Field Monitoring, Transmission and Influences of Immunosuppression on Ranaviral Infections in Native North American Amphibian Species

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Abstract

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Amphibian species are in decline around the globe and infectious diseases are thought to be a large contributor to these declines. The Ranaviruses, a group of viruses belonging to the Iridoviruses, are emerging infectious diseases in amphibian populations. The Ranavirus has been associated with mass mortality events in ranid tadpoles in Ontario. This thesis examines the progression of Ranaviral infections in the wild in three aquatic amphibian communities. It is the first study to examine the presence of Ranaviral infections in multiple amphibian species in the same physical space. The mode of transmission of frog virus 3, a Ranavirus, is also examined in wood frog (*Rana sylvatica*) tadpoles. It was found that both vertical and horizontal transmission of a Ranavirus could occur.

The effect of exposure to both frog virus 3 and an immunosuppressant pesticide, Malathion, was examined in both wood frog and bullfrog (*Rana catesbeiana*) tadpoles. This study supports the immunosuppression hypothesis. When assessing the mortality, as well as the abnormal behaviour data, it appears that the effects of combined exposure to an immunosuppressant and an infectious disease was more severe than in either treatment alone. From the field data, the information about the mode of transmission, and the immunosuppression data, a model of viral transmission for the Ranavirus in aquatic amphibian communities is proposed.

Key Words: Ranavirus, amphibians, field monitoring, transmission

Preface

This thesis has been written in chapters in manuscript format that will be adapted into publications. Chapter one is a literature review that examines the causes of amphibian declines, the Iridoviruses, amphibian immunology, the natural history of wood frogs (*Rana sylvatica*) and bullfrogs (*Rana catesbeiana*). Chapters two through four are written for development into possible publications. Chapter five is the development of a model of transmission for the Ranavirus which will be included in a publication

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Chapter 1: General Introduction

Amphibian Declines

It is now an accepted fact that amphibian species around the world are in decline (Stuart *et al.* 2004). Although there are numerous potential reasons for these declines many, if not all, are caused by direct or indirect interference by humans. These reasons or factors include toxic substances in the environment, disease, and interactions between these factors (Alford and Richard 1999). Emerging infectious diseases, including *Batrachochytrium dendrobatidis*, the chytrid fungus (Carey *et al.* 1999; Pounds *et al.* 2006) and Iridoviruses (Carey *et al.* 1999; Chinchar 2002) are thought to be large contributors to amphibian declines. Viral emerging infectious diseases, such as the infections produced by Iridoviruses have not been researched to the extent that the chytrid fungus has been, even though the total distribution of the Iridoviruses pathogens is unknown. Many other aspects of the Iridoviruses, including their biology are unknown, such as the interactions with naturally occurring stressors (e.g. desiccation of ponds), or anthropogenic stressors (e.g. pesticide applications).

Environmental Toxins

Amphibian populations tend to be less dense in highly farmed areas. An example of this occurs in the Holland Marsh in Ontario (Bishop *et al*. 1997). Reduced anuran species diversity was found in the Holland Marsh, along with reduced hatching rates and developmental delays. These changes have been attributed to nutrient rich run-off from the agricultural fields. Organophosphorus pesticide levels, phosphate levels, nitrogen levels, and total ammonia levels, as well as the biological oxygen demand were measured

leading to the possibility of multiple interactions between the compounds/chemicals. It is likely that nitrates from the fertilizers probably are not responsible for the developmental problems (de Wijer *et al.* 2003) that Bishop *et al.* (1997) observed. Both pesticides and herbicides are likely to be the most harmful form of agricultural/anti-pest pollution. They are widely used to control a variety of organisms that threaten to reduce the profits from harvested crops. Chemicals such as organochlorine pesticides (e.g. DDT and DDE), and polychlorinated biphenols (PCBs) were once widely used (Russell *et al*. 1995; Clark *et al.* 1998).

Organochlorine pesticides act as endocrine disruptors, which affect the sexual development of amphibian larvae. For example, DDT (dichlorodiphenyltrichloroethane) negatively interacts with estrogen in females and causes a reduced developmental rate of the gonads (Clark *et al.* 1998). DDT is no longer used as a pesticide in North America, but because of bioaccumulation in food chains, it is still found in North America (Russell *et al*. 1995). DDT is considered to be one of the main contributing factors in the local extinction of the Blanchard's cricket frog (*Acris crepitans*), the eastern grey treefrog (*Hyla versicolor*), the bullfrog (*Rana catesbeiana*) and Fowler's toad *(Bufo fowleri*) from Point Pelee, Ontario (Russell *et al*. 1995). As recently as 1995, residues of DDT have been found in spring peepers (*Pseudacris crucifer*) in the Point Pelee area (Russell *et al*. 1995). The DDT problem is slowly going away in North America, but the damage that this pesticide and other organochlorine compounds have caused may be permanent in some areas and within some species. DDT is still used in some undeveloped countries around the globe.

Polychlorinated biphenols (PCBs) are another group of organochlorine pesticides that have left a scar on amphibians. Exposure to PCBs result in increased time required to reach metamorphic climax, increased mortality and lower body mass (Gutelb *et al.* 2000). The effect of PCB exposure becomes more intense during long-term exposures, as would be the case with tadpoles when they burrow into the sediments and eat detritus in PCB contaminated sediments. PCBs interfere with the proper functioning of the thyroid hormone, which results in physical malformations. Interestingly, mortality due to PCB exposure is delayed in cases of prolonged exposure for several weeks when compared to acute exposure (Gutelb *et al.*1999). PCBs also accumulate in fatty tissues as well as in the environment (Glennemeier and Begnoche 2002). Ranid eggs have been found to be extremely sensitive to PCB residues from sediments (Glennemeier and Begnoche 2002). Also, animals that have been exposed to contaminated sediments carry PCB residues within their tissues. The legacy of the organochlorine pesticides in North America is one that has not yet been fully realized, however, the number of pesticides used keeps increasing as more modern pesticides are developed.

Embryos and tadpoles of *Rana sylvatica*, *R. pipiens*, *R. clamitans*, *Bufo americanus*, and embryos and larvae of *Ambystoma maculatum* showed behavioural changes when they were exposed to permethrin or fenvalerate (pyrethroid insecticides) (Berrill *et al.* 1993). Despite differences in the length of the exposure and the concentration of the pesticides, there was no significant mortality in any of the species observed. However, tadpoles and larvae showed abnormal behaviour that lasted longer for animals that were exposed to higher concentrations of the chemicals. The species of amphibian that was the most sensitive to pesticide exposure was *A. maculatum*, indicated

by the length of time that the larvae took to recover from exposure (Berrill *et al.* 1993). In a related experiment, embryos and tadpoles of *R. pipiens*, *R. clamitans* and *R. castbeiana* were exposed to different concentrations of either a pesticide or one of two herbicides which are commonly used in forests to control unwanted biomass (Berrill *et al.* 1994). The embryos and tadpoles were exposed to either: the pesticide fenitrothion, the organochlorine herbicide triclopyr, or the herbicide hexazione (Berrill *et al.* 1994). Exposure to fenitrothion did not affect the hatching success of any species when they were exposed as eggs. However, when tadpoles were exposed to fenitrothion at high concentrations they developed abnormal behaviour and either died or were paralyzed. The tadpoles that were exposed to lower concentrations took between one to three days to recover and regain normal behaviour, similar results were obtained with triclopyr exposures. Embryos exposed to triclopyr did not experience a reduction in hatching success, however, when tadpoles were exposed to higher concentrations they were either paralyzed or died. When tadpoles were exposed to hexazione, there were no effects observed in any of the species on either hatching rates or tadpoles. Species vary in their sensitivity to exposure. Bullfrogs appear to be the most sensitive species, green frogs are the second most sensitive species, and leopard frogs are the least sensitive of the species used (Berrill *et al.* 1994). This trend is interesting because bullfrogs and green frogs usually breed in more permanent water bodies, which would likely be larger than those used by leopard frogs and therefore have a larger dilution effect on chemicals applied. Leopard frogs tend to breed in vernal pools, which are not necessarily large or deep, reducing their ability to dilute any chemicals applied. It is likely that leopard frogs have

had to deal with higher concentrations of contaminants over many generations than either the bullfrogs or green frogs have had to, making them more resistant to contaminants.

Rana sylvatica, *R. pipiens*, *R. clamitans*, *R. catesbeiana, Bufo americanus*, *Ambystoma maculatum* tadpoles/larvae were used to assess the effects of both single and pulsed applications of fenitrothion (Berrill *et al.* 1995). There were differences in the sensitivity of the species to fenitrothion. Both green frogs and bullfrogs were more sensitive to exposure than either wood frogs or leopard frogs. Bullfrog and green frog tadpoles experienced effects of chemical exposure at lower concentrations than the other two ranid species. American toads did not show symptoms of exposure until high concentrations, however, tadpoles that did show symptoms rapidly recovered. Spotted–salamanders showed a similar reaction to exposure as the green frogs and bullfrogs did, and paralysis occurred at low pesticide concentrations. Wood frogs, leopard frogs, green frogs, bullfrogs, or American toads when exposed to fenitrothion twice, did not show a greater effect than if they had only been exposed once. However, the spotted salamander larvae were more sensitive to the second exposure, and the mortality rate increased in a concentration dependent manner. The increased sensitivity of bullfrog and green frog tadpoles to chemical exposure may be the result of their smaller sized eggs and their earlier developmental stage at hatching (Berrill *et al.* 1995). However, there are many other types of pesticides that are commonly used near aquatic amphibian habitats.

Rana sphenocephala, the southern leopard frog, tadpoles were exposed to carbaryl at several stages of development to determine the effects of exposure (Bridges 2000). Embryos, both in and out of the egg, and tadpoles were exposed to carbaryl. Neither the hatching rate nor embryo survival was affected by the presence of carbaryl.

The age at metamorphosis was not significantly affected by the presence of carbaryl, however the control treatment produced metamorphs sooner than any other treatment. Mass at metamorphosis was affected by the developmental stage at which the embryo/tadpole was exposed to carbaryl. When the tadpoles were exposed as an egg/early embryo, the metamorphs produced were significantly smaller than those produced from other treatments. Also, when tadpoles were chronically exposed to carbaryl significant mortality observed (Bridges 2000).

There are compounds in the environment other than organochlorine pesticides, that affect sexual development of amphibian larvae. These compounds are termed endocrine disrupting compounds (EDCs). When leopard frog (*Rana pipiens*) and wood frog (*R. sylvatica*) tadpoles were exposed to estrogen mimicing compounds (estradiol, ethylestradiol or nonylphenol) or estrogen inhibiting compounds (flavone or ICI 182780) at environmentally relevant concentrations, a significant alteration occurred in normal reproductive tissue development (MacKenzie *et al*. 2003). The abnormalities observed ranged from oocyte atresia in females to the complete feminization of males. There was also a difference in the susceptibility of the species: leopard frogs experienced a higher rate of sex reversal and inter-sexed gonads than wood frogs. Wood frogs frequently experienced deformed testicles, early germ cell maturation and oocyte atresia (MacKenzie *et al*. 2003). This indicates that the concentrations of EDCs which are commonly found in waste water can negatively affect gonad development and influence the future reproductive potential of a population of amphibians.

Another group of widely used herbicides, which is based on glyphosate, has the potential to negatively interact with tadpole development at environmentally relevant

concentrations. When *Rana clamitans*, *R. pipiens*, *R. sylvatica* and *Bufo americanus* tadpoles were exposed to environmentally relevant concentrations of glyphosate-based herbicides and its component parts, it was found that different species had different sensitivities to the formulations (Howe *et al.* 2004). POEA, polyethoxylated tallowamine surfactant, had the largest effect on the development of tadpoles. POEA caused high toxicity in green frog tadpoles. Leopard frog tadpoles, which were chronically exposed to POEA or a product containing this surfactant, showed a large degree of abnormality, including gonadal abnormalities. This indicates that there was a disruption in the process of gonadal development, likely caused by an interference with thyroid hormone receptors (Howe *et al.* 2004). Therefore, the use of surfactant chemicals such as POEA may cause long term damage to amphibian populations through decreases in population size as the result of reproductive failure, even though the herbicide itself, glyphosate, may be relatively harmless.

Exposure to pesticides or herbicides at many different stages of development in amphibians can cause mortality, alter life history traits and behavioural modification to both individual animals and populations of amphibians. The effect of pesticide exposure appears to be dependent on the developmental stage and species of the amphibian larvae. However, the effect of pesticide exposure at any stage of development is detrimental to amphibian larvae.

Disease

Infectious diseases are now recognized as a large contributing factor to amphibian declines on a global scale. The leading hypothesis to explain the apparent increase in the

effects of infectious diseases is associated with climate change (Harvell *et al.* 2002; Pounds *et al*. 2006). Pathogens are sensitive to a variety of environmental factors, the majority of which are influenced by temperature (Harvell *et al.* 2002). When the temperature is increased, as in global warming, pathogens benefit with increased survivorship, increased transmission rates and most importantly their hosts may become more susceptible. This situation may result in increases in the range of the pathogen, increased species of hosts and host species, and declines in the host populations which may result in extinction of the host (Harvell *et al.* 2002).

Anthropogenic environmental modifications are associated with the appearance of new emerging infectious diseases (Daszak *et al.* 2001). An emerging infectious disease has been defined as a disease caused by a pathogen which is currently increasing in geographical range, and/or the diversity of hosts, or which has recently evolved (Daszak *et al.* 2000). Agents responsible for emerging wildlife diseases in amphibian populations around the world include parasites, *Batrachochytrium dendrobatidis* (chytrid fungus), and the Iridoviruses.

Parasites

Parasites are often overlooked when it comes to emerging infectious diseases. In recent years, there has been a large increase in the number of limb deformities and other abnormalities that are found in anuran amphibian populations (Cohen 2001; Kiesecker *et al.* 2004). Perhaps the most infamous outbreak of anuran limb deformities occurred in Minnesota in 1995, in which thousands of frogs were deformed (Kiesecker *et al.* 2004). The infamous trematode, *Ribeiroia,* has been implicated in these frog deformities (Cohen

2001; Kiesecker *et al.* 2004; Johnson 2005). *Ribeiroia* are trematodes that have the ability to form metacerearіae, otherwise known as cysts (Cohen 2001; Kiesecker *et al.* 2004). These cysts embed themselves into the developing limb buds in the tadpoles and physically disrupt the development of the limbs (Cohen 2001). This results in mirror image limb duplications. Since the *Ribeiroia* use snails as one of their hosts, there is a correlation of limb deformities with the number of snails present (Johnson 2005).

Chytrid Fungus

The chytrid fungus has received more attention than parasites or other infectious agents. Chytrid fungus is an emerging infectious disease that may cause high mortality in amphibian populations. It has been implicated in the decline of many amphibian species (Rollins-Smith *et al.* 2002a,b; Hanselmann *et al.* 2003) and is currently recognized to be a pandemic amphibian pathogen, present on every continent except Asia (Weldon *et al.* 2004). With the global trade in amphibians, there is the potential of diseases, which were once isolated geographically, to spread around the world. This is thought to be what happened with the chytrid fungus: Weldon *et al.* (2004) examined specimens collected from 1871 to 2001 for the presence of chytrid fungus, and found that the first positive animal for the fungus occurred in a specimen of the African clawed frog, *Xenopus laevis* collected in 1938 in South Africa. In fact, the chytrid fungus was found to infect both *Xenopus laevis* and *Xenopus tropicalis*, (Family: Pipidae) two species of totally aquatic frogs which are both common in the pet trade and commonly used as model systems by researchers. The occurrence of the chytrid fungus in North America and Europe seems to be associated with the import of the *Xenopus* species (Weldon *et al.* 2004).

Chytrid fungus targets areas on the body of both adult and larval amphibians that contain keratin (Rollins-Smith *et al.* 2002a,b), and therefore it affects the outer layer of the epidermis and the mouthparts in larval amphibians. In larval anurans, the effects of a chytrid infection are visible to the naked eye, for example the mouthparts appear white and lack the normally black labial teeth which have been completely de-keratinized by the fungus (Fellers *et al*. 2001). However, in adult amphibians diagnosing a chytrid infection is more complicated and usually requires the use of histological and/or molecular techniques.

In North America, the first known chytrid infection was found in a preserved green frog (*Rana clamitans*) from Quebec, Canada collected in 1961 (Ouellette *et al.* 2005). Since this initial discovery of chytrid fungus in North America, there have been many other amphibian species that have been found to harbour chytrid fungus infections in natural populations. These afflicted species include the endangered mountain yellow-legged frog (*Rana mucosa*) (Fellers *et al.* 2001), tiger salamanders (*Ambystoma tigrinum stebbinis*) (Davidson *et al.* 2003), and boreal toads (*Bufo boreas boreas*) (Muths *et al.* 2003). However, the mechanism by which the chytrid fungus causes mortality remains unknown (Cohen 2001). Because a mechanism has not yet been elucidated for chytrid fungus to cause death, it may not be the main factor involved in population declines. It is highly likely that animals that are under stress from other factors are more susceptible to chytrid fungus infections. Chytrid infections may cause additional stress on the already weakened animal and contribute to mortality in that way. Burrowes *et al.* (2004) invoke chytrid infections as a mechanism of decline for the amphibians that are present in Puerto Rico but also describe many other possible reasons for the declines which have been

observed. Burrowes *et al*. (2004) neglected the fact that these factors could actually be interacting to result in the observed declines and they provide no argument for why chytrid fungus was singled out as a main contributor to the declines in Puerto Rico.

Recently, a hypothesis associating range expansion of chytrid fungus with global warming has been proposed based on both temperature and altitudinal patterns of chytrid fungus prevalence of the Harlequin frogs (*Atelopus* species) and their declines (Pounds *et al.* 2006). This group of species show maximum diversity at altitudes between 200m and 2400m and maximal species loss occurs at mid-altitudes. Temperatures at altitudes below 200m and above 2400m are thought to impede the growth and development of the chytrid fungus, therefore restricting it to mid-elevations. The temperatures that are experienced at mid-altitudes are thought to have increased through global warming. Other species of amphibians (including some *Atelopus* species) that are affected by the chytrid fungus and are outside of these optimal altitudes are spared from extinction even though they tend to have smaller species ranges (Pounds *et al.* 2006). Although this hypothesis may apply to amphibians in tropical regions, it does not necessarily explain chytrid associated die-offs that occur in temperate regions during the hottest time of year. However, it is possible that in temperate climates, the hottest temperatures experienced are similar to those experienced at mid-altitudes in the tropics.

The Iridoviruses

The Iridoviruses are a group of viruses that affect ectothermic vertebrates and invertebrates. The criteria used to define the viral family of the *Iridoviridae* are both morphological and structural. Iridoviruses are enclosed by an isohedral capsid 120 –

200nm in diameter and possess a lipid membrane, with linear double stranded DNA genomes containing 100 – 210kbp (Chinchar 2002). Within the *Iridoviridae* there are four genus designations: *Ranavirus*, *Lymphocystivirus*, *Iridovirus*, and *Chloridiovirus*. Ranaviruses and Lymphocystiviruses are groups of viruses that affect ectothermic vertebrates, whereas the Iridoviruses and Chloridioviruses affect invertebrates (Chinchar 2002). Therefore, in all cases when the term Iridovirus is used in this paper it refers to the viral family and not the genus unless otherwise specified.

The Lymphocystiviruses are only known to infect fish (Ahne *et al*. 1997; Chinchar 2002) and will not be discussed further because there is no known potential for these viruses to infect amphibians at this time. The Ranaviruses, however, infect amphibians, reptiles and fish (Ahne *et al*. 1997; Chinchar 2002), and will be the focus of further discussion. The Ranaviruses are distinguished from the rest of the *Iridoviridae* by several structural and clinical criteria. Structurally, the Ranaviruses encompass a large range of virion diameter, from 130nm (un-enveloped particle) to 160 – 200nm (an enveloped particle) (Chinchar 2002). The viral genome contains over 50% cytosine-guanine (CG) content and is highly methylated (Ahne *et al*. 1997; Chinchar 2002). On a clinical level, the Ranaviruses are distinct because they cause systemic infections with tissue necrosis and a large range of mortality (which in some cases can be 100%) (Chinchar 2002).

There is only a small amount of information available about Ranaviral infections in reptiles (Chinchar 2002). The most well documented cases of Ranaviral infections in reptiles actually occurred in the soft-shelled turtle (*Trionyx sinensis*) (Chen *et al.* 1999) and in Hermann's tortoises (*Testudo hermanni*) (Marschang *et al.*1999). The infections

were first noted as 'red neck disease' in farm raised turtles in China (Chen *et al.* 1999). The disease typically targeted small turtles and incurred a high mortality rate. Infections resulted in haemorrhages on the surface of the liver (Chen *et al.* 1999) as is found in other types of Ranaviral infections (Bollinger *et al.* 1999; Chinchar 2002: Docherty *et al.* 2003). The study by Chen *et al.* (1999) fulfilled Koch's postulates and used electron microscopy to morphologically identify the virus. However, it is not known if this virus can cross the species barrier and is not as of yet a concern in North American turtle populations. The Ranaviruses have been the cause of many amphibian morbidity and mortality events across North America (Green *et al*. 2002; Docherty *et al*. 2003; Greer *et al*. 2005).

The Iridovirus isolated by Marschang *et al.* (1999) is closely related to frog virus 3 (FV3). FV3 has been designated as the type species in the genus Ranavirus and infects a large number of amphibian species (Chinchar 2002; Green *et al*. 2002; Docherty *et al*. 2003). The pathology of the virus isolated is also similar to what occurs in an FV3 infection with gastro-intestinal ulcerations and hepatic necrosis (Marschang *et al.*1999). The virus that was isolated may be a threat to amphibian species because of its close relationship to FV3, but there has been no attempt made to ascertain this experimentally.

The Ranaviruses appear to predominantly affect fish and amphibians. Many of the Ranaviruses that affect fish are similar to FV3 (e.g. Mao *et al.* 1997 and Tamai *et al.* 1997). Within the Ranaviruses, it appears that some viruses may in fact be able to infect both amphibians and fish. For example, a virus was isolated from both the three–spined stickleback (*Gasterostelus aculeatus*) and sympatric tadpoles of the red-legged frog (*Rana aurora*) (Mao *et al*. 1999). The virus isolated from the sticklebacks was named the

stickleback virus (SBV), whereas the virus that was isolated from the red-legged frog tadpoles was called tadpole virus 2 (TV2). The viruses that were isolated were cultured in Fathead minnow cells, and further analysis was performed. When protein isolates, restriction length polymorphisms, and molecular characterization of the major capsid protein (MCP) were examined for both SBV and TV2, they were identical, indicating that, in fact, they were just different isolates of the same virus. Also, both of the viruses that were isolated are thought to be closely related to FV3 (Mao *et al*. 1999). Although it is likely that other Ranaviruses infect both fish and amphibians, there are no other studies that have examined this possibility.

The nature of the pathogen influences its ability to remain present in a population. When a pathogen is highly virulent and kills its host rapidly, like Ranaviral infections in some amphibians, it needs to be transmitted between hosts quickly to avoid the effects of illness on the host that would reduce the pathogen's ability to move from host to host (Day 2002; Day 2003). An evolutionary stable strategy for a pathogen involves trade-offs that optimize the rate of transmission as well as the time in which the pathogen can be transmitted between individuals (Day 2003). A successful pathogen should be highly virulent and have a long period where it can be transmitted from host to host. However, pathogens which are highly virulent and cause high mortality are likely to run out of hosts when the hosts are in short supply, as would be found in a community of amphibian larvae. When the pathogen has the ability to use an intermediate host (a vector of disease), which is not necessarily affected by the pathogen's presence, transmission of the disease is not necessarily impeded by the mortality that it causes in the primary host (Day 2002). Therefore, the pathogen may remain in a community even if its primary host has

been seasonally extirpated. Ranaviruses are thought to use intermediate hosts to persist in natural populations (Brunner *et al.* 2004). A community of amphibian species that vary in sensitivity to Ranavirus infection should provide enough alternative hosts to be a classic example of interspecies transmission of an infectious disease.

Infectious diseases likely contribute to amphibian declines on a larger scale than is currently recognized. Therefore, it is necessary to continue to research their effects on amphibian populations and their potential interactions with other stressors.

Interactions between Stressors

The study of how different stressors interact with one another to influence declines of amphibian populations is becoming an increasingly important avenue of research. Many stressors are naturally occurring, such as predation or crowding, whereas, others are anthropogenic, such as pesticides. Although it seems reasonable to expect that exposure to multiple stressors will have a greater effect than exposure to only one stressor, at this time few experiments have examined the interaction between such stressors.

One recent set of experiments has explored this question and is worth more detailed comment. Relyea (2004a) examined the effects of multiple pesticide exposure on the growth and survival of tadpoles of the anurans *Rana pipiens*, *R. catesbeiana*, *R. clamitans*, *Bufo americanus* and *Hyla versicolor*. These species were collected as eggs and were hatched in outdoor pools. In the exposure experiment, 1mg/L and 2mg/L treatments of each of carbaryl, diazinon, malathion and glyphosate were used as controls, but when the chemicals were paired to examine their effects, only 1mg/L of each

pesticide was used. A significant decrease in growth occurred in all species when tadpoles were exposed to two pesticides in combination. As well, in four of five species mortality was greater when exposed to paired pesticides (Relyea 2004a).

The mass of the tadpoles at the end of the experiment was also used to indicate the effects of multiple pesticide exposure (Relyea 2004a). The tadpoles were fed a strict ration of food each day. This small ration of food may not have been enough for all of the different species of tadpoles that were used, especially since the amount of food given did not change over the course of the experiment. Tadpoles are exceptionally voracious feeders, different stages and different species require different amounts of food, and there is a large amount of competition for food. This may result in a density-dependent effect, where in treatments with lower tadpole density more food is available per tadpole leading to increased growth and development. Therefore, the mass of the tadpoles is likely also to be affected by the amount of food that the tadpoles were able to obtain, as well as the tadpole's own competitive ability, and not necessarily by the presence of a pesticide.

The same lab has attempted to assess the interactive effects of 'predation' stress and the presence of a pesticide on amphibian tadpoles. The study assessed the effect of the presence of the predatory salamander, *Ambystoma maculatum*, and exposure to the pesticide carbaryl on grey tree frog (*Hyla versicolor*) tadpoles (Relyea and Mills 2001). The salamander larvae were kept in cages within the tanks where the tadpoles were held. Water chemistry was only taken once over the entire 16 day experiment and was not reported. Relyea and Mills (2001) base many observations on the activity levels of the tadpoles, but they never quantify the scale that was used to make these observations making statistical analysis unreliable.

Furthermore, when only carbaryl was present there were large reductions in tadpole survivorship, especially at the end of the experimental period, regardless of predator presence (Relyea and Mills 2001) and the severity of the decline in survivorship appears to be more dependent on the concentration of carbaryl used than on the presence of a predator. Only at low levels of carbaryl, was survivorship of tadpoles significantly less when a predator was present than when the predator was absent. However, because the declines in survivorship first appear after the tenth day of the experiment, the lower dissolved oxygen levels found in the carbaryl treatments may have confounded the results, especially since it does not appear that any attempt was made to regulate oxygen levels in the water. No effort was made to quantify the effects of the pesticide exposure on the predator, and if the predator died over the course of the treatment, it was simply replaced by another (Relyea and Mills 2001). Dead predators indicate that there was a problem in the experiment, perhaps the result of poor water quality.

The second predator/pesticide interaction study by the Relyea lab involved six different anuran tadpole species (*Rana pipiens*, *R. sylvatica*, *R. catesbeiana*, *R. clamitans*, *Bufo americanus* and *Hyla versicolor*), five different concentrations of malathion, and the predator in this case was *Notophthalmus viridescens*, the eastern spotted newt (Relyea 2004b). The newts were housed in cages and were fed tadpoles every second day (Relyea 2004b). In only one species (*Hyla versicolor*) and at only one treatment (5mg/L malathion) was any significant difference between the 'predator present' and the 'predator absent' treatments found. The analysis excludes a large body of information that explains the effects of malathion on amphibians (e.g. Christin *et al*. 2003; Gilbertson *et al.* 2003).

Assuming the evidence of interaction reported is a result of that interaction, other factors could be involved. The pesticides used, many of which are organophosphorus pesticides (e.g. carbaryl and malathion), are known to be immunosuppressants (Cooper and Parrinello 1996; Galloway and Handy 2003). As well, there is a possibility that the reductions in survivorship observed are the result of infections passed between the predator and the prey. In both studies the predator species are known in field studies elsewhere to be infected with or carriers of Iridoviruses and the anuran species used are susceptible to Iridoviruses (Bollinger *et al.* 1999; Green *et al.* 2002; Docherty *et al.* 2003). Without the elimination of disease as a potential cause of the reduction in survivorship, the results from Relyea (2004b) or Relyea and Mills (2001) remain unconfirmed. Until more complete studies are done to examine the interaction between a predator and a pesticide stress, no conclusions about the existence of interactive or synergistic effects can be made.

Fortunately, there are more convincing experiments which examine the interactions between different stressors. A notable experiment, done in ponds, using southern leopard frog (*Rana sphenocephala*) tadpoles, examined the interactive effects of UV-B radiation and an organophosphorus pesticide and showed unexpectedly that the combination of the UV-B radiation and carbaryl did not increase the toxic effects of the carbaryl (Bridges and Boone 2003).

Three further studies examine the interaction of pesticide and parasite or pathogen exposure. The first study examined the interaction between UV-B radiation and the fungus, *Saprolegnia* on hatching rates of two species of ranids (Kiesecker and Blaustein

1995). When the eggs were exposed *in situ* to both the fungi and natural UV-B radiation, the hatching rates were significantly reduced when compared to either treatment alone.

The second experiment that examined the effects of pesticides and a parasite, looked at a combination of pesticides commonly found in surface water and *Rhadbias ranae*, a nematode (Christin *et al.* 2003). Leopard frog (*Rana pipiens*) tadpoles were collected from ponds free of any agricultural pesticides and were raised in the lab until metamorphosis. After the tadpoles had metamorphosed, they were exposed to the following cocktail of pesticides in their water for 21 days: atrazine (21μg/L); metribuzin (0.56 μg/L); aldicarb (17 μg/L); deldrin (15 ng/L); endosulfan (0.02 ng/L); and lindane (0.33 ng/L). After the 21 day exposure, half of the metamorphs were euthanized to examine different immunological parameters, e.g. T-cell proliferation. The other half of the frogs were exposed to 30 nematode larvae for 24 hours, then transferred to clean water. The frogs were then kept for 21 days and their feces were examined to determine the amount of time required for the nematode infection to become established and begin reproducing. After 21 days, the frogs were then euthanized, the nematode infection was quantified and immunological parameters such as T-cell proliferation were measured. Animals that were exposed only to the pesticide cocktail showed a significant decrease in T-cell proliferation when compared to the control frogs. T-cells are important in the immune response to parasites which reside outside of cells: when T-cells proliferate at low rates, the activation of B-cells is reduced and therefore antibody production is also reduced, which would facilitate proliferation of disease. The infection severity increased with the concentration of pesticide cocktail. After exposure to the highest concentration of pesticides for 21 days, leopard frogs showed a decrease in their ability to react to

infections and had the highest nematode infection rates (Christin *et al.* 2003). Thus, exposure to pesticides increased the likelihood and the severity of nematode infections.

The third study examined the effects of exposure to *Ambystoma tigrinum* virus (ATV) and atrazine in the long-toed salamander (*Ambystoma macrodactylum*). Salamander larvae, aged six weeks, were exposed to environmentally relevant concentrations of atrazine and ATV (Forson and Storfer 2005). The salamander larvae that were exposed to atrazine alone did not develop ATV infections. However, larvae that were exposed to both stressors had reduced mortality and ATV infection rates when compared to the control treatments of ATV only and reduced mortality when compared to atrazine exposure alone. Atrazine at higher environmentally relevant concentrations accelerated the transformation of the salamanders and resulted in smaller metamorphs. However, the ATV infection rates in the salamanders were lower than expected and this has been attributed to the fact that the long-toed salamander is not the regular host–species of ATV which reduced the virulence of the virus. The interaction of ATV and atrazine may be beneficial for long-toed salamanders, because the presence of atrazine appears to reduce the infectivity of ATV, but atrazine does promote the prevalence of traits that have the potential to reduce fitness in adults (Forson and Storfer 2005). Therefore, even though the initial interaction of a pesticide and a pathogen may be positive, the long term effects of exposure may in fact be detrimental.

These studies indicate that anthropogenically caused stressors have the potential to negatively interact with naturally occurring or other anthropogenic stressors. However, more research is needed to determine the nature and the extent of interactions between

stressors before definite conclusions can be made about the effects of more than one stressor.

The Amphibian Immune System

In amphibians, there are two branches of the immune system that are similar to those of other vertebrates. However, the immune system of amphibians, unlike that of other vertebrates, has two distinct phases, the larval and the adult stages, where the immune system differs in both composition and function.

The Innate Immune System

The innate immune system is involved with the cellular response to a pathogen and is often termed the cellular response system. In many ways the innate immune system is the first line of defence against infections. This system is non-specific, reacts rapidly to infection and continues to function even after the adaptive immune system develops a response against the pathogen (Rollins–Smith and Smits 2005). The innate immune system is primarily comprised of phagocytic cells that interact with the chemicals which are produced by tissues (e.g. complement, a chemical which increases the number of cells responding to the infection as well as many other functions) and/or chemicals that are produced by the pathogen itself (e.g. the waste products which are produced by bacteria) (Parham 2005; Rollins–Smith and Smits 2005).

The way in which the innate immune system responds to a viral infection is mediated through chemicals produced by infected cells (Parham 2005), since the viral particles on their own do not produce any form of chemical while in the blood stream.

Tissues and cells that are infected by a virus produce and secrete a chemical called interferon. Interferon serves two purposes in the innate immune system: it interferes with the viral replication process, slowing the production of viral particles (Parham 2005) and it signals to the Natural Killer cells of the immune system that an infection exists (Parham 2005; Rollins–Smith and Smits 2005). The Natural Killer cells are important in amphibians as they respond to virally infected cells (Rollins-Smith and Smits 2005).

The Adaptive Immune System

The adaptive immune system is the primary system involved in dealing with viral infections after an initial response from the innate immune system. The adaptive immune system is made up of four principal components: immunoglobulins, T-cell receptors, B-lymphocytes and T-lymphocytes (Parham 2005). Immunoglobulins and T-cell receptors bind to antigens with a specific conformation and are therefore said to be antigen specific. The diversity of immunoglobulins and T-cell receptors present that is primarily due to the genetic make up of the individual. Therefore, an individual with more genetic diversity (or heterozygosity) at the loci which code for the immunoglobulins and the T-cell receptors will produce more varied end products. This genetic variability is enhanced by genetic re-arrangements. B–lymphocytes and T–lymphocytes are two fundamentally important cell types in the adaptive immune response. The actions of B- and T-lymphocytes are quite different. B–cells secrete antibodies that bind to specific pathogens or antigens (Parham 2005).

T–cells operate in conjunction with the major histocompatibility complexes (MHC), which are present on the surfaces of plasma membranes of cells (Parham 2005).

MHC molecules present portions of proteins from foreign particles to which the T-cells can bind (Parham 2005). B-cells interact directly with the pathogen through antibodies, whereas T-cells interact with the pathogen through cells via the presentation of foreign peptides by MHC on the cell membranes.

MHC molecules are important in the immune response of T-cells. MHC molecules belong to one of two classes: MHC I or MHC II. MHC I molecules are responsible for the presentation of protein particles from pathogens which replicate within the cells, such as viruses (Parham 2005). MHC I particles interact with cytotoxic T-cells which eliminate cells infected with (i.e. presenting) foreign protein particles. MHC I proteins are expressed in almost every tissue type, because all nucleated cells have the potential to become virally infected. MHC II molecules, on the other hand, have a more complex action. MHC II molecules present peptides from pathogens that originate in the extracellular space. These peptides are taken into T-cells via phagocytosis and are then presented by the MHC II molecules. MHC II molecules interact with Helper T-cells, which are cells that only interact with B-cells and phagocytic cells in the immune system. After the helper T-cell has been activated by its interaction with the MHC II molecule presenting a foreign peptide, it begins to secrete cytokines that activate B-cells and/or macrophages and T-cells (Parham 2005).

The Adaptive Immune Response to Infection

When an individual is exposed to a pathogen for the first time, a primary immune response is triggered. A primary immune response activates both T- and B-cells that have not yet been exposed to the specific pathogen/antigen that its receptors are for

(Parham 2005). The T-cells are mobilized from the thymus and the B-cells are mobilized from the bone marrow. T-cells have the ability to respond to cytokines that are produced by the innate immune response. The activation of the T-cells results in one of two responses: a continuation of the inflammatory response that was initiated by the innate immune response or an antibody mediated response (Parham 2005).

The antibody mediated response primarily occurs in the lymph tissues and takes seven to ten days to develop in mammals (Parham 2005). In the early stages of infection, macrophages and other cells in the lymph present peptides of the pathogen through MHC molecules on the cell surface. Both B- and T-cells are present in the lymph system. B-cells aggregate in the lymph nodes and T-cells circulate in the lymph. The T-cells secrete cytokines that trigger the B-cells to both proliferate and differentiate (Parham 2005).

B-cells go through several phases in an immune response. In the beginning of the immune response, some B-cells are present in the lymph organs and produce the first wave of antigen specific antibodies (Parham 2005). These B-cells act as temporary plasma cells. In the spleen, B-cells proliferate rapidly and in three to five days differentiate into antibody secreting plasma cells. Another group of B-cells form germinal centers in the lymph tissues, where they undergo somatic hypermutation to create a large variety of antibody products. As the infection progresses, the antibody that is the most effective against the pathogen is selected for and the cells which do not produce these antibodies are eliminated. The B-cells that produce the most effective antibodies against the pathogen then produce the second wave of antibodies (Parham 2005). After the infection has been terminated, an immunological memory is developed. This protects that individual from future infections with the same pathogen.

