

**LIFE-HISTORY TRADE-OFFS IN NORTHERN LEOPARD FROG  
(*LITHOBATES [RANA] PIFIENS*) TADPOLES: INTERACTIONS OF  
TRACE METALS, TEMPERATURE, AND RANAVIRUS**

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## ABSTRACT

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Emerging infectious diseases, pollution and climate change are associated with amphibian extinction events worldwide; however, direct causation is often obscured by the interactions of these stressors. Elucidating the possible synergies between metal contamination and disease is, therefore, critical in advancing our knowledge of the co-evolutionary mechanisms in host-pathogen systems and helping with the ability to better forecast the spread of diseases in metal-stressed environments. Additionally, increasing ecotoxicological research has improved our understanding of the complex influence trace metals may have on the physico-chemical nature of aquatic systems; however, the discrepancy in concentration-response within the toxicological literature makes it difficult to accurately define the range of toxicity, often due to the variability in media used in experimentation. The first chapter of this thesis reports an evaluation of copper, nickel and copper/nickel concentrations on several Northern Leopard Frog (*Lithobates [Rana] pipiens*) larvae life history traits within field collected smelting effluent water. Overall, results indicated that copper had a stronger negative impact on survival than nickel. However, tadpoles exposed to copper displayed increased growth and developmental patterns while those exposed to nickel demonstrated opposing life history traits. These results indicate that tadpoles are displaying different fitness strategies, in terms of survival and life history, in the presence of increased copper and/or nickel stress.

In the second chapter, I studied the combined effects of copper and temperature on the *L. pipiens-ranavirus* system to determine if these two environmental stressors have separate, synergistic or antagonistic impacts on host-pathogen systems. Tadpoles infected by ranavirus displayed accelerated growth and development with a decreased survival while those exposed to copper showed decreased growth, development and survival. These results are possibly due to the absence of food and the type of media in this experiment, whereby the added copper is readily available for absorption by the tadpoles due to the low ionic content of the selected medium. However, the combination of copper and the ranavirus led to an antagonistic reaction with the result that the tadpole growth and development were not different from the control group. In addition, temperature strongly influenced life history/survival trade-offs in *L. pipiens* tadpoles. Tadpoles reared at 14°C grew and developed faster but had a lower survival rate than tadpoles raised at 20°C regardless of the treatment. In the context of increasing environmental changes to our aquatic ecosystems this study demonstrates that *L. pipiens* larvae may be able to tolerate stressful conditions through plastic adaptations in life history traits, but these rapid phenotypic responses are associated with a fitness cost. Together, these results will benefit conservationists, ecotoxicologists, industry and government in recognizing how human-induced stressors can impact and alter the life history of amphibians and the ecology of wetland ecosystems. My work ultimately highlights the need for more interdisciplinary research to understand and mitigate the effects of trace metals and diseases on aquatic vertebrates, especially in disturbed ecosystems.

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## GENERAL INTRODUCTION

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### **Global amphibian declines**

Amphibians are currently experiencing alarming declines throughout the world. Nearly 2,110 (32%) species are on the brink of extinction while 120 (2%) have already gone extinct (McDiarmid and Mitchell 2010, Robert 2010). Several factors are associated with these declines (Blaustein and Kiesecker 2002; Kiesecker 2011). However when added together, those stressors can have compounding effects leading to even higher environmental pressure, especially in the case of anthropogenic stressors (e.g. habitat destruction, climate change, metal contamination, invasive species and overexploitation) combined with natural stressors (e.g. resource availability, drought, disease and predation) which are a highly variable pressure on these ectothermic vertebrates (Alford and Richards 1999, Stuart et al. 2004, Blaustein et al. 2011, Kerby et al. 2011). Therefore, amphibians can be considered excellent models for evolutionary ecology research as they display rapid physiological, phenotypic and behavioural responses to environmental constraints while also having short life spans (Newman 1992, Laurila et al. 2002, Van Buskirk 2002).

Amphibians are ectothermic vertebrates that rely on the environment to help stabilize and maintain their homeostatic equilibrium (Rome et al. 1992). Due to their unique life style, amphibians are important bioindicators for wetland ecosystem health (Blaustein and Kiesecker 2002, Venturino et al. 2003). First, most amphibians have both aquatic and terrestrial phases, spending their larval period in the water while their adult stage is terrestrial or semi-terrestrial (Blaustein and Belden 2003). Second, amphibians complete several physiological and metabolic processes such as the exchange of gases, ions, osmolytes, heat and water through their semi-permeable skin (Boutilier et al. 1992, Spotila et al. 1992). Environmental stressors generated by increased anthropogenic activity can severely disrupt the physiology and ecology of amphibians (Relyea and Hoverman 2006, St-Amour et al. 2008, Leduc et al. 2012).

The Northern Leopard Frog (*Lithobates [Rana] pipiens*) has a wide range geographic distribution throughout North America (Figure 1). *L. pipiens* is considered a rather sensitive anuran species and chemical pollution by acid depositions, pesticides, and trace metal emissions appear to have affected many Northern and Western Canadian *L. pipiens* populations (Fournier et al. 2005, Wilson et al. 2008, COSEWIC 2009). Northern Leopard Frog populations are typically found in lower densities in agricultural and mining landscapes in the presence of high pesticides and metal contaminants (Chistin et al. 2003, Greer et al. 2005, St-Amour and Lesbarrères 2006, Blaustein and Bancroft 2007, Duffus et al. 2008, Kerby et al. 2010). However, a previous study indicated that *L. pipiens* populations are now slowly beginning to recolonize and are reproducing in abundance in metal-stressed wetland ecosystems (Leduc et al. 2012) thus making this species an excellent model species to test the interaction between trace metals and other stressors.



**Figure 1:** Northern Leopard Frog (*Lithobates pipiens*) adult and its distribution range across North America (<http://biology.mcgill.ca/undergra/c465a/biodiver/2001/northern-leopard-frog/northern-leopard-frog.htm>).

## **Amphibian population declines**

Emerging Infectious Diseases (EIDs) appear to be causing widespread amphibian population decline with reports indicating increased occurrences of chytridiomycosis (*Batrachochytrium dendrobatidis*; Daszak et al. 1999 Briggs et al. 2005) and ranavirus (Blaustein et al. 2012, Lesbarrères et al. 2012). The Chytrid fungus, *B. dendrobatidis* (Bd), an aquatic fungal pathogen has also become a pervasive and prevailing infectious disease reported in amphibian populations worldwide (Bosch et al. 2001, Briggs et al. 2005, Voyle et al. 2009, Gahl et al. 2011, Searle et al. 2011). Although historically regarded as less of a cause for concern as Bd, *ranaviruses* are also a serious threat to amphibian populations (Lesbarrères et al. 2012). Ranaviruses are considered one of the five genera from the Family *Iridoviridae* that aggressively infects amphibians (Daszak et al. 1999, Kerby et al. 2011), reptiles (Chen et al. 1999, Johnson et al. 2007, Jancovich et al. 2010) and osteichthyan fish (Mao et al. 1997, Whittington et al. 2010). The first ranavirus strain was first detected in an anuran population (i.e. *Lithobates pipiens*) during the 1960's (Granoff et al. 1965), and in 2007, *ranaviruses* were recognized as emerging infectious diseases by the World Organisation for Animal Health (IOE) due to its importance as an ecological driver in amphibian extinction events (Blaustein et al. 2012, IOE 2012). Frog Virus 3 (FV3) was first discovered within anurans and is one of the most occurring ranavirus strains (Chinchar 2002, Chinchar et al. 2009). FV3 replication occurs within 2-3 hours of exposure and within 12-32°C range (Chinchar 2002, Rojas et al. 2008) whereby the replicate rate is most prolific between 20-24°C (Ariel et al. 2009). Ranavirus virulence can be extremely aggressive because of its ability to disperse through horizontal transmission (Duffus et al. 2008, Shock et al. 2008), cannibalism (Harp and Petranka 2006), and contact with contaminated environment (Jancovich et al. 2004). Amphibian larvae are the most highly susceptible life stage to ranavirus infection because they have rudimentary immunological systems. In fact, a large proportion of the metabolic investment of a tadpole is allocated to growth and development, where the endpoint is metamorphosis (Rollins Smith 1999, Warne et al. 2010). Individuals who died of ranavirus infection

present behavioural (i.e. lethargy, erratic swimming, and unresponsiveness) and physiological symptoms (i.e. swelling, erythema, and lordosis) (Figure 2; reviewed in Gray et al. 2009).

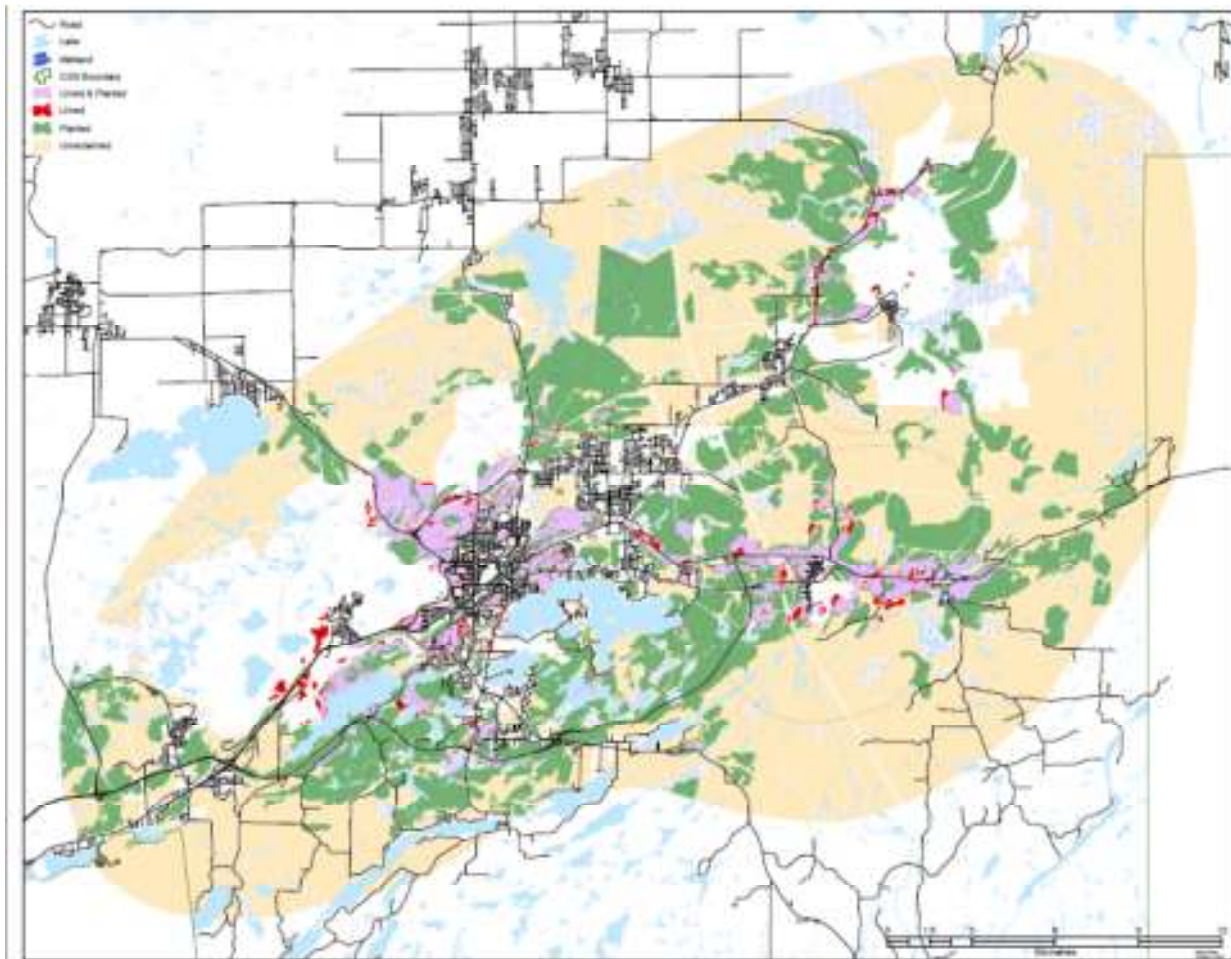


**Figure 2:** Ranavirus virion particle (left) and various physiological effects of a ranavirus infection in Northern Leopard Frog tadpoles at various stages of larval development (i.e. swelling (centre) and internal hemorrhaging (right)).

### **Ecological footprint of trace metals**

Mining and smelting industries are important sources of trace metal pollution of aquatic environments (Snucins et al. 2001, Keller et al. 2007). Over the past century, Sudbury, Ontario, Canada, has been one of the world's largest producers of copper and nickel ore (Shuhaimi-Othman et al. 2006, Keller et al. 2007). Since the 1960's, irresponsible smelting approaches have left Sudbury natural ecosystem with extensive trace metal contamination (Keller et al. 2007). At full production, the smelters emitted over 2 million tonnes of sulfur dioxide (SO<sub>2</sub>) and 1.5 thousand tonnes of trace metal particulates into Sudbury's surrounding ecosystem (Leduc et al. 2012) acidifying over 7000 lakes and wetlands while contaminating over 17000 km<sup>2</sup> of soils and waters (Dixit et al. 1992; Keller et al. 2007) (Figure 3). Since the 1990's, abatement restrictions and refined smelting approaches have been implemented to reduce SO<sub>2</sub> and trace metal emissions by over 90% (Shuhaimi-Othman et al. 2006, Leduc et al 2012). However, contaminated wastewater is another by-product of the milling process of ore. After several chemical treatment procedures, the treated wastewater is released into the natural aquatic ecosystem which is regulated by Provincial (MISA, 2012) and Federal (MMER, 2012) legislations.

Among the multiple trace metals and elements found in smelting effluent wastewater, copper ( $\text{Cu}^{2+}$ ) and nickel ( $\text{Ni}^{2+}$ ) are often encountered in high concentrations (Eisler 1998, Handy 2003). Once released into ecosystems, these trace metals contaminate the surrounding waters and soils, presenting damaging and long-lasting impacts on the local wildlife (Márquez-Ferrando et al. 2009). At high concentrations, some inorganic metals (e.g. Cu, Ni, Zn, Fe, Ca, S, Mg, ect.) toxic to plant and vertebrate organisms, but at low concentrations, they represent essential micronutrients (Festa and Thiele 2011). For amphibians, they have several exposure routes (i.e. skin, nostrils, mouth and/or gills) which can uptake bioavailable metals depending on their lifecycle stages (i.e. egg, larvae or adult) and taxonomy (i.e. *Anura*, *Caudata* or *Gymnophiona*) (Linder et al. 2010).



**Figure 3: Trace metal contamination footprint (in yellow) produced by the emissions of the smelters in Sudbury, Ontario, Canada (VETAC, 2010).**

Aquatic organisms depend on copper for activation of cellular aerobic respiration proteins (i.e. cytochrome c oxidase and NADH dehydrogenase; Hoshi et al. 1993, Festa and Thiele 2011), metabolic Cu-dependent proteins (Opazo et al. 2003) and transcriptional regulators (Festa and Thiele 2011). In addition, Cu also increases the presence of neutrophils and proliferation of epithelial cells in the gills and intestines (Handy 2003). However, when Cu contamination becomes very high in natural systems, it is toxic to aquatic organisms (Handy 2003). For instance, copper sulphates are components of several herbicidal, algaecidal, bacteriocidal and fungicidal agents; however, these Cu-based deterrents are also detrimental to all other aquatic plant and animal life (Nor 1987, Festa and Thiele 2011). In fish and molluscs, acute and chronic Cu exposure studies have demonstrated adverse effects on morphological, behavioural, immunological, endocrinal, respiratory, and olfactory systems (Meyer et al. 1999, Handy 2003, Green et al. 2010). Moreover, acute and chronic exposure studies have shown that *Lithobates pipiens* tadpoles suffer from reduced survival, abnormal behaviour and reduced life history traits when copper concentrations increased above 50 µg/L (Landé and Guttman 1973, Reddick and LaPoint 2004, Chen et al. 2007, Lance et al. 2012).

Vertebrates also depend on nickel (Ni) for specific optimal enzymatic functioning (Thauer 2001) but Ni becomes carcinogenic to vertebrates when it is highly bioavailable (Costa 1991, Eisler 1998, Pane et al. 2003, ATSDR 1988). Additionally, Ni has a high affinity for bioaccumulating within the lungs and kidneys producing cardiovascular, pulmonary and renal disorders. At the cellular level, nickel has the ability to enter the cell, accumulate within the nucleus, and disrupt DNA metabolism causing mutations and cancerous cells (Costa 1991, Eisler 1998). Much of the Ni toxicity has been conducted using teleost fish (Ptashynski et al. 2001, Pane et al. 2003), while the effects of Ni on other aquatic groups such as amphibians have been rarely investigated. Sublethal Ni-exposure produced congenital malformations, reduced life history traits, aberrant behaviour, and increased mortality in various anuran species (i.e. *Bufo melanostictus*, Khangarot and Ray 1987; and *Xenopus laevis*, Hopfer et al. 1991).

