CURIOUS CASE OF THE COASTAL TAILED FROG (*ASCAPHUS TRUEI*) NEAR THE NORTHERN EXTENT OF ITS RANGE

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DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
NATURAL RESOURCES AND ENVIRONMENTAL STUDIES

UNIVERSITY OF NORTHERN BRITISH COLUMBIA

June 2020

ABSTRACT

Ascaphus truei, an ancient species of frog, migrated into British Columbia, Canada, following the last ice age. *A. truei* is of conservation interest because forestry practices, and the associated infrastructure, may reduce habitat quality. There is also concern that a warmer and drier climate will change the distribution and abundance of this species. I used two genetic methods, microsatellites and next-generation sequencing, to compare the genetic diversity of *A. truei* from the northern extent of its range in British Columbia, CA to southern Washington, USA. Both methods suggested a dramatic reduction in diversity across the northern portion of the species' range. The phylogeography suggests a northern range expansion from a single refugium. I used DNA metabarcoding to compare the gut contents of larvae across three stream reaches and two development classes near the northern extent of *A. truei*'s range. Gut contents differed between stream reach but did not differ among development class. Wetted width and ultimately stream volume may influence differences in gut content among stream reaches. I also quantified the relationship between an index of larvae density and environmental factors hypothesized to influence population density near the northern extent of the range. I assessed the segregation of larvae at various developmental stages. Wetted width and wetted depth correlated with differences in the abundance of larvae. Older developmental stages were captured in stream reaches with greater slopes than younger stages. Management should minimize modifications of stream structure, such as in-stream siltation due to road building. We should maintain habitat connectivity and gene flow to ensure the continued migration and adaptive capacity of *A. truei*.

"The mountains are very precipitous, with numerous small, dashing streams, snow-fed and spring-fed, seldom more than a foot or so wide and a few inches deep. Collecting in these creeks was an arduous task; they were very swift, with many falls and miniature rapids, filled with rocks, with great tangles of devil's club and fallen trees along their banks, and the water was extraordinarily cold, usually under 40° even on the warmest days.

It was under the rocks in these little creeks that *Ascaphus* lived."

~ Helen Thompson Gaige, 1920

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ACKNOWLEDGEMENTS

Thank you to the women who have researched *A. truei* before me, and to all who fight to understand species that exist in-between the lines. You are the light guiding me along my path.

Merci to the balancing forces that are Dr. Chris Johnson and Dr. Brent Murray. Chris, you challenged me. I didn't always understand or appreciate the challenge, learning more due to my confusion. I am a better writer because of you and will forever be grateful. Brent, I adored our unexpected exploratory conversations. It was during one of these I recognized I was truly capable of being a PhD. I will miss our exchanges. Merci to both of you for all you did for me, seen and unseen.

Danke to Dr. Mark Shrimpton, Dr. Philippe Henry, and Melissa Todd for being on my committee. I was beyond fortunate to have learned from, and worked with, researchers in different fields. Special thanks to Mark for making space for me in your office. Thank you to Dr. Andrew Storfer for evaluating this dissertation as an external examiner. Your insights helped evolve the work and the writer.

Thank you to everyone who worked under the umbrella of the Biodiversity Monitoring and Assessment Program and thank you to the coordinators, especially Dr. Michael Gillingham and Dr. Jeanne Robert, for providing funding to me.

Gracias to friends, in academia and out, who helped keep the creeping darkness from taking over. Aynsley and Kristin, you have such big hearts. Nicole, without knowing it, you are the silver-lining that everyone needs in their lives. Aija and Tammy, thank you for the purple-stained teeth and the laughter. Angel, your community saved me. Riley, I always want to go herping with you. Luke, I have learned so many new things from listening to you. Leland, I met you during this process. You were my fun friend in the beginning and are my partner in life at the finish. I'm so grateful. Jeannine, how do I even begin to thank you? We will find a way to change the world and be confident. Gracias for exploring with me.

Thank you to my family. Christine, you have been so supportive. I'm grateful that our relationship grew stronger even as the physical distance between us increased. Dad, you have always pushed my intellectual pursuits. Thank you for giving me my sense of exploration.

And thank you to my Bjorn. You were with me before this was a thought in my mind. I lost you before it was over. Thanks for taking this eccentric journey with me.

PROLOGUE

The Setting

A long stretch of fog and rain-drenched mountains follows the north Pacific coast of North America. Situated between the Pacific Ocean and Interior Plateau, the coast and Cascade mountains of the Pacific Northwest are dominated by large coniferous trees and mosses, fungi, and ferns. The forest floors filled with decaying humus and tree snags. These forests grow on mountainsides and fill valleys gouged from moving ice. Freshwater streams originating from ice, snow, and rain carve their way down the mountainsides. A geography created, in large part, by ice-age glaciers then whittled by flowing water (Heusser, 1960).

The fauna of this region was also influenced by the last ice age. Glaciers and icefields of the Pleistocene began to recede in the Coast Mountains of British Columbia (B.C.) around 11,000 years ago. Since then, the current distribution of species was established. Species migrated into the area from one or more refugia along the coast, south of the ice coverage, or north of the ice.

Around 20 species of amphibian occur in the coastal and Cascade Mountains of Washington, USA, and B.C., Canada (Nussbaum et al., 1983). Roughly half have ranges that extend north into the Coast Mountains of B.C. These amphibians have migrated into the region, and have unique adaptations for life in northern climes. *Ascaphus truei* (coastal tailed frog) is one such species. Specially tailored for life in this region.

The Frog

A tadpole is enveloped by streaming water during fall, winter, spring, and summer. She remains in her natal stream's riffle, cascade, and pool for as many as three more seasonal

cycles. Streamlined for swimming in fast-flowing water while also adapted to hang from river stone. Forelimbs slowly develop in a special chamber of her body cavity (Brown, 1990), eventually visible through a thin layer of tissue. Appearing as a perpetual self-hug. Slowly, hindlimbs develop hinges and toes while they remain streamline with the body to protect them from damage.

Relative to the previous slow development, metamorphosis happens rapidly. She emerges from the stream to an environment of air. Intimately connected to the cool freshwater of her Pacific Northwest habitat. She likely stays close to her natal stream as a juvenile and adult frog, feeding on small arthropods. Coarse woody debris protects, feeds, and keeps her cool and damp. Although, it is difficult for us to understand the life and movement of *A. truei* throughout her complex and often subterranean terrestrial environments. We do not know the amount of time before she reaches adulthood and sexual maturity (possibly two years); every indication suggests it is a slow development for a frog.

She has no tail as an adult, unaware of the common name given to her species (the coastal tailed frog). The males have a cloacal extension ('tail') for internal fertilization, because external fertilization is not likely to happen in fast-flowing streams. They do not vocalize to attract a mate, as the species is voiceless and has limited hearing. Communication through sound being difficult when copulexus happens while males and females are crawling along the bottom of a stream (side note: tailed frogs do not frog kick, an interesting thing for a frog not to do). She is unaware of the creativity humans have used to describe intromission in her species (Noble and Putnam, 1931; Metter, 1964; Bull and Carter, 1996; Stephenson and Verrell, 2003).

The summer after intromission, she deposits her paired-strands of fertilized eggs (the largest of the North American frogs) on the underside of an instream stone. Yet again, development is slow. Her offspring emerge as tadpoles and the life cycle of *A. truei* continues for another generation as it undoubtedly has since near the Jurassic period (Green and Cannatella, 1993).

The Studies

A. truei is an ancient species in the comparatively young habitat of the Coast Mountains in B.C. Their current range extends north to Nisga'a Lands (Figure 1.1). How close their range gets to Lisims (the 'Nass River') is unknown, as access to streams within this area is limited due to the remoteness of the region. Even so, we know *A. truei* has established populations near the northern extent of their range since the glaciers receded after the last ice age.

Ritland et al. (2000) used random amplified polymorphic DNA (RAPD) to examine the post-glacial range expansion of *A. truei* in B.C. They included three geographic regions along the coastal mountains (labelled North coast, Midcoast, and South coast). The North coast region referred to in this study (King Island, north of Bella Coola) lies south of the northern extent of *A. truei's* range.

Estimates of genetic distance clustered the North coast and the Midcoast geographic regions together. However, these geographic regions had higher genetic diversities than the South coast. A surprising result as we would expect reduced diversity due to northern expansion. RAPD markers were ultimately dropped due to their lack of reproducibility among studies, as the quality of DNA template and the competing effects of coamplifying loci affected the merit of the results.

In the United States, Nielson et al. (2006) used allozyme and mitochondrial DNA to identify evolutionary significant units (ESUs) for *A. truei* and the other species of *Ascaphus, A. montanus*. ESUs emphasize the historical structure within a species; anything that is considered 'significant' has monophyletic mtDNA and shows a divergence in the alleles of nuclear loci (Moritz, 1994). For *A. truei*, they found significant differentiation between the Olympic Mountains (Washington USA) and the Siskiyou Mountains (California and Oregon USA).

At the landscape scale, Spear and Storfer (2008) compared the influence of forest age on population genetic structure. They found more genetic connectivity in the harvested forests than in the unmanaged forests, though they postulated that those differences may be related to dissimilarities in slope between the two forest types. Spear et al. (2012) used population genetics to describe the recolonization of *A. truei* after the eruption of the Mount St. Helens volcano. Their results suggested recolonization from outside source populations within one generation; interestingly, salvage logging and replanting appeared to limit gene flow when compared to natural regeneration. As with Spear and Storfer (2008), they found that increased topographic slope was positively correlated with gene flow. Gene flow was also associated with terrestrial habitats and not aquatic ones.

Dispersal among local populations results in gene flow that helps to maintain genetic diversity within a species. There is clear evidence from a variety of species that the quality of the interstitial environment (e.g. humidity, terrain, ruggedness, temperature) influences the degree of gene flow via dispersal or migration (Rothermel and Semlitsch, 2002). Gene flow and drift are influenced by the local biotic and abiotic environments that influence the possibility 'to stay or to go'.

Pitfall trap data showed that the abundance and distribution of post-metamorphic *A.truei* was related to seasonal changes in local climate, canopy closure, and the distance to a stream (McEwan 2014). Post-metamorphic frogs are known to wander more than 100 meters from streams when conditions are wet (Wahbe et al. 2004), though they appear to have a high fidelity to an area within a stream channel (Burkholder and Diller 2007). Wahbe et al. (2004) found a mean movement distance from streams of about 24 meters for females and about 17 for males in B.C.

Hayes et al. (2006), in southwestern Washington, USA, determined that adult frogs make at least two "large-scale" movements along the stream over the course of a year. They also showed that the median position of adults and second-year larvae in late summer was upstream of their position in early summer. They postulated that adults lay their eggs downstream and move upstream post-breeding, downstream larvae may metamorphose before upstream larvae, and larvae may move upstream as stream drying occurs in the peak of the summer. The upstream-downstream movements may be an opportune time for dispersal events (Wahbe et al., 2004; Spear et al., 2012). Others have proposed that frogs move further from their streams during rainy weather (Noble and Putnam, 1931; Metter, 1967).

The density of larvae in a stream varies greatly, ranging from 0.1 to 10.0 per square meter (Dupuis and Steventon, 1999). However, there is a positive relationship between the number of post-metamorphic frogs found in pitfall traps near streams and the number of larvae caught in the corresponding stream (Matsuda and Richardson, 2005). The density of larvae is likely influenced by adult distribution, aggregate ovipositioning, and stream characteristics.

Near the northern extent of *A. truei's* range, Dupuis and Friele (2003) found larvae uncommon in north-facing basins and they presumed it was due to water temperatures that were too cold for embryo development. Larval *A. truei* selected temperatures between 0°C and 22°C in a laboratory experiment (de Vlaming and Bury, 1970). This study also determined that 1st year larvae selected temperatures lower than 2nd year larvae. In the wild, the density of larvae (measured as captures/m^2) on the Olympic Peninsula, Washington, USA, had a significant relationship to elevation, gradient, and aspect (Adams and Bury, 2002). Studies focused on the relationship between stream morphology and abundance of larvae suggest a preference for riffle-pool (i.e., step-pool) habitat (Karraker et al., 2006). Currently, there is little understanding of the relative use of in-channel habitats by different cohort ages of *A. truei*.

Wahbe and Bunnell (2001) found that tadpoles followed the 'colonization cycle' described by Muller (1974) where stream populations were maintained through interplay between downstream drift and upstream dispersal. According to Wahbe and Bunnell (2001), there was significant downstream movement by larvae. However, the relationship between downstream movement of tadpoles and upstream movement of post-metamorphic individuals is still unclear.

My Studies

A. truei is of conservation interest because large portions of its range are managed for timber harvest. Human-caused disturbance, including forest removal and road building can reduce the quality or availability of habitat (Hayes and Quinn, 2015). There is increasing concern that warmer and drier climate will act in tandem with other anthropogenic activities to limit the abundance and ultimately the distribution of *A. truei*. I conducted a series of

studies designed to understand the genetic connectivity and spatial ecology of *A. truei* across the northern portion of its range. Each chapter focuses on a particular ecological and spatial scale ranging from the phylogeography of the species to the ecology of larvae found in streams.

The first chapter details a phylogeography of *A. truei* across the northern half of the species' range. Previous research on the genetic variation of *A. truei* was narrow in geographic focus or used low resolution marker systems. My phylogeography used higher resolution marker systems and increased our knowledge of the relatedness of the species across the central and northern portion of its geographic range.

I used a well-established method of genotyping, microsatellites, as well as a newer method of genotype-by-sequencing (GBS). Microsatellites use a small number of DNA regions to represent differences in genomes, and the advent of GBS increases the coverage across the genome. However, more data can have more noise. I compared the variability of GBS to that of microsatellites and determined if the GBS method provided better insight into the genetic diversities within and between geographic regions.

Phylogeographic studies use genotyping technologies to describe long axes of time and space to better understand the historical relationships of populations. Genotyping technologies can also be used to characterize relationships along shorter time and space scales. As the technology evolves, we use genetics to better understand a wider range of ecological relationships. Metabarcoding combines the mass amplification of DNA (high-throughput sequencing) and the use of DNA to identify the taxonomy of an organism within a sample (DNA barcoding).

In my second chapter, I used metabarcoding to move beyond morphological identification of prey in the stomachs of *A. truei* larvae. I extracted the DNA found in gut contents of larvae and sequenced the region of the cytochrome c oxidase I (COI) gene of mitochondrial DNA. I used the COI sequences to generate a survey of the taxonomies of organisms in the gut contents. I compared larvae from three different stream reaches and at two development stages in non-fish bearing streams near Terrace, B.C. (just south of the northern extent of *A. truei*'s range). This work adds to our knowledge of the predator-prey dynamics of cool, mountain lotic systems as well as the feeding ecology of *A. truei*.

For my third chapter, I quantified the relationship between an index of larval density and environmental factors measured within natal streams. At the northern periphery of their range, larvae likely average a 4-year pre-metamorphic period (Brown, 1990). I examined if variable larval age-classes, categorized by size and development, differed in their use of aquatic habitat. These results increase our understanding of the ecology of *A. truei* near the northern extent of its geographic distribution and provide insights into the age-specific distribution of larvae.

The study of a species' ecology should consider a range of biological and ecological factors that vary across space and time (Levin, 1992). For this dissertation, each chapter focuses on a particular ecological and spatial scale. Our lack of understanding of specific habitat requirements, and identifying the potential impacts of habitat loss and fragmentation, create obstacles to the conservation of this species from the patch to the landscape. As global climates warm and precipitation patterns change, greater understanding of *A. truei*'s evolutionary and spatial ecology will help guide appropriate conservation actions.

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CHAPTER 1

Tracking the northern expansion of an ancient amphibian species with high habitat-specialization using two types of genetic markers

ABSTRACT

Reconstruction of historical relationships between geographic regions within a species' range, determined through phylogeographic study, can indicate dispersal patterns and help predict future responses to shifts in climate. *Ascaphus truei* (coastal tailed frog) is an indicator species of the health of forests and perennial streams in the Coastal and Cascade Mountains of the Pacific Northwest of North America. I used two genetic techniques — microsatellite and genotype-by-sequencing (GBS) — to compare the within region genetic diversity of populations near the northern extent of the species' range (British Columbia, Canada) to two geographic regions in British Columbia and two in Washington, USA, moving towards the core of the range. Allelic richness and heterozygosity declined substantially as latitude increased. The northernmost region had the lowest mean observed heterozygosities for both techniques (microsatellite, M = 0.19, SE = 0.073; GBS, M = 0.028, SE = 0.0011) and the southernmost region had the highest (microsatellite, M = 0.86, SE = 0.072; GBS, M = 0.18, SE = 0.0027). For both genetic techniques, geographic regions (N=5) separated into 4 genetic clusters with the two most northern regions clustering together. My discovery of reduced diversity may have important conservation and management implications for population connectivity and the response of *A. truei* to climate change.

INTRODUCTION

Studying populations known to have been established following major climatic shifts helps us understand the historical processes that influenced a species' current geographic distribution. Populations established after the last glacial extent will likely have experienced more genetic drift than populations in ice-free habitats, resulting in lower genetic diversity and greater genetic differentiation due to founders' or bottleneck effects (Sagarin and Gaines 2002; Eckert et al. 2008; Shafer et al., 2010; D'Aoust-Messier and Lesbarrères, 2015). However, areas colonized from multiple refugia may have higher than expected genetic diversities (Petit et al. 2003). The intensity of genetic drift is contingent on population dynamics, the number of historical refugia, the proximity to refugia, the way in which a species radiates out from refugia, and the founding population size (Brunsfeld et al., 2001; Dudaniec et al., 2012). Knowing the extent of genetic drift of populations in geographic regions with historically unsuitable habitat, and how those populations relate to regions near refugia will help us predict how species will react to future climate shifts.

The distribution of amphibians in northern North America is greatly influenced by the Pleistocene glaciation; most amphibians migrated into what is now Canada after the Last Glacial Maximum (Riedel, 2017). The ease of northern expansion of a given amphibian species is tied to its dispersal ability, the number and distribution of historical refugia, and the availability of suitable habitat (Lee-Yaw, 2007). A narrow band of usable habitat may limit the opportunity for dispersal, decreasing genetic variation during northern expansion, and increasing the likelihood of founders' or bottleneck effects.

Previous studies of the postglacial phylogeography of amphibians in Canada have focused on species with broad distributions. For example, *Rana sylvatica*, the wood frog,

typically breeds in vernal ponds and uses a range of habitat types. Mitochondrial DNA (mtDNA) and autosomal DNA suggest a rapid and contiguous post-glacial range expansion of the species with radiation out of, and admixture from, four to five putative refugia (Lee-Yaw, 2008; D'Aoust-Messier and Lesbarrères, 2015). Similar results have been shown for *Pseudacris crucifer*, the spring peeper (Austin et al., 2002), and *Ambystoma maculatum*, the spotted salamander (Zamudio et al., 2003). A broad distribution of suitable habitat and several refugia allowed for, not only northern, but eastern and western expansion of these species.

Unlike many other North American frogs, *Ascaphus truei*, the coastal tailed frog, has a narrow geographic range (Fig. 1.1). *A. truei*, along with the other species of the genus (*Ascaphus montanus*), is adapted for life in cold, mountain streams (Nussbaum et al., 1983). The western-eastern extent of their ranges is often limited by appropriate mountain habitat (Dupuis and Friele, 2003), while the southern and northern limits are related to their physiology (i.e. thermal minimum and maximum) and the availability of perennial streams (Bury 1968; Dupuis and Friele, 2003). For *A. truei*, usable habitat during northern expansion may have been narrow from west-to-east in some locations, potentially limiting the scope of radiation from its glacial refugia as suitable habitat became available (Fig. 1.1).

Previous research on the genetic variation of *A. truei* was narrow in geographic focus or used low resolution marker systems. RAPD markers were used for a phylogeography of the two species of *Ascaphus* in British Columbia, Canada, including three geographic regions of *A. truei* (mainly separated by latitude and referred to as 'north', 'mid', and 'south'). Genotypes for *A. truei* clustered into two major groups with the two most-northern

geographic regions (north and mid) clustering into a single genetic group (Ritland et al. 2000).

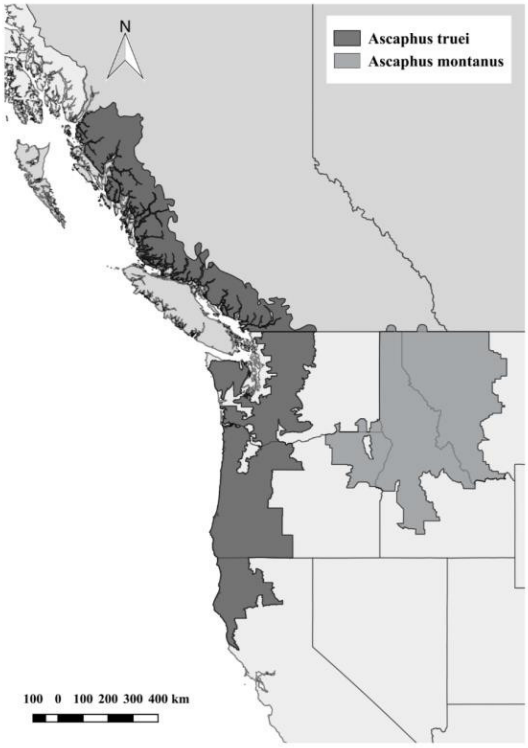

Figure 1.1. Map of northwestern North America showing the distributions of the two *Ascaphus* species from the International Union for Conservation of Nature and Natural Resources (IUCN).

At the landscape scale, a study in Washington, USA, demonstrated that gene flow was extensive in *A. truei* across forested habitats (Spear and Storfer, 2008). They found land cover and topography influenced population connectivity, with little influence based on stream connectivity. Spear et al. (2012) also analyzed the genetic structure among *A. truei* populations following the eruption of Mt. St. Helens, a dramatic and rapid landscape alteration. High genetic diversity and low genetic differentiation suggested prompt recolonization from outside source populations and widespread gene flow across the blast area.

Our understanding of the genetic relatedness of many species across their geographic range, including *A. truei*, may be improved by the advancement of new marker systems and corresponding analyses. A continual concern with a prominent marker system (microsatellites) has been the low number of DNA regions used to represent differences in genomes. The advancement of genotype-by-sequencing (GBS), specifically the quick sequencing of DNA fragments across the nuclear and mitochondrial DNA (mtDNA) genomes, increases the coverage. The following is a phylogeography of *A. truei* in the northern half of its range using three genetic markers; microsatellite genotyping, GBS using nextRAD derived genomic libraries for single-nucleotide polymorphism (SNP) genotyping, and mtDNA haplotypes isolated from the nextRAD genomic libraries.

I compared the variability of SNP genotypes in *A. truei* to that of microsatellites to determine if the nextRAD method provides greater detail for the within and between geographic region genetic diversity due to the greater amount of data across a broader range of the genome. I determined the genetic differentiation within and between regions using three genetic methods, and related the genetic diversity of northern populations to populations near the core of the geographic range of *A. truei*. This work provides important insights into the historical relationships of populations across the northern portion of *A. truei*'s range.

METHODS

Sampling

A. truei is a cryptic, long-lived frog whose larvae remain in cold, fast-flowing streams for 1-4 years before metamorphosis (Brown, 1990; Bury and Adams 1999). Larvae use a modified oral disc to cling to the underside of substrate within their natal streams, thus I

targeted this life stage for tissue collection due to the relative ease of locating them. I employed an opportunistic non-random sampling scheme for tissue collection. Streams were included based on accessibility by road while also representing the broad study area. Larvae were caught using a dipnet while flipping over rocks. I targeted several locations along a stream reach in an effort to minimize the collection of siblings (~100m apart; Wahbe and Bunnell, 2001). Tissue samples consisted of skin clipped from the posterior of the tail; I avoided the muscle as much as possible. Larvae were retained in a bucket of stream water until they were comfortably swimming, and any bleeding had subsided. They were returned slightly upstream of their capture location. Tissue samples were preserved in 95% ethanol and stored at -80 °C.

Sampling was concentrated in the 'Northcoast' (NC) region, around Terrace BC, in 2014. The 'Midcoast' (MC) region, around Bella Coola BC, and the 'Southcoast' (SC), around Chilliwack BC, were sampled during the summer of 2015 (Fig. 1.2). I received purified DNA from stream reaches in the Olympic National Forest and Olympic National Park; referred to as the 'Olympic Peninsula' (OP) region (Fig. 1.2). I also received DNA from the area around Mt. St. Helens and the lower Columbia river and referred to these as from the 'Cascade Mountain' (CM) region. DNA was extracted from tail clips using the DNeasy Blood and Tissue kit (Qiagen, Inc., Toronto, ON) following the manufacturer's instructions.

Molecular methods

Microsatellite markers

I used ten polymorphic microsatellite DNA markers for the microsatellite analysis (Spear et al. 2008; Table 1.1). PCR thermal cycling included an initial denaturation at 95 °C

for 15 minutes, followed by 35 cycles at a locus specific annealing temperature for 30 seconds, an extension at 72 °C for 30 seconds, and a further denaturation at 95 °C for 30 seconds. The cycles were followed by an additional elongation at the annealing temperature for 60 seconds. One primer per pair was labeled with a fluorescent tag (FAM, PET, or VIC).

