Evolutionary Responses of Amphibian Populations Exposed to Mining-Impacted Environments and Climate Stress

by

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Abstract

Environments impacted by pollution can often be characterized by numerous compounding stressors. These pollutants, and their interactions with environmental variables, present unique challenges for wildlife, often resulting in profound evolutionary changes. However, evolutionary mechanisms such as local adaptation to multiple anthropogenic and environmental selection pressures are still poorly understood. I conducted a laboratory experiment with Northern leopard frog (*Rana pipiens*) tadpoles from three populations to test if they are adapted to mining-impacted environmental conditions. I also assessed the potential role of global warming on the evolutionary outcome of these different populations by using two different temperature regimes. Variability in survival was largely consistent with life-history trade-offs associated with local adaptation to environments impacted by metals. Specifically, tadpoles from the environment with high levels of toxic metals showed no difference in mortality, but had slower growth rates, when raised in environments with medium and low levels of toxic metals. By contrast, tadpoles from environments with medium and low levels of toxic metals displayed a higher risk of mortality but no changes in growth rate when raised in an environment with high levels of toxic metals. Unexpectedly, a warmer environment (25°C) led to higher survival for tadpoles from the high toxic metal environment when raised in medium and low toxic metal environments compared to tadpoles born in medium and low toxic metal environments. My study demonstrates the potential for amphibian populations to adapt to mining-impacted environments and provides evidence that such environmental conditions can influence life-history traits. This work also highlights that multiple stressors associated with pollution and climate change can produce a greater effect in isolation than in combination, which is an unlikely scenario according to current climate change research.

Keywords

Local Adaptation, Environmental Contaminants, Metals, Climate Change, Multiple Stressors, Evolution

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Chapter 1

1 Introduction

1.1 Environmental Pollution

Environmental pollution is defined as “the contamination of the physical and biological components of the earth/atmosphere system to such an extent that normal environmental processes are adversely affected” (Muralikrishna & Manickam, 2017, p. 1). As a result of environmental pollution, 220 billion chemical pollutants are released into the environment each year, greatly impacting wildlife and plant species worldwide (Naidu *et al*., 2021). Chemical contaminants can, for example, disrupt metabolic processes, alter endocrine/neural signalling, and impair sensory and cognitive abilities in animals (Saaristo *et al*., 2018). These contaminants also present indirect effects, like changes in predator avoidance (McCallum *et al*., 2017) and reproduction (Clotfelter *et al*., 2004) in exposed species, which can adversely affect community interactions (Rohr *et al*., 2006). Anthropogenic activities which cause environmental pollution have also caused a rise in greenhouse gas emissions, resulting in global climate change (IPCC, 2013). Since 2012, global average land and sea temperatures have increased and represent the greatest warming period in the northern hemisphere in the last 1400 years (IPCC, 2013; Noyes *et al*., 2015). Such temperature changes can profoundly affect wildlife and plants by altering rainfall indexes (Westra, 2013), altering prey and predator abundances, changing pathogen and host distributions (MacLeod *et al*., 2007; Patz *et al*., 2008), and affecting the uptake and fate of persistent organic pollutants (Ma *et al*., 2016). Further, increases and fluctuations in temperature can negatively affect the physiology of organisms by triggering immediate physiological stress, reducing reproduction, and causing death (Buckley & Huey, 2016).

1.2 Adaptations to Multiple Stressors in Polluted Environments

The suite of chemical stressors present in polluted environments can have negative consequences for wildlife, which may become exacerbated by global warming (Nordstrom, 2009; Foulds *et al*., 2014; Northey *et al*., 2017). For instance, a rise in temperature can influence the physiological and developmental impacts of some chemical pollutants (Relyea, 2012) or alter their biological uptake and disposition, resulting in generally higher toxicity among exposed taxa (Noyes *et al*., 2009; Holmstrup *et al*., 2010). The effects of climate change may also affect organisms' sensitivity to chemical pollutants, resulting in increased chemical bioactivity and physiological dysfunction (Hooper *et al*., 2013). Consequently, exposure to chemicals may induce physiological and developmental defects in the short term, in turn impairing the ability to cope physiologically or behaviourally with environmental change in the future (Noyes *et al*., 2015; Dutilleul *et al*., 2017). Accordingly, evaluating the interactions between chemical stressors and environmental stressors associated with climate change (e.g., temperature) is crucial for understanding the challenges wildlife and plant communities will face when affected by environmental pollution in the context of a variable climate (Northey *et al*., 2017). Although the impact of pollutants may negatively alter the sensitivity and susceptibility of organisms to stress (Fischer *et al.*, 2013; Hooper *et al*., 2013; Boelee *et al*., 2019; Häder *et al*., 2020), adaptive processes may also mitigate these negative effects (Bell & Collins, 2008; Lavergne *et al*., 2010; Sih *et al*., 2011; Niinemets *et al*., 2017). In some cases, populations can adapt to their local environmental conditions through the emergence of specialized genotypes whose relative survival and/or fitness are higher in their local habitat than genotypes from other habitats (local adaptation; Kawecki & Ebert, 2004; Hereford, 2009). Additionally, genetic adaptation to one stressor may lead to an increase in tolerance to another (co-tolerance; Bubliy & Loeschcke, 2005), which is likely since genetic mechanisms underlying tolerance to stressors are usually conserved (Sikkink *et al*., 2015). However, acute chemical exposure can entail evolutionary trade-offs in which selection on developmental or metabolic pathways that make populations more tolerant to toxicants can also delay evolutionary responses to climatic changes (Noyes *et al*., 2015). Furthermore, the combined selective pressures could reduce population genetic diversity, in turn resulting in genetic bottlenecks, lack of adaptability and eventual local extinctions (Moe *et al*., 2013). Evolution of chemical tolerance may therefore be associated with fitness costs that reduce populations' capacity to adapt to climate-related stress (Moe *et al*., 2013).

1.3 Response to Metals and High Temperature in Amphibians

Metals are a major concern for amphibians because of their persistent nature and complex interactions which can negatively affect traits associated with survival and fitness (Noyes *et al*., 2009; Holmstrup *et al*., 2010; Hooper *et al*., 2013). For example, exposure to metals in amphibians can reduce embryonic and larval survival (Sparling *et al*., 2010; Metts *et al*., 2012; Lance *et al*., 2013; Flynn *et al*., 2015) and cause malformations (Haywood *et al*., 2004; Peles, 2013; Perez-Alvarez *et al*., 2018). In addition, amphibians can be at a greater risk as their high energy demands during metamorphosis can make them more sensitive to contaminants. This increased sensitivity can directly or indirectly impact metamorphosis, which can have detrimental effects on amphibian growth and development such as longer developmental periods during pre- and post-metamorphosis, lowered body mass/SVL and reduced metamorphic success (Egea-Serrano *et al*., 2012; Hill *et al*., 2022).

Temperature changes can also affect amphibian development and survival (Blaustein *et al*., 2010). In amphibians, development rates are generally faster as temperatures rise, up to a threshold which often varies by species and population (Blaustein *et al*., 2010). An increase in temperature may affect larval amphibians by reducing time to metamorphosis and decreasing size at metamorphosis (Li *et al*., 2013). Survival of amphibians is also influenced by temperature, however, the effects of temperature on survival is complex (Blaustein *et al*., 2010; Li *et al*., 2013). For example, shorter larval periods can increase chances of survival in adverse environments, by increasing the chance of successful emergence from said environment (Morand *et al*., 1997; Capellán & Nicieza, 2007; Dahl *et al*., 2012) and this shorter tadpole phase can be detrimental, as amphibians that metamorphose at larger size typically have higher fitness (Altwegg & Reyer, 2003; Allentoft & O'Brien, 2010) and survival (Cabrera-Guzmán *et al*., 2013). They are less likely to be preyed upon, have a lower metabolic rate, and can maintain temperature and hydration more easily than smaller amphibians (Levy & Heald, 2015). Ultimately, it appears that there is likely a trade-off between survival and growth which may become exacerbated by climate change.

