RESTORATION OF WAVY-RAYED LAMPMUSSEL (*LAMPSILIS FASCIOLA*), SPIKE (*EURYNIA DILATATA*), AND RAINBOW MUSSEL (*VILLOSA IRIS*) TO THEIR NATIVE RANGE IN THE OCONALUFTEE RIVER BASIN OF CHEROKEE, NORTH CAROLINA

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Biology

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ABSTRACT

RESTORATION OF WAVY-RAYED LAMPMUSSEL (*LAMPSILIS FASCIOLA*), SPIKE (*EURYNIA DILATATA*), AND RAINBOW MUSSEL (*VILLOSA IRIS*) TO THEIR NATIVE RANGE IN THE OCONALUFTEE RIVER BASIN OF CHEROKEE, NORTH CAROLINA

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Western Carolina University (March 2020)

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The Wavy-rayed Lampmussel (*Lampsilis fasciola*) and Spike (*Eurynia dilatata*) are state Species of Special Concern and Rainbow Mussels (*Villosa iris*) are Threatened in North Carolina. Once common in their native range, which reached across most of the Eastern United States, agricultural pollution, siltation, and river impoundments have led to sharp declines in abundance of these invertebrates. A previous feasibility study confirmed that *L. fasciola* and *V. iris* could survive and grow in enclosures in the Oconaluftee River within the Qualla Boundary, and therefore concluded that these species would be good candidates for restoration in that system. This study pursued the next step in the efforts to restore populations of these organisms by introducing individuals of *L. fasciola, V. iris,* and *E. dilatata,* back into the Oconaluftee. Juveniles of *V. iris* and *L. fasciola* were obtained from the North Carolina Wildlife Resource Commission's Conservation Aquaculture Center in Marion, NC; these juveniles were raised from the glochidia of adults collected from the Little Tennessee River, where some populations of both species persist. Adult *E. dilatata* were collected directly from the Little Tennessee River, as this species is not currently cultured under hatchery conditions. The individuals of all three species were marked and stocked at four study sites chosen based on adequate substrate types for mussel survival. Adequate substrate included small cobbles, some fines and sand, but low siltation. Sampling took place over the course of one growing season (May to October 2019) to record survival and growth. Additionally, measurements of the remaining silo populations from the previous feasibility study were continued, as well as monitoring of an additional three new silos at each site, to allow comparison of growth in free-living mussels and those in the enclosures. We concluded that the free-living mussels could survive, and that they showed significantly greater growth than those held in enclosures. All three species had near-perfect survival when stocked into the substrate or placed in silos, with the exception of those in silos lost during storm events. Additionally, we detected positive growth for all species, which differed among sites with individuals growing less at upstream sites for both *L. fasciola* and *V. iris.* Furthermore, free-living individuals showed significantly lower valve damage than those held in silos.

INTRODUCTION

Invertebrate species constitute nearly 99% of global diversity, yet biological research and publicity is heavily skewed to focus on other, larger taxa (Lydeard et al. 2004). As a result, there are many deficits in knowledge of many of these species, including non-marine mollusks. One thing that does appear quite evident, however, is that these organisms play a vital role as ecosystem engineers, altering their habitat both physically and chemically, and even providing habitat for other organisms on their own bodies (Vaughn, Nichols, and Spooner 2008). Freshwater mussels (Order Unionida) are represented by 298 recognized extant species in the United States and Canada alone (Williams et al. 2017). Alarmingly, over 2/3 of these species are thought to be critically imperiled, imperiled, or vulnerable (Bouska et al. 2018, Lopes-Lima et al. 2018). Further, at least 29 North American species have become extinct in the last 100 years (Haag and Williams 2014).

There is a clear global decline in freshwater mollusk populations, even those that are still presumably healthy in some areas of their range (Lopes-Lima et al. 2018, Spooner and Vaughn 2006, Lydeard et al. 2004). Habitat modification, specifically river impoundment, increased point and nonpoint source pollution, and the introduction of invasive mussel species (primarily *Dreissena polymorpha* and *Corbicula fluminea*) are among the potential causes of decline (Haag and Williams 2014, Vaughn and Taylor 1999). Three species facing decline in western North Carolina are the Wavy-rayed Lampmussel (*Lampsilis fasciola*) and Spike (*Eurynia dilatata*), which are listed as State Species of Special Concern, and the Rainbow Mussel (*Villosa iris*) which is listed as State Threatened in North Carolina (Ratcliffe et. al 2018). These species are regularly found in multi-species assemblages elsewhere in the Little Tennessee drainage, but are not known to be found in the Oconaluftee. Each of the three species is unique in its distribution, but share similar habitat preferences. *Lampsilis fasciola* are found as far north as the Great Lakes, inhabiting small to medium sized rivers in the Ohio, Mississippi, and Tennessee River basins (Bogan 2002). They are currently found in the French Broad, Hiwassee, and Little Tennessee River systems of western North Carolina (Bogan 2002). They are hardy, able to withstand slow moving water and fine sediments that most lotic Unionid species cannot tolerate, but they are found in their highest densities in shallow rivers (depths less than 1 meter) with stable gravel streambeds (Bogan 2002, Alderman, Johnson, and McDougal 2001). *Villosa iris* inhabit the Tennessee, Cumberland, Ohio, and Mississippi River basins, in medium sized rivers (Bogan 2002). Found in the Hiwassee, Little Tennessee, and French Broad river systems of North Carolina, these mussels are most numerous in shallow, clearwater riffles with gravel substrate and strong flows (Bogan 2002, Alderman, Johnson, and McDougal 2001). *Eurynia dilatata* (also *Elliptio dilatata* or *Unio dilatata*) is found in the vast majority of the Mississippi River drainage, as well as the Ohio River basin as far north as the Great Lakes and south to the Tennessee River basin (Alderman, Johnson, and McDougal 2001). In North Carolina, it can be found in the Hiwassee, Little Tennessee, French Broad, and New River drainages (Bogan 2002). These mussels are more generalized in habitat preference, living at a variety of depths and substrate types, but are thought to grow best in firm gravel substrates and moderate water flows (Bogan 2002, Elderkin et al. 2010).

Freshwater mussels, including *L. fasciola, E. dilatata* and *V. iris,* fill highly important roles in the river ecosystems they occupy. Not only do these large, long-lived invertebrates (potentially living over 20 years) contribute significantly to the trophic web themselves, they promote the survival and abundance of other macroinvertebrates (Vaughn and Hakenkamp

2001). These bivalves are primarily filter feeders, removing suspended particulate organic matter and meio-/micro-organisms such as phytoplankton and bacteria from the water column. They help regulate the level of primary production and abate eutrophication in polluted systems while simultaneously increasing elemental nutrient content of the substrate (Spooner and Vaughn 2006). The mussels also increase the nutrient content of the substrate by removing particles from the water column and transferring them to the sediment in the form of biodeposits, many of which are practically unaffected by the digestion of the mussel and act as valuable food sources for other organisms (Howard and Cuffey 2006). Not only this, but their bioturbation of sediments oxygenates streambeds, encouraging growth and survival of diverse benthic communities of microbes and invertebrates (Vaughn and Hakenkamp 2001). These activities of the mussels can help to make a lotic ecosystem more resilient and speed recovery following disturbance, but only if they have a significant biomass and a healthy, diverse population (Howard and Cuffey 2006). Established mussel beds provide stability for the substrate, preventing bedload shift and lessening the effect of high flow events and allowing for easier re-establishment of benthic organisms after disturbance (Cowie et al. 2017). This could be of particular importance in mountainous streams, such as the Oconaluftee, where elevational changes and unique tributary topography can contribute to rapid fluctuations in water flow. By providing hard substrate, the valves of living mussels are regularly colonized by other organisms (such as tardigrades and bacteria); they enhance the habitat quality around them and provide habitat on and between their shells (Spooner and Vaughn 2006). Diverse and abundant macroinvertebrate assemblages have positive effects on numerous fish species and increases the overall health of the ecosystem.

While most larger streams of the Upper Tennessee River Basin in western North Carolina (Nolichucky, Watauga, French Broad, Pigeon, Tuckaseegee, Little Tennessee, and Hiwassee) have at least some populations of native mussels, the Oconaluftee River in the Qualla Boundary of North Carolina, the major tributary to the Tuckaseegee has no recorded occurrence of mussels. The exact cause of their extirpation is not fully known, but this drainage did experience dramatic changes in the twentieth century including impoundment, rapid riparian development, and heavy trout stocking. All of these are thought to be factors leading (directly or indirectly) to the loss of mussel populations, and are widespread issues in many watersheds (Haag and Williams 2014). The timber industry exploded in the southern Appalachians beginning in the late 19th century and timber rights to thousands of hectares of tribal land were sold. In the early 20th century an introduced blight reached the remaining chestnut-dominated forests of the Qualla Boundary, and an estimated additional 9000 ha of forest was lost (EBCI Natural Resources Department 2013). The town of Bryson City constructed a hydroelectric dam near the confluence of the Oconaluftee and Tuckaseegee Rivers in 1924-25 which essentially isolated the Oconaluftee from the rest of the Little Tennessee watershed. With the establishment of the Great Smoky Mountains National Park and Blue Ridge Parkway in the 1930s, the Qualla Boundary became the most visited Native American lands in the United States and led to rapid development targeting tourists (Beard-Moose and Paredes 2009). In the late 1990s a casino, hotel, and conference center opened along Soco Creek, a major tributary of the Oconaluftee and introduced a new wave of development.

