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Chemical ecology and genetics of rough-skinned newts, Taricha granulosa

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Chemical ecology and genetics of rough-skinned newts, *Taricha granulosa*

by

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Abstract

Interactions between predator and prey have played a crucial role in adaptive evolutionary processes; however, phenotypic and genetic variation may also be driven by many other spatially variable biotic and abiotic factors. Rough-skinned newts, *Taricha granulosa*, possess a neurotoxin known as tetrodotoxin (TTX), which acts as an antipredator defense and was originally presumed to be a result of reciprocal coevolutionary interactions with resistant garter snakes across a geographic mosaic. In this thesis, I investigate several aspects of the chemical ecology and genetics of rough-skinned newts and consider how these factors play out on Vancouver Island, an interesting study region due to its isolation from mainland populations and recent non-native species introductions. By characterizing toxicity both within and among 23 populations of newts on Vancouver Island, I found significant variation in TTX and evidence for a previously unidentified hotspot, indicating selection pressures besides reciprocal coevolution may contribute to the observed patterns. Next, I present the first investigation into molecular mechanisms of tetrodotoxin expression in newts using an RNA-sequencing approach. By creating a *de novo* transcriptome assembly and annotation, I was able to identify novel differentially expressed genes putatively related to endogenous sources of TTX. Amphibians are also facing worldwide population declines due to factors such as negative impacts by non-native species, and Vancouver Island has experienced a recent introduction of signal crayfish and American bullfrogs. I reviewed the potential impacts of these species on rough-skinned newts and found a negative correlation between their presence and newt relative abundance, but no effect on body condition or toxicity. Using the aforementioned transcriptome assembly, I identified

thousands of single nucleotide polymorphisms in 32 newts from BC and Oregon and characterized the population genetic structure at two spatial scales. Vancouver Island newts were found to belong to a cluster genetically distinct from Oregon with lower heterozygosity while displaying a lack of population structure across the island. Collectively, these results improve our understanding of the spatial variation and genetics of chemical defense in rough-skinned newts while integrating these findings with conservation implications for Vancouver Island populations.

Preface

This thesis is the original work by the author (me), Haley Cathleen Glass, with collaborations as described below.

Chapter 2 has been formatted for publication, with some changes from the thesis version as:

Glass, H. C. & Vamosi, S. M. (2020). Evidence for previously unidentified hotspot in rough-skinned newt toxicity. Manuscript in preparation.

An earlier version of Chapter 3 without the differential expression analysis was uploaded as a pre-print to bioRxiv as:

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Dedication

To my late grandmother Nonnie, for always inspiring my love of animals and nature.

Table of Contents

List of Tables

List of Illustrations, Figures, and Graphics

List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ANOVA	Analysis of variance
BC	British Columbia
BIC	Bayesian information criterion
bp	Base pairs
BP	Biological Process (Gene Ontology category)
BUSCO	Benchmarking Universal Single-Copy Ortholog
℃	Degrees Celsius
CC	Cellular Component (Gene Ontology category)
CIEIA	Competitive Inhibition Enzymatic Immunoassay
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
DAPC	Discriminant Analysis of Principal Components
DNA	Deoxyribonucleic acid
DU	Designatable Unit
ESU	Evolutionarily Significant Unit
FDR	False discovery rate
F_{IS}	Inbreeding coefficient
F_{ST}	Genetic distance
g	Gram
GO	Gene Ontology
H_O	Observed heterozygosity
H_S	Expected heterozygosity
hr	Hour
IUCN	International Union for the Conservation of Nature
K	Number of clusters
MCMC	Markov Chain Monte Carlo (statistical algorithm)
MF	Molecular Function (Gene Ontology category)
min	Minute

MS-222	Tricaine methanesulfonate
mtDNA	Mitochondrial DNA
MU	Management Unit
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
nr	NCBI non-redundant protein database
PCA	Principal components analysis
qPCR	Quantitative (real-time) polymerase chain reaction
QTL	Quantitative trait loci
RAD-seq	Restriction side associated DNA sequencing
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
SMI	Scaled mass index
SNP	Single nucleotide polymorphism
SVL	Snout-vent length
STX	Saxitoxin
TTX	Tetrodotoxin
VCF	Variant call format
µg	Micrograms
µL	Microliters

Epigraph

The wildlife and its habitat cannot speak, so we must and we will.

-Theodore Roosevelt

CHAPTER ONE – GENERAL INTRODUCTION

1.1 Background

1.1.1 Coevolution

The evolutionary processes that take place within an environment are often complex and may be impacted by both abiotic and biotic factors. Many changes in species can be attributed to alterations in the physical environmental, and major trends such as mass extinctions are likely a result of abiotic components (Raup 1986). However, biological interactions have undoubtedly played a crucial role in the patterns of evolutionary history that have shaped the world into what it is today (Benton 2009, Voje et al. 2015). The most important agents of selection on the adaptive evolution of species can sometimes be the organisms themselves, and interactions between co-occurring species have influenced the adaptive patterns observed throughout the course of evolutionary history. Populations respond to this type of selective pressure within their biotic environment by developing behavioral, morphological, or physiological adaptations in order to gain increased fitness (Williams 1966). When two or more interacting species experience reciprocal selection pressures and evolve in response to changes in the other, this is known as the process of coevolution (Futuyma & Slatkin 1983). While the concept of coevolution goes as far back as Darwin's hypothesis that the Madagascar star orchid with a long nectary must be pollinated by a moth with a specialized proboscis (Arditti et al. 2012), the theory was later given its name by Ehrlich and Raven (1964). A coevolutionary "arms race" occurs when defenses and counter-defenses in two lineages continually increase over time due to constant mutual selection pressures (Dawkins & Krebs 1979).

Individuals in interspecific relationships such as plants and pollinators, hosts and parasites, and predators and prey exert varying degrees of selection pressures on one another across the landscape (Thompson 1994, Thompson 2005). Because interactions and their consequences can vary from one locality to another, the outcomes of coevolution and the adaptive processes that drive it may vary geographically, leading to spatially variable traits. This is known as the geographic mosaic theory of coevolution (Thomson 2005). Given variation in the strength of selection between interacting organisms, populations can evolve along different trajectories across a landscape. A classic example of coevolution across a geographic mosaic is the obligate mutualism between yucca plants (*Agavaceae*) and yucca moths (*Prodoxidae*). Adult moths are sole pollinators of the yuccas, and in return moth larvae eat the seeds of the yucca to complete their development (Riley 1872). Across their range, different species of yucca plants and moths have coevolved traits that enable this interaction to occur only between given pairs of species in order for their life cycles to complete (Pellmyr 2003).

1.1.2 Predator-Prey Dynamics

Throughout the course of evolutionary time, predation has influenced patterns in the history of life on this planet and has been especially influential in defensive adaptations, divergence, and diversification of prey (Vermeij 1987, Vamosi 2005). Adaptive traits in predators and prey are under strong selection if their fitness-related benefits exceed the costs (Abrams 2000). Predator species may develop traits which enable them to increase their chances of capturing prey, therefore exerting selection pressures on their prey. Subsequently, prey species evolve specific antipredator defenses as a result of selection pressures due to long-term association with their sympatric

predators. These defense mechanisms include body armor, increased speed or agility, camouflage coloration, venom, and toxins (Arbuckle & Speed 2015).

Selection on prey by predators tends to produce greater evolutionary responses than selection on predators by prey, and not all predator-prey interactions lead to coevolution (Vermeij 1987). Spatially variable traits across a landscape may also result from neutral genetic diversity (Holderegger et al. 2006), or due to differences in selective environments with spatially heterogeneous selection pressures (Kawecki & Ebert 2004). Predator-prey interactions often do not lead to reciprocal coevolution so it is important to consider other sources of selection that may contribute to patterns of local adaptation seen in associated species (Nuismer et al. 2010, Gomulkiewicz et al. 2007). Crab predators and their gastropod prey in Lake Tanganyika, Africa, for instance, were once believed to be in a coevolutionary arms race where crabs evolved larger, stronger crushing claws and gastropods developed thicker shells (West et al. 1991). Nevertheless, this correlation does not unequivocally establish reciprocal selection and it is possible the crabs are responding to selection pressure from their own predators as they also use their claws defensively rather than just to capture prey (Dietl & Kelley 2002). The idea of unilateral, enemy-driven selection is known as the escalation hypothesis in which prey respond to their predators, but instead of predators reciprocally responding to their prey, they are more likely to respond to their own enemies (Vermeij 1987).

Multiple predators may contribute additional selection pressures to either enhance or reduce risks to prey. If predators are competing with one another, pressures on prey are reduced, but they may also collectively increase the predation rate due to the multiple predator effect (Sih et al. 1998). Furthermore, non-native predators can have an even

greater detrimental effect than native predators on prey populations. When prey have coexisted with their predators for long periods of time, they are able to develop antipredator defenses over time. In the case of non-native predators, prey may be naive to novel predators and subsequently unable to evolve defenses quickly enough (Cox & Lima 2006, Salo et al. 2007). This might happen when novel selective agents cause a decrease in population size, thus reducing genetic variability too low to allow for adaptive responses to occur (Strauss et al. 2006).

One mode of antipredator defense is for prey to secrete noxious chemicals to become unpalatable or even deadly to potential predators. Neurotoxins, for example, are used by some species to block voltage-gated sodium channels and can cause paralysis, seizures, or cardiac arrest in the exposed individual. One of the most potent of these toxins, known as tetrodotoxin (TTX), is used as a chemical defense by several species including puffer fish, blue-ringed octopus, moon snails, and salamanders (Moczydlowski 2013). The most toxic salamander species, the rough-skinned newt (*Taricha granulosa*), was historically hypothesized to be in a predator-prey coevolutionary arms race with the common garter snake (*Thamnophis sirtalis*) (e.g., Brodie & Brodie 1990, Brodie et al. 2005). Garter snakes are the main predators of these dangerous newts and over time have developed a resistance to TTX. In response to the increasing effects of predation on populations, the newts have consequently been under selection to produce higher skin TTX concentrations in some areas where these two species co-occur (e.g., Hanifin et al. 1999, Motychak et al. 1999). The toxin resistance in garter snakes is enabled by allelic variations causing structural changes to the voltage-gated sodium channels targeted by TTX, thus decreasing the binding affinity of the toxin (McGlothlin et al. 2014). While the

genes related to snake TTX resistance have been identified, the source of chemical defense in newts has been widely debated and not yet resolved (Chau et al. 2011, Moczydlowski 2013).

1.1.3 Genomics of Adaptation

The connection between phenotype and genotype is crucial to our understanding of the mechanisms of adaptive evolution. Processes such as new mutations, gene flow among populations, and random genetic drift contribute to the geographic mosaic of coevolution by shifting the spatial distributions of genes and traits that may be involved in coevolutionary adaptations (Thompson 2005). Traditionally, biologists made inferences about adaptative traits based on phenotypic information. Adaptations could be studied by measuring morphological features, observing behaviors, and analyzing physiological conditions of organisms. The analysis of trait variation within and between species is known as the comparative method, which can reveal phenotypes that are adaptive across different environments (Clutton-Brock & Harvey 1984). However, in the past several decades, scientists have become increasingly interested in investigating the molecular basis of adaptation (Radwan & Babik 2012).

Investigating the genetics of adaptive traits beyond phenotype-based comparative methods alone has not been possible until recent advances in genome sequencing technologies and analysis tools. The ability to identify polymorphisms across the genome has greatly contributed to our understanding of the relationship between genotype and phenotype, providing a more complete picture of the underlying processes affecting adaptation (Rieseberg 1998, Whitlock 2015). As a result, the field of ecological genetics was created, which emphasizes the study of genetic characters associated with

adaptations and ecological interactions (Via 2002). Massively parallel sequencing, or next generation sequencing (NGS), has made it possible to obtain large amounts of sequence data quickly and relatively cheaply (Mardis 2008), making it easier now more than ever to identify the loci of adaptation. Threespine stickleback (*Gasterosteus aculeatus*), for example, were long considered a model organism to study phenotypic adaptation and divergence, but it was not until recently that the genes responsible for certain morphological adaptations were discovered. Stickleback populations exhibit significant variation in their morphology, with reduced armor plates and pelvic structures observed in postglacial-lake forms as compared to marine forms (Bell & Foster 1994). Using a method known as quantitative trait loci (QTL) mapping on crosses between lake and marine forms, the genes *Pitx1* and *EDA* were found to be responsible for pelvic reduction and armor plate reduction in stickleback, respectively (Shapiro et al. 2004, Colosimo et al. 2005).

Despite the successes in this field, several limitations to studying the genomics of adaptation still exist. For example, organisms such as salamanders can have large, repetitive genomes, estimated to be around ten times the size of the human genome. In one study, an attempted full genome assembly on the Mexican axolotl (*Ambystoma mexicanum*) failed due to memory limitations of the computer (Keinath et al. 2015). Additionally, some studies are still restricted by the lack of a reference genome for non-model organisms, thus limiting the possibility of comprehensively investigating the genomics of adaptation in many species. As genomic and computational techniques are being continually refined and made more affordable and accessible, this will allow for a greater number of traits in more populations and species to be studied.

One method that may be a feasible solution for studies of non-model organisms without reference genomes is a transcriptomics or RNA-sequencing (RNA-seq) approach (da Fonseca et al. 2016). RNA-seq utilizes high-throughput sequencing data to characterize the functional elements of the genome by mapping and quantifying the transcriptome (Wang et al. 2009). Because transcriptomics only encompasses expressed genes, this method is a practical solution for studying genes from highly repetitive genomes or diverged species. Moreover, this approach allows the *de novo* assembly and analysis of the transcriptome without mapping to a reference genome and such methods can even outperform genome-guided assemblies in non-model species (Grabherr et al. 2011, Huang et al. 2016). RNA-seq studies also aid in the identification of genes of adaptation by comparing genes that are differentially expressed in various tissue types or treatment groups (Costa-Silva et al. 2017). For instance, a study on thermal adaptation of redband rainbow trout (*Oncorhynchus mykiss gairdneri*) discovered differentially expressed genes that control respiration (Garvin et al. 2015).

RNA-seq also enables studies of population genomics in absence of a reference genome by identifying patterns of molecular variation (Gayral et al. 2013). Population genetic studies utilizing transcriptomic data may be used to understand local adaptation (adaptation in response to spatially variable selection) and identify the molecular basis to differing phenotypic adaptations between populations across a landscape (Tiffin & Ross-Ibarra 2014). Investigating patterns of adaptation guides predictions of how well populations may be able to respond to future environmental change, such as red abalone (*Haliotis rufescens*) along the California coast which were found to be locally adapted to stressors including hypoxia, heat, and pathogens (De Wit & Palumbi 2012). Such

approaches may also be useful in elucidating trait variation in different populations of coevolving species across a geographic mosaic. Another goal of population genetics is to characterize population structure to identify patterns of genetically distinct subgroups and uncover demographic history (Chakraborty 1993). For example, a population transcriptomics approach enabled the identification of founder events and invasion patterns of a hyperinvasive weed, *Mikania micrantha*, as well as provided insight to the molecular and adaptive processes underlying its successful establishment and rapid spread (Yang et al. 2017). Additionally, it is important to monitor the population genetic structure of species that are at risk of declines, such as those impacted by non-native species. Understanding the structure of populations across a landscape will ultimately enable management decisions to be made at the proper spatial scale (Manel et al. 2003).

1.2 Study System

1.2.1 Rough-skinned newts

In this thesis, I focus on a subject of many predator-prey coevolutionary studies, the rough-skinned newt (*Taricha granulosa),* with the aim of investigating several aspects of the chemical ecology and genetics of the species while integrating findings with conservation implications. The rough-skinned newt is an urodelan amphibian that is currently listed by the International Union for the Conservation of Nature (IUCN) as a species of Least Concern and inhabits a range spanning the western coast of North America from central California to northern British Columbia (IUCN SSC Amphibian Specialist Group 2015). In the wild, their life span averages 12 years and their adult diet consists of aquatic and terrestrial invertebrates (i.e., crustaceans, arachnids, and insects)

as well as the eggs and larvae of other amphibian species (Evenden 1948, Efford & Mathias 1969). Newts migrate seasonally between aquatic habitats such as ponds, lakes, and slow-moving streams, and terrestrial habitats of burrows and deadfall in old-growth forests (Lanoo 2005). They spend most of their time in aquatic habitats with males staying up to 10 months in permanent water bodies, and females usually remaining no longer than 8 months (Pimentel 1960). While there is little insight on their total home range size, rough-skinned newts exhibit breeding site fidelity in which they return year after year to the same site in which they were born (Pimentel 1960, Efford & Mathias 1969). During peak breeding season, reproductive males develop significant morphological changes including smoother skin, heightened tail crest, and swollen cloaca, while females remain relatively unchanged (Oliver & McCurdy 1974).

Rough-skinned newts also have aposematic coloration and can assume a posture known as the unken reflex in which they arch backwards to display their bright orange ventral side to predators, indicating unpalatability (Johnson & Brodie 1975). As previously mentioned, *T. granulosa* possesses a potent neurotoxin known as tetrodotoxin, making it the most toxic amphibian species in North American (Brodie et al. 1974). TTX is primarily excreted from their dorsal skin but is also found in high concentration in newt eggs, in which females may invest toxin production in their eggs to defend against egg predation (Hanifin et al. 2003, Gall et al. 2014). Newts remain toxic throughout their larval and juvenile stages (Gall et al. 2011), and even in long term captivity, adult newts excrete toxin from their skin (Hanifin et al. 2002) and produce TTX-laden eggs (Gall et al. 2012a). Tetrodotoxin not only works directly on the nervous system of those who prey on newts, but there is evidence it may also serve other functions such as a chemical cue to

alert conspecifics of a predator's presence (Zimmer et al. 2006) and protection from parasitic infections (Johnson et al. 2018). Recent work suggests chemical defense in California newts, *Taricha torosa*, is inducible over time (Bucciarelli et al. 2017) and the same may be true of *T. granulosa*. Although captive newts were able to increase their toxin defense over the course of several days in response to simulated predator attacks (i.e., the effects of captivity and tissue sampling acted as a proxy for failed predation), newt toxicity is maintained at a baseline concentration in wild populations (Bucciarelli et al. 2017). The ability to secrete tetrodotoxin has also led to newt skeletal muscles being unaffected by TTX self-intoxication. Both TTX-producing and non-toxic species from the Salamandridae family were found to have some degree of TTX resistance, indicating that resistance is ancestral to the full TTX-bearing phenotype in these lineages (Hanifin & Gilly 2015).

Patterns of tetrodotoxin levels in rough-skinned newts suggest that a geographic mosaic structure is an important component in coevolutionary interactions throughout their range. Rough-skinned newts share a common selective agent, their main predators, garter snakes (*Thamnophis sirtalis*), with varying degrees of predation pressure leading to high variation in toxin levels across sympatric populations. This results in so-called "hotspots" and "coldspots" of toxicity and matched toxin resistance across the landscape, with certain hot localities displaying resistance up to 100 times higher than others (Brodie et al. 2002). Hotspots with the highest TTX concentration were found to include sites in Oregon and northern California, and there is evidence of significant variation within and among populations across their entire range (e.g., Hanifin et al. 2008, Stokes et al. 2015, Hague et al. 2016). There are several known genes that enable tetrodotoxin resistance in

snakes, but the allelic variants do not confer discrete levels of resistance or solely account for the resistance (Feldman et al. 2010, McGlothlin et al. 2014, Hague et al. 2018). Rather, TTX resistance in garter snakes is measured on a continuous scale by conducting a bioassay of whole animal performance (Brodie & Brodie 1990). The lethality of rough-skinned newts to garter snakes is therefore dependant upon the snake's degree of resistance, as well as the toxin concentration of the newt. For example, a highly toxic newt from the hotspot in Oregon would not affect garter snakes inhabiting the same region but would be deadly to a snake from any other region that had a lower level of resistance (Brodie et al. 2002).

Although the toxicity of rough-skinned newts has been widely studied over the past few decades, the source of toxin production remains unknown. It was first suggested that TTX is bio-accumulated as a result of diet, but newts kept in captivity and fed TTX-free diets are able to retain their toxin concentrations over time (Hanifin et al. 2002, Cardall et al. 2004, Gall et al. 2012a). Another hypothesis was that bacterial endosymbionts are responsible for toxicity in newts due to the presence of symbiotic bacteria in many other TTX-producing taxa (Chau et al. 2011). Lehman et al. (2004) suggested bacterial endosymbionts are not responsible for the observed toxicity after discovering that there were no bacteria present on newt skin via polymerase chain reaction (PCR) amplification, but this could have been an erroneous result due to the higher proportion of newt DNA interfering with amplification of bacterial sequences and the potential role of bacteria should not yet be ruled out (Chau et al. 2011).It is therefore still plausible that endosymbiotic bacteria play a role in toxin production, or that tetrodotoxin biosynthesis is under genetic control and toxins are synthesized within

individual newts (Chau et al. 2011, Moczydlowski 2013). However, a satisfactory determination of the molecular mechanisms responsible for TTX expression remains lacking.

Further complicating this system is the fact that other species besides garter snakes have been shown to prey on or impact rough-skinned newts. River otters (*Lontra canadensis*) (Stokes et al. 2015) and barred owls (*Strix varia*) (Medina et al. 2018) have been observed preying on adult newts, and caddisfly larvae are known to consume newt eggs (Gall et al. 2012b). Additionally, crayfish are believed to affect newt species both directly and indirectly, which can result in significant negative impacts on populations. They prey upon eggs and larvae, attack adult newts, compete for prey invertebrates, and disrupt littoral habitat (Gamradt & Kats 1997, Nystrom et al. 2001). In Crater Lake National Park in Oregon, the Mazama newt (*Taricha granulosa mazamae*), a subspecies of the rough-skinned newt, is facing serious declines as a result of non-native crayfish presence (Girdner et al. 2018). American bullfrogs (*Lithobates catesbeianus*), which are a globally pervasive alien species, are also known to outcompete native amphibians as tadpoles and prey on rough-skinned newts as adults (Kats & Ferrer 2003, Jancowski & Orchard 2013).

1.2.2 Vancouver Island, British Columbia

Vancouver Island is the largest island off the western coast of North America, representing a substantially sized region of the rough-skinned newt range and providing an interesting region to study newts due to several reasons. First, it is geographically isolated, thus preventing gene flow with mainland populations. It is also a relatively young island that was recolonized by many species after the retreat of the Cordilleran ice

sheet 10,000 years ago (Mann & Hamilton 1995). Finally, it is faced with many conservation concerns including presence of non-native species (Bondar et al. 2005, Jancowski & Orchard 2013), habitat fragmentation (Vellend et al. 2008), deforestation of old growth forests (Gilani & Innes 2020), and climate change (Gerick et al. 2014). Despite these unique characteristics, Vancouver Island has been overlooked in many previous studies of rough-skinned newt chemical ecology and genetics. Previous research has suggested that newts from this region excrete uniformly low levels of TTX. However, very small sample sizes of six (Brodie & Brodie 1991), one (Hanifin et al. 1999), and 16 newts (Hanifin et al. 2008) were used to come to these conclusions, and therefore these studies do not provide a robust representation of toxicity across the island. Additionally, there is very little known about the genetics of rough-skinned newts and to date, no population genetic studies of the species have included data from Vancouver Island (i.e., Jones et al. 2001, Kuchta & Tan 2004, Ridenhour et al. 2007, Mebs et al. 2016, Hague et al. 2016, Hague et al. 2019).

Vancouver Island is also a region of interest to study antipredator adaptation in newts because in the last century it has been faced with introductions of signal crayfish (*Pacifastacus leniusculus*) (Bondar et al. 2005) and American bullfrogs (*Lithobates catesbeianus*) (Jancowski & Orchard 2013), both of which are known to cause negative effects on newts and other amphibian species in different regions (e.g., Nystrom et al. 2001, Girdner et al. 2018). Non-native signal crayfish have already been implicated in the collapse of the benthic and limnetic threespine stickleback species pair (*Gasterosteus aculeatus*) in Enos Lake on Vancouver Island (Taylor et al. 2006), but their impacts on rough-skinned newts have not been considered. The variation in toxicity of newts may be

further altered by different overlapping ranges and selection pressures from these non-native predators in addition to garter snakes. Moreover, it is important to consider population structure and genetic diversity among populations of newts. Raw genetic diversity is the means by which adaptation may progress (Frankham 2005), and this will indicate if certain populations with lower genetic diversity might be less resilient to adapting to the presence of non-native predators. As of March 2020, rough-skinned newts have been identified by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) as a mid-priority candidate species for assessment, meaning they could be at risk of extinction or extirpation in Canada (COSEWIC 2020). Although this indicates the Committee has significant concerns about rough-skinned newts in BC, I was unable to find any published information on specific risk factors.

1.3 Objectives

The overall goal of my thesis was to address several aspects of the chemical ecology, genetics, and conservation of rough-skinned newts. The majority of my work investigated questions focused on Vancouver Island, BC, a large island that provides a unique region to study newts due to its isolation from mainland populations and recent introduction of non-native species. My first objective was to reassess a previous hypothesis in which newts from the island were presumed to have uniformly low toxin levels, a finding based on small sample sizes and broad interpolation across the landscape (Brodie & Brodie 1991, Hanifin et al. 2008). In Chapter 2, I analyzed a robust dataset of rough-skinned newts to (i) characterize variation in baseline TTX levels of newts across Vancouver Island, and (ii) determine if there were significant differences in the observed

variation among populations. This work revealed evidence of a previously undetected hotspot of rough-skinned newt toxicity on the island and helps further elucidate the chemical ecology of this species across the entirety of the geographic mosaic.

The objective of Chapter 3 was to identify genes related to high toxicity adaptations rough-skinned newts using a transcriptomics approach. Previous studies have investigated other potential sources of TTX such as diet (Hanifin et al. 2002) or endosymbiotic bacteria (Lehman et al. 2004, Vaelli et al. 2020), but no other work had attempted to identify molecular mechanisms of chemical defense in newts. To address this, I presented the assembly and characterization of the rough-skinned newt transcriptome and identified differentially expressed genes between dorsal and ventral tissue in individuals from known high toxicity populations in Oregon. Not only did this chapter describe potentially adaptive genes related to endogenous sources of newt TTX, but it serves as a resource for future genetic studies in this species, including the population genetics analyses in Chapter 5.

In Chapter 4, I provided an overview of the potential impacts on rough-skinned newts of two non-native species currently spreading across Vancouver Island. Both signal crayfish and American bullfrogs are known to cause detrimental effects on native amphibians (e.g., Nystrom et al. 2001, Jancowski & Orchard 2013, Girdner et al. 2018), so I predicted that their presence would also negatively impact rough-skinned newts on the island. My objective was to characterize how the presence of non-native crayfish and bullfrogs on Vancouver Island impacts newt (i) relative abundance, (ii) body condition, and (iii) tetrodotoxin concentration (based on results from Chapter 2). Although it is difficult to disentangle the complex effects of non-native species on populations, this

study established a baseline of the current impacts of crayfish and bullfrogs on Vancouver Island rough-skinned newts as the species continue to spread and may cause more significant effects in the future.

Incorporating findings from the previous three chapters, Chapter 5 investigated the population genetics and conservation implications of rough-skinned newts on Vancouver Island. My objectives were to utilize single nucleotide polymorphisms (SNPs) identified from RNA-seq data to study the population genetic structure and heterozygosity both among newt populations in Oregon and BC, and at smaller scale across Vancouver Island. The transcriptome assembly from Chapter 3 served as a reference sequence for aligning sequences and identifying SNPs, the patterns of tetrodotoxin variation in Chapter 2 are reconsidered based on the lack of population genetic structure of Vancouver Island, and the genetic consequences of islands faced with non-native species concerns were discussed based on Chapter 4.

Finally, the main findings of my thesis were summarized and synthesized in Chapter 6, where I also discussed the implications and future directions for studies of rough-skinned newts.

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CHAPTER TWO – EVIDENCE FOR PREVIOUSLY UNIDENTIFIED HOTSPOT IN ROUGH-SKINNED NEWT TOXICITY

2.1 Introduction

Interactions between co-occurring species have played a crucial role in the adaptive patterns observed throughout the course of evolutionary history (Vermeij 1987, Schluter 1996). Predators, for example, are especially influential in the defensive adaptations of their prey (Vermeij 1987, Vamosi 2005, Przeczek et al. 2008, Beston et al. 2019). Associations between predator and prey are responsible for evolution of certain traits which increase the ability of predators to capture prey, and subsequently enable prey to better evade predation. Such associations may lead to coevolution in which interacting species reciprocally evolve in response to changes in one another (Futuyma & Slatkin 1983). There are many axes along which prey may adapt to predators and one of them is chemical defenses. Both vertebrate and invertebrate taxa have the ability to produce a wide range of toxic compounds which cause various physiological effects (Berenbaum 1995, Utkin 2015). Neurotoxins, for instance, are a class of chemicals which act on the nervous system of affected individuals, causing paralysis, seizures, and even death (Adams & Olivera 1994).

Tetrodotoxin (TTX) is a particularly potent neurotoxin which acts by blocking the outer pore of voltage gated sodium channels of neurons and muscles (Narahashi et al. 1967). The effects of TTX were alluded to as far back as 2500 BC by the Egyptians (Fuhrman 1986), but it was first formally extracted and described in 1909 in pufferfish (*Fugu rubripes*), and thus given its name after the fish order Tetrodontiformes (Hashimoto 1979). Since then, this toxin has been identified in almost 140 species worldwide, ranging from marine fish and mollusks to terrestrial flatworms and

amphibians. In each of these species, TTX may serve a specific function such as an olfaction cue or communication signal, but it is most commonly utilized as an antipredator defense (Lorentz et al. 2016). Moreover, the source of TTX also varies among taxa. Tetrodotoxin may be bioaccumulated through the food chain, produced by bacterial endosymbionts, synthesized endogenously through unknown mechanisms, or by a combination of these processes (Jal & Khora 2015).

One tetrodotoxin-bearing species that has been the subject of many studies is the rough-skinned newt (*Taricha granulosa*). Amphibians are the only class of terrestrial metazoans in which TTX is found and rough-skinned newts are the most toxic species of amphibian, containing up to 14,000 μg of TTX per individual (Hanifin 2010). Rough-skinned newts primarily excrete TTX from their granular skin glands as a chemical defense against predators, with the highest concentrations found in their dorsal skin (Hanifin et al. 2004). Additionally, significant quantities of TTX are found in the ovaries of female newts, who are then able to produce TTX-laden eggs, presumably to defend against egg predation (Hanifin et al. 2003, Gall et al. 2014). Despite the chemical ecology of rough-skinned newts being widely studied, the source of TTX in newts remains unknown. Previous studies have tested whether TTX is accumulated through diet (Hanifin et al. 2002), or bacterial endosymbionts (Lehman et al. 2004, Vaelli et al. 2020), but ultimately the source of newt toxicity remains unresolved.

Another interesting aspect of the chemical ecology of tetrodotoxin in newts is the defensive role it plays in interactions with their main predators, common garter snakes (*Thamnophis sirtalis*). For many decades, rough-skinned newts and garter snakes were a textbook example of predator-prey coevolution in which they were believed to exhibit

reciprocal selection pressures on one another (i.e., Brodie & Brodie 1990, Brodie et al. 2005). Snakes developed a resistance to TTX through allelic changes in skeletal muscle voltage-gated sodium channel genes (McGlothlin et al. 2014), whereas higher toxicity in newts led to reduced predation (Williams et al. 2010). Nevertheless, the increasing magnitude of reciprocal selection does not transpire in every sympatric population of newts and garter snakes. Across the range where these species co-occur, there exist "hotspots" and "coldspots" of newt toxicity and snake resistance (Brodie et al. 2002), and this extreme spatial variation in traits may represent a geographic mosaic of coevolution (Thompson 2005).

The geographic mosaic theory of coevolution (Thompson 2005) suggests that predator and prey phenotypes should be closely matched, with selection in the absence of a TTX-resistant predator favoring reduced toxicity in newts. However, local adaptations in populations may be driven by neutral genetic divergence or other spatially variable selection pressures outside of a seemingly reciprocal predator-prey interaction (Kawecki & Ebert 2004, Holderegger et al. 2006). Phenotypic mismatch in newt toxicity and snake resistance across the mosaic was first detected in which snakes were able to escape the arms race in some localities through the rapid evolution of extreme TTX resistance (Hanifin et al. 2008). Newts in allopatry with garter snakes at the northern extreme of their range also exhibit fine-scale geographic variation in toxicity, suggesting newts face selection pressures other than just predation by resistant snakes (Hague et al. 2016). At present there is lack of consensus regarding whether neutral genetic divergence may best predict TTX variation, rather than coevolution (Hague et al. 2016, Hague et al. 2019). Newt and snake predator-prey dynamics are considerably more complex than originally

thought and variation across sympatric populations of these species could be due to other biotic or abiotic factors besides coevolution contributing to local adaptation (Nuismer et al. 2010).

One region in the geographic mosaic of rough-skinned newt and garter snake coevolution that was previously thought to be a coldspot with low newt toxicity is Vancouver Island, British Columbia (i.e., Brodie & Brodie 1991). Extrapolating from small sample sizes of six (Brodie & Brodie 1991), one (Hanifin et al. 1999), and 16 newts (Hanifin et al. 2008), previous studies claimed that newts on Vancouver Island produce uniformly low levels of TTX. There are several factors that likely minimize the ability of low sample sizes to accurately predict realized variation across the island. First, Vancouver Island is in the top 15% of islands by land area (31,285 km^2), making it comparable to Belgium (30,688 km^2) and Taiwan (36,193 km^2). Second, there is considerable variation in environments, with the central mountain range separating a wet west coast from a relatively dry east coast. These environments introduce the potential for differing selective pressures, both biotic and abiotic. Third, newts generally migrate only within 550 m of breeding ponds (Efford & Mathias 1969), setting the stage for local adaptation. Indeed, fine-scale geographic variation in newt TTX levels was detected in Alaska, in which sites separated by a short geographic distance of 15 km had significantly different toxin concentrations (Hague et al. 2016), and similar patterns may be present on Vancouver Island.

Here, I analyze the baseline toxicity of a large number of rough-skinned newts across Vancouver Island to test the robustness of prior conclusions regarding the lack of toxicity in the region (Brodie and Brodie 1991, Hanifin et al. 2008). Based on the reasons

cited above, I predict greater geographic variation in TTX on the island than previously suggested, including the potential for previously undetected hotspots to exist on the island. On the other hand, if low toxicity with low variation across the island is detected, this would support the hypothesis regarding the loss of toxicity in island populations (Brodie and Brodie 1991). Characterizing the variation in toxin defense among populations of rough-skinned newts on Vancouver Island will promote further investigation into the chemical ecology of this species across an isolated island system.

2.2 Methods

2.2.1 Sample Collection

A total of 308 rough-skinned newts from 28 sites across British Columbia, Canada and Oregon, USA were sampled in spring of 2017 and 2018, coinciding with the peak of their breeding season (Figure 2.1). I chose these sites to represent a robust sample of locations across Vancouver Island with different characteristics, in addition to reference sites in Oregon and Mainland BC (Appendix A). At each study site, adult newts were collected in unbaited minnow traps set for up to 24 hr, with hand nets, or a combination of these methods. Juvenile or larval newts were not collected. I sampled one to 18 individuals from each location and considered sites as distinct populations due to breeding site fidelity. For each individual newt collected, body mass and snout-vent length (SVL) were measured on a scale and sex was physically determined based on cloaca and tail shape. Following a non-lethal sampling method (Bucciarelli et al. 2014), I used a 2mm skin biopsy tool (Robbins Instruments, USA) to remove a skin sample from the posterior dorsolateral area of each individual, and then returned the newts were to their original habitat. Biopsy samples were taken from roughly the same region in each

newt, and previous studies found no significant difference in toxin quantification results from samples taken across different regions of the dorsal skin (Hanifin et al. 2004, Bucciarelli et al. 2014). I immediately placed skin samples were in a tube of 300 μL of 0.1M acetic acid and kept them in a liquid nitrogen dewar at -190℃ before transportation back to the University of Calgary for storage at -80℃. Sample collection was performed in accordance with BC Wildlife Act Permit NA17-263401, Oregon Department of Fish and Wildlife Scientific Taking Permit 064-18, and University of Calgary Animal Use Protocol AC15-0033.