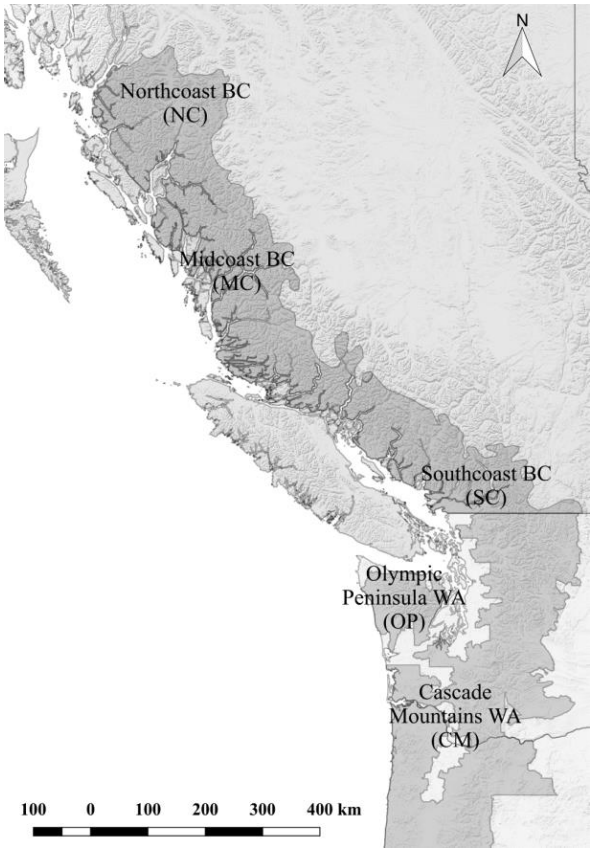

Figure 1.2. Sampled regions along the northern portion of *A. truei's* geographic distribution. 'NC' ('Northcoast') represents sampled stream reaches around Terrace BC. 'MC' ('Midcoast') is the area surrounding Bella Coola BC, 'SC' ('Southcoast') is the area around Chilliwack BC, 'OP' ('Olympic Peninsula') represents the Olympic Peninsula, and 'CM' ('Cascade Mountains') is the area surrounding Mount St. Helens.

Table 1.1. Forward and reverse primer sequences, annealing temperature (T_A), allelic size ranges, and GenBank Accession numbers for 10 microsatellite loci for *A. truei* (from Spear et al., 2008). The fluorescent dye label for each forward primer is indicated before the sequence.

Locus	Primer sequence	T_A	Size range	GenBank no.
A1	F: FAM-GGAAACGAAGACGGAGAACAG R: TTCAGAGCCAGTGAAAGTGC	53	108-216	EU271857
A3	F: PET-GGAACGAGTTGAACCTATCTGG R: CGGATAGGTAGCAGAGATGG	60	145-287	EU271859
A4	F: PET-CGCGCTACATTTATGCAGGA R: TGCTGTTTCTTTGCTGTTGC	53	123-284	EU271860
A12	F: VIC-TGTGAAAGGCGGTCTGATAG R: CCCAACCAGTTCCCAGAATC	53	192-232	EU271861
A13	F: VIC-GCCAGCAGGTACATACAGCAC R: CCTAGAGAGCAGGCAGCAAG	60	172-228	EU271862
A15	F: FAM-ATCACTGTGGCAGGTTCATC R: AATGCATTGCAGAGCATAGAT	53	123-214	EU271864
A17	F: FAM-GATCCCAGCATTATTAGTGAGG R: TGGACAGTTTGCACCCTAATATC	60	176-294	EU271865
A24	F: VIC-TATCCCTTGTCCCACCCTTC R: CAATAAATACACAGATGTAGCATACCC	53	207-304	EU271866
A26	F: VIC-GCTTTGCTGGTTTCTACAAGTG R: TGTGGAGAGAAACAGGTACAGAT	53	153-338	EU271867
A31	F: FAM-TCCTGCAATTAGAGAAGGACAG R: GCAATATGTGGACCCCAATC	53	137-211	EU271869

Each PCR reaction included 1 μl of purified DNA, 0.1 μl of the forward primer, 0.2 μl of a fluorescent tag, 0.2 μl of the reverse primer, 3.5 μl of RNase-free H_2O, and 5 μl of multiplex PCR master mix (Qiagen, Inc., Toronto, ON). I created 4 amplicon pools based on size and generated multilocus genotypes using fragment analysis with the Applied Biosystems 3130xL (Burlington, ON). I scored microsatellite genotypes with GeneMapper (Applied Biosystems).

nextRAD sequencing

I sent purified DNA for nextRAD library preparation, sequencing, and initial filtering to SNPsaurus, LLC (Eugene, OR). Several samples were sent for sequencing in duplicate and triplicate to determine the efficacy of the sequencing method as compared to the microsatellite analysis. SNPsaurus converted genomic DNA into nextRAD genotyping-by-sequencing libraries as in Russello et al. (2015). Genomic DNA was randomly fragmented with Nextera reagent (Illumina, Inc), which also ligates short adapter sequences to the ends of the fragments. The Nextera reaction was scaled for fragmenting 25 ng of genomic DNA, although 50 ng of genomic DNA was used for input to compensate for any degraded DNA in the samples and to increase fragment sizes.

Fragmented DNA was then amplified for 27 cycles with 74 °C extension, with one of the primers matching the adapter and extending 10 nucleotides into the genomic DNA with the selective sequence GTGTAGAGCC. Thus, only fragments starting with a sequence that could be hybridized by the selective sequence of the primer were efficiently amplified. SNPsaurus sequenced the nextRAD libraries on a HiSeq 4000 with two lanes of 150 base pair (bp), single reads (University of Oregon) and a 20x depth of coverage.

Data Analyses

Microsatellite markers

In each geographic region, I checked for null alleles and allelic dropout using MICRO-CHECKER v2.2.3 (van Oosterhout et al., 2004). I tested for linkage disequilibrium between pairs of loci, and for significant deviations from Hardy-Weinberg equilibrium in each region using ARLEQUIN v.3.5.2.2 (Excoffier and Lischer, 2010). Expected heterozygosity (H_e) and observed (H_o) heterozygosity were used to determine the genetic diversity of each region, calculated in POPPR v.2.8.0 for R v.3.4.0 (Kamvar et al., 2014, 2015). I determined the number of repeated microsatellite genotypes per region using GENALEX v.6.5 (Peakall and Smouse, 2006).

nextRAD sequencing

SNPsaurus, LLC used custom scripts that trimmed the sequence reads based on bbduk (BBMAP TOOLS; Bushnell et al., 2017): bash bbmap/bbduk.sh in=$file out=$outfile ktrim=r k=17 hdist=1 mink=8 ref=bbmap/resources/nextera.fa.gz minlen=100 ow=t qtrim=r trimq=10. A reference adapter was matched to the reads and all bases to the right were trimmed (as these were single-end reads), allowing for one mismatch. Reads were filtered based on an average Phred quality score, or estimated probability of a miscalled base, of Q10 (i.e. bases with a 10% chance or less that the call was wrong were retained). Reads shorter than 100 base pairs were removed.

A de novo reference was created by collecting 10 million reads in total, evenly from the samples, and excluding clusters that had counts fewer than 20 or more than 1000. The remaining clusters were then aligned to each other to identify allelic loci and collapse allelic haplotypes to a single representative. For each sample, all reads were mapped to the

reference with an alignment identity threshold of 95% using bbmap (BBMAP TOOLS). Genotype calling was done using SAMTOOLS v1.8 and BCFTOOLS v1.8 (samtools mpileup -gu -Q 10 -t DP, DPR -f ref.fasta -b samples.txt | bcftools call -cv - > genotypes.vcf) (Li et al., 2009; Li, 2011), generating a vcf file. Bases were included if they had a minimum base quality score of 10. Only sites with variants were retained.

The vcf file was filtered to remove alleles with a population frequency of less than 3% (referred to as minor allele frequency). Loci were removed that were heterozygous in all samples or had more than 2 alleles in a sample (suggesting collapsed paralogs). SNPsaurus, LLC checked for the absence of artifacts by counting SNPs at each read nucleotide position and determining that SNP number did not increase with reduced base quality at the end of the read. I removed loci that were variable due to base insertions or deletions using VCFTOOLS v0.1.14 (Danecek et al., 2011).

nextRAD triplicate comparison

I generated a final SNP dataset with all samples from the three British Columbia regions (SC, MC, and NC), including those genotypes in triplicate and duplicate. I converted the SNP calls into a pairwise individual-by-individual genetic distance matrix, with interpolated missing data, using GENALEX. Per geographic region, I compared mean and 95% confidence intervals of within triplicate genetic distances against the mean and 95% confidence interval for non-triplicate genetic distances. I also created a neighbor-joining phylogeny using MEGA7 (Kumar, 2016) to compare the relatedness of replicates compared to non-replicates.

For the final filtering steps, I randomly retained one of the triplicates or duplicates. I generated text files from the filtered vcf file for additional per region filtering, as the

heterozygosity and levels of missing data varied between regions. I removed loci with ≥ 40 % missing data in at least one geographic region and loci with an excessive heterozygosity p-value of ≤ 0.005 per region to further reduce the potential impact of paralogs (GENALEX). I tested for significant deviations from Hardy-Weinberg equilibrium in each geographic region using GENALEX. H_e and H_o were calculated for the final SNP dataset in POPPR.

Genetic structure

I used ADEGENET v2.1.1 package for R v.3.4.0 (Jombart and Ahmed, 2011; Jombart, 2008) to calculate F-statistics and Nei's estimator of pairwise F_{ST} for both molecular marker datasets (ADEGENET relies on HIERFSTAT v0.04.22 for F-statistics). I used 10,000 bootstraps with a lower and upper quantile for confidence intervals of 0.25 and 0.975 to determine pairwise F_{ST} between sets of geographic regions (boot.ppfst).

A population that experienced a recent bottleneck will likely have a lower effective population size than a population that experienced equilibrium or expansion (Nei and Tajima, 1981). I approximated the long-term effective population size per geographic region with the microsatellite dataset. I calculated a population parameter, Θ, using mean allele frequency per microsatellite locus under a stepwise mutation model (Haasl and Payseur, 2010) with PEGAS v0.13 for R (theta.msat; Paradis, 2010). I calculated effective population size per geographic region using an assumed mutation rate of 4.98×10^{-3} (Bulut et al., 2008) and the mean value of Θ per region across all microsatellite loci ($\Theta = 4N_e\mu$, μ is the mutation rate and N_e is the effective population size; Beerli and Felsenstein, 2001).

Population structure was modelled in two ways. I used the Bayesian-based clustering method STRUCTURE v2.3.4 (Pritchard et al., 2000), and also used a multivariate analysis method (discriminant analysis of principle components; DAPC; Jombart et al. 2010). I used

DAPC, in addition to STRUCTURE, as it is free of the Hardy-Weinberg equilibrium assumptions of the more commonly used method. DAPC was conducted in the ADEGENET package.

STRUCTURE assigned individuals to different clusters (*K*) ranging from 2 to 10, without prior sample site location and with admixture. It performed 10 iterations with a burn-in of 100,000 and a running length of 100,000 steps. I included *K*'s based on Ln *P*(D) and ΔK values, scored using STRUCTURE HARVESTER (Evanno et al., 2005; Earl and von Holdt, 2012). As I had multiple runs, I generated consensus alignments of clusters for the top *K*'s using CLUMPAK (Kopelman et al. 2015).

For the DAPC, I used find.clusters in ADEGENET to determine the placement of genotypes within clusters. I used spline interpolation α-score, also in ADEGENET, to determine the number of principal components to retain. All discriminant functions were retained (N=4).

Phylogenetics using mtDNA

I used the previously published *Ascaphus* mitochondrial genome from GenBank (a.c. AJ871087.1) with the nextRAD sequencing data to extract mtDNA reads. The mtDNA genome was indexed, each genotype was separated into individual files, and each file was aligned to the mtDNA genome with the Burrows-Wheeler Aligner algorithm BWA-MEM (BWA; Li and Durbin, 2010). Using SAMTOOLS v1.8, alignments were removed if they had a MAPQ score ($-10 \log_{10}$Pr {mapping position is wrong}) of ≤ 30.

I removed duplicate sequences and merged files using PICARD, retaining a single sample from those sent in triplicate or duplicates for sequencing. Files were sorted and indexed using SAMTOOLS. SNPs were called using FREEBAYES v0.9.10 (Garrison and

Marth, 2012). The ploidy was set to 1, and the defaults were used for all other settings. Loci that had more than 5% missing calls across samples were not included in the analysis, and samples with greater than 40% missing calls were removed. I generated a phylogenetic, minimum spanning network using POPART v1.7 (Bandelt et al., 1999; Leigh and Bryant, 2015).

RESULTS

Microsatellite markers

For the microsatellite analysis, I analyzed four samples from each of 12 streams, a total of 48 genotyped *A.truei* per geographic region for each of the five regions. Locus A13 showed evidence for a null allele and significant deviation from Hardy-Weinberg equilibrium and was dropped from the final analysis. No linkage disequilibrium was found between pairs of loci within each region.

Twelve of the 48 *A. truei* samples in the NC region were identical in their microsatellite genotypes to at least one other (Fig. 1.3). Only two of the four matching genotypes came from the same stream reach, while most were found in separate streams, some as great as 60 km apart. Three matching genotypes had one heterozygotic locus and one had two heterozygotic loci. No other geographic region had identical genotypes.

nextRAD sequencing

De novo assembly and initial filtering of nextRAD sequences produced 8,690 polymorphic loci. The dataset was reduced to 8,213 loci after I removed SNPs based on insertions and deletions. The number of loci were further reduced to 4,249 after I removed loci with excessive heterozygosity and excessive missing data per region. I eliminated

additional loci because of significant deviations from Hardy-Weinberg equilibrium in two or more regions, yielding a final dataset of 4,228 loci.

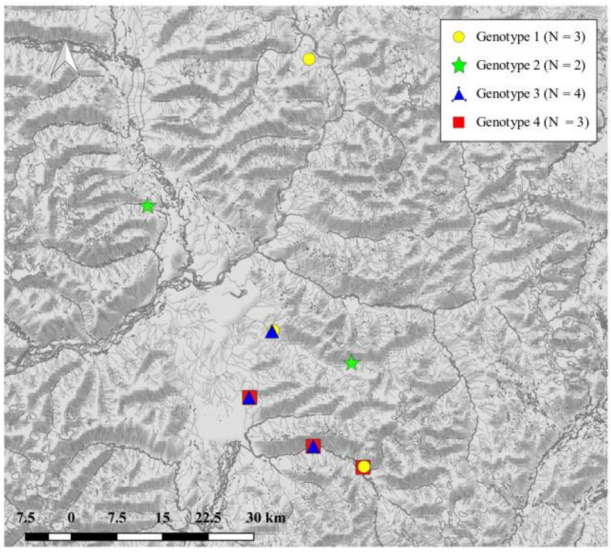

Figure 1.3. Repeated *A. truei* genotypes for 9 microsatellite loci of the 'NC' region (near Terrace BC). Genotype 3 had two of the same genotype from the same stream reach.

nextRAD triplicate comparison

Fifteen samples were processed from library construction to SNP genotyping in triplicate or duplicate; seven samples in triplicate from the NC region and three samples in triplicate and one in duplicate from both the MC and SC geographic regions. A phylogeny of the filtered nextRAD dataset (4,228 loci) showed triplicates and duplicates as separate monophyletic clusters in the SC region. However, repeat genotypes did not cluster together for the MC and NC regions (Appendix 1).

In the MC and NC regions, mean pairwise genetic distance for triplicates was either greater than or similar to the mean genetic distance for non-triplicates (Fig. 1.4). One triplicate in the NC region (135.67 ± 11.76) and one in the MC region (146.61 ± 31.57) had a lower genetic distance than that of the mean non-triplicates (NC = 188.64 ± 1.05; MC =

227.13 ± 1.92), without overlap in their 95% confidence intervals. In contrast, the pairwise genetic distance for non-triplicates in the SC region (844.65 ± 1.05) was greater than the pairwise distances for the triplicates. I randomly selected and retained one of the replicates for further analysis. Thirty-five genotypes were randomly selected from each geographic region for a total of 175 genotypes.

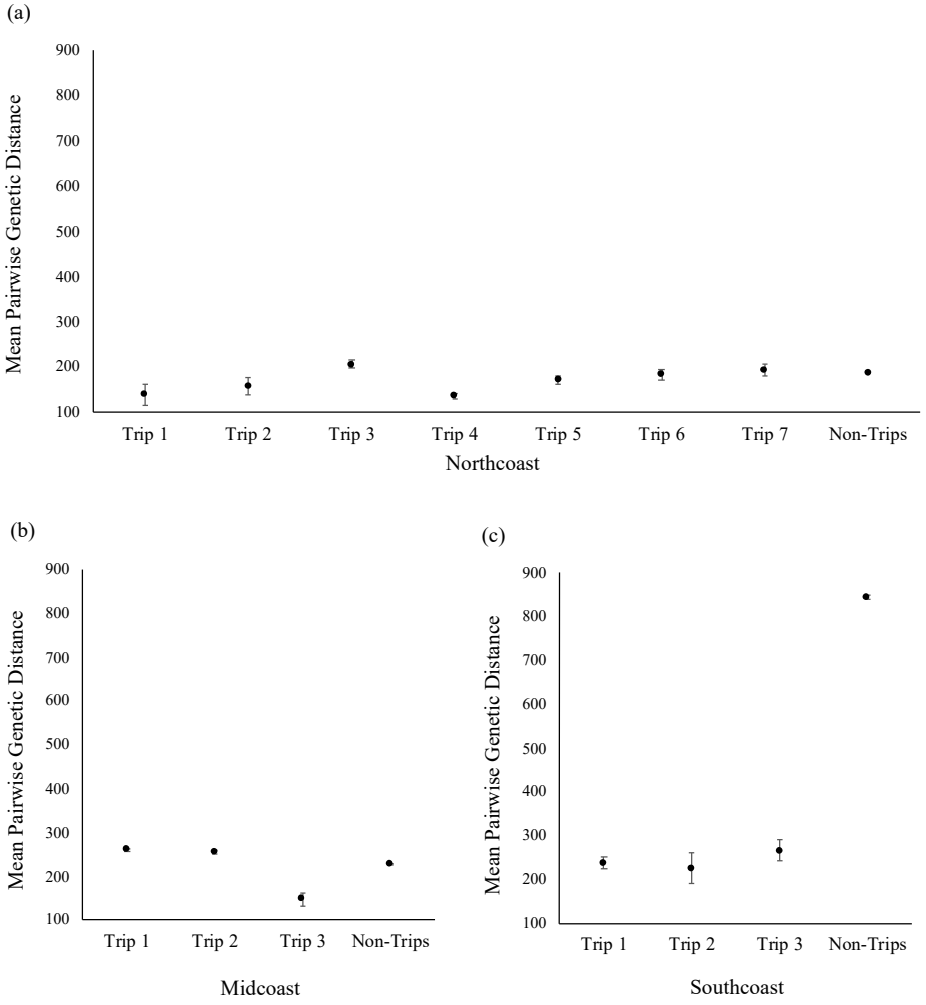

Figure 1.4. Mean pairwise genetic distance comparison between triplicate and non-triplicate nextRAD genotypes for *A. truei* in three geographic regions; (a) 'Northcoast' (NC) around Terrace, BC, (b) 'Midcoast' (MC) around Bella Coola, BC, and (c) 'Southcoast' (SC) around Chilliwack, BC. Error bars represent 95% confidence intervals around the mean. 'Northcoast' (NC) is the northernmost geographic region in British Columbia, CA and 'Southcoast' (SC) is the southernmost. The 'Non-Trips' mean is for all pairwise genetic distance scores that were not associated with a replicated genotype.

Genetic variability

For the microsatellite analysis, mean allelic richness across loci provides a measure of genetic variability as loci can have many alleles. The mean allelic richness across geographic regions was 12.27 ± 1.54 and ranged within regions from 3.33 (NC) to 23.56 (CM) (Table 1.2). Two loci were monomorphic in the NC region and one locus was monomorphic in the MC region. The greatest number of alleles for a locus was sampled from the OP region (43). Mean H_o was 0.60 and ranged from 0.19 (NC) to 0.86 (CM).

For the nextRAD sequencing, a percentage of polymorphic loci provides a measure of genetic variability as loci have fewer potential alleles than microsatellites (likely two). NC region had 20.65% polymorphic loci. The MC region had 23.53% polymorphic loci and the SC had 39.50%. The CM region had the highest percent of polymorphic loci (70.98%), followed by the OP region with 61.49%. Mean H_o ranged from 0.028 (NC) to 0.18 (CM) (Table 1.2).

Genetic structure

The global F_{ST} was 0.26 for the microsatellites and 0.63 for the nextRAD (Table 1.3). Pairwise F_{ST} varied between pairs of regions for the microsatellite and nextRAD datasets (Table 1.4). All pairwise F_{ST} values were significantly different of zero. The genetic distance between the NC and MC geographic regions was largest in the microsatellite dataset (Fig. 1.5). The nextRAD dataset had the smallest genetic distance between the NC and MC regions and the largest between the OP and CM

The population parameter Θ ranged from 82.06 ± 22.68 in the CM region to 1.72 ± 1.31 in the NC region (an almost fiftyfold difference; Table 1.2). The value of Θ decreased from near the core of the geographic range in Washington to the northern edge (Fig. 1.6).

Table 1.2. Measurements of genetic variation of *A. truei* by geographic region for microsatellite and nextRAD sequencing data sets. Number of alleles is represented by mean number of alleles per loci for the microsatellite data and total number of alleles across all genotypes for the nextRAD sequencing data. H_e represents expected heterozygosity; H_o, observed heterozygosity. Mean Θ is a population parameter based on mean allele frequency per locus for calculating effective population size. Standard errors given in parentheses.

	Microsatellite dataset (N loci = 9)				nextRAD sequencing dataset (N loci = 4228)		
Region	Mean N alleles	Mean H_e	Mean H_o	Mean Θ	N alleles	Mean H_e	Mean H_o
OP	18.00 (2.43)	0.89 (0.021)	0.84 (0.033)	45.92 (12.78)	6828	0.14 (0.0027)	0.14 (0.0027)
CM	23.56 (3.63)	0.88 (0.054)	0.86 (0.072)	82.06 (22.68)	7229	0.20 (0.0029)	0.18 (0.0027)
SC	11.11 (2.62)	0.72 (0.074)	0.70 (0.080)	21.78 (11.50)	5898	0.088 (0.0022)	0.079 (0.0021)
MC	5.33 (1.56)	0.40 (0.096)	0.39 (0.099)	5.50 (3.81)	5223	0.029 (0.0011)	0.033 (0.0012)
NC	3.33 (0.91)	0.20 (0.080)	0.19 (0.073)	1.72 (1.31)	5101	0.025 (0.0010)	0.028 (0.0011)

Table 1.3. F-statistics of 5 geographic regions along the northern half of *A. truei's* range for microsatellite and nextRAD sequencing data.

	Microsatellite (9 Loci)	nextRAD (4228 Loci)
F_{ST}	0.26	0.63
F_{IT}	0.29	0.66
F_{IS}	0.043	0.074

Estimates of effective population size, N_e, were over 2000 individuals in the geographic regions closer to the core of the range and under 300 in the northernmost regions.

Table 1.4. Nei's estimator of pairwise F_{ST} between pairs of regions for microsatellite and nextRAD sequencing data for *A. truei* along the northern half of *A. truei's* distribution. 'NC' (northcoast) represents the northernmost geographic region, followed by 'MC' (midcoast) and 'SC' (southcoast). 'OP' (Olympic Peninsula) and 'CM' (Cascade Mountains) represent the two southernmost regions. Nei's estimator of pairwise F_{ST} was performed in ADEGENET. An * represents a value significantly different of zero using the HIERFSTAT bootstrapping over loci method.

Microsatellite (9 Loci)				
	OP	CM	SC	MC
CM	0.043*			
SC	0.097*	0.067*		
MC	0.21*	0.18*	0.079*	
NC	0.29*	0.26*	0.19*	0.16*

nextRAD (4228 Loci)				
	OP	CM	SC	MC
CM	0.40*			
SC	0.57*	0.30*		
MC	0.66*	0.41*	0.21*	
NC	0.66*	0.40*	0.20*	0.0062*

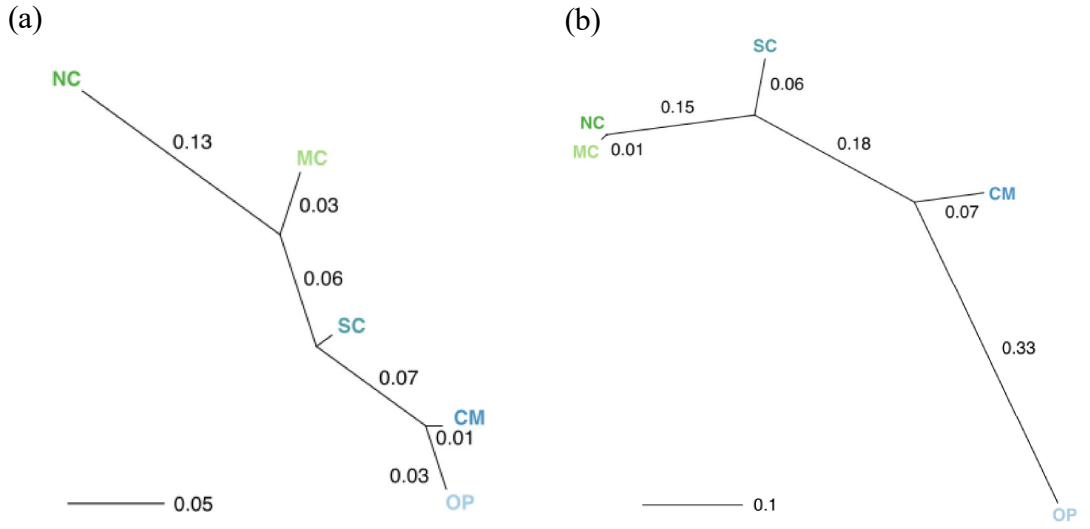

Figure 1.5. Tree of genetic distance between 5 geographic regions along the northern half of *A. truei's* distribution, based on pairwise F_{ST}, for the (a) microsatellite data and (b) nextRAD sequencing data. 'NC' (northcoast) represents the northernmost geographic region, followed by 'MC' (midcoast) and 'SC' (southcoast). 'OP' (Olympic Peninsula) and 'CM' (Cascade Mountains) represent the two southernmost regions. Nei's estimator of pairwise F_{ST} and tree construction were performed in ADEGENET.