Finigan (2019) explored the feasibility of restoring *L. fasciola* and *V. iris* in the Oconaluftee by placing individuals in enclosures at three sites in the river and found that they survived well and grew at all three locations. These individuals were kept in enclosures, rather than living freely in the river substrate. I conducted a study to measure the survival and growth of wavy-rayed lampmussels, spike, and rainbow mussels stocked into the Oconaluftee outside of enclosures. I used PIT tags to aid in relocation of individuals for data collection to track survival and growth. I also continued to monitor growth and survival of the mussels in enclosures from the previous study in order to compare their growth and survival over time with those that are free-living in the river with PIT tags.

The introduction of three species establishes some mussel diversity at the study sites. These mussels are known to aggregate in multi-species assemblages, and while the exact ecological function of each species is not fully known, their tendency to form diverse benthic communities indicates that each species uses resources sufficiently differently to allow coexistence (Vaughn and Hakenkamp 2001). Therefore, preserving biodiversity of these bivalves helps to ensure not only their persistence, but also the benefits they provide to their ecosystems.

METHODS

Site Selection

We selected sites for introduction of the Wavy-rayed Lampmussel (*Lampsilis fasciola*), Spike (*Eurynia dilatata*), and the Rainbow Mussel (*Villosa iris)* based on used in the previous feasibility study (Finigan 2019, Table 1), and on the availability of adequate substrate types for the establishment of a new mussel bed. Site 1 was located upstream of the Ela reservoir in the Oconaluftee River and downstream of the Cherokee Wastewater Treatment Plant. Site 2 was located immediately upstream of the treatment plant, and Site 3 was located even farther upstream, above the confluence of Soco Creek and the Oconaluftee. Site 4, not used in the previous feasibility study, was added further upstream above town and the popular tourist area near the border between the Qualla Boundary and Great Smoky Mountains National Park and the Blue Ridge Parkway (Figure 1). I chose these sites because they each provide slightly different habitat conditions associated with their location in the watershed and proximity to development. In addition to expected temperature increases from upstream to downstream, Sites 3 and 4 were positioned above the confluence of a tributary with significant development in its riparian zone (Soco Creek). Site 2 was downstream of this confluence but above the water treatment plant and therefore would avoid any additional nutrients or pollutants from the waste treatment plant. Site 1, then, would experience any introductions from either Soco Creek or the treatment plant in addition to any changes resulting from close proximity to the reservoir resulting from impoundment. All three of the lower sites exhibited success in mussel growth and survival during the earlier feasibility study, so I hypothesized that they would exhibit similar success for mussel reintroduction.

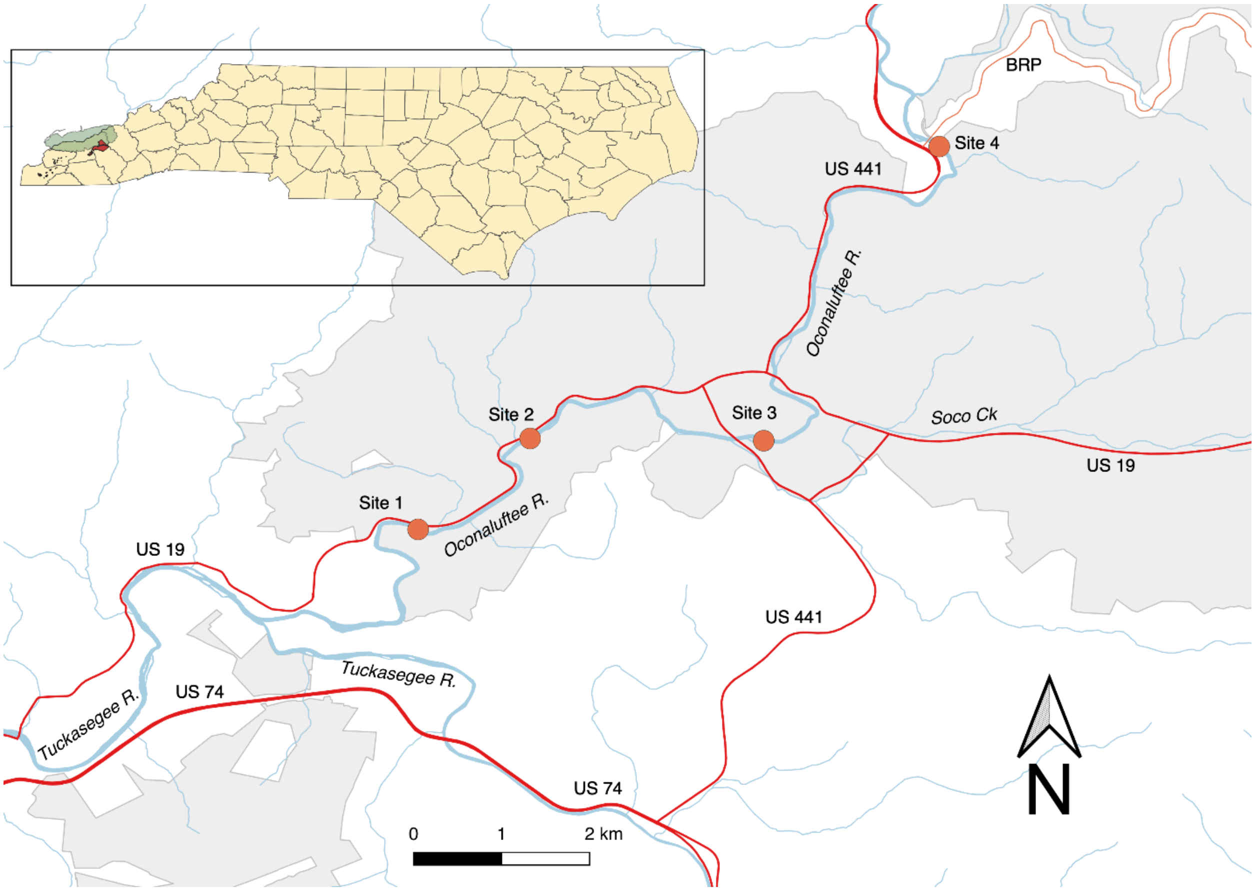


Figure 1. Map showing site locations on the Oconaluftee River within the Cherokee Qualla Boundary. On the smaller map, the red area represents the Qualla, and the green represents the Great Smokey Mountains National Park.

Table 1. Coordinates of each study and river kilometers from the confluence with the Tuckaseegee River.

Site	Latitude	Longitude	River Km
1	35.4574	-83.3642	3.9
2	35.4688	-83.3502	6.1
3	35.4685	-83.3210	9.4
4	35.5053	-83.2990	15.8