2.2.2 TTX Extraction and Quantification

I extracted tetrodotoxin from the collected skin samples using a new micro-extraction method developed by Bucciarelli et al. (2014). Each skin biopsy sample, along with ~300 mL of 0.1 M acetic acid it was previously stored in were transferred to a new tube and macerated with a 1 mL glass tissue grinder (Kimble Kontes Duall). The mixture was then heated in a ThermoMixer at 100°C for 5 min and cooled in an ice bath for 5 min. Next, samples were centrifuged for 20 min at 13000rpm to allow sedimentation, and the supernatant was transferred to 0.5 mL filter tubes (Amicon Ultra 10K) before being spun again at 13000 rpm for 20 min. The centrifugal filter was washed by adding 100 μL of 0.1 M acetic acid and centrifuged for 20 min at 13000 rpm. Finally, I diluted the extracts to a final volume of 800 μL with 0.1 M acetic acid.

Extracted samples were sent to Dr. Amber Stokes at California State University Bakersfield for quantification in her lab following the Competitive Inhibition Enzymatic Immunoassay (CIEIA) method outlined in Stokes et al. (2012). Values below detectable limits are treated as zero in subsequent analyses. The concentration of TTX present in each sample was estimated based on the standard curve of the assay, yielding micrograms

(μg) of TTX per milliliter of extract solution. Finally, micrograms of TTX per cm^2 of newt skin was calculated using the diameter of 2 mm from the initial skin biopsy taken. I used toxin concentrations in $\mu g/cm^2$ in this study rather than estimates of whole newt TTX (see Appendix B for discussion of how estimates using whole newt mass may bias TTX calculation).

2.2.3 Statistical Analyses

To analyze variation in newt toxicity between populations, I used R with the R Studiov1.1.163 interface (R Core Team 2018). The ggplot2 package (Wickham 2009) was used to create a boxplot to display TTX concentration for each population. I performed a Kruskal-Wallis test (non-parametric one-way ANOVA) and subsequent Wilcoxon pairwise comparisons with Bonferroni correction to identify significant differences in the mean toxicity among all study sites, including Oregon and Sunshine Coast. Because the *post hoc* test does not allow for comparisons between groups containing only one data point, four lakes where only one newt was collected had to be removed from the analysis: Maple Lake, Loon Lake, Dougan Lake, and Martlet Pond. I also performed a two-way ANOVA to investigate variation in TTX by population and by sex. Finally, I conducted a permutation-based Mantel test in the R vegan package (Oksanen et al. 2019) with 10,000 permutations to test whether differences in TTX concentrations increase between samples when study sites are farther apart. Only study sites on Vancouver Island were included in the Mantel test because Oregon sites might bias the results due to the substantial geographic distance from the island.

2.2.4 Visualizing Geographic Variation

To visualize variation in TTX among populations across the landscape, I generated isocline maps by inverse distance-weighted (IDW) interpolation (Shepard

1968) in ArcMap 10.6.1. The analyses used input of latitude and longitude coordinates of each study site, along with the mean TTX concentration at each site. First, to confirm accuracy of the IDW interpolation analysis, I pulled whole newt TTX data in mg from Hanifin et al. (2008) and followed their methods as closely as possible to ensure reproducibility of results (Fig. 2.2). The neighborhood search radius was set to 500 km with a power of 2. Interpolated TTX concentrations were (i) classified into seven color categories according to the manual intervals set by Hanifin et al. (2008), (ii) reclassified into seven equal intervals to represent proportionate changes in interpolated newt toxicity between each of the seven color classes, and (iii) displayed using a continuous/stretched symbology because TTX is a continuous variable (Fig. 2.3). The manually classified map (Fig. 2.3a) roughly aligns with the previously published figure (Fig. 2.2), but slight discrepancies may be due to changes in the software versions and algorithms. By changing the color classification method, it becomes clear that patterns of toxicity can be interpreted in vastly different ways (Fig. 2.3). Hanifin et al. (2008) biased their color classifications towards the low end of TTX values, with the lower five of seven classes spanning the same absolute range of TTX values as the sixth class alone (Fig. 2.2). Consequently, the seventh class (TTX > 2 mg) contains 57% of the interpolated values.

To generate a more robust representation of TTX variation across Vancouver Island alone, I created an IDW interpolation map with a 500 km neighborhood search radius using all populations sampled on the island, including the aforementioned study sites with only one sample. I excluded Oregon and Sunshine Coast study sites because the interpolation method would not accurately reflect the presence of the Pacific Ocean separating these sites from Vancouver Island. Interpolated TTX values were classified

using a continuous/stretched symbology rather than classified color scale to more accurately represent newt toxicity being a continuous variable. I also created a map showing the color based on the mean toxicity at each study site individually for comparison to color classifications from the IDW interpolation analysis. For both maps, I have chosen to utilize a diverging color ramp symbology rather than rainbow which can be misleading when visually interpreting data and is widely considered bad practice (e.g., Moreland 2016) (however, see Appendix C for comparison between the rainbow and diverging color scales). Finally, the use of the term "hotspot" in based on the theory of hotspots and coldspots of coevolution across a geographic mosaic where hotspots represent regions with strong reciprocal selection between interacting species (Thompson 2005), not to be confused with the hotspot analysis method for spatial analysis in GIS (e.g., Yu et al. 2014). Here, a hotspot represents a region of exaggerated toxicity with the assumption of matched resistance in snakes based on the idea of reciprocal selection pressures (Brodie et al. 2002).

2.3 Results

2.3.1 Statistical Analyses

I quantified baseline newt toxicity levels in μg of TTX per cm^2 of skin in 308 rough-skinned newts from 28 populations across British Columbia and Oregon (Table 2.2, Fig. 2.4). Of the 28 study sites, 23 were on Vancouver Island, allowing for a more robust, fine-scale analysis of the region. I included the four study sites in Oregon to compare to previous work and benchmark whether I would obtain similar toxin quantification results (i.e., if the TTX concentration results for Oregon were very low compared to previous reports then that would indicate there was possibly an error in the

extraction or quantification steps). Benton County, Oregon is a previously identified hotspot in the geographic mosaic with newts from the county having an average toxicity of 16.9 μg/cm^2 (Hague et al. 2019). However, my study found even higher mean TTX concentrations at two sites from Benton County: 33.59 μg/cm^2 at Cronemiller Lake and 23.70 μg/cm^2 at Bald Hill. Additionally, the two study sites in Tenmile County, Oregon (Tenmile Lake and Shore Acres) as well as four sites on Vancouver Island (Dougan Lake, Martlet Pond, Mary Lake, Queen Alexandra Centre) had greater mean toxicity than the previously reported hotspot with 16.9 μg/cm^2 (Hague et al. 2019). Several study sites from Vancouver Island also displayed high concentrations of toxicity and high within-population variation, falling within the range of the four Oregon sites (Table 2.2, Fig. 2.2).

Significant differences in mean toxicity were detected among all study sites (Kruskal-Wallis; χ^2=170.65, $p < 2e^{-16}$, df=27) and exclusion of Oregon sites from the analysis did not change the results (Kruskal-Wallis; χ^2=91.706, $p < 1.8e^{-10}$, df=22). Several populations were found to have significant differences in TTX concentration, with the sites in Oregon having the highest number of significant comparisons (Wilcox test; Table 2.2). Specifically, Shore Acres in Tenmile County, Oregon had a greater mean toxicity than 17 out of the 23 comparisons, and it also had the highest mean toxicity of 41.97 μg/cm^2. TTX levels did not vary by sex (ANOVA; F=1.937, p=0.165, df=1). The Mantel test did not indicate any correlation between toxicity and distance among sites on Vancouver Island (Mantel test; r=0.0508, p=0.0792).

2.3.2 Geographic Visualization

The isocline map of Vancouver Island displays fine-scale variation in baseline TTX levels between rough-skinned newt populations across the landscape; furthermore,

it is not entirely a coldspot of toxicity (Fig. 2.5). When visualizing the interpolated toxicity using a continuous color scale, there is a hotspot of toxicity in the southern region of the island, with the northern half being mainly blue, representing lower interpolated toxicity (Fig. 2.5). The color scale in the IDW interpolation map is generally concordant with colors based on the mean population toxicity at each study site (Fig. 2.6).

2.4 Discussion

For several decades, rough-skinned newts on Vancouver Island, BC were portrayed as having uniformly low levels of tetrodotoxin and the island was assumed to be a coldspot of coevolution with their garter snake predators (Brodie & Brodie 1991). However, the robustness of this conclusion was limited by small sample sizes, sparse geographic coverage, and the way in which TTX values were interpolated. In this study, my objective was to perform a robust analysis of fine-scale variation in newt toxicity from 23 study sites across Vancouver Island, as well as four reference sites in Oregon and one on the Sunshine Coast, BC. In addition to furthering our knowledge of chemical defense in rough-skinned newts inhabiting a unique island system, this work provides better resolution of the patterns of toxicity across the geographic mosaic.

My study reveals that Vancouver Island does not represent a homogenous, low-toxicity part of the range and has a hotspot in the southern region of the island that rivals the toxicity of other known hotspots in Oregon. Several island populations fell within the range of the previously presumed “hottest” spot of toxicity in Benton County, Oregon (Fig. 2.4). The high variability in newt toxicity both within and among populations on Vancouver Island is concordant with similar patterns detected in other study areas (e.g.,

Hanifin et al. 2008, Stokes et al. 2015, Hague et al. 2016). Newt populations at the northern end of their range, where they live in allopatry to garter snakes, were found to contain individuals with very high TTX levels, similar to those of highly toxic newts co-occurring with resistant garter snake predators in Oregon (Hague et al. 2016). Such patterns of within-population variation indicate toxic phenotypes are not yet fixed in specific populations, possibly because selection pressures by garter snakes are not strong enough to favor a certain phenotype. I also did not find a significant association of sex and toxicity, but the interpretation of these results may be limited by the low number of females collected (Table 2.1). There also seems to be no consistent patterns of such an association in previous work, with some studies having found a correlation between sex and toxicity (e.g., Hague et al. 2019) whereas others did not (e.g., Cardall et al. 2004, Hague et al. 2016).

I would like to note that by using a different combination of toxin extraction and quantification methods, an important caveat is the ability to compare toxin levels reported in previous studies is limited to relative rather than absolute comparisons. First, I used a refined micro-extraction method capable of isolating approximately 50% more TTX than previous methods (Bucciarelli et al. 2014), which was not utilized in several other recent analyses of rough-skinned newt toxicity (e.g., Stokes et al. 2015, Hague et al. 2016, Hague et al. 2019). For example, the Cronemiller Lake study site in Benton County, Oregon from my study had an average toxicity of 33.59 $\mu g/cm^2$, nearly double the previously reported concentration of 16.9 $\mu g/cm^2$ in Benton County (Hague et al. 2019). The use of the new CIEIA toxin quantification method in another study (Stokes et al. 2015) also nearly doubled the mean toxin concentration detected in Benton County

compared to that detected using high performace liquid chromatography (HPLC) (Hanifin et al. 2008). Nevertheless, inclusion of the previously established hotspot in Oregon in my study allowed the southern portion of Vancouver Island to be identified and assigned as a hotspot by relative comparison.

The high TTX levels detected on Vancouver Island may be the result of historical or evolutionary processes. Earlier observations led to the hypothesis that the island was colonized by newts from less toxic populations, and founder effects contributed to the loss of toxicity on the island (Brodie & Brodie 1991). Subsequent phylogenetic analyses, however, suggested that newts from Oregon and Washington recolonized the northern parts of their range after the retreat of the Cordilleran ice sheet approximately 10,000 years ago (Kuchta & Tan 2005). In this scenario, it is possible that newts on Vancouver Island may also have been colonized by individuals from southerly high toxicity lineages.

The observed variation in TTX levels among populations may also result from other sources of selection. Highly toxic newts, for instance, may be experiencing selection from predators other than garter snakes. There have been multiple recorded instances of novel predators consuming rough-skinned newts, including river otters (*Lontra canadensis*) (Stokes et al. 2015), barred owls (*Strix varia*) (Medina et al. 2018), bullfrogs (Jancowski & Orchard 2013), and caddisfly larvae (predation on eggs; Gall et al. 2012). On the other hand, newts with relatively low TTX concentrations may be more strongly influenced or constrained by other unidentified ecological or genetic factors (Hanifin et al. 2008). Garter snakes with TTX resistant phenotypes are known to experience fitness trade-offs, whereby their locomotor performance is reduced (Brodie & Brodie 1999). It is unknown whether newts experience explicit fitness consequences as a

result of investing resources into toxin production or what those consequences may be. However, the synthesis of defensive chemical compounds is believed to be metabolically costly (Longson & Joss 2006) and such fitness effects are postulated in California newts (Bucciarelli et al. 2017). If this is the case, rough-skinned newts with relatively low toxicity could be investing resources into other fitness-related traits affected by selection from factors besides predation.

There was an impression in the literature of newts across the entirety of Vancouver Island having relatively low toxicity, largely based on an inverse distance-weighted (IDW) interpolation map (Hanifin et al. 2008). By having a greater density of sampling locations on Vancouver Island, I was able to provide a clearer depiction of interpolated toxicity across the island. Visual representation of interpolated toxicity across Vancouver Island from my study indicates the island is not entirely a coldspot (Fig. 2.5). The isocline map of the island visually displays a gradient ranging from higher toxicity populations in the southern tip of the island to lower toxicity in the north (Fig. 2.5). Although the Mantel test on the association of distance between study sites and TTX concentrations was not significant, it is possible selection is influencing the observed patterns. One hypothesis is that non-native species prevalent at the southern end of Vancouver Island could be contributing to the hotspot seen in that region. Both signal crayfish (*Pacifastacus lenisculus*) and American bullfrogs (*Lithobates catesbeianus*) were introduced to the southern portion of the island in the last century and are continuing to spread northwards (Bondar et al. 2005, Orchard 2011). Crayfish and bullfrogs are both known to negatively affect rough-skinned newt populations, but the impacts of non-native species on newt toxicity is currently unknown (see Chapter 4 for a consideration of

invasive species). The southern tip of the island also has the highest urban population density, where more than half of the people living on the island are within the Greater Victoria metropolitan area.

Despite the simplicity of visually representing variation in toxicity (or other continuous traits) across a study region, IDW interpolation methods should be used with caution. First, when input data is not sufficiently dense across the geographic area of interest, the isocline map will not appropriately represent the desired surface (Watson and Philip 1985), as was the case in previous interpolation maps (Hanifin et al. 2008). Even within my study of the island, the northern portion of the island was mostly inaccessible and only contained a few study sites, which likely contributes to the lack of potential hotspots detected in that region. Another problem with the IDW interpolation method is that values that are input into the analysis are sites means; thus, it does not take into consideration the substantial variation within study sites. Additionally, the choice of classification method used for the color classes of interpolated TTX values may significantly alter the conclusions made with reference to the figure (Fig. 2.2, Fig 2.3). When the symbology of the original map (Fig. 2.2; Hanifin et al. 2008) was changed to equal interval classification to represent equal intervals of interpolated toxicity there remained only higher toxicity areas in Oregon with nearly all other localities being represented as low toxicity areas (Fig. 2.3). A continuous color scale should be used to more accurately display the interpolated values rather than classified symbology that can influence the interpretation of the figure.

Overall, this study provides further evidence that reciprocal coevolution is not solely responsible for toxicity in rough-skinned newts, and external selection pressures

from biotic or abiotic factors may contribute to observed patterns of toxicity. The significant variation in tetrodotoxin concentrations both within and among populations of newts on Vancouver Island indicates it is not a coldspot of coevolution as once believed, and it displays patterns inconsistent with theories of garter snake matched resistance (Brodie & Brodie 1991, Hanifin et al. 2008). Such findings align with recent other work on chemical defense in *Taricha*, indicating toxicity is associated with other evolutionary processes or environmental conditions such as simulated predator attacks (i.e., the effects of captivity and tissue sampling acted as a proxy for failed predation; Bucciarelli et al. 2017), elevation, and novel predators (Stokes et al. 2015). Closely related newts from the genus *Notophthalmus* are also known to produce low concentrations of TTX but are not associated with any potentially resistant predators (Yotsu-Yamashita et al. 2012), further implicating a non-coevolutionary explanation for the chemical defense. Additionally, there is evidence of phenotypic mismatch between newt toxicity and snake resistance in one third of sites (Hanifin et al. 2008) as well as substantial variation in toxicity outside the range of garter snake predators (Hague et al. 2016). The chemical ecology of tetrodotoxin in rough-skinned newts is much more complex than originally thought and an understanding is emerging that we cannot adequately explain variation in toxicity across the range of *Taricha granulosa*.

The possibility of other selection pressures influencing the rough-skinned newt and garter snake system has parallels with another previous example of predator-prey interaction, which may also no longer be considered reciprocal coevolution. In Lake Tanganyika, Africa, predatory crabs evolved larger, stronger claws to presumably crush the shells of their prey, with gastropods evolving thicker shells in response (West et al.

1991). However, it is equally likely that the size increase seen in the crab claws could be from selection pressures of their own enemies, because crabs also use their claws defensively (Dietl & Kelley 2002). Similarly, garter snakes with higher toxin resistance have decreased locomotor performance (Brodie & Brodie 1999), so slower crawl speed may subsequently make it more difficult for snakes to evade predators such as raptors or large birds (Sparkman et al. 2013). Garter snakes are also known to be generalist predators, further calling into question how tightly their interaction is correlated with rough-skinned newts when they are capable of predating other non-toxic species (Kephart et al. 1982). Furthermore, although there are 12 recognized subspecies of *Thamnophis sirtalis*, I have not come across any reference to their existence or potential impacts in the rough-skinned newt literature. The Puget Sound garter snake (*Thamnophis sirtalis pickeringii*) is the only subspecies found on Vancouver Island, whereas Oregon is home to the valley garter snake (*Thamnophis sirtalis fitchi*) and red-spotted garter snake (*Thamnophis sirtalis concinnus*) (Janzen et al. 2002). Whether different garter snake subspecies are more or less specialized, or differ in their impacts, on rough-skinned newts remains an open question at this time.

Moving forward, it is crucial for researchers to come to a consensus on the methods used in studies of tetrodotoxin in rough-skinned newts. Incorporating different combinations of toxin extraction and quantification methods can yield TTX concentrations that differ by orders of magnitude (e.g., Hanifin et al. 2008, Bucciarelli et al. 2014, Stokes et al. 2015, Hague et al. 2019). Additionally, the units used to present TTX concentration data vary among studies (e.g., Hanifin et al. 2008, Hague et al. 2019) and can be biased by estimates utilizing measurements of newt body mass (Appendix B).

Such discrepancies confound the ability to compare toxicity data among studies. Inverse distance-weighted interpolation methods must also be used with caution due to the risk of interpolating with low sampling densities across a study area and using inappropriately classified values. Once those differences are resolved, there is great potential with experiments in controlled environments (e.g., mesocosms) to investigate different variables that may impact rough-skinned newt toxicity, and further studies of garter snakes to better understand phenotype (mis-) matching of snake resistance and newt toxicity.

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Figures

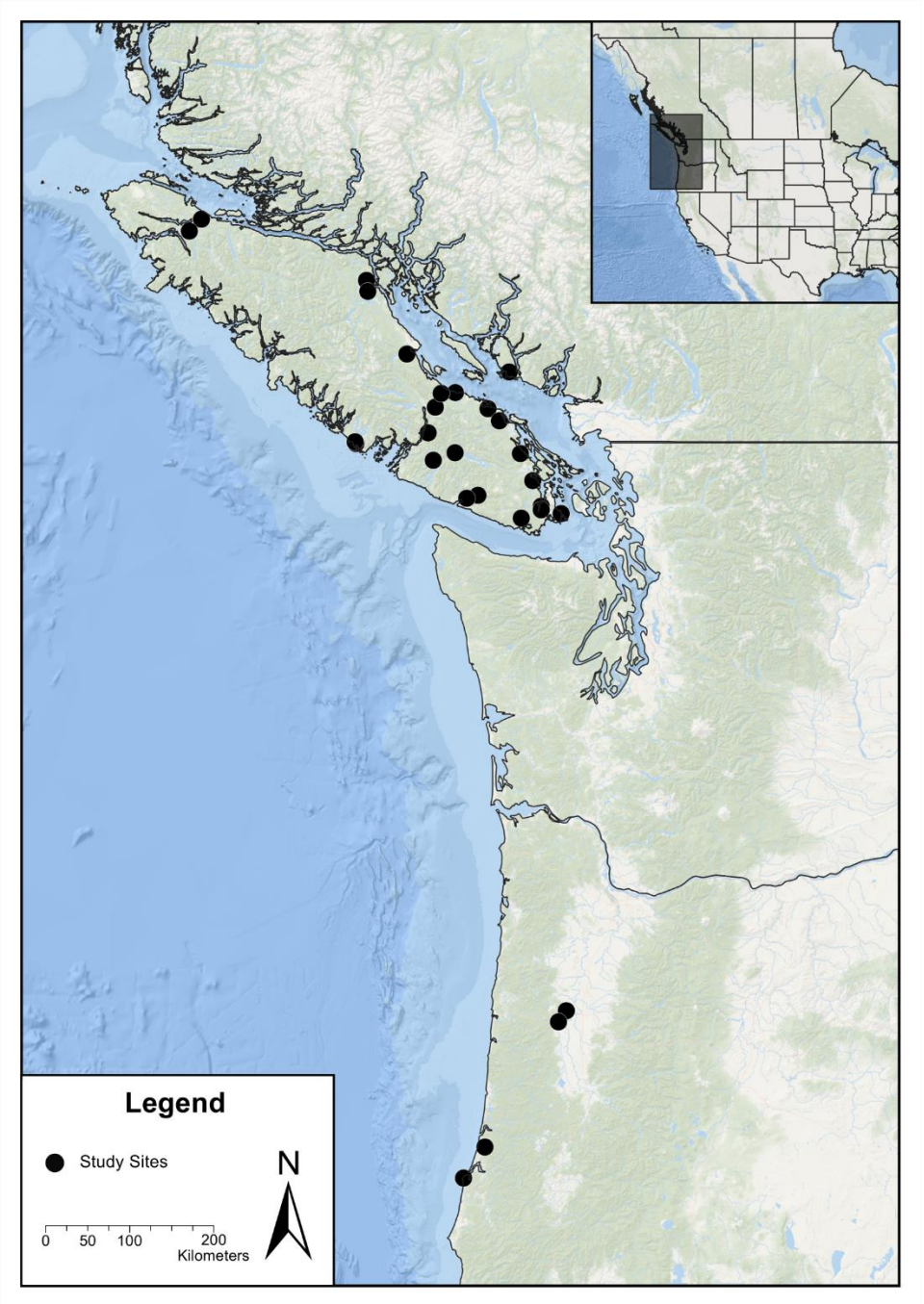

Figure 2.1: Map of the Pacific Northwest displaying locations of the 28 sampling sites in Vancouver Island and Sunshine Coast, BC, Canada and in Oregon, USA. Inset shows the map region relative to Canada and the United States.

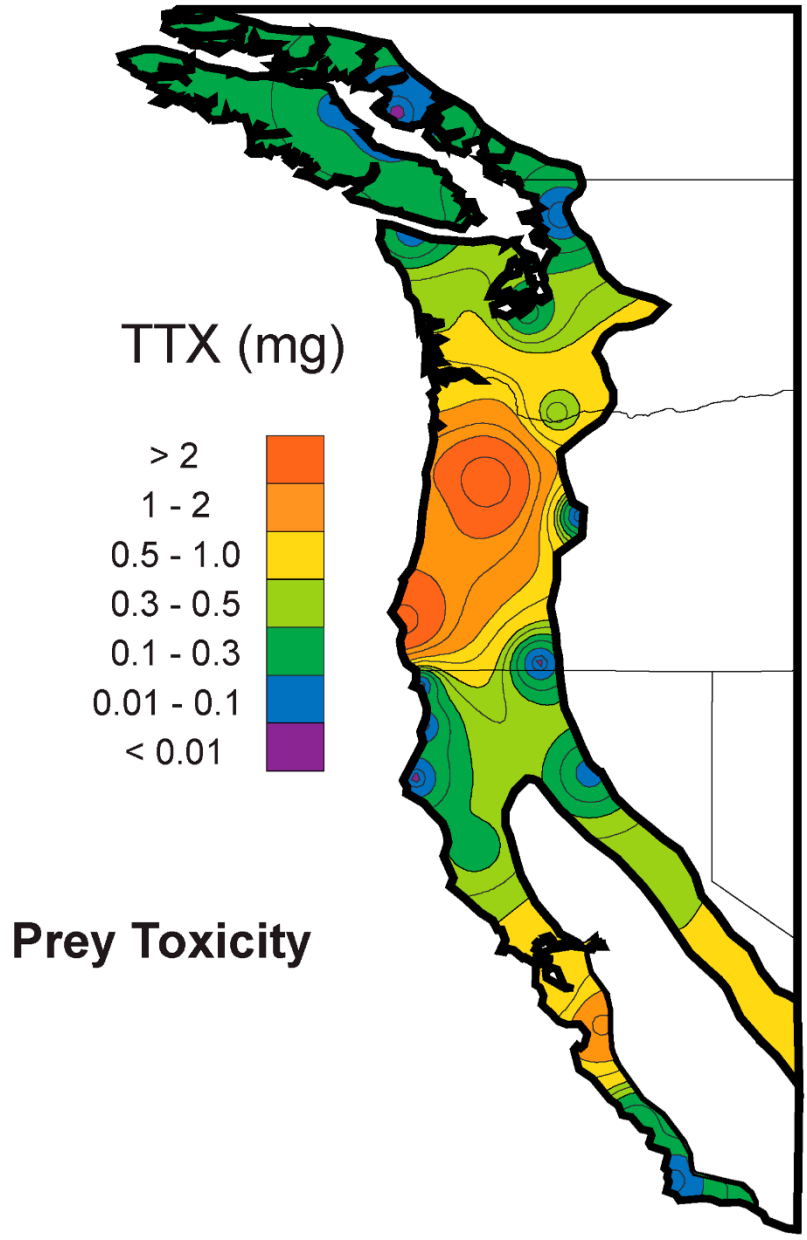

Figure 2.2: Inverse distance-weighted (IDW) interpolation map of whole newt TTX in mg. This figure is from Hanifin et al. (2008) and is published under the Open Access Creative Commons Attribution License (CC BY).

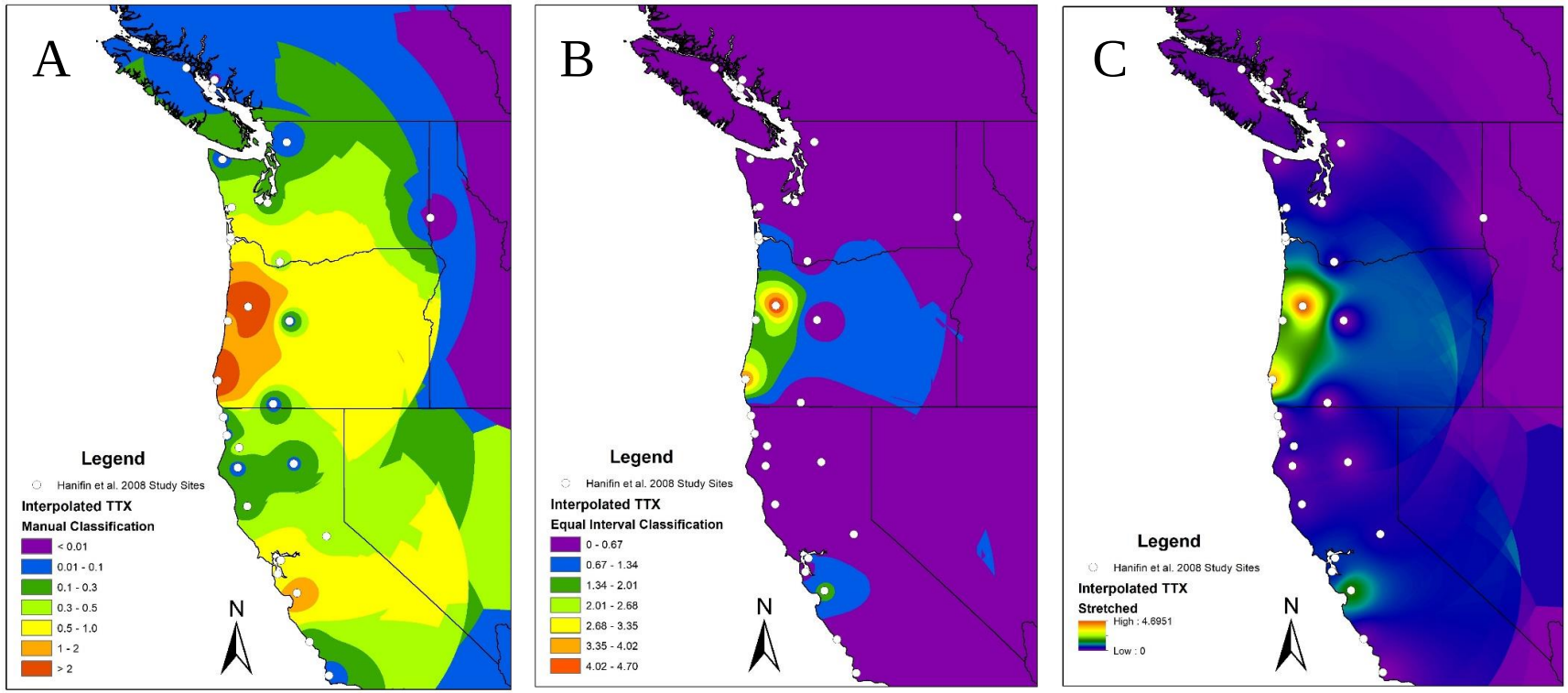

Figure 2.3: Maps recreated using whole next toxicity data in mg from Hanifin et al. (2008) and the same IDW interpolation parameters used in their study. White points represent study sites and were included to display geographic locations used as input for the interpolation analysis. Interpolated TTX values were represented by: (A) manual classification to align with the previous study, (B) equal interval classification, and (C) stretched symbology.

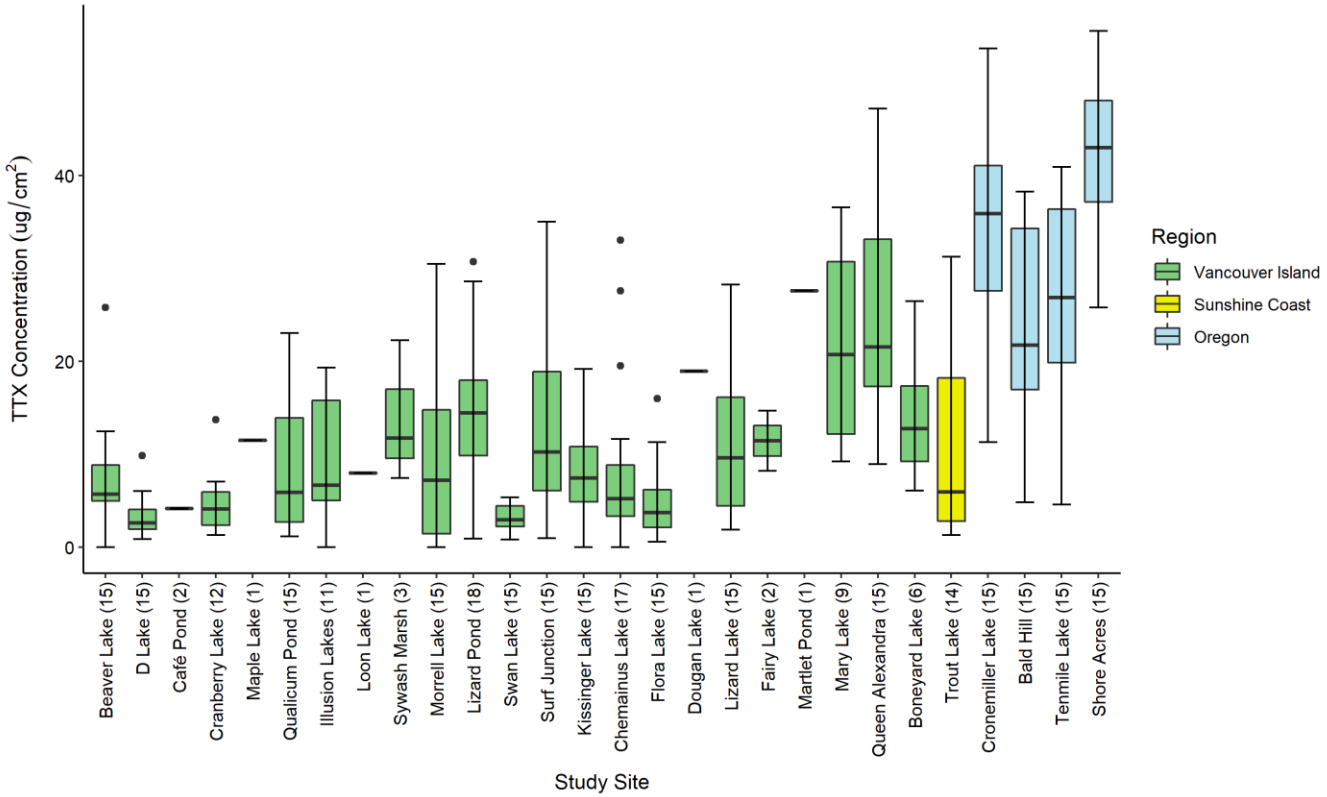

Figure 2.4: Boxplot showing whole newt TTX levels detected at each of 28 study sites from three regions: Vancouver Island, BC, Canada, Sunshine Coast, BC, Canada, and Oregon, USA. Each boxplot shows the distribution of TTX data from a given study site. Whiskers represent the minimum and maximum values, colored boxes show the interquartile range with thick horizontal line for the mean, and black points are outliers. Study sites displaying a single horizontal line had only one sample, which is therefore displayed as the mean. Values in parentheses on the x-axis represent sample size for each boxplot.

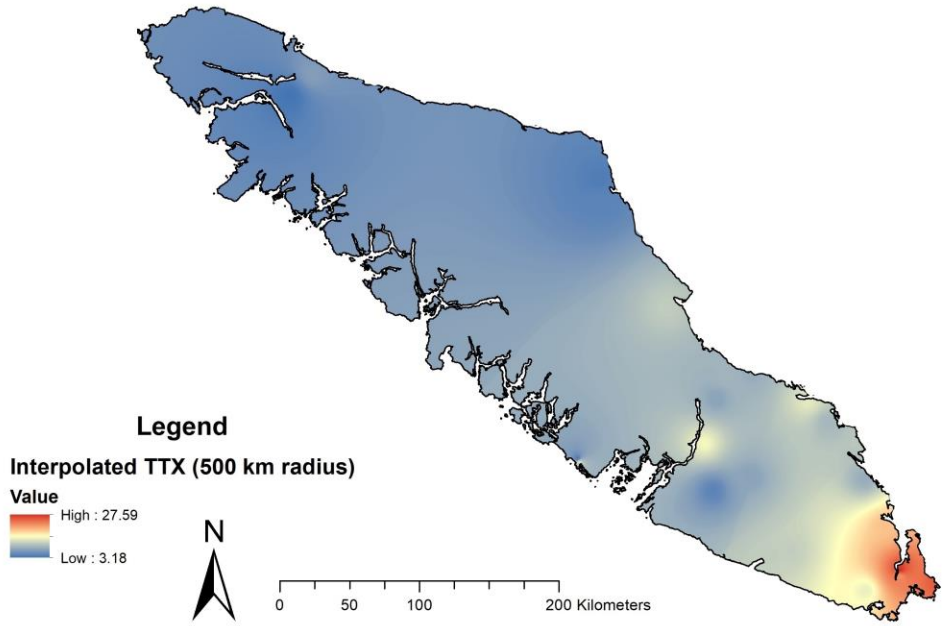

Figure 2.5: Map of Vancouver Island, BC showing inverse distance-weighted interpolation of newt toxicity in µg/cm^2 for 23 study sites. Interpolated TTX values have been classified using a continuous color scale.