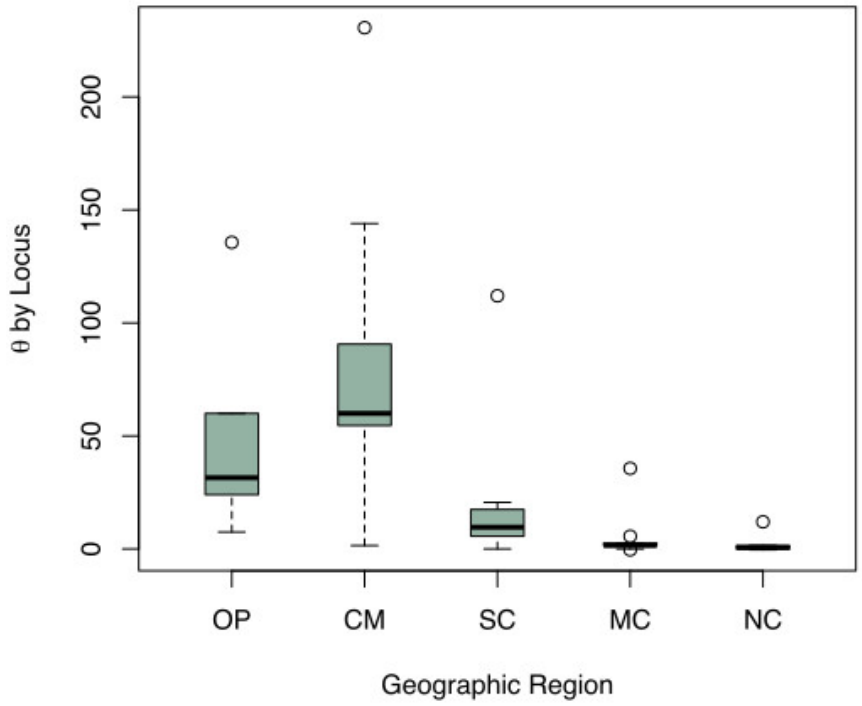

Figure 1.6. Box plots of a population parameter, Θ, calculated using allele frequency per microsatellite locus under a stepwise mutation model for 5 geographic regions along the northern half of *A. truei's* distribution. The black line is the median, upper and lower limits of the box are the 75th and 25th percentiles, respectively, the whiskers extend up to 1.5 times the interquartile range, and outliers are represented as points.

In the models of population structure using STRUCTURE, four clusters ($K = 4$) had the highest ΔK and Ln P(D) values (Appendix 1) for the microsatellite analysis, with NC and MC clustering together (Fig. 1.7). The results for nextRAD were more inconsistent, as the ΔK value suggested 2 clusters and the Ln P(D) suggested 4 (Appendix 1). In both analyses, the northernmost regions (NC and MC) clustered together even as the allotted number of clusters increased. STRUCTURE plots with clustering from 2-5 were included as informative patterns emerged from the data with increasing total clusters (Fig. 1.7 and 1.8).

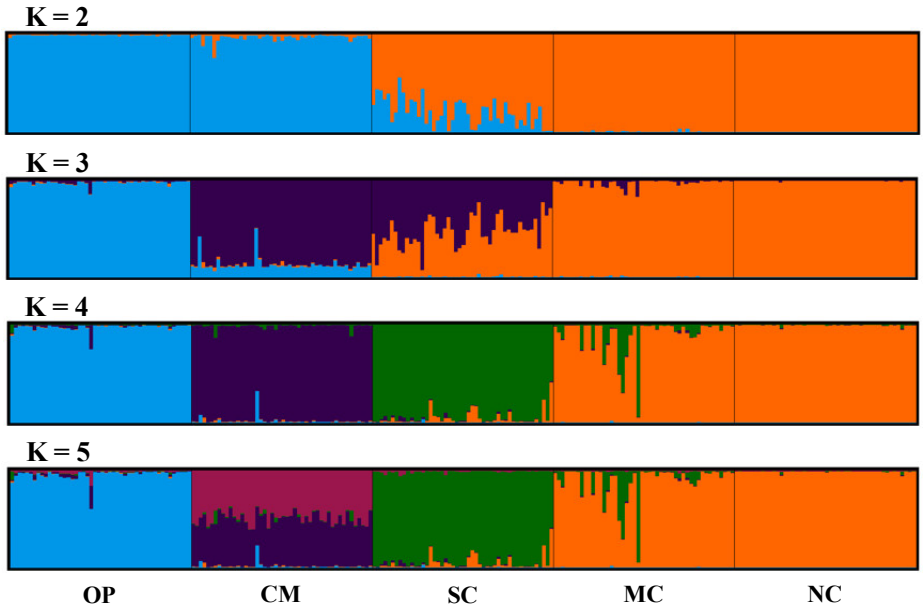

Figure 1.7. Bayesian STRUCTURE plots of microsatellite genotypes (N=9) for *A. truei* across 5 regions in Washington USA and British Columbia CA. Plots represent 2-4 assigned clusters (K) and are partitioned by region. 'OP' (Olympic Peninsula) and 'CM' (Cascade Mountains) represent the two southernmost regions in Washington USA. 'SC' (southcoast), 'MC' (midcoast), and 'NC' (northcoast) represent geographic regions in British Columbia. K=4 had the highest ΔK and Ln *P*(D) values.

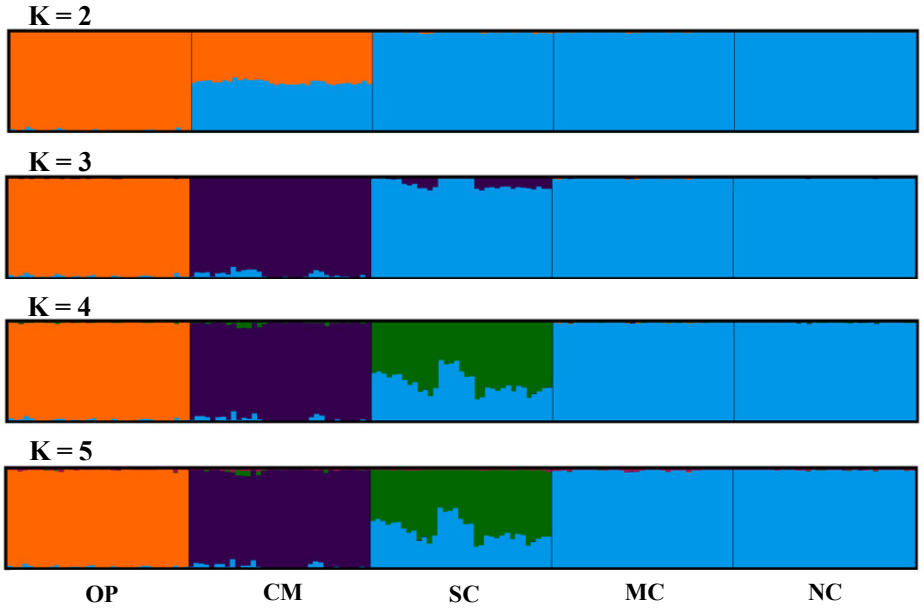

Figure 1.8. Bayesian STRUCTURE plots of nextRAD genotypes (N=4228) for *A.truei* across 5 regions in Washington, USA and British Columbia, CA. Plots represent 2-4 clusters (K) and are segregated based on region. 'OP' (Olympic Peninsula) and 'CM' (Cascade Mountains) represent the two southernmost regions in Washington USA. 'SC' (southcoast), 'MC' (midcoast), and 'NC' (northcoast) represent geographic regions in British Columbia. K=2 had the highest ΔK and Ln *P*(D) suggested K=4.

For DAPC plots, I retained 10 principal components for the microsatellite dataset and 8 for the nextRAD dataset (Appendix 1). The DAPC plots showed similar trends between the two datasets (Fig. 1.9). OP and CM were distinct from each other and from the British Columbia regions. Genotypes from the northern regions (MC and NC) clustered more tightly together than those from the more southern regions in both DAPC plots, and showed little distinction between each other.

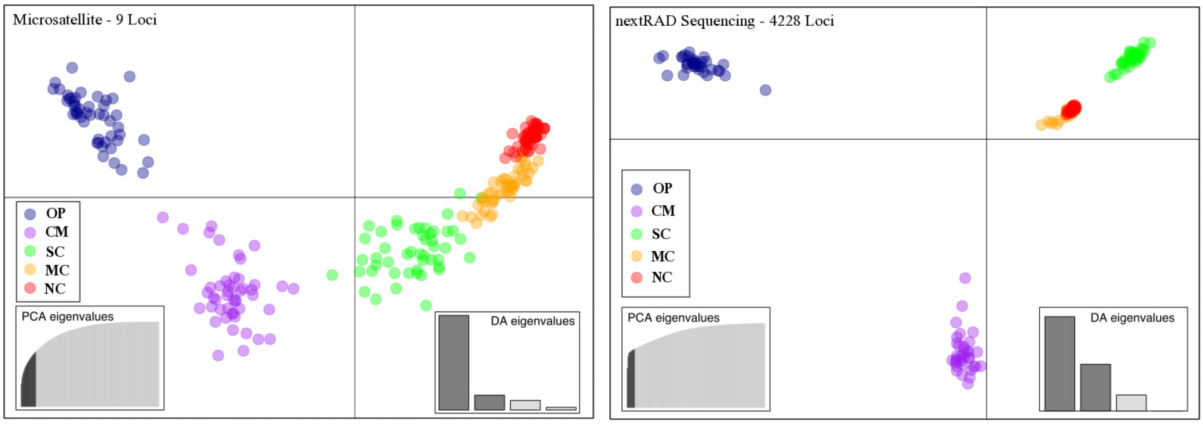

Figure 1.9. Plots of discriminant analysis of principal components for (A) microsatellite and (B) nextRAD sequencing across five regions along the northern half of *A. truei's* range. Individual genotypes are represented by dots and regions are colour coded. 'OP' represents the Olympic Peninsula and 'CM' the area surrounding Mount St. Helens (Washington, USA). 'SC', 'MC', and 'NC' are three geographic regions (south to north) in British Columbia, CA.

mtDNA haplotypes with nextRAD data

Several individuals were excluded from the minimum spanning network of mtDNA haplotypes due to large amounts of missing data. The final dataset included 27 individuals from the CM geographic region, 21 from the OP region, 20 from both SC and MC, and 26 from NC. The network had 6 parsimony-informative sites, a nucleotide diversity of $\pi = 0.10$, and five haplotypes (A-E; Fig. 1.10). One haplotype (A) was found in all three BC regions (NC, MC, and SC). The OP region had two haplotypes (B and C; 13 and 8 individuals

respectively) and the CM region had two haplotypes (D and E; 20 and 7 individuals respectively).

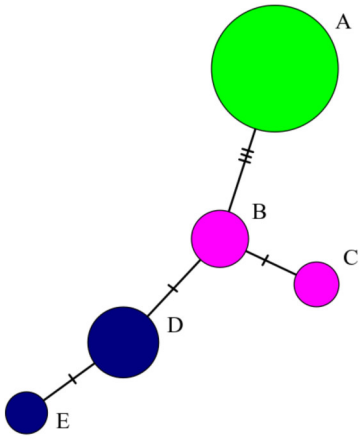

Figure 1.10. Minimum spanning network for mtDNA from five geographic regions along the northern half of *A.truei*'s range. The haplotype from British Columbia, Canada is in green (representing three geographic regions). Haplotypes from Olympic Peninsula, USA are in pink and the area surrounding Mt. St. Helens, USA are in blue.

DISCUSSION

The results of this study provide new insights into the northern expansion of an amphibian species with habitat specialization. I found unexpectedly sharp reduction in genetic diversity in the northernmost geographic regions. Additionally, this study provided new information on the efficacy of a next-generation sequencing technique for a non-target species. My results have implications for future molecular marker selection when researching species with low genetic diversities, and the conservation and management of *A. truei* along the northern portion of its range.

Phylogeography

As expected, there was reduced genetic diversity along the northern extent of *A. truei's* range as compared to geographic regions more central in its range. This is shown in mtDNA and allozyme genetic markers of other amphibian species (Eckert et al., 2008; Funk et al., 2008; Goebel, 2009; Metzger et al., 2015; Cortázar-Chinarro et al., 2017). In the Pacific Northwest of North America, those species restricted to the coastal and Cascade Mountains had fewer mtDNA and allozyme haplotypes in their northern populations than species with broader ranges (Brunsfeld et al., 2001; Kuchta and Tan, 2005; Steele and Storfer, 2006). For example, *Taricha granulosa* is a newt species whose range follows the northwestern coast of North America. Kuchta and Tan (2005) found one allozyme group from Washington USA to Alaska USA and a single mtDNA haplotype from northern California USA to Alaska (with a single satellite haplotype in Washington USA).

I found a single mtDNA haplotype throughout British Columbia. This haplotype is likely the same as the 'Zed' haplotype sequenced in northern Washington (Nielson et al., 2000) and northern BC samples (Murray, *personal communication*), potentially having a similar northern expansion as that of *T. granulosa*. The low number of mtDNA haplotypes throughout geographic regions sampled in the north suggests the combined effect of limited dispersal ability and a single refugium, or a single founding population recolonizing the Coast Mountains post ice sheet retreat (Shafer et al., 2010).

The degree of diversity in more variable, genomic DNA is not reflected in mtDNA and allozyme markers, as they have low mutation rates (McCartney-Melstad, 2018). I found an unexpectedly dramatic reduction in genomic DNA diversity along the northern half of *A. truei's* range compared to in the core of the geographic range. The estimated effective

population size for the southernmost region was almost fiftyfold that of the northernmost region, suggesting genetic drift during the establishment of the northern populations in British Columbia (Kimmel et al., 1998).

Though the relative effective population sizes among sampled regions suggests genetic drift, the calculated sizes may not be accurate. The mutation rate I used was based on the eastern tiger salamanders (*Ambystoma tigrinum tigrinum*; Bulut et al., 2008), and may not reflect the mutation rate in *A. truei*. Exploring fluctuations in effective population size over time will reveal important patterns in future range expansion and relationships between the populations of *A. truei* throughout its range.

These results reiterate the likelihood of a northern range expansion following the Pleistocene (~10,000 years ago), most likely from a single refugium. Future research is needed to determine if the extremely low genetic diversity is due to a founder's effect from low dispersal rates, a bottleneck effect due to a partial barrier somewhere between the southcoast region (Chilliwack BC) and midcoast region (Bella Coola BC), or other influences on dispersing frogs not yet known.

There may have been at least two refugia for *A. truei* during the Pleistocene glaciations. One located in the Klamath-Siskiyou Mountains (Nielson et al. 2001; Nielson et al. 2006; Shafer et al. 2010) and one in the Columbia River area of Washington USA. My results may reflect a post-Pleistocene northern expansion from a Columbia River refugium similar to that described in Steele and Storfer (2006). The high levels of genetic diversity in the Washington USA regions may reflect admixture from the two refugia following the northern range expansion into British Columbia. Examining the genetic structure of *A. truei* throughout Washington, Oregon, and northern California USA will provide a more

comprehensive understanding of the number of refugia during the Pleistocene and the potential mixing between them following glacial retreat.

Historical relationships between populations can aid in distinguishing geographic regions that are important for conservation (Petit et al., 1998). I suggest three to four evolutionarily significant units (ESU) for conservation of *A. truei* across the northern half of its range, based on the uniqueness of allele frequencies (Moritz, 1999). The two southern geographic regions included in this study, the area surrounding Mount St. Helens and the Olympic Peninsula, are important to conservation due to the high levels of genetic diversity and warrant separate ESU designation.

Populations in mid- to northern British Columbia have low levels of diversity; however, they have a unique historical lineage. Additionally, it is important to conserve areas at both the center and edge of a geographic distribution, as expansion can come from the edge or center of the distribution. My southcoast region, the area surrounding Chilliwack BC, may warrant an additional ESU designation. This geographic region shows greater genetic diversity with strong relatedness to the other BC regions. Studies that clarify the phylogeographic relationship among populations found across the species' range could reveal responses to a warming climate and future habitat refugia (Françoso et al., 2019).

Marker comparison

Microsatellite genotypes (i.e. simple sequence repeats) and nextRAD genotypes (i.e. SNPs from across the genome) showed similar spatial genetic patterns for *A. truei* along the northern half of its range. The DAPC plots for both molecular markers resembled each other, with genotype clusters of the two northernmost regions having little distinction between them. Both markers showed similar trends across geographic regions in number of

alleles, heterozygosities, and global F-statistics. There was one exception, that of the pairwise F_{ST} estimates among geographic regions.

The greatest genetic distance (F_{ST}) for the microsatellite markers was between the northcoast and midcoast regions. This was the smallest genetic distance in the nextRAD dataset. Conversely, the smallest distance for the microsatellites was between the Cascade Mountains and Olympic Peninsula regions of Washington USA; the greatest for the nextRAD markers. Based on the STRUCTURE and DAPC results, I would expect the pairwise F_{ST} estimates from the nextRAD marker to be a more accurate representation of the genetic relationships of these geographic regions.

Microsatellite markers have the potential for a large number of alleles per locus. For my analysis, the mean number of alleles per locus varied considerably among geographic regions (~3 to ~24). A few new alleles in populations that have extremely low diversity (such as the northcoast and midcoast) may hyper-inflate the difference between populations. Conversely, a large number of alleles per locus in a population leads to high within-population differences, limiting the between population difference that can be calculated with F-statistics (Charlesworth, 1998; Allendorf and Luikart, 2007; Tishkoff et al., 2009; Jakobsson et al., 2013).

The dramatic difference in the number of alleles between regions could explain why there was a much greater genetic distance between the northcoast and midcoast regions as compared to the distance between the Olympic Peninsula and Cascade Mountain regions for the microsatellite dataset. Low allelic diversity within and between the two most northern regions amplified their pairwise genetic difference. High allelic diversity within the two most southern regions minimized the corresponding genetic difference. My study shows an

overestimation of the true genetic difference in pairwise F_{ST} measurements among populations with reduced genetic variation.

In comparison to microsatellites, SNPs have few potential alleles per loci; therefore, the pairwise F_{ST} measurements are less constrained by large differences in the number of alleles at a single locus. Though there may be issues in relation to the frequency of the most common allele (Jakobsson et al., 2013), the limited number of alleles per loci may provide more accurate estimates of genetic distance when there are extreme differences in genetic diversity among sampled areas.

SNPs discovered through GBS include both neutral and adaptive variation, where microsatellites are expected to be neutral (Narum et al., 2013). Differences among the microsatellite and SNP genotypes may be due to SNPs within the nextRAD dataset that represent adaptive genetic variations amongst geographic regions. Further research of *A. truei* should explore the representation of selection in GBS data across their geographic range.

Although SNP data provides more accurate estimates of between population differences, caution should be taken when comparing individuals within populations with low genetic diversity. Manual data handling is nearly impossible with next-generation sequencing data and there are several potential biases contained within the data; including genotype call errors, missing data, and paralogous loci (Hodel et al., 2017). Several studies have shown that the impact of these potential biases is minimal in comparison to the information provided by several thousand loci across the genome (Attard et al., 2017; Hodel et al., 2017; McCartney-Melstad et al., 2018; Zhang et al. 2018). However, the degree of genetic variability was so low in our two northern geographic regions that the mean pairwise

genotypic distance within triplicates was no different than the distance between non-triplicate. This suggests potential limitations in the use of next-generation sequencing for populations with extremely low genetic diversity, even with thousands of loci across the genome.

I suggest using an additional check, such as analysing samples in triplicate, if using next-generation sequencing techniques with species that are known to have or may have extremely low genetic diversity. This study demonstrates there are limits in how fine the geographic scale can be for low diversity species (including many endangered species), where the genetic signal is difficult to differentiate from potential biases in the data. Checking the noise to signal ratio will ensure the accuracy and efficacy of any population, landscape, or spatial genetic study using genotype-by-sequencing.

Conclusions

New methodologies and technologies in genetic and genomic research may deliver vastly more data, however, they also deliver more noise. My research shows that genotype-by-sequencing requires a check to ensure that the genetic diversity within the studied species is great enough to overpower the noise in the data. However, if operating at the appropriate geographic scale, genotype-by-sequencing data provides a more accurate picture of the genetic distance among populations compared to microsatellites.

My results reflected a northern range expansion into British Columbia following the late Pleistocene glaciations. It is likely that *A. truei* had a very low dispersal ability during northern range expansion. However, my results along the northern portion of the range may not reflect the total number of refugium nor *A. truei's* recent dispersal ability in the southern and central portions of its range.

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CHAPTER 2

Exploration of the gut contents of *Ascaphus truei* larvae using metabarcoding

ABSTRACT

Coastal tailed frog (*Ascaphus truei*) larvae are a top predator in non-fish bearing streams in the Cascade and coastal mountains. Their interactions, including through predator-prey relationships, influence their aquatic habitats and the availability of downstream nutrients. I used metabarcoding to compare differences in the gut contents among *A. truei* of two age-classes and larvae sampled from three stream reaches. I used invertebrate-specific primers targeting the COI sequence of mitochondrial DNA. Searches against sequence databases revealed 105 taxonomies from 12 different phyla. For the phylum Arthropoda, 66 of the operational taxonomic units (OTUs) were identified to, at least, the taxonomic rank of order. Diptera and Ephemeroptera were represented by the greatest number of OTU counts. Average species diversity and species composition of gut contents differed among stream reaches and did not differ between age-classes. The availability of prey for individual *A. truei*, nested within variation amongst stream reaches, may have influenced the variation in diet of the larvae. Future research on diet variations and tropic position should reflect the range of cohort ages found across the study area. Changes in water flow, temperature, and siltation may have important implications for the prey and diet of *A. truei* and ultimately the persistence of the species across these high-elevation ecosystems.

INTRODUCTION

Ecosystem structure and function is influenced, in part, by predator-prey relationships. Predators exert selection pressures on prey and prey influence the distribution and densities of predators, with cascading effects across the organic and inorganic characteristics of an ecosystem (Swain et al., 2015). Our ability to observe the interaction or examine the trace evidence of predation determines who is labelled a predator, who is labelled a prey, and how the relationship is defined.

Perennial, headwater streams (first-, second-, and third-order) typically have low primary productivity and high detrital input, leading to unique flora and fauna adapted to fast-flowing water (Weins, 2002; Meyer et al., 2007). Predators and prey of non-fish bearing, headwater streams are often unobvious, as their biomass is a function of the sheer number of individuals instead of their total body size (Smith and Smock, 1992; Peterson et al., 2007); however, the sum of trophic interactions in headwater streams influences the availability of downstream nutrient and detrital matter (Gomi et al., 2002).

Amphibians are an important, but rarely considered predator in headwater streams. Most amphibians have an aquatic larval stage and larvae can be herbivorous, omnivorous, or carnivorous (Hoff et al., 1999). They often exist at a relatively high abundance at multiple trophic levels; thus, a reduction in the density of amphibian larvae can have a cascading effect for headwater ecosystems. For example, food-webs of small, tropical streams in Australia were more complex when amphibians were present compared to when they were absent (Schmidt et al., 2017). Excluding larvae from stream reaches in Panamá reduced organic and inorganic sediments while also shifting the composition of periphyton species (Ranvestel et al., 2004).

Headwater streams comprise 80% of the streams within drainage basins found across the Pacific Northwest (PNW) of North America (Oregon USA, Washington USA, and British Columbia Canada; Dunne and Leopold, 1978). These streams are vulnerable to disturbances, such as increased sediment load and the deposition of coarse woody debris from forestry practices (England and Rosemond, 2004; Olson et al. 2007). Three amphibian species have been considered good indicators for ecosystem health of headwater streams in the PNW because of their sensitivity to changes in water temperature and percent of fine sediments (*Dicampton tenebrosus*, *Rhyacotriton variegatus*, and *Ascaphus truei*; Welsh and Hodgson, 2008), though most of the past research on their trophic interactions focused on correlations between their presence and other organic and inorganic matter in the streams.

A. truei, the coastal tailed frog, inhabits headwater streams, and the surrounding forest, of the coastal and Cascade Mountains. They have a larger geographic distribution than *D. tenebrosus* and *R. variegatus*, thus are the single lotic amphibian species throughout most of their distribution in British Columbia, Canada. Adults move between aquatic and terrestrial habitats and are believed to feed on small arthropods (Bury, 1970; Jones et al., 2005).

Larvae of *A. truei* rely on flowing streams and metamorphize after 1–4 years, spending at least one winter in their larval form. They have an oral disc used to cling to the surfaces of substrate and graze on periphyton from those surfaces (Altig and Brodie, 1972). Research on the feeding ecology of larvae has focused on tropic relationships, primary production, and invertebrate grazers. For example, occupancy of *A. truei* larvae within a stream reach reduced periphyton standing crop in southwestern British Columbia (Mallory and Richardson, 2005). Benthic invertebrate biomass decreased in the presence of *A. truei*

larvae (Lamberti et al., 1992). The decrease in benthic invertebrates could have been due to direct predation by *A. truei* larvae or to indirect pressures from the larvae.

Metter (1964) used morphological identification of the gut contents of larvae as part of a detailed description of the ecology of *A. truei*. Diatoms, filamentous algae, desmids, pollen, and invertebrate larvae were included in the description. However, materials within a gut are often highly fragmented and degraded, limiting the taxonomic resolution through morphological identification (Berry et al., 2015). New genetic techniques, including DNA barcoding, are providing increased resolution for identifying prey items that are no longer identifiable through visual inspection (Herbert et al., 2003).

