Spring 2020

Toadal Isolation: Genetic Connectivity of the Western Toad (Anaxyrus boreas) along I-90 in the Snoqualmie Pass Area of Washington State

Anneliese Myers
anneliesemy@gmail.com

TOADAL ISOLATION: GENETIC CONNECTIVITY OF THE WESTERN TOAD (*ANAXYRUS BOREAS*) ALONG I-90 IN THE SNOQUALMIE PASS AREA OF WASHINGTON STATE

A Thesis

Presented to

The Graduate Faculty

Central Washington University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Biology

---

by

Anneliese Katherine Myers

June 2020

CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

We hereby approve the thesis of

Anneliese Katherine Myers

Candidate for the degree of Master of Science

APPROVED FOR THE GRADUATE FACULTY

_____ _____
Dr. Jason Irwin, Committee Chair

_____ _____
Dr. Alison Scoville

_____ _____
Dr. Linda Raubeson

_____ _____
Dean of Graduate Studies

ABSTRACT

TOADAL ISOLATION: GENETIC CONNECTIVITY OF THE WESTERN TOAD (*ANAXYRUS BOREAS*) ALONG I-90 IN THE SNOQUALMIE PASS AREA OF WASHINGTON STATE

by

Anneliese Katherine Myers

June 2020

Single-nucleotide polymorphisms (SNPs) were used to assess the genetic connectivity of western toad (*Anaxyrus boreas*) breeding populations along Interstate-90 near Snoqualmie Pass, WA. Sites north and south of the freeway were sampled during the breeding season of 2019. SNP loci were subsequently generated using the proprietary DArTseq^{TM} (Canberra, ACT, Australia) method. A total of 15,468 SNPs were used to calculate pairwise F_{ST} values and three distinct breeding populations were identified, two north and one south of I-90. All pairwise F_{ST} values between these sites were low (<0.02) but significantly different from 0. The lowest pairwise F_{ST} was between the two sites that were furthest apart (11.6 km), indicating higher levels of connectivity along than across the freeway. A *de novo* discriminant analysis of principal components (DAPC) confirmed this division between sites on either side of I-90. Although I-90 is the most prominent potential barrier on the landscape, the Yakima River may also be contributing to this division. An *a priori* DAPC was able to distinguish between all populations with enough confidence to assign toads that were randomly encountered in the summer of 2019 to their most likely population of origin and will be a useful tool in future studies.

ACKNOWLEDGMENTS

soli Deo gloria

I would like to thank my advisor, Dr. Jason Irwin, without whose help this project would not have been possible. His guidance and critiques in designing and carrying out this project were invaluable, as was his help with collecting samples in the field during breeding season itself. Thank you for advocating for and encouraging me; it is in large part due to your support that I saw this program through to completion.

I would also like to extend my gratitude to my other committee members, Dr. Alison Scoville and Dr. Linda Raubeson, who also gave me valuable feedback on my study design and analysis. Dr. Scoville, thank you for helping me figure out how to use R to wade through a mess of statistics. Dr. Raubeson, thank you for your input into the genetic protocols that were used in this study. Both of your perspectives on this project greatly enhanced the quality of the questions I asked and the analyses I performed.

I am also grateful for the funding support I received from CWU Department of Graduate Studies, CWU Department of Biological Sciences, and the Washington State Department of Transportation I-90 project.

Thank you also to Dr. Adam Leaché, professor at the University of Washington and director of the Burke museum. Thank you for responding to my e-mails, even during a time of global pandemic, and for sharing your knowledge and experience with

me. The programs and analyses I explored at your suggestion all furthered my understanding of population genetics and led to better interpretations of my data.

A great big thank you to all who volunteered to brave the cold, wet, late nights during toad breeding season. Without you, I would not have had nearly enough data! Lauren Segarra, Alex McCarrel, Jordan Ryckman, Josh Perry (thanks for sending his help my way, Dana Whitmore!), Adam Hess, Adrian Slade, and Tyler Larsen – with your help, we were able to collect over 80 samples in just 2 nights! I was (and am!) overwhelmed by your eager willingness to lend a hand during this time. Many of the pictures featured in this thesis also came from you during those nights, so thank you for remembering to document the event!

Two individuals in particular went above and beyond to further this project. Tyler and Adrian, thank you so much for spending so many late nights with me, even outside of your contracted hours, to try for just one more sample. I don't think I'll ever listen to 95.3 again without thinking of all those times "working on the night shift, baby"! "I'm going to need a pair of whiskey glasses…" just so that I can raise a toast to the two of you.

Thank you also to the rest of my cohort. It definitely helps to have a tribe of people who are going through the same crazy as you. Thank you for your listening ears, goofy conversations in the grad office, joint rants about the perils and frustrations of school and research, and for creating a supportive and safe environment. I'm so proud of all of us, and I look forward to seeing where life takes each of you.

Thank you to all those working on the I-90 project – Dr. Kris Ernest, Dr. Paul James, Dr. David Darda, Dr. Dan Beck, Patty Garvey-Darda, Dana Stringer, Josh Zylstra, Mark Norman, Kelly McAllister, the rest of the wildlife working group, and many others. Your feedback on study design and results, reported sightings of toads, support, and general knowledge of the project area were vital to the success of this study. It has been a privilege to work with such an enthusiastic and collaborative group!

To Mari, Kari, Stacey, Mark, and Jonathan, thank you for all you do to keep the department running. You have found answers to questions, booked rooms, processed forms, procured supplies, and gone above and beyond to support my labs, research, and studies.

An extra big thank you to Mari and Forrest for going through a mountain of paperwork with me to ensure my samples would successfully be shipped internationally!

Thank you to my past Riverside teachers and undergraduate professors from Whitworth University, who all fostered my love of learning and gave me such a valuable academic experience that I was willing to continue it. Special mention goes to:

-Mrs. Cunnington, who sparked my voracious love of reading

-Mrs. Shmidt, in whose class I began learning about the anatomy of both biological organisms and of poetry

-Mrs. Bellamy, who saw the teacher in me before I did

-Mrs. Van Beek, who taught me as much about kindness and generosity as she has about English and grammar

-Mrs. Bos, who taught me to perform for an audience, which was a joy and has been useful in my presentations and classroom management!

-Mr. Golladay, for extending grace towards an angry, yet comical, outburst – and for telling the story of it over and over again

-Mrs. Horsley (and her twin!), I'm still working on that 12-inch drop from the head to the heart

-Mr. Benson, for teaching me that there are many ways to approach a problem

-Mrs. Emmans, for making me laugh until my sides hurt and telling me to "sit in the back of class and take a nap" on those days when I needed it

-Mrs. Lambertson, for helping me to fall in love with the subject of biology, and with knowledge bowl!

-Mr. Herring, for challenging me to explore what I believe and why

-Dr. Thom Caraway, for giving me a taste of editing and for helping me to become a better writer

-Dr. Laurie Lamon, for guiding me in my efforts to find the language to bridge my love of science and of poetry

-Dr. Mike Sardinia, for your always open door. Without your encouragement, I don't know if I would have gone back to graduate school – certainly not so soon

-Dr. Grant Casady, for being the very best advisor to guide me through my undergraduate career. Thank you for not pressuring me to have it all figured out, and for helping me see the gift a diversity of interests can be. Thank you for the words you spoke to a distraught, anxious undergrad in your office "God didn't tell Abraham where he was going, just to go. Abraham took the step that was before him, trusting that there would be a step to take after that."

To the friends who have been on my side for well over a decade now – Kelle, Debbie, Shama, and Courtney – you are the embodiment of loyalty and lovingkindness. Though we have often been far apart and out of touch, we continue to share some of the big griefs and celebrations of life with one another. Thank you, each one of you, for the phone calls, the prayers, the real and warm human connection you have continued to offer freely in this season of life. It is a gift.

To the Dance Syndicate of Yakima, there are too many of you to count! JJ and Jake, Andrea and Zach, Christina and Phil…Bethany and Luis, Sara and Dillon, Jared and Janelle, Dan and Amanda, Skyler and Heather….Bethany and David,.…and so many, many others. To anyone who has danced or spoken with me on a Friday night, thank you for helping me escape the stress of graduate school for a moment or two!

To my church community, thank you for welcoming my husband and me with open arms. Thank you for entrusting us with your youth, and for blessing us with a

physical home during this time. Thank you to Pastor Dean Nelson and Rob Rife for your friendship and guidance, and to the rest of the church staff and congregation for your support and encouragement. A huge thank you to the youth and their families for your patience with us as we learn to lead, your flexibility and compassion with our work and school schedules, and the enthusiasm and vitality that you bring into the youth group! It is a joy to walk alongside you all.

Thank you to my family for your unconditional love and support. To my in-town relatives - Grandma and Grandpa Immel, Aunt Faye and Uncle Tim, Aunt Kim and Uncle Ken, Kimberly and Daylen, Lindsey, Victoria, K.C., and Kaitlyn, and the Myers Clan: Terry and Nancy, John and Jade, Joy and Daniel, Beth and Trenton, David and Leanne, and all our nieces and nephews - thanks for understanding my busy life, for your interest in what I've been working on, and for putting up with me on the rare occasions when I show up to family events to grab a quick bite and sneak away again. To the rest of my extended family both near and far, thank you for the interest, care, and empathy you have expressed whenever we cross paths. All of your encouragement, patience, and prayer in this busy season has truly been a saving grace.

And to my nuclear family, thank you for jumping right in with me and supporting me in every way conceivable! You know it has been a struggle at times, but I have truly enjoyed being able to share what I do with you. Thank you for wanting to be such an important part of it.

Mom and Dad, thank you for always being on my side. You have shown me so much love and have eased so many of my burdens. Whatever I need, if you can provide it you offer it freely, often before I even ask. I can't count all the home-cooked meals, help with emergency errands, listening ears, or access to Amazon prime and streaming accounts you have gifted me. Mom, I'm sorry we didn't find any salamanders on our midnight hike. Dad, thanks for spotting Margot for me! I love you both so much.

Moriah, thank you for letting me crash at your apartment after so many of my late-night escapades. Not having to drive all the way back to Yakima made night field work much more bearable. I so appreciate you putting up with me in your space on short notice, at any given hour of the day. Thank you for braving the dark with me so I didn't need to work alone that night when all the chorus frogs were singing. It was a pretty magical moment, and I'm glad I got to share it with you.

Sarah, thank you for all the tea. And home baked scones. And finger sandwiches. And Tangled episodes. You just know how to make a bad day better. I can't wait to have more Spokane adventures with you!

Isaac, thanks for always asking how things are going, and always offering to play a game. Even with my busy schedule that made me say 'no' so many times, you helped me to take breaks when I needed them! You were a champ in the field; if you ever come out with me again, I promise to make sure we go somewhere with less spiders.

And, of course, I would like to thank my truly AMAZING husband Jim Myers for standing by me through thick and thin. We had a lot of conversations about whether-or-not continuing grad school was the right option for us, but in all that time I never doubted your support of me. Thank you for putting up with my wonky and ever-changing schedule, for making sure I ate, for picking up my slack around the home and in our other areas of responsibility again and again and again. Thank you for letting me rant and cry and tell you way more about toads and genetics than you've ever wanted to know. Thanks for being my forever partner in crime. I love you so very much.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

Figure Page

I

INTRODUCTION

Habitat Fragmentation – a Major Source of Amphibian Decline

We are in an era that many researchers (e.g., Leakey and Lewin 1995; Thomas et al. 2004; Wake and Vredenburg 2008) are calling "the sixth mass extinction." The last few centuries have been characterized by a decline across all phylogenic classes of organisms (Pievani 2014); however, no group is declining as quickly as that of amphibians. At least 146 species have gone extinct since the year 1900 alone (Ceballos et al. 2015), and it is estimated that up to one-third of all known amphibian species are currently threatened with extinction (Wake and Vredenburg 2008).

Explanations for the current amphibian extinction crisis are varied and complex, but most stem from human activities (Ceballos et al. 2015). Human modification and destruction of landscapes have been heavily implicated in the decline of many species (Pereira et al. 2010). After modification to a landscape has been completed, resulting anthropogenic structures may break up surrounding area that was once continuous, natural habitat. The effects of such habitat fragmentation have been noted world-wide and have been increasing in severity (Haddad et al. 2015).

Amphibians can be negatively affected by fragmentation in a variety of ways (Cushman 2006). Many species of amphibian have a biphasic life history, requiring an aquatic habitat for breeding and early development, and a terrestrial habitat in which to spend their adult life (Schoch 2009). If fragmentation cuts a population off from either

of these key habitat features, the population may not survive to mature and reproduce the next generation of individuals.

In the case where a population does have access to all necessary habitat requirements, fragmentation poses other risks. Fragmentation reduces dispersal success and the survival of juveniles for many amphibian species (Cushman 2006). If populations are cut off from each other, fragmentation can also lead to a loss of genetic diversity, and, consequently, an increased vulnerability to disease and harmful recessive allelic traits (Couvet 2002; McCallum and Dobson 2002). In the case of extreme isolation, when such a population becomes locally extirpated, it may be impossible for their habitat patch to be re-colonized by other individuals (Antolin and Schoettle 2001).

Though many kinds of human developments can break a landscape up into smaller patches, roads are of particular concern, due to their length and abundance. It is estimated that 70% of forested area worldwide is within 1 km of a road (Haddad et al. 2015), and road networks continue to grow each year. Not only do roads contribute to direct mortality of amphibians (Fahrig et al. 1995; Mazerolle 2004) but, when traffic volumes are high, they can also pose a nearly impenetrable barrier to these small organisms (Fahrig et al. 1995; Mazerolle 2004), effectively fragmenting entire landscapes.

A nation-wide study conducted in the United States using citizen science was able to show that, for all species included in the study, road disturbance had a negative effect on amphibian species richness and distribution (Cosentino et al. 2014). Fragmentation caused by roads has also been shown to negatively impact amphibian

density (Fahrig et al. 1995) and connectivity (Cushman 2006). Even common and hardy amphibian species have been noticeably affected in some regions (e.g., Dixo et al. 2009), including the species under consideration in this study, the western toad (*Anaxyrus boreas*, formerly *Bufo boreas)* (COSEWIC 2012).

The Western Toad – Life History and Study Site Habitat

The western toad belongs to the family of the "true toads," *Bufonidae.* The western toad is a relatively common and hardy species, able to thrive under a wide variety of conditions, as can be seen from the extent of their range. The western toad can be found in the Western United States and Canada, from southern Alaska to Baja California, and as far east as Colorado (IUCN…2015).

