**CAUSES AND CONSEQUENCES OF VARIATION IN PATTERNS OF
PARASITISM IN ANURAN AMPHIBIANS**

By

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ABSTRACT

My thesis is principally an exploration of ideas about trade-offs between life history traits and susceptibility to parasitism, and about the evolution of parasite specificity. I observed how changes in developmental rates and sizes in frogs, induced by varying rearing density, influenced establishment of parasites and altered the temporal synchrony of hosts and parasites. I also observed a relationship between sex-specific developmental trajectories of metamorphs and susceptibility to parasitism that was independent of host investment in size. I assessed the potential for adaptation of lungworms in two host species, and explored further the occurrence of sex differences in developmental rate as it relates to susceptibility to infection. I provided evidence for host-parasite adaptation among two previously confused species of lungworm, and demonstrated sex-linked variation in susceptibility to infection that appears to relate to sex differences in developmental trajectories in a host species not expected to demonstrate such a bias. I then explored how my results from experimental studies might bear on parasitism in frog populations. My field surveys revealed patterns of infection among host sexes that were not predicted by controlled studies, and pointed to other hypotheses explaining the observed natural variation. Results from these surveys lent further support for the occurrence of host-parasite adaptation. I also examined patterns of parasitism among natural populations of an invasive host species to test predictions based on the hypothesis of parasite release as a mechanism of enhanced competitive

fitness. I found little evidence for parasite release as a regulating factor for an invasive host species. My research largely concerned parasites interacting with hosts, but in a final study, I explored the potential for parasite-parasite interactions by observing patterns of segregation of two parasites that exploit similar niches within a host. My thesis underscores the importance of host life history and parasite specificity in creating variation in host susceptibility to parasitism and variation in host adaptation by parasites. While these two factors are important, they are not alone in determining variation in parasitism and its consequences.

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TABLE OF CONTENTS

Abstract.....	i
Acknowledgements.....	iv
Table of Contents.....	ix
List of Tables.....	x
List of Figures.....	xiii
List of Appendices	xiv
Chapter 1: General Introduction.....	1
Glossary of Terms.....	18
Chapter 2: Rearing density and susceptibility of <i>Rana pipiens</i> metamorphs to cercariae of a digenetic trematode.....	19
Chapter 3: Rates of development in male and female Wood frogs and patterns of parasitism by lung nematodes.....	38
Chapter 4: Nematode lungworms of two species of anuran amphibians: evidence for adaptation.....	64
Chapter 5: Patterns of infection by two genera of lungworms in a population of Northern leopard frogs.....	90
Chapter 6: Testing the predictions of parasite release: species richness and species- specific parasitism in invasive bullfrog hosts.....	111
Chapter 7: General Discussion.....	135
References.....	146
Appendix chapters	170

LIST OF TABLES

2- 1 Results from a series of Nested ANOVAs where the effect of rearing density on log ₁₀ -transformed developmental variables (Mass, SVL, and Time to metamorphosis) was assessed after controlling for the effect of tank nested within the density treatments.....	35
2- 2 Backtransformed means $\pm$ 1 standard error (SE) for log-transformed Mass, SVL and Time to metamorphosis.....	36
2- 3 Backtransformed means (ranges $\pm$ 1 standard error (SE) of the proportion of parasites established).	37
3- 1 Developmental trajectories of male and female <i>R. sylvatica</i> . Means $\pm$ SE of mass (g), snout-vent length (SVL) (cm) and time to metamorphosis (d).....	59
3- 2 Median abundance and intensity of female worms (interquartile ranges in parentheses) established in male and female metamorphs. T refers to the test statistic.....	60
4- 1 Distribution of male and female <i>Lithobates pipiens</i> metamorphs reared among 13 tanks and subsequently exposed to infective <i>Rhabdias</i> sp. larvae.....	86
4- 2 Associations between parasite species and host sex on mass (g), day of metamorphosis (d), and snout to vent length (cm). Data shown include mean $\pm$ SE.....	87

LIST OF TABLES (cont.d)

4- 3 Effects of parasite species on worm establishment (range) and worm length (mm), controlling for sex of host. Data shown include mean $\pm$ SE.	88
5- 1 Prevalence % (CI) of <i>Rhabdias ranae</i> (R.) and <i>Haematoloechus</i> (H.) of breeding and non-breeding adult, and juvenile frogs (N).....	107
5- 2 Prevalence (%), mean abundance $\pm$ SE and intensity $\pm$ SE of <i>R. ranae</i> infections in male and female adult (breeding and non-breeding) and juvenile frogs.....	108
5- 3 Prevalence (%), mean abundance $\pm$ SE and intensity $\pm$ SE of <i>Haematoloechus</i> infections in male and female adult (breeding and non-breeding) and juvenile frogs.....	109
5- 4 Grand mean lengths of <i>R. ranae</i> worms collected from female and male adult (breeding and non-breeding) and juvenile Northern Leopard frogs.....	110
6- 1 Numbers of juvenile and adult frogs sampled from Bishops Mills, ON and Victoria sites, BC.....	132
6- 2 Median abundance, interquartile range (IQR) and actual range of <i>Haematoloechus</i> spp. lung flukes from bullfrogs collected among 5 sites in Victoria, BC.....	133
6- 3 Median abundance, interquartile range (IQR) and actual range of kidney cysts (echinostome) from bullfrogs collected among 5 sites in Victoria, BC.....	134

LIST OF TABLES (cont.d)

Table A1- 1 <i>Rhabdias</i> prevalence (%) with Clopper-Pearson confidence intervals (Zar 1996) on binomial proportions (CI), abundance, intensity and worm length (μm) of male and female frogs collected in the first week of April, 2007.....	182
Table A2-1 Numbers of frogs carrying lungworm infections (<i>Haematoloechus</i> spp., <i>Rhabdias</i> spp. or both).....	192
Table A2-2 <i>Haematoloechus</i> spp. prevalence (%) with Clopper-Pearson confidence intervals on binomial proportions (CI), abundance and intensity of breeding male Northern leopard frogs and male Wood frogs collected in early and late April, 2007.	193

LIST OF FIGURES

3- 1 Representative stages of <i>Rhabdias ranae</i>	61
3- 2 Relationships between developmental rate (mean number of days to metamorphosis) and parasite establishment (mean numbers of female worms that established in lungs) for male and female metamorphs, on a per tank basis.....	62
4- 1 Single most parsimonious tree inferred from branch-and-bound analysis of ITS/5.8S rDNA sequence data (CI=0.98). Tree was mid-point rooted. Sequences include DQ264773 (<i>Rhabdias bakeri</i>), DQ845738 (<i>Rana pipiens</i>), DQ264769 (<i>Rhabdias ranae</i>), and DQ845741 (<i>Rana sylvatica</i>).....	89

LIST OF APPENDICES

Appendix I:	Patterns of parasitism of <i>Rhabdias</i> lungworms in two sympatric ranid hosts.....	170
Appendix II:	Observations on trematode and nematode co-infections in lungs in ranid hosts.....	183
Appendix III:	Generalized Life Cycles of Helminths of Interest.....	194

CHAPTER I. GENERAL INTRODUCTION

The field of Parasitology has matured from the elementary reporting and characterization of life cycles of parasites and their hosts, to an acknowledgement of the multi-faceted dimensions in which parasites and hosts interact within ecosystems. Modern ecological parasitologists and evolutionary biologists now recognize the important role of parasites in driving species interactions and have turned their focus to the underpinnings of the patterns of parasitism originally documented by classical parasitologists, and the consequences of such variation for species interactions in an ecological framework.

Our understanding of the mechanisms that drive variation is still very much in its infancy, and each step forward reveals ever more layers of complexity in interactions that may never be fully explained. Yet with the advancement of modern technical tools and molecular techniques we now are able to elucidate subtleties that were previously impossible to decipher. Further study will undoubtedly result in both the re-assessment of original assumptions about the nature of host-parasite interactions in the environment, and the development of new hypotheses to explain patterns observed in nature.

My thesis stems from an interest in two main arms of parasitological research that address evolutionary relationships between hosts and parasites that are thought to result in variation in patterns of parasitism. I have addressed several questions in parasite-host ecology from a broad perspective. I will first

introduce the basic tenets of present research exploring causes of variation in parasitism. I will then introduce the general ideas underlying each of my chapters and outline specific objectives and questions addressed therein. I will also introduce the experimental or observational design of each study as it was used to test the proposed hypotheses, and discuss some of the design challenges that I faced.

Causes of variation in parasitism

The over-dispersed pattern of parasites in host populations has continued to intrigue parasitologists, who have asked both specific and broad questions about the causes of such variation in an attempt to understand the nature of the interactions between hosts and parasites. Of particular interest are those host individuals that serve as primary drivers of subsequent cohorts of parasites in populations. Two main branches of parasitological research have arisen as a result of the different approaches to explaining variability in parasitism.

One approach explores specific interactions between hosts and parasites. Variation in parasitism is thought to arise as a result of a reciprocal evolutionary arms race between hosts and parasites in which each player, host or parasite, evolves and maintains an advantage over the other (the Red Queen hypothesis, Van Valen 1973). From the viewpoint of a parasite, transmission and establishment may be improved through the evolution of traits that permit the evasion of more general immune responses of hosts and/ or by targeting the few individuals in a population that are genetically predisposed to infection. Hosts, in

turn, respond to the pressure of parasitism and evolve evasive traits and/or resistance to parasitic infection. And thus it is believed that this specific co-evolution, modulated by a dynamic host immune system, may lead to the observed variability in host susceptibility within and among populations.

However, variable or stochastic environmental conditions may also influence exposure to infection and cause variation in the prevalence of infection in host populations.

Despite the large amount of study on this topic, there still remains a lack of information about the ecological consequences of adaptation from the perspective of the parasite. One important question, on which little empirical study has been focused, is the extent to which adaptation influences patterns of parasitism among different host species in natural systems. Parasite **fitness**, and potentially the influence of parasites on host population demography, may hinge on the closeness of the relationship between parasite and host species.

The other main branch of parasitological research explores variation in parasitism from an evolutionary perspective, in which individual hosts are constrained in their response to infection by trade offs between immune function and other life history traits. Such tradeoffs between specific immune response and other life history traits are difficult to demonstrate because the responses of specific components of the immune system are difficult to isolate. However, the negative relationships observed between levels of parasitism and investment in traits such as growth or reproduction suggest that there can be significant costs

to investment in immune function (Lochmiller and Deerenberg 2000; Schmid-Hempel and Ebert 2003).

Variation in response to parasitism is thought to arise as a result of individual differences in investment between somatic and reproductive functions and immune response. However, the degree and direction of investment are in turn evolved responses to selection pressures, such that patterns of allocation may be dictated by life history strategies adopted, for example, by one or another sex or, at different life stages of development. Thus, an understanding of the constraints imposed by selection can shed light on the causes of variation in investment in immune response, and enable the prediction of individuals that are more susceptible to infection. The picture is expected to become clearer for species that display more extreme plasticity in investment in certain life history traits including growth, developmental rate and reproductive output, such as is observed in many anuran amphibians. For such species, tradeoffs in immune response may become more pronounced as a result of greater (or less) investment in other functions. Few empirical studies have attempted to induce such tradeoffs under controlled conditions. And very little study of amphibians has been conducted to examine these relationships between immune response and developmental trajectories.

These two main thrusts of research (adaptation and life history tradeoffs) have fueled other branches of parasitological study. For example, one area of exploration involves the role of parasites in driving host species interactions at the community level. Research on the species composition and distribution

patterns of parasites has revealed clear linkages between host association with habitat (endemic or introduced ranges) and the dynamics of parasitic infection. These studies are important because of the recent increase in potential for species introductions into new habitats; researchers are examining factors that may influence the establishment of introduced host species in an attempt to predict the potential for species to become invasive. Parasites may act as regulators of establishment success of hosts in newly colonized habitats, and more importantly, play a key role in the ability of hosts to become invasive. Thus variation in parasitism may explain the competitive advantage that some host species appear to possess in some habitats. However, empirical evidence for direct connections between the development of competitive advantage and infection is lacking.

Thesis structure

In the stand-alone Chapters 2, 3 and 4, I pose and test hypotheses to explain variation in establishment and other measures of fitness of parasites in hosts. These chapters contain common elements exploring the influence of host development and susceptibility. In my fifth chapter and Appendix I, I describe field surveys with objectives that follow closely with my third and fourth chapters. Chapter 6 is a test of predictions of the parasite release hypothesis. Appendix II is a first glance at interspecific interactions between parasites that exploit a similar niche in a common host species. Finally, Appendix III describes the life cycles of helminths of interest for my studies.

Chapter 2- Parasite exposure and transmission; developmental trajectories and susceptibility to infection

The success and timing of development of amphibians to metamorphosis is influenced by environmental factors such as hydro period or crowding (Denver 1997a; Loman 2001; Browne et al 2003). Much evidence exists for adaptive or phenotypic plasticity in development (rate or size) of amphibians in response to variable environmental conditions and perturbation (Werner 1986; Smith 1987). As such, this plasticity is thought to be a life history trait that has evolved in response to natural selection (Denver 1997a).

One might also argue that variability in development arises as a result of parasite-mediated selection, whereby hosts that vary developmental rate experience reduced exposure to stage-specific parasites. Yet, such plasticity likely incurs fitness costs later on the amphibian life cycle (Semlitsch et al 1988). As well, developmental changes in response to variable environmental conditions are thought to be mediated by stress responses (Denver 1997b). Such responses can also negatively affect the development and natural remodeling of a robust immune system in metamorphs (Rollins-Smith 1998). One goal of this study was to determine whether there was any association between variability in host development and susceptibility to infection. I induced variation in development by rearing amphibians under varying density conditions.

Parasites are adapted to exploiting a host in its most vulnerable state. Most parasites with **indirect life cycles** depend on temporal, as well as spatial

overlap of infective stages with hosts at stages of development such as metamorphosis, when immune response is inhibited, in order for transmission to occur (see Trematode life cycle see below). Thus synchrony with the development of a suitable host species is imperative for the completion of a parasite's life cycle. Parasites must be astutely sensitive to the kinds of environmental cues that can influence the rate of development of the host (Pietroock and Marcoglise 2003). Yet hosts may respond to exogenous factors or sources of stress, such as high density conditions, that parasites may not be able to detect. As such, a shift in developmental timing of the host may alter the period of overlap between infective parasites and susceptible hosts. Thus such exogenous interactions may produce patterns of infection in nature that are not easily predicted by theoretical or experimental models. Thus, a second goal of this chapter was to test the hypothesis that variation in investment in amphibian developmental rate and size, induced by crowding, relates to variation in response to infection.

Chapter 3- Sex-specific developmental trajectories and susceptibility to infection

At the outset of my thesis, I was unaware of potential differences in developmental trajectories among the sexes that could be manifested at tadpole and juvenile stages of development in amphibians. This simply has not been reported in the literature. However, many studies attribute differences in growth trajectories of adults to differential prioritization of reproductive and somatic

functions to maximize lifetime reproductive fitness between the sexes (Woolbright 1983, Rolff 2002). It was reasonable therefore to expect sex differences in investment in growth or development to impact immune function particularly at periods of greatest investment in development, such as at metamorphosis. I thus turned my attention to this gap in the literature.

For the third chapter of my thesis, I explored relationships between sex-specific developmental trajectories of metamorphs and susceptibility to parasitism in a host species that displays dimorphism in size at adulthood (Wood frog: *Rana sylvatica*, now *Lithobates sylvaticus*). Sexual size dimorphism is a common phenomenon across vertebrate classes (see Greenwood and Wheeler 1985). The differential prioritization of functions may have significant consequences for the development of immune response in each sex. Sexually dimorphic species may disparately allocate resources towards immune response (Poulin 1996; Moore and Wilson 2002; Rolff 2002). This has been suggested in mammalian species, in which larger males tend to carry significantly greater parasite loads than smaller females (see reviews by Poulin 1996 and Klein 2004).

To a lesser extent, the relationship between reverse or female-biased (larger females) sexual dimorphism and sex-biased susceptibility to parasitism also has been examined in mammalian models (Poulin 1996; Schalk and Forbes 1997). A consistent negative pattern of association between susceptibility to parasitism and investment in growth also has been observed (Moore and Wilson; 2002). That is, larger females experience higher rates of parasitism than males.

Thus, larger mammals, regardless of sex, appear to be more susceptible to parasitism. Further, the degree of dimorphism relates to the degree of parasitism, such that greater differences between the sexes in size are associated with greater differences in infection levels (Poulin 1996). Certainly, it is possible that the relationship between size dimorphism and susceptibility to parasitism may not be direct, but instead due to other factors that also co-vary with investment in development. Nevertheless, a significant inverse correlation between development and susceptibility to infection suggests that greater investment in development occurs at the cost of reduced immune function; and, investment in development can be influenced by life history strategies employed by different sexes. Interestingly, mine was the first known study to test these relations between development and susceptibility to infection among male and female amphibians, and at subadult stages of development. Yet reverse sexual size dimorphism is common among anurans.

Chapter 4- Parasite adaptation and fitness

The results from my third chapter provided the impetus for a subsequent study on whether sexes showed differences in susceptibility to parasitism for a species that does not show sexual size dimorphism at any stage of development, to determine if similar patterns to Chapter 3 might be observed.

However, in the course of carrying out preparatory pilot studies using a different species of frog (Northern leopard frog: *Rana pipiens*, now *Lithobates pipiens*), I observed differences in the gross morphology (size) of worms

removed from the lungs of different host species and incubation time of infective larvae. I also had observed immature stage parasites that persisted in the body cavity of the metamorph hosts, an observation that previously has been anecdotally conveyed by other researchers, but which is almost entirely absent from the literature. This presumably is because these latter observations are not originally-described characters of the life cycle of this nematode lungworm.

However, variation in such characteristics has significance in terms of the host range of nematode parasites; variable fitness of a worm may determine the extent to which parasites can successfully exploit different host species. Variation in host adaptation of parasites might also cause variation in the cost of infection to the host as a result of differential tissue damage and pathology of infection. These peripheral observations lead to the suspicion that adaptation of this parasite might occur and result in developmental and establishment traits that cause differences in parasitemia among the worm's two frog host species.

Thus my fourth thesis chapter assumed the two-fold purpose of ascertaining the occurrence of adaptation of lungworms, and exploring the generality of sex differences in developmental rate as they relate to susceptibility to infection in metamorphs by examining metamorphs of a species that does not display sexual size dimorphism.

I followed my experimental research on adaptation of lungworms in Northern leopard frogs and Wood frogs with an examination of sympatric populations of these two host species to determine whether differences in establishment and worm size among the lungworms in natural populations could

be detected (Appendix I). Results showed clear differences in establishment and growth among the worms, as well as variation in timing of transmission and life cycles that are likely tied to variation in natural history and behaviour among the two host species.

Chapter 5- Patterns of parasitism in a Northern leopard frog population

One limitation of studies that aim at understanding ecological relationships between species is the myriad of factors in natural systems that can confound or veil effects of interest. Few studies attempt to follow controlled experimentation with observational studies of natural populations. My experimental studies demonstrated a clear relationship between the developmental rates of males and females, and susceptibility to infection. However, the extent to which this relationship might be manifested as sex differences in parasitism in natural host populations was unknown. I thus selected a population of Northern Leopard frogs with known migratory routes (to be sure individuals were indeed from one population) and examined samples of different age classes of the population over 3 seasons in a year for lungworm (trematode and nematode) infections. I was interested in observing patterns of infection among male and female juvenile frogs, but I also was interested in determining the extent to which any patterns observed in juveniles were also observed among adult cohorts.

Chapter 6: Testing the predictions of parasite release: species richness and species- specific parasitism in invasive Bullfrog hosts.

Parasite release is a controversial hypothesis that explains the mechanism through which introduced species develop the ability to out-compete other species and become invasive (Torchin et al 2001; Keane and Crawley 2002). The removal of the pressure of parasitism in introduced habitats is thought to enable invasive hosts to invest more resources in developmental traits that enhance fitness and therefore competitiveness (Colautti et al 2004). However, direct empirical evidence supporting (or refuting) this idea in vertebrate invasive species is very limited (see Torchin et al 2003). Furthermore, few studies have examined species introduced to new habitats that are in close geographic range and within the same climatic gradient as endemic habitats. Thus observations of variation in parasitism among hosts in endemic and introduced habitats are often confounded by differences in latitudinal gradients. Support for the hypothesis is inferred from observational studies that have shown that the prevalence of parasite infection and species richness of parasites in host species in introduced habitats is comparatively lower than that observed in host species in native habitats.

For this study, I examined whether there was any evidence for parasite release among invasive American bullfrogs in their introduced range in the greater Victoria area in British Columbia. I measured levels of parasitism for specific parasites and determined species richness of parasites in juvenile and

adult bullfrogs. These measures were compared with richness and levels of parasitism in bullfrogs from 2 endemic areas in Canada.

It is clear that hosts represent extended phenotypes, and although my thesis is largely concerned with host-parasite interactions there is also expected to be parasite-parasite interactions in determining susceptibility of hosts to infections. In the process of necropsy, I often noticed infections of trematode helminths (*Haematoloechus* spp.) in the lungs of frogs; in some cases dual infections with *Rhabdias* spp. were observed. In Wood frogs, comparatively high numbers of trematodes were often found. However the worms appeared to occupy different regions of the lung when they co-occurred, which lead me to examine the potential for competitive interactions leading to niche segregation in these lungworms. My observations of Wood frogs were compared with a sympatric population of Northern leopard frogs, to determine the generality of the spatial segregation of these two types of lungworm.

Study Designs

For my second chapter, I reared tadpoles in several mesocosms under varying density conditions and exposed emerged metamorphs to parasites. I mimicked natural temporal patterns of exposure to infection by repeatedly sampling parasites from a naturally infected local pond. The availability of parasites from the natural ponds diminished over the development period of the tadpoles into metamorph frogs. This allowed me to investigate temporal overlap of infective stages of parasites with appropriate staged hosts.

For the third and fourth chapters of my thesis, I again reared tadpoles in mesocosms and exposed metamorphs to parasites. However, I distinguished developmental rates, sizes achieved and levels of infection between males and females. I also measured fitness parameters of worms. These latter measurements enabled subtleties in susceptibility to infection among sexes to be elucidated and highlighted the importance of parasite size as an index of infection success that can explain variation in parasitism among host species.

The fourth chapter of my thesis involved a partial cross experimental design in which a single recipient host species was exposed to parasites from two (conspecific and heterospecific) donor host species. Here, the original intent was to elucidate differences in infection success that might exist between parasites from different host (donor) species origins. Donor host species were collected to obtain parasites from different geographic locations with the goal of exaggerating any differences among the worms from the two host sources. This design also allowed the examination of relationships between development and susceptibility to infection in a frog species that does not display sexual size dimorphism. Furthermore, genetic sequencing of parasite DNA allowed the separation of parasite species that were morphologically indistinct.

The design of the experiments that involved tank rearing of tadpoles required me to address a problem that is common to many parasitological studies; the issue of autocorrelation of sampling units (see Paterson and Lello 2003). The considerable logistical effort required in rearing and conducting

controlled infections (e.g. for Chapter 2, I reared a total of 1200 tadpoles in 30 tanks) precluded an alternative design of large numbers of independent rearing tanks. Thus for those projects involving tank husbandry, I reared several larval frogs in each tank in an attempt to mimic variation that would be observed among natural ponds/populations, with the expectation that any treatment effects would result in greater variation than observed between tanks. For some of the response variables of interest, the data were analyzed at the level of tank. However in some cases, individual frogs were treated as sample units. The reasons for this are described in greater detail in each chapter.

This issue of pseudo replication required the statistical testing of tank effects on response variables of interest to determine whether source of rearing tank contributed to significant variation in the measured responses of the individuals. In cases where tank effects were significant, individual tadpoles were nested within tank in mixed model analyses to separate (and account for) tank-associated variation from treatment effects (Paterson and Lello 2003). Further details of these analyses are included in the two respective chapters.

Another challenge emerged following the discovery of different species of nematode lungworm between Northern leopard frogs and Wood frogs. Without being able to decipher morphological distinctions between species, I was unable to confirm that a single species was found only in one host, and that it was a different species from that found in the other host. Thus, I was unable to conclude with certainty that the patterns that I observed in my population survey for Appendix I were due to adaptation of parasite and host, or that I was

examining a single parasite species in Northern leopard frogs in Chapter 5. At present, the general assumption is that each lungworm species exclusively infects a single host species in nature. However, genetic testing of all worms from each sampled frog would need to be done in order to confirm the exclusive use of each worm species.

The recent use of genetic typing to characterize parasite species has revealed the difficulty and confusion in identifying cryptic species based solely on morphology; several genera of trematodes are presently undergoing taxonomic revision as a result of recently discovered genetic distinctions. The challenge of identification also arose in my study on Bullfrogs in Chapter 6, in which lung and kidney trematodes could not be distinguished to species by morphological features. As such, only general trends could be discussed without a direct comparison of species-specific parasitism.