DNA barcoding is the use of specific gene sequences to identify the taxonomy of an organism within a given sample (Herbert et al., 2003). High-throughput sequencing uses massively parallel sequencing technologies such as ion semiconductor sequencing-by-synthesis to quickly generate millions of sequence reads from a single sample (Reuter et al., 2015). Metabarcoding combines DNA barcoding with high-throughput sequencing to generate a survey of the taxonomies of an unknown number of organisms within a sample, thus providing an opportunity to move beyond morphological identification of prey in the gut-contents of predators.

I used metabarcoding to compare the gut-content of *A. truei* larvae from three different stream reaches and at two development stages. I generated a preliminary metabarcoding analysis of the diet of a top predator in non-fish bearing streams. I assessed differences in diet among *A. truei* of two size-classes and larvae sampled from three stream reaches. This work will add to our knowledge of the predator-prey dynamics of cool, mountain lotic systems as well as the feeding ecology of *A. truei*.

METHODS

I collected larvae from three stream reaches northwest of Terrace B.C., Canada, during August of 2016 (Fig. 2.1); Shames Feeder (SF; 9U 503158m E 6037593m N), Kalum Feeder (KF; 9U 516437m E 6050850m N), and Erlandsen Creek (EC; 9U 516969m E 6050849m N). I measured wetted width at the start location of the survey for larvae and moved up stream. Larvae were caught using a dipnet while flipping over rocks.

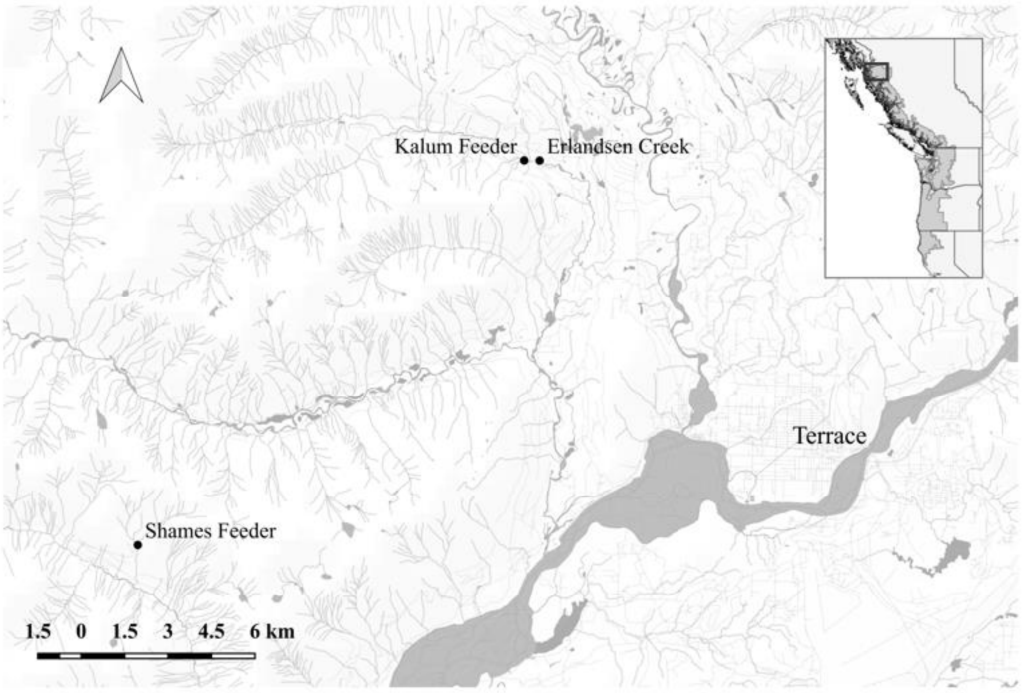

Fig. 2.1. Locations of the 3 stream reaches where *A. truei* larvae were captured for gut content analysis in August of 2016, in northwestern British Columbia, Canada. Inset illustrates the global distribution of *A. truei*.

A. truei larvae will remain in streams from 1 to 4 years; longer larval periods are correlated with inland or high elevations, and with shorter growing seasons (Bury and Adams, 1999). At the northern periphery of their range, larvae develop more slowly than in more southern latitudes, likely averaging a 4-year pre-metamorphic period. A cohort is

determined by length, mass, and developmental stage (Metter, 1967; Brown, 1990; Bury and Adams, 1999). Due to the exploratory nature of this study I separated larvae into two groups; "younger larvae" had no visible limbs and "older larvae" had visible limbs.

Five larvae with no visible, external limbs and five with visible limbs were retained from each sampled stream. The larvae were immediately euthanized with tricaine methanesulfonate (MS222), preserved in 95% ethanol, and stored at -20 ºC until processing. I also used captive-raised larvae as positive controls. They were acclimated to the lab for three weeks and were fed boiled spinach. Diatoms were observed growing on the tiles that lined the tanks. The captive-raised larvae were euthanized in MS222 similarly to the wild-caught larvae.

DNA extraction

I weighed each larva and extracted the gastrointestinal (GI) tract using a clean and sanitized scalpel and tweezers under a dissecting microscope. The GI tract was placed in a sterile 2 ml tube and preserved in 95% ethanol. I made no attempt to remove the gut-contents due to the small size of the GI tract.

All DNA extractions were completed in a dedicated low-DNA room to avoid contamination. A negative control was included with each round of extractions to monitor for contamination. I added two 5/32" steel grinding beads to each 2 ml tube and homogenized the samples using a Genogrinder for two minutes at 1500 oscillations min^{-1}. Grinding beads were removed using sterile tweezers. Excess ethanol was evaporated overnight.

I used the DNeasy Blood and Tissue Kit (Qiagen, Inc., Toronto, ON) to extract DNA from the homogenate. I followed the manufacturer's instructions and applied a 1.5hr

incubation at 56 ºC. I eluded the purified DNA into 75 µl AE buffer twice, for a final elution of 150 µl. DNA concentrations were quantified using a Qubit 2.0 Fluorometer (dsDNA BR assay).

Primer selection and Ion Torrent Sequencing

Due to the exploratory nature of this study, I used general invertebrate primers. I targeted a 157 base pair fragment at the 5' end of the cytochrome c oxidase subunit I (COI) using the COI forward primer (ZBJ F) published by Zeale et al. (2011; Table 2.1). The reverse primer was designed to capture lotic arthropod taxa known to exist in streams near Terrace BC (EPTD; O'Dell et al., 2020; Table 2.1).

I used a two-step PCR amplification method with each sample run in triplicate. The ZBJ F and EPTD primers were bound to target sequences during the first PCR (PCR1). Attached to the forward and reverse primers were fusion, two-tailed end sequences (UniA and UniB) designed at the University of Windsor, ON (Table 2.1; O'Dell et al., 2020). The second PCR (PCR2) attached an IonA adaptor and a unique 10 base-pair sequence, for sample identification, to the UniA-tailed ZBJ F primer. PCR2 also bound a P1 adaptor to the UniB-tailed EPTD primer (Table 2.1; O'Dell et al., 2020). The P1 adaptor attaches to the priming sites on the Ion Torrent chip.

PCR1 thermal cycles included an initial denaturation at 95 ºC for 15 minutes, followed by 30 cycles with a denaturation at 94 ºC for 30 seconds, an annealing at 46 ºC for 60 seconds, and an extension at 72 ºC for 60 seconds. The cycles were followed by a final extension at 72 ºC for 10 minutes. I aimed for 20-30 ng of purified DNA in each reaction and used 1X Multiplex MasterMix (Qiagen Inc., Toronto, ON) for PCR. Each run included a negative control.

Table 2.1. Forward and reverse primer sequences (ZBJ-F and EPT-R respectively), UniA and UniB were adapter sequences attached to the primer sequences for the second round of PCR (PCR2). P1 (and a sample identifier) were attached during PCR2. P1 attaches to the priming site on the Ion Torrent chip.

Name	Sequence
ZBJ-F	AGATATTGGAACWTTATATTTTATTTTTGG
EPT-R	ACTAAYCARTTNCCRAAHCCHCC
UniA	ACCTGCCTGCCG
UniB	ACGCCACCGAGC
P1	CCTCTCTATGGGCAGTCGGTGAT

I ran PCR1 product through 2% agarose gels stained with ethidium bromide to verify the presence of bands around 250 base pairs, the size of the targeted CO1 sequence with the attached primers. When present, I purified PCR1 product using Agencourt AMPure XP purification beads following the manufacturers instruction (Beckman Coulter, Mississauga, ON), then quantified concentrations using a Qubit.

PCR2 had an initial denaturation at 95 ºC for 2 minutes, 5 cycles with a denaturation at 94 ºC for 30 seconds, an annealing at 60 ºC for 30 seconds, and an extension at 72 ºC for 60 seconds. The cycles were followed by an extension at 72 ºC for 5 minutes. I aimed for 20-40 ng of purified PCR1 product in each reaction and created my own master mix (Table 2.2).

I determined the concentration of PCR2 product of one of each sample's triplicate using the Experion electrophoresis system (DNA 1K Analysis Kit; Bio-Rad, Mississauga, ON) to ensure at least 3 ng/µl. I pooled the triplicates, purified using AMPure purification beads (Beckman Coulter, Brea, CA), and determined a final concentration using the Experion system.

Table 2.2. The mix for the second step (PCR2) in a two-step PCR amplification method targeting a 157 base pair fragment at the 5' end of the cytochrome c oxidase subunit I (COI) sequence in the gut contents of larval *A. truei*. UniA and UniB are primer sequences and pPCR1 is the product from the first step (PCR1).

Component	Volume
10x Buffer	3.6 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTPs (10 mM)	0.75 µl
Platinum Taq	0.15 µl
UniA	0.75 µl
UniB	0.75 µl
pPCR1	Aimed for 20 ng of DNA
ddH_2O	Varied; to get to a total of 22.5 µl

Samples were normalized based on the equimolar ratios of each and pooled, including 5 µl of each negative control. I ran two 40 µl aliquots of the pooled samples and controls in a 1.5% TAE agarose gel stained with ethidium bromide to separate out the 295 base pair bands. I extracted the appropriate bands with sterile scalpels using a UV-light box. PCR product was extracted from the gel using a Qiagen MinElute Gel extraction Kit (Toronto, ON). I determined a final concentration of the aliquot using the Experion system. The Great Lakes Institute for Environmental Research (GLIER) Lab at the University of Windsor, Ontario, Canada completed the library creation. Sequencing was done on a PGM Ion Torrent Sequencer (Thermo Fisher Scientific, Waltham, MA) using an Ion Torrent 318 chip. The Ion Torrent Sequencer generate "single-end" reads with a target length of 295 base pairs.

Data Preparation

I used USEARCH v8.1 to complete the initial data screening (Edgar, 2010). I removed the barcodes and truncated sequences to 157 base pairs in order to align to the

appropriate CO1 sequence (-fastq_strip_barcode_relabel2.py; -fastq_trunclen). All sequences shorter than 157 base pairs were discarded. I filtered sequences using an expected error threshold of 0.5 (-fastq_maxee). The 'maxee' value is the sum of each base quality score for a sequence read and suggests the likelihood of erroneous calls on the read. This generated a filtered fasta file.

Operational taxonomic units (OTUs) are clusters of highly similar sequences, likely with the same taxonomic classification. To generate OTUs, I first identified unique sequences and added an annotation for the abundance of sequences within a cluster using USEARCH (-derep_fulllength; -sizeout). The subsequent file was sorted based on the annotation, and singletons were discarded (-sortbysize; -minsize).

I created OTUs by grouping clusters with 97% similarity and removing chimeras (-cluster_otus). Chimeras are sequence reads that may be a combination of two separate genetic sequences. I binned sequences in the filtered fasta file using my generated OTUs and a 97% identity threshold with the UPARSE algorithm (Edgar, 2013). I created an OTU table with OTU calls separated out based on sample identification using a python script in USEARCH (-uc2otutab.py).

After checking for signs of contamination, I removed extraction and PCR controls from the dataset. The OTU count for a gut was recorded as '0' if only one of the triplicates had sequence counts. I rarified the sequence counts in the OTU table to reduce the potential impacts of differences in amplification between taxonomic groups (Weiss et al. 2017). I rarified to the total read count of the sample with the lowest count, repeated ten times, using the GUniFrac package in R v2.1, and averaged across the ten runs (Chen, 2012). I merged the triplicates and summed OTU counts.

Taxonomic assignment

I conducted a nucleotide BLAST search (blastn; NCBI) with representative sequences for each OTU in the OTU table, using the default parameters (Altschul et al., 1997). A single taxonomic line, to the lowest available level, was determined using a ≥ 97% match in MEGAN v6.5.8 (Huson et al., 2016). All OTUs with unassigned taxa were confirmed using the Biodiversity of Life Database (BOLD v3; Rantnasingham and Herbert, 2007). OTUs with taxonomic results of *Ascaphus* were removed. I analyzed three datasets: all OTUs, OTUs with taxonomic assignment, and OTUs with a taxonomic assignment within Arthropoda.

Data analysis

I used PC-Ord v6.08 to run a hierarchical cluster analysis to determine the similarity between laboratory-raised and wild *A. truei* larvae for all samples and all OTUs (McCune and Grace, 2002). For the hierarchical cluster analysis, I used proportional city-block distance measures (specifically Sørensen). The Sørensen distance measure double-weights shared abundance as a proportion of the total abundance (Cha, 2007). Samples were assigned to groups based on similarities according to the Sørensen distance measure. Group spacing was based on a flexible beta value of -0.25. I created a cluster dendrogram from the results of the hierarchical cluster analysis. The laboratory-raised samples were removed from the datasets for all further analyses.

To understand the degree to which taxa were detected, I generated a species accumulation curve using all OTUs with the *specaccum* command ('random' method) in the package VEGAN v2.5 for R v2.1 (Oksanen et al., 2017). I also created species accumulation curves for OTUs with taxonomic assignment, and OTUs with a taxonomic assignment within

Arthropoda. I calculated alpha diversity, or mean OTU count, by sampled location and cohort age for all three data sets (all OTUs, OTUs with known taxonomy, and OTUs in Arthropoda). Datasets were transformed to presence/absence using PC-Ord v6.08; a '1' represented the presence of an OTU in a gut and a '0' represented an absence of an OTU in a gut for that dataset. I evaluated the differences in alpha diversity amongst sampled location and between cohort ages using one-way ANOVA in R. Since there were three sampled locations, when a *p* value was less than 0.05 I used the Tukey post hoc test to determine which locations differed from each other.

I calculated the differences in species composition, or beta diversity, between pairs of sampled locations using the *betadiver* function in VEGAN v2.5 for R (*betadiver*; Oksanen et al., 2017; Whittaker, 1960). Beta diversity was also calculated between cohort groups. The presence/absence data were combined by category, i.e. sampled location or cohort group, for each dataset (all OTUs, OTUs with known taxonomy, and OTUs in Arthropoda). A category received a score of '1' for an OTU if present in any of the samples in that category. I used the equation:

$$\beta = \frac{b + c}{2a + b + c}$$

where *a* represents all OTUs shared by two categories (i.e., two sampled locations or cohort groups), *b* represents the unique OTUs to one sampled location, and *c* represents unique OTUs to the other.

I ran a Bray-Curtis ordination for the three datasets (all OTUs, OTUs with a known taxonomy, and OTUs in Arthropoda). I used the Sørensen distance measure and city-block

method for calculating ordination scores and residual distances. Axis I was plotted with axis II and colour-coded by location. Ordinations and plots were done in PC-Ord.

I ran a distance-based, multivariate analysis of variance (PerMANOVA; Anderson and Walsh, 2013) on the three datasets. PerMANOVA allowed me to run an F-test on the difference between means of groups, and I defined groups based on 'location' and 'development age'. I used a distance measure of Sørensen and a one-way design with 999 randomizations.

RESULTS

Shames Feeder had a wetted width of 5.2 m at the start location of the survey for larvae and the Kalum Feeder and Erlandsen Creek had wetted widths of 1.4 m and 19.6 m respectively. The average mass for larvae with no visible limbs was 0.20 g (s.d. 0.012) and for larvae with visible limbs was 0.47 g (s.d. 0.034).

Forty-five samples were sent for sequencing. Included were samples from four larvae raised in a laboratory (positive controls), four controls from the DNA extractions (negative controls), seven controls from the PCR runs (negative controls), and 30 from larvae capture in the wild. All samples were sent in triplicate. No contamination was revealed in the extraction and PCR controls and they were removed. One laboratory-based, positive control was dropped as it had too few sequences.

Ion Torrent sequencing generated 7,792,367 sequences. Initial filtering, and the removal of *Ascaphus* sequences, reduced the number of sequences to 132,890 (Table 2.3). UPARSE generated 435 OTUs. Rarefaction reduced the OTU count to 218 (Table 2.3).

The samples from the laboratory-raised larvae clustered together in the dendrogram from the hierarchical cluster analysis (Appendix 2). The laboratory-raised controls had a

total of 20 OTUs, and 16 of those were unique to the control samples. The searches of BLAST and BOLD revealed one arthropod in the laboratory-raised control samples (Astigmata); that OTU was not identified in the wild-caught samples. Control samples were removed for the rest of the analysis.

Table 2.3. Total sequences and OTU counts after each step of processing the 5' end of the cytochrome c oxidase subunit 1 (CO1) sequences in the gut contents of *A. truei* larvae.

	Sequences	OTUs
Raw data	7,792,367	-
After initial filtering	132,890	435
After rarefaction	25,176	218
With taxonomic identification (≥ Class)	18,372	124
Without laboratory-raised samples	16,911	105
Within phylum Arthropoda	14,925	72

Taxonomic assignment

The searches of BLAST and BOLD revealed 105 taxonomic identifications from 12 different phyla (any OTU that did not have a known taxonomy beyond 'Eukaryota' was not included; Table 2.4). For the phylum Arthropoda, 66 of the OTUs were identified to, at least, the taxonomic rank of order. Diptera and Ephemeroptera were represented by the greatest number of OTU counts (51 and 14 respectively).

Seventeen of the OTUs within Diptera did not include taxonomic classification below order. Nine OTUs within Diptera included genera. All OTUs within Ephemeroptera included taxonomic classification to family, and ten OTUs had classification to genus. Ixodida and Sarcoptiformes were represented by one OTU each.

Table 2.4. OTUs from the guts of *A. truei* larvae with taxonomic identification to, at least, Class based on searches against USEARCH and BOLD databases. Gut contents were sampled from three stream reaches near Terrace BC in August of 2016. Included are the total number of classified OTUs per Class.

Phylum	Class	OTUs
Ameobozoa	Tubulinea	1
Arthropoda	Arachnida	2
Arthropoda	Insecta	70
Bacillariophyta	Mediophyceae	1
Chlorophyta	Mamiellophyceae	1
Heterokonta	Eustigmatophyceae	1
Heterokontaphyta	Oomycetes	3
Nematoda	Chromadorea	1
Nematomorpha	Gordioida	1
Rhodophyta	Florideophyceae	6
Rotifera	Bdelloidea	11
Rotifera	Monogononta	3
Tardigrada	Eutardigrada	3
Tardigrada	Heterotardigrada	1

Data analysis

The species accumulation curve did not reach an asymptote for all OTUs, suggesting more samples would have revealed more taxa (Figure 2.2). The curves for OTUs with taxonomic assignment and for OTUs with assignment in Arthropoda did not reach asymptote (Appendix 2). The total number of OTUs (i.e., alpha diversity) varied among sampled locations (Figure 2.3). There was a statistically significant difference in alpha diversity between sampled locations for all three datasets (all OTUs, $F = 9.68$, $p < 0.001$; OTUs with taxonomic assignment, $F = 3.94$, $p = 0.032$; and OTUs with assignment in Arthropoda, $F = 3.82$, $p = 0.035$). There was no difference in the alpha diversity between development stage of larval *A. truei* for the three datasets (Figure 2.3; all OTUs, $F = 0.24$, $p = 0.63$; OTUs with

taxonomic assignment, $F = 0.565$, $p = 0.46$; and OTUs with assignment in Arthropoda, $F = 0.79$, $p = 0.38$).

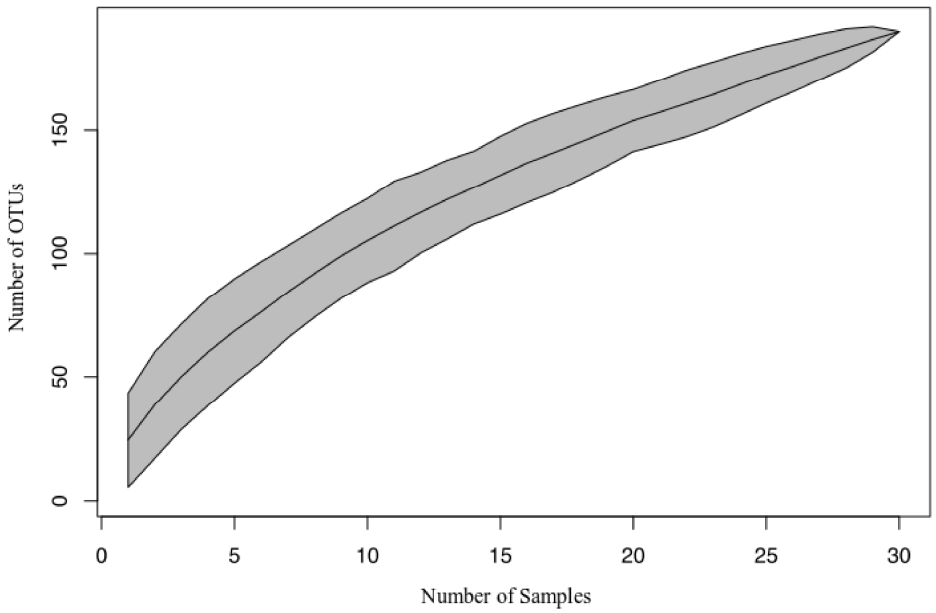

Figure 2.2. Species accumulation curve and 95% confidence interval for the gut contents of wild-caught *A. truei* samples with all operational taxonomic units (OTUs). Larvae were collected from three stream reaches northwest of Terrace, British Columbia, Canada, during August 2016. The 5' end of the cytochrome c oxidase subunit 1 (COI) was targeted using insect-specific primers.

For all OTUs, a post hoc Tukey test showed that Kalum Feeder and Erlandsen Creek differed significantly ($p < 0.05$) in total number of OTUs; Shames Feeder was not significantly different from the other two sampled locations. Shames Feeder differed significantly ($p = 0.025$) in total number of OTUs from Erlandsen Creek in the dataset of OTUs with taxonomic assignment; Kalum Feeder was not significantly different from the other two sampled locations in this dataset. In the dataset of OTUs in Arthropoda, Kalum Feeder and Erlandsen Creek again differed significantly ($p = 0.05$) and Shames Feeder was

not significantly different from the other two sampled locations (though Shames Feeder/Erlandsen Creek had a *p* value of 0.074).

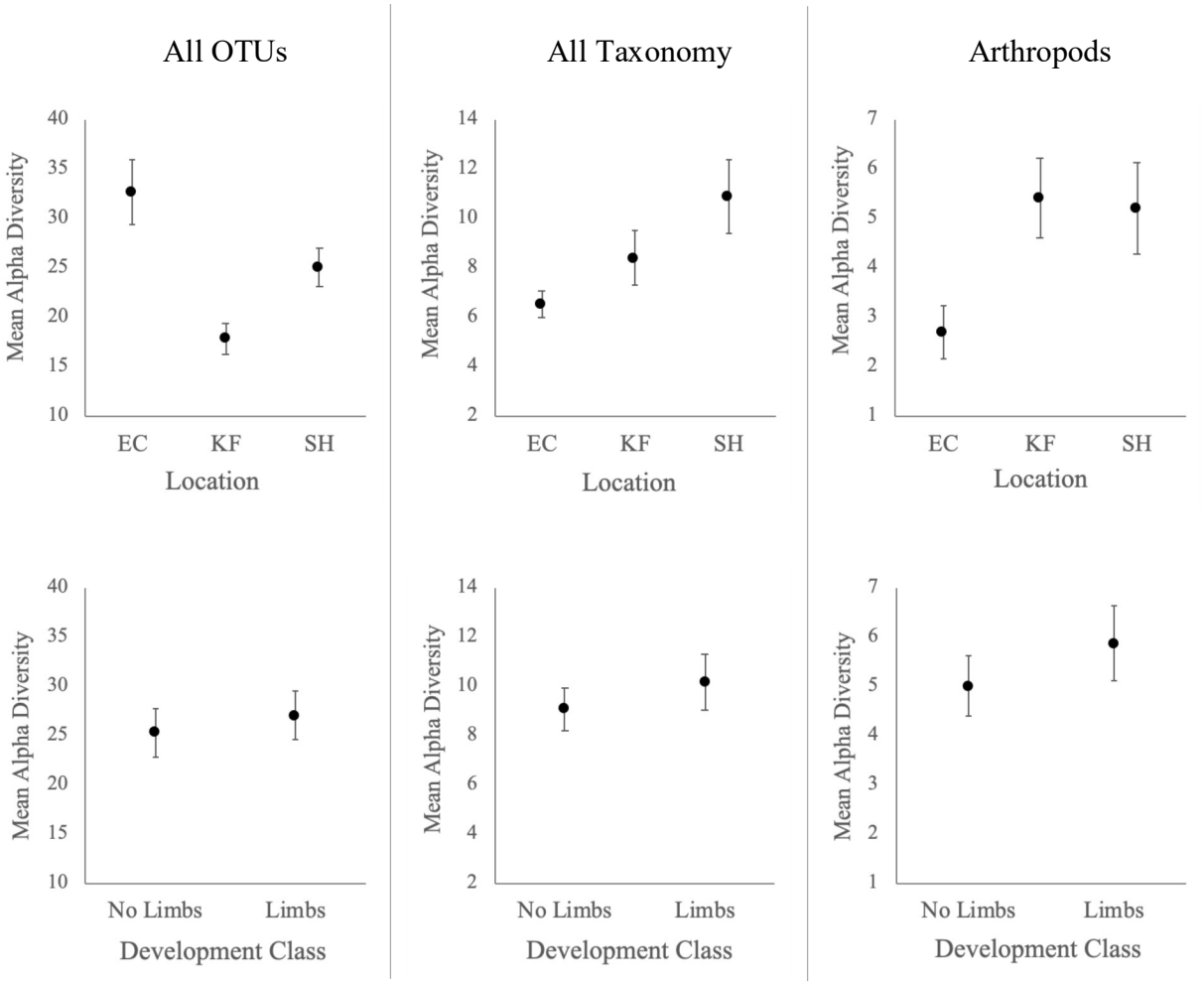

Figure 2.3. Alpha diversities and standard errors for three datasets of operational taxonomic units (OTUs) representing the gut contents of *A. truei* larvae sampled in August of 2016 near Terrace, British Columbia, Canada. Samples were grouped based on sampled stream reach and larvae development class. 'Development class' was based on observations at the time of capture. 'No limbs' category represents larvae with no visible, external limbs and the 'limbs' category represents larvae with hindlimbs extending beyond the anal flap. Included are three levels of data filtering; all OTUs generated by UPARSE, OTUs with some level of taxonomy based on USEARCH and BOLD databases, OTUs with a taxonomy within the Arthropoda phylum.