Although widely distributed, the International Union for Conservation of Nature (IUCN) reports that the western toad exhibits a trend of population decline along its range (IUCN…2015). Some of these declines are reported to be quite dramatic in number (Drost and Fellers 1996; Muths et al. 2003). The extirpations of entire populations on Vancouver Island (Davis and Gregory 2003), as well as in New Mexico (Jackson 2004) and Colorado (Carey 1993**;** Livo and Yeakley 1997), are alarming and could indicate that other amphibian populations may face similar risks. It is therefore critically important to better understand what factors are contributing to the decline of common, hardy species like the western toad, and how to mitigate them.

As stated previously, habitat fragmentation is one of the major causes for the decline of the western toad (Stuart et al. 2004; COSEWIC 2012). The site selected for this study, located in Central Washington and known as the "I-90 corridor" is a stretch

of habitat in the Cascade Mountains which is bisected east to west by I-90: a freeway with heavy traffic volumes. The western toad is common to this area; five breeding locations in the study site are currently known, some north and some south of the freeway (personal communication, Dr. Irwin).

The western toad exhibits strong breeding site fidelity, returning to the same breeding grounds year after year (Carpenter 1954; Tracy and Dole 1969). Standing water is required for successful breeding to occur, and the western toad has an affinity for wetland areas and shallow, vegetated margins of lakes (Maxell et al. 2002; Bull 2006; personal observation). In the study area, breeding generally occurs sometime between mid-April to early-May, once the snow has begun to melt off of the breeding habitat surface and temperatures stay above freezing (personal communication, Dr. Irwin). The breeding period is short, lasting no more than a week. Females lay strings of up to ~12,000 eggs, which hatch after 3-12 days (Samollow 1980).

Tadpoles spend 4-12 weeks feeding and growing in their aquatic environment before metamorphosing into juvenile toads (Hayes et al. 1993; Wood and Richardson 2009). As juveniles, they disperse into the surrounding habitat to forage, grow, and mature. A male will reach sexual maturity between 2-3 years of age, while a female will reach maturity between 4-6 years (Olson 1988; Carey 1993; Matsuda et al. 2006). Mature males may breed annually, and even multiple times per breeding season, while mature females generally will not breed over consecutive breeding seasons, and only breed once per season (Olson 1988; Bull and Carey 2008).

Western toads spend the winters hibernating in underground hibernacula (Mullally 1952; Browne and Paszkowski 2010; Palmeri-Miles 2012). In the time intervening breeding and hibernation, western toads are largely terrestrial, and can range great distances from their breeding site in search of forage. Females are generally known to range farther than males and to have larger home ranges (Muths 2003). Males have been recorded to move over 0.9 km from their breeding site to summer home range, while females are recorded to move up to 2.4 km from breeding site to home range (Muths 2003; Bartelt et al. 2004). western toads are also capable of long-distance dispersal movements – Schmetterling and Young (2008) observed movements as great as 13km, with a median total travel distance of 2.9 km, over the course of 6 weeks.

Home-range movements have been reported in previous thesis work for the area of interest in this study. Toads were found to move between 0.25-290 meters daily, and up to 1976 meters within a month, with no significant differences observed between males and females (Palmeri-Miles 2012). Combined with published values, it is possible that migration or dispersal between breeding locations under study may be possible, as distances between breeding sites range from <0.5 km – 11.6 km. However, I-90 may pose an impassible barrier between some of the sites. In all previous telemetry work done at the study site, no western toad has been observed to cross I-90, although juvenile toads have been observed in in two culverts under I-90 (Swamp Lake and Price Creek; personal communication, Dr. Irwin).

I-90 – a Brief History

I-90 has been in place since 1905, when a rough road was established upon a footpath that had been developed by indigenous peoples. It was not until the 1930s that this road was paved and maintained for passage through the winter months. In the 1950s the road was widened to four lanes and significant traffic (4,000-7,000 vehicles a day) began to be seen along the then-highway. Since then, many improvements of I-90 have been made (I-90…History c2020). Traffic volumes have increased steadily and are currently around 30,000 vehicles a day. This number is projected to grow to 39,000 vehicles per day by 2040 (I-90…2019 c2020).

The latest improvements to I-90 are focused on accommodating these volumes, through the expansion of a 15-mile stretch on the East side of Snoqualmie Pass between Easton and Hyak (I-90…2019 c2020). This section of road has been identified to bisect important movement routes for animals in the north Cascade Ecosystem (Singleton and Lehmkuhl 2000; Shirk 2009). Because of this, in addition to widening the road, stabilizing slopes, and adding chain-up areas, WSDOT has partnered with the Forest Service and other organizations (see I-90…Statement c2020) to facilitate the crossing of wildlife from one side of the freeway to another through the construction of culverts, overcrossings, and underpasses (I-90…Statement c2020). When combined with fencing, these structures have been shown to reduce large wildlife-vehicle collisions and improve the safety of roads (Bruinderink and Hazebroek 1996). Crossing structures are also widely assumed to mitigate the effects of habitat fragmentation caused by

roadways, though few before/after studies exist that are rigorous enough to support this claim (Corlatti et al. 2009).

Wildlife Bridges – A Potential Solution to Habitat Fragmentation

Both underpasses and overpasses have been constructed around the globe in attempts to reconnect habitats across roadways. While these efforts are widely assumed to increase road permeability for a variety of animals, it is difficult to quantify this effect. Because construction of these structures may span decades, "before" and "after" studies that compare pre- and post- construction populations are rare (Glista et al. 2009). Such comparative studies are necessary to assess the degree to which a wildlife bridge has contributed to connectivity (Rytwinski et al. 2015).

One popular method of pre/post construction is camera trapping. Though wildlife cameras can be relatively easy and cheap to operate, their use tends to focus studies on benefits to individual organisms, rather than populations. This is because it is often difficult or impossible to tell from footage whether different individuals are genetically related (Clevenger and Huijser 2011). This method does not reveal if individuals from different populations are coming into contact with each other or whether the number of migrant mating individuals is enough to reach a threshold where the overall health of the populations will be improved (Corlatti et al. 2009). Additionally, this method is biased toward evaluating only the crossing of large animals, chiefly mammals, that are both easily identified and capable of tripping the movement trigger on a monitoring device. Smaller, more obscure organisms, such as amphibians, often go unrecorded and unidentified.

With their small home range and restricted dispersal capabilities compared to larger animals, it is unclear whether amphibians will be able to effectively utilize a land bridge at all. Along the portion of the I-90 corridor considered in this study, 11 crossing structures (culverts, underpasses, and an overpass) have been constructed or expanded to date. These structures are intended to reduce wildlife-vehicle interactions and to fulfill the goal of the US Forest Service to support overall biodiversity and ecosystem function in the corridor (I-90 Corridor…2008), making it a priority to assess the effects of crossings structures on a wide variety of species, including amphibians. This is in contrast to other projects, where the main goal is to prevent collisions or to increase connectivity for specific species (Glista et al. 2009).

For this study, the connectivity of the western toad is of particular concern since it is able to disperse such large distances (up to 13 km) compared to other anurans in the study site, such as the Cascades frog (*Rana cascadae*, up to 1.3 km; Garwood 2009) and the Pacific chorus frog (*Pseudacris regilla*, < 0.5 km; Jameson 1956). I-90 is expected to pose less of a threat to the latter species, as the distances between most breeding sites and the freeway is greater than an individual's dispersal ability (Burton 2002). However, breeding sites separated by I-90 are within the dispersal ability of western toads, and it is unclear what specifications are required to allow them to move over or under a freeway. Therefore, monitoring of this species is critical to determine if I-90 has historically posed a barrier to the species.

In recent years, advanced genetic techniques have been used to assess the effects of fragmentation and isolation on populations (e.g., Gutiérrez-Rodríguez et al. 2017).

Though so-called "landscape genetics" studies have been used to assess amphibian fragmentation across roadways, a literature search revealed that no studies have characterized genetic structure of amphibian populations before and after construction of crossing structures, as has been done for charismatic organisms such as sugar glider, bear, and deer populations (Kuehn et al. 2007; Van Manen et al. 2012; Soanes et al. 2017). Such studies are necessary to determine if crossing structures may also benefit less vagile organisms, and to what extent.

Landscape Genetics – High-resolution SNPs

Landscape genetics is a field of growing interest which examines the effect that landscape features have on the genetic composition of populations over time (Epps and Keyghobadi 2015). In a typical landscape genetics study, molecular markers in the species of interest are used to compare unique alleles and ratios of allelic occurrence between populations. Most commonly, genetic structuring of amphibian populations has been assessed by looking at microsatellites (Schaffer et al. 2014).

Microsatellites are short, tandem repeat sequences of DNA that are generally multi-allelic and have relatively high mutation rates compared to point mutations (Gemayel et al. 2012), which allows this technique to be used to detecting recent barriers to gene flow (Takahata & Nei 1984; Safner et al. 2011). For example, Richardson (2012) used microsatellites to show that both roads and Euclidean distance between populations affect the genetic structuring of wood frog populations. Another study by Peterman et al. (2015) examined microsatellites of ringed and spotted salamander (*Ambystoma annulatum, A. maculatum*) populations and determined that

dispersal propensity and ability are factors that play a role in genetic differentiation between subpopulations.

Though this method has been used with some success, the use of microsatellite markers gives less resolution than other so called next-generation sequencing (NGS) methods, such as the identification of Single Nucleotide Polymorphisms (SNPs) (Lemopoulos et al. 2019). SNPs are single-nucleotide loci that are polymorphic across individuals in a population. Thousands of SNP loci can be identified and analyzed in a study, compared to the average of 10 markers that are used in a microsatellite studies of amphibian populations (Lawrence et al. 2019). The higher resolution of SNPs reduces the number of individuals that must be sampled to detect population structuring (Willing et al. 2012; Nazareno et al. 2017) and has more power to detect weak population structure arising from recent or incomplete barriers within the landscape (Landguth et al. 2012). Furthermore, no *a priori* knowledge of a species' genome is needed to generate SNP loci, making this a particularly useful method when examining the population structure of non-model organisms (Davey et al. 2011; Andrews et al. 2016).

A recent study by McCartney-Melstad et al. (2018) demonstrated the utility of SNPs by examining populations of eastern tiger salamanders (*Ambystoma tigrinium*) on Long Island, New York. A previous study using 12 microsatellites was unable to detect any significant population structuring associated with human development (Titus et al. 2014). Using SNP loci, McCartney-Melstad et al. (2018) were able to demonstrate that

the populations were highly structured, and that both Euclidean distance and presence of roads were predictors of the genetic variance that was observed.

Though offering an increase in resolution, SNP markers are not yet widely used in studies of amphibian connectivity. One explanation for this could be that the cost of SNP sequencing may be prohibitive for many studies. Though the per-loci cost of SNPs have decreased drastically with advancing technology, the thousands of loci generated for SNPs result in more expensive costs per individual sample when compared with the use of microsatellite panels (Lemopoulos et al. 2019). Even for this study, the cost of generating SNP data was prohibitively expensive at most facilities. However, the methods employed by Diversity Arrays Technology Sequencing (DArTseq, Canberra, ACT, Australia), along with the academic discount they provide, offered a quality and affordable sequencing option for this research project.

Their proprietary DArTseq™ methods make use of restriction-site-associated DNA sequencing (RADseq), wherein endonucleases are used to target low repeat sections of a genotype, creating a subsample of the genome that is likely to contain variable nucleotides of interest. Only this subsample is sequenced, reducing overall cost (Andrews et al. 2016). The DArTseq™ method has recently been validated for use in vertebrates through the examination of case studies involving phylogeny and hybridization (Melville et al. 2017). It has been used to assess the structuring of animal populations such as tuna (*Thunnus albacares*), oyster (*Pinctada margaritifera*), and lobster (*Panulirus homarus*) (Grewe et al. 2015; Lal et al. 2017; Al-Breiki et al. 2018; respectively), among others.

The DArTseq™ method has primarily been used in amphibian species to identify sex-linked loci to assess sexual preference, determinism, ratios, etc. Only a few studies were found directly evaluating landscape genetics (e.g., Cummins et al. 2019) other than the validation study previously mentioned, and none focused on the effects of roads. Nevertheless, these sources imply that DArTseq™ may be a cost-effective, high-resolution method for monitoring the structure of amphibian and other vertebrate populations.

One measure of population structuring that may be assessed using SNP datasets is F_{ST} – a variable ranging from 0 to 1 that describes the amount of genetic differentiation between subpopulations. A value of 0 indicates that the subpopulations are freely interbreeding, while 1 indicates the subpopulations are totally separated.

Although it is difficult to compare F_{ST} values across different species, locations, and marker types, for reasons described below, it should be noted that a previous study of the western toad using a panel of 12 microsatellite markers found no statistical differentiation ($F_{ST} = 0$) between eastern WA populations ~70 km from each other (Switzer et al. 2009). In contrast to this, a similar number of microsatellites has been used to detect statistically significant structuring of tailed frog (*Ascaphus truei*, $F_{ST} = 0.01$) and Cope's giant salamander (*Dicamptodon copei*, $F_{ST} = 0.033$ to 0.127) populations within the Olympic Peninsula (Spear et al. 2011).This could be an indication that the higher vagility of the western toad, which has a home-range of 0.002 to 0.43 km^2 in the study area (Palmeri-Miles 2012), compared to the tailed frog and Cope's giant salamander (see Daugherty and Sheldon 1982, Johnston 1999), enables it

to maintain elevated levels of gene flow across large distances. An increased number of genetic markers, which SNPs provide, is likely needed to pick up any subtle differentiation that may exist between western toad populations in Washington.

F_{ST} – A Measure of Genetic Differentiation

The concept of F_{ST} was first developed by Sewell Wright, who defined it as "the correlation between random gametes within subdivisions, relative to gametes of the total population" (Wright 1950; Wright 1965). Later geneticists would interpret this 'total population' as representative of either the combination of the two subpopulations of interest (e.g., Nei 1973), or the ancestral population from which both subpopulations of interest had arisen (e.g., Weir and Cockerham 1984). This latter definition has been the most widely used, as it allows F_{ST} to explain evolutionary processes, rather than merely describe current population parameters (Bhatia et al. 2013**).**

Wright's research was conducted in a time when most alleles were thought to be bi-allelic, following Mendelian principles of genetics. Since the development of electrophoresis and subsequent discovery of multi-allelic markers, new parameters have been described which may better deal with these markers, such as the standardized G'_{ST} and Jost's D (Hedrick 2005; Jost 2008). However, the classic F_{ST} parameter described by Wright (1965) is applicable to bi-allelic SNP data (Equation 1**).**

$$F_{ST} = \mathrm{var}\{p\}/[\bar{p}(1-\bar{p})]$$

Equation 1. Equation for F_{ST}, where $\mathrm{var}\{p\}$ is variance in allele frequency among subdivisions, and $\bar{p}$ is the overall mean allele frequency in the total population (Wright, 1965).

