# EPIDEMIOLOGY OF AN EMERGING VIRUS IN WESTERN TIGER SALAMANDERS (*AMBYSTOMA MAVORTIUM*) IN SOUTHWESTERN ALBERTA

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ABSTRACT

Emerging infectious diseases are one of the factors that have contributed to global amphibian population declines over the past few decades. The recent emergence of the highly virulent *Amybstoma tigrinum* virus (ATV) in tiger salamanders at three sites in Alberta has led to concerns regarding the population status of this prairie icon. Results from my population surveys showed that tiger salamanders occurred in approximately 50% of 15 sites in southwestern Alberta, but densities were low. In a longitudinal survey of a salamander population in Livingston Lake, Alberta in 2013 and 2014, ATV was strongly seasonal, increasing from 0 to 100% prevalence between early July and metamorphosis in mid-August. Despite a consistent seasonal pattern of exposure to ATV, host mortality was high in one year, but low in the next. These results suggest that ecological factors (i.e. triggers) act in addition to ATV exposure to contribute to ATV outbreaks and to variability in infection outcome in host populations.

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| Abbreviation | Definition |
|---|---|
| AESRD | Alberta Environment and Sustainable Resource Development |
| ATV | *Ambystoma tigrinum* virus |
| *Bd* | *Batrachochytrium dendrobatidis* |
| bp | Base pair |
| CI | Confidence interval |
| COSEWIC | Committee on the Status of Endangered Wildlife in Canada |
| CPUE | Catch per unit effort |
| DNA | |
| FV3 | Frog Virus 3 |
| iiPCR | Insulated isothermal polymerase chain reaction |
| IUCN | International Union for Conservation of Nature and Natural Resources |
| MCP | Major capsid protein |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative polymerase chain reaction |

# CHAPTER ONE

## General Introduction

Global amphibian populations have been declining since the 1960's (Houlahan *et al.* 2000). These population declines have been occurring simultaneously across several amphibian taxa (Collins & Storfer 2003). A review by Houlahan *et al.* (2000) found significant declines in a global dataset on amphibian populations from the 1960's onward, with the strongest declines in North America and Australia/New Zealand. The number of amphibian species classified as critically endangered almost doubled in the period between 1980 and 2004 (review by Stuart *et al.* 2004) and the rates of extinction significantly increased (review by McCallum 2007). Compared to all other classes of vertebrate, amphibians currently have the highest extinction rates (Regan *et al.* 2001), with an estimated increase >200 times that of background levels (McCallum 2007). Almost one third of the approximately 6000 extant amphibian species are at risk of extinction in the near future (IUCN 2014). This phenomenon is especially significant considering that some of the population declines have occurred in pristine habitats and protected areas that were previously thought to be a refuge for threatened species (Collins & Storfer 2003, Stuart *et al.* 2004). The severity of these recent changes led to the first global assessment of amphibian species and populations by the International Union for Conservation of Nature and Natural Resources (IUCN) in 2004. The results of this report showed that >30% of amphibian species are threatened globally (Stuart *et al.* 2004).

Taken together, these results emphasize that amphibian biodiversity is threatened at global, regional, and local scales. Nevertheless, ecologists and conservation biologists continue to recognize the inherent variability of amphibian population sizes and the

notorious difficulty in distinguishing significant population-level declines from natural fluctuations caused by factors such as variation in water availability. Results from a 12-year monitoring study involving daily captures of *Ambystoma spp.* breeding adults showed that annual variation in the return of adult females to their natal ponds varied 90-fold (Pechmann *et al.* 1991). Similarly, Harte & Hoffman (1989) reported a population decline of 65% of adult tiger salamanders over 6 consecutive years, followed by recovery in juvenile recruitment in the 7$^{th}$ year. These results and others reviewed by Blaustein & Kiesecker (2002) highlight the importance of detailed field-monitoring studies in the assessment of population-level declines of amphibians.

As one of the many factors implicated as causative agents of observed declines in amphibian populations (e.g. habitat alteration, increased ultraviolet radiation, introduced predators, climate change, aquatic toxicants), emerging infectious diseases have been shown to play a key role that clearly acts in addition to natural population fluctuations (Daszak *et al.* 2003, Stuart *et al.* 2004). The chytrid fungus *Batrachochytrium dendrobatidis*, and some members of the ranavirus clade of viruses (Family *Iridoviridae*) have been characterized as the two main mortality-inducing pathogens that infect amphibian populations worldwide (Daszak *et al.* 1999, Daszak *et al.* 2003, Stuart *et al.* 2004, Lips *et al.* 2006). Since chytridiomycosis was first described in 1998, chytrid has caused massive declines and recurrent epidemics throughout Central America, Australia, and North America (Daszak *et al.* 1999, Daszak *et al.* 2003, Lips *et al.* 2006).

Ranaviruses have linear, double-stranded DNA genomes and infect a wide variety of species of amphibian, reptile, and fish (review by Chinchar 2002). Although the effects of these pathogens on individuals and populations are often highly variable

and context-dependent, some ranavirus-induced outbreaks have caused 100% mortality within their host populations (Chinchar 2002, Green *et al.* 2002). Over the past two decades, the incidence of ranaviruses decimating local populations in North America, Europe, and Asia has steadily risen (Daszak *et al.* 2003, Lips *et al.* 2006, Chinchar & Waltzek 2014, Price *et al.* 2014). Some ravaviruses, such as the Frog Virus 3 (FV3), can infect a variety of amphibian and reptile species (Chinchar 2002, Chinchar *et al.* 2009), whereas others such as *Ambystoma tigrinum* Virus (ATV) have a much narrower host spectrum (Chinchar 2002).

The ATV-*Ambystoma* interaction is a key model system that has increased our understanding of the complex dynamics of aquatic host-pathogen systems. One key advantage lies in the ability of researchers to combine results from detailed field monitoring studies with empirical studies in laboratory, mesocosm, and field settings to uncover important mechanisms leading to virus-induced outbreaks. Thus, results involving this host-pathogen interaction have demonstrated the complex dynamics associated with how and when new pathogens invade naïve host populations (Jancovich *et al.* 1997, Bollinger *et al.* 1999), their consequences on host populations (Jancovich *et al.* 1997), the conditions under which variation in exposure is associated with variation in host mortality (Brunner *et al.* 2007), and how pathogen characteristics can influence outcomes (Schock *et al.* 2009).

One current shortcoming of ongoing studies involving the ATV-*Ambystoma* system is that the geographical scope of field monitoring studies is highly restricted. Most epidemiological studies have focused on the initial outbreak area in the southwestern United States, namely Arizona and Colorado (Collins 1988, Jancovich *et al.* 1997,

Brunner *et al.* 2004, Greer *et al.* 2009). Almost nothing is known about the occurrence of ATV in host populations at the northern edges of their range, or the extent of variation in parameters associated with transmission in host populations. For example, ATV was reported to cause outbreaks in populations of tiger salamanders over 10 years ago at sites in Saskatchewan and Manitoba (Bollinger e*t al.* 1999, Jancovich *et al.* 2005). Since then, no studies have further investigated the occurrence of ATV in tiger salamander populations on the Canadian prairies. Anecdotal reports suggest that ATV is present, but not reported, in additional populations in Alberta (C. Goater, personal communication). This highlights the need for further studies, as the occurrences of ATV in northern and western edges of the tiger salamander range in Canada are not well known.

A further shortcoming is that there is little information on annual or seasonal variation in transmission dynamics within and between local sites. Thus, we have a coarse understanding of temporal and spatial variation in rates of transmission at particular sites. It follows that we have only a crude understanding of the frequency of ATV-induced outbreaks, and whether these might be annual, cyclic, or episodic. Greer *et al.* (2009) evaluated changes in prevalence of ATV among and within 4 adjacent wetlands over 4 years, showing a pattern of highly complex and unpredictable transmission, especially between years. Greer *et al.* (2009) also showed that outbreaks were highly context-dependent, such that high-prevalence years were not necessarily concordant with high-mortality years. The degree to which such complexity is generalizable to other sites is not known based upon the results of this single study.

A third shortcoming is that most other studies have focused on sites that involve various degrees of anthropogenic disturbance. Thus, most of the study sites in the

southwestern United States were conducted in artificial wetlands (e.g. dugouts), many of which were highly impacted by features such as livestock grazing and/or eutrophication (Collins 1988, Jancovich *et al.* 1997, Greer *et al.* 2009). The damage to shallow vegetation from grazing livestock has been shown to increase the ATV transmission rates in some host populations (Hoverman *et al.* 2012). Additionally, the presence of certain pesticides in water bodies can work synergistically with ATV to increase the rates of larval mortality (Kerby *et al.* 2011). What's more, other sites are impacted by the fish-bait industry, where salamander larvae are transported widely between sites in support of a freshwater fishing economy (Jancovich *et al.* 2005). Overall, ATV transmission dynamics and outbreak dynamics are unknown in relatively pristine wetlands.

In this study, I report for the first time the occurrence of ATV in tiger salamander populations at the northwest edge of their range in southwest Alberta, Canada. My emphasis is on describing transmission and outbreak dynamics over two years in a wetland that has experienced periodic ATV-induced outbreaks for at least the past 10 years.

### 1.1. Model system – The host

The *Ambystomatid* tiger salamanders (or mole salamanders) represent one of the largest families of salamanders in North America. The tiger salamander, *Ambystoma tigrinum*, was first described by Baird in 1850, and was considered for many years to be a widely distributed species occurring across much of North America (Gehlbach 1967). Formerly designated as the barred tiger salamander, *Ambystoma tigrinum mavortium*, the western tiger salamander has recently been elevated to species status, *Ambystoma mavortium,* based on morphological, molecular, and phylogenic evidence (Shaffer &

McKnight 1996, Irschick & Shaffer 1997, Crother 2012). Under this designation the western tiger salamander is distributed in Canada from western Manitoba to British Columbia, while the eastern tiger salamander, *A. tigrinum*, ranges from eastern Manitoba to the east coast (Crother 2012, Figure 1.1). According to some authorities, the western tiger salamander can be further differentiated into 5 subspecies; *A.m. diaboli, A.m. melanostictum, A.m. nebulosum, A.m. stebbinsi, A.m. mavortium* (Crother 2012, Figure 1.1). Since the results of my study are not affected by subspecies designations, the study population in southwest Alberta will be referred to in this thesis as the western tiger salamander, *A. mavortium.*

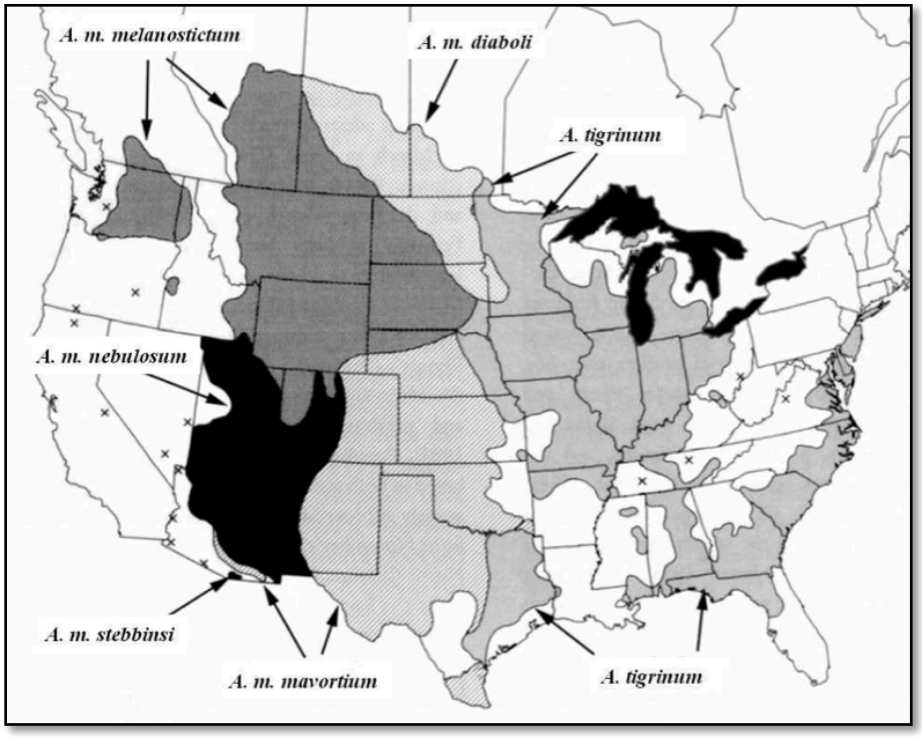


Figure 1.1. Distribution of *A. tigrinum* and *A. mavortium* in North America, including subspecies designations proposed by Crother (2012). Figure modified from COSEWIC (2012).

The life cycle of the tiger salamander and other north-temperate ambystomids involves the emergence of adults from subterranean overwintering sites in very early spring. The majority of overwintering adults return to natal ponds each year, while some may disperse to adjacent wetlands (Trenham *et al.* 2000, Pechmann *et al.* 2001). Females lay clutches of eggs onto aquatic macrophytes or other aquatic debris and larvae hatch approximately three weeks later (Russell & Bauer 2000). Larvae are generalist, nocturnal, aquatic carnivores. Metamorphosis occurs in mid to late summer, depending on water temperature, at which time they reabsorb their brachiated gills and dorsal fin to assume the characteristic terrestrial body form. Dispersal of larvae or metamorphs from the water is completed by early fall when individuals begin to dig underground or use abandoned mammal burrows for overwintering (Madison & Farrand 1998). In some populations of the western tiger salamander, a proportion of individuals within a clutch of eggs will overwinter as larvae and metamorphose the following summer (Bizer 1978). In still others, these larvae will retain the larval body morphology up to and including the reproductive phase. These facultative paedomorphic characteristics can be permanent in some individuals (review by Sprules 1974).

The conservation status of the western tiger salamander varies throughout its range. In the United States this species is listed federally as 'secure', although the last status review was completed in 1996 prior to the taxonomic split of the eastern and western tiger salamanders (NatureServe 2014). Individual states classify the western tiger salamander as 'secure' in Arizona and Idaho, 'apparently secure' in Wyoming, 'vulnerable' in Colorado and Washington, and 'unranked' in Oregon (NatureServe 2014). The Sonora tiger salamander (*Ambystoma mavortium stebbinsi*), is listed federally as

'endangered' with less than 100 breeding populations known in the United States (Gerst 1997). It is clear that the status of the western tiger salamander varies greatly across western North America and may no longer be a secure species relative to conservation.

In 2012, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) listed the Prairie/Boreal population of the western tiger salamander, which ranges through Alberta, Saskatchewan, and Manitoba, as a species of 'Special Concern'. This shift from the previous 'Not at Risk' designation highlights the major threats facing amphibians in Canada. Habitat loss, invasive fish species, emerging infectious diseases, and other factors affecting breeding and dispersal are cited as the major threats to the Prairie/Boreal population (COSEWIC 2012). Although Alberta Environment and Sustainable Resource Development (AESRD) currently classifies this species among the 30% of 'secure' amphibian species in the province, no population-level monitoring has been completed. Current concerns regarding the potential decline of the Alberta population in particular is due to the expanding oil and gas industry affecting habitat and migration, large scale fish stocking into many water bodies, and emerging infectious diseases (COSEWIC 2012). Anecdotal evidence suggests that large populations of western tiger salamander once existed across the prairies and into the foothills region prior to major fish stocking events, however, many of these large populations have been greatly reduced or even extirpated (C. Goater, unpublished data).

**1.2. Model system - The pathogen**

The *Ambystoma tigrinum* virus (ATV) has a large, double-stranded DNA genome that infects tiger salamanders (Jancovich *et al.* 1997). ATV is part of the ranavirus group (Family *Iridoviridae*), which has been implicated as a factor in global amphibian

population declines (Daszak *et al.* 2003). First described in an Arizona population of *A.m. stebbinsi* by Collins (1988) and Jancovich *et al.* (1997), ATV has now been reported in tiger salamanders through the western United States and into parts of southern Canada (Bollinger *et al.* 1999, Green *et al.* 2002, Brunner *et al.* 2004, Jancovich *et al.* 2005). Limited by its host range, ATV has only been reported in tiger salamanders in North America (Lesbarreres *et al.* 2011).