For beta diversity, 0 suggests complete overlap in taxonomic composition and 1 suggests no taxa in common. Beta diversity was lowest when grouped by cohort for all datasets (Figure 2.4). Erlandsen Creek and Kalum Feeder, closest in geographic proximity, had the lowest estimates of taxonomic overlap across all datasets (Figure 2.4). Taxonomic overlap was lowest (had the highest beta diversity scores) in the Arthropod OTUs for all sampled reaches.

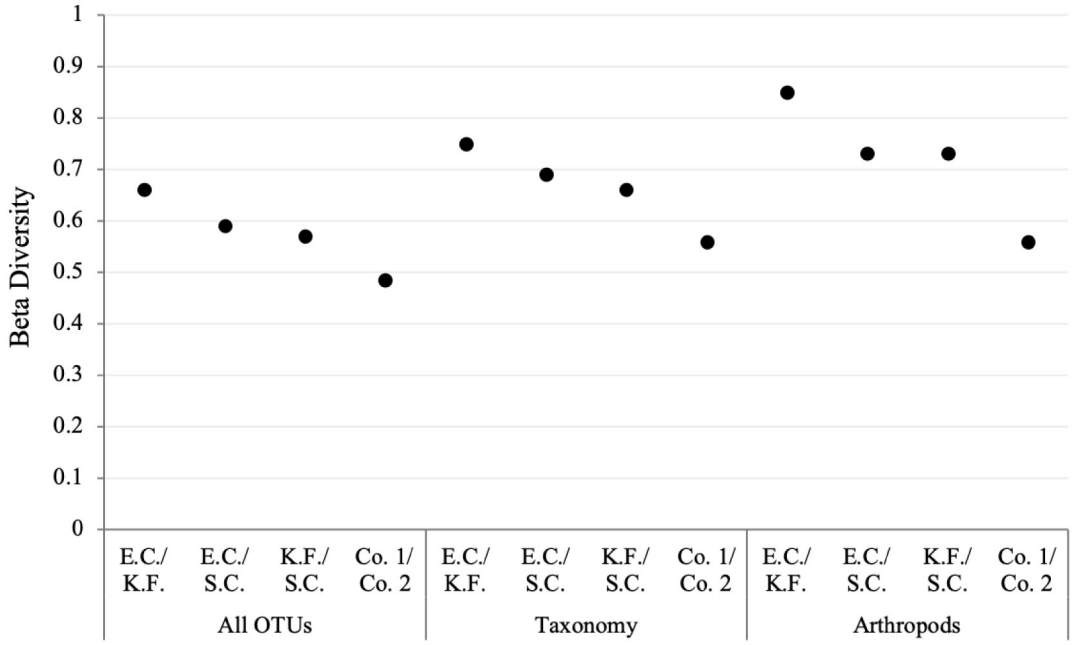

Figure 2.4. Beta diversities for three datasets of operational taxonomic units (OTUs) representing the gut contents of *A. truei* larvae sampled in August of 2016 near Terrace, British Columbia, Canada. Beta diversity was calculated between pairs of sampled stream reaches and between larvae development classes. 'E.C.' is Erlandsen Creek, 'K.F.' is Kalum Feeder, and 'S.C.' is Shames Creek. 'Co. 1' represents larvae with no visible, external limbs and 'Co. 2' represents larvae with hindlimbs extending beyond the anal flap. Included are three levels of data filtering; all OTUs generated by UPARSE, OTUs with some level of taxonomy based on USEARCH and BOLD databases, OTUs with a taxonomy within the Arthropoda phylum.

The first two axes in the Bray-Curtis ordination of all OTUs in wild-caught samples represented 71.56% of the variation (Figure 2.5). There was a difference between sampled location in the distance-based, multivariate analysis of variance (PerMANOVA; $F = 4.40$; $p < 0.001$), and no difference between the stage of development of larvae ($F = 0.98$; $p = 0.42$). The first two axes in the ordination of OTUs with taxonomic identification extracted 66.81% of the variance (Figure 2.4). The results from the PerMANOVA were similar to the results from all OTUs; a difference between sampled locations ($F = 3.89$; $p < 0.001$) and no difference between development stage class ($F = 1.15$; $p = 0.26$). The first two axes in the Bray-Curtis ordination of the Arthropod OTUs extracted 56.67% of the variation (Figure 2.5). As with the previous two datasets, there was a difference between sample location ($F = 3.56$; $p < 0.001$) and no difference between development-stage classes ($F = 1.23$; $p = 0.22$).

Arthropods

Seventy-two percent of the OTU calls of the phylum Arthropoda were represented by a single sample within a sampled location, and 20% were represented by two samples (Table 2.5). Twenty-one OTUs within Arthropoda had a classification to 11 genera, with 5 represented by multiple OTUs (Table 2.5). Multiple OTUs representing a single genus were found together in at least one sampled reach. Sixty-six percent of the OTUs classified to genus were present in no more than one sample per sampled reach (Table 2.5). One OTU was found in 6 of the 10 samples from Shames Feeder, and one was present in all ten samples from the Kalum Feeder (*Epeorus* and *Rheotanytarsus* respectively; Table 2.5).

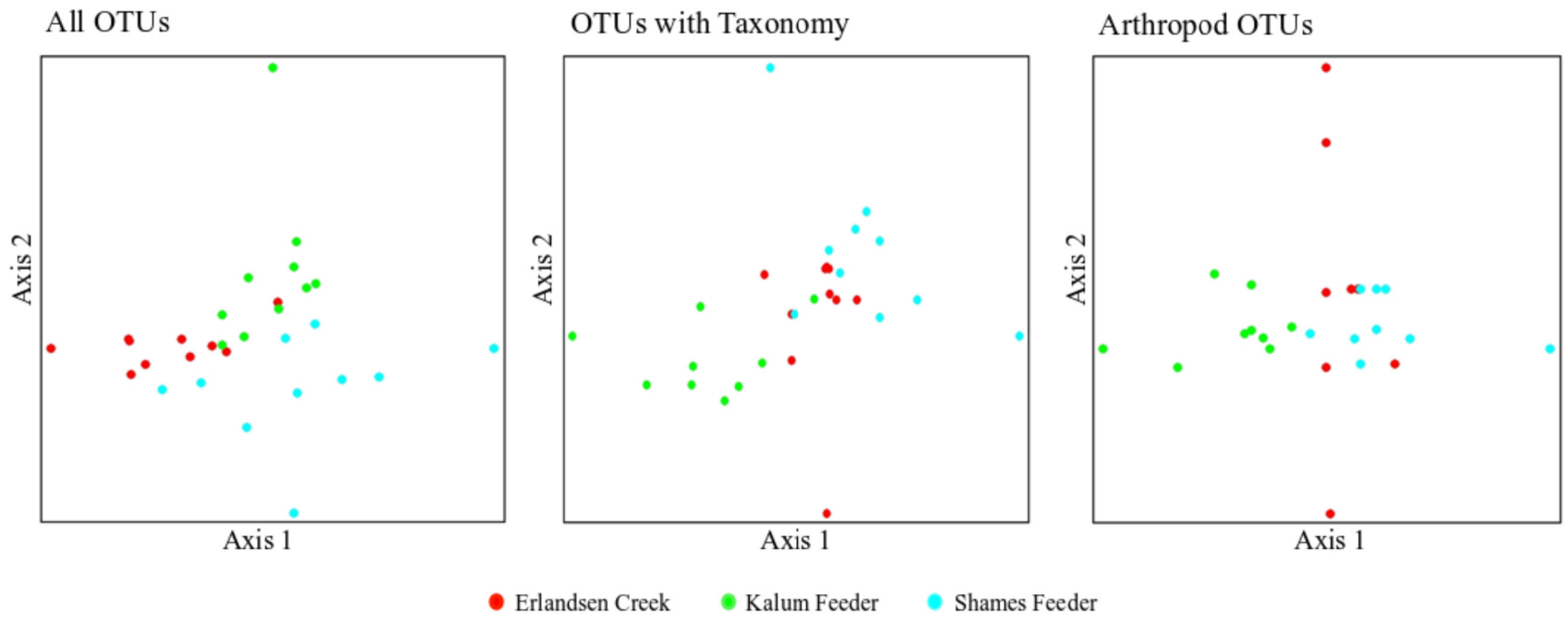

Figure 2.5. Plots from the Bray-Curtis ordinations for three datasets of operational taxonomic units (OTUs) representing the gut contents of *A. truei* larvae sampled in August of 2016 near Terrace, British Columbia. The datasets are (from left to right); all OTUs from UPARSE, OTUs with some level of taxonomy based on USEARCH and BOLD databases, and OTUs with a taxonomy within the Arthropoda phylum. Axis 1 is the distance representing the greatest variance from the original matrix, and axis 2 represents the second greatest. Points are color-coded based on sampled stream reach.

Table 2.5. Classifications to genus of OTUs within phylum Arthropoda, based on comparisons to USEARCH and BOLD databases, for gut contents of *A. truei* larvae from three stream reaches (10 guts per stream). Not included are the 2 OTUs without taxonomic assignment beyond 'Arthropoda'. 'Total OTUs' refers to the number of OTUs within that classification across all sampled reaches, and 'OTUs' refers to the count of OTUs per sampled reach. A checkmark represents the presence of that classification within a stream reach; '1' denotes presence within a single sample, '2' designates presence within two, and '>2' denotes presence in three or more guts. Larvae were collected in August of 2016 near Terrace, BC.

				Erlandsen Creek				Kalum Feeder				Shames Feeder			
Order	Family	Genus	Total OTUs	OTUs	1	2	> 2	OTUs	1	2	> 2	OTUs	1	2	> 2
Diptera	Chironomidae	*Brillia*	1	0	–	–	–	1	✓			0	–	–	–
Diptera	Chironomidae	*Eukiefferiella*	1	0	–	–	–	1	✓			1		✓	
Diptera	Chironomidae	*Orthocladius*	4	2	✓			1	✓			3	✓		
Diptera	Chironomidae	*Rheotanytarsus*	3	1	✓			3	✓			0	–	–	–
Diptera	Tipulidae	*Tipula*	1	0	–	–	–	0	–	–	–	1	✓		
Ephemeroptera	Ameletidae	*Ameletus*	3	0	–	–	–	2	✓			1	✓		
Ephemeroptera	Baetidae	*Baetis*	1	1		✓		1		✓		0	–	–	–
Ephemeroptera	Ephemerellidae	*Ephemerella*	1	0	–	–	–	1	✓			0	–	–	–
Ephemeroptera	Heptageniidae	*Epeorus*	3	1		✓		1	✓			3	✓	✓	✓
Ephemeroptera	Leptophlebiidae	*Paraleptophlebia*	2	2	✓			0	–	–	–	0	–	–	–
Ixodida	Ixodidae	*Ixodes*	1	1		✓		1		✓		0	–	–	–

DISCUSSION

The larvae of *A. truei* are considered an influential feeder in non-fish bearing streams throughout their range, and a potential top predator north of the geographic range of *D. tenebrosus* (Hawkins et al., 1988). This study was an exploration of the gut contents of larvae across stream reaches and cohort-age classes. It was the first to look at invertebrates as potential prey, and to assess their feeding ecology using metabarcoding.

Differences in average species diversity and species composition of gut contents for *A. truei* could be due to feeding or habitat preferences of individual larvae or the availability of different prey species among stream reaches. Past survey work has reported variation in the abundance of periphyton species among geographically clustered stream reaches in British Columbia, Canada. As examples, reduced canopy closure and greater light increased the abundance of algae and periphyton (Mallory and Richardson, 2005). An increase in dissolved nutrients, particularly phosphate, resulted in a greater abundance of periphyton and lentic invertebrates (Kiffney and Richardson, 2001).

The 'Kalum Feeder' eventually drains into Erlandsen Creek. Erlandsen Creek and Kalum Feeder had the lowest overlap in taxonomic composition. This is surprising given the geographic proximity of the two sampled reaches. Wetted width and ultimately stream volume may have influenced taxonomic composition as Kalum Feeder had the narrowest width and Erlandsen Creek had the widest. Site characteristics, such as vegetation cover, depth, and dissolved oxygen levels, might explain variation in larvae diet for *A. truei*.

Amphibian larvae can be opportunistic feeders (Hoff et al., 1999). In Australia, the diet of larvae varied based on nutrient quality and food availability (Schmidt et al., 2017). *Lithobates sylvaticus* (wood frog) larvae were generalist feeders, and individual

specialization increased with the width of the trophic niche of the population (Schriever and Williams, 2013). The availability of prey for individual *A. truei* likely varied among stream reaches and may have influenced the variation in diet of the larvae that I sampled. Metabarcoding the gut contents of more larvae from additional dissimilar stream reaches will increase our understanding of variation in diet including the trophic position of diet items.

Paraleptophlebia, *Ephemerella*, *Epeorus*, *Baetis*, and *Ameletus* are genera within Ephemeroptera that cling to substrate in lotic systems, though they may occupy different microhabitats within their streams. Only one genus (*Epeorus*) was found in more than two guts within a sampled stream reach. Most often an OTU was found in one or two of the ten larvae sampled per stream reach, suggesting presence within the gut as opposed to aquatic environmental exposure.

The diet of amphibian larvae is poorly understood, most reports infer diet according to the morphological characteristics of the mouthparts and guts. The disc-shaped mouth of *A. truei* larvae suggest that the primary method of feeding is scraping substrate. Even so, Metter (1964) found invertebrates in the guts of larvae and my results suggest lotic invertebrates as likely prey. Future research should focus on confirming this result and assessing the relative importance of invertebrates as prey for the larvae of *A. truei* across the species' range.

I separated larvae into two cohort groups based on limb development, no visible limbs and visible limbs. This classification approximately represented larvae that were younger or older than at least two years across the northern distribution of the species. I found no difference in the gut contents of the two groups. Brown (1990) described five categories of larvae age-classes in the northern Cascade Mountains of Washington, USA.

Larvae in three of the categories had limbs that did not extend beyond the anal flap (no visible limbs). Two of the categories had visible hindlimbs. Ideally, I would have used Brown's categorizations and included five age-classes. However, this would have required a much larger sample size. Future research on diet variations and tropic positions should reflect the range of cohort ages found across the study area.

This research represented an exploration of the gut contents of *A. truei*. As such, I used invertebrate-specific primers targeting the CO1 sequence of mitochondrial DNA. The study was not a complete picture of the feeding behaviour of the larvae, nor a full picture of everything that was contained within their guts. Primer bias can impact the invertebrate taxa that are represented (Elbrechet and Leese, 2017), and *A. truei* larvae are known to feed on a variety of species (Metter, 1964). Further work using a variety of DNA primer sequences targeting different taxonomic groups, supported through morphological identification when appropriate, will improve our understanding of *A. truei's* feeding ecology.

A. truei larvae are a top predator in non-fish bearing streams in the Cascade and coastal mountains, often with poor primary production. As with many frogs, they interact with their aquatic environment as larvae to build up energy, transform, and leave for terrestrial systems. How they interact with their lotic systems, through predator-prey relationships, influences their aquatic habitats and the availability of downstream nutrients. Thus, the foraging ecology of *A. truei* could have cascading effects for the productivity and community ecology of these high-elevation, fast-flowing streams.

This species of frog is sensitive to environmental disturbance and alteration resulting from human-caused land clearing and future climate change (Welsh and Ollivier, 1998; Stoddard and Hayes, 2005; Welsh and Hodgson, 2008; Pollett et al., 2010). Although past

research has focused on the direct effects of environmental change for both larval and post-metamorphic *A. truei* (Kroll et al. 2008 and 2010; Pollett et al. 2010), there is a strong likelihood that such changes could fundamentally influence community composition and dynamics. Thus, effective conservation of *A. truei* may benefit from a better understanding of the secondary impacts of environmental disturbance. Changes in water flow, temperature, and siltation may have important implications for the prey and diet of *A. truei* and ultimately the persistence of the species across these high-elevation ecosystems.

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CHAPTER 3

Environmental influences on the density and age-class distribution of *Ascaphus truei* larvae near the northern extent of its range

ABSTRACT

Complex environmental factors influence the distribution of species within lotic systems, requiring comprehensive habitat models that can fully guide management practices. *Ascaphus truei*, the coastal tailed frog, are sensitive to changes in their environment including to headwater streams where their larvae will remain for up to 4 years. I compared the abundance of larvae near the northern extent of the species range to a collection of environmental factors hypothesized to influence population density. I also assessed spatial segregation at various developmental stages where relatively short summers and cool climates result in a long residence for larvae. The top ranked model for larvae abundance included covariates representing the wetted width and wetted depth of the stream. Capture rates decreased in a nonlinear fashion as wetted width increased, and rates increased in streams wider than 6 m. Capture rates nonlinearly increased when stream depth exceeded 20 cm. Older larvae were associated with greater slopes than younger larvae. My results suggest little evidence for a relationship between elevation and cohort distribution. I recommend considering micro-scale influences on the presence and movement patterns of *A. truei* larvae within streams.

INTRODUCTION

Species are non-randomly distributed within their habitats due to the combination of biological limitations (i.e., physiological tolerance to temperature), resource availability, and

natural and human-caused disturbances. The influence of these factors can vary with the spatial or temporal scale of the feature and the scale of investigation (Rettie and Messier, 2000). Thus, the study of a species' habitat ecology should consider a range of biological and ecological factors that potentially differ across space and even the life-history of the organism.

Characterizing the habitat-ecology of amphibians can be especially challenging as they have distinct, biphasic life-stages optimized to aquatic and terrestrial environments. Ideal models of the distribution of amphibians incorporate the often very different resources required for developing eggs and rearing of larvae (often aquatic), the growth of juvenile and the maturation of adults (often terrestrial) (Heyer et al., 1994). Though formidable, habitat inventories that incorporate all life-stages for amphibian species are necessary for building comprehensive habitat models that can fully guide management practices.

Ascaphus truei (coastal tailed frog) are endemic to the Cascade and coastal ranges of North American's Pacific Northwest, from near sea level to almost 2,000 m in elevation (Dupuis and Bunnell 1997). This species is distinctive among North American amphibians due to its unique evolutionary heritage and habitat specialization (Gaige, 1920; Noble, 1925; Noble and Putnam, 1931; Green et al., 1989; Haas and Richards, 1998). Post-metamorphic frogs are found across relatively wet, forested landscapes. During the terrestrial phase of their life history, *A. truei* are intolerant of warm and dry environmental conditions and are associated with coarse woody debris and wet microsites (Hayes and Quinn, 2015). In a recent study from the northern extent of their range, adult frogs were trapped more frequently near streams than juveniles (82% within 30 m), and juveniles were found in more variable habitats and further from streams than adults (McEwan, 2014). Similar results were reported

from southern BC, where immature frogs were more common in open, recently deforested habitats (Matsuda and Richardson, 2005).

The larvae of *A. truei* occupy perennial, fast-flowing montane streams with large diameter substrate and relatively little sand or silty sediments (Hawkins et al., 1988; Wahbe, 1996; Dupuis and Steventon, 1999). Water temperature is variable across the range of the species, and the temperature minimum and maximum for embryonic development ranges from 5 °C to 18.5 °C (Brown, 1975). *A. truei* will remain in streams from 1 to 4 years; longer larval periods are correlated with inland or high elevations, and with shorter growing seasons (Bury and Adams, 1999). At the northern periphery of their range, larvae develop more slowly, likely averaging a 4-year pre-metamorphic period.

Larvae are ventrally flattened and possess an oral disc that serves as a suction cup for clinging to rocks. *A. truei* use the interstitial space between rocks for refuge. Several studies have found that inorganic substrates influence the presence and abundance of larvae in their natal streams (Altig and Brodie, 1972; Adams and Bury, 2002). In British Columbia (B.C.), 65–256 mm cobble was correlated with an increase in density (Wahbe, 1996), and fine sediments with a decrease in density (Hawkins et al., 1988; Wahbe, 1996; Dupuis and Steventon, 1999). Dupuis and Steventon (1999) found that stream width, in addition to inorganic substrate, correlated with density of larvae at the northern extent of the species' range.

Stream width and composition is spatially nested within a broader set of factors that may influence the distribution and abundance of *A. truei*. For example, Diller and Wallace (1999) associated larvae presence with geologic formation (landscape), gradient (stream reach), and substrate type (sampled location). They postulated that substrate size had the

greatest relative influence on the presence of larvae and that geologic formation and stream gradient at the larger scales were necessary to produce and maintain the required substrate. Similarly, Stoddard and Hayes (2005) reported a correlation between stream width and elevation, and gradient. They suggested that these were key factors influencing larval drift down stream. They also found a relationship between larval abundance and size of substrate at the sampled location, heat load index and forest age at the scale of the stream reach, and width of the surrounding forest at the 'landscape' scale. In total, their results suggest that a complex, multi-scalar set of factors influence the distribution of larval *A. truei* both within and across stream gradient.

In this study, I quantified the relationship between an index of larval density and environmental factors measured within natal streams found at the northern extent of the species' distribution. Based on observations from previous studies, I predicted that substrate size and wetted width would influence abundance. I expected larvae to be found more frequently with larger substrate sizes and at median stream widths. I used those data to determine if different larval cohorts, as determined by size and development, differed in their use of aquatic habitat. I predicted that younger larvae would be captured more frequently in narrower streams and at higher elevations than older larvae. In total, these results increase our understanding of the ecology of the species at the northern periphery of its geographic distribution and provide new insights into the age-specific distribution of larvae.

METHODS

Stream survey

Surveyed streams were located near Terrace and Kitimat, B.C., Canada in the Nass Ranges Ecoregion. The area included the Kitimat Ranges, and the Nass and Bulkley Ranges

of the Hazelton Mountains. The Skeena River is the primary drainage in the region (May 4, 2016 maximum discharge: 2530 m^2/s); sampled streams were north and south of the river (Fig. 3.1). The Nass Ranges Ecoregion is characterized by moist, oceanic air lifting over the height of the mountains (Demarchi, 2011). Annual precipitation is approximately 960 mm per year. Watersheds in this area are predominately Type I (Hogan and Luzi, 2010) with a concave-up longitudinal profile. Streams are primarily riffle-pool morphology in the lower, wider gradients, and step-pool morphology in the higher, narrower gradients.

Stream investigation and corresponding surveys were conducted between August and mid-November of 2014. I employed an opportunistic non-random sampling scheme, accessing only streams that crossed operational roads. I only sampled streams that we assumed served as habitat for *A. truei*.

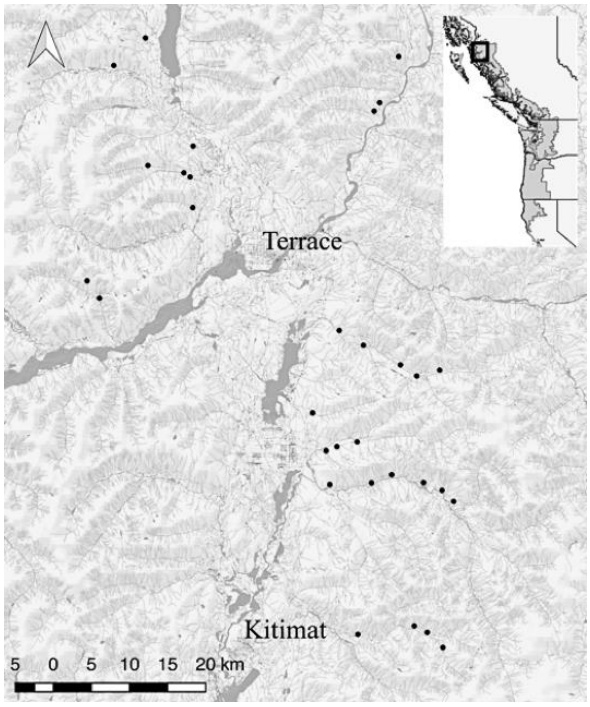

Fig. 3.1. Locations of the 32 streams where *A.truei* larvae were surveyed and captured between mid-June and mid-November, 2014, in northwestern British Columbia, Canada. Inset illustrates the global distribution of *A. truei*.

Streams were not surveyed if they were ephemeral or it appeared they would dry out during the summer. Surveyed sites were excluded or moved if accessibility was limited or if the mouth of the stream was <200 m from the road crossing. I did not sample two immediately adjacent streams (<100 m distance) unless they emptied into different watersheds. The goal was to sample as many streams as were accessible while also ensuring there was adequate time to visit streams throughout the study area (Fig. 3.1).

Streams were surveyed at several locations along a stream reach, with the goal of 5 locations per reach. First, I inspected the stream from just below the road crossing (0 m site) to determine if *A. truei* larvae were present. If they were present within a 60-minute inspection, I, and a field assistant, surveyed the stream at 200 and 100 m downstream, and 100 and 200 m upstream (Appendix 3). Water temperature and time were recorded at the start point of each surveyed site.

