Epidemiology of the amphibian pathogen *Batrachochytrium dendrobatidis*, across multiple spatial scales

by

Kirsten Marie McMillan

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy (PhD) in Boreal Ecology

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

"The pathogen is nothing. The terrain is everything."

Louis Pasteur (1822 – 1895)

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis Titre de la thèse	Epidemiology of the amphibian pathogen Batrachochytrium dendrobatidis, across multiple spatial scales
Name of Candidate Nom du candidat	McMillan, Kirsten Marie
Degree Diplôme	Doctor of Philosophy
Department/Program Département/Programme	Boreal Ecology
Date of Defence Date de la soutenance	June 15, 2017

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. David Lesbarrères
(Co-Supervisor/Co-directeur de thèse)

Dr. Trenton W.J. Garner
(Co-Supervisor/Co-directeur de thèse)

Dr. Albrecht I. Schulte-Hostedde
(Committee member/Membre du comité)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
Monsieur David Lesbarrères
Dean, Faculty of Graduate Studies
Doyen, Faculté des études supérieures

Dr. Robert Puschendorf
(External Examiner/Examinateur externe)

Dr. Andrew J. Tanentzap
(Internal Examiner/Examinateur interne)

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

I, **Kirsten McMillan**, hereby grant to Laurentian University and/or its agents the non-exclusive license to archive and make accessible my thesis, dissertation, or project report in whole or in part in all forms of media, now or for the duration of my copyright ownership. I retain all other ownership rights to the copyright of the thesis, dissertation or project report. I also reserve the right to use in future works (such as articles or books) all or part of this thesis, dissertation, or project report. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that this copy is being made available in this form by the authority of the copyright owner solely for the purpose of private study and research and may not be copied or reproduced except as permitted by the copyright laws without written authority from the copyright owner.

ABSTRACT

Emerging infectious diseases are increasingly recognized as key threats to wildlife. *Batrachochytrium dendrobatidis* (*Bd*), the causative agent of chytridiomycosis, has been implicated in mass mortalities, population declines, and local and global extinctions of many species of amphibians around the world. As such, it is currently the largest infectious disease threat to biodiversity. Understanding the distribution and spatial dynamics of *Bd* is crucial to predicting spread to new geographic areas, revealing the history of infection, and developing appropriate management strategies. One of the most striking features of *Bd* is the variability in outcome of infection that has been observed within a species, among populations. By identifying and comparing differences in variables that co-vary between populations exhibiting different infection characteristics, we can start to disentangle the mechanisms allowing for parasite persistence and proliferation. However, infection dynamics operate across nested levels of biological organization: within-host processes underlie among-host processes within a population. As such, this thesis works within the classical themes of spatial epidemiology to consider: 1) the distribution of *Bd* and the evidence for spatial heterogeneity in both the prevalence and intensity of infection, and 2) the role of individual- and population-level traits in defining infection outcome.

The research presented, identifies that *Bd* functions endemically within *Rana pipiens* populations in Ontario. Outbreaks of chytridiomycosis are not observed, but infection dynamics show significant interannual fluctuations related to stable geographic factors and local climatic nuances experienced at particular host life history stages. However,

Rana pipiens also display variation in resistance to the pathogen, mediated by thermoregulation, dispersal behaviour, and phenotypic properties. Comparisons between host populations show variation in skin-associated bacterial communities, which may mediate susceptibility to chytridiomycosis. These bacterial communities are found to vary across latitude and between sites experiencing different levels of anthropogenic disturbance. Additionally, individual level traits, such as amphibian body temperature and body size are reported to influence bacterial community. Hence, this research highlights the importance of considering context-dependent individual- and population-level environmental heterogeneity, when attempting to predict the infection risk of *Bd*.

Keywords

Batrachochytrium dendrobatidis, *Rana pipiens*, Ontario, host-parasite interaction, life history, environmental heterogeneity, climate, phenotype, thermoregulation, dispersal, microbiome, host-resistance, ectotherm, Illumina sequencing, mixed-effects models, MCMCglmm.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisory team: David and Trent. David, without you I would never have seen Ontario. It truly is a beautiful province, and I feel very lucky to have had the opportunity to experience the best (and the worst) that Ontario towns and cities have to offer. Trent, thanks for all the whiskies, salted caramel chocolate puddles, hilarious and/ or tear-drenched chats, and for allowing me to run away when I needed to the most. James Oppenheim once said: "The foolish man seeks happiness in the distance, the wise man grows it under his feet". You helped me realise this – and now I'm happier than I've ever been. Thanks. To both of you, the road trip continues to scar me, and any image of Dolly Parton and/ or Dollywood still brings about red mist – however, it was an experience I will never forget. Additionally, I would like to thank my committee and all those involved in my comprehensive exam, including: Albrecht Schulte-Hostedde, Tom Johnston and John Gunn.

Xav, you have been more important in this process than you will ever know. In all sincerity, this PhD would never have been completed without your help. You have always made yourself available to me - for discussing ideas, chapter direction, research design and statistical help, despite being overwhelmingly busy yourself. You never made me feel foolish or shortsighted, despite me asking some ludicrous questions. You have taught me so much about focus and determination: and you gave me real guidance, strength and confidence when I needed it the most. For that, I will be forever grateful.

My works pals: Xav, Claresbear, Lols, Monni, Jon, Kevin, Franks, Matt, Stephen, Dada and Gonçalo – thanks for the wise words, reassurance, kind counsel, and for helping me

drown my stresses/ sorrows when needed most. Additionally, thanks to everyone involved in the field or laboratory work, including: Becca Grainger-Wood; Alice Fitch; Amanda Colles; Sean Boyle; Sam Cruickshank; Matt Perkins and Brandon Varela. In particular, thanks to Andrée-M. D'Aoust Messier who has been by my side from the very beginning. When I hurt my back, she ensured that I would still have a project by carrying on the fieldwork without me. Without you Andrée, these chapters wouldn't have been possible.

Thanks to my family and friends, who provided constant support and reassurance. YM and YP, you encouraged my scientific curiosity and love of the natural world from as early as I can remember. You put up with me wasting vast amounts of shampoo, toothpaste, toilet roll and talcum powder, just so I could create "espermints". You didn't panic when I brought home pink-winged stick insects, frogspawn, or even a rehomed corn snake. You let me spend 99 % of my childhood making dens in bushes and falling out of trees. In short, you encouraged me, to be me. Thanks.

Lisa and Carolyn: you are the best people I know. You are the strongest and most capable women I have ever met, and I couldn't be prouder to be your sister. Also, you have excellent husbands and children. Well done. Additionally, Lisa you deserve an enormous thank you for patiently sitting in front of R with me (for hours), teaching me how to use ggplot and introducing me to the world of Wes Anderson colour palettes. This thesis wouldn't be nearly as beautiful without your exceptional datavis expertise.

Red, with you, I have more fun than it makes sense for me to be having. You taught me to fill my life with adventure, not things. I look forward to making memories all over the world with you.

To my favourite ladies: Lindsay, Parks, Linzi, Christina, Louise, Annalise, Rachel, Sophie, Kirsten, Emily and Clare – you are all fantastic people, and I LOVE that you are in my life.

Finally, thanks to the Ontario Trillium Scholarship, which made this doctoral research possible. Also, thanks to the Laurentian University, the rare Charitable Research Reserve and the Institute of Zoology, Zoological Society of London, who all funded the work in Ontario, presented in this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: GENERAL INTRODUCTION

.....

1. 1 Emerging infectious diseases and mycoses

Recent studies suggest that infectious diseases in wildlife populations are emerging at unusually high rates (Harvell *et al*. 1999, 2002; Epstein 2001). Emerging infectious diseases (EIDs) are caused by pathogens that: (i) have increased in incidence, geography or host range; (ii) have changed pathogenesis; (iii) have newly evolved; or (iv) have been discovered or newly recognized (Lederberg *et al*. 1992; Daszak *et al*. 2000). In recent years, it has become increasingly apparent that EIDs pose a major threat to the conservation of global biodiversity (Harvell *et al*. 1999; Daszak *et al*. 2000; Cleaveland *et al*. 2001; Jones *et al*. 2008). Indeed, infectious diseases can mediate community dynamics, shrink host ranges (McCallum & Dobson 1995; Lafferty 2003; Walsh *et al*. 2003), and extirpate local populations (Holmes 1996; Daszak *et al*. 2000; Harvell *et al*. 2002). Traditionally, infectious disease was not considered a plausible cause of local extinction because, in many cases, the ability of pathogens to be transmitted between hosts was expected to reduce as the number of susceptible hosts decayed (McCallum & Dobson 1995). However, recent modeling of pathogens with multiple hosts, small host-population sizes, and biotic or abiotic reservoirs, have shown that extinctions of rare species caused by infectious disease may be more common than first considered (McCallum & Dobson 1995; De Castro & Bolker 2005; Crawford *et al*. 2010).

Relative to other types of pathogens such as bacteria, viruses, and protozoa, the number of emerging diseases caused by fungi, also known as mycoses, has risen

steeply during the last two decades (Fisher *et al*. 2012). However, the causes for this bias are unclear (Fisher *et al*. 2012). Mycoses were not previously considered a real threat to the persistence of populations, but this perception was recently felled due to the occurrence of several high-profile declines in wildlife caused by the emergence of previously unknown fungi (e.g. White-Nose Syndrome, see Blehert *et al*. 2009; Snake Fungal Disease, see Rajeev *et al*. 2009; Sea Fan Disease, see Smith *et al*. 1996 and Geiser *et al*. 1998). Among these emerging fungal diseases is the amphibian-killing chytrid fungus *Batrachochytrium dendrobatidis* (hereafter *Bd;* Longcore *et al*. 1999). This fungus, which causes amphibian chytridiomycosis, has been implicated in the global increase in amphibian mortality and extinctions worldwide (Stuart *et al*. 2004; Skerratt *et al*. 2007; Fisher *et al*. 2009; Kilpatrick *et al*. 2010).

1.2 Global impact of *Batrachochytrium dendrobatidis (Bd)* and amphibian decline

Since it was first described in the late 1990s (Berger *et al*. 1998; Longcore *et al*. 1999), *Bd* has been detected within 48 % of all localities surveyed (Olson *et al*. 2013), across 54 countries (Fisher *et al*. 2009; *Bd*-Maps). It is regarded as an extreme generalist, infecting close to 700 species (Olson *et al*. 2013; Olson & Ronnenberg 2014) across all three-amphibian orders (*Anura*, *Urodela* and *Apoda*; Gower *et al*. 2013). Amongst these hosts, there exists a great deal of variation in the susceptibility to and the costs of parasite exposure (Lips *et al*. 2006; Olson *et al*. 2013).

The Global Amphibian Assessment (http://www. iucnredlist.org/amphibians) recently suggested that the 7400+ species of amphibians are one of the most threatened vertebrate lineages, with 41 % of species threatened (Monastersky 2014). *Bd* has contributed to the threatened status of almost 400 amphibian species: a greater impact

than predation by established and well-known invasive species that affect all classes of vertebrates (Bellard *et al*. 2016). Additionally, 92.5 % of amphibian species labeled as 'critically endangered' are undergoing 'enigmatic declines' that have been linked to the presence and occurrence of *Bd* (Stuart *et al*. 2004; Bielby *et al*. 2008). It has been definitively tied with the collapse of amphibian populations in eastern Australia (Berger *et al*. 1998), Panama (Lips *et al*. 2006), California (Vredenburg *et al*. 2010), and Peru (Catenazzi *et al*. 2011). As such, *Bd* has been called the "worst infectious disease ever recorded among vertebrates in terms of the number of species impacted, and its propensity to drive them to extinction" (Gascon *et al*. 2007).

1.3 The amphibian-killing chytrid fungus *Batrachochytrium dendrobatidis (Bd)*

Bd is a member of a basal group of fungi, the *Chytridiomycota*. This is an early diverging lineage of true fungi whose members are characteristically aquatic and reproduce by forming motile, flagellate zoospores (James *et al*. 2006). Many species of chytrid have been described in aquatic environments and soils, as free-living or commensal organisms, and as parasites of algae, invertebrates, fungi, and plants (Gleason *et al*. 2008; Fisher *et al*. 2012). Of these, *Bd* is near unique in that it is one of only two of its order (the *Rhizophydiales*) to parasitize and kill amphibians. The second is *Batrachochytrium salamandrivorans (Bsal)*, which causes chytridiomycosis in salamanders and newts (order: *Urodela*; Martel *et al*. 2013). *Bsal* is morphologically, genetically, and functionally distinct from *Bd* (Martel *et al*. 2013). The discovery of *Bsal* pushes the association of the *Batrachochytrium* genus as an amphibian parasite to an age of at least 25 million years, showing that the emergence of *Bd* is not associated with a recent host jump to amphibians (James *et al*. 2015). Recent studies have shown that *Bd* is actually comprised of several genetically

distinct lineages that vary in virulence and phenotype (Farrer *et al*. 2011). Of these lineages, the Global Pandemic Lineage (GPL) is clearly an invasive species that has spread across the globe and caused mass mortality, population declines and extinctions (Farrer *et al*. 2011).

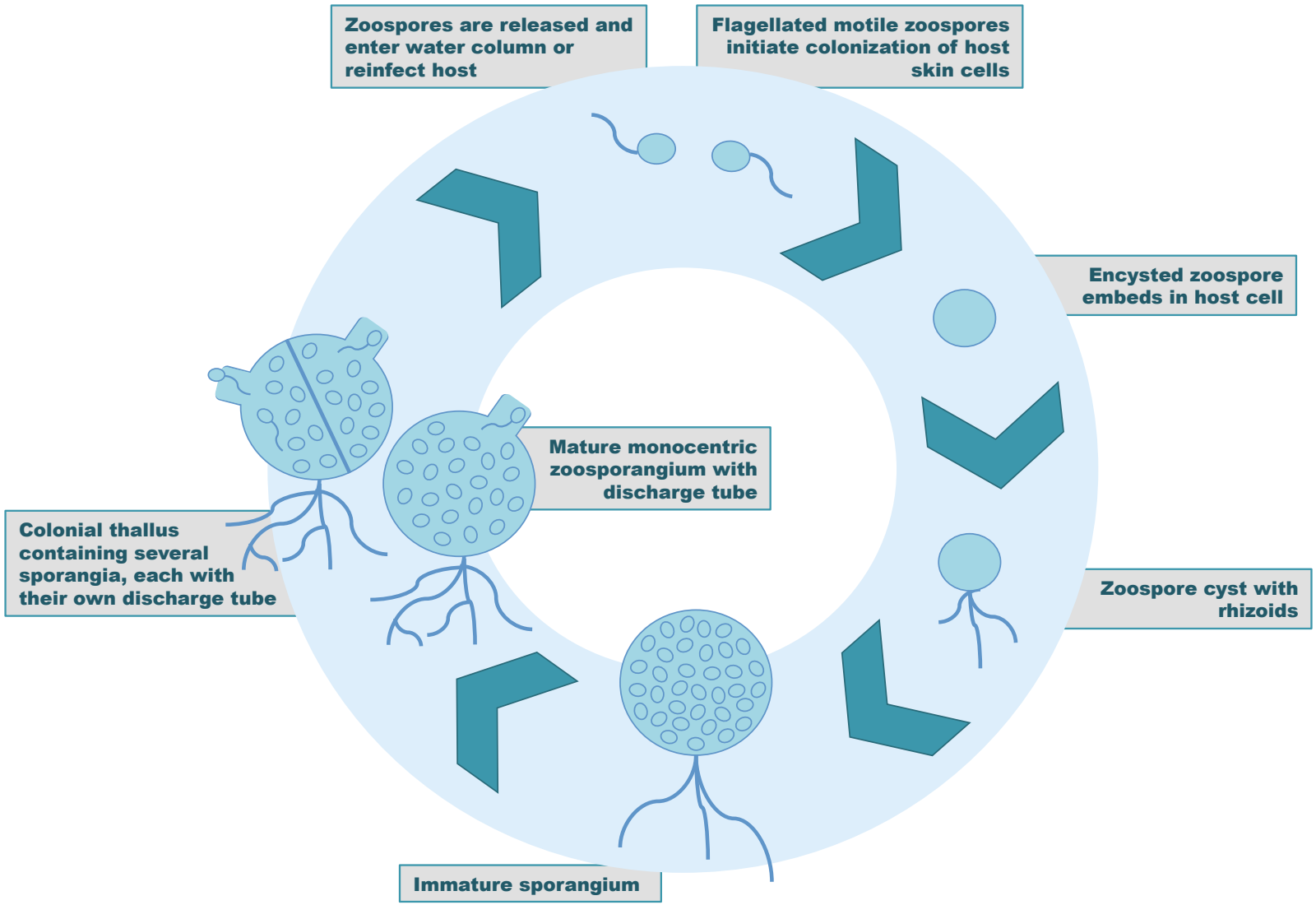
1.3.1 *Bd* life cycle

The *Bd* life cycle consists of two distinct life stages: the infectious flagellate zoospore of approximately 2 μm in diameter; and the thallus, where the zoospores develop. The thallus can produce one or more sessile spherical reproductive bodies called zoosporangia, of 10 μm - 40 μm in diameter (Longcore *et al*. 1999; Berger *et al*. 2005a). At 22 °C, the life cycle in vitro takes 4 to 5 days to complete (Berger *et al*. 2005a; Figure 1.1). Zoospores are surrounded by a membrane and are motile. They may swim using a flagellum but spread in water largely via passive movement. They initiate the colonization of frog skin by entering a keratinized skin cell within the stratum granulosum or stratum corneum of the epidermis. Upon colonization of the host epidermis, the zoospores encyst, the flagellum is absorbed and a cell wall is formed (Longcore *et al*. 1999; Berger *et al*. 2005a). The zoospore cyst germinates and develops a germ tube that invades the host epidermis (Greenspan *et al*. 2012). The way in which zoospores evade potential host immune defences while invading cells is not known, but cytoplasmic extensions have been observed projecting and retracting from *Bd* zoospores (Longcore *et al*. 1999). Once a zoospore encysts inside a skin cell, it begins development into the second stage of the *Bd* life cycle, the thallus, which produces the zoosporangium (Longcore *et al*. 1999). The thallus may be monocentric, developing into a single zoosporangium, or colonial, where internal septa develop and each thallus segment then develops into a zoosporangium (Longcore *et al*. 1999). The

development of sporangia matches the turnover of epidermal cells, so that by the time sporangia are mature they have been carried to the skin surface (Berger *et al*. 2005a). From each zoosporangium, thread-like rhizoids protrude (Longcore *et al*. 1999).

Members of this Rhizophydiales order have a chitin-walled zoosporangium plus rhizoids that function as anchorage to the sub-stratum and also increase the surface area for enzyme excretion and nutrient absorption (Alexopoulos *et al*. 1996). Numerous new zoospores, the asexual reproductive spores, form within the zoosporangium following mitosis. Meanwhile, one or more plugged discharge tubes extend from the zoosporangium to the skin cell surface (Longcore *et al*. 1999). Once the zoospores are mature, the plug dissolves or decays and the zoospores are released through the discharge tube to the epidermal surface and into the surrounding water (Longcore *et al*. 1999).

Figure 1.1: Life cycle of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis (Bd)*. At 22 °C, the life cycle in vitro takes 4 to 5 days to complete (Berger *et al*. 2005a). *(Figure: author's own, modified from Berger et al. 2005a and Van Rooij et al. 2015).*



1.3.2 Transmission of *Bd*

Bd appears to lack an efficient mechanism to transmit between cells within an infected host (Berger *et al*. 2005a). Thus, increasing intensity of infection depends on reinfection from zoospores released onto the skin surface and infection from aquatic zoospores (Longcore *et al*. 1999). Infected amphibians may shed considerable loads of zoospores into waterbodies, making them potential environmental reservoirs (Reeder *et al*. 2012; Kolby *et al*. 2014). These zoospores can survive for up to 24 hours in water in the absence of an amphibian host (Berger 2001; Woodhams *et al*. 2008a). Furthermore, *Bd* is able to saprobically grow and survive upon non-amphibian taxa, such as sterile bird feathers, arthropod exoskeletons, keratinous toes of waterfowl, and may survive in the gastrointestinal tract of crayfish (Longcore *et al*. 1999; Johnson & Speare 2003, 2005; Garmyn *et al*. 2012; McMahon *et al*. 2013). Additionally, *Bd* DNA has been found on wild Panamanian lizards and snakes (Kilburn *et al*. 2011), albeit it is not clear if *Bd* can persist on reptile skin under natural conditions. Given that *Bd* has an unusually broad host range, can survive on and be transported by non-amphibian taxa and that infectious zoospores can survive in the absence of an amphibian host, it seems unlikely that we will prevent the spread of the fungus into countries in which it is currently absent (Olson *et al*. 2013). In light of this, it seems prudent to work on improving our understanding of the factors that influence long-term parasite persistence, and host response to endemic infection.

1.3.3 Clinical signs of *Bd* infection

Bd infects the superficial, keratin-containing layers of amphibian skin (Berger *et al*. 1998). In frog tadpoles, only the mouthparts are keratinized and susceptible to *Bd* infection (Berger *et al*. 1998). Although infection of mouthparts is often associated

with depigmentation and damage or loss of tooth rows and jaw sheaths, mortality has not been observed in infected larvae (Vredenburg & Summers 2001; Fellers *et al*. 2001; Rachowicz 2002; Blaustein *et al*. 2005). However, *Bd* may cause sub-lethal effects in tadpoles, including lethargy or poor swimming abilities, leading to low foraging efficiencies, exacerbated by damaged mouthparts, which is reflected in reduction in body size (Parris 2004; Hanlon *et al*. 2015). This aclinical presence of *Bd* in amphibian larvae implicates this life-cycle stage as a reservoir host for the pathogen, and may enable *Bd* to persist in reduced amphibian populations (Daszak *et al*. 1999). During metamorphosis, the amphibian skin becomes increasingly keratinized. The fungal infection is then able to spread throughout the skin in froglets of susceptible species (Marantelli *et al*. 2004; Rachowicz & Vredenburg 2004), and as such, newly metamorphosed froglets appear to suffer the highest mortality (Lamirande & Nichols 2002).

In metamorphosed amphibians, clinical signs are variable and range from sudden death without obvious disease to significant skin disorder: however, infection may nonetheless elapse asymptomatically (Van Rooij *et al*. 2015). Common signs of chytridiomycosis include: thickening of the skin (hyperkeratosis); excessive epidermal sloughing; erythema (redness); and/ or discoloration of the skin (Pessier 2008). Other common signs of infection include anorexia, lethargy, abnormal posture with hind legs extended, and lack of righting reflex (Berger *et al*. 2005b; Pessier 2008). Clinical examinations of dead or dying diseased individuals, have also reported: epidermal ulcerations; gross lesions; haemorrhages in the skin, muscle or eye; hyperaemia of digital and ventrum skin; and congestion of viscera (Berger *et al*. 1999; Figure 1.2).

The tough outer protective layer of amphibian skin, the integument, is a site of regulated transport for respiratory gases, water and ions (Deyrup 1964; Heatwole & Barthalmus 1994; Jørgensen 1997). Physical disruption of the epidermis directly affects the osmoregulatory function of the skin, impairing electrolyte transport. This is further accompanied by a reduction in trans-epithelial resistance and leakage of ions, leading to ion imbalances, and a reduced ability for frogs to osmoregulate or rehydrate. Individuals heavily infected with *Bd* present abnormally low blood levels of certain electrolytes, such as sodium, magnesium, chloride and potassium (Voyles *et al*. 2007, Voyles *et al*. 2009), and in particular, low plasma potassium concentrations (or hypokalemia) have been linked to abnormal cardiac electrical activity. Thus, cardiac arrest is thought to be the proximate cause of death in diseased amphibians (Voyles *et al*. 2009).

Figure 1.2: Possible clinical signs associated with *Bd* infection. These individuals were found to be *Bd* positive via qPCR analysis. However, no post-mortem or histological examination was completed, thus clinical signs of disease are observational, not definitive. Naturally infected Northern Leopard frogs (*Rana (formerly Lithobates) pipiens*) showing signs of: **(A)** excessive epidermal sloughing; **(B)** ventral discoloration of skin; **(C)** erythema (redness) on hind limbs; **(D)** anorexia; **(E)** lethargy (hind legs extended); **(F)** abnormal posture (abducted hind legs); **(G)** ulceration of the right hindfoot; **(H)** severe ulceration of the right hindfoot with loss of digits; **(I)** ventral epidermal ulcerations; **(J)** scarring characteristic of healed ulcerated skin; **(K)** lesion between external nares; and **(L)** hemorrhages in the eye. *(Images: author's own).*


G
H
I
J
K
L

1.3.4 Within- and among-population variation in *Bd* infection outcomes

One of the most striking features of *Bd* is the variability in outcome of infection that has been observed among species (Kilpatrick *et al*. 2010; Briggs *et al*. 2010). Chytridiomycosis leads to the rapid death of individuals of some species (Berger *et al*. 1998; Rachowicz *et al*. 2006; Lips *et al*. 2006), while individuals of other species develop only minor infections and suffer little or no negative effects (Daszak *et al*. 2004; Weldon *et al*. 2004). Consequently, *Bd* has the potential to significantly alter community composition through unequal impacts on species. This is especially true if species diversity dilutes or amplifies disease risk, altering the response of a community to the infection (Keesing *et al*. 2006; Fernández-Beaskoetxea *et al*. 2016). However, the lack of information on the ecology of many amphibian species makes predicting community response difficult.

Within a species, individual hosts and separate populations also exhibit highly heterogeneous responses to *Bd* infection (Bradley *et al*. 2002; Davidson *et al*. 2003; Daszak *et al*. 2004; Retallick *et al*. 2004; Woodhams & Alford 2005; Blaustein *et al*. 2005; Rachowicz *et al*. 2006, Puschendorf *et al*. 2011; Daskin & Alford 2012;). For example, the spread of *Bd* within some amphibian populations has led to rapid, local extirpation, while other populations coexist alongside *Bd* with no evidence of disease (Bosch *et al*. 2001; Rachowicz *et al*. 2006; Vredenburg *et al*. 2010; Walker *et al*. 2010; Tobler *et al*. 2012). This level of variability is the raw material for the possible evolution of resistance or tolerance (Roy & Kirchner 2000) to *Bd*, and hence population persistence (Spielman *et al*. 2004; Miller & Vincent 2008; Bell & Collins 2008).

1.4 The Northern Leopard Frog (*Rana pipiens)* in Ontario

Figure 1.3: The Northern Leopard Frog (*Rana pipiens*), are typically green or brown in colouration, with dark spots on their dorsal side. *(Images: author's own).*

The Northern Leopard Frog (*Rana pipiens*, *R. pipiens* from this point forward, formerly *Lithobates pipiens;* Yuan *et al*. 2016) is a medium-sized, semi-terrestrial frog that is currently considered globally secure. They are characterised by dark spots on their dorsal side, light coloured dorsolateral folds and pearly-white undersides. These frogs are typically green or brown, as a result of genetic variation at a single locus (Fogleman *et al*. 1980; Figure 1.3), and both colour morphs can co-occur (Volpe 1955; Fogleman *et al*. 1980). In addition to the two colour morphs, individuals have a highly variable degree of black spot patterning on the dorsal surface, and thus exhibit varying degrees of melanism (Moore 1942; Merrell 1965). The snout-to-vent length of most adult *R. pipiens* ranges from 50 - 100 mm, with females displaying larger snout-vent lengths (Seburn & Seburn 1998). Maximal adult dispersal ranges of 8 – 10 km have been documented, and migrations to and from upland and breeding sites within the leopard frog home range occur seasonally (Dole 1971; Seburn & Seburn 1997; Romanchuk & Quinlan 2006).

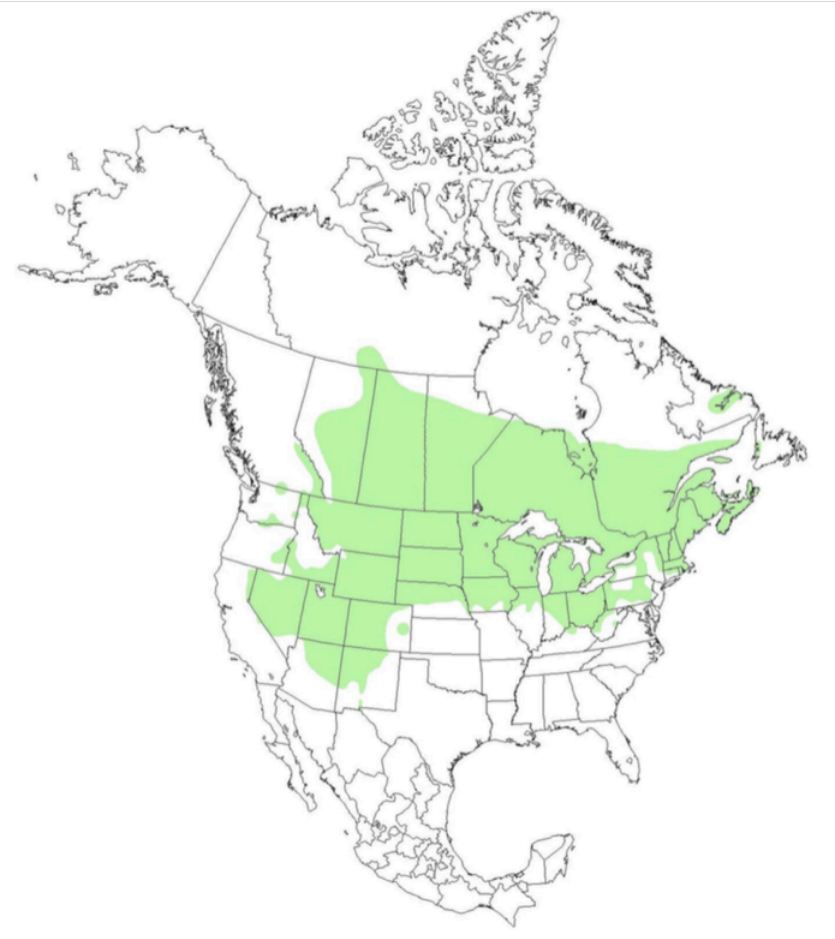


Figure 1.4: Probable historic North American range (highlighted in green) of the Northern Leopard Frog (*Rana pipiens*). *(Figure: extracted from Smith & Keinath 2007, as synthesized from Stebbins 2003; Hillis 1988; Conant & Collins 1991).*

R. pipiens was once locally common throughout its expansive range of central North America (Leonard *et al*. 1999: Figure 1.4) and was previously common throughout parts of western Canada until declines started occurring in the 1970s. Over the following decade, most western North American populations suffered varying degrees of decline (Leonard *et al*. 1999; Lannoo 2005) while eastern populations remained relatively intact (Seburn & Seburn 1998). In some areas, *R. pipiens* has disappeared completely (Corn & Fogleman 1984; Bulle & Wales 2001; Werner 2003). As there was little monitoring during that time period, the spatial and temporal spread of the decline is unclear.

Although common and widespread throughout southern Ontario, *R. pipiens* appears to have declined in northern Ontario (Weller *et al*. 1994; Seburn & Seburn 1998; Figure 1.5). Yet, this species is not listed on the 'Species at Risk in Ontario' (SARO) list. In 1997, a survey of areas from Sudbury to Geraldton failed to locate any presence of *R. pipiens* north of Sault Ste. Marie (Seburn & Seburn 1997; Figure 1.5).

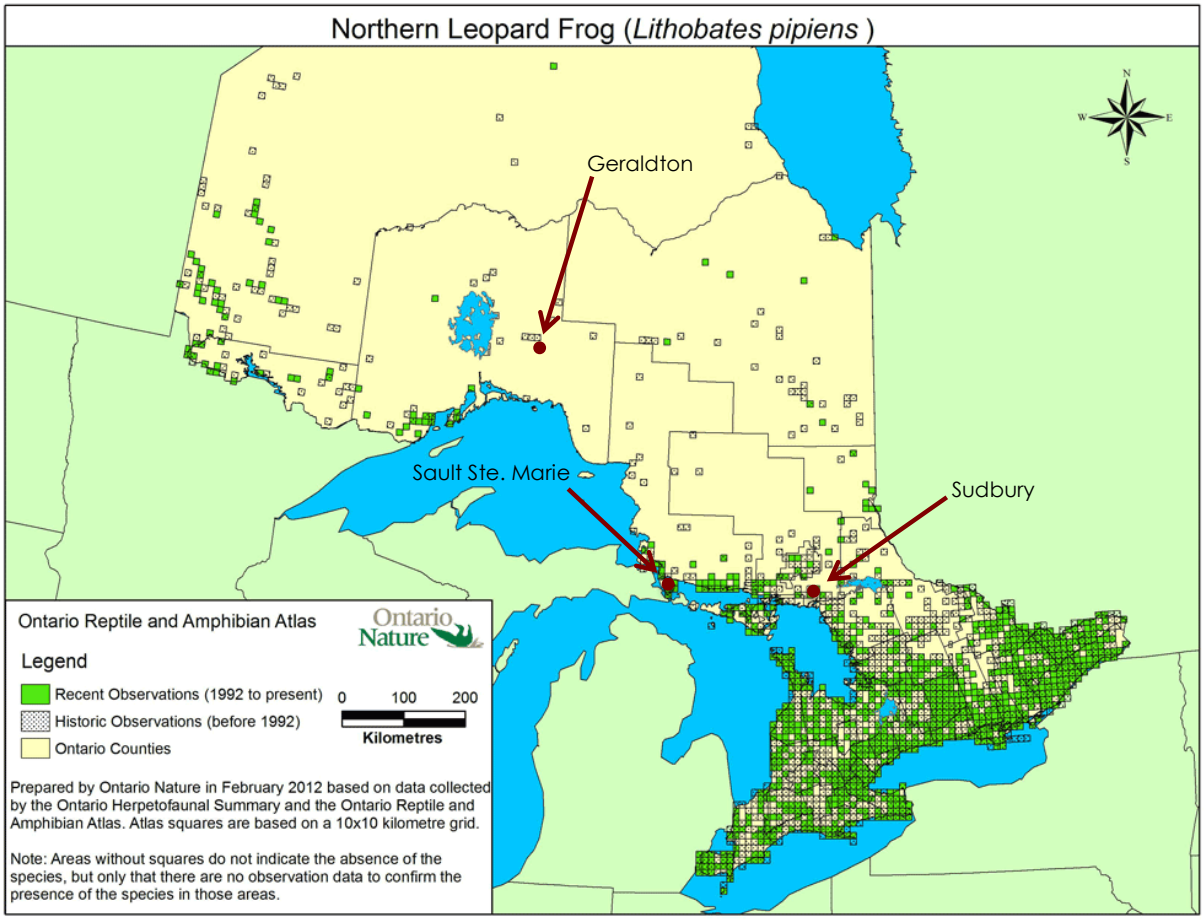


Figure 1.5: The distribution of the Northern Leopard Frog (*Rana pipiens)* throughout Ontario, recorded between 1992 - 2012. Note the absence within Northern Ontario. *(Figure: extracted from Ontario Nature 2012, annotated by author).*

Carey *et al*. (1999) suggested that northern *R. pipiens* population declines observed in the late 1960s - early 1970s (Gibbs *et al*. 1971; Rorabaugh 2005), might have been caused by the emergence of chytridiomycosis. In the nearby province of Quebec, 10 - 20 % of *R. pipiens* specimens collected between 1960 and 2001 were infected with *Bd* (Ouellet *et al*. 2005), and the incidence of infection did not vary between 1960 – 1969 and 1990 - 2001 (Rollins-Smith *et al*. 2006). Consequently, *Bd* infection appears to be enzootic within *R. pipiens* populations (Ouellet *et al*. 2005). Despite the long-term presence of *Bd* (Ouellet *et al*. 2005), chytridiomycosis-driven mass mortality events and species declines are yet to be definitively reported. Consequently, it has been suggested that *R. pipiens* may serve as a reservoir of infection within amphibian communities (Woodhams *et al*. 2008b). However, *Bd* infection may cause sublethal effects in *R. pipiens* populations, such as weight loss and other metrics that indicate reduced fitness, which can affect susceptibility to other stressors, such as food availability, predation and variable environmental conditions (Davidson *et al*. 2003; Parris & Beaudoin 2004; Garner *et al*. 2006; Retallick & Miera 2007). As the pathogen has been detected within *R. pipiens* populations in Ontario (St-Amour *et al*. 2008; D'Aoust-Messier *et al*. 2015), this system provides a unique environment in which to assess the influence of micro, meso, and macro environmental heterogeneity on the spread of *Bd*.

1.5 Host parasite interactions across scales

Within- and among- population variation in *Bd* infection outcome has been implicated with a number of factors including: temperature and moisture (Berger *et al*. 2004; Seimon *et al*. 2007; Kriger *et al*. 2007); habitat composition (Lips *et al*. 2003); host behaviour (Richards-Zawacki 2010; Rowley & Alford 2013; McMahon *et al*. 2014),

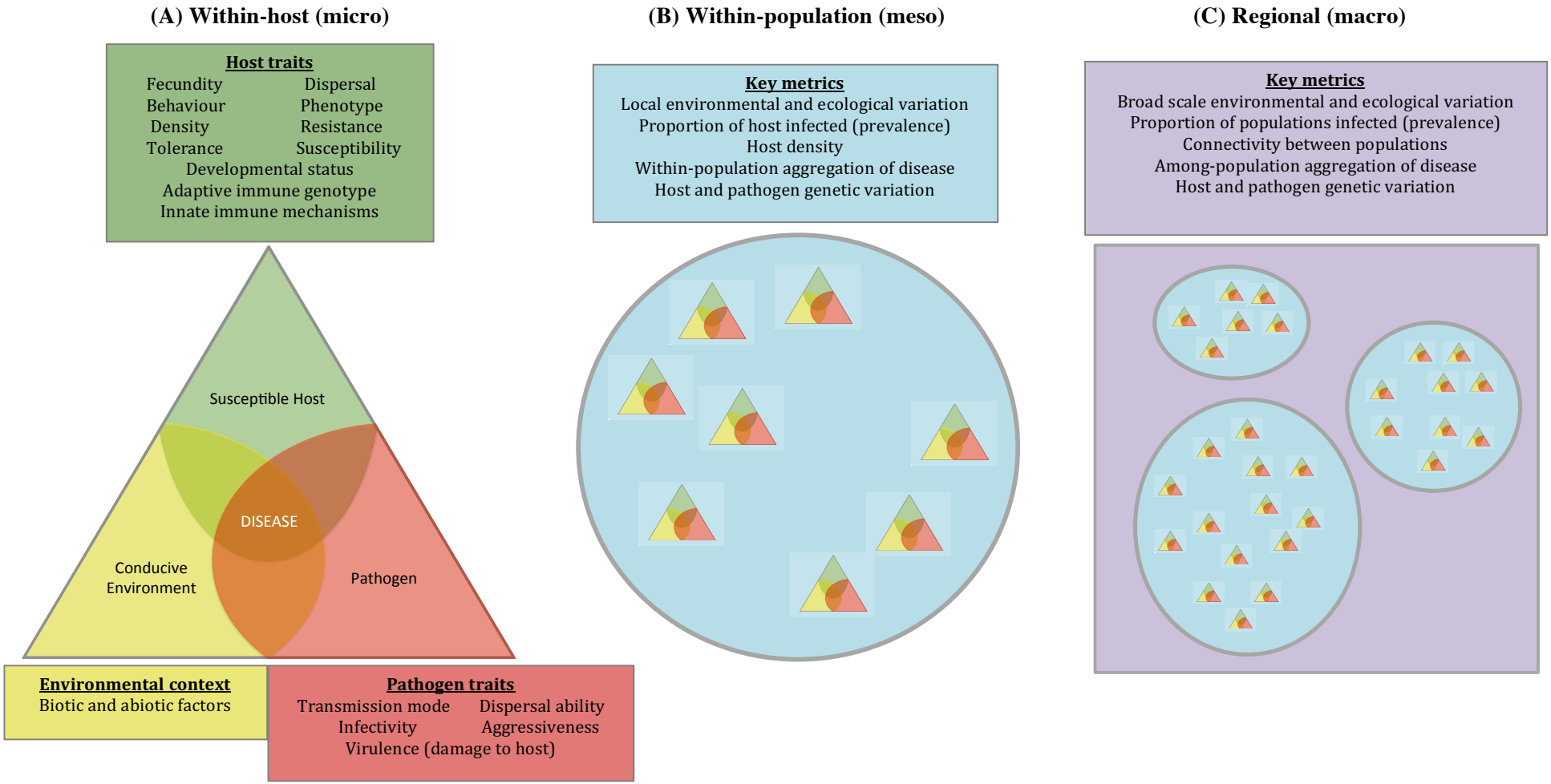
host ecology (Kriger & Hero 2007a; Bielby *et al*. 2008); host innate immune mechanisms such as antimicrobial peptides (Woodhams *et al*. 2014) and skin bacteria (Harris *et al*. 2009; Kueneman *et al*. 2014; Jani & Briggs 2014); host adaptive immune genotype (Savage & Zamudio 2011); and pathogen genotype and virulence (Farrer *et al*. 2011; Schloegel *et al*. 2012; Bataille *et al*. 2013; Berger *et al*. 2016). Thus, as our understanding of the factors that influence *Bd* infection improves, it is becoming more apparent that infection outcome arises from the interaction between the ecology and evolutionary history of the host (e.g. resistance and tolerance), the genotype and phenotype of the fungus (e.g. infectivity and virulence), and the surrounding abiotic and biotic environment (e.g. environmental heterogeneity). However, these factors operate across nested levels of biological organization: within-host processes underlie among-host processes within a population. Consequently, studies looking to gain insight into epidemiological processes of *Bd* must consider within-host up through population-level dynamics.

Despite this fact, very few studies have simultaneously tested for *Bd* infection dynamics occurring across scales. At present, the focus of most studies falls into one of three categories: (1) within-host (micro), (2) within-population (meso), and (3) regional (macro). At the individual level, the host may be thought of as an ecosystem in which parasites, commensals and immune components interact and compete for resources (Rynkiewicz *et al*. 2015). As such, this concept is best considered within a disease triangle framework (Figure 1.6(A)), which is commonly used to explain how variation in environmental factors, host susceptibility, and pathogen virulence lead to varying infection outcomes, yet only a small parameter space in the model results in disease (Scholthof 2007; Gurr *et al*. 2011). At the population (meso) level, the spatial distribution of uninfected and infected hosts typically varies greatly over space and

time. This variation may be due to both host and parasite genetic differentiation throughout the population, as well as local biotic and abiotic conditions (Wolinska & King 2009). These small-scale spatiotemporal variations may alter infection processes, such as host and parasite dispersal ability, and parasite transmission mode (Figure 1.6(B); Anderson & May 1978, Anderson & May 1981). At the regional (macro) scale, comparative methods from macroecology are implemented in order to explore the drivers of parasite distributions (Dunn *et al*. 2010). For example, patterns of infection occurrence among populations can be studied to evaluate how parasite occurrence and environmental heterogeneity interact, and how environmental heterogeneity may determine which parasite populations persist and proliferate (Figure 1.6(C)). Studies focusing on one scale often ignore the dynamics occurring at higher and lower scales. In reality, it appears that dynamic interactions occur in both directions (Tompkins *et al*. 2011; Chesson 2012).

The study of host–parasite interactions across scales is increasingly important as there is building evidence that these scales are changing due to human-mediated factors including climate change, habitat fragmentation (Opdam & Wascher 2004) and increased dispersal (Altizer *et al*. 2013; Alexander *et al*. 2014). Through phenotypic plasticity, genotypes will interact with local changing environments (Nussey *et al*. 2007), producing disparity in the intensity of selection on host and parasite, leading to different phenotypes for both (Mitchell *et al*. 2005). As a result, changing habitats can have profound effects on host–pathogen evolutionary dynamics (Altizer *et al*. 2013; Echaubard *et al*. 2014). Only by studying host–parasite interactions at spatial scales ranging from micro, to meso, to macro; and at temporal scales ranging from single seasons to multiple years, may we gain key insights into epidemiological processes, and assess the potential for (co)evolution of hosts and their parasites.

Figure 1.6: Schematic of hierarchical spatial scales of *Bd* host–parasite interactions. **(A)** Within-host (micro): the 'disease triangle framework' (Wobeser 2006; Scholthof 2007). The interaction between host ecology and evolution, parasite genotype and phenotype, and environmental conditions, are thought to be key drivers of disease intensity, which ultimately determine individual mortality and host response to infection. Only a small parameter space in the model results in disease. **(B)** Within-population (meso): the prevalence and spatial distribution of disease in a population, and ecological and evolutionary consequences of infection, are shaped by variation in host traits, pathogen traits and environmental factors over small spatial scales. **(C)** Regional (macro): within-host and among-host processes interact with larger-scale environmental variation to determine the prevalence and spatial structure of disease at the regional level. *(Figure: author's own, modified from Penczykowski et al. 2015).*



1.6 Project description

My project focused upon the infection dynamics of *Bd*, an amphibian emerging infectious disease, and the circumstances that contribute to increased infection risk. I used amphibians as they represent a global model for disease emergence, providing a study system in which to investigate the role of ecological processes on infection emergence and spread. Previous studies have questioned the additive, competitive, or nonlinear interactions of biotic and abiotic factors on amphibian host populations, across a range of spatial scales. Consequently, this wealth of knowledge provided *a priori* reasons to question particular variables. Building on this information, along with a combination of field surveillance and monitoring, and lab-based diagnostics, I hoped to investigate the following two questions and their associated objectives:

A. Are spatiotemporal patterns of *Bd* infection regulated by environmental heterogeneity, whether naturally or anthropogenically driven? The objective was to develop our knowledge regarding meso and macro environmental drivers of infection risk, in order to improve future predictions of infection spread and parasite persistence.

B. Within a species, what modulates heterogeneous host population-responses to *Bd* infection? The objective was to elucidate whether environmental heterogeneity (meso-scale), host-specific traits (micro-scale), or intrinsic epidemiological dynamics (micro-scale), regulated heterogeneous responses to infection. This would provide us with the appropriate tools with which to assess the risk that *Bd* presents to a naïve population.

1.6.1 Chapter Two: Environmental correlates of *Bd* infection intensity and prevalence

A key result emerging from the literature is that environmental heterogeneity at each spatial and temporal scale can strongly shape *Bd* host–parasite interactions and the mode of coevolution (Olson *et al*. 2013). This is because environmental conditions affect disease dynamics by physiologically limiting vital processes of both host and parasite: including growth, dispersal and survival (Parmesan & Yohe 2003; Ostfeld *et al*. 2005). However, the current literature often reveals complex and even conflicting patterns. For example, at the meso scale, higher *Bd* prevalence in Puerto Rico was associated with drier months (Longo *et al*. 2010), while in Central America, epidemics occurred in in the middle or end of the rainy season (Lips 1998). At the macro scale, temperature is likely responsible for a positive correlation between prevalence and both elevation (Gründler *et al*. 2012) and latitude (Kriger *et al*. 2007). However, in the Sierra Nevada mountain range, there was no reported relationship between *Bd* prevalence and elevation or temperature (Knapp *et al*. 2011). Thus, individually, meso or macro environmental factors currently fail to provide clear and consistent patterns. As such, we clearly need to incorporate biotic and abiotic characteristics from both scales, within one modelling framework, in order to attempt to accurately translate ecological theory into predictions regarding *Bd* presence and intensity. By identifying and comparing spatiotemporal variables that co-vary between populations exhibiting different infection characteristics, we can start to disentangle the mechanisms allowing for parasite establishment, persistence and proliferation. This will be the focus of Chapter Two.

1.6.2 Chapter Three: Extrinsic and intrinsic traits effect adaptive changes in host resistance during an ecological interaction

Variation in population-level *Bd* infection prevalence and host mortality is often correlated with ambient environmental temperature. This is especially true for ectotherms as extrinsic factors constrain the physiological temperatures of both host and pathogen. At the meso level, warmer or drier areas serve as refugia from *Bd* (Puschendorf *et al*. 2009, 2011), while seasonal low temperatures were significantly related to higher *Bd* prevalence in areas where *Bd* is endemic (Woodhams & Alford 2005). At the micro level, preferences for higher temperatures correlate with reduced probability of *Bd* infection (Richards-Zawacki 2010; Rowley & Alford 2013). A temporary rise in host body temperature may negatively affect the pathogen, as the thermal regime will be suboptimal for fungal growth and survival. These acute changes in body temperature may be due to a physiological response driven by pathogen recognition (so-called 'behavioural fever'; Parris *et al*. 2004; Richards-Zawacki 2010); or a 'selective sweep' in which individuals that attain higher temperatures for other reasons are more likely to survive (Witters & Sievert 2001). In either case, the functional mechanisms underlying these effects, within natural conditions, are not well understood (Carey *et al*. 1999; Jackson & Tinsley 2002).

Host response to *Bd* infection can be roughly categorized as susceptible (infection resulting in disease, either followed by clinical recovery or mortality), tolerant (persistent infection in absence of disease) or resistant (inhibition or fast clearance of infection). General consensus is that host response is fixed at the beginning of a host-pathogen interaction (Bull 1994; Ewald 1994; Frank 1996; Kraaijeveld *et al*. 1998; Fenner & Fantini 1999; Dieckmann *et al*. 2002). However, this view has been

challenged as several studies have reported a change in resistance during an ecological interaction, due to intrinsic changes in the state of one of the organisms (Taylor & Read 1997; Pels & Sabelis 1999; Sokurenko *et al*. 1999; De Jong & Janss 2002). For example, despite a wealth of knowledge regarding the temperature dependent relationship between *Bd* infection and disease outcome, experimental infections of the toad *Alytes obstetricans* show that *Bd* survival varies among individuals and populations under constant environmental conditions (Tobler & Schmidt 2010). These idiosyncrasies suggest that intrinsic traits may be, in part, responsible for variation in resistance to *Bd* infection. Identifying host-specific determinants of *Bd* resistance has the potential to enhance the success of amphibian conservation efforts (Woodhams *et al*. 2011). Thus, a clearer understanding of the links between individual phenotype, body temperature and the outcome of host – pathogen interactions, will provide valuable insights into host – pathogen coevolution and epidemiology (Lambrechts *et al*. 2006; Klass *et al*. 2007). This will be the focus of Chapter Three.

1.6.3 Chapter Four: Landscape and host characteristics influence amphibian skin microbiome

All multicellular organisms are host to microbial symbionts, termed the microbiome. This diverse resident bacterial community, inhabiting the amphibian skin (Belden & Harris 2007) may interact with pathogens via resource competition, contact-dependent antagonism, release of antimicrobial compounds, or modulation of the host immune response (Round & Mazmanian 2009). The successful completion of the *Bd* life cycle (Figure 1.1) requires the achievement of several parasite-specific processes, including: attachment of zoospores to the host skin, zoospore germination, penetration

into the skin cells, and finally invasive growth in the host skin. In order to achieve these goals, *Bd* zoospores will first have to interact with the amphibian microbiome (Belden & Harris 2007).

Previous studies have demonstrated that amphibian skin microbial communities mediate susceptibility to chytridiomycosis (Woodhams *et al*. 2007a, 2007b; Harris *et al*. 2009), while others have characterized the probiotic role of skin bacteria with regards to the intensity and severity of *Bd* infection (Vredenburg *et al*. 2011; Bletz *et al*. 2013; Woodhams *et al*. 2014). These studies suggest that an "imbalanced" microbiome may leave the host more susceptible to *Bd* infection (Croswell *et al*. 2009; Khosravi & Mazmanian 2013); and/ or *Bd* may disrupt the microbiome (Stecher *et al*. 2007; Barman *et al*. 2008; Round & Mazmanian 2009; Winter *et al*. 2010), leading to a shift in the relative proportions of bacterial species present (Nichols *et al*. 2008; Vartoukian *et al*. 2010). Consequently, the microbiome may play a substantial role in disease resistance, or may itself, be disturbed or altered by invading pathogens. The importance of this latter point has only recently become apparent, as recent research suggests that many phenotypic traits, once thought to be the sole product of host genetics, are influenced by host-microbial associations, including: immune response, metabolism, mate-choice and behaviour (Bravo *et al*. 2011; Neufeld *et al*. 2011; Sharon *et al*. 2011; Chung *et al*. 2012). However, the specific characteristics required to: (1) maintain a "healthy" microbial diversity or, (2) trigger disease, are still not well understood in natural populations (McKenzie *et al*. 2012; Loudon *et al*. 2014a; Kueneman *et al*. 2014).

Within a species, members of the same population tend to exhibit more similar microbiomes than individuals across populations (Kueneman *et al*. 2014). This

suggests that different populations either select for a relatively specific microbiome, which may be cultivated and influenced by environmental reservoirs (McKenzie *et al*. 2012; Kueneman *et al*. 2014; Walke *et al*. 2014), or environmental conditions select for the persistence of particular bacterial taxa on the host skin (Vartoukian *et al*. 2010). However, very little is known regarding how aspects of the micro (individual level) and meso (within-population level) environments influence host-associated microbial communities. This underexplored topic may become increasingly relevant with increasing climate change and environmental disturbance (Belden & Harris 2007). Field studies that assess the influence of micro and meso level characteristics on the amphibian microbiome, can improve our understanding of the differential effects of local disease resistance, which in turn, will contribute to the design and application of microbial therapeutics or determine the implications of microbiome disruption. This will be the focus of Chapter Four.

1.6.4 Chapter Five: Summary, conclusions and perspectives

Chapter Five offers an over-arching discussion of findings, attempting to place these within the greater global context of *Bd* epidemiology. A review of the limitations of this work is provided, together with suggestions for future avenues of research.

CHAPTER TWO: ENVIRONMENTAL CORRELATES OF *BD* INFECTION INTENSITY AND PREVALENCE

2.1 INTRODUCTION

2.1.1 Ectothermic host-pathogen interactions at the local scale

Epidemiological processes are embedded within ecological landscapes. Landscape structure and climatic patterns are forms of spatial heterogeneities known to influence the physiology and demography of parasitic species (Walther *et al*. 2002), with effects on host development, behaviour, fecundity and mortality (Parmesan & Yohe 2003; Ostfeld *et al*. 2005). This is especially true for ectothermic hosts as spatial heterogeneities contribute towards the physiology of both host and pathogen. As such, ectotherms are particularly vulnerable to the potential impacts of ongoing climate change (Carey & Alexander 2003; Araújo *et al*. 2006; Chamaille-Jammes *et al*. 2006; Buckley *et al*. 2012).

Most studies focusing on the ecological consequences of climate change have focused on multidecadal variability of environmental parameters (Gilman *et al*. 2010). In order to obtain that wealth of data, many have applied interpolated measures of climatic variables from broad geographic scales such as continent- or global-wide (Hudson *et al*. 2002; Bosch *et al*. 2007, Walker *et al*. 2010, Olson *et al*. 2013). However, as climate change is projected to alter the frequency of extreme weather events across multiple timescales (Easterling *et al*. 2000; Rohr & Raffel 2010; Screen 2014; Screen & Simmonds 2014), fine-scale climatic variability may play a significant role in structuring ecological communities. The influence of this variability on ectotherms and their pathogens will certainly depend on the local scales at which

organisms experience these climatic conditions. For example, local ambient conditions will directly influence an ectotherm's activity pattern, as climate acts as a proximate driver for daily activity. Thus, the nature and intensity of a particular host-parasite interaction will be contingent upon the precise spatio-temporal patterns of both host and parasite (Real & McElhany 1996; Hess *et al*. 2001). As such, infection must be placed in the context of the local environment.

Several studies have produced clear evidence that changes in climate, such as temperature or precipitation, alter the behaviour and susceptibility of ectothermic hosts and/ or the virulence of their pathogens (Carruthers *et al*. 1992; Blanford *et al*. 2003; Woodhams *et al*. 2003, 2008a; Lazarro *et al*. 2008; Paaijmans *et al*. 2013). However, there are often difficulties in interpreting which aspects of the host-parasite system are being influenced by such changes. Hosts and parasites may respond to multiple, interacting, and often nonlinear climatic variables in very different ways (Altizer *et al*. 2013). Thus, the challenge remains to accurately determine whether climatic changes impact the host, parasite, or their interaction. This challenge is further exacerbated by the disturbance of habitats. Beyond climate change, habitat change, such as agricultural development, urbanization and sprawl, road development, and deforestation, may cause a cascade of factors that exacerbate infectious disease emergence (Patz *et al*. 2008). These factors include: pathogen introduction, pollution, human migration and landscape fragmentation. In particular, landscape fragmentation may potentially dampen or mask the effect of climate upon infection dynamics, by altering social interactions amongst hosts. A key component of disease dynamics is that the likelihood and impact of an epidemic increases with host density (Anderson & May 1986). Outside stressors that depress population density or limit frequency of contact, should reduce the chance of an epidemic occurring. This dependence of

infection upon density is one of the key intersections of epidemiology and conservation biology. Thus, landscape fragmentation may function to limit social interactions, which alters fundamental infection dynamics by restricting and altering transmission routes.

2.1.2 *Batrachochytrium dendrobatidis (Bd)* and amphibian declines

Chytridiomycosis is a potentially lethal skin infection of amphibians caused by the fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*; Longcore *et al*. 1999). The fungus infects keratinized tissues of amphibians, specifically the skin of post-metamorphic stages and mouthparts of larval stages of most anurans (Longcore *et al*. 1999; Berger *et al*. 2005a), and is transmitted via an aquatic, flagellated zoospore (Berger *et al*. 2005a; Piotrowski *et al*. 2001). Zoospores are thought to infect cells within the stratum granulosum either directly or via a germ tube and then develop into sporangia (Longcore *et al*. 1999; Berger *et al*. 2005a). After a temperature-dependent number of days, the sporangium releases zoospores through a discharge papilla (Berger *et al*. 2005a; Woodhams *et al*. 2008a; Figure 1.1).

Bd is associated with population declines and mass-mortality events in over 200 species (Skerratt *et al*. 2007; Scheele *et al*. 2017), across several continents (Berger *et al*. 1998; Lips 1999; Bosch *et al*. 2001; Weldon & du Preez 2004; La Marca *et al*. 2005). Despite its expansive distribution, the localities of lethal outbreaks have been restricted to a few regions, notably Eastern Australia, Central America, the western United States and Europe (Bosch *et al*. 2001; Lips *et al*. 2006; Skerratt *et al*. 2007; Jones *et al*. 2008; Murray *et al*. 2009; Bielby *et al*. 2009). Very few die-offs have been reported in the eastern three-quarters of North America, even though *Bd* infections have been widespread since the early 1960s (Ouellet *et al*. 2005).

Within a laboratory setting, species exposed to *Bd* exhibit varying degrees of mortality (Garcia *et al*. 2006; Blaustein *et al*. 2015). This is further supported by observations from the field, where the effects of *Bd* on amphibian populations appear to vary among species and locations. For example, mass-mortality events due to chytridiomycosis have occurred in Colorado and California (U.S.A.) (Muths *et al*. 2003; Vredenburg *et al*. 2010), Queensland (Berger *et al*. 1998), and throughout Panama (Lips *et al*. 2006). In contrast, in other locations including South Carolina (U.S.A.) (Daszak *et al*. 2005) and some areas of Queensland (Kriger & Hero 2006), populations appear to be coexisting with endemic chytridiomycosis. This heterogeneous response to *Bd* infection may be due to species-specific factors, such as host behaviour (Richards-Zawacki 2010; Rowley & Alford 2013), host life history (Kriger & Hero 2007a; Bielby *et al*. 2008); and host innate immune mechanisms (Woodhams *et al*. 2007a, 2007b). Given the differential susceptibility to *Bd* observed amongst species, and the multiple temporal and spatial scales over which chytridiomycosis occurs (www.bd-maps.net; Fisher *et al*. 2009; Aanensen *et al*. 2012), I would suggest that understanding the external forcing factors of infection dynamics may be more easily addressed using ecological data from a single species.

The Northern Leopard Frog (*Rana pipiens;* Yuan *et al*. 2016; Figure 1.3) is exposed to *Bd* throughout its expansive range of North America (Ouellet *et al*. 2005; Voordouw *et al*. 2010). The species is threatened and its range is contracting (Smith & Keinath, 2007; Voordouw *et al*. 2010). In Canada, western populations are now listed as endangered (COSEWIC 2009), while eastern populations have remained relatively stable (Seburn & Seburn 1998; Ouellet *et al*. 2005; Longcore *et al*. 2007; Woodhams *et al*. 2008b). In eastern populations, chytridiomycosis-driven mass

mortality events and species decline are yet to be reported, despite the long-term presence of *Bd* (Ouellet *et al*. 2005). Consequently, it has been suggested that *R. pipiens* may serve as a reservoir of infection within amphibian communities (Ouellet *et al*. 2005; Longcore *et al*. 2007; Woodhams *et al*. 2008b). However, other leopard frog populations appear more susceptible to *Bd*, as chytridiomycosis is the cause, or suspected cause, of mass mortality events (Carey *et al*. 1999; Green *et al*. 2002; Voordouw *et al*. 2010) and individual mortality (Adama *et al*. 2004). Gahl *et al*. (2012) suggested that this lack of documented declines despite the presence of the pathogen, may be due to environmental factors inhibiting *Bd* infection. This implies that much of the spread of *Bd* within eastern populations of *R. pipiens* occurred decades ago (when it was epidemic) and that *Bd* is now endemic (Rachowicz *et al*. 2006; Kinney *et al*. 2011).

2.1.3 The effect of spatial heterogeneities on *Bd*

Shifting climate results in the decline of some amphibian species, due to changes in reproductive behaviour and timing (Alford & Richards 1999; Collins & Storfer 2003). In recent decades, several temperate frog species have initiated their breeding seasons earlier, presumably in response to higher mean daily temperatures and the earlier onset of spring (Beebee 1995; Gibbs & Breisch 2001; Oseen & Wassersug 2002; Saenz *et al*. 2006). For *R. pipiens*, the onset of spring triggers their emergence from overwintering sites. Once emerged, they become highly active in the terrestrial realm, travelling up to 1.6 km to their breeding habitats (Hine *et al*. 1981; Wershler 1991; Souder 2000). This period of heightened activity may result in expanded opportunities for the successful transmission of *Bd* between individuals (Lampo *et al*. 2006). Breeding season starts when daytime air temperatures average 15 ºC, at which point

male *R. pipiens* begin calling (Seburn 1992). Both sexes exhibit strong site fidelity and limited movement throughout the breeding period (Waye & Cooper 2001).

In micro-parasite systems, once an individual is infected, re-exposure of the infected host to the pathogen does not normally affect disease progression (Carey *et al*. 2006; Mitchell *et al*. 2008). However, *Bd* appears to lack an efficient mechanism to transmit between cells within an infected host (Berger *et al*. 2005a). Thus, increasing intensity of infection depends mainly on reinfection from zoospores released within the skin and onto the skin surface, or infection from aquatic zoospores. These zoospores can survive for up to 24 hours in water in the absence of an amphibian host (Berger 2001; Woodhams *et al*. 2008a). For parasites with environmental transmission stages, first principles suggest that infection progression should depend more strongly on temperature, relative to a host-pathogen system where environmental transmission stages do not occur. Indeed, temperature is thought to be an important factor in the epidemiology of chytridiomycosis. Laboratory experiments suggest that *Bd* in culture has a defined thermal optima: with maximum growth occurring between 17 and 25 °C; peak growth and pathogenicity occurring at 23 °C; and growth ceasing at 28 °C (Piotrowski *et al*. 2004; Woodhams *et al*. 2008a; Richards-Zawacki 2010). Temperature-dependence of infection *in vivo*, however, varies among host species. A positive multi-decadal correlation between increasing air temperature and amphibian extinctions at several locations across the globe led many authors to conclude that climate change drives amphibian declines by increasing *Bd* infection risk (Pounds *et al*. 2006; Bosch *et al*. 2007; Laurence 2008; D'Amen & Bombi 2009). However, in some tropical regions, *Bd* outbreaks occurred in upland sites where temperatures were cool (Berger *et al*. 2004; Lips *et al*. 2006), and seasonal low temperatures have been significantly related to high *Bd* prevalence in areas where *Bd* is endemic (Woodhams

& Alford 2005). Although the mechanisms of thermal effects on both amphibian and pathogen are still the subject of investigation, it is widely considered that temperature is a key factor for disease development.

Water must be present for *Bd* to infect new hosts or to re-infect current hosts as the obligate aquatic chytrid zoospores require moisture for movement, survival and colonization (Piotrowski *et al*. 2004; Kriger 2009). Consistent with these observations, several field studies have reported correlations between *Bd* infection and moisture-related variables, such as precipitation, humidity, aquatic connectivity and host water usage (Kriger 2009; Liu *et al*. 2013; Hossack *et al*. 2013; Sapsford *et al*. 2013; Terrell *et al*. 2014; Holmes *et al*. 2014). However, no published studies have linked infection with a reduction in either the size of the water basin, or the density of river networks. Amphibian populations concentrated within a small drainage basin (hydroshed) may facilitate rapid proliferation and transmission of zoospores, as increasing amphibian population density increases transmission rates (Briggs *et al*. 2005). Additionally, amphibian populations scattered across a large hydroshed may experience diluted zoospore concentrations, as zoospores may be carried away with water currents (Piotrowski *et al*. 2004). Streams also serve as likely vectors for the waterborne zoospores of *Bd* (Kriger & Hero 2007a), and a reduction in the number of flowing streams may hinder the spread of the fungus.

Roads serve as barriers to individual movements, via behavioural road avoidance or presenting a physical obstacle. These barrier effects can make certain resources (e.g. mates, food, and breeding sites) inaccessible, which will affect individual fitness. Road mortality has a similar barrier effect, but can additionally reduce population sizes directly. Furthermore, the road network fragments the landscape, resulting in

small and more or less isolated habitat patches (Balkenhol & Waits 2009). In combination, the effect of roads on amphibian taxa may include: (1) altered movement and social interactions at very fine spatial scales (Gibbs 1998); (2) increased genetic structure and decreased genetic diversity (Lesbarrères *et al*. 2003, 2006; Marsh *et al*. 2008); and (3) reduced abundance and diversity (Vos & Chardon 1998; Houlahan & Findlay 2003). Thus, habitat change due to road development may directly influence infection dynamics by altering transmission routes, host population viability, and amphibian community structure and function (St-Amour *et al*. 2008).

2.2 RESEARCH AIMS AND OBJECTIVES

In this study, I detail the surveillance and analysis of the distribution of *Bd* infection in populations of *R. pipiens* throughout Ontario, Canada. I first assess whether there is heterogeneity in the distribution of infection and second, whether environmental variables account for heterogeneity in:

(1) The proportion of individuals affected within a site (prevalence), and

(2) The mean intensity of infection within a site.

Specifically, I hypothesize that: (i) due to the suggested endemic nature of *Bd* within the region, cooler temperatures and greater precipitation will increase prevalence and infection intensity; (ii) these climatic variables will have a greater impact on infection dynamics during the active period due to an increased opportunity for successful *Bd* transmission; (iii) as water basins and rivers serve as likely vectors for the waterborne zoospores (Kriger & Hero 2007a), an increase in basin size will lower intensity (genomic equivalents; GE) due to diluted zoospore concentrations, while a reduction in river density will limit transmission nodes, thus lowering prevalence and infection

intensity; and (iv) increased road density will intensify landscape fragmentation leading to isolated habitat patches and dense host populations, which will allow for an increase in overall prevalence and intensity. A clearer understanding of the links between environmental parameters and the outcomes of ectothermic host-pathogen interactions will provide valuable insights into host-pathogen epidemiology, as well as more fundamental aspects of the ecology and evolution of interspecific interactions (Lambrechts *et al*. 2006; Klass *et al*. 2007).

2.3 METHODS

2.3.1 Sampling for *Bd*

I surveyed 41 *R. pipiens* population localities in Ontario, Canada. Sites were defined as a circular area with a 2 km radius, each separated by a minimum of 10 km from their nearest neighbor. As maximum dispersal distances of 8 – 10 km have been documented for *R. pipiens* (Dole 1971; Seburn & Seburn 1997; Romanchuk & Quinlan 2006), I considered sites to be distinct populations with limited (to zero) gene flow between them and their nearest neighbor. Each study site was geo-referenced using GPS and sampled at least once during the summer months (May – August) from 2012 to 2014 (Figure 2.1; Table 2.1). Site selection was based on the known whereabouts of *R. pipiens* populations (Figure 2.2). As latitudinal spans were large in my study (42 °N - 47 °N), I opted to divide the landscape into three distinct units based on the latitudinal spans of established terrestrial ecoregions (Olson *et al*. 2001). Ecoregions are defined as relatively large units of land containing a distinct assemblage of natural communities, sharing a large majority of species, dynamics, and environmental conditions. They provide a logical framework for the development of large-scale conservation strategies and ecological analyses. Within the study area,

the landscape was separated into the following ecoregions: (1) the Southern Great Lakes forest (latitude < 43.5 °N), henceforth 'south region'; (2) the Eastern Great Lakes lowland forest (43.5 °N > latitude < 44.5 °N), henceforth 'central region'; and (3) the Eastern forest-boreal transition (latitude > 44.5 °N), henceforth 'north region'.

Each site was sampled within a three-week period in order to limit within-season variation due to local environmental conditions. At each site, 30 post-metamorphic frogs were captured using a dip net, and sampled. In an infinite population, a sample size of 30 is sufficient to detect at least one infected individual with a 95 % probability if the underlying prevalence is 10 % (Cannon & Roe 1982). In localities where *R. pipiens* were rare (n < 24), the population was sampled for 30 person-hours. I toe-clipped each individual (first phalange of the longest toe of the front right foot) using sterile dissecting scissors following standardized protocols (Hyatt *et al*. 2007). Tissue samples were stored in 70 % ethanol at 4 °C until processing (Hyatt *et al*. 2007). To prevent pathogen transmission, all frogs were handled with non-powdered disposable vinyl gloves, and standard biosecurity measures were followed (Phillott *et al*. 2010). Toe clipping also facilitated identification of previously sampled individuals in subsequent years. Those found to have a clipped longest toe on their right front foot, were immediately released. Consequently, there were no recaptured individuals and I treated each sampling year as independent.

All animals were handled and released according to an approved Laurentian University Animal Care and Use Committee protocol #2009-03-04. Field surveys and specimen collection were conducted under the Ontario Ministry of Natural Resources Wildlife Scientific Collector's Authorization #1068178. Amphibian handling and sampling within protected areas (including National Parks, Provincial Parks,

Conservation Areas and Management Areas) followed permit guidelines awarded by: Parks Canada Agency (#BPF-2013-13913); Ontario Ministry of Natural Resources and Forestry (#4534); Nature Conservancy of Canada (#AG-ON-2012-144055); and St. Clair Region Conservation Authority (#SCRCA-2012-28-05).

Figure 2.1: *Bd* infection prevalence (%) in *R. pipiens* populations collected from 41 sites in Ontario from 2012 to 2014. Pie chart denotes prevalence (red: *Bd* +ve, blue: *Bd* -ve), with sample size noted in brackets. Data loggers symbolized by orange marker, weather stations symbolized by green marker. *(Figure: author's own, created in ArcGIS version 10.2.2).*

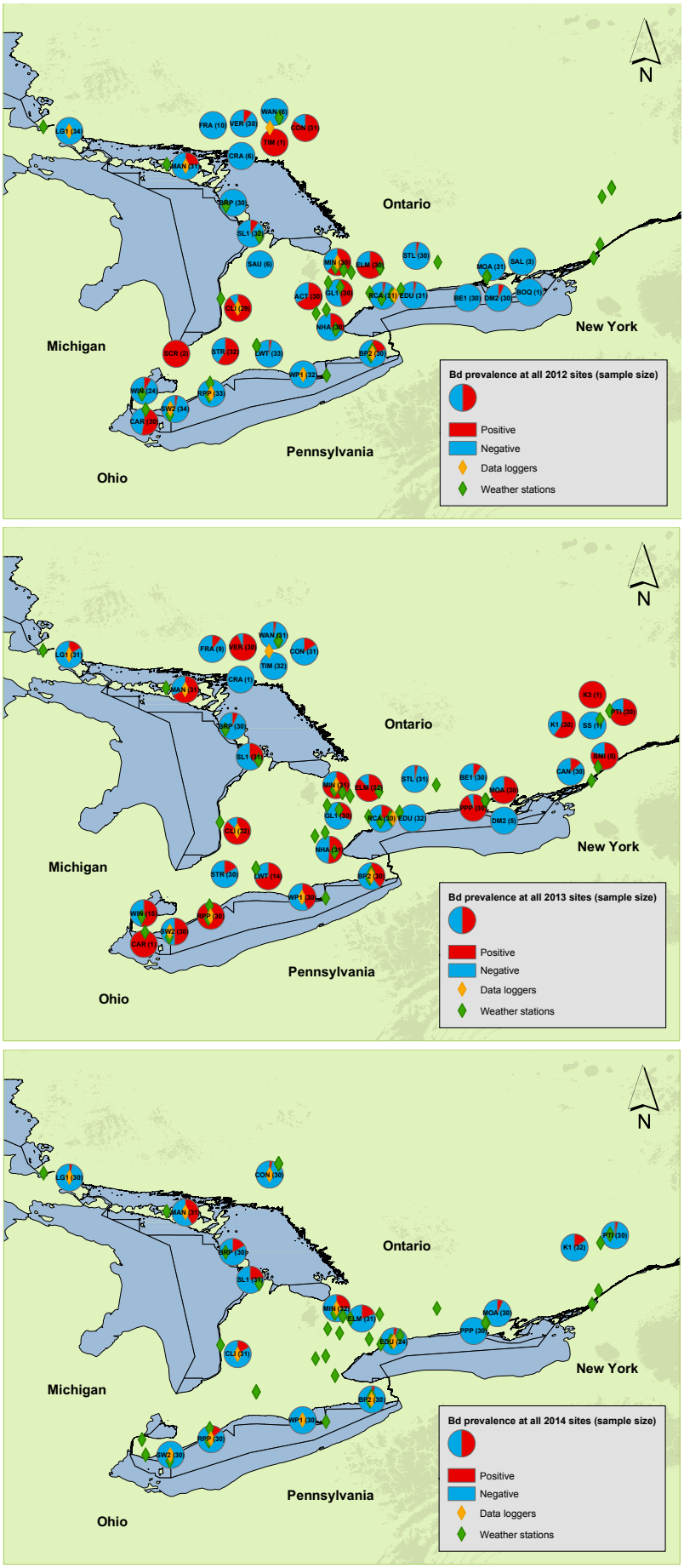


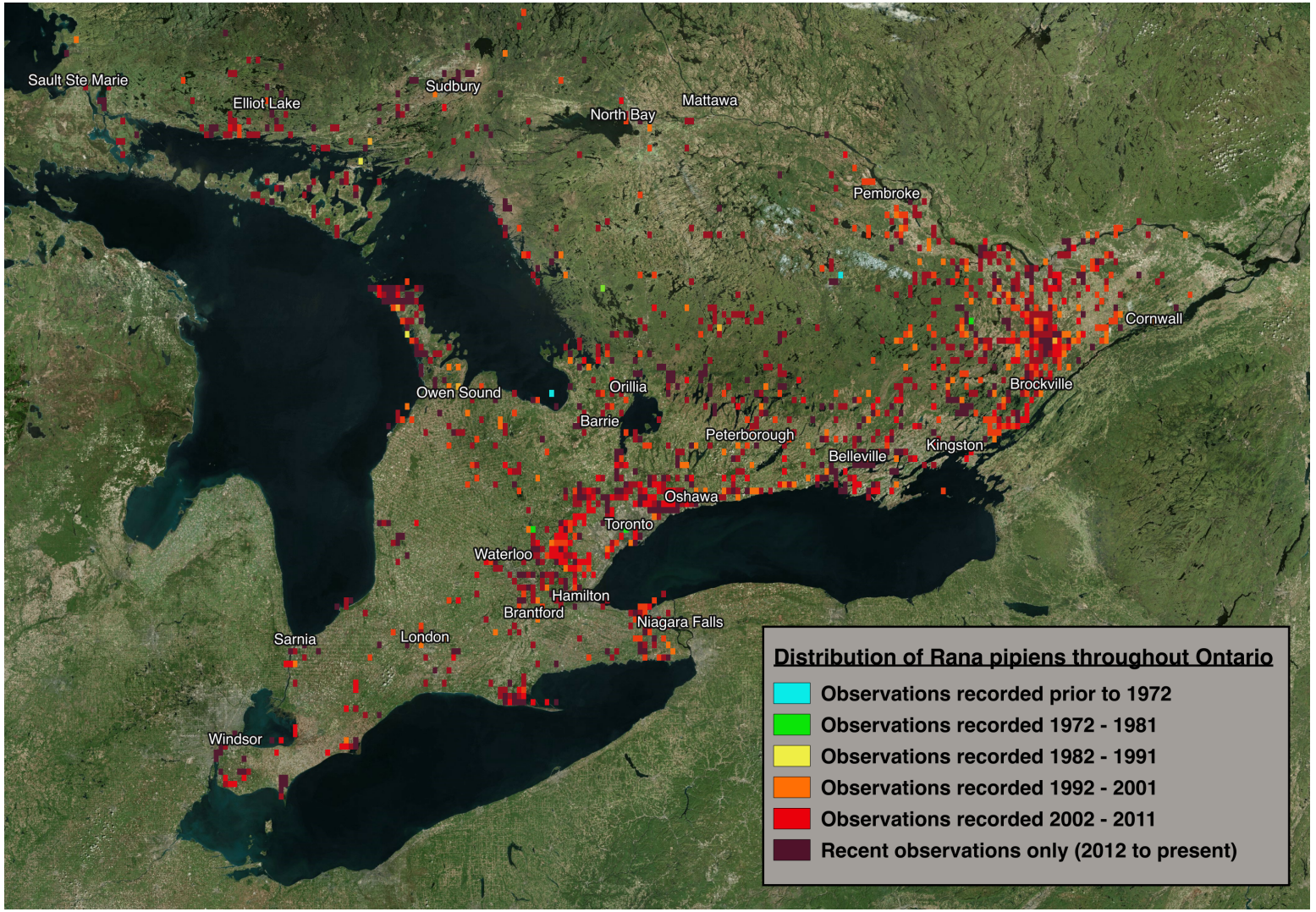
Table 2.1: *Bd* prevalence (%) and infection intensity (GE) in *R. pipiens* populations collected from 41 sites in Ontario from 2012 to 2014. *(Table continues on next pages)*.