The Amphibian Immune System

Although the amphibian immune system is similar to that of mammals, the immune systems of larval and adult amphibians are different from each other, as are the immune responses that are produced (Flajnik 1996; Rollins-Smith 1998). As the limb-buds of the larva begin to develop, the thymus and spleen develop characteristics and functions of the lymph system (Rollins-Smith 1998). During larval development the lymphocytes proliferate rapidly, but at metamorphic climax, the levels of lymphocytes recede. This decline in the lymphocyte population may provide some of the energy necessary for metamorphosis. The lymphocytes in anuran amphibians can be divided into two distinct classes, pre- and post-metamorphic, which may be indicative of the physical changes between larval and adult amphibians. However, there is an immunological memory that is transferred from the larval stage to the adult stage. These changes in the lymphocyte populations between larval and adult anurans may be the result of the hormonal mechanisms that are responsible for metamorphosis. Some of the hormones which control metamorphosis act as triggers for apoptosis in mammalian lymphocytes and a similar situation is thought to occur in the case of anuran amphibians. The group of hormones responsible for apoptosis induction in anuran amphibians is thought to be the corticosteroid hormones. Lymphocytes in larval anuran amphibians are approximately ten times more sensitive to the corticosteroid hormones than lymphocytes present in adults (Rollins-Smith 1998).

The two phase immune development of anuran amphibians is correlated with changes in the tissue composition that occur during metamorphosis. In metamorphosing amphibians, adult tissues replace larval tissues and if the larval lymphocytes were present

in high numbers during this transformation, an immune response would be triggered, the new adult tissue would be destroyed and the animal may die (Rollins-Smith 1998). The removal of the majority of larval lymphocytes during metamorphosis avoids this potentially lethal situation (Rollins-Smith 1998). There are also differences in the MHC molecules expressed in larval and adult amphibians. In larval amphibians there are only low levels of MHC I and II expressed, whereas in adults there are much higher levels present (Flajnik 1996). The low levels of MHC expressed may be sufficient to provide tadpoles with protection against pathogens, yet too low to interfere with development (Flajnik 1996).

There are potentially lethal consequences if late stage or metamorphosing anuran amphibian tadpoles are stricken with an infection for they are likely to be particularly susceptible. When Ranaviral infections occur, mortality appears to be restricted to larval stages and recently metamorphosed froglets (Green *et al.* 2002; Greer *et al.* 2005).

Exposure to sub-lethal levels of immunosuppressant chemicals such as malathion, result in the disruption of both humoral and innate immune function in mammals, fish and amphibians (Cooper and Parrinello 1996; Galloway and Handy 2003). Therefore, studying animals under the influence of an immunosuppressant chemical is important since such a chemical may increase the effects of naturally occurring infections.

Natural History of Wood Frogs (*Rana sylvatica*) Bullfrogs (*R. catesbeiana*) and Ambystomids

Ranid frogs are important species to consider in studies of Ranavirus infections because many mortality events in these species have been associated with the presence of Ranaviruses (e.g. Green *et al.* 2002; Greer *et al.* 2005). Therefore, it is useful to understand the complex life history characteristics of the ranid species considered in this thesis.

*Wood Frogs (*Rana sylvatica*)*

Wood frogs have the largest species range of any North American anurans, encompassing most of North America (Oliver 1955). Wood frogs are found throughout Ontario (M^{ac}Cullough 2002) and are the only amphibians that have its species range extend beyond the Arctic Circle (Oliver 1955). They are small frogs (6.5 cm) and spend the majority of their lives on the forest floor in the leaf litter eating insects (M^{ac}Cullough 2002; Homan *et al*. 2004). Adult wood frogs have poor dispersal abilities and are totally dependant upon a forest habitat where they over-winter in the leaf litter (Regosni *et al.* 2003; Homan *et al*. 2004). However, juvenile wood frogs have greater dispersal abilities than adults (M^{ac}Cullough 2002) and this ability allows for gene flow between established populations, and the establishment of new populations. It also allows for the transfer and spread of pathogens.

Wood frogs are explosive breeders, breeding in the early spring when about 95% of the ice has melted from vernal pools (Oliver 1955; M^{ac}Cullough 2002; Homan *et al*. 2004; Stevens and Paszkowski 2004). Sex recognition in wood frogs occurs only after a

male has grasped another individual (Oliver 1955). A male wood frog will clasp an individual of any species, but if he clasps another male wood frog, the male being clasped will emit a warning croak and will be released. Male wood frogs rely on size differences between gravid and non-gravid females to determine whether to continue amplexus. If a female has been clasped and is not gravid, she will be immediately released. However, if the female is gravid the male will hold her in amplexus until she has laid her eggs, ensuring that he is the father of her brood (Oliver 1955). Female wood frogs tend to deposit their eggs in roughly spherical clusters and groups of females lay their eggs in the same area (Oliver 1955; M^{ac}Cullough 2002). The chorus size of male wood frogs is correlated with the number of broods of eggs that will be deposited in a pond (Stevens and Paszkowski 2004). These aggregations of adult frogs provides an opportunity for pathogens to be transferred between adult frogs and possibly to the eggs and between egg broods as more dense populations create more opportunities for pathogens to be transferred between individuals.

As in all amphibians, wood frog larval development is temperature dependant. Increased temperatures will result in shorter incubation periods and faster developmental rates (Oliver 1955). Animals found in more northern areas tend to remain in the egg for longer periods of time than animals of the same species in the south. Not surprisingly, wood frog embryos can tolerate a large range of temperatures (2˚C to 24˚C) (Oliver 1955). In the first two to three weeks of development after they hatch, tadpoles tend to aggregate in groups. This could also facilitate the transmission of disease between individuals through horizontal transmission of the virus.

Adult wood frogs are also freeze tolerant, meaning that they have the ability to become frozen solid (Matutte *et al*. 2000; Regosni *et al.* 2003). It is likely that these frogs experience a large amount of physiological stress from rapid thawing, and with the further stress of the breeding season they may be particularly more susceptible to disease.

*Bullfrogs (*Rana catesbeiana*)*

Bullfrogs are a widely distributed species in Eastern North America (M^{ac}Cullough 2002). This species has been distributed far beyond its natural range because of its high commercial value as the provider of frog legs (a delicacy) (Oliver 1955) and has been introduced to many places, such as Argentina, Venezuela and British Columbia where it may be a vector of disease and is now considered to be a pest species. However, bullfrogs are native to Ontario. Adults tend to live only in permanent bodies of water (M^{ac}Cullough 2002).

Bullfrogs are among the largest frog species on the planet with males reaching a maximum snout to vent length (SVL) of 20cm and females reaching a maximum SVL of 16cm (Oliver 1955). They are long - lived, taking up to seven years to grow to their maximum size, with a record lifespan of 16 years in captivity (Oliver 1955). The primary diet of adult bullfrogs consists of insects, aquatic invertebrates and other frogs (M^{ac}Cullough 2002).

In Ontario, bullfrogs breed over a period of six to eight weeks beginning in June and extending into August (M^{ac}Cullough 2002). A contributing factor to the late breeding period of bullfrogs may be that they emerge from hibernation much later than other amphibian species and they feed and grow for several weeks before the breeding season

(Oliver 1955). Bullfrogs prefer to breed in deep, permanent bodies of water (M[ac]Cullough 2002). They are sexually dimorphic with males having larger tympanums than females. Males are territorial; their calls function to attract females and to disperse other males, and a female bullfrog chooses her mate based on the quality of the territory that he occupies (M[ac]Cullough 2002). After the female has chosen a mate, she lays a brood of between 4000 and 8000 eggs (Oliver 1955, M[ac]Cullough 2002). The embryos can tolerate temperatures between 15˚C and 32˚C, taking 11 days to hatch at 20˚C (Oliver 1955). Bullfrog tadpoles usually over-winter for two winters before they metamorphose (M[ac]Cullough 2002). The dispersal of bullfrogs is affected by both density and sex ratio with males having a higher dispersal rate than females (Ireland 2004). The greater dispersal of males may increase their reproductive success and reduce inbreeding since females do not appear to disperse.

Since bullfrogs are a long lived species, there is the potential for the adults to act as reservoirs of diseases, such as the Ranavirus. Also, since the tadpoles spend up to two years in the pond, they, too, have the potential to act as reservoirs of the virus for the next year's tadpoles, the tadpoles of other species, uninfected adult bullfrogs, and adults of other species, and may have carried pathogens into sites where the frogs have been introduced.

Ambystomids

The ambystomids are a group of urodele species that include blue-spotted salamanders (*Ambystoma laterale*) and yellow-spotted salamanders (*Ambystoma maculatum*). The eastern–spotted newt (*Notophthalmus viridescens*) will also be considered in this section, even though it is not technically an ambystomid.

Blue-spotted and yellow-spotted salamander adults are terrestrial, living in the leaf-litter or just under the soil, and requiring a moist habitat. Both species are nocturnal and feed on insects and other invertebrates (M^{ac}Cullough 2002). Like wood frogs, they breed in the early spring when about 95% of the ice has melted from vernal pools. In yellow-spotted salamanders, unlike blue-spotted salamanders, breeding is triggered by the occurrence of several rainy nights. The larvae of both blue and yellow-spotted salamanders are active predators, preying on tadpoles and aquatic invertebrates (M^{ac}Cullough 2002).

The eastern-spotted newt also breeds early in the spring (M^{ac}Cullough 2002), often in the same ponds as ambystomids. The species has a complex life history, for after metamorphosis, the newts spend one to three years as terrestrial efts before becoming aquatic adults (M^{ac}Cullough 2002).

The life history characteristics of ambystomids make them ideal reservoirs of disease. As larvae they co-exist with the tadpole stages of many other amphibian species. Since the larvae are active predators, they come into contact with many other species of tadpoles and unsuccessful predation attempts may spread disease. They also appear to be carriers of the viral diseases and are thought to be reservoirs of Ranaviral disease for the

next generation of ambystomids as well as other species of amphibians (Brunner *et al.* 2004).

Efts provide an interesting opportunity for disease dynamics. Since they are absent from the aquatic amphibian community for one to three years, they are absent from the aquatic amphibian population for a longer amount of time than the ambystomid larvae. If there are carriers of disease among them when they return, they may re-introduce diseases to these populations.

Table 1.1 summarizes life history stages and information about reproductive timing of the ambystomids and the potentially cohabiting anuran species included in this thesis.

Table 1.1. Important life history characteristics of native North American species of amphibians that were used in the following thesis. (Information obtained from Oliver (1955) and M^{ac}Cullough (2002).)

Species	Time of Reproduction	Number of Eggs/Brood	Length of Egg Incubation	Larval Period	Additional Information
Ambystoma laterale	Early Spring	1 – 10	21 – 28 Days	Ends Mid - Summer	Terrestrial Adults Congregate to Breed
Ambystoma maculatum	Early Spring	50 – 100	~ 28 Days	Ends Mid - Summer	Terrestrial Adults Congregate to Breed
Notophthalmus viridescens	Early to Mid Spring	Eggs Laid Singly	25 – 25 Days	2 Months	Terrestrial Eft Stage lasting 1 – 3 years Aquatic Adults
Hyla versicolor	Late Spring	5 – 30	4 – 5 Days	Late Summer	Terrestrial Adults Males Congregate near ponds and call during the breeding season
Pseudacris crucifer	Early Spring	5 – 70	14 – 21Days	3 Months	Terrestrial Adults Males Congregate near ponds and call during the breeding season
Pseudacris triseriata	Early Spring	5 - 70	~14 Days	Ends Mid - Summer	Terrestrial Adults Males Congregate near ponds and call during the breeding season
Rana sylvatica	Early Spring	250 - 400	10 – 30 Days	Ends Mid-Summer	Terrestrial Adults Males Congregate near ponds and call during the breeding season
Rana catesbeiana	Early to Mid Summer	4 000 – 8 000	5 – 20 Days	2 – 3 years	Adults are primarily aquatic Males congregate and call during the breeding season

Objectives of Thesis and Chapter Outlines

Infectious diseases are thought to be important contributing factors to global amphibian declines. Ranaviruses are group of pathogens that are considered to be the cause of an emerging infectious disease in Wood frogs (*Rana sylvatica*). The objectives of this thesis are as follows: 1) track Ranavirus infections in aquatic amphibian communities near Peterborough, ON; 2) examine the mode(s) of transmission of the Ranavirus; 3) examine possible interactions of an immunosuppressant on laboratory induced frog virus 3 infections in Wood frogs and Bullfrogs (*Rana catesbeiana*); and 4) propose an initial model of Ranaviral transmission in amphibian populations in Ontario.

Chapter 2: Field Monitoring of Ranaviral Infections in 3 Larval Amphibian Communities in South Central Ontario.

Field monitoring of Ranaviral infection in pond communities of amphibian larvae is important in order to determine the diversity of species infected as well as when the virus appears in the community. Three ponds with diverse amphibian communities were sampled over several months to determine when the Ranavirus appeared in wood frog tadpoles and whether co-existing amphibian species were also infected. One of the ponds, Parker Pond, was sampled extensively, and all species of amphibians present in the pond were sampled repeatedly to search for the possible reservoir of the virus. This appears to be the first field data set from one aquatic amphibian community that documents the occurrence of the Ranavirus in both time and the all of the affected species.

Chapter 3: Vertical, Horizontal, and Interspecies Transmission of the Ranavirus in Wood Frog (Rana sylvatica) Tadpoles.

The mode of transmission of Ranaviruses in many amphibian species remains unknown. The mode of transmission of frog virus 3 (FV3) in Wood frogs was examined because of the extremely high mortality that the infections can cause in the tadpoles. Experiments reported are designed to assess the possibility of transmission of the virus between the parents and the offspring (vertical transmission - defined here as the transfer of a pathogen from parent to offspring) and/or through the water (horizontal transmission) between or within species. The mortality rates and infection rates were determined.

Chapter 4: Interactive Effects of Malathion Exposure and Ranaviral Infections in Wood Frog (Rana sylvatica) and Bullfrog (Rana catesbeiana) Tadpoles.

There are many different chemicals that are applied to the environment to control pests. Many of these chemicals have adverse effects on species that are not their intended target, and include immunosuppressant effects that may make the animals more susceptible to disease. The interaction between malathion (a known immunosuppressant) and frog virus 3 (FV3) is examined in wood frog and bullfrog tadpoles.

Chapter 5: Model of Ranaviral Transmission in Aquatic Amphibian Communities

A model of Ranaviral transmission in aquatic amphibian communities is developed based on the field study and experiments performed. The model takes into account potential complex interactions between species as well as the effects caused by the application of an immunosuppressant.

Appendices

In the appendices the PCR analysis techniques are described (Appendix A). Detailed methods for dissection of tadpoles and molecular analysis for the presence of the Ranavirus are also described (Appendix A). The methodology for propagation and quantification of frog virus 3 are explained (Appendix B). There is also a preliminary inter-year phylogenetic analysis of the Ranavirus present in Oliver Pond intended to determine if the virus is changing on a yearly basis (Appendix C). A note submitted to the Herpetological Review about terrestrially amplexing pairs of wood frogs is included (Appendix D). Also tables with the stage and infection status of all animals analyzed are included along with images of the gels (Appendix E).

Chapter 2: Field Monitoring of Ranaviral Infections in 3 Larval Amphibian Communities in South Central Ontario.

Introduction

Iridoviruses are pathogens which are associated with emerging infectious diseases that affect many species of amphibians and are associated with large scale morbidity and mortality events throughout North America. The first outbreak reported in Canada occurred in Saskatchewan in 1997 and was reported by Bollinger *et al.* (1999). This outbreak of the Ranaviral infection was restricted to tiger salamanders (*Ambystoma tigrinum diaboli*) and a small geographical area which encompassed only four ponds (Bollinger *et al.* 1999). Both tiger salamander larvae and adults were affected by this Iridoviral outbreak and the disease was widespread in the ponds that were affected. Some of the sick adult salamanders were brought to the lab and further examined for visible symptoms of the disease. The symptoms included a variety of gastrointestinal manifestations including vomiting and bloody stool. There were also epidermal manifestations of the viral infection which included ulcerations and increased shedding of the skin. Upon the development of bloody stools, death usually occurred within the next 48 hours. In laboratory infection trials, when infections with severe clinical symptoms were seen, death usually occurred within 13 days post exposure (Bollinger *et al.* 1999). However, the length of time in the wild for the development of a viral infection producing such severe symptoms remains unknown.

In the tiger salamanders, there were also various internal symptoms of the viral infection that could only be observed at the time of necropsy. In the abdominal cavity, there was frequently the presence of a clear fluid (Bollinger *et al.* 1999). The livers of

infected animals were discoloured and upon microscopic examination the hepatocytes were found to contain viral inclusions. Also, there were lesions in the intestinal tracts of the animals that had bloody stool, and it is thought that these lesions were the source of the blood. Upon molecular examination of the virus, it was concluded that the virus was indeed an Iridovirus and belonged to the Ranavirus genus. The virus was identified as a close relative to frog virus 3 (FV3) and designated as the Regina Ranavirus (Bollinger *et al.* 1999). This was the first Ranavirus to be described in Canada.

There have been a large number of Iridoviral outbreaks documented in the United States in recent years (Green *et al*. 2002; Docherty *et al*. 2003). Docherty *et al*. (2003) describe three different mortality events, all of which affected tiger salamander larvae. The areas affected were geographically distinct with one outbreak occurring in each of the following states: North Dakota, Utah and Maine. The outbreaks in Utah and North Dakota affected two subspecies of tiger salamanders, *Ambystoma tigrinum diaboli* and *A. tigrinum melanostictum*, whereas the outbreak that occurred in Maine affected *A. maculatum*, the blue spotted salamander. All of the affected populations had extremely high mortality rates. The manifestations of viral infections in the salamanders that were necropsied by Docherty *et al*. (2003) exhibited classic signs of Iridoviral infections: the external symptoms included ventral haemorrhages and subcutaneous and intramuscular edema, and the internal symptoms were similar to those described by Bollinger *et al.* (1999) with the Regina Ranavirus outbreak and included observations of viral inclusions in hepatocytes. However, the internal symptoms observed in these three outbreaks also included distended gastrointestinal tracts, which may have had haemorrhages and/or contained blood or a clear fluid (Docherty *et al*. 2003). The virus was cultured from

multiple tissues from salamanders collected in each location and examined with molecular methods to determine the identity of the virus(es) involved. The viruses isolated from both the Utah and North Dakota outbreaks were similar to the Regina Ranavirus, and the virus that was isolated from the outbreak in Maine was an FV3–like virus (Docherty *et al*. 2003).

Ranaviruses affect more than just salamanders. Green *et al*. (2002) examined 64 amphibian morbidity and mortality events in the United States between 1996 and 2001. Iridoviruses were associated with 21 of the 64 mortality events and were a contributing factor in 4 other die-off events that involved other diseases. The following anuran species were reported to have experienced a mortality event attributed to an Iridoviral outbreak: mink frogs (*Rana septentrionalis*), northern leopard frogs (*R. pipiens*), wood frogs (*R. sylvatica*), pickerel frogs (*R. palustris*), green frogs (*R. clamitans*), and spring peepers (*Pseudacris crucifer*). The following urodele species were affected by Iridoviral outbreaks: tiger salamanders (*Ambystoma tigrinum diaboli*), blue-spotted salamanders (*Ambystoma laterale*), and eastern spotted newts (*Notophthalmus viridescens*).

Currently, the distribution of Iridoviruses in Canada remains relatively unknown. Apart from the salamander populations in Saskatchewan (Bollinger *et al.* 1999), the virus has now been reported in populations of wood frogs (Greer *et al.* 2005; Charbonneau 2006) and leopard frogs in Southern Ontario (Greer *et al.* 2005; Duffus *et al.* in review). (Table 2.1). Wood frogs appear to be particularly affected in Southern Ontario (Greer *et al.* 2005; Charbonneau 2006) as well as across much of the Eastern United States. (Table 2.2)

Table 2.1. The known distribution of the Ranaviruses in Canada that have been reported in the literature since 1999, the species affected, the life history stages affected and the publication in which the infection was reported.

Species Affected	Latin Binomial	Location	Affected Life History Stages	Citation
Tiger Salamanders	*Ambystoma tigrinum diaboli*	Southern Saskatchewan	Larvae and Adults	Bollinger *et al.* (1999)
Wood Frogs	*Rana sylvatica*	South Central Ontario	Tadpoles	Greer *et al.* (2005); Charbonneau 2006
Leopard Frogs	*Rana pipiens*	South Central Ontario	Metamorphs and Adults	Greer *et al.* (2005); Duffus *et al.* (in review)

Table 2.2. All known wood frog mortality events which have been associated with an Iridovirus in North America, the location of the event, and the publication in which it was reported.

Location	Date	Citation
Maine	July 1991	Green *et al.* (2002)
North Dakota	July 1998	Green *et al.* (2002)
North Carolina	April 2000	Green *et al.* (2002)
Massachusetts	June 2000	Green *et al.* (2002)
Tennessee	February 2001	Green *et al.* (2002)
Maryland	May 2001	Green *et al.* (2002)
Ontario	June 1999 – June 2001	Greer *et al.* (2005)

There are no reports of die-off events associated with an Iridovirus involving multiple species in one location when the duration of the die off was short term (two to five days). The longer the duration of the mortality event, the more likely it was that other species were involved (Green *et al*. 2002). The potential exists for interspecies transmission of Ranaviruses between taxonomically distinct animals: the same Iridovirus was isolated from sympatric three–spined stickleback (*Gasterostelus aculeatus*) and tadpoles of the red-legged frog (*Rana aurora*) (Mao *et al*. 1999). Therefore, having multiple species of amphibians affected by a Ranaviral outbreak caused by a single species of the Ranavirus is not unlikely. Brunner *et al.* (2004) suggest that adult salamanders are reservoirs of the virus for the larval salamanders and this is the reason for the long term persistence of Ranaviruses observed in salamander populations.

However, there is an important phenology of mortality events that have been attributed to Iridoviral outbreaks. Mortality events tend to begin in June and continue through to August, when the majority of amphibian species are nearing the end of their aquatic development (Green *et al*. 2002; Greer *et al.* 2005). The onset of the mortality events can be extremely quick (i.e. in less than 24 hours) and there tends to be an extremely high mortality rate when an outbreak occurs (Green *et al*. 2002). Greer *et al.* (2005) suggests that the outbreaks occur at a time when there is significant desiccation of wetlands that causes crowding and therefore increases the rate of viral transmission between individuals. This is partially supported by lab experiments performed with leopard frogs (*Rana pipiens*) where frogs kept in dense populations had higher Ranaviral infection rates than those that were kept isolated (Duffus *et al.* in review). However, in

Ontario, in June, the ponds are still quite full of water, tadpoles are spread throughout the entire pond, and crowding is unlikely to be a stressor.

In 1999 the first Ranavirus outbreak was recorded in Ontario at Oliver Pond in the James Oliver M^{ac}Lean Centre, a field station maintained by Trent University. This outbreak killed almost an entire population of wood frog (*Rana sylvatica*) tadpoles and similar outbreaks continued to occur for several years (Greer *et al*. 2005). The strain of Ranavirus that is thought to be involved in these outbreaks in Southern Ontario is frog virus 3 (FV3) or an FV3–like virus. Oliver Pond is likely to have the most lengthy data set in Ontario with respect to Ranavirus outbreaks and is becoming an increasingly important study site for the Ranavirus and its ecology. These large scale outbreaks continued from 1999–2001 and only appeared to affect wood frog (*Rana sylvatica*) tadpoles (Greer *et al.* 2005). However, no other species were examined to determine the presence of asymptomatic Ranaviral infections, but Greer *et al.* (2005) report a mortality event at another location in Ontario that affected Leopard frog (*R. pipiens*) metamorphs. Therefore, a more in-depth examination of the amphibian species affected by the Ranavirus in Ontario is warranted.

In this study I examine the timeline of the initial Ranavirus appearance in wild amphibian communities, and in the developmental stage of infected animals, in three different pond locations. If the Ranavirus is present in multiple species at the ponds in which multiple species were sampled, then there is the potential for interspecific transmission and reservoirs of the virus. If multiple species are affected, ambystomid larvae are probably included. Because salamanders are widely reported to be infected with the Ranavirus, they may be the most likely reservoir of the virus. Another potential

reservoir of the virus is the adult eastern spotted newts because they have also been involved in mortality events associated with Iridoviruses (Green *et al.* 2002). Therefore, examining the community dynamics of the Ranavirus in aquatic amphibian communities is important to determine the probable reservoir(s) of the virus and the total number of amphibian species affected within the given community.

Methods

Field Collections

Wood frog (*Rana sylvatica*) tadpoles were collected at 10 day intervals from Oliver Pond, the pond on Crowe's Line Road, and Parker Pond at the beginning of the development season. (Note: Parker Pond was included in the study to observe a pond that was known to be infected with the Ranavirus in the previous year.) All of these ponds are located near Bobcaygeon Ontario, Canada (44°33N 78°33W). The collection interval was then reduced to four days to ensure the collection of many stages of tadpole development. The collections from Oliver Pond and the pond on Crowe's Line Road were made from May 16th, 2005 to June 17th, 2005 (collections at ten day intervals from May 16 to June 6, then at four day intervals from June 6 to June 17.) One collection of ambystomid larvae at Oliver Pond was made on June 13th, the last day of collection from that site. In order to observe when a Ranaviral infection occurred in a pond that was infected with the Ranavirus the previous year, wood frog tadpoles from Parker Pond were collected from May 16th until June 29th. Ambystomid larvae and *Pseudacris* species tadpoles were also collected at Parker Pond from June 13th to June 29th. On June 30th and July 9th, *Hyla versicolor*, grey tree frog, tadpoles were collected from Parker Pond. Five adult

Notophthalmus viridescens, eastern-spotted newts, were also collected from Parker Pond. All animals were euthanized and preserved in 70% ethanol to be tested for the presence of the Ranavirus. Each sample consisted of 15–30 animals, from which 10 individuals were randomly selected for PCR analysis for the presence of the Ranavirus. The rest of the samples have been archived

Biosafety Procedures

To prevent the trafficking of the virus between field sites, after each pond sampling all equipment was sprayed with a 30% bleach solution to eliminate any pathogens from the surfaces of the equipment. All of the equipment was then air dried. These precautions were developed at Arizona State University by the Collins Lab. All procedures followed were approved by the Animal Care Committee and the Biosafety Committee at Trent University.

Analysis

Animals were dissected and a small sample of hepatic tissue was removed from each animal that was analyzed for the presence of the Ranavirus. Please see Appendix A:- Dissection Procedures and Molecular Analysis for the Presence of the Ranavirus. An individual was considered to be infected with the Ranavirus if it tested positively.

Results

Length of Aquatic Period

Tadpoles and larvae from five species of coexisting amphibian species were collected at regular intervals from Oliver Pond, Crowe's Line Road and Parker Pond to determine the phenology of Ranaviral emergence in amphibian populations. The length of the aquatic period of amphibian species present in Parker Pond can be found in Figure 2.1A. The length of the aquatic period of amphibian species present in both Oliver Pond and Crowe's Line Road are similar, however there were fewer species of amphibians present in each of these ponds (Figure 2.1 B and C).

Figure 2.1. A) Length of the aquatic period of amphibian species present of tadpoles/larvae of different species of amphibians in Parker Pond. There were also adult *Notophthalmus viridescens* (eastern–spotted newts) present in May, but none were found after mid to late June. B) Length of the aquatic period of amphibian species of amphibian species in Oliver Pond. C) Length of the aquatic period of wood frog tadpoles, the only species present, in the pond on Crowe's Line Road.

Wood Frog Infection Rates

Wood frog tadpoles from Oliver Pond, the pond on Crowe's Line Road, and Parker Pond were extensively sampled over the course of their development. At both Oliver and Parker Ponds, the infections of wood frogs were more prevalent in the earlier stages of development (Gosner stages 25–26). However, the infections continued at Oliver Pond until the tadpoles reached Gosner stages 37-39, whereas the infection rate at Parker Pond the infections did not continue past Gosner stage 26. Only the wood frog population at the pond on Crowe's Line Road showed no Ranaviral infections over the course of the season. (Table 2.3) The negative controls did not show any contamination and the positive controls were positive for the Ranavirus. The gels can be found in Appendix E.

Infection Rates in Multiple Species

To determine the number of different amphibian species infected with the Ranavirus in Oliver Pond and Parker Pond different amphibian species were sampled. In Oliver Pond, ambystomid larvae were sampled on a single occasion (n=9), and 22% (two of nine) of these larvae were infected with the Ranavirus. Parker Pond was sampled over time, as were all species present in the pond (Table 2.4). The infection rates of ambystomid larvae collected remained relatively high and constant. However, the anuran larvae sampled did not show the same trend as the ambystomid larvae. The *Pseudacris* species tested positive for the Ranavirus on only two occasions - June 17 and June 25 - with infection rates of 60% and 10% respectively. The *Hyla versicolor* tadpoles tested positive for the Ranavirus on only one occasion. (Table 2.4) The gels can be found in

Appendix E. The negative controls did not show any contamination and the positive controls were positive for the Ranavirus. Tadpoles from Parker Pond often had soft bellies that can be indicative of a Ranaviral infection and there were also 10 dead wood frog tadpoles found in that pond in late June.

In Parker Pond, in a sample of 5 aquatic adult *Nophthalmus viridescens*, collected in May 2005, one was infected with the Ranavirus. Also, yellow-spotted salamander eggs were collected from Parker Pond in late April 2005; these were tested for the presence of the Ranavirus and none were found to be infected (n=5). The gels can be found in Appendix E. The negative controls did not show any contamination and the positive controls were positive for the Ranavirus.

Table 2.3. The Ranavirus infection rate (%) and Gosner stage of wood frog tadpoles collected from 3 ponds in May and June 2005 (n=10 animals/date).

	May 16	**May 26**	**June 6**	**June 13**	**June 17**	**June 21**	**June25**	**June 27**
Crowe's Line								
Gosner Stages	*25 - 26*	*25 - 29*	*28 - 34*	*37 - 39*	*39 - 40*			
Infection Rate	0%	0%	0%	0%	0%			
Oliver Pond								
Gosner Stages	*25*	*25 - 27*	*30 - 32*	*35 - 37*	*37 -39*			
Infection Rate	80%	40%	10%	30%	0%			
Parker Pond								
Gosner Stages	*25 - 26*	*26 - 28*	*30 - 38*	*31 – 38*	*38 - 40*	*40 - 41*	*41 – 43*	*38 - 45*
Infection Rate	20%	0%	0%	0%	0%	0%	0%	0%

Table 2.4. The infection rate (%) and Gosner stage of amphibian tadpoles/larvae infected with the Ranavirus collected from Parker Pond in June 2005. (n=10 per species per date)

	June 13	**June 17**	**June 21**	**June 25**	**June 29**	**June 30**	**July 9**
Ambystomid Species	50%	90%	50%	40%	70%		
Gosner Stages	*35 - 39*	*33 - 41*	*34 - 43*	*30 - 41*	*30 - 40*		
Pseudacris Species	0%	60%	0%	10%	0%		
Gosner Stages						*31 - 40*	*31 - 40*
Hyla versicolor						10%	0%

Discussion

Extensive field monitoring of Ranavirus infections of wood frog tadpoles over their developmental period in Oliver Pond, the pond on Crowe's Line Road, and Parker Pond showed that infection rates of wood frog tadpoles can vary over their development and appear to be independent of the infection history of the pond.

At Oliver Pond, the wood frog tadpoles were found to be infected, and the tadpoles had a high initial infection rate (80%). Over time, the infection rate in this population of wood frogs decreased to zero. There are two possibilities that can explain this trend. Either the wood frog tadpoles that were infected with the Ranavirus were able to clear the infection, or infection-induced mortality eliminated the infection from the wood frog population leaving only resistant phenotypes. Outbreaks of the Ranavirus in wood frog tadpoles have been occurring on an annual basis at Oliver Pond since 1999 (Greer *et al.* 2005). There is a high mortality in wood frog tadpoles when they become infected with the virus (Green *et al.* 2002; Greer *et al.* 2005) although the mortality rate in a population is unlikely to be 100%. There is evidence that exposure to a Ranavirus at low levels can result in the infection of animals but not necessarily cause symptoms of disease or death (Brunner *et al*. 2005). It is also possible that wood frog tadpoles that are infected with the Ranavirus are more likely to be caught than uninfected tadpoles. This would result in artificially inflated tadpole/larvae infection rates. A third possibility that cannot be discounted is that sample sizes were too small to accurately reflect infection rates in the population.

Since Ranaviral infections in Oliver Pond have occurred for 6 years, the original breeding wood frog adults would have died at least several years ago from either natural

causes or from Ranaviral infections themselves. The infection rate of the Ranavirus in adult wood frogs remains unknown. Since the mortality rate from Ranaviral infections is not likely to be 100% in wood frog tadpoles, it is possible that a portion of the current population of breeding adult wood frogs at Oliver Pond are phenotypically resistant to Ranaviral infections. The presence of the Ranavirus in a population of wood frogs for several years in a row would likely act as a selection pressure where only animals who were phenotypically resistant to the Ranavirus metamorphosed into adults. These animals later became breeding adults in that population. For this to persist over many generations of breeding adults, the basis of resistance is likely to be genetic. There is also the possibility that the virus has become less virulent over time, resulting in the reduced severity of the mortality events over time but consistent annual infections of tadpoles.

The disappearance of infected wood frog tadpoles over the course of the summer could also be due to density factors. When wood frog tadpoles hatch, they remain in large groups and are largely sedentary. It is only after the tadpoles have developed sufficiently that they disperse throughout the pond (approximately late Gosner Stage 25). When the density of a population is high, there is likely to be an increased rate of transmission, and highly virulent pathogens would be selected for, because there is a large degree of contact between individuals. The ratio of susceptible individuals to infected individuals should be high and facilitate transmission of disease. This would result in the population being purged of susceptible individuals via infection-induced mortality and a lowered infection rate over time because only individuals who are not susceptible to the disease remain in the population. Desiccation of the pond would also have the same effect of increasing the density of tadpoles since this would likely result in crowding and increased viral

transmission. Large scale desiccation did not occur in the ponds that were observed in this study, but, it still could be a large factor in amphibian communities which persist in roadside ditches that are full in the spring but dry out quickly in the summer sun killing any tadpoles which have not metamorphosed.

In larval amphibians there are only low levels of the major histocompatibility complex (MHC) I and II expressed, whereas in adults there are much higher levels present (Flajnik 1996). Flajnik (1996) suggests that the low levels of MHC expressed should provide sufficient protection against low level infection by pathogens and that the expression of these molecules at low levels does not interfere with development. This situation leads to potentially lethal consequences if a metamorphosing anuran amphibian is stricken with an infection. When chytrid infections occur, the recently metamorphosed froglets are highly susceptible to the pathogen (cited in Rollins-Smith 1998), and the same could be true for Ranavirus infections. When Ranaviral infections occur, mortality appears to be restricted to late larval stages and recently metamorphosed froglets (Green *et al.* 2002; Greer *et al.* 2005). Therefore, the later stages of larval development and recent metamorphs are likely to be more susceptible to diseases. This trend is not seen in the wood frog tadpoles at Oliver Pond, where the earlier developmental stages have higher infection rates than later developmental stages, increasing the likelihood of the presence of wood frogs which are resistant to the Ranavirus. The population of wood frog tadpoles in Oliver Pond did not show symptoms of Ranavirus infections even though there were infected tadpoles present for several weeks. This fact also increases the likelihood that there are asymptomatic carriers of the virus within the population, which

could carry sub-detectable levels of the Ranavirus, similar to the tiger salamanders which Brunner *et al.* (2004) observed.

The hypothesis of the presence of wood frog tadpoles resistant to the Ranavirus is supported by the presence of ambystomid larvae with high Ranavirus infection rates. Interspecies transmission of the Ranavirus appears to be possible (Mao *et al*. 1999). Therefore, it is possible that interspecies transmission of the Ranavirus can occur between different anuran species and between salamanders and anurans, that individuals may be susceptible or resistant, and that an intermediate host or reservoir species may exist.

The absence of infected wood frog tadpoles at the pond at Crowe's Line Road is probably, in fact, not surprising. FV3 outbreaks that cause mortality and re-occur for several years are usually localized to a small geographical area, which may be restricted to only one pond in an area (Carey *et al.* 1999; Greer *et al.* 2005). Even though the pond on Crowe's Line Road is not very far from Oliver Pond (less than 1km), the dispersal ability of adult wood frogs is poor (Homan *et al*. 2004) and juvenile wood frogs which have great dispersal abilities (M^{ac}Cullough 2002) would be very few during a heavy mortality year, and unlikely to carry the Ranavirus to a new population.

Although transmission of the Ranavirus could be from infected wood frogs or ambystomids, other sources of the virus are possible. Oliver Pond is less than 500m from Pigeon Lake on the Trent-Severn Water Way. Several populations of leopard frogs (*Rana pipiens*) in this system have been found to be infected with the Ranavirus (Greer *et al*. 2005; Duffus *et al*. in review) and it is at least possible that the initial source of the

Ranavirus was from Pigeon Lake. The infection could have been brought into the pond from leopard frogs that had migrated from the lake to the pond in the spring to breed.

However, the pattern of infected wood frog tadpoles in Parker Pond is puzzling. Only very young (Gosner stage 25) wood frog tadpoles were observed to be infected with the Ranavirus. There is no long term data set available for Parker Pond, but in 2004 wood frog tadpoles from this population were infected with the Ranavirus (Charbonneau 2006). Therefore, the pattern of infection in the amphibian community at Parker Pond cannot necessarily be explained by the long term persistence of a Ranavirus within the wood frog population - another reservoir species appears to be necessary.