1.4 Trade-offs Resulting from Adaptations to Stressors

Stress, and its different forms, is considered a selection pressure to which organisms can adapt under appropriate circumstances (Sibly & Calow, 1989; Blanquart *et al*., 2013; Hawkins & Storey, 2020). However, adaptation can come at a cost, be it increased sensitivity to additional stressors (Wirgin & Waldman, 2004; Dutilleul *et al*., 2017; Flynn *et al*., 2021), or poorer performance when not in the presence of the stressor (Bergelson & Purrington, 1996; Klerks *et al*., 2011; Coninck *et al*., 2014). This trade-off, also known as the physiological cost theory, stems from the fact that defence or resistance to stressors is generally associated with metabolic or energetic costs (Sibly & Calow, 1989), particularly when there is a finite supply of energy and resources available for physiological processes. For example, a greater expenditure of metabolic energy on defense may reduce mortality to some extent (Huot *et al*., 2014; Pancic & Kiorboe 2018). However, energy used towards defence cannot be used for growth and thus there may be a trade-off between mortality and growth (Wright *et al*., 2010; Fitzgerald-Dehoog *et al*., 2012; Fan *et al*., 2022). Variations in fitness may thus be driven by environmental and genotypic factors. (Pfennig *et al*., 2010; Hu & Barrett, 2017). For example, adaptation to chemical stressors may occur as a result of the natural selection of genotypes that are more tolerant of those stressors (Medina *et al*., 2007; Sih *et al*., 2011; Niinemets *et al*., 2017). However, such adaptation can result in loss of genetic diversity with negative long-term consequences, including reduced ability to cope with additional stressors (Moe *et al*., 2013), decreased fitness after the stressor has been removed (Coutellec & Barata, 2011), and increased extinction risk (Bijlsma & Loeschcke, 2012).

1.5 Evidence of Local Adaptation and Effects of Temperature on its Outcomes

Adaptation to pollutants, such as road salt (Bell & Gonzalez, 2011; Brady, 2012; Albecker & McCoy, 2017, 2019), pesticides (Hua *et al*., 2013, 2017; Uwizeyimana *et al*., 2017; Richmond *et al*., 2018), and metals (Roelofs *et al*., 2009; Lance *et al*., 2012, 2013; Nakamura *et al*., 2020; Petitjean *et al*., 2021) have often been examined in isolation and few studies have comprehensively investigated the ability of organisms to adapt to multiple stressors. In fact, focusing on the response induced by specific stressors may not accurately represent the complexity of stress faced in the wild (Cadmus *et al*., 2016; Girotto *et al*., 2020). In this study, I am investigating the evidence for local adaptation to metal-polluted environments in Northern leopard frogs (*Rana pipiens*) and evaluating the additional influence of high temperatures on trade-offs in life-history traits associated with local adaptation. A secondary question I would like to address is how high temperatures resulting from climate change affect the tadpoles' ability to cope with changing environments. To this end, I performed a lab-based experiment at ambient and high temperatures on three populations of Northern leopard frogs originating from wetlands in Sudbury, Ontario, Canada with varying levels of toxic metal pollution. I characterized patterns of survival and growth in larvae from each population by recording mortality, length and weight measurements over 125 days. I expected populations to display trade-offs in survival and growth consistent with local adaptation when raised in environments with different levels of toxic metals (Fasola *et al*., 2015; Bachmann & Buskirk, 2021). Therefore, I predicted tadpoles from populations with low levels of toxic metals in their origin environment to exhibit a decrease in growth rate, a decrease in body mass and a higher risk of mortality when raised in foreign environments with higher levels of toxic metals, and vice versa. I also expected novel environments and increased temperatures to result in a compounding negative effect on tadpole survival, in comparison to the effect of the two stressors in isolation.

2 Materials & Methods

2.1 Study Species and Populations

Northern leopard frogs (*Rana pipiens*) are widely distributed across North America where they are found in most provinces and states excluding the pacific coast (Green & Taylor, 2009). During breeding, male *R. pipiens* call to attract females and fertilization occurs externally in aquatic habitats (Green & Taylor, 2009). Females lay egg masses typically containing 3000–7000 eggs which are fertilized by a single male. Eggs hatch after 9-12 days and tadpoles metamorphose approximately 6-12 weeks later (Green & Taylor, 2009).

The three populations of *R. pipiens* studied were sampled at separate wetlands. Coniston and Jerry are lacustrine wetlands, located 1.0 km from each other in an area with continuous mining activity for the last century (Lavoie *et al.,* 2018). Long Lake is a riverine wetland with no recorded historical mining or smelting (Figure 1). Due to the proximity of the Coniston and Jerry wetlands, there is a possibility for gene flow between the populations at each wetland. Yet, notable gene flow between Coniston and Jerry is unlikely given the two breeding sites are separated by over one kilometre of elevated rocky/semi-barren terrain, with no suitable wetland or riparian corridors in between, in a landscape which has remained largely unchanged for decades (Winterhalder, 1995). Similarly, Long Lake is 20 km from Coniston and Jerry, making genetic connectivity between these populations very unlikely (Smith & Green, 2005). Furthermore, amphibians have high site fidelity, with most adults remaining within 200 meters of their breeding sites and juvenile dispersal limited to less than 1 km for most species (Dole, 1968; Semlitsch, 2000).

A surface water analysis was conducted to assess the water quality (pH, conductivity, dissolved oxygen) and concentration of trace elements at each wetland. The water chemistry at all wetlands showed exceedingly high specific conductance and low dissolved oxygen (Appendix A). Additionally, numerous toxic metals were found in the wetlands, which are metals and metal compounds that cause negative health effects for organisms (Ali *et al*., 2019). I performed a principal component analysis (PCA) in R (R Core Team, 2013) to provide a more comprehensive analysis involving water chemistry and metals among the three wetlands. Based on standard PCA procedures involving chemical analysis, data below the minimum detection limit (MDL) were considered by defining half of the MDL value (Hornung, 1990; Croghan & Egeghy, 2016; Fernández-Ayuso *et al*., 2023). In addition, only variables for which at least 2 out of 3 wetlands had levels above the MDL were included. The PCA analysis revealed that the variance in the original dataset was sufficiently explained by PC1 and PC2 (93.4%). The water parameters with the highest loadings (i.e., the water parameters with the most influence on where the wetlands were placed on Figure 2) were copper, spc (specific conductance), iron, nickel, selenium, pH, barium, arsenic, and manganese, in sequential order (Figure 2, Appendix B). Considering the most toxic metals loaded heavily on PC1, I differentiated wetlands based on PC1 as Low (Long Lake), Medium (Coniston) and High (Jerry) (Figure 2, Appendix C). Hereafter, the 3 population will be referred to as a Low, Medium, and High.

2.2 Experimental Design

From each population, five recently laid *R. pipiens* egg clutches were sampled, and 24 eggs were carefully removed from each clutch and transported to Laurentian University in plastic containers filled with pond water and placed on ice. Four eggs from each clutch were randomly assigned to one of six groups following a 3 x 2 design, such that each group of eggs was raised in the water of each wetland (Low, Medium, and High at two temperatures [21°C and 25°C]). I chose 21 degrees as the ambient temperature based on Sudbury seasonal averages from May to September in 2018 and 2019. In order to replicate the 3-5 degree temperature increase expected due to climate change in the next century, the high temperature was set at 25 degrees (Huang *et al.*, 2020). Each treatment was replicated five times at the clutch level, resulting in 20 eggs per treatment, 120 eggs per wetland and 360 eggs in total. All experimental procedures were approved by the Laurentian Animal Care Committee (AUP 2019-04-01).

2.3 Animal Husbandry

All tadpoles were housed in individual containers divided between two rooms based on temperature treatments; a thermostat, along with heating/cooling lamps were used to keep temperatures constant. Tadpole containers were placed on a 91.5 x 35.6 x 138.4 cm 3-shelf storage rack with LED lights and timer to replicate a photoperiod of 14h:10h. The position of tadpole containers were haphazardly repositioned once a day during the experiment. Containers received a static water renewal (250 ml, 1/3 of the original 750 ml) of UV-treated water every 4 days. The agitation of changing the water every 4 days could cause tadpoles to emit stress hormones such as ACTH and corticosterone (Santymire *et al*., 2018; Forsburg *et al*., 2019). However, according to a previous study, hormone release rates declined after 2 hours and peaked after 6 hours in agitation tests, indicating a relatively rapid recovery (Forsburg *et al*., 2019). Further, tadpoles reared individually did not omit any stress response compared to those reared in groups (Forsburg *et al*., 2019). Therefore, the water changes and resulting stress hormones are not likely to affect survival and growth in this experiment. A dissolved oxygen (DO) meter was used to monitor DO levels once a day to ensure the tadpoles environment had adequate oxygen levels (0.7–6.0 mg L^{-1}; Mansano *et al*.,2019). Tadpoles were given 4 mg of standard dried tadpole food every 2 days; this amount was doubled every two weeks and capped at 16 mg as tadpoles were unable to finish the remaining food beyond this amount.