Site	Year	Sample size	% Prevalence (N = Individuals *Bd* positive)	Mean infection intensity (SE, range)	Repeat sampled (2012, 2013 & 2014)
ACT	2012	30	66.7 (20)	104.92 (89.9, 0.21 - 2708.28)	
BE1	2012	30	0 (0)	0	
	2013	30	10 (3)	0.24 (0.17, 0.56 - 4.83)	
BMI	2013	5	80 (4)	22.34 (18.82, 0.56 - 97.31)	
BOQ	2012	1	0 (0)	0	
BP2	2012	30	20 (6)	1.0 (0.49, 0.52 - 11.63)	*
	2013	30	40 (12)	20.25 (7.99, 1.98 - 199.09)	*
	2014	30	3.3 (1)	4.01 (4.01, 120.44 - 120.44)	*
BRP	2012	30	0 (0)	0	*
	2013	30	6.7 (2)	0.08 (0.07, 0.32 - 2.09)	*
	2014	30	16.7 (5)	2.42 (1.39, 2.93 - 35.79)	*
CAN	2013	30	13.3 (4)	0.74 (0.49, 0.47 - 11.41)	
CAR	2012	30	53.3 (16)	3.9 (1.79, 0.14 - 42.39)	
	2013	1	100 (1)	0.32 (0, 0.32 - 0.32)	
CLI	2012	29	89.7 (26)	51.47 (22.75, 0.52 - 622.49)	*
	2013	32	87.5 (28)	158.27 (54.84, 0.65 - 1323.62)	*
	2014	31	16.1 (5)	0.78 (0.4, 0.98 - 7.72)	*
CON	2012	31	83.9 (26)	127.8 (46.65, 0.92 - 1022.6)	*
	2013	31	16.1 (5)	0.25 (0.18, 0.3 - 5.49)	*
	2014	30	3.3 (1)	0.09 (0.09, 2.6 - 2.6)	*
CRA	2012	6	0 (0)	0	
	2013	1	0 (0)	0	
DM2	2012	30	6.7 (2)	1.45 (1.45, 0.09 - 43.47)	
	2013	5	0 (0)	0	
EDU	2012	31	3.2 (1)	0.05 (0.05, 1.52 - 1.52)	*
	2013	32	0 (0)	0	*
	2014	24	4.2 (1)	1.27 (1.27, 30.47 - 30.47)	*
ELM	2012	30	73.3 (22)	8.46 (2.63, 0.12 - 58.69)	*
	2013	32	87.5 (28)	484.97 (141.23, 3.61 - 3371.34)	*
	2014	31	19.4 (6)	5.95 (4.23, 0.24 - 116.57)	*

Site	Year	Sample size	% Prevalence (N = Individuals *Bd* positive)	Mean infection intensity (SE, range)	Repeat sampled (2012, 2013 & 2014)
FRA	2012	10	0 (0)	0	
	2013	9	11.1 (1)	0.08 (0.08, 0.72 - 0.72)	
GL1	2012	30	46.7 (14)	21.7 (9.86, 0.27 - 256.42)	
	2013	30	30 (9)	5.7 (3.43, 0.83 - 96.61)	
K1	2013	30	60 (18)	42.24 (15.66, 0.29 - 426.67)	
	2014	32	15.6 (5)	0.92 (0.51, 1.29 - 14.77)	
K3	2013	1	100 (1)	20.76 (0, 20.76 - 20.76)	
LG1	2012	34	0 (0)	0	*
	2013	31	16.1 (5)	0.43 (0.31, 0.02 - 9.25)	*
	2014	30	3.3 (1)	0.04 (0.04, 1.13 - 1.13)	*
LWT	2012	33	3 (1)	0.45 (0.45, 14.97 - 14.97)	
	2013	14	71.4 (10)	25.12 (15.72, 1.15 - 221.86)	
MAN	2012	31	22.6 (7)	16.39 (8.33, 2.58 - 167.84)	*
	2013	31	67.7 (21)	36.01 (12.98, 0.84 - 307.94)	*
	2014	31	41.9 (13)	7.05 (3.93, 0.57 - 117.91)	*
MIN	2012	30	63.3 (19)	9.34 (3.8, 0.18 - 100.78)	*
	2013	31	74.2 (23)	39.87 (14.28, 1.48 - 424.68)	*
	2014	32	25 (8)	0.72 (0.35, 0.35 - 7.93)	*
MOA	2012	31	0 (0)	0	*
	2013	30	73.3 (22)	149.52 (85.62, 0.58 - 2407.03)	*
	2014	30	6.7 (2)	10.43 (10.41, 0.67 - 312.27)	*
NHA	2012	30	30 (9)	2.31 (1.16, 0.27 - 26.43)	
	2013	31	51.6 (16)	121.64 (51.47, 4.69 - 1400.91)	
PPP	2013	30	93.3 (28)	1001.34 (292.82, 1.08 - 7427.23)	
	2014	30	0 (0)	0	
PTI	2013	30	80 (24)	486.15 (176.71, 0.43 - 4166.62)	
	2014	30	3.3 (1)	2.6 (2.6, 78.09 - 78.09)	
RCA	2012	31	3.2 (1)	4.4 (4.4, 136.48 - 136.48)	
	2013	30	20 (6)	63.39 (58.13, 2.61 - 1746.99)	
RPP	2012	33	0 (0)	0	*
	2013	30	93.3 (28)	216.69 (60.06, 3.79 - 1230.34)	*
	2014	30	13.3 (4)	0.93 (0.59, 0.82 - 15.19)	*
SAL	2012	3	0 (0)	0	
SAU	2012	6	0 (0)	0	
SCR	2012	2	100 (2)	343.73 (342.78, 0.95 - 686.51)	

Site	Year	Sample size	% Prevalence (N = Individuals *Bd* positive)	Mean infection intensity (SE, range)	Repeat sampled (2012, 2013 & 2014)
SL1	2012	32	9.4 (3)	6.6 (4.12, 41.12 - 117.01)	*
	2013	31	38.7 (12)	6.16 (3.1, 0.16 - 91.78)	*
	2014	31	22.6 (7)	9.09 (4.92, 0.41 - 138.03)	*
SS	2013	1	0 (0)	0	
STL	2012	30	3.3 (1)	0.02 (0.02, 0.59 - 0.59)	
	2013	31	3.2 (1)	0.1 (0.1, 3.22 - 3.22)	
STR	2012	32	59.4 (19)	65.95 (46.1, 0.6 - 1479.94)	
	2013	30	16.7 (5)	3.88 (3.36, 1.33 - 101.02)	
SW2	2012	34	2.9 (1)	9.79 (9.79, 332.92 - 332.92)	*
	2013	30	50 (15)	45.84 (20.67, 3.37 - 528.43)	*
	2014	30	0 (0)	0	*
TIM	2012	1	100 (1)	0.85 (0, 0.85 - 0.85)	
	2013	32	0 (0)	0	
VER	2012	30	10 (3)	0.41 (0.33, 0.87 - 9.89)	
	2013	30	93.3 (28)	1062.49 (290.73, 4.14 - 6221.99)	
WAN	2012	6	0 (0)	0	
	2013	31	3.2 (1)	1.64 (1.64, 50.99 - 50.99)	
WIN	2012	24	8.3 (2)	0.46 (0.43, 0.69 - 10.41)	
	2013	15	53.3 (8)	5.87 (2.3, 0.77 - 29.9)	
WP1	2012	32	0 (0)	0	*
	2013	30	43.3 (13)	45.49 (22.89, 2.7 - 601.2)	*
	2014	30	0 (0)	0	*
TOTAL		2223	28.9 (644)	61.79 (7.82, 0.02 - 7427.23)	14 sites

Figure 2.2: The distribution of the *R. pipiens* throughout Ontario. Includes both historic and recent observations compiled from data recorded by Bird Studies Canada: Marsh Monitoring Program, and Ontario Nature: Ontario Reptile and Amphibian Atlas and the Original Herpetofaunal Summary. Atlas squares are based on a 5 x 5 km grid. Areas without squares do not indicate the absence of the species, but only that there are no observation data to confirm the presence of the species in those areas. *(Figure: author's own, created in QGIS version 2.18).*



2.3.2 Laboratory analysis of samples

All toe-clip samples were stored at 4 °C until processing (Hyatt *et al*. 2007). DNA was extracted using QIAGEN DNeasy Kits following the standard protocol. The presence⁄ absence and quantity of *Bd* was then assessed using the real-time PCR protocol described by Boyle *et al*. (2004). Extractions were diluted 1/10 and all assays were performed in duplicate, to ensure reliability of results. Samples were considered *Bd* positive when a clear log-linear amplification was evident and both repeats indicated a detection limit of > 0.1 mean genomic equivalents (GE).

2.3.3 Acquisition of environmental data

Each site locality was assigned to a drainage basin based on HydroSHED 30 arc seconds (~1 km) basin delineations, downloaded from USGS HydroSHEDS (Lehner *et al*. 2008). Mean monthly precipitation was obtained as environmental layers at 30 arc seconds (~1 km) resolution, which were downloaded from the WorldClim data set (version 1.4; Hijmans *et al*. 2005). Hydroshed range size and mean monthly precipitation were obtained by extracting the raw value of each variable, at all site localities from their raster source. River and road cartographic boundary files were obtained from the 2011 Statistics Canada census (Statistics Canada 2011a) and the National Road Network for Ontario Geobase (Government of Canada 2015), respectively. Both were downloaded at 1 km^2 resolution. The density of rivers and roads surrounding each site locality was obtained by calculating the extent to which each spatial line dataframe overlaps a cell within a spatially explicit (1 km^2) blank raster, and subsequently extracting the mean value of all raster cells found in a radius of 10 km and 50 km around each site locality, respectively. The apportioned radius

for calculating road density was increased from 10 km to 50 km, in order to include maximum variation recorded in the dataset, with regards to habitat fragmentation and local disturbance. All data extractions were implemented using the R package 'raster' (Hijmans *et al*. 2010).

Mean daily air temperature (˚C), was compiled from data loggers activated at 10 sites (HOBO U23 Pro v2 Data Logger (U23-001)) and weather stations in close proximity to the remaining 31 sites (Saino *et al*. 2011; Figure 2.1). Data were collected from January 2011 to October 2015, in order to assess climatic trends occurring ± 1 year from sampling point. In an effort to achieve completeness of data, any gaps in the hourly records were filled with data from the second closest weather station (range: 1.4 – 44.69 km, mean: 17.4 km). This dataset allowed me to estimate the following annual variables (for 2011 to 2015): '*onset of spring*': first occasion on which the mean daytime temperature exceeded zero degrees Celsius for fourteen consecutive days; '*calling date*': first occasion on which the maximum daytime temperature reached fifteen degrees Celsius for ten consecutive days (Seburn 1992); '*active period*': period between onset of spring and calling date; and '*breeding period*': period between the calling date and the last day of June (MMP 2003; Stebbins 2003). I then quantified mean daily air temperature (˚C) and mean precipitation (mm) during both the active and breeding periods.

As all *R. pipiens* were sampled post active period, variables measured throughout the active period could be considered predictor variables. However, several individuals were sampled during the breeding period: ergo, mean daily air temperature (˚C) during breeding period included variation recorded post sampling. Consequently, it could not be considered a predictor variable, and was not included in the analysis. On

the other hand, mean precipitation (mm) throughout breeding period was based on monthly interpolations of observed data, representative of 1960 - 1990. This monthly figure remained static across years, and as such, could be considered a predictor variable.

I tested the following predictor variables based on the above hypotheses: hydroshed size (km^2), river density (within 10 km buffer from site centroid), mean precipitation (mm) throughout breeding period, mean precipitation (mm) throughout active period, mean daily air temperature (˚C) throughout active period, length of active period (days) and road density (within 50 km buffer from site centroid). Details regarding the variations (per grid cell), raw resolutions, year of record, unit and source for all spatial predictors are listed in Table 2.2. All variables were re-projected and re-sampled to the same equal area grid as the site localities, using spatial tools from the statistical software R (version 3.1.2; R Core Team 2015). All variables were z-transformed [(x-mean)/SD] prior to analysis to have a mean of 0 and standard deviation of 1. This put all predictors on a common scale and made main effects interpretable in the presence of interactions (Schielzeth 2010).

Table 2.2: Six environmental and one anthropogenic predictor variables were selected for use in model building based on their suitability for hypothesis testing.

Predictor	Variations (per grid cell)	Raw resolution	Year recorded	Unit	Prediction	Source
Hydroshed range size	total	30 arc seconds (~1 km)	2008	km^2	-	'USGS HyrdoSHEDS' http://hydrosheds.cr.usgs.gov Lehner *et al*. 2008
River density	mean	1 x 1 km (10 km mean extracted)	2011	km	+	'2011 Census -Rivers (lines)' https://www12.statcan.gc.ca/census-recensement/2011/geo/bound-limit/bound-limit-2011-eng.cfm Statistics Canada 2011a
Precipitation (during breeding period)	mean	30 arc seconds (~1 km)	1950 - 2000	mm	+	'Current Conditions: Precipitation' http://www.worldclim.org Hijmans *et al*. 2005
Precipitation (during active period)						
Air temperature (during active period)	mean	N/A	Jan. 2011 - Oct. 2015	°C	-	Genetics and Ecology of Amphibian Research Group
Length of active period (days between spring onset and calling date)	total			days	+	
Road density	mean	1 x 1 km (50 km mean extracted)	2015	mm	+	'National Road Network, ON' http://geogratis.gc.ca/api/en/nrcan-rncan/ess-sst/c0d1f299-179c-47b2-bcd8-da1ba68a8032.html Government of Canada 2015

2.3.4 Statistical analysis

All statistical analyses were implemented in R (version 3.1.2; R Core Team 2015). I defined *Bd* prevalence as the number of *Bd* positive *R. pipiens* within a site, divided by the total number of *R. pipiens* sampled within that site. I compared variation of prevalence and estimated differences in infection intensity between sites and years with χ^2 tests and univariate analysis of variance (ANOVA), respectively. Any significant results from ANOVA testing were further tested using Tukey post hoc test in order to determine which categorical groupings were different from the others. Presence of infection was compared between sites using a Fisher's exact test for count data. A Pearson correlation coefficient was used to determine the relationship between annual spring onset and first date of calling. Generalised Linear Models (GLM) were used to model *Bd* presence (Binomial errors) and intensity (Negative Binomial errors) in response to latitude. These results were then compared with intercept only models to determine the statistical significance of increasing latitude. Linear regression was also used to assess the relationships between spring onset or calling date, with each of the predictor variables, as well as for spring onset or calling date across years. The Potthoff Whittinghill test statistic (Potthoff & Whittinghill 1966) was used to assess the null hypothesis of homogeneity among all the relative risks. Under the null hypothesis, the number of cases in each site is Poisson distributed whereas the alternative hypothesis is one of over dispersion, represented by the negative binomial distribution. I implemented this using pottwhitt.stat in the R package 'DCluster' (Gómez-Rubio *et al*. 2015).

Due to the sampling method, not all sites were sampled each year. Consequently, I restricted the analysis to a subset of sites for which I had at least 24 post-metamorphic

individuals repeat sampled in each of the following years: 2012, 2013 and 2014 (n = 14 sites; Table 2.1). I constructed two separate generalized linear mixed models (GLMM), using a Binomial error structure for the *Bd* prevalence model (R package 'lme4'; Bates *et al*. 2015) and a Negative Binomial error structure to the *Bd* intensity of infection model (mean genomic equivalents) (R package 'glmmADMB'; Fournier *et al*. 2012). I accounted for possible non-independence of samples collected at the same site locality by including a random intercept effect for site ID (n = 14), and added year as a fixed effect. I did not expect serial autocorrelation to be present in the data as sampling occurred at yearly intervals. Excluding all models that included confounding pairs of covariates (absolute correlation coefficient > 0.5: Table 2.3), I constructed a set of 26 competing candidate models based on the above hypotheses. These were tested against both response variables at site level: *Bd* prevalence and *Bd* intensity of infection (mean genomic equivalents). I used an information-theoretic model selection process to rank models based on their Akaike's information criterion value (quasi-Akaike information criterion was used with regards to the *Bd* prevalence model in order to correct for overdispersion; Burnham & Anderson 2002). After applying the 'nesting rule', in which models that are more complex versions of models with better support (lower AIC) are removed (Richards 2008; Richards *et al*. 2011), I considered models under a delta-2 threshold (Burnham & Anderson 2002). The nesting rule prevents the retention of overly- complex models that do little to improve the fit to the data (Arnold 2010). R^2 values were calculated with the rsquared.glmm function (R package 'MuMIn', Barton 2011). Unfortunately, the rsquared.glmm function does not recognize the glmmADMB package, and as such, there are no R^2 values for the *Bd* intensity of infection model. I used the 'sim' function (R package 'arm'; Gelman & Hill 2007) to simulate values of the posterior

distribution of the model parameters, in order to plot predicted relationships. Ninety-five per cent credible intervals (CI) around the mean were extracted based on 1000 simulations (Gelman & Hill 2007). All graphs were created using ggplot (R package 'ggplot2; Wickham 2009).

Table 2.3: Absolute correlation coefficient for environmental predictor variables. All combinations with an absolute correlation coefficient of > 0.5 are excluded from future modelling (see 'Retained'). **Active period length**, number of days between onset of spring and calling date; **Active period mean temperature**, mean daily air temperature (˚C) throughout active period; **Active period mean precipitation**, mean precipitation (mm) throughout active period; **Breeding period mean precipitation**, mean precipitation (mm) throughout breeding period; **River density**, river density within 10 km buffer from site centroid; **Hydroshed range size**, size of hydroshed (km^2); **Road density**, road density within 50 km buffer from site centroid.

Predictor #1	**Predictor #2**	**cor**	**Retained**
Active period length	Active period mean temperature	0.22	✓
Active period length	Active period mean precipitation	0.01	✓
Active period mean temperature	Active period mean precipitation	0.09	✓
Active period length	Breeding period mean precipitation	0.16	✓
Active period mean temperature	Breeding period mean precipitation	0.19	✓
Active period mean precipitation	Breeding period mean precipitation	0.85	✗
Active period length	River density	0.03	✓
Active period mean temperature	River density	-0.09	✓
Active period mean precipitation	River density	-0.15	✓
Breeding period mean precipitation	River density	-0.22	✓
Active period length	Road density	-0.06	✓
Active period mean temperature	Road density	-0.03	✓
Active period mean precipitation	Road density	-0.26	✓
Breeding period mean precipitation	Road density	-0.18	✓
River density	Road density	0.71	✗
Active period length	Hydroshed range size	0.21	✓
Active period mean temperature	Hydroshed range size	0.14	✓
Active period mean precipitation	Hydroshed range size	0.04	✓
Breeding period mean precipitation	Hydroshed range size	0.27	✓
River density	Hydroshed range size	-0.06	✓
Road density	Hydroshed range size	-0.13	✓

2.4 RESULTS

I collected and processed 2223 toe clips from post-metamorphic *R. pipiens*, captured from 41 sites in Ontario. Of the 2223 *R. pipiens* sampled: 833 were sampled in 2012; 878 were sampled in 2013; and 512 were sampled in 2014. Overall prevalence was 28.9 %, and strength of infection indicates that detections were unambiguous (Table 2.1). Infection was detected across a broad geographic range (36 of 41 sites were infected; Figure 2.1) and despite presence of strong infections (maximum GE = 7427.23: Table 2.1) no mortality was detected.

2.4.1 Variation in annual *Bd* infection

Prevalence in 2013 was significantly higher than 2012 and 2014 ($\chi^2 = 69.7$, $p < 0.0001$, df = 1; $\chi^2 = 149.2$, $p < 0.0001$, df = 1, respectively; Figure 2.3). I recorded a 79.8 % increase in prevalence between 2012 and 2013, and a 73.1 % decrease in prevalence between 2013 and 2014. Infection intensity (GE) was also greatest in 2013 (mean GE [SE] = 138.8 [19.1], maximum GE = 7427.2, $F_{2, 2220} = 32.8$, $p < 0.0001$) than 2012 (mean GE [SE] = 16.9 [4.4], maximum GE = 2708.3; $p < 0.0001$) or 2014 (mean GE [SE] = 2.7 [0.8], maximum GE = 312.3; $p < 0.0001$; Figure 2.4).

In order to evaluate interannual variation of infection metrics and the relationship between prevalence and intensity, I categorized all sites repeat sampled in 2012 and 2013 into three separate groups: 1) those that exhibited an increase in prevalence (n = 9 of 22 sites); 2) those changing from *Bd* absent (prevalence = 0) to *Bd* present (prevalence ≥ 1) (n = 6 of 22 sites); and 3) those that exhibited a decrease in prevalence (n = 7 of 22 sites). There was significant variation in mean infection loads between the three categories ($F_{2, 670} = 10.1$, $p < 0.0001$; Table 2.4). While there was no

variation between sites exhibiting an interannual decrease in prevalence and those transitioning between *Bd* absent to present, sites displaying an interannual increase in prevalence presented much greater mean infection loads in 2013, than the other two categories ($p < 0.0001$ and $p < 0.01$, respectively). This was also carried out for all sites repeat sampled in 2013 and 2014, but due to low prevalence and infection loads in 2014, there was no significant difference between categories (Table 2.4).

2.4.2 Spatial distribution of *Bd* infection

I modelled *Bd* prevalence as a function of *Bd* intensity (GE; both linearly and as a 2nd order polynomial), and found a positive relationship between the two variables (GLM: $F = 62.43$, $p < 0.0001$; Figure 2.5). In support of this relationship, site localities exhibiting high prevalence (> 66.6 %), exhibited higher infection loads (mean [SE] = 143.6 [23.1], maximum = 3371.3) than regions with moderate (33.3 % - 66.6 %; mean [SE] = 22.2 [5.4], maximum = 601.2; $p < 0.001$) or low prevalence levels (< 33.3 %; mean [SE] = 2.6 [0.7], maximum = 332.9; $p < 0.001$).

The Potthoff Whittinghill test statistic (T) was significant for both 2012 and 2013 allowing me to reject the null hypothesis of homogeneity of relative risks (T_{2012} = 95776.5, p = 0.01; T_{2013} = 221734.2, p = 0.01) (Wakefield *et al*. 2000). However, the spatial scan statistic detected no clusters of infection. In complement to this observation, 2012 and 2013 sites varied in prevalence ($\chi^2 = 255.2$, $p < 0.001$, df = 13; $\chi^2 = 165.3$, $p < 0.001$, df = 13, respectively) and intensity ($F_{33, 799} = 2.7$, $p < 0.001$; $F_{33, 842} = 7.3$, $p < 0.0001$; Figure 2.6 and Figure 2.7). However, neither prevalence nor intensity varied between sites in 2014, as both infection measures remain consistently low (Figure 2.3 and Figure 2.4). Additionally, there was no relationship between *Bd* prevalence or intensity, and latitudinal gradient ($p = 0.9$; $p = 0.4$, respectively).

Figure 2.3: Stacked barcharts representing *Bd* prevalence (%) and infection intensity (GE), by year. Individual counts of *Bd* intensity (categorized by GE) are labeled in blue; proportion of infected and non-infected individuals are labeled as percentages; and samples sizes are denoted on top. Note significantly greater prevalence and higher infection intensity (GE) recorded in 2013.

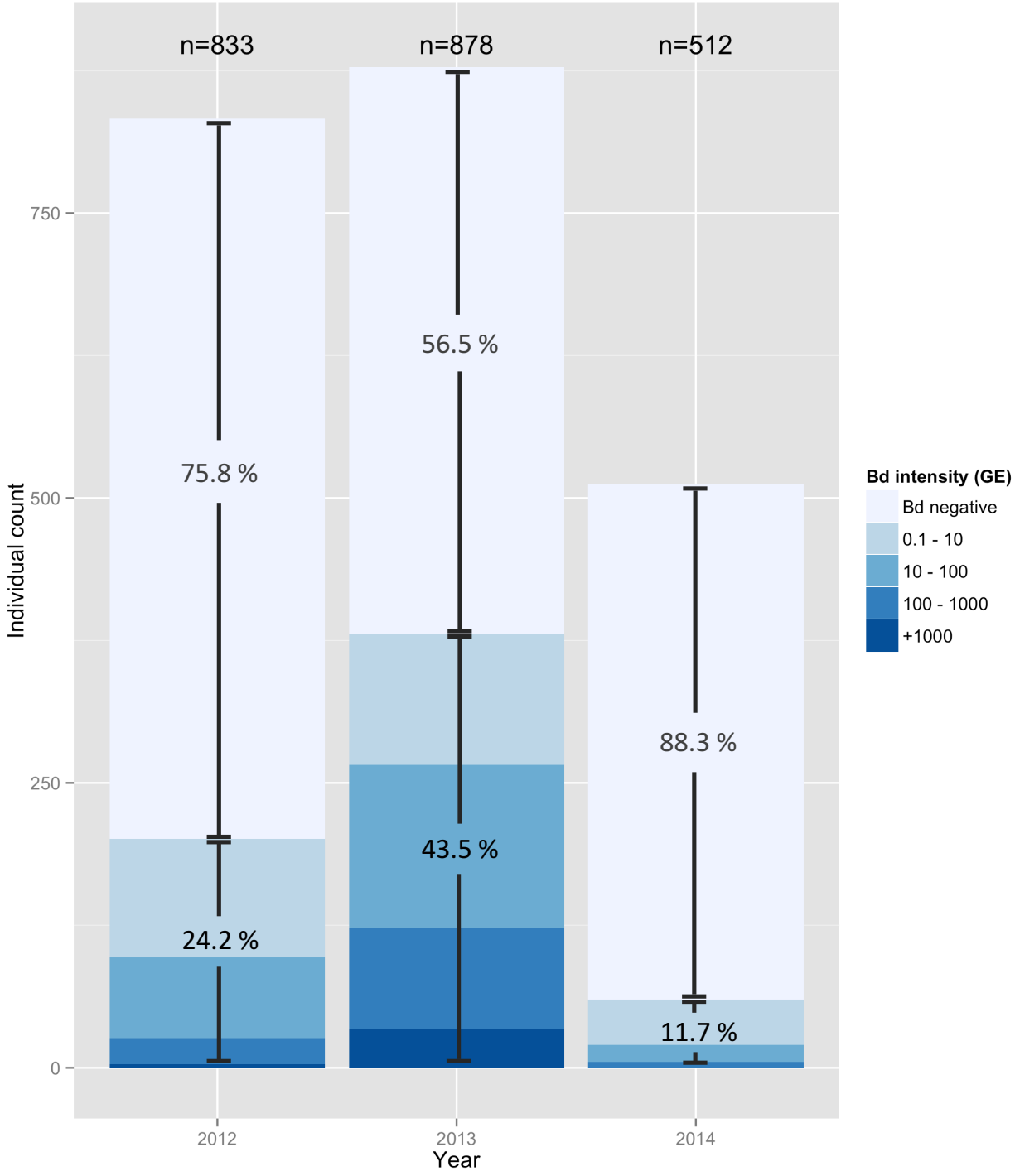


Figure 2.4: Density plot of log *Bd* intensity (GE), by year. Note the lower density of zero/ low infections in 2013, in comparison to 2012 and 2014, and again a greater density of strong infections in 2013, in comparison to 2012 and 2014.

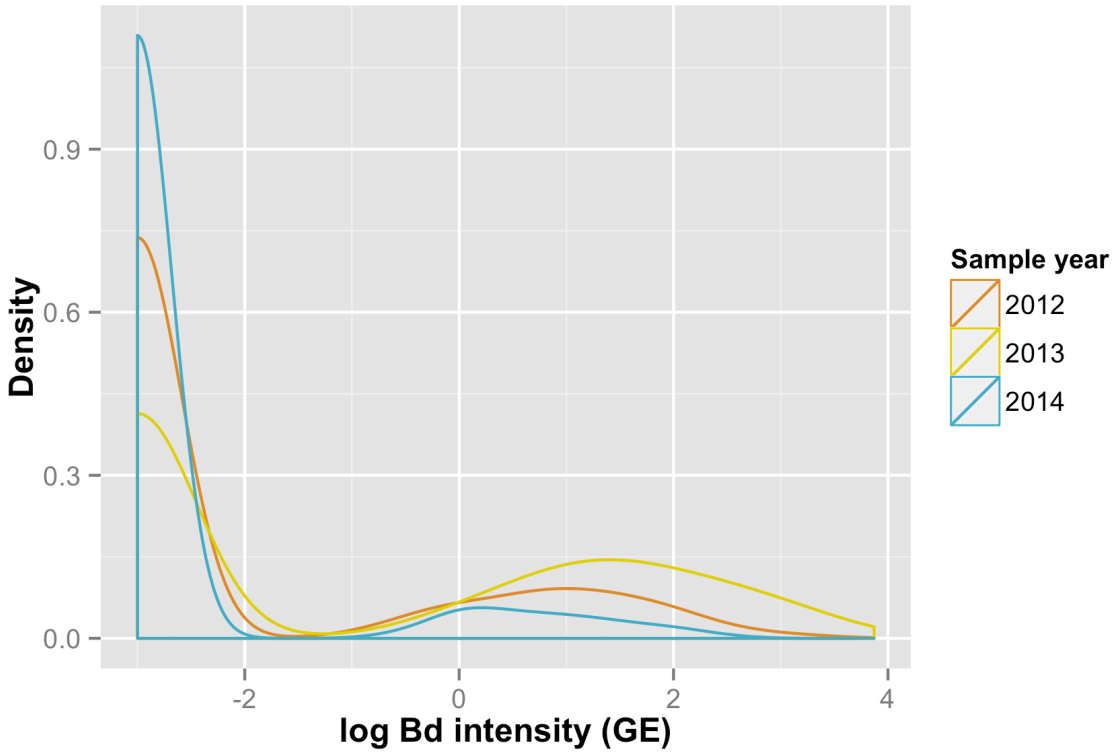


Table 2.4: Mean *Bd* intensity (GE) for sites exhibiting one of the following: an interannual increase in prevalence; an interannual decrease in prevalence; a transition from *Bd* absence to presence; or a transition from *Bd* presence to absence. Between 2012 and 2013, sites displaying an interannual increase in prevalence presented much greater mean infection loads in the latter year, than the other two categories. Between 2013 and 2014, there was no significant difference between categories.

	***Bd* intensity (GE) in the later year**			
	n = Individuals (Sites)	**$\bar{x}$**	**se**	**max**
2012-2013				
Increase in prevalence	276 (9)	208.7	41.3	6222.0
Absence to presence	181 (6)	68.4	18.6	2407.0
Decrease in prevalence	216 (7)	30.7	9.1	1323.6
TOTAL	673 (22)	113.8	18.2	6222.0
2013-2014				
Presence to absence	90 (3)	0	0	0
Decrease in prevalence	368 (12)	3.5	1.1	312.3
TOTAL	458 (15)	2.8	0.9	312.3

Figure 2.5: Scatterplot representing positive relationship between *Bd* prevalence (%) and mean *Bd* intensity (GE; 2nd order polynomial) at the 14 repeat sampled sites (black dots). After an initial rapid exponential increase, the mean infection intensity reaches a plateau. Shaded area spans the 95% credible intervals for the fitted means.

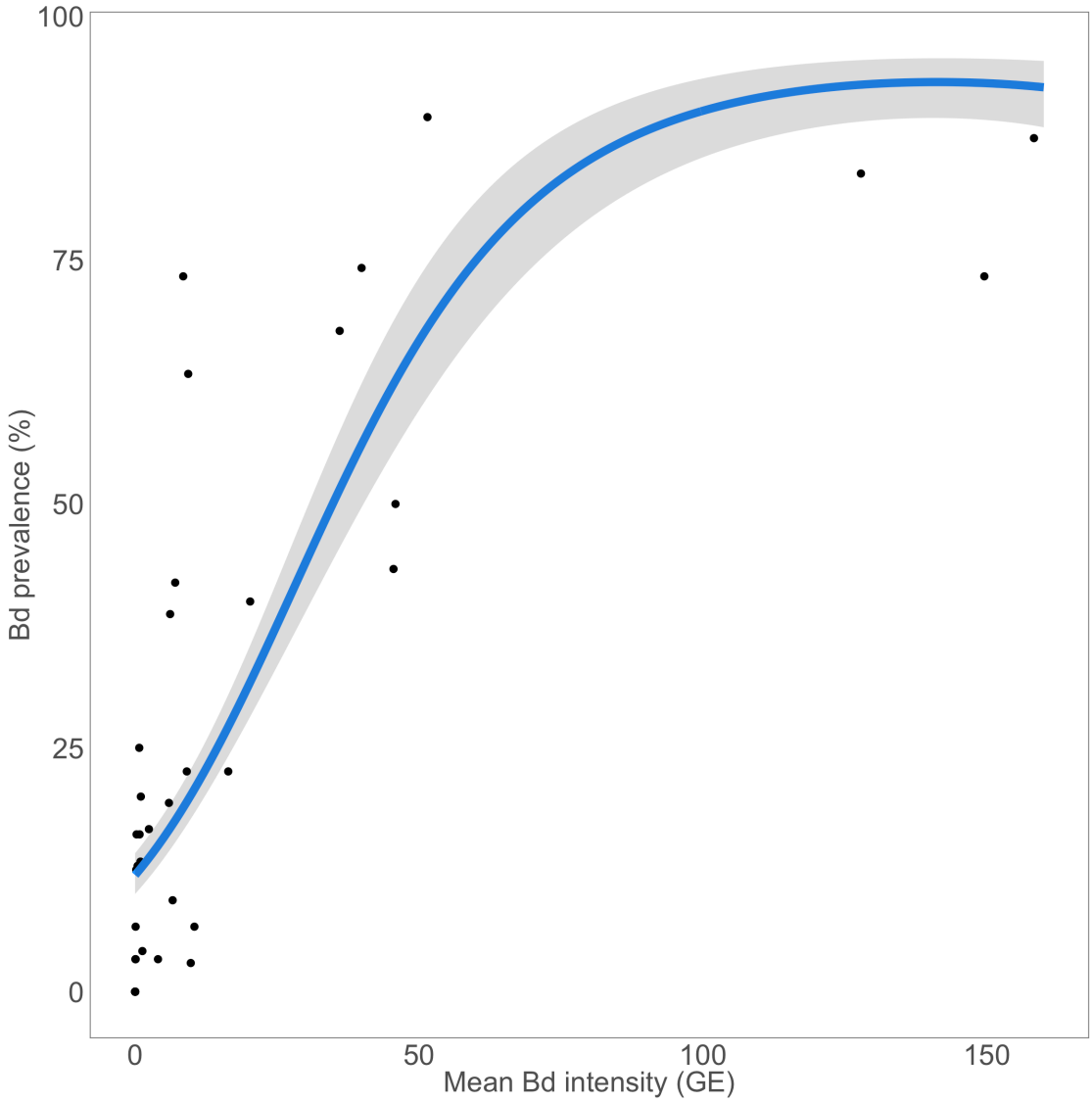


Figure 2.6: Stacked barcharts representing the *Bd* prevalence (%) and infection intensity (GE), by year and site. Sites have comparable sample sizes, thus proportions are comparable. Within a site, % of individuals are grouped by *Bd* intensity (GE) categories (including *Bd* negative), which are labeled in blue. Note significantly greater prevalence and higher infection intensity (GE) recorded in 2013. 11 of the 14 sites exhibited an increase in prevalence between 2012 and 2013, and 12 of the 14 sites exhibited a decrease in prevalence between 2013 and 2014. Oddities include: BRP (steady increase); CLI (dramatic decrease in 2014); CON (dramatic decrease in 2013); and EDU (overall very low infection, but lowest prevalence in 2013).

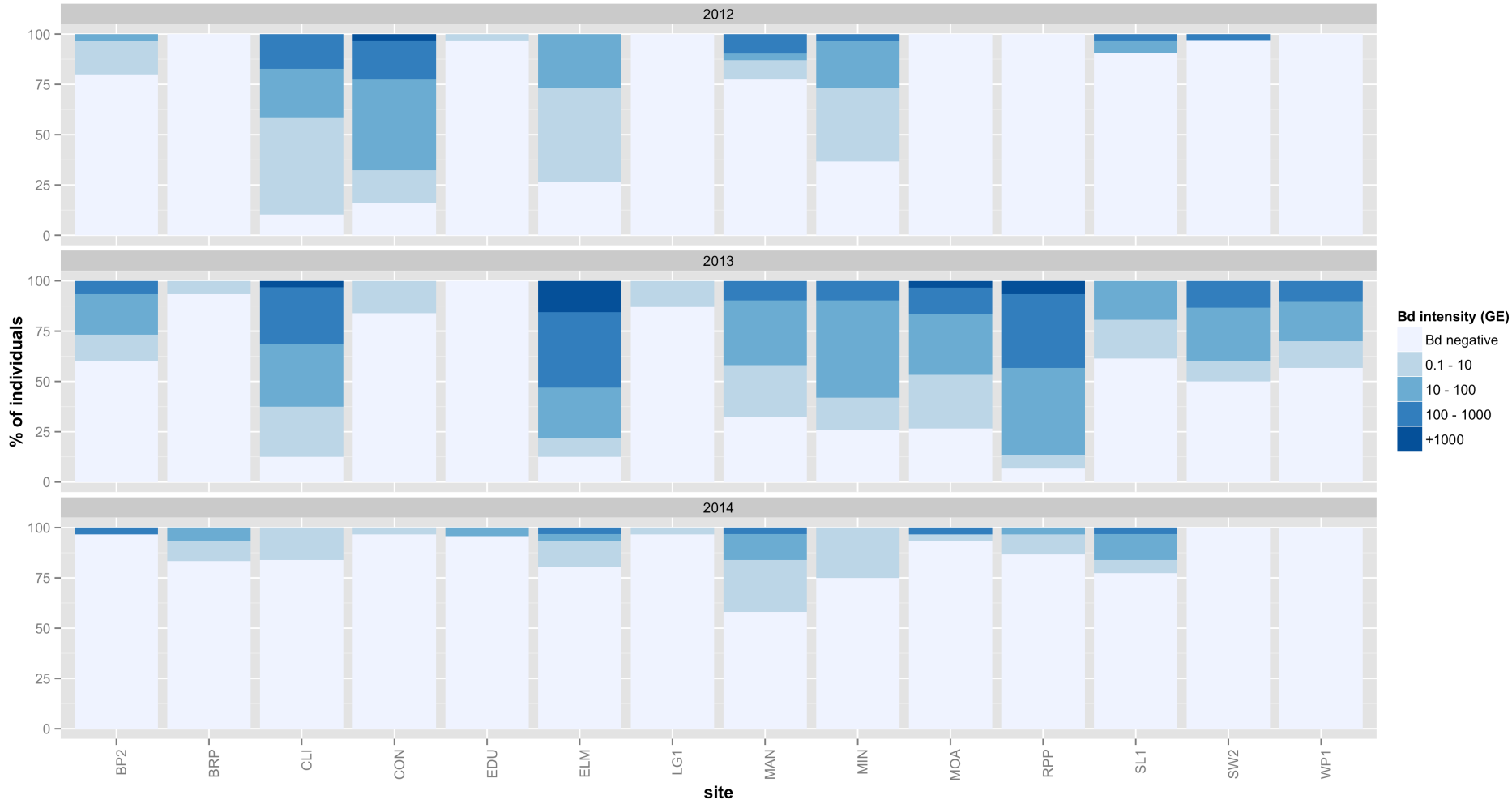
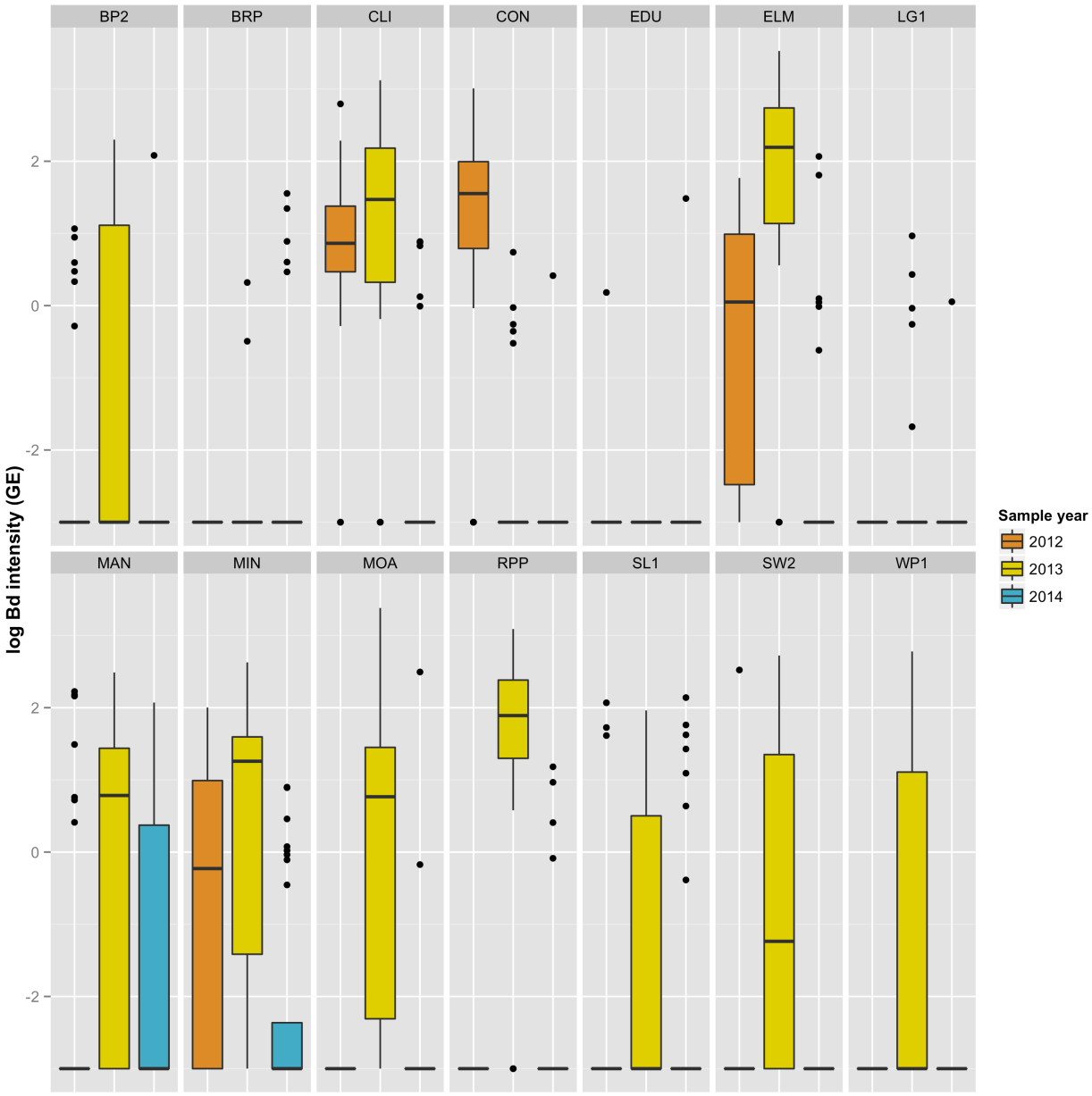


Figure 2.7: Boxplots representing log *Bd* intensity (GE) variation between sites, per year. All sites repeat sampled (24 + individuals) in 2012, 2013 and 2014. Note the high infection rate in 2013 in comparison to 2012 and 2014.



2.4.3 Environmental covariates

Compiling all years, I calculated that spring typically started in week 13, which falls within the month of March. However, the specific date differed significantly between regions. Spring commenced significantly later in the north than in the central or southern regions ($F_{2, 162} = 12.1$, $p < 0.0001$; Table 2.5), while the latter regions presented similar seasonal foundations. The maximum delay between regional onset of spring was 8 weeks in length (between north and south). Within a region, spring onset had the capacity to change considerably between years. The south and central regions exhibited similar inter-annual fluctuations (range = - 12.7 to 19.7 decimal days; range = - 17.1 to 24.8 decimal days, respectively; Table 2.6), while the north presented the greatest level of inter-annual variation (range = - 27.4 to 38.6 decimal days; Table 2.6). Averaging across all years, the north exhibited a move towards an earlier spring date, with an advancement of 0.35 days per annum ($F_{4, 50} = 41.2$, $p < 0.0001$); while the south and central regions exhibited a move towards a later spring date, with an average delay of 0.30 ($F_{4, 45} = 7.9$, $p < 0.0001$) and 0.63 days per annum ($F_{4, 55} = 45.3$, $p < 0.0001$), respectively (Table 2.6). The annual spring onset and first date of calling were weakly, but significantly correlated ($r = 0.2$, $p < 0.01$).

Calling date fell within the month of May, and again, the specific date differed between regions ($F_{2, 162} = 5.9$, $p < 0.01$). Week of calling was calculated to be significantly later in the north, than in the central or southern sites ($p < 0.05$; $p < 0.01$, respectively; Table 2.5), while the latter regions exhibited similar temporal breeding foundations. The maximum difference between calling dates was 14 weeks in length (between north and south). Within a region, calling date also changed considerably between years. The north and central regions exhibited similar inter-

annual fluctuations (range = - 14.6 to 9.7 decimal days; range = - 21.7 to 16.2 decimal days, respectively), while the south presented the greatest level of inter-annual variation (range = - 46.4 to 24.5 decimal days; Table 2.7). Averaging across all years, each region showed a significant shift towards an earlier breeding season. The south exhibited an advancement of 2.05 days per annum ($F_{4, 45} = 10.9$, $p < 0.0001$); while the north and central regions exhibited more substantial advancements of 6.40 ($F_{4, 50} = 6.8$, $p < 0.001$) and 6.53 days per annum ($F_{4, 55} = 10.3$, $p < 0.0001$), respectively (Table 2.7).

The active period typically lasted for 41 days (SE = 1.6, maximum = 94; Table 2.5). However, having established that regions may experience substantial shifts in both spring onset and calling date, one would assume that the length of active period would vary across latitudes. Yet, when all years are compiled, the three regions exhibited similar active period lengths (Table 2.5). Additionally, averaging across years, all regions exhibited a shortening of the active period (Table 2.8). The south exhibited the least inter-annual variation regarding active period length, and revealed a shortening of the period with an average reduction of 2.35 days per annum. In contrast, the central and northern regions exhibited a substantial shortening of the active period, with an average reduction of 7.18 and 6.05 days per annum, while also experiencing significantly different interannual variations (Table 2.8).

Table 2.5: Summary statistics for spring onset (decimal week), calling date (decimal week) and activity period (days), by region, from 2011 to 2015.

	Spring onset (decimal week)				Calling date (decimal week)				Activity period (days)			
	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range
NORTH	13.5	0.3	17	7	19.5	0.3	24	8	41.3	2.9	94	89
CENTRAL	12.5	0.2	15	5	18.2	0.3	24	13	39.1	2.3	85	83
SOUTH	11.9	0.2	15	6	17.9	0.4	22	12	41.6	3.0	80	78

Table 2.6: Spring onset (decimal date) variation between regions, by year. $\mathbf{Y_D}$, difference in decimal dates between current year and the previous year; $\mathbf{T_V}$, total inter-annual variation in decimal days irrespective of directionality; $\mathbf{T_{VD}}$, total inter-annual variation in decimal days respective of directionality; $\mathbf{T_{VD} / Y}$, average variation per annum in decimal days, respective of directionality.

	Spring onset (decimal date), by region and year					
	NORTH		CENTRAL		SOUTH	
	Decimal date	Y_D	Decimal date	Y_D	Decimal date	Y_D
2011	98.4		88.1		84.3	
2012	71.0	- 27.4	71.0	- 17.1	71.6	- 12.7
2013	109.6	+ 38.6	95.8	+ 24.8	91.3	+ 19.7
2014	101.6	+ 8.0	96.1	+ 0.3	88.4	- 2.9
2015	97.0	- 4.6	90.6	- 5.5	85.5	- 2.9
$\mathbf{T_V}$		78.6		47.7		38.2
$\mathbf{T_{VD}}$		- 1.4		+ 2.5		+ 1.2
$\mathbf{T_{VD} / Y}$		- 0.35		+ 0.63		+ 0.30

Table 2.7: Calling date (decimal date) variation between regions, by year. $\mathbf{Y_D}$, difference in decimal dates between current year and the previous year; $\mathbf{T_v}$, total inter-annual variation in decimal days irrespective of directionality; $\mathbf{T_{VD}}$, total inter-annual variation in decimal days respective of directionality; $\mathbf{T_{VD}}$ **/ Y,** average variation per annum in decimal days, respective of directionality.

	Calling date (decimal date), by region and year					
	NORTH		**CENTRAL**		**SOUTH**	
	Decimal date	$\mathbf{Y_D}$	**Decimal date**	$\mathbf{Y_D}$	**Decimal date**	$\mathbf{Y_D}$
2011	150.6		142.8		143.4	
2012	139.1	- 11.5	121.1	- 21.7	97.0	- 46.4
2013	129.9	- 9.2	120.0	- 1.1	121.5	+ 24.5
2014	139.6	+ 9.7	136.2	+ 16.2	132.2	+ 10.7
2015	125.0	-14.6	116.7	- 19.5	135.2	+ 3.0
$\mathbf{T_V}$		45.0		58.5		84.6
$\mathbf{T_{VD}}$		- 25.6		- 26.1		- 8.2
$\mathbf{T_{VD}}$ **/ Y**		- 6.40		- 6.53		- 2.05

Table 2.8: Variation in length of active period (days) between regions, by year. $\mathbf{Y_D}$, difference in length (days) between current year and the previous year; $\mathbf{\%_V}$, percentage change in length (days) between current year and the previous year; $\mathbf{T_v}$, total inter-annual variation in days irrespective of directionality; $\mathbf{T_{VD}}$, total inter-annual variation in days respective of directionality; $\mathbf{T_{VD}}$ **/ Y,** average variation per annum in days, respective of directionality.

	Active period (days), by region and year								
	NORTH			**CENTRAL**			**SOUTH**		
	Days	$\mathbf{Y_D}$	$\mathbf{\%_V}$	**Days**	$\mathbf{Y_D}$	$\mathbf{\%_V}$	**Days**	$\mathbf{Y_D}$	$\mathbf{\%_V}$
2011	52.2			54.8			59.1		
2012	68.1	+ 15.9	+ 30.5	50.1	- 4.7	- 8.6	25.4	- 33.7	- 57.0
2013	20.3	- 47.8	- 70.2	24.3	- 25.8	- 51.5	30.2	+ 4.8	+ 18.9
2014	38.0	+ 17.7	+ 87.2	40.1	+ 15.8	+ 72.8	43.8	+ 13.6	+ 45.0
2015	28.0	- 10.0	- 26.3	26.1	- 14.0	- 24.9	49.7	+ 5.9	+ 13.5
$\mathbf{T_V}$		91.4			60.3			58	
$\mathbf{T_{VD}}$		- 24.2			- 28.7			- 9.4	
$\mathbf{T_{VD}}$ **/ Y**		- 6.05			- 7.18			- 2.35	

Compiling all regions, the length of active period differed significantly between years ($F_{4, 160} = 15.9$, $p < 0.0001$) with 2013 exhibiting a shorter active period (mean [SE] = 24.7 [2.2], maximum = 64) than 2012 (mean [SE] = 48.6 [4.8], maximum = 94; $p < 0.0001$) or 2014 (mean [SE] = 40.5 [1.7], maximum = 62; $p = 0.3$; $p < 0.01$). This pattern was mostly consistent between regions: the north and central regions depicted a significant drop in length of active period from 2012 to 2013 (- 70.2 % and – 51.5 %, respectively), and an increase between 2013 and 2014 (87.2 % and 72.8 %, respectively). However, the south saw a steady increase in length of active period from 2012 to 2014 (Table 2.8).

In general, *R. pipiens* experienced cooler temperatures during their active period in 2013 in comparison to 2012 ($F_{1, 77} = 7.9$, $p < 0.01$; Table 2.9). As temperatures recorded in 2014 fall between the means recorded in both 2012 and 2013, there was no significant difference between these years, nor was there a significant difference between regions. On the other hand, mean precipitation (mm) throughout the active or breeding period did not differ between years ($p = 0.8$ and $p = 0.8$, respectively; Table 2.9), but both measures differed between regions ($F_{2, 87} = 38.9$, $p < 0.0001$; $F_{2, 87} = 45.2$, $p < 0.0001$, respectively; Table 2.10). Within each year, the south consistently experienced significantly wetter conditions than the north or central regions, during both the active and breeding period. The north and the central regions experienced similar levels of precipitation, throughout.

The density of rivers, within a 10 km buffer surrounding each site, was greater in the central region than in the north ($F_{1, 100} = 8.7$, $p < 0.05$). However, river densities in the south were not significantly different from the northern ($p = 0.1$) or central ($p = 0.2$) sites (Table 2.11). Size of hydroshed (km^2) was also greater in the central region, than

the south ($F_{1, 88} = 10.3$, $p < 0.01$). However, hydroshed sizes in the north were not significantly different from the central ($p = 0.1$) or southern ($p = 0.1$) sites (Table 2.11). This suggests that the central region has both the largest water bodies, and the greatest number of tributaries leading to and from each watershed. Additionally, river density was lowest in the northern sites, while watershed size was smallest in the southern sites. Finally, road density, measured within a 50 km buffer of all site localities, differed significantly between the north and south ($F_{1, 86} = 18.8$, $p < 0.0001$) and the north and central ($F_{1, 100} = 38.0$, $p < 0.0001$) regions. While the southern and central regions exhibited similar high road densities ($p = 0.1$), the northern sites had a significantly lower concentration of roads (Table 2.11).

Table 2.9: Summary statistics for mean daily air temperature (˚C) throughout active period, and mean precipitation (mm) throughout both: active period and breeding period, within all sample years.

	Mean daily air temperature (˚C) throughout active period				**Mean precipitation (mm) throughout active period**				**Mean precipitation (mm) throughout breeding period**			
	x̄	**se**	**max**	**range**	**x̄**	**se**	**max**	**range**	**x̄**	**se**	**max**	**range**
2012	7.7	0.2	10.2	4.4	66.3	1.0	76.5	20.5	72.6	0.9	81.0	18.5
2013	6.9	0.2	11.7	6.8	69.5	1.3	88.0	31.0	73.0	1.1	86.5	23.0
2014	7.5	0.2	9.8	4.1	66.3	1.2	76.5	17.5	72.0	1.3	80.0	17.5

Table 2.10: Summary statistics for mean precipitation (mm) during both the active period and the breeding period, across the three regions (north, central and south), within all sample years.

	Mean precipitation (mm)											
	2012				**2013**				**2014**			
	x̄	**se**	**max**	**range**	**x̄**	**se**	**max**	**range**	**x̄**	**se**	**max**	**range**
Active period												
NORTH	61.4	1.0	67.5	11	64.9	1.7	74.5	16.5	63.1	1.0	66.0	7.0
CENTRAL	65.4	1.4	72.5	16.5	66.2	1.5	73.0	16.0	66.5	2.1	73.5	13.5
SOUTH	72.1	1.3	76.5	11.5	77.0	1.5	88.0	17.5	72.6	1.4	76.5	6.0
Breeding period												
NORTH	70.5	2.0	81.0	18.5	70.5	1.8	82.0	18.5	69.6	1.8	77.5	15.0
CENTRAL	70.6	1.0	79.0	16.0	68.0	0.9	76.0	12.0	70.7	1.3	76.5	9.5
SOUTH	77.4	0.7	80.0	7.0	80.4	1.1	86.5	11.5	78.8	0.6	80.0	2.5

Table 2.11: Summary statistics for road density (within 50 km buffer from site centroid), river density (within 10 km buffer from site centroid), and size of hydroshed (km^2), by region.

	Road density (within 50 km buffer)				**River density (within 10 km buffer)**				**Size of hydroshed (km^2)**			
	x̄	**se**	**max**	**range**	x̄	**se**	**max**	**range**	x̄	**se**	**max**	**range**
NORTH	6.5	0.6	13.6	12.7	0.8	0.1	2.9	2.9	5273.7	2008.1	60759.4	60451.5
CENTRAL	14.2	1.1	28.0	20.7	1.7	0.3	5.6	5.6	1872.5	173.4	4565.7	4410.3
SOUTH	11.5	1.1	24.6	20.0	1.2	0.2	3.8	3.8	1076.2	166.1	3629.0	3517.1

2.4.4 Outputs from GLMMs

The highest ranked model predicting for *Bd* prevalence comprised of an interaction between two variables: mean daily air temperature (˚C) during active period and length of active period (days; Table 2.12). Both variables were found to have a similar but mirrored effect upon the global mean for *Bd* prevalence (Figure 2.8). The marginal r^2 (variance explained by the fixed effects) of the top model was 16 %. All models within the delta-2 threshold (Burnham & Anderson 2002) included climatic variables measured throughout the active period; with only one model referring to the breeding period (Table 2.12, Model 'm19'); and zero models including geographic variables, such as: hydroshed area, river density or road density. Prevalence of *Bd* was negatively correlated with mean daily air temperature (˚C) during active period (Figure 2.9(A)), but prevalence also increased significantly as the length of active period was extended (days) (Figure 2.9(B)). More specifically, as length of active period extends, the negative relationship between *Bd* prevalence and mean daily air temperature lessens (Figure 2.10); while the variation in *Bd* prevalence remains relatively constant throughout the range of temperatures. Conversely, as length of active period shortens, the relationship between *Bd* prevalence and mean daily air temperature (˚C) becomes more negative, while variation in *Bd* prevelance fluctuates dependent on temperature. The highest prevalence outcome was predicted to occur when the active period has been shortened and when mean air temperature was coolest.

Table 2.12: Akaike's information criterion model rankings for the candidate models explaining *Bd* prevalence (%) at site level. Quasi-Akaike information criterion (QAIC) was used in order to correct for overdispersion. The top model for *Bd* intensity of infection (mean GE) at site level is highlighted in bold. ***k***, number of parameters; **logLik,** log likelihood; **QAIC**, Akaike's information criterion corrected for small sample size; **ΔQAIC**, difference in QAIC compared with the model with the lowest QAIC; ***wi***, model weight; **Retained**, models in the Δ2 QAIC set (grey shaded rows) are not retained if they are more complex versions of nested (simpler) models with better QAIC support (higher up in the table); **year**, year of sample date; **site**, accounted for possible non-independence of toe-clips collected at the same site locality by including this random intercept effect; **active period length**, number of days between onset of spring and calling date; **active period mean temperature**, mean daily air temperature (°C) throughout active period; **active period mean precipitation**, mean precipitation (mm) throughout active period; **breeding period mean precipitation**, mean precipitation (mm) throughout breeding period; **river density**, within 10 km buffer from site centroid; **hydroshed area**, (km^2); **road density**, within 50 km buffer from site centroid.

Model	Model description	*k*	logLik	QAIC	ΔQAIC	*wi*	Retained
m1	factor(year) + active period length * active period mean temperature + (1 | site)	7	-177.36	72	0	0.127	✔
m20	factor(year) + active period mean temperature + (1 | site)	5	-190.84	72.26	0.259	0.111	✔
m25	factor(year) + (1 | site)	4	-198.65	72.73	0.725	0.088	✔
m2	factor(year) + active period mean temperature + active period mean precipitation + (1 | site)	6	-186.32	72.83	0.831	0.084	✗
m5	factor(year) + active period length * active period mean precipitation + (1 | site)	7	-180.77	73.08	1.079	0.074	✔
m21	factor(year) + active period mean precipitation + (1 | site)	5	-195.40	73.7	1.698	0.054	✔
m19	factor(year) + breeding period mean precipitation + (1 | site)	5	-195.71	73.8	1.795	0.052	✔
m4	factor(year) + active period mean temperature + river density + (1 | site)	6	-190.23	74.06	2.064	0.045	
m3	factor(year) + active period mean temperature + hydroshed area + (1 | site)	6	-190.24	74.07	2.067	0.045	
m18	factor(year) + active period mean temperature + road density + (1 | site)	6	-190.79	74.24	2.241	0.041	
m24	factor(year) + hydroshed area + (1 | site)	5	-197.64	74.41	2.405	0.038	
m22	factor(year) + river density + (1 | site)	5	-198.12	74.56	2.555	0.035	
m23	factor(year) + road density + (1 | site)	5	-198.58	74.7	2.703	0.033	
m12	factor(year) + breeding period mean precipitation + hydroshed area + (1 | site)	6	-194.19	75.32	3.317	0.024	
m6	factor(year) + active period mean precipitation + hydroshed area + (1 | site)	6	-194.50	75.41	3.413	0.023	
m7	factor(year) + active period mean precipitation + river density + (1 | site)	6	-195.09	75.6	3.601	0.021	
m17	factor(year) + active period mean precipitation + road density + (1 | site)	6	-195.17	75.62	3.624	0.021	
m11	factor(year) + river density + breeding period mean precipitation + (1 | site)	6	-195.53	75.74	3.74	0.02	
m16	factor(year) + road density + breeding period mean precipitation + (1 | site)	6	-195.54	75.74	3.743	0.019	
m15	factor(year) + road density * breeding period mean precipitation + (1 | site)	7	-193.44	77.08	5.078	0.01	
m9	factor(year) + river density + breeding period mean precipitation + hydroshed area + (1 | site)	7	-193.95	77.24	5.241	0.009	
m14	factor(year) + road density + breeding period mean precipitation + hydroshed area + (1 | site)	7	-194.10	77.29	5.286	0.009	
m10	factor(year) + river density * breeding period mean precipitation + (1 | site)	7	-194.34	77.36	5.362	0.009	
m13	factor(year) + road density * breeding period mean precipitation + hydroshed area + (1 | site)	8	-191.82	78.57	6.568	0.005	
m8	**factor(year) + river density * breeding period mean precipitation + hydroshed area + (1 | site)**	**8**	**-192.30**	**78.72**	**6.717**	**0.004**	
m26	1 + (1 | site)	2	-289.60	97.44	25.442	0	

Figure 2.8: Effect sizes of predictor variables from QAIC top model, explaining variation in *Bd* prevalence (%) at site level. Black dotted line depicts the global mean for *Bd* prevalence when all predictor variables are averaged. Red point represents plus one standard deviation for each predictor variable, blue point represents minus one standard deviation for each predictor variable. **Length of active period** (number of days between onset of spring and calling date) and **mean daily air temperature (˚C) during active period**, were found to have similar but mirrored effects, upon the global mean for *Bd* prevalence. Combined, the interaction had a slightly lesser effect upon the global mean for *Bd* prevalence.

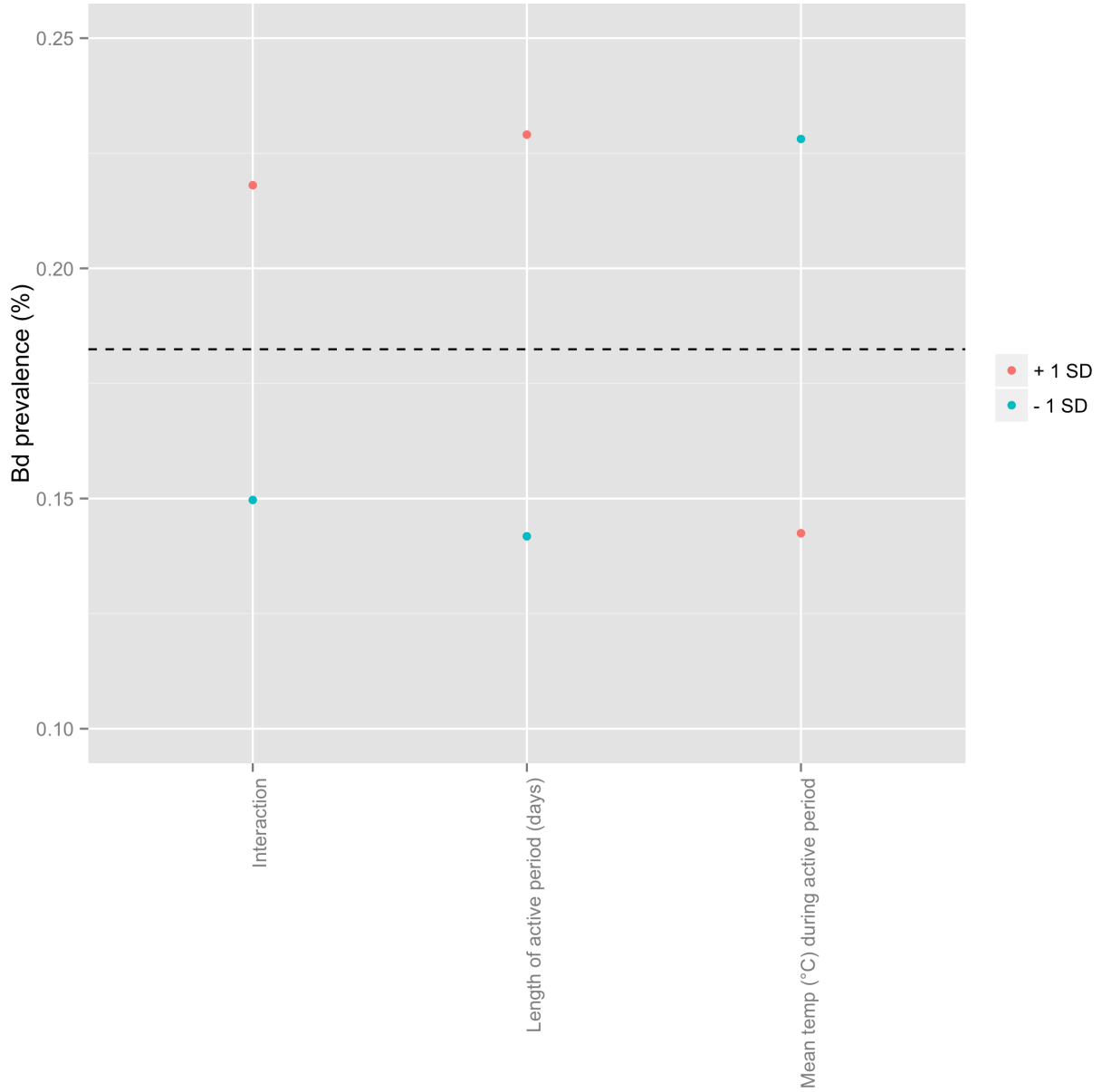
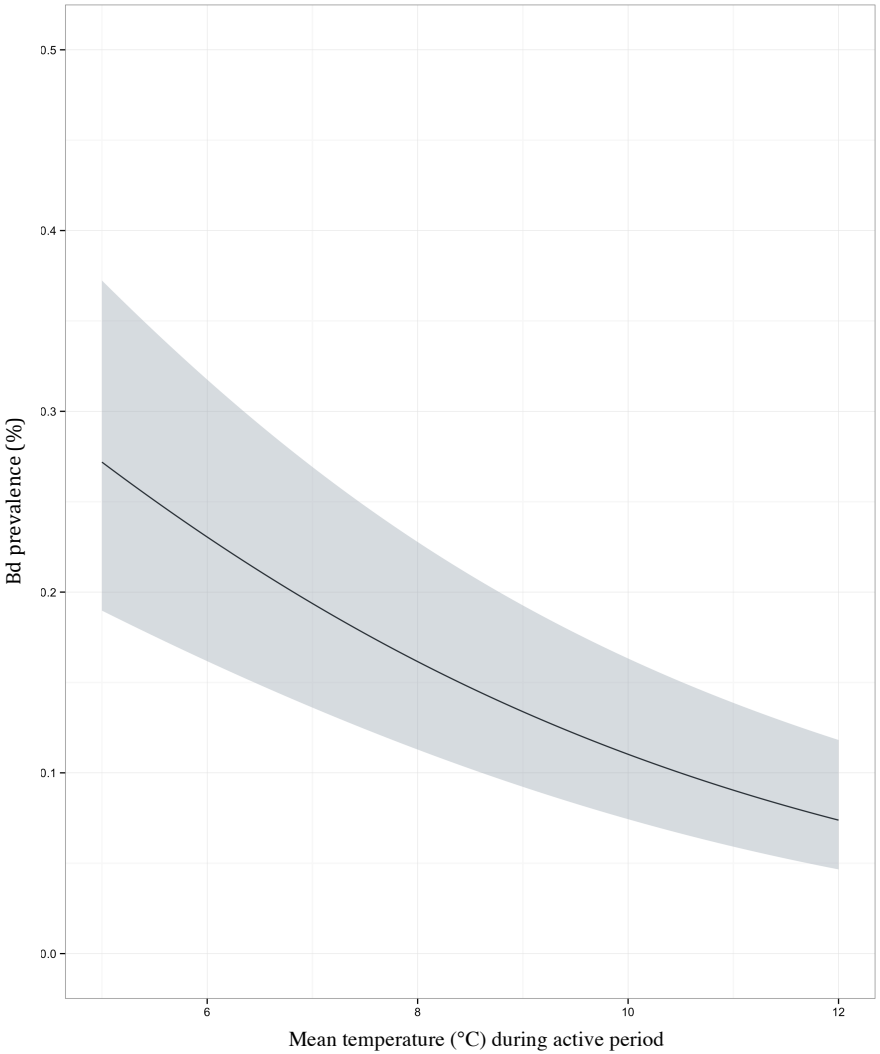


Figure 2.9(A) & 2.9(B): Model predicted relationship between *Bd* prevalence (%) and **(A)** mean daily air temperature (˚C) during active period, and **(B)** length of active periods (number of days between onset of spring and calling date). Shaded areas span the 95 % credible intervals for the fitted means.

(A)



(B)

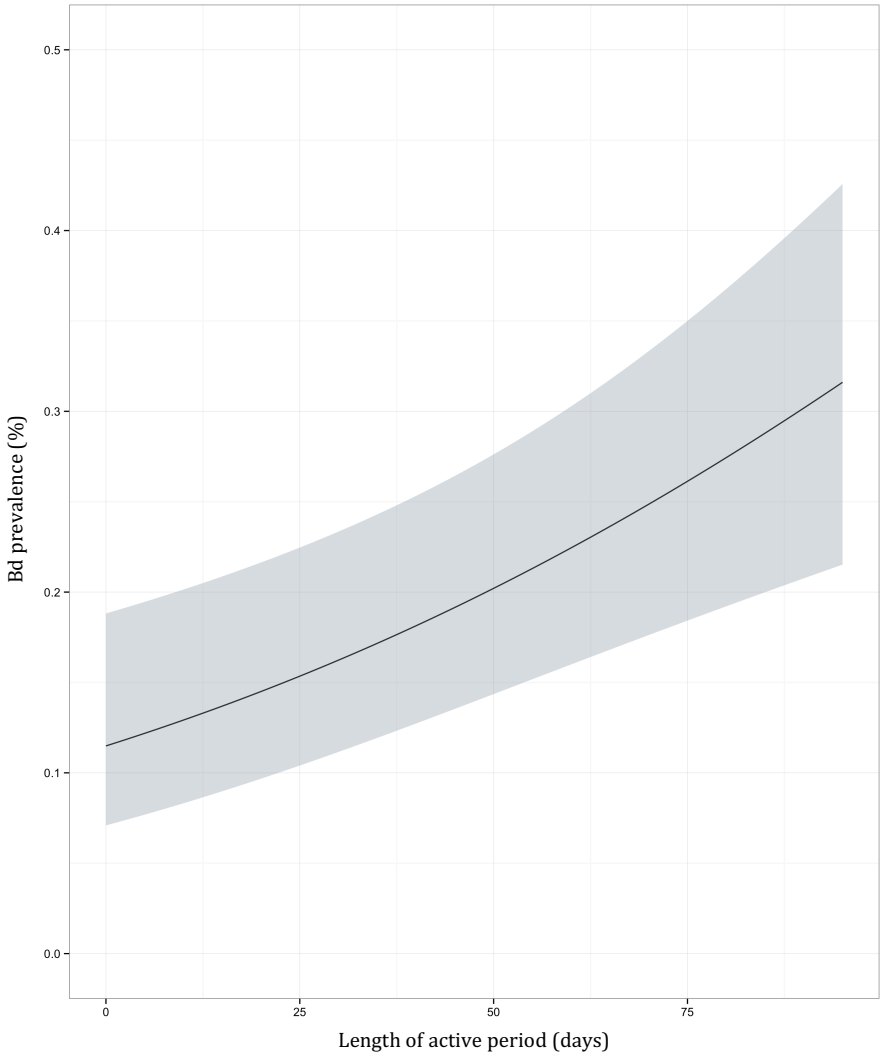
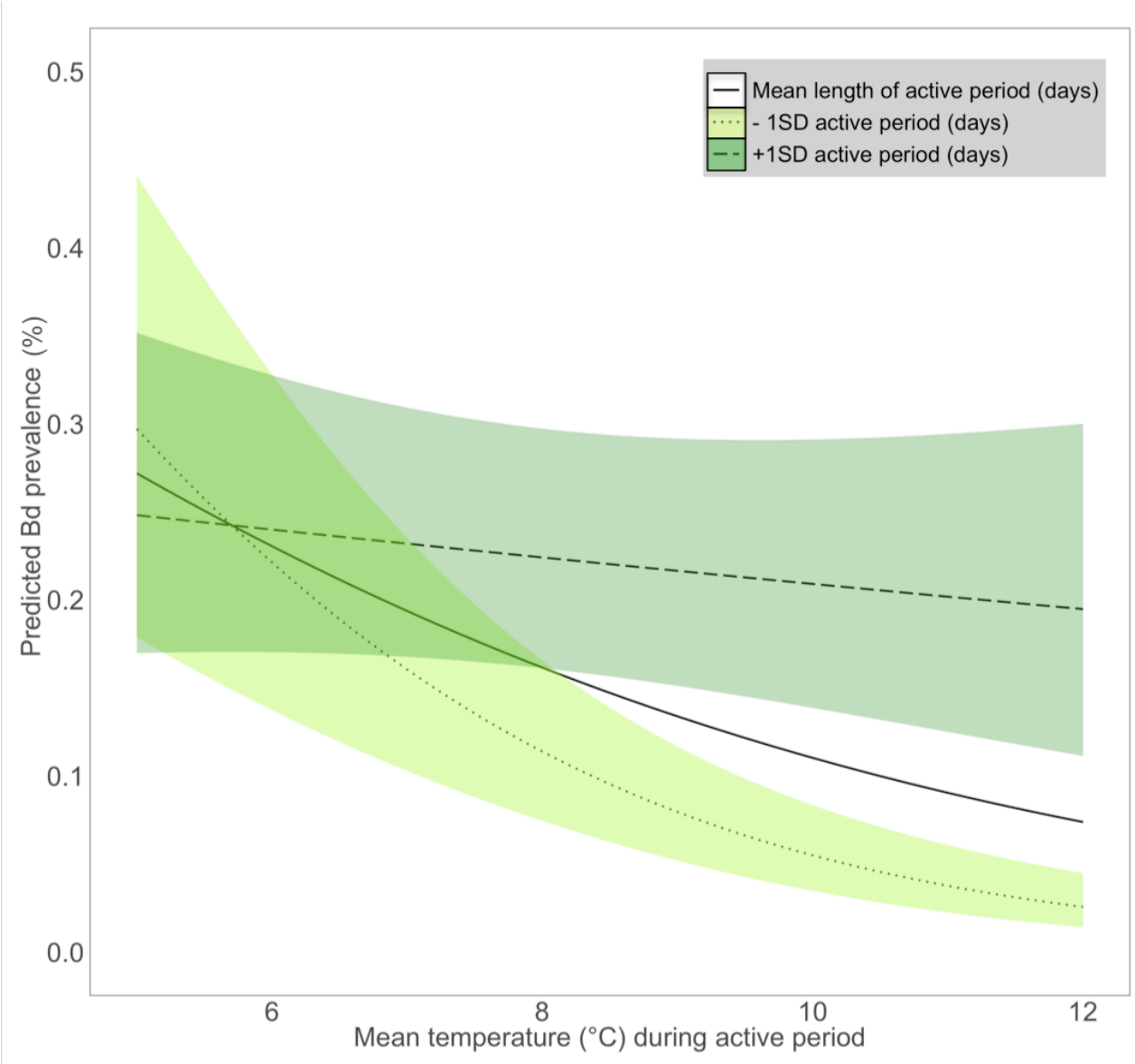


Figure 2.10: Model predicted relationship between *Bd* prevalence (%) and the following interaction: mean daily air temperature (˚C) during active period (negatively correlated) and length of active period (days; positively correlated). The three lines represent the relationship between *Bd* prevalence and mean daily air temperature (˚C) during the active period when length of active period (days) is held at: i) its global mean (**sold black line**), ii) one standard deviation below the global mean (**dotted line, light green shaded area spans the 95 % credible intervals for the fitted means**), iii) one standard deviation above the global mean (**long dashed line, dark green shaded area spans the 95 % credible intervals for the fitted means**). Variation in *Bd* prevalence is greatest when active periods are shortened and temperatures are cool. If active periods are extended, the relationship between *Bd* prevalence and mean daily air temperature (˚C) diminishes. However, if active periods are shortened, this relationship becomes more negative.



