FEASIBILITY STUDY FOR RESTORATION OF FRESHWATER MUSSELS (*VILLOSA IRIS* AND *LAMPSILIS FASCIOLA*) INTO THE UPPER OCONALUFTEE RIVER IN 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.

By

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ABSTRACT

FEASIBILITY STUDY FOR RESTORATION OF FRESHWATER MUSSELS (*VILLOSA IRIS* AND *LAMPSILIS FASCIOLA*) INTO THE UPPER OCONALUFTEE RIVER IN NORTH CAROLINA

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Anthropogenic influences have contributed to the decline of many freshwater mussel species, with many listed as endangered, threatened, or of special concern. Suitable water quality, proper substrate habitat, and the presence of fish hosts are needed for mussel survival. Freshwater mussels have not previously been recorded in the Oconaluftee River in North Carolina upstream of the Bryson Dam despite suitable water quality, the presence of proper fish hosts, and the historically rich mussel fauna of the Little Tennessee River drainage. Two species of freshwater mussel, *Lampsilis fasciola* and *Villosa iris*, were placed in enclosures at three locations along the Upper Oconaluftee River. Growth and survival were monitored over the course of a growing season (March through November) to determine if the Oconaluftee River is suitable for restoration of these species. Throughout the experiment, four *L. fasciola* died, all *V. iris* survived, and both species grew at all three sites. Mussels grew the most and had the best survival at the farthest downstream site, which had the highest temperature and specific conductivity. Despite this difference both species flourished at all sites, suggesting the Upper Oconaluftee River is an ideal location for introduction of *L. fasciola* and *V. iris* and the water conditions associated with the river can be used as a reference for future restoration projects. As a secondary study, three

tag types (*i.e.* Hallprint shellfish tags, laser etching, and queen bee tags) were evaluated to determine long term legibility. Hallprint shellfish tags were the only tag to remain readable throughout experiment, closely followed by the queen bee tags, indicating laser tags were the least effective.

INTRODUCTION

Anthropogenic influences have led to detrimental effects on many ecosystems, resulting in the need for increased conservation efforts. The phylum Mollusca is one of the most threatened groups of animals, accounting for 42% of all extinctions since the 1500s (Lydeard *et al.,* 2004). Freshwater mussels, superfamily Unionoidea, are of particular interest because 69% of known modern species are listed as vulnerable, imperiled, critically imperiled, or presumed extinct (Master *et al.,* 2000). While mussels are internationally at-risk, an abundance of endemic species make their conservation particularly important in North America. The United States is home to approximately 300 recognized freshwater mussel species, of which 28% are listed as federally imperiled and 65% are listed as imperiled from independent assessments (Haag and Williams, 2014). Southeastern states are particularly at-risk due to the large species diversity and endemism, particularly the Tennessee River watershed, which contains the most species rich assemblage in the United States (Bogan, 2008; Jones and Neves, 2010). For example, Muscle Shoals, a city in the Tennessee River watershed, lost 32 species since the early 1900s, when the construction of multiple dams changed the habitat structure of the river (Lydeard *et al.,* 2004). This kind of habitat alteration, spread of invasive species, exploitation, and climate change have contributed to declines in freshwater mussel populations (Cowie *et al.,* 2017).

Mussels are mostly sedentary, remaining in nearly the same location throughout their often long, adult lifetime, some over 100 years, meaning they are unable to take refuge from a suddenly changing environment (Vaughn and Taylor, 1999). Freshwater mussels are filter feeders, taking in food from the water column; therefore, environmental changes that influence the water column composition via sedimentation affect food intake (Vaughn *et al.,* 2008). A

variety of human activities lead to increased sedimentation, including mining, agriculture, and dams (Wood, 1997). Increased total suspended solids (TSS) causes a decrease in clearance rates, the volume of water cleared of particles over a particular time frame, of both juvenile and adult freshwater mussels (Tuttle-Raycraft *et al.,* 2017). Feeding can also be impacted by nutrient availability in the water column, which can be altered by human activities, such as agriculture. Elevated specific conductivity, a measure of ion concentration in the water column, is positively associated with nutrient availability, and therefore food availability for freshwater mussels (Krawczyk and Ford, 2006).

In addition to altering feeding rate, human activities can increase pollutants that can be toxic to freshwater mussels. Freshwater mussels are highly sensitive to changes in water quality because their burrowing and filter feeding behaviors expose them to pollutants in both the sediment and water column (Augspurger *et al.,* 2003). In acute and chronic sensitivity tests, freshwater mussels are consistently sensitive to a variety of pollutants, including ammonia, copper, cadmium, zinc, and lead (Keller and Zam, 1991; Augspurger *et al.,* 2003; Mummert *et al*., 2003; Gillis *et al*., 2008; Wang *et al.* 2010). This sensitivity is associated with young, early stage mussels, and is a contributor of low juvenile recruitment in populations (Keller and Zam, 1991; Gillis *et al*., 2008).

Mussels play a critical role in freshwater ecosystems and are often considered ecosystem engineers due to their filter feeding and burrowing activities. Because mussels are epibenthic and filter feeders, they directly link the nutrients in the water column and benthos. Excretion provides important limited nutrients, such as phosphorus and nitrogen, into the sediment from the water column for use by other benthic organisms and algal communities (Spooner and Vaughn, 2006; Vaughn *et al.,* 2008; Francoeur *et al.,* 2017). Excretion also provides fine particulate organic

matter (FPOM) for consumption by macroinvertebrates (Howard and Cuffey, 2006). The influx of nutrients and FPOM can stimulate bacteria growth, accelerating decomposition of organic matter, and provide food for detritivores and grazers (Howard and Cuffey, 2006).

After mussels die, the remaining shell provides habitat for other organisms, particularly in areas where the substrate is small, such as gravel and sand (Vaughn and Hakenkamp, 2001; Gutierrez *et al.,* 2003; Vaughn *et al.,* 2008). The areas between the shells of mussel beds fill with organic matter and provide refuge for colonization and food for various organisms (Vaughn and Hakenkamp, 2001). In addition, the shells of living mussels provide a surface for periphyton to flourish, leading to increased food availability, which results in increased abundance of various macroinvertebrate larvae (*e.g.* caddisflies, mayflies, and mites) (Vaughn and Hakenkamp, 2001; Howard and Cuffey, 2006; Spooner and Vaughn, 2006).

Mussel burrowing behavior helps stabilize the stream bed during large rain events, which creates refugia for other benthic species (Strayer *et al.,* 2004; Vaughn *et al.,* 2008). The movement of mussels in the sediment is also helpful for the ecosystem through bioturbation. The movement from burrowing causes particles to shift, allowing oxygen and nutrients into the sediment, creating a more suitable habitat for burrowing macroinvertebrates (Vaughn and Hakenkamp, 2001; Vaughn *et al.,* 2008).

Various freshwater mussel species native to Western North Carolina are considered rare and require conservation. North Carolina species of special concern, those which require monitoring by the Wildlife Resources Commission, include *Lampsilis fasciola* Rafinesque, 1820, the wavyrayed lampmussel, and *Villosa iris* (I. Lea, 1829), the rainbow mussel, (Bogan, 2002; Radcliff *et al.,* 2016). Both species are also ranked as S2 in North Carolina, meaning they are imperiled and at-risk of extirpation (Radcliff *et al.,* 2016).

Lampsilis fasciola inhabit medium sized rivers from the Great Lakes to the Ohio and Mississippi River basins (Bogan, 2002). In western North Carolina, these mussels are found in the French Broad, Hiwassee, and Little Tennessee River systems (Bogan, 2002; Fraley, 2002). While *L. fasciola* are able to survive in slow currents with fine substrate, conditions unfavorable for other species, populations reach their largest numbers in stream beds with stable gravel floors at depths less than 3 feet (Bogan, 2002). *Villosa iris* are found in medium sized rivers from the St. Lawrence, Ohio, and Mississippi River basins (Bogan, 2002). In western North Carolina, these mussels currently inhabit only the Hiwassee and Little Tennessee River systems, however they were historically also located in the Watauga and French Broad River systems (Bogan, 2002; Fraley, 2002). *Villosa iris* are found in multiple habitats, including sand and gravel substrates of moderate to strong current streams and the edges of emerging vegetation in shallow riffles; and populations reach their highest numbers in depths less than 3 feet (Bogan, 2002; Fraley, 2002).

Freshwater mussels require proper habitat and specific fish hosts in order to thrive due to their parasitic stage (Bogan and Roe, 2008; Vaughn *et al.,* 2008: Haag, 2012). After fertilization, female mussels hold their eggs in brood pouches until they develop into larvae, or glochidia, which are then released into the water column (Fraley, 2002; Haag, 2012). Within 6 hours, glochidia will attach to the gills and fins of fish hosts and become encapsulated by their epithelial tissue, feeding off the host's blood as a parasite until metamorphosis into a juvenile mussel (O'Connell and Neves, 1999; Fraley, 2002; Haag, 2012). While the duration of the parasitic stage varies depending on water temperatures, both *L. fasciola* and *V. iris*, are fully metamorphosed after 2-6 weeks (Haag, 2012). The new juvenile mussels then detach from the host and begin to grow into free living adults on the river floor (Fraley, 2002; Bogan and Roe,

2008; Haag, 2012). Mussel species vary greatly in which host species they are able to infect, with glochidia being rejected by a non-suitable host's immune system (Haag, 2012). Some species use a specific fish host, while others are generalists. *Lampsilis fasciola* primarily uses Smallmouth Bass (*Micropterus dolomeiu*) and Largemouth Bass (*Micropterus salmoides*) as hosts (Fraley, 2002). *Villosa iris* is known to use a wider range of hosts, such as the Smallmouth Bass, Largemouth Bass (*Micropterus salmoides*), Rock Bass (*Amblophlites rupestris*), and Western Mosquitofish (*Gambusia affinis*) (O'Connell and Neves, 1999; Bogan, 2002; Fraley, 2002).

With freshwater mussel populations becoming increasingly at-risk due to human activities, their conservation relies on not only preserving current population, but also establishing new populations. The first step in establishing self-sustainable populations is to find a suitable habitat and determine if species are able to survive and grow in that habitat. While reintroduction efforts have been initiated in North Carolina watersheds (Layzer and Scott, 2006; Rooney, 2010), no attempts have been made in the Oconaluftee River.

The Oconaluftee River is a tributary of the Tuckasegee River and forms from the confluence of three streams, the Kephart Prong, Smith Branch, and Kanati Fork, at the eastern edge of Great Smoky Mountain National Park (Stowe, 2014; Davis, 2015). A hydroelectric dam previously operated by Duke Energy with ownership currently transitioning to Northbrook Energy, known as the Bryson Dam, creates a full pool reservoir, called Lake Ela (Fraley, 2002; Stowe, 2014; Davis, 2015). The river upstream of Lake Ela, hereinafter referred to as the Upper Oconaluftee River, is characterized by low levels of sedimentation, large particulate substrate (*e.g.* boulders and cobble), and deep pools (Stowe, 2014; Davis, 2015). There are no historical records of mussels occurring upstream of the reservoir, however the temperature, substrate,

water quality, and presence of fish hosts indicate that the Upper Oconaluftee could be an ideal location for restoration (Fraley, 2002). In addition, the habitat is comparable to other tributaries of the Little Tennessee River, where freshwater mussels are found (Fraley, 2002). Generally, streams become warmer and carry more nutrients as one moves downstream. Rooney (2010) found that *L. fasciola* in the Pigeon River had higher growth rates at downstream sites, where water temperatures and nutrient concentrations were higher; therefore, on the Upper Oconaluftee River, water closer to the reservoir may be more suitable for restoration than upstream sites.