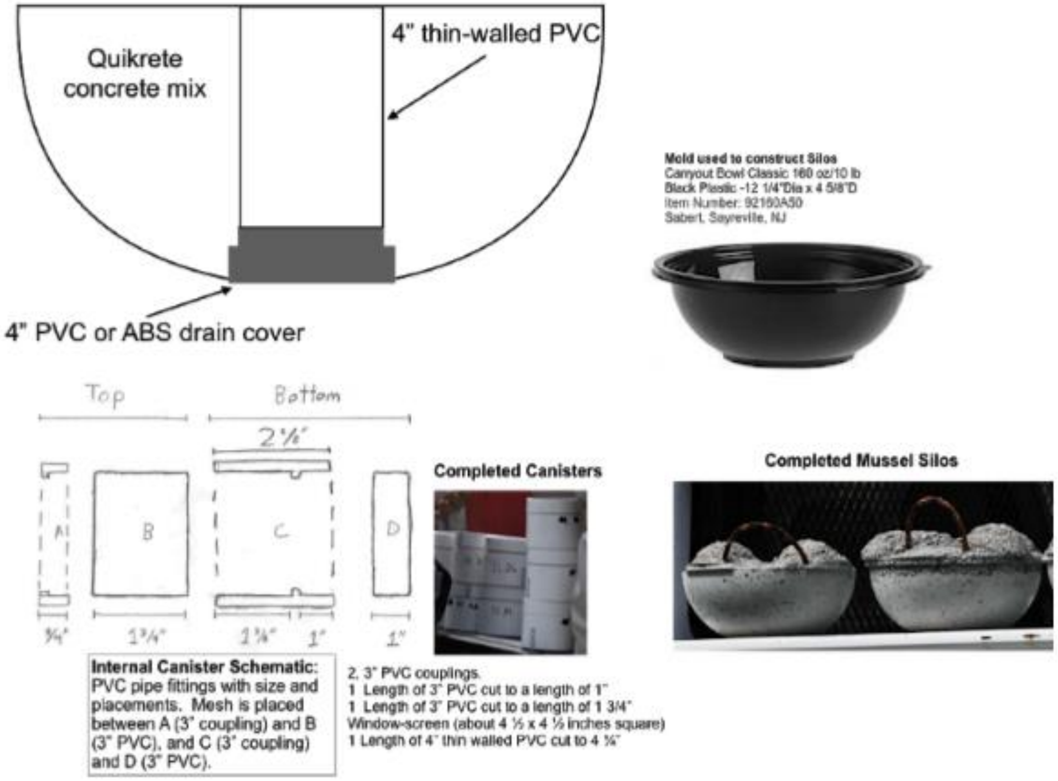
Mussel Tagging and Placement

The NCWRC Conservation Aquaculture Center in Marion, NC provided Wavy-rayed Lampmussels and Rainbow mussels that had been reared from gravid females collected out of the Tuckaseegee River in 2016. Largemouth Bass (*Micropterus salmoides*) were used as the host fish to support them during their obligate parasitic phase as glochidia larvae. After transformation, they were transferred from a recirculating tank to a flow-through system supplied by pond water (Rachael Hoch, NCWRC, personal communication). One hundred-twenty mussels of each species were marked using Hallprint Shellfish tags (27 Commerce Crescent, Hindmarsh Valley, South Australia, 521) fixed to their shells using waterproof super glue (Loctite Ultra Gel, Henkel Corporation, Rocky Hill, CT) and sealed with clear brush-on nail glue (Omega Labs USA, Robanda International, Inc., San Diego, CA), (Lemarié et. al 2000). Additionally, sixty juveniles that were large enough (18 mm or larger) when collected were tagged using a Passive Integrated Transponder (PIT) tags (Oregon RFID, 8mm FDX-B, 2421 Southeast 11th Avenue, Portland, OR 97214) , so that 20 of each species could be stocked into the substrate at each of the first three sites. I fixed PIT tags to the right valve with superglue, and then covered them with JB Water Weld (P.O. Box 483 Sulphur Springs, TX 75483) molded to the shell. I smoothed out the edges of the epoxy evenly, to prevent the tag from being scraped off. The 2016 cohort of *Villosa iris* did not contain enough individuals to be used in both the free-living group and in the silo enclosures, so those used in the silos were from the 2017 cohort.

We did not place free-living mussels at site four because this area of stream bed was almost exclusively large cobbles in the areas of the stream that were accessible, and therefore did not appear to be good habitat for these. The use of PIT tags allowed for much easier location of individuals (particularly juveniles) in the field using a water-resistant PIT tag reader (Biomark

Identification Solutions, HPR Plus, 705 S. 8th Street, Boise, Idaho 83702), with the Hallprint tags allowing for easy identification of individuals at first glance. The remaining 60 *L. fasciola* and *V. iris* were placed in enclosures (silos), with three silos containing five individuals of each species placed at each site. Silos are dome-shaped concrete enclosures with a hollow column in the center; the hollow center contains a PVC chamber enclosed with screen on both sides so that mussels have access to the water column to feed (Barnhart et al. 2007, Figure 2). Water flows over the dome, creating negative pressure and flow through the center column, allowing continuous access to oxygen and food particles so long as the chambers are not allowed to clog with sediments and do not become buried. To prevent movement of silos during times of high flow, we arranged the enclosures in an arrow-shaped cluster with the upstream facing sides angled down, encouraging water to flow over the domes and not beneath them (Figure 3). The initial introductions of these mussels took place in May 2019, with monthly surveys following over the course of the growing season. For each data collection event, I collected, identified, and measured the mussels (length, width, and height in mm) with digital calipers. The USGS river gauge (USGS 03512000) near site two at Birdtown was used as a baseline for water chemistry parameters.

The Spike (*Eurynia dilatata*) were not acquired from the Conservation Aquaculture Center, as they do not survive well in hatchery conditions (Rachael Hoch, personal communication). We collected adults directly from the Little Tennessee River and translocated them to the Oconaluftee in June. I completed collection with the help of wildlife technicians from the North Carolina Wildlife Resources Commission. The adult spike were collected, tagged in the field with both PIT and Hallprint Shellfish tags similarly to the other two species, and initial measurements taken prior to transport. We then immediately transported mussels in a cooler of river water and placed them at sites one through three in the Oconaluftee. Translocation was completed in one day in order to prevent prolonged stress and loss of life from being held in containers outside of the river. We placed thirty-two *E. dilatata* in the already established mussel beds present at the first three sites; none of them were placed in silos due to their large size.



Internal Canister Schematic: PVC pipe fittings with size and placements. Mesh is placed between A (3" coupling) and B (3" PVC), and C (3" coupling) and D (3" PVC).

2, 3" PVC couplings.
1 Length of 3" PVC cut to a length of 1"
1 Length of 3" PVC cut to a length of 1 3/4"
Window-screen (about 4 ½ x 4 ½ inches square)
1 Length of 4" thin walled PVC cut to 4 ¾"

Figure 2. Mussel silo construction modified from an original design by Dr. M. Christopher Barnhart at Missouri State (Barnhart et al. 2007, Huffstetler and Russ 2008).

Figure 3. Mussel silos arranged in a streamlined shape with upstream sides angled down to prevent movement during high flow events. The arrow indicates direction of water flow.

Valve Damage Scores

Within the first few sampling events, I noticed that the mussels held in silos appeared to have scrapes, chipping, and even deep gouge marks in their valves that were not present in most of the free-living individuals. In order to quantify this damage, I developed a rubric to provide a numeric score for each individual mussel based on physical wear and tear present on its valves (Table 2). I assigned scores for all mussels collected either from the substrate or from silos in October, the final month of sampling. I took photos of the left and right valves of each mussel in the field, then scored based each of them on the rubric (Figure 4). Mild surface-level wear on and around the umbo of a mussel was not considered to be damage, as this area is naturally worn down by the regular activity of the animals.

Table 2. Grading criteria for the valve damage scores assigned to each individual mussel collected in the final sampling event.

Score	Group	Criteria
1	No Damage	no obvious scrapes, chips, or gouges into the valves
2	Mild Damage	visible scraping on valves, no chips out of valve edges or deep gouges
3	Damaged	visible scrape marks and deep gouging into the valves or edges chipped
4	Severely Damaged	valve is cracked, broken, or crushed in; some part of the animal inside may be visible.

Figure 4. Examples of mussels that received valve damage scores of a one, two, and three (from left to right). Note that the mild wear around the umbo of C047 is not considered damage, but rather simply normal valve condition.

Data Analysis

I compared length measurements of individual mussels over time in order to examine growth for individuals from the original size measurements taken at stocking. Sampling events were always conducted in such a way so as to limit the emersion time of mussels as much as possible, to reduce potential mortality from handling. In order to lower emersion time, collected mussels were held in mesh bags submerged in the river's edge until time for their processing, and then immediately placed back into submerged mesh bags or their silo columns following measurements. Taking measurements during the growing season when air temperature and water temperature were more similar also aided in limiting shock caused to the animals due to dramatic differences in air and water temperatures (Bartsch et al. 2000; Ohlman and Pegg 2019). Differences in growth among sites were compared using a repeated measures ANOVA (linear mixed-effects model analysis). Type II SS and the Kenward-Rogers approximation for denominator degrees of freedom were used to estimate p-values. The Kenward-Rogers approximation is thought to be one of the more conservative methods for estimating significance. Unfortunately, a test for differences in growth between individuals stocked outside enclosures with those inside silos was only possible for *L. fasciola* as differences in growth for *V. iris* were confounded by different cohorts. Data analysis was conducted in R (v. 3.6.1, R Core Team 2019) using the lme4 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2017) packages. The relationship between valve damage scores and location (treatment and site) were tested using G-tests of independence from the DescTools package (Signorell et al. 2020).

RESULTS

All three species had near perfect survivorship, with little evidence of mortality in mussels stocked freely into the substrate or in silos. Those in silos that did not wash away in heavy rains had very few losses as well, but silos that washed away were often buried and the mussels within smothered. The only observed mortalities not associated with the loss of a silo were four *Villosa iris*, two *Lampsilis fasciola*, and four *Eurynia dilatata*. There was no observed mortality at sites two and four.

All three species showed significant positive growth at all four sites (Tables 3, 4, and 5, Figures 5, 6, 7, and 8). The rate of growth differed among sites with individuals growing less at upstream sites for both *L. fasciola* and *V. iris* (Tables 3 and 4, Figures 4 and 5). Slightly higher water temperature (differing by an average of approximately 0.64℃ between sites 1 and 3 according to Finigan 2019) and potentially higher availability of suspended algae and microbes are likely the cause of more rapid growth at sites 1 and 2. I observed the greatest growth rate at the farthest site downstream in both *V. iris* and *L. fasciola*. Growth of free-living *L. fasciola* mussels was significantly greater than for those inside silos at all three sites (Table 3). This was also true for *V. iris*, but because this effect was confounded by differing cohorts, we cannot make this comparison with confidence (Table 4). There was a significant difference in mussel growth over time between sites as well as treatments, with free-living mussels at down stream sites showing the greatest success in both *V. iris* and *L. fasciola*. I did not detect any differences among sites in growth of *E. dilatata* and they grew much slower than the other species, likely due to the slower growth rates in adult mussels (Table 5). This species showed the greatest growth at site two, rather than site 1 as in the others (Figure 8).

