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Population level responses to direct application liming in Gyrinophilus porphyriticus

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POPULATION LEVEL RESPONSES TO DIRECT APPLICATION LIMING IN *GYRINOPHILUS PORPHYRITICUS*

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DEDICATION

I would like to dedicate this work to my mother Gwen Stevenson-Maurer for her sacrifices, for inspiring me, and for giving me the confidence to pursue my dreams.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 1

CHAPTER 2

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

APPENDIX

ABSTRACT

Direct application liming (DAL) has been used to neutralize acidified streams to restore aquatic biota. This mitigation technique has been used globally for decades, yet little data exist on its effects on amphibian populations. My study investigated the effects of liming on amphibians by measuring variability in life histories of larval *Gyrinophilus porphyriticus.* I collected larvae from six streams in the Monongahela National Forest, West Virginia. I examined the effects of DAL on age structure, and I failed to detect a treatment effect. I used ANCOVAs to examine differences in body condition, body size, and gape size. I observed that larvae located directly below DAL reached significantly larger body sizes at younger ages and appeared to have higher body conditions. Larvae below DAL had significantly smaller gape sizes than larvae in the treatment reference. By identifying the impacts of DAL on amphibian life history strategies, biologists can better manage aquatic habitats.

CHAPTER 1

POPULATION LEVEL RESPONSES TO DIRECT APPLICATION LIMING IN *GYRINOPHILUS PORPHYRITICUS*

INTRODUCTION

Anthropogenic stream acidification, from industrial SO_2 emissions or acid mine drainage, is a global concern due to the negative impacts it has on aquatic ecosystems (Driscoll et al. 2003). To mitigate acidification, liming, that is, the addition of calcium carbonate into aquatic systems, has been used in many industrialized nations to neutralize pH levels and improve water chemistry, with a majority of attention on game fisheries restoration (Eggleton et al. 1996; Mckie et al. 2006). Multiple liming methods have been used to mitigate acidic conditions, including dosers, full catchment, and direct application liming (Menendez et al. 1996; Menendez et al. 2000; Clair & Hindar 2005). Although liming has successfully increased pH and improved overall water chemistry (Clayton et al. 1998; Menendez et al. 1996; Clair & Hindar 2005), the mitigation practice itself acts as an ecological perturbation and could have broad ecological impacts and unexpected emergent effects.

Emergent effects are often scale dependent and arise when simple mechanisms interact at broad spatial scales (Harfoot et al. 2014) and can result when there are alterations to community structure (Carey & Wahl 2010; Reynolds & Elliott 2012). Fine sediments associated with liming can fill interstitial spaces within the stream, changing the physical structure of the stream substrate and likely stressing biota (Keener & Sharpe 2005). Liming also has the potential to alter trophic structure by changing species and predator composition, which may result in shifts in optimal life history strategies for biota. The interaction of these changes may produce unexpected emergent effects (Carey & Wahl 2010; Reynolds & Elliott 2012). These unexpected consequences, such as alterations in habitat and trophic structure, are important considerations

given that economies of scale often encourage large-scale mitigation practices, such as liming (Carey & Wahl 2010; Reynolds & Elliott 2012).

Previous research has demonstrated that liming can have mixed effects on stream biota, particularly directly downstream of lime application, although results vary by stream (Clayton & Menendez 1996; LeFevre & Sharpe 2002; Clair & Hindar 2005; Keener & Sharpe 2005; McClurg et al. 2007). In general, liming increases fish abundance and survival and increases the presence of acid-sensitive macroinvertebrates (Clair & Hindar 2005; Mant el al. 2013); however, numerous studies have observed a decrease in overall macroinvertebrate abundance and diversity below lime application sites, which was often the direct result of increased sedimentation (LeFevre & Sharpe 2002; Keener & Sharpe 2005; Simmons et al. 2006; McClurg et al. 2007). Macroinvertebrate and amphibian abundance can also be negatively affected by non-native trout introduction (Finlay & Vredenburg 2007; Hartel et al. 2007), which often follows lime application because reestablishing game fisheries is one of the primary goals of this mitigation (Downey et al. 1994). Such variation in population-level responses to direct and indirect effects of liming indicates the need for further investigation on the possible impacts of this mitigation practice on aquatic biota. It is prudent to examine the utility of this mitigation practice with emergent effects in mind to ensure that there are no unintentional negative impacts on non-target wildlife. Specifically, there is a lack of information on how liming affects amphibians, which is necessary to understand broader impacts, such as ecological integrity (Welsh & Droege 2001).

Amphibians are considered indicators of ecosystem health and integrity (Corn & Bury 1989; Welsh & Olliver 1998; Welsh & Droege 2001). Sedimentation is a major perturbation in many streams, leading to the loss of microhabitat (Waters 1995), and has been shown to negatively affect stream salamander abundance and survival (Welsh & Olliver 1998; Bonin

1999; Lowe & Bolger 2002). In addition to their sensitivity to sedimentation, salamander larvae have permeable skin, limited dispersal, and cannot escape aquatic conditions until metamorphosis, which make them good models for my study (Petranka 1998; Lowe 2003). Specifically, *Gyrinophilus porphyriticus*, the Spring Salamander, is an ideal study species due to its highly plastic life history with an aquatic larval stage ranging from three to six years, which varies depending on a number of environmental conditions (Bruce 1980; Resetarits 1995). *Gyrinophilus porphyriticus* is an appropriate biological indicator because it relies on interstitial habitats, which can be greatly reduced below lime application sites (Bishop 1941; Bruce 1980; Keener & Sharpe 2005). In its larval stage, *G. porphyriticus* serves as a gape-limited, intermediate-to-apex predator in aquatic systems and can operate as a bellweather to trophic perturbations (Bruce 1980; Maret & Collins 1996; Welsh & Ollivier 1998).

Phenotypic divergence is typically low in *G. porphyriticus* from populations in close proximity along the stream corridor, compared to populations separated by equivalent distances over land (Lowe et al. 2012); therefore, if environmental and trophic conditions are similar throughout the stream, life history strategies should not differ. The reaction norm, which is the ability of an organism's genetics to exhibit different phenotypes in a range of environments, reflects tradeoffs and is maintained when a species is exposed to a variety of environmental conditions throughout its geographic range and several potential life history traits are more beneficial than producing only a single, canalized trait (Stearns 1989; DeWitt et al. 1998; Ghalambor et al. 2007). In *G. porphyriticus*, phenotypic divergence can be induced in novel or stressful environments and apparent shifts in life history strategies can be quantified to detect environmental changes, such that could be manifested after liming (Stearns 1992; DeWitt et al. 1998). Plasticity is adaptive when it increases fitness, neutral if there is no effect with respect to

fitness, or maladaptive if environmental information is unreliable/rapidly changing or if the level of plasticity needed for a particular environment cannot overcome physiological or genetic constraints, leading to decreased fitness (Smith-Gill 1983; DeWitt et al. 1998; Ghalambor et al. 2007). Life history shifts are often associated with tradeoffs (Hereford 2009), which reflect constraints on resources, such that an increase in one trait necessitates a decrease in another (Pease & Bull 1988). Tradeoffs occur when shifts in resource allocations are necessary to better fit local environments (Luquet et al. 2015). Major tradeoffs that are common in amphibians are variations in activity level, mortality, and growth rate and variations in size and timing of metamorphosis (Berven & Gill 1983; Stearns 1992; Werner and Anholt 1993; Roff 2000). For example, amphibian fitness is often positively correlated with body size, which is affected by larval period, growth rates, and gape size (Forsman 1994). Individuals with rapid growth often mature or complete metamorphosis earlier than individuals that have a longer larval period with slower growth. Rapid growth is often selected for when environmental conditions are stressful or unstable and greater fitness is achieved by maturing and reproducing at younger ages. Slower growth, and therefore longer larval periods, occurs in stable environments and results in larger, higher fecundity adults with increased fitness. However, a tradeoff for rapid growth may contribute to higher mortality due to the need for increased activity levels, whereas a tradeoff for slower growth would be forgoing reproduction until later in life (Stearns 1992; Arendt 1997). Tradeoffs may also be caused by physical or physiological limitations from increased stress after an environmental perturbation (Smith-Gill 1983). Therefore, optimal life history strategies vary in different environments; however, optimal strategies under stressors from environmental perturbations, such as liming, may not be equivalent with respect to fitness (Welsh & Olliver 1998; Teplitsky et al. 2007).

Direct application liming (DAL) is the instream bulk application of sand-sized limestone and has become a widely-used method (Downey et al. 1994; Keener & Sharpe 2005) for increasing stream pH. It is the primary method used in West Virginia and throughout Appalachia due to its affordability, ease, and its effectiveness compared to other methods (Ivahnenko et. al 1988; Clayton et al. 1998). Stream flow continuously distributes limestone particles downstream until the limestone pile is eroded away, at which time more lime is added (2-3 times per year). Limestone dissolution rates vary depending on particle size and initial pH (Sverdrup 1986; Menendez et al. 2000). Undissolved particles can embed the natural substrate, reducing the hyporheic zone, which is the primary habitat for many biota, including salamander larvae (Ivahnenko et. al 1988; Waters 1995; Lowe & Bolger 2002). Reduction of microhabitat from sedimentation can increase stress in larvae and limit predator avoidance, potentially leading to life history shifts (Welsh & Ollivier 1998; Resetarits 1995).

Little data exist on the effects of liming on amphibian populations; therefore, my study will examine variations in size and age of *G. porphyriticus* from limed and un-limed streams. Direct application liming has the potential to either promote longer larval periods due to improved conditions and decreased stress levels, or could lead to selection for early metamorphosis if stress levels increase, shifting optimal life history strategies (Stearns 1992; Arendt 1997). I used active DAL streams and un-limed control streams to examine the effects of liming on larval *G. porphyriticus* life history strategies, specifically population age structure, and individual body condition, growth, and gape size. I hypothesized that I would detect a tradeoff between life history parameters resulting from increased sedimentation from DAL. Specifically, I expected larvae downstream of the DAL sites to have younger populations, indicating selection for earlier metamorphosis, and smaller individual body conditions, body size, and gape size from

increased stress and alterations in trophic structure. By identifying the impacts of liming on amphibians, biologists can better manage aquatic habitats and determine if liming is beneficial to overall stream diversity. Understanding how liming affects non-target species will aid with improvements to methodology or bring into question the efficacy of this mitigation practice to achieve conservation and management objectives. My research will promote future comprehensive studies on various mitigation practices in efforts to reduce unexpected emergent effects.

METHODS

Study Sites

I collected *G. porphyriticus* larvae from multiple populations in the Gauley River watershed in Pocahontas and Greenbrier counties, near Richwood, WV, which is in the southern portion of the Monongahela National Forest. I sampled six first- and second-order streams, which included three treatment streams and three control streams (Figure 1). I sampled Sugar Creek of the Williams River (treatment stream; first-order) and its neighboring unnamed tributary (control stream; first-order), Bear Run of the North Fork Cherry River (treatment stream; first-order) and its neighboring unnamed tributary (control stream; first-order), and Dogway Fork of the Cranberry River (Figure 1; treatment stream; second-order) and its neighboring unnamed tributary (control stream; first-order). Because DAL relies on a road-crossing to apply lime fines, I selected three treatment streams and three control streams that included an unpaved road-crossing. Streams were characterized by rocky substrates, shallow stream depth (<1 m), and forested riparian zones. Direct application liming was applied to treatment streams several times yearly using dump trucks. Each lime application was applied by the West Virginia Division of Natural Resources directly into the stream to mitigate for acid

precipitation. The rate and volume of liming applications varied among sites. Sugar Creek was treated twice yearly in the spring and fall, totaling 45 metric tons of limestone fines. Bear Run was treated three times yearly in the spring, summer, and fall with a target limestone fines of 66 metric tons and Dogway Fork is limed throughout the spring, summer, and fall with nearly 250 metric tons of limestone fines. The rates and amount of application were dependent on drainage size and original pH and was designed to bring each stream up to a neutral pH. The West Virginia Division of Natural Resources used limestone fines that were 90% calcium carbonate or higher. Control streams were not treated with lime and had similar stream characteristics as treatment streams. Each treatment stream had a paired control stream to control for intra-study variability and to increase power to detect treatment effects (Sokal & Rohlf 1981; Figure 2). Of the available limed streams in the area, I selected streams that included large *G. porphyriticus* populations, had a paired control stream, and had a sufficiently long stream channel (~0.8 km) to incorporate three independent sampling sites.