ATV is closely related to other ranaviruses including tiger frog virus and frog virus 3 (Jancovich *et al.* 2003). The ranaviruses are thought to have originated from a host shift event involving a fish iridovirus followed by radiation into various amphibian species (Jancovich *et al.* 2010). ATV has been fully sequenced with a genome size of 106, 332 bp (Jancovich *et al.* 2003). A highly conserved region of the ranavirus major capsid protein (MCP) gene is used to identify the virus in host tissue (Mao *et al.* 1996, Mao *et al.* 1997). Sequencing of a 531 bp region of the MCP gene isolated from tiger salamander tissue indicates the presence of ATV in an individual (Mao *et al.* 1997, Greer & Collins 2007).

ATV can be transmitted through direct contact between individuals, ingestion of infected tissue, and exposure to water or substrate previously in contact with an infected individual (Jancovich *et al.* 1997, Brunner *et al.* 2005, Brunner *et al*. 2007). Studies have shown that ATV can persist in water and substrate samples for more than 30 days, although the virus may become inactive long before it becomes undetectable (Brunner *et al.* 2007, Nazir *et al.* 2012, Johnson & Brunner 2014). Currently, there is no evidence that ATV uses a vector to persist over winter or between outbreaks (Brunner *et al.* 2004). Brunner *et al.* (2004) suggest that overwintering adult tiger salamanders with sublethal

infections can act as a vector between years, transmitting the virus to larvae each spring/summer.

Evidence from experimental infections show that larvae can begin to exhibit clinical signs of ATV in as little as 5 days post-exposure (Jancovich *et al.* 1997). Clinical signs of ATV include skin sloughing, lethargy, loss of appetite, internal organ hemorrhaging, vomiting, excretion of bloody stool and mucus, erosions and ulcers on the skin (Jancovich *et al.* 1997, Bollinger *et al.* 1999). Laboratory experiments have shown >50% mortality in individuals exposed to ATV through a water bath, with the time from exposure to death being approximately 2 - 3 weeks (Brunner *et al.* 2004). In some cases host mortality is observed in as little as 10 days post-exposure (Bollinger *et al.* 1999). Similar experiments in outdoor mesocosms have also shown that ATV causes mortality in larvae, even when other pathogens are present (Jancovich *et al.* 1997). Jancovich *et al.* (1997), Bollinger *et al.* (1999), and Green *et al.* (2002) provide evidence of ATV-induced mortality in natural populations by testing dead and dying individuals collected during mass mortality events. These results provide direct evidence that ATV causes mortality in larval salamanders.

**1.3. ATV in Alberta, Canada**

Prior to my study, ATV was not formally documented in salamander populations in Alberta. However, anecdotal evidence from host surveys following a mass mortality event in 2002 at Livingston Lake, approximately 20 km northeast of Crowsnest Pass, Alberta, raised concerns that ATV may be present in the region. The discovery was serendipitous, following the observation by landowners of a family of grizzly bears (*Ursus arctos*) feeding on salamander carcasses. A second mortality event occurred in

Livingston Lake in summer 2005, and ranavirus was confirmed via molecular diagnosis and sequencing of the MCP in 15 moribund/dead individuals (C. Goater, B. van Paridon, & T. Bollinger, personal communications). Virus and salamander populations have been monitored at this site annually between 2005 and the start of my study in 2012.

ATV was discovered at a second site in Waterton Lakes National Park in 2011 after a mortality event involving over 1000 salamander larvae (B. Johnston, personal communication). Ranavirus was confirmed at the Canadian Cooperative Wildlife Health Centre in three individuals from this outbreak (T. Bollinger, personal communications). A third mortality event involving tiger salamanders and ATV was observed in a pond in Fish Creek Provincial Park in 2012 (D. Whiteside, personal communication). Taken together, these anecdotal observations strongly suggested that ATV is present, and possibly widespread, in southwestern Alberta and, given the pattern of ATV-induced mortality observed at well-studied sites in Arizona (Jancovich *et al.* 1997, Brunner *et al.* 2004, Greer *et al.* 2009), was likely associated with high host mortality.

**1.4. Thesis objectives**

There are three data chapters in this thesis. Chapter Two provides the first population-level assessment of the occurrence and density of tiger salamanders at selected sites within a 4800 $km^2$ region of southwestern Alberta. These amphibians are assumed in the literature and in status reports to occur commonly throughout this region (COSEWIC 2012), but supportive evidence is not available. Information on occurrence and density is essential to understanding the population status of this species and to ultimately understanding the overall role of ATV in influencing its regional distribution. Chapter Three is a methods-based chapter that examines the relative specificity and

sensitivity of a new tool, insulated isothermal polymerase chain reaction (iiPCR), for virus detection that could improve the speed and accuracy of ATV diagnosis, and compares this with diagnostic methods involving conventional PCR. Current diagnostic procedures are time-consuming, can be prohibitively expensive, and can result in misdiagnosis in newly-infected individuals. A new user-friendly diagnostic platform may address these issues and provide advantages for field-based surveys that require rapid ATV detection. These new methodologies may also be generalizable to other host-pathogen interactions involving wild animals. Chapter Four provides the first assessment of spatial (between pond), seasonal, and annual variation in ATV transmission into western tiger salamanders in Canada. I also use crude assessments of host survival to evaluate the linkage between ATV transmission and salamander mortality. My focus in this chapter is on the ecological epidemiology of ATV in the salamander population in Livingston Lake.

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# CHAPTER TWO

Population and life history characteristics of the western tiger salamanders (*Ambystoma mavortium*) in southwestern Alberta

## 2.1. Introduction

The 32 species of ambystomatidae salamanders (the mole salamanders) are among the most recognizable amphibians in North America. However, relative to other amphibian taxa, as a single group, they are among the most threatened amphibians in the world, showing greater than average declines in population and range sizes (review by Stuart *et al.* 2004). The IUCN Red List of Threatened Species (2014) lists 11 of the 32 species of ambystomatidae salamanders as 'endangered' or 'critically endangered'. For example, the results of long-term monitoring studies have documented catastrophic declines of the Sonora tiger salamander (*Ambystoma tigrinum stebbinsi*) in Arizona (U.S. Fish and Wildlife Service 2002) and the Mexican axolotl (*Ambystoma mexicanum*) (Contreras *et al.* 2009). While it is difficult to detect true declines in population size due to the inherent annual variability in reproduction and survival of ambystomatidae salamanders (Pechmann *et al.* 1991), there is strong evidence of declines across the taxa as a whole. The ambystomatidae family shows a decreasing population trend in North America (Adams *et al.* 2013), with 48% of species decreasing and another 19% lacking the data to accurately assess trends in population sizes (IUCN 2014).

The population status of the western tiger salamander, *Ambystoma mavortium*, is poorly known throughout its range in western North America. Published range maps indicate that the species occurs extensively, but at unknown densities, in the central parts of its range (Keinath & Andersen 2010). However, long-term population and distribution

studies of *A. mavortium* are rare. Much of the current knowledge related to assessments of changes in *A. mavortium* population sizes come from focused, small-scale studies. For example, Spear *et al.* (2006) used evidence based upon genetic variation in microsatellite markers to indicate historic and recent declines in the *A. mavortium* populations in Colorado and Wyoming. These limited data, together with recent changes in the status of the Canadian population (COSEWIC 2012), lead to justifiable concerns regarding the overall status of this species throughout its range.

The formal population status of *A. mavortium* within the northwest of its range varies from Secure (Idaho) to Special Concern (Alberta) to Vulnerable (Washington) to Endangered (British Columbia) (COSEWIC 2012, NatureServe 2014). A recent assessment of the Prairie/Boreal population across Alberta, Saskatchewan, and Manitoba identified numerous potential threats to population sizes including habitat fragmentation, invasive predators, and emerging infectious diseases (COSEWIC 2012). Information on the distribution and population sizes of *A. mavortium* in Alberta is extremely limited (COSEWIC 2012). Anecdotal evidence suggests that widely scattered and high density populations of *A. mavortium* existed across the prairies and into the foothill region prior to major fish stocking events, however, many of these large populations have been greatly reduced or extirpated from some water bodies (Goater, unpublished data). The lack of recent data on the population status and distribution of *A. mavortium* throughout Alberta and the northwest edge of its range is a fundamental knowledge gap.

There are two aims of the current study. The first is to assess the occurrence and relative population density of *A. mavortium* within selected sites in southwestern Alberta. The second is to assess larval life-history traits, such as growth rate and size and age at

metamorphosis, for one site where *A. mavortium* was known to consistently occur at relativly high population densities. I am especially interested to assess the length of the larval period of *A. mavortium* populations in mid-elevation sites that are characteristic of foothills regions along the eastern slopes of the Rocky Mountains. Although 3-4 month larval periods are characteristic in populations located within prairie wetlands (Deutschman & Peterka 1988) and 1-2 year larval periods are common in high elevation sites in the Rocky Mountains (Bizer 1978, Sexton & Bizer 1978), little is known about life-history variation of *A. mavortium* in foothills regions.

## 2.2. Methods

### 2.2.1. Site selection

My survey was focused on the southwestern corner of Alberta (Figure 2.1), an area located within the subalpine, montane, and foothills fescue natural sub-regions (Downing & Pettapiece 2006). Vegetation in this region is dominated by grasses (e.g. *Festuca campestris*, mountain rough fescue) and characteristic prairie forbs (e.g. *Potentilla frutilla,* shrubby cinquefoil) interspersed with stands of aspen (*Populus tremuloides*), cottonwood (*Populus deltoides*), and lodgepole pine (*Pinus contorta*) (Downing & Pettapiece 2006). The sites within the survey area ranged between UTM zone 11U (695693E) and zone 12U (299120E) and between 5439194 and 5512211N. Elevation in this area ranges from 1000 to 1900m above sea level.

Selected wetlands were chosen using ArcGIS software. Shapefiles of all water bodies within the study area, totaling over 3000, were obtained from GeoGratis (Natural Resources Canada). Using the program ArcMap (ESRI), an initial group of 100 water bodies was selected at random from this larger sample. These 100 sites consisted of 28

wetlands (0.5-1.0ha), 60 ponds/small lakes (0.5-20ha), and 12 dugouts (0.3-0.5ha). Each site was then assessed for general location, accessibility, and landowner permission. Acceptable sites were required to be less than 1 km from an access road and more than 1 km from an adjacent site. A final list of 25 water bodies was targeted for surveying. However, once the sites were initially visited, not all were suitable for trapping due to factors such as cattle density and/or inaccessibility. In total, a subset of 13 sites was surveyed for larval salamanders. Prior surveys completed by our laboratory since 2005 indicated that *A. mavortium* was consistently present in two additional sites. These two sites, Waterton Pond and Livingston Lake, were targeted for more intensive sampling.

Habitat assessments were completed at each site during the initial visits. Data was collected on the location, elevation, type of water body (pond, lake, wetland, or dugout), surface area, dominant surrounding vegetation (coniferous forest, deciduous forest, or grassland), dominant substrate composition (gravel, silt, or macrophytes), distance to nearest water body (<1 km or >1 km), fish presence/absence, cattle access, permanency (temporary or year-round), and presence of other amphibians. Data collected during the habitat assessments is in Appendix 6.1.

**2.2.2. Survey methods**

Four modified funnel traps (Mushet *et al.* 1997) were used to assess the presence and density of *A. mavortium* within each water body. Mushet *et al.* (1997) compared the efficiency of this modified trap (45 x 50 x 100cm steel frame covered with 0.32cm galvanized steel mesh) to other standard methods for detecting larval salamanders (e.g. minnow traps, seine nets, dip nets). They showed that the modified funnel traps captured more salamanders with less variance than minnow traps and were much more effective

during periods of the summer when there was excessive macrophyte growth. Overall, the design of this trap allows for the live-capture of larval and adult salamanders by sampling a large portion of the water column over a defined period of time. Funnel traps have also been shown to detect a greater diversity of amphibian life-stages than dip netting (Wilson & Dorcas 2003). Benoy *et al.* (2005) and Balas *et al.* (2012) used this trapping method to survey populations of *A. tigrinum* in prairie wetlands.

At each site, traps were placed at varying depths (15-100cm) parallel to the shoreline in order to target nocturnally foraging salamanders that tend to move into the water column at night (Hassinger 1970). When possible, the four traps were placed at random positions along the shoreline. However, in some situations, inaccessibility due to dense macrophytes, uneven substrate, or impassible obstacles forced alternative placement.

Each site was sampled at least once between June and August in 2012 and 2013. A small number of sites were sampled more than once in a season and/or in multiple years (Appendix 6.2). The two focus sites were sampled 2 to 5 times each year between 2012 and 2014. In each instance, traps were left in place over a consecutive 48-hour period. Information on the date, location, and outcome of individual trapping events are in Appendix 6.2 and 6.3.

Following the placement and retrieval of each funnel trap, salamanders were removed and placed into 2L plastic containers with adequate water for short-term holding. Each salamander was then individually processed with clean, sterilized equipment. Data on weight and general condition were recorded, as well as a photograph taken. Following processing (maximum duration = 30 minutes), individuals were

released at the point of capture. Total body length, from snout to tail, was determined from the digital image with ImageJ software (Abramoff *et al.* 2004). Traps and handling equipment were cleaned and sterilized (3% sodium hypochlorite) following each trapping event (Phillott 2010).

**2.2.3. Analyses**

The low number of sites that contained larval salamanders placed constraints on analyses. Fisher's exact tests were used to assess associations between selected habitat characteristics and the occurrence of *A. mavortium.* This statistical test produces an exact probability that two variables are statistically independent when one or more frequencies are less than five (Sokal & Rohlf 1981).

Salamander density in each pond was estimated as catch per unit effort (CPUE), where one unit equals the effort of one trap for one hour. Therefore, CPUE can also be quantified as larvae caught per trap per hour. Analyses of differences in CPUE between sites were not possible due to the small number of sites that contained salamanders and due to unequal sampling effort between sites. These semi-quantitative data are presented as relative differences in CPUE between sites that contained salamanders.

The growth rates (± 95% confidence intervals) of larval salamanders at Livingston Lake were determined by the formula: Growth Rate = $(V_{present} - V_{past}) / N$, where $V_{present}$ = value present, $V_{past}$ = value past, and N = number of days between sampling events $V_{past}$ and $V_{present}$, following standard procedures (Cook 2000). Size at metamorphosis was determined by the average weight at the last trapping event in mid-August each year. While not all individuals had completely metamorphosed at that time, most were in the early to mid stages of metamorphosis. Comparison of growth rates and

size at metamorphosis between years for data collected at Livingston Lake was completed using a standard t-test.

## 2.3. Results

### 2.3.1. Distribution and density of *Ambystoma mavortium* in southwest Alberta

Salamander larvae were detected in 8 of the 15 sites surveyed in 2012 and 2013 (Figure 2.1). At seven sites, larvae were caught in funnel traps, while at an eighth site dead larvae were observed adjacent to the traps. Sites that contained salamanders were between 1309 and 1457m in elevation. There were no significant associations between the presence of salamanders and permanency of the water body (Fisher's Exact Test, $p=0.22$), distance to nearest water body ($p=0.22$), fish present ($p=0.22$), livestock access ($p=1.00$), or other amphibians present ($p=0.15$). Other amphibian species present at the sites are listed in Appendix 6.1.