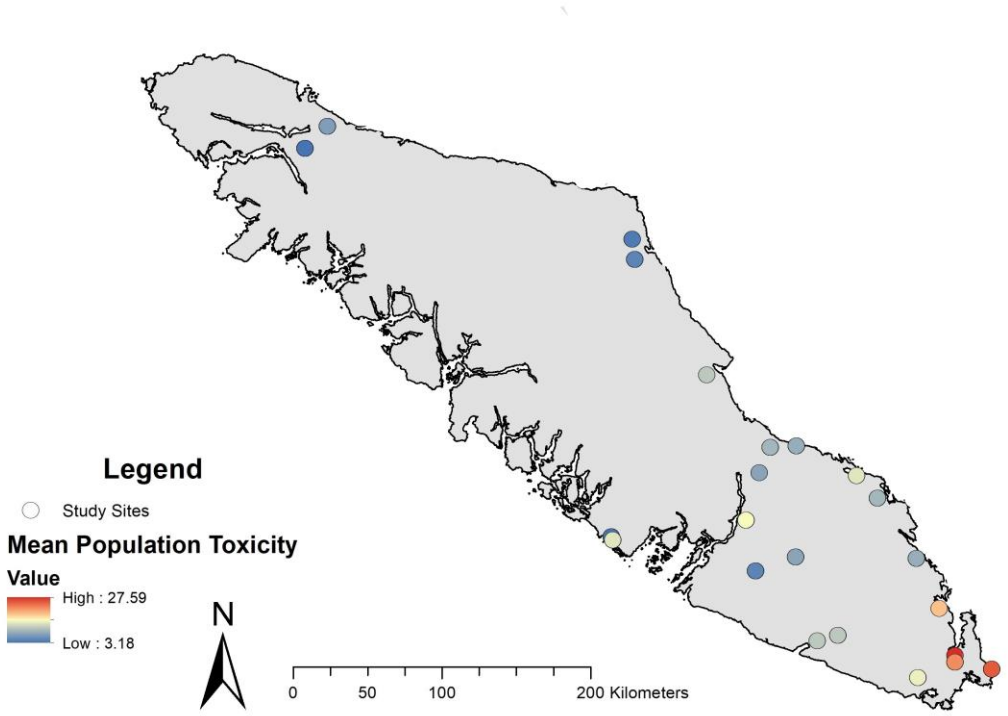

Figure 2.6: Map of 23 study sites on Vancouver Island representing mean newt toxicity in µg/cm^2 at each site based on a continuous color scale.

Tables

Table 2.1: Tetrodotoxin concentration results from this study for 28 study sites sampled in Vancouver Island, Sunshine Coast, and Oregon. Sites are grouped by region and ordered north to south by latitude.

Region	Study Site	N Total	N Males	N Females	Mean TTX (μg/cm^2)	Standard Error of the Mean
Vancouver Island	Beaver Lake	15	14	1	7.55	1.55
	D Lake	15	14	1	3.41	0.60
	Café Pond	2	1	1	4.18	0.06
	Cranberry Lake	12	6	6	4.79	0.99
	Maple Lake	1	0	1	11.52	N/A
	Qualicum Pond	15	8	7	8.72	1.97
	Illusion Lakes	11	7	4	9.48	2.00
	Loon Lake	1	1	0	8.00	N/A
	Sywash Marsh	3	2	1	13.82	4.40
	Morrell Lake	15	14	1	9.65	2.56
	Lizard Pond	18	13	5	14.89	1.84
	Swan Lake	15	12	3	3.18	0.38
	Surf Junction	15	13	2	13.67	2.82
	Kissinger Lake	15	14	1	7.91	1.31

	Chemainus Lake	17	14	3	8.72	2.28
	Flora Lake	15	10	5	4.83	1.08
	Dougan Lake	1	1	0	18.96	N/A
	Lizard Lake	15	13	2	11.49	2.25
	Fairy Lake	2	2	0	11.46	3.23
	Martlet Pond	1	1	0	27.59	N/A
	Mary Lake	9	8	1	21.69	3.54
	Queen Alexandra	15	3	12	24.93	2.96
	Boneyard Lake	6	4	2	14.18	3.06
Sunshine Coast	Trout Lake	14	11	4	10.35	2.55
Oregon	Cronemiller Lake	15	11	4	33.59	3.27
	Bald Hill	15	15	0	23.70	2.76
	Tenmile Lake	15	12	3	26.51	2.82
	Shore Acres	15	13	2	41.97	2.23

Table 2.2: Wilcoxon pairwise comparisons with Bonferroni corrected p-values. Cells with significant p-values (< 0.05) are shaded. Four study sites containing only one sample were removed from this analysis: Maple Lake, Loon Lake, Dougan Lake, and Martlet Pond. An asterisk (*) indicates populations from Oregon, and a plus (+) indicates the Sunshine Coast, BC population.

	Beaver Lake	D Lake	Café Pond	Cranberry Lake	Qualicum Pond	Illusion Lakes	Sywash Marsh	Morrell Lake	Lizard Pond	Swan Lake	Surf Junction	Kissinger Lake	Chemainus Lake	Flora Lake	Lizard Lake	Fairy Lake	Mary Lake	Queen Alexandra	Boneyard Lake	Trout Lake+	Cronemiller Lake*	Bald Hill*	Tenmile Lake*
D Lake	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Café Pond	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cranberry Lake	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Qualicum Pond	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Illusion Lakes	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sywash Marsh	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Morrell Lake	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lizard Pond	0.4295	0.0112	1	0.0532	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Swan Lake	0.3275	1	1	1	1	1	0.6765	1	0.0014	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Surf Junction	1	0.2894	1	1	1	1	1	1	1	0.0481	-	-	-	-	-	-	-	-	-	-	-	-	-
Kissinger Lake	1	1	1	1	1	1	1	1	1	0.7457	1	-	-	-	-	-	-	-	-	-	-	-	-
Chemainus Lake	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-	-	-	-
Flora Lake	1	1	1	1	1	1	1	1	0.0209	1	1	1	1	-	-	-	-	-	-	-	-	-	-
Lizard Lake	1	0.4465	1	1	1	1	1	1	1	0.3886	1	1	1	1	-	-	-	-	-	-	-	-	-
Fairy Lake	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	-	-	-	-	-	-	-	-
Mary Lake	0.0583	0.0228	1	0.0225	1	1	1	1	1	0.0004	1	0.2035	0.5856	0.0080	1	1	-	-	-	-	-	-	-
Queen Alexandra	0.0018	0.0011	1	0.0002	0.0594	0.0832	1	0.2150	1	3.56e-6	1	0.0013	0.0510	6.76e-5	0.3275	1	1	-	-	-	-	-	-
Boneyard Lake	1	0.2577	1	0.8920	1	1	1	1	1	0.0101	1	1	1	0.6510	1	1	1	1	-	-	-	-	-
Trout Lake+	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.3694	1	-	-	-	-
Cronemiller Lake*	6.76e-5	0.0009	1	6.35e-e5	0.0010	0.0099	1	0.0158	0.0253	3.56e-6	0.0197	0.0002	0.0055	2.49e-5	0.0043	1	1	1	0.6510	0.0065	-	-	-
Bald Hill*	0.0155	0.0020	1	0.0014	0.0893	0.2270	1	0.4465	1	1.42e-5	1	0.0155	0.1063	0.0002	1	1	1	1	1	0.6243	1	-	-
Tenmile Lake*	0.0056	0.0017	1	0.0021	0.0197	0.1082	1	0.1164	0.6542	2.49e-5	1	0.0043	0.0592	0.0002	0.1595	1	1	1	1	0.1423	1	1	-
Shore Acres*	3.56e-6	0.0009	1	3.18e-5	3.56e-6	7.14e-5	0.6765	0.0017	6.39e-6	3.56e-6	0.0001	3.56e-6	0.0009	3.56e-6	2.49e-5	1	0.0410	0.0893	0.0203	2.85e-5	1	0.0073	0.0594

CHAPTER THREE – TRANSCRIPTOME ANALYSIS REVEALS NOVEL DIFFERENTIALLY EXPRESSED GENES RELATED TO NEWT TOXIN DEFENSE

3.1 Introduction

In recent years, there has been rapid progress in massively parallel sequencing technologies, but many challenges remain for genomic studies of non-model organisms (Gayral et al. 2013, da Fonseca et al. 2016). It is especially difficult to study the genomics of species that have highly repetitive genomes, particularly amphibians, and it can be insurmountable to produce genome assemblies due to computing resource limitations (Funk et al. 2018). To date, there are only 17 amphibian genomes published in the NCBI database, compared to e.g., 808 mammal and 725 insect genomes (National Center for Biotechnology Information 2020a). A solution to this problem is to utilize a transcriptomics or RNA-sequencing (RNA-seq) approach from high-throughput sequencing data built from high-quality RNA. One goal of RNA-seq is to interpret the functional elements of the genome, which makes this a practical method for studying genes from species with highly repetitive genomes (Wang et al. 2009). RNA-seq also allows the *de novo* assembly and analysis of the transcriptome without mapping to a reference genome (Grabherr et al. 2011). *De novo* transcriptome assembly methods are especially useful for highly diverged species or those with complex genomes, and can even outperform genome-guided assemblies in non-model organisms (Huang et al. 2016). Moreover, this approach enables the identification of differentially expressed genes between different samples, allowing the genetic study of phenotypic characteristics (Costa-Silva et al. 2017).

An RNA-seq approach would be useful to studies of the genomics of adaptation in the rough-skinned newt, *Taricha granulosa*; it is amphibian species that has been the subject of much historical and contemporary work and is a textbook story of predator-prey interaction. This urodelan amphibian is famous for its deadly phenotype in which a potent neurotoxin known as tetrodotoxin (TTX) is primarily excreted through its dorsal skin as a predator deterrent (Brodie 1968, Hanifin et al. 2004). There is significant variation in TTX concentration among populations of newts, and researchers had previously attributed this variation to predation pressure from garter snakes (*Thamnophis sirtalis*), which have developed a resistance to this toxin (e.g., Brodie & Brodie 1990, Brodie et al. 2002, Brodie et al. 2005).

Despite the chemical ecology of rough-skinned newts being widely studied over the past several decades, researchers have not yet been able to determine the source of toxin production in this species. The use of TTX as a defense mechanism has also convergently evolved in several other taxa including pufferfish, blue ringed octopus, and moon snails (Moczydlowski 2013), in which tetrodotoxin may be bioaccumulated through the food chain, produced by endosymbiotic bacteria, or synthesized endogenously through unknown mechanisms (Jal & Khora 2015). In rough-skinned newts, TTX bioaccumulation though diet was first tested in captivate newts fed a TTX-free diet, but this hypothesis was rejected as newts retained their toxin concentrations over time (Hanifin et al. 2002). Moreover, newts are still capable of excreting TTX from their skin and producing TTX-laden eggs even in long-term captivity (Cardall et al. 2004, Gall et al. 2012). Endosymbiotic bacteria were also considered as a possible source of TTX, but one study failed to detect any bacteria in newt skin (Lehman et al. 2004). More

recently, a study on the skin microbiome of rough-skinned newts did find evidence of the presence of bacteria capable of producing tetrodotoxin (Vaelli et al. 2020). However, it is still unclear if the bacteria are solely capable of producing such high concentrations of TTX, if they respond to the selection pressures experienced by newts, and how newts may transport and sequester TTX produced by the bacteria in their granular skin glands. The same study also indicated that they administered antibiotics to the newts, without significant reduction in skin toxicity (Vaelli et al. 2020).

The possibility remains that TTX in rough-skinned newts is synthesized, whether partially or fully, within the individual and may therefore be under genetic control. Chau et al. (2011) postulated that the biosynthesis of tetrodotoxin may involve similar mechanisms to that of saxitoxin (STX) in which two of the major genes in the STX biosynthetic pathway, *NRPS* and *PKS*, may encode enzyme complexes to act as substrates for pre-cursors in the synthesis of TTX. This idea was based on the finding that some TTX producing species, including the pufferfish *Fugu pardalis* and *Takifugu oblongus*, are also capable of producing STX, which is synthesized through a cluster of 26 genes (Jang & Yotsu-Yamashita 2006, Kellman et al. 2008, Ngy et al. 2009). Other studies of the tetrodotoxin biosynthetic pathway in toxic newts have suggested TTX is derived from a terpene precursor (Kotaki & Shimizu 1993, Kudo et al. 2016). Although previous studies have hypothesized the existence of an endogenous origin of tetrodotoxin in rough-skinned newts, investigations of the molecular mechanisms responsible for adaptations of chemical defense in the species remain lacking (Chau et al. 2011). Recent work has also suggested that TTX variation in rough-skinned newts may instead be related to neutral population divergence, whereas snake resistance is an evolutionary

response to prey toxicity rather than reciprocal coevolution, further implicating a genetic basis to newt toxicity (Hague et al. 2019). Regardless of whether TTX is produced endogenously within newts, by endosymbionts, or otherwise, newts presumably must possess adaptations to withstanding such high levels of the toxin in their skin and concentrating it in their granular skin glands and other tissues such as eggs.

The goal of this chapter is to make a crucial step towards elucidating the molecular mechanisms of adaptation to high concentrations of tetrodotoxin by (i) characterizing the transcriptome of rough-skinned newts from populations known to produce high quantities of TTX, and (ii) identifying genes that are differentially expressed between the dorsal and ventral tissue of newts. A previous study found that the dorsal skin in newts has approximately five times higher TTX concentration than the ventral skin within an individual (Hanifin et al. 2004). Therefore, by comparing gene expression in these two dermal tissue samples from the same individuals, I will be able to account for potential confounding variables that might occur when comparing samples between tissue types. Here, I present the transcriptome characterization and differentially expressed genes of *Taricha granulosa* using Illumina RNA-sequencing technology and bioinformatics tools, while combining a hypothesis-driven and exploratory approach to identify genes related to newt toxicity. This work enables further study of chemical defense in *T. granulosa* and will document the genes expressed in the skin of newts, including any putatively responsible for tetrodotoxin synthesis or adaptations to retaining such high concentrations in their skin. In addition, this work will expand our understanding of salamander genes in general and will provide a valuable resource for

future genetic studies in this species, such as population genetics research (Chapter 5, this thesis) and comparative transcriptomics.

3.2 Methods

3.2.1 Sample Collection

I collected seven adult male rough-skinned newts from sites in Benton County and Coos County Oregon, USA following methods described in Chapter 2. I only included males in this study to eliminate the potential for any sex-related genetic differences. Individuals were euthanized by submersion in a water bath with a lethal concentration of tricaine methanesulfonate (MS-222) for over 30 minutes. I used a 3mm skin biopsy tool (Robbins Instruments, USA) to remove dorsal and ventral skin samples from each newt. The skin samples were immediately placed in individual tubes of RNAlater solution (Thermo Fisher Scientific, USA) and kept at room temperature for approximately 24 hours. I then placed the tubes in a liquid nitrogen dewar at -190°C before transportation back to the University of Calgary for storage at -80°C. Sample collection was performed in accordance with Oregon Department of Fish and Wildlife Scientific Taking Permit 064-18 and University of Calgary Animal Use Protocol AC15-0033.

3.2.2 RNA Extraction and Sequencing

Total RNA was isolated and purified with the RNeasy Micro Extraction Kit (Qiagen, USA) including on-column DNase digest (Qiagen, USA) using a modified protocol that I optimized in which the tissue samples were macerated with a glass tissue grinder without freezing in liquid nitrogen (Appendix D). Individual libraries were

constructed using the TruSeq Stranded mRNA Library Prep kit (Illumina, USA). Whole transcriptome sequencing (100-bp paired-end) was performed on an Illumina HiSeq 4000 at a sequencing depth of approximately 16-40M reads per library. Library preparation and sequencing was performed at the Genome Quebec Innovation Centre.

3.2.3 De novo Transcriptome Assembly and Annotation

Raw reads from the seven individuals were first pooled into two fastq files, one for all forward reads and another for all reverse reads. To remove low quality reads, I performed quality control with Trimmomatic v0.36 (Bolger et al. 2013) using the following parameters: (i) Trimming of bases at the leading and trailing ends of sequences with a phred+33 quality score below 20, (ii) a four-base sliding window scan to remove read fragments with an average quality per base below 20, (iii) removal of reads below 36 base pairs long, and (iv) clipping of Illumina adapter sequences. After trimming, only reads with both forward and reverse reads remaining were used for the assembly because the Trinity program does not support a mix of paired and unpaired reads. I used the quality-filtered reads to perform a *de novo* transcriptome assembly with Trinity v2.8.5 (Grabherr et al. 2011, Haas et al. 2013) with the default parameters.

Assembly statistics were generated using a perl script within the Trinity package to summarize the length of the assemblies and transcripts produced. I then mapped the cleaned reads to the assembly with Bowtie2 (Langmead & Salzberg 2012). Using BUSCO v3.0.2, I assessed the transcriptome completeness via the number of complete, duplicated, fragmented, and missing Benchmarking Universal Single-Copy Orthologs (BUSCOs) in the assembly (Simão et al. 2015). The presence of single-copy orthologs was detected by determining the conserved ortholog content with Tetrapod single-copy

orthologs from the OrthoDB v9.1 database (Zdobnov et al. 2016). Fragmented genes are those that were only partially recovered, while missing genes are those with no significant matches in the ortholog database (Simão et al. 2015). I also compared the similarity between the rough-skinned newt dorsal tissue assembly and that of retinal tissue from the Japanese fire belly newt, *Cynops pyrrhogaster*, (Nakamura et al. 2014). *C. pyrrhogaster* was chosen because it had the most comparable assembly statistics and is also capable of producing low concentrations of TTX (Tsuruda et al. 2001). Orthologs between the rough-skinned newt and Japanese fire belly newt transcriptome assemblies were identified using a reciprocal best-hit BLAST approach (Moreno-Hagelseib & Latimer 2007).

I annotated the *de novo* assembly with the Trinotate v3.1.1 pipeline, which incorporates several different programs (Bryant et al. 2017). TransDecoder v5.5.0 was first used to locate candidate coding regions and identify those with the longest open reading frame (TransDecoder 2020). Contigs were characterized via blastx and blastp sequence homology searches against the UniProt/SwissProt NCBI *nr* protein database, using BLAST+ v2.4.0 with an e-value cutoff of $1e^{-5}$ (Camancho et al. 2009). I based the choice of e-value following methods for the annotation of the axolotl transcriptome by the authors of the Trinotate pipeline (Bryant et al. 2017). Next, I completed an HMMER v3.0 (Eddy 2019) search that uses hidden Markov models to identify sequence homologs, which are proteins with shared ancestry to the transcript sequences (Eddy 2019). Next, TmHMM v2.0 also uses hidden Markov models to predict transmembrane domains, which are helix structures in the membranes of proteins (Sonnhammer & Krogn 1998). Finally, I used SignalP v4.1 to predict the presence of signal peptides that are responsible

for translocating proteins across the endoplasmic reticulum membrane (Petersen et al. 2011). The results from these programs along with the Trinity assembled transcriptome file were loaded into a Sqlite database and Trinotate v3.1.1 was used to carry out the functional annotation. I then assigned Gene Ontology (GO) terms to the annotated sequences using a perl script within Trinity (Grabherr et al. 2011, Haas et al. 2013), based on matches to homologous proteins from TrEMBL/SwissProt databases (Bairoch & Apweiler 2000).

3.2.4 Differential Gene Expression Analysis and Comparison to Saxitoxin Genes

I used the program RSEM v1.3.1 (Li & Dewey 2011) to estimate expression values for transcripts and genes by aligning separate sample read pairs back to the Trinity assembly with Bowtie2 (Langmead & Salzberg 2012). These results were then converted into a matrix of counts and a matrix of normalized expression values to be used in downstream analyses of differential expression. Next, I used edgeR v3.28.0 (Robinson et al. 2010) to identify genes with a false discovery rate (FDR) threshold of 0.05 that were at least two-fold differentially expressed (log ratio of gene expression values between the dorsal and ventral samples to identify over and under expressed genes). Pairwise comparisons were performed among each of the dorsal and ventral replicates. Using custom perl scripts from the Trinity package, I extracted and clustered the differentially expressed transcripts by their patterns of expression among the samples. Gene Ontology enrichment analysis was also performed using GO-Seq v1.38.0 (Young et al. 2010) to classify significantly enriched and depleted GO categories from the sample comparisons. To further investigate potential function of upregulated differentially expressed genes, I performed *post hoc* blastx searches to identify matches in the *nr* protein database in the NCBI BLAST Web

Interface (Johnson et al. 2008). Finally, I searched for sequences from two of the major genes (*NRPS* and *PKS*) in the known biosynthetic pathway of saxitoxin in the rough-skinned newt transcriptome. The genes from the UniProt database were compared to the Trinity transcripts and TransDecoder predicted peptides.

3.3 Results

3.3.1 De Novo Transcriptome Assembly, Assessment, and Functional Annotation

A total of 353,375,308 raw reads were filtered and assembled into 361,968 transcripts using Trinity and represent an average length of 784.48 bp, median length of 335, N50 length of 1874, and GC content of 44.66% (Table 3.1, Fig. 3.1). After aligning the reads to the assembly, approximately 95.4% of read pairs were properly mapped. Next, I assessed the assembly completeness by identifying BUSCOs, which revealed that 84.3% of vertebrate BUSCO genes were recovered in the *T. granulosa* transcriptome [48.2% complete and single-copy (N=1904), 36.1% complete and duplicated (N=1427)], with 5.2% fragmented (N=207) and 10.5% missing (N=412).

I benchmarked the relative *de novo* assembly quality via comparison to transcriptome assemblies of other urodelan amphibian species. Assembly statistics from the rough-skinned newt transcriptome were compared to those of seven other species (Table 3.2). I selected these studies for comparison in order to represent a variety of urodelan amphibian species with completed transcriptome assemblies. The *de novo* transcriptome assembly of *T. granulosa* yielded comparable assembly and annotation statistics to other salamander assemblies. Specifically, it had similar BUSCO results to *C. phyrrogaster* and *A. mexicanum.* Additionally, orthologs between the rough-skinned newt

and Japanese fire belly newt transcriptome assemblies were identified using a reciprocal best-hit BLAST approach. A total of 30,556 orthologous sequences between the two transcriptomes were detected with a query coverage above 70%, which represented approximately 12.9% of *C. pyrrhogaster* transcripts.

To better understand putative functions of assembled transcripts, I conducted functional annotation with the Trinotate v3.1.1 pipeline (Bryant et al. 2017). Trinotate reported 111,666 annotated transcripts for *T. granulosa*, representing about 30.8% of the 361,968 *de novo* assembled transcripts. A total of 62,762 transcripts were assigned at least one GO term, which covered various functional pathways (Table 3.3). Of those, approximately 48.6% were classified as biological processes, 23.0% as molecular functions, and 28.4% as cellular components. Many GO terms could be assigned to one transcript, so there was overlap between the three categories (Fig. 3.2).

3.3.2 Differential Gene Expression

There were significant differences in gene expression detected between dorsal and ventral dermal tissue of rough-skinned newts. Expression profiles of transcripts across all samples revealed strong positive correlation within dorsal and ventral replicates, and negative correlation between sample types (Fig. 3.3). A total of 234 genes were detected with FDR < 0.05 that were at least two-fold differentially expressed, with 177 genes upregulated in ventral samples and 57 genes upregulated in dorsal samples (Fig. 3.4). GO enrichment analysis identified terms enriched and depleted in both dorsal and ventral differentially expressed genes, where multiple GO terms could be annotated to a single gene (Table 3.4). Enriched terms in one tissue type are not inherently depleted in another; they are identified by comparing the observed frequency of a GO term with the frequency

expected by chance (Huang et al. 2008). Genes enriched, or "over-represented", in dorsal samples are particularly of interest due to the significantly higher concentration of TTX known to occur in this tissue type (Hanifin et al. 2004). There were 109 GO terms enriched in the dorsal sample replicates (Appendix E), representing 19 of the 57 upregulated genes. The remaining 38 genes were not annotated by GOseq, which could be due to quality filtering or the absence of functional annotation for homologous genes in the TrEMBL/SwissProt databases. Fifteen of the 38 genes did not have any matches in the NCBI *nr* protein database, and the remaining genes corresponded to proteins covering a wide range of taxa and quality scores (Appendix F). The 15 novel genes could be unique to rough-skinned newts, and possibly associated with adaptations to retaining TTX in their dorsal skin.

Of the annotated genes over-represented in dorsal tissue, a majority of the most significantly enriched GO terms were related to melanin and pigmentation. This was expected due to the dorsal skin being a dark brown color compared to the bright orange ventral side of rough-skinned newts, therefore serving as a positive control of the effectiveness of this analysis. However, the analysis also identified additional sets of GO terms revealing potential adaptations to tetrodotoxin in newts. There were 12 GO terms associated mainly with membrane potential and permeability (Table 3.5), all of which were associated with the same two Trinity assembled genes: *TRINITY_DN1094_c0_g1* and *TRINITY_DN113557_c0_g1*. The nucleotide sequences from these genes were then blasted against the *nr* protein database in the NCBI BLAST Web Interface (Johnson et al. 2008). They both matched sequences for the heme-binding protein 2 (*HEBP2*), but only with a maximum percent identity of 54% for *TRINITY_DN1094_c0_g1* and 38% for

TRINITY_DN113557_c0_g1. Additionally, there were three GO terms related to terpene biosynthesic and metabolic processes that matched to the *TRINITY_DN17261_c0_g1* gene (Table. 3.6), but the only matches in the NCBI database were uncharacterized proteins. Finally, I blasted the genes *NRPS* and *PKS* from the biosynthetic pathway of saxitoxin against the assembled Trinity transcripts and TransDecoder predicted peptides. I did not detect either of these genes, which were hypothesized to encode enzyme substrates for the synthesis of TTX (Chau et al. 2011), in the transcriptome of the rough-skinned newt.

3.4 Discussion

Tetrodotoxin (TTX) is a potent neurotoxin used in antipredator defense by several aquatic species, including the rough-skinned newt, *Taricha granulosa*. While the source of chemical defense in newts remains unresolved, newts should nevertheless possess molecular adaptations allowing them to retain high concentrations of TTX in their skin. Here, I used an RNA-seq approach to present the *de novo* transcriptome assembly and annotation of dorsal skin samples from the tetrodotoxin-bearing species *T. granulosa*, and subsequent analysis of differentially expressed genes putatively related to adaptations to toxicity or biosynthetic pathways of TTX. To my knowledge, this is the first report of transcriptome analysis of this species, and the data provided here are not only a crucial first step in identifying novel genes in newt dorsal skin potentially associated with toxin defense, but will also serve as a valuable resource to expand our knowledge of rough-skinned newt genetics.

Overall, the rough-skinned newt transcriptome yielded assembly and annotation statistics comparable to other salamander assemblies (Table 2), suggesting that my

assembly is on par with or exceeds the quality of other published transcriptomes. The Trinity assembled transcriptome consisted of 361,968 transcripts, in which approximately 30.8% of the transcripts were annotated based on sequences of orthologous function. The annotation rate is similar to the 36.7% of 237,120 transcripts annotated for the Japanese fire belly newt, *Cynops phyrroghaster* (Nakamura et al. 2014). Unannotated transcripts could indicate artifacts of mis-assemblies or non-coding RNAs, although many are likely novel genes with unknown function due to the general lack of knowledge about amphibian proteins. The rough-skinned newt and Japanese fire belly newt assemblies also shared 13.2% orthologous sequences (N=31,370) based on a reciprocal best-hit blast approach. The number of orthologous sequences detected is higher than in other salamander species reciprocal comparisons; for example, the comparison between *Bolitoglossa ramosi* and *Ambystoma mexicanum* identified 13,065 shared orthologs (Gomez et al. 2018). The large difference in orthologs detected could be because *T. granulosa* and *C. phyrroghaster* both belong to the Salamandridae family and share a more recent common ancestor than the cross-family comparison between *B. ramosa and A. mexicanum*.

The analysis of Benchmarking Universal Single-Copy Orthologs (BUSCOs) indicated that I recovered approximately 84% of vertebrate orthologs in the *T. granulosa* transcriptome based on the total number of complete and duplicated BUSCOs. The duplication seen in the BUSCO results is also similar to that of *C. pyrrhogaster* (Nakamura et al. 2014) and *A. mexicanum* (Bryant et al. 2017) (Table 3.2), which could be due to a couple of reasons. First, amphibians are known to have complex, highly duplicated genomes (Mable et al. 2011). Second, the Trinity assembly may also include

isoforms of the same gene or allelic heterozygote transcripts from different individuals in which molecular variants at a given site are assembled into separate but similar transcripts. Higher proportion of complete BUSCOs would also be expected in transcriptome assemblies of multiple tissue types and life stages (Waterhouse et al. 2017). Therefore, a relatively low percentage of complete BUSCOs recovered in targeted samples may still be indicative of a high-quality transcriptome assembly due to the lower number of tissue-specific genes expressed (Waterhouse et al. 2017).

Differential gene expression analysis and Gene Ontology enrichment revealed novel genes that may be associated with rough-skinned newt adaptations to resisting high concentrations of tetrodotoxin in their dorsal tissue. Tetrodotoxin functions by blocking the voltage-gated sodium channels on cell membranes of neurons and muscles, thus preventing action potential generation and propagation (Lee & Ruben 2008). There was a set of 12 GO terms enriched in dorsal replicates that were mostly related to membrane potential and permeability, which could reveal adaptations allowing newts to withstand TTX concentration in their skin (Table 3.5). The GO terms were all mapped to the same two differentially expressed Trinity genes, *TRINITY_DN1094_c0_g1* and *TRINITY_DN113557_c0_g1*, and a blastx search revealed the sequences most closely match with heme-binding protein 2 (*HEBP2*). In humans, *HEBP2* is thought to play a role in the collapse of mitochondrial membrane potential prior to necrotic cell death (National Center for Biotechnology Information 2020b). By using TTX to block sodium channels, researchers found that activation of voltage gated sodium channels and entry of sodium ions are crucial events required for cell death (Banasiak et al. 2004). The enriched GO terms such as “Positive regulation of necrotic cell death” and “Positive regulation of

membrane permeability" indicate activity is taking place that allows cell death in newt dorsal tissue to proceed despite TTX potentially blocking such events from occurring. Newts are known to have some degree of resistance to TTX thereby preventing self-intoxication (Hanifin & Gilly 2015), so these genes may be associated with TTX resistance. Nevertheless, the Trinity genes still had a relatively low match with *HEBP2*, with a maximum percent identity of 54% for *TRINITY_DN1094_c0_g1* and 38% for *TRINITY_DN113557_c0_g1*. The two differentially expressed genes associated with the 12 membrane potential and permeability GO terms are likely unique homologs serving similar function in other species.

The differential gene expression analysis also provided insight into potential biosynthetic pathways of tetrodotoxin. Another set of three GO terms indicated the gene *TRINITY_DN17261_c0_g1* is related to terpene biosynthetic and metabolic processes (Table. 3.6). Terpenes are organic hydrocarbon compounds that were one of several proposed precursors thought to form the carbon backbone during the synthesis of tetrodotoxin (Kotaki & Shimizu 1993, Kudo et al 2016). Although the Trinity gene only matched uncharacterized proteins in the *nr* database, this provides potential evidence that TTX biosynthesis may indeed utilize terpene precursors as previously hypothesized. It remains unclear whether newts use the terpenes to synthesize TTX endogenously, or if bacterial endosymbionts may uptake such molecules from newt dorsal skin to further synthesize tetrodotoxin. Moreover, the *NRPS* and *PKS* genes from the synthesis of STX were not located in the rough-skinned newt transcriptome, despite the functional similarity of TTX to STX. Saxitoxin is found in a variety of marine species through a cluster of 26 genes (Kellman et al. 2008), and TTX production may similarly come from

a cluster of multiple genes, whether synthesized endogenously in newts or by endosymbiotic bacteria. If bacteria are responsible for producing TTX and newt toxicity is subject to selection, then this would require heritability of the skin microbiome across generations (Vaelli et al. 2020). The heritability of such bacterial communities remains unknown, and further calls into question whether the bacteria would be capable of responding to selection pressures from predators for increased toxicity and producing such deadly concentrations of TTX in a single newt. On the other hand, individual variation in newt skin microbiomes without heritability might explain the high variation of TTX concentration observed in rough-skinned newt populations, further suggesting a non-coevolutionary explanation for the substantial variation detected across their range (e.g., Hanifin et al. 2008, Stokes et al. 2015, Hague et al. 2016; Chapter 2, this thesis).

It is also important to consider the differentially expressed genes that were unannotated, as these genes could be unique to the rough-skinned newt and possibly related to other unidentified adaptations to chemical defense. Of the 57 differentially expressed genes in dorsal versus ventral replicates, 23 of them were unannotated by GOseq but had potential matches in the *nr* protein database, and 15 were not similar to any known proteins. As previously mentioned, unannotated transcripts may be artefacts of mis-assembly, but it is more likely that they are novel genes with unknown function because the estimated mis-assembly rate for Trinity *de novo* transcriptomes assembled with paired-end sequencing data is only 1.31% (Celaj et al. 2014), and it is common for unannotated genes to be differentially expressed (e.g., Denoeud et al. 2008, Trapnell et al. 2010). The 15 differentially expressed genes lacking a match to any homologous proteins in annotated protein databases may represent genes unique to newts that are involved in

adaptations to dorsal skin toxicity or are potentially related to endogenous sources of TTX.

The possibility remains that TTX concentration in rough-skinned newts is a phenotypically plasticity trait. Phenotypic plasticity occurs when multiple phenotypes arise from a single genotype due to changes in the biotic or abiotic environment (Pigliucci 2001) and is primarily regulated by differential changes in gene expression (Schlichting & Smith 2002). Plasticity is also known to be widespread in amphibians (Levis & Pfennig 2018) and can be predator-induced (e.g., Middlemis Maher et al. 2013). One example of phenotypic plasticity of chemical defense is when common toads, *Bufo bufo,* displayed plasticity of bufadienolide toxin production in response to predation risk (Hettyey et al. 2019). Recent work also suggests tetrodotoxin defense in the rough-skinned newt's congener, *Taricha torosa,* may be an inducible, plastic trait in which California newts are able to increase their toxicity from baseline levels in response to simulated predator attacks (Bucciarelli et al. 2017). If this were also the case in rough-skinned newts, the same genes may be responsible for observed toxin levels in different individuals and populations, with changes in expression patterns of TTX-related genes corresponding to variation in toxicity.