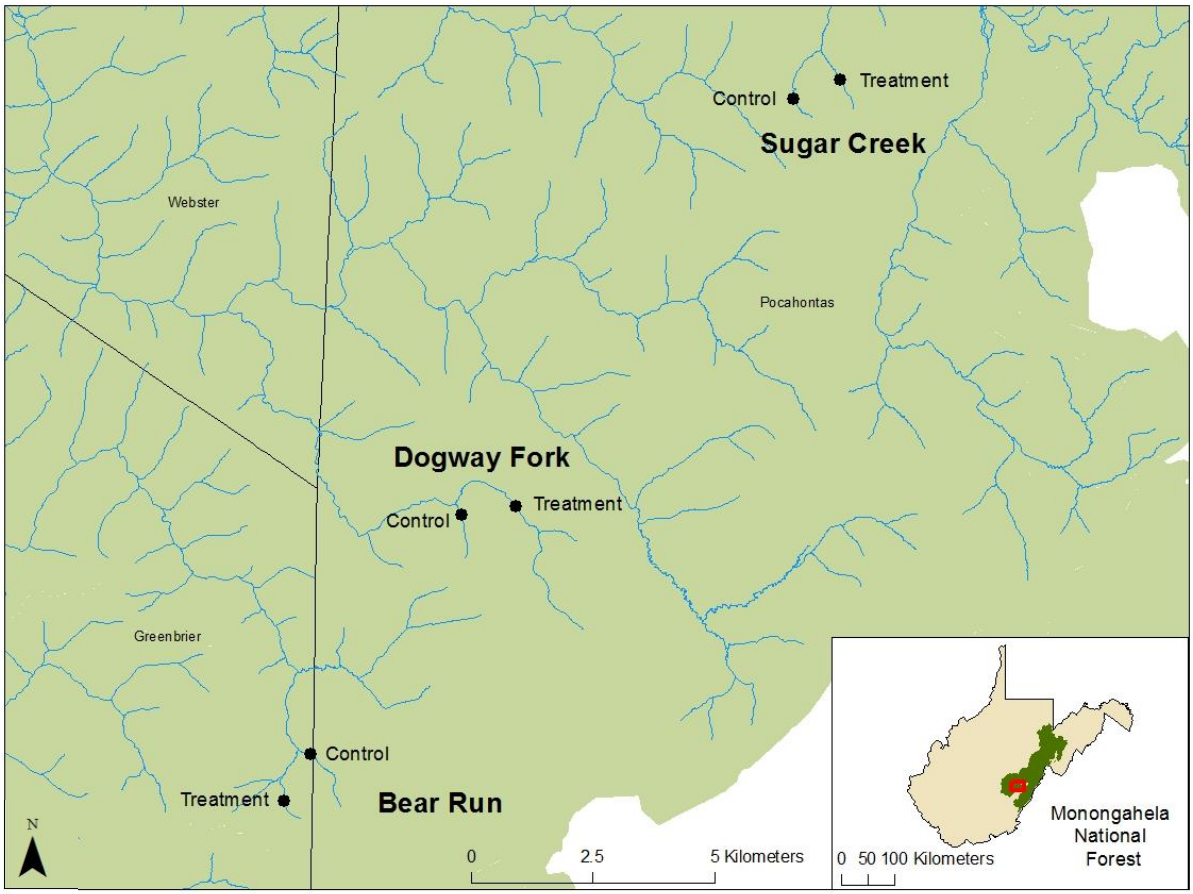


Figure 1: Study site locations in the Monongahela National Forest, WV. Sites were located in Greenbrier and Pocahontas counties.

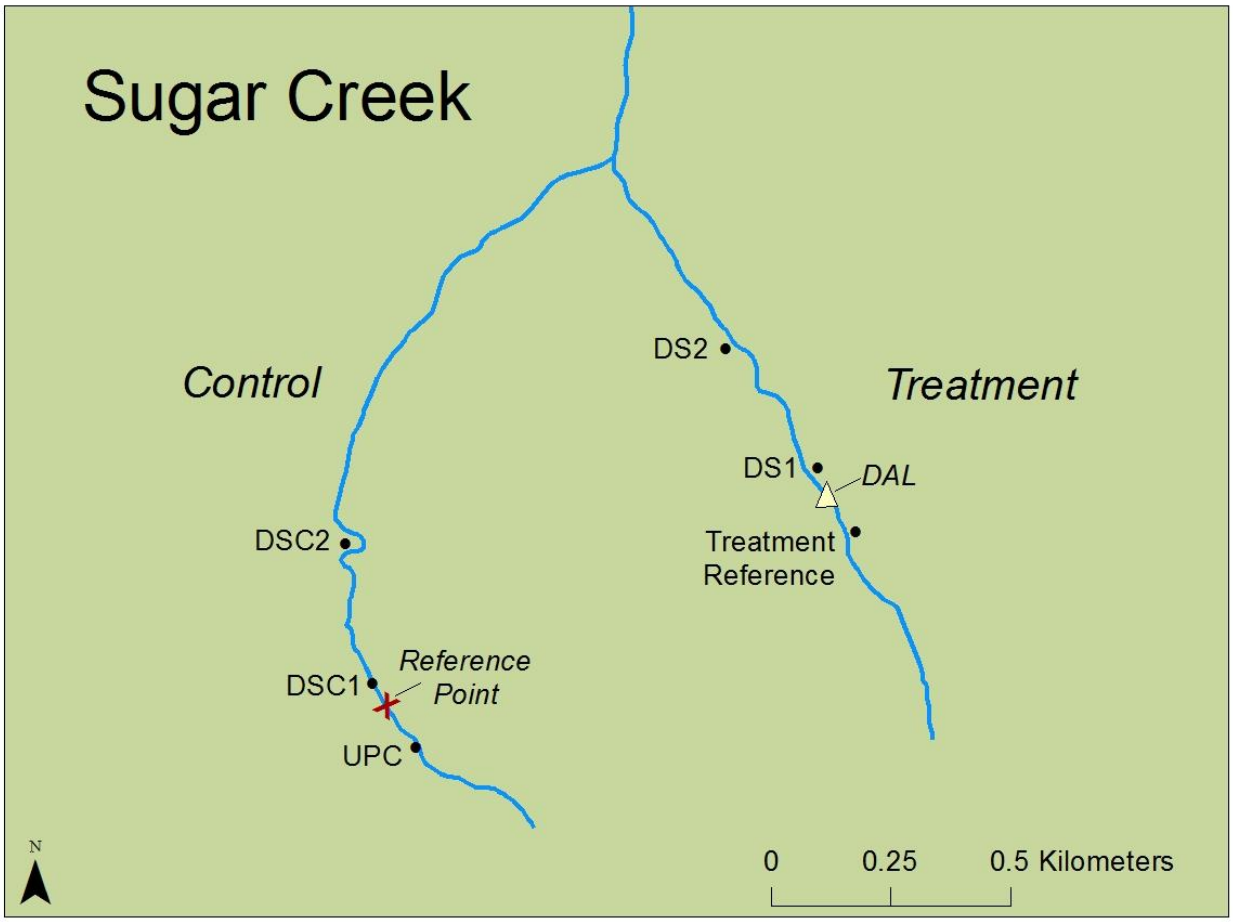


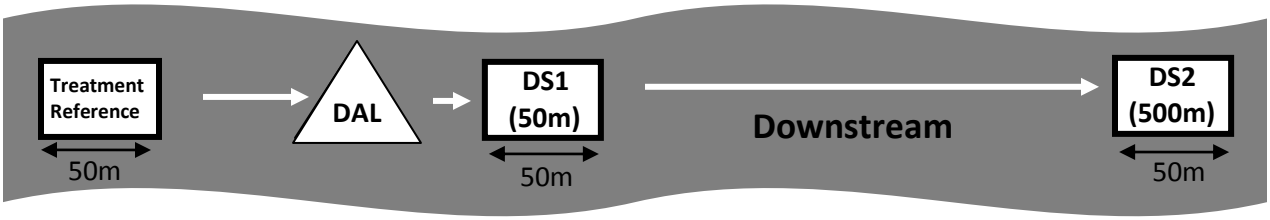
Figure 2: Sampling site locations showing paired treatment and control streams at Sugar Creek. Each stream had three sites. The control stream sites were measured from the "Reference Point" with one site 100 m upstream (UPC) of the reference point and two sites downstream. The first site below the reference point is 50 m downstream (DSC1) and the other site is 500 m downstream (DSC2). Sites in the treatment stream were measured from the direct application lime site (DAL) with one site 100 m upstream (Treatment Reference) and two sites downstream. The first site below DAL was 50 m downstream (DS1) and the other site was 500 m downstream (DS2).

Data Collection

Within each stream, I collected *G. porphyriticus* larvae from three independent locations relative to a road crossing (N=18 sites). Sample locations within treatment streams included a treatment reference located 100 m upstream of the DAL application site (i.e., road crossing; Figure 3A). The first treatment site was located 50 m downstream of the DAL site (DS1) and the second

treatment site was located 500 m downstream of the DAL site (DS2). Control streams were designed with the same site layout, but because there was no DAL site, the control sites were measured from a reference point located at the road-crossing to mirror road-crossings in treatment streams (Figure 3B). Control streams had one site located 100 m upstream of the reference point (UPC) and two sites located downstream of the reference point at 50 m below (DSC1) and 500 m below (DSC2). For treatment and control streams, each sampling site was 50 m long and at least 100 m from any other collection site to maintain independent larval populations (Lowe 2003). I collected larvae on multiple sampling occasions from April 25, 2014 to August 23, 2014 until at least eight individuals were collected from each site. I selected a sample size of 8-12 individuals per site to increase statistical power, while not overharvesting from a single population. I collected larvae using a flip and search method during diurnal surveys (Lowe & Bolger 2002). All cover objects were flipped throughout the site until eight individuals were captured. *Gyrinophilus porphyriticus* larvae were most often found in the interstitial spaces under cobble sized rocks (between 64 mm and 256 mm diameter; Lane 1947) that were not embedded in the substrate and were generally located in riffles or pools (Lowe 2005). After I collected larvae, they were euthanized in MS-222 and transferred to 70% ethyl alcohol. I recorded pH on each sampling occasion, and fish and crayfish presence. The pH of each site was measured on at least two occasions with a pHTest ® Series, pH Testr30 meter, measured to the nearest tenth.

A) Treatment stream site layout



B) Control stream site layout

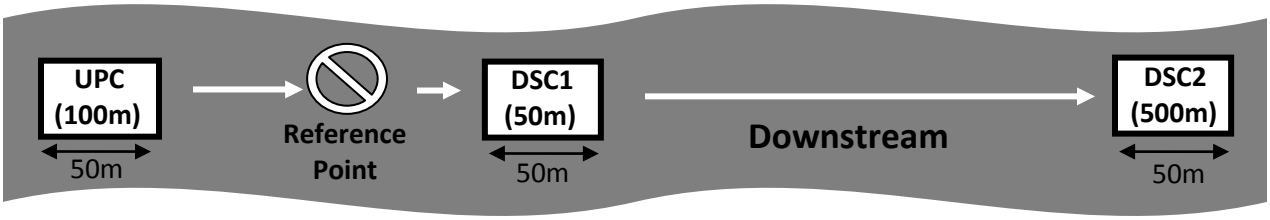


Figure 3: A) Treatment stream site layout. Sites relative to the direct application lime site (DAL). DS1= first downstream site; DS2= second downstream site. The treatment reference site was located 100 m above lime application and was not impacted by lime. Downstream site 1 (DS1) was located 50 m below the DAL site and DS2 was located 500 m below the DAL site. Each site was 50 m in length. **B)** Control stream site layout. Sites relative to random reference point. UPC=upstream control site; DSC1= first downstream control site; DSC2= second downstream control site. Upstream control site (UPC) was located 100 m above the reference point. Downstream control site 1 (DSC1) was located 50 m below the reference point and DSC2 was located 500 m below the reference point. Each site was 50 m in length.

Size and Age Determination

I measured snout-vent length (SVL) of preserved larvae using calipers to the nearest 0.05 mm from the snout to the posterior edge of the cloacal slit. Wet mass was measured using a digital scale to the nearest milligram. Body condition was calculated using a ratio index of body mass divided by SVL so that the values could be compared across populations (Jakob et al. 1996). Gape size was measured for each larva using calipers to the nearest 0.05 mm. Gape was measured across the width of the head from the edges of the mouth (Ohdachi 1994).

I used skeletochronological analysis of femurs for larval age determination. Age can be determined in amphibians by counting the number of lines of arrested growth (LAGs) in the

cross-sections of the diaphysis of long bones or phalanges (Castanet et al. 1993; Smirina 1994). Lines of arrested growth are deposited annually in temperate regions during winter when growth slows and individuals are less active. The areas of bone between LAGs are laid during periods of active osteogenesis and are distinguishable from the darker staining LAGs (Castanet et al. 1993). Because LAGs are generally laid annually during the winter, they provide a reliable means of determining age (Castanet et al. 1993; Smirina 1994). This method has been verified using amphibians of known age and is most reliable in young, rapidly growing individuals, such as larvae (Smirina 1994). Age estimation in older individuals can be less accurate because early LAGs can be resorbed and slowed growth rates after sexual maturity result in LAGs that are very close together and difficult to distinguish. Thus, in this study, I limited my analysis to larval *G. porphyriticus* (Smirina 1994).