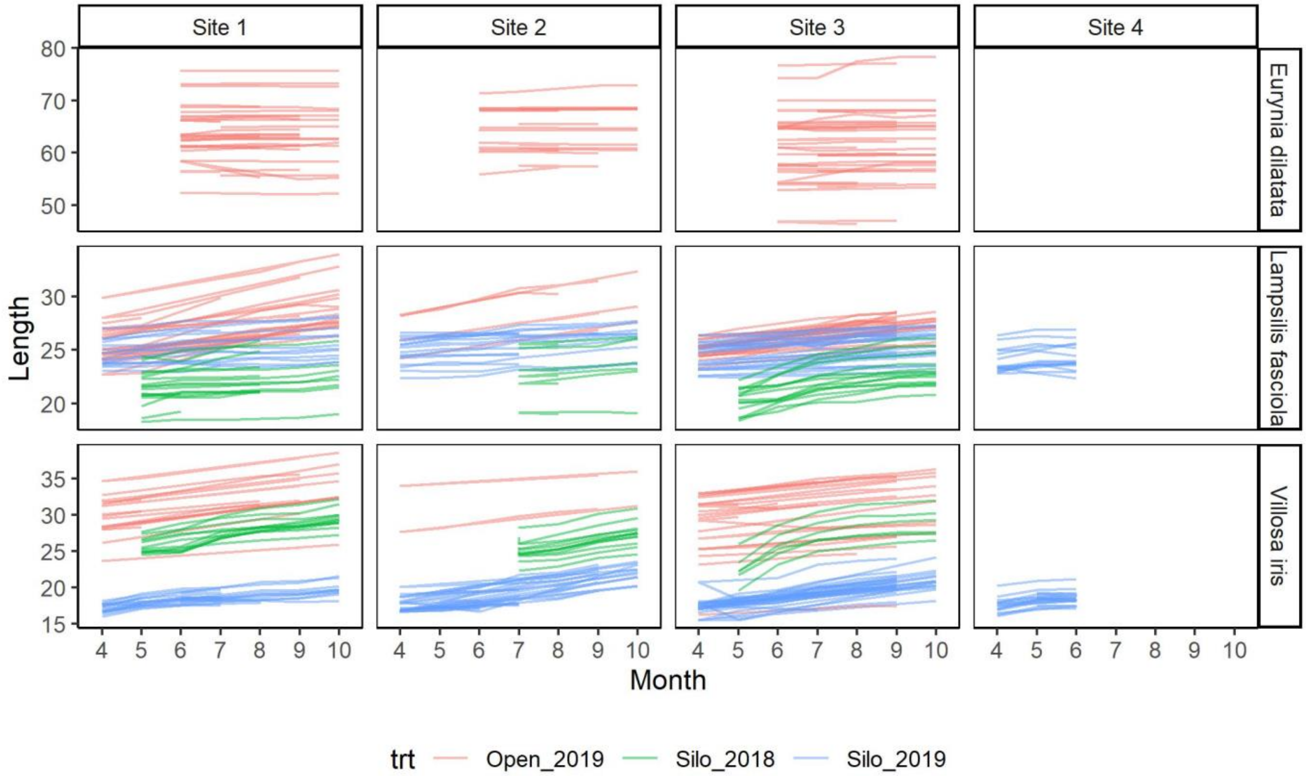


Figure 5. Growth trajectories of individual mussels over the course of the entire study by site and species. “Open_2019” refers to individuals stocked into the stream substrate in spring 2019; “Silo_2018” refers to individuals held in silos in the river since spring of 2018; and “Silo_2019” refers to individuals held in silos since spring 2019. Site 4 data ends in June, because all of the silos at this site washed away during a rain event between the June and July samples, and were not successfully recovered with snorkel surveys.

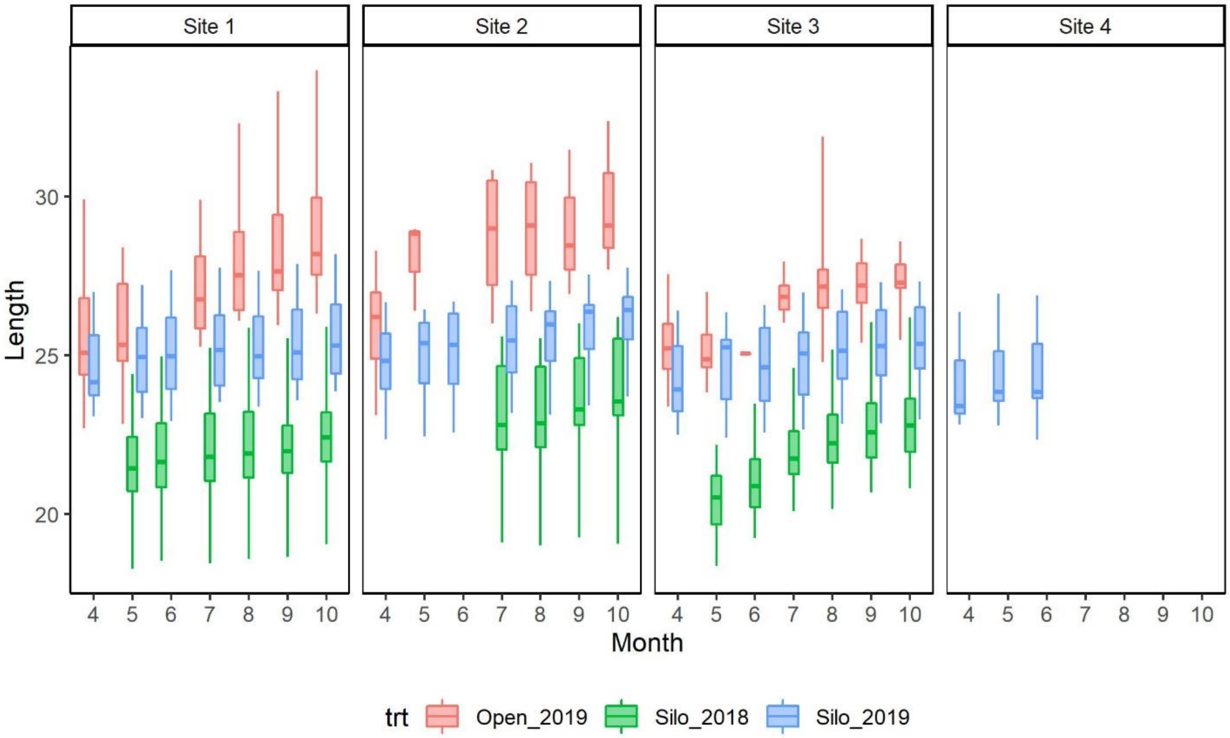


Figure 6. Boxplots of *L. fasciola* length. “Open_2019” refers to individuals stocked into the stream substrate in spring 2019; “Silo_2018” refers to individuals held in silos in the river since spring of 2018; and “Silo_2019” refers to individuals held in silos since spring 2019.

Table 3. Linear mixed-effects model analysis results for *Lampsilis fasciola* length. Type II SS and the Kenward-Rogers approximation for denominator degrees of freedom were used to estimate p-values.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Site	0.251	0.125	2	103.9	1.25	0.2907
Month	207.018	34.503	6	336.7	344.43	0.0000
trt	4.377	4.377	1	100.2	43.70	0.0000
Site:Month	4.597	0.383	12	336.8	3.82	0.0000
Site:trt	0.021	0.011	2	116.2	0.11	0.8994
Month:trt	42.886	7.148	6	337.7	71.35	0.0000
Site:Month:trt	5.862	0.586	10	337.2	5.85	0.0000