The infection rate among ambystomid species in Parker Pond remains fairly consistent throughout the summer making it a likely candidate as the reservoir of the virus in Parker Pond. This may be due to the fact that some species of ambystomids can clear Ranaviral infections (Brunner *et. al.* 2004). No dead salamander larvae were ever observed at Parker Pond. Nor were any salamander larvae observed with buoyancy problems, haemorrhages, or lethargy, symptoms which have been observed where there have been Ranavirus-associated ambystomid larvae mortality events (reported by Docherty *et al*. 2003). It is possible that the infections observed in ambystomids were sub-lethal and that the ambystomids passed the virus on to other individuals. This is supported by findings by Brunner *et. al.* (2004) in experiments which exposed tiger salamander (*Ambystoma tigrinum nebulosum*) larvae and recent metamorphs to the *Ambystoma tigrinum* virus (ATV). Larval salamanders were more likely to recover from ATV infections and were also more likely to carry sub-lethal viral loads after they had metamorphosed (Brunner *et. al.* 2004). When these carriers were allowed contact with

susceptible sub-adult salamanders, transmission of the virus to the susceptible animals occurred (Brunner *et. al.* 2004). Also, ATV infected sub-adult tiger salamanders emerging from ponds in field studies, supports the hypothesis that ambystomids are a reservoir of ATV (Brunner *et. al.* 2004). Combined with evidence that Ranaviruses can easily cross the species barrier (Mao *et al*. 1999), it is likely that ambystomids are the reservoir of the Ranavirus in Parker Pond.

Of the adult *Notophthalmus viridescens* (eastern spotted newts), only one of five carried the Ranavirus and it showed no symptoms. Like the salamanders, the newts may be able to carry sub-lethal infections and experience no adverse effects from the presence of the virus. The discovery of infected eastern spotted newts is not surprising, as infected individuals have been previously documented (Green *et al.* 2002). The newts could also be an alternative or additional possible reservoir of the Ranavirus.

The infection of *Pseudacris* species tadpoles in Parker Pond is not unexpected because an Iridoviral mortality event involving a *Pseudacris* species has been reported previously (Green *et al*. 2002). The highest infection rate (60%) in the *Pseudacris* species tadpoles occurred at a time when the infection rate of the ambystomid larvae was 90%. *Hyla versicolor*, breeding later than the other species in Parker Pond, had a low infection rate, but this is the first infection reported for this species. It appears that the Ranavirus passes easily between amphibian species in a larval amphibian community and that all species of amphibians in the community may be susceptible to infection. Considering the ability of Ranaviruses to infect taxonomically distinct animals (Mao *et al*. 1999), this should not be surprising.

The results of this study may greatly underestimate the extent of Ranavirus infection rate in the three ponds that were examined. PCR analysis, even at this scale, is expensive and therefore small sample sizes were used. The sample sizes used in this experiment are too small to give an accurate representation of the true infection rates within the population and certainly larger sample sizes (e.g. n=100) would be very useful. Also, although the current method of PCR analysis used can detect the presence/absence of the Ranavirus, it cannot quantify the amount of the virus present. To quantify the amount of virus that is present in an animal, real time PCR analysis is needed. This would permit conclusions about how great the viral load needs to be to produce symptomatic infections.

Conclusions

Ranavirus infection rates of aquatic amphibian larvae and adults appear to vary from pond to pond, even within a small geographic area. The infection rates in wood frog tadpoles is independent of the infection rates in other species. This is especially evident in Parker Pond, where all species of amphibians surveyed, except for wood frogs, had some level of Ranaviral infection.

The aquatic amphibian community at Parker Pond provides insights into the community dynamics of Ranaviral infections. The infection rate among ambystomid larvae remained relatively constant throughout the entire period of time in which the larvae were surveyed. This is consistent with the hypothesis that ambystomid larvae are the reservoir of the Ranavirus for the amphibian community in Parker Pond, and by implication, other anurans in other ponds.

Chapter 3: Vertical, Horizontal, and Interspecies Transmission of the Ranavirus in Wood Frog (Rana sylvatica) Tadpoles.

Introduction

Amphibians are in decline on a global scale (Stuart *et al.* 2004). A multitude of factors that are thought to be contributing to this situation includes habitat modification, increasing ultra-violet radiation levels, predation, climate change, environmental contaminants, and emerging infectious diseases, as well as interactions between these factors (Alford and Richards 1999). Emerging infectious diseases are credited to be a major contributing factor to global amphibian decline and anthropogenic environmental modifications are probably responsible for their appearance (Daszak *et al.* 2001; Pounds *et al.* 2006). An emerging infectious disease is defined as a disease caused by a pathogen which is currently increasing in geographical range, is infecting an increased diversity of hosts, and/or has recently evolved (Daszak *et al.* 2000). Examples of emerging wildlife diseases in amphibian populations around the world include those caused by *Batrachochytrium dendrobatidis*, also known as the chytrid fungus, and the Iridoviruses. The Iridoviruses appear to be a particularity virulent group of pathogens.

The Iridoviruses are a group of viruses that affect ectothermic vertebrates and invertebrates (Chinchar 2002). The Ranaviruses are a group within the Iridoviruses that infect aquatic insects, fish and amphibians (Ahne *et al*. 1997; Chinchar 2002). Ranavirus infections have been correlated with amphibian mortality events in North America, involving both urodeles (Bollinger *et al.* 1999; Green *et al.* 2002; Docherty *et al.* 2003) and anuran amphibian larvae (Green *et al.* 2002; Greer *et al.* 2005).

A strain of the Ranavirus, frog virus 3 (FV3) has been identified as the infectious agent in salamander mortality events in North America and it has recently been fully sequenced (Tan *et al*. 2004). High mortality rates in wood frog (*Rana sylvatica*) tadpoles have been correlated with FV3 and other Iridoviral infections (Green *et al.* 2002; Greer *et al.* 2005). Mortality events associated with FV3 infections have a tendency to re-occur for several years and are usually localized to a very small geographic area (Bollinger *et al.* 1999; Carey *et al.* 1999; Greer *et al.* 2005).

Interspecies transmission of the Ranaviruses between taxonomically distinct species may occur. For example, an Iridovirus isolated from the three–spined stickleback (*Gasterostelus aculeatus*) and from tadpoles of the red-legged frog (*Rana aurora*) was determined by molecular analysis to be the same virus (Mao *et al*. 1999). It is likely that interspecies transmission of the Ranavirus can occur between different amphibian species.

Pathogens which are highly virulent and cause high mortality (such as Ranaviruses) are likely to run out of susceptible hosts, as would be found in a community of amphibian larvae which is finite. When the pathogen has the ability to use an intermediate host (a vector of disease), which is not affected by the pathogen's presence, transmission of the disease is not impeded by the mortality that it causes in the primary host (Day 2002). Therefore, the pathogen may remain in a community even if its primary host has been seasonally extirpated. Recently, it has been suggested that ambystomids are the reservoir of Ranaviruses in aquatic amphibian communities (Brunner *et. al.* 2004).

There are two possible ways that the Ranavirus may be transmitted between individual amphibians: vertical transmission and horizontal transmission. Vertical

transmission involves passing the virus from parent to offspring and not all of the progeny are necessarily infected (Brauer 1995). In contrast, horizontal transmission involves passing the pathogen from individual to individual, regardless of their relationship or age, through direct contact or through the water.

A horizontal transmission model has been proposed for the *Ambystoma tigrinum* virus (ATV), a Ranavirus, which can be passed to both larval and recently metamorphosed tiger salamanders (*Ambystoma tigrinum nebulosum*) by exposure to the virus through the water (Brunner *et. al.* 2004). Recent metamorphs experienced higher mortality rates than larval salamanders when they were exposed to ATV in this way (Brunner *et. al.* 2004). Also, salamanders that were exposed to ATV as larvae were more likely to become ATV carriers as adults and pass the virus on to other uninfected adults (Brunner *et. al.* 2004).

Wood frogs (*Rana sylvatica*) provide another species for exploring methods of Ranavirus transmission. When a population of wood frogs are infected with a Ranavirus, mortality rates are high (Green *et al.* 2002; Greer *et al.* 2005). Wood frog tadpoles are relatively easy to raise from eggs in the lab, making laboratory exposure experiments possible. Experiments were performed to determine whether the mode of transmission of FV3 in wood frogs is vertical, horizontal or both. If vertical transmission of the Ranavirus occurs, the virus should be transmitted from parent to egg. Therefore, if infected parents produce offspring, then some, if not all, of their offspring will be infected as eggs with the Ranavirus. If uninfected parents produce offspring, then none of their offspring will be infected as eggs with the Ranavirus. If broods collected from the wild within a few hours after being laid are infected with the Ranavirus, then the infection is likely due to

vertical transmission of the virus because it is assumed that such a short period of time spent in pond water is insufficient for horizontal transmission of the virus to occur.

Further experiments, similar to those that had been done with the *Ambystoma tigrinum* and the *Ambystoma tigrinum* virus (ATV) (Brunner et al 2005), were performed to test the hypothesis that higher concentrations of FV3 in the water would increase the infection rate and decrease the survivorship in wood frog tadpoles. If horizontal transmission of the virus does occur, then higher concentrations of the Ranavirus in the water should result in a greater infection rate in wood frog tadpoles.

Experiments were also performed to determine if interspecies transmission of the Ranavirus occurs between wood frog tadpoles and ambystomid larvae. If interspecies transmission of the virus does occur between ambystomid larvae and susceptible wood frog tadpoles, then the wood frog tadpoles should become infected with the Ranavirus shed from the ambystomids.

Methods

Eggs Collected From Natural Ponds

Broods of wood frog eggs were collected from three ponds within 15km of Peterborough, ON (ponds are defined as Barb's Marsh, Division Road at Donwood Road, and County Road 6). These broods were collected early in the morning after they were deposited and the eggs had not begun to rotate (Gosner stage 1). Broods were also collected directly from two ponds near Bobcaygeon, ON (ponds are defined as Oliver Pond and Crowe's Line Road) at night and within 1–2 hours of being laid (Gosner stage 0–1). The broods were brought back to the lab and placed in aged tap water. As the

tadpoles hatched from the eggs, the jelly was removed to avoid death induced by the decomposition of the jelly masses in the aquaria.

Eggs Laid in Captivity

On April 17th, 2005, three amplexing pairs of wood frogs were collected from Crowe's Line Road (Duffus and Ireland, in review). Wood frogs normally undergo amplexus only in the water, so finding three amplexing pairs before reaching the pond was unlikely, but fortuitous, making it possible to eliminate any contact with pond water as a possible source of Ranavirus exposure. The amplexing pairs were collected and placed into containers partially filled with aged tap water. The pairs remained in amplexus throughout transportation back to the lab; if there was a break between the members of a pair, they returned swiftly to the amplectic position. Back at the lab the pairs were placed into a temperature controlled room (at 15°C) in constant dark conditions to facilitate breeding in captivity. Pairs were originally provided with long grasses to act as oviposition sites, but no eggs were laid. However, eggs were laid after anchored twigs were provided as oviposition sites. The first egg mass was laid three days after capture and the second egg mass was laid four days after capture. Both egg masses were viable, developed normally and had a hatching rate of over 90%. A non - viable egg mass was produced by the third pair after six days in captivity - since no egg rotation was observed of these eggs, fertilization had failed. Each pair of wood frogs remained in amplexus until an egg mass was laid.

After the eggs had been laid, they were removed from the tank that contained their parents and transferred to 5-gallon aquaria, partially filled with aged tap water and

permitted to develop and hatch. The parents were then sexed, euthanized, preserved in 70% ethanol and tested for the presence of the Ranavirus.

General Care of Eggs and Tadpoles

Egg masses were placed into tap water that had been aged for 2–3 days, to avoid contamination from an untreated water source such as filtered river water. The use of aged tap water also eliminated the possibility of diseases being inadvertently introduced into the water and negatively affecting the experiments. Tadpoles were kept in 5-gallon tanks in a 15°C environmental chamber for several weeks to ensure normal development. Partial water changes were performed when the water began to cloud during the first weeks of development after hatching (approximately once per week). Tadpoles were fed shredded, boiled spinach as needed (usually every 1-2 days). After the tadpoles began feeding, the water was then changed every three days to avoid the build up of waste products. Also, the spinach fed to the tadpoles decomposed quickly and needed to be removed on a regular basis.

As the tadpoles developed, the biomass of animals quickly increased. Broods were therefore divided into multiple aquaria when the tadpoles were between 1 and 2 cm long. Each brood was divided into two 5 gallon aquaria and transferred to a room kept at 20˚C. The resulting density was approximately 100–150 tadpoles per aquarium.

Vertical Transmission Experiments

The youngest brood collected from each of Oliver Pond, the pond at Crowe's Line Road, Barb's Marsh, County Road 6 and Division at Donwood Road, as well as one of the broods that was laid in the lab, were selected for this experiment, for a total of six

broods. The most recently deposited broods prior to the collection of the naturally laid broods were selected because they had spent less time in contact with the water at the ponds and were, therefore, less likely to be infected with the Ranavirus via the water. (The broods were Gosner stage 0-1 when collected.)

Samples (n=10) of the eggs from each brood were taken as soon as possible after being laid. To examine the progression of a Ranaviral infection potentially derived from a parent, samples of ten tadpoles from each brood were taken every ten days thereafter. These tadpoles were euthanized and preserved in 70% ethanol. The sampling occurred from the end of April until July 19th, 2005. At the end of the experiment, the remaining tadpoles were euthanized and placed into 70% ethanol. However, to reduce the number of samples for molecular analysis, only samples from the beginning, middle and end of the experiment were analyzed for the presence of the Ranavirus. The remaining samples have been archived.

Wood frog FV3 Infection Trial Procedures

To test whether the FV3 acquired from the American Type Culture Collection (ATCC) was able to infect wood frog (*Rana sylvatica*) tadpoles via the water, five tadpoles were placed into 150mL of water and 10µL of FV3 stock (final concentration of the virus in each beaker was 6.7 x 10^2 Plaque Forming Units (PFU)/mL). The tadpoles ranged in Gosner stage from 25–30. A control sample of five tadpoles was placed in water without FV3. The control tadpoles ranged in Gosner stage from 25–40. The tadpoles were left in 150mL of water overnight and the next morning the water level was increased to 300mL of water to increase the amount of oxygen present and to dilute waste

products. The tadpoles were fed boiled spinach during this experiment. They also remained in the same water until the end of the experiment to ensure viral transmission could occur efficiently (t=5 days). All animals were then euthanized and preserved in 70% ethanol.

Bullfrog FV3 Infection Trial Procedures

This experiment was intended to test whether or not FV3 in low concentrations can cause infection through exposure via the water in bullfrog (*Rana catesbeiana*) tadpoles. 250mL beakers were partially filled with 75mL of aged tap water. This water was then inoculated with 10µL of FV3 (viral concentration of ~13 PFU/mL). Control animals (n=5) were euthanized on the first day of experimentation. All tadpoles used were Gosner stage 25. The experiment had five replicates with one tadpole in each replicate. The water was left un-aerated for four hours. After 16 hours, the water in the beakers was increased to 150mL to increase the water volume for the tadpole, dilute waste products and provide more oxygen. The experiment was terminated after five days of exposure to the virus. The tadpoles were then dissected and analyzed for the presence of FV3.

Horizontal Transmission of the Ranavirus

Wood frog tadpoles from a brood laid in the lab that previously tested negative for the presence of the Ranavirus were used in the experiment to assess horizontal transmission. Tadpoles were exposed to one of three different concentrations of FV3 or a control that contained no virus. The FV3 used in this experiment was grown in Fathead Minnow Cells, harvested on June 23, 2005. The titre of the FV3 used in the experiment

was measured to be 10^7 virions per mL. The final viral concentrations in the treatments were 67 PFU/mL, 6.7 x 10^2 PFU/mL, and 6.7 x 10^3 PFU/mL, respectively (See Appendix B). The tadpoles used in this experiment ranged in Gosner stage from 25–46. Each treatment consisted of two replicates with 15 individuals in each. To ensure the maximal exposure to the Ranavirus, the tadpoles were placed into 1L beakers containing 150mL aged tap water with the prescribed amount of FV3 overnight. The water level was increased to 350mL of water the next morning to increase the water volume for the tadpoles, dilute waste products and provide extra oxygen to the tadpoles. The beakers were left un-aerated for 5-6 hours at the beginning of the experiment to increase the likelihood of viral transmission, but were aerated for the remainder of the experiment. The tadpoles were fed boiled spinach and the water was changed every 72 hours. No virus was added to the water after the water changes occurred. Animals that died over the course of the experiment were removed and preserved in 70% ethanol for analysis for the presence of the Ranavirus. At day 9, the experiment was terminated and the remaining tadpoles were euthanized and placed into 70% ethanol. For PCR analysis of the presence of an FV3 infection, a set of 10 individuals from each treatment was taken at random.

Interspecies Transmission of the Ranavirus

Blue-spotted salamander (*Ambystoma laterale*) larvae were collected from Parker Pond, near Bobcaygeon, ON, a population known previously to be infected with the Ranavirus (See Chapter 2). This site also exhibited signs of a Ranaviral infection in 2005: tadpoles from the site often had soft bellies which can be indicative of a Ranaviral infection and there were also five to ten dead wood frog tadpoles found in late June. Ten

ambystomid larvae were euthanized upon return to the lab to serve as controls. Wood frog tadpoles from lab populations, known from previous experiments to be free of the Ranavirus, were used in this experiment, twenty of which were euthanized to serve as uninfected experimental controls. Ten of the twenty tadpoles that were euthanized were randomly selected for Ranaviral testing and the rest of the animals have been archived in case further testing is warranted.

Five replicates of the experiment were set up in 5-gallon aquaria. The aquaria were partially filled with aged tap water. Five ambystomid larvae were placed in each tank. Wood frog tadpoles were then evenly distributed between the treatments, resulting in 25-30 individuals per treatment. As well as being test subjects, the surplus number of wood frog tadpoles in the aquaria were to act as food for the ambystomid larvae as well as test subjects. The end points of the experiment were defined when there were approximately 10 wood frog tadpoles left in the aquarium or after 15 days, whichever came first. The wood frogs were fed boiled spinach and the water was changed every seven days. The long period of time between water changes was to permit the establishment of virions in the water to facilitate interspecies transmission, just as would occur in nature.

At the end of the experiment the remaining tadpoles and all of the ambystomid larvae were euthanized and placed into 70% ethanol. The animals were then tested for the presence/absence of the Ranavirus (only 2 trials and the controls were tested to reduce the costs associated with analysis). In treatments which had more than ten wood frog tadpoles remaining at the time of termination, 10 tadpoles were randomly selected for Ranaviral testing and the remaining tadpoles were euthanized and preserved in 70%

ethanol. All ambystomid larvae and sub-adults were euthanized and tested for the presence of the Ranavirus.

Biosafety Procedures

Field Precautions

To prevent the trafficking of the virus between field sites, all equipment was sprayed with a 30% bleach solution and permitted to air dry after each site was visited to eliminate any pathogens from the surfaces of the equipment. These precautions were developed at Arizona State University by the Collins Lab. All procedures followed were approved by the Animal Care Committee and the Biosafety Committee at Trent University.

Laboratory Precautions

In the lab, preventing the spread of the virus from potentially infected animals to uninfected animals, as well as to other areas, was also essential. Aquaria were subjected to a three day wash cycle before re-use: on the first day the aquaria contained 30% bleach solution, and on the second and third days the aquaria were filled with clean tap water. All animals were kept in a limited access facility and all equipment used in the experiments was also decontaminated before being used in any other experiments or removed from the laboratory. Upon entrance and exit of the facility, the use of a disinfectant footbath was used to prevent viral spread. All materials of animal origin were disposed of through the Trent University Animal Care Facility's waste disposal program.

Experiments that involved direct manipulation of the virus were conducted in a Level 2 Biohazard Laboratory. Wastewater from these experiments was disinfected with a bleach solution (minimum 30%) and was disposed of through the municipal sewage system. Equipment used was also disinfected using the three day cycle, this time using a minimum of 50% bleach solution. After disinfection with the bleach, the equipment was again washed with a caustic home cleaner. The exterior of the glassware used was wiped down with 100% ethanol. All procedures followed were approved by the Animal Care Committee and the Biosafety Committee at Trent University.

Analysis

Animals were dissected and a small sample of hepatic tissue was removed for PCR analysis to test for the presence of FV3. Please see Appendix A: - Dissection Procedures and Molecular Analysis for the Presence of the Ranavirus. An individual was considered to be infected with the Ranavirus if it tested positively.

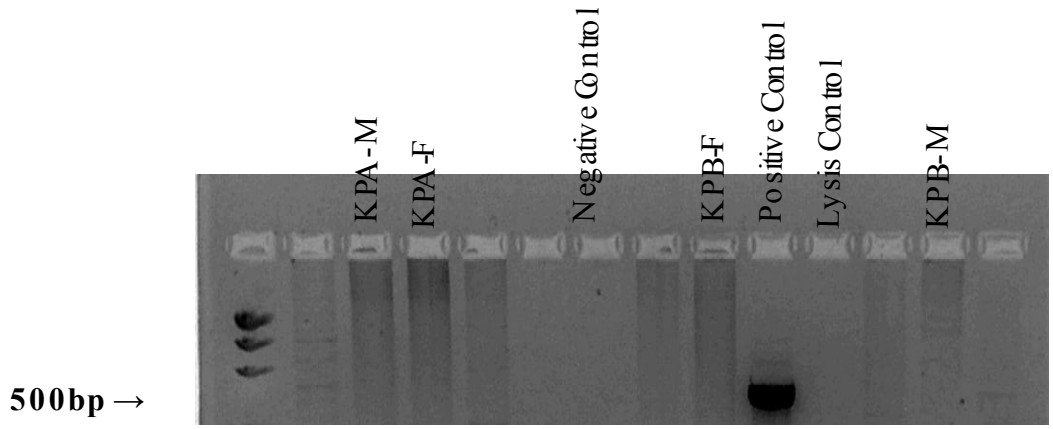
Results

Vertical Transmission

Infection of Parents of Broods Laid in Captivity

Three amplexing pairs of wood frog adults, captured on the road prior to reaching their pond ensuring that their offspring were not exposed to any virus that might be in the pond water, were tested for the presence of the Ranavirus. They are designated as the parents of broods A, B, and C respectively. Both parents for brood A and B tested negative for the presence of the Ranavirus (Figure 3.1 A). However, although the female parent of brood C tested negative, the male parent of brood C tested positive for the presence of the Ranavirus, indicating that adult wood frogs can carry the virus (Figure 3.1 B).

A)



B)

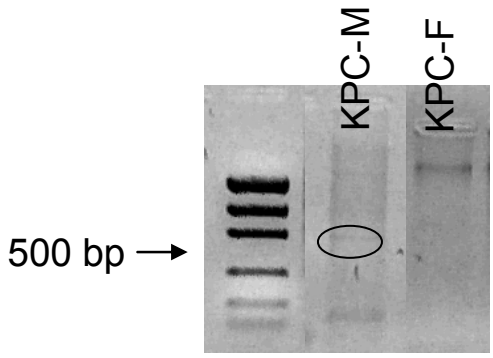


Figure 3.1. A) The molecular analysis for the presence of the Ranavirus for the parents of broods A and B. KPA are the parents from brood A, KPB are the parents from brood B. B) KPC are the parents of brood C. (This image is a composite of 3 separate gel images.) Sexes are noted by – M for the male and – F for the female. The band has been circled because it is faint. The presence of a band at the 500 base pair (bp) level indicates the presence of the Ranavirus. The positive control indicates that the PCR reaction functioned properly and the negative and lysis controls indicate no contamination occurred.

Infections of Eggs and Tadpoles from Laboratory Raised Wood Frog Broods

Eggs and tadpoles from five broods laid naturally and the three broods laid in captivity were assessed for the presence of FV3. Four of five broods collected from the wild were positive for the Ranavirus (Table 3.1), indicating that at least one of the parents of each of these broods was probably infected with the Ranavirus. In only one naturally collected brood did the Ranaviral infection persist beyond the egg stage: in the brood from Crowe's Line Road the Ranavirus persisted until tadpoles had reached Gosner stage 26. It is not known if the infected individuals died or were able to clear the virus.

Of the three broods that were laid in the lab, one had Ranaviral infections in the egg stage. This infection occurred in the progeny of Known Parents C, the pair in which the male was infected with the Ranavirus (Figure 3.2). Broods produced by Known Parents A and B did not have any infections in their offspring, nor were any of the parents infected with the Ranavirus. The gels can be found in Appendix E. The negative controls did not show any contamination and the positive controls were positive for the Ranavirus.

Table 3.1. FV3 infection rate over time of eggs and tadpoles from the five lab raised wood frog populations from wild collected broods and the brood of Known Parents A that was laid in the lab, and infection rate of the eggs from the two of the three broods laid in the lab (n = 5 eggs or tadpoles/sample/date).

	Gosner Stage			
Population	**Eggs**	**24 - 26**	**25 - 35**	**38 - 44**
Country Road 6	0%	0%	0%	0%
Oliver Pond	25%	0%	0%	0%
Crowe's Line Road	20%	33%	0%	0%
Division at Donwood	20%	0%	0%	0%
Barb's Marsh	20%	0%	0%	0%
Known Parents A	0%	0%	0%	0%
Known Parents B	0%			
Known Parents C	20%			

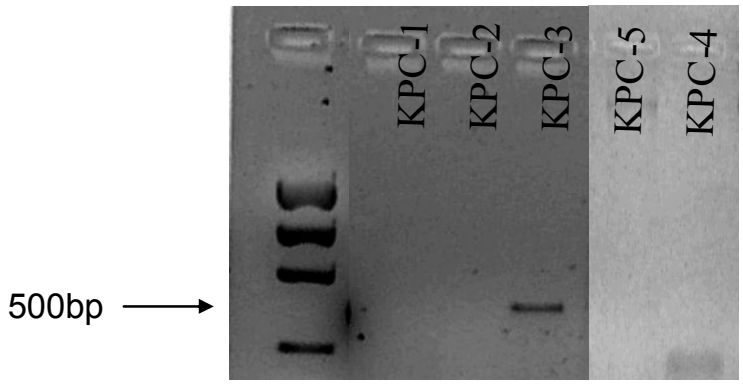


Figure 3.2. Molecular analysis of eggs of captive bred brood C. The presence of a band at the 500 base pair (bp) level indicates the presence of the Ranavirus. KPC indicates the parentage of the brood and the number represents the sample number (n=5). (This image is a composite of three separate gels.)

FV3 Infection Trials

To determine if wood frog tadpoles can be infected with FV3 through the water, wood frog tadpoles were exposed to 670 PFU/mL of FV3 in water. The 670 PFU/mL infection trial resulted in 100% infection rate of individuals exposed to the virus, while the controls were free from the virus (Figure 3.3). None of the tadpoles died during this exposure and none of the tadpoles in the 670 PFU/mL FV3 treatment exhibited any clinical symptoms of illness, such as haemorrhages. Even though the banding in the gels for the infected tadpoles varied in signal strength, it does not necessarily indicate that the tadpole carried a varying viral load; it is only possible to indicate the presence/absence of the virus. When bullfrog tadpoles were exposed to low levels of FV3 via the water, none of the tadpoles became infected with the virus. This indicates that at low concentrations, FV3 do not necessarily cause infections in ranids. The gels can be found in Appendix E. The negative controls did not show any contamination and the positive controls were positive for the Ranavirus.

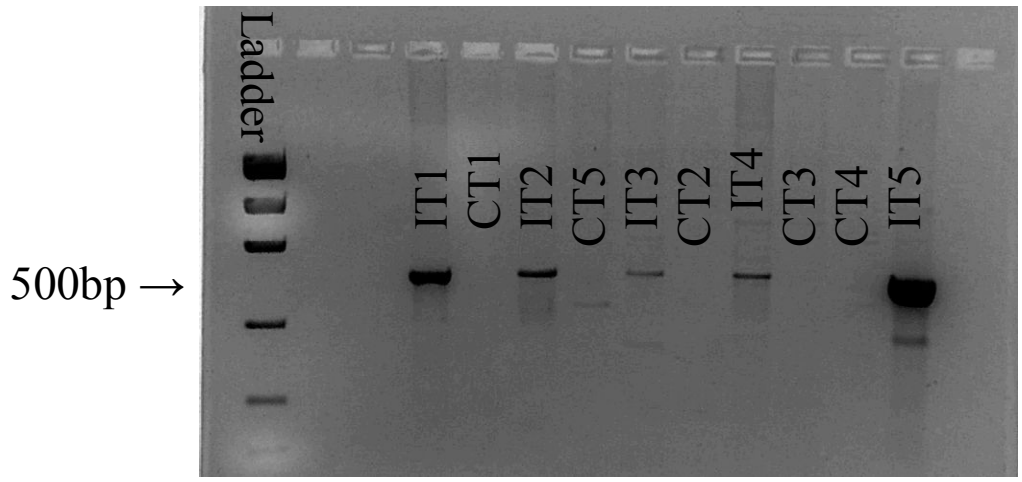


Figure 3.3. FV3 infection of wood frog tadpoles exposed to water inoculated with 670 PFU/mL of FV3 for five days. Presence of a band at the 500 base pair (bp) level indicates the presence of FV3. IT are the tadpoles from the FV3 treatment (all are infected) and CT are the tadpoles from the control treatments (none are infected) (n = 5 for each treatment).

Horizontal Transmission

Wood frog tadpoles were exposed to different concentrations of FV3 to determine the effect of concentration on both survivorship and the infection rate. Both the survivorship and infection rates tend to be correlated with the concentration of FV3 to which the tadpoles were exposed. As the concentration of FV3 increased, the survivorship decreased (Figure 3.4). Until day 5, the survivorship for all treatments is comparable, but then the mortality of tadpoles exposed to the 6700 PFU/mL FV3 treatment increased sharply. In the control, 67 PFU/mL and 670 PFU/mL treatments, survivorship shows the same trends until day 7, when the control treatment stabilized and the other treatments continue to decline. The infection rate of the wood frog tadpoles exposed to the different concentrations of FV3 increased as the concentration of FV3 increased (Figure 3.5). Mortality in the controls and 67 PFU/mL FV3 treatment cannot be explained by disease induced mortality, the cause of death is unknown and may be associated with water quality. The sample sizes were 20 tadpoles/exposure level. The gels can be found in Appendix E. The negative controls did not show any contamination and the positive controls were positive for the Ranavirus.

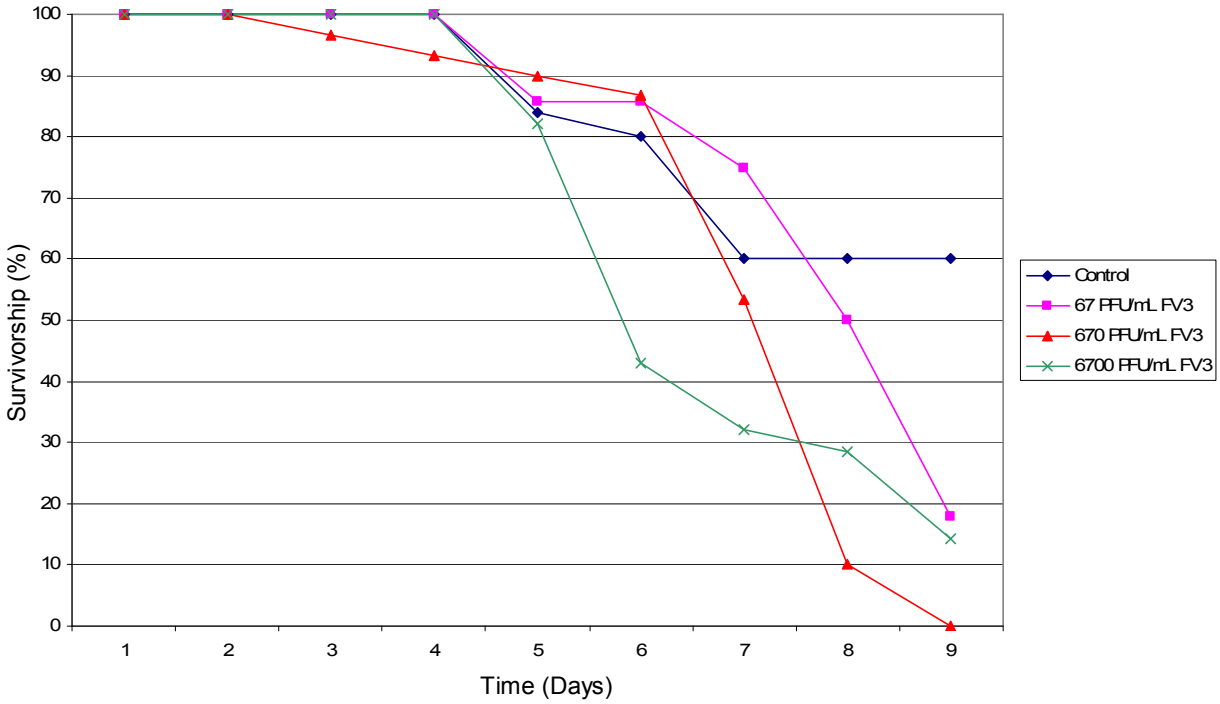


Figure 3.4. Survivorship of wood frog tadpoles exposed to different concentrations of FV3. The concentrations of FV3 used were 67 PFU/mL, 6.7 x 10^2 PFU/mL, and 6.7 x 10^3 PFU/mL, respectively. (n = 30)

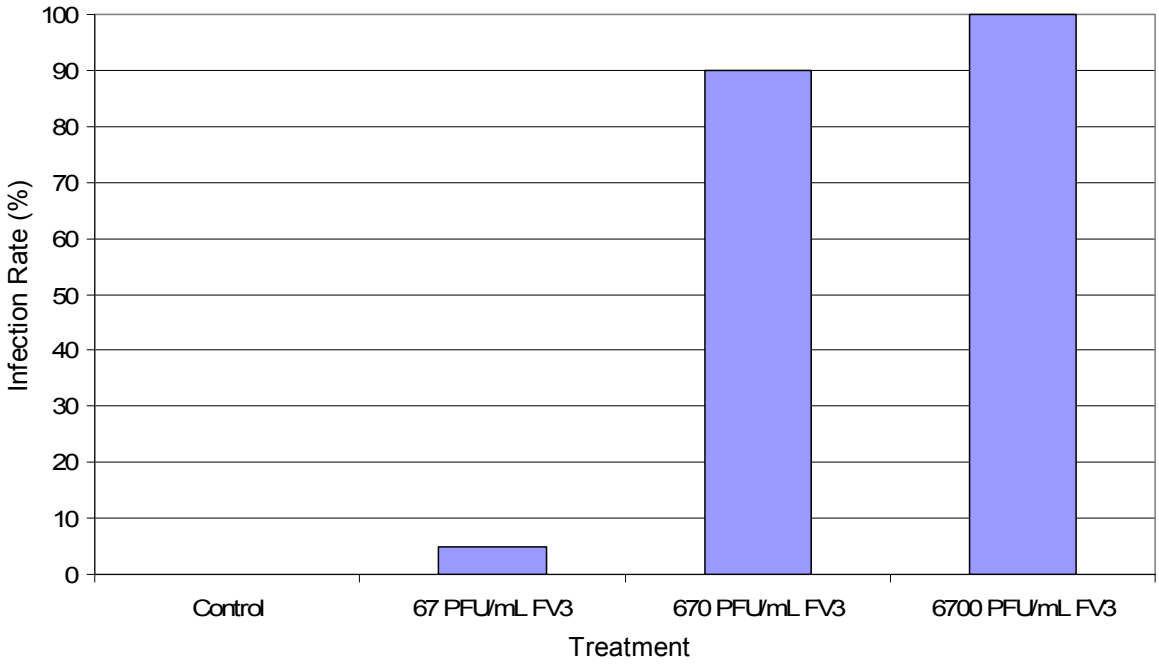


Figure 3.5. Infection rate of wood frog tadpoles exposed to the different concentrations of FV3. Tadpoles that were tested were randomly chosen from tadpoles that died over the course of the experiment and those that were euthanized at the end of the experiment. The concentrations of FV3 are 67 PFU/mL, 6.7 x 10^2 PFU/mL, and 6.7 x 10^3 PFU/mL, respectively. (n = 20)

Interspecies Transmission

Wood frog tadpoles that were raised in the lab were exposed to blue-spotted salamander larvae collected from Parker Pond, in this pond the tadpoles of other anuran species were exhibiting some signs of Ranaviral infections, such as remaining close to the surface of the water and having softer bellies. Tadpoles in all trials exhibited symptoms commonly observed in the wild. Tadpoles remained at the surface of the water in the aquaria, which is typical of tadpoles infected by the Ranavirus, whereas the salamander larvae remained at the bottom of the tank. The control wood frog tadpoles had an infection rate of 0% (n=10). Initially the infection rate in the salamander larvae was 43%, but over the course of the experiment, they transformed into sub-adults, and the infection rate dropped to 0% (n=5). The wood frogs that were exposed to the salamander larvae developed infection rates of 25% and 33%.

Some contamination occurred in the DNA extraction process, as the negative controls were positive for the Ranavirus, and the tadpoles involved were eliminated from the data.

Discussion

Vertical Transmission

This experiment provides the first evidence that vertical transmission of the Ranavirus may be occurring in wood frogs. The eggs of one of the three broods produced in the lab were infected with the virus. Because of the extreme precautions that were taken to avoid contact with any external sources of the virus, it is likely that this brood was exposed to the Ranavirus through the infected male parent. This evidence of vertical transmission is further supported by the fact that the two broods that were not infected with the virus also had uninfected parents. There remains the possibility that the eggs could have become infected through contact with the virus through the water from the male and not directly from the male gametes. However, the broods of eggs that were produced in the lab were quickly removed from the water containing their parents soon after they were deposited, and such transmission is unlikely.