While the differential expression and Gene Ontology enrichment analyses identified genes that may be related to adaptations enabling newts to retain TTX in their dermal tissue or synthesize TTX, I wanted to discuss some potential limitations of the study. First, the analyses may have failed to detect genes differently expressed at lower levels in the tissues studied or those filtered out by the stringency parameters utilized. Unfortunately, one caveat of transcriptomics is that highly differentially expressed and

conserved genes are prioritized over those which cannot be annotated or only undergo moderate fold-changes (Evans 2015). Second, differentially expressed genes between dorsal and ventral tissue may be related to other unknown functions rather than TTX expression. Moreover, the functional role of genes identified in this chapter are not yet validated and require further experimental investigation e.g., via quantitative real-time polymerase chain reaction (qPCR; Derveaux et al. 2008). Additional work is needed to clarify or rule out the potential contribution of bacterial endosymbionts.

The transcriptome data provided here help advance the understanding of potential molecular mechanisms of chemical defense in rough-skinned newts. Novel differentially expressed genes that may be related to toxicity in their dorsal skin were successfully identified, representing one step on a long journey to fully elucidating the source of the toxin in newts. The transcriptome assembly will be useful to other studies of rough-skinned newts for different purposes, such as population genetics analyses (Chapter 5, this thesis) or comparative transcriptomics. Another goal may be to characterize the differential expression patterns of the genes identified in this chapter among different populations of newts with varying TTX levels to study the phenotypic plasticity of toxin defense. Future work should compare differential expression not only within *T. granulosa* in relation to their toxin levels and skin microbiome, but to closely related species that do not synthesize TTX and to other TTX-bearing species such as pufferfish. The work will also advance the availability of amphibian genomic resources and enable researchers to continue expanding our knowledge of amphibian genes. Due to the sensitivity of amphibians to invasive species (Chapter 4, this thesis), as well as other

anthropogenic disturbances, it will be crucial to have more robust genetic resources of rough-skinned newts available.

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Figures

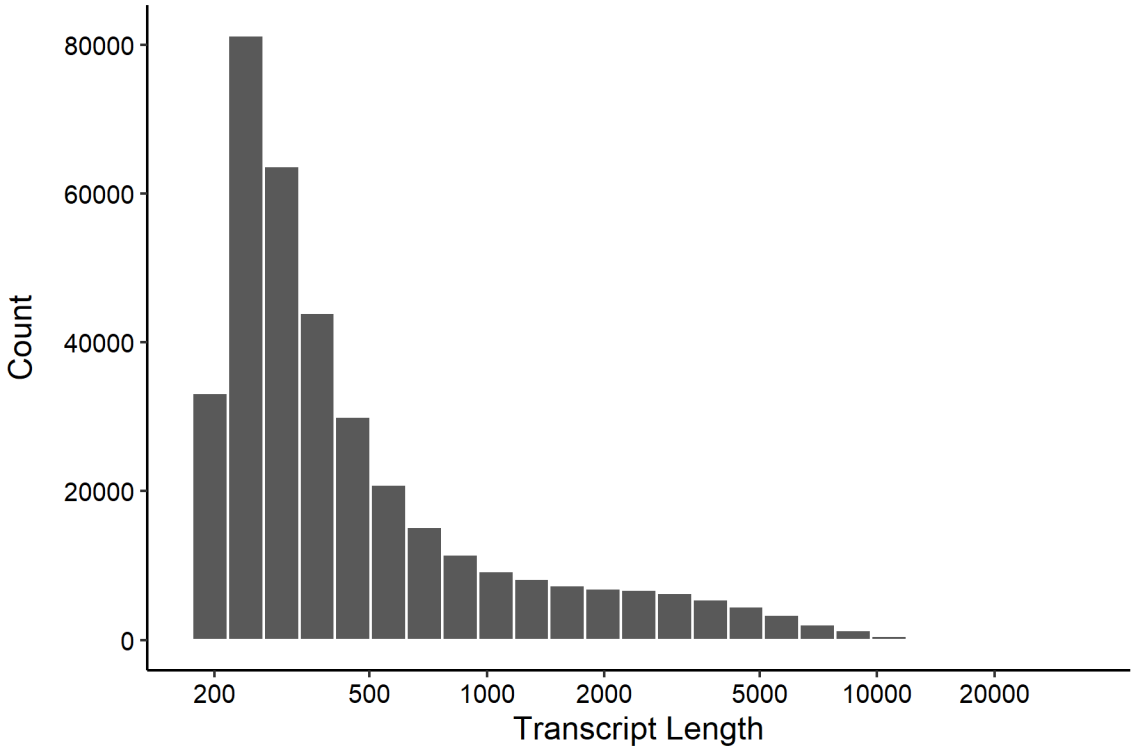

Figure 3.1: Histogram showing the distribution of transcripts assembled by Trinity v2.8.5 (Grabherr et al. 2011, Haas et al. 2013), binned by length.

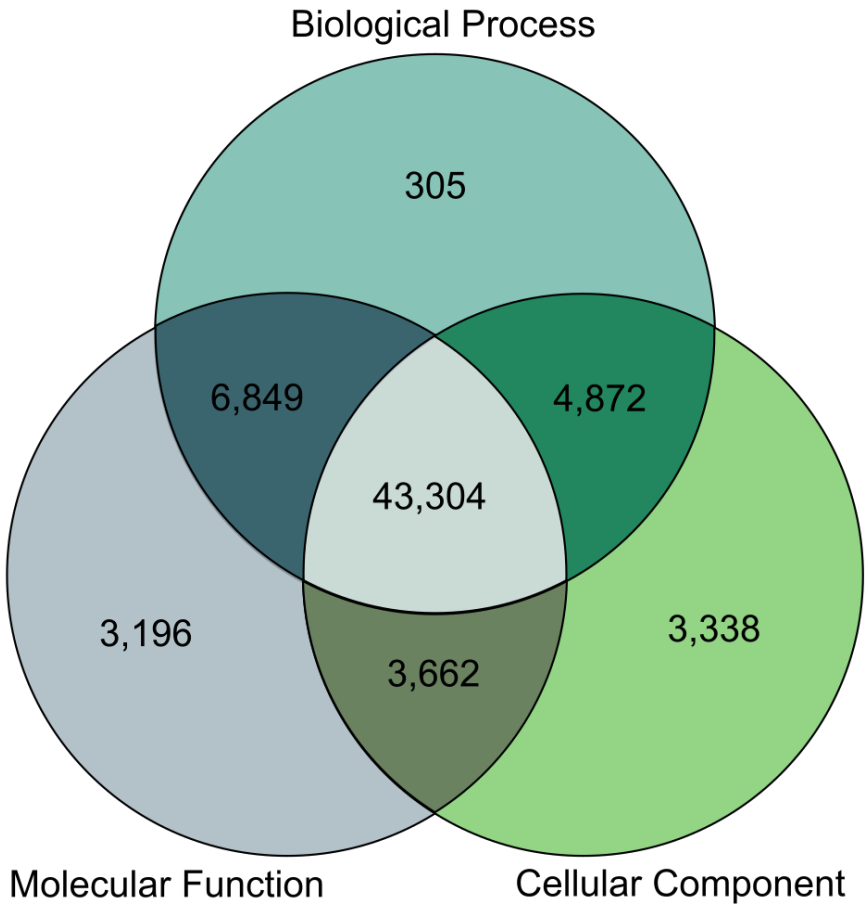

Figure 3.2: Venn diagram of Gene Ontology (GO) categories of the annotated transcripts, assigned using the Trinotate pipeline (Bryant et al. 2017). The values represent the number of transcripts with a GO assignment from each of three categories: Biological Process, Molecular Function, and Cellular Component.

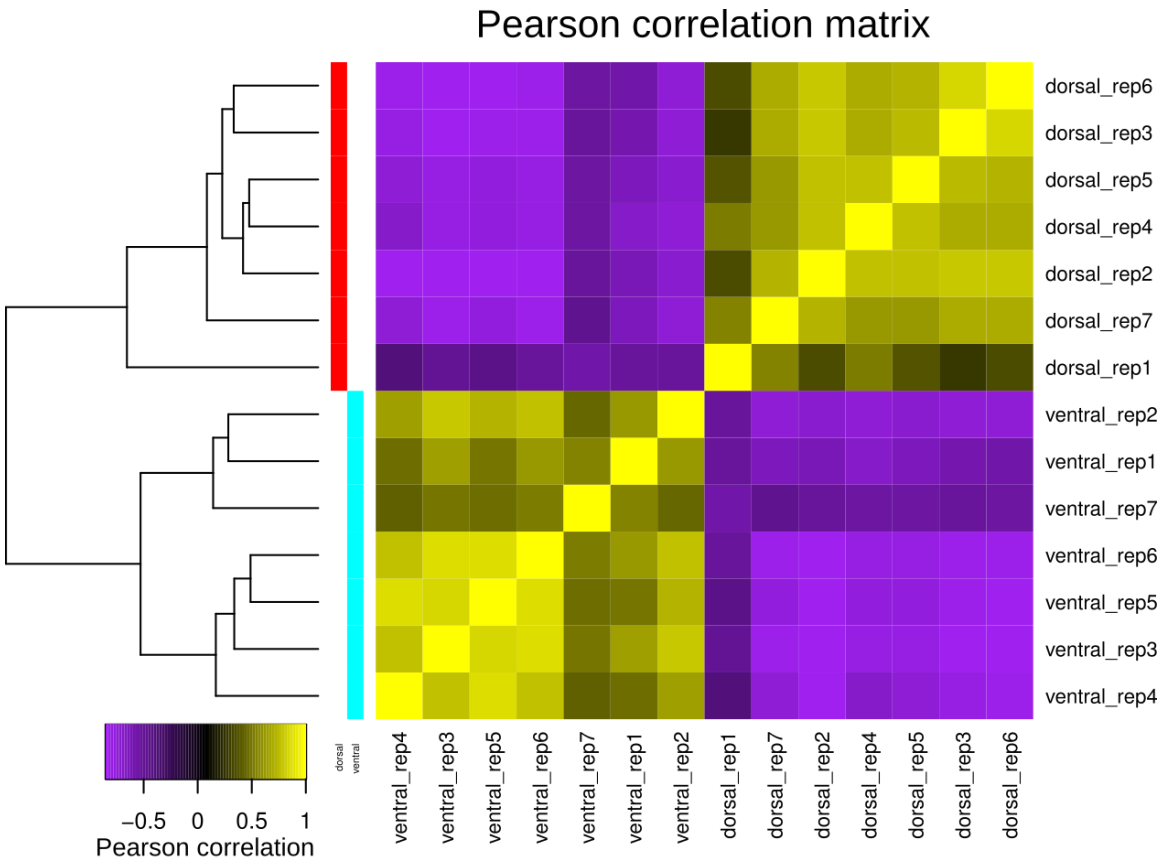

Figure 3.3: Clustered heatmap visualized from Pearson correlation matrix for pairwise sample comparisons of differential gene expression between dorsal and ventral replicates. Yellow indicates positive correlation between samples whereas purple indicates negative correlation. Figure was generated using a perl script within Trinity v2.8.5 (Grabherr et al. 2011, Haas et al. 2013).

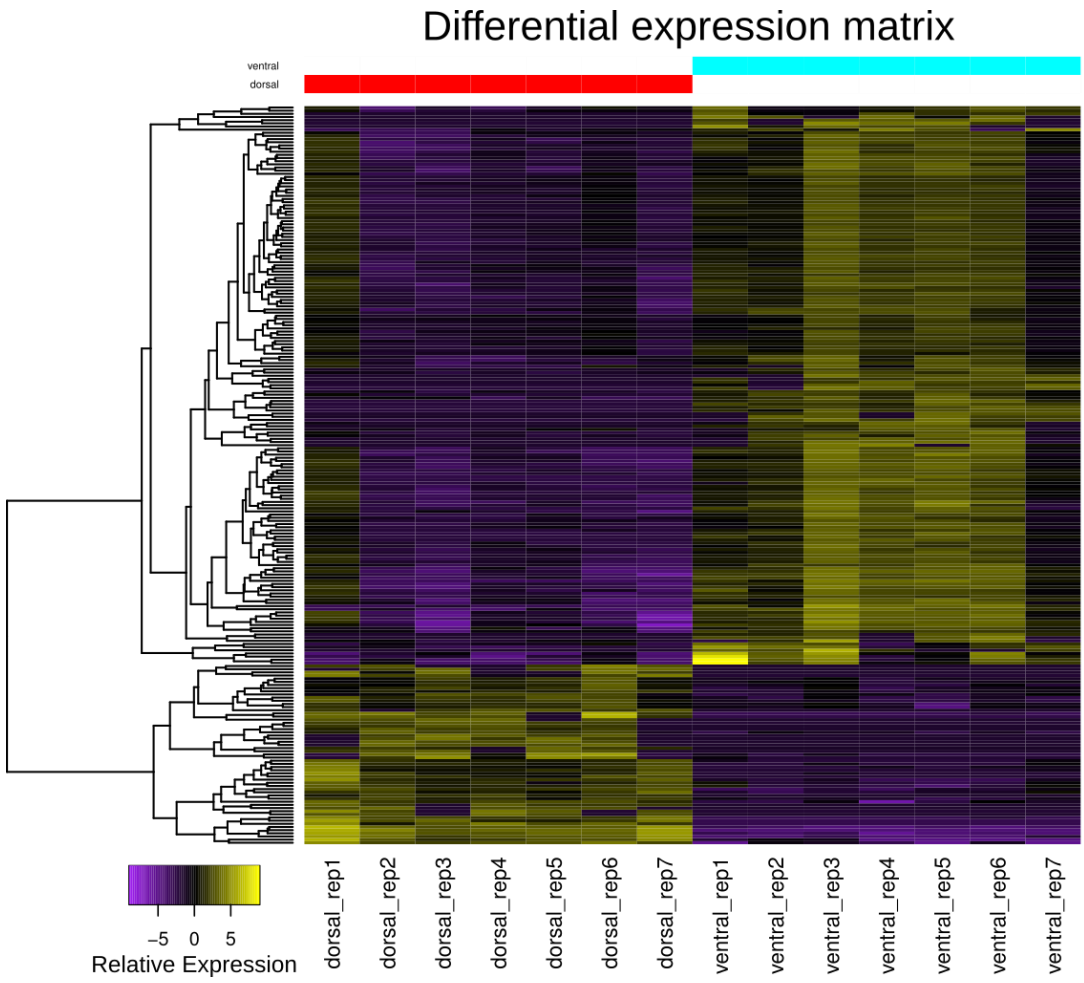

Figure 3.4: Clustered heatmap showing differentially expressed genes vs. sample replicates. Each row represents one differentially expressed gene and is aligned to a branch of the dendrogram on the left. Figure was generated from a perl script within Trinity v2.8.5 (Grabherr et al. 2011, Haas et al. 2013).

Tables

Table 3.1: Assembly statistics for the *de novo* assembled transcriptome of the rough-skinned newt.

Assembly Statistics	
Number of Read Pairs	353,375,308
Assembly Size (bp)	283,958,001
Number of Trinity Transcripts	361,968
Number of Trinity Genes	262,505
GC (%)	44.66
Contig N10	6904
Contig N20	4991
Contig N30	3750
Contig N40	2754
Contig N50	1874
Median Contig Length (bp)	335
Average Contig Length (bp)	784.48

Table 3.2: *De novo* assembly statistics and BUSCOs (C: Complete, D: Duplicated, F: Fragmented, M: Missing) of the *T. granulosa* transcriptome together with the Trinity assembled transcriptomes of related species. Complete BUSCOs include both complete & single-copy sequences and complete & duplicate sequences.

Species	Tissue used for assembly	Assembled Bases (bp)	Number of Transcripts	Number of Genes	BUSCOs
Rough-skinned newt, *Taricha granulosa* (This study)	Skin	283,958,001	361,968	262,505	C: 84.3% [D: 36.1%], F: 5.2%, M: 10.5%
Eastern newt, *Notophthalamus viridescens* (Looso et al. 2013)	Iris, heart, limbs	Not reported	120,922	Not reported	C: 30% [D: 7.0%], F: 10%, M: 58%
Japanese fire belly newt, *Cynops pyrrhogaster* (Nakamura et al. 2014)	Retina	Not reported	237,120	Not reported	C: 82% [D: 34%], F: 4.6%, M:13%
Axolotl, *Ambystoma mexicanum* (Bryant et al. 2017)	Multiple tissues	Not reported	1,554,055	1,388,798	C: 88% [D: 53%], F: 4.5%, M: 7.3%
Chinese giant salamander, *Andrias davidianus* (Huang et al. 2017)	Organs	128,175,999	158,103	132,912	Not reported
Bosca's newt, *Lissotriton boscai* (Nourisson et al. 2017)	Liver	173,736,688	153,270	Not reported	Not reported

Ramos' mushroomtongue salamander Bolitoglossa ramosi (Gomez et al. 2018)	Limbs, gut, skin	654,673,506	577,037	433,809	C: 78.2% [D: 2.4%], F: 9.9%, M: 11.9%
Yenyuan stream salamander, Batrachuperus yenyuanensis (Xiong et al. 2019)	Liver, spermary, muscle, spleen	88,019,795	115,495	Not reported	C: 40.1% [D: 7.2%], F: 8.4%, M: 51.5%

Table 3.3: Top ten Gene Ontology terms from each GO category for the annotated transcripts.

GO Category	GO Term	Count
Biological Process	Transcription, DNA-templated	9,004
	Regulation of transcription, DNA-templated	6,518
	DNA recombination	4,580
	DNA integration	4,082
	Positive regulation of transcription from RNA polymerase II promoter	2,183
	Transposition, RNA-mediated	1,935
	Negative regulation of transcription from RNA polymerase II promoter	1,826
	Small molecule metabolic process	1,706
	Innate immune response	1,581
	Nucleic acid phosphodiester bond hydrolysis	1,522
Cellular Component	Nucleus	18,499
	Cytoplasm	15,598
	Integral component of membrane	9,465
	Plasma membrane	8,312
	Cytosol	6,775
	Nucleoplasm	5,954

	Extracellular exosome	5,752
	Membrane	5,293
	Mitochondrion	3,297
	Nucleolus	2,428
Molecular Function	Metal ion binding	13,479
	DNA binding	8,266
	RNA-directed DNA polymerase activity	7,760
	ATP binding	6,216
	Zinc ion binding	6,099
	Transcription factor activity, sequence-specific DNA binding	4,171
	RNA binding	3,251
	Aspartic-type endopeptidase activity	3,096
	Nucleic acid binding	3,082
	Poly(A) RNA binding	2,960

Table 3.4: Enriched and depleted Gene Ontology terms annotated to differentially expressed genes in dorsal and ventral dermal tissue samples from rough-skinned newts. Enriched terms are those found expressed more than expected in the sample type, and depleted terms are expressed less than expected by chance. The total number of terms adds up to more than the total number of differentially expressed genes (N=234) because a given gene can be assigned to multiple GO terms.

Sample Type	Representation	Number of GO Terms
Dorsal	Enriched	109
	Depleted	19
Ventral	Enriched	515
	Depleted	51

Table 3.5: **Gene Ontology terms related primarily to membrane potential and permeability that were enriched in the differentially expressed genes between dorsal and ventral samples. All of these GO terms fall under the same GO Category of Biological Process and were all associated with the same two Trinity assembled genes: *TRINITY_DN1094_c0_g1* and *TRINITY_DN113557_c0_g1*.**

GO ID	GO Term	p-value
GO:0010917	Negative regulation of mitochondrial membrane potential	1.1E-5
GO:0045837	Negative regulation of membrane potential	2.4E-5
GO:0010940	Positive regulation of necrotic cell death	2.5E-5
GO:0035794	Positive regulation of mitochondrial membrane permeability	6.8E-5
GO:1905710	Positive regulation of membrane permeability	8.2E-5
GO:0010939	Regulation of necrotic cell death	5.3E-4
GO:0051881	Regulation of mitochondrial membrane potential	1.1E-3
GO:0046902	Regulation of mitochondrial membrane permeability	1.2E-3
GO:0090559	Regulation of membrane permeability	1.5E-3
GO:0007006	Mitochondrial membrane organization	2.8E-3
GO:0042391	Regulation of membrane potential	1.6E-2
GO:0007005	Mitochondrion organization	2.9E-2

Table 3.6: **Gene Ontology terms related primarily to terpenoid biosynthetic and metabolic processes that were enriched in the differentially expressed genes between dorsal and ventral samples. All of these GO terms fall under the same GO Category of Biological Process and were all associated with the same Trinity assembled gene: *TRINITY_DN17261_c0_g1*.**

GO ID	GO Term	p-value
GO:0006714	Sesquiterpenoid metabolic process	4.3E-3
GO:0016114	Terpenoid biosynthetic process	7.4E-3
GO:0006721	Terpenoid metabolic process	0.0495

CHAPTER FOUR – POTENTIAL IMPACTS OF NON-NATIVE SPECIES ON ROUGH-SKINNED NEWTS OF VANCOUVER ISLAND

4.1 Introduction

Species ranges are dynamic through time and are constantly shifting, expanding, or contracting (Brown et al. 1996). Around the globe, species have been spreading outside their original habitats due to factors such as response to changing environmental conditions, or intentional or accidental introduction (Sexton et al. 2009). Such range expansions are evident throughout the fossil record (i.e., Davis & Shaw 2001, Kaustuv et al. 2001), but in the past few centuries as human settlements have continued to proliferate worldwide, this has led to further opportunities for species to spread. Human-assisted species introductions are now occurring at an alarming rate greater than any other time in evolutionary history (Ricciardi 2007, Boivin et al. 2016). Not all introduced species are able to establish themselves in a new environment, and it is even thought that non-native species may be beneficial to conservation objectives, specifically those able to persist despite the effects of climate change and habitat modification (Schlaepfer et al. 2011). Nevertheless, the spread of non-native species more often than not leads to significant environmental, economic, and sociological consequences (Pimentel et al. 2005, Simberloff et al. 2013). Species introductions now pose a major threat to global biodiversity and may soon be even more detrimental than habitat loss and fragmentation (Crooks & Soule 1999).

Amphibians around the globe are facing population declines from factors such as habitat destruction, global warming, and disease but many are also particularly vulnerable to the presence of non-native species (Stuart et al. 2005). According to the IUCN Red

List of Threatened Species, 40% of amphibian species are threatened with extinction, a proportion higher than any other group of taxa (IUCN 2019). Non-native species can cause detrimental effects on amphibian populations through predation, competition, spreading disease, compromising the immune system of native amphibians in a community, hybridizing with them, or by a combination of these factors (Bucciarelli et al. 2014). Introduced species of fish, crayfish, and bullfrogs were found to be the major contributors to amphibian population declines worldwide (Kats & Ferrer 2003). Aquatic prey such as amphibians are also more susceptible to the effects of novel predators than their terrestrial or marine counterparts due to heterogeneity in predation regimes promoting predator naivety at multiple spatial scales (Cox & Lima 2006, Salo et al. 2007). For example, trout introduced into naturally fishless high elevation lakes in the Sierra Nevada mountains led to near elimination of most amphibians in the lakes (Knapp et al. 2001). In another instance, green and golden bell frog tadpoles (*Litoria aurea*) in Australia were naïve to introduced mosquitofish (*Gambusia holbrooki*) and their size at metamorphosis was also impacted, therefore affecting their fitness (Hamer et al. 2002).

One amphibian species that may be facing future declines as a result of non-native species presence is the rough-skinned newt (*Taricha granulosa*). These newts excrete a potent neurotoxin from their skin called tetrodotoxin (TTX) and were historically believed to be in a coevolutionary arms race with their primary predators, garter snakes (*Thamnophis sirtalis*), who over time have developed a resistance to TTX (i.e., Brodie & Brodie 1990, Brodie et al. 2005, see also: Chapter 2, this thesis). Rough-skinned newts span a large range across the western coast of North America and are currently listed by the IUCN as a species of Least Concern (IUCN 2015), but newts on Vancouver Island,

British Columbia may be threatened by the impacts of non-native species. Islands are known to experience a heightened sensitivity to the presence of invasive species due to factors such lower genetic variation (Frankham 1997; see also Chapter 5, this thesis) and increased predator naivety of island populations (Cox & Lima 2006). While BC is home to many introduced plants and animals, two species present on the island that are particularly concerning for rough-skinned newt populations are signal crayfish (*Pacifastacus leniusculus*) and American bullfrogs (*Lithobates catesbeianus*).

Signal crayfish are native to some regions sympatric with newts, including Oregon, Washington, and mainland BC, but it is believed they were introduced by humans to Vancouver Island about a century ago. One account suggests that crayfish were brought from Oregon to Shawnigan Lake on the island sometime between 1908 and 1929 (Carl & Guiguet 1957), and as of 2005 they were widespread throughout the southern end of Vancouver Island in many freshwater habitats (Bondar et al. 2005). There is still a high degree of uncertainty about the status of this crayfish in BC and little work so far has been completed to assess their impacts on Vancouver Island. Signal crayfish have been implicated in the collapse of the stickleback species pair in Enos Lake (Kraak et al. 2001), but their impacts on rough-skinned newts on the island are currently unknown.

Crayfish are known to affect newt species both directly and indirectly, which can result in significant negative impacts on populations by predation on eggs and larvae, attacks on adult amphibians, competition for prey invertebrates, and disruption of littoral habitat (Nystrom et al. 2001). In Crater Lake in Oregon, the Mazama newt (*Taricha granulosa mazamae*), a subspecies of the rough-skinned newt, is facing serious declines

as a result of crayfish presence. Signal crayfish were introduced to the lake in 1914 to feed stocked fish and have since competed with the newts for habitat and invertebrate food sources, as well as directly attacking newts. As of 2018, crayfish occupy over 80% of lakeshore sites in Crater Lake, and Mazama newts are unable to co-occur with crayfish in any of the sites (Buktenica et al. 2015, Girdner et al. 2017). In another study in the Santa Monica Mountains of southern California, invasive red swamp crayfish (*Procambarus clarkii*) were found to cause population declines of the California newt (*Taricha torosa*). In streams previously inhabited by California newts where red swamp crayfish invaded, newts were absent due to aggressive crayfish attacks deterring breeding in those streams (Gamradt & Kats 1996, Gamradt et al. 1997). California newts were found to increase their toxin defenses from baseline levels in response to simulated predator attacks (Bucciarelli et al. 2017) and there is also evidence that crayfish could develop a resistance to TTX. White river crayfish (*Procambarus acutus*) displayed functional resistance to TTX when offered toxic newt eggs in controlled feeding trials, but they lacked physiological resistance when TTX was administered as an intramuscular injection (Wilson et al. 2014).

American bullfrogs (*Lithobates catesbeianus*) are another non-native species present on Vancouver Island that may pose a threat to rough-skinned newts. While native to the eastern United States, bullfrogs were introduced to western North America in the early 20th century primarily as a food source and are now located in every western continental state, Hawaii, Europe, Asia, the Caribbean, and South America (Palen 2006, Snow & Whitmer 2010). Bullfrogs made their way to southern Vancouver Island near the city of Victoria in the mid to late 1980's and have since invaded countless ponds and

lakes on the island (Jancowski & Orchard 2013). Measures to eradicate bullfrogs on the island have been proposed, but to my knowledge there are currently no active management programs in place, and to this day bullfrogs continue to spread northwards up the island (Govindarajulu et al. 2005, Orchard 2011). American bullfrogs are regarded as one of the most successful vertebrate invaders due to their ability to adapt to a wide range of habitats, rapid population growth, and spread pathogens including chytrid fungus, making it difficult, if not impossible, to eradicate them (Adams & Pearl 2007).

Bullfrogs are responsible for population declines of native amphibians through both direct and indirect effects including competition, predation, and habitat displacement (Adams & Pearl 2007). In Oregon, invasive American bullfrogs were implicated in population declines of both the Northern red-legged frog (*Rana aurora*) and the Oregon spotted frog (*Rana pretiosa*), with more severe impacts observed in the Oregon spotted frog (Pearl et al. 2004). Overwintered bullfrog tadpoles also negatively impact metamorphosis of native amphibians, causing shorter larval periods and lower mass at metamorphosis of spotted salamanders (*Ambystoma maculatum*) (Boone et al. 2004). One study on Vancouver Island found evidence of rough-skinned newts in the stomach content of bullfrogs (Jancowski & Orchard 2013), but there is currently no evidence for how their presence may impact newt populations. Moreover, there have been reported cases of bullfrogs on the island testing positive for the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), a pathogen that infects amphibians and is responsible for many species declines and extinctions worldwide (Garner et al. 2006, Stuart et al. 2004). American bullfrogs can be infected with *Bd* but remain asymptomatic, making them highly efficient vectors for the spread of this disease among amphibians (Schloegel et al.

2010). Rough-skinned newts were found to have a 100% mortality rate when exposed to chytrid fungus (Martel et al. 2014), so the spread of *Bd* through American bullfrogs in the Pacific Northwest may have significant detrimental effects on newt populations if bullfrog habitat expansion is left uncontrolled.

Both signal crayfish and American bullfrogs are known to negatively affect amphibians and may be particularly harmful in aquatic habitats on Vancouver Island (e.g., Nystrom et al. 2001, Adams & Pearl 2007, Jancowski & Orchard 2013, Girdner et al. 2018). Previous studies have found that non-native crayfish can significantly impact newt populations in other regions, so this might also be the case on the island. Moreover, bullfrogs on Vancouver Island are known to consume rough-skinned newts and other amphibians, as well as carry *Bd* which is lethal to newts (Garner et al. 2006, Jancowski & Orchard 2013). I predict that the presence of these non-native species will cause detrimental effects to rough-skinned newts on the island. To test this, my objectives are to investigate how the presence of non-native species on Vancouver Island impacts newt (i) relative abundance, (ii) body condition, and (iii) dorsal skin toxicity. I first predicted that newts would have a lower relative abundance in sites with non-native species present due to increased competition and predation. Because TTX is an antipredator defense, in locations where crayfish or bullfrogs have been introduced, newts might display increased toxin levels in response if they are able to increase their defenses quickly enough in response to novel predators. Alternatively, if there is no significant difference in toxicity, this could be due to predator naivety and different selection pressures contributing to observed patterns of toxicity. The presence of crayfish or bullfrogs is also known to cause decreased amphibian body condition, which is thought to be a result of

increased time spent trying to avoid predation (Kiesecker et al. 2001, Nystrom et al. 2001, Boone et al. 2004). Body condition is a measure of amphibian population health and habitat quality parameters (Bancila et al. 2010), so newts displaying decreased fitness as a result of coexistence with non-native crayfish or bullfrogs would display lower body condition indices. Overall, there is no previous work on the impacts of non-native signal crayfish and American bullfrogs on rough-skinned newts of Vancouver Island. This study will contribute to a greater understanding of their current impacts, serving as a snapshot in time and reference for future studies on the potential effects of non-native species on Vancouver Island newts.

4.2 Methods

4.2.1 Sample Collection

Rough-skinned newts were collected from Vancouver Island in the spring of 2017 and 2018 using methods detailed in Chapter 2. I excluded the additional study sites from Oregon and Sunshine Coast mentioned in Chapter 2from analyses in this chapter because signal crayfish are native to both those regions. A total of 26 sites on Vancouver Island are studied in this chapter (Fig. 4.1, Table 4.1), and each study site is considered a distinct population due to breeding site fidelity. I collected one to 15 newts at each site, with 15 being the maximum per year due to time constraints, and to prevent significant impacts to very low-density populations. Presence or absence of non-native crayfish or bullfrogs in each site were determined by (i) whether I found evidence of them in the sites I sampled, and (ii) if I did not find any, I researched published papers and reports which may indicate they have been detected other times (i.e., Jancowski & Orchard 2013,

Klinkenberg 2018). However, it is possible there could still be crayfish or bullfrogs present in a site labeled as "none detected" where I did not detect them myself or find any evidence of them in the literature, as they may have recently colonized a site or remained undetected.

Tetrodotoxin was extracted and quantified from dorsal skin samples of each newt, as outlined in Chapter 2. I also took measurements of mass in grams and snout-vent length (SVL) in millimeters of each individual. As a reliable indicator of body condition, the scaled mass index (SMI), was calculated from mass and SVL measurements as described in Peig & Green (2009). Body condition estimates are correlated with an animal's health and fitness, and SMI values also reflect rough-skinned newt energy stores (MacCracken & Stebbings 2012).

4.2.2 Analyses

To investigate the relative abundance of newt populations, I developed a method which could be considered a modified catch per unit effort measure. I was unable to sample in a consistent manner between sites because I had to capture some newts by hand and some by setting traps, in the interest of time as well as lake accessibility issues. Therefore, I could not utilize catch per unit effort, but instead considered sampling efficiency as an insight into relative abundance of newts. Sampling efficiency scores on a scale of 1 to 4 were assigned to each study site as an estimate for the relative effort needed to collect the maximum number of newts (N=15) from each site. Visual representations of sampling efficiency are provided in Figure 4.2 to justify how I assigned the scores to each lake. During my field work, I had been keeping track of effort required to capture individuals since I had strict catch limits and I was monitoring how

quickly I approached that limit. I subsequently turned this into a four-point scale reflecting how efficiently I was able to capture newts from each population. Sites with confirmed presence of newts in the past where I was unable to collect any newts during my study (N=0) were also included in this analysis. Sampling efficiency scores were assigned as follows:

1= Very easy to collect newts, could catch up to 15 in a few hours or less

2= Collected enough by setting traps overnight, but it would have been difficult to collect all newts in one day

3= Set traps overnight for approximately 24 hours but did not collect as many newts as needed

4= Set traps overnight or for several days and only caught 0-3 newts

Analyses were carried out in R with the R Studio interface v1.1.163 (R Core Team 2018). I used the ggplot2 package (Wickham 2009) to create a stacked bar plot of sampling efficiency scores as well as boxplots to display newt TTX concentration and SMI values by study site. A Fisher's exact test was conducted to test for independence in sampling efficiency scores between sites with and without non-native species. Using the R package lme4 (Bates et al. 2015), I also performed two linear mixed effects models to test for a relationship between non-native species presence and log-transformed (i) SMI values and (ii) newt TTX levels. The models used non-native species presence as the predictor variable with study site as a random effect, and model significance was checked by determining the p-value via the lmerTest package (Kuznetsova et al. 2017). While I am unable to consider the potential effects of garter snake predators on patterns of newt

toxicity or body condition, for the sake of these analyses I am assuming any potential differences in the presence of non-native species would be relative to if garter snakes were the only predator presumably present. Finally, I used a linear regression to test whether there is a relationship between newt toxicity and SMI.

4.3 Results

A total of 26 study sites were sampled on Vancouver Island, 16 with no non-native species detected and ten with crayfish and/or bullfrogs present (Fig. 4.1, Table 4.1). There was not an equal representation of sites with and without non-native species due to the difficulty of collecting newts at sites where crayfish or bullfrogs were known to be present. Sampling efficiency scores were assigned to each study site and eight out of the ten (80%) sites with non-native species had a sampling efficiency score of four, indicating the extreme effort it took to capture newts from those sites (Fig. 4.3). One noteworthy example is Dougan Lake which was inhabited by both crayfish and newts in the past, but I could only collect one newt after sampling for three days in two separate years (Fig. 4.2B). Sampling efficiency was negatively correlated with presence of non-native species (Fisher's Exact; $p=0.0013$).

Generally, SMI values visually appeared lower in newts coexisting with non-native species (Fig. 4.4), and baseline TTX concentrations seemed higher (Fig. 4.5). However, there were no statistically significant relationships between SMI (Linear mixed effect model; $p=0.131$) or TTX concentration (Linear mixed effect model; $p=0.751$) in the presence or absence of non-native species. There was also no significant association

between newt toxicity and body condition (Linear model; p=0.225, df=232), which aligns with findings from previous studies (e.g., Hanifin et al. 2004, Bucciarelli et al. 2016).