For my fifth chapter, I surveyed local populations of Northern leopard frogs and Wood frogs for lungworms over the period of one year. I examined a population of Northern leopard frogs with the intent of determining the extent to which the results of my controlled experiments could predict patterns of lungworm parasitism among adult (non-breeding and breeding) and juvenile frogs in nature. Of course, the limitation of this study to a single host population begs the argument of limited inference in conclusions drawn; if my observations followed my predictions from experimental work, it would be difficult to generalize among different populations of Northern leopard frogs. However, the use of a

single host population was sufficient to demonstrate that sex differences in susceptibility may not explain natural variation in parasitism.

For my study on invasive bullfrogs, I sampled 5 populations in the greater Victoria area of BC. Although the lakes and ponds from which the frogs were sampled were separated by highways and land, across which the frogs were unlikely to cross, only one collective invasive site was used (N=1). For most of the statistical analyses, the mean of responses from the 5 populations was tested against a native site in Ontario and also compared with a second native region in New Brunswick. Thus inferences about the role of parasite release in facilitating invasiveness of bullfrogs are limited to the BC populations. Further, bullfrogs from the native and invasive locations were sampled at different times, thereby introducing an additional issue of temporal variation that may have influenced the results observed. Nevertheless, this study provides evidence that does not support the parasite release hypothesis and points towards other hypotheses that might explain the invasive properties of this vertebrate species. Further details of the methods and analyses are reported in the chapter.

Although I use a common format throughout my thesis for much of the text, idiosyncrasies between chapters reflect the fact that each data chapter has been formatted to fit the journal to which a manuscript was submitted.

GLOSSARY OF TERMS

Fitness	An individual's cumulative contribution to reproduction, relative to others within a population (Roff 1992).
Intrapopulation	A population consisting of all members of a single parasite species in one host (Bush et al 2001).
Indirect life cycle	A life cycle in which multiple hosts are required for completion. Intermediate, paratenic and vector host species carry a parasite, often across different trophic levels, to its definitive host (see Trematode lifecycle).
Overdispersed	Pattern of parasite aggregation in which the majority of infection is clumped among few individual hosts in a population.
Stress	Environmental stressors are forces that induce changes in physiology or behaviour beyond normal homeostatic limits that result in changes in mortality or natality of the individual experiencing the stress, which in turn alters the density of the population. Crowding which can alter food resource availability and predation risk for an individual, can be considered a stressor if it is experienced at levels beyond that which an individual has a genetically based life strategy to counteract or "cushion" (Esch et al 1975).
Prevalence	The number of individuals (proportion or percentage) in a sample that are infected with a single parasite species (Bush et al 1997).
Abundance	The number of parasites of a single species found in a single host, regardless of whether or not the host is infected. That is, this measure includes hosts with zero parasites (Bush et al 1997).
Intensity	The number of parasites of a single species found in a single infected host (Bush et al 1997).

CHAPTER 2: REARING DENSITY AND SUSCEPTIBILITY OF *RANA PIFIENS* METAMORPHS TO CERCARIAE OF A DIGENETIC TREMATODE

Results from this chapter form the basis for the following publication:

O.K. Dare, Rutherford, P.L. and Forbes, M.R. 2006. Rearing density and susceptibility of *Rana pipiens* metamorphs to cercariae of a digenetic trematode. Journal of Parasitology 92: 543-547.

ABSTRACT: Cercariae of many digenean trematodes target particular developmental stages of their hosts. For some digeneans that are parasites of amphibians, infection appears timed to host metamorphosis. The success and timing of metamorphosis is itself affected by a number of factors, including host density. I investigated the degree to which rearing density of *Rana pipiens* larvae influenced time to metamorphosis and snout-to-vent length and mass at metamorphosis, as well as establishment of cercariae of the trematode *Manodistomum syntomentera* Stafford, 1905. As expected, individuals were smaller and weighed less at metamorphosis if they were reared under intermediate to high densities as compared to low densities, in experimental outdoor mesocosms. Cercariae establishment was higher in smaller metamorphs that took longer to metamorphose within the low-density treatment. Additionally, cercariae establishment was lower in larvae from the low-density tanks compared to larvae from the intermediate- to high-density tanks. However, more tadpoles had failed to metamorphose in the intermediate to high rearing densities by the time cercariae were no longer available from natural collections of first intermediate hosts, *Physa* spp. Larval amphibians under crowded conditions should experience increased susceptibility to trematode establishment in nature, but only if they metamorphose within the time period when cercariae are still available.

INTRODUCTION

Digenean trematodes typically have complex lifecycles that include both sexual and asexual reproductive stages (Erasmus, 1972). Cercariae can infect both vertebrate and invertebrate hosts. As the cercaria stage typically survives for less than 48 hours (Esch et al., 2001), infection success is highly dependent on close spatial and temporal overlap with appropriate stages of the second intermediate host. Species parasitizing amphibian second intermediate hosts often display strong host-stage specificity. Some species infect amphibians prior to metamorphosis and before emigration from the pond (Schotthoefer et al., 2003).

Targeting amphibian hosts at metamorphosis is likely important for cercariae (Combes et al., 1994; Toledo et al., 1999), given that this is a time when individuals may experience reduced immunity because of the dramatic loss and reorganization of immune tissues (Rollins-Smith, 1998). Consequently, metamorphosing individuals may be susceptible to infection regardless of contribution of energetic resources to the immune system. Notwithstanding, variation in the degree to which metamorphosing individuals can resist cercarial infections likely exists, and relates to factors influencing timing and probability of successful metamorphosis. One such factor is density.

Density influences developmental trajectories and the survival of amphibians, particularly during larval and metamorph stages (Newman, 1998; Loman, 2001; Browne et al., 2003). Density is thought to exert its influence on individuals through increased frequency or intensity of agonistic interactions

between conspecifics, competition for resources, and social stresses (Semlitsch and Caldwell, 1982; Alford, 1999; Glennemeier and Denver, 2002). Further, increased intraspecific competition through increased density is known to be associated with changes in growth rates of amphibians (Glennemeir and Denver, 2002; Lea et al., 2002). These factors could negatively affect individual condition and perhaps also immune system function (Alford, 1999), thereby leaving hosts more susceptible to parasitic infection. However, comparatively little is known about the direct and indirect role(s) that host density plays in the susceptibility of amphibians to parasitic infection.

The complexity of these dynamics increases when density-dependent effects on parasite establishment and fecundity are also considered. Others (Goater et al., 1993; Goater and Vandebos, 1997) have reported negative impacts of nematode infections on the pulmonary capacity of juvenile amphibians, but the magnitude of the effect is itself dependent on the density of parasites that establish within a host.

Host density is also important for reasons other than its expected effects on immune function. For parasites with direct life cycles, higher host density elevates horizontal transmission of parasites between hosts (Price, 1990). In some species, hosts may be 'primed' under high-density conditions to respond to the threat of parasitic infection (Wilson et al., 2002). Whether host density affects susceptibility of hosts to parasites with *indirect* life cycles is less often studied. However, hosts reared or housed under high densities are expected to have altered developmental trajectories for reasons detailed above. One important

point of such alteration is that it may result in less temporal overlap with free-living infective stages of parasites. For example, an increase or decrease in developmental rate of larval amphibians could enable individuals to avoid infection by stage-specific parasites, such as trematodes, by circumventing the 'window of release' of cercariae.

The present study was undertaken to explore how rearing density of hosts influences susceptibility of *Rana pipiens* metamorphs to trematode cercariae, and to establishment of trematode cysts. I expected that susceptibility might be mediated by changes in timing, and size or mass at metamorphosis. I approached this objective by determining the susceptibility of metamorphs, raised under 3 different density treatments, to experimentally controlled challenges with trematode cercariae. Frog tadpoles can aggregate at high densities in nature due to a number of factors including limited suitable habitats for breeding or limited habitats with sufficient food resources, or because of predator avoidance, social attraction, and/or thermoregulation (Alford, 1999; Hoff et al., 1999; Smith et al., 2003). One ancillary objective arose as a consequence of my experiment, i.e. I was able to address how density might influence the temporal overlap of cercariae and metamorphs in nature. For each density treatment, I assessed how many individuals had developed sufficiently to be challenged with cercariae obtained from field-collected first intermediate hosts (*Physa* spp. snails).

MATERIALS AND METHODS

Mesocosms

Thirty 378.5-L stock tanks were established in an enclosed outdoor compound at Carleton University, Ontario, Canada (45°25'N, 75°42'W) to which larval *R. pipiens* were later added (see below). My set-up was similar to that of other researchers (Relyea 2001). To each tank, I first added 8.75 g Purina® Rabbit Chow and 100 g of dried leaves from a nearby mixed-deciduous forest. I also seeded each tank with *Daphnia* spp., obtained from a local pond on campus. Water levels were established initially at 300 L, but allowed to fluctuate marginally with natural precipitation and evaporation, as would occur in nature. On 3 occasions, I removed water from the tanks to prevent overflow due to heavy rainfalls. Each tank was covered with 40% shade cloth to prevent predation of tadpoles by wildlife, to prevent oviposition by invertebrates, and to preclude escape of *R. pipiens* metamorphs.

Rearing of larval frogs

I collected portions of 7 *R. pipiens* egg masses from local ponds in Old Chelsea, Quebec, Canada (39°50'N, 36°42'W) and randomly assigned them to lab aquaria. I allowed eggs to hatch in the lab and then introduced tadpoles (Gosner stage 25) (Gosner, 1960) to tanks 14 days later (May 18, 2004). Ten tanks were assigned to each of the following 3 density treatments: low (20 tadpoles/300 L or 0.07 tadpoles/L), intermediate (40 tadpoles/300 L or 0.13 tadpoles/L) and high (60 tadpoles/300 L or 0.2 tadpoles/L). My density treatments were within natural ranges reported for *R. pipiens* (Smith et al., 2003).

Equal proportions of tadpoles from each egg mass were distributed among each tank. Tadpoles were allowed to develop in the tanks until they metamorphosed (Gosner stages 42-45) at which time they were removed and challenged with cercariae of the digenean *Manodistomum syntomentera*, as described below. *Manodistomum syntomentera*, preferentially targets the musculature and connective tissues of developing limbs and tails in pre-metamorph frogs (Prudhoe and Bray, 1982). For each tank, I thus obtained the numbers of tadpoles that had metamorphosed and their time to metamorphosis. In total, I reared 123 metamorphs, measured and weighed them, and challenged them successfully with cercariae.

Cercariae challenges and susceptibility of metamorphs to establishment of cysts

To obtain cercariae, I collected snails (*Physa* spp.) from a local pond near the Queen's University Biological Research Station, Ontario, Canada (44°35'N, 76°19'W). Upon return to the lab, each snail was housed individually in Petri dishes with pond water in the dark for 24-48 hr to encourage cercarial release. Following release, snails were placed in natural light during the day, during which time cercariae release diminished. Although the infective properties of cercariae may alter over time, for the present experiment it was necessary to use cercariae from different snails to more closely match patterns of cercarial release in the pond. Thus, snail collections were performed several times over the course of the summer. Prevalence of *M. syntomentera* was at least 40% on each collection day at the beginning of the experiment; however, the proportion of infected snails

diminished over the course of the experiment. Reference specimens of cercariae obtained from the snails, and metacercariae from host tissue, were preserved in 95% EtOH. Live and preserved specimens were examined and identified (following keys in Prudhoe and Bray, 1982) with the aid of a compound microscope. Voucher specimens of *Physa* sp. (# CMNML 93777) and cercariae (# CMNPA 2005:0008-00010) were contributed to the Canadian Museum of Nature (Ottawa, Ontario).

Frog metamorphs were removed from tanks as they became available, weighed (to the nearest 0.01 g), and exposed to 20 cercariae. Each metamorph was confined individually to shallow Petri dishes to minimize movement and lessen behavioural avoidance of contact with parasites. After 6 hr of exposure, I measured snout-to-vent length or SVL (to the nearest 1 mm) then transferred each metamorph to aquaria and housed them in isolation for 3 days. I recorded any cercariae that remained in Petri dishes following challenge. Following this 3-day period to allow for parasite establishment, I euthanized the metamorphs using an overdose of MS-222. I froze the specimens for future necropsy to determine the proportion of penetrated cercariae that established as viable cysts.

The experiment was terminated when further collections of snails and housing them in the lab under the same conditions did not produce cercariae for infections. The last metamorph was challenged on day 114 (September 13, 2004). After this date, cercariae were no longer available. All snails collected on each of 5 days (starting on 13 September) were dissected to confirm that they were not infected with cercariae that could be shed.

Specific objectives and statistical analyses

I first examined the effects of rearing density on proportion of tadpoles reaching metamorphosis. I arcsin $\sqrt{}$ transformed these proportions to normalize the data, following the recommendation of Zar (1996). Here, each tank was treated as a replicate.

I next examined whether rearing density, or both rearing density and tanks affected time to metamorphosis, mass, and SVL. I first $\log_{10}$ -transformed these data (transformed data were normally distributed and did not show a dependence of variance on mean values for tanks). I then used MANOVA as a single test where only the influence of rearing density was considered. Following this test, I determined whether tanks accounted for significant variation in developmental variables (SVL, Mass, and Time to metamorphosis) with each density treatment, using a series of ANOVAs where tank was a random factor. Finding significant tank effects, I then explored whether rearing density influenced any of the developmental variables, using a series of nested ANOVAs to test for, and factor out, tank effects. In the nested ANOVAs the individual tadpole was the unit of replication for assessing the tank effect, whereas each tank was a replicate for the density effect.

I also tested whether proportion of cercariae that established as cysts in metamorphs was related to rearing density. Again, I arcsin $\sqrt{}$ transformed the proportional data and used a randomization (or distribution-free) test based on ANOVA (Simon, 1999). The proportional data were the number of cysts established divided by numbers of cercariae used to challenge. The number

used to challenge was the difference between 20 cercariae used for exposure minus the number remaining in the Petri dish following challenge. I used a randomization test because although the transformed data met the assumption of normality, there was a dependence of variance in proportions of cysts established on the mean proportion of cysts established for metamorphs per tank ($r = -0.65$; $P < 0.001$). For this result, I analyzed metamorphs from 20 tanks that produced 2 or more metamorphs.

Finally, I used multiple regression analysis to explore more fully the degree to which susceptibility to parasitism was related to log-transformed developmental variables (SVL, Mass and Time to metamorphosis). I chose to do this analysis for only the low-density treatment *a priori* because it has the greatest range in proportion of cercariae that established as cysts (from 0-100% establishment).

Analyses were performed using Resampling Statistics version 5.0 (2003), Statistica data analysis software version 6.1 (2003) and JMP version 4.0 (2002). For all tests, statistical significance was established at $P < 0.05$.

RESULTS

The 30 tanks produced 0-10 metamorphs per tank for experimental challenges. Two tanks from the intermediate density treatment failed to produce metamorphs by the time the experiment was terminated; another 2 tanks were high-density tanks that produced 1 and 2 metamorphs, respectively, that died prior to infection.

I found a highly significant effect of rearing density on the mean transformed proportion of metamorphs ($F_{2,26} = 43.96$; $P < 0.0001$) and this effect was due largely to greater likelihood of metamorphosis for individuals reared in the low-density tanks. Tukey Cramer post hoc tests showed differences between low and intermediate, and low and high density treatments ($P < 0.05$), but not between intermediate and high density treatments. More specifically, 78 tadpoles or 43.1% of the 200 tadpoles placed in the 10 low-density tanks metamorphosed (back-transformed estimates ± 1 SE ranged from 37.4% to 73.7%). In comparison, 24 tadpoles metamorphosed in the 10 intermediate density tanks, which represented 5.1% of 400 tadpoles placed in those tanks (estimated from 3.1% to 30.9%), and 24 tadpoles metamorphosed in the 10 high-density tanks, which represented 3.7 % of 600 tadpoles placed in those tanks (from 3.1% to 24.4%).

The MANOVA showed that rearing density had an affect on log-transformed SVL, Mass and Time to metamorphosis when all were considered together as developmental response variables (Wilk's Lambda=17.1, $P < 0.0001$). Even within a rearing density, tanks accounted for significant variation in developmental variables. For SVL, tank effects were seen at all three densities (F -values ranged from 7.1 to 25.9; P -values from 0.001 to 0.0001). Similarly, for Mass, tank effects were seen at all three densities (F -values ranged from 7.1 to 25.9; P -values from 0.001 to 0.0001). For Time to Metamorphosis, tank effects were seen only at the low density ($F=3.27$, $P < 0.005$). Tanks did not account for significant variation in Time to metamorphosis at the intermediate and high

rearing densities (F -values ranged from 0.98-1.2, P -values from 0.37 to 0.48).

Thus, tank effects were very common and had to be considered explicitly, hence my use of the nested ANOVA.

Using the nested ANOVAs, metamorphs weighed less if they were reared from intermediate- or high-density tanks as compared to those reared from low-density tanks, after controlling for the significant effect of tank nested within density treatment (Tables 2-1, 2-2). Metamorphs were also smaller if they were reared from intermediate- or high-density tanks as compared to those reared from low-density tanks, after controlling for the significant effect of tank nested within density treatment (Tables 2-1, 2-2). Finally, tadpoles reared under low density metamorphosed significantly sooner than those in either intermediate or high density, after controlling for the significant effect of tank nested within density treatment (Tables 2-1, 2-2).

Rearing density had a significant effect on mean proportion of cercariae that established as cysts ($F_{2,23}=4.19$, $P=0.021$; $r^2=0.25$), after controlling for the significant effect of tank nested within density treatment ($F_{23,97}=2.19$, $P=0.004$). Using the randomization test, I did not find any F -value that was as great as or greater than the observed F -value for density, based on 1,000 randomizations of data on proportion of successful establishment among density treatments, followed by ANOVAs ($P < 0.001$). Metamorphs reared from low-density tanks were less susceptible to cyst establishment than were metamorphs reared from either intermediate- or high-density tanks (Table 2-3). Cyst establishment in

metamorphs from intermediate- and high-density tanks was not significantly different.

Finally, I investigated linkages between developmental variables and susceptibility to parasitism for metamorphs from the low-density tanks (overall, 0-100% of cercariae established as cysts in these individuals). This comparison was chosen a priori because the range of successful establishment was considerable and required explanation. None of the 2-way or the 1, 3-way interaction(s) between developmental variables was significant (F -values ranged from 0.19-0.48, P -values ranged from 0.48-0.66). There also was no significant effect of SVL on susceptibility to parasitism ($F=0.01$, $P=0.90$). However, heavier metamorphs had lower successful establishment of cysts ($F=6.11$, $P=0.01$) as did individuals that reached metamorphosis sooner ($F=4.88$, $P=0.03$).

DISCUSSION

My results show that increased rearing density of larval amphibians increases their susceptibility to trematode infection, although the precise effects of crowding on susceptibility are still unclear. Other factors, such as behavioral avoidance of parasites may also influence susceptibility to infection (Thiemann and Wassersug, 2000); however, my experimental design minimized the expression of such behavior in individuals. While this study does not explicitly measure immune parameters, it does raise questions for further research on the immunological responses of amphibians reared under varying density conditions. The initiation of metamorphosis triggers a series of endocrine responses that

leads to the apoptotic disassembly of some components of the larval amphibian immune system (Kikuyama et al., 1993; Rollins-Smith, 1998). Tadpoles forced to alter timing of metamorphosis experience even more significant losses of immune tissues than normal, and are thereby expected to show further compromises in their ability to resist infection (Rollins-Smith, 1998).

The difference in mass of metamorphs reared in low and intermediate to high rearing densities was not surprising as the reduction of body mass in response to increasing density has previously been reported for several species (e.g., Goater et al. 1993; Browne et al., 2003). Nonetheless, mass reduction with increased density is of particular significance to the survival of post-metamorphic individuals (Semlitsch et al., 1988; Scott, 1994), and mating success in adult males and fecundity of females (Berven and Gill, 1983; Smith, 1987). My results further showed that metamorphs that weighed less carried higher parasite burdens. Taken together, my findings show that elevated density leaves metamorphs more susceptible to infection and decreases the potential for post-metamorphic survival.

As mentioned, individuals reared under higher density also displayed a retarded developmental rate. This delay may have implications for the temporal overlap of hosts and parasites because the successful establishment of cercariae is dependent on infecting the appropriate host stage (Sessions and Ruth, 1990; Schotthoefer et al., 2003). Such stage-specificity has critical consequences for the demographic distribution of parasites within amphibian populations, whereby

certain stages of tadpoles can experience low-level chronic infections while others experience between 80-100% mortality (Schotthoefer et al., 2003).

In general, the ability of cercariae to infect and establish in larval amphibians diminishes with host maturity, with older pre-metamorphic tadpoles possessing the greatest resistance to infection. This reduced susceptibility may be due to a more developed immune system (Du Pasquier et al., 1989) or behavioral changes in more mature stages that decrease contact with parasites (Kiesecker and Skelly, 2001). However, as mentioned, my confinement of individuals reduced behavioral variation among metamorphs. In contrast, later metamorphs actually carried significantly higher numbers of *M. syntomentera* cysts than did those that metamorphosed earlier in my experiment. One explanation for the greater parasitism of later metamorphs is that those individuals less able to develop reasonably fast and maintain mass are also less able to ward off challenges. It is interesting in this regard that only mass and time to metamorphosis were associated with susceptibility to parasitism for metamorphs reared from the low-density treatment. Although SVL was reduced for metamorphs from higher rearing densities, I think it has little direct relation to susceptibility.

My findings have implications for parasite-mediated selection on hosts. In the case of *M. syntomentera*, one might conclude that delaying metamorphosis is a viable means of reducing likelihood of parasitism. However, this would depend on the degree of host-stage specificity of the parasite. In nature, individuals might evade exposure to some parasites by delaying development and avoiding the

window of release of infective stages of parasites, but this strategy is employed at the cost of greater susceptibility to infection should contact with cercariae still occur. Delayed metamorphosis also is not likely to be a viable strategy if reduced growth and delayed development is detrimental to post-metamorphic survival and reproduction, even in the absence of parasitism.

Table 2- 1. Results from a series of Nested ANOVAs where the effect of rearing density on $\log_{10}$ -transformed developmental variables (Mass, SVL, and Time to metamorphosis) was assessed after controlling for the effect of tank nested within the density treatments. In all cases, there is a significant effect of rearing density, and also a significant tank effect. Distributions of log-transformed Mass, SVL and Time to metamorphosis were not statistically different from normal distributions in 10, 8 and 8 tanks, respectively (P-values ranged from 0.01 to 0.98, Shapiro-Wilk's test) for 11 tanks from which 5 or more metamorphs were obtained. There also was no dependence of the variance on the tank mean for any of those variables (r-values ranged from -0.021 to 0.12; P values ranged from 0.77 to 0.95).

<u>Variable</u>	<u>R²</u>	<u>Treatment</u>	<u>Df</u>	<u>F</u>	<u>P</u>
Mass	0.75	Tank (Density)	23	15.43	0.0001
		Density	2	3.91	0.03
SVL	0.72	Tank (Density)	23	13.04	0.0001
		Density	2	5.21	0.012
Time	0.48	Tank (Density)	23	1.8986	0.016
		Density	2	20.4114	<0.0001

Table 2- 2. Backtransformed means $\pm$ 1 standard error (SE) for log-transformed Mass, SVL and Time to metamorphosis. The least squares means were each derived from a Nested ANOVA where the effect of rearing density was assessed, after controlling for tank effects (see text and Table I for details). For each variable, treatment means with the same letter designation as superscripts are not significantly different from one another based on post hoc contrasts ($P < 0.05$).

	<u>Density treatment</u>		
	<u>Low</u>	<u>Intermediate</u>	<u>High</u>
Mass (g)	1.33 (1.24-1.38) ^a	0.99 (0.91-1.08) ^b	1.01 (0.93-1.10) ^b
SVL (cm)	2.31 (2.29-2.35) ^a	2.13 (2.07-2.21) ^b	2.07 (2.02-2.12) ^b
Time (days)	66.7 (65.1-68.4) ^a	92.1 (89.3-95.0) ^b	91.9 (89.4-94.6) ^b

Table 2- 3. Backtransformed means (ranges $\pm$ 1 standard error (SE) of the proportion of parasites established). Least squares means were obtained from arcsin $\sqrt{}$ transformed data and a randomization test based on ANOVA (see text for details). Parasite establishment in metamorphs from the low-density treatment was significantly lower than in metamorphs from intermediate- and high-density treatments. For each treatment, means with the same letter designation as superscripts are not significantly different from one another ($P < 0.05$).

<u>Density Treatment</u>	<u>Parasite Establishment (%)</u>
Low	65.9 (61.2-70.3) ^a
Intermediate	86.1 (82.5-89.4) ^b
High	78.7 (74.3-82.7) ^b

CHAPTER 3: RATES OF DEVELOPMENT IN MALE AND FEMALE WOOD FROGS AND PATTERNS OF PARASITISM BY LUNG NEMATODES

Results from this chapter form the basis of the following publication:

O.K. Dare and Forbes, M.R. 2008. Rates of development in male and female wood frogs and patterns of parasitism by lung nematodes. *Parasitology* 135: 385-393.