Many estimators have been developed for Wright's F_{ST}, the most cited of which is Weir and Cockerham's (1984) θ, (hereafter, WC). An ANOVA-like approach is used to calculate this estimator as a ratio of the variance between populations relative to the variance of the total population (Equation 2).

$$\mathrm{WC} = \frac{a}{a + b + c}$$

Equation 2. Equation for the WC estimator, where *a* is the variance in allele frequency between populations, *b* is the variance in allele frequency between individuals within populations, and *c* is the variance in allele frequency between gametes within individuals (Weir and Cockerham, 1984).

The WC estimator assumes that both subpopulations have experienced the same amount of genetic drift since dividing from the ancestral population. Bhatia et al. (2013) point out that this can lead to inflated values of F_{ST} when sample sizes from populations are unequal. They recommend using instead the approach described by Hudson et al. (1992), which allows for each population to have a unique amount of genetic drift. They created an explicit equation (Equation 3) to calculate this F_{ST} estimator, which they named Hudson's estimator:

$$\hat{\mathrm{F}}_{\mathrm{ST}}^{Hudson} = 1 - \frac{H_w}{H_b} = \frac{(\bar{p}_1 - \bar{p}_2)^2 - \frac{\bar{p}_1(1-\bar{p}_1)}{n_1 - 1} - \frac{\bar{p}_2(1-\bar{p}_2)}{n_2 - 1}}{\bar{p}_1(1-\bar{p}_2) - \bar{p}_2(1-\bar{p}_1)}$$

Equation 3. Equation for an F_{ST} estimator using H_w, the mean number of differences within populations, and H_b, the mean number of differences between populations (Hudson et al. 1992). These were explicitly defined by Bhatia et al. (2013) in terms of sample size, n_i , and allele frequency, p_i , in population i for $i \in \{1,2\}$.

The use of F_{ST} has received some critique from the scientific community. The parameter is based on an infinite island model, where subpopulations are assumed to be discrete, infinitely large, and have an equal chance of receiving migrants from all other populations. Clearly, natural populations do not exhibit such characteristics, but F_{ST} estimators have proven robust to violations of these assumptions (Neigel 2002). Estimates of F_{ST} are dependent on the species and system under study, as well as the molecular marker being used (Meirmans and Hedrick 2010) and are subject to mathematical limitations (see Jakobsson et al. 2013). When structuring is subtle, F_{ST} values have a large variance, and only provide a coarse measurement of population differentiation (Neigel 2002, Jost 2008). However, after decades of use these limitations are generally well understood, and F_{ST} is still widely viewed as a useful measure of population structure (Neigel 2002). It is suggested that other methods of data analysis be used in conjunction with F_{ST}, such as the estimation of the closely related G'_{ST} and Jost's D (Ma et al. 2015**;** Whitlock 2011), or visual exploration of the data through multivariate tools such as Principle Components Analysis (PCA), Discriminant Analysis of Principle Components (DAPC), and other clustering methods (Jombart et al. 2010; Balzarini et al. 2011; Alhusain and Hafez 2018).

F_{ST} has previously been useful in revealing amphibian population structure associations with isolation-by-distance, roads, and other landscape features (Vos et al. 2001; Lesbarrères 2006; Bartoszek and Greenwald 2009; McCartney-Melstad et al. 2018). Simulation studies have shown that F_{ST} responds more rapidly to landscape modification than other related measures (Kalinowski 2002; Lloyd 2013). For species

with short generation times and relatively small effective population sizes, an increase in F_{ST} due to the addition of a barrier may be detected after only a few generations (Hoffman et al. 2017), although equilibrium will take longer to establish (Mech and Hallett 2001**;** Landguth et al. 2010**;** Alcala et al. 2013).

In the current study area, I-90 has received significant traffic since the 1950s, representing over ten generations of the western toad. If the road has been acting as a barrier to toad movement, F_{ST} values may reflect this. Similarly, F_{ST} has been shown to equilibrate rapidly (1-15 generations) following barrier removal (Landguth et al. 2010), making it an appropriate measure to use in future studies to detect the effect of crossing structures.

Study Objectives

The goal of my study was to use DArTseq generated SNPs to assess which western toad populations along the I-90 corridor were most closely connected. Although several crossing structures have already been constructed in the study area, the time-lag associated with genetic population parameters (Landguth et al. 2010) allows my study to approximate a "pre-construction" snapshot of western toad connectivity along I-90. Pairwise F_{ST} values were quantified to determine whether I-90 has been acting as a barrier to gene flow for this species.

I predicted that I-90 poses a total barrier to gene flow, and that the populations north and south of I-90 on Snoqualmie Pass would be distinct in their genetic structuring, having relatively high pairwise F_{ST} values. In contrast, I predicted that sites near each other on the same side of the freeway would be less structured due to

unimpeded migration and dispersal between proximal sites, which would be indicated by relatively low pairwise F_{ST} values. Additionally, I predicted that pairwise F_{ST} values involving Mardee Lake, the most removed breeding location in the study site, would consistently exhibit the highest values, because Euclidean distance between it and all other sites is more than twice the distance between any other pair of breeding locations. Finally, I predicted that populations would be structured enough to discriminate between using a DAPC, allowing individuals of unknown origin to be assigned to their most likely population.

By using F_{ST} values and DAPC results as a proxy for population structure prior to land bridge construction, this research will provide a foundation for later, comparative studies to assess whether these structures have any positive effect on gene flow for the western toad. It is hoped that the methodology employed here will provide a useful template for the assessment of other connectivity projects involving amphibian species of concern.

II

METHODS

Study Location

This study took place from April-September 2019, in Washington State, over a fifteen-mile stretch of the I-90 corridor between Easton and the Snoqualmie Pass Summit. As mentioned previously, this location was selected for its known western toad breeding sites and its inclusion in the I-90 Eastbound road improvement project. All toads incidentally encountered in the research area over the course of the study were sampled. Several specific locations were surveyed periodically during the western toads' breeding season (April – May) in order to obtain a representation of breeding populations.

These efforts focused on five wetland habitats where toads have previously been observed to breed (personal communication, Dr. Jason Irwin). Three of these wetland habitats are on the north side of the freeway (Mardee Lake – northwest, Townsend Pond – northcentral, Swamp Lake – northeast) and two (Keechelus Dam Ponds) are on the south side of the freeway (Fig. 1).

It is unclear whether the two southern wetlands would have existed prior to construction of the Keechelus Dam in 1917; at the very least they would have looked much different than they do today and would not necessarily have been suitable to support western toad populations. Both Mardee Lake (NW) and Swamp Lake (NE) are presumed to be historical breeding areas for the western toad.

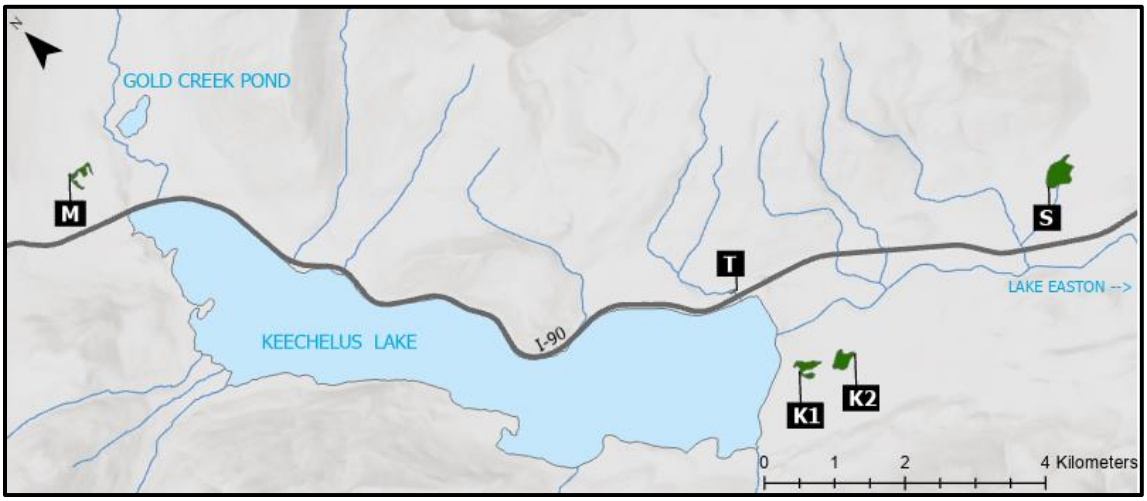

Fig. 1 Map of known breeding locations of the western toad (*Anaxyrus boreas*) along a 15-mile improvement stretch of I-90 in Washington State. Sites, from left to right, include Mardee Lake, Townsend Pond (wetland outline not visible), Keechelus Dam Ponds 1 and 2, and Swamp Lake. The study area was digitized in ArcGIS Pro and utilizes a hillshade base layer (version 2.5.0, ESRI c2020).

The fifth site, Townsend (NC) mitigation area, is a special case. It was historically a wetland area, but the establishment of the Sunset Highway in 1913 cut through the site, reducing and degrading available habitat area. A portion of this highway was removed in 2016, and mitigation measures were taken to restore a portion of wetland habitat (Mohagen 2019). A culvert near the wetland area, running beneath I-90, was expanded in 2017.

One western toad was observed to establish itself in the restored area in 2018. No toads had been observed in the area prior to that time, either before or during the restoration process. The summer of 2018 was the first time western toad tadpoles were observed at this site (personal communication, Dr. Irwin). It is unclear whether toads

migrated to this location from the nearby Keechelus Dam Ponds (S) via the new culvert or from the more distant Swamp (NE) or Mardee Lake (NW) populations.

Euclidean distances between these breeding locations were calculated using ArcGIS Pro (version 2.5.0, ESRI c2020). Wetland data for the upper Yakima Basin were obtained from the U.S Fish & Wildlife Service's National Wetland Inventory (NWI) (NWI…2019). Four out of the five breeding locations fell under 'freshwater emergent wetland' habitat patches in the NWI. The margin of these and any connected 'freshwater emergent wetland' habitat patches were traced as the boundary of these four breeding locations. The restored Townsend wetland does not appear on the NWI. Satellite imagery from Septermber 2018 (ESRI, 2018) was used to trace the visible water's edge around the identified breeding location. The "Near" tool was then run in ArcGIS Pro to determine the shortest distances between breeding margins. The "Measure Feature" tool was used to calculate total area of each breeding site.

In addition to the five known breeding locations, two sites were identified as areas of interest for the species. Lost Lake, on the south side of the freeway, has historical reports of western toad observations (Patricia Garvey-Darda, pers. comm.). Swamplands north of Lake Easton, on the north side of the freeway, appear to have good potential breeding habitat. Due to these characteristics, the two sites were also included in breeding season surveys.

Sample Collection

Beginning in April, Swamp Lake (NE) was surveyed every few nights to detect the movement of male toads into the area prior to the peak breeding event. Activity at

this location served as an indicator for the other focal sites. After May 1, when male toads were sighted at Swamp Lake (NE), nightly sampling was conducted by a team of researchers and volunteers. Samples of breeding populations of the western toad were collected from May 2 – May 4, 2019.

During the course of these nights, each focal site was surveyed to assess whether toads were present. If none were found, the area was resurveyed 1-2 nights later. If toads were present, the area was surveyed for egg masses to determine if breeding had occurred, then toads were caught by hand for sampling. The goal was to sample up to 30 adult toads per site, or as many adult toads as were found.

Each captured toad was weighed and measured from snout to vent. The toad was scanned for passive integrated transponder (PIT) tags; if none was present, a PIT tag with a unique identification code was injected into the individual (Fig. 2). A small amount of tissue from one of the toad's hind toes was clipped using sterilized scissors, and then the individual was released near its point of capture. The toe-tip tissue was stored in 95% denatured ethanol in a freezer until it could be sent out for analysis. After the breeding season was completed, toads incidentally encountered in the study area during the summer of 2019 were also sampled using the procedure outlined above. All samples were collected under Central Washington University Institutional Animal Care and Use Protocol A061602 and Washington Department of Fish and Wildlife Scientific Collection permit IRWIN 18-314.

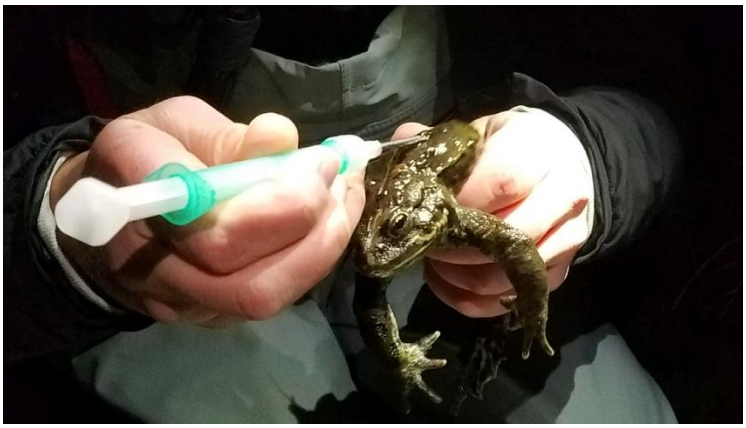

Fig. 2 A western toad (*Anaxyrus boreas*) being injected with a PIT tag by a trained researcher. Photo Credit: Adrian Slade

Genetic Sequencing and Filtering

Tissue samples were sent to Diversity Arrays Technology Sequencing (DArTseq, Canberra, ACT, Australia) for DNA extraction and DArTseq™ genotyping. The DArTseq™ method begins with a "complexity reduction" step, wherein an endonuclease set is used to target low repeat sections of a genotype. This creates a subsample of the genome that is likely to contain variable nucleotides of interest. Afterwards, next generation sequencing (NGS) takes place on Illumina (San Diego, CA, United States) platforms (Sansaloni et al. 2011; Kilian et al. 2012; Courtois et al. 2013; Cruz et al. 2013; Raman et al. 2014).

DArTseq selected the enzyme combination of *PstI-SphI* to subsample the genome of *Anaxyrus boreas.* A digestion/ligation mixture was prepared, containing the two enzymes, as well as (forward) *PstI*- and (reverse) *SphI*-compatible adaptors, as per Kilian et al. (2012). Both adaptors included a flowcell attachment sequence from Illumina. The *PstI*-compatible adaptor additionally included a sequencing primer and a

barcode region of varying length. This barcode sequence was unique to each sample, similar to the barcode used by Elshire et al. (2011).