4.4 Discussion

Within the last century, two non-native aquatic predators have been introduced by humans to Vancouver Island, BC. Both signal crayfish and American bullfrogs were first present at the southern end of the island and continue to spread northwards, threatening many native species including amphibians which are already a particularly vulnerable group (i.e., Bondar et al. 2005, Jancowski & Orchard 2013). In this chapter, my objective was to identify the impacts of these non-native species on sampling efficiency, body condition, and tetrodotoxin concentration of Vancouver Island's rough-skinned newts. While crayfish and bullfrogs have been known to cause other negative consequences on the island (e.g., Kraak et al. 2001), no previous work had identified their impacts on newts in the region. This study helps establish a baseline of the current state of potential impacts of non-native crayfish and bullfrogs on rough-skinned newts from Vancouver Island, and documents sites where they are currently present to serve as a reference for future research.

There is a significant negative correlation between presence of non-native species and the ability to capture newts on Vancouver Island. In general, it was very difficult to collect enough samples from sites with confirmed presence of crayfish or bullfrogs (Fig. 4.2B, Fig 4.3). One plausible explanation for the difficulty of collecting such low densities of newts could be that they were trap shy, or larger lakes could have created more opportunities for niche partitioning and allowed newts to hide in certain areas away

from where I was able to sample. Another possibility is that they were hiding on land, but this comes at a cost since they would have missed breeding opportunities and rough-skinned newts are highly aquatic, spending up to 10 months of the year in permanent water bodies (Pimentel 1960). Moreover, there were three lakes with non-native species present that newts were known to inhabit in the past, but I was unsuccessful at capturing any newts in those sites (Enos Lake, Elk/Beaver Lake, and Mesachie Lake). The possibility remains that newts were present in those sites, although none were captured at the time. Conversely, they might have become locally extinct if they were unable to coexist with the non-native species. In support of the latter possibility, newts were previously abundant in Enos Lake along with the threespine stickleback benthic and limnetic species pair (Taylor et al. 2006). Signal crayfish are now pervasive in the lake and have been implicated in the collapse of the stickleback species pairs, while newts are thought to be completely extirpated from the lake (Taylor et al. 2006).

Scaled mass index (SMI) scores of rough-skinned newts on Vancouver Island did not have a significant relationship with the presence of non-native species (Fig. 4.4). Although there currently appears to be no impact of crayfish and/or bullfrogs on newt body condition, the presence of these species might affect newt fitness and population health in the future. Fish are another possible predator that I was unable to take into consideration in this study. Stocking lakes with species such as trout for recreational fishing is common practice on Vancouver Island, which might also affect newt body condition or reproductive ability. The presence of stocked rainbow trout (*Oncorhynchus mykiss*) in lakes in inland BC was found to be negatively correlated with amphibian abundance and body condition (Hirner & Cox 2007). The presence of trout, crayfish,

and/or bullfrogs in addition to garter snakes could lead to the multiple predator effect, which occurs when several predators are coexisting in a given habitat and collectively increase the predation rate (Sih et al. 1998). For example, the interactive effects of both signal crayfish and rainbow trout significantly reduced the size at metamorphosis and proportion of froglets surviving to adulthood (Nystrom et al. 2001), and their additive effects could also cause similar impacts on rough-skinned newts.

In addition to the multiple predator effect, naivety to novel predators could further influence newt populations. One study in Tuscany, Italy found that the presence of introduced trout and crayfish led to a drastic decrease in the probably of egg occurrence of the Northern spectacled salamander (*Salamandrina perspicillata*) in the region, while native crayfish did not have an effect (Piazzini et al. 2011). Similar patterns might be evident in variation in rough-skinned body condition across their range. Even though signal crayfish were recently introduced to Vancouver Island, they are native to other regions of the newt's range including mainland BC, Washington, and Oregon. Benton County, Oregon, for example, is known to have highly toxic newts (i.e., Hanifin et al. 2008; Chapter 2, this thesis) with much higher SMI scores than those seen on the island (Fig. 4.4), despite co-occurring with signal crayfish. Such patterns indicate that newt body condition is not affected by native crayfish, but could be impacted by selection pressures from novel predators they lack an evolutionary history with (Salo et al. 2007). It is also possible that as bullfrogs continue to spread *Bd* and infect rough-skinned newts, this could lead to further impacts on newt body condition because SMI is an indicator of population health and fitness (MacCracken & Stebbings 2012). At present, due to the short time frame since the introduction of crayfish and bullfrogs, newts currently do not

show an evolutionary response in body condition SMI scores, but this could change in the future as the effects of these non-native species continue to proliferate.

There was also no significant association between the presence of non-native species and the tetrodotoxin concentration of rough-skinned newts. There remain many gaps in knowledge regarding the chemical ecology of newts, and it is unclear how quickly newts may be able to evolve increased toxicity or if they are even able to evolve in response to selection pressures from predators other than garter snakes (see Chapter 2, this thesis). One explanation for the current lack of association between newt toxicity and non-native species presence could be that newts are still naive to predation by crayfish and bullfrogs, which may have not been present on the island long enough for newts to increase their toxic defenses. However, in the future newts might respond to these novel predation pressures by continuing to evolve increased toxicity. Recent evidence is starting to suggest that patterns in newt toxicity across their range are not a result of reciprocal coevolution with garter snake predators, but could be due to other selective pressures also at play (i.e., Hague et al. 2016, Hague et al. 2019; Chapter 2, this thesis), such as novel predators (Stokes et al. 2015). While non-native species are currently not a significant factor in patterns of TTX concentration, chemical defenses may increase when newts can reliably detect a predator or stimulus (Bucciarelli et al. 2017).

Overall, it is difficult to disentangle the possible factors contributing to the observed variation in newt SMI and toxicity on Vancouver Island. While the results of this chapter yielded non-significant associations between SMI/TTX values and non-native species presence, this does not discount the potential for serious negative impacts to occur in the future as crayfish and bullfrogs become more widespread across the

island, or the possibility that other interactions exist that are not currently documented in this chapter. The conservation status of rough-skinned newts on Vancouver Island may also be further impacted by their population genetic structure; island populations are known to have lower genetic diversity and higher extinction rates (Frankham 1997, Frankham 1998; Chapter 5, this thesis). Populations with low measures of genetic diversity may be less resilient to adapting to the impacts of crayfish and bullfrogs, since raw genetic diversity is the means by which adaptations may progress (Frankham 2005). Historical data on rough-skinned newts of Vancouver Island was previously lacking, so this work is a critical milestone in identifying newt population health and toxicity, as well as documenting sites where newts and non-native species are currently present to serve as a baseline for future studies. Future work should prioritize monitoring and managing the spread of non-native signal crayfish and American bullfrogs on Vancouver Island to prevent further impacts on rough-skinned newts and other native aquatic species. Long term monitoring and controlled experiments will also enable researchers to investigate the impacts of non-native species and elucidate the complex selection pressures affecting rough-skinned newts on Vancouver Island.

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Figures

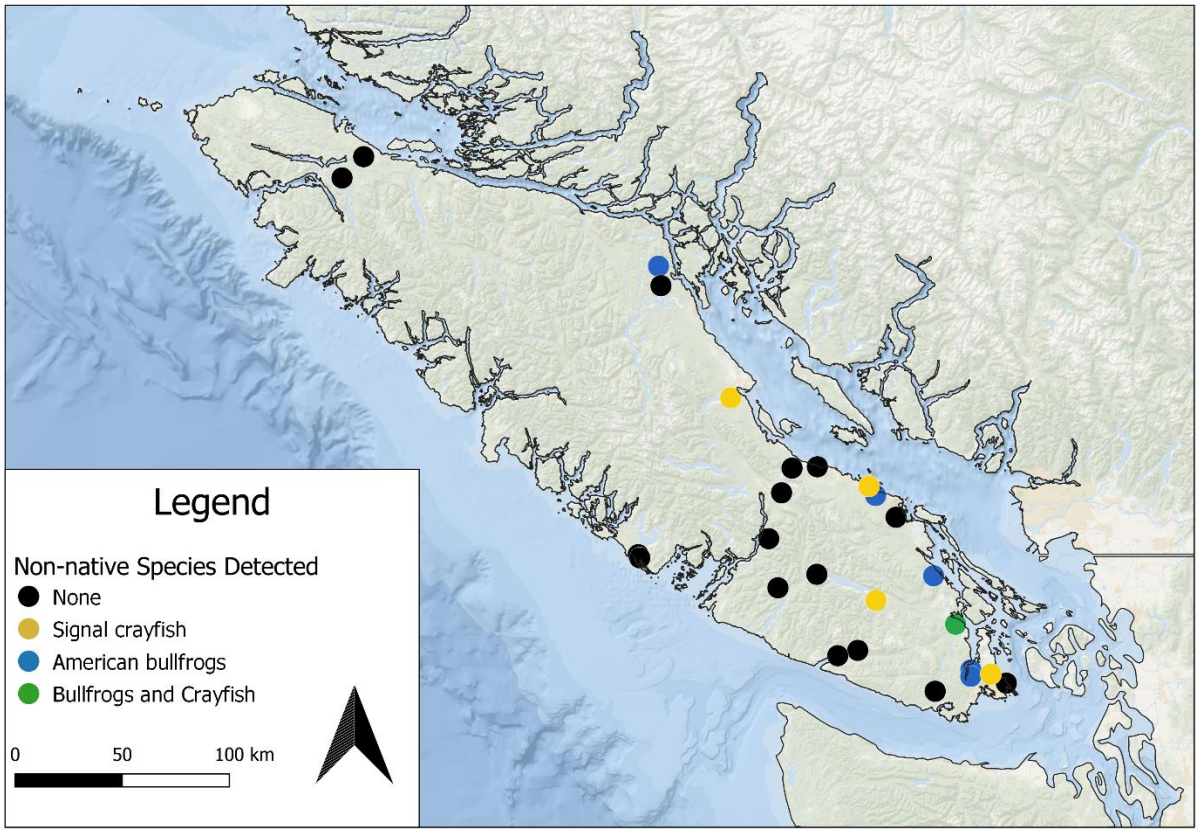
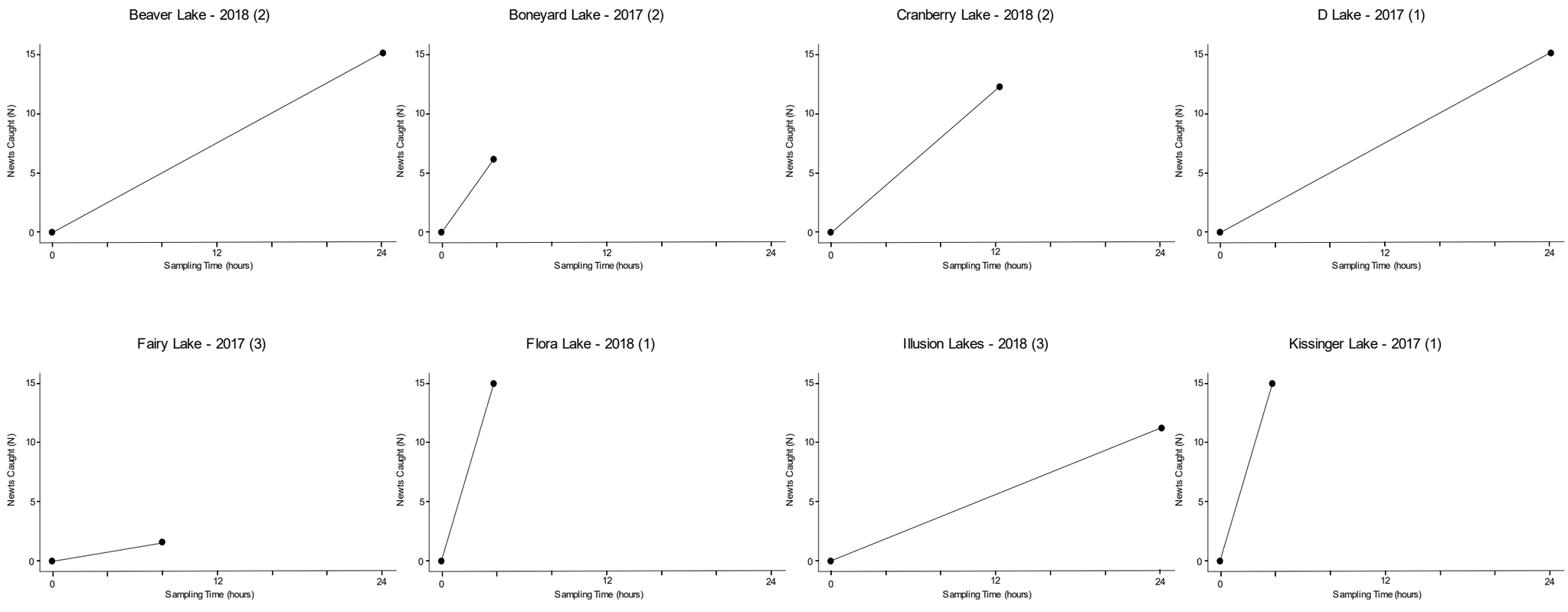


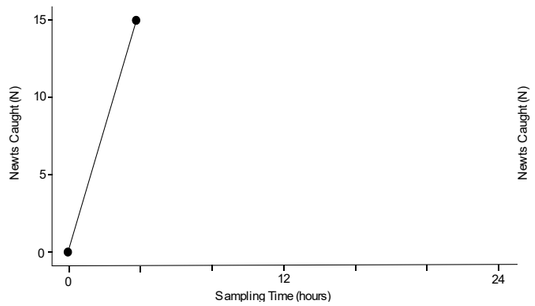
Figure 4.1: Map of 26 study sites on Vancouver Island, BC showing non-native species detected. Five sites had American bullfrogs, four had signal crayfish, and one had both bullfrogs and crayfish.

Figure 4.2: Line plots for each study site to represent the total number of newts caugh (N) up to the catch limit of N=15, by sampling time, up to a 24 hour period. Study sites are separated by (A) sites without non-native species detected, and (B) sites with non-native signal crayfish and/or American bullfrogs detected, each organized in alphabetical order. Plot titles indicate the site name, followed by the year it was sampled, and the sampling efficiency score on a scale of 1 to 4 in parentheses. Three study sites were sampled across both 2017 and 2018 and therefore have more than one plot: Dougan Lake, Maple Lake, and Sywash Marsh.

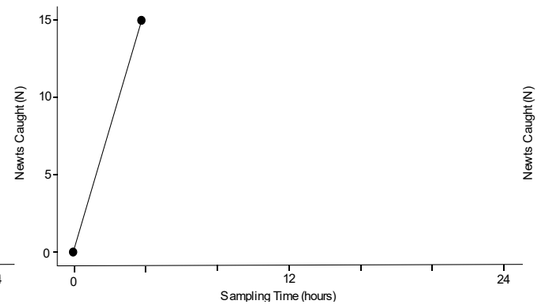
A. Sites without non-native species detected



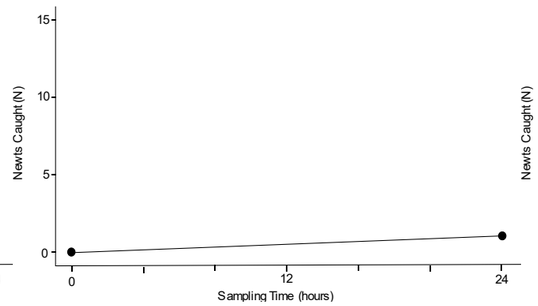
Lizard Lake - 2017 (1)



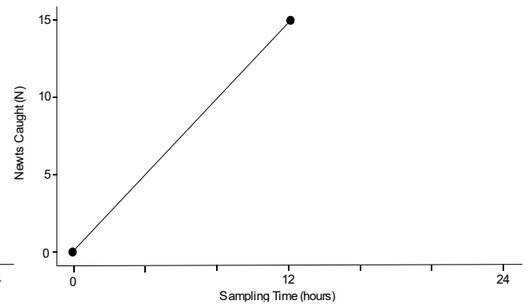
Lizard Pond - 2017 (1)



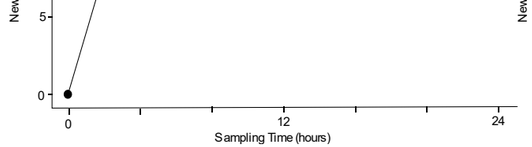
Loon Lake - 2017 (4)



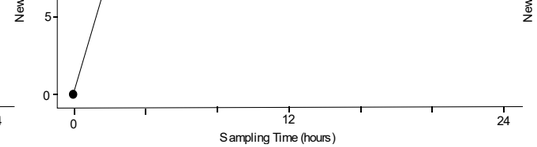
Morrell Lake - 2017 (2)



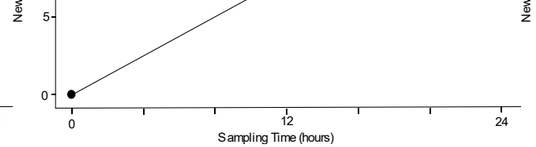
Qualicum Pond - 2018 (1)



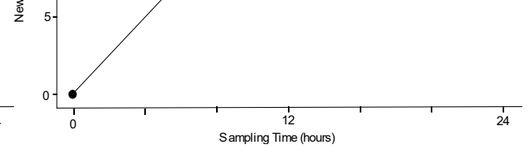
Queen Alexandra Centre - 2017 (1)



Surf Junction - 2018 (2)

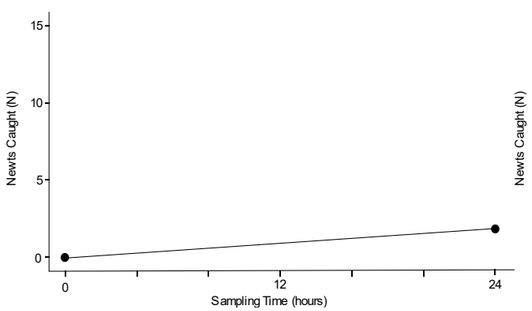


Swan Lake - 2018 (2)

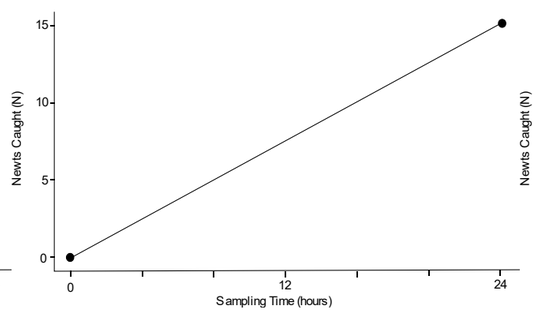


B. Sites with non-native species detected

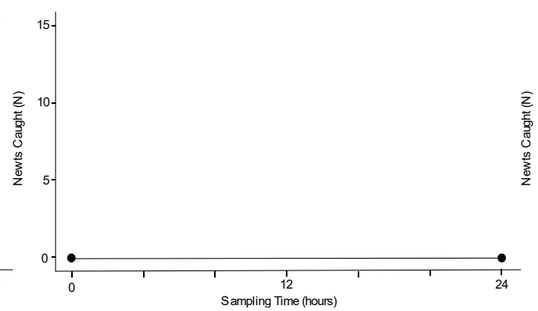
Cafe Pond - 2017 (4)



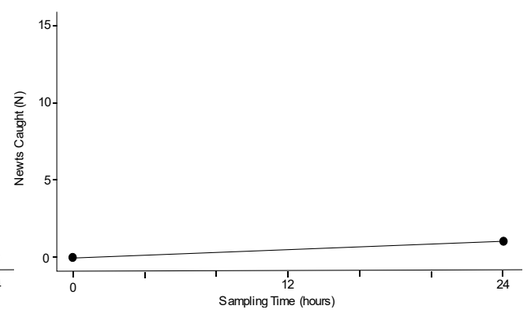
Chemainus Lake - 2018 (2)



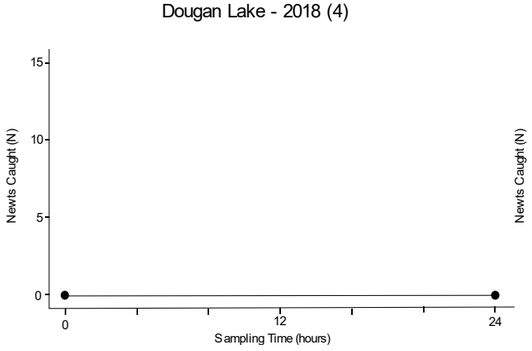
Dougan Lake - 2017a (4)



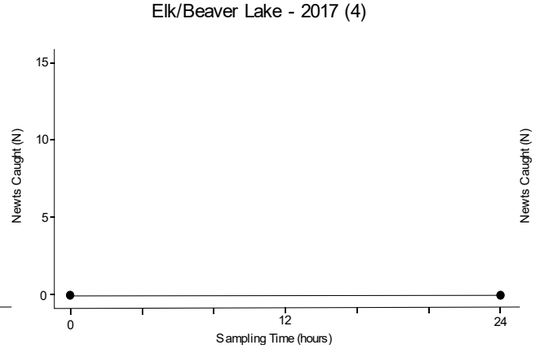
Dougan Lake - 2017b (4)



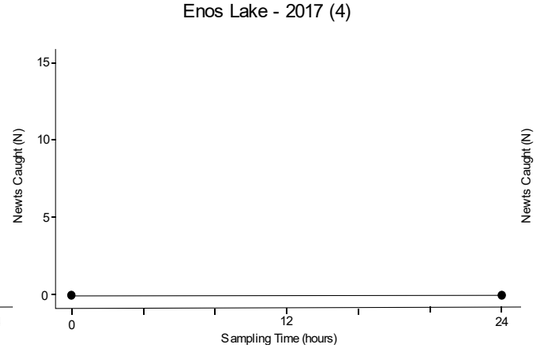
Dougan Lake - 2018 (4)



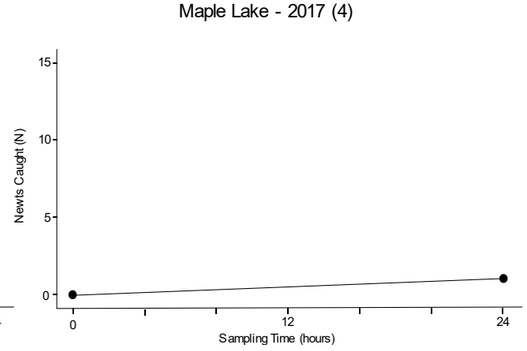
Elk/Beaver Lake - 2017 (4)



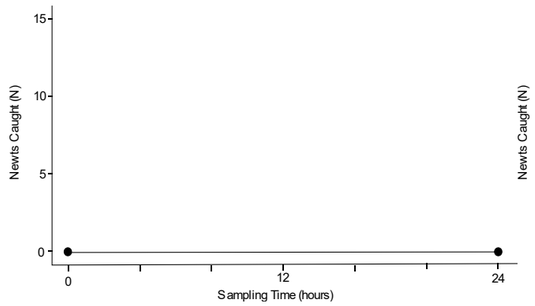
Enos Lake - 2017 (4)



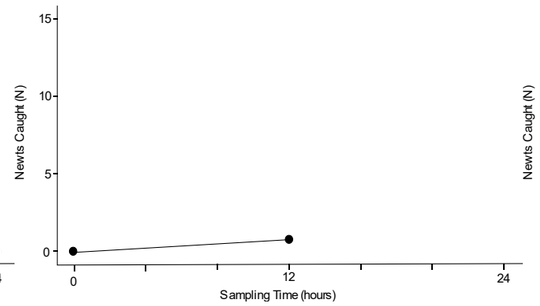
Maple Lake - 2017 (4)



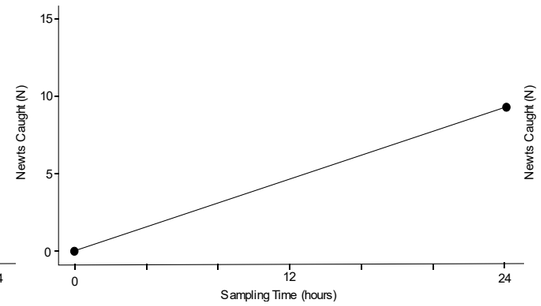
Maple Lake - 2018 (4)



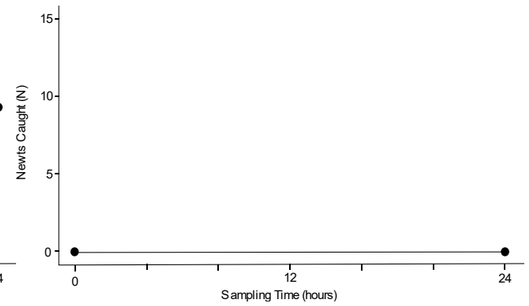
Martlet Pond - 2017 (4)



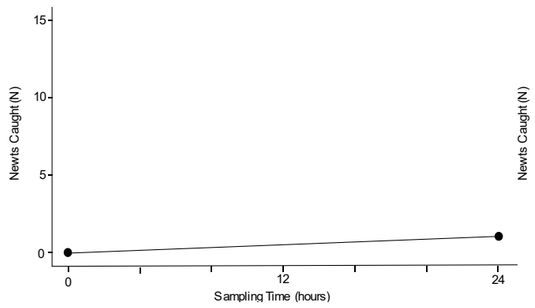
Mary Lake - 2018 (3)



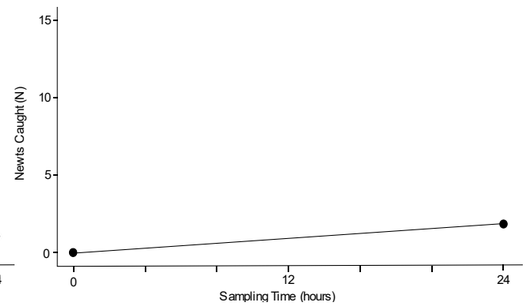
Mesachie Lake - 2017 (4)



Sywash Marsh - 2017 (4)



Sywash Marsh - 2018 (4)



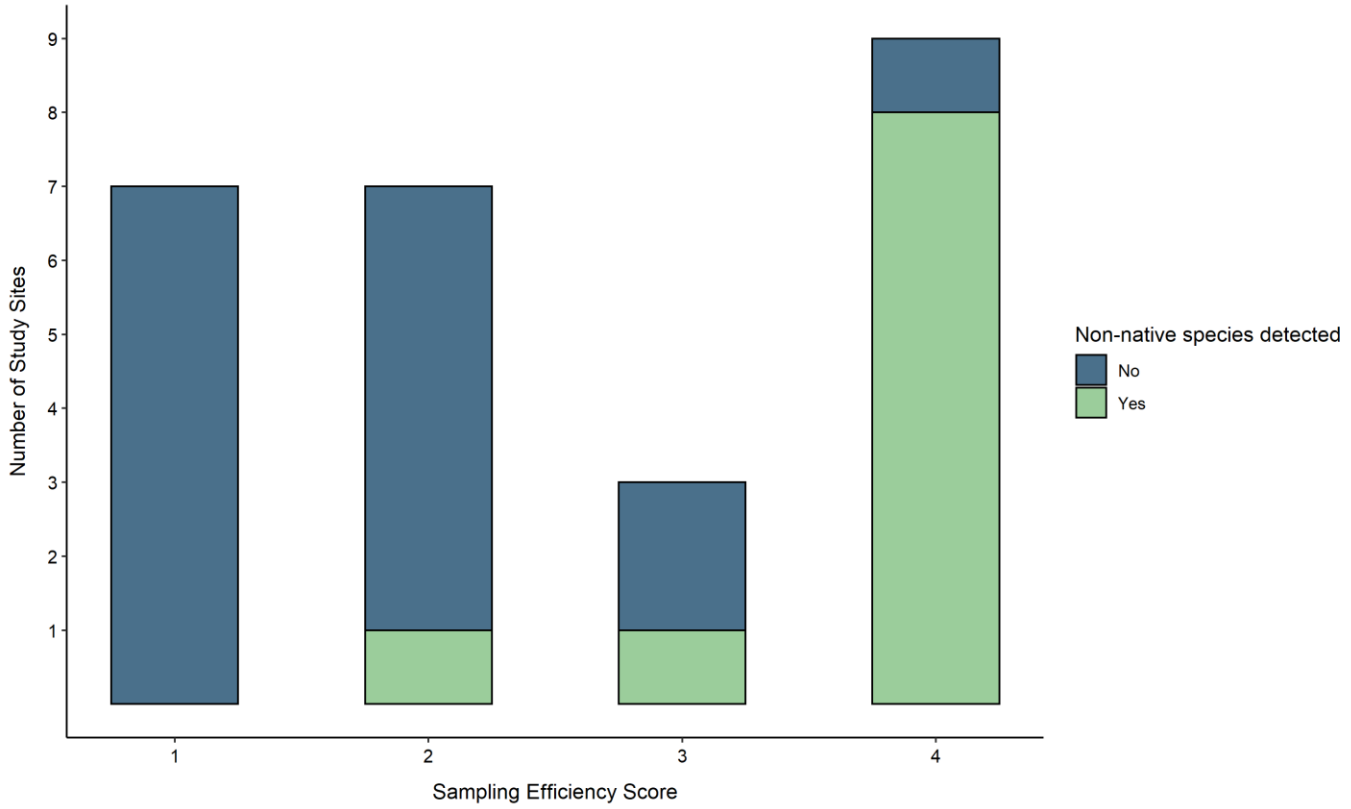


Figure 4.3: Barplot showing sampling effort for study sites with and withput non-native species detected. Sampling efficiency scores were assigned based on the relative amount of effort needed to collect rough-skinned newts from a given site, 1 being the least effort to 4 being the most.

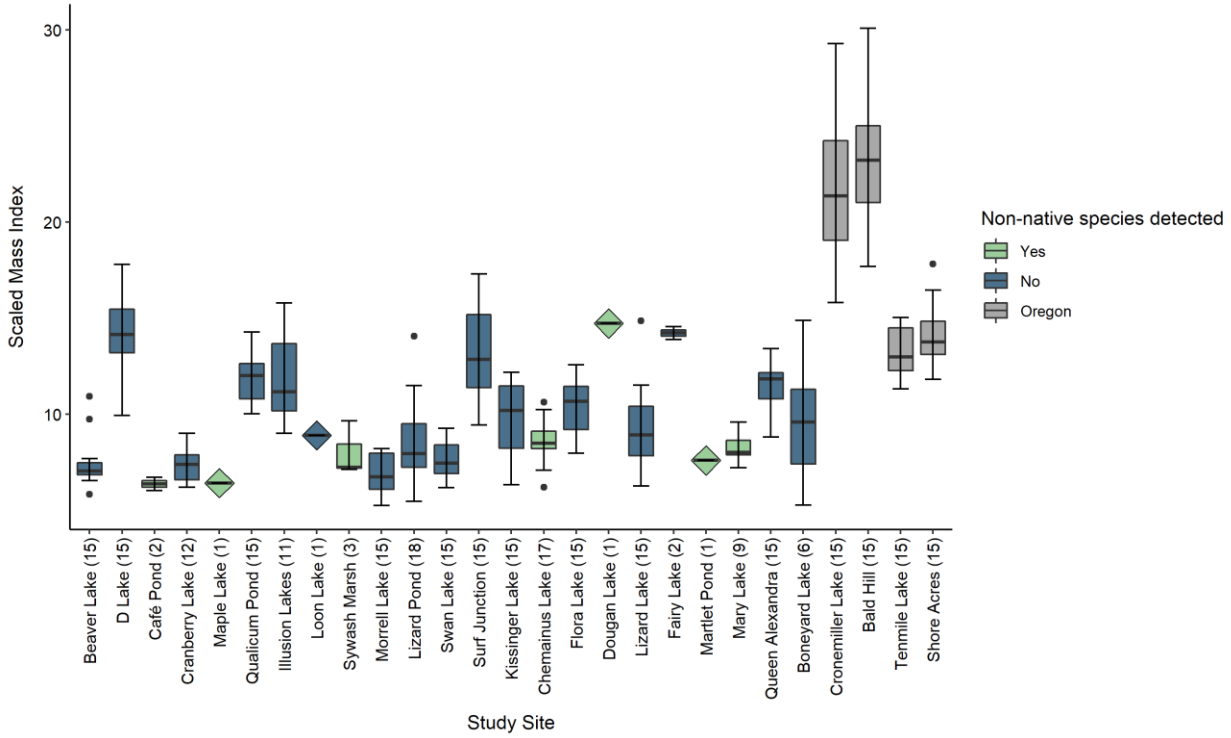


Figure 4.4: Boxplots showing variation in rough-skinned newt scaled mass index (SMI) in the presence or absence of non-native species by each study site, ordered north to south by latitude. Whiskers represent the minimum and maximum values, colored boxes show the interquartile range with thick horizontal line for the mean, and black points are outliers. Study sites displaying a single horizontal line had only one sample, which is therefore displayed as the mean, with colored diamond shapes to display presence or absence of non-native species. Oregon study sites (shaded gray) were not used in the analyses but are included for comparative purposes. Values in parentheses on the x-axis represent sample size for each boxplot.

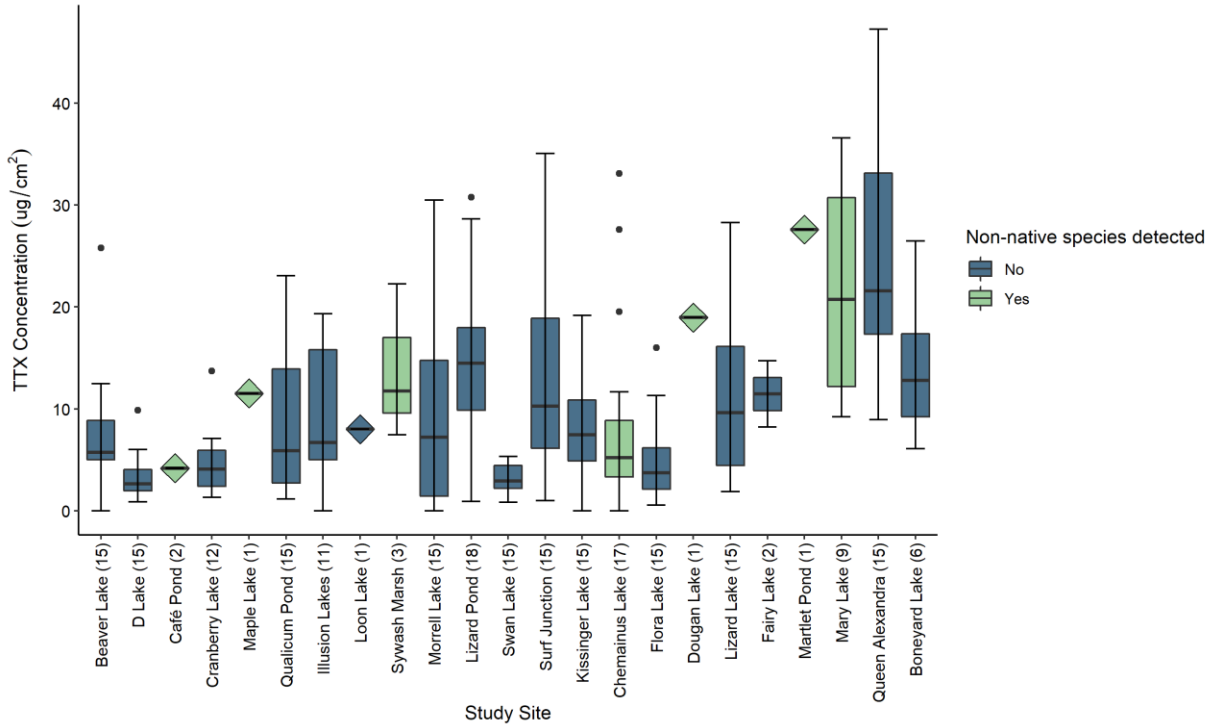


Figure 4.5: Boxplots showing variation in rough-skinned newt dorsal skin TTX concentration in µg/cm^2 in the presence or absence of non-native species by each study site, ordered north to south by latitude. Whiskers represent the minimum and maximum values, colored boxes show the interquartile range with thick horizontal line for the mean, and black points are outliers. Study sites displaying a single horizontal line had only one sample, which is therefore displayed as the mean, with colored diamond shapes to display presence or absence of non-native species. Values in parentheses on the x-axis represent sample size for each boxplot.