The density of larvae was <0.1 CPUE at all but Livingston Lake, where densities tended to be at least 3 times higher (Figure 2.2). Overall, 59% of the 121 traps set over the 3 years contained no larvae. At Livingston Lake, 1 – 62 larvae were captured within a single trap. At all sites, the highest capture rates occurred in July (0.24 CPUE), followed by diminishing rates in August (0.12 CPUE) and September (0.03 CPUE).

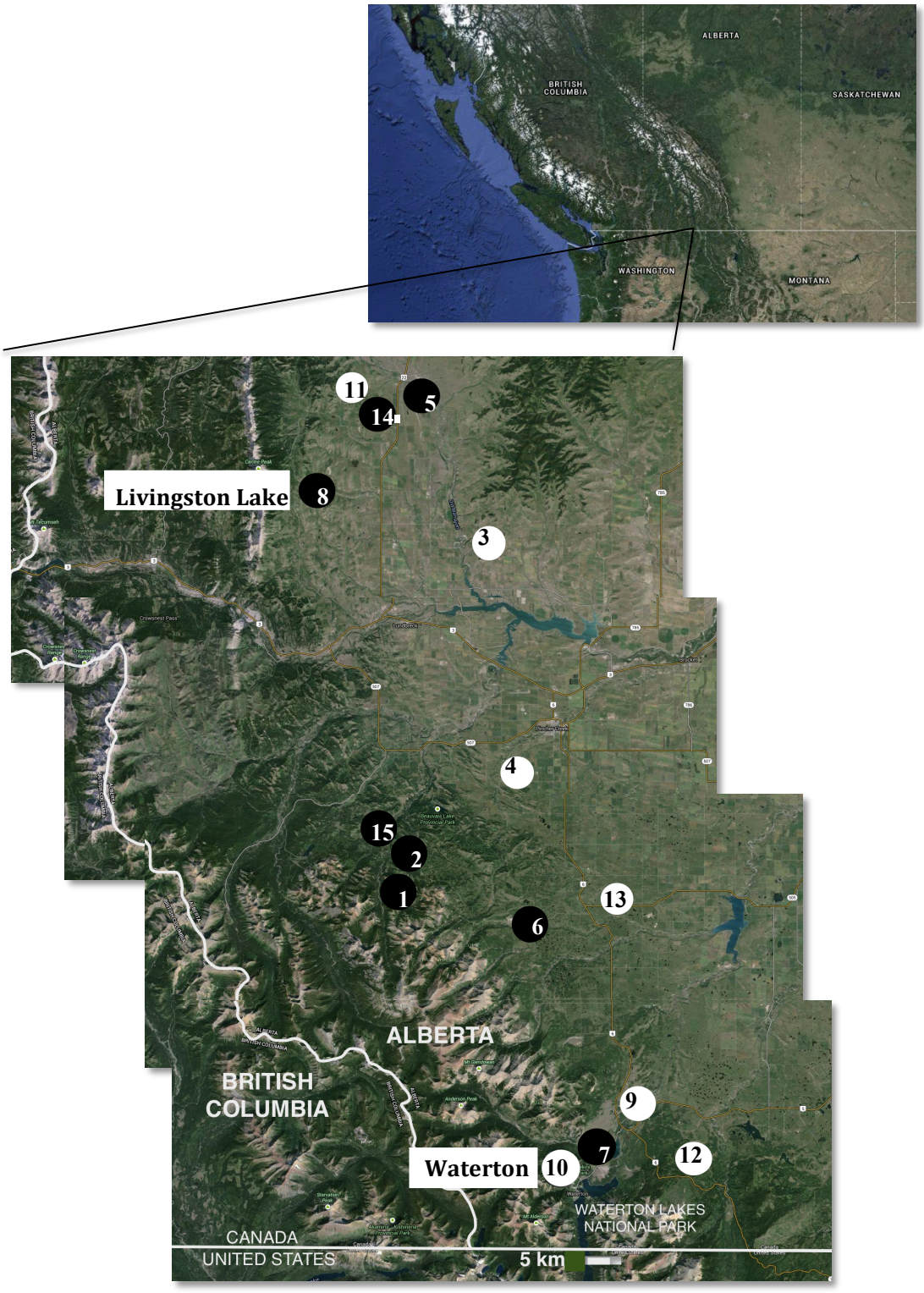


Figure 2.1. Occurrence of *A. mavortium* in 15 selected wetlands in southwestern Alberta. Black points show sites where *A. mavortium* was present; white points show sites where *A. mavortium* was absent. Numbers correspond to site names listed in Appendix 6.1.

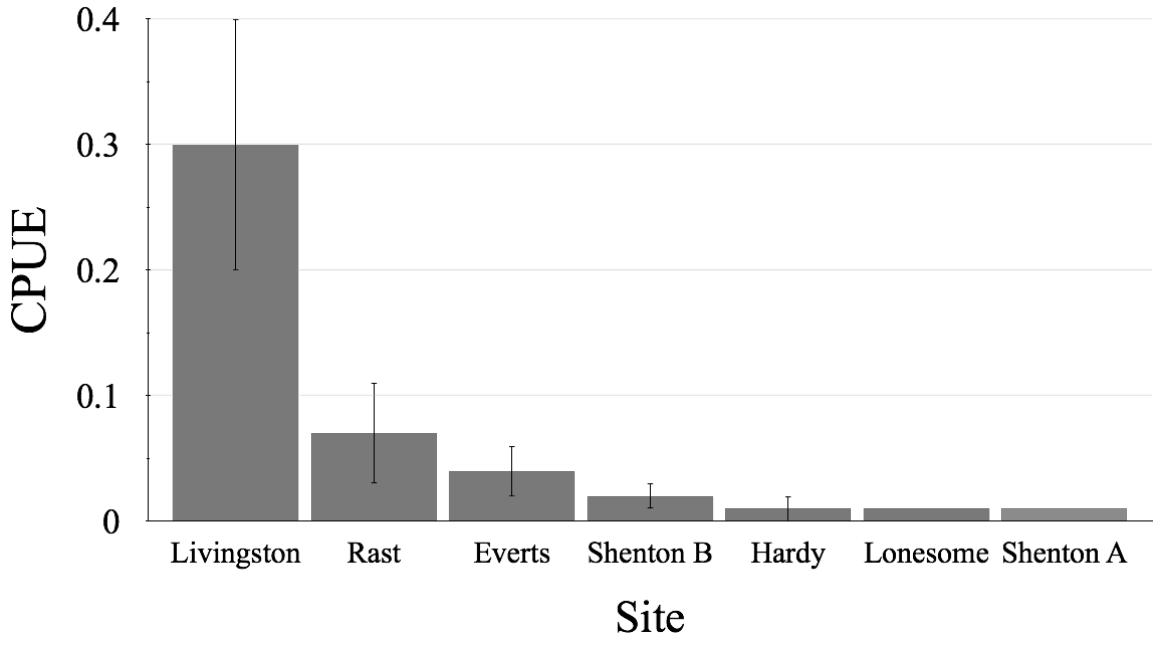


Figure 2.2. CPUE of *A. mavortium* at seven sites in southwestern Alberta. CPUE at eight other target sites was zero. Error bars indicate a 95% confidence interval, based on sample size, from sites that were visited more than once.

### 2.3.2. Larval growth rates & size at metamorphosis

Larval salamanders captured at Livingston Lake in mid-July (2013 n=29, 2014 n=170), late July (2013 n=52, 2014 n=195), and mid-August (2013 n=12, 2014 n=132), were used to determine annual variation in growth rate. A fourth sampling event was attempted in late August each year, however, due to low sample size (2013 n=1, 2014 n=3), these data were excluded. Frequency distributions of the length of larval *A. mavortium* (Figure 2.3) show a similar pattern of growth between years. In both 2013 and 2014, larvae were first detected in the traps in mid-July, when individuals averaged 8-8.5 cm in length. Approximately 2 weeks later, the larvae had grown substantially, averaging 12.8-13 cm in length. A further 2 weeks later, they had increased another 2-3 cm. At the beginning of metamorphosis, larvae averaged 14.8 cm in 2013 and 16 cm in 2014. Although the pattern of growth was consistent between years, growth rate in 2014 was

significantly higher compared to 2013. The larval growth rates based on weight and length were 61% higher (t-test, $t = 11.77$, $p < 0.01$) and 36% higher ($t = 19.59$, $p < 0.01$), respectively, in 2014 than in 2013. Similarly, mass at metamorphosis was 39% greater in 2014 than in 2013 (t-test, $t = 4.57$, $p < 0.05$). Data on length at metamorphosis were more similar between the two years, with larvae metamorphosing 8% longer in 2014 than 2013 (t-test, $t = 3.34$, $p < 0.05$). Regardless of variation in length or mass, the timing of metamorphosis was consistent, occurring in mid-August in both 2013 and 2014.

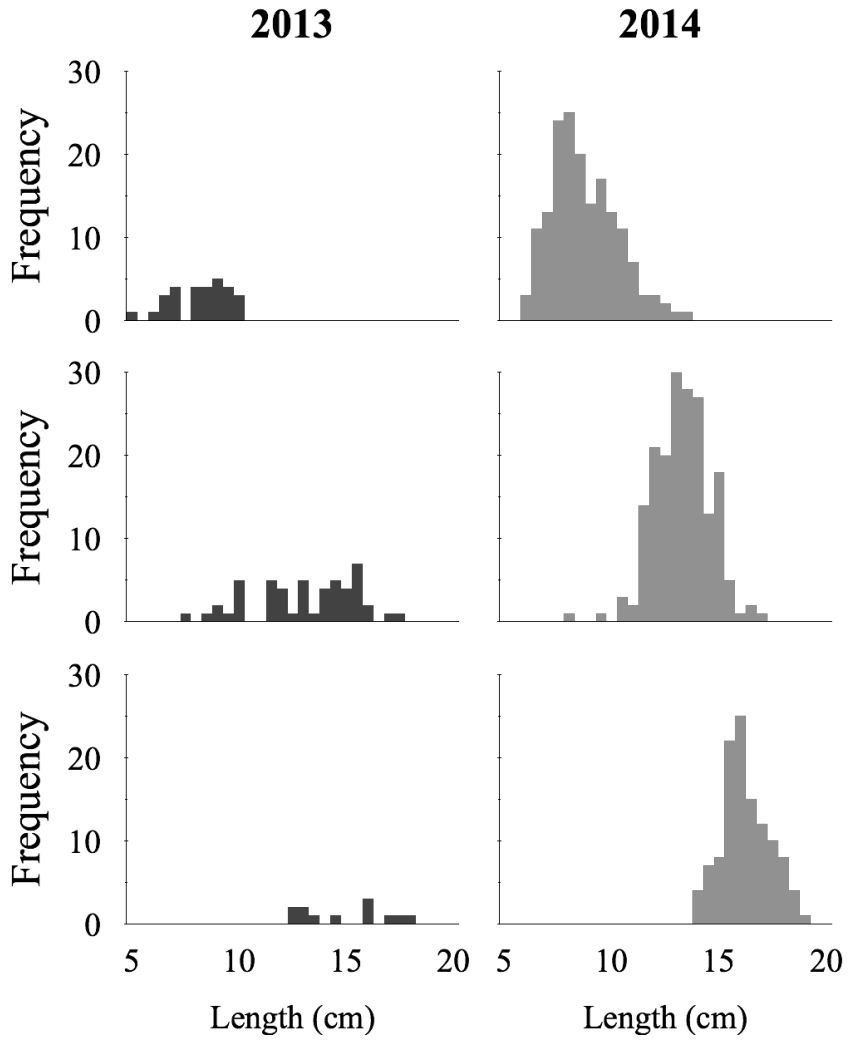


Figure 2.3. Frequency distributions of *A. mavortium* lengths in Livingston Lake in 2013 and 2014. Graphs correspond to sampling events in 2013 (top to bottom; July 10, July 30, August 16) and 2014 (top to bottom; July 15, July 31, August 12).

## 2.4. Discussion

My survey results show that breeding populations of *A. mavortium* are present in southwestern Alberta, but throughout most of this region, larval densities are low. Over a two-year period between 2012 and 2013, *A. mavortium* were detected in 53% ± 26% (95% CI) of water bodies surveyed. Although no comparable studies have been completed in Alberta, this occupancy rate is within the reported range for this species. The results from three other comparable studies in wetlands in Wyoming and the Prairie Pothole Region showed that the occupancy rate of *A. mavortium* was 24 ± 4.8%, 54 ± 37%, and 75-80% respectively (Benoy 2002, Corn *et al.* 2005, Balas 2008). Unfortunately, it is not possible to compare larval densities with other studies due to variation in trapping methods and effort. In southwestern Alberta, aside from one site, all larval densities ranged between 0.01 and 0.07 larvae caught per trap per hour. The results suggest that either *A. mavortium* larval densities are extremely low in southern Alberta, or that sampling occurred over three years when breeding and recruitment were low.

There was no detectable pattern of occurrence relative to the wetland characteristics that I measured. Thus, salamanders were present in both the largest and smallest wetlands (Livingston Lake and Rast Dugout, respectively), and occurred in wetlands with predaceous fish (Livingston Lake) and those without. These results imply that salamanders in this region are capable of breeding and developing through the larval stage within all of the wetland types that are available in southwest Alberta. However, many suitable wetlands appear not to be colonized by breeding pairs of adults, at least not in the years that I sampled. This pattern of occurrence is similar to the pattern reported for *A. mavortium* in 154 wetlands in Colorado, distributed across 7 habitat types (Collins

1981). Elsewhere within its range, salamander occurrence is associated more frequently with grassland than cropland habitats (Ghioca & Smith 2008), and with undisturbed riparian buffer strips of at least 600m (Trenham & Shaffer 2005). It is possible that the occurrence of *A. mavortium* in the study area are associated with some of these factors, however, low sample sizes severely constrained detection of any patterns, if present.

*A. mavortium* populations in the study area displayed a standard life history, where salamanders metamorphosed at the end of the first season. At most sites, each sampling event revealed evidence of only one cohort of similar size and development. However, at two sites (Hardy & Rast), there was limited evidence for the presence of two co-occurring cohorts. At these sites, young of the year were shorter (<13.5cm in late July), weighed less and were noticeably further behind in limb development than a small number of others that were longer (>16.5cm in late July), heavier, and had fully developed limbs. It is possible that these populations were neotenic, where larger individuals were larvae that had over wintered at least one year in the ponds. It is also possible that the larger larvae were paedomorphic. Paedomorphism and neoteny have been reported frequently in *A. mavortium* populations in other parts of its range (Bizer 1978, Sexton & Bizer 1978, Routman 1993), and paedomorphism is known to dominate at certain sites in southern Alberta (Cormie 1975). Bizer (1978) suggests that *A. mavortium* populations at mid to high elevations respond to environmental pressures by adapting to longer or permanent larval stages. Based upon observations from this study, paedomorphism and/or neoteny appear to be rare life history adaptations for *A. mavortium* in these mid-elevation, foothills sites. In this area, selection pressures appear to favor the standard life history, which utilizes both aquatic and terrestrial habitats.

Livingston Lake was the only site that contained *A. mavortium* in all three years. The population size at this site was much higher than at any other site, with a maximum of 195 individuals captured in a single event. The highest number captured in a single event at any other site was 19. Thus, Livingston Lake is an anomaly, with densities more than triple that of the seven other sites where *A. mavortium* was detected. Similarly, when compared with information reported in the literature, the population at Livingston Lake remains an outlier. Larval *A. mavortium* in Livingston Lake, especially in 2014, had the largest size at metamorphosis compared to other mid to high-elevation sites throughout the species range (Tanner 1971, Bizer 1978). Only one site, a lower elevation lake in North Dakota, reported growth rates of *A. mavortium* that were slightly higher than those at Livingston Lake (1.90 and 1.15 ± 0.50 g/day, respectively), and were associated with years of high prey density (Deutschman & Peterka 1988). My anecdotal observations suggest that aquatic secondary productivity is atypically high in Livingston Lake, even compared to adjacent wetlands that are of a similar size and depth. Productivity of the crustaceans, *Gammarus* and *Hyallela* are consistently high throughout the year, as are population sizes of zooplankton that are dominated by cladocerans and ostracods (Goater, unpublished observations). It would be worthwhile to assess annual changes in prey productivity in Livingston Lake to determine if the high growth rates and large size at metamorphosis of larvae in 2014 was a response to unusually high availability of prey.