It is interesting that the virus was apparently passed onto the offspring by the male parent. Since the reproductive tract in amphibians shares common anatomy with the urinary and gastro-intestinal tracts, there are several opportunities for sperm or eggs to come into contact with tissue potentially infected with the Ranavirus. An Iridovirus, which was associated with salamander mortality events in Utah, was cultured and isolated from the testes of affected salamanders (Docherty *et al*. 2003). The virus was also isolated from the gut, liver and kidneys of affected salamanders in the same mortality event (Docherty *et al*. 2003). It is, therefore, possible that the testes of the infected wood frog were also infected with the Ranavirus. Therefore, the initial exposure

to the virus may have occurred during spermatogenesis. Further exposure to the virus could be a result of the shared ureter/gonaduct. Since sperm is usually accompanied by some sort of seminal fluid, there is a chance that shed virus will be present in these secretions. Upon fertilization, the sperm comes into direct contact with the egg before the egg membranes absorb water. Without the protective membranes around the egg, the virus could likely pass easily from the sperm or the seminal fluid to the egg.

Two of the five broods of eggs that were laid in ponds, and were collected soon after they were laid, were also infected. The eggs would have been exposed to pond water which was cold because of the early spring conditions and where the virus, if present, would have been at low levels and unlikely able to infect via waterborne transmission because of the low temperatures. The wood frog population present at Oliver Pond has been experiencing yearly Ranaviral infections since the first outbreak occurred in 1999 (Greer *et al*. 2005). Since outbreaks of the Ranavirus have been occurring on a yearly basis since 1999, even though the occurrence of mass mortality events has diminished, it is not surprising that wood frog eggs were infected with the Ranavirus. The wood frogs in Barb's Marsh are also known to have been infected with Ranavirus in 2003 (Charbonneau 2006).

Therefore, it is likely that at least one of the parents of the broods collected from those two ponds were infected with the Ranavirus and that some of the breeding adult wood frogs in these populations are also infected with the virus. Although vertical transmission is difficult to confirm, these observations suggest that it does occur.

The infection rate of adult wood frogs is unknown, however it is possible that adults can carry a viral load and not show symptoms of Ranaviral infections as has been

found in other species of amphibians. Leopard frogs (*Rana pipiens*) collected from Gannon's Narrows (44°29', 78°28') carried Ranaviral loads and exhibited no symptoms of infection (Greer *et al*. 2005; Duffus *et al*. in review). Tiger salamanders (*Ambystoma tigrinum nebulosum*) with sub-lethal infections of *Ambystoma tigrinum* virus (ATV) can also transmit the virus to other animals (Brunner *et al*. 2004). Also, the male parent of brood C did not show any symptoms of Ranaviral infection. Hence it is not unlikely that adult wood frogs could carry sub-lethal viral loads and pass the virus on either through vertical transmission or through contact with the water that is absorbed into the egg membranes.

Although, there are no long term data available for the populations of wood frogs in the ponds on Crowe's Line Road, Division at Donwood Road, or Country Road 6, other wood frog populations in adjacent ponds have been found to carry the virus but show no symptoms (Charbonneau 2006). Therefore, it is not unlikely that the wood frog populations from the two other ponds contained infected breeding adults. The possibility of vertical transmission of the virus in wood frogs cannot be rejected.

FV3 Infection Trials in Wood Frogs and Bullfrogs

Although other studies have examined the presence of Ranaviral infections in the wild (e.g. Bollinger *et al.* 1999; Green *et al.* 2002; Docherty *et al.* 2003; Greer *et al*. 2005), few have examined the possible mechanisms or modes of transmission of the Ranaviruses. Brunner *et al*. (2004) examined the potential for Ranaviruses to have intraspecific reservoirs, such as ambystomid salamanders. To determine this, ambystomid larvae were exposed to *Ambystoma tigrinum* virus (ATV) (a Ranavirus) via water baths

to create infections in larval salamanders - efficient transfer of the virus occurred (Brunner *et al*. 2004).

The current study provides concrete evidence that FV3 is also a waterborne disease for wood frogs (*Rana sylvatica*) tadpoles. Since all wood frog tadpoles that were exposed to 670 PFU/mL of FV3 became infected with FV3, transmission of Ranaviruses through the water is likely to be a common mode of transmission. Bullfrog tadpoles that were exposed to low concentrations of FV3 via the water did not develop FV3 infections indicating the concentration of FV3 was likely below the critical level required to cause infection in bullfrog tadpoles. Although more experiments are needed to clarify this point, the results suggest that even at 20˚C, virus concentrations in ponds need to reach a critical level for infection to occur.

Since two different species of ranid tadpoles were used, there is a possibility that they differ in susceptibility to FV3. Also the small sample size used may not be representative of what occurs in the wild, since it is not known what proportion of the tadpoles in either species must be infected to maintain the presence of the Ranavirus in these populations.

Horizontal Transmission

Horizontal transmission of disease is considered the most common method of transmission between individuals. This appears to be true of salamanders infected with ATV where mortality and infection rates of tiger salamander larvae and metamorphs increased with water concentration of ATV up to 10^4 PFU/mL, after which there was no increase in the mortality and infection rates (Brunner *et al*. 2005). Wood frog tadpoles

exhibited a similar trend when they were exposed to different concentrations of FV3 in the present study. As the concentration of the virus in the water increased, the mortality and infection rates tended to increase. However, because of the mortality experienced in the control treatment, the dose effect on mortality is suggestive but not conclusive.

However, the data on infection rates in the wood frog tadpoles exposed to increasing concentrations of FV3 show a tendency for increased infection rates with increased concentrations of FV3. Between the 67 PFU/mL and 6700 PFU/mL treatments of FV3 the infection rate increased from 5% to 100%. Therefore, as was observed in salamanders (Brunner *et al*. 2005), the higher the concentration of virus particles, the greater the likelihood of a virus particle penetrating the animal's immune defences, up to some saturation point. After that point has been reached there is a large enough viral concentration in the surrounding water, and the tadpole becomes overwhelmed and unable to deal with the virus effectively. At low FV3 concentrations, infection remains relatively unlikely. This further supports the possibility that vertical transmission also occurs in wood frogs because it is unlikely that the concentration of the virus is high enough in the surrounding water in early spring conditions to infect eggs.

Interspecies Transmission

In tiger salamanders, it is thought that adults act as a reservoir of ATV as recently metamorphosed salamanders carried sub-detectable levels of the ATV and remained infective to others (Brunner *et al.* 2004). In the current study, wood frog tadpoles that were exposed to infected blue–spotted salamander larvae became infected with the Ranavirus. Inter-species transmission of Ranaviruses between taxonomically distinct

animals has also been established by Mao *et al*. (1999). Therefore, it is likely that that the Ranaviruses can jump between amphibian species and it is possible that ambystomids may act as a reservoir of the virus in many amphibian communities.

Conclusions

FV3 can be transmitted through the water to wood frogs. However, the likelihood that the virus will cause infections in wood frogs is highly dependant on the concentration of FV3 that the tadpoles are exposed to. There is also evidence that vertical transmission of the Ranavirus from parent to offspring may occur. However, since larval amphibians spend all of their time in the water, the likelihood of coming into contact with the virus that has been shed by infected individuals should be quite high. Also, susceptible individuals may come into contact with infected individuals and the virus may be passed through this contact within and between species. Infections and re-infection of wood frog tadpole populations can therefore occur through both vertical and horizontal transmission. Sub-lethally infected wood frog adults or ambystomids may act as reservoirs of the Ranavirus.

Chapter 4: Interactive Effects of Malathion Exposure and Ranaviral Infections in Wood Frog (*Rana sylvatica*) and Bullfrog (*Rana catesbeiana*) Tadpoles.

Introduction

Amphibian populations are declining on a global scale (Stuart *et al.* 2004). There are many potential causes of these declines such as habitat modification, increasing ultra-violet radiation levels, predation, climate change, chemicals, and emerging infectious diseases, as well as interactions between these factors (Alford and Richards 1999). Since amphibians are a critical link between aquatic and terrestrial ecosystems, their decline on a global scale is likely to have resonating repercussions in both ecosystems. Emerging infectious diseases are beginning to receive a large amount of attention for their roles in these declines. Pathogens such as *Batrachochytrium dendrobatidis*, the chytrid fungus (Carey *et al.* 1999) and Iridoviruses (Carey *et al.* 1999; Chinchar 2002) are having very negative effects on amphibian populations around the world (Carey et al. 1999; Chinchar 2002). Larval anuran amphibians that inhabit aquatic ecosystems are subject to many types of chemical stressors that originate from human sources; the main ones include pesticides, herbicides, and nitrates. The interaction of these anthropogenic stressors with naturally occurring stressors is becoming an important avenue of research. In this study, I assess the interaction of a Ranavirus (Family: *Iridoviridae*), frog virus 3 (FV3), and a known immunosuppressant, the organophosphorus pesticide malathion, on mortality and abnormal behaviour of exposed wood frog (*Rana sylvatica*) and bullfrog (*R. catesbeiana*) tadpoles.

Only a few experiments have examined the interaction between stressors. For example when amphibians in ponds were exposed to UV-B radiation and an organophosphorus pesticide, the combination of the UV-B radiation and carbaryl unexpectedly did not increase the toxic effects of exposure to carbaryl alone (Bridges and Boone 2003). The effects of multiple pesticide exposure on the growth and survival of *Rana pipiens*, *R. catesbeiana*, *R. clamitans*, *Bufo americanus* and *Hyla versicolor* tadpoles was assessed through exposure to carbaryl, diazinon, malathion and glyphosate and controls (Relyea 2004a). Growth decreased when the tadpoles were exposed to the pesticides in combination (Relyea 2004a). However, the species responded in different ways to exposure to two pesticides on survivorship and one species, *Rana pipiens*, did not experience a significant reduction in survivorship (Relyea 2004a). In another experiment the effect of the presence of a predator (*Ambystoma maculatum* larvae, the yellow spotted salamander) and a pesticide (Carbaryl) on *Hyla versicolor*, grey tree frog tadpoles, was examined (Relyea and Mills 2001). When low levels of carbaryl were used, the survivorship of tadpoles was significantly less when a predator was also present than when the predator was absent, however, when high concentrations of carbaryl were used, this difference was not observed (Relyea and Mills 2001). In a related experiment, six different species (*Rana pipiens*, *R. sylvatica*, *R. catesbeiana*, *R. clamitans*, *Bufo americanus* and *Hyla versicolor*) were exposed to different concentrations of malathion, with and without, the predator *Notophthalamus viridescenc*, the eastern spotted newt (Relyea 2004b). In only one species and at only one concentration of malathion did the presence of the predator have any effect (Relyea 2004b). No generalized conclusions about the likelihood of interactive effects can be drawn from these studies.

A more reliable experiment examined the effects of exposure to a pathogen, *Rhadbias ranae*, a nematode, in combination with exposure to a pesticide in leopard frogs (*Rana pipiens*) (Christin *et al.* 2003). Animals that were exposed only to the pesticide cocktail showed a significant decrease in T-cell proliferation when compared to the control frogs (Christin *et al.* 2003). When T-cells proliferate at low rates, the activation of B-cells is also reduced and therefore antibody production is reduced, which would facilitate proliferation of disease (Christin *et al.* 2003). The infection severity increased with the concentration of pesticide cocktail (Christin *et al.* 2003). After exposure to the highest concentration of pesticides for 21 days, leopard frog metamorphs showed a decrease in their ability to react to infections and had the highest nematode infection rates (Christin *et al.* 2003). Thus, exposure to pesticides increased the likelihood and the severity of nematode infections.

Another experiment examined the interaction between atrazine and the *Ambystoma tigrinum* virus (ATV) infection in long-toed salamander (*Ambystoma macrodactylum*) larvae (Forson and Storfer 2006). The larvae that were exposed to both the ATV and atrazine showed lower levels of mortality than those which had been exposed to ATV alone, suggesting that atrazine reduces ATV's infectivity (Forson and Storfer 2006). Therefore, certain environmental contaminants may reduce the virulence of some Ranaviruses. In further support of the probability of interactions between stressors, when the eggs of two ranid species were exposed *in situ* to both the fungus, *Saprolegnia*, and natural UV-B radiation, the hatching rates were significantly reduced when compared to either treatment alone (Kiesecker and Blaustein 1995). This result indicated that anthropogenically caused stressors have the potential to negatively interact

with naturally occurring stressors. However, in my experimental exposures, only limited signs of pathogenesis occurred that could be attributed to an FV3 infection (See Chapter 3). Therefore, I tested whether an FV3 infection, in combination with an immunosuppressant chemical, malathion, can induce the same degree of symptoms that are seen in natural outbreaks of FV3. The experiment is also designed to test the immunosuppression hypothesis of amphibian declines. This hypothesis states that environmental factors, such as increasing temperatures or the presence of anthropogenic chemicals, suppress the amphibian immune system and make the affected amphibians more susceptible to disease. Ultimately, population decline and the extinction of affected amphibian species may ensue.

Frog virus 3 (FV3) is an Iridovirus (Chinchar 2002). This group of viruses has been responsible for many amphibian mortality events in North America: the species affected include ambystomids (Bollinger *et al.* 1999; Green *et al.* 2002; Docherty *et al.* 2003) and anuran amphibian larvae (Green *et al.* 2002; Greer *et al.* 2005). FV3 infection has a high mortality rate in wood frog (*Rana sylvatica*) tadpoles (Green *et al.* 2002; Greer *et al.* 2005). When an FV3 outbreak occurs, the mortality events usually continue to re-occur for several years and are usually localized (Carey *et al.* 1999; Greer *et al.* 2005). The symptoms of an FV3 infection are quite dramatic. External symptoms involve endema in the abdominal area and ventral haemorrhages, while internal symptoms present themselves as distended gastrointestinal tracts, which may have haemorrhages and/or contain blood or clear fluid, and the livers that are often discoloured (Bollinger *et al.* 1999; Docherty *et al.* 2003).

Mass mortality events in natural populations of wood frogs and bullfrogs (as well as many other species of amphibians) have been attributed to FV3 and other Ranaviral outbreaks (Bollinger *et al.* 1999; Green *et al.* 2002; Docherty *et al.* 2003; Greer *et al.* 2005).

It is well-known that many different pesticides are toxic to amphibians (e.g. Berrill *et al*. 1993; Berrill *et al*. 1994; Berrill *et al*. 1995). Malathion is an organophosphorus pesticide, which is commonly used in both rural and agricultural areas to control insect pests, especially mosquitoes. As a group, the organophosphorus pesticides were introduced to replace the organochlorine pesticides (e.g. DDT) (Galloway and Handy 2003). However, this group organophosphorus pesticides also has high acute toxicity in many non-target species (Galloway and Handy 2003). The use of malathion was the cause of mass mortality in fishes in US coastal areas in the 1980s (Key *et al.* 1998). The primary mode of action of organophosphorus pesticides is through the inhibition of acetylecholinesterase activity, which leads to disrupted neural function (Galloway and Handy 2003). Malathion is able to target insects preferentially because vertebrates possess high levels of carboxyesterase enzymes in their cells, which convert the pesticide and its metabolites by hydrolysis into non-toxic compounds that are easily extruded from cells (Blasiak *et al.* 1999; Venturino *et al.* 2001). Malathion exposure can also have genotoxic effects both *in vitro* (humans) (Blasiak *et al.* 1999) and *in vivo* (mice) (Giri *et al*. 2002). However, it is the breakdown products of malathion that are responsible for these genetic effects *in vitro* situations (Blasiak *et al.* 1999) and likely *in vivo* as well. Increasing the amount of malathion that an individual is exposed to

increases the genetic damage that results from exposure (Blasiak *et al.* 1999; Giri *et al.* 2002).

In anuran amphibians, malathion has a tendency to accumulate quickly in tissues, especially in adipose tissue (Venturino *et al.* 2001). In *Bufo arenarum* tadpoles, the action of carboxylesterase enzymes is inhibited (Venturino *et al.* 2001). Malathion exposure also affects the immune system, for it also acts as an immunotoxic/suppressive agent (Galloway and Handy 2003). Exposure to some chemicals, including malathion, at sub-lethal levels results in the disruption of both humoral and innate immune function in mammals, fish and amphibians (Cooper and Parrinello 1996). The humoral branch, as well as the cell-mediated response of the immune system, are key in the defence against specific pathogens, especially viruses. In tadpoles, the immune system appears mainly to protect the animal against pathogens because otherwise there would be problems with autoimmunity during metamorphosis (Flajnik 1996). Therefore, any disruption in the function of the humoral immune system is likely to leave a tadpole more vulnerable to pathogens. When adult northern leopard frogs (*Rana pipiens*) were injected with malathion, the immune system lost the ability to produce any significant number of antigen specific antibodies (Gilbertson *et al.* 2003). However, malathion exposure also affects the innate immune response in amphibians (Gilbertson *et al.* 2003). Hence, exposure to malathion affects both aspects of the immune system and should increase the susceptibility of amphibians to infection by pathogens. For example, juvenile leopard frogs that were exposed first to a mixture of agricultural chemicals and then to a nematode, had reduced lymphocyte proliferation as well as increased nematode infection rates (Christin *et al*. 2003).

This study investigated the possible interactive effects of malathion exposure and concurrently induced infections of the emerging infectious disease, frog virus 3 (FV3) in wood frog (*Rana sylvatica*) and bullfrog (*Rana catesbeiana*) tadpoles. Exposure to an immunosuppressant in combination with FV3 should increase tadpole mortality, increase both the onset and severity of abnormal behaviours, and increase infection rates in tadpoles. It also will test the plausibility of the immunosuppression hypothesis in a controlled setting.

Methods

Wood Frog (Rana sylvatica) Experiment

Collection of Tadpoles

Three terrestrially amplexing pairs of wood frogs (*Rana sylvatica*) were captured near Bobcaygeon, Ontario Canada (44°33N 78°33W) (Duffus and Ireland, in review). The animals were returned to the lab and provided with an anchored twig as an oviposition site. Two of these pairs produced viable broods of eggs. After the broods were laid, they were then transferred to 5-gallon aquaria, partially filled with aged tap water, and permitted to develop and hatch.

Rearing of Tadpoles

As the tadpoles hatched from the eggs, the jelly was removed to avoid death induced from the decomposition of the jelly masses in the aquaria. Throughout the first weeks of development, water changes occurred whenever the water attained a light

greyish tinge. After the tadpoles began feeding, the water was then changed every three days to avoid the build up of toxic waste products. Tadpoles were kept in 5-gallon tanks in a 15°C environmental chamber for about two weeks to ensure normal development and then moved to a 20˚C lab for experimental exposures. Tadpoles were fed boiled and shredded spinach daily. When the experimental exposures began, the tadpoles varied in extent of development from Gosner Stage 25 to Gosner Stage 44, the majority of tadpoles being between Gosner stage 30 and 40.

Exposure to FV3 and Malathion

Tadpoles were placed in 1L beakers for 12 hours containing 150mL aged tap water with the concentration of 670 PFU/mL of FV3 and/or 3mg/L or 10mg/L of malathion. The beakers were left un-aerated for four to five hours at the beginning of the experiment to allow for maximal exposure to FV3, but were aerated for the remainder of the experiment. The water volume was increased to 350mL 16 hours post-infection. The initial low water volume was intended to expose tadpoles to high enough levels of FV3 to cause infections. At 48 hours, all treatments that involved malathion were re-inoculated with the pesticide because of malathion's short half-life. The volume of stock malathion added to each treatment was adjusted to accommodate the new water volume of 350mL. At 72hrs, tadpoles were moved to fresh water, free from both malathion and FV3; the water was changed again on day six; and on day eight the experiment was terminated. A treatment was terminated earlier when only one replicate remained in which there was less than a third of the original number of animals still alive. Animals that died over the course of the experiment were removed and preserved in 70% ethanol to be analyzed for

evidence of FV3 infections. Throughout the experiment observations about the abnormal behaviour and appearances of the tadpoles were taken. These observations included abnormalities in feeding behaviour, avoidance response and swimming behaviour. The experiments were performed in duplicate with 15 animals in each treatment.

Bullfrog (Rana catesbeiana) Tadpole Experiment

Animal Collection and Husbandry

Bullfrog tadpoles were raised from eggs in outside enclosures at Trent University for use in other experiments unrelated to this study. Excess tadpoles were obtained from these enclosures and the tadpoles were transferred to 5-gallon aquaria filled with aged tap water, with approximately 150 animals per aquarium. They were fed a combination of spinach and fish food (to act as a protein supplement) as needed and the water in the aquaria was replaced every three days.

Exposure to Malathion and FV3

Tadpoles (Gosner Stage 25) were placed in 200mL of aged tap water in 1L beakers. Five tadpoles were placed in each beaker, with three replicates per treatment. Exposure treatments consisted of malathion concentrations of 1mg/L, 3mg/L, 7mg/L and 10mg/L, both with and without FV3 at 80 PFU/mL in the initial water concentration; FV3 only; and a control without either FV3 or malathion.

The beakers were left unaerated for approximately six hours at the beginning of the experiment, but were aerated for the remainder of the experiment. The water level was increased to 400mL of water 16 hours post-infection. At 48 hours, all treatments

which involved malathion were re-inoculated with the pesticide because of malathion's short half-life. The volume of stock malathion added to each treatment was adjusted to accommodate the new water volume of 400mL. After 72 hours, the tadpoles were given fresh water, free from malathion or FV3. The water was changed again on day six and the experiment was terminated on day nine. Animals that died over the course of the experiment were removed and preserved in 70% ethanol for analysis for the presence of FV3. Throughout the experiment observations about abnormal appearance and behaviour of the tadpoles were taken. These observations included abnormalities in feeding behaviour, avoidance response and swimming behaviour. Tadpoles were fed SeraMicron (Sera) on a daily basis.

Biosafety Procedures

Field Precautions

To prevent the trafficking of the virus between field sites, all equipment was sprayed with a 30% bleach solution and permitted to air dry after each site was visited to eliminate any pathogens from the surfaces of the equipment. These precautions were developed at Arizona State University by the Collins Lab. All procedures followed were approved by the Animal Care Committee and the Biosafety Committee at Trent University.

Laboratory Precautions

In the lab, preventing the spread of the virus from potentially infected animals to uninfected animals, as well as to other areas, was also essential. Aquaria were subjected

to a three day wash cycle before reuse: on the first day the aquaria contained 30% bleach solution, on the second and third days the aquaria were filled with clean tap water. All animals were kept in a limited access facility. All equipment used in the experiments was also decontaminated before being used in any other experiments or being removed from the laboratory. Upon entrance and exit of the facility, the use of a disinfectant footbath was used to prevent viral spread. All materials of animal origin were disposed of through the Trent University Animal Care Facility's waste disposal program.

Experiments that involved direct manipulation of the virus were conducted in a Level 2 Biohazard Laboratory. Wastewater from these experiments was disinfected with a bleach solution (minimum 30%) and was disposed of through the municipal sewage system. Equipment used was also disinfected with the three day cycle, using a minimum of 50% bleach. After disinfection with the bleach, the equipment was again washed with a caustic home cleaner. The exterior of the glassware used was wiped down with 100% ethanol. All procedures followed were approved by the Animal Care Committee and the Biosafety Committee at Trent University.

PCR Analysis of FV3 Infections

Tadpoles were dissected and a small sample of hepatic tissue was removed to be analyzed for the presence of the FV3 using PCR. Please see Appendix A: - Dissection Procedures and Molecular Analysis for the Presence of the Ranavirus. An individual was considered to be infected with the Ranavirus if it tested positively.

Results

Wood frogs

Exposure to FV3 and Malathion

Wood frog tadpoles were exposed to malathion, FV3 or both. Exposure to both FV3 and malathion appeared to result in increased mortality compared with either treatment in isolation. Malathion inoculations were performed at zero and 48 hours. Mortality was greatest in the first 24 hours following each inoculation with malathion in the 10mg/L Malathion + FV3 treatment (Figure 4.1). The same trend occurred in the 3mg/L Malathion + FV3 treatment, except the increase in mortality was not as severe at the 24 hour mark. The greatest mortality for all treatments (except the 10mg/L Malathion + FV3) occurred between 48 and 72 hours. Greater mortality appears to be associated with the 24 hour time period after the malathion inoculations. Mortality in the FV3 only treatment was similar to that observed in the controls. Mortality in the control treatment increased to 64% at 120 hours, suggesting that other factors than treatment differences could be causing subsequent mortality. Analysis of the data after 120 hours was not attempted.

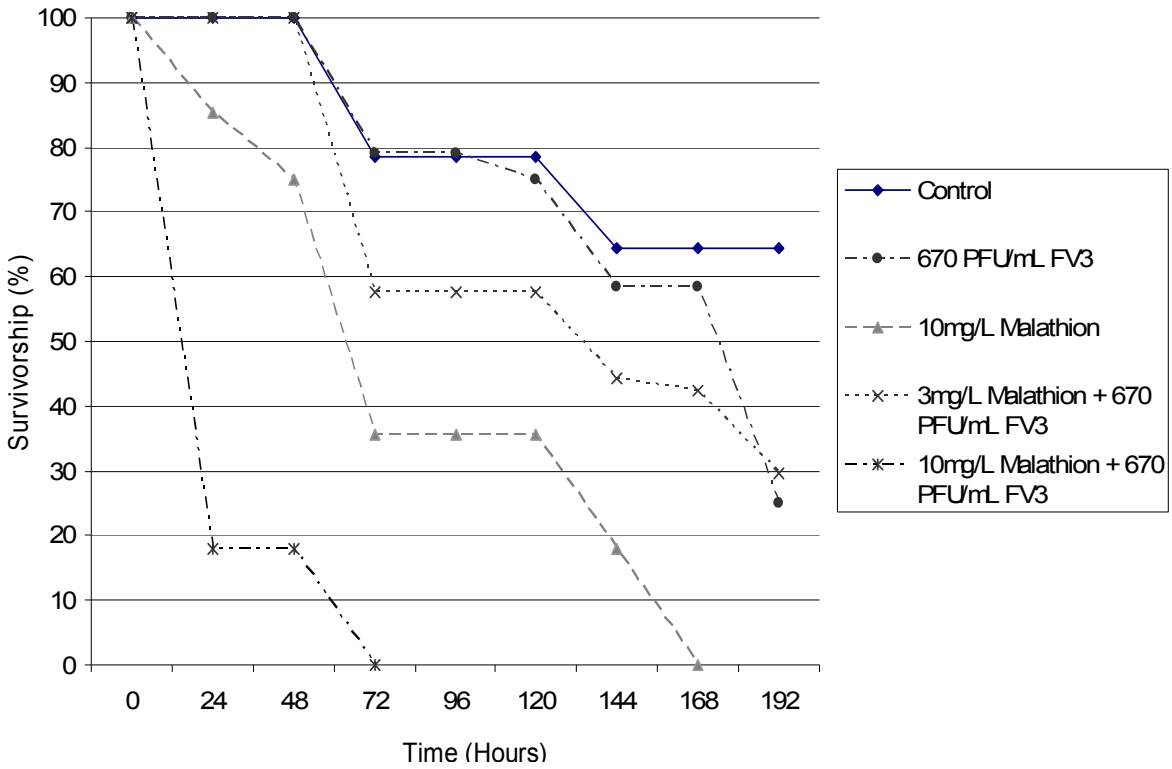


Figure 4.1. Mortality rates of wood frog tadpoles associated with malathion and FV3 exposure. Tadpoles were exposed to combinations of either 3mg/L or 10mg/L Malathion and/or 6.7 x 10^2 Plaque Forming Units (PFU)/mL in 1L beakers with 150mL of aged tap water. After 16hrs, the water level was increased to 350mL. Treatments which involved malathion were re-inoculated with malathion after 48hrs because of the short half-life of malathion. Animals that died over the course of the experiment were removed from the beakers. The experiment was run with two replicates per treatment and the data from each treatment was pooled (n = 30). The 3mg/L Malathion only exposure was eliminated from analysis because of an unexpected experimental catastrophe.

Observations of Abnormality in Experimental Tadpoles

Throughout the exposure to malathion and/or FV3, tadpoles were observed for evidence of the development of clinical symptoms of an FV3 infection and the effects of malathion exposure. The day when more than half of the tadpoles in a treatment exhibited a particular symptom was considered to be the day of onset for that symptom. The following symptoms were observed:

- disturbed swimming - the tadpoles swam in circles and were unable to maintain equilibrium
- lethargy - the tadpoles did not exhibit much movement and usually sat on the bottom of the beaker
- reduction in feeding - the tadpoles did not actively seek out food and their waste output was diminished
- loss of avoidance behaviour - the tadpoles lost the ability to respond by darting around when the beaker was gently shaken

A final symptom likely to be associated with developing FV3 infections because of its later onset (i.e. they do not appear within hours of malathion inoculation) is:

- ventral haemorrhage - blood spots which were likely the result of burst capillaries in the skin.

These symptoms are not additive but the tadpoles have probably passed from one symptom group to the next, even if the transitions occurred in rapid succession. I constructed the following scale of degree of abnormality in order to provide an estimation of the level of abnormality that the tadpoles were experiencing:

Level 1 – Lethargy/Disturbed swimming

Level 2 - Reduction in feeding

Level 3 – Loss of avoidance behaviour

Level 4 – Ventral haemorrhaging.

The level of abnormality was classified when two thirds of all animals in the treatment exhibited the symptoms. Observations were made once per 24 hour period.

Tadpoles in the 10mg/L Malathion + 670 PFU/mL FV3 treatment exhibited a rapid onset of symptoms, with the most severe symptoms occurring within only 24 hours. Tadpoles in the treatments that included exposure to malathion exhibited symptoms of chemical exposure only a few minutes after inoculation with the chemical. Only in the treatments that included both FV3 and malathion did the tadpoles exhibit the most severe symptoms. Tadpoles in the treatments that were only FV3 or malathion only experienced a loss of avoidance behaviour near the end of the experiment. Tadpoles in the control treatment did not exhibit any symptoms. (Figure 4.2 A - D)

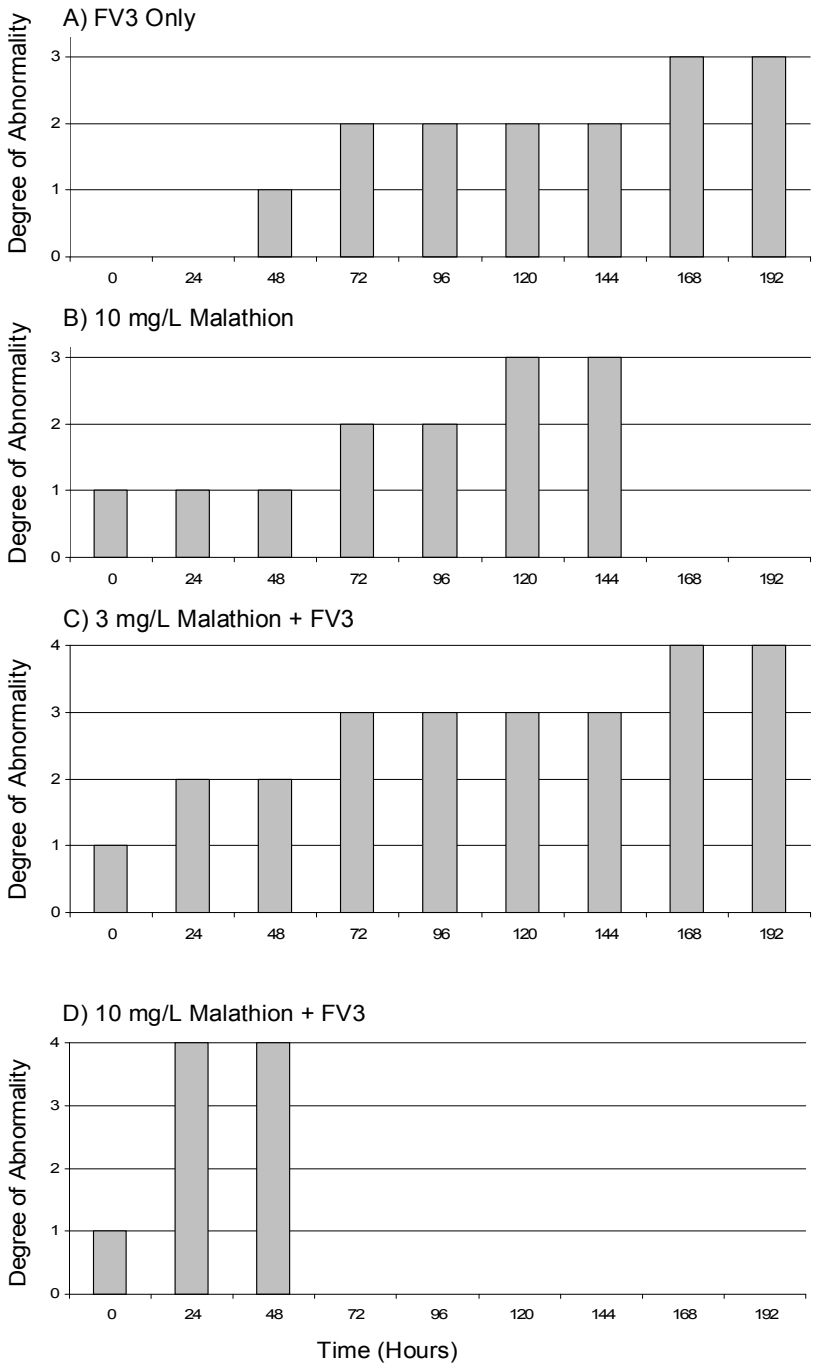


Figure 4.2. Observations of the onset of clinical symptoms of wood frog tadpoles exposed to malathion and/or FV3. For detail on the symptoms and levels, see the text.

Infection Rates of Exposed Tadpoles

To determine if tadpoles were infected with FV3, PCR was used to assay for the presence of the virus. All tadpoles that were analyzed from both the control and 10mg/L Malathion treatments tested negatively for presence of FV3 (n=20). Eighty percent of the tadpoles tested from the 670 PFU/mL FV3 treatment tested positive for the presence of an FV3 infection. All of the tadpoles from the 3mg/L Malathion + 670 PFU/mL FV3 treatment tested positive for the presence of FV3. Unexpectedly, only 35% of tadpoles from the 10mg/L Malathion + 670 PFU/mL FV3 treatment tested positive for FV3, but, most had probably died from acute malathion exposure (Figure 4.3). In the treatments in which the tadpoles lived the longest they were more likely to be infected, resulting in the higher infection rates. The gels can be found in Appendix E. The negative controls did not show any contamination and the positive controls were positive for the Ranavirus.

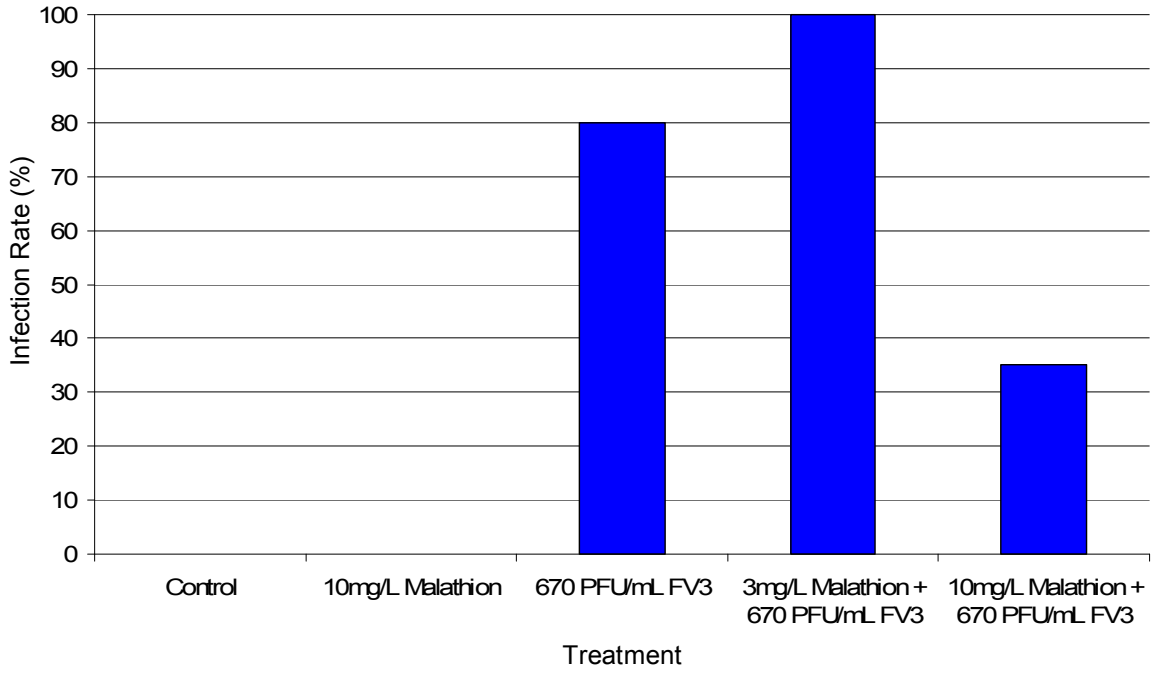


Figure 4.3. Infection rate of wood frog tadpoles used in experimental exposure period - 20 of 30 tadpoles from each treatment were chosen randomly to be tested for the presence of FV3. A sample of hepatic tissue was dissected out of each tadpole and the DNA was extracted from this sample. The presence of an FV3 infection was determined by PCR with a primer targeting a 500 base pair sequence of the major capsid protein (MCP) of the FV3 genome.