Hallprint shellfish tags (Hallprint; type FPN 8x4; Hindmarsh Valley, South Australia) are commonly used for freshwater mussel tagging. Hallprint tags have been compared to other techniques (*e.g*. fingerling and visible implant) and shown as the longest lasting when used on freshwater mussels (Lemarié *et al*., 2000). Queen bee tags (The Bee Works, Oro-Medonte, Ontario) are smaller than Hallprint tags, so may be a viable option for smaller mussels, and while they have been used in studies (Hoch, 2012), their longevity has not been compared to Hallprint tags. Both Hallprint and queen bee tags are adhered with superglue, so may detach from the mussel. An alternative marking technique is laser etched tags, which will not detach, but may erode over time. Hallprint tags have been compared to laser etched tags and micromarkers when used on the great scallop (*Pecten maximum*) and were considered most effective and easier to apply (Ross *et al.,* 2001). However, laser tags have not been experimentally compared to queen bee tags or Hallprint tags on freshwater mussels.

The primary purpose of this study was to determine which areas along the Upper Oconaluftee River would be most suitable for introduction of two freshwater mussel species of special concern, *L. fasciola* and *V. iris* by comparing the survival and growth of caged juvenile mussels held at different sites in the Upper Oconaluftee River. The secondary purpose was to

contribute to our knowledge of the growth patterns. A third goal was to compare different marking techniques to determine long term legibility of Hallprint shellfish tags, laser etching tags, and queen bee tags.

METHODS

Study Site and Animal Collection

The Oconaluftee River begins within The Great Smoky Mountains National Park at an elevation of approximately 850 m where Beech Flats prong merges with Kephart Prong and Kenati Fork. It flows for approximately 30 km through the Qualla Boundary to its confluence with the Tuckasegee River at an elevation of approximately 500 m. It drains an area of approximately 480 km^2. Mussels were placed at three sites along the Upper Oconaluftee River in western North Carolina (Table 1). The first site was at river kilometer 3.9 near the Bird Town community and upstream of the Ela Reservoir. The second site was placed at river kilometer 6.1 directly upstream of the Cherokee Wastewater Treatment Plant, and a third site was placed at river kilometer 9.4 upstream of the confluence with Soco Creek. All three sites were located in the Qualla boundary of the Eastern Band of Cherokee Indians. A fourth control site was placed on the Tuckasegee River, representing the habitat of the source populations, for comparison (Table 1).

Juvenile *Lampsilis fasciola* and *Villosa iris* mussels were acquired from the North Carolina Wildlife Resources Commission. Mussels were cultured from 11 *L. fasciola* and 9 *V. iris* gravid females collected from the Little Tennessee River on 25 May 2016 and reared at the Conservation Aquaculture Center at the Marion State Fish Hatchery in Marion, NC. Largemouth bass purchased from Foster Pond and Lake Management, Inc. in Garner, NC were used as a fish host for glochidia. Newly metamorphosed juveniles were reared in a recirculating system with fine substrate. Juveniles were fed a mix of commercially purchased microalgae and diatoms (Nanno 3600, *Nannochloropsis* and Shellfish Diet 1800, *Isochrysis*, *Pavlova*, *Testraselmis*,

Thalassiosira weissflogii, and *Thalassiosira pseudonana* from Reed Mariculture Inc., Campbell, CA). The mussels were moved from this system to a single-pass flow through tub with coarsely filtered pond water and substrate at age one. On 8 March 2018, *V. iris* averaged 19.5 mm and *L. fasciola* averaged 18.7 mm in length.

Table 1: Locations of each site described by river, GPS coordinates, and river kilometers.

Site	River	GPS Coordinates	River Kilometers
S1	Oconaluftee	35.457411 N, -83.364221 W	3.9
S2	Oconaluftee	35.468788 N, -83.350204 W	6.1
S3	Oconaluftee	35.468508 N, -83.320973 W	9.4
T1	Tuckasegee	35.347798 N, -83.237598 W	53.4

Experimental Design

My methods were adapted from Rooney (2010), a project involving introduction of *L. fasciola* into the Pigeon River downstream of Canton, North Carolina. I constructed mussel enclosures (silos) following the design initially developed by Dr. M. Christopher Barnhart at Missouri State University and modified by the Virginia Department of Game and Inland Fisheries (VDGIF) (Barnhart *et al*., 2007) (Figure 1). Each silo consisted of an inner chamber made with a PVC pipe and window screen that fits into a concrete dome. Water flowing under the dome, creates a Bernoulli effect, which draws water up through the pipe and over the mussels

in a continuous flow. This structure allowed me to easily access the mussels for data collection, but also allowed for the constant flow of water and food.

I marked each mussel on 9 January 2018 with three tags to monitor individual growth throughout the experiment, an alphanumeric glue-on shellfish tag (Hallprint; type FPN 8x4; Hindmarsh Valley, South Australia), a queen bee tag (The Bee Works, Oro-Medonte, Ontario), and a laser etched tag with a 30 watt, 115 v, Epilog Zing 16 laser engraver (Epilog Laser, Golden, CO) (Figure 2). I adhered Hallprint and bee tags with Loctite Ultragel Control (Henkel Corporation, Stamford, CT) and the laser etchings were sealed with brush-on clear nail glue (Omega Labs USA, San Diego, CA). Multiple tag types provided a comparison for readability over the course of the experiment and ensured long-term identification of individuals. I haphazardly selected mussels for each silo and deployed at each site on 3 April 2018. Each site had three replicates of each species, resulting in six silos at each site, and a total of 24 silos. A single silo contained six mussels of a single species; therefore, there were 18 mussels of each species at a site and 72 individuals of each species total.

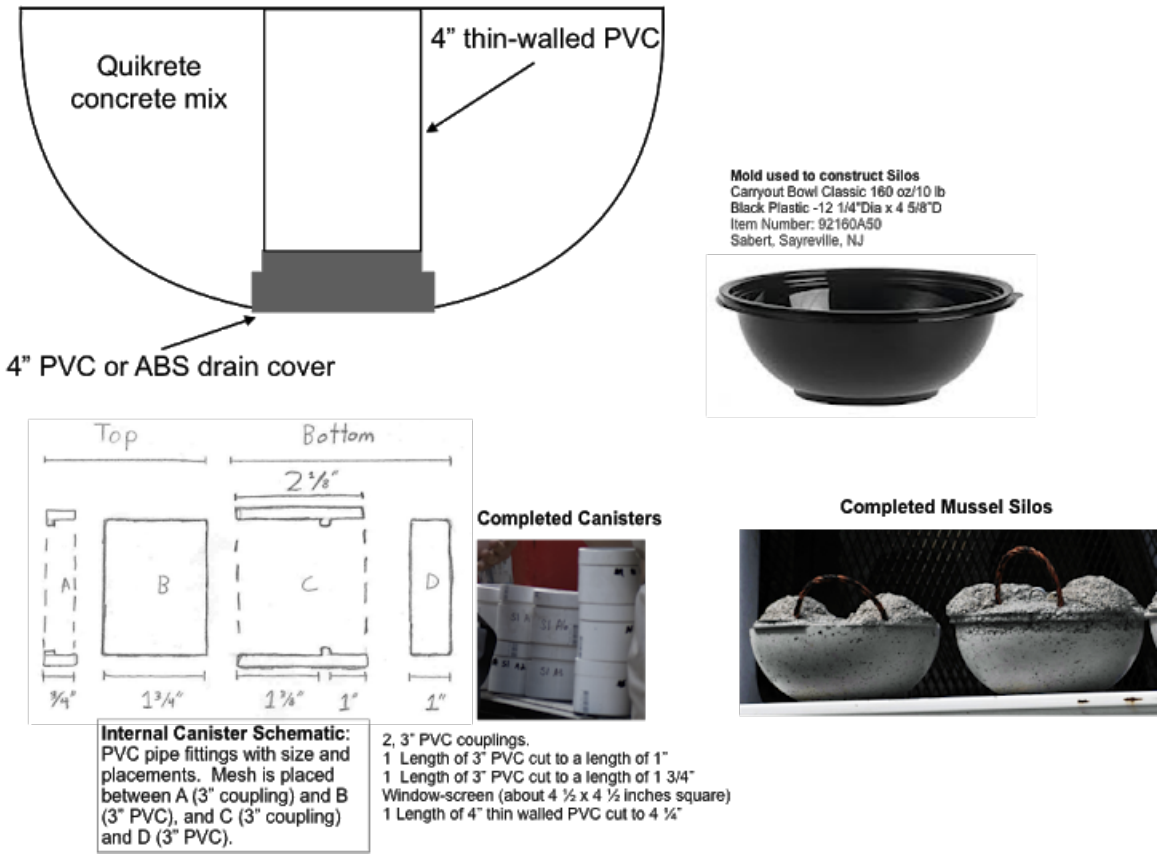

Figure 1: Mussel silo developed by Dr. M. Christopher Barnhart at Missouri State University and modified by the Virginia Department of Game and Inland Fisheries (VDGIF) (Barnhart *et al.*, 2007).

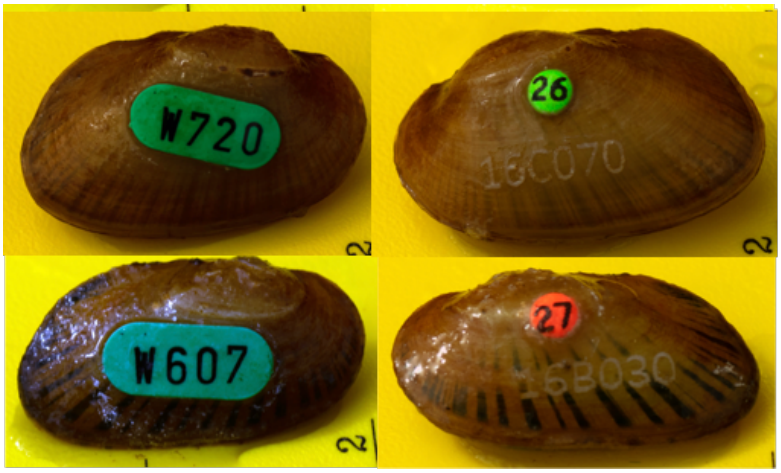

Figure 2: Hallprint shellfish tags (left), queen bee tags (right top of mussel), laser etched tags (right bottom of mussel) on *L. fasciola* (top) and *V. iris* (bottom).

Data Collection

Before stocking in enclosures, I measured length, height, and width to the nearest tenth of a millimeter on 8 March 2018 at the Conservation Aquaculture Center. After enclosure installation, I measured length, height and width once each month from May 2018 to November 2018. Because mussels slow or cease growth during the cold winter months, I concluded this study on 21 November 2018.