My data are too restricted in spatial and temporal scope to assess whether the low densities and low rates of occurrence in wetlands in southern Alberta are indicative of a general decline in *A. mavortium* in this region. Similar constraints associated with sampling and with inherent variability in ambystomid life histories are characteristic of

this and other ambystomid species (Pechmann *et al.* 1991, Semlitsch *et al.* 1996, Trenham *et al.* 2000, COSEWIC 2012). Anecdotal evidence suggests that at least 2 high-density *A. mavortium* populations have been extirpated from sites in southern Alberta. Tyrrell Lake, located in south-central Alberta, was widely known for its large paedomorphic population of *A. mavortium* (Cormie 1975). Despite intensive, annual sampling, salamanders have been absent since 1998 (Goater, unpublished data). In this case, annual stocking of introduced rainbow trout is thought to have lead to a predator-induced crash in salamander population sizes. At a site approximately 70 km south of Livingston Lake in Waterton Lakes National Park, over 1000 dead larvae were recovered, 3 which were confirmed to be infected with ATV (Parks Canada, personal communications). There has been no recovery at this site since the mass die-off in 2011. These observations indicate that *A. mavortium* populations have declined precipitously at some sites in this region, without recovery. While there are sites where *A. mavortium* occurs annually at high densities, the standard situation across southwestern Alberta is haphazardly distributed, low-density populations of *A. mavortium*.

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# CHAPTER THREE

Sensitivity comparison of two molecular diagnostic methods for *Ambystoma tigrinum* Virus in *Ambystoma mavortium*

## 3.1. Introduction

Since the mid-1980's many tiger salamander populations in North America have declined or have been extirpated due to exposure to a potentially fatal amphibian pathogen (Daszak *et al.* 1999, Collins *et al.* 2004). The first suspected outbreak of the *Ambystoma tigrinum* virus in tiger salamanders was observed at three sites in southern Arizona in 1985 (Collins 1988, Jancovich *et al.* 1997). Salamanders affected by this outbreak showed symptoms similar to certain bacterial pathogens, but the causative agent was unknown. Testing by Jancovich *et al.* (1997) identified the cause of these mortality events as a new species of iridovirus, *Ambystoma tigrinum* virus, or ATV. Through a series of virus isolation assays and experimental infections, the authors identified, characterized, and described ATV as the causative agent of virus-induced outbreaks in salamanders. Tissue and cells from diseased animals were observed using microscopy, and infected cells were identified by a characteristically enlarged nucleus (Jancovich *et al.* 1997). Prior to the development of accurate molecular diagnosis, less precise methods of detection, such as identification of general clinical signs, observation of infected cells using light or transmission electron microscopy, and fluorescent antibody tests, were used to characterize the virus (Chinchar & Mao 2000). However, these tools did not permit viral species-specific identification.

A specific and sensitive molecular tool for diagnosing ATV and other ranaviruses was first developed by Mao *et al.* (1996) using conventional PCR methodologies. The

gene encoding the major capsid protein (MCP) is similar between all iridoviruses, both in structure and function (Mao *et al.* 1996, Tidona *et al.* 1998). This discovery led to the development of the first set of molecular primers aimed at identifying and distinguishing various iridoviruses in PCR reactions. Mao *et al.* (1997) synthesized six PCR primers based on the MCP gene from the Frog Virus 3 (FV3). Their results showed that one set of primers (MCP4 and MCP5) were able to detect 10 species of iridovirus (Mao *et al.* 1997). Since the development of these specialized PCR primers, many follow-up studies have used MCP4 and MCP5 to diagnose ATV in tiger salamanders (Bollinger *et al.* 1999, Jancovich *et al.* 2001, Rojas *et al.* 2005, Brunner *et al.* 2005, Brunner *et al.* 2007).

Following the pioneering work by Mao *et al.* (1997), iridovirus diagnoses within natural salamander populations has commonly involved molecular tools. One of the most common methods of diagnostic testing is to use conventional PCR to amplify a viral gene sequence within DNA extracted from host tissues. While PCR is widely used in many fields, there are some drawbacks associated with cost, training, and optimization. Most importantly, the need for liquid transfer during post-amplification processing for visualization risks potential contamination of samples and work areas. An alternative diagnostic method is real-time (quantitative) polymerase chain reaction (qPCR), which does not require post-amplification processing and results are obtained in real-time. qPCR uses primers, as well as a probe or dye, to detect a desired product. This method has been shown to be much more sensitive at detecting target sequences than conventional PCR (Allendar *et al.* 2013). Allender *et al.* (2013) developed a qPCR assay able to detect viral concentrations of FV3 at levels 1000 times lower than were detectable with conventional PCR. However, specialized equipment, time-consuming assay

development, and cost are three major limitations for the widespread use of qPCR in iridovirus diagnostics.

Convective PCR is a method of nucleic acid amplification that relies on a single, constant heat source to drive convection throughout a reaction cell. This method relies on the principal that if a liquid is heated from below, the warmer bottom layer will rise while the cooler upper layer sinks (Krishnan *et al.* 2002). In this way, template denaturation, primer annealing, and product extension can all occur at varying stages of convection. The temperature profile created allows the reaction volume to cycle multiple times, similar to conventional PCR (Braun 2004). During the development of convective PCR equipment, inconsistent heating throughout the reaction could be problematic (Krishnan *et al.* 2002). Instruments with thermal baffles were developed to deal with the issue of temperature consistency within the reaction vessel (Tsai *et al.* 2012.b). This technique is termed insulated isothermal polymerase chain reaction (iiPCR). iiPCR has been shown to significantly reduce the reaction time due to the elimination of temperature ramping (time necessary to heat and cool the sample to specific temperatures), while returning results with accuracy comparable with those of conventional PCR (Tsai *et al.* 2012.b).

The use of early convective PCR significantly decreased the time for reactions when compared to conventional PCR (Braun 2004), but still required gel electrophoresis methods (or similar) in order to visualize results. Developments have been made that pair a TaqMan probe technique with iiPCR to create a one-step diagnostic process (Tsai *et al.* 2012.a, Tsai *et al.* 2012.b, Chung & Chang 2011). The TaqMan probe works by detecting the presence or absence of a fluorescent signal attached to target sequences after amplification, and comparing these to signals before amplification to determine a specific

ratio (Tsai *et al.* 2012.b). When the required result is only the presence or absence of a target sequence, this probe design eliminates the need for post-amplification processing. Rather than using a conventional PCR reaction and analyzing the results subsequently on a gel, detection occurs during the reaction and the results are available in real-time in the same reaction chamber.

Recently, portable diagnostic systems employing the use of iiPCR have been developed (Tsai *et al.* 2012.b). One example is POCKIT™ (GeneReach Biotechnology Corporation), a diagnostic platform using iiPCR amplification and a TaqMan probe system for one-step processing. The POCKIT™ platform is currently being used to diagnose a variety of animal pathogens. Chung & Chang (2011) were able to detect and diagnose a virulent Porcine Reproductive and Respiratory Syndrome virus. Tsen *et al.* (2013) used POCKIT™ to detect *Salmonella* in a shorter time period than when using conventional PCR. Tsai *et al.* (2012.a) were able to consistently detect white spot syndrome virus in whiteleg shrimp using POCKIT™. Finally, Balasuriya *et al.* (2014) used the portable iiPCR platform to detect the equine influenza virus at a concentration that is 100-fold more sensitive than qPCR. The extent to which similar tools can be used to diagnose viral infections in wildlife hosts, including ATV in tiger salamanders, is unknown.

The aim of this study is to use the POCKIT™ iiPCR platform to detect the *Ambystoma tigrinum* virus (ATV) in a population of western tiger salamanders that periodically undergoes ATV-induced mass mortalities (Livingston Lake, Chapters 2 and 4) and then compare the diagnostic and analytic sensitivity of diagnoses with those of conventional PCR. To determine the relative performance of the two diagnostic methods,

three tests were performed. A comparison of diagnostic sensitivity was completed using DNA extracted from salamander tissue samples collected throughout the study area. The samples were tested using both methods, and results compared according to the number testing positive for ATV. A limit-of-detection and analytical sensitivity test was then performed using both methods to assess the minimal number of copies of viral DNA template within a sample that will produce an ATV-positive result. The third test used an ATV positive field sample, diluted to various concentrations and tested on both platforms, to determine which method could detect the virus at a lower concentration.

## 3.2. Methods

### 3.2.1. Sample collection

Hosts were collected using the trapping and handling methods outlined in Chapter 2. A 1cm$^2$ tissue sample ('tail clip') was removed from the tail of each salamander using a fresh razor blade on a surface sterilized with 90% ethanol (Greer & Collins 2007). Tail clipping has no effect on larvae survival (Polich *et al.* 2013) and individuals regenerate tissue lost within 30-days post-incision (Greer & Collins 2007). Tissue samples were preserved in 90% ethanol in a 2mL tube and stored at -4°C for approximately two weeks, until they could be transported to -80°C for long-term storage. Collection sites and sample sizes are listed in Table 3.1.

Table 3.1. Site of origin and collection year of tiger salamander tissue samples used in the diagnostic sensitivity comparison test.

| Site Name | UTM Coordinates | Year Collected | Number of Samples |
|---|---|---|---|
| Livingston Lake | 11U 695693E 5503494N | 2005 | 9 |
| | | 2009 | 3 |
| | | 2010 | 27 |
| | | 2011 | 3 |
| | | 2013 | 94 |
| Lonesome Lake | 12U 288971E 5439388N | 2012 | 1 |
| Rast Dugout | 11U 703961E 5512211N | 2012 | 19 |
| Shenton B Pond | 11U 705644E 5474331N | 2012 | 7 |
| Everts Pond | 11U 706017E 5478966N | 2013 | 23 |
| Hardy Pond | 12U 284424E 5464196N | 2013 | 2 |
| **Total** | | | **188** |

### 3.2.2. DNA extraction

DNA extraction from salamander tissue was completed with a commercially available DNA isolation kit and accompanying protocols (Qiagen DNeasy Blood and Tissue Kit Handbook, 2011). This kit uses the enzyme Proteinase K in a buffer solution to break down the tissue and lyse cells to obtain nuclear, mitochondrial, and pathogen DNA. Following lysing of the cells, DNA was bound to a filter, washed to remove any unwanted material, and then eluted for use in PCR and iiPCR reactions.

To begin this procedure, previously frozen tissue samples (stored in 90% ethanol at -80°C) were thawed and cut on a sterile surface with a fresh razor blade in order to trim the tissue to the appropriate size (<25mg). Tissue was trimmed to approximately $2mm^2$ and then placed into a 2mL locking microcentrifuge tube, along with 20µl of Proteinase K and 180µl ATL buffer. To prevent contamination, the cutting surface and tweezers were sterilized with 95% ethanol between each sample. As a control against cross contamination of DNA between samples, a "bench negative" was obtained after the processing of every fourth sample. The cutting surface and tweezers were sterilized with

95% ethanol, and then tweezers were rubbed on the cutting surface before being placed in the Proteinase K solution. If sterilization were effective, all bench negatives would show no evidence of DNA contamination.

All samples were incubated at 56°C overnight on a rocking platform. The following morning, samples were removed from the incubator and vortexed for 10 seconds. In cases where a tissue sample had not been fully digested, the tube was returned to the rocking platform and incubated at 56°C for another 4 hours. The samples of eluted DNA (approximately 300 to 400µl each) were stored at either 4°C for immediate use or at -20°C for long-term storage.

### 3.2.3. Conventional PCR

Conventional PCR was carried out with the use of *Taq* DNA Polymerase from two commercially available kits. Invitrogen™ *Taq* DNA Polymerase was used on 69 samples taken from *A. mavortium* between 2005 and 2012. QIAGEN™ *Taq* DNA Polymerase was used on 119 samples collected in 2013. Testing was completed at two different laboratories, leading to the use of two sources for *Taq* DNA polymerase. Custom primers MCP4 (5' GACTTGGCCACTTATGAC 3') and MCP5 (5' GTCTCTGGAGAAGAAGAA 3') were obtained from IDT™ (Integrated DNA Technologies). All PCR and iiPCR reactions included a positive, negative, and "no template" control. Positive controls, template material previously confirmed to be ATV positive, were used to ensure all aspects of the PCR process were working properly and to identify false negative results. Negative controls, template material previously confirmed to be ATV negative, were used to identify false positive results. Finally, no

template controls, water in place of template material, were used to identify false positives and ensure no cross contamination had occurred during sample preparation.

Primers MCP4 and MCP5 (10μM) were used to amplify a 531 bp segment of the major capsid protein coating region during the PCR reaction. Template DNA (2μl), extracted from host tissue, was added just prior to the beginning of the thermal cycling process. A full list of reagents and measurements is shown in Table 3.2.

Table 3.2. List of reagent volumes used in the conventional PCR diagnostic tests for ATV in tiger salamander tissue.

| Reagent | Amount |
|---|---|
| Ultrapure water | 15.5μl |
| 10x PCR Buffer –Mg | 2.5μl |
| 10nM dNTP's | 1.0μl |
| 50nM$MgCl_2$ | 1.0μl |
| 10uM MCP 4 | 1.0μl |
| 10uM MCP 5 | 1.0μl |
| *Taq* DNA polymerase (5 U/μl) | 1.0μl |
| Template | 2.0μl |
| **Total** | **25μl** |

Temperatures for the thermal cycling process followed protocols used in previous work on ATV samples (Goater lab, unpublished data) and began with an initialization step of 94°C for 5 minutes, then cycled 35 times at 94°C, 55°C, and 72°C (30 seconds for each step), before concluding with a final extension period of 72°C for 2 minutes. Once amplification was completed, all samples were either visualized immediately or stored at 4°C until visualization.

PCR amplifies target regions in a sample, but analysis of these results cannot be completed without some form of visual representation. PCR product visualization refers to the process by which fragments of DNA are separated according to size, and then

depicted for interpretation. When diagnosing the presence of ATV, a 1.0% agarose gel was run at 100V for 45 minutes. Using a 1kb ladder, each sample was checked for the presence of a 531 bp fragment. Positive and negative controls were also checked for the presence and absence of a 531 bp fragment, respectively. No-template controls were checked to ensure no contamination had occurred throughout the PCR and visualization process.

### 3.2.4. POCKIT™ iiPCR

The primers and probe used for iiPCR POCKIT™ assays, which targeted sequences of the major capsid protein-coding region, were signed and sold by GeneReach Biotechnology Corporation. To extend the shelf life, the primers, probe, dNTP's, and enzyme are lyophilized into a small dry pellet that must be suspended in a buffer solution prior to use. 50µl of Premix Buffer B was added to each of the tubes containing the lyophilized reagents. 5µl of template DNA was added to each tube. Samples were vortexed to mix and then spun down in a centrifuge for 10 seconds. 50µl of the reaction solution was transferred into an R-tube, and then capped. The R-tube was then spun down for 10 seconds. Tubes were checked for the presence of any air bubbles in the solution, and if found, samples were spun down for another 10 seconds. All samples were run on the POCKIT™ using the 520nm setting. Once the program was completed, indicator lights displayed the infection status as positive or negative. During a session of testing, positive and negative controls were added to the first, third, and final runs of the day. By running the controls at the beginning and end of each session, detection capabilities were kept consistent day to day.