After digestion/ligation was completed, only "mixed" fragments, cut by one end at *PstI* and at the other end by *SphI*, were amplified by PCR under the following conditions: a one minute initial denaturation at 94°C, followed by 30 cycles of 20 seconds at 94°C, 30 seconds at 58°C, and 45 seconds at 72°C, with a final seven minute extension at 72°C. After this, amplification products of all samples were pooled in equimolar amounts for cluster generation through C-bot (Illumina) bridge PCR.

Briefly, bridge PCR is a process in which single-stranded amplification products are annealed to short, complementary sequences bound to a flow-cell surface. The bound sequence is extended from the 3' end as a copy of the amplification product, which is subsequently removed by denaturation. The copied strand has a flow-cell adaptor sequence at its 3' end, which binds to a new flow-cell sequence. This forms a bridge and provides another site for synthesis, after which denaturation can occur. Multiple cycles, followed by cleavage of one adaptor sequence, result in clonal clusters of DNA fragments across the flow-cell (Bentley et al. 2008). Genetic sequences were then generated on an Illumina Hiseq2500 platform using 77 single read cycles.

These raw sequences were filtered in DArT's primary analytical pipeline. Higher filtering thresholds were applied to the barcode region (minimum phred pass score 30, minimum pass percentage 75) compared to the rest of the sequence (minimum phred pass score 10, minimum pass percentage 50). The more stringent treatment of the barcode enabled reads to be accurately de-multiplexed. After filtering, approximately

2,500,000 sequences per sample remained. Identical sequences were grouped into fastqcoll files. DArT PL's proprietary algorithm was used to identify and correct low-quality base pairs. This resulted in "groomed" fastqcoll files, which were moved into DArT PL's secondary pipeline.

In the secondary pipeline, proprietary calling algorithms (DArTsoft14) identified low error-rate SNP markers based on several technical parameters, with scoring consistency of technical replicates used as the main selection criteria. The Mendelian distribution of identified loci was assessed to remove paralogous sequences from the dataset. On average, there was a read depth of over 50 reads per SNP locus, ensuring high calling quality.

The SNP loci that were returned from DArTseq were subjected to additional filtering (95% call rate of loci, 95% call rate of individuals, 100% reproducibility, minor allele frequency of 5%, only one SNP retained per locus, removal of monomorphic loci) in R using the package dartR (Gruber and Georges 2019; R Core Team 2019). Using a missing data (call rate) threshold ensures that poorly genotyped SNPs are removed from the dataset, as well as low-quality individual samples (Alhusain and Hafez 2018). Filtering based on the reproducibility of technical replicates removes potentially erroneous sequences. The potential for erroneous genotype calling increases as the minor allele frequency (MAF) decreases; this is especially true of small population sizes, such as those obtained in this study, justifying the stringent threshold of 0.05 that was used (Coleman et al. 2016). This filtered set of SNPs was used in all downstream population genetic analysis.

Inclusion of full siblings may bias the results of population structure analyses (Goldberg and Waits 2010; O'Connell et al. 2019)**,** therefore, individuals from breeding sites were assessed in COLONY (Jones and Wang 2010) for familial relationships. Only SNPs with an MAF > 0.35 were used during this filtering process, due to the guided user interface (GUI) program restraints. For each full-sibling group identified, only one individual was retained in the dataset for downstream population genetic analysis.

Population Genetic Analysis

The within-population measure of expected heterozygosity (H_E) was calculated as an indicator of overall population genetic health. H_E is a common measure of genetic variability and represents the proportion of genotypes that are expected to be heterozygous under Hardy-Weinberg equilibrium (Nei 1973). H_E was calculated using the *gl.Hs* function of the R package adegenet (Gruber and Georges 2019).

Pairwise F_{ST} values between populations were calculated using Weir and Cockerham's estimator (1984), using the function *stammpFst* in the package StAMPP (Pembleton et al. 2013). Results were bootstrapped over all loci 100 times to obtain 95% confidence intervals. To check for bias of F_{ST} estimates due to uneven sample sizes, pairwise F_{ST} values were also calculated using Hudson's estimator, per Bhatia et al. (2013), using the *fst.hudson* function of the package KRIS (Chaichoompu et al. 2018).

An initial calculation of the Weir and Cockerham (1984) estimator (W-C) resulted in an F_{ST} value of -0.0013 for the Keechelus Dam Ponds (S) population pair,

indicating that they are one fully admixed population. Individuals from these two areas were subsequently pooled into one population dataset called “Keechelus Ponds (S)” and pairwise F_{ST} values were recalculated.

Pairwise F_{ST} values were also calculated using the Hudson estimator (data not shown). Though all values were slightly inflated compared to those obtained using the W-C estimator, they did not change the qualitative nature of the results. W-C is the more commonly cited estimator (Bhatia et al. 2013) and is presented in this study to facilitate comparison with previous literature.

The SNP dataset was subsequently explored using DAPC in the R package adegenet (Jombart 2008; Jombart and Ahmed 2011). DAPC is a multivariate analysis that first transforms the SNP dataset using a principal component analysis (PCA), which generates a set of uncorrelated variables that fit the assumptions needed to subsequently perform a discriminant analysis (DA). This DA partitions a selected number of principal components (PCs) into within- and between-group variances, creating new weighted variables to maximize the between-group differences while minimizing variance within groups. Data can either be discriminated into pre-defined groups or K-means clustering can be used to identify groups that minimize within-group variation for the dataset (Jombart et al. 2010).

To prevent over- or under-discrimination, the number of PCs retained for each DAPC was determined by using the *optim.a.score* function (10 replicate alpha-scores), which predicts the maximum alpha-score for each number of PCs retained. A DAPC was first run with no *a priori* grouping (*de novo*), using the *find.clusters* function to

predict the most likely number of distinct genetic clusters (K) via K-means clustering to calculate a Bayesian information criterion (BIC) value for each value of K (Jombart et al. 2010), with a lower BIC indicating a better model fit.

A DAPC was then run using *a priori* population assignments as described by Jombart et al. (2010). The posterior population assignments of all individuals were then assessed, to determine how well the discriminant functions (DFs) produced were able to discriminate between groups. Subsequently, individuals from unknown populations were introduced to the model for population assignment using the *predict.dapc* function.

III

RESULTS

Study Location

In the spring of 2019, breeding behavior was observed at Swamp Lake (NE), the Keechelus Dam Ponds (S), and Mardee Lake (NW). At Swamp Lake (NE), only males were observed and no egg masses on the night of sampling (May 2nd). At both Keechelus Dam Pond (S) sites, the majority of toads sampled were male, with a few females. No eggs were observed at these sites on the night of sampling (May 2nd and 3rd, respectively). At Mardee Lake (NW), one female was sampled; the rest were males. Egg masses were observed at this site on May 3rd.

No toads were observed at Townsend Pond, Lost Lake, or swamplands above Lake Easton during any of the surveys conducted over the course of the breeding season. Over the course of the summer field season, six additional adult and two subadult toads were incidentally encountered across the study area (Fig. 3).

Samples

From the sites where breeding was observed, samples were successfully collected from each: 30 from Swamp Lake (NE), 30 from Keechelus Dam Pond 1 (S), 12 from Keechelus Dam Pond 2 (S), and 11 from Mardee Lake (NW). Two of the individuals from Mardee Lake (NW) were recaptures from previous years (PIT# 3D6 AC9 D303, PIT# 3D6 AC9 D109).

The eight other toads incidentally encountered over the summer included two adults near Twin Lakes (south of I-90), one adult at Gold Creek (north, near Mardee

Lake), one adult and one subadult at Townsend (north), one subadult at Price Creek (crossing structure beneath I-90), and two adults on the roads surrounding the Keechelus wetlands (south) (Fig. 3).

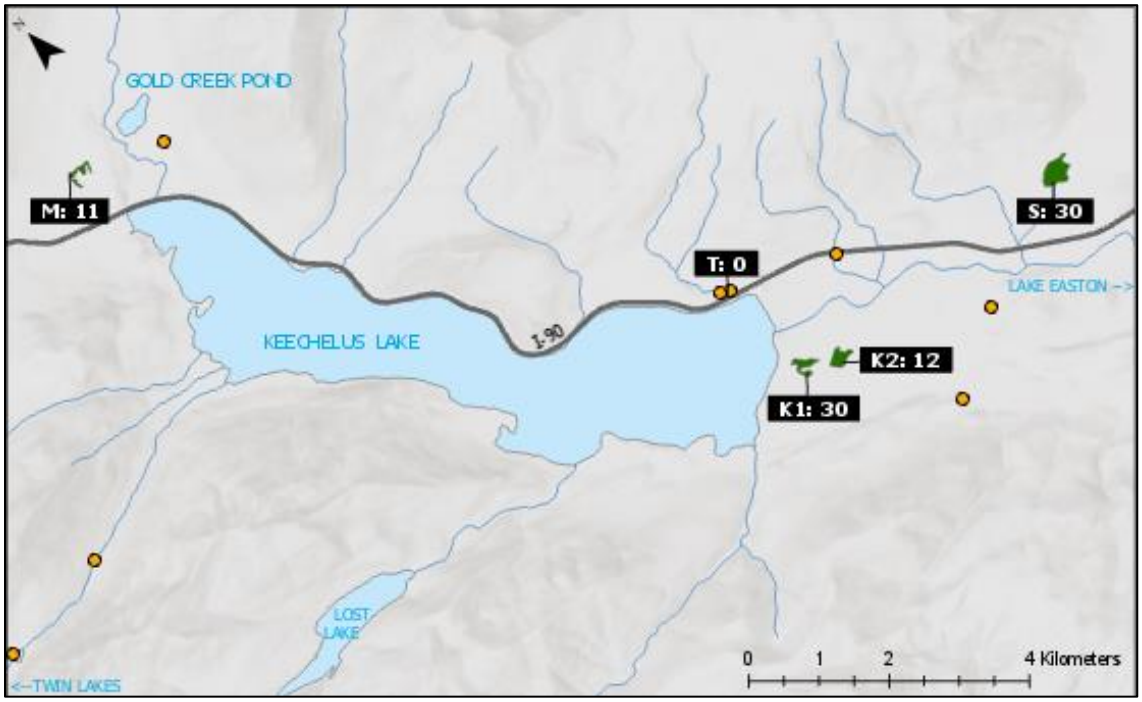

Fig. 3 Map of western toad (*Anaxyrus boreas*) samples collected from the I-90 Snoqualmie Pass East project area in 2019. Black markers indicate known Western toad breeding locations, with letter representing site ID and number indicating the number DNA samples taken during the spring breeding season of 2019. Yellow points indicate individual toads encountered and sampled in the study area over the summer field season of 2019. The map was digitized in ArcGIS Pro and utilizes a hillshade base layer (version 2.5.0, ESRI c2020).

Genetic Sequencing

Ninety-four samples were sent to DArTseq for extraction and sequencing (Table 1). Several collected samples were smaller than was recommended by DArTseq (< 5mg); however, most of these samples were included in the shipment to have the largest

sample sizes possible. All 11 and 12 samples collected from Mardee Lake (NW) and Keechelus Dam Pond 2 (S), respectively, were included. Additionally, seven samples collected from Mardee Lake (NW) over previous breeding seasons (Dr. Jason Irwin, 2017, 2018) were included in order to enhance this population's sample size.

Due to well-plate restrictions, only 94 samples total could be sent. To accommodate the extra samples for Mardee Lake (NW), samples from Swamp Lake (NE) and Keechelus Dam Pond 1 (S) populations were reduced to 29 and 28, respectively. In keeping with DArTseq's size recommendations, the smallest tissue samples from these populations were selected for removal. Six of the seven samples from incidentally encountered individuals were also sent. The seventh was a juvenile sample from Townsend weighing <5mg. It was deemed too valuable to risk using, based on uncertainty regarding the origins of this newly established breeding population.

Table 1 Western toad DNA samples collected between 2017-2019 and sent to DArTseq (Canberra, ACT, Australia) for sequencing. The # below 5mg column shows how many tissue samples fell beneath the lab's recommended weight.

Breeding Group	Year	# Samples	# Below 5mg
Mardee Lake	2017-19	18	8
Swamp Lake	2019	29	13
Keechelus Dam Pond 1	2019	28	23
Keechelus Dam Pond 2	2019	12	7
Unknown Origin	2019	7	4

Of the 94 samples, only one (PIT# 003 D474 B9F) failed to amplify. This sample was a female from the Keechelus Dam Pond 2 (S) population, thus reducing this

site's sample size to 11 individuals. It should be noted that this tissue sample was within DArTseq's recommended weight guidelines. Furthermore, the group of samples beneath 5mg (0.5-4.9mg) did not have a greater proportion of missing data, on average (0.174 ± 0.001), than the group of samples within the recommended guidelines (0.171 ± 0.002) ($t_{0.05(2),68.6}$=1.28, p=0.205).

From the 93 samples that did amplify, 131,762 SNPs were generated from DArTseq's proprietary analytical pipeline. Subsequent filtering of SNPs with dartR (Gruber and Georges 2019) resulted in 15,468 SNPs, which were retained for all downstream analyses.

When the set of breeding individuals was introduced to COLONY (Jones and Wang 2010), 10 sets of full siblings were identified. Four pairs occurred within the Keechelus Ponds (S) population, and six within Swamp Lake (NE). For each full-sibling group, only one individual was randomly retained for all downstream analyses.

Population Genetic Analysis

H_E values of 0.266 ± 0.140, 0.261 ± 0.136, and 0.263 ± 0.138 were calculated for Mardee Lake (NW), Keechelus Ponds (S), and Swamp Lake (NE) populations, respectively.

All pairwise W-C F_{ST} estimates calculated for the study area were generally low in value (< 0.02), though 95% CIs show all are significantly different than 0. Interestingly, the pairwise F_{ST} value calculated for Swamp (NE) – Keechelus (S) populations, even though representative of the shortest Euclidean distance, was as large or larger than all other F_{ST} values representing greater distances. Pairwise F_{ST} values of

Mardee (NW) – Swamp (NE) and Mardee (NW) – Keechelus (S) were not significantly different from each other (Table 2).

Table 2 Weir and Cockerham (1984) pairwise F_{ST} estimator for western toad populations along I-90. All pairwise F_{ST} values are significantly different than zero. F_{ST} subscripts are used to indicate significant differences between groups. 95% CIs were generated by 100 rounds of bootstrapping over loci. Sample size for Mardee Lake, Keechelus Ponds, and Swamp Lake populations is equal to 18, 39, and 29, respectively.