After preservation, the right hind limb of each individual was removed and placed directly into 5% nitric acid for decalcification (Bruce & Castanet 2006). Typically, the femur is separated from the surrounding tissue and muscle before it is placed into nitric acid (Leclair & Castanet 1987; Socha & Ogielska 2010; Ashkavandi et al. 2012); however, after trying both methods, removing the tissue was deemed unnecessary (Figure 4). Leaving the surrounding tissue intact made the bones easier to handle and prevented them from being damaged during processing. Bones were decalcified for three hours then rinsed three times in deionised water and placed in 30% sucrose. If bones were not immediately cut, they were refrigerated at 4°C. To prepare the bone for cross-sectioning, I placed the leg upright in Tissue-Tek OCT Compound freezing medium. I then made 15 um cross-sections (Bruce & Castanet 2006) using a Leica CM 1950 cryostat (freezing microtome) at -15° C. I placed cross-sections from the diaphysis of the femur directly onto Superfrost Plus microscope slides (Wake & Castanet 1995; Bruce &

Castanet 2006; Socha & Ogielska 2010). The slides were stained in Ehrlich's Hematoxylin for 10 minutes and then rinsed in deionised water for five minutes and permanently mounted with Clear-Mount, an aqueous mounting medium. Ehrlich's Hematoxylin was used because LAGs are highly chromophilic with hematoxylin dyes (Castanet et al. 1993). Once stained, I examined the slides under a light microscope and counted the number of LAGs for each individual. Because each LAG was laid annually (Castanet et al. 1993; Smirina 1994), LAGs were translated directly to age. Larvae that lacked LAGs were considered young of the year and were assigned to the zero age class. If the sections were unclear the other femur was used to determine age.

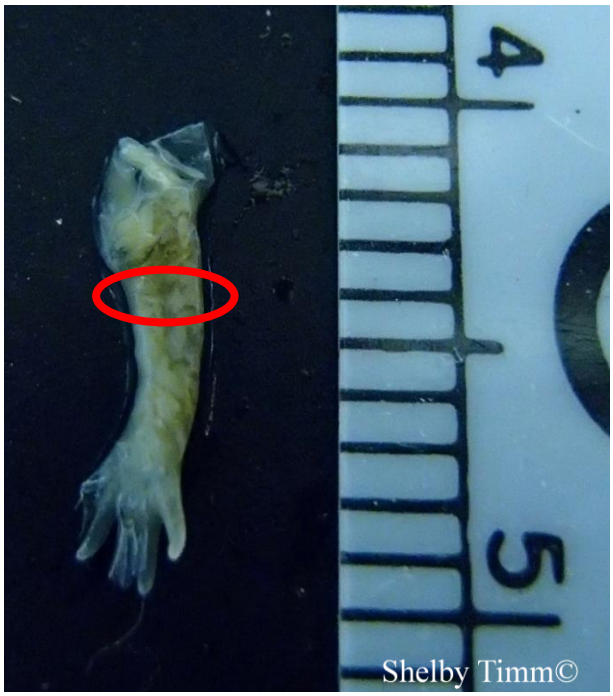


Figure 4: Hind limb removed from larva for skeletochronological analysis. Cross-sections from the diaphysis of the femur (circled) were used for age determination.

Analysis

I determined each individual's age and did not have to exclude any individuals from analysis due to unclear LAGs or regenerated limbs. I aged each individual on three separate occasions using blind analysis to prevent bias (Castanet et al. 1993). In the event that more than

one age was estimated for an individual, I retained the most recurring age for analysis. Any age discrepancies for an individual were within one year of other estimates, and no individual was estimated to be three different ages; therefore individual age estimates were consistent.

All statistical analyses were performed in SAS® [9.4] (Copyright © 2013). I used an ANOVA to compare variations in pH across sampling sites in both treatment and control streams. I used Fisher's exact tests to examine whether age frequency distributions differed among sites and stream (i.e., DAL treatment). Because young individuals (0-2) were underrepresented in my sample due to their secretive nature and greater use of interstitial spaces (Resetarits 1995), and because my goal was to examine differences in metamorphic age (Bishop 1941; Bruce 1980), I excluded age classes 0-2 in the age structure analysis. I used analysis of covariance (ANCOVA) to examine differences in body condition (dependent variables) in treatment and control stream sites (independent variables), while controlling for variation caused by age (covariate). I also used ANCOVAs to examine differences in SVL (dependent variable) in treatment and control stream sites (independent variables), while controlling variation caused by age (covariate). Differences in gape size (dependent variable) in treatment and control stream sites (independent variables), while controlling for variation cause by SVL (covariate), were also tested using ANCOVAs. I ran each ANCOVA with only two factors (i.e., treatment) to increase power because my sample size was small (McDonald 2014). I tested for homogeneity of slopes ($\alpha = 0.05$) prior to running each ANCOVA. If slopes were homogenous, then the interaction statement was removed from the final ANCOVA (Engqvist 2005). If slopes were heterogeneous, then further analyses were not conducted. Significant covariates ($\alpha = 0.05$) were determined using Type III of sum of squares. I used ANCOVAs to test for differences in body condition within treatment stream sites and within control stream sites (Table 1). I also used

ANCOVA to examine differences in SVL within treatment stream sites and within control stream sites (Table 1). Snout-vent lengths from control streams were squared to meet the normality assumption. I used the body size ANCOVAs as a proxy for larval growth rate, because the analysis showed the increase in size relative to age. I analyzed gape size with ANCOVAs to test for differences within treatment stream sites and within control stream sites (Table 1).

Table 1: Treatment combinations used in ANCOVAs to examine differences in body condition, SVL, and gape size. TR=Treatment Reference (100 m above DAL); DS1=first downstream treatment (50 m below); DS2= second downstream treatment (500 m below); UPC=upstream control (100 m above); DSC1= first downstream control (50 m below); DSC2= second downstream control (500 m below).

	ANCOVAs	
	Treatment Streams	**Control Streams**
Body Condition	TR vs. DS1	UPC vs. DSC1
	TR vs. DS2	UPC vs. DSC2
SVL	TR vs. DS1	UPC vs. DSC1
	TR vs. DS2	UPC vs. DSC2
Gape Size	TR vs. DS1	UPC vs. DSC1
	TR vs. DS2	UPC vs. DSC2

I mirrored the distribution of sample sites within control streams to those in the treatment streams. Although redundant, my analysis depended heavily on multiple ANCOVAs and this approach allowed me to use comparisons among sampled sites to test specific hypotheses and control for potential longitudinal effects (Table 1). For example, by comparing treatment reference to DS1, I examined direct effects of DAL. By examining treatment reference to DS2, I assessed the effects of distance from DAL. Similar analyses were performed on the control stream with the expectation that I would not detect longitudinal differences and thus provide further support for treatment effects. Sites from control streams were not tested against sites in treatment streams due to fact that *G. porphyriticus* populations from different streams inherently

have greater phenotypic divergence, because dispersal is typically along the stream corridor and not over land, meaning that there is decreased gene flow between streams (Lowe et al. 2012).

RESULTS

I collected 158 *G. porphyriticus* larvae. Individuals that were collected at the beginning of the sampling season had LAGs located at the periphery of the bone section (Figure 5A), whereas larvae collected later in the summer had growth behind the most recent LAG, so the LAG was not at the periphery of the bone (Figure 5B). Larvae ranged from zero (young of the year) to six years in treatment and control streams; however, I failed to detect 6-year individuals below DAL sites (Table 2). Average age was lowest at the DS1 site (Table 2; Figure 6). Younger age classes (0-2) were rarely encountered during sampling, likely because younger larvae inhabit interstitial spaces, the areas between substrate particles, and are more secretive (Resetarits 1995).

A) **B)**

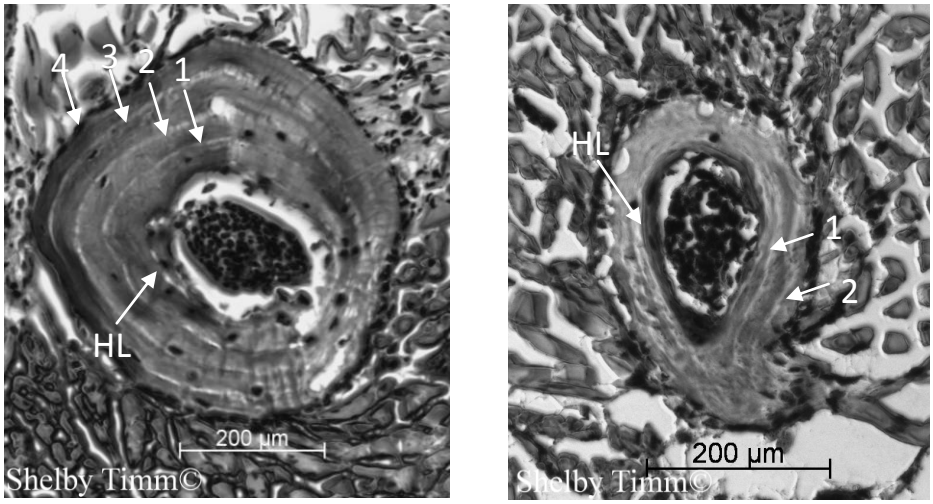


Figure 5: A) Larva collected at the beginning of the sampling season, with a LAG located along the peripheral edge of the bone. **B)** Larva collected at the end of the sampling season with growth behind the final LAG.

Table 2: Descriptive statistics for snout-vent length (SVL) and age for treatment and control streams. Treatment sites include the treatment reference located upstream of lime application, the first downstream site (DS1), and the second downstream site (DS2). Control sites include upstream control site (UPC) located above the reference point, the first downstream control site (DSC1), and the second downstream control site (DSC2). The range and mean for SVL ± standard deviation, and the range and mean age ± standard deviation was calculated for each sample (n).

		SVL (mm)		Age (years)	
Treatment	**n**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
Treatment Reference	27	26.50-61.67	47.13 ± 9.25	0-6	3.56 ± 1.22
DS1	27	25.70-67.20	47.85 ± 12.16	0-5	2.96 ± 1.37
DS2	26	28.10-67.75	49.08 ± 10.41	1-5	3.27 ± 1.00
Control	**n**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
UPC	24	31.05-59.85	47.23 ± 8.08	1-6	3.25 ± 1.03
DSC1	27	35.20-58.75	48.83 ± 7.00	2-6	3.67 ± 1.21
DSC2	27	29.30-59.00	48.40 ± 7.11	1-5	3.70 ± 1.02

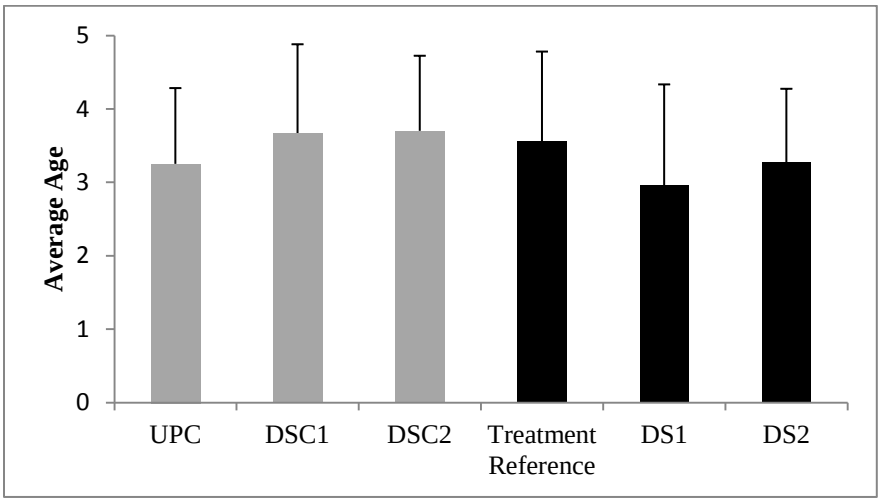


Figure 6: Average age in years and standard deviation for control (gray) and treatment (black) streams. Treatment sites include the treatment reference located upstream of lime application, the first downstream site (DS1), and the second downstream site (DS2). Control sites include upstream control site (UPC) located above the reference point, the first downstream control site (DSC1), and the second downstream control site (DSC2).

I detected a difference in pH between treatment and control streams, with a significantly higher pH below the DAL site ($F_{5,12} = 8.30$; $p = 0.001$; Figure 7). The average pH in control streams and in the treatment reference was 4.8 and the average pH below lime was 7.0.