## **The rising threat of temperature**

Amphibians occupy every continent other than Antarctica (Alford et al. 1999) and display adaptations to a wide range of temperature conditions (Carey et al. 1999). Amphibian population declines have been recently associated with climate change (Hof et al. 2011). Global warming stemming partly from the reduction of the ozone layer of the Earth's atmosphere due to human-induced pollution and greenhouse effects (Noyes et al. 2009). Global warming can be considered a threat to amphibians since their behavioural, physiological and ecological traits are specifically associated to temperature changes within the environment (Blaustein et al. 2001). In warm temperatures, amphibians experience increased metabolic functioning whereby their metabolic rate increase 2 fold with every 10°C increase in temperature (i.e.  $Q_{10}$ , Pelster 1999). Northern Leopard Frogs incubated at 22°C have displayed increased lymphocyte proliferation, leukocyte abundance and serum complement levels (Maniero et al. 1997). However in colder temperatures, amphibians exhibit immunosuppressive tendencies, especially closer to hibernation temperature ranging from 4-8°C. Leopard Frogs demonstrated reductions in serum and lymphocytes production and antibody synthesis when held at 5°C for several months (Maniero et al. 1997, Rojas et al. 2005). Since temperature can greatly affect the immunocompetency of amphibians, it is critical to understand the role of temperature combined with other stressors (e.g. trace metal pollution and disease). With regard to disease, temperature has a significant effect on the virulence, growth, replication and infectivity of pathogens (Ostfeld 2009). Increasing temperature can strongly change the dynamics of host-pathogen systems as foreign pathogens have the ability to invade new ecosystems and can have devastating impacts on amphibian populations (Garner et al 2011, Hof et al. 2011). Furthermore, the toxicology of trace metals can be greatly altered by global warming. As trace metal behaviour (e.g. speciation, complexation, and toxicity) is highly dependent on environmental variables, climate change could influence environmental pollution in two ways: (1) trace metal toxicity can increase with temperature; or (2) an increase in temperature can result in a faster rate of contaminant elimination into the water (Benitez et al 2006, Noyes et al. 2009). Finally, global warming can impose further negative

effects because trace metal uptake through respiration will increase in response to higher metabolic activity in warmer conditions (Handy 2003, Schiedek et al. 2007).

### **Interdisciplinary approach for a better understanding of the amphibian crisis**

Considering the sensitivity of amphibian communities within northern landscapes, especially those already challenged by metal stress, it is expected that the emergence of novel diseases in these landscapes will be highly detrimental to amphibian populations. Moreover, the synergistic or antagonistic interactions between trace metal pollution, disease, and climate change can be challenging to disentangle because they not only impose singular direct effects but they also interact amongst each other to create compounding indirect effects. An interdisciplinary approach integrating evolutionary biology and ecotoxicology provides a framework to better understand the roles of these stressors. In this context, the scope of my thesis was to investigate the combined effects of trace metals, disease and temperature increase on the survival and growth of Northern Leopard Frog (*Lithobates pipiens*) tadpoles. In a first chapter, I will evaluate the dose-response concentration ranges of copper, nickel and a combination of copper/nickel on *L. pipiens* tadpoles as both metals constitute an important part of the ecological footprint left by Sudbury mining and smelting industries. In a second chapter, I will investigate whether the combined effect of trace metal and temperature stressors on host-ranavirus system would have direct or indirect effects on survival or life history of *L. pipiens* tadpoles. Little to nothing is known on the role of trace metal contamination on the dynamics of host-pathogen interactions so this study will improve our understanding of ranavirus disease in stressed environments while bridging the gap between ecotoxicology and evolutionary biology.



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## CHAPTER 1

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### **Copper and Nickel Effects on the Growth and Mortality of *Lithobates pipiens* Tadpoles in a Field-Collected Smelting Effluent Water**

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**Abstract**— Water contamination by trace metals has contributed significantly to the global biodiversity crisis. Trace metals can have a subtle, yet chronic impact on an organism by inducing physiological stress that reduces their survival or impedes their ability to tolerate additional environmental stresses. However, toxicity tests indicate that aquatic organisms react differently to trace metals depending on the environments in which they are present. The concentration-response variability within the toxicological literature can for example be attributed to the difference in medium being used for experimentation. In this context, the objective of this study was to understand the variation in life-history traits of Northern Leopard Frog (*Lithobates pipiens*) larvae to ionic copper, nickel and their combination within a field collected effluent medium. Tadpoles were randomly assigned to either one of 8 copper concentrations (8-200 µg/L), 7 nickel concentrations (160-1200 µg/L), or 8 Cu/Ni combined concentrations (Cu/Ni; 8/160-200/1200 µg/L) in effluent water collected downstream of a wetland tailings treatment area. The results indicate significant differences in survival and life history traits among treatments. In Cu and Cu/Ni combined treatments, tadpole survival decreased with increased copper exposure and stabilized at [Cu] = 65 µg/L while in the Ni treatment, tadpoles exhibited a continuous decrease in survival as Ni concentration increased. Surprisingly, all Cu-exposed treatments induced an increase in growth rate as the concentration increased while tadpoles showed a significant decrease in growth rate in Ni treatments. These contrasting outcomes suggest a rapid plastic response by Northern Leopard Frog tadpoles to trace metals whereby tadpoles allocate their energy reserves towards either escaping or a coping with the metal-stressed environment. Finally, these responses generated by the interactions trace metals have upon *L. pipiens* tadpole within the aquatic ecosystem further indicate the need to study the impacts multiple stressors can have on amphibian populations.

**Keywords**— Copper, Nickel, Tailing Wetlands, Northern Leopard Frog, Life History Traits, LC<sub>40</sub>, LC<sub>100</sub>

## Introduction

Ecotoxicology is defined as the study of the effects of natural or anthropogenic contaminants (e.g. metals and pesticides) on the disruption of an organism's homeostasis in relationship to its environment (Truhaut 1977, Moriarty 1988, Chapman 2001, Sparling et al. 2011). In particular, concentration responses for terrestrial and aquatic vertebrates continue to be investigated in both experimental and natural settings (Relyea and Hoverman 2006). Predicting the toxicity of metal contaminants that are biologically hazardous can be difficult in natural systems because their concentrations and chemical forms (e.g. speciation) are highly variable and greatly depend on the chemical composition of the aquatic medium (Bridges and Semlitsch, 2000, Hopkins 2007). The concentration-response literature is therefore scrutinized as many extraneous factors (i.e. time, temperature, pH, DOC, water hardness) can dramatically influence the speciation and chemical nature of trace metals that affect organisms (Eggen et al. 2004). In fact, there are numerous discrepancies throughout the scientific literature when surveying for the toxicological impact on particular species (Hecnar 1995, Bridges and Semlitsch 2000). The addition of a toxicant will disrupt and change the physicochemical properties of the environmental medium and directly affect all organisms within the aquatic system (Eggen et al. 2004). Depending on the severity of the adverse changes within the system, these contaminants can alter specific metabolic components of an organism and in turn elicit either subtle or strong physiological and/or phenotypic trade-offs (Carey and Bryant 1995, Rosseland et al. 2001). Therefore, the variation in trace metal behaviour, and thus the extent of the resulting trade-off is difficult to predict because both are contingent on a multitude of factors including the experimental media being used in laboratory or semi-natural studies (Rowe and Dunson 1994; Walker et al. 2006).

Along with recent advances in the field of ecotoxicology, researchers are beginning to address the reoccurring problems stemming from the difference between *in-situ* and *ex-situ*-based toxicological findings (Hopkins and Rowe 2010). Ecotoxicological research is an interdisciplinary field studying the link between chemical and other ecological stressors (e.g. biochemical components of mediums,

competition, drought, predation, disease, etc.) in order to predict the physiological or behavioural impacts contaminants have on living organisms within realistic ecological settings (Boone and James 2005, Hopkins 2007, Hopkins and Rowe 2010). For instance, semi-ecological experiments, such as microcosms and other semi-static experiments are a critical step before manipulating complete natural systems as these are controlled environments allowing ecotoxicologists to better understand the complexity of natural systems (Rowe and Dunson 1994).

Past studies have been performed on concentration-response relationships on vertebrate taxa such as fish (66.7%), mammals (19.9%), birds (8.8%), amphibians (3.8%) and reptiles (0.8%; Sparling et al. 2010). In aquatic systems, fish are considered the keystone taxon for toxicological studies since they are of commercial importance, cover a large range of prey to predator species, and their life histories and development are well known for many species (Braunbeck et al. 1998; Sparling et al. 2010). While understanding the effects of a contaminant on higher consumers within the food web is important (Braunbeck et al. 1998), relatively little is yet known about how lower consumers are affected by contaminants. Furthermore, high concentrations of contaminants are often studied to observe varying degree of toxicity although more subtle physiological or phenotypic effects could occur when exposed to lower concentrations (Eggen et al. 2004). By contrast with fish, relatively little toxicological research has been performed on amphibians and reptiles even though these taxa are considered highly sensitive to anthropogenic pollutants (Gibbons et al. 1997; Sih et al. 2004; Sparling et al. 2010). Amphibian species demonstrate a great variation in responses toward high pollutant levels depending on the methodology used (i.e. *in-situ* or *ex-situ*, Rowe et al. 2003). In fact, most of the toxicological research performed on amphibians has focused on synthetic organic contaminant (i.e. pesticides) in wetland ecosystems in direct contact with agricultural lands (Gilbertson et al. 2003, Relyea and Hoverman 2006, Cothran et al. 2013). Less work has been reported for trace metal effects however trace metals have been described to be both naturally prevalent (Walker et al. 2006) and the result of anthropogenic activity (Leduc et al. 2012) within natural wetland ecosystems. Therefore, more research is needed to clarify the toxicological impacts of

inorganic pollutants (particularly when considering the various forms of a given metal in a complex environmental system and referred to as the chemical speciation) on lower-classed vertebrates that demonstrate lower tolerance and greater susceptibility to these contaminant stressors (Cairns 1992; Rowe et al. 2003).

Amphibians have been declining at an alarming rate making this group one of the most severely threatened vertebrates in the world (Bridges and Semlitsch 2000, Pimms and Raven 2000, Stuart et al. 2004). Amphibians are key wetland species and they are considered important bioindicators of the overall environmental health of aquatic ecosystems (McDiarmid and Mitchell 2000, Kiesecker 2011). For instance, trace metal contamination resulting from industrialization, such as mining and smelting are associated with large-scale declines of Northern Leopard Frogs because of widespread use of trace metal and pesticide contaminants in North America (Gilbertson et al. 2003, Karasov et al. 2005). Tailings wetlands are engineered rehabilitation strategies that are implemented to help degrade and precipitate the smelting waste material, known as tailings, through both aerobic and anaerobic processes (Kalin and van Everdingen 1990, Michelutti and Wiseman 1995). With time and moderate rehabilitation, tailings wetlands can constitute potential breeding sites for several amphibian species (Leduc et al. 2012). While such wetlands are effective at reducing metals from being exported, trace metal activity within the wetlands is largely unknown. Presumably, their concentrations are much higher than in the outflow and thus local amphibian populations encounter a myriad of bioavailable trace metals, such as copper ( $\text{Cu}^{2+}$ ) and nickel ( $\text{Ni}^{2+}$ ) ions, that can yield serious adverse morphological, behavioural, and physiological effects (Nor 1987, Meyer et al. 1999, Handy 2003, Relyea and Hoverman, 2006). Chronic nickel exposures studies have indicated that nickel has been shown to be a limiting stressor in aquatic organisms, such as teleost fish, whereby nickel affected gas exchange processes and respiratory functioning through accumulation in the gills, kidneys and plasma (Eisler 1998, Pane et al. 2003, 2004). For instance, acute and chronic copper exposure studies conducted in artificial media (i.e. dechlorinated, deionized, ground well, or synthetic water) have reported that Northern Leopard Frog, *Lithobates [Rana] pipiens*, tadpoles

exposed to metals at toxic concentrations exhibit elevated mortality, abnormal behaviour and reduced life history traits as copper concentrations increase (Landé and Guttman 1973, Reddick and LaPoint 2004, Chen et al. 2007; Lance et al. 2012). In addition, chronic copper exposures in teleost fish have demonstrated that copper can be severely toxic to aquatic vertebrates by inhibiting deficiencies in metabolism, swimming speeds, reproductive strategies, ionic regulation, immunology, enzymatic activity, nervous system, and respiratory system (Handy 2003, Chen et al. 2007).

The aim of our study is to assess the effects of trace metal effects on species living and breeding in disturbed metal-stressed wetlands near mining areas. In this context, we investigated Cu, Ni and their combined effect on survival and life history traits of Northern Leopard Frog tadpoles in field-collected effluent water which mimics a treated metal-contaminated environment. We predicted that both survival and growth would be negatively affected by an increase in trace metals concentrations (Cu, Ni, and Cu/Ni combined). Moreover, based on available literature we expected anuran larvae to tolerate and survive more efficiently in a nickel rather than copper-exposed medium due to the stronger toxic nature of available copper on the metabolism and homeostasis of aquatic organisms.

## **Material and Methods**

### *The Northern Leopard Frog (*Lithobates [Rana] pipiens*)*

Northern Leopard Frogs have been observed to live and reproduce in metal-contaminated ephemeral wetlands (Burger and Snodgrass 2001, Leduc et al. 2012). In this experiment, two clutches of *L. pipiens* larvae were produced through hormone-induced breeding procedure (e.g. Amphiplex) (Trudeau et al. 2010) in March 2011. The parents of these tadpoles were wild-caught Northern Leopard Frog adults originating from non-contaminated areas near Ottawa, Ontario, Canada (Trudeau et al. 2010). The tadpoles were separated into four 20L aquarium and left to acclimate for 48h before being subjected to the metal treatments.

### *Trace metal medium*

We manipulated copper, nickel, and copper and nickel combined concentrations in a field collected effluent water to explore the variation of dose responses in Northern Leopard Frog tadpoles. This first experiment consisted of twenty six treatments concentrations (8 for copper, 7 for nickel, 8 for Cu/Ni combined and 3 controls with no added metal) in the effluent water). Copper stock solution was prepared using a 3.802g/L solution of  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$  to create eight experimental Cu concentrations (8, 22, 40, 65, 70, 110, 160, and 200 $\mu\text{g/L}$ ). Nickel stock solution was prepared using a 4.955g/L solution of  $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  to create seven experimental Ni concentrations (160, 360, 500, 650, 800, 1000, and 1200 $\mu\text{g/L}$ ). The Cu/Ni combined metal treatment was created by mixing the Cu and Ni concentrations together making eight doses (8/160, 22/360, 40/500, 65/650, 70/1400, 160/800, 200/1000, 250/1200 $\mu\text{g/L}$  for Cu/Ni respectively). The selected concentration ranges for copper and nickel were based from previous toxicological studies performed on anuran species. Each of these solutions was mixed with 20L of tailings effluent water collected from Sudbury Integrated Nickel Operations (Sudbury INO) – A Glencore Company (Falconbridge, Ontario, Canada). Effluent water was collected at the site where the tailing water discharged after it had been limed and polished by flowing through a 150 ha wetland area. The effluent water was chemically monitored weekly and generally met provincial and federal environmental standards before leaving the Sudbury INO property and reaching the Coniston watershed (Table 1; MISA 2011, MMER 2011). We used tailings effluent water from tailings wetlands outflow of Sudbury INO because this water is discharged into a natural aquatic system, where it could directly impact aquatic organisms. Moreover, Northern Leopard Frogs have been observed recolonizing and reproducing in these metal-stressed sites and their interaction with trace metals has been previously questioned (Leduc et al. 2012).

### *Experimental design*

In this study, 12 Northern Leopard Frog tadpoles (Gosner Stages (GS) 23-25, Gosner 1960) were randomly assigned to each one of 130 3L plastic tanks (i.e. 26 dose treatments x 5 replicates) for a total of 1560 tadpoles. The density in each experimental unit was standardized to 250mL of water per tadpole (Karasov et al. 2005) and water volume was regulated throughout the entire experiment as individuals died the appropriate amount of water was controlled for the surviving tadpoles. Every four days, all tanks received a static renewal whereby tadpoles received a water change with their appropriate trace metals mixture and food. Food given was based at a rate of 120mg of dried tadpole food/ tadpole in each tank (Carolina Biological Supply Company, Burlington, NC). Photoperiod and temperature were fixed at 12h:12h (Light:Dark) and 21°C for the duration of the experiment.

### *Monitoring and data collection*

All tanks were monitored daily for dead tadpoles. Each dead tadpole was promptly removed and processed for four life history traits (i.e. length, weight and developmental stage) then individually preserved in plastic vials filled with 70% (v/v) ethanol. Every 4-5 days, all tanks were cleaned and refilled with fresh water (metal-treated effluent water) and the appropriate metal concentration. At the same time, tadpoles were fed dried food (120mg/tadpole; Carolina Biological Supply Company, Burlington, NC). The length of the experiment extended for a total of 70 days at the end of which surviving tadpoles were euthanized by a gradual three-step ethanol-based full submersion procedure (i.e. 20%, 50%, 80% (v/v) ethanol) (Wright and Whitaker 2001). Once euthanized, all tadpoles were processed for life history traits. Body length was measured using VWR electronic calliper ( $\pm 0.005\text{mm}$ ) and body weight was assessed using a Metler Toledo balance ( $\pm 0.001\text{g}$ ). Developmental stage was identified using a dissecting microscope and Gosner's developmental nomenclature (Gosner 1960). Furthermore, the lethal concentration (LC, the dose response of a specific metal on a target population) was calculated as the metal concentration that affects a desired percentage of a study population (e.g.  $\text{LC}_{40} = 65 \mu\text{g/L}$  indicates

that 40% of the population will die when exposed to a concentration of 65 µg/L). All experimental procedures followed protocol #2011-01-01 approved by the Laurentian University Animal Care Committee

### *Statistical analyses*

We performed statistical analyses in *R* statistical computing environment (version 2.13.0, The *R* Foundation for Statistical Computing, 2011) with the *Agricolae*, *Nlme*, *Survival* packages for general linear model and non-parametric analyses. We computed a factorial model where the type of trace metal was a fixed factor, and survival, length, weight and developmental stage associated growth rates were assigned as dependant variables. Metal concentrations were considered nested factors because the concentration range were different within each metal. For each individual, we estimated the growth rates for the three life-history traits measured by computing:  $y = ((x-\bar{x})/t)$ ; where  $y$  is the growth rate,  $x$  is the trait measured (length, weight or developmental stage),  $\bar{x}$  is the trait mean across all samples and  $t$  is the time before death. Growth rate estimators were used in place of the actual measure of life-history traits in order to correct for the differences between the time periods for each treatment. To test whether the metal concentrations differed from the control, we first performed a GLM followed by an *a posteriori* Tukey HSD test on length, weight, and developmental stage growth rates. Finally, we analyzed tadpole survival using a survival analysis for each trace metal concentration ranges and water medium comparisons based on a Kaplan-Meier Survival Distribution. Within the survival analyses, surviving tadpoles were classified as censored to explain the lack of information about their time of death while tadpoles that died during the experiment are registered as complete (Leung et al. 1997).

