

Biological Control of Disease Vectors: A Case  
Study Evaluating the Efficacy of Fathead Minnows  
(*Pimephales promelas*) for Mosquito Control in  
Northeast Wyoming

by

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## **Author's Declaration**

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public

## Abstract

West Nile virus (WNV) has become a significant and increasing threat to wildlife populations and human health throughout North America. Mosquito control is an important and potentially effective means of controlling the spread of WNV, as the virus is primarily spread between avian and mosquito vectors. This is of particular concern for avian host species such as the Greater sage-grouse (*Centrocercus urophasianus*), where WNV has been documented to negatively affect sage-grouse survival to the point of possible local extirpation. The most common mosquito control methods focus on controlling mosquitoes at their larval life stages. Efforts have primarily been limited to larvicide pucks or sprays, which require repeated application and could have potentially negative ecological consequences. Here, my primary objective is to test the efficacy of using fathead minnows (*Pimephales promelas*) as a biological control for mosquito populations in northeastern Wyoming. Specifically, I address two main questions: 1) does the presence of fathead minnows influence mosquito larva density within reservoirs, and 2) what pond and water quality characteristics support viable populations of fathead minnows? In the summers of 2013, 2014, I introduced minnows into 9 of 16 monitored reservoirs. The presence of fathead minnows, mosquito larva density, and adult mosquito populations were monitored at all sites on a weekly basis during 2013. During the 2014 and 2015 seasons, tissue samples were collected for stable isotope analysis, and fathead minnow population density was measured and recorded at the beginning and end of each season. Results indicate that minnows are a promising alternative to controlling mosquito larvae density within these reservoir environments. The presence of fathead minnows lowered and suppressed temporal variation in mosquito population densities throughout the summer seasons. Additionally, self-sustaining minnow populations established at the majority of sites. Water quality parameters measured did not influence survivability at the ranges detected in these reservoir environments. Overall, the efficacy, life history traits and tolerance ranges of the fathead minnow make them ideal biological control option in cattle reservoirs within arid rural environments.

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# 1 General Introduction

This paper provides a brief overview of biological control methods with a focus on their importance for mitigating the increasing threats of vector-borne diseases. Specifically, I will draw on my Master's thesis research to exemplify the efficacy of biological control as it relates to mosquito control and West Nile virus (WNV) in northeast Wyoming, USA. I will begin with a general review of the literature as it relates to my research, which will be comprised of three main sections. First, I outline the history of biological control beginning by defining key terms associated with biological control and control theory. Second, I describe the theory and practice of biological control, including an evaluation of the perceived safety of such practices. Third, I outline the increasing and significant threats brought by vector-borne diseases and how biological control has and continues to play a major role in regulating various disease vectors. Following this review, I outline the research questions and objectives of my Master's thesis research which specifically evaluates the efficacy of using a larvivorous fish (*Pimephales promelas*) for mosquito control in northeast Wyoming. Wyoming is a critical study area for WNV disease control for many reasons. Specifically for this paper, I will focus on the Greater sage-grouse (*Centrocercus urophasianus*; hereafter, sage-grouse), a shrub-steppe species of western North America which currently only occupies half of its historic range (Aldridge et al., 2008; Turnbull and Chant, 1961). Sage-grouse population trends suggest declines in most major portions of their range. They are of current interest as discussions are being held to list the sage-grouse as an endangered species under the US Federal Act (Schroeder et al., 2004). WNV has emerged as an important threat to sage-grouse population persistence. WNV is an important source of mortality in low and mid-elevation populations throughout the west (Walker et al. 2011) and severe impacts have been documented in northeastern Wyoming (D E Naugle et al., 2005; David E Naugle et al., 2004). WNV reduces sage-grouse survival and can result in local and regional population declines (D E Naugle et al., 2005; David E Naugle et al., 2004). This becomes particularly important in high abundance population centers (based primarily on lek counts), referred to as core areas

(Doherty, Naugle, Copeland, Pocewicz, & Kiesecker, 2011). Taylor et al. (2013) suggested that WNV is one of the primary factors impacting sage-grouse populations in northeastern Wyoming. For this region, WNV outbreaks are referred to as the “wild card” in sage-grouse and core area management (Taylor et al., 2013). Population viability analysis including WNV outbreaks predicted functional extinction for the species in north-eastern Wyoming and suggested that many local populations “may be one bad WNV year away from extirpation” (Taylor et al., 2013). Sage-grouse are extremely susceptible to WNV (Clark et al., 2006) and management of sage-grouse – particularly in northeastern Wyoming – needs to address this important threat.

In addition to the ecological threats of the virus, WNV poses some significant threats to rural communities. WNV poses a serious threat to overall rural health and productivity, bringing with it significant financial costs. Since the introduction of WNV into the United States, there have been over 3 million people infected with the virus, with approximately 40,000 serious clinical cases and over 1,600 deaths (Barrett, 2014). It is estimated that the total cumulative costs for hospitalization between 1999 and 2012 was close to \$800 million USD (Barrett, 2014a), and these statistics are human cases alone. WNV can have serious debilitating effects on horses, with veterinary bills adding up well over \$1,000 for each infected individual. Economic losses increase dramatically if a horse should die or need to be put down as a result of infection. However, when it comes to both humans and livestock, it is difficult to place an absolute cost on the repercussions after infection. In humans, the virus is capable of rendering a person unfit to work and perform various types of agricultural activities. Livestock such as horses face similar threats from the virus with many cases going undetected for some period of time. Horses may lose tremendous value and ability if WNV should cause West Nile neuroinvasive disease (WNND), which typically manifests as encephalitis, meningitis, or acute flaccid paralysis (AFP). In these cases, the animals will likely have lost their original use, with symptoms severely affecting their abilities. These conditions, in both humans and horses, take a significant toll on long term health and can often lead to death. Vaccinations exist for horses, but veterinary records suggest that many horses with the vaccinations

are still able to contract the virus, and cases have begun to increase over the last 2 years (United States Department of Agriculture, 2013). The prevalence of WNV is variable and unpredictable (Beasley et al., 2013) making a pre-emptive approach to mitigation necessary to effectively control the spread of WNV.

This thesis has 2 data chapters. Chapter 4 evaluates the effectiveness of fathead minnows as a vector control species in northeastern Wyoming. Chapter 5 investigates what reservoir and water quality characteristics support viable populations of fathead minnows. Promising results are accumulating alongside increasing challenges to biological control efforts around the world. Through presenting a brief history of the literature along with my thesis research, this thesis aims to provide a better understanding of biological control for the purposes of mitigating the negative effects of vector-borne diseases in contemporary/novel environments, specifically WNV in rural northeast Wyoming.

## 2 Literature Review

### 2.1 Biological Control - Terminology

Perhaps self-explanatory, the word ‘biological’ implies that a living organism is responsible for the control. In contrast to other methods of pest control or species management (which may rely on the use of toxic chemicals, human force etc.), biological control often seeks to find efficient natural enemies to a selected pest species relying on natural processes of population dynamics to control their abundance (Hokkanen and Fimentel, 1989; Murdoch and Briggs, 1996). The term “biological control” was first coined by Smith (1919) to imply the use of natural enemies for insect pests (Huffaker and Messenger, 1976). For the purposes of this review I will be specifically referring to insect pest species. Biological control efforts around the world have largely focused on the field of entomology, as insect populations can affect a wide spectrum of human activities and impact population health (Lemon et al., 2008; Turnbull and Chant, 1961). Furthermore, with specific interest in the control of vector-borne diseases, insect pest species become particularly important along with their respective controls. Given that the primary objective of biological control is to *control* a target pest population, how we define the words ‘pest’ and ‘control’ becomes particularly important. In the field of economic entomology, any species that negatively impacts commodities that humans need or desire is considered a pest (Turnbull and Chant, 1961). Control in this context specifically refers to limiting the abundance of such pest species. Depending on the situation and type of commodity certain levels of damage may be tolerated. When damage thresholds are crossed and loss of a desired commodity becomes intolerable in the given context, reductions in pest species abundance is seen to be required (Turnbull and Chant, 1961). In this sense, we aim to ‘control’ the level of damage caused by the pest species to within tolerable levels. What is considered to be tolerable depends on a variety of other factors, but this question is beyond the scope of this review. This definition of control, however, may not be the most suitable as it does have some notable ambiguity. According to the economic definition, it is implied that a pest is controlled when the economic damages it causes are within a tolerable limit. This has very little to do with exact values of

population abundance and more importantly ignores the biological significance of control outside of economic values. Biological control for the purposes of ecological conservation, for example, would need an alternative definition and means to monitor success. This definition came around the time of Malthus in the 18<sup>th</sup> century as increasing attention was being given to the concept of population ecology (University of California, 2014). In this context, control referred to the various processes of population dynamics, controlling abundance at various temporal or spatial scales (Turnbull and Chant, 1961). Control, as defined in population ecology, is then successful to a certain extent if population dynamics can be influenced in a desired way; not only referring to the desires of man (Turnbull and Chant, 1961).

For the purposes of this thesis, both uses of the term control are relevant. However, attempts to clarify the definition have been a subject of debate in the literature for decades. For instance, Franz (1960) suggested control refers to any case where the number of individuals was reduced by man. Franz also stated that one cannot claim to be engaged in control work until it proves to be successful. In this sense, any reduction could be considered successful control. This opinion, however, was not shared by other scholars of the 20<sup>th</sup> century. As suggested by Turnbull and Chant (1961), we should reserve the term *control* for the more economic concept, referring to the control of unwanted damage or harm. Since the vast majority of biological control work deals with economic or health related concerns, this use of the term seems most suitable and appropriate. For cases in population ecology that focus on altering population dynamics, we may wish to refer to terminology used by David Lack, one of the most influential people on the development of ideas for the population regulation of birds (Newton, 2003). Lack (1954) suggests the use of the term “regulation”. With a clearer definition and context for biological control, we are still left with defining success. As this could likely be a separate paper of its own, I will simply suggest that success in control for the purposes of this thesis, be conceptualized as any demonstrable reduction in damage or harm. The *level* of success needed to make control projects worth-while (in agriculture for example) is highly case specific and cannot be easily generalized.

## 2.2 A Brief History

Biological control of pest species is by no means a new idea. In fact, the documented history of biological control dates back over 4000 years through ancient Egyptian records which depicted cats as useful agents for rodent control (University of California, 2014). At this time, biological control was fairly simple in design in that it applied the basic functions of predator-prey relationships to pest management. Ecological theory has thus always played a crucial role in applied biological control efforts even long before ecological theory was fully described. Established biological control mechanisms (naturally occurring or implemented by man) are direct applications of population dynamics focusing on either a predator or parasitoid population maintaining a prey or host population over time (Murdoch and Briggs, 1996). Predators implement control by consuming the pest species, while parasitoids thrive and live off the host organism (pest) throughout their lifetime ultimately causing death. Some of the first noted applications of biological control date back to early population in Asia, where insect predation was observed as a useful tool in the beginnings of sophisticated agricultural activities. The Citrus ant (*Oecophylla smaragdina*), for example, has been used in Chinese agriculture for roughly 1700 years to protect citrus growers from pest insects (Peng, 1983). Carnivore behaviour was thus one of the first phenomena to be observed by ancient people in terms of biological control. However, ancient peoples also noted observations of parasitic insects as early as 1196 (Peng, 1983). Although there was no record of the implications of this relationship in early records, these observations would eventually lead to one of the most influential methods of biological control; insect parasitism.

Insect parasitism and its useful applications in biological control were not fully recognised until the beginning of the 17<sup>th</sup> century. Italian Doctor Ulisse Aldrovandi published the first book on insects “De Animalibus Insectis Libri VII” (1602) describing parasitoid relationships, although his descriptions were somewhat inaccurate (Capinera, 2008; University of California, 2014). Throughout the 18<sup>th</sup> century, various entomologists such as Antoni van Leeuwenhoek (1700) and Vallisnieri (1706) recognized a more accurate depiction of insect parasitoid relationships. During this time, further developments were also

being made with predator based control methods. In 1762, the mynah bird was imported from India onto the island of Mauritius to control locusts there by becoming the first successful importation of a foreign species for biological control (University of California, 2014). But it was not until the latter writings of Erasmus Darwin (1800) that these parasitoid and predator relationships were discussed for their usefulness in regulating insect pests (University of California, 2014). The development of more modern biological control theories, however, did not come until after Malthus and the ideas of population ecology towards the end of the 18<sup>th</sup> century (Berryman, 1992).

Biological control gained momentum throughout the 19<sup>th</sup> century when the westward expansion of American agriculture saw rapid growth and opened up new opportunities for biological control. Charles Valentine Riley, Chief Entomologist of the U. S. Department of Agriculture (also known as the father of modern biological control), became the first person to successfully transfer insect predators from one country to another. Riley also was one of the first people to identify the origin (Australia) of the cottony cushion scale. One of the first major successes was the control of the cottony cushion scale (*Icerya purchasi*) in the California Orange groves in 1889 by the Vedalia beetle (*Rodolia cardinalis*) (Turnbull and Chant, 1961). The project was such a great success, that many agriculturalists and entomologists were convinced that biological control was the solution to all insect based problems. In hindsight, however, this was a dangerous time in North American history as large quantities of insects were imported from around the world and released without thorough screening or consideration of adverse non-target consequences (Turnbull and Chant, 1961; Yasuda, 2006). It was also seen that many efforts to find effective natural enemies were often ill-conceived. In what some would say was a stroke of luck, most of these importations did not result in serious consequences besides wasted money and time (Turnbull and Chant, 1961; Yasuda, 2006). The cottony cushion scale success did however inspire some 200 successful control projects around the world throughout the century (University of California, 2014). It also helped to make the University of California in Riverside the most famous institution in the world for biological control research by 1962.



### 2.3 Theory and Practice

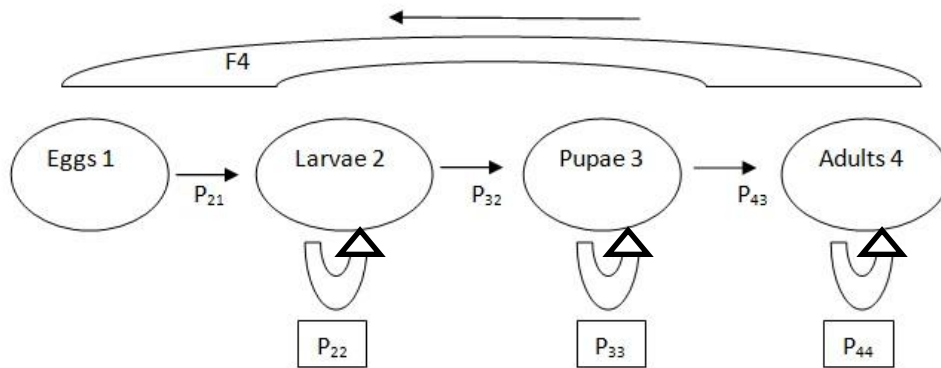
Classical biological control theory relies on the thought that various natural enemies, typically from a pest's area of origin, will be successful in limiting the abundance of the target pest population (Murdoch et al., 1985). This theory has many sources of guidance, but perhaps the most significant source has stemmed from ecological theory. Ecological theory in the context of biological control generally focuses on factors which influence the stability of a single population, pest-enemy system (Murdoch and Briggs, 1996). Literature concerning the relevance of ecological theory to biological control has generally emphasized the importance of population dynamics in pest-enemy systems (Huffaker and Messenger, 1976; Murdoch, 1973; Murdoch, 1979). It has been argued that the key to control is to understand the density dependence (of predator species) required to stabilize a target pest population at low equilibrium density (Huffaker and Messenger, 1976; Murdoch, 1973; Murdoch, 1979). The likelihood of success is thus improved when the natural enemy species selected possesses the following attributes: 1) host or prey specificity, 2) is synchronous with the pest, 3) potential for rapid population increase when host population increases, 4) needs to kill only a few pests to complete its life cycle, and 5) a high rate of successful searches (Murdoch and Briggs, 1996; Murdoch et al, 1985). General predators or *opportunistic feeders* may be considered poor candidates for biological control, as they often do not synchronize with target pest species and carry the potential to deplete beneficial insect species in the system. This however is a general rule and there may be some exceptions in closed or small systems as will be discussed later.

Models used to describe the success of biological control are often deterministic equations that are locally stable and focused on a single predator-prey relationship. In practice, these models most often involved empirical measurements in discrete-time models meant for annual insects with no within-year generational overlap. Most of the non-metapopulation models have used the Nicholson-Bailey framework and operate under the assumptions that predator-prey encounters are random and there is a one-generation development delay (Murdoch and Briggs, 1996; Murdoch et al, 1985). This would then imply that stability and control of a pest population is heavily dependent on the spatial aggregation of predator

species, which leads to some theoretical controversy. Since the original Nicholson Bailey models assume a one-generation development delay, stability might arise solely due to the fact that predators aggregating towards denser patches at the start of a generation cannot re-aggregate in response to changes in pest distribution within that generation. This limitation affects predator efficiency in the sense that their attack rate becomes density dependant. Continuous time models such as the Lotka-Volterra model avoid this problem by allowing predators to re-aggregate to local prey densities within generations (Murdoch and Briggs, 1996; Murdoch et al, 1985). These models do not typically stabilize, but have shown to lead to lower mean pest densities suggesting that natural enemies which concentrate attacks on patches of higher pest density are most effective towards control.