With regards to mean *Bd* intensity of infection (GE), the highest ranked model included hydroshed area (km^2), and an interaction between river density (within 10 km buffer from site centroid) and mean precipitation (mm) throughout breeding period (Table 2.13). Hydroshed area was found to have the greatest effect upon *Bd* intensity of infection (Figure 2.11). Individually, mean precipitation throughout breeding period and river density presented similar weak effect sizes. However, combined, the effect upon *Bd* intensity of infection was much greater, than the sum of both its parts (Figure 2.11).

Mean GE was negatively correlated with hydroshed area (km^2) (Figure 2.12), but positively correlated with both river density (Figure 2.13) and mean precipitation throughout breeding period (Figure 2.14): this latter interaction was plotted in Figure 2.15. As river density increases, the relationship between infection intensity and mean precipitation during breeding period becomes significantly more positive. As both predictor variables increase, infection intensity grows exponentially. Within site localites with low surrounding river densitites, mean infection intensity will only increase (during the breeding period) when precipitation levels are low. The top model for *Bd* intensity of infection was ranked 25^{th} out of the 26 competing models tested against *Bd* prevalence at site level (Model 'm8', highlighted bold in Table 2.12). Simialrly, the top model for *Bd* prevalence was ranked 16^{th} out of the 26 competing models tested against *Bd* intensity of infection at site level (Model 'm1', highlighted bold in Table 2.13).

TABLE 2.13: Akaike's information criterion model rankings for the candidate models explaining *Bd* intensity of infection (mean GE) at site level. The top model for *Bd* prevalence (%) at site level is highlighted in bold. ***k***, number of parameters; **logLik,** log likelihood; **AIC**, Akaike's information criterion corrected for small sample size; **ΔAIC**, difference in AIC compared with the model with the lowest AIC; ***wi***, model weight; **Retained**, models in the Δ2 AIC set (grey shaded rows) are not retained if they are more complex versions of nested (simpler) models with better AIC support (higher up in the table); **year**, year of sample date; **site**, accounted for possible non-independence of toe-clips collected at the same site locality by including this random intercept effect; **active period length**, number of days between onset of spring and calling date; **active period mean temperature**, mean daily air temperature (˚C) throughout active period; **active period mean precipitation**, mean precipitation (mm) throughout active period; **breeding period mean precipitation**, mean precipitation (mm) throughout breeding period; **river density**, within 10 km buffer from site centroid; **hydroshed area**, (km^2); **road density**, within 50 km buffer from site centroid.

Model	Model description	*k*	logLik	AIC	ΔAIC	*wi*	Retained
m8	factor(year) + river density * breeding period mean precipitation + hydroshed area + (1 | site)	9	-140.05	298.1	0	0.297	✔
m24	factor(year) + hydroshed area + (1 | site)	6	-143.39	298.77	0.668	0.212	✔
m3	factor(year) + active period mean temperature + hydroshed area + (1 | site)	7	-143.21	300.42	2.318	0.093	
m12	factor(year) + breeding period mean precipitation + hydroshed area + (1 | site)	7	-143.25	300.49	2.388	0.09	
m6	factor(year) + active period mean precipitation + hydroshed area + (1 | site)	7	-143.26	300.51	2.408	0.089	
m14	factor(year) + road density + breeding period mean precipitation + hydroshed area + (1 | site)	8	-143.21	302.43	4.324	0.034	
m9	factor(year) + river density + breeding period mean precipitation + hydroshed area + (1 | site)	8	-143.23	302.45	4.346	0.034	
m25	factor(year) + (1 | site)	5	-146.60	303.21	5.104	0.023	
m10	factor(year) + river density * breeding period mean precipitation + (1 | site)	8	-143.80	303.6	5.5	0.019	
m13	factor(year) + road density * breeding period mean precipitation + hydroshed area + (1 | site)	9	-143.21	304.42	6.314	0.013	
m20	factor(year) + active period mean temperature + (1 | site)	6	-146.28	304.55	6.446	0.012	
m19	factor(year) + breeding period mean precipitation + (1 | site)	6	-146.33	304.66	6.554	0.011	
m21	factor(year) + active period mean precipitation + (1 | site)	6	-146.43	304.86	6.76	0.01	
m23	factor(year) + road density + (1 | site)	6	-146.51	305.02	6.914	0.009	
m22	factor(year) + river density + (1 | site)	6	-146.52	305.04	6.932	0.009	
m1	**factor(year) + active period length * active period mean temperature + (1 | site)**	**8**	**-144.92**	**305.83**	**7.728**	**0.006**	
m5	factor(year) + active period length * active period mean precipitation + (1 | site)	8	-144.93	305.85	7.746	0.006	
m18	factor(year) + active period mean temperature + road density + (1 | site)	7	-146.14	306.28	8.18	0.005	
m2	factor(year) + active period mean temperature + active period mean precipitation + (1 | site)	7	-146.23	306.46	8.356	0.005	
m4	factor(year) + active period mean temperature + river density + (1 | site)	7	-146.24	306.48	8.376	0.005	
m11	factor(year) + river density + breeding period mean precipitation + (1 | site)	7	-146.28	306.57	8.462	0.004	
m16	factor(year) + road density + breeding period mean precipitation + (1 | site)	7	-146.29	306.58	8.476	0.004	
m7	factor(year) + active period mean precipitation + river density + (1 | site)	7	-146.40	306.81	8.702	0.004	
m17	factor(year) + active period mean precipitation + road density + (1 | site)	7	-146.41	306.81	8.71	0.004	
m15	factor(year) + road density * breeding period mean precipitation + (1 | site)	8	-146.21	308.42	10.312	0.002	
m26	1 + (1 | site)	3	-154.50	315	16.9	0	

Figure 2.11: Effect sizes of predictor variables from AIC top model, explaining variation in *Bd* intensity of infection (mean GE) at site level. Black dotted line depicts the global mean for *Bd* intensity of infection when all predictor variables are averaged. Red point represents plus one standard deviation for each predictor variable, blue point represents minus one standard deviation for each predictor variable. **Size of hydroshed area (km^2)** was found to have the greatest effect upon *Bd* intensity of infection. Individually, **breeding period mean precipitation (mm)** and **river density (within 10 km buffer from site centroid)** presented similar weak effect sizes. However, combined, the effect upon *Bd* intensity of infection was much greater.

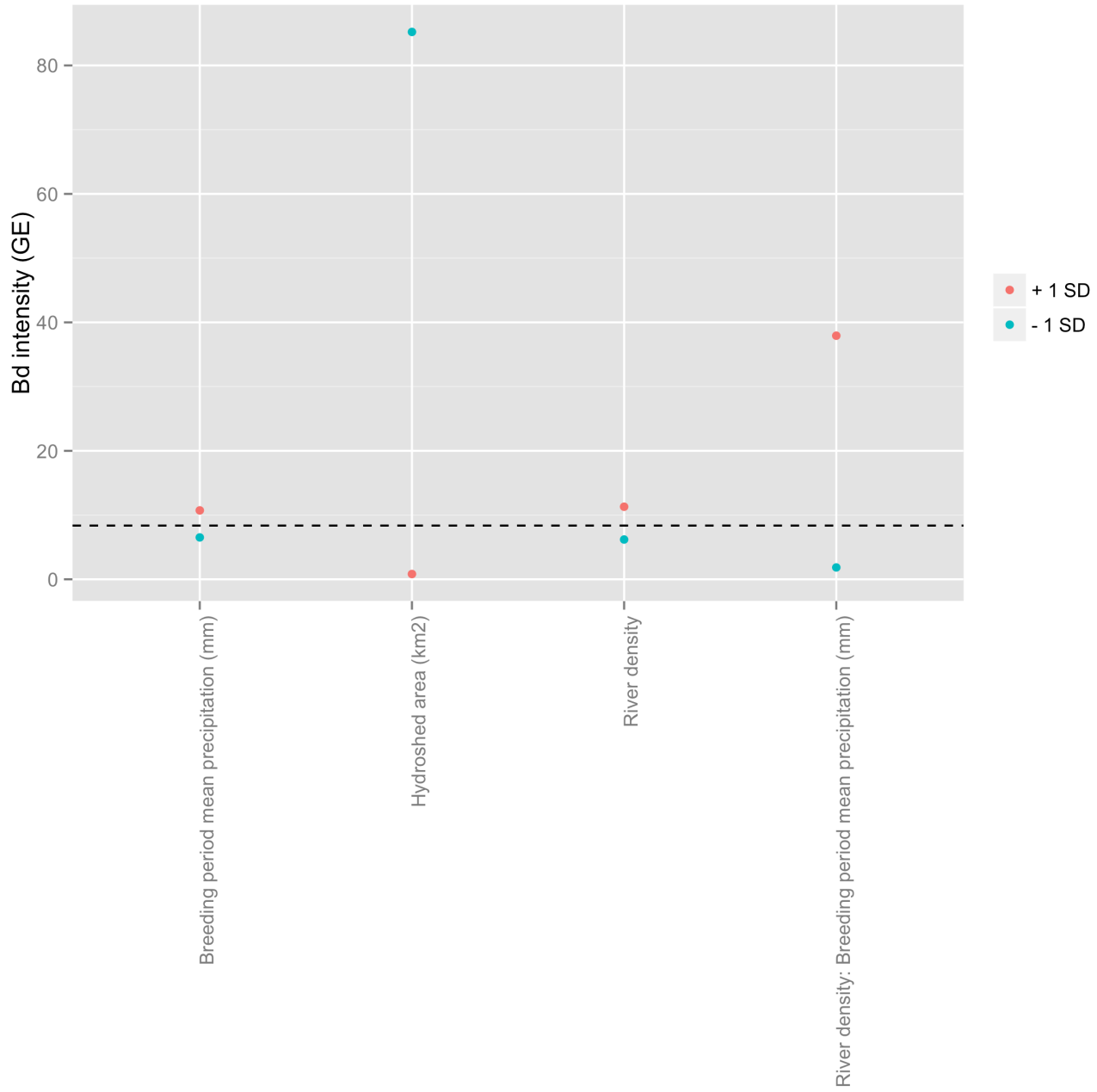


Figure 2.12: Model predicted relationship between *Bd* mean intensity (GE) and size of hydroshed area (km^2). After an initial rapid exponential decrease, mean infection intensity reaches a plateau at very low intensities/ zero infection at approximately 20,000 km^2.

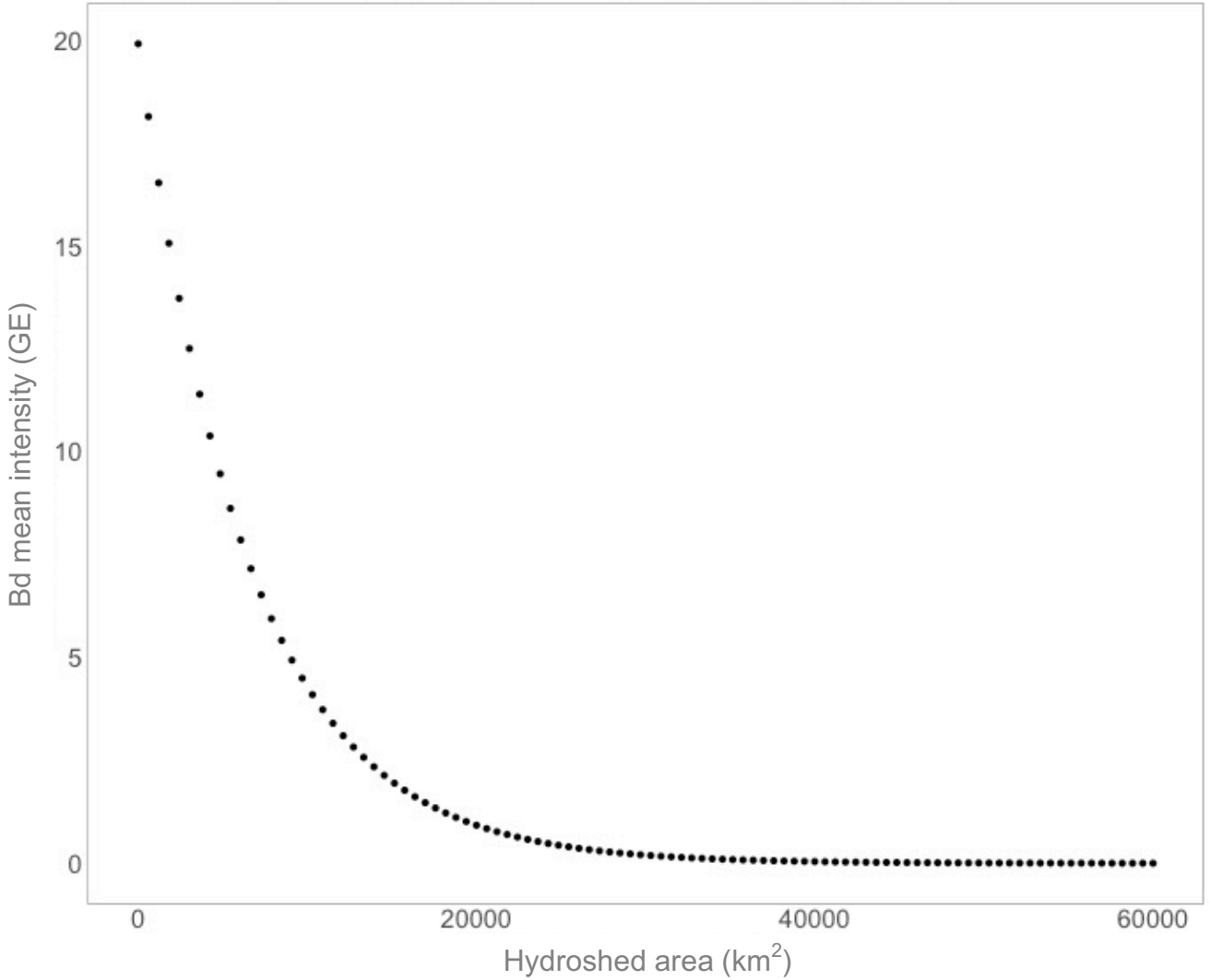


Figure 2.13: Model predicted positive relationship between *Bd* mean intensity (GE) and river density (within 10 km buffer from site centroid).

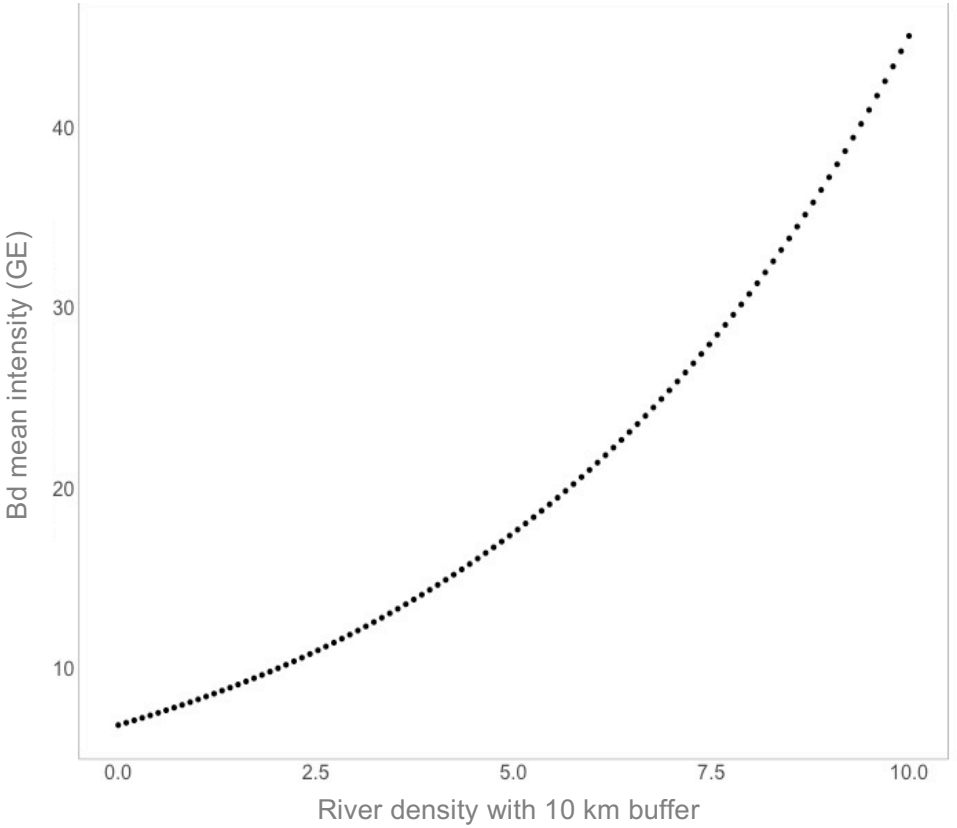


Figure 2.14: Model predicted positive relationship between *Bd* mean intensity (GE) and mean precipitation (mm) throughout breeding period.

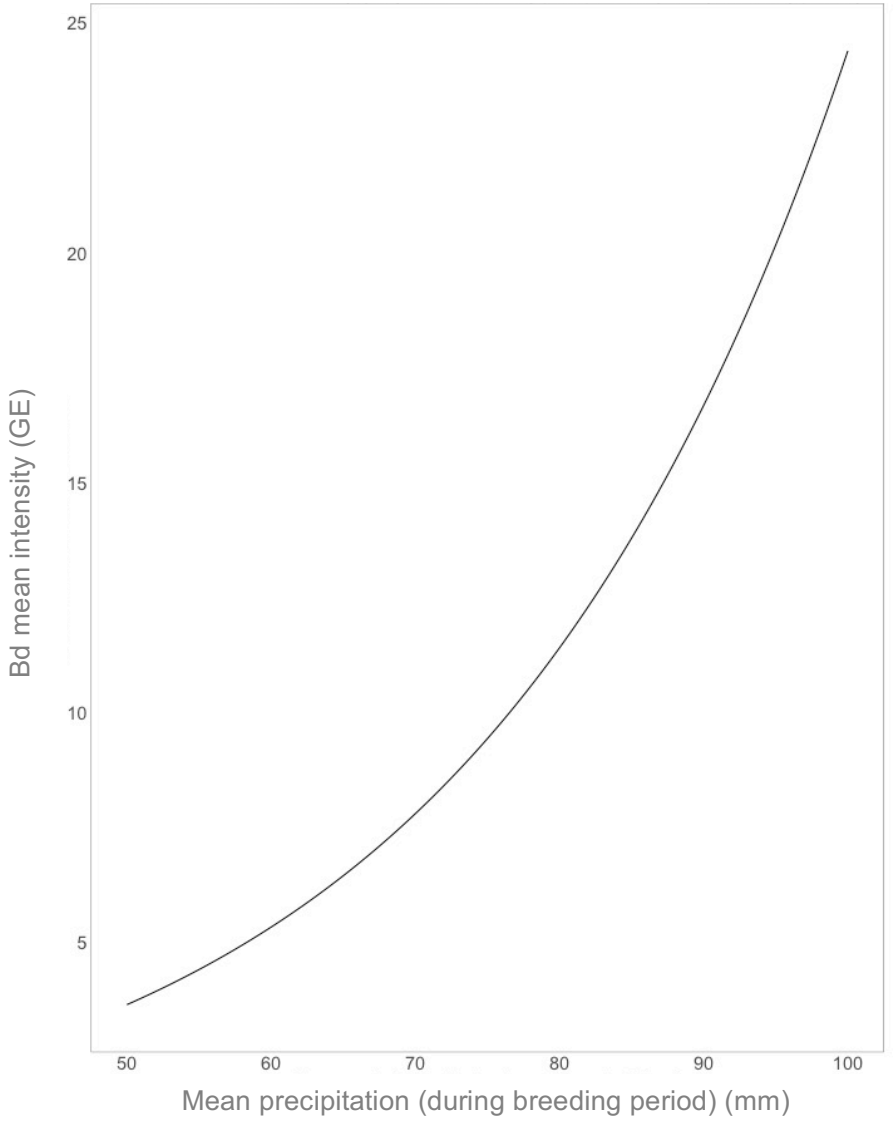
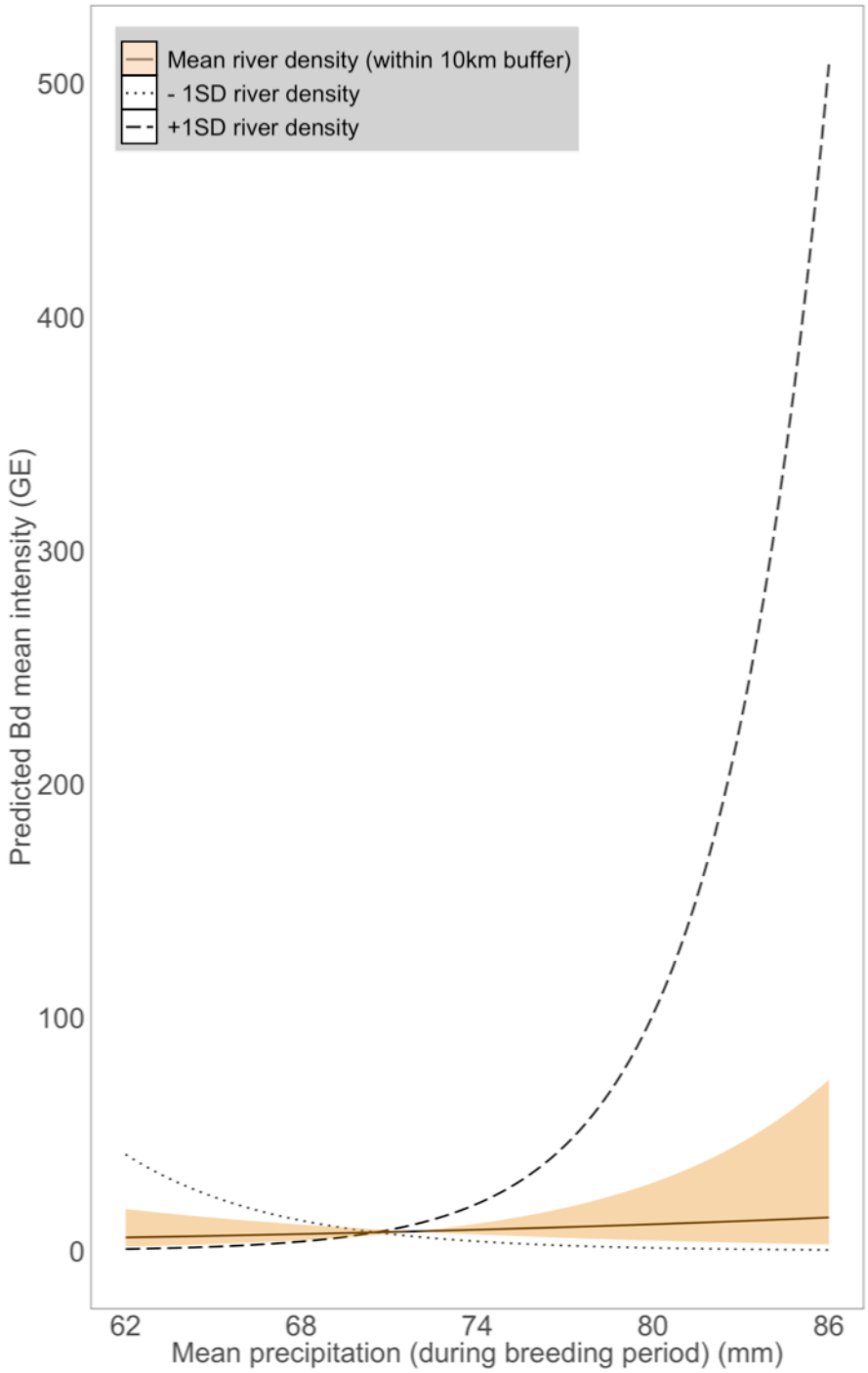


Figure 2.15: Model predicted relationship between *Bd* mean intensity (GE) and the following interaction: river density (within 10 km buffer from site centroid; positively correlated) and mean precipitation (mm) throughout breeding period (positively correlated). The three lines represent the relationship between *Bd* mean intensity (GE) and mean precipitation (mm) throughout breeding period, when river density is held at: i) its global mean (**sold black line, orange shaded area spans the 95% credible intervals for the fitted means**), ii) one standard deviation below the global mean (**dotted line**), iii) one standard deviation above the global mean (**long dashed line**). Mean *Bd* intensity is greatest when a site locality is in close proximity to a dense network of rivers, and precipitation is high. If river densities are low, mean GE increases slightly when precipitation levels are low.

.



2.5 DISCUSSION

I demonstrate that *Bd* is consistently present in *R. pipiens* populations throughout Ontario, with weak infection loads and relatively low prevalence. There is no evidence of clustering across the landscape, but *Bd* does display substantial local interannual fluctuations of both prevalence and intensity of infection (Figure 2.6). At such times, infection metrics can escalate dramatically. An increase in prevalence from one year to the next coincides with a significant increase in infection load, greater than that observed from a site exhibiting infection for the first time (Table 2.4). This suggests that at certain site localities, when local conditions allow, *Bd* can establish, spread and proliferate rapidly within a susceptible population. For most micro-parasite models, re-exposure of the infected host to a pathogen does not affect disease progression. However, for *Bd*, infection load strongly determines the infectivity of individuals and the definitive outcome (Carey *et al*. 2006; Voyles *et al*. 2009). As such, one may expect dramatic spikes in infection metrics, between years, to impact upon *R. pipiens* populations. Despite this fact, chytridiomycosis-driven mass mortality events and species declines have not been observed within this system.

There are three opposing hypotheses that explain this lack of *R. pipiens* decline (despite presence of *Bd*):

(1) *Bd*-caused declines are present but not detected due to a lack of long-term population data (Houlahan *et al*. 2000),

(2) *Bd* has recently spread into a new geographic area, encountering naïve host individuals (Alford 2001) but has yet to firmly establish or reach a critical threshold where more detectable costs to hosts may be detected, or

(3) *Bd* is endemic within the system, and either virulence has dropped or the host species exhibits increased tolerance/ resistance.

With regards to the first hypothesis, several studies have reported modest declines in *R. pipiens* in eastern Canada (Oldham & Weller 1992; Gilbert *et al*. 1994; Russell *et al*. 1995; Hecnar 1997; Hecnar & M'Closkey 1997; Gilbertson *et al*. 2003). Yet, the causes of these declines are attributed to species introductions, habitat loss and pesticide use. No published study has linked *Bd* infection to a reduction in *R. pipiens* populations in eastern Canada. Furthermore, Hoffman *et al*. (2004) found that the genetic structure of *R. pipiens* populations in southern Ontario was stable across a substantial period of time (22 – 30 years). This suggests that these populations were not undergoing frequent extinctions and recolonisation. Despite this fact, I cannot entirely eliminate the possibility of *Bd*-declines occurring, as infection and mortality is often cryptic. However, I would suggest that chytridiomycosis-driven mass mortality events and *Bd*-related declines are very unlikely within *R. pipiens* populations throughout Ontario, as I did not observe any mortality. With regards to the second hypothesis, Carey *et al*. (1999) suggested that population declines observed in *R. pipiens* in central Canada in the late 1960s - early 1970s (Gibbs *et al*. 1971; Rorabaugh 2005) may have been caused by the emergence of chytridiomycosis. Additionally, a histological survey of *Bd* infections in *R. pipiens* collected in the Quebec province of Canada between 1960 and 2001 revealed a prevalence of 10 – 20 % (Ouellet *et al*. 2005). These historical findings suggest that much of the spread of *Bd* within eastern populations of *R. pipiens* occurred decades ago (Rachowicz *et al*. 2006; Kinney *et al*. 2011). This allowed me to disregard hypothesis two, as it relies on *Bd* recently spreading into a new geographic areas. My results thus support the third hypothesis: *Bd* is endemic within the system, and either virulence has dropped and/ or

the host species exhibits increased tolerance/ resistance. Within my study system, the pathogen did not cluster across the landscape, nor was the pathogen more or less likely to be present within a particular region. All regions presented positive infection metrics. This suggests that *Bd* is endemic within Ontario. While no published study has assessed virulence of *Bd* throughout Ontario, several publications have suggested that *R. pipiens* may have developed resistance to chytridiomycosis, and are thus acting as a reservoir or carrier species for *Bd* (Ouellet *et al*. 2005; Longcore *et al*. 2007; Woodhams *et al*. 2008b). Consequently, I can confidently suggest that my findings are concurrent with hypothesis three.

My results suggest that interannual variation of local climatic regimes interact with stable geographic factors (such as hydrology), to preclude the build-up of infection load and prevalence. More specifically, my analyses show evidence for: (1) a state of endemism in Ontario; (2) both temporal and spatial heterogeneity in infection; (3) evidence for a relationship between *Bd* prevalence and thermal regimes prior to breeding; (4) evidence for a relationship between infection load and aquatic conditions throughout breeding period; and (5) no evidence for a relationship between *Bd* prevalence/ infection load, and road density. Thus, despite apparent coupling of infection metrics (Figure 2.5), *Bd* prevalence and intensity of infection are governed, at least in part, by different environmental factors operating during different life history events.

Once *R. pipiens* emerge from their overwintering sites, local climatic conditions may determine whether *Bd* is able to establish within a population. Localities that experienced short warm climates prior to breeding were unlikely to support the establishment of *Bd,* while sites that experienced cold temperatures, irrespective of

duration, were most likely to harbour *Bd* infections (Figure 2.10). This result supports both bioclimatic predictive models (Ron 2005) and laboratory studies (Longcore *et al*. 1999; Johnson *et al*. 2003; Piotrowski *et al*. 2004) that point to *Bd* as favouring cooler temperatures, especially within endemic systems. However, the temperatures experienced during the active period are well below the optimal thermal range of the fungus (17 – 25 °C; Longcore *et al*. 1999; Johnson *et al*. 2003). Woodhams and colleagues (2008a) identified that at lower temperatures, growth rate slows but fecundity and infectivity (dependent upon the life span of the zoospore) increases, hereby supporting my results, as populations experiencing cooler climates exhibited higher prevalence.

Length of active period was shortened by 5.3 days per annum, or 21.3 days over the four-year period. As this period shortens, the model predicts a lower prevalence rate. Consequently, it would seem reasonable to conclude that this annual curtailment would be beneficial to *R. pipiens* with regards to infection risk, but 2013 experienced the shortest active period and the greatest prevalence. However, 2013 also experienced the coolest mean temperatures during the active period. The model predicts that the extension or shortening of the active period has little affect on *Bd* prevalence when mean temperatures are low (between 5 – 8 °C) (Figure 2.10). Extending this period when temperatures are warm (10 – 12 °C), generates an increase in prevalence, while limiting this period leads to a reduction in prevalence. I suggest that this pattern may be due to expanded opportunities for the successful transmission of *Bd* between individuals, when the active period is elongated and warmer.

All variables that correlate with *Bd* mean intensity (GE) are framed within hydrology: size of hydroshed (km^2), surrounding river density, and mean precipitation (mm) throughout the breeding period. This corroborates predictions that *Bd* should be more abundant in wetter areas (Ron 2005; Kriger *et al*. 2007), and field studies indicating that *Bd* outbreaks might be more likely under wet conditions (Piotrowski *et al*. 2004; La Marca *et al*. 2005). However, no published study has linked infection load with a reduction in either: (1) the size of water basin (hydroshed), or (2) the density of river networks. While mean precipitation (mm) throughout breeding period is likely to vary with region and year, size of water basin and river density are unlikely to change, unless anthropogenic alterations occur. Consequently, the mean *Bd* infection model predicts a negative relationship with a stable metric (hydroshed area) and a complex interaction between a relatively unstable metric (mean precipitation (mm) throughout breeding period) and more predictable one (river density). I found that as hydroshed area increased, infection load decreased slightly (Figure 2.12). I suggest that *R. pipiens* populations concentrated within a small drainage basin (hydroshed) may facilitate the proliferation and transmission of zoospores, as increasing host population density increases transmission rates (Briggs *et al*. 2005). Additionally, larger hydrosheds may allow for greater dilution of zoospores, thus the frequency with which a zoospore interacts with a host may decrease with increasing spatial context. However, the predicted effect of hydroshed size on mean *Bd* intensity (predicted *Bd* mean intensity range: 0 – 20 GE; Figure 2.12) is significantly less than the predicted effect of the complex interaction on mean *Bd* intensity (predicted *Bd* mean intensity range: 0 – 500 GE; Figure 2.15). I predicted that during the breeding season, frogs at sites that exhibit a dense concentration of rivers, within a 10 km buffer, would experience exponential growth of mean infection load when

precipitation levels are high. However, when precipitation levels are low, high river density sites will elicit no variation from the global mean, while frogs at sites surrounded by very few rivers, will experience a small increase in mean infection load (Figure 2.15). There are two hypotheses that can explain these patterns. First, rivers serve as likely vectors for the waterborne zoospores of *Bd* (Kriger & Hero 2007a). A high concentration of rivers within a 10 km^2 area suggests an increase in transmission channels via aquatic nodes, or feasible amphibian movement between catchments. The connectivity of river networks may prove particularly influential as zoospores may be carried away with water currents (Piotrowski *et al*. 2004), thus expanding the spatial reach of infection. When precipitation levels are high, moisture levels will be elevated within the terrestrial environment, along with the aquatic, which increase zoospore movement, survival and colonization (Piotrowski *et al*. 2004; Kriger 2009). This is especially important for this host-pathogen interaction, as *R. pipiens* is a semi-terrestrial anuran. Consequently, when individuals are active within the terrestrial realm, increased precipitation allows for the continual reinfection from zoospores released within the skin and onto the skin surface, in turn aiding the significant increase in infection load. Alternatively, low precipitation levels allow for an increase in hydrological desiccation, especially when rivers are not well connected. This may force individuals to congregate in smaller pools, thus increasing opportunity for successful *Bd* transmission. However, as precipitation levels are low, when *R. pipiens* enter the terrestrial realm, zoospores cannot access the moisture they require. Consequently, zoospore growth will be limited (Johnson *et al*. 2003). The shared theme in these two hypotheses is the role of re-infection. I suggest that the increase in strength of infection in the system is largely attributable to within-host reinfection

(from zoospores released within the skin and onto the skin surface) rather than among-host transmission.

As climate change is projected to alter the frequency of extreme weather events across multiple timescales (Easterling *et al*. 2000; Rohr & Raffel 2010; Huntingford *et al*. 2013; Screen 2014), short-term climatic variability may play a significant role in structuring infection dynamics. High-resolution interpolation of climate scenarios by 2100 for Ontario suggest an increase in mean annual temperature ranging from 3.0 °C to 5.0 °C for the minimum and from 2.5 °C to 4.5 °C for the maximum, and an increase in winter minima by as much as 4.0 °C to 7.0 °C (depending on scenario; Price *et al*. 2011). This projected shift towards a warmer climate may have significant implications for both host and pathogen. While a positive shift in thermal regime may prove advantageous to *R. pipiens*, as infection risk decreases, it may also result in the decline of *R. pipiens*, due to changes in reproductive behavior and timing (Alford & Richards 1999; Collins & Storfer 2003). Changes in seasonal and annual precipitation patterns are more difficult to predict, as precipitation is inherently more variable than temperature, both temporally and spatially. However, Price *et al*. (2011) have projected an increase in total annual precipitation of 5 - 13 % by 2100. This increase is distributed unevenly, with the largest increases occurring in winter and spring. Furthermore, interannual variability in rainfall events is projected to increase year-round. As the model predicts the greatest variability in infection loads when precipitation levels are high, this illustrates the potential risk of chytridiomycosis to populations of *R. pipiens* across Ontario. However, while there may be an increase in mean annual temperature and precipitation across the study region, the extent to which microclimates are altered will vary between habitats. These local shifts will directly influence the activity patterns of local *R. pipiens*, as climate acts as a

proximate driver for phenology and daily activity. Consequently, the populations may not respond uniformly to the projected changes and we may see greater variation in *Bd* outcomes across the landscape. This cautions against generalizations about the outcome of changing climate on phenology, infection dynamics and population persistence.

Transportation infrastructures such as roads can have major environmental impacts (Balkenhol & Waits 2009). In addition to fragmenting the landscape, interrupting movement and social interactions, increasing erosion and pollution, and lowering local abundance and diversity, several studies have reported that the spread of infectious diseases and invasive species have been facilitated by the road network (Pauchard & Alaback 2004; Hansen & Clevenger 2005; Miura 2007; Archie *et al*. 2009). They do so by disrupting native communities, changing physical habitats and providing movement corridors for pathogens (Trombulak & Frissell 2000). As such, I expected *Bd* prevalence and intensity of infection to be higher within sites at close proximity to road networks. However, I detected no relationship between road density (within a 50 km^2 buffer from site centroid) and either *Bd* prevalence or intensity. I would suggest that this is because habitat disturbance is such a complex issue that a single indicator is insufficient to grasp its multifaceted aspects. As such, I would suggest that future studies incorporate an index of habitat change, rather than focus on one form of disturbance. This index should include agricultural development, urbanization and sprawl, deforestation and road development (Leu *et al*. 2008). By creating this human-footprint score, one would develop a disturbance gradient from wild and inaccessible areas, to areas that have experienced high levels of habitat change. Thus, instead of suppressing the complexity of this natural system, one would

explicitly account for the different forms of habitat disturbance, rather than ignoring their impacts.

Several sites gained infection in 2013, only to exhibit dramatic declines of infection metrics in 2014. Although a wide geographic distribution may be difficult to reconcile with unstable pathogen presence, there is substantial capacity for repeat pathogen introductions within the system. For example, the region supports a diverse amphibian community providing *Bd* with a range of alternative hosts (Johnson & Speare 2005; Garmyn *et al*. 2012; McMahon *et al*. 2013) and while *Bd* may be lethal to infected metamorphs for some species of anurans (Berger *et al*. 1998), infection does not directly increase mortality in tadpoles (Parris & Baud 2004; Parris & Cornelius 2004). Consequently, sublethally infected tadpoles may serve as an intraspecific pathogen reservoir for newly metamorphosing froglets and adults (Brunner *et al*. 2004). Both options provide routes for repeat pathogen introductions, which function to limit the stability of infection within the site.

2.6 CONCLUSION

To fully understand the impacts of *Bd* on amphibians, it is necessary to examine the interaction between life history patterns and environmental variables that exacerbate *Bd* prevalence and intensity (Lips *et al*. 2003). Consequently, I fixed climatic variables to behavioural time points: 'active period' (period between the onset of spring and the commencement of breeding) and 'breeding period' (period between the commencement of breeding and the last day of June). This allowed me to incorporate the dynamic relationship between host and environment, which is altered annually due to seasonal fluctuations. Interannual variation may play a significant role in structuring infection dynamics, as local climatic nuances will alter the way in which a

host operates within its environment. This adjustment in behaviour may be forced upon the host, for example: annual increases in average air temperature have been shown to correlate with earlier initiation of the breeding season for several temperate taxa, including amphibians (Beebee 1995; Blaustein *et al*. 2001; Brodman 2009). Alternatively, the host may actively alter their behaviour to buffer themselves against negative effects, for example: ectotherms choose particular microclimates within a spatially and temporally variable environment, in order to regulate their body temperature (Bartholomew 1966; Huey 1991). It is imperative that we follow these behavioural alterations, and assess the environment in which these infection dynamics operate, as the value of predictive modelling for infection risk increases substantially when parameters affecting local host species-specific infection dynamics are considered at a local scale (Paaijmans *et al*. 2009).

Akin to other ectotherms, the fitness of amphibians is particularly sensitive to ambient temperatures (Raffel *et al*. 2006). Despite this fact, the role of climate change in the unprecedented decline of ectothermic biodiversity and emergence of infectious diseases remains controversial (Daszak *et al*. 2000; Harvell *et al*. 2002; Rohr *et al*. 2008; Lafferty 2009; Rohr & Raffel 2010). Impacts of climate change on host and pathogen dynamics are expected to be particularly strong for ectotherms, as host metabolism and activity patterns are closely linked to environmental temperatures, which in turn, directly influence the establishment of the pathogen. However, less attention has been devoted to the consequences of changes in precipitation and water availability. A strong impact is expected for host and pathogen, as both species rely on humid environments, require water for reproduction, and are particularly active during wet periods. However, in the absence of quantitative summaries across multiple studies, it is difficult to identify general patterns. These results of differential,

context-dependent host susceptibility to *Bd* is supported by Doddington *et al*. (2013) and may be a pattern exhibited by other fungal pathogens that threaten wildlife hosts (Fisher *et al*. 2012). This observation highlights a crucial need for long-term ecological studies that examine the consequences of climate-disease interactions within local communities, as changing environmental conditions could shift the balance from co-existence to significant mortality in some populations, but not in others (Phillott *et al*. 2013). This knowledge will directly affect the framing and development of conservation efforts to mitigate infections. Furthermore, studying the patterns of local infections may be crucial to understanding how infection dynamics affect biodiversity at larger spatial scales. Hence, I stress the exigency to identify how local factors may exacerbate or reduce the impact of an infectious disease.

In conclusion, my research highlights the importance of: (1) measuring both infection parameters (*Bd* prevalence (%) and intensity of infection (GE)) in order to gain insight into *Bd* infection dynamics; (2) setting environmental heterogeneity into a host-behavioural context; and (3) incorporating species-specific micro-climatic data in predictive models. Given that *Bd* has an unusually broad host range (Olson *et al*. 2013), can survive on and be transported by non-amphibian taxa (McMahon 2013), and that infectious zoospores can survive for up to 24 hours in water in the absence of an amphibian host (Berger 2001; Woodhams *et al*. 2008a), it seems unlikely that we will prevent the spread of the fungus into countries in which it is currently absent (Olson *et al*. 2013). In light of this, studies such as this may help guide conservation strategies, by helping to predict infection risk at a local scale, based on both 'stable' and 'unstable' environmental factors. Such measures may permit some individuals to survive the initial epidemic, and allow the population to persist within an endemic system. However, ecological events are explained through the detection of patterns at

a given spatial scale. Within this system, environmental heterogeneity explained 16 % of the total variation regarding *Bd* prevalence, at the site level. Infectious diseases often emerge from interactions across nested levels of spatial organization. From a pathogen's point of view, each host represents a highly complex and rich environment where, in addition to nutrients, the pathogen will encounter varied, and at times, inhospitable physical conditions, e.g. non-optimal thermal regimes (Richards-Zawacki 2010; Rowley & Alford 2013) or defensive resident microorganisms (Woodhams *et al*. 2014; Jani & Briggs 2014). Consequently, smaller spatial units need to be investigated (Walker *et al*. 2010; Baláž *et al*. 2013). Elucidating possible drivers of heterogeneities among individuals will aid targeting of control strategies, and will help to forecast dynamic changes in the system due to ongoing climate change.

CHAPTER THREE: EXTRINSIC AND INTRINSIC TRAITS EFFECT ADAPTIVE CHANGES IN HOST RESISTANCE DURING AN ECOLOGICAL INTERACTION

3. 1 INTRODUCTION

3.1.1 Host–pathogen evolution, biodiversity, and disease risks for natural populations

Emerging infectious diseases are increasingly recognized as playing important roles in ecological systems. Pathogens introduced into previously unexposed host populations can spread quickly, cause high fatality rates and lead to dramatic reductions in host abundance (Anagnostakis 1987; Osterhaus & Vedder 1988; Sherald *et al*. 1996). Although documented cases of pathogen-driven host extinction are limited (Roelke-Parker *et al*. 1996; Hochachka & Dhondt 2000; Funk *et al*. 2001; Jensen *et al*. 2002; Smith *et al*. 2006), simple models show that parasites and infectious diseases may, in some circumstances, be capable of significantly contributing to extinction (De Castro & Bolker 2005).

There is substantial evidence that parasites significantly reduce host fitness in the wild (Hudson *et al*. 2002), interact with other population processes and shape community structure (May & Anderson 1983; Dobson & Crawley 1994; Hiers & Evans 1997). Host-pathogen interactions can generate a number of evolutionary outcomes, ranging from the maintenance of genetic variation within the community (Hudson *et al*. 2006), to significant shifts in the genetic composition of both host (Burdon & Thompson 1995; Dybdahl & Lively 1998; Little & Ebert 2001) and parasite (Schrag

& Wiener 1995; Hudson *et al*. 2002; Altizer *et al*. 2003). As such, parasites and infectious disease have become a major concern in conservation biology (Harvell *et al*. 1999; Daszak *et al*. 2000; Lafferty & Gerber 2002).

3.1.2 Host resistance versus host tolerance

Defense against pathogenic micro-organisms and other parasites can be divided into two conceptually different components: (1) 'resistance', traits that prevent infection or limit its extent, and (2) 'tolerance', traits that do not reduce or eliminate infection, but instead reduce or offset its fitness consequences (Simms & Triplett 1994; Fineblum & Rausher 1995; Fornoni *et al*. 2004; Restif & Koella 2004). By definition, resistance has a negative effect on parasites while tolerance may not. It is important to distinguish between these two components, as their relative importance will have a significant effect upon the ecology and evolution of disease dynamics (Roy & Kirchner 2000; Rausher 2001). Here, I will focus on host resistance alone.

3.1.2.1 Evolutionary dynamics of host resistance, an intrinsic biotic trait

Until recently, evolutionary processes had been deemed to be operating on timescales that were too slow to be of immediate concern for species facing imminent extinction risks (Lande 1988; Thompson 1998). However, a catastrophic event, e.g. an epidemic, may lead to strong selection and rapid change of intrinsic biotic traits. For example, when pathogen prevalence is high within a host population, traits conferring resistance are predicted to increase in frequency (Altizer *et al*. 2003). These traits may include: humoral and cell-mediated immune defenses; physiological and innate responses to infection; and behavioural defenses to avoid exposure or remove parasites (Clayton & Moore 1997).

Several studies have highlighted the importance of genetic variation in host resistance, with regards to population-wide disease dynamics, in both field and experimental settings (Jokela & Lively 1995; Alexander *et al*. 1996; Gaffney & Bushak 1996; Dwyer *et al*. 1997; Paterson *et al*. 1998; Coltman *et al*. 1999; Culloty *et al*. 2001). However, this body of work has focused on adaptive changes in host resistance over evolutionary time-scales. The general assumption is that over ecological time-scales, host resistance is fixed (Bull 1994; Ewald 1994; Frank 1996; Kraaijeveld *et al*. 1998; Fenner & Fantini 1999; Dieckmann *et al*. 2002). This view has been challenged, as several studies have reported a change in host resistance within a decade or shorter. For example, multi-year studies of plant pathogens have shown rapid changes in resistant phenotypes in populations following short-term selection by rust pathogens (Burdon & Thompson 1995; Burdon & Thrall 1999). Dube *et al*. (2002) reported that high mortality of sea fan corals, caused by a fungal pathogen (*Aspergillus sydowii*), could be shaping spatial variation in anti-fungal resistance in the Florida Keys. Thus, resistant phenotypes can evolve surprisingly quickly in natural communities. On an even shorter time-scale, several studies have reported a change in host resistance during an ecological interaction, due to intrinsic changes in the state of one of the organisms (Taylor & Read 1997; Pels & Sabelis 1999; Sokurenko *et al*. 1999; De Jong & Janss 2002). This suggests that evolution can be studied as an ecological process (Reznick *et al*. 1997; Thompson 1998; Scheiner & Callahan 1999), and highlights the fact that host-parasite interactions may provide a rich showcase of co-evolution.

3.1.2.2 Effects of extrinsic factors on host resistance

Extrinsic biotic and abiotic factors are generally viewed as the 'backdrop' for host-pathogen interactions. Most studies do not consider them to play an explicit role in the development of disease outcome (Steinhaus 1960; Lewis & Tumlinson 1988; Karban & Myers 1989; Agrawal *et al*. 1999; Tollrian & Harvell 1999; Elliot *et al*. 2000). As a result, few studies have considered the effect of extrinsic factors on host resistance (see Martens *et al*. 1967; Kraaijeveld & Godfray 1997; Ostfeld & Keesing 2000; Johnson *et al*. 2008; Raffel *et al*. 2013; García-Ramos *et al*. 2015). Given the strong selective pressures imposed by parasites and the benefits of host resistance traits, it is important to investigate both intrinsic and extrinsic factors that maintain intra- and inter- population variation in resistance to pathogens. Thus, I wish to consider a system in which a fluctuating climate (extrinsic abiotic factor), mediated by host behaviour (intrinsic biotic factor) and phenotype (intrinsic biotic factor), determines the course of a host–pathogen interaction.

3.1.3 The chytrid fungus *Batrachochytrium dendrobatidis (Bd)*

Chytridiomycosis is a potentially lethal skin infection of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*; Longcore *et al*. 1999). *Bd* is transmitted via an obligate aquatic flagellated zoospore (Berger *et al*. 2005a; Piotrowski *et al*. 2001; Figure 1.1), and infects keratinized tissues of amphibians, specifically the skin of post-metamorphic stages and mouthparts of larvae (Longcore *et al*. 1999; Berger *et al*. 2005a). It is associated with population declines and mass-mortality events in over 200 species (Skerratt *et al*. 2007; Scheele *et al*. 2017), across several continents (Berger *et al*. 1998; Lips 1999; Bosch *et al*. 2001; La Marca *et al*. 2005). Despite this fact, individual species can exhibit highly heterogeneous

responses to *Bd* infection (Daskin & Alford 2012; Puschendorf *et al*. 2011; Woodhams & Alford 2005; Retallick *et al*. 2004; Blaustein *et al*. 2005; Rachowicz *et al*. 2006, Bradley *et al*. 2002; Davidson *et al*. 2003; Daszak *et al*. 2004). For example, the spread of *Bd* in mountain yellow-legged frog (*Rana muscosa*) populations in the Sierra Nevada mountains of California has led to rapid, local extirpation of some populations, but persistence of others at low frog densities (Briggs *et al*. 2010; Vredenburg *et al*. 2010). This pattern is extended in Europe within common midwife toads (*Alytes obstetricans*), where some populations have experienced high rates of mortality and population crashes due to chytridiomycosis, while other comprehensively infected populations appear stable (Bosch *et al*. 2001; Walker *et al*. 2010; Tobler *et al*. 2012). This variation in population-level infection prevalence and host mortality is often correlated with extrinsic abiotic factors, such as temperature and moisture.

3.1.3.1 Effects of temperature and moisture on survival of *Bd*

Bd displays maximum growth in laboratory culture under cool conditions (17 - 25 °C). Peak growth and pathogenicity occurs at 23 °C, and *Bd* growth ceases at 28 °C (Piotrowski *et al*. 2004; Woodhams *et al*. 2008a; Richards-Zawacki 2010). Incubation of *Bd* cultures at 30 °C for 8 days killed 50 % of colonies (Piotrowski *et al*. 2004), and 100 % mortality occurred within 96 hours at 32 °C, or within 4 hours at 37 °C (Johnson & Speare 2003). Additionally, *Bd* does not persist in amphibian hosts above certain temperature thresholds. In laboratory experiments, short-term exposure to temperatures between 27 and 37 °C successfully cleared *Bd* infections from five species of adult frogs with no reported side effects (Woodhams *et al*. 2003, 2012; Berger *et al*. 2004; Retallick & Miera 2007; Chatfield & Richards-Zawacki 2011).

The link between disease and cooler temperatures is further supported by field studies conducted in disparate geographic regions, which show that *Bd* infections are generally more severe in winter months, and when hosts are located at cooler temperatures (Bradley *et al*. 2002; Berger *et al*. 2004; Retallick *et al*. 2004; Murray *et al*. 2009; Voordouw *et al*. 2010; Kilpatrick *et al*. 2010). This relationship is also supported by my findings in Chapter 2, where *Rana pipiens* populations experiencing short warm climates prior to breeding were unlikely to support the establishment of *Bd,* while populations facing cold climates prior to breeding were most likely to harbour *Bd* infections (see Figure 2.10). Although it is widely accepted that temperature is a key factor for chytridiomycosis (Knapp *et al*. 2011), the functional mechanism(s) linking ambient conditions to epidemiological patterns remain poorly understood.

Bd is highly susceptible to drying, as obligate aquatic chytrid zoospores require moisture for movement, survival and colonization (Piotrowski *et al*. 2004; Kriger 2009). In culture, *Bd* is killed after 3 hours of drying at room temperature (Johnson *et al*. 2003). Furthermore, Murphy *et al*. (2011) reported that the endangered toad, *Anaxyrus boreas*, had higher survival and lower *Bd* zoospore loads in drier and warmer laboratory terraria. Field studies further support this relationship, showing that *Bd* is less pathogenic in drier habitats (Kriger & Hero 2007b; Puschendorf *et al*. 2011; Terrell *et al*. 2014). Additionally, my findings in Chapter 2 also support this relationship, as site-level *Bd* mean intensity (Genomic Equivalents; GE) is influenced by hydrological variables (see Figure 2.11).

3.1.4 Amphibian thermoregulation and disease dynamics

Amphibians are active behavioural thermoregulators. They routinely increase their body temperature (T_b) by basking in sunlight (Freed 1980; Duellman & Trueb 1986) and/ or selecting for high-temperature microclimates within a spatially and temporally variable environment. In doing so, they buffer themselves against the negative effects of temperature (Bartholomew 1966; Huey 1991). If T_b affects an amphibian's vulnerability to *Bd*, individuals may be able to avoid or reduce the severity of infection by behaviorally manipulating their body temperatures (Woodhams *et al*. 2003). Richards-Zawacki (2010) demonstrated that during an epidemic, mean body temperatures of frogs increased, which reduced the negative effects of the pathogen. Rowley and Alford (2013) showed that an individual's probability of infection declined rapidly as they spent more time above the pathogen's upper optimum temperature. These increases in mean body temperature, at the population level, could reflect adaptive responses of individuals to infection, shifting thermal preferences in order to produce a 'behavioural fever' (Parris *et al*. 2004; Richards-Zawacki 2010). However, it could also result from 'selective sweeps' in which individuals that attain higher temperatures for other reasons (Witters & Sievert 2001) are more likely to survive during outbreaks of chytridiomycosis. In either case, it suggests that direct or indirect selection for higher thermal preferences could reduce susceptibility to this pathogen. This relationship may explain the highly heterogeneous response to *Bd* infection recorded within a single species, and as such the population-level patterns of prevalence in nature.

3.1.4.1 Individual phenotypic traits and thermal capacity

Ectothermic animals are characterized by possessing physiological and behavioural states, which are temperature dependent. It has therefore been suggested that constraints on the range of body temperatures available to individuals, imposed by individual phenotypic properties, may translate into differential fitness and selection against thermally inferior phenotypes. For instance, within species that are polymorphic for dorsal colour pattern, dark morphs generally increase their body temperature more rapidly, and achieve a higher body temperature than their paler counterparts (Watt 1968; Gibson & Falls 1979; Forsman 1995, 1997; Jong *et al*. 1996). This is because darker pigmentations (e.g. forest green, indigo and brown) absorb more solar irradiance and have lower reflectance (Gates 1980) than paler pigments (light green, pale grey and yellow), which leads to higher heating rates and equilibrium temperatures (Kalmus 1941; Watt 1968). Such variation in thermal capacity has been noted to play a significant role in various fitness-related traits, such as: effects on activity periods, energy budgets, escape capability, dispersal, mating success and fecundity (Gibson & Falls 1979; Kingsolver & Watt 1983; Willmer 1991; Adolph & Porter 1993; Jong *et al*. 1996; Gilchrist 1996; Kingsolver 1996; Forsman & Appelqvist 1998).

3.1.4.2 *Rana pipiens* morphotypes and disease dynamics

A number of studies have demonstrated that dark pigmentation in amphibians can aid individuals in thermoregulating at higher temperatures (Carey 1978; Hoppe 1979; Garcia *et al*. 2003). The Northern Leopard Frog, (*Rana pipiens;* Yuan *et al*. 2016; Figure 1.3) is a medium-sized, semi-terrestrial frog that is currently considered globally secure. The species is characterised by dark spots located on their dorsum

and conspicuous dorsolateral ridges, which border the spots. They are typically green or brown, as a result of genetic variation at a single locus at which the green allele is dominant (Fogleman *et al*. 1980). In addition to the two colour morphs, there are two dominant spot-pattern mutants: (1) 'burnsi', conspicuous by the absence of the customary leopard-like spots (Moore 1942), and (2) 'kandiyohi', which exhibit unique vermiculate mottlings in the interspaces between the dorsal spots (Volpe 1955). The two mutant genes are non-allelic and express their effects independently when combined together in the same genotype (Volpe 1960; Volpe & Dasgupta 1962). Thus, individuals display varying degrees of melanism across four classes of progeny: wild type, kandiyohi, burnsi and double dominant (Merrell 1965; Figure 3.1(A) & 3.1(B)). Given that pigmentation can aid individuals in thermoregulating at higher temperatures, and evidence suggests that warmer individuals harbor reduced infection levels: perhaps measures of pigmentation in *R. pipiens* directly influence disease dynamics, by depressing individual infection load or clearing the infection entirely.

Figure 3.1(A): The Northern Leopard Frog, *Rana pipiens*. Green colour morph, depicting the range of spot patterning. *(Images: author's own)*.

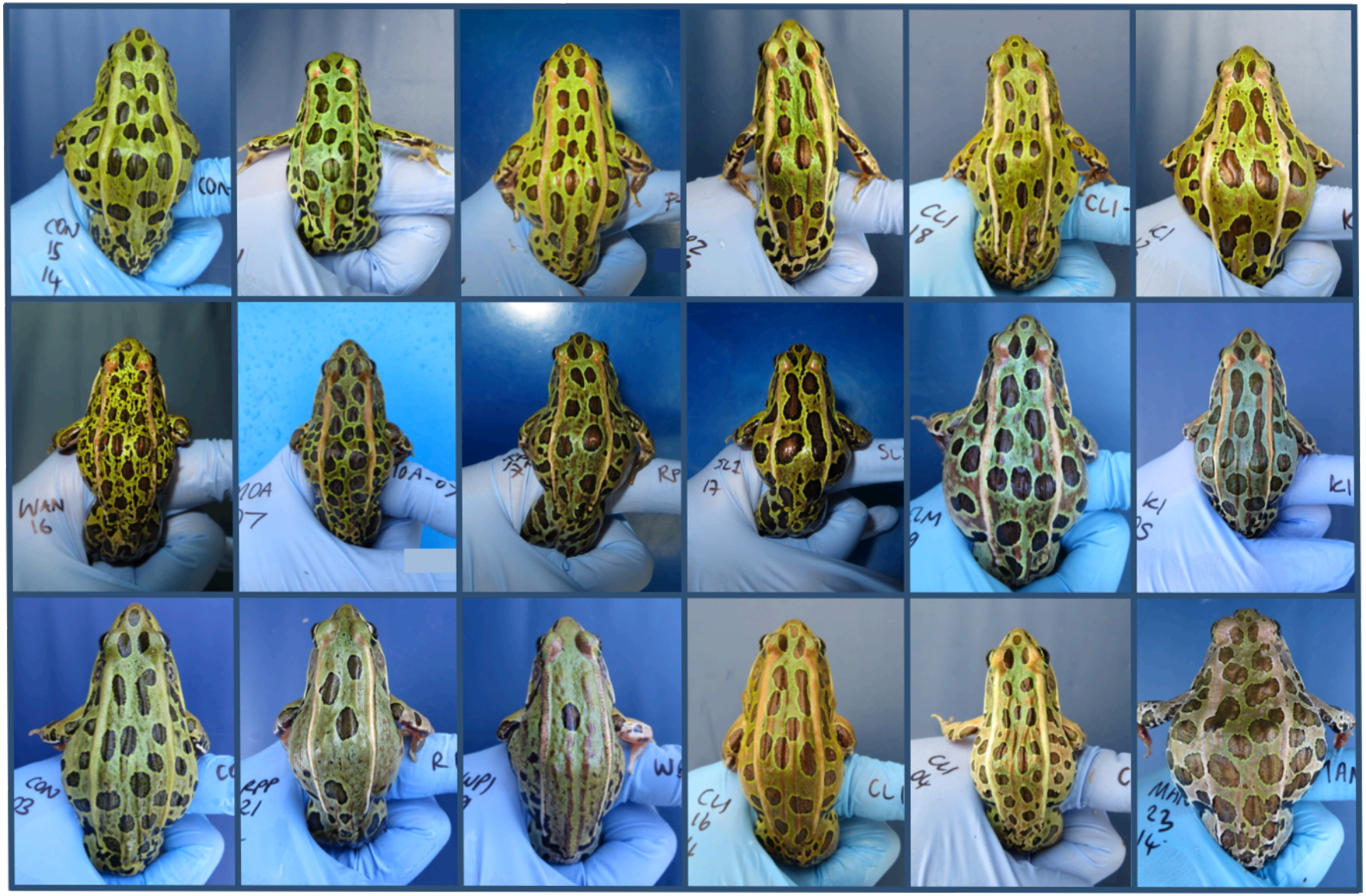


Figure 3.1(B): The Northern Leopard Frog, *Rana pipiens*. Brown colour morph, depicting the range of spot patterning. *(Images: author's own)*.

3.1.4.3 *Rana pipiens* dehydration, dispersal and disease dynamics

The outermost layer of frog skin, the stratum corneum, is composed of a thin layer of keratinized cells, and offers very little resistance to movement of water between internal and external environments (Lillywhite 2006). As such, most amphibians readily lose water across their moist, permeable skins. Increasing ambient temperatures will exacerbate evaporative losses (Spotila 1972), and limit water economy (Pounds & Crump 1994). However, among anurans, the survival limits for dehydration tolerance range from 25 to 60 % of total body water lost (Thorson & Svihla 1943; Hoar 1986; Shoemaker 1992), which is generally positively correlated with the degree of their terrestriality (Thorson & Svihla 1943; Thorson 1955; Farrel & MacMahon 1969).

Once *R. pipiens* emerge from their overwintering sites, they become highly active in the terrestrial realm. Post-metamorphic individuals commonly travel up to 800 m from pond of origin (Dole 1971). However, maximum dispersal distances of 8 – 10 km have been documented (Dole 1971; Seburn & Seburn 1997; Romanchuk & Quinlan 2006). This suggests that individuals will experience varying degrees of dehydration, as a function of distance from free water. However, *R. pipiens* is a semi-terrestrial anuran, and as such, the species can survive a loss of up to 50 % of total body water (approximately 40 % of body mass; Churchill & Storey 1995). In contrast, chytrid zoospores cannot survive desiccation (Johnson *et al.* 2003). Consequently, increased dispersal rates (measured in distance from water body) could alter disease dynamics by exposing zoospores to a greater level of dehydration, especially when ambient temperatures are high.

Alternatively, increased dispersal of *R. pipiens* individuals could also lower pathogen transmission by depressing population density and/ or limiting frequency of host-host contact (Briggs *et al*. 2005). Transmission of *Bd* is likely influenced by both density- and frequency- dependent components (Ryder *et al*. 2007). Controlled experimentation has demonstrated that route of *Bd* transmission is through contact with water that previously contained an infected tadpole or adult (Berger *et al*. 1998; Retallick 2002, Parris & Cornelius 2004; Rachowicz & Vredenburg 2004). However, infective *Bd* zoospores are present on, and can be recovered from, the skin surfaces of infected animals (Berger *et al*. 1998; Pessier *et al*. 1999), and *Bd* DNA has been detected on wet rocks and leaves at a site during an epidemic (Lips *et al*. 2006; Kolby *et al*. 2015). Becker *et al*. (2014a) found that amphibian host diversity decreased *Bd* infection due to changes in species interactions, specifically by reducing shared habitat use and transmission among hosts. It therefore seems likely that *Bd* transmission occurs via contact with infected individuals and/ or contaminated environmental substrates. Dispersal that is seasonal- and density- dependent has been found to directly affect host-host contact rates in wildlife populations (Sandell *et al*. 1990; Pusenius & Viitala 1993; Loughran 2006). As such, increased dispersal of *R. pipiens* individuals could lower pathogen transmission by depressing population density and/ or limiting frequency of host-host contact (Briggs *et al*. 2005).

3.2 RESEARCH AIMS AND OBJECTIVES

In this study, frog body temperature, phenotype (colouration and spot pattern), individual dispersal, air temperature and *Bd* infection intensity (Genomic Equivalents; GE) were used to test the following hypotheses:

(1) Individuals thermoregulating at the high end of the thermal scale, will have lower *Bd* infection loads, than those thermoregulating at the low end of the thermal scale,

(2) Individuals will be constrained on the range of body temperatures available to them, due to their phenotypic properties. Individuals with a greater percentage of spots or those exhibiting low green colouration (and conversely, a high brown colour content) will be able to achieve higher body temperatures than those with a lower percentage of spots or those exhibiting high green colour content,

(3) Particular phenotypes will be more likely to harbour high infection loads due to their inability to reach body temperatures that may restrict pathogen growth on the host,

(4) Individuals that disperse more widely from their breeding habitat are more likely to exhibit lower infection intensities, due to: (i) evaporative dehydration, which will minimize zoospore mobility and viability, and/ or (ii) lowered host-host pathogen transmission owing to depressed contact rates,

(5) Air temperature will play an explicit role in the development of the above interactions, by directly influencing the body temperature of the host.

Thus, I wish to demonstrate the adaptive value of host thermoregulation in terms of infection intensity, mediated by phenotype.

3.3 METHODS

3.3.1 Sampling wild *Rana pipiens* populations

I surveyed 26 *R. pipiens* population localities in Ontario, Canada. All sites were geo-referenced using GPS and sampled at least once during the summer months (May –

August) of 2013 and/ or 2014 (Table 3.1; Figure 3.2). Each site was sampled within a three-week period in order to limit within-season variation due to local environmental conditions. Sites were defined as a circular area with a 2 km radius, each separated by a minimum of 10 km from their nearest neighbour. As maximum dispersal distances of 8 – 10 km have been documented for *R. pipiens* (Dole 1971; Seburn & Seburn 1997; Romanchuk & Quinlan 2006), sites may be considered as distinct populations with limited (to zero) gene flow between them and their nearest neighbour. The 26 population localities were selected based on the known distribution of *R. pipiens* populations (Figure 2.2).

As latitudinal spans were large in my study (42 °N – 47 °N), I opted to group the site localities into three distinct categories based on the latitudinal spans of established terrestrial ecoregions (Olson *et al*. 2001). Ecoregions are defined as relatively large units of land containing a distinct assemblage of natural communities, sharing a large majority of species, dynamics, and environmental conditions. The three categories included: (1) the Southern Great Lakes forest (latitude < 43.5 °N), henceforth 'south region'; (2) the Eastern Great Lakes lowland forest (43.5 °N > latitude < 44.5 °N), henceforth 'central region'; and (3) the Eastern forest-boreal transition (latitude > 44.5 °N), henceforth 'north region'.

Within each site, 30 post-metamorphic frogs were captured using a dip net, and sampled. Snout-vent length (SVL) was measured to the nearest 0.01 mm (using electronic callipers). Individuals with a SVL of less than 45 mm were considered recent metamorphs, those with an SVL > 52 mm were considered adults, and those with an SVL between 45 - 52 mm were considered juveniles (Wright & Wright 1949; Leduc & Lesbarrères, unpublished data). I did not include recent metamorphs in the

analysis as natal dispersal and behaviour is dissimilar to that observed in juveniles and adults (Dole 1965a; Merrell 1977). In localities where *R. pipiens* were rare (n < 24), the observed population was sampled exhaustively for 30 person-hours. In an infinite population, a sample size of 30 is sufficient to detect at least one infected individual with a 95 % probability if the underlying prevalence is 10 % (Cannon & Roe 1982).

At point of capture, frog temperature was recorded using a Fluke 561 Multi-purpose Infra-red Thermometer (emissivity set to 0.95; Tracy 1976; Carroll *et al*. 2005; Rowley *et al*. 2006; Rowley & Alford 2007a) providing T_b readings within 0.5 °C of cloacal temperatures (Rowley & Alford 2007a). Water, air and substrate temperatures (°C) were recorded at point of capture using K-type Thermocouple probes (Fluke 80PK-22, 80PK-24 and 80PK-27). Distance from nearest water body (m) from point of capture was recorded. However, only water bodies with a minimum depth of 6 cm were considered, as accurate measurement requires the probe to be inserted to this depth (FLUKE Corporation 2009). Air and frog body temperatures were recorded to the nearest 0.1 °C while water and substrate temperatures were measured to the nearest 1 °C.

I toe-clipped each individual (first phalange of the longest toe of the front right foot) using sterile equipment following standardized protocols (Hyatt *et al*. 2007). Tissue samples were stored in 70 % ethanol at 4 °C until processing (Hyatt *et al*. 2007). To prevent pathogen transmission, all frogs were handled with non-powdered disposable vinyl gloves, and standard biosecurity measures were followed (Phillott *et al*. 2010). There were no recaptured individuals, thus I treated each sampling year as independent.

All animals were handled and released according to an approved Laurentian University Animal Care and Use Committee protocol #2009-03-04. Field surveys and specimen collection were conducted under the Ontario Ministry of Natural Resources Wildlife Scientific Collector's Authorization #1068178. Amphibian handling and sampling within protected areas (including National Parks, Provincial Parks, Conservation Areas and Management Areas) followed permit guidelines awarded by: Parks Canada Agency (#BPF-2013-13913); Ontario Ministry of Natural Resources and Forestry (#4534); Nature Conservancy of Canada (#AG-ON-2012-144055); and St. Clair Region Conservation Authority (#2012-28-05).

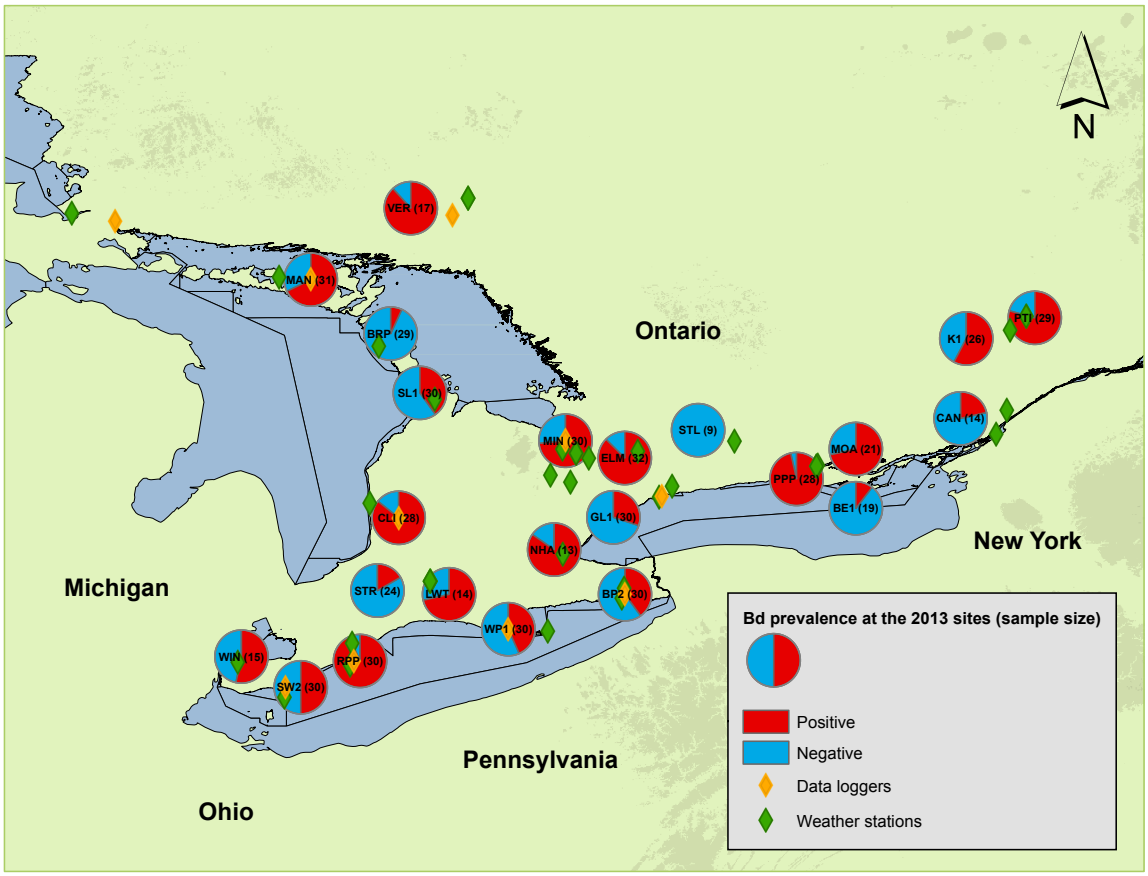
Table 3.1: Summary statistics for adult and juvenile (SVL > 45 mm) *R. pipiens* individuals, sampled at 26 sites in 2013 and 2014. *(Table continues on next pages)*.