## **Results**

### *Survival*

In the Cu treatment, survival was not linearly related to metal concentrations but appeared to be actually enhanced at low concentrations of exposure (Figure 4A). Survival was significantly different among the



eight Cu<sup>2+</sup> concentrations ( $\chi^2=282.7$ ,  $P<0.001$ ). The Cu concentrations could be separated into three categories with the lower concentrations associated with significantly elevated survival as compared to the control treatment (8µg/L = 100%,  $Z_{8\mu\text{g/L}}=-7.36$ ,  $P<0.001$ ; 22µg/L = 100%,  $Z_{22\mu\text{g/L}}=-7.48$ ,  $P<0.001$ ; and 40µg/L = 100%,  $Z_{40\mu\text{g/L}}=-7.62$ ,  $P<0.001$ ; Control = 86.7%). For the intermediate dose, similar survival patterns to the control treatment were observed (65µg/L = 73.3%,  $Z_{65\mu\text{g/L}}=-0.64$ ,  $P=0.52$ ; 70µg/L = 60%,  $Z_{70\mu\text{g/L}}=-1.51$ ,  $P=0.13$ ; and 110µg/L = 58.3%,  $Z_{110\mu\text{g/L}}=-0.94$ ,  $P=0.34$ ). For the higher concentrations, the tadpoles displayed significantly lower survival rates than the control (160µg/L = 0%,  $Z_{160\mu\text{g/L}}=9.48$ ,  $P<0.001$ ; 200µg/L = 0%,  $Z_{200\mu\text{g/L}}=8.85$ ,  $P<0.001$ ). Therefore, the lethal concentrations for Cu were: LC<sub>30</sub>=65 µg/L, LC<sub>40</sub>=110 µg/L and LC<sub>100</sub>=160 µg/L (Figure 4A).

In the Ni treatment, tadpoles displayed a significantly increasing mortality as the concentrations increased (Figure 4B). Survival analyses indicated significant differences between the seven Ni<sup>2+</sup> concentrations ( $\chi^2=281.8$ ,  $P<0.001$ ). The lower Ni concentration ranges demonstrated significantly elevated survival patterns as compared to the control (160 µg/L = 100%,  $Z_{160\mu\text{g/L}}=-6.32$ ,  $P<0.001$ ; 360 µg/L = 98.3%,  $Z_{360\mu\text{g/L}}=-6.87$ ,  $P<0.001$ ; 500 µg/L = 95%,  $Z_{500\mu\text{g/L}}=-5.79$ ,  $P<0.001$ ; Control =96.7%). Meanwhile, the intermediate dose (650 µg/L) and higher doses displayed significantly lower survival rates than the control treatment (650 µg/L = 16.7%,  $Z_{650\mu\text{g/L}}=8.05$ ,  $P<0.001$ ; 800 µg/L = 21.7%,  $Z_{800\mu\text{g/L}}=5.90$ ,  $P<0.001$ ; 1000 µg/L = 8.3%,  $Z_{1000\mu\text{g/L}}=8.60$ ,  $P<0.001$ ; 1200 µg/L = 10%,  $Z_{1200\mu\text{g/L}}=8.95$ ,  $P<0.001$ ). Therefore, the lethal concentrations for Ni were: LC<sub>40</sub>=650 µg/L, LC<sub>50</sub>=800 µg/L; no Ni concentration reached 100% mortality although the 1200 µg/L dose affected over 93% of the study sample (Figure 4B).

In the combined copper and nickel (Cu/Ni) treatment, tadpoles exhibited significant mortality effects similar to those demonstrated by the Cu treatment doses (Figure 4C). Survival analyses indicated significant differences between the eight combined Cu<sup>2+</sup>/Ni<sup>2+</sup> concentrations ( $\chi^2=410.2$ ,  $P<0.001$ ). The lower combined concentrations demonstrated significantly elevated survival patterns as compared to the

control (8/160  $\mu\text{g/L}$  = 100%,  $Z_{8/160\mu\text{g/L}}=-7.69$ ,  $P<0.001$ ; 22/360  $\mu\text{g/L}$  = 100%,  $Z_{22/360\mu\text{g/L}}=-8.89$ ,  $p<0.001$ ; 40/500  $\mu\text{g/L}$  = 100%,  $Z_{40/500\mu\text{g/L}}=-9.62$ ,  $P<0.001$ ; Control = 91.7%). Meanwhile, the intermediate dose (65/650  $\mu\text{g/L}$ ) and higher doses displayed significantly lower survival rates than the control treatment (65/650  $\mu\text{g/L}$  = 33.3%,  $Z_{65/650\mu\text{g/L}}=8.00$ ,  $P<0.001$ ; 70/1400  $\mu\text{g/L}$  = 35.5%,  $Z_{70/1400\mu\text{g/L}}=3.04$ ,  $P<0.001$ ; 160/800  $\mu\text{g/L}$  = 3.3%,  $Z_{160/800\mu\text{g/L}}=3.04$ ,  $P<0.001$ ; 200/1000  $\mu\text{g/L}$  = 0%,  $Z_{200/1000\mu\text{g/L}}=9.87$ ,  $P<0.001$ ; 250/1200  $\mu\text{g/L}$  = 0%,  $Z_{250/1200\mu\text{g/L}}=10.51$ ,  $P<0.001$ ). In fact, as the Cu concentrations in the combined treatment surpass 160  $\mu\text{g/L}$ , the combined trace metal mixture exhibited similar lethal characteristics as those presented in the Cu treatment alone. Therefore, the lethal concentrations for Cu/Ni combined were:  $\text{LC}_{25}=65/650$   $\mu\text{g/L}$  and  $\text{LC}_{100}=160/800$   $\mu\text{g/L}$  (Figure 4C).

### *Growth rates*

In the Cu treatment, we detected significant differences in growth rates based on the linear growth rate of length (LGR) ( $F_{(8,549)}=59.14$ ,  $P<0.001$ ), weight (WGR) ( $F_{(8,549)}=29.93$ ,  $P<0.001$ ) and developmental stage (DSGR) ( $F_{(8,549)}=42.96$ ,  $p<0.001$ ) as the Cu concentration increased. Tadpoles in intermediate (65-110  $\mu\text{g/L}$ ) and higher (160-200  $\mu\text{g/L}$ ) Cu concentrations showed significant increase in LGR as the trace metal concentrations increased (*a posteriori* Tukey test  $\text{HSD}=0.179$ ,  $P<0.05$ ; Figure 5 A-1). Additionally, Leopard Frog larvae in the intermediate ranges (65-110  $\mu\text{g/L}$ ) exhibited the highest significant increases in WGR and those in the higher concentrations (160-200  $\mu\text{g/L}$ ) doses also displayed significantly elevated WGR as compared to those in the control treatment (*a posteriori* Tukey test  $\text{HSD}=0.007$ ,  $P<0.05$ ; Figure 5 A-2). Furthermore, *L. pipiens* tadpoles exhibited significantly elevated DSGR ( $\text{HSD}=0.024$ ,  $P<0.05$ ) in 6 out of the 8 copper concentrations as compared to the control treatment, suggesting that tadpoles subjected to elevated Cu concentrations were developing significantly faster than those in a no-Cu treatment (Figure 5 A-3).

In the Ni treatment, we observed significant differences in growth rates based on LGR ( $F_{(7,416)}=5.53$ ;  $P<0.001$ ), WGR ( $F_{(7,416)}=8.05$ ;  $P<0.001$ ) and DSGR ( $F_{(7,416)}=25.75$ ;  $P<0.001$ ) as the Ni concentration increased. Based on *a posteriori* Tukey tests for LGR (HSD= 0.068) and WGR (HSD= 0.002), we found significant differences in life history traits as the nickel ranges increases in concentration. Leopard Frog tadpoles in the intermediate (650  $\mu\text{g/L}$ ) and higher (800-1200  $\mu\text{g/L}$ ) nickel concentrations had significantly lower LGR (*a posteriori* Tukey test HSD=0.068,  $P<0.05$ ) while those in the lower (160-500  $\mu\text{g/L}$ ) doses displayed no comparable difference than the control (Figure 2 B-1). Additionally, tadpoles in mostly all nickel concentrations (160-1200 $\mu\text{g/L}$ ) displayed significantly lower WGR than the control group (*a posteriori* Tukey test HSD=0.002,  $P<0.05$ ) and the WGR decreased as the Ni concentration increased (Figure 5 B-2). Furthermore, *L. pipiens* tadpoles displayed significantly lower DSGR (HSD=0.018,  $P<0.05$ ) in 5 out of the 7 the Ni concentrations as compared to the control treatment while the tadpoles in the lower concentrations (360 and 500  $\mu\text{g/L}$ ) presented higher DSGR at the time of death than tadpoles in the control treatment (Figure 5 B-3).

In the Cu/Ni combined treatment, we found significant differences in growth rates based on LGR ( $F_{(8,479)}=27.34$ ,  $P<0.001$ ), WGR ( $F_{(8,479)}=22.21$ ,  $P<0.001$ ) and DSGR ( $F_{(8,479)}=5.72$ ,  $P<0.001$ ). Leopard frog tadpoles in the higher (160/800-250/1200  $\mu\text{g/L}$ ) concentration displayed significantly elevated LGR while those in the lower (8/160-40/500  $\mu\text{g/L}$ ) and intermediate (65/650-70/1400  $\mu\text{g/L}$ ) ranges had no significant differences (*a posteriori* Tukey test HSD=0.272,  $P<0.05$ ) (Figure 5 C-1). Similarly, the tadpoles in higher combined ranges (70/1400-200/1200  $\mu\text{g/L}$ ) had significantly elevated WGR and tadpoles in the lower ranges (8/160-65-650  $\mu\text{g/L}$ ) had significantly lower WGR as compared to tadpoles in the control treatment (*a posteriori* Tukey test HSD=0.003,  $P<0.05$ ; Figure 5 C-2). Finally, *L. pipiens* larvae in 3 out of the 8 Cu/Ni combined concentrations exhibited significantly higher DSGR (HSD=0.034,  $P<0.05$ ) while the remaining 5 out of the 8 concentrations displayed no significant differences compared to the control (Figure 5 C-3).

## Discussion

Overall, our results indicate that anuran larvae can be highly sensitive to inorganic trace metals (e.g. copper and nickel) that are present in wetland ecosystems. Our study also reveals a significant variation in concentration-responses by Northern Leopard Frog tadpoles within our field collected medium, resulting in metal-specific responses in terms of growth and survival. The effects of copper were only observed after a specific concentration threshold (65  $\mu\text{g/L}$ ) while the impact of nickel increased linearly with the concentration. In addition, our multi-factorial design supports that *L. pipiens* larvae tolerate and react differently to the stress imposed by increased copper and nickel trace metals within their aquatic environment.

### *Phenotypic response to trace metals*

Specific survival and growth responses to trace metals support the prediction that Cu imposes a stronger adverse effect on tadpoles as compared to Ni with a 7-fold difference in  $\text{LC}_{100}$  (1200  $\mu\text{g/L}$  vs. 160  $\mu\text{g/L}$  for Ni and Cu respectively). Although uncommonly tested in lower vertebrates (Eisler 1998, Pane et al. 2003, ADSTR 2010), the Ni  $\text{LC}_{100}$  was expected to be much larger than that of Cu as revealed by a study on *Bufo melanostictus* tadpoles withstanding a Ni concentration of 25300  $\mu\text{g/L}$  (Khengarot and Ray 1987). Our results demonstrate that Northern Leopard Frog larvae can withstand relatively high concentrations of Ni but may not be as tolerant as other species such as *Bufo*. Moreover, tadpoles within the field collected medium displayed significant decreases in growth rate which is consistent with other anuran toxicological studies (i.e. *Xenopus laevis*, Hopfer 1991). By contrast, *L. pipiens* tadpoles demonstrated significant increases in growth rate when exposed to Cu; whereas in previous toxicological copper studies, *L. pipiens* tadpoles have displayed significant reduction in growth rate as Cu concentration increased to 160  $\mu\text{g/L}$  (Landé and Guttman 1973, Chen et al. 2007, Lance et al. 2012). Additionally, the response to the combined Cu/Ni treatment was similar to the trend observed by the Cu treatment

suggesting that the latter is the driving metal stressor in this binary mixture (Tomasik et al. 1995, Xue et al. 2010).

Three hypotheses could explain such positive and negative changes in growth rate within different trace metal-stressed environment. First, anuran larvae undergo an adaptive phenotypic trade-off or phenotypic response to deal with environmental stressors (e.g. temperature (Merilä et al. 2004), contaminants (Jansen et al. 2011), pH (Räsänen et al. 2003), drought (Denver 1997) and disease (Kerby et al. 2011) and these adaptive plastic changes come at a cost by inducing a fitness trade-off between traits (Stearns 1989). Our study suggests that tadpoles assign excessive energy supplies into metabolic functioning to increase growth and development when exposed to a stressful larval environment (Werner et al. 1993, Kerby et al. 2011). Pollutants can generate highly variable phenotypic plastic responses to exposed organisms (Kammenga and Risken 1996). In most metal-exposure studies incorporating standardized experimental conditions, anuran larvae develop an adaptive stress tolerance to trace metals which enabled them to cope with the metal but consequently results in decreased body size (Landé and Guttman 1973, Reddick and LaPoint 2004, Chen et al. 2007; Lance et al. 2012). While this phenotypic trade-off appeared to occur in the Ni treatment, tadpoles in the Cu-stressed treatments presented higher growth rates as compared to their control counterparts. The introduction of selective toxic chemical pressure may elicit different responses for both trace metals. When trace metals are detrimental to an organism, the stress signal can induce physiological reactions to tolerate such stressor (DeWitt et al. 1998, Moberg 2000). For anurans and other vertebrate species, Ni exposure is likely to be subtle yet long-lasting under a chronic exposure where bioaccumulation occurs within the organism (Eisler 1998). The slower growth rate observed in the nickel treatment may suggest a trade-off between growth and the resistance. By contrast, Cu-stressed tadpoles achieved further developmental stages suggesting that when the exposure to a metal stress is severe enough, it may trigger a plastic response allowing the organism to further escape the stressful environment (DeWitt et al. 1998, Moberg 2000, Kerby et al. 2011).

Second, a hormetic dose response may explain the increased growth rate observed in the Cu treatment. In theory, the intended hormetic response to a subtle increase in metal concentration is an inverted U-shaped growth response curve where low and moderate trace metal concentrations would be considered beneficial to the organism and display an increase in growth; while higher concentrations would be presented as stressful and lead to decreases in body size (Chapman 2001, Calabrese and Baldwin 2002, Calabrese 2005). On one hand, bioaccumulation of inorganic trace metals within the body can cause severe physiological damage as increased Cu uptake leads to morphological, behavioural, immunological, endocrinal, respiratory, and olfactory system disruptions (Green et al. 2010, Handy 2003, Meyer et al. 1999); meanwhile, elevated Ni exposure results in cardiovascular, pulmonary and renal disorders (ATSDR 1988, Pane et al. 2003, Eisler 1998). On the other hand, trace metal absorption at low/moderate exposures can be beneficial to functional metabolic processes whereby an organism will overcompensate for a sudden change in its homeostasis (Chapman 2001). In fact, trace amounts of Cu are biologically essential for vertebrates, and used for optimal activations of cellular aerobic respiration protein, cytochrome c oxidase (Hoshi et al. 1993) and metabolic metal-binding proteins (Opazo et al. 2003). In small concentrations, Ni is also an important macronutrient for optimal specific enzymatic functioning within living organisms (Thauer 2001).

Third, the mechanisms by which Cu and Ni enter and persist within the body are different and could result in the physiological differences observed between the two treatments: accelerated growth and development for tadpoles exposed to Cu as opposed to a decrease in survival and growth for tadpoles exposed to Ni. Free Cu can be absorbed into the body through inhalation and absorption within the gastrointestinal tract where it can compete with sodium in Na/K pumps by binding to proteins such as ceruloplasmin, albumin, transthyretin and copper-amino acid complexes before being stored in either the liver or kidney (Laurie and Pratt 1986, Margerison and Mann 2005). Similarly, Ni ions also enter the body through both inhalation and absorption; but while it accumulates primarily in the lungs and kidneys, it is poorly absorbed through the gastrointestinal tract and a large portion is excreted through urine or

feces (ATSDR 1988). This ability for an organism to be able to uptake and metabolize Cu more readily than Ni suggests that Cu may have a stronger physiological effect than Ni. Other trace metals have also produced changes in tadpole life-history. Carew and Helbing (Carew 2013) found that chronic sublethal nanosilver (nAg) exposure inhibited accelerated metamorphic timing in premetamorphic *Rana catesbeiana* larvae. The authors concluded that chronic nAg exposure was found to act directly on the thyroid hormone and subsequently stimulate the production of thyroid hormone (TH) which is a precursor to metamorphosis in premetamorphic amphibian larvae (Hinter et al. 2010). In this case, increased TH production induced faster metamorphosis in bullfrog tadpoles when exposed to sublethal nAg concentrations. In our study, Cu exposure may act similarly to nAg on the thyroid in turn increasing growth and development in *L. pipiens* tadpoles.