Regardless of the models used to evaluate population dynamics, the existence of an equilibrium state is not necessary or sufficient on its own to bring about control. Locally stable systems or local regulation does not necessarily imply globally stable systems or stable metapopulations. In fact, the degree of stability we observe in these models is often a function of scale (Murdoch et al., 1985). The focus of biological control often relies more on pest density which must remain below certain tolerable thresholds throughout time or at least for significant time periods. For regional control, this means placing a primary focus on pest density while also acknowledging that successful biological control will rely on limiting the temporal variability of pest densities. The importance of understanding continuous changes in population dynamics and species densities highlights the need for stage-structured population models (see Figure 2.3-1 below). Mosquitoes for example, a common insect pest, typically pass through a variety of life stages (egg, larvae instars, pupae, and adult) each with unique properties that can be assumed constant within each stage (Darsie and Ward, 2005; Murdoch and Briggs, 1996). The inclusion of stage structured models invites increased accuracy and realism to biological control models offering a relatively simple layout to complex interactions in population dynamics. Most of the parameters included can be measured and therefore expressed in the form of probabilities or values such as survivorship, fecundity, or the duration of time spent within each stage (Meisch, 1985; Smith, 1985). These models can be used not only to

project populations but to evaluate elasticities within each life stage using population matrix models to determine the proportional change in growth rate ( $\Lambda$ ) for every unit change in survivorship caused by predation. In this sense, the comparative use of such models could help screen potential control agents (natural predators) selecting those which “choose” the most vulnerable life stages influencing their population projections (Murdoch and Briggs, 1996).



**Figure 2.3-1:** Example of a stage structured population model for mosquitoes (not including instar larval stages).

## 2.4 Is Biological Control Safe?

As previously mentioned, some of the first attempts at implementing biological controls involved some significant and unnecessary risks. Classical biological control attempts acquired natural enemies which generally came from the pest species place of origin. This meant that throughout the 20<sup>th</sup> century (and in some cases, earlier) insect species and other natural enemies were being relocated around the world in large numbers with little screening for their overall efficacy or safety. This in part, was due to the fact that biological control was advanced for quite some time under the notion that it was the ‘green’ alternative to potentially harmful chemical control options (Simberloff and Stiling, 1996). Many argued that there was no inherent reason that biological control should be seen as risky behaviour, especially under the classical definition. However, cause for concern increased with the implementation of “neoclassical biological control”, which refers to new associations of enemies and pests. In contrast to classical biological control, these new associations come about when the antagonist of an indicated native pest species is an

introduced (non-native) species (Hokkanen and Pimentel, 1989). Despite the obvious concerns to this alternative approach, Hokkanen and Pimentel (1989) hypothesize that it is superior to classical control in the sense that the pest has not had the opportunity to co-evolve with the enemy. Attempts to quantify any advantage over classical control have been challenged however, generally coming down to numbers, definitions of success, and choices of methodology.

Despite any possible advantages, neo-classical control comes with some significant risks which should be recognized. Many of these risks are also shared with even the classical approach. Non-target species effects have been at the forefront of debate regarding the safety of biological controls. This is especially the case when generalized predators or herbivores are released for control. For example, the Indian mongoose (*Herpestes auropunctatus*), upon its release to the West Indies, Hawaiian Islands and Fiji, greatly diminished native bird populations when its original intention was for the control of rats in agricultural fields. Herbivores introduced for biological control can also have large-scale negative effects on native populations. Freshwater fish, for example, released for weed control or other purposes, have some of the worst track records in biological control with many cases of large-scale ecosystem disturbances (Simberloff and Stiling, 1996). Aside from direct interactions, there is also concern over potential competition for resources, especially food. Since competition for resources is extremely difficult to document and quantify in the field, these impacts are often neglected and remain poorly understood (Simberloff and Stiling, 1996). Potential for community-level alterations, ecosystem effects, population dispersal, and evolution of the introduced species are among other primary concerns for biological control projects. The apparent lack of literature documenting negative impacts or outcomes relative to the above mentioned concerns is not however, in any way, evidence of safety. One of the most significant challenges preventing a legitimate evaluation of the safety of biological control is the lack of monitoring of non-target, long term effects (Simberloff and Stiling, 1996). Non-target effects are generally only documented when they reach critical levels, at which point reversal of the damage is unlikely. Strong consideration should be given towards any possible impacts that pests or natural enemy species might

have on subjected ecosystems with emphasis on a cost benefit analysis which recognized more than human interests and immediate commercial values (Simberloff and Stiling, 1996).

## **2.5 Vector-Borne Diseases**

Although biological control has a long history of use in agriculture, its effectiveness in the prevention of vector-borne diseases has become increasingly recognized as a priority use. Put simply, a vector is any organism that carries and transmits infectious pathogens to another organism. Although once limited by the geographic range of a host, many vector-borne diseases are currently spreading rapidly around the world. For the purposes of this thesis, I will focus primarily on West Nile virus (WNV) and mosquito vectors, as this is the central focus of my Masters research.

West Nile virus (WNV) is primarily transmitted between avian and mosquito vectors. It was originally detected in Uganda in 1937, but in August of 1999 the virus was detected in New York state, having since spread throughout North America (Lemon et al., 2008). This rapid dispersal has been largely due to anthropogenic factors such as population growth, globalized transportation, and large scale land use changes, but has also been influenced by factors of climate change (Lemon et al., 2008). Vector-borne diseases are extremely sensitive to environmental changes that affect vector distribution and abundance as well as contact with other hosts (Fish, 2008). During the early 1900s, vector-borne diseases were emerging as one of the most significant public health problems (Gubler, 2008). Since the spread of disease is directly related to factors of vector population dynamics and abundance, efforts towards disease prevention have historically focussed on directly eliminating vector populations within an environment (Fish, 2008). Biological control played a dominant role in vector-borne disease control throughout the early 1900s, however, with the advent and success of pesticides post World-War II, ecological research and focus on biological control rapidly declined (Fish, 2008). Unfortunately, the rapid success of pesticides for the control of vector-borne diseases such as malaria, yellow fever, and dengue fever, ultimately led to major problems including vector resistance and adverse ecological effects (Fish, 2008;

Lemon et al., 2008). Rather than revert focus back to ecological studies, research expanded towards new alternative methods such as insect sterilization, genetic manipulation, and translocation (Fish, 2008; Dame, 1985). But perhaps the most significant problem was the false assumption that these issues were under control. Investments into control declined, and less effective chemicals were implemented often being aimed at inappropriate vector life stages. As a result of these misguided decisions, as well as the previously mentioned anthropogenic influences, diseases once under effective control re-emerged with rapid geographic expansion (Fish, 2008; Lemon et al., 2008).

WNV has become a particularly significant threat in North America since its original detection in August of 1999. It is suggested to have arrived here by a human host from Israel, which was experiencing an outbreak of the virus at that time. Between 1999 and 2005, WNV had spread all the way to the west coast, north into Canada, south into Mexico and throughout Central America. WNV is known to cause severe and often fatal neurological problems as well as flu-like symptoms in both humans and other animals. Many bird species (which are the primary host/vectors) have high rates of fatality (Lemon et al., 2008). As the virus primarily spreads between birds and mosquitoes, it is thought that migratory birds have played an important part in the spread of the virus throughout North America (Lemon et al., 2008). Landscape alterations and the resulting impacts of activities such as oil and gas development throughout the western United States has also been suggested to contribute to WNV dispersal and increased infection rates in birds such as the sage-grouse (Taylor et al., 2013; Walker et al., 2011). Another important factor that has contributed to the rapid spread of WNV throughout North America is the broad range of vectors and host species. There are roughly 174 species of mosquito in North America (Darsi and Ward, 2005), 62 of which have been isolated to carry the virus. As for vertebrate hosts, there are currently around 317 species of birds and over 30 non-avian hosts of the virus (Lemon et al., 2008).

## 2.6 Research Question, Objectives and Hypothesis

WNV has emerged as an important threat to sage-grouse. WNV is an important source of mortality in low and mid-elevation populations throughout the west (Walker et al., 2011) and severe impacts have been documented in northeastern Wyoming (Naugle et al., 2004, 2005). WNV reduces sage-grouse survival and can result in local and regional population declines (Naugle et al., 2004, 2005). Taylor et al. (2013) suggested that WNV is one of the primary factors impacting sage-grouse populations in northeastern Wyoming. For this region, WNV outbreaks are the “wild card” in sage-grouse and core area management (Taylor et al., 2013). Population viability analysis including WNV outbreaks predicted functional extinction for the species in north-eastern Wyoming and suggested that many local populations “may be one bad WNV year away from extirpation” (Taylor et al., 2013). Sage-grouse are extremely susceptible to WNV (Clark et al., 2006) and management of sage-grouse – particularly in northeastern Wyoming – needs to address this important threat.

Infected mosquitoes are the primary vector for WNV. The most effective approach to controlling WNV and limiting its impacts on sage-grouse populations will likely involve mosquito control. In particular, eliminating mosquito breeding habitat, or controlling mosquito larval populations in anthropogenic water sources is crucial for reducing impacts (Zou et al., 2006, Walker and Naugle, 2011). One of the primary anthropogenic water sources that serves as breeding habitat for mosquitoes in north-eastern Wyoming are livestock reservoirs. Mosquito control through the application of larvacides is one option. However, treating large areas with larvacides is expensive and can potentially have detrimental effects (Marra et al., 2004). Thus, a complimentary and more cost-effective option is required in high-density sage-grouse areas where risk is highest.

Fathead minnows (*Pimephales promelas*) can function as effective biological control agents of mosquitoes in the larval stage (Paskewitz 2009). The reproductive biology of fathead minnows is well studied, and individuals mature rapidly, and can tolerate a wide range of environmental conditions including wide variation in water quality conditions (Scott and Crossman, 1973). Additionally, several

studies have revealed the fathead minnow's ability to survive low oxygen levels during winter months. These life history traits likely facilitate the establishment of self-sustaining populations in a wide-range of environmental conditions.

In the following chapters, I evaluate the efficacy of using fathead minnows for mosquito control in northeast Wyoming and the suitability of local environments for self-sustaining minnow populations in the hopes of minimizing the threat of WNV and the significant impacts it can have on sage-grouse population persistence. My research objectives are broken down into two chapters addressing two fundamental questions: Chapter 1 asks - does the presence of fathead minnows influence mosquito larva density within selected reservoirs? Chapter 2 asks - what pond and water quality characteristics support viable populations of fathead minnows?

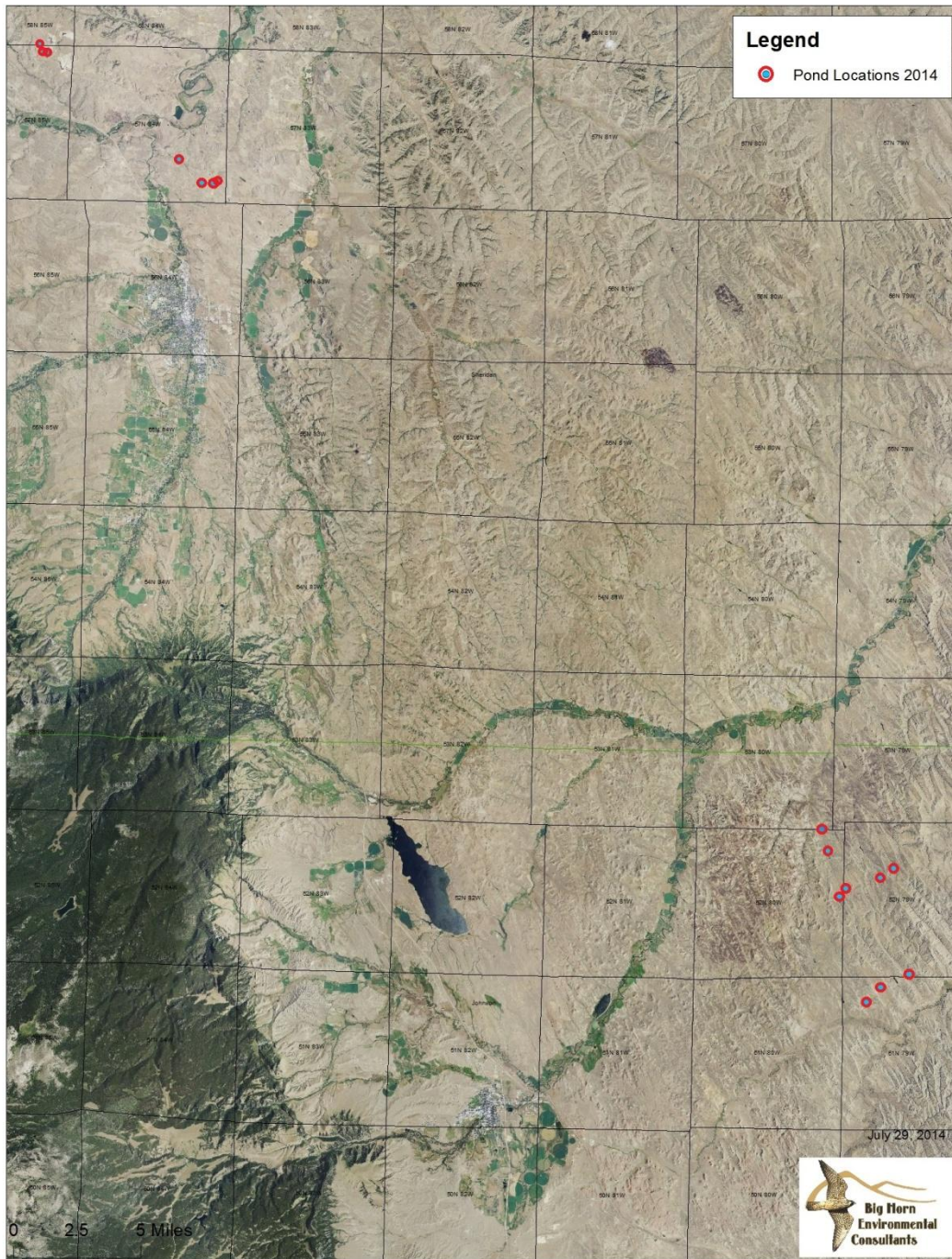


### 3 Study Site

The research for my Master's thesis was conducted over three summer field seasons spanning 2013-2015. The study region falls within the Powder River Basin (PRB), a geologic structural basin which spans across the northeast of Wyoming and into the southeast of Montana, United States. The PRB includes some of the most significant wildlife habitat in Wyoming, especially concerning sage-grouse populations. However, the PRB also contains some of the finest coal bed natural gas (CBNG) opportunities in North America, being the largest coal producing basin in the United States. This, on top of the fact that it is also one of North America's largest natural gas fields, has led to rapid CBNG development over the past two decades (Walker and Naugle, 2011).

This study was conducted in and around Sheridan County, northeast Wyoming USA (geographic location 44.7967° N, 106.9589° W). There were 16 reservoirs/research sites selected for this study (figure 2.6-1). Of these 16 reservoirs, six were designated as controls and ten were assigned as treatments. Reservoir assignment was done prior to larva sampling due to the time frame of the initial study season, and available land access at that time. Sites ranged in size from 0.24 to 1.33 surface hectares (ha) with a mean of 0.5 ha. Pond depths (at the deepest point) ranged from 0.60 to 5.2 meters, with a mean of 1.72m. Sites selected to be in the study were all contained in an arid rural environment, and were all currently or recently used as livestock reservoirs. These reservoirs were either originally created for livestock by blocking off geographic drainages, or have been created as a bi-product of oil and gas development. These reservoirs and surrounding landscapes of the intermountain west become ideal habitat for many species of wildlife as they provide a vital water source and abundant supply of vegetation that would otherwise not grow in such an arid environment. The surrounding plains are made up primarily of sagebrush habitat, which include sage brush (*Artemisia tridentate*) as well as a variety of tall grasses, flowering plants and cacti.

The reservoirs themselves are closed systems, primarily rainwater fed with the exception of some groundwater/spring water inflow. These reservoirs are not connected to any waterway making them ideal controlled environments. Water quality at these sites varies, but they all have similar environmental influences leading to their observed conditions. Being closed systems in agricultural landscapes, these reservoirs are generally eutrophic. Cattle utilize the reservoirs during the day, often excreting bodily waste into the water increasing nutrient levels. Physical structure and pond morphology also greatly influence the water conditions, specifically, water depth, benthic layer contours, and surface hectares. Vegetation present at these sites generally consisted of emergent, submersed, and free-floating plants as well as algae at certain points of the season. These reservoirs may be recognized as novel ecosystems as they were introduced to this arid environment as a result of anthropogenic activities and land use changes resulting in relatively new community compositions, at least at the current frequency, within this region.



**Figure 2.6-1:** Location map for Sheridan and Johnson county research sites showing site dispersion. Blue dots indicate the location of reservoirs used in the study.

