Effect of the neonicotinoid imidacloprid on embryogenesis and anuran survivorship in frog virus 3 infected tadpoles

A thesis submitted to the Committee of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science in the Faculty of Arts and Science

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ABSTRACT

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Morgan A. Hrynyk

Exposure of pre-metamorphic amphibians to neonicotinoid insecticides may be contributing to the global decline in amphibian populations. In this study, anuran embryos and tadpoles of the African clawed frog (Xenopus laevis) and the North American leopard frog (Lithobates pipiens) were used to determine the effects of embryonic exposure to neonicotinoids. In addition, Xenopus was used to determine if prolonged exposure to neonicotinoids influenced tadpole sensitivity to frog virus 3 (FV3). Exposure of anuran embryos to concentrations of the neonicotinoid insecticide, imidacloprid, ranging from 1 -20 ppm induced a concentration dependent increase in malformations of the retina in Xenopus embryos. However, similar responses were not observed with embryos of leopard frogs. Exposure of Xenopus tadpoles to 500 ppb concentration of imidacloprid followed by challenge with FV3 showed that pesticide exposure unexpectedly decreased the rates of mortality, although total mortalities by the end of the experiment were not significantly different from controls. This unexpected observation may be attributed to a reduced inflammatory response induced by exposure to imidacloprid. Despite the low acute toxicity of neonicotinoid insecticides to vertebrates, these studies indicate that exposure to this class of insecticides causes sublethal effects in anuran species during early life stages.
**Key words:** Neonicotinoid, imidacloprid, embryogenesis, *Ranavirus*, Frog virus 3, amphibian, amphibian conservation, *Xenopus laevis*, Leopard frog, tadpole, malformation, virus
Preface

This dissertation has been prepared in manuscript format for publication submission. Chapters 2 and 3 have been prepared according to the requirements of the Journal of Environmental Toxicology and Chemistry. Chapters 2 and 3 are the result of a collaborative effort between myself, Dr. Chris Metcalfe and Dr. Dennis Murray. I conducted experimental manipulations, data analysis and wrote the manuscript which was critically review by Dr. Chris Metcalfe and my committee members Dr. Leslie Kerr and Dr. Craig Brunetti.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>Acetylcholine esterase</td>
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<tr>
<td>a.i.</td>
<td>Active ingredient</td>
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<tr>
<td>ATV</td>
<td>Ambystoma tigrinum virus</td>
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<tr>
<td>BIV</td>
<td>Bohle Iridovirus</td>
</tr>
<tr>
<td>CCD</td>
<td>Colony Collapse Disorder</td>
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<tr>
<td>CCME</td>
<td>Canadian Council of Ministers of the Environment</td>
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<tr>
<td>DI</td>
<td>Deionized</td>
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<tr>
<td>dpi</td>
<td>Days post-infection</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post-fertilization</td>
</tr>
<tr>
<td>ECV</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>FETAX</td>
<td>Frog embryo teratogenesis assay - <em>Xenopus</em></td>
</tr>
<tr>
<td>FV3</td>
<td>Frog Virus 3</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin hormone</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post-fertilization</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin – one beta</td>
</tr>
<tr>
<td>IMI</td>
<td>Imidacloprid</td>
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<tr>
<td>INF-γ</td>
<td>Interferon – gamma</td>
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<tr>
<td>IU</td>
<td>International units</td>
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<tr>
<td>LD₅₀</td>
<td>Lethal dose for 50% of the population</td>
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Leopard frog  North American leopard frog (*Lithobates pipiens*)

LOEC  Lowest observable effect concentration

LOD  Limits of detection

LOQ  Limits of quantitation

MHC  Major histocompatibility complex

MME  Mass mortality event

nAChR  Nicotinic acetylcholine receptor

NCLDV  Nucleocytoplasmic large DNA viruses

NK  Nieuwkoop and Faber (developmental stage)

NOEC  No observable effect concentration

PBS  Phosphate buffer solution

pf  post fertilization

PFU  Plaque forming units

PMRA  Pest Management Regulatory Agency

ppb  parts per billion

ppm  parts per million

SCRV  Santee Cooper *Ranavirus*

TNF-α  Tumor necrosis factor – alpha

*Xenopus*  African clawed frog (*Xenopus laevis*)

5H-IMI  5-hydroxy imidacloprid

6-AN  6-aminonicatinomide
Chapter 1: Introduction

Some experts claim that the amphibian decline began globally in the late 1950s (Houlahan et al., 2000), while others argue that the decline originated as recently as the early 1990s and has been restricted to North America (Alford et al., 2001). What both parties can agree on is that amphibian populations are in decline, and that the rate of this decline has accelerated in the last 25 years. According to The International Union for Conservation of Nature (2004), amphibians are currently more threatened than any other class of vertebrate, with approximately 2,468 species (43.2%) experiencing recognized declines as of 2004. This unprecedented decline has led amphibians to the foreground of the global biodiversity crisis conversation (Beebee et al., 2005).

The causes of amphibian decline can be broken down into two groups of hypotheses. Group I hypotheses relate to mechanisms that have a known direct effects on amphibian populations, such as habitat alteration (e.g. destruction of habitat), introduction of invasive species, and over-exploitation of amphibian populations. Group II hypotheses involve more complex and intricate mechanisms that are less understood, such as climate change, chemical contamination, and emerging infectious diseases. However, potential and known causes of amphibian decline are unlikely to act in isolation, and it is more probable that they act in concert with one another creating additive or multiplicative effects (Collins and Storfer, 2003). Therefore, the study of individual components, while useful, does not provide a comprehensive view of the interacting variables impacting amphibian populations at any given time. Moreover, the focus on singular direct effects has led to the perception that indirect, sub-lethal, or long range anthropogenic impacts do not play prominent roles in amphibian decline.
Wetland ecosystems are increasingly threatened by anthropogenic stressors, such as fertilizer runoff and pesticide contamination (Mann et al., 2009). The intensification of agricultural practices has increased demand for chemical pest management, which has in turn led to higher levels of contaminants in surface waters and groundwater (Smaliling et al., 2015). A 10-year study provided by the U. S. Geological Survey’s National Water Quality Assessment Program from 1992-2001 found that 97% of water samples collected from agricultural areas tested positive for the presence of pesticides (Gilliom, 2007). Of the samples collected, 57% had pesticide concentrations greater than the US national aquatic-life protection benchmark (Gilliom, 2007). With continued exposure at these levels, amphibians are at risk for sub-lethal effects, including alterations in growth (mass, and length), time to metamorphosis, immune function, sexual development, limb development, or endocrine function (Mann, et al., 2009). However, studies on the relationship between the sub-lethal effects of these compounds and chronic exposure in amphibians are still limited (Collins and Storfer, 2003).

Emerging pathogens can play a role in amphibian population declines and extirpation events (Echaubard et al., 2010). Pathogens can alter the structure of host communities and influence population dynamics by affecting host fitness traits, or resulting in mass mortality (Echaubard et al., 2010). The susceptibility of amphibians to pathogens can vary depending on life stage, species, con-specific density, temperature, habitat and habitat biodiversity (Miller et al., 2011; Kolby et al., 2010). It has also been suggested that high levels of environmental stressors, such as contaminants or predation, can increase vulnerability to infection (Kerby et al., 2011). Moreover, transcontinental and international commercial trade of live amphibians and improper disposal of untreated wastewater has increased the transmission of detrimental pathogens to novel host environments (Kolby et al., 2014).
The study of the impacts of anthropogenic stressors, including exposure to agrochemical contaminants and natural stressors such as pathogens, are commonly studied in isolation and are rarely examined concurrently. However, given that the severity of pathogen infection is often dependent on habitat quality (Echaubard et al., 2010), it is important to understand if and how contaminant exposure at sub-lethal levels can influence the risk of amphibian mortality. The present study focuses on the potential impacts of the family of water soluble insecticides known as neonicotinoids. Neonicotinoids are considered the most important class of chemical insecticides of the present day, and are favoured for their versatility of application and low risk to non-target vertebrates (Jeschke et al., 2010). This study will examine if exposure to a model neonicotinoid insecticide, imidacloprid, at sub-lethal concentrations increases pathogen susceptibility when early life stage amphibians are exposed to the common ranavirus strain, frog virus 3 (FV3). Ranaviruses are considered a globally emerging pathogen family that infect a wide range of ectothermic vertebrate hosts and have been connected to amphibian mass mortality events on four continents (Lesbarrères et al., 2012). Therefore, this study will investigate the impact of environmentally relevant concentrations of neonicotinoids in Ranavirus infected tadpoles. In addition, the acute toxicity thresholds for embryonic stage amphibians will be determined using malformation and mortality thresholds.

**Neonicotinoids**

**History and usage**

Before 1970, attempts to modify the structure of the natural insecticide, nicotine, in order to improve its insecticidal activity were either unsuccessful, or only applicable to niche markets. A major innovation was accomplished in the 1970s when Nihon Tokushu Noyaku Seizo Inc. in
Japan (now Bayer Crop Science Japan) produced the prototype neonicotinoid compound which increased compound potency, but yet still failed to maintain stability when exposed to ultraviolet radiation. However, by changing the nitromethylene component to a nitroguanidine or cyanoamide, photostability was attained, resulting in the development of imidacloprid (IMI); the first neonicotinoid compound effective in field conditions (Tomizawa and Casida, 2005).

Neonicotinoids are a family of water-soluble systemic insecticides that can be used to control pests in urban and agricultural environments or to treat for ectoparasites in domestic animals. They act via the insect nicotinic acetylcholine receptor (nAChR), a previously underexploited target receptor. After their development in the mid 1980s, neonicotinoids first became available for commercial application in 1991 (Jeschke et al., 2010). Neonicotinoids have now been registered in over 120 countries, compromise 27% of the global market, and are beginning to replace previously used major pesticide families such as the organophosphates, pyrethroids and carbamates (Jeschke and Nauen, 2008; Jeschke et al., 2010). Neonicotinoids are favoured for their high efficacy against a wide spectrum of biting and sucking insect pests, ease of application, low dose requirements, and low toxicity to vertebrates (Anderson et al., 2015; Main et al., 2014). There are currently eight patented neonicotinoid insecticides; nithiazine (1977), imidacloprid (1985), thiacloprid (1985), thiamethoxam (1992), nitenpyram (1988), acetamiprid (1989), clothianidin (1989), and dinotefuran (1994; Tomizawa and Casida, 2005).

Of this insecticide family, imidacloprid is the most commonly applied worldwide, accounting for 45% of the $2.63-billion-dollar neonicotinoid market in 2009 (Jeschke et al., 2010) and with an estimated 25,702 tonnes exported worldwide from China in 2015 (CCME International, 2016).

The development of neonicotinoids is considered a milestone in insecticide research, as neonicotinoids are now dominating the insecticide market due to their efficacy and perceived
low environmental risk (Jeschke et al., 2010). However, following their connection with honey bee colony collapse disorder (CCD) as described by Spivak et al. (2011), and subsequent restrictions of use in the European Union, there is a greater demand to understand the long and short term impacts of neonicotinoids on non-target organisms and ecosystems. As such, neonicotinoids are currently under re-evaluation by Health Canada Pest Management Regulatory Agency (PMRA) as the impact they have on non-target organisms and ecosystem health is largely unknown (Anderson et al., 2015).