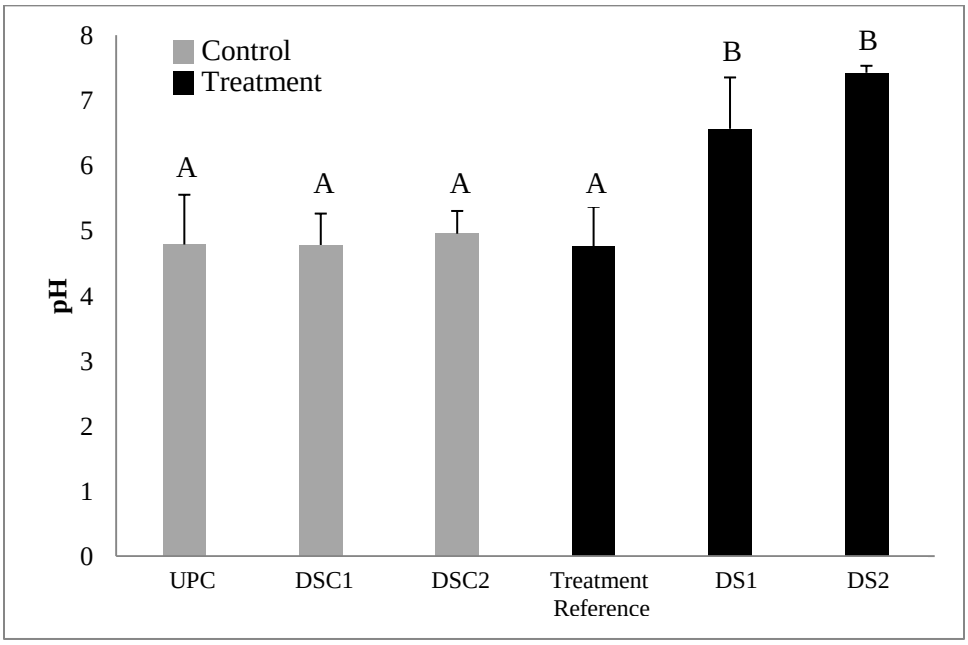
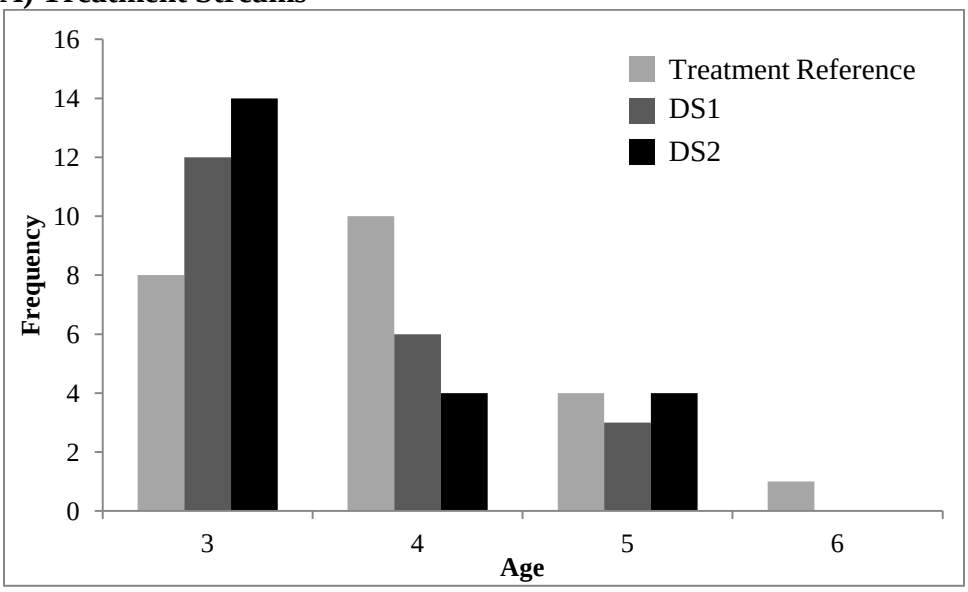


Figure 7: Average pH and standard deviation for control (gray) and treatment (black) streams. UPC=upstream control (100 m above); DSC1= first downstream control (50 m below); DSC2= second downstream control (500 m below). Treatment reference is located 100m above lime application. DS1=first downstream treatment (50 m below); DS2= second downstream treatment (500 m below). "B" indicates that pH is significantly different than "A" (ANOVA).

I failed to detect a significant shift in age structure between sites in treatment ($p = 0.35$) and control streams ($p = 0.20$) using the Fisher's exact test (Figure 8). Although I failed to detect a significant treatment effect on age structure, the predominant age class for larvae below the DAL site was three years (57.1% in the DS1 and 63.6% in the DS2; Figure 8A) compared to larvae in the treatment reference, which were predominantly four years old (52.2%; Figure 8B).

A) Treatment Streams



B) Control Streams

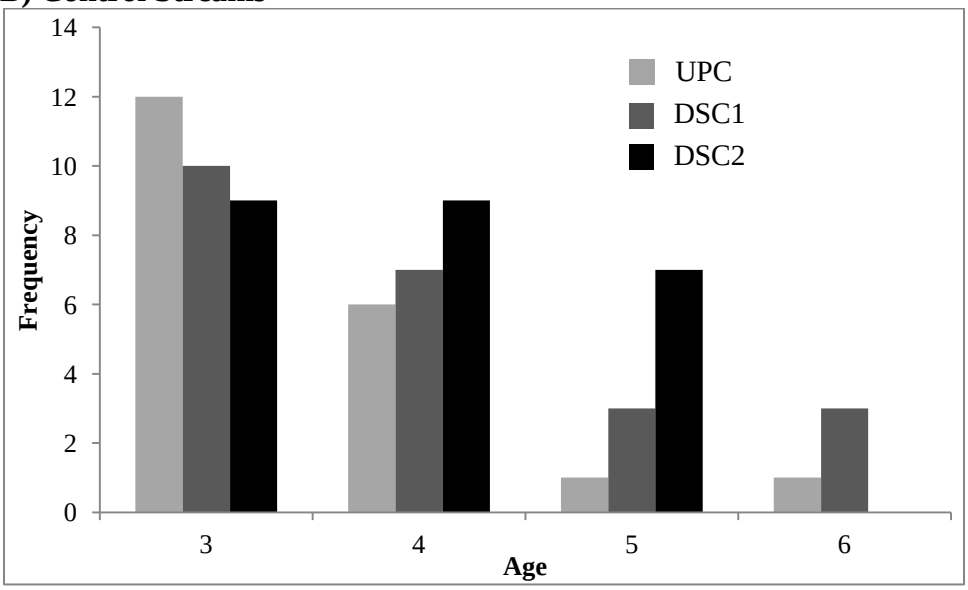


Figure 8: Age frequency distributions for 3-6 year larvae. I failed to detect a difference in treatment or control stream age structures. Treatment sites include the treatment reference located upstream of lime application, the first downstream site (DS1), and the second downstream site (DS2). Control sites include upstream control site (UPC) located above the reference point, the first downstream control site (DSC1), and the second downstream control site (DSC2).

I failed to detect significant differences in larval body condition between sites in treatment and control streams. However, the analysis for the treatment reference and DS1 approached significance ($F_{1,51} = 3.31$; $p = 0.07$; Figure 9). Larvae from DS1 in treatment streams appeared to reach higher body conditions than larvae in the treatment reference, compared to control stream larvae from UPC and DSC1, which did not differ ($F_{1,48} = 0.32$; $p = 0.58$). I also failed to detect a longitudinal effect in body conditions between the treatment reference and DS2 sites ($F_{1,49} = 2.04$; $p = 0.16$) and between the UPC and DSC2 sites ($F_{1,48} = 0.61$; $p = 0.44$).

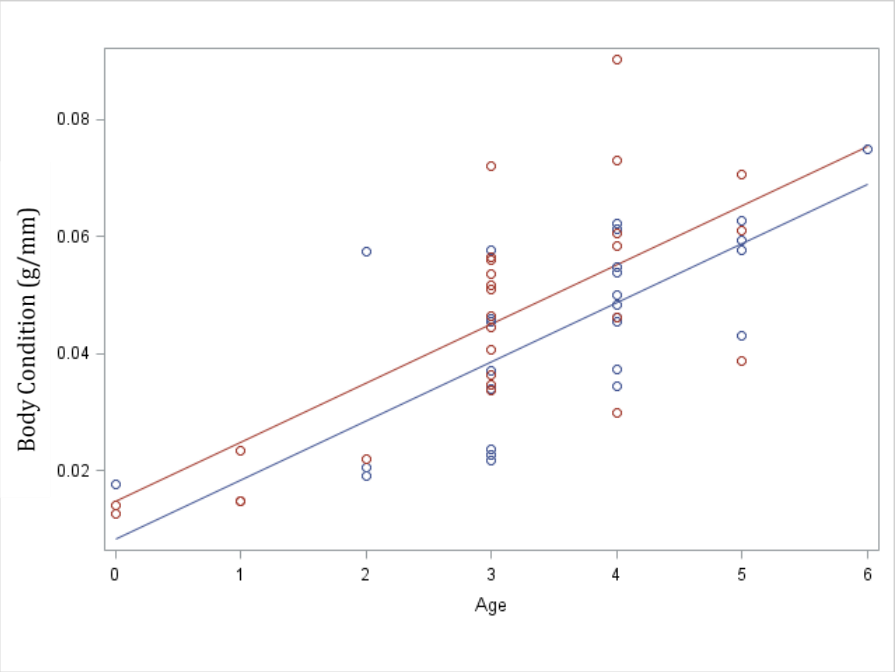


Figure 9: Body condition ANCOVA for treatment stream. Includes downstream site one (DS1; red), which is located 50 m below the lime application, and the treatment reference (blue), which is located 100 m upstream of the lime application.

I detected a significant treatment effect on body size. Specifically, body size differed between the treatment reference and DS1 (ANCOVA; $F_{1,51} = 5.75$; $p = 0.02$; Figure 10). Larvae from DS1 in treatment streams reached larger body sizes (SVL) at younger ages than individuals that were located above the DAL site in the treatment reference. In the ANCOVA analysis for UPC and DSC1 in the control streams, I failed to detect a difference in body size ($F_{1,48} = 0.03$;

p = 0.86; Figure 11). In the treatment streams, larval body size from DS2 did not significantly differ from larvae in the treatment reference**,** however a similar trend to the DS1 site was present with larvae from DS2 reaching larger body sizes at younger ages than individuals from the treatment reference (ANCOVA; $F_{1,50} = 2.74$; p = 0.10; Figure 12). In the ANCOVA analysis for UPC and DSC2 in the control streams, I failed to detect a difference in larval body sizes ($F_{1,48} = 0.42$; p = 0.52; Figure 13)**.**

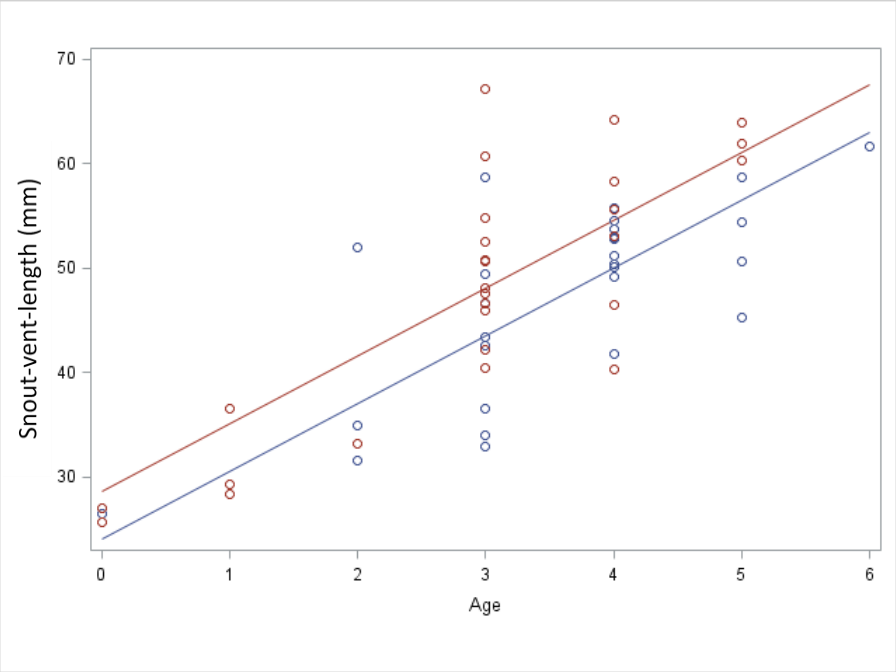


Figure 10: Treatment stream ANCOVA for snout-vent-length. Includes downstream site one (DS1; red), which is located 50 m below the lime application, and the treatment reference (blue), which is located 100 m upstream of the lime application.