2.4 Measurements

Growth rate (mm/day), survival and mass at mortality (g) were calculated at the end of the experiment. Tadpoles were photographed every 4 days and individuals were measured to the nearest 0.1 mm using the open-source software package ImageJ (Radersma *et al*., 2018). Length measurements continued until day 125 of the experiment or when the tadpoles had developed into Gosner Stage (GS) 39 (Gosner, 1960). Tadpole weight was only recorded for tadpoles that survived until day 125. The final mass of tadpoles was measured by using the wet weight of individual tadpoles and subtracting mass at hatching from mass at mortality. Tadpoles presenting signs of physical or behavioural deformations (n = 8) were euthanized and removed from the study.

2.5 Survival Analysis

The survival package in R (Therneau & Grambsch, 2000) was used to fit mixed-effects Cox's proportional hazard models and test variability of larval mortality amongst populations, environments, and temperatures. Larval mortality was modelled as a binary response (1 = died, 0 = survived or reached GS Stage 39) using a binomial (logit-link) distribution. Population, rearing environment and their interaction were included as fixed effects while clutch number (1-5) was included as a random effect. Time to mortality was analyzed by plotting the Hazard Ratios (HR) and Confidence Intervals (CI) amongst the tadpole populations using ggstatsplot (Patil, 2021). The coefficients in a Cox regression relate to hazard with a positive coefficient indicating a higher risk of mortality and a negative coefficient indicating a lower risk of mortality.

2.6 Growth Analysis

Differences in tadpole growth rate and final mass among populations and environments were analyzed using linear mixed effects models with lme4 (Bates *et al*., 2015) in R. Significance of fixed effects was determined using linear model coefficients which were converted to F coefficients and tested with Wald F tests and Kenward Roger Degrees of Freedom, using car (Fox & Weisberg, 2019). Post-hoc analyses were conducted using Tukey pairwise comparisons with multcomp (Hothorn *et al*., 2008). Population, rearing environment and their interaction were included as fixed effects while clutch number (1-5) was included as a random effect. Hedge’s g was calculated as a measure of effect size based on the pooled mean differences and standard deviations for length growth rate (mm) and mass (grams) across all datasets. A g of 1 indicates a difference of 1 standard deviation between the two groups and a g of 2 indicates a difference of 2 standard deviations (Hedges, 1981). When interpreting hedges g, the following rule applies: small effect = 0.2, medium effect = 0.5, large effect = 0.8 (Hedges, 1981). Differences in effect size can mean different things in different fields of study, therefore caution should be taken when interpreting the terms “small” and “large” in consideration to effect size (Hedges, 1981; Durlak, 2009). The growth data associated with tadpoles at 25°C were not included in this study because of the high probability of type II errors due to the small sample size of surviving tadpoles at 25°C (0% survival at day 150/GS 39) relative to 21°C (18.6% survival at day 150/GS 39; Columb & Atkinson, 2016). Continuous response variables were plotted and visually analyzed to confirm normality of residuals with ggplot2 (Wilkinson, 2011).

3 Results

3.1 Mortality

Tadpole mortality was significantly related to their rearing environment and temperature. At 21°C, Medium tadpoles showed a 3 times greater risk of mortality when reared in High [HR 4.06; 95% CI: 0.45 to 2.35; $p = 0.004$], compared to their original water. Medium tadpoles also had a 1.5 times greater risk of mortality when reared in Low [HR 2.5; 95% CI: 0.11 to 1.72; $p = 0.03$], as compared to their original water (Table 1, Figure 3A). In comparison, tadpoles from High and Low populations did not show a difference in risk of mortality in foreign environments (Table 1, Figure 3A, C). When comparing environments, Medium and Low tadpoles presented a significantly higher risk of mortality when reared in High (Table 1, Figure 3A, B, C). Medium tadpoles had a 4.7 times higher risk of mortality [HR 5.59; 95% CI: 0.82 to 2.64; $p < 0.001$] and Low tadpoles had a 12 times higher risk of mortality [HR 12.7; 95% CI: 1.52 to 3.6; $p < 0.001$], compared to tadpoles from High (Figure 3B). By contrast, the mortality of tadpoles from High was not different from Medium and Low tadpoles when raised in Medium and Low respectively (Table 1, Figure 3A, C).

At 25°C, Medium tadpoles did not display differences in risk of mortality in foreign environments compared to their original water and Low tadpoles only displayed a significantly higher risk of mortality when reared in High, with a 61% reduction in risk of mortality [HR 0.39; 95% CI: -1.62 to -0.25; $p = 0.008$] compared to their original water. Interestingly, tadpoles from High demonstrated a lower risk of mortality when reared in Medium, with a 91% reduction in risk of mortality [HR 0.09; 95% CI: -3.15 to -1.6; $p < 0.001$] compared to Medium tadpoles and a 20% reduction in risk of mortality when reared in Low [HR 0.20; 95% CI: -2.77 to -0.91; $p < 0.001$] in comparison to Low tadpoles (Table 2, Figure 3E, F). Additionally, in the High environment, Medium tadpoles displayed a 6.5 times higher risk of mortality [HR 7.53; 95% CI: 1.33 to 2.71; $p < 0.001$] and Low tadpoles displayed a 1.35 times higher risk of mortality [HR 2.35; 95% CI: 0.19 to 1.52; $p = 0.01$], in comparison to tadpoles from High (Table 2, Figure 3D).

3.2 Growth Rate

The interaction of population and rearing environment had a significant effect on tadpole growth rate ($F_{4,57} = 18.00$, $p = 0.002$; Table 3, Figure 4). Tadpoles from High displayed a lower growth rate in Medium, compared to Medium tadpoles with a very large effect size ($p < 0.001$, $g = 4.2$; Table 5). Yet, this growth rate was higher than when they were raised in their original environment, with a large effect size ($p = 0.02$, $g = 1$; Table 5, Figure 4). Tadpoles from Medium displayed a higher growth rate in High, compared to Tadpoles from High with a large effect size ($p = 0.03$, $g = 0.87$; Table 5, Figure 4). There were no significant differences in growth rate among the other tadpole groups (Table 5, Figure 4).

3.3 Mass

The interaction of population and rearing environment had a significant effect on tadpole mass (Table 4; Figure 5). Differences in mass were observed between tadpoles from High raised in Medium, compared to tadpoles from Medium and High raised in their origin environments (Table 6, Figure 5). Specifically, tadpoles from High had a lower mass when raised in Medium, compared to tadpoles from Medium and High raised in their origin environments, with very large effect sizes ($p < 0.001$, $g = 2.38$; $p = 0.03$, $g = 1.5$, respectively; Table 6, Figure 5). No significant difference in mass was observed within or among tadpole groups in any other comparison (Table 6, Figure 5).

4 Discussion

Overall, my results demonstrate life-history trade-offs consistent with local adaptation to environments contaminated with toxic metals. Tadpoles from the environment with the highest levels of toxic metals (High) displayed changes in growth rate and mass, but their survival was not altered when transplanted into environments with a medium level of toxic metals (Medium) or a low level of toxic metals (Low). By contrast, tadpoles from Medium and Low were more likely to experience mortality when raised in High but showed little change in growth rate or mass. Furthermore, I observed that Tadpoles from High’ survival was more affected by the rearing environment or the increased temperature alone than in combination. These results suggest that populations from High are adapted to stressful conditions which can allow them to deal with additional stressors, highlighting the importance of considering organisms' evolutionary background and the interactions among multiple stressors when investigating the effects of pollution. In addition, my study corroborates previous research detailing the complex interactions between chemicals and climate on the life histories of organisms.