We visually inspected the aquatic substrate and flipped over rocks while sweeping with small aquarium nets. Larvae were usually captured by placing a net downstream from a rock, then rotating the rock up and towards the net, and netting the larvae as it released and moved downstream. A metal pin with flagging tape was then placed underneath the rock to which the larva was clinging (Appendix 3). The goal was to collect 4 larvae from a surveyed site or to search for a maximum of 60 minutes. I recorded the time till detection, the number of larvae, the distance in relation to the start point, and the cohort of each larva (based on size and limb-development) captured at each pin. I measured the length and mass of each captured larva.

I recorded wetted width, wetted depth, and stream profile over the pin where the larva was found. Profile was related to three classes: pool, riffle, and cascade. I used a

gravelometer, placed within a 0.4 x 0.4 m quadrat, to estimate the percent cover of each size class of substrate (≤45mm, 64mm, 90mm, 128mm, 180mm, >180mm; Appendix 3). Stones were placed in size classes based on their total size, but only the portion of the stone that was within the plot was included in the estimate of percent cover. 'Embeddedness' was used to reflect the amount of exposed bedrock, as opposed to the degree to which coarse substrates were embedded in fine materials. High embeddedness represented 76–100% exposed bedrock in the quadrant, medium was 26–75%, and low was 1–25%.

I used a spherical crown densiometer to measure the percent cover of shade due to vegetation over-story. I averaged measures directed downstream, at the left stream bank, and right stream bank. I used a digital elevation model (DEM; GeoBC 2017) to assign elevation, aspect (northness and eastness; Clark et al., 1999), and slope (percent) to the GPS location of each sampled reach (QGIS Desktop, 1991).

Data Analysis

Combinations of environmental variables served as hypotheses representing ecologically plausible explanations for the influence of environmental features on (a) the rate of larvae captured-per-hour and (b) the spatial distribution of different cohort classes. Sets of hypotheses represented three broad explanatory factors: temporal, topographical, and habitat features (Table 3.1). I developed those hypotheses after reviewing previous studies that examined the relationship between the distribution or abundance of *A. truei* larvae and the stream environment (de Vlaming and Bury, 1970; Wahbe, 1996; Diller and Wallace, 1999; Dupuis, 1999; Dupuis and Steventon, 1999; Dupuis et al., 2000; Adams and Bury, 2002; Karraker et al., 2006; Kroll et al., 2008; Stoddard and Hayes, 2005).

Table 3.1. Independent variables used to model the influence of temporal, topographical, and habitat factors on the relative abundance and distribution of cohorts of *A. truei* larvae in northwestern British Columbia, Canada.

Category	Variable	Symbol	Description
Temporal	Field assistant	FA	Categorical variable for three field assistants
	Julian date	JD	Date of sampling recorded as Julian day
	Temperature	T	Temperature (C°) at each reach, prior to the start of the survey
Topographical	Elevation	EV	Elevation above sea level (m) derived from 1:250,000 DEM
	Aspect	A	Topographic aspect derived from 1:250,000 DEM, converted into 'northness' and 'eastness'
	Slope	SL	Topographic slope (%) derived from 1:250,000 DEM
Habitat	Vegetation cover	VC	Average percent canopy cover at capture location
	Width*	W	Stream wetted width (m) at capture location
	Depth*	D	Stream depth (cm) at capture location
	Profile	P	Categorical variable representing the stream profile: 1) pool; 2) riffle; 3) cascade
	Substrate cover	SC	Percent cover of the instream substrate across a 0.4 x 0.4m centered on the capture location. Cover was classified into 6 size classes: 1) ≤ 45mm; 2) 64mm; 3) 90mm; 4) 128mm; 5) 180mm; 6) >180mm
	Embeddedness	EM	Categorical variable of exposed bedrock: 0) None; 1) 1-25%; 2) 26-75%; 3) 76-100%

*Both linear and quadratic terms

I calculated the rate of larvae collected per hour as the number of larvae captured at a surveyed site divided by the total time at a site. The rate was rounded to the nearest whole number. I averaged environmental data recorded per flagged location across a surveyed site. Mode, instead of mean, was used for the categorical variables (profile and embeddedness). I used negative binomial regression models to correlate the measured environmental variables with the larvae capture rate (Stata, ver. 12.1, StataCorp, 2011). For each model I included a categorical variable for 'field assistant'; this accounted for potential differences in search effort by different field assistants (N = 3). A number of variables were fitted as two-term quadratic functions to account for a nonlinear relationship with capture rate. I used a clustering algorithm to correct the confidence intervals for the repeated sampling of sites within the same stream reach (Williams, 2000).

A larval cohort is determined by length, mass, and developmental stage (Metter, 1967; Brown, 1990; Bury and Adams, 1999). *A. truei* larva range in length from approximately 11 mm to over 50 mm (Brown, 1975; Bury and Adams, 1999), and mass from 0.10 g to above 1.5 g. Larvae develop a functional oral disc, then hindlimbs grow gradually external of the body cavity. Metamorphosis begins when the forelimbs breach through the body wall and is completed with the reabsorption of the tail. The growth of larvae varies with latitude, elevation, and season resulting in imprecision in the assignment of larvae into cohort classes (Daugherty and Sheldon, 1982; Bury and Adams, 1999).

I used published classification schemes and personal observation to identify six cohort classes (Brown, 1990; Table 3.2). Cohort 1, or "new emerge", represented the smallest larvae with an average length of 20.0 mm (S.E.=0.40) and mass of 0.16 g (S.E.=0.0070). Cohort 2 had an average length and weight of 28.8 mm (S.E.=0.22) and 0.35

g (S.E.=0.0060) and were dubbed "svelte" as they had no external limbs and a smaller body size than cohort class 3 (Fig. 3.2). Cohort 3 were designated "robust" (average of 31.0 mm ± 0.20 and 0.51 g ± 0.0070) due to a larger body cavity than "svelte", yet no obvious external limbs.

Table 3.2. Cohort categories for sampled *A. truei* in northwestern British Columbia, Canada, and total captures per cohort. 'Pool' represents the ways in which the data were combined for logistic regression analysis.

				Pool	
#	Cohort Name	Description	Captures	1	2
1	New Emerge	≈ 20.0 mm, ≈ 0.15 g	21	-	-
2	Svelte	≈ 28.0 mm, ≈ 0.35 g	191	1	1
3	Robust	≈ 31.0 mm, ≈ 0.50 g	200	1	2
4	Feet Peeking	Hind-limbs extend beyond anal cover	84	2	3
5	Legs	Hind-limbs clearly visible	32	2	3
6	Knees	Joint in hind-limbs clearly visible	3	-	-

Cohorts 4–6 were separated based on limb development. Cohort 4, "feet peeking", had visible hindlimbs, being long enough to extend just beyond the length of a flap of skin that covered the anal opening. Cohort 5, "legs", had hindlimbs well beyond the anal-membrane flap. Cohort 6, "legs with knees", had external hindlimbs with obvious joints. The average mass and total lengths differed between cohorts 4, 5, and 6 (Fig. 3.2). I further combined classes into cohort pools to address the small number of samples within some cohorts and to minimize the potential impacts of different cohort classes not reflecting the true annual stages (Table 3.2).

I used logistic regression to calculate the likelihood of capturing one cohort pool relative to another and used a multinomial model when > 2 pools were identified (Stata, ver. 12.1, StataCorp, 2011). Variation in the distribution of cohorts was related to the

environmental variables measured at each stream reach (Table 3.1). As with the analysis of larvae abundance, I used a variance estimator that accommodated correlated samples since multiple stream segments were surveyed in a reach (Williams, 2000). Each model included the Julian date of the survey to control for larvae growth over time.

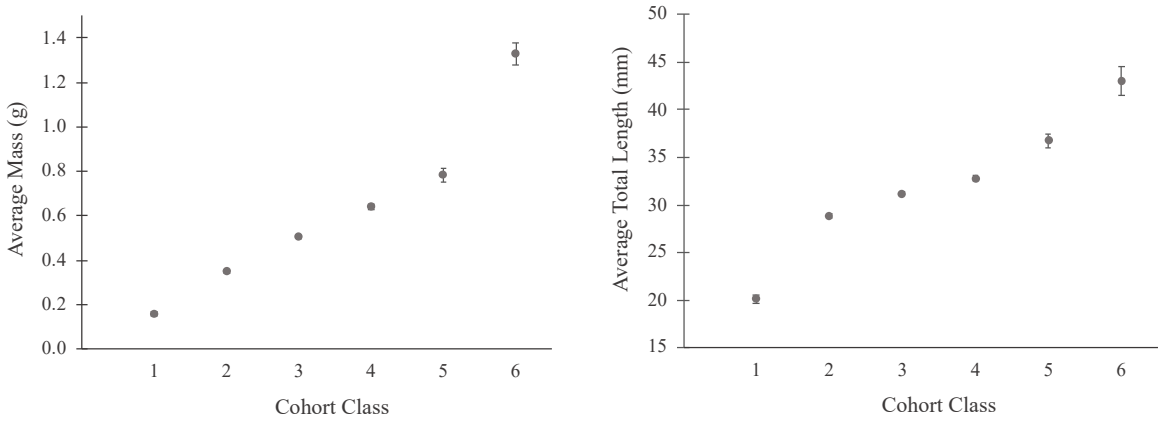

Fig. 3.2. Average mass (g; ± standard error) and average length (mm; ± standard error) of cohort classes for *A. truei* larvae surveyed in northwestern British Columbia, Canada. Cohorts 1, 2, and 3 have no externally visible limbs. Cohorts 4, 5, and 6 have visible limbs.

Model Selection

I used Akaike's Information Criterion (AIC_c) for small sample sizes to select the most parsimonious model from the set of ecologically plausible models. The Akaike weight (w_i) represented the approximate probability that the model was the best of the set. I gave priority to the model with the fewest parameters when models were nearly equivalent (i.e., $\Delta AIC_c < 2$) (Johnson et al., 2006). I reported all models that represented 95% of the AIC_c weight (Mazerolle, 2006), and used 95% confidence intervals to assess the strength of the coefficients within the top-ranked models. When intervals did not overlap zero, covariates were considered as a significant factor influencing the density of larvae or the distribution of cohort pools. Information theoretic approaches provide a relative measure of model

suitability but do not address the absolute fit of the model. I used a bootstrapping method to independently cross-validate the predictive accuracy of the most parsimonious models (Bridger et al., 2017). Each record was withheld sequentially from the model fitting progression and the resulting model (N-1), along with the withheld record, was used to predict a larval count or the probability of the record occurring within a cohort pool.

I used the Wilcoxon matched-pairs signed-rank test to assess differences in the observed and predicted capture rates of larvae. The signed-rank test assumes a difference of zero between the observed and predicted scores, with symmetry in the distribution around zero (Stata, ver. 12.1, StataCorp, 2011). For the analysis of cohort pools, I used the Receiver Operating Characteristic (ROC) to calculate the area under the curve (AUC) for the independently predicted probabilities. The higher the predictive accuracy of the model, the greater the AUC value (between 0 and 1.0). A score between 0.5 and 0.7 is considered a "poor model", between 0.7 and 0.9 is a "good model", and between 0.9 and 1.0 a "highly predictive model" (Swets, 1988).

RESULTS

I captured larvae in 31 of 49 streams surveyed from August to November across a 4,800 km^2 area (Fig. 3.1). Elevation of surveyed streams ranged from 130 m to 697 m (376.62 ± 12.59) with a mean stream wetted width of 2.79 m (S.E.=0.078; Fig. 3.3). A total of 120 larvae-per-hour capture events and a maximum of 531 cohort records (dependent on cohort pools) were used in the analysis.

Substrate percent cover and stream profile were not highly variable across the sites surveyed. There was greater variation in percent cover of the ≤ 45 substrate and > 180 size

classes, but the remaining classes had similar percent covers (Fig. 3.4). For profile, over 70% of the surveyed locations occurred within the 'riffle' category.

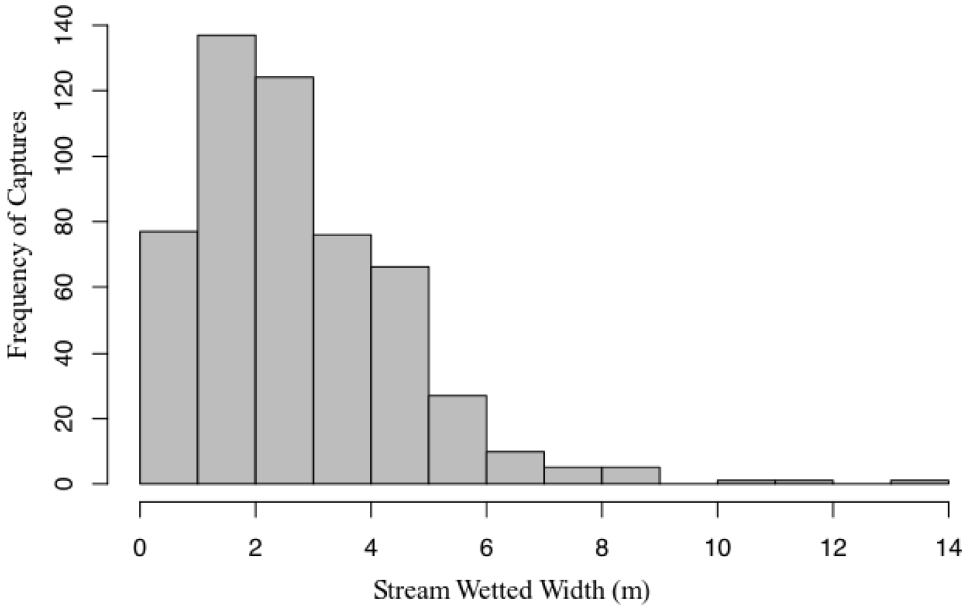

Figure 3.3. Histogram of the frequency of capture for *A. truei* larvae across stream wetted widths surveyed in northwestern British Columbia, Canada.

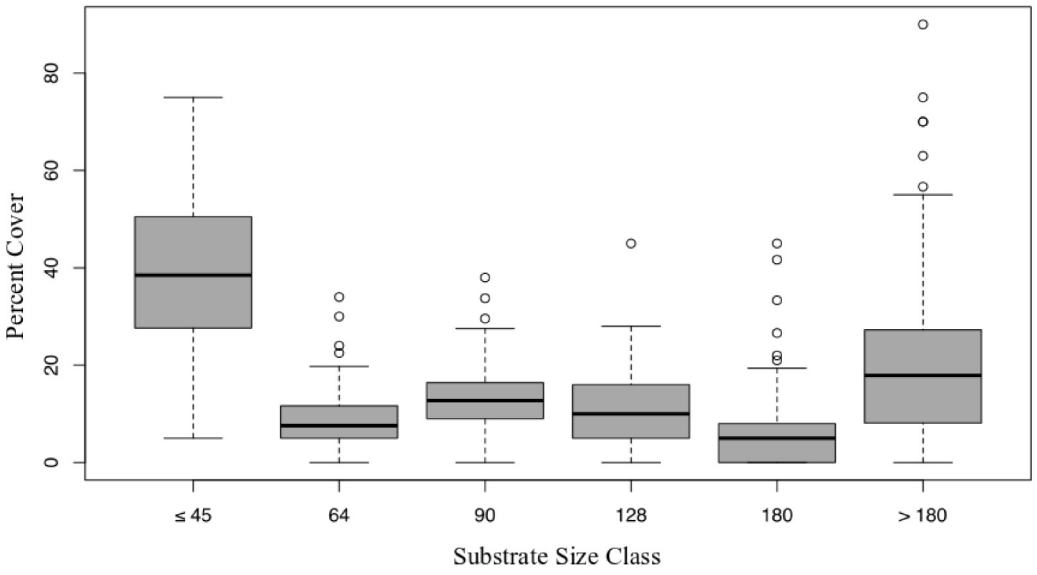

Figure 3.4. Box plots of percent cover categories for *A. truei* larvae surveyed in northwestern British Columbia, Canada. The black line is the median, upper and lower limits of the box are the 75th and 25th percentiles, respectively, the whiskers extend up to 1.5 times the interquartile range, and outliers are represented as points.

Larvae capture rate

Two records were considered outliers and removed from the larvae capture rate analysis. One due to eight larvae collected in a single sweep of the net, and the other because it had obvious differences in ecological conditions (mainstem of watershed). The rate of capture ranged from 2 to 75 larvae per survey hour, with a mean of 13.63 per hour (S.E. = 1.10) (Fig. 3.5).

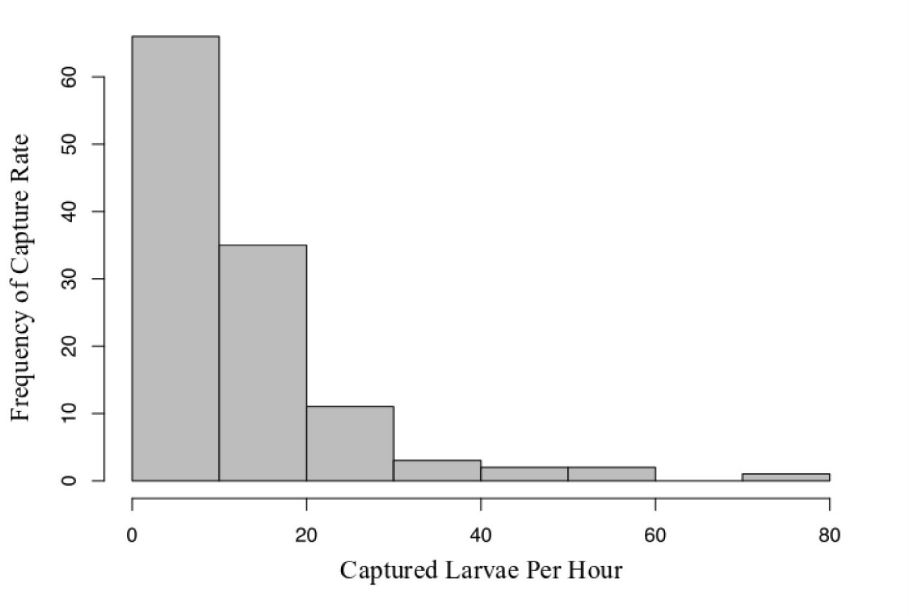

Figure 3.5. Histogram showing the frequency of capture rates for *A. truei* larvae surveyed in northwestern British Columbia, Canada.

The top-ranked negative binomial regression model included 'field assistant' (in all models), percent slope, and both wetted width and depth (linear and quadratic) (Table 3.3). The second-ranked model marginally differed ($\Delta AIC_c = 0.53$) and was a subset of the top model (Fig. 3.6). The two top models accounted for 96% of the w_i. The observed and expected capture rates did not significantly differ suggesting that the two top-ranked models had good predictive accuracy (Table 3.3).

Table 3.3. Summary of AIC results for negative binomial models representing the number of *A. truei* larvae sampled per-hour in northwestern, British Columbia, Canada. The citation represents original study that inspired model formulation. Italicized coefficients had 95% confidence intervals that crossed zero. Included are the p-values for the Wilcoxon matched-pairs signed-rank test comparing the independent predicted and observed samples for the top ranked models.

Model	Rank	k	AIC_c	ΔAIC_c	w_i	Sign Test p-value
* + **Width2** + **Depth2** + ***Slope*** - Field Observation$^{\propto}$	1	8	814.74	0.00	0.543	0.055
* + **Width2** + **Depth2** - Dupuis and Steventon 1999$^{\propto}$	2	7	815.27	0.53	0.417	0.035
* + ***Substrate Cover*** + **Slope** + **Temp** - Diller and Wallace 1999	3	10	820.46	5.72	0.031	
* + ***Elevation*** + **Width2** - Kroll et al. 2008$^{\propto}$	4	6	824.91	10.17	0.003	
* + ***Substrate Cover*** + **Temp** + ***Aspect*** - BC Manage. Plan 2015	5	11	825.55	10.81	0.002	
* + **Slope** + **Depth2** + **Embed** - Diller and Wallace 1999$^{\propto}$	6	9	827.19	12.45	0.001	
* + **Temp** + ***Profile*** - Dupuis 1999 and Karraker et al. 2006	7	6	828.17	13.43	0.001	
* + **Veg Cover** + ***Temp*** - Vlaming and Bury 1970	8	5	828.60	13.86	0.001	
* + **Width2** + ***Profile*** + ***Embed*** - Field Observation$^{\propto}$	9	10	829.13	14.40	<0.001	
* + **Width2** + ***Substrate Cover*** - Dupuis and Steventon 1999$^{\propto}$	10	10	830.74	16.00	<0.001	
* + ***Substrate Cover*** + ***Profile*** + ***Slope*** - Diller and Wallace 1999	11	11	843.22	28.48	<0.001	
* + ***Substrate Cover*** + ***Profile*** + ***Embed*** - Dupuis et al. 2000 and Wahbe 1996	12	13	846.69	31.95	<0.001	
* + ***Elevation*** + ***Aspect*** + ***Slope*** - Adams and Bury 2002	13	7	847.35	32.61	<0.001	
* + ***Veg Cover*** + ***Slope*** - Stoddard and Hayes	14	5	849.23	34.49	<0.001	
* + ***Slope*** + ***Profile*** - Diller and Wallace 1999	15	6	850.59	35.85	<0.001	
* + ***Profile*** + ***Aspect*** - BC Manage. Plan 2015	16	7	857.13	42.39	<0.001	

*Field assistant, included in all models

$^{\propto}$Both linear and quadratic terms used in models

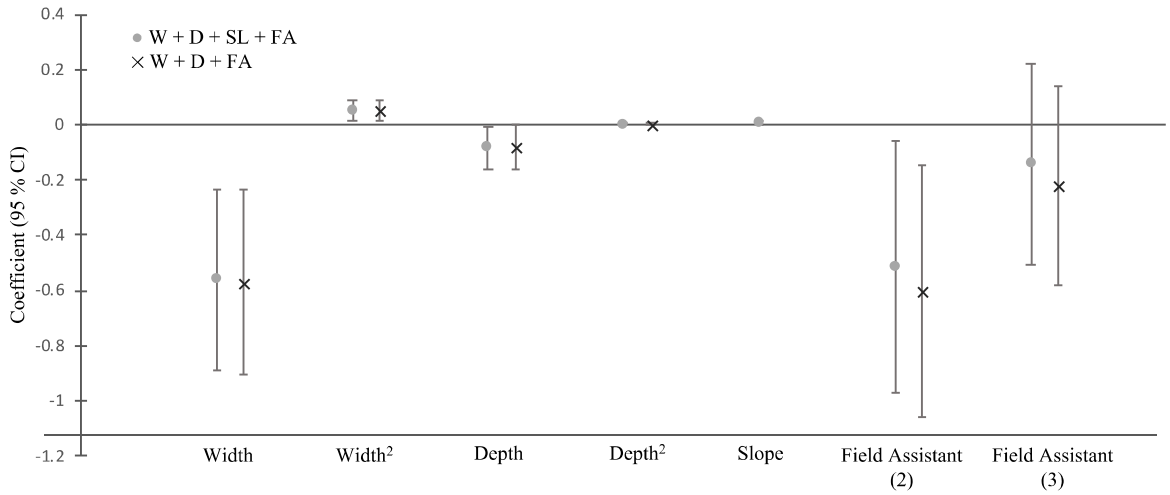

Figure 3.6. Coefficients and 95% confidence intervals of the top-ranked negative binomial regression models ($\Delta AIC_c < 2.0$) representing environmental influences on the capture rates for *A. truei* in northwestern British Columbia, Canada.

The nonlinear coefficients for stream width and depth were statistically significant in the two top-ranked models. The capture rate of larvae decreased in a nonlinear fashion as stream width increased, with an increased rate of captures in streams larger than 6 m (range: 0.6 m to 8.3 m). In contrast, the capture rate of larvae increased when stream depth exceeded 20 cm (range: 5.3 cm to 40 cm). One field assistant had a positive influence on the rate of capturing larvae. Julian date was collinear with field assistant, and was removed.

Distribution of cohorts

Cohort 1 and cohort 6 were excluded from the analysis due to low total numbers. Twenty-nine percent of cohort 1 captures occurred at one stream, and cohort 6 had only three captures. The 'field assistant' variable was not included due to collinearity with date, and date was included in all models as there was variation in rates of capture among different cohorts over time (Fig. 3.7).

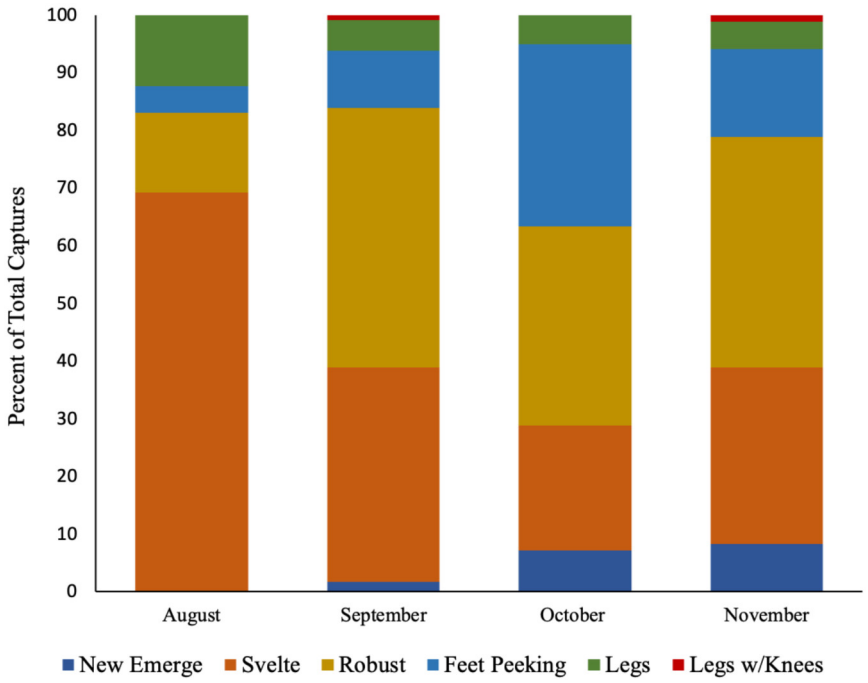

Figure 3.7. Larvae cohort captures per month of *A. truei* across surveyed reaches in Northwestern British Columbia, Canada. Bars represent the percent a cohort class represents of the total and are colour-coded based on cohort type.