Application and aquatic environmental contamination

Neonicotinoids are used in both rural and urban settings and are primarily applied as a seed or soil treatment, but can also be applied as a foliar spray, where 1.7 to 312g active ingredient per hectare (a.i./ha) is applied depending on crop type and formulation (CCME, 2007; Sánchez-Bayo and Hyne, 2014; Hladik and Kolpin, 2015). After application, the active ingredient is taken up by the growing plant and transported through the vascular system into all plant tissues where it will be directly consumed by plant-predating insects (CCME, 2007). Depending on the method of application and crop type, between 1.6% and 28% of the total neonicotinoid active ingredient (a.i.) applied will be taken up by the plant (Sur and Stork, 2003). The remaining insecticide residue will either photodegrade, or as result of high water solubility (e.g. $6.1 \times 10^5$ ppb for IMI), dissipate into the soil, ground water, or nearby surface waters (Anderson et al., 2015). Once in the environment, neonicotinoids are known to have highly variable half-lives, ranging from 3 to 6,931 days in soil, and 4 to 129 days in water (Anderson et al., 2015; Sánchez-Bayo and Hyne, 2014). The half-life range can be influenced by numerous abiotic and biotic factors such as ultraviolet (UV) radiation, turbidity, pH, temperature, and microbial action (Morrissey et al., 2015; Anderson et al., 2015).
Monitoring neonicotinoids in aquatic environments is limited both in Canada and internationally. Furthermore, there is currently no central registry of pesticide use in Canada, resulting in uncertainty over the volume of neonicotinoids applied annually (Anderson et al., 2015). The Pest Management Regulatory Agency (PMRA) and the national pesticide sales data base can be utilized to estimate insecticide use patterns and have shown that clothianidin and thiamethoxam, were among the top 10 insecticides sold in Canada in 2010 (Somers and Chung, 2014). However, using sales estimates to infer application rates assumes that all purchased active ingredient was used, which may provide a skewed representation. Several studies have begun to report neonicotinoids in Canadian aquatic environments. A study in the prairie pothole region of Saskatchewan found that 16 - 91% of seasonally sampled wetlands from 2012-2013 contained neonicotinoids, with a maximum reported concentration of 3.11 ppb (Main et al., 2014). Well water samples collected in 2008 and 2009 in potato producing regions of Quebec found that 62% of the 77 samples collected contained imidacloprid with concentrations reaching up to 6.1 ppb (Government of Quebec, 2011).

Neonicotinoids have also been detected in the United States, Europe and Australia. A nationwide study of streams in the US conducted in 2013-2014, found that at least one neonicotinoid compound was present in 63% of the 38 samples collected, where the maximum concentration recorded was 0.45 ppb in a single sample. Imidacloprid was the most commonly detected neonicotinoid, present in 37% of samples, and appeared most frequently in urbanized areas at 90% of samples (Hladik and Kolpin, 2015). A study performed in three Californian regions from 2010 to 2011 found that 85 to 100% of samples taken, depending on region, contained imidacloprid. The maximum cumulative neonicotinoid concentration found in a single sample was 3.29 ppb (Starner and Goh, 2012). In the Netherlands, almost half of the 9,037 water
samples taken from 2004-2012 have exceeded the $1.3 \times 10^{-2}$ ppb maximum permissible risk limit for the Netherlands, and the highest recorded sample concentration was 320 ppb (Van Dijk et al., 2013). In a survey of river waters around Sydney, Australia, found that 93% of samples taken in 2013 from 13 locations contained two or more neonicotinoids, where the maximum concentration was 4.5 ppb (Sánchez-Bayo et al., 2014).

The above mentioned cumulative neonicotinoid concentrations range from 0.45 ppb to 320 ppb, and all exceed the Canadian interim water quality limit for the protection of aquatic life (i.e. 0.23 ppb IMI) and the National Institute for Public Health and the Environment (NIPHE) Environmental Risk Limits in the Netherlands (i.e. 0.2 ppb for acute exposure and 0.067 ppb for chronic exposure) and many exceed the United States Environmental Protection Agency (EPA) Aquatic Life Benchmark (i.e. 1.05 ppb IMI; CCME, 2007; Van Dijk et al., 2013; Main et al., 2014). The maximum environmental concentrations do not represent environmental norms, rather, they reflect worst case scenario for levels in surface waters following heavy rainfall or in small shallow water bodies. However, it can be predicted that as neonicotinoids continue to replace previously used insecticides the resulting average environmental concentrations are subject to increase.

*Mode of neonicotinoid action and its effect on non-target organisms*

The nicotinic acetylcholine receptor (nAChR) is a pentameric transmembrane ligand-gated ion channel responsible for rapid post-synaptic neurotransmission (Tomizawa and Casida, 2005). These receptors can be found in the insect central nervous system, and in the vertebrate central and peripheral nervous systems (Jeschke et al., 2011). The chemical ligand acetylcholine (ACh) is the primary neurotransmitter that acts on the nAChR, resulting in membrane
depolarization and propagation of action potentials. After binding to the nAChR, ACh is rapidly hydrolyzed by the enzyme acetylcholine esterase (AChE), effectively removing the receptor stimuli and allowing the receptor to return to a resting state. Similar to ACh, neonicotinoids also bind to the nAChR. However, in insects they bind irreversibly and as an agonist, resulting in membrane depolarization, centrally mediated toxicity, downstream excitation at the neuromuscular junction, and death (Tomizawa and Casida, 2005).

Neonicotinoids display selectivity for insects, which can be attributed to the difference between the insect and vertebrate nAChR. The nACh receptors are composed of five subunits, and the diversity of subunits and subunit combinations allows for changes in sensitivity to acetylcholine and other neurotransmitters (Tomizawa and Casida, 2005). Subunits also display variation in amino acid residues, which can alter the pharmacological properties of each subunit (Tomizawa and Casida, 2005). When comparing insect and vertebrate nAChRs, there is variation in the amino acid residues in the D-loop of the α7 subunit (Tomizawa et al. 2007). In the insect nAChR D-loop, the amino acids present are non-charged, such as valine, proline, threonine, alanine or serine. On the other hand, this subunit in vertebrates contains glutamic acid, an amino acid with a negatively charged side chain at physiological pH. Neonicotinoids, unlike nicotine, are non-protonated at physiological pH, and instead are substituted with an electronegative cyan- or nitro- pharmacophore. In insects, the uncharged amino acids in the D-loop of the α7 subunit and the electronegative pharmacophore form a strong attraction, thus increasing binding strength. Conversely, the slightly negatively charged α7 subunit found in vertebrates repels the electronegative component of the neonicotinoid compound. This translates into a selective binding mechanism which allows for strong agonistic binding in insects, and low affinity partial agonist binding in vertebrates (Tomizawa et al. 2007).
Despite the lower binding affinity of neonicotinoids to vertebrate nACh receptors, studies have shown that sub-lethal doses of the insecticide can impact vertebrate body condition, growth, development and reproduction, and can induce neurobehavioral disorders, and reduce immune function (Gibbons et al., 2015). For example, grainivorous birds have been known to consume neonicotinoid-coated seeds, resulting in endocrine system disruption and immune suppression of offspring suggesting potentially heritable effects from parental exposure to neonicotinoids (Lopez-Antia, 2013). In another study, Devan et al. (2014) showed that Wistar rats, a common experimental rodent model, displayed significantly decreased macrophage function and impaired cell signaling when animals were administered 10% of the lethal dose (LD$_{50}$) of the neonicotinoid, acetamiprid. Similarly, Duzguner and Erdogan (2012) found that chronic imidacloprid exposure at 1 mg/kg/bw-day induced oxidative stress and prompted exaggerated inflammatory cytokine release in rats. To date, several studies have shown a connection between neonicotinoids and impaired immune function, suggesting that increased neonicotinoid use and exposure may increase pathogen susceptibility in non-target organisms.

According to the United States Environmental Protection Agency (US EPA), neonicotinoid insecticides range from slightly toxic to practically non-toxic in amphibians, yet little toxicological data beyond LD$_{50}$ levels and direct hazard effects has been investigated (Gibbons et al., 2015). The study of sub-lethal effects caused by neonicotinoid contaminant exposure may provide a better understanding of agrochemical impacts on amphibian populations and ultimately ecosystem stability (Mann, et al., 2009). Previous studies examining sub-lethal toxicity resulting from exposure to chemicals with similar targets has been shown to influence growth (mass and length), time to metamorphosis, or cause abnormal sexual development, limb abnormalities, and endocrine disruption in amphibians (Mann, et al., 2009). However, studies on
the sub-lethal effects of pesticides on amphibians, or their impact on pathogen susceptibility, are limited in number.

**Ranaviruses**

*Ranavirus phylogeny and geographical range*

Nucleocytoplasmic large DNA viruses (NCLDV) are an apparent monophyletic group comprised of six families of virus (Koonin and Yutin, 2010). NCLDVs have some of the largest known viral genomes, reaching approximately 1.2 million base pairs in size, and are able to infect a wide range of eukaryotic hosts from animals to unicellular organisms (Raoult *et al.*, 2004; Yutin and Koonin, 2012). The six families comprising NCLDV are *Poxviridae, Asfarviridae, Mimiviridae, Ascoviridae, Phycodnaviridae,* and *Iridoviridae* (Yutin and Koonin, 2012). Virions of the family *Iridoviridae* share 26 core genes and are structurally complex. They display icosahedral symmetry, can reach diameters up to 350nm, and possess a terminally redundant single linear dsDNA molecule of 140-303kbp (King *et al.*, 2012). The five genera comprising the family *Iridoviridae* can be divided into two categories; viruses that infect invertebrate hosts such as the *Iridovirus* and *Chloriridovirus*, and viruses that infect ectothermic vertebrates such as *Megalocytivirus, Lymphocystivirus* and *Ranavirus*.

*Ranaviruses* are globally emerging pathogens known to infect at least 175 species of ectothermic vertebrates from fish, to reptiles and amphibians (Brunner, 2015). As of 2015, *Ranaviruses* have been reported in 32 countries across North America, South America, Europe, Africa, and Asia (Duffus *et al.*, 2015). However, the true geographic range is likely unknown due to limitations in sampling effort and detection. There are currently six recognized species of *Ranaviruses; Ambystoma tigrinum virus* (ATV), Bohle iridovirus (BIV), Epizoonotic
hematopoietic necrosis virus (EHNV), European catfish virus (ECV), Santee Cooper ranavirus (SCRV), and the type species, Frog Virus 3 (FV3).

**Ranaviruses and amphibians**

Ranaviruses are increasingly implicated in the global amphibian decline, with over 100 species of amphibians known to contract *Ranavirus* infections (Duffus *et al.*, 2015). Signs of *Ranavirus* morbidity in amphibians are varied and can include gross lesions, loss of pigmentation, ulcerations, epithelial sloughing, erythema, edema, swollen liver, internal hemorrhaging and swelling of extremities. The severity of these pathologic responses are dependent on the extent of infection, developmental stage, and the presence of additional stressors (Jancovich *et al.*, 1997; Robert *et al.*, 2005; Miller *et al.*, 2009; Gray *et al.*, 2009). Movement and normal behavior can also be impeded, as infected animals often show signs of lethargy, erratic swimming, anorexia, and loss of equilibrium in the water column (Jancovich *et al.*, 1997; Miller *et al.*, 2015).

Mass mortality events (MME) associated with *ranavirus* often occur in the summer months, with rapid onset and high rates of mortality (Miller *et al.*, 2015). Reports of apparently normal amphibian populations experiencing >90% mortality rates within days are not uncommon (Green *et al.* 2002). Amphibian MME have been reported around the globe on five major continents, but are most commonly reported in North America and Europe. A study performed by Green *et al.* (2002) found that ranavirus infection was the sole cause of 48% of amphibian mass mortality events in the United States from 1996-2001, and contributed to 9% of mortality events with multiple etiologies. A similar study by Muths *et al.* (2006) conducted from 2000-2005 found that 43% of reported MME in the United States were due to ranaviral infection while
a study based in the Netherlands found that 35% of sites sampled from 2011-2014 exhibited ranavirus MME (Rijks et al., 2016). However, it has been proposed that many MME go unreported, and therefore the total extent of these events and their impact on amphibian populations may be underestimated (Brunner et al., 2015; Miller et al., 2015). Commercial ranaculture facilities also experience extreme mortality events despite long term experience and rearing success. For instance, a United States commercial bullfrog breeding facility experienced >50% onsite frog mortalities due to ranavirus infection in 2006 (Miller et al., 2007). While no species extinctions have been directly attributed to ranavirus outbreaks, local extirpations and amphibian population fluctuations have occurred (Green et al. 2002).

Adult vs. larval amphibian immune function

Ranavirus susceptibility varies with the amphibian developmental stage (Gray et al. 2009) such that adult *Xenopus laevis* mount an effective immune response to viral infection and are able to recover in approximately 21 days, while tadpoles often succumb to infection (De Jesus Andino et al., 2012). Adult *X. laevis*, and other adult stage amphibian species possess immune systems comprised of interacting innate and adaptive components that are fundamentally comparable to the mammalian immune system (Du Pasquier et al., 1989). Adult amphibians possess natural killer cells and leukocytes, including neutrophils, basophils, eosinophils, monocytes and macrophage-type cells which can be found circulating in the blood or within the peritoneal fluid (Robert and Ohta, 2009; Chen and Robert, 2011). T cells are differentiated in the thymus and primarily accumulate with B cells in the white pulp of the spleen but both lymphocytes can also be found in the liver, kidney and intestine (Du Pasquier, et al. 1989; Chen and Robert, 2011). Major histocompatibility complex (MHC) class I restricted
cytotoxic T cells, and MHC class II restricted helper T cells are also found in the adult amphibian adaptive immune system (Robert and Ohta, 2009). In adults the presence of a ranavirus infection is followed by an immediate innate inflammatory response as a result of infiltration of the peritoneal leukocytes. This is then followed by an effective cytotoxic T cell response, which plays a major role in efficiently controlling and subduing the ranavirus infection (Chen and Robert, 2011; De Jesus Andino et al., 2012).