4.1 Evolutionary Responses to Stressors

Different evolutionary responses were observed across *R. pipiens* tadpoles raised in novel conditions. Survival and growth patterns differed between populations from High compared to Medium and Low. Tadpoles from Medium and Low had lower survival when transferred to High, a finding consistent across numerous taxa, where naïve organisms survive significantly less in contaminated environments compared to individuals that had prior exposure to contaminants (Xie & Klerks, 2004; Roelofs *et al*., 2009; Agra *et al*., 2011; Hangartner *et al*., 2011; Hua *et al*., 2013; Coldsnow *et al*., 2017). Tolerance to contaminants can occur at the population level, resulting in local adaptation, where genotypes are better adapted to their native environments than those from other populations (Hereford, 2009; Blanquart *et al*., 2013). For example, in Moor frog (*Rana arvalis*) populations, phenotype-environment correlations revealed that embryonic acid tolerance and metamorphic size diverged most strongly with pH in breeding ponds (Hangartner *et al*., 2011). As a result, embryonic and larval acid tolerance was higher (higher survival/unaffected larval period), larval growth was higher, and larvae metamorphosed at a larger size in populations originating from acid ponds (Hangartner *et al*., 2011). Insecticides can also affect the genes of individuals previously exposed, compared to non-exposed individuals (Poupardin *et al*., 2012). For instance, Wood frog (*Lithobates sylvaticus*) populations exposed to sublethal concentrations of insecticides in early development exhibited higher survival rates after exposure to a sublethal dose of insecticides later in life, compared to populations without prior exposure (Hua *et al*., 2013).

In this study, tadpoles from High displayed a slower growth rate and lower mass when raised in Medium and Low, in line with previous research indicating that exposure to metals can result in the evolution of physiological tolerance that results in trade-offs in fitness in the absence of these metals (Wirgin & Waldman, 2004; Medina *et al*., 2009; Klerks *et al*., 2011; Coninck *et al*., 2014; Oziolor *et al*., 2014; Flynn *et al*., 2021). These findings support the notion of the physiological cost theory (Sibly & Calow, 1989) suggesting that organisms coping with toxic stress have individual costs associated with altered physiological processes. Among these processes are detoxification mechanisms that can hinder growth or reproduction in unpolluted environments by diverting energy from other fitness traits, such as growth and/or reproduction, but improve survival under polluted conditions (Sibly & Calow, 1989; Lopes *et al*., 2005). For example, the higher respiration rates of copper-resistant *Daphnia longispina* clones were associated with increased metabolic demands due to detoxification processes (i.e., metallothionein synthesis; Agra *et al*., 2011). Similarly, the rapid evolution of salt tolerance in *Daphnia pulex* occurs with the tradeoff of suppressed circadian function which can affect species interactions, having profound consequences on individuals, populations, and aquatic food webs (Coldsnow *et al*., 2017). Here, tadpoles originating from High demonstrated small trade-offs in life-history traits when raised in a foreign environment. Rearing tadpoles from High in Medium and Low did not affect their survival but it did slow their growth. Therefore, it is likely that tolerance to metal environments could be associated with potentially costly delays in growth under non-polluted environments, which could reduce the viability of populations from mining-impacted areas when there is a high degree of temporal/spatial variation in pollutant presence and concentrations.

4.2 Impacts of Global Warming on Adaptations to Metals

In comparison to ambient temperatures (21°C), Medium and Low tadpoles showed fewer changes in survival at high temperatures (25°C) in High compared to their original environment. However, Medium, and Low tadpoles survived less in High, compared to their original environment. These results do not coincide with previous ecotoxicity experiments which have demonstrated that chemical toxicity generally increases with increasing temperature, negatively affecting organisms (e.g., Messiaen *et al*., 2013; Barbosa *et al*., 2015; Ali *et al*., 2019; Soliman & Moustafa, 2020). Interestingly, tadpoles from High showed lower mortality when raised at high temperatures (25°C) in Medium and Low suggesting that amphibian populations may respond differently to changes in temperature based on their history of exposure to toxic metals. This contrasting result was unexpected because most detoxification processes that might alter toxic effects, like substance uptake pathways and metabolic oxygen demand, are temperature dependent (Honkanen & Kukkonen, 2006). Tadpoles with a tolerance to one stressor may gain tolerance to other stressors via shared pathways (Fasola *et al*., 2015). This co-tolerance may explain why Tadpoles from High' survival was less affected by increased temperatures, compared to Medium and Low tadpoles, as heavy metals, and high temperatures both act on the same biological pathway in response to cell stress (Heikkila, 2017; Steurer *et al*., 2018). Known as the heat shock response, this pathway stimulates the synthesis of heat shock proteins by activating transcriptomes such as HSF1 (Heat Shock Factor 1) (Heikkila, 2017; Steurer *et al*., 2018). Under stressful conditions, heat shock proteins function as molecular chaperones that ensures normal protein function, such as folding, assembly, and translocation (Heikkila, 2017; Steurer *et al*., 2018). Furthermore, if both heavy metals and high temperatures produce a similar response to cell stress and act on the same biological pathway, it appears possible that the genetic or physiological changes that occur to produce tolerance to one of these stressors could also produce tolerance to the other. Co-tolerance was previously observed in invertebrates (Lopes *et al*., 2005) and in amphibians (Marquis *et al*., 2009; Fasola *et al*., 2015) for pollutants with similar adverse effects. Alternatively, the vast range of possible interactions among stressors suggests that some stressor combinations may pose a greater threat than others (Heard *et al*., 2015; Piggott *et al*., 2015; Brady *et al*., 2019). For example, although Ranavirus causes large die-offs in amphibian populations exposed to this pathogen (Lesbarrères *et al*., 2012), previous studies have demonstrated antagonistic interactions between copper and Ranavirus (Leduc, 2013), whereby the growth and development of tadpoles exposed to both copper and Ranavirus were not different from tadpoles exposed to each stressor in isolation (Leduc, 2013). Likewise, predator-cues have also been shown to act antagonistically with metals in larval amphibians (Rumrill *et al*., 2016).

While this study underscores that mining-impacted *R. pipiens* populations can adapt to multiple stressors in polluted environments, the physiological processes that are involved in eliminating the pollutants or detoxifying them may require additional resources, reducing amphibians' ability to cope with additional environmental stressors (e.g., rising temperatures; Hooper *et al*., 2013; Alton & Franklin, 2017). Additionally, this study was conducted under controlled laboratory conditions in the absence of environmental factors typically present under natural conditions. Accordingly, I cannot report on the effect of mining-impacted environments broadly, but rather on the effects associated with the toxic and trace elements present in these environments. Future laboratory studies should attempt to analyze the effects of multiple toxic elements, as well as other environmental stressors associated with polluted environments and provide insight into the specific physiological mechanisms underlying their responses.

5 Conclusion

Anthropogenic threats such as the chemical contamination of natural habitats, have increased in frequency and intensity throughout the world and are expected to continue to do so in the future (Tilman & Lehman, 2001; Bell & Collins, 2008). Simultaneously, rising temperatures from climate change continue to alter the earth's climate, causing spikes in global average land and sea temperatures (IPCC, 2013). Thus, understanding evolutionary responses to multiple stressors, specifically chemical and climate stressors, is crucial for the conservation of natural populations. Even though wetlands in areas with a century of continuous copper and nickel smelting can produce unique selective pressures on amphibians, my results demonstrate that local adaptation can alleviate some of the fitness costs typically incurred in these environments, thus supporting previous literature showing that tolerance to these chemical stressors can cause life-history trait variation and trade-off costs in the absence of these stressors (Hughes *et al*., 2007; Hereford, 2009; Moe *et al*., 2013; Bono *et al*., 2017). In addition, I provide evidence that amphibians from mining environments can experience more adverse effects from stressors in isolation than in combination, potentially leading to less fitness costs when adapting to climate change, compared to amphibians from non-impacted environments. To this end, I recommend that future studies investigating evolutionary responses to multiple stressors continue to develop a framework using a life-history trait-based approach along with the integration of genomics, behavioural and population demographic analyses.

Table 1. Variation in larval mortality among populations and environments in *Rana pipiens* populations at 21°C determined using a multivariate Cox regression model. Column 1 and 2 represents the population/environment identifier; the first 1-letter code is the origin pond the tadpoles came from and the second 1-letter code is the pond the tadpoles were raised in for the duration of the experiment: Low (L), Medium (M) and High (H).