Finally, the fluctuating differences in larval development and growth in the Cu-stressed treatments in our study may be the result of larval developmental stages at the time of exposure and food availability. Previous exposure studies using Cu have usually used younger tadpoles (GS19, Landé and Guttman 1973; GS19, Chen et al. 2007) than those in our study (GS23-24), suggesting that the immune or tolerance response of tadpoles used in our experiment was possibly more developed than in other studies. Moreover, tadpoles are usually fed during long-term exposure experiments (Landé and Guttman 1973, Chen et al. 2007, Lance et al. 2012). Therefore, although it was standardized for the number of tadpoles in each aquarium, the provision of food in our experiment may have resulted in magnifying the phenotypic plasticity observed in *L. pipiens* growth rate.

### *The importance of water media in ecotoxicology*

The definition of ecotoxicology implies the study of specific environmental pollutant interaction and the homeostatic response of the residing organism within the entire functional ecosystem (Truhaut 1969, Moriarty 1988, Eggen et al. 2004). Although an artificial medium allows for controlling any undesired effect stemming from a natural system, there are no other relevant ecological findings that can be interpreted from this type of study except for the direct toxicological effect of the manipulated trace metal (Low and Braude 2010). In order to gain a better understanding of trace metal contaminant impacts in ecological settings, the observed response differences by *L. pipiens* support the ecological importance of using field-collected water in experimental-based research. Furthermore it allows ecotoxicologists the opportunity for a better understanding of the life history responses (i.e. survival and phenotypic) *L. pipiens* tadpoles produced in response to increases in copper, nickel and combined concentrations. In effluent water, the concentration range used for Cu (0-160 µg/L) was comparable to other toxicological studies that have reported concentration-response ranges of up to 150 µg/L in dechlorinated water (Landé and Guttman 1973, Redick and LaPoint 2004)) and 100 µg/L in both slightly ionic (Chen et al. 2007) and synthetic water (Lance et al. 2012). Although different artificial media have been used in previous toxicological experiments, the field collected medium (effluent water) manipulated in our experiment contained physicochemical properties which could benefit tadpole growth and survivorship.

Trace metal bioavailability and behaviour are key roles in natural systems but the extent of their effects is highly dependent on their interaction with the physicochemical properties of the water (Engel et al. 1981; Table 2). First, water hardness is associated with the stress tolerance of aquatic organisms as it influences not only the toxicity of trace metals in the water (Khangarot and Ray 1987) but biological functioning of organisms which utilize the base cations (eg. Ca and Mg) associated with water hardness (Wurts and Durborow 1992). Many inorganic trace metals (i.e. zinc, copper and cadmium) are observed to be more toxic in soft water (<60 mg/L) rather than hard water (>120 mg/L) (Playle et al. 1992, Ebrahimpour et al. 2010) because in soft water there are less ions which leads to a deficiency in



ionoregulation, which is a disruption in performance of ion exchange at a cellular level because of competing toxic inorganic ions (McDonald and Rogano 1986). In addition, productive wetlands, in which amphibians and small fish species reside, are moderately stagnant bodies of water that can have poorly mineralization and low hardness levels ( $< 20 \text{ mg CaCO}_3/\text{L}$ ) although total hardness of wetlands can greatly fluctuate (Wurts and Masser 2004). In our study, the effluent water had a hardness of  $406.81 \pm 30.65 \text{ mg CaCO}_3/\text{L}$  representing a much harder medium due to the liming and polishing processes it undertakes before being reintroduced to the natural system. Environmental calcium is essential for bone formation, muscle contraction, but also for osmoregulation (Wurts and Masser 2004). Thus, the effluent water may have potentially alleviated the physiological stress imposed by the added copper ions.

Furthermore, dissolved organic carbon (DOC) is important in determining the bioavailability of free metal ions within aqueous mediums. Due to the high affinity of DOC for binding, it rapidly complexes with free ions, thus enhancing metal solubility and transport in cell membranes, but reduces the potential for trace metal uptake by organisms as it competes with other free metal ions to biotic binding sites (Playle et al. 1993, Doig and Liber 2007). In fact, free trace metals ions generally display their greatest toxicity potential under low DOC concentrations. Since we used a field-collected medium, DOC likely had a reducing effect on the bioavailability of both Cu and Ni (Playle et al. 1993, Doig and Liber 2006, 2007), especially within the low concentration range. Therefore, although there may be confounding variables with the use of a field-collected medium, researchers can make stronger ecological inferences and allow for greater understanding of high fluxes of a particular trace metals. In natural systems exposed to anthropogenic contamination, anuran communities may be experiencing elevated metal stress thresholds resulting in lower fitness levels than their counterparts that are living in less undisturbed ecosystems (Linder et al. 2010).

## Conclusion

Copper and nickel are valuable precious metals that are released into the natural environment by anthropogenic activities and can have devastating effects on aquatic communities. For both metal type and regardless of the medium used, exposure to high concentrations led to detrimental effects on the physiology and survival of Northern Leopard Frog tadpoles. For copper, tadpoles were rapidly affected by low concentrations; while for nickel, tadpoles could withstand greater concentration ranges before being overwhelmed by the toxicity. Our study also revealed that tadpoles exposed to copper showed increases in growth and development while those exposed to nickel displayed reduced growth and development over time as compared to the control. Therefore, depending on the trace metals, *L. pipiens* larvae were potentially displaying phenotypic trade-offs to either: 1) hasten toward metamorphosis by increasing their growth and developmental response to copper or 2) increase their metal-stress tolerance to nickel by decreasing their growth response. Finally, our results support that increases in copper and nickel concentrations can promote significant changes in fitness responses (i.e. survival and life history) and provide insights on the adaptability of anurans in wetland ecosystems disturbed by anthropogenic sources. In fact, investigating the synergistic functionalities imposed by multiple stressors is becoming increasingly relevant because the current loss of biodiversity is evidently not explained by one factor but by many.

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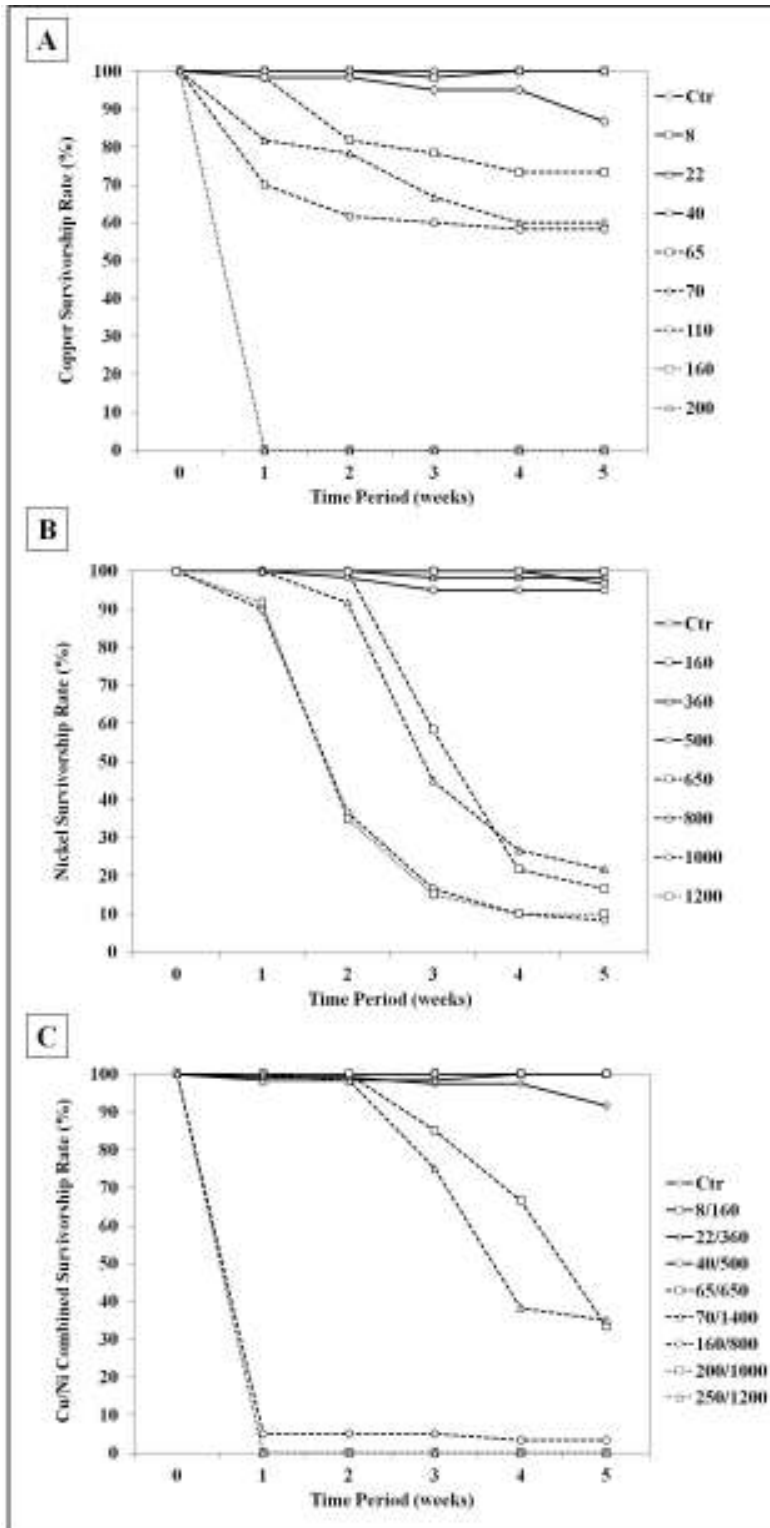
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**Table 1:** Water chemistry from field collected effluent water collected between March 01, 2011 and March 30, 2012. The effluent water collected from Sudbury INO, Falconbridge, Ontario, Canada, is controlled by MMER (Federal) and MISA (Provincial) regulations. Effluent water was used in a semi-static experiment to study the effects of copper, nickel and copper/nickel combined treatments on Northern Leopard Frog (*Lithobates pipiens*) tadpoles in a metal-stressed aquatic environment. The mean and S.E. of all measured trace metal elements are quantified in  $\mu\text{g/L}$ .

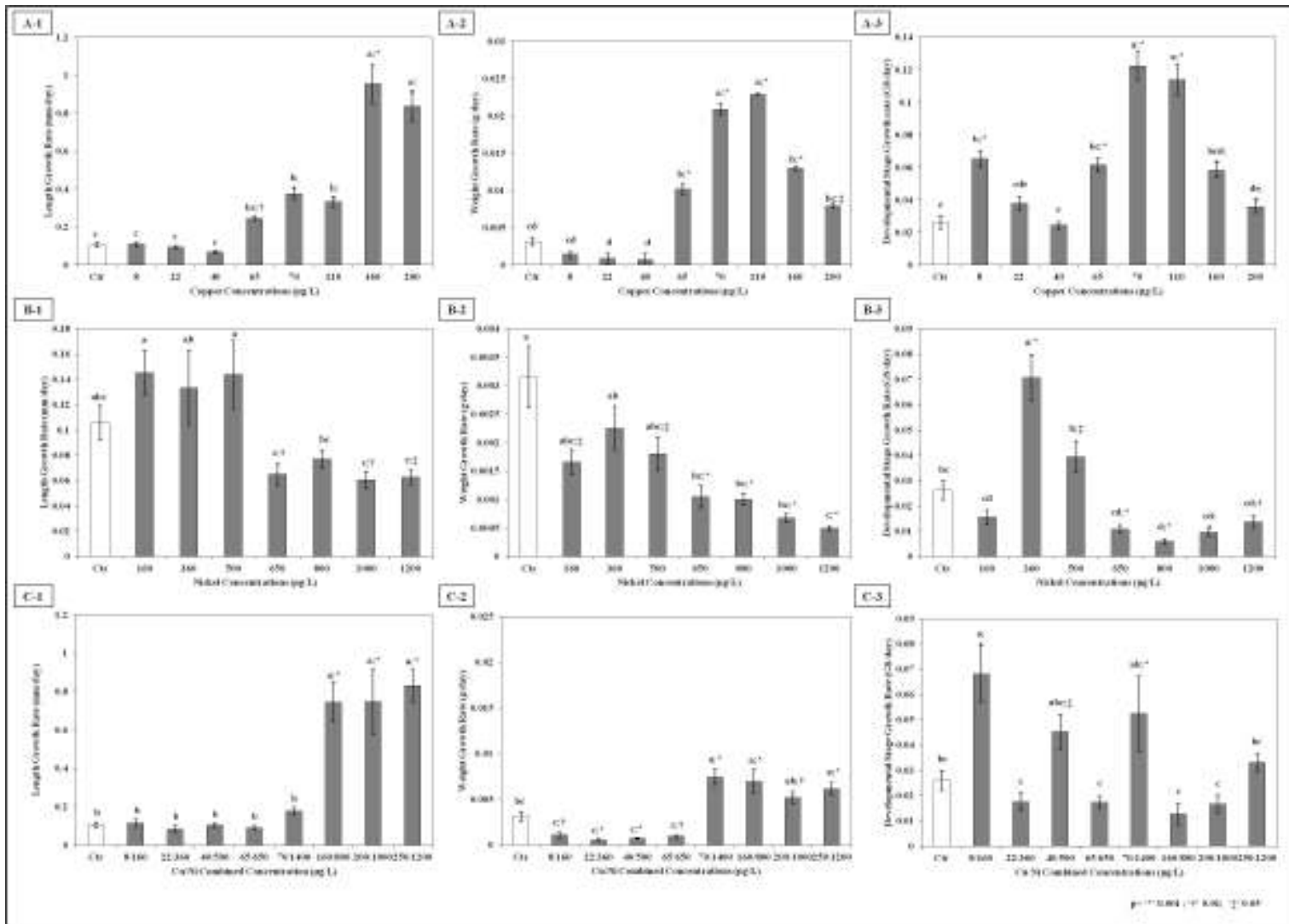
<b>Trace Metal Element</b>	<b>Concentration (<math>\mu\text{g/L}</math>)</b>
Cu	$6.4 \pm 2.4$
Ni	$87.6 \pm 5.4$
Al	$17.8 \pm 2.1$
As	$5.2 \pm 1.9$
Co	$3.7 \pm 1.9$
Cd	$0.3 \pm 8.4$
Cr	$1.9 \pm 1.9$
Ca	$182100 \pm 320$
Fe	$811.0 \pm 222.6$
Hg	$0.01 \pm 0.0$
Ld	$0.9 \pm 1.8$
Mg	$11840.0 \pm 1007.8$
Zn	$6.7 \pm 2.8$
pH	$7.1 \pm 0.2$
Hardness (mg $\text{CaCO}_3/\text{L}$ )	$502.0 \pm 3.5$

**Table 2:** Water chemistry for a water change period (4 days) for field collected effluent water media with added copper [eH<sub>2</sub>O], which was used in a semi-static experiment to study the effects of copper exposure on Northern Leopard Frog (*Lithobates pipiens*) tadpoles. The effluent water collected from Sudbury INO, Falconbridge, Ontario, Canada, is controlled by MMER (Federal) and MISA (Provincial) regulations. The concentration means and standard deviations of all measured trace metal elements are quantified in µg/L.

Water Type	Day	Cu	Ni	Fe	Pb	Ca	Mg	Conductivity (S/m)	NH3-N	pH	Hardness (mgCaCO <sub>3</sub> /L)
eH <sub>2</sub> O	1	74.5 ± 2.7	60.7 ± 8.0	19.7 ± 8.9	15 ± 8.2	149000 ± 10825	8440 ± 882	1.10 ± 0.08	200.0 ± 0.0	7.0 ± 0.1	406.8 ± 30.6
	2	72.8 ± 6.1	63.8 ± 5.4	36.2 ± 22.5	15.8 ± 5.3	155833 ± 15613	8576 ± 605	1.14 ± 0.08	200.0 ± 0.0	7.2 ± 0.2	424.4 ± 41.3
	3	72.0 ± 6.2	65.5 ± 0.6	31.2 ± 29.8	9.0 ± 9.1	151500 ± 8962	8587 ± 469	1.11 ± 0.08	200.0 ± 0.0	7.1 ± 0.1	413.7 ± 24.4
	4	82.8 ± 14.34	64.0 ± 2.2	63.2 ± 22.2	7.5 ± 7.6	151250 ± 6551	8637 ± 495	1.11 ± 0.02	200.0 ± 0.0	7.2 ± 0.3	413.2 ± 18.4



**Figure 4:** *Lithobates pipiens* larvae survivorship when exposed to metal-treated effluent water treatments and the control group (no added Cu). Tadpoles were exposed to one of two trace metal treatments: A) 8 copper concentrations, B) 7 nickel concentrations, and C) 8 Cu/Ni combined concentrations. Survivorship (%) is determined by the decrease in survivors through the 5 week experiment.