In comparison to adults, tadpoles are highly susceptible to ranavirus infection and exhibit mortality rates approximately four fold greater than the adults (Chen and Robert, 2011). Tadpoles lack an effective adaptive immune response and rely primarily on an immature innate immune system to eradicate ranaviral infection (De Jesus Andino et al., 2012). The tadpole immune system possesses limited MHC class I expression, weak B cell responses, no detectable natural killer cells, weak helper T cells, inactive cytotoxic T cells, as well as a weak but detectable antibody response at Nieuwkoop and Faber (NK) stage 49 (10-13 days post-fertilization; Robert and Ohta, 2009). The tadpole immune response to a ranavirus infection is comprised primarily of a 6-day delayed, moderate up-regulation of the pro-inflammatory cytokines; TNF-α, IL-1β, and IFN-γ from peritoneal leukocytes (De Jesus Andino et al., 2012). Research conducted by De Jesus Andino et al. (2012) suggests that this delay in immune response is likely due to a virally induced suppression of normal immune function.

_Ranavirus transmission and persistence in the environment_

Immunocompetent adult amphibians are often able to sustain a subclinical infection, acting as an intra-class reservoir and introducing the virus to novel hosts or environments (Miller et al., 2007). Furthermore, the explosive breeding nature of many temperate amphibian species
and high density at breeding sites likely results in intra-class transmission. Subclinical infections have also been found in overwintering green frog tadpoles which suggests that susceptible host species contribute to the persistence of the virus in the ecosystem (Miller et al., 2009).

*Ranaviruses* also have the capacity for inter-class transmission between fish, reptiles and amphibians (Jancovich *et al.*, 2010).

*Ranavirus* infection can occur through direct contact with or consumption of infected host tissue. Alternatively, infection may also occur passively through environmental transmission routes such as exposure to water or sediment (Jancovich *et al.* 1997). Research performed by Brunner *et al.* (2007) found that ranavirus can transmitted between conspecifics in the same aquatic environment without physical contact. Furthermore, studies have suggested that active virions are relatively persistent in freshwater aquatic environments (Hooverman *et al.*, 2012).

**Variation in susceptibility due to stressors**

Variation in ranavirus susceptibility can be attributed to numerous natural and anthropogenic stressors. Natural stressors include changes in the environment such as temperature shifts, resource limitations, predation risk, reproduction and host density, or may include physical characteristics such as development stage or co-pathogen infections (Gray *et al.* 2009; Blaustein *et al.*, 2012). Anthropogenic stressors can include loss of habitat, genetic isolation, introduction of invasive species, or environmental contamination such as the introduction of nitrogenous wastes, fertilizer, heavy metals or pesticides to the environment (Gray *et al.* 2009; Blaustein *et al.*, 2012). Moreover, it has been suggested that anthropogenic stressors are a stronger predictive factor of ranaviral outbreaks compared to natural stressors (Reeve *et al.*, 2013). Stressors are recognized as internal or environmental factors that increase
the circulation of glucocorticoid stress hormones, such as corticosterone in amphibians, which may alter behaviour or non-vital physiological functions (Blaustein et al., 2012). In the case of chronic stress, immune suppression occurs as glucocorticoids reduce cytokine production as well as antibody response (Blaustein et al., 2012). This modulation of normal immune responses and related peptides likely increases ranavirus susceptibility, and reduces host immune response leading to higher rates of mortality.

The relationship between amphibian host exposure to pesticides and enhanced ranavirus virulence is a frequently stated hypothesis and has been documented in several studies. For example, it has been shown that passive exposure to environmentally relevant concentrations of the herbicide, atrazine in tiger salamanders (Ambystoma tigrinum) resulted in elevated rates of ranavirus (Ambystoma tigrinum virus [ATV]) infection in both larval and adult salamanders exposed to the virus passively through water bath (Forson and Storfer, 2006; Kerby and Storfer, 2009). The elevated infection rate in adult salamanders was attributed to the fact that exposure to atrazine directly affected immune function by reducing leukocyte levels (Forson and Storfer, 2006). In another study, exposure to the insecticide chlorpyrifos was also found to increase larval tiger salamander susceptibility to ATV, as well as increase mortality rates (Kerby and Storfer, 2009). A third study performed by Sifkarovski et al., (2014) found that Xenopus laevis tadpoles had a reduced ability to elicit an innate immune response to frog virus 3 when exposed to atrazine.

Despite these studies, further empirical investigation is required to better understand the potential impact of pesticides on amphibian ranavirus susceptibility as only a limited number of pesticides have been studied. Moreover, experimental designs often involve simultaneous exposure to pesticide and virus, yet the immunosuppressive or deleterious effects of pesticides on
amphibians may take a longer period of time to develop. Therefore, prolonged or chronic exposure would provide a more environmentally relevant understanding of contaminant impacts on viral infection (Brunner et al. 2015). In addition, experimental manipulations also involve viral oral inoculation which may not accurately reflect natural exposure or infection.

**Experimental direction**

Amphibians are oviparous and reliant on their environmental conditions to maintain broods. Thus, developing amphibian eggs are both dependent on the resources provided by their environment and often exposed to a variety of biotic and abiotic stressors. Despite the low binding affinity of neonicotinoids to the vertebrate nAChR, sub-lethal doses have been shown to impact vertebrate body condition, growth and development (Gibbons et al., 2015). Therefore, it is hypothesised that amphibian embryos will be developmentally hindered by prolonged exposure to the neonicotinoid insecticides. To test this hypothesis, Chapter 2 investigates the impact of imidacloprid on growth and morphology of two species of amphibian; the African Clawed frog (*Xenopus laevis*) and the North American leopard frog (*Lithobates pipiens*).

*Ranavirus* related mortality events are considered a leading cause of amphibian decline, but, their impact can be compounded by exposure at early development stages and by exposure to anthropogenic stressors. The impact of the combined factors of *ranavirus* infection and chronic neonicotinoid exposure to early life stages of amphibians is currently unknown. However, given the reductive impact of neonicotinoids on immune function in mammals and birds, it is hypothesized that amphibians chronically exposed to neonicotinoids will be more susceptible to ranavirus infection. Chapter 3 examines this hypothesis by testing the interactive effects of neonicotinoids on ranavirus infection.
Chapter 2: Effects of chronic imidacloprid exposure on anuran embryogenesis in the African clawed frog (*Xenopus laevis*) and the Northern Leopard frog (*Lithobates pipiens*)

**Abstract**

Neonicotinoids are now one of the most commonly utilized insecticides on an international scale. Because of their high water solubility (0.61 g L\(^{-1}\) for imidacloprid), they are susceptible to transfer into surface waters, including ponds and wetlands that are habitat for early life stages of anurans. Neonicotinoids have low acute toxicity in vertebrates. However, early life stages of anurans may be sensitive to this class of insecticides because of the dependence of the critical role of the nicotinic acetylcholine receptor in several developmental processes. When African clawed frog (*Xenopus laevis*) embryos were exposed to the neonicotinoid, imidacloprid, at concentrations of 1 - \(10^4\) ppb for 96 hrs (NK stage 8-46) malformations were observed; most frequently in the retina. Furthermore, malformation frequency appeared dose dependent. These effects were observed at nominal concentrations (i.e. potential variation surrounding stated concentrations) comparable to the water quality guidelines for the protection of aquatic life established in Canada and Europe. Comparatively, exposures of North American leopard frog (*Lithobates pipiens*) embryos to imidacloprid showed elevated malformations, but the frequency of abnormalities did not increase with exposure concentration. Future research regarding the impacts of neonicotinoids on the development of anurans should consider variations in sensitivity between species.

**Key Words:** Embryo, embryogenesis, neonicotinoid, imidacloprid, *Xenopus laevis, Lithobates pipiens*
Introduction

Amphibians are the fastest declining vertebrate taxon worldwide, with the most recent comprehensive global assessment estimating that 43.2% of the world’s amphibian species are experiencing declines (IUCN, 2004). The potential causes of these declines are wide ranging and have been heavily debated. There have been recent studies of indirect effects associated with exposure to environmental contaminants that could potentially contribute to amphibian declines (IUCN, 2004; Mann et al., 2009, Ezemonye and Ilechie, 2010) Although, research on the effects of neonicotinoid exposure on developing amphibians is limited.

Neonicotinoids are a broadly used class of systemic insecticides that can be applied as a foliar spray, soil drench or seed coating in both urban and agricultural environments (Sur and Stork, 2003; Canadian Council of Ministers of the Environment, 2007). Due to their high usage over the last 20 years, high water solubility, and a long half-life, neonicotinoids have been widely detected in aquatic environments (Canadian Council of Ministers of the Environment, 2007; Anderson et al., 2015). Studies conducted across North America and Europe have reported detectable levels of neonicotinoids in freshwater samples ranging from 0.45 ppb to 320 ppb in concentration (Starner and Goh, 2012; Van Dijk et al., 2013; Sánchez-Bayo and Hyne, 2014; Hladik and Kolpin, 2015). While the highest reported within Canada was 3.1 ppb (Main et al., 2014) These maximum concentrations represent extreme scenarios, often involving periods of high precipitation and discharge, that are unlikely to remain consistently high thorough the year. However, since the half-life of these compounds in can exceed 6,000 days in soil and 120 days in water, the frequency of detection as well as maximum concentrations of neonicotinoids in aquatic environments are likely to increase with increased use (Starner and Goh, 2012; Anderson et al., 2015).
Neonicotinoids bind agonistically to the insect nicotinic acetylcholine receptor (nAChR), but have a lower binding affinity to the vertebrate nAChR (Tomizawa and Casida, 2005). This lower binding affinity translates into a reduced risk of acute toxicity. For example, the lethal concentration for 50% (LC\textsubscript{50}) of the black spotted frog, Western chorus frog, and American toad are $1.29 - 2.19 \times 10^5$, $1.94 \times 10^5$, and $2.34 \times 10^5$ ppb respectively (Gibbons \textit{et al.}, 2015). However, several studies with vertebrate models have shown that exposure to neonicotinoids at sublethal concentrations during embryogenesis can impact development. For instance, a study by Gu \textit{et al.} (2013) reported that exposure to 500 µM imidacloprid had a drastic impact on blastocyst formation, and delayed normal embryonic development in mice. A similar study found that chick embryo injection of 10µg – 40µg of imidacloprid resulted in an increased mortality and malformation rate compared to the control treatment (Hussein \textit{et al.}, 2014). However, the effects of neonicotinoids on amphibian embryonic development are yet to be determined.

Amphibians breed and deposit egg clutches in freshwater areas such as drainage basins, reservoirs or estuaries. Due to agricultural run-off, these areas are potentially contaminated with neonicotinoids, thus resulting in amphibian exposure during sensitive embryo developmental stages. Understanding how this exposure affects embryogenesis and embryo survivorship is therefore important for ensuring stable amphibian populations.

The frog embryo teratogenesis assay – \textit{Xenopus} (ie. FETAX) is a widely used method to detect toxicants that can affect amphibian development (American Society for Testing and Materials, 2003; Hoke and Ankley, 2005, Isidori \textit{et al.}, 2016). Moreover, this method has been proposed as a tool for routine ecological risk assessments (Hoke and Ankley, 2005). Indeed, this assay has been utilized to determine detrimental effects for a number of environmental contaminants, including pharmaceuticals (Isidori \textit{et al.}, 2016; Chae \textit{et al.}, 2015), and commercial
textile dyes Birhanli and Ozmen, 2005). However, FETAX has not been used as a tool to evaluate effects on embryonic development in the context of assessing contaminants potentially contributing to amphibian declines.

*Xenopus* is utilized as an amphibian model species in the FETAX due to the ease of captive breeding, embryo manipulations, rapid development, as well as the solid base of developmental biology knowledge for this species. In addition, *Xenopus* is considered a robust amphibian model for the effects on wild native amphibians (Hoke and Ankley, 2005). However, the long-term laboratory rearing and non-native origin have called into question the ecological relevance of using *Xenopus* as a model for environmental studies (Hoke and Ankley, 2005). Therefore, in the present study, the effects of chronic exposure to a model neonicotinoid insecticide, imidacloprid, was studied in both developing *Xenopus* and the common North American native anuran, the leopard frog (*Lithobates pipiens*).