I categorized the cohorts into two different pool sets (Table 3.2). The first (Pool 1) was a comparison of larvae with no external limbs against larvae with external limbs. In the second (Pool 2), 'svelte' and 'robust' were retained as unique classes, while the cohorts with limbs were pooled. Logistic regression was used for the former as the explanatory variable included two pool categories, and multinomial logistic regression for the later to accommodate three categories.

The top-ranked model for the comparison of larvae with no external limbs against larvae with external limbs included slope, vegetation cover, and date ($w_i = 0.53$; Table 3.4).

Table 3.4. Summary of AIC results for logistic regression models representing the distributions of *A. truei* larvae with visible limbs and no visible limbs in surveyed streams of northwestern, British Columbia, Canada. Italicized coefficients had 95% confidence intervals that crossed zero. Included is the predictive accuracy estimate (ROC AUC) for the top ranked models.

Model	Rank	k	AIC_c	ΔAIC_c	w_i	AUC (95% Conf.)
* + ***Veg Cover*** + **Slope**	1	4	535.96	0.00	0.53	0.78 (0.60–0.97)
* + ***Elevation*** + ***Aspect*** + **Slope**	2	6	539.05	1.74	0.22	0.59 (0.39-0.79)
* + **Slope** + ***Profile***	3	5	529.20	2.90	0.12	0.67 (0.48-0.86)
* + **Slope** + $Depth^2$ + $Embed^{\propto}$	4	8	550.09	4.18	0.07	0.69 (0.58-0.79)
* + $Width^2$ + $Depth^2$ + $Slope^{\propto}$	5	7	537.33	4.72	0.05	0.67 (0.54-0.80)
* + ***Elevation*** + $Width^{2\propto}$	6	5	530.35	8.50	0.01	
* + ***Substrate Cover*** + **Slope** + ***Temp***	7	9	527.45	9.87	<0.001	
* + ***Veg Cover*** + ***Temp***	8	4	540.98	11.60	<0.001	
* + ***Substrate Cover*** + ***Profile*** + **Slope**	9	10	545.38	12.20	<0.001	
* + $Width^2$ + ***Profile*** + $Embed^{\propto}$	10	10	531.63	12.93	<0.001	
* + ***Temp*** + ***Profile***	11	5	539.65	13.46	<0.001	
* + $Width^2$ + $Depth^{2\propto}$	12	6	540.92	13.53	<0.001	
* + ***Profile*** + ***Aspect***	13	6	542.83	15.38	<0.001	
* + $Width^2$ + $Substrate\ Cover^{\propto}$	14	9	532.17	17.93	<0.001	
* + ***Substrate Cover*** + ***Profile*** + ***Embed***	15	12	546.83	19.38	<0.001	
* + ***Substrate Cover*** + ***Temp*** + ***Aspect***	16	10	540.38	22.63	<0.001	

*Date, included in all models

$^{\propto}$Both linear and quadratic terms used in models

The second-ranked model included slope, aspect, elevation, and date ($\Delta AIC_c = 1.74$, $w_i = 0.22$). Five models accounted for 98% of the w_i, and all included a coefficient for slope. Slope and date were the only statistically significant coefficients in the top models. For the top-ranked model, the slope and date coefficients were positively correlated with the presence of limbed larvae (Fig. 3.8). Conversely, vegetation cover was negatively correlated; however, it was not statistically significant ($p > 0.05$). Slope and date were also positively correlated with limbed larvae in the second ranked model (Fig. 3.8). The AUC score for the top model was 0.78 (S.E. = 0.094), suggesting a "good" model. The second ranked model had an AUC score of 0.59 (S.E. = 0.10), suggesting poor predictive accuracy (Table 3.4).

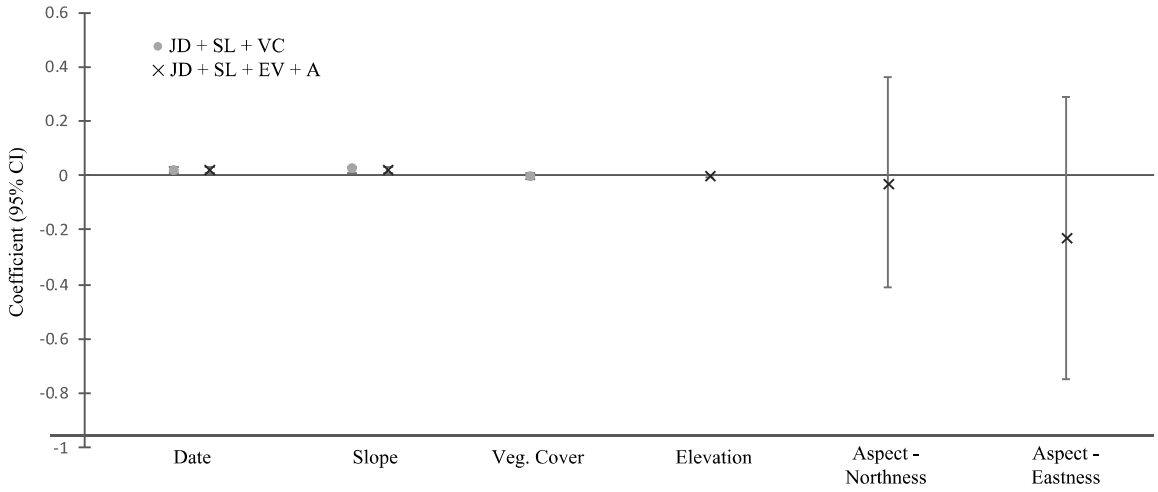

Figure 3.8. Model coefficients and 95% confidence intervals of the top-ranked logistic regression models ($\Delta AIC_c < 2.0$) representing environmental influences on the distribution within streams of *A. truei* larvae with visible limbs compared to no visible limbs in northwestern, British Columbia, Canada.

The second comparison (Pool 2) contained the ‘svelte', 'robust', and 'limbed' cohort groups. ‘Robust’ served as the comparison cohort for all models. The multinomial logistic regression resulted in a single best model ($w_i = 0.61$; Table 3.5), and included slope,

elevation, aspect, and date (Fig. 3.9). Date and elevation were statistically significant for the 'svelte' cohort, and negatively correlated with the observed distribution of the ‘robust’ cohort. Slope was statistically significant and positively correlated with the distribution of ‘limbed’ larvae. The AUC scores were 0.69 (S.E. = 0.030) when differentiating ‘svelte' from the ‘robust’ cohort, 0.72 (S.E. = 0.035) for ‘svelte' versus ‘limbed’, and 0.66 (S.E. = 0.064) for ‘robust’ versus ‘limbed’ (Table 3.5).

Three additional models accounted for the remaining w_i for Pool 2, though ΔAIC_c scores for those models were greater than 2. The second-ranked model included date, substrate cover, profile, and embeddedness ($\Delta AIC_c = 2.67$, $w_i = 0.16$). Of the models that explained 95% of the w_i, this was the only model that did not include slope. Date and low embeddedness (1–25%) were negatively and positively correlated with the distribution of the ‘svelte’ cohort, respectively. The 'limbed' pool was positively correlated with all categories for embeddedness (low, medium, and high) compared to the observed distribution of the ‘robust’ cohort. AUC scores were 0.71 for ‘svelte' versus ‘robust’, 0.68 for ‘svelte' versus ‘limbed’, and 0.68 for ‘robust’ versus ‘limbed’ (Table 3.5).

The third-ranked model included date, slope, depth (quadratic), and embeddedness ($\Delta AIC_c = 2.79$, $w_i = 0.15$). This model had the same statistically significant coefficients as the second-ranked model; however, all embeddedness categories were statistically significant and positively correlated with the distribution of the ‘svelte' cohort. AUC scores were; 0.68 (S.E. = 0.030) for ‘svelte' versus ‘robust’, 0.68 (S.E. = 0.034) for ‘svelte' versus ‘limbed’, and 0.68 (S.E. = 0.035) for ‘robust’ versus ‘limbed’. The fourth top model had a delta AIC_c of 5.28 with a w_i of 0.04, suggesting considerably less support.

Table 3.5. Summary of AIC results for multinomial logistic regression models representing the distributions of *A. truei* larvae categorized as 'svelte', 'robust', and 'limbed'. Streams were surveyed in northwestern, British Columbia, Canada. Symbols for variables are defined in Table 3.1. Italicized coefficients had 95% confidence intervals that crossed zero. Included is the predictive accuracy estimate (ROC AUC) based on logic regression of binary components for the top ranked models; components are: (1) 'svelte', (2) 'robust', (3) 'limbed'.

Model	Rank	k	AIC_c	ΔAIC_c	w_i	AUC (95% Conf.) 1v2	AUC (95% Conf.) 2v3	AUC (95% Conf.) 1v3
* + ***EV*** + ***A*** + **SL**	1	6	1035.47	0.00	0.61	0.69 (0.63-0.75)	0.66 (0.53-0.78)	0.72 (0.65-0.79)
* + ***SC*** + ***P*** + ***EM***	2	12	1038.14	2.67	0.16	0.71 (0.65-0.76)	0.68 (0.61-0.75)	0.68 (0.62-0.75)
* + **SL** + D^2 + $EM^{\propto}$	3	8	1038.26	2.79	0.15	0.68 (0.62-0.74)	0.66 (0.59-0.73)	0.68 (0.16-1.20)
* + ***SC*** + ***P*** + **SL**	4	10	1040.75	5.28	0.04	0.65 (0.59-0.71)	0.62 (0.53-0.70)	0.65 (0.57-0.74)
* + W^2 + ***P*** + $EM^{\propto}$	5	9	1041.62	6.15	<0.001			
* + ***SC*** + **SL** + ***T***	6	9	1047.21	11.74	<0.001			
* + ***SC*** + ***T*** + ***A***	6	10	1047.21	11.74	<0.001			
* + ***EV*** + $W^{2\propto}$	7	5	1047.55	12.08	<0.001			
* + **SL** + ***P***	8	5	1052.35	16.88	<0.001			
* + ***P*** + ***A***	9	6	1053.53	18.06	<0.001			
* + W^2 + $SC^{\propto}$	10	9	1053.66	18.19	<0.001			
* + ***VC*** + **SL**	11	4	1053.81	18.34	<0.001			
* + W^2 + D^2 + $SL^{\propto}$	12	7	1056.15	20.68	<0.001			
* + ***T*** + ***P***	13	5	1062.55	27.08	<0.001			
* + ***VC*** + ***T***	14	4	1064.98	29.51	<0.001			
* + W^2 + $D^{2\propto}$	14	6	1064.98	29.51	<0.001			

*Date, included in all models

$^{\propto}$Both linear and quadratic terms used in models

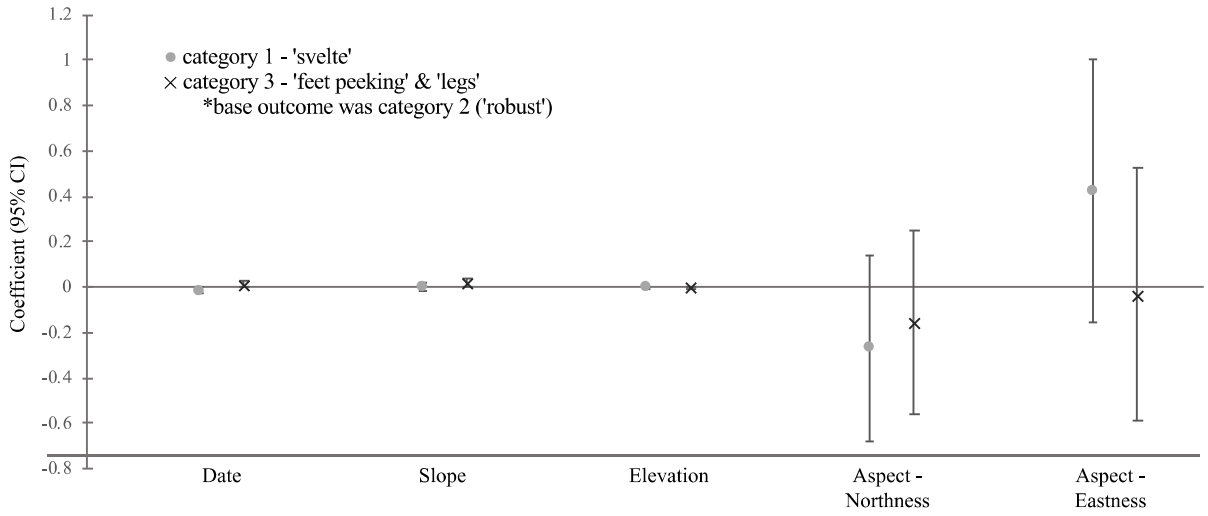

Figure 3.9. Model coefficients and 95% confidence intervals of the top-ranked multinomial logistic regression model ($\Delta AIC_c < 2.0$) representing environmental influences on the distribution within streams of *A. truei* larvae categorized as 'svelte', 'robust', and 'limbed'. Streams were surveyed in northwestern, British Columbia, Canada.

DISCUSSION

A. truei are sensitive to changes in their environment, specifically to the structure of streams and to forest overstory; thus they have been well studied throughout their range. Larvae are the most well-studied life stage, as they occur at a higher density and they are easier to survey (de Vlaming and Bury, 1970; Wahbe, 1996; Diller and Wallace, 1999; Dupuis and Steventon, 1999; Dupuis et al., 2000; Adams and Bury, 2002; Karraker et al., 2006; Kroll et al., 2008; Stoddard and Hayes, 2005). My study is one of the few to relate an index of abundance to a collection of environmental factors hypothesized to influence population density. This is one of the first studies to assess the spatial segregation of larvae at various developmental stages. This is especially important at the northern extent of *A truei's* range where relatively short summers and cool climates result in a long period of stream residence for larvae (likely 4 years).

Abundance of Larvae

The top-ranked model explaining variation in larval abundance included a covariate representing the wetted width of the stream. While controlling for all other covariates in that model, capture rates decreased in a nonlinear fashion as wetted width increased. Capture rates increased in streams greater than 6 m, though we sampled few streams that exceed 6 m in width (range: 0.6 m–8.3 m).

Wetted width has been related to the distribution and abundance of larvae, though width was considered a proxy for other environmental factors (Wahbe, 1996; Dupuis and Steventon, 1999; Kroll et al., 2008). For example, width may be correlated with drainage size, or the combined complexity and size of a network of streams (Hayes et al., 2006). Kroll et al. (2008) observed a nonlinear relationship between stream width and occupancy, and suggested that peak occupancies in mid-sized streams was the product of small drainages having few perennial streams and large drainages having flow-rates that were too high for *A. truei* larvae.

I assessed streams of a similar size as reported by Kroll et al. (2008; 0.2–6.0 m). My results also reflected a nonlinear relationship, but abundance was greater in narrow and wide streams. This discrepancy in results could be a function of differences in sampling protocol. I recorded width at the location of capture while Kroll et al. (2008) recorded it once per surveyed stream. Also, I related width to a measure of relative abundance, not stream occupancy as recorded by Kroll et al. (2008). Potentially, their results reflect the ease of surveying in smaller and mid-sized streams, though the increased capture rates in larger streams contradicts that explanation.

It is not uncommon for the ecology of amphibians to differ across their range. As examples, the larvae of *Rana boylii* (foothill yellow-legged frog) developed faster in inland compared to coastal rivers, and *Lithobates sylvaticus* (wood frog) selected smaller and larger wetlands depending on the surrounding terrestrial environment (Cunningham et al., 2007; Groff et al., 2017). Similarly, *A. truei* may demonstrate differences in ecology across their roughly 3000 km south to north distribution. These inherent differences in ecology may account for observed variation in the use of streams by larvae. For example, a top predator species, *Dicamptodon tenebrosus* (coastal giant salamander) has been shown to influence larvae activity and distribution, but is not present near the northern extent of *A. truei*'s range (Feminella and Hawkins, 1994). And as noted previously, the length of the larval phase varies from 1–4 years depending on latitude and elevation. Residence time in the stream may influence the habitat ecology of larvae.

At the northern extent of its distribution, the density of *A. truei* larvae increased with stream bank width, and that relationship was associated with substrate composition (Dupuis and Steventon, 1999). Abundance, measured as larvae biomass and density, decreased with increasing wetted width near Squamish BC (Wahbe, 1996). Wahbe (1996) suggested that this relationship reflected the covariation of stream width with changes in water temperature and solar exposure. She hypothesized that larvae abundance increased with low temperatures and greater sunlight, or higher temperatures and less sunlight. It is unclear if Wahbe (1996) tested for a nonlinear relationship between width and abundance, but the raw data (Wahbe 1996, Fig. 3) suggest that biomass was greatest in narrow and wide streams, similar to my results.

The rate of larvae captures increased as the sampled stream narrowed and deepened, suggesting a change in abundance with a change in the rate of water flow. As with my work, past studies of *A. truei* used categorical variables (i.e., cascade, riffle, and pool) to quantify stream profile, a coarse or approximate measure of water flow. The coarseness of this measure was apparent in my data as the majority of capture locations were recorded as 'riffle'. A variable representing the combined measurements of width, depth, and slope at the point of capture may have provided a more comprehensive depiction of stream profile. The rate of water flow may be an additional determinant of the abundance of larvae; although, this variable has not been reported in the literature. Future microsite research should include flow meter measurements to confirm the importance of water flow.

Relative Distribution of Larval Cohorts

The slope of the sampled stream influenced the distribution of cohort ages with older cohorts being captured in reaches with greater slope. One potential explanation is that larger-sized larvae can maintain their position in streams with greater slope and corresponding flow. They may select these locations for enhanced concealment, or as a means to reduce competition with younger larvae that are not physically able to occupy high-flow environments.

The interspecific and intraspecific interactions of *A.truei* are among the most poorly understood aspects of this species' ecology. Several studies have demonstrated that larvae of different species will compete for food (Kupferberg, 1997, McDiarmid and Altig 1999). Relative to intraspecific competition, Petranka and Sih (1986) reported that *Ambystoma texanum* (small-mouth salamander) larvae grew faster, larger, and had more food in their stomachs when there were less *A. texanum* larvae in the river.

A. truei grazing significantly reduced periphyton crop in the northern half of its range (Lamberti et al., 1992; Kiffney and Richardson, 2001; Mallory and Richardson, 2005). Larvae selected the largest substrate at night for foraging and either the same substrate or slightly smaller adjacent substrate for concealment during the day (Altig and Brodie, 1972; Hawkins et al. 1988). My observation of cohort-specific selection of stream slope may be a strategy by which older and larger larvae reduce foraging competition by occupying stream reaches with greater flow velocity, and this stratification is evident in the substrates they use for daylight concealment.

Wahbe and Bunnell (2001) proposed that larvae of *A. truei* drift downstream and the adults migrate upstream. Downstream drift occurred in the Oregon Coast Range of Oregon USA, but steeper gradient streams had less downstream movement (Chelgren and Adams, 2017). However, Hayes et al. (2006) found older larvae (2nd year) higher along a watershed than younger larvae (1st year) in southwestern Washington, USA. My data provided little evidence of downstream drift for older cohorts in the northern extent of its range, as elevation was not a highly influential factor. This may reflect a limited range of surveyed elevations in my study (135-700m).

Future research should look to the home range of *A. truei* larvae within their natal streams throughout a 4-year larval stage. There may be an area of given size that larvae exist in between egg clutch site and the location of post-metamorphic emergence. I recommend a consideration of greater micro-scale movements in *A. truei* larvae than merely downstream drift.

Implications for Habitat Management

A. truei is of conservation concern across much of its North American distribution. A slow life history and reliance on forests and fast-flowing streams suggest the species is sensitive to forest harvest, other forms of land clearing, and obstructions or modifications to streams. In B.C., current management objectives focus on forest practices and run-of-the-river hydroelectric development (Environment and Climate Change Canada, 2018).

My objective was to quantify the relationship between the density of *A. truei* larvae and environmental factors previously hypothesized to affect their distribution. There is still much to be learned from examining that relationship; however, my findings and those of others consistently suggest that optimal habitat is related to stream width and depth. Also, I revealed intraspecific differences in distribution. Most notably, limbed and larger larvae were more often found in steeper slopes relative to younger cohorts.

This work is a foundation for future research on the distribution of *A. truei* larvae near the northern extent of their range. Research should include the relationship between the use of microhabitat and water velocity and, if possible, dissolved oxygen. Studies on larger stream reaches to confirm the lack of downstream drift (Chelgren and Adams, 2017) will increase our understanding of the population dynamics of larval and post-metamorphic frogs. This work will help inform where and how riparian management zones and habitat reserves are established, and the potential impacts of road and pipeline crossings along a stream reach.

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EPILOGUE

A. truei is an ancient species of frog, adapted for life associated with cool mountain streams in the northwest of North America. This species is of conservation interest because large portions of its range are managed for timber harvest, and disturbances associated with forestry may reduce habitat quality (Hayes and Quinn, 2015). The impacts of forestry and other disturbances may be amplified by warming climates. Comprehensive studies of the habitat and movement ecology of the species will aid in appropriate conservation actions to maintain the long-term persistence of *A. truei*.

I performed three studies at varying spatial and temporal scales near the northern extent of *A. truei*'s geographic range. It is important to disentangle the influences of the distant past (e.g., glaciation) on population structure from the more recent (e.g., availability of prey species). Understanding specific habitat requirements helps identify key aspects of their environment to target for protection. Additionally, constructing the patterns and extent of genetic divergence at different points along a geographic range is crucial for developing effective conservation and management plans (Allendorf and Luikart, 2007).

The Phylogeography

At the broadest scale, I completed a phylogeography of *A. truei* across the northern half of its range. Herptofauna, generally speaking, have lower dispersal abilities and narrower temperature requirements compared to mammals and birds. *A. truei*, specifically, has high habitat specialization and one of the narrowest temperature requirements of any species of North American frog. I found a reduction in genetic diversity along the northern

half of its range. My results indicate a northern expansion of the species' range following the Pleistocene (~10,000 years ago), most likely from a single refugium.

Populations of many species were restricted and often isolated during the Pleistocene glaciations, becoming genetically differentiated over time (e.g. Avise, 2001). Species colonized newly available habitats as the glaciers receded, resulting in a reduction of genetic variation along the leading edge of expansion. In the Pacific Northwest, those species specialized to habitats across the coastal and Cascade mountains had lower genetic diversity at the northern periphery of their distribution when compared to species with broader ranges (Brunsfeld et al., 2001; Kuchta and Tan, 2005; Steele and Storfer, 2006).

Mitochondrial haplotypes are the most commonly used genetic marker for studies on northern range expansion following the Pleistocene glaciations (e.g., Shafer et al., 2010). As examples, mtDNA was used to understand the range expansion of *Thamnophis sirtalis* (common garter snake; Janzen et al., 2002), a widely distributed reptile throughout the northwest of North America, and *Taricha granulosa* (rough-skinned newt; Kutcha and Tan, 2005), an amphibian whose range is restricted to the coastal and Cascade mountain ranges. The phylogeography of *T. sirtalis* suggested three geographic clades, though the historical relationships of the northern clades could not be fully resolved. The results of Janzen et al. (2002) suggested multiple refugia, with a possible island refuge on Haida Gwaii, followed by admixture across the northern portion of their range from the refugia.

T. granulosa also had three geographic clades (Kutcha and Tan, 2005). Conversely, the northern clade covered greater than 900 km and was a tight grouping. The results of Kutcha and Tan (2005) suggested only southern refugia and a northern expansion. My results for *A. truei* suggest a similar range expansion as observed for *T. granulosa*. I found a

single mtDNA haplotype throughout B.C., and my mtDNA results suggested a northern range expansion from a southern refugium.

Mitochondrial DNA has a very low (negligible) rate of recombination, a mostly maternal inheritance and a relatively rapid rate of evolution (Rubinoff and Holland, 2005). The degree of diversity in more variable genomic DNA is not always reflected in mtDNA (McCartney-Melstad, 2018). Analyzing nuclear DNA, in addition to mtDNA, provides a more informative and thorough reconstruction of the relationships between populations. A single haplotype throughout B.C. limits the resolution of mtDNA; therefore, I also used genomic DNA to better understand the phylogeography of *A. truei* in B.C. I found an unexpectedly dramatic reduction in the diversity of genomic DNA along the northern half of *A. truei*'s range. My southcoast region, the area surrounding Chilliwack B.C., showed greater genetic diversity with strong relatedness to the other B.C. regions. It would be interesting to see if *T. granulosa* has a similar, sharp reduction in genomic diversity and if there are indications of bottleneck effect or founders' effect in that species as well.

The phylogeographies of *T. granulosa* and *A. truei* suggest a northern range expansion into B.C. from a single refugium. The Klamath-Siskiyou Mountains is a likely refugium for *A. truei* (Nielson et al., 2001, 2006) and *T. granulosa* (Kutcha and Tan, 2005), along with many other species of flora and fauna. The Columbia River valley has been suggested as a refuge for several species of amphibian, including *Anaxyrus boreas* (western toad; Goebel et al., 2009), *Batrachoseps wrighti* (Oregon salamander; Miller et al., 2005), *Plethodon larselli* (Larch Mountain salamander, Wagner et al., 2005), *Dicamptodon tenebrosus* (Pacific giant salamander; Steele and Storfer, 2006), and *Dicamptodon copei* (Cope's salamander; Steele and Storfer, 2007). Future research should explore the Columbia

River valley as a second refugium for *A. truei* and determine if coalescence between the two refugia has occurred in Oregon and Washington.