Tables

Table 4.1: Sample size (N), invasive species presence, mean TTX, mean SMI, and sampling efficiency score for 26 study sites sampled on Vancouver Island. Sites listed as "None detected" for non-native species indicate that I did not find evidence of crayfish or bullfrog presence during my sampling efforts and did not find any mention of their presence in published literature. Lakes with N=0 were only used for the Fisher's exact test for the sampling efficiency analysis.

Study Site	N	Invasive Species	Mean TTX ($\mu g/cm^2$)	Mean SMI	Sampling Efficiency Score
Beaver Lake	15	None detected	7.55	7.42	2
D Lake	15	None detected	3.41	14.07	1
Café Pond	2	American bullfrog, *Lithobates catesbeianus*	4.18	6.38	4
Cranberry Lake	12	None detected	4.79	7.38	2
Maple Lake	1	Signal crayfish, *Pacifastacus leniusculus*	11.52	6.42	4
Qualicum Pond	15	None detected	8.72	11.90	1
Illusion Lakes	11	None detected	9.48	11.88	3
Enos Lake	0	Signal crayfish, *Pacifastacus leniusculus*	N/A	N/A	4
Loon Lake	1	None detected	8.00	8.90	4
Sywash Marsh	3	American bullfrog, *Lithobates catesbeianus*	13.82	9.01	4
Morrell Lake	15	None detected	9.65	6.91	2
Lizard Pond	18	None detected	14.89	8.51	1

Swan Lake	15	None detected	3.18	7.70	2
Surf Junction	15	None detected	13.67	13.21	2
Kissinger Lake	15	None detected	7.91	9.76	1
Chemainus Lake	17	American bullfrog, *Lithobates catesbeianus*	8.72	8.64	2
Flora Lake	15	None detected	4.83	10.45	1
Mesachie Lake	0	Signal crayfish, *Pacifastacus leniusculus*	N/A	N/A	4
Dougan Lake	1	American bullfrog, *Lithobates catesbeianus;* Signal crayfish, *Pacifastacus leniusculus*	18.96	14.73	4
Lizard Lake	15	None detected	11.49	9.27	1
Fairy Lake	2	None detected	11.46	14.23	3
Martlet Pond	1	American bullfrog, *Lithobates catesbeianus*	27.59	7.60	4
Elk/Beaver Lake	0	Signal crayfish, *Pacifastacus leniusculus*	N/A	N/A	4
Mary Lake	9	American bullfrog, *Lithobates catesbeianus*	21.69	8.23	3
Queen Alexandra	15	None detected	24.93	11.51	1
Boneyard Lake	6	None detected	14.18	9.65	2

CHAPTER FIVE – UNIQUE GENETIC CLUSTER OF ROUGH-SKINNED NEWTS ON VANCOUVER ISLAND IDENTIFIED USING RNA-SEQ

5.1 Introduction

Genetic population subdivision may occur when various biotic and abiotic factors, including changes in community composition, geographic barriers, or anthropogenic effects, lead to non-random mating or geographic isolation (Hartl & Clark 1997). Variation in genetic diversity may also result from processes such as population bottlenecks, drift, mutation, or selection (Hughes et al. 2008). The study of population structure and genetic variation allows the characterization of population demography across a landscape and can provide insights regarding the ability of populations to respond to various selective pressures as well as their susceptibility to extirpation (Chakraborty 1993, Manel et al. 2003, Frankham 2005, Allendorf et al. 2012). Such studies may also inform management decisions to guide conservation efforts (Pearse & Crandall 2004, Shafer et al. 2015) and help with the identification of meaningful units of conservation (de Guia & Saitoh 2007, Shaffer et al. 2015).

Traditionally, markers such as microsatellites, mitochondrial DNA (mtDNA), and nuclear markers were used to study population genetic processes (Abdul-Muneer 2014). Recent technological advances, however, have also enabled the study of independent loci through methods such as restriction site-associated DNA sequencing (RAD-seq) and RNA sequencing (RNA-seq) (e.g. Davey & Blaxter 2010, Andrews et al. 2017, Todd et al. 2017). Neither of these high-throughout methods require prior genome information, thus making population genetics studies more feasible for a greater number of non-model organisms (Hohenlohe et al. 2012, Gayral et al. 2013). An RNA-seq, or transcriptomics,

approach is advantageous because not only does it allow identification of thousands of single nucleotide polymorphisms (SNPs) in expressed genes, but the same dataset can also be used to study differentially expressed genes among different phenotypes (Thorntenson et al. 2020, see also Chapter 3, this thesis). Focusing on expressed genes also enables greater accuracy of functional annotation than reduced representation genomic DNA methods like RAD-seq (De Wit et al. 2015).

A transcriptomics approach for population genetic studies would be particularly beneficial for studies of non-model taxa such as amphibians (Shaffer et al. 2015). Around the globe, amphibian populations are rapidly declining while robust assessments of population trends remain lacking, with many amphibian species classified as data deficient (Pinya et al. 2017). One amphibian species that may be at risk of future declines is the rough-skinned newt, *Taricha granulosa*. These newts are known to possess an antipredator defense whereby they excrete a potent neurotoxin known as tetrodotoxin (TTX) primarily from their dorsal skin (Brodie 1968). While their main predators, garter snakes, have evolved resistance to TTX (e.g., Brodie & Brodie 1990, Brodie et al. 2005; see also Chapter 2, this thesis), newts may face future threats from other novel predators (Chapter 4, this thesis). Rough-skinned newts are presently listed as a species of Least Concern (IUCN SSC Amphibian Specialist Group 2015). However, non-native signal crayfish and American bullfrogs are rapidly spreading across Vancouver Island, British Columbia, causing conservation concern in the region (see Chapter 4, this thesis).

Vancouver Island is an interesting region to study rough-skinned newts due to the special considerations of island ecosystems. Island populations are known to have lower genetic diversity, higher chances of inbreeding depression, and elevated extinction rates

compared to mainland conspecifics (Frankham 1997, Frankham 1998). Such characteristics of islands are further impacted by their heightened sensitivity to the effects of non-native species (Frankham 1997, Cox & Lima 2006). A majority of plant and animal extinctions in the past several centuries occurred on islands, in most cases due to the impacts of non-native species (Sax & Gaines 2008). It is therefore crucial to consider population genetics for the conservation of endemic island populations threatened by non-native species. For example, Pacific wrens native to islands in the North Pacific Rim of Alaska are made up of several subspecies that are impacted by predation from Norway rats, which were first introduced to the area around 200 years ago (Bond & Eggleston 2015, Pruett et al. 2017). The westernmost island in the chain, Attu Island, was found to contain a genetically distinct population of wrens of the subspecies *Troglodytes pacificus meligerus,* with the lowest observed genetic diversity out of nine populations sampled throughout Alaska and BC (Pruett et al. 2017). Potential impacts by non-native rats may cause conservation concern for the unique island population, which is an important region for high-latitude biodiversity (Pruett et al. 2017).

Previous population genetic studies of rough-skinned newts are lacking in robust data and have not included any samples from Vancouver Island. Studies utilizing traditional population genetics methods to investigate population structure in this species used only one mitochondrial marker, *cytochrome-B* (Kuchta & Tan 2005), one mitochondrial plus one ribosomal marker, *COI* and *16S* (Mebs et al. 2016), or a few microsatellites (Jones et al. 2001, Ridenhour et al. 2007, Hague et al. 2016). Another recent study performed RAD-seq to identify SNPs from newts in Oregon and Washington, USA (Hague et al. 2019). Despite the generally low number of genes

analyzed and low geographic coverage, some of these studies were able to detect unique genetic clusters and patterns of isolation by distance (IBD) at various spatial scales in rough-skinned newts (e.g., Kuchta & Tan 2005, Ridenhour et al. 2007, Hague et al. 2016, Hague et al. 2019). However, the ability to discern genetic divergence patterns among populations may also be influenced by the sequencing method chosen. Using microsatellites, Hague et al. (2016) concluded there was no evidence for a relationship between neutral genetic divergence and newt toxicity, whereas their RAD-seq dataset implied there was such a relationship (Hague et al. 2019). Conversely, in some cases the use of genome-wide SNP data may indicate more genetic admixture than previously found with microsatellites (e.g., Hundsdoerfer et al. 2019).

Here, I investigate the population genetics of rough-skinned newts by identifying thousands of SNPs using an RNA-seq approach. The goals of this chapter are to (i) characterize the previously unknown population genetic structure among newts from Vancouver Island and mainland populations in Oregon, and (ii) determine if there is any population sub-structuring evident across Vancouver Island. Previous studies of the population genetics of rough-skinned newts did not include Vancouver Island, a region that may face future conservation concerns because of the presence of non-native signal crayfish and American bullfrogs (see Chapter 4, this thesis). Due to the oceanic barrier between island and mainland populations, I expect Vancouver Island newts to be genetically distinct from their Oregon counterparts. Breeding site fidelity may also lead to differentiation and population clustering of newts within the island, as rough-skinned newts tend to return multiple years to the same breeding site in which they were born (Pimentel 1960). Such behavior may result in decreased gene flow between watersheds

with distinct breeding sites, while suggesting a more complex population genetic structure among populations with low diversity within populations. It is crucial to characterize the population genetic structure of rough-skinned newts to help inform management plants at the proper spatial scale in the face of amphibian declines, especially for island populations that are particularly susceptible to the impacts of non-native species (Frankham 1997, Cox & Lima 2006).

5.2 Methods

5.2.1 Sample Collection and Sequencing

I collected dorsal tissue samples from rough-skinned newts across 28 sites in Vancouver Island, BC, Sunshine Coast, BC, and Oregon, USA (see Fig. 2.1 in Chapter 2, this thesis), as described in Chapter 2. The samples were preserved, extracted, and sequenced following methods from Chapter 3. I randomly chose one individual from each of the 24 sites in BC for sequencing, with 23 being from Vancouver Island and one from the Sunshine Coast. There were also eight samples from sites in Oregon (seven dorsal tissue samples used for differential expression analysis in Chapter 3 plus one additional sample), for a total of 32 samples in this study. RNA sequencing on an Illumina HiSeq 4000 (Illumina, USA) yielded a sequencing depth of approximately 16M-69M reads per library. Multiple samples per site in BC were not sequenced to keep costs down while still allowing for a broad geographic representation of study sites. Within-site genetic variation was also expected to be low due to high breeding site fidelity in rough-skinned newts and previous studies finding evidence of low within-population genetic diversity (e.g., Ridenhour et al. 2007, Mebs et al. 2016).

5.2.2 SNP Calling

I trimmed raw sequencing reads with Trimmomatic v0.36 (Bolger et al. 2013) using the following quality control parameters: (i) trimming of bases at the leading and trailing ends of sequences with a phred+33 quality score below 20, (ii) a 4-base sliding window scan to remove read fragments with an average quality per base below 20, (iii) removal of reads below 36 base pairs long, and (iv) clipping of Illumina adapter sequences. The *de novo* transcriptome assembly generated in Chapter 3 was further processed to identify SuperTranscripts, which is a feasible alternative to reference genome alignment for non-model organisms. SuperTranscripts are constructed from a set of transcripts where genes with multiple transcripts are represented by a single sequence, thereby creating a linear representation of the transcriptome for variant detection (Davidson et al. 2017). I carried out the SuperTranscript pipeline via a Python script within Trinity v2.8.5 (Grabherr et al. 2011, Haas et al. 2013).

Single nucleotide polymorphisms (SNPs) were identified following the GATK Best Practices Workflow for short variant discovery (Van der Auwera et al. 2013). First, I individually aligned trimmed reads from all 32 samples to the SuperTranscript reference sequence using the STAR v2.6.1a RNA-seq aligner in two-pass mode (Dobin et al. 2013). After alignment, reads were processed using Picard v2.20.6 by removing duplicates, splitting cigar reads, and recalibrating base quality (Broad Institute 2020). HaplotypeCaller in GATK v3.8 (McKenna et al. 2010) called the variants, and SNPs were then subject to stringent filtering parameters in both GATK and vcftools v0.1.14 (Danecek et al. 2011). Filtering parameters included: (i) filtering clusters of at least three SNPs that are within a window of 35 bases between them, (ii) maximum Fisher Strand

value of 30, (iii) minimum Qual by Depth value of 2, (iv) removal of indels, (v) minimum Genome Quality of 30, (vi) minimum depth of 10 reads per SNP, and (vii) exclusion of sites with missing genotypes in more than 50% of the samples. The final filtering parameter regarding missing data is less conservative than other studies which exclude sites that are not present in 100% of samples (De Wit & Palumbi 2012, Marques et al. 2017, Thorstensen et al. 2020). I combined the individual variant call format (VCF) files for each sample into merged VCFs using bcftools (Li 2011), and then converted them to STRUCTURE format via PGDSpider v2.1.1.5 (Lischer & Excoffier 2012). Two datasets were created for analyses: (1) all 32 samples including Vancouver Island, Sunshine Coast, and Oregon (hereafter referred to as the "complete dataset"), and (2) 23 samples from Vancouver Island alone (the "Vancouver Island dataset").

5.2.3 Population Structure

To analyze population structure, I used the program STRUCTURE v2.3.4to identify the number of genetic clusters (K) via a Bayesian clustering method (Pritchard et al. 2000). Analyses used the admixture model with correlated allele frequency, without using sampling location as a prior. Each STRUCTURE run had a burn-in period of 10,000 repetitions with 100,000 subsequent Markov Chain Monte Carlo (MCMC) repetitions and ten independent replicates at each value of K from K=1 to 7. STRUCTURE runs were performed with the SNP data separately for the complete dataset and the Vancouver Island dataset. The optimal values of K were inferred according to the Evanno method (Evanno et al. 2005), using the R package pophelper v2.3.0 (Francis 2016). Resulting STRUCTURE plots were also visualized in pophelper.

I performed additional analyses of population structure in R with the R Studio interface v1.1.163 (R Core Team 2018). I first used the vcfR v1.10.0 package (Knaus & Grünwald 2017) to convert the merged VCF files into the proper format for subsequent analyses. Using Adegenet v 2.1.2 (Jombart 2008), principal components analyses (PCA) were performed for both the complete dataset and the Vancouver Island dataset. PCAs are widely used to identify population structure from large SNP datasets without any assumptions about underlying population genetics models, by reducing the dimensionality of the dataset (Patterson et al. 2006, Lee et al. 2009). The analysis in Adegenet uses an input of a matrix of dimension *N x P* with N rows of individual samples and P columns of SNPs, where the genotypes within the matrix are coded as 0, 1, and 2 based on the allele combination at each site. That matrix is transformed into a matrix of centered and scaled allele frequencies for each SNP by assigning the frequency of a coded genotype value per allele in each column (Jombart 2008). Then the PCA was performed to calculate eigenvalues that explain and visualize genetic variation among individuals. I also identified genetic clusters for both datasets in Adegenet based on the *find.clusters* function, which uses the K-means clustering procedure to find the best supported number of clusters based on the lowest Bayesian information criterion (BIC) value. The maximum number of clusters used was 10, with 15 principal components retained. To describe the relationship between the clusters, I also performed Discriminant Analysis of Principal Components (DAPC) (Jombart et al. 2010) in Adegenet. Finally, I used the package hierfstat v0.04-22 (Goudet 2005) to calculate basic statistics between the Oregon and British Columbia population clusters. The fixation index (F_{ST}) was calculated to characterize genetic differentiation via the Weir and Cockerham method

(Weir & Cockerham 1984), with 95% confidence intervals generated over 1000 bootstrap iterations. Observed heterozygosity (H_O), expected heterozygosity (H_S), and inbreeding coefficients (F_{IS}) were also calculated separately for Oregon and BC clusters.

5.3 Results

A total of 305,863 SNPs were identified from RNA-seq data aligned to the *de novo* assembled transcriptome for rough-skinned newts. After strict quality filtering parameters were implemented, a total of 33,659 SNPs remained in the complete dataset and 41,240 SNPs in the Vancouver Island dataset. More SNPs were likely present in the Vancouver Island dataset because the quality filtering parameter excluding variants with missing data in more than 50% of the samples may have filtered out more variant sites in the complete dataset (i.e., newts on the island likely have more SNPs in common with each other than with individuals from Oregon).

There was strong evidence of population structure among Oregon and British Columbia newts with each geographic region belonging to a distinct genetic cluster. STRUCTURE runs from K=1 to 7 identified the best supported number of clusters as K=2, which clearly separated the newts from Oregon and BC (Fig. 5.1, Appendix G). The single sample from Sunshine Coast clustered with the Vancouver Island samples, with a 0.088 probability of belonging to the Oregon cluster. The PCA plotted individual coordinates along axes of genetic variation for the first two principal components (PC) and displayed similar patterns of clustering between Oregon and BC, with PC1 explaining 8.6%, and PC2 explaining 4.3%, of the genetic variance among individuals (Fig. 5.2). There is no defined threshold upon which a PC explains a significant amount

of variance, but PC1 in my study explained higher genetic variance than similar population genetic studies with large SNP datasets on songbirds (PC1=4%; Tan et al. 2018), deep-sea mussels (PCA=4.9%, Xu et al. 2017), and Chinese cattle breeds (PC1=6.9%; Li et al. 2019). Furthermore, K-means clustering in Adegenet resulted in the lowest BIC score of 199.66 corresponding to K=2 (Fig. 5.3). The DAPC results also aligned with the STRUCTURE findings, with the Oregon and BC samples belonging to two different clusters (Fig. 5.4). Pairwise F_{ST} between the two clusters was 0.1026 (95% confidence interval=0.1003-0.1049), indicating strong genetic differentiation. The Oregon cluster had a higher observed heterozygosity than the BC cluster, and both clusters had negative inbreeding coefficients which is indicative of an excess of heterozygotes (Table 4.1).

In contrast to the patterns between the two regions, there does not appear to be significant population substructure across Vancouver Island. STRUCTURE analysis found the best supported number of clusters to be K=5 via the Evanno method (Appendix G). However, when inspecting the STRUCTURE plots from K=1 to 7, there is clearly no clustering evident and the results rather point to a true value of K=1 (Fig. 5.5, Appendix G). Unfortunately, K may be overestimated in the case of minor model departures, such as some inbreeding, and the Evanno method does not consider K=1 in the delta K plot used to select the value of K (Pritchard et al. 2000). PCA and K-means clustering results also suggest a lack of population structure exists in newts across the island (Fig. 5.6, Fig. 5.7). DAPC was not performed with the Vancouver Island SNP dataset because the K-means clustering in Adegenet suggested all 23 samples belong to a single genetic cluster (K=1) and therefore the analysis was not applicable. Despite the STRUCTURE results

suggesting there are K=5 clusters, the visual plots and additional clustering analyses lead to the conclusion that there is a lack of population genetic structure across the island.

5.4 Discussion

Understanding the population genetic structure is crucial for the conservation of at-risk populations, especially on islands, which tend to have lower genetic diversity and are more susceptible to the effects of invasive species (Frankham 1997, Cox & Lima 2006). In this chapter my objective was to utilize SNPs identified from RNA-seq data to characterize the population genetic structure of rough-skinned newts at two spatial scales: among newt populations in Oregon and BC, and within newts across Vancouver Island. There was strong support for significant population clustering among newts from Oregon and BC with evidence that a unique genetic cluster exists on Vancouver Island. However, there was weak population structure apparent across Vancouver Island, indicating they may represent a single panmictic population. Observed heterozygosity (H_O) was lower in Vancouver Island than in Oregon, as expected for island populations. This work characterizes the population structure of rough-skinned newts on Vancouver Island, which is a large, geographically isolated region of the newt range that faces conservation concerns as non-native signal crayfish and American bullfrogs are rapidly spreading across the island (Chapter 4, this thesis).

There was significant population clustering detected among Oregon and BC samples, and results were consistent across the multiple clustering methods performed. Bayesian clustering in STRUCTURE, as well as PCA, K-means clustering, and DAPC in Adegenet, identified two distinct genetic clusters in the complete dataset (Fig. 5.1, Fig.

5.2, Fig. 5.3, Fig. 5.4). The two clusters corresponded to BC and Oregon samples each belonging to different respective groups. Although the single sample from Sunshine Coast, BC clustered with the Vancouver Island samples, it still had a small probability (0.088) of belonging to the Oregon cluster. If more samples from Sunshine Coast were available, perhaps they would have been assigned to a third separate cluster if the sample size was large enough. The high level of genetic differentiation between Oregon and BC clusters (F_{ST}=0.1026) further suggests that newts from the two regions are genetically distinct. Rough-skinned newts from Bella Coola, BC and from the northern edge of their range in Alaska were also found to be clustered separately by STRUCTURE, although they had lower genetic differentiation (F_{ST}=0.0705, Hague et al. 2016) than the two clusters in my study. Similarly, the Mazama newt, *Taricha granulosa mazamae,* is reported to be genetically distinct from other Pacific Northwest *T. granulosa* populations ("unpublished results" mentioned in Girdner et al. 2017). The data remain unpublished, so I am unable to compare their quantitative results with those from this study.

Within Vancouver Island, there was evidence of admixture across the island with no apparent population structure. STRUCTURE, PCA, and K-means clustering all suggest that the 23 samples from the island belong to a single genetic cluster. Due to the high number of SNPs detected, the large size of the island, and life-history traits such as breeding site fidelity, I expected to find some degree of population sub-structuring across the island. The lack of structure may suggest that watershed connectivity in the region is high, or newts are able to migrate further distances than formerly thought. Previous studies also found evidence of little genetic differentiation in rough-skinned newts from Northern California to Washington (F_{ST}=0.031) (Ridenhour et al. 2007) and in Alaska

(F_{ST}=0.0185) (Hague et al. 2016), despite their limited dispersal capabilities. This suggests that while newts in potentially connected habitat may have low genetic differentiation, the geographic isolation of newts on Vancouver Island has enabled them to evolve into a genetically distinct group overall. Another possibility is that upon recolonization of the island after retreat of the Cordilleran ice sheet approximately 10,000 years ago (Mann & Hamilton 1995), little molecular evolution has taken place in the Vancouver Island populations due to the relatively slow rate of amphibian genome evolution (Liedtke et al 2018).

Additionally, the lack of genetic differentiation in newts on Vancouver Island is interesting given the significant variation in toxicity detected in the same populations (Chapter 2, this thesis). As previously discussed, there is a lack of consistency in results regarding whether rough-skinned newt toxicity is best predicted by neutral genetic divergence rather than coevolution with their garter snake predators (Hague et al. 2016, Hague et al. 2019). Two different studies by the same group of researchers first found that there was no correlation between neutral genetic divergence and newt toxicity (Hague et al. 2016), but later found evidence for a relationship with a different dataset (Hague et al. 2019). The phenotypic variation in TTX identified on Vancouver Island in Chapter 2 does not appear to align with the lack of genetic structure found in this chapter, suggesting that my findings are more in line with the first hypothesis regarding a lack of relationship (Hague et al. 2016). Unfortunately, I was unable to perform the same analyses with my dataset, so future studies may approach this problem again with a similar study design to Hague et al. (2019), but on Vancouver Island. Nevertheless, such results indicate that variation in newt toxicity may be due to phenotypic plasticity and

could be regulated through environmentally induced changes in gene expression (e.g., Levis et al. 2017) or predation risk (e.g., Hettyey et al. 2019) rather than neutral genetic divergence.

Measures of observed heterozygosity suggest that newts from the BC cluster (H_O=0.4516) have substantially lower genetic diversity than those from Oregon (H_O=0.7692) (Table 5.1). Low genetic variation is expected on islands compared to mainland populations due to processes such as greater impact of genetic drift and higher levels of inbreeding, which ultimately leads to a higher extinction risk (Frankham 1997). Island size and effective population size are also found to be correlated with genetic diversity of islands (Frankham 1996). Using microsatellites, another study found small islands in the northern edge of the rough-skinned newt range in Alaska had H_O estimates from 0.29 to 0.39 (Hague et al. 2016). Vancouver Island is a very large island, which may explain the slightly higher observed heterozygosity than the small Alaskan sites, although compared to Oregon it still has much lower variation. Raw genetic diversity provides evolutionary potential for future adaptations to progress (Frankham 2005), so rough-skinned newts on Vancouver Island may be less resilient to adapting to the presence of non-native predators such as signal crayfish and American bullfrogs (Chapter 4, this thesis).

Because newts on Vancouver Island are genetically distinct from their Oregon counterparts and have a significant decrease in genetic diversity, this may inform future conservation management decisions. Species-level conservation is often not the best approach when trying to preserve intraspecific genetic diversity, which has led to various proposed intraspecific units of conservation, including management units (MUs),

evolutionarily significant units (ESUs), and designatable units (DUs) (Green 2005, Shaffer et al. 2015). Criteria for defining ESUs are that populations have reciprocal monophyly of mtDNA and show significant divergence of allele frequencies at nuclear loci, whereas MUs are intra-ESU groups that are not reciprocally monophyletic but have diverged allele frequencies (Moritz 1994). The concepts of ESUs and MUs are employed worldwide, but in Canada, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) specifically uses DUs for species status assessment (Green 2005). COSEWIC identifies designatable units for subspecies or varieties, or discrete and evolutionarily significant populations (COSEWIC 2018). Population discreteness includes meeting one or more criteria related to genetic distinctiveness and/or geographic isolation, and then significance is determined based on local adaptation, genetic divergence, evidence that a population is the only surviving occurrence in its native range, and/or that loss of the population would result in a gap in the species range in Canada (COSEWIC 2018). Geographic isolation in addition to evidence of the unique genetic cluster of newts on Vancouver Island may warrant *Taricha granulosa* to be considered as a DU. However, DNA data were not obtained in this study so future work is needed to definitively address this question. Morphological differentiation can also be used to identify units of conservation. For example, the black-chested spiny-tailed iguana, *Ctenosaura melanosterna*, in Honduras has two identified ESUs, with one group having a smaller body size (Pasachnik et al. 2012). The newts on Vancouver Island were significantly smaller than newts from Oregon (Fig. 5.8, Kruskal-Wallis; χ^2=125.53, $p<2e^{-16}$, df=1), and this physical difference may lend further support for designation as a distinct DU or ESU. The Mazama newt was even classified as a subspecies of rough-

skinned newts when differences in tail morphology and ventral skin coloration were identified (Myers 1942).

In this chapter, there are some limitations worth noting. Despite its accuracy in SNP calling and usefulness for other purposes, transcriptomics is still not widely used for population genetics studies (Rogier et al. 2018). This is likely due to high sequencing costs, although the use of RNA-seq should continue to become more widespread as sequencing costs decrease and analytical methods are further refined. One limitation of using RNA-seq data for SNP calling is that certain types of variants are not represented in the dataset or are screened out at low read depths. Because the transcriptome only includes expressed genes, SNPs such as those that are not expressed or those with monoallelic expression will be missed (Piskol et al. 2013). Another shortcoming is that the stringent filtering parameters may have also excluded potentially relevant variants that were instead coded as missing data. For instance, because SNPs were called individually compared to the reference transcriptome, a potential population-wide variant would not be identified in one sample if the SNP matched the reference, but if another sample had a different allele from the reference then that variant would be identified. However, such occurrences are expected to be randomly distributed and unlikely to alter inferences about population structure. Filtering out variant sites that are not present in at least 50% of samples also prevents the study of potentially adaptive SNPs that are only present in a few individuals. Overall, the cost of sequencing also prevented us from studying multiple samples per site on Vancouver Island, which subsequently did not allow certain population genetics analyses such as quantifying IBD or between-site genetic diversity and *F*-statistics (Rousset 1997). Nevertheless, the RNA-seq data utilized

here may be repurposed for other studies beyond this thesis, which ultimately outweighs the downfalls of this approach.

I hope this work will (i) serve as further evidence that rough-skinned newts of Vancouver Island should have special consideration, and (ii) help guide conservation management efforts to decrease the extinction risk of the unique island population, especially due to threats from non-native species (Chapter 4, this thesis). Future work should include mtDNA analyses to determine whether Vancouver Island newts may qualify for designation as an ESU due to the definition of reciprocal monophyly (Moritz 1994). Other objectives may include sequencing multiple samples per site on Vancouver Island, analyzing additional mainland populations or other coastal islands in British Columbia, and comparing genetic differentiation to that of Mazama newts. It would also be interesting to analyze the same mitochondrial or microsatellite markers as in previous research (e.g., Ridenhour et al. 2007, Hague et al. 2016) to study divergence among populations of newts on the island when such broad SNP coverage is not used. Ultimately, more work on the impacts of invasive species on Vancouver Island is needed to guide conservation management efforts and decrease the extinction risk of its populations.

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Figures

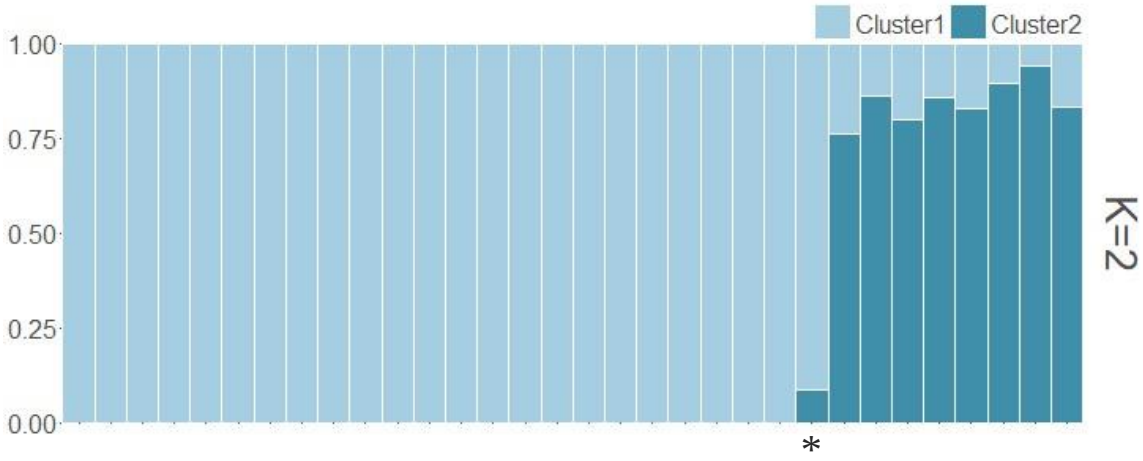

Figure 5.1: STRUCTURE plot for K=2 of 32 rough-skinned newt samples from Vancouver Island, BC (N=23), Sunshine Coast, BC (N=1), and Oregon (N=8). Each vertial line along the x-axis corresponds to a single individual with each color representing a unique cluster. The individual marked with an asterisk (*) is the single sample from Sunshine Coast; samples to the left are from Vancouver Island and samples to the right are from Oregon. The y-axis displays probability of each individual belonging to a given cluster.

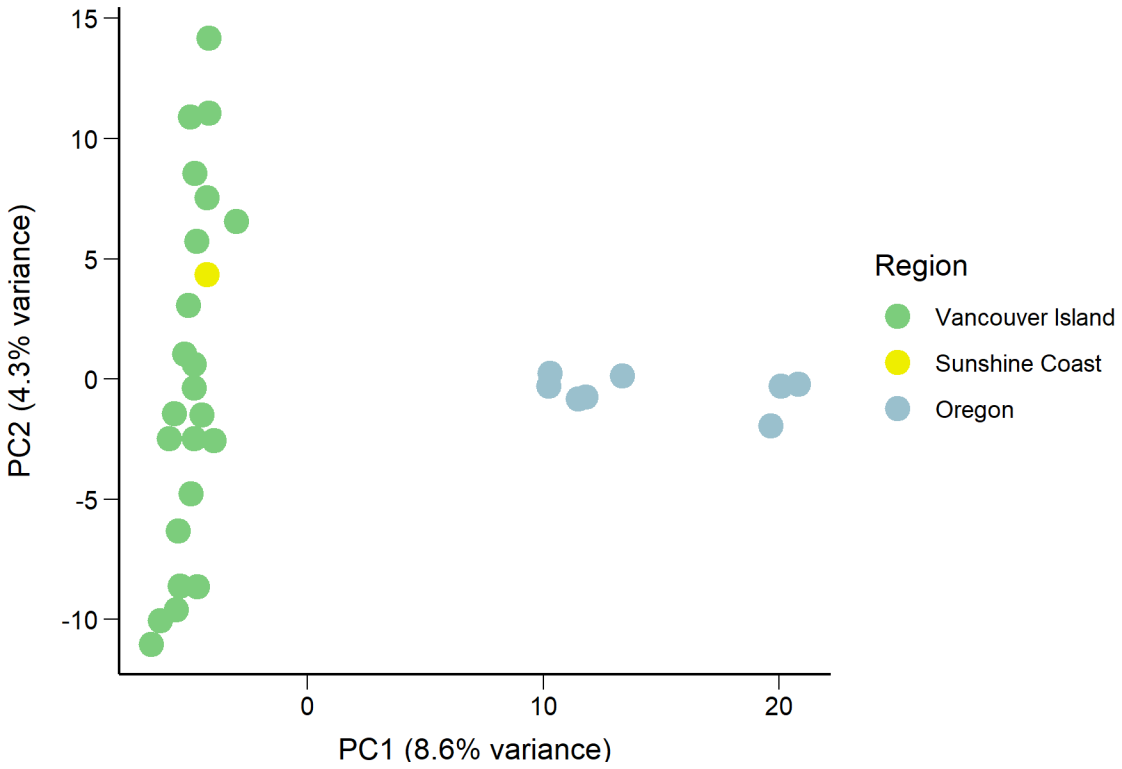

Figure 5.2: Principal Components Analysis of 33,659 SNPs from 32 samples in British Columbia and Oregon, with points colored by sampling region. Principal Component 1 (PC1) represents 8.6% of the genetic variance among individuals and Principal Component 2 (PC2) represents 4.3% of the variance among individuals.

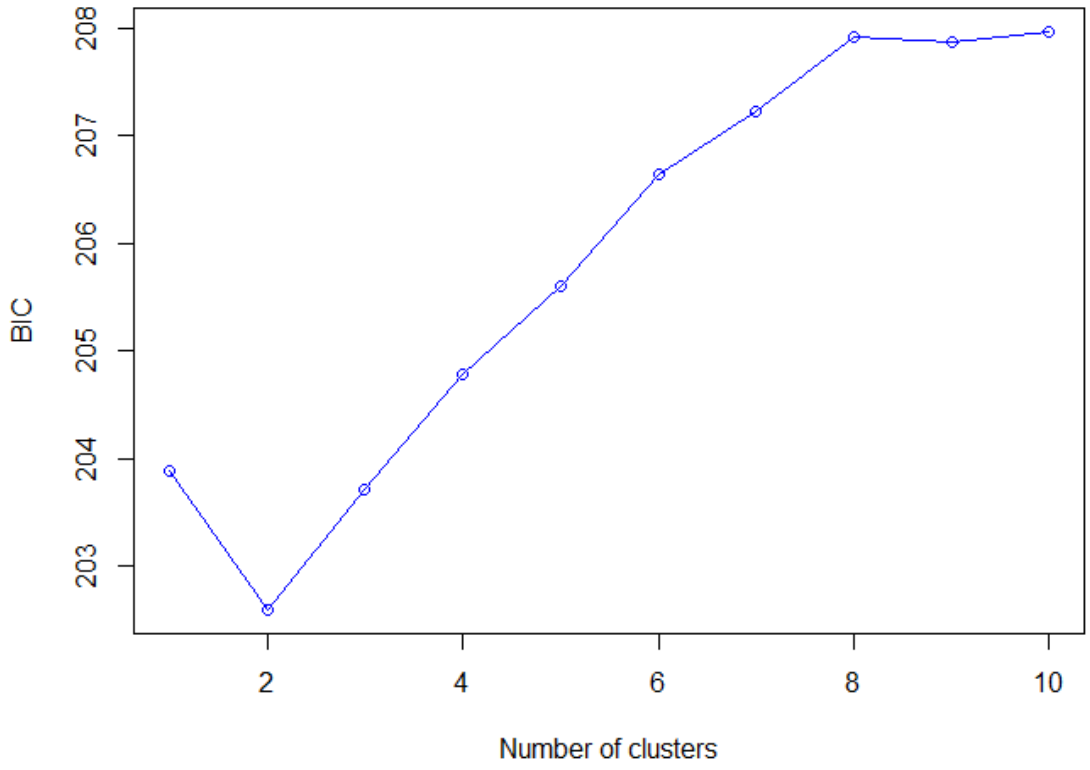

Figure 5.3: BIC values from K-means clustering of K=1 to 10 of 33,659 SNPs in 32 samples from British Columbia and Oregon. K=2 corresponds to the lowest BIC score of 199.66, indicating two genetic clusters in the data. Figure generated from adegenet v2.1.2 (Jombart et al. 2010).