Bullfrogs

Exposure to FV3 and Malathion

Bullfrog tadpoles (Gosner stage 25) were exposed to malathion, FV3 or both, in three replicates of five tadpoles. Despite the differences in treatments, little mortality occurred. Of the four tadpoles that died over the course of the experiment, two were exposed to 10mg/L Malathion + 80 PFU/mL FV3 and one died following the second inoculation with Malathion in the 7mg/L Malathion + 80 PFU/mL FV3 treatment. (Table 4.1) The fourth tadpole died immediately when exposed to 80 PFU/mL FV3 only and such a rapid death is unlikely to be a result of exposure to FV3. Though these infection rates are not significant, they at least support the hypothesis that infection should be greatest in exposure to both stressors.

Table 4.1. Cumulative mortality of Gosner stage 25 bullfrog tadpoles exposed to malathion, FV3 or both over a 10 day period. Tadpoles were exposed in groups of five to combinations of either 1mg/L, 3mg/L , 7mg/L or 10mg/L Malathion and/or 80 PFU/mL of FV3 in 1L beakers in 200mL of aged tap water. The experiment was run in triplicate (n = 15/treatment). After 16hrs, the water level was increased to 400mL. All treatments that involved malathion were re-inoculated with malathion after 48hrs because of the short half-life of malathion. The initial exposure period lasted 72hrs, after which all tadpoles were kept in uncontaminated water. Animals that died over the course of the experiment were removed from the beakers.

Time (Hours)	**24**	**48**	**72**	**96**	**120**	**144**	**168**	**192**	**216**	**240**
Control	0	0	0	0	0	0	0	0	0	0
1 mg/L Malathion	0	0	0	0	0	0	0	0	0	0
3 mg/L Malathion	0	0	0	0	0	0	0	0	0	0
7 mg/L Malathion	0	0	0	0	0	0	0	0	0	0
10 mg/L Malathion	0	0	0	0	0	0	0	0	0	0
FV3 Only	1	1	1	1	1	1	1	1	1	1
1 mg/L Malathion + FV3	0	0	0	0	0	0	0	0	0	0
3 mg/L Malathion + FV3	0	0	0	0	0	0	0	0	0	0
7 mg/L Malathion + FV3	0	0	0	1	1	1	1	1	1	1
10 mg/L Malathion + FV3	0	0	2	2	2	2	2	2	2	2

Observations of Abnormality in Exposed Tadpoles

Throughout the exposure to malathion and/or FV3, tadpoles were observed for evidence of the development of clinical symptoms of an FV3 infection and for the effects of malathion exposure. The day when more than half of the tadpoles in a treatment exhibited a particular symptom was considered to be the day of onset for that symptom. A finer scale of symptoms was developed for the bullfrog tadpoles after the completion of the exposure experiment with the wood frog tadpoles. The following symptoms were observed:

- disturbed swimming - the tadpoles swam in circles and were unable to maintain equilibrium
- lethargic but still responsive to food - the tadpoles did not exhibit much movement and usually sat on the bottom of the beaker, but would actively seek out food when fed
- lethargic but not responsive to food - the tadpoles did not seek out food actively when fed.

Symptoms that were likely to be associated with developing FV3 infections because of their later onset are:

- loss of avoidance response to vibration - tadpoles remained immobile when the beaker was gently shaken
- loss of avoidance response to physical disturbance - tadpoles did not avoid or move rapidly after physical contact

- tadpoles floating on or near the surface and slow to respond to disturbance - tadpoles floated at the surface of the water as seen in wild infected populations of tadpoles

The level of abnormality was classified when two thirds of all animals in the treatment exhibited the symptoms. Observations were made once per 24 hour period.

These symptoms are not additive but the tadpoles have probably passed from one symptom group to the next, even if the transitions occurred in rapid succession. I constructed the following scale of degree of abnormality in order to provide an estimation of the level of abnormality that the tadpoles were experiencing:

Level 1 – Disturbed swimming/chemical effects

Level 2 – Lethargic but still responsive to food

Level 3 – Lethargic but not responsive to food

Level 4 – Loss of avoidance response to vibration

Level 5 – Loss of avoidance response to physical disturbance

Level 6 – Tadpoles floating on or near the surface and slow to respond to disturbance.

During the first two days of exposure, the tadpoles in all treatments with malathion exhibited the typical symptoms of chemical exposure, such as disturbed swimming and lethargy. On day three the degree of abnormality of the tadpoles exposed to malathion only peaked, with lethargic animals that were not responsive to food. After that gradual recovery occurred. However, tadpoles that were exposed to 7mg/L Malathion with FV3, as well as 10mg/L Malathion and FV3, increased in the degree of abnormality and did not recover. The tadpoles exposed to 7mg/L Malathion with FV3

exhibited a loss of avoidance behaviour to physical disturbance. The tadpoles exposed to 10mg/L Malathion with FV3 increased in the degree of abnormality at the most rapid pace and reached the most severe abnormality by 192 hours, which involved tadpoles floating at the surface of the water and which were slow to respond to stimulation. Tadpoles that were exposed to FV3 only began to show symptoms after 120 hours of exposure, exhibiting lethargy though still able to feed, and this did not change over the course of the experiment. (Figure 4.4 A - E) Tadpoles in the control treatment, not exposed to either FV3 or malathion, exhibited no symptoms of abnormality.

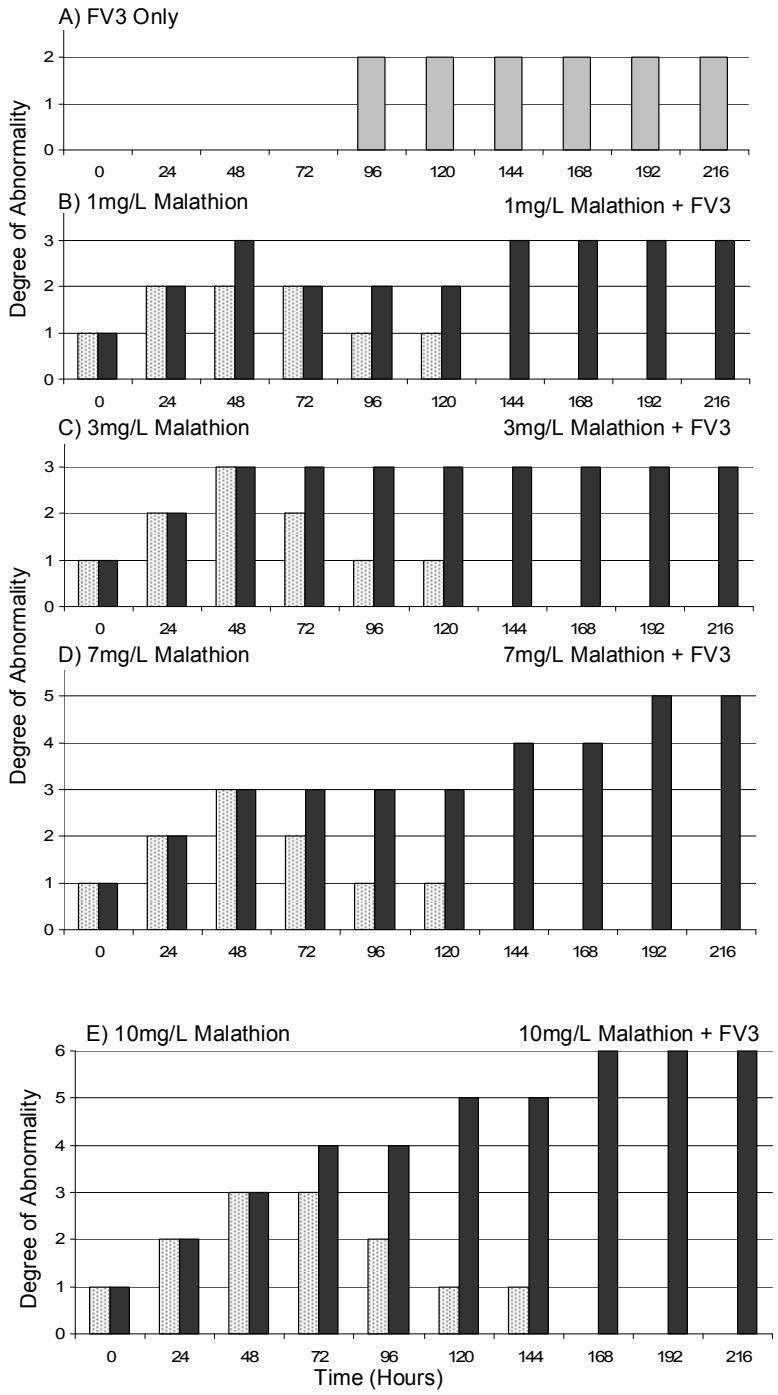


Figure 4.4. Observations of the onset of clinical symptoms in bullfrog tadpoles exposed to FV3, increasing levels of Malathion, or both. See the text for detail on the symptoms and levels. The light bars represent the FV3 only treatment (A). The stippled bars represent Malathion only treatments (B-E) and the black bars represent the Malathion + FV3 treatments (B-E). Tadpoles in the control treatment, not exposed to either FV3 or malathion, exhibited no symptoms of abnormality

Infection Rates of Exposed Tadpoles

To determine if tadpoles were infected with FV3, PCR was used to assay for the presence of the virus. This revealed that most tadpoles, including most controls and most exposed only to malathion, were unexpectedly infected with FV3 (range 74 – 100%). Treatments with 100% infection rates included the highest malathion concentrations and four of five of the treatments with FV3 (Figure 4.5). The gels can be found in Appendix E. The negative controls did not show any contamination and the positive controls were positive for the Ranavirus confirming that the infections were not the result of contamination during analysis and that the tadpoles must have been infected prior to the experimental exposures. The tadpoles did not exhibit any symptoms of Ranaviral infections, again leading to the possibility of asymptomatic carriers of the virus. The source of the unexpected infections is unknown.

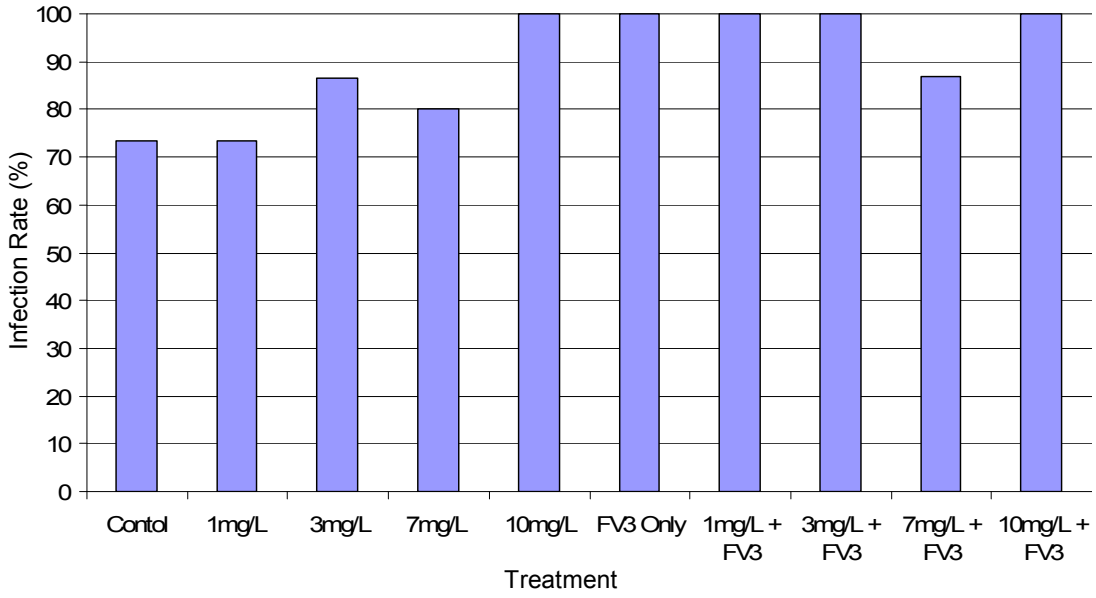


Figure 4.5. Infection rate of bullfrog tadpoles. All 15 tadpoles from each treatment were tested for the presence of FV3. A sample of hepatic tissue was dissected out of each tadpole and the DNA was extracted from this sample. The presence of an FV3 infection was determined by PCR with a primer targeting a 500 base pair sequence of the major capsid protein (MCP) of the FV3 genome.

Discussion

This study suggests that possible interactive effects of malathion exposure and concurrently induced infections of the emerging infectious disease, Frog virus 3 (FV3) occurred in wood frogs (*Rana sylvatica*) and bullfrog (*Rana catesbeiana*) tadpoles.

Assessing Initial Possible Infections

Wood Frogs

The parents of the wood frog tadpoles used in the experiment that examined the interaction of malathion and FV3 infections were free from FV3 infections. This is critically important because the possibility of vertical transmission of FV3 was completely eliminated. Although there still remains the potential for vertical transmission of the virus under natural conditions, the fact that neither the parents nor the brood of eggs were ever in contact with FV3 contaminated water from the wild eliminates any possibility of pre-experimental exposure to FV3 from external sources.

Bullfrogs

The source of the bullfrog tadpoles is unknown. They were collected as eggs in the wild and raised in tubs outside in a semi-enclosed environment. It is possible that the FV3 infection existed throughout their development and infection occurred in the eggs. However, it is also possible that tadpoles came into contact with the virus because they were initially raised in filtered river water, though there is no evidence yet that the virus is carried by river water.

Malathion Toxicity

When the wood frog tadpoles were exposed to only 10mg/L of Malathion, the survivorship decreased over time, and greater mortality occurred in the 24 hour period following malathion inoculation events. Bullfrog tadpoles that were exposed to 10mg/L Malathion did not die. This may be due to differences in susceptibility to chemical exposure of tadpoles of different species and in different stages of development, as indicated in lab studies on pesticide exposures of a number of anuran species (Berrill *et al.*1993; Berrill *et al.* 1994; Berrill *et al.* 1995; Bridges 2000; Boone *et al.* 2001; Greulich and Pflugmacher 2003). Older green frog tadpoles were more sensitive to permethrin than younger tadpoles (Berrill *et al.*1993). The wood frog tadpoles in this study were of a generally later developmental stage (Gosner stage 25–44) than the bullfrog tadpoles (Gosner stage 25) and may have been more sensitive to malathion exposure.

Malathion accumulates in tissues (especially adipose tissue) and inhibits the action of the carboxylesterase enzyme that detoxifies it and its intermediates into breakdown products which can be removed from the cells (Blasiak *et al.* 1999; Venturino *et al.* 2001). Since malathion bio-accumulates, the effects of exposure are likely to continue after initial exposure. However, the bullfrog tadpoles were able to recover from the effects of malathion exposure. Malathion also inhibits the proper functioning of the cholinerginic nerves in the nervous system through the inhibition of acetylecholinesterase which results in impaired signal transduction between the nerve cells (Galloway and Handy 2003). Since the proper function of neurons is key in feeding behaviour, malathion exposure may inhibit the ability of tadpoles to feed. As time progresses, the animals would begin to use the energy stored in adipose tissues releasing the malathion

which had accumulated in those tissues, prolonging the effects of exposure. Bullfrog tadpoles were able to recover from exposure to the highest concentrations malathion, whereas wood frog tadpoles exposed to the same malathion concentration experienced an increasing degree of abnormality and most of them died. Since malathion is a known immunosuppressant, exposure may also have resulted in an impaired immune system, making the tadpoles more susceptible to FV3 infection.

Viral Toxicity

When wood frog tadpoles were exposed to 670 PFU/mL of FV3 without the presence of malathion, there was an apparent decrease in survivorship when compared to the control treatment. In wood frogs, Ranavirus infections result in a high mortality rate in nature (Green *et al.* 2002; Greer *et al.* 2005), and therefore these results were not unexpected. In wood frogs, little is known about the length of time that FV3 infections take to develop symptoms. Infection trials (see Chapter 2) indicated that asymptomatic infections can occur within seven days after the initial exposure to FV3 in the same concentration used in this experiment, but no mortality was observed. With bullfrog tadpoles, it is impossible to assign mortality from the virus alone, since mortality was so low and there was an extensive initial infection rate. Knowing the viral load of an individual would be helpful in assessing the degree of infection, but the PCR analysis used to test for the presence of the virus in this study can only determine presence/absence of the virus. For the amount of virus present in an individual to be properly quantified, real time PCR is needed. It is, for example, possible that the initial infection rate in the bullfrog tadpoles was due to a low viral load, as suggested by the

lack of symptoms, but this remains unknown because of the use of a presence/absence test. If the viral load in the animal is too low, the PCR reaction will not function because there is not enough template DNA for the primers to anneal to and proceed successfully with the reaction.

Infection Rate

The infection rates for the wood frog tadpoles which were exposed to 670 PFU/mL of FV3 and to 10mg/L Malathion + 670 PFU/mL FV3 were not 100%. Of particular interest, the tadpoles in the 10mg/L Malathion + 670 PFU/mL FV3 treatment had an infection rate of only 35%. Since all of the tadpoles in this treatment died within three days, and there is no consistency as to the date of death and the infection status of the tadpole, it is possible that FV3 infections were not detectable in the liver, but did occur (data not shown, but see Appendix E). When both the spleen and liver tissues are examined, infections that have resulted in natural deaths are detected more frequently in the spleen than in the liver (Duffus *et al.* unpublished data). Therefore, it is possible that since only the livers of the tadpoles were analysed, not all infections were detected. This result is also surprising because the tadpoles in the 10mg/L Malathion + 670 PFU/mL FV3 showed the most severe symptoms after only 24 hours. However, this mortality is still likely to be mostly due to increased response to the higher levels of malathion.

In contrast, wood frog tadpoles exposed to 3mg/L Malathion + 670 PFU/mL FV3 showed an infection rate of 100%. This suggests that when sufficient time has elapsed, the combination of FV3 and malathion result in a greater detectable infection rate. Since the effects of malathion are concentration dependant (Christin *et al.* 2003), it is possible

that when exposed to low concentrations of malathion, the tadpoles do not experience extensive immunosuppression or prolonged severe neurological effects permitting the tadpoles to live. As these tadpoles continue to survive, the FV3 infections should increase because of the reduced immune response and therefore it is possible that the FV3 infections in the wood frog tadpoles became lethal because of the effects of malathion on the immune system of the animal.

The trends in infection rate of wood frog tadpoles appear to support the hypothesis that exposure to malathion and FV3 results in larger infection rates than when either treatment is applied alone.

Interaction between Malathion and Frog virus 3 Infections

Mortality

Mortality was most rapid and extensive when wood frog tadpoles were exposed to 10mg/L Malathion and 670 PFU/mL of FV3, compared to 10mg/L Malathion or 670 PFU/mL of FV3 alone. The effect of pesticide exposure on the function of the immune system appears to be concentration dependent (Christin *et al.* 2003). Therefore it is possible that malathion exposure at 10mg/L inhibited the immune system of the tadpoles from mounting any significant response to the FV3 infection due to the negative effects of malathion on immune function in anuran amphibians (Gilbertson *et al*. 2003). Since all of the wood frog tadpoles had died after only 72 hours, it is unlikely that bio-accumulation of malathion played a significant role in mortality.

Bullfrog tadpole mortality was too low to draw any strong conclusions, but three of the four tadpoles that died were in Malathion + FV3 treatments and are consistent with the wood frog tadpole mortality results.

Quantification of Abnormality

Tadpoles of both species exhibited a more rapid onset of abnormal symptoms when exposed to malathion, regardless of whether FV3 was present or not. However, when malathion was combined with FV3, the severity of the symptoms was greater over time. The fast onset of symptoms in the 10mg/L of Malathion + 670 PFU/mL FV3 treatment may be the result of the malathion virtually eliminating the tadpole's ability to mount an immune response to the virus. Malathion affects the ability of anuran immune system to produce specific antibodies (Gilbertson *et al*. 2003) which are important in the immune defence against viruses. However, the antibody response takes more time than permitted here to develop. The lack of an immune response or minimal immune response would permit the virus to infect the tadpole without having to contend with the defences of the immune system and could have resulted in the emergence of symptomatic FV3 infections after only 24 hours. Further support for the immunosuppression hypothesis comes from the fact that the high concentration of malathion in combination with FV3, caused more severe symptoms than other treatments. In the bullfrog tadpoles, the most severe abnormalities (loss of avoidance response to physical disturbance and tadpoles floating on or near the surface) would make the tadpoles vulnerable to predation in natural conditions. Also, when tadpoles float at the surface of the water in natural ponds this usually indicates that the FV3 infection is in its final stages and death is eminent.

The symptoms that were noted on the first day of the exposures are probably solely due to the effects of malathion on the nervous system: the lethargy and abnormal swimming observed only occurred in treatments that involved malathion, and these symptoms did not appear in either the controls or in the 670 PFU/mL FV3 treatment. Nothing is known about how the immediate behavioural effects of malathion exposure influence the progression of FV3 infections. Because tadpoles become lethargic or develop disturbed swimming patterns, the spread of FV3 infections between individuals may in fact be aided by increased contact between the tadpoles.

Bullfrog and wood frog tadpoles that were exposed to 7mg/L or 10mg/L Malathion exhibited clinical symptoms of pesticide exposure shortly after the malathion was added to the water. The progression in the severity of symptoms may be due to the accumulation of malathion in the tissues of the tadpoles, combined with the neurological effects of exposure. When these symptoms persist for long periods of time the tadpoles are unable to acquire the nutrition necessary for survival and begin to metabolize tissues to sustain themselves.

The fact that bullfrog tadpoles in the control treatment showed no symptoms of infections suggests that the viral load was very low. Increasing the effect of FV3 exposure as seen in infection rates, mortality rates, and development of abnormality in experiments with increasing concentrations of malathion further suggests that interaction is occurring. Only the use of real-time PCR would resolve this ambiguity.

When wood frog tadpoles were exposed to 670 PFU/mL of FV3 slight symptoms developed after two days. Symptoms took more time to develop than in treatments which combined Malathion and FV3 or 10mg/L Malathion only. The development of

symptoms/abnormality is likely to be the result of the time required for FV3 to replicate and create a sufficient number of virions to produce a symptomatic infection. There are no data available on the length of time required for FV3 infections to become symptomatic in wood frog or bullfrog tadpoles. However, the replication cycle of FV3 in Fathead minnow cells in culture is relatively slow and takes several days.

Conclusions

This study appears to provide support for the immunosuppression hypothesis. When assessing the mortality, as well as abnormal behaviour data, it seems that the effects of combined exposure to an immunosuppressant and an infectious disease was more severe than in either treatment alone. Also, the effects of exposing wood frog tadpoles to both malathion and FV3 increased the speed at which symptoms appeared, as well as the severity of the symptoms, in a concentration dependant manner with respect to malathion. A similar trend was shown in experiments with bullfrog tadpoles despite the unexpected initial infection rates of tadpoles with FV3. However, for the immunosuppression hypothesis to be tested adequately, the effects on the immune system must be directly measured. Both the effects of malathion exposure on the immune system and the immune response to FV3 need to be determined, as well as the immune response when the animal has been exposed to both malathion and FV3. A measure of the effect of malathion on the immune system could be indicated by lymphocyte proliferation (Christin *et al*. 2003) and also the immune system's ability to produce pathogen specific antibodies (Gilbertson *et al.* 2003). However, in the latter study the leopard frogs were injected with malathion and it is therefore difficult to relate the results to natural

exposures. To measure the combined effects of malathion and FV3 exposure on the immune system, a measure of the production of FV3 specific antibodies could be used. More research is needed into how the immune system of anuran amphibians responds to an infectious disease when there is a concurrent exposure to an immunosuppressant. Investigations into natural immunosuppression should be studied, e.g. the susceptibility of newly emerged over-wintered adult wood frogs to disease. Also, experiments using environmentally relevant levels of malathion should be used. Efforts to determine environmentally relevant concentrations of FV3 are needed to make realistic experimentation possible.

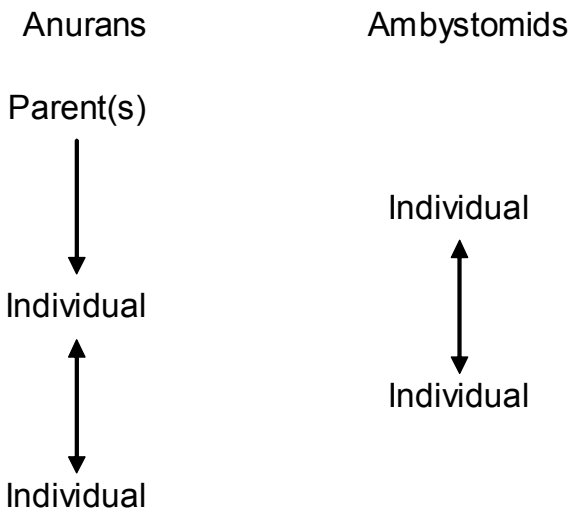
Chapter 5: Model of Ranaviral Transmission in Aquatic Amphibian Communities

The mode of Ranaviral transmission in aquatic amphibian communities is relatively unexamined. It has been suggested that ambystomids are the reservoir of Ranaviruses in wild communities of amphibians (Brunner *et al.* 2004). However, the situation is likely to be more complex than a single species interaction. There are a number of assumptions which are inherent to the development of this model of Ranaviral transmission in aquatic amphibian communities, none of which are contradicted by the results reported in this thesis or published elsewhere:

- the same strain of the Ranavirus is present in all of the amphibian species
- the strain of the Ranavirus can be transmitted between amphibian species
- vertical transmission of the Ranavirus can occur in wood frogs
- horizontal transmission of the virus is likely in all cases
- there are no external stressors such as desiccation, density
- human induced transmission of the virus between ponds
- amphibian species vary in their susceptibility to the virus
- there are no density dependant factors associated with Ranaviral transmission
- asymptomatic carriers of the virus are present in multiple amphibian species
- immunosuppression may influence the infection rate

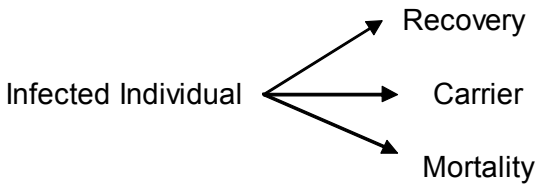
- ambystomids are a likely reservoir of the Ranavirus in amphibian communities.

The model developed in this chapter was inspired by a model of viral dynamics developed by Day and Proulx (2004). There are two possible modes of transmission in which the Ranavirus moves in wood frog (*Rana sylvatica*) tadpole populations; horizontally, either through the water or through physical contact between individuals, or vertically from parent(s) to offspring. It is likely that the same mechanisms act to spread the virus in other species of anuran amphibians. There are no data available to support vertical transmission in ambystomids (Brunner *et al.* 2005a), therefore the following scenario of viral transmission is the only one that is supportable: (Note: The direction of the arrow indicate the direction of viral transmission.)



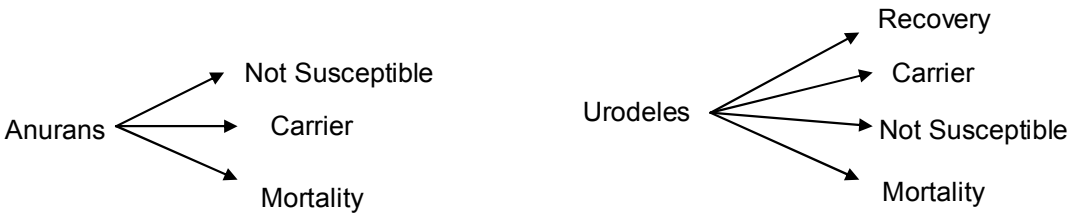
In wood frogs, the infection rate decreases over time, as seen in both laboratory raised broods and in wild populations. This trend also appears in wild populations of other species of anuran amphibian species. It is important to note that even though there

is currently no evidence for recovery from Ranavirus infections in anuran amphibians, this possibility cannot be ignored. There is also the possibility that anuran amphibians can be asymptomatic carriers of the virus after they have been exposed to the virus: these animals will simply be referred to as carriers in the model. This leads to the following possibilities for infected anuran amphibians:

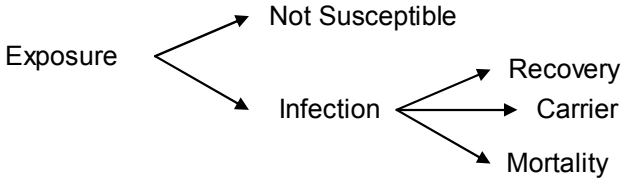


It is likely that mortality contributes greatly to the decrease in the infection rates over time in anuran amphibian tadpoles. There are amphibian communities where the infection rate for a population of a species within that community is zero, and there are two ways this could happen. Firstly, the community may have been previously repeatedly exposed to the Ranavirus, which would have purged the community of the majority of susceptible individuals. For natural selection to act in this way, Ranavirus outbreaks would have to have occurred for several years, long enough for the survivors of the initial Ranaviral outbreaks (who either were naturally resistant to or developed immunity specific to the Ranavirus) to become the breeding individuals in subsequent years. The second explanation for a zero infection rate in a population of a species of amphibian within an infected amphibian community is that the Ranavirus exists only at sub-detectable levels, making these individuals asymptomatic carriers of the Ranavirus. It is not known if anuran amphibians can recover from Ranavirus infections, but since the

mortality rate in some species of anuran amphibian tadpoles is extremely high, it is likely that the number of recovered individuals is negligible. It is known that Ambystomids can clear the virus or be asymptomatic carriers of the virus (Brunner *et al*. 2004). Therefore the following general models for both anurans and ambystomids are possible:

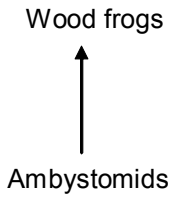


In laboratory trials with amphibians the following general model can occur when they are exposed to a single source of the virus:



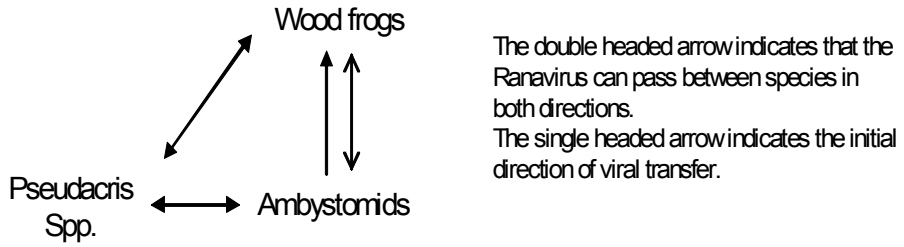
These outcomes are easy to predict because in lab exposures there is one point source of the virus and the larvae have been exposed to a known quantity of the virus. In the field the situation is more complicated because the number of sources of the virus changes over time. In the following development of the transmission model, data from Parker Pond will be used. In Parker Pond the ambystomid larvae demonstrate a consistent and fairly high infection rate of the Ranavirus. This makes them the likely candidate for

the reservoir and initial source of the virus, so they are placed in the center of the model. Wood frogs breed at the same time as the ambystomids. Assuming that the ambystomids are the reservoir of the virus, they must pass it on to the wood frog tadpoles. There is, however, another species present in many aquatic amphibian communities at the same time as the ambystomids; the eastern spotted newts (*Notophthalmus viridenscens*) are present in both larval and aquatic adult forms. It is known that both adult and larval eastern spotted newts can be infected with the Ranavirus (Green *et al*. 2002). Since both the larvae and adults have the potential to participate in the viral dynamics of the aquatic amphibian community, it is possible that the adult eastern spotted newts act as an additional reservoir of the Ranavirus in aquatic amphibian communities. In Parker Pond, one out of five adult eastern spotted newt tested positively for the presence of the Ranavirus. It may be possible that adult eastern spotted newts carry a sub-detectable viral load and can transmit the virus to susceptible individuals of other species, similar to what has been observed by Brunner *et al.* (2004) in ambystomids. Therefore, eastern spotted newts will be placed in the category of ambystomids (the other urodele species present), and the following potential situation results:

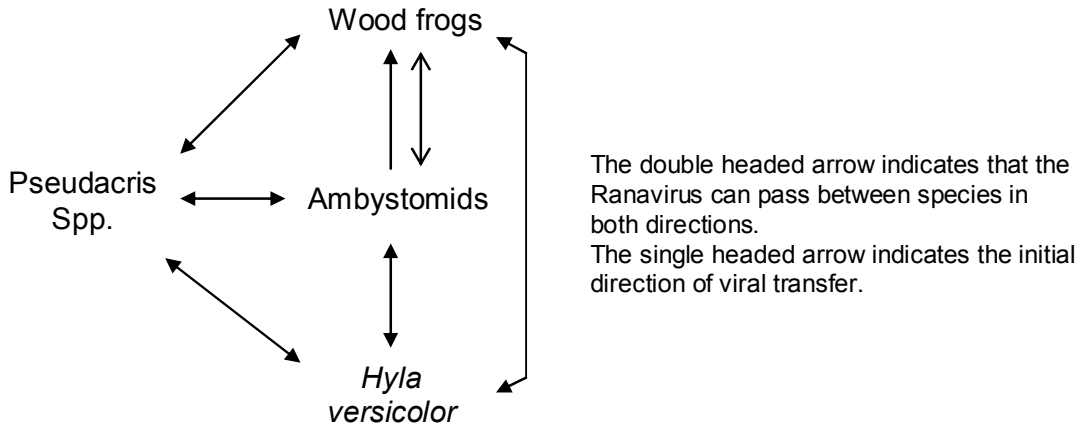


As the amphibian breeding season progresses, more species breed and their larvae are susceptible to the Ranavirus. The next species to breed are the *Pseudacris* species,

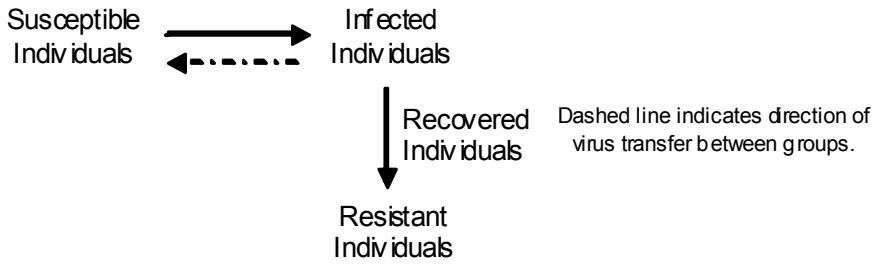
which overlap with the end of the wood frog and ambystomid breeding seasons. The *Pseudacris* species present include the spring peeper (*Pseudacris crucifer*) and the chorus frog (*P. triseriata*). The *Pseudacris* species in Parker Pond are infected with the Ranavirus. The *Pseudacris* tadpoles have two potential sources of the Ranavirus, the wood frog tadpoles and the ambystomids. This results in the following possible dynamic:



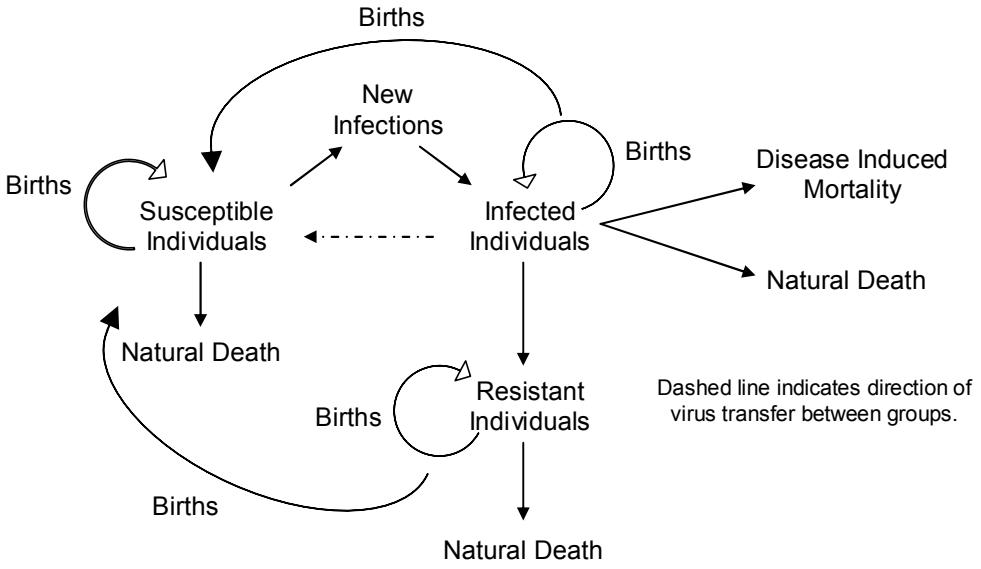
Since each species is assumed to act as a source and a sink for the virus for each of the other species, the infection can now perpetuate itself between and within all species present. At the end of the breeding season, the grey tree frogs (*Hyla versicolor*) are one of the last species of anuran amphibians to breed in the pond. The grey tree frog tadpoles exist in the pond with all of the larvae of other amphibian species, resulting in the following situation of viral transmission:



The model now becomes more complex because each species of amphibians present in the pond has a susceptible, an infected group of individuals, and a resistant group of individuals. It is now possible to combine the susceptible groups of all species into one group, all infected individuals into a second group, and all resistant individuals into a third group. Individuals that carry the disease and can transmit the virus to susceptible individuals, regardless of whether or not they exhibit symptoms, are considered to be a part of the pool of infected individuals. For any one population of a species in the amphibian community the following dynamic can occur:

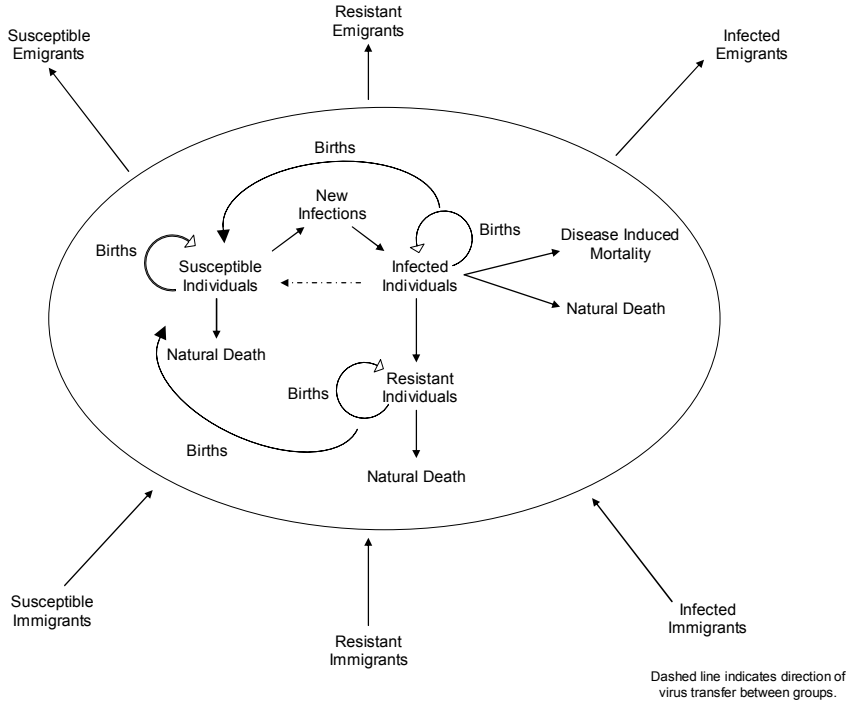


Resistant individuals do not interact with the viral dynamics because they do not return to either the susceptible or infected groups in the community. However, if enough time has elapsed the resistant group may take over the entire community through the action of natural selection. It is important to realize that not all amphibian species may be equally susceptible to the Ranavirus. In a natural population the situation is made more complex because of births and deaths. Deaths may be due to Ranaviral infections (disease induced mortality). Natural deaths result from predation and other natural causes such as developmental abnormalities. Therefore, the following situation arises:



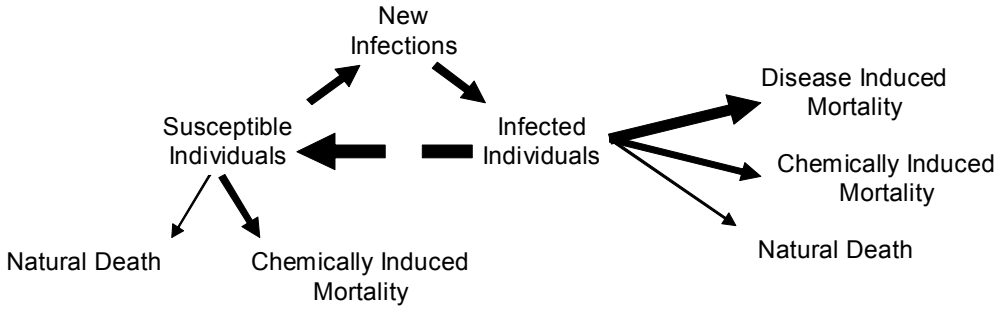
It is not known if vertical transmission can occur in all amphibian species that are affected by the Ranavirus. Where vertical transmission does occur, if an infected individual mates with a susceptible individual, it is likely that at least some of the offspring are going to be infected with the Ranavirus. If a resistant individual mates with a susceptible individual, the result depends on the mechanism of resistance. If the resistance has been obtained by exposure to the virus, the offspring will be susceptible. However, if the resistance is genetic, it is likely that at least some of the offspring will be resistant to the Ranavirus. A similar situation occurs when a resistant individual and an infected individual mate. If the resistance is acquired through exposure, then it is likely that some or all of the offspring will be infected. If the resistance is genetic, some, if not all, of the offspring will be resistant to the virus: those offspring not resistant to the virus may be infected with the Ranavirus through the infected parent or they may be susceptible to later viral infection.