### 3.2.5. Relative sensitivity

First, the 188 field samples were tested on each of the two platforms, and then results were compared by looking for any discrepancy in the diagnosis of ATV between the two methods. Second, an analytical sensitivity test was determined using serial dilutions of a plasmid template containing ATV sequence of known quantity. Due to the fact that different primer pairs were used for the conventional and iiPCR, different plasmids containing ATV sequences were used for the two assays. LLC10, a PCR product cloned from a known positive sample obtained from hosts collected at Livingston Lake and confirmed by sequencing, was used for the conventional PCR assay. The plasmid sample $FV3^{+}$, provided by GeneReach Biotechnology Corporation, was used in the iiPCR assay. The DNA concentration in each of the two stock samples was determined prior to the start of testing. The starting concentration of the $FV3^{+}$ sample was 10,000 copies of DNA per µl (GeneReach Biotechnology Corporation, personal communication). The following formula was used to calculate the number of copies of DNA in the sample LLC10, following methods described by the URI Genomics & Sequencing Center (2004). DNA concentration was obtained using the NanoDrop Spectrophotometer.

$$\text{Number of copies of DNA} = \frac{(\text{DNA conc } {}^{ng}/_{\mu l})(\text{Avogadro's number})}{(\text{Length }_{bp})((1\text{x}10^{9})(650))}$$

$$= \frac{(290.5^{ng}/_{\mu l})(6.022\text{x}10^{23 \text{ molecules}}/_{\text{mole}})}{(4050_{bp})((1\text{x}10^{9})(650))}$$

$$= 6.5\text{x}10^{10} \text{ copies of DNA}/\mu l$$

Next, each sample was diluted with ultrapure water from the starting concentration, down to 2 copies of DNA per µl. Each dilution was then tested for the presence of sequences from the MCP gene, indicating an ATV positive sample.

A third test was conducted to determine the extent that an ATV positive field sample can be diluted, while still returning a positive result when tested using conventional PCR and iiPCR methods. Similar to the limit of detection test, a known ATV-positive sample was serially diluted using water, and then tested on both platforms. LL.20.08.13.01, a tissue sample obtained during the 2013 outbreak at Livingston Lake, was used. Each of the 12 dilutions (including one neat sample) was tested for the presence of ATV using POCKIT™ and conventional PCR.

The sensitivity and specificity of each platform were calculated using the diagnostic test, a procedure that calculates characteristics such as sensitivity, specificity, and disease prevalence using the number of positive and negative results (Zhou *et al.* 2002).

### 3.2.6. Gel extraction and sequencing

Two *A. mavortium* tissue samples testing positive for ATV were subjected to DNA sequencing. DNA sequencing is a procedure that determines the exact placement of nucleotides within a DNA molecule. In the case of this study, DNA sequencing was used to compare the nucleotide arrangement of known ATV positive samples from the literature, with that of samples obtained during this study. The comparison would confirm that the disease affecting *A. mavortium* in southwestern Alberta was, in fact, ATV.

The two samples were amplified using conventional PCR and MCP4/MCP5 primers, then run on a 1% low melt agrose gel at 100V for 45 minutes plus 110V for 15

minutes, before the specific band was excised and the target DNA extracted. To obtain the DNA fragment, each band was manually cut from the gel using fresh razor blades and transferred to a fresh microcentrifuge tube. Following the gel excision process, the actual extraction of DNA was completed using the procedure outlined in the commercially available kit "QIAEX II: DNA Extraction from Agarose Gels".

Eurofins MWG Operon (Louisville, Kentucky) completed sequencing of the amplicon using the MCP4 and MCP5 primers. The computer program BioEdit was used to compare sequencing results with those of known ATV sequences.

**3.3.0. Results**

Of the 188 field samples tested, 176 gave identical conventional and iiPCR results. Of the remaining 12 samples, all were ATV positive with iiPCR, and ATV negative with conventional PCR.

Samples tested from 2005, 2009, 2011, and 2012 showed consistent results between the two methods. All 2005 samples were ATV positive, while samples from 2009, 2011, and 2012 were negative. The greatest discordancy between conventional PCR and iiPCR was found in samples collected in 2010, when conventional PCR identified 12 of the 27 samples from Livingston Lake as positive for ATV, whereas 19 of the 27 samples were positive via iiPCR. Discordant results between the two methods were also observed in samples collected in 2013. 20 of the 119 samples were ATV positive with conventional PCR, while 25 of the 119 samples were positive with iiPCR. Overall, the two methods had 93.6% agreement, and 6.4% discordance (Table 3.3). The sensitivity and specificity of the iiPCR relative to conventional PCR is 77.4% ± 13.6% and 100% ± 3.7% (95% CI), respectively.

Table 3.3. Comparison of the sensitivity and specificity of ATV diagnostic tests using iiPCR and conventional PCR.

| | | iiPCR | |
|---|---|---|---|
| | | Positive (+) | Negative (-) |
| Conventional PCR | Positive (+) | **21.8%** (41/188) | **0%** (0/188) |
| | Negative (-) | **6.4%** (12/188) | **71.8%** (135/188) |

The limit of detection comparison used two stock solutions with known concentrations of template DNA to determine the number of copies of DNA needed to give a positive result. Conventional PCR was able to detect a positive result at $2x10^5$ copies of DNA per reaction, with 2µl of template. Testing showed that conventional PCR would return a strong positive result down to $2x10^7$ copies of template DNA per reaction. A weak positive result was detected at $2x10^6$ and $2x10^5$ copies of template DNA per reaction (Figure 3.1). In contrast, iiPCR was consistently able to detect an ATV positive sample down to 25 copies of template DNA per reaction using the assay with 5µl of template (Table 3.4). The limit of detection test was completed on each platform in triplicate to ensure results were accurate and repeatable.

The analytical sensitivity of the two assays was also compared using one field sample. iiPCR was able to detect an ATV positive sample consistently at 1 million times dilution. At 5 million times dilution, iiPCR could no longer produce a positive result (Table 3.5). Conventional PCR was able to detect a positive sample at 100,000 times dilution, but failed to do so at 1 million times dilution. The dilution comparison test was completed in duplicate; with both runs using returning identical results. Genomic sequencing confirmed the virus present in tiger salamanders in southwestern Alberta is

the *Ambystoma tigrinum* virus, with >96% alignment to known samples (Ridenhour & Storfer 2008).

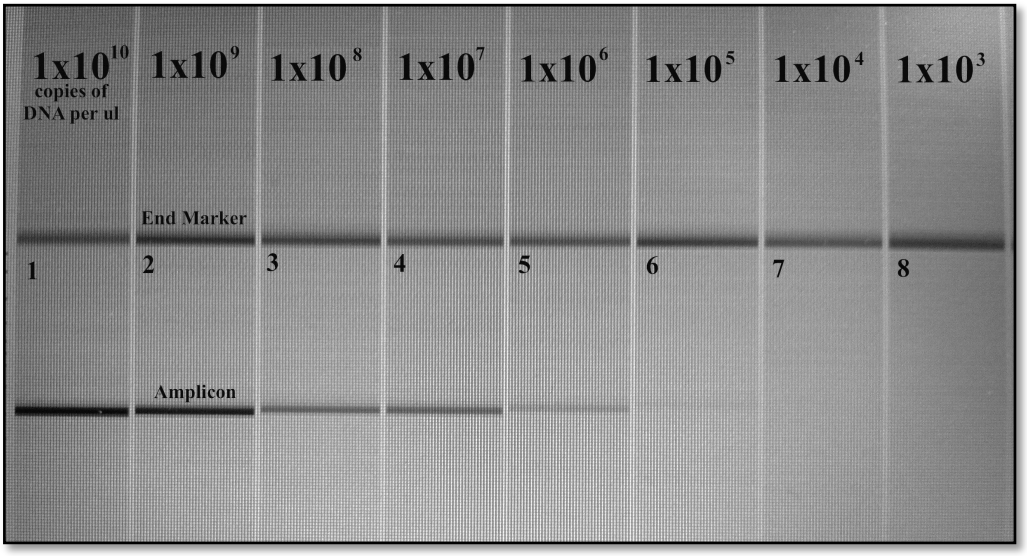


Figure 3.1. Analytical sensitivity of the conventional PCR for ATV in tiger salamander tissue. Lanes 1, 2, 3, and 4 all show a strong positive result, while lane 5 shows a fairly weak positive result, and lane 6 shows an extremely weak positive result. Lanes 7 and 8 show a negative result.

Table 3.4. Results of the limit of detection test comparing the analytical sensitivity of iiPCR and conventional PCR in detecting ATV in serially diluted plasmid samples.

| Copies of DNA in conventional PCR test using 2ul of template | PCR Result | Copies of DNA in iiPCR test using 5ul of template | POCKIT™ Result |
|---|---|---|---|
| $2x10^{10}$ | + | $5x10^{10}$ | n/a |
| $2x10^{9}$ | + | $5x10^{9}$ | n/a |
| $2x10^{8}$ | + | $5x10^{8}$ | n/a |
| $2x10^{7}$ | + | $5x10^{7}$ | n/a |
| $2x10^{6}$ | + | $5x10^{6}$ | n/a |
| $2x10^{5}$ | + | $5x10^{5}$ | n/a |
| $2x10^{4}$ | - | $5x10^{4}$ | + |
| $2x10^{3}$ | - | $5x10^{3}$ | + |
| $2x10^{2}$ | - | $5x10^{2}$ | + |
| $2x10^{1}$ | - | $5x10^{1}$ | + |
| 10 | - | 25 | + |
| 4 | - | 10 | - |

Table 3.5. Analytical sensitivity of iiPCR and conventional PCR when detecting ATV in a diluted field sample.

| Dilution | Conventional PCR Results | iiPCR Results |
|---|---|---|
| Neat | + | + |
| 10x | + | + |
| 50x | + | + |
| 100x | + | + |
| 500x | + | + |
| 1000x | + | + |
| 5000x | + | + |
| 10,000x | + | + |
| 100,000x | + | + |
| 1,000,000x | - | + |
| 5,000,000x | - | - |
| 50,000,000x | - | - |

### 3.4. Discussion

iiPCR has shown results that yield specificity and sensitivity equal to or greater than that of conventional PCR (Tsen *et al.* 2013, Tsai *et al.* 2012.b, Tsai *et al.* 2014). My results are the first to demonstrate that a compact portable device using a TaqMan probe and iiPCR system can provide a diagnostic test for the *Ambystoma tigrinum* virus that is equally specific and more sensitive than conventional PCR methods. iiPCR outperformed conventional PCR in each of the three sensitivity comparison tests. ATV positive samples were detected more frequently in field samples and had lower detection limits when using iiPCR, as compared to conventional PCR. The limit of detection test carried out in this study produced results comparable to others when using the POCKIT™ diagnostic platform. ATV was detected at 25 copies of DNA/reaction, compared to 11 copies of RNA/reaction for the equine influenza virus (Balasuriya *et al.* 2014) and 17 copies of DNA/reaction for the white spot syndrome virus (Tsai *et al.* 2014).

The POCKIT™ system has many advantages over conventional PCR diagnostics, including its ease of use. However, the iiPCR assay used in this study had to be validated with a larger panel of target and non-target sequences to determine specificity of the primers and probes prior to use (GeneReach Biotechnology Corporation 2012). This validation process to determine the best primers and probe for each disease diagnostic test may be time consuming, depending on the complexity of the target sequences and required specificity. The POCKIT™ instrument used in this study can test a maximum of 8 samples at one time, although a larger instrument that can process up to 24 samples is now available. The lower capacity may increase the processing time when testing a large number of samples, particularly when compared to most thermal cyclers, which are capable of processing 96 samples per run.

A cost comparison shows that the processing cost per sample was similar between the two methods. For this study, reagents for POCKIT™ cost $5.28 per sample reaction, while reagents for conventional PCR totaled $4.35 per sample reaction. However, it is important to note that this reflects the total cost for POCKIT™ testing, while conventional PCR required additional funds and equipment to complete post-amplification processing. Unlike conventional PCR, iiPCR does not need post-amplification processing to complete a diagnostic test. Overall, considering equipment, reagents, and man-hour input, cost is most likely higher when testing is completed using conventional PCR. Additionally, iiPCR's higher sensitivity means that pooling samples is possible and would further increase efficiency.

POCKIT Express™, which includes iiPCR machine, centrifuge, and pipets, is designed as a compact portable diagnostic system that can be used at the point-of-care

(eg. medical or veterinary clinic). However, it is field-deployable and can be powered outdoors with a stable AC power supply, on level ground, between 15-35°C (GeneReach Biotechnology Corporation 2012). All necessary equipment can be carried in a single case and reagents can be kept stable at room temperature (GeneReach Biotechnology Corporation 2012). This means ecologists, potentially, could conduct diagnostic testing anywhere that is accessible by vehicle or by foot. Results can be obtained from extracted nucleic acid in less than 60 minutes (GeneReach Biotechnology Corporation 2012). The portability of this system would allow for the earliest possible detection and confirmation of an ATV outbreak on-site.

Following the results of this study, ecologists and epidemiologists now have an additional tool for the highly sensitive diagnosis of an important amphibian pathogen. The primer/probe design used in this study can be applied to other ranaviruses (GeneReach Biotechnology Corporation, personal communication) and there is vast potential to develop assays to tests for other wildlife diseases. The POCKIT™ iiPCR system is much easier to use than conventional PCR, and is also more sensitive, uses lower cost instruments, is extremely fast, and portable. This combination of features will allow for rapid, on location diagnostic testing for ATV and, potentially, other wildlife diseases.

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# CHAPTER FOUR

Seasonal transmission and outbreak dynamics of invasive *Ambystoma tigrinum* virus in a western tiger salamander, *Ambystoma mavortium*, population in southwestern Alberta, Canada

## 4.1. Introduction

Emerging infectious diseases are one of the most frequently observed causes of worldwide amphibian declines (review by Daszak *et al.* 2003; Chapter 1). Although these pathogens do not always cause detectable changes in host population sizes, they have been implicated to cause significant declines, local extirpations, and possible extinctions (Daszak *et al.* 2003). As discussed in Chapter 1, ranaviruses (family *Iridoviridae*) and the fungus *Batrachochytrium dendrobatidis* (*Bd*), are the main emerging pathogens associated with amphibian mortalities (Collins & Storfer 2003, Daszak *et al.* 2003). *Bd* and ranaviruses have caused the decline of amphibian species following mass mortality events in areas of high amphibian species richness (Lips *et al.* 2006, Price *et al.* 2014). Lips *et al.* (2006) reported biodiversity loss across multiple families of frogs and salamanders following a *Bd* outbreak in Central America. Price *et al.* (2014) described devastating losses in northern Spain involving two ranaviruses that caused mass mortalities across multiple sites and species. These studies highlight the recent concerns regarding the effects of emerging infectious diseases in amphibian populations.

The ranavirus, *Ambystoma tigrinum* virus (ATV), is an emerging pathogen of tiger salamander populations in North America (review in Chapter 1; Collins 1988, Jancovich *et al.* 1997, Bollinger *et al.* 1999). First identified in an Arizona population of Sonora tiger salamanders (*Ambystoma tigrinum stebbinsi*) in 1985, ATV has been documented to cause recurrent epidemics in populations throughout much of its broad

geographical range. ATV has a large, double-stranded DNA genome and causes systemic infections that can persist within a population for a period of time without noticeable effects, yet when an outbreak occurs the mortality rate can vary between 40 and 90% (Collins *et al.* 2004, Brunner *et al.* 2005). Results from experimental studies have shown that infection can arise in naïve individuals following ingestion of infected tissue, brief skin to skin contact, exposure to water previously occupied by an infected individual, or exposure to infected substrate (Chapter 1; Jancovich *et al.* 1997, Brunner *et al.* 2005, Brunner *et al.* 2007). Further evidence from field and laboratory studies involving the exposure ATV in naïve hosts has shown that it can be highly infectious and virulent (Jancovich *et al.* 1997). Brunner *et al.* (2004) showed that more than 50% of larvae that were exposed to ATV through a water bath became infected and then died within 40 days post exposure. Similar outcomes were demonstrated for salamanders exposed within outdoor mesocosms (Jancovich *et al.* 1997). These results demonstrate that ATV can cause direct, additive mortality of larval salamanders. Thus, mortality events that are associated with the diagnosis of ATV in large numbers of dead and dying salamanders are highly likely to be caused by exposure to this pathogen.