ABSTRACT

Researchers are becoming interested in testing whether investment in growth and/or development trades off against investment in parasite defence. I tested this idea by examining relations between development of Wood Frogs (*Rana sylvatica*) and susceptibility to lung nematodes (*Rhabdias ranae*). Male and female frogs reared in outdoor mesocosms were the same length and mass at metamorphosis. However, males metamorphosed sooner than did females. Lung nematodes were no more likely to penetrate male versus female metamorphs following controlled exposures, but males had higher intensities of adult female worms and the largest worms per host were on average of larger size in male metamorphs. Males that took longer to metamorphose carried higher numbers of worms in their lungs than males that metamorphosed early. In comparison, females that developed faster harboured more worms in their lungs than females that took longer to reach metamorphosis. My results suggest that variation in susceptibility to lung nematodes is influenced by host sex and possibly also by sex-specific relations with developmental rate. Further, male hosts might prove to be a more important source of infective stages of worms than female hosts.

INTRODUCTION

Sexual size dimorphism is common among species from various vertebrate classes and can reflect sex differences in investment of energy or resources to growth for maximizing lifetime reproductive fitness (Greenwood and Wheeler, 1985). Sexes also can differ in their developmental trajectories (e.g., Leclair *et al.* 2000). Importantly, greater investment in growth and/or development is thought to affect the ability of individuals to invest in behavioural or somatic defences against parasites (Sheldon and Verhulst, 1996; Moller *et al.* 1998; Soler *et al.* 2003). The evolution of resource allocation will be influenced not only by fitness gains of increased size or faster development rates, but also by fitness losses associated with parasitism, should parasitism occur (Schmid-Hempel, 2003; Tschirren and Richner, 2006).

One might expect certain species to show sex-biased parasitism because of sexual dimorphism in investment in immune defence (Moller *et al.* 1998). Testing for sex differences in susceptibility to parasites, as predicted by theory, is best done with experimental infections, where exposure to parasites is controlled (Schalk and Forbes, 1997). Male-biased parasitism is often seen in mammals, where males are often the larger sex (Poulin, 1996a; Moore and Wilson, 2002). However, male-biased parasitism is not a general rule in birds (McCurdy *et al.* 1998) or arthropods (Sheridan *et al.* 2000). Little is known for amphibians.

Sex differences in growth and development are expected to be detectable even at young ages, when parasites are first encountered (Tschirren *et al.* 2003). This is because energetic trade-offs towards faster development or greater

growth at earlier life stages can reduce the ability to control parasitic infection (Isomursu *et al.* 2006). From the viewpoint of the parasite, infecting a host with reduced potential for immune response can result in increased establishment and growth. For some species of parasitic worms, increased size in females relates to greater egg output (Poulin, 1996b). The fitness potential of such parasites might therefore be expected to differ between parasites infecting male and female hosts because of the existence or expression of fewer defences in one sex of host.

Anuran amphibians are a good model for exploring whether sex biases in parasitism, or measures of parasite fitness, are associated with sex biases in patterns of host growth or development. Approximately 90% of anurans display sexual size dimorphism with males being smaller than females (Shine, 1979). However, research on growth patterns has centred on adult frogs just prior to reproductive maturity, and not at earlier stages in development (Howard, 1985). Yet amphibian metamorphosis is a particularly vulnerable life-stage for amphibians, during which time there are gross changes in physiology and tissue development (Rollins-Smith, 1998; Tata, 1999). At this stage, individuals also find themselves at the interface between aquatic and terrestrial environments each of which contains novel suites of parasites. Furthermore, plasticity in development is expected to vary both between and within species (Leclair *et al.* 2000); and slower development can result in increased susceptibility to trematode parasites within one anuran species (Dare *et al.* 2006).

For this project, I experimentally challenged Wood Frog, *Rana sylvatica* (= *Lithobates sylvaticus*) metamorphs with the lungworm parasite, *Rhabdias ranae* (Nematoda). My principal objectives were threefold: first, I explored relations between sex and measures of size and developmental rate for metamorphs. Wood Frogs show sexual size dimorphism at sexual maturity with larger females than males (Berven, 1982; Bastien and Leclair, 1992; Leclair *et al.* 2000), but it is unknown whether any sex differences in growth or development occur for larvae. Second, I examined one measure of expected fitness for worms (maximum size attained) infecting male and female metamorphs, following experimental infections with *R. ranae*. Third, I determined whether hosts showed sex-specific relations between rate of development and numbers of worms establishing in the lungs. To meet these objectives, I reared male and female *R. sylvatica* metamorphs from larvae in outdoor mesocosms.

MATERIALS AND METHODS

General considerations

All protocols for housing frogs and for experimental challenges using nematode larvae were done in accordance with guidelines observed by Carleton University's Animal Care Committee. All statistical analyses were performed using JMP (Version 4. SAS Institute Inc., Cary, NC) and SPSS for Windows (Version 10.0.7 SPSS Inc, Chicago, IL).

Growth and development of male and female *R. sylvatica*

To rear frogs to metamorphosis, ten tanks (Rubbermaid[®]; 132 cm X 78 cm X 63 cm) were established as outdoor mesocosms in a fenced-off compound. Each tank was filled with 300L of water and covered with 40% shade cloth to prevent oviposition by invertebrates, to prevent predation of frog tadpoles by wildlife, and also to prevent frogs from escaping. Water levels fluctuated naturally with precipitation and evaporation. On two occasions, water was removed to prevent overflow. Each tank was seeded with 100g of leaf litter collected from a mixed deciduous forest stand and 8.75g of Purina Rabbit Chow[®] two weeks prior to the introduction of the tadpoles. By this time, there also was sufficient growth of algae to provide food. *Daphnia* spp. were collected using D-frame dipnets from a local pond, and introduced to a “stock” tank with 300L of water. Once the *Daphnia* had established in the stock tank, 750 mL of water containing *Daphnia* were added to each rearing tank to control excessive algal growth.

Portions of three *R. sylvatica* egg masses were collected from Casselman, Ontario (45°19' N, 75°05' W) on April 11, 2005. Each egg mass was housed indoors in a separate aquarium until hatching, with pond water from their natal habitats and a bubbler. Tadpoles were fed a granular mixture of tadpole food (Ward's Nat. Sci. Tadpole Food # 88V 6534) and Hagen fish flakes (Nutrafin Max Complete Flake Food #668672) until they had developed to Gosner stage 25 (Gosner, 1960). Five stage-25 tadpoles reared from eggs from each of the 3 egg masses were added together for a total of 15 individuals in each of the 10 tanks.

Rana sylvatica metamorphosed from June 12-June 26, 2005, coinciding with metamorphosis of wild conspecifics in the Ottawa region (O. Dare, *personal observations*). I recorded the first day I removed a metamorph as day 0 to metamorphosis (DM = 0) because I was interested in the timeframe associated with exposure to infection. Metamorphs were removed from tanks when they developed forelimbs (Gosner stage 42). At this stage, I measured snout-to-vent lengths (SVL) to the nearest 0.1cm, and also weighed each metamorph to the nearest 0.1g.

Parasite fitness metrics

Filariform, infective (L₃) *Rhabdias* larvae emerge from the mother and penetrate the skin of amphibians (see Rhabditean lifecycle described in thesis Appendix). These larvae travel through various tissues, lymph and blood towards the lungs. As they move into the lungs they first develop into adult males and then into functional females (Baker, 1979). Thus, I used the number of established adult worms in the lungs of hosts relative to the success of larval worms penetrating hosts as a metric of fitness potential. This is equivalent to numbers infecting hosts.

In *R. sylvatica*, adult fecund *R. ranae* worms can be found in the lungs after about 1 wk post-infection (Baker, 1979; Goater and Vandenbos, 1997). The number of females present in the lungs $\geq$ 1 wk post-infection gives an indication of the success of worms in establishing and developing to their reproductive potential. Since developmental rate influences the time of first reproduction and potentially length of egg laying over the parasite's lifetime, any delay in becoming

a female could reduce the parasite fitness potential. Another measure of parasite fitness is the size of fecund females. High mortality during juvenile stages should result in strong selection for high egg output and thus larger body sizes of adult females (Poulin and Morand, 1997). In nematodes, female worm length is a strong predictor of fecundity (Goater, 1992; Marcogliese, 1997; Tompkins and Hudson, 1999). Thus, maximum female size attained in hosts after a set period of time can index potential fecundity for those individuals. These two measures of parasite fitness, developmental rate and size attained, are known to be independent of each other in *Rhabdias ranae* (Gendron *et al.* 2003).

Host sex and metrics of parasite success

Of the initial 150 tadpoles reared, only 93 metamorphs were used for the experiment, due in part to mortality, but more to logistic limitations on the numbers of lungworm larvae available for infection. Metamorphs were brought indoors into a quiet room. Frogs were housed in square Ziploc[®] plastic containers (12cm X 12cm X 5.5cm (ca. 946 ml)) with perforated lids. The bottom of each plastic container was lined with a wet paper towel to provide traction and prevent drowning of individuals.

To obtain parasites for controlled challenges, I caught 12 wild adult *R. sylvatica* in Bishops Mills Ontario (44 ° 53' N 75 °40'W) and brought them back to the laboratory. I housed each frog individually in 2.5 L glass aquaria in the laboratory. Each aquarium was lined with a damp paper towel and an overturned Petri dish was placed at one end to provide a dry post. Temperature was maintained between 23°C and 26°C. I fed each frog three to five large crickets

daily and dusted the crickets in amphibian multivitamin mix (Herptivite, Rep-Cal Research Labs, CA, USA) every other day.

I cultured infective stage *R. ranae* larvae in a manner similar to Goater and Vandebos (1997). Briefly, I incubated faeces from the wild-caught adult frogs at room temperature (23° – 25°C) in Petri dishes lined with moist, unbleached, coarse-grade coffee filter paper (#2 Presidents Choice Green coffee filters®) for three to five days. When infective larvae were observed (a minimum of 72 hrs), I rinsed the contents of the Petri dish with water, and isolated and counted larvae using a dissecting microscope.

Metamorphs were challenged only after they had completely resorbed their tails, i.e., at Gosner stage 46. I thus controlled for stage of development, although the metamorphs developed at different rates and achieved different sizes by this stage. I placed each metamorph in a Petri dish (10.0 cm X 1.5 cm) and exposed it to 30 infective larvae (10 larvae from each of three adult frogs) for 24 hrs. This exposure in a Petri dish was done to minimize parasite avoidance by metamorphs and thereby enable worm larvae to find their host. After 24 hrs, the Petri dishes and damp filter paper were rinsed thoroughly three times and any remaining larvae were counted to index the numbers of worms that penetrated. I allowed a period of 10-11 days before killing the metamorphs in an overdose of MS-222, and later necropsied each. Thus, infections were staggered across all individuals and done for each individual according to its timing of metamorphosis.

Following challenges, metamorphs were each fed two or three crickets (from ca. 3.2-6.4 mm) every day. The number of crickets fed to each individual

was determined by satiety. Crickets were dusted with amphibian multivitamin mix every other day. The containers housing metamorphs were rinsed every three days with water and 100% EtOH.

During necropsies, I counted the number of worms in the lungs and body cavity, and preserved samples in 70% glycerol. Each worm was identified as subadult or adult. Adult worms were identified to sex based on morphological features (ovaries and characteristic darkened digestive tube and the sheath on adult females, Fig. 3-1). I measured the length of the largest female worm from each infected male and female metamorphs, using the scaling function of a digital microscope (Zeiss Axioplan 2ie) which is accurate to ± 0.01 mm. Although this measurement does not represent the mean or median length of worms in the population within a host, I used this length to index the maximum potential reproductive output of a worm, because size of the worm likely relates to fecundity. An accurate measure of egg output per day or per gram of feces as a direct measure of worm fecundity in *Rhabdias* was not possible. Some eggs hatch as they move out from the lung into the intestines before they are shed as L₁ stage larvae along with the feces. At present, it is not known if there is substantial variation in proportions of larvae versus eggs that are voided. Specimen vouchers of subadults, males and females were catalogued at the Canadian Museum of Nature, Ottawa, Ontario (voucher numbers: CMNPA 2005 0011-0019).

I identified the sex of each metamorph during necropsy by the presence of either ovaries or testes. Because my necropsies were done 10-11 days later and

sex was determined last during necropsy, my parasite counts and later my measures of parasite size, were taken blind relative to sex of the hosts. I examined whether the numbers of worms that successfully penetrated hosts varied with host sex. I also compared the number of worms that established successfully in lungs as females, given that numbers penetrating were not different between male and female hosts (see Results). I used median abundance and median intensity of worms (*sensu* Bush *et al.* 1997). I also computed a mean number of established worms for each sex on a per tank basis and examined whether the sexes differed in these mean numbers of established worms using a paired t-test. Unlike the original counts, means are amenable to parametric tests. I then compared the mean sizes of the largest female worms obtained from male and female hosts (using ANOVA) and also the differences in mean sizes of the largest female worms from males and females, on a per tank basis (using a paired t-test).

Sex-specific patterns of infection in relation to developmental trajectories

My initial questions were whether there were sex differences in host developmental trajectories and also sex differences in measures of parasite success. I therefore examined whether differences in developmental rates within a sex were related to differences in establishment of female worms. I did these analyses to determine whether there were sex-specific patterns of worm establishment in relation to developmental rate.

I examined whether time to metamorphosis related to establishment of female worms for all metamorphs, separately by sex. I also examined whether SVL or mass related to establishment of female worms for all metamorphs, separately by sex. These analyses were done using non-parametric correlation. I also calculated a mean Date of Metamorphosis for males and females in each tank separately as well as mean abundance of worms. I compared whether these two measures were related for each sex, weighted by the number of individuals used to calculate each mean. Data were analyzed by sex separately (1-way ANOVA) because sexes differed in developmental rate and this precluded other approaches (e.g., analyses of covariance with time to metamorphosis as a covariate, following Zar, 1996).

I compared numbers of male and female metamorphs produced by each tank to test whether certain tanks might differentially contribute individuals of one sex to subsequent analyses. In studies such as this one, there is often significant variation in variables among rearing tanks, despite the tanks having identical rearing regimens. I tested for an effect of tank on time to metamorphosis for both males and females. I did this to address the potential problem of pseudo replication. If certain tanks were more likely to contribute early metamorphs of one sex, then subsequent sex differences in developmental rate might be reasonably ascribed to tank effects. Finding no significant tank effects, I considered frogs in tanks to be quasi-replicates and compared sexes across all tanks for differences in mean SVL, mean mass, and mean time to metamorphosis, using ANOVA. I also controlled for the potential problem of

pseudoreplication by taking tank means of SVL, mass, and time to metamorphosis for each sex and tested for differences between sexes in these measures, using paired t-tests. These considerations of tank effects also were assessed prior to other analyses examining relations between host sex and metrics of parasite success.

RESULTS

Growth and development of male and female *R. sylvatica*

I found that there was no propensity for tanks to contain a particular sex (pooled $SE=0.58$, $F=2.53$, $df=1,9$; $P=0.13$). When all 10 tanks were emptied, I removed 40 male and 53 female stage 46 metamorphs (a result that is not statistically different from 50:50, $P>0.05$, sign test). At metamorphosis, male mass and SVL were very similar to females (Table 3-1). However, males and females did differ in the average time it took to metamorphose, with females taking almost 2 days longer than it took males (Table 3-1). Thus, although males and females were not size dimorphic at metamorphosis, the sexes still showed different developmental trajectories with males showing more rapid development. I found no effect of tank on time to metamorphosis for males ($F=1.04$, $df=9,30$; $P=0.43$) or for females ($F=1.41$, $df=9,43$; $P=0.21$). Using mean time to metamorphosis per tank for each sex and comparing these grand means, I still found that males took significantly shorter duration to reach metamorphosis (*paired* $t=2.7$, $df=9$, $P=0.02$). This sex difference in developmental time on a per tank basis occurred,

again despite males not being different from females in terms of mean mass or mean SVL (*paired t-values* ranged from 1.2-2.0, $df=9$, P ranged from 0.07-0.28).

Host sex and metrics of parasite success

On average, both males and females had *ca.* 20 of the 30 infective larvae penetrate (males: 20.2 ± 0.95 ; females: 20.1 ± 0.83 ; $F=0.009$, $df=1,91$; $P=0.92$). In total, 15 worms were subadults, found in either lung (8) or in the body cavity (7). In comparison, 90.6% of 159 worms dissected out during necropsies were adults. Of these 144 worms, 11 were males, carried by 7 metamorphs (5 females and 2 males); male worms are not considered further. I found no effect of tank on numbers of worms establishing in male metamorphs ($F=0.42$, $df=9,30$; $P=0.91$) or in females, although this latter result approached significance ($F=1.96$, $df=9,43$; $P=0.07$).

The number of worms that established in lungs of hosts ranged from 0-10 for females and 0-13 for males. A large number of male and female hosts did not have any worms establish (20 of 40 males and 27 of 53 females). For this reason, I first examined whether there were differences in median abundance of worms between males and females. I found that 20 of 40 male frogs had more than the median number of 0.5 worms as compared to a near equal proportion (25 of 53) of females ($Z=0.27$, $P=0.78$, Median test, Table 3-2). I then examined only hosts that were infected with adult female worms. The results here were clear. Eleven of 20 male frogs had more than the median number of 2 worms as compared to 4 of 25 females ($Z= -2.72$, $P<0.01$, Median test, Table 3-2). I also

compared mean numbers of female worms established for each host sex per tank and compared them using a paired t-test, which required only that the differences of matched pairs be normally distributed (Zar 1996). I found males again averaged higher numbers of worms (*paired t* = -2.76, *P*=0.02) for the 8 tanks where individuals of both sexes were infected with female worms.

Finally, I found that the largest female worm was, on average, larger in size in male metamorphs than in female metamorphs, despite male hosts harbouring more worms than female hosts (Table 3-2). However, when I examined mean lengths of the largest female worms for each sex on a per tank basis, I found that there was only a trend for larger female worms in male metamorphs (*paired t* = -1.81, *P*=0.11). There was significant variation among male metamorphs in sizes of female worms that were attributable to tank effects (*F*=4.30, *df*=1,9, *P*=0.02), but not so for female metamorphs (*F*=1.18, *df*=1,9, *P*=0.38). Worm intensity did not influence the size of the largest female worm (*F*=0.91, *df*=1,35, *P*=0.35).

Sex-specific patterns of infection in relation to developmental trajectories

Time to metamorphosis was unrelated to number of female worms that established for either male or female metamorphs, when individuals were considered replicates (*Spearman rho* ranged from -0.14 to -0.18, *P* values ranged from 0.19 to 0.33). However, other patterns emerged between mean time to metamorphosis and mean number of female worms that established for each host sex, on a per tank basis. For males, I found these two variables related

positively to one another ($r^2=0.52$, $F=8.97$, $P=0.02$) whereas they were inversely related for females ($r^2=0.39$, $F=5.12$, $P=0.05$) (Fig. 3-2). Thus, males that developed slower on average compared to other males, and females that developed faster compared to other females, had higher numbers of established worms. I found no significant nor consistent relations between either SVL or mass and number of female worms that established for either male or female hosts (*Spearman rho* ranged from 0.056 to 0.26, P values ranged from 0.10 to 0.73).

DISCUSSION

The salient results of my study are fourfold. First, sexual differences in developmental rate can occur without an associated sex difference in size or mass (a result supported by tests based on individuals as replicates, and also tests based on tank means for each sex). Second, male and female hosts did not differ in the numbers of worms that penetrated, but male hosts harboured more female worms in their lungs than did female hosts in both types of tests. Third, the size of the largest adult female worms was larger in male than female hosts, but this result was restricted to tests where individuals were considered replicates and was only a strong trend when tank means of worm lengths for male and female metamorphs were compared with paired tests. Fourth, patterns of worm establishment in relation to developmental rate differed between sexes and between tests. Relations using individuals as replicates were not significant. However, relations based on tank means showed that as average time to

metamorphosis increased for males, more female worms established. The opposite pattern was seen for females. Qualitatively similar results were found for susceptibility to parasites and its relation to developmental rate for the sexes when transformed data were used: i.e., $\arcsin \sqrt{\text{(proportion of penetrating worms that established as females, data not presented)}}$.

Although several species of amphibians display sexual size dimorphism at adulthood, there is still some uncertainty about how the differences in sizes of adult males and females are reached. In *R. sylvatica*, males achieve reproductive maturity faster, but have lower longevity than females (Leclair *et al.* 2000). The timepoint in development at which a noticeable divergence in growth rate and/or size between sexes occurs is still largely unknown for sexually size dimorphic amphibians. However, the examination of natural populations of *R. sylvatica* (Howard, 1985) has revealed that size-specific growth rates of adult males (just prior to reproductive maturity) are slower than females. Furthermore, females continue to grow for approximately a year longer than males before achieving the minimum size for reproduction. My study demonstrates that differences in developmental rate occur between males and females of *R. sylvatica*, as early as metamorphosis (consistent with earlier maturity of adult males). Developmental rate is a life history trait that is thought to trade off against avoidance of parasites or investment in immunity (e.g., Lochmiller and Deerenberg, 2000). This idea can be tested experimentally with amphibians at early life stages.

Most anurans take 2-4 years to achieve reproductive maturity, thus, it can be difficult to establish appropriate laboratory protocols for experimental

infections using adults. Most controlled studies may necessitate the use of juveniles. Importantly, amphibians experience the highest rate of mortality during the aquatic phase of their lifecycle. For instance, *Rana pipiens* survivorship in the first stages of life is usually very low with less than 10% of oviposited eggs achieving metamorphosis (Hine *et al.* 1981). Rapid development through larval stages might confer survival advantages for terrestrial juvenile and adult stages (Werner, 1986). Thus, amphibians may adopt the strategy of rapid development to metamorphosis for predation avoidance, or as an adaptation to ephemeral breeding ponds. For my study species there was still a reasonable amount of variation in developmental rate against which susceptibility to parasitism could be examined, even though this species can display highly synchronous, rapid development to metamorphosis (Waldman, 1982).

For adult amphibians, I might expect that increased allocation to larger size would result in inhibited immune function. For anurans, females should therefore carry higher parasite burdens than conspecific males, and larger individuals within a sex should carry higher burdens than smaller individuals of the same sex. The same prediction should apply to rate of development, that is, individuals that invest in faster rates of development should show greater susceptibility to parasite infection. Alternatively, the relationship between investment and immune function may be positive, whereby individuals of superior fitness invest more in faster rates of development and also display heightened resistance to parasitic infection.

For larval *R. sylvatica*, there are no differences in body size or mass at metamorphosis. However, sex-specific patterns of development appear to exist. This might explain why males were generally more susceptible to parasites and on average carried female worms of larger size, when the largest worm in each host was considered. Among infected males, those that developed the fastest seem less parasitized than other males that developed more slowly. Whereas for females, those with the slowest development had the lowest worm burdens (Fig. 2).

My data suggest that in amphibians, time to metamorphosis may be a determinant of susceptibility to worm infection (although different relations may exist within the sexes). This existence of apparent sex-specific relations between development and susceptibility to parasitism is intriguing. It appears that individuals that do not have a developmental trajectory characteristic of their sex, are the ones most susceptible to parasitism (Fig. 3- 2). This finding is somewhat at odds with theoretical predictions that different developmental trajectories shown by different sexes can result in differential susceptibility to infection between sexes. It is important that there is still considerable within-tank variation in both time to metamorphosis and susceptibility to nematodes for both males and females (Fig. 3- 2).

Susceptibility to parasitism is a host-centred metric, but also relates to the parasite's expectation of fitness. The largest female worm from each host was on average larger in male metamorphs than in female metamorphs. It was not possible to isolate live female worms from the lungs to determine egg output or

establish fecundity; however, larger worms might reasonably be expected to have higher fecundity. Several studies have reported a positive relationship between per capita fecundity and per capita worm size (see Tompkins and Hudson, 1999). Larger-sized helminths in male hosts may be a generality; it has been observed in the majority of studies in which helminth size has been measured (Poulin, 1996b). Furthermore, egg production by individual worms infecting male hosts has been observed to be higher than that observed in female hosts (Molan and James, 1984; Swanson *et al.* 1984). Thus studies examining sex-bias in parasitism should include lengths of worms obtained from male and female hosts.

The nematodes studied herein are expected to exact fitness costs on their hosts. Each female worm spanned approximately 2/3 the length of a host's lung (O. Dare-*unpublished observations*), thereby occupying a significant portion of the lung cavity. Also, adult female *R. ranae* acquire blood meals from their host. Thus, these worms may inhibit the mechanical functioning of the lung, may cause damage to the lining of the lung or cause blockages of the blood vessels and therefore impose a considerable debilitating effect on hosts if even few worms are carried (see Goater *et al.* 1993). As many as 92 adult female worms have been observed in the lungs of a single wild *R. sylvatica* individual (O. Dare-*unpublished observations*). *Rhabdias bufonis*, a congener lungworm of similar natural history and infective characteristics, is also known to cause anorexia and reduce survival, growth and physical performance of the toad, *Bufo bufo* (Goater and Ward, 1992). Similarly, it has been suggested that infection with *R. ranae*

may impede chorusing ability in male *R. sylvatica* during the breeding season and reduce reproductive success (Goater and Vandebos, 1997). Intensity of infection in the host did not affect the size of the largest female worms, or developmental rate, likely because of the low infection dosage used in my experimental protocol. I suspect increased susceptibility to infection in males might contribute to the higher mortality levels observed in wild male amphibians (Leclair *et al.* 2000).