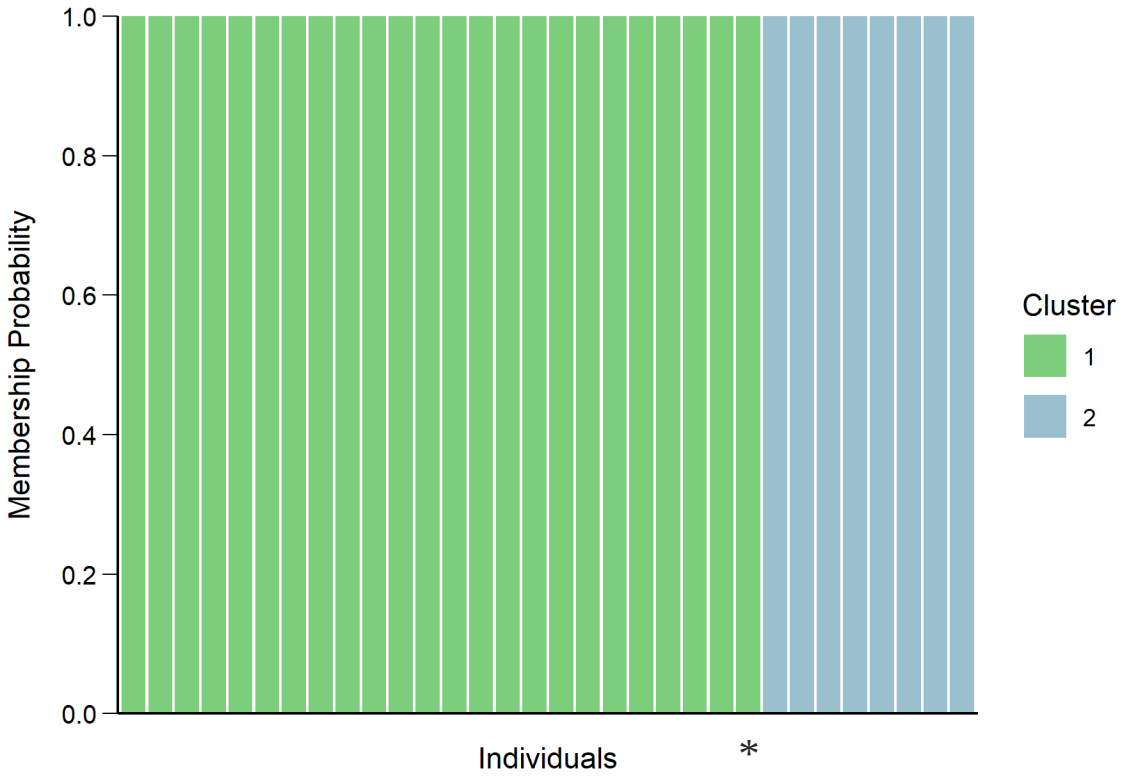

Figure 5.4: Membership probability from Discriminant Analysis of Principal Components using 33,659 SNPs in 32 samples from British Columbia and Oregon. Similar to the STRUCTURE plot, each vertial line along the x-axis corresponds to a single individual with each color representing a unique cluster. The individual marked with an asterisk (*) is the single sample from Sunshine Coast; samples to the left are from Vancouver Island and samples to the right are from Oregon. The y-axis displays probability of each individual belonging to a given cluster.

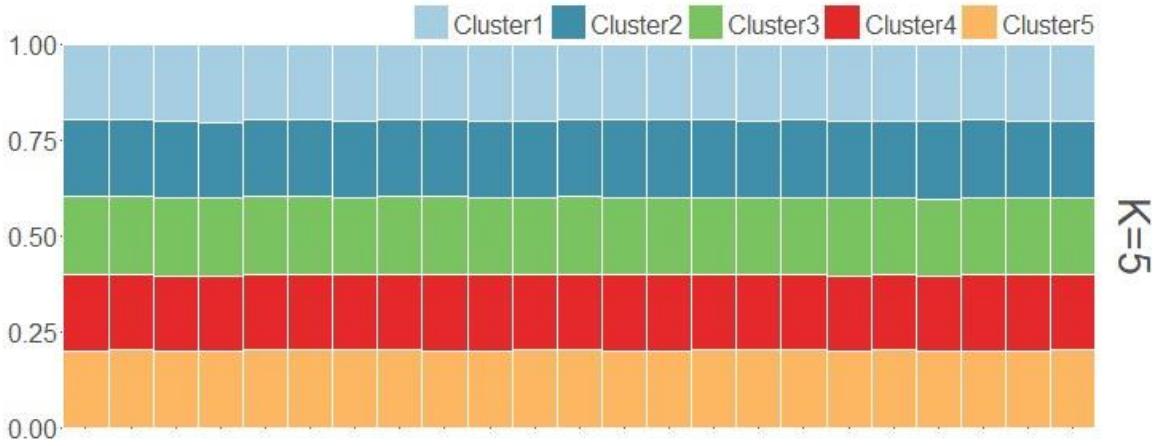

Figure 5.5: STRUCTURE plots for K=5 of 23 rough-skinned newt samples from Vancouver Island. Each vertial line along the x-axis corresponds to a single individual with each color representing a unique cluster. The y-axis displays probability of each individual belonging to a given cluster.

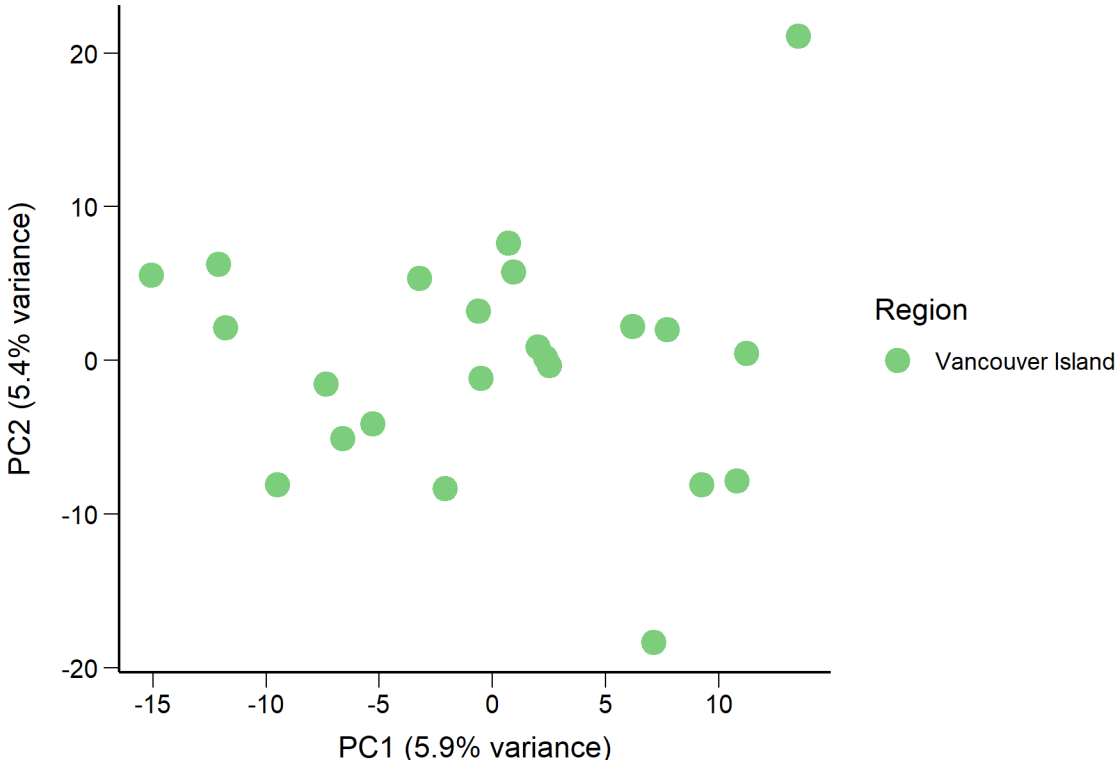

Figure 5.6: Principal Components Analysis of 41,240 SNPs from 23 samples on Vancouver Island. Principal Component 1 (PC1) represents 5.9% of the genetic variance among individuals and Principal Component 2 (PC2) represents 5.4% of the variance among individuals.

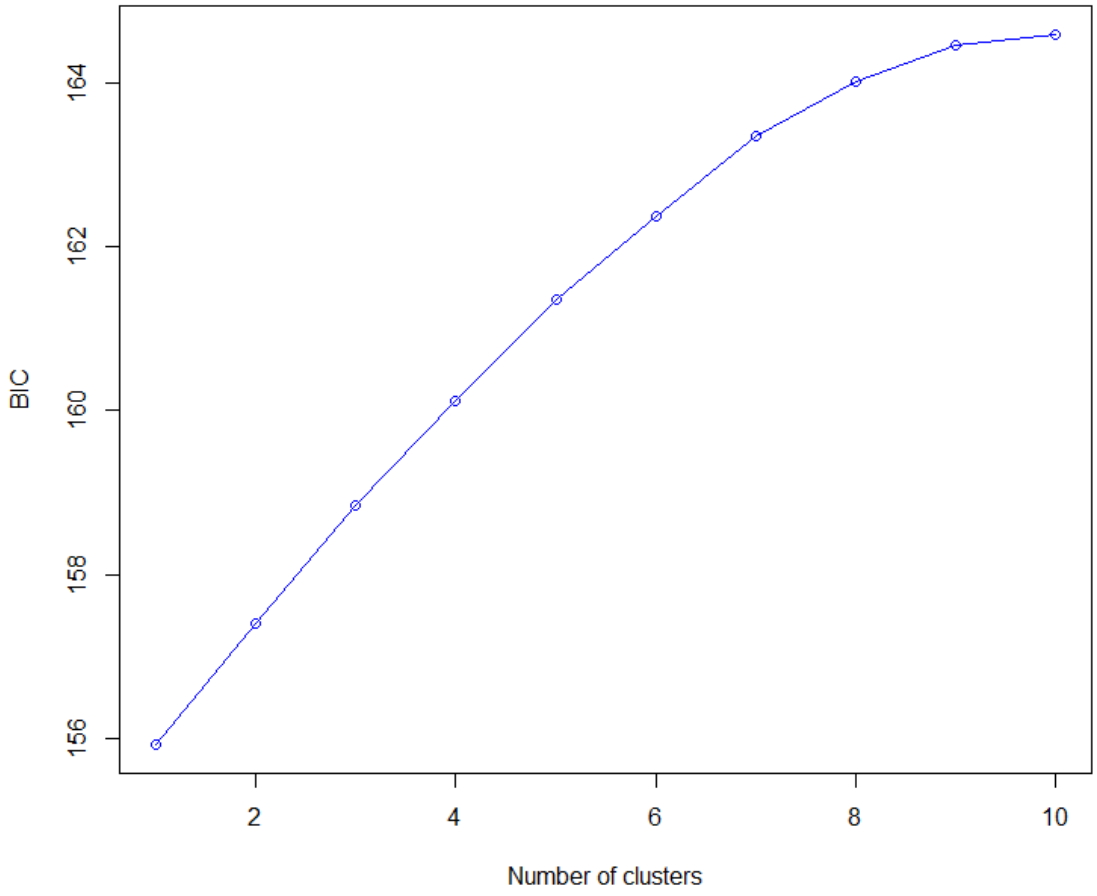

Figure 5.7: BIC values from K-means clustering of K=1 to 10 of 41,240 SNPs in 23 samples from Vancouver Island, BC. K=1 corresponds to the lowest BIC score of 155.94, indicating one genetic cluster in the data. Figure generated from adegenet v2.1.2 (Jombart et al. 2010).

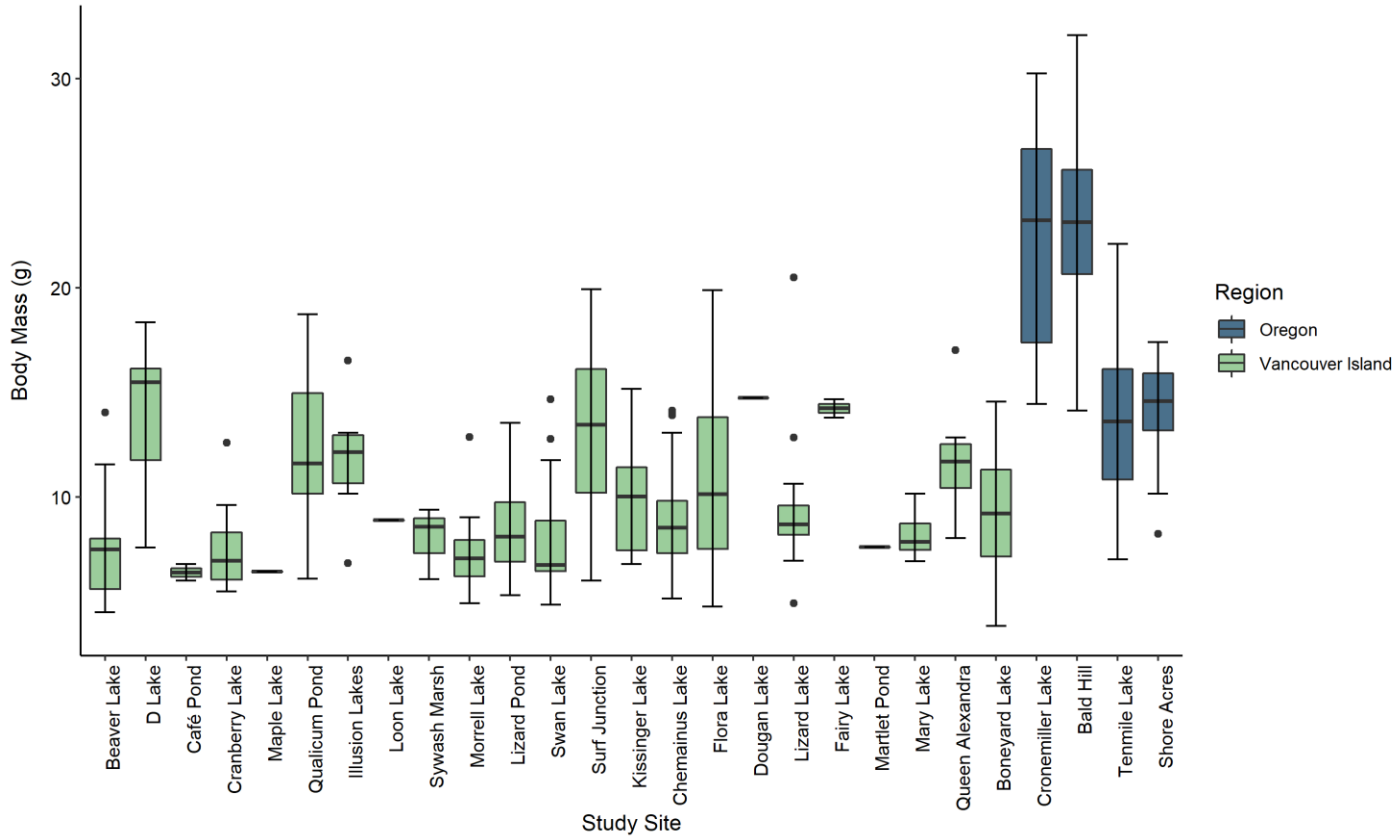

Figure 5.8: Boxplots showing variation in rough-skinned newt body mass in grams by region, ordered north to south by latitude. Whiskers represent the minimum and maximum values, colored boxes show the interquartile range with thick horizontal line for the mean, and black points are outliers. Study sites displaying a single horizontal line had only one sample, which is therefore displayed as the mean.

Tables

Table 5.1: Genetic diversity statistics calculated for the two identified clusters. All samples from British Columbia (N=24) and all samples from Oregon (N=8) were clustered separately. Observed heterozygosity (H_O), expected heterozygosity (H_S), and inbreeding coefficients (F_{IS}) were calculated for each cluster.

Cluster	N	Region	H_O	H_S	F_{IS}
1	24	British Columbia	0.4516	0.3009	-0.5007
2	8	Oregon	0.7692	0.4567	-0.6842

CHAPTER SIX – GENERAL CONCLUSIONS

6.1 Findings and Implications

In this thesis, I set out to address several aspects of the chemical ecology and genetics of rough-skinned newts (*Taricha granulosa*). This work was motivated by an interest in the spatial distribution of newt chemical defense, whether this defense is genetically regulated, and how these factors play out in an island system that represents a large part of the species range and is faced with conservation concerns. By analyzing variation in tetrodotoxin concentration within and among populations of newts on Vancouver Island, I was able to identify the southern region of the island as a hotspot of toxicity, a finding inconsistent with previous hypotheses suggesting uniformly low toxicity on the island (Brodie & Brodie 1991, Hanifin et al. 2008). Next, I utilized a transcriptomics approach to identify novel differentially expressed genes putatively related to endogenous sources of TTX. Potential impacts of non-native signal crayfish and American bullfrogs on Vancouver Island were then reviewed, with findings emphasizing the difficulty of capturing newts in sites with confirmed presence of non-native species despite no significant impacts on newt body condition or toxicity. Finally, the population genetic structure of rough-skinned newts revealed Vancouver Island populations belong to a genetically distinct cluster with low genetic diversity, which may inform future conservation strategies, particularly in the face of non-native species threats. While each chapter represents a robust, novel contribution to knowledge of the chemical ecology, genetics, and conservation of rough-skinned newts, the implications are strongest when integrating the findings with one another.

The interactions between rough-skinned newts and their main predators, garter snakes, were long believed to be a classic example of a coevolutionary arms race (e.g.,

Brodie & Brodie 1990, Brodie et al. 2005). This thesis provides further evidence that there may be additional external factors at play. In Chapter 2, I characterized variation in toxicity within and among populations of newts on Vancouver Island and found that fine-scale spatial variation is an important characteristic of the region, but the patterns are inconsistent with a coevolutionary explanation. Other external selection pressures on the island including the presence of non-native species (Chapter 4), deforestation of old growth forests (Gilani & Innes 2020), and habitat fragmentation (Vellend et al. 2008) may contribute to variation in newt toxicity. The ability to quantify the magnitude of influence that garter snakes have on rough-skinned newt toxicity is confounded in such multidimensional systems with various biotic and abiotic factors at play. Patterns of newt toxicity alone do not unequivocally provide evidence for reciprocal coevolution in response to garter snake predation, and this thesis contributes to the understanding that the chemical ecology of rough-skinned newts is more nuanced than once believed.

This thesis also highlights the promise of genetics in elucidating the molecular basis of adaptive phenotypes in non-model organisms such as rough-skinned newts. Genetic resources for *T. granulosa* were previously sparse, and the use of a transcriptomics approach in my thesis made it possible to (i) characterize differentially expressed genes potentially associated with chemical defense in rough-skinned newts (Chapter 3) and (ii) identify SNPs to study the population genetic structure of newts (Chapter 5). The ability to connect phenotype to genotype will provide insight to the source and maintenance of chemical defense in newts and improve our understanding of adaptive evolutionary processes (Stapley et al. 2010, Savolainen et al. 2013, Whitlock 2015). However, there does not appear to be a relationship between phenotypic variation

in newt toxicity and genetic structure on Vancouver Island, indicating that phenotypic plasticity regulated by differences in gene expression is another plausible explanation for the observed variation. Consideration of genetics will also be vital in informing effective conservation management plans by enabling the study of population structure, barriers to gene flow, and patterns of historic dispersal (Manel et al. 2003, Frankham et al. 2005, Allendorf et al. 2010, Shafer et al. 2015). As next generation sequencing and analysis techniques continue to advance and become more feasible (Mardis 2008, da Fonseca et al. 2016), we will continue to uncover insights into the molecular basis of newt toxicity.

Furthermore, my work emphasizes the importance of assessing the conservation status of rough-skinned newts, particularly on Vancouver Island. Amphibian populations around the globe are rapidly declining, and non-native species invasions represent a global threat to biodiversity (Stuart et al. 2005, Bucciarelli et al. 2014). While the findings in Chapter 4 regarding the impacts of crayfish and bullfrogs on newts should be viewed as preliminary, the potential for additional significant impacts to occur in the future is worrisome. By identifying Vancouver Island as a genetically distinct group of rough-skinned newts with low genetic diversity (Chapter 5), this further suggests that newts in the region warrant special protection because local extirpation of unique genetic units may represent a significant overall loss to genetic diversity in the species (Frankham 2005). To date, rough-skinned newts in Canada have not yet been assessed by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC 2020). However, it will be crucial to develop conservation approaches at appropriate spatial and temporal scales to prevent further declines of Canadian herpetofauna (Lesbarrères et al. 2014).

6.2 Future Directions

While this thesis has greatly improved our understanding of the chemical ecology, genetics, and conservation concerns of rough-skinned newts, there remain many additional research avenues to be explored. Ultimately, this thesis sets the foundation for future studies to build upon, and I believe that each chapter presented here could subsequently evolve into separate, standalone theses.

Although studies of wild populations are critical in establishing patterns of baseline variation in phenotypic traits, I would first recommend that future work focuses on disentangling the possible factors contributing to variation and potential heritability of rough-skinned newt toxicity by performing long-term, laboratory-controlled experiments in captive bred individuals. There is also more work to be done on investigating potential match or mismatch in newt toxicity and garter snake resistance of Vancouver Island and on studying the magnitude of selection pressure snakes may exert on newts such as through a diet analysis of garter snakes. While *Taricha granulosa* has long been a model species for studies on the chemical ecology of tetrodotoxin, incorporating other TTX-bearing Salamandrids into future studies will help clarify the possible ecological roles of chemical defense.

To further validate genes related to endogenous sources of tetrodotoxin and the potential role of phenotypic plasticity, differential expression of the candidate genes identified in Chapter 3 could be compared (i) among different individuals with known TTX levels (using RNA-seq data already available from Chapter 5), (ii) across different tissue types within an individual, (iii) among closely related salamander species also known to produce TTX, and (iv) in relation to differing skin microbiomes on newts.

Moreover, further studies are needed on the potential role of endosymbiotic bacteria in TTX production in newts and on how newts may sequester the toxin from bacteria in their granular skin glands.

Additional investigations into the population genetic structure of rough-skinned newts could compare populations from the mainland, Vancouver Island, other coastal islands, and the Mazama newt subspecies. This would also provide the opportunity to reassess the correlation between newt toxicity with neutral genetic divergence, considering previous discrepancies in identifying such relationships (Hague et al. 2016, Hague et al. 2019).

Ultimately, the ability to study rough-skinned newt chemical ecology and genetics as described above will be hindered by population declines if conservation efforts are not undertaken. Close monitoring of native newt populations and of the spread of non-native crayfish and bullfrogs across Vancouver Island should be prioritized to gather more information about their ecological impacts. Future conservation goals may also include management plans at a smaller scale than the entire species level, and assessment by COSEWIC may be necessary to preserve populations of rough-skinned newts in BC facing unique conservation concerns.

To conclude, my work has contributed to our overall understanding of the spatial variation and genetics of chemical defense in rough-skinned newts while integrating these findings with conservation implications for Vancouver Island populations, ultimately laying the foundation upon which future studies may emerge.

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APPENDIX A – SUMMARY INFORMATION OF BRITISH COLUMBIA AND OREGON ROUGH-SKINNED NEWT POPULATIONS

Table A.1: Metadata for 31 study sites sampled in Vancouver Island, Sunshine Coast, and Oregon. Sites are grouped by region and ordered north to south by latitude. Sites listed as "None detected" for non-native species indicate that I did not find evidence of crayfish or bullfrog presence during my sampling efforts and did not find any mention of their presence in published literature. Measures of mean tetrodotoxin concentration (TTX) and scaled mass index (SMI) are reported as the mean of all individuals sampled (N) at a given study site. Sampling efficiency scores were assigned based on the amount of effort needed to capture newts as a proxy for relative abundance on a scale from 1 representing the least effort to 4 being the most. (*Lakes with N=0 were only used for the sampling efficiency analysis in Chapter 5).

Region	Study Site	Coordinates (Latitude, Longitude)	N	Non-Native Species	Mean TTX ($\mu g/cm^2$)	Mean SMI	Sampling Efficiency Score
Vancouver Island	Beaver Lake	50.6011, -127.3098	15	None detected	7.55	7.42	2
	D Lake	50.5169, -127.4447	15	None detected	3.41	14.07	1
	Café Pond	50.1661, -125.4687	2	American bullfrog, *Lithobates catesbeianus*	4.18	6.38	4
	Cranberry Lake	50.08791, -125.453446	12	None detected	4.79	7.38	2

Maple Lake	49.637375, -125.01771	1	Signal crayfish, *Pacifastacus leniusculus*	11.52	6.42	4
Qualicum Pond	49.3581, -124.477051	15	None detected	8.72	11.90	1
Illusion Lakes	49.352663, -124.635176	11	None detected	9.48	11.88	3
Enos Lake	49.278962, -124.155700	0*	Signal crayfish, *Pacifastacus leniusculus*	N/A	N/A	4
Loon Lake	49.25226, -124.70078	1	None detected	8.00	8.90	4
Sywash Marsh	49.241169, -124.113241	3	American bullfrog, *Lithobates catesbeianus*	13.82	9.01	4
Morrell Lake	49.1527, -123.9858	15	None detected	9.65	6.91	2
Lizard Pond	49.065136, -124.780785	18	None detected	14.89	8.51	1
Swan Lake	48.999816, -125.595659	15	None detected	3.18	7.70	2
Surf Junction	48.9871, -125.586	15	None detected	13.67	13.21	2
Kissinger Lake	48.919488, -124.48019	15	None detected	7.91	9.76	1
Chemainus Lake	48.9135, -123.7512	17	American bullfrog, *Lithobates catesbeianus*	8.72	8.64	2
Flora Lake	48.864238, -124.72323	15	None detected	4.83	10.45	1
Mesachie Lake	48.812653, -124.11037	0*	Signal crayfish, *Pacifastacus leniusculus*	N/A	N/A	4

	Dougan Lake	48.714573, -123.61364	1	American bullfrog, *Lithobates catesbeianus;* Signal crayfish, *Pacifastacus leniusculus*	18.96	14.73	4
	Lizard Lake	48.607, -124.225	15	None detected	11.49	9.27	1
	Fairy Lake	48.585, -124.3499	2	None detected	11.46	14.23	3
	Martlet Pond	48.526385, -123.5188	1	American bullfrog, *Lithobates catesbeianus*	27.59	7.60	4
	Elk/Beaver Lake	48.508915, -123.393426	0*	Signal crayfish, *Pacifastacus leniusculus*	N/A	N/A	4
	Mary Lake	48.49984, -123.519137	9	American bullfrog, *Lithobates catesbeianus*	21.69	8.23	3
	Queen Alexandra	48.4717, -123.2972	15	None detected	24.93	11.51	1
	Boneyard Lake	48.483106, -123.741691	6	None detected	14.18	9.65	2
Sunshine Coast	Trout Lake	49.507832, -123.876205	13	None detected	10.35	8.26	2
Oregon	Cronemiller Lake	44.663874, -123.23913	15	Rusty crayfish, *Procambarus clarkii*	33.59	21.90	1
	Bald Hill	44.575794, -123.326539	15	None detected	23.70	23.27	1

Tenmile Lake	43.573123, -124.147535	15	Rusty crayfish, *Procambarus clarkii*	26.51	13.24	1
Shore Acres	43.322015, -124.385656	15	None detected	41.97	14.12	1

APPENDIX B – CONSIDERATIONS OF METHODS USED TO REPORT TETRODOTOXIN CONCENTRATION IN ROUGH-SKINNED NEWTS

At present there is currently no standardized method for reporting units of tetrodotoxin concentration in rough-skinned newts. When quantifying TTX extracted from the dorsal skin of rough-skinned newts, the first value obtained is the concentration of the extract solution itself, in μg TTX per mL of solution. Next, this value is converted to an estimate of TTX over the surface area of the skin based on the diameter of the biopsy punch used, in μg TTX per cm^2 of skin. This estimate is appropriate to use when comparing data between studies using different biopsy punches. For instance, I used a 2 mm diameter biopsy punch to collect tissue samples from newts, and another study I compare my data to in Chapter 2 used a 5 mm biopsy punch (Hague et al. 2019).

A further calculation would be to estimate the amount of TTX present in the newt's entire skin, based on the skin surface area and body mass of an individual (Hanifin et al. 2004). This value is useful when considering the potential effects of newt toxicity on predators such as garter snakes who would consume them whole and ingest all TTX contained in the newt's body. However, the estimate of whole newt toxicity relies on questionable assumptions about the scaling effects of mass. The formula assumes that newt mass is proportional to the surface area of each newt according to:

$$\log S = 0.945 + 0.719 \log W$$

where S is the surface area in cm^2 and W is the mass in grams (Whitford & Hutchison 1967). To obtain the estimate of whole newt toxicity in mg, Hanifin et al. (2004) derived the formula:

total skin TTX = (0.3(S))(dorsal TTX) + (0.45(S))(0.208(dorsal TTX)) + (0.25(S))(0.502(dorsal TTX))

where S is the previously estimated skin surface area in cm^2 and dorsal TTX is the concentration in mg/cm^2.

Within my study, there is substantial variation in the body condition of newts sampled. For example, there were two newts both with a snout-vent length (SVL) of 70 mm (Fig. B.1). One of them had a mass of approximately 10 grams (red circle), and the other was approximately 30 grams (yellow circle). This shows newts of the same SVL may vary in body condition with masses that can be nearly triple the lowest mass for that given length. If these values were used in the formulas above assuming they both had a dorsal TTX concentration of 1, the 10 gram newt is estimated to have 23.95 mg TTX while the 30 gram newt would have 52.76 grams. Clearly variation in body condition leads to significantly different estimates of TTX concentration, indicating mass is not an accurate predictor in the estimation of whole newt toxicity. Using this estimate may be appropriate for comparing individuals within lakes who show similar body condition measurements, but it should be excluded from comparisons between sites displaying significant variation in newt body condition. Because of this, I have chosen to only use newt toxicity in the units of $\mu g/cm^2$ for my study.

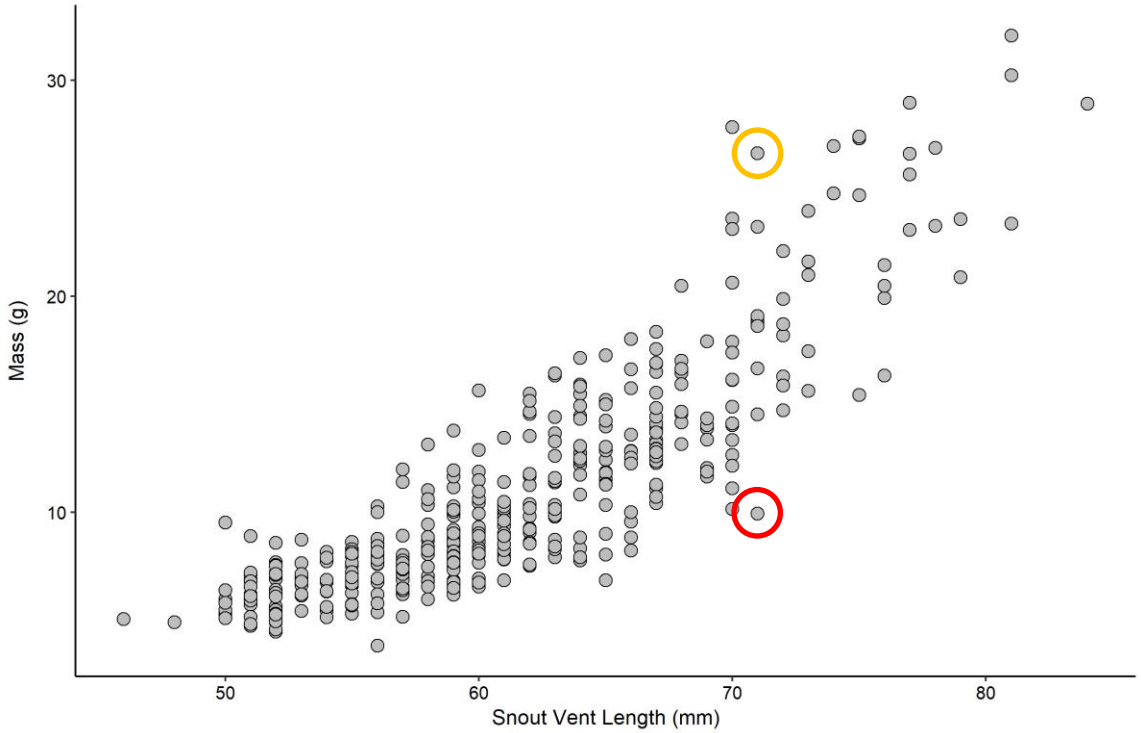

Figure B.1: Scatter plot of Snout Vent Length (SVL) in millimeters versus Mass in grams of 308 rough skinned newts collected from Vancouver Island, BC, Sunshine Coast, BC, and Oregon, USA. Yellow and red circles show an example of two different individuals with the same SVL, but the yellow circled individual has nearly triple the mass of the red.

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APPENDIX C – COMPARISON OF COLOR SCALE FOR IDW INTERPOLATION OF NEWT TOXICITY ON VANCOUVER ISLAND

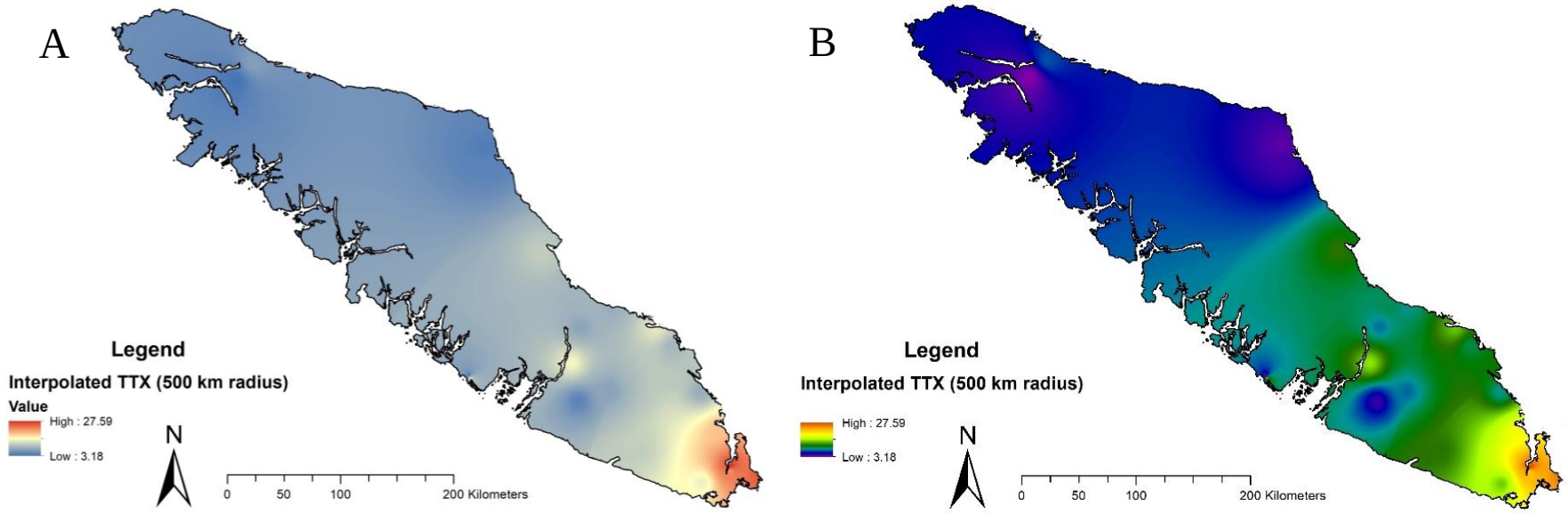

Figure C.1: Inverse distance-weighted (IDW) interpolation of newt toxicity in µg/cm^2 using a 500 km neighborhood search radius with continuous color classifications. Interpolated toxicity is displayed using (A) diverging color ramp chosen for use in Chapter 2 of this thesis, and (B) rainbow color scale used by Hanifin et al. (2008).