Wakeley (1999) described two phases in genetic data when considering a migration model: the collecting phase and the scattering phase. The collecting phase reflects an older historical time dimension within the genetic signatures. That phase occurs when interbreeding groups (demes) are separated from each other. In my phylogeography, refugia were separated by unsuitable habitat. Migration events occur until a deme is established by migrants from different groups and coalesces.

This pattern of population establishment may explain the high variability in genomic DNA in the Washington populations. During the collecting phase, those populations are drawing on genomic DNA from multiple refugia. At the scale of sampling across B.C., demes have not yet had the opportunity for coalescence from multiple refugia and are still reflecting the collecting phase.

The second phase is the scattering phase and it depends on the current number of demes, the sizes of demes, and the current migration rate (Wakeley, 1999). The length of the scattering phase depends mostly on the carrying capacity of the sampled geographic region. Habitat fragmentation alters the relative length of the scattering and collecting phases (Mona et al., 2014). However, long distance dispersal can shorten the scattering phase and counteract the potential deleterious effect of fragmentation.

In Washington, USA, gene flow was extensive across terrestrial habitats with no indication of connectivity based on watershed (Spear and Storfer, 2008). Juveniles are the suggested dispersers for populations of *A. truei* (Daughtery and Sheldon, 1982; McEwan, 2014). Near the northern extent of its range, canopy cover influenced the movements of

adult and juvenile frogs as fewer frogs were caught in locations with open canopies (McEwan, 2014). When considering conservation and management plans for *A. truei*, we should minimize habitat fragmentation and maximize opportunities for migration by maintaining linked forested habitats. These actions will help maintain historic gene flow and maintain genetic diversity in local populations.

The Ecology of Larvae

As with many frogs, *A. truei* larvae interact with their aquatic environment to build up energy, transform, and leave for terrestrial systems. Changes in environmental characteristics of their streams may have important implications for the health of *A. truei* populations. I compared the gut-contents of larvae from stream reaches near the northern extent of the frog's range. I also related a set of environmental factors to the abundance of larvae and the spatial segregation of various developmental stages.

The interspecific and intraspecific interactions of larvae are among the most poorly understood aspects of this life stage. I found little evidence for downstream drift in older cohorts as they were captured in stream reaches with greater slopes than younger cohorts. Also, I did not find variation in the diets of different development classes; however, my results may reflect the limited number of samples and the inaccurate representation of the number of cohort classes near the northern extent of *A. truei*'s range.

Wetted stream width, likely one proxy for the flow rate of water within a stream reach, correlated with differences in the abundance of larvae and the contents of their guts. The capture rate of larvae increased as a sampled stream reach narrowed, though the relationship was nonlinear. When all OTUs, OTUs with taxonomic identification, or OTUs of phylum Arthropoda were included gut contents differed between sampled stream reaches.

Changes in water flow and substrate size will have important implications for larvae. Human activity that creates more uniform stream structure, or minimizes the ability of larvae to make multiple movements throughout their development, may have adverse impacts on the success of larvae. Climate change may intensify the impacts of humans on stream health.

According to Mote and Salathé (2010), annual mean temperature is expected to rise 0.1°C per decade. Leung et al. (2004) predicted a reduction in precipitation west of the Cascades and increases in extreme daily precipitation. Most streams in the Coastal Mountains of B.C. are fed by snowpack accumulated during the winter months and are strongly impacted by how rapidly water levels rise and fall with precipitation during the driest months. Impacts of climate change for this geographic region will include a reduction in snowpack, and changes in the frequency and intensity of precipitation.

It is unclear exactly how future climate change will affect streams in the Coast Mountains of B.C. However, we should prevent the direct modifications of stream structure, such as in-stream siltation and debris accumulation due to cross-stream yarding. Such activities can be managed, and that management will ultimately minimize the cumulative changes to stream dynamics. I also suggest plans that protect the connectivity of terrestrial habitats to ensure the continued large-scale movement over time of *A. truei*.

We are continually discovering fascinating new information about this ancient species. From its role within headwater streams and mountainside coniferous forests to its genome and genetic expression. As global climates warm and precipitation patterns change, more informed management plans will help ensure appropriate actions to protect this unique frog species.

Conservation and Management

Management suggestions presented in this dissertation confirm recommendations made by others, including those detailed in a plan released by the government of Canada (Environment and Climate Change Canada, 2018). Congruous recommendations to those previously outlined include: avoid stream sedimentation, avoid cross-stream yarding, develop road designs that minimize impacts to streams, maintain natural water flow, and maintain structural complexity of terrestrial habitats. Difficulties lie in the implementation, and the key to the conservation of *A. truei* is to ensure strong links between new research findings, government regulation, and forestry practices.

The government of B.C. has regulations related to riparian retention around fish-bearing streams (Riparian Areas Regulation, 2019), and unclear guidelines related to riparian management around non-fish-bearing streams. Currently, there is a disconnect between management recommendations for *A. truei* and government regulation as the focus is on fish-bearing streams. Terrestrial habitats that are optimal for *A. truei* include coarse woody debris (CWD), and the Forest and Range Practices Act sets out legal minimums for CWD. These regulations may not ensure appropriate structural complexity available for the large-scale movements of *A. truei*.

Opportunities exist to provide requirements or incentives for the protection of this frog species, and will benefit other species that rely on non-fish-bearing streams and structurally complex forests. Regulations on the retention of riparian zones can be extended to include permanent streams with no reported presence of fish. The legal minimums for retained CWD in the Forest and Range Practices Act can be increased. Alternatively, certification programs can be created that focus on under-protected streams or current

certification programs can be adjusted to include them (such as the Canadian Standards Association; Clark and Kozar, 2011). Certification programs may also be an excellent option for incentivizing the retention of CWD. Additionally, multinational certification programs, such as the Sustainable Forestry Initiative, provide an opportunity to extend requirements related to non-fish-bearing streams and CWD across international and state borders in Canada and the United States. Any incentives or new regulations should be supported by well-funded monitoring programs and enforcement.

A. truei is a figurehead for the protection of non-fish bearing streams and the understory of forests in the Pacific Northwest. Developing incentives or regulations for the protection of this species will help maintain habitats for a variety of other species. We must maintain a natural mosaic of clean fast-flowing streams and forests across these fog and rain-drenched mountains.

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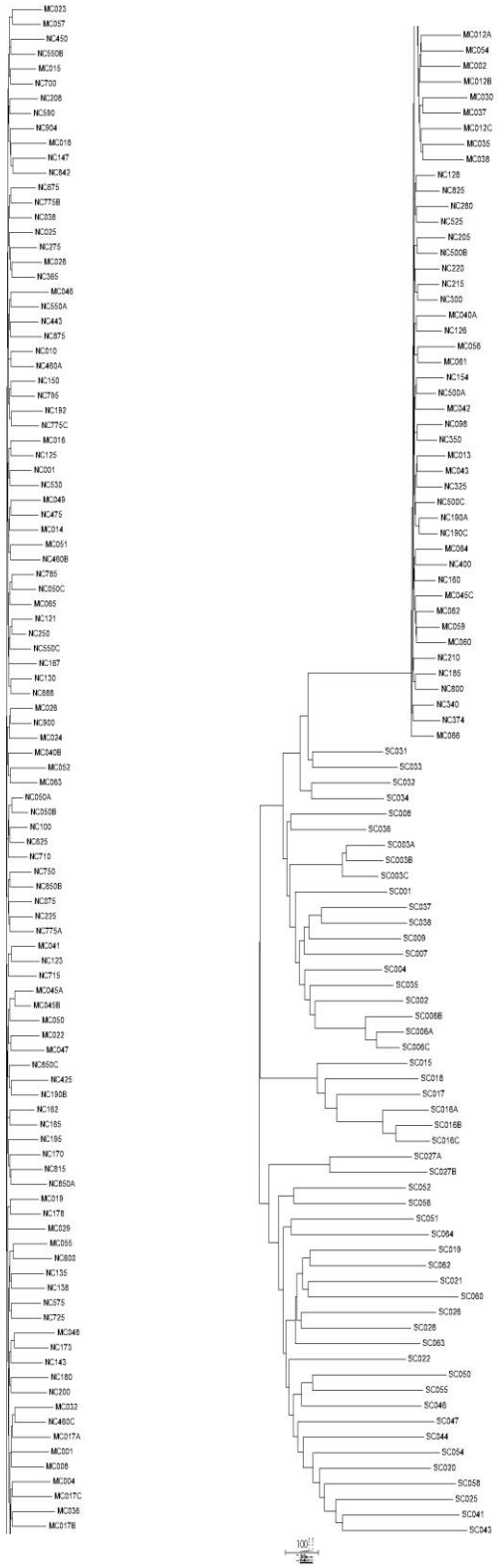
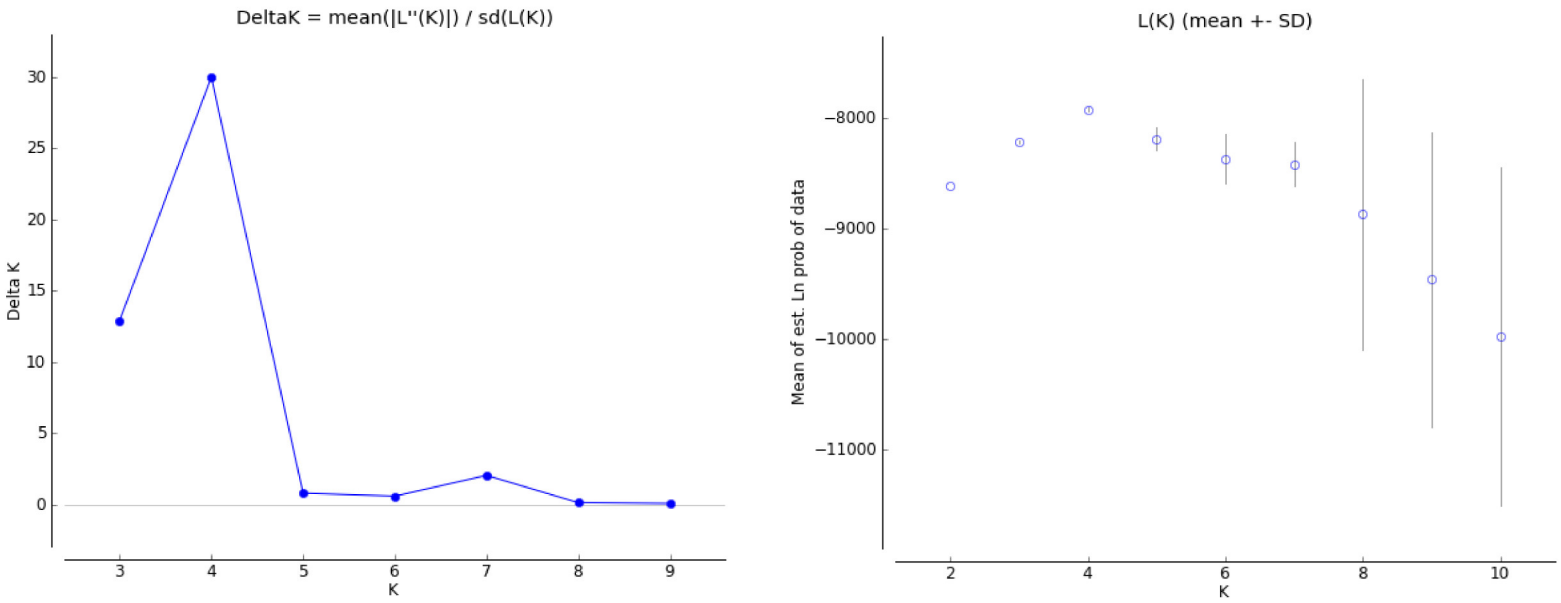
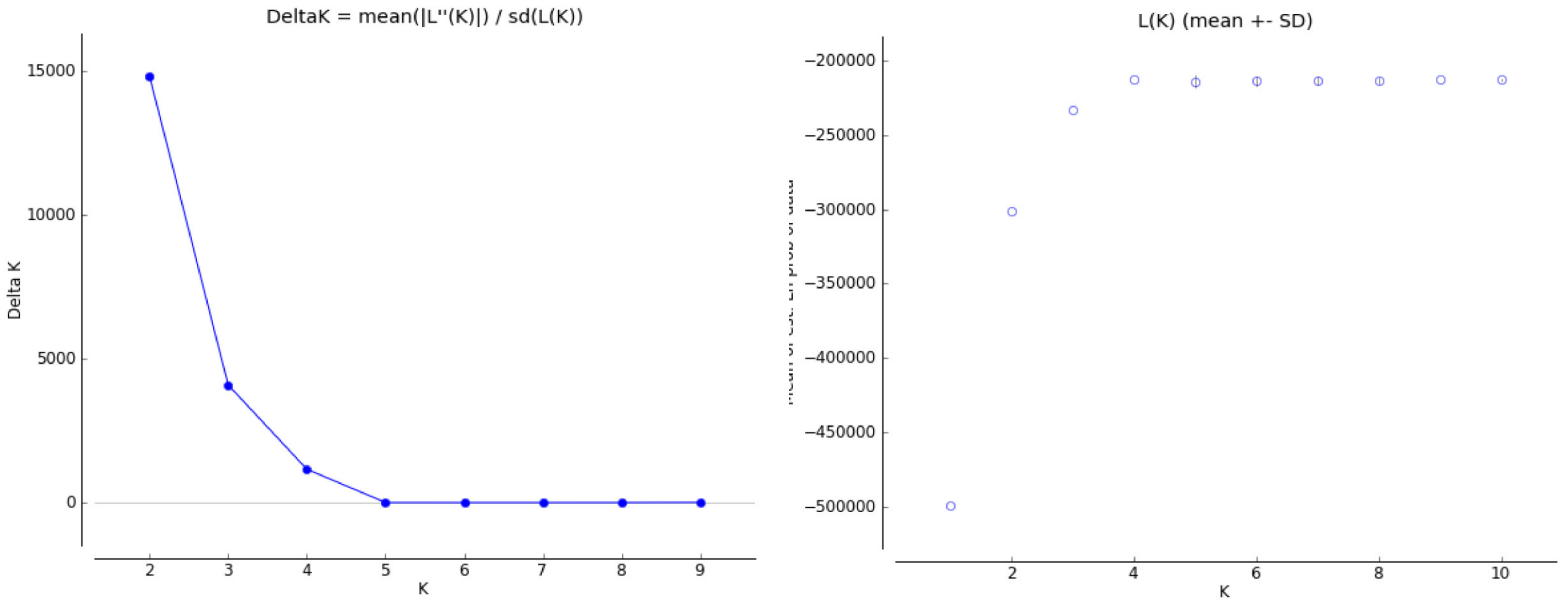
APPENDIX 1



A1.1. Phylogeny of triplicate and non-triplicate nextRAD genotypes for *A. truei* in three geographic regions in B.C. 'NC' designates genotypes from around Terrace, BC, 'MC' from around Bella Coola, BC, and 'SC' around Chilliwack, BC. Triplicates have the same genotype identification and are distinguished by an 'A', 'B', or 'C'.

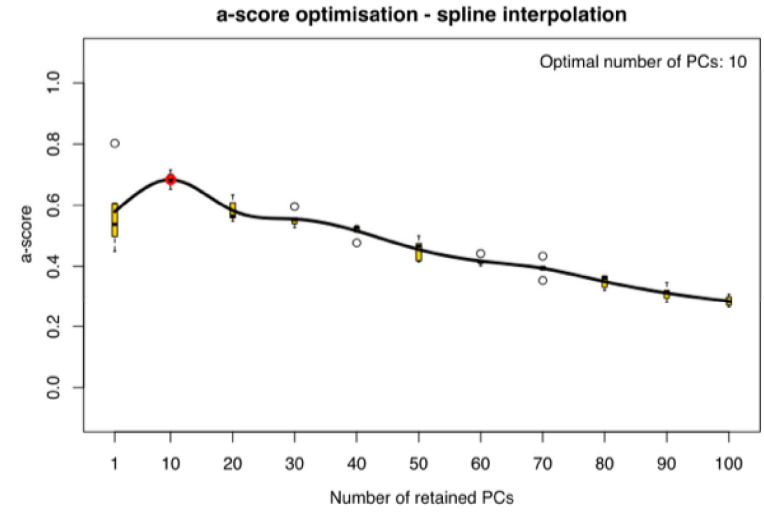


A1.2. The delta *K* and Ln *P*(D) values from the program STRUCTURE and compiled by the program STRUCTURE HARVESTER for microsatellite genotypes of *A. truei* from 5 geographic regions along the northern half of its distribution. Genotypic clusters (K) ranged from 2 to 10.

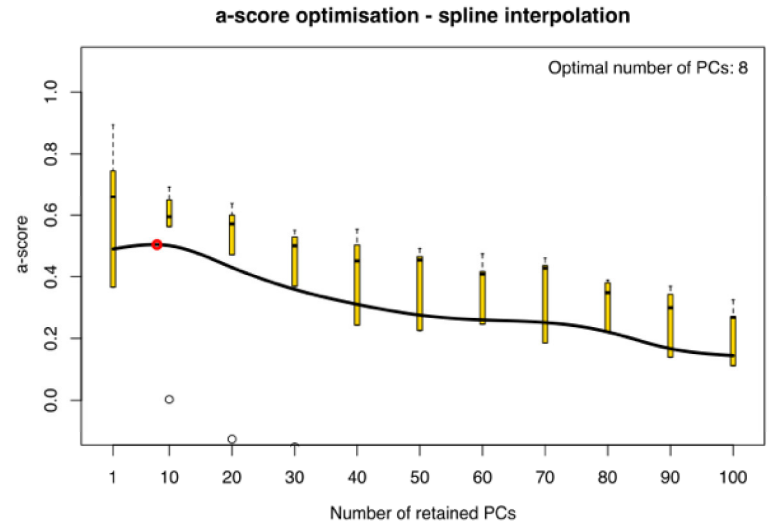


A1.3. The delta *K* and Ln *P*(D) values from the program STRUCTURE and compiled by the program STRUCTURE HARVESTER for nextRAD sequencing genotypes of *A. truei* from 5 geographic regions along the northern half of its distribution. Genotypic clusters (K) ranged from 2 to 10.

(a)

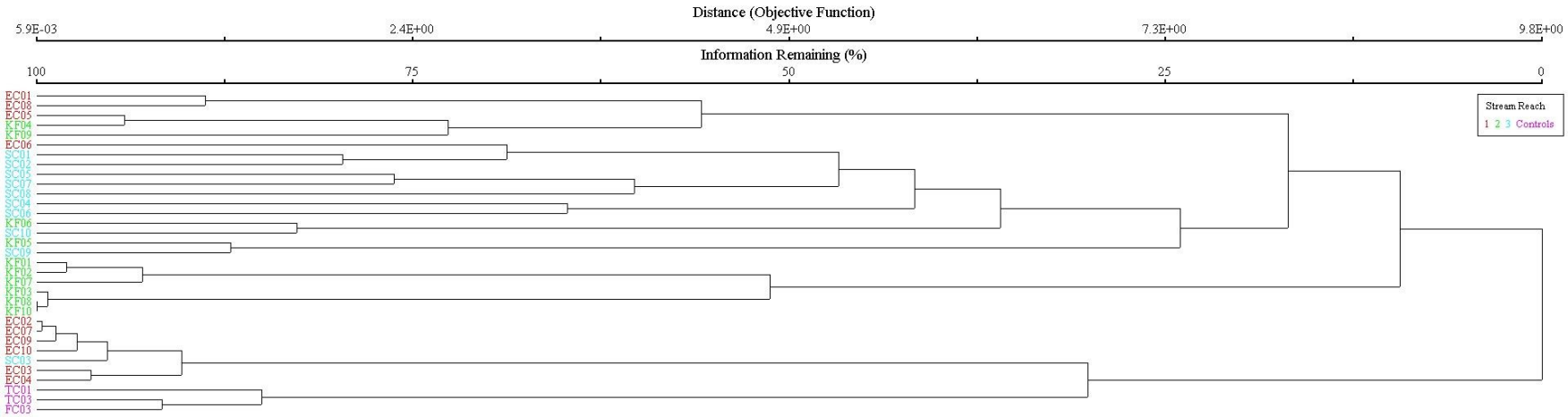


(b)



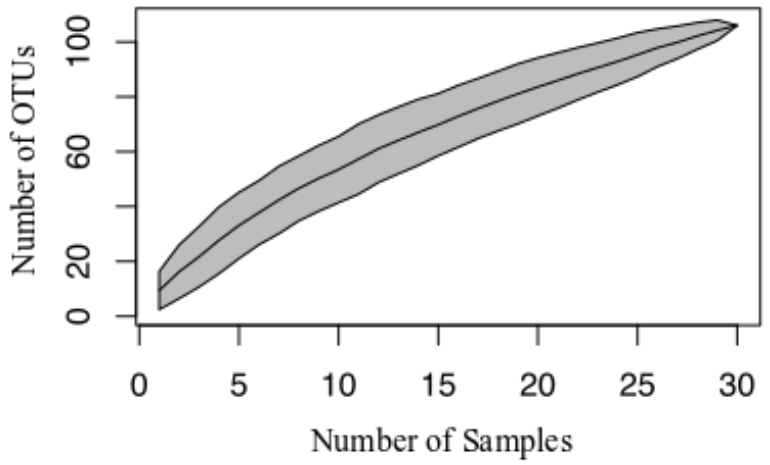
A1.4. The a-scores of principle components for (a) microsatellite genotypes and (b) nextRAD sequencing genotypes for *A. truei* across the northern half of its range. The red circle represents the optimal number of PCs to retain for discriminant analysis of principle components.

APPENDIX 2

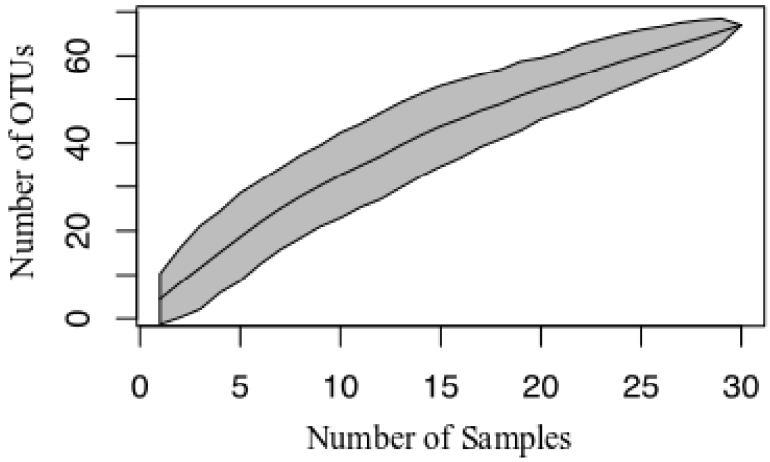


A2.1. Dendrogram of the 5' end of the cytochrome c oxidase subunit 1 (COI) in gut contents of lab-raised and wild-caught larvae of *A. truei*. Pink represents the guts from lab-raised larvae. The rest are color coded based on their natal stream reaches; red for 'Erlandsen Creek', green for 'Kalum Feeder', and blue for 'Shames Creek'.

(a)

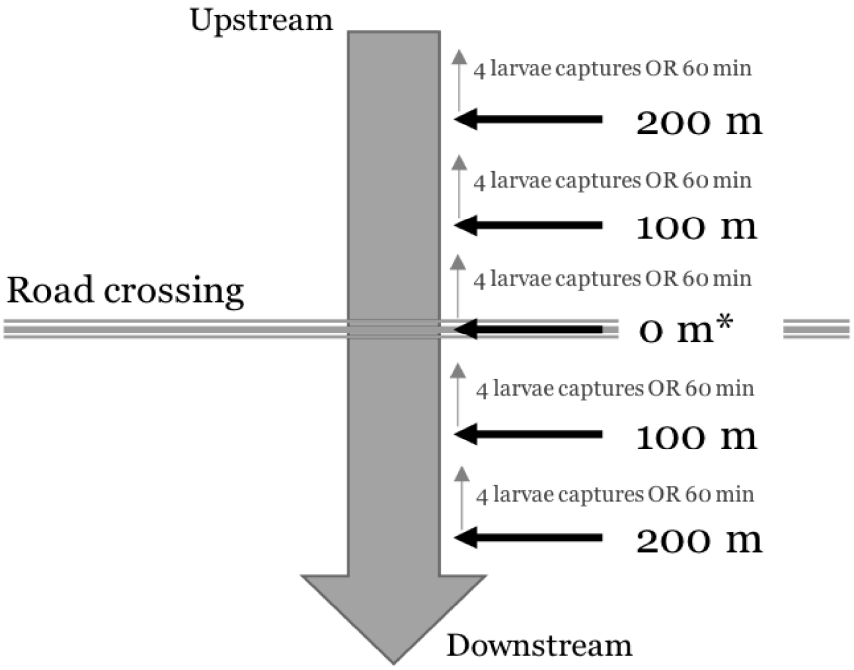


(b)



A2.2. Species accumulation curve and 95% confidence interval for the gut contents of wild-caught *A. truei* samples with (a) operational taxonomic units (OTUs) with taxonomic assignment and (b) OTUs with taxonomic assignment in the phylum Arthropoda. Larvae were collected from three stream reaches northwest of Terrace, British Columbia, Canada, during August 2016. The 5' end of the cytochrome c oxidase subunit 1 (COI) was targeted using insect-specific primers.

APPENDIX 3



A3.1. Generalized schematic of survey method for *A. truei* larvae in northwestern British Columbia, Canada. The 0 m mark was the first surveyed location.

A3.2. Fine-scale habitat assessment array used to measure percent cover of substrate sizes over the point of capture for *A. truei* larvae in northwestern British Columbia, Canada.