In summary, my study demonstrates that differential developmental trajectories between sexes can occur in an amphibian species independently of differences in size. I also show that variation in host development is associated with variation in parasite establishment, maximum size attained by worms and their potential reproductive output. One epizootiological implication of sex-bias in immune response is that parasite burdens may be over-dispersed within hosts of a particular sex because of that sex's increased susceptibility to infection. As such, individuals of that sex may become the main contributors of subsequent cohorts of parasites, and may drive the dynamics of parasite transmission and establishment throughout a host population. This problem has been suggested by others (Ferrari *et al.* 2004; Skorpington and Jensen, 2004), but will of course depend on exposure of individuals of each sex to parasites, and whether there is disparate pathology or mortality in the sex with overdispersed parasite burdens. Such sex differences in exposure and impact of parasites are worthy of further investigation.

Table 3-1. Developmental trajectories of male and female *R. sylvatica*. Means $\pm$ SE of mass (g), snout-vent length (SVL) (cm) and time to metamorphosis (d) are presented. Time to metamorphosis is the number of days for larvae to develop to metamorphosis following the appearance of the first metamorph (day 0 was the first day in which a metamorph was seen). F-values (F), associated degrees of freedom (df) and P-values (P) are also presented. The text also describes matched analyses based on means for each sex from each tank.

	Males	Females	F	Df	P
Mass	0.61 $\pm$ 0.017	0.60 $\pm$ 0.015	0.13	1,91	0.72
SVL	1.82 $\pm$ 0.018	1.83 $\pm$ 0.016	0.26	1,91	0.61
Time	1.6 $\pm$ 0.47	3.20 $\pm$ 0.40	6.37	1,91	0.01

Table 3-2. Median abundance and intensity of female worms (interquartile ranges in parentheses) established in male and female metamorphs. T refers to the test statistic. For tests comparing abundance and intensity between host sexes, the Z-value is the test statistic ($N=93$ for abundance and $N=45$ for intensity). For tests comparing lengths of the largest female worms established in host sexes, the *T*-test is used with 36 degrees of freedom. P-values (*P*) are also presented. The text also describes matched analyses based on mean numbers of female worms established for each sex from each tank.

	Male	Female	T	P
Abundance	0.5 (0-3)	0 (0-1)	0.27	0.78
Intensity	3 (1.25-5.75)	1 (1-2)	-2.72	0.02
Worm length (mm)	3.38 ± 0.19	2.80 ± 0.16	5.08	0.03

Fig. 3-1. Representative stages of *Rhabdias ranae*. A: L₃ infective larva, B: Subadult, C: male adult and D: female adult. L₃ larvae were obtained from fecal samples contributed by wildcaught *Rana sylvatica*. Subadults were removed from the body cavity, while male and female adult worms were removed from the lungs of infected *R. sylvatica* metamorphs. Females were identified by presence of ovaries, darkened digestive tube and sheath, all shown in the photograph. The ranges of lengths of the worms (mm) I measured are indicated on each stage.

Fig. 3-2. Relationships between developmental rate (mean number of days to metamorphosis) and parasite establishment (mean numbers of female worms that established in lungs) for male and female metamorphs, on a per tank basis. Standard errors of the means are also shown indicating that there was much within-tank variation in both time to metamorphosis and parasite abundance for each sex. A significant positive correlation between the two variables was found for males and a significant negative correlation for females (when weighted by numbers of individuals used to obtain mean values such that single observations received little weight). These results suggested slight, but nonetheless, important sex-specific differences in relations between development and susceptibility to infection by lung nematodes. See text for further details.

CHAPTER 4: NEMATODE LUNGWORMS OF TWO SPECIES OF ANURAN AMPHIBIANS: EVIDENCE FOR ADAPTATION

Results from this chapter form the basis of the following paper:

O.K. Dare, Nadler, S.A. and Forbes, M.R. 2008. Nematode lungworms of two species of anuran amphibians: evidence for adaptation. International Journal for Parasitology: doi:10.1016/j.ijpara.2008.05.004

All molecular analyses and the creation of the phylogenetic tree were done by S.A. Nadler.

ABSTRACT

Genetic studies have indicated that some parasite species formerly thought to be generalists are complexes of morphologically similar species, each appearing to specialize on different host species. Studies on such species are needed to obtain ecological and parasitological data to address whether there are fitness costs in parasitizing atypical host species. I examined whether lungworms from two anuran host species, *Lithobates sylvaticus* and *Lithobates pipiens*, differed in measures of infection success in *L. pipiens* recipient hosts. I also determined if the worms from the two host species were sources of genetically-resolvable species of morphologically similar nematodes. Sequences of internal transcribed spacer and 18S rDNA regions of adult lungworms from each host species indicated that worms from *L. sylvaticus* matched *Rhabdias bakeri*, whereas worms from *L. pipiens* matched *Rhabdias ranae*. My work suggested that these morphologically similar species are distant non-sibling taxa. I infected male and female metamorphs experimentally with lungworm larvae of the two species. I observed higher penetration, higher prevalence and higher mean abundance of adult worms in lungs of male and female metamorphs exposed to *R. ranae* larvae than in lungs of metamorphs exposed to *R. bakeri* larvae. Furthermore, metamorphs exposed to *R. ranae* larvae carried larger adult female worms in their lungs. Some variation in infection measures depended on host sex, but only for one parasite species considered. Overall, the differential establishment and reproductive potential of *R. ranae* and *R. bakeri* in *L. pipiens* suggests adaptation.

INTRODUCTION

The recent thrust of research on host-parasite co-evolution has been bolstered by the advancement of molecular and genetic tools that complement morphological and ecological observations. Armed with this combination of tools and perspectives, it has become possible to examine the occurrence of new (and possibly cryptic) species and how they arise (Blaxter, 2003; Herre, 2006). The use of genetic tools has allowed the elucidation of differences between species that otherwise would not be apparent from investigations based on morphology alone. For example, specializations among species within complexes of (sibling) species have been revealed (e.g., among phytophagous insects, Dres and Mallet, 2002), spurring investigations into other species with broad host ranges, which have been presumed to be single generalist species (e.g., Smith et al. 2006). Sibling or cryptic species are typically indistinguishable morphologically, but are reproductively, ecologically and often behaviourally distinct from each other. The existence of cryptic or different parasite species is important because it might explain the variation in parasitism, or in fitness of parasites, that occurs among host species.

Cryptic or different species are often found infecting different host species, although they can also be associated with the same host species (Emelianov, 2007). Parasites might not undergo speciation even when they have a history of infecting different host species. The parasites might nonetheless adapt to the different host species and their environments (Shostak and Dick, 1987; Szalai and Dick, 1989). One intermediate step in the evolution of species may be the

development of host races or populations whose individuals show adaptation to host species typically used. Host races form when generalist parasites retain some gene flow between populations, but differentially exploit various host species (Jaenike, 1981). McCoy (2003) has noted that parasite speciation might also occur in the absence of geographic isolation, when parasites exploit different microhabitats within hosts leading to reproductive isolation. For both parasite races and different species, host specificity can arise when parasites adapt further to a particular host species, to circumvent general host defenses and/or better exploit the host's resources. Parasites can also be more successful at establishing in atypical hosts that may not recognize or respond immunologically to infection (i.e. the parasites show a form of maladaptation to a more familiar host).

The recent discovery and description of *Rhabdias bakeri* (a nematode lungworm) in Wood frogs by Tkach et al. (2006) has raised questions about host specificity of *Rhabdias* spp. in amphibians. *Rhabdias bakeri* has likely been misidentified as *Rhabdias ranae* (see Kuzmin et al., 2001, 2003; Tkach, 2006) and there is some controversy regarding the accuracy of the original life cycle description for *R. ranae* (see Tkach et al. 2006). *Rhabdias ranae* had been assumed to be a broad generalist infecting hosts in the genus *Rana*. These recent discoveries suggest that *Rhabdias* may include several host-specific species and also races that are morphologically difficult to distinguish. However, the potential fitness differences for different species of *Rhabdias* in different host species have not been explored.

Rhabdias ranae was believed to commonly infect two anuran host species in Canada, *Lithobates sylvaticus* (syn. *Rana sylvatica*) (Wood frog) and *Lithobates pipiens* (syn. *Rana pipiens*) (Northern Leopard frog). These host species occupy different habitats for a large part of the year, although their spatial distributions within aquatic environments overlap during breeding and migration periods. It is therefore plausible that different *Rhabdias* species or host races exist and that they have developed host specificity. It is also possible that races or different species can infect both hosts, but show differing levels of adaptation, with higher relative success in their typical host species. The main objective of this study was to determine whether there was evidence of host adaptation for worms originating from *L. sylvaticus* and *L. pipiens*. I compared nuclear rDNA sequence data for worms obtained from each host species for the purpose of molecular characterization and species identification. As indicated below, I found two different parasite species infecting the two different anuran host species (*R. ranae* in *L. pipiens* and *R. bakeri* in *L. sylvaticus*). I then assessed how closely related these two species were, based on phylogenetic considerations. Finally, I assessed whether there was any evidence of adaptation. Adaptation might exist if a parasite species is more successful in the host it typically infects than in an atypical host species. Obviously, the best way to assess the extent of adaptation is with reciprocal experimental infections. However in this experiment, I only compared the infection success of larval *R. ranae* reared from *L. pipiens* host sources in recipient *L. pipiens* metamorphs, and compared these measures to those for larval *R. bakeri* from *L. sylvatica* host

sources. I was unable to rear *L. sylvatica* metamorphs simultaneously and thus could not do the reciprocal experiment. Higher infection success of *R. ranae* larvae compared to *R. bakeri* larvae (in recipient *L. pipiens*) could mean greater adaptation, whereas the reverse would suggest maladaptation. Complete experiments would be needed to see if both species show the same level of adaptation.

To address my main objective, I compared the penetration, establishment, and size of worms in experimental infections of *L. pipiens* metamorphs that were reared in outdoor mesocosms and subjected to controlled exposures. I also compared male and female frogs with respect to size and developmental rate, and in relation to whether they were exposed subsequently to infective *R. ranae* versus *R. bakeri*. Importantly, host size, developmental rate and sex are thought to be important determinants of infection in other vertebrates generally (Gonzalez and Acuna, 2000; Ferrari et al. 20007) and in ranid frogs specifically (Dare and Forbes, 2008).

MATERIALS AND METHODS

All experimental protocols were carried out in accordance with the Carleton University Animal Care Committee guidelines.

Frog collection and tank rearing

Portions of four *L. pipiens* egg masses were collected from Old Chelsea, (39° 50'N, 36° 42'W) on May 1, 2005. Each egg mass was individually housed in

aquaria with pond water that was aerated by an airstone in the laboratory. Tadpoles were fed a granular mixture of tadpole food (Ward's Nat. Sci. Tadpole Food # 88V 6534) and Hagen Fishflakes (Nutrafin Max Complete Flake Food #668672) until they had developed to Gosner stage 25 (Gosner, 1960). At stage 25 they were introduced to outdoor Rubbermaid[®] tanks. Equal numbers of tadpoles from each egg mass were allocated to each tank to a total of 30 individuals in each of 13 tanks. Tanks were established in a fenced compound on the Carleton University campus (45° 25'N, 75° 42'W). Each tank was filled with 300 L of water and covered with 40% shade cloth to prevent oviposition by invertebrates, eliminate predation of the tadpoles by wildlife, and prevent the escape of the frogs from the tanks. Each tank was seeded with leaf litter collected from a mixed deciduous forest stand and populations of *Daphnia* spp. collected from a local pond. Water levels were maintained at 300 L by the removal of water after heavy rainfalls.

Frog husbandry

Metamorphs were removed from the tanks when they developed forelimbs (Gosner stage 42) and brought indoors into a quiet room. Details of individual frog husbandry and feeding during the experiment are outlined in Dare and Forbes (2008).

To obtain worm larvae for infection experiments, five wild-caught adult *L. pipiens* from Ottawa (45° 25'N, 75° 42'W) and five *L. sylvatica* from Bishops Mills (44° 53' N 75° 40'W) were housed in the laboratory. I collected worms from

different host species from different locations to increase the chances of my finding genetically different worms. I housed each frog individually in 2.5 L glass aquaria in the laboratory. Each aquarium was lined with a damp paper towel and an overturned Petri dish at one end as a dry post. The temperature in the room was maintained between 23 and 25⁰ C. I fed each frog three to five large crickets daily and dusted the crickets in amphibian multivitamin mix (Herptivite, Rep-Cal Research Labs, CA, USA) every other day. Two additional adult frogs of each species (and locality) were captured and necropsied to obtain adult worms for PCR and sequencing. These worm specimens were preserved in 95% ethanol and stored at -20 °C prior to nucleic acid extraction.

PCR, sequencing and species characterization

Adult nematodes from *L. sylvaticus* and *L. pipiens* hosts were digested and nucleic acids prepared using the sodium hydroxide method (Floyd et al., 2002); PCR amplifications were performed on DNA from single adult *Rhabdias*. Nematode DNA concentrations were not determined prior to use of extractions in PCR amplifications, instead, 1.5-2 µl of the extract was used for each reaction.

Partial *lsrDNA* was amplified and sequenced using primers that anneal to the 5'-end of the large subunit (LSU) and include a region containing the D2/D3 divergent domains. These primers were #500 (5'-ACTTTGAAGAGAGAGTTCAAGAG) and #501 (5'-TCGGAAGGAACCAGCTACTA). Twelve individual *Rhabdias* specimens were sequenced for this large subunit region (eight from *L. sylvaticus* and four from *L.*

pipiens hosts). rDNA from the internal transcribed spacers (ITS-1 and ITS-2) and 5.8S regions was amplified using primers #93 (5'-TTGAACCGGGTAAAAGTCG) and #94 (5'-TTAGTTTCTTTTCCTCCGCT). Ten individual *Rhabdias* specimens were sequenced for this ITS region (six from *L. sylvaticus* and four from *L. pipiens* hosts). Amplification reactions (25 µl) consisted of 0.5 µM of each primer, 200 µM deoxynucleoside triphosphates, and 3mM MgCl₂. Proofreading polymerase (0.5 units, Finnzymes DyNAzyme EXT) was used for amplification. For both the LSU and ITS amplifications, PCR cycling parameters included denaturation at 94 °C for 3 min, followed by 36 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 s, followed by a post-amplification extension at 72 °C for 7 min.

One µl of each PCR product was used for agarose gel electrophoresis to confirm amplicon size and yield. PCR products were prepared for direct sequencing using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation). Sequencing reactions were performed using dye-terminator cycle sequencing chemistry and reaction products were separated and detected using an ABI 3730 automated DNA Sequencer. Sequences were completely double-stranded for verification using reactions primed from the PCR primers. Contig assembly and sequence conflict resolution was performed with the aid of CodonCode Aligner (CodonCode Corp., Dedham, MA). Internal transcribed spacer/5.8S sequences were aligned using Clustal X Version Qt (Thompson et al., 1997). Phylogenetic analysis was performed by maximum parsimony (MP) using the branch-and-

bound option of PAUP* 4.0b10 (Swofford, 1998) with gaps treated as missing data. Tree interpretation relied upon midpoint rooting because appropriate (and alignable) outgroup sequences were not available.

Parasite collections and metamorph infections

I cultured infective *Rhabdias* larvae in a manner similar to Goater and Vandenbos (1997). Briefly, I incubated faecal contributions from adult frogs at room temperature (23-25 °C) in Petri dishes lined with moist, unbleached, coarse-grade coffee filter paper (#2 Presidents Choice Green coffee filters®) for 3-5 days. When infective larvae were observed on the faeces (a minimum of 72 h), I rinsed the contents of the Petri dish with water, and separated (and counted) the larvae using a dissecting microscope.

I exposed each metamorph to infective larvae obtained from wild-caught adult frogs, only after the metamorphs had completely absorbed their tails (Gosner stage 46). Thus, the exposures were staggered over time to accommodate varying developmental times of individuals. I randomly selected fecal contributions (from three of the five adult host frogs of a particular species) to serve as the source of infective larvae to be used on any given infection day. I obtained equal numbers of infective larvae from each of these three host frogs, for a total of 30 larvae. Equal numbers of metamorphs were simultaneously exposed to infective larvae from *L. sylvatica* and *L. pipiens*. During exposure, metamorphs were isolated in a Petri dish containing damp filter paper and infective larvae for 24 h. Metamorphs were weighed prior to exposure, and snout-

to-vent-length (SVL) measured before housing them individually in plastic containers. The number of larvae remaining in the Petri dish following the infection period was used to determine the number of larvae that penetrated each host.

Rhabdias ranae undergoes protandrous hermaphroditism, developing from a functional male into adult fecund female in about a week. I allowed 14 days following infection before necropsy of each metamorph. The number of adult *R. ranae* and *R. bakeri* worms in the lungs was counted, and samples were preserved in 70% glycerol, diluted to 70% with 70% ethanol, for subsequent measurement using a compound microscope. Only females were recovered from the metamorphs. Two adult worms were damaged in the process of extraction from the lungs, and thus measurements were not obtained for these individuals. Specimen vouchers have been deposited in the Canadian Museum of Nature, Ottawa, Ontario (voucher numbers: CMNPA 2005 0011-0019). I determined the sex of each metamorph by examining the gonads.

Statistical analyses

Multivariate analysis of variance (MANOVA) was used to assess if the groups of trials, in which different parasite species were used for infections, differed by chance in mass, SVL, and day of metamorphosis for female and male metamorphs. This cautionary analysis was done because variables such as developmental rate have been suggested to influence infection outcomes for females and males differently (Dare and Forbes, 2008). I also tested for

differences in the ability of infective larvae of *R. ranae* and *R. bakeri* to penetrate metamorphs upon exposure (using ANOVA). I did this to assess whether differences in parasitism might be explained simply by differences in penetration by *R. ranae* and *R. bakeri*. To determine if sex of host and parasite species influenced likelihood of infection, I used χ^2 tests of association. I then assessed whether abundance or intensity of infection by *R. ranae* and *R. bakeri* varied for males and females separately. Data for numbers of adult female worm numbers did not conform to the assumptions of normality or homogeneity of variances, thus Wilcoxon/Kruskal-Wallis tests were performed to determine if the numbers of worms that established differed if exposures were done with *R. ranae* versus *R. bakeri*. Chi-square approximations were used where sample sizes were lower than 10, whereas *Z* and *P* values for normal approximations were reported where sample sizes were large. Length of adult female nematodes is known to be a strong predictor of fecundity (Goater, 1992; Tomkins and Hudson, 1999); I therefore used ANOVA to test whether lengths of adult worms recovered from lungs of male and female metamorphs differed for *R. ranae* and *R. bakeri*.

I examined whether the numbers of larval *R. ranae* and *R. bakeri* that successfully established as adult females related to numbers of larvae *R. ranae* and *R. bakeri* penetrating. For tests of sex differences in establishment of *R. ranae* and *R. bakeri* in metamorphs, I used absolute worm numbers, since dosage of larval infection did not influence subsequent establishment (see below).

I used paired *t*-tests to determine whether any tank-associated biases existed in sex-ratio of metamorphs reared; and to check for any tank effects on measurements of development that differed between the sexes that could influence my interpretation of susceptibility to infection.

RESULTS

Frog collection and tank rearing

Frogs metamorphosed from July 11 to August 22, 2005 from day 0 to day 42, respectively. Individuals developed at times that coincided with development of wild conspecifics (personal observations). In total, 54 male metamorphs and 45 female metamorphs were used for the experiment, a proportion that does not significantly differ from a 50:50 ratio (sign-test least likelihood ratio: $P = 0.37$; Table 4-1).

Sequencing and species characterization

I next outline my evidence for two parasite species being used, even though my genetic analyses followed my experimental infections. ITS *lsrDNA* sequences from individual *Rhabdias* specimens have been deposited in GenBank (Accession numbers **EU360823-360844**). No intra-individual sequence polymorphisms were detected. All individual nematodes obtained from a single frog species had identical sequences for each respective gene (LSU and ITS). In contrast, *lsrDNA* sequences representing eight nematodes from *L. sylvaticus* hosts had two differences compared with sequences representing four

nematodes from *L. pipiens* hosts. These two sequence differences were both transition substitutions ($A \leftrightarrow G$). Internal transcribed spacer sequences representing six nematodes from *L. sylvaticus* hosts had 27 differences compared with sequences representing four nematodes from *L. pipiens* hosts. These sequence differences included 20 transition substitutions ($A \leftrightarrow G$; $C \leftrightarrow T$), four transversions (two types: $T \leftrightarrow A$; $G \leftrightarrow T$), and three gap versus nucleotide (indel) changes. Approximately 75% of these substitutions occurred in ITS-1, with the remainder in ITS-2. The ITS/5.8S sequences from these two hosts were identical to sequences previously deposited in GenBank. The *Rhabdias* individuals from *L. sylvaticus* (Wood frog) matched *R. bakeri* (e.g. DQ264774), whereas the *Rhabdias* specimens from *L. pipiens* (Northern leopard frog) matched *R. ranae* (e.g. DQ264769).

The mid-point rooted parsimony analysis of ITS/5.8S sequences yielded a single tree of 48 steps (Consistency Index 0.98) and depicted a sister-taxon relationship between *R. bakeri* and *Rhabdias pseudosphaerocephala* with *R. ranae* occupying the branch external to the three other *Rhabdias* species analyzed (Fig. 4-1). I thus continue to report worms from *L. pipiens* and *L. sylvaticus*, as *R. ranae* and *R. bakeri*, respectively.

Metamorph infections

I examined whether experimental infections were likely to be influenced unduly by other variables. Multivariate analyses of parasite species (*R. ranae* and *R. bakeri*) and host sex on mass, SVL and date to metamorphosis revealed

only a significant host sex effect (parasite species: $F = 0.23$, $df = 3, 93$, $P = 0.88$; host sex: $F = 2.95$, $df = 3, 93$, $P = 0.04$; host sex*parasite species: $F = 1.36$, $df = 3, 93$, $P = 0.36$). I found no a priori chance association between mass of metamorphs and subsequent exposure to infective *R. ranae* or *R. bakeri* larvae, nor were there differences in mass between sexes (Tables 4-2, 4-3): the interaction between these two factors was not associated with variation in mass at metamorphosis.

I found no effect of host sex on SVL ($P = 0.12$, Table 4-2) and no chance association with parasite species on SVL ($P = 0.68$, Table 4-2). The interaction between sex and parasite species was not associated with variation in SVL at metamorphosis ($F = 2.10$, $df = 1, 95$, $P = 0.15$). However, I found a clear effect of sex on developmental rate (Table 4-2), where male metamorphs took on average 11.7 days to develop to metamorphosis, which was significantly less time than female metamorphs (average of 17.9 days). There was no chance association between day to metamorphosis and parasite species used (Table 4-3): the interaction between host sex and parasite species used was not associated with variation in day to metamorphosis ($F = 1.17$, $df = 1, 95$, $P = 0.28$). Thus male and female metamorphs differed only in developmental rate, but not size.

There were 28 male and 21 female metamorphs exposed to infection from *R. ranae* larvae, whereas 26 male and 24 female metamorphs were exposed to infection from *R. bakeri*. The proportion of male or female metamorphs recovered did not vary significantly among tanks (pooled Standard Error (SE) = 0.58, t -statistics = -2.05, $df = 12$, $P = 0.063$).

Infective *R. ranae* larvae were more successful at penetrating metamorphs than infective *R. bakeri* larvae ($F = 18.26$, $df = 1, 97$, $P < 0.0001$), with fewer *R. ranae* larvae remaining in the Petri dish following the exposure period. I next determined if the dosage of infective larvae related to establishment of adult worms in the hosts' lungs. If the number of infective larvae that penetrated hosts was predictive of the number of adults that ultimately established, dosage would have to be controlled for statistically before examining whether there was an influence of either host sex or parasite species. However, none of the four relations between number of larvae used for exposures and proportion of worms that established as adult (i.e. for female and male metamorphs with two species of worm) approached significance (r ranged from 0.028-0.200, P ranged from 0.36-0.88). I also determined no greater propensity for either male or female metamorphs to acquire infection, regardless of the species of the worms (*R. bakeri*: $\chi^2 = 0.07$, $P = 0.79$; *R. ranae*: $\chi^2 = 0.03$, $P = 0.87$).

I found that the species of infective larvae had a very significant impact on establishment of worms. More of the metamorphs infected with *R. ranae* larvae carried established adult worms than metamorphs infected with *R. bakeri* larvae. Ten of the 21 female metamorphs exposed to infective *R. ranae* larvae had at least one worm establish, whereas only five of the 24 female metamorphs exposed to *R. bakeri* larvae had worms establish. Similarly, 12 of the 28 male metamorphs had at least one worm establish if the infections were with *R. ranae*. In stark contrast, only six of the 26 male metamorphs had established worms when exposed to *R. bakeri* larvae.

