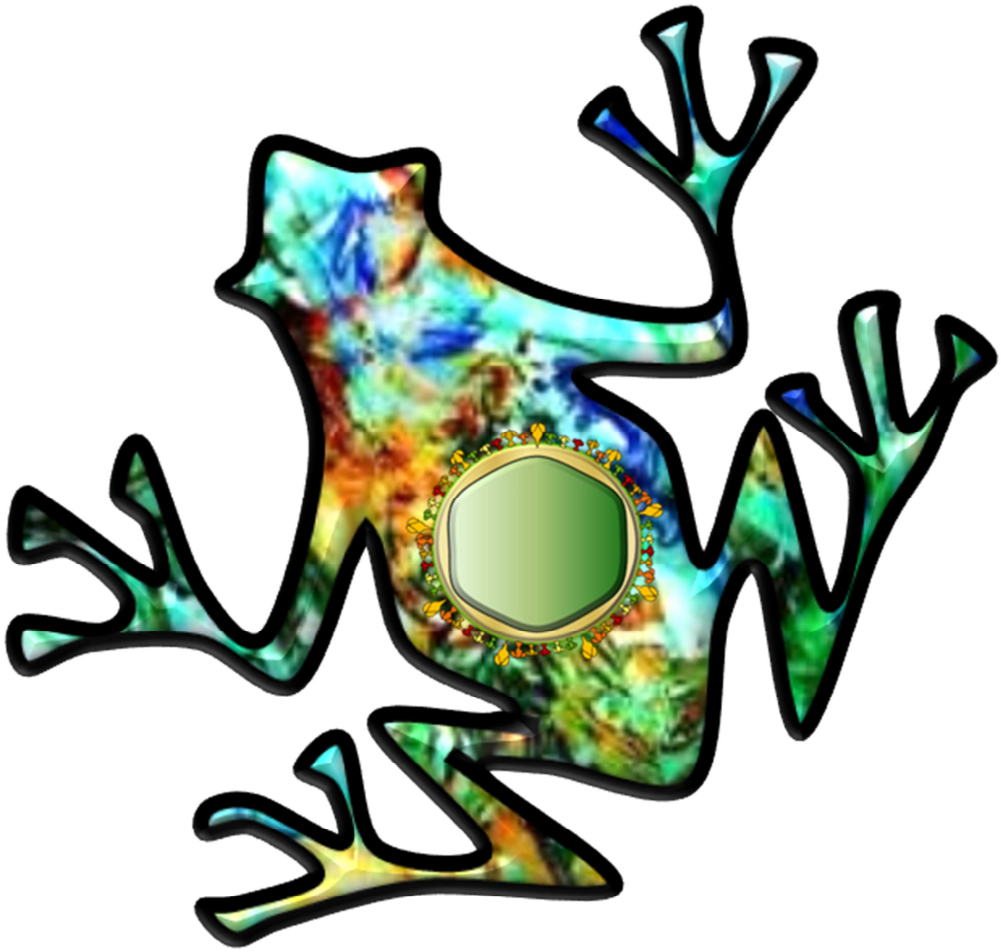
[…] promoting the dialogue between Host-Pathogen Evolutionary-Ecology and Disease applied Epidemiology in order to allow the achievement of an integrative eco-evolutionary framework that stimulate a novel epidemiological approach with regards to Emerging Infectious Diseases.

Evolution and Ecology of an Amphibian Emerging Infectious Disease: a context-dependant approach of ranavirus virulence in *Lithobates (Rana) pipiens*



Pierre Echaubard

Thesis presented as a partial requirement in the Doctor of Philosophy (Ph.D) in Boreal Ecology

School of Graduate Studies
Laurentian University
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Title of Thesis
Titre de la thèse **EVOLUTION AND ECOLOGY OF AN AMPHIBIAN EMERGING INFECTIOUS DISEASE: A CONTEXT-DEPENDANT APPROACH OF RANAVIRUS VIRULENCE IN *LITHOBATES (RANA) PIPIENS***

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Nom du candidat **Echaubard, Pierre**

Degree
Diplôme **Doctor of Philosophy**

Date of Defence
Date de la soutenance JUN 29 2012

Department/Program
Département/Programme **Boreal Ecology**

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October 16, 2012
Date

ABSTRACT

Host-pathogen investigations have conceptually evolved during the last two decades, from a basic and descriptive approach to a current hypothesis-driven and a more theoretical discipline shaped by evolutionary biology. Our deeper understanding of the elements influencing the mutual selective pressures that the host and the pathogens exert on each other, together with recent conceptual advances, currently position this field of research at the frontier between ecology and evolution. Recent theoretical considerations define host-pathogens systems as an evo-eco mosaic comprised of evolutionary and ecological attributes in turn underlying the context-dependent nature of the system dynamic. Therefore, investigations of host-pathogen interactions should integrate the diversity of the systems drivers by using an integrative approach in order to elucidate both coevolutionary trajectory and epidemiological dynamic of the system. In this thesis, such a framework is used to investigate Amphibian/ranavirus interactions. Ranaviruses are emerging pathogens known to have caused amphibian die-offs on five continents with the greatest number of reported mortality events documented in North America and Europe. Despite an increasing understanding of ranaviral disease properties, ranavirus disease dynamics in the environment remain poorly understood. For instance, the influence of potential abiotic and biotic mechanisms including temperature, local landscape features, larval developmental stages, host density and genetic variability as well as genotypic interactions between the host and the pathogen has on the prevalence and virulence of the virus remains to be elucidated. In order to improve our knowledge regarding these specific determinants of ranaviral disease, I designed a combination of manipulative laboratory experiments and a field mensurative survey using the ranid amphibian *Lithobates (Rana) pipiens* as the host model for this system.

I observed that populations of amphibian hosts inhabiting urbanized landscapes suffered from significant decline in genetic diversity in turn promoting the accrued infection by the ranavirus (manuscript 1). Complementary analysis using two amphibian host species, *L.pipiens* and *L.sylvaticus*, and three ranavirus strains revealed significant variation among hosts for their susceptibility to ranavirus, and significant variation among ranavirus strains for infectivity. I also showed that specific amphibian/ranavirus interactions might have a tighter coevolutionary history than other combinations, resulting in sharper mutual coadaptations and the potential for frequency-dependent selection to operate in this system. However, the coevolutionary trajectories in this host-pathogen system are dependent on the temperature conditions in which the interaction takes place. Amphibian/ranavirus interactions outcomes

are therefore temperature, host, and pathogen genotype-dependent suggesting that the range of infection outcomes in this system is potentially large (manuscript 2). Further, I observed that increasing animal holding density is detrimental for host fitness as mortality rate is higher, day of death earlier, development longer, and growth rate significantly lower when tadpoles are experimentally exposed to ranavirus in high holding density situations. These results paralleled a linear increase of detrimental effects when ranavirus doses increased in low density conditions, with control tadpoles having a significantly higher overall relative fitness. However, this pattern was not observed in high density conditions, where the effects of increasing ranavirus dose were limited, revealing non-trivial density-dependence of virulence expression (manuscript 3). Finally, ranavirus infection rate varied with the host developmental stage as the host immune system clears the infection over the course of individual host development. However the intensity of the clearing depends on both the timing and number of ranavirus exposures (manuscript 4). Overall the results described in my thesis suggest that ranavirus virulence depends on a diversity of ecological, epidemiological, and evolutionary determinants. The underlying complexity of ranavirus epidemiological dynamics clearly shows the relevance of a context-dependent approach.

ACKNOWLEDGEMENTS

I would like to express my warmest thanks and appreciation to David for his excellent guidance throughout my Phd studies. His mind was open enough to accept me the way I was, and established a very balanced and transparent dialogue contributing to the concretisation of this work. Not only these ideal working conditions promoted the success of my research but also were at the origin of our friendship. Thanks for all of this. Thanks to Bruce Pauli for his supervision and insights. Coming from a different background than me he was instrumental in complementing my reasoning with more applied aspects, giving my project a broader reach. I thank Albrecht and Charles, members of my thesis committee, for their patience and advices during the last 4 year. Thanks to my two external reviewer Dr Francisco Diaz-Mitoma and Dr Matthew Fisher for their promptness in reviewing my thesis and their very relevant feedbacks.

Working towards a Phd degree is a long run and often necessitates the complementary expertise of labmates or colleagues. Similarly the Phd journey is challenging emotionally and unexpected results or situations can easily discourage a Phd or MSc candidates. The presence of companions committed to the same objective is very appreciated as we can provide mutual support and encouragements. With this respect I would like to address a very warm thank you to all my lab-mates and friends, members of the GEARG family, Andrée, Jöel, David, Vincent as well as Kirsten. Jöel, thank you for your reliability and impressive organisation, it was very very relieving to know I could count on you, whatever the situation, to insure the proper finallisation of the experiments. Despite the fact that we came from relatively different backgrounds you managed to adapt yourself to my approach and open your mind enough to make our collaboration and friendship work. Little David, I consider your friendship a very privilegiate gift and your expertise a very valuable one. I admire you for your immense knowledge about nature and the humility you express despite this. As a rather theoretical scientist but nature lover, I was really stimulated by your naturalistic inspiration and you encouraged me to pursue in my quest to better know our surroundings in order to better know myself. Many thanks. Vincent, it is amazing how much potential you have. Not only in research but as an explorer of life in general. Your openness and curiosity are obviously the ingredients that will make you succeed in your journey…and that re-ignited my flame. Thank you for that. Andrée, your friendship and enthusiasm were such a support and inspiration. Your ability to always place your research (in and outside the lab) in context, the link to the whole, is amazing and make you being an wonderful friend and a very valuable collegue…you reminded me to always look beyond the academic finality of my work and see how my research should be applied and used for the sake of the community! Gracias para

todo! Thanks Kirsten, Darryl and Sophie for the constructive discussions and the share of our graduate preocupations.

Despite all the support from my inspired lab mates and supervisors, nothing could have happened without the logistic support and relevant advices of our wonderful biology staff. Tom, Lorraine, Brigitte, Luc, Suzanne and Diane, thank you very very much for your availability, support, experience and supervision! You make the biology department running and you deserve my Phd as much as me.

Retrospectively, it appears that a Phd could be seen as a dual journey: academic and personal, that brings a lot of emotions, unceretainties, doubts, hopes, amazing opportunities, wonderful encounters, human and non human. A constant emotional support is needed in order to keep surviving, and for that aspect I would like to express my recognition to my family, for their support and love: Brigitte, Mamie Jeanne, maman Nicole, les frero et soeurettes, Christiane, Pierrette, Daniel, Dadou, Christine, Jean-Pierre, Katia et tous les cousins et cousines! Words are not enough to express, to the extent required, my recognition to 'mes peres', Michel et Jean-Jacques who provided me with self-confidence, the thirst for knowldege and the enthusiasm for discovery, ingredients required for an emancipated life… an unvaluable treasure.

All my friends from here and abroad are part of this achievement as they represent or have represented at some point a source of inspiration and love and therefore have helped to built who I am. The French connection: Ben, Jenny, Fx, Fanny, Bertrand, Baptiste, Yohan, Arnaud, Loon, Sybille et Fred, Perrette, Quentin, Laura, Emeric, Brieuc, Hugo, Christophe, Emilie, Sylvain, Claude, Hortense, Kherveen; The swiss alpine team: Tobi, Camillo, Nat, Otti, Ben, Léonie, Igor; The maple leaf family: Nico, Claire et Guigui, Mat et Krista, Fabio, Taus, Lindsay, Deb and Dan, Mirna, Pauline, Erin, Greg, Raph, Mathilde, Pierre, Kevin, Allan, Joe, Roach, Chelsea, Margaret, Angèle, Kylie, Kayla, Nina and Mike, Jamie and Sarah, Sophie, Kornel et Justine et bien sur Julie, Dominique, Marion, Arielle et Lolo!

Last but certainly not least. While I was defending my thesis, talking under everyone`s attention, someone was sitting silently at the back of the auditory. Galie, my wonderful wife deserves at least twice as more as me this Phd. She followed me across the ocean in a completely unknown academic world, in a place at first that was not necessarily keeping all its promises. Despite all of that, she stayed, supported me with her love and balanced my rational mind with her limitless and healing creativity and intuition. At some point her intuition was to offer us an ultimate source of inspiration, our baby luna who day after day make this life so enjoyable…what an amazing journey.

Merci

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CHAPTER 1:

General Introduction

1. Context-dependent explanatory framework for host-pathogen interactions: a conceptual baseline

The integration of ecology and evolutionary biology has been approached several times over the last few decades but remains an "elusive synthesis" (Sterelny 2005). The advantages of a union of the two sciences are, however, clear. For community ecologists, incorporating evolutionary mechanisms in their studies, either conceptually or in mathematical models (Day 2005), simply may allows more variation in community structure and dynamics to be explained. From the point of view of evolutionary biology, considering ecological context provides more dimensions for understanding the outcomes of interactions among species. While evolutionary theory largely deals with the potential consequences of fitness differences among individuals and populations, the source of these fitness differences lies within the ecological interactions of a community (Sober 1984 cited in Sterelny 2005). In Hutchinson's (1965) words the evolutionary play exists within an ecological theater and without the context of community ecology, the ideas of evolutionary biology lack a real-world test.

In this context, if there are such advantages to a union of evolutionary biology and community ecology, why has this synthesis proven so elusive? One of the main reasons is that evolutionary and ecological processes exist at very different time scales (Holt 2005, but see Carroll *et al.* 2007) and also at very different spatial scales, what Sterelny (2005) calls the "grain problem." The differences between evolutionary biology and ecology in terms of both time and spatial scales are perhaps the most commonly identified reasons for a lack of synthesis between the two disciplines. Rapid evolution may quickly change the frequency of traits in a population but for the most part, traits emerge and are shaped over many generations. In contrast, ecological processes largely occur within the scale of a single generation.

Nevertheless, while the fitness benefits of traits might be the end result of tuning over the long-term,the main tool of evolution, natural selection, is the integrated process of many ecological events. In the lives of individuals there are many competing constraints that may be affected by different traits and the integration over this multidimensional matrix in the long term is part of the process that may allow fitness advantages to accrue for particular traits. Furthermore, species do not exist in isolated populations but in metapopulations that are interconnected to varying degrees. Even for environmental conditions that appear to be broad

scale, there is no guarantee that selective pressures are the same across different metapopulations or even within the sub-habitats in the area of a single population. The result of this graininess (Sterelny 2005) is that immigration among metapopulations may dilute the effects of local selection by introducing alleles that were either neutrally selected or perhaps were selected in different ways.

The practical result of these differences in time and spatial scales is a separation in the focuses of evolutionary biologists and community ecologists. Evolutionary biologists tend to study traits in isolation of as many ecological interactions as possible that might dilute the fitness effects that are their focus. Community ecologists, on the other hand, tend to think of traits as fixed because, within the myriad of simultaneous ecological interactions in which they work, evolutionary change in any trait is unlikely to be manifest. The resulting compartmentalization of the disciplines might even result in questioning the actual importance of bridging community ecology and evolutionary biology as there is a lack of clear demonstration under what circumstances it is important for biologists to take into account both community interactions and evolutionary theory (Johnson and Stinchcombe 2007).

I suggest that host-parasite interactions may provide this closer linkage and serve as an ideal model for the synthesis of evolutionary biology and community ecology. Host-parasite investigations (H-P hereafter) have conceptually evolved during the last two decades, from a basic and descriptive approach to the current hypothesis-driven and more theoretical discipline shaped by evolutionary biology (Poulin 2007). Our deeper understanding of the mutual selective pressures that the host and the parasites exert on each other, together with recent conceptual advances, currently position this field of research at the frontier between ecology and evolution. In particular hosts and their parasites are different species thus are independent units of natural selection, yet their lives are strongly interwined. The parasite is indirectly subject to the same myriad of day-to-day ecological interactions that affect the host. Thus, ecological realities for the host strongly and at short time scales affect the parasite. In other words, the strength and specificities of the selective pressures involved in a given interaction may promote rapid evolution, within the timeframe of ecology, thus allowing the interplay between evolution and ecologically significant processes to be more clearly seen (Neuhauser *et al.* 2003) possibly circumventing Sterelny's (2005) grain problem. Such a convergence between evolution and ecology renders H-P interactions very dynamic over time and space, fluctuating along a continuum ranging from mutualism to strict parasitism (Renaud and de Meeûs 1991) depending on given ecological conditions. For these reasons, HP systems should be seen as evo-eco mosaic made of a heterogeneous mix of ecological and

evolutionary determinants resulting in a context-dependent coevolutionary dynamic (Fig. 1; the complete argument for this conceptual framework is provided in manuscripts 5 and 6 (see Appendix)). In this thesis, I use this generalist context-dependent framework as a conceptual guideline for the specific investigation of the ranavirus/*Lithobates pipiens* interaction.

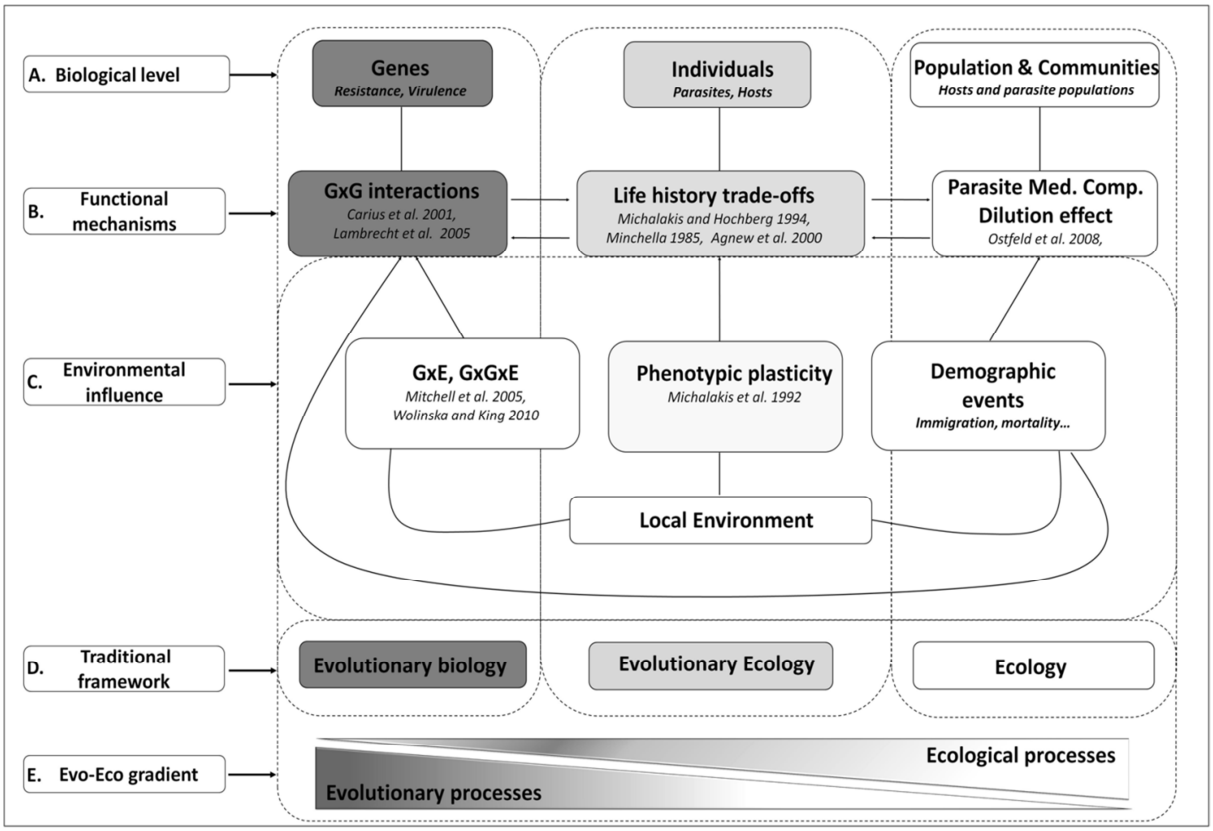

Fig. 1. Schema of the context-dependent approach. Each of the three biological levels (A) under which H-P interactions can be investigated is characterized by specific functional mechanisms determining the outcome of the interaction between the host and the parasite (B). The influence of such mechanisms can in turn be modulated by external environmental features (C) so that the traditional framework under which investigations regarding the different levels of organization are carried (D) is now reconsidered as a conceptual evo-eco gradient (E).

2. The Amphibian model

a. Amphibian populations declines and extinction

According to the Millennium Ecosystem Assessment, the greatest threat facing biodiversity is the combined effect of accelerate climate change and landscape modification due to agricultural development, urbanization and forestry practices (MA 2005, Lee and Jetz 2008). Rapid population declines and extinctions of species following the widespread destruction of natural habitat have been reported with respect to biodiversity across the natural

world (Brook *et al.* 2003) and up to 50% of species are predicted to be lost in the next 50 years (Pimm and Raven 2000, Thomas 2004). As part of this overall biodiversity crisis, many amphibian populations are in decline accross the world (Blaustein *et al.* 1994). The severity and the large geographic scale of the amphibian decline in conjunction with their ecological importance make the subject a conservation topic of high priority which has been suggested to be one of the greatest issues of the 21st century (Daszak *et al.* 1999).

The Global Amphibian Assessment (GAA) has shown that over 1856 (32%) of the 5743 amphibian species known worldwide are at risk of going extinct, 2468 (43%) are experiencing some form of population decrease and 1552 (25%) are stable. A reported 122 amphibian species have become extinct since 1980 (Stuart *et al.* 2004). These observations underline the extent of the problem, reinforcing the necessity for action. In addition, these reports suggest that many unknown causes are involved in addition to well-known threats to biodiversity (Houlahan *et al.* 2000, Pounds *et al.* 2006, Fig 2).

Alford and Richards (Alford and Richards 1999) attempted to review and summarize the causes of amphibian declines. They recognized 6 major causes plus their interactions. Among them, ultraviolet radiation has been investigated as a cause that reduces survival or hatching success of amphibian embryos (Ovaska *et al.* 1997). In particular it seems that significant variation among species in levels of photolyase, a photoreactivating DNA repair enzyme that repairs UV-B damage, is correlated with hatching success (Blaustein *et al.* 1994).

Second, introduction of invasive alien species has been shown to impact amphibian communities through ecological interactions. For example, predation by introduced predatory fish in ponds can lead to amphibian extinction (Fisher and Shaffer 1996).

Third, habitat modification and fragmentation is well documented and has been viewed as the major threat to biodiversity and especially to amphibian populations (Becker *et al.* 2007). Fragmentation of habitats and the subsequent gene flow interruption is recognized as a major threat to amphibian populations. For instance, fragmentation of habitats by a highway drastically diminished genetic diversity and polymorphism of local *Rana dalmatina* populations (Lesbarrères *et al.* 2006). Furthermore, general urban building leads inevitably to population perturbations or even extinction when not appropriately done. The dramatic declines of Limon Harlequin frog populations in Ecuador, is an example where unsuitable improvement of a road, continues to weaken this threatened species (La Marca *et al.* 2005).

Fourth, the specific physiology and anatomy of Amphibians also makes them susceptible to water quality changes. Water pollution and acidity have been shown to have major impacts

on amphibian distribution, reproduction, embryo and larval development and mortality (Alford and Richards 1999).

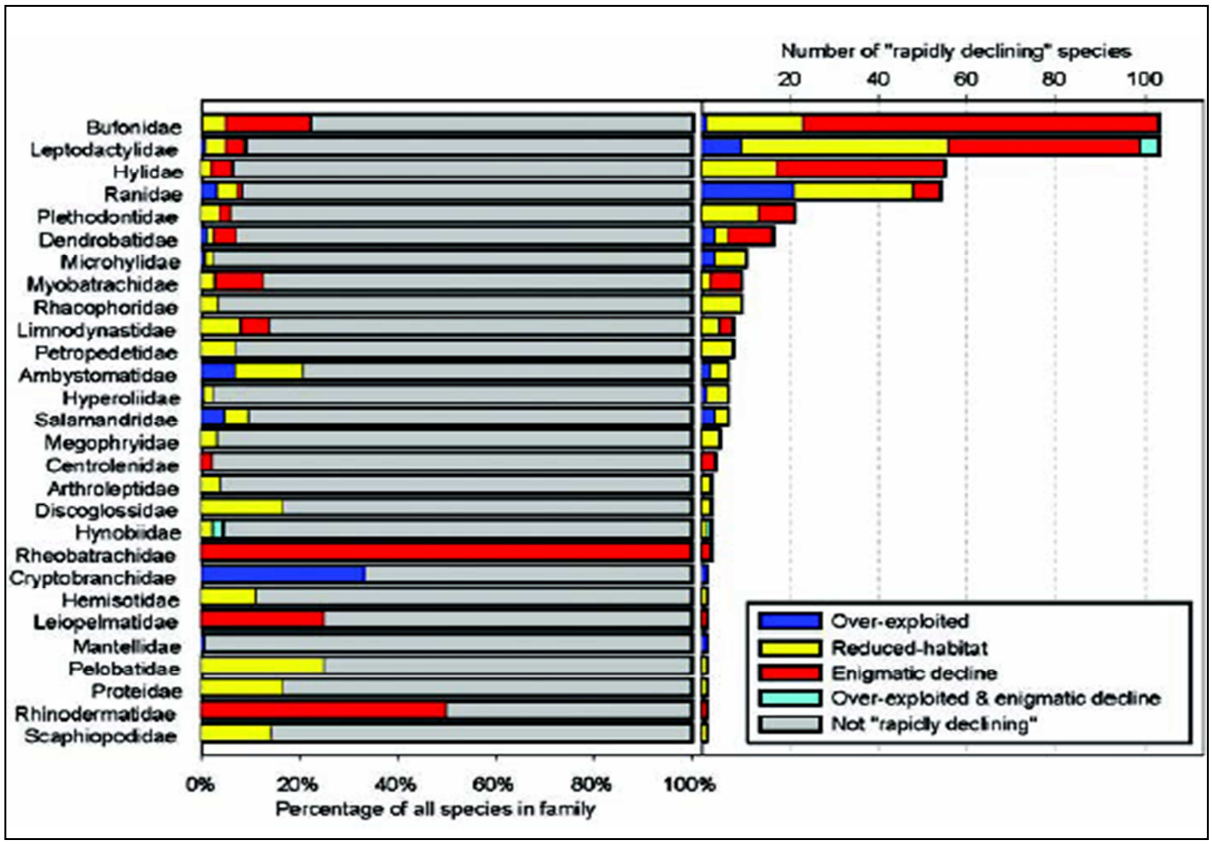

Fig. 2. Percentage of Amphibian species per family that are facing rapid declines. Causes for decline are also detailed. Notice the significant amount of enigmatic declines (in red). (from Stuart *et al.* 2004)

Fourth, the specific physiology and anatomy of Amphibians also makes them susceptible to water quality changes. Water pollution and acidity have been shown to have major impacts on amphibian distribution, reproduction, embryo and larval development and mortality (Alford and Richards 1999).

Fifth, climate change (i.e. modification of temperature, precipitation and associated changes in hydrology) has profound impacts on amphibian populations. Increased temperature and decreases in summer precipitation may affect amphibians in Canada (Ovaska *et al.* 1997) and increasing solar radiation may directly affect amphibian mortality and modify amphibian ecology and life history (Donnelly 1998). Climate change acts as a leading process that combines or influences all factors together and multiplies their own independent effects (Plowright *et al.* 2008, Brook *et al.* 2008).

Sixth, in recent decades some declines have been characterized as enigmatic (Fig. 2). We know now that the common cause for these declines is was linked to Emerging Infectious

Diseases (EIDs), including chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* and infection by ranavirus. Both have been since recognized as causing unprecedented mass die-off in amphibian populations.

b. Amphibian EIDs and the study system

i. Amphibian EIDs

Evidence has shown that Emerging Infectious Diseases (EIDs); diseases which have recently increased in range or incidence in a given area (Daszak *et al.* 1999, Daszak 2000); particularly the Chytrid fungus *Batrachochytrium dendrobatidis (Bd)* and the *Ranavirus (Rv)*, are causing mass die-offs in amphibian populations (Cheng *et al.* 2011, Miller *et al.* 2011). *Bd*, responsible for the chytridiomycosis, is suggested to have been distributed worldwide either by human induced translocation of hosts (the novel pathogen hypothesis; (Laurance *et al.* 1996, Weldon *et al.* 2004, Rachowicz *et al.* 2005) or through the expansion of its infectious potential (the endemic pathogen hypothesis; (Carey 1993). Chytridiomycosis is considered as one of the biggest threats faced by amphibian species as chytridiomycosis-caused mass die-offs have been observed in all continents where amphibians are found (Daszak *et al.* 1999, Fisher *et al.* 2012). The disease has been reported to occur since 1960 in North America and has been implicated in population declines of the Northern Leopard frog (Carey *et al.* 1999, Muths *et al.* 2003). An increasing number of studies are dealing with the effect of this pathogen on the life history of its amphibian hosts, and many are also considering human-induced modifications as promoter of its spread (St Amour *et al.* 2008). Although knowledge on *Bd* is growing, little is known about its mode of transmission, its epidemiology within amphibian communities, its physiology, its survival in the wild, or factors that precipitate amphibian casualties (Piotrowski *et al.* 2004). However the mechanism by which it becomes a fatal infection has been recently elucidated: the Chytrid induces a severe electrolyte imbalance that cause the frog's heart to stop (Voyles *et al.* 2009). While historically less investigated than Bd, a group of iridoviruses in the genus *Ranavirus* is currently becoming increasingly surveyed and studied as it is believed to be responsible for an increasing number of die-offs in amphibian populations (Lesbarrères *et al.* 2011). The later is the focus of my thesis.

Ranaviruses were first isolated from *Lithobates* (formerly *Rana) pipiens* in the mid-1960s (Granoff *et al.* 1965). Viruses in the family *Iridoviridae*, which contains five genera, infect invertebrates (*Iridovirus* and *Chloriridovirus)* and ectothermic vertebrates (*Ranavirus*, *Megalocytivirus*and *Lymphocystivirus*; (Chinchar *et al.* 2009). Ranaviruses are large, double-

stranded DNA viruses (ca. 105 kbp, 150 nm diameter; Williams *et al.* 2005), with a distinctive icosahedral shape that is frequently visible in the cytoplasm of infected cells as paracrystalline arrays in electron microscopic images (, see Gray *et al.* 2009 and Miller *et al.* 2011 for recent reviews). Despite recent methodological advances, precise taxonomic identification of ranavirus based on morphology or serology is difficult and a consensus is still needed (Chinchar 2002). While specialists agree on the presence of three distinctive species of ranavirus (Frog virus 3 (FV3), Regina ranavirus (RRV, ATV), Santee-Cooper ranavirus (SCRV), it is worth noting that observations of ecological niches have to be considered in order to resolve whether two isolates should be strains of the same species (for example FV3), or actually different species, despite their proximity at the molecular level. For example, FV3 and RRV are 90% identical within parts of several major genes, but they infect different animal species, suggesting potential relevant ecological divergences.

Ranaviruses as emerging pathogens are known to have caused amphibian die-offs on five continents (Gray *et al.* 2009, Miller *et al.* 2011). The greatest number of reported mortality events has been in North America and Europe, resulting in population declines in several cases (Teacher *et al.* 2010). Ranaviruses are known to infect at least 72 amphibian species in 14 families (Miller *et al.* 2011). The majority of cases have been in the family *Ranidae*. Susceptibility to ranavirus infection varies widely among species (Schock *et al.* 2008, Hoverman *et al.* 2010, Echaubard *et al.* manuscript 2). Of 19 North American species tested, wood frog (*Lithobates sylvaticus*), gopher frog (*L. capito*) and Eastern spadefoot toads (*Scaphiopus holbrookii*) were the most susceptible to ranavirus (Hoverman *et al.* 2010, Haislip *et al.* 2011). Ranavirus-induced mortality is rare in adult amphibians whose immune system is more developed than in larvae (Robert *et al.* 2005, Miller *et al.* 2011). Susceptibility of larvae to ranavirus varies depending on the developmental stage of the larvae (Haislip *et al.* 2011, Echaubard *et al.* manuscript 4). The maturation of the immune system together with the number and severity of virus exposures influence the severity of the resulting disease (Echaubard *et al.* manuscript 4).

In terms of transmission, ranavirus can transmit horizontally among individuals via indirect and direct routes (Gray *et al.* 2009). Transmission of ranaviruses has been documented via exposure to contaminated water (Brunner *et al.* 2004, 2005, Pearman *et al.* 2004), by direct contact with infected individuals (Brunner *et al.* 2007), and by exposure to fomites such as virus-contaminated sediment (Harp and Petranka 2006). Ingestion of infected tissue either through necrophagy, coprophagy or cannibalism is another effective transmission route (Jancovich *et al.* 1997). Exposure to infected individuals in water for three hours without

contact can result in transmission (Robert *et al.* 2011), and only brief direct contact is needed to cause infection (Brunner *et al.* 2007). Typically, ingestion of the virus results in faster mortality than exposure via virus particles in the water (Hoverman *et al.* 2010). During an outbreak, it is likely that ranavirus infects hosts via multiple routes of horizontal transmission; although vertical transmission of iridoviruses has been shown in invertebrates (Hunter *et al.* 2001), it has not been demonstrated for ranaviruses infecting vertebrates (Drennan *et al.* 2006). Attempts to test for vertical transmission have yielded mixed results (Brunner *et al.* 2004, Duffus *et al.* 2008).

ii. Lithobates (Rana) pipiens

The Northern Leopard frog, *L. pipiens*, is distributed widely North-America (Fig. 3), but declines in Western Canada and Ontario started occurring during the 1970s (Wilson *et al.* 2008).

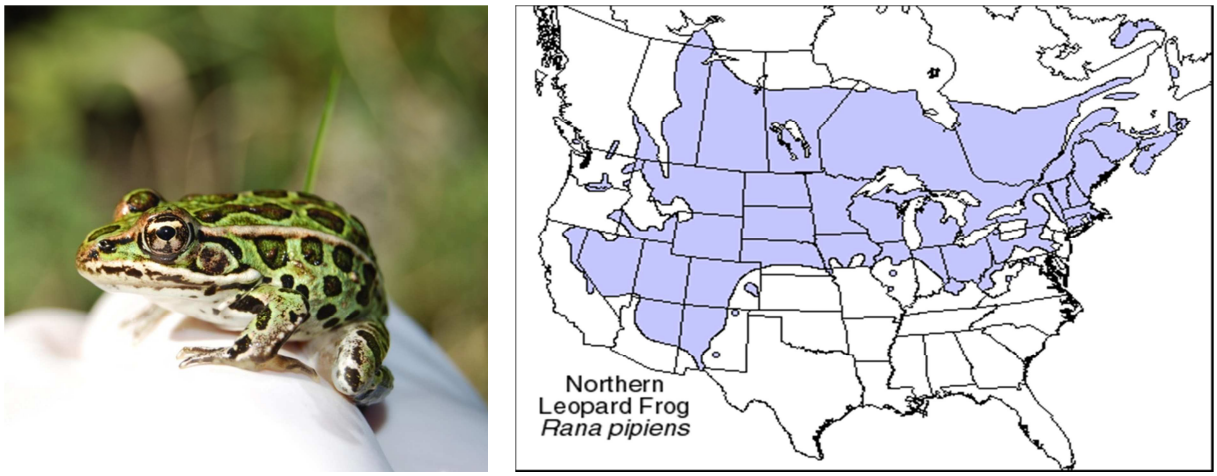

Fig 3. The Northern Leopard Frog *Lithobates pipiens* is widely distributed in North America (shaded areas). Recently the species has suffered a decline in the western part of its range.

The decline is thought to have been caused by airborne pollution from the United States falling in the form of acid rain. Many populations of Northern Leopard Frogs have not yet recovered from these declines in Ontario, and the western populations are COSEWIC-listed. *L. pipiens* is common and widespread throughout southern areas but appears to have declined in northern parts of the province (Wilson *et al.* 2008).The species is normally found in a variety of habitats, from permanent ponds, swamps, marshes, and slow moving streams throughout forested to open and urban areas. Both *Batrachochytridium dendrobatidis* and ranavirus are known to infect *L. pipiens* (St Amour *et al.* 2008) and ranavirus was first described in this species, however little is known about their epidemiology. Furthermore, the ecology and behavior of *L. pipiens*, especially its dispersion, its co-occurrence with other

species which act as reservoirs for pathogens (i.e., species that carry the pathogen but do not suffer clinical signs of infection (Brunner *et al.* 2004, Duffus *et al.* 2008, Schock *et al.* 2008)), its sensitivity to human modification (i.e. especially road density (Eigenbrod *et al.* 2008) and its large geographic distribution, make this species a good model for the study of ranavirus epidemiology.

iii. Ranavirus

Ranaviruses are members of the genus *Ranavirus* which belongs to the family Iridoviridae. Iridoviridae are large viruses (120-200nm) possessing icosahedral symmetry and linear, double-stranded DNA genomes (Williams *et al.* 2000). The viral genome encodes approximately 100 proteins and, reminiscent of some bacteriophage genomes, is circularly permuted and terminally redundant. In contrast to other virus families, both enveloped and non-enveloped (naked) virions are infectious, although enveloped virions possess a higher specific infectivity (Braunwald *et al.* 1979). The family Iridoviridae is currently divided into five genera (Table 1). The five iridovirid genera can be partitioned into two groups based on the hosts they infect and the level of genomic methylation (Chinchar et al. 2005). Members of the genera *Iridovirus* and *Chloriridovirus* infect invertebrates (i.e., insects, crustaceans, etc.) and lack a highly methylated genome. In contrast, members of the *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus* genera infect cold-blooded vertebrates such as fish, amphibians, and reptiles and possess genomes in which approximately 25% of the cytosine residues are methylated by a virus encoded DNA methyltransferase (Willis and Granoff 1980). However, there is at least one ranavirus, Singapore grouper iridovirus (SGIV) lacking the DNA methyltransferase gene and cannot methylate its DNA (Song et al. 2004).

The division of the family into genera was initially based on biological properties of the viruses (e.g., host range, GC content of the genome, serology, virion morphology, particle size, histopathology, and clinical signs of disease). GC content varies markedly and ranges from 27%–29% (irido- and lymphocystiviruses) to 48%–55% (chloriridо-, rana- and megalocytiviruses) and does not correspond to either the GC content of the host or the methylation status of the virus. Not unexpectedly, codon usage is influenced by the overall GC content, but the basis for the marked difference in GC content among different viral genera is unknown (Schackelton et al. 2006; Eaton et al. 2007; Tsai et al. 2007). Recent analyses of the amino acid sequences of the major capsid protein (MCP) and other viral proteins confirmed these taxonomic divisions and indicated that species within a genus generally shared high levels of identity/similarity.

Table 1. Taxonomy of the family Iridoviridae (Chinchar *et al.* 2009)

Genus	Viral species (strain*)	Tentative species
Iridovirus	*Invertebrate iridescent virus 6* (IIV–6), IIV–1	Anticarsia gemmatalis iridescent virus (AGIV), IIV–2, –9, –16, –21, –22, –23, –24, 29, –30, –31
Chloriridovirus	*Invertebrate iridescent virus* 3 (IIV–3)	
Ranavirus	*Frog virus 3* (FV3), [tadpole edema virus, TEV; tiger frog virus, TFV] *Ambystoma tigrinum virus* (ATV), [Regina ranavirus, RRV] *Bohle iridovirus* (BIV) *Epizootic haematopoietic necrosis virus* (EHNV) *European catfish virus* (ECV), [European sheatfish virus, ESV] *Santee-Cooper ranavirus*, [Largemouth bass virus, LMBV; doctor fish virus, DFV; guppy virus 6, GV–6]	Singapore grouper iridovirus (SGIV); Grouper iridovirus (GIV) Rana catesbeiana virus-Z (RCV-Z)
Megalocytivirus	*Infectious spleen and kidney necrosis virus* (ISKNV) [Red sea bream iridovirus, RSIV; African lampeye iridovirus, ALIV; Orange spotted grouper iridovirus, OSGIV; Rock bream iridovirus, RBIV]	
Lymphocystivirus	*Lymphocystis disease virus 1* (LCDV–1)	
Unclassified	White sturgeon iridovirus (WSIV)	LCDV–2, LCDV-C, LCDV-RF

Typically, members of the same viral genus show more than 70% similarity within the major capsid protein (MCP) at the amino acid level, whereas species from different genera show less than 50% similarity (Do et al. 2005a, 2005b). Although identification of iridovirid genera has been relatively straightforward, identification of individual viral species has proven to be more difficult because of high levels of sequence identity/similarity within the MCP and other highly conserved proteins among members of the same genus. For example, several ranavirus species show greater than 90% amino acid identity within the highly conserved MCP. Thus, differentiation of viral species is based on multiple criteria including viral protein profiles, DNA restriction fragment length polymorphisms (RFLPs), host species infected, clinical signs (i.e., histopathology and gross pathology), and differences in nucleotide and amino acid sequences (Mao et al. 1997; Chinchar and Mao 2000; Chinchar et al. 2005).

Most of what is known about iridovirus replication is based on studies of frog virus 3 (FV3), the type species of the genus *Ranavirus*. Excellent reviews provide detailed description of the infection mecanics (Chinchard 2002. Chinchard 2009) and therefore only a

brief summary of the main steps of the ranavirus replication cycle are given hereafter. There are two routes by which the virus can enter the cellular cytoplasm of its host. The virions are either enveloped by receptor mediated endocytosis of the cellular membrane, or naked (lacking membrane structure) virion particles enter by fusion between the lipid bilayer of the cellular membrane. Once inside the cytoplasm the virions shed their cellular membrane and their DNA is transported into the nucleus of the host cell. Replication of FV3 DNA occurs within the host nucleus. Viral DNA then exits the nucleus and concatemers form while inside the host cytoplasm. Viral mRNA and protien synthesis also occurs within the host cytoplasm and capsids form around the new viral DNA at the assembly site. The new virions will either build up within the host cytoplasm or exit the cytoplasm via budding in order to spread to other host cells (FIG 4).

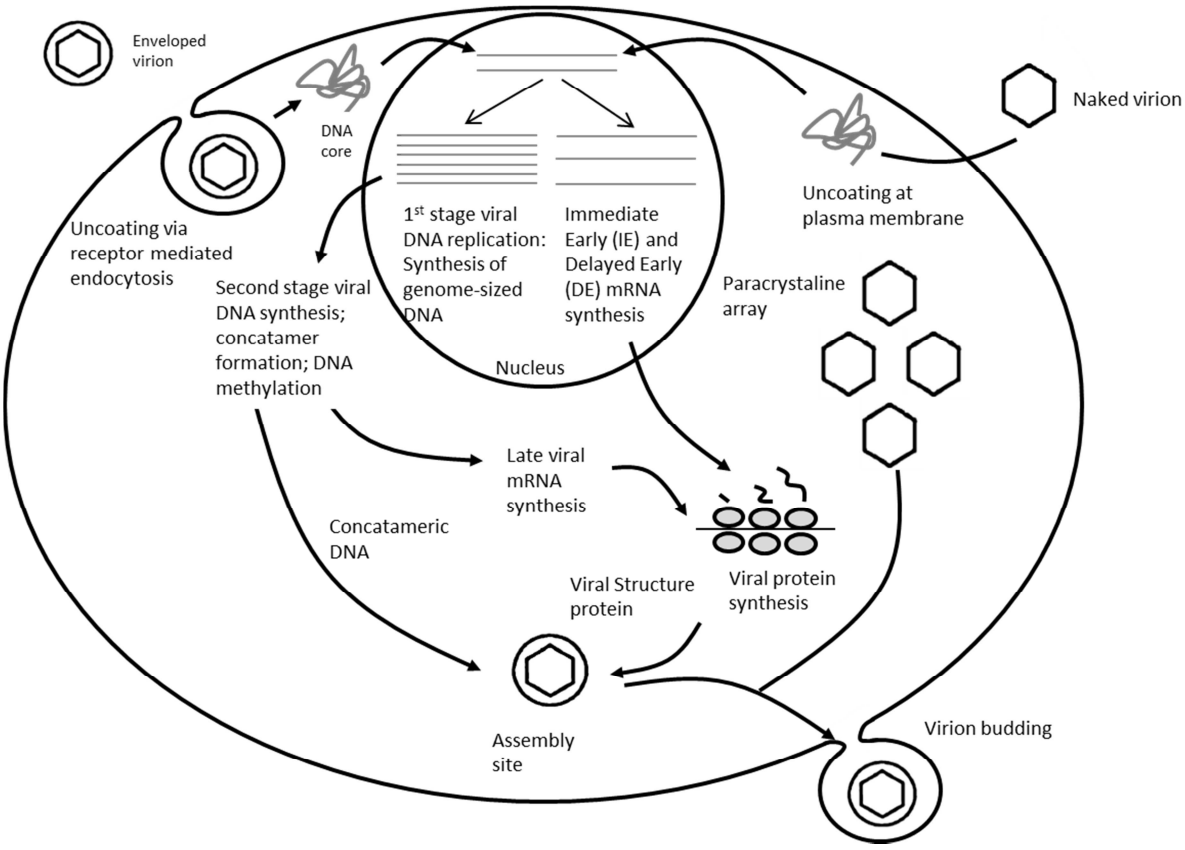

Fig. 4. Summary of the ranavirus replication cycle (adapted from Chinchar 2002).

Although there is little specific information about the host immune response to iridovirid infection, both humoral and cell-mediated immunity likely play roles in the prevention of, and recovery from, virus infection. For example, *Xenopus* mount effective B cell and T cell responses against FV3 infection (Morales and Robert 2007; Maniero et al. 2006), and

antibodies targeted to other ranaviruses can be detected in infected frogs (Zupanovic et al. 1998a). Moreover, vaccination is effective in preventing disease due to RSIV infection (Caipang et al. 2006a, 2006b), and prior infection of bullfrog tadpoles with relatively avirulent FV3 protects against subsequent challenge with virulent RCV-Z (Majji et al. 2006). At the molecular level, ISKNV infection has been shown to induce in mandarin fish a variety of putative antiviral proteins, including homologs of a VHSV-induced protein, Gig2, viperin, Mx, CC chemokines, the immunoglobulin heavy chain etc. (He et al. 2006). As the immune systems of lower vertebrates become better understood, it is likely that their role in protecting fish, amphibians, and reptiles from iridovirid infections will become clearer and utilized to develop more effective vaccination strategies.

iv. Relevance of a context-dependent approach for the study of amphibian ranavirus

Despite an increasing understanding of ranaviral disease determinants, ranavirus dynamics in the environment remain to be elucidated. Our understanding of ranavirus ecology is obscured by environmental contingencies that result in context-dependant disease dynamics (Lesbarreres *et al.* 2011, Daskin and Alford 2012). The interdependent nature of disease determinants renders the investigation of ranavirus-induced mortality a challenge and the influence of potential abiotic and biotic mechanisms such as temperature, larval development, density and competition for resources on the prevalence and virulence of the virus remain to be explored (Lesbarreres *et al.* 2011). Amphibian ranaviral disease appears to be related to ecological change and therefore can be mediated through complex and large scale processes that are not amenable to traditional reductionist approaches regarding causal inference (Plowright *et al.* 2008). Consequently, it is necessary to apply an integrative approach where ecological, evolutionary and epidemiological concepts are used together for the understanding of ranavirus/amphibian interactions (Daskin and Alford 2012). The explanatory framework developed at the beginning of the current chapter therefore becomes a relevant conceptual tool to use in order to elucidate ranaviral disease dynamics and predict coevolutionary trajectories. This framework proposes to bridge conceptual compartments and to bring together ideas from different backgrounds (i.e. ecology, evolutionary biology and epidemiology) in order to encompass the multidimensionality that characterizes host-pathogen relationships.

3. Objectives and organization of the thesis

In line with the conceptual considerations described in the above sections, I developed a combination of multifactorial manipulative and mensurative experiments in order to improve

our understanding of ranavirus ecology and evolution. The diagram shown in figure 4 represents the current state of our understanding with regard to Amphibian/ranavirus interactions and incorporates the specific research objectives of this thesis. Specifically, I articulated my research around two main objectives:

1- To determine how host genetic variability in the wild correlates with ranavirus occurrence and how host-ranavirus genotypic interactions are modulated by the environment (e.g., temperature; Chapter 2).

2- To determine the relationships between amphibian host life history and ranavirus epidemiological parameters (Chapter 3).

This thesis consists of four chapters, of which this introduction (Chapter 1) and the final conclusion (Chapter 4) provide the context for the research and highlight the main findings and implications. In chapter 2, manuscript 1 investigates the tripartite interconnection between habitat fragmentation, *L. pipiens* genetic diversity and ranavirus occurrence based on incidence of infection in *L. pipiens*. The hypothesis underlying this study is that the fragmentation of habitats leads to a decrease in genetic variability by genetic drift and gene flow interruption which in turn might increase Northern leopard frog population susceptibility to diseases.

Manuscript 2, examines how genotype by genotype interactions between hosts and their pathogens (G_H x G_P) are modulated by the temperature in which the infection develops. The role of the environment in modulating host-pathogen genotypic interactions is described then as G_H x G_P x E interactions. For the purpose of this investigation, I designed a fully factorial laboratory experiment to investigate the outcome of the interaction between two common North American frog species (*L. pipiens* and *L. sylvaticus*) and three strains of the ranavirus in a variable environment.

Chapter 3 of the thesis is composed of manuscripts 3 and 4. In the research described in manuscript 3, I investigated the influence of varying host density on ranavirus virulence. In a factorial experiment, I exposed *L. pipiens* tadpoles to different concentrations of ranavirus and analyzed the effect of host holding density on certain life-history traits, namely survival, growth rate, developmental stage and number of days from virus exposure to death. This experiment was designed to document how the net fitness of organisms may be shaped by ecological context and emphasized the necessity of examining the direct/indirect costs and

benefits balance to fully understand host-pathogen interactions. In manuscript 4, I described research examining the susceptibility of *L. pipiens* embryos to infection by ranavirus and quantified the hatchling infection rate. I investigated the infection carry-over rate between hatchlings and later stage tadpoles and assessed the virulence of the virus in relation to the time of infection and number of exposures.