Population Pair	Euclidean Dist	F_{ST}	95% CI
Mardee-Keechelus	10.0 km	$0.0119^{a,b}$	0.0111 - 0.0129
Mardee-Swamp	11.6 km	0.0105^{a}	0.0096 - 0.0114
Swamp-Keechelus	2.5 km	0.0123^{b}	0.0117 - 0.0130

For the DAPC analysis without *a priori* population assignment, the *find.clusters* function considering K values 1-15 indicated that BIC scores were lowest for K=1 (Fig. 4). However, it is important to realize that the notion of a "true K" is largely hypothetical, and this function often provides a range of K values that may be useful in describing the data (Jombart and Collins 2015). Additionally, K-means clustering often fails to identify differing groups when structuring is subtle (Stift et al. 2019**;** Maigret et al. 2020). Therefore, K values between 2 and 5 were explored without *a priori* population assignment.

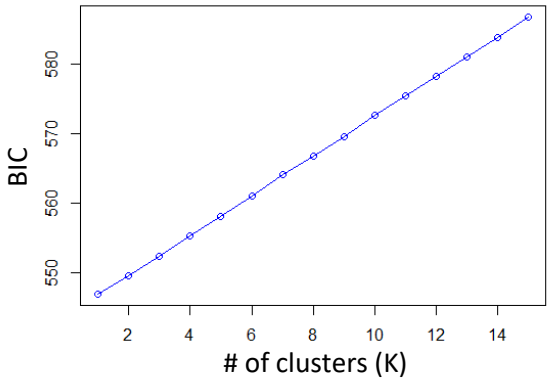

Fig. 4 Bayesian information criterion (BIC) values for different levels of K, predicted by the *find.clusters* DAPC function of adegenet (Gruber and Georges 2019).

The *optim.a.score* function indicated the first 8 PC-axes, which represent 15.8% of the total genetic variation, should be used in the DAPC analysis for K=2 (alpha-score mean = 0.399, sd = 0.170). Clustering of two groups resulted in a nearly complete split of the Keechelus Ponds (S), population from the northern Mardee Lake (NW) and Swamp Lake (NE) populations along the first discriminant function (DF) (Fig. 5).

Increased K values of 3-5 utilized 6, 7, and 17 PCs, respectively (alpha-score mean = 0.544, sd = 0.203; alpha-score mean = 0.601, sd = 0.179; alpha-score mean = 0.566, sd = 0.170), representing 12.3 - 30.2% of the total genetic variation. K=3 resulted in further subdivision of the Keechelus Ponds (S) population, while still grouping nearly all northern Mardee Lake (NW)/Swamp Lake (NE) individuals together. K=4 also consisted of two groups representing subdivisions of the Keechelus Ponds (S) population. However, in this case the remaining two groups did largely separate the Swamp Lake (NE) and Mardee Lake (NW) populations from each other. For K=5, all populations become more subdivided and become less distinguishable from each other (Fig. 5).

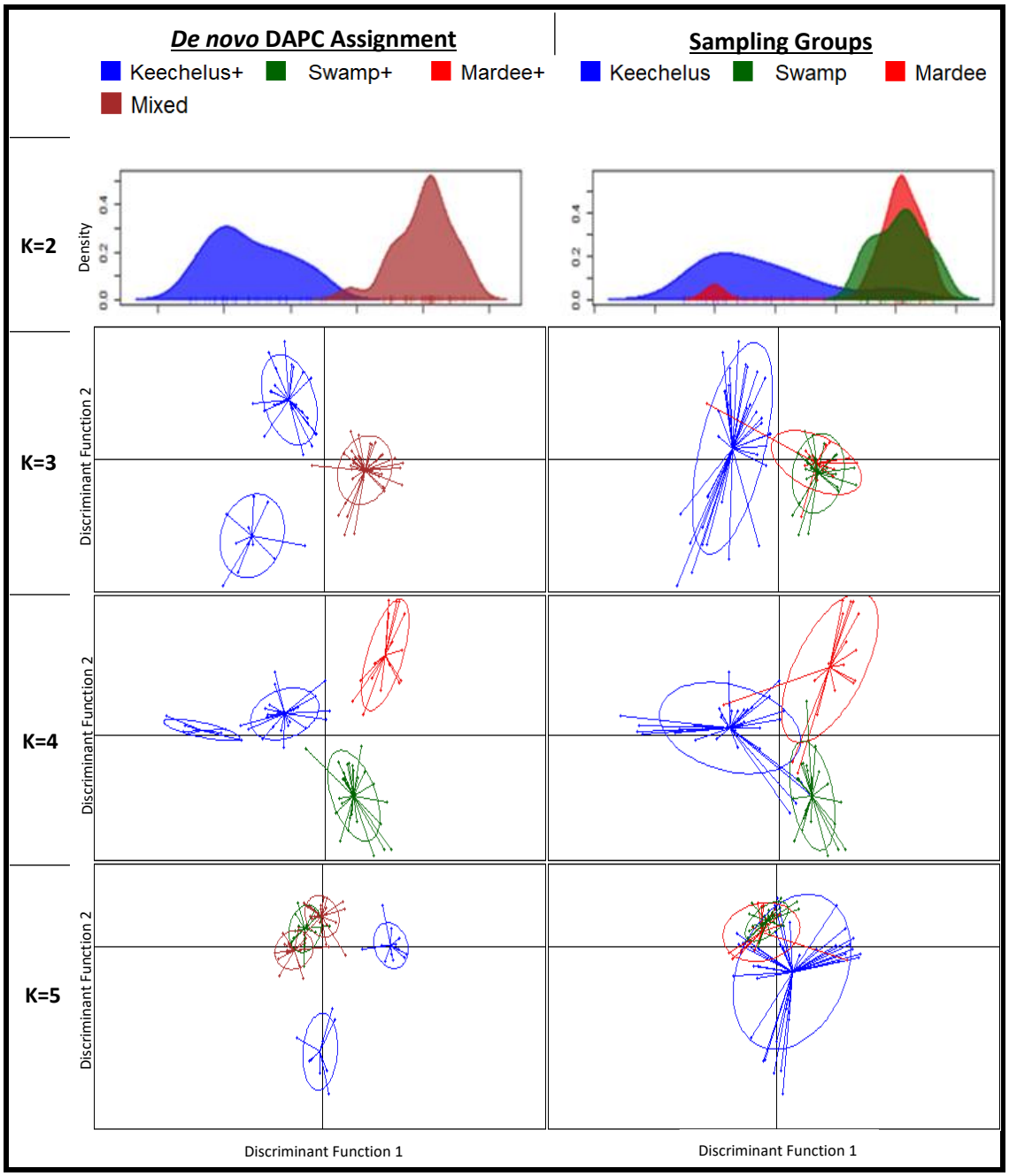

Fig. 5 *De novo* DAPC assignment (left) of western toad (*Anaxyrus boreas*) genetic samples where K=2 to 5, as compared to the sampling group (right) of each individual. In the left column, groups are colored according to whether they have >75% membership from one of the breeding populations. The right column contains the same discriminant function spaces, but individuals are color-coded according to their population of sampling origin. For K = 2-5, 8, 6, 7 and 17 PCs were used, respectively, as determined by the *optim.a.score* function of adegenet (Gruber and Georges 2019).

Three PCs were retained for the DAPC test with *a priori* population assignment (alpha-score mean = 0.364, sd = 0.111), representing 6.6% of the total genetic variation. As in the previous analyses, the first DF appears to discriminate the southern (Keechelus Ponds) population from the two northern (Mardee Lake, Swamp Lake) populations. In this case, the second DF partially separates Mardee Lake (NW) and Swamp Lake (NE) populations, though some overlap remains (Fig. 6). A "correct assignment" was defined as an individual with a >50% posterior assignment probability associated with their actual sampling site. A likely migrant was considered to be an individual with >80% posterior assignment probability associated with a site they were not sampled from. Under this model, the DAPC was able to assign 93.5% of individuals to the population from which they were originally sampled, and two likely migrants (Keechelus to Mardee, Swamp to Mardee) were identified (Fig.7).

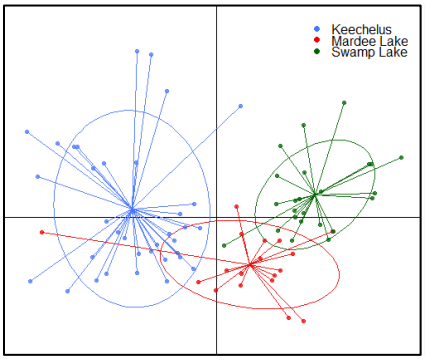

Fig. 6 *A priori* DAPC of Western toad (*Anaxyrus boreas*) genetic samples. Three PCs were included in this analysis, as determined by the *optim.a.score* function of adegenet (Gruber and Georges 2019).

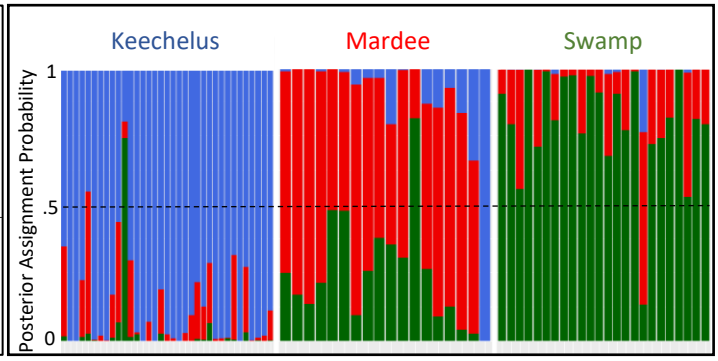

Fig. 7 Posterior assignment probabilities for Western toad (*Anaxyrus boreas*) genetic samples as determined by an *a priori* DAPC. Each vertical bar represents a sampled individual, grouped by sampling location. The fill color of each bar represents the posterior probability that the individual belongs to each breeding group, as predicted by the DAPC.

Incidentally encountered individuals were then introduced into this DAPC for population assignment. The results indicate that the Gold Creek individual most likely came from the Mardee Lake (NW) population, the Townsend Pond individual from a northern (Swamp or Mardee Lake) population, and the two roadside individuals and the Price Creek individual from the Keechelus Ponds (S) population. The two Twin Lakes individuals have strong probabilities associated with both the Keechelus Ponds (S) and Mardee Lake (NW) populations (Fig. 8).

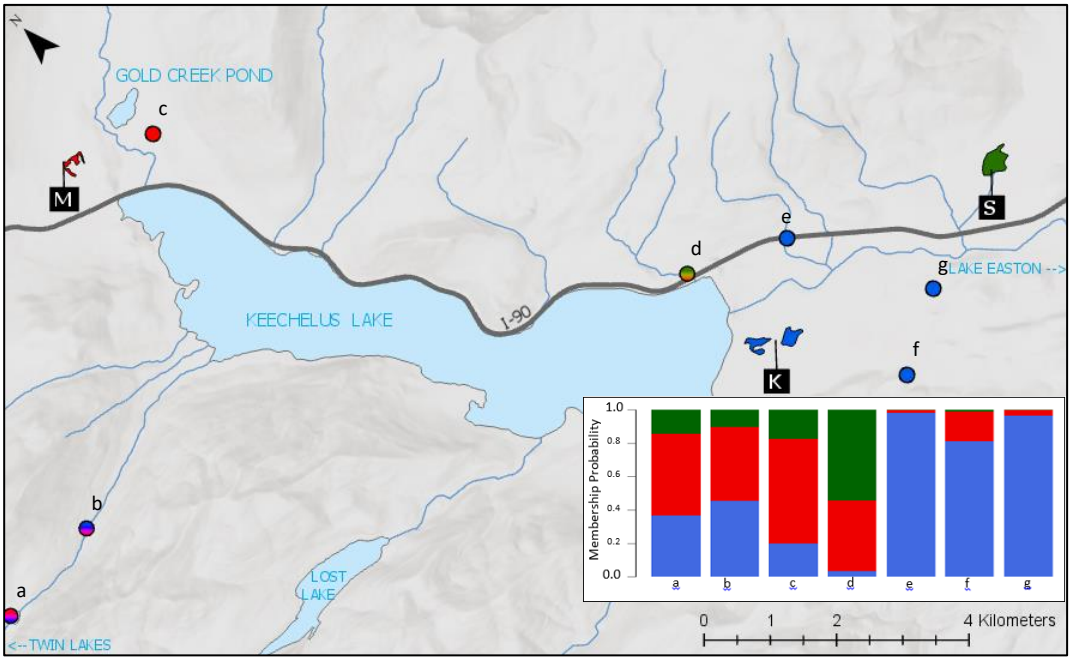

Fig. 8 Map of most likely breeding population membership of incidentally encountered western toads (*Anaxyrus boreas*). Breeding wetlands are represented with colored polygons and labeled by site ID (red = Mardee, blue = Keechelus, green = Swamp). Points labeled a-g represent incidentally encountered toads and are colored according to most likely breeding population membership. Two colors were used when the top two probabilities were within 15% of each other. The map insert contains a graph of these probabilities, as predicted by a DAPC using *a priori* groupings of n=18, n=39, and n=29 individuals from Mardee Lake, Keechelus Dam Ponds, and Swamp Lake, respectively.

IV

DISCUSSION

Breeding Status of Focal Sites

Toads were observed to breed in four out of five previously known breeding locations, showing that that viable breeding populations are present both north and south of the freeway. Although tadpoles were observed at Townsend in summer of 2018 for the first time (Fig. 9), no breeding or tadpole presence was observed in this area in 2019. This does not, however, exclude the possibility of a breeding population remaining in the area, especially since females do not breed every year. Townsend should continue to be observed to detect future breeding events of this newly established population.

Fig. 9 Western toad (*Anaxyrus boreas*) tadpoles observed for the first time at the Townsend mitigation area, in summer 2018.

Though no breeding toads were encountered at Lost Lake**,** Twilight Lake, or the swamplands near Lake Easton, a previous breeding sighting at Lost Lake has been confirmed (personal communication, Patty Garvey-Darda). Breeding toads were not found at Lost Lake during the course of the study, but this site should be surveyed in the future to add to the current dataset. Potential breeding sites at Twilight Lake and the swamplands near Lake Eastern may have been similarly missed, as western toads have exhibited a tendency to use only a small, easily overlooked patch of the available habitat for breeding. These locations have generally been characterized by shallower waters with plenty of grassy vegetation, and such areas should continue to be identified and monitored.