Origin Pond - Transfer Pond	**Origin Pond - Transfer Pond**	**Coefficient (*bi*)**	**HR [exp(*bi*)]**	**95% CI**	**p-value**
L - L	L - M	0.46	1.58	(-0.25-1.16)	0.20
L - L	L - H	0.64	1.89	(-0.14-1.41)	0.11
L - L	M - L	-0.18	0.84	(-0.89-0.54)	0.63
L - L	H - L	-0.49	0.62	(-1.23-0.26)	0.20
M - M	M - H	1.4	4.06	(0.45-2.35)	**0.004**
M - M	M - L	0.92	2.5	(0.11-1.72)	**0.03**
M - M	H - M	0.44	1.55	(-0.42-1.29)	0.32
M - M	L - M	1.61	5	(0.73-2.49)	**< 0.001**
H - H	H - M	0.16	1.17	(-0.63-0.95)	0.69
H - H	H - L	0.44	1.55	(-0.36-1.23)	0.28
H - H	M - H	1.72	5.59	(0.8-2.64)	**< 0.001**
H - H	L - H	2.56	12.7	(1.52-3.6)	**< 0.001**

Table 2. Variation in larval mortality among populations and environments in *Rana pipiens* populations at 25°C determined using a multivariate Cox regression model. Column 1 and 2 represents the population/environment identifier; the first 1-letter code is the origin pond the tadpoles came from and the second 1-letter code is the pond the tadpoles were raised in for the duration of the experiment: Low (L), Medium (M) and High (H).

Origin Pond - Transfer Pond	**Origin Pond - Transfer Pond**	**Coefficient (*bi*)**	**HR [exp(*bi*)]**	**95% CI**	**p-value**
L - L	L - M	-0.50	0.61	(-1.14-0.14)	0.13
L - L	L - H	-0.93	0.39	(-1.62-(-0.25)	**0.008**
L - L	M - L	-0.31	0.73	(-0.94-0.32)	0.33
L - L	H - L	-1.59	0.20	(-2.27-0.91)	**<0.001**
M - M	M - H	0.08	1.08	(-0.56-0.72)	0.81
M - M	M - L	-0.29	0.75	(-0.92-0.33)	0.36
M - M	H - M	-2.38	0.09	(-3.15-(-1.6)	**<0.001**
M - M	L - M	-0.51	0.60	(-1.17-0.15)	0.13
H - H	H - M	-0.18	0.83	(-0.79-0.43)	0.56
H - H	H - L	0.38	1.46	(-0.25-1.01)	0.24
H - H	M - H	2.02	7.53	(1.33-2.71)	**<0.001**
H - H	L - H	0.85	2.35	(0.19-1.52)	**0.012**

Table 3. Effect of origin and transfer ponds on mean growth rates (cm/day) for *Rana pipiens* tadpoles for two-way ANOVA.

Predictor	**df**	**df residuals**	**F**	**p-value**
Origin Pond	2	49	18	**<0.001**
Transfer Pond	2	55	0.16	0.86
Origin Pond × Transfer Pond	4	57	4.85	**0.002**

Table 4. Effect of origin and transfer ponds on mass (grams) for *Rana pipiens* tadpoles for two-way ANOVA.

Predictor	**df**	**df residuals**	**F**	**p-value**
Origin Pond	2	55	8.11	**<0.001**
Transfer Pond	2	55	2.42	0.1
Origin Pond × Transfer Pond	4	57	2.82	**0.03**

Table 5. Growth rates (mm/day) response amongst *Rana pipiens* populations and environments. Column 1 and 2 represents the population/environment identifier; the first 1-letter code is the origin pond the tadpoles came from and the second 1-letter code is the pond the tadpoles were raised in for the duration of the experiment: Low (L), Medium (M) and High (H). P-values are calculated using Tukey HSD Post-hoc Test for Multiple Comparisons of means.

Origin Pond (T) - Transfer Pond (R)	**Origin Pond (T) - Transfer Pond (R)**	**Mean Difference (R-T)**	**Std.Error**	**95% CI**	**p-value**	**Hedges' g**
L - L	L - M	0.01 –0.01	0.0007	(-0.002-0.0018)	0.99	0.08
L - L	L - H	0.01 – 0.01	0.0007	(-0.0006-0.003)	0.33	1.43
L - L	M - L	0.01 – 0.02	0.0008	(-0.002-0.0018)	0.99	0.49
L - L	H - L	0.01 – 0.01	0.0009	(-0.003-0.002)	0.93	0.44
M - M	M - H	0.01 – 0.01	0.0005	(-0.0008-0.002)	0.83	0.41
M - M	M - L	0.013- 0.01	0.0006	(-0.0003-0.003)	0.15	1.80
M - M	H - M	0.013 – 0.01	0.0005	(0.002-0.004)	**<0.001**	**4.15**
M - M	L - M	0.013 – 0.01	0.0014	(-0.00005-0.003)	0.06	1.70
H - H	H - M	0.011 – 0.01	0.0005	(0.0002-0.003)	**0.02**	**1.10**
H - H	H - L	0.01 – 0.01	0.0007	(-0.003-0.0009)	0.61	0.53
H - H	M - H	0.01 – 0.01	0.0005	(-0.003-(-0.0001)	**0.03**	**0.87**
H - H	L - H	0.01 – 0.01	0.0005	(-0.0006-0.002)	0.50	0.50

Table 6. Mass (grams) response amongst *Rana pipiens* populations and environments. Column 1 and 2 represents the population/environment identifier; the first 1-letter code is the origin pond the tadpoles came from and the second 1-letter code is the pond the tadpoles were raised in for the duration of the experiment: Low (L), Medium (M) and High (H). P-values are calculated using Tukey HSD Post-hoc Test for Multiple Comparisons of means.

Origin Pond (T) - Transfer Pond (R)	**Origin Pond (T) - Transfer Pond (R)**	**Mean Difference (R-T)**	**Std.Error**	**95% CI**	**p-value**	**Hedges' g**
L - L	L - M	1.8 - 1.34	0.24	(-0.17-0.99)	0.24	1.01
L - L	L - H	1.8 - 1.47	0.22	(-0.25-0.87)	0.50	1.14
L - L	M - L	1.8 - 1.48	0.25	(-0.36-0.87)	0.70	0.43
L - L	H - L	1.8 - 1.48	0.27	(-0.6-0.72)	1	0.35
M - M	M - H	1.64 - 1.53	0.16	(-0.28-0.49)	0.93	0.34
M - M	M - L	1.64 - 1.48	0.2	(-0.32-0.65)	0.87	0.34
M - M	H - M	1.64 - 0.85	0.15	(-0.39-1.15)	**<0.001**	**2.38**
M - M	L - M	1.64 - 1.34	0.18	(-0.13-0.77)	0.27	0.78
H - H	H - M	1.34 - 0.85	0.16	(0.02-0.82)	**0.03**	**1.46**
H - H	H - L	1.34 - 1.66	0.23	(-0.09-0.19)	0.31	1.35
H - H	M - H	1.34 - 1.53	0.16	(-0.65-0.16)	0.41	0.92
H - H	L - H	1.34 - 1.47	0.16	(-0.56-0.24)	0.76	0.69