Each month, I also assessed water quality. Temperature (°C), specific conductivity (µs/cm at 25°C), and dissolved oxygen (mg/L) were measured using a Yellow Springs Professional Plus Instrument (YSI Incorporated, 1700/1725 Brannum Lane, Yellow Springs, OH 45387, USA). Nitrate (0 – 50 mg/L range), ammonia (0-2.5 mg/L range), free chlorine (0-3.4 mg/L range), total chlorine (0-3.4 mg/L range), and phosphate (0-50 mg/L range) concentrations were measured using a HACH field kit (HACH Company, P.O Box 389, Loveland, CO 80539, USA). Flow velocity was measured at 5 locations (4 surrounding the silos, 1 in the center) using a Swoffer 2100 meter (Swoffer Instruments, Inc, 1112 South 344th Street, Suite 302, Federal Way, Washington 98003, USA) and averaged at each site.

I determined degree of urbanization, or environmental impacts from human development, visually using the procedure described by Lyons *et al.* (2007). Land use (buildings and roads), riparian zone condition, riparian bank modification, dams and spillways, erosion, and human trash were ranked (0-3) to determine an overall degree of urbanization, with higher scores corresponding to a higher degree of urbanization. For each category, a rank of 0 indicates no impact from urbanization, 1 indicates a slight impact from urbanization, 2 indicates a moderate impact from urbanization, and 3 indicates an extreme impact from urbanization. For example, 0 would mean no buildings are visible, 1, some buildings can be seen away from the river, 2, there

are some buildings immediately along the river, and 3, there is high density of buildings immediately along the river.

I evaluated tag readability each month using a scale adapted from Lemarié *et al*. (2000). A numeric score of 0 through 2 was assigned: 0, the tag is unreadable (*e.g.* fallen off or severely eroded), 1, the tag requires some work to read, and 2, the tag can be read immediately.

While appropriate fish hosts are known to reside in the river (Eastern Band of Cherokee Indians Natural Resources, personal communication), I performed a local assessment of each site by snorkeling each site for a visual estimation of fish hosts previously recorded in the Upper Oconaluftee River: Rock Bass, Largemouth Bass, and Smallmouth Bass. Starting downstream, longitudinal transects were snorkeled by 2 people for 10 minutes. The transect length, habitat types (*e.g.* pools, riffles, snags, undercut banks, backwaters, detritus, and aquatic weeds), and substrate types (*e.g.* sand, silt, cobble, gravel, boulder, bedrock) observed were recorded. Number of each species was recorded as a catch per unit effort (CPUE).

Analysis

R statistical software version 3.4.3 and associated packages, including lme4 and lmerTest, were used to analyze the data (R Core Team, 2017; Kuznetsova *et al*., 2017; Bates *et al*., 2015). Analysis of variance (ANOVA) was used to determine if initial lengths at the beginning of the experiment differed among sites. Monthly growth was calculated from initial measurements in March. Differences in mean lengths each month was determined using repeated measures ANOVA. Due to equipment failure in May, that data was removed from the analysis. Analysis of covariance (ANCOVA) was used to determine differences in temperature, specific conductivity, and dissolved oxygen, using measurements taken continuously at a USGS gauge station 03512000 at Birdtown, NC (U.S Geological Survey, 2016) as a covariate to correct for

season and time of day. Outliers were removed from the analysis for dissolved oxygen and temperature. Repeated measures ANOVA was also used to determine differences in flow velocity among sites and differences in readability of tag type over the course of the study.

RESULTS

Initial Length, Survival, and Growth

Initial lengths of both species were homogeneous across sites at the beginning of the experiment, with the range of sizes broadly overlapping (Figure 3; Table 2). All *V. iris* mussels survived at all four sites through November (Figure 4). Beginning in July, four *L. fasciola* died, three of these deaths occurred at site 3, the farthest upstream site, and one occurred at site 2, the middle site (Figure 4). All mussels grew over the course of the experiment, increasing in length relatively rapidly through the summer months, then leveling off in autumn for both *V. iris* and *L. fasciola* (Figure 6; Figure 8). *V. iris* grew the most at site 1, followed by site 2 and site 3, with the difference developing over time (Figure 5; Figure 6; Table 3; Site:Month interaction; p=0.0041). Differences in *L. fasciola* growth were not significant among sites (Figure 7; Figure 8; Table 4). During handling, three *L. fasciola* were lost (one at site 3 and two at the control site) and not included in this analysis.

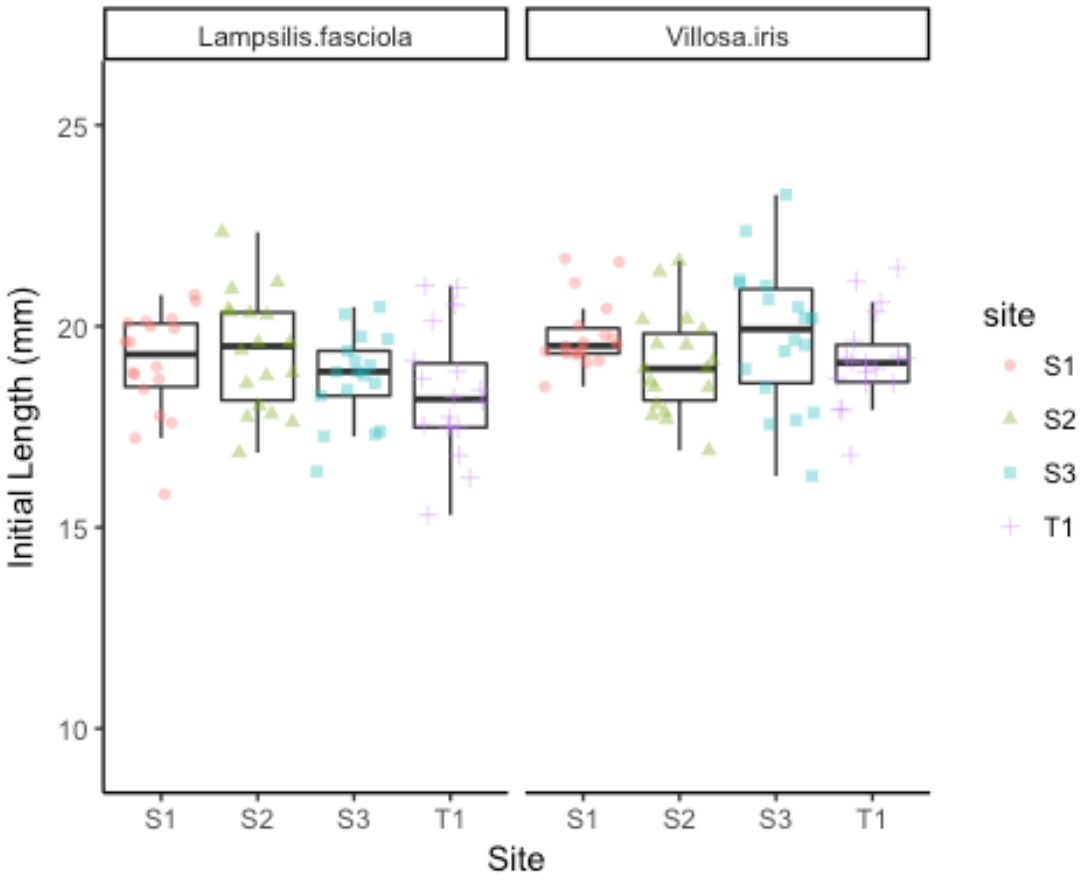

Figure 3: Initial length (mm) of *L. fasciola* and *V. iris* at site 1 (red circle), site 2 (green triangle), site 3 (blue square), and control (purple cross).

Table 2: ANOVA summary for tests of differences in initial length of *V. iris* and *L. fasciola*.

Species	Source	df	SS	MS	F	P
V. iris	site	3	1.30	0.44	2.31	0.1529
	residuals	8	1.51	0.19		
L. fasciola	site	3	1.75	0.58	1.28	0.3445
	residuals	8	3.63	0.45		

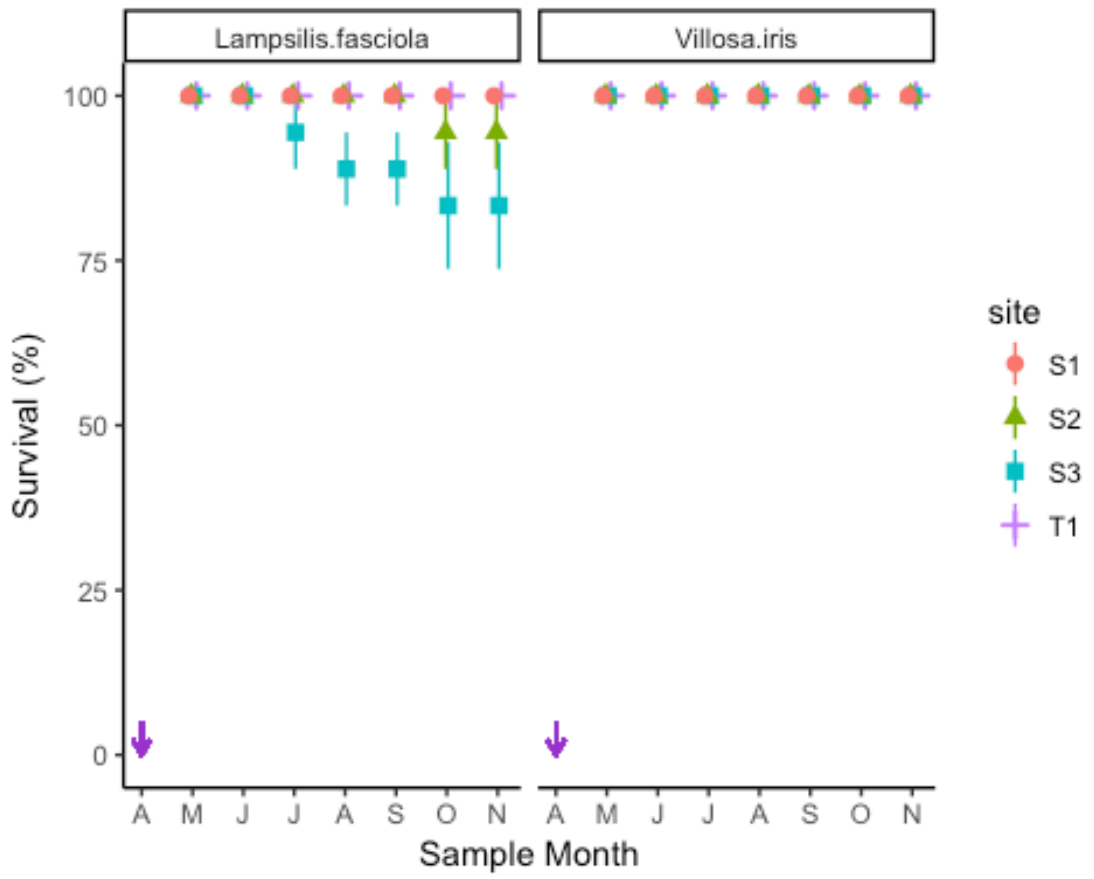

Figure 4: Percent survival of *L. fasciola* (left) and *V. iris* (right) at sites 1 (red circle), 2 (green triangle), 3 (blue square), and control (purple cross) from May to November. The purple arrow shows experiment installation month, when no measurements were taken.