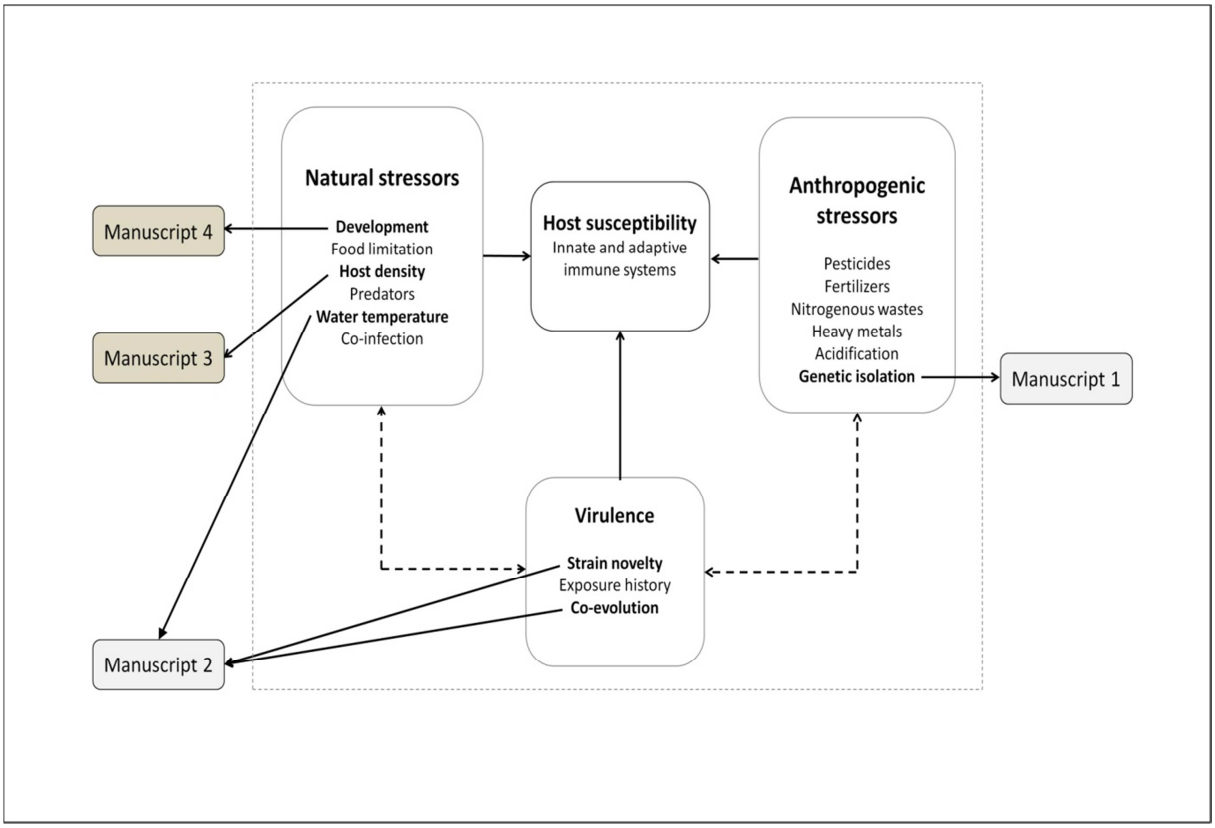

Fig. 5. Conceptual model of ranavirus ecology and research objectives. The diagram represents factors influencing host susceptibility and pathogen virulence for which further investigations are needed for a proper understanding of amphibian/ranavirus interactions. Solid and dotted lines are known and unknown effects respectively. The research objectives of this thesis along with their corresponding manuscripts are inserted and linked by black arrows to the specific topics they are investigating (adapted from Gray *et al.* 2009).

The main findings of the thesis are discussed in Chapter 4, which highlights the key factors that modulate ranavirus virulence and describes the significance and the implications of the research conducted. At the end of the thesis, the Supplemental Material section includes manuscripts 5 and 6 to complement the Introduction and present in details the conceptual foundations of this thesis. In manuscript 5, I advocate the application of a context-dependant approach for the investigation of host-pathogen interactions. Manuscript 6 presents a bibliometric analysis I conducted in order to document the fact that host-parasite investigations as a field of research stands at the frontier between ecology and evolution, further advocating the proposed context-dependent approach I undertook in this thesis.

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CHAPTER 2:

Host genetic variability and host-pathogen genotypic interactions in amphibian ranaviral disease

Manuscript 1

Habitat fragmentation, host genetic diversity and pathogen prevalence: a landscape genetics approach in the Leopard Frog-ranavirus system

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Contributions:

PE: design, data collection, analysis and writing

LW: data collection, analysis

BP: writing

DL: design, analysis, writing

Abstract

Amphibians are the vertebrate group facing the most severe decline worldwide. Habitat fragmentation and the occurrence of Emergent Infectious Diseases have been suggested to be two of the main determinants associated with population declines. Considering both the individual severity of each of these two threats and the potential for synergistic effects between them, the objective of the present study was to investigate the tripartite interconnection between habitat fragmentation, genetic diversity and ranavirus occurrence in Ontario populations of *Lithobates (Rana) pipiens*.

We sampled *L. pipiens* populations in 18 Ontario locations for toe-clips from which we extracted DNA. We then typed each samples at seven polymorphic microsatellite loci (Rpi100, Rpi101, Rpi102, Rpi 103, Rpi 105, Rpi 106, Rpi 108) and quantitified both population genetic diversity and genetic structure. Each individals was also screened for ranavirus presence by PCR**.** Additionally, in order to quantify habitat quality with regard to frog biology, we built a landscape matrix incorporating seven indexed habitat quality variables and five fragmentation /connectivity estimators for each location sampled. We used GIS as a tool for merging geographic information on road density, buildings and forest cover, rail presence, types of aquatic habitats, amount of water edges and land use layers. Canonical Correspondence Analyses and multiple regressions were used to quantify the relationships between environmental variable, genetic diversity and structure and ranavirus occurrence.

Our results indicate that leopard frog genetic diversity is higher when the habitat is characterized by a lower fragmentation degree but also by a high density of forest, and an overall high habitat quality, suggesting that fragmentation is not soly responsible for the diminution of the genetic diversity but habitat suitability play a significant role in *L. pipiens* population genetics dynamic. Additionally we observed that significant environmental variables retained as predictors, such as railway and measures of landscape fragmentation induced non-trivial patterns of allelic frequencies. Finally, while we did not observed significant direct relationship between ranavirus occurrence and environmental variables, we noted a higher prevalence of ranavirus in population of Leopard Frog characterized with low genetic diversity. Altogether our result suggest that the extent of landscape fragmentation and habitat deterioration, in addition to have direct consequences in terms of individual survival, might also result in free-ranging populations having lower genetic diversity and higher risk of extinction, particularly upon future exposure to emerging pathogens.

Introduction

Decades of investigations have shown both empirically and theoretically that local extinction and recolonization processes can have significant consequences for the genetic structure of populations. In the metapopulation context (Levins 1969), defined here as a group of local populations connected by dispersing individuals (Hanski 1998), movements of individuals are of primary importance as they allow allelic migration (i.e gene flow) among subpopulations. Gene flow is a fundamental evolutionary force that contributes to the introduction of new alleles in a population, which can counterbalance population genetic differentiation by via selection, reducing inbreeding depression and countering allelic diversity depletion due to genetic drift, especially in small fragmented populations (Williams *et al.* 2003; Keller & Largiader 2003). Thus, landscape connectivity and the maintenance of gene flow between subpopulations serve together to promote high genetic diversity at the metapopulation level.

The loss of genetic diversity has detrimental effects on individual fitness components such as survival, growth, fecundity and developmental stability (Britten 1996; David 1998; Reed *et al.* 2003; Lesbarreres *et al.* 2005), and may also have important implications for populations susceptible to emergent diseases (Altizer *et al.* 2003). Genetic variability has been shown to reduce host susceptibility to pathogens in captive fish species (Hedrick *et al.* 2001) and to increase pathogen resistance in ants and bumble-bees (Baer & Schmid-Hempel 1999; Hughes & Boomsma 2004). Genetically diverse populations of the topminnow fish are less susceptible to pathogens (Lively *et al.* 1990), and evidence in California sea lion populations that inbred animals have a higher susceptibility to a suite of pathogens (Acevedo-Whitehouse *et al.* 2003). Habitat fragmentation and isolation can thus affect host evolution, pathogen prevalence and host disease susceptibility through the depletion of host genetic diversity. Similarly, host and parasite movement among habitat fragments could be crucial to both parasite persistence, and the spread and maintenance of host resistance alleles (Hess 1996; Thrall & Burdon 2003). These considerations emphasize the complexity of understanding pathogen epidemiology and host susceptibility in natural populations, and suggest thatcareful investigation of host genetic diversity and the proper determination of the environmental factors that modulate such diversity, is required to understand them.

Among vertebrates, amphibians are reported to have the most severe population declines worldwide, with half of the roughly 6000 species described having at least a threatened status (Stuart 2004). Amphibian die-offs and extinctions are mainly due to habitat loss and the

occurrence of Emergent Infectious Diseases (Alford & Richards 1999; Daszak *et al.* 1999). In particular, local anuran presence and abundance has been shown to be affected by forest cover and road density, particularly high traffic roads (Fahrig *et al.* 1995; Lesbarrères *et al.* 2006), as many species require different habitat types for parts of their life cycle and good habitat connectivity for their annual migrations (Wilbur 1980). The necessity to move between habitats to complete their their life cycle means that amphibians are vulnerable to roads. In addition, anuran populations are likely to exhibit metapopulation dynamics (Marsh & Trenham 2000; Pope *et al.* 2000, but see Smith & Green 2005), suggesting a strong potential for gene flow reduction and genetic diversity depletion when landscape connectivity decreases (Johansson *et al.* 2007, Dixo *et al.* 2009).

Additionally, amphibians are known to be particularly sensitive to ranaviruses, virulent pathogen known to infect fish (Mao *et al.* 1997), reptiles (Hyatt *et al.* 2002) and a wide range of amphibian species (Jancovich *et al.* 1997; Daszak *et al.* 1999; Docherty *et al.* 2003). Effects of ranaviruses on amphibians are widespread; they cause disease and mortality at various locations worldwide (Miller *et al.* 2011). The pathogen has been suggested to be synergistically associated with other causes of declines (Plowright *et al.* 2008). Among them, habitat fragmentation leading to population genetic diversity depletion is thought to be a critical factor promoting ranavirus emergence (Pearman *et al.* 2005). For example, Pearman et al (2005) experimentally compared susceptibility of *Rana latastei* populations upon exposure to an emerging strain of ranavirus using a range of natural populations with various degrees of genetic variability. The authors were able to demonstrate the causal link between genetic diversity depletion and mortality risk from the ranavirus, documenting indirectly the link between habitat fragmentation, genetic diversity depletion and pathogen occurrence.

The objective of the present study was to investigate the relationship between habitat fragmentation, genetic diversity and ranavirus occurrence. We hypothesized that the fragmentation of habitats leads to a decrease of genetic variability by genetic drift and gene flow interruption which in turn increases Northern leopard frog population susceptibility to diseases. Considering this hypothesis and what is actually known about the biology of the Northern leopard frog and the epidemiology of the amphibian ranavirus, we make the following two predictions: (1) a positive relationship between degrees of habitat fragmentation and the extent of genetic diversity depletion and, (2) a positive relationship between populations that harbor low genetic variability and pathogen occurrence.

Material and methods

1. Study species

In Ontario, the Northern Leopard Frog, *Lithobates (Rana) pipiens* is probably the most familiar species as it is distributed widely from south to north. Leopard Frogs are found in a variety of habitats from permanent ponds, swamps, marshes and slow moving streams from forest to open and urban areas. This species was once quite common through parts of western Canada until declines started occurring during the 1970s (Wilson *et al.* 2008). In Ontario, *L. pipiens* is common and widespread throughout the southern part but appears to have declined in northern Ontario, as it has in western Canada, presumably due to habitat alteration (Wilson *et al.* 2008). Additionally, Leopard Frogs are known to be vagile, dispersing annually up to 5–6 km (Seburn & Seburn 1998) which makes this species relatively vulnerable to habitat fragmentation by roads and high traffic densities (Carr & Fahrig 2001; Eigenbrod *et al.* 2008) but also by conversion of favourable habitat to pasture or cropland (Mazerolle *et al.* 2005).

Ranaviruses belong to the family *Iridoviridae*, which is composed of viruses able to infect diverse species of ectothermic animals such as amphibians, fish and reptiles (Chinchar 2002; Hyatt *et al.* 2002; Gray *et al.* 2009). Within the anurans, frog virus 3 (FV3) was first isolated from *Lithobates pipiens* (Granoff *et al.* 1965), and is used to study the ecology and mechanisms of the ranavirus group (Gantress *et al.* 2003) in this taxon. Although studies involving ranavirus have helped gather new information, much remains to be discovered regarding ranavirus ecology, effects, transmission and specific interactions with their host (Gray *et al.* 2009). Previous studies have shown the influence of host genetic diversity on ranavirus prevalence (Pearman & Garner 2005) and, with regard to ranavirus transmission, the ecology and behavior of *L. pipiens*, especially its dispersion, its co-occurrence with other species which act as reservoirs for pathogens (*i.e.* species that carry the pathogen but do not suffer the clinical signs of infection, Schock *et al.* 2008), its sensitivity to human modification (i.e. especially habitat fragmentation, Carr & Fahrig 2001), its large geographic distribution, and its tendency to move consistently between sites, the northern leopard frog is a good model for the study of ranavirus epidemiology and how epidemiology is related to *L. pipiens* genetic structure in the wild.

2. Study area and sampling

We conducted the present study in rural areas of the Ottawa region and the Greater Sudbury region distant of approximately 485kms, in Ontario, Canada. This area contains the

dispersal range of most amphibian species in Ontario (Seburn & Seburn 1998) including the Northern leopard frog. Sampling sites were located within 100 kms of both regions and we were able to find Leopard Frogs in 7 and 11 locations visited in these areas, respectively, during the 2009 breeding season (Table 1). Sites were at least 3 kms apart to avoid overlap in landscape analysis (see below) and pseudoreplication (Hurlbert 1984).

At each site, frogs were caught by hand with disposable gloves. This method is preferred to the net-catching method because it has been suggested that cross contamination could occur via the net (Hyatt *et al.* 2007). Gloves were changed between each animal capture. Each individual was toe clipped (following the protocol #2009-03-04 approved by the Laurentian University Animal Care Committee) for tissue sample collection.

Table 1. Northern leopard frog populations studied along with their geographic coordinates, number ofindividuals sampled (N),and ranavirus infection rate

Pop. #	Location	abbreviation	Lat	Long	N	Rv+	Infection rate (%)
1	Ottawa_A2,	O_A2	45.3897	-76.3121	4	0	0
2	Ottawa_Bishop Mills, main road,	O_BM	44.9025	-75.6816	50	10	20
3	Sudbury_Conservation	S_CA	46.4607	-80.9418	28	2	7
4	Bruce peninsula NP, Horse lake,	S_HLP	45.2384	-81.5213	25	5	20
5	Ottawa_K1,	O_K1	45.3186	-76.1075	28	3	10.7
6	Ottawa_K3,	O_K3	45.2502	-75.9631	30	2	6.6
7	Sudbury_Kill. Camprground	S_KC	46.0143	-81.3986	13	0	0
8	Sudbury_Killarney Light House	S_KLH	45.9679	-81.4997	9	2	22.2
9	Ottawa_Limerick road, Bishop Mills,	O_LR	44.8773	-75.6479	28	11	39.3
10	Sudbury_Moonlight beach	S_MB	46.4696	-80.9065	6	5	83.3
11	Sudbury_Manitoulin Island	S_MO	45.9018	-82.2587	21	21	100
12	Sudbury_Richelieux	S_R	46.532	-81.3344	29	13	44.8
13	Ottawa_Stony swamp, beaver trail,	O_SS	45.2932	-75.8231	24	12	50
14	Sudbury_Mississagi PP, SWC	S_SWC	46.5771	-82.6985	9	8	88.9
15	Thunder bay	ThB	48.8387	-88.4943	9	6	66.6
16	Sudbury_Timberwolf	S_TW	46.5376	-80.9476	13	3	23.1
17	Sudbury_Capreol lake road	S_WA	46.7138	-80.8723	16	6	46.15
18	Sudbury_Xstrata	S_X	46.5939	-80.7988	18	11	61.1

3. Genetic markers and infection verification

a. Ranavirus infection verification

From individual toe clips, genomic DNA was extracted using QIAmp DNeasy Kit following the standard protocol (Qiagen). After extraction, samples were sent to Pisces Molecular (Boulder, Colorado, USA) for ranavirus screening. At Pisces Molecular a double blind PCR was performed using a primer known to successfully amplify ranavirus,

specifically Frog Virus 3: MCP-ranavirus-F (5'-GACTTGGCCACTTATGAC-3') and MCP-ranavirus-R (5'- GTCTCTGGAGAAGAAGAA), following the PCR conditions listed in Mao *et al.* (Mao *et al.* 1997). This specific primer has been used in other studies and is known to amplify a portion of the major capsid protein within the Frog Virus 3 genome. Along with a qualitative screening, Pisces Molecular provided a semi-quantitative assessment of the infection intensity by looking at the PCR signal. Only individuals that were found infected in both screenings were considered infected.

b. Microsatellite genotyping

Each individual was genotyped at the seven polymorphic microsatellite loci (Rpi100, Rpi101, Rpi102, Rpi 103, Rpi 105, Rpi 106, Rpi 108) described by Hoffman *et al.* (2003), following the same amplification protocol but using one IRDye-labeled M13 primer per locus. Amplification products were pooled together according to annealing temperature, forming a post-PCR triad (3.75 µL of PCR product from each microsatellite amplification, brought to a volume of 15 µL with PCR-grade water), enabled by the use of forward primers within each triad labeled with the distinct fluorescent dyes FAM, NED, VIC, and PET (Applied Biosystems). Pooled products were sent to Génome Québec Innovation Centre at McGill University in Montréal for genotyping analysis.

4. Data analysis

a. Population genetics data

First, we used MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.* 2004) to detect null alleles and scoring errors. Following this, variability at each microsatellite locus was tested for deviation from Hardy–Weinberg equilibrium (HWE) using an exact test based on a Markov chain approach using ARLEQUIN v3.5.1.3 (Excoffier *et al.* 2005). We also calculated the mean number of alleles (Nmean), the allelic richness corrected for sample size (Ar), the observed and expected heterozygosity (Ho and He), the allelic range (Alr), and the number of different alleles (Na) of each loci and each population. We also used the Garza-Williamson index of gene diversity (G-W, Garza and Williamson 2001, Excoffier *et al.* 2005) which corresponds to the ratio of the number of alleles at a given loci in a population sample divided by the allelic range. Low G-W statistic scores are reported for populations with low genetic diversity and due to its characteristics, the G-W statistic has been used to test for population bottlenecks; in the context of this study we only use it as a measure of genetic diversity (Excoffier *et al.* 2005). We also calculated the average gene diversity across loci (GD) using

the molecular diversity option available in ARLEQUIN v3.5.1.3 (Excoffier *et al.* 2005). The inbreeding coefficient (F_{IS}) was also used as an estimator of genetic diversity. Differences between populations regarding the above mentioned measures of genetic diversity per loci was assessed using General Linear Models (GLM; MANOVA) with location as a fixed independent variable and any given measurements of genetic diversity as dependent variables. When the assumptions of the GLM were not met, we computed Generalized Linear Models (GLZ) using a log link function. To test the significance of the model the GLZ function used the Likelihood Type 1 test which is based on the asymptotic normality property of maximum likelihood estimates. The analyses were performed using Statistica 8.1 (Statsoft 2007).

Population structure was assessed using a Bayesian clustering algorithm implemented in the program STRUCTURE v2.3.3, with population identifiers used as prior information (Pritchard *et al.* 2000; Hubisz *et al.* 2009). We used the admixture model with correlated allele frequencies to account for any migrants in the dataset (Francois & Durand 2010). STRUCTURE was run for populations belonging to the whole dataset as well as separately for the SUDBURY group and the OTTAWA group. We set the cluster (''k'') value incrementally from 1 to 30 with five independent runs at each k value. A burn-in period of 100000 steps was followed by Markov Chain Monte Carlo (MCMC) sampling for 500,000 steps. After determining the k value with the lowest log-likelihood score (k = 9, k = 5 and k= 5 for the complete data set, the SUDBURY populations or the OTTAWA populations respectively), the 5 independent runs at k = 9, 5 and 5 were summarized using the program CLUMPP (Jakobsson & Rosenberg 2007) with the LargeKGreedy algorithm and 10,000 permutations. The STRUCTURE analysis was also run using both SUDBURY and OTTAWA populations together, with three iterations at each k and an additional 10 iterations at k = 9.

In addition, population differentiation based on microsatellite genetic variation was measured using pairwise F-statistics (F_{ST}), and an analysis of molecular variance (AMOVA) in ARLEQUIN. F_{ST} was measured using two metrics of genetic variation, the allelic frequencies and the corrected pairwise difference based on the sum of squared differences in the number of repeats (Schoville *et al.* 2011). Geographical partitioning of microsatellite genetic variation was assessed using an AMOVA (Excoffier *et al.* 1992). Genetic variation was partitioned hierarchically at four levels: within individuals, among individuals within populations, among populations within regions, and among regions. Differentiation at these hierarchical levels was assessed for statistical significance by permuting the data 1000 times in ARLEQUIN. Calculations such as F_{ST} and AMOVA are often sensitive to deviations from

HWE resulting in less robust measures of population structures (Schoville *et al.* 2011) but FSTAT software enables tests for genetic structure that do not assume HWE within samples (*e.g.* log-likelihood G; Goudet *et al.* 1996). These values were thus used to assess the differentiation between each pair of localities if deviations from HWE were to be found. For the AMOVA, jackknifing was used to verify the weight of the disequilibrium (Morin *et al.* 2009).

b. Landscape genetics

i. The approach

Canonical Correspondence Analysis (CCA) is a multivariate analysis developed to relate community composition to known variation in the environment based on an eigenvalue ordination technique (ter Braak 1986). Contrasting with conventional ordination techniques, CCA integrates a regression in the ordination model resulting in the ordination axes appearing in order of variance explained by linear combinations of independent variables (ter Braak 1988a). The multiple regression used in the ordination model thus constrains the ordination scores (ter Braak 1988a). Additionally, as the tests implemented in CCA are based on Monte-Carlo permutations, there are no specific assumptions regarding data distribution. Therefore, CCA has been suggested to provide an efficient way to empirically relate variation of genetic diversity and descriptive environmental variables (Angers *et al.* 1999). In the present analysis, genetic diversity and genetic structure estimators act as dependent variables and were related separately to a set of environmental independent variables.

ii. Dependent variables: genetic diversity and genetic structure.

To fully take advantage of the CCA, genetic data were structured into 15 separate matrices (Angers *et al.* 1999, Storfer *et al.* 2007). Genetic structure among populations was inferred from the variation of the relative abundance of each allele at a given locus (allelic frequencies) and a different matrix was constructed for each of the seven loci ("alleles at a given locus by population" matrix). In addition, genetic diversity (9 matrices) was inferred from the variation of the average number of alleles (Nmean), the allelic richness corrected for sample size (Ar), the observed and expected heterozygosity (Ho and He), the allelic range (Alr), the Garza-Williamson index of gene diversity (G-W), the number of different alleles (Na), the inbreeding coefficient (F_{is}), and the average gene diversity (GD) per locus. Thus, genetic diversity within populations took the form of a "level of variation of loci by population" matrix. While both genetic diversity and genetic structure estimators are

calculated from allelic frequencies, they present different information since two populations may carry the same intrapopulational diversity level without sharing any common allele. Furthermore, the number and frequency of alleles may vary substantially for the same expected heterozygosity values in situations where populations are not at mutation-drift equilibrium (Angers & Bernatchez 1997). The investigation of genetic structure patterns in relation to environmental characteristics provides additional information regarding how physical barriers may prevent random movement of alleles that are otherwise expected to be found equally distributed (due to the neutral nature of the microsatellite markers).

iii. Independent variables: landscape metrics.

In order to quantify habitat quality with regard to Leopard Frog ecology, we built a landscape matrix incorporating indexed landscape variables for each location sampled. We used GIS as a tool for merging geographic information on railway, road, building and forest densities, types of aquatic habitats, length of water edges, and land use layers. Using arcMap we created a 2 kms buffer zone around each sampling location within which we inserted all the chosen specific geographic layers (railways, roads, buildings, etc.). The data contained in the geographic information layers is made discrete in multiple rasterized polygons made of vector data, themselves composed of discrete coordinates that can be used to precisely delineate the boundaries of each polygon. Consequently, the surface area of each polygon per layer and per buffer zone can be calculated, in turn providing a precise measure of the surface area for a given data type (e.g. roads, buildings…etc.) within each buffer zone. We used this information for the calculation of the landscape variables. Geographic data layers were obtained through the Ontario Ministry of Natural Resources (OMNR) and Landscape Information Ontario (LIO).

Two types of environmental variables were determined using the available information. The first type corresponds to specific measures of landscape fragmentation as developed by Jaeger (2000). In this category, five variables were determined: 1. The degree of coherence (C), defined as the probability that two animals placed in different areas somewhere in the region of investigation might encounter each other. 2. The degree of landscape division (D), defined as the probability that two randomly-chosen places in a given sampled location are not situated in the same undissected area, 3. The splitting index (S), defined as the number of patches resulting from the division of the total region into parts of equal size leading to the same degree of landscape division, 4. The effective mesh size (m_{eff}) which denotes the size of the areas when the region under investigation is divided into S areas with the same degree of

landscape division (D). 5. The effective mesh density (S_{eff}) gives the effective number of meshes per km^2, in other words the density of the meshes. The effective mesh density value rises when fragmentation increases (Jaeger 2000).

The second type of environmental variables represent complementary measures of environment quality for each sampled location as suggested by Jaeger (2000). We determined 7 variables that likely affect Leopard Frog movements such as railways, roads, buildings and forest densities, type of aquatic habitat, water edge length and land use type. In order to calculate specific values of each variable for each location, we multiplied the total area represented by the feature of interest within each buffer (*e.g.* rail, road), by a specific ordinal factor determined in relation to the particular features' capacity to reduce or enhance Leopard Frog movement and ability to sustain its biological activity. For example, the Road variable scores were calculated by multiplying the roads' length by the road respective number of lanes, and added 1 if the road was paved in order to account for higher traffic rate. This calculation was performed for each road in each location and then summed as the overall Road index for a given location. The detail of all the procedures and raw data tables are given in Appendix 1.

iv. Statistics

The statistical approach used was similar to Angers *et al.* (1999). In order to determine the variation of dependent variables (genetic data) related to independent variables (environmental data), each of the matrices encoding for population genetic diversity and genetic structure was related to the environmental variables separately by CCA, using CANOCO (ter Braak 1988b; program available from C. J. F. ter Braak, Agricultural Mathematics Group, TNO Institute for Applied Computer Science, Wageningen, The Netherlands). For environmental variables, the variables that contributed most to the explanation of the variation were selected using a forward selection procedure available in CANOCO, with a cut-off point of $P = 0.10$, based on 1000 Monte Carlo permutations (see ter Braak 1988b). The contribution of each set of variables was estimated independently using the sum of canonical eigenvalues and the statistical significance was assessed by Monte Carlo permutation tests of the sum of all eigenvalues, using 1000 permutations (ter Braak 1990). To correct for multiple uses of the same set of observations (environmental variables) we applied the sequential Bonferroni correction (Rice 1989) starting at a/k where $a = 0.05$, $k = 7$, with the number of different genetic matrices simultaneously tested against the environmental matrix.

The influence of both the environmental and genetic diversity variables on ranavirus prevalence was assessed using multiple regressions under the GeneraLiZed linear model approach (GLZ). To test the significance of model function, we used the Type 1 likelihod ratio test.

Results

1. Genetic variability in Sudbury and Ottawa localities

Overall, 376 individuals were genotyped with 98.2% success, ranging from 90% (Rpi105) to 100% (Rpi100, Rpi101 and Rpi106) success by locus. Several populations exhibited statistically significant deviations from HWE even after Brookfield null allele frequency estimation and subsequent genotype adjustment. However, there is no clear trend of deviation from HWE at specific loci across all populations and there is no clear predominance of deviations in specific populations, which suggests that HWE deviations are not a result of null alleles or admixture. For measures of population differentiation (STRUCTURE, FST, and AMOVA) that might be sensitive to deviations from HWE, we computed Bonferroni adjustments of 95% confidence interval. The results were qualitatively similar to those of the initial analyses, so all individuals were used in subsequent computations.

The seven loci were 100% polymorphic, the mean rarefied allelic richness in the localities over all loci being 1.801 ± 0.013, ranging from 1.88 (S_HLP and O_K1) to 1.71 (O_LR, S_SWC and ThB; Table 2). No significant differences in measures of genetic diversity were found between the Sudbury and the Ottawa region except with regards to the total rarefied allelic richness (averaged per loci) that was significantly higher in the Ottawa region (8.68 vs 10.42 for Sudbury and Ottawa respectively; GLZ, $X^2 = 3.56$, $p=0.05$). Within region comparison of the rarefied allelic richness resulted in significant differences between localities both within the Sudbury area (GLZ, $X^2 = 32.23$, $p < 0.001$) and the Ottawa area (GLZ, $X^2 = 58.18$, $p < 0.001$). Differences in the observed and expected heterozygosity, as measured in the fixation index, showed that most populations did not have a deficit of heterozygotes. However, two populations from the Ottawa region (O_K1 and O_K3) presented a small but significant deficit in heterozygotes (0.097 and 0.053 for O_K1 and O_K3 respectively; Table 2).

2. Population genetic structure

The analysis of microsatellite variation of the entire set of populations revealed a distinct plateau for nine clusters (Fig. 1a). Additionally, STRUCTURE analysis within the Sudbury region and the Ottawa region revealed 5 clusters (Fig. 1b and 1c), despite noticeable heterogeneity in LnP(D) estimates. Population differentiation based on pairwise genetic distances (F_{ST}) revealed a significant population differentiation in all population comparisons except for a set of contrasts (including some inter-regional contrasts; O_A2 vs. S_CA, O_A2 vs. S_TW, O_LR vs. S_MB, O_LR vs. S_MO, O_SS vs. S_TW, O_SS vs. S_MB and O_BM vs. S_CA; Table 3).

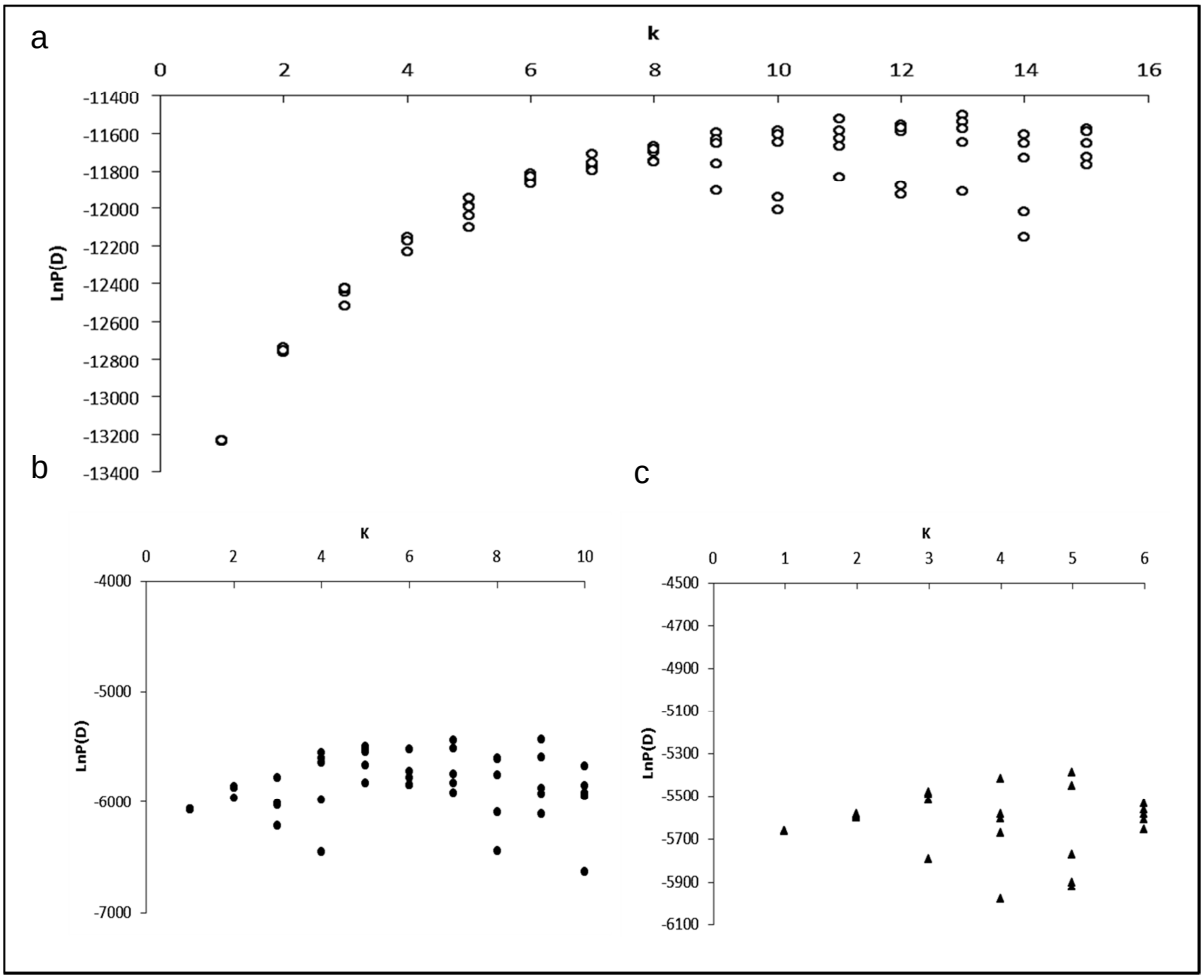

Fig. 1. Maximal number of clusters among populations of Northern leopard frogs. The log probability of the data [Ln P(D)] is plotted as a function of the numbers of clusters (K) in a) the entire set of populations, b) the SUDBURY populations and c) the OTTAWA populations. Data probabilities were calculated by STRUCTURE v. 2.3.1 (Pritchard *et al.* 2000; Hubisz *et al.* 2009).

Table 2. Genetic variability at 7 microsatellite loci in 18 populations of Northern leopard frogs in Ontario.

Pop. #	Area	Nmean	Ar	Ho	He	Alr	GW	Na	Fixation index	GD
1	***O_A2***									
	Mean	5.29	1,86	0.82	0.86	50.29	0.11	0.90	-0.121	0.851
	Se	0.52	0.05	0.05	0.05	8.45	0.01	0.56	-	0.05
2	***O_BM***									
	Mean	16.29	1,85	0.81	0.85	96.29	0.18	0.82	0.00185	0.849
	Se	1.58	0.04	0.05	0.04	16.06	0.02	0.46	-	0.04
3	***S_CA***									
	Mean	11.57	1,86	0.82	0.86	90.86	0.15	0.83	-0.00749	0.857
	Se	1.48	0.02	0.06	0.02	17.40	0.02	0.46	-	0.02
4	***S_HLP***									
	Mean	12.71	1,88	0.82	0.88	84.86	0.18	0.87	0.04507	0.886
	Se	1.61	0.02	0.04	0.02	27.61	0.02	0.48	-	0.02
5	***O_K1***									
	Mean	14.43	1,88	0.82	0.88	108.71	0.18	0.88	0.09685*	0.884
	Se	1.59	0.02	0.04	0.02	41.01	0.02	0.48	-	0.02
6	***O_K3***									
	Mean	14.14	1,86	0.82	0.86	87.14	0.19	0.84	0.05316*	0.857
	Se	1.42	0.02	0.03	0.02	22.66	0.02	0.46	-	0.02
7	***S_KC***									
	Mean	7.57	1,75	0.82	0.75	64.29	0.15	0.76	-0.11775	0.748
	Se	0.84	0.07	0.03	0.07	15.67	0.03	0.43	-	0.07
8	***S_KLH***									
	Mean	7.57	1,80	0.82	0.80	65.14	0.15	0.80	-0.09747	0.801
	Se	1.17	0.06	0.07	0.06	22.94	0.03	0.45	-	0.07
9	***O_LR***									
	Mean	9.43	1,71	0.83	0.71	75.14	0.18	0.66	-0.21990	0.706
	Se	1.78	0.09	0.10	0.09	19.01	0.04	0.38	-	0.09
10	***S_MB***									
	Mean	6.14	1,83	0.84	0.83	48.57	0.15	0.84	-0.18367	0.823
	Se	0.63	0.03	0.07	0.03	9.21	0.02	0.49	-	0.03
11	***S_MO***									
	Mean	8.43	1,74	0.83	0.74	55.43	0.21	0.70	-0.11503	0.745
	Se	1.11	0.08	0.09	0.08	19.18	0.03	0.40	-	0.08
12	***S_R***									
	Mean	12.00	1,81	0.85	0.81	72.57	0.18	0.80	-0.06675	0.811
	Se	2.11	0.06	0.02	0.06	18.82	0.02	0.44	-	0.06
13	***O_SS***									
	Mean	10.57	1,77	0.85	0.77	68.57	0.19	0.69	-0.38693	0.769
	Se	2.77	0.07	0.07	0.07	21.88	0.04	0.40	-	0.07
14	***S_SWC***									
	Mean	4.43	1,71	0.85	0.71	50.57	0.17	0.71	-0.48018	0.698
	Se	0.84	0.06	0.07	0.06	19.37	0.05	0.40	-	0.06
15	***ThB***									
	Mean	4.86	1,71	0.85	0.71	54.57	0.14	0.68	-0.3565	0.700
	Se	0.77	0.05	0.08	0.05	14.60	0.05	0.40	-	0.05
16	***S_TW***									
	Mean	7.29	1,75	0.85	0.75	61.14	0.16	0.73	-0.37207	0.754
	Se	1.63	0.07	0.08	0.07	18.31	0.04	0.42	-	0.07
17	***S_WA***									
	Mean	9.71	1,83	0.86	0.83	86.86	0.13	0.82	-0.18098	0.828
	Se	1.36	0.04	0.03	0.04	24.65	0.02	0.46	-	0.04
18	***S_X***									
	Mean	8.14	1,77	0.81	0.77	68.57	0.16	0.71	-0.27915	0.765
	Se	1.74	0.07	0.07	0.07	20.07	0.04	0.41	-	0.07

Nmean = average number of alleles; Ar = allelic richness corrected for sample size; H_o = observed heterozygosity; H_e = expected heterozygosity; Alr = allelic range; GW = Garza-Williamson statistic; Na = number of different alleles; Fixation index = inbreeding coefficient based on permutation procedure; GD = averaged Gene Diversity.

Within the Ottawa region, all contrast were significant except between O_A2 vs. O_BM and O_A2 vs. O_SS. Similarly, in the Sudbury area all contrasts were significant except for 4 combinations of populations, 3 out of 4 involving S_MO (S_MO vs. S_HLP, S_MO vs. S_MB, S_MO vs. S_R and S_TW vs. S_WA; Table 3).

Table 3. Pairwise genetic distances (F_{ST}) for *Lithobates pipiens* populations of the Sudbury and Ottawa region[a].

	O_A2	O_BM	S_CA	S_HLP	O_K1	O_K3	S_KC	S_KLH	O_LR	S_MB	S_MO	S_R	O_SS	S_SWC	ThB	S_TW	S_WA	S_X
O_A2		-0.033	0.003	**0.081**	**0.096**	**0.099**	**0.054**	**0.049**	**0.059**	**0.049**	**0.030**	**0.098**	0.020	**0.121**	**0.084**	0.001	**0.059**	**0.125**
O_BM	-0.122		**0.024**	**0.070**	**0.103**	**0.110**	**0.063**	**0.055**	**0.057**	**0.051**	**0.050**	**0.093**	**0.044**	**0.141**	**0.112**	**0.045**	**0.088**	**0.151**
S_CA	0.028	0.096		**0.102**	**0.116**	**0.105**	**0.068**	**0.063**	**0.085**	**0.076**	**0.053**	**0.117**	**0.067**	**0.157**	**0.129**	**0.062**	**0.106**	**0.165**
S_HLP	**0.255**	**0.275**	**0.417**		**0.095**	**0.171**	**0.144**	**0.125**	**0.015**	**0.015**	**0.020**	**0.032**	**0.042**	**0.146**	**0.105**	**0.082**	**0.094**	**0.152**
O_K1	**0.324**	**0.427**	**0.491**	**0.340**		**0.175**	**0.134**	**0.116**	**0.101**	**0.075**	**0.074**	**0.124**	**0.033**	**0.041**	**0.017**	**0.027**	**0.008**	**0.060**
O_K3	**0.445**	**0.480**	**0.479**	**0.719**	**0.752**		**0.105**	**0.095**	**0.147**	**0.127**	**0.114**	**0.154**	**0.141**	**0.197**	**0.174**	**0.125**	**0.181**	**0.172**
S_KC	**0.252**	**0.267**	**0.301**	**0.618**	**0.579**	**0.479**		**0.042**	**0.095**	**0.074**	**0.060**	**0.117**	**0.080**	**0.140**	**0.130**	**0.071**	**0.121**	**0.159**
S_KLH	**0.223**	**0.228**	**0.275**	**0.517**	**0.488**	**0.426**	**0.181**		**0.090**	**0.060**	**0.057**	**0.101**	**0.079**	**0.118**	**0.108**	**0.062**	**0.102**	**0.132**
O_LR	**0.225**	**0.235**	**0.375**	**0.046**	**0.380**	**0.667**	**0.427**	**0.396**		-0.002	**0.012**	**0.020**	**0.022**	**0.146**	**0.099**	**0.064**	**0.088**	**0.147**
S_MB	**0.199**	**0.208**	**0.338**	**0.040**	**0.268**	**0.579**	**0.330**	**0.263**	-0.008		0.004	**0.016**	0.014	**0.099**	**0.063**	**0.042**	**0.060**	**0.120**
S_MO	**0.131**	**0.193**	**0.231**	0.038	**0.249**	**0.517**	**0.264**	**0.249**	0.034	0.013		0.007	**0.023**	**0.104**	**0.079**	**0.043**	**0.074**	**0.119**
S_R	**0.350**	**0.386**	**0.507**	**0.109**	**0.470**	**0.664**	**0.509**	**0.423**	**0.069**	**0.050**	0.002		**0.058**	**0.132**	**0.120**	**0.097**	**0.116**	**0.155**
O_SS	**0.077**	**0.179**	**0.286**	**0.157**	**0.124**	**0.640**	**0.349**	**0.342**	**0.089**	0.053	**0.082**	**0.228**		**0.062**	**0.030**	0.005	**0.014**	**0.092**
S_SWC	**0.451**	**0.634**	**0.747**	**0.547**	**0.139**	**0.925**	**0.659**	**0.533**	**0.600**	**0.392**	**0.405**	**0.510**	**0.256**		**0.024**	**0.039**	**0.025**	**0.099**
ThB	**0.307**	**0.484**	**0.588**	**0.385**	**0.055**	**0.797**	**0.595**	**0.479**	**0.392**	**0.240**	**0.298**	**0.466**	**0.120**	**0.084**		**0.018**	**0.007**	**0.072**
S_TW	0.006	**0.184**	**0.271**	**0.299**	**0.091**	**0.564**	**0.315**	**0.269**	**0.259**	**0.168**	**0.172**	**0.379**	0.020	**0.153**	**0.067**		-0.001	**0.066**
S_WA	**0.208**	**0.369**	**0.468**	**0.345**	**0.026**	**0.833**	**0.541**	**0.442**	**0.347**	**0.229**	**0.279**	**0.455**	**0.054**	**0.092**	**0.027**	-0.003		**0.065**
S_X	**0.468**	**0.685**	**0.794**	**0.576**	**0.207**	**0.788**	**0.764**	**0.608**	**0.607**	**0.484**	**0.474**	**0.618**	**0.389**	**0.356**	**0.264**	**0.263**	**0.246**	

[a] FST calculated based on the allelic frequencies (above diagonal) and the corrected average pairwise difference (below diagonal), (PiXY-(PiX+PiY)/2) where (PiXY) is the average number of pairwise difference between populations and (PiX) is the average number of pairwise difference within populations. Bold type indicates statistical significance corrected for multiple tests.

In addition, the hierarchical AMOVA of populations from the Sudbury and the Ottawa region showed evidence of significant genetic structure among populations within each region (6.3% of the total variation). Within-individual variation was also significant, accounting for 90% of the total variation while only 2.05% of the variation was attributed to the regional groupings (Sudbury vs Ottawa) suggesting that the genetic structure is mostly influenced at an intra-regional scale in this area (Table 4).

Table 4. Hierarchical AMOVA based on regional (Sudbury and Ottawa) groupings; fixation indices that are significant with $p < 0.001$ are marked with an *.

Source of variation	df	Sum of squares	Variance components	% of variation	Fixation Indices
Among groups	1	55.032	0.063	2.052	0.021*
Among pop. Within groups	16	156.928	0.195	6.314	0.064*
Among ind. Within pop.	356	968.095	0.0255	0.823	0.009^{NS}
Within ind.	376	1017	2.81	90.81	0.092*
Total	751	2197.056	3.09429	100	

3. Influence of Landscape structure on genetic diversity, genetic structure and ranavirus prevalence

a. Influence of landscape characteristics on population genetic diversity

The CCA analyses of genetic diversity indicated a significant effect of both fragmentation and habitat quality variables (Table 5). The forward selection procedure retained seven out of 12 environmental variables as being significant predictors of genetic diversity, explaining 25.3 – 48.5% of the total variation ($0.006 < p < 0.03$; Table 5). Railway density was selected in three separate instances (i.e., as significantly explaining three separate genetic variables), while forest and road densities and m_{eff} significantly explained two separate genetic variables (Table 5). Building density, S and S_{eff} each significantly explained one genetic variable. Environmental influence, as estimated by the seven selected variables, was similar across the four estimators of genetic diversity as a general decrease of habitat quality and/or an increase of habitat fragmentation resulted in a significant decrease of genetic diversity as measured by the Garza-Williamson index of genetic diversity (GW; $p = 0.006$, 48.9% of the variance explained), the allelic range (Alr; $p = 0.039$, 27.5 % of variance explained), the observed heterozygotsity (Ho; $p = 0.01$, 25.3% of the variance explained) and inbreeding (F_{IS}; $p = 0.01$, 40.6% of the variance explained; Table 5, Fig. 2). More specifically, components of genetic diversity, as measured by the Garza-Williamson index, were discriminated both on the first and second axes of the CCA. On axis 1, variation was mainly captured by railway and road densities while variation explained by axis 2 was mainly the result of forest density and m_{eff}. Components of the genetic diversity for GW exhibited negative relationships with railway and road densities and positive relationships with m_{eff} and forest density (Fig 2a). Both Alr and Ho showed negative relationships with railway, road and building densities as observed in CCA ordination triplots (Fig. 2c and 2d, respectively). Components of genetic diversity for Alr and Ho appeared to be mainly discriminated along the first axis of the CCA where railway and road densities (Alr) and building and railway densities (Ho) explained most of the variation. Values for Alr and Ho exhibited a negative relationship with the environmental variables selected (Fig 2c and 2d). Finally, measures of inbreeding were discriminated both on the first and second axes of the CCA. On axis 1, variation was mainly captured by forest density while fragmentation variables such as m_{eff}, S and S_{eff}. explained most of the variation on axis 2. In fact, we observed high F_{is} values (high inbreeding) when forest density and m_{eff} were low, and when S and S_{eff} were high (Fig. 2b).

