were log averaged. There is an obvious negative correlation between growth and age. As the mussel ages, there is a noticeable decrease in growth noticeable between the relic shell specimens from both sites.

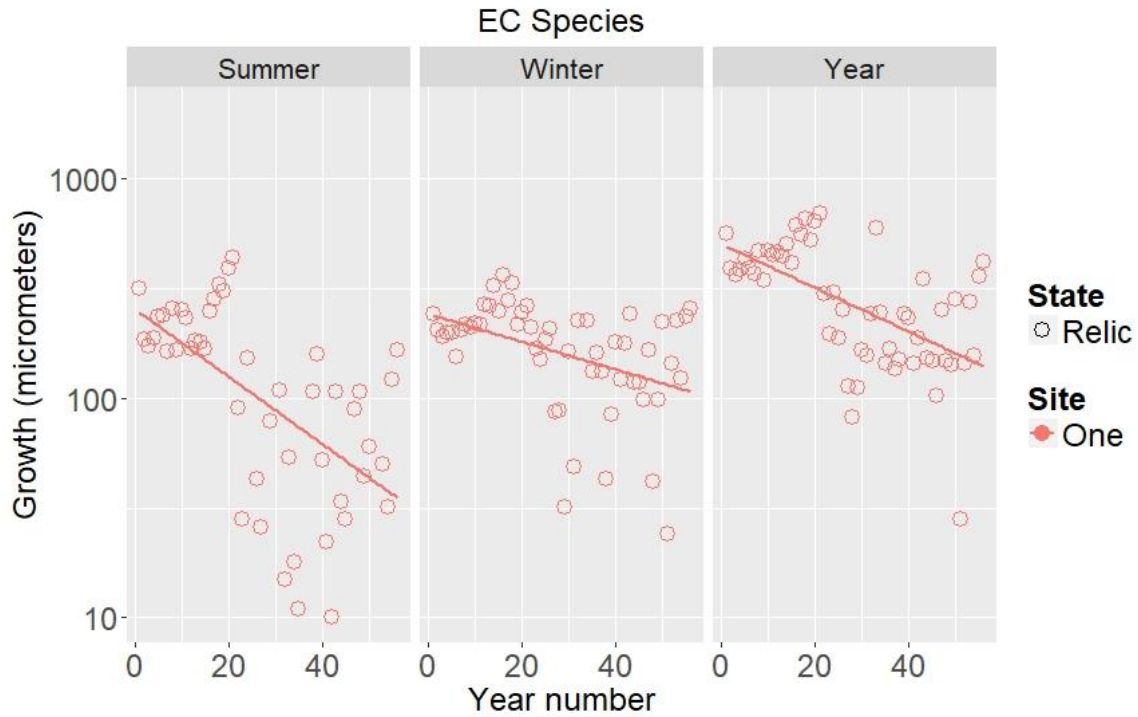


Figure 13: Regression of Growth: *Elliptio crassidens*

All data were log averaged. There is an obvious negative correlation between growth and age. As the mussel ages, there is a noticeable decrease in growth noticeable between the relic shell specimens from site 1. There is no data for site 2, as no shell material for this species was available.