The mean abundance of worms in metamorphs also differed considerably depending on species of infective larvae. Metamorphs infected with *R. ranae* carried higher numbers of adult worms than did those infected with *R. bakeri*. This worm species effect was significant among males, and showed a similar strong trend for females (Table 4-3). However, male and female metamorphs did not differ in abundance of worms, regardless of worm species (*R. ranae*: $Z = -0.89$, $P = 0.38$; *R. bakeri*: $Z = -0.31$, $P = 0.76$).

When I examined only those individuals harboring at least one adult worm (i.e., intensity, see Bush et al., 1997), I observed no differences in numbers of established worms in metamorphs (male or female) infected with *R. ranae* or *R. bakeri* larvae (Table 4-3). That is, worm species did not influence worm intensity within each sex (Table 4-3). However, male metamorphs infected with *R. ranae* had significantly more worms than females infected with the same worm species (males: 2.93 ± 0.52 SE, females: 1.6 ± 0.43 SE; $Z = -2.11$, $P = 0.04$). This sex bias in intensity was not observed for metamorphs exposed to infective *R. bakeri* larvae.

I also measured worm length as a proxy for fecundity, a variable that I expect to be responsive to adaptation (see Dare and Forbes, 2008). Worm length was influenced by worm species. *R. ranae* removed from male metamorphs, were on average $5.20 \text{ mm} \pm \text{SE} = 0.22$ long, while the average length of worms removed from male metamorphs that had been exposed to *R. bakeri* larvae was $3.86 \text{ mm} \pm \text{SE} = 0.21$ (Table 4-3). Worms removed from female metamorphs that had been exposed to *R. ranae* were on average 5.17 mm in length $\pm \text{SE} = 0.24$,

while worms removed from female metamorphs exposed to *R. bakeri* larvae were $3.45 \text{ mm} \pm \text{SE} = 0.35$ (Table 4-3). There was no difference between male and female metamorphs in the lengths of adult *R. ranae* worms ($F = 0.009$, $df = 1, 20$, $P = 0.92$) or *R. bakeri* worms ($F = 1.11$, $df = 1, 9$, $P = 0.32$).

DISCUSSION

ITS and 18S rDNA sequence comparisons of *Rhabdias* from *L. sylvaticus* and *L. pipiens* hosts were consistent with the presence of two different nematode species in the donor frog hosts. In particular, ITS sequences of multiple individuals from each frog host match GenBank sequences of *Rhabdias* identified by genotype (Tkach et al., 2006) as *R. bakeri* and *R. ranae*. Thus, these ITS sequence tags strongly suggest that the *L. sylvaticus* source worms were *R. bakeri* and the *L. pipiens* source worms were *R. ranae*. These ITS sequences provide a relatively simple method to discriminate between these species and this approach could be extended to all life history stages of the parasites. In contrast to species identification using sequence tags, species delimitation should include more comprehensive population sampling and phylogenetic analysis of character data, preferably using a multilocus approach (Nadler et al., 2000; Nadler, 2002). Phylogenetic analysis of *Rhabdias* ITS sequences depicts each of the four morphospecies analyzed as an independent lineage based on autapomorphies (data not shown). The phylogenetic relationships among these species suggests that *R. bakeri* and *R. ranae* are not sister taxa, with *R. bakeri* more closely related to *R. pseudosphaerocephala*, a

parasite of *Bufo marinus*. Mid-point rooted distance analysis of these sequences failed to recover a sister-group relationship between *R. bakeri* and *R. ranae* (not shown), although the sister taxon of *R. bakeri* is different in the distance tree. However, if this general result is reliable, it indicates that the experimental infections performed in this study were not between closely related parasites. Future studies of the fitness effects of cross infections would benefit from an experimental design that considers phylogenetic relationships.

The species of infective worm larvae played a clear role in susceptibility of metamorphs to infection. Metamorphs exposed to infective *R. ranae* from conspecific donor host sources had more larvae penetrate upon exposure and harboured more and larger adult worms than when exposed to *R. bakeri* larvae from a heterospecific donor host source. This observation points towards adaptation of *R. ranae* that is manifested in establishment and reproductive potential of adult worms. Tkach et al. (2006) observed that only a few young *L. pipiens* metamorphs harboured *R. bakeri* and thus concluded that this species, found principally in *L. sylvaticus*, is a specialist, while *R. ranae* is a generalist. My experimental infections, using these two *Rhabdias* species, suggest *R. ranae* may in fact be closely adapted to *L. pipiens*. Additional experiments are necessary to determine the level of adaptation, that is, whether it also occurs consistently for *R. bakeri*.

Although *R. bakeri* has previously been distinguished from *R. ranae* by morphological differences in the pseudolabia, buccal capsule and esophageal bulb (Tkach et al., 2006), the errors in identification that have only been

elucidated through recent gene sequencing clearly demonstrate that these morphological differences have not been distinct enough to effectively distinguish working specimens. *Rhabdias ranae* grew to larger sizes in the *L. pipiens* metamorphs than did *R. bakeri*. My results show these two species also differ in rate of development and reproductive potential. A re-characterization of the life cycles of these two species might also reveal differences from the cycle previously described by Baker (1979) for *R. ranae*. For example, it has previously been assumed that only those worms that reach the lungs establish in the host (Baker, 1979). Differences in the migratory pathways and duration of infective larvae in the body cavities of different host species might also exist, although this remains to be tested.

Sex bias in parasitism has been observed in *L. sylvaticus* (Dare and Forbes, 2008), a species that is known to be sexually dimorphic in size at adulthood with females larger than males (Berven, 1982; Leclair et al., 2000). Although Dare and Forbes (2008) did not observe size dimorphism in *L. sylvaticus* at metamorphosis, disparate rates of development to metamorphosis between the sexes were observed that related to susceptibility to infection; individuals that digressed from the trajectory of development typical for their sex experienced greater susceptibility to infection. More specifically, males that developed more slowly and females that developed more rapidly carried higher worm burdens than expected. As expected, there were no differences in size between male and female *L. pipiens* metamorphs. However developmental rates differed significantly between the sexes. And those differences in host sex and

development related to sex differences in parasitism by *R. ranae* but not by *R. bakeri*. Furthermore, the contrast between my observations of *L. pipiens* and those of the previous study on *L. sylvaticus* metamorphs (Dare and Forbes, 2008) suggest that relationships between developmental rate and susceptibility to infection are parasite species-specific.

It was also important to confirm that variation in developmental rate between male and female metamorphs was not associated with the source of the rearing tank from which the metamorphs were obtained. I reared the metamorphs in several tanks to create variation that would reflect normal variation observed among metamorphs had they been obtained from natural ponds. Any biases in developmental rate attributable to the rearing tank would need to be controlled for in my analyses. However, tanks did not influence rate of development among the male and female metamorphs; thus I am confident that observed effects were real.

My work provides empirical evidence for an interaction between host sex and parasite species in determining measures of infection success. The larger size of worms from *L. pipiens* host sources, combined with higher intensities of infection in male metamorphs exposed to worms from a conspecific host source suggests that male metamorphs may play a more significant role than females in terms of parasite contributions to a population. I also report that parasite fitness is influenced by host sex for *R. ranae*.

My original intent for this study was to examine whether host species origin of a parasite influenced measures of parasitism. The finding of different

species still allowed us to examine evidence of adaptation in the same manner that one explores whether host races exist. The problem of how well parasite species exploit atypical host species is an important one for understanding constraints on parasite evolution and ecological barriers in the use of different host species. Variation in fitness among different hosts has been shown for generalist parasite (phytophagous) species (Fry, 1990; Agrawal, 2000). My results demonstrate clear differences in penetration, establishment and reproductive potential of *R. ranae* and *R. bakeri* in *L. pipiens*. Ideally, a reciprocal experiment in which both *R. ranae* and *R. bakeri* are used to infect *L. sylvaticus* would permit discussion of the relative success of both worm species in the alternate host species. The fitness of phylogenetically closely related or distant parasites, and the extent to which hosts can tolerate these different parasites should they establish, is likely to be important as ecological and temporal boundaries between species continue to be removed.

Table 4-1. Distribution of male and female *Lithobates pipiens* metamorphs reared among 13 tanks and subsequently exposed to infective *Rhabdias* larvae from *Lithobates pipiens* and *Lithobates sylvaticus* donor host species.

Tank	<i>Lithobates pipiens</i> donors			<i>Lithobates sylvaticus</i> donors			Tank Total
	F	M	Total	M	F	Total	
3	1	5	6	4	3	7	13
11				1		1	1
12	2	1	3	1	1	2	5
16	2		2	2		2	4
17	2	2	4		2	2	6
18	5	3	8	1	1	2	10
19				1		1	1
22	5	3	8	2	4	6	14
24	2	5	7	4	3	7	14
25				1	2	3	3
30	1	4	5	2	2	4	9
32	1	4	5	4	7	11	16
33		1	1	1	1	2	3
Grand Total	21	28	49	24	26	50	99

Table 4-2. Associations between parasite species and host sex on mass (g), day of metamorphosis (d), and snout to vent length (cm). Data shown include mean $\pm$ SE. See text for details of tests used.

	<i>R. ranae</i>	<i>R. bakeri</i>	F	df	P
Mass	0.77 $\pm$ 0.03	0.82 $\pm$ 0.64	0.48	3,93	0.49
Day of metamorphosis	14.37 $\pm$ 1.71	14.64 $\pm$ 1.70	0.14	3,93	0.91
Snout to vent length	2.05 $\pm$ 0.03	2.07 $\pm$ 0.04	0.17	3,93	0.68
<hr/>					
	Male	Female	F	df	P
Mass	0.75 $\pm$ 0.03	0.85 $\pm$ 0.07	1.87	3,93	0.18
Day of metamorphosis	11.65 $\pm$ 1.44	17.9 $\pm$ 1.90	7.14	3,93	0.009
Snout to vent length	2.02 $\pm$ 0.03	2.10 $\pm$ 0.04	2.47	3,93	0.12

Table 4-3. Effects of parasite species on worm establishment (range) and worm length (mm), controlling for sex of host. Data shown include mean $\pm$ SE. See text for details of tests used.

Host species	<i>R. ranae</i>	<i>R. bakeri</i>	Test stat.	df	P
Worm abundance (females)	0.76 $\pm$ 0.27	0.25 $\pm$ 0.11	Z = 1.93	NA	0.053
Worm abundance (males)	1.46 $\pm$ 0.38	0.39 $\pm$ 0.16	Z = -2.27	NA	0.02
Worm intensity (females)	1.6 $\pm$ 0.43	1.2 $\pm$ 0.20	χ^2 = 0.31	NA	0.58
Worm intensity (males)	2.93 $\pm$ 0.52	1.67 $\pm$ 0.3	χ^2 = 1.24	NA	0.26
Worm length (females)	5.17 $\pm$ 0.24	3.45 $\pm$ 0.35	F= 18.90	1,13	<0.001
Worm length (males)	5.20 $\pm$ 0.22	3.86 $\pm$ 0.21	F= 13.002	1,16	0.002

Fig. 4-1. Single most parsimonious tree inferred from branch-and-bound analysis of ITS/5.8S rDNA sequence data (CI=0.98). Tree was mid-point rooted. Sequences include DQ264773 (*Rhabdias bakeri*), DQ845738 (*Rana pipiens*), DQ264769 (*Rhabdias ranae*), and DQ845741 (*Rana sylvatica*).

CHAPTER 5: PATTERNS OF INFECTION BY TWO GENERA OF LUNGWORMS IN A POPULATION OF NORTHERN LEOPARD FROGS

Results from this chapter have been submitted for review to the Journal of Parasitology in a manuscript of the same title.

ABSTRACT

I examined a population of Northern leopard frogs to determine whether sex biases in investment in immunity, previously reported for this host species under controlled exposures to lung nematodes, was predictive of patterns of parasitism in nature. I examined *Rhabdias ranae* and *Haematoloechus* spp. infections in 74 breeding adult, 28 non-breeding adult and 53 juvenile frogs. Contrary to my predictions, *R. ranae* prevalence and abundance were higher in breeding female frogs than breeding male frogs, although no sex bias was observed among non-breeding adults, or among juvenile frogs. Female frogs also carried larger *R. ranae* worms than did male frogs regardless of age or breeding condition. I observed no sex-linked patterns of parasitism by *Haematoloechus* spp. worms in either adult or juvenile frogs. Alternative hypotheses explaining the observed female bias in parasitism by nematode lungworms in nature need to be considered.

INTRODUCTION

The occurrence of sex bias in parasitism in natural populations of amphibian hosts remains equivocal. Most observational studies examining sex bias in parasitism within vertebrates have found sex biases in species that are sexually dimorphic in size, and in cohorts of individuals in breeding condition or just prior to breeding (reviews by Poulin, 1996a; Schalk and Forbes 1997; Moore and Wilson, 2002). In such studies, there are often confounds of size differences and reproductive status between males and females. Such factors might alone or together contribute to a bias between sexes in parasitism that is not attributable to sex *per se*. This is important as researchers are beginning to investigate which sex is more important from the viewpoint of parasite population dynamics (Ferrari et al., 2004). Some assessment during the lifetime of individuals is needed, as well as when differences in growth rates or breeding condition are manifested.

There are also other important considerations about host use by parasites. For example, larger individuals, regardless of sex, may present more available resources for more parasites (Esch et al., 1990), while individuals in reproductive condition might invest less in resistance to infection in favour of investment of resources in reproduction. The extent to which sex biases in susceptibility to parasites is seen among juveniles or adults in non-breeding condition is not well studied. This problem of not examining sex biases in parasitism in juveniles or non-breeding individuals is inherent in many observational studies (Schalk and Forbes, 1997) and limits the ability of researchers to identify those individuals

that might be most important in driving infection dynamics through natural host populations.

Differences between sexes in parasitism following controlled exposure to infective larvae is likely mediated by investment in parasite resistance (Dare and Forbes, 2008); such sex-linked differences in parasitism are found in amphibian species that do not display size dimorphism and can be observed as early as metamorphosis (Dare et al., 2008). Laboratory experiments using *Rhabdias ranae* (Nematoda) lungworms to infect juvenile Northern leopard (*Rana pipiens* syn. *Lithobates pipiens*) and Wood (*Rana sylvatica* syn. *Lithobates sylvaticus*) frogs, have shown that male and female hosts do not differentially prevent infective larval penetration; however once inside the host, more adult worms establish in lungs of male hosts than female hosts (Dare and Forbes, 2007; Dare et al., 2008). Northern leopard frogs do not display sexual size dimorphism; however, juvenile male leopard frogs develop faster than females, and variation in *R. ranae* parasitism among male and female Northern leopard frogs has been linked to variation in investment in developmental rate (Dare et al., 2008). As well, lungworms that successfully penetrate and establish in juvenile Wood frog hosts achieve larger sizes in male frogs than in female frogs. For nematodes, larger sizes confer greater fecundity (Poulin, 1996b; Tompkins and Hudson, 1999). Collectively, these findings suggest a reduced immunological response to lungworm infection in juvenile male frogs than in juvenile female frogs. There is a need for studies on sex biased patterns of parasitism in relation to host age and breeding state in natural populations of species that do not display sexual

dimorphism, where researchers can distinguish among different age cohorts of frogs, to determine the extent to which differences in investment in resistance that have been recorded in controlled experiments are apparent in patterns of parasitism in nature.

In the present study, I examined patterns of parasitism of two lung helminths in a natural population of Northern leopard frogs. My objectives were to compare male and female adult (breeding and non-breeding) and juvenile frogs for *R. ranae* and *Haematoloechus* spp. (Trematoda) lung infections. Based on observations from experimental post-metamorphic leopard frogs, I predicted greater susceptibility of male frogs to infection than female frogs, controlling for age. I also predicted that breeding frogs would be more susceptible to infection than non-breeding frogs, because of the energetically high costs associated with breeding activities.

MATERIALS AND METHODS

Frog collections and necropsy

I collected road-killed Northern leopard frogs from Bishops Mills, Ontario (44 52 60.0 N 75 40 0.0W). The site of collection is an extensive wetland area sectioned by highways. Levels of vehicle-related mortality are high in this region, particularly during seasonal migration periods when cohorts move *en masse* over a few short days. I collected frogs in the spring (April 2- 26, 2007) as they migrated from their hibernation sites to breeding ponds, and again in the late summer/early fall (August 11-26, 2007) when they were moving between feeding

grounds and hibernation sites. A long warm summer followed by a sharp drop in temperature resulted in the absence of a typical fall mass movement of frogs back towards their hibernation sites for the winter. Thus I was not able to collect adult or juvenile frogs later in the fall. However, I did collect a small number of metamorphs (8) on a single day in the fall (September 11, 2007).

I do not believe that there was an influence of infection on migration/travelling abilities of frogs. The roads on which carcasses were collected divided a large wetland area, sometimes separating bodies of water; thus frogs had not travelled far from their ponds before being killed on the roads. I also do not believe that escape response to the approach of a vehicle was affected by infection, as Northern leopard frogs respond to approaching vehicles by assuming a crouched stance, rather than by leaping away (Dare, *personal obs.*).

I brought the frog carcasses back to the laboratory for necropsy and identified the age of each individual (juveniles/yearlings and adults). I also determined the sex of individuals by the presence of secondary sex characteristics (swollen and darkened thumb in males) and by examining the gonads when present. Only frogs that had both intact lungs were used for analyses. *R. ranae* (nematodes) found in the lungs were removed and enumerated. I report prevalence, abundance and intensity as response parameters for parasitological counts (according to Bush et al., 1997). *R. ranae* worm lengths were measured with the aid of a dissecting microscope and computerized measurement software (Carl Zeiss, Axio vision Rel. 4.6). Although

all worms removed were intact, some were too damaged to measure lengths accurately. These damaged worms (24 out of 230 worms in total from all adults and juveniles) were not included in my analyses of worm length, although they were retained in my tests of abundance and intensity. I did not measure length of *Haematoloechus* because the same association between length and egg output has not been reported for fluke worms.

Statistical analyses

For both juveniles and adults, differences in prevalence of *R. ranae* and *Haematoloechus* lungworm infections between sexes, or between age/ breeding cohorts (adults and juveniles; adult breeding and non-breeding), were analyzed with tests of equality of proportion.

I tested for differences between juvenile frog sexes in intensity of *Rhabdias* infections using analysis of variance (ANOVA); I used Kruskal-Wallis non-parametric tests to measure sex differences where data did not conform to normal distributions or pass Levene's test for homogeneity of variances. I tested for sex effects on length of *R. ranae* worms obtained from different individual juvenile frogs using nested ANOVA, to control for variation in the number of worms carried by different individual frogs.

For adult frogs, I tested for sex and breeding condition effects on abundance and intensity of *R. ranae* infections using ANOVA. I used a 3-factor partial nested ANOVA to test for host breeding condition (fixed), sex (fixed) and individual host (random) effects on mean length of worm obtained from different

individual frogs. Different numbers of worms were obtained from each individual frog. I therefore tested for sex and breeding condition effects, controlling for any differences among individual hosts that could influence the mean length of worms, by using the partial nested ANOVA model. Thus, breeding condition was crossed with sex and individual was nested within breeding condition (b.cond) and sex in the following model: **worm length = sex + b.cond. + individual host(sex, b.cond.) + sex*b.cond.** I then tested male and female frogs separately for effect of breeding condition on mean worm length of *Rhabdias* worms using ANOVA.

Tests for sex differences in *Haematoloechus* spp. intensity and abundance were performed with ANOVA (Kruskal-Wallis tests were used for data that were not normally distributed and/or did not pass Levene's test of homogeneity of variances).

RESULTS

Frog collections

Spring collections consisted of 74 adult frogs, while there were 28 adult and 45 yearling (juvenile) frogs collected in the summer. I also collected 8 metamorphs in the fall.

The difference in the sample size between spring and summer adult collections is due to the synchronized migrations that occur in the spring, resulting in higher mortality on nights when movement occurs. Juvenile frogs do not migrate to breeding ponds in the spring, so I did not observe any road-killed

yearling frogs in the spring. Adult movements in late summer were less synchronized than in the spring and thus fewer road-killed adults were found on the roads on a given night.

Thirty-two of the 74 adult frogs collected in the spring were infected with *Rhabdias ranae*. Twelve of the 28 adults collected in the early fall were infected and 17 of the 45 yearling frogs collected in the early fall carried *R. ranae* worms. None of the 8 metamorphs collected in the fall were infected with *R. ranae*.

Twenty-three of the 74 adult frogs in the spring were infected with *Haematoloechus*, while 10 of the 28 adults collected in the early fall were infected. Nine of the 45 yearlings were infected. Four of the 8 metamorphs were infected with *Haematoloechus*. I detail the numbers of male and female adult and juvenile frogs infected with *R. ranae* and *Haematoloechus* spp (Table 5-1) and I summarize the prevalence, abundance and intensity of *Rhabdias* infections among adult and juvenile frogs of both sexes (Tables 5-2 and 5-3).

I observed only 13 frogs with dual infections of *Haematoloechus* and *Rhabdias*, but 77 frogs carried one or the other lungworm.

Juvenile frog infections

The prevalence of *R. ranae* in yearling frogs collected did not differ between the sexes (test for equality of proportions: $Z = -0.89$, $p = 0.37$). The abundance of *R. ranae* also did not differ between male and female juvenile leopard frogs (K-W: $\chi^2 = 1.62$, $p = 0.20$). When I examined those individuals that carried one or more parasite (i.e. intensity), males tended to carry more worms ($F = 3.68$, $df = 1, 15$,

$p=0.07$). However, the length of worms from female frogs was greater than the length of worms from male frogs ($F=10.03$, $df=1,14$, $p<0.01$). No differences between the sexes were observed in *Haematoloechus* prevalence ($Z=0.75$, $p=0.45$), abundance ($F=0.56$, $df=1,43$, $p=0.46$) or intensity ($F=0.17$, $df=1,7$, $p=0.69$).

Adult frog infections

The prevalence of *R. ranae* was higher in female than in male frogs collected in the spring (just prior to breeding) ($Z=2.30$, $p=0.02$). However, there was no significant difference in prevalence of *R. ranae* in female and male frogs collected in the early fall (non-breeding condition) ($Z=0.66$, $p=0.51$). No effect of breeding condition on prevalence was observed when I examined each sex separately (Males: $Z= -0.17$, $p=0.87$; Females: $Z=0.84$, $p=0.40$).

Sex and breeding condition did not significantly influence *R. ranae* abundance (ANOVA: $F=2.41$, $df=3,98$, $p=0.07$). I then examined individual effects because this overall model was borderline significant, and found that females carried significantly more worms than males ($F=6.84$, $df=1,98$, $p=0.01$), although breeding condition did not significantly affect abundance ($F=0.17$, $df=1,98$, $p=0.68$). The interaction of effects was also not significant ($F=0.66$, $df=1,98$, $p=0.42$). Sex and breeding condition also did not significantly influence *R. ranae* intensity (ANOVA: $F= 1.94$, $df=3,29$, $p= 0.15$).

The length of *R. ranae* worms differed significantly between male and female frogs and also among frogs in different breeding condition (Table 5-4).

Female frogs carried larger worms than males in both breeding conditions (partial nested ANOVA: Sex $F=4.92$, $df=1,85.78$, $p=0.03$). Worms from breeding frogs (collected in the spring) were significantly larger than those from non-breeding frogs collected in the early fall (partial nested ANOVA: breeding condition $F=4.96$, $df=1,86.62$, $p=0.03$). The interaction between sex and breeding condition was not significant ($F=0.61$, $df=1, 86.87$, $p=0.44$). When I tested whether breeding condition influenced worm length for each frog sex separately, I found that breeding female frogs carried larger worms than non-breeding females ($F=8.25$, $df=1,89$, $p=0.01$). However length did not vary significantly among breeding and non-breeding male frogs ($F=0.84$, $df=1,41$, $p=0.37$).

The prevalence of *Haematoloechus* did not differ between male and female adults in either breeding or non-breeding condition (breeding: $Z=1.01$, $p=0.313$, non-breeding: $Z=0.57$, $p=0.57$). Sex and breeding condition also did not influence the abundance or intensity of fluke infection of adult frogs (abundance: $F=1.15$, $df=3,98$, $p=0.33$; intensity: $F=1.33$, $df=3,29$, $p=0.29$).

Adult versus juvenile infections

The prevalence of *R. ranae* in male or female frogs did not differ among yearlings and adults (females: $Z= -1.06$, $p=0.29$; males: $Z= 0.45$, $p=0.65$). *R. ranae* abundance was significantly influenced by the overall model (ANOVA: $F=2.83$, $df=3,69$, $p=0.04$), however individual effects tests revealed this was driven by the interaction between the two effects (sex: $F=1.80$, $df=1, 69$, $p=0.18$; age: $F=2.64$,

df=1,69, $p=0.11$, sex*age: $F=5.81$, df=1,69, $p=0.02$). *R. ranae* intensity did not vary with sex and age (ANOVA: $F=2.25$, df=3,25, $p=0.11$).

The prevalence of *Haematoloechus* did not significantly vary with age in either female or male frogs (females: $Z= -1.10$, $p=0.27$; males: $Z= -1.17$, $p=0.24$). As well, age and sex did not significantly influence *Haematoloechus* abundance (ANOVA: $F=0.27$, df=3,69, $p=0.85$) or intensity (ANOVA: $F=0.36$, df=3,15, $p=0.78$).