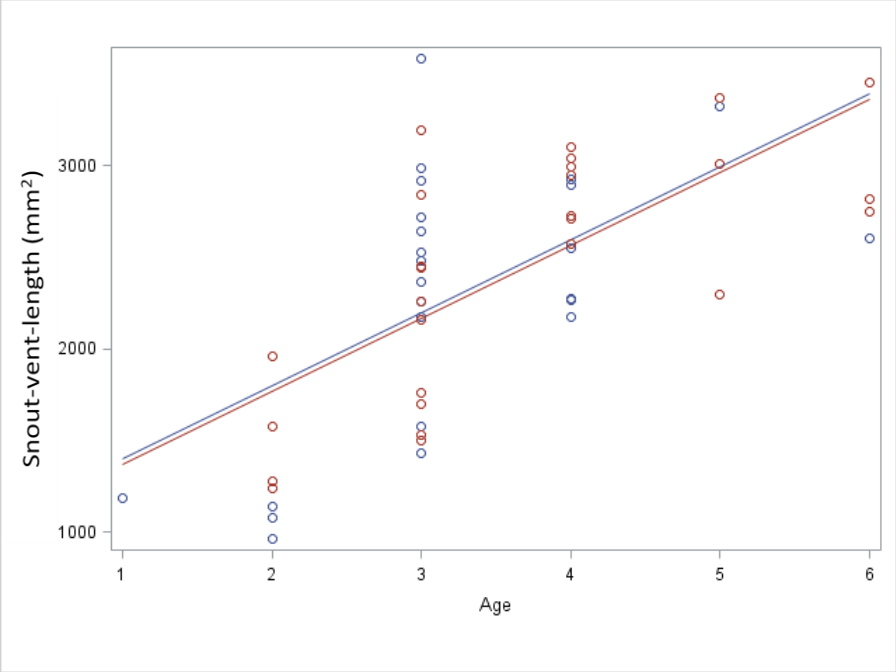


Figure 11: Control stream ANCOVA for snout-vent-length squared. Includes downstream control site one (DSC1; red), which is located 50 m below the reference point, and the upstream control site (blue), which is located 100 m upstream of the reference point.

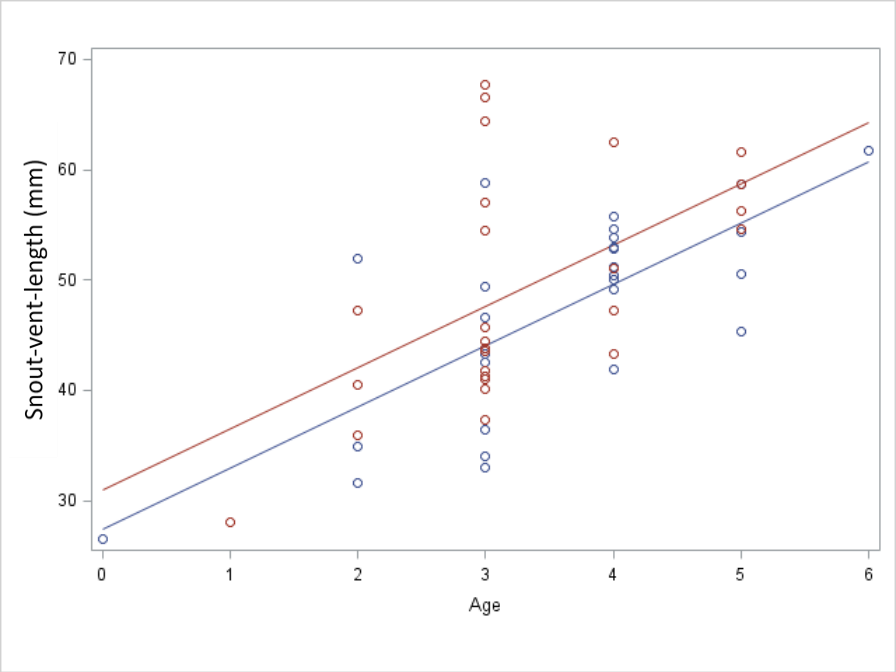


Figure 12: Treatment stream ANCOVA for snout-vent-length. Includes downstream site two (DS2; red), which is located 500 m below the lime application, and the treatment reference (blue), which is located 100 m upstream of the lime application.

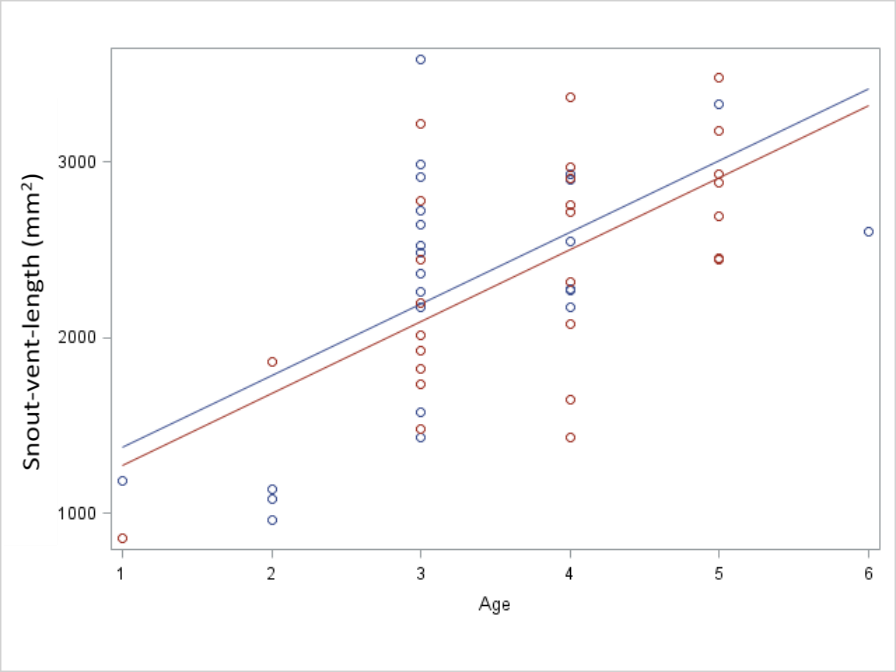


Figure 13: Control stream ANCOVA for snout-vent-length squared. Includes downstream control site two (DSC2; red), which is located 500 m below the reference point, and the upstream control site (blue), which is located 100 m upstream of the reference point.

I detected a significant treatment effect on gape size. Specifically, gape size differed between the treatment reference and DS1 (ANCOVA; $F_{1, 51} = 9.48$; $p = 0.003$; Figure 14) and between the treatment reference and DS2 (ANCOVA; $F_{1, 50} = 5.01$; $p = 0.03$; Figure 15). Larvae from the treatment reference had larger gape size at shorter SVLs than individuals from DS1 and DS2. In the ANCOVA analysis for UPC and DSC1 ($F_{1,47} = 2.57$; $p = 0.12$; Figure 16) and for UPC and DSC2 ($F_{1,48} = 1.85$; $p = 0.18$; Figure 17) in the control streams, I failed to detect a difference in gape size.

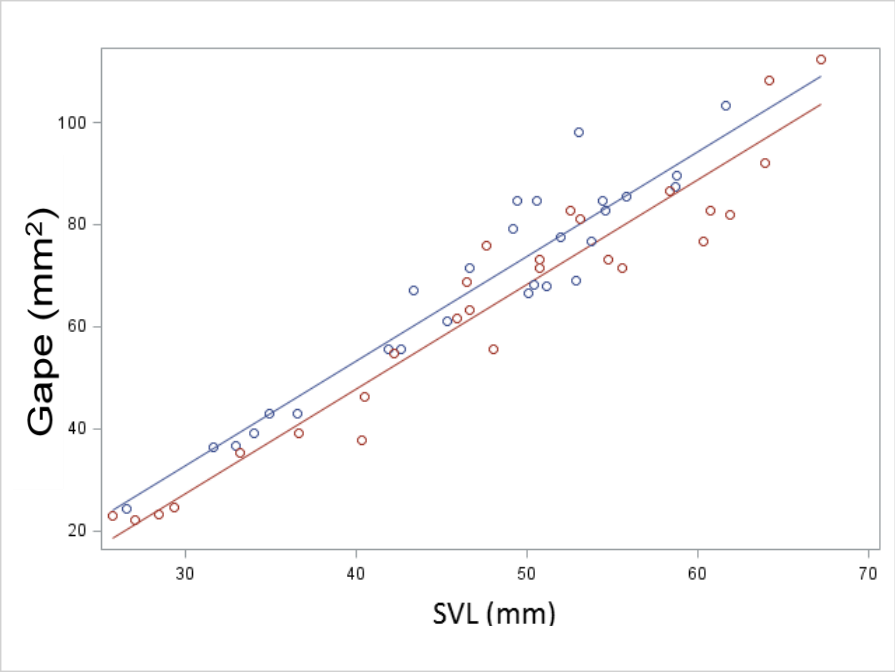


Figure 14: Treatment stream ANCOVA for gape size squared. Includes treatment reference (blue), which is located 100 m upstream of DAL, and the first downstream treatment site (DS1; red), which is located 50 m downstream of DAL.

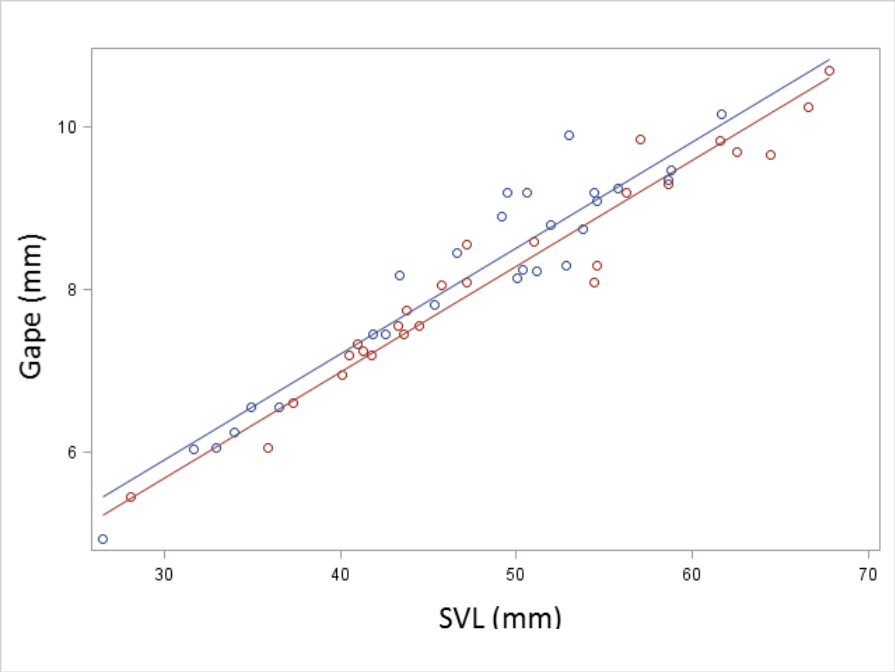


Figure 15: Treatment stream ANCOVA for gape size. Includes treatment reference (blue), which is located 100 m upstream of DAL, and the second downstream treatment site (DS2; red), which is located 500 m downstream of DAL.

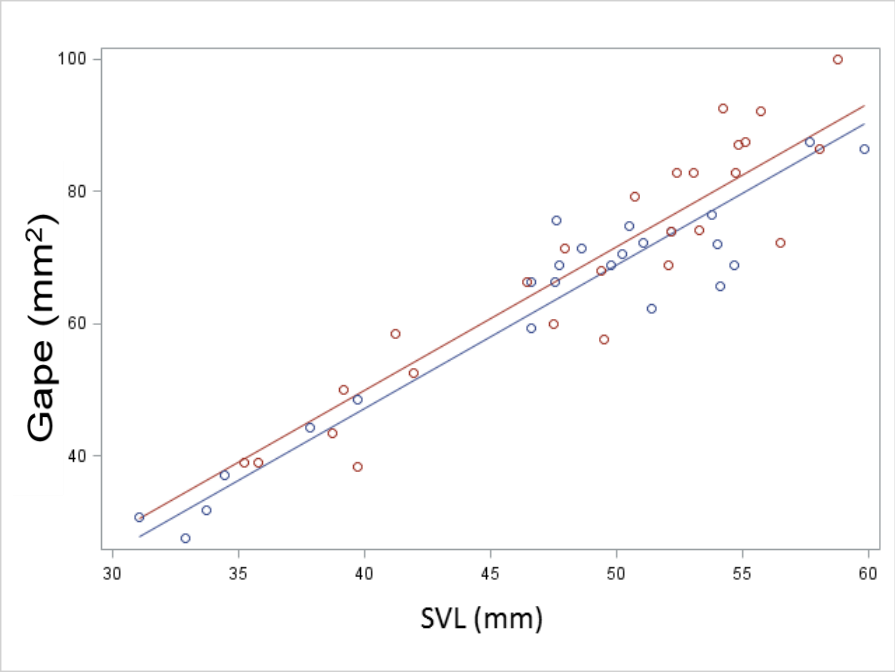


Figure 16: Control stream ANCOVA for gape size. Includes upstream control (UPC; blue), which is located 100 m upstream of the reference point, and the first downstream control site (DSC1; red), which is located 50 m downstream of the reference point.