APPENDIX D – RNA EXTRACTION PROTOCOL

This is a protocol based on the RNeasy Micro Extraction Kit (Qiagen, USA), modified for the purpose of extracting RNA from skin biopsy tissue samples from rough-skinned newts.

Pre-Procedure

- Follow Biological Safety Cabinet Preparation protocol to ensure decontaminated work space.
- Prepare solutions in RNeasy kit by adding the appropriate amounts of ethanol and 2-mercaptoethanol, following the manufacturers protocol.
- Prepare the RNase-free DNase Set following the manufacturers protocol.
- Wear lab coat with cuffed sleeves, goggles, face mask and long gloves throughout the whole procedure.
- Ensure the centrifuge is set between 20-25°C.
- Bleach and acids are NOT compatible with materials used in this protocol.
- Set up BSC/clean room work station with the following:

❑ **Qiagen RNeasy micro kit**	❑ Samples	❑ Microcentrifuge
❑ *Buffer RLT*	❑ QIAshredder columns	❑ Biological Safety Cabinet
❑ *Buffer RW1*	❑ Sterile 1.5 ml microcentrifuge tubes	❑ Pipettes (1000, 200, and 20 uL)
❑ *Buffer RPE*	❑ Filter tips (1000, 200, and 20 uL)	❑ Container for liquid waste
❑ *RNase free water*	❑ Sterile tweezers	❑ Container for solid waste
❑ *RNeasy columns*	❑ Sterile glass or plastic pestles	❑ Racks for 1.5/2.0 mL tubes
❑ *Sterile 2 ml collection tubes*	❑ **Qiagen RNA-free DNase Set**	❑ Balances for centrifuge
❑ Absolute ethanol	❑ *DNase I Stock Solution*	❑ Timer
❑ 2-mercaptoethanol	❑ *Buffer RDD*	

❑ 70% ethanol	❑ Needle and syringe (to fit 550 uL)	

Note: Italicized materials come with a kit (bolded) and do not need to be purchased separately.

Procedure

1. Remove tissue sample from tube using sterile tweezers and place in 1.5 mL micro tube.
2. Add 300 uL of Buffer RLT to the tube and thoroughly grind the tissue with a sterile glass or plastic pestle. It is ok if the tissue is not able to fully dissolve.
3. After grinding, add another 300 uL of buffer RLT then pipette the lysate including the buffer and ground tissue from step 2 into a QIAshredder spin column placed in a 2mL collection tube.
4. Centrifuge the QIAshredder column for 5 min at full speed. Remove the flow through and transfer it to a new 1.5 mL micro tube. Discard the QIAshredder column.
5. Add 600uL of 70% ethanol to the lysate and mix immediately by pipetting. Do not centrifuge.
6. Transfer up to 700uL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2mL collection tube. Close the lid gently and centrifuge for 15s at 8000g. Discard the flow through using a pipette and reuse the collection tube for the following steps.
7. Repeat step 6 using the same spin column for the remaining sample.

8. Add 350uL of Buffer RW1 to the RNeasy spin column. Close the lid gently and centrifuge for 15s at 8000g. Discard the flow through with a pipette and use the collection tube for the following step.
9. In a separate tube, add 10 uL DNase I Stock Solution to 70 uL Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
10. Carefully add the 80 uL mix from step 9 directly to the RNeasy spin column membrane, and place on the benchtop (20–30°C) for 15 min.
11. Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at 8000g Discard the flow-through with a pipette and use the collection tube for the following step.
12. Add 500uL Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 15s at 8000g. Discard the flow through with a pipette and use the collection tube for the following step.
13. Add 500uL Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 2 min at 8000g.
14. Place the RNeasy spin column in a new 2mL collection tube and discard the old collection tube and flow-through. Close the lid gently and centrifuge at full speed for 1 min.
15. Place the RNeasy spin column in a new 1.5mL collection tube. Carefully add 25-30uL RNase-free water directly to the spin column membrane (not the walls of the tube) and incubate at room temperature for 5 min. Close the lid gently and centrifuge for 1 min at 8000g.

16. Remove eluted water with a pipette and place back on spin column. Centrifuge again for 1 min at 8000g. This is the RNA elute. Immediately Qubit the extracted RNA or store at -80C until further analyses.

APPENDIX E – GENE ONTOLOGY TERMS ENRICHED IN DIFFERENTIALLY EXPRESSED GENES

Table E.1: 109 Gene Ontology terms enriched in the differentially expressed genes between dorsal and ventral dermal tissue samples from the rough-skinned newt.

GO ID	GO Term	GO Category	p-value
GO:0033162	Melanosome membrane	CC	0
GO:0042438	Melanin biosynthetic process	BP	6.76E-12
GO:0006582	Melanin metabolic process	BP	1.41E-11
GO:0044550	Secondary metabolite biosynthetic process	BP	3.15E-11
GO:0046189	Phenol-containing compound biosynthetic process	BP	9.56E-11
GO:0019748	Secondary metabolic process	BP	6.94E-10
GO:0018958	Phenol-containing compound metabolic process	BP	3.08E-09
GO:0046148	Pigment biosynthetic process	BP	5.05E-09
GO:0042440	Pigment metabolic process	BP	1.81E-08
GO:1901617	Organic hydroxy compound biosynthetic process	BP	1.67E-07
GO:0016716	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, another compound as one donor, and incorporation of one atom of oxygen	MF	2.18E-07
GO:0034493	Melanosome lumen	CC	8.66E-07
GO:0044433	Cytoplasmic vesicle part	CC	9.57E-07
GO:0030659	Cytoplasmic vesicle membrane	CC	1.21E-06
GO:0012506	Vesicle membrane	CC	1.64E-06
GO:0010917	Negative regulation of mitochondrial membrane potential	BP	1.06E-05
GO:1901615	Organic hydroxy compound metabolic process	BP	1.45E-05
GO:0042470	Melanosome	CC	1.55E-05

GO:0048770	Pigment granule	CC	1.55E-05
GO:0045837	Negative regulation of membrane potential	BP	2.44E-05
GO:0010940	Positive regulation of necrotic cell death	BP	2.53E-05
GO:0035794	Positive regulation of mitochondrial membrane permeability	BP	6.81E-05
GO:1905710	Positive regulation of membrane permeability	BP	8.19E-05
GO:0060205	Cytoplasmic vesicle lumen	CC	1.39E-04
GO:0031983	Vesicle lumen	CC	1.73E-04
GO:0016021	Integral component of membrane	CC	1.75E-04
GO:0031224	Intrinsic component of membrane	CC	2.18E-04
GO:0098805	Whole membrane	CC	3.45E-04
GO:0004497	Monooxygenase activity	MF	3.63E-04
GO:0010939	Regulation of necrotic cell death	BP	5.27E-04
GO:0005302	L-tyrosine transmembrane transporter activity	MF	7.15E-04
GO:0015828	Tyrosine transport	BP	7.15E-04
GO:0044711	NA	NA	7.22E-04
GO:0051881	Regulation of mitochondrial membrane potential	BP	1.12E-03
GO:0046902	Regulation of mitochondrial membrane permeability	BP	1.16E-03
GO:0006716	Juvenile hormone metabolic process	BP	1.18E-03
GO:0006718	Juvenile hormone biosynthetic process	BP	1.18E-03
GO:0016106	Sesquiterpenoid biosynthetic process	BP	1.18E-03
GO:0047886	Farnesol dehydrogenase activity	MF	1.18E-03
GO:0016705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	MF	1.18E-03
GO:0005502	11-cis retinal binding	MF	1.21E-03
GO:0005507	Copper ion binding	MF	1.49E-03
GO:0090559	Regulation of membrane permeability	BP	1.49E-03
GO:0004578	Chitobiosyldiphosphodolichol beta-mannosyltransferase activity	MF	1.89E-03

GO:0019187	Beta-1,4-mannosyltransferase activity	MF	1.89E-03
GO:0006726	Eye pigment biosynthetic process	BP	1.91E-03
GO:0042441	Eye pigment metabolic process	BP	1.91E-03
GO:0043324	Pigment metabolic process involved in developmental pigmentation	BP	1.91E-03
GO:0043474	Pigment metabolic process involved in pigmentation	BP	1.91E-03
GO:0015173	Aromatic amino acid transmembrane transporter activity	MF	1.92E-03
GO:0004167	Dopachrome isomerase activity	MF	2.21E-03
GO:0030246	Carbohydrate binding	MF	2.61E-03
GO:0007006	Mitochondrial membrane organization	BP	2.83E-03
GO:0016491	Oxidoreductase activity	MF	3.01E-03
GO:0009758	Carbohydrate utilization	BP	3.05E-03
GO:0050053	Levansucrase activity	MF	3.05E-03
GO:0044425	Membrane part	CC	3.64E-03
GO:0098588	Bounding membrane of organelle	CC	3.76E-03
GO:0006714	Sesquiterpenoid metabolic process	BP	4.25E-03
GO:0009881	Photoreceptor activity	MF	4.32E-03
GO:0015801	Aromatic amino acid transport	BP	4.56E-03
GO:0016918	Retinal binding	MF	5.98E-03
GO:0005576	Extracellular region	CC	6.64E-03
GO:0016114	Terpenoid biosynthetic process	BP	7.38E-03
GO:0004503	Monophenol monooxygenase activity	MF	8.27E-03
GO:0018298	Protein-chromophore linkage	BP	8.61E-03
GO:0005501	Retinoid binding	MF	1.19E-02
GO:0019840	Isoprenoid binding	MF	1.31E-02
GO:0003857	3-hydroxyacyl-CoA dehydrogenase activity	MF	1.33E-02
GO:0005922	Connexin complex	CC	1.35E-02
GO:0016758	Transferase activity, transferring hexosyl groups	MF	1.37E-02

GO:0006855	Drug transmembrane transport	BP	1.43E-02
GO:0045087	Innate immune response	BP	1.45E-02
GO:0042391	Regulation of membrane potential	BP	1.57E-02
GO:0016863	Intramolecular oxidoreductase activity, transposing C=C bonds	MF	1.60E-02
GO:0033018	Sarcoplasmic reticulum lumen	CC	1.62E-02
GO:0015893	Drug transport	BP	1.78E-02
GO:0015238	Drug transmembrane transporter activity	MF	1.78E-02
GO:0031410	Cytoplasmic vesicle	CC	1.83E-02
GO:0005618	Cell wall	CC	1.86E-02
GO:0044444	Cytoplasmic part	CC	2.02E-02
GO:0090484	NA	NA	2.02E-02
GO:0044710	NA	NA	2.04E-02
GO:0004790	Thioether S-methyltransferase activity	MF	2.07E-02
GO:0030748	Amine N-methyltransferase activity	MF	2.07E-02
GO:0000030	Mannosyltransferase activity	MF	2.09E-02
GO:0030318	Melanocyte differentiation	BP	2.11E-02
GO:0050931	Pigment cell differentiation	BP	2.11E-02
GO:0030312	External encapsulating structure	CC	2.17E-02
GO:0097708	Intracellular vesicle	CC	2.33E-02
GO:0031974	Membrane-enclosed lumen	CC	2.33E-02
GO:0043233	Organelle lumen	CC	2.33E-02
GO:0070013	Intracellular organelle lumen	CC	2.33E-02
GO:0007602	Phototransduction	BP	2.64E-02
GO:0008172	S-methyltransferase activity	MF	2.65E-02
GO:0009583	Detection of light stimulus	BP	2.71E-02
GO:0031090	Organelle membrane	CC	2.74E-02
GO:0008299	Isoprenoid biosynthetic process	BP	2.79E-02

GO:0042446	Hormone biosynthetic process	BP	2.81E-02
GO:0007286	Spermatid development	BP	2.89E-02
GO:0044432	Endoplasmic reticulum part	CC	2.90E-02
GO:0016757	Transferase activity, transferring glycosyl groups	MF	2.91E-02
GO:0007005	Mitochondrion organization	BP	2.94E-02
GO:0055085	Transmembrane transport	BP	3.53E-02
GO:0006955	Immune response	BP	3.73E-02
GO:0006820	Anion transport	BP	3.81E-02
GO:0015179	L-amino acid transmembrane transporter activity	MF	4.08E-02
GO:0015297	Antiporter activity	MF	4.66E-02
GO:0006721	Terpenoid metabolic process	BP	4.95E-02

APPENDIX F – UNANNOTATED DIFFERENTIALLY EXPRESSED GENES

Table F.1: 37 differentially expressed genes between dorsal and ventral tissue in rough-skinned newts which were unannotated and subsequently blasted to the *nr* protein database. The top blastx hit for each gene is reported, along with the corresponding query cover, e-value, and percent identity.

Trinity Gene	Top blastx hit	Query Cover	E-value	Percent Identity
TRINITY_DN1888_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN24409_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN194047_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN43043_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN13975_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN171534_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN209977_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN39325_c0_g3	No significant similarity found	N/A	N/A	N/A
TRINITY_DN92039_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN208925_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN78533_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN17773_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN23458_c0_g1	No significant similarity found	N/A	N/A	N/A

TRINITY_DN13932_c1_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN65251_c0_g1	No significant similarity found	N/A	N/A	N/A
TRINITY_DN166711_c0_g1	nuclear protein 1 [*Xenopus tropicalis*]	60%	1E-9	79.07%
TRINITY_DN243443_c0_g1	melanoma antigen recognized by T-cells 1 isoform X2 [*Rhinatrema bivittatum*]	86%	3E-8	58.46%
TRINITY_DN9962_c0_g1	hypothetical protein [*Paenibacillus sinopodophylli*]	29%	2.2	34%
TRINITY_DN8986_c0_g1	nicotinamide N-methyltransferase-like [*Pelodiscus sinensis*]	14%	4E-23	39.58%
TRINITY_DN24278_c0_g1	PREDICTED: prostate stem cell antigen-like [*Latimeria chalumnae*]	10%	3E-24	55.81%
TRINITY_DN223485_c0_g1	hypothetical protein [*Euryarchaeota archaeon*]	68%	2E-3	41.07%
TRINITY_DN179760_c0_g1	zinc finger protein 280D isoform X2 [*Rhinatrema bivittatum*]	87%	2E-18	64.86%
TRINITY_DN8609_c0_g1	cartilage acidic protein 1 isoform X1 [*Rhinatrema bivittatum*]	81%	0	84.84%
TRINITY_DN1668_c0_g1	CFBD-1 beta-defensin precursor [*Cynops fudingensis*]	23%	9E-22	89.58%
TRINITY_DN821_c2_g1	hypothetical protein DUI87_25654 [*Hirundo rustica rustica*]	32%	0.21	46.94%
TRINITY_DN260754_c0_g1	PREDICTED: uncharacterized protein LOC108786480 [*Nanorana parkeri*]	98%	6E-24	75.76%
TRINITY_DN28778_c0_g1	uncharacterized protein LOC112846917 [*Oreochromis niloticus*]	91%	1E-14	48.53%
TRINITY_DN206366_c0_g1	PREDICTED: uncharacterized protein LOC107106395 [*Gekko japonicus*]	98%	7E-9	35.37%

TRINITY_DN207_c1_g1	non-classical arabinogalactan protein 31-like [*Neophocaena asiaeorientalis asiaeorientalis*]	94%	2E-11	53.85%
TRINITY_DN4432_c0_g1	PREDICTED: uncharacterized protein LOC108786480 [*Nanorana parkeri*]	61%	2E-130	72.2%
TRINITY_DN9011_c0_g1	PREDICTED: gap junction alpha-5 protein [*Nanorana parkeri*]	26%	3E-148	70%
TRINITY_DN127866_c0_g1	S8 family serine peptidase [*Paenibacillus aquistagni*]	61%	0.92	33.96%
TRINITY_DN14606_c0_g1	elafin [*Xenopus tropicalis*]	56%	7E-6	33.62%
TRINITY_DN220374_c0_g1	aminopeptidase P family protein [*Sphingobacterium paucimobilis*]	42%	3	51.72%
TRINITY_DN207_c0_g1	Xin actin-binding repeat-containing protein 1 [*Cricetulus griseus*]	29%	4E-3	41.82%
TRINITY_DN10690_c0_g1	hypothetical protein XENTR_v10008315 [*Xenopus tropicalis*]	64%	2E-5	35.98%
TRINITY_DN138945_c0_g1	calumenin isoform X2 [*Ornithorhynchus anatinus*]	62%	1E-17	82.22%
TRINITY_DN26681_c0_g1	PREDICTED: uncharacterized protein LOC108680075 [*Hyalella azteca*]	64%	2.9	42.31%

APPENDIX G – SUPPORTING INFORMATION FOR POPULATION GENETIC STRUCTURE RESULTS

Using the results from the Bayesian clustering analysis of population structure in STRUCTURE v2.3.4 (Pritchard et al. 2000), the most likely number of genetic clusters (K) were identified in the R package pophelper v2.3.0 (Francis 2016) via the Evanno method (Evanno et al. 2005). The analysis of the complete dataset with 32 samples from Vancouver Island (N=23), Sunshine Coast (N=1), and Oregon (N=8) revealed K=2 (Fig. G.1). The Vancouver Island dataset with 23 samples was found to have K=5 clusters (Fig. G.2). In both figure G.1D and G.2D, the peak of delta K (the second order rate of change in the log probability of the data) indicates the most likely number of genetic clusters. While K=2 is well supported for the complete dataset (Fig. G.3, see also Chapter 4), K=5 for the Vancouver Island dataset appears to be an overestimation of the number of clusters (Fig. G.4, see also Chapter 4). First, by comparing the variance in the mean log probability of the data in Fig. G.1A and G.2A, there is clearly substantially higher variance with significant overlap between estimates of K for the Vancouver Island dataset. The plots of first and second order rates of change in the log probability of the data (Fig. G.2B and G.2C – intermediate steps to calculating delta K) have a similar pattern of variance. Finally, the magnitude of delta K on the y-axis in Fig. G.1D ranges from 0 to 300, while in Fig. G.2D it only goes up to 1.6, which is another indication of the uncertainty for the estimate of K=5 in the Vancouver Island dataset. Such results are possible when the true value of K is 1, as the Evanno method does not consider this possibility in the estimation based on delta K (Pritchard et al. 2000, Evanno et al. 2005)

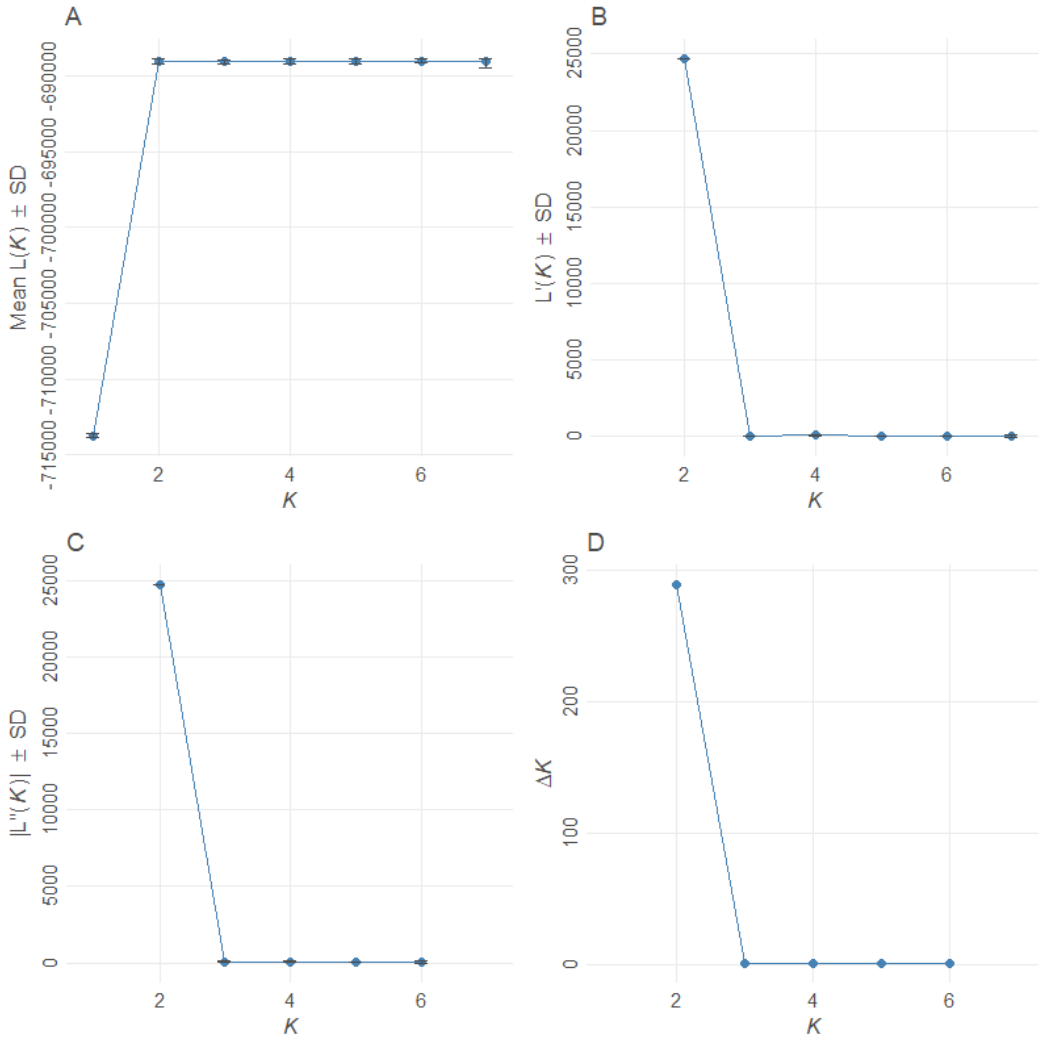

Figure G.1: Results from the Evanno method (Evanno et al. 2005) to identify the most likely K, or number of genetic clusters, from STRUCTURE results for K=1 to 7 for 32 samples from Vancouver Island, BC, Sunshine Coast, BC, and Oregon. In panel D, the peak of Delta K, the second order rate of change in the log probability of the data, supports K=2 genetic clusters.

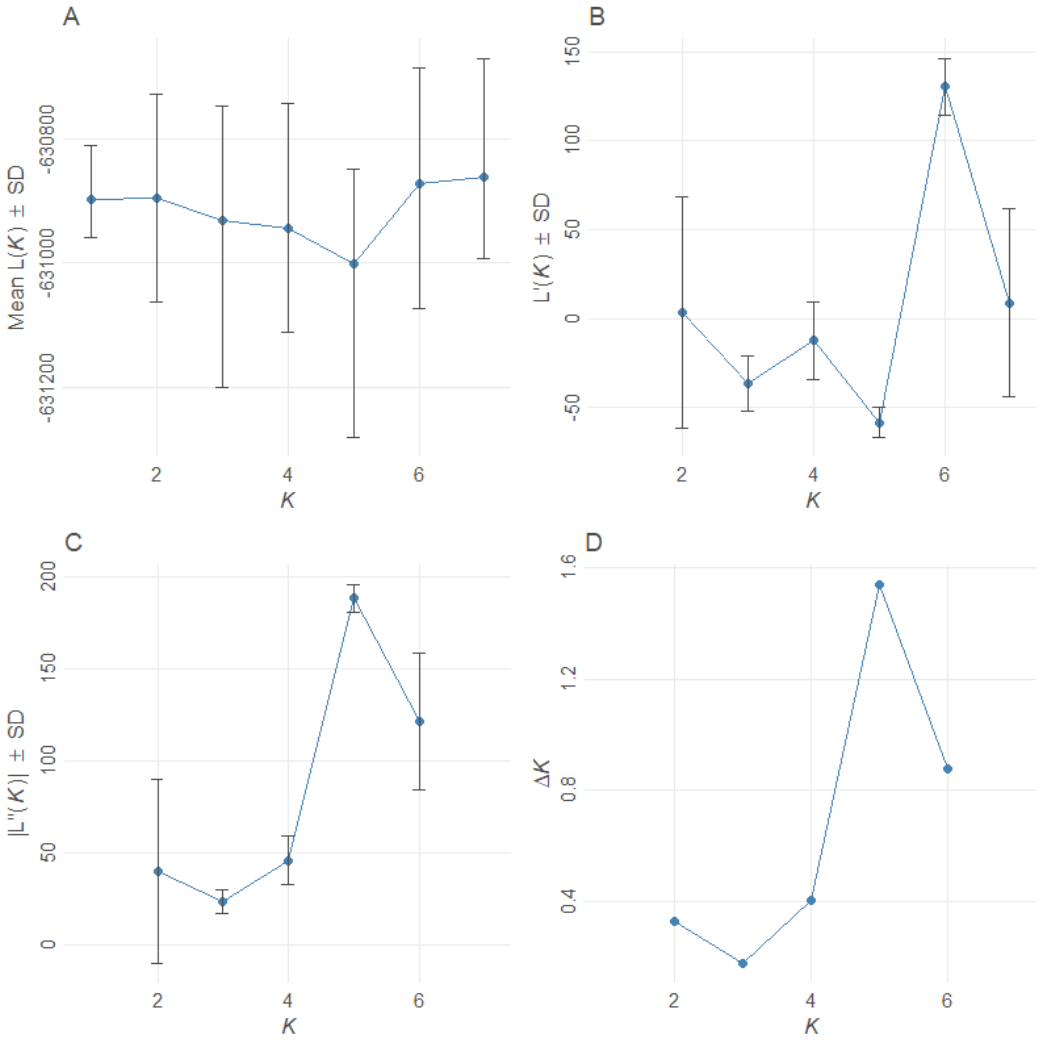

Figure G.2: Results from the Evanno method (Evanno et al. 2005) to identify the most likely K, or number of genetic clusters, from STRUCTURE results of K=1 to 7 for 23 samples from Vancouver Island, BC. In panel D, the peak of Delta K, the second order rate of change in the log probability of the data, supports K=5 genetic clusters. However, there is more support that the true value of K for this dataset is K=1.

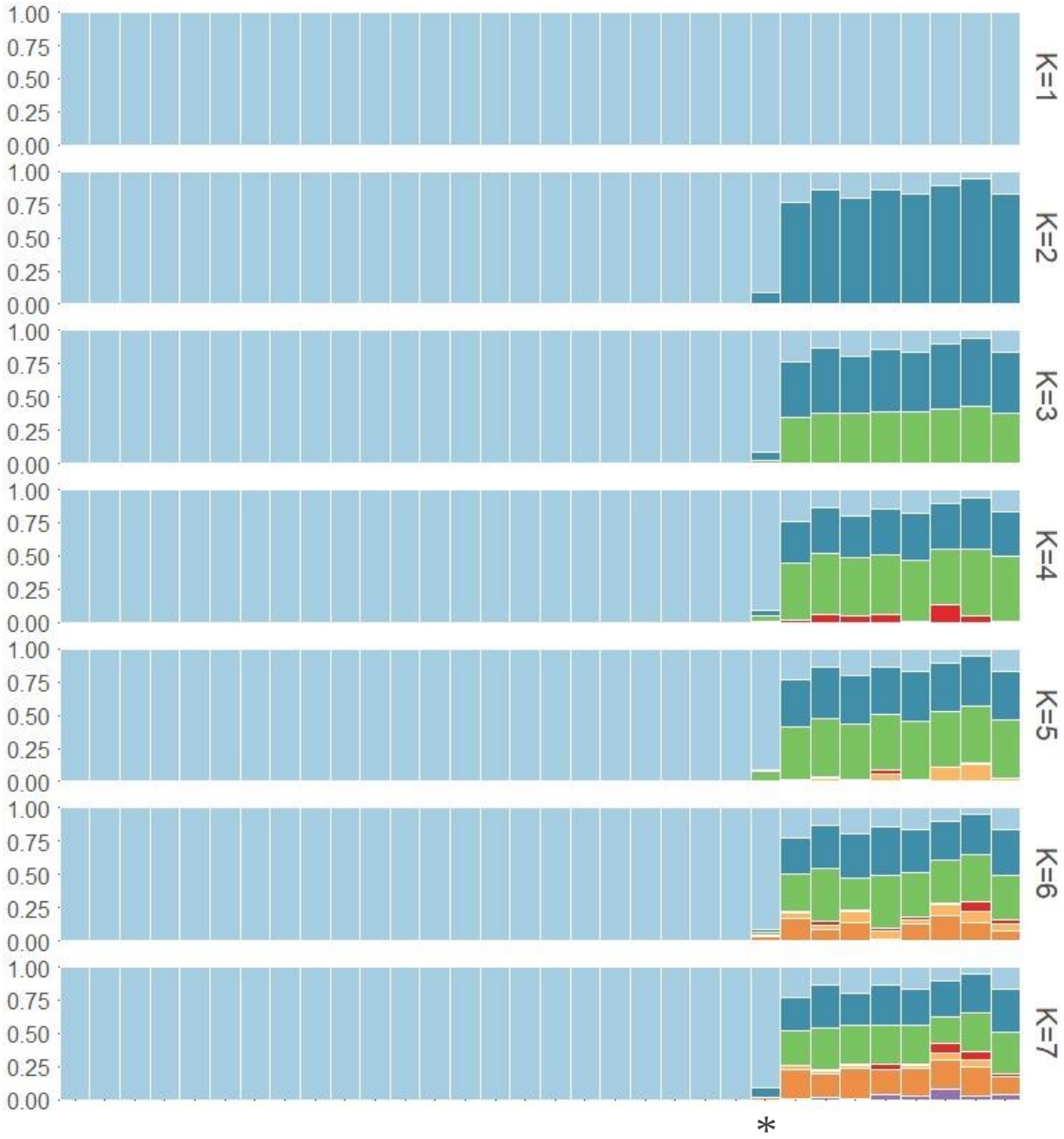

Figure G.3: STRUCTURE plots for clusters K=1 to 7 of 32 rough-skinned newts from Vancouver Island, Sunshine Coast, and Oregon. Each vertial line along the x-axis corresponds to a single individual with each color representing a unique cluster. The individual marked with an asterisk (*) is the single sample from Sunshine Coast; all samples to the left are from Vancouver Island and all samples to the right are from Oregon. The y-axis displays probability of each individual belonging to a given cluster. K=2 was found to represent the most likely number of clusters.

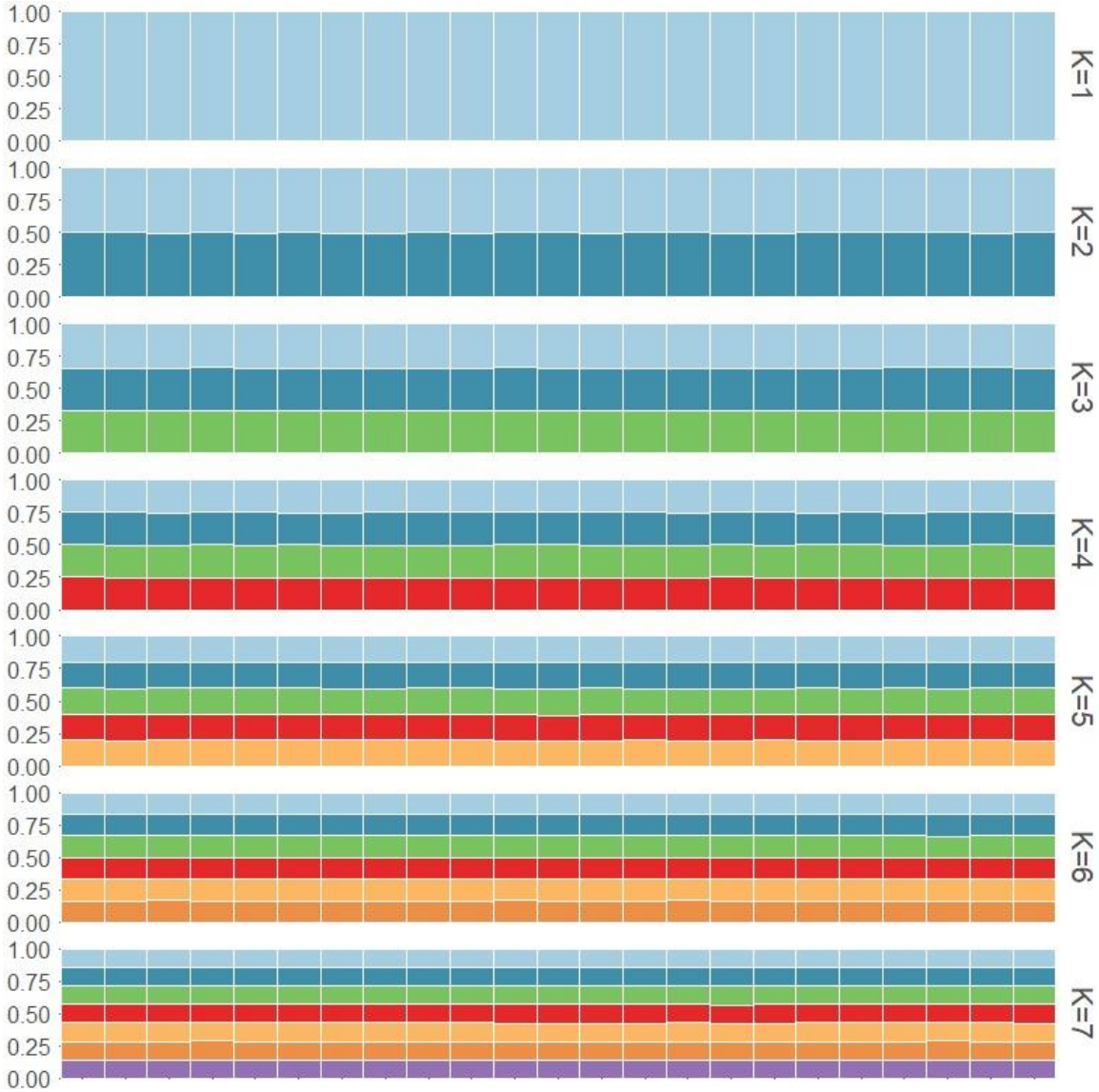

Figure G.4: STRUCTURE plots for clusters K=1 to 7 of 23 rough-skinned newt samples from Vancouver Island, BC. Each vertial line along the x-axis corresponds to a single individual with each color representing a unique cluster. The y-axis displays probability of each individual belonging to a given cluster. K=5 was found to represent the mostly likely number of clusters based on the Evanno method, however the lack of clustering evident in this plot indicates there is more support that the true value of K for this dataset is K=1.

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