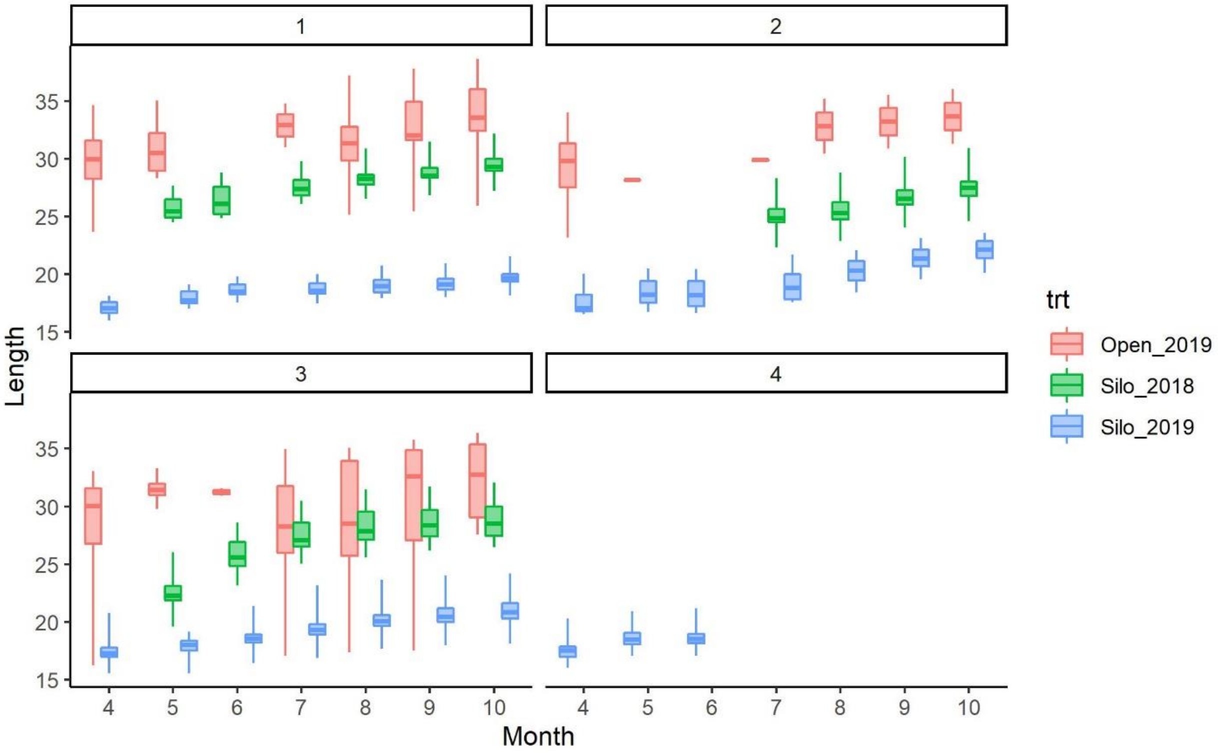


Figure 7. Boxplots of *V. iris* length. “Open_2019” refers to individuals stocked into the stream substrate in spring 2019; “Silo_2018” refers to individuals held in silos in the river since spring of 2018; and “Silo_2019” refers to individuals held in silos since spring 2019.

Table 4. Linear mixed-effects model analysis results for *Villosa iris* length. Type II SS and the Kenward-Rogers approximation for denominator degrees of freedom were used to estimate p-values.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Site	0.251	0.125	2	103.9	1.25	0.2907
Month	207.018	34.503	6	336.7	344.43	0.0000
trt	4.377	4.377	1	100.2	43.70	0.0000
Site:Month	4.597	0.383	12	336.8	3.82	0.0000
Site:trt	0.021	0.011	2	116.2	0.11	0.8994
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Site:Month:trt	5.862	0.586	10	337.2	5.85	0.0000

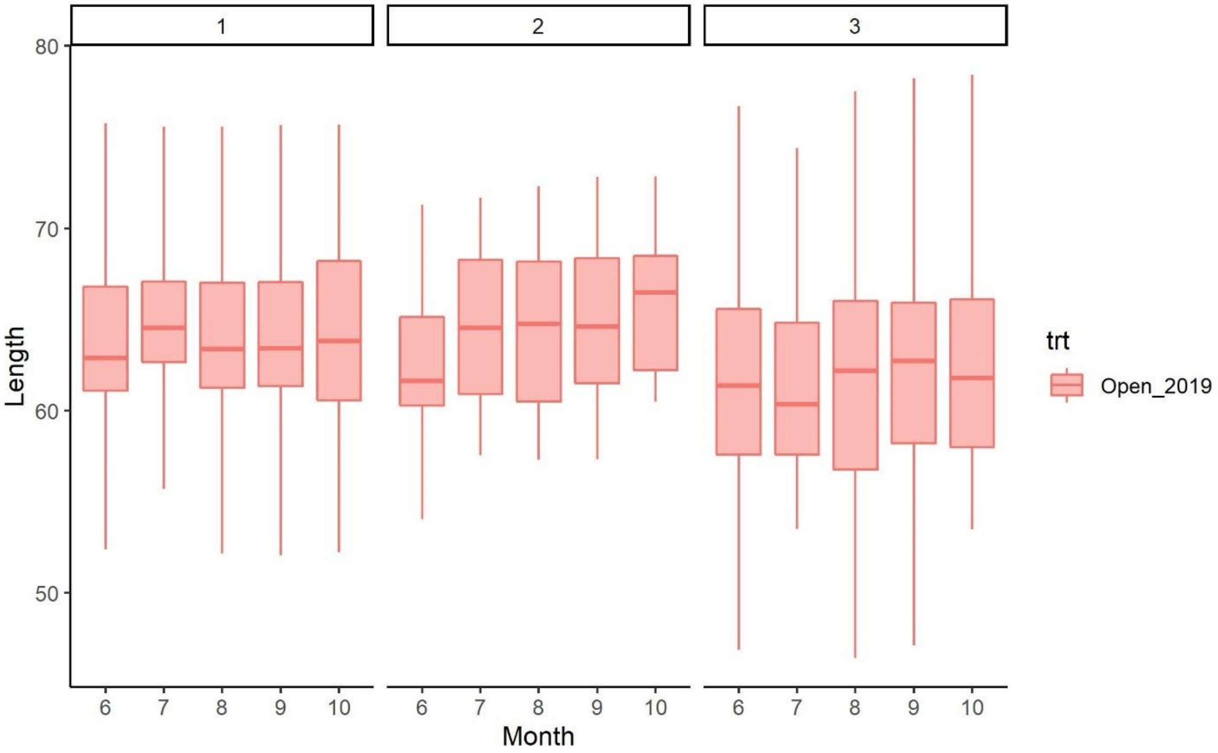

Figure 8. Boxplots of *E. dilatata* length. “Open_2019” refers to individuals stocked into the stream substrate in spring 2019; “Silo_2018” refers to individuals held in silos in the river since spring of 2018; and “Silo_2019” refers to individuals held in silos since spring 2019.

Table 5. Linear mixed-effects model analysis for *Eurynia dilitata* length. P-value estimated using Type II SS and the Kenward-Rogers approximation for denominator degrees of freedom

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Site	0.096	0.048	2	229.3	0.18	0.8341
Month	4.259	1.065	4	198.4	4.02	0.0037
Site:Month	4.232	0.529	8	198.4	2.00	0.0483

The valve damage scores for both *L. fasciola* and *V. iris* were significantly lower in the free-living individuals than those in silos at all three sites (Figures 9 and 11). This was true both when individuals in silos from 2018 were included and when they were excluded from the calculation (Figures 10 and 12).

Other qualitative observations that I recorded included that the stocked animals were often found with numerous macroinvertebrates, including caddis flies and mayflies, inhabiting their valves. Mussels stocked into the substrate were always found congregated closely together, even when they were placed far from one another. The animals in silos at site one occasionally presented with black staining around the umbo and interior of the valves, which occurs when the animals are exposed to low dissolved oxygen levels.

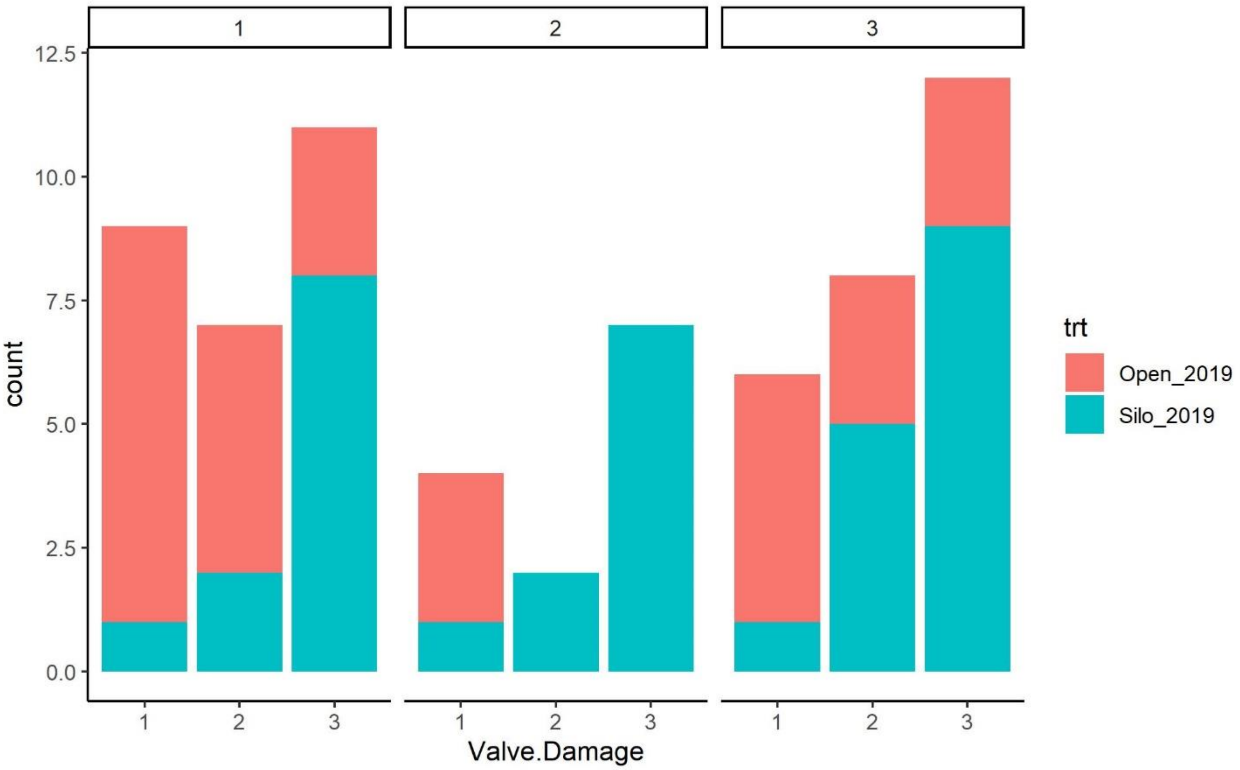

Figure 9. Valve damage scores for *Lampsilis fasciola* individuals that are free-living ("Open_2019") and those that are held in silos ("Silo_2019"). For the G test of valve damage score by site and treatment, G = 133.52, df = 35, $p < 0.0001$.