## **4 Assessing the Efficacy of Fathead Minnows (*Pimephales promelas*) for Mosquito Control in Northeast Wyoming, USA.**

### **4.1 Introduction**

Sagebrush (*Artemisia spp.*) ecosystems spanning the western portions of the United States and Canada hold high conservation value, hosting many species of conservation interest (Knick, Steven T et al., 2003; Schrag, Konrad, Miller, Walker, & Forrest, 2010). These habitats and their associated obligate species are in peril as a result of significant degradation, fragmentation, and loss of native sagebrush (Knick, Steven T et al., 2003). The sagebrush landscapes of Wyoming have undergone significant habitat changes over the last few decades (Aldridge et al., 2008; Doherty et al., 2011; Naugle, Doherty, Walker, Holloran, & Copeland, 2011). Energy development and agricultural production have become important factors influencing this arid landscape through the creation of additional water sources, either as a by-product of development or necessity for ranching activities (David E Naugle et al., 2004, 2011; Taylor, Tack, Naugle, & Mills, 2013). These additional water sources then become breeding grounds for certain disease vectors such as the mosquito. Mosquitos are primary vectors for a variety of diseases such as Malaria, Dengue, and West Nile virus (WNV) which can result in major ecological and economic consequences in disease-endemic locations (Hemingway, Beaty, Rowland, Scott, & Sharp, 2006). WNV has become a particularly significant threat in North America since its original detection in August of 1999 (Lemon et al., 2008). Infected mosquitoes are the primary vector for WNV. There are roughly 174 species of mosquito in North America (Darsie and Ward, 2005), 62 of which have been isolated to carry the virus. As for vertebrate hosts, there are currently around 317 species of birds and over 30 non-avian hosts of the virus such as horses and humans (Lemon et al., 2008). WNV has since emerged as an important threat to species such as the Greater sage-grouse (*Centrocercus urophasianus*; hereafter, sage-grouse) within these landscapes (Naugle et al., 2004). Sage-grouse has become a species of prominent

interest for conservation efforts as it can act as an umbrella species for other sagebrush obligate, and associated species (Rowland, Wisdom, Suring, & Meinke, 2006). WNV has become an important source of mortality in low and mid-elevation sage-grouse populations throughout the west (Walker et al. 2011) and severe impacts have been documented in northeastern Wyoming (Naugle et al. 2004, 2005). WNV reduces sage-grouse survival and can result in local and regional population declines (Naugle et al., 2004, 2005). This becomes particularly important in high abundance population centers referred to as core areas, as they host vulnerable breeding populations of sage-grouse (Doherty et al., 2011). Taylor et al. (2013) suggested that WNV could potentially be one of the primary factors impacting sage-grouse populations in northeastern Wyoming. For this region, WNV outbreaks are the “wild card” in sage-grouse and core area management (Taylor et al., 2013). Population viability analysis including WNV outbreaks predicted functional extinction for the species in north-eastern Wyoming and suggested that many local populations “may be one bad WNV year away from extirpation” (Taylor et al., 2013). As sage-grouse are extremely susceptible to WNV, it is essential for management strategies to address this important threat. Northeast Wyoming thus becomes an ideal test case for WNV control efforts, as much great work has been done in northeast considering low to mid-elevation populations of sage-grouse and the identification of current and future threats to population persistence.

The most effective approach to controlling WNV and limiting its impacts on sage-grouse populations will likely involve mosquito control. In particular, eliminating mosquito breeding habitat, or controlling mosquito larval populations in anthropogenic water sources is crucial for reducing impacts (Zou et al. 2006, Walker and Naugle, 2011). One of the primary anthropogenic water sources that serves as breeding habitat for mosquitoes in north-eastern Wyoming are livestock reservoirs. These reservoirs also create local mesic areas, which provide high quality habitat important for breeding populations of sage-grouse as females with broods choose wetter areas (Aldridge & Brigham,

2002; Fedy et al., 2012) and are likely foraging in proximity to these water bodies. During this life stage when they are at high risk. Mosquito control through the application of larvacides is one option. However, treating large areas with larvacides is expensive and can potentially have detrimental effects (Marra et al., 2004). Thus, a complimentary and more cost-effective option is required in high-density sage-grouse areas where risk is highest.

Various species of larvivorous fishes have been used around the world for biological control of disease vectors through trophic interactions (Aditya, Pal, Saha, & Saha, 2012; Fletcher, Teklehaimanot, & Yemane, 1992; Griffin & Knight, 2012; Howard, Zhou, & Omlin, 2007; Irwin & Susan, 2009; Tranchida et al., 2010). Fathead minnows (*Pimephales promelas*) can function as effective biological control agents of mosquitoes in the larval stage (Irwin & Susan, 2009). The reproductive biology of fathead minnows is well studied, and individuals mature rapidly, and can tolerate a wide range of environmental conditions including wide variation in water quality conditions (Scott and Crossman, 1973). Additionally, several studies have revealed the fathead minnow's ability to survive low oxygen levels during winter months (Klinger et al., 1982). These life history traits likely facilitate the establishment of self-sustaining populations in a wide-range of environmental conditions. Here, I test the efficacy of using fathead minnows for mosquito control in northeastern Wyoming, in the hopes of minimizing the threat of WNV and the significant impacts it can have on sage-grouse population persistence. Specifically, I test this efficacy in actual livestock reservoirs using a case-control matched design across multiple years with diverse moisture regimes.

## **4.2 Materials and Methods**

This study was conducted at multiple sites within Sheridan and Johnson counties, in northeastern Wyoming USA (geographic location 44.7967° N, 106.9589° W) (Figure 2.6-1). There were 16 reservoirs selected as research sites for this study. Monitoring at 15 of these sites began in 2013, with the addition of one site in 2014. All sites were located in arid rural environments dominated by sagebrush (*Artemisia* spp.) and were actively utilized as livestock reservoirs. All reservoirs selected as study sites were formed by dammed drainages or old mining operations and relied solely on naturally available water. Sites ranged in size from 0.02 to 1.33 surface hectares (ha) with a mean of 0.5 ha. Reservoir depths (at the deepest point) ranged from 0.60 to 5.2 m, with a mean of 1.72m. Sites were selected based on representativeness of reservoirs in the region, land access, and to ensure similar pond morphology across both treatments and controls. Sites were paired based on pond morphology (e.g., surface ha, max depth, shoreline vegetation) and one of each pair was randomly assigned to the treatment group prior to larvae sampling in the first year of the study.

### **4.2.1 Baseline Site Conditions and Fish Introduction**

Reservoir morphology was quantified by measuring total surface hectares, perimeter distance, and depth at the deepest point. Reservoir size and perimeter distance were calculated in *All Topo* using waypoints collected from a handheld Garmin GPS unit. Waypoints were taken around the end of June by walking the reservoir perimeter at the water's edge. Depth was measured from a boat using a weighted measuring line. Measurements were recorded by an assistant on shore, who also aligned the appropriate contour depth points. Outer contour depths were taken 2m from shore, while inner contours were taken 5m from shore in the direction towards the deepest point. Vegetation coverage and composition were also sampled and recorded. This focused primarily on emergent and surface vegetation around the water edge (shore) and was calculated as percent coverage within a circular

quadrat 1 meter in diameter. Observations were recorded at 10 meter sampling intervals and an average percent coverage was then calculated for each reservoir.

Captive-raised fathead minnows were transported by an aerated tanker truck and released into the reservoirs identified as treatment sites (n=10). The stocking rate for each site was set at 2,500 minnows per 0.4 surface hectares (1 surface acre), measured in late June. Due to annual changes in surface area the total number of fry required changed between years. The total number of fry used for stocking in 2013 was 25,200, while 2014 required 32,500. Fry were introduced to all treatment reservoirs in 2013 and 2014. Fry were not introduced to treatment reservoirs in 2015.

#### **4.2.2 Population Monitoring**

*Mosquitoes* - Larval sampling was conducted during each season using a stratified random sampling design to address changes in larval habitat along the reservoir shores. Samples were taken from both treatment and control sites on weekly bases throughout both field seasons accounting for sample times 1 through 7. Samples times between all years of the study remained consistent with calendar dates and were accurate to within 1 week between years. Count data of both larvae and larvae exoskeletons were obtained using the standard 350ml dip cup method (Chandra, Bhattacharjee, Chatterjee, & Ghosh, 2008; Irwin & Susan, 2009). Sampling was stratified based on emergent vegetation coverage and live larval presence. Dip samples were taken from the water edge every 10m for the non-vegetated edge. Along the edge with emergent vegetation, dip samples were taken every 5m utilizing a *stalking-method* approach to avoid disturbance or possible larval avoidance. The *stalking-method* for larval sampling requires researchers to approach the water edge with as little disturbance as possible and to avoid casting shadows onto the water where samples are taken. Along the edge with submersed vegetation, dip samples were taken every 7.5m in the same manner. Sampling intervals would tighten to every 2.5m when live larvae were found in the previous sample regardless of vegetation coverage until no more live larvae were present in the dip. Water edge in this



study refers to the edge of the dominant open-water body of the reservoir. Where drainages lead to long shallow vegetated tails to the reservoir, sampling would be conducted 10m in to that drainage. In this study (dependant on year/precipitation levels) approximately 5 sites contained shallow vegetated tails off of the open water body at the beginning of the season. Dependant on reservoir morphology and precipitation levels, some of these sites would lose the vegetated tails, while others began to exhibit these features due to draw-down.

In 2013, adult mosquito populations were sampled bi-weekly using CO<sub>2</sub> night traps to determine species abundance and distribution (Oli, Jeffery, & Vythilingam, 2005). Night traps were positioned at the water edge, held in place by a 1m wooden stake (Appendix 1, Figure 1). The night traps utilized a dry ice container which gradually released CO<sub>2</sub> as an attractant to adult mosquitoes. Individuals drawn in to the trap were blown by an electric fan into a sampling net. Trapped individuals were frozen, placed in labelled vials and shipped to a lab for identification. All intact individuals were identified to the species level by researchers at the entomology lab, Montana State University.

Presence or absence of fathead minnows was recorded weekly based on sightings and minnow trapping success using baited steel traps when sightings were not conclusive. Relative densities were determined using standard electrofishing techniques at the beginning (early July) and end (late August) of the 2014-2015 seasons using the Smith-Roots LR-24 or HallTeck HT-2000 backpack shocker. The electrofishing protocol consisted of shocking 10% of the total reservoir perimeter between 0.5-2m from shore in the littoral zone. Total number of transects used at each site was dependant on reservoir size with each transect spanning approximately 6m (formula:  $n \text{ transects} = (\text{Reservoir perimeter (m)} * 0.10) / 6\text{m}$ ). Each transect was shocked for 90 seconds. Time was tracked by a field assistant on shore. Personnel operating the electrofisher would begin shocking at the start

of each transect, moving forward at a steady pace throughout the duration of the shocking period. Due to difficulty moving through the substrate of the reservoir, fish counts were based on observation rather than collection. Only fish *turned* by the electrofisher would be counted (*turning* a fish consisted of any fish visibly affected by the current to the point of flipping over or surfacing, or becoming temporarily stunned). All observational data was collected by the same individual to minimize observation bias. Voltage and general output settings such as duty-cycle were specific to each site dependant on water conditions (primarily conductivity) to allow for a comparable sample. The LR-24 automatic pre-set was used as a baseline in 2014, with voltage and frequency outputs being increased as needed based on vegetation densities and conductivity. These values were replicated specific to each site in 2015.

#### **4.2.3 Stable Isotope Analysis**

In 2014, tissue samples from mosquito larvae, adults, and minnows were collected for stable isotope analysis to assess the predator-prey relationship between minnows and mosquitoes. Adult mosquito samples were collected using night traps as described above. Larvae tissue samples were obtained from dip cup samples throughout each season and placed into labelled vials which indicated their capture region. Minnow tissue samples were collected at the beginning and end of the seasons. Minnows were obtained using baited steel minnow traps at 5 treatment sites. Five minnows were euthanized according to ethics protocols (AUPP 13-12, University of Waterloo), and tissues samples were taken from side fillets of muscle and bone (not including internal organs) and placed into labelled vials with demineralised water for transport. Five minnows from each site at each intra-annual sampling time period were utilized for tissue samples in 2014. All animal tissues collected were transported back to a drying facility where they were removed from their transport vials and placed into a drying oven. Adult mosquito and larva tissue samples were placed in a drying oven for a minimum of 12 hours at 50-75 degrees Celsius. Minnow tissue samples were placed in a drying

oven for a minimum of 24 hours at 50-75 degrees Celsius. Dried samples were then pulverized to a homogenate with a ceramic mortar and pestle. Approximately 0.3mg (300mcg) of prepared material was used for stable isotope analysis (SIA) completed with a Delta Plus Continuous Flow Stable Isotope Ratio Mass Spectrometer (Thermo Finnigan, Bremen, Germany) coupled to a Carlo Erba elemental analyzer (CHNS-O EA1108, Carlo Erba, Milan, Italy). Analyses were conducted at the Environmental Isotope Laboratory, University of Waterloo (Waterloo, Ontario) with an analytical precision of  $\pm 0.2\text{‰}$  ( $\delta^{13}\text{C}$ ) and  $\pm 0.3\text{‰}$  ( $\delta^{15}\text{N}$ ). Samples weights necessary for SIA were obtained from a high precision ultra microbalance (Model XP2U, Mettler-Toledo GmbH, Greifensee, Switzerland).

#### **4.2.4 Data Analysis**

Data analysis was conducted in R (version 3.2.2). Binary data, considering presence-absence of mosquito larvae or exoskeletons were used to create a proportional data set (proportion of positive dips). By using proportional data ( $X_{\text{positive dips}} / N_{\text{dips}}$ ), variation in reservoir size throughout the season can be accounted for. The proportion of positive dips at each site was used as the main response variable accounting for both differences in initial reservoir size as well as intraseasonal size variation at each site.

Model testing was done using Generalized Linear Models in the lme4 package in R. Models were run using the *lmer* function. Site (pond\_id) was included as a random effect in each model to account for random variation between sites. The top 6 models in the set (of 15 candidate models) were used to select key variables for the general model. There were a total of 7 predictor variables used in the creation of the complete model set. A test plot for the top model (Model 1) including a regression curve with added 95% confidence intervals was included along with visual inspection of a histogram of model residuals, a normal Q-Q plot of residual values, and plots of residual vs. fitted values.

## 4.3 Results

### 4.3.1 Field Data

Open water coverage at the reservoirs changed throughout the summer season due to decreased precipitation and snow melt throughout the summer. Two reservoirs included in the study proved to be ephemeral in that by the end of the season no open water remained. Emergent vegetation coverage near reservoir shores ranged from 0% to 100% with a mean of 25.13%. Vegetation coverage changed throughout the year and between years, likely in response to inter-annual changes in water depth (Table 4.3-1).

**Table 4.3-1: Reservoir size, morphology and vegetation measures for all 3 study seasons from 2013-2015 including stocking rates**

Pond ID	Max Depth in m			Emergent Vegetation (%cover)			Hectares			Minnows Stocked	
Year	2013	2014	2015	2013	2014	2015	2013	2014	2015	2013	2014
<b>C2</b>	2.8	2.8	2.8	12.93	20.69	46.28	0.92	0.92	0.92	0	0
<b>C3</b>	0.6	0.6	0.6	0.00	0.00	0.00	0.22	0.22	0.22	0	0
<b>C4</b>	0.3	1.5	1.5	0.00	0.00	0.00	0.13	0.40	0.40	0	0
<b>C5</b>	2.3	2.4	2.4	58.33	47.29	57.61	0.55	0.55	0.46	0	0
<b>C6</b>	2.2	2.2	2.5	0.00	6.67	0.00	0.07	0.07	0.16	0	0
<b>C7</b>	1.3	0.9	1.3	0.00	61.76	2.94	0.42	0.10	0.77	0	0
<b>C8</b>	0.8	0.8	1.1	11.25	22.66	75.78	0.47	0.47	0.67	0	0
<b>T2</b>	2.1	2.1	2.1	0.00	62.46	32.00	1.33	1.33	1.33	8,200	8,200
<b>T3</b>	0.4	1.3	0.4	0.00	36.29	34.52	0.14	0.43	0.43	1,000	1,500
<b>T4</b>	1.8	1.4	1.1	0.00	20.31	2.94	0.63	1.16	0.63	3,900	6,400
<b>T5</b>	1.1	1.1	1.1	0.00	0.00	0.00	0.38	0.38	0.38	2,400	2,400
<b>T6</b>	1.2	1.2	1.2	0.00	3.99	63.04	0.91	0.91	0.91	5,700	7,500
<b>T7</b>	1.5	1.5	2.1	48.61	14.58	9.09	0.31	0.31	0.40	2,000	2,000
<b>T8</b>	5.2	5.2	5.2	46.79	36.90	100.00	0.22	0.22	0.22	1,500	1,500
<b>T9</b>	0.6	0.6	na	100.00	100.00	na	0.02	0.02	Na	500	500
<b>T10</b>	Na	1.5	1.5	na	0.00	33.33	0.26	0.26	0.26	0	2,500
<b>Total</b>										<b>25,200</b>	<b>32,500</b>

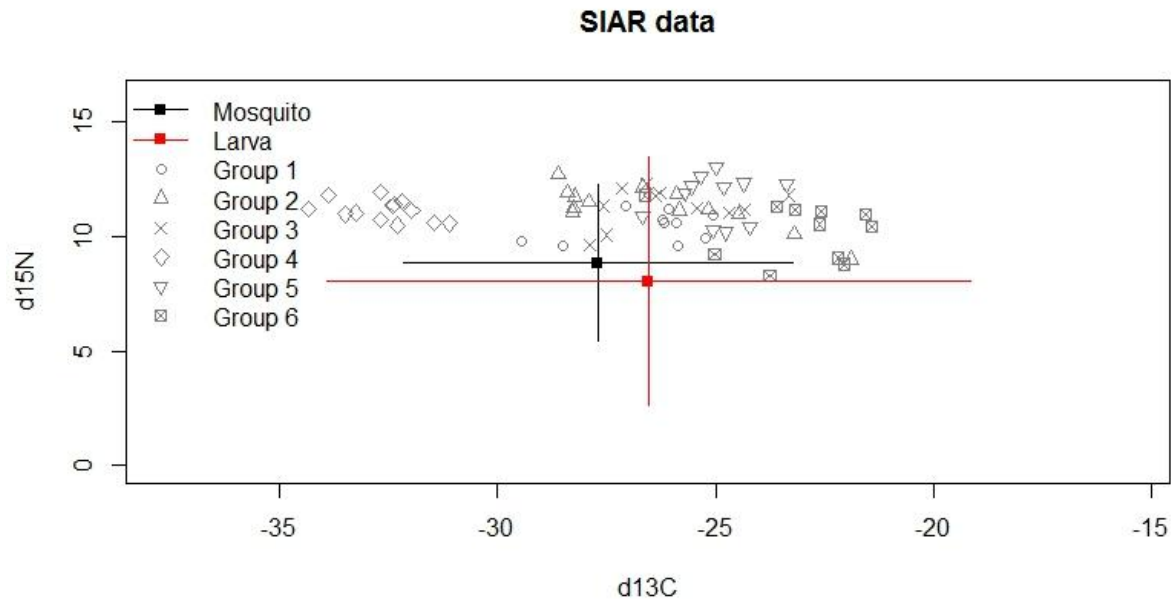
Adult mosquitoes were sampled over 44 trap nights across all sites in 2013. A total of 1,399 adult mosquitoes were collected and identified. There were 14 different species confirmed with 1 individual being unidentified (Table 4.3-2). The *Culex tarsalis* was the most abundant species in the sample.