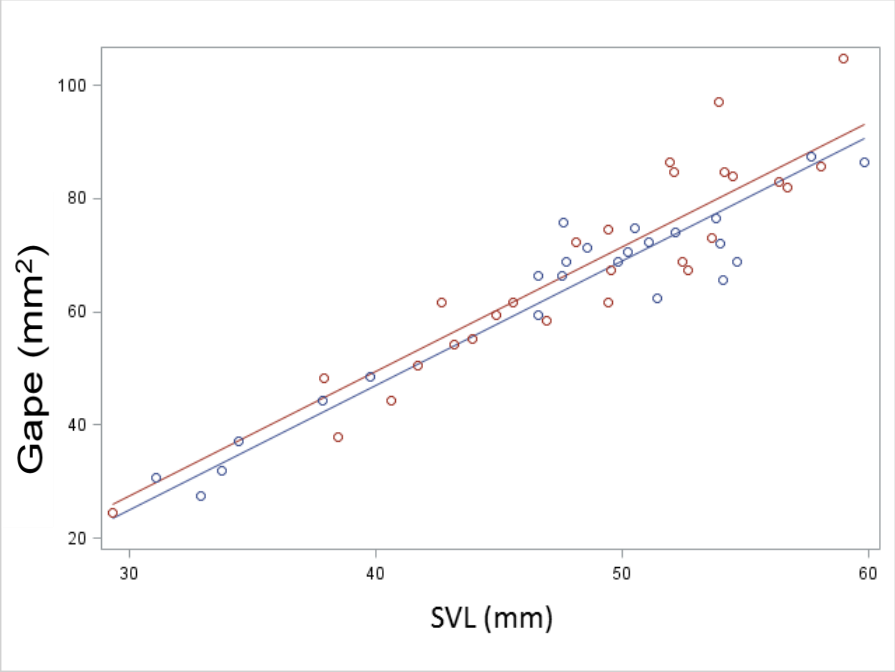


Figure 17: Control stream ANCOVA for gape size. Includes upstream control (UPC; blue), which is located 100 m upstream of the reference point, and the second downstream control site (DSC2; red), which is located 500 m downstream of the reference point.

DISCUSSION

The effects of DAL are complex and may have positive and negative effects on aquatic biota. In this study, I failed to detect an effect of DAL on larval *G. porphyriticus* age structure and body condition. However, liming significantly increased body size and decreased gape size in larval *G. porphyriticus* at the DS1 site, indicating population-level effects that potentially reflect life history shifts (Stearns 1992). Optimal life history strategies should maximize survival and fitness. Larvae from DS1 reached larger body sizes at younger ages compared to the treatment reference larvae; therefore larval growth rates were increased downstream of DAL sites. I failed to detect significant effects in control streams, suggesting that changes in body size were not the result of longitudinal differences in the stream.

Fish, which have been shown to decrease growth rates in *G. porphyriticus* through interference competition (Resetarits 1991, 1995; Lowe et al. 2004), were encountered in both treatment and control streams, and thus it is unlikely that observed differences in growth reflected fish presence below DAL sites, especially because the effect was not present at the DS2 site. Furthermore, faster growth rates, suggest that effects were not result of fish presence because predators typically decrease growth rates in *G. porphyriticus* (Resetarits 1991, 1995; Sih et al. 1988; Lowe et al. 2004; Currens et al. 2007). However, there is the potential for increased interactions with predators directly downstream of DAL due to decreased habitat availability from sedimentation (Sih & Kats 1991). Increased interactions with gape limited predators could select for increased growth rates until a size refugia is met, which is when larvae become less susceptible to predation (Wilbur 1980; Abrams & Rowe 1996; Chase 1999; Urban 2008). The effect of liming on body size decreased at sites located farther downstream, likely because the habitat is less physically altered by sedimentation due to dissolution of the lime, providing greater habitat availability than sites located closer to lime application. The failure to detect a

significant treatment effect on growth rates at DS2 supports that increased nutrient availability from improved water quality was not the selective factor and that reduced refugia from sedimentation directly below the DAL may increase predation risk. Previous research has demonstrated that amphibians with varying phenotypes and growth rates exhibited tradeoffs that did not affect survival or reproduction (Clobert et al 2000). In this study, *G. porphyriticus* larvae exhibited plastic growth rates, which in theory, should be a mechanism to maximize overall fitness in the given environment and may or may not have negative tradeoffs (Hereford 2009).

Metamorphic size in *G. porphyriticus* occurs within a narrow range, with little plasticity in this trait (Wilbur & Collins 1973; Bruce 1980), suggesting that metamorphosis is size dependent. Rapid growth in *G. porphyriticus* larvae directly below the DAL site may indicate a life history tradeoff for earlier metamorphosis, because larvae are reaching minimum metamorphic size quickly. By quickly morphing into adults, which are less reliant on aquatic habitats than larvae, individuals can potentially become less susceptible to aquatic stressors (Wilbur & Collins 1973; Rohr et al. 2004). For many amphibians, the timing of metamorphosis can vary by both age and size depending on a number of factors (Wilbur 1980; Arendt 1997; Bruce 2005). Some of the factors affecting metamorphic timing are temperature, resources, predator density, and habitat stability, such as water permanence or the absence of perturbations (Bruce 2005). The optimal life history strategy for an individual should therefore maximize growth, survival, and reproduction for a specific environment within the species range of plasticity (Arendt 1997; Bruce 2005).

Disturbed habitats, such as DAL sites, can induce rapid growth rates and can reduce time spent in stressful conditions (Wilbur & Collins 1973; Arendt 1997; Hereford 2009). Because growth did not differ across sites in control streams, it is likely that favorable stream conditions

contributed to delayed metamorphosis, increasing overall time that growth occurred prior to reproductive age (Bruce 1980; Arendt 1997; Rowe & Ludwig 2001). However, acidic conditions, like those in control stream and the treatment reference, can reduce amphibian growth rates, indicating that it too may act as a stressor (Freda & Dunson 1985; Pierce 1985; Skei & Dolmen 2006).

Gape size was also impacted by lime treatment. Larvae below DAL sites, from both DS1 and DS2, had smaller gape sizes relative to SVL than larvae in the treatment reference. This effect was not seen in control streams. *Gyrinophilus porphyriticus*, like other salamanders, are gape-limited predators, which affects the type of prey they can ingest (Zaret 1980). Larvae with narrower gape sizes have a smaller range of available prey items (Maret & Collins 1996). Because gape size is positively correlated with body size, large larvae have the largest range for prey selection. However, according to my results larvae downstream of DAL had smaller gape sizes at relative sizes and were therefore more limited in their prey selection. The differences in gape size could indicate a change in prey composition below DAL, although this was not measured.

I failed to detect a significant effect of DAL on age structure or body condition. Although there was not a significant treatment effect on age structure, the predominant age class for larvae downstream of the DAL site was three years compared to larvae in the treatment reference, which were predominantly four years old. Below DAL sites, a majority of individuals could be completing metamorphosis at three years, versus four years upstream of the DAL site. I failed to locate six year larvae below DAL sites and average larval age was lowest at the DS1 site (Table 2; Figure 6). The failure to detect the oldest age class below DAL sites does not equate to absence; however, if larvae downstream of DAL completed metamorphosis at younger

ages, I would expect to find fewer larvae in the older age classes. Although not significant, larval *G. porphyriticus* populations tended to have higher body conditions at the DS1 site, but this effect was not present at the DS2 site, suggesting that the effect of liming on body condition decreased.

Because there was no difference in body condition in control stream sites, I assumed that any effects detected below DAL sites were not the result of longitudinal stream differences. Higher body conditions are positively correlated with growth rates (Lowe et al. 2006) and generally indicate higher energy reserves and habitat quality (Pope & Matthews 2002; Schulte-Hostedde et al. 2005); however, higher body condition may be confounded with the increased growth rates of larvae from the same site. Higher body conditions could also be the result of increased prey densities (Beachy 1994, 1995) or decreased larval densities below DAL sites (Wilber & Collins 1973; Edwards 2014), limiting intraspecific competition. Although increased body condition generally indicates increased prey availability (Beachy 1994; Pope & Matthews 2002), sites located downstream of the DAL appeared to have fewer macroinvertebrates and previous research has shown that liming generally decreases the total biomass of macroinvertebrates (Okland & Okland 1986; Keener & Sharpe 2005). Because increased growth rates and body condition require higher energy reserves, I assumed that gape size would also increase to facilitate a greater range of prey capture. By capturing large, high-energy prey, less time would be required for foraging (Walls et al. 1993; Forsman 1996). However, my results show that larvae with increased growth rate and body conditions have smaller relative gape sizes, which would greatly increase the time needed for foraging. By increasing foraging time, activity levels, and therefore mortality rates due to predation risks, are also increased; however, mortality was not measured (Stearns 1992; Werner and Anholt 1993; Arendt 1997; Roff 2000). Previous

research on the effects of liming on *G. porphyriticus* abundance, although not significant, indicated that distance from lime and liming frequency were the most important covariates for abundance estimates, with lower abundance in sites closer to DAL sites and in sites with greater liming frequencies (Edwards 2014). The trend for lower abundance downstream of DAL suggests lower densities that could have resulted from increased mortality through predation. Decreased abundance would lower intraspecific competition and increase resource availability.

Although liming significantly increased the pH as intended, I observed other differences in stream characteristics below the DAL site, such as increased sedimentation (Figure 18), compared to control streams (Figure 19) and the presence of a grayish precipitate on the stream substrate (Figure 20). The grayish substance observed was likely a precipitate of dissolved aluminum, which occurs when pH levels increase and has been found below lime application in other streams (LeFevre & Sharpe 2002). Aluminum precipitate decreases macroinvertebrate abundance, with the greatest impact directly below lime in the mixing zone (Okland & Okland 1986; Gensemer & Playle 1999; LeFevre & Sharpe 2002; Simmons et al 2006), which would further explain why macroinvertebrate abundance appeared lower downstream of the DAL; however, this was not quantified.

Figure 18: Direct application lime site in Dogway Fork.

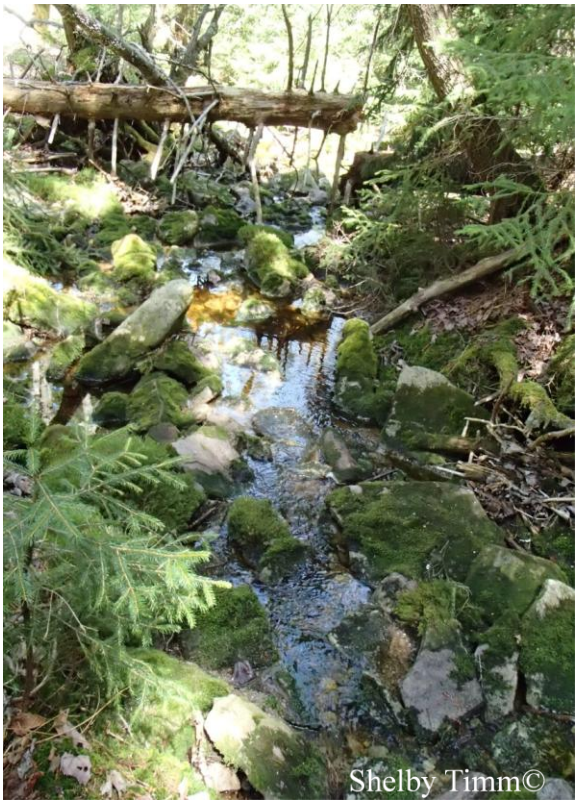


Figure 19: Control stream site with numerous moss covered rocks providing good habitat for larvae.

Figure 20: Photos from DS1 site on Bear Run showing grayish precipitate.

My results may indicate that strong selection, where slower growing individuals are removed from the population, results in lower abundances and reduced intraspecific competition, which possibly explains both faster growth and higher body condition downstream of DAL. Plasticity is a trait that allows many species, such as *G. porphyriticus*, to occupy a range of environmental conditions. The adaptive nature of the plastic trait is evidenced by the reaction norm, which is often predictable across populations and characteristic of specific populations (Stearns 1989; DeWitt et al. 1998). My research demonstrates that *G. porphyriticus* exhibits population-level responses to DAL mitigation, which may cause life history shifts in larvae. Further research is needed to identify specific mechanisms to understand if my observations are the direct effects of liming, such as increased sedimentation and changes in water chemistry, or caused by indirect effects, such as alterations in trophic structure, and how these changes contribute to emergent effects. It is important to further investigate the tradeoffs that are occurring within individuals with increased growth rates and smaller gape sizes, as well as differences in diet using gut content analysis to determine if trophic structure has been altered.

CHAPTER 2

GYRINOPHILUS PORPHYRITICUS LIFE HISTORY NOTE

Gyrinophilus porphyriticus is a stream associated species and its distribution ranges from Southern Quebec to Alabama, throughout the Appalachian Mountains (Petranka 1998). Their distribution covers a large range of latitudes, with southern populations experiencing different climates than northern populations, contributing to different life history strategies. Previous research on *G. porphyriticus* populations in the southern Blue Ridge Mountains reported that there was no correlation between SVL and age, and suggested that skeletochronology may be an unreliable technique for aging these populations, which may remain active throughout the year, due to indistinguishable lines of arrested growth (n=17; Bruce & Castanet 2006).