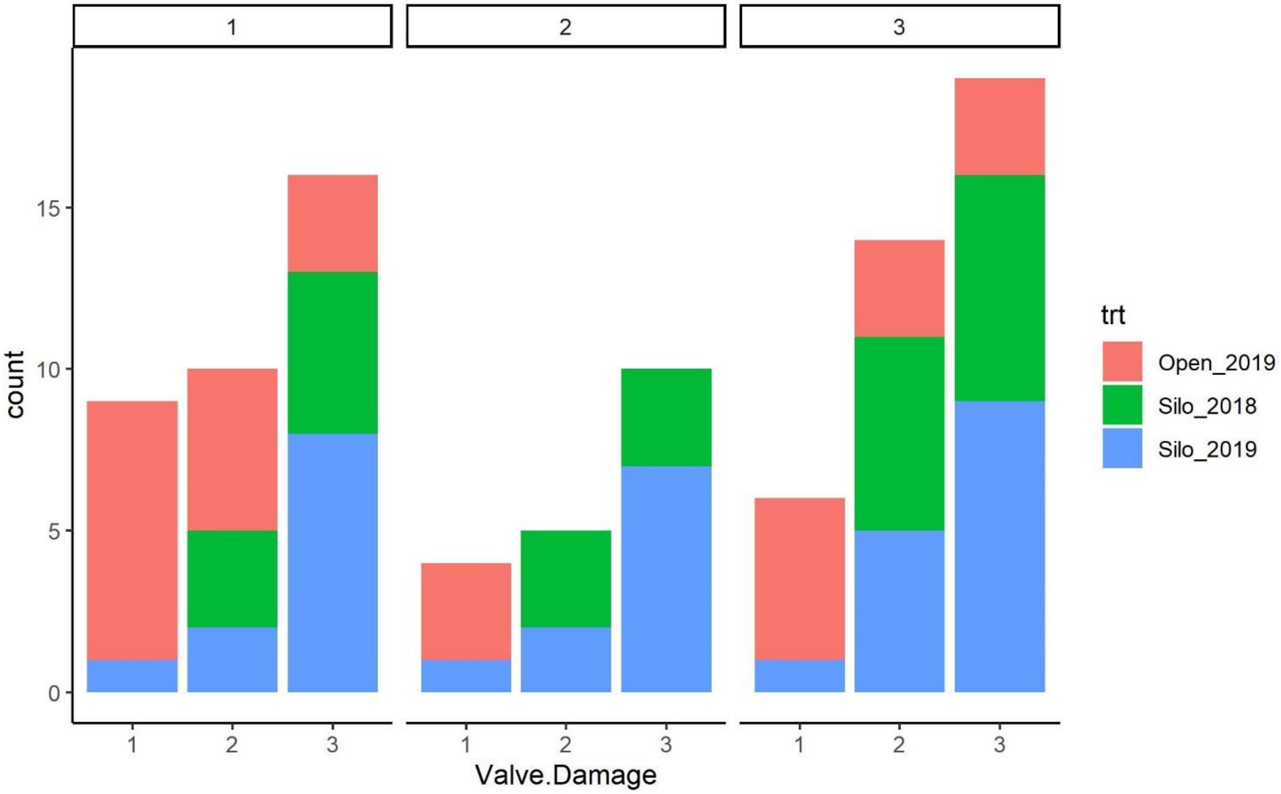

Figure 10. Valve damage scores for *Lampsilis fasciola* individuals that are free-living ("Open_2019") and those that are held in silos for both years ("Silo_2019", and "Silo_2018"). All of these mussels are from the same cohort, but the "Silo_2018" group has been held inside the silos for one year longer than the others. For the G test of valve damage score by site and treatment, G = 121.61, df = 35, $p < 0.0001$

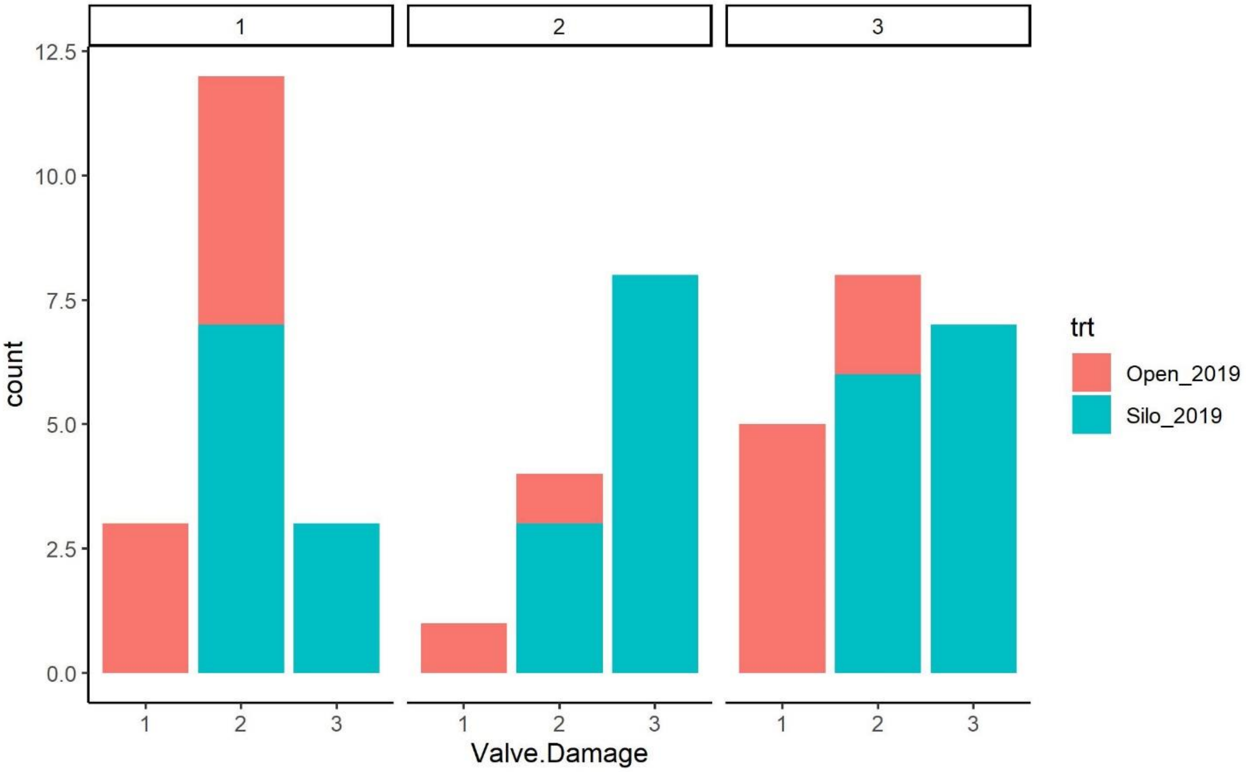

Figure 11. Valve damage scores for *Villosa iris* individuals that are free-living (“Open_2019”) and those that are held in silos (“Silo_2019”). Individuals in the “Silo_2019” group are one year younger than the others. For the G test of valve damage score by site and treatment, G = 128.47, df = 35, p < 0.0001.