DISCUSSION

Contrary to my expectations, adult female Northern leopard frogs had higher levels of *R. ranae* infections than male frogs, and breeding males did not appear to be more susceptible to infection than non-breeding males. Exposure to infective worms over late spring and summer was likely similar for both host sexes, as no difference in prevalence was observed between male and female juvenile frogs, or between male and female non-breeding adults collected in early fall. However, in the spring collections, a higher proportion of adult breeding female frogs were infected with *R. ranae* worms than adult breeding male frogs. I also observed a higher abundance of *R. ranae* in female frogs than in male frogs. Furthermore, the worms removed from female frogs were larger than those removed from male frogs regardless of age or breeding condition. Since the worms recovered from frogs in the spring were infections carried over from the previous year, it could be that the variation observed is due to differences in

immune response between host sexes. However, I did not measure immunity in this study.

Greater susceptibility to infection is expected to occur for adults just prior to and during the breeding season when energetic costs of breeding are high. Breeding males invest considerable resources towards advertising (chorusing) and active combat against competitors to increase reproductive success (Emlen, 1976; Judge and Brooks, 2001). Female frogs also expend energy in the production of eggs. On average, a Northern leopard frog can lay up to 7000 eggs (Seburn and Seburn, 1998). The high costs associated with mating behaviours and egg production may trade off against resources for other functions including defense against parasite infection. My observations were consistent with this expectation, although other differences that influence exposure, such as behaviour also may have produced these differences independent of immunity-based susceptibility. Yet, I found no difference in worm size in adult males regardless of breeding condition, although worms in breeding female frogs were larger than those in non-breeding females. This latter finding suggests a greater cost of reproduction for females than males in terms of trade-offs against immunity.

Differences in immune response as a result of differential investment in development were expected. Previous work has shown that male Northern leopard frogs develop faster than female frogs (Dare et al., 2008). Although my small sample size of metamorphs did not permit comparisons to be made between observations on metamorphs in the previous controlled experiment and

this natural population, I did find a tendency for male yearling frogs to harbour higher numbers of worms than females, which is consistent with previous results using experimental infections.

A limitation of observational studies is that they permit limited speculation about the mechanisms driving the patterns observed. Nevertheless, observations from previous experimental work and my present field study indicate occurrences of sex biases in parasitism of *Rhabdias* infections in Northern leopard frogs, and these biases are independent of investment in size regardless of host age.

The proportion of breeding adult frogs infected with *Rhabdias* in the spring was similar to the proportion of infected non-breeding frogs in the fall; however infected frogs collected in the spring harboured twice as many worms as frogs collected in the early fall. The similarity in proportions of infected individuals across seasons indicates similar exposure from one year to the next. However, the difference in numbers of worms indicates a loss of infection, probably as a result of senescence of the mature worms that were carried over the winter, and/or an increase in investment in immune response after the breeding season ended. *R. ranae* is believed to undergo only one generational cycle in a year in natural populations, with a peak in transmission of new larval infections occurring in the late summer and early fall (Baker, 1979). Thus, it is likely that the frogs I sampled in the early fall were still acquiring a new generation of larval worms to be carried over the winter. The damaged condition of the road-killed carcasses precluded a survey of immature worms in the body cavities of the hosts, and therefore I could not compare rates of worm development among seasons.

Juvenile frogs and non-breeding adults did not differ in proportion of infected individuals or levels of infection. However, I observed a significant interaction between sex and age effects on *Rhabdias* abundance and intensity between sex and age that reflected the female bias in infection for adults and the male bias in infection for juveniles.

Patterns of *Haematoloechus* spp. infection were not associated with sex, age or breeding condition. The lack of association between levels of parasitism and sex or breeding condition might be due in part to my inability to distinguish between species of fluke worm. *Haematoloechus* is a genus complex of morphologically similar species that are difficult to identify without genetic testing (Kennedy, 1981; Bolek and Janovy, 2007). Furthermore, co-infections of *Haematoloechus* species are common. The similar proportions of infected males and females might reflect similar feeding habits or dietary preferences of hosts. Northern leopard frogs are indiscriminate feeders with prey choices determined by gape width of the frog (Seburn and Seburn, 1998). *Haematoloechus* lung flukes infect a number of different dragonfly and damselfly species, and are transmitted when frogs prey on the odonate intermediate hosts. Thus without distinguishing among species, biases in infection cannot be determined.

My study revealed that the sex-bias in susceptibility to *R. ranae* infection that I have observed in juvenile frogs is reversed in adult Northern leopard frogs from Bishops Mills. I speculate that the female bias in susceptibility among adult frogs may be as a result of variation in immune response to parasitism. Differences in immune response in turn could be driven by differential costs of

reproduction between the sexes that might inhibit the capacity for females to mount an effective response to infection. However, other hypotheses besides differential reproductive costs, or growth rate among the sexes might explain the opposing patterns of infection among juvenile and adult frogs.

The thermal clines of winter hibernacula selected by adult male and female leopard frogs could result in a sex-bias in parasitism among adults but not in reproductively immature frogs. Northern leopard frogs select hibernation ponds that do not freeze and excavate shallow depressions in the mud in areas of heavy algal growth or around dead stumps (Emery et al., 1972). The frogs rest in these depressions, but are capable of limited movement including swimming during the winter. In the spring, adult male leopard frogs emerge from these hibernation ponds and migrate to breeding ponds to chorus, 5-14 days *ca.* before female frogs (Seburn and Seburn, 1998). This early movement in the spring may necessitate male habitation of warmer sites in the hibernation pond than those chosen by adult females, in order to permit faster warm-up and earlier seasonal movement of the males. Choice of thermal cline may also influence response to parasitic infection. Sustained low temperatures are known to dramatically depress immune response by reducing lymphocyte and leukocyte proliferation, and inhibit the activity of several key components of the immune system in leopard frogs (Cooper et al., 1992; Maniero and Carey, 1997).

Parasites that infect cold-blooded hosts also face the challenge of surviving lower temperatures; but a reduction in host temperature may inhibit the ability to mount an effective immune response to infection. Thus, host sex differences in optimal

thermal climate during the winter could result in variation in immune capabilities, which in turn would affect the growth and survival of parasitic worms. Indeed, the lower temperature of hosts over the winter is thought to be important for the longevity and survival of the *Rhabdias* in hosts (Baker, 1979). All *Rhabdias* worms that I found in the breeding adult frogs in the spring were gravid females, indicating that they had been acquired late in the previous year and had persisted over the winter (Baker, 1979). My observations support the idea that females were not able to fight off *Rhabdias* infection over the winter as well as males. Although I could not detect differences between the sexes in *Haematoloechus* spp. infections, these flukes also overwinter in the host and are therefore subjected to the same changes in temperature that their hosts experience. Further studies on hibernation behaviours of Northern leopard frogs and sex specific immune responses to lower temperatures are needed to test this alternative hypothesis.

Table 5-1. Prevalence % (CI) of *Rhabdias ranae* (R.) and *Haematoleechus* (H.) of breeding and non-breeding adult, and juvenile frogs (N). Clopper-Pearson 95% confidence limits (CI) are provided (following Zar, 1996). Numbers of *R. ranae* (N_R) and *Haematoleechus* (N_H) worms are also indicated.

	R. ranae		Haematoleechus		
	N	Prev. % (CI)	N _R	Prev.% (CI)	N _H
Breeding adults	74	43.2 (31.7-55.3)	124	31.08 (20.8-42.9)	113
Non-breeding adults	28	42.8 (24.4-62.8)	64	35.7 (18.6-55.9)	28
Juveniles	45	37.8 (23.7-53.4)	42	20.0 (9.6-34.6)	44
Metamorphs	8	0	0	50.0 (15.7-84.3)	9

Table 5-2. Prevalence (%), mean abundance $\pm$ SE and intensity $\pm$ SE of *R. ranae* infections in male and female adult (breeding and non-breeding) and juvenile frogs. N refers to number of frogs sampled and Ni to number of frogs infected.

	N	N _i	Prev. %	Abundance	Intensity
Breeding females	20	13	39.4 (22.9- 57.9)	3.05 $\pm$ 0.85	4.69 $\pm$ 1.05
Breeding males	54	19	26.0 (16.5-37.6)	1.17 $\pm$ 0.52	3.31 $\pm$ 1.37
Non-breeding females	16	6	50.0 (13.3-59.0)	4.33 $\pm$ 2.63	8.66 $\pm$ 4.79
Non-breeding males	12	6	37.5 (10.7-50.2)	0.75 $\pm$ 0.34	2.00 $\pm$ 0.63
Juvenile females	25	8	32.0 (14.9-53.5)	0.48 $\pm$ 0.21	1.50 $\pm$ 0.50
Juvenile males	20	9	45.0 (23.1-68.5)	1.50 $\pm$ 0.51	3.33 $\pm$ 0.78

Table 5-3. Prevalence (%), mean abundance $\pm$ SE and intensity $\pm$ SE of *Haematoloechus* infections in male and female adult (breeding and non-breeding) and juvenile frogs. Symbols as in Table 5-2.

	N	N_i	Prev. (%)	Abundance	Intensity
Breeding females	20	8	40.0 (19.1-63.9)	2.45 $\pm$ 0.94	6.13 $\pm$ 1.67
Breeding males	54	15	27.8 (16.5-41.6)	1.19 $\pm$ 0.36	4.26 $\pm$ 0.93
Non-breeding females	12	5	41.7 (15.2-72.3)	0.83 $\pm$ 0.42	2.0 $\pm$ 0.77
Non-breeding males	16	5	31.3 (11.0-58.7)	1.13 $\pm$ 0.64	3.6 $\pm$ 1.63
Juvenile females	25	6	24.0 (9.4-45.1)	1.36 $\pm$ 0.99	5.67 $\pm$ 3.89
Juvenile males	20	3	15.0 (3.2-37.9)	0.50 $\pm$ 0.29	3.33 $\pm$ 0.88

Table 5-4. Grand mean lengths of *R. ranae* worms collected from female and male adult (breeding and non-breeding) and juvenile Northern Leopard frogs. Data presented are means (μm) $\pm$ SE. Note that actual nested analyses included the effect of individual on worm length.

	Female	Male
Breeding adults	6407.38 $\pm$ 153.80	5198.42 $\pm$ 131.09
Non-breeding adults	5324.62 $\pm$ 269.15	4694.89 $\pm$ 439.76
Juvenile	5685.68 $\pm$ 576.94	4501.92 $\pm$ 759.90

**CHAPTER 6: TESTING THE PREDICTIONS OF PARASITE RELEASE:
SPECIES RICHNESS AND SPECIES- SPECIFIC PARASITISM IN INVASIVE
BULLFROG HOSTS.**

Results from my sixth chapter have been submitted for review to
Biological Invasions.

ABSTRACT

In this study, I examined invasive bullfrogs sampled near Victoria, British Columbia for evidence of parasite release. I compared measures of helminth species richness, and parasitism in bullfrogs from Victoria, with bullfrogs from an endemic site in Ontario and from a previous study of bullfrogs sampled in New Brunswick. Helminth species richness in bullfrogs from Victoria was lower than bullfrogs from the Ontario site, but comparable to New Brunswick bullfrogs. The prevalence of lung flukes (*Haematoloechus* spp.) in invasive bullfrogs was twice as high as that of Ontario bullfrogs and higher than reported from New Brunswick. In bullfrogs from 4 of the 5 sample sites from Victoria, numbers of echinostome cysts were lower than observed in Ontario frogs. The lung flukes and echinostomes studied are expected to impose costs on host survival, growth and reproductive output. However, parasite release does not appear to have contributed to the invasiveness of bullfrogs in BC.

INTRODUCTION

Researchers have argued that hosts introduced to new habitats might experience relatively high fitness, because of a loss of most parasite species they would normally encounter in endemic habitats (Torchin et al. 2002). This loss can even include parasites that travel with a host to a new habitat, but fail to establish. Hosts also generally experience a low acquisition of new parasite species in newly colonized habitats (Torchin et al. 2002). For these reasons, researchers expect that hosts in introduced habitats are relatively free from parasitic infection and therefore might demonstrate enhanced competitiveness and invasion success once there (Torchin et al. 2001; Keane and Crawley 2002).

Evidence for parasite release comes mainly from observational studies. For example, introduced host populations can have reduced numbers of helminth species per host (parasite species richness) compared to endemic hosts (Dobson 1988; Torchin et al. 2002). Additionally, hosts in species-poor communities or within restricted areas are found to harbour fewer parasite species than those hosts in larger communities or found within broader ranges (Morand and Guegan 2000; Guegan et al. 2005). Researchers expect that measures of parasitism, like species richness, will be different for hosts sampled from sites in regions where introductions occurred, and hosts from sites in endemic regions; and whether introductions occur in site-restricted areas or species-poor habitats is likely important.

Parasite species richness is not the only metric of parasitism considered in observational studies. Prevalence of parasite species (the proportion of sampled

individuals that are infected by a single parasite species, Bush et al. 1997) is another measure. Prevalence of parasites tends to be higher in host populations at higher densities largely due to density-dependent transmission rates (Poulin and Guegan 2002). Thus, one might surmise that introduced hosts should have lower parasite prevalence because host species introductions generally involve the translocation of small numbers of individuals into new habitats. However, parasites have been found to achieve levels of prevalence among hosts in introduced habitats comparable to those of endemic populations (see review by Torchin et al. 2005).

Notably, the parasites that are lost during the translocation of hosts into introduced habitats tend to be those of low prevalence in endemic habitats. Furthermore, hosts acquire, on average, only 4 new parasite species in introduced habitats, and these parasites do not achieve higher prevalence in introduced hosts than in native hosts (Torchin et al. 2005). In the case of repeated introductions of few individuals collected from various endemic host populations, parasite prevalence has been observed to vary among the different introduced host populations depending on the prevalence of source endemic populations (Kulisic et al. 2004). Thus, distribution patterns of parasites are influenced by their species-specific patterns of association with hosts in endemic habitats.

The impact of parasite release on host fitness may influence invasiveness of hosts. Hosts with weak or no defence against parasites are expected to experience a direct "regulatory release" from fewer parasites, whereby fitness

traits such as survival and fecundity are enhanced (Colautti et al. 2004). However, for hosts that launch a defence against infection, the removal of fitness costs associated with resistance to infection and convalescence, allows more resources to be directed towards somatic and reproductive development, that is, a "compensatory release" is expected (Colautti et al. 2004). The two forms of release may not be exclusive, yet ecologists studying invasive species have focused on the compensatory form of release because developmental traits, such as growth rate and size, are known to influence fitness and competitive ability of hosts (Goater et al. 1993; Lampo and Bayliss 1996). Such developmental traits also have been linked to enhanced establishment success and superior competitiveness of introduced species (Lampo and De Leo 1998; Wolfe 2002; Campbell and Echternacht 2003; Grosholz and Ruiz 2003). Thus parasites that carry greater fitness costs are of greater importance in determining the establishment success of hosts.

There remain gaps in our understanding of parasite release. Most studies of parasite release have examined herbivorous insects and fungi (Beckstead and Parker 2003; Mitchell and Power 2003; Torchin et al. 2003). Furthermore, comparisons of parasite richness between introduced and endemic habitats tend to be made at scales that extend across transcontinental ranges, where latitudinal differences in climate and geography can impose gross and confounding effects. Few studies have examined parasite richness of hosts colonized in ranges possessing similar local environmental conditions, or within similar latitudinal gradients as their endemic habitats (Torchin et al. 2003).

I tested two predictions of the parasite release hypothesis: helminth species richness in introduced American bullfrogs (*Lithobates catesbeianus* syn. *Rana catesbeiana*) would be lower; and measures of infection for specific parasite species expected to cause fitness reductions in the hosts also would be lower, compared to endemic bullfrogs. My study is timely. Bullfrogs are known to be super-competitive and invasive, based on research on demographic patterns of bullfrog spread on Vancouver Island, British Columbia (hereafter BC) (Govindarajulu et al., 2005). Bullfrogs also are known to carry high numbers of parasite species, some of which have been associated with fitness costs in Anuran amphibians (see Discussion). However, bullfrogs introduced to BC have not been examined to determine whether parasite composition and richness, or levels of prevalence, abundance and intensity for specific parasites differ from endemic populations. Abundance is the number of parasites of a given species (or taxa) in a host, whereas intensity is the number of parasites of a given species (or taxa) in an *infected* host (Bush et al. 1997). Notwithstanding, other introduced bullfrogs are known to carry very different helminth fauna from bullfrogs from endemic populations (Goldberg et al. 1998). My study is also relevant because it is based on collections done at introduced sites near to one another, which are compared with sites from comparatively close range, even though climate is likely to differ.

I also tested for sex- and/or age-biases in parasitism, two factors that might influence parasitism (Forbes 2007). The diet, behaviour, and ecology of juvenile bullfrogs differ considerably from that of adults (Govindarajulu et al.

2005) and might influence exposure and susceptibility to parasites. Most reports on parasite burdens of bullfrogs, both in native and other introduced ranges, have not made distinctions among ages or sexes of frogs in samples (Andrews et al. 1992; Bursey and De Wolf 1998; Goldberg 1998).

Study system

Bullfrogs were introduced from southeastern Canada to BC in the early 1900's to support the gourmet culinary and garden ornamentation industries (Culley 1981). Bullfrogs have since been introduced to various backyard ponds, irrigation pools and lakes where they rapidly have become invasive and are a pest species in the province (Govindarajulu et al. 2005). The invasiveness of bullfrogs has been associated with the large size of adults (up to 750g), low mortality through predation, high reproductive output, and voracious appetites of tadpoles and adults. Interestingly, Govindarajulu et al. (2005) showed that population growth rate is most sensitive to alterations in survival rates of the postmetamorphic age cohorts, rather than fecundity or larval (tadpole) survival. Higher fitness also might be associated with parasite release and might manifest itself as both higher growth and reproduction, thereby enabling bullfrogs to out-compete other species and alter communities. Native frog species that are forced to live sympatrically with bullfrogs might become vulnerable to extinction (Pearl et al. 2004).

Other aspects of bullfrog natural history are relevant to this study.

Bullfrogs are highly aquatic and stay closely affiliated with their natal lakes

throughout their lives. Furthermore, adults prefer microhabitats within bodies of water and form reproductive leks within the perimeter of a lake (Pearl et al. 2004). Significant migrations (to a maximum of about 2 km) are reported only along watersheds (Willis et al. 1956; Semlitsch and Bodie 2003). Thus for this species, migrations between bodies of water separated by land tend to occur rarely. As such, the suites of parasite species among bullfrogs from different sites are expected to be distinct and varied.

METHODS

Bullfrog collections: sampling sites

Adults (> 3 yrs old) and juveniles (1-2 yrs post metamorphosis) were collected with dip and seine nets from five sites in the greater Victoria area, Vancouver Island, BC, in the summer and fall of 2003. They were euthanized with MS-222 and frozen for future necropsy.

The 5 sites around Victoria were 2.5-7 km apart from one another. Trevlac pond (48 29 44.6N 123 26 37.6W) was a peat bog/wetland that was mined and converted into a permanent lake. Bullfrogs recently colonized Trevlac in the 1990's; it is the most recent introduction of this host species among my samples. Florence lake (48 27 31.8N 123 30 43.9W) and Eagle lake (48 30 32.2N 123 27 40.4W) are natural lakes. Wade pond is a man-made agricultural pond (48 33 13N 123 26 58.0W). The Interurban pond (48 31 11.87N 123 25 34.45W) is a dugout pond located near a highway.

I also collected road-killed bullfrogs from Bishops Mills, Kemptville, Ontario (44 52 60.0N 75 40 0.0W) in the summer and fall of 2007. This location consists of an extensive wetland area sectioned by highways. High vehicle-induced amphibian mortalities are experienced in this area, indicating significant movement across roads that divide bodies of water. Consequently, I expect that my samples were from a large meta-population of bullfrogs in the area that provided a broader profile than would be observed from a single isolated lake or pond population. I collected adult and juvenile frogs over the summer and fall to coincide with seasonal times of collection of bullfrogs from the Victoria sites.

Necropsies

I examined all frogs from Bishops Mills and Victoria sites (N total = 76 bullfrogs, see Table 6- 1) for trematodes found in lungs and kidneys. However, one road-killed frog from Bishops Mills did not have kidneys present upon collection, and thus my sample size when enumerating kidney cysts was twenty-five.

I performed full necropsies on haphazardly selected sub-samples of 7 and 13 bullfrogs from the Bishops Mills and Victoria samples, respectively, to determine general parasite species numbers in the various organs (Table 6- 1). I selected only fully intact individuals from Bishops Mills for full necropsy. No juveniles from Bishops Mills were intact enough to examine all tissues, thus I examined only adults for the full necropsies. However, both juveniles and adults from introduced sites were examined. Helminths were identified to genus on the basis of morphological characteristics with the aid of identification keys.

I restricted further examinations of the bullfrogs to the lungs and kidneys because these two organs carried substantially higher intensities of trematode infection than other organs (see results below). Echinostome infections that exceeded 1000 cysts (e.g., in bullfrogs from Bishop's Mills) were difficult to count and therefore I recorded numbers as 1000. Parasites were identified at necropsy.

Statistical analyses

A t-test for samples with unequal variance was performed on helminth species richness among frogs from Victoria and Bishops Mills. For prevalences of lung fluke and echinostome infections, I performed 2-sample tests of equality of proportions. This test of equality directly compares the two proportions and generates confidence intervals on the differences in proportions. I reported results of non-parametric univariate tests (Wilcoxon/Kruskal-Wallis) for data that did not pass Levene's test for homogeneity of variances when testing for differences in abundance and intensity of lung flukes and echinostome infections. Z and p statistics are reported for normal approximations where samples are greater than 10 (when comparing among Victoria and Bishops Mills sites) and Chi-square approximations are reported for samples lower than 10 (when comparing among the five sites in Victoria).

I tested for age effects on lung fluke and echinostome infections among the Victoria and Bishops Mills sites using 1-way analyses of variance (ANOVA). All analyses were conducted with SAS (V 9.1.3, 2008).

RESULTS

I examined 50 individuals in total from the 5 sites in Victoria (ranging from 1-32 individuals per site, see Table 6-1). I observed a total of 9 unidentified helminth species from the 13 necropsied adult and juvenile bullfrogs. I recovered 1 nematode (from the gastrointestinal or GI tract), 1 acanthocephalan (GI tract) and 5 trematode species (1 in mouth, 1 in lungs and 3 in GI tract). I also observed trematodes of the genus *Haematoloechus* spp. (lungs) and *Echinostoma* spp. (kidney cysts). Species from these last two genus complexes are difficult to distinguish solely from morphological characteristics (Kennedy 1981; Fried and Toledo 2004; Bolek and Janovy 2007).

I collected 15 adult and 11 juvenile frogs from Bishops Mills. Of the 7 adult frogs I necropsied, I observed a total of 19 helminth species. I recovered 11 nematodes (2 in muscle, 1 in body cavity, 1 in urinary bladder, 1 in liver, 6 adults/larvae in GI tract), 1 cestode in the GI tract and 7 trematode species (3 species in the GI tract including *Megalodiscus temperatus*, *Glypthelmins quieta*, 1 unidentified species; 1 in body cavity; *Gorgoderina* sp. in urinary bladder; *Haematoloechus* spp. in lungs and *Echinostoma* spp. in kidneys).

Frogs from Victoria carried on average $1.46 \pm 0.31\text{SE}$ helminth species per individual (range 0-3), whereas frogs from Bishops Mills carried on average 5.6 ± 0.99 ; mean $\pm$ SE helminth species (range 2-9) (t-stat. unequal variances=4.087, $p=0.004$, $df=1,8$).

Overall, 90% of bullfrogs from the Victoria sites had *Haematoloechus* spp. in their lungs (45/50 infected; 18 adults and 27 juveniles). In contrast, 42.3% of

the frogs from Bishops Mills carried *Haematoloechus* spp. (11/26 infected: 7 adults and 4 juveniles) (test of equality of proportions: $Z=4.48$, $p<0.0001$, CI: 0.27 to 0.68) (Table 6-2). The overall prevalence of echinostomes cysts in the kidneys of bullfrogs the Victoria sites was 34% (17/50 individuals infected: 7 adults and 10 juveniles), whereas all 25 bullfrogs from Bishops Mills with kidneys present harboured cysts (test of equality of proportions: $Z= -5.43$, $p<0.0001$, CI: -0.791 to -0.53).

Frogs from the Victoria sites carried significantly higher abundances and intensities of lung flukes than did frogs from Bishops Mills (abundance: $Z= -5.925$, $p<0.0001$; intensity: $Z= -4.66$, $p<0.0001$). The grand median abundance of worms carried by the 50 frogs from the Victoria sites was 27 (Interquartile range [IQR]=11-40; range of intensity 29-136 worms/frog) (for median abundance on a per site basis see Table 6-2), while the median abundance of worms carried by the 26 frogs from Bishops Mills was 0 (IQR=0-1; range of intensity: 1-23 worms/frog).