Since communities are not closed to immigrants and emigrants, we must also consider immigration and emigration of individuals from infected, susceptible and resistant individuals. This consideration results in the following situation:



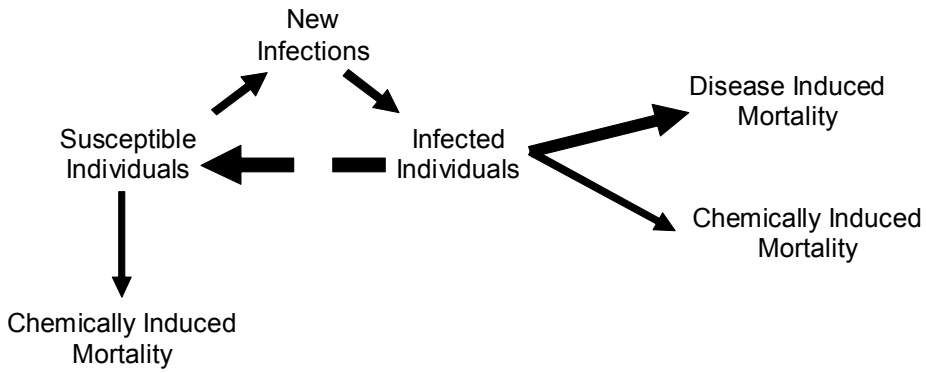
This becomes a complicated model of viral dynamics within an amphibian community. Resistant individuals cannot be eliminated because if their resistance to the virus is acquired, then they will produce susceptible offspring. Also, the persistence of resistant individuals may impede the transmission of the virus once they have reached a certain percentage of the populations (this number is unknown).

The above model assumes that the environment is constant and that there are no perturbations. Consider the following scenario: There is an environmental perturbation, such as the application of an immunosuppressant chemical (e.g., a pesticide such as malathion). This results in a rapid increase in the number of infected individuals within the population and a proportional decrease in the number of susceptible individuals. Since agricultural chemicals are usually applied after the breeding season of amphibians and the life stages that are present in the ponds are larvae, the contributions to the susceptible and infected individuals through births are non-existent. Also, immigrants usually arrive just prior to or during the breeding season, and emigration usually occurs after the amphibians have metamorphosed. Therefore, the following scenario of viral dynamics occurs in an immunosuppressed amphibian population:



The thickness of the arrows indicates the relative number of individuals that would suffer that particular fate. The thick dashed arrow indicates an increased transmission of the virus between infected and susceptible individuals. Depending on the concentration of the immunosuppressant chemical, the interaction between the susceptible individuals and the spread of the disease changes slightly. Increased concentrations of the immunosuppressant chemical will increase the transmission rate of

the virus to the susceptible individuals. At high concentrations, death will be highly correlated with the infection status of the individual and chemical exposure, permitting us to eliminate natural deaths as a significant contributor to the population dynamics. The following situation results:



Thus, in situations where there is an immunosuppressant chemical involved, the viral dynamics are altered. The number of infected individuals increases over time and has the potential to eliminate all susceptible individuals. Also, the number of disease - induced deaths will increase, chemically induced mortality will occur at a low rate, and recovery will not be significant.

Since this model depends on a number of assumptions, it is likely that it may still not indicate the extent of complexity of Ranaviral transmission in aquatic amphibian communities.

Future Directions That Would Strengthen the Model

Since the in-depth study of emerging infectious diseases in amphibians is a relatively new area of study, there are many intriguing avenues of research to explore.

Extent of Infections

In Ontario, there needs to be a wide-scale sampling project undertaken to establish the full extent and distribution of the Ranavirus in amphibian populations. Sampling should include multiple species, and not be restricted to amphibians. Fish and aquatic insects should also be sampled because they, too, can be infected with Iridoviruses. Ideally, this study would be expanded to cover the entire range of amphibian species and their ranges in Canada.

Modes of Transmission

Viral transmission is an important area of exploration and the mode of transmission of the Ranavirus in wood frogs was explored in this thesis. However, there are many other species of amphibians that are affected by the Ranavirus. Further research into the modes of transmission in other amphibian species, especially in anurans, is needed. There also needs to be further investigation into the potential for the Ranaviruses to spread between different species of amphibians and other fish and insect species that are present in the aquatic communities.

Configuration of the Ranavirus Strain Present in Ontario

In Ontario, a complete investigation into the strain of Ranavirus is needed. This would involve the testing of Koch's Postulates of infectious diseases. Even though the preliminary investigations into the identity of the Ranavirus have pointed to frog virus 3 as the strain of the Ranavirus present in amphibian populations, there is a possibility that there is a unique strain of the virus in Ontario. Full sequencing and characterization of the virus from wild populations of amphibians should be performed. Studies from viral isolates from sympatric and taxonomically distinct species have isolated identical strains of Ranavirus (Mao *et al*. 1999), therefore it is likely that the virus present in Ontario affects multiple amphibian species. Concrete establishment of cross-species transfer of the virus from different amphibian species needs to be performed in the laboratory, for until then all models of transmission will remain uncertain and based on a multitude of assumptions. Therefore, the determination of the strain of the Ranavirus present in Ontario, is required as well as determining if it is the actual cause of the disease being observed.

Action of Immunosuppressant

This thesis provides preliminary evidence that exposure to an immunosuppressant chemical influences the progression of Ranaviral infections in wood frog and bullfrog tadpoles. However, much has been left unanswered in this preliminary investigation. For example, the effect of exposure to an immunosuppressant needs to be quantified in amphibian tadpoles. Measures such as the number of circulating white blood cells, specific Ranaviral antibody production, and/or natural killer cell numbers would provide

insight into the actual effects of the immunosuppressant on the immune system. When compared to mammalian systems, the immune system in amphibians is complex and poorly understood. Also, it is important to examine the effect of both the length of the exposure to an immunosuppressant and the length of time required to recover from that exposure. The effect of Ranaviral infection on amphibian tadpoles should be examined, measuring the production of antibodies and establishing quantifiable measures of disease progression of the Ranaviral infection. Knowing which symptoms are attributed to the viral infection and those that can be attributed to exposure to chemicals will aid in determining the nature of the interaction between the immunosuppressant and the Ranaviral infection. More investigation into the interactive effects between an immunosuppressant chemical and infections are needed before any concrete conclusions can be made.

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Appendix A: Dissection Procedures and Molecular Analysis for the Presence of the Ranavirus

Dissection Procedures:

All specimens were preserved in 70% ethanol. The dissection area was kept exceptionally clean and covered with bench protector which was changed frequently to prevent cross-contamination. Each animal was dissected on its own separate piece of bench protector. Instruments were cleaned with a high concentration solution of DECON 75 (Decon Laboratories Limited) and rinsed with water between each use to remove any possible contaminants from them. Both the DECON 75 solution and the rinse water were changed frequently. Hepatic tissue samples were collected and placed into 500µL of lysis buffer. Lysis buffer control samples were taken every 5 to 10 samples to enable tracking of both the source and route of contamination should it occur.

Analysis for the Presence of Ranavirus:

Hepatic tissue samples were incubated at 37^{o}C overnight in 500µL of 1x lysis buffer (4M urea, 0.2M NaCl, 0.5% n-lauroyl sarcosine, 10mM 1,2-cyclohexanediaminetetraacetic acid, 0.1M Tris-HCl, pH 8.0). After overnight incubation 25µL of Proteinase K (Roche Diagnostics Corporation) was added and samples were incubated for 2 hours at 65^{o}C, followed by an additional spike of 25µL Proteinase K incubated at 37^{o}C overnight. DNA was extracted following QIAamp DNeasy Extraction Kit protocol (Qiagen). The DNA obtained from the hepatic tissue was analyzed for the presence of the Ranavirus using primers which target a 500 base pair section of DNA in

the coding region of the major capsid protein (MCP) of Frog virus 3 (FV3) as per Greer *et. al* (2005). The viral DNA in the samples was amplified in a 10 μL polymerase chain reaction (PCR) with 2 μL extracted DNA, 1U Taq DNA polymerase (Invitrogen), 1x PCR buffer (Invitrogen), 1.5mM $MgCl_2$ (Invitrogen), 0.2 uM each dNTP (Amersham Biosciences), and 0.2 uM of each primer. PCR was performed on MJ Research PTC-225 DNA Engine Tetrad thermocyclers (MJ Research Incorporated) using the following cycles: initial denaturation at 94ºC for 5 minutes followed by 35 cycles of 30 seconds at 94ºC, 30 seconds at 60ºC and 30 seconds at 72ºC followed by a final extension of 2 minutes at 72ºC. (Greer *et al*. 2005). The PCR products were run on a 1.5% agarose gel stained with ethidium bromide. Presence of a band at an approximate length of 500 base pairs indicated the presence of the Ranavirus in samples.

Even though the banding for the infected tadpoles varies in signal strength, it does not necessarily indicate that the tadpole carried a varying viral load. The PCR analysis method used can only indicate the presence/absence of the virus.

Appendix B: Frog virus 3 (FV3) Culture and Titer Methods

Culture of Fat Head Minnow cells:

Fat Head Minnow (FHM) cells (American Tissue Type Collection) are cultured at 30˚C in Minimum Essential Media (MEM) (Gibco) with 10% foetal bovine serum (FBS) in air.

Frog virus 3 Culture:

A sub-confluent 75cm^2 flask of FHM cells with approximately 80% of the bottom of the flask covered with cells was used. The media was replaced the media with MEM + 1% FBS. Approximately 0.1 Plaque Forming Units/mL of Frog virus 3 (FV3), was added and incubation at 30 ˚C was continued until the FHM cells were bulbous, but still attached to the plate (this is called the cytopathic effect). The cells were then scraped from the plate. The solution obtained was then transferred to a 50mL centrifuge tube and spun at 7000 revolutions per minute for 5 minutes. The majority of the excess media was removed, leaving approximately 0.5mL of media to re-suspend the pellet. The mixture was then freeze–thawed rapidly three times. The mixture was frozen at – 80˚C, then thawed rapidly and vortexed for 30 seconds. The FV3 was then stored at – 80˚C until use.

FV3 Titer Methods:

Serial dilutions between 10^{-3} and 10^{-8}mL of the virus in 1mL of MEM + 1% FBS in a 6 well tissue culture tray were used. The tray was then incubated at 30˚C, after 24 hours, 2mL MEM + 1% FBS was added to each well. The tray was incubated at 30˚C until plaques were seen. The media was then removed and 2mL of crystal violet were

added to the wells to stain the cells. They were then incubated at 30˚C for approximately 2 minutes. The wells were then washed with water and the number of plaques were quantified and the number of plaque forming units were calculated. e.g. If there are 45 plaques in the well with the dilution of 10^{-6}, then the titer of the virus is 4.5×10^{7} Plaque forming Units (PFU)/mL.

Appendix C: Preliminary Phylogenetic Analysis of a Ranavirus Implicated in Mass Mortality Events in Central Ontario and a Comparison to Frog virus 3.

Purpose:

To determine if inter-year evolutionary changes occur in the Ranavirus responsible for amphibian mass mortality events in Oliver Pond. The virus will be compared from infected tadpoles collected from 2001 to 2004, as well as to the sequence of Frog virus 3 (Accession Number AY548484: Tan *et al.* 2004).

Methods:

Using primers for the Major Capsid Protein, developed by Greer *et al.* (2005), viral DNA from infected wood frog tadpoles from outbreaks of the Ranavirus at Oliver Pond from 2001 – 2004 was amplified. (See Appendix A: Dissection and Analysis Methods for further detail.) The DNA was sequenced by Kristyne Wosney at the Natural Resources DNA Profiling and Forensics Center (NRDPFC). The sequence of the major capsid protein of FV3 (Accession Number AY548484: Tan *et al.* 2004) and the Grouper Iridovirus (Accession Number AY666015: Tsai *et al.* 2005) were obtained from GenBank.

Sequences were aligned using BioEdit and the tails of the sequences which were not aligned were removed. Phylogenetic analyses were conducted using *MEGA* version 3.1 (Kumar *et al.* 2004). Both sequence and amino acid trees were created. The Grouper Iridovirus was used as the out-group. The neighbour joining method was used to create a phylogenetic tree with the DNA and amino acid sequences based upon the

number of nucleotide or amino acid differences. The bootstrap values were based on 2000 repetitions.

Results and Discussion:

Molecular analysis of the virus was inconclusive due to the fact that the DNA sequences were rather short, less than 250 base pairs long. The phylogenetic tree which is based upon the DNA sequence (Figure 1), shows that FV3 and the virus present in 2002 group together with a bootstrap value of 100%. The viruses present in 2004 were the most divergent, but did not group together. Therefore, no solid conclusion about the identity of the virus or the rate of change can be made based on these analyses alone.

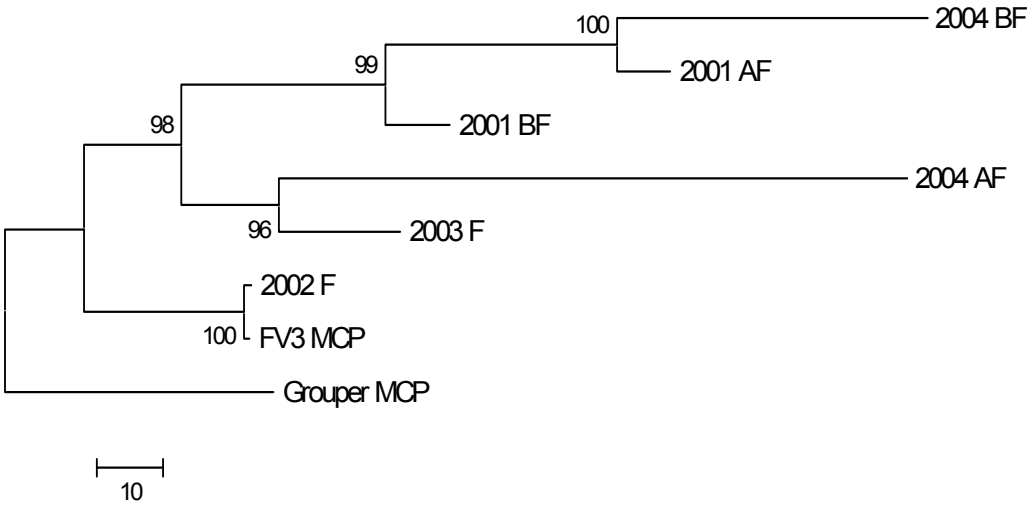

Figure 1. Phylogenetic tree of viral DNA sequences from Oliver Pond, FV3 and the Grouper Iridovirus major capsid proteins. The outgroup is the Grouper Iridovirus. The tree was made using the neighbour – joining method, and bootstrap values are based on 2000 repetitions. The scale bar in the lower left hand corner represents the distance on the tree equivalent to a 10 base pair difference.

The phylogenetic tree which is based upon the amino acid sequence (Figure 2), again shows that FV3 and the virus present in 2002 group together with a bootstrap value of 100%. In this tree, all of the bootstrap values are lower than for the DNA sequence, which decreases the reliability of the results even further. The viruses present in 2004 were the most divergent, but did not group together.

Even though both the DNA and amino acid sequence trees resemble one another, because the results are only based on a single short sequence, solid conclusion about the identity of the virus cannot be made.

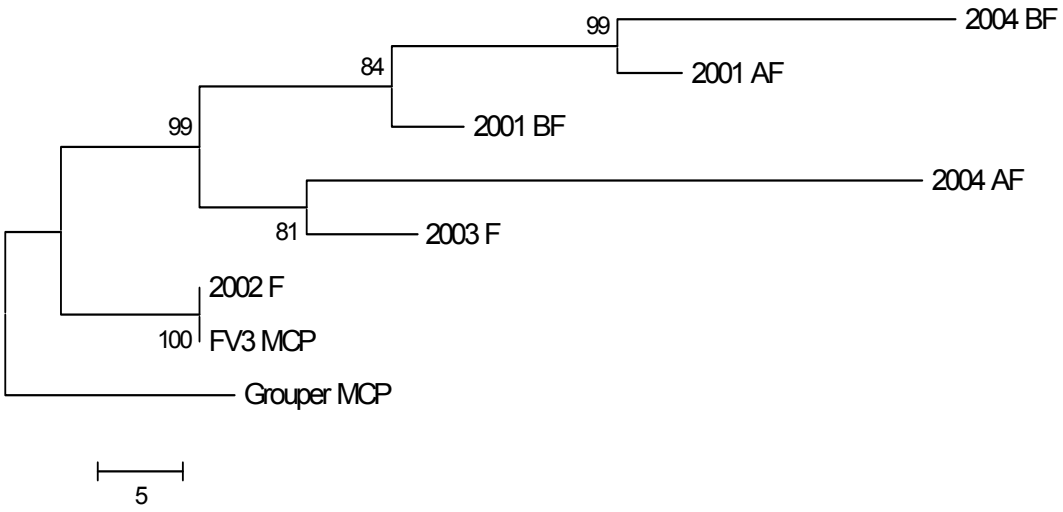

Figure 2. Phylogenetic tree of viral amino acid sequences from Oliver Pond, FV3 and the Grouper Iridovirus major capsid proteins. The outgroup is the Grouper Iridovirus. The tree was made using the neighbour – joining method, and bootstrap values are based on 2000 repetitions. The scale bar in the lower left hand corner represents the distance on the tree equivalent to a 5 base pair difference.

Appendix D: Terrestrially Amplexing Pairs

RANA SYLVATICA (Wood Frog) **TERRESTRIAL AMPLEXING PAIRS**.

On the evening of the 17th of April 2005, at approximately 21:00hrs, 3 amplexing pairs of wood frogs were found on Crowe's Line Road near Bobcaygeon Ontario, Canada (44°33N 78°33W). The pairs were likely heading toward a large chorus of 200-300 male wood frogs (as well as two other chorusing species) in the wetland on the other side of the road (the direction in which the female of each pair was oriented). Upon further investigation of the wetland, ~25–30 freshly deposited wood frog egg masses were found. The air temperature was 10-12°C, and the water temperature in the wetland was 12°C. The sky was clear and three-quarters of the moon was visible; the humidity was low; and the road was not moist nor were the ditches particularly wet.

The terrestrial amplexing pairs of wood frogs suggest that male wood frogs have an alternative mating strategy to attending and participating in a chorus for ensuring their reproductive success, amplexing a female on land and being piggy-backed by the female to a suitable oviposition site.

These specimens are currently reposed at Trent University, Peterborough, ON and are part of an ongoing study of emerging wildlife diseases in Ontario. The authors would like to thank Michael Berrill and Jean Duffus for comments on earlier versions of this note.

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Appendix E: Tables of Stage Information and Gel Images

Table 1. The results from the field monitoring of wood frogs. The date indicates the day in which the tadpole was collected. The stage value is the Gosner Stage of development. The code and number correspond to the identification of the sample on the gel.

Collection Date	Population	Code	Number	Stage	Infected?
16-May	Crowe's Line Rd. (Wild)	CWC -	1	25	No
16-May	Crowe's Line Rd. (Wild)	CWC -	2	25	No
16-May	Crowe's Line Rd. (Wild)	CWC -	3	25	No
16-May	Crowe's Line Rd. (Wild)	CWC -	4	25	No
16-May	Crowe's Line Rd. (Wild)	CWC -	5	25	No
16-May	Crowe's Line Rd. (Wild)	CWC -	6	25	No
16-May	Crowe's Line Rd. (Wild)	CWC -	7	25	No
16-May	Crowe's Line Rd. (Wild)	CWC -	8	25	No Data
16-May	Crowe's Line Rd. (Wild)	CWC -	9	26	No
16-May	Crowe's Line Rd. (Wild)	CWC -	10	25	No
26-May	Crowe's Line Rd. (Wild)	CWC -	11	25	No
26-May	Crowe's Line Rd. (Wild)	CWC -	12	26	No
26-May	Crowe's Line Rd. (Wild)	CWC -	13	26	No
26-May	Crowe's Line Rd. (Wild)	CWC -	14	28	No
26-May	Crowe's Line Rd. (Wild)	CWC -	15	25	No
26-May	Crowe's Line Rd. (Wild)	CWC -	16	27	No
26-May	Crowe's Line Rd. (Wild)	CWC -	17	25	No
26-May	Crowe's Line Rd. (Wild)	CWC -	18	26	No
26-May	Crowe's Line Rd. (Wild)	CWC -	19	28	No
26-May	Crowe's Line Rd. (Wild)	CWC -	20	29	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	21	28	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	22	31	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	23	32	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	24	31	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	25	34	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	26	31	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	27	30	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	28	34	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	29	34	No
6-Jun	Crowe's Line Rd. (Wild)	CWC -	30	32	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	31	38	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	32	39	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	33	38	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	34	37	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	35	36	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	36	38	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	37	38	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	38	36	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	39	38	No
13-Jun	Crowe's Line Rd. (Wild)	CWC -	40	36	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	41	40	No

Collection Date	Population	Code	Number	Stage	Infected?
17-Jun	Crowe's Line Rd. (Wild)	CWC -	42	37	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	43	38	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	44	39	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	45	40	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	46	39	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	47	38	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	48	40	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	49	40	No
17-Jun	Crowe's Line Rd. (Wild)	CWC -	50	38	No
16-May	Oliver Pond (Wild)	OPW -	1	25	No
16-May	Oliver Pond (Wild)	OPW -	2	25	No
16-May	Oliver Pond (Wild)	OPW -	3	25	Yes
16-May	Oliver Pond (Wild)	OPW -	4	25	Yes
16-May	Oliver Pond (Wild)	OPW -	5	25	Yes
16-May	Oliver Pond (Wild)	OPW -	6	25	Yes
16-May	Oliver Pond (Wild)	OPW -	7	25	Yes
16-May	Oliver Pond (Wild)	OPW -	8	25	Yes
16-May	Oliver Pond (Wild)	OPW -	9	25	Yes
16-May	Oliver Pond (Wild)	OPW -	10	25	Yes
26-May	Oliver Pond (Wild)	OPW -	11	26	Yes
26-May	Oliver Pond (Wild)	OPW -	12	27	Yes
26-May	Oliver Pond (Wild)	OPW -	13	26	No
26-May	Oliver Pond (Wild)	OPW -	14	25	Yes
26-May	Oliver Pond (Wild)	OPW -	15	25	Yes
26-May	Oliver Pond (Wild)	OPW -	16	25	No
26-May	Oliver Pond (Wild)	OPW -	17	25	No
26-May	Oliver Pond (Wild)	OPW -	18	25	No
26-May	Oliver Pond (Wild)	OPW -	19	25	No
26-May	Oliver Pond (Wild)	OPW -	20	26	No
6-Jun	Oliver Pond (Wild)	OPW -	21	30	No
6-Jun	Oliver Pond (Wild)	OPW -	22	31	No
6-Jun	Oliver Pond (Wild)	OPW -	23	31	No
6-Jun	Oliver Pond (Wild)	OPW -	24	31	No
6-Jun	Oliver Pond (Wild)	OPW -	25	30	No
6-Jun	Oliver Pond (Wild)	OPW -	26	30	No
6-Jun	Oliver Pond (Wild)	OPW -	27	30	No
6-Jun	Oliver Pond (Wild)	OPW -	28	31	No
6-Jun	Oliver Pond (Wild)	OPW -	29	31	Yes
6-Jun	Oliver Pond (Wild)	OPW -	30	32	No
13-Jun	Oliver Pond (Wild)	OPW -	31	35	No
13-Jun	Oliver Pond (Wild)	OPW -	32	35	No
13-Jun	Oliver Pond (Wild)	OPW -	33	36	Yes
13-Jun	Oliver Pond (Wild)	OPW -	34	36	Yes
13-Jun	Oliver Pond (Wild)	OPW -	35	36	No
13-Jun	Oliver Pond (Wild)	OPW -	36	36	No
13-Jun	Oliver Pond (Wild)	OPW -	37	37	No
13-Jun	Oliver Pond (Wild)	OPW -	38	37	No

Collection Date	Population	Code	Number	Stage	Infected?
13-Jun	Oliver Pond (Wild)	OPW -	39	36	Yes
13-Jun	Oliver Pond (Wild)	OPW -	40	37	No
17-Jun	Oliver Pond (Wild)	OPW -	41	38	No
17-Jun	Oliver Pond (Wild)	OPW -	42	37	No
17-Jun	Oliver Pond (Wild)	OPW -	43	37	No
17-Jun	Oliver Pond (Wild)	OPW -	44	38	No
17-Jun	Oliver Pond (Wild)	OPW -	45	38	No
17-Jun	Oliver Pond (Wild)	OPW -	46	39	No
17-Jun	Oliver Pond (Wild)	OPW -	47	38	No
17-Jun	Oliver Pond (Wild)	OPW -	48	38	No
17-Jun	Oliver Pond (Wild)	OPW -	49	38	No
17-Jun	Oliver Pond (Wild)	OPW -	50	38	No
16-May	Parker Pond (Wild)	PPW -	1	25	No
16-May	Parker Pond (Wild)	PPW -	2	25	No
16-May	Parker Pond (Wild)	PPW -	3	25	No
16-May	Parker Pond (Wild)	PPW -	4	26	No
16-May	Parker Pond (Wild)	PPW -	5	25	Yes
16-May	Parker Pond (Wild)	PPW -	6	25	No
16-May	Parker Pond (Wild)	PPW -	7	25	Yes
16-May	Parker Pond (Wild)	PPW -	8	25	No
16-May	Parker Pond (Wild)	PPW -	9	25	No
16-May	Parker Pond (Wild)	PPW -	10	25	No
26-May	Parker Pond (Wild)	PPW -	11	26	No
26-May	Parker Pond (Wild)	PPW -	12	26	No
26-May	Parker Pond (Wild)	PPW -	13	27	No
26-May	Parker Pond (Wild)	PPW -	14	26	No
26-May	Parker Pond (Wild)	PPW -	15	26	No
26-May	Parker Pond (Wild)	PPW -	16	27	No
26-May	Parker Pond (Wild)	PPW -	17	25	No
26-May	Parker Pond (Wild)	PPW -	18	28	No
26-May	Parker Pond (Wild)	PPW -	19	26	No
26-May	Parker Pond (Wild)	PPW -	20	27	No
6-Jun	Parker Pond (Wild)	PPW -	21	33	No
6-Jun	Parker Pond (Wild)	PPW -	22	30	No
6-Jun	Parker Pond (Wild)	PPW -	23	34	No
6-Jun	Parker Pond (Wild)	PPW -	24	32	No
6-Jun	Parker Pond (Wild)	PPW -	25	34	No
6-Jun	Parker Pond (Wild)	PPW -	26	32	No
13-Jun	Parker Pond (Wild)	PPW -	27	38	No
13-Jun	Parker Pond (Wild)	PPW -	28	36	No
13-Jun	Parker Pond (Wild)	PPW -	29	38	No
6-Jun	Parker Pond (Wild)	PPW -	30	32	No
13-Jun	Parker Pond (Wild)	PPW -	31	38	No
13-Jun	Parker Pond (Wild)	PPW -	32	38	No
13-Jun	Parker Pond (Wild)	PPW -	33	36	No
13-Jun	Parker Pond (Wild)	PPW -	34	37	No
13-Jun	Parker Pond (Wild)	PPW -	35	38	No

Collection Date	Population	Code	Number	Stage	Infected?
13-Jun	Parker Pond (Wild)	PPW -	36	38	No
6-Jun	Parker Pond (Wild)	PPW -	37	32	No
6-Jun	Parker Pond (Wild)	PPW -	38	31	No
6-Jun	Parker Pond (Wild)	PPW -	39	32	No
13-Jun	Parker Pond (Wild)	PPW -	40	38	No
17-Jun	Parker Pond (Wild)	PPW -	41	39	No
17-Jun	Parker Pond (Wild)	PPW -	42	38	No
17-Jun	Parker Pond (Wild)	PPW -	43	39	No
17-Jun	Parker Pond (Wild)	PPW -	44	39	No
17-Jun	Parker Pond (Wild)	PPW -	45	39	No
17-Jun	Parker Pond (Wild)	PPW -	46	39	No
17-Jun	Parker Pond (Wild)	PPW -	47	39	No
17-Jun	Parker Pond (Wild)	PPW -	48	39	No
17-Jun	Parker Pond (Wild)	PPW -	49	40	No
17-Jun	Parker Pond (Wild)	PPW -	50	38	No
21-Jun	Parker Pond (Wild)	PPW -	51	40	No
21-Jun	Parker Pond (Wild)	PPW -	52	41	No
21-Jun	Parker Pond (Wild)	PPW -	53	41	No
21-Jun	Parker Pond (Wild)	PPW -	54	41	No
21-Jun	Parker Pond (Wild)	PPW -	55	41	No
21-Jun	Parker Pond (Wild)	PPW -	56	41	No
21-Jun	Parker Pond (Wild)	PPW -	57	41	No
21-Jun	Parker Pond (Wild)	PPW -	58	41	No
21-Jun	Parker Pond (Wild)	PPW -	59	41	No
21-Jun	Parker Pond (Wild)	PPW -	60	40	No
25-Jun	Parker Pond (Wild)	PPW -	61	43	No
25-Jun	Parker Pond (Wild)	PPW -	62	41	No
25-Jun	Parker Pond (Wild)	PPW -	63	41	No
25-Jun	Parker Pond (Wild)	PPW -	64	41	No
25-Jun	Parker Pond (Wild)	PPW -	65	42	No
25-Jun	Parker Pond (Wild)	PPW -	66	41	No
25-Jun	Parker Pond (Wild)	PPW -	67	41	No
25-Jun	Parker Pond (Wild)	PPW -	68	41	No
25-Jun	Parker Pond (Wild)	PPW -	69	41	No
25-Jun	Parker Pond (Wild)	PPW -	70	41	No
29-Jun	Parker Pond (Wild)	PPW -	71	43	No
29-Jun	Parker Pond (Wild)	PPW -	72	45	No
29-Jun	Parker Pond (Wild)	PPW -	73	42	No
29-Jun	Parker Pond (Wild)	PPW -	74	43	No
29-Jun	Parker Pond (Wild)	PPW -	75	42	No
29-Jun	Parker Pond (Wild)	PPW -	76	42	No
29-Jun	Parker Pond (Wild)	PPW -	77	41	No
29-Jun	Parker Pond (Wild)	PPW -	78	38	No
29-Jun	Parker Pond (Wild)	PPW -	79	43	No
29-Jun	Parker Pond (Wild)	PPW -	80	44	No

Table 2. The results from the field monitoring of other amphibians. The date indicates the day in which the tadpole was collected. The stage value is the Gosner Stage of development. The code and number correspond to the identification of the sample on the gel.