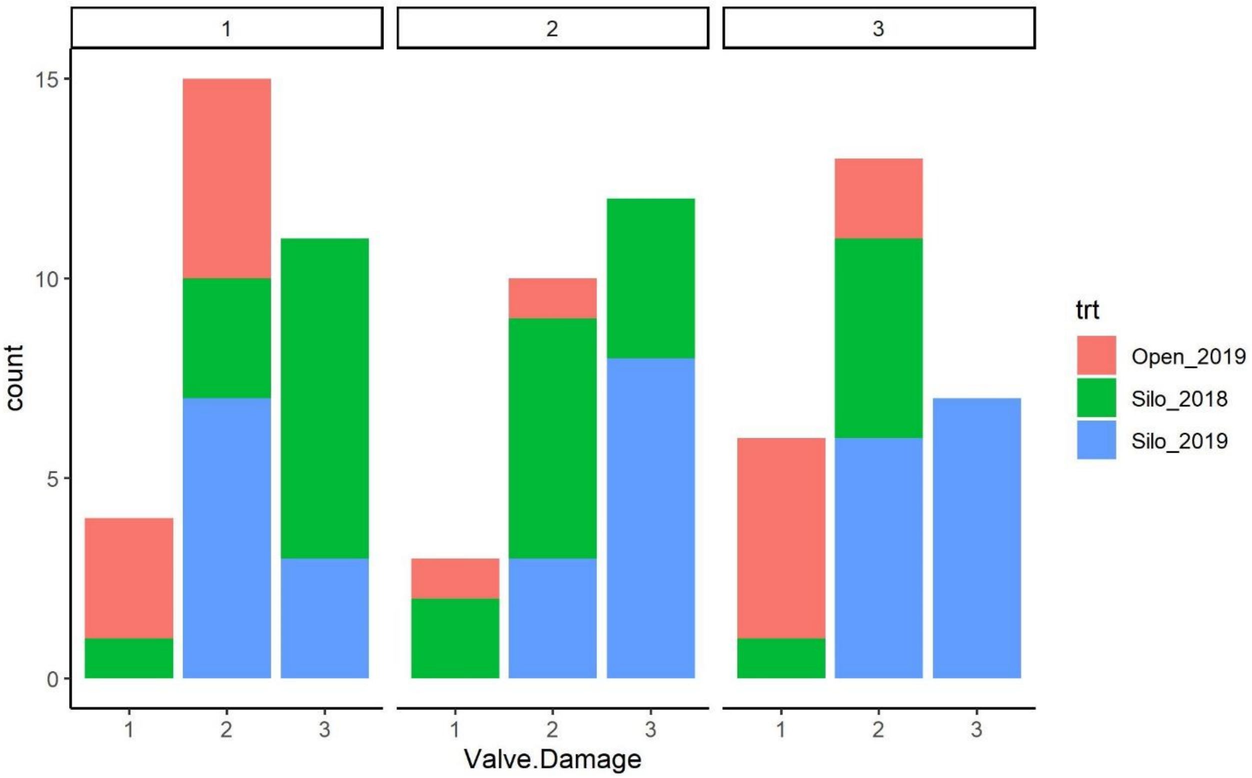

Figure 12. Valve damage scores for *Villosa iris* individuals that are free-living ("Open_2019") and those that are held in silos for both years ("Silo_2019", and "Silo_2018"). Individuals in the "Silo_2019" group are one year younger than the others. For the G test of valve damage score by site and treatment, G = 123.94, df = 35, p < 0.0001.

DISCUSSION

All three species survived and grew at all study sites, which suggests that the Oconaluftee River is a suitable location for restoration of these three species. Not only that, but the mussels appeared to be performing ecological functions in the river. Both free-living and silo mussels provided habitat to macroinvertebrates, which inhabited their valves. The mussels displayed normal growth patterns as observed by other researchers over the course of the growing season, with higher growth rates during warmer months and slower towards the end of the season (Finigan 2019, Rooney 2010, Beaty and Neves 2004, Augspurger et al. 2003). The tendency for higher growth rates at slightly warmer temperatures would explain why the *V. iris* and *L. fasciola* showed higher growth at the two most downstream sites. The mussel beds established at the first three sites remained in their original locations throughout the study, even when the silos at these same sites were washed hundreds of meters downstream. The movement of silos and other enclosure types is not uncommon during in situ mussel studies, and has been reported by many researchers (Elderkin et al. 2008, Haag and Commens-Carson 2008, Bartsch et al. 2000, Huehner 1987). The fact that the mussel beds remained in consistent locations in spite of bedload shifting may indicate that in particularly unstable river systems (as in the Oconaluftee and many others in this region), keeping mussels in silos may place them at a greater risk for suffocation, as they have no means of escape if the enclosures are buried during adverse weather events. Those individuals kept in silos often had greater wear and tear on their valves that those outside of enclosures. Even when free-living mussels were placed away from one another when returned to the substrate after a data collection event, the mussels were always found in close proximity to one another the following month. Uryu et al. (1996) found that juvenile freshwater mussels, *Limnoperna forunei* (Dunker), moved to aggregate and form small clumps in a controlled laboratory experiment, but Perles et al. (2003) were unable to detect similar behavior in Fatmucket mussels, *Lampsilis siliquoidea* (Barnes 1823). The mussels in the Perles et al. study were stocked into the artificial channels used for the experiment at densities higher than observed in the field which may explain the lack of further movement to form aggregates. The behaviors and conditions that result in aggregate formation is area of active research (e.g. Liu et al. 2014, van de Koppel et al. 2008).

Free-living mussels appeared to naturally form multi-species beds, as is consistent with what other researchers have found (Bogan and Roe 2008, Alderman et al. 2001). Multi-species mussel beds allow for greater resilience and positive ecological impact, as each species performs a unique ecological function (Liu et al. 2014, Mitchell et al. 2018, Cowie et al. 2017). Restoration efforts for multiple species in the same location, as in this study, may help to increase success.

Site two consistently had noticeably higher velocity flows than at the other sites, likely because the channel was narrower at this site. This site also had the greatest incidence of silo displacement from high flow events, as is evident in that all but two of the silos were washed away completely at some point in the study. The two most easily recovered silos were still found over 10 meters downstream; the others had to be located by snorkel survey and were hundreds of meters downstream. The number of recovered mussels in the open treatment group was much lower at this site than at the others. The mussels buried themselves deeper in the substrate at this site than at the other sites, and this decreased our ability to locate them with the PIT tag reader. Mussels at this site were regularly found 20-30 cm below the substrate, while those at other sites were rarely deeper than 15 cm. This may be due to greater penetration of oxygenated water into the substrate from the higher flows. I noticed that the bedload at this site shifted even between monthly sampling events, indicating that this site was consistently unstable.

Site three had some unique traits and changed significantly over the course of the study. Initially, this site was a fast-moving, shallow, wide section in the river. Late in May, a large tree washed downstream and lodged in the river just downstream of the mussel site. Over the course of the growing season, this tree accumulated debris and formed a partial dam, resulting slower velocity and deeper water. The mussels at this site were often recorded sitting on top of the substrate rather than buried or partially buried. The only known instance of predation also occurred at this site; the empty, partially crushed valves of one *E. dilatata* individual were found on the bank with tracks and scat of a river otter close by.

The greatest challenge throughout the study was not the location of juvenile mussels within the river substrate as was originally anticipated, but rather the repeated loss of silos due to high flow events. In total, 6 silos washed away at some point in the study and were not able to be found or were found buried with all of the mussels inside smothered. One such silo was found nearly one kilometer downstream from its original placement site. Several other silos were washed downstream and were successfully recovered with snorkel surveys. This movement of enclosures, not predation or water conditions, was the most dangerous factor for the mussels in our study. The increase in damage to the valves of the mussels inside the silos may limit growth rate, rather than simply the limitation of feeding to the water column alone as some researchers have speculated (Barnhart et al. 2007, Bouska et al. 2018). The design of the silos funneling water through the column could cause them to become inhospitable during higher flows, as the water flows rapidly through the column and the mussels within have no means to escape impacts from sediment or even other mussels inside the silo. This results in scraping, chipped edges, and deep scars in the valves of the mussels. Others have found that simple handling such as measurement with calipers can cause scars and stunted growth in the annual rings of mussel valves in Unionids (Haag and Commens-Carson 2008, Ohlman and Pegg 2019). Exposure to high levels of movement inside silos with each rainfall could cause similar internal damages as well, while the free-living mussels can escape much of this disturbance by digging down into the substrate.

The overall success of these three mussel species stocked into the open substrate of the Oconaluftee River is encouraging, and it provides evidence that there can be success both in translocating adult individuals and in introductions of hatchery-raised mussels into this watershed. Based on our results, the Oconaluftee River should be an excellent candidate for establishment of reproducing populations of *Lampsilis fasciola, Villosa iris, and Eurynia dilatata*. Efforts towards re-establishing populations of Unionid mussels are extremely important in light of the dramatic declines seen other parts of their range. While the exact causes of much of the losses are not known, there are increasing efforts to understand the physiological tolerance of different mussel species worldwide to better understand the potential causes. The general rise in pollutants caused by human population increase and urbanization, coupled with rising global temperatures and weather extremes resulting from climate change cause freshwater mussel populations decline (Mitchell et al. 2018, Cowie et al. 2017, Alderman et al. 2001, Lyons et al. 2007). Translocation and establishment of new populations in better protected river systems remains one of the best options for protecting this group of organisms in the future (Cowie et al. 2017, Bogan and Roe 2008, Neves 1999, Lyons et al. 2007). The Oconaluftee River on the Eastern Band of Cherokee Indians (EBCI) Qualla Boundary is a prime location for such an effort; it has protected headwaters within the Great Smoky Mountains National Park, and the

EBCI Natural Resources Department has made significant efforts to protect its watershed (including the establishment of new, rigorous water quality standards in 2019, EPA Eastern Band of Cherokee Indians Water Quality Standards established 2019). Further efforts should be focused on establishing populations of reproductive adults of all three species, as the only introduced individuals of reproductive maturity were *E. dilatata*. This site may be an option for introductions of other species as well. Rather than using concrete silos, which may limit the success of stocked mussels, PIT tagging would be an excellent alternate method for any future feasibility studies.