The insecticide, imidacloprid was chosen for this study as it is the most frequently utilized and most widely studied neonicotinoid (Morrissey et al., 2015). Furthermore, all neonicotinoids act and bind irreversibly on the nAChR, therefore individual neonicotinoids are assumed to be additive in relative toxicity (Main et al., 2014). The neonicotinoid concentrations utilized in this study were based on the current maximum environmental concentrations or 3.1 ppb and 320 ppb found within Canada and Europe (the Netherlands) respectively. The ecological water quality guidelines for neonicotinoids vary by country. For example, the current freshwater benchmark limits for Canada, the United States of America, Europe, and the Netherlands are 0.23, 1.05, 0.2, and 0.0083 ppb, respectively (Morrissey et al., 2015).

The objective of this study was to ascertain if exposure to imidacloprid impacts anuran embryogenesis. In addition, studies with two genetically distinct species will assist in
understanding if susceptibility varies across amphibians. It was hypothesized that exposure to imidacloprid would result in no mortalities or developmental malformations in the anuran test species at environmentally relevant concentrations (ie. \( \leq 320 \text{ ppb} \)). In addition, we hypothesized that there would be no differences in developmental responses between the two test species.

**Methods**

*Adult Xenopus husbandry*

Adult *Xenopus* originally purchased from Boreal Science (St. Catharines, ON, Canada) were maintained in large plastic basins with 40 L aged (ie. de-chlorinated) tap water at 21°C with a 16h light, 8h dark photoperiod. They were fed crickets, night crawlers, and turtle pellets to satiation twice weekly and water changes occurred once weekly on the day following the second feeding. *Xenopus* were handled and induced to breed according to the protocols described by Sive *et al.* (2010). Human chorionic gonadotropin hormone (hCG) lyophilized powder (Sigma-Aldrich, Saint Louis, MO, USA) was reconstituted for injection with 0.9% NaCl in deionized (DI) water. Females were primed with injections of 50 IU of hCG one week prior to induction. Females and males were induced to breed with injections of 500 IU, and 150 IU of hCG, respectively and placed in a plastic bin with 15 L aged tap water to breed overnight.

All procedures were approved by the Animal Care Committee of Trent University.

*Embryo collection, selection and treatment*

*Xenopus* embryos were collected immediately post-breeding and transferred to a 1 L holding container. In addition, a large mass of leopard frog eggs was collected in April 2016 from a naturally occurring population located in non-agricultural pond near Peterborough,
Ontario Canada (44°362’N, 78.299’W). Leopard frog embryos were then stored in pond water from the collection site until experimental manipulation, approximately 3 hours.

At Nieuwkoop and Faber (NK) stage 8, embryos from both species were de-jellied with a FETAX solution diluted 2% L-cysteine solution (97% pure L-cysteine [(R)-2-Amino-3-mercapto-propionic acid]; Sigma-Aldrich, Oakville, On, Canada) with pH adjusted to 8.1 using 1M NaOH. This was done to separate eggs from each other for selection purposes and to prevent fouling of the water due to decay of unfertilized eggs. The FETAX solution was composed of 625mg NaCl, 96 mg NaHCO₃, 30 mg KCl, 15 mg CaCl₂, 60 mg CaSO₄, 75 mg MgSO₄ per liter of deionized (DI) water and pH adjusted to 7.6-7.9 as described by ASTM International (American Society for Testing and Materials, 2003). All unfertilized or malformed embryos were removed from the experiment before NK gastrula stage 11 (American Society for Testing and Materials, 2003). All embryos were maintained in plastic petri dishes containing 10 mL of FETAX solution.

Between NK stages 8 and 11, de-jellied embryos were randomly allocated to the appropriate treatments. The imidacloprid used in treatments, henceforth referred to as IMI (1-[6-chloro-3-pyridin-3-methyl] N-nitroimidazolidin-2-yliden-amine) was a PESTANAL analytical grade standard purchased from Sigma-Aldrich. A known amphibian teratogen, 6-aminonicotinamide (6-AN; 6-Aminopyridine-3-carboxamide, 99% purity; Sigma-Aldrich) purchased was used as a positive control treatment at a test concentration of \(2.23 \times 10^6\) which is the LC₅₀ for this compound (henceforth referred to as positive control) (American Society for Testing and Materials, 2003). Stocks of all compounds were diluted with FETAX solution.

*Xenopus* embryo treatments included a vehicle control, IMI concentrations of 1 ppb, 10 ppb, 10² ppb, 10³ ppb, 10⁴ ppb IMI, as well as the positive control treatment (\(2.23 \times 10^6\) 6-AN).
Treatments were achieved through serial dilution from a stock solution of \( 5 \times 10^6 \) ppb IMI. These treatment concentrations were not confirmed using mass spectrometry and exact values may fluctuate around the value stated, therefore all treatments for this experiment are considered nominal. The concentrations of 1 ppb to \( 10^3 \) ppb were based on global environmentally relevant neonicotinoid concentrations in wetland habitats (Starner and Goh, 2012; Van Dijk et al., 2013; Sánchez-Bayo and Hyne, 2014; Hladik and Kolpin, 2015) [8, 9, 10, 11].

Leopard frog embryo treatments included a vehicle control, IMI concentrations of 5 ppm, 10 ppm, 15 ppm, and 20 ppm, and positive control treatment (\( 2.23 \times 10^6 \) 6-AN). These IMI concentrations were chosen to represent potential environmental concentrations of neonicotinoids should the trend of high application and environmental accumulation continue (Sanchez-Bayo, 2016).

Each treatment consisted of four replicates of 25 embryos placed in 60 mm plastic petri dishes containing 10 mL of vehicle control or FETAX solution containing IMI or 6-AN treatment at 23°C. Water changes were performed every 24 hours, and dead embryos were removed every 12 hours. The experiment was terminated at NK stage 46. Development rate is varied between *Xenopus* and leopard frogs, therefore the duration to reach NK 46 was 4 and 7 days, respectively. Euthanizing was performed using 99% pure benzocaine [4-Aminobenzoic acid ethyl ester], and embryos were subsequently fixed in a 3% buffered formalin solution (American Society for Testing and Materials, 2003). Malformation type, malformation frequency and survivorship were recorded at termination of the experiment.

*Statistics*

*Xenopus* embryo malformations were divided into two categories; eye related malformation or non-eye related malformation. Frequencies of non-eye related malformations
such as edema, spinal curvatures, or an improperly coiled digestive tract were pooled together due to infrequent occurrence. Malformations were not mutually exclusive, as a single malformed embryo may present one or more abnormalities and these were accounted for in each category.

Survivorship and malformation data distribution were tested for assumptions of normality and homoscedasticity. If these assumptions were met, a one-way analysis of variance (ANOVA) was utilized to determine if the independent treatment variable resulted in variation of the dependent variable (i.e. survivorship and malformation frequency). When significant difference was found, a post-hoc Tukey Honest Significant Differences test was performed. If the data did not meet the assumptions, a Kruskal-Wallis Rank Sum test was performed to determine variation between treatments. If variation between treatments were present, a non-parametric post-hoc analysis was conducted using a Dunn’s Test of Multiple Comparisons.

A comparison between *Xenopus* and leopard frog survivorship and the numbers of malformed embryos was conducted across the three overlapping treatments (i.e. vehicle control, 10ppm IMI and positive control treatments). Due to due to violations of normality and homogeneity of variance a non-parametric aligned rank transformation was conducted. Species and treatment were considered potentially interacting variables.

All statistical analyses were performed using R Version 3.3.2 - © 2009-2017 R Studio, Incorporated (https://cran.r-project.org).

**Results**

**Xenopus**

No significant difference in survival was found among the different IMI exposure concentrations in *Xenopus* embryos ($\chi^2_6 = 7.382, p=0.287$; Figure 1). However, there was a
significantly different number of malformations observed among treatments (F$_{6,16}$ = 9.524, P<0.05; Figure 2). As illustrated in Figure 2, the embryo treatments of positive control, and concentrations of 10$^3$ ppb and 10$^4$ ppb IMI resulted in significantly more malformations than those exposed to no IMI (vehicle control; p<0.05 for each). Embryo treatment with 10$^4$ ppb IMI also resulted in a significantly greater number of malformed embryos when compared the IMI treatments at concentrations of 1, 10 ppb and 10$^2$ ppb (p<0.05 for each). Whereas treatment with 10$^3$ ppb IMI produced a significantly greater frequency of malformations than the 10 ppb IMI treatment (p<0.05). Malformations displayed by *Xenopus* included improper digestive coiling, edema, spinal abnormalities, and eye malformations such as irregular retinal pigment epithelium and split retinas (Figure 3).

The frequency of eye malformations in exposed *Xenopus* embryos are illustrated in Figure 2. Eye malformations included retinal fracturing, irregularly pigmented retinal epithelium, and shape variations from spherical eye (Figure 3). A significant difference in eye malformation frequency was found among treatments (F$_{6,16}$=3.103, p<0.05). The treatment of 6-AN resulted in significantly greater proportion of eye malformations compared to the vehicle control treatment (p<0.05 for each).

Overall, these data indicate that the lowest observed effect concentration (LOEC) for induction of malformations by IMI was 10$^2$ ppm and the no observable effect concentration (NOEC) for IMI was 10 ppb. Both of these values are useful for ecological risk assessment related to the sublethal effects of IMI.

*Leopard Frog*

Analysis of leopard frog embryo survivorship indicated that there a significant differences among treatment groups were present ($\chi^2_5 = 14.157, p<0.05$; Figure 4). Exposure to
the positive control resulted in a significantly lower embryo survivorship when compared to the vehicle control treatment and the IMI concentrations of 5, 10, and 15 ppm treatments ($z=2.713$, $z=2.915$, $z=2.045$, $z=3.371$, $p<0.05$ respectively) treatments. Embryos treated with 20 ppm IMI had a significantly lower survivorship than the embryos treated with 15 ppm IMI ($z=1.784$, $p<0.05$).

As illustrated in Figure 5, a significant difference in the number of malformed leopard frog embryos was found among treatments ($\chi^2 = 21.384$, $p<0.05$). IMI treatments at concentrations of 5, 15, and 20 ppm resulted in significantly more of malformations relative to vehicle control ($z=-2.068$, $z=-2.782$ and $z=-2.910$, $p<0.05$ respectively). Exposure to the positive control resulted in a significantly more of malformations compared to vehicle control and the IMI treatments at 5 and 10 ppm IMI ($z=-4.033$, $z=-1.702$, $z=-2.564$, $p<0.05$ respectively). The frequency of malformations in embryonic leopard frogs did not appear correlated to IMI treatment concentration (Figure 5).

Unlike Xenopus embryos, ocular malformation did not predominate over other types of malformations in leopard frog embryos. Therefore, the leopard frog data were not analyzed according to the numbers of eye and non-eye malformations. The LOEC for elevated numbers of malformations in leopard frog embryos exposed to IMI was 5ppm, but no NOEC could be estimated because lower concentrations of IMI were not tested.

**Comparing effects on early life stages of Xenopus and leopard frogs**

As illustrated by Figure 6, treatment significantly affected embryo survival of both *Xenopus* and leopard frog embryos ($F_{2,18}=10.148$, $p<0.05$). Survivorship at 10 ppm IMI was not significantly different than vehicle control ($p=0.271$). However, survivorship in embryos treated
with positive control \((2.23 \times 10^6 \text{ 6-AN})\) was significantly lower than both the vehicle control treatment and the IMI treatment at a concentration of 10ppm \((p<0.05\) for each).

Amphibian species and treatment had a significant effect on numbers of malformations observed in surviving embryos \((F_{1,18} = 38.448, p<0.05, \text{ and } F_{2,18} = 25.595, p<0.05\) respectively). A significant interaction between species and treatment was also present \((F_{2,18} = 16.329, p<0.05\). The malformation frequency in the 10 IMI mg L\(^{-1}\) and positive control treatments were significantly different from the vehicle control across species \((p<0.05\) for each), as shown in Figure 7. Malformation frequencies in the positive control treatment were not significantly different across species \((p=0.294)\). However, there was a significant difference between species in the frequency of malformations observed in the 10 mg L\(^{-1}\) IMI treatments \((p<0.05)\).