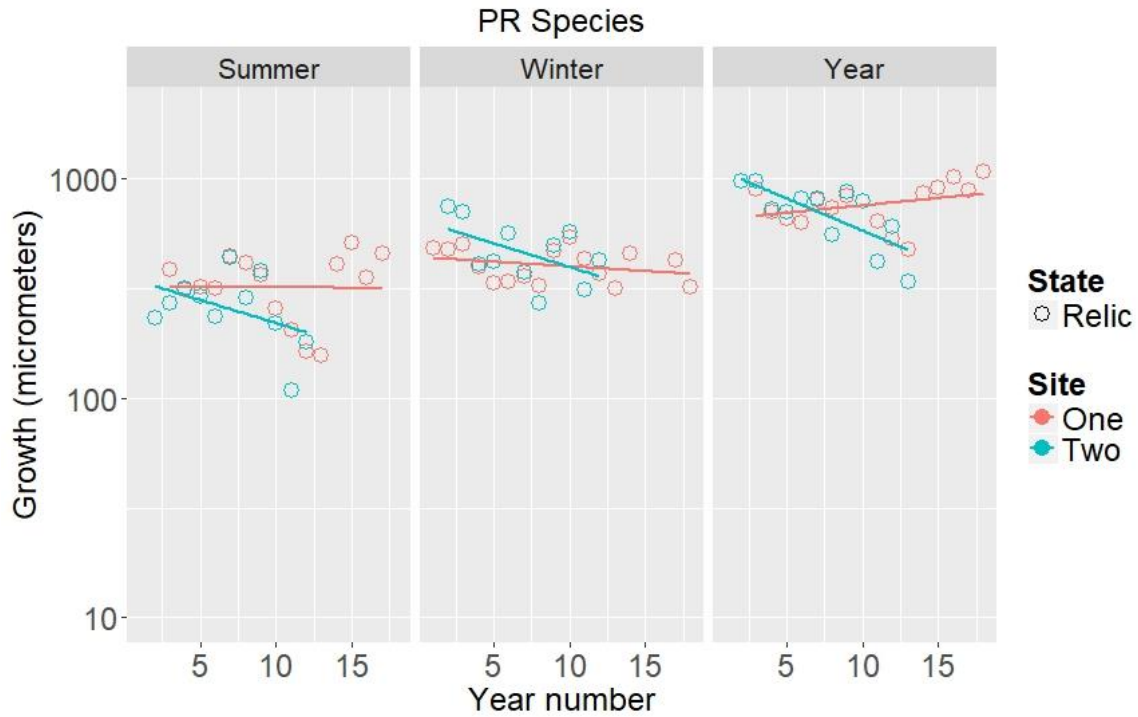


Figure 14: Regression of Growth: *Pleurobema rubrum*

All data were log averaged. There is an obvious negative correlation between growth and age. As the mussel ages, there is a noticeable decrease in growth noticeable between the relic shell specimens from site 1. There is no data for site 2, as no shell material for this species was available.

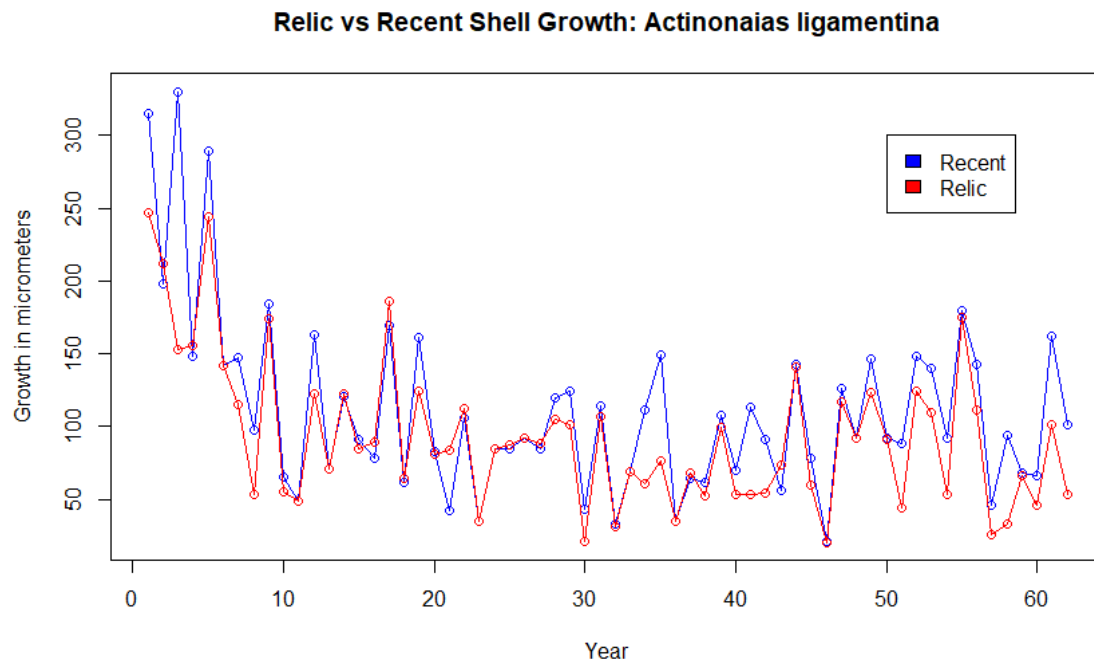


Figure 15: Growth Comparison of Recent and Relic *Actinonaias ligamentina*

This is a growth comparison in micrometers per year between 1 relic and 1 recent specimen of *Actinonaias ligamentina* of the same age from site 1 (McBee Island). There is a consistent increase in average yearly growth rate for the recent specimens when compared to relic specimens from this site.

VITA

Ashley Slater attended the University of Florida upon the completion and graduation from the International Baccalaureate Program in the year of 2009 from Forest Hill High School in West Palm Beach, FL. She majored in anthropology with a specialization in biological and forensic anthropology whilst also minoring in classical studies. During her time at the university, she participated in the excavation of Kingsley Plantation, located on Fort George Island near Jacksonville, Florida, as a student and then as a teaching assistant for two seasons. During this time, she focused on graveyard excavation, sediment analysis, artifact documentation and GIS mapping of several sites within the plantation. In addition to her archaeological interests, she volunteered as an assistant at the Pound Human Identification Lab, located in Gainesville, Florida under the direction of Dr. Michael Warren. After receiving her Bachelor's degree, she joined AmeriCorps as a water quality technician and public servant for the Town of Farragut, located near Knoxville, Tennessee under the CAC AmeriCorps Program. During this time, she participated in the mapping of the waterways of Turkey Creek and Little Turkey Creek and helped implement the adopt-a-stream program by leading several clean-up events with groups from the local area. After she served with the CAC AmeriCorps program for a year, she was accepted into the Earth and Planetary Sciences graduate program to work with Dr. Michael McKinney on freshwater mussels, ecological preservation and archaeological dating techniques. Upon completion of her Master's research, she plans to pursue a career in environmental archaeology and/or biological anthropology in conjunction with museum studies.