**Table 4.3-2: List of adult mosquito species collected over 44 trap nights across all sites for 2013.**

Species	Total Count
Cx. Tarsalis	538
Ae. Campestris	17
Ae. Dorsalis	440
Ae. Flavescens	3
Ae. s. idahoensis	12
Ae. Increpitus	1
Ae. Melanimon	25
Ae. nigromaculis	5
Ae. Sollicitans	59
Ae. s. spencerii	26
Ae. Trivittatus	4
Ae. Vexans	160
Cq. Perturbans	99
Ps. Signipennis	8
Unidentified	1
<i>Total # Mosquitoes.</i>	<i>1399</i>

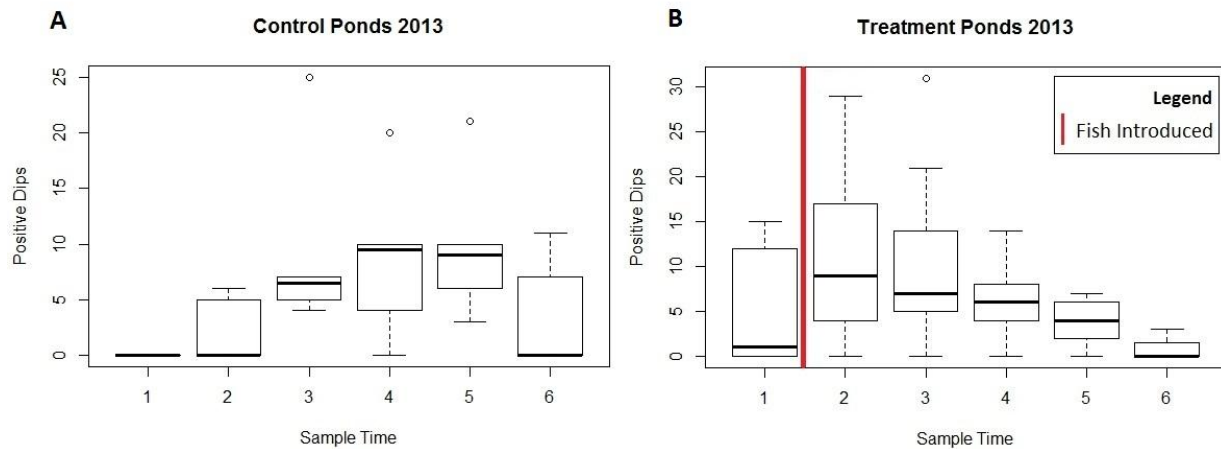
Stable isotope analysis indicated predation at the predicted trophic level when adjusted for an average trophic enrichment factor of 3.4‰ for  $d^{15}N$ , and 0.4‰ for  $d^{13}C$  (Post & Mar, 2002). Groups were based on site to account for variation between sites in reservoir structure and environmental conditions, which can influence prey selection (Scott and Crossman, 1973).

Treatment site T8 (group 4), displayed a slightly lower  $\delta^{13}\text{C}$  average, possibly indicating different prey selection at this site (France, 1995) (Figure 4.3-1).



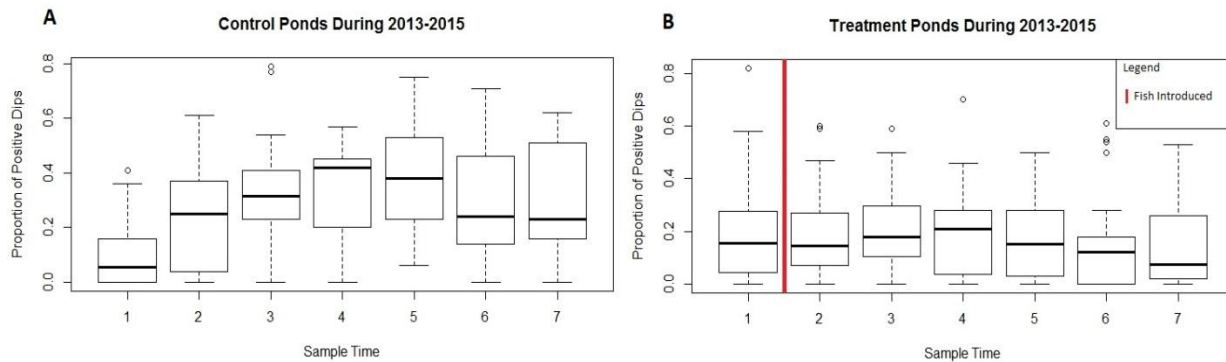
**Figure 4.3-1:** Stable Isotope Analysis in R (SIAR) plot displaying carbon and nitrogen ratios for 6 predator (fathead minnow) groups and their 2 potential prey sources (Adult Mosquitoes and Mosquito Larvae). Predator groups are based on study site with groups 1-5 corresponding to sites C5, T2, T7, T8 and T5 respectively. Group 6 is from an un-stocked supply directly from a hatchery.

A total of 10,492 dip samples were collected between all sites across 7 within-season time periods from 2013-2015. In the initial year of the study, treatment sites only appeared to display increasing larvae densities up until the time of treatment (during week 2 of the study) (Figure 4.3-2). Following treatment, trajectories in larvae populations appeared opposite between control and treatment sites, with treated sites declining in larvae presence over time and controls increasing respectively. Because treatment sites were assigned at random, the initial lower levels of larvae at control sites was due to chance and not any systematic difference between the control and treatment sites.



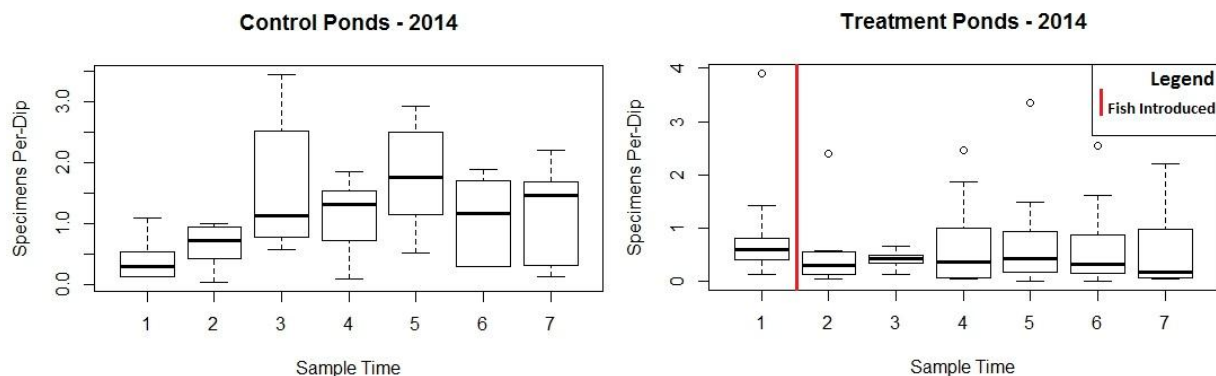
**Figure 4.3-2** – Boxplots displaying the distribution of count (of positive dips) data in control (A) and treatment (B) reservoirs across 6 sampling periods for the 2013 data set. A positive dip indicates a sample dip, using a 350ml dip cup that contained either live mosquito larva or exoskeletons. Sample times ran from June 27 to August 16 and correspond to approximately 1 week intervals during sample times 1 through 6.

Reservoir sizes varied considerably, so the proportions of positive dips were considered to partially control for this variation. Combined data from the 2013 and 2015 seasons reveals similar trends in the proportion of positive dips. Treatment sites displayed less temporal variation and remained suppressed relative to controls. Additionally, the median value and overall spread for the proportion of positive dips among treatment sites began above those values within controls but ended lower in comparison by the end of the season (Figure 4.3-3).



**Figure 4.3-3 :** Boxplots displaying the distribution of proportional positive dip counts in control (A) and Treatment (B) reservoirs across 7 sampling periods for the 2013-2015 combined year data set. A positive dip indicates a sample dip, using a 350ml dip cup that contained either live mosquito larva or exoskeletons. Sample times ran from June 17 to August 20 and correspond to approximately 1 week intervals during sample times 1 through 7.

Larva densities reached their highest in control sites during 2014 as higher water levels were witnessed throughout the season due to above average precipitation levels. As a result, 2014 was used to display the potential in larva density fluctuations under elevated precipitation conditions (Figure 4.3-4).



**Figure 4.3-4:** Boxplots displaying the distribution of average larva counts per dip in treatment and control reservoirs across 7 sampling periods using the 2014 data set. Dips were made using a 350ml dip cup that contained either live mosquito larva or exoskeletons, each counting as “specimens” of larva in that dip. Sample times ran from June 17 to August 19 and correspond to approximately 1 week intervals during sample times 1 through 7.



### 4.3.2 Model Testing

The top 6 models accounted for the majority of explanatory power (combined model weights > 0.95) with the top 2 models accounting for the majority of the weight (combined  $w_i \geq 0.81$ ) (table 4.3-4).

**Table 4.3-3:** Environmental and general predictor variables quantified at each study site and their respective models.

Variable	Abbreviation	Description	Range	Models
Fathead Minnows	Treatment	Reservoir stocked with fathead minnows at a rate of 2500 individuals per 0.4 ha	0/1	1, 2, 3, 4, 5, 6
Vegetation	Veg_cover	Average percent-coverage around Reservoir perimeter either by surface or emergent vegetation	0-100%	4
Perimeter Distance	Perim	Reservoir perimeter measure in feet by GPS waypoint maps	237 ft – 2,870 ft	5,6
Surface Hectares	SH	A measure of open-water surface hectares of a reservoir, not including marshy drainages or shallow pools	0.02 – 1.33 ha	3, 6
Max Depth	Depth	Depth in feet at deepest point	2 ft – 16.9 ft	na
Sample Time	Sample	Corresponds to 1-week sampling Intervals (1-7) between July and August	1-7	1, 2, 3, 4, 5, 6
Site	pond_id	Individual study site ID's Indicating treatment or control	T2 – T10 (n=9) C2-C8 (n= 7)	1, 2, 3, 4, 5, 6
			Total N = 16	

na = variable not included in top model selections ( $w = >95\%$ )

**Table 4.3-4:** Model rankings for main hypotheses concerning the efficacy of fathead minnows for controlling mosquito population in northeast Wyoming. The response variable used was proportion of positive dips. Variables defined in Table 4.3-4:  $k$ , number of parameters in the model; AIC, Akaike's information criterion;  $\Delta AIC$ , difference in AIC from the model with the lowest AIC; rank, model's rank within the set;  $w_i$ , model's weight within the set.

Model #	Models	$K$	AIC <sub>c</sub>	$\Delta AIC_c$	Rank	$W_i$
1	Treatment + Sample + ( <i>Treatment x Sample</i> ) + e(pond_id)*	3	-166.7	0	1	0.569
2	Treatment + Sample + e(pond_id)	2	-165.1	1.6	2	0.256
3	SH + ( <i>Treatment x Sample</i> ) + e(pond_id)	4	-162.1	4.6	3	0.057
4	Veg_cover + ( <i>Treatment x Sample</i> ) + e(pond_id)	4	-161.5	5.2	4	0.043
5	Perim + ( <i>Treatment x Sample</i> ) + e(pond_id)	4	-159.6	7.1	5	0.017
6	SH + Perim + ( <i>Treatment x Sample</i> ) + e(pond_id)	5	-159.4	7.3	6	0.015

\*(treatment x sample) accounts for treatment + sample + (treatment x sample) in following models.

Response = Proportion of positive dips

*Presented models account for >95% of the model weight in the original model set (N = 15)*

**Table 4.3-5:** Beta coefficients and associated standard errors for variables included in the top 95% model set. Values rounded to 2 decimal places.

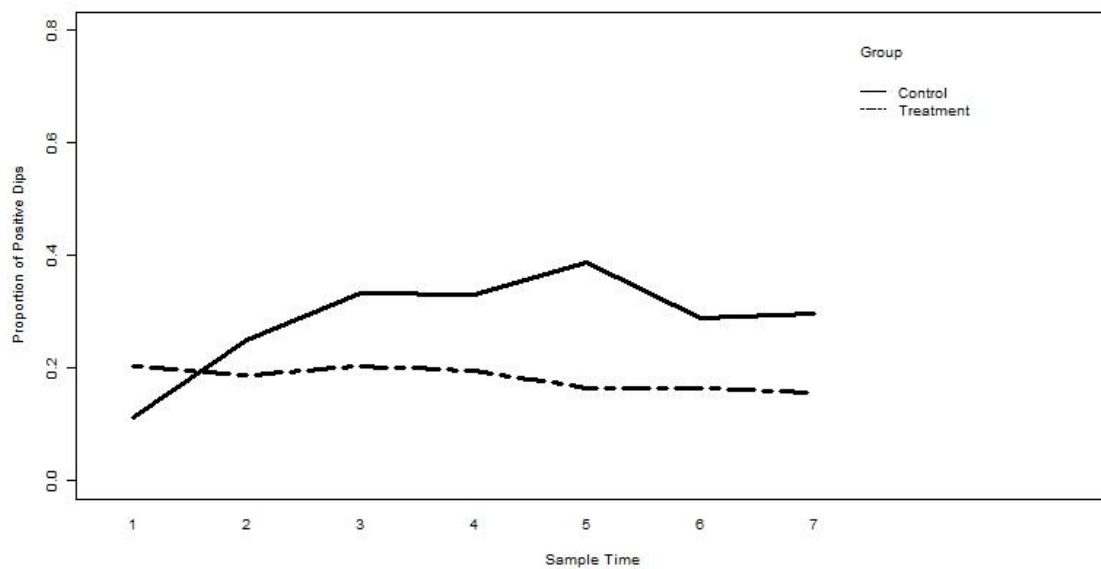
<b>Model</b>	<b>Variable in Model</b>	<b>Metric</b>	<b>B<sub>i</sub></b>	<b>SE</b>
1	Treatment	Presence/Absence	0.07	0.07
	Sample	Time Intervals	0.03	0.01
	<i>Treatment x Sample</i>	Interaction Term	-0.03	0.01
	pond_id	Site		
2	Treatment	Presence/Absence	-0.06	0.05
	Sample	Time Intervals	0.01	0.01
	pond_id	Site		
3	SA	Surface Hectares (scaled)	-0.04	0.02
	Treatment	Presence/Absence	0.07	0.06
	Sample	Time Intervals	0.03	0.01
	<i>Treatment x Sample</i>	Interaction Term	-0.03	0.01
	pond_id	Site		
4	Veg_cover	Mean Cover (%)	0.03	0.01
	Treatment	Presence/Absence	0.04	0.06
	Sample	Time Intervals	0.03	0.01
	<i>Treatment x Sample</i>	Interaction Term	-0.03	0.01
	pond_id	Site		
5	Perim	Perimeter distance (scaled)	-0.02	0.02
	Treatment	Presence/Absence	0.07	0.07
	Sample	Time Intervals	0.03	0.01
	<i>Treatment x Sample</i>	Interaction Term	-0.03	0.01
	pond_id	Site		
6	SA	Surface Hectares (scaled)	-0.12	0.05
	Perim	Perimeter distance (scaled)	0.10	0.06
	Treatment	Presence/Absence	0.09	0.07
	Sample	Time Intervals	0.03	0.01
	<i>Treatment x Sample</i>	Interaction Term	-0.03	0.01
	pond_id	Site		

Model fit was assessed and confirmed through visual inspection of a histogram of model residuals, a normal Q-Q plot of residual values, and plots of residual vs. fitted values (Appendix 1, Figures 4, 5). Fitted vs residual values indicate some non-constant variance, however the absolute value of the residuals is strongly positively correlated with the fitted values in the top model (Appendix 1, Figure 6). A test plot for the top model (Model 1) including a regression curve with added 95% confidence intervals confirms model fit (Figure 4.3-5).