**Figure 5:** Northern Leopard Frog (*Lithobates pipiens*) larvae phenotypic response when exposed to metal-treated effluent water treatments and the control group (no added copper). Tadpole growth rate was estimated with three life-history traits: body length (mm/day; A1, B1, C1), body weight (g/day; A2, B2, C2) and developmental stage (Gosner Stage/day; A3, B3, C3). P-values (\* 0.001; † 0.01; ‡ 0.05) are from a multivariate analysis for their interactions ( $P < 0.05$ ).

## CHAPTER 2

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### **Life History Trade-offs Induced by Copper and Temperature in the *Lithobates pipiens* - *Ranavirus* System.**

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**Abstract**— Emerging infectious diseases, pollution and climate change are directly or indirectly associated with amphibian extinctions worldwide. However, identifying causation is often obscured by the interplay of environmental and anthropogenic stressors. In this context, understanding interactions between evolutionary, ecological and epidemiological determinants of disease is critical to improve our knowledge of host-pathogen co-evolution. Due to the increased effects of climate change and anthropogenic disturbances in northern landscapes, pathogens, whose replication rate is often enhanced in warmer conditions, are increasing their range of occurrence, migrating northward and threatening many aquatic vertebrate communities. We designed a factorial experiment where Northern Leopard Frog (*Lithobates [Rana] pipiens*) tadpoles were subjected to a combination of three experimental conditions: (1) a toxic copper-spiked medium, equal to an LC<sub>40</sub>; (2) an acute exposure to the ranavirus pathogen; and (3) two temperature regimes (14.0 and 20.2°C) to assess the interactive effects of metals, pathogen exposure and temperature. We hypothesized that trace metal and pathogen stressors when combined will induce a trade-off in *L. pipiens* where an increase in development and growth will reduce the time to metamorphosis but severely affect survival. We further predicted that temperature would have a direct impact on this trade-off with more significant differences in a warmer environment. In the presence of ranavirus only, infected hosts displayed increased growth and development at both 14.0°C and 20.2°C temperatures as compared to the non-infected tadpoles. Additionally, when exposed to copper ions at 14.0°C, tadpoles had smaller body sizes and experienced reduced developmental rates as compared to the control tadpoles; whereas at 20.2°C, copper exposed tadpoles had an increase growth with no difference in development. Our results also showed that host and pathogen responses are temperature-dependent with lower FV3 virulence at 20.2°C than 14.0°C suggesting differential trade-offs between the host and pathogen fitness with regards to temperature. Finally, rather than additive effects, we observed antagonistic effect of the stressors whereby tadpoles exposed to both the pathogen and the metal combined grew and developed at a similar rate to the control tadpoles. Given these observed life history trade-offs induced by copper and ranavirus, we suggest that long term exposures to trace metal pollutants should be considered, especially in the context of infectious diseases.

**Keywords** Host-Pathogen Interaction, Northern Leopard Frog, Life-History Traits, Copper, Ranavirus

## Introduction

Host-parasite systems display high sensitivity to changes in environmental factors (Woodhams et al. 2008), which can significantly alter the evolutionary trajectories within host and/or parasite, thus changing the dynamic nature of the co-adaptive arms race between hosts and parasites, and resulting in an environment-dependent genotypic interaction (Thomas and Blanford 2003, Lazzaro and Little 2009, Wolinska and King 2009). In nature, organisms experience environmental variability through both biotic and abiotic stresses such as temperature, predation, competition, disease, and chemical contamination among others (Lafferty and Kuris 1999). Phenotypic plasticity is an adaptive response to selective environmental pressures whereby various phenotypes coexist, with different fitness potentials (Schoeppner and Relyea 2009). Recent empirical research has stressed that temperature-dependency is a major environmental factor impacting the genetic, physiological and behavioural processes of both hosts and/or parasites (Lazzaro and Little 2009, Wolinska and King 2009). For instance in the chestnut tree, *Castanea* spp, temperature differences directly affect the immunology, growth and tolerance to infection by multiple strains of *Cryphonectria parasitica* hypovirus-1 (Bryner and Rigling 2011).

Host-parasite interactions driven by environmental influences are most commonly studied in plants (Bryner and Rigling 2011) and invertebrates (Fellowes et al. 1999, Vale and Little 2009) because such taxa are often important dispersal vectors for vertebrate diseases (Elliot et al. 2003). However, amphibians can also be considered as important models for studying the environmental dependencies of host-pathogen systems in light of the worldwide declines of amphibian populations (Blaustein et al. 2011; Hof et al. 2011) where emerging infectious diseases, such as chytridiomycosis (*Batrachochytrium dendrobatidis*; Daszak et al. 2003, Briggs et al. 2010) and ranavirus (Robert 2010; Lesbarrères et al. 2012), appear to play important roles in biodiversity loss. Ranaviruses (*Iridoviridae*) are considered one of the five genera that targets ectothermic vertebrates such as amphibians (Daszak et al. 1999, Kerby et al. 2011), reptiles (Chen et al. 1999, Johnson et al. 2007, Jancovich et al. 2010) and osteichthyan fish (Mao et al. 1997, Whittington et al. 2010). The first ranavirus isolate was detected in *Lithobates [Rana] pipiens*



populations during the 1960's (Granoff et al. 1965) and research has since focused on two major strains, *Frog Virus 3* (FV3) and *Ambistoma tigrinum Virus* (ATV) infecting anurans and salamanders respectively (Chinchar 2002, Lesbarrères et al. 2012). Although vertical transmission of the virus has been suggested as a possible infection pathway (Duffus et al. 2008; Gray et al. 2009), the most definitive exposure route for ranaviruses are through horizontal transmission such as direct contact with infected individuals (Shock et al. 2008), cannibalism of infected individuals (Harp and Petranka 2006) or contact with infected aquatic environment (Jancovich et al. 2001). Amphibian larvae are deemed to have relatively low pathogen resistance capabilities because at his life stage they have rudimentary immune systems due to the high metabolic cost of growth and development (Rollins-Smith 1998). In fact, ranavirus infections most often occur at early larval to pre-metamorphic developmental stages when anurans appear to be most vulnerable (Echaubard et al. 2010). Within just a few hours after infection occurs, FV3 replication begins at 10°C and the replication rate is most prolific between a temperature range of 20-28°C (Chinchar 2002, Rojas et al. 2005, Ariel et al. 2009) suggesting that the amphibian-ranavirus interaction is sensitive to temperature changes.

In northern landscapes, emerging infectious diseases are expected to migrate northward (Harvell et al. 2002, Ostfeld 2009) as anthropogenic disturbance and climate change increase (St-Amour et al. 2008, Woodhams et al. 2008, Rohr et al. 2011). The ability of emerging infectious diseases to infect host populations is contingent upon the host immune response, which in turn is altered by environmental conditions (Garner et al. 2011). In amphibians, several physiological defense mechanisms exist (e.g. resistance, tolerance or avoidance strategies) depending on the type of environmental stressors present within the aquatic ecosystem (Parris and Baud 2004, Voyles et al. 2009, Warne et al. 2011). While the literature addressing amphibian population declines most often reports potential causes (e.g. climate change, pollution, habitat destruction, diseases) separately, there is much interest in studying the potential synergistic interaction of multiple factors (Lips et al 2008, Wake and Vredenburg 2008, Lesbarrères et al 2012, Reeve et al 2013). For instance, little research has been performed on the combined effects of

disease and pollutants. Kerby et al. (2011) studied the interaction of pesticides, predation and ranavirus on larval tiger salamanders (*Ambystoma tigrinum*) and found that all three stressors separately reduced growth and development; however, no clear interaction was observed when the stresses were combined. Similar to pesticides, inorganic trace metals can have unpredictable effects on organisms in nature (Kerby et al. 2010). In high concentrations, the bioavailable forms of trace metals can impose severe physiological and survival effects on amphibians (Lefcort et al. 1998, Lance et al. 2012). Conversely, in low concentrations, trace metals are often considered essential and beneficial to vertebrate organisms, as they activate and complete metabolic processes including enzymatic and protein functioning (Hoshi et al. 1993, Thauer, 2001, Opazo et al. 2003).

Among these trace metals, copper is well known to exert toxic effects on larval amphibians (Chen et al. 2007, Lance et al. 2012) and studies have also shown that copper can reduce virus survival by inhibiting viral proliferation and cellular membrane mechanisms (Sagripanti et al. 1997, Miyamoto et al. 1998). However, the form of the copper present must also be considered because it will act and be speciated differently depending on the physicochemical and organic properties of the aqueous medium (Carvalho and Fernandes 2006, see Chapter 1). Copper toxicity is also highly dependent on water temperature (Engel et al 1981, Carvalho and Fernandes 2006) with temperature-related changes in the rate of metal absorption, accumulation, and secretion within the organism (Cairns et al. 1975). Most researchers have shown that toxicity of copper increases with increasing temperature and that it causes significant increases in oxygen consumption due to heightened physical activity and competition with oxygen binding site in amphibian/fish gills (Cairns et al. 1975, Playle et al. 1993).

It is clear that amphibian population declines may be associated with a variety of stressors but to date most epidemiological studies tend to focus on these stressors separately rather than addressing their interactions. Therefore to make advances in addressing this problem, an integrative approach is clearly required to better understand host-pathogen dynamics in the wild and help forecast and control disease outbreaks in host populations where multiple stressor interactions are common (Blaustein et al. 2011, Sih

et al. 2004). The Northern Leopard Frog (*Lithobates [Rana] pipiens*) was selected for this study. It is considered a rather sensitive species that is typically not present in polluted ecosystems (Blaustein and Bancroft 2007, Kerby et al. 2010). However, we have found that abundant reproducing populations of *L. pipiens* can occur in metal-contaminated wetlands in the mining region of Sudbury Ontario (Leduc et al. 2012). Although there are therefore some uncertainties about metal tolerance in situ, it is clear that *L. pipiens* is a species that is highly susceptible to ranavirus, from findings in both agricultural and industrial ecosystems (Chistin et al. 2003, Greer et al. 2005, St-Amour and Lesbarrères 2008, Duffus et al. 2008) making it an excellent candidate to test the interaction of disease with environmental variables for our study. Following the assumption that phenotypic plasticity is high at the tadpole stage, we hypothesized that multiple stressors will have additive adverse effects on the *L. pipiens* – ranavirus system inducing changes in both the host life-history traits (i.e. growth, development, survival and resistance) and the virus epidemiological characteristics (i.e. virulence). Furthermore, we expected FV3 virulence to be greater in cold as compared to warmer temperatures due to decreased immunological and metabolic activity of the host at reduced temperatures (Cairns et al. 1975, Kiesecker 2011).

## **Material and Methods**

### *Host-pathogen system*

#### *a. The Northern Leopard Frog (Lithobates [Rana] pipiens)*

For this experiment, Northern Leopard Frog tadpoles originated from an artificial breeding of adults captured in a natural population near Ottawa, Ontario, Canada (Trudeau et al. 2010). To collect fertilized eggs, a procedure was implemented an induced artificial off-season breeding procedure based on a combinational gonadotropin-releasing hormone agonist and a dopamine antagonist hormone injection (e.g. Amphiplex; Trudeau et al. 2010) at an aquatic breeding facility located at the University of Ottawa. In February 2011, we received and used one large clutch of *L. pipiens* larvae to control for genetic

background (full siblings). When hatchlings reached Gosner Stages (GS) 21 (Gosner 1960), they were fed dried food (120mg/tadpole; Carolina Biological Supply Company, Burlington, NC) for 30 days until they reached GS 24 (Gosner 1960).

*b. The ranavirus*

At the beginning of the experiment, Northern Leopard Frog tadpoles were infected with FV3 through a 12 hour inoculation period. *L. pipiens* tadpoles were placed in 50mL of dechlorinated water with a viral concentration of 10000 pfu/mL designed to induce a sub-lethal infection as viral replication of FV3 starts rapidly within only a few hours of direct contact (Gantress et al. 2003, Echaubard et al. 2010).

*Trace metal medium*

Copper stock solution was prepared using a 3.802g/L solution of  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ . This solution was mixed into the artificial soft water medium (Laurentian University water with low ionic concentration; Table 4) 30min before the tadpoles were introduced. The concentration of copper used for this exposure was based on the results of preliminary copper dose-response studies for Northern Leopard Frogs, that revealed that 0.5  $\mu\text{g/L}$  of Cu was equivalent to the  $\text{LC}_{40}$  levels of toxicity in a 96 h static toxicity test. A sublethal  $\text{LC}_{40}$  of copper was used in order to assess the effects of copper on life-history traits rather than inducing high mortality (i.e. > 50%) in each replicate. Low ionic strength water was chosen as our experimental medium to maintain Cu in a bioavailable form throughout the exposure periods.

*Experimental design*

To investigate the toxicological response to copper, ranavirus and temperature conditions, we designed a fully factorial experiment with twenty-four static renewal aquaria equally with a balanced design involving two temperature regimes (14 or 20°C; Figure 6). Temperature was maintained constant using climatic chambers (Thermo Incubator Model 3740) and photoperiod was fixed at 12h:12h. The temperature of rearing water was measured twice daily and remained constant ( $14.0 \pm 0.1^\circ\text{C}$  or  $20.2 \pm 0.1^\circ\text{C}$ , respectively) over the course of the experiment. Within each temperature, each aquarium received

2L of one of two experimental water media (i.e. with or without copper). Three replicate aquariums in each metal condition received a FV3 infection while 3 were used as control (no infection). In total, 144 *L. pipiens* tadpoles were used in this study with eight tadpoles per aquarium (8 tadpoles\*6 treatments [2 viral conditions, 2 trace metal conditions, 2 temperature conditions] \*3 replicates). To control for density, each tadpole received 250mL of experimental medium (Echaubard, 2010). No food was administered during the study in order to account for direct changes in tadpole growth resulting from the effects of the metal, pathogen and temperature and to prevent discrepancies in individual food intake. The experiment was terminated after 15 days and all surviving tadpoles were euthanized using a gradual three-step ethanol submersion procedure (using 20%, 50% and 80% (v/v) ethanol; Wright and Whitaker, 2001) and individually preserved in vials with 70% (v/v) ethanol for further ranavirus infection and life-history trait measurements. All experimental procedures followed protocol #2011-01-01 approved by the Laurentian University Animal Care Committee.

### *Monitoring and data collection*

#### *a. Daily monitoring and care*

All tanks were monitored three times daily (i.e. 8h00, 12h00 and 17h00) for tadpole mortality. Each dead tadpole was promptly removed, processed for life history traits (i.e. weight and developmental stage), and preserved within a plastic vial filled with 70% (v/v) ethanol. Every second day of the experiment, water chemistry samples were taken. For water chemistry sampling, 75mL of water medium was taken daily from two randomly selected aquariums within each treatment. Using both 25 and 50mL pipettes, water was sampled from the middle area of the aquaria and placed into a 100mL Fisherbrand Amber HDPE Narrow-Mouth Bottles. Pipettes were thoroughly rinsed and cleaned with distilled water after every sample. For water sample preservation, concentrated nitric acid was added to each sample to fix trace metals at pH < 2 for further chemical analyses. Water samples were then preserved at room temperature until analyzed at Sudbury INO (Falconbridge, Ontario, Canada) using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis with the following detection limits in mg/L: Cu: 0.006, Ni: 0.003, Fe:

0.009, Pb: 0.04, Ca: 0.12, Mg: 0.03. The chemical analysis of the rearing water is provided in Table 5. Additionally, water change occurred every five days to preserve the semi-static environment of the experiment.

*b. Life history trait measurements*

Within a week of death, all tadpoles were processed for specific life history traits, including body weight and developmental stage at time of death. As feeding stopped at the beginning of the experiment, tadpole weight increase likely resulted from grazing on the biofilm collected on the tank walls and/or water intake. Each tadpole was placed on a plastic disc and blotted dry with a Kimwipe before being weighed on a top loading Denver balance ( $\pm 0.001$  g) to determine final wet weight. For each individual, growth rate was calculated using  $y = ((x - x_0)/t)$  where  $x$  is the final wet weight,  $x_0$  is the average weight of a subsample of tadpoles taken at day 0 within each tank and  $t$  is the number of days the individual tadpole lived (with a maximum of 15 for tadpoles surviving the whole experiment). Final developmental stage was estimated using Gosner's Anuran Larvae Identification Manual (1960). Developmental rate, the number of developmental stages reached by time was calculated similarly to growth rate with the original developmental stage = 24. All growth rate data were  $\log_{10}$  transformed to ensure normality. For survival, each dead individual was assigned a time of death and survival rates were calculated using daily mortality data within each replicate aquarium for each experimental treatment.

*Infection screening*

Once processed for all life history traits, each tadpole was dissected and their visceral organs preserved in 70% (v/v) ethanol. Tissues were then denatured, mixed thoroughly into 1.5mL Eppendorf tubes, and DNA was extracted using QIAmp DNeasy Kit following standard protocol (Qiagen). After extraction, a double blind PCR (Polymerase Chain Reaction), which is a molecular technology used for amplifying DNA, was performed using a primer that amplifies the Frog Virus 3 Ranavirus strain: MCP-ranavirus-F (5'-GACTTGGCCACTTATGAC-3') and MCP-ranavirus-R (5'-GTCTCTGGAGAAGAAGAA), following PCR protocol by Mao et al. (1997) with slight modification to the amplification protocol, which

consisted of 94°C for 8 min, 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min for 30 cycles followed by a final annealing period of 72°C for 5 min and a resting period of 4°C. Since most ranavirus research has focused on the FV3 strain, this specific primer has been proven to amplify the capsid protein in the genome and detect infection through PCR analysis (Mao et al. 1997). To be considered infected, individuals had to show consistent positive amplification for both PCR tests.