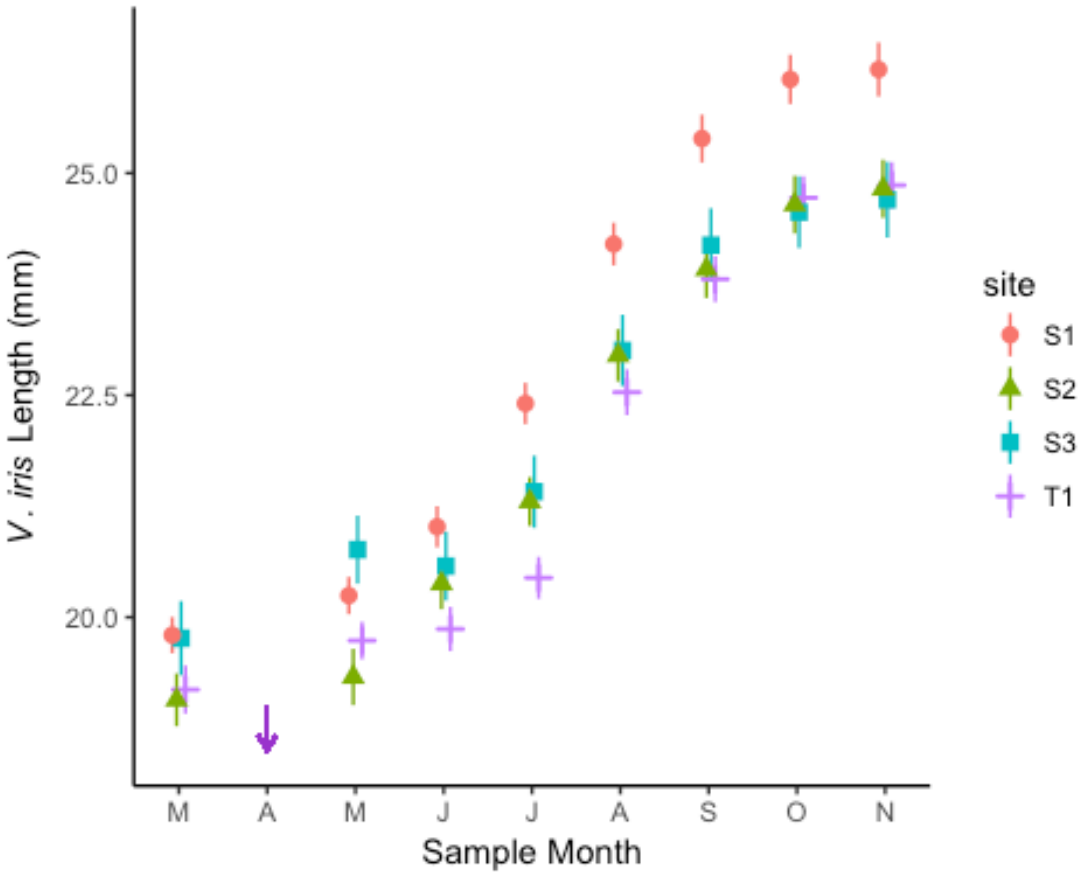

Figure 5: Average length (mm) of *V. iris* at sites 1 (red circle), 2 (green triangle), 3 (blue square), and control (purple cross) from March to November. Initial measurements were taken in March and the purple arrow shows experiment installation month, when no measurements were taken. May data was removed from the analysis.

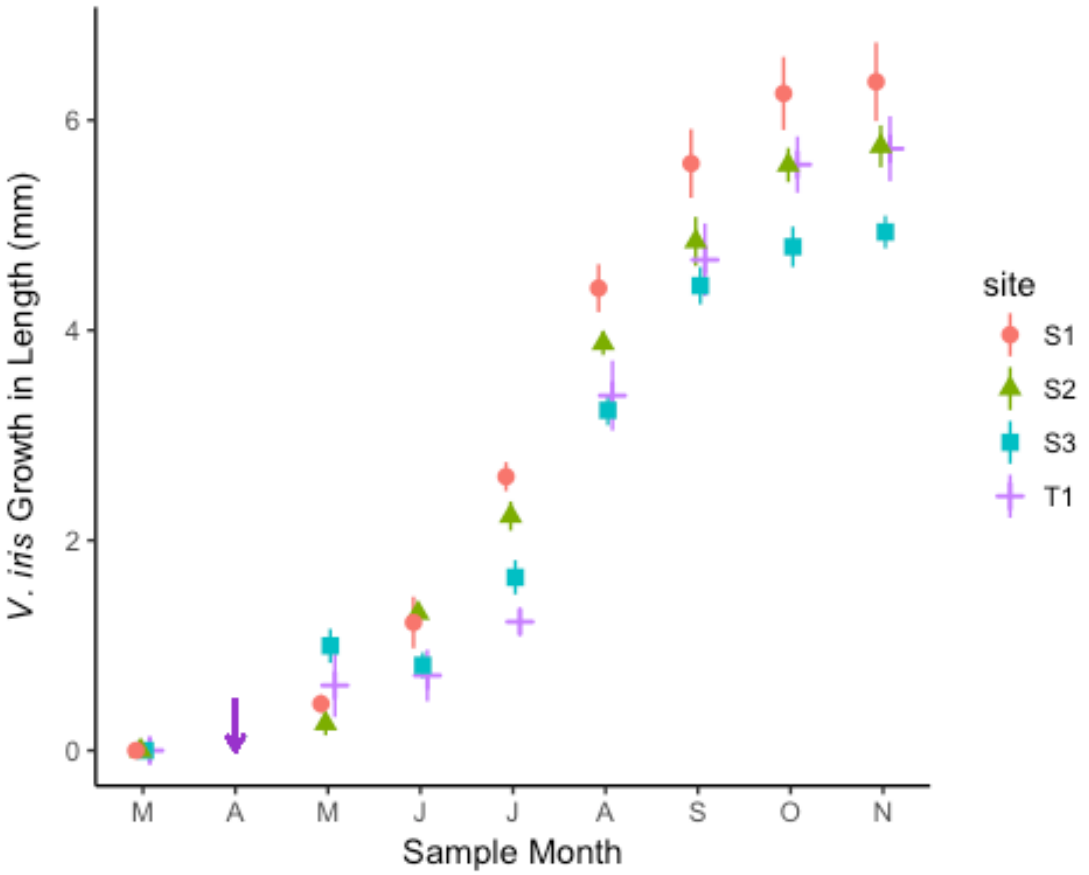

Figure 6: Average growth in length (mm) of *V. iris* at sites 1 (red circle), 2 (green trangle), 3 (blue square), and control (purple cross) from March to November. Initial measurements were taken in March and the purple arrow shows experiment installation month, when no measurements were taken. May data was removed from the analysis.

Table 3: ANOVA summary for tests of differences in maximum length of *V. iris*.

Source	SS	MS	df_{num}	df_{den}	F	P
Site	1.24	0.41	3	7	6.24	0.0217
Month	229.29	45.86	5	40	693.61	<0.0001
Initial Length	0.59	0.59	1	7	8.92	0.0203
Site:Month	2.83	0.19	15	40	2.86	0.0041

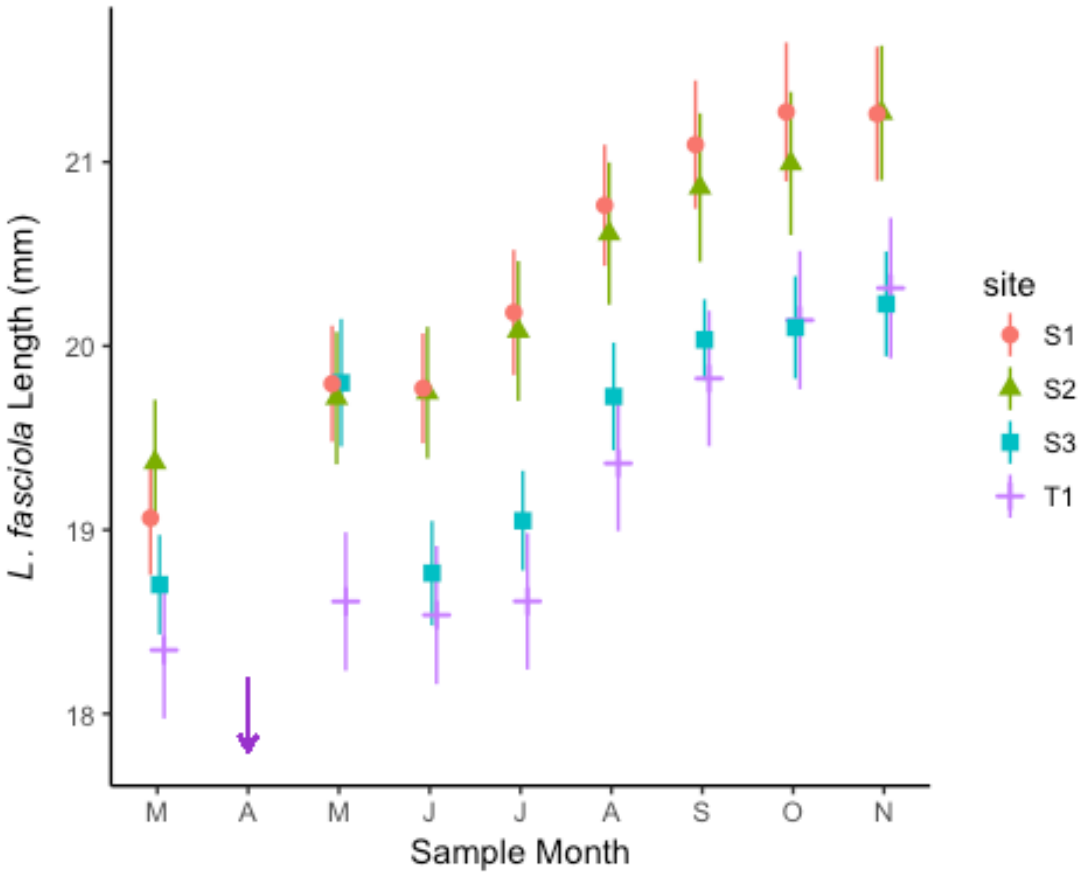

Figure 7: Average length (mm) of *L. fasciola* at sites 1 (red circle), 2 (green triangle), 3 (blue square), and control (purple cross) from March to November. Initial measurements were taken in March and the purple arrow shows experiment installation month, when no measurements were taken. May data was removed from the analysis.