**Figure 4.3-5:** Model test plot for the top model (model 1) including regression curves and 95% confidence intervals.

An interaction plot was created for a visual representation of the interactions between time ( $Sample_i$ ) and treatment group ( $Treatment_i$ ) for the proportion of positive dips (Figure 4.3-6). An analysis of variance for the top model structure indicated the influence of treatment was significant ( $p=0.02$ ) and that the interaction between treatment and time was also significant within groups ( $p=0.001$ ).



**Figure 4.3-6:** Interaction plot of the proportion of positive dips from a combined 2013-2015 data set. A positive dip indicates a sample dip, using a 350ml dip cup that contained either live mosquito larva or exoskeletons. Sample times ran from June 17 to August 20 and correspond to approximately 1 week intervals during sample times 1 through 7. (See Model 1)

Although depth was not included as a key predictor in the top models, a histogram of reservoir depth from all seasons indicated that T8 (5.2m or 16.9ft) was an outlier (Appendix 1, Figure 3).

#### 4.4 Discussion

WNV has emerged as a significant threat to human health, wildlife conservation, and the economies around the world (Barrett, 2014b; Hemingway et al., 2006; Zohrabian et al., 2004). In North America, energy development and agricultural activities have increased the number of water sources within many arid landscapes, creating mesic areas that become prime breeding grounds for disease vectors like the mosquito. Cattle reservoirs are one specific example of these environments. My research denotes the first study to date evaluating the efficacy of fathead minnows as a biological control agent against mosquito vectors in these environments. Importantly, I evaluate their efficacy across multiple years and moisture regimes, revealing the possibility of implementing fathead minnows as a long term control option.

Mosquito night trapping results indicate adult mosquito presence at both treatment and control sites. Out of the 14 different species identified, the *Culex tarsalis*, identified as a primary vector of WNV in Wyoming (Zou, Miller, & Schmidtman, 2006), was the most abundant species in the samples indicating regional potential for WNV. Weekly WNV activity surveys from the Wyoming Department of Health reported avian, equine, and human cases of WNV in Wyoming and the study region during all 3 years of the study period (2013-2015). As these reports rely largely on the public to report avian cases, it becomes difficult to predict the actual number of infected birds. As a result, it is important to consider veterinary and human cases which are more likely to be recorded and may be useful in detecting regional outbreaks. Larvae sampling revealed their presence at every study site, although densities were respectively lower at controls near the beginning of the seasons purely by chance as site designation took place prior to larvae sampling. Due to this occurrence, temporal changes in larvae density trajectories become increasingly important within groups, rather than between groups as they began with different baseline densities.

As predicted, the introduction of fathead minnows produced a detectable influence on mosquito larvae populations in treatment sites compared to controls. An analysis of variance using the top model ( $W=0.57$ ) shows that treatment is a significant predictor in larvae abundance ( $p = 0.02$ ). The presence of fathead minnows suppressed temporal variation in mosquito population densities throughout the summer season. Fathead minnows had a detectable effect in all sites including ephemeral reservoirs which did not carry fathead minnow populations over the winter months. A critical observation can be made with the interaction plot (Figure 4.3-6) which looks at the interaction between treatment ( $Treatment_i$ ) and time ( $Sample_i$ ). This interaction is also the most critical predictor in the top model for proportion of positive dips (Table 4.3-5), and displays high significance within groups under an analysis of variance ( $p=0.001$ ). Control reservoirs began with few larvae present (~50%) respective to reservoirs selected as treatments. By the end of the season (and at various sampling times), control sites had more than double the larval presence compared to treatments. If treatment sites followed the same temporal trajectories as control sites, one would expect a possible two-fold increase in the proportion of positive dips respective to observed treatment proportions.

The interaction between treatment ( $Treatment_i$ ) and time ( $Sample_i$ ) appears as the most critical component in determining the efficacy of the fathead minnow for mosquito larvae control. Although treatment alone had a detectable influence on abundance, it is the suppression of temporal changes in abundance that is particularly important. Similar observations were made concerning the interaction between time ( $Sample_i$ ) and treatment group ( $Treatment_i$ ) with respect to density, expressed as specimen count per dip (See Appendix 1, Figure 2). Other variables that appeared in the top 6 models were surface hectares, vegetation coverage and perimeter distance. Both surface hectares and perimeter distance were negatively correlated with larva abundance ( $B_i = -0.04$  and  $B_i = -0.02$

respectively). As expected, vegetation coverage is positively correlated with larva abundance ( $B_i = 0.03$ ).

Arguably, the key to biological control is to understand the source of predator density dependence required to stabilize a target pest population at a low equilibrium density (Huffaker and Messenger, 1976; Murdoch, 1973; Murdoch, 1979). In this study, density dependence (for fathead minnows) was controlled at a minimum of 2,500 minnows per 0.4 surface hectares (1 acre), while equilibrium density in the pest population was expressed as the proportion of positive dips. As suggested in the literature, successful control relies on the selected natural enemy species possessing host or prey specificity, potential for rapid population increase when host population increases, and a high rate of successful searches (Murdoch and Briggs, 1996; Murdoch et al., 1985). Although fathead minnows are opportunistic feeders, previous studies indicate their ability to consume mosquito larva at a substantial rate under controlled conditions (Irwin and Paskewitz, 2009). In order to examine dietary preferences in these semi-natural systems, stable isotopes were included in this study. Stable isotopes provide a means of quantifying dietary relationships by tracking and measuring isotopic signatures through the food chain. Here, the addition of isotopic analyses shows there is an existing relationship between the predator and prey. Although stable isotope results from this study are unable to determine dietary proportions, they do indicate predation from the appropriate trophic level. When paired with autoregressive models (Figure 4.3-6) looking at temporal variation of mosquito larvae densities in both treatment and control sites, there is strong evidence that the minnows are feeding on larvae in most cases. The fathead minnow's ability to be synchronous with mosquito larvae populations is enhanced by their opportunistic feeding habits, as opportunistic feeders will tend to prey on what is available or in high abundance. Therefore, when mosquito populations are high (more available) in comparison to other potential prey, fathead minnows may be more likely to consume mosquito larvae. The potential for rapid population increases and the high fecundity of



fathead minnows also makes them a suitable candidate for mosquito pest control (Gale & Buynak, 1982; Thomson and Hasler, 1944).

As displayed in control sites, mosquito populations tend to fluctuate throughout the summer season.

This may be attributed to fluctuations in temperature, precipitation, and a variety of other local

weather factors. Treatment sites did not express the same temporal variation in mosquito density,

suggesting that feeding habits of the fathead minnow may be synchronous with spikes in mosquito

larvae abundance and availability. Fathead minnows also have the potential to reproduce

continuously throughout the season, with females laying an average of 391-480 eggs during a single

spawning event (Gale & Buynak, 1982). Evidence of reproductive activity was observed at most

treatment sites during the entire duration of each year of the study (June through August). This

evidence was based on observation of breeding activity, or the presence of eggs on introduced

breeding habitat. Sites which did not show evidence of breeding throughout the entire duration did

however display evidence of breeding activity until approximately the second week of August. The

fecundity and breeding success of fathead minnows allows their population to increase rapidly when

resources are available and environment conditions are suitable (Gale & Buynak, 1982; Markus,

1934). Environmental condition within the majority of the reservoirs included in the study proved to

be sufficient for viable fathead minnow populations. Lastly, successful control is said to rely on high

rates of successful searches. Although difficult to quantify, this can be determined by the influence of

treatment, demonstrated by the models, as well as field observations. One of the greatest challenges

to mosquito control comes from their ability to reproduce in the smallest quantities of water and the

shallowest of pools. Mosquito larvae thus tend to be congregated around the reservoir perimeters,

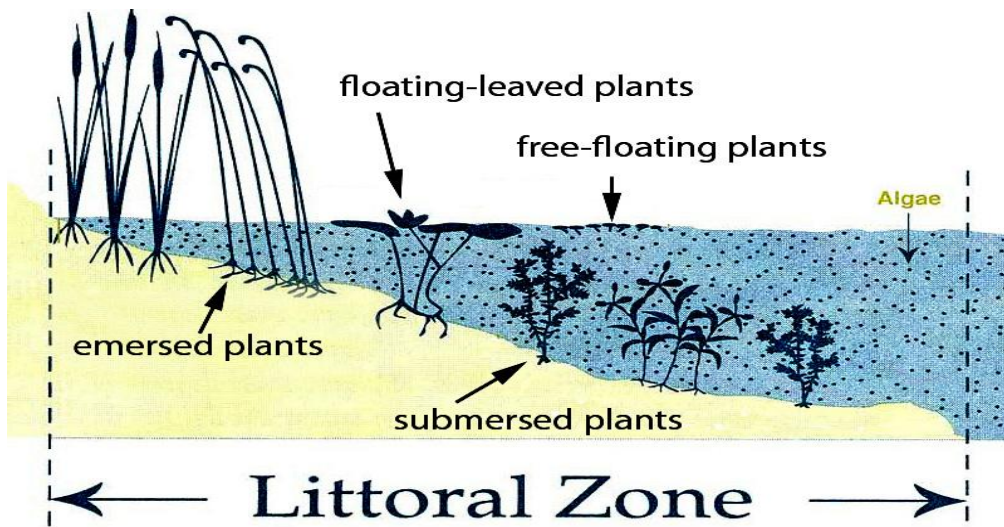
often in shallow littoral zones around emergent and submerged vegetation for protection against

predators and direct sunlight (Figure 4.4-1). Fathead minnow fry are small enough to be able to swim

into the shallow areas of the littoral zone, with some observed fry in this zone of treatment sites being

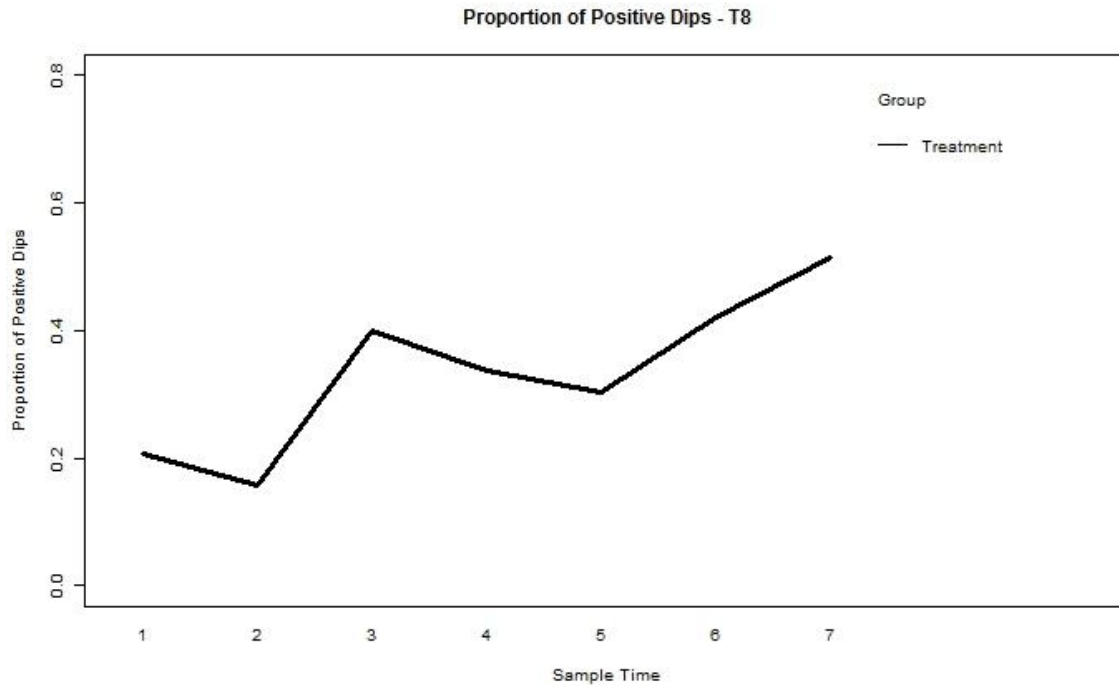
no more than 1cm in length. Observational data also indicates fry presence in the shallow tails that

branch off the littoral zones near reservoir drainage areas where larvae presence tends to increase. The small size of the fathead minnow increases the potential of successful searches, as well as the potential for predation on early in-star life stages of the mosquito.



**Figure 4.4-1:** Cross section view of the littoral zone commonly found in livestock reservoirs where mosquito larvae are most likely to be found. Source: Minnesota Dept. of Natural Resources <[http://www.dnr.state.mn.us/shorelandmgmt/apg/where\\_grow.html](http://www.dnr.state.mn.us/shorelandmgmt/apg/where_grow.html)>.

Although this study displayed promising results in treatment sites as a whole, challenges exist to the overall efficacy of control in certain environments. The primary concern in determining the intraseasonal efficacy of this control effort is intraseasonal predation effect, which is the most critical element in the control of mosquito populations. In the majority of treatments, the difference in larva population trajectories (as indicated through analysis of variance and interactions, Figure 4.3-6), suggest a significant difference from controls. However, some treatment sites did not show the same effect when analyzed on their own. T8 for example displayed very different trends when compared with other treatments or treatment site trajectories as a whole (Figure 4.4-2).



**Figure 4.4-2:** Interaction plot of the proportion of positive dips for treatment site 8 only (T8) from 2013-2015. A positive dip indicates a sample dip, using a 350ml dip cup that contained either live mosquito larva or exoskeletons. Sample times ran from June 17 to August 20 and correspond to approximately 1 week intervals during sample times 1 through 7.

Temporal changes and abundance of larva populations in T8 more closely resembled that of control sites, yet minnow populations were present and seemingly abundant in trap counts. These higher larval densities suggests that the fathead minnows may be selecting for other prey when environmental conditions either restrict access, or present opportunities to feed on alternative prey sources. When combined with vegetation cover, depth may have an indirect influence on the efficacy of fathead minnows as a control agent through alternative prey opportunities. This hypothesis is supported by isotope data, with T8 (group 4) being the only site that deviated in carbon ( $\delta^{13}\text{C}$ ) ratios for the fathead minnow (Figure 4.3-1, Group 4). Previous studies have shown significant  $^{13}\text{C}$  enrichment in littoral compared to pelagic consumers in lake environments (France, 1995). Similar studies on prey consumption in freshwater lake species have also confirmed these differences in  $^{13}\text{C}$  levels, indicating differences in benthic reliance (Vander Zanden & Vadeboncoeur, 2002). The

isotope data in this study suggests slightly different prey selection in deeper reservoirs such as T8, likely due to features of reservoir morphology leading minnows to feed primarily on pelagic prey sources. Reasons for such deviations likely revolve around 2 main components: 1) emergent vegetation cover, and 2) reservoir depth. First, vegetation cover at T8 was consistently at 100% throughout the season. Vegetation cover at T8 was almost exclusively emergent vegetation with thick submerged vegetation communities which hosts ideal cover and suitable environmental conditions for larval sheltering. Vegetation coverage of this type greatly decreases the chances for successful searches by predatory fishes such as the fathead minnow, as there is no free water access to the prey. Secondly, T8 was also by far the deepest reservoir at 5.2m with an extremely steep benthic gradient (Appendix 1, Figure 3). Limited access to the near shore littoral zone likely shifted feeding opportunities to pelagic zooplankton found in deeper waters, or at the surface. Reservoir/lake morphology and composition can have an important impact on various zooplankton community structures and populations, leading to different prey source opportunities that may be more appealing to opportunistic feeders such as the fathead minnow (Cottenie, Nuytten, Michels, & Meester, 2001). Overall, it was found that dense vegetation and extreme reservoir depth contributed challenges to the fathead minnow's efficacy as a control agent. In such environments, other options of control may be required alongside fathead minnow introduction.

#### **4.5 Conclusions**

This study evaluates and proves the efficacy of fathead minnows as a useful control agent in closed man-made ecosystems. Although there is an abundance of other wildlife at these reservoirs, these systems are novel and only exist as a result of anthropogenic activities. It is important to recognize that due to the fathead minnow's life history traits, stocking interconnected water systems must take into account different considerations. In high abundance, the fathead minnow has the potential to significantly influence aquatic ecosystems (Scott and Crossman, 1974). For example, a study

conducted by Eaton et al. (2005) found that small bodied fishes such as the fathead minnow have the potential to suppress certain amphibian populations such as the Wood frog (*Rana sylvatica*) in Boreal Alberta lakes. Other research conducted by Zimmer et al. (2001, 2002) found that fathead minnows can be an important determinant of many biotic factors in prairie pothole wetlands, including aquatic insect levels and salamander populations. It was also found that fathead minnows can increase turbidity and total phosphorous levels in these systems. Although these are important consideration in other ecosystems, the sites used in this study were all eutrophic water bodies used as cattle reservoirs where turbidity and nutrient levels had extremely high baselines (Appendix1, Figure 7). No other fish species inhabited the reservoirs prior to treatment, as site conditions would only support survival of the most enduring of species.