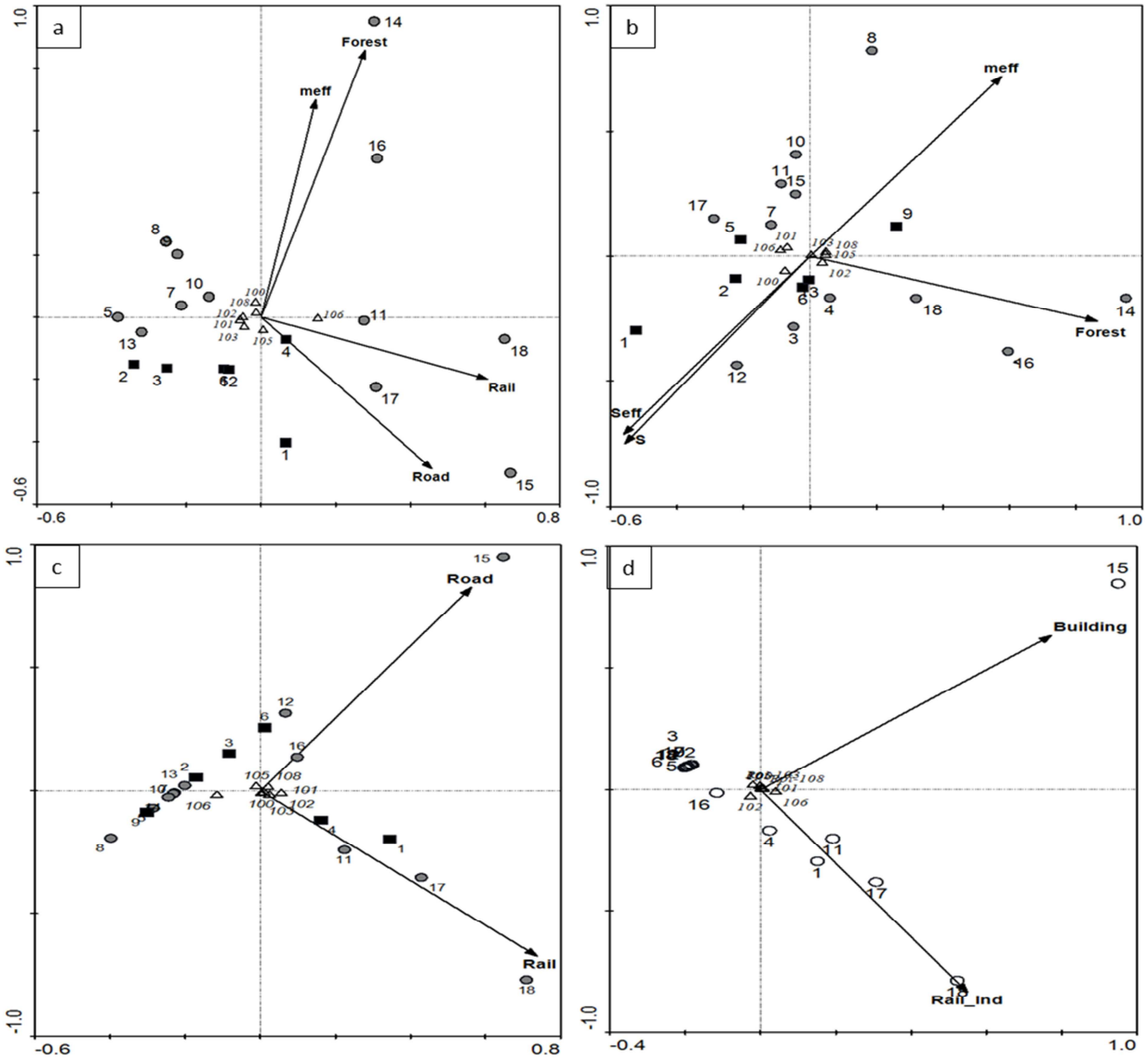

Fig. 2. Canonical correspondence ordination triplot of populations (grey circles for Sudbury populations, black squares for Ottawa populations; numbering as in Table 1), loci (open triangles), and selected environmental variables (arrows) for the number of alleles per population for a. the Garza-Williamson index, b. F_{is}, c. the allelic range, and d. Ho. The length of the arrows, drawn from the centroid of population dispersion, represents the strength of the correlation between population variation and the ordination axes (ter Braak 1995). Loci appear tightly clustered at the center of the diagram, reflecting the low levels of variation at individual loci.

b. Influence of landscape characteristics on population genetic structure

The CCA revealed a significant influence of landscape characteristics on the genetic structure of Leopard Frog populations. The forward selection procedure retained 10 out of 12 environmental variables as being significant predictors of genetic diversity, explaining 32.9 – 76.9% of the total variation ($0.01 < p < 0.04$; Table 5). Railway density explained variation in allelic frequencies in three separate instances, forest density and length of water edges in two instances, and S, S_{eff}, D, m_{eff}, C, type of aquatic habitat, and building density explained variation in one instance.

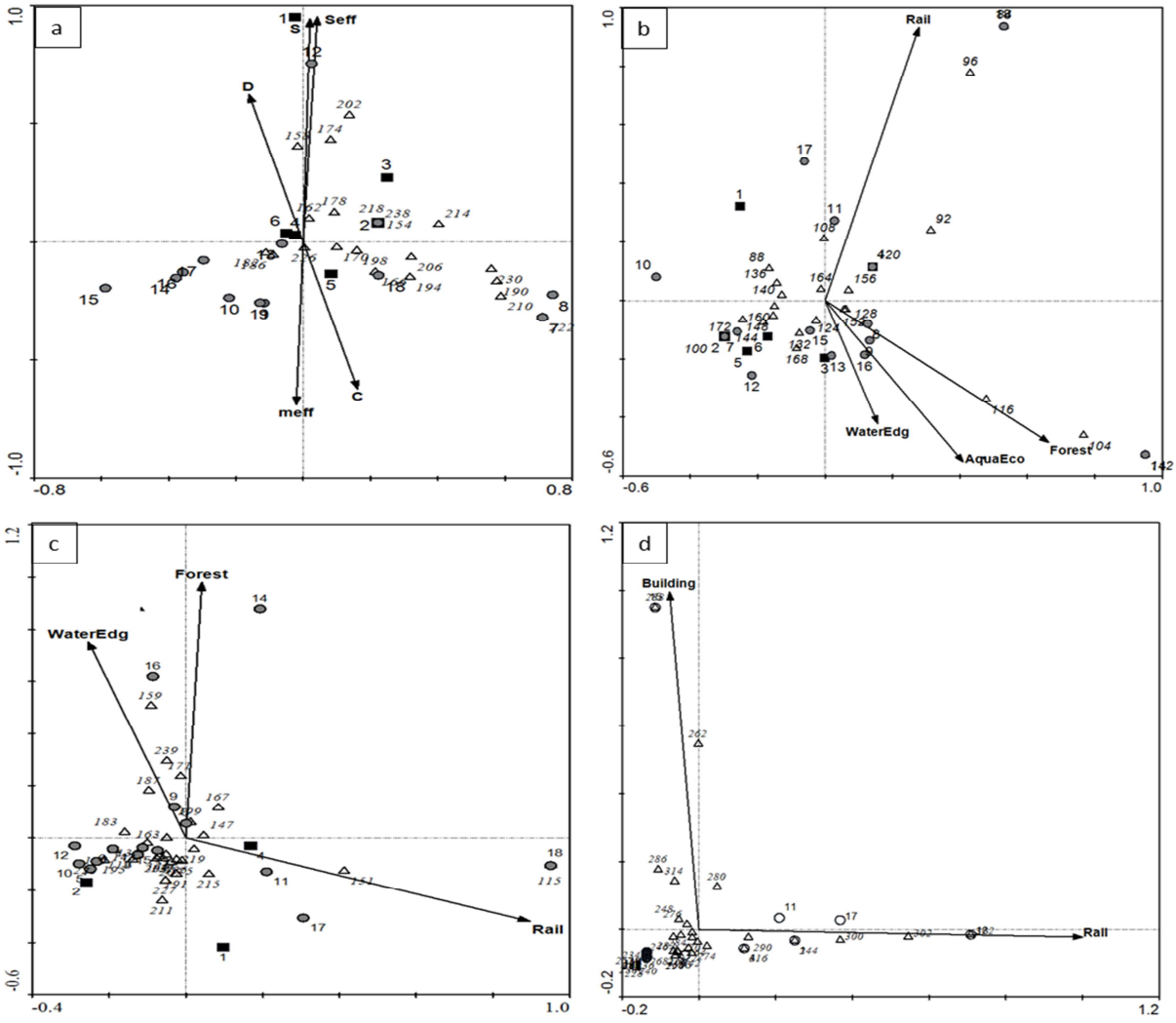

Fig. 3. Canonical correspondence ordination triplot of populations (grey circles for Sudbury populations, black squares for Ottawa populations; numbering as in Table 1), loci (open triangles), and selected environmental variables (arrows) for the number of alleles per population for a. Rpi-100, b. Rpi-102, c. Rpi-103, d. Rpi-108. The length of the arrows, drawn from the centroid of population dispersion, represents the strength of the correlation between population variation and the ordination axes (ter Braak 1995). Loci appear tightly clustered at the center of the diagram, reflecting the low levels of variation at individual loci

Environmental influence on variation in allelic frequencies was significant for Rpi100 ($p = 0.014$, 76.9% of the variance explained), Rpi102 ($p = 0.037$, 70.2% of the variance explained), Rpi103 ($p = 0.035$, 63.7% of the variance explained) and Rpi108 ($p = 0.04$, 32.9% of the variance explained; Table 5). For these four loci, the allelic frequencies were influenced by human-induced disturbance such as railway and building densities as well as by natural features such as forest density, water edge length and the type of aquatic habtitat (Table 5). Interestingly however, it appeared that a greater proportion of alleles, across all 4 loci, were distributed in locations characterised by higher forest density, more suitable aquatic habitats and longer water edge and/or by lower road, railway and building densities (Fig.3).

Furthermore, the fragmentation variables (S, S_{eff}, D, m_{eff} and C) were only selected for Rpi-100 where, in that case, a greater proportion of alleles were distributed in locations characterized by lower fragmentation measures (Fig. 3a). Therefore, these results confirm the patterns observed for population genetic diversity as well as the relative sensitivity of allelic frequencies to habitat characteristics.

Table 5. Summary of statistics for canonical correspondence analysis of genetic diversity genetic structure and environmental variables. For each locus, eigenvalues are given in parentheses. See Appendix for details on environmental variables

	Canonical coefficients			
	Axis 1	Axis 2	*P* model	Explained variation (%)
Genetic structure				
Rpi-100	***(0.241)***	(0.191)	0.014	76.9
S	43.638	-3.1156		
S_{eff}	-43.042	4.5719		
D	0	0		
m_{eff}	-9.3911	1.1779		
C	10.7407	-0.6444		
Rpi-102	***(0.310)***	(0.226)	0.037	70.2
Forest	0.5204	-0.3811		
Rail	0.48	1.6094		
Water_Edge	0.1350	-0.3651		
Aqua_Eco	0.2354	0.3176		
Rpi-103	***(0.174)***	(0.158)	0.035	63.7
Rail	0.85	1.32		
Forest	0.62	-0.046		
Water Edge	-0.67	-0.45		
Rpi-108	***(0.358)***	(0.204)	0.04	32.9
Rail	1.7264	-0.038		
Buildings	-0.2484	3.2248		
Genetic diversity				
F_{is}	***(0.032)***	(0.008)	0.011	40.6
Forest	2.4021	-1.1686		
m_{eff}	-1.1451	0.5166		
S	0.4943	-5.1609		
S_{eff}	-0.1451	4.5713		
Allelic Range	***(0.019)***	(0.003)	0.03	33.5
Rail	0.68	0.75		
Road	0.84	-0.57		
GW	***(0.059)***	(0.006)	0.006	48.9
Rail	0.8656	-0.0813		
Road	0.7319	-0.4085		
Forest	0.3030	0.7218		
m_{eff}	0.3863	0.2378		
Ho	***(0.049)***	(0.002)	0.01	25.3
Buildings	0.8402	0.5523		
Rail	0.6372	-0.7778		

c. Influence of landscape structure and genetic diversity on ranavirus prevalence

Ranavirus prevalence varied extensively from location to location, from absence in all animals (O_A2 and S_KC) to detected in all animals (S_MO; Table 1). We did not find significant influence of environmental variables on ranavirus prevalence except a tendency for railway density to be positively correlated to ranavirus occurrence (GLZ, $X^2 = 3.01$, $p = 0.082$). By contrast, we observed a significant decrease of ranavirus prevalence when allelic range (Alr) and the number of different alleles (Na) increased (GLZ, $X^2 = 11.77$, $p < 0.001$; $R^2 = -0.352$ and GLZ, $X^2 = 3.26$, $p = 0.014$; $R^2 = -0.565$, respectively; Fig. 4).

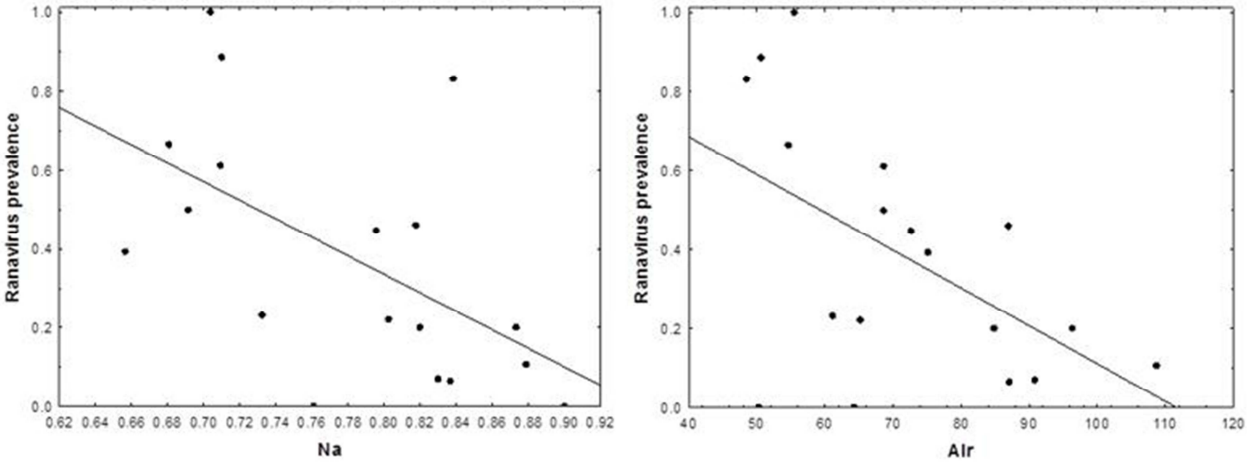

Fig. 4. Linear regressions between ranavirus prevalence and (a) the number of different alleles (Na), (b) the allelic range (Alr).

Discussion

1. Genetic variability and geographic structure in Leopard frog populations

Genetic diversity within the sampled populations of *L. pipiens* as measured by heterozygosities (Ho = 0.83 and He = 0.80) is relatively high and accords with previously published results (He ranging between 0.721–0.970, Hoffman and Blouin 2004). Other measures of genetic diversity such as the allelic range (Alr) reflects high genetic diversity as well. Additionally, only two populations showed a significant but low levels of inbreeding. Comparisons between Ottawa and Sudbury populations yielded only a weak difference in number of rarefied alleles; Ottawa populations were characterized by a higher diversity. Intra-regional comparisons showed higher population differences suggesting that, in the context of this study, small scale habitat specificities influence genetic structure to a greater extent than large scale geographic patterns do. Moreover, STRUCTURE analysis, pairwise genetic distance (F_{ST}) analysis and an AMOVA confirmed that significant genetic structure occurs,

particularly between populations of the same region. Altogether, the significant genetic structure observed in our data confirm the influence of landscape fragmentation and environmental specificities on the metapopulational dynamics of amphibian populations.

2. Influence of landscape variables on amphibian genetic diversity and genetic structure

a. Genetic diversity

The analysis revealed a significant effect of several landscape variables on intra-population genetic diversity, as represented by the Garza-Williamson (GW) index of genetic diversity, the allelic range (Alr) and heterozygosity (Ho). Interestingly, in the case of inbreeding (F_{IS}), 3 out of 5 environmental variables retained were direct measures of habitat fragmentation suggesting the role of landscape fragmentation in increasing inbreeding in populations (Andersen *et al.* 2004). The GW statistic, Alr and Ho showed similar patterns, where an increase of landscape fragmentation and/or a decrease of habitat quality reduces the amount of genetic variability in leopard frog populations.

Noteworthy, the environmental variables associated with low levels of genetic diversity were S, S_{eff}, m_{eff}, rail, road, forest and aquatic habitat, which, with the noticeable exception of road, were also all retained by the models for the investigation of genetic structure. For instance, for all measures of genetic diversity, we observed an opposite relationship between road and rail variables and measures of fragmentation on one hand and forest on the other hand. The significance of the relationship between GW or the allelic range and the environmental variables is particularly due to the loci Rpi100 and Rpi106, while in the case of the F_{is}, the relationship with the environmental variables is due to loci Rpi100, Rpi101 and Rpi106. In the case of Ho, loci Rpi102 is particularly associated with the environmental variables. For these loci and these variables, there was a negative correlation with rail, road, building, S and S_{eff} and a positive correlation with forest, the general index and m_{eff}. These results indicate that leopard frog genetic diversity is higher when the habitat is characterized by a lower fragmentation degree but also by a high density of forest, and an overall high habitat quality, suggesting that fragmentation is not entirely responsible for the diminution of genetic diversity. While our analyses demonstrated a clear relationship between landscape structure, genetic diversity and genetic structure, the extinction–recolonization dynamics characterising amphibian populations (Marsh & Trenham 2000) advocate for the role of historical effects (bottlenecks) in determining genetic patterns. Hence, the patterns of intrapopulation genetic diversity described in this study may be partly representative of

historical diversity rather than resulting from contemporary demographic factors and we must remain relatively cautious in interpreting these results.

b. Genetic structure

Results of the CCA indicate that most of the environmental variables selected significantly influence the variation of allelic frequencies among populations. Among these environmental factors, railway density was significant for 3 out of 4 loci and measures of fragmentation (m_{eff}, S, S_{eff}, C, D) were associated with allele frequencies for locus Rpi-100. Significant environmental variables retained as predictors, such as railway and measures of landscape fragmentation, could be interpreted as a potential source of gene flow interruption within locations resulting in non-trivial patterns of allelic frequencies and apparent differences among population genetic diversity. Railway density forward selection was accompanied by the co-selection of either forest density (Rpi-102 and Rpi-103) or building density (Rpi-108), with forest density inducing opposite effects on allelic frequencies than did railway or buildings. This suggests in turn that railway presence is systematically associated with a decrease of forest density and/or an increase of building presence. Railways effect appears therefore to be indicative of landscape fragmentation; its negative influence on gene flow among populations of amphibians has been documented in the case of *Rana arvalis*, where barriers such as roads and railways emerged as significant factors that reduced gene flow and metapopulation dynamics (Vos *et al.* 2001). Surprisingly, we did not detect any significant effects of roads on the allelic frequencies among populations despite their expected influence (Trombulak & Frissell 2000; Lesbarrères *et al.* 2006).

3. Ranavirus prevalence and conservation insights

Our findings suggest that a decrease of leopard frog host genetic diversity (as measured by Alr and Na) is related to an increasing prevalence of ranavirus in populations. Interestingly, we found Alr to be significantly and negatively correlated with the presence of rails and roads. Therefore, Alr is likely a critical parameter around which both environmental variables and ranavirus prevalence interact, illustrating an indirect link between fragmentation and ranavirus prevalence mediated through host genetic diversity. Consequently, landscape fragmentation may facilitate a lowering of *L. pipiens* genetic diversity and facilitate the occurrence of ranavirus. Although such interpretation is supported by a recent study by Pearman & Garner (2005), other factors might result in similar relationships. For instance, pathogens may select for high host genetic diversity through balancing selection (Coltman *et*

al. 1999), which would result in a positive correlation between population genetic diversity and the occurence of sympatric pathogens (Wegner *et al.* 2003). Our study demonstrated that *L. pipiens* populations potentially harbor high loads of ranavirus representing a significant source of selection (Echaubard *et al.* unpublished data). While our study provides significant elements supporting the negative effect of habitat fragmentation on genetic diversity, our results cannot invalidate an hypothesis suggesting that ranavirus might be a significant source of balancing selection. Additional studies are thus needed to disentangle the respective weight of each hypothesis with respect to *L. pipiens* genetic variability in the wild.

4. Conclusion

Many amphibian populations are in decline around the world (Blaustein & Kiesecker, 2002) and the severity and large geographic scale of such declines in conjunction with their ecological importance make this issue a high conservation priority, possibly the greatest of the 21st century (Daszak *et al.*, 1999). Several factors including infectious diseases and habitat fragmentation have been identified as major threats to amphibian populations (Stuart *et al.* 2004) with poorly known synergies (Plowright *et al.* 2008). Our study is one of the few investigating the link between habitat fragmentation and emerging infectious diseases, illustrating the connection between these two threats. Moreover, our results suggest that reducing landscape fragmentation will result in free-ranging populations with a higher level of genetic diversity and lower risk of extinction, particularly upon exposure to emerging pathogens (Daszak *et al.* 2000, Pearman et al 2005).

Appendix: Procedure for the determination of environmental quality variables.

In order to quantify habitat quality with regard to Leopard Frog biology we built a landscape matrix incorporating indexed landscape variables for each location sampled. We used GIS as a tool for merging geographic information on road density, buildings and forest cover, rail presence, types of aquatic habitats, amount of water edges and land use layers. Using arcMap we created a 2km buffer zone around each sampling location within which we inserted all the chosen specific geographic layers (road, rail, forest cover…etc; Fig. A1). The data contained in the geographic information layers is discretized in multiple rasterized polygons made of vector data themselves composed of discrete coordinates that can be used to precisely delineate the boundaries of each polygon. Consequently, the surface area of each polygon per layer and per buffer zone can be calculated, in turn providing a precise measure of the surface area or linear length for a given data type (e.g. roads, building…etc.) within each buffer zone. We used this information to design variables that characterized environment quality and complement measures of fragmentation as suggested by Jaeger (2000). We determined 8 variables that incorporated significant features of the landscape that were susceptible to affect Leopard Frog movements such as road, rail, buildings, forest, aquatic habitat, water edge, land use and a general index that combines all 7 previous variables.

a. The Road index

The Road index was calculated by multiplying each identified road length (in meters) by its respective number of lanes + 1 if the road was paved in order to account for higher traffic rate (Eingenbrod *et al.* 2008). The sum of all scores was taken as the overall Road index for a given buffer area surrounding a sampled location. Higher scores of the Road index suggest fragmentation of the habitat and potentially that amphibian movements may be impeded.

b. The Rail index

The rail index was calculated following the same principles as for the Road index. We multiplied each identified railway length (in meters) by either 1 or 0.1 if the railway was identified as abandoned. We down-weighted 10-fold the scores for abandoned railway in order to account for their relatively low detrimental effect on wildlife. Abandoned railways have been suggested, to be even beneficial for wildlife as they are usually characterized by a significant cover of native plant communities favorable, in particular, to amphibian movements (Box 1999). The sum of all scores was taken as the overall Rail index for a given

buffer area surrounding a sampled location. Similarly to the Road index, higher scores of the Rail index suggest fragmentation of the habitat and potentially that amphibian movements may be impeded.

c. The Building Index

The Building index per buffer area was calculated as the sum of all built areas (in square meters) within the buffer. Higher scores of the Building index represent areas characterized by poor habitat quality for amphibians

d. The Forest cover index

The Forest cover index relates to the amount of woodland present per buffer area providing an idea of the degree of natural landscape remaining in the buffer zone and consequently an estimate of the habitat permeability to amphibian movements. The Forest cover index corresponds to the sum of wooden parcels (in square meters) within a given location. Landscapes with high forest cover are critical for amphibian population maintenance (Findlay and Houlahan 1997, Eigenbrod *et al.* 2008) and high score of the Forest cover index represents area of good habitat quality with regards to amphibian ecology.

e. The AquaEco index

The AquaEco index documents the prevalence of various aquatic habitats within the buffer zone. Each type of habitat has been assigned a specific score in relation to its suitability for amphibians. In the classification of aquatic habitats provided by GIS, there are 4 main types of habitats: shoreline, streams, lake and wetland. Within each of these habitats a certain number of sub-categories are also provided to further describe a given aquatic habitat. The combination of all of these categories resulted in the characterization of 126 specific aquatic habitat types each assigned to a semi-quantitative score of habitat suitability (Table A1, A2, A3 and A4). The philosophy underlying the scores assignment was as follow: we first classify the 4 main types of aquatic habitats with regards to their suitability for amphibians. Among the 4 habitat types Wetlands are by far the most suitable habitat type for amphibian including Leopard Frogs. This habitat was assigned a score of 4 (out of 4). After wetlands, the most suitable habitat for amphibian and particularly the Leopard Frogs, was Lake which was assigned a score of 3. Streams can host amphibians and to a certain extent Leopard Frogs (Echaubard pers. obs.) but are usually less appropriate than lakes or wetlands. The Stream

category was therefore assigned a score of 2. Finally shorelines were assigned a score of 1 as they represent, in the GIS classification, open water habitats less appropriate for amphibians.

Once the main aquatic habitat types have been assigned with a score, we then classified sub-categories within each main habitat types according to their appropriateness with regards to amphibian ecology and movements following a nested categorical scoring. For example, among the two categories within Shoreline, drainage patterns and shoreline type, we considered that the drainage pattern characteristics had the potential to influence amphibians to a greater extent than the shoreline type. In the following nested categorical scoring, the drainage patterns sub-category was therefore assigned with a higher rank than the shoreline type sub-category. Consequently, a polygon classified as "Shoreline, Large streams, Abrupt shoreline gradient" (Type code C02; Table A1) was assigned a score of 1.21 because the main level of classification (Shoreline) had the smallest rank out of 4 class (score 1), the primary sub-category (drainage pattern) had the second lowest rank out of 4 (score 0.2; a small stream is better than a large one for amphibians; Table A1) and the secondary sub-category (shoreline type), in the current example "Abrupt shoreline", was assigned with the lowest score out of 6 (score 0.01; an abrupt shoreline is less appropriate than a gently sloping shoreline for amphibians; Table A1). Similarly, a polygon classified as « wetland, marsh, large, connected » was assigned a score of 4.234 because the main level of classification (Wetland) has the highest rank out of 4 class and thus received a 4, the secondary level (Connectivity) has the highest rank out of two and scored 2, the third level (Size) is represented by 3 categories (Large, medium and small), Large being assigned to a score of 3. Finally the last level is represented by 4 categories (Marsh, Swamp, Fen, Bog), Marsh corresponding to the highest score possible out of 4 (Table A4). Once each type of habitat has been assigned with a specific score, we calculated the total surface area (in square meters) represented by a given habitat within a buffer area and multiplied it by its score. Finally we summed all the scored surface areas within a buffer zone to obtain the overall AquaEco index for a given buffer.

f. The WaterEdge index

The WaterEdge index represents the total length (in meters) of shorelines present in a given buffer.

g. The LandCover index

The LandCover index assesses the quality of the landscape for the frogs based on 28 land use categories. Each category was assessed with regards to amphibian ecology (Table A5). The LandUse index for a given buffer was obtained by summing all scored land use categories surface areas (in square meters)

Table A1. Shoreline classification, type codes and scores

Drainage pattern	Shoreline type	Type code	Score
Small streams	Abrupt shoreline gradient	C01	1.41
Large streams	Abrupt shoreline gradient	C02	1.21
Large and small streams	Abrupt shoreline gradient	C03	1.31
no streams	Abrupt shoreline gradient	C04	1.11
small streams	Gently sloping shoreline gradient	C05	1.42
large streams	Gently sloping shoreline gradient	C06	1.22
large and small streams	Gently sloping shoreline gradient	C07	1.32
no streams	Gently sloping shoreline gradient	C08	1.12
small streams	Low riverine coastal plain	C09	1.43
large streams	Low riverine coastal plain	C10	1.23
large and small streams	Low riverine coastal plain	C11	1.33
no streams	Low riverine coastal plain	C12	1.13
small streams	Open shoreline wetlands	C13	1.46
large streams	Open shoreline wetlands	C14	1.26
large and small streams	Open shoreline wetlands	C15	1.36
no streams	Open shoreline wetlands	C16	1.16
small streams	Semi-protected wetlands	C17	1.45
large streams	Semi-protected wetlands	C18	1.25
large and small streams	Semi-protected wetlands	C19	1.35
no streams	Semi-protected wetlands	C20	1.15
small streams	Artificial or unclassified	C21	1.44
large streams	Artificial or unclassified	C22	1.24
large and small streams	Artificial or unclassified	C23	1.34
no streams	Artificial or unclassified	C24	1.14
	Low permeability	C25	1.003
	Intermediate permeability	C26	1.002
	High permeability	C27	1.001

Table A2. Stream classification, type codes and scores.

Watershed position	Gradient	Permeability	Type code	**Score**
Headwater	Steep	High	S01	**2.111**
Headwater	Steep	High	S02	**2.111**
Headwater	Medium	High	S03	**2.121**
Headwater	Steep	Medium	S04	**2.112**
Headwater	Gentle	High	S05	**2.131**
Headwater	Medium	High	S06	**2.121**
Headwater	Steep	Medium	S07	**2.112**
Headwater	Medium	Medium	S08	**2.122**
Headwater	Steep	Low	S09	**2.123**
Headwater	Medium	Medium	S10	**2.122**
Headwater	Gentle	Medium	S11	**2.132**
Headwater	Steep	Low	S12	**2.113**
Headwater	Medium	Low	S13	**2.123**
Headwater	Gentle	Low	S14	**2.133**
Headwater	Gentle	High	S15	**2.131**
Headwater	Gentle	Medium	S16	**2.132**
Headwater	Medium	Low	S17	**2.123**
Headwater	Gentle	Low	S18	**2.133**
Middle tributary	Gentle	Low	S19	**2.333**
Middle tributary	Medium	Low	S20	**2.323**
Middle tributary	Gentle	Low	S21	**2.323**
Middle tributary	Gentle	Medium	S22	**2.332**
Middle tributary	Steep	Low	S23	**2.313**
Middle tributary	Gentle	High	S24	**2.331**
Middle tributary	Medium	Medium	S25	**2.322**
Middle tributary	Gentle	Medium	S26	**2.332**
Middle tributary	Medium	Low	S27	**2.323**
Middle tributary	Steep	Low	S28	**2.313**
Middle tributary	Steep	Medium	S29	**2.312**
Middle tributary	Medium	High	S30	**2.321**
Middle tributary	Gentle	High	S31	**2.331**
Middle tributary	Steep	High	S32	**2.311**
Middle tributary	Medium	Medium	S33	**2.322**
Middle tributary	Steep	Medium	S34	**2.312**
Middle tributary	Medium	High	S35	**2.321**
Middle tributary	Steep	High	S36	**2.311**
Mainstream	Steep	High	S37	**2.211**
Mainstream	Medium	High	S38	**2.221**
Mainstream	Steep	High	S39	**2.211**
Mainstream	Steep	Medium	S40	**2.212**
Mainstream	Gentle	High	S41	**2.231**
Mainstream	Steep	Low	S42	**2.213**
Mainstream	Medium	High	S43	**2.221**
Mainstream	Medium	Medium	S44	**2.222**
Mainstream	Steep	Medium	S45	**2.212**
Mainstream	Gentle	High	S46	**2.231**
Mainstream	Gentle	Medium	S47	**2.232**
Mainstream	Medium	Low	S48	**2.223**
Mainstream	Steep	Low	S49	**2.213**
Mainstream	Gentle	Low	S50	**2.233**
Mainstream	Medium	Medium	S51	**2.222**
Mainstream	Gentle	Medium	S52	**2.232**
Mainstream	Medium	Low	S53	**2.223**
Mainstream	Gentle	Low	S54	**2.233**

Table A3. lake classification, type codes and scores

Connectivity	Size	Shape	Permeability	Type code	**Score**
Unconnected	Large	Irregular	High	L01	**3.1321**
Connected	Large	Irregular	High	L02	**3.2321**
Unconnected	Large	Irregular	Medium	L03	**3.1322**
Unconnected	Large	Round	High	L04	**3.1311**
Unconnected	Large	Irregular	Low	L05	**3.1323**
Connected	Large	Irregular	Medium	L06	**3.2322**
Connected	Large	Round	High	L07	**3.2311**
Unconnected	Large	Round	Medium	L08	**3.1312**
Connected	Large	Irregular	Low	L09	**3.2313**
Unconnected	Large	Round	Low	L10	**3.1313**
Connected	Large	Round	Medium	L11	**3.2312**
Connected	Large	Round	Low	L12	**3.2313**
Connected	Medium	Round	Low	L13	**3.2213**
Connected	Medium	Round	Medium	L14	**3.2212**
Connected	Medium	Irregular	Low	L15	**3.2223**
Unconnected	Medium	Round	Low	L16	**3.2213**
Connected	Medium	Irregular	Medium	L17	**3.1212**
Unconnected	Medium	Round	Medium	L18	**3.1212**
Connected	Medium	Round	High	L19	**3.2211**
Unconnected	Medium	Irregular	Low	L20	**3.1213**
Unconnected	Medium	Irregular	Medium	L21	**3.1222**
Connected	Medium	Irregular	High	L22	**3.2221**
Unconnected	Medium	Round	High	L23	**3.1211**
Unconnected	Medium	Irregular	High	L24	**3.1221**
Unconnected	Small	Irregular	High	L25	**3.1121**
Connected	Small	Irregular	High	L26	**3.2121**
Unconnected	Small	Irregular	Medium	L27	**3.1122**
Unconnected	Small	Round	High	L28	**3.1111**
Unconnected	Small	Irregular	Low	L29	**3.1123**
Connected	Small	Irregular	Medium	L30	**3.2122**
Connected	Small	Round	High	L31	**3.2112**
Unconnected	Small	Round	Medium	L32	**3.1112**
Connected	Small	Irregular	Low	L33	**3.2123**
Unconnected	Small	Round	Low	L34	**3.1113**
Connected	Small	Round	Medium	L35	**3.2112**
Connected	Small	Round	Low	L36	**3.2113**

Table A4. Wetland classification, type codes and scores.

Type	Size	Connectivity	Type code	Score
Bog	Null	Connected	W10	**4.211**
Bog	Null	Unconnected	W09	**4.111**
Fen	Null	Connected	W08	**4.212**
Fen	Null	Unconnected	W07	**4.112**
Marsh	Large	Connected	W04	**4.234**
Marsh	Large	Unconnected	W03	**4.134**
Marsh	Small	Connected	W02	**4.224**
Marsh	Small	Unconnected	W01	**4.124**
Muskeg	Large	Connected	W12	**4.232**
Muskeg	Large	Unconnected	W11	**4.132**
Swamp	Null	Connected	W06	**4.213**
Swamp	Null	Unconnected	W05	**4.113**

Table A5. LandCover classification, type code and scores.

Type code	Type	Score (1-10)
24	Settlement and Developed Land	2.1
23	Bedrock / Sand / Mine Tailings	2.5
12	Tundra Heath	2.8
15	Coniferous Plantation	3.1
14	Dense Coniferous Forest	3.4
18	Sparse Coniferous Forest	3.7
17	Mixed Forest, Mainly Coniferous	4
13	Dense Deciduous Forest	4.3
16	Mixed Forest, Mainly Deciduous	4.6
19	Sparse Deciduous Forest	4.9
21	Recent Burns	5.2
20	Recent Cutovers	5.5
22	Old Cutovers and Burns	5.8
25	Pasture and Abandoned Fields	6.1
26	Cropland	6.4
27	Alvar	6.7
1	Water	7
2	Coastal Mudflats	7.3
3	Intertidal Marsh	7.6
4	Supertidal Marsh	7.9
9	Treed Fen	8.2
8	Open Fen	8.5
11	Treed Bog	8.8
10	Open Bog	9.1
7	Conifer Swamp	9.4
6	Deciduous Swamp	9.7
5	Freshwater Coastal Marsh / Inland Marsh	10
28	Unclassified	

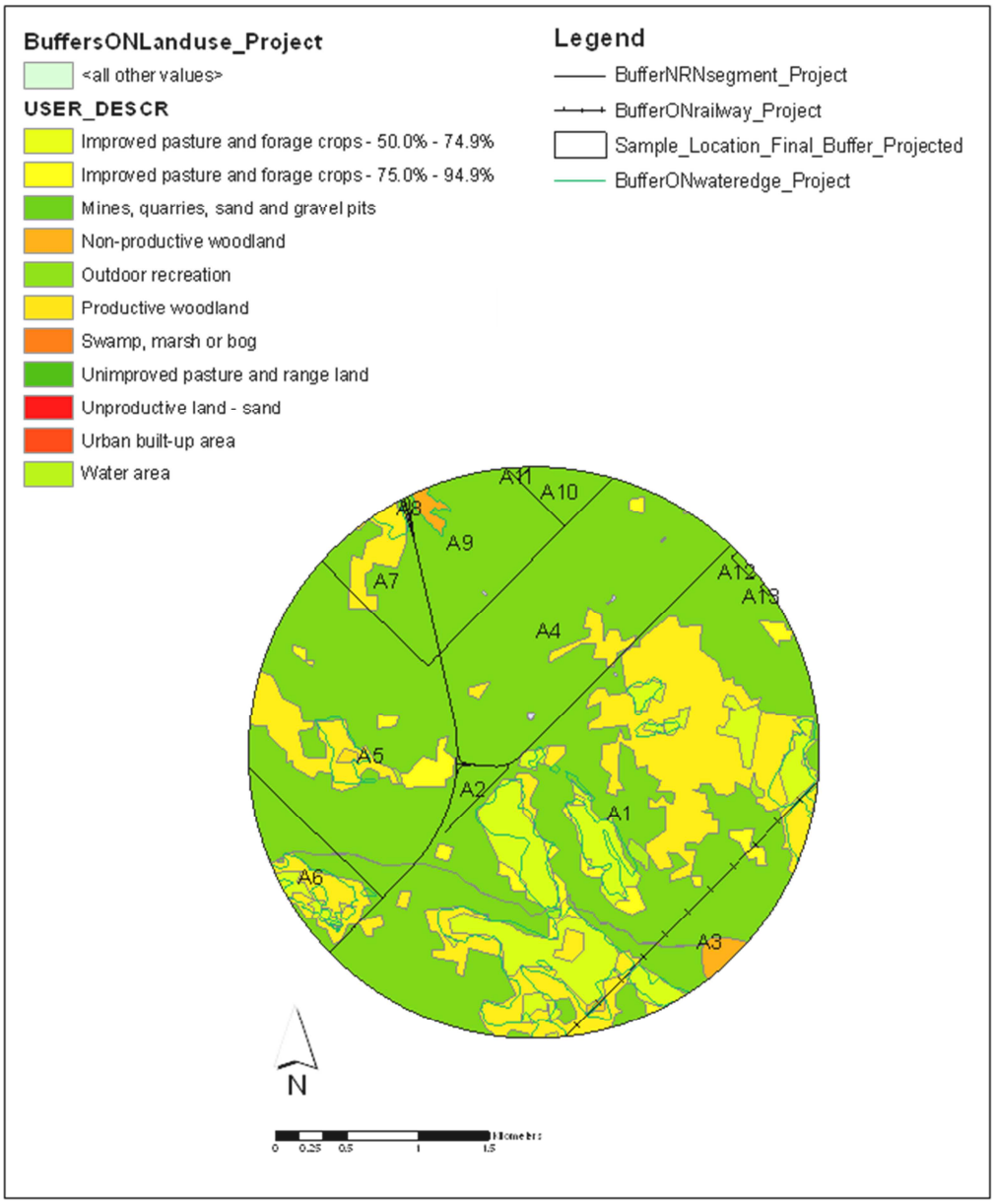

Fig. A1. Example of buffer that includes layer of geographic information and the delimitation of patches (A1 to A11) of non-fragmented land.

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Manuscript 2

Environmental dependency of host-pathogen genetic interactions in the Amphibian-ranavirus system: an experimental evidence

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DL: design, analyses and writing

Abstract

Interactions between host and pathogen genotypes (G_HxG_P) are important determinants of infection outcome and ultimately are critical determinants of host-pathogen coevolutionary dynamics. Environmental conditions such as temperature, however, can affect host immune responses and pathogen virulence, in turn modulating the reciprocal interactions of host/pathogen genotypes resulting in complex G_HxG_PxE interactions. Investigations of G_HxG_PxE interactions have the potential to explain variations in fitness related traits in host-pathogen systems with greater accuracy as they account for both genetic and environmental influences. Using two common North American frog species (*Lithobates pipiens* and *Lithobates sylvaticus*) and three strains of frog virus 3 (FV3) at different temperatures, we designed a fully factorial laboratory experiment to investigate the potential for G_HxG_PxE interactions. Our results revealed significant variations in host susceptibility and strain infectivity, suggesting the potential for frequency-dependent selection in this system. However, our results also suggest that the strength of the mutual selective pressure exerted by the host and the pathogen is temperature-dependent, revealing for the first time in a vertebrate-pathogen system the occurrence of G_HxG_PxE interactions. Finally, our study suggests that using a reaction norm approach might help explain variation in gene frequencies in response to selective forces in host-pathogen systems.

Key words: genotype by genotype interactions, genotype-environment interactions, coevolution, host-pathogen, *Lithobates pipiens*, *Lithobates sylvaticus*,

Introduction

Genotype by genotype interactions in a host-parasite system are the interactive effects of the host and parasite genotypes on the outcome of infection. When assuming no environmental influences, the phenotype of infection traits in a given host-parasite interaction (such as host resistance or parasite virulence) is expected to be determined by the host and parasite genotypes (G_H and G_P respectively; see Lambrechts *et al.* 2006 for a discussion). While most empirical and theoretical studies of the evolution of host-parasite systems consider that infection traits are governed exclusively by the genotype of either the host or the pathogen, a growing portion of published work acknowledges that epidemiological traits are controlled by their interaction (Restif and Koella 2003, Lambrechts *et al.* 2006). Such interaction may lead to counterintuitive observations whereby increasing the background mortality rate of the host, may not necessarily lead to an increase in parasite's virulence (Restif and Koella 2003). Furthermore, it is likely that the evolutionary response of selection on an allele will induce changes, either positive or negative, in all the traits with which it is genetically correlated. For fitness related traits, this can lead to an evolutionary trade-off where an increase in effectiveness of one trait will have a cost of decreased effectiveness of another, and vice versa, preventing fitness to be maximized for all combinations of traits. Furthermore, when the genetic correlations are under shared control of the host and the parasite, we can predict variability in a given evolutionary trade-off (Salvaudon *et al.* 2005, Lambrechts *et al.* 2006, De Roode and Altizer 2010), thus emphasising the relevance and need for an integrative approach in the investigation of host-parasite interactions. Additionally, the dynamic nature of the adaptation and counter-adaptation between the molecular arsenals of the host and parasite (antagonistic co-evolution) may be particularly sensitive to environmental influences. Environmental variables may affect the strength and response to selection, resulting in host or/and pathogen Genotype by Environment (E) interactions (G_HxE, G_PxE, or G_HxG_PxE), possibly resulting in condition-dependent pathogen virulence (Thomas and Blanford 2003, Lazzaro and Little 2009, Wolinska and King 2009).

Among other factors, temperature has been documented to greatly affect the host's biochemical, physiological, and behavioural processes. Temperature directly modulates host immunity, host development, and pathogen virulence (Thomas and Blanford 2003). The direction and extent of temperature effects on genotypic interactions resulting in a range of different phenotypes is known as the reaction norm (see Scheiner (1993) for a review). The influence of temperature on host and parasite genotypes is a critical process that should be

accounted for when investigating host-pathogen interactions (Wolinska and King 2009), and an increasing number of studies incorporate the influence of temperature on either host susceptibility or pathogen infectivity (G_HxE or G_PxE interactions; Mitchell *et al.* 2005, Vale and Little 2009). However very few studies have investigated the three-way interaction between host genotype, pathogen genotype, and temperature, despite clear conceptual relevance (Lazzaro and Little 2009, Wolinska and King 2009; but see Tétard-Jones *et al.* 2007).

Most studies investigating genotype-environment (GxE) interactions have used either invertebrate (Fellowes *et al.* 1999, Vale and Little 2009) or plant (Laine 2007) hosts although some work has been conducted with protozoans (Fels and Kaltz 2006). In vertebrates, molecular interplay between the parasite's antigens and host cell receptors or circulating antibodies are the strongest determinants of an infection (Frank and Schmid-Hempel 2008). The highly polymorphic Major Histocompatibility Complex (MHC) alleles vary among hosts causing each individual to have a particular spectrum of presentation efficiencies for different parasitic antigens. Thus the strength of a host's immune response to a particular antigen depends substantially on its MHC genotype. From the parasite's point of view, a particular antigenic variant may be better able to be displayed by particular host haplotype than others. The ability for a parasite to avoid detection by the host's immune system depends on several mechanisms including random mutations during replication to generate novel antigens or switching expression between archived variants. The variability of both the host's MHC alleles and the parasite's antigenic variants results from a mutation-selection balance and suggests that G_HxG_P interactions, particularly in vertebrates, are a critical mechanism shaping the outcome of H-P interactions and leading to antagonistic co-evolution. Moreover, the high complexity of vertebrates, including multiple levels of molecular interactions and gene expression regulation (i.e., epigenetics; see Bossdorf *et al.* 2008 for discussion of epigenetic processes in an ecological context) allows the influence of direct (within the organism) and indirect (e.g., habitat influence on host physiology) environmental variability on the outcome of the interaction between the host and pathogen genotypes. Such considerations therefore suggest that the investigation of G_HxG_PxE interactions in vertebrate host systems might increase our understanding of co-evolutionary processes.

Ranaviruses (*Iridoviridae*) are highly virulent pathogens known to infect fish (Mao *et al.* 1997), reptiles (Jancovich *et al.* 2010), and a wide range of amphibian species (Jancovich *et al.* 1997, Daszak *et al.* 1999, Docherty *et al.* 2003) . Ranaviruses are widespread, and cause disease and mass mortality at various locations worldwide especially in amphibian

populations (Daszak *et al.* 1999). Ranaviruses are recognized as important pathogens and ranaviral disease is now reported by the World Organization for Animal Health (OIE) (http://www.oie.int/eng/maladies/en_classification2010.htm?e1d7). Our current understanding of ranaviral infections is very limited and no real consistency between outbreak determinants has been detected (Lesbarrères *et al.* 2011). The causes of this variability are difficult to study because many ecological drivers are involved (Plowright *et al.* 2008). Additionally, amphibian species differ in their susceptibility to ranaviruses, and non-trivial molecular interplays as well as multiple isolates of a given species have been documented (Hyatt *et al.* 2000, Wang *et al.* 2003). Finally, amphibian development and immune response are highly sensitive to temperature (Wabl and Du Pasquier 1976, Jackson J. and Tinsley R. 2002, Lazzaro and Little 2009, Robert and Ohta 2009) giving the investigation of G_HxG_PxTemperature interactions a real potential for improving our understanding of ranavirus virulence and host susceptibility.

In order to study such G_H x G_P x Temperature interactions, we designed a fully factorial laboratory experiment using two common North American frog species, three ranaviruses (wt FV3, an azacytidine resistant mutant of FV3, and an FV3-like virus isolated from *Ambystoma maculatum*) and two temperature settings. The five questions we addressed were: (1) does pathogen infectivity vary among strains? (2) Does susceptibility vary among hosts? (3) Is the outcome of the interaction specific to a host-strain combination? (4) Does temperature influence pathogen virulence and/or host susceptibility? (5) Does temperature modulate host-strain genotypic interactions resulting in G_HxG_PxE interactions?

Material and Methods

1. Hosts

The Northern Leopard Frog (*Lithobates pipiens*) and the Wood Frog (*Lithobates sylvaticus*) have been shown to be highly susceptible to ranavirus and mass die-offs associated with ranaviral disease have been observed for both species (St-Amour and Lesbarrères 2006, Duffus *et al.* 2008). While using different habitats during the summer months (i.e., grasslands for the Northern Leopard Frog, woodlands for the Wood Frog) both species can be found spawning in the same wetlands during the spring months suggesting potential for horizontal transmission of the disease. In July 2010, we received tadpoles, approximately Gosner stage

25 (Gosner 1960), of both species from the Environment Canada Atlantic Laboratory for Environmental Testing in Moncton, NB., courtesy of Paula Jackman.

2. *Ranavirus* strains

We used three different ranaviruses: (1) Wild type frog virus 3 (wt-FV3) infecting frogs, including *L. pipiens* and *L. sylvaticus* and which is expected to be the most virulent. (2) An azacytidine (azaC)-resistant mutant that is thought to be less virulent because its unmethylated genome may trigger an early innate immune response (Essani *et al.* 1987). In the latter case, unmethylated DNA is seen by the host cell as a danger signal and, acting through TLR 9 or one of the intracellular DNA sensors, triggers the production of IFN and another pro-inflammatory cytokines (Akira *et al.* 2006); (3) SsMeV, isolated from a spotted salamander in Maine, USA. Initial sequence study of the MCP suggests it is a FV3-like virus (Gregory Chinchar, pers. obs.). High titer stocks of ranavirus strains were elaborated by Prof. Gregory Chinchar at the University of Mississipi Medical Center (Jackson, MS, USA) and stored at -80°C. As titer accuracy may be lost after few freeze/thaw cycles, we split the entire volume solution of each viral strain into several 1ml "single-use" vials. Consequently only "fresh" virus solution was used for experimental inoculation.

3. Experimental design

To investigate variability in the interactions between *Lithobates sp*. hosts and ranavirus strains, we designed a full factorial experiment including one *L. pipiens genotype* (from one egg mass) and two *L.sylvaticus* genotypes (from two eggs masses; Fig. 1). Tadpoles of each genotype were exposed to all three ranaviruses plus a control (no infection) resulting in 12 possible host genotype-ranavirus combinations, each replicated three times. Every treatment consisted of 10 to 12, *L. pipiens* or *L. sylvaticus* tadpoles aged at GS 21 (Gosner Stage, Gosner 1960). In addition, a temperature treatment (14 °C and 22 °C) representing a relevant range for species in similar latitudes (Laugen *et al*. 2003) was used to investigate the potential environmental influence. A total of 400 tadpoles (200 of each species) were used in this experiment. Tadpoles were placed in 2 L plastic containers filled with 1 L of dechlorinated water (aged for three days) and were randomly assigned to their respective ranavirus and temperature treatments. The host density (number of tadpoles per volume of water) was adjusted to 1 tadpole per 250 mL of water to avoid any effect of density on tadpole development (Echaubard *et al.* 2010).

For ranavirus exposure, the tadpoles assigned to the infected treatment were placed within 50 mL of infected water containing 10000 pfu/mL of a particular ranavirus (wt-FV3, azaC or SsMeV, accordingly). The administered ranavirus dose was chosen to induce a sub-lethal effect therefore enabling the measurement of the virulence variation between isolates (Gantress *et al.* 2003, Chinchar pers. obs.). The tadpoles were left within the infected solution overnight (12 h) before they were transferred along with the 50 mL of infected water into their respective tanks.