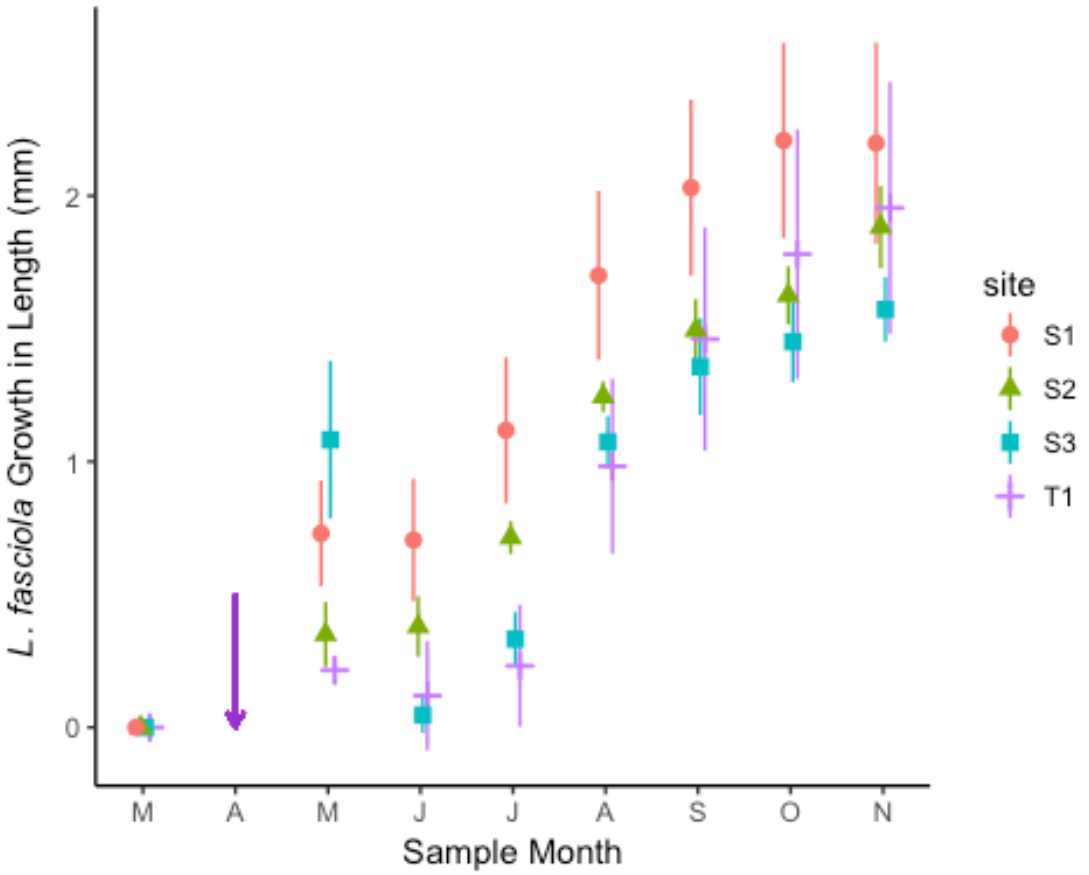

Figure 8: Average growth in length (mm) of *L. fasciola* at sites 1 (red circle), 2 (green triangle), 3 (blue square), and control (purple cross) from March to November. Initial measurements were taken in March and the purple arrow shows experiment installation month, when no measurements were taken. May data was removed from the analysis.

Table 4: ANOVA summary for tests of differences in maximum length of *L. fasciola*.

Source	SS	MS	df_{num}	df_{den}	F	P
Site	0.31	0.10	3	7	2.48	0.1455
Month	25.27	5.05	5	40	121.54	<0.0001
Initial Length	0.52	0.52	1	7	12.47	0.0096
Site:Month	0.66	0.04	15	40	1.06	0.4191

Water Quality, Urbanization, and Snorkel Surveys

Nitrate (mg/L), ammonia (mg/L), free chlorine (mg/L), total chlorine (mg/L), and phosphate (mg/L) were never found to be above detection limits and are not reported here. As expected, specific conductivity (µs/cm) increased from upstream to downstream, reaching its highest levels downstream of the wastewater treatment plant (Figure 9; Table 5). The mean difference of specific conductivity between sites 1 and 2 was 2.54 µs/cm, sites 1 and 3 was 5.50 µs/cm, and sites 2 and 3 was 2.96 µs/cm. Dissolved oxygen (mg/L) was highest at sites 1 and 2 and lowest at site 3 (Figure 10; Table 6). The mean difference of dissolved oxygen between sites 1 and 2 was 0.002 mg/L, sites 1 and 3 was 0.12 mg/L, and sites 2 and 3 was 0.12 mg/L. Temperature (°C) increased from upstream to downstream (Figure 11; Table 7). The mean difference of temperature between sites 1 and 2 was 0.37°C, sites 1 and 3 was 0.64°C, and sites 2 and 3 was 0.27°C. As expected due to site selection, no significant difference in flow velocity (m/s) was observed among sites (Figure 12; Table 8). Urbanization was very similar across all sites, with the only differences on the Oconaluftee River being more trash at site 1 and slightly less river bank modification at site 3 (Figure 13). The control site on the Tuckasegee was more urbanized, with more river bank modification and erosion (Figure 13). We did not observe the targeted fish hosts during snorkeling surveys despite evidence of populations from surveys performed by the Eastern Band of Cherokee Indians Natural Resources (personal communication).

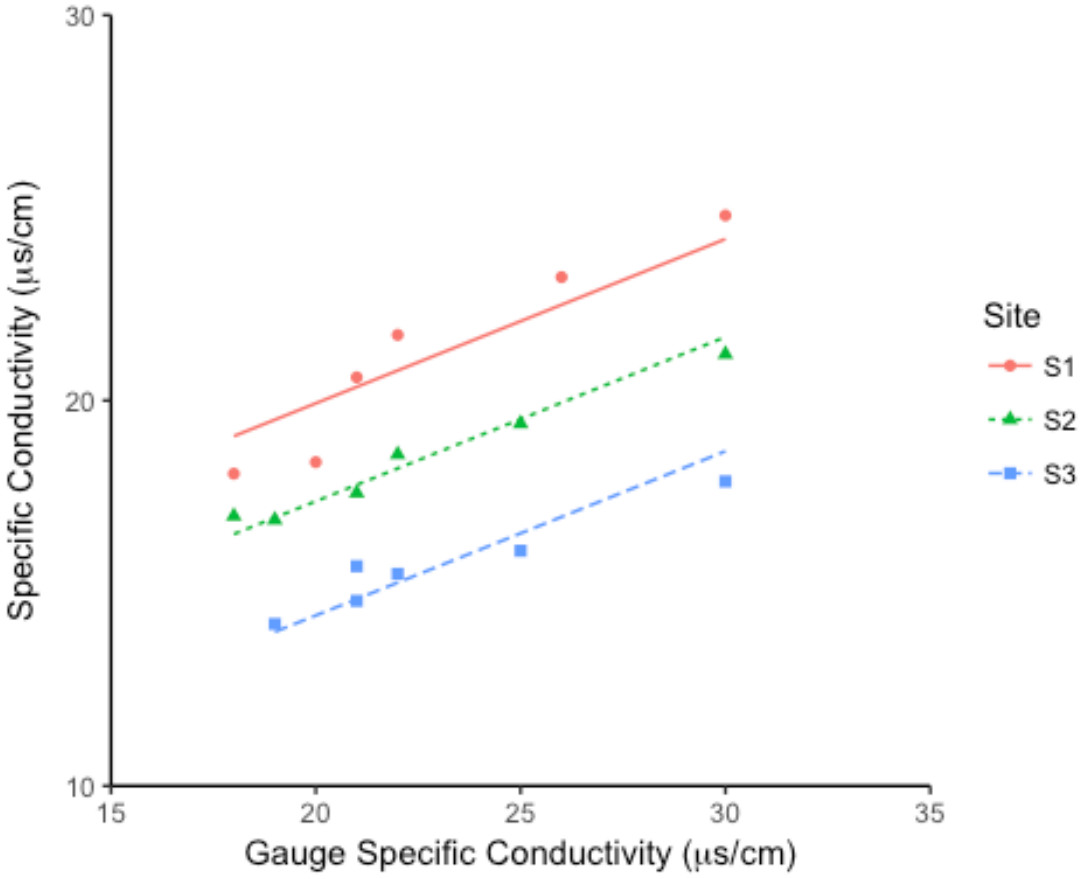

Figure 9: Specific conductivity (µs/cm) of sites 1 (red circle), 2 (green triangle), and 3 (blue square) plotted against USGS gauge specific conductivity (µs/cm).

Table 5: ANCOVA summary table of specific conductivity (µs/cm).

Source	df	Sum Square	Mean Square	F	P
Site	2	88.58	44.28	54.22	<0.0001
Gauge SC	1	45.37	45.37	55.56	<0.0001
Residuals	14	11.43	0.82		

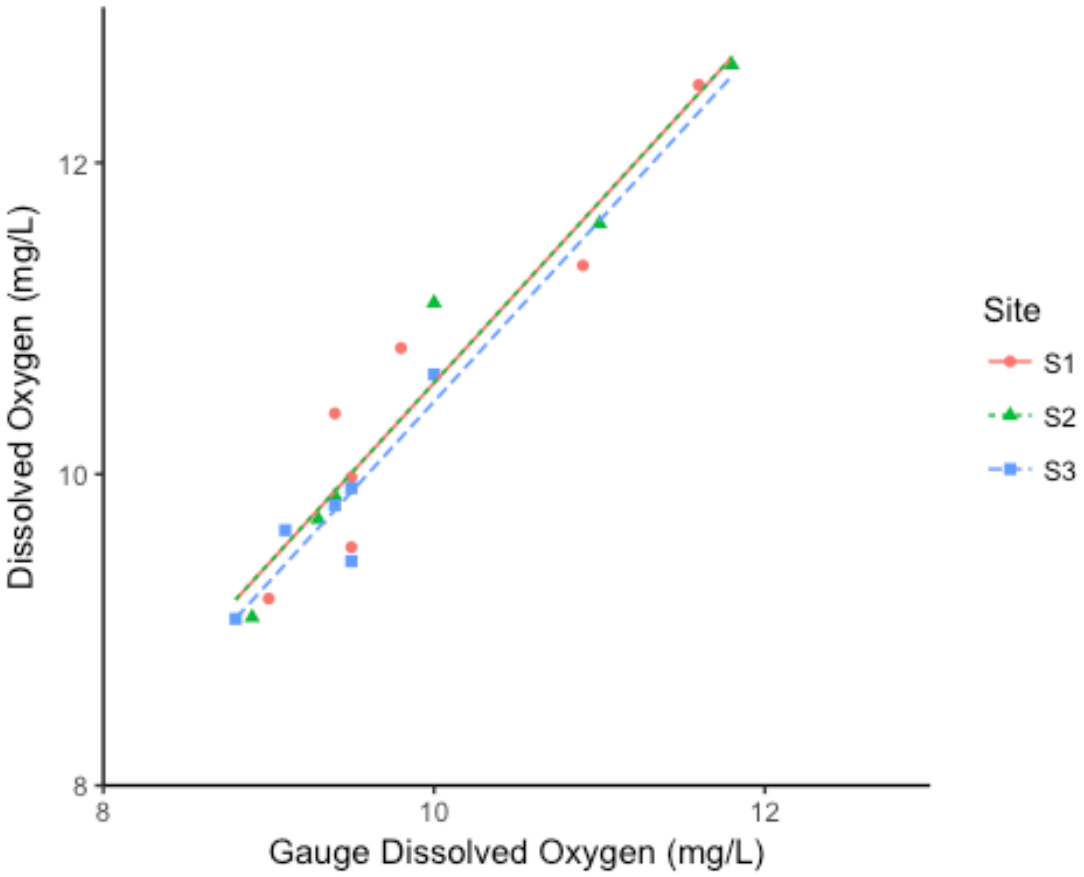

Figure 10: Dissolved oxygen (mg/L) of sites 1 (red circle), 2 (green triangle), and 3 (blue square) plotted against USGS gauge dissolved oxygen (mg/L).

Table 6: ANCOVA summary table of dissolved oxygen (mg/L).