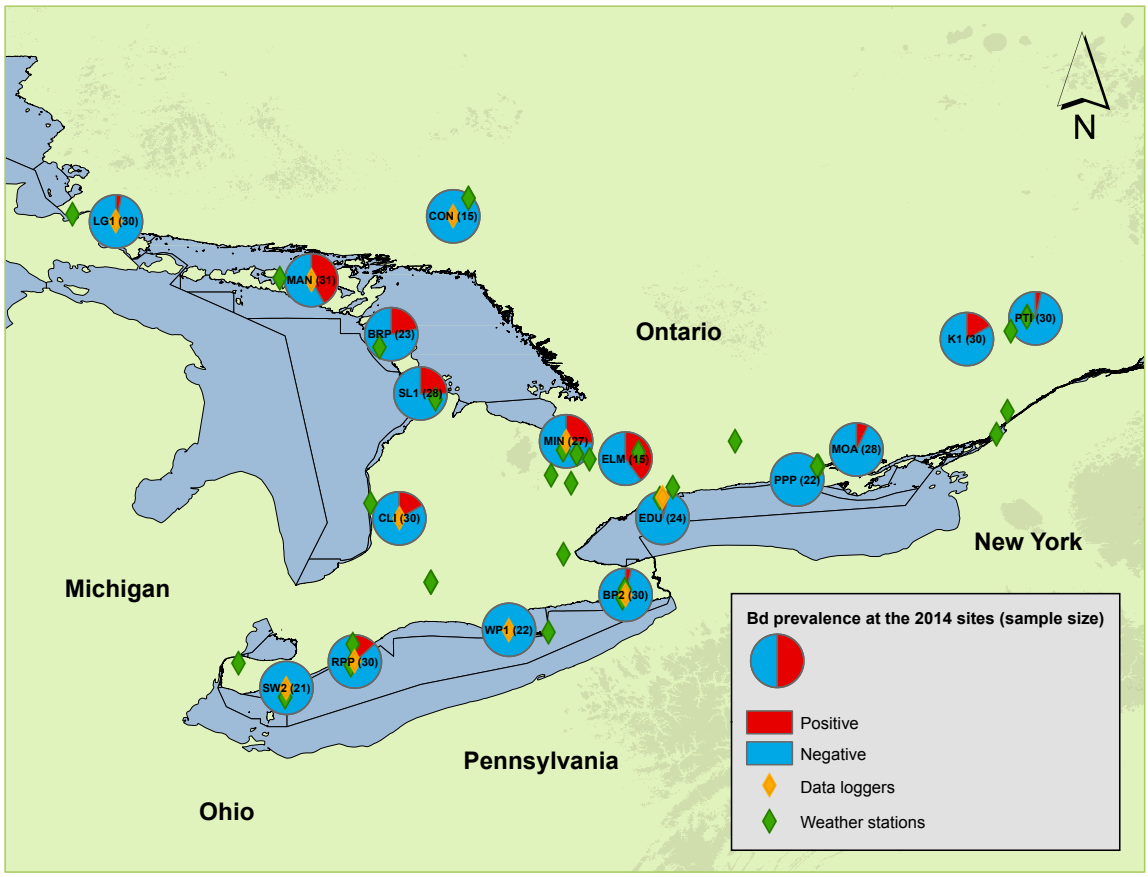
Site	Year	Sample size	% Prevalence (N = Individuals *Bd* positive)	Mean infection intensity (GE) (SE, range)	Mean distance from water (m) (SE, range)	Mean green pigmentation (%) (SE, range)	Mean spot cover (%) (SE, range)	Mean frog temperature (T_b) (°C) (SE, range)	Mean air temperature (LOG_A) (°C) (SE, range)
BE1	2013	19	10.5 (2)	3.3 (0.3, 1.8 - 4.8)	17.0 (2.6, 3.1 - 41.1)	24.5 (4.3, 0 - 54.6)	31 (1.6, 19.1 - 42.7)	26.3 (0.5, 19.5 - 28.6)	28.8 (1.0, 17 - 34)
BP2	2013	30	40 (12)	50.6 (8.0, 2.0 - 199.1)	2.3 (0.2, 0.1 - 4.1)	32.3 (3.6, 0.4 - 66.3)	32.9 (0.8, 27.0 – 49.0)	25.7 (0.4, 21.0 - 29.5)	27.6 (0.3, 24 - 32)
	2014	30	3.3 (1)	120.4 (4.0, 120.4 - 120.4)	21.0 (4.8, 2.3 - 100.8)	39.3 (3.7, 2.2 - 65.9)	31.1 (0.8, 22.6 - 40.6)	23.6 (0.2, 20.9 – 26.0)	25.1 (0.4, 22 - 28)
BRP	2013	29	6.9 (2)	1.2 (0.1, 0.3 - 2.1)	16.4 (2.0, 1.0 - 47.2)	26.6 (4.7, 0.3 - 63.6)	31.1 (1.3, 16.9 - 48.8)	27.2 (0.2, 25.1 - 29.3)	30.0 (0.3, 28 - 35)
	2014	23	21.7 (5)	14.5 (1.8, 2.9 - 35.8)	27.8 (3.3, 5.2 - 61.1)	38.3 (4.6, 1.7 - 71.2)	31.8 (1.2, 22.9 - 46.8)	24.5 (0.6, 19.9 - 32.3)	26.7 (0.5, 23 - 30)
CAN	2013	14	21.4 (3)	7.2 (1.0, 0.5 - 11.4)	34.1 (9.4, 5.8 - 119)	38.2 (5.0, 4.6 - 71.6)	32.5 (1.9, 23.7 - 45.5)	27.7 (0.4, 25.6 - 30.1)	29.5 (0.4, 28 - 33)
CLI	2013	28	85.7 (24)	162.7 (56, 0.7 - 1323.6)	0.1 (0, 0 - 1)	42.5 (2.9, 11.6 - 67.5)	33.6 (1.1, 23.3 - 44.4)	21.3 (0.4, 17.8 - 25.3)	21.0 (0.2, 19 - 23)
	2014	30	16.7 (5)	4.9 (0.4, 1.0 - 7.7)	12.0 (2.3, 1.8 - 43.7)	51.1 (2.7, 16.8 - 73.5)	30.2 (1.1, 19.4 - 45.9)	19.9 (0.2, 17.1 - 22.2)	21.8 (0.3, 19 - 25)
CON	2014	15	0 (0)	0 (0, 0 – 0)	64.3 (13.9, 0 - 159.1)	53.8 (3.4, 39 - 77.7)	32.1 (1.2, 22.8 - 42.5)	25.3 (0.6, 19.4 - 28.1)	28.5 (0.8, 19 - 32)
EDU	2014	24	4.2 (1)	30.5 (1.3, 30.5 - 30.5)	28.4 (2.9, 5.2 - 51.8)	58.4 (3.6, 21.0 – 80.7)	34.1 (1.0, 24.5 - 44.1)	23.4 (0.6, 17.4 - 28.1)	25.8 (0.6, 22 - 30)
ELM	2013	32	87.5 (28)	554.2 (141.2, 3.6 - 3371.3)	2.1 (0.4, 0 - 11.5)	31.5 (3.5, 1.2 - 64.9)	32.2 (1.0, 21.2 – 45.0)	26.2 (0.3, 23.5 - 29.7)	26.6 (0.3, 24 - 30)
	2014	15	40 (6)	30.7 (8.6, 0.2 - 116.6)	5.6 (0.9, 0.6 - 10.5)	47.9 (4.2, 22.0 - 77.2)	32.8 (1.9, 22.8 – 47.0)	24.4 (0.5, 21.4 – 27.0)	30.7 (0.6, 25 - 33)
GL1	2013	30	30 (9)	19.0 (3.4, 0.8 - 96.6)	42.6 (6.3, 0 - 130)	47.1 (3.5, 12.6 - 79.3)	32.2 (1, 21.8 - 42.3)	26.5 (0.5, 21.7 - 32.3)	32.1 (0.6, 27 - 39)
K1	2013	26	57.7 (15)	78.8 (17.9, 0.3 - 426.7)	11.4 (4.6, 0 - 97)	51.9 (2.1, 34.4 - 71.9)	29.4 (1.0, 19.4 - 43.2)	23.7 (0.6, 17.8 - 27.7)	23.5 (0.5, 19 - 28)
	2014	30	16.7 (5)	5.9 (0.5, 1.3 - 14.8)	46.6 (9.8, 0.6 - 148.5)	56.1 (2.5, 13.1 - 76.2)	31.3 (0.7, 24.6 - 39.1)	25.4 (0.4, 21.4 - 28.8)	29.3 (0.6, 22 - 34)

Site	Year	Sample size	% Prevalence (N = Individuals *Bd* positive)	Mean infection intensity (GE) (SE, range)	Mean distance from water (m) (SE, range)	Mean green pigmentation (%) (SE, range)	Mean spot cover (%) (SE, range)	Mean frog temperature (T_b) (°C) (SE, range)	Mean air temperature (LOG_A) (°C) (SE, range)
LG1	2014	30	3.3 (1)	1.1 (0, 1.1 - 1.1)	153.3 (15.4, 14 - 288.4)	31.8 (3.4, 1.0 - 59.5)	33.6 (1.5, 21.9 - 64.1)	25.5 (0.3, 22.2 - 29.2)	31.4 (0.3, 29 - 34)
LWT	2013	14	71.4 (10)	35.2 (15.7, 1.2 - 221.9)	67.2 (15.0, 1.5 - 124)	42.1 (5.7, 3.6 - 70.4)	35.5 (1.9, 25.3 - 50.4)	24.4 (0.8, 20.4 - 29.4)	26.1 (0.9, 20 - 30)
MAN	2013	31	67.7 (21)	53.2 (13, 0.8 - 307.9)	78.2 (9.1, 0 - 197)	41.8 (3.2, 1.1 - 65.4)	37 (0.9, 25.2 - 47.8)	25.9 (0.5, 21.0 - 29.5)	28.6 (0.5, 23 - 33)
	2014	31	41.9 (13)	16.8 (3.9, 0.6 - 117.9)	91.7 (4.8, 36.4 - 153)	41.7 (3.3, 2.8 - 63.5)	33.3 (1, 23.3 - 47.2)	23.2 (0.4, 19.0 - 26.6)	27.2 (0.5, 22 - 34)
MIN	2013	30	73.3 (22)	55.3 (14.7, 1.5 - 424.7)	2.1 (0.7, 0 - 10.3)	41.1 (3.6, 5.7 - 65.9)	33.6 (1.1, 22.6 - 46.7)	23.4 (0.3, 20.3 – 26.0)	22.9 (0.1, 21 - 25)
	2014	27	25.9 (7)	3.2 (0.4, 0.4 - 7.9)	7.3 (1.4, 0.6 - 31.5)	53.8 (2.3, 25.2 - 79.8)	34.3 (0.8, 27.5 – 43.0)	25.2 (0.5, 20.1 - 28.6)	28.3 (0.5, 24 - 34)
MOA	2013	21	71.4 (15)	186.5 (114, 0.6 - 2407)	0.1 (0, 0 - 0.6)	54.0 (3.7, 7.5 - 76.5)	30.0 (1.3, 21.5 - 43.4)	23.7 (1.2, 13.6 – 30.0)	25.4 (1.3, 15 - 32)
	2014	28	7.1 (2)	156.5 (11.2, 0.7 - 312.3)	0 (0, 0 - 0)	53.3 (2.9, 12.3 - 76.9)	34.2 (1.0, 26.2 - 45.5)	23.8 (0.3, 19.6 – 27.0)	25.9 (0.3, 21 - 29)
NHA	2013	13	84.6 (11)	161.9 (53.9, 4.7 - 659.2)	10.7 (9.5, 0 - 124)	51.0 (7.4, 4.3 - 80.1)	30.6 (1.2, 23.7 - 38.1)	21.8 (0.8, 16.5 - 25.3)	23 (0.7, 18 - 26)
PPP	2013	28	96.4 (27)	1045.3 (310.6, 1.1 - 7427.2)	3.1 (1.3, 0 - 29.6)	28.8 (2.6, 11.2 - 65.4)	30.6 (1.2, 19.7 - 51.2)	21.7 (0.6, 17.7 – 29.0)	21.1 (0.4, 18 - 27)
	2014	22	0 (0)	0 (0, 0 – 0)	66.1 (12.6, 0.6 - 177.6)	37.7 (3.3, 12.5 - 66.4)	31.9 (1.3, 23.1 - 45.8)	22.8 (0.6, 18.0 - 28.8)	24.5 (0.4, 19 - 28)
PTI	2013	29	79.3 (23)	452.9 (127.3, 0.4 - 2691.5)	0.4 (0.2, 0 - 4.2)	33.2 (3.0, 9.3 - 62.4)	33.4 (0.8, 25.8 - 44.9)	17.8 (0.2, 15.7 - 20.5)	18.8 (0.1, 18 - 20)
	2014	30	3.3 (1)	78.1 (2.6, 78.1 - 78.1)	33.9 (7.9, 1.8 - 137.5)	38.3 (2.8, 0.4 - 64.8)	33.5 (0.9, 25.7 - 43.6)	22.9 (0.2, 20.1 - 24.4)	22.5 (0.1, 21 - 24)
RPP	2013	30	93.3 (28)	232.2 (60.1, 3.8 - 1230.3)	6.5 (1.3, 0 – 32.0)	21.1 (4.0, 1.8 - 68.4)	29.00 (1.1, 14.9 - 41.9)	26.4 (0.3, 23.9 - 28.4)	27.2 (0.3, 24 - 31)
	2014	30	13.3 (4)	7.0 (0.6, 0.8 - 15.2)	11.7 (1.1, 0.6 - 29.1)	29.9 (2.7, 1.7 – 79.0)	29.1 (1.6, 1.9 – 40.0)	26 (0.3, 22.6 - 28.5)	29.2 (0.3, 26 - 32)
SL1	2013	30	40 (12)	15.9 (3.2, 0.2 - 91.8)	8.4 (1.5, 0 - 36.8)	31.0 (3.8, 2.0 - 67.9)	39.7 (1.6, 23.6 - 63.1)	26.8 (0.2, 24.3 - 29.4)	30.6 (0.2, 28 - 33)
	2014	28	25 (7)	40.2 (5.4, 0.4 - 138)	9.2 (1.9, 0.7 - 28)	39.4 (3.3, 11.4 - 66.7)	40.8 (1.3, 27.8 - 54.6)	24.2 (0.4, 20.9 – 33.0)	26.5 (0.7, 19.3 - 33)

Site	Year	Sample size	% Prevalence (N = Individuals *Bd* positive)	Mean infection intensity (GE) (SE, range)	Mean distance from water (m) (SE, range)	Mean green pigmentation (%) (SE, range)	Mean spot cover (%) (SE, range)	Mean frog temperature (T_b) (°C) (SE, range)	Mean air temperature (LOG_A) (°C) (SE, range)
STL	2013	9	0 (0)	0 (0, 0 – 0)	15.3 (2.4, 0 - 21.5)	62.5 (2.5, 53.2 - 76.2)	28.5 (1.3, 21.9 - 33.3)	26.7 (0.7, 23.1 - 29.8)	26.8 (1.0, 22 - 31)
STR	2013	24	16.7 (4)	28.7 (4.2, 2.0 – 101.0)	13.4 (1.6, 4.9 - 28.2)	54.0 (3.2, 0.9 - 71.4)	32.1 (1.1, 25.3 - 53.5)	27.5 (0.3, 24.7 - 30.2)	29.2 (0.6, 25 - 37)
SW2	2013	30	50 (15)	91.7 (20.7, 3.4 - 528.4)	7.1 (0.9, 0.1 - 23)	33.4 (4.2, 1.3 - 77.9)	26.8 (1.1, 6.9 - 40.9)	27.1 (0.6, 20.4 - 32.2)	26.3 (0.6, 21 - 31)
	2014	21	0 (0)	0 (0, 0 – 0)	17.0 (5.1, 4.1 - 103.6)	35.5 (4.2, 5.3 - 66.9)	30.4 (1.3, 20.1 - 44.4)	22.9 (0.5, 18.4 - 29.7)	25.2 (0.4, 23 - 30)
VER	2013	17	88.2 (15)	767.0 (285.5, 4.1 - 3950.9)	0 (0, 0 - 0.2)	42.3 (3.3, 19.5 - 72.1)	34.9 (1.8, 18.0 – 45.0)	22.2 (0.5, 19.7 - 25.9)	26.4 (0.5, 21 - 30)
WIN	2013	15	53.3 (8)	11.0 (2.3, 0.8 - 29.9)	7.8 (1.7, 0 - 27.6)	34.7 (7.0, 1.0 – 77.6)	28.5 (1.2, 17.8 - 34.6)	24.3 (0.4, 21.5 - 26.2)	21.5 (0.6, 18 - 26)
WP1	2013	30	43.3 (13)	105.0 (22.9, 2.7 - 601.2)	4.9 (1.0, 0 - 25.9)	20.4 (3.5, 0 - 57.9)	30.9 (1.1, 18.0 - 43.9)	25.5 (0.3, 22.6 - 29.2)	24.7 (0.4, 21 - 30)
	2014	22	0 (0)	0 (0, 0 – 0)	12.8 (2.2, 4.2 - 40.2)	33.0 (3.9, 8.3 – 70.0)	31.8 (1.8, 5.5 - 46.9)	24.6 (0.4, 21.5 – 28.0)	26.8 (0.2, 25 - 29)

Figure 3.2: *Bd* infection prevalence (%) in adult and juvenile (SVL >45 mm) *R. pipiens* collected from 26 sites in Ontario from 2013 and 2014. Pie chart denotes prevalence (**red: *Bd* +ve, blue: *Bd* -ve**), with sample size noted in brackets. Data loggers symbolized by orange marker, weather stations symbolized by green marker. *(Figure: author's own, created in ArcGIS version 10.2.2).*





3.3.2 Laboratory analysis of samples

All toe-clip samples were stored at 4 °C until processing (Hyatt *et al*. 2007). DNA was extracted using QIAGEN DNeasy Kits following the standard protocol (Qiagen). The presence⁄ absence and quantity of *Bd* was then assessed using the real-time PCR protocol described by Boyle *et al*. (2004). Extractions were diluted 1/10 and all assays were performed in duplicate, to ensure reliability of results. Samples were considered *Bd* positive when a clear log-linear amplification was evident and both repeats indicated a detection limit of > 0.1 mean genomic equivalents (GE).

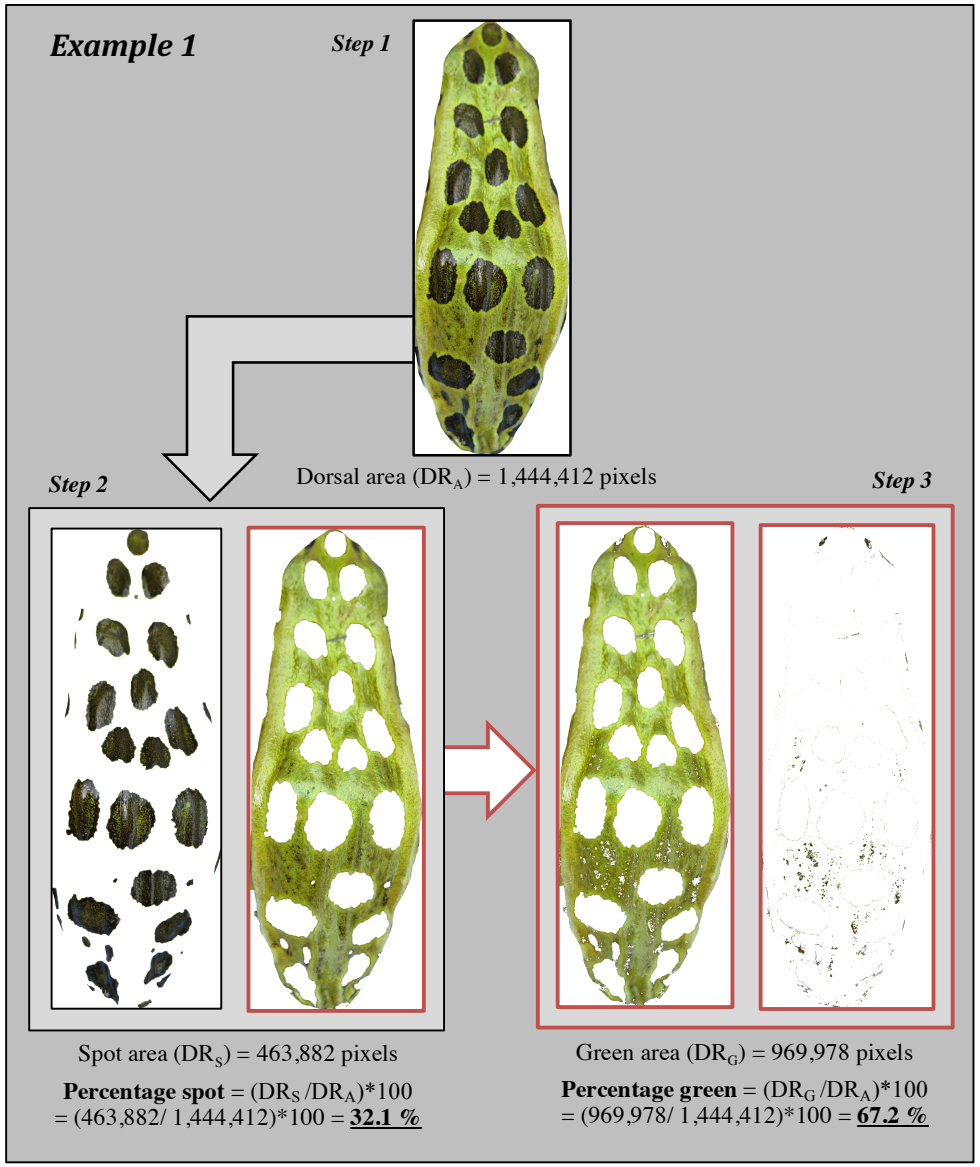
3.3.3 Acquisition of environmental data/ annual climatic variation

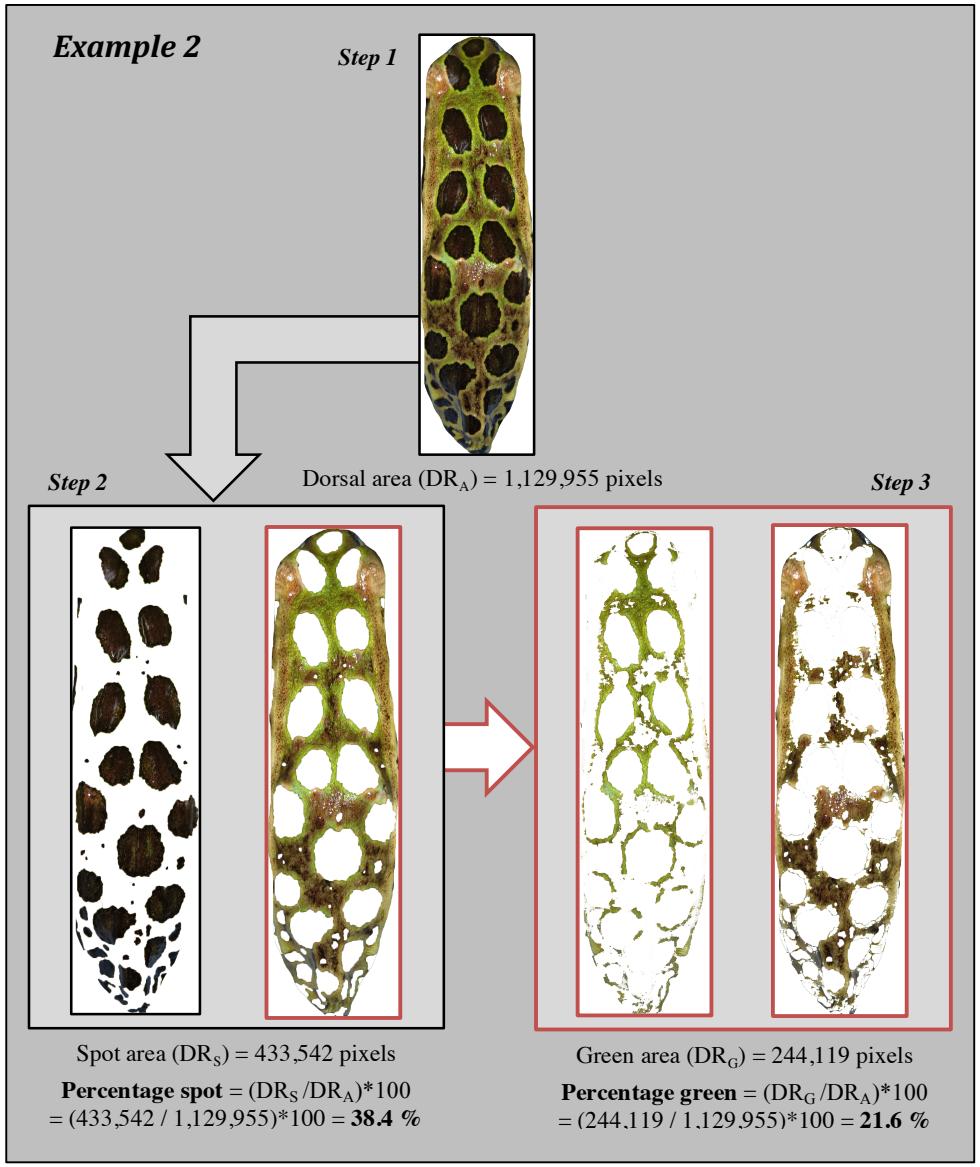
In order to assess climatic variation throughout 2013 and 2014, data regarding air temperature (˚C), relative humidity (Φ), and wind speed (km/h) were downloaded from the Government of Canada (2016) historic climate database, for weather stations in closest proximity to site centroids (range: 1.4 – 42.3 km, mean: 18.9 km; Figure 3.2; Saino *et al*. 2011). In an effort to achieve completeness of data, any gaps in the hourly recordings (between January 2013 and December 2014) were filled with data from the next closest weather stations (range: 6.6 – 39.3 km, mean: 24.9 km). Additionally, 10 of the 26 sites were fitted with data loggers (HOBO U23 Pro v2 Data Logger (U23-001); Figure 3.2). These loggers recorded air temperature (˚C) and relative humidity (Φ) at site centroid, on an hourly basis, throughout the sampling period. If more than one record existed for a particular time point at a particular site, prominence fell to the record at closest proximity to site centroid.

3.3.4 Photographic analysis

To quantify *R. pipiens* morphotypes, unmodified and uncompressed jpeg image files of all individuals were accessed in Adobe Photoshop CC (version 2015.5.1; Figure 3.3). Multiple images of each individual were taken with the flash, against a standard blue card in order to capture a high-quality image with minimal reflection and glare (Figure 3.1(A) & 3.1(B)). Total dorsal area (pixels; DR_A), bordered by the snout, vent and dorso-lateral folds, was calculated using the 'magnetic lasso tool'. Any obvious debris or glare that could hinder selection was removed from the image by selection (via lasso tool) and deletion from the DR_A. Brightness and contrast were not altered, and no filters (e.g. sharp or unsharp mask) were applied. Numbers of dorsal spots, defined as spherical cells with clearly defined smooth edges, were quantified. Spots that overlapped were counted as individual entities if 75 % of each spot was visible. If not, they were identified as one spot. All dorsal spots within the DR_A were selected via the 'quick selection tool' and the area recorded (pixels; DR_S). Spot cover as a percentage of DR_A was then calculated as: $(DR_S/DR_A)*100$. To allow unbiased selection of colouration and increase the throughput of analysis, a representative green colour range selection mask was created using five randomly chosen images from each of the 26 sites (n = 130). A colour range mask is a palette of manually selected colour tones, which when applied to an image serves as a command to select those tones. In this case, the mask contained a comprehensive and representative panel of green colour tones that were specifically observed in *R. pipiens*. Having selected the green colour tones (DR_G), green pigmentation as a percentage of DR_A was then calculated as: $(DR_G/DR_A)*100$.

Figure 3.3: Photographic analysis of uncompressed jpeg image files for all *R. pipiens* individuals (assessed in Adobe Photoshop CC version 2015.5.1). *(Images: author's own)*. ***Step 1:*** **DR_A**, dorsal area is recorded (pixels) by selecting the desired area via 'magnetic lasso tool'. ***Step 2:*** **DR_S**, spot area, is calculated by selecting spots via 'quick selection tool' and recording area (pixels). **Percentage spot** is then calculated as: $(DR_S/DR_A)*100$. ***Step 3:*** A green colour mask is then applied to the DR_S image, minus dorsal spots, to select for all green colour tones, **DR_G**. **Percentage green pigmentation** is then calculated as: $(DR_G/DR_A)*100$. The remaining percentage represents percentage brown colouration.





3.3.5 Statistical analysis

All statistical analyses were implemented in R (version 3.2.3; R Core Team 2015). I did not expect serial autocorrelation to be present, with respect to amphibian life history and infection status data, as sampling occurred at yearly intervals. All variables were z-transformed [(x-mean) / SD] prior to analysis to have a mean of 0 and standard deviation of 1. This put all predictors on a common scale and made main effects interpretable in the presence of interactions (Schielzeth 2010).

3.3.5.1 Univariate models for data description and assessing bias

I estimated differences in: infection intensity; phenotype; distance from water; body temperature; and climate, between regions (north, central and south), months and years with univariate analysis of variance (ANOVA). Any significant results from ANOVA testing were further tested using Tukey post hoc test in order to determine which categorical groupings were different from the others. In order to assess confounding pairs of covariates, a Pearson correlation coefficient was used (absolute correlation coefficient > 0.5). In order to assess the dissimilarity of climates between years, I used the R package 'pdc' to carry out permutation distribution cluster analysis (Brandmaier 2015).

3.3.5.2 Multivariate mixed model framework

I used a bivariate-response mixed effects model to quantify the relationship between *Bd* intensity and frog temperature while simultaneously evaluating support for predictors such as phenotype (percentage green and percentage spot), air temperature, and distance from water; and controlling for confounding effects such as sample year. Frog temperature cannot simply be used as a predictor of *Bd* intensity as both traits

may be driven by the same processes (e.g. air temperature), and so including frog temperature as a predictor would result in high correlations in the fixed effects model structure. Use of a bivariate model instead prevents the need to use predicted values from prior models in subsequent models, which can cause bias in results (Wilson *et al*. 2010; Houslay & Wilson 2017).

In order to carry out the multivariate mixed model analysis, I used the R package 'MCMCglmm' (Hadfield 2010) to fit a bivariate response model with *Bd* intensity (GE, rounded to nearest integer) and frog temperature (°C) as Poisson- and Gaussian-distributed responses, respectively. While running this package, the two response variables are passed as a matrix. The rows of this matrix are indexed by the reserved variable 'units', which refers to each observation at the site or individual level. The columns of this matrix are indexed by the reserved variable 'trait', referring to both response variables (*Bd* intensity and frog temperature). The terms 'unit' and 'trait' are not part of the original data set: rather, they are reserved terms in the MCMCglmm package. I specified an unstructured variance-covariance matrix of the residuals. I fitted the fixed effect 'trait' to ensure that the two response variables could have different intercepts. I suppressed the intercept so that the second coefficient represents the trait specific intercept, not just the difference between the intercept for the first (*Bd* intensity) and the second level trait (frog temperature). I modelled frog temperature as a function of air temperature (both linearly and as a 2^{nd} order polynomial), distance from water and the interaction between percentage green and percentage spot. I modelled *Bd* intensity as a function of distance from water. I accounted for possible non-independence of samples collected at the same site locality by including a random intercept for site ID (n = 26), and fitted an unstructured variance-covariance matrix for the levels of the outcome. Finally, I

added year as a fixed effect (n = 2). I then estimated the posterior correlation between frog temperature and *Bd* intensity following Harrison *et al*. (2011), whereby a significant positive correlation (95 % credible intervals do not cross zero) is representative of frog temperature significantly influencing *Bd* intensity, after controlling for other confounding factors. Significance of fixed effects was assessed by whether the 95 % credible intervals for their parameter estimates crossed zero. I did not perform model simplification and comparison among models, as the Deviance Information Criterion is unreliable for mixed models containing non-Gaussian responses. Models were run for 110,000 iterations after a burn in of 5000 and using a thinning interval of 50. Convergence was assessed by running two separate MCMC chains with overdispersed starting values and applying the Gelman-Rubin diagnostic to the two sets of posterior samples (Gelman & Rubin 1992). All parameters indicated adequate convergence (potential scale reduction factors all < 1.01). The posterior correlation between frog temperature and *Bd* intensity was calculated from the stored values in the Markov chain as the posterior mode of the covariance (frog temperature, *Bd* intensity) divided by the product of the standard deviations of frog temperature and *Bd* intensity. All graphs were created using R package 'ggplot2' (Wickham 2009).

3.4 RESULTS

I collected and processed 995 toe clips from adult and juvenile *R. pipiens* from 26 sites in Ontario (Table 3.1). Of these 26 sites, 9 were located in the north (latitude > 44.5 ˚ N), 9 in the central belt (43.5 ˚N < latitude < 44.5 ˚N), and 8 in the south (latitude < 43.5 ˚N). Due to the sampling strategy, not all sites were sampled in both years. Of the 995 individuals sampled: 559 were sampled in 2013, and 436 were

sampled in 2014. Infection was detected across a broad geographic range: in 2013, 22 out of the 23 sites sampled showed presence of *Bd*; in 2014, 13 of the 17 sites showed presence of *Bd* (Figure 3.2). Overall prevalence was 37.9 %, and despite presence of strong infections (mean GE [SE] = 90.3 [13.6], maximum GE = 7427.2; Table 3.1) no mortality was detected. Air temperature, distance from water, percentage green and percentage spot, did not display an absolute correlation coefficient > 0.5, consequently all predictor variables were included in future modelling (Table 3.2).

3.4.1 Univariate models

3.4.1.1 Annual and monthly climatic variation

Air temperature (LOG_A) was recorded as a continuous time series at each of the 26 sites via loggers or weather stations. This allowed for permutation distribution cluster analysis, which indicated dissimilarity between 2013 and 2014 ($p < 0.0001$). Assessing the sample period as one time frame per year (May – August), 2013 was significant warmer than 2014 ($F_{1, 237463} = 2753$, $p < 0.0001$; Table 3.3, Figure 3.4(A)). May, July and August were warmer in 2013 than their 2014 counterparts ($F_{1, 53328} = 114.1$, $p < 0.0001$; $F_{1, 64084} = 3804$, $p < 0.0001$; $F_{1, 65267} = 391.9$, $p < 0.0001$, respectively), although June 2013 was cooler in comparison to June 2014 ($F_{1, 54778} = 318.3$, $p < 0.0001$; Table 3.4). This pattern was also evident throughout the regions (Table 3.5). May consistently experienced the coolest temperatures, while July experienced the warmest (2013: $F_{3, 104776} = 8534$, $p < 0.0001$; 2014: $F_{3, 132681} = 10047$, $p < 0.0001$; Table 3.4). This pattern was also evident throughout the regions (Table 3.5). The southern sites experienced the warmest temperatures throughout the two years ($p < 0.0001$), and the central sites were warmer than northern sites ($p < 0.0001$; Table 3.5, Figure 3.4(B)). As patterns in air temperature remained constant

throughout month and region, across the two years, I decided not to include either month or latitude as predictors in further modelling.

3.4.1.2 Variation in *Bd* infection intensity

Intensity of infection (GE) did not vary between adults (SVL > 52 mm) and juveniles (45 mm < SVL < 52 mm), however, it did vary significantly between years ($F_{1, 993} = 32.9$, $p < 0.0001$; Table 3.3) with a greater intensity of infection recorded in 2013 than in 2014. In 2013, infection intensity differed between months ($F_{3, 555} = 11.3$, $p < 0.0001$). Infection loads in May were much greater than those recorded in June ($p < 0.01$), July ($p < 0.0001$) or August ($p < 0.001$). Similarly, June exhibited higher infections loads than July and August ($p < 0.01$). However, there was little variation in infection loads between July and August, which was also evident in 2014. Assessing intensity of infection between the same months, across the two years: infection intensity was greater in July 2013 than July 2014 ($F_{1, 461} = 7.6$, $p < 0.01$). However, infection intensity did not differ between August 2013 and August 2014 (Table 3.6). Unfortunately, no individuals were sampled in May or June of 2014, thus no direct comparisons can be made between these months, across the two years.

Table 3.2: Absolute correlation coefficient for environmental predictor variables. All combinations were included in analysis as absolute correlation coefficient for all pairs of covariates < 0.5. **Air temperature,** air temperature at point of amphibian capture (˚C); **Distance from water,** amphibian proximity to water at point of capture (m); **Percentage green,** amount of green pigmentation between snout-vent and dorso-lateral folds, for each amphibian; **Percentage spot,** total area, between snout-vent and dorso-lateral folds, covered in dark spots.

Predictor #1	**Predictor #2**	**cor**
Air temperature	Distance from water	0.27
Air temperature	Percentage green	-0.01
Distance from water	Percentage green	0.01
Air temperature	Percentage spot	0.06
Distance from water	Percentage spot	0.09
Percentage green	Percentage spot	-0.12

Table 3.3: Annual summary statistics, for all variables recorded in 2013 and 2014. **LOG_A,** mean air temperature (°C); **T_b**, frog temperature (°C).

	2013	**2014**
Sample size (juvenile/ adult)	559 (88/471)	436 (117/319)
Mean infection intensity (SE, range)	158.2 (23.8, 0.16 – 7427.23)	3.22 (1.0, 0.24 – 312.27)
Mean LOG_A from May – August (SE, maximum)	18.5 (0.02, 39.9)	17.3 (0.02, 37.9)
Mean T_b (SE, maximum)	24.8 (0.1, 32.3)	23.9 (0.1, 33.0)
Mean percentage green pigmentation (SE, range)	37.0 (0.9, 0 - 85.1)	43.2 (0.9, 0.4 – 87.4)
Mean percentage spot cover (SE, range)	32.2 (0.3, 6.9 – 63.1)	32.8 (0.3, 1.9 – 64.1)

Figure 3.4(A): Mean daily air temperature (LOG_A; °C) across 26 sites, separated by region, between January 2013 and December 2014. The **red data points** highlight sampling period (May – August). Note the higher temperatures recorded in 2013, across all regions.

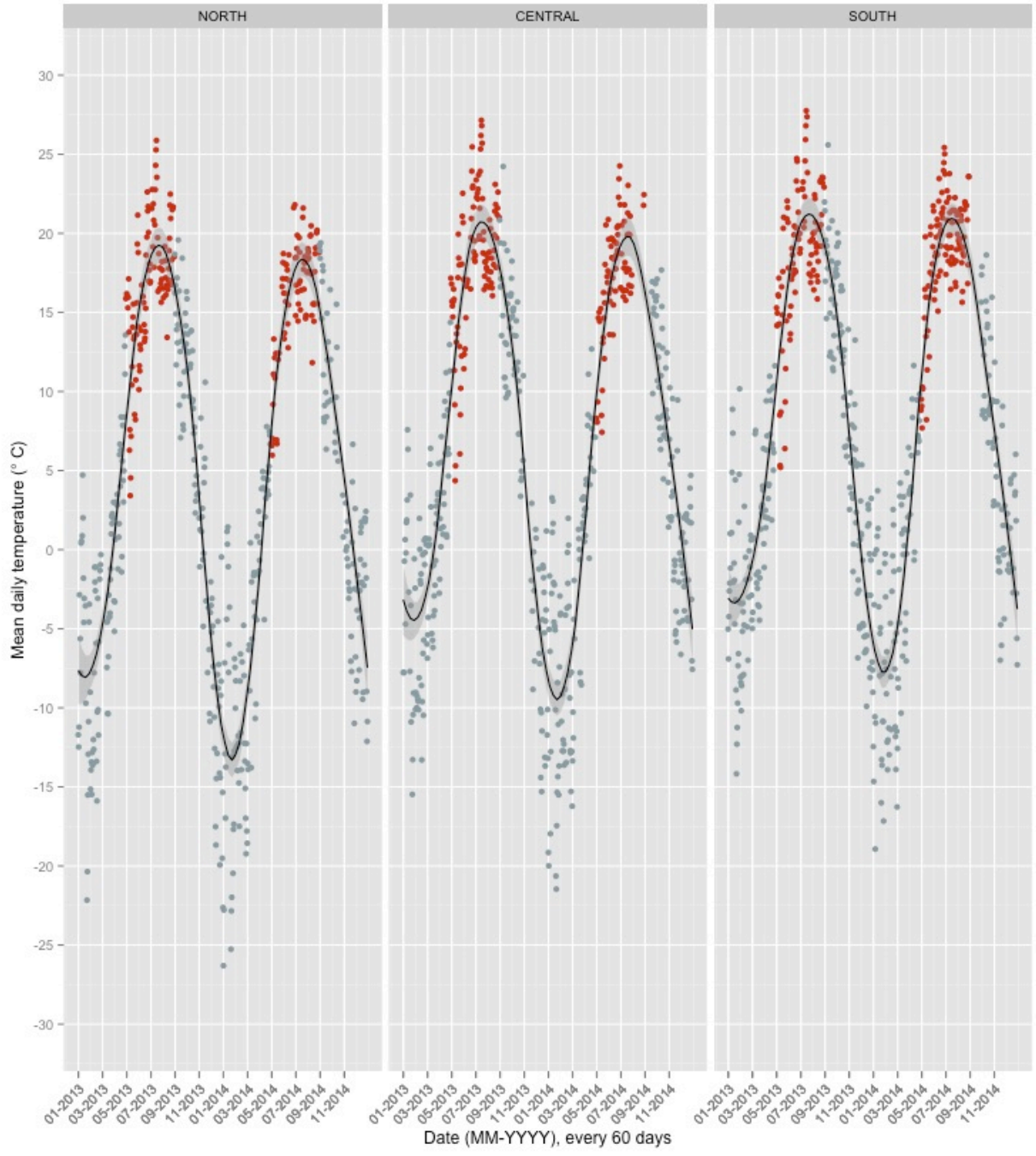


Figure 3.4(B): Mean daily air temperature (LOG_A; °C) by region (across 26 sites), between January 2013 and December 2014. Each region experiences differing temperature regimes, with the northern sites (**red line**) consistently experiencing cooler temperatures than the southern (**yellow line**) or central sites (**green line**).

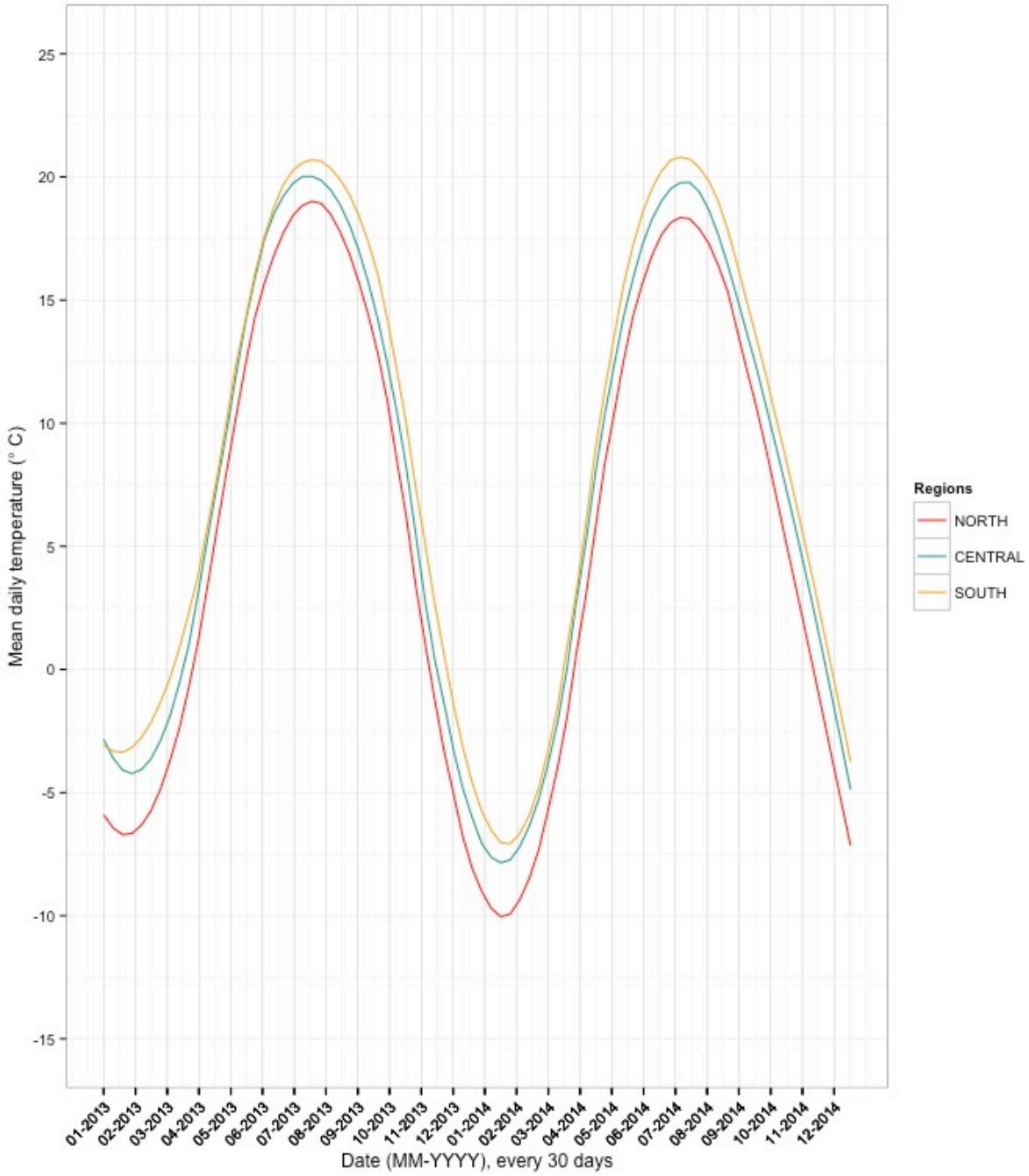


Table 3.4: Summary statistics per month, for air temperature (LOG_A; °C) across 26 sites sampled in 2013 and 2014.

	2013 & 2014 COMBINED Air temperature (°C)				2013 Air temperature (°C)				2014 Air temperature (°C)			
	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range
May	13.3	0.3	37.9	42.5	13.7	0.5	31.5	34.6	13.1	0.3	37.9	42.5
June	18.7	0.2	38.1	36.3	17.9	0.3	38.1	36.3	18.7	0.3	37.1	34.9
July	19.9	0.2	39.9	35.8	21.1	0.3	39.9	35.8	18.8	0.3	35.3	31.2
August	19.1	0.2	37.0	33.0	19.4	0.3	35.3	30.1	18.7	0.3	37.0	33.0

Table 3.5: Summary statistics for all regions, per month, for air temperature (LOG_A; °C) across 26 sites sampled in 2013 and 2014.

	2013 Air temperature (°C)												2014 Air temperature (°C)											
	NORTH				CENTRAL				SOUTH				NORTH				CENTRAL				SOUTH			
	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range
May	12.6	0.8	31.5	33.5	13.9	0.8	30.9	34.0	14.6	0.8	30.4	30.9	11.6	0.6	36.8	41.5	13.6	0.6	37.9	39.8	13.9	0.5	35.9	37.1
June	16.1	0.6	30.9	28.9	17.9	0.6	38.1	36.3	19.3	0.5	33.6	29.3	17.2	0.5	32.0	29.9	18.6	0.5	37.1	33.6	20.1	0.4	33.2	27.0
July	19.8	0.5	34.9	30.9	21.2	0.5	39.9	33.1	21.9	0.4	34.4	26.2	17.5	0.4	31.3	27.2	18.7	0.4	35.3	28.5	20.1	0.3	34.3	26.3
August	18.3	0.5	34.6	29.4	19.3	0.5	35.3	29.7	20.5	0.4	34.3	27.9	17.5	0.4	33.2	29.2	18.5	0.4	37.0	30.5	20.2	0.4	33.0	26.5

Table 3.6: Summary statistics per month, for *Bd* infection intensity in adult and juvenile (SVL > 45 mm) *R. pipiens*, recorded in 2013 and 2014.

	2013 & 2014 COMBINED		2013		2014	
	Sample size (juvenile/ adult)	**Mean infection intensity (SE, range)**	**Sample size (juvenile/ adult)**	**Mean infection intensity (SE, range)**	**Sample size (juvenile/ adult)**	**Mean infection intensity (SE, range)**
May	88 (20/68)	417.04 (111.7, 0.58 - 7427.23)	88 (20/68)	417.0 (111.7, 0.58 - 7427.23)	-	-
June	282 (21/261)	177.70 (30.0, 0.29 - 3950.87)	282 (21/261)	177.7 (30.0, 0.29 - 3950.87)	-	-
July	463 (100/363)	5.12 (1.3, 0.16 - 312.27)	153 (27/126)	10.1 (2.9, 0.16 – 307.94)	310 (73/237)	2.7 (1.2, 0.24 – 312.27)
August	162 (64/98)	4.22 (1.4, 0.41 - 138.03)	36 (20/16)	3.0 (2.8, 7.94 – 101.02)	126 (44/82)	4.6 (1.6, 0.41 – 138.03)

3.4.1.3 Variation in frog body temperature

Over the course of this study, frog body temperature was significantly associated with substrate temperature (Pearson: $0.57 < R < 0.70$, Bonferroni-corrected $p < 0.05$) and air temperature (Pearson: $0.64 < R < 0.76$, Bonferroni-corrected $p < 0.01$). However, substrate and air temperature correlated ($R > 0.75$). As air temperature fluctuated with both month and latitude, only air temperature was used in further analysis. By contrast, frog body temperatures (daily mean, minimum and maximum) were not significantly correlated with wind speed (Pearson: $-0.35 < R < -0.14$), relative humidity (Pearson: $0.05 < R < 0.23$) or water temperature (Pearson: $0.45 < R < 0.49$).

Body temperature did not vary between adults and juveniles. Compiling both years, *R. pipiens* were found to thermoregulate at 24.4 °C (SE = 0.1 °C, maximum = 33.0 °C). Additionally, adults and juveniles did not vary in their ability to thermoregulate consistently above (> 25 °C) or within (17 – 25 °C) the pathogen's optimum temperature. For any given sample date and year, 45.9 % of all *R. pipiens* sampled were found to have a higher T_b than the daily maximum air temperature; 97.9 % were found to have a higher T_b than the daily mean air temperature; and 100 % of individuals were found to thermoregulate above the daily minimum air temperature.

With respect to *Bd* growth in laboratory cultures, 1.4 % of individuals were found to be operating above the range where *Bd* can no longer persist (30 °C); 10.7 % exhibited a T_b high enough to stop the fungus from growing (28 °C); 35.5 % were found to be above the thermal optima for *Bd* (25 – 28 °C); 50.8 % were found to be within the range where *Bd* achieves peak growth and infectivity (17 – 25 °C); and only 1 % were operating below the thermal optima (< 17 °C) (Figure 3.5(A) & 3.5(B)).

Body temperature differed between the two years ($F_{1, 993} = 19.7$, $p < 0.0001$) with body temperatures recorded in 2013 being higher than those recorded in 2014 (Table 3.3). Frogs sampled in July 2013 and August 2013 were warmer than their 2014 counterparts ($F_{1, 461} = 175$, $p < 0.0001$; $F_{1, 160} = 25.1$, $p < 0.0001$, respectively; Table 3.7). Within years, T_b differed between months (2013: $F_{3, 555} = 57.2$, $p < 0.0001$; 2014: $F_{1, 434} = 8.5$, $p < 0.01$). Following the same pattern as air temperature (LOG_A), T_b was cooler in May than June ($p < 0.0001$), July ($p < 0.0001$) and August ($p < 0.0001$); and cooler in June than July ($p < 0.0001$) and August ($p < 0.0001$). In 2013, there was no significant difference in T_b between July and August, yet in 2014, T_b was cooler in July than August ($p < 0.01$; Table 3.7). T_b varied by region ($F_{2, 992} = 18.9$, $p < 0.0001$): the frogs sampled in the south were warmer than those in the central ($p < 0.0001$) or northern regions ($p < 0.001$; Table 3.8).

3.4.1.4 Variation in distance from water

Adults and juveniles did not vary in their proximity to water. However, as their movement patterns are defined by season (Dole 1965a, 1965b, 1967, 1968; Merrell 1970, 1977), it is unsurprising that distance from water varied between months increasing from May to August on average ($F_{3, 991} = 77.6$, $p < 0.01$; Table 3.7). Yet, distance from water also varied between years ($F_{1, 993} = 73.7$, $p < 0.0001$): frogs sampled in July 2013 were located farther from the water body, than those sampled in July 2014; and frogs sampled in August 2013 were located closer to the water body, than those sampled in August 2014 (Table 3.7).

Figure 3.5(A) & 3.5(B): Distribution of frog body temperatures with respect to daily air temperature (LOG_A: maximum, mean and minimum) for **(A)** 2013 and **(B)** 2014. Body temperatures (**black circles**) were almost exclusively above the mean daily air temperature (**green line**) and often above the daily maximum temperature (**red line**). This is especially true for 2013. All body temperatures were above the minimum daily air temperature (**blue line**). *Bd* grows best from 17 to 25°C (**dark green band**); at 28°C the fungus stops growing (**yellow band**) and at 30 °C it dies (**red band**); at lower temperatures, growth rate slows but fecundity and infectivity increases (**blue band**).

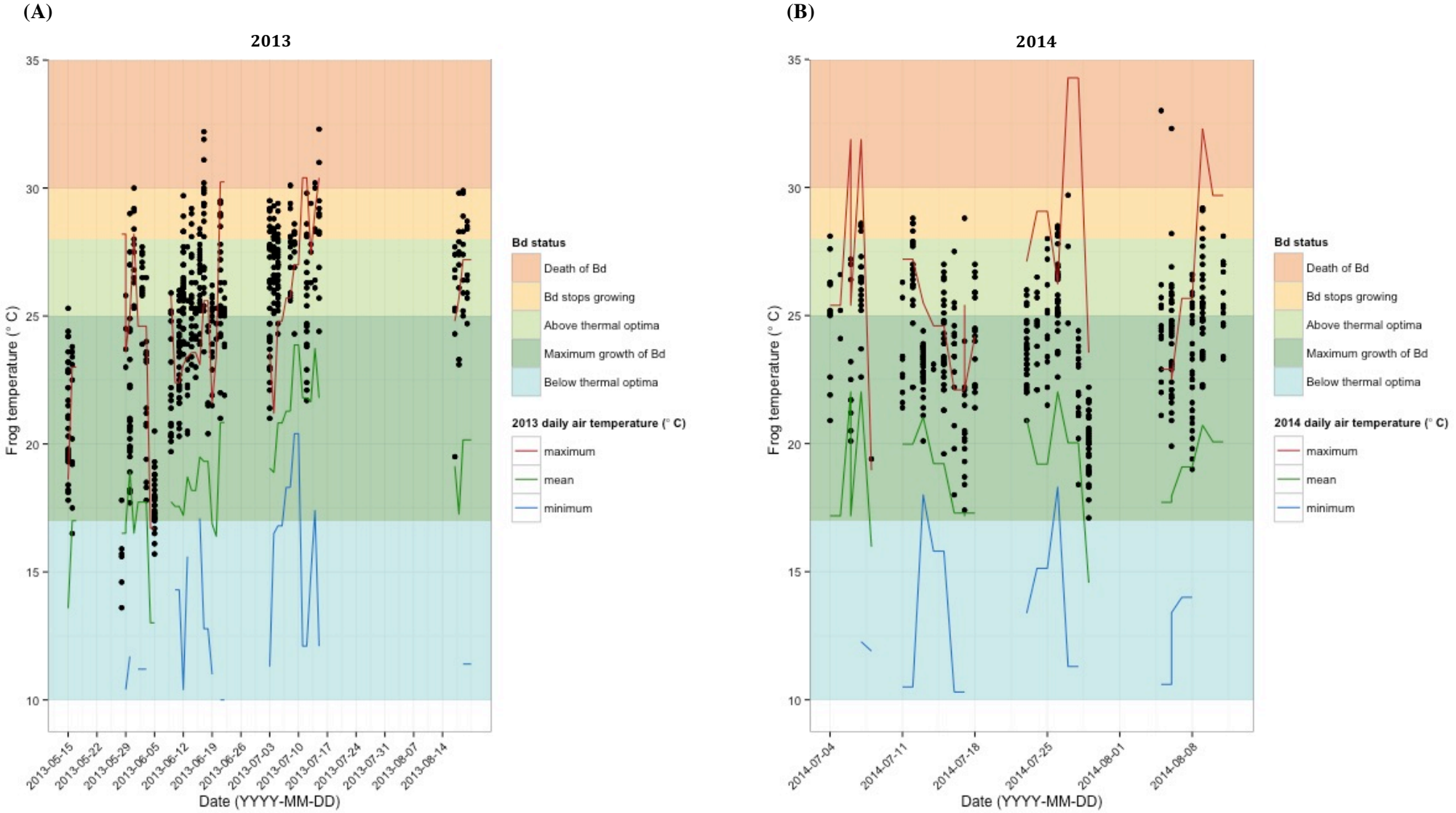


Table 3.7: Summary statistics per month, for frog body temperature (°C) and distance from nearest water body (m), across 26 sites sampled in 2013 and 2014.

	2013							2014						
	Distance from nearest water body (m)			Frog body temperature (°C)				Distance from nearest water body (m)			Frog body temperature (°C)			
	$\bar{x}$	se	max	$\bar{x}$	se	max	range	$\bar{x}$	se	max	$\bar{x}$	se	max	range
May	1.3	0.5	29.6	21.9	0.4	30	16.4	-	-	-	-	-	-	-
June	6.7	1.1	124.0	24.3	0.2	32.2	16.5	-	-	-	-	-	-	-
July	36.7	3.3	197.0	26.9	0.2	32.3	11.3	21.9	1.9	177.6	23.7	0.1	29.7	12.6
August	11.9	1.2	27.6	26.7	0.3	29.9	10.4	73.8	6.4	288.4	24.5	0.2	33.0	14.0

Table 3.8: Summary statistics per month within each region, for frog body temperature (°C) across 26 sites sampled in 2013 and 2014 combined.

	2013 & 2014 COMBINED Frog body temperature (°C)											
	NORTH				CENTRAL				SOUTH			
	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range	$\bar{x}$	se	max	range
May	-	-	-	-	22.1	0.4	30	16.4	20.9	0.9	23.8	7.3
June	20.9	0.4	27.7	12.0	24.8	0.3	29.7	9.4	25.7	0.2	32.2	11.8
July	25.8	0.2	30.1	10.7	23.9	0.2	32.3	15.2	24.7	0.2	30.2	11.8
August	24.5	0.2	33.0	14	26.1	0.6	29.8	10.3	27.2	0.4	29.9	5.2

3.4.1.5 Spatio-temporal variation of phenotypic properties

Frogs tended to be greener in 2014 than 2013 ($F_{1, 993} = 23.0$, $p < 0.0001$; Table 3.3; Figure 3.6); this was also true between regions within years (2013: $F_{2, 556} = 3.7$, $p < 0.05$; 2014: $F_{2, 433} = 26.2$, $p < 0.0001$; Table 3.9). In 2013, frogs located in the central region were significantly greener than those sampled in the south ($p = 0.01$). There was no significant difference between the north and central regions, nor the north and southern regions. In 2014, the pattern is repeated: frogs sampled in the central area were greener than the northern or southern areas ($p < 0.0001$). There was no variation in spot cover (%) between the years (Table 3.3). However, compiling both years, spot cover is higher in the north (mean [SE] = 34.1% [0.4], maximum = 64.1%) than in the central (mean [SE] = 32.3% [0.3], maximum = 51.2%; $p < 0.001$) or southern regions (mean [SE] = 30.5% [0.4], maximum = 53.5%; $p < 0.0001$). This is further supported by the fact that there is a significant positive correlation between spot cover (%) and latitude ($p < 0.0001$, slope = 0.05; Figure 3.7).

3.4.2 Multivariate models

3.4.2.1 Predictors of *Bd* intensity

Bd intensity was significantly influenced by sample year and distance from water. Individuals sampled in 2014 exhibited a less intense infection load (mean = -5.39 [95% credible interval -6.1 to -4.63]; Table 3.10), which supports the univariate findings stated above (see subsection 3.4.1.2; Table 3.3). Intensity of infection was also significantly negatively influenced by distance from water (m) (mean = -0.96 [95% credible interval -1.49 to -0.44]; Table 3.10): as distance from water increased, mean *Bd* intensity decreased (Figures 3.8(A) & 3.8(B)). Within highly prevalent sites,

this relationship is evident. The model also predicts large credible intervals around the correlation at minimal distance from water, which reflects the large variation observed in infection load at the waters edge (Figures 3.8(A)). However, this relationship is not apparent within an average site (i.e. marginalising with respect to site ID) as infection-free conditions predominate (Figures 3.8(B)).

3.4.2.2 Predictors of frog body temperature

Frog body temperature was not influenced by distance from water (m), but did significantly differ between years (mean = -1.45 [95% credible interval -1.73 to -1.18]; Table 3.10). *R. pipiens* sampled in 2014 exhibited cooler body temperatures, which supports the univariate findings stated above (see subsection 3.4.1.3: Table 3.3 & Table 3.7). Both a linear and 2nd order polynomial term for air temperature also influenced frog body temperature (mean = -0.51 [95% credible interval -0.61 to -0.39]; Table 3.10). As air temperatures rise, frog body temperatures are predicted to increase exponentially until approximately 25 °C, when the relationship begins to plateau (Figure 3.9). Individuals sampled in 2013 are also predicted to present higher mean body temperatures, for any given air temperature, than individuals sampled in 2014. For example, maintaining an air temperature of 25 °C: the model predicts that an individual sampled in 2013 will exhibit a mean T_b that is 1.5 °C greater than that of an individual sampled in 2014. Furthermore, as air temperatures increase, the discrepancy between mean frog temperatures exhibited in both years is predicted to widen (Figure 3.9).

Figure 3.6: Density plot of green pigmentation (%), by year. Note the lower density green pigmentation in 2013 (**yellow line**), in comparison to 2014 **(blue line**). The density plot is bi-modal due to the interaction between colour and spot patterning.

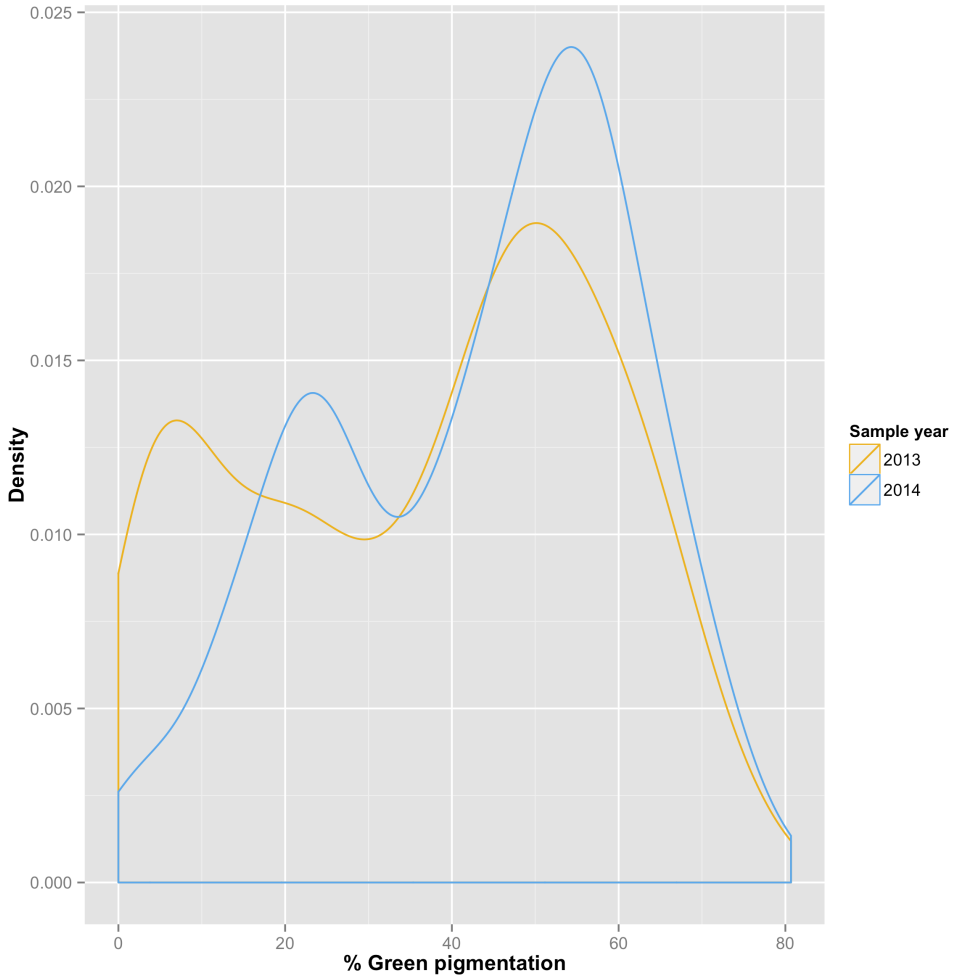


Figure 3.7: Significant positive correlation between spot cover (%) and latitude (grey shading represents 95 % CI). Northern populations are more likely to exhibit higher spot coverage.

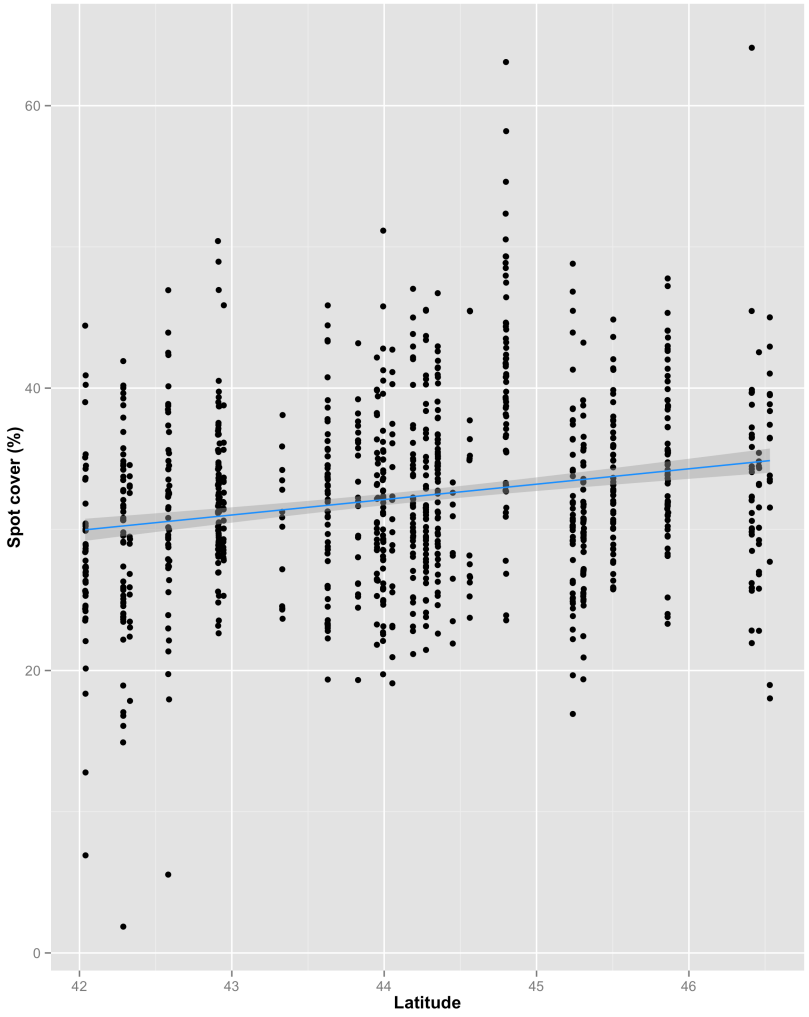


Table 3.9: Summary statistics per month within each region, for frog phenotype (green pigmentation and spot cover; %) across 26 sites sampled in 2013 and 2014.

	2013								2014							
	Green pigmentation (%)				Spot cover (%)				Green pigmentation (%)				Spot cover (%)			
	$\bar{x}$	se	min	max	$\bar{x}$	se	min	max	$\bar{x}$	se	min	max	$\bar{x}$	se	min	max
NORTH	37.3	1.5	0.3	72.1	34.2	0.5	16.9	63.1	42.0	1.4	0.4	77.7	33.9	0.5	21.9	64.1
CENTRAL	39.7	1.4	0	79.3	31.9	0.4	19.1	51.2	50.9	1.3	12.3	87.4	32.9	0.5	19.4	47.0
SOUTH	33.8	1.8	0.05	85.1	30.5	0.4	6.9	53.5	34.4	1.8	1.7	79.0	30.5	0.7	1.9	46.9

Table 3.10: Results from a Bayesian bivariate-response mixed effects model investigating the posterior correlation between *Bd* intensity and frog body temperature. All significant results (95 % credible intervals do not cross zero) are highlighted in bold. Frog temperature was significantly affected by sample year, a 2nd order polynomial term for air temperature and frog phenotype (interaction between percentage green and percentage spot). Sample year and distance from water significantly affected *Bd* intensity. Once controlling for these predictors, there was a significant posterior correlation between frog temperature and *Bd* intensity, at the site level only (not at the individual level). **Mean,** modal estimates from 2,100 models; **Lower 95 % CI,** lower 95% confidence interval from 2,100 models; **Upper 95 % CI,** upper 95% confidence interval from 2,100 models; **us(trait),** unstructured variance-covariance matrix of the residuals. **Notes on model**: 110,000 iterations with 5,000 burn-in and thinning interval of 50; Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ', 1

	Mean	**Lower 95 % CI**	**Upper 95 % CI**	
G-structure: ~us(trait): site				
Bd intensity Variance	7.85	3.06	13.76	
***Bd* intensity/ Frog temperature Covariance**	**-1.25**	**-2.76**	**-0.03**	
***Bd* intensity/ Frog temperature Covariance**	**-1.25**	**-2.76**	**-0.03**	
Frog temperature Variance	0.92	0.36	1.59	
G-structure: ~us(trait): individual				
Bd intensity Variance	12.45	10.49	14.56	
Bd intensity/ Frog temperature Covariance	-0.05	-0.59	0.48	
Bd intensity/ Frog temperature Covariance	-0.05	-0.59	0.48	
Frog temperature Variance	3.35	3.04	3.64	
Predictors				**pMCMC**
Bd intensity intercept	-0.08	-1.19	1.12	
Frog temperature intercept	26.19	24.95	27.39	***
***Bd* intensity: Sample year 2014**	**-5.39**	**-6.1**	**-4.63**	***
Frog temperature: Sample year 2014	**-1.45**	**-1.73**	**-1.18**	***
Frog temperature: Air temperature	**2.23**	**2.05**	**2.37**	***
Frog temperature: poly(Air temperature)^2	**-0.51**	**-0.61**	**-0.39**	***
***Bd* intensity: Distance from water**	**-0.96**	**-1.49**	**-0.44**	***
Frog temperature: Distance from water	0.01	-0.14	0.18	
Frog temperature: Percentage green	0.07	-0.07	0.20	
Frog temperature: Percentage spot	0.08	-0.04	0.22	
Frog temperature: Percentage green: Percentage spot	**0.12**	**0.02**	**0.24**	*
Posterior Correlation				
G-structure: ~us(trait): site				
***Bd* intensity/ Frog temperature**	**-0.47**	**-0.78**	**-0.09**	
G-structure: ~us(trait): individual				
Bd intensity/ Frog temperature	-0.008	-0.09	0.08	

Figure 3.8(A) & 3.8(B): Model predicted negative relationship between *Bd* mean intensity (GE) and distance from water (m) within **(A)** a highly prevalent site (site MAN; see Table 3.1), and **(B)** an average site. Pink shaded areas span the 95% credible intervals for the fitted means.

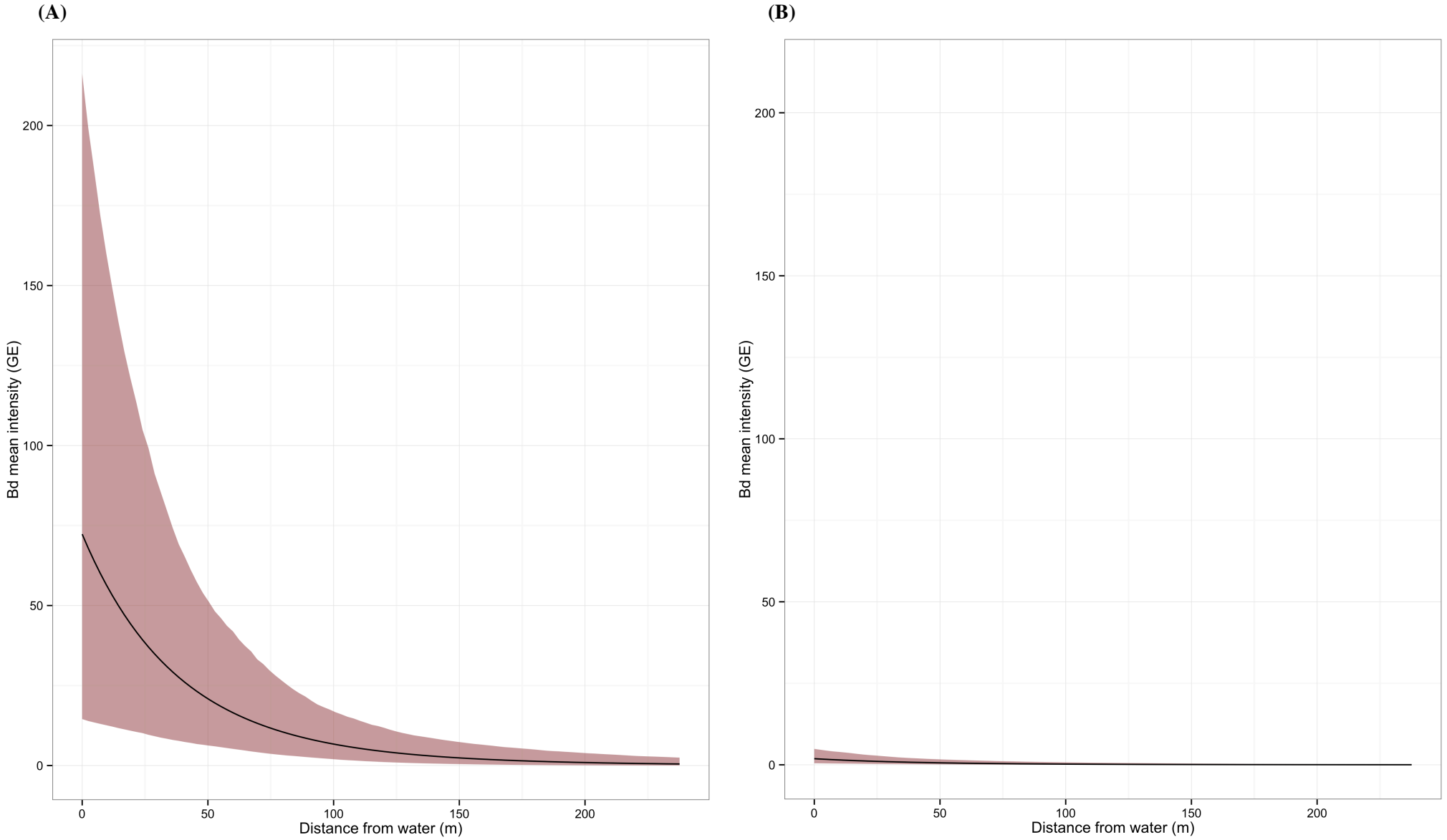
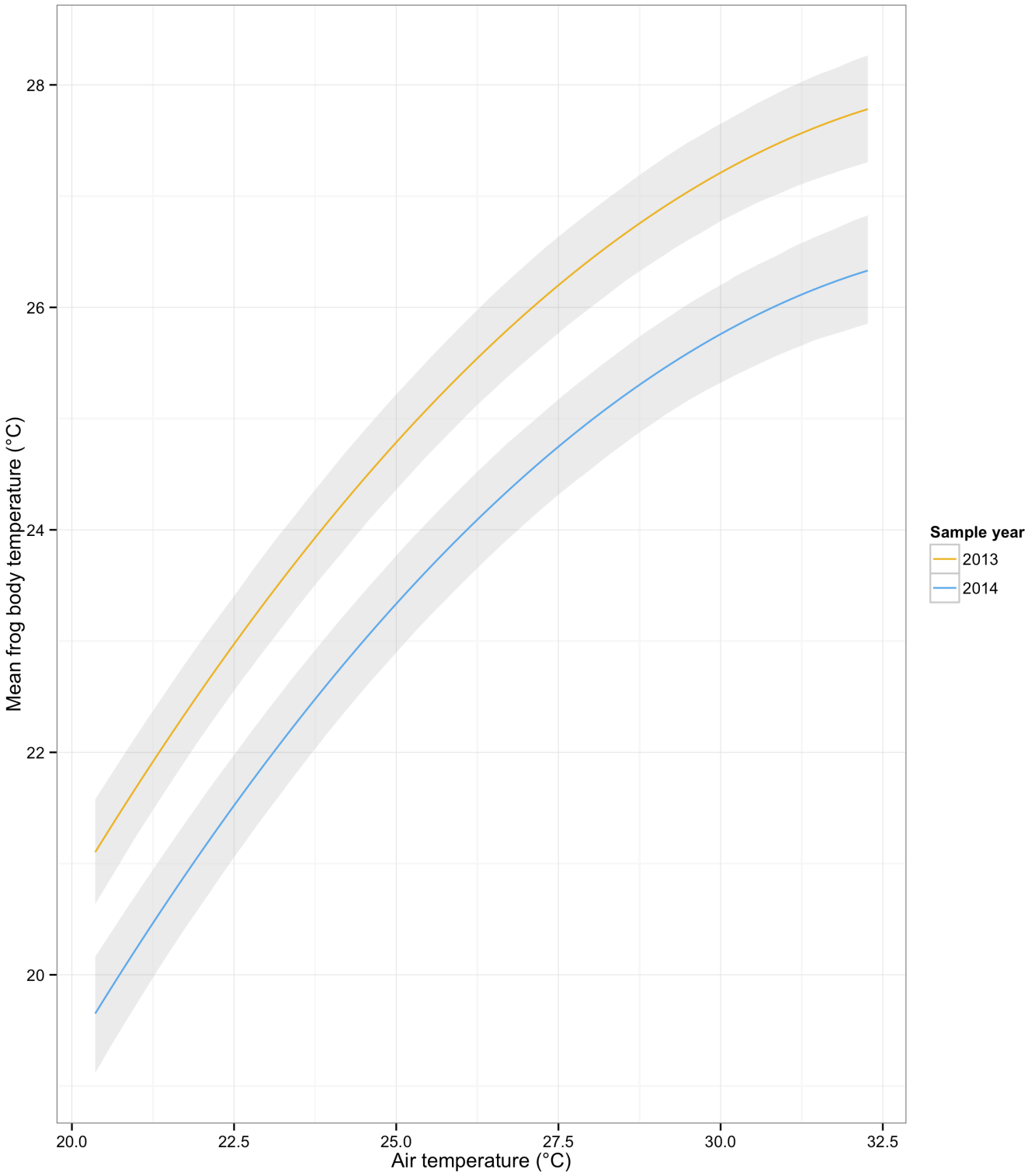


Figure 3.9: Model predicted relationship between mean frog body temperature (˚C) and air temperature (˚C; 2nd order polynimial). In 2013 (**yellow line**), mean frog body temperatures are predicted to be higher, given any air temperature, than in 2014 (**blue line**). Shaded areas span the 95% credible intervals for the fitted means.



Phenotype (percentage green interacting with percentage spot) significantly positively influenced frog body temperature (mean = 0.12 [95% credible interval 0.02 to 0.24]; Table 3.10). Frogs exhibiting high green pigmentation and low dorsal spot cover were predicted to exhibit a lower mean T_b than individuals exhibiting high green pigmentation and high dorsal spot cover (Figure 3.10). This is also illustrated in Figure 3.11, where I found that an increase in spot cover from 10 – 30 % when green pigmentation is high (70 %), allows for a 1 ˚C increase in T_b. However, when green pigmentation is low (30 %, thus brown pigmentation is high) an increase in spot cover has little influence on T_b. Furthermore, individuals exhibiting low green pigmentation thermoregulated consistantly at, or above, the maximum T_b that a high green coloured frog could achieve (Figure 3.11).