**Discussion**

The development of early life-stage amphibians is dependent on the quality of their aquatic environment and thus are susceptible to effects from exposure to waterborne contaminants (Melvin and Trudeau, 2012). Neonicotinoids have been widely detected at ppb concentrations in surface waters in Canada and elsewhere (Main *et al.*, 2014; Anderson *et al.*, 2015; Morrissey *et al.*, 2015). However, there is limited information available on the sublethal effects of neonicotinoid exposure on developing anuran embryos. Although neonicotinoids are considered relatively non-toxic to vertebrates due to their low binding efficiency to the vertebrate nicotinic acetylcholine receptor (nAChR), previous studies have shown that some neonicotinoid binding does occur in vertebrates (Tomizawa and Casida, 2000). Additionally, in embryonic amphibians, as well as other vertebrates, the nAChR serves several key roles in developmental, such as mediating cell maturation, differentiation, migration and apoptosis, in addition to the
commonly recognized role of neurotransmission seen in mature amphibians (Atluri et al., 2001; Maneu et al. 2010).

Exposure of early life stages of *Xenopus* and leopard frog to IMI did not affect overall survival relative to the vehicle control, indicating that the exposure concentrations were sublethal. Despite not affecting survivorship, larval IMI exposure in *Xenopus* did cause malformations including improper digestive coiling, edema, spinal abnormalities. However, in this species, eye malformations were the most frequent and consisted of irregular retinal pigment epithelium and split retinas. Eye malformations were also prominent in the positive control treatment.

During vertebrate embryogenesis the eye originates from the anterior neural plate which then through a combination of genetic determination and complex inductive signals differentiates into the presumptive eye region. The presumptive eye region, which is composed of retinal precursor cells, gives rise to the optic vesicle and subsequently develops in to the bi-layered optic cup (Martinez-Morales and Wittbrodt, 2009; Viczian, 2013). The optic cup is composed of retinal progenitor cells that proliferate to form the retinal pigment epithelium and the neural retina (Viczian, 2013).

Retinal progenitor cells express a variety of membrane receptors that integrate extrinsic signals, allowing for cell proliferation and differentiation. Cholinergic neurotransmitters, such as the nicotinic AChR, are one such group of receptors expressed by retinal progenitor cells (Das et al., 2005). The nAChR subunits currently known to be present in retinal progenitor cells are $\alpha_1$, $\alpha_4$, $\beta_1$, $\beta_2$. While several combinations of these subunits are possible, one of the most common is the $\alpha_4\beta_2$ nAChR, a receptor previously shown to be upregulated by up to eightfold greater than normal expression by chronic IMI exposure (Tomizawa and Casida, 2000). A process which
releases Ca\(^{2+}\), a secondary signalling messenger, is also known to impact developmental processes, including apoptosis, cell proliferation, differentiation and neurite extension (Atluri et al., 2001). Therefore, it is possible that IMI exposure in *Xenopus* embryos increased the activity of the \(\alpha4\beta2\) nAChR, leading to increased levels of cytosolic Ca\(^{2+}\) which may prevent the natural formation of retinal pigment epithelium and the neural retina. In addition, the positive control (6-AN) also acts on the nAChR and corresponded with a high proportion of eye malformations potentially adding support for this hypothesis. However, further research is required to validate this possibility.

The frequency of malformations observed in leopard frogs was consistent across the IMI treatments of 5, 15 and 20 ppm. Yet, the proportion of malformed embryos was greater than observed in vehicle control. This suggests that there is a potential time point for sensitivity to environmental neonicotinoid contamination, rather than an impact from prolonged exposure. However, future studies must be explored to determine if this is indeed the case.

Early life stages of *Xenopus* showed a greater sensitivity than leopard frogs to IMI exposure as evident by the significantly higher frequency of malformations in embryos exposed to concentrations of 10 ppm IMI. In addition, unlike *Xenopus*, ocular malformations were not prominent in leopard frog embryos. The variations in malformation frequency between the two species may be due to variation in development rate, genetic diversity or a difference in sensitivity to neonicotinoid contaminants. However, it would be beneficial to test leopard frog embryos at lower concentrations than 10 ppm in order to define an NOEC for this species native to North America. *Xenopus* embryos develop rapidly, reaching NK stage 46 after 4 days, while leopard frogs take approximately 7 days after fertilization to reach the same stage (American
Society for Testing and Materials, 2003). This indicates that rapid development may play a more critical role than previously thought.

Embryo development time is a highly adaptive trait commonly associated with natural selection (Hopkins et al., 2012). Where more rapid amphibian embryonic development is seen in warmer habitats and slower development is observed in more temperate regions such as North America (Hopkins et al., 2012). While the short development time of Xenopus embryos may be advantageous in their natural environment, the associated rapid cell proliferation may lead to a higher risk of developmental error in the presence of neonicotinoids. Rapidly proliferating cells in embryogenesis utilize anaerobic glycolysis for developing adenosine triphosphate (a common molecule for extracellular energy transfer) which leads to the build-up of lactate and other glycolytic intermediates (Leese, 2002: Krisher and Prather, 2012). This process has also been observed in the proliferation of retinal progenitor cells in amphibians (Agathocleous et al., 2012). The produced lactate and glycolytic intermediates are then recycled by the embryo to generate glucose through a process known as gluconeogenesis (Kessi et al., 1996). This process normally occurs in the liver of fully formed animals, however in amphibian embryos this process occurs on the cellular level and is still poorly understood (Kessi et al., 1996).

It is therefore possible that the partial agonist binding of IMI to the nAChR during embryogenesis may impact the normal regulation of gluconeogenesis within embryonic cells. This may lead to a buildup of lactate, which would then increase cellular pH levels and increase the risk of acidosis within developing embryos. If this is the case, then the rapid development rate of Xenopus may result in more lactate build up compared to the slower developing leopard frog, thus leading to more frequent ocular and somatic malformations. However, this theory must be further investigated.
Genetic diversity may also contribute to variation in malformation susceptibility under chronic IMI exposure. The leopard frog brood used was collected from a naturally occurring wetland, in which we can assume some degree of natural selection and genetic diversity. In contrast, the *Xenopus* adults used to produce embryos were purchased from a commercial retailer. Although several cultures of *Xenopus* are maintained across North America, there is currently no effort to maintain genetic diversity through outbreeding between laboratories or commercial colonies (Hoke and Ankley, 2005). In addition, *Xenopus* are tetraploid, which when compared to diploid anurans may result in higher rates of genetic divergence. A factor that may be further exacerbated by isolated breeding populations (Hoke and Ankley, 2005). Although no malformations or abnormalities were observed in the *Xenopus* control treatments, the potential genetic divergence and lack of genetic variation in *Xenopus* may be a confounding factor contributing to malformation susceptibility in neonicotinoid treatments.

Environmentally relevant concentrations of neonicotinoid insecticides in aquatic environments are difficult to gauge due to irregular sampling, potential environmental accumulation, and increased commercial use (Starner and Goh, 2012; Main *et al.*, 2014; Anderson *et al.*, 2015). Internationally, the highest cumulative neonicotinoid concentration reported to date was in the Netherlands at 320 ppb, while the highest reported within Canada was 3.1 ppb (Main *et al.*, 2014; Sánchez-Bayo and Hyne, 2014). The concentration in the Netherlands is above the NOEC of 10 ppb for malformations in *Xenopus* exposed to IMI in this study. The lowest observed effect concentration (LOEC) for *Xenopus* (10³ ppb) and leopard frog (5×10⁴ ppb) is greater than the currently implemented freshwater benchmark limits for Canada (0.23 ppb), the United States of America (1.05 ppb), Europe (0.2 ppb), and the Netherlands (0.0083 ppb).
Given the higher sensitivity of *Xenopus* to IMI, this species may not be an ideal surrogate for native amphibians with respect to neonicotinoid toxicity tests. Therefore, the notion that neonicotinoids cause anuran embryo malformations must be interpreted with caution.

The present study describes some of the sublethal impacts of neonicotinoid contamination on developing anurans. While these biological responses were sublethal over the exposure period, developmental abnormalities reducing normal vision and motility are likely to impact organism behaviour and survivorship over time. Malformation, and eye abnormalities were induced in a concentration dependent manner in *Xenopus* embryos exposed to IMI. Leopard frog embryos displayed substantially lower malformation rates that did not appear to be concentration dependent. It is recognized that the toxicological thresholds reported for this study are nominal in concentration, and future work should include mass spectrometry analysis to determine accuracy of applied treatment. It is possible that the lack of an observed concentration-dependent trend in the leopard frog tests was due to variations in persistence of IMI in the various treatments.

The variation between species indicates that future research on neonicotinoid compounds should include multiple anurans species in order to determine variation in sensitivity. In addition, future research should include the study of neonicotinoid metabolites, such as the desnitro metabolite of IMI, a breakdown component with a higher affinity for the nAChR than its parent compound (Tomizawa and Casida, 2000). Finally, more toxicological data are needed on the effects of other classes of neonicotinoid insecticides, including third generation compounds, such as dinetofuran, and the effects of mixtures of neonicotinoids on anuran development.
**Figures**

**Figure 1.** Bar graph showing the mean percentage (± SE) of surviving Xenopus embryos (NK stage 46) after 96h of exposure.
Figure 2. Bar graph showing the percentage (± SE) of malformed Xenopus embryos (NK stage 46) displaying eye and non-eye malformations after 96h of exposure. Asterisk indicate significant difference (p<0.05).
Figure 3. Xenopus embryos displaying malformations caused by exposure to imidacloprid. (A) Vehicle control treatment animal displaying no signs of malformation. (B) Vehicle control treatment animal displaying properly coiled digestive tract. (C) $10^4$ ppb IMI treatment animal
displaying edema, non-spherical eye, improperly coiled digestive tract and oral malformations. 
(D) $10^3$ ppb IMI treatment animal displaying craniofacial malformations, eye abnormalities, spinal abnormalities, and edema. (E) $10^3$ ppb IMI treatment animal displaying edema, and an improperly coiled digestive tract. (F) $10^2$ ppb IMI treatment animal displaying spinal abnormalities. (G) $10^2$ ppb IMI treatment animal displaying a retinal split. (H) $10^2$ ppb IMI treatment animal displaying pigment epithelium abnormality.
Figure 4. Bar graph showing the percentage (± SE) of surviving leopard frog embryos (NK stage 46) after 168 h of exposure. Asterisk indicate significant difference (p<0.05).
Figure 5. Bar graph showing the percentage (± SE) of surviving leopard frog embryos (NK stage 46) displaying malformations after 168 h of exposure. Asterisk indicate significant difference (p<0.05).
Figure 6. Bar graph showing the percentage (± SE) of surviving leopard frog and Xenopus embryos (NK stage 46) after 168 and 96 h of exposure, respectively.
Figure 7. Bar graph showing the percentage (± SE) of surviving leopard frog and Xenopus embryos (NK stage 46) displaying malformations after 168 and 96 h of exposure, respectively.
Chapter 3: Effect of imidacloprid on the survival of *Xenopus* tadpoles challenged with wild type Frog Virus 3

*Abstract*

Ranavirus related mass mortality events have occurred throughout North American and on four other contents. Amphibian sensitivity to this pathogen genus has been suggested to increase with the presence of environmental stressors such as habitat degradation and environmental contamination. Neonicotinoids, a family of systemic insecticides, currently comprises 27% of the global insecticide market a factor which has led to an increase in reported incidence of neonicotinoids in wetlands. This study investigates the effects of the neonicotinoid, imidacloprid (IMI), on premetamorphic *Xenopus laevis* sensitivity to the common *Ranavirus*, frog virus 3 (FV3). It was hypothesized that increased environmental concentrations of IMI would increase virus related mortality. However, it was found that IMI reduced the rate of mortality over time, a phenomenon likely caused by modulation of the inflammatory cytokine response to viral infection commonly observed in tadpoles. Despite this observation, there were an equal number of mortalities found among treatments indicating the IMI presence does not impact total survivorship.