Source	df	Sum Square	Mean Square	F	P
Site	2	52.99	1.50	15.51	0.0002
Gauge DO	1	16.67	16.67	172.10	<0.0001
Residuals	15	14.44	0.10		

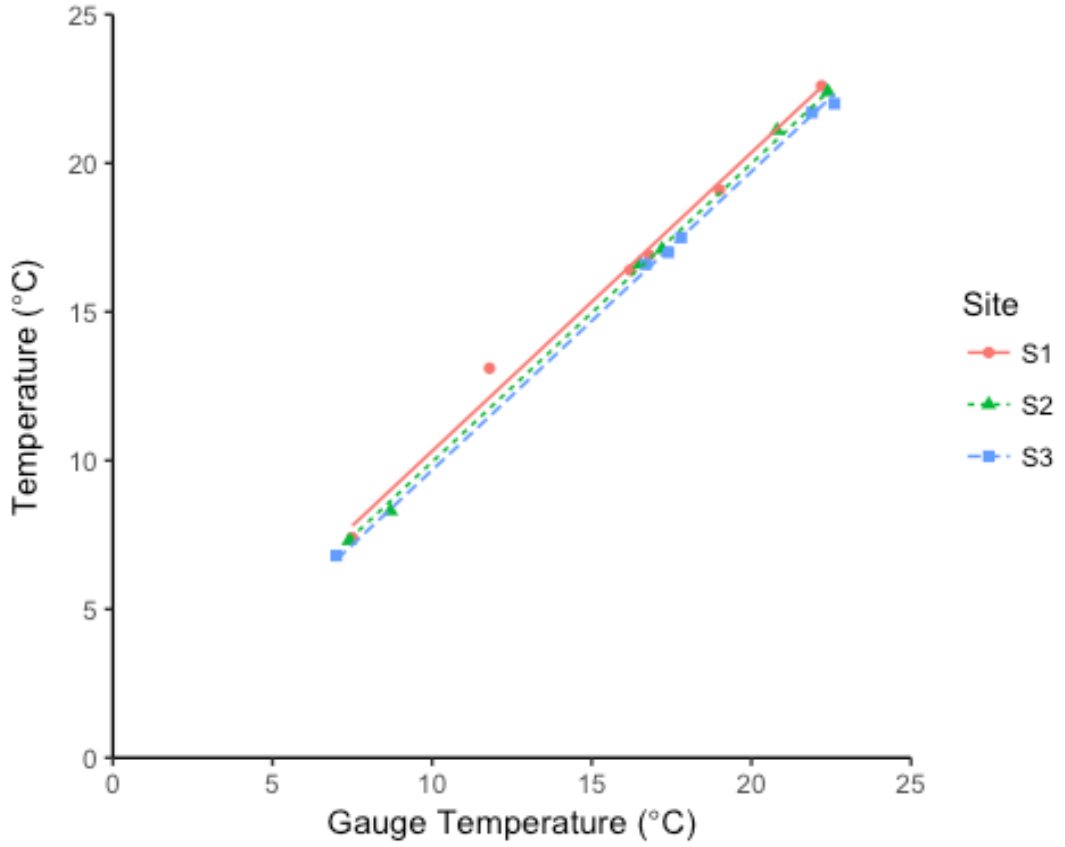

Figure 11: Temperature (°C) of sites 1 (red circle), 2 (green triangle), and 3 (blue square) plotted against USGS gauge temperature (°C).

Table 7: ANCOVA summary table of temperature (°C).

Source	df	Sum Square	Mean Square	F	P
Site	2	6.77	3.39	28.24	<0.0001
Gauge Temperature	1	488.12	488.12	4069.22	<0.0001
Residuals	14	1.70	0.12		

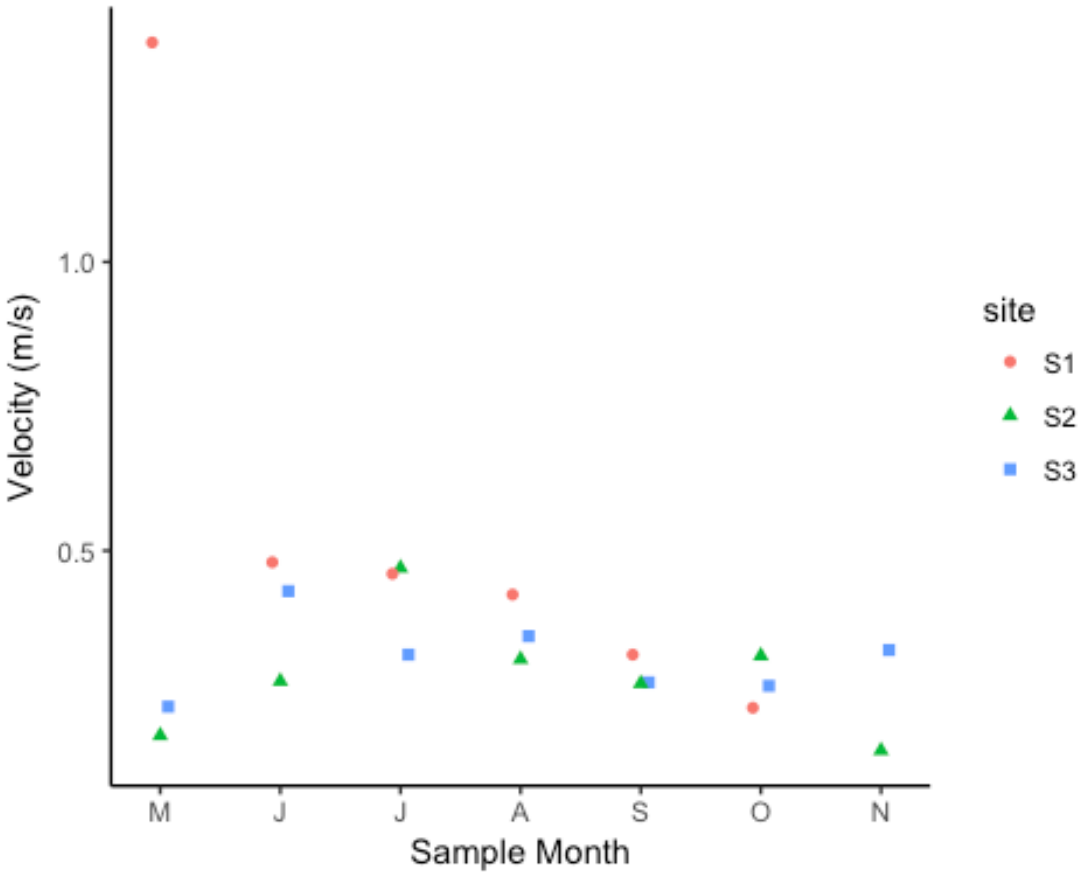

Figure 12: Velocity (m/s) from May to November at sites 1 (red circle), 2 (green triangle), and 3 (blue square).

Table 8: ANOVA summary for analysis of velocity (m/s).

Source	df	Sum Square	Mean Square	F	P
Site	2	0.27	0.13	2.66	0.1009
Date	1	0.16	0.16	3.22	0.0915
Residuals	16	0.80	0.05		

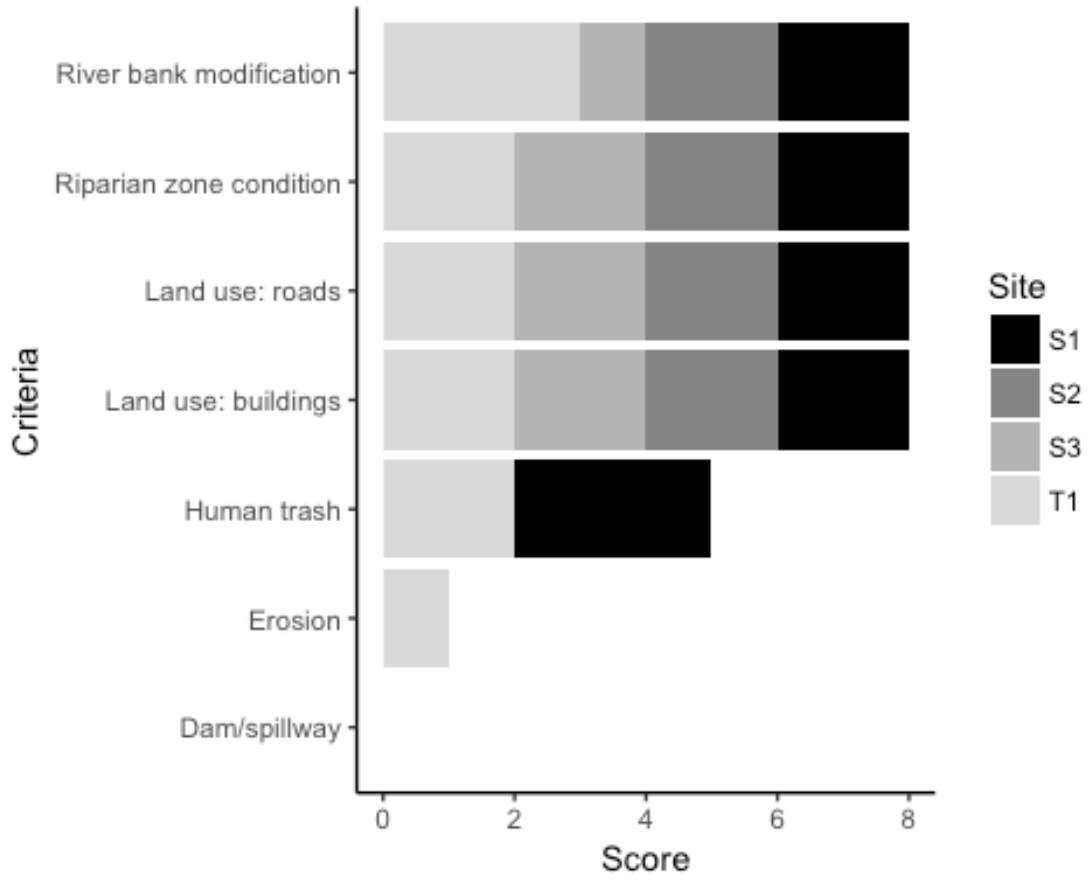

Figure 13: Urbanization criteria scores at sites 1, 2, 3, and the control site.

Tags

Each tag type differed in how long it remained legible and longevity differed between species (Table 9). The queen bee tags remained on *L. fasciola* longer than *V. iris*, while laser etched tags remained legible on *V. iris* longer than *L. fasciola* (Figure 14). Hallprint shellfish tags remained adhered to the mussels and easily legible throughout the experiment (Figure 15A). Queen bee tags began to fall off some mussels in July; however, those that remained attached to

the mussels were always legible (Figure 15B). Laser etched tags began eroding the earliest, in June, and the average legibility score was the lowest by the end of the experiment (Figure 15C).