Fathead minnows prove to be an effective method of control for mosquito larvae population densities under certain reservoir conditions. Fathead minnows also demonstrate the ability to suppress temporal variation in larvae abundance. However, the existence of an equilibrium state or suppressed larvae population is not sufficient on its own to bring about successful control in the context of biological control theory and practice. The reservoirs monitored in this study are closed local systems, and locally stable systems or local regulation does not necessarily imply stable metapopulations. Northeast Wyoming has numerous livestock reservoirs, drainages and other man-made water sources which can become prime habitat for mosquito populations. A landscape level approach to mosquito population control may be required if control efforts are to be successful. Although some water sources may not be suitable for fathead minnow introduction, many of the reservoirs in rural northeast Wyoming are comparable to those featured in this study and would be prime candidates for possible minnow introduction as part of a larger control effort. Successful control impacting disease and disease vectors often relies most on pest density which must remain below certain tolerable thresholds throughout time or, at least, for significant time periods. For

regional mosquito control, this means placing a primary focus on mosquito density while also acknowledging that successful biological control for the purposes of reducing disease outbreaks will rely on limiting the temporal variability of mosquito larva densities on a landscape level. In the majority of my study sites, fathead minnows have demonstrated the ability to suppress larval densities by up to 200%. They have also demonstrated the ability to significantly suppress temporal variation in both density and abundance, making the fathead minnow a suitable control option in many rural reservoirs.

## **5 The Effects of Reservoir Morphology and Water Quality on Fathead Minnow (*Pimephales promelas*) Survival and Population Viability in Northeast Wyoming, USA.**

### **5.1 Introduction**

The biology and life history traits of the fathead minnow (*Pimephales promelas*) have been well studied in the literature (Ankley & Villeneuve, 2006; Scott and Crossman, 1973). Individuals mature rapidly, and can tolerate a wide range of environmental conditions (Ankley and Villeneuve, 2006; Scott and Crossman 1973). As a result, fathead minnows have a long history as subjects in toxicology and water quality experiments (Ankley & Villeneuve, 2006; Brungs, 1971; Doudoroff & Katz, 1953; Mount, 1973). Several studies have shown the fathead minnow's ability to survive in low oxygen level environments, even during winter months (McCarraher & Thomas, 1968) . These life history traits are likely to facilitate the establishment of viable, self-sustaining populations in a wide-range of environments.

Investigating population viability under certain environmental conditions becomes especially important when the species is to be implemented as a biological control for disease vectors such as the *Culex* mosquito. In the previous chapter, I tested the efficacy of using fathead minnows (*Pimephales promelas*) for mosquito control in rural cattle reservoirs in northeastern Wyoming in the hopes of minimizing the threat of West Nile virus, a disease primarily spread by mosquito vectors (Zou et al., 2006). As fathead minnows proved to be beneficial for suppressing mosquito populations in these reservoir environments, it is now critical to understand whether or not these environments foster the establishment of self-sustaining populations. In this chapter, I evaluate effects of reservoir morphology and various water quality characteristics on fathead minnow survival and population viability in cattle reservoirs in northeast Wyoming. Population viability undoubtedly dictates their

utility as a sustainable control option. Specifically, this chapter addresses two main questions: 1) what features of pond morphology affect fathead minnow survival and population viability, and 2) what water quality characteristics within these cattle reservoirs impacts survival and population densities?

## **5.2 Methods**

### **5.2.1 Water Chemistry**

During each field season, primary components of water chemistry were analyzed using water samples taken from each treatment site. Sample bottles were filled at or near the deepest point of the reservoirs, below the water surface (bottles were only opened after they were completely submerged in the reservoir). General parameters of pH, alkalinity, hardness, and nitrogen were analysed in-lab with samples being delivered to the lab for inspection no more than 24 hours from collection time. All lab testing was conducted according to approved standard methods (SM) through the United States Environmental Protection Agency (EPA) (2015). pH was measured using the SM 4500 H B method. Alkalinity was calculated as total  $\text{CaCO}_3$ , measured in mg/L and analysed using the SM 2320B method. Water hardness was a measure of calcium/magnesium as  $\text{CaCO}_3$  and measured in mg/L using the SM 2340B method. Nitrogen, or ammonia, was taken as a measure of N in mg/L using the EPA 350.1 method.

Anions and cations were measured in mg/L, using the EPA 353.2 method and EPA 200.7 method respectively. Anions were taken as a measure of Nitrate-Nitrite (as N) and cations included calcium, magnesium and sodium. Dissolved and total metals were measured in mg/ml and also followed the EPA 200.7 method. This included Iron, Magnesium, and Zinc with total metals measured by Phosphorus. Water temperature and dissolved oxygen (DO) was also recorded with a YSI meter, along with water conductivity using a handheld Hanna *combo* pH and conductivity meter. Dissolved oxygen content was monitored over the winter of 2013-2014 at sites which had available road access.



For winter dissolved oxygen readings, a hole was drilled into the ice at the center of the reservoir and measurements were taken at 0.3m intervals beginning at the surface.

### **5.2.2 Reservoir Morphology**

Features of reservoir morphology included reservoir size measured by surface hectares (ha), perimeter distance, as well as depth measured at the deepest point. Reservoir size and perimeter distance were calculated in *All Topo* using waypoints collected from a handheld GPS unit. Waypoints were taken near the end of June by walking the reservoir perimeter at the water's edge. Depth was measured from a boat using a weighted measuring line. Vegetation coverage and composition were also sampled and recorded. This focused primarily on emergent and surface vegetation around the water edge and was calculated as percent coverage within a circular quadrat 1 meter in diameter. Observations were recorded at 10 meter sampling intervals and an average percent coverage was then calculated for each reservoir. Captive-raised fathead minnows were transported and released into the reservoirs identified as treatment sites (n=10). The stocking rate for each site was 2500 minnows per 0.4 ha (equivalent to 1 surface acre). Due to annual changes in reservoir size the total number of minnows introduced to each reservoir varied between treatment years. The total number of fry used for stocking in 2013 was 25,200, while 2014 required 32,500. Stocking was conducted in 2014 regardless of overwinter survival from 2013 or reproductive success.

### **5.2.3 Population Monitoring**

Presence or absence of fathead minnows was recorded weekly based on sightings and trapping using baited steel traps. In particular, traps were used most often when sightings were not conclusive. Reproductive efforts were assessed by the presence or absence of eggs on artificial breeding substrates introduced at the beginning of the 2013 season. Standard wooden shipping pallets were used as artificial substrates. Observations of male nesting activity were also used as indicators of

reproductive effort. Relative densities were determined using standard electrofishing techniques at the beginning (early July) and end (late August) of the 2014-2015 seasons using the Smith-Roots LR-24 or HallTeck HT-2000 backpack shocker. The electrofishing protocol consisted of shocking 10% of the total reservoir perimeter between 0.5-2m from shore in the littoral zone. Total number of transects used at each site was dependant on reservoir size with each transect spanning approximately 6m (formula:  $n \text{ transects} = (\text{Reservoir perimeter (m)} * 0.10) / 6\text{m}$ ). Each transect was shocked for a total of 90 seconds. Personnel operating the electrofisher would begin shocking at the start of each transect, moving forward at a steady pace throughout the duration of the shocking period. Fish counts were based on observation rather than collection due to the extreme difficulty walking in the reservoirs. Only fish *turned* by the electrofisher would be counted (*turning* a fish consisted of any fish visibly affected by the current to the point of flipping over or surfacing, or becoming temporarily stunned). All observational data was collected by the same individual to minimize observation bias. Voltage and general output settings such as duty-cycle were specific to each site dependant on water conditions (primarily conductivity).

At the beginning of the second and third seasons (2014-2015), overwinter survival was assessed using baited minnow traps (2 traps/0.4 ha - or 1 surface acre) to detect presence or absence of fathead minnows.

#### **5.2.4 Data Analysis**

General Linear Models were used to identify key features of pond morphology that impacted relative densities (expressed as catch per unit effort). Model testing was done using Generalized Linear Models in the lme4 package in R using the *lm* function. Each potential covariate influencing relative density was initially inspected using univariate models for each covariate, and then included in multivariate models. Out of 12 candidate models, the top model was chosen based on model weight and the top variables in the set. The top model was then used to identify the key predictor variables. There were a total of 4 predictor variables for pond morphology used in the creation of the

complete model set. Standard X-Y plots including a regression curve were included for visual inspection of the top 2 predictors including the  $R^2$  value. Water quality indicators were assessed using the same standard X-Y plots including a regression curve and  $R^2$  value. Data analyses were conducted in R (version 3.2.2).

## **5.3 Results**

### **5.3.1 Field Data**

Reservoirs ranged in size from 0.02 to 1.33 ha with a mean of 0.5 ha. Reservoir depths (at the deepest point) ranged from 0.60 to 5.2 m, with a mean of 1.72m. Emergent vegetation coverage near reservoir shores ranged from 0% to 100% with a mean of 25.13%. Vegetation coverage changed throughout the year and between years likely in response to inter-annual changes in water depth. Almost all of the treatment reservoirs overwintered between all 3 field seasons. During the winter of 2013-14, all reservoirs overwintered minnow populations except treatment reservoirs T3 and T9. These reservoirs were also the two smallest reservoirs during this time period. During the following winter (2014-15), T4 was the only site which did not display overwinter survival, with T9 being removed from the study (Table 5.3-1). Reproductive activity was witness throughout the entire season at all sites that overwintered. Average drawdown across all sites reached 36.59%, with a range between 0% - 100%.

**Table 5.3-1:** Reservoir size, morphology and vegetation measures for all 3 study seasons from 2013-2015 including stocking rates and overwinter survival

Pond ID	Max Depth (m)			Emergent Vegetation (%cover)			Hectares			Minnows Stocked		Overwintering	
	2013	2014	2015	2013	2014	2015	2013	2014	2015	2013	2014	2013-14	2014-15
<b>T2</b>	2.1	2.1	2.1	0.00	62.46	32.00	1.33	1.33	1.33	8,200	8,200	Yes	Yes
<b>T3</b>	0.4	1.3	0.4	0.00	36.29	34.52	0.14	0.43	0.43	1,000	1,500	No	Yes
<b>T4</b>	1.8	1.4	1.1	0.00	20.31	2.94	0.63	1.16	0.63	3,900	6,400	Yes	No
<b>T5</b>	1.1	1.1	1.1	0.00	0.00	0.00	0.38	0.38	0.38	2,400	2,400	Yes	Yes
<b>T6</b>	1.2	1.2	1.2	0.00	3.99	63.04	0.91	0.91	0.91	5,700	7,500	Yes	Yes
<b>T7</b>	1.5	1.5	2.1	48.61	14.58	9.09	0.31	0.31	0.40	2,000	2,000	Yes	Yes
<b>T8</b>	5.2	5.2	5.2	46.79	36.90	100.00	0.22	0.22	0.22	1,500	1,500	Yes	Yes
<b>T9</b>	0.6	0.6	Na	100.00	100.00	Na	0.02	0.02	Na	500	500	No	No
<b>T10</b>	Na	1.5	1.5	Na	0.00	33.33	0.26	0.26	0.26	0	2,500	NA	Yes

Overwinter dissolved oxygen levels were reported at sites with available road access (n=5). Ice thickness ranged from 0.5-0.6m. Surface levels of dissolved oxygen were relatively high, with dissolved oxygen decreasing with increased water depth. Deeper sites such as T1 and T8 experienced higher overall dissolved oxygen levels. The average dissolved oxygen for sites with overwinter access was 6.3 mg/L across all sites and depth marks with a standard deviation of 1.8mg/L (Table 5.3-2).

**Table 5.3-2:** Winter 2014 Dissolved oxygen levels between January 24, 2014 and February 10, 2014. Measurements were only taken at sites with available road access. Ice sheet thickness was between 0.5 and 0.6 meters for all sites. This thickness was included in depth marks for DO readings.

Site	Dissolved Oxygen (mg/L) at Specified Depth in Meters								
	Surface	0.3m	0.6m	0.9m	1.2m	1.5m	1.8m	2.1m	2.4m
t1*	10.5	4.1	3.4	2.6	2.3	2.1	2	2	NA
t2	11.1	10.9	10.9	6.4	2.4	2	NA	NA	NA
c5	9.2	8.9	8.8	8.3	5	4.5	3.8	NA	NA
t7	5.6	5.7	6.8	2.6	NA	NA	NA	NA	NA
t8	11.2	11.3	11.3	10.4	10.1	9.8	8.1	7.3	6.2
Average	9.5	8.2	8.2	6.1	5.0	4.6	4.6	4.7	6.2

*\*Treatment site 1 was included in winter DO readings even though this site did not end up in the study. This was done strictly to obtain DO readings at given depths, as many sites became inaccessible during the winter due to harsh road conditions.*

Relative densities expressed as catch per unit effort revealed increasing density between years at 7 of the 10 study sites (Table 5.3-3). Two of the sites displayed no survival as of 2015 including T9, which was removed from the study as it did not prove to support survival in 2014. Although supporting relatively high densities in 2014, T4 dried up in 2015 and did not support any survival.

**Table 5.3-3: End of year catch per unit effort for all treatment sites (2014-15) with change in catch per unit effort between years.**

<b>End of Year Catch Per Unit Effort*</b>			
<b>Site</b>	<b>2014</b>	<b>2015</b>	<b>Change in CPUE*</b>
<b>T2</b>	73.3	26.5	-46.8
<b>T3</b>	0	3.3	+3.3
<b>T4</b>	35.1	0	-35.1
<b>T5</b>	4.6	25.3	+20.7
<b>T6</b>	3.6	12.5	+8.9
<b>T7</b>	4.3	16	+11.7
<b>T8</b>	2	4.7	+2.7
<b>T9</b>	0	na	na
<b>T10</b>	14	35	+21
<b>C5</b>	16.2	28.2	+12

**\*Catch per unit effort (CPUE) refers to fish turned per transect (averaged between all transects). 10% of the total reservoir perimeter was used to standardize sample.**

Only 1 site (T2) decreased in relative density while supporting survival. Although T2 had identical pre-season water levels, GPS reading from the end of the 2015 season revealed that it experienced significant draw-down losing 69% of its water holdings between June and August. The average seasonal draw-down across all sites was 36.59% between the middle of June and the end of August.

Water quality parameters averaged across study sites are within tolerable ranges for fathead minnow survival (Table 5.3-4). Water temperatures between mid-June and the end of August in most reservoirs were within the optimal range with the exception of a 5 sites during 2014 (which was a slightly cooler season. In one case (T9), lower temperatures were a result of shade cover from surrounding trees keeping temperatures cooler. These sites were still however within the tolerable range.

**Table 5.3-4:** Table of water quality indicators measured in the field and in laboratory. Values include the mean and standard deviation for treatment sites included in this study, with optimal range and maximum and/or minimum tolerances derived from the literature.

Parameter	Treatment			
	Reservoirs	Optimal Range(s)*	Max/Min Tolerance*	Literature
	Mean $\pm$ STDev			
		20.9 – 29°C (Ideal)		
<b>Water Temperature (°C)</b>	22.83 $\pm$ 3.64	25°C)	max = 40.2	(Denny, 1987; Lyons et al., 2009)
<b>Dissolved Oxygen (mg/L)</b>	8.51 $\pm$ 3.52	>7 mg/l	min = 1-4mg/L	(Brungs, 1971; Denny, 1987; Wares and Igram, 1979)
<b>pH</b>	8.65 $\pm$ 1.38	7.4 - 8.2	min = 6	(Mount, 1973; Rahel and Magnuson, 1983)
<b>Conductivity (<math>\mu</math>s)</b>	1284 $\pm$ 985	ND	ND	
<b>Total Alkalinity (as CaCO<sub>3</sub>) (mg/L)</b>	104.21 $\pm$ 38.15	42mg/l	max = 2000 mg/L	(Denny, 1987)
<b>Hardness</b>	413.68 $\pm$ 239.49	40 - 300 mg/l	ND	(Denny, 1987)
<b>Ammonia Nitrogen</b>	0.08 $\pm$ 0.2	<2.19 mg/l	Max = 2.19 **	(Armstrong et al., 2012)
<b>Calcium (mg/L)</b>	76 $\pm$ 40.75		max = 300-1000mg/L	(Doudoroff & Katz, 1953)
<b>Magnesium (mg/L)</b>	54.63 $\pm$ 41.52		max = 100-400mg/L	(Doudoroff & Katz, 1953)
<b>Sodium (Na<sup>+</sup>, mg/L)</b>	80.84 $\pm$ 138.61		max = 8,000mg/L (NaCl)	(Burnham & Peterka, 1975)
<b>Dissolved Iron (mg/L)</b>	0.07 $\pm$ 0.17		max = 1mg/L	(Doudoroff & Katz, 1953)
<b>Dissolved Manganese (mg/L)</b>	0.01 $\pm$ 0.03		max = 50mg/L	(Doudoroff & Katz, 1953)
<b>Total Phosphorus (mg/L)</b>	0.17 $\pm$ 0.39		ND	(Doudoroff & Katz, 1953)

\*Optimal Ranges and Tolerance limits were derived from the literature on fathead minnows, or closely associated species.