3.4.2.3 Relationship between response variables: frog body temperature and *Bd* intensity

I observed a significant, negative posterior correlation between frog temperature and *Bd* intensity at the site level (mean = -0.47 [95% credible interval -0.78 to -0.09], Table 3.10), after controlling for the effects of sample year, air temperature, distance from water, and frog phenotype (percentage green and percentage spot). Thus, sites exhibiting higher mean frog body temperatures are predicted to have lower infection loads (Figure 3.12). Additionally, Figure 3.12 highlights the fact that this relationship does not seem to be driven by latitude, as there is no pattern regarding region. Finally, I did not observe a significant posterior correlation between frog temperature and *Bd* intensity at the individual level, as the credible intervals crossed zero (mean = -0.008 [95% credible interval -0.09 to 0.08], Table 3.10).

Figure 3.10: (A) Model predicted relationship between mean frog body temperature (˚C) and observed phenotypes from the tail-end of the distribution. Frogs exhibiting high green pigmentation and high dorsal spot coverage are predicted to exhibit a much higher mean body temperature range, than frogs exhibiting high green pigmentation and low doral spot coverage. Bars represent one standard deviation. **(B)** Difference in mean frog body temperatures (˚C) between the two phenotypes is significant, as the 95 % credible intervals do not cross zero (dashed line).

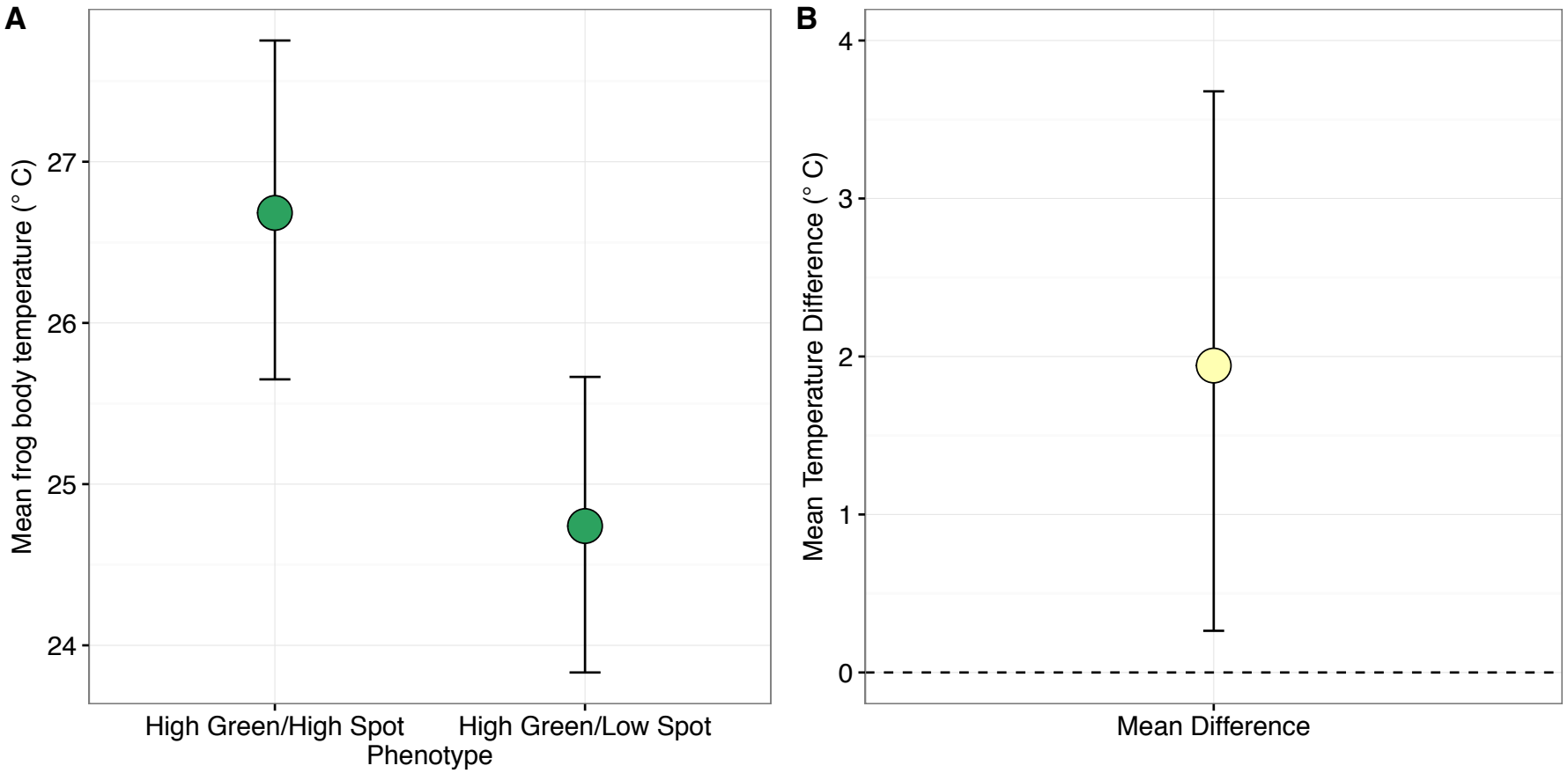


Figure 3.11: Model predicted relationship between mean frog body temperature (˚C) and spot cover (%) when percentage green pigmementaion is maintained at 70 % (high green; green shadow 95 % CI) or 30 % (low green; green shadow 95 % CI). When green pigmementation is high, frogs exhibiting high spot cover (30 %) are predicted to achieve ~ 1 ˚C greater T_b than those exhibiting low spot cover (10 %). When green pigmementation is low (i.e. brown pigmentation is high), spot cover has very little influence on T_b.

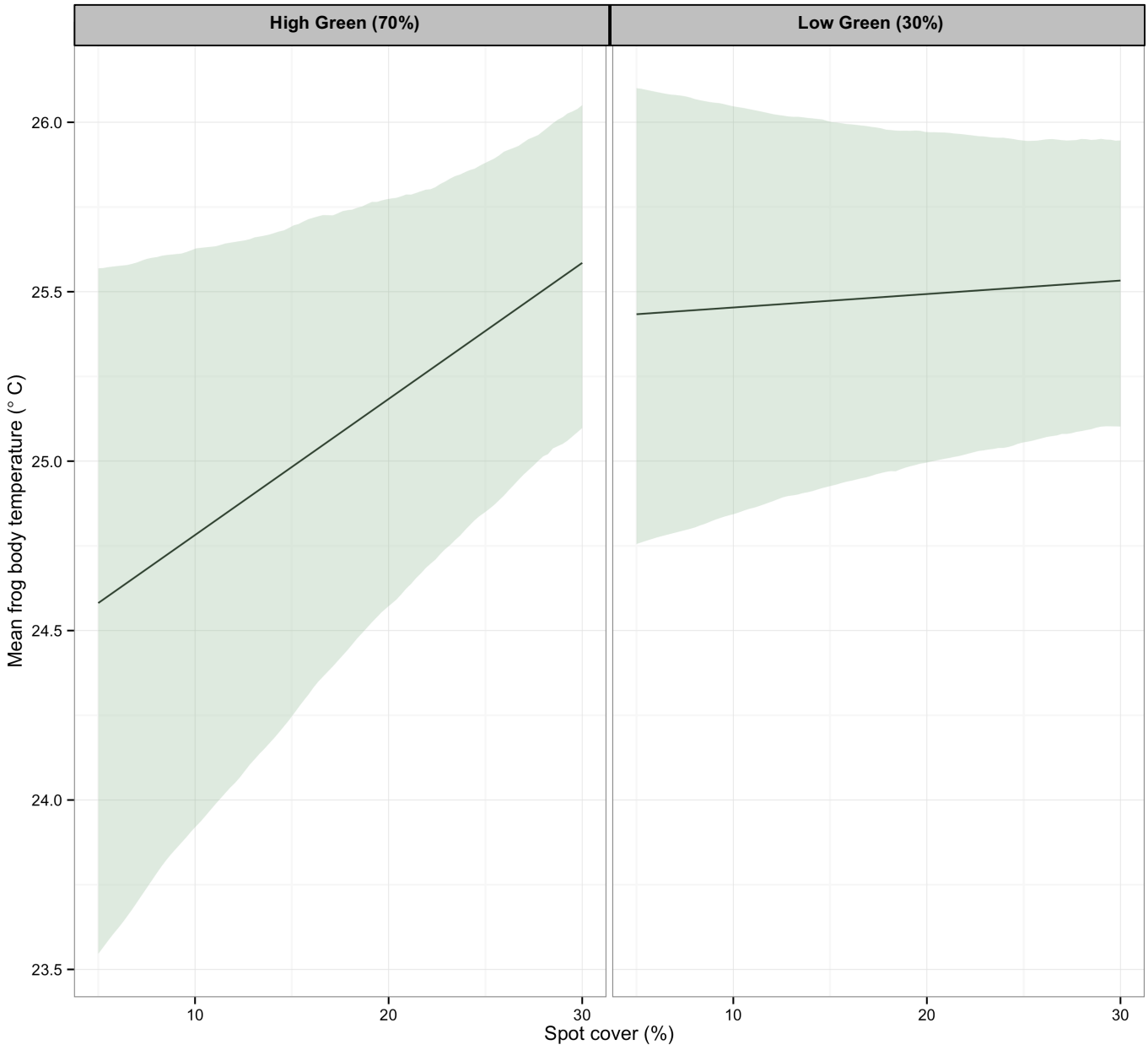
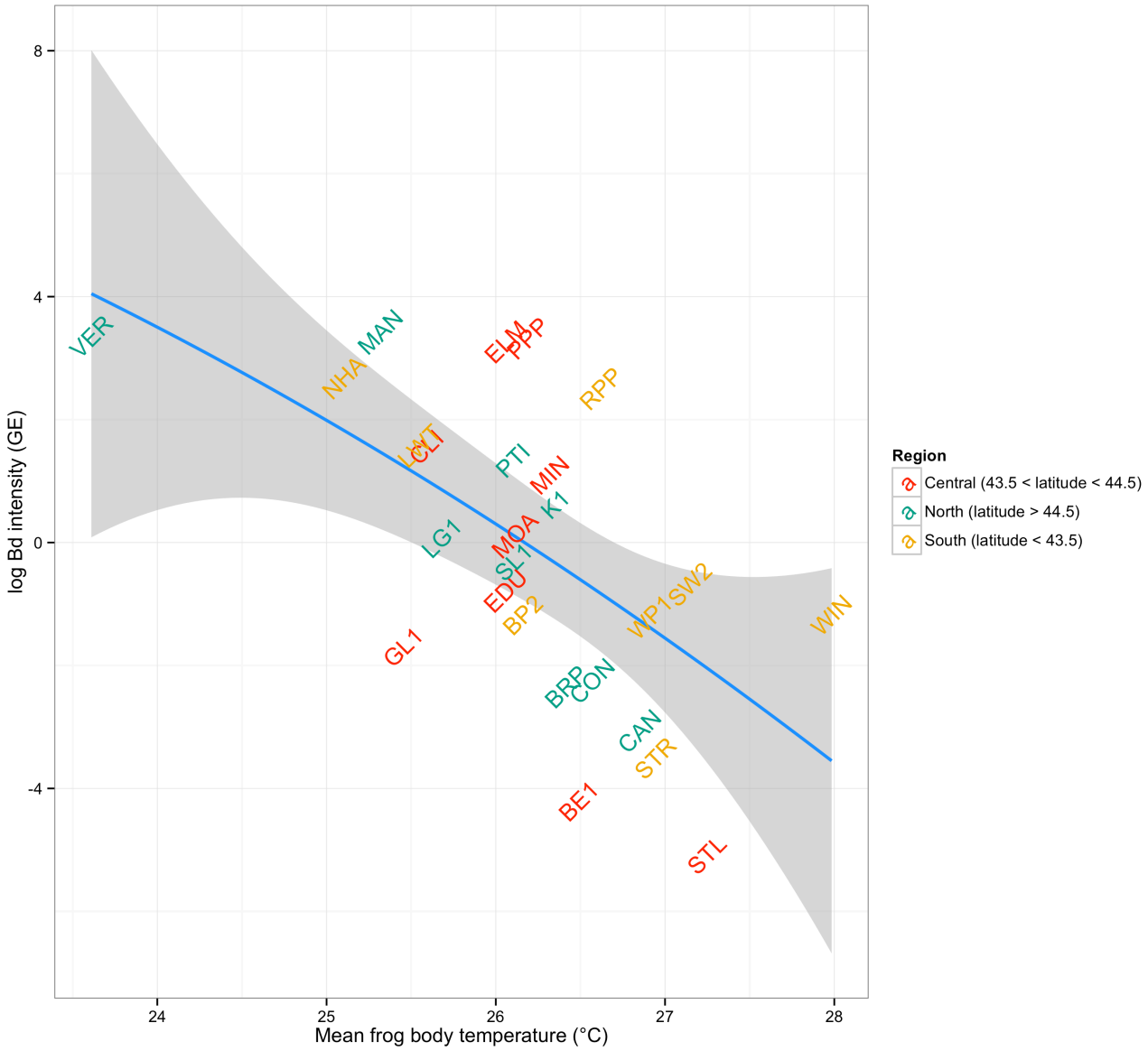


Figure 3.12: Significant negative posterior correlation between mean frog body temperature (°C) and log *Bd* intensity (GE), at the site level (grey shading represents 95 % CI). Sites are referred to by their three-letter code, and are grouped by region in order to highlight the lack of latitudinal pattern.



3.5 DISCUSSION

These results provide evidence that *Bd* infection intensity (GE) of *R. pipiens* is a function of frog body temperature, but that the strength of this effect is modified by air temperature, distance from water, and frog phenotype. Sites exhibiting higher mean frog body temperatures have lower infection loads. However, this may only be achieved when: 1) the local ambient conditions are warm, 2) individuals are located far from water, and/ or, 3) individuals exhibit high levels of overall dark pigmentation. These three hypotheses are not mutually exclusive and may be operating simultaneously. However, as the relationship between phenotype and frog body temperature was observed along with a significant, negative posterior correlation between frog body temperature and *Bd* intensity at the site level, these data suggest that particular phenotypes provide additional survival advantages to the host, above and beyond normal thermoregulatory behaviour.

My results suggest that a fluctuating thermal environment (air temperature; extrinsic abiotic factor) and individual dispersal (distance from water; intrinsic biotic factor) partly determine the course of the host–pathogen interaction, by altering host resistance during an ecological interaction. Air temperature positively influences frog body temperature. Within this system, given suitable environmental conditions, *R. pipiens* will actively thermoregulate at 24.4 °C (+/- 0.1 °C) for large parts of the day. As the upper threshold for maximum *Bd* growth (in a laboratory setting) is 25 °C (Piotrowski *et al*. 2004), maintenance of such T_b through thermoregulation may restrict pathogen growth on the host, leading to substantial delays in fungus-induced mortality. Furthermore, *R. pipiens* are capable of reaching a body temperature of 30 – 33 °C. These temperatures are lethal to *Bd* in culture (Piotrowski *et al*. 2004), and

may elicit increased activity of the innate immune system (Raffel *et al*. 2006; Terrell *et al*. 2013). The negative relationship between frog body temperature and *Bd* intensity is apparent within my study system. However, as host thermoregulation is dependent upon ambient temperatures, the pathogen still has the potential to damage the host if ambient temperatures fall. Therefore, infection intensity is partly determined by daily temperature fluctuations. More specifically, individual infection load is a function of the balance between negative growth or decay of *Bd* during daylight hours, and positive growth or proliferation at night when hosts cannot thermoregulate.

Individual dispersal (distance from water) was significantly negatively correlated with *Bd* intensity, but was not significantly correlated with frog body temperature, suggesting that the mechanism behind this negative correlation is aridity, not temperature. This result supports both laboratory studies (Johnson *et al*. 2003; Murphy *et al*. 2011) and field studies (Kriger & Hero 2007b; Puschendorf *et al*. 2011; Terrell *et al*. 2014) that point to *Bd* exhibiting less pathogenic dynamics in drier habitats. My findings in Chapter 2 also support this relationship, as site-level *Bd* mean intensity (Genomic Equivalents; GE) correlated with hydrological variables (see Figure 2.11). However, desiccation only minimises zoospore mobility and viability: it does not eradicate the intracellular sporangia (Johnson *et al*. 2003). Consequently, although host dehydration may play an explicit role in the development of this host-pathogen interaction, it may only provide temporary resistance. However, I previously reported that frog body temperature (daily mean, minimum and maximum) was not significantly correlated with site-level relative humidity (Pearson: $0.05 < R < 0.23$), which contradicts the above argument. I would argue that relative humidity recorded at a weather station is not indicative of the variation in humidity levels experienced by

an amphibian at ground level. For example, dense herbaceous and woody-stemmed plants will form an insulate layer, which will maintain high humidity levels: whereas, a thinned environment will present a substantially different microclimatic (Weng *et al*. 2007; Brooks & Kyker-Snowman 2008). Consequently, I do not consider the relative humidity measure recorded at weather stations to be a reliable source of data with regards to local climatic nuances experienced by the host. I would suggest that future research gathers data regarding the aridity of the host's immediate environment, in order to elucidate the mechanism behind decreasing *Bd* intensity with increasing distance from water.

Alternatively, increased host dispersal may influence disease dynamics by limiting *Bd* transmission rates. There is some evidence that host density influences *Bd* prevalence: suggesting that direct, or at least close contact between amphibians, has a role in generating new infections (Rachowicz & Briggs 2007; Becker *et al*. 2014a). However, as *Bd* zoospores can persist in the environment (Johnson & Speare 2003; Lips *et al*. 2006; Chestnut *et al*. 2014; Kolby *et al*. 2015), there is also potential for transmission to occur in a frequency-dependent manner, providing that the density of infectious zoospores in the environment is sufficient to compensate for a reduction in host number (Anderson & May 1991). This effect is observed in vector borne infection dynamics (Antonovics 1995). By de-coupling transmission and host density, pathogens capable of frequency-dependent transmission are more likely to drive a population to extinction (Ryder *et al*. 2007). The risk of host extinction is especially high when pathogens are capable of saprophytic reproduction, as this provides a continued source of infection entirely independent from the host. At present, there is a lack of empirical evidence regarding a saprophytic lifecycle for *Bd* (Longcore *et al*. 1999; Piotrowski *et al*. 2004). However, as *Bd* is culturable on nutrient media in the

absence of keratin, and is able to grow limitedly on snake skin, sterile bird feathers, the keratinous toes of waterfowl, and arthropod exoskeletons: reproduction outside the host may well be possible (Longcore *et al*. 1999; Johnson & Speare 2003, 2005; Garmyn *et al*. 2012; McMahon *et al*. 2013).

Phenotype may constrain the range of body temperatures available to individuals, which in turn, may translate into differential fitness. This is because individuals exhibiting greater levels of dark pigmentation will absorb more solar irradiance and have lower reflectance (Gates 1980) than those with limited pigmentation. Such dark overall phenotypes, will lead to higher equilibrium temperatures (Kalmus 1941; Watt 1968), which will restrict pathogen growth on the host. This relationship is evident at the site level, as higher mean frog body temperatures were correlated with both lower infection intensities and darker phenotypes, within a singular modelling framework. While there have been a handful of studies which demonstrate that individual thermal histories affect the probability of infection by *Bd* of frogs in nature (Richards-Zawacki 2010; Rowley & Alford 2013; Cohen *et al*. 2017), none have identified a mechanism in which amphibians achieve upper levels of thermal tolerance, thus increasing their probability of survival. Consequently, this study is the first to demonstrate that individual phenotypic traits i.e. colouration and melanism, alters the survival of an amphibian host against the "worst infectious disease ever recorded among vertebrates" (Gascon *et al*. 2007). Within the field of disease ecology, I am only aware of one other study, which has reported a correlation between vertebrate host morphotype and disease outcome. Nolan *et al*. (1998) reported that host size and male plumage predicted survival of the house finch (*Carpodacus mexicanus)*, following an epidemic outbreak of a new bacterial disease (*Mycoplasma gallisepticum; MG)*. This resulted in directional selection on reduced body size and increased plumage redness.

Since then, although the bacterial disease has stabilized to endemic levels, males in some populations remain redder than they were prior to the epidemic (Hill 2002). Additionally, in subsequent controlled infection experiments, redder males from unexposed populations showed more rapid clearance of infection than the alternative yellow males (Hill & Farmer 2005). This suggests possible underlying molecular mechanisms mediating the abilities of more and less ornamented males to respond to and survive infection. However, the molecular mechanisms that might connect plumage colouration to disease resistance are poorly understood (Hill & Johnson 2012; Hill 2014). In contrast, the physiological mechanism connecting amphibian pigmentation to *Bd* infection resistance is founded upon the widely accepted theory of thermal melanism, which states that melanistic ectothermic individuals should heat faster and reach higher equilibrium temperatures than lighter ones, under the same environmental conditions and assuming a similar body size (Gates 1980; Trullas *et al*. 2007).

There are two hypotheses that can explain the pattern between *Bd* intensity and amphibian host body temperature. First, presence of infection causes individuals to thermoregulate at higher levels than normal in order to retard the pathogen's growth. This is consistent with the 'behavioural fever' hypothesis, whereby amphibians alter their behaviour in response to infection (Parris *et al*. 2004; Richards-Zawacki 2010). Alternatively, amphibians thermoregulating at higher levels than normal are less likely to become infected. This revives the 'selective sweep' hypothesis, whereby amphibians that attain higher temperatures for other reasons, ultimately allows them to survive outbreaks of chytridiomycosis (Witters & Sievert 2001). There is a subtle difference between these two effects, but it is important to distinguish their components, as their relative importance will have a significant effect upon the

ecology and evolution of disease dynamics. I would like to extend these hypotheses to better suit my study system, and then discuss their feasibility:

(1) *Reactive strategy*: individuals alter their thermoregulatory behaviour to sustain a higher-than-normal body temperature in response to infection, and their range of body temperatures available to them is constrained by their phenotype.
(2) *Neutral strategy*: individuals exhibiting darker phenotypes are fundamentally capable of casually attaining higher body temperatures, and are therefore more likely to survive during outbreaks of chytridiomycosis.

These hypotheses are differentiated due to their management strategies. A reactive approach is based on responding to events after they have happened, and a neutral approach focuses on eliminating problems before they have a chance to appear. Richards-Zawacki (2010) demonstrated that during an epidemic, mean body temperature of wild frogs increased, which reduced the negative effects of *Bd*. This suggests that amphibians are capable of altering their thermoregulatory behaviour in response to an epizootic. My results may support this statement as individuals sampled in 2013 presented significantly greater infection loads and higher mean body temperatures, for any given air temperature, than individuals sampled in 2014. However, recent evidence suggests that amphibians do not actively thermoregulate to counter *Bd* infection (Han *et al*. 2008; Rowley & Alford 2013). Furthermore, I do not have the pre- and post- infection data required to assess this relationship, as individuals were sampled at one time point only. Consequently, I cannot support nor refute the hypothesis that *R. pipiens* levy a reactive strategy in response to infection. Alternatively, Rowley and Alford (2013) reported that an individual's probability of infection declined rapidly as they spent more time above the pathogen's upper

optimum temperature. Suggesting that individuals that attain higher temperatures for other reasons may have coincidentally decreased their probability of *Bd* infection. My results more firmly support this statement. I would suggest that an increase in mean body temperature, within *R. pipiens* populations, has resulted from a neutral strategy in which individuals that attain higher temperatures due to greater levels of dark pigmentation, have higher survival probabilities.

Increased temperature profiles may not only provide additional host survival advantage by minimizing pathogen viability: but may also influence the differential growth and/ or proliferation of bacteria living on the host skin, which in turn may offer disparate levels of host protection. Previous studies have demonstrated that amphibian skin microbiome mediates susceptibility to chytridiomycosis (Woodhams *et al*. 2007a, 2007b; Harris *et al*. 2009). Infectivity and severity of disease in susceptible amphibians have been reduced via antifungal compounds produced by particular bacteria (Bletz *et al*. 2013). Thus, skin bacteria likely play a role in *R. pipiens* host resistance, along with host life history, genetics, behaviour and environmental conditions. If we can harness the power of these microbes, we may be able to prevent disease (Rebollar *et al*. 2016a). Despite this fact, very little research attention has been devoted to assessing wild variation in amphibian skin microbiome, and/ or the contributive roles of host-phenotype and environmental heterogeneity in influencing bacterial community structure (Kueneman *et al*. 2014). This area of research warrants substantial media and scientific attention.

An increasing number of studies indicate that pathogens represent powerful selective agents in natural populations (Daszak *et al*. 2000; Roelke-Parker *et al*. 1996; Jensen *et al*. 2002; Hochachka & Dhondt 2000). The observation that existing and emerging

pathogens might both rapidly change and be mitigated by the genetic composition of host populations, gives new significance to the role of host genetic diversity in species conservation. Given that *Bd* can cause drastic amphibian declines, this could lead to a potential thermal arms race between the fungus and its amphibian host, and strong selection pressure on phenotype. I did not find a latitudinal effect of infection dynamics within this study system; however, I did find variation in phenotype across latitude. Percentage spot coverage increased with increasing latitude i.e. the northern populations presented a significantly higher spot coverage. This finding supports the thermal melanism hypothesis, or Bogert's rule, which is based on the basic biophysical principle that darker ectotherms should predominate in low-temperature environments, as they will achieve higher fitness (Bogert 1949; Trullas *et al*. 2007). This higher fitness will be due to darker individuals realising operating temperatures more rapidly at a given level of solar radiation, which allows for longer periods of activity (Lusis 1961; Norris 1967; Watt 1968; Kettlewell 1973; Luke 1989). These results suggest that darker *R. pipiens* will also obtain higher fitness due to lowered *Bd* infection risk. Both hypotheses represent powerful selective agents and thus, we may see underlying shifts in the genetic composition of host populations, towards higher thermal norms and darker phenotypes. This may be especially true for the most northern populations, where combined selective pressure may be strongest.

I only observed a significant posterior correlation between frog temperature and *Bd* intensity at the site level, not the individual level. I would suggest that this is a result of sampling individuals at only one time point, making the individual variance-covariance matrix equivalent to an observation level random effect. Consequently, there are few data at the individual level to allow the estimation of the covariance between frog temperature and disease residuals once controlling for the effects of

sample year, air temperature, distance from water, and frog phenotype. Similarly, as individuals were not sampled multiple times per annum, or across years, I lack the ability to estimate how the strength of the posterior correlation between frog body temperature and *Bd* intensity fluctuates in concert with seasonal fluctuations. This may be of particular importance to this study system, as infection intensity differs between months and years and individual dispersal fluctuates with breeding phenology and microclimates. Consequently, one might not expect the strength of correlation between traits to be uniform across the year or between years. By employing multiple observations of individuals from both within and across years, future work will be able to focus on quantifying the degree to which these relationships fluctuate with seasonal dynamics, and how phenotype alters the strength of this effect, at both the individual and site level. Elucidating how processes from different periods of the annual cycle interact to influence the thermal history of individuals will have important implications for our understanding of the forces regulating the infection dynamics of *Bd*.

3.6 CONCLUSION

The thermal dependency of the relationship between *Bd* and its amphibian host demonstrates how disease outcome can be influenced by complex interactions between host and pathogen phenotypes and the environments in which they occur. A key finding from this work is that individual phenotype i.e. colouration and melanism, appears to be an important intrinsic variable explaining variation in susceptibility to *Bd* infection within a species. However, the role of colouration and melanism in maintaining the negative relationship between thermal history and infection risk will not be consistent across amphibian families. This is due to two facts: (1) interspecific

variation in host thermal tolerances places constraints on the ability of some species to warm themselves to temperatures that inhibit *Bd* growth; and (2) the outcome of this host-pathogen interaction is likely to differ among host species due to interspecific variation in phenotype and other aspects of natural history (e.g. microhabitat associations, climate, and breeding phenology). Additionally, very little is known about the temperature optima, growth rates, and morphology of various *Bd* genotypes or isolates. Recent research has reported that the optimum growth temperature of the newly described salamander parasite *Bsal* (Martel *et al*. 2013) is markedly lower (15 °C) than that of *Bd* (17–25 °C). We now know that there is a much greater diversity of *Bd* than was previously recognized (Farrer *et al*. 2011), and consequently the growth rates and temperature optima for *Bd* strains may be vastly disparate. A clearer understanding of the expected relationship between pathogen load and body temperature during a chytridiomycosis infection, and how this might vary among host taxa and *Bd* strains, could be gained in a laboratory setting by allowing infected frogs to thermoregulate in a temperature gradient while monitoring their infection status. These types of studies have the potential to elucidate the extent to which amphibians must raise their body temperatures, both incrementally and temporally, in order to combat *Bd* infection, and the role that host phenotype plays in this interaction. This research focus may become increasingly important as the climate changes. It is generally assumed that parasites may be more adaptable to climate change than their hosts due to their shorter generation times and faster acclimation rates (Raffel *et al*. 2013). However, the adaptability of host skin-microbiomes to climate change remains an elusive subject, despite knowing that antifungal activity of commensal bacteria living on amphibian skin is temperature dependent (Daskin *et al*. 2014). Thus, host species able to thermoregulate at temperatures favourable to these bacteria may

benefit from improved function of defensive microbial symbionts. Thus, it is critical that researchers focus on the thermal mismatch between host and parasite performance, in order to more precisely predict the distribution and severity of this pathogen's effect.

CHAPTER FOUR: LANDSCAPE AND HOST CHARACTERISTICS INFLUENCE AMPHIBIAN SKIN MICOBIOME

.....

4.1 INTRODUCTION

4.1.1 Amphibian skin: first line of defense against pathogen invasion

The amphibian skin plays a key role in the everyday survival of amphibians and their ability to exploit a wide range of habitats and ecological conditions (Clarke 1997). It serves a variety of essential biological functions, including osmoregulation, respiration and thermoregulation (Duellman & Trueb 1986), in addition to acting as a physical barrier to infection. The skin protects amphibians from numerous pathogens in the environment via two skin-associated immune defense traits: the microbial communities (microbiome) inhabiting the skin surface (Belden & Harris 2007) and the anti-microbial peptides, found within the natural peptide secretions produced by granular glands within the host's skin (Rollins-Smith & Conlon 2005; Rollins-Smith *et al*. 2005). While there is some recent evidence of adaptive immunity in amphibians (Robert *et al*. 2005; McMahon *et al*. 2014; Price *et al*. 2015), these two skin-associated traits act as a first line of defense against pathogen invasion (Rollins-Smith 2009). Here, I will focus on the amphibian microbiome only.

4.1.2 Amphibian skin microbiome and chytridiomycosis

Considering hosts as habitats for microbial communities expands our understanding of the roles microbes contribute to host functions. Recent studies show that microbial symbionts of animals can largely affect the physiology, development and fitness of their animal hosts (McFall-Ngai *et al*. 2013). More specifically, changes in host

physiology can be brought about by differences in the presence or absence of microbial taxa or in their relative abundances (Turnbaugh *et al*. 2006; Kohl *et al*. 2014, 2015). Yet the symbionts of amphibian hosts are understudied, and basic ecological principles of host-associated community assembly remain unknown for all but a few species (McKenzie *et al*. 2012; Kueneman *et al*. 2014; Krynak *et al*. 2016).

Research on amphibian skin microbiomes is greatly motivated by the desire for a deeper understanding of the interaction between the skin microbiome and amphibian disease. Of particular focus is the disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*; Longcore *et al*. 1999). The degree of susceptibility to *Bd* varies greatly between and within species (Daszak *et al*. 2004; Retallick *et al*. 2004; Blaustein *et al*. 2005; Woodhams & Alford 2005; Rachowicz *et al*. 2006; Garcia *et al*. 2006; Puschendorf *et al*. 2011; Daskin & Alford 2012) and is linked to multiple factors including environmental conditions (supported by my results in Chapter 2), host physiology (supported by my results in Chapter 3), and the skin microbial communities (Bletz *et al*. 2013; Bielby *et al*. 2015). Of these factors, skin microbial communities have come to the forefront of research on *Bd* susceptibility due to the role symbiotic bacteria play in host resistance.

Previous studies have demonstrated that amphibian skin microbiome mediates susceptibility to chytridiomycosis (Woodhams *et al*. 2007a; Harris *et al*. 2009). Additionally, infectivity and severity of disease in susceptible amphibians have been reduced via antifungal compounds produced by particular bacteria (Bletz *et al*. 2013). This brings about the possibility of implementing microbial therapeutics against the establishment and spread of *Bd*. The approach that is currently being developed is probiotic bioaugmentation, which entails the establishment and augmentation of

protective microbes that naturally occur on, at least some, individuals in an amphibian population or community (Bletz *et al*. 2013). Several laboratory and field based trials have reported success in this active area of research, as bioaugmentation has prevented *Bd* morbidity and mortality in some amphibian species (Harris *et al*. 2006, 2009; Bletz *et al*. 2013). However, application of probiotics has been ineffective in other amphibian species (Becker *et al*. 2011, 2015; Küng *et al*. 2014). This mixed success of probiotics could be, in part, due to the selection of ineffective probiotic candidates arising from our lack of knowledge regarding: 1) microbial diversity within wild amphibian populations, and /or 2) the ecological interactions occurring within wild amphibian populations (Reid *et al*. 2011; Hawkes & Keitt 2015). Thus, investigating the basic ecology structuring skin microbiota, within wild amphibian populations, will inform the design and application of microbial therapeutics.

4.1.3 Amphibian skin microbiome and their host

The relationship between symbiotic bacterial communities and their hosts can be highly complex. In recent years, it has become clear that amphibians host a diverse array of cutaneous microbes (Culp *et al*. 2007; Lauer *et al*. 2007, 2008; Walke *et al*. 2011; McKenzie *et al*. 2012), and several studies have aimed to characterize variation in microbiota across different amphibian species and populations (Kueneman *et al*. 2014). Researchers have found that host species identity is one of the most important factors influencing the amphibian skin microbiome (McKenzie *et al*. 2012; Kueneman *et al*. 2014; Walke *et al*. 2014). Conspecific individuals exhibit microbial communities that are more similar to one another than to those present on cohabiting individuals of different species. This may be because the skin of different amphibian species varies with regards to the presence and types of anti-microbial peptides and

mucosal secretions, which can affect the establishment and maintenance of resident microbial communities (Walke *et al*. 2014). Additionally, to ensure the continued integrity of the skin surface, amphibians undergo a cyclic moulting process whereby an entire stratum of skin is shed, known as 'slough' (Jørgensen & Larsen 1961; Budtz & Larsen 1973; Smith 1975; Herman 1992; Alibardi 2003). This process is regulated hormonally (Taylor & Ewer 1956; Jørgensen & Larsen 1960, 1961, 1964; Jørgensen *et al*. 1965; Budtz 1979; Jørgensen 1988), however, the period of time between the recurrent shedding events, known as the intermoult interval, varies from days to weeks depending on the species (Bouwer *et al*. 1953; Jørgensen & Larsen 1960, 1961, 1964; Joørgensen *et al*. 1965; Budtz 1979; Jørgensen 1988). Until recently, very little was known about how sloughing influenced cutaneous microbial abundance. However, it is now understood that sloughing may result in up to 100 % reduction in cutaneous microbial abundance, suggesting that sloughing may act as a component of the innate immune system of amphibian species (Meyer *et al*. 2012).

Within one species, members of the same population exhibit more similar microbiomes than individuals across populations. This suggests that different populations either harbor a relatively specific microbiome, which may be cultivated and influenced by environmental reservoirs (McKenzie *et al*. 2012; Kueneman *et al*. 2014; Walke *et al*. 2014), or environmental conditions select for the persistence of particular bacterial taxa on the host skin (Vartoukian *et al*. 2010). The mechanism by which amphibians acquire symbiotic bacteria is not well understood. Microbes can be transmitted vertically, i.e. parent to offspring (Banning *et al*. 2008; Walke *et al*. 2011). However, this route of transmission is unlikely for ranid species, as they do not exhibit parental care (Smith & Keinath 2007). Microbes can also be transmitted horizontally, i.e. host to host, both directly through contact among hosts and indirectly

from host, to environment, to host (Rebollar *et al*. 2016b). As stated above, amphibians may obtain their skin microbes directly from the environment in each new generation or following disturbances, such as skin sloughing (Meyer *et al*. 2012). Alternatively, the environmental microbial pool may contribute to the host's gut microbiome, which may serve as a reservoir for skin microbes (Kohl *et al*. 2013). Consequently, the available environmental microbial pool may be immensely important for maintaining the host's microbiome either directly or indirectly (Loudon *et al*. 2014a, 2014b).

4.1.3.1 Environmental correlates of amphibian skin microbiome variation

The hypothesis that the environment may alter microbial community structure is not new. Local environmental conditions, such as soil and sediment, are known to influence the community structures of environmental microbiomes (Yergeau *et al*. 2012; Sharp *et al*. 2014). Studies conducted in culture have shown that the environment affects which bacterial species can persist on a particular media, at differing temperatures, pH, and nutrient concentrations (Vartoukian *et al*. 2010). Furthermore, within these changing environments, bacterial species compete for space and nutrients and this in turn can shift the relative proportions of species present (Nichols *et al*. 2008; Vartoukian *et al*. 2010). Despite this fact, few studies have tested whether the environment influences inter-population variation in the skin-associated microbiome of a single amphibian species (McKenzie *et al*. 2012; Becker *et al*. 2014b; Fitzpatrick & Allison 2014; Kueneman *et al*. 2014; Loudon *et al*. 2014a; Krynak *et al*. 2015).

In nature, anthropogenic disturbance such as environmental contamination, habitat destruction, landscape fragmentation, human population encroachment and the

introduction of invasive pathogens (Daszak *et al*. 2003), could cause a change in the local pool of microbial colonists: thereby affecting the microbiome of the amphibian skin (Fitzpatrick & Allison 2014). Alternatively, biological stress associated with anthropogenic disturbance is known to cause immune suppression across many taxa (Morimoto *et al*. 2011), which has been shown to negatively impact the gut microflora (Moloney *et al*. 2014). For example, several laboratory studies focusing on mammalian species have shown that maternal separation, restraint conditions, crowding, heat stress and acoustic stress, alter the composition of gut microbiota (Bailey & Coe 1999; Bailey *et al*. 2011; Collins & Bercik 2013; Garcia-Rodenas *et al*. 2006; O'Mahony *et al*. 2009; Suzuki *et al*. 1983; Tannock & Savage 1974; Timoveyev *et al*. 2002). Wild amphibian populations may experience chronic sublethal stress associated with drought, temperatures, food shortages, chemical contaminants or pathogen presence, which will alter host physiological traits such as corticosterone levels (Gendron *et al*. 1997; Homan *et al*. 2003; Pounds *et al*. 2006; Liesenjohann *et al*. 2013; Gabor *et al*. 2015). Such stressors elicit selection pressure on the relative proportions of the host's microbial colonists, which will ultimately shift the microbiome structure. Thus, field studies that assess the influence of environmental characteristics on amphibian skin microbiome, across populations, may provide rationale for altering land-management practices to better protect wildlife health. This area of research may become increasingly relevant with increasing anthropogenic disturbance (Belden & Harris 2007).

4.1.3.2 Individual traits influencing amphibian skin microbiome variation

Several studies have identified a distinct microbiome signature across life stages in numerous species. In *Rana cascadae*, adults and subadults have similar microbiomes, which are significantly distinct from the microbiomes of tadpoles (Kueneman *et al*. 2014), suggesting an ontogenetic pattern in microbiome variation. The reason for this difference remains unclear. However, it is known that as amphibian's age, their immune systems change, which could lead to the cultivation of a specific skin microbiome between life stages (Longo *et al*. 2015). Alternatively, pre- and post-metamorphic individuals occupy separate environmental niches, which will alter the available environmental microbial pool. Larval development occurs within a single pond, and as such, premetamorphic individuals will have a known environmental origin and shared history. Post-metamorphic amphibians are capable of limited dispersal, thus they will gain a community of skin microbes from terrestrial inocula, and will share influences on the microbiome, such as climate, water chemistry, soil type and food availability (Kueneman *et al*. 2014, 2015).

Body temperature has been understudied in relation to microbial community structure. To date, most microbiome studies have been conducted on mammals, such as laboratory rodents and humans, which maintain consistent body temperatures in the face of environmental variation. Roughly, 99 % of animal species and 75 % of vertebrate species are considered ectotherms (Hammond 1992). Temperature effects have a strong influence on aspects of amphibian behaviour and physiology because of their ectothermy (Hutchison & Dupré 1992; Harvell *et al*. 2002). As amphibians are active behavioural thermoregulators, they routinely increase their body temperature by basking in sunlight (Freed 1980; Duellman & Trueb 1986) and/ or selecting for

high-temperature microclimates within a spatially and temporally variable environment. In doing so, they buffer themselves against the negative effects of temperature (Bartholomew 1966; Huey 1991). Culture-based studies have demonstrated that environmental temperature alters the gut community structure of amphibians (Carr *et al*. 1976; Gossling *et al*. 1982). However, the effect of body temperature on skin-associated microbial communities has not been investigated using high-throughput sequencing techniques. Several studies have reported that relatively low temperatures reduce sloughing frequency in many amphibian species (Taylor & Ewer 1956; Meyer *et al*. 2012; Cramp *et al*. 2014). Furthermore, Meyer *et al*. (2012) reported that sloughing substantially reduced the abundance of cultivable cutaneous bacteria and fungi by up to 100 %, and Cramp *et al*. (2014) suggested that at cool temperatures, an extended intermoult interval allowed microbial abundance to reach higher levels than at warmer temperatures (when the intermoult interval is significantly reduced). Thus, microbes could have greater success at establishing and/ or maintaining themselves upon the epidermis of amphibian hosts that thermoregulate at lower temperatures, where microbe numbers are possibly less strongly regulated by frequent host sloughing regimens. However, this may not necessarily equate to higher bacterial diversity. Microbial studies have reported that bacterial species compete for space and nutrients within their environment (Nichols *et al*. 2008; Vartoukian *et al*. 2010) and as such, bacteria that are relatively abundant in amphibian skin microbial communities are likely to be competitively dominant (Hibbing *et al*. 2010). Thus, lower temperatures and reduced sloughing frequency, may support the proliferation of dominant phyla, which in turn, may shift the relative proportions of species present and decrease overall bacterial diversity. This suggestion is supported by studies that report the inhibition of many bacterial taxa, within amphibian microbiomes, at low

temperatures (Matutte *et al*. 2000; Rollins-Smith *et al*. 2002; Ribas *et al*. 2009). The strong dependence of both host and its microbiome upon temperature highlights the importance of measuring key environmental parameters at an appropriate scale to understand dynamics within the system.

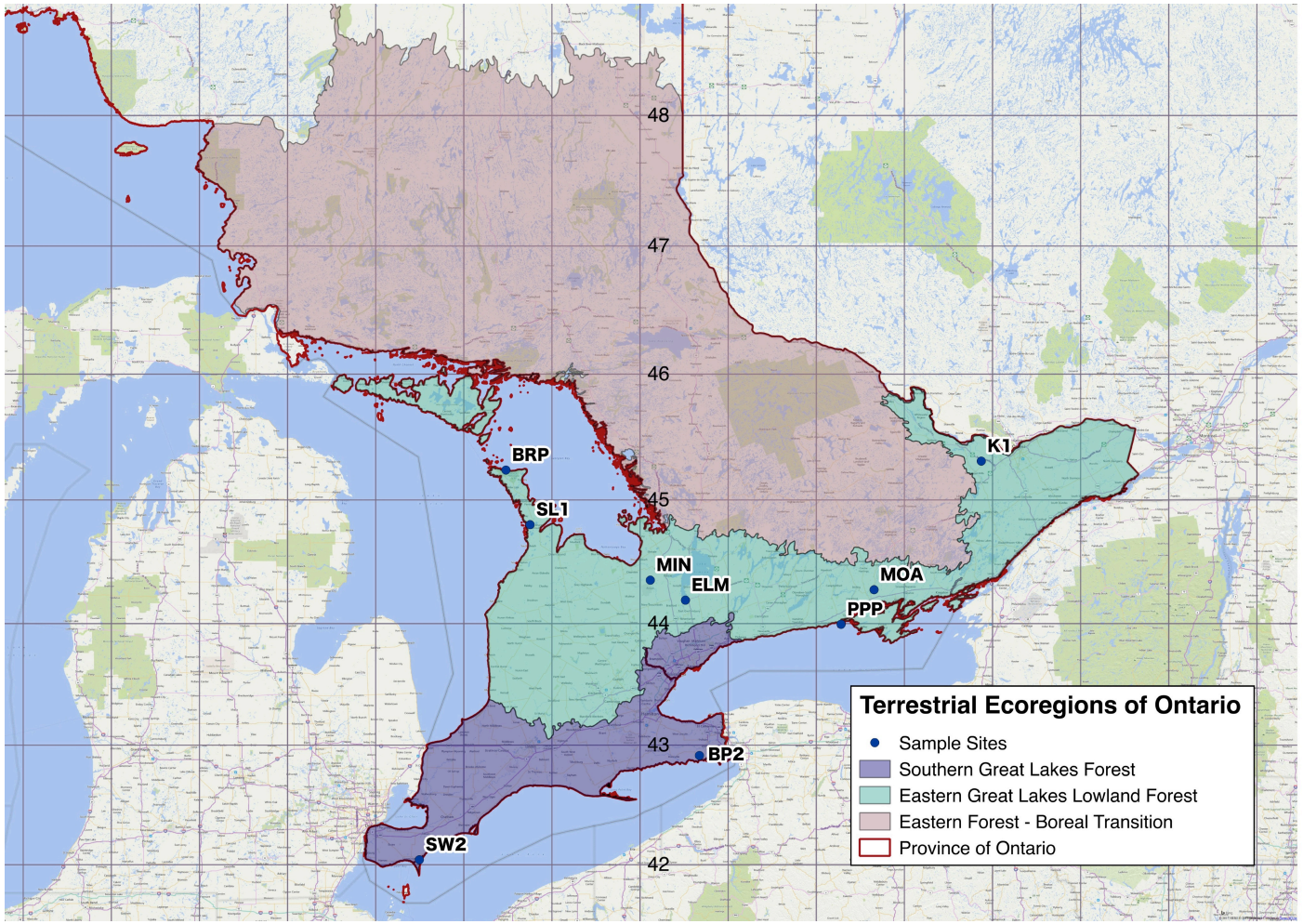
4.1.4 *Rana pipiens* and their skin micobiome

As amphibians have limited dispersal capabilities, they may be particularly vulnerable to disease associated mortality and subsequent decline if changes in their environment depress immune function. The Northern Leopard Frog (*Rana pipiens*, Yuan *et al*. 2016; Figure 1.3) is one such species as the maximum dispersal range for juveniles and adults has been suggested to be 8 – 10 km (Dole 1971; Seburn & Seburn 1997; Romanchuk & Quinlan 2006). Natal dispersal is recorded to be significantly lower than that observed in juveniles and adults, with the young frogs travelling up to a maximum of 5 km from their pond of origin (Dole 1965, 1971; Merrell 1977). Within Ontario, although common and widespread throughout the southern region, *R. pipiens* appears to have declined in northern Ontario (Figure 1.5; Weller *et al*. 1994; Seburn & Seburn 1997, 1998). A variety of anthropogenic environmental alterations have been hypothesized to be the cause of these declines, including habitat loss, fragmentation, acidification, and chemical contamination (Bishop *et al*. 1999; Carr & Fahrig 2001; Gilbertson *et al*. 2003; Cushman 2006). In addition, disease outbreaks, including those caused by *Bd*, have been suspected as having a potential role in these declines (Gibbs *et al*. 1971; Carey *et al*. 1999; Rorabaugh 2005; Greer *et al*. 2005). Thus, synergistic interactions between environmental change and disease are likely, especially as *R. pipiens* have highly vascularized skin, which may enhance the effects of anthropogenic disturbance and disease susceptibility (Beasley *et al*. 2005).

The southern area of Ontario is separated into ecoregions (Figure 4.1; Olson *et al*. 2001), which are defined by their distinct assemblage of natural communities. These ecoregions occur within distinctive bedrock that differs in origin and chemistry from the bedrock domain immediately adjacent to it: as such, the sediment type, pH, nutrient concentrations, and topography are dissimilar. In the southern area of Ontario, three distinct ecoregions have been defined: (1) the Southern Great Lakes forest (latitude < 43.5 °N); (2) the Eastern Great Lakes lowland forest (43.5 °N > latitude < 44.5 °N); and (3) the Eastern forest-boreal transition (latitude > 44.5 °N) (Olson *et al*. 2001). These ecoregions exhibit distinct latitudinal ranges, and have a major influence on the ecosystem processes and biota occurring there, including bacterial microbes.

The potential anthropogenic sensitivity of *R. pipiens*, along with their suspected disease susceptibility and declining status within Ontario, make this species an excellent model for examining the influence of both environmental (latitude and anthropogenic disturbance) and individual level traits (amphibian body size and body temperature) on the skin-associated microbiome.

Figure 4.1: Map of the nine Northern Leopard Frog (*Rana pipiens*) populations included in the micobiome study, within their terrestrial ecoregions. **Blue dots** represent site centroids: note the wide latitudinal range. Transparent colouring highlights the terrestrial ecoregions of Ontario: the Southern Great Lakes Forest (**purple**), the Eastern Great Lakes Lowland Forest (**blue**); and the Eastern Forest – Boreal Transition (**pink**). *(Figure: author's own, created in ArcGIS version 10.2.2).*



4.1.5 Measures of alpha and beta diversity

Within microbiome analyses, Operational Taxonomic Units (OTUs) take the place of 'species', as named species genomes are often unavailable for particular marker sequences. The assignment of sequences to OTUs is referred to as 'binning', and it can be performed by: A) unsupervised clustering of similar sequences (Schloss *et al*. 2009); B) phylogenetic models incorporating mutation rates and evolutionary relationships (Hamady *et al*. 2010); or C) supervised methods that directly assign sequences to taxonomic bins based on labelled training data (Wang *et al*. 2007). The binning process allows a community to be analysed in terms of OTUs, opening up a range of computationally tractable representations for biological analysis. However, an important concept when dealing with OTUs is that of 'population diversity', i.e. the number of distinct bins in a sample or in the originating population. When quantifying population diversity, two mathematically well-defined questions arise:

1) Given that x bins have been observed in a sample of size y, from a population of size z, how many bins are expected to exist in the population?

2) Given that x bins exist in a population of size z, how big must the sample size y be, to observe all of them at least once?

Within a community, several estimators exist for calculating alpha diversity, the number (richness) and distribution (evenness) of taxa expected within a single population. These give rise to rarefaction curves, since increasing numbers of sequenced taxa allow increasingly precise estimates of total population diversity (Colwell & Coddington 1994). When comparing multiple populations, beta diversity measures, including absolute or relative overlap, describe how many taxa are shared

between them (Morgan & Huttenhower 2012). An alpha diversity measure thus acts like a summary statistic of a single population, while a beta diversity measure acts like a similarity score between populations. Many alpha- and beta-diversity measures have been developed that each reveal slightly different aspects of community ecology (for reviews see Legendre & De Cáceres 2013; Chao *et al*. 2014; Chao & Chiu 2016).

4.2 RESEARCH AIMS AND OBJECTIVES

This study investigates the natural diversity and distribution of skin microbes across wild *R. pipiens* populations from Ontario, in order to answer the following question: do disparate amphibian populations have unique microbial communities, and are differences in bacterial richness and community structure linked to environmental and/ or host-specific factors? Single time point measurements are not sufficient to capture abiotic environmental signals that would correlate with the skin community variation across sites. Consequently, I have chosen to focus on site-level predictors that are non-transient, and individual level predictors. Specifically, I hypothesize that:

(i) *R. pipiens* populations located at northern latitudes will exhibit substantially different skin-associated microbiomes than southern populations, because of variation in physical environmental factors such as sediment type, pH, nutrient concentrations, and topography, as defined by ecoregions (Vartoukian *et al*. 2010; Yergeau *et al*. 2012; Sharp *et al*. 2014). Additionally, as temperature decreases with increasing latitude, northern latitudes will present a lower overall bacterial diversity, as cooler temperatures will inhibit the growth of certain bacterial taxa, thus limiting the available colonizing microbes within the environment (Matutte *et al*. 2000; Rollins-Smith *et al*. 2002; Ribas *et al*. 2009).

(ii) *R. pipiens* individuals thermoregulating at low body temperatures will exhibit lower bacterial diversity than individuals thermoregulating at high body temperatures, as colder individuals will exhibit: (a) an extended host intermoult interval (Meyer *et al*. 2012), thus allowing for interspecific competition among microbial species, selecting for the persistence and proliferation of dominant bacterial taxa (Vartoukian *et al*. 2010); and/ or (b) inhibit the growth of certain bacterial taxa upon their body (Matutte *et al*. 2000; Rollins-Smith *et al*. 2002; Ribas *et al*. 2009). As *R. pipiens* are active behavioural thermoregulators, their body temperatures are not solely influenced by air temperature experienced at any given latitude (supported by my results in Chapter 3). Consequently, amphibian body temperature must be considered separate to latitude.

(iii) *R. pipiens* populations located in close proximity to urban environments will present lower bacterial diversity than populations located far from urban environments, as anthropogenic disturbance will: (a) limit the local pool of microbial colonists (Sutton *et al*. 1991; Sjöling *et al*. 2005; Berga *et al*. 2012; Tsiafouli *et al*. 2015); and/ or (b) increase host-specific chronic sublethal stress (Janin *et al*. 2012) which will depress the hosts ability to maintain a natural protective skin microbiota (Moloney *et al*. 2014).

(iv) As the amphibian microbiome is in constant contact with the environment and undergoes a continual process of microbial exchange, large post-metamorphic individuals will present a greater bacterial diversity than small post-metamorphic individuals, as they exhibit wider dispersal ranges, which will increase their encounter rate of additional colonizing microbes. Furthermore, during metamorphosis, amphibians undergo substantial structural and

immunological changes in the skin (Robinson & Heintzelman 1987; Faszewski & Kaltenbach 1995; Rollins-Smith 1998, Faszewski *et al*. 2008; Rollins-Smith *et al*. 2011). Consequently, recently metamorphosed individuals (i.e. small post-metamorphic individuals) will exhibit significantly different skin bacterial communities in comparison with adult individuals (i.e. large post-metamorphic individuals).

4.3 METHODS

4.3.1 Field sampling

I sampled nine *R. pipiens* population localities in Ontario, Canada (site names BP2, BRP, ELM, K1, MIN, MOA, PPP, SL1 and SW2), covering a wide latitude range (42.04 °N - 45.31 °N; Figure 4.1; Table 4.1). All sites were geo-referenced using GPS and sampled once within a five-week period, during the months of July and August 2014. Sites were defined as a circular area with a 2 km radius, each separated by a minimum of 10 km from their nearest neighbour. As maximum dispersal distances of 8 – 10 km have been documented for *R. pipiens* (Dole 1971; Seburn & Seburn 1997; Romanchuk & Quinlan 2006), sites may be considered as distinct populations with limited (to zero) gene flow between them. Additionally, all sites were located in separate watersheds. The nine population localities were selected based on the known distribution of *R. pipiens* populations (Figure 2.2).

Within each site, 8 - 10 post-metamorphic frogs were captured and sampled within 36 hours. At point of capture, frog body temperature was recorded using a Fluke 561 Multi-purpose Infra-red Thermometer (emissivity set to 0.95; Tracy 1976; Carroll *et al*. 2005; Rowley *et al*. 2006; Rowley & Alford 2007a) providing body temperature

readings within 0.5 °C of cloacal temperatures (Rowley & Alford 2007a). Snout-vent length (SVL) was measured to the nearest 0.01 mm (using electronic callipers). Individuals with a SVL < 45 mm were considered recent metamorphs, those with an SVL > 52 mm were considered adults, and those with an SVL between 45 - 52 mm were considered juveniles (Wright & Wright 1949; Leduc & Lesbarrères, unpublished data). Sampling location and sample size for each *R. pipiens* population is given in Table 4.1, along with mean snout-vent length (SVL; mm), and mean frog temperature (°C).

Table 4.1: Summary statistics for sites included in microbiome analysis. **Site**, circular area with a 2 km radius, each separated by a minimum of 10 km from their nearest neighbor; **N (adult/ juv/ meta),** total number of amphibians sampled (total number of adults sampled / total number of juveniles sampled/ total number of metamorphs sampled)**; Distance from large urban population**, proximity (km) to a large urban population centre (population of 100,000 or more); **Latitude,** degrees north**; Mean SVL**, mean snout-vent length (mm); **Mean frog temperature,** mean body temperature of amphibian at point of capture (˚C).

Site	**N (adult/ juv/ meta)**	**Distance from large urban population (km)**	**Latitude (°N)**	**Mean SVL (mm) (SE, range)**	**Mean frog temperature (°C) (SE, range)**
BP2	10 (10/ 0/ 0)	3.75	42.91	61.11 (1.9, 55.26 – 73.72)	23.9 (0.3, 22.0 – 26.0)
BRP	10 (4/ 3/ 3)	129.11	45.24	49.27 (1.9, 40.78 – 59.9)	24.8 (0.9, 21.8 – 32.0)
ELM	10 (9/ 1/ 0)	5.07	44.19	56.40 (1.3, 50.96 – 66. 89)	24.4 (0.5, 22.0 – 26.7)
K1	10 (6/ 3/ 1)	17.49	45.31	52.46 (2.4, 36.92 – 66.52)	25.9 (0.5, 22.7 – 28.6)
MIN	9 (4/ 4/ 1)	13.94	44.35	51.93 (2.0, 43.73 – 64.82)	25.5 (0.8, 21.7 – 28.5)
MOA	10 (9/ 1/ 0)	66.92	44.27	56.31 (1.1, 50.26 – 60.65)	23.9 (0.6, 21.3- 27.0)
PPP	9 (6/ 3/ 0)	105.04	43.99	56.93 (2.4, 46.87 – 68.43)	22.4 (0.9, 18.4 – 27.5)
SL1	8 (3/ 4/ 1)	145.97	44.80	51.55 (2.5, 44.47 – 61.76)	23.8 (0.6, 20.9 – 26.2)
SW2	8 (6/ 2/ 0)	30.42	42.04	55.72 (2.0, 49.72 – 66.38)	21.8 (0.7, 18.4 – 24.1)
TOTAL	**84 (57/ 21/ 6)**				
MEAN (range)		**57.52 (3.75 - 145.97)**	**44.12 (42.04 – 45.31)**	**54.63 (36.92 – 73.72)**	**24.0 (18.4 – 32.0)**

All *R. pipiens* were captured using a dip net, and each individual was handled with non-powdered disposable vinyl gloves (Phillott *et al*. 2010). Prior to specimen sampling, each individual was rinsed with 100 mL of sterile water three times to ensure that the skin sample primarily included skin-associated microbes rather than environmental material, including pond water, sediment and transient microbes (Culp *et al*. 2007; Lauer *et al*. 2007; McKenzie *et al*. 2012). Earlier studies by Culp *et al*. (2007) and Lauer *et al*. (2007) demonstrated that the composition of bacteria obtained from amphibian skin versus rinse water differ substantially, suggesting that most of the bacteria observed in this study are associated with the amphibians and not transient bacteria from the environment. Immediately following rinsing, each individual was sampled using three sterile cotton-tipped swabs brushed over the: (1) right side of abdomen for 20 seconds, (2) left side of abdomen for 20 seconds, and (3) dorsum (snout to vent) and ventral femur for 20 seconds. The three swabs were then placed in a sterile vial and stored on ice for transfer to a - 20 °C freezer for storage until DNA extraction. There were no recaptured *R. pipiens*, thus we treated each individual as independent.

All animals were handled and released according to an approved Laurentian University Animal Care and Use Committee protocol #2009-03-04. Field surveys and specimen collection were conducted under the Ontario Ministry of Natural Resources Wildlife Scientific Collector's Authorization #1068178. Amphibian handling and sampling within protected areas (including National Parks, Provincial Parks, Conservation Areas and Management Areas) followed permit guidelines awarded by: Parks Canada Agency (#BPF-2013-13913) and the Ontario Ministry of Natural Resources and Forestry (#4534).

4.3.2 Acquisition of environmental data

In order to assess the proximity of each site locality to an urban landcape, I downloaded data regarding the location of population centres throughout Ontario (Statistics Canada 2011b). Distance to large urban population centres, with a population of 100,000 or more, was calculated by finding the shortest distance to a large urban population centres for site centroid, using the 'dist2Line' function (R package 'geosphere', Robert 2015).

4.3.3 DNA extraction/ sample processing

Genomic DNA was extracted from microbiome swabs using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. A pre-treatment with mutanolysin was included to enhance recovery of DNA from gram-positive bacteria (Yuan *et al*. 2012). DNA extracted from the three swabs per individual was pooled, and used to amplify the V4 region of the 16S rRNA gene using custom barcoded primers and PCR conditions adapted from (Kozich *et al*. 2013). PCR conditions consisted of a denaturing step of 95 °C for 15 min, followed by 28 cycles of 95 °C for 20s, 50 °C for 60s, 72 °C for 60s and a final extension step of 72 °C for 10 min. Each PCR was performed in triplicate including a negative water control. Amplicons were visualized on a 2 % agarose gel and pooled yielding a final per sample volume of 24 μl. Pooled amplicon DNA was purified using an Ampure XP PCR purification kit (Beckman Coulter). Following purification, 1 µl of each combined sample was pooled into a preliminary library and the concentration was determined using Qubit fluorometric quantification (Life Technologies). Amplicon quality and incidence of primer dimer was assessed using an Agilent 2200 TapeStation system (Agilent Technologies). A titration run of 50 sequencing cycles was performed on a MiSeq

instrument (Illumina) to quantify the number of reads yielded per sample from the preliminary library. An equimolar concentration of each sample was then pooled into a final composite library based on the index representation from the titration run and subsequently sequenced on a 500 cycle run with a 250 bp paired-end strategy.

4.3.4 Sequence analyses/ bioinformatics

Raw 16S metagenomic sequence data were analysed using *mothur* v1.36.1 (Schloss *et al*. 2009) following an established method (Kozich *et al*. 2013). Demultiplexed 250bp paired end reads were assembled into contigs by aligning consensus bases at read overlaps. Unique contigs were then aligned to a SILVA reference alignment (Pruesse *et al*. 2007) and reads falling outside of the target V4 region were removed. Sequences with ambiguous bases and homopolymer runs > 6 were also removed. Sequences were then pre-clustered by combining abundances of sequences that differed by 2 or fewer nucleotides from a more abundant sequence. Each barcoded sample was then screened for chimeric sequences using UCHIME with default parameters (Edgar *et al*. 2011). Sequences were clustered into Operational Taxonomic Units (OTUs) at a threshold level of 97 % similarity. OTUs were then classified by taxonomy using a Bayesian classifier (Wang *et al*. 2007) and the SILVA reference database with a minimum bootstrap confidence threshold of 80 %. OTU abundances, taxonomic assignments and sample metadata were then exported from *mothur* as a 'biom' object and read directly into the R package 'phyloseq' (McMurdie & Holmes 2013).

4.3.5 Statistical analyses

All statistical analyses were implemented in R (version 3.2.3; R Core Team 2015). All variables were z-transformed prior to analysis to have a mean of 0 and standard deviation of 1, ensuring that all predictors were on a common scale and that main effects would be interpretable in the presence of interactions (Schielzeth 2010). Sites with fewer than 8 individuals, and samples with fewer than 25,000 sequences per sample, were removed from the analysis, yielding 84 *R. pipiens* samples, from 9 sites. All samples were rarified to 25,000 sequences per sample to remove sample heterogeneity, which impacts α- and β-diversity metrics (Lozupone *et al*. 2011).

Within-community (α-diversity) metrics were calculated using the Chao1 estimator (Chao 1984; Colwell & Coddington 1994), which calculates the estimated true species diversity of a sample using the equation:

$$S_1 = S_{obs} + \frac{F_1^2}{2F_2}$$

where S_{obs} is the number of species in the sample, F_1 is the number of singletons (i.e. the number of species with only a single occurrence in the sample) and F_2 is the number of doubletons (the number of species with exactly two occurrences in the sample). The idea behind the estimator is that if a community is being sampled, and rare species (singletons) are still being discovered, there is likely still more rare species not found. When all species have been recovered at least twice (doubletons), there is likely no more species to be found. Tests of the estimator have shown that it does provide reasonable estimates, at least for modern data sets (Chao 1984; Colwell & Coddington 1994; Chazdon *et al*. 1998). No other measures of alpha diversity were included (for example Shannon and Simpson diversity indices; Hill 1973) as this

would result in multiple testing. Furthermore, I chose to implement the Chao1 estimator over any other measure of alpha diversity, as this measures species richness from the rarefaction of observed sequences, thus emphasizes the importance of rare OTUs (Chao 1984, Chao *et al*. 2009). As there was equal variance among sites and developmental status (adult, juvenile and metamorph; Levene's test), alpha diversity was compared by analysis of covariance (ANCOVA). OTU richness per frog served as the response variable (n = 84) and site and development status served as two categorical covariables. The data were normally distributed as determined by a Shapiro–Wilk W goodness-of-fit test.

In order to compare the composition of different communities (β-diversity), I applied Nonmetric Multi-Dimensional Scaling (NMDS; Kruskal 1964) based on the Bray–Curtis measure of dissimilarity (Bray & Curtis 1957) in OTU relative abundances across samples. Bray-Curtis values were calculated using the 'vegdist' function (R package 'vegan', Oksanen *et al*. 2017). The goal of the Bray–Curtis dissimilarity measure is to utilize community distance matrices, in order to visually compare community composition. This matrix is computed using the following equation:

$$\overline{C} = 1 - \frac{2w}{a + b}$$

where w is the sum of the of the lesser scores for only those species which are present in both communities, a is the sum of the number of taxa in one community, and b is the sum of the number of taxa in the other community. β-diversity patterns were visualized using a NMDS ordination approach (Clarke & Warwick 2001). This is the recommended method for ordination of next generation sequencing (NGS) bacterial community data as NMDS is non-parametric, free of assumptions, and can reduce the

data into fewer axes than principal coordinates analysis (PCoA) (Quinn & Keough 2002; Ramette 2007). To test for significant differences in community composition, I used a two-way nested ANOSIM (analysis of similarity) to examine whether host site and/ or development status were significant predictors of variability in bacterial communities across individual amphibian samples. I ran this analysis with Bray–Curtis similarity as the response variable.

I constructed two separate linear mixed-effect models (R package 'lme4', Bates *et al*. 2015) in order to test multiple predictor variables against two response variables: (1) α-diversity (Chao1), and (2) β-diversity (NMDS1). I applied a gaussian error structure to both models, and included 'site' as a random effect to account for nestedness of samples from the same locality. There were no confounding pairs of covariates (absolute correlation coefficient > 0.5; Table 4.2), consequently I constructed a set of 23 competing candidate models (including the intercept only model), based on the above hypotheses. I used an information-theoretic model selection process to rank models based on their Akaike's information criterion value. After applying the 'nesting rule', in which models that are more complex versions of models with better support (lower AIC) are removed (Richards 2008; Richards *et al*. 2011), I considered models under a delta-2 threshold (Burnham & Anderson 2002). R^2 values that account for random effects were calculated with the rsquared.glmm function (R package 'MuMIn', Barton 2011). I used the 'sim' function (R package 'arm'; Gelman & Hill 2007) to simulate values of the posterior distribution of the model parameters. Ninety-five per cent credible intervals (CI) around the mean were extracted based on 1000 simulations (Gelman & Hill 2007). Effects were considered to be significant when the 95 % CI did not overlap with 0. All graphs were created using ggplot (R package 'ggplot2; Wickham 2009).

Table 4.2: Absolute correlation coefficient for all microbiome predictor variables. All combinations can be included in analysis as absolute correlation coefficient for all pairs of covariates < 0.5. **Latitude,** degrees north**; Distance from large urban population**, proximity (km) to a large urban population centre (population of 100,000 or more); **SVL**, snout-vent length (mm); **Frog temperature,** body temperature of amphibian at point of capture (˚C).

Predictor #1	**Predictor #2**	**cor**
Latitude	Distance from large urban population	0.39
Latitude	SVL	- 0.37
Distance from large urban population	SVL	- 0.26
Latitude	Frog temperature	0.37
Distance from large urban population	Frog temperature	0.12
Snout-vent length	Frog temperature	0.20

4.4 RESULTS

4.4.1 Variation in SVL, frog body temperature and anthropogenic disturbance, between sites

Across the nine sites, there was substantial variation in proximity to large urban population (mean km = 57.5, range = 3.75 – 145.97; Table 4.1); and latitude (mean °N = 44.12, range = 42.04 – 45.31; Table 4.1). Snout-vent length varied significantly between sites ($F_{8, 75} = 3.5$, $p < 0.01$). *R. pipiens* sampled in BP2 were larger than those sampled at BRP, K1, MIN and SL1 ($p < 0.01$; $p < 0.05$; $p < 0.05$; $p < 0.05$, respectively). All remaining sites showed similar variance in mean amphibian body size (Figure 4.2; Table 4.1). All sites were sampled within a five-week period in order to limit within-season variation due to local environmental conditions. However, due to the large latitudinal range covered within this study, I expected to see variation in frog body temperature due to local climatic nuances. Accordingly, mean frog body

temperature varied between sites ($F_{8, 75} = 3.4$, $p < 0.001$). *R. pipiens* sampled in SW2 were cooler than amphibians sampled in K1 or MIN ($p < 0.01$; $p < 0.05$, respectively). All remaining sites showed similar variance in mean frog body temperature (Figure 4.3; Table 4.1).

4.4.2 Core skin microbiome

From the rarefied 25,000 sequences per sample (n = 84), we found between 33 and 126 unique OTUs per sample (mean [SE] = 69.9 [2.4]), representing 22 different bacterial phyla. Diversity results thus apply only to the dominant members of the community, and it is possible that sequences scored as absent are in fact present at lower abundance; however, the communities are substantially different from one another when sampled at this depth. The dominant phylum across all samples was *Proteobacteria*. However, some sites showed limited presence of *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* (Figure 4.4). The dominant genera across all samples (in order) were *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas*, *Acinetobacter*, and *Chryseobacterium*. Limited presence of *Brevundimonas*, *Massilia* and *Neorhizobium* were also sampled across all sites. However, *Psychrobacter* was only dominant in two sites: ELM and MIN (Figure 4.5).

Figure 4.2: Snout-vent length (SVL; mm), by site. Site BP2 was found to harbour larger amphibians than BRP; K1; MIN and SL1. All other sites showed similar variances in frog body size.

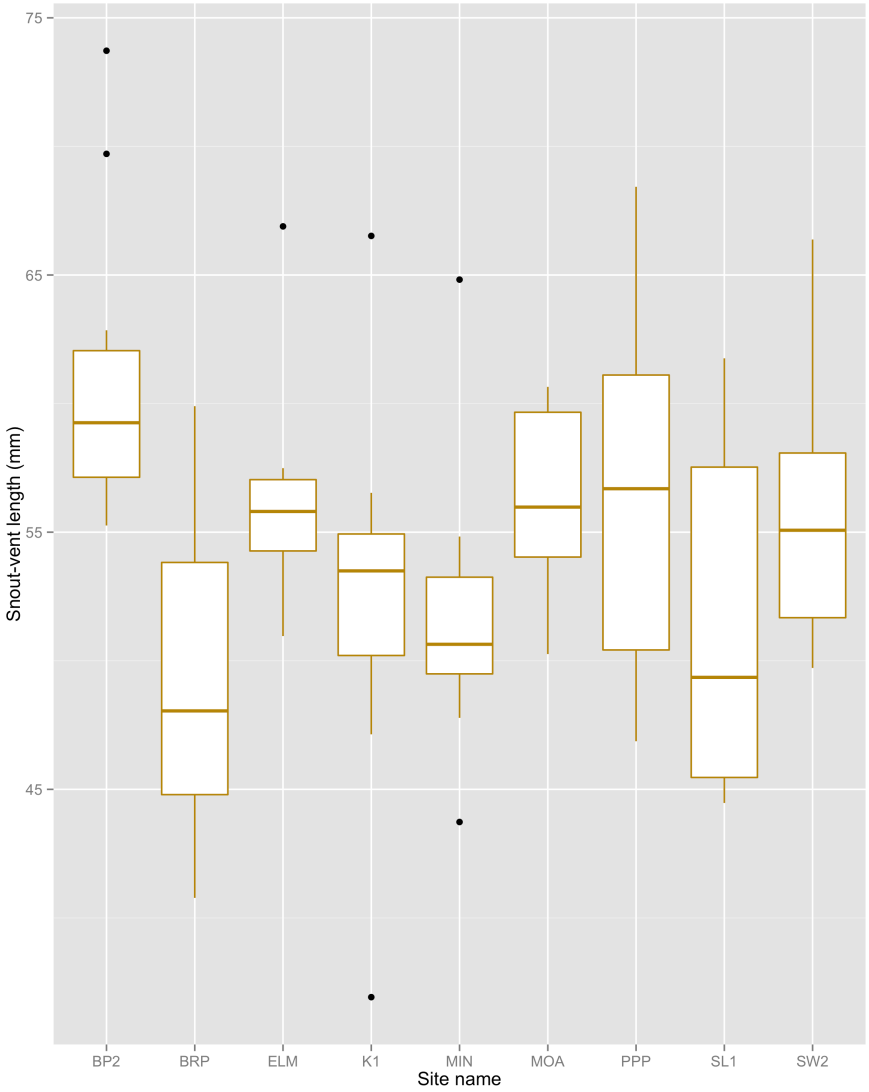


Figure 4.3: Frog body temperature (°C), by site. Site SW2 was found to harbour significantly cooler amphibians than sites K1 and MIN. All other sites showed similar variances in frog body temperature.

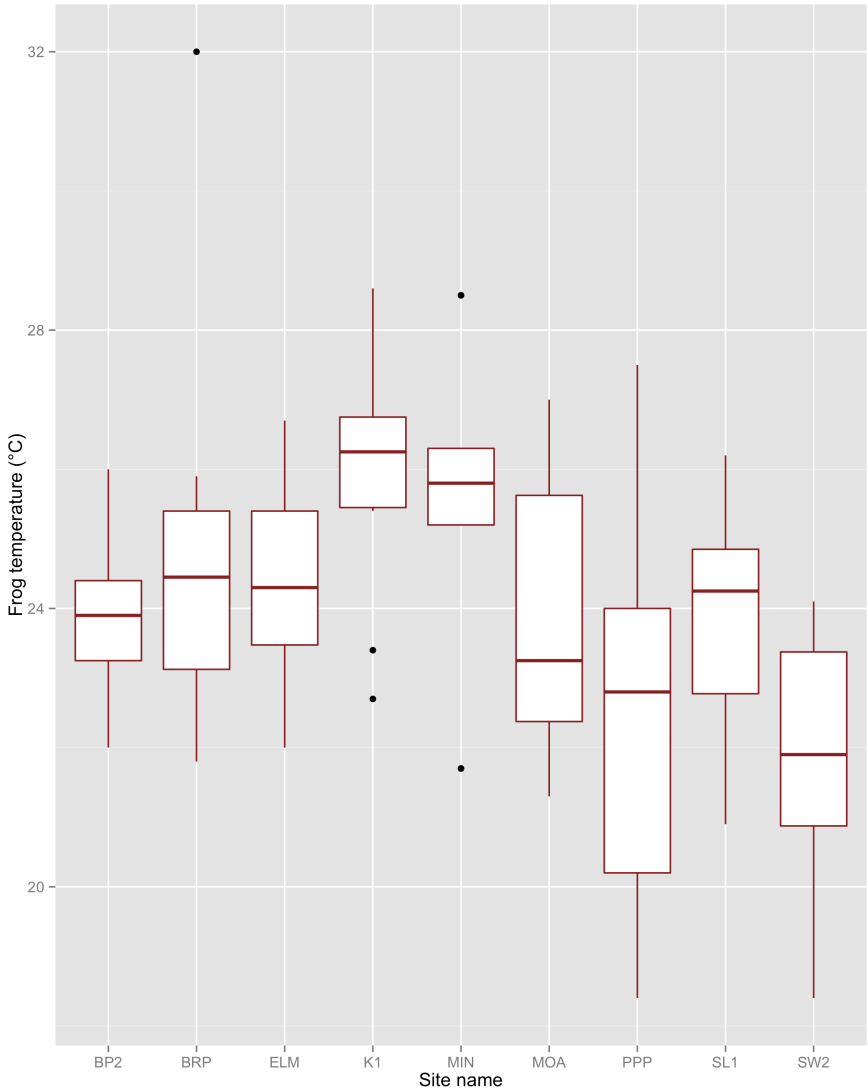


Figure 4.4: The relative abundance of sequences assigned to major bacterial phyla in the data set (colour coordinated by site). The dominant phyla across all samples was *Proteobacteria*. However, some sites showed limited presence of *Bacteroidetes*, *Actinobacteria* and *Firmicutes*.