REFERENCES

Alderman, J.M., Johnson J.A., McDougal. L.A. (2001). North Carolina atlas of freshwater mussels and endangered fish.

Augspurger, T., Keller, A. E., Black, M. C., Cope, W. G., Dwyer, F. J. (2003). Water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. *Environmental Toxicology and Chemistry*, *22*(11), 2569–2575.

Bates, Douglas, Martin Mächler, Ben Bolker, and Steve Walker. 2015. “Fitting Linear Mixed-Effects Models Using lme4.” *Journal of Statistical Software* 67 (1): 1–48. https://doi.org/10.18637/jss.v067.i01.

Beaty, B. B., & Neves, R. J. (2004). Use of a natural river water flow-through culture system for rearing juvenile freshwater mussels (Bivalvia: Unionidae) and evaluation of the effects of 40 substrate size, temperature, and stocking density. American Malacological Bulletin, 19(1–2), 15–23.

Barnhart, M. C., Fobian, T. B., Whites, D. W. and Ingersoll, C. G. (2007). Mussel silos: Bernoulli flow devices for caging juvenile mussels in rivers. *In Fifth Biennial Symposium*. of the Freshwater Mollusk Conservation Society, Little Rock, AR.

Bartsch M.R., Waller D.L., Cope W.G., Gutreurter S. (2000). Emersion and Thermal Tolerances of three Species of Unionid Mussels: Survival and Behavioral Effects. Journal of Shellfish Research. 9(1):233–240.

Beard-Moose, C.T., Paredes, J.A. (2009). Public Indians, private Cherokees: Tourism and tradition on tribal ground. University of Alabama Press. 186 pp.

Bogan, A. E. (2002). Workbook and key to the freshwater bivalves of North Carolina. North Carolina Freshwater Mussel Conservation Partnership, Raleigh, NC 101 pp, 10 color plates.

Bogan, A. E., Roe, K. J. (2008). Freshwater bivalve (Unioniformes) diversity, systematics, and evolution: status and future directions. *Journal of the North American Benthological Society*, *27*(2), 349–369.

Bouska, K.L., Rosenberger, A., McMurray, S.E., Lindner, G.A., Key, K.N. (2018) State-level freshwater mussel programs: Current status and a research framework to aid in mussel management and conservation. Fisheries 43(8):345-360.

Cowie, R., Régnier, C., Fontaine, B., Bouchet, P. (2017). Measuring the Sixth Extinction: what do mollusks tell us? *The Nautilus*, *131*, 3–41.

EBCI Natural Resources Department. (2013). Integrated Resource Management Plan "Legacy Plan." Eastern Band of Cherokee Indians, 143 pp.

Elderkin C.L., Christian A.D., Metcalfe-Smith J.L., Berg D.J. (2008). Population genetics and phylogeography of freshwater mussels in North America, Elliptio dilatata and Actinonaias ligamentina (Bivalvia: Unionidae). Molecular Ecology 17:2149–2163.

Finigan, R.E. (2019) Feasibility study for restoration of freshwater mussels (*Villosa iris* and *Lampsilis fasciola*) into the upper Oconaluftee River in North Carolina. Western Carolina University MS Thesis.

Haag W.R, Commens-Carson A.M. (2008).Testing the assumption of annual shell ring deposition in freshwater mussels. Canadian Journal of Fisheries and Aquatic Sciences. 65(3):493–508. doi:10.1139/f07-182.

Haag, W.R., Williams, J.D. (2014). Biodiversity on the brink: an assessment of conservation strategies for North American freshwater mussels. Hydrobiologia 735:45-60.

Howard, J.M., Cuffey, K. (2006). Functional role of freshwater mussels in the benthic environment. Freshwater Biology, 51, 460–474.

Huehner M.K. (1987). Field and laboratory determination of substrate preferences of Unionid mussels. Ohio Journal of Science 87:29–32.

Huffstetler, D., Russ, T.W., (2008) NCWRC Mussel Silo Construction Guidelines

Kuznetsova, Alexandra, Per B. Brockhoff, and Rune H. B. Christensen. 2017. “lmerTest Package: Tests in Linear Mixed Effects Models.” *Journal of Statistical Software* 82 (13): 1–26. https://doi.org/10.18637/jss.v082.i13.

Lemarié, D., Smith, D., F. Villella, R., & A. Weller, D. (2000). Evaluation of tag types and adhesives for marking freshwater mussels (Mollusca: Unionidae). Journal of Shellfish Research, 19(1), 247-250.

Liu, Q.-X., Herman, P.M.J., Mooij, W.M., Huisman, J. Scheffer, M. Olff, H. van de Koppel, J. (2014). Pattern formation at multiple spatial scales drives the resilience of mussel bed ecosystems. Nature Communications 5:5234 doi: 10.1038/ncomms6234

Lopes-Lima, M., Bulakova, L.E., Karatayev, A.Y., Mehler, K., Seddon, M., Sousa, R. (2018). Conservation of freshwater bivalves at the global scale: diversity, threats and research needs. Hydrobiologia 810:1-14.

Lydeard, C., Cowie, R., Ponder, W., Bogan, A., Bouchet, P., Clark, S., Thompson, F. (2004). The Global Decline of Nonmarine Mollusks. BioScience, 321–330.

Lyons, M. S., Krebs, R. A., Holt, J. P., Rundo, L. J., & Zawiski, W. (2007). Assessing Causes of Change in the Freshwater Mussels (Bivalvia: Unionidae) in the Black River, Ohio. *The American Midland Naturalist*, *158*(1), 1–15.

Mitchell Z.A., McGuire J., Abel J., Hernandez B.A., Schwalb A.N. (2018). Move on or take the heat: Can life history strategies of freshwater mussels predict their physiological and behavioral responses to drought and dewatering? Freshwater Biology 63:1579–1591.

Ohlman L.M., Pegg M.A. (2019). Handling effects on survival and growth of plain pocketbook *Lampsilis cardium* (Rafinesque, 1820) freshwater mussels. Hydrobiologia. 2019 Oct 25. doi:10.1007/s10750-019-04106-y

Perles, S.J., Christian, A.D., Berg, D.J. (2003). Vertical migration, orientation, aggregation, and fecundity of the freshwater mussel, *Lampsilis siliquoidea*. Ohio Journal of Science 103(4):73-78.

Neves, R. J. (1999). Conservation and commerce: management of freshwater mussel (Bivalvia: Unionidae) resources in the United States. *Malacologia*, *41*(2), 461–474.

Ratcliffe, J., Hall, S. P, McRae, S. E., Finnegan, J. T. (2018). Natural Heritage Program list of the rare animal species of North Carolina. NC Natural Heritage Program, NC Department of Environment and Natural Resources, Raleigh, NC. 38-42p.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Signorell. A. et mult. al (2020). *DescTools: Tools for Descriptive Statistics*. R package version 0.99.32, https://cran.r-project.org/package=DescTools.

Spooner, D. E., Vaughn, C. C. (2006). Context-dependent effects of freshwater mussels on stream benthic communities. Freshwater Biology, 51(6), 1016–1024.

Uryu, Y., Iwasaki, K, Hinoue, M. (1996). Laboratory experimentals on behaviour and movement of a freshwater mussel, Limnoperna fortunei (Dunker). Journal of Molluscan Studies, 62:327-341.

van de Koppel, J., Gascoigne, J.C., Theraulaz, G. Rietkerk, M., Mooij, W.M. Herman, P.M.J. (2008). Experimental evidence for spatial self-organization and its emergent effects in mussel bed ecosystems. Science 322:739-742.

Vaughn, C. C., Hakenkamp, C. C. (2001). The functional role of burrowing bivalves in freshwater ecosystems. Freshwater Biology, 46(11), 1431–1446.

Vaughn, C. C., Nichols, S. J., Spooner, D. E. (2008). Community and food web ecology of freshwater mussels. Journal of the North American Benthological Society, 27(2), 409–423.

Vaughn, C. C., Taylor, C. M. (1999). Impoundments and the decline of freshwater mussels: A case study of an extinction gradient. Conservation Biology, 13(4), 912–920.

Williams, J.D., Bogan, A.E., Butler, R.S., Cummings, K.S., Garner, J.T., Harris, J.L., Johnson, N.A., & Watters, G.T. (2017). A revised list of the freshwater mussels (Mollusca: Bivalvia: Unionida) of the United States and Canada. Freshwater Mollusk Biology and Conservation 20:33-58.