### *Statistical analyses*

We performed statistical analyses in *R* statistical computing environment (version 2.13.0, The *R* Foundation for Statistical Computing, 2011) with the *Agricolae*, *Nlme*, *Survival* packages for general linear model and non-parametric analyses. To test the effects of copper, ranavirus and temperature treatments, we first performed a General Linear Model (GLM) followed by *a posteriori* Tukey HSD test on  $\log_{10}$ -transformed growth and developmental rates. Additionally, we analyzed tadpole survival using a survival analysis for each treatment based on a Kaplan-Meier Survival Distribution. Surviving tadpoles were marked as censored to describe an unknown time of death while tadpoles that died during the experiment were noted as complete (Leung et al 1997).

Pathogenic exposure can severely impact host fitness by eliciting a stronger immune response, in turn leading to virulence and resistance trade-offs (Read et al. 2008; Rohr et al. 2010; Hatcher et al. 2012). We thus analyzed the FV3 virulence and host resistance using General Linear Model (GLM) analyses followed by an *a posteriori* Tukey HSD test. All GLM used here are multivariate analyses of variance (MANOVA) with virulence and resistance as dependent variables and copper, ranavirus and temperature as independent variables. For the Leopard Frog/Ranavirus interaction, we assessed FV3 virulence by dividing the number of dead infected tadpoles by the number of infected tadpoles at the end of the experiment (Brunner et al. 2005). Resistance to infection was calculated as the number of tadpoles that were not infected while exposed to FV3 divided by the total number of FV3-exposed tadpoles (Rohr et al. 2010).

Lastly, a Principal Component Analysis (PCA) was used to determine the stability of experimental media in both temperature conditions (i.e. 14.0°C and 20.2°C) throughout the experiment as well as the variation attributed to the difference in copper concentration between media (Table 6).

## Results

### *Survival*

Temperature had a strong influence on *L. pipiens* tadpole survival with larvae dying significantly more at 14.0°C than at 20.2°C ( $\chi^2=55.24$   $P<0.001$ ; Table 3). We also observed significant interaction between copper and temperature on infected tadpole survival ( $\chi^2=77.57$   $P<0.001$ ; Figure 7). Although the interaction between copper and virus was not significant ( $\chi^2=3.05$   $P=0.38$ ), infected tadpoles which were also exposed to copper presented a lower survival at 14.0°C (16.7%) than at 20.2°C (62.7%). In general, tadpoles infected with FV3 showed a low survival (16.7-62.7%) but more so at 14.0°C (16.7-25.0%) than at 20.2°C (54.2-62.7%). Furthermore, non-infected tadpoles survived better than infected tadpoles in the presence of copper at 20.2°C (No FV3: 66.7% vs. FV3: 54.2-62.7%) but not at 14.0°C (No FV3: 0-20.8% vs. FV3: 16.7-25.0%;  $\chi^2=77.57$   $P<0.001$ ; Figure 7).

### *Laval life history traits*

#### *a. Growth*

Tadpoles reacted positively to FV3 with infected tadpoles showing a trend for higher growth than non-infected ones ( $F_{(1,77)}=3.03$   $P<0.07$ ; Figure 8A). By contrast, copper-exposed tadpoles were significantly smaller than non-exposed tadpoles ( $F_{(1,77)}=13.15$   $P=0.001$ ; Figure 9A). In addition, when FV3 and copper were combined, tadpoles exhibited similar growth to the control groups ( $F_{(2,77)}=1.73$   $P<0.19$ ); Figure 8A).

Temperature directly affected tadpole growth over the short time of this experiment ( $F_{(1,77)}=53.63$   $P<0.001$ ) with tadpoles displaying higher growth at 14.0°C than at 20.2°C. We also observed a significant



interaction between copper exposure and temperature ( $F_{(2,77)}=3.77$   $P=0.05$ ; Tukey HSD=1.07,  $P<0.05$ ) as both copper-exposed and non-exposed tadpoles grew larger at 14.0°C as compared to 20.2°C. Finally, the interaction of FV3 infection, copper exposure and temperature was nearly significant ( $F_{(2,77)}=3.40$ ;  $P<0.07$ ; Tukey test HSD=1.35,  $P<0.05$ ). At 14.0°C, tadpoles exposed to copper were significantly smaller than those infected with FV3 or the control group (Figure 9-A1) but tadpoles that were both infected by FV3 and exposed to copper presented similar growth than the control group (Figure 9-A1). At 20.2°C however, tadpoles exposed to copper were larger than the control group but those exposed to FV3 alone or tadpoles exposed to both copper and FV3 did not show any difference in growth with the control group (Figure 9-A2).

*b. Development*

In the analysis of the developmental rate (DR) of *L. pipiens* tadpoles, we observed significant effects for all of the three main variables. Tadpoles placed at 14.0°C presented a faster DR than those at 20.2°C ( $F_{(1,173)}=496.08$   $P<0.001$ ). Copper-exposed tadpoles displayed lower DR ( $F_{(1,173)}=115.93$   $P<0.001$ ) while FV3-exposed tadpoles exhibited a faster DR ( $F_{(1,173)}=33.93$   $P<0.001$ ) than their respective control counterparts. By contrast, tadpoles exposed to the combination of FV3 and copper displayed no significant difference in DR as compared to control tadpoles (Figure 8B). We also detected no significant difference in tadpoles development when analyzing the interaction between FV3 and copper exposures ( $F_{(1,173)}=0.35$   $P=0.55$ ).

Furthermore, FV3-infected tadpoles showed significant differences in DR when exposed to copper with respect to temperature ( $F_{(2,173)}=6.93$   $P<0.01$ ; Tukey test HSD=0.40,  $P<0.05$ ). At 14.0°C, infected tadpoles developed faster than the control group while those that were either exposed to copper only or both copper and FV3 developed at a slower rate than the control (Figure 9-B1). Similarly, infected tadpoles that were also exposed to copper showed a faster DR than tadpoles exposed only to copper. At 20.2°C, only FV3 exposed tadpoles developed faster than the control group while tadpoles exposed to

copper only and FV3 and copper in combination displayed reduced development as compared to the control group (Figure 9-B2).

### *Virulence*

Ranavirus virulence was significantly influenced by both temperature ( $\chi^2=7.21$ ,  $P<0.01$ ) and the presence of copper ( $\chi^2=6.2$ ,  $P<0.05$ ). In general, ranavirus virulence was higher at 14.0°C (85%) than at 20.2°C (48.0%) and in the presence of FV3, copper-exposed tadpoles died less (56.0%) due to the virus than unexposed tadpoles (78.0%). Furthermore, the interaction between copper and temperature had significant effect on virulence ( $\chi^2=7.75$   $P=0.05$ ; Figure 10-A) as ranavirus displayed stronger virulence at 14.0°C (Cu: 75.0% and No Cu: 100%) as compared to 20.2°C (Cu: 60.0% and No Cu: 40.0%).

### *Resistance*

Host resistance against ranavirus infection was significantly influenced by both temperature ( $\chi^2=23.58$   $P<0.001$ ) and copper ( $\chi^2=23.56$   $P<0.001$ ). Overall, fewer tadpoles were infected at 14.0°C (41.7%) than at 20.2°C (61.0%) and a smaller proportion of copper-exposed tadpoles (40.0%) were free of infection as compared to tadpoles non-exposed to copper (59.1%). Furthermore, we observed a significant interaction between temperature and copper ( $\chi^2=24.39$   $P<0.001$ ; Figure 10-B). Tadpoles non-exposed to copper at 20.2°C presented a lower resistance (29.0%) than both copper-exposed tadpoles (50.0%) at 20.2°C and either the non-exposed (50.0%) or copper-exposed (67.0%) tadpoles at 14.0°C.

### *Water chemistry*

Water chemistry in the experimental medium for both temperatures was initially the same (Table 4) with regards to trace amounts of major elements (i.e. Cu, Ni, Fe, Pb, Ca and Mg) and physico-chemical properties (i.e. conductivity, ammonia-nitrogen, pH and hardness). A PCA confirmed that most of the variance (31.9%) was explained by copper and hardness (including the presence of Ca and Mg). A second component associated with ammonia and pH explained a further 18.0% of the variance (Table 5). When the tadpoles were introduced to each media, trace metal concentrations slightly increased before a general

decrease was observed over time (Table 6). Neither time nor temperature influenced water conductivity levels (ranging between 0.1-0.11 across treatments). Similarly, temperature did not influence pH (ranging between 6.2-6.7 across treatments). Ammonia-nitrogen levels were typically higher in the copper – exposed medium as compared to the control medium. Additionally, the average hardness of the experimental media was consistently soft (ranging between 0.93-2.41 mgCaCO<sub>3</sub>/L across treatments) regardless of temperature (Table 6).

## **Discussion**

Our results strongly suggest that multiple stressors can alter the risk of disease in amphibian populations as both host life history strategies and virus epidemiology were influenced by temperature and the presence of copper ions. In fact, even sublethal concentrations of copper and virus can result in direct and indirect effects within the *L. pipiens*-ranavirus system. Therefore, our study stresses the importance of addressing the interactions between multiple factors when investigating host-parasite dynamics and ultimately the multiple causes of amphibian population declines.

### *Temperature effects on L. pipiens - ranavirus interactions*

Overall, *L. pipiens* tadpoles had lower survival in the cold (14.0°C) treatment regardless of the presence of copper or ranavirus as compared to the warmer (20.2°C) treatment. These two temperature conditions were selected to represent temperatures that *L. pipiens* larvae are naturally exposed to; 14.0°C is indicative of early spring after the snowmelt while 20.2°C is common in mid to late summer periods (Gilbert et al. 1994). In cold water, the slow growth and development of tadpoles ultimately affect their ability to survive (Álvarez and Nicieza 2002) while tadpoles raised at 20.2°C showed reduced mortality rates. When exposed to either copper or virus, tadpole growth and development as well as pathogen-related traits were also affected by temperature. Tadpoles showed strong survival differences when exposed to sublethal ranavirus infection within the different temperature regimes. At 14.0°C, survival of

all FV3-infected groups were low (ranging between 0.0% and 25.0%) while tadpoles raised at 20.2°C showed higher survival (ranging between 54.0% and 67.0%). Water temperature has been shown to increase susceptibility to *ranaviruses* in amphibian populations (Gray et al. 2009) most likely due to suppression of the immune function in colder temperatures. For instance at 5°C, *L. pipiens* were more susceptible to infection than at 22°C following a lower T-lymphocyte production and serum complement activity in turn decreasing B-lymphocytes signaling and antibody production (Maniero and Carey 1997). Furthermore, while the viral proliferation of *Ambystoma Tigrinum Virus* (ATV) peaks at 26°C and is minimal at 10°C, *Ambystoma* larvae mortality was lower at 26°C most likely due to a higher immune response of the host at this temperature rather than the inability of ATV to proliferate (Rojas et al. 2005). In another study by Ariel et al. (2009), the authors studied *in vitro* propagation and isolation of multiple ranavirus isolates, including FV3 in various thermal conditions (10, 15, 20, 24 and 28°C) and showed that the optimal temperature was 24°C. Unfortunately, these results were obtained on cell cultures while we observed different temperature effects on virulence using living animals. Thus, although ranavirus replication is higher in warm temperatures (Rojas et al. 2005; Ariel et al. 2009), our findings suggest that virulence is higher in colder conditions suggesting more complex temperature-dependent responses to FV3 infection. Similar studies have reported that several species of *Lithobates* larvae within the United States are more prone to *ranavirus* infection in the winter months (Gray et al. 2007, Hoverman et al. 2012).

Typically, enhanced immune function and resistance to infection are developed as temperature increases (Elliot et al. 2002). Amphibians have immunological constituents comparable to the mammalian system (Du Pasquier et al. 1989, Du Pasquier 1993) and their immunological response to stress is similar (Carey et al. 1999). The survival of amphibians is compromised by contaminant, pathogens and other environmental stressors because their immunological systems are overwhelmed and cannot adequately function. Especially for larvae, alterations to the environment, such as temperature increase can stress the organism and stimulate the production of stress hormones resulting in immunosuppression and higher

susceptibility to other sublethal stressors (Carey et al. 1999). In colder temperatures, anuran immunosuppression is induced to help cope with temperature stress by decreasing the constituents (leukocytic and/or integumentary defenses) of the innate and adaptive immune system. In turn, this generally results in a lower resistance or tolerance to stressors including pathogens whose virulence is then increased (Carey and Bryant 1995, Carey et al. 1999). Thus, at 14.0°C, fewer tadpoles were infected but the virulence was high while at 20.2°C, more tadpoles were infected but few of them died. These results suggest that the trade-offs observed between the hosts and the pathogen fitness are temperature-dependant (Mitchell et al. 2007, Vale et al. 2008) and the effect of temperature on their interaction may lead to different outcomes within a population.

#### *Copper effects on L. pipiens larvae*

Trace metals react differently when confronted with changes in physicochemical properties of the aquatic ecosystem, especially with temperature (Cairns et al. 1975, Schiedek et al. 2007). Our results indicate that the toxic effects of copper on tadpole survival were strongest at 14.0°C than at 20.2°C. The increased mortality at 14.0°C is likely to be a by-product of the temperature effect rather than a direct influence of copper. It has been suggested that the toxicity of copper increases with temperature due to an increase in cell membrane solubility as temperature increases, thus allowing for greater ion uptake across the cell membrane in warmer temperatures (Cairns et al. 1975, Spurgeon and Hopkins 1996). Furthermore, Cu uptake and absorption through the gills increases with temperature via a change in metabolism. In colder temperatures, ectothermic organisms are less active and thus trace metal uptake and toxicity is reduced as opposed to warmer conditions (Handy 2003, Schiedek et al. 2007). Therefore, temperature most likely had a greater direct influence on tadpole survival while copper induced an indirect effect on the host-pathogen interaction. In the presence of copper, tadpole growth and development were also lower than the control group at both temperatures, and this decrease was stronger at 20.2°C than at 14.0°C. These results are consistent with other copper exposure whereby both acute and chronic exposure to copper impairs tadpole osmoregulation, neuroendocrinal processes, and enzymatic and immunological activity leading to

decreased survival, reduced growth rates and embryonic deformities when exposed to increased copper concentrations (Landé and Guttman 1973, Reddick and LaPoint 2004, Chen et al. 2007, Lance et al. 2012).

In addition, throughout the observations of the trace metal analyses of the water during the experiment, there was a noticeable change in the copper concentration between the initial spike of copper and its concentration present during the first day of the experiment where the study organisms were present within the water, especially for the 20°C treatment. At the start of the experiment, all cupric water treatments were taken from the same original source of copper-spiked water media. However, we noted that total copper concentration within the media was no longer the same during the first experimental day indicating that the added copper ions were residing elsewhere. The possible explanation is that the free cupric ions were absorbed by the tadpoles (Landé and Guttman 1973). The water media consisted of low ionic trace metal concentrations allowing the free cupric ions to be readily available for absorption by organisms as opposed to becoming non- or less available molecules through speciation with other available ions and ligands within the medium (Meyer et al. 1999). A second explanation could be that free cupric ions bonded to the side of the plastic containers which made the cupric ions non- or less available for absorption by the study organisms. Although addressing the question as to where to copper ions went once they were added to the water media was beyond the scope of this research, these potential implications can be further studied in future research.

#### *Influence of multiple stressors on *L. pipiens* – ranavirus interactions*

When combined, multiple stressors affect survival and life history differently as opposed to the response observed for the same stressors studied separately. Our findings did not support our initial predictions that multiple stressors would have additive effects on Northern Leopard Frog tadpoles. In fact, tadpoles exposed to copper and FV3 in combination did not display differences in life-history traits as compared to the control group, suggesting antagonistic effects of the stressors at both temperatures. Similar effects

have been measured in salamanders, *Ambystoma tigrinum* exposed simultaneously to ranavirus, carbaryl, and predation (Kerby et al. 2011). In their study, Kerby et al. (2011) observed that *A. tigrinum* larvae displayed increased growth when infected with the virus; but when the host-pathogen system was further exposed to an environmental stressor (i.e. predator cues), there was no difference in growth as compared to the control group. In addition, a study by Reeve et al. (2013) examined the effect of multiple natural stressors (i.e. density, predator cues and resource availability) on susceptibility to ranavirus infection in wood frog (*Lithobates [Rana] sylvaticus*) tadpoles and determined that simultaneous exposure to the stressors did not increase viral susceptibility. There are two potential explanations for how a pathogen and a trace metal can influence one another. First, FV3-infected tadpoles may display an increased immunological response against the infection associated with an increase in metabolic function (Pelster 1999) that could in turn reduce the negative effects of copper. Alternatively, sublethal copper concentrations could benefit tadpoles exposed to FV3 infection through different enzymatic pathways: (1) copper increases enzymatic and cellular activity, as well as protein and neutrophil synthesis (Handy 2003), which could improve the immunological response of tadpoles; (2) free ionic copper may induce toxic effects on the pathogen at a cellular level by altering enzyme activation site, deteriorating nucleic acid and oxidizing the cell membrane (Cervantes and Gutierrez-Corona 1994); and (3) copper can inhibit viral replication and proliferation through anti-apoptotic effects (Miyamoto et al. 1998). More research is thus required to determine the process by which exposure to low concentrations of copper can have a toxic effect on the viral particles while maintaining normal growth and developmental rates in tadpoles.