DArTseq™ Results in High-quality, High-quantity SNPs

The high-density DArTseq™ method proved to be effective for this study, resulting in a large number of high-quality SNPs. Though it is always best to supply the minimum recommended tissue weight whenever possible, sending in western toad samples that were as small as 0.5 mg, well below the 5 mg minimum recommendation, did not result in a loss of data. It seems reasonable that the two Townsend Pond samples held in reserve, as well as additional samples from previous years and future underweight samples, could be sent in for sequencing to add to the current dataset.

Of the returned SNPs, 15,468 were of high enough quality to use in all downstream analyses. This is many times greater than the number of SNPs needed to adequately distinguish between populations. For example, random sets of ~250-500

SNPs have been sufficient to identify weak spatial structure in studies of tiger salamander (*Ambystoma tigrinum*) and copperhead snake (*Agkistrodon contortrix*) populations (McCartney-Melstad et al. 2018; Maigret et al. 2020), while a panel of just 96 SNPs has been developed to assess parentage and relatedness in gray whales (*Eschrichtius robustus)* (DeWoody et al. 2017). Jahner et al. (2016) found that for Greater sage-grouse populations (*Centrocercus urophasianus*), precision in estimating F_{ST} initially increased with the number of SNPs, but plateaued at around 4000 SNPs. Similarly, a study using both high and low-density DArTseq to assess *Litoria ewingii – Litoria paraewingi* frog hybridization showed that the qualitative results obtained in the study did not vary with method (Melville et al. 2017). It seems likely that the more cost-effective, low-density sequencing could be sufficient to continue monitoring western toad populations in the I-90 study area, and this option should be explored in future studies.

Within-population Genetic Variation

Values of H_E were similar across all three sites (~0.26). These were comparable to other H_E values obtained in other SNP marker studies of amphibians, such as *Euproctus platycephalus* (0.20-0.30), *Rana italica* (0.21-0.29), and *Bufo andrewsi* (~0.26) (Guo et al. 2016; Rovelli et al. 2018). These data suggest that western toad populations in the study area exhibit a typical level of genetic diversity for amphibians, indicating they have not recently undergone a genetic bottleneck.

Between-population Differentiation

F_{ST} analysis confirmed that the two southern breeding ponds are representative of the same population, referred to in this study as the Keechelus Ponds (S). Though all pairwise F_{ST} values between Mardee Lake (NW), Swamp Lake (NE), and the Keechelus Ponds (S) populations were significantly different from zero, they were all less than 0.02. This is considered to be quite a low value. In comparison, Hartl and Clark (1997) classify F_{ST} values below 0.05 as representative of little genetic differentiation. F_{ST} values of 0.02 are considered "low" in a studies using microsatellite markers to assess ornate chorus frog (*Pseudacris ornate*) and jaguar (*Panthera onca*) populations (Degner et al. 2010; Menchaca et al. 2019), while a synthesis of amphibian microsatellite-based studies found a mean population F_{ST} of 0.106 ± 0.015 (Lawrence et al. 2019). In some cases, the significance of low F_{ST} values can be artifacts of sampling error, and may not truly represent biologically meaningful differences between populations (Wapples 1998).

However, given that the generation time of the western toad is ~6 years (COSEWIC 2012) and the fact that I-90 only began receiving heavy traffic 60-70 years (10+ toad generations) ago, any consequent genetic differentiation between populations is likely to be subtle at present. While a barrier effect may begin to be detected after relatively few generations have passed (5-10) (e.g., Lesbarréres et al. 2006; Clark et al. 2010), it may take hundreds of generations for F_{ST} values to reach equilibrium (Landguth et al. 2010). It is therefore unclear from F_{ST} values alone whether the subtle

statistical differences found between western toad populations in this study are biologically meaningful.

Increasing the spatial extent of sampling and repeated sampling across time have been suggested to increase confidence in low but significant F_{ST} values (Wapples 1998). Likewise, mark-recapture methods have been used to corroborate the significance of such values (e.g., Knutsen et al. 2010). Recently, the use of multivariate analyses in some studies has also been able to highlight biologically meaningful F_{ST} values as low as 0.023 in sage-grouse populations (Jahner et al. 2016) and 0.0037 in populations of coastal Atlantic cod (Knutsen et al. 2010). Multivariate analyses offered similar insight when applied to the current study.

When a *de novo* DAPC was forced to split the data into two groups (K=2), a clear pattern was observed. The first group consisted of mostly northern Swamp Lake (NE) and Mardee Lake (NW) individuals. The second group contained 29 of the 35 Keechelus Ponds (S) individuals, plus one Mardee Lake (NW) individual. This geographic pattern lends support to the possibility of biologically significant differences between northern and southern groups, indicating that I-90 or other landscape features may be acting as a barrier.

When this process was repeated for K=3, one group still consisted of Swamp Lake (NE) and Mardee Lake (NW) individuals, while the other two groups both contained mainly Keechelus Ponds (S) individuals. This was not representative of a geographic pattern. Such a division within a known breeding population is unlikely to be biologically meaningful. The fact that this separation occurred before the

discrimination of Swamp Lake (NE) and Mardee Lake (NW), at K=4, signifies that high levels of connectivity are maintained between the two populations.

Even with this high connectivity, a DAPC with *a priori* groups defined was able to discriminate between all three populations, confirming they are distinct breeding groups. The first axis separated Keechelus Ponds (S) from the northern populations very cleanly and highlighted a clear migrant individual, while the second axis separated Swamp Lake (NE) and Mardee Lake (NW), with some overlap between the two. Again, this shows that the difference between Keechelus Ponds (S) and the northern populations is greater than the difference between Swamp Lake (NE) and Mardee Lake (NW), even though the latter are representative of the greatest Euclidean distance (11.6 km). This result is quite striking, given that the distance between Swamp Lake (NE) and Keechelus Ponds (S) is less than a quarter of this length (2.5 km).

Evidence of Migration

The level of discrimination provided by the *a priori* DAPC allows evidence of migration to be assessed. Looking at the posterior assignment probabilities (Fig. 7), one clear migrant, sampled at Mardee Lake (NW) but given a 100% assignment to the Keechelus Ponds (S) population, stands out. This indicates that at least one individual has made it from Keechelus Ponds (S) to Mardee Lake (NW). Unfortunately, due to the timing of this study, it is impossible to say whether the undercrossings at Gold Creek and Hyak, constructed in 2012, may have facilitated this movement. Other underpasses in the study area have been more recently constructed (e.g., at Townsend Pond, mile 60.9, Price Creek, and Noble Creek) as well; however, these are more centrally located.

Were these structures responsible for facilitating migration between Keechelus Ponds (S) and Mardee Lake (NW), evidence of Keechelus Ponds (S)/Swamp Lake (NE) migration would be expected as well. If western toads at the Keechelus Ponds (S) use the margin of Keechelus Lake to the west to disperse, they would be likely to encounter the older crossing structures near Mardee Lake (NW), but far from Swamp Lake (NE). This or some other factor may be enabling toad movement between Keechelus Ponds (S) and Mardee Lake (NW) over Keechelus Ponds (S) and Swamp Lake (NE).

One other likely migrant, with an incorrect posterior assignment greater than 80%, was sampled at Swamp Lake (NE) but identified as a Mardee Lake (NW) individual. This suggests migration is possible between the two sites. An alternative explanation is that another northern breeding population exists which contributes migrants to both Swamp and Mardee (NW) Lakes, resulting in the observed evidence of genetic connectivity. However, the area between the two populations has been well surveyed and there are no other breeding populations between Swamp Lake (NE) and Mardee Lake (NW) (personal communication, Dr. Irwin), making direct migration between these two sites the more likely explanation. Movement across this 11.6 km distance seems plausible, given that the average western toad movement per month in the study area is 371 m, with a maximum monthly movement of 1976 m (Palmeri-Miles, 2012). Additionally, a study by Schmetterling and Young (2008) has documented individuals moving up to 13 km in under six weeks.

The other individuals incorrectly assigned by the *a priori* DAPC are less clearly defined, with assignment probabilities less than 80%. They may represent other

migrants, admixed individuals, or genetic outliers within their sampled population. It is interesting that no likely Keechelus Pond (S)/Swamp Lake (NE) migrants were observed in this study, as might be expected from their greater proximity and larger sample sizes, compared with Mardee Lake (NW). The recent expansion of underpasses at mile 60.9, Price Creek, and Nobel Creek (2018) and a culvert at Townsend Pond (2017), all near Swamp Lake (NE), may result in the observation of Keechelus Pond (S) /Swamp Lake (NE) migrants in future studies.

Incidental Assignments

Toads incidentally encountered near Twin Lakes show that this area is part of some individuals' home ranges and could potentially include a breeding site. Twin Lakes and nearby wetlands and water features (including Lost Lake) should be surveyed during the breeding season. All other incidentally encountered toads were found near known breeding sites.

When the *a priori* DAPC was used to predict the population assignment of these incidentally encountered individuals, assignments were in line with the previously discussed north/south split (Fig. 8). Surprisingly, the northern individual *d*, from Townsend Pond, though only 1.6 km away from the Keechelus Ponds (S), more likely came from a northern population. Swamp Lake (NE) is 3.5 km from Townsend Pond, while Mardee Lake (NW) is 8.4 km distant. Similarly, the southern individual *g*, though closest spatially to Swamp Lake (NE), was assigned to the Keechelus Ponds (S) population.

The northern individual *c*, sampled near Gold Creek, was assigned to the Mardee Lake (NW) population. This was an expected result, as previous telemetry work has shown that other toads found in the Gold Creek area breed at Mardee Lake (NW) (Palmeri-Miles 2012). Individuals *e* and *f* were assigned to the breeding site nearest them, Keechelus Ponds (S). Individual *f* was located south of the freeway; individual *e* was a subadult found directly beneath I-90. It was using the Price Creek undercrossing, which was constructed in the summer of 2018. While it is unclear which direction this individual was coming from or whether it made a complete crossing, this suggests that the structure is suitable for western toad movement and may be used by the species within a year of completion.

Southern individuals *a* and *b* did show high probabilities associated with both Mardee Lake (NW) and the Keechelus Ponds (S). While this may be indicative of migration and/or admixture between Mardee Lake (NW) and Keechelus Ponds (S), they may also represent genetic outliers belonging to one or the other population. Alternatively, the DAPC's low ability to discriminate the origin of these two individuals may be associated with the presence of a separate, unsampled breeding population to which these individuals belong. As stated previously, the Lost Lake area is known to have a breeding population which was not located in this study. Sampling should be conducted to determine whether individuals *a* and *b* belong to this population. The Twin Lakes area should also be surveyed for other overlooked breeding groups to which they may belong.

Additional Considerations and Future Work

It is possible that I-90 is contributing to the suggested north/south divide; however, other geographic features exist that may also pose a barrier to toad movement, such as the Yakima River and Keechelus Lake. The Yakima River experiences low flow volumes during the early spring and fall seasons and is unlikely to pose a year-round barrier to toad movement. Keechelus Lake would only separate Mardee Lake (NW) and Keechelus Ponds (S) breeding sites, and could theoretically be traveled around, unlike I-90.

The newly constructed crossing structures along I-90 are anticipated to reduce any barrier effect associated with it. As F_{ST} responds more rapidly barrier removal than to barrier placement (Landguth et al. 2010), replicating the current study design in a couple of toad generations – approximately12 years (COSEWIC 2012) – could indicate which feature is causing the divide. If the north/south split is still present, this could suggest that the crossing structures do not facilitate toad movement across I-90, or that the lake and river are the major cause of the divide. However, if the north/south split is not evident in a future DAPC (and F_{ST} values decrease), this could point to effective mitigation of I-90 by the new crossing structures.

In the intervening years, intensive mark/recapture studies could complement this work to get a better idea of current migration and use of crossing structures (Neigel 2002). Additionally, the sampling of Townsend Pond if a breeding event occurs here again, as well as locating other breeding sites in the study area to sample, will be useful in obtaining a more complete picture of genetic connectivity in the region.

Another consideration is that the majority of the individuals sampled in this study were male. Though no statistically significant difference has been observed between male and female movement at the study site (Palmeri-Miles 2012), other studies have indicated that females move greater distances and have larger home ranges (e.g., Muths 2003). It is likely that sex-linked dispersal is operating in this system, as has been noted for other amphibian species (Helfer et al. 2012; Wang et al. 2012), which could result in biased estimates of genetic differentiation (Prugnolle and Meeus 2002; Tucker et al. 2017; Sawaya et al. 2019). Future studies should prioritize collecting more samples from female toads to discern whether the effects of barriers on females are similar to those seen in males.

V

CONCLUSION

Although the level of genetic differentiation between western toad populations in the study area is slight, it does appear to be biologically meaningful and geographically representative of a north/south split. Whereas I-90 is the most obvious potential barrier, we cannot yet distinguish the effects of the freeway from other potential barriers, such as the Yakima River. It is recommended that this study design be replicated in the future to determine if the crossing structures recently installed in the area have an observable mitigating effect.

The use of SNP markers has shown to be an effective method to resolve subtle differences between western toad populations. As the high resolution provided by these markers allowed populations to be distinguished by DAPC, the use of SNP markers would be useful in studies of home-ranges. Toads incidentally encountered over the next several years can be introduced to the DAPC developed here for population assignment. SNP analyses may be suitable for application to other species in the study area, such as salamanders, alligator lizards, and small mammals, as well as for monitoring other crossing structure projects.

As with most landscape genetic research, this study would have been more informative if sampling had taken place sooner. The Gold Creek and Hyak undercrossings were constructed roughly one toad generation ago – enough time to change dispersal and migration patterns between Mardee Lake (NW) and Keechelus Ponds (S) sites which, if they exist, will have gone unobserved. However, continuing to

collect a time series of population samples and F_{ST} values should reveal if population structure in the study area is shifting. Combining this with more intensive mark/recapture and radio telemetry efforts could be a powerful means of monitoring the current state of western toad migration.

REFERENCES

Al-Breiki RD, Kjeldsen SR, Afzal H, Hinai MSA, Zenger KR, Jerry DR, Al-Abri MA, Delghandi M. 2018. Genome-wide SNP analyses reveal high gene flow and signatures of local adaptation among the scalloped spiny lobster *(Panulirus homarus*) along the Omani coastline. BMC Genomics. 19:690.

Alcala N, Streit D, Goudet J, Vuilleumier S. 2013. Peak and persistent excess of genetic diversity following an abrupt migration increase. Genet. 193(3):953–971.

Alhusain L, Hafez AM. 2018. Nonparametric approaches for population structure analysis. Hum Genomics. 12(1):25.

Andrews KR, Good JM, Miller MR, Luikart G, Hohenlohe PA. 2016. Harnessing the power of RADseq for ecological and evolutionary genomics. Nat Rev Genet. 17(2):81–92.