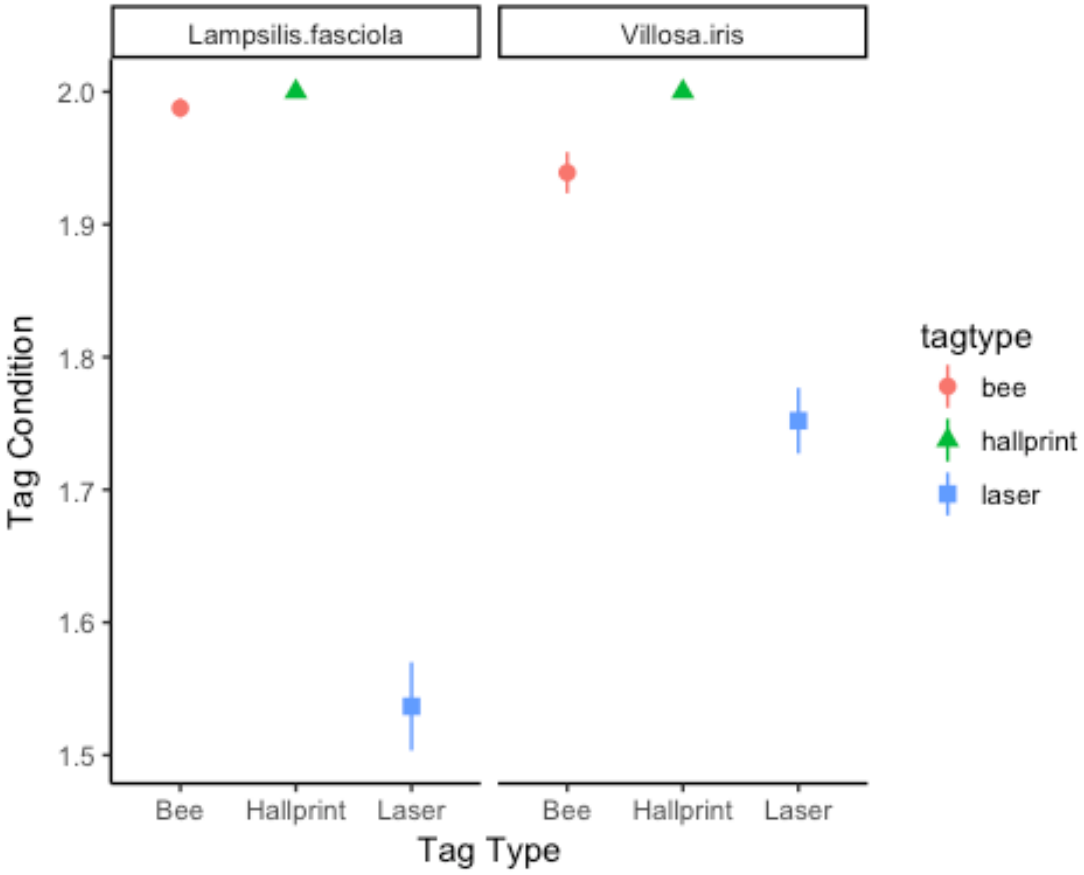

Figure 14: Average tag condition of queen bee tags (red circle), Hallprint tags (green triangle), and laser tags (blue square) of *L. fasciola* (left) and *V. iris* (right).

Table 9: ANOVA summary for tests of differences in tags.

Source	Sum Square	Mean Square	df_{num}	df_{den}	F	P
Tag Type	33.60	16.80	2	4212.77	135.89	<0.0001
Species	2.70	1.35	2	37.39	10.94	0.0002
Tag Type:Species	20.74	5.18	4	4206.70	41.93	<0.0001

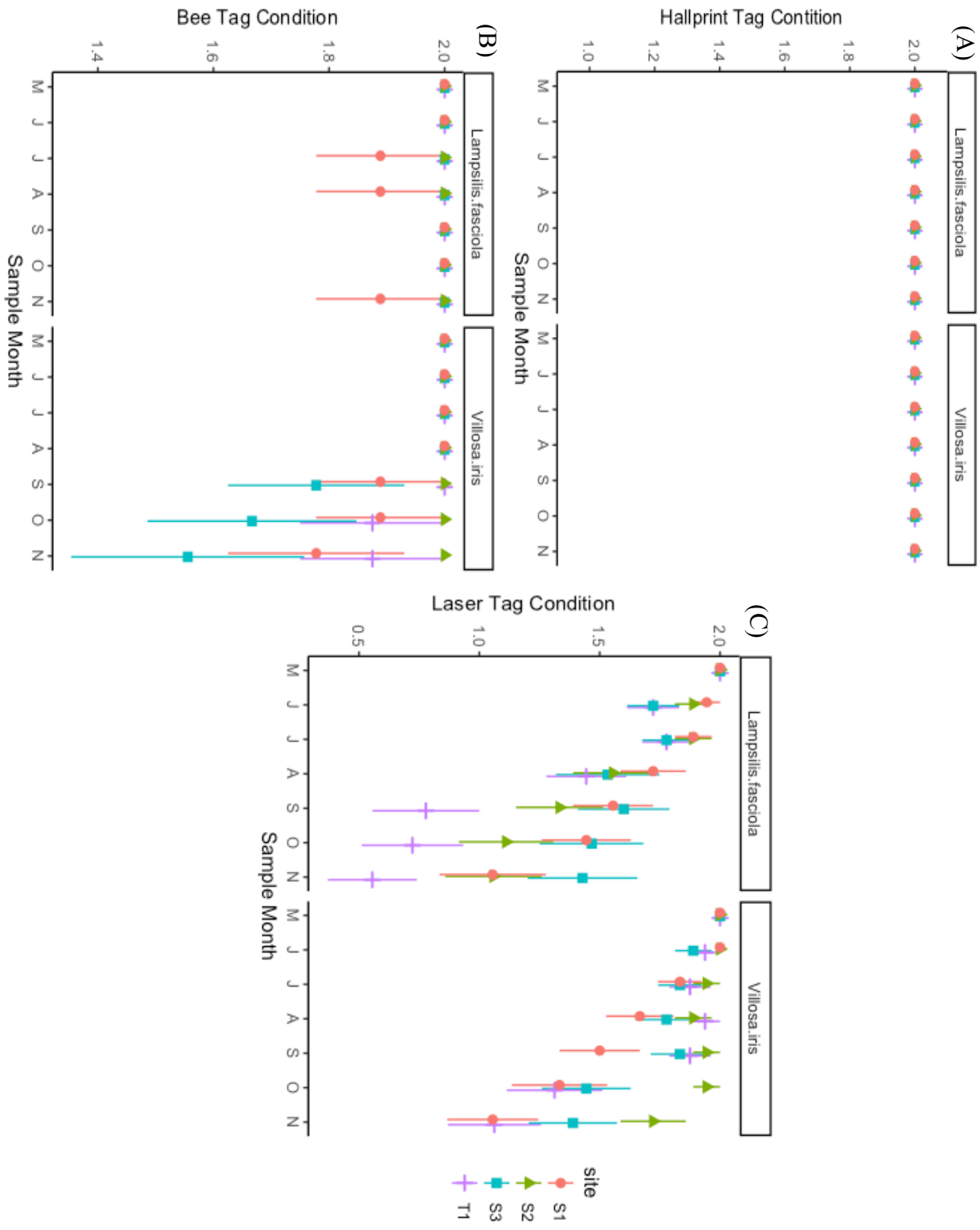

Figure 15: Average Hallprint (A), queen bee (B), and laser tag (C) condition *of L. fasciola* and *V. iris* at sites 1 (red circle), 2 (green triangle), 3 (blue square), and control (purple cross) from May to November.

DISSCUSSION

Survival and Growth

Survival of all *V. iris*, minimal mortality of *L. fasciola*, and positive growth provides evidence that both species are able to survive in the Upper Oconaluftee River, suggesting that the river is suitable for reestablishing populations. *Villosa iris* exhibited the most growth farthest downstream, and growth decreased moving upstream. Similarly, while there are no differences in growth among sites for *L. fasciola*; the mortality that occurred was primarily upstream. Both trends support reintroduction at all reaches, but show better potential in the downstream reaches of the Upper Oconaluftee River.

Temperature is positively correlated with annual growth rates of freshwater mussels, potentially explaining the increased survival and growth of *V. iris* and *L. fasciola* at site 1 (Schöne *et al.,* 2004). This trend has previously been observed for *L. fasciola* growth in natural river systems (Pigeon River), and both *L. fasciola* and *V. iris* in artificial systems (fish hatchery raceways and flow through culture system) (Beaty and Neves, 2004; Hanlon and Neves, 2006; Rooney, 2010). Rooney (2010) split *L. fasciola* into two cultures; culture 1 was raised at higher temperatures for about four months and ended up approximately 10 mm longer than culture 2 before being placed in the river. In addition, when left in the Pigeon River for one year, the two cultures of *L. fasciola* grew to an average of about 36 mm and 24 mm respectively downstream, where water reached about 25ºC, compared to about 34 mm and 21 mm respectively upstream, where water reached about 21ºC (Rooney, 2010). In addition to differences among sites, temperature may also be a factor in explaining differences in growth among rivers. *Lampsilis fasciola* reintroduced into the Pigeon River grew approximately 5-7 mm (Rooney, 2010), while

L. fasciola in the Upper Oconaluftee River grew approximately 2 mm over the same time frame (March to November). This difference could be related to the 4-5ºC higher water temperature in the Pigeon River (Rooney, 2010) compared to the Oconaluftee River.

The connection between growth and temperature can also be observed temporally, in that both mussel species grew most during the warm summer months and growth slowed as temperatures began to decrease in October and November. Beaty and Neves (2004) also observed this trend with *V. iris* raised in an artificial system supplied by natural river water that fluctuates temperature with the river. In this system, *V. iris* exhibited better survival and growth during the warm months of summer (Beaty and Neves, 2004). This increase in growth is associated with higher feeding and respiration rates at warmer temperatures (Schneider, 1992).

Different mussel species have different optimal temperatures. For example, a more heat sensitive species is the marine mussel *Mytilus edulis*, which increases growth rate until approximately 20ºC, when growth rate begins to decline, while *V. iris* is more heat tolerant and cold sensitive, ceasing growth until temperatures exceed 15ºC (Almada-Villela *et al.,* 1982; Beaty and Neves, 2004). Similarly, laboratory propagated *L. fasciola*, *Epioblasma brevidens*, and *Epioblasma capsaeformis* have slower growth at lower temperatures, about 20ºC, and reach maximum growth at about 26ºC (Carey *et al*., 2013).

While there is evidence for slightly elevated temperatures positively influencing mussel growth and survival, extreme temperatures caused by climate change or human industry can be detrimental and should be taken into consideration when choosing restoration locations. Temperature influences sensitivity to toxic contaminants and physiological activities, so can be lethal with tolerance limits often reached between 30-35ºC (Mummert *et al*., 2003; Rajagopal *et al*., 2005; Archambault *et al.,* 2013; Archambault *et al.,* 2014). Various freshwater mussel

species, including *L. fasciola, Lampsilis abrupta,* and *Lampsilis radiate,* have a median lethal temperature between 29.9 and 35.7ºC (Archambault *et al.,* 2013; Archambault *et al.,* 2014). These extreme temperatures do not appear to be a factor in the Upper Oconaluftee River, where the highest temperature reached was 22.6ºC, well below known tolerance limits of freshwater mussels.

Elevated specific conductivity at site 1 may also be associated with the superior survival and growth. A study involving the marine mussel *Mytilus edulis* grown off the coast of California provided evidence that food availability, shown via phytoplankton biomass, is positively correlated to mussel growth (Page and Hubbard, 1987). While my study did not measure phytoplankton biomass, the elevated specific conductivity at site 1 is proportional to ion concentration in the water column and is also associated with elevated nutrients in the water column (Krawczyk and Ford, 2006). This trend is supported by growth of *L. fasciola* also increasing with specific conductivity in the Pigeon River (Rooney, 2010). The higher specific conductivity may be explained by the wastewater treatment plant located upstream of site 1. Wastewater treatment plants increase the concentration of limited nutrients in the water column, increasing mussel growth rate (I and Hu, 1996; Carey and Migliaccio, 2009). Similarly, *L. fasciola* in the Pigeon River had the most growth downstream on the paper mill wastewater treatment plant (Rooney, 2010). Despite the elevated specific conductivity, nutrient concentrations (*e.g.* nitrates, ammonia, phosphate, free chlorine, and total chlorine) were never above detection limits.