## **Conclusion**

Environmental stressors can influence the co-evolutionary dynamics of a host-parasite system (Wolinska and King 2009). In order to compensate for environmental changes and optimize its fitness, a host must induce necessary physiological trade-offs. Phenotypic plasticity in amphibians is widely documented (Newman 1992, Denver 1997, Relyea 2001, 2003, Laurila et al. 2002, Van Buskirk 2009, Lind and Johansson 2011) and highlights the resilience of these taxa to both subtle and drastic

environmental changes (Van Buskirk 2002, Kerby et al. 2010). However, such plasticity comes at a physiological cost (DeWitt et al. 1998). In our study, *L. pipiens* larvae displayed life history trade-offs that were significantly influenced by temperature. Regardless of infection or metal exposure, tadpole growth and development, as well as, pathogen virulence and host resistance decreased as temperature increased; while tadpole survival increased with temperature. These observations are consistent with herpetological studies indicating that in colder environments, anuran species display increased body size at time of metamorphosis but to the detriment of their survival (Smith-Gill and Berven 1979, Álvarez and Nicieza 2002). In warmer conditions however, more resources are allocated to survival by decreasing growth and development (Carey and Bryant 1995, Álvarez and Nicieza 2002).

Disease, climate change, and pollution are all factors contributing to worldwide amphibian population declines. Although it is clear that further research is required to understand the interactions between multiple causal factors, our study sheds some light on these interacting threats. In the presence of copper and ranavirus, life history trade-offs between survival, growth and development allow tadpoles to maximize their fitness in stressful environments. Furthermore, host pathology and ranavirus epidemiology are both strongly influenced by temperature. Unexpectedly, exposure to copper at different temperatures also revealed antagonistic effects on the dynamics of host-ranavirus interactions warranting further investigation. Finally, the variation in pathogen-related traits (i.e. virulence and resistance) observed here suggest that environmental stressors are also potentially affecting the efficacy of the pathogen.



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**Table 3:** Survival and life-history trait (i.e. final wet weight and developmental stage) means  $\pm$  standard errors Northern Leopard Frog (*Lithobates pipiens*) larvae are measured at the end of the 15 day experiment for all surviving tadpoles in the trace metal, ranavirus, and temperature mixed treatments. The initial wet weight and developmental stage means  $\pm$  standard errors were taken from a subsample (N=20) of *L. pipiens* tadpoles at the onset of the experiment.

Temperature (°C)	Trace Metal	Virus	N	Survival (%)	Wet Weight (g)	Developmental Stage (GS)
14.0	Control	FV3	24	25.0 $\pm$ 19.1	0.056 $\pm$ 0.006	25.8 $\pm$ 0.1
		No FV3	23	20.8 $\pm$ 8.3	0.053 $\pm$ 0.007	25.7 $\pm$ 0.1
	Copper	FV3	24	16.7 $\pm$ 16.7	0.053 $\pm$ 0.006	25.8 $\pm$ 0.1
		No FV3	24	0.0 $\pm$ 0.0	0.077 $\pm$ 0.009	25.8 $\pm$ 0.1
20.2	Control	FV3	20	54.2 $\pm$ 12.5	0.053 $\pm$ 0.008	25.8 $\pm$ 0.1
		No FV3	23	66.7 $\pm$ 0.0	0.046 $\pm$ 0.006	25.7 $\pm$ 0.1
	Copper	FV3	21	62.7 $\pm$ 12.5	0.064 $\pm$ 0.007	26.0 $\pm$ 0.1
		No FV3	22	66.7 $\pm$ 15.0	0.066 $\pm$ 0.011	26.0 $\pm$ 0.1
Initial Tadpole Weight and Developmental Stage (N=20)					0.039 $\pm$ 0.001	24.0 $\pm$ 0.0

**Table 4:** Water chemistry from low ionic water collected during March 2012. The low ionic water collected from Laurentian University, Sudbury, Ontario, Canada. Low ionic water was used in a semi-static experiment to study the effects of ranavirus and copper treatments in both 14.0°C and 20.2°C temperature conditions on Northern Leopard Frog (*Lithobates pipiens*) tadpoles. The averages and standard errors of all measured trace metal elements are quantified in µg/L. Water chemistry was analyzed by the Chemistry Department of Sudbury INO, Falconbridge, Ontario, Canada.

<b>Trace Metal Element</b>	<b>Concentration (µg/L)</b>
Cu	7.2 ± 0.6
Ni	3.5 ± 1.0
Fe	9.8 ± 6.8
Pb	8.3 ± 6.3
Ca	291.3 ± 19.8
Mg	29.8 ± 14.0
Conductivity	12.8 ± 6.5
NH <sub>3</sub> -N	200.0 ± 0.0
pH	6.2 ± 0.0
Hardness (mg CaCO <sub>3</sub> /L)	0.8 ± 0.1

**Table 5:** Principal Component Analysis (PCA) of water chemistry samples taken from both media (slightly ionic water with and without copper) during the experiment. Samples were taken daily and 95% of water was changed every 4 days.

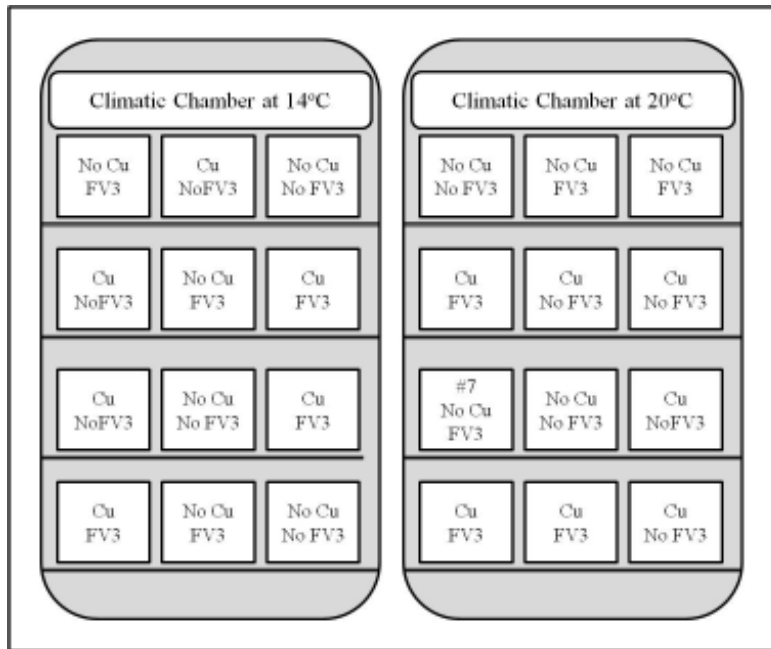
<b>Total Variance Explained</b>				
<b>Initial Eigen Values</b>				
<b>Component</b>	<b>Total</b>	<b>% of Variance</b>	<b>Cumulative %</b>	
1	3.19	31.87	31.87	
2	1.80	17.99	49.86	
3	1.18	11.76	61.62	
4	1.12	11.17	72.79	
5	0.93	9.26	82.05	
6	0.70	7.00	89.01	
7	0.54	5.42	94.43	
8	0.41	4.10	98.53	
9	0.15	1.48	100.00	
10	1.78x10 <sup>-17</sup>	1.78x10 <sup>-16</sup>	100.00	

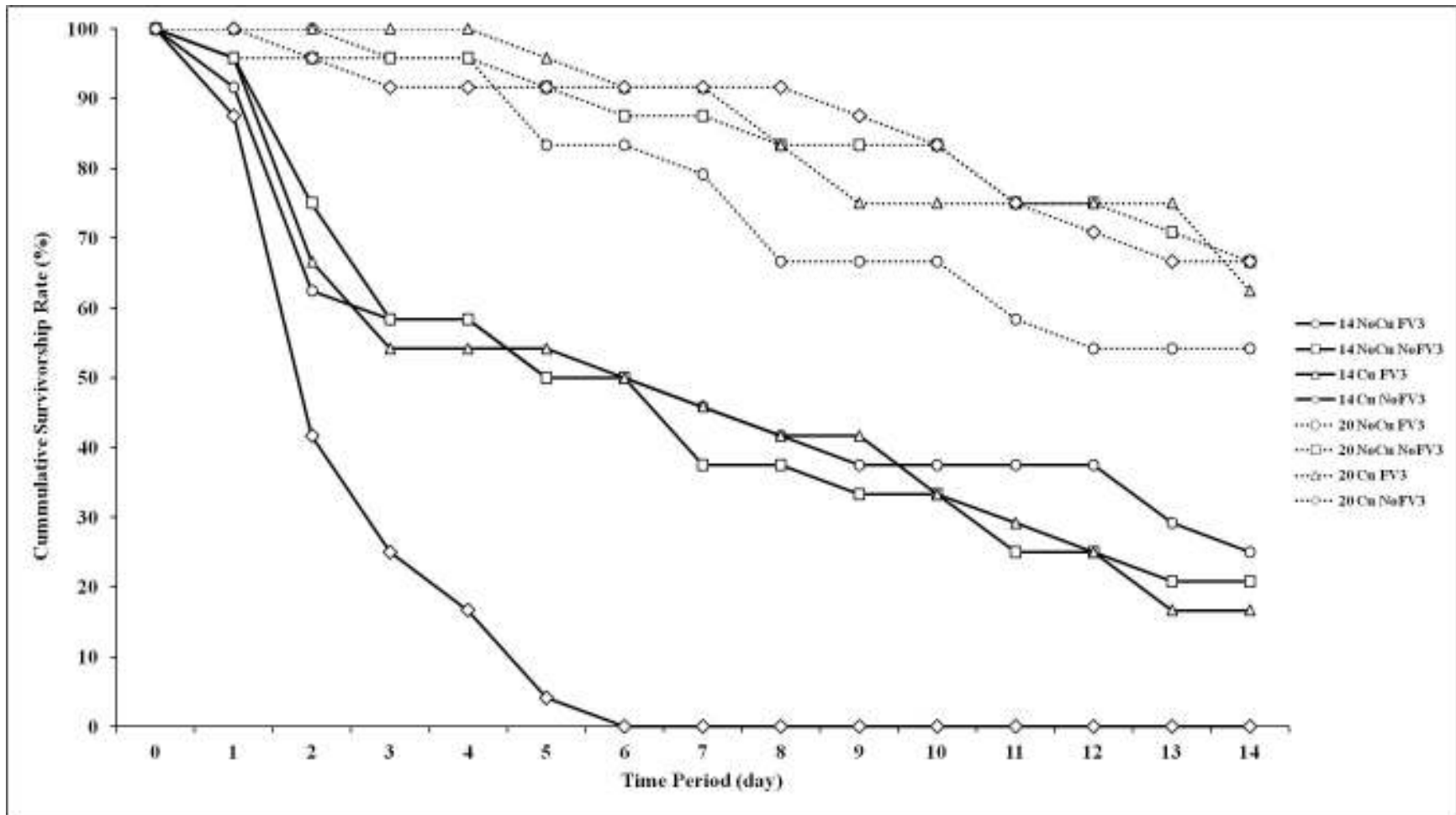
<b>Component Matrix</b>				
	<b>Component</b>			
	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
Cu	0.79	0.34	0.23	0.08
Ni	0.46	-0.12	-0.12	0.68
Fe	0.09	-0.47	-0.43	0.51
Pb	0.46	-0.11	0.83	-0.13
Ca	0.69	-0.61	-0.11	-0.26
Mg	0.88	0.24	-0.02	0.13
Conductivity	0.40	0.10	-0.31	-0.24
NH <sub>3</sub> -N	0.29	0.77	-0.02	2.31
pH	0.44	0.42	0.29	-0.37
Hardness	0.82	-0.49	-0.10	-0.20

**Table 6:** Water chemistry was performed for a 14 day period where water changes occurred every 4 days. Water samples were taken twice daily for four days following water change. Data represent the average for identical days (#1, #2, #3 or #4) across each period. Concentrations ( $\mu\text{g/L}$ ) are noted for both media (i.e. slightly ionic water [Control] and slightly ionic water with added copper [ $\text{dH}_2\text{O} + \text{Cu}$ ]) used to study the effects of copper, ranavirus and temperature differences on Northern Leopard Frog (*Lithobates pipiens*) tadpoles. Water chemistry was analyzed by the Chemistry Department of Sudbury INO, Falconbridge, Ontario, Canada.

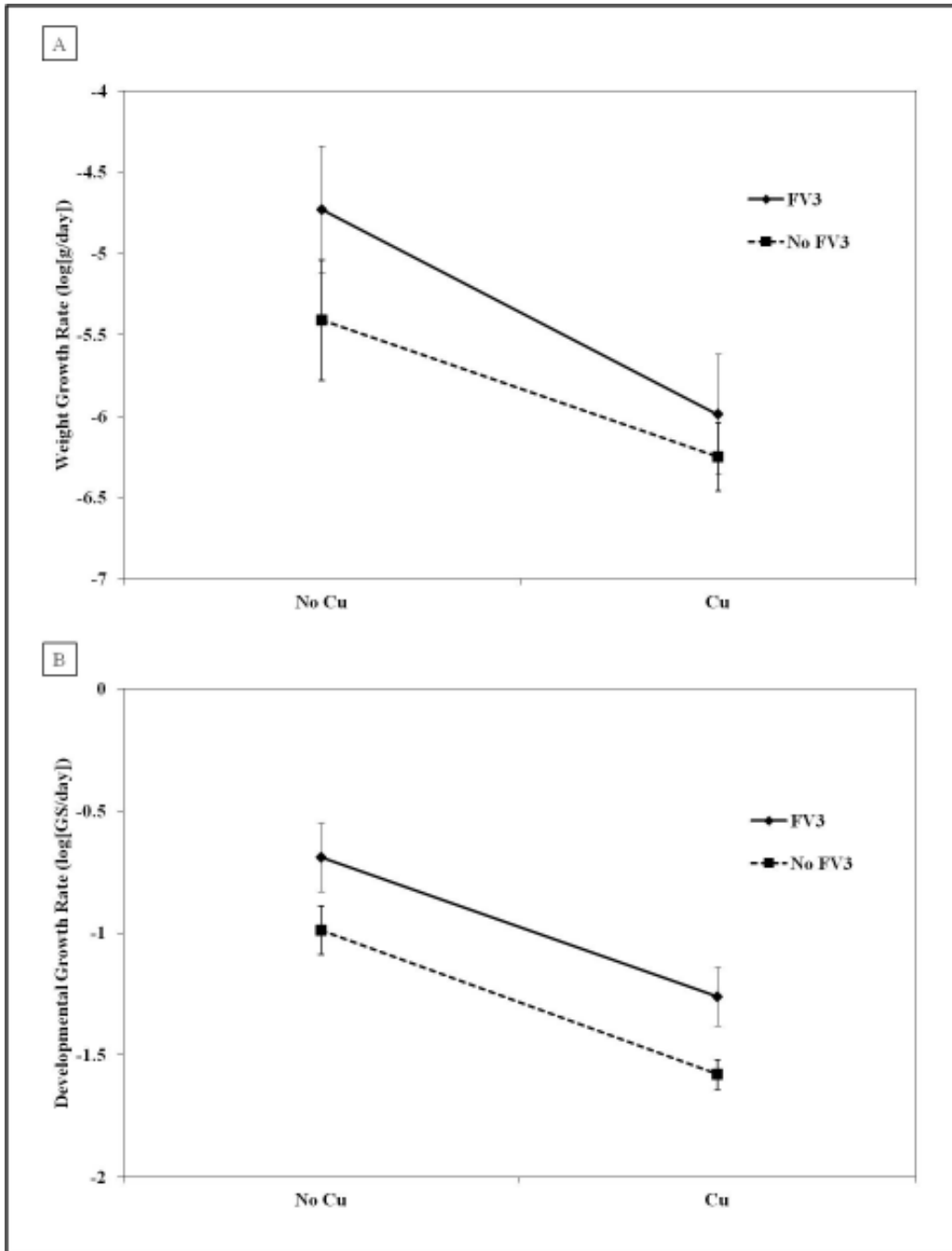
Temp	Water Type	Day	Cu	Ni	Fe	Pb	Ca	Mg	Conductivity (S/m)	NH3-N	pH	Hardness (mgCaCO <sub>3</sub> /L)
14°C	Control	1	13.7 ± 3.3	6.1 ± 0.6	9.2 ± 5.1	4.2 ± 1.7	358.5 ± 53.2	31.07 ± 4.5	3.5 ± 0.0	300.0 ± 63.3	6.5 ± 0.1	1 ± 0.1
		2	11.6 ± 2.7	6.5 ± 0.5	1.8 ± 1.6	4.5 ± 2.3	876.5 ± 341.5	53.1 ± 13.0	2.6 ± 0.0	406.7 ± 227.7	6.6 ± 0.2	2.4 ± 0.9
		3	20.25 ± 0.5	9.0 ± 3.0	13.0 ± 11.0	1.6 ± 2.4	1100.0 ± 40.0	68.0 ± 2.0	3.0 ± 0.0	200.0 ± 0.0	6.6 ± 0.2	3.0 ± 0.2
		4	16.3 ± 3.7	7.2 ± 1.2	7.0 ± 4.2	6.7 ± 3.6	326.5 ± 70.4	36.7 ± 5.2	2.0 ± 0.0	275.0 ± 47.9	6.4 ± 0.2	1.0 ± 0.2
	dH <sub>2</sub> O + Cu	1	44.2 ± 8.6	11.9 ± 3.2	15.3 ± 9.4	5.2 ± 2.0	477.2 ± 20.1	130.0 ± 26.0	4.0 ± 0.0	833.3 ± 370.3	6.8 ± 0.2	1.7 ± 0.1
		2	44 ± 8.0	15.0 ± 2.8	24.4 ± 12.5	9.0 ± 4.6	539.3 ± 16.7	133.8 ± 28.8	3.0 ± 0.0	720.0 ± 332.3	6.7 ± 0.2	1.9 ± 0.1
		3	30.6 ± 5.7	13.9 ± 3.6	1.3 ± 0.8	12.3 ± 6.5	603.2 ± 83.8	101.0 ± 19.3	2.0 ± 0.0	300.0 ± 100.0	6.6 ± 0.1	1.9 ± 0.3
		4	39.9 ± 10.6	11.7 ± 2.3	16.5 ± 15.8	1 ± 0.6	647.3 ± 217	140.3 ± 31.9	5.0 ± 0.1	350.0 ± 95.7	6.7 ± 0.2	2.2 ± 0.6
20°C	Control	1	15.2 ± 2.8	12.8 ± 3.2	8.7 ± 5.9	4.8 ± 2.5	491.0 ± 75	60.8 ± 9.7	2.0 ± 0.0	350.0 ± 131	6.5 ± 0.3	1.5 ± 0.2
		2	6.5 ± 3.2	6.7 ± 2.1	19.5 ± 8.5	5.6 ± 2.9	487.5 ± 152	50.5 ± 11.7	5.0 ± 0.0	200.0 ± 0.0	6.2 ± 0.5	1.4 ± 0.4
		3	12.5 ± 4.3	20 ± 2.5	24 ± 10.4	2.5 ± 0.5	598.0 ± 143.6	43.8 ± 8.4	2.0 ± 0.0	225.0 ± 25.0	6.3 ± 0.2	1.7 ± 0.4
		4	10.3 ± 3.3	19.3 ± 3.6	28 ± 12.8	2.5 ± 0.5	371 ± 83.8	42.3 ± 6.7	1.0 ± 0.0	450.0 ± 150.0	6.6 ± 0.4	1.1 ± 0.2
	dH <sub>2</sub> O + Cu	1	13.2 ± 1.8	12.8 ± 3.5	5.3 ± 2.2	5.8 ± 3.0	422.0 ± 62.4	92.33 ± 13	3.0 ± 0.0	516.7 ± 155.8	6.6 ± 0.3	1.4 ± 0.2
		2	7.8 ± 2.6	4.0 ± 1.3	4.7 ± 2.4	2.8 ± 1.4	252.5 ± 91.8	52.33 ± 17.3	2.0 ± 0.0	566.7 ± 294	6.2 ± 0.2	0.9 ± 0.3
		3	13 ± 0.4	21.3 ± 0.6	10.78 ± 5.2	2.0 ± 0.0	475.8 ± 57.05	80.3 ± 22.5	3.0 ± 0.0	400.0 ± 200	6.3 ± 0.2	1.5 ± 0.2
		4	15 ± 3.1	23.5 ± 3.6	16.8 ± 10.9	2.5 ± 0.5	550.0 ± 209.6	101 ± 21.2	2.0 ± 0.0	450.0 ± 184.8	6.2 ± 0.1	1.8 ± 0.6