Collection Date	Population	Species	Code	Number	Stage	Infected?
13-Jun	Oliver Pond	Ambystomid	OAM -	1	46	No
13-Jun	Oliver Pond	Ambystomid	OAM -	2	46	Yes
13-Jun	Oliver Pond	Ambystomid	OAM -	3	46	No
13-Jun	Oliver Pond	Ambystomid	OAM -	4	46	No
13-Jun	Oliver Pond	Ambystomid	OAM -	5	46	No
13-Jun	Oliver Pond	Ambystomid	OAM -	6	46	No
13-Jun	Oliver Pond	Ambystomid	OAM -	7	46	Yes
13-Jun	Oliver Pond	Ambystomid	OAM -	8	46	No
13-Jun	Oliver Pond	Ambystomid	OAM -	9	46	No
13-Jun	Parker Pond	Pseudacris	PPS -	1	39	No
13-Jun	Parker Pond	Pseudacris	PPS -	2	39	No
13-Jun	Parker Pond	Pseudacris	PPS -	3	39	No
13-Jun	Parker Pond	Pseudacris	PPS -	4	39	No
13-Jun	Parker Pond	Pseudacris	PPS -	5	39	No
13-Jun	Parker Pond	Pseudacris	PPS -	6	33	No
13-Jun	Parker Pond	Pseudacris	PPS -	7	37	No
13-Jun	Parker Pond	Pseudacris	PPS -	8	37	No
13-Jun	Parker Pond	Pseudacris	PPS -	9	38	No
13-Jun	Parker Pond	Pseudacris	PPS -	10	35	No
17-Jun	Parker Pond	Pseudacris	PPS -	11	38	No
17-Jun	Parker Pond	Pseudacris	PPS -	12	40	Yes
17-Jun	Parker Pond	Pseudacris	PPS -	13	37	Yes
17-Jun	Parker Pond	Pseudacris	PPS -	14	37	Yes
17-Jun	Parker Pond	Pseudacris	PPS -	15	30	No
17-Jun	Parker Pond	Pseudacris	PPS -	16	36	No
17-Jun	Parker Pond	Pseudacris	PPS -	17	33	No
17-Jun	Parker Pond	Pseudacris	PPS -	18	36	Yes
17-Jun	Parker Pond	Pseudacris	PPS -	19	39	Yes
17-Jun	Parker Pond	Pseudacris	PPS -	20	41	Yes
21-Jun	Parker Pond	Pseudacris	PPS -	21	41	No
21-Jun	Parker Pond	Pseudacris	PPS -	22	41	No
21-Jun	Parker Pond	Pseudacris	PPS -	23	34	No
21-Jun	Parker Pond	Pseudacris	PPS -	24	27	No
21-Jun	Parker Pond	Pseudacris	PPS -	27	34	No
21-Jun	Parker Pond	Pseudacris	PPS -	28	39	No
21-Jun	Parker Pond	Pseudacris	PPS -	29	34	No
21-Jun	Parker Pond	Pseudacris	PPS -	30	43	No
25-Jun	Parker Pond	Pseudacris	PPS -	31	38	No
25-Jun	Parker Pond	Pseudacris	PPS -	32	30	No
25-Jun	Parker Pond	Pseudacris	PPS -	33	32	No
25-Jun	Parker Pond	Pseudacris	PPS -	34	39	No
25-Jun	Parker Pond	Pseudacris	PPS -	35	36	No

Collection Date	Population	Species	Code	Number	Stage	Infected?
25-Jun	Parker Pond	Pseudacris	PPS -	36	30	No
25-Jun	Parker Pond	Pseudacris	PPS -	37	34	No
25-Jun	Parker Pond	Pseudacris	PPS -	38	37	No
25-Jun	Parker Pond	Pseudacris	PPS -	39	41	Yes
25-Jun	Parker Pond	Pseudacris	PPS -	40	33	No
29-Jun	Parker Pond	Pseudacris	PPS -	41	40	No
29-Jun	Parker Pond	Pseudacris	PPS -	42	41	No
29-Jun	Parker Pond	Pseudacris	PPS -	43	43	No
29-Jun	Parker Pond	Pseudacris	PPS -	44	29	No
29-Jun	Parker Pond	Pseudacris	PPS -	45	41	No
29-Jun	Parker Pond	Pseudacris	PPS -	46	36	No
29-Jun	Parker Pond	Pseudacris	PPS -	47	38	No
29-Jun	Parker Pond	Pseudacris	PPS -	48	42	No
29-Jun	Parker Pond	Pseudacris	PPS -	49	36	No
29-Jun	Parker Pond	Pseudacris	PPS -	50	31	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	1	32	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	2	37	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	3	36	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	4	31	Yes
30-Jun	Parker Pond	Hyla versicolor	PHV -	5	33	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	6	37	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	7	40	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	8	34	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	9	37	No
30-Jun	Parker Pond	Hyla versicolor	PHV -	10	37	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	11	38	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	12	38	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	13	37	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	14	37	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	15	37	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	16	40	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	17	36	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	18	31	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	19	37	No
9-Jul	Parker Pond	Hyla versicolor	PHV -	20	38	No
13-Jun	Parker Pond	Ambystomids	PAS -	1	46	Yes
13-Jun	Parker Pond	Ambystomids	PAS -	2	46	No
13-Jun	Parker Pond	Ambystomids	PAS -	3	46	No
13-Jun	Parker Pond	Ambystomids	PAS -	4	46	Yes
13-Jun	Parker Pond	Ambystomids	PAS -	5	46	Yes
13-Jun	Parker Pond	Ambystomids	PAS -	6	46	Yes
13-Jun	Parker Pond	Ambystomids	PAS -	7	46	No
13-Jun	Parker Pond	Ambystomids	PAS -	8	46	No
13-Jun	Parker Pond	Ambystomids	PAS -	9	46	Yes
13-Jun	Parker Pond	Ambystomids	PAS -	10	46	No
17-Jun	Parker Pond	Ambystomids	PAS -	11	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	12	46	Yes

Collection Date	Population	Species	Code	Number	Stage	Infected?
17-Jun	Parker Pond	Ambystomids	PAS -	13	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	14	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	15	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	16	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	17	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	18	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	19	46	Yes
17-Jun	Parker Pond	Ambystomids	PAS -	20	46	No
21-Jun	Parker Pond	Ambystomids	PAS -	21	46	Yes
21-Jun	Parker Pond	Ambystomids	PAS -	22	46	Yes
21-Jun	Parker Pond	Ambystomids	PAS -	23	46	No
21-Jun	Parker Pond	Ambystomids	PAS -	24	46	Yes
21-Jun	Parker Pond	Ambystomids	PAS -	25	46	No
21-Jun	Parker Pond	Ambystomids	PAS -	26	46	Yes
21-Jun	Parker Pond	Ambystomids	PAS -	27	46	No
21-Jun	Parker Pond	Ambystomids	PAS -	28	46	No
21-Jun	Parker Pond	Ambystomids	PAS -	29	46	No
21-Jun	Parker Pond	Ambystomids	PAS -	30	46	Yes
25-Jun	Parker Pond	Ambystomids	PAS -	31	46	No
25-Jun	Parker Pond	Ambystomids	PAS -	32	46	No
25-Jun	Parker Pond	Ambystomids	PAS -	33	46	No
25-Jun	Parker Pond	Ambystomids	PAS -	34	46	No
25-Jun	Parker Pond	Ambystomids	PAS -	35	46	Yes
25-Jun	Parker Pond	Ambystomids	PAS -	36	46	Yes
25-Jun	Parker Pond	Ambystomids	PAS -	37	46	No
25-Jun	Parker Pond	Ambystomids	PAS -	38	46	Yes
25-Jun	Parker Pond	Ambystomids	PAS -	39	46	Yes
25-Jun	Parker Pond	Ambystomids	PAS -	40	46	No
29-Jun	Parker Pond	Ambystomids	PAS -	41	46	Yes
29-Jun	Parker Pond	Ambystomids	PAS -	42	46	Yes
29-Jun	Parker Pond	Ambystomids	PAS -	43	46	No
29-Jun	Parker Pond	Ambystomids	PAS -	44	46	No
29-Jun	Parker Pond	Ambystomids	PAS -	45	46	Yes
29-Jun	Parker Pond	Ambystomids	PAS -	46	46	Yes
29-Jun	Parker Pond	Ambystomids	PAS -	47	46	No
29-Jun	Parker Pond	Ambystomids	PAS -	48	46	Yes
29-Jun	Parker Pond	Ambystomids	PAS -	49	46	Yes
29-Jun	Parker Pond	Ambystomids	PAS -	50	46	Yes
21-Jun	Parker Pond	Notophthalmus viridescens	ANV	1	Adult	No
21-Jun	Parker Pond	Notophthalmus viridescens	ANV	2	Adult	Yes
21-Jun	Parker Pond	Notophthalmus viridescens	ANV	3	Adult	No
21-Jun	Parker Pond	Notophthalmus viridescens	ANV	4	Adult	No
21-Jun	Parker Pond	Notophthalmus viridescens	ANV	5	Adult	No

Collection Date	Population	Species	Code	Number	Stage	Infected?
23-Apr	Crowe's Line Rd.	Rana sylvatica	KPC	F	Adult	No
23-Apr	Crowe's Line Rd.	Rana sylvatica	KPC	M	Adult	Yes
17-Apr	Oliver Pond	Rana sylvatica	OAW	1	Adult	No
17-Apr	Oliver Pond	Rana sylvatica	OAW	2	Adult	No
17-Apr	Oliver Pond	Rana sylvatica	OAW	3	Adult	No
22-Apr	Crowe's Line Rd.	Rana sylvatica	KPB	1	Egg	No
22-Apr	Crowe's Line Rd.	Rana sylvatica	KPB	2	Egg	No
22-Apr	Crowe's Line Rd.	Rana sylvatica	KPB	3	Egg	No
22-Apr	Crowe's Line Rd.	Rana sylvatica	KPB	4	Egg	No
22-Apr	Crowe's Line Rd.	Rana sylvatica	KPB	5	Egg	No
24-Apr	Crowe's Line Rd.	Rana sylvatica	KPC	1	Egg	No
24-Apr	Crowe's Line Rd.	Rana sylvatica	KPC	2	Egg	No
24-Apr	Crowe's Line Rd.	Rana sylvatica	KPC	3	Egg	Yes
24-Apr	Crowe's Line Rd.	Rana sylvatica	KPC	4	Egg	No
24-Apr	Crowe's Line Rd.	Rana sylvatica	KPC	5	Egg	No
22-Apr	Parker Pond	Ambystoma maculatum	EAM	1	Egg	No
22-Apr	Parker Pond	Ambystoma maculatum	EAM	2	Egg	No
22-Apr	Parker Pond	Ambystoma maculatum	EAM	3	Egg	No
22-Apr	Parker Pond	Ambystoma maculatum	EAM	4	Egg	No
22-Apr	Parker Pond	Ambystoma maculatum	EAM	5	Egg	No

Table 3. The results for the vertical transmission experiment in the lab. The date indicates the day in which the tadpole was collected. The stage value is the Gosner Stage of development. The code and number correspond to the identification of the sample on the gel. Stage 1 indicates that the sample analyze is an egg.

Collection Date	Population	Code	Number	Stage	Infected?
16-Apr	County Rd. 6	CRV -	1	1	No
16-Apr	County Rd. 6	CRV -	2	1	No
16-Apr	County Rd. 6	CRV -	3	1	No
16-Apr	County Rd. 6	CRV -	4	1	No
16-Apr	County Rd. 6	CRV -	5	1	No
2-May	County Rd. 6	CRV -	6	25	No
2-May	County Rd. 6	CRV -	8	25	No
2-May	County Rd. 6	CRV -	9	25	Yes
2-May	County Rd. 6	CRV -	10	25	No
26-May	County Rd. 6	CRV -	25	26	No
5-Jun	County Rd. 6	CRV -	27	25	No
5-Jun	County Rd. 6	CRV -	28	31	No
5-Jun	County Rd. 6	CRV -	30	25	No
15-Jul	County Rd. 6	CRV -	46	44	No
15-Jul	County Rd. 6	CRV -	47	41	No

Collection Date	Population	Code	Number	Stage	Infected?
15-Jul	County Rd. 6	CRV -	48	43	No
15-Jul	County Rd. 6	CRV -	49	38	No
15-Jul	County Rd. 6	CRV -	50	38	No
18-Apr	Oliver Pond	OLV -	1	1	No
18-Apr	Oliver Pond	OLV -	2	1	No
18-Apr	Oliver Pond	OLV -	3	1	No
18-Apr	Oliver Pond	OLV -	4	1	Yes
28-Apr	Oliver Pond	OLV -	6	25	No
28-Apr	Oliver Pond	OLV -	7	25	No
28-Apr	Oliver Pond	OLV -	8	25	No
28-Apr	Oliver Pond	OLV -	9	24	No
28-Apr	Oliver Pond	OLV -	10	24	No
7-Jun	Oliver Pond	OLV -	27	27	No
7-Jun	Oliver Pond	OLV -	28	28	No
7-Jun	Oliver Pond	OLV -	29	34	No
17-Jul	Oliver Pond	OLV -	47	40	No
17-Jul	Oliver Pond	OLV -	48	44	No
17-Jul	Oliver Pond	OLV -	50	44	No
18-Apr	Crowe's Line Rd.	CLV -	1	1	No
18-Apr	Crowe's Line Rd.	CLV -	2	1	No
18-Apr	Crowe's Line Rd.	CLV -	3	1	Yes
18-Apr	Crowe's Line Rd.	CLV -	4	1	No
18-Apr	Crowe's Line Rd.	CLV -	5	1	No
28-Apr	Crowe's Line Rd.	CLV -	6	24	Yes
28-Apr	Crowe's Line Rd.	CLV -	8	25	No
28-Apr	Crowe's Line Rd.	CLV -	9	24	No
7-Jun	Crowe's Line Rd.	CLV -	27	25	No
7-Jun	Crowe's Line Rd.	CLV -	28	36	No
7-Jun	Crowe's Line Rd.	CLV -	29	35	No
7-Jun	Crowe's Line Rd.	CLV -	30	34	No
7-Jul	Crowe's Line Rd.	CLV -	41	45	No
7-Jul	Crowe's Line Rd.	CLV -	42	43	No
7-Jul	Crowe's Line Rd.	CLV -	43	35	Yes
7-Jul	Crowe's Line Rd.	CLV -	44	41	No
7-Jul	Crowe's Line Rd.	CLV -	45	35	No
18-Apr	Division @ Donwood	DDV -	1	1	No
18-Apr	Division @ Donwood	DDV -	2	1	No
18-Apr	Division @ Donwood	DDV -	3	1	No
18-Apr	Division @ Donwood	DDV -	4	1	Yes
18-Apr	Division @ Donwood	DDV -	5	1	No
28-Apr	Division @ Donwood	DDV -	6	24	No
28-Apr	Division @ Donwood	DDV -	7	24	No
28-Apr	Division @ Donwood	DDV -	8	25	No
28-Apr	Division @ Donwood	DDV -	10	24	No
7-Jun	Division @ Donwood	DDV -	26	29	No
7-Jun	Division @ Donwood	DDV -	28	36	No
7-Jun	Division @ Donwood	DDV -	30	25	No

Collection Date	Population	Code	Number	Stage	Infected?
17-Jul	Division @ Donwood	DDV -	46	43	No
17-Jul	Division @ Donwood	DDV -	47	40	No
17-Jul	Division @ Donwood	DDV -	49	38	No
21-Apr	Barb's Marsh	BMV -	1	1	No
21-Apr	Barb's Marsh	BMV -	2	1	No
21-Apr	Barb's Marsh	BMV -	3	1	Yes
21-Apr	Barb's Marsh	BMV -	4	1	No
21-Apr	Barb's Marsh	BMV -	5	1	No
1-May	Barb's Marsh	BMV -	6	24	No
1-May	Barb's Marsh	BMV -	7	24	No
1-May	Barb's Marsh	BMV -	8	24	No
1-May	Barb's Marsh	BMV -	9	24	No
1-May	Barb's Marsh	BMV -	10	24	No
9-Jun	Barb's Marsh	BMV -	26	29	No
9-Jun	Barb's Marsh	BMV -	27	25	No
9-Jun	Barb's Marsh	BMV -	28	30	No
9-Jun	Barb's Marsh	BMV -	29	25	No
9-Jun	Barb's Marsh	BMV -	30	31	No
19-Jul	Barb's Marsh	BMV -	46	37	No
19-Jul	Barb's Marsh	BMV -	47	41	No
19-Jul	Barb's Marsh	BMV -	48	44	No
19-Jul	Barb's Marsh	BMV -	49	41	No
19-Jul	Barb's Marsh	BMV -	50	36	No
21-Apr	Known Parents A	KPA -	1	1	No
21-Apr	Known Parents A	KPA -	2	1	No
21-Apr	Known Parents A	KPA -	3	1	No
21-Apr	Known Parents A	KPA -	4	1	No
21-Apr	Known Parents A	KPA -	5	1	No
1-May	Known Parents A	KPA -	6	24	No
1-May	Known Parents A	KPA -	7	25	No
1-May	Known Parents A	KPA -	8	25	No
1-May	Known Parents A	KPA -	9	25	No
1-May	Known Parents A	KPA -	10	25	No
9-Jun	Known Parents A	KPA -	26	25	No
9-Jun	Known Parents A	KPA -	28	31	No
9-Jun	Known Parents A	KPA -	30	31	No
29-Jun	Known Parents A	KPA -	36	44	No
29-Jun	Known Parents A	KPA -	37	36	No
29-Jun	Known Parents A	KPA -	38	39	No
29-Jun	Known Parents A	KPA -	39	43	No
29-Jun	Known Parents A	KPA -	40	38	No

Table 4. The experimental results for the FV3 concentration experiment. The date indicates the day in which the tadpole died or was euthanized. The stage value is the Gosner Stage of development. The code and number correspond to the identification of the sample on the gel. Please note that the code of this experiment often appears as FUV on the gel.

Date	Experiement	Trial	Death Type	Code	Number	Stage	Infected?
11-Jul	Control	A	Natural	FVC -	1	29	No
13-Jul	Control	A	Natural	FVC -	2	25	No
13-Jul	Control	A	Natural	FVC -	3	41	No
13-Jul	Control	A	Natural	FVC -	4	41	No
13-Jul	Control	A	Natural	FVC -	5	39	No
15-Jul	Control	A	Euthanized	FVC -	6	27	No
15-Jul	Control	A	Euthanized	FVC -	7	38	No
15-Jul	Control	A	Euthanized	FVC -	8	29	No
15-Jul	Control	A	Euthanized	FVC -	9	39	No
15-Jul	Control	A	Euthanized	FVC -	10	35	No
11-Jul	Control	B	Natural	FVC -	11	44	No
11-Jul	Control	B	Natural	FVC -	12	46	No
11-Jul	Control	B	Natural	FVC -	13	44	No
15-Jul	Control	B	Euthanized	FVC -	14	30	No
15-Jul	Control	B	Euthanized	FVC -	15	28	No
15-Jul	Control	B	Euthanized	FVC -	16	28	No
15-Jul	Control	B	Euthanized	FVC -	17	33	No
15-Jul	Control	B	Euthanized	FVC -	18	35	No
15-Jul	Control	B	Euthanized	FVC -	19	34	No
15-Jul	Control	B	Euthanized	FVC -	20	31	No
11-Jul	1uL FV3	A	Natural	FVC -	21	44	Yes
11-Jul	1uL FV3	A	Natural	FVC -	22	45	Yes
13-Jul	1uL FV3	A	Natural	FVC -	23	31	Yes
13-Jul	1uL FV3	A	Natural	FVC -	24	34	Yes
14-Jul	1uL FV3	A	Natural	FVC -	25	31	Yes
14-Jul	1uL FV3	A	Natural	FVC -	26	37	Yes
15-Jul	1uL FV3	A	Natural	FVC -	27	38	Yes
15-Jul	1uL FV3	A	Natural	FVC -	28	34	Yes
15-Jul	1uL FV3	A	Euthanized	FVC -	29	31	Yes
15-Jul	1uL FV3	A	Euthanized	FVC -	30	27	No
11-Jul	1uL FV3	B	Natural	FVC -	31	45	No
14-Jul	1uL FV3	B	Natural	FVC -	32	38	No
14-Jul	1uL FV3	B	Natural	FVC -	33	38	No
14-Jul	1uL FV3	B	Natural	FVC -	34	37	No
15-Jul	1uL FV3	B	Natural	FVC -	35	36	No
15-Jul	1uL FV3	B	Natural	FVC -	36	38	No
15-Jul	1uL FV3	B	Natural	FVC -	37	37	No
15-Jul	1uL FV3	B	Euthanized	FVC -	38	30	No
15-Jul	1uL FV3	B	Euthanized	FVC -	39	35	No
15-Jul	1uL FV3	B	Euthanized	FVC -	40	32	No
9-Jul	10uL FV3	A	Natural	FVC -	41	44	No
11-Jul	10uL FV3	A	Natural	FVC -	42	46	No

Date	Experiement	Trial	Death Type	Code	Number	Stage	Infected?
13-Jul	10uL FV3	A	Natural	FVC -	43	38	No
13-Jul	10uL FV3	A	Natural	FVC -	44	36	No
13-Jul	10uL FV3	A	Natural	FVC -	45	38	No
14-Jul	10uL FV3	A	Natural	FVC -	46	35	No
14-Jul	10uL FV3	A	Natural	FVC -	47	25	No
15-Jul	10uL FV3	A	Natural	FVC -	48	38	No
15-Jul	10uL FV3	A	Natural	FVC -	49	32	No
15-Jul	10uL FV3	A	Natural	FVC -	50	34	No
10-Jul	10uL FV3	B	Natural	FVC -	51	44	Yes
12-Jul	10uL FV3	B	Natural	FVC -	52	38	No
13-Jul	10uL FV3	B	Natural	FVC -	53	34	No
13-Jul	10uL FV3	B	Natural	FVC -	54	38	Yes
13-Jul	10uL FV3	B	Natural	FVC -	55	38	Yes
13-Jul	10uL FV3	B	Natural	FVC -	56	29	Yes
14-Jul	10uL FV3	B	Natural	FVC -	57	30	Yes
14-Jul	10uL FV3	B	Natural	FVC -	58	35	Yes
14-Jul	10uL FV3	B	Natural	FVC -	59	32	Yes
14-Jul	10uL FV3	B	Natural	FVC -	60	34	Yes
11-Jul	100uL FV3	A	Natural	FVC -	61	43	Yes
11-Jul	100uL FV3	A	Natural	FVC -	62	43	Yes
12-Jul	100uL FV3	A	Natural	FVC -	63	44	Yes
15-Jul	100uL FV3	A	Natural	FVC -	64	35	Yes
15-Jul	100uL FV3	A	Natural	FVC -	65	37	Yes
15-Jul	100uL FV3	A	Natural	FVC -	66	35	Yes
15-Jul	100uL FV3	A	Euthanized	FVC -	67	41	Yes
15-Jul	100uL FV3	A	Euthanized	FVC -	68	37	Yes
15-Jul	100uL FV3	A	Euthanized	FVC -	69	38	Yes
15-Jul	100uL FV3	A	Euthanized	FVC -	70	38	Yes
11-Jul	100uL FV3	B	Natural	FVC -	71	43	Yes
12-Jul	100uL FV3	B	Natural	FVC -	72	38	Yes
12-Jul	100uL FV3	B	Natural	FVC -	73	25	Yes
12-Jul	100uL FV3	B	Natural	FVC -	74	26	Yes
12-Jul	100uL FV3	B	Natural	FVC -	75	34	Yes
12-Jul	100uL FV3	B	Natural	FVC -	76	36	Yes
12-Jul	100uL FV3	B	Natural	FVC -	77	33	Yes
12-Jul	100uL FV3	B	Natural	FVC -	78	32	Yes
12-Jul	100uL FV3	B	Natural	FVC -	79	31	Yes
12-Jul	100uL FV3	B	Natural	FVC -	80	41	Yes

Table 5. The results for the interspecies transmission experiment between wood frog tadpoles and Ambystomid larvae. The date indicates the day in which the tadpole died or was euthanized. The stage value is the Gosner Stage of development. The code and number correspond to the identification of the sample on the gel.

Date	Treatment	Code	Number	Stage	Infected?
9-Jul	Wood Frog Tadpole Control	AMB -	1	30	No
9-Jul	Wood Frog Tadpole Control	AMB -	2	31	No
9-Jul	Wood Frog Tadpole Control	AMB -	3	27	No
9-Jul	Wood Frog Tadpole Control	AMB -	4	34	No
9-Jul	Wood Frog Tadpole Control	AMB -	5	34	No
9-Jul	Wood Frog Tadpole Control	AMB -	6	28	No
9-Jul	Wood Frog Tadpole Control	AMB -	7	28	No
9-Jul	Wood Frog Tadpole Control	AMB -	8	32	No
9-Jul	Wood Frog Tadpole Control	AMB -	9	28	No
9-Jul	Wood Frog Tadpole Control	AMB -	10	29	Yes
9-Jul	Ambystomid Larvae Control	AMB -	11	46	Yes
9-Jul	Ambystomid Larvae Control	AMB -	12	46	Yes
9-Jul	Ambystomid Larvae Control	AMB -	13	46	Yes
9-Jul	Ambystomid Larvae Control	AMB -	14	46	No
9-Jul	Ambystomid Larvae Control	AMB -	15	46	No
9-Jul	Ambystomid Larvae Control	AMB -	16	46	No
9-Jul	Ambystomid Larvae Control	AMB -	17	46	Yes
9-Jul	Ambystomid Larvae Control	AMB -	18	46	Yes
9-Jul	Ambystomid Larvae Control	AMB -	19	46	No
9-Jul	Ambystomid Larvae Control	AMB -	20	46	Yes
24-Jul	Tadpoles from Exposure 1	AMB -	21	38	Yes
24-Jul	Tadpoles from Exposure 1	AMB -	22	39	No
24-Jul	Tadpoles from Exposure 1	AMB -	23	39	Yes
24-Jul	Tadpoles from Exposure 1	AMB -	24	32	Yes
24-Jul	Tadpoles from Exposure 1	AMB -	25	41	Yes
24-Jul	Tadpoles from Exposure 1	AMB -	26	34	Yes
24-Jul	Tadpoles from Exposure 1	AMB -	27	36	No
24-Jul	Tadpoles from Exposure 1	AMB -	28	37	No
24-Jul	Tadpoles from Exposure 1	AMB -	29	36	
24-Jul	Tadpoles from Exposure 1	AMB -	30	38	Yes
16-Jul	Tadpoles from Exposure 3	AMB -	41	38	Yes
16-Jul	Tadpoles from Exposure 3	AMB -	42	36	No
16-Jul	Tadpoles from Exposure 3	AMB -	43	37	No
16-Jul	Tadpoles from Exposure 3	AMB -	44	34	Yes
16-Jul	Tadpoles from Exposure 3	AMB -	45	36	Yes
16-Jul	Tadpoles from Exposure 3	AMB -	46	43	Yes
16-Jul	Tadpoles from Exposure 3	AMB -	47	37	No
16-Jul	Tadpoles from Exposure 3	AMB -	48	39	No
16-Jul	Tadpoles from Exposure 3	AMB -	49	33	Yes
16-Jul	Tadpoles from Exposure 3	AMB -	50	44	
24-Jul	Ambystomid Larvae from Exp. 1	AMB -	71	Sub - adult	
24-Jul	Ambystomid Larvae from Exp. 1	AMB -	72	Sub - adult	Yes
24-Jul	Ambystomid Larvae from Exp. 1	AMB -	73	Sub - adult	Yes

Date	Treatment	Code	Number	Stage	Infected?
24-Jul	Ambystomid Larvae from Exp. 1	AMB -	74	Sub - adult	No
24-Jul	Ambystomid Larvae from Exp. 1	AMB -	75	Sub - adult	Yes
16-Jul	Ambystomid Larvae from Exp. 3	AMB -	81	Sub - adult	No
16-Jul	Ambystomid Larvae from Exp. 3	AMB -	82	Sub - adult	Yes
16-Jul	Ambystomid Larvae from Exp. 3	AMB -	83	Sub - adult	No
16-Jul	Ambystomid Larvae from Exp. 3	AMB -	84	46	Yes
16-Jul	Ambystomid Larvae from Exp. 3	AMB -	85	Sub - adult	No

Table 6 .The experimental results for the FV3 and Malathion experiment. The date indicates the day in which the tadpole died or was euthanized. The code and number correspond to the identification of the sample on the gel.

Date	Treatment	Trial	Death Type	Code	Number	Stage	Infected?
14-Jul	Control	A	Euthanized	IMN -	1	40	No
9-Jul	Control	A	Natural	IMN -	2	34	No
9-Jul	Control	A	Natural	IMN -	3	25	No
12-Jul	Control	A	Natural	IMN -	4	43	No
12-Jul	Control	A	Natural	IMN -	5	30	No
12-Jul	Control	A	Natural	IMN -	6	29	No
14-Jul	Control	A	Euthanized	IMN -	7	39	No
14-Jul	Control	A	Euthanized	IMN -	8	35	No
14-Jul	Control	A	Euthanized	IMN -	9	35	No
14-Jul	Control	A	Euthanized	IMN -	10	35	No
9-Jul	Control	B	Natural	IMN -	11	33	No
14-Jul	Control	B	Euthanized	IMN -	12	39	No
14-Jul	Control	B	Euthanized	IMN -	13	30	No
14-Jul	Control	B	Euthanized	IMN -	14	35	No
14-Jul	Control	B	Euthanized	IMN -	15	38	No
14-Jul	Control	B	Euthanized	IMN -	16	37	No
14-Jul	Control	B	Euthanized	IMN -	17	43	No
14-Jul	Control	B	Euthanized	IMN -	18	32	No
14-Jul	Control	B	Euthanized	IMN -	19	33	No
14-Jul	Control	B	Euthanized	IMN -	20	39	No
9-Jul	10uL FV3 Only	A	Natural	IMN -	21	25	No
9-Jul	10uL FV3 Only	A	Natural	IMN -	22	25	No
9-Jul	10uL FV3 Only	A	Natural	IMN -	23	29	No
9-Jul	10uL FV3 Only	A	Natural	IMN -	24	33	No
12-Jul	10uL FV3 Only	A	Natural	IMN -	25	39	Yes
14-Jul	10uL FV3 Only	A	Natural	IMN -	26	41	Yes
14-Jul	10uL FV3 Only	A	Natural	IMN -	27	33	Yes
14-Jul	10uL FV3 Only	A	Natural	IMN -	28	34	Yes
14-Jul	10uL FV3 Only	A	Euthanized	IMN -	29	34	Yes
14-Jul	10uL FV3 Only	A	Euthanized	IMN -	30	40	Yes
11-Jul	10uL FV3 Only	B	Natural	IMN -	31	25	Yes
12-Jul	10uL FV3 Only	B	Natural	IMN -	32	29	Yes
12-Jul	10uL FV3 Only	B	Natural	IMN -	33	27	Yes
14-Jul	10uL FV3 Only	B	Natural	IMN -	34	39	Yes

Date	Treatment	Trial	Death Type	Code	Number	Stage	Infected?
14-Jul	10uL FV3 Only	B	Natural	IMN -	35	41	Yes
14-Jul	10uL FV3 Only	B	Natural	IMN -	36	39	Yes
14-Jul	10uL FV3 Only	B	Euthanized	IMN -	37	38	Yes
14-Jul	10uL FV3 Only	B	Euthanized	IMN -	38	35	Yes
14-Jul	10uL FV3 Only	B	Euthanized	IMN -	39	35	Yes
14-Jul	10uL FV3 Only	B	Euthanized	IMN -	40	34	Yes
7-Jul	3mg/L Malathion Only	A	Natural	IMN -	41	43	Yes
7-Jul	3mg/L Malathion Only	A	Natural	IMN -	42	34	No
7-Jul	3mg/L Malathion Only	A	Natural	IMN -	43	31	Yes
7-Jul	3mg/L Malathion Only	A	Natural	IMN -	44	30	No
7-Jul	3mg/L Malathion Only	A	Natural	IMN -	45	33	No
8-Jul	3mg/L Malathion Only	A	Natural	IMN -	46	41	Yes
9-Jul	3mg/L Malathion Only	A	Natural	IMN -	47	38	Yes
9-Jul	3mg/L Malathion Only	A	Natural	IMN -	48	35	No
9-Jul	3mg/L Malathion Only	A	Natural	IMN -	49	37	No
9-Jul	3mg/L Malathion Only	A	Natural	IMN -	50	37	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	51	39	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	52	34	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	53	29	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	54	35	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	55	32	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	56	40	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	57	25	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	58	25	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	59	35	No
7-Jul	3mg/L Malathion Only	B	Natural	IMN -	60	34	No
7-Jul	10mg/L Malathion Only	A	Natural	IMN -	61	34	No
9-Jul	10mg/L Malathion Only	A	Natural	IMN -	62	35	No
9-Jul	10mg/L Malathion Only	A	Natural	IMN -	63	27	No
9-Jul	10mg/L Malathion Only	A	Natural	IMN -	64	41	No
12-Jul	10mg/L Malathion Only	A	Natural	IMN -	65	34	No
12-Jul	10mg/L Malathion Only	A	Natural	IMN -	66	34	No
12-Jul	10mg/L Malathion Only	A	Natural	IMN -	67	31	No
12-Jul	10mg/L Malathion Only	A	Euthanized	IMN -	68	38	No
12-Jul	10mg/L Malathion Only	A	Euthanized	IMN -	69	34	No
12-Jul	10mg/L Malathion Only	A	Euthanized	IMN -	70	41	No
7-Jul	10mg/L Malathion Only	B	Natural	IMN -	71	28	No
7-Jul	10mg/L Malathion Only	B	Natural	IMN -	72	28	No
8-Jul	10mg/L Malathion Only	B	Natural	IMN -	73	31	No
8-Jul	10mg/L Malathion Only	B	Natural	IMN -	74	34	No
8-Jul	10mg/L Malathion Only	B	Natural	IMN -	75	25	No
9-Jul	10mg/L Malathion Only	B	Natural	IMN -	76	38	No
9-Jul	10mg/L Malathion Only	B	Natural	IMN -	77	38	No
9-Jul	10mg/L Malathion Only	B	Natural	IMN -	78	41	No
9-Jul	10mg/L Malathion Only	B	Natural	IMN -	79	37	No
9-Jul	10mg/L Malathion Only	B	Natural	IMN -	80	37	No
9-Jul	3mg/L Malathion & 10ul FV3	A	Natural	IMN -	81	29	Yes

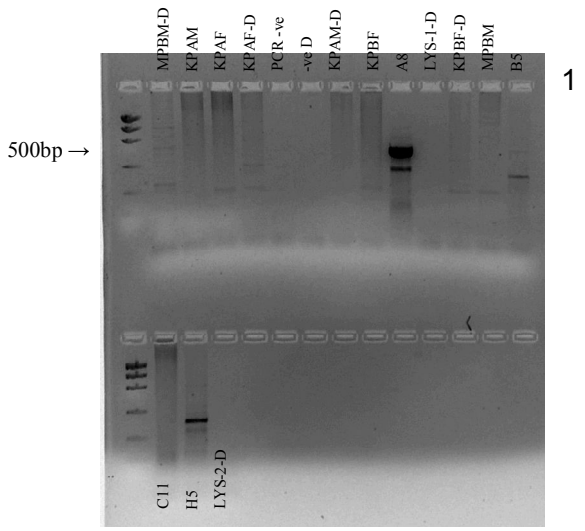
Date	Treatment	Trial	Death Type	Code	Number	Stage	Infected?
9-Jul	3mg/L Malathion & 10ul FV3	A	Natural	IMN -	82	30	Yes
9-Jul	3mg/L Malathion & 10ul FV3	A	Natural	IMN -	83	34	Yes
12-Jul	3mg/L Malathion & 10ul FV3	A	Natural	IMN -	84	35	Yes
12-Jul	3mg/L Malathion & 10ul FV3	A	Natural	IMN -	85	40	Yes
12-Jul	3mg/L Malathion & 10ul FV3	A	Natural	IMN -	86	44	Yes
14-Jul	3mg/L Malathion & 10ul FV3	A	Euthanized	IMN -	87	39	Yes
14-Jul	3mg/L Malathion & 10ul FV3	A	Euthanized	IMN -	88	35	Yes
14-Jul	3mg/L Malathion & 10ul FV3	A	Euthanized	IMN -	89	37	Yes
14-Jul	3mg/L Malathion & 10ul FV3	A	Euthanized	IMN -	90	37	Yes
9-Jul	3mg/L Malathion & 10ul FV3	B	Natural	IMN -	91	32	Yes
9-Jul	3mg/L Malathion & 10ul FV3	B	Natural	IMN -	92	30	Yes
9-Jul	3mg/L Malathion & 10ul FV3	B	Natural	IMN -	93	30	Yes
9-Jul	3mg/L Malathion & 10ul FV3	B	Natural	IMN -	94	38	Yes
14-Jul	3mg/L Malathion & 10ul FV3	B	Natural	IMN -	95	38	Yes
14-Jul	3mg/L Malathion & 10ul FV3	B	Natural	IMN -	96	35	Yes
14-Jul	3mg/L Malathion & 10ul FV3	B	Natural	IMN -	97	36	Yes
14-Jul	3mg/L Malathion & 10ul FV3	B	Euthanized	IMN -	98	38	Yes
14-Jul	3mg/L Malathion & 10ul FV3	B	Euthanized	IMN -	99	35	Yes
14-Jul	3mg/L Malathion & 10ul FV3	B	Euthanized	IMN -	100	34	Yes
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	101	43	No
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	102	25	Yes
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	103	35	Yes
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	104	37	Yes
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	105	26	No
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	106	41	Yes
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	107	38	Yes
7-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	108	43	No
9-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	109	30	No
9-Jul	10mg/L Malathion & 10ul FV3	A	Natural	IMN -	110	38	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	111	25	Yes
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	112	28	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	113	25	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	114	31	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	115	34	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	116	38	Yes
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	117	25	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	118	41	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	119	40	No
9-Jul	10mg/L Malathion & 10ul FV3	B	Natural	IMN -	120	38	No

Table 7. Experiment, population and code information of animals for the gels present below.

Experiment	Population	Species	Code
Vertical Transmission	County Rd. 6	*Rana sylvatica*	CRV
Vertical Transmission	Oliver Pond	*Rana sylvatica*	OLV
Vertical Transmission	Crowe's Line Rd.	*Rana sylvatica*	CLV
Vertical Transmission	Division at Donwood Rd	*Rana sylvatica*	DDV
Vertical Transmission	Barb's March	*Rana sylvatica*	BMV
Vertical Transmission	Known Parents A	*Rana sylvatica*	KPA
Wild Collections	Crowe's Line Rd.	*Rana sylvatica*	CWC
Wild Collections	Oliver Pond	*Rana sylvatica*	OPW
Wild Collections	Parker Pond	*Rana sylvatica*	PPW
Wild Collections	Oliver Pond	*Ambystomid* Species	OAM
Wild Collections	Parker Pond	*Pseudacris* Species	PPS
Wild Collections	Parker Pond	*Hyla versicolor*	PHV
Wild Collections	Parker Pond	*Ambystomid* Species	PAS
Wild Collections	Parker Pond	*Notophthalmus viridescens*	ANV
Wild Collections	Parker Pond	*Ambystoma maculatum* Eggs	EAM
Known Parents	Crowe's Line Rd.	*Rana sylvatica*	KPC
Known Parents	Crowe's Line Rd.	*Rana sylvatica*	KPB
Wild Collections	Oliver Pond	*Rana sylvatica*	OAW
Horizontal Transmission	Lab	*Rana sylvatica*	FVC
Immunosupression	Lab	*Rana sylvatica*	IMN
Interspecific Transmission	Parker Pond	*Rana sylvatica*	AMB
Interspecific Transmission	Parker Pond	*Ambystomid* Species	AMB
Horizontal Transmission	200uL Trial	*Rana sylvatica*	
Lysis Buffer Controls		All	LYS

Table 8. Legend to the experiment and on which gel the samples can be found.