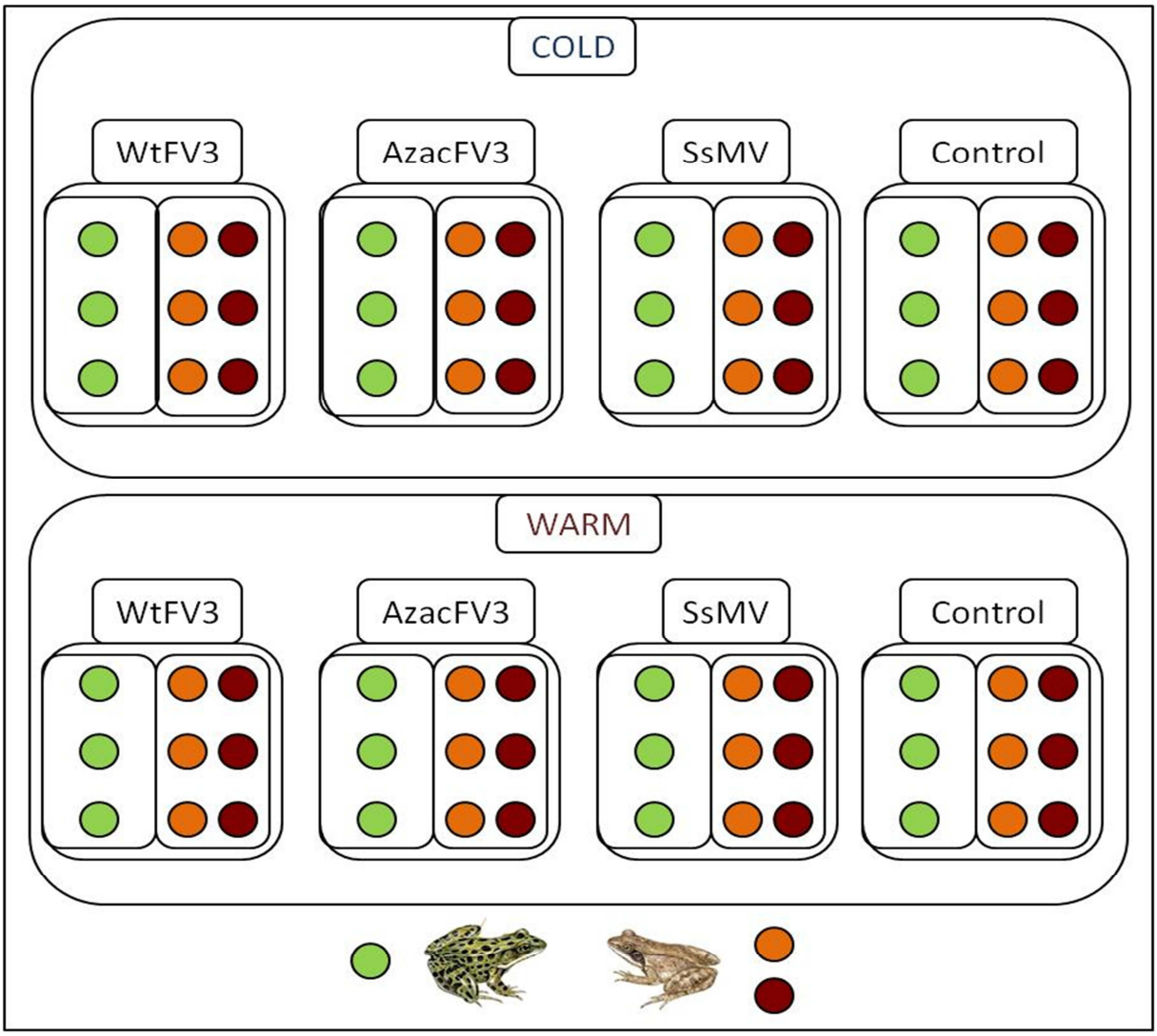

Fig. 1. Experimental design. Three replicates of *L. pipiens* and *L. sylvaticus* tadpoles were exposed to three strains of ranavirus in two temperature conditions

4. Animal monitoring

a. Daily monitoring and care

Tanks were monitored on a daily basis. Dead tadpoles were removed using assigned disposable plastic pipettes to prevent any scavenging. Upon removal dead tadpoles were

measured for body mass and length (see below) and their developmental stage was estimated using the nomenclature proposed by Gosner (Gosner, 1960). Tadpoles were then kept at -25 ^{o}C in individual plastic vials with ethanol for subsequent analyses. Euthanized individuals were subjected to the same measurements, the water in each tank was replaced with clean dechlorinated water aged for 24 hours on a weekly basis starting on week 3. We consider that the amount of time the tadpoles were in contact with contaminated water (12 hours in close proximity plus 3 weeks in a larger volume) was long enough to simulate natural viral exposure conditions. At the end of the experiment, all contaminated water was treated with 5% bleach and left to sit for 2-3 days to kill off any remaining viral particles before being discarded. Food was administered to each tank after each weekly water change. Tadpoles were fed on a weekly basis with standard tadpole food (Carolina Biological Supply Company, Burlington, NC) at 30 mg/tadpole for week 1, 60 mg/tadpole for week 2, and 120 mg/tadpole for week 3 until the end of the experiment (Echaubard *et al.* 2010). The experiment terminated when all the individuals died or reached metamorphosis. The procedures used in this experiments follow protocol #2010-04-02 approved by the Laurentian University Animal Care Committee.

b. Life history traits measurement

Specific life history traits were used as indicators of host fitness following infection. Final body weight (Travis 1984), final length and width (Semlitsch *et al.* 1988) and final developmental stage were recorded as estimators of growth in turn representing proxies for fitness (Semlitsch *et al.* 1988). The final body length (nose to tail) and width (behind the eyes at the level of the operculum) of the tadpole (nose to tail) were measured using an electronic caliper (VWR, model 12777.830 ± 0.005 mm). Final body weight was measured to the nearest 0.001 g using a precision balance (Denver Instrument). Final developmental stage was determined using Gosner's anuran development nomenclature (Gosner 1960). Developmental rate was calculated by dividing the developmental stage at death by the total number of days the individual survived. Data on weekly mortality per tank were used to estimate the mortality rate over time.

We also calculated several components of pathogen and host fitness directly related to infection. We defined virulence of a given virus strain as the proportion of individuals that died from infection out of the total number of infected individuals (number of dead individuals that were infected / number of infected individuals). We defined resistance of the host as the proportion of individuals that were successful at preventing an infection despite

being exposed or individuals that were able to clear the infection (number of non-infected individuals / number of exposed individuals). Finally, we defined tolerance of the host as the proportion of individuals that survived despite being infected (number of infected survivors / number of infected individuals). Some authors have assumed that individuals exposed to pathogens but that are not infected would have the same fitness as control individuals not exposed to pathogens (Read *et al.* 2008). However, exposure to the pathogen could be costly to the animals even when infection is successfully prevented revealing a cost of exposure/resistance and the need for a distinction between resistance and tolerance (Rohr *et al.* 2010).

5. Infection screening

Upon death, all animals (including euthanized ones) were dissected to remove the liver which was then crushed into a 1.5 ml Eppendorf tube. The resulting tissue mixture was used for DNA extraction. DNA was extracted using QIAmp DNeasy Kit following the standard protocol (Qiagen). After extraction, a double blind PCR was performed using a primer known to successfully amplify ranavirus**,** specifically Frog Virus 3: MCP-ranavirus-F (5'-GACTTGGCCACTTATGAC-3') and MCP-ranavirus-R (5'- GTCTCTGGAGAAGAAGAA), following the PCR conditions listed in Mao *et al.* (1997), using 1.5 µl of template DNA and cycled 40 times**.** This specific primer has been used in other studies and is known to amplify a portion of the major capsid protein within the Frog Virus 3 genome (Mao *et al.* 1997). Individuals showing two positive amplifications for both PCRs were considered infected.

6. Statistical analyses

Data on host body weight, length and width and development were analyzed using General Linear Models (GLM). We computed a full factorial MANOVA model, with temperature, host species, and virus strain as fixed factors and virulence, resistance, tolerance, day of death, length, width, weight, developmental stage, and developmental rate as dependant variables. When the standard assumptions of analysis of variance were not met, even after BoxCox transformation (Sokal and Rohlf 1995), we used Generalized Linear/non-linear models (GZM) with a log link function because residuals of the dependant variables in the parametric GLM followed a gamma distribution (McCullagh and Nelder 1989). To test the significance of GZM function model, we used the Wald statistics which is based on the asymptotic normality property of maximum likelihood estimates.

We analyzed host survival using a survival analysis and failure time analysis using the Kaplan & Meier product limit method associated with Chi square and Gehan's Wilcoxon tests (multiple and two sample comparisons respectively; Gehan 1965). Individuals surviving to the end of the experiment were censored to account for our lack of information about their true time to death (Leung *et al.* 1997).

Data on virulence, tolerance and resistance were analyzed using a log-linear analysis of frequency tables based on a Maximum Likelihood Chi-square calculation. The log-linear analysis deals with multi-way frequency tables in terms that are very similar to ANOVA through logarithmic transformations. Thus allows the exploration of the structure of the categorical variables included in the table. All statistical analyses were performed using Statistica 8.0 (Statsoft 2007).

Results

1. Mortality patterns

Temperature had a strong overall effect on the mortality rate with individuals held at low temperature (i.e., 14 ^{o}C) dying more rapidly than individuals at higher (i.e., 22 ^{o}C) temperature conditions ($Z = -3.51$, $p < 0.001$). With regard to species, a greater percentage of Wood Frog (WF) tadpoles died following viral challenge than Northern Leopard Frog tadpoles (LF, $X^2 = 12.64$, $p<0.005$). However, among WF genotypes there was no difference in mortality ($X^2 = 0.63$, $p=0.43$). With regard to the pathogens, we noticed significant differences in strains effect on tadpole mortality rates ($X^2 = 10.39$, $p<0.01$). Tadpoles exposed to wt-FV3 were characterized by the highest mortality rate (98%) followed by SsMeV (92%), azaC (88%) and controls (75%; Fig. 2, Table 1).

Differences in mortality between host species was influenced by the temperature (G_HxE, $X^2 = 20.13$, $p<0.001$). Under warmer conditions, fewer LF tadpoles died than the two genotypes of WF tadpoles (60% vs. 96% for WF1, $X^2 = 23.76$, $p<0.001$, and 94% for WF2, $X^2 = 29.1$, $p<0.001$) while no differences in mortality were observed between WF hosts (WF1 vs. WF2, $X^2 = 0.5$, $p=0.47$). At 14 ^{o}C, however, no differences in mortality were observed among the three host genotypes ($X^2 = 2.77$, $p=0.24$). With regard to viral strains at low temperature, tadpoles exposed to azaC, SsMeV or wt-FV3 tend to experience similar mortality rates ($X^2 = 2.17$, $p=0.23$) but this did not hold true at 22 ^{o}C where tadpoles exposed to wt-FV3 died at a higher rate than tadpoles exposed to SsMeV and tadpoles exposed to azaC (G_PxE; $X^2 = 45.04$, $p<0.001$; Fig. 2, Appendix 1).

Table 1. Results of survival analysis. Only results for the main factors are shown. Results for factor interactions are provided in supplemental material. Abbreviations: Leopard Frog (LF), Wood Frog (WF), WF1, WF2. Significant ($p<0.005$) *a posteriori* differences between levels of factors are indicated by letters.

Effect	Treatment	Dead	Survivors	N Total	% Dead
	Statistics		(Z = -3.51, p<0.001)		
TEMPERATURE	COLD	196	11	207	0.95
	WARM	160	33	193	0.83
	Statistics		(X^2 = 12.64, p<0.005)		
SPECIES	LF	129	36	165	0.78 [a]
	WF1	107	4	111	0.96 [b]
	WF2	120	4	124	0.97 [b]
	Statistics		(X^2 = 10.39, p<0.01)		
STRAINS	AzaC	87	12	99	0.88 [ab]
	Control	78	22	100	0.78 [a]
	SsMeV	91	8	99	0.92 [bc]
	wt-FV3	100	2	102	0.98 [c]

We also observed strain x species interactions (G_H x G_P) for mortality rate (X^2 = 37.22, p<0.001) with host species showing different patterns of mortality depending on which strain they have been exposed to. Leopard frog tadpoles exposed to wt-FV3 displayed the highest mortality rate as compared to the other strains whereas no differences between strains were found to explain mortality patterns in WF tadpoles (Fig. 2, Appendix 1).

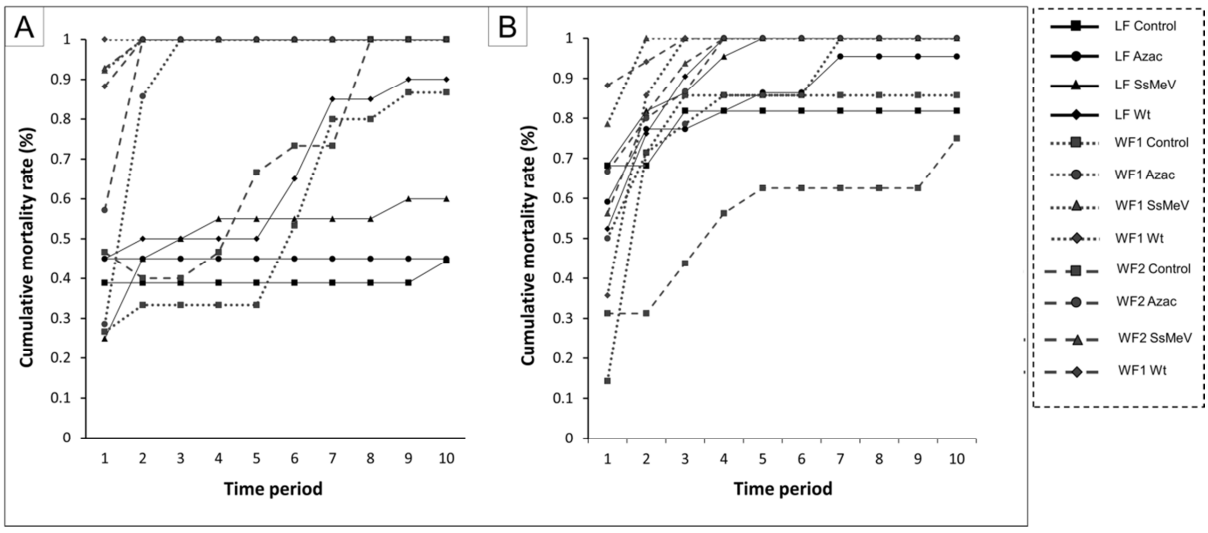

Fig. 2. Mortality rate over time in (A) cold (14 °C) and (B) warm (22 °C) conditions.

In addition to G_H x G_P interactions, we observed a synergistic effect of temperature, host genotype and parasite genotype on the mortality rate, (G_HxG_PxE; X^2 = 45.04, p<0.001). The

greatest source of variation observed for this triple interaction was with LF tadpoles that experienced significantly less mortality than wood frog tadpoles at 22 ^{o}C and that the pattern of mortality of the LF tadpoles exposed to the different strain was different that in WF. At 14 ^{o}C such differences were not observed (Fig. 2, Appendix 1).

2. Infection related traits

a. Virulence

Overall, ranavirus virulence (all strains combined), defined as the proportion of individuals that died from infection out of the total number of infected individuals, was influenced by temperature (X^2 = 134.66, p<0.001) whereby the proportion of individuals dying from infection was greater at 14 ^{o}C (96% vs. 86% at 14 ^{o}C and 22 ^{o}C respectively). Although we did not observe differences between strains (X^2 = 34.9, p=0.69) there was a trend for wt-FV3 to induce higher levels of mortality (97.5%), followed by SsMeV (91.43%) and azaC (87.23%). Additionally, we observed that the virulence of ranavirus (all strains combined) varied according to the host species (X^2 = 141.52, p<0.001) as a greater proportion of individual died from infection in WF1 (95%) and WF2 (100%) as compared to LF (80%; Table 2).

No temperature x strain interaction was observed (X^2 = 23.19, p=0.87) although azaC had a tendency to kill a smaller proportion of tadpoles at 22 ^{o}C compared to the two other strains. This pattern was not observed at 14 ^{o}C where azaC was as lethal as the other strains. However, we observed an interaction between temperature and host species (X^2 = 130.81, p<0.001). Although equivalent levels of tadpoles from each host species died from infection at 14 ^{o}C (X^2 = 10.6, p=0.78), a significantly smaller proportion of LF tadpoles (56%) died from infection as compared to WF1 and WF2 tadpoles (both 100%; X^2 = 56.3, p<0.001) at 22 ^{o}C. No interaction between temperature, strains and species (G_HxG_PxE) was observed for virulence (X^2 = 3.9, p=0.94; Appendix 2).

b. Tolerance

We define tolerance as the proportion of tadpoles that survived despite the infection. Tolerance was greatly influenced by temperature (X^2 = 94.4, p<0.001). Approximately 20% of tadpoles that were infected survived at 22 ^{o}C as compared to less than 2% at 14 ^{o}C. We observed significant differences between species in their ability to tolerate an infection (X^2 = 93.45, p<0.001). LF tadpoles were the only host able to tolerate the infection during this experiment, with 27% of them surviving despite the presence of the virus (Table 2).

We also noticed that tolerance by the hosts depended on the type of strain they were infected with (X^2 = 70.1, p<0.002). LF tadpoles were able to tolerate infection with azaC and SsMeV to a greater extent than an infection with wt-FV3 (respectively 13%, 11.5% and 2.5%). Consequently, due to the contrast between WF and LF tolerance and the significant strain effect, we observed a G_H x G_P interaction (X^2 = 276.87, p<0.001; Appendix 2).

Table 2. Results of log-linear analysis for virulence, tolerance, and resistance in response to temperature, host species, and virus strains. Only results for the main factors are shown. Results for factor interactions are provided in supplemental material. Abbreviations: Temperature (Temp.), Leopard Frog (LF), Wood Frog (WF), WF1, WF2, number of individuals infected (# inf.), number of individuals that died from infection (# died inf.), number of individuals that survived despite being infected (# surv. inf.), number of individuals exposed (# exp.) and number of individuals non infected but exposed to infection (# non.inf.). Significant (p<0.005) *a posteriori* differences between levels of factors are indicated by letters.

		VIRULENCE			TOLERANCE			RESISTANCE		
Effects	**Treatment**	**# inf**	**# died inf.**	**% virulence**	**#inf.**	**# surv. Inf.**	**% tolerance**	**#exp.**	**# non inf.**	**% resistance**
TEMP.	***Statistics***		(X^2 = 134.66, p<0.001)			(X^2 = 94.4, p<0.001)			(X^2 = 275.34, p<0.001)	
	COLD	71	68	95.77	71	1	1.41	155	84	54.19
	WARM	51	44	86.27	51	10	19.61	145	94	64.83
SPECIES	***Statistics***		(X^2 = 141.52, p<0.001)			(X^2 = 93.45, p<0.001)			(X^2 = 251.18, p<0.001)	
	LF	41	33	80.49 [a]	41	11	26.83 [a]	125	84	67.20 [a]
	WF1	42	40	95.24 [b]	42	0	0 [b]	82	40	48.78 [b]
	WF2	39	39	100 [b]	39	0	0 [b]	109	54	49.54 [b]
STRAINS	***Statistics***		(X^2 = 34.9, p=0.69)			(X^2 = 70.1, p<0.002)			(X^2 = 37.44, p=0.57)	
	AzaC	47	41	87.23 [b]	47	6	12.77 [c]	99	52	52.53 [a]
	Control	0	0	0 [a]	0	0	0 [a]	0	0	0 [a]
	SsMeV	35	32	91.43 [b]	35	4	11.43 [b]	99	64	64.65 [c]
	Wt-FV3	40	39	97.50 [b]	40	1	2.5 [b]	102	62	60.78 [b]

Temperature had a strong influence on the host ability to tolerate an infection illustrating a G_H x E interaction (X^2 = 148.82, p<0.001). The ability of LF tadpoles to tolerate the infection was significantly higher at 22 ^oC (62.5%) than at 14 ^oC (4%). Additionally a significant G_PxE interaction was observed (X^2 = 50.1, p = 0.02). At 14 ^oC, a small fraction of infected LF individuals were able to tolerate the infection with azaC (4.3%) but not infection with the other strains. In warm conditions, LF tadpoles were able to tolerate infection with all strains, particularly SsMeV (30.8%) and azaC (20.3%). Infection with wt-FV3 was less well tolerated by LF tadpoles (7%; Appendix 2).

We also observed a G_H x G_P x E interaction **(**X^2 = 247.52, p < 0.001) suggesting that the tolerance of a given host species is contingent on the temperature setting and the strains responsible for infection (Appendix 2).

c. Resistance

Resistance, the proportion of tadpoles that have not been infected despite being exposed to ranavirus or individuals that were able to clear the infection, was influenced by the

temperature at which the interaction took place (X^2 = 275.34, p<0.001). At 14 °C, a smaller proportion of individuals were free of infection (54% vs. 64% in warm conditions) than at 22 °C. Resistance to the infection also varied depending on the host species (67.2%, 48.8% and 49.6% for LF, WF1, and WF2 respectively, X^2 = 251.18, p<0.001). Patterns of resistance to infection did not vary depending on the strain involved in the infection (X^2 = 37.44, p=0.57) although the hosts (both species accounted) had a tendency to be able to resist infection with SsMeV (64%) more than with wt-FV3 (61%) and azaC (52.5%; Table 2).

We found no significant genotypic interaction between strains and host species despite the noticable difficulty for LF to resist wt-FV3 (58.5%) relatively to the other strains. This trend contrasted with what was observed for WF tadpoles that were mostly having difficulty to resist azaC (35.7% for WF1 and 48.3% for WF2) as compared to the other strains. Our results revealed however a G_HxE interaction (X^2 = 249.84, p<0.001). Both at 14 °C and 22 °C, a larger proportion of LF tadpoles resist the infection but the extent of the difference between WF and LF tadpoles' ability to resist was greater at 14 °C (40% and 42% vs 62% for WF1, WF2 and LF respectively) than at 22 °C (58%, 60% and 73% for WF1, WF2 and LF respectively). No strain x temperature interaction was observed (X^2 = 34.9, p=0.33) although a significant G_H x G_H x E interaction was revealed (X^2 = 40.13, p=0.02; Appendix 2).

3. Host life history traits

a. Size and mass

Temperature had a strong influence on host body length, width and weight. For these traits, tadpoles kept at 22 °C were bigger as compared to those held at 14 °C (GZL, $W_{(df = 1)}$ = 43.21, p<0.001; $W_{(df = 1)}$ = 35.09, p<0.001; $W_{(df = 1)}$ = 22.56, p<0.001 for length, width and weight, respectively). We also observed significant differences between species for these traits (GZL, $W_{(df = 2)}$ = 14, p<0.001; $W_{(df = 2)}$ = 55.13, p<0.001; $W_{(df = 2)}$ = 70.36, p<0.001 for length, width and weight, respectively). LF tadpoles were significantly heavier, longer and wider than WF tadpoles which in turn did not show differences between WF1 and WF2. With regard to strain effect, we observed significant differences between controls and infected tadpoles for length, width and weight (GZL, $W_{(df = 3)}$ = 40.85, p<0.001; $W_{(df = 3)}$ = 65.16, p<0.001; $W_{(df = 3)}$ = 25.07, p<0.001 respectively) with controls being heavier, larger and wider than infected individuals. We also found significant or marginally significant differences between virus strains with respect to their effects on host body width and weight (GZL, $W_{(df = 2)}$ = 9.06, p = 0.02; $W_{(df = 2)}$ = 5.04, p=0.08, respectively) but not on body length (GZL, $W_{(df = 2)}$ = 2.65, p=0.26).

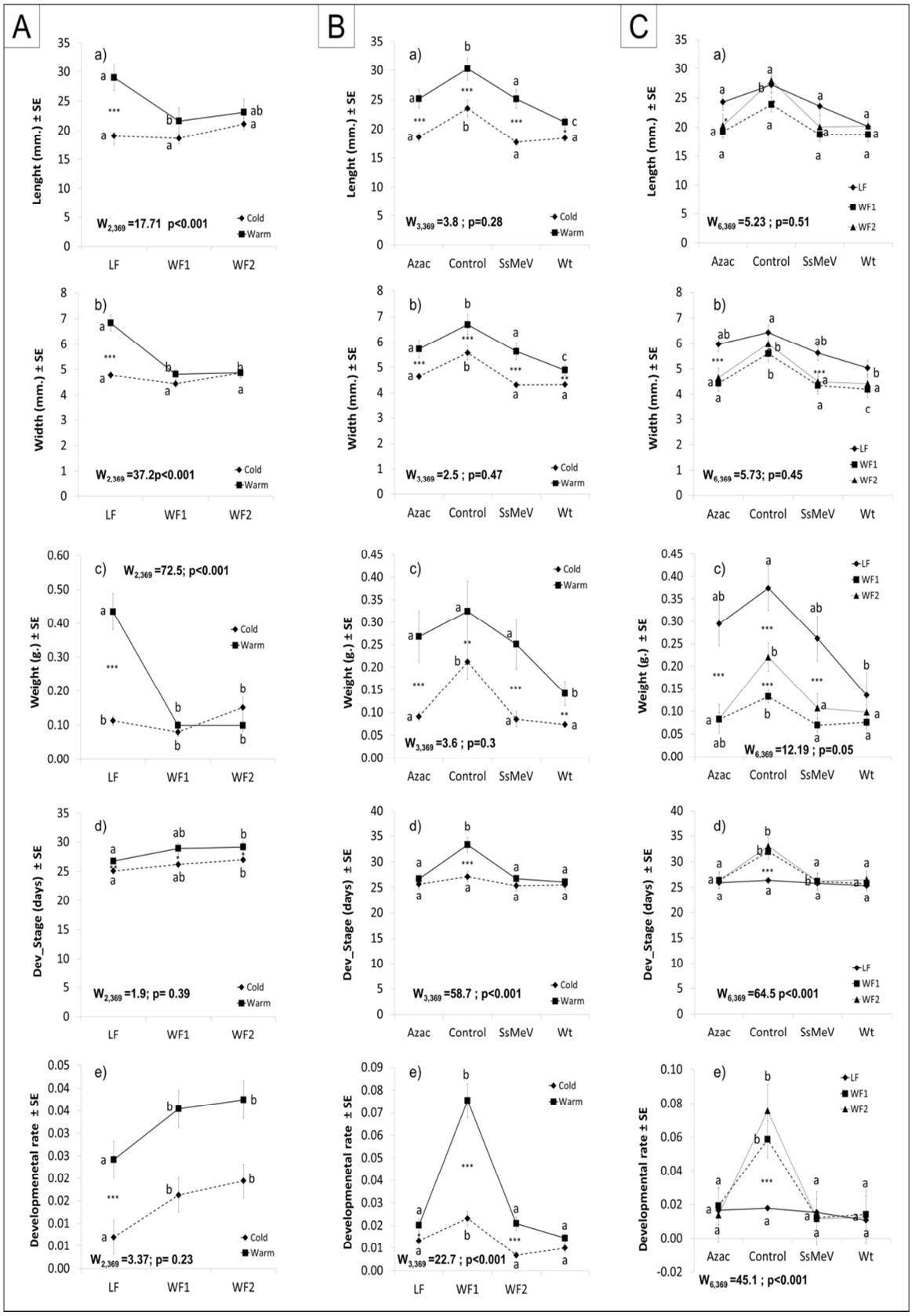

Fig. 3. Results of generalized linear model ANOVAs (log link and gamma error distribution) on (A) the effects of the interactions of Temperature and Species (G_H x E), (B) Temperature and Strains (G_P x E), and (C) Species and Strains (G_H x G_P) on LF and WF tadpole life history traits: (a) length, (b) width, (c) weight, (d) developmental stage, and (e) developmental rate. Values are least square means ± 1SE.

For width and weight, all tadpoles no matter the species, were smaller when infected by wt-FV3 (Table 3).

Additionally, we observed a significant G_H x E interaction for body length, width, and weight (GZL, $W_{(df = 2)} = 17.71$, $p<0.001$; $W_{(df = 2)} = 37.2$, $p<0.001$; $W_{(df = 2)} = 72.52$, $p<0.001$ respectively). The main source of variation for this interaction was represented by LF tadpoles that were heavier, larger, and wider than WF tadpoles at 22 ^{o}C but not at 14 ^{o}C. By contrast, no G_P x E interaction (temperature x strains) was observed (Table 3, Fig. 3).

Furthermore, we documented a marginally significant G_H x G_P interaction (strains x species) for body weight (GZL, $W_{(df = 6)} = 12.19$, $p=0.05$), but not for either length or width. While no difference in weight was noted among WF tadpoles infected by the three different strains, LF tadpoles infected with wt-FV3 were significantly lighter than LF tadpoles infected with either SsMeV or azaC (Table 3, Fig. 3).

Finally, we observed a G_H x G_P x E interaction for body width and weight (GZL, $W_{(df = 6)} = 14.56$, $p=0.02$; $W_{(df = 6)} = 27.3$, $p<0.001$ respectively) but not for length (Table 3, Fig. 4).

b. Growth

Temperature had a strong influence on the final developmental stage reached by the tadpoles and their developmental rate (GZL, $W_{(df = 1)} = 59.1$, $p<0.001$; $W_{(df = 1)} = 70.45$, $p<0.001$, resp ectively). For these traits, tadpoles held at 22 ^{o}C developed faster compared to individuals raised at 14 ^{o}C. Similarly, we also observed significant differences between species for these traits (GZL, $W_{(df = 2)} = 45$, $p<0.001$; $W_{(df = 2)} = 20.41$, $p<0.001$ for developmental stage and developmental rate, respectively). LF tadpole developmental rate was much slower than WF although there was no significant difference between WF1 and WF2 for these traits. We also noticed a significant strain effect on the growth variables (GZL, $W_{(df = 3)} = 167.1$, $p<0.001$; $W_{(df = 3)} = 114.2$, $p<0.001$ for developmental stage and developmental rate, respectively). Control tadpoles grew faster than infected ones, and comparisons of infected individuals suggest that tadpoles infected with wt-FV3 had an overall tendency to develop slower than tadpoles infected with azaC. Tadpoles infected with SsMeV presented an intermediate developmental rate (Table 3).

While there was no interaction between species and temperature for growth, we observed significant temperature x strain interactions (G_PxE, GZL, $W_{(df = 3)} = 58.7$, $p<0.001$; $W_{(df = 3)} = 22.72$, $p<0.001$) for developmental stage and developmental rate, respectively).

Table 3. Results of generalized linear model ANOVAs (log-link and gamma error distribution) showing variation in LF tadpole life history traits in response to temperature, species, strains, and their interactions. Significant effects based on the asymptotic normality property of maximum likelihood estimates correspond to p<0.005. Significant (p<0.005) *a posteriori* differences between levels of factors are indicated by letters.

Effect	Stat. and factors	Length	Width	Weight	Dev_Stage	Developmental rate
TEMP.	***Statistics***	**$W_{1,369}$ =43.21; p<0.001**	**$W_{1,369}$ =35.1, p<0.001**	**$W_{1,369}$ =22.56, p<0.001**	**$W_{1,369}$ =59.1, p<0.001**	**$W_{1,369}$ =70.45, p<0.001**
	COLD	19.58	4.72	0.12	25.97	0.013
	WARM	25.22	5.69	0.24	28.06	0.031
SPECIES	***Statistics***	**$W_{2,369}$ =14, p=0.01**	**$W_{2,369}$ =55.1, p<0.001**	**$W_{2,369}$ =70.36, p<0.001**	**$W_{2,369}$ =45, p<0.001**	**$W_{2,369}$ =20.41, p<0.001**
	LF	23.77 ^{a}	5.76 ^{a}	0.27 ^{a}	25.89 ^{a}	0.015 ^{a}
	WF1	20.07 ^{b}	4.62 ^{b}	0.09 ^{b}	27.49 ^{b}	0.025 ^{b}
	WF2	22.07 ^{ab}	4.87 ^{b}	0.13 ^{b}	28.00 ^{b}	0.028 ^{b}
STRAINS	***Statistics***	**$W_{3,369}$ =40.8, p<0.001**	**$W_{3,369}$ =65.2, p<0.001**	**$W_{3,369}$ =25.07, p<0.001**	**$W_{3,369}$ =167.1, p<0.001**	**$W_{3,369}$ =114.2, p<0.001**
	Azac	21.76 ^{a}	5.17 ^{b}	0.18 ^{b}	26.20 ^{a}	0.017 ^{b}
	Control	26.54 ^{b}	6.07 ^{c}	0.26 ^{c}	29.91 ^{b}	0.046 ^{c}
	SsMeV	21.20 ^{a}	4.93 ^{ab}	0.16 ^{ab}	26.05 ^{a}	0.013 ^{a}
	Wt	19.80 ^{a}	4.61 ^{a}	0.11 ^{a}	25.85 ^{a}	0.012 ^{a}
Temp*Sp	***Statistics***	**$W_{2,369}$=17.7, p<0.001**	**$W_{2,369}$ =37.2, p<0.001**	**$W_{2,369}$ =72.52, p<0.001**	**$W_{2,369}$ =1.9, p= 0.39**	**$W_{2,369}$ =17.9, p= 0.22**
	COLD_LF	19.06 ^{ab}	4.79 ^{a}	0.11 ^{a}	25.10 ^{c}	0.007 ^{d}
	COLD_WF1	18.64 ^{a}	4.45 ^{a}	0.08 ^{a}	26.20 ^{bc}	0.016 ^{b}
	COLD_WF2	21.14 ^{ab}	4.87 ^{a}	0.15 ^{a}	26.98 ^{ab}	0.019 ^{bc}
	WARM_LF	29.02 ^{c}	6.84 ^{b}	0.44 ^{b}	26.76 ^{ab}	0.024 ^{ac}
	WARM_WF1	21.64 ^{ab}	4.82 ^{a}	0.10 ^{a}	28.90 ^{a}	0.035 ^{a}
	WARM_WF2	23.12 ^{bc}	4.87 ^{a}	0.10 ^{a}	29.14 ^{a}	0.037 ^{a}
Temp*Strains	***Statistics***	**$W_{3,369}$ =3.7, p=0.28**	**$W_{3,369}$ =2.5, p=0.47**	**$W_{3,369}$ =3.6, p=0.3**	**$W_{3,369}$ =58.7, p<0.001**	**$W_{3,369}$ =22.7, p<0.001**
	COLD_Az	18.61 ^{ab}	4.65 ^{ab}	0.09 ^{bc}	25.73 ^{ab}	0.013 ^{a}
	COLD_C	23.53 ^{bcd}	5.57 ^{ac}	0.21 ^{ac}	27.15 ^{b}	0.023 ^{a}
	COLD_Ss	17.73 ^{a}	4.32 ^{b}	0.09 ^{b}	25.42 ^{a}	0.007 ^{b}
	COLD_Wt	18.43 ^{ab}	4.33 ^{b}	0.07 ^{b}	25.58 ^{ab}	0.010 ^{b}
	WARM_Az	25.17 ^{cd}	5.73 ^{ac}	0.27 ^{a}	26.72 ^{ab}	0.020 ^{a}
	WARM_C	30.27 ^{d}	6.69 ^{c}	0.32 ^{a}	33.33 ^{c}	0.075 ^{c}
	WARM_Ss	25.13 ^{cd}	5.63 ^{ac}	0.25 ^{ac}	26.76 ^{ab}	0.021 ^{a}
	WARM_Wt	21.19 ^{abc}	4.89 ^{ab}	0.14 ^{abc}	26.14 ^{ab}	0.014 ^{a}
Sp*Strains	***Statistics***	**$W_{6,369}$ =5.2, p=0.51**	**$W_{6,369}$ =5.7, p=0.45**	**$W_{6,369}$ =12.2, p=0.05**	**$W_{6,369}$ =64.5, p<0.001**	**$W_{6,369}$ =45.1, p<0.001**
	LF_Az	24.30 ^{abcd}	5.97 ^{bcd}	0.29 ^{bc}	25.95 ^{a}	0.017 ^{a}
	LF_C	27.20 ^{bd}	6.44 ^{d}	0.37 ^{c}	26.40 ^{a}	0.018 ^{a}
	LF_Ss	23.59 ^{abcd}	5.62 ^{abcd}	0.26 ^{abc}	25.86 ^{a}	0.015 ^{ab}
	LF_Wt	20.14 ^{ac}	5.03 ^{abcd}	0.14 ^{ab}	25.36 ^{a}	0.011 ^{b}
	WF1_Az	19.26 ^{ab}	4.43 ^{a}	0.08 ^{ab}	26.39 ^{a}	0.019 ^{a}
	WF1_C	23.87 ^{abcd}	5.60 ^{abcd}	0.13 ^{abc}	31.96 ^{b}	0.059 ^{c}
	WF1_Ss	18.73 ^{ab}	4.35 ^{ab}	0.07 ^{a}	26.19 ^{a}	0.012 ^{ab}
	WF1_Wt	18.73 ^{ab}	4.20 ^{a}	0.08 ^{a}	25.78 ^{a}	0.014 ^{ab}
	WF2_Az	20.29 ^{abcd}	4.65 ^{abcd}	0.08 ^{ab}	26.41 ^{a}	0.014 ^{ab}
	WF2_C	27.93 ^{cd}	5.98 ^{cd}	0.22 ^{abc}	33.00 ^{b}	0.076 ^{c}
	WF2_Ss	20.05 ^{abcd}	4.49 ^{abc}	0.11 ^{ab}	26.21 ^{a}	0.012 ^{ab}
	WF2_Wt	20.22 ^{abcd}	4.42 ^{abc}	0.10 ^{a}	26.53 ^{a}	0.012 ^{ab}
Temp*Sp*Strains	***Statistics***	**$W_{6,369}$ =8.8, p=0.18**	**$W_{6,369}$ =14.6, p=0.02**	**$W_{6,369}$ =27.3, p<0.001**	**$W_{6,369}$ =24.2, p<0.001**	**$W_{6,369}$ =15.7, p=0.01**
	C_LF_Azac	19.22 ^{abc}	4.92 ^{abcd}	0.11 ^{ab}	25.35 ^{ab}	0.008 ^{abcd}
	C_LF_C	21.09 ^{abc}	5.29 ^{abcde}	0.17 ^{abcd}	25.23 ^{ab}	0.008 ^{ef}
	C_LF_Ss	17.56 ^{a}	4.41 ^{ab}	0.08 ^{ab}	24.77 ^{a}	0.006 ^{abcd}
	C_LF_Wt	18.33 ^{ab}	4.51 ^{ab}	0.09 ^{ab}	25.05 ^{ab}	0.005 ^{abcd}
	C_WF1_Az	17.38 ^{ab}	4.33 ^{abc}	0.07 ^{ab}	25.71 ^{ab}	0.019 ^{abcd}
	C_WF1_Cl	21.10 ^{abcd}	4.98 ^{abcde}	0.13 ^{abcd}	27.29 ^{abcd}	0.022 ^{abcd}
	C_WF1_Ss	17.22 ^{ab}	4.18 ^{ab}	0.06 ^{a}	25.86 ^{abc}	0.006 ^{de}
	C_WF1_Wt	18.85 ^{abc}	4.30 ^{abc}	0.07 ^{ab}	25.93 ^{abc}	0.018 ^{a}
	C_WF2_Az	18.84 ^{abc}	4.53 ^{abcd}	0.08 ^{ab}	26.36 ^{abc}	0.015 ^{abc}
	C_WF2_C	28.99 ^{bcd}	6.49 ^{bcde}	0.34 ^{bcde}	29.69 ^{cde}	0.045 ^{abcd}
	C_WF2_Ss	18.41 ^{abc}	4.31 ^{abc}	0.12 ^{ab}	25.94 ^{abc}	0.008 ^{fg}
	C_WF2_Wt	18.20 ^{abc}	4.13 ^{a}	0.06 ^{a}	25.94 ^{abc}	0.009 ^{cde}
	W_LF_Az	30.14 ^{bcd}	7.18 ^{cde}	0.51 ^{de}	26.65 ^{abc}	0.026 ^{ab}
	W_LF_C	34.67 ^{d}	7.84 ^{e}	0.62 ^{e}	27.83 ^{bcd}	0.030 ^{g}
	W_LF_Ss	30.23 ^{cd}	6.95 ^{de}	0.46 ^{cde}	27.05 ^{abc}	0.025 ^{g}
	W_LF_Wt	21.95 ^{abc}	5.55 ^{abcde}	0.18 ^{abcd}	25.67 ^{ab}	0.016 ^{g}
	W_WF1_Az	21.14 ^{abcd}	4.53 ^{abc}	0.09 ^{abcde}	27.07 ^{abcd}	0.019 ^{abcde}
	W_WF1_C	27.40 ^{abcd}	6.38 ^{abcde}	0.14 ^{abc}	37.91 ^{e}	0.105 ^{abcde}
	W_WF1_Ss	20.35 ^{abcd}	4.53 ^{abcd}	0.08 ^{ab}	26.54 ^{abc}	0.019 ^{abcd}
	W_WF1_Wt	18.6 ^{abc}	4.09 ^{a}	0.08 ^{ab}	25.62 ^{abc}	0.010 ^{abcde}
	W_WF2_Az	21.84 ^{abcd}	4.78 ^{abcde}	0.09 ^{abcd}	26.46 ^{abc}	0.012 ^{ab}
	W_WF2_C	26.62 ^{abcd}	5.36 ^{abcde}	0.07 ^{ab}	37.08 ^{de}	0.113 ^{bcde}
	W_WF2_Ss	22.07 ^{abcd}	4.7 ^{abcde}	0.09 ^{abcd}	26.54 ^{abc}	0.016 ^{abcd}
	W_WF2_Wt	22.23 ^{abcd}	4.7 ^{abcd}	0.14 ^{abcd}	27.12 ^{abcd}	0.015 ^{cde}

At 22 ^{o}C, tadpoles infected with wt-FV3 developed slower than tadpoles infected with either azaC or SsMev, whereas at 14 ^{o}C no differences were observed (Table 3, Fig. 3).

We observed a G_H x G_P interaction (GZL, $W_{(df = 6)}$ = 64.5, $p<0.001$; $W_{(df = 6)}$ = 45.1, $p<0.001$ for developmental stage and developmental rate, respectively). LF tadpoles developed less and slower than WF tadpoles when infected with wt-FV3 but not with azaC or SsMeV (Table 3, Fig. 3).

Finally, we observed a G_H x G_P x E interaction for growth (GZL,$W_{(df = 6)}$ = 24.2, $p<0.001$; $W_{(df = 6)}$ = 15.7, $p=0.01$ for developmental stage and developmental rate, respectively). The interaction suggests that the tadpole growth was contingent on both the temperature at which the interaction took place and the virus strain responsible for the infection (Table 3, Fig. 4).

Discussion

Overall, our results revealed significant variation among hosts in their susceptibility to ranavirus and significant variation among ranavirus strains with regards to their infectivity. We also showed that some specific interactions (i.e., LF-Wt) might have tighter coevolutionary histories than other combinations, resulting in sharper mutual co-adaptations. Our findings therefore suggest that the prerequisites for frequency-dependent selection to occur are met in this system with host and strain genotypes being mutually influenced by each other. However, our results also suggest that the strength of these mutual selective pressures are also influenced by the temperature at which the interaction takes place, revealing for the first time in a vertebrate pathogen system the occurrence of G_H x G_P x E interactions.

1. Genotypic interactions between hosts and strains

We observed significant statistical interactions between host and virus strains for host mortality, tolerance, weight, final developmental stage, and developmental rate, suggesting the reciprocal influence of host and pathogen specificities for the determination of the outcome of the infection.

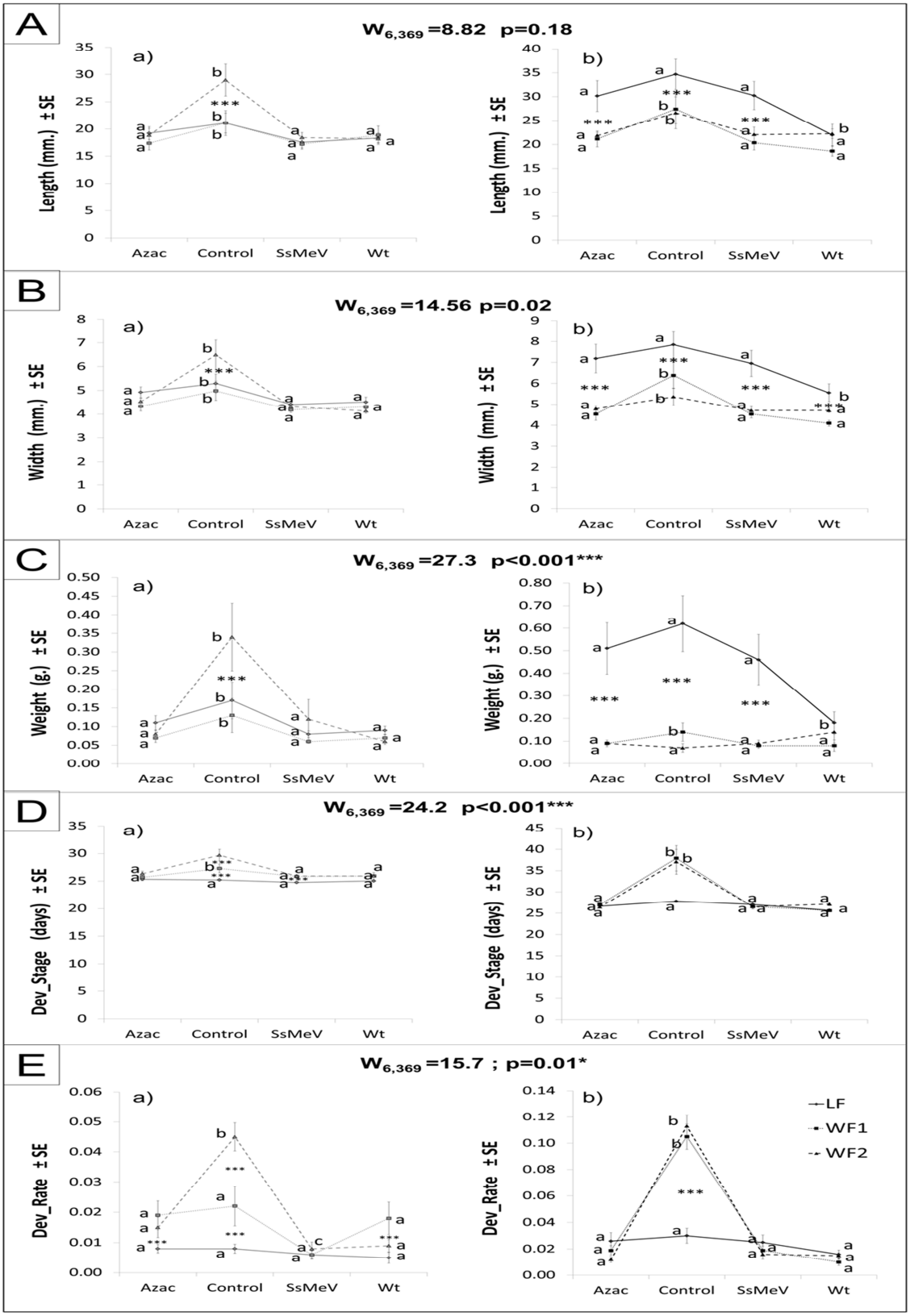

Fig. 4. Results of generalized linear model ANOVAs (log link and gamma error distribution) showing variation in life history traits in response to the interaction between Host and Pathogen genotypes and the temperature (G_H x G_P x E) interactions. To improve readability the results for each temperature condition have been separated into two different graphics, (a) cold (14 °C), and (b) warm (22 °C). The traits analysed were (A) length, (B) width, (C) weight, (D) developmental stage, and (E) developmental rate. Values are least square means ± 1SE.

The differences in strain infectivity and disease severity can possibly be explained by the structural components of these viruses. AzaC is known to be a mutant virus that is resistant to azacytidine. It presumably possesses a mutated DNA methyltransferase gene and cannot methylate its genomic DNA. The properties of SsMeV are less well known. The initial sequence study of the MCP suggested it to be a FV3-like virus with the ability to replicate in spotted salamanders, or to be a close relative to the FV3 that replicates in salamanders (Gregory Chinchar, pers. obs.). Here, we observed that hosts infected by SsMeV had a higher relative fitness than hosts infected by wt-FV3, the wild type strain. A potential explanation for this difference is that SsMeV is mostly adapted to replicate in salamanders and not in anurans, and that the latter is most likely to encounter wt-FV3 rather than the other strains. Ranaviruses are virulent pathogens inducing high mortality rate in their host species and from an evolutionary perspective, there should be no selection to reduce virulence in this system as they are horizontally transmitted (Ebert and Herre 1996); in other words, killing their host rapidly will not prevent transmission as necrophagy by other hosts can sustain it. Therefore, selection should favour virulent strains over mild ones and considering that wt-FV3 is more virulent than SsMe and azaC, wt-FV3 should be selected for and be more prevalent in the wild. Consequently, it is likely that the LF-wt-FV3 interaction is more prevalent than the other LF-ranavirus combinations in nature resulting in tighter coevolution for this specific interaction. Since the replication rate of the virus is faster than the replication of its host, it is very likely that the pathogen would have some selective advantage in this arms race and that in turn the host would have a harder time to resist and tolerate the pathogen. This interpretation needs further investigation but our results suggest that, under natural conditions, certain strains are better adapted to specific hosts resulting in high variability in infectivity and virulence.