ATV outbreaks occur when larval salamanders are active in the aquatic environment, typically between May and October (Bollinger *et al.* 1999), with most mortality events occurring in mid to late summer approximating the timing of metamorphosis (Collins 1988, Green *et al.* 2002, Docherty *et al.* 2003, Brunner *et al.* 2004). However, the dynamics of ATV outbreaks are complex. Some salamander populations experience annual ATV outbreaks involving larvae (Brunner *et al.* 2004, Collins *et al.* 2004), whereas others tend to skip years between detectable mortality

episodes (Greer *et al.* 2009). In still other host populations, mortality is undetectable, even during years when rates of exposure are high (Greer 2009). Brunner *et al.* (2004) suggest that the terrestrial adult salamanders act as a reservoir for ATV through the winter, potentially exposing naïve larvae during the spring breeding season. These results show that our limited understandings of the factors that determine variation in exposure and in virus-induced mortality within host populations are key gaps in our understanding of ATV epidemiology in salamanders.

Epidemiological characteristics of ranaviruses in populations of amphibians in Western Canada are poorly known (e.g. Schock *et al.* 2010). In Alberta, Canada, ATV-induced die-offs have been suspected to occur in southern regions of the province, but the evidence is anecdotal. A mortality event involving 1000's of larvae occurred in a pond in Waterton Lakes National Park in 2011 (B. Johnston, personal communication). Ranavirus (probably ATV) was confirmed in a sample of dead individuals at the Canadian Cooperative Wildlife Health Centre (T. Bollinger, personal communication). A similar sequence of mortality, followed by ranavirus diagnosis, was confirmed at a wetland in Fish Creek Provincial Park, Alberta (D. Whiteside, personal communication). Periodic mortality events have been documented at Livingston Lake, a small wetland north of Crowsnest Pass, Alberta, Canada. The first mortality was observed in 2002 involving 1000's of carcasses (Chapter 1; C. Goater, unpublished observations). Subsequent mortality events were observed in 2005 and 2013. Sequencing of ranavirus from 18 hosts sampled from Livingston Lake during the 2005 and 2013 outbreaks confirmed a >96% nucleotide identity with the 531 bp region of the MCP gene in the *Ambystoma tigrinum* virus (B. van Paridon & C. Goater, unpublished observations). In addition to these

mortality events at Livingston Lake, ATV has been detected in asymptomatic individuals during years when no die-offs occurred.

These anecdotal observations indicate that ATV is present in *A. mavortium* in southwestern Alberta, causing periodic outbreaks similar to those reported in Saskatchewan, Manitoba, and Arizona (Bollinger *et al.* 1999, Brunner *et al.* 2004, Jancovich *et al.* 2005). At Livingston Lake, ATV-induced mortality appears to be a common outcome, but it is intermittent in its occurrence. One problem is that we have a poor understanding of fundamental rate parameters that underlie ATV/salamander dynamics, such as transmission rate and rate of ATV-induced mortality. Our understanding is also poor relative to how these rates vary within a season, between years, and between sites. One aim of this longitudinal field study in Livingston Lake is to monitor seasonal transmission and rates of ATV-induced host mortality over two consecutive years. To determine if patterns of ATV infection in Livingston Lake are characteristic of other sites in the region where salamanders are known to occur (Chapter 2), I also determined general patterns of ATV occurrence in 14 other sites.

## 4.2. Methods

### 4.2.1. Study site

Livingston Lake is located in southwestern Alberta, northeast of Crowsnest Pass, in UTM Zone 11U 695693E 5503494N, elevation 1456m. It is a permanent water body covering 3.96ha, with an average depth of 0.8m and a maximum depth of 2.7m. Although this privately-owned lake is located within the Native Rangeland/Grazing Land zone (Government of Alberta 2010), cattle have not been present in the surrounding watershed for at least 15 years. A small herd of 8 – 10 horses are pastured within the watershed, but

fencing prohibits access to the lake and its immediate riparian area. The area surrounding Livingston Lake is part of the subalpine, montane, and foothills fescue natural sub-regions (Downing & Pettapiece 2006). The surrounding vegetation primarily consists of grasses (e.g. *Festuca campestris*, mountain rough fescue), characteristic forbes and shrubs (e.g. *Potentilla frutilla*, shrubby cinquefoil). Small stands of aspen (*Populus tremuloides*) and lodgepole pine (*Pinus contortal)* occur at higher elevations within the watershed. Within the lake, the macrophyte community is dominated by rushes (*Juncus* spp.), horsetails (*Equisetum* spp.), bulrush (*Scirpus* spp.), sedges (*Carex* spp.), and pondweeds (*Potamogeton* spp.). Annual precipitation (440 mm p.a., Strong 1992), and melting snowpack in the spring are the main water sources and there is no natural outflow or inflow. The lake is naturally fishless. However, rainbow trout (*Oncorhynchus mykiss*) fingerlings have been stocked intermittently between 1994 and 2012. Stocking operations are challenging, and generally unsuccessful, due to annual winterkill (R. Brinkmann, personal communication).

**4.2.2. Host collection**

Salamanders were sampled using the live-trapping methods described in Chapter 2. A full description of the sampling sites, sampling periods, and catch per unit effort are provided in Appendix 6.2 and 6.3. In brief, I used four modified funnel traps (Mushet *et al.* 1997) to sample larval and adult *A. mavortium* populations at 15 sites. The traps were placed parallel to the shore, in water 15 – 100 cm deep. During each collection, traps were left in place over a 48-hour period. Each site was sample at least once between June and August in 2012 and 2013. At Livingston Lake, where I knew salamander densities

were relatively high, I sampled biweekly through early July and late August in 2013 and 2014.

All salamanders captured were measured and photographed, and then a 1cm$^2$ tissue sample from the tail was obtained using a sterile razor blade (Chapter 2). Tissue samples were preserved in 90% alcohol at -80°C until processing. Salamanders were held for a maximum of 30 minutes, and then released at the point of capture. Equipment was sterilized between sites to avoid the spread of pathogens. In total, 216 individual tissue samples were obtained from Livingston Lake, and an additional 59 were obtained from the other 14 sites.

**4.2.3. Tissue collection**

DNA was extracted from the tissue samples following methods described in Chapter 3. In brief, DNA was extracted from the preserved tissue samples, then stored at -20°C until diagnostic tests were preformed. Two types of molecular diagnostic methods were used. First, conventional PCR procedures were completed with the use of primers MCP4 and MCP5 (Mao *et al.* 1997) to amplify a 531 bp segment of the major capsid protein-coding region. Specific reaction conditions are listed in Chapter 3. PCR products were visualized using agarose gel electrophoresis. The presence of a 531 bp band was an indication of a positive ATV diagnosis. Second, insulated isothermal PCR (iiPCR) technology using a probe and primer design, also targeting sequences of the major capsid protein coding region, was used to test all samples. Results in Chapter 3 indicate that ATV detection using iiPCR is equally specific and more sensitive than diagnostic testing using conventional PCR. Therefore, the number of positive hosts detected using iiPCR determined the prevalence of ATV. During all diagnostic testing, positive and negative

controls were implemented to detect any false positives or false negatives within the samples.

#### 4.2.4. Shoreline surveys

Shoreline surveys were completed twice each week at Livingston Lake in August 2013 and 2014 to detect signs of ATV-induced mortality. The entire shoreline of the lake was surveyed to detect any dead or dying salamanders in the water or on shore, similar to visual encounter survey techniques developed by Heyer *et al.* (1994). Individuals that were dead or moribund were observed for visible signs of ATV (internal organ hemorrhaging, skin sloughing, and bloody exudates), and then collected as a whole body sample to be tested for ATV. Shoreline surveys were competed at 14 other sites using the same methods. At these sites, the water body was surveyed once at the time traps were in place.

#### 4.2.5. Response variables and analyses

ATV prevalence was estimated as the number of positive hosts divided by the total number of hosts collected during each sampling period. This estimate represents minimum ATV prevalence of the sample, since recently exposed hosts may not be detected. Greer & Collins (2007) showed that testing individuals within 5 days post exposure to ATV can lead to the underestimation of overall prevalence when using conventional PCR diagnostic methods. Thus, my estimates of prevalence are conservative minimums. Seasonal and annual changes in ATV prevalence were assessed with standard chi-squared analyses. These changes were assessed separately for samples collected in 2013 and 2014. Confidence intervals (95%) involving prevalence were estimated based on sample sizes (Dohoo *et al.* 2003).

## 4.3. Results

### 4.3.1. Patterns of infection in Southern Alberta

Other than at Livingston Lake, ATV was detected at only one of the 14 sites in southwestern Alberta. At this site, Everts pond, 2 of 28 individuals tested positive for ATV in August 2013. During trapping events and shoreline surveys, no individuals, including the two infected with ATV, showed clinical symptoms associated with the virus. Everts pond occurs at the edge of a wetland complex approximately 25 km south of Livingston Lake.

### 4.3.2. Seasonal pattern of infection

The seasonal pattern of ATV transmission at Livingston Lake was very similar between years (Figures 4.1 & 4.2). Only 7% of the 58 larvae (8.0 – 8.5 cm) collected during the first sample in early to mid July in both years tested positive for ATV. Over the next 28 - 38 days, ATV prevalence increased to 15 -19% in late July, and then quickly rose to at least 96% by mid-August. At the time of metamorphosis in mid-August when larvae were 14.8 to 16.0 cm long, ATV was detected in 100% of juveniles in 2013 and 96% of juveniles in 2014. Chi-square tests on the 2013 ($\chi^2 = 50.30$, df = 3, $p < 0.0001$) and 2014 ($\chi^2 = 67.51$, df = 3, $p < 0.0001$) data showed that the increases in ATV prevalence each season were highly significant.

The frequency distribution of host length showed a similar pattern between the two years (Figure 4.1, see also Chapter 2). Thus, young-of-the-year reached a size when they could be detected in the traps in mid-July and then they grew rapidly through the larval stage for approximately the next 30 – 40 days. Metamorphosis occurred in mid-August in both years and there was no evidence that a proportion of larvae remained in

the lake through their first winter. When the occurrence of ATV in each individual was superimposed on these host-length frequency distributions (Figure 4.1), there was no evidence that ATV was associated with larval size. For samples collected at the end of July in both lakes, there was no significant difference in host length between infected and uninfected hosts (2013: $t = 0.49$, $df = 48$, $p = 0.62$; 2014: $t = 1.80$, $df = 38$, $p = 0.08$).

### 4.3.3. Host mortality

In 2013, mortality was observed in 8 of the 13 individuals collected in the traps between 16-20 August and clinical symptoms (skin sloughing, bloody exudates and lethargy) of ATV were observed in all 13 individuals. Also, 10 dead individuals were observed during shoreline surveys between 16 – 20 August, 2013. All tissue samples collected during this 5-day period tested positive for ATV. The larval ATV infection rate in 2014 was similar to the pattern observed in 2013, but very little mortality was observed during mid-August, and no dead individuals were detected during the shoreline surveys at Livingston Lake. 17 out of the 132 individuals collected in the traps on August 12, 2014 were dead, and only 25 of the 132 individuals showed clinical signs of ATV

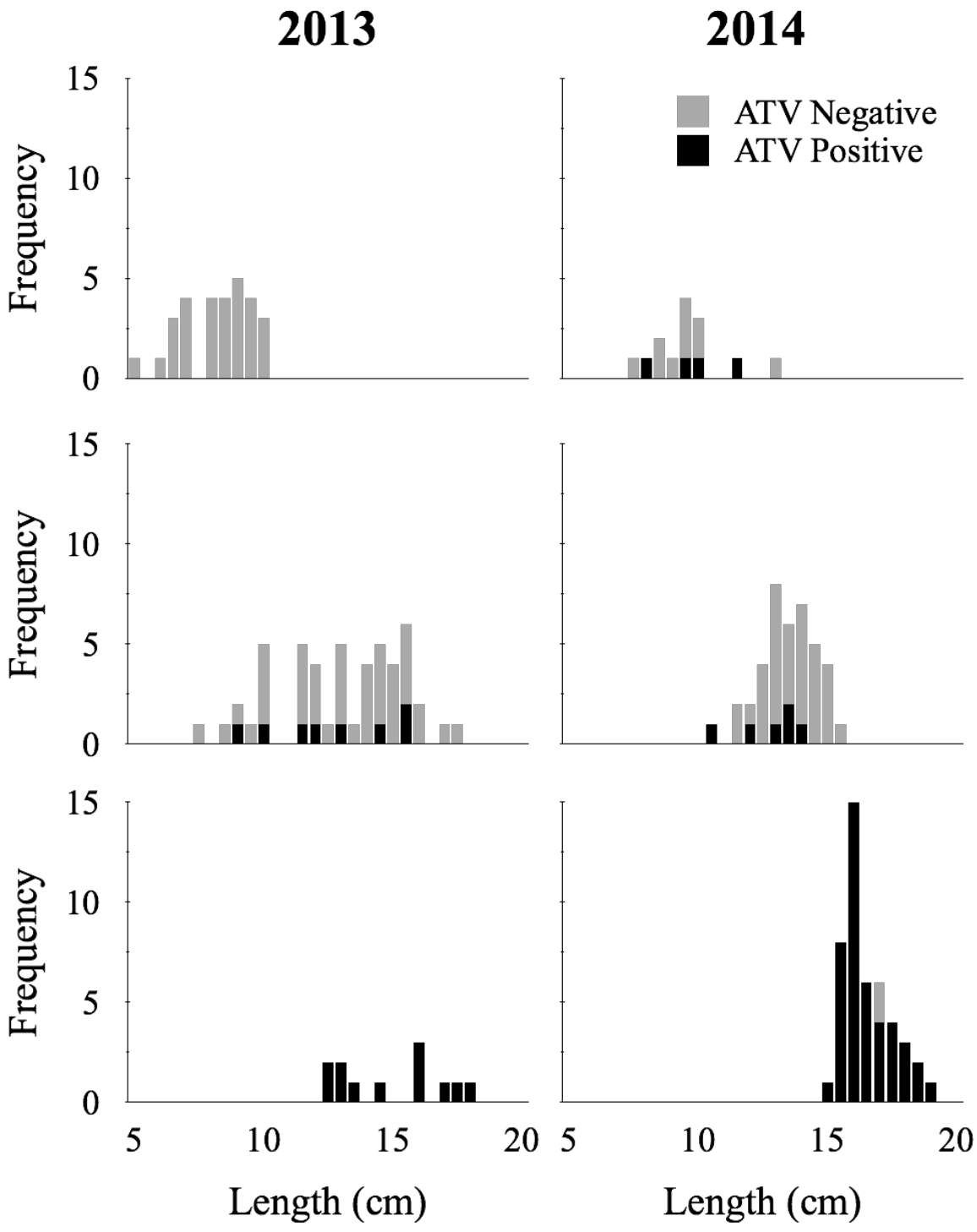


Figure 4.1. Seasonal distribution of ATV-positive salamander larvae within host samples collected at Livingston Lake in 2013 and 2014. Sampling dates (from top to bottom) in 2013 were July 10th, July 30th, and August 16th, and in 2014 were July 15th, July 31st, and August 12th.