I collected *G. porphyriticus* larvae between April 25, 2014 to August 23, 2014 from multiple populations in the Gauley River watershed in Pocahontas and Greenbrier counties, WV, in the southern portion of the Monongahela National Forest. I used skeletochronological analysis of femurs for larval age determination. Age can be determined in amphibians by counting the number of lines of arrested growth (LAGs) in the cross-sections of the diaphysis of long bones or phalanges (Castanet et al. 1993). Lines of arrested growth are deposited annually in temperate regions during winter when growth slows and individuals are less active. My research shows that there is a significant positive correlation between SVL (mm) and age (years) in *G. porphyriticus* (Figure 1; $p < 0.0001$, $R = 0.669$). However, there is a substantial amount of overlap in SVL between age classes (Table 1), supporting that size classes should not be used to estimate age. My research indicated that skeletochronology is a reliable method *G. porphyriticus* in West Virginian populations that experienced colder winters and therefore experienced a period of reduced growth that produced reliable and well defined LAGs.

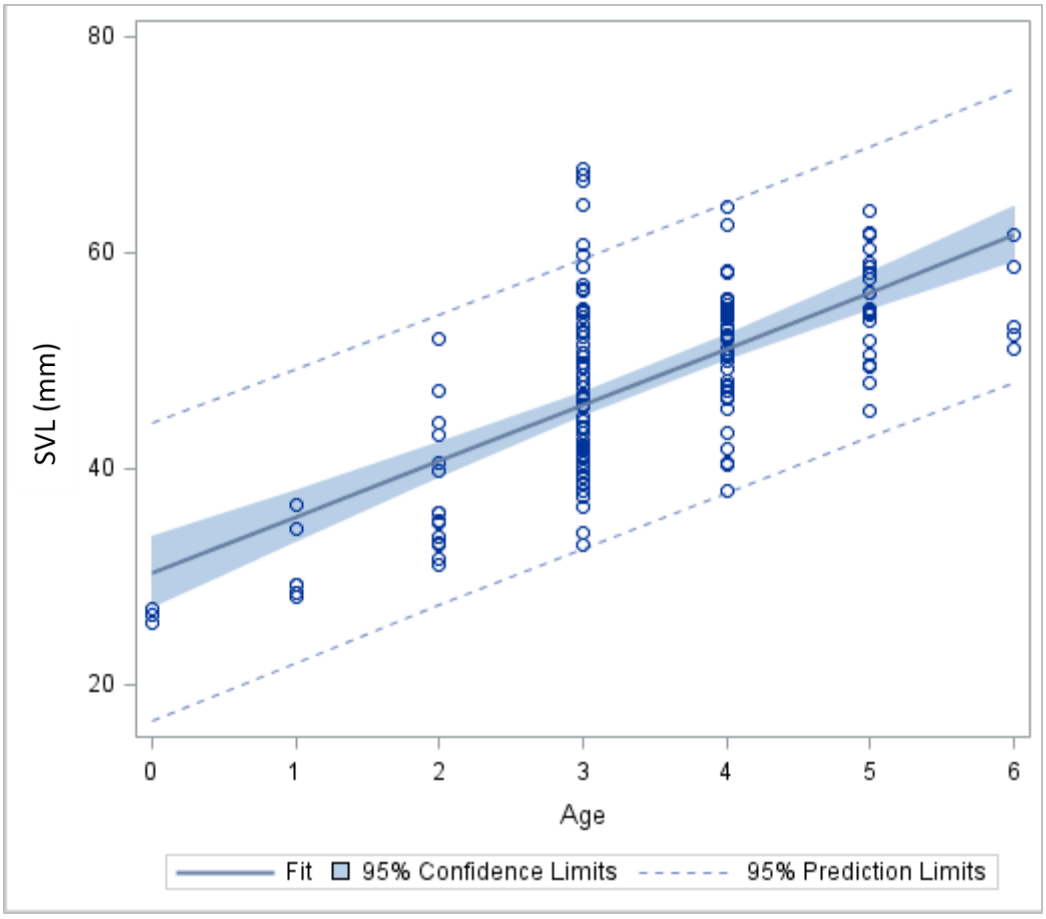


Figure 1: Linear correlation for SVL with age, including 95% confidence intervals.

Table 1: Descriptive statistics for larval *G. porphyriticus* populations in the Monongahela National Forest. The range and mean for SVL ± standard deviation was calculated for each age. The percentage of each age group from the entire sample (n = 158) is reported.

	AGE						
Indicators	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Range (SVL, mm)	25.70-27.00	28.10-36.60	31.05-51.95	32.95-67.75	37.90-64.20	45.35-63.90	51.05-61.67
Mean (SVL, mm)	26.40±0.66	31.03±3.58	38.08±6.19	47.92±7.90	51.26±5.52	55.23±4.90	55.38±4.58
% of Sample	1.91%	3.82%	9.55%	41.40%	26.75%	13.38%	3.18%

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APPENDIX A

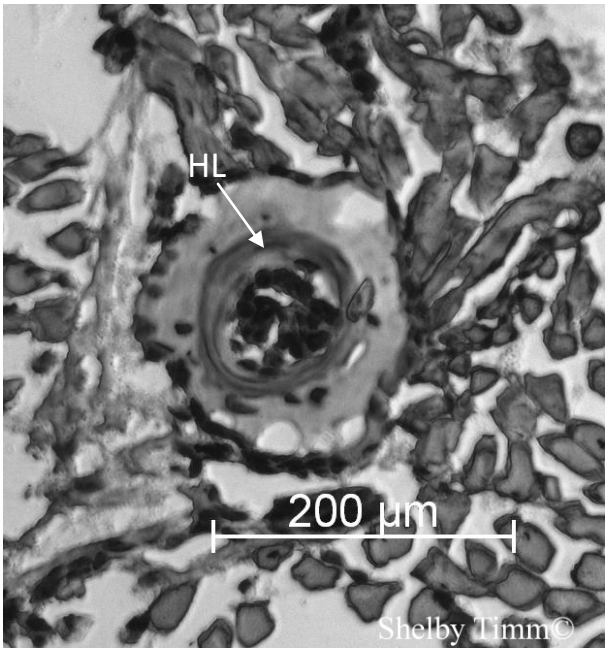

Figure 1: Cross section. No LAGs are present, but the hatching line (HL) is clearly seen. This larva is a young of the year (0 years).

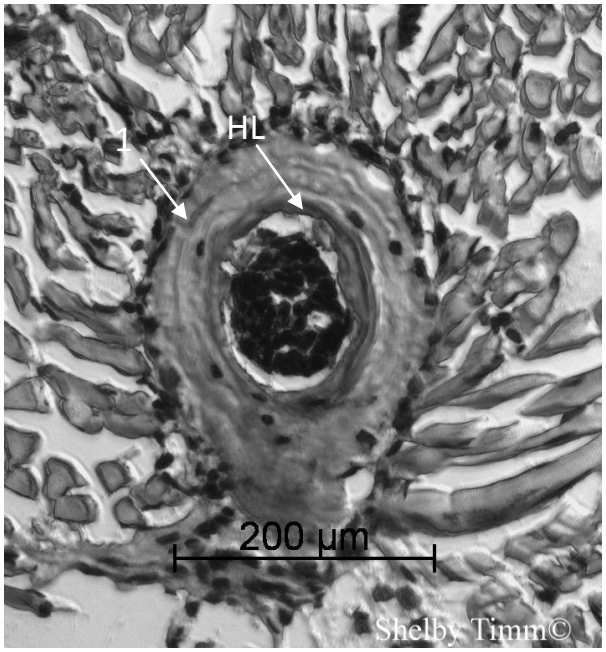

Figure 2: Cross section. One LAG is present, with the HL clearly seen. This individual was collected at the end of the sampling season; therefore, there is growth behind the LAG, and the peripheral edge is not considered a LAG. This larva is one year.

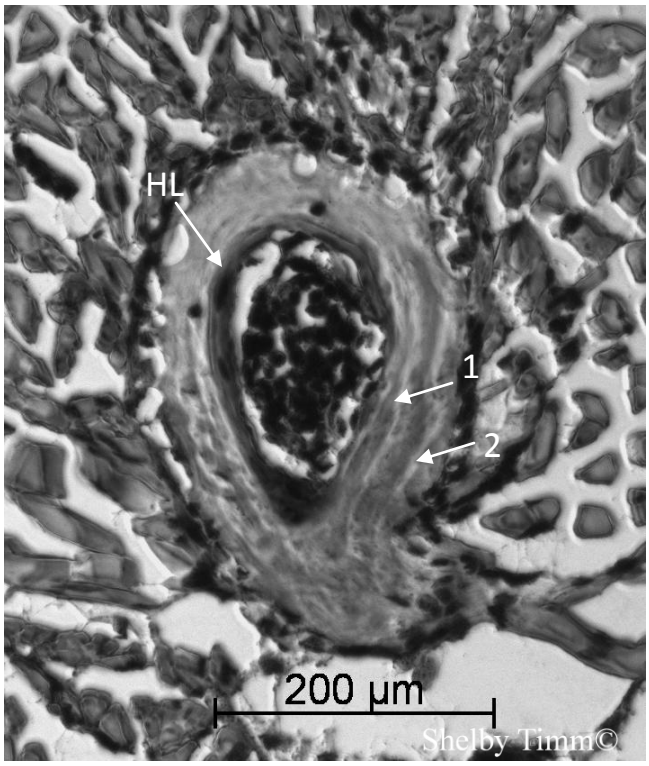

Figure 3: Cross section. Two LAGs are present, with the HL clearly seen. This individual was collected at the end of the sampling season; therefore, there is growth behind the LAG, and the peripheral edge is not considered a LAG. This larva is two years.

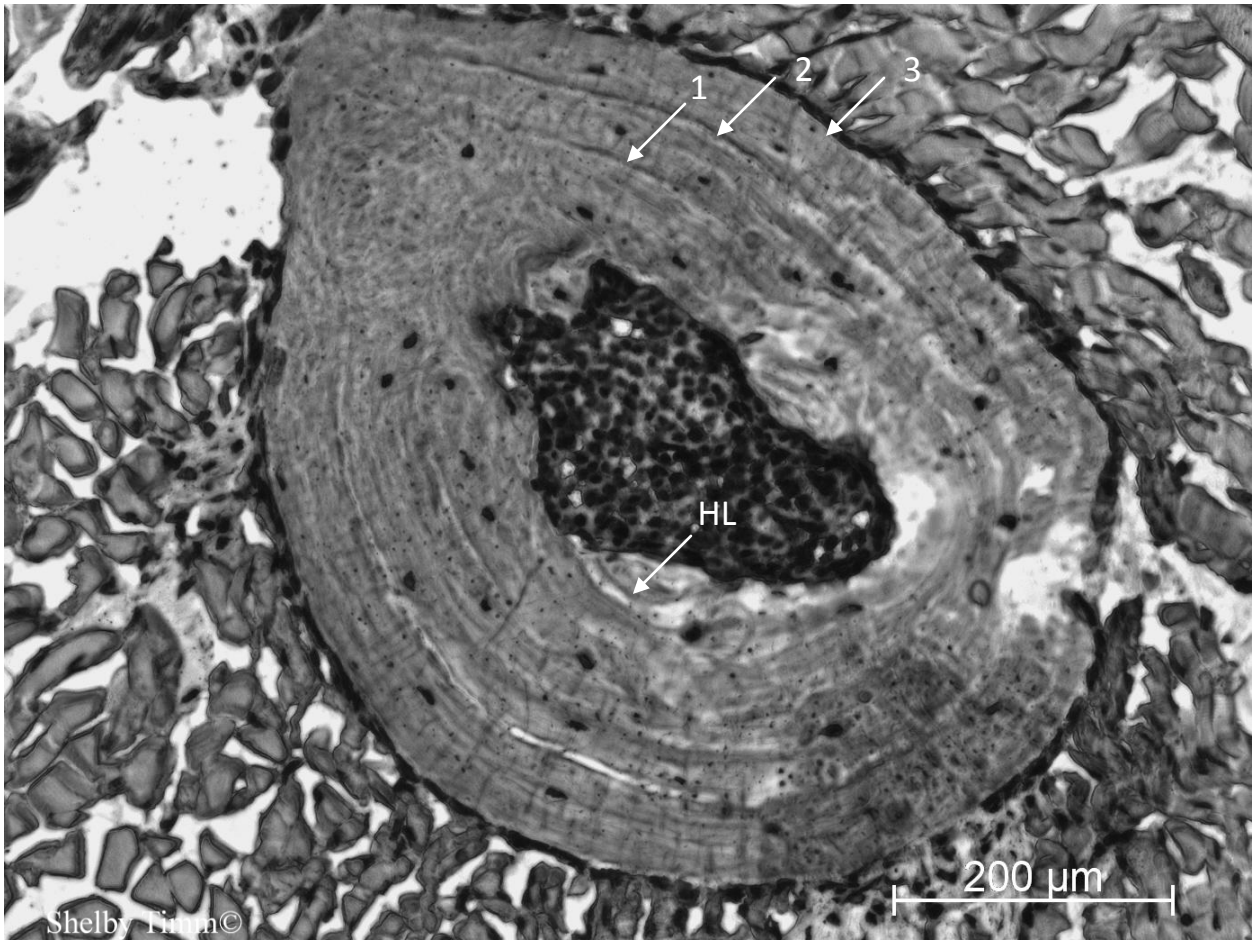

Figure 4: Cross section. Three LAGs are present, with the HL clearly seen. This individual was collected at the beginning of the sampling season; therefore, there is no growth behind the most recent LAG so the peripheral edge of the bone is considered a LAG. This larva is three years.