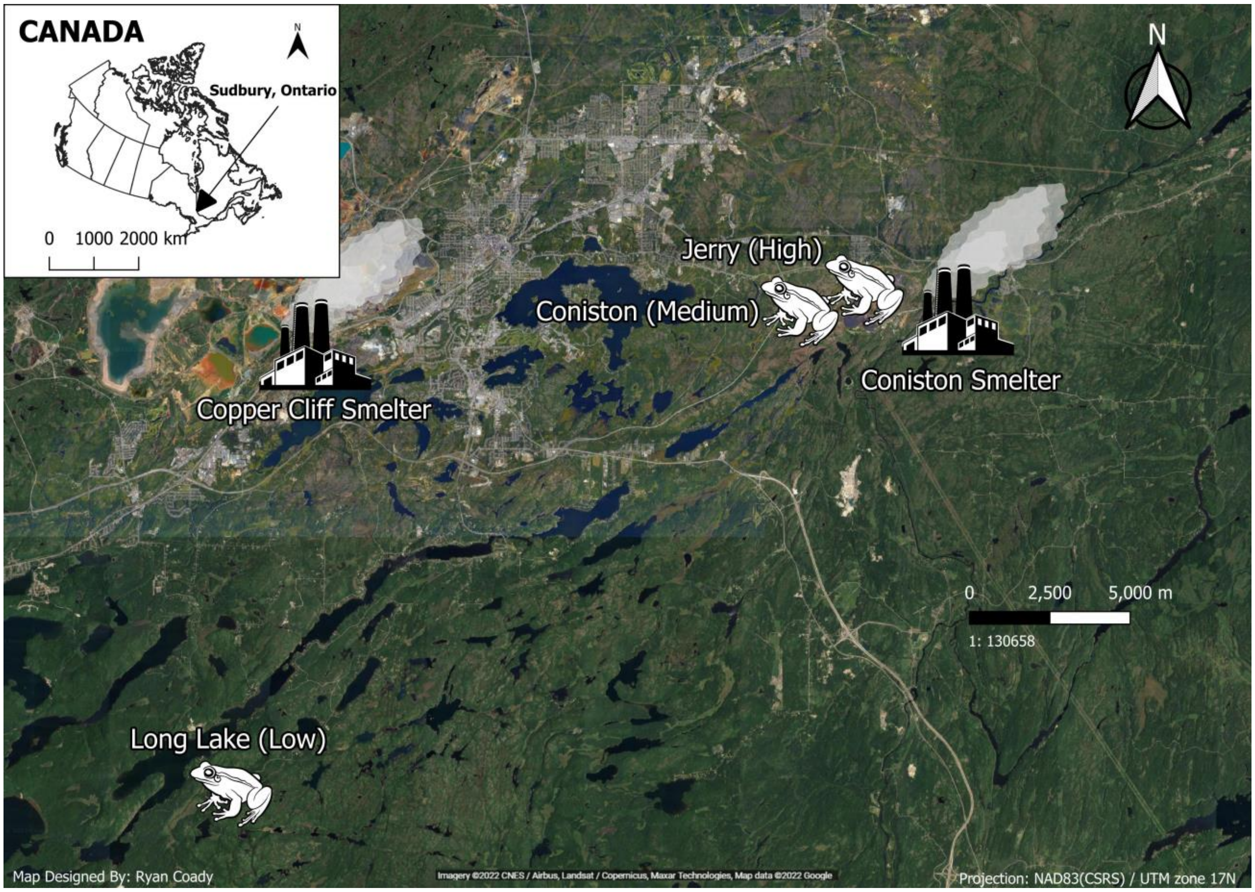

Figure 1. Locations of wetlands with different levels of toxic metals where tadpole populations were sampled, within the Greater Sudbury region. Tadpole populations are indicated with frog symbols: Long Lake (Low), Coniston (Medium) and Jerry (High). Smelter locations are indicated with factory symbols.

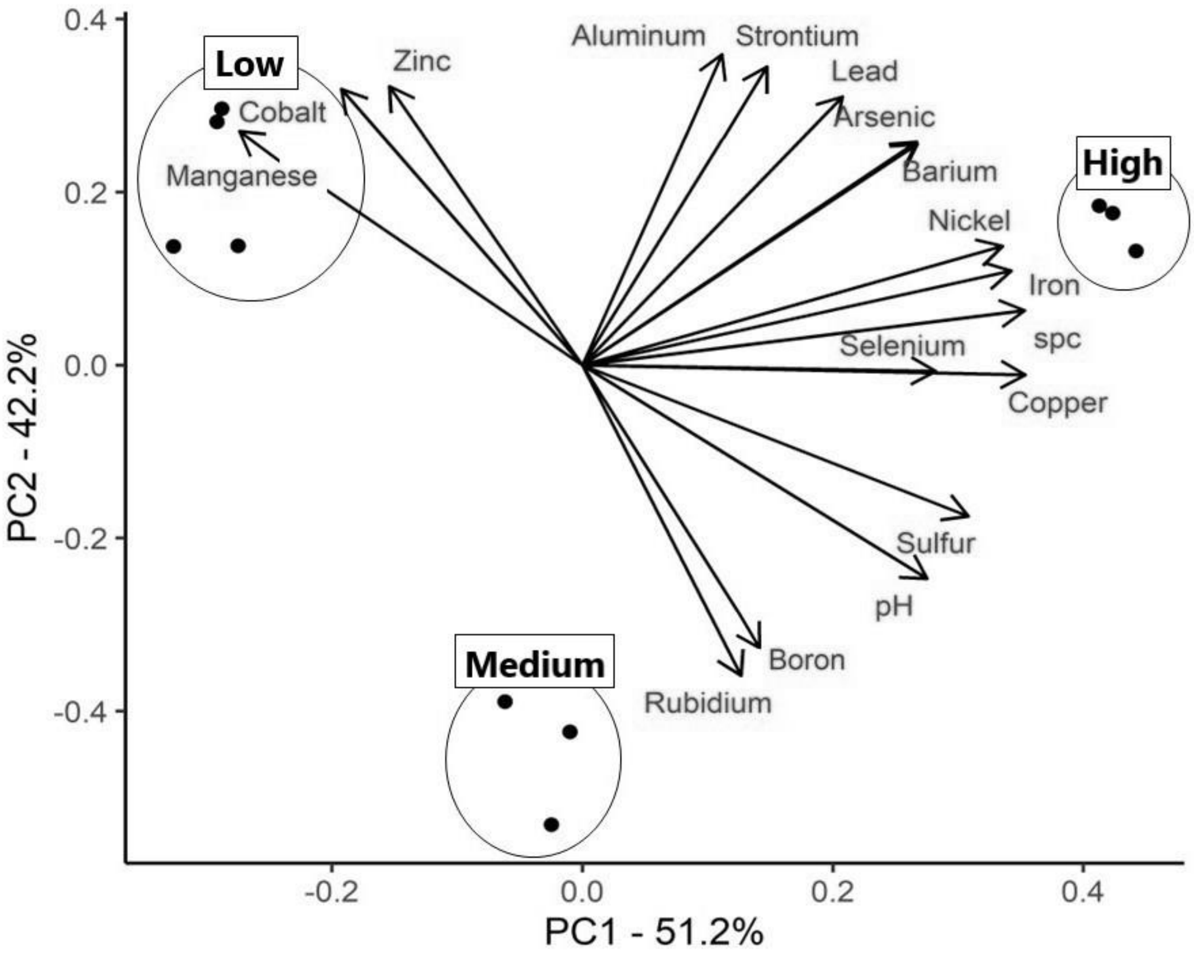

Figure 2. Plot of the first two PC-axes associated with metals and water parameters at the three wetlands. PC1 explains 51.2% and PC2 42.2% of the variance in trace element and water quality of the aquatic environments (93.4% total). The two rearing environments Low and Medium are primarily separated from the third rearing environment High along PC1, which was associated with copper, spc (specific conductance), iron, nickel, selenium, pH, barium, arsenic, and manganese, in sequential order. See Appendix D for PC eigenvalues, variance, and loading scores.