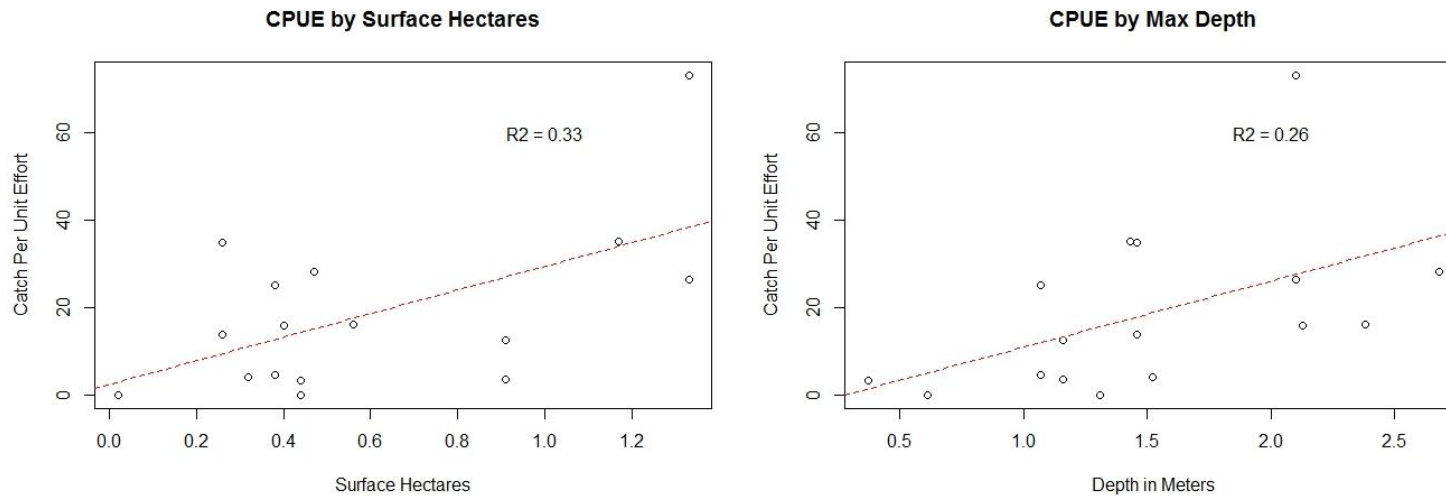
ND= No published data specific to particular parameter tolerance available.

\*\* Max tolerance of Ammonia Nitrogen before experiencing negative effects on reproduction.



### 5.3.2 Data Analysis

General Linear Models identified 2 key features of pond morphology that influenced relative densities of minnows (expressed as catch per unit effort). The top model in the set (N=12) identified surface hectares and depth as key predictor variables influencing relative densities. T8 was removed from the analysis as it was an outlier with respect to pond depth (See Appendix 2, Figure 3). Standard X-Y plots of CPUE by surface hectares and depth ( $R^2 = 0.33$  and  $0.26$  respectively) illustrate a positive relationship (Figure 5.3-1).



**Figure 5.3-1:** Standard X-Y plots displaying regression curves and associated  $R^2$  values for surface hectares (A) and depth (B), the top two pond morphology predictors of relative density using catch per unit effort as the response.

None of the water quality indicators measured in-lab had a strong relationship with relative density (see appendix 2, Figure 1). Other features of pond morphology as well as temperature and conductivity measured in field also lacked any relationship with CPUE (see appendix 2, Figure 2). Survival was witnessed across all levels of water quality parameter variation.

## **5.4 Discussion**

In the previous chapter, I evaluated and quantified the efficacy of fathead minnows for mosquito control within cattle reservoirs in northeast Wyoming. This chapter addresses fathead minnow survival and population viability to better evaluate the utility of fathead minnows as a sustainable control option. This is the first study to evaluate the sustainability of fathead minnows as a biological control agent against mosquito vectors in arid environments. Importantly, I evaluated population viability across multiple years and moisture regimes, revealing the possibility of implementing fathead minnows as a long-term control option within these environments.

### **5.4.1 Pond characteristics**

During the winter of 2013-14, minnow populations survived winter in all but two reservoirs (T3 and T9). These reservoirs were the two smallest reservoirs included in the study during this time period (0.14 and 0.02 ha respectively). T3 experienced die off of fathead minnow populations as a result of complete freeze-through during the 2013-14 winter. The smallest water body included in this study (T9), was removed as of 2014 as it did not display any potential to support survival for more than 2 weeks due to ephemeral water conditions. During the following winter (2014-15), T4 was the only site which did not display overwinter survival. Pre-season measurements of both surface hectares and maximum depth decreased in T4 between 2014 and 2015. This reservoir also experienced rapid drawdown during the 2015 season (100%) with no water remaining by the end of August suggesting changes in local run-off, drainage features and water holding capacity from previous seasons.

Overwinter dissolved oxygen (DO) levels in reservoirs with winter sampling access proved to be suitable for fathead minnow survival with an overall average of 6.35mg/L (Denny, 1987; Igram & Wares, 1979). Oxygen levels were higher near the surface, especially at sites with greater depths. Larger, specifically deeper ponds had higher dissolved oxygen reading on average.

#### **5.4.2 Relative Densities**

Surface hectares and maximum depth were the key features of pond morphology influencing relative densities. Densities fluctuated between 2014 and 2015. Between both study years, relative densities increased within 7 of 10 reservoirs. Only one site (T4), as of 2015, displayed no evidence of fathead minnow survival. Although supporting relatively high densities in 2014, T4 experienced a total loss of all water holdings in the 2015 season which seemed to have killed off its residing minnow population before sampling began in late June of 2015. Only one site included in the study (T2) demonstrated a decrease in relative density while overwintering between years. This decrease in relative density followed a 69% drawdown in water holdings during the 2015 season, also accompanied by heavy cattle presence throughout this time. As cattle frequently occupy the shallow edges of the littoral zone, minnows are forced into deeper waters. Heavy cattle presence also removed vegetation communities and the associated biomass from the littoral zone which may have had a negative impact on both predation and reproductive efforts as fathead minnows generally feed and reproduce along shorelines (Divino & Tonn, 2008; Grant & Tonn, 2002; Scott & Crossman, 1973). All other reservoirs displayed an increase in relative density between years.

#### **5.4.3 Water Quality**

None of the water quality parameters (n=13) measured seemed to influence minnow population densities. Reservoir T8 was removed from all water quality and density analyses. As mentioned in chapter 1, T8 was an outlier with respect to reservoir depth (see Appendix 2, Figure 2). Relative densities utilizing

electrofishing techniques were taken in the littoral zone of the reservoirs. Sites with very steep benthic gradients (generally very deep ponds such as T8) do not allow for the standard electrofishing protocols (described in the methods) to be consistently implemented. Furthermore, the previous chapter revealed that minnow populations within these extremely deep reservoirs congregate and feed primarily in the pelagic zone, making littoral zone density estimates at these sites incomparable to other reservoirs in the study where minnows primarily feed and congregate in the littoral zones.

All parameters measured within these treatment reservoirs were within tolerance limits and often within optimal ranges for fathead minnows. However, previous studies that manipulated nutrient concentrations (such as Phosphorous and Nitrogen) have shown that increased nutrient concentrations in freshwater systems can significantly increase the production of zooplankton and algae, which can be an important food sources for fathead minnows at certain life stages (Grant & Tonn, 2002). In response to increased biomass, fathead minnows have been observed to lay more eggs and experience greater survival of age-0 fish during the breeding season which would influence overall population size (Grant & Tonn, 2002). This may become important for population viability when other food sources are not present, or when populations of fathead minnows are forced into different zones of the reservoirs shifting feed opportunities. Reservoir conditions in northeast Wyoming can change annually based on water levels and cattle presence. As discussed in the previous chapter, vegetation density in the littoral zone can cause fathead minnows to shift feeding activities to the pelagic zone. However, in sites where feeding is primarily based in the littoral zone, introducing cattle could push fathead minnows into deep waters as a result of disturbance.

Reproductive activity and success may be the most sensitive factors to water quality indicators and nutrient concentrations recorded in this study. Since fathead minnows will spawn on a range of natural and artificial substrates (Benoit & Carlson, 1977; Clemment & Stone, 2004; Grant & Tonn, 2002;

Jensen, Korte, Kahl, Pasha, & Ankley, 2001), spawning activity was made easily detectable using introduced habitat. Here, standard wooden shipping pallets were utilized as breeding substrate and to detect breeding activity. During spawning, males actively defend their nest sites which are generally on the underside of submerged objects (Gale & Buynak, 1982). Nest defense generally entails the male circling below the nest site rubbing against the substrate with the sponge-like pad on the top of their head to clear the surface (Divino & Tonn, 2008). After eggs are laid, this scrubbing behaviour continues to clean and aerate the eggs. At sites where submerged breeding substrates do not exist (or are not abundant), fathead minnows are known to excavate under stones at the bottom of the water body (Gale & Buynak, 1982). Males can spawn with multiple females producing large nests of up to 12,000 eggs (Gale & Buynak, 1982; Markus, 1934). Females will spawn multiple times per season (as many as 26 times) with an average clutch size of around 400-500 eggs, although this number is highly variable between females (Gale & Buynak, 1982). Fecundity of females has been reported at over 10,000 eggs during a season, but averaged around 8,600 in a study conducted by Gale & Buynak, (1982). Although clutch size was not calculated for this study, fathead minnows seemed to lay eggs consistently throughout June, July and August in dense patches on the introduced substrates (Appendix 2, Figure 4). Through observation of these substrates and other natural surfaces, egg deposits were successful and consistent in treatment reservoirs.

Even within tolerance ranges, temperature can have a significant influence on fathead minnow populations. The temperature range in which fathead minnows will engage in spawning activity is between 18-30 °C (Smith, Schreck, & Maughan, 1978). The treatment reservoirs included in this study all displayed temperature readings within this range, which coincides with breeding efforts being witnessed across all sites that overwintered and displayed continued survival. However, temperature also has an effect on growth which may impact the rate at which fry mature and can engage in courtship. Fathead minnows raised at colder temperatures ( $\geq 21$  °C) grow slower than those raised at warmer, more optimal temperatures (around 25 °C) (Denny, 1987; Smith et al., 1978). Slower growth rates in colder

environments may cause fry to fail at reaching spawning condition by the end of one year, impacting population growth potential. Under ideal temperatures, fathead minnows may reach spawning condition in as little as 4 months (Smith et al., 1978). Although dissolved oxygen levels across study sites were within the tolerable range, levels on the low end of the scale also have the potential to impact population growth. The absolute minimum level for dissolved oxygen is suggested to be 1 mg/L, at which point no spawning will occur and survival is reduced (Brungs, 1971; Denny, 1987; Wares & Igram, 1979). At a concentration of 2mg/L, egg production is significantly reduced in females. While minnow fry survival decreases at 4mg/L, growth rates of minnow fry can be significantly reduced at all concentrations below 7.9 mg/L lengthening the time it takes for fry to reach spawning condition and for fertilized eggs to hatch (Brungs, 1971; Smith et al., 1978). A study by Mount (1973) revealed that although lower pH concentrations did not always affect survival, it did however have potential to cause detrimental impacts on growth rates and reproduction. For example, at pH 4.5 and 5.2, fish behaviour became abnormal and individuals were found deformed. At a pH of less than 5.9, egg production and egg hatchability were reduced with all eggs being abnormal. As seen in my results, all sites had pH levels within the tolerable range suggesting it did not have a negative effect on survival, density or reproduction in these environments.

## **5.5 Conclusions**

With the exception of very small water bodies ( $\leq 0.02\text{ha}$ ,  $< 0.3\text{m}$ ), rural cattle reservoirs in northeast Wyoming seem to have the appropriate environmental conditions to support viable populations of fathead minnows. Surface area and maximum depth were selected as the two key features of reservoir morphology affecting minnow survival and relative densities within these environments. Fathead minnows survived in all reservoirs that held water between all three years of the study. Populations were able to successfully overwinter at these sites. The only cause of total die-off witnessed in the study was either complete drawdown between June and August, or complete freeze-

over during the winter months. Reproduction was witnessed throughout the entire season (June-August) in reservoirs which overwintered and held water throughout this time period. Out of the reservoirs that overwintered between study years, all experienced increasing relative densities with the exception of one site (T2). This reduction in relative density was linked to extreme drawdown during the 2015 season, which may be seen as the primary cause for population declines in these environments. None of the water quality indicators analyzed had a significant or detectable influence on survival or relative densities of minnow populations. Although factors such as temperature, pH, dissolved oxygen and various nutrient levels can influence population viability, the ranges detected in these reservoir environments were all within tolerable levels. Extreme conditions at which point survival or reproduction would be affected in any critical way were not detected for any of the parameters.

The life history traits of the fathead minnow make them an ideal species for population establishment in tough environments. With consideration given to the key features above, many rural cattle reservoirs in northeast Wyoming host the appropriate environmental conditions to establish and support sustainable populations of fathead minnows. However, in order for fathead minnows to be implemented as a sustainable control option, economic costs must be considered alongside their efficacy. Given the low direct cost of stocking fathead minnows in cattle reservoirs, an in depth cost analysis and comparison to the use of larvacide pucks and spraying should be done. This should be done with consideration to both single year, and multi-year survival of fathead minnows.

## **6 Overall Conclusions and Future Research**

This study evaluates and proves the efficacy of using fathead minnows for mosquito control in northeast Wyoming. Fathead minnows demonstrate the ability to reduce mosquito larvae densities at treatment sites through predation. Fathead minnows also suppress temporal variation in larvae abundance, which suggests that fathead minnows may be selecting mosquito larvae as a primary food source when larvae densities are high and more available. As fathead minnows are opportunistic feeders, environmental characteristics can influence feeding behaviour and opportunities. Stable isotope data indicated that fathead minnows shift towards feeding in the pelagic zones when reservoirs are extremely deep, and/or possess thick littoral zone vegetation cover. Limited access to the near-shore littoral zone likely shifted feeding efforts to the deeper waters of the pelagic zone, or at the surface. As a result, the efficacy of using fathead minnows for mosquito control is reduced when emergent/submerged vegetation becomes prohibitive of successful searches for larvae in the littoral zone. Their efficacy as a control agent may also be reduced by extreme reservoir depth, as this tends to shorten the littoral zone area and provides more feeding opportunity in the pelagic zone.

The reservoirs included in this study host the appropriate environmental conditions to support viable populations of fathead minnows. Surface area and maximum depth were noted as the two key features of reservoir morphology affecting minnow survival and relative densities within these environments. Other aspects of pond morphology such as vegetation coverage and perimeter distance did not prove to influence densities or survival. Complete die-off within a single year only resulted from total drawdown or complete freezing over the winter. None of the water quality indicators analyzed had a significant or detectable influence on survival or relative densities of minnow populations, suggesting that the parameter ranges witnessed within these environments were all within acceptable limits for fathead minnow population viability.



The life history traits of the fathead minnow make them an ideal species for population establishment in tough environments. With consideration given to the key features above, many rural cattle reservoirs in northeast Wyoming host the appropriate environmental conditions to establish and support sustainable populations of fathead minnows. This study has demonstrated and proven the fathead minnow's ability to suppress mosquito population within treated reservoirs. Combined with appropriate site selection, the introduction of fathead minnows can make a positive contribution to mosquito control efforts in northeast Wyoming in the hopes of reducing the risk of WNV infection and the significant impacts it can have on sage-grouse population persistence.

At a local level, more detailed isotope analyses would be useful in providing further insight towards dietary preferences and prey consumption. This would be beneficial in determining the direct impacts fathead minnows have on mosquito life cycles and population growth potential. Reservoir design and management becomes an important determinant of the fathead minnow's success towards mosquito predation, and should be considered when introducing minnows for control or future studies. Further research is needed to determine the utility of fathead minnows as a biological control agent at the landscape level. Integration with other potential management options may create more optimal outcomes for widespread control of mosquito populations and WNV. Regardless of project size, economic costs must also be considered alongside fathead minnow efficacy. The low direct cost of stocking fathead minnows in cattle reservoirs would however promote their implementation as a wide-spread control option. An in-depth cost analysis needs to be conducted to compare fathead minnows to the use of larvacide pucks and aerial spraying. This should be done with consideration to both single year, and multi-year survival of fathead minnows.