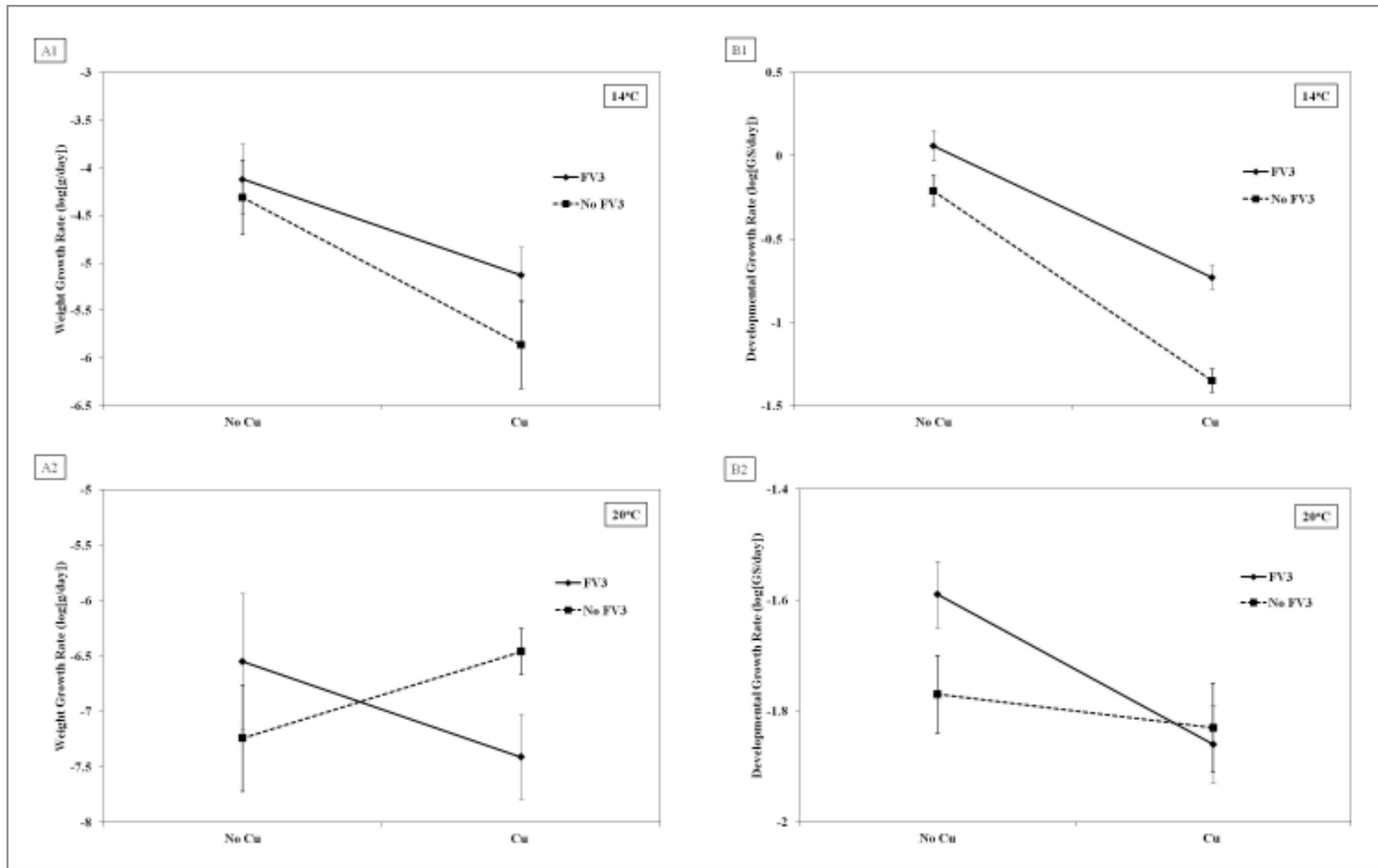
**Figure 6:** Experimental design. Twenty four aquaria were randomly assigned trace metal (Copper or No Copper), ranavirus (FV3 or No FV3) and temperature (14.0 or 20.2°C) treatments. Each treatment had three replicate aquaria each with eight Northern Leopard Frog (*Lithobates pipiens*) larvae of approximately equal body size and developmental stage. Aquaria were located in climatic chambers (Thermo Incubator Model 3740) to maintain temperature constant. During the 14-day experiment, no food was provided to the tadpoles and the water was replaced every four days. Water samples were taken twice daily for water chemistry.



**Figure 7:** Northern Leopard Frog (*L. pipiens*) larvae survival curves for the interaction between trace metal, virus, and temperature treatments. Tadpoles were exposed to sublethal concentration of copper (i.e. Cu or NoCu) and virus (i.e. FV3 or NoFV3) treatments at one of three temperature conditions (14.0 or 20.2°C) for a 14-day time period.

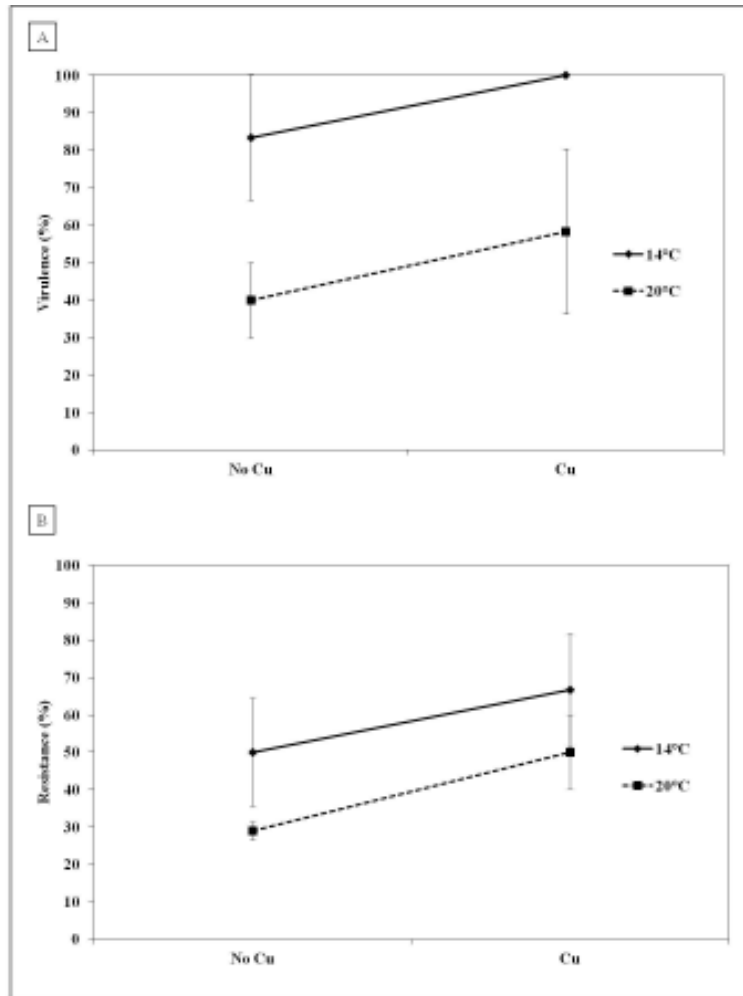


**Figure 8:** Differences in Northern Leopard Frog (*L. pipiens*) tadpole log-transformed weight (A) and development (B) established by the interaction between virus and copper treatments.



**Figure 9:** Differences in Northern Leopard Frog (*L. pipiens*) tadpole weight [14.0°C (A1); 20.2°C (A2)] and development [14.0°C (B1); 20.2°C (B2)] growth rates determined by the triple interaction of virus, trace metal and temperature stressor treatments.





**Figure 10:** Differences virulence (A) and resistance (B) in the ranavirus-exposed Northern Leopard Frog (*L. pipiens*) - ranavirus system when tadpoles were exposed to copper at two temperature conditions (14.0 and 20.2°C) for a 14-day time period.

## GENERAL CONCLUSION

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In disturbed ecosystems, environmental factors can strongly influence life history traits such as the survival, growth and development of amphibian larvae (deSolla et al. 2002, Relyea 2003, Parris and Baud 2004, Robert 2010, Warne et al. 2011). In particular, bioaccumulation of trace metals can have toxic and chronic respiratory, behavioural, morphological, endocrinal, neurological and metabolic adverse effects (Eisler 1998, Thauer 2001, Handy 2003, Opazo et al. 2003, Festa and Thiele 2011). Acute and chronic exposure to copper (Landé and Guttman 1973, Reddick and LaPoint 2004, Chen et al. 2007, Lance et al. 2012) and nickel ions (Khangarot and Ray 1987, Hopfer et al. 1991) have shown reduced survival, decreased growth and development, as well as, abnormal behaviour in various anuran species.

Infectious diseases are also being increasingly associated with amphibian population declines (St-Amour et al. 2008, Buck et al. 2012). For example, individuals infected by sublethal doses of ranavirus show reduced survival and growth as well as accelerated development (St-Amour 2010, Warne et al. 2010, Kerby et al 2011, Echaubard et al. 2012). Water temperature is also a strong determinant of life history trait variation in amphibian larvae. With increased water temperatures, amphibian larvae exhibit increased survival, growth, development, and metabolic features with increasing temperature (Maniero and Carey 1997, Pelster 1999, Blaustein et al. 2001, Rojas et al. 2005). Furthermore, extreme temperatures can influence the interaction of other environmental stressors and thus elicit rapid phenotypic adaptations (Maniero and Carey 1997, Álvarez and Nicieza 2002, Carey and Alexander 2003).

Crucial epidemiological forecasting is beginning to be performed through the assessment of the various factors suspected to increase the impact of infectious diseases on amphibians (Berngruber et al. 2013). This thesis examined two stressors associated with amphibian population declines, trace metals and infectious diseases, and addressed the following questions: (1) what are the effects of increased copper and nickel concentrations on *Lithobates pipiens* larvae, and (2) do multiple stressors demonstrate synergistic effects in a *Lithobates pipiens-ranavirus* system?

In the first part of this thesis, I demonstrated that the tadpole phenotypic response to copper and nickel was dependant of metal concentration and that *L. pipiens* tadpoles are more sensitive to elevated concentrations of Cu than similar concentrations of nickel. When exposed to increased nickel concentrations, tadpoles showed a slow and continuous decrease in survival over time and displayed reduced growth and development; however, when exposed to copper alone or a combination of copper and nickel, larvae exhibited elevated growth and development rates when compared to a control group, however, their survival decreased with an increase in copper concentration. These results indicate opposing responses, in terms of energy allocation for growth, with either a faster time to metamorphosis when exposed to copper or a slower development and growth rate toward metamorphosis when exposed to nickel. My results also suggest that copper can potentially presents a greater threat to survival than nickel since tadpoles succumbed to lower concentrations of copper than nickel. To gain a clearer perspective of how these trace metals are impacting the fitness of *L. pipiens* larvae, further studies should investigate the interaction (i.e. speciation, binding, free ions) of the added copper and nickel ions with base cations (Ca, K, Mg, etc.) and DOC within the effluent water. In depth water chemistry analyses should provide a clearer indication of what chemical species are available and being absorbed by the study organisms.

In the second part of this thesis, I confirmed that amphibians are highly plastic organisms, often able to adapt and tolerate either acute or chronic stressors within the natural environment (Newman 1992, Lind and Johansson 2011). By exposing the *L. pipiens-ranavirus* system to temperature and Cu in very dilute media with elevated metal toxicity conditions, my study revealed that while host survival was influenced by the ranavirus, environmental stressors had separate and antagonistic effects on the host and its pathogen. In absence of food, Leopard Frog tadpoles present plastic responses such as an accelerated growth and development when infected by the ranavirus or the opposite effects when tadpoles were exposed to copper regardless of the temperature they were reared at. Interestingly, the combination of copper and ranavirus displayed an antagonistic response whereby, at both 14°C and 20°C, tadpoles

showed no significant difference in growth and development as compared to the control group. This apparent antagonistic effect suggests that copper may influence the infectivity of ranavirus and future research should be dedicated to this relationship. These current findings can perhaps help understand and forecast the spread of the ranavirus on a global and local scale, Globally, the study demonstrates that perhaps forecasting the presence of FV3 maybe more difficult than projected since trace metals, such as copper, can cancel the effects of the pathogen even though organisms are still infected and able to transmit and disperse the virus. Locally, perhaps Leopard Frogs living in metal-stressed environments are at an advantage because they do not suffer from all of the effects of the virus.

### **Implications for future research**

The goal of ecotoxicological research is to uncover adverse effects associated with chemicals and metal contaminants on biological organisms and environmental systems. This problem-driven science is further complicated by the fact that contaminants not only impact local biodiversity directly but they also interact indirectly with other environmental stressors (e.g. temperature, pathogens, invasive species, UV radiation) resulting in both small and large-scale ecosystem effects (Eggen et al. 2004, Hermens et al. 2004). Most often, ecotoxicological studies are striving to validate the laboratory exposure limits recorded for various contaminants that pollute our environment (Hermens et al. 2004). My research argues for further ecotoxicological research in disturbed environments because trace metals (i.e. copper and nickel) are not only toxic at various thresholds depending on the physicochemical properties of the aquatic environment but also induce subtle morphometric changes. In this context, my results will help guide future experimental field studies to assess real world concentration exposures and the effects of trace metals on host-pathogen interactions through the use of quasi-experimental methods (e.g. mesocosm).

Ranavirus is recognized as a threatening infectious disease affecting ectothermic vertebrates (IOE 2012) and further investigation is required to understand how surrounding environmental dependency is

altering the ranavirus-host dynamics (Echaubard et al. 2010; Lesbarrères et al. 2012), thus improving our current knowledge of how trace metals affect parasitic relationships. In this study, I showed that both *L. pipiens* resistance and ranavirus virulence were less affected by warmer (20°C) than colder (14°C) temperatures. Furthermore, copper had a negative effect on both resistance and virulence, suggesting that this trace metal is affecting both biological entities within this host-parasite system. Future research should be aimed at understanding how various bioavailable trace metals and elements (e.g. copper, nickel, cadmium, selenium, and aluminum) present in disturbed aquatic ecosystems, impact ranavirus biology. Finally, my findings show how sub-lethal ranaviral infections and copper exposure can contribute to subtle alterations in the morphology of anuran larvae and thus impact their survival and fitness. Further investigations are therefore required to fully address the effects of ranavirus and copper exposure on immunological (e.g. MHC and leukocyte counts), physiological (e.g. protein, enzymatic and hormone productions), and behavioural (e.g. predator avoidance and competition abilities) components of anuran fitness.

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