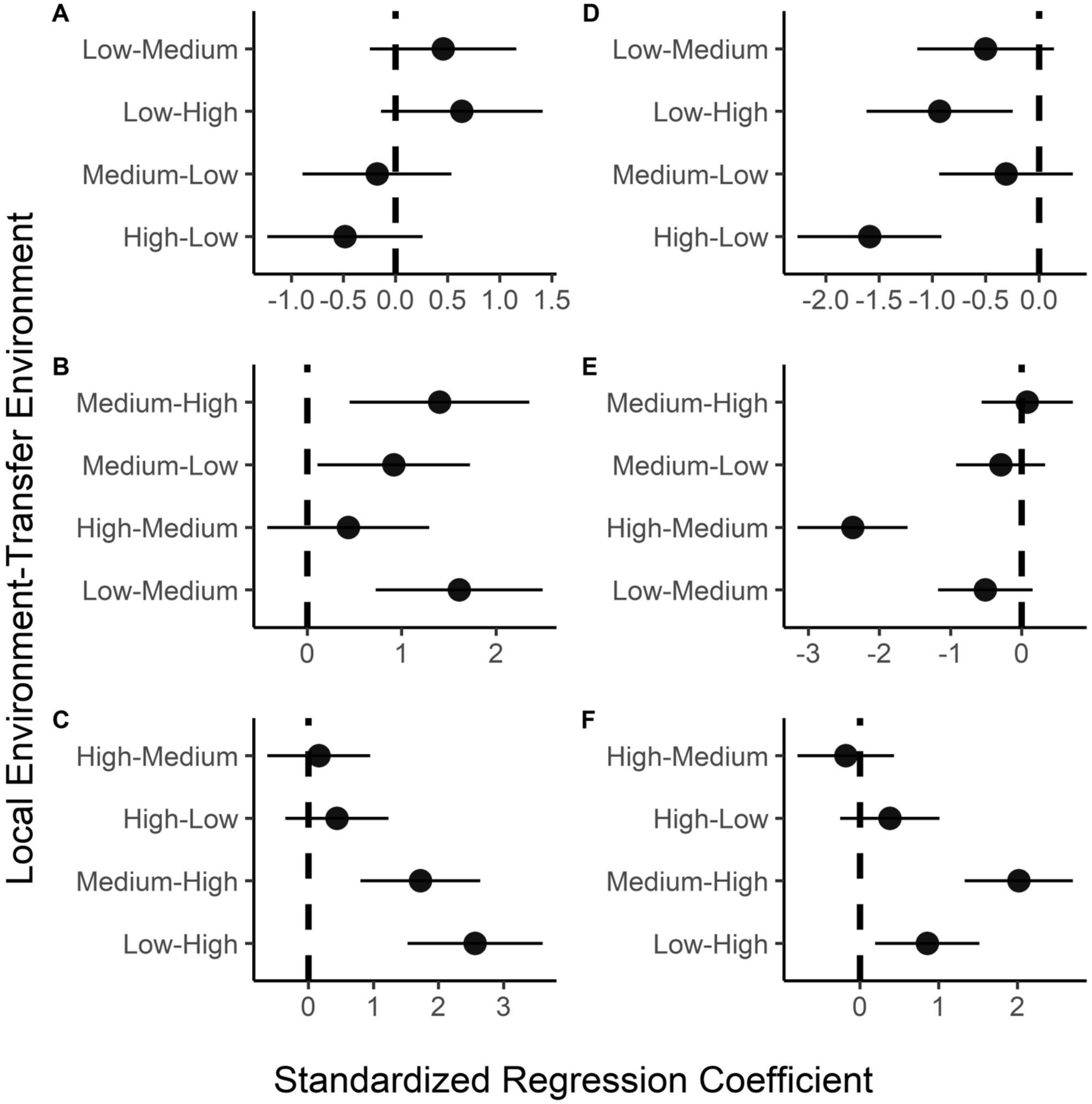

Figure 3. Risk of mortality for tadpoles with regards to their origin vs. transfer environment using multivariate cox regression models. The Standardized Regression Coefficient (β) and the 95% CI relate to hazard, with a positive coefficient indicating a higher risk of mortality and a negative coefficient indicating a lower risk of mortality. The dotted line on each figure represents the reference group. The environment where tadpoles were transferred to and the origin populations are represented as Low, Medium, and High. The letter at the left corner of each individual figure represents the temperature of the transfer environment; A, B and C represent 21°C, while D, E and F represent 25°C.

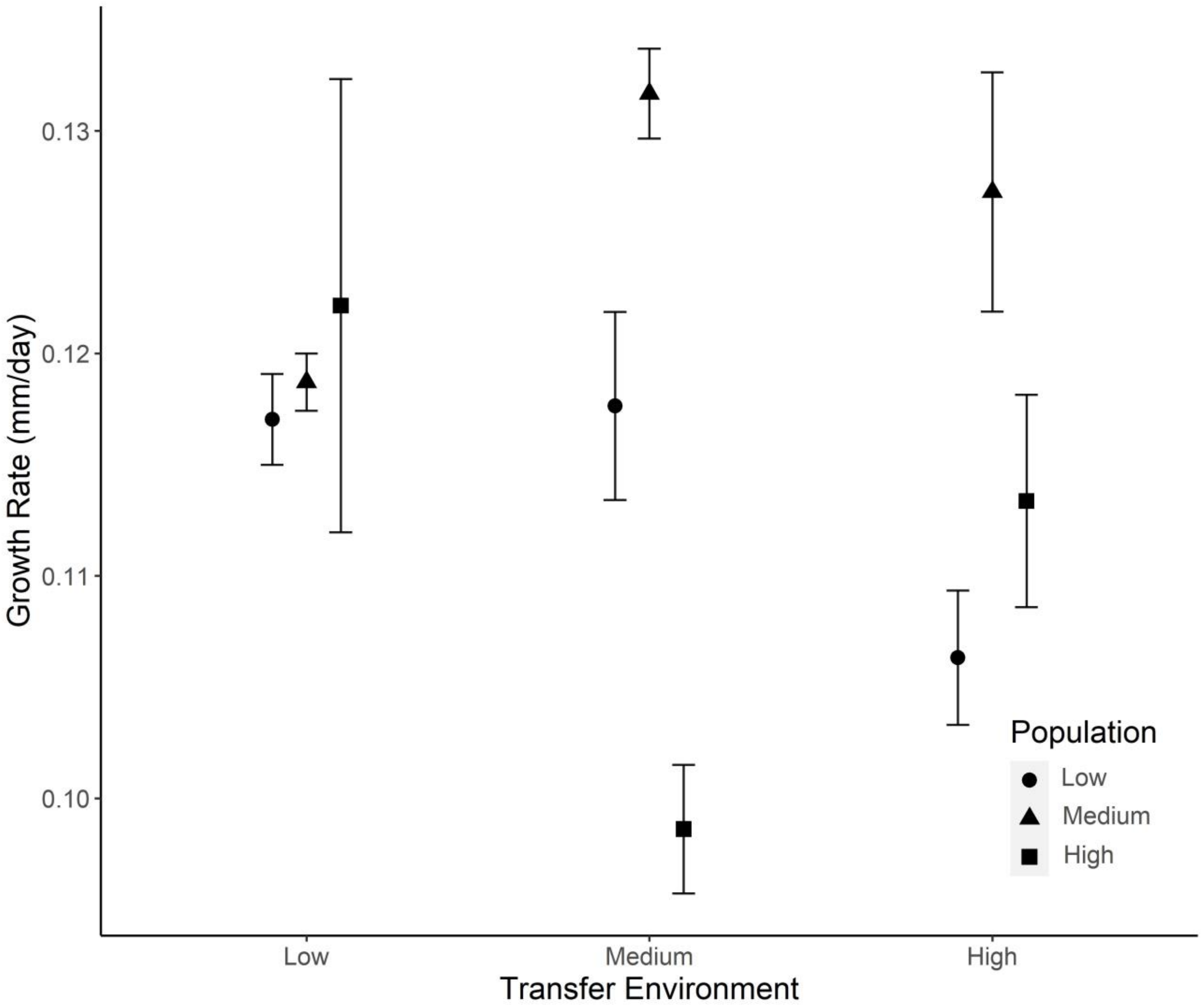

Figure 4. Tadpole length (SVL) mean growth rates (mm/day) and standard error between populations and transfer environments. The x-axis depicts the environment where tadpoles were transferred to Low, Medium and High. The three origin populations are represented as Low (circle), Medium (triangle) and High (square). Horizontal mean comparisons represent tadpoles from a single population transferred into the three different transfer environments. Vertical mean comparisons represent tadpoles from each of the three populations transferred into one of the transfer environments.