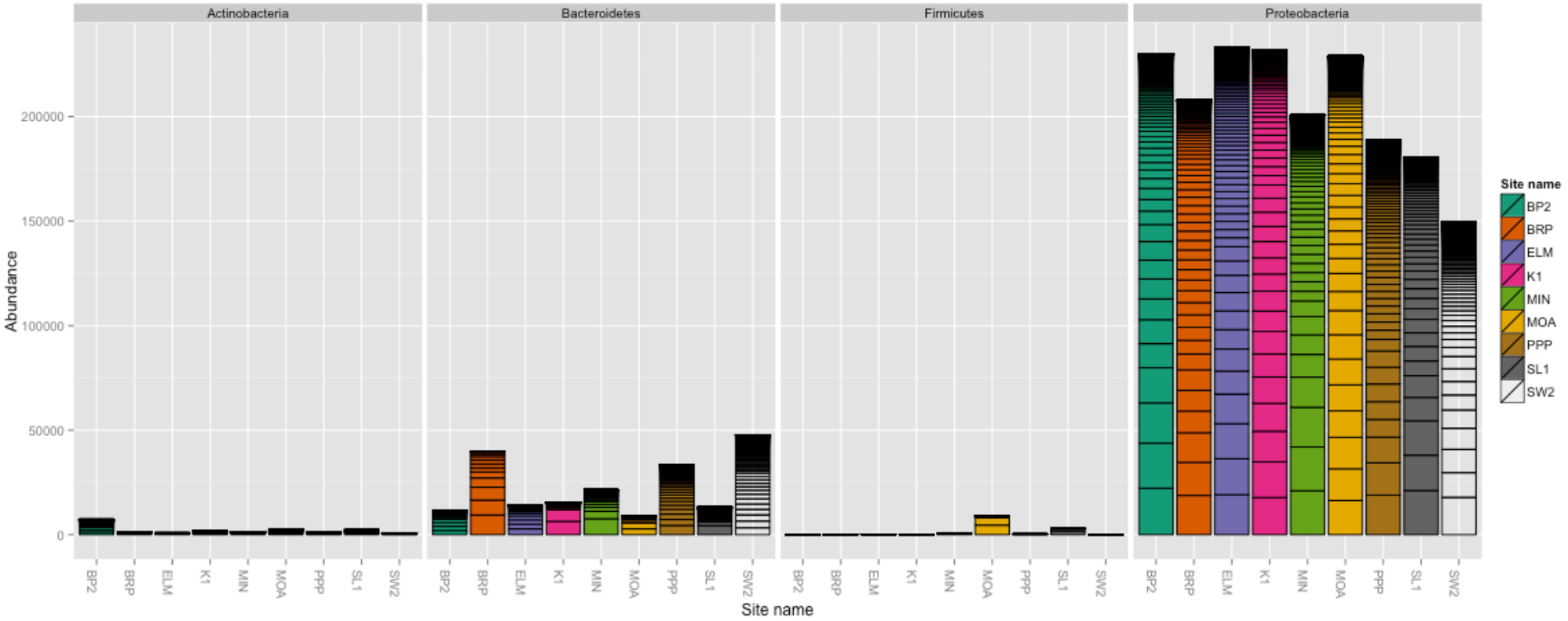
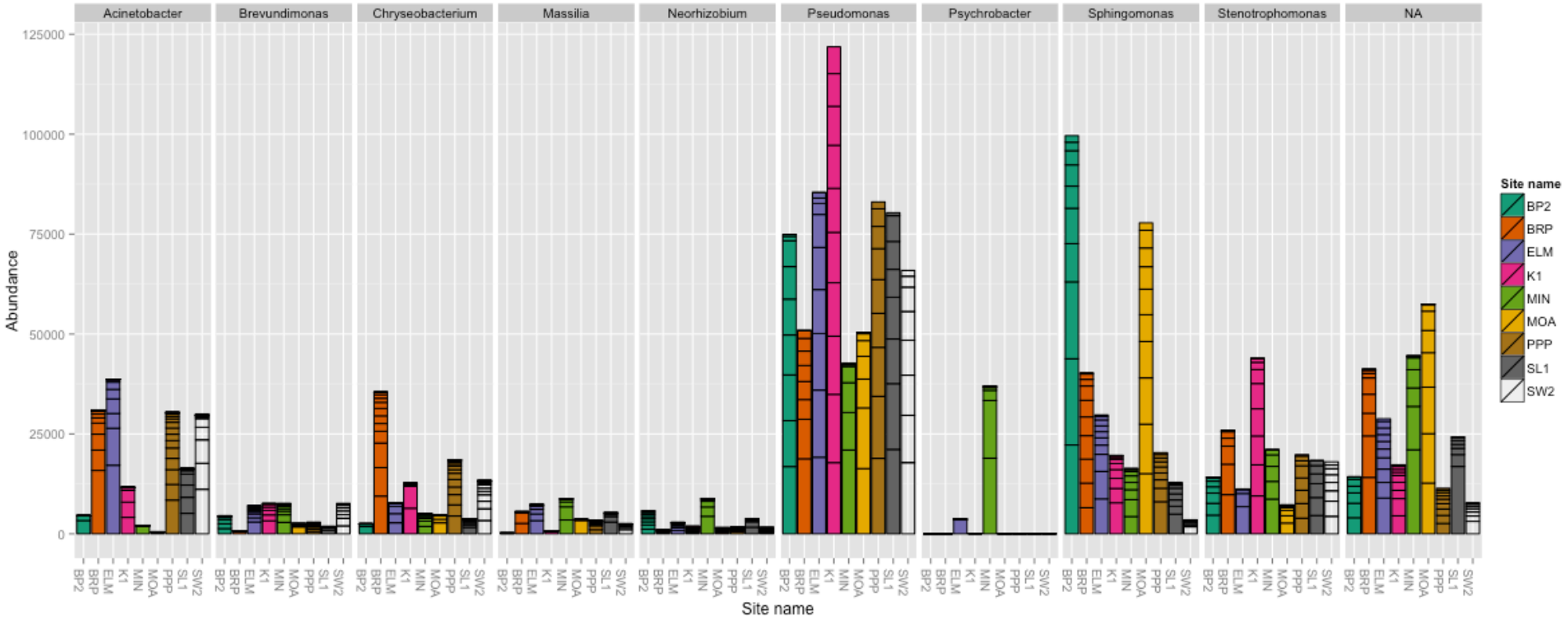


Figure 4.5: The relative abundance of sequences assigned to major bacterial genera in the data set (colour coordinated by site). The dominant genera were *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas*, *Acinetobacter*, and *Chryseobacterium*. Limited presence of *Brevundimonas*, *Massilia* and *Neorhizobium* were also sampled across all sites. *Psychrobacter* was only dominant at two sites: ELM and MIN.



4.4.3 Variation in OTU richness (α-diversity)

Levene's test indicated equal variance among sites ($F = 0.4$, $p = 0.9$) and developmental status (adult, juvenile and metamorph; $F = 0.8$, $p = 0.5$). OTU richness did not vary between development status (adult, juvenile, and metamorph; $p = 0.3$: Figure 4.6), but did vary significantly between sites ($p < 0.0001$, $r^2 = 0.27$; Figure 4.7), as determined by an analysis of covariance. The greatest levels of mean α-diversity were recorded at sites SW2, SL1 and PPP, while sites K1 and ELM had the lowest α-diversity (Table 4.3). Consequently, I decided to include 'site' as a random effect, and exclude the categorical variable 'development status' in all further modelling.

The highest ranked model predicting for OTU richness (α-diversity), comprised of two variables: euclidean distance from large urban population (population of 100,000 or more; km) and latitude (˚N) (Table 4.4). Having applied the 'nesting rule', only the top model was retained with $\Delta AIC < 2$. The marginal r^2 (variance explained by the fixed effects) of the top model was 28 %. OTU richness was positively correlated with distance from large urban population (km): sites located far from population centres with 100,000 or more human inhabitants had greater OTU richness than those located in close proximity to large urban populations (Figure 4.8). OTU richness was also negatively correlated with latitude: as latitude increased, α-diversity decreased (Figure 4.9). Latitude was found to have a greater effect on upon the global mean for OTU richness than distance from large urban population centre (Figure 4.10).

Figure 4.6: Boxplots representing non-significant variation in mean OTU richness (α-diversity) between developmental status: adult, juvenile, and metamorph.

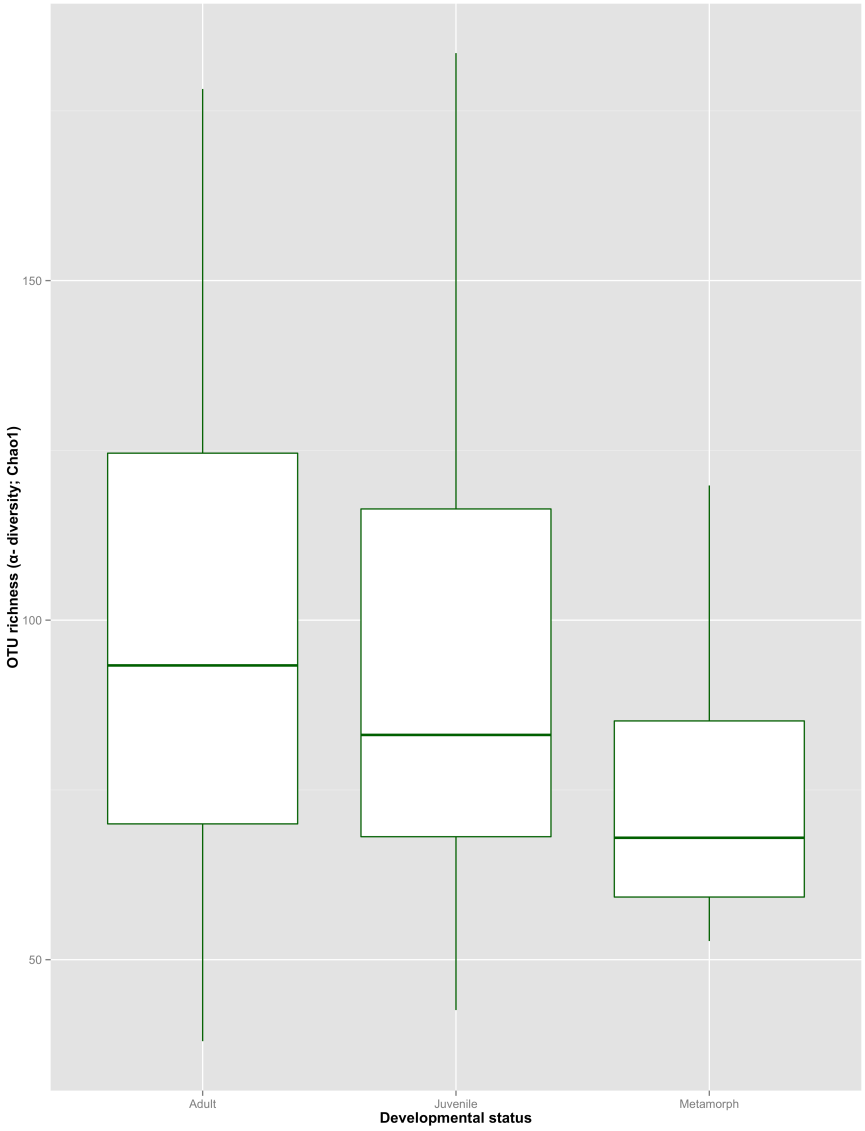


Figure 4.7: Boxplots representing significant variation in OTU richness (α-diversity) between sites. SW2, SL1 and PPP present the greatest levels of mean α-diversity, while sites K1 and ELM show the lowest.

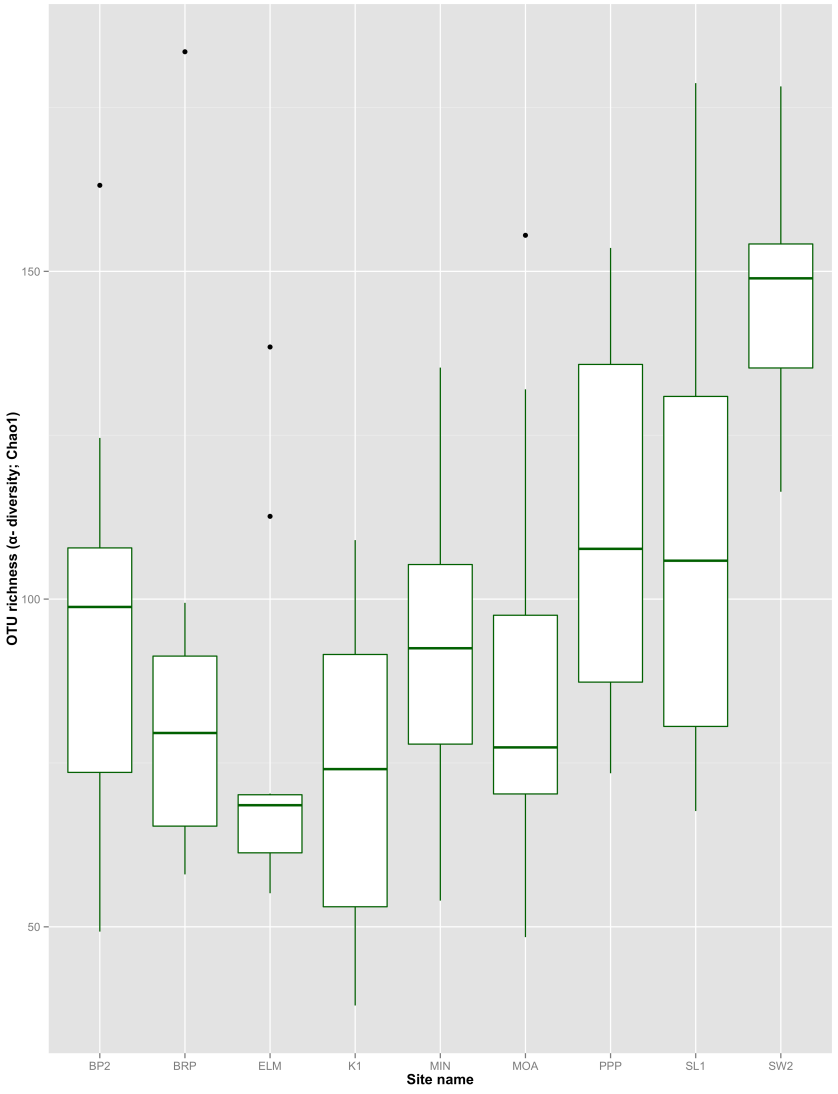


Table 4.3: Summary statistic regarding OTU richness (α-diversity), by site.

	OTU richness (α-diversity)			
SITE	$\bar{x}$	**se**	**min**	**max**
BP2	96.7	10.3	49.3	163.1
BRP	87.9	11.5	58.0	183.5
ELM	76.8	8.5	55.1	138.5
K1	72.0	7.9	38.0	109.0
MIN	90.7	9.1	54.0	135.3
MOA	88.3	10.3	48.4	155.5
PPP	111.6	9.7	73.4	153.6
SL1	110.2	13.2	67.7	178.7
SW2	146.8	6.8	116.4	178.2

Table 4.4: Akaike's information criterion model rankings for the candidate models explaining within-community OTU richness (α-diversity). Within-community axis scores are based on the Chao1 estimator. The top model for predicting differences in bacterial composition between communities (β-diversity) is highlighted in bold. ***k***, number of parameters; **logLik,** log likelihood; **AIC**, Akaike's information criterion; **ΔAIC**, difference in AIC compared with the model with the lowest AIC; w_i, model weight; **Retained**, models in the Δ2 AIC set (grey shaded rows) are not retained if they are more complex versions of nested (simpler) models with better AIC support (higher up in the table); **distance from large urban population**, proximity (km) to a large urban population centre (population of 100,000 or more); **latitude,** degrees north**; site**, accounted for possible non-independence of toe-clips collected at the same site locality by including this random intercept effect; **frog temperature,** body temperature of amphibian at point of capture (˚C); **SVL**, snout-vent length (mm).

Model	Model description	k	logLik	AIC	ΔAIC	w_i	Retained
m16	distance from large urban population + latitude + (1 | site)	5	-404.03	818.06	0	0.252	✔
m10	distance from large urban population + latitude + frog temperature + (1 | site)	6	-403.42	818.84	0.779	0.171	✗
m8	distance from large urban population * latitude + (1 | site)	6	-403.62	819.25	1.192	0.139	✗
m12	distance from large urban population + latitude + SVL + (1 | site)	6	-404.03	820.06	2	0.093	✗
m5	distance from large urban population * latitude + frog temperature + (1 | site)	7	-403.15	820.3	2.247	0.082	
m3	latitude * frog temperature + distance from large urban population + (1 | site)	7	-403.41	820.81	2.755	0.064	
m9	distance from large urban population + latitude + frog temperature + SVL + (1 | site)	7	-403.41	820.82	2.758	0.064	
m6	distance from large urban population * latitude + SVL + (1 | site)	7	-403.62	821.24	3.184	0.051	
m2	distance from large urban population * latitude + frog temperature + SVL + (1 | site)	8	-403.13	822.27	4.21	0.031	
m1	latitude * frog temperature + distance from large urban population + SVL + (1 | site)	8	-403.39	822.79	4.731	0.024	
m13	frog temperature + latitude + (1 | site)	5	-407.33	824.67	6.609	0.009	
m20	latitude + (1 | site)	4	-408.75	825.5	7.444	0.006	
m7	latitude * frog temperature + (1 | site)	6	-407.11	826.22	8.164	0.004	
m11	**latitude + frog temperature + SVL + (1 | site)**	**6**	**-407.32**	**826.65**	**8.589**	**0.003**	
m17	latitude + SVL + (1 | site)	5	-408.74	827.48	9.422	0.002	
m4	latitude * frog temperature + SVL + (1 | site)	7	-407.11	828.22	10.164	0.002	
m19	frog temperature + (1 | site)	4	-410.4	828.79	10.737	0.001	
m23	1 + (1 | site)	3	-412.16	830.32	12.26	0.001	
m15	frog temperature + SVL + (1 | site)	5	-410.18	830.36	12.306	0.001	
m14	distance from large urban population + frog temperature + (1 | site)	5	-410.19	830.39	12.332	0.001	
m22	SVL + (1 | site)	4	-411.78	831.55	13.493	0	
m21	distance from large urban population + (1 | site)	4	-411.94	831.88	13.826	0	
m18	distance from large urban population + SVL + (1 | site)	5	-411.45	832.91	14.851	0	

Figure 4.8: Model predicted positive relationship between OTU richness (α-diversity) and euclidean distance from large urban population centre (population of 100,000 or more). Shaded area spans the 95 % credible intervals for the fitted means.

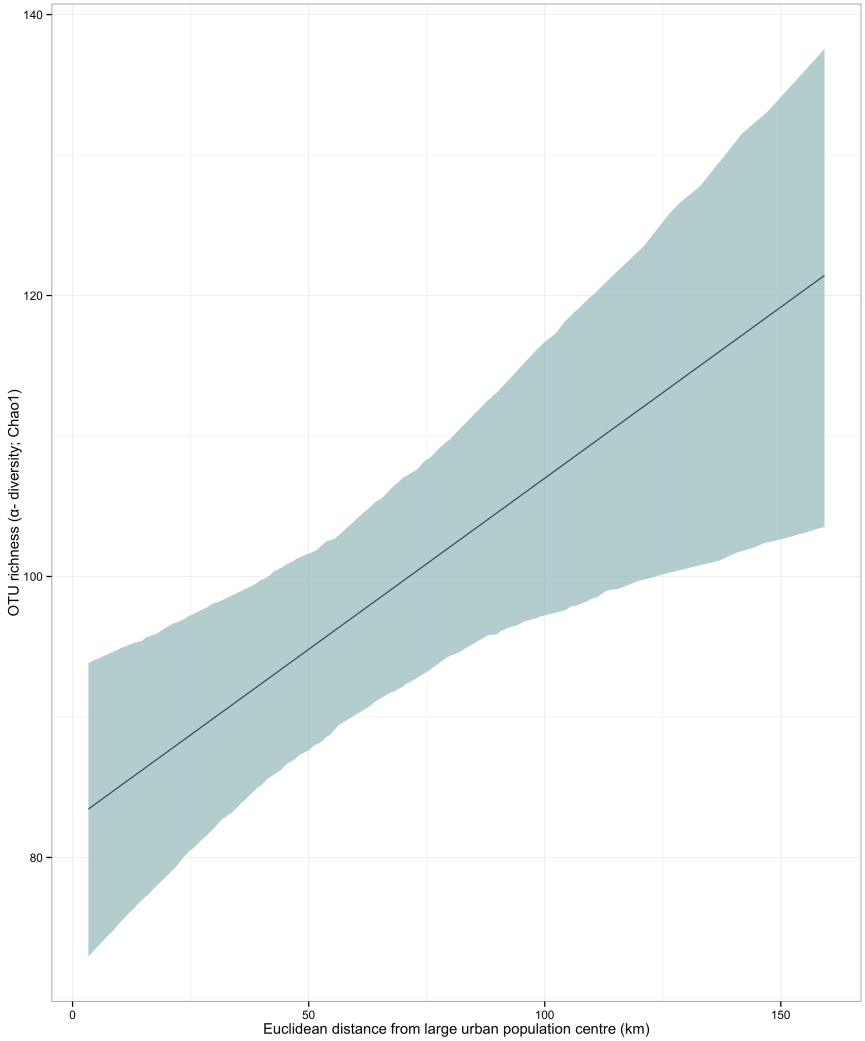


Figure 4.9: Model predicted negative relationship between OTU richness (α-diversity) and latitude (˚N). Shaded area spans the 95 % credible intervals for the fitted means.

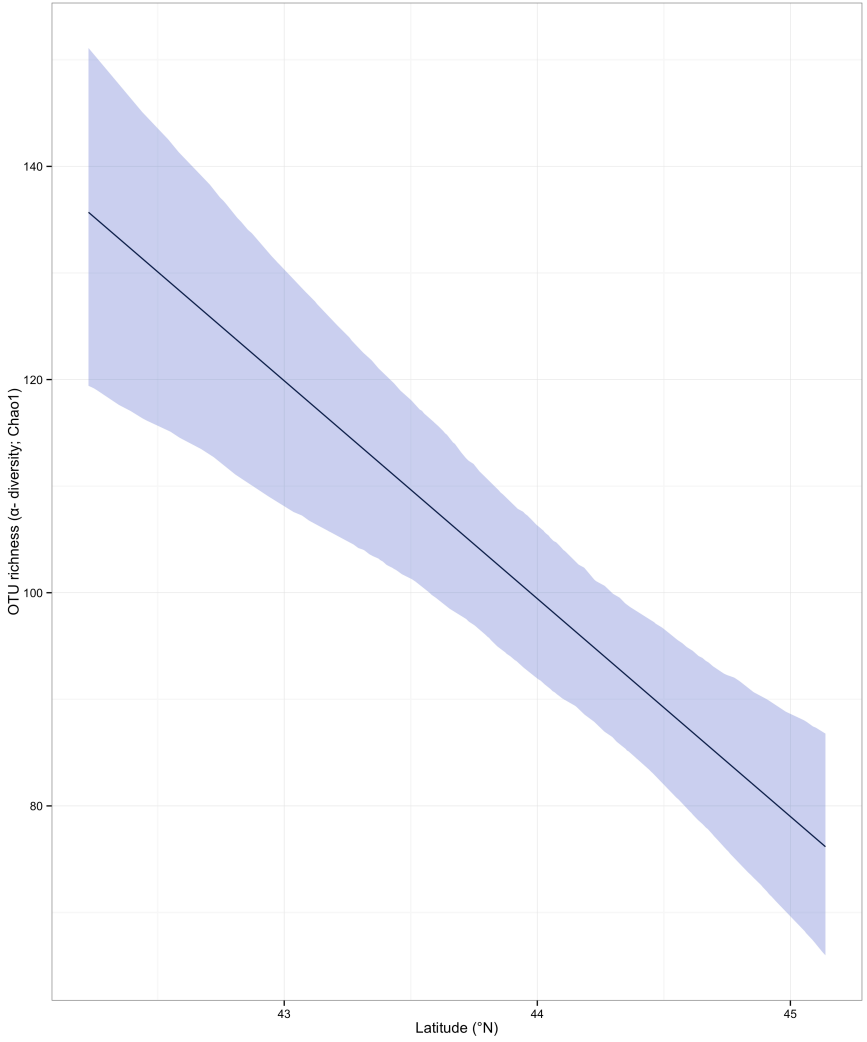
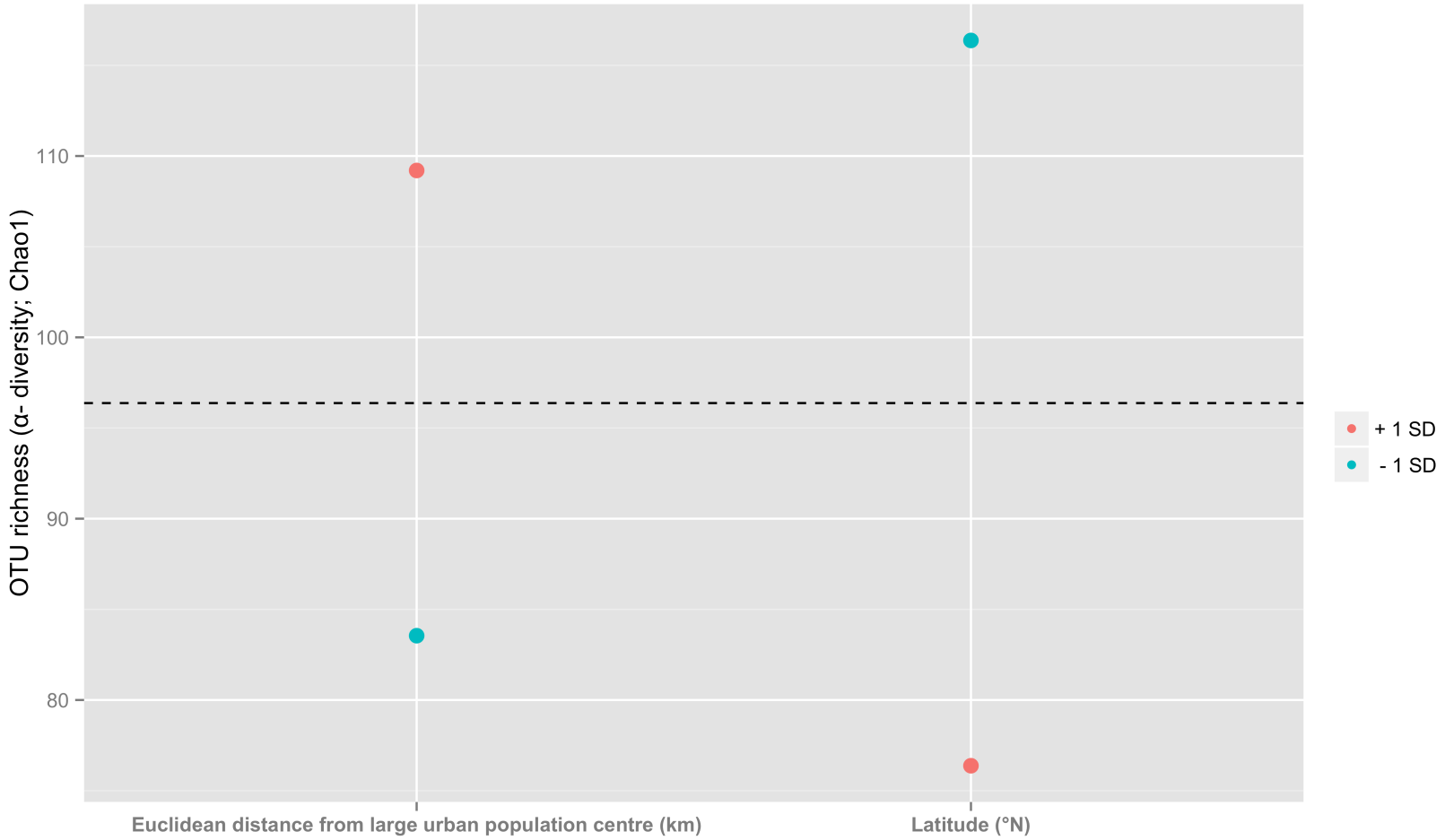


Figure 4.10: Model predicted effect sizes of predictor variables from AIC top model explaining variation in OTU richness (α-diversity). Black dotted line depicts the global mean for OTU richness when all predictor variables are averaged. Red point represents plus one standard deviation for each predictor variable, blue point represents minus one standard deviation for each predictor variable. Latitude (°N) has a greater impact upon the global mean for OTU richness, than euclidean distance from large urban population centre (population of 100,000 or more; km).



4.4.4 Variation in bacterial community composition (β-diversity)

There were strong differences in the composition of the skin-associated bacterial communities found between sites, a pattern evident in the ordination plot (Figure 4.11). These patterns are confirmed by the ANOSIM analyses using the taxon-based Bray–Curtis distance metric, site was a significant predictor of skin bacterial community uniqueness, explaining 19 % of the variance ($p = 0.001$; Table 4.5). Development status was not significant predictor of skin bacterial communities ($p = 0.3$; Table 4.5). Consequently, I decided to include 'site' as a random effect, and exclude development status in all further modelling.

The highest ranked model predicting for community composition (β-diversity), comprised of three variables: latitude (˚N), frog temperature (˚C) and snout-vent length (mm) (Table 4.6). Having applied the 'nesting rule', six models were retained within $\Delta AIC < 2$. The marginal r^2 (variance explained by the fixed effects) of the top model was 21 %. Bacterial community composition was influenced by latitude (Figure 4.12); frog temperature (Figure 4.13); and snout-vent length (Figure 4.14). As the microbial community axis scores were based on NMDS ordination solution, these results highlight the structural differences in bacterial community composition between northern and southern populations; amphibians thermoregulating at warm and cooler temperatures; and large and small amphibians. Thus, the directionality of the correlation is immaterial. However, latitude was found to have a greater effect on upon the global mean for variation in community composition (β-diversity), than snout-vent length (mm) and frog temperature (˚C) (Figure 4.15). Thus, community structure was at its most diverse between northern and southern latitudes.

As stated above (subsection 4.4.1), *R. pipiens* sampled within site BP2 were larger (SVL; mm), than those sampled at BRP, K1, MIN and SL1 ($p < 0.01$; $p < 0.05$; $p < 0.05$; $p < 0.05$, respectively; Figure 4.2). Additionally, BP2 exhibited one the greatest deviations in bacterial community structure from the global mean (Figure 4.16). As such, a lack of sample variation in body size within this one site, may have had a strong effect upon the model outcome. However, in removing this site from the available data, and re-running the 23 competing candidate models, the same model is top ranked. Similarly, as stated above (subsection 4.4.1), *R. pipiens* sampled in SW2 were found to have cooler body temperatures, on average, than those sampled in sites K1 and MIN ($p < 0.01$; $p < 0.05$, respectively; Figure 4.3). Furthermore, SW2 was the most southerly site within the dataset (Figure 4.1; Table 4.1). As frog body temperature and latitude were found to be significant predictors with regards to bacterial community composition, and site SW2 exhibits one the greatest departures from the global mean in community structure (along with BP2; Figure 4.16), it is, again, possible that this one site strongly influences the model outcome. However, in removing SW2 from the available data, and re-running the 23 competing candidate models, the top model is still within delta 2. Consequently, we can assume that the model selection is robust.

Figure 4.11: Ordination plot of β-diversity patterns within and between sites. Each point represents the skin bacterial community of an individual amphibian, connected to their site locality (three letter code). β-diversity patterns were visualized using a NMDS ordination approach with skin-associated bacterial community differences represented as Bray–Curtis distances. The 2D distance between points in the ordination plot is a good representation of the degree of similarity between each sample's bacterial community.

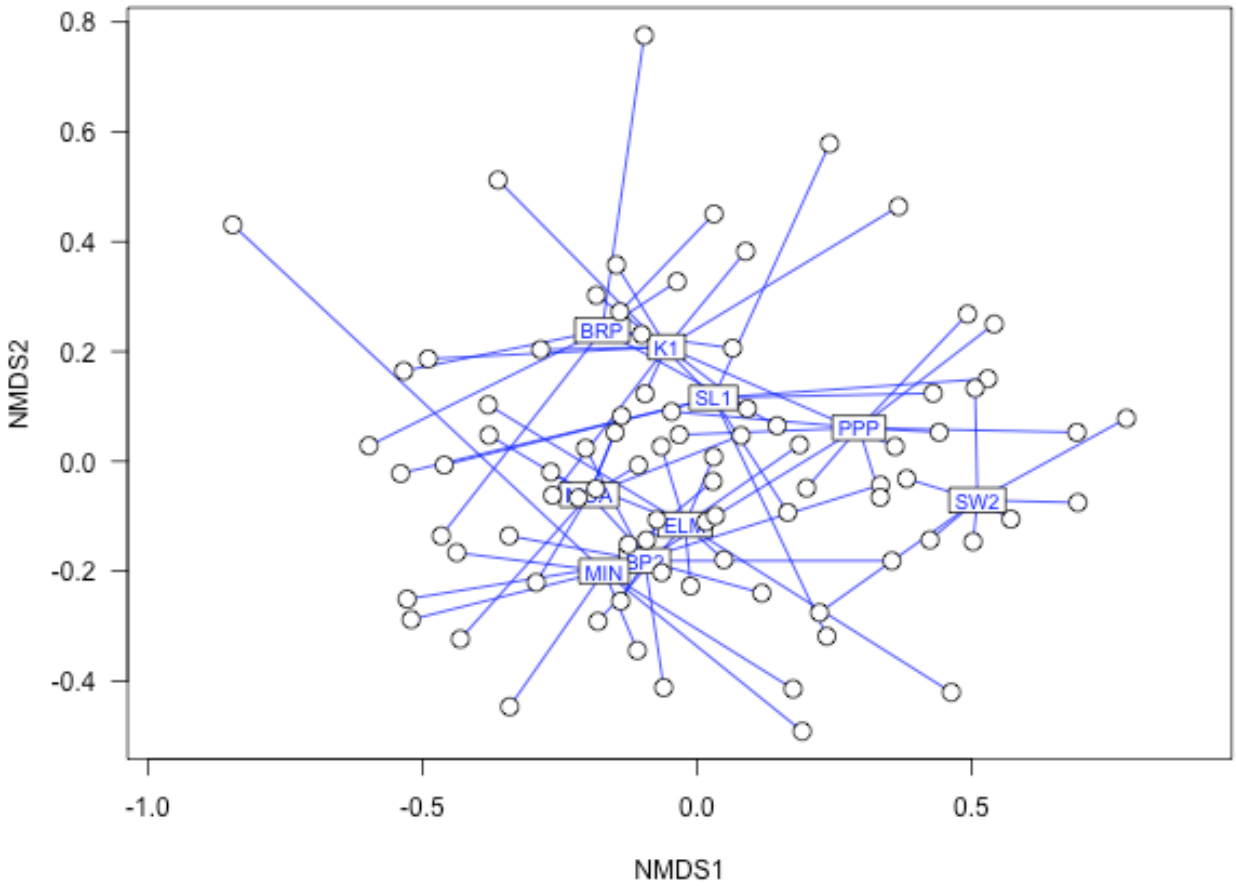


Table 4.5: Results of the two-way nested analysis of similarity (ANOSIM). I examined the differences among bacterial communities from different development status within different sites using Bray-Curtis similarity measurements.

	Bray-Curtis similarity	
	Global R	**Significance**
Between sites	0.19	p = 0.001
Between development status	0.03	p = 0.3

Table 4.6: Akaike's information criterion model rankings for the candidate models explaining differences in bacterial composition between communities (β-diversity). Microbial community axis scores are based on NMDS ordination solution. The top model for predicting within-community OTU richness (α-diversity) is highlighted in bold. ***k***, number of parameters; **logLik,** log likelihood; **AIC**, Akaike's information criterion; **ΔAIC**, difference in AIC compared with the model with the lowest AIC; w_i, model weight; **Retained**, models in the Δ2 AIC set (grey shaded rows) are not retained if they are more complex versions of nested (simpler) models with better AIC support (higher up in the table); **latitude,** degrees north**; frog temperature,** body temperature of amphibian at point of capture (˚C); **SVL**, snout-vent length (mm); **site**, accounted for possible non-independence of toe-clips collected at the same site locality by including this random intercept effect; **distance from large urban population**, proximity (km) to a large urban population centre (population of 100,000 or more).

Model	Model description	k	logLik	AIC	ΔAIC	w_i	Retained
m11	latitude + frog temperature + SVL + (1 | site)	6	-13.70	39.40	0.000	0.118	✔
m15	frog temperature + SVL + (1 | site)	5	-14.76	39.51	0.111	0.111	✔
m2	distance from large urban population * latitude + frog temperature + SVL + (1 | site)	8	-11.77	39.54	0.139	0.110	✗
m6	distance from large urban population * latitude + SVL + (1 | site)	7	-12.93	39.86	0.460	0.094	✔
m9	distance from large urban population + latitude + frog temperature + SVL + (1 | site)	7	-13.00	40.00	0.601	0.087	✗
m12	distance from large urban population + latitude + SVL + (1 | site)	6	-14.32	40.63	1.231	0.064	✔
m17	latitude + SVL + (1 | site)	5	-15.33	40.66	1.261	0.063	✔
m4	latitude * frog temperature + SVL + (1 | site)	7	-13.53	41.05	1.653	0.051	✗
m22	SVL + (1 | site)	4	-16.68	41.36	1.963	0.044	✔
m5	distance from large urban population * latitude + frog temperature + (1 | site)	7	-13.71	41.41	2.014	0.043	
m13	latitude + frog temperature + (1 | site)	5	-15.87	41.74	2.346	0.036	
m1	latitude * frog temperature + distance from large urban population + SVL + (1 | site)	8	-12.87	41.75	2.349	0.036	
m8	distance from large urban population * latitude + (1 | site)	6	-15.14	42.29	2.889	0.028	
m10	distance from large urban population + latitude + frog temperature + (1 | site)	6	-15.32	42.64	3.243	0.023	
m18	distance from large urban population + SVL + (1 | site)	5	-16.57	43.14	3.744	0.018	
m19	frog temperature + (1 | site)	4	-17.72	43.44	4.042	0.016	
m7	latitude * frog temperature + (1 | site)	6	-15.84	43.68	4.283	0.014	
m20	latitude + (1 | site)	4	-17.90	43.80	4.399	0.013	
m16	**distance from large urban population + latitude + (1 | site)**	**5**	**-16.99**	**43.99**	**4.586**	**0.012**	
m3	latitude * frog temperature + distance from large urban population + (1 | site)	7	-15.30	44.61	5.209	0.009	
m14	distance from large urban population + frog temperature + (1 | site)	5	-17.72	45.44	6.042	0.006	
m23	1 + (1 | site)	3	-20.18	46.36	6.956	0.004	
m21	distance from large urban population + (1 | site)	4	-20.17	48.33	8.936	0.001	

Figure 4.12: Model predicted relationship between community composition (β-diversity) and latitude (˚N). Shaded area spans the 95 % credible intervals for the fitted means.

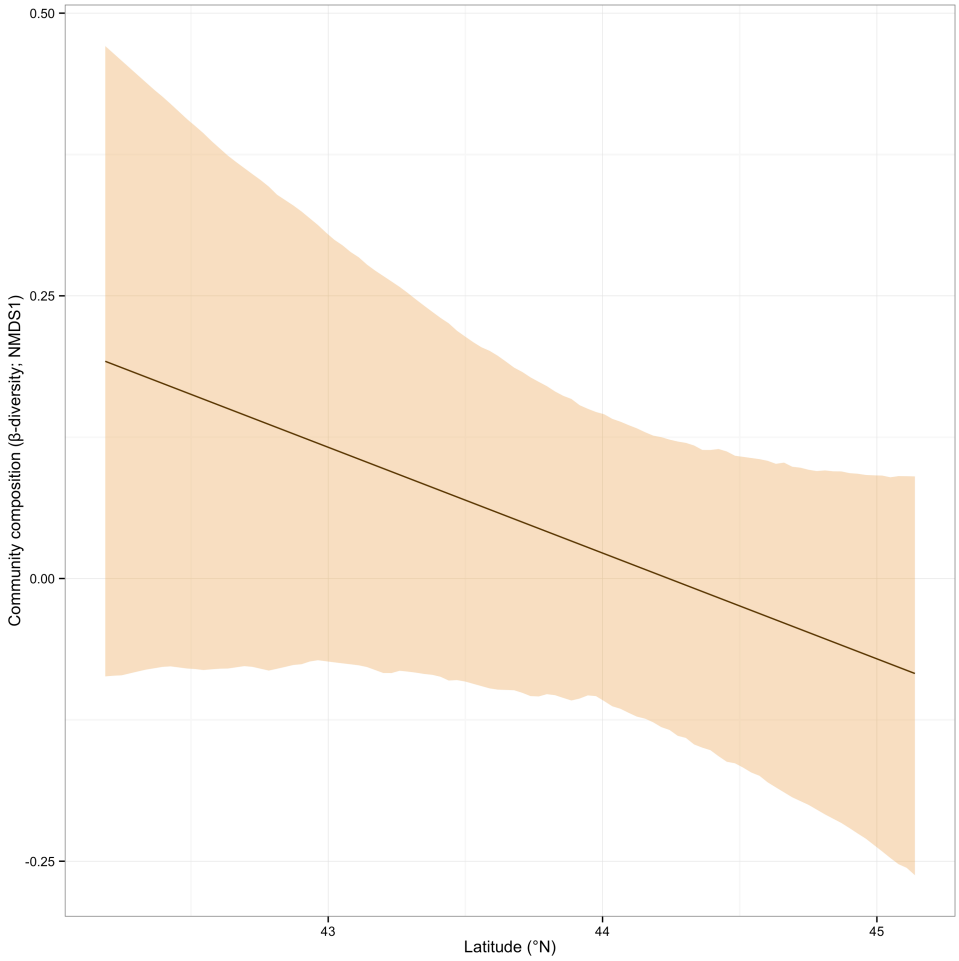


Figure 4.13: Model predicted relationship between community composition (β-diversity) and frog temperature (˚C). Shaded area spans the 95 % credible intervals for the fitted means.

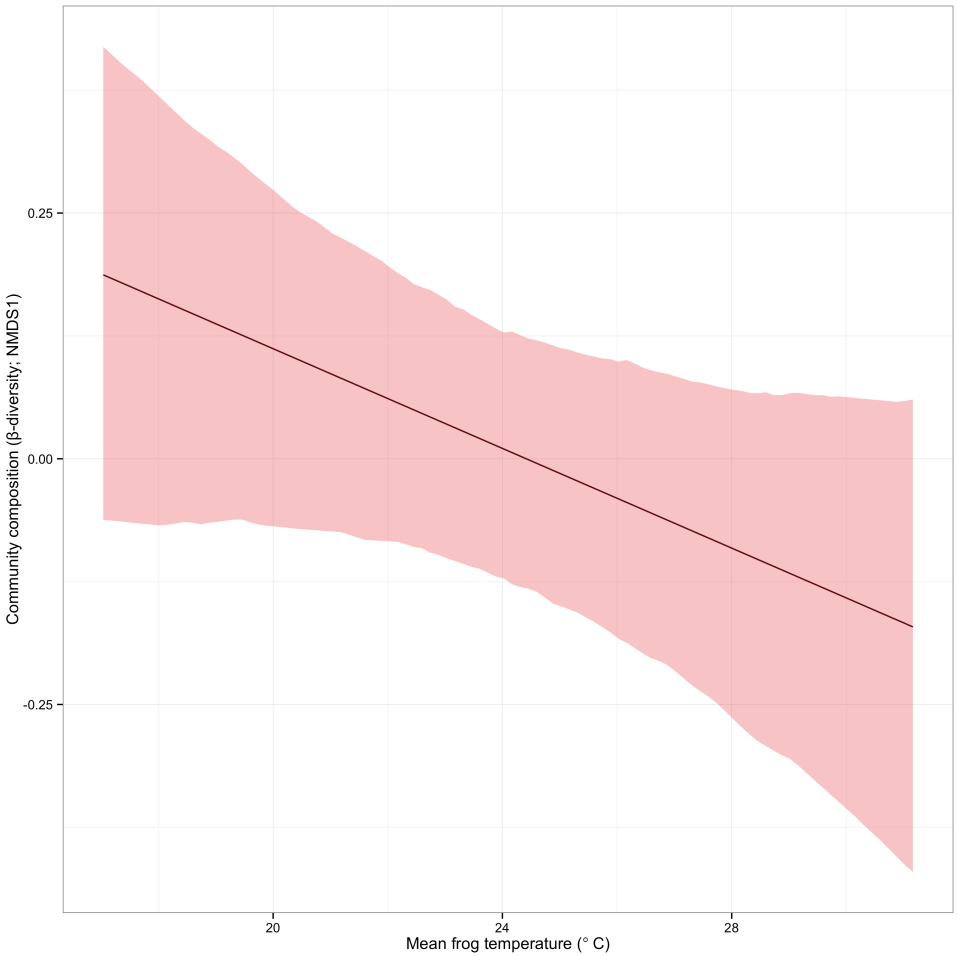


Figure 4.14: Model predicted relationship between community composition (β-diversity) and snout-vent length (SVL; mm). Shaded area spans the 95 % credible intervals for the fitted means.

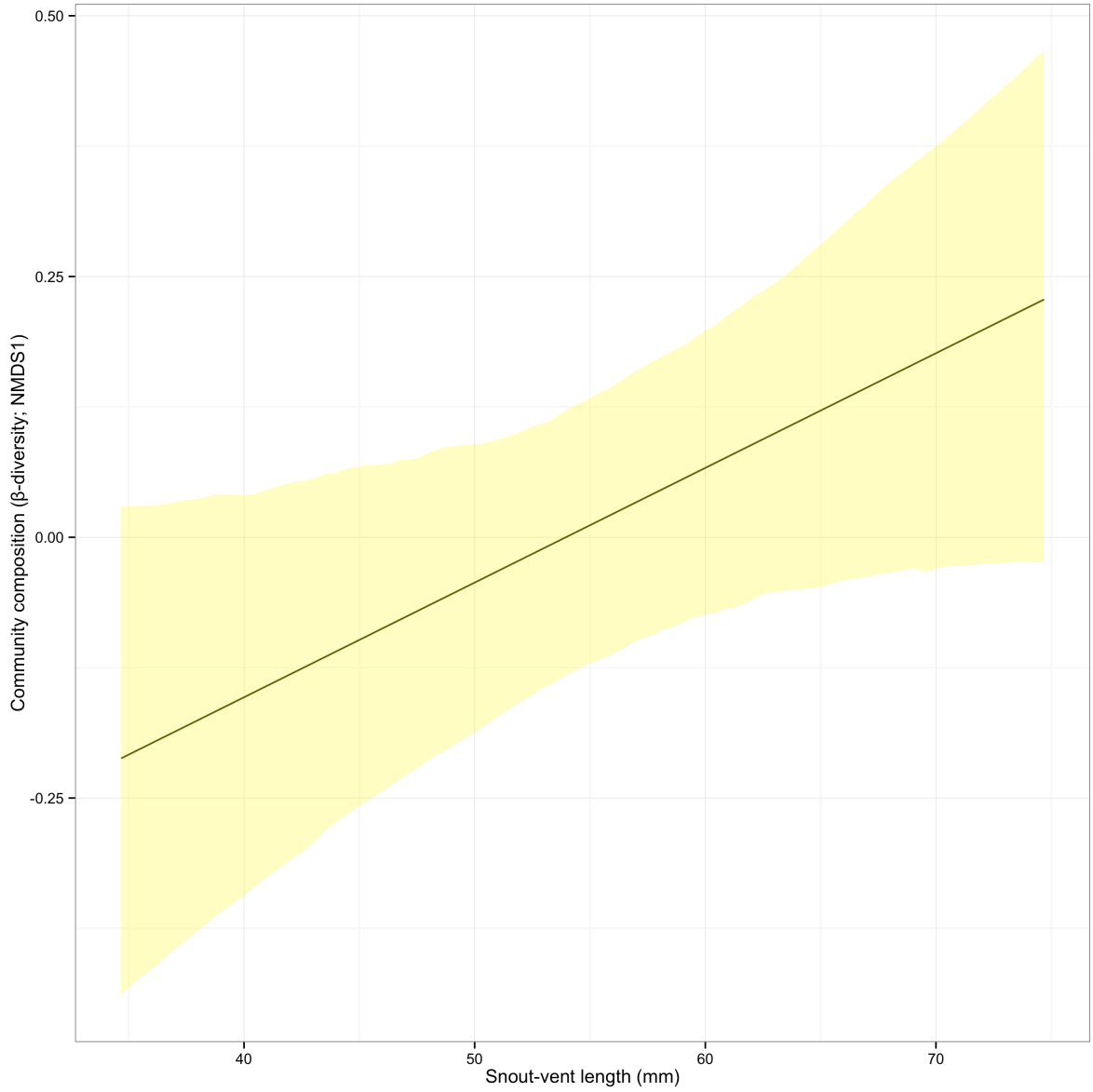


Figure 4.15: Effect sizes of predictor variables from AIC top model explaining variation in community composition (β-diversity; NMDS1). Black dotted line depicts the global mean for community composition when all predictor variables are averaged. Red point represents plus one standard deviation for each predictor variable, blue point represents minus one standard deviation for each predictor variable. Latitude (˚N) has the greatest impact upon the global mean for community composition, then snout-vent length (mm) and frog temperature (˚C).

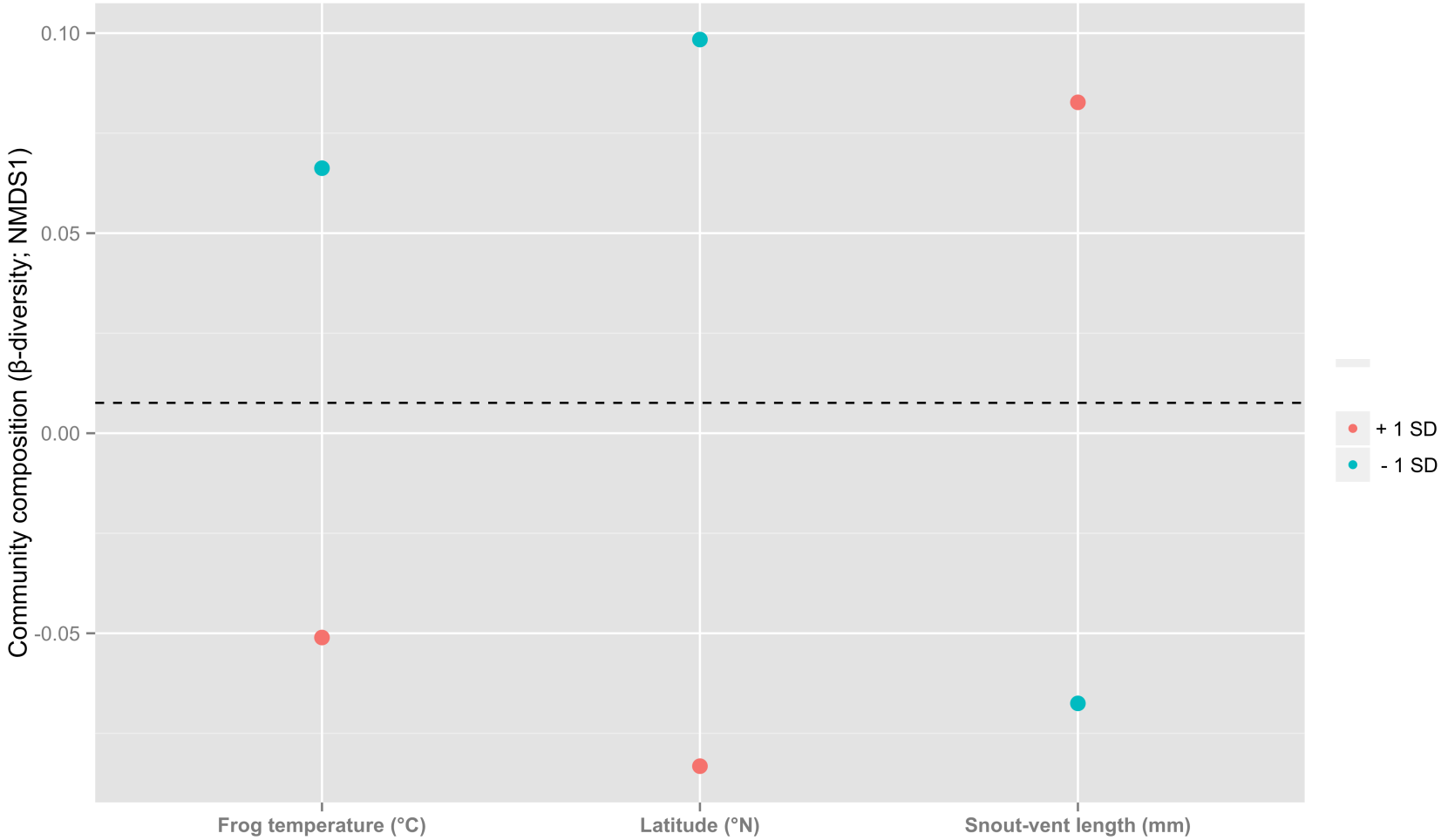
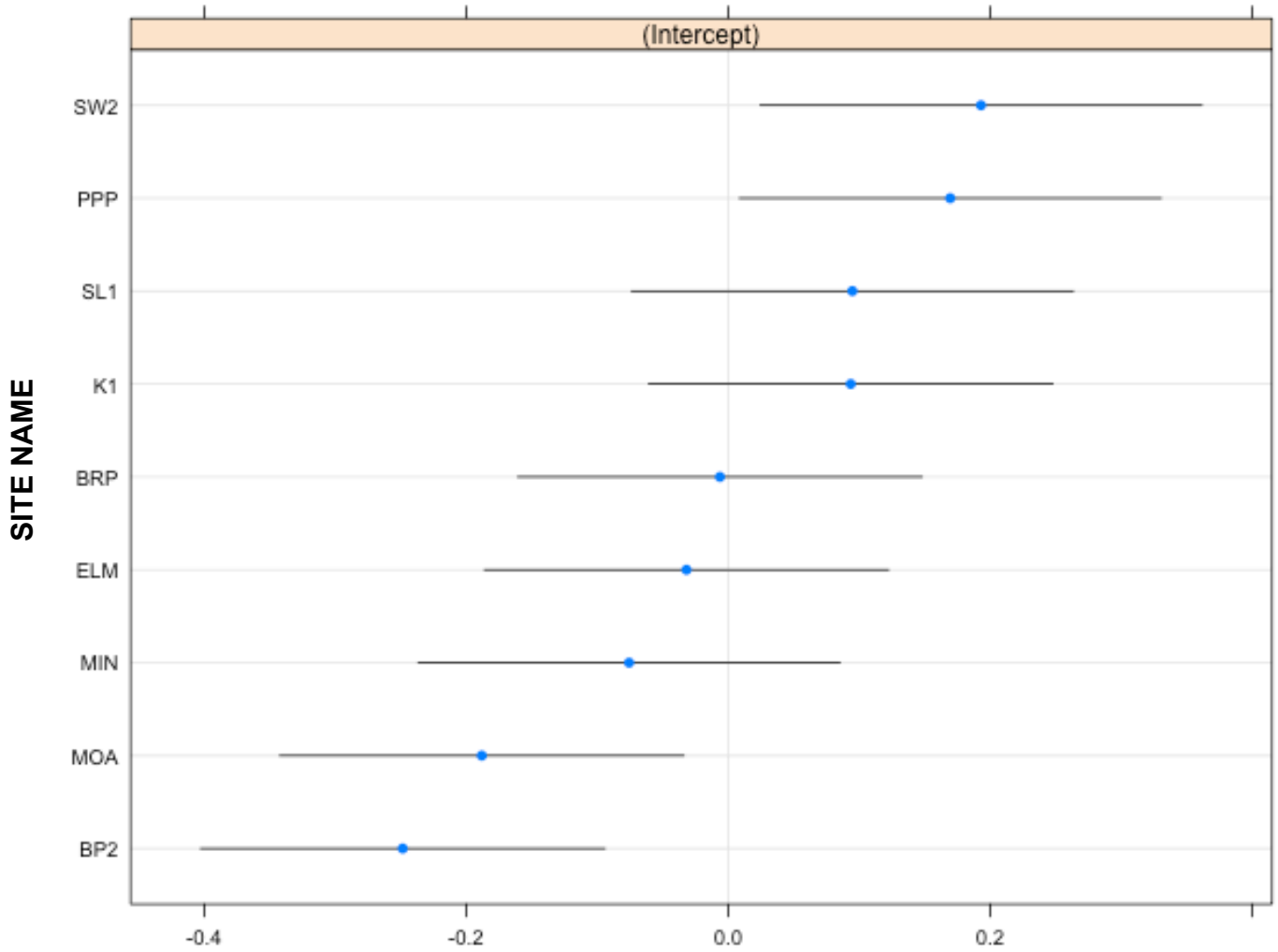


Figure 4.16: Model predicted intercept for each site ID, along with their standard errors, with regards to difference in community composition (β-diversity) from the global mean (intercept = 0). Sites BP2 and SW2 represent the greatest divergence in bacterial community structure, in comparison to the remaining seven sites.



4.5 DISCUSSION

4.5.1 Landscape and host characteristics influence amphibian skin micobiome

This study demonstrates that, within a population, frogs have similar microbiome structure; and that both site and individual level characteristics are important factors in determining skin bacterial diversity and community structure in an amphibian species that appears to be coexisting with endemic *Bd* (as supported by my findings in Chapter 2). Within *R. pipiens* populations, overall bacterial diversity was significantly higher in populations located at: (1) lower latitudinal ranges; and (2) far from large urban populations, while bacterial community structure was found to be highly divergent between: (3) northern and southern populations; (4) warmer and cooler amphibian individuals; and (5) larger and smaller amphibian individuals. However, the influence of latitude on both bacterial diversity and community composition was greater than the proximity to large urban populations, or individual-level characteristics, indicating that latitude plays the most important role in shaping microbial occurrence on the skin.

The hypothesis that the environment may alter amphibian microbial community structure is not new; however, only one published study has investigated whether latitude alters the skin-associated microbiome of amphibians (Krynak *et al*. 2016). The authors found that amphibian bacterial community composition (β-diversity) was significantly different between northern and southern populations (latitudinal range: 39.5 ˚N – 42 ˚N). However, they did not test α-diversity, thus variation in overall diversity across the latitudinal gradient is unclear. My results demonstrate that OTU richness decreases with increasing latitude. This may be due to two broad scale

factors: (1) cooler climatic conditions at northern latitudes inhibit the growth of certain bacterial taxa (Matutte *et al*. 2000; Rollins-Smith *et al*. 2002; Ribas *et al*. 2009), or (2) local physical characteristics, such as sediment type, pH, and nutrient concentrations, define the available colonizing microbes within the environment (Vartoukian *et al*. 2010; Yergeau *et al*. 2012; Sharp *et al*. 2014;). While I cannot identify the mechanism by which latitude alters these immune defenses, this is a promising area of future research.

Outside of amphibian research, Bálint *et al*. (2015) investigated how the foliar endophytic fungal microbiome of the North American balsam poplar (*Populus balsamifera*) reacted to the human-assisted relocation of trees to high latitudes. They found that the relocation of hosts to the north, from the south, influenced microbiome structure. However, the reactions of the microbiome to relocation were strongly dependent on host genetic identity. This suggests that the interaction of environmental factors and the population genetic processes of the hosts, mediated the latitudinal effect of host–microbiome systems. In my study system, sites were located across a wide latitude range (42.04 ˚N - 45.31 ˚N) and were separated by a minimum of 36.5 km. As this distance is above the maximum dispersal range abilities of *R. pipiens* (8 – 10 km; Dole 1971; Seburn & Seburn 1997; Romanchuk & Quinlan 2006), I acknowledge that genetic variation may play a significant role in explaining variation in bacterial communities between sites. However, I would argue that we must first understand the direct effects of the environment on the amphibian microbiome, before examining the potential interactions between environmental and genetic factors.

Latitudinal differences in microbiome of *R. pipiens* may also reflect differences in pathogen resistance among populations across the species' range. *R. pipiens* appear to

have declined in northern Ontario (Figure 1.5; Weller *et al*. 1994; Seburn & Seburn 1998), and this may have been caused by the emergence of chytridiomycosis (Gibbs *et al*. 1971; Carey *et al*. 1999; Rorabaugh 2005; Greer *et al*. 2005). If the bacterial community composition in the northern latitudes caused depressed immune function, this may have led to these declines (Oldham & Weller 1992). Conversely, the destructive presence of *Bd* in northern latitudes may have altered bacterial community composition, and the surviving *R. pipiens* populations represent microbial communities that may be considered the "Ghosts of Epizootics Past" (James *et al*. 2015). However in Chapter 2, I established that there was no relationship between *Bd* prevalence or intensity, and latitudinal gradient in 2012 or 2013 ($p = 0.9$; $p = 0.4$, respectively). Consequently, if chytridiomycosis did play a role in defining variation in bacterial community structure between northern and southern populations (either directly or indirectly), this occurred prior to 2012. I would suggest that future studies focus on quantifying the microbiomes of *R. pipiens* populations at similar latitudes in the presence and absence of *Bd,* repeated across a wide latitudinal range, in order to elucidate the roles that pathogen presence and environmental factors play in shifting bacterial community structure. This will allow us to fully characterize how latitude affects microbial colonization and persistence, within a system where *Bd* now seems to function endemically.

Inter-population differences, and the relationship between OTU diversity and site-level characteristics, suggest that variation in frog skin microbial communities may be due to the availability of colonizing microbes within the environment, rather than differences in frog physiology. This is supported by other studies reporting that land use and local environmental factors are the primary predictors of microbial communities in host and non host-associated environmental samples, such as soil and

water (Yao *et al*. 2000; Fierer & Jackson 2006; Lozupone & Knight 2007; Costello *et al*. 2009; Carrino-Kyker *et al*. 2011). Conversely, my results also suggest that amphibian thermoregulation alters bacterial communities. This result may be due to cooler host conditions: (1) inhibiting the growth of certain bacterial taxa (Matutte *et al*. 2000; Rollins-Smith *et al*. 2002; Ribas *et al*. 2009), or (2) extending the host intermoult interval (Meyer *et al*. 2012), thus allowing for interspecific competition among microbial species, selecting for the persistence and proliferation of dominant bacterial taxa (Vartoukian *et al*. 2010). Consequently, I would suggest that synergistic interactions between local environmental factors and frog physiology are likely to influence skin bacterial diversity and community structure in *R. pipiens*. Unfortunately, as individuals were not sampled multiple times per year, or across years, I lack the ability to estimate how the microbiome fluctuates in concert with individual thermoregulation history and seasonal fluctuations. Longo *et al*. (2015) observed significant seasonal changes in skin microbial communities of adult *Rana (Lithobates) yavapaiensis,* occurring in North America. The authors found that skin microbial communities increased in diversity from summer to winter, which was attributed to possible temperature-mediated changes in host immune function and/ or bacterial growth. Conversely, Kohl & Yahn (2016) reported that tadpoles reared at cool temperatures exhibited a significantly lower OTU richness in comparison to those reared at the warm temperatures. While these studies show that the directionality of this pattern is inconsistent, and the mechanism of thermal effects on microbial communities is still the subject of investigation, it is widely considered that temperature is a key factor in amphibian immune defenses. Interestingly, *Bd* dynamics also change seasonally (Kriger & Hero 2007b; Bosch *et al*. 2007; Ruggeri *et al*. 2015). By employing multiple observations of individuals across years, and

within both *Bd* infected and uninfected sites, future work will be able to focus on quantifying the degree to which the amphibian microbiome and pathogen presence fluctuates with seasonal dynamics, and how individual traits (e.g. thermoregulation), alter the strength of this effect.

My results show that the skin-associated bacterial community of *R. pipiens* does not differ between different age classes (metamorph, juvenile, and adult). This is a surprising result as amphibians undergo dramatic physical and biochemical changes during metamorphosis, such as immune system development, and keratinization of the skin tissue (Robinson & Heintzelman 1987; Faszewski & Kaltenbach 1995; Rollins-Smith 1998; Faszewski *et al*. 2008; Rollins-Smith *et al*. 2011). As such, one would expect recently metamorphosed individuals to exhibit significantly different skin bacterial communities as compared to adult individuals. Despite no significant differences between age classes, there was a trend for lower mean and lower variation of OTU richness found in metamorphs, in comparison to juveniles and adults (Figure 4.6). Thus, I would suggest that the non-significant relationship between OTU richness and age category is a result of small sample size. The sample size for metamorphs (n = 6) was substantially smaller than juveniles (n = 21) or adults (n = 57). This provides low statistical power and reduces the chance of detecting a true effect. Thus, snout-vent length (a continuous variable), provides a much more accurate recording of the variation observed in the data. This study demonstrates that snout vent length influences variation in frog microbiome structure across NMDS axis 1. Thus, the microbiome observed upon larger frogs from one population, was most similar to the microbiome on other large frogs from other populations (and the same for smaller individuals). However, as all individuals sampled within this study were post-metamorphic, I cannot suggest that the process of metamorphosis is wholly

responsible for the observed variation in bacterial community composition. A recent study on the direct-developing frog *Eleutherodactylus coqui* reported variation in alpha diversity amongst post-metamorphic individuals: OTU richness was significantly higher in juveniles than in adults (Longo *et al*. 2015). While my results do not suggest that small frogs exhibit greater OTU richness than large frogs (or vice versa), it does highlight significant variation in community structure between small and large frogs. I would suggest that this pattern is due to changing life histories as the individuals age. For example, dispersal for *R. pipiens* metamorphs is significantly lower than that observed in juveniles and adults (Dole 1965a, 1971; Merrell 1977; Seburn & Seburn 1997; Romanchuk & Quinlan 2006). Thus, older (and larger) *R. pipiens* will exhibit wider dispersal ranges, which may increase their encounter rate of additional colonizing microbes. Furthermore, research focusing on the human gut microbiome, have reported changes in gut microbial composition alongside age: with a high degree of variability at the two extremes of infancy and old age, punctuated by comparative stability during adulthood (for reviews, see Woodmansey *et al*. 2004; Woodmansey 2007; O'Toole & Claesson 2010). The gut of amphibians also harbors a diverse microbial community, and it has been suggested that this acts as a reservoir for skin-associated microbes (Kohl *et al*. 2013). Consequently, as amphibians senesce, their microbiome may become structurally altered due to physiological changes or variation in individual behaviour. Future research should sample individuals multiple times within a year and across years, in order to observe microbial shifts during the development of *R. pipiens*.

Due to the nondestructive sampling method (use of sterile cotton-tipped swabs) it seems likely that I explored the amphibian skin community superficially, i.e. the mucus and potentially only the top layer of amphibian skin. For example, despite

finding significant overlap of bacterial presence between human tissue layers, not all operational taxonomic units (OTUs) found in the skin are associated with the superficial layers (Grice *et al*. 2008). Thus, I may not have captured members of the bacterial community that specialize in deeper layers of amphibian tissue, such as granular or mucus glands. Furthermore, despite rinsing the individuals prior to sampling, it is still possible that not all of the bacteria obtained from the swab are skin symbionts. However, as I found a significant difference in α- and β-diversity between sites and individual groupings, it seems likely that a significant proportion of the sequences must be host-associated and not simply transient microbes from the environment.

4.5.2 Description of bacterial phyla and genera

Previous studies focusing on the bacteria located on amphibian skin, have observed: (1) the same phyla found in the present study, including: *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*, and (2) the same genera found in the present study, including: *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas* and *Psychrobacter* (Culp *et al*. 2007; Woodhams *et al*. 2007a; Lauer *et al*. 2007, 2008; Lam *et al*. 2010). Closer examination of the composition of the bacterial communities revealed that, for seven of the nine sites sampled in this study, *Pseudomonads* dominated their skin bacterial communities, while the remaining two sites exhibited *Sphingomonas* as their dominated genus. *Pseudomonas* is a large, ecologically diverse group, and members of this genus include known pathogens, common environmental bacteria, and commensals. Due to a wealth of culture-dependent work, including cultivation of amphibian-associated microbes, it is widely accepted that many *Pseudomonads* grow quickly and produce antimicrobials that can inhibit bacteria and fungi including *Bd*

(Lauer *et al*. 2007; Woodhams *et al*. 2007b). All sites exhibited significant abundance of *Pseudomonads*, which may help *R. pipiens* defend against pathogen establishment and maintain homeostasis through association and cultivation of pathogen-inhibiting microbes. *Stenotrophomonas* species are broadly present in the environment, including freshwater habitats, sewage, plankton samples, and soil (Piccini *et al*. 2006). They are known to cause nosocomial infections in human patients and have been found to be resistant to a broad-spectrum of antibiotics (Denton & Kerr 1998). *Stenotrophomonas* species have been isolated from amphibian skin (Woodhams *et al*. 2007b; Flechas *et al*. 2012), and an isolate from harlequin toads (*Atelopus elegans*) inhibited *Bd* growth in laboratory tests (Flechas *et al*. 2012). The abundant presence of both *Pseudomonas* and *Stenotrophomonas* genera throughout the study system may help to explain the relatively low *Bd* infection prevalence and intensity levels recorded in 2014, in comparison with 2012 and 2013 (Figure 2.3, Figure 2.4, Figure 2.6, Figure 2.7).

The two other more abundant genera found on the amphibians: *Sphingomonas* and *Psychrobacter* also match sequences of bacterial strains that are previously known as symbionts living with hosts. *Sphingomonas* (*Alphaproteobacteria*) is reported to be a symbiont with plant hopper insects (Tang *et al*. 2010) and is not known to produce anti-fungal compounds (CLSI 2006). In support of this latter comment, Becker and colleagues (2015b) reported that *Sphingomonas* was a dominant bacterial family located on dead, *Bd* positive Panamanian golden frogs (*Atelopus zeteki*). Despite the reported presence of *Sphingomonas* within several amphibian skin-microbiome studies (McKenzie *et al*. 2012; Longo *et al*. 2015; Becker *et al*. 2015b) very little is known regarding its life history, morphology and competitive dominance. *Psychrobacter* is part of the normal skin and gut microbiotas of fish (Ringø *et al*.

2006; Bowman 2006), and is known to inhibit the growth of *Saprolegnia australis* and *Mucorhiemalis*, two important aquatic fungal pathogens. The presence of this genus is rarely reported within amphibian microbiome studies. However, interestingly, Miller *et al*. 2009 found *Psychrobacter* to be prominent in larval amphibians inhabiting farm ponds in Tennessee. Within this study, *Psychrobacter* was only found to be abundant within two sites, ELM and MIN, both of which are located in close proximity to farming.

4.6 CONCLUSION

Amphibians have undergone dramatic disease-associated declines in recent years and these declines are expected to increase due to the ease of global transportation and introduction of novel diseases (Daszak *et al*. 2003). This hypothesized increase in pathogen introduction, coupled with changing climate and anthropogenic disturbance make understanding how the amphibian skin microbiome is altered by changing environments crucial for successful long-term conservation efforts (Lips *et al*. 2008; Rohr *et al*. 2008). I provide evidence that host-associated bacterial communities can be dynamic and that it is likely that their performance on the host's skin depends on environmental conditions and individual traits. Considering the variation in community structure of these bacterial communities across sites, I suggest that further characterization of the functions of these communities could be a promising area of research, to elucidate how bacterial diversity influences function across latitudes and environmental gradients.

As the study of the amphibian skin microbiome is relatively new, few conservation applications of this research are available, but the future possibilities are promising. The microbiome can be a useful tool throughout the conservation process, from

identifying populations of concern, to the management of captive populations. At present, conservation strategies related to the skin microbiome have focused on combatting *Bd,* by applying probiotic-based strategies (Rebollar *et al*. 2016a). We now know host microbiota can affect host phenotype and may partially explain variability in *Bd* infection susceptibility observed across hosts, as susceptibility may be, in part, microbially mediated (Harris *et al*. 2009; Bletz *et al*. 2013). However, Antwis *et al*. (2015) reported that only a small proportion of candidate probiotics exhibited broad-spectrum inhibition across *Bd* isolates. Furthermore, while some bacterial genera showed significantly greater inhibition than others, overall, bacterial genus and species were not particularly reliable predictors of *Bd* inhibitory capabilities. Thus, a bacterial consortium approach is likely to offer more comprehensive protection of hosts from *Bd* (Antwis *et al*. 2015). This suggests that increased OTU richness at the site-level may prove vital to future *Bd* mitigation strategies, as rare OTUs may contribute to the overall success of a bacterial consortia approach. In support of this statement, previous studies have reported that lowered bacterial diversity decreases defensive function to pathogens throughout a range of vertebrate and invertebrate hosts (Cariveau *et al*. 2014; Theriot *et al*. 2014; Kueneman *et al*. 2016). The role of bacterial community structure towards limiting pathogen invasion and proliferation is more difficult to consider: two amphibian populations may present equal bacterial richness while posing divergent bacterial community structures. Only by understanding functional redundancy in amphibian skin microbiomes, and elucidating the additive, antagonistic or synergistic effects of OTUs operating within a bacterial consortium, will we develop a deeper understanding of how community structure inhibits *Bd* establishment and growth. Studies of survivor populations hosting microbes known to exhibit and produce anti-pathogen compounds

may help to focus future research efforts, and support the concept that immunity provided by microbiota can function as an extended phenotype (Woodhams *et al*. 2007b; Rollins-Smith & Woodhams 2012). I suggest that ongoing work focuses on: (1) disentangling the contributive roles of the host and the environment, as this is key to understanding the process of microbial colonization and assembly in and on hosts, and (2) consider the context-dependent function of probiotic candidates within the range of local environmental conditions, in order to maximize the success of amphibian conservation efforts.

CHAPTER FIVE: SUMMARY, CONCLUSIONS AND PERSPECTIVES

.....

5.1 INTRODUCTION

The extensive and on-going worldwide loss of biodiversity has drawn increasing attention to the process of extinction (Soulé 1985; Wilcove *et al*. 1986). The Living Planet Index indicates a global population decline of 58 % in vertebrate species between 1970 and 2012 (McRae *et al*. 2016). However, this decline is not constant throughout vertebrate groups, as freshwater populations have declined by 81 %, marine populations by 36 %, and terrestrial populations by 38 % (McRae *et al*. 2016). Threats and pressures upon the natural world continue to increase (Dirzo *et al*. 2014; Tittensor *et al*. 2014) and species extinction rates are likely to rise around 10,000 times the background rate (De Vos *et al*. 2015). Extinction is more than the loss of a species. With the extinction of any species, we also lose unique features and detailed adaptations, which cannot be replaced. In the careless and excessive ways in which we have managed Earth's finite resources, the human species has pushed most other species to the side. We are losing whole swathes of irreplaceable evolutionary history, and certain ancient evolutionary lineages are struggling for survival (Baillie *et al*. 2010).

Modern amphibians are the oldest extant group of terrestrial vertebrates. They present vast diversity with 7400 + species presently known, and an estimated 8,000 - 12,000 in existence (Alford 2011). They occupy every terrestrial habitat except for Antarctica and the high Arctic, and have a vital role in the energy flows between terrestrial and freshwater systems, nutrient dynamics, and the control of pest insects.

However, among vertebrates, amphibians are in particularly serious decline worldwide (Blaustein & Kiesecker 2002). According to the International Union for Conservation of Nature (IUCN), amphibians have the highest proportion of threatened species, the highest proportion of data deficient species and the lowest proportion of least concern species among the vertebrate groups (Baillie *et al*. 2010).

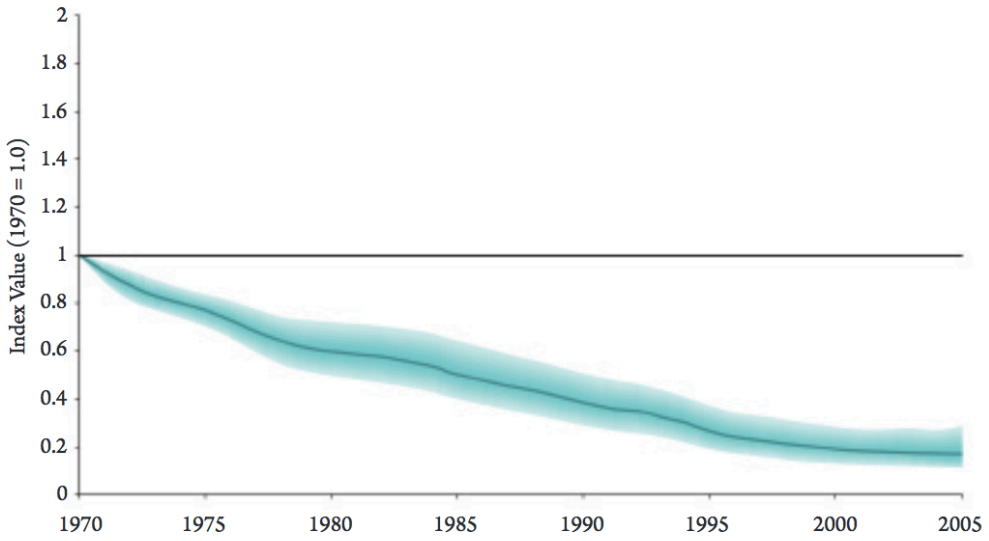


Figure 5.1: Living Planet Index for amphibians (357 populations of 162 species). Index value shown in bold line, shaded area shows 95 % confidence intervals. *(Figure: extracted from Baillie et al. 2010).*

The Global Amphibian Assessment (GAA; http://www. iucnredlist.org/amphibians) has shown that in recent decades, at least 43 % of amphibian species have declined, 41 % are globally threatened (Monastersky 2014), 37 species are extinct and an additional 88 species are possibly extinct (Stuart *et al*. 2004). Additionally, there is a great deal of supporting evidence to suggest that amphibians have experienced a major global population decline since he 1970s (Figure 5.1; Stuart *et al*. 2004; Bielby *et al*. 2008; Baillie *et al*. 2010). These figures highlight the rapid occurrence of declines and the immediate danger of extinction unless urgent action is taken.