Location also significantly influenced both abundance and intensities of echinostomes cysts. However, the pattern of association was opposite to that observed for the lung flukes. That is, frogs from the Victoria sites harboured fewer kidney cysts than did frogs from Bishops Mills (abundance: $Z=6.13$, $p<0.0001$; intensity: $Z= -2.79$, $p=0.005$). The overall median number of cysts carried by frogs from Victoria was 0 (IQR=0-6; range of intensity: 1-500 cysts/frog), while the median number of cysts carried by frogs from Bishops Mills was 128 (IQR=100-500; range of intensity: 16-1000 cysts/frog). The 4 infected

bullfrogs from the single agricultural pond (Wade pond) carried an average of $260 \pm \text{SE } 81.24$ cysts/frog, compared with an average of $35.38 \pm \text{SE } 13.75$ cysts/frog observed in frogs from the other 4 locations (Table 6-3).

I also checked for differences in abundance and intensity of lung flukes and echinostomes among the 5 Victoria sites. Location significantly influenced both the abundance and intensity of lung flukes (abundance: $\chi^2 = 10.78$, $p = 0.02$; intensity: $\chi^2 = 10.22$, $p = 0.02$). Location also influenced abundance and intensity of echinostomes (abundance: $\chi^2 = 22.54$, $p = 0.002$; intensity: $\chi^2 = 10.38$, $p = 0.03$).

There were roughly similar proportions of juvenile and adult frogs from across my samples (Table 6-1). Furthermore, age did not have a significant effect on abundance or intensity of lung flukes in frogs from the Victoria sites (ANOVA: abundance: $F = 1.53$, $df = 1, 48$, $p = 0.22$; intensity: $F = 1.78$, $df = 1, 78$, $p = 0.19$) or Bishops Mills (abundance: $F = 1.59$, $df = 1, 24$, $p = 0.22$; intensity: $F = 3.19$, $df = 1, 9$, $p = 0.11$). Age also did not significantly affect abundance or intensity of echinostome cysts in the kidneys of frogs from the Victoria sites (abundance: $F = 0.00$, $df = 1, 48$, $p = 0.99$; intensity: $F = 0.01$, $df = 1, 15$, $p = 0.93$) sites or Bishops Mills (abundance/intensity-all hosts were infected: $F = 8.32$, $df = 1, 23$, $p = 0.008$).

I also tested whether there was any association between sex and parasitism, because sex-bias in lung fluke infections in bullfrogs has on rare occasions been reported (Whitehouse 2002). However, sex was not significantly associated with abundance or intensity of either lung fluke or echinostome in hosts from either location (p values ranged from 0.12 to 0.82).

DISCUSSION

I tested two predictions of the parasite release hypothesis for invasive bullfrogs. The first was that parasite species richness would be lower for invasive compared to endemic bullfrogs. I examined 13 frogs from the Victoria sites and seven from Bishops Mills, and therefore likely underestimated true species richness at each site. Parasite species richness in bullfrogs in another endemic site has been found to increase up to a host sample size of about fifty before leveling off (McAlpine 1997). My observed average of 5.6 helminth species per individual at Bishops Mills falls within the range reported for another endemic population in New Brunswick. McAlpine (1997) reported an average of 2.5 helminth species per individual (2.7 for juveniles), with up to 21 helminth species per host. Thus bullfrogs in Victoria, with an average of 1.5 helminth species, have significantly lower parasite species richness than bullfrogs in Ontario, but have comparable average numbers of parasite species to those of bullfrogs from other endemic populations. I note however that the highest numbers of helminth species that some individuals in New Brunswick harbour far exceeds any numbers observed in bullfrogs from Victoria.

At first glance, the species richness data alone suggest little evidence for parasite release in invasive frogs. However, there were no parasites from the body cavity, liver, muscle, or urinary bladder, reported for frogs collected at Victoria. Also, 2 of the 13 fully necropsied frogs from the Victoria sites had no parasites whatsoever and 7 frogs only had fluke worms in their lungs.

The second prediction of the parasite release hypothesis that I tested was that levels of parasitism by those species thought to cause fitness reductions in their hosts, would be lower for invasive than for endemic frogs. I am confident that this was not the case for *Haematoloechus* spp., because infections in bullfrogs from Victoria were markedly higher than those observed for endemic bullfrogs. Other studies have reported prevalence levels of various species of *Haematoloechus* spp. in endemic populations of bullfrogs that range between 1%-50% (Kennedy 1981; Andrews et al. 1992; Bursey and DeWolf 1998). Furthermore, bullfrogs from Victoria carried much higher intensities of lung flukes than has been reported in the literature (Brandt 1936; Goldberg et al. 1998; Goldberg and Bursey 2002). This contrasts with the range of intensity of infection that I observed in the endemic population. The intensity of lung flukes in bullfrogs from Bishops Mills corresponds to levels reported for populations in New Brunswick (McAlpine and Burt 1998). Thus it appears that levels of *Haematoloechus* spp. infection observed around Victoria are uniquely high. This may well be a reflection of patterns of infection among odonate intermediate hosts.

Haematoloechus flukes are acquired when frog hosts consume infected naiad or teneral anisopteran and zygopteran odonates. These flukes display a wide range of patterns of odonate host specificity, from specialist to broad generalist (Snyder and Janovy 1996; Snyder and Tkach 2001). Odonates comprise 25% of juvenile bullfrog diets in Vancouver populations (Govindarajulu et al. 2006); adult bullfrogs also consume odonates, but also include a wide

variety of other prey items. It is possible that the infections I observed were acquired *Haematoloechus* species from the colonized sites, rather than those transported with hosts from native regions. Future studies that identify *Haematoloechus* species, and focus on patterns of bullfrog infections as they relate to the distribution patterns of odonates, are needed to explain the unusual levels of intensity and prevalence of lungworms in bullfrogs in Victoria.

Haematoloechus worms destroy the integrity lung parenchyma; the burrowing of the blood-feeding worms into the alveoli results in alveolar compression and dead worms result in the formation of cysts that extend through the lung and puncture the wall of the lung (Shields 1987; *personal obs.*). Other blood-feeding lung helminths are known to negatively impact lung function, stamina, growth and survival, and cause anorexia of anuran hosts (Goater and Ward 1992; Goater et al. 1993). Such damage to lungs may also impede chorusing ability of males during breeding season, thereby lowering reproductive success of infected individuals. Thus the high infections of this parasite likely impose significant fitness costs on hosts.

The second prediction also was not unequivocally supported for echinostome infections. There was overall a lower prevalence of echinostome infection among the bullfrogs from Victoria relative to bullfrogs from Bishops Mills. Previous studies have reported that parasites that achieve high prevalence in endemic habitats are likely to experience the same distribution among hosts introduced to new environments (Torchin et al. 2002). The high prevalence of echinostome infections among the Bishops Mills population is consistent with

reports of prevalences of 80% or higher in New Brunswick populations of bullfrogs (McAlpine and Burt 1998), but different from Victoria sites. Without knowing the source populations of bullfrogs in Victoria, I cannot match endemic prevalence levels with those of introduced sites. It is clear, however, that echinostome intensity varied among bullfrogs from Victoria. Interestingly, the highest infections were found in bullfrogs collected from the only agricultural pond (Wade Pond). Although based on small samples, this observation supports other studies that have shown clear linkages between agricultural activity and susceptibility of larval amphibians to trematode (Koprivnikar et al. 2006; Johnson et al. 2007), and in particular, echinostome infections (King et al. 2007). The ability to achieve such high prevalence is likely connected to density and mode of transmission of the infective cercarial stage of echinostomes. Population densities of bullfrogs in Victoria (4.1-530 frogs/ha) (Govindarajulu et al. 2006) fall within the range of densities observed in Ontario populations (4.2-1,377 frogs/ha) (Shirose et al. 1993). Therefore, differences in infection levels might be due to density- and/or distribution-dependent attributes of suitable intermediate gastropod hosts (Johnson et al. 2007).

Echinostomes also are parasites expected to impose fitness costs on their hosts. Echinostome cercariae swim up the cloaca of hosts and travel to the renal system. Infection occurs primarily during the host's larval stages, but postmetamorphic stage amphibians can also acquire infections (Schotthoefer et al. 2003). Echinostome infections have been associated with low juvenile amphibian recruitment (Beasely et al. 2003). Mortality rates between 40-100%

have been reported with echinostome infections of larval frogs (Schotthoeffer et al. 2003; Holland et al. 2007). Tadpoles of other species are known to exhibit edema and inhibited growth (Fried et al. 1997), although older frogs do not display the same lack of resistance to infection (Schotthoeffer et al. 2003). The lower intensity of cysts among the introduced populations suggests that any regulatory effect of echinostome infection on early tadpole stage individuals is likely lower than that experienced by hosts in Ontario. In such a manner, introduced bullfrogs from most Victoria sites may experience parasite release from this one trematode species.

My observations support other studies that have shown no differences between sexes in helminth species richness or abundance in adult or juvenile bullfrogs (McAlpine 1997). I did not observe any association between host sex and degree of parasitism by either *Haematoloechus* spp. or echinostome cysts, although host size and sex biases in intensity of *Haematoloechus* spp. have been documented (Dronen 1977; Kennedy 1981). Interestingly, juvenile bullfrogs in New Brunswick are known to be more heavily parasitized than adults, with higher abundances and higher numbers of helminth species (McAlpine 1997). However, I observed no differences in any parasite measure between juvenile and adult bullfrogs from either Victoria or Bishops Mills.

Although the parasite release hypothesis as a mechanism for determining host establishment and regulating invasiveness is widely debated, the actual extent to which parasites have an impact (direct or indirect) on host invasiveness is still unknown. It is important to note that not all parasite species impose heavy

fitness costs on their hosts (Rigby et al. 2002). Notwithstanding, anurans typically carry high parasite burdens; bullfrogs are known to carry exceptionally high numbers and intensities of metazoan parasites (Brandt 1936; Muzzall 1991; McAlpine 1997). In some amphibian populations, the prevalence of trematode infections can be as high as 85% and at intensities high enough to impose regulatory effects on the populations (Zelmer and Esch 2000). Thus the release from parasites that impose high fitness costs could have a considerable positive effect on survival, to the extent that in the absence of this regulatory factor, competitive species can rapidly increase and displace other species.

Future research needs to address the effects of release from parasite species for which there are known fitness costs. Hosts that harbour reduced parasite species and lower numbers of parasites may still incur fitness costs, yet the magnitude of the cost will depend both on parasite virulence (Drake 2003) and nature of trade-offs associated with defence. While some parasite species may exert a more benign impact on hosts, others may exert a regulatory effect on hosts. For highly virulent parasites, fitness costs of infection are likely to be high, even with non-lethal infections (Read 1994). Thus measures of parasite abundance, prevalence and/ or intensity, provide only a partial picture. However, research to date has only provided profiles of abundance, intensity and prevalence of parasitic infection in endemic and introduced habitats. Still needed are measures of the fitness costs associated with those translocated parasite species that are able to establish in new habitats, and importantly, the ultimate effect these parasites have on the establishment of their hosts.

A recent study of the bullfrog populations in Vancouver revealed that the ecology of frogs present today does not differ from that of bullfrogs from populations in endemic ranges. Govindarajulu et al. (2006) revealed that life history traits including, but not limited to, breeding times, length of winter torpor and timing of spring emergence, are similar to Ontario populations. It is presumed that accelerated growth rates and enhanced body sizes that are characteristic of invasive species, are likely only apparent in bullfrogs within the first 3 years following colonization. This is because of the high reproductive capacity of the species that enables rapid population explosions, which in turn produce intraspecific density-dependent pressures that regulate developmental rates and sizes of post metamorphic individuals (Govindarajulu et al. 2005). Thus, any gross responses of bullfrog populations to parasitism are likely only apparent within the first 5 years of introduction into a new habitat when rapid growth and increase in population density is more sensitive to the impact of infection. It is important to note however, that the influence of parasite release may act synergistically with other factors and contribute to the post-invasion establishment of introduced hosts, though parasitism may not by itself be a regulating factor. Other confounding intraspecific competitive or predation pressures associated with higher population densities may mask the influence of parasitism at a later time.

My study thus suggests that there is little direct support for parasite release influencing invasive spread of bullfrogs in BC. However, as mentioned above, there are issues associated with timing of the study and sampling area. I

nevertheless have identified unusual levels of lung flukes and echinostome infections in frogs from one or more Victoria sites that likely impact host fitness. Despite the expected inhibitory effects of *Haematoloechus* spp. and echinostome infections, bullfrog populations appear able to compensate for infection and bullfrogs still successfully invade new habitats. This study highlights the importance of species-specific approaches to determining if parasite release occurs.

Table 6- 1. Numbers of juvenile and adult frogs sampled from Bishops Mills, ON and Victoria sites, BC. The number of frogs selected for full parasite richness is indicated in parenthesis.

Location	Juv. (necrop.)		Adults (necrop.)		N Total
	M	F	M	F	
Bishops Mills, ON	5 (0)	6(0)	8(4)	7(3)	26
Victoria, BC					
<i>Eagle Lake</i>	1(0)	1(0)	-	1(1)	3
<i>Florence Lake</i>	14(1)	7(1)	6(3)	5(3)	32
<i>Interurban pond</i>	-	-	-	1(0)	1
<i>Trevlac</i>	3(0)	1(0)	2(0)	3(1)	9
<i>Wade Pond</i>	3(2)	-	1(0)	1(1)	5

Table 6- 2. Median abundance, interquartile range (IQR) and actual range of *Haematoloechus* spp. lung flukes from bullfrogs collected among 5 sites in Victoria, BC. The number of bullfrogs infected at each site is also indicated.

Location	Median	IQR	Actual Range	N Infected
<i>Eagle Lake</i>	34	14-47	14-47	3
<i>Florence Lake</i>	26.5	16-40	0-136	28
<i>Interurban pond</i>	0	0	0	0
<i>Trevlac</i>	38	31-53	5-90	9
<i>Wade pond</i>	5	4-5	3-27	5

Table 6- 3. Median abundance, interquartile range (IQR) and actual range of kidney cysts (echinostome) from bullfrogs collected among 5 sites in Victoria, BC. The number of bullfrogs infected at each site is also indicated.

Location	Median	IQR	Actual Range	N Infected
<i>Eagle Lake</i>	0	0-60	0-60	1
<i>Florence Lake</i>	0	0	0-200	4
<i>Interurban pond</i>	-	-	100	1
<i>Trevlac</i>	9	1-14	0-23	7
<i>Wade pond</i>	300	200-300	0-500	4

CHAPTER 7: GENERAL DISCUSSION

My main goal for this thesis was to further explore the prevailing ideas about the causes of parasitism. I chose to use model hosts for which ample information on patterns of parasitism is available. This was done for the dual purpose of logistical ease that sampling and rearing frogs lent to my studies, and also to test the generality of predictions developed for other vertebrate hosts. The concepts that I explore in each chapter of this thesis are not new; however, my research involving amphibians offers unique perspective and presents new information on the relationships between host investment in life history trajectories and variation in parasite fitness that explain more subtle interactions between host tradeoffs between immune and other somatic functions. Amphibians have offered the opportunity for novel approaches to testing hypotheses, and in elucidating interactions that have not been observed in other host models.

Below, I discuss the main findings of each chapter and suggest further avenues for research pursuit.

In Chapter 2, I confirmed other reports of the effects of higher densities on Northern leopard frog development (Glennemeier and Denver 2002); individuals reared under higher density conditions exhibited slower developmental rates and were of smaller size than individuals reared under low density conditions. I also discovered that the period of infectivity of the parasite ended before individuals reared under high density conditions had developed to metamorphosis.

Slower developmental rate may therefore offer the benefit of avoidance of infection until infective stages of parasites are no longer a threat. However, the controlled infection experiments demonstrated that the effects of higher density conditions left metamorphs less able to fight off infections when they were exposed to infective larvae. Smaller metamorphs, regardless of rearing regime, and individuals that developed slower were more susceptible to trematode infection when exposed, than those of larger size and with faster developmental rates.

From a broader conservation perspective, the habitat loss and changing hydroperiod conditions that are closely linked to the dramatic decline of amphibians in North America (Hecnar 2004) may also force high density rearing conditions for larval amphibians (Holmes 1996), and create patterns of interactions among hosts and parasites that may be very difficult to predict or regulate.

Theory suggests that differences among sexes in tradeoffs in development and investment in immunity can only be observed in species that display sexual size dimorphism. My results from Chapter 3 revealed relationships between investment in developmental rate and susceptibility to infection that are not predicted by current theory. I demonstrated that sex-linked relationships between investment in development and investment in immunity can occur independently of selective pressures that produce sexual dimorphism in size. Prior to this study, no information was available for amphibians about the earliest

life stage for detection of differential investment in development among the sexes. I discovered clear differences in developmental rates as early as metamorphosis, although size differences among the sexes were not discernable. This demonstrated that size dimorphism is not a pre-requisite for sex-linked differentiation in investment in growth trajectories of amphibians.

My results suggest that individual susceptibility to parasitism is determined by the degree of congruence of an individual's growth trajectory with what is characteristic for that individual's sex. My results do not support the assumption that developing quickly through certain life stages such as larval stage, in which high mortality is experienced, might be an adaptive strategy for improving survivorship and fitness for both sexes. Apparently, evolutionary constraints imposed on each sex may counter fitness benefits of displaying plasticity in an individual's trajectory of development.

I also found that worms that established in male hosts tended to achieve larger maximum sizes than worms that established in female hosts. Although only a trend, this observation holds strong biological significance as it suggests that there may be fitness costs to the parasite that are associated with infecting one or another host sex. Better establishment and enhanced growth of parasites in male hosts point to this sex as the primary driver of infection and contributor of subsequent cohorts of infection in a population (Skorping and Jensen 2004). Even slight differences in measures of parasite fitness may have large consequences in terms of transmission and movement of parasites through host populations.

The importance of this latter result segued directly into the subsequent study (Chapter 4) of my thesis, in which I determined whether sex-linked differences in parasitism were also observable in a host species that does not display dimorphism in size at any life stage. I was also interested in measuring parasite fitness as an indicator of benefits conferred from host adaptation.

In Chapter 4, I elucidated effects of host-parasite adaptation on parasite establishment by measuring variation in parasite traits; an approach which highlights the ecological consequences of such adaptation. This study was complemented by genetic analysis of phylogenetic relationships between two parasites that have previously been confused as a single species. The prediction of adaption for parasites is that greater host specialization confers enhanced fitness, or conversely, that fitness costs are associated with parasitizing atypical host species, which may limit host ranges (Fry 1990). However empirical studies testing these predictions are lacking.

My findings become even more complex and interesting from an epizootiological context, when differentiation in parasite fitness occurs among individuals within a different host species population. Results in Chapter 4 also demonstrated connections between differential investment in development in a species that does not display sexual size dimorphism, host susceptibility to infection, and fitness consequences for parasites that infect one or the other sex. Here I found that male Northern leopard frogs developed to metamorphosis at a faster rate than female frogs, without measureable differences in size. Further,

the male trajectory of development appears to carry the cost of greater susceptibility to infection than females; however such a consequence was only measureable when hosts are exposed to parasites to which they are more closely-adapted. Adaptation between hosts and parasites may exaggerate costs associated with faster development in male hosts that otherwise would be imperceptible.

In Chapter 5, I found male juvenile frogs displayed a tendency to carry higher numbers of worms than female juveniles; however, the sex-bias in adults was opposite to that observed in experimental results. The switch in patterns of host sex bias in susceptibility to infection from earlier to later life stages implicates other selection pressures on adult frogs that may override investment in development. The higher burdens and larger worms found in breeding adult females suggest that there may be higher costs of reproduction for female frogs than for males, resulting in a decreased investment in immune function in breeding females. This study also highlights an imperative qualification for parasitological study: a strong understanding of the patterns of parasite acquisition and transmission, and the natural history of their hosts, in order to be able to interpret unexpected observations and provide plausible hypotheses for patterns revealed.

I also found differences in establishment of 2 rhabditean lungworms in natural populations of Wood frogs and Northern leopard frogs (Appendix I). Host specificity is predicted to be a major source of variation in patterns of parasitism

(Schmid-Hempel 2003): a proposition my work supports. There is a need for ecological studies to complement genetic delineations of specialist parasites. A more integrative approach to the study of these relationships will likely yield greater insight into the role of parasites themselves influencing variation in infection among host species.

In Chapter 6 I provided some evidence that challenges the idea that ecological release from parasites influences host competitive advantage and enables success of invasive species. I found that neither of the 2 predictions of the parasite release hypothesis that I tested were unequivocally supported. Contrary to prediction, I discovered that the intensity of infection of one of the two focal groups of parasites examined was considerably higher than observed in endemic habitats, and highly variable for the second focal group. However, introduced frogs had fewer organs infected with parasites, and had mean species numbers that overlapped only with the lower range of numbers observed in endemic populations. These observations would appear to support the idea of parasite release; however, high levels of infection do occur with certain parasites but do not appear to have limited the invasiveness of hosts at this site. Furthermore, my results demonstrate the need for studies to approach parasite release on a parasite species-specific basis in order to be able to elucidate fitness costs or benefits in hosts.

Interactions among parasites may also influence variation in patterns of parasitism in hosts. My observations in Appendix II showed that these interactions may take the form of competitive exclusion or niche partitioning that may affect the fitness traits of parasites themselves.

I have in each of my chapters, provided novel contributions to the field of modern parasitology through the integration of field, experimental and molecular approaches, and I encourage the testing of hypotheses from this more multifaceted perspective. However, if there is a singular weakness throughout this thesis, it is the issue of scope. The examination of only one or two host species to test hypotheses that have broad underpinnings, limits the extent of inferences that can be drawn from each chapter, and likely reflects simpler interactions than actually occur in natural systems. Observations in my field studies following experimental work were counter-intuitive and aptly demonstrate this point. Further, logistical limitations confined my examinations to small sample sizes and resulted in issues of pseudo replication; these two issues are common to many parasitological studies and are difficult to circumnavigate.

As is also commonly observed in parasitological studies, the data obtained often violated assumptions of normality and/ or in a number of cases, the assumption of homogeneity of variances. As well, a lack of independence of response variables and the dependence of the variances of some response parameters on rearing tank necessitated the assessment of interactions between variables. Altogether, these somewhat difficult data sets required the use of more

involved transformations of data, complex multivariate analyses of variance, and non-parametric and mixed model hierarchical statistical tests.

My thesis paves the way for many fruitful avenues of study and exploration.

Obviously, the considerable variation in life history strategies among amphibians warrants the examination of more species to determine the generality of effects of environmental stresses on host-parasite temporal overlap. As well, examining a broader suite of parasites to which hosts may be exposed will provide greater insight into the potential for parasite-mediated selection pressures to influence developmental trajectories of hosts. Such studies would also be improved by isolating components of the process of infection to determine fitness consequences for parasites that are associated with timing of infection. This could be done, for example, by determining the viability or success of various stages of establishment of parasites that infect hosts earlier or later in the period of infectivity. Further, an explicit examination of the host immune responses that are associated with infection would lead to a greater understanding of the extent to which tradeoffs between development and investment in immunity occur.

My initial studies suggest that differential patterns of investment in immunity that relate to developmental trajectories between sexes are likely species-specific.

The generality of sex-linked differences in developmental trajectories at earlier life stages among amphibian species still remains unknown. Species can now be examined for tradeoffs in investment and development without the assumed pre-

requisite of differential investment in size. Such studies also have important ecological application from the perspective of larval amphibian survival in variable rearing pond conditions; susceptibility to parasitism may vary with acceleration or slowing of developmental rate depending on the conditions to which a species is evolutionarily adapted. Other important considerations for future studies on sex-biases in parasitism include an examination of differential exposure to parasites, and differential impacts of parasitism among sexes.

There is the need for studies to include an examination of parasite fitness attributes associated with specialist versus generalist approaches to host exploitation. At present the one-sided focus on genetic delineation of parasite species and/ or complexes, without associated consequences for host-parasite interactions, neglects the ecological importance of adaptation as an important source of variation in patterns of parasitism in nature. Further, my results in Chapter 4 suggest that there may be interactions between parasite adaptation and host sex-linked investment in immune function that may have important consequences for parasite distributions among host populations. Future studies could examine the fitness consequences of infecting male or female hosts by known specialist and generalist parasite species.

My field observations pointed to other hypotheses, such as behavioural differences between host sexes, which may cause variation in patterns of parasitism among frogs. The observed reversal of the sex-bias in susceptibility to

parasitism from juvenile to adult life stages may be explained by thermoregulatory preferences that only become important at the onset of reproductive maturity. This remains to be explored. Further study of physiological constraints that parasites face in hosts that inhabit extreme environments will also contribute to our understanding of general parasite adaptations and transmission biology.

Parasite release as a mechanism for enhanced competitiveness of host species introduced to new habitats remains poorly explored through empirical studies. My preliminary results demonstrate the need for a closer examination of parasite species- species fitness costs. Future research directions could include an examination of genetic profiles of introduced and endemic populations to determine whether there are any associations between patterns of parasitism and genetic closeness among populations. That is, do more-closely related endemic and introduced populations have more similar patterns of parasitism than more distantly related populations?