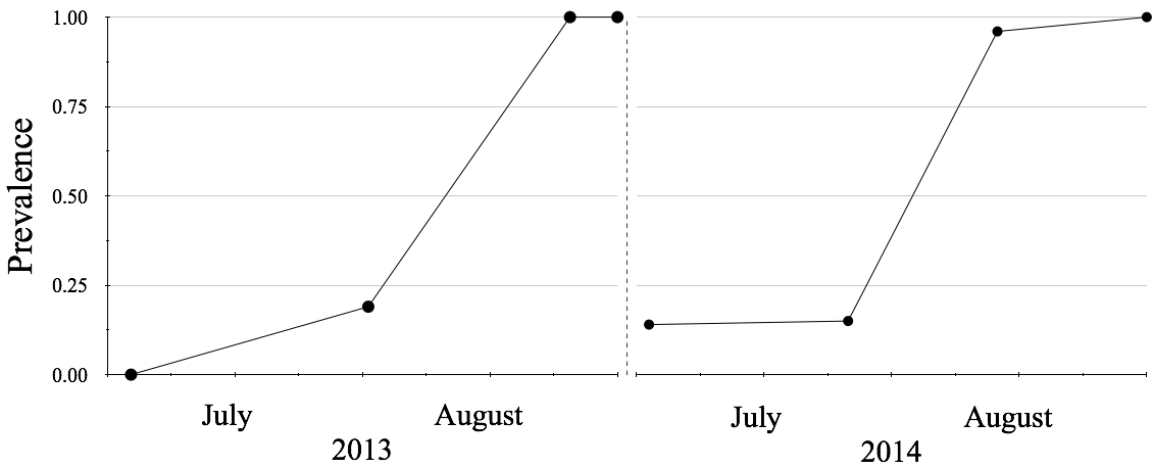


Figure 4.2. Seasonal changes in prevalence of ATV in samples of larval *A. mavortium* from Livingston Lake between July 1 and August 31 in 2013 and 2014. The dashed line represents transition between ATV prevalence in 2013 and 2014.

**4.4. Discussion**

Using tissue samples collected from larval *A. mavortium* at Livingston Lake, Alberta, I found that the timing of initial exposure to ATV and the prevalence of ATV infection were highly similar between years. In both years, initial exposure occurred in mid-July, with prevalence increasing to <20% by late July, then to 96 – 100% by mid-August. However, high host mortality was only detected in a single year, despite a prevalence of >95% in the larval population in both years. In mid-August 2013, 18 individual larvae were found dead with clear clinical signs of ATV. Additionally, variation in the proportion of symptomatic individuals varied between 100% in 2013 and 19% in 2014 (mid-August assessment). Therefore, the seasonal dynamics of exposure were consistent between years, while the outbreak dynamics varied.

The overall pattern of seasonal transmission in Livingston Lake was highly consistent between years. Transmission of ATV into young-of-the-year larvae occurred in early to mid summer when the larvae were approximately 45-60 days old. Following this initial period of exposure, there was a rapid increase in prevalence, from 0 to >95%

within approximately 5 weeks. This seasonal pattern of transmission into developing larval salamanders is consistent with the results of the other longitudinal field surveys involving ATV (Collins *et al.* 1988, Jancovich *et al.* 1997, Bollinger *et al.* 1999). The overall pattern suggests that two important transmission events occur each year in Livingston Lake. Since ATV does not survive long enough to overwinter in the water or substrate (Jancovich *et al.* 1997, Nazir *et al.* 2012, Johnson & Brunner 2014), or use a vector (Collins *et al.* 2004), annual re-introduction of infective stages must occur each spring. Following single or multiple introductions of ATV early in the summer, there must also follow a period of larvae-to-larvae transmission in mid-summer that results in exposure within >95% of the population. Future epidemiological studies should focus on quantifying these two important rate parameters.

The source of initial exposure is likely metamorphosed individuals with sub-lethal infections that return to the lake to breed in spring. Brunner *et al.* (2004) showed that overwintering juvenile and adult salamanders could transmit ATV into larvae in subsequent years. Larvae can become infectious within 2 days following exposure to ATV, resulting in a high potential for larvae-to-larvae transmission (Brunner *et al.* 2007). There is also evidence that ATV becomes increasingly infectious over time within individual hosts, further contributing to rapid transmission among hosts within a pond (Brunner *et al.* 2007). Taken together, these results indicate that the pattern of seasonal transmission of ATV into larval salamanders, while consistent between years, is likely determined by complex introduction of the virus and factors such as water temperature and macrophyte cover that may affect rates of exposure among individual hosts in mid-summer.

A pattern of consistent seasonal transmission was not paralleled by a consistent pattern of host mortality. Thus, even though ATV prevalence reached close to 100% in both years of my study, mortality was fairly high in one year, and virtually absent in the next. Results showing unpredictable annual host mortality in 2013 and 2014 are consistent with the results of previous surveys on salamanders at Livingston Lake (C. Goater & B. van Paridon, unpublished data). In surveys completed between 2005 and 2011, at least three patterns of host mortality have been shown to occur in the lake; low prevalence/no host mortality, high prevalence/low host mortality, and high prevalence/high host mortality. My results show that this range of outcomes can occur in consecutive years. Thus, there was a narrow, but distinctive window of observed mortality that occurred over 4 days in summer 2013, a year when prevalence reached 100%. During the following year, low mortality was observed despite >95% prevalence. These results suggest that even in the face of consistent year-to-year transmission into virtually all larvae up to the point of metamorphosis, patterns of ATV-induced host mortality are unpredictable and complex. Similarly, complex and episodic patterns of ATV-induced host mortality have been observed at other sites (Brunner *et al.* 2004, Greer *et al.* 2005).

One explanation for the inconsistency in annual mortality is that ATV-induced mortality in Livingston Lake is context dependent, meaning outbreaks occur when infection interacts synergistically or interactively with other potential stressors. A possible explanation for the differences in host mortality between 2013 and 2014, despite equally high rates of transmission, is that overall host quality was higher in the latter year. My results presented in Chapter 2 show that larval growth rates were 36-61%

higher in 2014 and larvae metamorphosed 8-39% larger. There is extensive evidence involving other host/parasite interactions that hosts in good overall condition can either better tolerate, or better compensate, for the potentially negative consequences of infection (review by Goater *et al.* 2014). Determine the underlying causes of the higher growth rates in 2014 would be a useful addition to future studies.

There have been few experimental tests of the context-dependent hypothesis. Greer *et al.* (2008) evaluated the interaction between larval salamander density and ATV transmission. Their results showed that while density influenced the time from infection to death in larval salamanders, density did not affect the likelihood of ATV exposure among individuals. Rojas *et al.* (2005) linked higher mortality rates with lower incubation temperatures in larval salamanders infected with ATV. Laboratory experiments have also shown increased ranavirus susceptibility in larvae when certain chemicals or pesticides are present (Forson & Storfer 2006, Kerby *et al.* 2011). Greer *et al.* (2008), Rojas *et al.* (2005), Forson & Storfer (2006) and Kerby *et al.* (2011) showed that multiple factors can influence the rates of transmission and mortality in larval salamander populations. Empirical studies that cross factors such as host density, water temperature, and aspects of water quality are needed to test the context-dependent hypothesis as an explanation for the outbreak dynamics observed in Livingston Lake.

Another explanation is that annual changes in the genetic structure of ATV directly or indirectly influence virulence. Experimental demonstration of a decline in virus virulence within 1-2 generations, independent of changes in host response, has been demonstrated for the myxoma virus in rabbits (review by Kerr 2012). Similarly, 3 isolates of the iridovirus, large-mouth bass virus, have shown varying degrees of virulence in

juvenile large-mouth bass (*Micropterus salmoides*) (Goldberg *et al.* 2003). Three Frog Virus 3 isolates have also been shown to differ in virulence when tested on northern leopard frog (*Lithobates pipiens*) tadpoles (Morrison *et al.* 2014). Schock *et al.* (2009) have shown that both viral strain and characteristics of the host population contribute to the virulence of ATV in *A. mavortium* populations. It is possible that multiple viral isolates have been introduced into Livingston Lake during the past decade, each of which may vary in virulence. It is also possible that the virulence of individual isolates may change over time, conceivably even between years (Kerr 2012). Modern genomics and proteomics tools could be used to assess existing salamander tissue samples for changes in the genomic structure of ATV during outbreak and non-outbreak years.

Few studies of ATV epidemiology in tiger salamander have been conducted within relatively pristine sites (Collins *et al.* 1988, Jancovich *et al.* 1997, Bollinger *et al.* 1999). Greer & Collins (2008) showed that *A. mavortium* populations infected with ATV are more commonly found in modified sites than in unaltered sites in Arizona. Livingston Lake is a minimally disturbed waterbody with very few grazing livestock present in the catchment area, no irrigation system diverting water, and little cultivated land in the surrounding area. Although stocked with trout in the past, the lake is not open to public fishing and there is little risk of ATV infected individuals being introduced via the bait trade (Jancovich *et al.* 2005). Evidence from Livingston Lake suggests that ATV outbreaks are not limited to altered sites, but can occur in relatively pristine habitats with no apparent anthropogenic influences.

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# CHAPTER FIVE

Conclusions

## 5.1. Key Advances

My thesis had three primary goals. The first was to describe the population characteristics of *A. mavortium* at selected sites in southwestern Alberta. While *A. mavortium* are known to occur throughout the area, data regarding density, growth rate, and size at metamorphosis were unknown prior to this study. The second goal was to compare the sensitivity and specificity of a new molecular diagnostic tool, iiPCR, with that of conventional PCR-based methods. This diagnostic tool has previously been shown to be more sensitive and specific for the diagnosis of some important animal pathogens in their hosts compared to conventional PCR methods (Tsai *et al.* 2012, Tsen *et al.* 2013, Balasuriya e*t al.* 2014), but the tool has not yet been tested on pathogen systems involving wildlife. The third goal was to describe the seasonal transmission and outbreak dynamics of ATV in a salamander population in southern Alberta.

One key advance from the salamander population survey showed that breeding populations are present throughout southwestern Alberta at approximately 50% of sites (Chapter 2), similar to occupancy rates reported in the literature (Collins 1981, Corn *et al.* 2005). The results of previous studies have suggested that tiger salamanders are irregularly distributed across the landscape (Collins 1981), a pattern consistent with my results in southwestern Alberta. Larval population sizes tend to be low (less than 20 individuals captured per water body, Chapter 2) at the majority of sites that I surveyed. In southwestern Alberta, salamander larvae occur at densities that are lower than at sites reported in the literature, where some breeding populations exceed hundreds or thousands

of adults in some years (e.g. Pechmann *et al.* 1991, Wissinger & Whiteman 1992). However, *Ambystoma spp.* population densities can fluctuate enormously from year-to-year, making it difficult to accurately assess populations in single-year surveys (Pechmann *et al.* 1991, Wissinger & Whiteman 1992).

Results from my population surveys also showed that tiger salamander populations in southwestern Alberta contain individuals that do not overwinter as larvae. This contrasts with the 2-3 year larval stage seen in many higher elevation populations (Bizer 1978, Sexton & Bizer 1978, Morrison & Hero 2003) and in some paedomorphic populations at low elevation sites on the prairies (Larson *et al.* 1999). Given the high primary and secondary productivity that is characteristic of my study sites, their lack of fish predators, and their relatively high altitude (>1250 m), the absence of paedomorphic individuals was surprising. Further sampling efforts at comparable sites along the eastern slopes of the Rocky Mountains are needed to evaluate the generality of this observation.

A second advance was my demonstration that a new molecular diagnostic platform is equally specific, yet more sensitive than conventional methods (Chapter 3). Currently, the most common diagnostic tool for ATV diagnosis is via analysis of host tissue samples for the major capsid protein by conventional PCR. However, the sensitivity of this process can be limited and can often result in false negative diagnoses in recently exposed individuals (Greer & Collins 2007). Using three comparison tests, I showed that a portable iiPCR unit was more sensitive for detecting ATV in host tissue than conventional PCR methods. In diluted test samples, ATV was detected at 25 copies of DNA per reaction when using iiPCR, compared to conventional PCR detecting ATV at 200, 000 copies of DNA per reaction. When testing a tissue sample from an infected

individual, iiPCR could detect an ATV positive sample at dilutions 4-fold higher than needed for the same diagnosis when using conventional PCR. These finding are consistent with other tests involving host/pathogen systems that compared results between iiPCR platforms and conventional PCR (Tsen *et al.* 2013, Tsai *et al.* 2012, Tsai *et al.* 2014). In addition to greater sensitivity, the iiPCR platform offers advantages of faster processing times and can be used in field settings to diagnose ATV outbreaks. The two main advances of this new and highly sensitive technology in the context of ATV (and potentially other ranavirus/amphibian systems) is the reduced cost of diagnosis and the ease with which infection status within isolated host populations can be rapidly assessed.

The results in Chapter 4 show that in each year of the study, virtually all salamander larvae in Livingston Lake were exposed to infective stages of ATV (in late June or early July each year), leading to 100% prevalence at metamorphosis approximately 6 weeks later. Seasonality is a hallmark of many pathogen/host and parasite/host interactions (Altizer *et al.* 2005), so these results are not surprising. The striking result from my study is that high and consistent rates of transmission did not lead to consistent outbreak dynamics. High rates of ATV transmission (prevalence = 100%) at metamorphosis led to an observed outbreak and host mortality in 2013. However, an equally high rate of transmission in the subsequent year (96% prevalence) was associated with very low host mortality. When considering findings at Livingston Lake previous to my study, where both ATV prevalence and observed mortality ranged between 0% and 100%, it is apparent that outbreak dynamics are highly complex and unpredictable. Few other studies have investigated the concordance between variation in annual ATV

transmission and annual variation in outbreaks. Greer *et al.* (2009) monitored 4 tiger salamander populations over 4 years and found that prevalence varied significantly between years, although no mortality episodes were observed. This supports the findings of my study that overall prevalence was not necessarily associated with an outbreak, as high mortality was observed in the population in one year but not the following when prevalence was nearly identical.

**5.2. Future Directions**

A more intensive survey at a broader spatial scale is necessary to accurately detect population-level changes in the *A. mavortium* population in southwestern Alberta. Past assessments have assumed the species is common and abundant in the area (COSEWIC 2012). Preliminary evidence from my limited survey suggests that this is not the case. The low density of larval *A. mavortium* at all but one study site suggests that most breeding populations are small in this region and are probably highly episodic in their occurrence (Chapter 2). Future studies should expand the spatial and temporal scale of my study, working towards the goal of long-term monitoring of key populations. The long-term population monitoring study conducted by Pechmann *et al.* (1991), and others like it, provide examples of appropriate tools that could be used to assess annual variation in population density at targeted sites (e.g. Livingston Lake). Seasonal population monitoring would also provide a source of host tissue for the continued assessment of ATV at this site.

Attempting to diagnose ATV on site using the mobile iiPCR system would be the next step in integrating this technology into formal diagnostic protocols. The mobile platform is designed for use within veterinary diagnostic facilities and at the scale of

individual ranches or livestock operations (GeneReach Biotechnology Corporation 2012). The field conditions surrounding water bodies and wetlands could produce new challenges (e.g. remote locations, environmental conditions) for using this platform. Determining the number of days after exposure for ATV to be detected in tissue samples is also an important step to support the use of the iiPCR system. Conventional PCR is less accurate in detecting ATV in individuals 0 – 5 days post-exposure than individuals 5+ days post-exposure (Green & Collins 2007). This results in a timeframe when false negatives could be diagnosed, potentially underestimating the prevalence of ATV in a population. Since the iiPCR system is more sensitive than conventional methods, ATV can be detected earlier and with fewer false negatives. This technology is an important advance in ATV diagnosis, but more tests are needed in order to determine the limits of its use.

The continued seasonal monitoring of ATV transmission dynamics and outbreaks at Livingston Lake should be a top priority in future studies. The existing dataset will be strengthened with additional data from subsequent years. To my knowledge, there are no comparable studies that are designed to concurrently monitor long-term changes in both salamander densities and ATV exposure. Doing so would provide exceptional insight into the nature of outbreaks. To address the context-dependency hypothesis, future studies should include data on annual variation in water availability and water temperature, prey density and composition, and other components of water quality such as primary productivity and perhaps the presence and concentration of pesticides. To test the hypothesis that pathogen genetics change over time continued collection and preservation of ATV for DNA analysis in both outbreak and non-outbreak years would also be

straightforward. Results that demonstrate whole genome-level or gene sequence-level changes in ATV between years would be groundbreaking. Combined tests of these two alternative hypotheses are feasible in the ATV-*Ambystoma* system in Livingston Lake.