Antolin MF, Schoettle AW. 2001. Fragments, extinction, and recolonization: the genetics of metapopulations. In: Joyce, D, Simpson JD, editors. Part 2. Genetic Resource Management: Building Strategies for the New Millennium; Proceedings of the 27th Biennial Conference of the Canadian Tree Improvement Association; August 15-17, 2000; Sault Ste. Marie, Ontario. Natural Resources Canada, Canadian Forest Service. p. 37-46.

Balzarini M, Teich I, Bruno C, Peña A. 2011. Making genetic biodiversity measurable: a review of statistical multivariate methods to study variability at gene level. Rev Fac Cienc Agrar. 43(1):261–275.

Bartelt PE, Peterson CR, Klaver RW. 2004. Sexual differences in the post-breeding movements and habitats selected by western toads (*Bufo boreas*) in southeastern Idaho. Herpetol. 60:455–467.

Bartoszek J, Greenwald K. 2009. A population divided: railroad tracks as barriers to gene flow in an isolated population of marbled salamanders (*Ambystoma opacum*). Herpetol Conserv Biol. 4(2):191-197.

Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, Hall KP, Evers DJ, Barnes CL, Bignell HR, et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. Nature. 456(7218):53-59.

Bhatia G, Patterson N, Sankararaman S, Price AL. 2013. Estimating and interpreting FST: The impact of rare variants. Genome Res. 23(9):1514–1521.

Browne CL, Paskowski CA. 2010. Hibernation sites of western toads (*Anaxyrus boreas*): characterization and management implications. Herpetol Conserv Biol. 5(1):49-63.

Bruinderink GG, Hazebroek E. 1996. Ungulate traffic collisions in Europe. Conserv Biol. 10(4):1059–1067.

Bull EL. Carey C. 2008. Breeding frequency of western toads (*Bufo boreas*) in northeastern Oregon. Sci Jo. 3(2):282-288.

Bull EL. 2006. Sexual differences in the ecology and habitat selection of western toads (*Bufo boreas*) in northeastern Oregon. Herpetol Conserv Biol. 1:27–38.

Burton J, Cutler D, Marsden J, Sherwood BR. 2002. Wildlife and roads: the ecological impact. World Scientific.

Carey C. 1993. Hypothesis concerning the causes of the disappearance of boreal toads from the mountains of Colorado. Conserv Biol. 7(2):355-362.

Carpenter CC. 1954. A study of amphibian movement in the Jackson Hole wildlife park. Copeia. 1954:197.

Ceballos G, Ehrlich PR, Barnosky AD, García A, Pringle RM, Palmer TM. 2015. Accelerated modern human–induced species losses: Entering the sixth mass extinction. Sci Adv. 1:e1400253.

Chaichoompu K, Abegaz F, Tongsima S, Shaw PJ, Sakuntabhai A, Pereira L, Van Steen KV. 2018. KRIS: Keen and Reliable Interface Subroutines for Bioinformatic Analysis. R package version 1.1.1. https://CRAN.R-project.org/package=KRIS

Clark RW, Brown WS, Stechert R, Zamudio KR. 2010. Roads, interrupted dispersal, and genetic diversity in timber rattlesnakes. Conserv Biol 24(4):1059-1069.

Clevenger AP, Huijser MP. 2011. Wildlife crossing structure handbook design and evaluation in North America. Lakewood Co.: U.S. Federal Highway Administration Central Federal Lands Highway Division. Publication No.: FHWA-CFL/TD-11-003.

Coleman JRI, Euesden J, Patel H, Folarin AA, Newhouse S, Breen G. 2016. Quality control, imputation and analysis of genome-wide genotyping data from the Illumina HumanCoreExome microarray. Brief Funct Genomics. 15(4):298-304.

Corlatti L, Hackländer K, Frey-Roos F. 2009. Ability of wildlife overpasses to provide connectivity and prevent genetic isolation. Conserv Biol. 23(3):548–556.

Cosentino BJ, Marsh DM, Jones KS, Apodaca JJ, Bates C, Beach J, Beard KH, Becklin K, Bell JM, Crockett C, et al. 2014. Citizen science reveals widespread negative effects of roads on amphibian distributions. Biol Conserv. 180:31-38.

COSEWIC (Committee on the Status of endangered Wildlife in Canada). 2012. COSEWIC assessment and status report on the western toad *Anaxyrus boreas* in

Canada. [Internet]. [accessed 2020 March 20]. COSEWIC, Ottawa, Ontario, Canada. xiv:1-71. https://www.registrelep-sararegistry.gc.ca/default_e.cfm

Courtois B, Audebert A, Dardou A, Roques S, Ghneim-Herrera T, Droc G, Frouin J, Rouan L, Gozé E, Kilian A, et al. 2013. Genome-wide association mapping of root traits in a japonica rice panel. PLoS One. 8(11):e78037.

Couvet D. 2002. Deleterious effects of restricted gene flow in fragmented populations. Conserv Biol. 16(2):369-376.

Cruz VMV, Kilian A, Dierig DA. 2013. Development of DArT marker platforms and genetic diversity assessment of the U.S. collection of the new oilseed crop Lesquerella and related species. PLoS ONE. 8(5):e64062.

Cummins D, Kennington WJ, Rudin-Bitterli T, Mitchell NJ. 2019. A genome-wide search for local adaptation in a terrestrial-breeding frog reveals vulnerability to climate change. Glob Chang Biol. 25(9):3151–3162.

Cushman SA. 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. Biol Conserv. 128:231–240.

Daugherty CH, Sheldon AL. 1982. Age-specific movement patterns of the frog *Ascaphus truei*. Herpetologica. 38(4):468-474.

Davey JW, Hohenlohe PA, Etter PD, Boone JQ, Catchen JM, Blaxter ML. 2011. Genome-wide genetic marker discovery and genotyping using next-generation sequencing. Nat Rev Genet. 12(7):499–510.

Davis TM, Gregory PT. 2003. Decline and local extinction of the western toad, *Bufo boreas*, on Southern Vancouver Island, British Columbia, Canada. Herpetol. 34:350–352.

Degner JF, Silva DM, Hether TD, Daza JM, Hoffman EA. 2010. Fat frogs, mobile genes: unexpected phylogeographic patterns for the ornate chorus frog (*Pseudacris ornata*). Mol Ecol. 19(12):2501-2515.

DeWoody JA, Fernandez NB, Brüniche-Olsen A, Antonides JD, Doyle JM, Miguel PS, Westerman R, Vertyankin VV, Godard-Codding CAJ, Bickham JW. 2017. Characterization of the gray whale *Eschrichtius robustus* genome and a genotyping array based on single-nucleotide polymorphisms in candidate genes. Biol Bull. 232(3):186–197.

Dixo M, Metzger JP, Morgante JS, Zamudio KR. 2009. Habitat fragmentation reduces genetic diversity and connectivity among toad populations in the Brazilian Atlantic Coastal Forest. Biol Conserv. 142:1560–1569.

Drost CA, Fellers GM. 1996. Collapse of aregional frog fauna in the Yosemite area of the California Sierra Nevada, USA. Conserv Biol. 10:414–425.

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS ONE. 6(5):e19379.

Epps CW, Keyghobadi N. 2015. Landscape genetics in a changing world: disentangling historical and contemporary influences and inferring change. Mol Ecol. 24(24):6021–6040.

ESRI. 2018. World Imagery. Redlands, CA: Environmental Systems Research Institute. https://services.arcgisonline.com/ArcGIS/rest/services/World_Imagery/MapServer/0

ESRI. c2020. ArcGIS Pro: 2.5.0. Redlands, CA: Environmental Systems Research Institute.

Fahrig L, Pedlar JH, Pope SE, Taylor PD, Wegner JF. 1995. Effect of road traffic on amphibian density. Biol Conserv. 73:177-182.

Garwood JM. 2009. Spatial ecology of the cascades frog: identifying dispersal, migration, and resource uses at multiple spatial scales [thesis]. Humboldt State University.

Gemayel R, Cho J, Boeynaems S, Verstrepen KJ. 2012. Beyond junk-variable tandem repeats as facilitators of rapid evolution of regulatory and coding sequences. Genes. 3(3):461–480.

Glista DJ, Devault TL, Dewoody JA. 2009. A review of mitigation measures for reducing wildlife mortality on roadways. Landscape Urban Plann. 91:1–7.

Goldberg CS, Waits LP. 2010. Quantification and reduction of bias from sampling larvae to infer population and landscape genetic structure. Mol Ecol Resour. 10(2):304–313.

Grewe PM, Feutry P, Hill PL, Gunasekera RM, Schaefer KM, Itano DG, Fuller DW, Foster SD, Davies CR. 2015. Evidence of discrete yellowfin tuna (*Thunnus albacares*) populations demands rethink of management for this globally important resource. Sci Rep. 5:16916.

Gruber B, Georges A. 2019. DartR: Importing and analysing snp and silicodart data generated by genome-wide restriction fragment analysis. R package version 1.1.11. https://CRAN.R-project.org/package=dartR

Guo B, Lu D, Liao WB, Merilä J. 2016. Genomewide scan for adaptive differentiation along altitudinal gradient in the Andrew's toad *Bufo andrewsi*. Mol Ecol. 25(16):3884-3990.

Gutiérrez-Rodríguez J, Gonçalves J, Civantos E, Martínez-Solano I. 2017. Comparative landscape genetics of pond-breeding amphibians in Mediterranean temporal wetlands: The positive role of structural heterogeneity in promoting gene flow. Mol Ecol. 26(20):5407–5420.

Haddad NM, Brudvig LA, Clobert J, Davies KF, Gonzalez A, Holt RD, Lovejoy TE, Sexton JO, Austin MP, Collins CD, et al. 2015. Habitat fragmentation and its lasting impact on Earth's ecosystems. Sci Adv. 1(2):1500052.

Hartl DL, Clark AG. 1997. Principles of population genetics. Sunderland, MA: Sinauer Associates.

Hayes T, Chan R, Light P. 1993. Interactions of temperature and steroids on larval growth, development, and metamorphosis in a toad (*Bufo boreas*). J Exp Zool. 266:206-215.

Hedrick PW. 2005. A standardized genetic differentiation measure. Evol. 59(8):1633-1638.

Helfer V, Broquet T, Fumagalli L. 2012. Sex-specific estimates of dispersal show female philopatry and male dispersal in a promiscuous amphibian, the alpine salamander (*Salamandra atra*). Mol Ecol. 21(19):4706–4720.

Hoffman JR, Willoughby JR, Swanson BJ, Pangle KL, Zanatta DT. 2017. Detection of barriers to dispersal is masked by long lifespans and large population sizes. Ecol Evol. 00:1-11. https://doi.org/10.1002/ece3.3470

Hudson RR, Slatkin M, Maddison WP 1992. Estimation of levels of gene flow from DNA sequence data. Genetics. 132:583-589.

IUCN SSC Amphibian Specialist Group. 2015. *Anaxyrus boreas*. The IUCN red list of threatened species. [accessed 2020 March 20]; 2015: e.T3179A53947725. https://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T3179A53947725.en.

I-90 corridor thin project, environmental assessment. 2008. United States Department of Agriculture [USDA] Forest Service; [accessed 2020 April 11]. https://www.fs.usda.gov/nfs/11558/www/nepa/38722_FSPLT1_018009.pdf

I-90 Snoqualmie Pass East Project, Environmental Impact Statement. 2008. Washington State Department of Transportation [WSDOT]; [accessed 2020 April 11]. https://www.wsdot.wa.gov/sites/default/files/2019/03/06/summary-eis-i-90-project.pdf

I-90 - Snoqualmie Pass East - Keechelus Dam Vicinity to Stampede Pass Interchange (Phase 2) - Complete October 2019. c2020. Washington State Department of Transportation [WSDOT]; [accessed 2020 March 21]. https://www.wsdot.wa.gov/Projects/I90/SnoqualmiePassEast/KeechelusDamtoCabinCreek/home

I-90 - Snoqualmie Pass East - Project History. c2020. Washington State Department of Transportation [WSDOT]; [accessed 2020 March 21]. https://www.wsdot.wa.gov/Projects/I90/SnoqualmiePassEast/History.htm

Jackson T. 2004. Report on the status and conservation of the boreal toad *Bufo boreas boreas* in the southern Rocky Mountains. [Internet]. [accessed 2020 March 20]. The Boreal Toad Recovery Team, Colorado Division of Wildlife. https://cpw.state.co.us/Documents/Research/Aquatic/pdf/BUBOstatus04.pdf

Jahner JP, Gibson D, Weitzman CL, Blomberg EJ, Sedinger JS, Parchman TL. 2016. Fine-scale genetic structure among greater sage-grouse leks in central Nevada. BMC Evol Biol. 16:127.

Jakobsson M, Edge MD, Rosenberg NA. 2013. The relationship between FST and the frequency of the most frequent allele. Genet. 193(2):515–528.

Jameson DL. 1956. Growth, dispersal and survival of the Pacific tree frog. Copeia 1956(1): 25-29.

Johnston B, 1999. Terrestrial Pacific giant salamanders (*Dicamptodon tenebrosus* Good) – natural history and their response to forest practices [thesis]. University of British Columbia.

Jombart T. 2008. adegenet: A R package for the multivariate analysis of genetic markers. Bioinformatics. 24(11):1403–1405.

Jombart T, Ahmed I. 2011. adegenet 1.3-1: New tools for the analysis of genome-wide SNP data. Bioinformatics. 27(21):3070–3071.

Jombart T, Collins C. 2015. A tutorial for discriminant analysis of principal components (DAPC) using adegenet 2.0.0. MRC Centre for Outbreak Analysis and Modelling.

Jombart T, Devillard S, Balloux F. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet. 11:94.

Jones OR, Wang J. 2010. COLONY: a program for parentage and sibship inference from multilocus genotype data. Mol Ecol Resour. 10(3):551–555.

Jost L. 2008. GST and its relatives do not measure differentiation. Mol Ecol. 17(18):4015–4026.

Kalinowski ST. 2002. Evolutionary and statistical properties of three genetic distances. Mol Ecol. 11(8):1263–1273.

Kilian A, Wenzl P, Huttner E, Carling J, Xia L, Blois H, Caig V, Heller-Uszynska K, Jaccoud D, Hopper C, et al. 2012. Diversity Arrays Technology: A generic genome profiling technology on open platforms. Methods Mol Biol. 888:67–89.