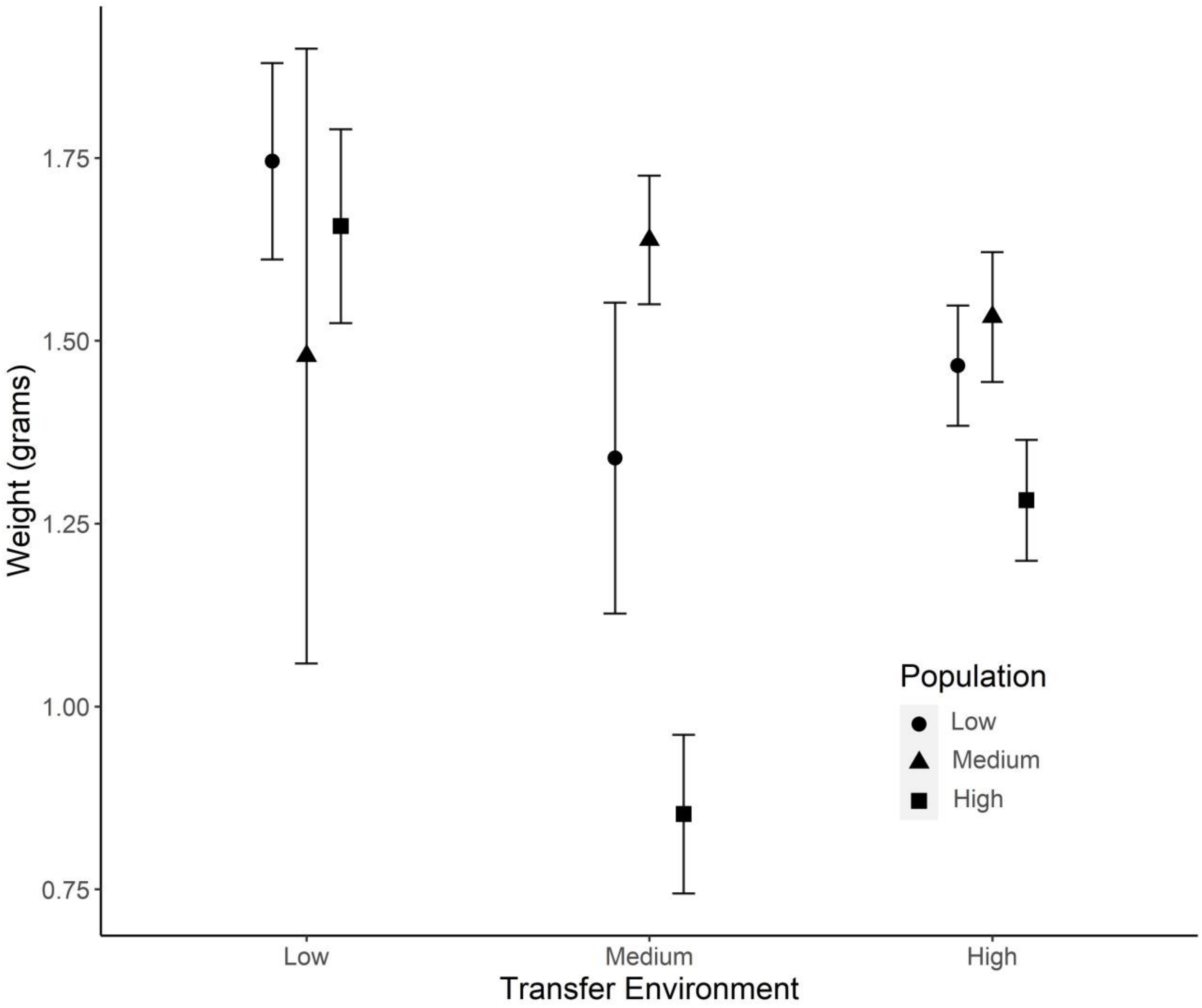

Figure 5. Tadpole mass (grams) means and standard error between populations and transfer environments. The x-axis depicts the environment where tadpoles were transferred to Low, Medium and High. The three origin populations are represented as Low (circle), Medium (triangle) and High (square). Horizontal mean comparisons represent tadpoles from a single population transferred into the three different transfer environments. Vertical mean comparisons represent tadpoles from each of the three populations transferred into one of the transfer environments.

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Appendices

Appendix A. Summary of water quality parameters at each wetland and major element concentration (ug/L – micrograms per litre). Specific Conductivity was measured in microsiemens per centimeter (uS/cm), temperature adjusted conductivity was measured in micrometres per centimeter (um/cm). Values for Coniston (Medium) and Jerry (High) are the average of 3 water samples, values for Long Lake (Low) are the average of 4 water samples.

	Low	**Medium**	**High**
pH	5.77	6.19	6.16
Dissolved Oxygen (%)	81.7	84.8	91.4
Salinity	0.1	0.11	0.16
Conductivity (uS/cm) - Actual	124	139	194
Conductivity (um/cm) - Adjusted for Temperature	205	229	328
Calcium (ug/L)	3333	3630	4837
Magnesium (ug/L)	930	2497	1973
Potassium (ug/L)	610	2467	1010
Sodium (ug/L)	2020	5150	32167

Appendix B. Principal component loadings of water quality parameters and dissolved metals in environmental water samples taken at each wetland. Specific Conductance (spc) was measured in µS/cm and trace element concentrations as ug/L.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**	**PC10**
Al	0.1069	0.3436	-0.1544	-0.2332	0.3023	0.2386	0.1810	0.0015	-0.6260	0.0537
As	0.2524	0.2440	-0.0257	0.0971	-0.2192	-0.2681	0.0962	-0.1659	0.0070	0.1367
Ba	0.2529	0.2415	-0.0515	0.1993	-0.1021	-0.2313	-0.2587	-0.4256	-0.0652	-0.1274
B	0.1344	-0.3086	-0.2736	-0.5196	-0.3886	-0.1285	-0.1104	0.0197	-0.1623	0.0718
Co	-0.1820	0.3016	-0.0448	-0.2870	-0.0064	0.1739	-0.8120	0.0885	0.1328	-0.1026
Cu	0.3344	-0.0109	0.0208	0.1101	0.3242	0.1101	-0.1141	-0.0277	0.1350	0.1175
Fe	0.3238	0.1030	0.0063	0.0751	0.2041	0.0064	-0.0822	0.4181	-0.1266	-0.1952
Pb	0.1965	0.2932	-0.0982	-0.0214	-0.2063	0.6640	0.1786	-0.2805	0.3758	0.1252
Mn	-0.2510	0.2485	-0.0039	-0.0461	-0.1773	0.1347	0.0685	-0.0411	-0.4067	0.1053
Ni	0.3171	0.1300	-0.0445	0.0597	0.0248	-0.0988	-0.0944	0.0153	-0.1467	-0.2270
Rb	0.1198	-0.3395	-0.2727	-0.1810	0.0676	0.0219	-0.0652	-0.5217	-0.1274	-0.0097
Se	0.2666	-0.0067	0.8084	-0.4730	-0.0898	0.0190	0.0719	-0.0728	-0.0080	-0.0481
Sr	0.1414	0.3302	-0.1432	-0.0595	-0.3793	-0.2632	0.0698	0.3312	0.1037	0.4110
S	0.2914	-0.1652	-0.3175	-0.1839	-0.0850	0.2197	0.1772	0.3326	0.1762	-0.3573
Zn	-0.1458	0.3047	-0.1848	-0.4706	0.4729	-0.3716	0.2427	-0.1231	0.3733	-0.0166
pH	0.2603	-0.2335	-0.0057	-0.0077	0.3004	0.0567	-0.2183	0.0885	-0.0043	0.6948
spc	0.3339	0.0595	-0.0227	0.0699	-0.0043	-0.1739	-0.0450	-0.0786	0.0030	-0.1854

Appendix C. Summary of trace element concentrations (ug/L) at each wetland: Low (L1, L2, L3 and L4), Medium (M1, M2, M3) and High (H1, H2, H3). Minimum detection limits (MDL) are also indicated.

	MDL	**L1**	**L2**	**L3**	**L4**	**M1**	**M2**	**M3**	**H1**	**H2**	**H3**
Al	1	44	48	28	29	12	10	14	46	46	47
Sb	0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
As	1	1	1	1	1	<1	<1	<1	2	2	2
Ba	1	8	8	8	8	5	5	6	14	13	14
Be	0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Bi	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
B	2	3	3	3	3	5	6	4	4	4	4
Cd	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Ce	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Cs	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Cr	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Co	0.1	0.4	0.4	0.3	0.3	0.1	<0.1	<0.1	0.2	0.1	0.2
Cu	1	6	6	4	5	13	11	15	24	24	24
Eu	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ga	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Fe	20	220	244	140	160	296	200	330	888	903	932
La	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Pb	0.1	0.2	0.2	0.1	0.2	<0.1	<0.1	<0.1	0.3	0.3	0.3
Li	5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Mn	1	20	21	18	19	2	1	2	4	3	4
Hg	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Mo	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ni	1	26	27	23	24	26	25	27	66	65	67
Nb	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Rb	1	2	2	2	2	4	5	4	3	3	3
Sc	1	<1	<1	<1	<1	<1	<1	<1	1	1	1
Se	0.2	<0.2	<0.2	<0.2	<0.2	0.3	<0.2	<0.2	0.3	0.3	0.2
Si	600	1500	1600	1400	1400	2200	2300	2200	2700	2700	2700
Ag	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Sr	1	21	21	20	20	13	14	12	24	24	25
S	800	1200	1200	930	1200	2100	2700	2000	2400	2600	2600
Te	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
TI	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Th	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Sn	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Ti	1	<1	<1	<1	<1	<1	<1	<1	2	2	2
W	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
U	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
V	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Y	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Zn	1	4	4	3	2	1	1	1	2	2	2
Zr	1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

Appendix D. Eigenvalues, Variance and Cumulative Variance of principal component loadings of water quality parameters and dissolved metals in environmental water samples taken at each wetland.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**	**PC10**
Eigenvalue	8.7041	7.1712	0.4591	0.3531	0.1889	0.0738	0.0411	0.0058	0.0028	<0.001
Variance	0.5120	0.4218	0.0270	0.0208	0.0111	0.0043	0.0024	0.0003	0.0002	0.0000
Cumulative Variance	0.5120	0.9338	0.9609	0.9816	0.9927	0.9971	0.9995	0.9998	1.0000	1.0000