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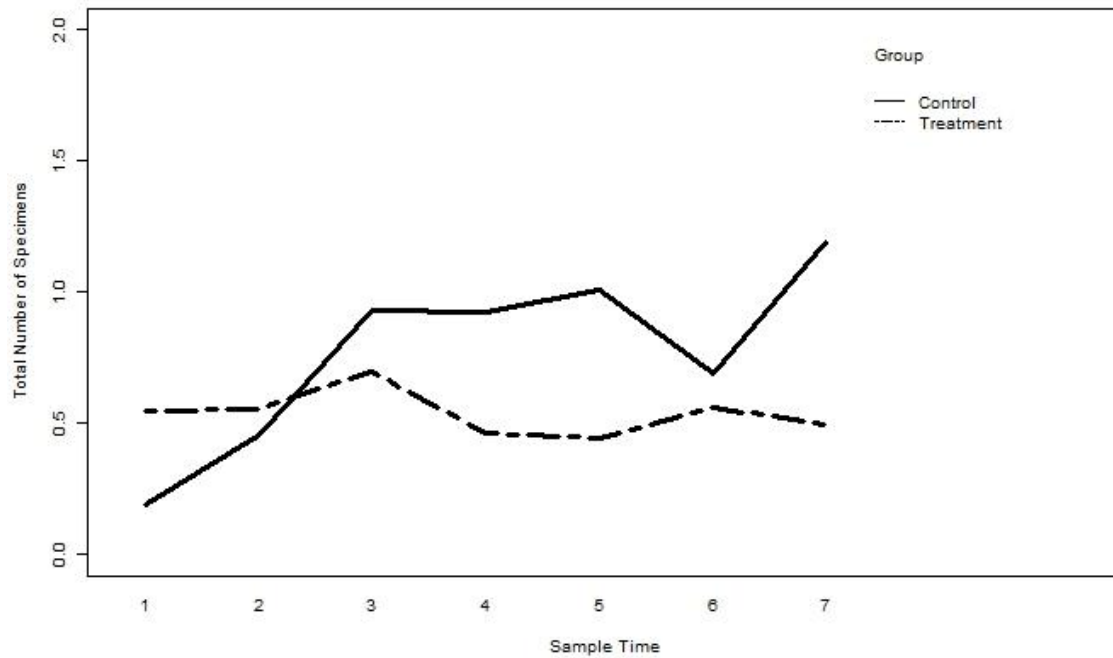
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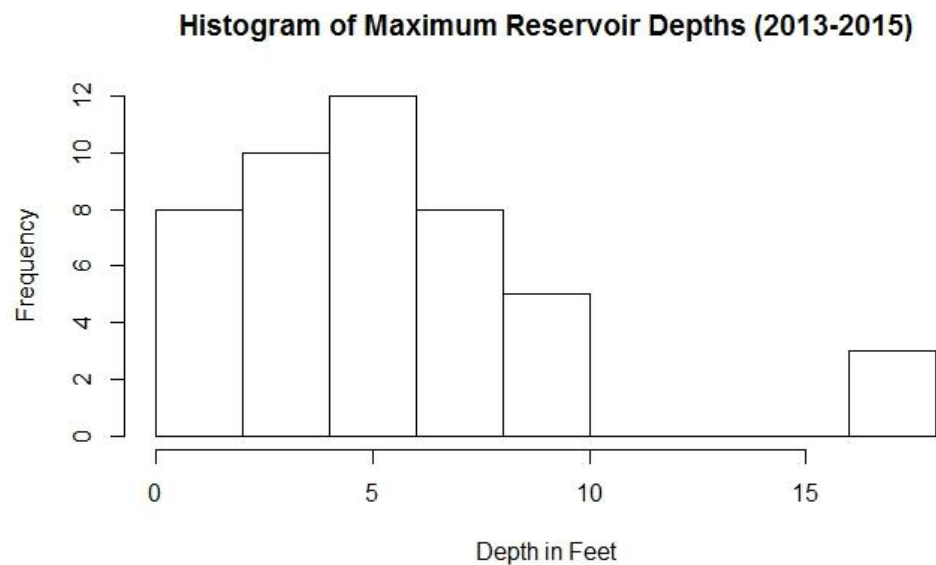
## Appendix 1



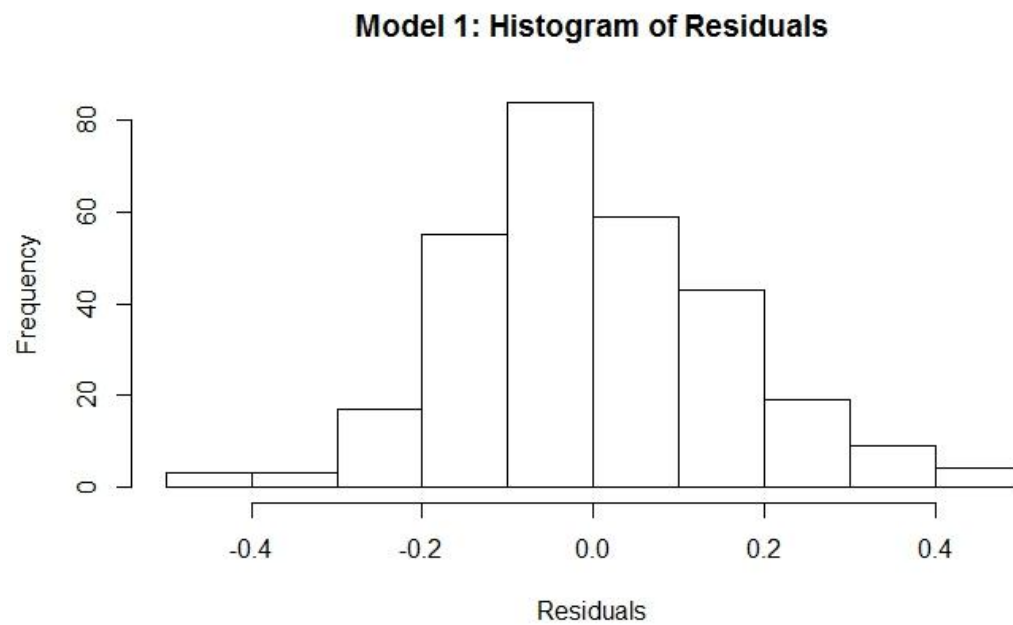
**Figure 1:** Image of adult mosquito night traps used to collect tissue samples around treatment and control reservoirs during the 2013 field season. Night traps utilized a dry ice container which gradually released CO<sub>2</sub> into the immediate atmosphere as an attractant to adult mosquitoes. Individuals drawn in to the trap were blown by an electric fan into a sampling net.



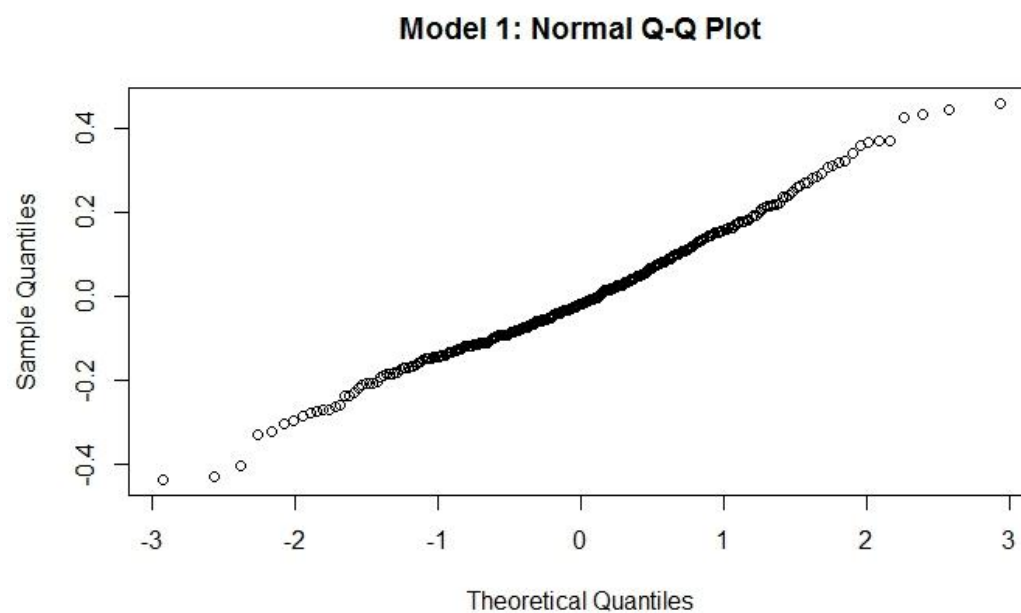
**Figure 2:** Interaction plot of average specimens per-dip (densities) from a combined 2013-2015 data set. A specimen count is derived from the number of individuals counted in a sample dip (either live larva, or exoskeletons), using a 350ml dip cup. Sample times ran from June 17 to August 20 and correspond to approximately 1 week intervals during sample times 1 through 7.



**Figure 3:** Histogram of maximum reservoir depths in feet over all three field seasons. As indicated by frequencies, T8 had an extremely high maximum depth suggestive that it may be seen as an outlier.

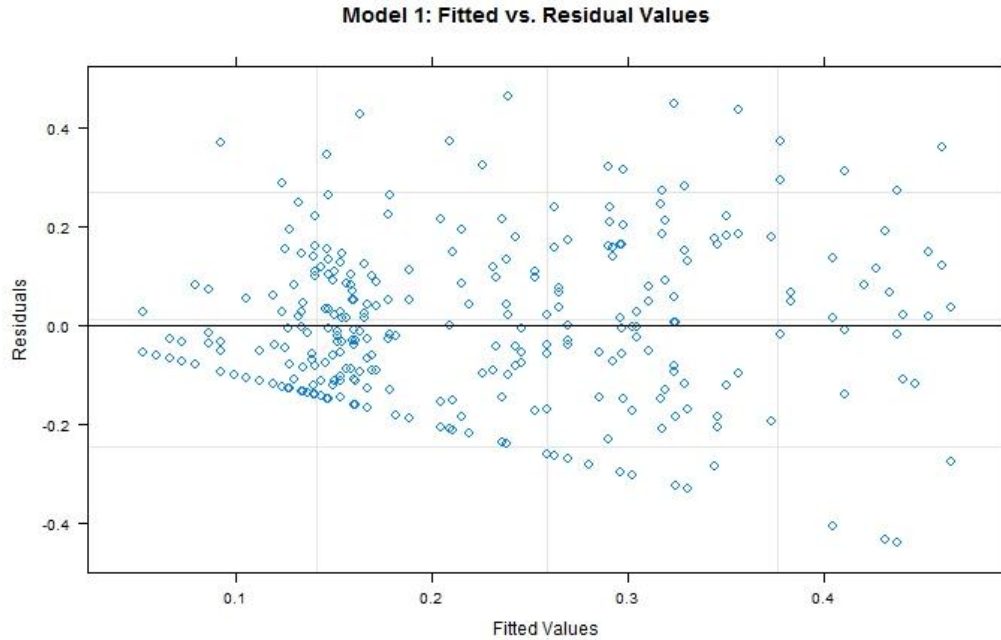


**Figure 4:** Histogram of residual values in the top model (Model 1) for model validation.



**Figure 5:** Sample and theoretical quantiles against a normal distribution indicating normally distributed residuals for the top model (Model 1).



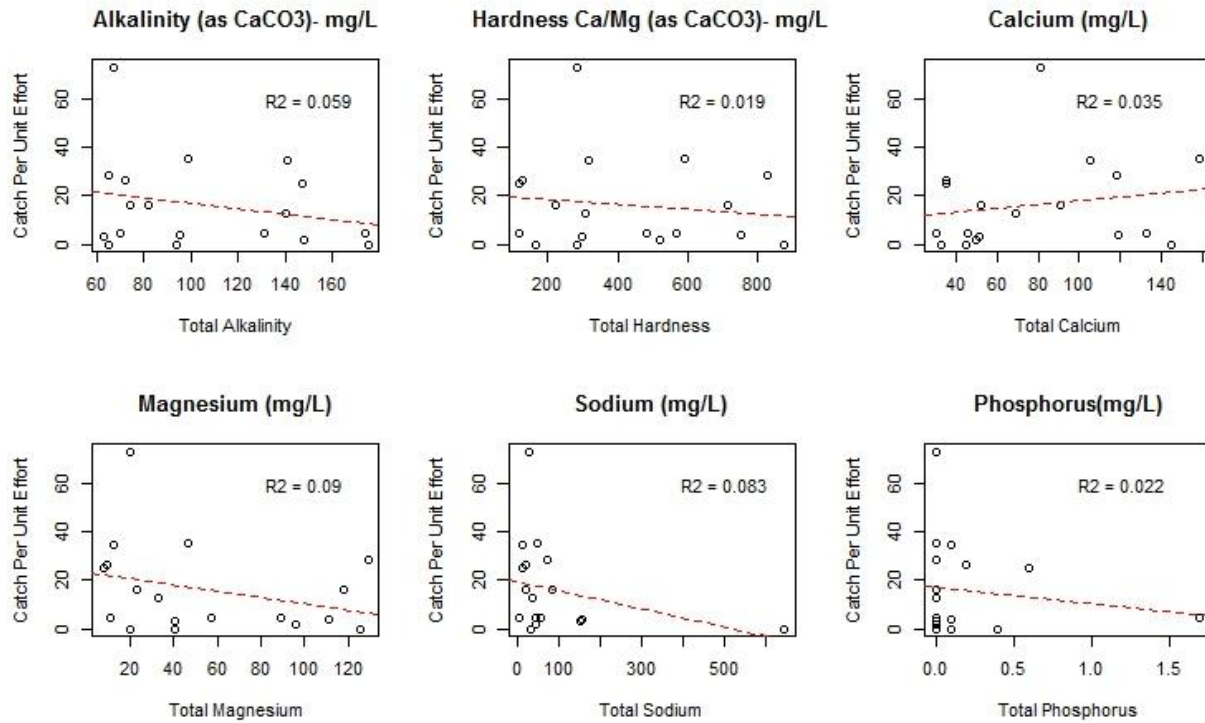


**Figure 6:** Fitted vs residual values in the top model (Model 1) indicating some non-constant variance; however the absolute value of the residuals is strongly positively correlated with the fitted values in the top model.

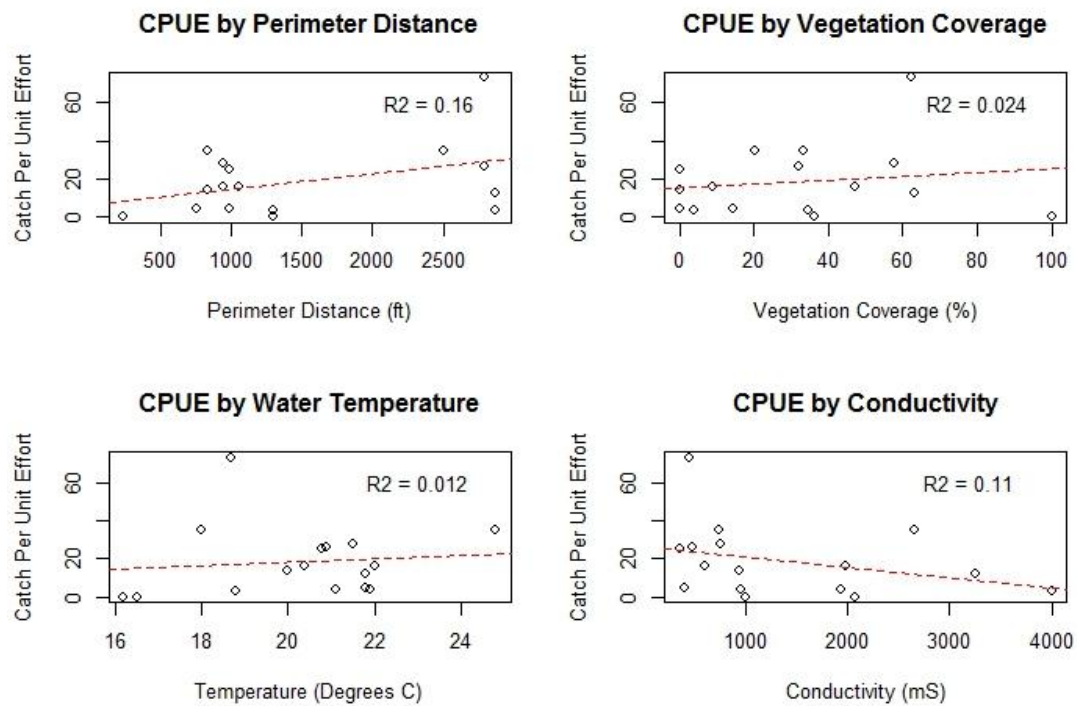


**Figure 7:** Picture of a treatment reservoir displaying heavy cattle use and disturbance. Daily use by cattle and other animals greatly increases turbidity and nutrient levels within these reservoirs. As a result, introduction of fathead minnows is not viewed as an additional concern regarding these factors.

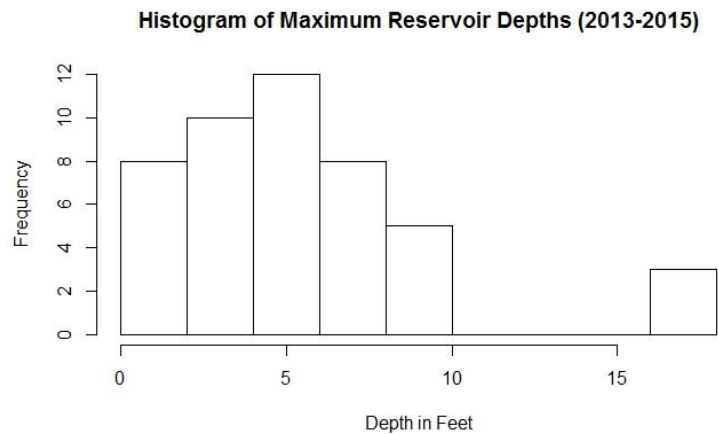
## Appendix 2



**Figure 1:** Standard X-Y plots including regression curves with  $R^2$  values for all lab evaluated water quality indicators included in the study that had detectable levels across the majority of sites. Water quality indicators that did not present detectable levels in study sites were removed from the analysis.



**Figure 2:** Standard X-Y plots including regression curves with  $R^2$  values for pond morphology indicators and in field readings not included in the top model.



**Figure 3:** Histogram of maximum reservoir depths in feet over all three field seasons. As indicated by frequencies, T8 had an extremely high maximum depth suggestive that it may be seen as an outlier.



**Figure 4:** Photograph of eggs laid on artificial breeding substrate. Eggs were deposited on these surfaces throughout the season, indicating constant breeding activity during summer months.