Hosts characteristics also contribute to the occurrence of the G_H x G_P interactions. While we found no difference between WF genotypes, LF tadpoles showed a greater ability to resist and tolerate an infection, had lower mortality, and were bigger than WF tadpoles. Interestingly however, WF tadpoles were reaching more advanced development stages and developed faster than LF tadpoles. Wood Frogs are the most northern frog species in North America and have a large distribution, experiencing a wide array of environmental conditions (e.g., temperature fluctuations), for which they have been shown to exhibit an extensive plasticity (Herreid II and Kinney 1967, Berven 1982). Because they often breed in vernal pools filled with snow melt, adult WF need to reproduce very early and their tadpoles must

develop rapidly because of the short reproductive and developmental season available in northern landscapes.

Despite evident benefit of being plastic in such variable conditions, our results suggest that WFs also experience some costs associated with such plasticity when infected. Phenotypic plasticity has been suggested to bear several types of costs, including genetic costs (Padilla and Adolph 1996, DeWitt *et al.* 1998). For instance, genes promoting plasticity may be linked with genes conferring low fitness or modifying expression of other genes through epistasis, or they may have negative pleiotropic effects on traits other than plastic traits (DeWitt *et al.* 1998). Therefore, epistatic or pleiotropic effects might limit the product efficiency of genes involved in immunity and pathogen resistance in WF, resulting in differences in their resistance and tolerance profiles.

Differences in host susceptibility and strain infectivity are the fuel for coevolution (Thompson 1994). In our system, host relative fitness with regard to their susceptibility changed with the infecting strain (tolerance, resistance). Reciprocally, strain infectivity (virulence and lethality) varied depending on the type of host, highlighting the existence of G_H x G_P interactions. Interestingly, although LF tadpoles were significantly more resistant and tolerant to infection than WF tadpoles, they were particularly sensitive to wt-FV3. In fact, LF tadpoles infected with wt-FV3 were smaller, developed slower and experienced greater mortality than when infected with either azaC or SsMeV. No such **s**train-specific variation was observed in the case of WFs, indicating specific host/ranavirus interactions and potential variation in host and strain gene frequencies. Such variation may be critical for ranavirus epidemiological dynamics at the population level, in turn suggesting the potential for frequency-dependent selection to operate in this system. For instance, their coevolution may be oscillatory as envisioned under the Red Queen hypothesis (Van Valen 1973). However, despite evidence for genetic variation in resistance or virulence, direct demonstrations of parasite-mediated selection in nature are rare (Little 2002, Woolhouse *et al.* 2002). One increasingly popular explanation for this lack of convincing evidence is that environmental variables, such as temperature, modulate the strength and potentially the direction of the selective pressures (this study, Lazzaro and Little 2009, Wolinska and King 2009).

2. Hosts reaction norms in response to temperature

Overall in our study, cold conditions negatively affected host body condition and increased their susceptibility to ranavirus. Leopard frog tadpoles were particularly sensitive to cold temperatures compared to WF tadpoles. In warm conditions, infected LF tadpoles had

significantly lower mortality than WF tadpoles, they were also less susceptible to infection (LF were more tolerant and resistant to infection), and LF tadpole body size and growth declined less than in WF tadpoles. In cold conditions however, for all fitness-related traits except resistance, LF tadpoles were not significantly different from WF tadpoles. Therefore, the reduction of fitness in WF tadpoles induced by cold temperatures was less drastic than for LF tadpoles which were more sensitive to temperature decrease (in WF, except for developmental rate and resistance, the difference between cold and warm conditions was not significant). This is supported by the plastic nature of WF phenotypes which gain a relative selective advantage when dealing with adverse environmental conditions. Consequently, selection in one environment could drive genetic change in the host population but may have no predictable effect in another environment, suggesting in turn that G x E interactions could prevent a strong response to selection and maintain polymorphism in this system (Mitchell *et al.* 2005, Lazzaro and Little 2009). This assumption is somehow supported by recent investigations on *L. pipiens* genetic structure in the wild which suggest that eastern North-American populations harbor a relatively high genetic diversity (Hoffman et al 2004, Echaubard *et al.* unpublished data). With regard to the pathogen, we also observed an effect of temperature on virulence. For example, the higher overall virulence observed for wt-FV3 infection as compared to the other strains was particularly prevalent in warm conditions but disappeared in cold conditions.

Our results revealed significant G_H x G_P x E interactions where the effect of a given pathogen is contingent on the host genotype and the environment in which the interaction takes place. To our knowledge this is the first time that this type of interaction has been documented in a vertebrate-pathogen system (but see Scott 2006 for G x E interactions in mice). For this interaction to occur, in the present study both the host susceptibility and the ranavirus infectivity must be influenced by temperature. Evidence for such influences on host traits is supported by an abundant literature showing temperature effects on biochemical, physiological, and behavioural processes resulting in host species or genotype specific reaction norms with regards to thermal performance (Huey and Kingsolver 1993, Mitchell *et al.* 2005). Of particular interest is the host's ability to sustain an effective immune response under different environmental and ecological conditions including temperature (Lazzaro and Little 2009). For instance, increasing temperature has been suggested to induce an increase in the absolute numbers of polymorphonuclear leukocytes (Cohen and Warren 1935), and enhance phagocytic leukocyte activity (Nahas *et al.* 1971) in turn increasing the survival of the lizard *Dipsosaurus dorsalis* after challenge with gram-negative bacteria (Kluger *et al.*

1975). Since *in vitro* bacterial growth rate was stable over the range of temperatures used in the study, the authors concluded that enhanced survival at higher temperature most likely resulted from the enhancement of host defense mechanisms (Kluger *et al.* 1975). Similarly, increasing temperature can induce a better antibody response in *Xenopus* (Wabl and Du Pasquier 1976) and promotes a more efficient T cell response as evidenced by faster skin graft rejection (Robert *et al.* 1995). By contrast, decreasing temperature has been documented to induce host immunosuppression and enhance infection in several systems such as winter saprolegniosis in channel catfish (Bly *et al.* 1993). In the context of our experiment, we can speculate that the increase of infection severity in cold conditions, especially for LF tadpoles might have been induced by immunosuppression in the host but further investigations are needed to clarify this relationship.

Finally we noticed that control mortality was higher than previously observed in similar conditions (Echaubard *et al.* 2010). However, differences in mortality between control tadpoles and tadpoles exposed to combinations of treatment was not statistically significant and did not affect the relative statistical ranking of tadpoles from different treatments, confirming in turn the validity of our conclusions.

3. Ranavirus reaction norms in response to temperature

Reaction norms in pathogen infectivity resulting from G_P x E interactions are far less documented and most of the information available comes from *in vitro* studies. For instance, temperature can regulate the kinetics of virus replication as temperature influences the rate of viral protein and nucleic acid synthesis (Stairs 1978). In general, lower temperature inhibits virus replication and infection is limited,while virus reproduction within the host increases with temperature until a particular threshold (Ghosh and Bhattacharyya 2007). This pattern has been documented in several virus genera including baculoviruses, NPV (nuclear polyhedrosis virus; Ribeiro and Pavan (1994) and WSSV (white spot syndrome virus; Du *et al.* (2006, 2008). In the case of ranaviruses, FV3 (Frog Virus 3) isolated from frogs in the UK grows *in vitro* between 8 °C and 30 °C with slower replication below 15 °C and the fastest replication observed at 30 °C. In salamanders, the ranavirus ATV (*Ambystoma tigrinum* Virus) replicates very rapidly at 26 °C, more gradually at 18 °C, and very slowly at 10 °C (Rojas *et al.* 2005). However, ATV fails to develop lethal infection at 26 °C but can cause 100% host mortality when infection occurs at 18 °C (Rojas *et al.* 2005). This observation suggests that low temperatures likely induce host immunosuppression while higher temperature enhances the host immune function enough to circumvent virus replication (Rojas

et al. 2005). Preliminary data about *in vitro* replication rate of the three strains used in this experiment, suggest that they follow similar temperature dependent patterns for replication and infection (Echaubard *et al.*, unpublished data). Furthermore, we observed that all strains reached their maximum (100%) virulence in colder temperatures, indicating that lowering the temperature potentially induces host immunosuppression without impeding virus replication. Interestingly, in warm temperatures, mortality due to infection varied with the different strains, suggesting strain-specific thresholds for replication rate maximization. Therefore, together with temperature-dependent susceptibility in the hosts, such strain-specific thresholds of virulence are likely the source of the G_H x G_P x E interactions we observed in this system.

4. Conclusion

The genetic specificity of amphibian host-ranavirus strain interactions (G_H x G_P) documented in this study reveals a strong potential for frequency-dependent selection (Carius *et al.* 2001). Ranaviruses have been involved in epizootics and mass die-offs highlighting both their roles as a strong source of selection and the existence of host-pathogen coevolution in this system (Collins and Storfer 2003, Teacher *et al.* 2009, Miller *et al.* 2011). Our study suggests that Red Queen dynamics and the potential coevolutionary trajectories in host-pathogen systems might be dependent on the environment in which the interaction occurs (Thomas and Blanford 2003, Lazzaro and Little 2009, Wolinska and King 2009). For the first time in a vertebrate-pathogen system, we reveal the occurrence of G_H x G_P x E interactions whereby amphibian host susceptibility to ranavirus is temperature, host, and pathogen genotype-dependent. Furthermore, the suite of outcomes is very likely to be larger in natural conditions where other environmental variables such as resource availability (Bedhomme *et al.* 2004) and developmental stage (Johnson *et al.* 2011) play roles. Thus, environmental heterogeneity significantly increases the difficulty of predicting host and pathogen fitness-related trait variation as the environment may alter both selection specificity and strength (Lazzaro and Little 2009, Wolinska and King 2009), leading to variation in specific gene frequencies in nature. We suggest that using a reaction-norm approach will be successful in explaining changes in gene frequencies as a response to selective forces in host-pathogen systems (Cousyn *et al.* 2001, Mitchell *et al.* 2005) and will lead to a better understanding of the epidemiology and evolution of ranaviral diseases especially in the current context of climate change (Lesbarrères *et al.* 2011, Daskin and Alford 2012).

Acknowledgements

We would like to thank Paula Jackman and Chris Blomme for technical assistance. This work was supported by the Natural Science and Engineering Research Council,he Canadian Fund for Innovation/Ontario Innovation Trust to DL, and by the STAGE program of Environment Canada to BP. The authors have declared that no competing interests exist

Appendix 1. Results of survival analysis for interaction between factors. Significant ($p<0.005$) a posteriori differences between levels of factors are indicated by letters. Abbreviations: Temperature (Temp.), Warm (W), Cold (C), Species (Sp.), Leopard Frog (LF), Wood Frog (WF), Strains (St.)

Effects	Treatment	Dead	Survivors	N Total	% Dead
Sp * Strains	***Statistics***		($X^2 = 37.22$, $p<0.001$)		
	LF Azac	30	12	42	0.71 [a]
	LF control	26	14	40	0.65 [a]
	LF SSM	34	8	42	0.81 [ab]
	LF FV3wt	39	2	41	0.95 [b]
	WF1 Azac	28	0	28	1.00 [b]
	WF1 control	25	4	29	0.86 [b]
	WF1 SSM	27	0	27	1.00 [b]
	WF1 FV3wt	27	0	27	1.00 [b]
	WF2 Azac	29	0	29	1.00 [b]
	WF2 control	27	4	31	0.87 [b]
	WF2 SSM	30	0	30	1.00 [b]
	WF2 FV3wt	34	0	34	1.00 [b]
Temp*Sp	***Statistics***		($X^2 = 20.13$, $p<0.005$)		
	COLD_LF	82	5	87	0.94 [a]
	COLD_WF1	54	2	56	0.96 [a]
	COLD_WF2	60	4	64	0.94 [a]
	WARM_LF	47	31	78	0.60 [b]
	WARM_WF1	53	2	55	0.96 [a]
	WARM_WF2	60	0	60	1.00 [a]
Temp*Strain	***Statistics***		($X^2 = 21.3$, $p<0.001$)		
	COLD_Azac	50	1	51	0.98 [a]
	COLD_Control	42	10	52	0.81 [b]
	COLD_SsMeV	52	0	52	1.00 [a]
	COLD_Wt	52	0	52	1.00 [a]
	WARM_Azac	38	11	49	0.78 [b]
	WARM_Control	32	16	48	0.67
	WARM_SsMeV	42	8	50	0.84 [ab]
	WARM_Wt	49	2	51	0.96 [a]
Temp*Sp*Strain	***Statistics***		($X^2 = 45.04$, $p<0.001$)		
	COLD_LF_Azac	21	1	22	95.45
	COLD_LF_Control	18	4	22	81.82
	COLD_LF_SsMeV	22	0	22	100.00
	COLD_LF_Wt	21	0	21	100.00
	COLD_WF1_Azac	14	0	14	100.00
	COLD_WF1_Control	12	2	14	85.71
	COLD_WF1_SsMeV	14	0	14	100.00
	COLD_WF1_Wt	14	0	14	100.00
	COLD_WF2_Azac	15	0	15	100.00
	COLD_WF2_Control	12	4	16	75.00
	COLD_WF2_SsMeV	16	0	16	100.00
	COLD_WF2_Wt	17	0	17	100.00
	WARM_LF_Azac	9	11	20	45.00
	WARM_LF_Control	8	10	18	44.44
	WARM_LF_SsMeV	12	8	20	60.00
	WARM_LF_Wt	18	2	20	90.00
	WARM_WF1_Azac	14	0	14	100.00
	WARM_WF1_Control	13	2	15	86.67
	WARM_WF1_SsMeV	13	0	13	100.00
	WARM_WF1_Wt	13	0	13	100.00
	WARM_WF2_Azac	14	0	14	100.00
	WARM_WF2_Control	15	0	15	100.00
	WARM_WF2_SsMeV	14	0	14	100.00
	WARM_WF2_Wt	17	0	17	100.00

Appendix 2. Measures of pathogen virulence and host tolerance and resistance in response to the interactions between species and strains (Sp*Strains), temperature and species (Temp.*Sp), temperature and strains (Temp.*Strains) and temperature, species and strains (Temp.*Sp*Strains).The analyze computed is a log linear analysis of frequency table, Differences between treatment are observed for $p<0.05$

		VIRULENCE			TOLERANCE			RESISTANCE		
Effects	**Treatment**	**# inf.**	**# died inf.**	**% virulence**	**# exp.**	**# surv. Inf.**	**% Tolerance**	**# exp.**	**# non inf.**	**% Resistance**
	Statistics		(X^2 = 26.46, p=0.33)			(X^2 = 276.87, p<0.001)			(X^2 = 43.1, p=0.19)	
Sp*Stains	LF Azac	14	8	57.14	14	6	42.86 ^{a}	42	28	66.67
	LF control	0	0	0	0	0	0	0	0	0
	LF SSM	10	9	90.00	10	4	40 ^{bc}	42	32	76.19
	LF FV3wt	17	16	94.12	17	1	5.9 ^{c}	41	24	58.54
	WF1 Azac	18	18	100	18	0	0	28	10	35.71
	WF1 control	0	0	0	0	0	0	0	0	0
	WF1 SSM	14	12	85.71	14	0	0	27	13	48.15
	WF1 FV3wt	10	10	100	10	0	0	27	17	62.96
	WF2 Azac	15	15	100	15	0	0	29	14	48.28
	WF2 control	0	0	0	0	0	0	0	0	0
	WF2 SSM	11	11	100	11	0	0	30	19	63.33
	WF2 FV3wt	13	13	100 ^{a}	13	0	0	34	21	61.76
	Statistics		(X^2 = 130.81, p<0.001)			(X^2 = 148.82, p<0.001)			(X^2 = 249.84, p<0.001)	
Temp.*Sp	C_LF	25	24	96 ^{a}	25	1	4 ^{b}	65	40	61.54 ^{a}
	C_WF1	25	23	92 ^{a}	25	0	0 ^{a}	42	17	40 ^{b}
	C_WF2	21	21	100 ^{a}	21	0	0 ^{a}	64	27	42 ^{ab}
	W_LF	16	9	56.25 ^{b}	16	10	62.5 ^{c}	60	44	73.33 ^{a}
	W_WF1	17	17	100 ^{a}	17	0	0 ^{a}	40	23	58 ^{b}
	W_WF2	18	18	100 ^{a}	18	0	0 ^{a}	45	27	60 ^{b}
	Statistics		(X^2 = 23.19, p=0.87)			(X^2 = 50.1, p=0.02)			(X^2 = 34.9, p=0.33)	
Temp.*Strains	C_Azac	23	22	95.65 ^{a}	23	1	4.35 ^{a}	51	28	54.9 ^{a}
	C_Control	0	0	0	0	0	0	0	0	0
	C_SsMeV	22	20	90.91 ^{a}	22	0	0	52	30	57.69 ^{b}
	C_Wt	26	26	100 ^{a}	26	0	0	52	26	50 ^{c}
	W_Azac	24	19	79.17 ^{a}	24	5	20.3 ^{b}	48	24	50 ^{d}
	W_Control	0	0	0	0	0	0	0	0	0
	W_SsMeV	13	12	92.31 ^{a}	13	4	30.77 ^{c}	47	34	72.34 ^{abcde}
	W_Wt	14	13	92.86 ^{a}	14	1	7 ^{a}	50	36	72 ^{e}
	Statistics		(X^2 = 3.9, p=0.94)			(X^2 = 247.52, p<0.001)			(X^2 = 40.13, p=0.02)	
Temp.*Sp*Strains	C_LF_Azac	8	7	87.50 ^{a}	8	1	12.5 ^{a}	22	14	63.64 ^{a}
	C_LF_C	0	0	0	0	0	0	0	0	0
	C_LF_Ss	6	6	100 ^{ab}	6	0	0	22	16	72.73 ^{a}
	C_LF_Wt	11	11	100 ^{a}	11	0	0	21	10	47.62 ^{ab}
	C_WF1_Az	11	11	100 ^{a}	11	0	0	14	3	21.43 ^{b}
	C_WF1_Cl	0	0	0	0	0	0	0	0	0
	C_WF1_Ss	8	6	75.00 ^{a}	8	0	0	14	6	42.86 ^{ab}
	C_WF1_Wt	6	6	100 ^{a}	6	0	0	14	8	57.14 ^{ab}
	C_WF2_Az	4	4	100 ^{a}	4	0	0	15	11	73.33 ^{a}
	C_WF2_C	0	0	0	0	0	0	0	0	0
	C_WF2_Ss	8	8	100 ^{a}	8	0	0	16	8	50 ^{ab}
	C_WF2_Wt	9	9	100 ^{a}	9	0	0	17	8	47.06 ^{ab}
	W_LF_Az	6	1	16.67 ^{b}	6	5	83 ^{b}	20	14	70 ^{a}
	W_LF_C	0	0	0	0	0	0	0	0	0
	W_LF_Ss	4	3	75.00 ^{a}	4	4	100 ^{b}	20	16	80 ^{ab}
	W_LF_Wt	6	5	83.33 ^{a}	6	1	17	20	14	70 ^{a}
	W_WF1_Az	7	7	100 ^{a}	7	0	0	14	7	50 ^{c}
	W_WF1_C	0	0	0	0	0	0	0	0	0
	W_WF1_Ss	6	6	100 ^{a}	6	0	0	13	7	53.85 ^{bc}
	W_WF1_Wt	4	4	100 ^{a}	4	0	0	13	9	69.23 ^{bc}
	W_WF2_Az	11	11	100 ^{a}	11	0	0	14	3	21.43 ^{c}
	W_WF2_C	0	0	0	0	0	0	0	0	0
	W_WF2_Ss	3	3	100 ^{a}	3	0	0	14	11	78.57 ^{ab}
	W_WF2_Wt	4	4	100 ^{a}	4	0	0	17	13	76.47 ^{ab}

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CHAPTER 3:

Relationships between amphibian host life history and ranavirus epidemiological parameters

Manuscript 3 (PLOS One 5(10):e13723)

Context-Dependent Effects of Ranaviral Infection on Northern Leopard Frog Life History Traits.

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DL: design, analyses and writing

Abstract

Pathogens have important effects on host life-history traits, but the magnitude of these effects is often strongly context-dependent. The outcome of an interaction between a host and an infectious agent is often associated with the level of stress experienced by the host. *Ranavirus* causes disease and mortality in amphibian populations in various locations around the world but most known cases of ranaviral infection have occurred in North America and the United Kingdom. While ranavirus virulence has been investigated, the outcome of *Ranavirus* infection has seldom been related to the host environment. In a factorial experiment, we exposed Northern Leopard Frog (*Lithobates pipiens*, formerly *Rana pipiens*) tadpoles to different concentrations of *Ranavirus* and investigated the effect of host density on certain life-history traits, namely survival, growth rate, developmental stage and number of days from virus exposure to death. Our results suggest a prominent role of density in driving the direction of the interaction between *L. pipiens* tadpoles and ranavirus. We showed that increasing animal holding density is detrimental for host fitness as mortality rate is higher, day of death earlier, development longer and growth rate significantly lower in high density tanks. We observed a linear increase of detrimental effects when ranavirus doses increased in low density conditions, with control tadpoles having a significantly higher overall relative fitness. However, this pattern was no longer observed in high density conditions, where the effects of increasing ranavirus dose were limited. Infected and control animals fitness were consequently similar. We speculate that the host may eventually diverts the energy required for a metabolic/immune response triggered by the infection (*i.e.*, direct costs of the infection) to better cope with the increase in environmental "stress" associated with high density (*i.e*, indirect benefits of the infection). Our results illustrate how the net fitness of organisms may be shaped by ecological context and emphasize the necessity of examining the direct/indirect costs and benefits balance to fully understand host-pathogen interactions.

Introduction

Pathogens are known to affect their hosts in a variety of ways (Poulin 2007). Most of the studies investigating the relationship between hosts and pathogens have focused on the direct effects that pathogens have on host life history traits, usually including measures such as body length, body weight, growth rate, and survival (Michalakis 2007). Traditionally, quantifying the variation in these traits following infection is used to assess pathogen virulence and host fitness effects. However, beyond the effect a pathogen can have on host-specific fitness traits, attention has also been given to the role that pathogens can play in structuring host communities and affecting population dynamics (Loreau and Tilman 2005). While local extinction due to pathogen exposure is rare (see Cunningham and Daszak (1998) for an example) the extent of detrimental effects caused by a parasite may depend on biological factors such as the pathogen's mode of transmission (Lipsitch *et al.* 1996), the host genotype (Carius *et al.* 2001), and the host condition (Seppälä *et al.* 2008, Brown *et al.* 2000). Some of these features have been reported to be highly context dependent. For instance, previous studies have suggested that the degree of differential mortality suffered by infected hosts is linked to the specific host-pathogen relationship, but may also be influenced by the type and level of stress experienced by the host (Wakelin 1989).

Relationships between pathogens, parasites and environmental disturbance have recently been addressed in human-modified systems (Lebarbenchon *et al.* 2008), whereby pesticides or other pollutant exposure has typically been found to enhance parasite virulence (Lafferty and Holt 2003, Coors *et al.* 2008) due to a reduction of the host immune function Yang and Glaser (2002). At the same time, natural environmental fluctuations can also interact with pathogen virulence. For instance, host population increase may lead to an increase in intraspecific competition for food resources (due to the reduction of per capita food availability) that may affect host traits such as body size, body weight, growth rate, and reproductive ability and in turn affect the pathogen virulence and epidemiology (Arneberg 2002). High density situations may also result in an increase in the contact rate between individuals that can be stressful (Renshaw and Service 1993), and may also boost pathogen transmission rate (*i.e.*, horizontal transmission (Arneberg *et al.* 1998)) and subsequent pathogen load and virulence. For example the Gray Treefrog (*Hyla versicolor*) can co-occur in temporary and permanent ponds with a snail (*Pseudosuccinea columella*) that is frequently infected with the digenetic trematode *Telorchis* spp., whose free-swimming cercariae infect *H. versicolor* tadpoles. One study (Kiesecker and Skelly 2001) has shown that the presence of infected *P. columella* had

strong negative effects on the performance of gray treefrog larvae. This effect, however, depended on whether ponds were temporary or permanent; temporary pond animals were exposed to higher rates of infection, suggesting an important role of snail and tadpole density on subsequent infection status (Kiesecker and Skelly 2001). Density fluctuations may therefore be a key component of host-pathogen interactions, evolution and epidemiology.

Ranaviruses are highly virulent pathogens known to infect fish (Mao *et al.* 1997), reptiles (Hyatt *et al.* 2002) and a wide range of amphibian species (Daszak et al 1999, Docherty *et al.* 2003, Jancovich *et al.* 1997). Effects of ranavirus seem to be widespread, as they cause disease and mortality at various locations worldwide (Daszak and Cunningham 1999). Most known cases of ranaviral infection that have been adequately studied have occurred in North America (Jancovich *et al.* 2001, St Amour *et al.* 2008, Bollinger *et al.* 1999) and the UK (Cunningham 1996, Cunningham 2001, Teacher *et al.* 2010). Ranaviruses are now recognized as important pathogens and ranaviral disease is acknowledged by the World Organization for Animal Health (OIE) (http://www.oie.int/eng/maladies/en_classification2010.htm?e1d7). This underlies the importance of studying what factors may affect the virulence and distribution of this pathogen.

We experimentally investigated whether ranavirus effects were modulated by environmental conditions and by inoculation doses. More specifically, we tested whether increased host density plays an important role in the outcome of the interaction between ranavirus and *L. pipiens* tadpoles. In particular, we predicted an increase of ranavirus effects (virulence) when host density and inoculation dose increase. We define virulence as the overall detrimental effect a parasite or pathogen has on the fitness of its host (see Poulin and Combes (1999) for a discussion).

Although our results demonstrate a density-condition expression of ranavirus virulence, they only partially support our prediction. We showed that increasing density is detrimental for the host fitness as mortality rate is higher, day of death earlier, development longer, and growth rate is significantly lower in high density tanks. However, while we observed a linear increase of virulence when ranavirus doses increased in low density conditions, the pattern disappeared in high density conditions where infected and control individuals had the same relative fitness. Direct costs of infection have potentially been balanced by indirect benefits in deteriorating environmental conditions, therefore sustaining the relative fitness of infected hosts.

Materials and methods

1. The Host-Pathogen System

a. The pathogen: ranavirus

Most of what is presently known about ranaviruses is based on studies of Frog Virus 3 (FV3), and the *Ambistoma tigrinum Virus* (ATV) the type strain of the *Ranavirus* genus for anurans and salamanders respectively (Brunner *et al.* 2007, Chinchar 2002). Amphibians are most vulnerable to ranavirus infection during the larval or early metamorphic stages of development, and mortality of infected animals also usually occurs during these developmental stages. While vertical transmission has been suggested (Duffus *et al.* 2008) but not verified, horizontal transmission of the virus is well known and can occur in three different ways: through direct contact with infected individuals (Schock *et al.* 2008), through cannibalism of infected individuals (Harp and Petranka 2006), or through exposure to infected water and sediment (Jancovich *et al.* 2001). Effects of ranavirus infection can sometimes be seen externally as skin ulcerations or systemic haemorrhaging (Drury *et al.* 1995). However signs of infection are not always noticeable (Brunner *et al.* 2005). For our study, we used a ranavirus (FV3) isolate derived from the wild type virus originally cultured by Granoff in 1965 (Granoff 1965). High titer stocks were kindly provided by Dr. Jacques Robert (University of Rochester Medical Center, Rochester, NY, USA) and stored at -80°C. As titer accuracy may be lost after few freeze/thaw cycles, we split the entire volume solution of the virus stock into several 1ml “single-use” vials. Consequently only “fresh” virus solution was used for experimental inoculation.

b. The host: the Northern Leopard Frog (*Lithobates (Rana) pipiens*)

In Ontario, Canada, the Northern Leopard Frog is distributed widely and can be found in a variety of habitats. This species was once quite common through parts of western Canada until declines started occurring during the 1970s (Wilson *et al.* 2008, Werner 2003). Many populations of Northern Leopard Frogs have not recovered from these declines (Wilson *et al.* 2008). Northern Leopard Frogs are a good model for the study of ranavirus epidemiology due to their wide distribution, presence with other species potentially acting as reservoirs for pathogens (Schock *et al.* 2008), and reported sensitivity to human influence (*e.g.*, pesticide exposure (Christin 2003).

2. Experimental Procedure

a. Experimental design

The tadpoles used in this experiment were obtained from Dr. Vance Trudeau (University of Ottawa, Ottawa, Ontario) in November 2008. These tadpoles were produced from a captive breeding trial of originally wild-caught *L. pipiens* adults that were captured in pristine areas near Ottawa, Ontario. Adult exposure to ranavirus prior to laboratory breeding can not be ruled out. However, there is no evidence for vertical transmission and as all tadpoles were bred from the same parental stock and under the same conditions, there should be no consistent difference between tadpoles used in our experiments. Thirty aquariums containing 3 L of dechlorinated water aged for three days were separated into one group of 12 low density tanks and another group of 12 high density tanks composed of the four dose treatments (Control, Dose 1, Dose 2. And Dose 3) replicated three times. Subsequently, 20 or 40 tadpoles, Gosner stage 25 (Gosner 1960) were randomly added into each of the low or high density tanks, respectively. In our experiment, the low density tanks correspond to a density of 6.6 tadpoles/L while the high density treatment corresponds to a density of 13.3 tadpoles/L. To our knowledge, there are no good data concerning a normal density of *L. pipiens* tadpoles in nature. However, 10 tadpoles/L is commonly used by amphibian rearing facilities to maximize tadpole metamorphosis (Paula Jackman, pers. com.). After 24 hours, all tadpoles from each tank were placed together in a plastic vial along with 100 mL of ranavirus -infected water containing a gradient of ranavirus doses. The four doses of virus were: 100 pfu/ml (Dose 1), 1,000 pfu/ml (Dose 2), 10,000 pfu/ml (Dose 3), plus a control dose (no virus). The tadpoles were left in the "infection solution" for five hours before they were transferred, along with the 100 ml of the virus-containing water, back into their respective tanks. Each tank was equipped with an approximately 16 cm long piece of 7.6 cm diameter PVC pipe cut in half to provide some cover for the tadpoles. The tadpoles were fed on a weekly basis with standard tadpole food (Carolina Biological Supply Company, Burlington, NC) at 45 mg/tadpole for week 1, 90 mg/tadpole for week 2, and 180 mg/tadpole for week 3 and for the duration of the experiment. A 12:12 L:D photoperiod was used in all experiments. Prior to ranavirus exposure, 10 tadpoles from each tank were randomly selected to be weighed and their body length (nose to tail) was measured using an electronic caliper (VWR, Catalogue Number 12777.830, ± 0.005 mm). This provided an average tadpole size and weight per tank at the beginning of the experiment and was further used to estimate growth rate (see below).

b. Daily monitoring

All tanks were monitored on a daily basis. Dead tadpoles were removed as soon as noticed using assigned disposable plastic pipettes and aquarium nets to avoid any scavenging. Upon removal, dead tadpoles were weighed and their body length and body weight measured as above. The developmental stage of each dead tadpole was recorded (Gosner 1960) and tadpoles were placed into individual plastic vials filled with 70% ethanol and stored at -25 ^{o}C for subsequent analyses.

Starting on week 3 the water in each tank was replaced once a week by clean filtered water that had been aged for 24 h. As a result, tadpoles were held in virus-containing water for three weeks. This was considered long enough for tadpoles to be in close proximity with residual infection therefore approximating natural virus exposure conditions. For instance, *L. clamitans and L. sylvaticus* tadpoles have been reported to show a behavioral response to avoid trematode parasites (Koprivnikar *et al.* 2006). It is therefore possible that tadpoles would avoid pathogen-contaminated water, providing relevance to this exposure scenario. Food was administered to each tank after weekly water changes. Removed contaminated water was treated with 5% bleach and left to sit for 2-3 days to kill off any remaining virus before being discarded. The experiment lasted 70 days, which provided enough time for surviving Northern Leopard Frog tadpoles to metamorphose into juveniles in our controlled laboratory conditions. At the end of the experiment, all the remaining individuals were euthanized using MS-222 following the protocol #2009-03-05 approved by the Laurentian University Animal Care Committee. All the other procedures used in this experiment follow the protocol # 2008-09-03 approved by the Laurentian University Animal Care Committee.

3. Life History Traits

In addition to initial body size and weight, final tadpole weight and size were recorded for each tadpole after their death. Percent mortality, average day of mortality, developmental stage, and growth rate was also determined. The percent mortality was calculated by determining the percentage of tadpoles that died from each tank at the end of the 70 day experiment. The average day of mortality was calculated as the average day tadpoles died in each tank. The growth rate was calculated for each tadpole by subtracting the average initial tadpole length (calculated from the initial ten tadpoles measured per tank) from the final tadpole length and dividing by the number of days the tadpole survived. Tadpole developmental stage was assessed using Gosner nomenclature (Gosner 1960)

4. Statistical Analysis

Data on host fitness traits were analyzed using a full factorial ANOVA model, with density and virus doses as fixed factors. When the standard assumptions of analysis of variance were not met, even after $\log_{10}$ transformation, we used the non-parametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test (*H* statistic; Sokal and Rohlf (1995)). Sums of squares based on rank transformed data were used. All statistical analyses were performed using JMP software version 8.0.1 (SAS institute Inc., USA).

5. Infection Screening

Post-experiment screening of infection was done by PCR. Animals were dissected, the liver extracted, crushed into 1.5 ml Eppendorf tubes and the resulting tissue mixture was used in the extraction protocol. DNA was extracted using QIAmp DNeasy Kit following the standard protocol (Qiagen). Extraction negatives, which consisted of lysis buffer and no DNA as well as samples from non-infected individuals, were used to determine if cross-contamination occurred while processing samples (Harp and Petranka 2006)**.** For virus detection, we used a primer known to successfully amplify ranavirus, specifically Frog Virus 3: MCP- ranavirus -F (5'-GACTTGGCCACTTATGAC-3') and MCP-*Ranavirus*-R (5'-GTCTCTGGAGAAGAAGAA), following the PCR conditions listed in Mao et al (Mao *et al.* 1997) and adapted according to (Duffus *et al.* 2008, Greeg *et al.* 2005**:** 94 °C for 5 min, 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. This was cycled 35 times and completed by a final extension of 2 min at 72 °C. This specific primer has been used in other studies (Pearman *et al.* 2004, St Amour and Lesbarrères 2007) and is known to amplify a portion of the major capsid protein within the frog virus 3 genome (Mao *et al.* 1997). Samples are then run on a 1% gel at 100V for 1 h. Gels were stained with ethidium bromide and virus presence was determined by the presence or absence of a band around 500 base pairs. A sample known to be infected from a previous study was used as a positive control (St Amour and Lesbarrères 2007)**.** Overall infection rate for Dose1 was 22%, 25% for Dose 2, and 28% for Dose 3. None of the control larvae were infected. In the field, ranavirus infection rates may oscillate between 0 and 63%, but mostly range between 0 and 30% (Duffus *et al.* 2008, St Amour *et al.* 2008) . Our infection rates were therefore in agreement with those found in the field suggesting the applicability of our results to field studies.

Results

From the 720 tadpoles originally entered into the experiment, 55 individuals were missing due to scavenging/cannibalism and therefore a total of 665 individuals were included in the analysis.

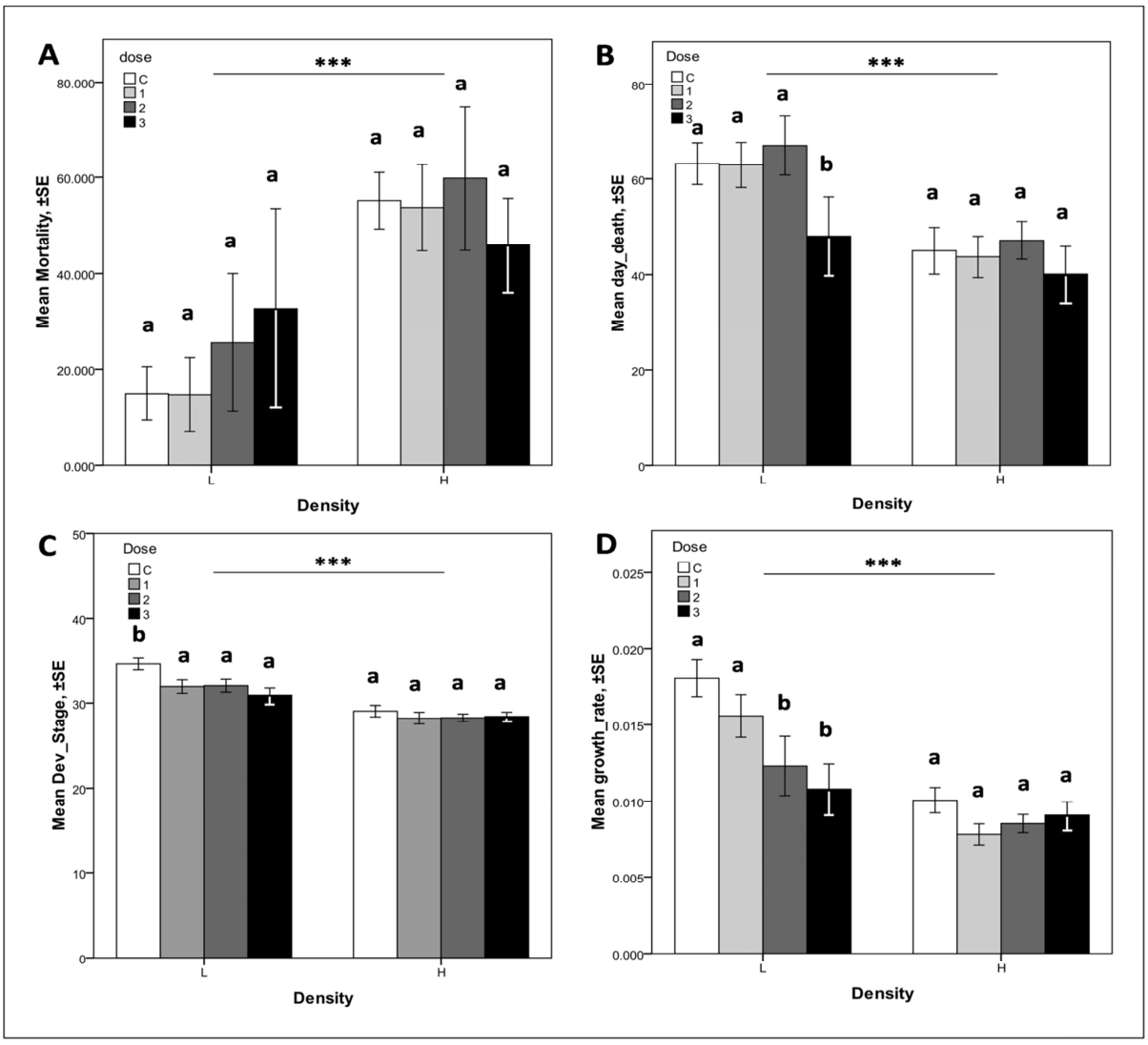

Fig 1. Interactions between tadpole density and exposure dose (Dose 1 = low dose, Dose 2 = medium dose, Dose 3 = high dose; control animals were not exposed to ranavirus). Mortality rate (A), day of death (B) developmental stage (C) and growth rate (D). Letters indicate grouping based on observed means in homogeneous subsets. Significant differences (α=0.05) between means imply grouping in different subsets (a or b) are based on a Tukey post hoc test.

1. Mortality patterns

Few deceased tadpoles showed external signs of ranavirus infection such as blood near the mouth or cloacal region. This observation seems to indicate a rather low virulence of the

ranavirus strain used. Nevertheless, the average percent mortality that occurred in the high density tanks was almost twice as high as the percent mortality observed in the low density tanks (Fig. 1A, Table 1). In the low density tanks, the high dose (Dose 3) caused the highest percent mortality, followed by Dose 2, then Dose 1, and the control (Fig. 1A). The same dose response was not seen in the high density tanks; although the results were not significant, with fewer tadpoles dying when exposed to Dose 3 as compared to tadpoles exposed to lower virus doses.

Table 1. Results of analysis of variance (*F* Ratio) and Sherrer-Ray-Hare extension of the Kruskal-Wallis test (*H* Ratio) representing the effect of dose, density as fixed effects and their interaction on mortality, day of mortality and growth rate of leopard frog tadpoles. All tadpoles were included to calculate percent mortality and day of death, but only tadpoles that survived until the end of the experiment were used to calculate growth rate. * indicates significance ($p<0.05$)

Responses/Effects	***Raw numbers***	***F*/*H* Ratio**	**Df**	**p value**
%Mortality				
Dose	*d1=34.20, d2=48.38, d3=38.41,c=35.03*	$F_{3, 624}$ =	3	0.9281
Density	*h=42.45, l=35.23*	$F_{1, 624}$ =	1	0.0054*
Dose x Density	*L1= 14.69, L2= 25.55, L3= 32.73,Lc= 14.91, H1= 53.71, H2= 59.79, H3= 46, Hc=*	$F_{3, 624}$ =	3	0.729
Day of Death				
Dose	*d1=50.42, d2=49.14 ,d3=42.58, c=51.28*	*H*= 13.494	3	0.0037*
Density	*h=46,l=59*	*H*= 13.125	1	0.0003*
Dose x Density	*L1=62.96, L2=63.38, L3=48.05, Lc=63.23, H1=43.7, H2=47.18, H3=40.18,*	*H*= 7.813	3	0.05*
Dev. Stage				
Dose	*d1=29.93, d2=29.10, d3=29.19, c=31.348*	$F_{3, 624}$ =	3	0.008*
Density	*h=28.47, l=32.382*	$F1_{, 624}$ =	1	0.0001*
Dose x Density	*L1=31.9, L2=32, L3=30.9, Lc=34.6, H1=28.2, H2=28.2, H3=28.4, Hc=29*	$F_{3, 624}$ =	3	0.153*
Growth Rate				
Dose	*d1=0.010,d2=0.009,d3=0.009,c=0.012*	*H* = 14.196	3	0.0027*
Density	*h=0.009,l*=0.014	*H* = 39.252	1	<0.0001*
Dose x Density	*L1= 0.015, L2= 0.012, L3= 0.010, Lc= 0.018, H1= 0.007, H2= 0.008, H3= 0.009,*	*H* = 12.860	3	0.0049*

2. Day of death.

The effect of virus dose on the tadpoles' day of mortality was statistically significant (Table 1; $H = 13.494$, df = 3, $p = 0.0037$). In low density tanks, tadpoles exposed to Dose 3 died on average on day 46 as compared to tadpoles in the control tanks that died on day 63 on average. Rearing Northern Leopard Frogs through metamorphosis in the laboratory can be difficult and a certain amount of mortality in control tanks is not unexpected. Density had a significant effect on the day of death for all tanks (Table 1), with tadpoles in the high density tanks dying, on average, earlier than tadpoles in the low density tanks (day 46 and 59 respectively; $H = 13.125$, df = 1, $p=0.0001$). The statistical interaction between dose and

density was marginally significant for day of death (H = 7.813, df = 3, p = 0.05; Fig. 1B) with tadpoles exposed to Dose 3 dying significantly earlier in low density tanks but not when held in high density conditions (Fig. 1B, Table 1).

3. Developmental stage at death

Overall, tadpoles in our experiment died on average at stage 30 but significant differences were observed between density treatments. In low density treatment tadpoles died on average at stage 32 whereas in high density conditions they died at stage 28 (F = 64.469, df = 1, p < 0.0001, Table 1, Fig. 1C). While the statistical interaction between dose and density is not significant (F = 1.763, df = 3, p=0.153, Table 1) it is worth noticing that control tadpoles in low density tanks reached a significantly more advanced stage of development than infected larvae (F = 4.252, df = 3, p = 0.006, stage 34 for control *vs.* 32 for dose 1, 32 for dose 2 and 30 for dose 3, Fig. 1C).

4. Growth rate

The average growth rate was significantly higher for tadpoles in low density tanks (0.009 g/day *vs*. 0.014 g/day for low and high density tanks, respectively; Table 1, Fig. 1D). A statistically significant interaction between density and virus dose (H= 12.860, df = 3, p = 0.0049) was also observed. In the low density tanks the tadpoles with the lowest growth rate were those exposed to the highest virus dose (Fig. 1D) indicating a dose response at this density: there were significant differences between Dose 3 and control, and Dose 2 and control (F = 14.64, df = 1, p < 0.001 and F = 6.07, df = 1, p = 0.0141, respectively). The difference in growth rate between Dose 1 and control was not statistically significant. In high density tanks however, no statistical differences were observed but tadpoles exposed to the highest dose tended to have a higher growth rate than tadpoles infected by either Dose 1 or the controls (Table 1, Fig. 1D).

Discussion

Our results revealed that ranavirus virulence is likely density-dependent, and that when compared to unexposed animals held under the same conditions, the overall effects of *Ranavirus* infection appears to be relatively more severe in animals held in low density as compared to animals held in high density.

1. Context-Dependent Virulence of Ranavirus

a. Doses

In the low density tanks, the effect of FV3 dose on host fitness was consistent with our prediction that an increase in FV3 dose would result in increased mortality, significantly earlier mortality, reduced developmental rate and a significantly decreased growth rate of leopard frog tadpoles (Fig. 1). This dose-response effect is supported by a number of previous studies. Duffus *et al.* (2008) showed that an increase in FV3 dose resulted in higher rates of virus infection in wood frog (*L. sylvaticus*) tadpoles. Brunner *et al.* (2005) observed that the odds of mortality increased approximately by a factor of 2.4 for every tenfold increase in ATV dose in tiger salamanders (*Ambystoma tigrinum*), with the greatest mortality at a dose of 10,000 pfu/mL. These authors also observed an earlier day of mortality as ATV dose increased (Brunner *et al.* 2005). Our results similarly suggest that an increase in FV3 dose reduces the fitness of leopard frog tadpoles. This is not unexpected as a deterioration of host fitness generally occurs when a pathogen load increases in a host since pathogen multiplication leads to resource depletion in the host potentially leading to death or morbidity if the process is not prevented by host immune defenses (Schmid-Hempel 2009).

b. Density

In high density tanks, tadpole mortality was higher, day of mortality was earlier, developmental rate was lower, and growth rate was lower than in low density tanks (Fig.1, Table 1). This suggests an overall increase of deleterious effects when population density increases. In our case, the increase in deleterious effects may be explained by at least three mechanisms that may act separately or synergistically: a decrease in resource availability (Joshi and Mueller 1996), an increase in contact rate, and/or pollution by conspecifics. In our study, tadpoles were fed *ad libitum* to avoid competition for resources and minimize the stress associated with resource appropriation; therefore food availability and stress related to resource appropriation potential should not have been influential in the current experiments. Second, increasing contact rate between individuals can be a stressful situation (Renshaw and Service 1993) and may also increase horizontal transmission of pathogens (Arneberg *et al.* 1998). As a result, the pathogen burden should be higher in individuals in high density conditions, resulting in increased deleterious effects of the pathogen. However, the pattern we observed did not completely support such a scenario. We did observe an overall decrease of host fitness but the relative fitness of tadpoles that had been exposed to higher doses of

ranavirus compared to tadpoles exposed to lower doses of ranavirus did not illustrate a clear effect of dose level on the amount of horizontal transmission (Fig. 1).

Finally, pollution by conspecifics has been suggested to be important factor in animal health in small aquatic systems (Bedhomme *et al.* 2005). The major nitrogen excretory product of tadpoles is ammonia, a compound which is highly soluble in water. In high density environments, environmental ammonia levels may become toxic to tadpoles. Effects of elevated levels of ammonia include disruptions in cerebral blood flow, interruptions in nerve conductance, modifications in the blood-brain barrier as well as alterations to fat and carbohydrate metabolism in a variety of tissues, potentially resulting in convulsions, coma or death of the organism (Burgett *et al.* 2007, Jofre and Karasov 1999, Wright 1995). While pollution by conspecifics may have been a factor involved in deteriorating tadpole fitness in our experiment, further investigation is needed to disentangle this hypothesis from others.

c. Interaction of dose and density

Both increasing virus doses and host density resulted separately in a deterioration of host fitness. However, the linearity of the relationship between virus dose and host fitness appears to be influenced by the density context in which the infection occurs. In high density tanks tadpoles exposed to the high dose (Dose 3) presented higher survival than tadpoles exposed to lower virus doses or no dose at all, although the results were not statistically significant (Fig. 1A). For the time of death, developmental stage, and the growth rate in high density tanks, the virus-exposed animals were essentially indistinguishable from the non-exposed animals (Fig. 1B-D). On the other hand, there was a trend for a dose-response relationship between virus exposure level and fitness in low density tanks: the higher the dose the more serious were the effects seen in the exposed animals. We propose that for the traits assessed in this experiment, being infected by a pathogen under high density conditions may be relatively less detrimental than expected, as its specific effects are masked and diluted by the overall increase of stressful conditions. The relative fitness of infected tadpoles in high density tanks therefore increased as compared to what occurred in animals in low density conditions, in turn leading to a status quo between control and infected individuals in terms of relative fitness. These results suggest a condition-dependence of ranavirus virulence in varying density environments whereby ranavirus observed relative virulence decreased as the environment induced more stress in the tadpoles. Several studies support the assumption that environmental stress aggravates the effects of infectious diseases and good examples are given in the context of toxic chemicals (Khan 1990), malnutrition , thermal stress (Bensadia *et al.* 2006, Harvell *et al.* 1999), UV-B

radiation (Guay *et al.* 2009) and population density increase. However, there are substantial theoretical and empirical reasons to expect that increasing environmental stress does not necessarily lead to increased pathogen virulence (Seppälä *et al.* 2008, Lafferty and Kuris 2005)

2. When Being Infected is no Longer Detrimental.

Our results suggest that for some traits that are directly linked to host fitness (mortality rate, day of death, growth rate), individuals with a substantial pathogen burden are no longer suffering a disadvantage relatively to less- or non-infected individuals in deteriorating or stressful conditions (*e.g.*, high density conditions) suggesting limited effects of increasing *Ranavirus* dose in high density conditions. There are several reasons why this may have occurred in the present experiments. First, upon being infected at the beginning of the experiment, the tadpole immune system was likely activated by ranavirus exposure (Gantress *et al.* 2003) and an associated general metabolic enhancement may have occurred. While amphibian larvae fail to express their MHC Class I immunity until metamorphosis, they do have CD8 T cells (Du Pasquier *et al.* 1989), and several other immune features are present in the larval immune arsenal early after hatching (see Du Pasquier *et al.* (1989) and Robert and Ohta (2009)). In *Xenopus laevis* liver, activity of Recombination Activating Genes (RAG) is detectable as early as three days after fertilization (Mußmann and Du Pasquier 1998), rearrangement of the Immunoglobulin heavy chain starts on day 5 and the larval type B-Cell Receptor (BCR) and T-Cell Receptor (TCR) repertoires are present within the first week after hatching. While no specific immune response targeting FV3 is likely to have occurred in the larvae (as there is only low or no surface MHC class I expression in tadpoles (Robert and Ohta 2009)), it seems nevertheless reasonable to assume that the tadpole's early-stage immune arsenal is activated as a reaction to FV3 infection (Flajnik 1996). Additionally, it is likely that a general metabolic enhancement occurred in response to infection during the average duration of tadpole development. In fact, tadpoles died at stage 30 on average, when independent feeding and normal metabolic functions are already set (Gosner 1960). Second, we observed a relatively low tadpole mortality rate as compared to similar studies (Gantress *et al.* 2003). This suggests a rather low virulence (defined as the detrimental effect on host fitness of a pathogen) of the ranavirus strain used or perhaps that the host developed some general immunity to this strain in nature. Moreover, the difference in mortality rates observed between our study and other similar studies may be associated with the condition in which the larvae were infected. In our study, we did not inject the tadpoles intraperitonealy with a

solution containing FV3 but bathed the tadpoles in FV3 solutions to better mimic natural conditions of exposure. It is likely that the amount of viral particles in each individual was therefore lower as compared to intraperitonealy-injected individuals, in turn explaining the relatively low mortality rate observed in our experiment.