Experiment	***Gel Numbers***
Field Monitoring	5, 6, 12, 13, 14, 15, 18, 21, 22, 23
Vertical Transmission	1, 2, 3, 4, 5, 6, 8, 9, 10
Horizontal Transmission	6, 7, 8, 11, 12, 19
Immunosuppression	16, 17, 18, 19, 20
Interspecies Transmission	5, 6
Infection Trial in Wood frogs	24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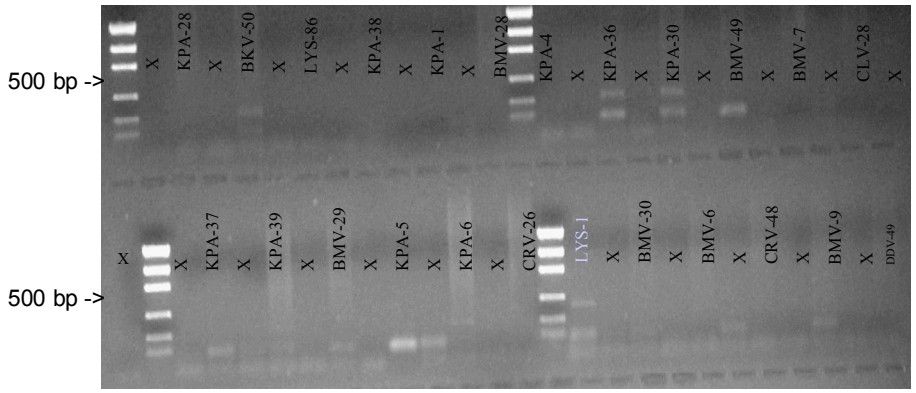
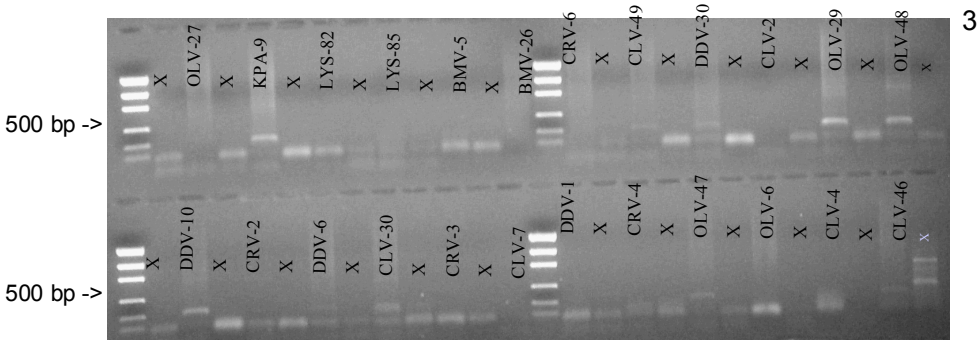
MPBM-D
KPAM
KPAF
KPAF-D
PCR -ve
-ve D
KPAM-D
KPBF
A8
LYS-1-D
KPBF-D
MPBM
B5
C11
H5
LYS-2-D

1


500 bp ->
500 bp ->
X
KPA-28
BKV-50
LYS-86
KPA-38
KPA-1
BMV-28
KPA-4
KPA-36
KPA-30
BMV-49
BMV-7
CLV-28
KPA-37
KPA-39
BMV-29
KPA-5
KPA-6
CRV-26
LYS-1
BMV-30
BMV-6
CRV-48
BMV-9
DDV-49

2


500 bp ->
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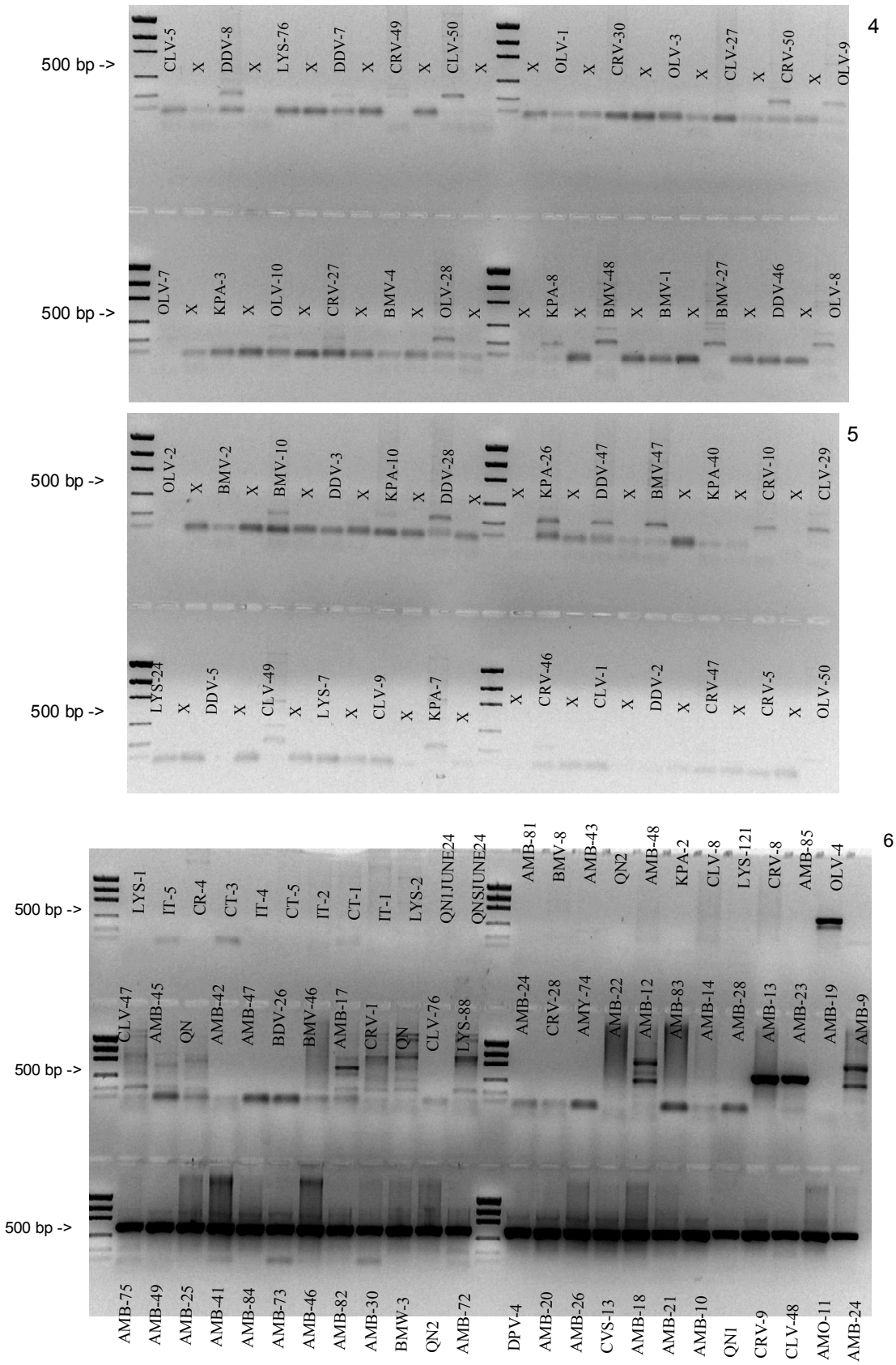
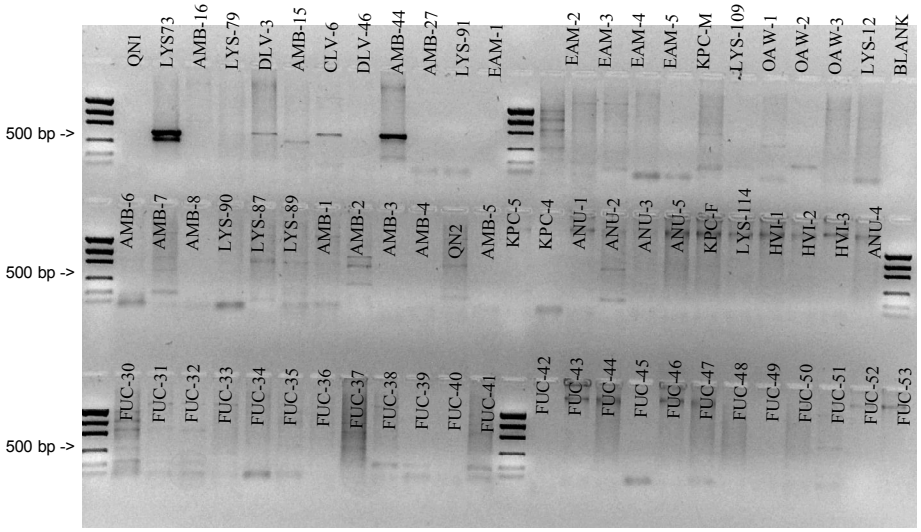
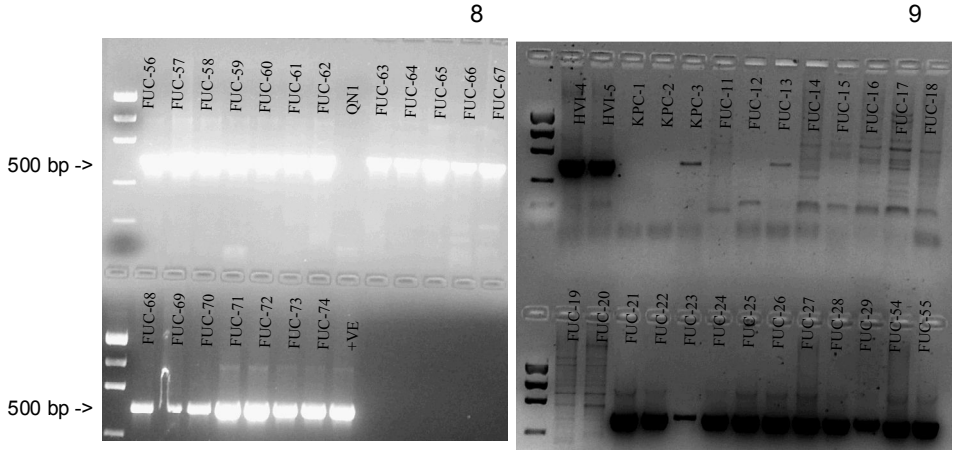
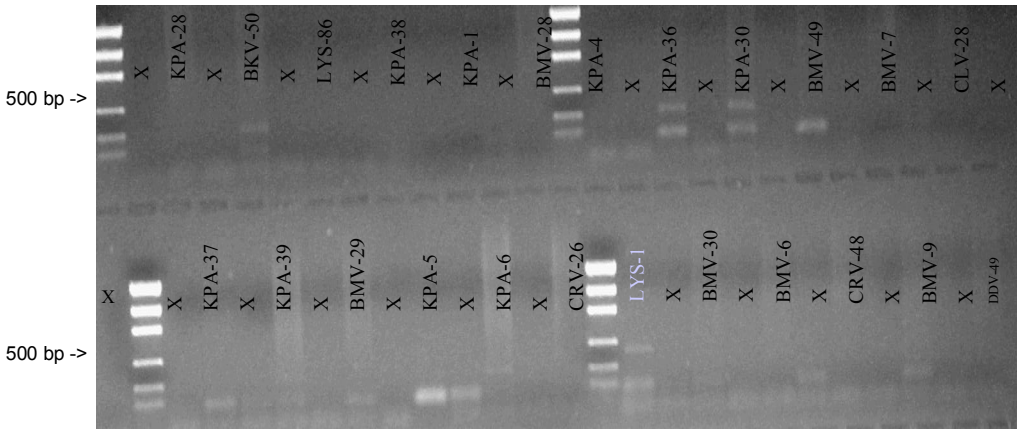
X
OLV-27
KPA-9
LYS-82
LYS-85
BMV-5
BMV-26
CRV-6
CLV-49
DDV-30
CLV-2
OLV-29
OLV-48
DDV-10
CRV-2
DDV-6
CLV-30
CRV-3
CLV-7
DDV-1
CRV-4
OLV-47
OLV-6
CLV-4
CLV-46

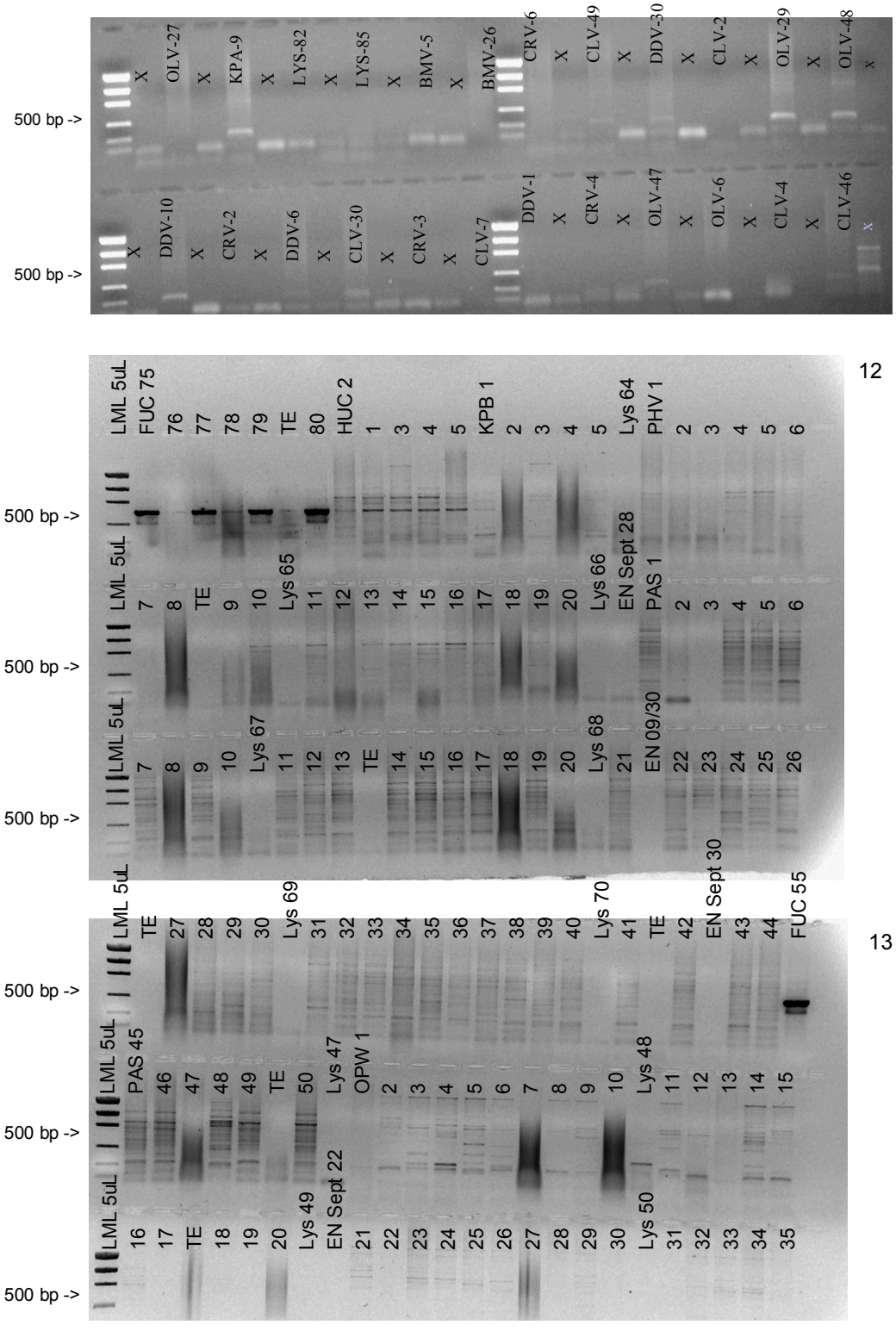
3

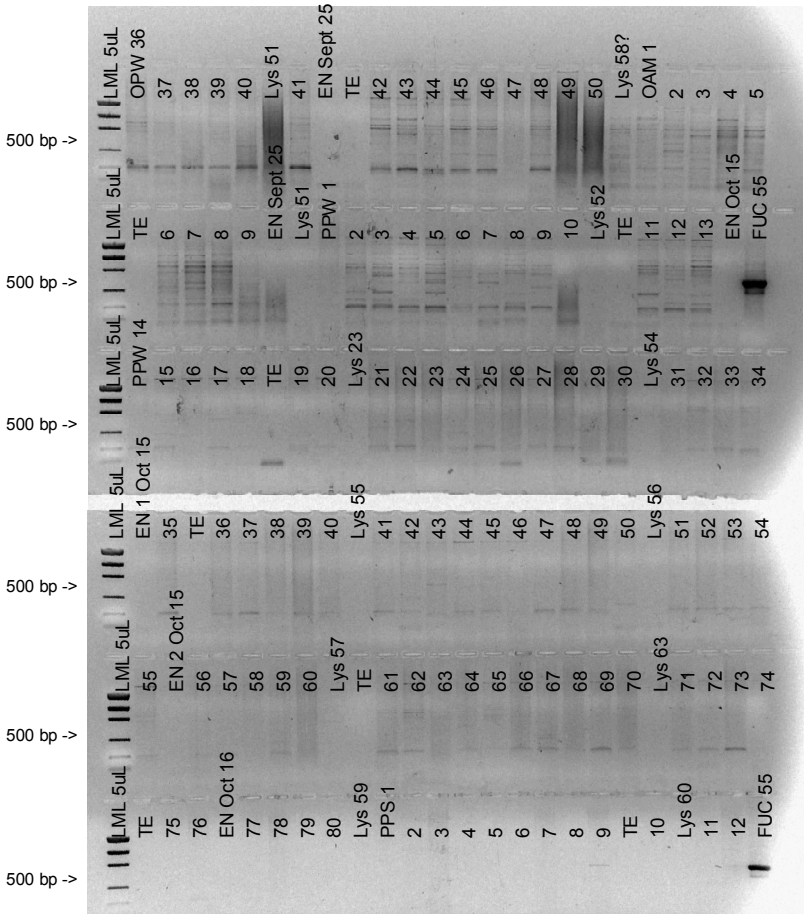

4
500 bp ->
500 bp ->
CLV-5 X DDV-8 X LYS-76 X DDV-7 X CRV-49 X CLV-50 X X OLV-1 X CRV-30 X OLV-3 X CLV-27 X CRV-50 X OLV-9
OLV-7 X KPA-3 X OLV-10 X CRV-27 X BMV-4 X OLV-28 X X KPA-8 X BMV-48 X BMV-1 X BMV-27 X DDV-46 X OLV-8
5
500 bp ->
500 bp ->
OLV-2 X BMV-2 X BMV-10 X DDV-3 X KPA-10 X DDV-28 X X KPA-26 X DDV-47 X BMV-47 X KPA-40 X CRV-10 X CLV-29
LYS-24 X DDV-5 X CLV-49 X LYS-7 X CLV-9 X KPA-7 X X CRV-46 X CLV-1 X DDV-2 X CRV-47 X CRV-5 X OLV-50
6
500 bp ->
500 bp ->
500 bp ->
LYS-1 IT-5 CR-4 CT-3 IT-4 CT-5 IT-2 CT-1 IT-1 LYS-2 QN1JUNE24 QNSJUNE24 AMB-81 BMV-8 AMB-43 QN2 AMB-48 KPA-2 CLV-8 LYS-121 CRV-8 AMB-85 OLV-4
CLV-47 AMB-45 QN AMB-42 AMB-47 BDV-26 BMV-46 AMB-17 CRV-1 QN CLV-76 LYS-88 AMB-24 CRV-28 AMY-74 AMB-22 AMB-12 AMB-83 AMB-14 AMB-28 AMB-13 AMB-23 AMB-19 AMB-9
AMB-75 AMB-49 AMB-25 AMB-41 AMB-84 AMB-73 AMB-46 AMB-82 AMB-30 BMW-3 QN2 AMB-72 DPV-4 AMB-20 AMB-26 CVS-13 AMB-18 AMB-21 AMB-10 QN1 CRV-9 CLV-48 AMO-11 AMB-24

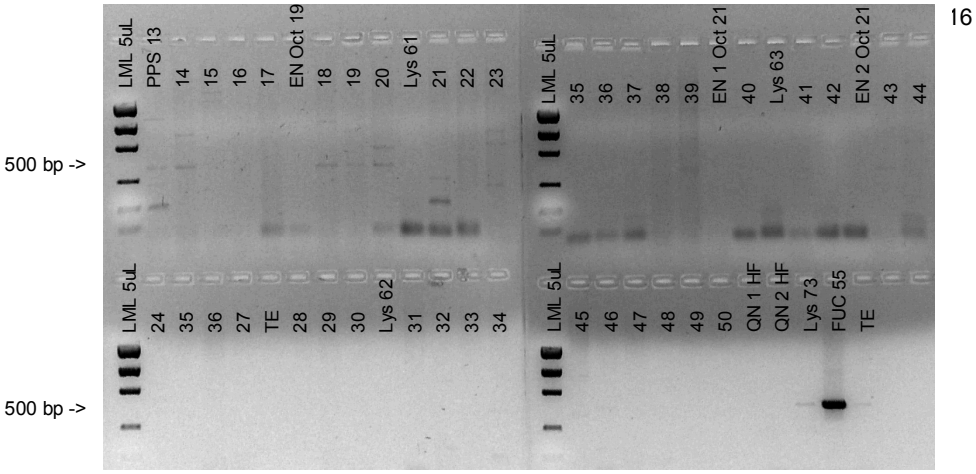

7
500 bp ->
500 bp ->
500 bp ->
QN1
LYS73
AMB-16
LYS-79
DLV-3
AMB-15
CLV-6
DLV-46
AMB-44
AMB-27
LYS-91
EAM-1
EAM-2
EAM-3
EAM-4
EAM-5
KPC-M
LYS-109
OAW-1
OAW-2
OAW-3
LYS-12
BLANK
-VE
AMB-6
AMB-7
AMB-8
LYS-90
LYS-87
LYS-89
AMB-1
AMB-2
AMB-3
AMB-4
QN2
AMB-5
KPC-5
KPC-4
ANU-1
ANU-2
ANU-3
ANU-5
KPC-F
LYS-114
HVI-1
HVI-2
HVI-3
ANU-4
FUC-30
FUC-31
FUC-32
FUC-33
FUC-34
FUC-35
FUC-36
FUC-37
FUC-38
FUC-39
FUC-40
FUC-41
FUC-42
FUC-43
FUC-44
FUC-45
FUC-46
FUC-47
FUC-48
FUC-49
FUC-50
FUC-51
FUC-52
FUC-53

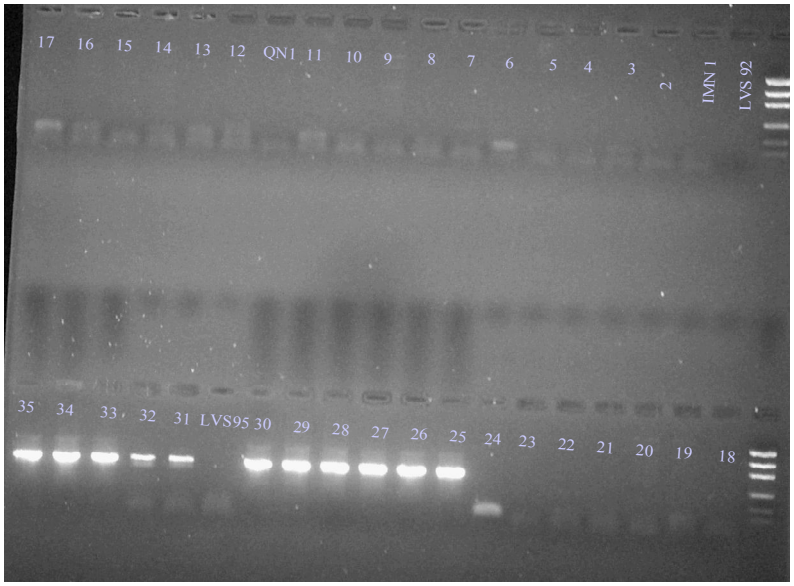

8
9
500 bp ->
500 bp ->
FUC-56
FUC-57
FUC-58
FUC-59
FUC-60
FUC-61
FUC-62
QN1
FUC-63
FUC-64
FUC-65
FUC-66
FUC-67
FUC-68
FUC-69
FUC-70
FUC-71
FUC-72
FUC-73
FUC-74
+VE
HVI-4
HVI-5
KPC-1
KPC-2
KPC-3
FUC-11
FUC-12
FUC-13
FUC-14
FUC-15
FUC-16
FUC-17
FUC-18
FUC-19
FUC-20
FUC-21
FUC-22
FUC-23
FUC-24
FUC-25
FUC-26
FUC-27
-FUC-28
FUC-29
FUC-54
FUC-55

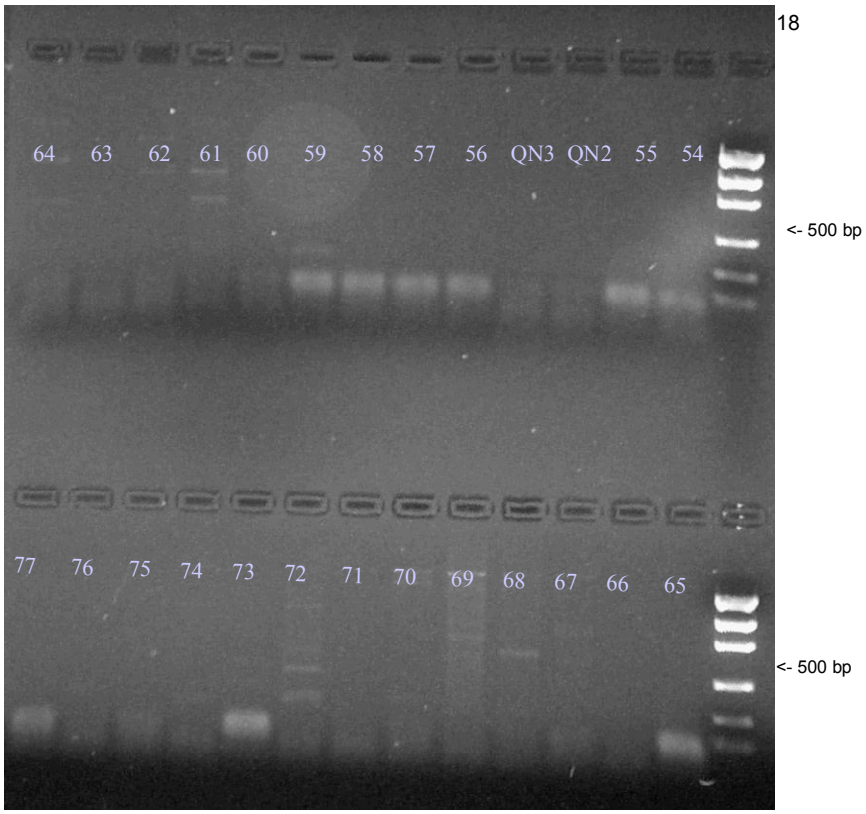

10
500 bp ->
500 bp ->
X
KPA-28
X
BKV-50
X
LYS-86
X
KPA-38
X
KPA-1
X
BMV-28
KPA-4
X
KPA-36
X
KPA-30
X
BMV-49
X
BMV-7
X
CLV-28
X
KPA-37
X
KPA-39
X
BMV-29
X
KPA-5
X
KPA-6
X
CRV-26
LYS-1
X
BMV-30
X
BMV-6
X
CRV-48
X
BMV-9
X
DDV-49

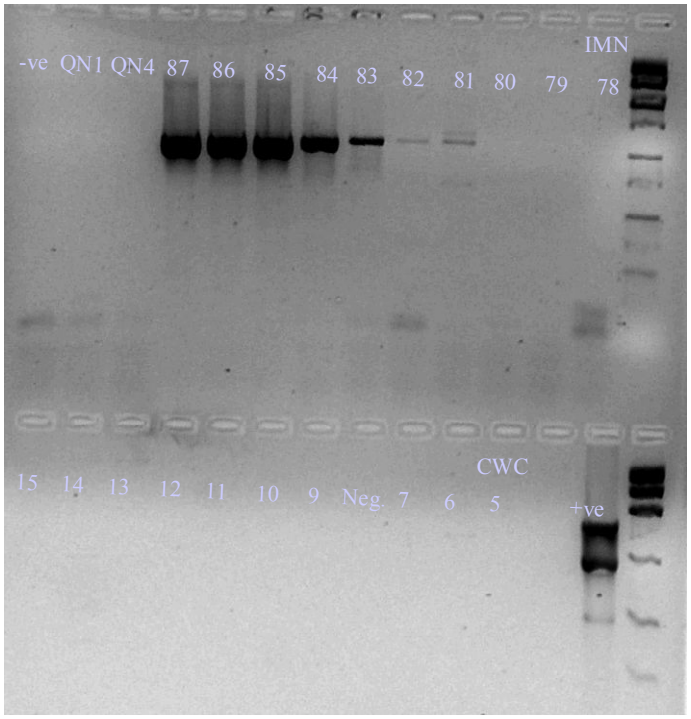

11
500 bp ->
500 bp ->
X
DDV-10
X
CRV-2
X
DDV-6
X
CLV-30
X
CRV-3
X
CLV-7
X
OLV-27
X
KPA-9
X
LYS-82
X
LYS-85
X
BMV-5
X
BMV-26
CRV-6
X
CLV-49
X
DDV-30
X
CLV-2
X
OLV-29
X
OLV-48
x
DDV-1
X
CRV-4
X
OLV-47
X
OLV-6
X
CLV-4
X
CLV-46
x
12
500 bp ->
500 bp ->
500 bp ->
LML 5uL
7
8
9
10
Lys 67
11
12
13
TE
14
15
16
17
Lys 68
18
19
20
Lys 66
21
EN 09/30
22
23
EN Sept 30
24
25
26
LML 5uL
7
8
TE
9
10
Lys 65
11
12
13
14
15
16
17
18
19
20
Lys 66
EN Sept 28
PAS 1
2
3
4
5
6
LML 5uL
FUC 75
76
77
78
79
TE
80
HUC 2
1
3
4
5
KPB 1
2
3
4
5
Lys 64
PHV 1
2
3
4
5
6
13
500 bp ->
500 bp ->
500 bp ->
LML 5uL
16
17
TE
18
19
20
Lys 49
EN Sept 22
21
22
23
24
25
26
27
28
29
30
Lys 50
31
32
33
34
35
LML 5uL
PAS 45
46
47
48
49
TE
50
Lys 47
OPW 1
2
3
4
5
6
7
8
9
10
Lys 48
11
12
13
14
15
LML 5uL
TE
27
28
29
30
Lys 69
31
32
33
34
35
36
37
38
39
40
Lys 70
41
TE
42
EN Sept 30
43
44
FUC 55

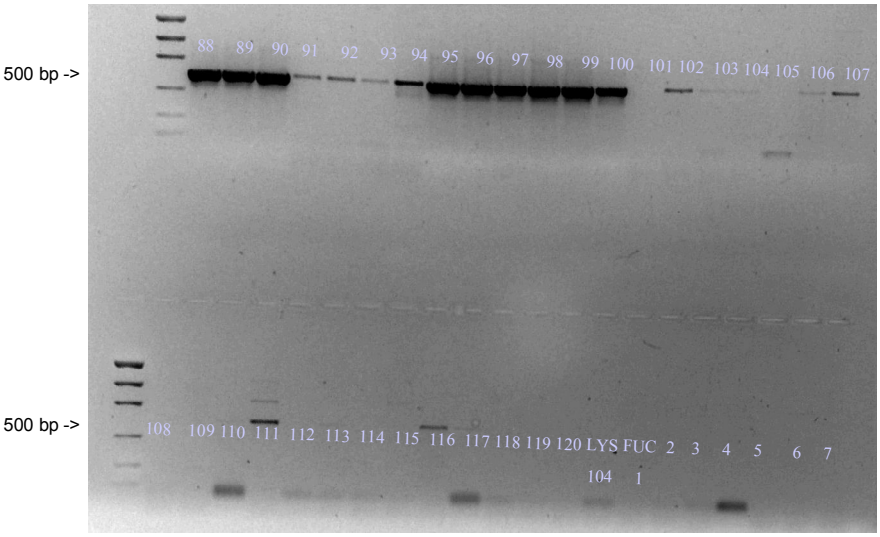

500 bp ->
500 bp ->
500 bp ->
500 bp ->
500 bp ->
LML 5uL
TE
EN Oct 16
Lys 59
PPS 1
FUC 55
EN 2 Oct 15
Lys 57
Lys 61
Lys 60
Lys 63
EN 1 Oct 15
Lys 55
Lys 56
PPW 14
Lys 23
Lys 54
EN Sept 25
Lys 51
PPW 1
Lys 52
EN Oct 15
OPW 36
Lys 58?
OAM 1
14
15

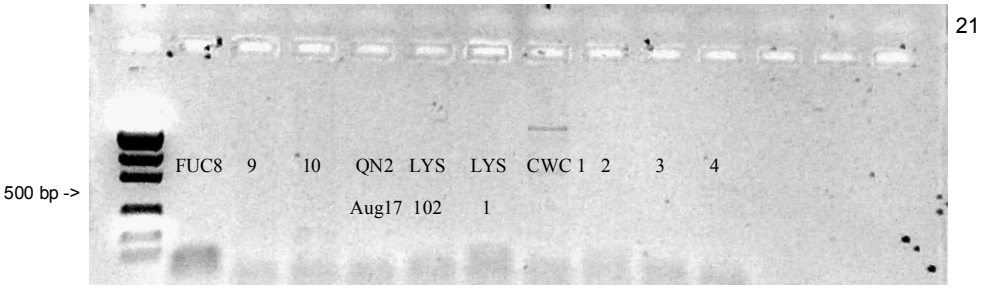

500 bp ->
500 bp ->
LML 5uL
PPS 13
EN Oct 19
Lys 62
Lys 61
EN 1 Oct 21
QN 1 HF
QN 2 HF
Lys 73
FUC 55
Lys 63
EN 2 Oct 21
16

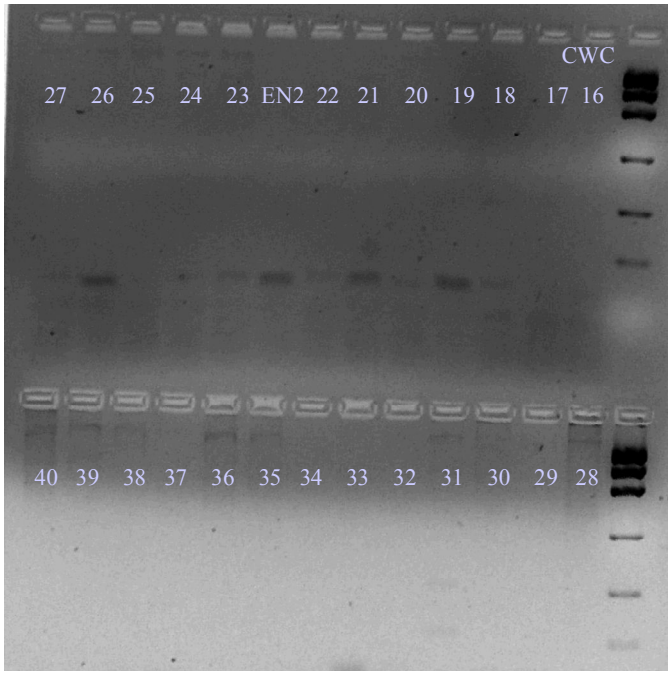

17
17 16 15 14 13 12 QN1 11 10 9 8 7 6 5 4 3 2 IMN 1 LVS 92
<- 500 bp
35 34 33 32 31 LVS95 30 29 28 27 26 25 24 23 22 21 20 19 18
<- 500 bp

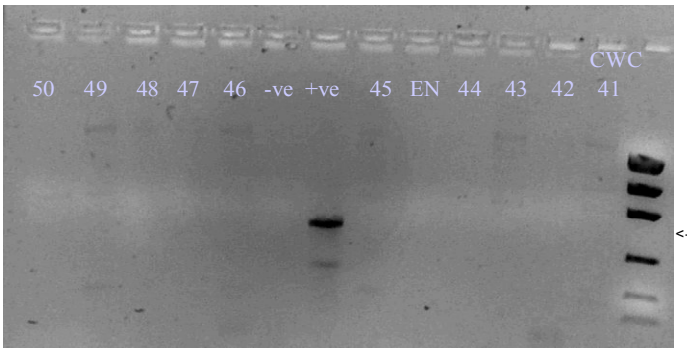

18
64 63 62 61 60 59 58 57 56 QN3 QN2 55 54
<- 500 bp
77 76 75 74 73 72 71 70 69 68 67 66 65
<- 500 bp

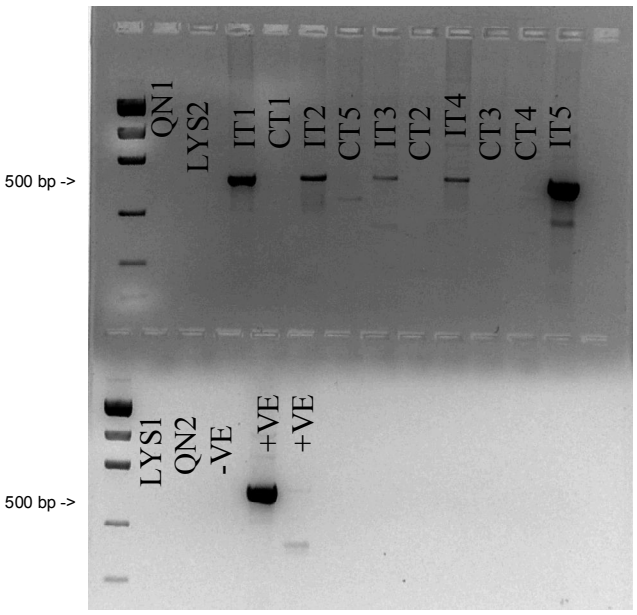

19
-ve QN1 QN4 87 86 85 84 83 82 81 80 79 78 IMN
<- 500 bp
15 14 13 12 11 10 9 Neg. 7 6 5 CWC +ve
<- 500 bp


20
500 bp ->
88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107
500 bp ->
108 109 110 111 112 113 114 115 116 117 118 119 120 LYS 104 FUC 1 2 3 4 5 6 7


21
500 bp ->
FUC8 9 10 QN2 Aug17 LYS 102 LYS 1 CWC 1 2 3 4


22
CWC
27 26 25 24 23 EN2 22 21 20 19 18 17 16
<- 500 bp
40 39 38 37 36 35 34 33 32 31 30 29 28
<- 500 bp


23
CWC
50 49 48 47 46 -ve +ve 45 EN 44 43 42 41
<- 500 bp


24
500 bp ->
QN1
LYS2
IT1
CT1
IT2
CT5
IT3
CT2
IT4
CT3
CT4
IT5
500 bp ->
LYS1
QN2
-VE
+VE
+VE