**Key Words:** *Ranavirus*, Frog Virus 3 (FV3), neonicotinoids, imidacloprid, *Xenopus laevis*
Introduction

Amphibian populations are currently more threatened than any other class of vertebrate, with approximately 43.2% of all species experiencing recognized declines as of 2004 (International Union for the Conservation of Nature, 2004). This unprecedented decline has led amphibians to the foreground of the conversation about the global biodiversity crisis (Beebee and Griffiths, 2005). Amphibian populations can be impacted directly by factors such as habitat alteration and introduction of invasive species, or by more complex indirect factors such as climate change, environmental contamination, and emerging infectious diseases. It is unlikely that such factors act independently to contribute to decline. Therefore, examining how these variables interact provides a more realistic view of the factors driving the trend toward amphibian population decline (Collins and Storfer, 2003).

Neonicotinoids, a family of insecticides introduced in the 1990s, are registered in over 120 countries, compromise 27% of the global insecticide market, and have taken over a significant market share of previously used insecticide classes such as the organophosphates, pyrethroids and carbamates (Jeschke and Nauen, 2008; Jeschke et al., 2011). Neonicotinoids have been favoured for their high efficacy against a wide spectrum of biting and sucking insect pests, ease of application, low dose requirements, and low toxicity to vertebrates (Main et al., 2014, Anderson et al., 2015).

Neonicotinoids are primarily applied as a seed or soil treatment, but can also be applied as a foliar spray (Main et al., 2014). After application, the active ingredient is taken up by the growing plant and transported through the vascular system into plant tissues where it will be directly consumed by plant-eating insects (Canadian Council of Ministers of the Environment, 2014). Depending on crop type and application method approximately 2 to 312 g of active
ingredient is applied per hectare, but only 1.6-28% of the applied treatment is taken up by the
plant (Canadian Council of Ministers of the Environment, 2014; Sánchez-Bayo and Hyne, 2014;
Gibbons et al., 2015; Hladik and Kolpin, 2015). The remaining insecticide residue will either
photodegrade, or as a consequence of high water solubility ($6.1 \times 10^5$ ppb for imidacloprid), be
transported from the soil into ground water, or nearby surface waters (Anderson et al., 2015). In
addition, neonicotinoids are known to have highly variable half-lives ranging up to 129 days in
water, thus increasing the risk of accumulation in the environment with repeated applications
(Sánchez-Bayo and Hyne, 2014; Anderson et al., 2015).

Worldwide neonicotinoid concentrations reported range from 0.45 ppb to 320 ppb, and in
many cases, these concentrations exceed the water quality limits for the protection of aquatic life
(Van Dijk et al., 2013; Hladik and Kolpin, 2015). For imidacloprid (IMI), the Canadian interim
water quality guideline is 0.23 ppb, the United States Environmental Protection Agency (EPA)
Aquatic Life Benchmark is 1.05 ppb IMI), and the National Institute for Public Health and the
Environment (NIPHE) Environmental Risk Limits in the Netherlands is 0.2 ppb for acute
exposure and 0.067 ppb for chronic exposure (CCME, 2007; Van Dijk et al., 2013; Main et al.,
2014). Furthermore, it can be predicted that as neonicotinoids continue to replace previously
used classes of insecticides, environmental concentrations are likely to increase.

Previous research has indicated that sublethal doses of the neonicotinoid insecticides can
influence vertebrate body condition, growth, development and reproduction, and can reduce
immune function (Sur and Stork, 2003). For example, granivorous birds have been known to
consume neonicotinoid-coated seeds, resulting in endocrine system disruption and immune
suppression (Lopez-Antia, 2015). In another study, Devan et al. (2014) showed that macrophage
function in rats was significantly decreased when animals were administered sublethal doses of
the neonicotinoid, acetamiprid. Similarly, Duzguner and Erdogan (2012) found that chronic exposure of rats to imidacloprid prompted exaggerated inflammatory cytokine release. These studies indicate that there is a connection between chronic sublethal neonicotinoid exposure and impaired immune function in non-target organisms. However, this connection has yet to be demonstrated in aquatic vertebrates.

*Ranaviruses* are a globally emerging genus of viruses currently known to infect 175 species of ectothermic vertebrates and have been connected to amphibian mass mortality events on five continents (Lesbarrères et al., 2012; Brunner et al., 2015). As of 2015, *Ranaviruses* have been reported in 32 countries across North America, South America, Europe, Africa, and Asia (Duffus et al., 2008). Sensitivity to *Ranavirus* in amphibians varies with amphibian developmental stage (Gray et al., 2009). *Xenopus*, which is a commonly used amphibian laboratory model and a surrogate for native amphibians, can mount an effective immune response to viral infection in the adult stage and are able to recover in approximately 21 days, while tadpoles often succumb to infection (De Jesús Andino et al. 2012). This variation can be attributed to the tadpole’s lack of an effective adaptive immune response and reliance on an immature innate immune system to eradicate *Ranavirus* infection (De Jesús Andino et al. 2012). The tadpole immune response to a *Ranavirus* infection is comprised of a 6-day delayed, moderate up-regulation of the pro-inflammatory cytokines; TNF-α, IL-1β, and IFN-γ originating from peritoneal leukocytes (De Jesús Andino et al. 2012). Research conducted by De Jesús Andino et al. (2012) suggests that this delay in immune response is likely due to a virally induced suppression of normal immune function.

Mortality events related to *Ranavirus* are considered a leading cause of amphibian decline. In addition, the impact of the virus can be influenced by exposure to anthropogenic
stressors. For example, it has been shown that exposure to the herbicide, atrazine resulted in elevated rates of \textit{Ranavirus (Ambystoma tigrinum virus; ATV)} infection in larval tiger salamanders \textit{(Ambystoma tigrinum)} (Forson and Storfer, 2006; Kerby and Storfer, 2009). Exposure to the insecticide chlorpyrifos was also found to increase the sensitivity of larval tiger salamander to \textit{Ranavirus}, and increase mortality rates associated with infection (Kerby and Storfer, 2009). A third study performed by Sifkarovski and colleagues found that \textit{Xenopus} tadpoles had a reduced ability to elicit an immune response to frog virus 3 when exposed to atrazine (Sifkarovski \textit{et al.}, 2014). However, the impact on amphibians of the combined factors of \textit{Ranavirus} infection and neonicotinoid exposure are currently unknown.

The present study focuses on the impact of neonicotinoids on the sensitivity of \textit{Xenopus} tadpoles to \textit{Ranavirus} susceptibility in amphibian tadpoles. In this study, IMI was used as a model neonicotinoid insecticide and the pathogen selected was the common \textit{Ranavirus} strain, frog virus 3 (FV3). IMI was selected as it is the most commonly applied neonicotinoid insecticide worldwide, accounting for 45\% of the neonicotinoid market in 2009 (Jeschke \textit{et al.}, 2011). FV3 was chosen as it is a \textit{Ranavirus} known to cause infection within many species of amphibians, and has been associated with amphibian mass mortality events within North America (Anderson \textit{et al.}, 2015). Tadpoles of the \textit{Xenopus} genus were used as the amphibian model as they are known to be susceptible to \textit{Ranavirus} and are fully aquatic, therefore increasing their risk of neonicotinoid exposure in the aquatic environment. The species used to test this interaction was the African clawed frog, \textit{Xenopus laevis} (henceforth referred to as \textit{Xenopus}), which is an amphibian model commonly utilized within laboratory studies because of the ease of captive breeding, larval manipulations, rapid development, and a strong base of
existing biological knowledge. In addition, *Xenopus* is considered a robust amphibian model for extrapolating responses to effects on wild native amphibians (Hoke and Ankley, 2005).

Given the impact of neonicotinoids on immune function in mammals and birds, it was hypothesized that exposure to IMI at sublethal concentrations will also impact immune function in *Xenopus* tadpoles. It is predicted that survivorship in tadpoles from treatments with IMI will be reduced in a dose dependent manner relative to control treatments when challenged with FV3 viral infection.

_Meth_ods_

_Chemicals_

The PESTANAL analytical standard IMI (1-[6-chloro-3-pyridin-3-methyl] N-nitroimidazolidin-2-yliden-amine) used for tadpole exposures was purchased from Sigma-Aldrich (Oakville, ON, Canada). Benzocaine (99% pure; [4-aminobenzoic acid ethyl ester]; Sigma-Aldrich Oakville, ON, Canada) was used to euthanize tadpoles. Analytical standards of IMI and 5-hydroxy imidaclorpid (5H-IMI) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

_Adult Xenopus husbandry_

Adult *Xenopus laevis* (Boreal Science, St. Catharines, ON, Canada) were maintained in large plastic basins with 40 L of aged (i.e. de-chlorinated) tap water at 21°C with a 16 h light, 8 h dark photoperiod. They were fed crickets, night crawlers, and turtle pellets twice weekly. Water changes occurred once weekly on the day following the second feeding. *Xenopus* were handled and induced to breed according to the protocols described by Sive _et al._ (2010). Human chorionic gonadotropin hormone (hCG) lyophilized powder (Sigma-Aldrich Saint Louis, MO,
USA) was reconstituted for injection with 0.9% NaCl in deionized (DI) water. Females were primed with injections of 50 IU of hCG one week prior to induction. Females and males were induced to breed with injections of 500 IU and 150 IU of hCG, respectively, and were then placed in a plastic bin with 15 L aged tap water to breed overnight.

**Embryo collection, selection and tadpole husbandry**

*Xenopus* embryos were collected immediately post-breeding and transferred to a 1 L holding container. Embryos were de-jellied with a 2% L-cysteine solution (97% pure L-cysteine [(R)-2-Amino-3-mercaptopropionic acid]; Sigma-Aldrich Oakville, ON, Canada), diluted with FETAX solution and pH adjusted to 8.1 using 1M NaOH. The FETAX solution was composed of 625mg NaCl, 96 mg NaHCO\(_3\), 30 mg KCl, 15 mg CaCl\(_2\), 60 mg CaSO\(_4\), 75 mg MgSO\(_4\) per liter of deionized (DI) and pH adjusted to 7.6-7.9 [27]. All unfertilized or malformed embryos were removed from the experiment before Nieuwkoop and Faber (NK) gastrula stage 11, which was approximately 11h 45min post-fertilization (pf) (American Society for Testing and Materials, 2003). After de-jellying, all embryos were maintained in plastic petri dishes containing 10 mL of FETAX solution. At NK stage 46 (96 hpf), the yolk sack is fully absorbed and tadpoles begin to filter feed. At this stage, animals were fed a finely ground combination of algae, freeze dried blood worms, and fish flakes every two days. Full water changes were performed prior to feeding.

**Frog Virus 3 culture**

Fathead minnow epithelial (*Epithelioma Papulosum Cyprini*; EPC) cells were cultured in a 75cm\(^2\) flask containing Leibovitz L-15 media (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Burlington, ON, Canada), 100 U/mL penicillin, and 100 µg/mL streptomycin at 23°C (Morrison *et al.*, 2014). When a confluent
cell monolayer (≥80%) formed, media was replaced with a 1% FBS Leibovitz L-15 media and EPC cells were infected with wild type frog virus 3 (wt-FV3) provided by C. Brunetti (Trent University, Peterborough, ON, Canada). After 48hrs, when cytopathic effects were present, the virus was harvested and purified. Virus solutions were centrifuged at 7×g for 5 minutes (Fisher Scientific centrifuge model 225A, Peterborough, ON, Canada), the supernatant was then removed and the precipitate was suspended with 300 µL of phosphate buffer solution (PBS; Invitrogen, Burlington, ON, Canada).

The solution was purified using three freeze thaw cycles at -80°C and 23°C, and then 15µL sample aliquots of the resulting homogenate were pipetted into cryovial tubes for future analysis of viral titer using a plaque assay. Plaque assays were performed using 100% confluent EPC cells in a 6-well flat bottom tissue culture plate, overlaid with 0.75% methylcellulose, and stained using crystal violet. After determining the number of plaque forming units, virus homogenate was diluted using PBS to achieve a viral titer of $1.25 \times 10^4$ PFU/mL and centrifuged immediately before tadpole exposure.

Preliminary wt-FV3 challenge

A viral challenge was conducted to determine the appropriate titer of the wildtype frog virus 3 strain (wt-FV3) required to infect larval *Xenopus*. A total of 60 animals were reared to NK Stage 49 using the methods described above, then exposed to one of three viral titers: $1.25 \times 10^4$, $1.25 \times 10^5$, or $1.25 \times 10^6$ PFU/mL of wt-FV3. Viral titers were selected based on a previous study which utilized a dose of $1.25 \times 10^5$ PFU/mL to successfully infect NK stage 54-56 *Xenopus* [23]. Animals were monitored daily for survivorship and signs of infection. The experiment concluded 21-days post infection (dpi), and the remaining live animals were
euthanized using a 0.1% benzocaine solution (using 99% pure benzocaine [4-Aminobenzoic acid ethyl ester]) in FETAX solution with pH adjusted to 7.6-7.9.