Given these three considerations, a potential scenario could be proposed to support the trade-off observed between infection and density stresses. The early activation of the infected tadpoles' immune system, together with an enhancement of their general metabolic state in response to FV3 infection, might have compensated for the detrimental physiological effect of density increases over the experiment. Such interaction could have maintained relatively similar "health" conditions of infected larvae as compared to non-infected larvae under our stressful holding conditions and may therefore reflect a subtle interplay between direct costs, compensatory byproducts (indirect benefits) of infection and stress effects. However, this scenario remains speculative and needs further investigation.

3. Conclusions

Our results, in line with theoretical considerations, suggest the importance of considering both direct and indirect effects of pathogen infection in estimating the fitness effects on the host (Pagan *et al.* 2009). While further quantitative assessments of factors such as tank pollution and virulence would be needed to better understand the underlying mechanisms of the host-pathogen system we studied under varying density conditions, our results illustrate the importance of considering such context-dependent processes for understanding the dynamics and coevolution of geographically structured populations evolving under different ecological pressures. In the current conceptual framework of the dynamics of host-pathogen evolutionary ecology, these condition-dependent processes need to be integrated by the broad community of pathogen researchers to focus study design and enlarge the scope of investigations. Only by investigating host-pathogen relationships in an integrative framework will researchers truly understand the evolutionary ecology of these relationships (Su *et al.* 2009).

Acknowledgments

We would like to thank Chris Blomme for providing excellent technical assistance and Drs. Gunn and Litzgus for comments on an earlier version of this paper. Thanks to Dr. A.L.J.

Duffus for helpful technical advices regarding ranavirus screening. We also thank the two anonymous reviewers for their very constructive comments.

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Manuscript 4

Ranavirus within-host infection dynamics in Northern Leopard Frogs: the timing and number of exposure matters

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Running title: Ranavirus within-host infection dynamics

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Abstract

Pathogen infection rate is a key parameter determining disease virulence and the potential for transmission. Consequently, accurate quantification of infection patterns is critical for a better understanding of disease epidemiological dynamic. Furthermore, while among-individual transmission of infection is important, within-host infection dynamics and the delineation of critical periods of disease vulnerability during host development remain relatively poorly understood. Our study investigated the susceptibility of *Lithobates (Rana) pipiens* hatchlings and larvae to ranavirus (FV3) and compared infection rates among developmental stages in a two-step laboratory experiment.

Our three objectives were: 1) to quantify the hatchling infection rate and to assess potential routes of infection, 2) to estimate the potential influence of hatchling exposure on tadpole infection rates and investigate the infection carry-over rates between hatchlings and tadpoles and 3) to assess the virulence of the virus with regard to the time of infection and number of exposures.

Our results indicate a varying susceptibility to ranavirus between developmental stages with hatchlings (Gosner stage 21 to 25) being more susceptible to infection than tadpoles (Gosner stage 26-35). Our study also reveals the potential for early hatchlings to bear a basal infection possibly acquired during the first hours post-hatching when they were feeding on the jelly (jelly surface transmission hypothesis), resulting in high infection rates. Additionally, the infection carry-over patterns (62% infection rate in hatchlings vs. 12-20% in tadpoles) suggest a significant clearing of the infection over the course of host development. The intensity of the infection clearing appears to depend on both the timing and number of ranavirus exposures.

Thus, our study highlights the critical importance of screening infection among individuals but also within individual developmental stages in order to accurately describe pathogens infection and host mortality patterns. More specifically our study emphasizes the potential for ranavirus to be the cause of unnoticed mortality events in amphibian communities leading to unexpected populations demographic declines.

Key words: Development, Embryos, Emerging Infectious Diseases, Epidemiology, Life-history traits, Northern Leopard Frog, ranavirus, Tadpoles,

Introduction

The rise and spread of emerging and re-emerging infectious disease is an increasing threat to humans, wildlife and domestic animals (Morens, Folkers, & Fauci 2004; Lebarbenchon *et al.* 2008). The pathogens associated with these diseases most often cause harm or even kill their host although they depend on them for their survival and transmission. A classic argument to explain such apparent paradox asserts that virulence is an unavoidable consequence of selection to maximize pathogen fitness (Anderson & May 1982; Ewald 1983) because pathogen must replicate within hosts in order to produce transmission stages or numerically increase their chances to enter into contact with a new host. Consequently, an intense replication of the pathogen leads to host resource depletion and potentially, immune clearance thereby shortening the infectious period, in turn compromising chances of transmission (de Roode, Yates, & Altizer 2008). Therefore, pathogens face a trade-off between the benefits of increased replication and transmission and the cost of potentially shortening the infection in their host (Messenger, Molineux, & Bull 1999; Jensen *et al.* 2006). According to this "trade-off hypothesis", a given value of replication for a pathogen requires a minimum of virulence and host recovery (Alizon *et al.* 2009; Froissart *et al.* 2010) which in turn might affect the overall disease epidemiology. In this context, it is particularly critical to accurately quantify host population infection rate in order to understand transmission patterns to model properly host-pathogen epidemiological dynamics (McCallum, Barlow, & Hone 2001) and generate quantitative predictions useful for epidemic management (Kao 2002).

Furthermore, while among-individual transmission of infection is important, it becomes more and more evident that the investigation of infection dynamic within host over the course of its development will promote proper delineation of critical windows of disease risk (Rohr, Raffel, & Hall 2010; Johnson, Kellermanns, & Bowerman 2011). Transfer of infection between life history stages of the same individual might be documented as an infection carry-over. Such a carry-over of infection is particularly subjected to fluctuations over time because it is exposed to the ontogenic change in host immune response. The intensity of the clearing exerted by the host depends on its developmental stage/immunity potential reached at the time of exposure and the frequency of exposure to the pathogen (Sadd & Schmid-Hempel 2006). In Amphibians for instance, metamorphosis is associated with a general reorganization of the immune system and often increased susceptibility to infection (Rollins-Smith 1998; Carey, Cohen, & Rollins-Smith 1999). It is thus critical to document differences of host stage susceptibility and the rate of infection carry-over between host life-history stages in order to

better understand infection rate fluctuation over time, host mortality and the associated pathogen transmission patterns (Johnson *et al.* 2011). Such an understanding is particularly important for the investigation of pathogens that infect hosts with complex life histories such as amphibians (Brunner *et al.* 2004).

Ranaviruses are virulent pathogens known to infect fish, reptiles and a wide range of amphibian species (Gray, Miller, & Hoverman 2009). Although mortality events have occurred at various locations worldwide, most known cases of ranaviral infection and mortality that have been adequately studied have occurred in North America and the UK (Cunningham *et al.* 1996; Teacher, Cunningham, & Garner 2010). In fact, ranavirus was recently recognized as an important pathogen and ranaviral disease is listed as a notifiable disease by the World Organization for Animal Health (OIE, http://www.oie.int/eng/maladies/en_classification2010.htm?e1d7). However, the transmission of the virus is still poorly understood. Indirect transmission of ranavirus from contaminated sediment to amphibian hosts can occur in less than 24 hours without any direct contact with naïve hosts (Greer, Briggs, & Collins 2008; Teacher *et al.* 2010), suggesting that virions shed into the aquatic environment can successfully infect new amphibian hosts. Direct transmission of ranaviruses is known to be highly effective in amphibians and can occur via scavenging and cannibalism (Harp & Petranka 2006; Brunner, Schock, & Collins 2007) or via direct skin contact (Cullen, Owens, & Whittington 1995; Brunner *et al.* 2007). While these examples support ranavirus horizontal transmission, we still lack solid evidence of vertical transmission where ranavirus is transmitted either transovarially from mother to offspring or through deposition of viral particle originating from parent skin on the embryos jelly (Docherty *et al.* 2003; Duffus *et al.* 2008). Additionally, embryos have been suggested to be a life stage susceptible to infection by ranavirus in one referential study (Tweedel & Granoff 1968) in which the authors inoculated the ranavirus by injections along the embryo's nephrogenic ridge. However, while maximizing the probability of infection, such procedure does not demonstrate the likelihood of egg infection in the wild and according to a recent study by Haislip et al (2011), it is likely that embryos would be naturally protected from infection by their jelly. Knowledge about natural embryo infection and the potential infection carry-over rates between subsequent hatchlings and larvae, is still limited and further investigation is required for a full understanding of ranavirus-host evolutionary ecology and ranaviral disease epidemiology (Lesbarrères *et al.* 2011).

Here, we investigated the susceptibility of *Lithobates (Rana) pipiens* hatchlings and larvae to ranavirus (FV-3) in a two-step laboratory experiment. Our objectives were threefold: 1) to

quantify hatchlings infection rate and to assess potential routes for their infection, 2) to estimate the potential influence of hatchling exposure on tadpole infection rates and investigate the infection carry-over rates between hatchlings and tadpoles and 3) to assess the virulence of the virus in relation to the time of infection and number of exposures.

Material and Methods

1. The host-pathogen system

a. The pathogen: ranavirus

Most of what is presently known about ranaviruses is based on studies of Frog Virus 3 (FV3) and *Ambystoma tigrinum Virus* (ATV) which are affecting anurans and salamanders respectively (Chinchar 2002; Brunner *et al.* 2007). Amphibians are most vulnerable to ranavirus infection during the larval or early metamorphic stages of development, and mortality of infected animals usually occurs during these developmental stages (Gray *et al.* 2009). Effects of ranavirus infection can sometimes be seen externally as skin ulcerations or systemic haemorrhaging; however signs of infection are not always noticeable (Brunner, Richards, & Collins 2005). In this study, we used a ranavirus (FV3) isolate derived from the wild type virus originally cultured by Granoff in 1965 (Granoff, Came, & Rafferty 1965). High titer stocks were kindly provided by Dr. Jacques Robert (University of Rochester Medical Center, Rochester, NY, USA) and stored at -80°C. As titer accuracy may be lost after few freeze/thaw cycles, we split the entire volume solution of the virus stock into several 1ml "single-use" vials. Consequently only "fresh" virus solution was used for experimental inoculation.

b. The host: the Northern leopard frog *(Lithobates (Rana) pipiens)*

Northern leopard frogs are a good model for the study of ranavirus epidemiology due to their wide distribution, their moderate susceptibility and their presence with other species potentially acting as reservoirs for pathogens (Schock *et al.* 2008). The eggs used in this experiment originated from an egg mass produced using the AMPHIPLEX method (Trudeau *et al.* 2010) from captive breeding (May 2009) of originally wild-caught *L. pipiens* adults that were captured in pristine areas near Ottawa, Ontario.

L. pipiens individuals were categorized into three general developmental stages according to Gosner (Gosner 1960): individuals from Gosner Stages 1 to 20 were defined as embryos, individuals from Gosner Stage 21 to 25 were considered hatchlings and individuals from

Gosner Stage 26 to 35 were defined as larvae (Haislip et al 2011). The experiment was terminated prior to metamorphosis, when animals were on average at Gosner Stage 35.

2. Experimental procedure

a. Egg stage and hatchlings infection

To quantify hatchlings infection rate and to assess potential routes of infection, we used a total of eight egg batches randomly assigned to either the Exposed (E, four batches) or the Non-Exposed (NE, four batches) treatments. Each batch consisted of 50 *L. pipiens* eggs housed in a 2.5L Pyrex beaker containing 750ml of dechlorinated aged water (aged for 2 days). Eggs masses were infected in plastic vials containing 100ml of ranavirus contaminated water. Ranavirus concentration was 10000pfu/ml and exposure duration was 12h. Such a concentration has been shown to provide a ranavirus infection rate of about 28% in tadpoles housed in similar conditions (Echaubard *et al.* 2010). In the field, ranavirus infection rates may oscillate between 0 and 63%, but mostly range between 0 and 30%, so our methods should represent infection rates in the wild (St Amour *et al.* 2008). Furthermore, such moderate dose of exposure does not lead to high mortality rates and allows the observation of variability in effects of infection on tadpole development and life history traits (Echaubard *et al.* 2010).

After exposure, the egg masses, together with the contaminated water were transferred back into the original beakers. Embryos were monitored twice daily during the duration of their development and prior to hatching (Gosner stage 20). Water in the exposed treatments was changed to clean water to prevent hatchlings to be in contact with virus-contaminated water, thus ensuring that potential infection occurred either during the egg exposure or after hatching through contact of the hatchlings with the jelly. Hatching of all individuals occurred within a 10 hours time-window. In each beaker, ten hatchlings (40 E and 40 NE in total) were measured for life history traits (see below), euthanized using MS-222, and screened by PCR for the presence of ranavirus (see below). The 40 remaining hatchlings in each beaker were then used in the tadpole-stage experiment.

b. Tadpole stage infections

In total, 320 tadpoles derived from the egg experiment were used in the second experiment to assess the transmission rate between eggs and tadpoles. Among these 320 tadpoles, half (160) were from previous exposed treatments (E1) and half from non-exposed treatments (NE1). In each of these two categories, four replicates of 20 tadpoles were further exposed to

ranavirus (E2), 48h post-hatching, and four replicates were not re-exposed (NE2). We therefore had 4 treatment combinations (NE1-NE2, NE1-E2, E1-NE2 and E1-E2), each replicated 4 times with 20 tadpoles per replicate. Tadpoles were maintained in 20L tanks containing 3L of dechlorinated aged water (6.7 tadpole/L) to minimize the influence of density on tadpole survival (Echaubard *et al.* 2010). Tadpoles to be exposed (E2) were placed in individual 125ml plastic vials together with 100ml of ranavirus contaminated water (10000pfu/ml) for 12h. All tadpoles were fed on a weekly basis with standard tadpole food (Carolina Biological Supply Company, Burlington, NC) at 15 mg/tadpole for week 1, 30 mg/tadpole for week 2, and 60 mg/tadpole thereafter. This amount of food corresponds to limited resources availability in these conditions (Echaubard *et al.* unpublished data). Limiting food availability promotes the potential for underlying resource allocation trade-off between host condition and immune response to occur, in turn enabling ranavirus effects to be more clearly observed and mimicking *in natura* conditions where resources are limited.

c. Daily monitoring

In the egg-stage experiment, embryo development was monitored twice a day for a period of 3 days post-exposure in our laboratory conditions (12L:12D photoperiod, 18 ^{o}C). Water in the infected treatments was changed when 50% of the embryos were at Gosner stage 19 (Gosner 1960). In the tadpole-stage experiment, all tanks were monitored on a daily basis and any dead tadpole was removed to avoid scavenging. Upon removal, dead tadpoles were processed for life history trait measurements as described below, placed into individual plastic vials filled with 70% ethanol, and stored at -25^{o}C for further analyses. Starting on week 3 the water in each tank was replaced once a week by clean dechlorinated aged water. As a result, exposed tadpoles were held in virus-containing water for 3 weeks, a period which is long enough for tadpoles to be in close proximity with residual infection (Echaubard *et al.* 2010). The experiment lasted 50 days when all the remaining individuals were euthanized using MS-222. All procedures follow the protocol #2009-03-05 approved by the Laurentian University Animal Care Committee.

3. Life history trait measurements

Upon death, tadpole skin was dried using absorbing paper, then each individual was weighed (Metler Toledo balance, ± 0.01g), and measured for body length and body width (VWR electronic caliper #12777.830, ± 0.005mm). Hatchlings were measured for length and width following the same procedure as for tadpoles but their weight was on average below our

scale threshold of accuracy. Consequently we did not record weight measurements for hatchlings. Tadpole developmental stage was assessed using Gosner nomenclature (Gosner 1960). For tadpoles, day of death of each individual was recorded and we calculated the growth rate per individual for several traits including body mass, length and developmental stage. To calculate the individual growth rate for each variable we divided the absolute differences between the treatment mean and individual measures by the number of days each individual survived.

4. Infection screening

For the egg-stage experiment, the whole body of the hatchlings was used for DNA extraction. For the tadpole-stage experiment, animals were dissected, the liver extracted and crushed into 1.5 ml Eppendorf tubes containing lysis buffer. DNA was extracted from the resulting tissue mixture using QIAmp DNeasy Kit following the standard protocol (Qiagen). After extraction, samples were sent to Pisces Molecular (Boulder, Colorado, USA) for ranavirus screening. They performed double blind PCR using validated primers for Frog Virus 3: MCP-ranavirus-F (5'-GACTTGGCCACTTATGAC-3') and MCP-ranavirus-R (5'- GTCTCTGGAGAAGAAGAA), following the PCR conditions listed in Mao *et al.* (Mao, Hedrick, & Chinchar 1997). This specific primer set has been used in other studies and is known to amplify a portion of the major capsid protein within the Frog Virus 3 genome. Along with their qualitative screening, Pisces Molecular provides a semi-quantitative assessment of the infection intensity by looking at the PCR signal. Only individuals that were found infected in both screenings were considered infected. Band intensity was evaluated against controls belonging to five categories: very strong positive signal (+++), strong positive signal (++), positive signal (+), weak positive signal (w+) or no signal/below limit of detection (-).

5. Statistical analysis

Data on hatchling and tadpole fitness traits were analysed using Generalized Linear Models (GZM) with treatment as a fixed factor. For tadpoles, in order to incorporate the potential influence of hatchling exposure on tadpole infection and avoid pseudo-replication, we used a nested Generalized Linear Model, where the second infection event was nested into the first infection event. Infection rate differences among treatments were analyzed using a Log-linear analysis of frequency tables based on a Maximum Likelihood Chi-square calculation. The relationship between infection and the observed mortality was investigated with a fixed non-linear regression model. In order to deal with non-uniform residuals

distribution of proportion data, we calculated the arcsin-square-root of the proportion dead for each tank and computed a factorial ANOVA with early and late infection as predictors of mortality. This procedure was used to assess the linearity of the relationship between mortality and infection, potentially revealing the role of development in explaining mortality patterns. Finally, mortality over time per treatments and differences among them were estimated using a Cox regression adapted for analysis of time-dependent covariates in order to incorporate the nested pattern of infection exposures. Individuals surviving to the end of the experiment were censored to account for our lack of information about their true time to death. Censoring is a standard technique that down-weights the influence of these individuals in the survival analysis (Leung, Elashoff, & Afifi 1997). All statistical analyses were performed using Statistica 8.0 (StatSoft.Inc. 2007).

Results

1. Egg-stage exposure

a.Hatchling's infection by the ranavirus

Among the 200 eggs that have been exposed to the ranavirus in the egg experiment, 75 of the resulting hatchlings (35 exposed, 40 controls) were tested for infection. Of the 35 exposed individuals, 62.85% (22/35) were positively infected but only weak ranavirus MCP target signals were observed (Fig. 1A). None of the 40 controls showed presence of ranavirus (Fig. 1A).

b. Hatchling life history traits

While we found no significant difference in body width between exposed individuals and controls (GZL, $W_{(df = 1)} = 2.65$, p=0.26), control hatchlings were significantly longer than hatchlings that had been exposed to ranavirus (GZL, $W_{(df = 1)} = 9.06$, $p = 0.02$). None of the hatchlings, either exposed or control, died.

2. Tadpole-stage exposure

From the 320 tadpoles used in the experiment, 22 were missing due to death and subsequent scavenging or possible cannibalism and therefore a total of 298 individuals from the four treatment combinations (NE1-NE2, NE1-E2, E1-NE2 and E1-E2) were included in the analysis.

a. Infection by the ranavirus

Screening for infection was performed on all tadpoles used in the experiment and significant differences between treatments were observed (Max likelihood Chi-square, X^2 = 35.62, $p < 0.001$, Fig. 1B). Among the four treatments, E1-E2 had the highest infection rate with 40% (31/75) of tadpoles infected, in great contrast with tadpoles from treatment NE1-NE2 where no infection was detected ($X^2_{(df = 1)}$ = 7.78, p = 0.005, Fig.1B). Infection rate in E1-E2 individuals was significantly higher than in NE1-E2 (20.5%; 15/73) and E1-NE2 individuals (12%; 9/75; $X^2_{(df = 1)}$ = 6.85, p = 0.009 and X^2 = 15.65, p<0.001 respectively, Fig. 1B). Interestingly, these two treatments tended to be different from each other with regards to infection rate ($X^2_{(df = 1)}$ = 3.45, p = 0.06, Fig. 1B) revealing a possible influence of the timing of infection on the resulting infection rate. Finally, tadpoles from both NE1-E2 and E1-NE2 treatments were significantly more infected than tadpoles from NE1-NE2 treatment ($X^2_{(df = 1)}$ = 16.72, $p < 0.001$ and X^2 = 9.33, p=0.002 respectively, Fig. 1B).

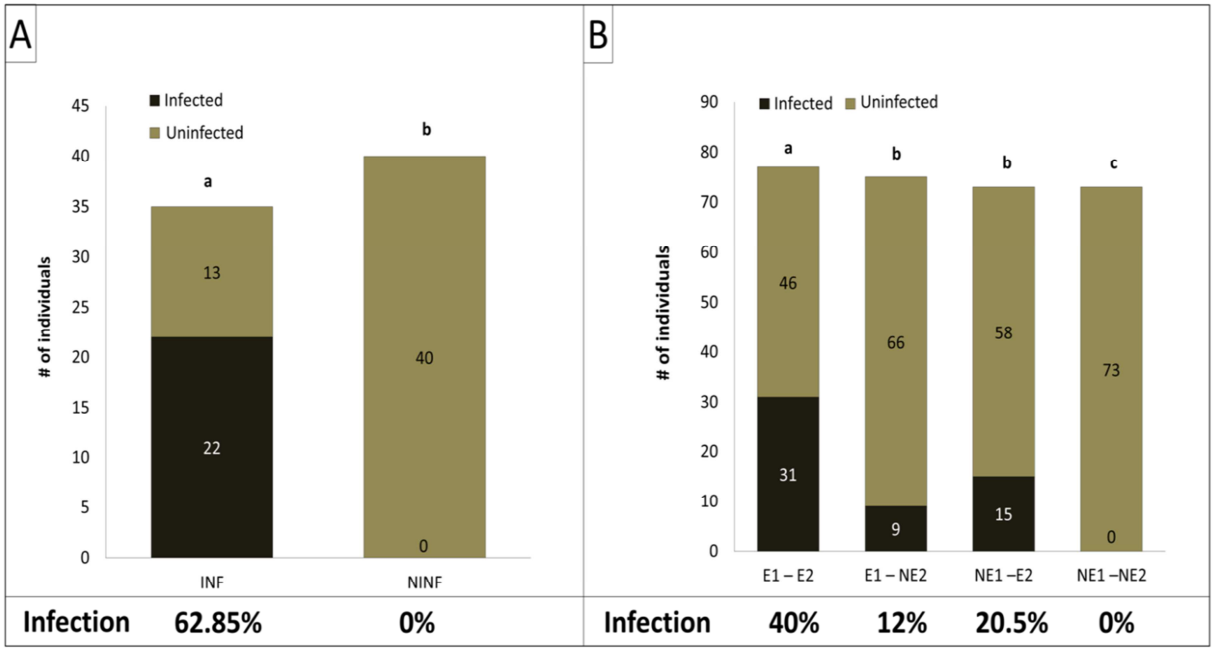

Fig. 1. Summary of infection rates and proportion of infected (dark grey) vs. non-infected (light grey) individuals among treatments. A- Hatchlings. B- Tadpoles. E1 – E2 refers to individuals that were exposed twice, E1-NE2 refers to individuals that were exposed as embryos but not as tadpoles, NE1-E2 refers to individuals that were not exposed as embryos but exposed as tadpoles, NE1-NE2 refers to individuals that were not exposed at all. Letters indicate significant differences (p<0.005) based on Log-linear analysis of frequency tables.

b. Mortality rate

Overall, we found a marginally significant difference between treatments ($X^2_{(df = 3)}$ = 6.40, p = 0.09). Tadpoles from treatment E1-E2 died at a higher rate as compared to tadpoles from treatments NE1-NE2 (16.8% vs. 5.4% respectively; $X^2_{(df = 1)}$ = 5.38, p = 0.02 Table 1, Fig.

2A). Additionally, tadpoles from treatment E1-E2 had a tendency to die more than tadpoles from E1-NE2 and NE1-E2 treatments ($X^2_{(df = 1)} = 3.07$, $p = 0.08$ and $X^2_{(df = 1)} = 2.89$, $p = 0.09$ respectively). The timing of infection had no effect on the rate of mortality as no significant difference in mortality between E1-NE2 and NE1-E2 tadpoles was observed (8 vs. 8.2% respectively, $X^2_{(df = 1)} = 0.29$, $p = 0.59$, Fig. 2A). We also investigated the differences between treatments for the proportion of individuals that died from infection and those that died without infection. The actual role of infection in explaining the observed mortality was supported by the fixed non-linear regression model ($R^2 = 0.95$, $p < 0.001$) although we only found a marginally significant difference between treatments ($X^2_{(df = 3)} = 6.12$, $p = 0.15$, Fig. 2B); dead individuals from treatment E1-E2

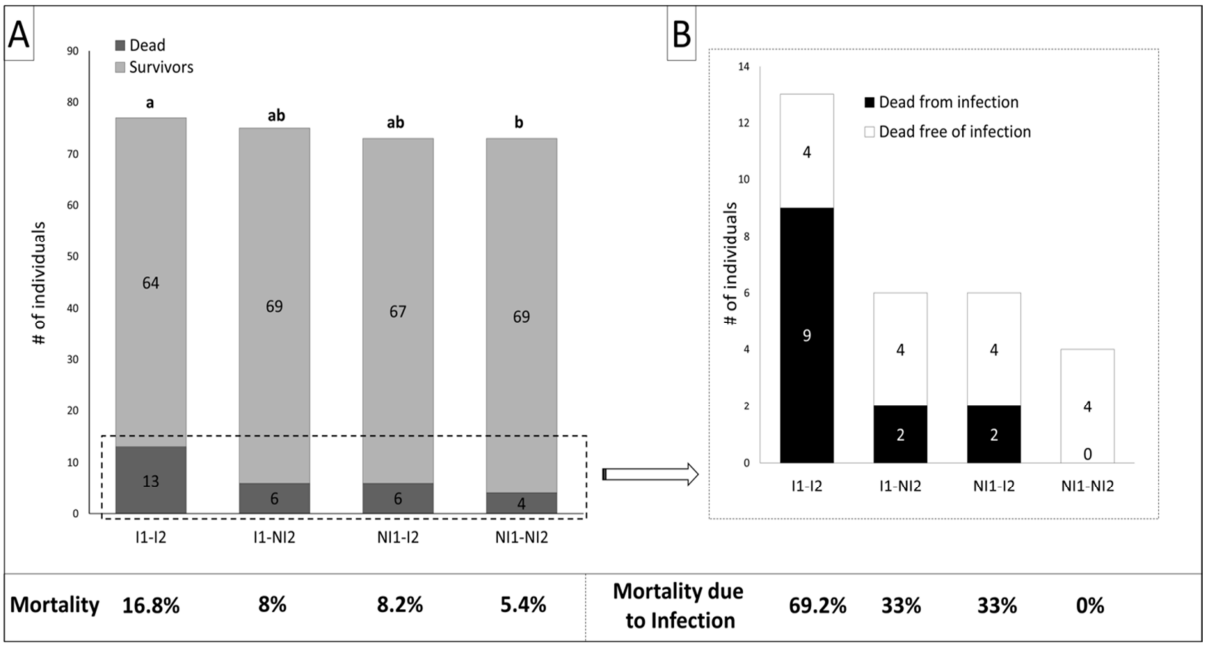

Fig. 2. Summary of mortality among treatments. A- Number of individuals dead (dark grey) and still alive (light grey) at the end of the 50 days experiment. B- Proportion of infected (black) vs. non-infected (white) individuals among the dead tadpoles. E1 – E2 refers to individuals that were exposed two times, E1-NE2 refers to individuals that were exposed as embryos but not as tadpoles, NE1-E2 refers to individuals that were not exposed as embryos but exposed as larvae, NE1-NE2 refers to individuals that were not exposed at all. Letters indicate significant differences in mortality ($p<0.005$) based on a Cox regression adapted for analysis of time-dependent covariates.

were infected twice as much (69.2%) than tadpoles from both E1-NE2 (33%) and NE1-E2 (33%) treatments ($X^2 = 7.02$, $p = 0.071$; Fig. 2B). Additionally, we computed a factorial ANOVA with early and late infection as predictors of mortality. The analysis revealed a significant interaction between early and late exposure ($F_{2,16} = 152.21$, $p < 0.001$) underlying the role of exposure timing in determining infection patterns and the non-linearity of the relationship between mortality and infection. Mortality rate and infection patterns over time are provided in Fig. 3.

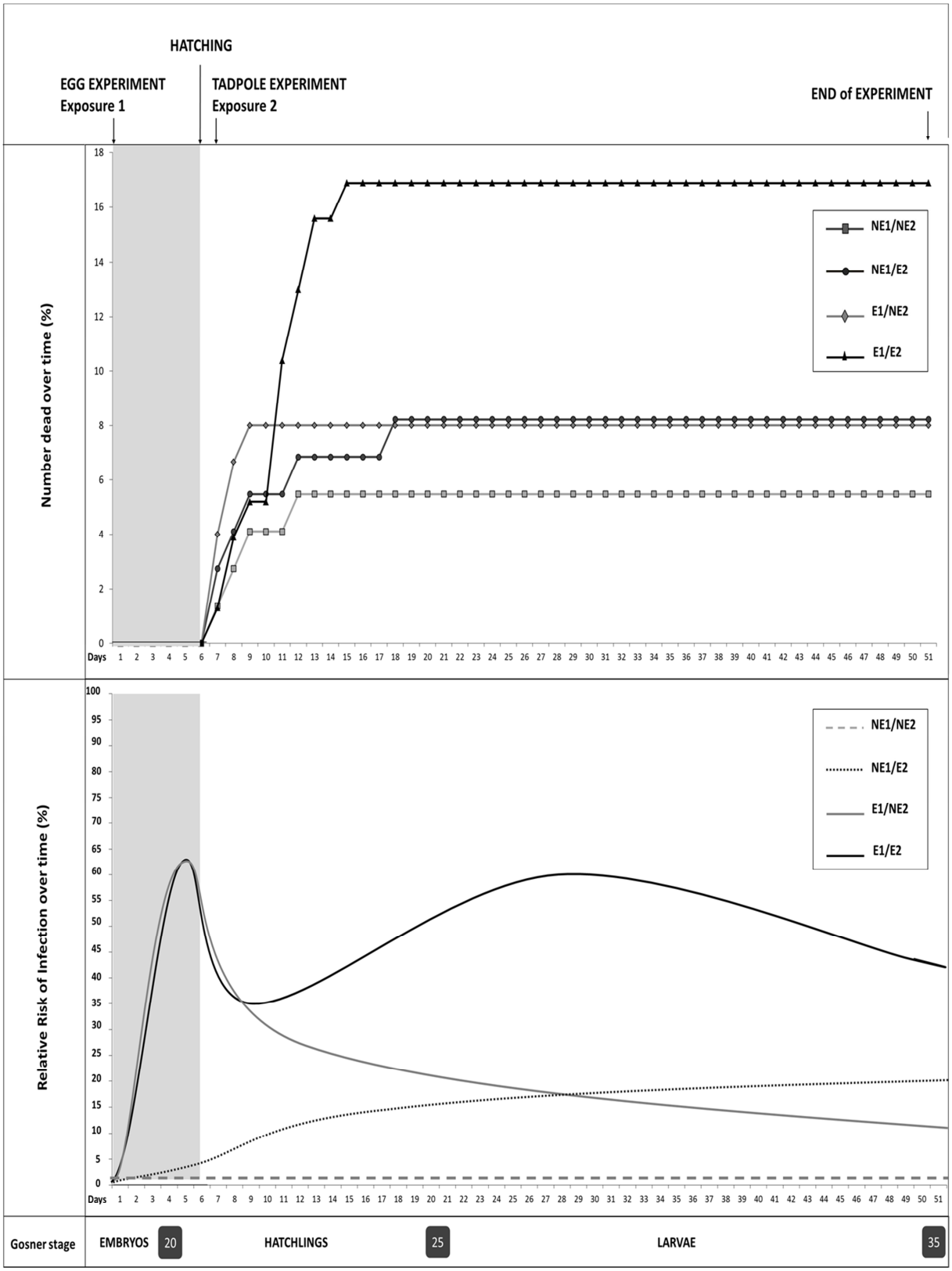

Fig. 3. Summary of number of dead and relative risk of infection over time. Infection rate over time between hatching and final day (where measure of infection rate have actually been done) is interpreted. Different timing and number of ranavirus exposures among the different treatments might induce different rate of replication, thus different slopes and final infection rate (see main text). E1 – E2 refers to individuals that were exposed two times, E1-NE2 refers to individuals that were exposed as embryos but not as tadpoles, NE1-E2 refers to individuals that were not exposed as embryos but exposed as larvae, NE1-NE2 refers to individuals that were not exposed at all.

c. Time of death and other traits

While no significant difference was observed between treatments for the time of death (GZL, $W_{(df = 3)} = 5.73$, $p = 0.12$, Table1), trends were consistent with mortality rates. Tadpoles from E1-E2 treatments had a tendency to die earlier than tadpoles from other treatments, particularly when compared to NE1-NE2 tadpoles (43.6 vs. 47.8 days respectively; Table 1).Similarly, there was a tendency for tadpoles from E1-E2 treatment to die on average three days earlier than tadpoles from E1-NE2 and NE1-E2 treatments (43.6 vs. 46.7 and 46.8 days respectively, Table1) suggesting an effect of the number of exposure events on survival time. On the other hand, the timing of

infection did not induce an effect on time to death as tadpoles from both NE1-E2 and E1-NE2 treatments showed similar day of death. Moreover, tadpoles from NE1-E2 and E1-NE2 treatments died on average one day before NE1-NE2 tadpoles that were kept free of infection (NE1-E2 and E1-NE2 vs. NE1-NE2; 46.8 and 46.7 vs. 47.8, Table1). Finally, there was no significant difference in body length, weight, width, developmental stage and growth rates among treatments (Table 1).

Table 1. Results of generalized linear model ANOVAs showing variation in leopard frog tadpole life history traits in response to infection. Significant effects based on the asymptotic normality property of maximum likelihood estimates correspond to $p<0.005$. *posteriori* differences between levels of factors are indicated by letters.

Life history traits	***E1_E2***	***E1_NE2***	***NE1_E2***	***NEI1_NE2***	***Statistics***
Length (mm)	28.84	29.19	29.37	29.28	$W_{(df = 3)} = 4.36, p=0.22$
Width (mm)	6.67	6.70	6.82	6.80	$W_{(df = 3)} = 5.20, p=0.15$
Weight (g)	0.38	038	0.40	0.37	$W_{(df = 3)} = 5.69, p=0.12$
Developmental Stage	27.74	27.50	27.45	27.68	$W_{(df = 3)} = 5.18, p=0.16$
Day of Death (day)	43.56	46.69	46.76	47.81	$W_{(df = 3)} = 5.73$, $p = 0.12$
Growth rate (weight)	27.74	27.51	27.45	27.68	$W_{(df = 3)} = 1.36, p=0.71$
Growth rate (length)	43.56	46.69	46.76	47.81	$W_{(df = 3)} = 0.64, p=0.96$
Growth rate (dev.)	0.17	0.08	0.08	0.05	$W_{(df = 3)} = 0.3.01, p=0.38$

Discussion

1. Embryos and Hatchlings infection

The results of the first experiment document a great proportion of the exposed individuals to be infected. Almost 65% of all hatchling screened tested positive for ranavirus but only

with weak or very weak infection signal. Two potential mechanisms could explain hatchlings infection as described below.

First, the evidence of ranavirus infection in early hatchlings might suggest that the thick jelly and the vitelline membrane encasing the developing embryo do not provide efficient protection against ranavirus infection. Data regarding the ability of ranavirus to reach amphibian eggs has been recently documented by Haislip *et al.* (2011) and their results indicate that embryos are not likely to be infected through the jelly. The thick *L. pipiens* egg jelly coat, is made of three to six biochemically different layers (Shivers & James 1970), and considering that the embryos' vitelline membrane lacks some required cell receptors for the virus to enter cells via receptor mediated endocytosis (Chinchar 2002), there may be limited porosity of the *L. pipiens* jelly coat for ranavirus to reach the embryo. Direct Infection of embryos through the jelly seems therefore unlikely.

A second scenario explaining hatchlings infection is related to their post-hatching behaviour and the capacity of ranavirus to remain viable in the water. The jelly, alongside its potential protection against ranavirus, has important nutritive significance for the embryos during their development and after hatching (Humphries Jr. 1966). If virions were to be in the jelly, this may have enabled ranavirus infection at this stage. We observed that upon removal of the hatchlings on average 10 hours after hatching, no significant remnants of the jelly were observed in the vial, suggesting that hatchlings fed on their jelly._This observation in turn suggests the possibility for hatchlings to have been infected with virions that were eventually deposited on the jelly surface. We refer to this mode of contamination as the "jelly surface transmission" hypothesis. A recent study by Haislip et al (2011) on *Lithobates sp*. embryo susceptibility to ranavirus infection supports this mode of infection; the vitelline membrane of the embryo and the jelly coat encapsulating the egg represented efficient protection against ranavirus and only hatchlings in contact with contaminated water become infected (Haislip *et al.* 2011). Additionally, our results revealed a trend for exposed embryos to result in smaller hatchlings suggesting that ranavirus might be detrimental to young individual development. Ranaviral macromolecular synthesis is readily detected 2h post-infection and the first cytopathic effects are observed about 6h post-infection (Goorha & Granoff 1974), immediately followed by a rapid inhibition of host cell DNA, RNA and protein synthesis and a marked re-arrangement of the cellular architecture (Murti *et al.* 1984). If our "jelly surface transmission" interpretation is correct, the time window for the virus to infect the hatchlings would be 10 hours when hatchlings fed on the jelly. This period would then be long enough to allow an infection and a subsequent altering of the hatchling normal metabolic activity

resulting in developmental differences between infected and non-infected individuals. This interpretation remains however speculative and further investigation is needed to validate this hypothesis.

2.Tadpole infection and mortality patterns: investigation of pseudo-vertical transmission

a. Infection patterns and carry-over

Trying to understand the rate of infection differences between stages relates to the investigation of how much of an infection is transferred from one stage to the next (infection carry-over rate). In our study, hatchlings that have been exposed to ranavirus during their embryo stage (E1) had an infection rate of about 65% but individuals deriving from these embryos (E1-E2 or E1-NE2) presented a much smaller infection rate, 40% and 12% respectively. It seems reasonable to assume that the reduction of the observed infection rate resulted from a subtle interplay between virus load, which depends on the exposure/re-exposure scenario, and the rise of the tadpole immune system over time. Over the course of its development, the amphibian larvae immune response potential increases in strength, complexity and diversity (Robert & Ohta 2009). In *Xenopus*, the larva gradually develops spleen B cells, Lymphopoiesis, lymphocytes and Immunoglobulin from Gosner stage 20 to 35. Maximal immunity is expected to be reached around Gosner stages 34-35 in this experiment. Using this immunological timeline, we propose that the drastic virus prevalence reduction in tadpoles from treatment E1-NE2 (from 62.85% to 12%) was likely the consequence of the increasing immune activity in the developing tadpoles. Based on these observations, we estimate the carry-over rate of infection (proportion of tadpoles infection vs. hatchlings infection) to be of approximately 19% ((12/62.85)*100). In tadpoles from treatment E1-E2, when the basic immune arsenal was progressively developed around Gosner stage 20-22, the individuals were exposed a second time to the virus, thus compensating for the earlier virus load reduction. In this treatment, the increased complexity and efficiency of the tadpole immune system might have been able to later fight the spread of the virus and reduce the number of viral particles in individuals resulting in an apparent reduction of the infection rate at the population level. Additionally, tadpoles from NE1-E2 treatments (20.5%) presented a higher final infection rate than tadpoles from E1-NE2 treatments (12%). This observation suggests that a greater proportion of individuals exposed to the infection as embryos but not re-exposed have been able to clear, to a certain extent, their infection. The timing of exposure, hence the conjunction of virus replication time and host immune development likely influences infection rates and appear therefore to be a key factor to

incorporate in epidemiological models, for the understanding of infection rate fluctuations (Ramsay, Speare, & Daley 2001; Schotthoefer *et al.* 2003).

b. Mortality patterns and the influence of infection episodes

Tadpoles that have experienced exposure to the ranavirus at either stage are dying more than those that remained uninfected. Although this finding is in line with other studies (Cunningham *et al.* 1996; Chinchar 2002), our study revealed some interesting characteristics of the ranavirus infection patterns over time, both within and among developmental stages. The relationship between infection and mortality follows a pattern where individual exposed twice died twice as much as individuals exposed once which in turn died more than non-exposed individuals. However, this relationship is not linear as suggested by the significant statistical interaction between early and late exposure, potentially resulting from physiological/metabolic thresholds that might be stage-dependent. The tadpoles from treatment E1-E2 are experiencing the highest rate of mortality with 16.8% of them dying along with the highest infection rate (40%). Interestingly, tadpoles from the two intermediate treatments (E1-NE2 and NE1-E2) experienced about the same mortality rate (8% and 8.2% respectively) but dissimilar infection rates (12% forE1-NE2 and 20.5% forNE1-E2 respectively). This observation suggests that the dose of inoculum is not the only feature responsible for ranavirus infection rate but that host characteristics, particularly the stage of development and the timing of infection might have influenced the establishment and development of the viral infection in the host (Hochberg 1991; Barlow 2000; Brunner *et al.* 2005). In particular, tadpoles from treatment NE1-E2 may tolerate infection to a greater extent than tadpoles from treatment E1-NE2, suggesting that tadpoles exposed later in ontogeny have accumulated enough resources prior to infection to tolerate the energetic cost of a sustained infection (Sheldon & Verhulst 1996). Furthermore, we observed that tadpoles from treatment that were exposed twice over time (E1-E2) died more from infection (65.2%) as compared to single exposure treatments (33% for either E1-NE2 or NE1-E2) and control treatments (0% for NE1-NE2). Together, these results support a relatively strong relationship, despite non- linear between infection and mortality rate, hence virulence of the virus. In fact, a dose-dependent mortality has also been shown in other species of amphibians such as in the ATV-*Ambystoma tigrinum* system (Brunner *et al.* 2005) and *Xenopus* infected by FV3 (Gantress *et al.* 2003).

3. Conclusion

Leopard frog embryos might be protected from ranavirus infection by their thick jelly coat and their vitelline membrane. We speculate however that encapsulated virions potentially trapped in the external layers of the jelly might be assimilated by early hatchlings while they eat the jelly in turn triggering the infection. More importantly, this study is the first to our knowledge to evaluate the effects of the number and timing of ranavirus exposures on infection rate variation with regard to host development. The non-linearity of the relationship observed between mortality and infection underlines the importance of accounting for timing of infection, host life history stage and number of exposures on host mortality if we are to understand the variability of ranavirus virulence and the real impact of epizootic events (Ebert 1999; Day 2002). Presence of virions in the sediment or presence of infected individuals at different period of the development of a target host might result in variable degrees of transmission and result in non-trivial mortality odds. Importantly, the infection carry-over patterns described in this study suggests a significant clearing of the infection by the immune system over the course of host development. The intensity of the infection clearing appears to be contingent of the timing of the infection and the dose of the inoculum, in turn leading to a variation in mortality or morbidity outcomes. Such variability in the virulence may render an epizootic difficult to detect as its severity may vary over time. Our results thus stress the importance of screening different life stages of hosts in order to better understand an infection timeline as well as die-off severity and variability in nature, especially when investigating host with complex life cycles such as Amphibians.

Acknowledgments

We would like to thank Dr. Schulte-Hostedde for comments on an earlier version of this manuscript. This work was supported by the Natural Science and Engineering Research Council and the Canadian Fund for Innovation/Ontario Innovation Trust to DL, and by the STAGE program of Environment Canada to BP.

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CHAPTER 4

Summary, Conclusions and perspectives

1. General summary and conclusions

Amphibians are facing dramatic population declines worldwide with over 32% of the 5743 species described being at risk of extinction (Stuart 2004). Among many causes for these declines, Emergent Infectious Diseases (EIDs), including disease associated with ranavirus infections, have been shown to be responsible for mass die-offs in amphibian populations. However, despite an increasing understanding of ranaviral disease determinants, ranavirus dynamics in the environment remain to be fully elucidated (Lesbarrères *et al.* 2011). Our understanding of ranavirus ecology is complicated by environmental contingencies reflecting a context-dependent disease dynamic (Daskin and Alford 2012). Therefore, understanding any synergies between evolutionary, ecological, and epidemiological determinants of ranaviral disease is critical in order to manage endangered host populations and forecast disease outbreaks.

From a more theoretical perspective, due to the complex and inter-dependent nature of the determinents and the infection outcome, investigating ranavirus-amphibian interactions is particularly useful for improving our understanding of coevolutionary dynamic and the underlying mechanisms of host-pathogen interactions in general. The work described here addresses several issues associated with our ability to develop a full epidemiological model with regards to ranavirus infecting amphibians. This thesis describes the influence of temperature, larval developmental stage, host density, as well as host and pathogen genetic backgrounds on the severity of the disease, using a context-dependent conceptual framework as described in the Introduction and more fully developed in manuscripts 5 and 6. While the studies described investigate specific determinants of ranavirus virulence and infection dynamics, the underlying mechanisms are linked in an epidemiological “loop” (Fig. 1).

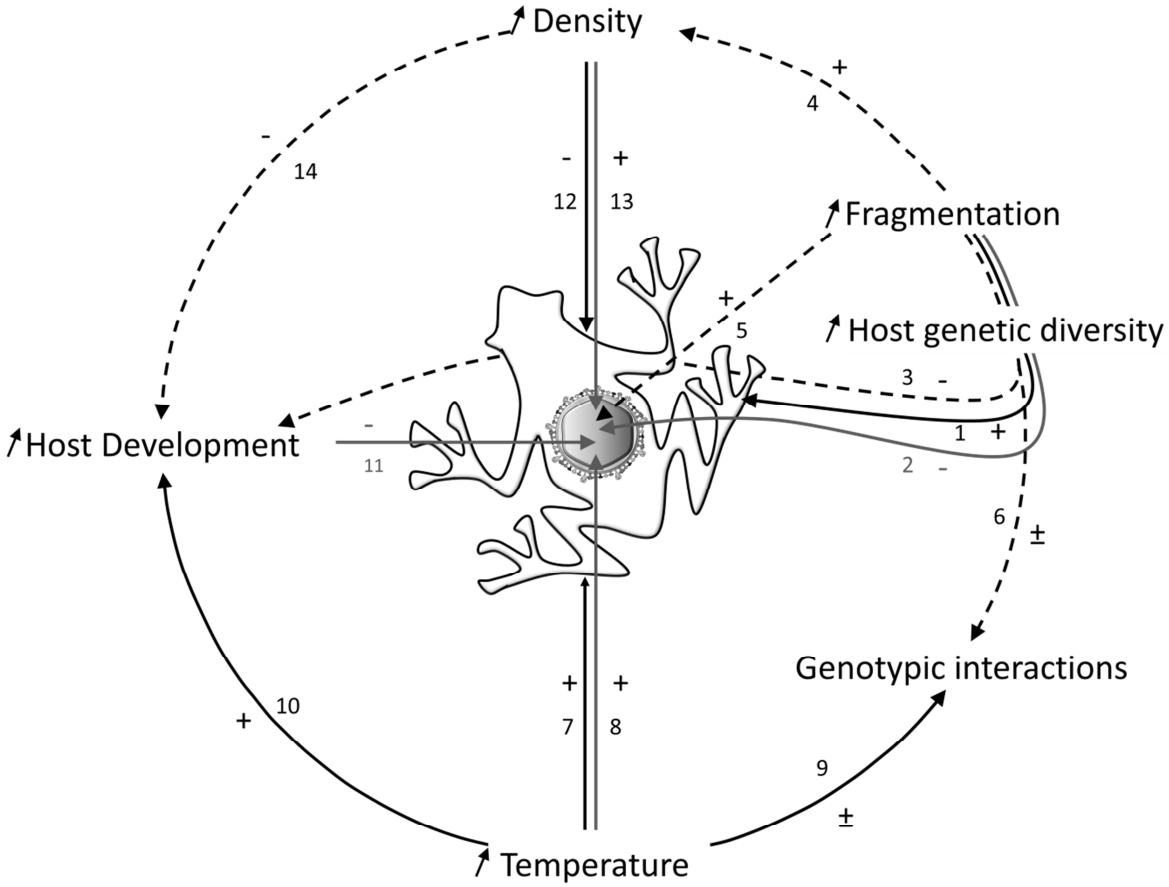

Fig. 1. Eco-epidemiological model of ranavirus-*L. pipiens* interactions based on the presented work. Black arrows represent documented effects on hosts; grey arrows represent documented effects on ranavirus; dashed lines represent effects that have not been documented in this work but known from the literature. Signs, + or -, represent beneficial or detrimental effects, respectively, and numbers are arrow numbers that are references to the text below.

a. The influence of habitat fragmentation on host genetic diversity and pathogen prevalence

Manuscript 1, presents an investigation of interconnection between habitat quality and fragmentation, genetic diversity, and ranavirus occurrence in Ontario populations of *Lithobates (Rana) pipiens*. The analysis indicated a significant effect of environmental variables on the genetic diversity among populations. Environmental variables having a significant impact on frog genetic diversity were railways, roads, forest and building densities as well as three fragmentation measures. The analysis revealed a significant negative relationship between these variables and the allelic range, the observed heterozygosity, the Garza-Williamson index of genetic diversity and a positive relationship with the inbreeding coefficient (Fis) suggesting a clear trend of genetic diversity reduction when fragmentation and habitat deterioration increase (Fig.1, arrow 1).