Habitat destruction, modification and fragmentation are arguably the most ubiquitous threats to amphibians, threatening 75 % of amphibian species (Stuart *et al*. 2008). However, while approximately half of amphibian species exposed to habitat loss are not threatened species, species exposed to infectious disease are almost comprehensively at risk (Stuart *et al*. 2008). Accordingly, Emerging Infectious Diseases (EIDs) are recognized as important contributors to the global decline in amphibian species (Bielby *et al*. 2008).

Batrachochytrium dendrobatidis (*Bd*) is an archetypal emerging infectious disease that is highly infectious, has a broad host range and is pathogenic across a wide diversity of amphibian species (Olson *et al*. 2013). Since it was first described in the late 1990s (Berger *et al*. 1998; Longcore *et al*. 1999), *Bd* has been detected within 48 % of all localities surveyed (Olson *et al*. 2013), across 54 countries (Figure 5.2; Fisher *et al*. 2009), indicating an astonishingly rapid epidemic, unless the organism has long been enzootic and previously unrecognized. The pathogen is hypothesized to be moving between countries via the international trade of amphibians (Daszak *et al*. 2003; Mazzoni *et al*. 2003; Pasmans *et al*. 2004; Fisher & Garner 2007; Schloegel *et al*. 2009). Furthermore, outbreaks of fatal chytridiomycosis (the disease caused by *Bd*) have been attributed with causing the greatest disease-driven loss of biodiversity ever recorded (Fisher *et al*. 2012) and in some parts of the world, infection-based mortality has resulted in the loss of 40 % of all amphibian species (Crawford *et al*. 2010).

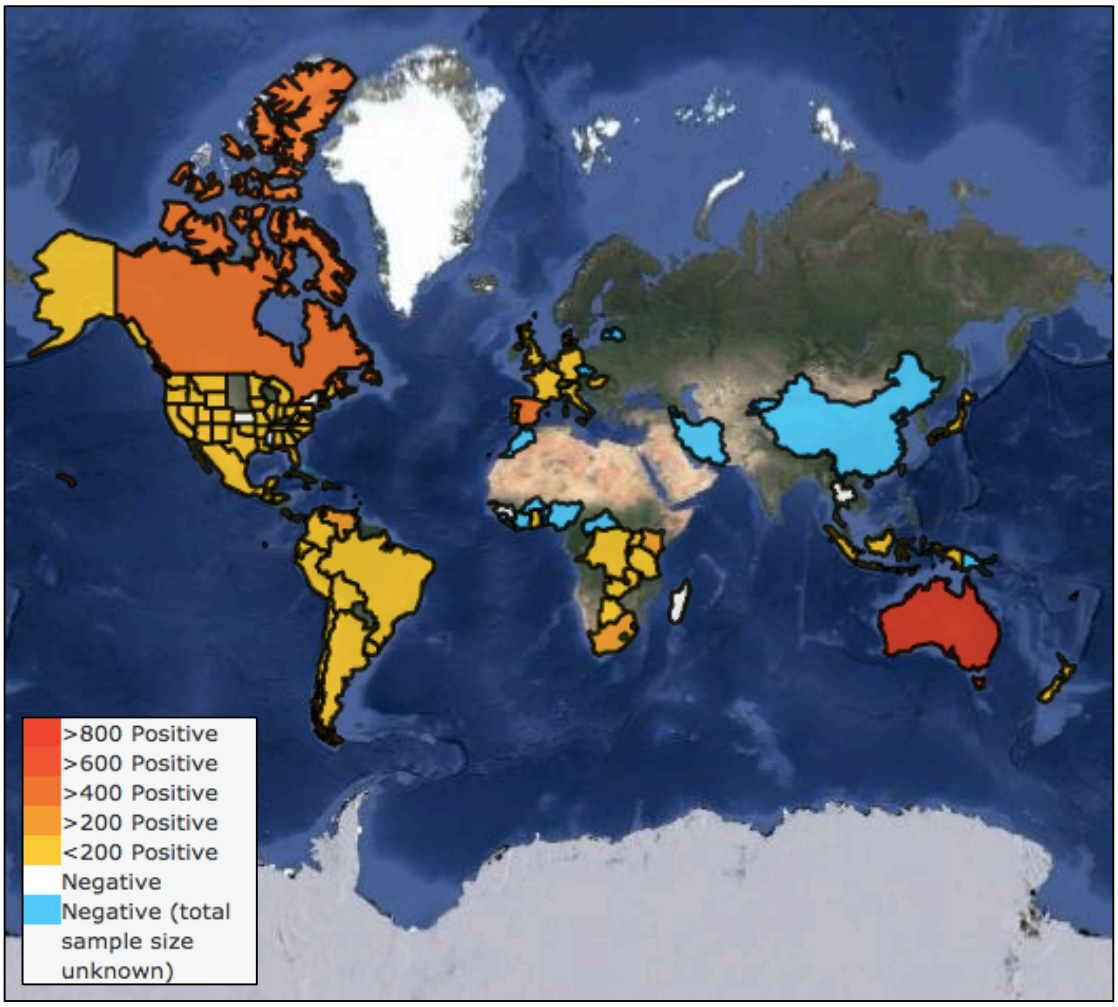


Figure 5.2: Global distribution of *Bd*. *(Figure: extracted from www.bd-maps.net; Fisher et al. 2009; Aanensen et al. 2012).*

5.2 MAIN FINDINGS

Despite the fact that *Bd* causes amphibian declines globally, host response is inconsistent, and *Bd* appears to be able to coexist with some amphibian species in a state of endemism (Bradley *et al*. 2002; Davidson *et al*. 2003; Daszak *et al*. 2004; Retallick *et al*. 2004; Woodhams & Alford 2005; Blaustein *et al*. 2005; Rachowicz *et al*. 2006; Puschendorf *et al*. 2011; Daskin & Alford 2012). It is widely accepted that the regional spread of *Bd* resembles an epidemic wave (Laurance *et al*. 1996, 1997). However, the results of this thesis do not support this statement. In Ontario, as in much of North America, infection is widespread but patchy, with no obvious spatial

clustering indicative of a single point of introduction or direction of spread. Furthermore, where *Bd* does occur, infection loads are weak, and prevalence is low. However, *Bd* does display substantial local interannual fluctuations of both prevalence and intensity of infection. Chapter 2 highlights that this is likely due to local climatic nuances altering the way in which a host operates within its environment, ultimately affecting pathogen persistence and proliferation. This suggests that the pathogen has long been present in Ontario, but has increased in pathogenicity because of environmental changes (Rachowicz *et al*. 2005). During years where conditions for pathogen survival, growth and transmission are favourable, i.e. cold moist climate prior to host breeding and heavy rainfall during the breeding period: *Bd* can establish, spread and proliferate rapidly within a susceptible population, especially when hosts harbour within a small well-connected waterbody. This is further supported by the fact that, as prevalence increases, so does intensity of infection. However, despite this apparent coupling, *Bd* prevalence and intensity of infection are governed, at least in part, by different environmental factors operating during different host life history events. Thus, Chapter 2 highlights the importance of: (i) measuring both infection parameters in order to gain insight into *Bd* infection dynamics; and (ii) examining the interaction between host life history patterns and environmental variables, when attempting to understand the impacts of *Bd* on an amphibian host.

As *Bd* infection load strongly determines the infectivity of individuals and the definitive disease outcome (Carey *et al*. 2006; Voyles *et al*. 2009), one would expect to see chytridiomycosis-driven mass mortality events and species declines throughout Ontario. However, although *Bd* infection has been reported (Ouellet *et al*. 2005; St-Amour *et al*. 2008; D'Aoust-Messier *et al*. 2015), and *Bd*-linked amphibian mortality

has been suggested (Carey *et al*. 1999), chytridiomycosis-driven declines have not yet been observed within Ontario. Several publications, have suggested that *R. pipiens* may have developed resistance to chytridiomycosis, and is thus acting as a reservoir or carrier species for *Bd* (Ouellet *et al*. 2005; Longcore *et al*. 2007; Woodhams *et al*. 2008b). Chapter 3 provides evidence for this statement, as *R. pipiens* were found to limit pathogen growth by maintaining an elevated body temperature through thermoregulation. Furthermore, the strength of this effect was modified by local climatic regimes, individual behaviour, and host phenotype. While there have been a handful of studies which demonstrate that individual thermal histories affect the probability of infection by *Bd* of frogs in nature (Richards-Zawacki 2010; Rowley & Alford 2013; Cohen *et al*. 2017), none have identified a mechanism in which amphibians achieve upper levels of thermal tolerance, thus increasing their probability of survival. This study is the first to demonstrate that individual phenotypic traits i.e. colouration and melanism, alter the survival of an amphibian host against *Bd*. However, as host thermoregulation is dependent upon ambient temperatures, the pathogen still has the potential to damage the host if ambient temperatures fall. Furthermore, individual behaviour (dispersal) may minimize *Bd* viability by exposing the pathogen to a more arid environment. However, this will not eradicate the pathogen entirely. Consequently, Chapter 3 provides evidence for a change in host resistance during the host-pathogen interaction, due to intrinsic changes in the host state.

Given that *Bd* can cause drastic amphibian declines, this could lead to a potential thermal arms race between the fungus and its amphibian host, and strong selection pressure on phenotype. The amphibian skin microbiome, which mediates *Bd* disease susceptibility (Woodhams *et al*. 2007a; Harris *et al*. 2009; Bletz *et al*. 2013) is also

temperature dependent (Daskin *et al*. 2014). Chapter 4 demonstrates that individual characteristics: frog body temperature and body size, influence bacterial community composition. As such, frog body temperature is interlaced in both infection dynamics (Chapter 3) and bacterial community composition (Chapter 4). Furthermore, as phenotype may constrain the range of body temperatures available to a host, individual variation in microbial community may be determined by host phenotype. Chapter 3 exhibits that percentage spot coverage increases with increasing latitude, and Chapter 4 exhibits that phylotype richness decreases with increasing latitude. Thus, perhaps frogs exhibiting a greater level of melanism have lower phylotype richness due to an increased ability to achieve upper levels of thermal tolerance and thus, preferentially favouring dominant taxa? Although I cannot test this relationship due to a lack of data, this is undoubtedly a promising area of future research. Chapter 4 also highlights that members of the same amphibian population exhibit more similar microbiomes than individuals across populations. This inter-population variation seems to be influenced by proximity to large urban environments and latitude. This suggests that different populations either select for a relatively specific microbiome, which may be cultivated and influenced by local environmental reservoirs (McKenzie *et al*. 2012; Kueneman *et al*. 2014; Walke *et al*. 2014), or local environmental conditions select for the persistence of particular bacterial taxa on the host skin (Vartoukian *et al*. 2010). In either case, site-level environmental heterogeneities may play a significant role in defining bacterial community structure and overall phylotype richness.

The overall aim of my thesis was to understand the site and individual levels drivers of pathogen persistence and proliferation in an amphibian species that appears to be coexisting with endemic chytridiomycosis. I aimed to gather data that would form an

evidence base, to clarify whether environmental heterogeneities have the potential to exacerbate or preclude infection load and pathogen establishment. In general, my results offer several explanations regarding the patchy distribution of *Bd* infection metrics observed in *R. pipiens* populations throughout Ontario, and highlight that: (i) infection outcome arises from the interaction between the ecology of the host and the surrounding abiotic and biotic environment, and (ii) *Bd* disease dynamics operate across nested levels of biological organization. Consequently, studies looking to gain insight into epidemiological processes of *Bd* must consider within-host up through population-level processes.

5.3 LIMITATIONS AND FUTURE RESEARCH

Site localities were initially chosen randomly, based on the known whereabouts of *R. pipiens* populations (Figure 2.2). However, due to difficult terrain and/ or limited road network, I was not able to sample all chosen site localities. Consequently, inaccessible sites were replaced with more accessible sites in close proximity. As a result, the reported prevalence and distribution of infected sites, together with any risk factors identified, might not be representative of Ontario as a whole. Sampling of amphibians was opportunistic and may have resulted in preferential selection of infected individuals, assuming an effect of infection on catch probability. Furthermore, all samples were collected exclusively from metamorphosed amphibians, and almost entirely from adults; however, *Bd* is known to affect hosts of different life stages in different ways. As a result, low levels of infection observed in the adult population may hugely underestimate the overall prevalence of *Bd,* and exclusion of larvae from this study has resulted in a significant knowledge gap.

Within this study system, I focused upon the ecology of the host, and the surrounding abiotic and biotic environment. I did not question the genotype and phenotype of the fungus (e.g. infectivity and virulence). Farrer *et al*. (2011) found that there is much greater diversity of *Bd* than was previously recognized. While the emergence and spread of chytridiomycosis is largely attributed to the globalization of the recently emerged hypervirulent recombinant lineage *Bd*-GPL, many enzootic lineages of *Bd* exist. For example, population genetics based on resequencing data indicated the existence of regional *Bd* lineages such as *Bd*-CAPE, which is likely specific to South Africa, and *Bd*-CH, which is likely specific to Switzerland (Farrer *et al*. 2011). Additionally, analyses of the rDNA internal transcribed spacer region (ITS) haplotypes exposed Asian *Bd* lineages in Japan (Goka *et al*. 2009), China (Bai *et al*. 2012), India (Dahanukar *et al*. 2013), and Korea (*Bd*-Korea; Bataille *et al*. 2013); and a Brazilian *Bd* lineage (*Bd*-Brazil), as well as a hybrid between *Bd*-GPL and *Bd*-Brazil (Schloegel *et al*. 2012). Such diversity challenges the notion of pathogen homogeneity. Enzootic genotypes may represent a pattern of historical genetic diversity that is in the process of being erased or outcompeted by the current panzootic. This is supported by infection experiments that demonstrate that strains of *Bd*-CAPE have lower virulence than the *Bd*-GPL (Farrer *et al*. 2011). However, due to a lack of research, I cannot generalize that certain lineages or genotypes are less virulent. Nevertheless, as I did not identify *Bd* lineage or genotype, there is a possibility that chytridiomycosis-related declines have not occurred in *R. pipiens* populations in Ontario, as the un-identified native strain is not particularly pathogenic (Gahl *et al*. 2012). More standardized studies are needed to quantify the pathogenicity of *Bd* genotypes across various hosts and continents (Kilpatrick *et al*. 2010; Langhammer *et al*. 2013). Other traits such as temperature optima, growth rates, and

morphology are worth investigating across *Bd* genotypes. For example, the optimum growth temperature of the newly described salamander parasite *Bsal* (Martel *et al*. 2013) is markedly lower (15 °C) than that of *Bd* (17 - 25 °C). Consequently, the growth rates and temperature optima for *Bd* strains may be vastly disparate.

Traditionally, epidemiologists have attempted to gain insight into the dynamics of a particular infection by focusing solely on the causative aetiological agent and the host, assuming no interaction with other parasites (Hassel & May 1973; Smyth & Smyth 1980; Clayton & Moore 1997). Yet, in natural systems, individual hosts often encounter multiple parasites concurrently, which can lead to additive, antagonistic, or synergistic effects on hosts (Petney & Andrews 1998; Bentwich *et al*. 1999; Jolles *et al*. 2008). These effects can increase infection prevalence and severity and have the potential to reduce host population size, as recently described in *Bison bison athabascae* (wood bison) affected by bovine tuberculosis and brucellosis (Joly & Messier 2005) and *Panthero leo* (African lions) impacted by canine distemper and babesiosis (Munson *et al*. 2008). Despite this fact, the significance of parasite interactions and the role that they may play in shaping host-parasite communities remain unclear. Rohani *et al*. (1998) proposed a mechanism that may contribute to interaction among unrelated acute infectious diseases: the 'interference model'. The mechanism is ecological (rather than immunological) and is based upon the temporary or permanent removal of potential hosts from the susceptible population for one parasite, following an acute infection by one of its direct competitors. This removal from the susceptible pool is predicted to strengthen the interaction between parasites. The significant prediction of the interference model (Rohani *et al*. 1998) is that the epidemics of competing infections would be temporarily segregated, with major outbreaks out of phase with each other. This may help to explain the substantial local

interannual fluctuations of both *Bd* prevalence and intensity of infection observed within my study system, as I have found evidence for the presence of two additional amphibian emerging infectious diseases within *R. pipiens* populations: *Ranavirus* (family *Iridoviridae,* Granoff *et al*. 1965; Rafferty 1965) and *Amphibiothecum* (formerly *Dermosporidium,* Densmore & Green 2007; Fiegna *et al*. 2016*)*. However, globally, there is a vast array of amphibian diseases such as: Red Leg Syndrome (*Bacterial Dermatosepticemia*; Pasteris *et al*. 2006, 2011); Flavobacteriosis (Olson *et al*. 1992; Green *et al*. 1999); Mycobacteriosis (Green 2001); Chlamydiosis (Bodetti *et al*. 2002); Lucke Herpesvirus (Lucké 1934; Lunger *et al*. 1965); Ranid Herpesvirus-2 (Essbauer & Ahne 2001); Saprolegniasis (Kiesecker *et al*. 2001); Mesomycetozoans (Pascolini *et al*. 2003; Feldman *et al*. 2005); Neoplasia (Green & Harshbarger 2001); and protozoan and metazoan parasites (Densmore & Green 2007). Individually, these diseases receive little attention in world of amphibian epidemiology. Furthermore, very few studies consider the distribution and spread of co-infecting amphibian emerging infectious diseases, or reflect upon the possible routes of disease interaction (however, see St-Amour *et al*. 2008; Schock *et al*. 2010; Kolby et al. 2014; D'Aoust-Messier *et al*. 2015; Warne *et al*. 2016; Rosa *et al*. 2017). Thus, systematic studies are required to elucidate the dynamical consequences of additive, antagonistic, or synergistic effects of multiple parasites on amphibian hosts in both the wild and laboratory settings.

Generally, epidemiological research suffers from a lack of long-term data on wildlife populations. This severely impedes the detection of epidemics, as there is inherent difficulty in separating population fluctuations from true declines (Adams *et al*. 2013). Baseline data need to be established, so that when declines and epizootics occur, there is a level for comparison. For example, as there was no long-term data

regarding my study area, I was not able to assess whether *R. pipiens* have declined since the discovery of regional *Bd*. However, baseline population and community data can be drawn from museum collections. Museum specimens not only yield locality records, but also allow for the development of genetic markers, which provide information regarding the introduction of particular pathogen genotypes, gene flow and a more precise migration history. Consequently, I would suggest that future studies utilize museum specimens to establish a baseline. Additionally, I believe it is the responsibility of the wildlife epidemiological community to release a publicly available standard operating procedure for preserving amphibian samples, in order to ensure the integrity of samples for pathogen DNA analysis and histology. This may be of particular importance in the future, as we continue to experience the adverse effects of climate change. The impacts of climate change on host and pathogen dynamics are expected to be particularly strong for ectotherms, as host metabolism and activity patterns are closely linked to environmental temperatures, which in turn, directly influence the establishment of the pathogen. Despite this fact, the role of climate change in the emergence of infectious diseases remains controversial (Daszak *et al*. 2000; Harvell *et al*. 2002; Lafferty 2009; Rohr *et al*. 2008, 2010). Museum and collector specimens may contribute significantly to long-term ecological studies that examine the consequences of climate-disease interactions within local communities, as changing climates could shift the balance from co-existence to significant mortality in some populations, but not in others. This knowledge will directly affect the framing and development of conservation efforts to mitigate infections in the future.

Finally, the next obvious step in this project would be to create a Species Distribution Model (SDM), predicting the distribution of *Bd* in geographic space, based on the known whereabouts of *R. pipiens* populations (Figure 2.2). However, SDMs tend to

identify regions of the world where we know *Bd* is already present at high prevalence. I suggest that this framework is inverted, and instead of considering 'hot-spots' i.e. areas of high infection risk, we consider 'cold-spots' i.e. areas where *Bd* is absent, or occurs at consistently low prevalence. By considering these areas, we can: (1) identify areas where *Bd* has never been, thus mapping the spread of the epidemic; and/ or (2) elucidate environmental conditions outside of *Bd*'s tolerance window, which raises questions regarding *Bd*'s physiological plasticity and propensity for local adaptation. Furthermore, if *Bd* was historically present but has recently faded out, we may: (3) identify amphibian populations that have evolved defensive mechanisms; and/ or (4) identify resistant amphibian species due to pathogen-driven selection. Thus, the existence of *Bd* cold spots will raise several important epidemiological questions, which will ultimately, allow us to aid targeting of control strategies.

REFERENCES

Aanensen, D. M., Spratt, B. G., Fisher, M. C., Olson, D. & Ronnenberg, K. (2012). *Bd-maps*. Available: http://www.bd-maps.net [accessed 2012 April 22].

Adama, D. B. & Beaucher, M. A. (2006). *Population monitoring and recovery of the northern leopard frog (Rana pipiens) in southeast British Columbia*. Report to the Columbia Basin fish and wildlife compensation program, Nelson, BC. 28 pages.

Adams, M. J., Miller, D. A., Muths, E., Corn, P. S., Grant, E. H. C., Bailey, L. L., Fellers, G. M., Fisher, R. N., Sadinski, W. J., Waddle, H. & Walls, S. C. (2013). Trends in amphibian occupancy in the United States. *PLoS ONE*, *8*(5): e64347.

Adolph, S. C. & Porter, W. P. (1993). Temperature, activity, and lizard life histories. *The American Naturalist*, 142(2): 273 - 295.

Agrawal, A. A., Laforsch, C. & Tollrian, R. (1999). Transgenerational induction of defences in animals and plants. *Nature,* 400: 60 - 63.

Alexander, H. M., Thrall, P. H., Antonovics, J., Jarosz, A. M. & Oudemans, P. V. (1996). Population dynamics and genetics of plant diseases: a case study of anther-smut disease. *Ecology,* 77(4): 990 - 996.

Alexander, H. M., Mauck, K. E., Whitfield, A. E., Garrett, K. A. & Malmstrom, C. M. (2014). Plant–virus interactions and the agro-ecological interface. *European Journal of Plant Pathology,* 138*:* 529 - 547.

Alexopoulos, C. J., Mims, C. W. & Blackwell, M. (1996). *Introductory mycology*. John Wiley & Sons, NY.

Alford, R. A. & Richards, S. J. (1999). Global amphibian declines: a problem in applied ecology. *Annual review of Ecology and Systematics*, 20: 133 - 165.

Alford, R. A. (2011) Ecology: bleak future for amphibians. *Nature,* 480: 461 - 462.

Alibardi, L. (2003). Adaptation to the land: the skin of reptiles in comparison to that of amphibians and endotherm amniotes. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, 298: 12 - 41.

Altizer, S., Harvell, D. & Friedle, E. (2003). Rapid evolutionary dynamics and disease threats to biodiversity. *Trends in Ecology and Evolution,* 18(11): 589 - 596.

Altizer, S., Otsfeld, R. S., Johnson, P. T., Kutz, S. & Harvell, C. D. (2013). Climate change and infectious diseases: from evidence to a predictive framework. *Science*, 341: 514 - 519.

Anagnostakis, S. L. (1987). Chestnut blight: the classical problem of an introduced pathogen. *Mycologia,* 79: 23 - 37.

Anderson, R. M. & May, R. M. (1978). Regulation and stability of host-parasite population interactions. I. Regulatory processes. *The Journal of Animal Ecology*, 47: 219 - 247.

Anderson, R. M. & May, R. M. (1981). The population dynamics of microparasites and their invertebrate hosts. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 291: 451 - 524.

Anderson, R. M. & May, R. M. (1986). The invasion, persistence and spread of infectious diseases within animal and plant communities. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 314(1167): 533 - 570.

Anderson, R. M. & May, R. M. (1991). *Infectious diseases of humans: dynamics and control*. Oxford University Press.

Antonovics, J. E. A. (1995). A generalized model of parasitoid, venereal, and vector-based transmission processes. *American Naturalist,* 145: 661 - 675.

Antwis R. E., Preziosi, R. F., Harrison, X. A. & Garner, T. W. J. (2015). Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of *Batrachochytrium dendrobatidis*. *Applied and Environmental Microbiology*, 81: 3706 - 3711.

Araújo, M. B. & Carsten, R. (2006). How does climate change affect biodiversity? *Science*, 313: 1396 - 1397.

Archie, E. A., Luikart, G. & Ezenwa, V. O. (2009). Infecting epidemiology with genetics: a new frontier in disease ecology. *Trends in Ecology and Evolution,* 24: 21 - 30.

Arnold, T. W. (2010). Uninformative parameters and model selection using Akaike's information criterion. *Journal of Wildlife Management*, 74: 1175 - 1178.

Bai, C., Liu, X., Fisher, M. C., Garner, T. W. J. & Li, Y. (2012). Global and endemic Asian lineages of the emerging pathogenic fungus *Batrachochytrium dendrobatidis* widely infect amphibians in China. *Diversity and Distributions*, 18(3): 307 - 318.

Bailey, M. T. & Coe, C. L. (1999). Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. *Developmental Psychobiology,* 35(2): 146 - 155.

Bailey, M. T., Dowd, S. E., Galley, J. D., Hufnagle, A. R., Allen, R. G. & Lyte, M. (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. *Brain, Behaviour and Immunity*, 25(3): 397 - 407.

Baillie, J. E. M., Griffiths, J., Turvey, S. T., Loh, J. & Collen, B. (2010). *Evolution lost: status and trends of the world's vertebrates*. Zoological Society of London, London.

Baláž, V., Kubečková, M., Civiš, P., Rozinek, R. & Vojar, J. (2013). Fatal chytridiomycosis and infection loss observed in captive toads infected in the wild. *Acta Veterinaria Brno*, 82(4): 351 - 355.

Bálint, M., Bartha, L., O'Hara, R. B., Olson, M. S., Otte, J., Pfenninger, M., Robertson, A. L., Tiffin, P. & Schmitt, I. (2015). Relocation, high-latitude warming and host genetic identity shape the foliar fungal microbiome of poplars. *Molecular Ecology*, 24: 235 - 248.

Balkenhol, N. & Waits, L. P. (2009). Molecular road ecology: exploring the potential of genetics for investigating transportation impacts on wildlife. *Molecular Ecology*, 18: 4151 - 4164.

Banning, J. L., Weddle, A. L., Wahl, G. W., Simon, M. A., Lauer, A., Walters, R. L. & Harris, R. N. (2008). Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum*. *Oecologia*, 156: 423 - 429.

Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N. & Salzman, N. (2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. *Infection and Immunity*, 76(3): 907 - 915.

Bartholomew, G. A. (1966). A field study of temperature relations in the Galapagos marine iguana. *Copeia*, 1966: 241 - 250.

Bataille, A., Fong, J. J., Cha, M., Wogan, G. O. U., Baek, H. J., Lee, H., Min, M. S. & Waldman, B. (2013). Genetic evidence for a high diversity and wide distribution of endemic strains of the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* in wild Asian amphibians. *Molecular Ecology*, 22(16): 4196 - 4209.

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1): 1 - 48.

Beasley, V. R., Faeh, S. A., Wikoff, B., Staehle, C., Eisold, J., Nichols, D., Cole, R., Schotthoefer, A. M., Greenwell, M. & Brown, L. E. (2005). Risk factors and declines in northern cricket frogs (*Acris crepitans*). In: Lannoo, M. (Ed.), *Amphibian declines: the conservation status of United States species*. University of California Press, Berkeley, Los Angeles, London, pp. 75 - 86.

Becker, C. G., Rodriguez, D., Toledo, L. F., Longo, A. V., Lambertini, C., Corrêa, D. T., Leite, D. S., Haddad, C. F. B. & Zamudio, K. R. (2014a). Partitioning the net effect of host diversity on an emerging amphibian pathogen. *Proceedings of the Royal Society B: Biological Sciences*, 281: 20141796.

Becker, M. H., Harris, R. N., Minbiole, K. P. C., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., Brucker, R. M., Domangue, R. J. & Gratwicke, B. (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *EcoHealth,* 8: 501 - 506.

Becker, M. H., Richards-Zawacki, C.L., Gratwicke, B. & Belden, L.K. (2014b). The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Biological Conservation*, 176, 199 - 206.

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., Minbiole, K. P. C., Harris, R. N., Belden, L. K. & Gratwicke, B. (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proceedings of the Royal Society B: Biological Science,* 282: 20142881.

Beebee, T. J. C. (1995). Amphibian breeding and climate. *Nature*, 374: 219 - 220.

Belden, L. & Harris, R. (2007). Infectious diseases in wildlife: the community ecology context. *Frontiers in Ecology and the Environment*, 5: 533 - 539.

Bell, G. & Collins, S. (2008). Adaptation, extinction and global change. *Evolutionary Applications*, 1: 3 - 16.

Bellard, C., Genovesi, P. & Jeschke, J. M. (2016). Global patterns in threats to vertebrates by biological invasions. *Proceedings of the Royal Society B: Biological Sciences*, 283(1823): 20152454.

Bentwich, Z., Kalinkovich, A., Weisman, Z., Borkow, G. & Beyers, N. (1999). Can eradication of helminthic infections change the face of AIDS and tuberculosis? *Immunology Today,* 20: 485 - 487.

Berga, M., Székely, A. J. & Langenheder, S. (2012). Effects of disturbance intensity and frequency on bacterial community composition and function. *PLoS ONE,* 7(5): e36959.

Berger, L., Speare, R., Daszak, P., Green, E. D., Cunningham, A. A., Goggin, C. L., Slocombe, R., Ragan, M. A., Hyati, A. D., McDonald, K. R., Hines, H. B., Lips, K. R., Marantelli, G. & Parkes, H. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America*, 95: 9031 - 9036.

Berger, L., Speare, R. & Hyatt, A. (1999). Chytrid fungi and amphibian declines: overview, implications and future directions. In: Campbell, A. (Ed.), *Declines and disappearances of Australian frogs*. Environment Australia, Canberra.

Berger, L. (2001). *Diseases in Australian frogs*. Thesis. School of Public Health and Tropical Medicine, James Cook University, Townsville, Australia.

Berger, L., Speare, R., Hines, H. B., Marantelli, G., Hyatt, A. D., McDonald, K. R., Skerratt, L. F., Olsen, V., Clarke, J. M., Gillespie, G., Mahony, M., Sheppard, N., Williams, C. & Tyler, M. J. (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Australian Veterinary Journal*, 82(7): 434 - 439.

Berger, L., Hyatt, A. D., Spear, R. & Longcore, J. E. (2005a). Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 68: 51 - 63.

Berger, L., Speare, R. & Skerratt, L. F. (2005b). Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. *Diseases of Aquatic Organisms*, 68: 65 - 70.

Berger, L., Roberts, A. A., Voyles, J., Longcore, J. E., Murray, K. A. & Skerratt, L. (2016). History and recent progress on chytridiomycosis in amphibians. *Fungal Ecology*, 19: 89 - 99.

Bielby, J., Cooper, N., Cunningham, A., Garner, T. W. G. & Purvis, A. (2008). Predicting susceptibility to future declines in the world's frogs. *Conservation Letters*, 1: 82 - 90.

Bielby, J., Bovero, S., Sotgiu, G., Tessa, G., Favelli, M., Angelini, C., Doglio, S., Clare, F. C., Gazzaniga, E., Lapietra, F. & Garner, T. W. J. (2009). Fatal chytridiomycosis in the Tyrrhenian Painted Frog. *EcoHealth*, 6: 27 - 32.

Bielby, J., Fisher, M. C., Clare, F. C., Rosa, G. M. & Garner, T. W. (2015). Host species vary in infection probability, sub-lethal effects, and costs of immune response when exposed to an amphibian parasite. *Scientific Reports*, 5: 10828.

Bishop, C. A., Mahony, N. A., Struger, J., Ng, P. & Pettit, K. E. (1999). Anuran development, density and diversity in relation to agricultural activity in the Holland River watershed, Ontario, Canada (1990 - 1992). *Environmental Monitoring and Assessment,* 57: 21 - 43.

Blanford, S., Thomas, M. B., Pugh, C. & Pell, J. K. (2003). Temperature checks the red queen? Resistance and virulence in a fluctuating environment. *Ecology Letters*, 6: 2 - 5.

Blaustein, A. R., Belden, L. K., Olson, D. H., Green, D. M., Root, T. L. & Kiesecker, J. M. (2001). Amphibian breeding and climate change. *Conservation Biology*, 15: 1804 - 1809.

Blaustein, A. R. & Kiesecker, J. M. (2002). Complexity in conservation: lessons from the global decline of amphibian populations. *Ecology Letters,* 5: 597 - 608.

Blaustein, A. R., Romansic, J. M., Scheessele, E. A., Han, B. A., Pessier, A. P. & Longcore, J. E. (2005). Interspecific variation in susceptibility of frog tadpoles to the parasitic fungus *Batrachochytrium dendrobatidis*. *Conservation Biology*, 19(5): 1460 - 1468.

Blehert, D. S., Hicks, A. C., Behr, M., Meteyer, C. U., Berlowski-Zier, B. M., Buckles, E. L., Coleman, J. T. H., Darling, S. R., Gargas, A., Niver, R., Okoniewski, J. C., Rudd, R. J. & Stone, W. B. (2009). Bat white-nose syndrome: an emerging fungal pathogen? *Science*, 323(5911): 227.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C. & Harris, R. N. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters*, 16(6): 807 - 820.

Bodetti, T. J., Jacobson, E. R., Wan, C., Hafner, L., Pospischil, A., Rose, K. & Timms, P. (2002). Molecular evidence to support the expansion of the host range of *Chlamydophila pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. *Systematic and Applied Microbiology*, 25: 146 - 152.

Bogert, C. M. (1949). Thermoregulation in reptiles, a factor in evolution. *Evolution*, 3(3): 195 - 211.

Bosch, J., Martínez-Solao, I. & García-Paris, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation*, 97: 331 - 337.

Bosch, J., Carrascal, L. M., Duran, L., Walker, S. & Fisher, M. C. (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society B: Biological Sciences*, 274: 253 - 260.

Bouwer, S., Ewer, D. W. & Shiff, C. (1953). Frequency of moulting in Anura. *Nature*, 172: 408 - 408.

Bowman, J. P. (2006). The genus Psychrobacter. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E. (Eds.), *The Prokaryotes*, 3rd edition, volume 3. Springer, New York, NY, pp. 920 - 930.

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. & Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, 60: 141 - 148.

Bradley, G. A., Rosen, P. C., Sredl, M. J., Jones, T. R. & Longcore, J. E. (2002). Chytridiomycosis in native Arizona frogs. *Journal of Wildlife Diseases*, 38(1): 206 - 212.

Brandmaier, A. M. (2015). pdc: an R package for complexity-based clustering of time Series. *Journal of Statistical Software*, 67(5).

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstick, J. & Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America*, 108(38): 16050 - 16055.

Bray, J. R. & Curtis, J. T. (1957). An ordination of upland forest communities of southern Wisconsin. *Ecological Monographs*, 27: 325 - 349.

Briggs, C. J., Vredenburg, V. T., Knapp, R. A. & Rachowicz, L. J. (2005). Investigating the population level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology*, 86(12): 3149 - 3159.

Briggs, C. J., Knapp, R. A. & Vredenburg, V. T. (2010). Enzootic and epizootic dynamics of the chytrid fungus pathogen of amphibians. *Proceedings of the National Academy of Sciences of the United States of America*, 107: 9695 - 9700.

Brodman, R. (2008). A 14-year study of amphibian populations and metacommunities. *Herpetological Conservation and Biology,* 4(1): 106 - 119.

Brooks, R. T. & Kyker-Snowman, T. D. (2008). Forest floor temperature and relative humidity following timber harvesting in southern New England, USA. *Forest Ecology and Management*, 254(1): 65 - 73.

Brunner, J. L., Schock, D. M., Davidson, E. W. & Collins, J. P. (2004). Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. *Ecology*, 85: 560 - 566.

Buckley, L. B., Hurlbert, A. H. & Jetz, W. (2012). Broad-scale ecological implications of ectothermy and endothermy in changing environments. *Global Ecology and Biogeography*, 21: 873 - 885.

Budtz, P. E. & Larsen, L. O. (1973). Structure of the toad epidermis during the moulting cycle. I. Light microscopic observations in *Bufo bufo* (L.). *Z Zellforsch Mikrosk Anat*, 144: 353 - 368.

Budtz, P. E. (1979). Epidermal structure and dynamics of the toad, *Bufo bufo*, deprived of the pars distalis of the pituitary gland. *Journal of Zoology*, 189: 57 - 92.

Bull, E. L. & Wales, B. C. (2001). Effects of disturbance on amphibians of conservation concern in eastern Oregon and Washington. *Northwest Science*, 75: 174 – 179.

Bull, J. J. (1994). Perspective: virulence. *Evolution,* 48: 1423 - 1437.

Burdon, J. J. & Thompson, J. N. (1995). Changed patterns of resistance in a population of *Linum marginale* attacked by the rust pathogen *Melampsora lini*. *Journal of Ecology*, 83: 199 - 206.

Burdon, J. J. & Thrall, P. H. (1999). Spatial and temporal patterns in coevolving plant and pathogen associations. *The American Naturalist*, S153: 15 - 33.

Burnham, K. P. & Anderson, D. R. (2002). *Model selection and multimodel inference: a practical information-theoretic approach*, 2nd edition. Springer-Verlag, New York.

Cannon, R. M. & Roe, R. T. (1982). *Livestock disease surveys – a field manual for veterinarians*. Australian Bureau of Animal Health, Canberra, Australia.

Carey, C. (1978). Factors affecting body temperatures of toads. *Oecologia*, 219: 197 - 219.

Carey, C., Cohen, N. & Rollins-Smith, L. (1999). Amphibian declines: an immunological perspective. *Developmental and Comparative Immunology,* 23: 459 - 472.

Carey, C. & Alexander, M. A. (2003). Climate change and amphibian declines: is there a link? *Diversity and Distributions*, 9: 111 - 121.

Carey. C., Bruzgul, J. E., Livo, L. J., Walling, M. L., Kuehl, K. A., Dixon, B. F., Pessier, A. P., Alfrod, R. A. & Rogers, K. B. (2006). Experimental exposures of boreal toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). *EcoHealth*, 3: 5 - 21.

Cariveau, D. P., Powell, J. E., Koch, H., Winfree, R. & Moran, N. A. (2014). Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*). *The ISME Journal*, 8(12): 2369 - 2379.

Carr, A. H., Amborski, R. L., Culley Jr, D. D. & Amborski, G. F. (1976). Aerobic bacteria in the intestinal tracts of bullfrogs (*Rana catesbeiana*) maintained at low temperatures. *Herpetologica*, 1976: 239 - 244.

Carr, L. W. & Fahrig, L. (2001). Effect of road traffic on two amphibian species of differing vagility. *Conservation Biology*, 15(4): 1071 - 1078.

Carrino-Kyker, S., Swanson, A. & Burke, D. (2011). Changes in eukaryotic microbial communities of vernal pools along an urban–rural land use gradient. *Aquatic Microbial Ecology*, 62: 13 - 24.

Carruthers, R. I., Larkin, T. S. & Firstencel, H. (1992). Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecology,* 73: 190 - 204.

Carroll, R. L., Irwin, J. & Green, D. M. (2005). Thermal physiology and the origin of terrestriality in vertebrates. *Zoological Journal of the Linnean Society*, 143(3): 345 - 358.

Catenazzi, A., Lehr, E., Rodriguez, L. O. & Vredenburg, V. T. (2011). *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper Manu National Park, southeastern Peru. *Conservation Biology,* 25: 382 - 391.

Chamaillé-Jammes, S., Massot, M., Aragón, P. & Clobert, J. (2006). Global warming and positive fitness response in mountain populations of common lizards *Lacerta vivipara*. *Global Change Biology*, 12(2): 392 - 402.

Chao, A. (1984). Non-parametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics*, 11: 265 - 270.

Chao, A., Colwell, R. K., Lin, C. W. & Gotelli, N. J. (2009). Sufficient sampling for asymptotic minimum species richness estimators. *Ecology*, 90(4): 1125 - 1133.

Chao, A., Chiu, C. H. & Jost, L. (2014). Unifying species diversity, phylogenetic diversity, functional diversity, and related similarity and differentiation measures through Hill numbers. *Annual Review of Ecology, Evolution, and Systematics*, 45: 297 - 324.

Chao, A. & Chiu, C. H. (2016). Bridging the variance and diversity decomposition approaches to beta diversity via similarity and differentiation measures. *Methods in Ecology and Evolution*, 7(8): 919 - 928.

Chatfield, M. W. H. & Richards-Zawacki, C. L. (2011). Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Diseases of Aquatic Organisms*, 94: 235 - 238.

Chazdon, R., Colwell, R., Denslow, J. & Guariguata, M. (1998). Statistical methods for estimating species richness of woody regeneration in primary and secondary rain forests of NE Costa Rica. In: Dallmeier, F. & Comiskey, J. (Eds.), *Forest biodiversity research, monitoring and modeling: conceptual background and old-world case studies*. Parthenon Publishing, Paris, pp. 285 - 309.

Chesson, P. (2012). Scale transition theory: its aims, motivations and predictions. *Ecological Complexity,* 10: 52 - 68.

Chestnut, T., Anderson, C., Popa, R., Blaustein, A. R., Voytek, M., Olson, D. H. & Kirshtein, J. (2014). Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. *PLoS ONE,* 9*:* e106790.

Chung, H., Pamp, S. J., Hill, J. A., Surana, N. K., Edelman, S. M., Troy, E. B., Reading, N. C., Villablanca, E. J., Wang, S., Mora, J. R., Umesaki, Y., Mathis, D., Benoist, C., Relman, D. A. & Kasper, D. L. (2012). Gut immune maturation depends on colonization with a host-specific microbiota. *Cell*, 149(7): 1578 - 1593.

Churchill, T. A. & Storey, K. B. (1995). Metabolic effects of dehydration on an aquatic frog, *Rana pipiens*. *Journal of Experimental Zoology*, 198: 147 - 154.

Clarke, B. T. (1997). The natural history of amphibian skin secretions, their normal functioning and potential medical applications. *Biological Reviews*, 72(3): 365 - 379.

Clarke, K. R. & Warwick, R. M. (2001). *Change in marine communities: an approach to statistical analysis and interpretation*, 2nd edition. PRIMER-E: Plymouth.

Clayton, D. H. & Moore, J. (1997). *Host-parasite evolution: general principles and avian models*. Oxford University Press, England, p. 473.

Cleaveland, S., Laurenson, M. K. & Taylor, L. H. (2001). Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 356(1411): 991 - 999.

CLSI Clinical Laboratory Standards Institute (2006). *Performance standards for antimicrobial disk susceptibility tests*, 9th edition. Wayne, PA: Clinical Laboratory Standards Institute.

Cohen, J. M., Venesky, M. D., Sauer, E. L., Civitello, D. J., McMahon, T. A., Roznik, E. A. & Rohr, J. R. (2017). The thermal mismatch hypothesis explains host susceptibility to an emerging infectious disease. *Ecology Letters*, 20(2): 184 - 193.

Collins, J. P. & Storfer, A. (2003). Global amphibian declines: sorting the hypotheses. *Diversity and Distributions*, 9: 89 - 98.

Collins, S. M. & Bercik, P. (2013). Gut microbiota: intestinal bacteria influence brain activity in healthy humans. *Nature Reviews, Gastroenterology and Hepatology,* 10(6): 326 - 327.

Coltman, D. W., Pilkington, J. G., Smith, J. A. & Pemberton, J. M. (1999). Parasite-mediated selection against inbred Soay sheep in a free-living island population. *Evolution,* 53: 1259 - 1267.

Colwell, R. K. & Coddington, J. A. (1994). Estimating terrestrial biodiversity through extrapolation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 345: 101 - 118.

Conant, R. & Collins, J. T. (1991). *A field guide to reptiles and amphibians: Eastern and Central North America*, 3rd edition. Houghton Mif in Company, Boston, MA, p. 450.

Corn, P. S. & Fogleman, J. C. (1984). Extinction of montane populations of the northern leopard frog (*Rana pipiens*) in Colorado. *Journal of Herpetology*, 18(2): 147 - 152.

COSEWIC: Committee on the Status of Endangered Wildlife in Canada (2009). *COSEWIC assessment and update status report on the northern leopard frog Lithobates pipiens, rocky mountain population, western boreal/prairie populations and eastern populations, in Canada*. Committee on the status of endangered wildlife in Canada, Ottawa, p. 69.

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I. & Knight, R. (2009). Bacterial community variation in human body habitats across space and time. *Science*, 326: 1694 - 1697.

Cramp, R. L., McPhee, R. K., Meyer, E. A., Ohmer, M. E. & Franklin, C. E. (2014). First line of defence: the role of sloughing in the regulation of cutaneous microbes in frogs. *Conservation Physiology*, 2(1): cou012.

Crawford, A.J., Lips, K.R. & Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences of the United States of America*, 107: 13777 - 13782.

Croswell, A., Amir, E., Teggatz, P., Barman, M. & Salzman, N. H. (2009). Prolonged impact of antibiotics on intestinal microbial ecology and susceptibility to enteric Salmonella infection. *Infection and Immunity*, 77(7): 2741 - 2753.

Culloty, S., Cronin, M. A. & Mulcahy, M. F. (2001). An investigation into the relative resistance of Irish flat oysters *Ostrea edulis* L. to the parasite *Bonamia ostreae* (Pichot *et al*. 1980). *Aquaculture*, 199: 229 - 244.

Culp, C. E., Falkinham III, J. O. & Belden, L. K. (2007). Identification of the natural bacterial microflora on the skin of eastern newts, bullfrog tadpoles and redback salamanders. *Herpetologica*, 63(1): 66 - 71.

Cushman, S. A. (2006). Effects of habitat loss and fragmentation on amphibians: a review and prospectus. *Biological Conservation*, 128(2): 231 - 240.

Dahanukar, N., Krutha, K., Paingankar, M. S., Padhye, A. D., Modak, N. & Molur, S. (2013). Endemic Asian chytrid strain infection in threatened and endemic anurans of the northern Western Ghats, India. *PLoS ONE*, 8(10): e77528.

D'Amen, M. & Bombi, P. (2009). Global warming and biodiversity: evidence of climate-linked amphibian declines in Italy. *Biological Conservation*, 142: 3060 - 3067.

D'Aoust-Messier, A. M., Echaubard, P., Billy, V. & Lesbarrères, D. (2015). Amphibian pathogens at northern latitudes: presence of chytrid fungus and ranavirus in northeastern Canada. *Diseases of Aquatic Organisms*, 113(2): 149 - 155.

Daskin, J. H. & Alford, R. A. (2012). Context-dependent symbioses and their potential roles in wildlife diseases. *Proceedings of the Royal Society B: Biological Sciences*, 279(1733): 1457 - 1465.

Daskin, J. H., Bell, S. C., Schwarzkopf, L. & Alford, R.A. (2014). Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians - implications for disease management and patterns of decline. *PLoS ONE*, 9: e100378.

Daszak, P., Berger, L., Cunningham, A. A., Hyatt, A. D., Green, D. E. & Speare, R. (1999). Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases,* 5(6): 735 - 748.

Daszak, P., Cunningham, A. A. & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife: threats to biodiversity and human health. *Science*, 287: 443 - 449.

Daszak, P., Cunningham, A. A. & Hyatt, A. D. (2003). Infectious disease and amphibian population declines. *Diversity and Distributions*, 9: 141 - 150.

Daszak, P., Strieby, A., Cunningham, A. A., Longcore, J. E., Brown, C. C. & Porter, D. (2004). Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetological Journal*, 14: 201 - 207.

Daszak, P., Scott, D. E., Kilpatrick, A. M., Faggioni, C., Gibbons, J. W. & Porter, D. (2005). Amphibian population declines at Savannah River site are linked to climate, not chytridiomycosis. *Ecology,* 86: 3232 - 3237.

Davidson, E. W., Parris, M., Collins, J. P., Longcore, J. E., Pessier, A. P. & Brunner, J. (2003). Pathogenicity and transmission of chytridiomycosis in tiger salamanders (*Ambystoma tigrinum*). *Copeia*, 3: 601 - 607.

De Castro, F. & Bolker, B. (2005). Mechanisms of disease-induced extinction. *Ecology Letters*, 8: 117 - 126.

De Jong, M. C. M. & Janss, L. L. G. (2002). Virulence management in veterinary epidemiology. In: Dieckmann, U., Metz, J. A. J., Sabelis, M. W. & Sigmund, K. (Eds.), *Adaptive dynamics of infectious diseases: in pursuit of virulence management*. Cambridge University Press, pp. 425 - 435.

De Vos, J. M., Joppa, L. N., Gittleman, J. L., Stephens, P. R. & Pimm, S. L. (2015). Estimating the normal background rate of species extinction. *Conservation Biology*, 29(2): 452 - 462.

Densmore, C. L. & Green, D. E. (2007). Diseases of Amphibians. *Institute of Laboratory Animal Resources Journal*, 48(3): 235 - 254.

Denton, M. & Kerr, K. G. (1998). Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. *Clinical Microbiology Reviews*, 11(1): 57 - 80.

Deyrup, I. J. (1964). Water balance and kidney. In: Moore, J. A. (Ed.), *Physiology of the amphibia*. Academic Press, New York, pp. 251 - 328.

Dieckmann, U., Metz, J. A. J., Sabelis, M. W. & Sigmund, K. (2002). *Adaptive dynamics of infectious diseases: in pursuit of virulence management*. Cambridge University Press.

Dirzo, R., Young, H. S., Galetti, M., Ceballos, G., Isaac, N. J. & Collen, B. (2014). Defaunation in the Anthropocene. *Science*, 345(6195): 401 - 406.

Dobson, A. & Crawley, M. (1994). Pathogens and the structure of plant communities. *Trends in Ecology and Evolution*, 9: 393 - 398.

Doddington, B. J., Bosch, J., Oliver, J. A., Grassly, N. C., Garcia, G., Schmidt, B. R., Garner, T. W. J. & Fisher, M. C. (2013). Context-dependent amphibian host population response to an invading pathogen. *Ecology,* 94: 1795 - 1804.

Dole, J. W. (1965a). Spatial relations in natural populations of the leopard frog, *Rana pipiens* Schreber, in northern Michigan. *American Midland Naturalist,* 74: 464 - 478.

Dole, J. W. (1965b). Summer movements of adult leopard frogs, *Rana pipiens* Schreber, in Northern Michigan. *Ecology*, 46: 236 - 255.

Dole, J. W. (1967). The role of substrate moisture and dew in the water economy of leopard frogs, *Rana pipiens*. *Copeia*, 1967: 141 - 149.

Dole, J. W. (1968). Homing in leopard frogs, *Rana pipiens*. *Ecology*, 49: 386 - 399.

Dole, J. W. (1971). Dispersal of recently metamorphosed leopard frogs, *Rana pipiens*. *Copeia*, 2: 221 - 228.

Dube, D., Kim, K., Alker, A. P. & Harvell, D. (2002). Size structure and geographic variation in chemical resistance of sea fan corals *Gorgonia ventalina* to a fungal pathogen. *Marine Ecology Progress Series*, 231: 139 - 150.

Duellman, W. E. & Trueb, L. (1986). *Biology of amphibians*. McGraw-Hill, New York.

Dunn, R. R., Davies, T. J., Harris, N. C. & Gavin, M. C. (2010). Global drivers of human pathogen richness and prevalence. *Proceedings of the Royal Society B: Biological Sciences*, 277: 2587 - 2595.

Dwyer, G., Elkinton, J. S. & Buonaccorsi, J. P. (1997). Host heterogeneity and the dynamics of infectious disease: tests of a mathematical model. *The American Naturalists*, 150: 685 - 707.

Dybdahl, M. F. & Lively, C. M. (1998). Host parasite coevolution: evidence for rare advantage and time-lagged selection in a natural population. *Evolution*, 52: 1057 - 1066.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changon, S. A., Karl, T. R. & Mearns, L. O. (2000). Climate extremes: observations, modeling, and impacts. *Science*, 289(5487): 2068 - 2074.

Echaubard, P., Leduc, J., Pauli, B., Chinchar, G., Robert, J. & Lesbarrères, D. (2014). Environmental dependency of amphibian-ranavirus genotypic interactions: evolutionary processes and applied perspectives. *Evolutionary Applications*, 7: 723 - 733.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics,* 27: 2194 - 2200.

Elliot, S. L., Sabelis, M. W., Janssen, A., van der Geest, L. P. S., Beerling, E. A. M. & Fransen, J. J. (2000). Can plants use entomopathogens as bodyguards? *Ecology Letters,* 3: 228 - 235.

Epstein, P. (2001). Climate change and emerging infectious diseases. *Microbes and Infection,* 3: 747 - 754.

Essbauer, S. & Ahne, W. (2001). Viruses of lower vertebrates. *Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Pubic Health*, 48: 401 - 476.

Ewald, P. W. (1994). *Evolution of infectious diseases*. Oxford University Press.

Farrell, M. P. & MacMahon, J. A. (1969). An eco-physiological study of water economy in 8 species of tree frogs (*Hylidae*). *Herpetologica,* 25: 279 - 294.

Farrer, R. A., Weinert, L. A., Bielby, J., Garner, T. W. J., Balloux, F., Clare, F., Bosch, J., Cunningham, A. A., Weldon, C., du Preez, L. H., Anderson, L., Kosakovsky Pond, S. L., Shahar-Golan, R., Henk, D. A. & Fisher, M. C. (2011). Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proceedings of the National Academy of Sciences of the United States of America*, 108(46): 18732 - 18736.

Faszewski, E. E. & Kaltenbach, J. C. (1995). Histology and lectin-binding patterns in the skin of the terrestrial horned frog *Ceratophrys ornata*. *Cell and Tissue Research*, 281: 169 - 177.

Faszewski, E. E., Tyrell, A., Guin, S. & Kaltenbach, J. C. (2008). Metamorphic changes in localization of sugars in skin of the leopard frog, *Rana pipiens*. *Journal of Morphology*, 269: 998 - 1007.

Feldman, S. H., Wimsatt, J. H. & Green, D. E. (2005). Phylogenetic classification of the frog pathogen *Amphibiothecum (Dermosporidium) penneri* based on small ribosomal subunit sequencing. *Journal of Wildlife Diseases,* 41: 701 - 706.

Fellers, G. M., Green, D. E. & Longcore, J. E. (2001). Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia*, 2001: 945 - 953.

Fenner, F. & Fantini, B. (1999). *Biological control of vertebrate pests: the history of Myxomatosis – an experiment in evolution*. Wallingford, UK: CAB International.

Fernández-Beaskoetxea, S., Bosch, J. & Bielby, J. (2016). Infection and transmission heterogeneity of a multi-host pathogen (*Batrachochytrium dendrobatidis*) within an amphibian community. *Diseases of Aquatic Organisms*, 118(11): 11 - 20.

Fiegna, C., Clarke, C. L., Shaw, D. J., Baily, J. L., Clare, F. C., Gray, A., Garner, T. W. & Meredith, A. L. (2016). Pathological and phylogenetic characterisation of Amphibiothecum sp. Infection in an isolated amphibian (*Lissotriton helveticus*) population on the island of Rum (Scotland). *Parasitology*, 1 - 13.

Fierer, N. & Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America*, 103: 626 - 631.

Fineblum, W. L. & Rausher, M. D. (1995). Trade-off between resistance and tolerance to herbivore damage in a morning glory. *Nature*, 377: 517 - 520.

Fisher, M. C. & Garner, T. W. J. (2007). The relationship between the emergence of *Batrachochytrium dendrobatidis,* the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews*, 21: 2 - 9.

Fisher, M. C., Garner, T. W. J. & Walker, S.F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual Review of Microbiology*, 63: 291 - 310.

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L. & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, 484(7393): 186 - 194.

Fitzpatrick, B. M. & Allison, A. L. (2014). Similarity and differentiation between bacteria associated with skin of salamanders (*Plethodon jordani*) and free-living assemblages. *FEMS Microbiology Ecology*, 88: 482 - 494.

Flechas, S. V., Sarmiento, C., Cárdens, M E., Medina, E. M., Restrepo, S. & Amézquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS ONE,* 7(9): e44832.

FLUKE Corporation (2009). *SureGrip immersion temperature probe instruction manual*. FLUKE Corporation, Everett, USA.

Fogleman, J. C., Corn, P. S. & Pettus, D. (1980). The genetic basis of color polymorphism in *Rana pipiens*. *Journal of Heredity*, 71: 439 - 440.

Fornoni, J., Núñez-Farfán, J., Valverde, P. L. & Rausher, M. (2004). Evolution of mixed strategies of plant defense allocation against natural enemies. *Evolution*, 58: 1685 - 1695.

Forsman, A. (1995). Heating rates and body temperature variation in melanistic and zigzag *Vipera berus*: does color make a difference? *Annales Zoologici Fennici*, 32: 365 - 374.

Forsman, A. (1997). Thermal capacity of different colour morphs in the pygmy grasshopper *Tetrix subulata*. *Annales Zoologici Fennici*, 34(3): 145 - 149.

Forsman, A. & Appelqvist, S. (1998). Visual predators impose correlational selection on prey color pattern and behaviour. *Behavioural Ecology*, 9: 409 - 413.

Fournier, D. A., Skaug, H. J., Ancheta, J., Ianelli, J., Magnusson, A., Maunder, M., Nielsen, A. & Sibert, J. (2012). AD Model Builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. *Optimization Methods and Software*, 27(2): 233 - 249.

Frank, S. A. (1996). Models of parasite virulence. *The Quarterly Review of Biology,* 71: 37 - 78.

Freed, A. N. (1980). An adaptive advantage of basking behavior in an anuran amphibian. *Physiological Zoology*, 53(4): 433 - 444.

Funk, S. M., Fiorello, C. V., Cleaveland, S., Gompper, M. E. (2001). The role of disease in carnivore ecology and conservation. In: Gittleman, J. L., Funk, S., Macdonald, D. & Wayne, R. K. (Eds.), *Carnivore conservation*. Cambridge University Press, pp. 241 - 281.

Gabor, C. R., Fisher, M. C. & Bosch, J. (2015). Elevated corticosterone levels and changes in amphibian behaviour are associated with *Batrachochytrium dendrobatidis* (*Bd*) infection and *Bd* lineage. *PLoS ONE*, 8(2): e5605410.

Gaffney, P. & Bushak, D. (1996). Genetic aspects of disease resistance in oysters. *Journal of Shellfish Research*, 15: 135 - 140.

Gahl, M. K., Longcore, J. E. & Houlahan, J. E. (2012). Varying responses of Northeastern North American amphibians to the chytrid pathogen *Batrachochytrium dendrobatidis*. *Conservation Biology,* 26: 135 - 141.

Garcia, T. S., Straus, R. & Sih, A. (2003). Temperature and ontogenetic effects on color change in the larval salamander species *Ambystoma barbouri* and *Ambystoma texanum*. *Canadian Journal of Zoology*, 715: 710 - 715.

Garcia, T. S., Romansic, J. M. & Blaustein, A. R. (2006). Survival of three species of anuran metamorphs exposed to UV-B radiation and the pathogenic fungus *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 72: 163 - 169.

García-Ramos, G., Dunoyer, L. A., Sasser, K. L. & Crowley, P. H. (2015). Evolution of resistance by a native competitor can lead to invasion collapse in disease-mediated invasions. *Biological Invasions*, 17(10): 2863 - 2879.

Garcia-Rodenas, C. L., Bergonzelli, G. E., Nutten, S., Schumann, A., Cherbut, C., Turini, M., Ornstein, K., Rochat, F. & Corthésy-Theulaz, I. (2006). Nutritional approach to restore impaired intestinal barrier function and growth after neonatal stress in rats. *Journal of Pediatric Gastroenterology and Nutrition*, 43(1): 16 - 24.

Garmyn, A., Van Rooij, P., Pasmans, F., Hellebuyck, T., Van Den Broeck, W., Haesebrouck, F. & Martel, A. (2012). Waterfowl: potential environmental reservoirs of the chytrid fungus *Batrachochytrium dendrobatidis*. *PLoS ONE*, 7(4): e35038.

Garner, T. W. J., Perkins, M., Govindarajulu, P., Seglie, D., Walker, S., Cunningham, A. A. & Fisher, M. C. (2006). The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biology Letters,* 2: 455 - 459.

Gascon, C., Collins, J. P., Moore, R. D., Church, D. R., McKay, J. E. & Mendelson. J. R. (2007). *Amphibian Conservation Action Plan (ACAP)*. Proceedings of the IUCN.SSC Amphibian Conservation Summit 2005 *(*World Conservation Union, Gland, Switzerland). Available: http://intranet.iucn.org/webfiles/doc/SSC/SSCwebsite/GAA/ACAP_Summit_Declaration.pdf [accessed 2009 November 1].

Gates, D. M. (1980). *Biophysical ecology*. Springer-Verlag, New York, NY.

Geiser, D. M., Taylor, J. W., Ritchie, K. B. & Smith, G. W. (1998). Cause of sea fan death in the West Indies. *Nature*, 394: 137 - 138.

Gelman, A. & Rubin, D. B. (1992). Inference from iterative simulation using multiple sequences. *Statistical Science*, 7(4): 457 - 472.

Gelman, A. & Hill, J. (2007). *Data analysis using regression and multilevel/ hierarchical models*. Cambridge University Press.

Gendron, A. D., Bishop, C. A., Fortin, R. & Hontela, A. (1997). In vivo testing of the functional integrity of the corticosterone-producing axis in mudpuppy (*amphibia*) exposed to chlorinated hydrocarbons in the wild. *Environmental Toxicology and Chemistry*, 16(8): 1694 - 1706.

Gibbs, E. L., Nace, G. W. & Emmons, M. B. (1971). The live frog is almost dead. *BioScience,* 21: 1027 - 1034.

Gibbs, J. P. (1998). Amphibian movements in response to forest edges, roads, and streambeds in southern New England. *The Journal of Wildlife Management,* 62(2): 584 - 589.

Gibbs, J. P. & Breisch, A. R. (2001). Climate warming and calling phenology of frogs near Ithaca, New York, 1900 - 1999. *Conservation Biology*, 15: 1175 - 1178.

Gibson, A. R. & Falls, B. (1979). Thermal biology of the common garter snake *Thamnophis sirtalis* (L.). II. The effects of melanism. *Oecologia*, 43: 99 - 109.

Gilbert, M., Leclair, R. & Fortin, R. (1994). Reproduction of the northern leopard frog (*Rana pipiens*) in floodplain habitat in the Richelieu River, P. Quebec, Canada. *Journal of Herpetology*, 28: 465 - 470.

Gilbertson, M-K., Haffner, G. D., Drouillard, K. G., Albert, A. & Dixon, B. (2003). Immunosuppression in the Northern Leopard Frog (*Rana pipiens*) induced by pesticide exposure. *Environmental Toxicology and Chemistry*, 22(1): 101 - 110.

Gilchrist, G.W. (1996). A quantitative genetic analysis of thermal sensitivity in the locomotor performance curve of *Aphidius ervi*. *Evolution*, 50: 1560 - 1572.

Gilman, S. E., Urban, M. C., Tewksbury, J., Gilchrist, G. W. & Holt, R. D. (2010). A framework for community interactions under climate change. *Trends in Ecology and Evolution*, 25(6): 325 - 331.

Gleason, F. H., Kagami, M., Lefevre, E. & Sime-Ngando, T. (2008). The ecology of chytrids in aquatic ecosystems: roles in food web dynamics. *Fungal Biology Reviews,* 22: 17 - 25.

Goka, K., Yokoyama, J. U. N., Une, Y., Kuroki, T., Suzuki, K., Nakahara, M., Kobayashi, A., Inaba, S., Mizutani, T. & Hyatt, A. D. (2009). Amphibian chytridiomycosis in Japan: distribution, haplotypes and possible route of entry into Japan. *Molecular Ecology*, 18(23): 4757 - 4774.

Gómez-Rubio, V., Ferrandiz-Ferragud, J. & Loópez-Quílez, A. (2015). *Package 'DCluster'; functions for the detection of spatial clusters of diseases*. R package. Available: https://cran.r-project.org/web/packages/DCluster/DCluster.pdf [accessed: 2016 January 3].

Gossling, J., Oesche, W. J. L. & Ace, G. W. N. (1982). Large intestine bacterial flora of nonhibernating and hibernating leopard frogs (*Rana pipiens*). *Applied and Environmental Microbiology,* 44: 59 - 66.

Government of Canada (2015). *National Road Network (NRN) - Ontario*. Available: http://geogratis.gc.ca/api/en/nrcan-rncan/ess-sst/c0db1b2e-bdaf-6998-8b8e-e569e5d39d6b.html [accessed: 2015 June 10].

Government of Canada (2016). *Historical climate data*. Available: http://climate.weather.gc.ca/historical_data/search_historic_data_e.html [accessed: 2015 June 10].

Gower, D. J., Doherty-Bone, T., Loader, S. P., Wilkinson, M., Kouete, M. T., Tapley, B., Orton, F., Daniel, O. Z., Wynne, F., Flach, E., Müller, H., Menegon, M., Stephen, I., Browne, R. K., Fisher, M, C., Cunningham, A. A., Garer, T. W. J. (2013). *Batrachochytrium dendrobatidis* infection and lethal chytridiomycosis in caecilian amphibians (*Gymnophiona*). *EcoHealth,* 10(2): 173 - 183.

Granoff, A., Came, P. E. & Rafferty, K. A. (1965). The isolation and properties of viruses from *Rana pipiens*: their possible relationship to the renal adenocarcinoma of the leopard frog. *Annals of the New York Academy of Sciences,* 126: 237 - 255.

Green, D. E. (2001). Pathology of amphibia. In: Wright, K. M., Whitaker, B. R. (Eds.), *Amphibian medicine and captive husbandry*. Malabar FL, Krieger Publishing Company, pp. 401 - 485.

Green, D. E. & Harshbarger, J. C. (2001). Spontaneous neoplasia in amphibia. In: Wright, K. M., Whitaker, B. R. (Eds.), *Amphibian medicine and captive husbandry*. Malabar FL, Krieger Publishing Company, pp. 335 - 400.

Green, D. E., Converse, K. A., Schrader, A. K. (2002). Epizootiology of sixty-four amphibian mortality and morbidity events in the USA, 1996 - 2001. *Annals of the New York Academy of Sciences*, 969: 323 - 339.

Green, S. E., Bouley, D. M., Tolwani, R. J., Waggie, K. S., Lifland, B. D., Otto, G. M., Ferrell, J. E. Jr. (1999). Identification and management of an outbreak of *Flavobacterium menigosepticum* infection in a colony of South African clawed frogs (*Xenopus laevis*). *Journal of the American Veterinary Medical Association*, 214: 1833 - 1838.

Greenspan, S. E., Longcore, J. E. & Calhoun, A. J. K. (2012). Host invasion by *Batrachochytrium dendrobatidis*: fungal and epidermal ultrastructure in model anurans. *Diseases of Aquatic Organisms*, 100: 201 - 210.

Greer, A. L., Berrill, M. & Wilson, P. J. (2005). Five amphibian mortality events associated with Ranavirus infection in south central Ontario, Canada. *Diseases of Aquatic Organisms*, 67: 9 - 14.

Grice, E. A., Kong, H. H., Renaud, G., Young, A. C., Bouffard, G. G., Blakesley, R. W., Wolfsberg, T. G., Turner, M. L. & Segre, J. A. (2008). A diversity profile of the human skin microbiota. *Genome Research*, 18(7): 1043 - 1050.

Gründler M. C., Toledo L. F., Parra-Olea G., Haddad C. F. B., Giasson L. O. M., Sawaya R. J., Prado, C. P., Araujo, O. G., Zara, F. J., Centeno, F. C. & Zamudio, K. R. (2012). Interaction between breeding habitat and elevation affects prevalence but not infection intensity of *Batrachochytrium dendrobatidis* in Brazilian anuran assemblages. *Diseases of Aquatic Organisms*, 97(3): 173 - 184.

Gurr, S., Samalova, M. & Fisher, M. (2011). The rise and rise of emerging infectious fungi challenges food security and ecosystem health. *Fungal Biology Reviews,* 25: 181 - 188.

Hadfield, J. (2010). MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. *Journal of Statistical Software*, 33(2): 1 - 22.

Hamady, M., Lozupone, C. & Knight, R. (2010). Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *The ISME Journal*, 4: 17 - 27.

Hammond, P. (1992). Species inventory. In: Groombridge, B. (Ed.), *Global biodiversity: status of the earth's living resources*. London, Chapman & Hall, pp. 17 - 39.

Han, B. A., Bradley, P. W. & Blaustein, A. R. (2008). Ancient behaviors of larval amphibians in response to an emerging fungal pathogen, *Batrachochytrium dendrobatidis*. *Behavioural Ecology and Sociobiology*, 63: 241 - 250.

Hanlon, S. M. & Parris, M. J. (2014). The interactive effects of chytrid fungus, pesticides, and exposure timing on Gray Treefrogs (*Hyla versicolor*) larvae. *Environmental Toxicology*, 33(1): 216 - 222.

Hansen, M. J. & Clevenger, A. P. (2005). The influence of disturbance and habitat on the presence of non-native plant species along transportation corridors. *Biological Conservation,* 125*:* 249 - 259.

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A. & Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth,* 3: 53 - 56.

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., Lam, B. A., Woodhams, D. C., Briggs, C. J., Vredenburg, V. T. & Minbiole, K. P. C. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal*, 3: 818 - 824.

Harrison, X. A., Bearhop, S., Inger, R., Colhoun, K., Gudmundsson, G. A., Hodgson, D., McElwaine, G. & Tregenza, T. (2011). Heterozygosity-fitness correlations in a migratory bird: an analysis of inbreeding and single-locus effects. *Molecular Ecology*, 20: 4786 - 4795.

Harvell, C. D., Kim, K., Burkholder, J. M., Colwell, R. R., Epstein, P. R., Grimes, D. J., Hofmann, E. E., Lipp, E. K., Osterhaus, A. D. M. E., Overstreet, R. M., Porter, J. W., Smith, G. W. & Vasta, G. R. (1999). Emerging marine diseases - climate links and anthropogenic factors. *Science,* 285(5433): 1505 - 1510.

Harvell, C. D., Mitchell, C. E., Ward, J. R., Altzier, S., Dobson, A. P., Otsfeld, R. S. & Samuel, M. D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science*, 296: 2158 - 2162.

Hassell, M. P. & May, R. M. (1973). Stability in insect host-parasite models. *Journal of Animal Ecology,* 42(3): 693 - 726.

Hawkes, C. V. & Keitt, T. H. (2015). Resilience vs. historical contingency in microbial responses to environmental change. *Ecology Letters,* 18: 612 - 625.

Heatwole, H. & Barthalamus, G. T. (1994). *Amphibian biology: the integument*. Surrey Beatty & Sons, Chipping Norton.

Hecnar, S. J. (1997). Amphibian pond communities in southwestern Ontario. In: Green, D. M. (Ed.), *Amphibians in decline: Canadian studies of a global problem. Herpetological Conservation*. Society for the Study of Reptiles, St. Louis, MO, USA, pp 1 - 15.

Hecnar, S. J. & M'Closkey, R. T. (1997). The effects of predatory fish on amphibian species richness and distribution. *Biological Conservation*, 79: 123 - 131.

Hess, G. R., Randolph, S. E., Arneberg, P., Chemini, C., Furlanello, C., Harwood, J., Roberts, M. G. & Swinton, J. (2001). Spatial aspects of disease dynamics. In: Hudson, P. J., Rizzoli, A., Grenfell, B. T., Heesterbeek, H. & Dobson, A. P. (Eds.), *The ecology of wildlife diseases*. Oxford, UK: Oxford University Press, pp. 102 - 118.

Hibbing, M. E., Fuqua, C., Parsek, M. R. & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology,* 8: 15 - 25.

Hiers, J. K. & Evans, J. P. (1997). Effects of anthracnose on dogwood mortality and forest composition of the Cumberland Plateau (USA). *Conservation Biology*, 11: 1430 - 1435.

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G. & Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, 25: 1965 - 1978.

Hijmans, R. (2010). *Package 'raster': geographic data analysis and modeling*. R package, version 1.6–15. Available: http://cran.r-project.org/web/packages/raster [accessed: 2010 November 7].

Hill, G. E. (2002). *A red bird in a brown bag*. New York: Oxford University Press.

Hill, G. E. & Farmer, K. L. (2005). Carotenoid-based plumage coloration predicts resistance to a novel parasite in the house finch. *Naturwissenschaften*, 92: 30 - 34.

Hill, G. E. & Johnson, J. D. (2012). The vitamin-A redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. *The American Naturalist*, 180: E127 - E150.

Hill, G. E. (2014). Cellular respiration: the nexus of stress, condition, and ornamentation. *Integrative and Comparative Biology*, 54(4): 645 - 657.

Hill, M. O. (1973). Diversity and evenness: a unifying notation and its consequences. *Ecology*, 54(2): 427 - 432.

Hillis, D. M. (1988). Systematics of the *Rana pipiens* complex: puzzle and paradigm. *Annual Review of Ecology and Systematics,* 19: 39 - 63.

Hine, R. L., Les, B. L. & Hellmich, B. F. (1981). *Leopard frog populations and mortality in Wisconsin, 1974 - 1976*. Wisconsin Department of Natural Resources, Tech Bull No 122. p. 39.

Hoar, W. S. (1986). *General and comparative physiology*, 2nd edition. Englewood Cliffs, NJ, Prentice Hall, pp. 382 - 389.

Hochachka, W. M. & Dhondt, A. A. (2000). Density-dependent decline of host abundance resulting from a new infectious disease. *Proceedings of the National Academy of Sciences of the United States of America*, 97: 5303 - 5306.

Hoffman, E. A., Schueler, F. W. & Blouin, M. S. (2004). Effective population sizes and temporal stability of genetic structure in *Rana pipiens*, the Northern Leopard Frog. *Evolution*, 58(11): 2536 - 2545.

Holmes, I., McLaren, K. & Wilson, B. (2014). Precipitation constrains amphibian chytrid fungus infection rates in a terrestrial frog assemblage in Jamaica, West Indies. *Biotropica*, 46: 219 - 228.

Holmes, J. C. (1996). Parasites as threats to biodiversity in shrinking ecosystems. *Biodiversity and Conservation,* 5: 975 - 983.

Homan, R. N., Regosin, J. V., Rodrigues, D. M., Reed, J. M., Windmiller, B. S. & Romero, L. M. (2003). Impacts of varying habitat quality on the physiological stress of spotted salamanders (*Ambystoma maculatum*). *Animal Conservation*, 6: 11 - 18.

Hoppe, D. M. (1979). The influence of color on behavioral thermoregulation and hydroregulation. In: Feder, M. E. & Burggren, W. W. (Eds.), *Environmental physiology of the amphibians*. Garland Publications Inc, New York, USA, pp. 37 - 63.

Hossack, B. R., Lowe, W. H., Ware, J. L. & Corn, P. S. (2013). Disease in a dynamic landscape: host behaviour and wildfire reduce amphibian chytrid infection. *Biological Conservation*, 157: 293 - 299.

Houlahan, J. E., Findlay, C. S., Schmidt, B. R., Meyer, A. H. & Kuzmin, S. L. (2000). Quantitative evidence for global amphibian population declines. *Nature*, 404: 752 - 755.

Houlahan, J. E. & Findlay, C. S. (2003). The effects of adjacent land use on wetland amphibian species richness and community composition. *Canadian Journal of Fisheries and Aquatic Sciences*, 60: 1078 - 1094.

Houslay, T. M. & Wilson, A. (2017). Avoiding the misuse of BLUP in behavioral ecology. *Open Research Exeter (ORE)*. Available: https://ore.exeter.ac.uk/repository/handle/10871/25386.

Hudson, P. J., Rizzoli, A., Grenfell, B. T., Heesterbeek, H. & Dobson, A. P. (2002). *The ecology of wildlife diseases*. Oxford University Press, Oxford.

Hudson, P. J., Dobson, A. P. & Lafferty, K. D. (2006). Is a healthy ecosystem one that is rich in parasites? *Trends in Ecology & Evolution*, 21(7): 381 - 385.

Huey, R. B. (1991). Physiological consequences of habitat selection. *The American Naturalist*, 137: S91 - 115.

Huntingford, C., Jones, P. D., Livina, V. N., Lenton, T. M. & Cox, P. M. (2013). No increase in global temperature variability despite changing regional patterns. *Nature,* 500: 327 - 330.

Hutchison, V. H. & Dupré, R. K. (1992). Thermoregulation. In: Feder, M. & Burggren, W. (Eds.), *Environmental physiology of the Amphibia*. University of Chicago Press, Chicago. pp. 206 - 249.

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., Dalton, A., Kriger, K., Hero, M., Hines, H., Phillott, R., Campbell, R., Marantelli, G., Gleason, F. & Colling, A. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 73: 175 - 192.