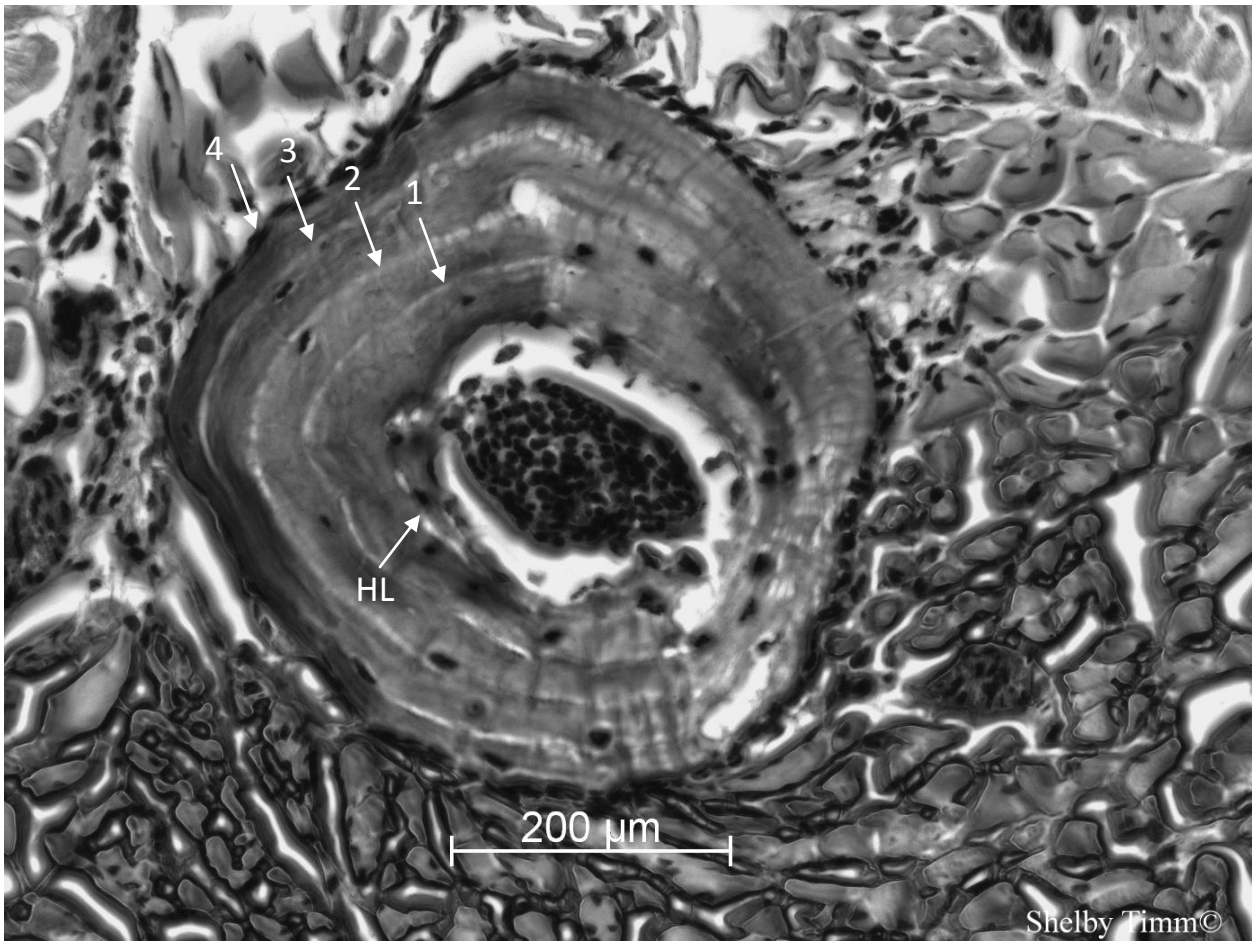

Figure 5: Cross section. Four LAGs are present, with the HL clearly seen. This individual was collected at the beginning of the sampling season; therefore, there is no growth behind the most recent LAG so the peripheral edge of the bone is considered a LAG. This larva is four years.

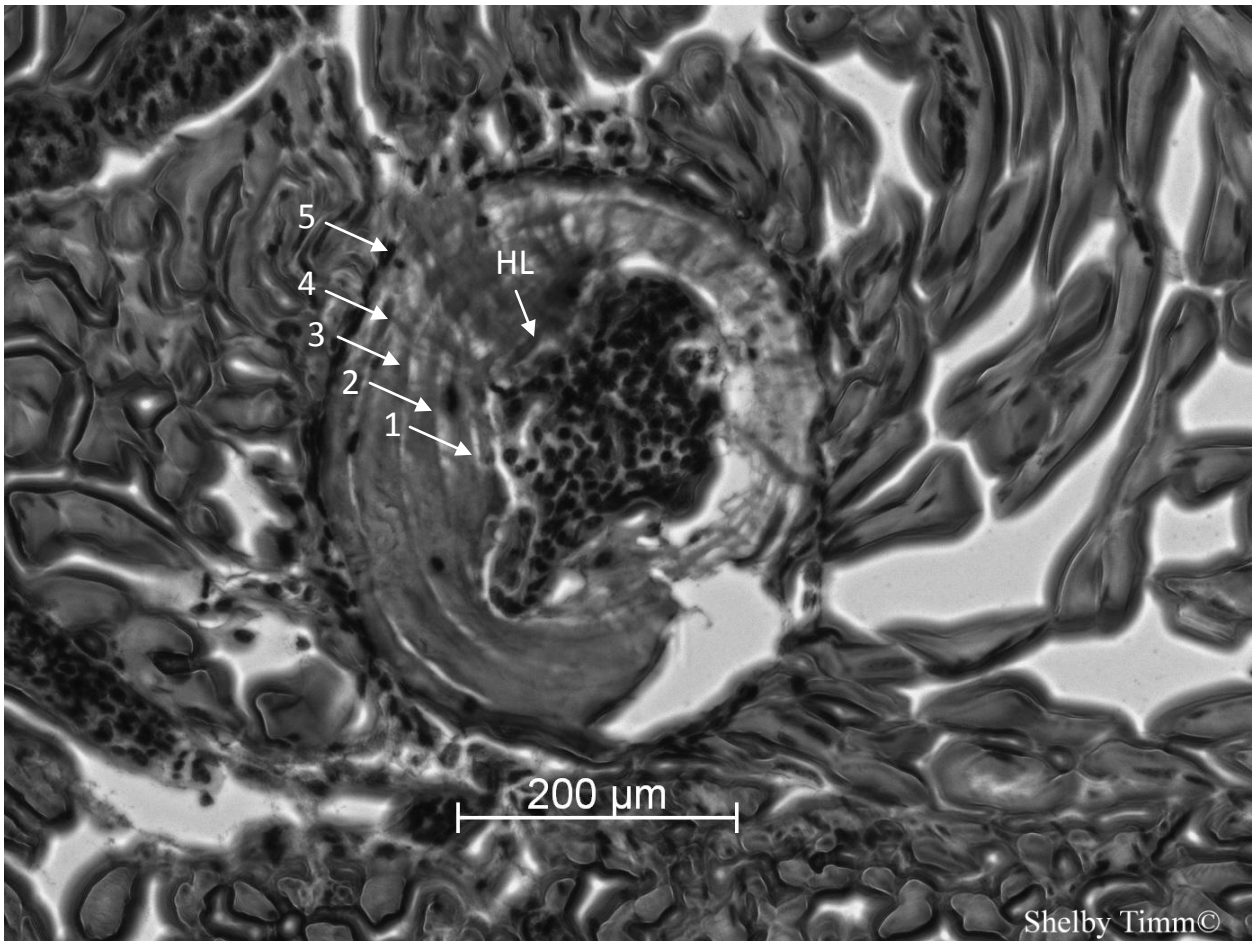

Figure 6: Cross section. Five LAGs are present, with the HL clearly seen. This individual was collected at the beginning of the sampling season; therefore, there is no growth behind the most recent LAG so the peripheral edge of the bone is considered a LAG. This larva is five years.

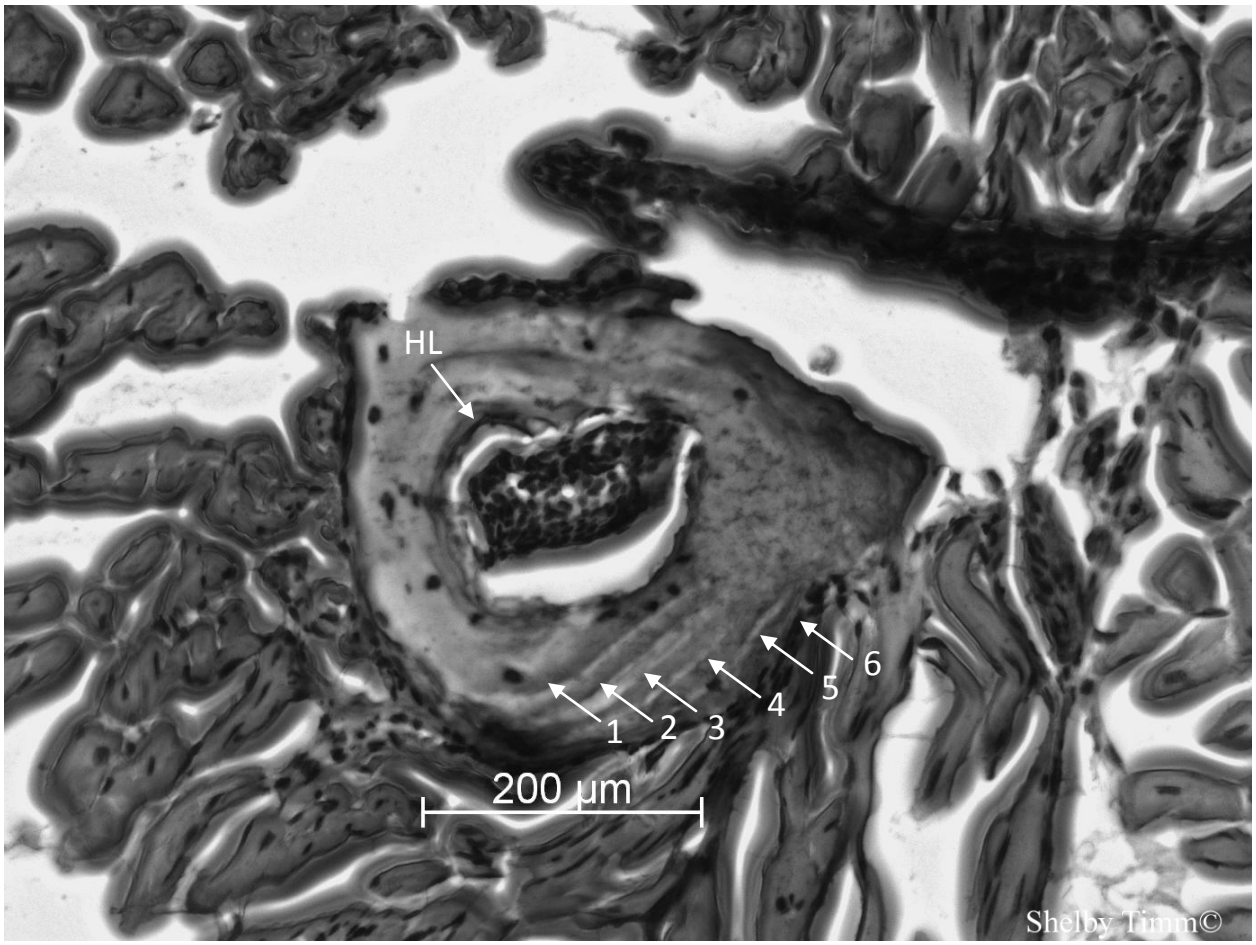

Figure 7: Cross section. Six LAGs are present, with the HL clearly seen. This individual was collected at the beginning of the sampling season; therefore, there is no growth behind the most recent LAG so the peripheral edge of the bone is considered a LAG. This larva is six years.

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DATE:	April 1, 2014
TO:	Jayme Waldron, PhD
FROM:	Marshall University IACUC
IACUC #:	572
PROJECT TITLE:	[573462-2] Life History Shifts and Trophic Perturbations as Emergent Effects of Large-scale Mitigation
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE:	April 1, 2014
EXPIRATION DATE:	October 1, 2014
REVIEW TYPE:	Full Committee followed by Designated Member Review of Revision

Thank you for your submission of Revision materials for this research project. The Marshall University IACUC has APPROVED your submission. All research must be conducted in accordance with this approved submission.

This submission has received Full Committee Review followed by Designated Member Review of the revision.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure.

Please report all NON-COMPLIANCE issues regarding this project to this committee.

This project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Monica Valentovic at (304) 696-7332 or valentov@marshall.edu. Please include your project title and reference number in all correspondence with this committee.

Monica A. Valentovic, Ph.D.
Chairperson, IACUC