Knutsen H, Olsen EM, Jorde PE, Espeland SH, André C, Stenseth NC. 2010. Are low but statistically significant levels of genetic differentiation in marine fishes 'biologically meaningful'? A case study of coastal Atlantic cod. Mol Ecol. 20(4):768–783.

Kuehn R, Hindenlang KE, Holzgang O, Senn J, Stoeckle B, Sperisen C. 2007. Genetic effect of transportation infrastructure on roe deer populations (*Capreolus capreolus*). J Hered. 98(1):13–22.

Lal MM, Southgate PC, Jerry DR, Bosserelle C, Zenger KR. 2017. Swept away: ocean currents and seascape features influence genetic structure across the 18,000 Km Indo-Pacific distribution of a marine invertebrate, the black-lip pearl oyster *Pinctada margaritifera*. BMC Genomics. 18:66.

Landguth EL, Cushman SA, Schwartz MK, McKelvey KS, Murphy M, Luikart G. 2010. Quantifying the lag time to detect barriers in landscape genetics. Mol Ecol Resour. 19:4179-4191.

Landguth EL, Fedy BC, Oyler-Mccance SJ, Garey AL, Emel SL, Mumma M, Wagner HH, Fortin M-J, Cushman SA. 2012. Effects of sample size, number of markers, and allelic richness on the detection of spatial genetic pattern. Mol Ecol Resour. 12(2):276–284.

Lawrence ER, Benavente JN, Matte J-M, Marin K, Wells ZRR, Bernos TA, Krasteva N, Habrich A, Nessel GA, Koumrouyan RA, et al. 2019. Geo-referenced population-specific microsatellite data across American continents, the MacroPopGen Database. Sci Data. 6(1):14.

Leakey, R., Lewin. R. (1995). The Sixth Extinction: Patterns of life and the future of humankind. New York (NY): Doubleday.

Lemopoulos A, Prokkola JM, Uusi-Heikkilä S, Vasemägi A, Huusko A, Hyvärinen P, Koljonen ML, Koskiniemi J, Vainikka A. 2019. Comparing RADseq and microsatellites for estimating genetic diversity and relatedness — Implications for brown trout conservation. Ecol Evol. 9(4):2106-2120.

Lesbarrères D, Primmer CR, Lodé T, Merilä J. 2006. The effects of 20 years of highway presence on the genetic structure of *Rana dalmatina* populations. Ecoscience. 13(4):531–538.

Livo LJ, Yeakley D. 1997. Comparison of current with historical elevational range in the boreal toad, *Bufo boreas*. Herpetol Rev. 28:143–144.

Lloyd MW, Campbell L, Neel MC. 2013. The power to detect recent fragmentation events using genetic differentiation methods. PLoS One. 8(5):e63981.

Ma L, Ji Y-J, Zhang D-X. 2015. Statistical measures of genetic differentiation of populations: Rationales, history and current states. Curr Zool. 61:886–897.

Maigret TA, Cox JJ, Weisrock DW. 2020. A spatial genomic approach identifies time lags and historical barriers to gene flow in a rapidly fragmenting Appalachian landscape. Mol Ecol. 29(4):673–685.

Matsuda BM, Green DM, Gregory PT. 2006. Amphibians and reptiles of British Columbia. R BC Mus Handbs.

Maxell BA, Nelson KJ, Browder S. 2002. Record clutch size and observations on breeding and development of the western toad (*Bufo boreas*) in Montana. Northwest Nat. 83(1):27-30.

Mazerolle MJ 2004. Amphibian road mortality in response to nightly variations in traffic intensity. Herpetologica. 60(1):45-53.

McCallum H, Dobson A. 2002. Disease, habitat fragmentation, and conservation. Proc R Soc Lond B. 269:2014-2049.

McCartney-Melstad E, Vu JK, Shaffer HB. 2018. Genomic data recover previously undetectable fragmentation effects in an endangered amphibian. Mol Ecol. 27(22):4430–4443.

Mech, S.G. and Hallett, J.G. 2001. Evaluating the effectiveness of corridors: a genetic approach. Conserv Biol. 15(2):467–474.

Meirmans PG, Hedrick PW. 2010. Assessing population structure: FST and related measures. Mol Ecol Resour. 11(1):5–18.

Melville J, Haines ML, Boysen K, Hodkinson L, Kilian A, Date KLS, Potvin DA, Parris KM. 2017. Identifying hybridization and admixture using SNPs: application of the DArTseq platform in phylogeographic research on vertebrates. R Soc Open Sci. 4(7):161061.

Menchaca A, Rossi NA, Froidevaux J, Dias-Freedman I, Caragiulo A, Wultsch C, Harmsen B, Foster R., Antonio de la Torre J, Medellin RA, et al. 2019. Population

genetic structure and habitat connectivity for jaguar (*Panthera onca*) conservation in Central Belize. BMC Genet. 20:100.

Mohagen T. 2019. I-90 Snoqualmie Pass East Project, Phase 1 (Sunset Highway) Mitigation Site. [Internet]. [accessed 2020 April 11]. Environmental Services Office, Washington State Depatment of Transportation [WSDOT]. Report No.: NWS-2007-2080. https://www.wsdot.wa.gov/sites/default/files/2019/03/28/Env-Wet-MonRpt-SunsetHwy2018.pdf

Mullally DP. 1952. Habits and minimum temperatures of the toad *Bufo boreas halophilus*. Copeia. 1952:274-276.

Muths E. 2003. Home range and movements of boreal toads in undisturbed habitat. Copeia. 2003(1):160–165.

Muths E, Corn PS, Pessier AP, Green DE. 2003. Evidence for disease-related amphibian decline in Colorado. Biol Conserv. 110:357–365.

Nazareno AG, Bemmels JB, Dick CW, Lohmann LG. 2017. Minimum sample sizes for population genomics: an empirical study from an Amazonian plant species. Mol Ecol Resour. 17(6):1136–1147.

Nei M. 1973. Analysis of gene diversity in subdivided populations. Proc Natl AcadSci. 70:3321–3323.

Neigel, J.E. 2002. Is F_{ST} obsolete? Conserv Genet. 3:167–173.

NWI data desktop/mobile viewer [Internet]. Updated 2019. National Wetlands Inventory, U.S. Fish & Wildlife Service. [accessed 2020 January 23]. https://www.fws.gov/wetlands/Data/Mapper.html

O'Connell KA, Mulder KP, Maldonado J, Currie KL, Ferraro DM. 2019. Sampling related individuals within ponds biases estimates of population structure in a pond-breeding amphibian. Ecol Evol. 9(6):3620-3636.

Olson DH. 1988. The ecological and behavioral dynamics of breeding in three sympatric anuran amphibians [thesis]. Oregon State University.

Palmeri-Miles A. 2012. Seasonal movement patterns and overwintering of western toads (*Anaxyrus Boreas*) near Snoqualmie Pass, WA [thesis]. Central Washington University.

Pembleton LW, Cogan NOI, Forster JW. 2013. StAMPP: An R package for calculation of genetic differentiation and structure of mixed-ploidy level populations. Mol Ecol Resour. 13(5):946–952.

Pereira HM, Leadly PW, Proença V, Alkemade R, Scharlemann JPW, Fernandez-Manjarrés JF, Araújo MB, Balvanera P, Biggs R, Cheung WWL, et al. 2010. Scenarios for global biodiversity in the 21st century. Sci. 330:1496–1501.

Peterman WE, Anderson TL, Ousterhout BH, Drake DL, Semlitsch RD, Eggert LS. 2015. Differential dispersal shapes population structure and patterns of genetic differentiation in two sympatric pond breeding salamanders. Conserv Genet. 16:59-69.

Pievani T. 2014. The sixth mass extinction: Anthropocene and the human impact on biodiversity. Rend. 25:85–93.

Prugnolle F, Meeus TD. 2002. Inferring sex-biased dispersal from population genetic tools: a review. Heredity. 88(3):161–165.

R Core Team. 2019. R: A language and environment for statistical computing. R Foundation for statistical computing, Vienna, Australia.

Raman R, Cowley RB, Raman H, Luckett DJ. 2014. analyses using SSR and DArT molecular markers reveal that Ethiopian accessions of white lupin (*Lupinus albus l.*) represent a unique genepool. Open J Genet. 4:87–98.

Richardson JL. 2012. Divergent landscape effects on population connectivity in two co-occurring amphibian species. Mol Ecol. 21(18):4437–4451.

Rovelli V, Ruiz-González A, Vignoli L, Macale D, Buono V, Davoli F, Vieites DR, Pezaro N, Randi E. 2018. Genotyping-by-Sequencing (GBS) of large amphibian genomes: A comparative study of two non-model species endemic to Italy [online]. Anim Biol. DOI: 10.1163/15707563-00001094

Rytwinski T, Ree RVD, Cunnington GM, Fahrig L, Findlay CS, Houlahan J, Jaeger JA, Soanes K, Grift EAVD. 2015. Experimental study designs to improve the evaluation of road mitigation measures for wildlife. J Environ Manage. 154:48–64.

Safner T, Miller MP, Mcrae BH, Fortin M-J, Manel S. 2011. Comparison of Bayesian clustering and edge detection methods for inferring boundaries in landscape genetics. Int J Mol Sci. 12(2):865–889.

Samollow PB. 1980. Selective mortality and reproduction in a natural population of *Bufo boreas*. Evol. 34(1):18-39.

Sansaloni, C., Petroli, C., Jaccoud, D., Carling, J., Detering, F., Grattapaglia, D., et al. 2011. Diversity Arrays Technology (DArT) and next-generation sequencing combined: genome-wide, high throughput, highly informative genotyping for molecular breeding of *Eucalyptus*. BMC Proc. 5:p54.

Sawaya MA, Clevenger AP, Schwartz MK. 2019. Demographic fragmentation of aprotected wolverine population bisected by a major transportation corridor. Biol Conserv. 236:616–625.

Schaffer HB, Gidid M, McCartney-Melstad E, Neal KM, Oyamaguchi HM, Tellez M, Toffelmier EM. 2015. Conservation genetics and genomics of amphibians and reptiles. Annu Rev Anim Biosci. 3:113-118.

Schmetterling DA, Young MK. 2008. Summer movements of boreal toads (*Bufo Boreas Boreas*) in two western Montana basins. J Herpetol. 42:111–123.

Schoch RR. 2009. Evolution of life cycles in early amphibians. Annu Rev Earth Planet Sci. 37:135-162.

Shirk, AJ. 2009. Mountain goat genetic structure, molecular diversity, and gene flow in the Cascade Range, Washington [thesis]. Western Washington University.

Singleton PH, Lehmkuhl JF. 2000. I-90 Snoqualmie Pass wildlife habitat linkage assessment. Final Report to the Washington State Department of Transportaion GCA1177. USDA Forest Service Pacific Northwest Research Station. PNW-98-0513-CC.

Soanes K, Taylor AC, Sunnucks P, Vesk PA, Cesarini S, Ree RVD. 2017. Evaluating the success of wildlife crossing structures using genetic approaches and an experimental design: Lessons from a gliding mammal. J Appl Ecol. 55:129–138.

Spear S, Baumsteiger J, Storfer A. 2011. Type N experimental buffer treatment study: Baseline measures of genetic diversity and gene flow of three stream-associated amphibians. Washington. Washington State Forest Practices Adaptive Management Program Cooperative Monitoring, Evaluation, and Research Committee (CMER) Report. Report No.: CMER 06-605.

Stift M, Kolář F, Meirmans PG. 2019. Structure is more robust than other clustering methods in simulated mixed-ploidy populations. Heredity. 123:429–441.

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Status and Trends of Amphibian Declies and Extinctions Worldwide. Sci. 306(5702):1783-6.

Swanson DL, Graves BM, Koster KL. 1996. Freezing tolerance/intolerance and cryoprotectant synthesis in terrestrially overwintering anurans in the Great Plains, USA. J Comp Physiol B. 166:110–119.

Switzer JF, Johnson R, Lubinski BA, King TL. 2009. Genetic structure in the *Anaxyrus boreas* species group (Anura, *Bufonidae*): an evaluation of the Southern Rocky Mountain population. A final report submitted to the U.S. Fish and Wildlife Service,

Mountain-Prairie Region. United States Geological Survey, Leetown Science Center, Kearneysville, West Virginia, USA.

Takahata, N., Nei, M. 1984. FST and GST statistics in the finite island model. Genet. 107(3):501-504.

Thomas, JA, Telfer MG, Roy DB, Preston CD, Greenwood JD, Asher J, Fox R, Clarke RT, Lawton JH. 2004. Comparative losses of British butterflies, birds, and plants and the global extinction crisis. Sci. 303(5665):1879-1881.

Titus V, Madison D, Green T. 2014. the importance of maintaining upland forest habitat surrounding salamander breeding ponds: case study of the eastern tiger salamander in New York, USA. Forests. 5(12):3070–3086.

Tracy CR, Dole JW. 1969. Orientation of displaced california toads, *Bufo boreas*, to their breeding sites. Copeia. 1969(4):693-700.

Tucker JM, Allendorf FW, Truex RL, Schwartz MK. 2017. Sex-biased dispersal and spatial heterogeneity affect landscape resistance to gene flow in fisher. Ecosphere. 8(6):e01839.

Van Manen FT, McCollister MF, Nicholson JM, Thompson LM, Kindall JL, Jones MD. 2012. Short-term impacts of a 4-lane highway on American black bears in eastern North Carolina. Wildl Monogr. 181:1-35.

Vos CC, Jong AGA-D, Goedhart PW, Smulders MJM. 2001. Genetic similarity as a measure for connectivity between fragmented populations of the moor frog (*Rana arvalis*). Heredity. 86(5):598–608.

Wake DB, Vredenburg VT. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Natl Acad Sci USA. 105:11466–11473.

Wang Y, Lane A, Ding P. 2012. Sex-biased dispersal of a frog (*Odorrana schmackeri*) is affected by patch isolation and resource limitation in a fragmented landscape. PLoS ONE. 7(10):e47683.

Wapples RS. 1998. Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. J Hered. 89(5):438-450.

Weir BS, Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. Evol. 38(6):1358-1370.

Whitlock MC. 2011. G'ST and D do not replace FST. Mol Ecol. 20(6):1083–1091.

Willing E-M, Dreyer C, Oosterhout CV. 2012. Estimates of genetic differentiation measured by fst do not necessarily require large sample sizes when using many SNP markers. PLoS One. 7:e42649.

Wood SLR, John SR. 2009. Impact of sediment and nutrient inputs on growth and survival of tadpoles of the western toad. Freshwater Biol. 54:1120–1134.

Wright S.1950. Genetical structure of populations. Nature. 166:247-249.

Wright S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. Evol. 19(3):395-420.