*Imidacloprid exposure and wt-FV3 challenge*

A three by two factorial design, with six unique treatments, each with 40 individually housed animal replicates (i.e. 240 animals in total) was utilized to examine the interaction between IMI and *wt*-FV3. Treatments consisted of PESTANAL analytical standard imidaclorpid (1-[6-chloro-3-pyridin-3-methyl] N-nitroimidazolidin-2-yliden-amine; henceforth IMI) were used at nominal concentrations of 1 and 500 ppb IMI along with a negative control (i.e. 0 ppb IMI) treatment. Treatments were achieved through a serial dilution and actual values may fluctuate around the value stated, all treatments for this experiment are considered nominal. These three IMI treatments without added virus were duplicated with IMI treatments in which tadpoles were exposed to *wt*-FV3 were then replicated with the addition of the *wt*-FV3. IMI treatments were achieved from dilution of an IMI stock (5 \( \times \) 10^6 ppb) with FETAX solution in 20 L source containers. Source containers were made of dark opaque plastic and kept away from light to prevent photolysis of IMI. Test solutions were renewed every 48 hrs. A total of 240 tadpoles were reared using the methods described above to NK blastula stage 11. From NK stage 11 to 46, embryos were maintained in groups of 30 in 100 mm plastic petri dishes in solutions dosed with IMI and control treatments. At NK stage 46, animals were transferred to 1L plastic containers, where they were individually housed in 0.25 L of IMI or control solution. Tadpoles were infected at NK stage 49 (12-15 dpf) when the first detectable antibody immune response occurs (Robert and Ohta, 2009). Viral infection with *wt*-FV3 was achieved by 1 h passive inoculation in a 60 mm plastic petri dish containing 10 mL of treatment solution at a viral titer 1.25 \( \times \) 10^5 PFU/ mL. Non-virus treated animals were mock infected under the same conditions.
with phosphate buffer solution (PBS) (De Jesús Andino et al., 2012). Animals were monitored for survivorship as well as physical and behavioral indications of infection. Upon completion of the experiment, all animals were euthanized with a 0.1% benzocaine solution.

*Analysis of IMI and degradation product*

Twenty-one 0.5 L water samples were randomly collected from source containers throughout the study and frozen at -20°C in plastic Nalgene bottles for later analysis using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Prior to analysis, samples were thawed and well shaken, before 300 µL aliquots were transferred into amber glass chromatography vials and allowed to reach room temperature. Samples from the highest IMI treatment of 500 ppb were diluted 3:1 with deionized water (milli-Q) prior to analysis. Two vials of 3 mL of methanol were used as method blanks.

Analysis by LC-MS/MS was conducted using an Applied Biosystems Sciex QTrap® 5500 instrument with an electrospray ionization (ESI) source, coupled with an Agilent 1100 HPLC (ABS Sciex, Missisagua, ON, Canada). The concentrations of IMI and its major degradation product, 5-hydroxy imidacloprid (5-H IMI) were determined using direct injection of 10µL volumes of the samples. Direct aqueous injection was made possible by the high sensitivity of the QTrap®5500, which allowed for removal of labor intensive sample preparation (Hao et al. 2016).

Analytes were separated chromatographically using a C18 column eluted with a binary mixture of solvents as the mobile phase. MS detection was preformed using multiple reaction monitoring (MRM) in positive ion mode. For chromatographic separation, the mobile phase consisted of an 80/20 binary mixture of Solvent A (milli-Q water, 2 mM ammonium acetate, 0.1% acetic acid) and Solvent B (acetonitrile, 0.1% acetic acid) eluted through a Genesis C18
column (100mm × 4.6 mm ID; 2.6 µm particle size) and a guard column of the same stationary phase; both purchased from Chromatography Specialities (Brockville, ON, Canada). The transition ions monitored by tandem mass spectrometry for IMI and 5H-IMI were 256>209 and 272>191, respectively.

Data was acquired using electrospray ionization mode with Analyst 1.6.2 software (AB Sciex). The target compound concentration in samples was calculated by comparison to a calibration curve, which was plotted using linear regression with a 1/x weighting and forced through the origin. Stock solutions of IMI and 5H-IMI were serial diluted from analytical standards using methanol to create the eight-point calibration curve (0.78 ppb- 200 ppb). The limits of detection (LOD) and limits of quantitation (LOQ) were determined using the signal to noise ratios of 3× and 10× the standard deviation of the baseline signal, respectively, monitored at the retention times of IMI and 5H-IMI.

Statistical analysis

Logistic regression was used to determine the significance of variations in tadpole mortality across all treatments over time, median survival time and to determine if an interaction was present between treatments. IMI treatment and viral titer were considered categorical variables, while the number of days post-infection was considered a continuous variable. Significant differences in logistic regression were further investigated using Tukey post-hoc analysis of mortalities between individual treatments. Differences in the proportions of surviving animals at experiment conclusion were determined using a Chi-squared test.

All statistical analyses were performed using R Version 3.3.2 - © 2009-2017 R Studio, Incorporated (https://cran.r-project.org).
Results

Preliminary wildtype Frog Virus 3 challenge

To determine the appropriate titer of *wt*-FV3 to infect *Xenopus* survivorship, NK stage 49 tadpoles were exposed to $1.25 \times 10^4$, $1.25 \times 10^5$, $1.25 \times 10^6$ PFU/mL titers of the virus (henceforth referred to as the low, medium, and high virus dose) and a non-virus control. As illustrated in Figure 9***, viral exposure resulted in larval mortalities at 6-days post infection (dpi) and reduced survivorship throughout the trial when compared to controls. A significant difference in mortalities was observed among viral titer treatments ($\chi^2 = 13.24$, p<0.05). A Chi-squared test revealed that the non-virus control had a significantly higher survivorship than survivorship in the low, medium, and high virus treatments ($\chi^2_1 = 17.8$, $\chi^2_1 = 36.2$, $\chi^2_1 = 46.2$, p<0.05, respectively). Larvae from the low virus treatment had a significantly higher survivorship than tadpoles from the medium and high virus treatments ($\chi^2_1 = 4.5$ and $\chi^2_1 = 6.9$, p<0.05, respectively). Medium and high dosages were not significantly different with respect to survivorship ($\chi^2_1 = 0$, p=0.926). Lethal time to 50% mortality ($LT_{50}$) for low, medium and high viral treatments were 17, 11, and 11 days respectively. By 21 dpi, animals from low, medium and high virus treatment groups showed 50%, 65%, and 75% total mortality respectively. There were no mortalities in the control treatment. FV3 infected tadpoles displayed signs of infection including discolouration, abdominal swelling, edema, hemorrhaging, and erratic swimming behaviours.

IMI exposure and *wt*-FV3 challenge

All *Xenopus* tadpoles treated with IMI that were not subsequently exposed to *wt*-FV3 survived until the conclusion of the experiment, which included 40 d of IMI exposure followed
by 25 d of mock infection with PBS. Animals exposed to wt-FV3 (1.25×10^4 PFU/mL) had significantly higher number of mortalities in control (χ²₁ = 51.5, p<0.05), 1 ppb IMI (χ²₁ = 18.4, p<0.05), and 500 ppb IMI (χ²₁ = 10, p<0.05) when compared to treatments in which the animals were not infected with wt-FV3. Because there were no mortalities observed in the treatments without wt-FV3 exposure, no further statistical analysis was conducted within this group.

A logistic regression analysis of virus infected animals showed no interactive effect of pesticide exposure and number of days post-infection (dpi) on tadpole mortality rates (χ² = 0.91, p = 0.34). The number of days after initial wt-FV3 exposure significantly increased the number of tadpole mortalities (χ² = 99.6, p <0.05, respectively). The probability of tadpole mortality increase by a factor of 9% for each day after initial wt-FV3 exposure (Figure 10). Logistic regression analysis indicated that exposure to IMI significantly decreased the rate of tadpole mortality rate over time (χ² = 49.0, p<0.05). Post hoc analysis showed that the rate of mortality in the IMI control treatment was significantly greater than mortality rates in both the low and high IMI treatments (z = -4.43, and z = -8.93, p<0.05 respectively). The mortalities in the low IMI treatment was significantly greater than the high IMI treatment (z = -4.81, p<0.05). After 25 dpi, FV3 treated animals in IMI control, and concentrations of 1 and 500 ppb IMI showed 37.5%, 27.5%, and 22.5% total mortality respectively (Figure 10). However, a Chi-squared analysis of these mortality data indicated that there was no significant difference between treatments in the number of tadpole mortalities by the end of the experiment at 25 (χ² = 3, p = 0.218; Figure 10).

The relationship between number of dpi and cumulative percent mortalities in the IMI control treatment group displayed a natural logarithmic trend line (i.e. y = 0.14ln(x) - 0.07; R² = 0.92). The mortality data for the low IMI treatment animals exhibited a similar logarithmic
relationship between dpi and percent mortality (i.e. $y= 0.12\ln(x)-0.09$; $R^2 = 0.84$). In contrast, mortality data from the high IMI treatment showed a linear relationship between dpi and percent mortality ($Y=0.01x; R^2 = 0.95$). A 20% mortality occurred for wt-FV3 treated IMI controls, and concentrations of 1 and 500 ppb IMI on days 6, 10 and 21 post FV3 infection respectively. FV3 infected tadpoles displayed signs of infection including discoloration, abdominal swelling, edema, hemorrhaging, and erratic swimming behaviours (Figure 11).

**IMI exposure**

The mean measured concentration of IMI in samples randomly collected from the low (1 ppb) and high (500 ppb) treatments were 0.90±0.59 (n=7) and 510.4±17.9 ppb (n= 7), respectively. The mean measured concentration of 5H-IMI in the low and high treatments were 0.74±0.48 and 35.9± 6.3 ppb respectively. IMI and 5H-IMI were not detected in the samples from the control treatment. The LOD and LOQ for IMI were 0.002 and 0.071 ppb, respectively. The LOD and LOQ for 5H-IMI were 0.150 and 0.500 ppb respectively.

**Discussion**

**Preliminary wildtype frog virus 3 challenge**

In the preliminary challenge study to determine the appropriate titers of wt-FV3 to use in the subsequent study on the interactive effects of IMI and virus, no mortalities were observed within the non-virus control treatment. This indicates that the test organisms were being raised with appropriate husbandry conditions and nutrition. Tadpole mortalities in treatments with viral exposure were significantly greater than mortalities in the control (i.e. mock infected with PBS) groups. Viral exposure resulted in larval mortalities in all wt-FV3 treatments within 5 to 6 dpi. This observed mortality corresponds to the previously documented rapid upregulation of the pro-
inflammatory cytokines TNF-α, IL-1β and IFN-γ, as well as an increase in the number of circulating peritoneal leukocytes associated with *Ranavirus* infection in pre-metamorphic tadpoles (De Jesús Andino *et al.*, 2012). It has been shown by other that this delayed immune response to *Ranavirus* infection, followed by the overproduction of inflammatory agents results in severe tissue damage, organ failure, and morbidities commonly associated with *Ranavirus* infection (De Jesús Andino *et al.*, 2012).

Tadpoles in the low viral treatment had a significantly greater number of mortalities compared to animals in the non-virus control, but significantly fewer mortality events compared to treatment with the medium and high virus dosages. High and medium viral treatments did not differ significantly in number of associated mortalities. The difference in mortalities between the low and medium viral treatments indicates that the rate of infection is dependent on the dose of inoculum, such that the number of viral particles within a consistent volume of water increases the probability that a tadpole will become infected. However, the rate of infection between medium and high viral titers did not differ significantly. This indicates that an infectivity threshold may be present at titers of around $1.25 \times 10^5$ PFU/mL, above which an increase in titer will not yield a higher rate of infectivity. A similar trend was reported in a study conducted by Brunner *et al.* (2005) on tiger salamander larvae exposed to *Ambystoma tigrium virus* (ATV), a *Ranavirus* similar to FV3. In this previous study, a threshold of infectivity was observed at $10^3$ PFU/mL for ATV, above which the viral dose was no longer a predictor of infectivity (Brunner *et al.*, 2005). Because greater numbers of mortalities were expected in treatments with combined stressors of IMI exposure and *wt-FV3* infection, the lowest dosage of virus (i.e. $1.25 \times 10^4$ PFU/mL) was selected for the second stage of this study aimed at determining the combined impact of neonicotinoid exposure on *Ranavirus* induced mortality.
The medium viral dose used in the present study \((1.25 \times 10^5 \text{ PFU/mL})\) is similar to the FV3 viral titer used in a previous study to infect NK stage 50 *Xenopus* with a 1 h passive exposure (Sifkarovski *et al.*, 2014). In that study, tadpole mortalities of 80% were observed at 50 dpi which is greater than the mortalities observed in the present study in tadpoles from both the medium and high viral treatments (Sifkarovski *et al.*, 2014). Factors which may explain the lower sensitivity in tadpoles from the present study include the duration of the experiment, the tadpole development stage, and the viral strain. The previous study extended to 50 dpi, so that tadpoles underwent metamorphosis, which typically occurs at 50-60 days-post fertilization. This development stage is associated with a higher risk of mortalities associated with *Ranavirus* infection (Reeve *et al.*, 2013). In addition, a different virus strain was used in the present study than in the previous study which may account for variation in infectivity and related host immune suppression (Morrison *et al.*, 2014).