The lower dissolved oxygen at site 3 is contrary to what is previously known about the relationship between temperature and dissolved oxygen concentrations. It would be expected that lower dissolved oxygen concentrations would be at the sites with higher temperatures, the

downstream sites, which is observed in other rivers, such as the Pigeon River (Badran, 2001; Rooney, 2010). While decreased dissolved oxygen concentrations leads to higher mortality of freshwater mussels due to decreased oxygen consumption, the levels reached at site 3 were not low enough to be considered a stressor (Sparks and Strayer, 1998; Chen *et al.,* 2001). Mussel mortality begins to increase at approximately 5 mg/L, while the lowest concentration measured at site 3 was 9.07 mg/L (Sparks and Strayer, 1998; Haag and Warren, 2008). Therefore, it is unlikely that dissolved oxygen concentrations at site 3 are related to the mortality and growth observed.

While the difference is minimal, it is interesting to note that site 1 had a higher urbanization score than the others due to the large amount of trash. Urbanization is highly correlated to a lack of freshwater mussel diversity (Lyons *et al.,* 2007), despite this, site 1 still had the highest survival and growth. Trash, particularly plastic and electronic waste, is known to release harmful chemicals into the environment (Engler, 2012; Man *et al.,* 2013). However, the trash concentrated at site 1 appears not to be at an extreme enough level to cause harm to the freshwater mussel populations.

Interestingly, mussels at the control site on the Tuckasegee River, where mussels are known to reside, had the least growth. This difference may be related to the higher urbanization score. The site contained some trash, although not as much as site 1, so was most likely not a factor in the poor growth. The difference may be associated with the increased riverbank modification and erosion compared to all sites on the Oconaluftee River. Modified riverbanks and erosion lead to increased sedimentation in the river column, which is associated with sub-lethal stress on freshwater mussel populations that may reduce growth rates while allowing the population to remain self-sustainable (Box and Mossa, 1999). Increased sedimentation is also

associated with increased discharge and flooding (Francke *et al*., 2008); therefore, daily fluctuations in water flow due to generating the Dillsboro Dam may have contributed to the reduced growth. Sedimentation can negatively affect a variety of freshwater mussel bodily functions, influencing populations, including their filter feeding and reproductive abilities (Landis *et al.,* 2013; Tuttle-Raycraft *et al.,* 2017). Decreased feeding ability could cause decreased growth, and both *V. iris* and *L. fasciola* have decreased feeding abilities when total suspended solids are elevated (Tuttle-Raycraft *et al.,* 2017).

Both *V. iris* and *L. fasciola* were about two years old when stocked, which due to their successful growth and survival, appears to be an appropriate age. *Lampsilis fasciola* stocked into the Pigeon River were also about two years old and had similar survival (Rooney, 2010). *Villosa iris* in an artificial recirculating aquaculture system were not as successful (O'Beirn *et al*., 1998). While the mussels grew similarly to *V. iris* in the Oconaluftee River over the same period of time, approximately 1.5 mm over 18 weeks, survival decreased over the same period, about 74% (O'Beirn *et al*., 1998). This difference may be due to older, larger mussels introduced into the Oconaluftee River (19.5 mm) compared to those grown in the recirculating system (2.7 mm) (O'Beirn *et al*., 1998); indicating older, larger juveniles are hardier and more likely to survive translocation.

The positive growth and survival of *V. iris* and *L. fasciola* observed is in stark contrast to another attempt to determine if the Oconaluftee River is suitable habitat for freshwater mussels. In a similar study determining the reintroduction feasibility of *Alasmidonta viridis* in the Upper Oconaluftee River, the mussels exhibited extreme mortality (Figure 16) (Ruigrok, 2019). The study took place at the same time (March to November 2018) as our study and had a similar design; five *A. viridis* were placed in mussel silos and substrate exposed enclosures at the same

sites as *V. iris* and *L. fasciola* on the Oconaluftee and Tuckasegee Rivers (Ruigrok, 2019). Out of the 96 individuals placed in the river, only three survived until November (Figure 16). This difference among species shows the importance of determining which species are able to survive in potential restoration locations before moving forward with introduction.

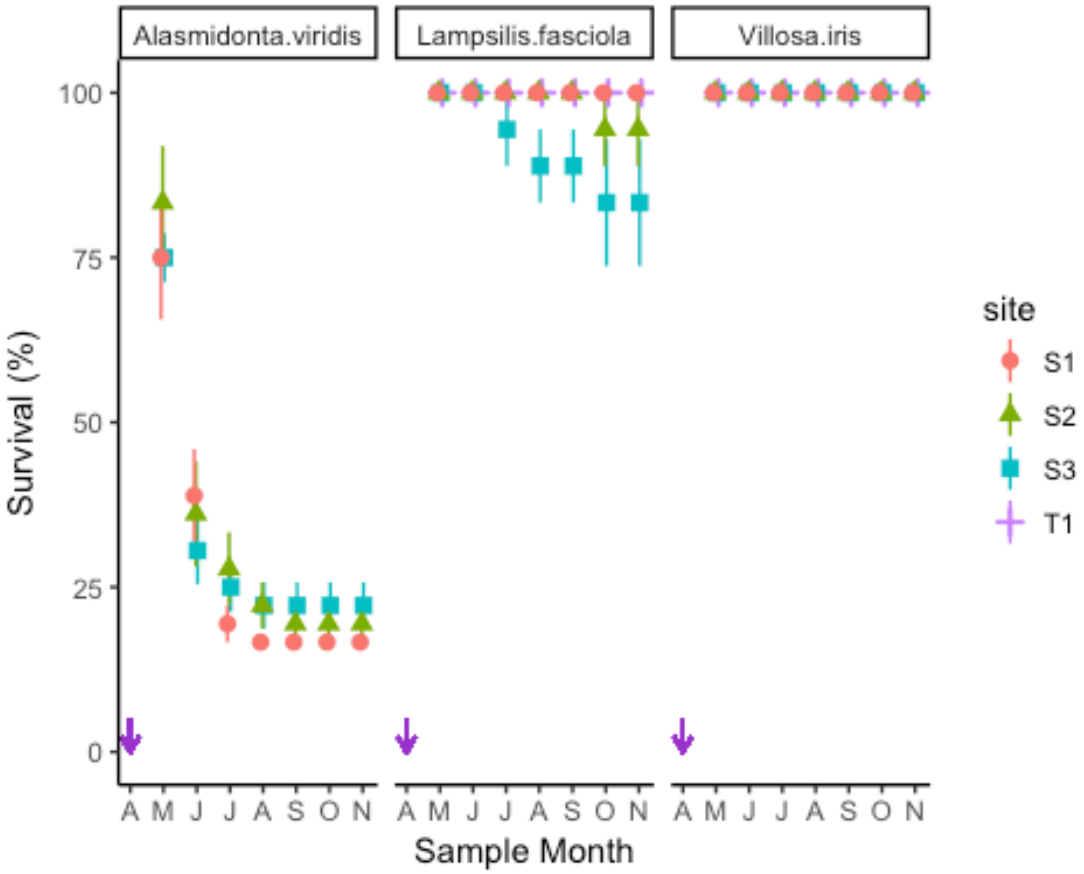

Figure 16: Percent survival of *A. viridis* (left), *L. fasciola* (middle), and *V. iris* (right) at sites 1 (red circle), 2 (green triangle), 3 (blue square), and control (purple cross) from May to November. The purple arrow shows experiment installation month, when no measurements were taken, *A. viridis* data obtained from Ruigrok (2019).

Tags

Ranking the tags allowed us to quantify when tag types began to erode and determine appropriate tag choice for future experiments in similar systems. Consistent with a previous study that compared shellfish tags to other tag types (*e.g*. fingerling, visible implant, laser etched, and micromarkers), the shellfish tags appear to be the superior choice (Lemarié *et al*., 2000; Ross *et al.,* 2001). The Hallprint shellfish tags were the longest lasting, with all tags remaining adhered to the mussels and no erosion or sediment buildup to the point of illegible numbers. Longer time in the water may result in the loss of tags, but over the 7 months of this study, Hallprint tags were superior.

The queen bee tags never eroded or discolored, remaining legible throughout the experiment. However, some did fall off the mussels after a few months. The queen bee tags are much smaller than the Hallprint tags and able fit on smaller mussels; therefore, are a good alternative when smaller tags are needed.

While laser etched tags are unable to fall off the mussels, they began eroding after only a few months. Despite brush-on nail polish applied to the etching as a precaution to avoid such deterioration, sediment filled the tags, making them unreadable. The turbulence of a natural river system may be too harsh for laser etched tags to be a long-term solution to glue on tags falling off the mussels. Contrary to our results, laser tags on mussels stocked in the Cheoah River, which is structurally similar to the Oconaluftee River, remained readable after two years (Luke Etchison, North Carolina Wildlife Resources Commission, personal communication). Therefore, the failure of the laser etched tags in our study could be the result of keeping the mussels in silos. Further studies comparing long term legibility of the three tag types in natural systems outside

enclosures is needed to make concrete conclusions regarding the effectiveness of laser etched tags.

Conclusions

While the evidence provided from this study supports further action by the North Carolina Wildlife Resources Commission and Eastern Band of Cherokee Indians Natural Resources to establish population of *V. iris* and *L. fasciola* in the Upper Oconaluftee River, further studies regarding the successful reintroduction of these species are needed. Both species are successfully able to thrive in silos, where they have access to the water column and some sediment. However, it is not yet known if they will be able to survive long term outside the silo, where they will have full access to the substrate and be exposed to other risks of the habitat, such as predation. In addition, the mussels used were juveniles, not yet of reproductive age and just beginning to show signs of sexual dimorphism. Introducing marked, mature individuals into the river and performing a mark recapture study will show if the adult mussels are able to survive outside the silo and if there is successful reproduction to allow the population to be self-sustainable.

In addition to determining what further action should be taken by the North Carolina Wildlife Resources Commission and Eastern Band of Cherokee Indians Natural Resources, this study aided in creating guidelines to assist in further efforts to help the plight of freshwater mussels. Freshwater mussels are an increasingly imperiled group due to a variety of anthropogenic influences, and understanding the specific factors that could be affecting the survival of populations helps deduce more specifically how human activities are influencing them, aiding in creating guidelines for management and conservation efforts (Neves, 1999; Master *et al.,* 2000; Cowie *et al*., 2017). Understanding the environmental conditions in which *V.*

iris and *L. fasciola* thrive aids in the search of future locations to establish populations of these at-risk species by narrowing what conditions are adequate. In this case, the sites significantly differed in temperature, specific conductivity, and dissolved oxygen, but both species were able to survive under all levels. However, *V. iris* and *L. fasciola* thrived most at the higher temperature and specific conductivity farthest downstream, meaning similar conditions should be sought out and cold headwaters may not be suitable choices for future restoration projects.

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