Future studies should also focus on additional monitoring approaches that are designed to evaluate key mechanisms relative to transmission. Brunner *et al.* (2004), monitored all the juveniles leaving natal ponds in the fall and adults arriving in the spring for ATV to determine if overwintering adults reintroduced the virus each spring. While I speculate that overwintering adults at Livingston Lake are the main source of annual introduction of ATV, supportive evidence is lacking. A monitoring study of this type could also provide data on the proportion of returning adults infected with the virus in outbreak and non-outbreak years. Overall, the ATV-*Ambystoma* system at Livingston Lake provides ideal opportunities to contribute to our understanding of the transmission and outbreak dynamics of ATV in tiger salamanders.

Future studies should also incorporate laboratory and mesocosm based experiments to test specific hypotheses regarding underlying causes of variation in ATV-induced mortality, particularly the role of additional stressors that might influence the course of host mortality. Greer *et al.* (2008) used laboratory experiments to test the role of host density on ATV transmission and outbreak dynamics. The potentially interactive effects of host characteristics, such as life history stage, host size, host immune status, and especially host quality, on the outcome of infection also need to be carefully assessed. It is intriguing that larval growth rates were higher in 2014 than in 2013, and in any other year prior (C. Goater, unpublished observations). Yet despite high rates of ATV transmission, mortality in this high host-quality year was very low. Experimental tests

that involve ATV exposure in samples of poor-quality versus high-quality hosts would be useful. Experimental approaches used by Brunner *et al.* (2005) and Robert *et al.* (2005) provide good examples.

### 5.3. Conservation Implications

Houlahan *et al.* (2000) and Stuart *et al.* (2004) provide clear evidence that global amphibian populations are declining. The same pattern is clear for amphibian populations in North America (Adams *et al.* 2013). This pattern is especially clear for populations of salamanders in the *Ambystomatidae* family, a group that consistently has shown higher population declines than other amphibian taxa (Stuart *et al.* 2004). The results of my study, although limited in scope, have important conservation implications for *A. mavortium* populations in western Canada at the northern and western extremes of its range.

The tiger salamander, the most wide-ranging species in the *Ambystomatidae* family, is commonly considered widely distributed through its range in Canada (COSEWIC 2012), although few recent studies provide supporting evidence. To ensure that provincial and federal conservation policies reflect the true status of this salamander in Alberta, I recommend that the overall population distribution and population size be determined to provide an accurate standard on which to base management decisions. Recent policy changes for this species in Alberta have been made in the absence of key data that describe the population status of known populations, but are based on an increase in the occurrence of factors known to cause significant population declines, including infection with ATV and chytrid fungus (COSEWIC 2012). This is a critical knowledge gap. Following the population assessment approaches of Dodd & Dorazio

(2004), a short-term, intensive survey of potential breeding sites should be conducted to assess site occupancy and relative density of *A. mavortium* in Alberta.

Long-term monitoring of selected populations is also a necessary component. Natural fluctuations in breeding and larval populations of *A. mavortium* and other *Ambystomid* salamanders can mask overall trends in short-term studies (Harte & Hoffman 1989, Pechmann *et al.* 1991). Long-term monitoring of multiple focus populations representing different regions and habitats will be an essential component, if the aim is to detect the roles of particular factors in determining variation in population sizes. Populations should be monitored for the number of breeding adults arriving each spring and the numbers of newly metamorphosed juveniles dispersing each fall, similar to Pechmann *et al.* (1991). While this type of study is difficult to maintain, it will produce critical data to inform conservation decisions. The Livingston Lake population would provide a logistically feasible model site for this type of approach.

I conclude that the data arising from this study is consistent with the notion that this salamander is at risk of future population declines, if they are not already occurring. Most populations were detected infrequently and in low densities, with the exception of the relatively high-density population in Livingston Lake. Yet the risk of ATV exposure at this site is high. The role of infected hosts in this population acting as a source for the spread of infection to other sites is unknown, but is potentially also high. Other sites in southern Alberta that once contained large populations of salamanders have been extirpated (e.g. Tyrrell Lake). Perhaps of most concern are the observations (Chapter 4) of the current extirpation of a tiger salamander population from a small pond in Waterton Lakes National Park following an ATV outbreak. Intensive sampling at this site for 3

consecutive years following a mortality event involving thousands of larval salamanders had yielded no evidence of returning individuals. Given that factors such as drought, increased water temperature, and introductions of piscivorous fish have been shown in empirical studies to cause direct additive mortality for salamanders, and these factors are known to occur in this region, the conservation of this species, and an assessment of the factors leading to its decline, should be a priority.

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## APPENDIX

Table 6.1 – Site characteristics and habitat data from 15 water bodies surveyed for *A. mavortium* in southwestern Alberta.

| Site | UTM | Elev. | Type | Permanency | Dominant Vegetation | Dominant Substrate | Surface Area ($m^2$) | Dist. To Nearest Water Body | Fish | Cattle Access | Other Amphibian Species |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 01 Shenton A | 11U 705000E 5473530N | 1452m | Pond | Year-round | Coniferous Forest | Silt | 562 | <1km | No | Yes | Long-toed Salamander |
| 02 Shenton B | 11U 705544E 5474331N | 1326m | Pond | Year-round | Coniferous Forest | Macrophyte | 958 | <1km | No | Yes | Long-toed Salamander |
| 03 Snake Trail | 11U 716206E 5505889N | 1357m | Pond | Year-round | Grassland | Macrophyte | 4901 | >1km | Yes | No | None |
| 04 Toney | 11U 712258E 5481218N | 1300m | Dugout | Year-round | Grassland | Silt | 337 | <1km | No | Yes | None |
| 05 Rast | 11U 703961E 5512211N | 1310m | Dugout | Year-round | Grassland | Silt | 355 | <1km | No | No | Long-toed Salamander |
| 06 Hardy | 12U 284656E 5464127N | 1452m | Wetland | Year-round | Deciduous Forest | Macrophyte | 12929 | <1km | No | Yes | Boreal Chorus Frog |
| 07 Lonesome | 12U 289279E 5439535N | 1309m | Lake | Year-round | Mixed Forest | Macrophyte | 74139 | <1km | No | No | Western Boreal Toad Long-toed Salamander Columbia Spotted Frog Boreal Chorus Frog |
| 08 Livingston | 11U 695693E 5503494N | 1456m | Lake | Year-round | Grassland | Macrophyte | 39552 | <1km | No | No | Boreal Chorus Frog |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 09 River A | 12U 293609E 5445691N | 1276m | Pond | Seasonal | Grassland | Macrophyte | 4866 | <1km | Yes | No | Long-toed Salamander |
| 10 Waterton | 12U 288358E 5439369N | 1326m | Water treatment pond | Year-round | Mixed Forest | Silt | 13599 | <1km | No | No | Western Boreal Toad |
| 11 Colwell | 11U 700989E 5510685N | 1396m | Dugout | Year-round | Grassland | Silt | 655 | <1km | No | No | None |
| 12 Birdseye | 12U 299120E 5439194N | 1512m | Pond | Year-round | Deciduous Forest | Macrophyte | 5992 | <1km | No | No | Columbia Spotted Frog Boreal Chorus Frog |
| 13 Marr | 12U 292924E 5467008N | 1351m | Lake | Year-round | Grassland | Macrophyte | 181625 | <1km | Yes | No | None |
| 14 Klein | 11U 700679E 5511919N | 1396m | Lake | Year-round | Grassland | Macrophyte | 99842 | <1km | No | Yes | None |
| 15 Everts | 11U 706017E 5478966N | 1384m | Wetland | Year-round | Mixed Forest | Silt | 6935 | <1km | No | Yes | Long-toed Salamander |

Table 6.2 – Information on trapping events targeting *A. mavortium* at 15 sites in southwestern Alberta. Catch per unit effort (CPUE) is calculated as the number of traps (1, 2, 3, or 4) multiplied by the number of hours set (24, 48, or 72).

| Site Name | Dates of Trapping Events | Effort | Number Captured | CPUE | Comments and Observations |
|---|---|---|---|---|---|
| 01 Shenton A | 12 July 5 | 96 | 1 | 0.010 | Long-toed salamander egg masses observed in May. One adult tiger salamander captured in July. |
| 02 Shenton B | 12 Aug 21 | 144 | 7 | 0.049 | One painted turtle spotted in the pond. Captured 7 larvae and took tail clips. |
| | 13 Aug 31 | 96 | 0 | 0 | No salamanders observed. |
| 03 Snake Trail | 12 July 15 | 144 | 0 | 0 | Previously stocked with trout. Two large fish observed. |
| 04 Toney | 12 June 11 | 72 | 0 | 0 | Observed many egg masses in May. |
| | 12 Aug 14 | 216 | 0 | 0 | Cattle now have access to dugout. |
| | 13 June 14 | 96 | 0 | 0 | No egg masses seen this season. |
| 05 Rast | 12 July 20 | 144 | 13 | 0.090 | Long-toed salamander eggs seen in May. Many adult sightings near dugout. Caught 13 large larvae. |
| | 12 July 31 | 144 | 19 | 0.132 | Caught 19 larvae, took tail clips. |
| | 13 June 19 | 96 | 0 | 0 | Water level significantly down from last year. |
| 06 Hardy | 12 July 8 | 144 | 2 | 0.014 | Tiger salamander egg masses observed in May. Two adults caught. |
| | 12 Aug 30 | 144 | 0 | 0 | Pond was very shallow (<30cm). Traps blown over from wind. |
| | 13 July 13 | 144 | 4 | 0.028 | Caught four larvae and took tail clips. |
| 07 Lonesome | 12 June 9 | 24 | 0 | 0 | Trap fell over, but net still upright. |
| | 12 July 13 | 144 | 1 | 0.007 | Saw lots of small toad tadpoles. Caught one large larva. |
| | 12 Aug 9 | 144 | 0 | 0 | Caught one YOY long-toed salamander larvae. |
| | 12 Sept 9 | 48 | 1 | 0.021 | Water level low. Caught one tiger salamander larvae and took tail clip. |
| | 13 June 5 | 96 | 0 | 0 | Saw many boreal toad tadpoles. |
| | 13 July 17 | 96 | 0 | 0 | Tadpoles observed, but no larvae. |
| 08 Livingston | 12 June 2 | 72 | 0 | 0 | All traps set at the north end of the lake. |
| | 12 July 20 | 144 | 78 | 0.542 | Caught 78 YOY larvae, all 3-7cm long. Smallest ones had no limbs, larger ones has thin limbs. |
| | 12 Aug 27 | 72 | 0 | 0 | No tiger salamanders observed in traps, lake, or shoreline. |
| | 12 Sept 19 | 144 | 0 | 0 | Water level slightly down from previous visits. |
| | 13 July 10 | 144 | 29 | 0.201 | All larvae are small, with non-functioning limbs. |
| | 13 July 30 | 96 | 52 | 0.542 | Observed two dead larvae, one near shore and one in trap. No others appeared sick. Getting close to metamorphosis. |

| | | | | | |
|---|---|---|---|---|---|
| | 13 Aug 16 | 192 | 12 | 0.063 | ATV outbreak. 7 of 12 larvae dead with obvious symptoms. Observed another 5 floating along the shore. |
| | 13 Aug 20 | 96 | 1 | 0.010 | ATV outbreak ending. One larvae dead in trap with obvious symptoms. No others observed. |
| | 13 Sept 10 | 192 | 0 | 0 | No signs of any larvae or metamorphosed adults. |
| | 14 July 15 | 192 | 170 | 0.885 | Water level much higher than in previous years. All larvae caught were small, between 2 and 8cm. 60 tail clips taken. |
| | 14 July 31 | 192 | 195 | 1.016 | All larvae caught of similar size. One dead in trap. One trap had many injured larvae, maybe as a result of attempted cannibalism. Tail clips from 40. |
| | 14 Aug 12 | 192 | 132 | 0.688 | 17 dead in traps. 25 total showing symptoms. 50 tail clips. Many looked close to morphing. |
| | 14 Aug 24 | 192 | 3 | 0.0156 | One dead in traps, one sickly looking, one normal. All larvae. Tail clips from all. |
| 09 River A | 12 June 9 | 24 | 0 | 0 | Caught one fish approx. 1.5” long. |
| 10 Waterton | 12 June 9 | 24 | 0 | 0 | Set perpendicular to shore. Very steep banks. |
| | 12 June 23 | 216 | 0 | 0 | Set parallel to shore. |
| | 12 July 29 | 144 | 0 | 0 | Lots of invertebrate productivity. One year post die-off. |
| | 13 June 20 | 144 | 0 | 0 | Boreal toad tadpoles observed. |
| | 13 July 28 | 192 | 0 | 0 | Two years post die-off. |
| | 14 July 29 | 192 | 0 | 0 | Boreal toad tadpoles observed. Three years post die-off. |
| | 14 Aug 15 | 192 | 0 | 0 | No salamanders observed. |
| 11 Colwell | 12 Aug 23 | 96 | 0 | 0 | Nothing caught in traps, but observed one dead tiger salamander larvae. No visible cause but was close to metamorphosis. |
| 12 Birdseye | 13 June 20 | 48 | 0 | 0 | Very rainy week. Caught four spotted frog tadpoles. Fairly high elev. |
| 13 Marr | 13 June 26 | 144 | 0 | 0 | Lake is similar size to Livingston. Caught hundreds of small minnows and chub. |
| 14 Klein | 13 July 24 | 144 | 0 | 0 | Within a few km’s from a dugout where tiger salamanders were observed. Large irrigation reservoir. |
| 15 Everts | 13 Aug 5 | 192 | 9 | 0.047 | Pond at the edge of wetland complex. Adult tiger salamanders observed nearby. Caught 9 larvae. Tail clips from all. |
| | 13 Aug 22 | 192 | 9 | 0.047 | Caught 9 larvae, one dead in trap. Tail clips from all. |
| | 13 Sept 4 | 192 | 10 | 0.052 | Caught 10 larvae, took tail clips from all. |
| | 13 Sept 19 | 192 | 1 | 0.005 | Caught one large larva, possible paedomorphic individual. Double the size of any larvae previously caught. |

Table 6.3. Capture data summary of *A. mavortium* at 6 sites in southwestern Alberta.

| Site | Date | No. of Larvae Captured | Average Length (cm) | Average Weight (g) |
|---|---|---|---|---|
| Rast | July 31 2012 | 19 | 18.10 | 42.2 |
| Shenton B | August 21 2012 | 7 | 14.15 | 23.3 |
| Lonesome | September 9 2012 | 1 | 15.97 | 27.4 |
| Livingston | July 10 2013 | 29 | 7.96 | 3.9 |
| Hardy | July 13 2013 | 4 | 9.41 | 6.6 |
| Livingston | July 30 2013 | 52 | 12.81 | 17.5 |
| Everts | August 5 2013 | 9 | 12.05 | 12.6 |
| Livingston | August 16 2013 | 12 | 14.76 | 27.1 |
| Livingston | August 20 2013 | 1 | 14.81 | n/a |
| Everts | August 22 2013 | 8 | 13.62 | 18.3 |
| Everts | September 4 2013 | 10 | 12.92 | 18.0 |
| Everts | September 19 2013 | 1 | 20.53 | 66.7 |
| Livingston | July 15 2014 | 170 | 8.46 | 5.16 |
| Livingston | July 31 2014 | 195 | 13.00 | 19.4 |
| Livingston | August 12 2014 | 132 | 15.99 | 37.7 |
| Livingston | August 24 2014 | 3 | 17.22 | 41.6 |