Virus infected animals displayed physical and behavioral signs of *Ranavirus* infection. Examples included loss of pigmentation, edema, internal bleeding, inability to maintain equilibrium in the water column and erratic swimming. Identical pathological effects have been observed in previous studies with tadpoles exposed to *Ranavirus* (Jancovich *et al.*, 1997; Robert *et al.*, 2005; Gray *et al.*, 2009; Miller *et al.*, 2007; Miller *et al.*, 2009, Miller *et al.*, 2015).

IMI exposed *Xenopus* that were not infected with virus displayed no mortalities or observable pathological changes. Therefore, IMI concentrations up to 500 ppb were sublethal for *Xenopus* tadpoles under these experimental conditions. However, further research is required to determine the effects of IMI exposure on later stages of amphibian development such as metamorphosis.
The analysis of randomly selected samples for levels of IMI indicated that the measured concentrations of IMI were similar to the target concentrations of 1 and 500 ppb. There were relatively low concentrations of the primary degradation product, 5H-IMI present in these samples, which indicates that transformation of the parent compound in the exposure containers was not a significant variable in these experiments.

Contrary to predictions, exposure to IMI did not increase mortalities in tadpoles infected with wt-FV3. In fact, animals treated with IMI and then virus challenged displayed lower rates of mortality when compared to animals from the control treatment. However, the total number of tadpoles that survived to the end of experiment did not significantly differ across the treatments. Therefore, it appears that IMI exposure increases the duration of time an animal can withstand a *Ranavirus* infection without succumbing to mortality, but does not improve overall survivorship. A potential mechanism for this observation is the interactive effect of neonicotinoids on the cholinergic anti-inflammatory pathway, which is a physiological mechanism mediated by the binding of acetylcholine to the α7 subunit of the nicotinic acetylcholine receptor (Wang *et al.*, 2003). This pathway inhibits the release of inflammatory cytokines through a post-transcriptional mechanism (Wang *et al.*, 2003). As mentioned previously, *Ranavirus* infections impact the normal tadpole immune response by initiating a drastic upregulation of inflammatory cytokines (primarily TNF-α, IL-1β and IFN-γ) approximately six days following initial viral infection (De Jesús Andino, 2012). This over production causes lethal systemic inflammation, tissue injury and organ failure (Gray *et al.*, 2009). Therefore, modulation or reduction of interstitial cytokine levels during this critical time frame may prolong the survivorship of infected tadpoles.

Mediation of pro-inflammatory cytokines using the cholinergic anti-inflammatory is a method currently studied in medicine to reduce risk of cytokine associated injury during patient
sepsis (Wang et al., 2004). Indeed, previous research has shown that application of nicotine, a cholinergic agonist against the nicotinic acetylcholine receptor, prevented the release of TNF-α from macrophage cells (Wang et al., 2004). Neonicotinoids, a derivative of nicotine, display a low binding affinity to the vertebrate nicotinic acetylcholine receptor, and act as a partial agonist (Tomizawa and Casida, 2005). Therefore, the treatment of IMI may have also activated the cholinergic anti-inflammatory pathway and prevented the abundant release of cytokines. This action could explain the reduction of rapid mortality observed within the IMI treatments.

Modulation of the inflammatory response did not appear to eradicate the viral infection, as similar total mortalities occurred by the end of the experiment across all virus infected animals. However, a relatively low number of animals died throughout the course of this study (i.e. <50%), indicating a low proportion of infected individuals. Therefore, further research involving a high viral titer or a more infective strain of Ranavirus would be beneficial to confirm the observations in the present study.

Growing concerns regarding the decline of amphibian populations have led to the study of both anthropogenic and naturally occurring impact factors. Previous research has identified environmental contaminants as stressors that could contribute to amphibian population declines (Collins and Storfer, 2003; Forson and Storfer, 2006; Kerby and Storfer, 2009; Sifarovski et al., 2014). However, this study showed that the relationship between amphibian declines and exposure to insecticides can be complex. Ranavirus kills 90-100% of infected tadpoles, and is considered a strong contributing factor to amphibian population declines and extirpations (Lesbarrères et al., 2012). Currently, no applications are known to reduce this rate of mortality. Future studies could further explore the potentially beneficial effects of exposure of amphibians to anti-inflammatory compounds. However, with regards to the use of neonicotinoid insecticides
as anti-inflammatory agents, the levels required to reduce inflammation in *Ranavirus* infected amphibians would be harmful to other sensitive non-target organisms, such as aquatic insects (Gibbons *et al.*, 2015), and therefore, this is not a viable option for field application.
Figure 4. Cumulative percent mortalities over 21 days post-infection for Xenopus tadpoles (NK stage 49-54) treated with low (1.25 × 10^4 PFU/mL), medium (1.25 × 10^5 PFU/mL) and high (1.25 × 10^6 PFU/mL) titers of wt-FV3.
Figure 5. Percent mortalities over 25 days post-infect for Xenopus tadpoles (NK stage 49-54) from control and nominal concentrations of 1 and 500 ppb in rearing solution that were subsequently treated with of wt-FV3 (1.25 × 10⁴ PFU/mL).
Figure 6. Xenopus laevis tadpoles (NK stages 50-54) displaying signs of Frog-Virus 3 (FV3) infection. (A) FV3 control and IMI control treatment, 25 dpi, animal exhibits no signs of infection. (B) FV3 infected, IMI control treatment, 20 dpi, animal displays edema, discolouration, swollen peritoneal cavity, and hemorrhaging within the tail. (C) FV3 infected, IMI control treatment, 21 dpi, animal displays discolouration and swollen peritoneal cavity. (D) FV3 infected, low IMI treatment, 9 dpi, Animal displays edema and inability to maintain equilibrium within the water column. (E) FV3 infected, low IMI treatment, 14 dpi, animal displays discoloration and edema. (F) FV3 treated, high IMI treatment, 19 dpi, animal displays discoloration, edema, and swollen peritoneal cavity.
Chapter 4: Conclusion

The goal of this thesis was to test the general hypothesis that neonicotinoid exposure affects premetamorphic anurans. By better understanding the impacts of neonicotinoid exposure on early life staged frogs, conservationists can better determine if neonicotinoid exposure acts as a contributing factor to amphibian declines. To test this general hypothesis, two experiments were conducted. The first involved the exposure of embryonic stage *Xenopus* and leopard frogs to the neonicotinoid, imidacloprid (IMI) to determine if exposure affects morphological development or survivorship. The second experiment involved the chronic exposure of premetamorphic *Xenopus* tadpoles to imidacloprid, and subsequent pathogen infection with Frog Virus 3 (FV3) to determine if environmental neonicotinoid presence affects viral pathogen sensitivity.

The environmental presence of pesticides has been shown to impact normal morphological development in developing fish and amphibians (Perkins *et al*., 2000; Zang *et al*., 2014; Fischer *et al*. 2015). Therefore, it was predicted that embryonic exposure to IMI would increase mortality and morphological malformations in *Xenopus* and leopard frog embryos in a dose dependent manner and that both species would yield similar results. However, the results of the experiment did not support this prediction. Exposure did not affect embryo survivorship, indicating that imidacloprid dosages up to 10 ppm for *Xenopus* and 20 ppm for leopard frog embryos are sublethal. Neonicotinoids have a greater affinity for the insect nicotinic acetylcholine receptor (nAChR) in comparison to the same receptor in vertebrates (Gibbons *et al*., 2015). Therefore, aquatic insects will be affected at much lower concentrations than amphibian embryos. However, indirectly the loss of insect biodiversity may reduce insect predation (i.e. dragonfly larvae) on tadpoles as well as reduce insect food availability for adult amphibians.
Therefore, research on the impacts of neonicotinoids on trophic interactions in aquatic environments is required.

With respect to the frequency of morphological malformations observed, *Xenopus* embryos responded in a dose dependent manner where an increase in environmental IMI concentration resulted in an increase in malformation presence. Conversely, leopard frog embryos did not display a dose dependent relationship with IMI concentration but did show an increased level of malformation in neonicotinoid treatments. These results indicate not only that neonicotinoid exposure can act as a potential teratogen, but that sensitivity to this compound can vary across species. This may be of concern given the common practice of utilizing a single species to represent a taxonomic class. Variation in contaminate sensitivity between species of anuran embryos has also been noted in exposures to the glyphosate based herbicide (Edginton *et al*, 2004).

The consistent level of malformation across all leopard frog treatments indicates that a critical time period for neonicotinoid exposure may be present. Where critical time period refers to a specific sequence or stage of development that is sensitive to contaminate presence. Future studies should investigate if variation in time of exposure alters the type and frequency of embryo malformations. In addition, research initiatives should further examine if variation in response is present among North American anuran species as well as among amphibian orders (i.e. Urodelia and Anura). Previous studies have found that salamanders can exhibit higher rates of malformations from trematode infection when compared to amphibians under similar conditions (Johnson *et al*. 2006). By determining if variation in sensitivity extends beyond the two species studied here, conservationists can better predict the impact that an environmental increase in neonicotinoid concentration would have on amphibian populations and biodiversity.
Overall, it appears that while neonicotinoids do have the capacity to impact normal anuran embryogenesis, the environmental concentrations required to do so are currently only found in extreme scenarios, such as small shallow water bodies or runoff following heavy rainfall (Hladik and Kolpin, 2015). However, should these extreme scenarios (i.e. 3 - 30 ppm) coincide with anuran embryogenesis, between 8-26% frequency of malformation can be expected depending on species, according to the results obtained from this study. Therefore, future research should investigate the impact that this malformation rate would have on amphibian population stability and impact on tadpole and adult survivorship stages as well as reproduction. Such as experiments may include exposing animals to neonicotinoid compounds during embryogenesis and then carrying them through to adult hood to determine how viability and behaviour are impacted.

Amphibians and other aquatic vertebrates have shown increased sensitivity to pathogens when exposed to agricultural runoff (Mann et al., 2009; Gahl and Calhoun, 2010; Kelly et al. 2010). Therefore, it was predicted that chronic exposure to imidacloprid would increase sensitivity to the common viral pathogen, FV3. However, the results indicated that this hypothesis could not be supported. Instead the exposure to imidacloprid reduced the rate of tadpole mortality over time, likely due to the modulation of the inflammatory cytokine response associated with *Ranavirus* mortality in tadpoles. However, neonicotinoid exposure ultimately did not affect the total number of animals that experienced mortality due to infection.

This is the first study that provides a potential mechanism for reduction in *Ranavirus* associated mortality rates in infected premetamorphic anurans. Given the frequency of mass mortality events (MME) in amphibian populations associated with *Ranavirus* infections (Collins
and Storfer, 2003), determining if the use of water soluble anti-inflammatory compounds is a viable method for treating infected animals should be further investigated.

The results of this study indicate that embryonic and premetamorphic stage anurans are relatively robust to the presence of neonicotinoids in the aquatic environment at moderate levels (i.e. < 100 ppb). However, risk of detrimental malformations are present at sublethal levels during embryogenesis. In addition, exposure to neonicotinoids does not increase sensitivity to \textit{Ranavirus} infection in anuran tadpoles. Therefore, based on the results of this thesis, it is unlikely that neonicotinoid exposure acts as a combined stressor at moderate environmental levels and therefore does not poses a substantial risk to amphibian populations or biodiversity. Further research in this area of study is required to confirm the consistency of results across species and to determine the true extent of long term sublethal impacts.
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