The analysis also revealed a significant influence of landscape structure on the genetic composition of Northern Leopard Frog populations. Among the 13 environmental variables

selected, 12 were found to be significant predictors of the variation in allele frequencies in four out of seven loci tested. Across the four loci for which we found significant relationships with environmental variables, the allelic frequencies were mostly influenced by human-induced disturbances such as railways and building densities, landcover, and fragmentation indexes suggesting that landscape fragmentation and habitat quality can influence the genetic composition of amphibian populations. Interestingly, among the environmental predictors retained in the analyses, the fragmentation variables were particularly important for one of the allelic frequencies, suggesting that some loci may be more sensitive to environmental determinants than others (Fig.1, arrow 1).

Furthermore, additional analyses revealed that when genetic diversity decreased, ranavirus prevalence significantly increased. Thus increasing the extent of landscape fragmentation and habitat deterioration, in addition to having direct consequences in terms of individual survival, may also result in natural populations having lower genetic diversity and higher risk of extinction, particularly upon future exposure to emerging pathogens (manuscript 1; Fig.1, arrow 2).

b. Importance of the coevolutionary dynamics

Alteration of landscape structure and the redistribution of high quality habitat in terms of ecological characteristics may not only reduce amphibian genetic diversity but also may influence ranavirus strain distribution through direct influences on the corridors of pathogen transmission which may modify the range of possible strain-host interactions (Hess 1996; Fig.1, arrow 5). Interactions between naïve hosts and virulent strains can potentially lead to amphibian population declines that are difficult to forecast, as they result from subtle underlying coevolutionary and epidemiological dynamics induced by habitat alteration (Thrall *et al.* 2007; Fig.1, arrow 6). Such considerations reveal the critical importance of investigating interactions between host and pathogen genotypes (G_H x G_P interactions), which may determine infection outcome and ultimately reflect host-pathogen coevolutionary dynamics. Micro-environmental (E) conditions such as temperature, however, can affect host immune responses and pathogen virulence, in turn modulating the interactions of host/pathogen genotypes (G_H x G_P x E interactions).

Investigations of G_H x G_P x E interactions have the potential to explain variations in fitness-related traits in host-pathogen systems with greater accuracy as they account for both genetic and environmental influences. In the experiment described in manuscript 2, the potential for G_H x G_P x E interactions between two common North American frog species (*Lithobates*

pipiens and *Lithobates sylvaticus*) and three strains of ranavirus at different temperatures, was investigated. The results revealed significant interactions between host type and virus strains for host mortality, host tolerance, final weight, final developmental stage, and developmental rate; all of which suggest that there are reciprocal influences among host and pathogen characteristics in determining the outcome of infection. In particular, Northern Leopard Frog tadpoles showed a greater ability to resist and tolerate an infection, presented a lower mortality, and grew bigger than did Wood Frog tadpoles, likely indicating the genetic cost of plastic development abilities in Wood Frogs when a ranaviral infection occurs.

Additionally, subsequent analyses demonstrated a significant effect of temperature on both host and ranavirus traits and on the genotypic interactions between them (Fig. 1, arrow 7, 8, and 9). Cold conditions negatively affected host body condition and increased tadpole susceptibility to ranavirus. In warm conditions, infected Northern Leopard Frog tadpoles suffered significantly lower mortality than did Wood Frog tadpoles, were less susceptible to infection, and their body size and growth rate declined less than they did in Wood Frog tadpoles. In cold conditions however, for all fitness-related traits except resistance, Northern Leopard Frog tadpoles were not significantly different from Wood Frog tadpoles. Therefore, the reduction of fitness in Wood Frog tadpoles induced by cold temperatures was less drastic than for Northern Leopard Frog tadpoles (Fig. 1, arrow 10 for the specific link between temperature and host development). The occurrence of these patterns was also contingent on ranavirus strain. Consequently, selection in one environment could drive genetic change in the host population but may have no predictable effect in another host and/or environment. To our knowledge, this is the first time that evidence for G_H x G_P x E interactions in a vertebrate-pathogen system has been provided revealing the relevance of using a context-dependent approach to investigate host-pathogen systems (manuscript 2).

c. Within-host infection dynamics

The host-pathogen genotypic interactions investigated in manuscript 2 provide potential mechanisms to explain differential host susceptibility and pathogen infectivity in natural systems. However, it becomes more and more evident that the investigation of an infection dynamics within hosts over the course of development could help identify critical windows of disease risk, and therefore further explain variation in host susceptibility (Johnson *et al.* 2011). Considering that pathogen infection rate is a key parameter in determining disease virulence and the potential for transmission, an accurate quantification of infection patterns

over the course of host ontogeny is critical for a better understanding of a disease's epidemiology and host mortality.

The study described in manuscript 4, investigated the susceptibility of *Lithobates (Rana) pipiens* hatchlings and larvae to ranavirus and compared infection rates among developmental stages. The results indicated a varying susceptibility to ranavirus between developmental stages with hatchlings being more susceptible to infection than tadpoles. This study also revealed the potential for early hatchlings to sustain high basal infection rates possibly acquired during the first hours post-hatch when they were feeding on the jelly or the egg mass. Additionally, the infection carry-over patterns (62% infection rate in hatchlings vs. 12% in E1-NE2 and 20% in E1-E2 treatments) suggest a significant clearing of the infection over the course of host development. The success of the infection clearing appears to depend on both the timing and number of ranavirus exposures. Thus, the results of our study highlight the critical importance of screening infection among individuals but also within individual developmental stages in order to accurately describe pathogen infection and understand host mortality patterns (manuscript 4). Finally, it is noteworthy that environmental parameters such as temperature (manuscript 2, Fig.1, arrow 10) and habitat fragmentation through the modulation of host genetic diversity (Lesbarrères *et al.* 2005, Fig.1, arrow 3) may influence tadpole development, in turn modulating indirectly ranavirus infection patterns.

d. The influence of host-density on ranavirus virulence.

Amphibian development can be strongly affected by density, and from the result of manuscript 4 summarized above, it appears that density can also indirectly alter ranavirus infection through modulation of the host immune system throughout the course of host ontogeny. Manuscript 3 (Echaubard *et al.* 2010) documents the investigation of the influence of host density on ranavirus infection following direct exposure to different virus concentrations. The outcome of the interaction between *L. pipiens* tadpoles and ranavirus appeared clearly influenced by the density at which hosts were held; increasing holding density was detrimental for host fitness as mortality rate was higher, day of death earlier, development longer, and growth rate significantly lower (Fig.1, arrow 14). In parallel, a linear increase of detrimental effects was observed when ranavirus doses increased in low density conditions, with control tadpoles having a significantly higher overall relative fitness. However, this pattern was no longer observed in high density conditions, where the effects of increasing ranavirus dose were limited and resulted in infected and control animals having similar fitness. It was speculated that the host may divert the energy required for a

metabolic/immune response triggered by the infection (*i.e.*, direct costs of the infection) to better cope with the increase in environmental "stress" associated with high density resulting in indirect benefits of the infection. These results illustrate how the net fitness of organisms may be shaped by ecological factors and emphasize the importance of examining the direct/indirect costs and benefits balance to fully understand host-pathogen interactions (manuscript 3; Fig.1, arrow 12 and 13).

At a community level, host density and pathogen transmission and virulence, may be influenced by landscape structure and habitat availability. For example, habitat fragmentation can contribute to a reduction of suitable habitats for amphibians, for instance where wetlands are decreased size and/or increased in drainage that could increase the incidence and length of drying periods (Weyrauch and Grubb Jr. 2004). In turn, these variations in hydroperiod may increase, temporarily at least, tadpole density in the remaining flooded areas (Fig.1, arrow 4) and indirectly influence ranavirus transmission and virulence.

2. Perspectives

Given the complexity of ranavirus disease emergence and the ever-increasing human-induced reduction and fragmentation of habitats, there is a critical need for integrative context-dependent investigations of ranavirus epidemiology. The present work presents an analysis of the influence of several factors determining ranavirus-amphibian outcomes in such a context-dependent framework and provides directions for future investigations.

Among the important issues that need to be addressed is the role of host immunity in controlling ranavirus infection. As demonstrated in manuscript 4, host susceptibility varies among developmental stages. Tadpoles are generally more susceptible than adults, and hatchlings are more susceptible than tadpoles (manuscript 3, Haislip *et al.* 2011). This trend has been attributed to the fact that tadpole immune system increases in efficiency and specificity over the course of ontogeny, with older tadpoles being better able to mount an effective response. However, the amphibian immune system is down-regulated during metamorphosis to facilitate the necessary changes in tissue development (Davis 2009). Despite the critical underlying role of immune variation among developmental stages in determining ranavirus infection outcome, there are no studies comparing changes in innate immune responses of tadpoles infected with ranavirus at different developmental periods.

It appears therefore critical to develop an eco-immunological approach that investigates immunity variation in amphibian hosts infected with ranavirus. The variations in immune function between developmental stages of individuals exposed to ranavirus can be estimated

through the measure of the amount of change in leukocyte numbers (Lymphocytes, Monocytes, Neutrophils, Eosinophils and basophils) at critical developmental stages and possibly under different temperature conditions to account for environmental influence. Blood from tadpoles can also be collected from a tail cut in order to isolate leukocytes that can be stained with Giemsa and recorded under a compound microscope (Davis 2009) and provide quantitative assessment of the tadpole immune response.

From the pathogen perspective, the effect of temperature on ranavirus replication rates needs to be better understood to disentangle the respective roles of the host and pathogen in determining infection outcomes. In particular, it appears critical to document how different strains with different virulences replicate in variable temperature conditions in order to understand the G_H x G_P x E interactions described in manuscript 2. *In vitro* laboratory experiments are ideal procedures to investigate these questions as all environmental variables, including temperature can be controlled. Specifically, it appears that experiments involving single step growth curves, which document the replication rate of a given virus at specific time intervals, would be relevant experiments to perform in order to acquire a precise description of ranavirus replication-tresholds in response to variable temperature conditions.

At the other end of the ecological continuum, ranavirus epidemiology needs to be investigated at the community level by investigating more specifically the link between landscape characteristics and ranavirus dynamics. One of the central questions regarding the dynamics and epidemiology of ranaviruses is to understand their pattern of spread. Most model-based projections of the spread of disease still treat the landscape as homogenous, failing to account for variation. Yet, as landscape features determine the abundance and spatial distributions of hosts and pathogen, it is probable that landscape heterogeneity may be instrumental in determining local disease risk, ranavirus persistence and spread. It appears therefore critical to first, implement the available knowledge on the environmental suitability, tolerance and transmission of ranavirus, in order to analyse risk patterns and factors; this approach would (1) allow predictions of disease spread; (2) reveal novel aspects of disease transmission such as critical metapopulation sizes and distributions; and (3) allow us to evaluate the competitive interactions that may occur between co-infecting pathogens, such as Chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus, in order to clarify pathogen impact on both host health and community level mechanisms.

Finally, as a corollary to the community level investigations, it appears critical to conduct phylogeographic analyses in order to investigate historical contingency in ranavirus occurrence and more clearly delineate ranavirus epidemiology. Emerging Infectious Diseases,

those that have recently been discovered, have recently increased in incidence, geography, host range or are newly evolved (Daszak et al. 2003) originate in two ways (Rachowicz *et al.* 2005). Ranaviruses may have recently spread into a new geographic area, encountering naïve host individuals highly susceptible to infection (the novel pathogen hypothesis) or the pathogen may have been present in the environment for a long time but recently increased its pathogenicity because of environmental changes (the endemic pathogen hypothesis). The results described in the present work suggest the likelihood of ranaviral disease being context-dependent but we still lack an extensive phylogenetical analysis to be able to delineate the respective part of historical vs environmental contingency.

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SUPPLEMENTAL MATERIAL

Manuscript 5

Host-parasite systems and the ecology-evolution synthesis: a new model for an old paradigm

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Contributions:

PE: original idea and writing

CWR: writing

DL: writing

Abstract

An increasing number of publications at the frontier between ecology and evolution are published every year, a trend enhancing the interconnectedness of ecological and evolutionary processes. Indeed a new synthesis between community ecology and evolutionary biology is emerging whereby genetic variation within populations has the potential to shape the ecological functioning of communities, and vice-versa. However, this synthesis is in its infancy and the research community has not yet convincingly demonstrated under which circumstances accounting for both community processes and evolution is critical. Here, we suggest that host-parasite interactions may provide a framework to investigate this link and may serve as an ideal model at the crossroads of evolutionary biology and community ecology. We discuss how specific evolutionary or ecological mechanisms may have cascading effects on each other and how local environments may influence these effects. We thus suggest that host-parasite interactions should be seen as a functional eco-evo mosaic, in turn advocating for an extended evolutionary-ecology conceptual playground to help investigate host-parasite relationships. In fact, both conceptual and methodological advances suggest that such integrative approaches could be the rule rather than the exception, comforting our idea that host-parasite evolutionary-ecology research has now evolved to a degree of maturity never reached before.

Key words: co-evolution, nested explanatory framework, multidisciplinarity, ecology, evolution, host-parasite

Introduction

An integration of ecology and evolutionary biology has been approached several times during the last few decades but still remains an "elusive synthesis" (Sterelny 2005). The advantages of a union of the two sciences are, however, quite clear. For community ecologists, incorporating evolutionary adaptation in their studies, either conceptually or in mathematical models (Day 2005), simply may allow more variation in community structure and dynamics to be explained. Many community ecologists conduct their work under conceptual models where species' traits are fixed. Thus the outcome of interactions among individuals and populations is decided by relationships that are non-deterministic based on stochasticity or contingency (Hubbell 2001). In reality, traits are not fixed but fluid and undergoing evolutionary change (Hairston Jr *et al.* 2005, Carroll *et al.* 2007). Mutation may be a rare source of novelty but epistasis (Weinreich *et al.* 2005), migration among metapopulations (Gilpin and Hanski 1991, Harrison and Hastings 1996), and re-emergence of past genotypes from seed and egg banks (Arnaud *et al.* 2011), for example, are significant agents of evolutionary change that can have considerable consequences for the outcome of ecological interactions.

From the point of view of evolutionary biology, considering ecological context provides both more dimensions for understanding the outcomes of interactions among species, and ecological realism. While evolutionary theory largely deals with the potential consequences of fitness differences among individuals and populations, the source of these fitness differences lies within the ecological interactions of a community (Sober 1993 cited in Sterelny 2005). In other words the evolutionary play exists within an ecological theater Hutchinson's (1965). Without the context of community ecology, the ideas of evolutionary biology lack a real-world test and "Arguably, its status as an empirical science is at risk" (Sterelny 2005).

If there are such advantages to a union of evolutionary biology and community ecology, why has a synthesis proven so elusive? Other authors have approached this topic in several excellent analyses (see Cuddington and Beisner 2005, Johnson and Stinchcombe 2007, Holt 2009). They have shown that among the possible reasons are historical ones such as the distractions of debates within evolutionary biology on issues like the adaptationist program and phylogenetic reconstruction that were of little relevance for community ecologists. However, several authors have also identified differences between evolutionary biology and community ecology that are more germane. For example, an adaptation in an ecological context may not involve adaptation in the evolutionary sense. As an example, organisms often

modify their environments as they occupy them over the long term. New recruits to the population essentially inherit the modified environment. Therefore even if traits in a population do not change over time (evolutionary adaptation), the functioning of the traits may change (ecological adaptation). A synthesis of evolutionary biology and community ecology should incorporate the idea that traits and environments may be shaped by each other.

Moreover, evolutionary and ecological processes exist at very different time scales (Holt 2005, but see Carroll *et al.* 2007) and also at very different spatial scales; the later Sterelny (2005) calls the "grain problem." The differences between evolutionary biology and ecology in terms of both time and spatial scales are perhaps the most commonly identified reasons for a lack of synthesis between the two disciplines. Rapid evolution may quickly change the relative frequency of different traits in a population but for the most part, traits emerge and are shaped over the long term. Evolutionary processes take place over many generations. In contrast, ecological processes largely occur within the scale of a single generation. Nevertheless, while the fitness benefits of traits might be the end result of tuning over the long term, the main tool of evolution, natural selection, is the integrated process of many, short-term ecological events. In the lives of individuals there are competing constraints that may be affected by different traits and the integration over this multidimensional matrix in the long term is part of the process that may allow fitness advantages to accrue for particular traits. Natural selection can have no goal and advantageous traits can emerge only along a bumpy road of ecological interactions. Evolutionary processes also contribute to the bumpiness of that road. Species do not exist in isolated populations but in metapopulations that are interconnected to varying degrees. Even for environmental conditions that appear to be broad scale, there is no guarantee that selective pressures are the same across different metapopulations or even within sub-habitats in the area of a single population (Ricklefs 2004). The result of this graininess is that immigration among metapopulations may dilute the effects of local selection by introducing alleles from different populations that were either neutrally selected or perhaps were selected in different ways.

The practical result of these differences in time and spatial scales is a divergence in focus between evolutionary biologists and community ecologists. Evolutionary biologists tend to study in isolation traits of as many ecological interactions as possible that might dilute the fitness effects that are their focus. Community ecologists, on the other hand, tend to think of traits as fixed because, within the myriad of simultaneous ecological interactions and short time scales in which they work, evolutionary change in any trait is unlikely to be manifest. Such compartmentalization of the disciplines can even result in a questioning of the actual

importance of bridging community ecology and evolutionary biology as there is a lack of clear demonstration under what circumstances it is important for biologists to take into account both community interactions and evolutionary theory (Johnson and Stinchcombe 2007).

Here, we suggest that host-parasite interactions may serve as an ideal model for the intersection of evolutionary biology and community ecology. Host-parasite investigations ("H-P" hereafter) have evolved conceptually during the last two decades, from a basic and descriptive approach to the current hypothesis-driven and more theoretical discipline shaped by evolutionary biology (Poulin 2007). A deeper understanding of the determinants of the mutual selective pressures that the host and the parasites exert on each other, together with recent conceptual advances, arguably position this field of research at the frontier between ecology and evolution. Additionally, current publication trends suggest an increasing tendency for H-P research to fit within both the evolutionary and ecological frameworks, further underlying the appropriateness of H-P systems when considering the synthesis of evolution and ecology (manuscript 6).

As hosts and their parasites are different species they are independent units of natural selection, yet their lives are strongly intermingled. The parasite is subject to most of the same myriad of day-to-day ecological interactions that affect the host. While these interactions cannot, of course, shape the parasite in the same evolutionary way as they shape the host, nevertheless it is the case that ecological realities for the host strongly and at short time scales affect the parasite. In other words, the strength and nature of the selective pressures encountered in the host's life may promote rapid evolution of the H-P system, within an ecological time frame. The interplay between evolution and ecologically significant processes may be thus more clearly seen in H-P systems, (Neuhauser *et al.* 2003) possibly avoiding Sterelny's (2005) grain problem. The convergence between evolution and ecology makes H-P interactions dynamic over time and space, and may even explain why H-P interactions can vary along a continuum from mutualism to strict parasitism, depending on given ecological conditions (Renaud and de Meeûs 1991).

From this perspective, we explore the ways that host-parasite relationships may be free of some of the issues that have thus far prevented a synthesis of evolutionary biology and community ecology. In the following sections, we explain our approach, describe the critical mechanisms affecting the outcome of H-P interactions, and identify under which conceptual framework (evolution or ecology) each mechanism may be explained. We then discuss the interactions between evolutionary and ecological mechanisms and investigate the role of local

environments in modulating such "eco-evolutionary" interactions. Finally, we propose an integrated, extended, H-P eco-evolutionary framework which may serve as a model for the emerging synthesis between ecology and evolution.

Functional mechanisms and the eco-evolutionary gradient

In H-P interactions a gradient across scale often exists in the strength of selective pressures exerted by each protagonist on the other. At the finest molecular scale, the co-evolutionary arms race between the host's immune system and the parasite's defenses is certainly under intense selective pressure. The outcome of this battle is strongly affected by interactions between the host's genotype and the parasite's genotype (hereafter referred to as GxG) with little direct influence of either environmental or ecological factors. At higher levels of organization the roles of both the internal and external environments become more relevant.

At the physiological level and above (i.e., individuals, populations, communities), the outcome of H-P interactions is determined not only by the specific, focused, co-evolutionary arms race of immune responses and defenses, but also by the environmental conditions both within and outside of the host's body (Bedhomme *et al.* 2004, Seppälä *et al.* 2008). For example, host condition such as the temperatures at which H-P interactions take place may affect allocation trade-offs by the host resulting in variable level of susceptibility to pathogens (Mitchell *et al.* 2005, Vale and Little 2009). At the population level, host density (Ebert *et al.* 2000, Bieger and Ebert 2009, Echaubard *et al.* 2010) and other factors are well known to affect pathogen transmission (Brunner *et al.* 2007). At the level of the whole community, the effects of parasites would be just one factor in a set of ecological challenges that may include foraging, predator avoidance, mate-seeking, and dealing with environmental contingencies. The end result is that the selective pressures shaping H-P interactions become more diluted along the path from molecules to community (Fig 1). Nevertheless, each of the critical mechanisms occurring at the different levels of biological organization, such as GxG interactions, allocation trade-offs, and community-based mechanisms are important for determining the outcome of H-P interactions at each of the relevant levels (genes, individuals, populations and communities). These mechanisms (hereafter referred to as "functional mechanisms") are therefore characterized in a range from primarily evolutionary to primarily ecological effects along the gradient of biological organization.

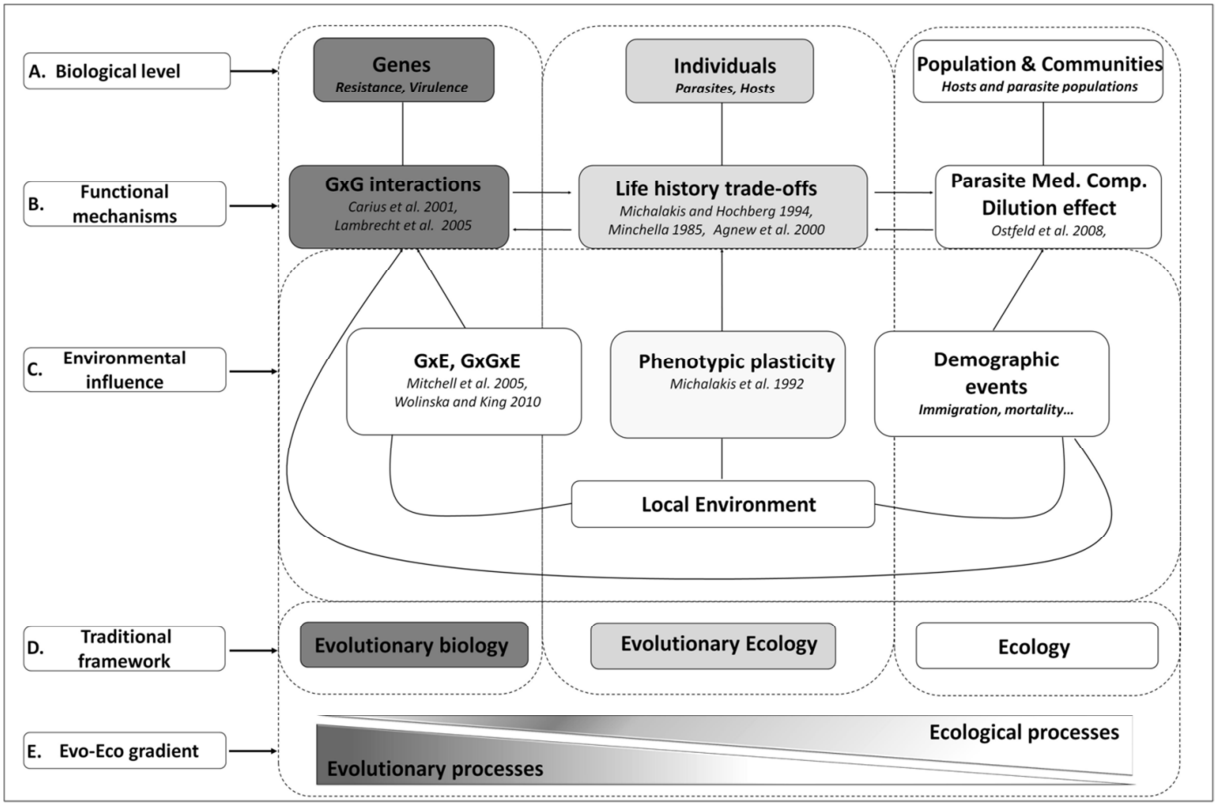

Fig. 1. New framework for the reintegration of ecology and evolutionary biology. Each of the three biological levels (A) under which H-P interactions can be investigated is characterized by specific functional mechanisms determining the outcome of the interaction between the host and the parasite (B). The influence of such mechanisms can in turn be modulated by external environmental features (C) so that the traditional frameworks under which investigations regarding the different levels of organization (D) is now reconsidered as a conceptual evo-eco gradient (E).

1. Genotypic interactions and evolutionary patterns

The molecular interaction between the parasite's epitope and the host's cell receptors and circulating antibodies are immunity battles, the outcomes of which are the strongest immediate determinants of an infection (Frank and Schmid-Hempel 2008). The highly polymorphic Major Histocompatibility Complex (MHC) alleles vary among hosts, causing each individual to have a particular spectrum of presentation efficiencies for the epitopes of different parasites. Thus the strength of a host's response to a particular epitope depends on its MHC genotype. From the parasite's point of view, a particular antigenic variant may be able to attack some host genotypes but not others. The ability of a parasite to avoid detection by the host's immune response depends on several mechanisms such as random mutation during replication which generates novel antigens, or switching expression between archived variants. The variability of both the host's MHC alleles and the parasite's antigenic variants results from a mutation-selection balance. Therefore both the host and pathogen genotypes (GxG) are important to consider as they both share control of the epidemiological parameters

of their relationship. and appear therefore to be critical mechanisms that shape the outcome of H-P interactions and are the fuel for antagonistic coevolution (Sorci *et al.* 1997, Carius *et al.* 2001). Coevolution is the result of a frequency-dependent reciprocal selection on host resistance and parasite infectivity (Thompson 1994). It requires genetic variation in resistance and infectivity as well as genotype-genotype specificity. Genotypic interactions between host and parasite have the potential to modulate both the strength and specificity of their mutual selective pressures resulting in non-trivial co-evolutionary dynamics. For example, Carius *et al.* (2001) found that interactions between a given *Daphnia magna* clone and a given *Pasteuria ramosa* isolate may result in different outcomes in comparison to other associations indicating the potential for frequency-dependent selection in this system. Good examples of the importance of GxG interactions in determining the outcome of H-P interactions and ultimately affecting the co-evolutionary process are also reported in anopheline mosquitoes (Lambrechts *et al.* 2005, 2006) and in amphibian/ranavirus systems (manuscript 2). Such co-evolutionary processes may, in turn, affect allocation trade-offs for other life-history traits.

2. Life history trade-offs

One common factor that links all classes of immune function is the need for resources that the host might use for other functions. Optimal resource allocation depends on balancing multiple demands for resources and their associated benefits (Sheldon and Verhulst 1996, Tschirren and Richner 2006), resulting in trade-offs at the individual level. For example, a trade-off between host immune function and reproduction has been documented in several species including birds, rodents and invertebrates (Ilmonen *et al.* 2000, Sanz *et al.* 2004, Ahtiainen *et al.* 2005, Schroderus *et al.* 2010). Theoretical analyses suggest that hosts would be favored by selection if they were able to reproduce earlier thus avoiding prolonged infection (Hochberg *et al.* 1992, Michalakis and Hochberg 1994). Furthermore, the effects of a given parasite on its host are often not immediate but rather increase with time from infection, reinforcing the need for a host to preferentially allocate resources toward reproduction, even at the expense of growth and survival (Forbes 1993, Agnew *et al.* 2000). Additionally, the responses of the hosts in modifying their reproductive schedule may be accomplished either by phenotypic plasticity, an essentially ecological response, (see section below) or genetic differentiation (Michalakis and Hochberg 1994), an essentially evolutionary response (genetic variation). A change in timing of reproduction has quite considerable consequences at the population and community levels. Early reproduction may increase population growth rate but at a cost in body size, an important factor in vulnerability to

predators (Emmerson and Raffaelli 2004) and competitive ability (Smith and Brown 1986). Additionally, a higher population density may more readily over-exploit food and other resources, in turn benefiting the parasite population except if the resources available for the parasite depend directly on the host condition (Seppälä *et al.* 2008). Clearly, even in H-P interactions that occur at the individual host level there will most likely be consequences at the population and community levels.

From the individual parasite's point of view, a fundamental property affecting the evolution of virulence is the trade-off between the virulence of an infection and the reproductive capacity of the parasite (Ewald 1987, Alizon *et al.* 2009, Froissart *et al.* 2010). Virulence is usually an unavoidable consequence of parasite reproduction in the host with higher parasite reproduction most often resulting in higher virulence (the baseline pathogen reproduction ratio, R0, is an inverse function of the host death rate, which is a measure of virulence; Anderson and May 1979). The parasite's fitness improves with an increase in its reproductive capacity, but is diminished by high virulence (which debilitates or kills the host), if transmission of the parasite requires the host to be alive, healthy and able to reproduce (i.e., vertical transmission). Such transmission-virulence trade-offs have been observed both in nature (Herre 1993, Ebert 1994) and in lab experiments (Ebert and Mangin 1997, Messenger *et al.* 1999).

3. Community-based functional processes: parasite-mediated competition and dilution effect

While many studies demonstrate that pathogens and parasites can have dramatic impacts on individual hosts, substantially fewer have explored the ecological consequences of parasite-induced changes in hosts and host populations. Pathogen effects on host behavior, reproduction, and mortality influence community interactions such as competition, facilitation, predation, and invasion and thus may have strong impacts on ecosystem dynamics (Ostfeld *et al.* 2008).

One of the better known mechanisms by which parasitism may affect the host community is through parasite-mediated competition which has received considerable attention since the reviews by Freeland (Freeland 1983) and Price *et al.* (Price *et al.* 1988). Parasite-mediated competition was first described by Park and his associates in the 1940s (Park 1948) when they conducted a series of laboratory experiments on competitive interactions with *Tribolium* beetles. The authors observed that a protozoan parasite (*Adelina tribolii*) could alter the competitive relationship between two species of *Tribolium*. Under certain conditions the

competitively superior beetle was prone to infection by *A. tribolii*, allowing the competitively inferior species to survive or even dominate (Park 1948). A considerable amount of mensurative and experimental data, as well as recent conceptual advances, suggest this mechanism to be particularly significant given the rise in emerging diseases (Palumbi 2001, Lebarbenchon *et al.* 2008) and the opportunity that pathogens have to directly affect host abundance (Hudson and Greenman 1998).

On the other hand, host populations and their associated ecological community can affect pathogen and parasite dynamics. The presence of more than one host species in a community may increase the pathogen's population size (amplification effect), and enhance its ability to persist (dilution effect) compared to situations where just one host species is present. While there is little evidence for an amplification effect or its prerequisites, strong evidence has been found for the dilution effect during the last decade (Ostfeld and Keesing 2000, LoGiudice *et al.* 2003, 2008). In essence, when more than one host species are present, a transmission event that might link an infectious and a susceptible individual of the competent host species may instead link, in greater proportions, infectious individuals to incompetent hosts. In consequence, this situation will generate far fewer new infections, modulating, in turn, the pathogen population dynamic and depleting its fitness (see Begon 2008 for a discussion).

Cascading influences and the eco-evolutionary mosaic

While all of the functional mechanisms described above may be crucial determinants of the outcomes of H-P interactions, they differ in their respective scale of occurrence and whether they are considered to be evolutionary or ecological processes. Historically, GxG interactions, allocation trade-offs, and community-based mechanisms have been, not necessarily deliberately, studied under the umbrella of either evolutionary biology or disease ecology/epidemiology. This epistemological/conceptual partitioning has increased the gap between reductionist evolutionary biologists and holistic community or disease ecologists leading to the current discontinuity in the evolutionary-ecology framework.

We believe that the conceptual partitioning between ecology and evolution is an obstruction in the study of H-P systems because these systems represent a mosaic of both types of influences. Furthermore, the current practice of studying evolutionary processes without considering the ecological setting, and studying ecological processes without considering adaptation potential, represents a detriment to enhanced understanding of the true

relationship between evolution and ecology. To address this, we propose to use the concept of cascading influences, which can be thought of as bi-directional (evolution to ecology and ecology to evolution) eco-evolutionary feedbacks (Post *et al.* 2009) between the functional mechanisms explained above. Each functional mechanism will, at its own level, affect the host and/or the parasite in terms of energy balance, susceptibility, density, and fitness. However, changes in parameters of the host and parasite populations will have implications for all other functional mechanisms. When researchers ignore this problem by compartmentalizing research at either end of an evolution-ecology gradient there might be a lack of understanding of their systems. Nature is a mosaic of evolutionary and ecological processes and incorporating this mosaic may be more productive than dissecting it.

Despite the somewhat abstract nature of the eco-evolutionary mosaic concept, and the difficulty to test it, several examples from the H-P literature support a synthesized view where ecological process affect evolutionary pattern and vice versa. The connection between H-P genotypic interactions and life-history trade-offs (see Fig 1), is particularly well illustrated in a recent study by Salvaudon *et al.* (2005). In a cross-infection experiment, the authors used five lines of the plant *Arabidopsis thaliana* and two strains of the oomycete pathogen *Hyaloperonospora parasitica*. They showed that three traits traditionally considered to result from the parasite transmission-lifespan trade-off differed among specific combinations of host and parasite lines. These findings are corroborated by the influence of genotypic interactions on life-history traits that may be involved in trade-offs, such as resistance to *Plasmodium falciparum* in *Anopheles gambiae*, the major vector of malaria in Africa (Lambrechts *et al.* 2006).

At higher levels of biological organization (Fig. 1), the links between individual trade-offs and community consequences are better known, particularly in the understanding of how host physiology affects epidemiological parameters at the community level. Such a connection has been observed in the yellow dwarf virus infecting wild grasses worldwide (Cronin *et al.* 2010). In this system, the physiological phenotype of the host and its associated trade-offs explain why hosts differ in susceptibility to infection, and ability to support vector populations. Ultimately, the authors suggest that the physiological phenotype of the host may explain pathogen transmission across ecological levels from the individual to the community (Cronin *et al.* 2010). Ultimately, while no study has directly tracked multi-level influences (e.g., from genotypes to phenotypes to community), a mechanistic continuum must underly any evo-eco gradient. Such intrinsic dependencies of ecological and evolutionary processes

argue strongly for the extension of the current evolutionary ecology framework to include community level mechanisms.

Environmental heterogeneity in host-parasite interactions: does it matter and to what extent?

In addition to the contrasting effects of more evolutionary vs. more ecological processes along the evo-eco gradient, other factors may come into play to determine the outcome of an H-P interaction. In this section, we explore a few of these ideas to illustrate how and when the local environment may modulate these processes. We start by presenting how the environment can modulate genotypic expression through phenotypic plasticity. We then discuss the influence of demographic events for both host condition and pathogen transmission at the population level and how these events can affect the genetic structure of host populations.

1. GxE, reaction norm and phenotypic plasticity

At the genotypic level, the dynamic nature of adaptation and counter-adaptation between the molecular arsenals of host and parasite (antagonistic co-evolution) may be particularly sensitive to environmental influence. In fact, environmental variables may affect the strength of selection and the type of response to selective pressures resulting in host and/or pathogen Genotype (G_H and G_P) by Environment (E) effects (*e.g.* G_HxE, G_PxE, or G_HxG_PxE). The result of these interactions may be condition-dependent virulence (Thomas and Blanford 2003, Wolinska and King 2009, Daskin and Alford 2012; Fig. 1). The direction and extent of environmental effects on genotypic interactions may result in the expression of different phenotypes as the reaction norm (see Scheiner 1993 for a review). Such influences by the environment on host and parasite genotypes have been documented widely in the last decade. For instance, the significant effects of environment on the specificity of selection in H-P system have been documented in 31 of 92 performed analysis reviewed by Wolinska and King (2009), who indicated that no single environmental optimum exists for a given H-P interaction and emphasized the critical role of the environment for the outcome of an infection (Wolinska and King 2009)

2. Demographic events

As a necessary consequence of populations and communities being comprised of disparate individuals, both genes and populations are prone to random fluctuations in abundance resulting in both genetic drift and population extinction (Vellend and Geber 2005). From a whole community point of view, natural contingencies in a given environment are instrumental in determining the mortality and birth rate of host and parasite populations. Among potential hosts, both inter- and intra-specific susceptibility to a pathogen or parasite will lead to differential host mortality, transmission rate, and infection pattern. The result is that variability among potential hosts in factors such as host social behavior and contact rates, susceptibility, and population size will affect the overall disease dynamic (see Altizer *et al.* (2006) for a review on the effect of environmental seasonality on infectious diseases dynamics). Parameters related to pathogen transmission such as the duration of infection and the probability of infection are critical for disease epidemiology and they may be directly related to ecological factors such as the densities of the susceptible and infected host populations (Anderson and May 1979, 1981, Hochberg and Holt 1990), as well as the so-called mass-action or density-dependent effect (see McCallum *et al.* 2001, for a discussion on alternative models of transmission). Any modification of a pathogen's transmission rate in the host community that results in differential mortality rates will alter host community structure (diversity, richness, abundance), and likely result in changes in the ecological interactions of the host species. Such changes will in turn influence the potential for a dilution effect and eventually modulate the intensity of the parasite-mediated competition (Fig. 1).

Furthermore, due to environmental stochasticity, drift and migration are the main regulators of neutral genetic diversity (Kimura 1985, Hubbell 2001) but these processes can also have important effects on non-neutral diversity (Lenormand 2002, Vellend and Geber 2005). Genetic drift, resulting from a decrease of gene flow among host populations may result in the loss of alleles useful for disease resistance. Several studies have indeed shown both empirically and theoretically that a genetically depauperate host population may be more susceptible to diseases and parasites (Lively *et al.* 1990, Coltman *et al.* 1999, Acevedo-Whitehouse *et al.* 2003, Garner *et al.* 2005). Migration may affect host and parasite genetic diversity through gene flow modulation (Thrall and Burdon 1997) whereby favorable resistance alleles are brought to the host populations by new migrants. The new molecular weapons may lead to new fitness outcomes in the host population(s) (Fig. 1).

From the evo-eco mosaic to a cohesive conceptual framework

As we have shown, three types of mechanisms influence the outcome of an H-P interaction, each of them occurring at different scales of biological hierarchy, and falling along a gradient from evolutionary biology to community ecology justifying in turn the view that H-P system are an eco-evolutionary mosaic. In the previous sections we provided the fundamental properties of these mechanisms and their inter-dependent influences and suggested that the compartmentalization between them is misleading. In this section we move beyond the mechanistic aspects and develop an epistemological justification for an extended eco-evolutionary framework.

The outcome of any infection clearly has multiple interacting dependencies at several biological levels. In other words, the set of potential and actual outcomes at a given level (genes, individual, population/communities) interact with conditions at the contiguous lower and upper levels of organization, through sets of many-to-one and one-to-many connections (Vepsäläinen and Spence 2000). The number of initial conditions and their permutations at the lower level (genes) define the potential states at the next level (individual). In turn, any given level (either genes or individuals) is constrained by the upper-level (communities) boundary conditions (Vepsäläinen and Spence 2000). In a newer conceptual approach, each mechanism at each level is investigated within a contextual framework that allows generalization based on multiple causations at this given level. Once the generalization is validated at the lowest level (genes) it allows relevant and specifically focused investigations at the next level up potentially leading to a generalization at a higher level (Fig. 2). Overall, this bottom-up contingency-based approach is a nested, continuous explanatory framework. Top-down influences, from communities to genes are also possible and may modulate existing lower levels mechanistic interactions leading to non-trivial system evolution. were Such a framework has the potential to incorporate and account for the great complexity and multitude of causations within, and among levels – the outcome of an infection, for example. This type of explanatory framework is likely to provide us with a greater understanding than simple generalizations linked to investigations at isolated levels (genes, individual, population, or communities; Vepsäläinen and Spence 2000). What we describe is elegantly summarized by Levins and Lewontin (1985): “We argue for a strategy that sees the unity of the general and the particular through the explanation of patterns of variations that are themselves higher-order generalities that in turn reveal patterns of variation”.

As a practical matter, we suggest the combinatory use of Causal Diagram and Structural Equation Modeling (SEM) for evaluating the entire plausible hypothesis space and the respective weight of potential causes within each focal level when determining the outcome of a given H-P interaction (Fig. 2). Both causal diagram and SEM are attributes of a strong inference approach which simultaneously use a full staff of working hypotheses (Chamberlain 1897).

The Causal diagram is a visual representation of the plausible mechanistic pathways, potential interactions, and confounders involved in a single outcome of interest (Greenland *et al.* 1999, Plowright 2008). The use of causal diagram promote communication among scientists and clarifies assumptions, foundations for analyses, generates clear testable hypotheses and identifies gaps in existing data (Hjorth and Bagheri 2006; Fig. 3).

Structural Equation Modeling is an advanced, multivariate, statistical process that can be used to construct theoretical concepts and establish causation links between manifest and latent variables. Latent variables are theoretical concepts that unite phenomena under a single term (e.g., genotypic interactions) while manifest variables are usually directly measurable quantities (Bollen 1989, Malaeb *et al.* 2000). The use of SEM in ecology and evolution is increasing due to its appropriateness for the investigation of multi-causal nested problems (Arhonditsis *et al.* 2006). SEM seems to be a robust technique for studying interdependencies among sets of correlated variables, and is well suited to providing insight into the relationships among the abiotic and biotic variables in ecological and evolutionary research. In this statistical technique, pre-conceptualizations that reflect research questions or available knowledge about a given system structure the initial framework for model development, while both direct and indirect effects and measurement errors are taken into account (Arhonditsis *et al.* 2006). Most SEM can be expressed through path/causal diagrams indicating the causal relationships between relevant variables which promote validation of a specific combination of explanatory hypotheses at each focal level. Generalization at an upper level requires concomitant generalization at the lowest level resulting in a nested validation process and the realization of a cohesive explanatory framework along the epistemological continuum as illustrated by the white arrow.

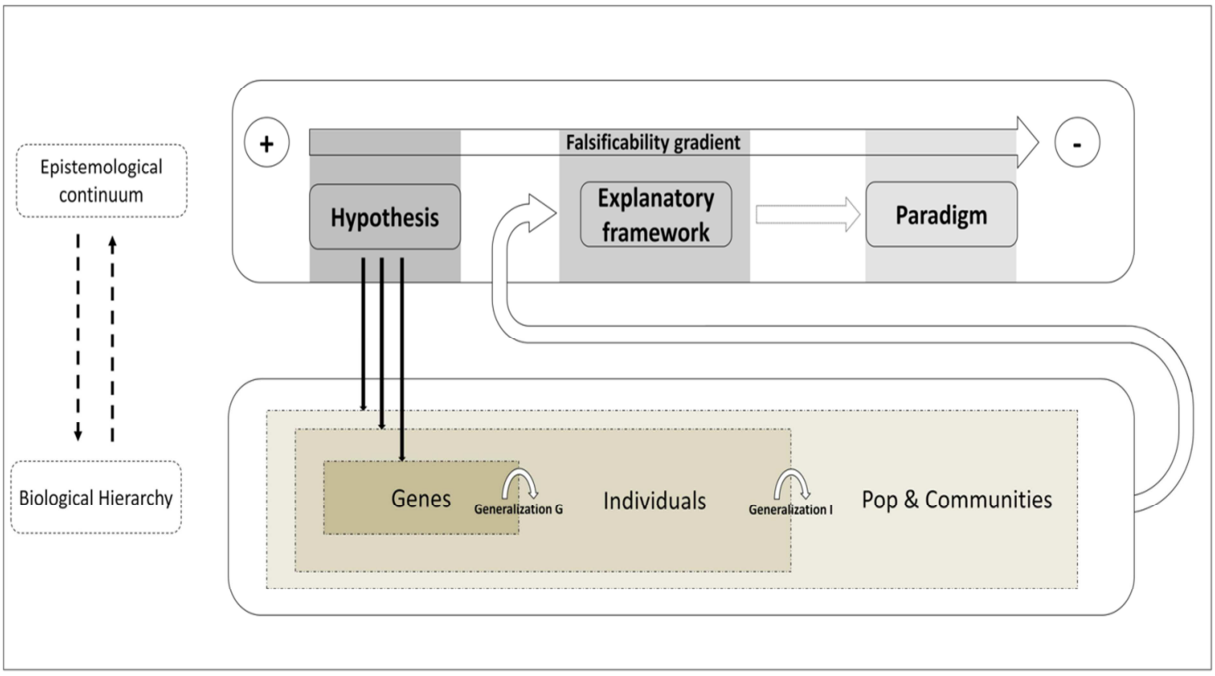

Fig.2. Epistemological continuum vs. biological hierarchy. Along the Epistemological continuum, researchers develop hypotheses that might be integrated in an explanatory framework and ultimately condition the validity of a paradigm. Hypotheses, explanatory framework and paradigm follow a falsifiability gradient in the context of the scientific method. From hypothesis to paradigm, the degree of falsifiability potential decreases in turn generating the observed bottom up validation procedure (plus and minus signs illustrate the gradient of falsifiability in the scientific process). In the biological hierarchy compartment, within each biological level, a set of hypotheses can be formulated (black arrows).

We believe that SEM is a powerful tool allowing the robust extrapolation of relationships within a focal level throughout the eco-evo framework. At a single focal level, relationships among a set of manifest variables can be analyzed in the form of a correlation matrix. This would allow determination of the role of each variable in explaining the variability of the others. A study of relationships among latent variables would delineate the critical linkages across focal levels. This would provide a rigorous means for discovering and evaluating the importance of, for example, genotypic interactions, life history trade-offs, and parasite-mediated competition. Practically, we agree with Arhonditis *et al.* (2006) that this multivariate statistical method can be supplemented with Bayesian analysis in an effective combination. Bayesian methods provide an *a posteriori* probability of accepting an ecologically meaningful specific hypothesis rather than providing a fixed threshold for rejecting an often meaningless null hypothesis, as is the case with frequentist methods. The joint use of SEM and Bayesian analysis in any complex system is more likely to identify plausible causative relationships in a robust manner.

Complex synergies and context-dependent dynamics in Amphibian-ranavirus interactions: an example

Amphibians are facing the most dramatic and enigmatic population declines worldwide with over 32% of the 5743 species described being at risk of extinction (Stuart *et al.* 2004). Among many causes for these declines, Emergent Infectious Diseases (EIDs) such as the one triggered by ranavirus infection have been shown to be responsible for mass die-offs in amphibian populations. Ranaviruses as emerging pathogens are known to have caused amphibian die-offs on five continents (Gray *et al.* 2009, Miller *et al.* 2011). The greatest number of reported mortality events has been in North America and Europe, resulting in population declines in several cases (Teacher *et al.* 2010). Ranaviruses are known to infect at least 72 amphibian species in 14 families (Miller *et al.* 2011).

Despite an increasing understanding of ranaviral disease determinants, ranavirus dynamics in the environment remain to be elucidated. Our understanding of ranavirus ecology is obscured by environmental contingencies that result in context-dependant disease dynamics (Lesbarreres *et al.* 2011, Daskin and Alford 2012). The interdependent nature of disease determinants renders the investigation of ranavirus-induced mortality a challenge and the influence of potential abiotic and biotic mechanisms such as temperature, larval development, density and competition for resources on the prevalence and virulence of the virus remain to be explored (Lesbarreres *et al.* 2011). Amphibian ranaviral disease appears to be related to ecological change and therefore can be mediated through complex and large scale processes that are not amenable to traditional reductionist approaches regarding causal inference (Plowright *et al.* 2008). Consequently, it is necessary to apply an integrative approach where ecological, evolutionary and epidemiological concepts are used together for the understanding of ranavirus/amphibian interactions (Daskin and Alford 2012). The explanatory framework developed through the present paper therefore becomes a relevant conceptual tool to use in order to elucidate ranaviral disease dynamics and predict coevolutionary trajectories. The case of ranavirus-amphibian interactions illustrates particularly well the benefits of incorporating conceptual developments, such as the eco-evolutionary mosaic framework coupled with techniques such as causal diagrams and SEM into applied approaches. In parallel, the application of the recommendations derived from the conceptual eco-evolutionary mosaic framework promote multidisciplinarity through the need of an extensive diversity of methodologies, from sequencing to large-scale mesocosm experiments through modeling and geographic data analyses.