Jackson, J. A. & Tinsley, R. C. (2002). Effects of environmental temperature on the susceptibility of *Xenopus laevis* and *X. wittei* (Anura) to *Protopolystoma xenopodis* (Monogenea). *Parasitology Research*, 88: 632 - 638.

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H. T., Rauhut, A., Reeb, V., Arnold, A. E., Amtoft, A., Stajich, J. E., Hosaka, K., Sung, G-H., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J. M., Slot, J. C., Wang, Z., Wilson, A. W., Schüβler, A., Longcore, J. E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P. M., Powell, M. J., Taylor, J. W., White, M. M., Griffith, G. W., Davies, D. R., Humber, R. A., Morton, J. B., Sugiyama, J., Rossman, A. Y., Rogers, J. D., Pfister, D. H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R. A., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R. A., Serdani, M., Crous, P. W., Hughes, K. W., Matsuura, K., Langer, E., Langer, G., Untereiner, W. A., Lücking, R., Büdel, B., Geiser, D. M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D. S., Lutzoni, F., McLaughlin, D. J., Spatafora, J. W. & Vilgalys, R. (2006). Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature*, 443: 818 - 822.

James, T. Y., Toledo, L. F., Rödder, D., Leite, D. S., Belasen, A., Betancourt-Román, C. M., Jenkinson, T. S., Soto-Azat, C., Lambertini, C., Longo, A. V., Ruggeri, J., Collins, J. P., Burrowes, P. A., Lips, K. R., Zamudio, K. R. & Longcore, J. E. (2015). Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. *Ecology and Evolution*, 5(18): 4079 - 4097.

Jani, A. J. & Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin micobiome during a natural epidemic and experimental infection. *Proceedings of the National Academy of Sciences of the United States of America*, 111(47): E5049 - E5058.

Janin, A., Lena, J. P., Deblois, S. & Joly, P. (2012). Use of stress-hormone levels and habitat selection to assess functional connectivity of a landscape for an amphibian. *Conservation Biology*, 26: 923 - 931.

Jensen, T., van de Bildt, M., Dietz, H. H., Andersen, T. H., Hammer, A. S., Kuiken, T. & Osterhaus, A. (2002). Another phocine distemper outbreak in Europe. *Science*, 297(5579): 209.

Johnson, M. L., Berger, L., Philips, L. & Speare, R. (2003). Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. *Disease of Aquatic Organisms*, 57(3): 255 - 260.

Johnson, M. L. & Speare, R. (2003). Survival of *Batrachochytrium dendrobatidis* in water: quarantine and disease control implications. *Emerging Infectious Diseases,* 9(8): 922 - 925.

Johnson, M. L. & Speare, R. (2005). Possible modes of dissemination of the amphibian chytrid *Batrachochytrium dendrobatidis* in the environment. *Diseases of Aquatic Organisms,* 65(3): 181 - 186.

Johnson, P. T., Hartson, R. B., Larson, D. J. & Sutherland, D. R. (2008). Diversity and disease: community structure drives parasite transmission and host fitness. *Ecology Letters*, 11(10): 1017 - 1026.

Jokela, J. & Lively, C. M. (1995). Spatial variation for infection by digenetic trematodes in a population of freshwater snails (*Potamopyrgus antipodarum*). *Oecologia*, 103: 509 - 517.

Jolles, A. E., Ezenwa, V. O., Etienne, R. S., Turner, W. C. & Olff, H. (2008). Interactions between macroparasites and microparasites drive infection patterns in free-ranging African buffalo. *Ecology,* 89: 2239 - 2250.

Joly, D. O. & Messier, F. (2005). The effect of bovine tuberculosis and brucellosis on reproduction and survival of wood bison in Wood Buffalo National Park. *Journal Animal Ecology,* 74: 543 - 551.

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L. & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, 451: 990 - 993.

Jong, P. W., Gussekloo, S. W. S. & Brakefield, P. M. (1996). Differences in thermal balance, body temperature and activity between non-melanic and melanic two-spot ladybird beetles (*Adalia bipunctata*) under controlled conditions. *The Journal of Experimental Biology*, 199: 2655 - 2666.

Jørgensen, C. B. & Larsen, L. O. (1960). Hormonal control of moulting in amphibians. *Nature*, 185: 244 - 245.

Jørgensen, C. B. & Larsen, L. O. (1961). Molting and its hormonal control in toads. *General and Comparative Endocrinology*, 1: 145 - 153.

Jørgensen, C. B. & Larsen, L. O. (1964). Further observations on molting and its hormonal control in *Bufo bufo* (L). *General and Comparative Endocrinology*, 17: 389 - 400.

Jørgensen, C. B., Larsen, L. O. & Rosenkilde, P. (1965). Hormonal dependency of molting in amphibians; effect of radiothyroidectomy in the toad *Bufo bufo* (L.). *General and Comparative Endocrinology*, 56: 248 - 251.

Jørgensen, C. B. (1988). Nature of molting control in amphibians: effects of cortisol implants in toads *Bufo bufo*. *General and Comparative Endocrinology*, 71: 29 - 35.

Jørgensen, C. B. (1997). 200 years of amphibian water economy: from Robert Townson to the present. *Biological Reviews of the Cambridge Philosophical Society*, 72: 153 - 237.

Kalmus, H. (1941). Physiology and ecology of cuticle colour in insects. *Nature*, 148: 428 - 431.

Karban, R. & Myers, J. H. (1989). Induced plant responses to herbivory. *Annual Review of Ecology and Systematics*, 20(1): 331 - 348.

Keesing, F., Holt, R. D. & Otsfeld, R. S. (2006). Effects of species diversity on disease risk. *Ecology Letters*, 9: 483 - 498.

Kettlewell, H. B. D. (1973*). The evolution of melanism: the study of a recurring necessity, with special reference to industrial melanism in the Lepidoptera*. Claredon Press, Oxford.

Khosravi, A. & Mazmanian, S. K. (2013). Disruption of the gut microbiome as a risk factor for microbial infections. *Current Opinion in Microbiology*, 16(2): 221 - 227.

Kiesecker, J. M., Blaustein, A. R. & Miller, C. L. (2001). Transfer of a pathogen from fish to amphibians. *Conservation Biology*, 15: 1064 - 1070.

Kilburn, V. L., Ibanez, R. & Green, D. M. (2011). Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama. *Diseases of Aquatic Organisms*, 97: 127 - 134.

Kilpatrick, A. M., Briggs, C. J. & Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology and Evolution*, 25(2): 109 - 118.

Kingsolver, J. G. & Watt, W. B. (1983). Thermoregulatory strategies in Colias butterflies: thermal stress and the limits to adaptation in temporally varying environments. *The American Naturalist*, 121: 32 - 55.

Kingsolver, J. G. (1996). Experimental manipulation of wing pigment pattern and survival in western white butterflies. *The American Naturalist*, 147: 296 - 306.

Kinney. V. C., Heemeyer, J. L., Pessier, A. P. & Lannoo, M. J. (2011). Seasonal pattern of *Batrachochytrium dendrobatidis* infection and mortality in *Lithobates areolatus*: Affirmation of Vredenburg's "10,000 zoospore rule". *PLoS ONE,* 6(3): e16708.

Klass, J. I., Blanford, S. & Thomas, M. B. (2007). Use of a geographic information system to explore spatial variation in pathogen virulence and the implications for biological control of locusts and grasshoppers. *Agricultural and Forest Entomology*, 9(3): 201 - 208.

Knapp, R. A., Briggs, C. J., Smith, T. C. & Maurer, J. R. (2011). Nowhere to hide: impact of a temperature sensitive amphibian pathogen along an elevation gradient in the temperate zone. *Ecosphere*, 2(8): 1 - 26.

Kohl, K. D., Cary, T. L., Karasov, W. H. & Dearing, M. D. (2013). Restructuring of the amphibian gut microbiota through metamorphosis. *Environmental Microbiology Reports*, 5(6): 899 - 903.

Kohl, K. D., Weiss, R. B., Cox, J., Dale, C. & Denise Dearing, M. (2014). Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecology Letters*, 17(10): 1238 - 1246.

Kohl, K. D., Stengel, A., & Dearing, M. D. (2015). Inoculation of tannin-degrading bacteria into novel hosts increases performance on tannin rich diets. *Environmental Microbiology,* 18(6): 1720 - 1729.

Kohl, K. D. & Yahn, J. (2016). Effects of environmental temperature on the gut microbial communities of tadpoles. *Environmental Microbiology*, 18(5): 1561 - 1565.

Kolby, J. E., Smith, K. M., Berger, L., Karesh, W. B., Preston, A., Pessier, A. P. & Skerratt, L. F. (2014). First evidence of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in Hong Kong amphibian trade. *PLoS ONE,* 9(3): e90750.

Kolby, J. E., Ramirez, S. D., Berger, L., Richards-Hrdlicka, K. L., Jocque, M. & Skerratt, L. F. (2015). Terrestrial dispersal and potential environmental transmission of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*). *PLoS ONE,* 10(4): e0125386.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq illumina sequencing platform. *Applied and Environmental Microbiology*, 79(17): 5112 - 5120.

Kraaijeveld, A. R. & Godfray, H. C. J. (1997). Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Nature*, 389: 278 - 280.

Kraaijeveld, A. R., van Alphen, J. J. M. & Godfray, H. C. J. (1998). The coevolution of host resistance and parasitoid virulence. *Parasitology,* 116: S29 - S45.

Kriger, K. M. & Hero, M. (2006). Survivorship in wild frogs infected with chytridiomycosis. *EcoHealth*, 3: 171 - 177.

Kriger, K. M. & Hero, J. M. (2007a). The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Diversity and Distributions*, 13: 781 - 788.

Kriger, K. M. & Hero, M. (2007b). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology*, 271(3): 352 - 359.

Kriger, K. M., Pereoglou, F. & Hero, M. (2007). Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. *Conservation Biology*, 21(5): 1280 - 1290.

Kriger, K. M. (2009). Lack of evidence for the drought-linked chytridiomycosis hypothesis. *Journal of Wildlife Diseases*, 45: 537 - 541.

Kruskal, J. B. (1964). Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika*, 29: 1 – 27.

Krynak, K. L., Burke, D. J., & Benard, M. F. (2015). Larval environment alters amphibian immune defenses differentially across life stages and populations. *PLoS ONE,* 10(6): e0130383.

Krynak, K. L., Burke, D. J. & Benard, M. F. (2016). Landscape and water characteristics correlate with immune defense traits across Blanchard's cricket frog (*Acris blanchardi*) populations. *Biological Conservation*, 193: 153 - 167.

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R. & McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology*, 23(6): 1238 - 1250.

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R. & McKenzie, V. J. (2015). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads *(Anaxyrus boreas)*. *The ISME Journal,* 10: 934 - 944.

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R. & McKenzie, V. J. (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proceedings of the Royal Society B: Biological Sciences,* 283(1839): 20161553.

Küng, D., Bigler, L., Davis, L. R., Gratwicke, B., Griffith, E. & Woodhams, D. C. (2014). Stability of microbiota facilitated by host immune regulation: informing probiotic strategies to manage amphibian disease. *PLoS ONE,* 9: e87101.

La Marca, E., Lips, K. R., Lotters, S., Puschendorf, R., Ibáñez, R., Rueda-Almonacid, J. V., Schulte, R., Marty, C., Castro, F., Manzanilla-Puppo, J., Garcia-Pérez, J. E., Bolaños, F., Chaves, G., Pounds, J. A., Toral, E. & Young, B. E. (2005). Catastrophic population declines and extinctions in Neotropical harlequin frogs (*Bufonidae: Atelopus*). *Biotropica,* 37: 190 - 201.

Lafferty, K. D. & Gerber, L. R. (2002). Good medicine for conservation biology: the intersection of epidemiology and conservation theory. *Conservation Biology*, 16(3): 593 - 604.

Lafferty, K. D. (2003). Is disease increasing or decreasing, and does it impact or maintain biodiversity*? Journal of Parasitology*, 89: s101 - s105.

Lafferty, K. D. (2009). The ecology of climate change and infectious diseases. *Ecology,* 90: 888 - 900.

Lam, B. A., Walke, J. B., Vredenburg, V. T. & Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biological Conservation*, 143(2): 529 - 531.

Lambrechts, L., Chavatte, J. M., Snounou, G. & Koella, J. C. (2006). Environmental influence on the genetic basis of mosquito resistance to malaria parasites. *Proceedings of the Royal Society B: Biological Sciences,* 273: 1501 - 1506.

Lamirande, E. W. & Nichols. D. K. (2002). Effects of host age on susceptibility to cutaneous chytridiomycosis in blue and yellow poison dart frogs (*Dendrobates tinctorius*). In: McKinnell, R. G. & Carlson, D. L. (Eds.), *Proceedings of the sixth symposium on pathology of reptiles and amphibians*. University of Minnesota Printing Services, Saint Paul, pp. 3 - 13.

Lampo, M., Rodriguez-Contreras, A., La Marca, E. & Daszak, P. (2006). A chytridiomycosis epidemic and a severe dry season precede the disappearance of *Atelopus* species from the Venezuelan Andes. *Herpetological Journal*, 16: 395 - 402.

Lande, R. (1988). Genetics and demography in biological conservation. *Science,* 241: 1455 - 1460.

Langhammer, P. F., Lips, K. R., Burrowes, P. A., Tunstall, T., Palmer, C. M. & Collins, J. P. (2013). A fungal pathogen of amphibians, *Batrachochytrium dendrobatidis*, attenuates in pathogenicity with in vitro passages. *PLoS ONE*, 8(10): e77630.

Lannoo, M. (2005). *Amphibian declines: the conservation status of United States species*. University of California Press, Berkeley, CA.

Lauer, A., Simon, M. A., Banning, J. L., André, E., Duncan, K. & Harris, R. N. (2007). Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia*, 3: 630 - 640.

Lauer, A., Simon, M. A., Banning, J. L., Lam, B. A. & Harris, R. N. (2008). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *The ISME Journal*, 2: 145 - 157.

Laurance, W. F., McDonald, K. R. & Speare, R. (1996). Epidemic disease and the catastrophic decline of Australian rain forest frogs. *Conservation Biology,* 10(2): 406 - 413.

Laurance, W. F., McDonald, K. R. & Speare, R. (1997). In defense of the epidemic disease hypothesis. *Conservation Biology*, 11: 1030 - 1034.

Laurance, W. F. (2008). Global warming and amphibian extinctions in eastern Australia. *Austral Ecology*, 33: 1 - 9.

Lazarro, B. P., Flores, H. A., Lorigan, J. G. & Yourth, C. P. (2008). Genotype-by-environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster*. *PLoS Pathogens*, 4: 1 - 9.

Lederberg, J., Oates, S. C. & Shope, R. E. (1992). *Emerging infections, microbial threats to health in the United States*. Institute of Medicine, National Academy Press, Washington, DC, USA, p. 294.

Legendre, P. & Cáceres, M. (2013). Beta diversity as the variance of community data: dissimilarity coefficients and partitioning. *Ecology Letters*, 16(8): 951 - 963.

Lehner, B., Verdin, K. & Jarvis, A. (2008). New global hydrography derived from spaceborne elevation data. *Eos, Transactions American Geophysical Union*, 89(10): 93 - 94.

Leonard, W. P., McAllister, K. R. & Friesz, R. C. (1999). Survey and assessment of northern leopard frog (*Rana pipiens*) populations in Washington State. *Northwestern Naturalist*, 80: 51 - 60.

Lesbarrères, D., Pagano, A. & Lodé, T. (2003). Inbreeding and road effect zone in a Ranidae: the case of Agile frog, *Rana dalmatina* Bonaparte, 1840. *Comptes Rendus Biologies*, 326: 68 - 72.

Lesbarrères, D., Primmer, C. R., Lodé, T. & Merilä, J. (2006). The effects of 20 years of highway presence on the genetic structure of *Rana dalmatina* populations. *Ecoscience,* 13(4): 531 - 538.

Leu, M., Hanser, S. & Knick, S. T. (2008). The human footprint in the west: a large-scale analysis of anthropogenic impacts. *Ecological Applications,* 18: 1119 - 1139.

Lewis, W. J. & Tumlinson, J. H. (1988). Host detection by chemically mediated associative learning in a parasitic wasp. *Nature*, 331: 257 – 259.

Liesenjohann, M., Liesenjohann, T., Palme, R. & Eccard, J.A. (2013). Differential behavioural and endocrine responses of common voles (*Microtus arvalis*) to nest predators and resource competitors. *BMC Ecology*, 13.

Lillywhite, H. B. (2006). Water relations of tetrapod integument. *Journal of Experimental Biology*, 209: 202 - 226.

Lips, K. R. (1998). Decline of a tropical montane amphibian fauna. *Conservation Biology,* 12: 106 - 117.

Lips, K. R. (1999). Mass mortality and population declines of anurans at an upland site in Western Panama. *Conservation Biology*, 13: 117 - 125.

Lips, K. R., Reeve, J. & Witters, L. (2003). Ecological traits predicting amphibian population declines in Central America. *Conservation Biology*, 17: 1078 - 1088.

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. & Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proceedings of the National Academy of Sciences of the United States of America*, 103(9): 3165 - 3170.

Lips, K. R., Diffendorfer, J., Mendelson III, J. R. & Sears, M. W. (2008). Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biology*, 6: 441 - 454.

Little, T. J. & Ebert, D. (2001). Temporal patterns of genetic variation for resistance and infectivity in a Daphnia-microparasite system. *Evolution,* 55: 1146 - 1152.

Liu, X., Rohr, J. R. & Li, Y. M. (2013). Climate, vegetation, introduced hosts and trade shape a global wildlife pandemic. *Proceedings of the Royal Society B: Biological Sciences*, 280(1753): 20122506.

Longcore, J., Pessier, A. & Nichols, D. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia*, 91(2): 219 - 227.

Longcore, J. R., Longcore, J. E., Pessier, A. P. & Halteman, W. A. (2007). Chytridiomycosis widespread in Anurans of Northeastern United States. *The Journal of Wildlife Management*, 71: 435 - 444.

Longo, A. V., Burrowes, P. A. & Joglar, R. L. (2010). Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Diseases of Aquatic Organisms*, 92: 253 - 260.

Longo, A. V., Savage, A. E., Hewson, I. & Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *Royal Society Open Science*, 2(7): 140377.

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V. & Harris, R. N. (2014a). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *The ISME Journal*, 8(4): 830 - 840.

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P. C. & Harris, R. N. (2014b). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Frontiers in Microbiology*, 5.

Loughran, M. F. E. (2006). Social organisation of female field voles *Microtus agrestis* in a population in Southern England. *Acta Theriologica,* 51: 233 - 242.

Lozupone, C. A. & Knight, R. (2007). Global patterns in bacterial diversity. *Proceedings of the National Academy of Sciences of the United States of America*, 104(27): 11436 - 11440.

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J. & Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *The ISME Journal*, 5: 169 - 172.

Lucké, B. (1934). A neoplastic disease of the kidney of the frog, *Rana pipiens*. *Cancer Research*, 20: 352 - 379.

Luke, C. A. (1989). *Color as a phenotypically plastic character in the side-blotched lizard, Uta stansburiana*. Ph.D. Thesis. University of California, Berkeley.

Lunger, P. D., Darlington, R. W. & Granoff, A. (1965). Cell-virus relationships in the Lucke renal adenocarcinoma: an ultrastructural study. *Annals of the New York Academy of Sciences,* 186: 289 - 314.

Lusis, J. J. (1961). On the biological meaning of colour polymorphism of lady beetle *Adalia bipunctata* L. *Latvijas Entomologs,* 4: 3 - 29.

McCallum, H. & Dobson, A. (1995). Detecting disease and parasite threats to endangered species and ecosystems. *Trends in Ecology & Evolution,* 10: 190 - 194.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., Dubilier,N., Eberl, G., Fukami, T., Gilbert, S. F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A. H., Kremer, N., Mazmanian, S. K., Metcalf, J. L., Nealson, K., Pierce, N. E., Rawls, J. F., Reid, A., Ruby, E. G., Rumpho, M., Sanders, J. G., Tautz, D. & Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America*, 110(9): 3229 - 3236.

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R. & Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *The ISME Journal*, 6: 588 - 596.

McMahon, T. A., Brannelly, L. A., Chatfield, M. W. H., Johnson, P. T. J., Joseph, M. B., McKenzie, V. J., Richards-Zawacki, C. L., Venesky, M. D. & Rohr, J. R. (2013). Chytrid fungus *Batrachochytrium dendrobatidis* has non-amphibian hosts and releases chemicals that cause pathology in the absence of infection. *Proceedings of the National Academy of Sciences of the United States of America,* 110: 210 - 215.

McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., Halstead, N. T., Lentz, G., Tenouri, N., Young, S., Civitello, D. J., Ortega, N., Scott Fites, J., Reinert, L. K., Rollins-Smith, L. A., Raffel, T. R. & Rohr, J. R. (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature*, 511(7508): 224 - 227.

McMurdie, P. J. & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, 8(4): e61217.

McRae, L., Freeman, R. & Marconi, V. (2016). The Living Planet Index. In: Oerlemans, N. (Ed.), *Living Planet Report 2016: Risk and resilience in a new era*. WWF International, Gland, Switzerland.

Marantelli, G., Berger, L., Speare, R. & Keegan, L. (2004). Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pacific Conservation Biology,* 10: 173 - 179.

Marsh, D. M., Page, R. B., Hanlon, T. J., Corritone, R., Little, E. C., Seifert, D. E. & Cabe, P. R. (2008). Effects of roads on patterns of genetic differentiation in red-backed salamanders, *Plethodon cinereus*. *Conservation Genetics*, 9: 603 - 613.

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F. & Pasmans, F. (2013). *Batrachochytrium salamandrivorans* sp nov causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences of the United States of America,* 110(38): 15325 - 15329.

Martens, J. W., McKenzie, R. I. H. & Green, G. J. (1967). Thermal stability of stem rust resistance in oat seedlings. *Canadian Journal of Botany*, 45: 451 - 458.

Matutte, B., Storey, K. B., Knoop, F. C. & Conlon, J. M. (2000). Induction of synthesis of an antimicrobial peptide in the skin of a freeze-tolerance frog, *Rana sylvatica*, in response to environmental stimuli. *FEBS Letters*, 483: 135 - 138.

May, R. M. & Anderson, R. M. (1983). Epidemiology and genetics in the coevolution of parasites and hosts. *Proceedings of the Royal Society B: Biological Sciences*, 219: 281 - 313.

Mazzoni, R., Cunningham, A. A., Daszak, P., Apolo, A., Perdomo, E. & Speranza, G. (2003). Emerging pathogen of wild amphibians in frogs (*Rana catesbeiana*) farmed for international trade. *Emerging Infectious Diseases*, 9: 995 - 998.

Merrell, D. J. (1965). The distribution of the dominant Burnsi gene in the leopard frog, *Rana pipiens*. *Evolution*, 19: 69 - 95.

Merrell, D. J. (1970). Migration and gene dispersal in *Rana pipiens*. *American Zoologist*, 10(1): 47 - 52.

Merrell, D. J. (1977). *Life history of the leopard frog, Rana pipiens, in Minnesota*. Occasional paper number 15. Bell Museum of Natural History. University of Minnesota, Minneapolis, Minnesota, USA.

Meyer, E. A., Cramp, R. L., Bernal, M. H. & Franklin, C. E. (2012). Changes in cutaneous microbial abundance with sloughing: possible implications for infection and disease in amphibians. *Diseases of Aquatic Organisms*, 101(3): 235 - 242.

Miller, D. L., Gray, M. J., Rajeev, S., Schmutzer, A. C., Burton, E. C., Merrill, A. & Baldwin, C. A. (2009). Pathologic findings in larval and juvenile anurans inhabiting farm ponds in Tennessee, USA. *Journal of Wildlife Diseases*, 45(2): 314 - 324.

Miller, M. P. & Vincent, E. R. (2008). Rapid natural selection for resistance to an introduced parasite of rainbow trout. *Evolutionary Applications*, 1(2): 336 - 341.

Mitchell, K. M., Churcher, T. S., Garner, T. W. J. & Fisher, M. C. (2008). Persistence of the emerging pathogen *Batrachochytrium dendrobatidis* outside the amphibian host greatly increases the probability of host extinction. *Proceedings of the Royal Society B: Biological Sciences*, 275: 329 - 334.

Mitchell, S. E., Rogers, E. S., Little, T. J. & Read, A. F. (2005). Host-parasite and genotype-by-environment interactions: temperature modifies potential for selection by a sterilizing pathogen. *Evolution*, 59: 70 - 80.

Miura, O. (2007). Molecular genetic approaches to elucidate the ecological and evolutionary issues associated with biological invasions. *Ecological Research,* 22*:* 876 - 883.

MMP (2003). *Marsh Monitoring Program: training kit and instructions for surveying marsh birds, amphibians and their habitats*. Bird Studies Canada in cooperation with Environment Canada and U.S. Environmental Protection Agency.

Moloney, R. D., Desbonnet, L., Clarke, G., Dinan, T. G. & Cryan, J. F. (2014). The microbiome: stress, health and disease. *Mammalian Genome*, 25(1): 49 - 74.

Monastersky, R. (2014). Biodiversity: Life – a status report. *Nature*, 516: 158 - 161.

Moore, J. A. (1942). An embryological and genetical study of *Rana burnsi* weed. *Genetics*, 27: 408 - 416.

Morgan, X. C. & Huttenhower, C. (2012). Human microbiome analysis. *PLoS Computational Biology*, 8(12): e1002808.

Morimoto, T., Kojima, Y., Toki, T., Komeda, Y., Yoshiyama, M., Kimura, K., Nirasawa, K. & Kadowaki, T. (2011). The habitat disruption induces immune-suppression and oxidative stress in honey bees. *Ecology and Evolution*, 1: 201 - 217.

Munson, L., Terio, K. A., Kock, R., Mlengeya, T., Roelke, M. E., Dubovi, E., Summers, B., Sinclair, A. R. E. & Packer, C. (2008). Climate extremes promote fatal co-infections during canine distemper epidemics in African lions. *PLoS ONE*, 3: e2545.

Murphy, P. J., St-Hilaire, S. & Corn, P. S. (2011). Temperature, hydric environment, and prior pathogen exposure alter the experimental severity of chytridiomycosis in boreal toads. *Diseases of Aquatic Organisms*, 95(1): 31 - 42.

Murray, K. A., Skerratt, L. F., Speare, R. & McCallum, H. (2009). Impact and dynamics of disease in species threatened by the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*. *Conservation Biology*, 32: 1242 - 1252.

Muths, E., Corn, P. S., Pessier, A. P. & Green, D. E. (2003). Evidence for disease-related amphibian decline in Colorado. *Biological Conservation*, 110: 357 - 365.

Neufeld, K. M., Kang, N., Bienenstock, J. & Foster, J. A. (2011). Reduced anxiety-like behaviour and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, 23(3): 255 - e119.

Nichols, D., Lewis, K., Orjala, J., Mo, S., Ortenberg, R., O'Connor, P., Zhao, C., Vouros, P., Kaeberlein, T. & Epstein, S. S. (2008). Short peptide induces an "uncultivable" microorganism to grow in vitro. *Applied Environmental Microbiology*, 74: 4889 - 4897.

Nolan, P. M., Hill, G. E. & Stoehr, A. M. (1998). Sex, size, and plumage redness predict house finch survival in an epidemic. *Proceedings of the Royal Society B: Biological Sciences*, 265: 961 - 965.

Norris, K.S. (1967). Color adaptation in desert reptiles and its thermal relationships. In: Milstead, W.W. (Ed.), *Lizard ecology: a symposium*. University of Missouri Press, Columbia, pp. 162 - 229.

Nussey, D. H., Wilson, A. J. & Brommer, J. E. (2007). The evolutionary ecology of individual phenotypic plasticity in wild populations. *Journal of Evolutionary Biology*, 20: 831 - 844.

O'Mahony, S. M., Marchesi, J. R., Scully, P., Codling, C., Ceolho, A. M., Quigley, E. M., Cryan, J. F. & Dinan, T. G. (2009). Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biological Psychiatry*, 65(3): 263 - 267.

O'Toole, P. W. & Claesson, M. J. (2010). Gut microbiota: changes throughout the lifespan from infancy to elderly. *International Dairy Journal*, 20: 281 - 291.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H. & Wagner, H. (2017). *Package 'vegan': community ecology package*. R package, version 2.4-2. Available: https://cran.r-project.org/web/packages/vegan/index.html [accessed 2017 January 15].

Oldham, M. J. & Weller, W. F. (1992). Ontario herpetofaunal summary: compiling information on the distribution and life history of amphibians and reptiles in Ontario. In: Bishop, C. A. & Pettit, K. E. (Eds.), *Declines in Canadian amphibian populations: designing a national monitoring strategy*. Canadian Wildlife Service, Occas. Paper 76, Environment Canada, Ottawa, pp. 21 - 22.

Olson, D. H., Aanensen, D. M., Ronnenberg, K. L., Powell, C. I., Walker, S. F., Bielby, J., Garner, T. W. J., Weaver, G., The *Bd* Mapping Group & Fisher, M. C. (2013). Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. *PLoS ONE*, 8(2): e56802.

Olson, D. H. & Ronnenberg, K. L. (2014). *Global Bd mapping project: 2014 update*. *Froglog*, 22: 17 - 21.

Olson, D. M., Dinerstein, E., Wikramanayake, E. D., Burgess, N. D., Powell, G. V. N., Underwood, E. C., D'amico, J. A., Itoua, I., Strand, H. E., Morrison, J. C., Loucks, C. J., Allnutt, T. F., Ricketts, T. H., Kura, Y., Lamoreux, J. F., Wettengel, W. W., Hedao, P. & Kassem, K. R. (2001). Terrestrial ecoregions of the world: a new map of life on Earth. *BioScience*, 51: 933 - 938.

Olson, M. E., Gard, S., Brown, M., Hampton, R. & Morck, D. W. (1992). *Flavobacterium indologenes* infection in leopard frogs. *Journal of the American Veterinary Medical Association*, 201: 1766 - 1770.

Ontario Nature (2012). *The Northern Leopard Frog* (*Lithobates pipiens*). Available: http://www.ontarionature.org/protect/species/reptiles_and_amphibians/images/map_Northern-Leopard-Frog-SO.gif [accessed: 2016 June 10].

Opdam, P. & Wascher, D. (2004). Climate change meets habitat fragmentation: linking landscape and biogeographical scale levels in research and conservation. *Biological Conservation,* 117(3): 285 - 297.

Oseen, K. L. & Wassersug, R. J. (2002). Environmental factors influencing calling in sympatric anurans. *Oecologia*, 133(4): 616 - 625.

Osterhaus, A. D. M. E. & Vedder, E. J. (1988). Identification of a virus causing recent seal deaths. *Nature,* 335: 20.

Ostfeld, R. S. & Keesing, F. (2000). Biodiversity and disease risk: the case of Lyme disease. *Conservation Biology*, 14: 722 - 728.

Ostfeld, R. S., Glass, G. E. & Keesing, F. (2005). Spatial epidemiology: an emerging (or re-emerging) discipline. *Trends in Ecology and Evolution*, 20: 328 - 336.

Ostfeld, R. S., Thomas, M. & Keesing, F. (2009). Biodiversity and ecosystem function: perspectives on disease. In: Naeem, S., Bunker, D. E., Hector, A., Loreau, M. & Perrings, C. (Eds.), *Biodiversity, ecosystem functioning, and human wellbeing: an ecological and economic perspective*. New York, NY: Oxford University Press, pp. 209 - 216.

Ouellet, M., Mikaelian, I., Pauli, B. D., Rodrigue, J. & Green, D. M (2005). Historical evidence of widespread chytrid infection in North America amphibian populations. *Conservation Biology*, 19: 1431 - 1440.

Paaijmans, K. P., Read, A. F. & Thomas, M. B. (2009). Understanding the link between malaria risk and climate. *Proceedings of the National Academy of Sciences of the United States of America,* 106(33): 13844 - 13849.

Paaijmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C. & Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global Change Biology*, 19: 2373 - 2380.

Parmesan, C. & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature*, 421: 37 - 42.

Parris, M. J. (2004). Hybrid response to pathogen infection in interspecific crosses between two amphibian species *(Anura: Ranidae). Evolutionary Ecology Research*, 6: 1 - 15.

Parris, M. J. & Baud, D. R. (2004). Interactive effects of a heavy metal and chytridiomycosis on Gray Treefrog larvae (*Hyla chrysoscelis*). *Copeia*, 2: 343 - 349.

Parris, M. J. & Beaudoin, J. G. (2004). Chytridiomycosis impacts predator-prey interactions in larval amphibian communities. *Oecologia,* 140: 626 - 632.

Parris, M. J. & Cornelius, T. O. (2004). Fungal pathogen causes competitive and developmental stress in larval amphibian communities. *Ecology*, 85: 3385 - 3395.

Parris, M. J., Davis, A. & Collins, J. P. (2004). Single-host pathogen effects on mortality and behavioural responses to predators in salamanders (*Urodela: Ambystomatidae*). *Canadian Journal of Zoology*, 82: 1477 - 1483.

Pascolini, R., Daszak, P., Cunningham, A. A., Tei, S., Vagnetti, D., Bucci, S., Fagotti, A. & DiRosa, I. (2003). Parasitism by *Dermocystidium ranae* in a population of *Rana esculenta* complex in Central Italy and description of *Amphibiocystidium* n. gen. *Diseases of Aquatic Organisms*, 56: 65 - 74.

Pasmans, F., Zwart, P. & Hyatt, A. D. (2004). Chytridiomycosis in the Central American bolitoglossine salamander (*Bolitoglossa dofleini*). *Veterinary Record*, 154: 153.

Pasteris, S. E., Bühler, M. I. & Nader-Macías, M. E. (2006). Microbiological and histological studies of farmed-bullfrog (*Rana catesbeiana*) tissues displaying red-leg syndrome. *Aquaculture*, 251(1): 11 - 18.

Pasteris, S. E., Guidoli, M. G., Otero, M. C., Bühler, M. I., Nader-Macías, M. E. (2011). *In vitro* inhibition of *Citrobacter freundii*, a red-leg syndrome associated pathogen in raniculture, by indigenous *Lactococcus lactis* CRL 1584. *Veterinary Microbiology*, 151(3): 336 - 344.

Paterson, S., Wilson, K. & Pemberton, J. M. (1998). Major histocompatibility complex (MHC) variation associated with juvenile survival and parasite resistance in a large ungulate population (*Ovis aries L.). Proceedings of the National Academy of Sciences of the United States of America*, 95: 3714 - 3719.

Patz, J., Olson, S. H., Uejio, C. & Gibbs, H. (2008). Disease emergence from global climate and land use change. *Medical Clinics of North America*, 92(6): 1473 - 1491.

Pauchard, A. & Alaback, P. B. (2004). Influence of elevation, land use, and landscape context on patterns of alien plant invasions along roadsides in protected areas of south-central Chile. *Conservation Biology,* 18*:* 238 - 248.

Pels, B. & Sabelis, M. W. (1999). Local dynamics, overexploitation and predator dispersal in an acarine predator–prey system. *Oikos,* 86: 573 - 583.

Penczykowski, R. M., Laine, A. L. & Koskella, B. (2016). Understanding the ecology and evolution of host–parasite interactions across scales. *Evolutionary Applications*, 9(1): 37 - 52.

Pessier, A. P., Nichols, D. K., Longcore, J. E. & Fuller, M. S. (1999). Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates spp*.) and White's tree frogs (*Litoria caerulea*). *Journal of Veterinary Diagnostic Investigations,* 11: 194 - 199.

Pessier, A. P. (2008). Amphibian chytridiomycosis. In: Fowler, M. E. & Miller, E. R. (Eds.), *Zoo and wild animal medicine*. Current therapy. St. Louis: Saunders Elsevier, pp. 137 - 143.

Petney, T. N. & Andrews, R. H. (1998). Multiparasite communities in animals and humans: frequency, structure and parasitic significance. *International Journal for Parasitology,* 28: 377 - 393.

Phillott, A. D., Speare, R., Hines, H. B., Skerratt, L. F., Meyer, E., McDonald, K. R., Cashiins, S. D., Mendez, D. & Berger, L. (2010). Minimising exposure of amphibians to pathogens during field studies. *Diseases of Aquatic Organisms*, 92: 175 - 185.

Phillott, A. D., Grogan, L. F., Cashins, S. D., McDonald, K. R., Berger, L. and Skerratt, L. F. (2013). Chytridiomycosis and seasonal mortality of tropical stream-associated frogs 15 years after introduction of *Batrachochytrium dendrobatidis*. *Conservation Biology*, 27: 1058 - 1068.

Piccini, C., Conde, D., Alonso, C., Sommaruga, R. & Pernthaler, J. (2006). Blooms of single bacterial species in a coastal lagoon of the southwestern Atlantic Ocean. *Applied and Environmental Microbiology*, 72(10): 6560 - 6568.

Piotrowski, J. S., Annis, S. L. & Longcore, J. E. (2001). Physiology, zoospore behavior, and enzyme production of *Batrachochytrium dendrobatidis*, a chytrid pathogenic to amphibians. *Phytopathology,* 91: S121.

Piotrowski, J. S., Annis, S. L. & Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, 96(1): 9 - 15.

Potthoff, R. F. & Whittinghill, M. (1966). Testing for homogeneity. I. The binomial and multinominal distributions. *Biometrika*, 53: 167 - 182.

Pounds, J. A. & Crump, M. L. (1994). Amphibian declines and climate disturbance: the case of the golden toad and the harlequin frog. *Conservation Biology*, 8(1), 72 - 85.

Pounds, J. A., Bustamante, M. R., Coloma, L. A., Consuegra, J. A., Fogden, M. P. L., Foster, P. N., La Marca, E., Masters, K. L., Merino-Viteri, A., Puschendorf, R., Ron, S. R., Sánchez-Azofeifa, G. A., Still, C. J. & Young, B. E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, 439(7073): 161 - 167.

Price, D. T., McKenney, D. W., Joyce, L. A., Siltanen, R. M., Papadopol, P. & Lawrence, K. (2011). *High-resolution interpolation of climate scenarios for Canada derived from general circulation model simulations*. Natural Resources Canada, Canadian Forest Service, Northern Forestry Centre, Edmonton, AB.

Price, S. J., Garner, T. W., Balloux, F., Ruis, C., Paszkiewicz, K. H., Moore, K. & Griffiths, A. G. (2015). A de novo assembly of the common frog (*Rana temporaria*) transcriptome and comparison of transcription following exposure to Ranavirus and *Batrachochytrium dendrobatidis*. *PLoS ONE*, 10(6): e0130500.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*, 35(21): 7188 - 7196.

Puschendorf, R., Carnaval, A. C., VanDerWal, J., Zumbado-Ulate, H., Chaves, G., Bolaños, F. & Alford, R. A. (2009). Distribution models for the amphibian chytrid *Batrachochytrium dendrobatidis* in Costa Rica: proposing climatic refuges as a conservation tool. *Diversity and Distributions*, 15: 401 - 408.

Puschendorf, R., Hoskin, C. J., Cashins, S. D., McDonald, K., Skerratt, L. F., Vanderwal, J. & Alford, R. A. (2011). Environmental refuge from disease-driven amphibian extinction. *Conservation Biology*, 25: 956 - 964.

Pusenius, J. & Viitala, J. (1993). Varying spacing behaviour of breeding field voles, *Microtus agrestis*. *Annales Zoologici Fennici,* 30: 143 - 152.

Quinn, G. P. & Keough, M. J. (2002). *Experimental design and data analysis for biologists*. Cambridge University Press; Cambridge, UK, p. 556.

R Core Team (2015). *R: A language and environment for statistical computing: R Foundation for Statistical Computing*. Vienna, Austria, 3(0). Available: http://www.r- project.org/ [accessed 2012 January 5].

Rachowicz, L. J. (2002). Mouthpart pigmentation in *Rana muscosa* tadpoles: seasonal changes without chytridiomycosis. *Herpetological Review*, 33: 263 - 265.

Rachowicz, L. J. & Vredenburg, V. T. (2004). Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Disease of Aquatic Organisms*, 61: 75 - 83.

Rachowicz, L. J., Hero, J., Alford, R. A., Taylor, J. W., Morgan, J. A. T., Vredenburg, V. T., Collins, J. P. & Briggs, C. J. (2005). The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology*, 19(5): 1441 - 1448.

Rachowicz, L. J., Knapp, R. A., Morgan, J. A. T., Stice, M. J., Vredenburg, V. T., Parker, J. M. & Briggs, C. J. (2006). Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology*, 87: 1671 - 1683.

Rachowicz, L. J. & Briggs, C. J. (2007). Quantifying the disease transmission function: effects of density on *Batrachochytrium dendrobatidis* transmission in the mountain yellow legged frog *Rana muscosa*. *Journal of Animal Ecology*, *76*(4): 711 - 721.

Raffel, T. R., Rohr, J. R., Kiesecker, J. M. & Hudson, P. J. (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Functional Ecology*, 20: 819 - 828.

Raffel, T. R., Romansic, J. M., Halstead, N. T., McMahon, T. A., Venesky, M. D. & Rohr, J. R. (2013). Disease and thermal acclimation in a more variable and unpredictable climate. *Nature Climate Change,* 3: 146 - 151.

Rafferty, K. A. (1965) The cultivation of inclusion-associated viruses from *Lucke tumor* frogs. *Annals of the New York Academy of Sciences,* 126: 3 - 21.

Rajeev, S., Sutton, D. A., Wickes, B. L., Miller, D. L., Giri, D., Van Meter, M., Thompson, E. H., Rinaldi, M. G., Romanelli, A. M., Cano, J. F. & Guarro, J. (2009). Isolation and characterization of a new fungal species, *Chrysosporium ophiodiicola*, from a mycotic granuloma of a black rat snake (*Elaphe obsoleta obsoleta*). *Journal of Clinical Microbiology*, 47(4): 1264 - 1268.

Ramette, A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiology Ecology,* 62(2): 142 - 160.

Rausher, M. D. (2001). Co-evolution and plant resistance to natural enemies. *Nature,* 411(6839): 857 - 864.

Real, L. A. & McElhany, P. (1996). Spatial pattern and process in plant-parasite interactions. *Ecology*, 77: 1011 - 1025.

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., Harrison, X. A., Hughey, M. C., Kueneman, J. G., Loudon, A. H., McKenzie, V., Medina, D., Minbiole, K. P. C., Rollins-Smith, L. A., Banning Walke, J., Weiss, S., Woodhams, D. C. & Harris, R. N. (2016a). Using "mics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Frontiers in Microbiology*, 7: 68.

Rebollar, E. A., Simonetti, S. J., Shoemaker, W. R. & Harris, R. N. (2016b). Direct and indirect horizontal transmission of the antifungal probiotic bacterium *Janthinobacterium lividum* on green frog (*Lithobates clamitans*) tadpoles. *Applied Environmental Microbiology*, 82(8): 2457 - 2466.

Reeder, N. M., Pessier, A. P. & Vredenburg, V. T. (2012). A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. *PLoS ONE*, 7(3): e33567.

Reid, G., Younes, J. A., Van der Mei, H. C., Gloor, G. B., Knight, R. & Busscher, H. J. (2011). Microbiota restoration: natural and supplemented recovery of human microbial communities. *Nature Reviews. Microbiology,* 9: 27 - 38.

Restif, O. & Koella, J. (2004). Concurrent evolution of resistance and tolerance to pathogens. *The American Naturalist*, 164(4): E90 - 102.

Retallick, R. (2002). *Using experimental translocations to learn about declines in Queensland's frog populations*. Final report. Queensland Parks and Wildlife Service, Atherton.

Retallick, R. W. R., McCallum, H. & Speare, R. (2004). Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLoS Biology*, 2: 1965 - 1971.

Retallick, R. W. R. & Miera, V. (2007). Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Diseases of Aquatic Organisms,* 75: 201 - 207.

Reznick, D. N., Shaw, F. H., Rodd, F. H. & Shaw, R. G. (1997). Evaluation of the rate of evolution in natural populations of guppies (*Poecilia reticulata*). *Science*, 275: 1934 - 1936.

Ribas, L., Li, M. S., Doddington, B. J., Robert, J., Seidel, J. A., Kroll, J. S., Zimmerman, L. B., Grassly, N. C., Garner, T. W. J. & Fisher, M. C. (2009). Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE,* 4: e8408.

Richards, S. A. (2008). Dealing with overdispersed count data in applied ecology. *Journal of Applied Ecology,* 45: 218 - 227.

Richards, S. A., Whittingham, M. J. & Stephens, P. A. (2011). Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework. *Behavioural Ecology and Sociobiology,* 65(1): 77 - 89.

Richards-Zawacki, C. L. (2010). Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. *Proceedings of the Royal Society B: Biological Sciences*, 277: 519 - 528.

Ringø, E., Sperstad, S., Myklebust, R., Refstie, S. & Krogdahl, Å. (2006). Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua L.*): the effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture*, 261: 829 - 841.

Robert, J., Morales, H., Buck, W., Cohen, N., Marr, S. & Gantress, J. (2005). Adaptive immunity and histopathology in frog virus 3-infected Xenopus. *Virology,* 332(2): 667 - 675.

Robert, J. H. (2015). *Package 'geosphere': spherical trigonometry*. R package, version 1.3-13. Available: https://cran.r-project.org/web/packages/geosphere.pdf [accessed 2015 November 2].

Robinson, D. H. & Heintzelman, M. B. (1987). Morphology of ventral epidermis of *Rana catesbeiana* during metamorphosis. *The Anatomical Record*, 217: 305 - 317.

Roelke-Parker, M. E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S. J., Pospischil, A., Hofmann-Lehmann, R., Lutz, H., Mwamengele, G. L. M., Mgasa, M. N., Machange, G. A., Summers, B. A & Appel, M. J. G. (1996). A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). *Nature*, 376: 441 - 445.

Rohani, P., Earn, D. J. D., Finkenstadt, B. F. & Grenfell, B. T. (1998). Population dynamic interference among childhood diseases. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* 265: 2033 - 2041.

Rohr, J. R., Raffel, T. R., Romansic, J. M., McCallum, H. & Hudson, P. J. (2008). Evaluating the links between climate, disease, and amphibian declines. *Proceedings of the National Academy of Sciences of the United States of America*, 105(45): 17436 - 17441.

Rohr, J. R. & Raffel, T. R. (2010). Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proceedings of the National Academy of Sciences of the United States of America,* 107(18): 8269 - 8274.

Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. *Immunological Reviews*, 166: 221 - 230.

Rollins-Smith, L. A., Carey, C., Longcore, J., Doersam, J. K., Boutte, A., Bruzgal, J. E. & Conlon, J. M. (2002). Activity of antimicrobial skin peptides from ranid frogs against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. *Developmental & Comparative Immunology*, 26(5): 471 - 479.

Rollins-Smith, L. A. & Conlon, J. M. (2005). Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Developmental & Comparative Immunology*, 29(7): 589 - 598.

Rollins-Smith, L. A., Reinert, L. K., O'Leary, C. J., Houston, L. E. & Woodhams, D. C. (2005). Antimicrobial peptide defenses in amphibian skin. *Integrative and Comparative Biology*, 45(1): 137 - 142.

Rollins-Smith, L. A., Woodhams, D. C., Reinert, L. K., Vredenburg, V. T., Briggs C. J., Nielsen, P. F. & Conlon, J. M. (2006). Antimicrobial peptide defenses of the mountain yellow-legged frog (*Rana muscosa*). *Developmental and Comparative Immunology*, 30: 831 - 842.

Rollins-Smith, L. A. (2009). The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1788(8): 1593 - 1599.

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L.K. & Woodhams, D. C. (2011). Amphibian immune defenses against chytridiomycosis: impacts of changing environments. *Integrative and Comparative Biology*, p. icr095.

Rollins-Smith, L. A. & Woodhams, D. C. (2012). Amphibian immunity: staying in tune with the environment. In: Demas, G. E. & Nelson, R. J. (Eds.), *Ecoimmunology*. Oxford University Press, New York, pp. 92 - 143.

Romanchuk, K.A. & Quinlan, R. W. (2006). *Magrath northern leopard frog reintroduction project: final report*. Alberta Sustainable Resource Development, Fish and Wildlife Division, Alberta Species at Risk Report No. 104. Edmonton, Alberta, p. 33.

Ron, S. (2005). Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the New World. *Biotropica*, 37: 209 - 221.

Rorabaugh, J. C. (2005). Northern Leopard Frog. In: Lannoo, M. (Ed.), *Amphibian declines: the conservation status of United States species*. University of California Press, Berkeley, CA, pp. 570 - 577.

Rosa, G. M., Sabino-Pinto, J., Laurentino, T. G., Martel, A., Pasmans, F., Rebelo, R., Griffiths, R. A., Stöhr, A. C., Marschang, R. E., Price, S. J., Garner, T. W. J. & Bosch, J. (2017). Impact of asynchronous emergence of two lethal pathogens on amphibian assemblages. *Scientific Reports*, 7(43260): 1 - 10.

Round, J. L. & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nature Review Immunology*, 9(5): 313 - 323.

Rowley, J. J. L., Alford, R. A. & Skerratt, L. F. (2006). The amphibian chytrid *Batrachochytrium dendrobatidis* occurs on freshwater shrimp in rain forest streams in Northern Queensland, Australia. *EcoHealth,* 3(1): 49 - 52.

Rowley, J. J. L. & Alford, R. A. (2007a). Non-contact infrared thermometers can accurately measure amphibian body temperatures. *Herpetological Review*, 38: 308 - 311.

Rowley, J. J. L. & Alford, R. A. (2007b). Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. *Diseases of Aquatic Organisms*, 77: 1 - 9.

Rowley, J. J. L. & Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in nature. *Scientific Reports*, 3: 1515.

Roy, B. A. & Kirchner, J. W. (2000). Evolutionary dynamics of pathogen resistance and tolerance. *Evolution,* 54: 51 - 63.

Ruggeri, J., Longo, A. V., Gaiarsa, M. P., Alencar, L. R. V., Lambertini, C., Leite, D. S., Carvalho-e-Silva, S. P., Zamudio, K. R., Toledo, L. F. & Martins, M. (2015). Seasonal variation in population abundance and chytrid infection in stream-dwelling frogs of the Brazilian Atlantic Forest. *PLoS ONE*, 10(7): e0130554.

Russell, R. W., Hecnar, S. J. & Haffner, G. D. (1995). Organochlorine pesticide residues in southern Ontario spring peepers. *Environmental Toxicology Chemistry*, 14: 815 - 817.

Ryder, J. J., Miller, M. R., White, A., Knell, R. J. & Boots, M. (2007). Host-parasite population dynamics under combined frequency- and density-dependent transmission. *Oikos*, 116(12): 2017 - 2026.

Rynkiewicz, E. C., Pederson, A. B. & Fenton, A. (2015). An ecosystem approach to understanding and managing within-host parasite community dynamics. *Trends in Parasitology*, 31: 1 - 10.

Saenz, D., Fitzgerald, L. A., Baum, K. A. & Conner, R. N. (2006). Abiotic correlates of anuran calling phenology: the importance of rain, temperature, and season. *Herpetological Monographs*, 20: 64 - 82.

Saino, N., Ambrosini, R., Rubolini, D., von Hardenberg, J., Provenzale, A., **H**ü**p**pop, K., **H**ü**p**pop, O., Lehikoinen, A., Lehikoinen, E., Rainio, K., Romano, M. & Sokolov, L. (2011). Climate warming, ecological mismatch at arrival and population decline in migratory birds. *Proceedings of the Royal Society B: Biological Sciences*, 278(1707): 835 - 842.

Sandell, M., Agrell, J., Erling, S. & Nelson, J. (1990). Natal dispersal in relation to population density and sex ratio in the field vole, *Microtus agrestis*. *Oecologia,* 83: 145 - 149.

Sapsford, S. J., Alford, R. A. & Schwarzkopf, L. (2013). Elevation, temperature, and aquatic connectivity all influence the infection dynamics of the amphibian chytrid fungus in adult frogs. *PLoS ONE*, 8: e82425.

Savage, A. E. & Zamudio, K. R. (2011). MHC genotypes associate with resistance to a frog-killing fungus. *Proceedings of the National Academy of Sciences of the United States of America,* 108(40): 16705 - 16710.

Scheele, B. C., Guarino, F., Osborne, W., Hunter, D. A., Skerratt, L. F. & Driscoll, D. A. (2014). Decline and re-expansion of an amphibian with high prevalence of chytrid fungus. *Biological Conservation*, 170: 86 - 91.

Scheele, B. C., Skerratt, L. F., Grogan, L. F., Hunter, D. A., Clemann, N., McFadden, M., Newell, D., Hoskin, C. J., Gillespie, G. R., Heard, G. W., Brannelly, L., Roberts, A. A. & Berger, L. (2017). After the epidemic: ongoing declines, stabilizations and recoveries in amphibians afflicted by chytridiomycosis. *Biological Conservation*, 206: 37 - 46.

Scheiner, S. M. & Callahan, H. S. (1999). Measuring natural selection on phenotypic plasticity. *Evolution*, 53: 1704 - 1713.

Schielzeth, H. (2010). Simple means to improve the interpretability of regression coefficients. *Methods in Ecology and Evolution,* 1: 103 - 113.

Schloegel, L. M., Picco, A. M., Kilpatrick, A. M., Davies, A. J., Hyatt, A. D. & Daszak, P. (2009). Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). *Biological Conservation*, 142: 1420 - 1426.

Schloegel, L. M., Toledo, L. F., Longcore, J. E., Greenspan, S. E., Viera, C. A., Lee, M., Zhao, S., Wangen, C., Ferreira, C. M., Hipolito, M., Davies, A. J., Cuomo, C. A., Daszak, P. & James, T. Y. (2012). Novel, panzootic, and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Molecular Ecology*, 21(21): 5162 - 5177.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J. & Sahl, J. W. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75(23): 7537 - 7541.

Schock, D. M., Ruthig, G. R., Collins, J. P., Kutz, S. J., Carrière, S., Gau, R. J., Veitch, A. M., Larter, N. C., Tate, D. P., Guthrie, G. & Allaire, D. G. (2010). Amphibian chytrid fungus and ranaviruses in the Northwest Territories, Canada. *Diseases of Aquatic Organisms*, 92(2-3): 231 - 240.

Scholthof, K. B. G. (2007). The disease triangle: pathogens, the environment and society. *Nature Reviews Microbiology*, 5: 152 - 156.

Schrag, S. J. & Wiener, P. (1995). Emerging infectious disease: what are the relative roles of ecology and evolution? *Trends in Ecology and Evolution*, 10: 319 - 324.

Screen, J. A. (2014). Arctic amplification decreases temperature variance in northern mid- to high-latitudes. *Nature Climate Change*, 4: 577 - 582.

Screen, J. A. & Simmonds, I. (2014). Amplified mid-latitude planetary waves favour particular regional weather extremes. *Nature Climate Change*, 4: 704 - 709.

Seburn, C. N. L. (1992). *Management plan for the Northern Leopard Frog in Alberta*. Unpublished report, Alberta Forestry, Lands and Wildlife, Edmonton, AB, p. 52.

Seburn, D. C. & Seburn, C. N. L. (1997). *Northern Leopard Frog survey of Northern Ontario: report on a declining amphibian*. Unpublished report to the Wildlife Assessment Unit of the Ontario Ministry of Natural Resources. Thunder Bay.

Seburn, C. N. L. & Seburn, D. C. (1998). COSEWIC status report on the Northern Leopard Frog *Rana pipiens* (Southern Mountain and Prairie populations) in Canada. In: *COSEWIC assessment and status report on the Northern Leopard Frog Rana pipiens in Canada*. Ottawa: Committee on the Status of Endangered Wildlife in Canada, pp. 1 - 40.

Seimon, T. A., Seimon, A., Daszak, P., Halloy, S. R. P., Schloegel, L. M., Aguilar, C. A., Sowell, P., Hyatt, A. D., Konecky, B. & Simmons, J. E. (2007). Upward range extension of Andean anurans and chytridiomycosis to extreme elevations in response to tropical deglaciation. *Global Change Biology*, 13(1): 288 - 299.

Sharon, G., Segal, D., Zilber-Rosenberg, I. & Rosenberg, E. (2011). Symbiotic bacteria are responsible for diet-induced mating preference in *Drosophila melanogaster*, providing support for the hologenome concept of evolution. *Gut Microbes*, 2: 190 - 192.

Sharp, C. E., Brady, A. L., Sharp, G. H., Grasby, S. E., Stott, M. B. & Dunfield, P. F. (2014). Humboldt's spa: microbial diversity is controlled by temperature in geothermal environments. *The ISME journal*, 8(6): 1166 - 1174.

Sherald, J. L., Stidham, T. M., Hadidian, J. M. & Hoeldtke, J. E. (1996). Progression of the dogwood anthracnose epidemic and the status of flowering dogwood in Catoctin Mountain Park. *Plant Disease*, 80: 310 - 312.

Shoemaker, V. H. (1992). Exchange of water, ions and respiratory gases in terrestrial amphibians. In: Feder, M. E. & Burggren, W. W. (Eds.), *Environmental physiology of the amphibians*. Chicago: University of Chicago Press, pp. 125 - 150.

Simms, E. L. & Triplett, J. (1994). Costs and benefits of plant responses to disease. *Evolution*, 48: 1973 - 1985.

Sjöling, S., Mohammed, S. M., Lyimo, T. J. & Kyaruzi, J. J. (2005). Benthic bacterial diversity and nutrient processes in mangroves: impact of deforestation. *Estuarine and Coastal Shelf Science*, 63: 397 - 406.

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., Hines, H. B. & Kenyon, N. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth*, 4: 125 - 134.

Smith, B. E. & Keinath, D. A. (2007). *Northern Leopard Frog (Rana pipiens): a technical conservation assessment [online]*. USDA Forest Service, Rocky Mountain Region. Available: http://www.fs.fed.us/r2/projects/scp/assessments/northernleopardfrog.pdf [accessed: 2010 June 22].

Smith, G. W., Ives, L, D., Nagelkerken, I. A. & Ritchie, K. B. (1996). Caribbean sea-fan mortalities. *Nature*, 383: 487.

Smith, K. F., Sax, D. F. & Lafferty, K. D. (2006). Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology,* 20(5): 1349 - 1357.

Smith, P. G. (1975). Aldosterone-induced molting in amphibian skin and its effect on electrical capacitance. *The Journal of Membrane Biology*, 22: 165 - 181.

Smyth, J. D. & Smyth, M. M. (1980). *Frogs as host parasite systems*. Macmillan Press, London, UK.

Sokurenko, E. V., Hasty, D. L. & Dykhuizen, D. E. (1999). Pathoadaptive mutations: gene loss and variation in bacterial pathogens. *Trends in Microbiology,* 7: 191 - 195.

Souder, W. (2000). *A plague of frogs*. New York, NY, Hyperion.

Soulé, M. E. (1985). What is conservation biology? *Bioscience,* 35: 727 - 734.

Spielman, D., Brook, B. W., Briscoe, D. A. & Frankham, R. (2004). Does inbreeding and loss of genetic diversity decrease disease resistance? *Conservation Genetics*, 5: 439 - 448.

Spotila, J. R. (1972). Role of temperature and water in ecology of lungless salamanders. *Ecological Monographs*, 42: 95 - 125.

St-Amour, V., Wong, W. M., Garner, T. W. J. & Lesbarrères, D. (2008). Anthropogenic influence on prevalence of 2 amphibian parasites. *Emerging Infectious Diseases,* 14: 1175 - 1176.

Statistics Canada (2011a). *Rivers (lines), 2011 Census – Boundary files (water file, ghy_000d11a_e.zip)*. Arc Info Edition. Available: https://www12.statcan.gc.ca/census-recensement/2011/geo/bound-limit/bound-limit-2011-eng.cfm [accessed: 2015 November 20]. System requirements: Arc Info Interchange for Windows.

Statistics Canada (2011b). *Population centres, 2011 Census – Boundary files (cartographic boundary file, ghy_000b11a_e.zip)*. Arc Info Edition. Available: https://www12.statcan.gc.ca/census-recensement/2011/geo/bound-limit/bound-limit-2011-eng.cfm [accessed: 2015 November 20]. System requirements: Arc Info Interchange for Windows.

Stebbins, R. C. (2003). *A field guide to western reptiles and amphibians*, 3rd edition. Houghton Mif in Company, Boston, MA, p. 533.

Stecher, B., Robbiani, R., Walker, A. W., Westendorf, A. M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A. J., Buer, J., Parkhill, J., Dougan, G., von Mering, C. & Hardt, W. (2007). *Salmonella enterica serovar typhimurium* exploits inflammation to compete with the intestinal microbiota. *PLoS Biology*, 5(10): 2177 - 2189.

Steinhaus, E. A. (1960). II. The importance of environmental factors in the insect-microbe ecosystem. *Bacteriological Reviews*, *24*(4): 365.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L. & Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science,* 306(5702): 1783 - 1786.

Stuart, S. N., Hoffman, M., Chanson, J. S., Cox, N. A., Berridge, R. J., Ramani, P. & Young B. E. (2008*). Threatened amphibian species of the world*. Lynx Edicions, Barcelona, Spain; IUCN, Gland, Switzerland, Conservational International, Arlington, Virginia, USA.

Sutton, S. D., Barrett, G. W. & Taylor, D. H. (1991). Microbial metabolic activities in soils of old-field communities following eleven years of nutrient enrichment. *Environmental Pollution*, 73: 1 - 10.

Suzuki, K., Harasawa, R., Yoshitake, Y. & Mitsuoka, T. (1983). Effects of crowding and heat stress on intestinal flora, body weight gain, and feed efficiency of growing rats and chicks. *Nihon Juigaku Zasshi,* 45(3): 331 - 338.

Tang, J., Li, J., Shao, Y., Yang, B. & Liu, Z. (2010). Fipronil resistance in the whitebacked planthopper (*Sogatella furcifera*): possible resistance mechanisms and cross resistance. *Pest management science*, 66(2): 121 - 125.

Tannock, G. W. & Savage, D. C. (1974). Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. *Infection and Immunity*, 9(3): 591 - 598.

Taylor, L. H. & Read, A. F. (1997). Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitology Today,* 13: 135 - 139.

Taylor, S. & Ewer, D. W. (1956). Moulting in the Anura: the normal moulting cycle of *Bufo regularis* Reuss. *Journal of Zoology*, 127: 461 - 478.

Terrell, K. A., Quintero, R. P., Murra, S., Kleopfer, J. D., Murphy, J. B., Evans, M. J., Nissen, B. D. & Gratwicke, B. (2013). Cryptic impacts of temperature variability on amphibian immune function. *The Journal of Experimental Biology*, 216(22): 4204 - 4211.

Terrell, V. C. K., Engbrecht, N. J., Pessier, A. P. & Lannoo, M. J. (2014). Drought reduces chytrid fungus (*Batrachochytrium dendrobatidis*) infection intensity and mortality but not prevalence in adult crawfish frogs (*Lithobates areolatus*). *Journal of Wildlife Diseases*, 50: 56 - 62.

Theriot, C. M., Koenigsknecht, M. J., Carlson Jr, P. E., Hatton, G. E., Nelson, A. M., Li, B., Huffnagle, G. B., Li, J. Z. & Young, V. B. (2014). Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nature Communications*, *5*.

Thompson, J. N. (1998). Rapid evolution as an ecological process. *Trends in Ecology and Evolution*, 13: 329 - 332.

Thorson, T. & Svihla, A. (1943). Correlation of the habitat of amphibians with their ability to survive the loss of body water. *Ecology*, 24: 374 - 381.

Thorson, T. B. (1955). The relationship of water economy to terrestrialism in amphibians. *Ecology* 36: 100 - 116.

Timofeyev, L., Loseva, E., Alekseeva, T. & Perminova, N. (2002). Stability to sound stress and changeability in intestinal microflora. *European Psychiatry*, 17(1): 200.

Tittensor, D. P., Walpole, M., Hill, S. L., Boyce, D. G., Britten, G. L., Burgess, N. D., Butchart, S. H., Leadley, P. W., Regan, E. C., Alkemade, R. & Baumung, R. (2014). A mid-term analysis of progress toward international biodiversity targets. *Science*, 346(6206): 241 - 244.

Tobler, U. & Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. *PLoS ONE,* 5: e10927.

Tobler, U., Borgula, A. & Schmidt, B. R. (2012). Populations of a susceptible amphibian species can grow despite the presence of a parasitic chytrid fungus. *PLoS ONE,* 7: e34667.

Tollrian, R. & Harvell, C. D. (1999). *The ecology and evolution of inducible defenses*. Princeton: Princeton University Press.

Tompkins, D. M., Dunn, A. M., Smith, M. J. & Telfer, S. (2011). Wildlife diseases: from individuals to ecosystems. *Journal of Animal Ecology*, 80(1): 19 - 38.

Tracy, R. (1976). A model of the dynamic exchanges of water and energy between a terrestrial amphibian and its environment. *Ecological Monographs*, 46(3): 293 - 326.

Trombulak, S. C. & Frissell, C. A. (2000). Review of ecological effects of roads on terrestrial and aquatic communities. *Conservation Biology*, 14(1): 18 - 30.

Trullas, S. C., van Wyk, J. H. & Spotila, J. R. (2007). Thermal melanism in ectotherms. *Journal of Thermal Biology*, 32: 235 - 245.

Tsiafouli, M. A., Thébault, E., Sgardelis, S. P., de Ruiter, P. C., van der Putten, W. H., Birkhofer, K., Hemerik, L., de Vries, F. T., Bardgett, R. D., Brady, M. V., Bjornlund, L., Jørgensen, H. B., Christensen, S., Hertefeldt, T. D., Hotes, S., Gera Hol, W.H., Frouz, J., Liiri, M., Mortimer, S. R., Setälä, H., Tzanopoulos, J., Uteseny, K., Pižl, V., Stary, J., Wolters, V. & Hedlund, K. (2015). Intensive agriculture reduces soil biodiversity across Europe. *Global Change Biology*, 21: 973 - 985.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122): 1027 - 1131.

Van Rooij, P., Martel, A., Haesebrouck, F. & Pasmans, F. (2015). Amphibian chytridiomycosis: a review with focus on fungus-host interactions. *Veterinary Research,* 45: 137.

Vartoukian, S. R., Palmer, R. M. & Wade, W. G. (2010). Strategies for culture of 'unculturable' bacteria. *FEMS Microbiology Letters*, 309: 1 - 7.

Venesky, M. D., Raffel, T. R., McMahon, T. A. & Rohr, J. R. (2014). Confronting inconsistencies in the amphibian chytridiomycosis system: implications for disease management. *Biological Reviews*, 89(2): 477 - 483.

Volpe, E. P. (1955). A taxo-genetic analysis of the status of *Rana kandiyohi* weed. *Systematic Zoology,* 4: 75 - 82.

Volpe, E. P. (1960). Interaction of mutant genes in the leopard frog. *Journal of Heredity*, 51(4): 150 - 155.

Volpe, E. P. & Dasgupta, S. (1962). Gynogenetic diploids of mutant leopard frogs. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 151(3): 287 - 301.

Voordouw, M. J., Adama, D., Houston, B., Govindarajulu, P. & Robinson, J. (2010). Prevalence of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, in an endangered population of northern leopard frogs, *Rana pipiens*. *BMC Ecology*, 10(6).

Vos, C. C. & Chardon, J. P. (1998). Effects of habitat fragmentation and road density on the distribution pattern of the moor frog *Rana arvalis*. *Journal of Applied Ecology* 35: 44 - 56.

Voyles, J., Berger, L., Young, S., Speare, R., Webb, R., Warner, J., Rudd, D., Campbell, R. & Skerratt, L. F. (2007). Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. *Diseases of Aquatic Organisms*, 77(2): 113 - 118.

Voyles, J., Yound, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., Cook, D., Webb, R., Alford, R. A., Skerratt, L. F. & Speare, R. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science*, 326: 582 - 585.

Vredenburg, V. T. & Summers, A. P. (2001). Field identification of chytridiomycosis in *Rana muscosa* (Camp 1915). *Herpetological Review,* 32: 151 - 152.

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S. & Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences of the United States of America,* 107(21): 9689 - 9694.

Vredenburg, V. T., Briggs, C. J. & Harris, R. N. (2011). Host-pathogen dynamics of amphibian chytridiomycosis: the role of the skin microbiome in health and disease. In: Olsen, L., Choffnes, E., Relman, D. A. & Pray, L. (Eds.), *Fungal diseases: an emerging challenge to human, animal, and plant health*. Washington, DC: The National Academies Press, pp. 342 - 355.

Wakefield, J. C., Kelsall, J. E. & Morris, S. E. (2000). Clustering, cluster detection and spatial variation in risk. In: Elliott, P., Wakefield, J., Best, N. & Briggs, D. (Eds.), *Spatial epidemiology: methods and applications*. Oxford University Press, Oxford, pp. 128 - 152.

Walke, J. B., Harris, R. N., Reinert, L. K., Rollins-Smith, L. A. & Woodhams, D. C. (2011). Social immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica*, 43(4): 396 - 400.

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V. & Belden, L. K. (2014). Amphibian skin may select for rare environmental microbes. *The ISME Journal*, 8: 2207 - 2217.

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D. A., Ginestet, C., Arthur, C-P. & Fisher, M. C. (2010). Factors driving parasitism vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters,* 13: 372 - 382.

Walsh, P. D., Abernethy, K. A., Bermejo, M., Beyers, R., De Wachter, P., Akou, M. E., Huijbregts, B., Mambounga, D. I., Toham, A. K., Kilbourn, A. M., Lahm, S. A., Latour, S., Maisels, F., Mbina, C., Mihindou, Y., Obiang, S. N., Effa, E. N., Starkey, M. P., Telfer, P., Thibault, M., Tutin, C. E. G., White, L. J. T. & Wilkie, D. S. (2003). Catastrophic ape decline in western equatorial Africa. *Science,* 422: 611 - 614.

Walther, G. R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T. J. C., Fromentin, J. M., Hoegh-Guldberg, O. & Bairlein, F. (2002). Ecological responses to recent climate change. *Nature*, 416: 389 - 395.

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73: 5261 - 5267.

Warne, R. W., LaBumbard, B., LaGrange, S., Vredenburg, V. T. & Catenazzi, A. (2016). Co-infection by chytrid fungus and ranaviruses in wild and harvested frogs in the Tropical Andes. *PLoS ONE,* 11: e0145864.

Watt, W. B. (1968). Adaptive significance of pigment polymorphisms in Colias butterflies. I. Variation of melanin pigment in relation to thermoregulation. *Evolution,* 22: 437 - 458.

Waye, H. L. & Cooper, J. M. (2000). *Status of the Northern leopard frog (Rana pipiens) in the Creston Valley Wildlife Management Area 1999*. In: Report to the Columbia Basin Fish and Wildlife Compensation Program. Nelson, BC, p. 56.

Weldon, C., du Preez, L. H., Hyatt, A. D., Muller, R. & Speare, R. (2004). Origin of the amphibian chytrid fungus. *Emerging Infectious Diseases*, 10: 2100 - 2105.

Weldon, P. J., Demeter, B. J., Rosscoe, R. (1993). A survey of shed skin-eating (dermatophagy) in amphibians and reptiles. Journal of Herpetology, 27: 219 - 228.

Weller, W. F., Oldham, M. J., Schueler, F. W. & Obbard, M. E. (1994). *Report of the historical database committee: report of the historical populations trends subgroup, Canadian working group - declines in Canadian amphibians identified using historical distributional data*. In: Fourth Annual Meeting of the Task Force on Declining Amphibian Populations in Canada. Proceedings of a workshop, Manitoba Museum of Man and Nature, Winnipeg.

Weng, S.-H., Kuo, S.-R., Guan, B.T., Chang, T.-Y., Hsu, H.-W. & Shen, C.-W. (2007). Microclimatic responses to different thinning intensities in a Japanese cedar plantation of northern Taiwan. *Forest Ecology and Management*, 241: 91 - 100.

Werner, J. K. (2003). Status of the Northern Leopard Frog (*Rana pipiens*) in Western Montana. *Northwestern Naturalist*, 84(1): 24 - 30.

Wershler, C. R. (1991). *Status of the Northern Leopard Frog in Alberta - 1990*. Unpublished report by Sweetgrass Consultants Ltd. for World Wildlife Fund Canada (Prairie for Tomorrow) and Alberta Forestry, Lands and Wildlife, Edmonton, AB, p. 47.

Wickham, H. (2009). ggplot2: elegant graphics for data analysis. Springer.

Wilcove, D. S., McLellan, C. H. & Dobson, A. P. (1986). Habitat fragmentation in the temperate zone. In: Soulé, M. E. (Ed.), *Conservation biology: the science of scarcity and diversity*. Sunderland, Massachusetts: Sinauer Associates.

Willmer, P. (1991). Thermal biology and mate acquisition in ectotherms. *Trends in Ecology and Evolution*, 6: 396 - 399.

Wilson, A. J., Réale, D., Clements, M. N., Morrissey, M. M., Postma, E., Walling, C. A., Kruuk, L. E. B. & Nussey, D. H. (2010). An ecologist's guide to the animal model. *Journal of Animal Ecology*, 79: 13 - 26.

Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L., Crawford, R. W., Russell, J. M., Bevins, C. L., Adams, L. G., Tsolis, R. M., Roth, J. R. & Bäumler, A. J. (2010). Gut inflammation provides a respiratory electron acceptor for Salmonella. *Nature,* 467(7314): 426 - 429.

Witters, L. R. & Sievert, L. (2001). Feeding causes thermophily in the woodhouse's toad (*Bufo woodhousii*). *Journal of Thermal Biology*, 26(3): 205 - 208.

Wobeser, G. A. (2006). *Essentials of disease in wild animals*. Blackwell Publishing, Ames, IA.

Wolinska, J. & King, K. C. (2009). Environment can alter selection in host–parasite interactions. *Trends in Parasitology,* 25: 236 - 244.

Woodhams, D. C., Alford, R. A. & Marantelli, G. (2003). Emerging disease of amphibians cured by elevated body temperature. *Diseases of Aquatic Organisms*, 55: 65 - 67.

Woodhams, D. C. & Alford, R. A. (2005). Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conservation Biology*, 19: 1449 - 1459.

Woodhams, D. C., Ardipradja, K., Alford, R. A., Marantelli, G., Reinert, L. K. & Rollins-Smith, L. A. (2007a). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defences. *Animal Conservation*, 10: 409 - 417.

Woodhams, D., Vredenburg, V., Simon, M., Billheimer, D., Shakhtour, B., Shyr, Y., Briggs, C. J., Rollins-Smith, L. A. & Harris, R. N. (2007b). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biological Conservation*, 138: 390 - 398.

Woodhams, D. C., Alford, R. A., Briggs, C. J., Johnson, M. & Rollins-Smith, L. A. (2008a). Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. *Ecology,* 89: 1627 - 1639.

Woodhams, D. C., Hyatt, A. D. & Rollins-Smith, L. A. (2008b). The Northern Leopard Frog *Rana pipiens* is a widespread reservoir species harboring *Batrachochytrium dendrobatidis* in North America. *Herpetological Review*, 39(1): 66 - 68.

Woodhams, D. C., Bosch, J., Briggs, C. J., Cashins, S., Davis, L. R., Lauer, A., Muths, E., Puschendorf, R., Scmidt, B. R., Sheafor, B. & Voyles, J. (2011). Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology*, 8: 8 - 31.

Woodhams, D. C., Geiger, C. C., Reinert, L. K. & Voyles, J. (2012). Treatment of amphibians infected with chytrid fungus: learning from failed trials with intraconazole, antimicrobial peptides, bacteria and heat therapy. *Diseases of Aquatic Organisms*, 98(1): 11 - 25.

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tibler, U., Davis, L. R., Schmidt, B. R., Bel, C., Hodel, S., Knight, R. & McKenzie, V. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS ONE*, 9(4): e96375.

Woodmansey, E. J., McMurdo, M. E. T., MacFarlane, G. T., & MacFarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Applied and Environmental Microbiol*ogy, 70: 6113 - 6122.

Woodmansey, E. J. (2007). Intestinal bacteria and ageing. *Journal of Applied Microbiology*, 102(5): 1178 - 1186.

Wright, A. H. & Wright, A. A. (1949). *Handbook of frogs and toads of the United States and Canada*, 3rd edition. Comstock Publishing Associates: Ithaca, New York.

Yao, H., He, Z., Wilson, M. J. & Campbell, C. D. (2000). Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. *Microbial Ecology*, 40: 223 - 237.

Yergeau, E., Lawrence, J. R., Sanschagrin, S., Waiser, M. J., Korber, D. R., & Greer, C. W. (2012). Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities. *Applied and Environmental Microbiology*, 78(21): 7626 - 7637.

Yuan, S., Cohen, D. B., Ravel, J., Abdo, Z. & Forney, L. J. (2012). Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PLoS ONE*, 7(3): e33865.

Yuan, Z., Zhou, W., Chen, X., Poyarkov, N. A., Chen, H., Jang-Liaw, N., Chou, W., Matzke, N. J., Iizuka, K., Min, M., Kuzmin, S. L., Zhang, Y., Cannatella, D., Hillis, D. M. & Che, J. (2016). Spatiotemporal diversification of the true frogs (genus Rana): a historical framework for a widely studied group of model organisms. *Systematic Biology*: syw055.