An example of causal diagram for ranaviral disease emergence in amphibian is given in Fig. 3. The last decade has seen an increasing interest for ranavirus disease dynamic and an important number of studies have contributed to narrow down the space of plausible hypotheses for an epidemic to occur and induce severe mortality in amphibian populations (Fig. 3). Susceptibility to ranavirus infection varies widely among species (Schock *et al.* 2008, Hoverman *et al.* 2010, manuscript 2). Of 19 North American species tested, wood frog (*Lithobates sylvaticus*), gopher frog (*L. capito*) and Eastern spadefoot toads (*Scaphiopus holbrookii*) were the most susceptible to ranavirus (Hoverman *et al.* 2010, Haislip *et al.* 2011). Modification of global cycles, hydroperiod and land use can alter patterns of ranavirus transmission through host population density change (Echaubard *et al.* 2010) and the modification of host species richness (Babbitt 2005). The reorganization of host species assemblages may also alter ranavirus-mediated competition and further modify host community composition (Price *et al.* 1988).

Ranavirus can transmit horizontally among individuals via indirect and direct routes (Gray *et al.* 2009). Transmission of ranaviruses has been documented via exposure to contaminated water (Brunner *et al.* 2004, 2005, Pearman *et al.* 2004), by direct contact with infected individuals (Brunner *et al.* 2007), and by exposure to fomites such as virus-contaminated sediment (Harp and Petranka 2006). Ingestion of infected tissue either through necrophagy, coprophagy or cannibalism is another effective transmission route (Jancovich *et al.* 1997). Exposure to infected individuals in water for three hours without contact can result in transmission (Robert *et al.* 2011), and only brief direct contact is needed to cause infection (Brunner *et al.* 2007). Typically, ingestion of the virus results in faster mortality than exposure via virus particles in the water (Hoverman *et al.* 2010). During an outbreak, it is likely that ranavirus infects hosts via multiple routes of horizontal transmission; although vertical transmission of iridoviruses has been shown in invertebrates (Hunter *et al.* 2001), it has not been demonstrated for ranaviruses infecting vertebrates (Drennan *et al.* 2006). Attempts to test for vertical transmission have yielded mixed results (Brunner *et al.* 2004, Duffus *et al.* 2008).

In parallel, land use modification and habitat fragmentation can alter host metapopulation dynamics and gene flow resulting in host genetic diversity depletion (manuscript 1) and potentially and higher sensitivity to perturbations including ranavirus infection (manuscript 1, Pearman *et al.* 2005). Ranavirus-induced mortality is rare in adult amphibians whose immune system is more developed than in larvae (Robert *et al.* 2005, Miller *et al.* 2011) and Susceptibility of larvae to ranavirus varies depending on the developmental stage of the

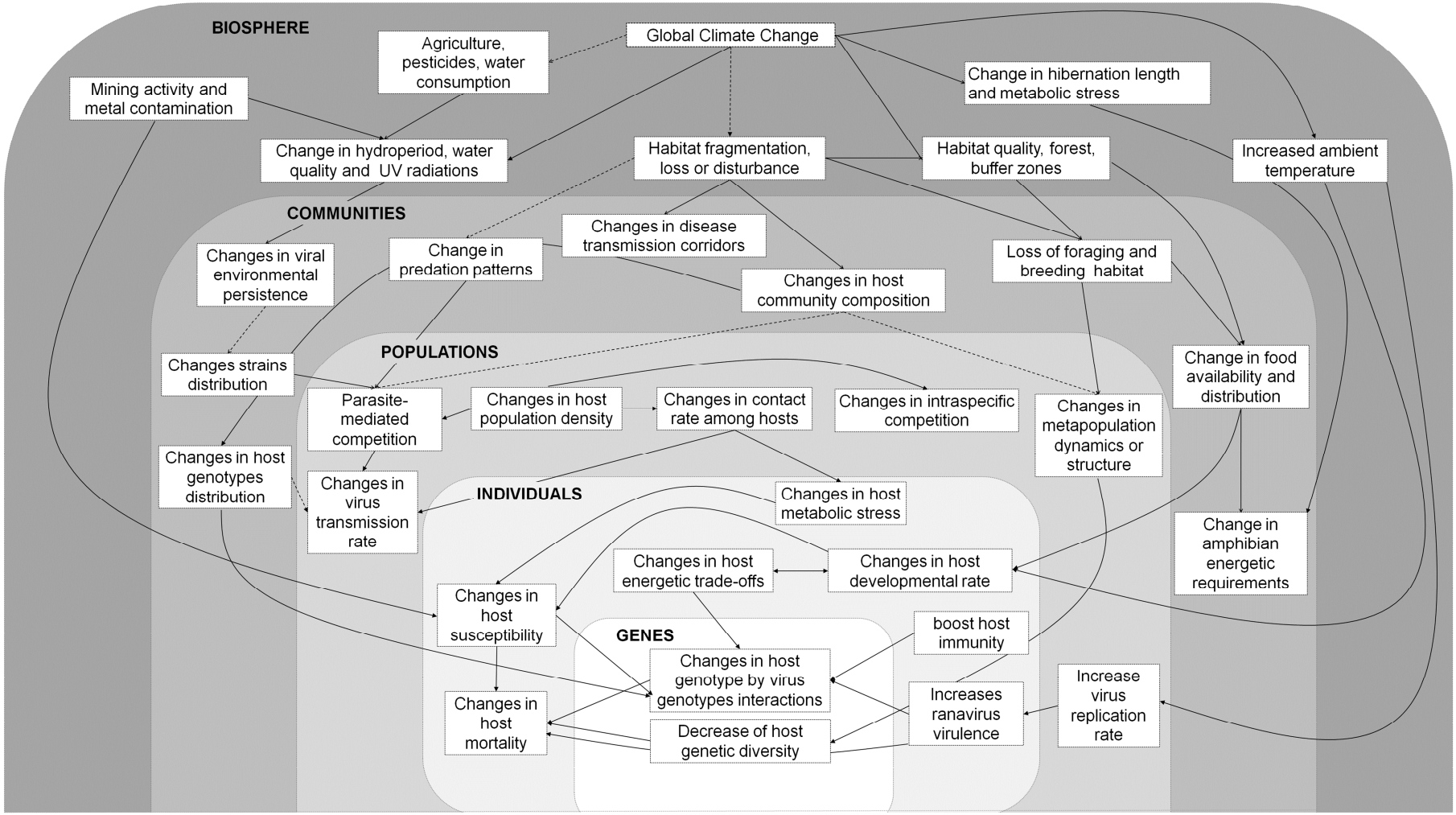

Fig 3. A causal diagram describing direct (solid arrows) and indirect (dashed arrows) effects and synergies of ranaviral disease determinants in amphibians. The figure illustrates the hierarchy of host and environmental factors that must be considered when investigating disease emergence, ranging from the level of the pathogen and host genotypes through individual hosts, populations, communities, landscape variables, and biosphere

larvae (Haislip *et al.* 2011, manuscript 4). The maturation of the immune system together with the number and severity of virus exposures influence the severity of the resulting disease (manuscript 4). Temperature increase is likely to modulate host developmental rate, immune response potential and genotypic interactions with frequent ranavirus strains resulting in non-trivial infection outcomes (manuscript 2).

The described investigations help to identify and validate potential causal pathways of ranaviral disease emergence, for most of them across several levels of the biological organization but many questions remain unanswered. In particular, clarifying the role of eco-evolutionary feedbacks between ecological and evolutionary determinants of ranaviral disease in a broad environmental context is required to assess hypotheses, and eliminating them whenever possible. The strong inference approach, concomitant of the application of the eco-evolutionary framework proposed, will eventually allow the examination of "parent" variables (such as land use and climate change), and ultimately lead to a better understanding of how ecological change drives disease emergence.

Host-parasite evolutionary ecology, towards a new paradigm?

Community ecology and evolutionary biology are disciplines typically studied in relative isolation from one another. Community ecology investigates how interactions among species and their environment affect the abundance, distribution, and diversity of the component species with limited reference to genetic variation and evolutionary change within species (but see landscape genetics, Manel *et al.* (2003). In contrast, evolutionary biology considers genetic variation and the mechanisms that result in genetic and phenotypic change within populations, without much regard to the ecological constraints that all populations are subjected to. Although there is a long tradition within evolutionary biology of investigating the effects of proximate ecological factors (phenotypic plasticity is an example), the role of the community in affecting evolutionary patterns, and vice versa, has received little attention. In fact, recent reviews at the frontier between community ecology and evolutionary biology stress the need for a new synthesis extending the current framework of evolutionary ecology to envelop community ecology. For instance, it is clear that in any community, the genetic potential within even one species can affect the ecological dynamics of the whole community and alternatively, community dynamics can govern evolutionary processes and patterns (Johnson and Stinchcombe 2007).

Our opinion is that a conceptual bridge between evolution and ecology even at the community level is particularly relevant when studying H-P interactions. In essence, we consider that mechanisms critical to H-P interactions (e.g., GxG, trade-offs, dilution effect, and parasite-mediated competition) occur at all scales of biological organization. Each level at which a given functional mechanism occurs may be affected more directly by ecological vs evolutionary processes. From the molecular scale of genotypic interactions to community-based processes, the ecological and evolutionary influences follow an antagonistic pattern. The intense selective trade-offs so often observed at the scale of genotypic interactions become more and more diluted by ecological influences as the biological realm broadens. The functional mechanisms here described have been traditionally investigated under the conceptual frameworks within which they originated. But our opinion is that the occurrence of synergies among these mechanisms augurs against conceptual compartmentalization and suggest an integrative approach is more appropriate, particularly for the study of H-P interactions. The knowledge available with regards to H-P interactions outcomes determinants that reveal bidirectional feedbacks among ecological and evolutionary processes suggest the relevance of a functional eco-evolutionary mosaic framework that incorporates community processes. As a corolary, the reductionist approach seems to have reached its limit and molecular studies are now able to relate back to the phenotype and address the relationship between gene, organism, and environment (Singh 2003). Barriers between disciplines also tend to be broken by an increasing degree of multidisciplinary collaboration. Such collaborations are also efficient in that they minimize methodological issues, as well as time and financial effort, to address broader research questions. Both these conceptual and methodological advances suggest that integrative approaches to investigate H-P interactions could, and even should, be the rule rather than the exception and will prove to be particularly valuable for the more applied investigations of Emerging Infectious Disease in the wild.

Finally, we believe that H-P evolutionary ecology as a field of research has now itself evolved to a degree of maturity where it can reach out to a novel paradigm which could unite evolutionary biology and community ecology.

Acknowledgements

We would like to thank Thierry Lefèvre for comments on an earlier version of this manuscript. This work was supported by the Natural Science and Engineering Research

Council and the Canadian Fund for Innovation/Ontario Innovation Trust to DL. The authors have declared that no competing interests exist.

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Manusript 6

Publication trends in Host-Parasite Evolutionary-Ecology: conceptual shifts and syntheses

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Contributions:

PE: design, data collection, analyses and writing

DL: analyses and writing

Abstract

The methodology associated with bibliometrics has been used to describe the evolution of a discipline and to document and monitor changes in science through output and citation analysis. At the same time, in the fields of ecology and evolution a synthesis linking community ecology and evolutionary biology is emerging but is still in its infancy.. Among all the potential research areas where the merging of the two fields could be examined, host-pathogens (HP) studies have been argued to be an ideal model:the strength and specificities of the selective pressures involved in a given interaction may promote the an investigation of the interplay between evolution and ecology. The purpose of the present study was to assess the tendency for ecological and evolutionary concepts to be published in tandem in HP studies and to underline the prominent role HP studies might have to promote such a synthesis of the two fields. To do this, a bibliometric analysis of the fields of Evolution (Ev), Ecology (Ec) Evolutionary Ecology (EvEc), and Host-parasite Evolutionary Ecology (HPEvEc) was conducted using indexed citations from the ISI web of Knowledge database.

Our analysis revealed that, in contrast with the three other studied fields, the output in HPEvEc publications is primarily growing in four ways: (1) in the number of HPEvEc indexed articles; (2) in the number of journals publishing work related to HPEvEc; (3) in the reach, quality and visibility of HPEvEc published articles (as measured by impact factor); and (4) in the number of reviews vs. research articles published in the field.

Consequently, we suggest that HPEvEc research is currently experiencing a marked maturity process whereby HP systems are relevant candidates to investigate and further achieve the synthesis of ecology and evolutionary biology.

Introduction

Bibliometrics is defined as “the use of statistical methods in the analysis of a body of literature to reveal the historical development of subject fields and patterns of authorship, publication and use” (Young and Belanger 1983). It offers a powerful set of methods and measures for studying the structure and process of scholarly communication (Borgman and Furner 2002). Traditionally, bibliometricians have concentrated their efforts on tracking visible and objective indicators of scholarly activity such as publications and citations, but the techniques have recently been applied to other outputs and processes illustrating the productivity of science (journal impact, federal funding, etc. ; Cronin 2001). As scientific publication is increasingly moving towards an online media, its attributes (citations number, number of readers, impact factors, Eigen factors., etc.) become progressively more available for investigation. Bibliometrics is therefore more than ever a relevant analytical tool for the investigation of trends within research areas. As Cronin (Cronin 2001) suggested: “the age of bibliometric spectroscopy is dawning” and the method is now steadily increasing in relevance with the potential to reach many scientific fields.

Bibliometrics methods have been used in a wide range of disciplines including psychology, pharmacology, health, education and medical informatics to describe the research and evolution of a discipline through output and citation analysis (Schloman 1997, Moorman and van der Lei 2003, García-García *et al.* 2008, Deshazo *et al.* 2009). To some extent, bibliometrics and the mapping of science have been logically suggested to be relevant approaches to document and monitor revolutionary changes in scientific areas in real time, thus quantifying potential paradigm shifts (Small 2003).

The fields of ecology and evolutionary biology are intricately linked together by shared concepts and ideas and parallel historical development (Collins *et al.* 1986). While “nothing makes sense in biology except in the light of evolution” (Dobzhansky 1973), evolution can in turn only be understood within the environment in which evolution occurs, suggesting that ecological understanding is a prerequisite to the understanding of evolution. The field of evolutionary ecology is at its core the study of variation within individuals, among individuals, and among populations and species, taking into account both the genetic constitution of individuals and the environment in which the individual lived. Evolutionary ecology essentially focuses on individual-centered interactions for which the investigation of phenotypic variation has been used as a proxy to capture the underlying genotypic variation and understand evolutionary dynamic. The emergence of key concepts such as trade-offs and

phenotypic plasticity results from strong conceptual combinations of ecological and evolutionary backgrounds. Despite a beneficial and legitimate interaction between ecology and evolution for the investigation of individuals' life history, a similar synthesis is lacking when it comes to the study of communities (Johnson and Stinchcombe 2007). In fact, community ecology and evolutionary biology are disciplines typically studied in relative isolation from one another (Johnson and Stinchcombe 2007). However encompassing community interactions and evolutionary processes together may provide new insight into questions typically asked by ecologist and evolutionary biologists.. Among all the potential candidates to demonstrate a synthesis of evolutionary and ecological concepts, host-parasite (HP) studies have been proposed to be an ideal model because the investigation of these systems requires the incorporation of both ecological and evolutionary influences (Thomas *et al.* 2009, Echaubard *et al.* unpublished). The strength and specificities of the selective pressures involved in a given interaction may promote rapid evolution, within an ecological timeframe, thus allowing the interplay between evolutionarily and ecologically processes to be more clearly noticeable (Neuhauser *et al.* 2003). Such a convergence between evolution and ecology, renders H-P interactions outcomes context-dependent and therefore very dynamic over time and space, fluctuating along a continuum ranging from mutualism to strict parasitism depending on given ecological conditions (Renaud and De Meeus 1991).

In ecology and evolution, meta-analyses (the sub-discipline of statistics that is designed for summarizing and analysing multiple independent studies) are used extensively (Arnqvist and Wooster 1995, Leimu and Koricheva 2005) to document general conclusions with regard to current theories within a field (Arnqvist and Wooster 1995, Leimu and Koricheva 2005). In comparison, analyses of citations and publication trends can be considered as external investigations of published work in order to detect numerical tendencies at a larger scale and to help ascertain key conceptual directions emerging from theories. While a reasonable number of bibliometric studies have appeared in the ecology-evolutionary biology literature (see Graham and Dayton 2002, Neff and Corley 2009), to our knowledge no studies have investigated the occurrence, based onpublication trends, of a synthesis between ecological and evolutionary concepts, and more particularly the potential leading role of H-P studies for the merging of these concepts.

Using a bibliometric analysis, the objectives of this study were (1) to document the tendency for ecological and evolutionary concepts to be published jointly and (2) to underline the prominent role of HP studies to promote such integration. We addressed the following questions to achieve our objectives:

1- Are there any differences in publication growth rate between the fields of evolutionary-ecology, ecology, evolutionary-biology and HP evolutionary ecology?

2- To what degree do publication trends in HP evolutionary ecology as opposed to evolutionary ecology in general exhibit linear or exponential growth?

3- What type of journals publish HP evolutionary ecology papers?

4- Is there a trend in HP evolutionary ecology research suggesting a maturity process and a wider audience?

Material and Methods

1. Search topics and relevance of the procedure.

For all bibliographic searches we used the academic citation indexing service ISI Web of Knowledge provided by Thomson Reuters. In October 2010, we searched for all documents assigned to terms related to the four topics of interest: “Evolution (Ev)”, “Ecology (Ec)”, “Evolutionary-Ecology (EvEc) and “Evolution and Ecology of HP systems (HPEvEc)”, for each year of from 1990-2009. In order to obtain the most accurate and relevant output from ISI Search Services for each of these topics, we used the following search criteria: “ecolog* NOT evolution*”; “evolution* NOT ecolog*”; “ecolog* and evolution*; and “(parasite* OR pathogen* OR disease*) AND ecolog* AND evolution*” for Ec, Ev, EvEc and HPEvEc respectively. All these requests were restricted to subject areas such as “Environmental science and Ecology” for “Ec ”, “Evolutionary Biology” for “Ev” or both subject areas for “EvEc” and “HPEvEc”. This procedure was used to better target relevant publication spheres thus avoiding out of topic published work (e.g. evolution of economic growth rate). The “non-specific” attribute (*) broadened the search to any words that include the root of the word written before * (*i.e.* ecolog* will return occurrences for ecology, ecological and ecologically). We described the literature using ISI Journal Citation Report and the searching algorithm of ISI Web of Knowledge. Although ISI does not necessarily include all journals in all fields, it nevertheless includes journals that contribute the most to the diffusion of high-standing scientific research and therefore can be considered as a highly relevant search engine for our study questions. We restricted our searches to the 1990-2009 study period and to key-words and titles which are the most consistent attributes for long-term bibliometric investigations (i.e. prior to 1995 no abstracts were usually available in the databases).

2. Growth rate

In order to assess the growth of the HPEvEc literature in contrast with the other fields, we calculated the annual growth rate (AGR) for the 1990-2009 study period for HPEvEc, EvEc, Ev and Ec. We defined AGR as the rate at which the number of the topic's publications increased yearly where AGR = (Current Year Total – Previous year Total)/Previous Year Total (Deshazo *et al.* 2009). Additionally, in order to investigate the temporal effect on the annual growth rate, we split the study period into decades (1990-1999 and 2000-2009) and included "Decadal Growth Rate" as fixed factor in a generalized linear model.

Several laws have been proposed to describe the dynamic of publication growth. For instance, in bibliometric analysis, it is common to statistically model the situation in which success breeds success (Price 1976).. Price's law following the so-called Cumulative Advantage Distribution (CAD) has been thus proposed to appropriately model bibliometric and diverse social science phenomena. Moreover, such a model has been shown to represent an appropriate underlying probabilistic theory for the Bradford law of publication and citation analysis (Price 1976). The CAD is governed by the Beta Function which may model a family of continuous probability distributions potentially mimicking an exponential type of growth. In order to assess whether the four topics' growth rate follows Price's Law, we fit their respective number of publications per year for the study period to both a linear and an exponential model. The goodness of fit of both models was evaluated by considering both the adjusted R^2 and the Akaike Information Criterion (AIC), which tests the significance of the difference between the functions of different model specifications (see Johnson and Omland 2004 for a review). Additionally, we computed a separate slope model to further investigate the significance of the differences among models that depict the topics' growth. We used "topics" as a categorical predictor and "year of publication" and "decades" as continuous predictors of publication counts. The separate slope design is more appropriate than a traditional analysis of covariance (ANCOVA) model for modeling the influences of the predictors' interactions on the response outcomes. The significance of the interaction between the categorical and the continuous predictors indicates significant slope differences between growth models.

3. Trends in journal and article types

In order to assess trends in journals, we applied Bradford's law of scattering to all journals publishing HPEvEc. Bradford's law estimates the exponentially diminishing returns of extending a search for references in science journals (Bradford 1934). In other words the

numbers of the groups of journals to produce nearly equal numbers of articles is roughly in proportion to 1: n: n2 …, where n is called the Bradford multiplier. Basically, Bradford's law states that a small core of journals has as many papers on a given subject as a much larger number of journals, n, which again has as many papers on the subject as n2 journals (Hjorland and Nicolaisen 2005). Although Bradford's law is not statistically accurate, librarians commonly use it as a guideline. We identified the number of journals publishing HPEvEc articles each year over the 1990-2009 period. We also investigated the number of individual journals publishing five or more HPEvEc indexed articles per year in order to document the growing relevance of HPEvEc for an increasing number of discrete subcategories of journals.

Part of our investigation included distinguishing between different types of published documents. In theory, a field characterized by a high proportion of reviews may indicate the time for new theories. Using the proportion of document types published annually, we compared the variation of the ratio of "research article" vs. "reviews" between the years 1990 and 2009 for each of the topics.

4. Impact factor

For each year from 1990 to 2009, we searched for the list of the top 25 journals (based on publication count) that published HPEvEc, EvEc, Ev and Ec papers. We then assigned each journal with their respective ISI annual Impact Factors. For each journal and for each year within each topic we also calculated a "Weighted IF" (W_IF) which takes into account the number of publications per journal. To do so, we divided the IF by the total number of papers for each journal for each year and then averaged the results. We then compared the Impact factor (IF) and the Weighted IF (W_IF) growth among topics, over the study period, using a separate slope model with "topic" as the categorical predictor and "year of publication" and "decade" as continuous predictors.

5. Statistical analyses

All statistical procedures were done using Statistica 8.0 (Statsoft 2007).

Results

The total number of publications obtained was 6,002, 48,293, 57,519, 15,117 for HPEvEc, EvEc, Ec and Ev respectively. In 1990 and 2009 there were 116 and 699 HPEvEc indexed

articles respectively indicating a 502% growth in annual publication over the 20-year time period. Over the same time period EvEc grew 94.6%, Ec 118% and Ev 255%.

1. Growth rate comparison and type of growth

HPEvEc indexed articles grew by an average of 15.13% each year over the study period (1990-2009) in comparison to the other fields of publication investigated (12.43%, 7.27% and 10.76% for EvEc, Ec and Ev, respectively)**.**The analysis also detected a significant interaction between decade and growth rate. For the 1990s, HPEcEv growth was significantly higher than the other fields (17.03% vs 9.94%, 3.40% and 10.76% for EvEc, Ec and Ev, respectively; H=10.45, p=0.015*).* For the 2000s, the difference in growth rate between fields was not significant (H1.576, p =0.665) but the respective growth rate of evolutionary ecology fields (including HP) was still higher than individual fields (HpEvEc = 15.48%, EvEc = 15.83%, Ec = 11.78%, and Ev = 10.67%).

The distribution of HPEvEc publication counts appeared to fit an exponential growth curve rather than a linear growth curve (exponential growth: R^2 = 0.9005, p <0.0001, linear growth: R^2 = 0.7815, p <0.0001; Fig 1). The exponential growth curve explained 90% of the variance in HPEvEc publications over the 20-year time period, while the linear equation explained 78.15%. Furthermore, the AIC goodness-of-fit test identified the exponential model as the best fit to describe the data (exponential model: AIC= 16.48, linear model: AIC= 388.68). We also observed that EvEc, Ec, and Ev growth ratesfollowed an exponential pattern (R^2: 0.897; 0.715; 0.991 for EvEc, Ec, and Ev, respectively ; p<0.0001 in all cases) which was confirmed by AIC scores to be the most relevant model fitting each fields' growth rate. When partitioning the growth dynamic for each decade of the study period, we observed that for all fields but Ev, the 2000s period was the most productive (Table 1), with a clear exponential growth for HPEvEc especially. Interestingly for the 1990s, Ec and Ev were in great contrast:Ev increased exponentially while Ec was barely growing. The separate slope model confirms the significance of the differences mentioned (Field*Year: X^2 = 1224.2, p < 0.001; Field*Decade: X^2 = 852, p < 0.0093; Topic: X^2 = 3.913, p = 0.271031).

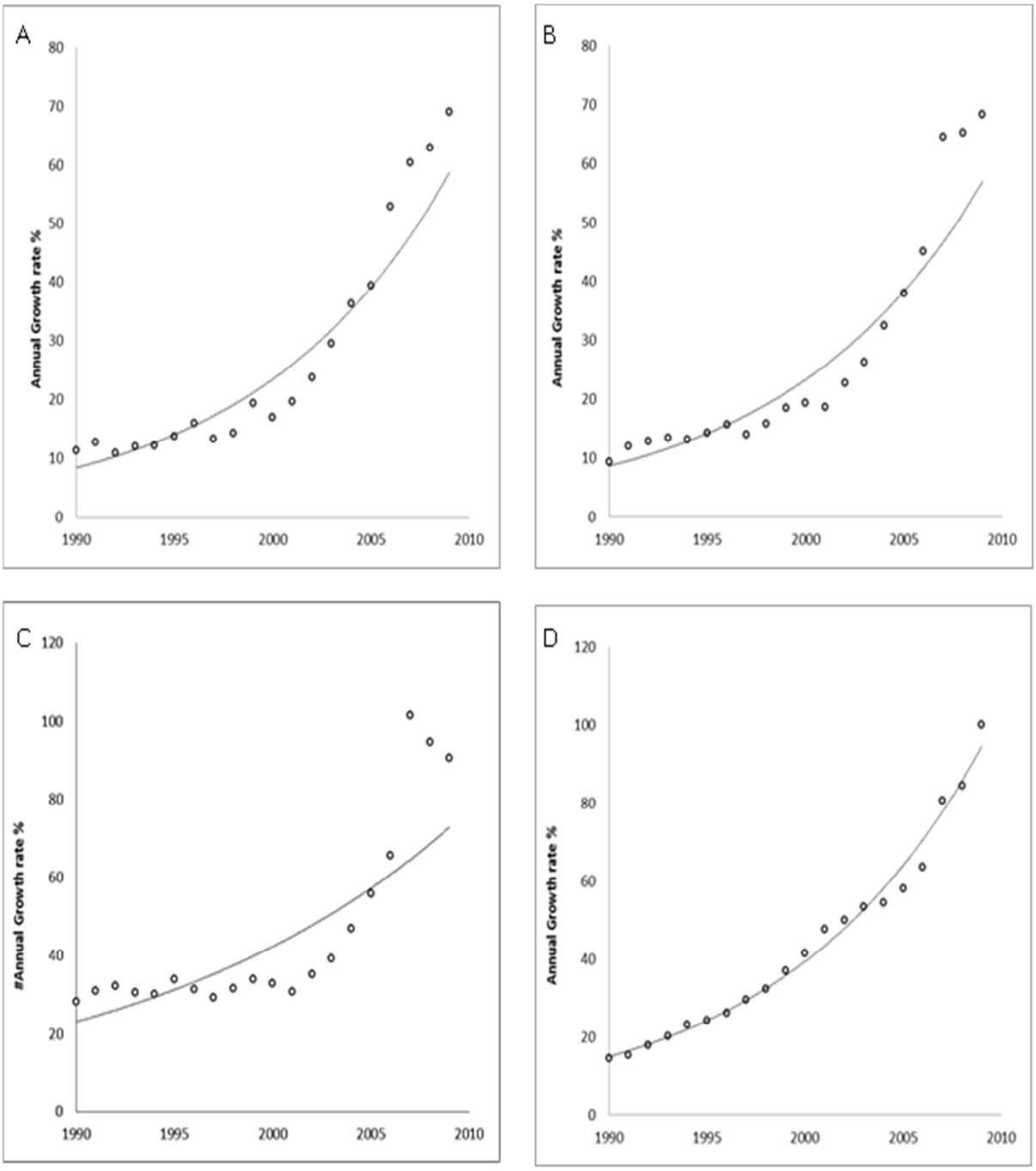

Fig. 1. Trends in annual growth rate for indexed articles in A. HPEVEc, B. EvEc, C. Ec and D. Ev.

Table 1.Summary of adjusted R^2 fitting exponential/linear growth by decade. Degrees of significance are * for p<0.05, ** for p <0.005 and *** for p<0.0005, NS = not significant

Field	*1990s*	*2000s*
HPEvEc	$0.558^{*}/0.5386^{*}$	$0.9783^{***}/0.9692^{***}$
EvEc	$0.7841^{**}/0.7854^{**}$	$0.9645^{***}/0.913^{***}$
Ec	$0.2391^{NS}/0.2402^{NS}$	$0.9186^{***}/0.8546^{***}$
Ev	$0.9913^{***}/0.9772^{***}$	$0.938^{***}/0.881^{***}$

2. Fluctuations in journal selection over time

The total number of unique journals (journals appearing every year are only counted once) over the twenty year period was 223, with an average of 2.54 HPEvEc-related papers per journal per year. We applied Bradford's Law of scattering to the data related to HPEvEc, and divided the output frequency of ranked journal into three groups representing approximately 1/3 of the 6002 articles published over the study period. Only 4 journals (Evolution, Molecular Ecology, the American Naturalist and Journal of Evolutionary Biology)represented one third (2000) of the total published HPEvEc articles. In comparison, 22 and 197 represented the tier two and three (Table 2).

Table 2. Results of Bradford's Law of scattering for the data related to HPEvEc published papers.

Field	***Journals***	***Articles***	***Cumulative total***
Top	4/1.80%	2000/33.32%	2000
Middle	22/9.86%	2012/33.52%	4012
Bottom	197/88.34%	1990/33.15%	6002
Total	223/100%	6002/100%	6002

The number of journal publishing HPEvEc papers increased over the study period from 54 in 1990 to 273 in 2009 (Fig. 2). The top five journals in 2009 ranked by citation count were Infection Genetics and Evolution (19), Proceedings of the Royal Society of London B (PROCS-B) and Molecular Ecology (17), PLoSOne (16), and American Naturalist (15; Table 3). These journals were particularly devoted to publishing evolutionary biology and ecology related work. We also identified only two journals publishing five or more HPEvEc indexed articles in 1990, while in 2009 there were 31. Also, within the 25 top journals (based on publication counts) in 1990, seven of them focused specifically on parasitology (International Journal for Parasitology, Journal of Parasitology, Parasitology, Parasitology Today, Annales de Parasitologie Humaine, Parazitologiya and Plant Pathology), while in 2009 only two of them were specifically focused on parasitology (Parasitology and Virus Research).

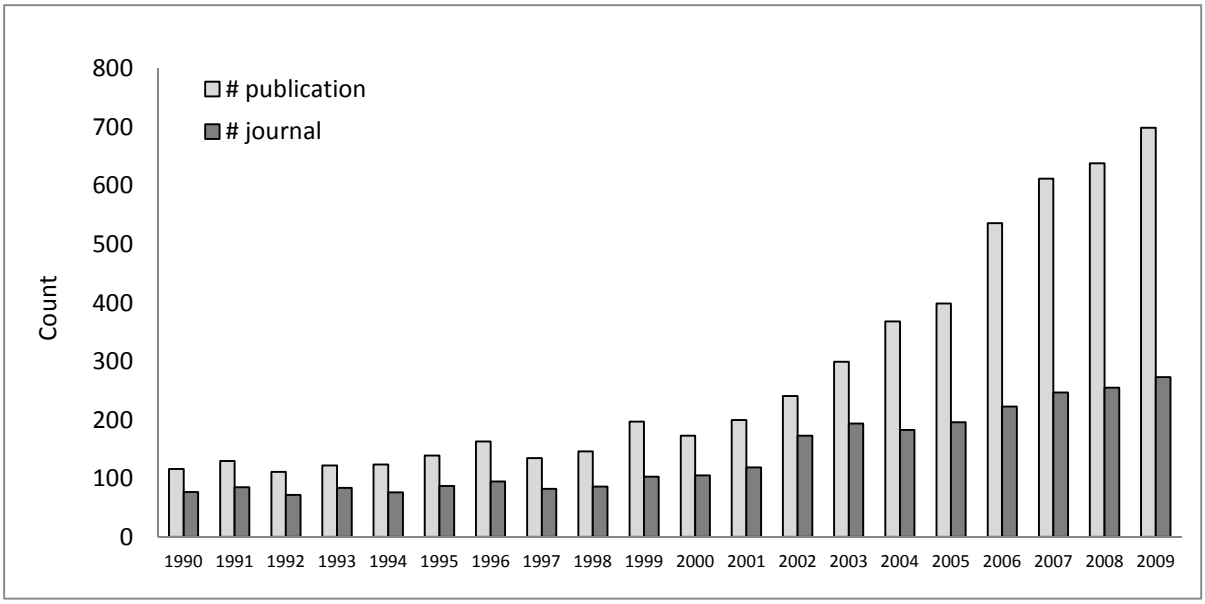

Fig 2. Trends in HPEvEc publication output over the study period by numbers of articles and number of journals which publish HPEvEc articles.

Table 3.Number of HpEvEC papers by journal and the percentage of the total number of papers published in 2009 (n=699)

Source Title	**# of HPEvEc papers**
INFECTION GENETICS AND EVOLUTION	19 (2.72%)
MOLECULAR ECOLOGY	17 (2.43%)
PROCEEDINGS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES	17 (2.43%)
PLoS ONE	16 (2.29%)
AMERICAN NATURALIST	15 (2.15%)
BMC EVOLUTIONARY BIOLOGY	13 (1.86%)
EVOLUTION	13 (1.86%)
ECOLOGY	11 (1.57%)
PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA	11 (1.57%)
BIOLOGICAL JOURNAL OF THE LINNEAN SOCIETY	9 (1.29%)
EVOLUTIONARY APPLICATIONS	8 (1.14%)
JOURNAL OF EVOLUTIONARY BIOLOGY	8 (1.14%)
NATURWISSENSCHAFTEN	8 (1.14%)
BEHAVIORAL ECOLOGY	7 (1.00%)
CURRENT BIOLOGY	7 (1.00%)
ISME JOURNAL	7 (1.00%)
JOURNAL OF THEORETICAL BIOLOGY	7 (1.00%)
PARASITOLOGY	7 (1.00%)
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES	7 (1.00%)
SCIENCE	7 (1.00%)
VIRUS RESEARCH	7 (1.00%)
BMC GENOMICS	6 (0.86%)
FARMING HUMAN PATHOGENS: ECOLOGICAL RESILIENCE AND EVOLUTIONARY PROCESS	6 (0.86%)
TRENDS IN ECOLOGY & EVOLUTION	6 (0.86%)
ANNUAL REVIEW OF ECOLOGY EVOLUTION AND SYSTEMATICS	5 (0.72%)

A comparison between 1990 and 2009 of the ratio of document types published in each of the fields showed that in 1990, an average of 84.52% of all work in all fields, was published as research articles and only 5.83% were reviews. In 2009, this ratio slightly decreased with 77.91% published as research articles vs. 8.77% as reviews. For both years, HPEvEc was characterized by the highest ratio of reviews to research articles in comparison to the other fields with 8.62% of all published work in 1990 (against 6.11%, 1.71% and 6.90% for EvEc, Ec, and Ev, respectively) and14.14% of all published work in 2009 (against 9.15%, 2.65% and 9.15% for EvEc, Ec, and Ev, respectively).

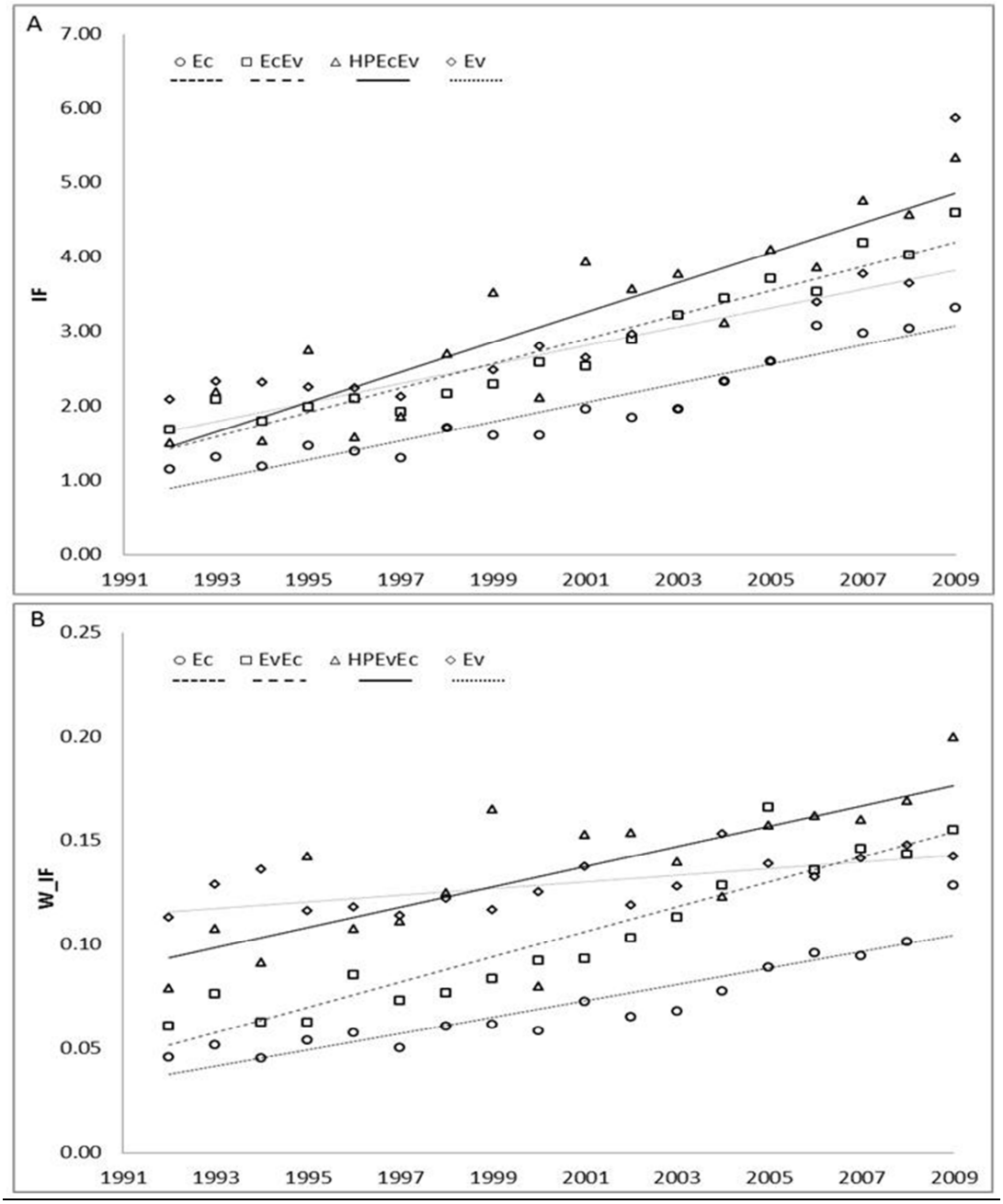

Fig. 3. Trends in A. annual Impact Factors (IF), and B. Weighed Impact Factors (W_IF) growth for each topic area over the 1990-2009 period.

3. Impact factor

Impact factors (IF) were identified for the 25 top journals (based on the number of publications) publishing HPEvEc research for each year of the study period. In 1990, the impact factor (IF) was 1.50, showing little increase during the first decade (2.12 in 2000). After 2000, we observed a continuous increase from 2.12 up to 5.34 in 2009. From 1990 to 2009, the impact factor of journals publishing HPEvEc studies grew by 256%. In comparison, Ec, Ev and EvEv articles were published in journals that were characterized by significantly smaller growth in their impact factors (180.8%, 187.9%, and 173.7%, respectively; separate slope model, "topic *year": $X^2 = 668.3$, $p < 0.001$; "topic*decade": $X^2 = 623.52$, $p < 0.001$; Fig. 3). Slightly different trends were observed for the weighed impact factor (W_IF). HPEvEc overall W_IF was significantly higher than for the other fields except Ev (0.072, 0.13, 0.104 and 0.135 for EC, Ev, EcEv, and HPEvEc, respectively; $X^2 = 11.05$, $p = 0.01$; Fig. 3) but its growth rate was similar to that of Ec and slightly lower than that of EvEc (154.7, 26.33, 179.13 and 152.67, respectively for Ec, Ev, EvEc and HPEvEc; $X^2 = 105.76$, $p < 0.001$; Fig. 3).

Table 4. Number of HPEvEc articles published in high impact factor (IF) journals (IF ≥15) per year.

Year	Journals (IF)	# of articles	Of total #	Ratio (%)
2008	NATURE (31.43)	5	638	0.78
2007	NATURE (28.75)	5	612	1.63
	NATURE Rev. microbio. (14.9)	5		
2006	NATURE (26.68)	4	536	0.75
2005	NATURE (29.27)	4	399	1.01
2004	none	0	368	0
2003	NATURE (30.97)	3	299	1
2002	SCIENCE (26.68)	3	241	1.24
2001	SCIENCE (26.58)	5	200	2.5
2000	none	0	173	0
Total		**34**	**3466**	**0.98**
1999	NATURE (29.49)	5	197	2.54
1998	none	0	146	0
1997	none	0	135	0
1996	none	0	163	0
1995	NATURE (27.07)	4	139	2.88
1994	none	0	124	0
1993	none	0	122	0
1992	none	0	111	0
1991	none	0	130	0
1990	none	0	116	0
Total		**9**	**1383**	**0.65**

In parallel we were also interested in documenting, if any journal among the 25 top ones would fit into the category of very high impact factor with an impact factors higher than 15.

We observed that such journals were mostly represented by the generalist publications, Nature and Science which had impact factors above 25. We noticed that there was a tendency between 2000 and 2009 for an increase in the number of HPEvEc publications in these journals with 34 papers published (0.98 % of the total number of articles). In contrast, only 9 HPEvEc papers were published (0.65 % of the total number of articles) in Nature and Science during the previous decade (Table 4).

Discussion

1. Topics' growth over time

Over the past 20 years, the specific field of Host-Parasite Evolutionary Ecology research (HPEvEc) was characterized by a higher average annual growth rate in publications than Evolutionary Ecology (EvEc), Ecology (Ec), and Evolutionary biology (Ev). This suggests that HP investigators tend to increasingly include a combination of ecological and evolutionary concepts in their studies. This supports the relevance of the HP system for concerted investigations involving both evolutionary and ecological theory. In other words "host-parasite investigations evolved from a basic and descriptive approach to the nowadays very conceptual discipline shaped by evolutionary-ecology" (Poulin 2007). We also observed a change in the relative growth rate differences through time. During the 1990s, HPEvEc publication growth rate was the highest of all fields investigated. During the 2000s, this difference was no longer observed as both evolutionary-ecology fields (HPEvEc and EvEc) were characterized by similar growth rates, albeit rates higher than the only ecology and only evolution fields (Ev and Ec). Three trends were therefore apparent. First, all fields but Ev showed a growth rate increase between the 1990s and the 2000s. Second, there was an initial tendency for HP investigations to incorporate evolutionary and ecological concepts to a greater extent than actual EvEc and the other fields suggesting the appropriateness and relevance of HP systems in encouraging the use of both ecological and evolutionary frameworks. Third, the absence of significant differences in growth rate over the last decade suggests that the leadership and relevance of the conceptual direction took by HP research a decade earlier.

Additionally, HPEvEc had the highest growth rate in the 90s but with noticeable variability from year to year. In the 2000s this field still increased but in a more stable fashion, resulting in a better fit to growth curves. The same dynamic was observed for EvEc but with a lower average AGR. With regard to Ec, the low fit to either a linear or an exponential growth curve

in the 90s may be explained by the low average AGR observed and the high variability of AGR scores from year to year. The growth rate for this field increased significantly in the 2000s, potentially explaining the better fit to the exponential curve. With regard to Ev, it had the second highest average AGR in the 90s and was characterized by a clear increase during this period resulting in a good fit to the exponential and linear growth curve. In the 2000s however, there was almost no increase of the average AGR for Ev resulting in a poorer fit to the growth curves. These observations may indicate that EvEc, Ev and particularly HPEvEc have been rapidly growing fields of research.

2. Fluctuations in journal selection over time

The important increase in the number of journals publishing HPEvEc research illustrates the overall popularity of investigating HP systems using a combination of ecological and evolutionary concepts. The increasing range of journals publishing articles in the field supports the idea of a diversification of publication targets in HPEvEc with more ecology or/and evolutionarily-oriented journals.

An increase in the relevance of the field of HPEvEc is evident by a 15-fold increase in the number of journals having at least five HPEvEc publications per year over the last 20 years. For an increasing number of journals, inclusion of HPEvEc articles reflects a real commitment towards the investigation of HP systems within an evolutionary ecology framework. The tendency of more journals with a conceptual, rather than descriptive, orientation publishing HPEvEc articles, as represented by the Bradford's law 1st tier journals (Evolution, Molecular Ecology, the American Naturalist and Journal of Evolutionary Biology), also emphasizes the maturation process of HP evolutionary ecology where theories are challenged and actively updated. At the same time, we noted that both evolutionary ecology and evolutionary biology alone stimulate production of an important volume of conceptual reviews and syntheses. This may be due to a recognition of the part of the research community to provide sound and highly-documented theories in these fields. Interestingly, HPEvEc as a particular field within evolutionary-ecology theory is characterized by an even greater proportion of reviews suggesting a higher demand for conceptual reflection and synthesis.

3. Impact factor

Impact factors have long been used as a proxy for journal excellence and to estimate author research quality. However this metric has often been used too reductively (Amin and Mabe 2000). Many subjective aspects emerge from calculated impact factors that may bias the community opinion on a given journal. It is necessary to address the fact that generalist and theoretical journals most often present higher average impact factors than specialized or applied journals, although this difference is not reflected in the overall scientific disparities between those journal categories. Furthermore, the impact factor should not be used without careful attention to the many phenomena that influence citation rates, as for example the average number of references cited in the average article (Amin and Mabe 2000). With this in mind, our results suggest a steadily growing visibility, breadth and attention to HPEvEc articles over the last 20 years, in significant contrast to the other topics. Additionally, the overall increase of impact factors observed, particularly during the 2000s, may be explained at least partially by the recurrent appearance and dominating effects of very high impact factor journals such as Science and Nature. Interestingly, this observation underlines further the increasing significance of publication in HPEvEc. However, considering the rather stable number of HPEvEc papers published yearly in Nature and Science (4±1), in the 2000s, we suggest that the constant increase of the calculated impact factor of journals that publish HPEvEc is not the result of a few publications in very high impact journals but rather the consequence of an increasing tendency of HPEvEc articles to be published in specific journals with good (but not as high) impact factors (e.g. Evolution, Trends in Ecology and Evolution, American Naturalist, Proceedings of the Royal Society of London B-Biological Sciences, etc.).

4. Conclusion

By describing and analyzing the peer-reviewed literature in the general and specific fields of Ecology (Ec), Evolutionary biology (Ev), Evolution Ecology (EvEc), and Host-Parasite Evolutionary Ecology (HPEvEc), our objective was to document conceptual changes that may have occurred during the last two decades in these areas of research. We compared the publication count growth rate and type of growth, journal range expansion, impact factor evolution, and the type of paper (research articles vs reviews) published during the last two decades in each of these four fields in order to illustrate the current conceptual revolution we suspected was occuring. More specifically, we wanted to highlight that host-parasite systems

are particularly relevant to provide evidence of a synthesis between evolutionary ecology and evolution.

We observed an overall tendency for steady growth of publication count of the fields under investigation, and particularly so for evo-eco fields. Our analysis revealed that the output in HPEvEc is primarily growing across four dimensions: (1) the number of HPEvEc-indexed articles, (2) the number of journals publishing work related to HPEvEc, (3) the quality and visibility of HPEvEc published articles (as measured by impact factor), and (4) the number of reviews vs. research articles published in the field. By contrast, the three other fields that we investigated presented less remarkable growth in these four dimensions, particularly with respect to the isolated ecology and evolution fields. From these observations we conclude that during the last two decades there has been a growing tendency to use a combination of ecological and evolutionary concepts, which highlight the developing synthesis between ecology and evolution. More specifically, a significant and remarkable growth rate increase of HPEvEc research output illustrates the overall appropriateness of using HP systems when considering the merging of evolution and ecology. Our results also strongly support the assumption that HPEvEc research is currently experiencing a marked maturation process whereby HP systems are relevant candidates to investigate and further achieve the evolutionary ecology synthesis. Along with current multidisciplinary trends and resulting holistic approaches, the current conceptual dynamic taken by HPEvEc research positions itself at the edge of scientific excellence among the other “ecological” disciplines and promises exciting discoveries to come.

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