Another question that is difficult to address with most studies of introduced species deals with the timing of introduction; in many cases it is not possible to determine when a species was introduced, or how long it has taken for that species to become invasive in its introduced habitat. An assessment of genetic divergence among populations may provide answers to this latter question, since the degree of divergence of introduced populations likely reflects the length of time since introduction.

Further study could also include measurements of relative growth patterns of hosts among endemic and introduced populations, to test for compensatory release of parasites. These measurements are easily obtained on amphibians through skeletochronology.

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APPENDIX I: PATTERNS OF PARASITISM OF *RHABDIAS* LUNGWORMS IN TWO SYMPATRIC RANID HOSTS.

Observations from this survey are in preparation for submission to a Journal for review.

ABSTRACT

Rhabdias ranae and *Rhabdias bakeri* are two nematode species that have until recently been confused as a single species. Experimental studies have shown that patterns of parasitism displayed by these species differ among their ranid hosts; however, the extent to which such divergent patterns are seen in nature has not been assessed. I measured lungworm parasitism of adult Wood frogs from a natural population, and compared my observations with parasitism of Northern leopard frogs sampled from the same geographic location. I observed differences in prevalence, abundance and intensity of lungworm infection, and worm length, among the two host species. Further, I observed no sex bias in parasitism among Wood frogs, although a female bias was observed in the Northern leopard frogs. I discuss my findings in the context of adaptation and the natural history of the two host species.

INTRODUCTION

Recent evidence suggests that the lungworm nematode, *Rhabdias bakeri*, a common parasite of *Rana sylvatica* syn. *Lithobates sylvaticus* (Wood frog), has been misidentified as a morphologically similar, but genetically distinct species (*R. ranae*) typically found in *Rana pipiens* syn. *Lithobates pipiens* (Northern leopard frog) (Tkach et al 2006). Until this discovery, experimental studies have used worms from both host sources interchangeably without an awareness of potential differences in lifecycles, pathology, life history traits such as growth rate or survivorship, and ecology of the worms. At present, the lifecycle of *R. bakeri* is uncharacterized and is assumed to be similar to that of *R. ranae*.

Experimental work has shown that both *Rhabdias* species will establish and reproduce successfully in either host under experimental conditions, although there are differences in establishment success of the two worm species among the two host species (Dare et al. 2008- In Press). *R. ranae* appears to be more closely co-adapted to Northern leopard frogs than is *R. bakeri*; the former species displays greater establishment and adult female worms reach larger sizes in Northern leopard frog hosts than in Wood frogs. It is possible that the two parasite species display some host specificity (Tkach et al 2006), however, the extent to which this is manifested among natural host populations is still undetermined.

Differential infection levels and establishment rates of parasites are also interesting from the point of view of ecological parasitology; the two parasites

might have disparate impacts on demography of their hosts. Infection by *Rhabdias* is known to cause anorexia, inhibit host growth and affect mobility in toads (Goater and Ward 1992, Goater et al 1993). It is also possible that infection impedes chorusing abilities and therefore reproductive success of adult male frogs. Thus, the cost of infection to a host may vary with *Rhabdias* species.

A re-characterization of the lifecycles and examination of the ecology of each of these previously confused species is needed. Wood and Northern leopard frogs share common ponds during breeding periods in the spring, but at other times of the year occupy different habitats, though still within the same local geographic areas. These two lungworms therefore provide a very good model in which to study parasite adaptation. Surveys of the natural patterns of establishment of *Rhabdias* lungworms in Wood frogs are required to determine the extent to which adaptation is displayed among the two lungworm species and the fitness consequences of differential parasite success in natural host populations.

In this study I examine *Rhabdias* parasitism among adult Wood frog hosts collected from a wooded area in Kemptville, Ontario. My study is unique because I sampled Wood frogs from the same wetland area from which I have documented patterns of *R. ranae* parasitism in Northern leopard frogs in the same season of collection (Dare and Forbes- In Review). Thus, direct comparisons can be made of prevalence, abundance, intensity and size of adult lungworms from both host species from the same area. In my previous survey of Northern Leopard frogs (Dare and Forbes- In review), I observed a female bias in

levels of infection; I therefore also checked whether a sex biases in parasitism can be observed in Wood frogs.

MATERIALS AND METHODS

All collections were conducted under permit and in accordance with Carleton University Animal Care guidelines. A small number of adult male (N=18) and female (N=16) Wood frogs were collected from wooded areas around a wetland in Bishops Mills, Kemptville, Ontario (44 52 60.0 N 75 40 0.0W) in the first week of April, 2007. These individuals were used to assess sex-associated variation in parasitism. Another 66 adult male Wood frogs were captured on 23 April, 2007. All frogs were killed by double pithing. Their lungs were excised and frozen in eppendorf tubes at -20C for later examination. The lungs were later dissected and all *Rhabdias* lungworms were removed and counted. Since worm length of nematodes is an index of fecundity (Goater, 1992; Marcogliese, 1997; Tompkins and Hudson, 1999), I also measured the length of each worm. However, some worms were damaged in the process of counting and therefore I was not able to obtain lengths for all worms observed. I measured 61 of 76 worms from female frogs, 184 of 186 worms from male frogs collected early in April and 251 of 268 worms from male frogs collected later in April. As well, I did not measure the lengths of the few (12) subadults found in two wood frogs, although they were included in my analyses of abundance and intensity of infection. All lung flukes (*Haematoloechus* spp.) also found in the lungs were removed and enumerated.

I tested for differences in prevalence of *Rhabdias* among male and female frogs in early April, and among male frogs from the two sample groups using tests of equality of proportions. I tested for differences in abundance and intensity of *Rhabdias* nematodes between female and male Wood frogs using ANOVA. I also checked for differences in intensity and abundance of lung nematodes among the two male frog sample groups however, my data did not pass Levene's test for homogeneity of variances, or conform to normal distribution, thus I used a Kruskal-Wallis non-parametric test. Data are reported as mean $\pm$ SE, however my statistical analyses comparing parasitism between groups of male Wood frogs were done using non-parametric tests, so I have reported median values for abundance and intensity of worms in parentheses. A nested ANOVA was used to test whether there were any differences between female and male Wood frogs in the length of the *Rhabdias* worms removed. I also used a nested ANOVA to test for differences in length of worms collected from the two sample groups of male frog hosts. I nested individual frogs within each sex to control statistically for variation in worm length attributable to host individual (that might have arisen because of differences in the numbers of worms carried by individual frogs).

RESULTS

Twelve out of the 16 female Wood frogs, and 14 out of the 18 male Wood frogs collected in early April were infected with a total of 76 and 186 *Rhabdias* worms respectively (Table I- 1). However, 52 of the 66 male Wood frogs collected on 23 April were infected with a total of 268 *Rhabdias* worms

(prevalence: 78.8%, Clopper-Pearson confidence interval on binomial proportions CI: 66.97-87.89). One male collected on April 23rd and one female Wood frog collected two weeks earlier each carried 1 and 11 subadult worms, respectively.

I observed 33 *Haematoloechus* spp. worms from 4 of the 16 female hosts, and 88 worms from 6 of the 18 male hosts. I also found 112 worms from 31 of the 66 male Wood frogs collected on 23 April (prevalence: 46.96%, CI: 34.56-59.65).

Rhabdias prevalence did not differ significantly between males and females in the first sample of frogs ($Z = -0.19$, $P = 0.85$) (Table I- 1). As well, the prevalence of *Rhabdias* worms in the male frogs collected later in April was similar to the prevalence of the male frogs collected 2 weeks earlier ($Z = 1.52$, $P = 0.13$). Abundance, intensity and worm length also did not differ between males and females, however the small sample size did not lend strong power to my analyses (Table I-1). The abundance of *Rhabdias* worms removed from males collected on 23 April was $4.06 \pm 0.59\text{SE}$ (median: 2.00), while intensity was 5.15 ± 0.67 (3.00). No significant differences between male frogs from the two sample groups were observed in either of these measures (abundance K-W: $Z = 1.17$, $P = 0.24$; intensity K-W: $Z = 1.78$, $P = 0.07$). The length of worms from male hosts collected early in April ($3927.84 \mu\text{m} \pm 74.49$) also did not differ significantly from the length of worms from males collected on 23 April ($\text{df} = 1$, 90.88 , $F = 0.53$, $P = 0.47$).

DISCUSSION

I now discuss my results in relation to what is known about *Rhabdias* infection in frogs. Levels of *Rhabdias* infection of the breeding Wood frogs were much higher than reported for breeding Northern leopard frogs that were collected at the same time of the year. The prevalence of infection of worms in Wood frogs was more than twice as high as that observed in the Northern leopard frogs (females 39.4% and male 26.0%) (Dare and Forbes, In Review). The abundance and intensity of *Rhabdias* infection in Wood frogs were also considerably higher than have been observed among the Northern leopard frogs collected at Bishops Mills. For the Northern leopard frogs I observed an average abundance of $3.05 \pm 0.85\text{SE}$ worms for female hosts and $1.17 \pm 0.52\text{SE}$ for male hosts, and intensities of $4.69 \pm 1.05\text{SE}$ for female hosts and $3.31 \pm 1.37\text{SE}$ for male hosts (Dare and Forbes, In Review). Yet the levels of infection that I observed in the Wood frogs are consistent with what has been reported for Wood frogs from other locations (Woodhams et al 2000; Baker 1979). Thus patterns of *Rhabdias* establishment appear to differ among the two host species.

My observation of immature worms in the lungs indicates that Wood frogs start to acquire infection early in the spring, when seasonal temperatures may still drop below zero degrees C. Yet, *R. ranae* is believed to undergo only one or two generational cycles in a year with peak transmission of new infective larvae presenting in the late summer and early fall (Baker 1979). That is, adult female *R. ranae* worms are thought to develop and over-winter in the host and lay eggs late in spring or early summer (Woodhams et al 2000, Baker 1979). Northern

Leopard frogs examined at the same time of year that my Wood frogs were sampled were never found to carry immature worms (Dare and Forbes-In Review). This suggests that although Northern leopard frogs also carry adult worms over the winter, eggs may not be shed and frogs may not begin to acquire new infections until later in the year than Wood frogs.

It is reasonable to expect differences between the two *Rhabdias* lungworms in their lifecycles and patterns of development under natural conditions. Wood frog hosts undergo physiologically more extreme conditions than leopard frogs in the winter; Wood frogs hibernate on land and will freeze solid along with their environments at temperatures as low as -6C (Storey and Storey 1984). On the other hand, Northern Leopard frogs occupy ponds that do not freeze and remain in a resting state in the mud under water with minimal mobility over the winter months (Emery et al 1972, Seburn and Seburn 1998).

The differences in the hibernation strategies of the two host frog species, and their associated physiological adaptations, likely necessitates strong adaptation of the parasites that over winter within them. Woodhams et al. (2000) reported cold hardiness in *Rhabdias* obtained from the lungs of Wood frogs, although the authors assumed that the worms they studied were *R. ranae*. The cold body temperatures of Wood frog hosts over the winter may contribute to longevity and survival of *R. bakeri* lungworms (Baker 1979), but may slow down developmental rate or result in smaller adult sizes compared to *R. ranae* in Northern Leopard frogs. It is important to note however, that developmental rate and size of worm at reproductive maturity are independent of each other

(Gendron et al 2003) and therefore, differences among worm species in these traits may not necessarily be observed at the same time. That is, the observation of differences in developmental rate of worms among *Rhabdias* species may not be accompanied by a concurrent difference in length of worm.

Other adaptive differences might exist among parasite species that enable survival in host species that occupy different habitats. Leopard frogs migrate to grassy feeding grounds around the ponds, whereas wood frogs move to the leaf litter in wooded areas (Bellis 1962). Thus the parasites that are carried to these different habitats for a large part of the year likely adopt different strategies to enhance transmission in each habitat and to deal with variable environmental conditions such as desiccation.

Rhabdias worms removed from the Wood frogs were on average half the size of the worms found in Northern leopard frogs. The mean length of worms removed from female and male Northern leopard frogs sampled at the same time of year was on average $6407 \pm 153.80\text{SE}$ and $5198 \pm 131.09\text{SE}$, respectively. These findings are consistent with results of an experimental study using Wood frogs and Northern leopard frog hosts that revealed that worm length differs among *Rhabdias* species (Dare et al. 2008). Intensity of infection has not been found to influence the length of *Rhabdias* worms (Dare and Forbes, 2008). Thus, the differences in length of worms found in the two hosts are not likely to be due to heightened competitive interactions between worms that may in turn have inhibited worm growth. Instead, the differences in worm length suggest real attributes of the two nematode species. Variation in fecundity among worms from

the two host species may have significant consequences for transmission success that explain the comparatively high prevalence of *Rhabdias* reported in Wood frog populations.

Contrary to my observations of female-biased parasitism of *Rhabdias* infections found in Northern leopard frogs, no sex bias was observed among Wood frogs. Wood frogs show sexual size dimorphism at sexual maturity, whereby females are the larger sex (Bastien and Leclair, 1992; Leclair *et al.* 2000). For dimorphic species, differences between the sexes in allocation of resources toward growth and/or development are expected to trade off against immune function and thereby result in variation in investment in immunity (Sheldon and Verhulst, 1996). Thus it is surprising that a sex bias would be detected among Northern leopard frogs that do not show sexual size dimorphism, but not in Wood frogs. However, my Wood frog samples were small, and my confidence in these results is limited.

My survey has shown clear differences between *Rhabdias* worms collected from Wood frog and Northern leopard frog hosts in establishment and growth patterns. The transmission period of infectivity of larval worms also appears to vary depending on host source, with *Rhabdias* larvae infecting Wood frogs earlier in the year than Northern leopard frogs. Presumably, the higher intensities of worms and the smaller size of Wood frogs may impose greater damage to the respiratory capabilities and/ or chorusing abilities in this host species than in the Northern leopard frog, although such effects may be offset by the smaller sizes of the worms.

These findings are only a first examination for differences in parasitism of *Rhabdias* worms in two congeneric amphibian hosts from the same location. My observations support the idea of adaptation of *Rhabdias* worms from Wood frogs with their hosts, however logistic limitations precluded genetic confirmation of the species of worms recovered from my samples. More studies that examine sympatric populations of Wood frogs and Northern leopard frogs, and that distinguish *Rhabdias* species with genetic testing are still required to elucidate differences between these parasites.

Table AI-1. *Rhabdias* prevalence (%) with Clopper-Pearson confidence intervals (Zar 1996) on binomial proportions (CI), abundance, intensity and worm length (μm) of male and female frogs collected in the first week of April, 2007. Data reported are mean $\pm$ SE, along with results of statistical analysis.

	Female	Male	df	F	p
	(N=16)	(N=18)			
Prevalence	75.0 (47.62-92.73)	77.8 (52.36-93.59)			
Abundance	4.75 $\pm$ 2.97	10.33 $\pm$ 5.02	1, 32	0.86	0.36
Intensity	6.33 $\pm$ 3.89	13.28 $\pm$ 6.27	1, 28	1.31	0.26
Worm Length	3037.36 $\pm$ 141.55	3445.30 $\pm$ 80.59	1, 73.89	2.27	0.10

APPENDIX II: OBSERVATIONS ON TREMATODE AND NEMATODE CO-INFECTIONS IN LUNGS IN TWO RANID HOSTS, NORTHERN LEOPARD FROGS (*LITHOBATES PIPPIENS*) AND WOOD FROGS (*LITHOBATES SYLVATICUS*).

Observations from this second appendix chapter have been submitted for review to the Journal of Helminthology.

ABSTRACT

Here, I report my observations of *Haematoloechus* spp. (Trematoda) and *Rhabdias* sp. (Nematoda) infections in two ranid host populations. I explored relationships between the two classes of lungworm to determine whether there was any evidence for competitive interactions. I found a strong negative relationship between the two types of lungworm in both Northern leopard frogs (*Lithobates pipiens*) and Wood frogs (*Lithobates sylvaticus*). Few hosts carried dual infections. Measures of prevalence, abundance and intensity of *Haematoloechus* spp. (Trematoda) were higher in breeding male Wood frogs and *Rhabdias* sp. (Nematoda) than in breeding male Northern leopard frogs. These latter observations likely reflect different suites of parasites among the two host species.

INTRODUCTION

Specialized feeding adaptations of endohelminths can lead to microhabitat restriction of species (see review by Rohde, 1994). Feeding strategies may also result in competitive exclusion, if damage to tissue by one species precludes the use of a microhabitat by other species. The timing of encounter with hosts thus becomes important for the successful transmission of a parasite, if establishment is contingent on the absence of a predecessor competitive species. Parasite species compositions of host populations (component populations) may therefore be determined by the order of encounter of parasites (Jackson et al 1998; Jackson et al 2006). The ultimate outcome of competitive interactions between parasites and establishment can pose serious consequences for the survival and success of hosts, since the cost of infection likely varies with parasite species (Booth *et al.*, 1993).

MATERIALS AND METHODS

Road-killed Northern leopard frogs were collected from Bishops Mills, Ontario (44° 52' 60.0" N 75° 40' 0.0" W). High vehicle-related mortality occurs particularly during seasonal migration periods when cohorts of frogs move *en masse* over a few short days. I collected Northern leopard frog samples in the spring (April 2- 26, 2007) as they migrated from their hibernation sites to breeding ponds, and again in the late summer/early fall (August 11-26, 2007) when they were moving between feeding grounds and hibernation sites. Northern leopard frogs were

identified to age (juveniles/yearlings and adults). Sex was determined by the presence of secondary sex characteristics and by examining the gonads when present. Wood frogs do not make these migrations across roads, and thus I captured adult male frogs from breeding ponds from the same local wetland area in Bishops Mills as the Northern leopard frogs. A small number ($n=18$) of Wood frog males were collected in the first week of April, 2008 and another larger sample group ($n=66$) on April 23, 2008.

Only frogs that had both intact lungs were used for my analyses. *Rhabdias* (nematodes) and *Haematoloechus* (flukes) found in the lungs were removed and enumerated. I report prevalence, abundance and intensity as response parameters for parasitological counts in breeding male Northern leopard frogs and Wood frogs (according to Bush *et al.*, 1997).

Statistical analyses

Dual infections of *Haematoloechus* and *Rhabdias* occurred less frequently than infections by either of the two classes of lungworms. I therefore performed chi-square tests on contingency tables to test for any relationships between *Haematoloechus* and *Rhabdias* infections. Tests were performed on data from individuals carrying infections by one or the other lungworm. For Northern leopard frogs, tests were performed separately for breeding adults, non-breeding adults and juveniles. For Wood frogs, the two male sample cohorts (early and late April) were tested separately.

I also tested for differences in intensity and abundance of lung nematodes and flukes among the two Wood frog sample cohorts using analyses of variance (ANOVA). Where my data did not pass Levene's test for homogeneity of variances, or conform to normal distribution, I used Kruskal-Wallis non-parametric tests.

RESULTS

Associations between lung helminths

Tests for association between *Haematoloechus* spp. and *Rhabdias* sp. infections in Northern leopard frogs revealed significant negative relationships between *Haematoloechus* spp. and *Rhabdias* spp. in both breeding adults and juveniles, but not in non-breeding adults (breeding: $\chi^2=4.30$, $p=0.04$, juveniles: $\chi^2=5.13$, $p=0.02$, non-breeding: $\chi^2=0.38$, $p=0.54$). Thirteen Northern leopard frogs had co-occurring *Haematoloechus* spp. and *Rhabdias* sp. infections, while 77 frogs carried one or the other type of parasite (Table II- 1). Prevalence, mean abundance and intensity of *Haematoloechus* spp. infection in breeding males are reported in Table II- 2).

A clear negative association between lungworms was also observed in both Wood frog sample groups (early collection ($n=18$): $\chi^2=9.14$, $p<0.002$; April 23 collection ($n=66$): $\chi^2=28.8$, $p<0.000$) (Table II- 1). Of the 18 Wood frogs sampled in early April, only 3 had dual infections, 14 had one or the other class of lungworm and 1 had no infection. Of the 66 Wood frogs sampled on 23 April,

24 individuals carried dual infections, 40 carried one or the other class of lungworm and 2 carried no infection.

There was no difference among male hosts from the two Wood frog sample groups in *Haematoloechus* spp. prevalence ($Z = 1.03$, $p = 0.30$) or abundance ($Z = -0.28$, $p = 0.77$) (Table II- 2). However, the intensity of flukes was significantly higher among male frogs collected on 23 April than in male frogs collected in the first week in April ($\chi^2 = 5.89$, $p = 0.02$).

Interestingly, I observed a segregation of *Haematoloechus* spp. and *Rhabdias* sp. worms within lungs when there were co-occurring infections in both Northern leopard frogs and Wood frogs. When both kinds of worms were present, *Rhabdias* sp. was almost always found at the base of the lung, whereas *Haematoloechus* spp. was located near the entrance of the lung at the bronchi. In contrast, worms in single infections were found throughout the lung.

DISCUSSION

This is a first study reporting relationships between two classes of lungworm commonly found in Northern leopard frogs and Wood frogs. Here I report three main findings: first, infections by one class of lung worm reduced the likelihood of infection by the other in both host species; second, on occasions in which I observed dual infections, there appeared to be some spatial partitioning of the worms, with *Rhabdias* sp. occupying the basal volume of the lung and *Haematoloechus* spp. occupying the top portion of the lungs; and third, numbers and prevalence of *Haematoloechus* spp. lungworms were higher in Wood frogs than in Northern leopard frogs.

It is possible that the damage done to the tissue by *Haematoloechus* spp. worms inhibits the ability of *Rhabdias* sp. to feed, resulting in the observed partitioning within the lung. *Haematoloechus* spp. worms are blood feeders that can cause significant damage to the pleural lining of the lung, and cause alveolar cysts that puncture the wall of the lung (Shields, 1987; personal obs.). A single study on 3 Northern leopard frogs deduced that the pathology of even high-level *Haematoloechus* spp. infections likely will not result in mortality of hosts (Hsu *et al.*, 2004), presumably because amphibians also respire through their skin. Further information on pathologies of infection by *Haematoloechus* spp. in Northern leopard frogs or Wood frogs, or any fitness costs of infection imposed by these lung flukes in amphibians is otherwise entirely lacking.

Rhabdias sp. also feeds on host blood, but infections do not result in gross disintegration of the integrity of the lung tissue. *Rhabdias* sp. lungworm infections are known to cause anorexia and reduce growth and survival of toad hosts (Goater & Ward, 1992). I presume that *Rhabdias* sp. infections likely affect Northern leopard frogs in a similar manner and may also impede adult male chorusing ability which in turn may result in reduced reproductive success. Nevertheless, competition for feeding surfaces likely explains why there were far fewer dual infections than single infections in a lung. Further research on this partitioning is needed to identify any competitive interactions among these parasites that may result in smaller realized niches.

Haematoloechus spp. infections are acquired by amphibian hosts when emergent dragonflies or damselflies are consumed by the frogs. The higher

prevalence of *Haematoloechus* spp. infection in male Wood frogs collected later in April, relative to intensities of the frog samples collected earlier likely reflects the seasonal increase in numbers of available odonate prey as seasonal temperatures rise over the month of April. The lower intensity later in April supports the expected development of immune response in hosts as exposure increases.

Interestingly, the prevalence, abundance and intensity of *Haematoloechus* spp. infections in male Wood frogs were higher than were been observed in breeding male Northern leopard frogs. These differences may reflect different suites of species within the genus that infect the different host species. Such variation in species composition could be attributed to host specificity of parasites, or simply different feeding habits of the two hosts that result in variation in exposure. The genus *Haematoloechus* is a species complex comprising morphologically indistinguishable species (Bolek & Janovy, 2007), thus I was unable to further distinguish worms without genetic testing.

As well, recent evidence has shown that two morphologically similar species of *Rhabdias* (*R. ranae* and *R. bakeri*) have previously been confused in Northern leopard frogs and Wood frogs hosts. It is likely that *R. ranae* only infects Northern leopard frogs, while *R. bakeri* only infects Wood frogs; however the host ranges and specificity of these two nematode species are undergoing revision to determine the extent of host adaptation and specificity of each species. Without genetic testing I was unable to distinguish whether my observations were of single or multiple species of either the trematodes or

nematodes. Thus, without identification of the fluke species, or an analysis of immune components I cannot further discuss the possible implications of patterns observed in the context of species-specific establishment or host resistance to infection.

Table All- 1. Numbers of host frogs carrying lungworm infections

(*Haematoloechus* spp., *Rhabdias* spp. or both). Data presented are single and dual infections in breeding male Wood frogs, and adult Northern leopard frogs in breeding and non-breeding condition, and juvenile frogs. Plus (+) and minus (-) signs indicate respectively, the presence or absence of *Haematoloechus* (H) or *Rhabdias* (R) infection. Tests for association between *Haematoloechus* and *Rhabdias* among cohorts frogs are reported in the text.

Leopard Frogs	H+, R-	H-, R+	H+, R+
Breeding adults	12	21	11
Non-breeding adults	9	11	1
Juvenile	8	16	1

Wood frogs (males)

Early April	3	11	3
(n=18)			
Late April	8	32	24
(n=66)			

Table All- 2. *Haematoloechus* spp. prevalence (%) with Clopper-Pearson confidence intervals on binomial proportions (CI), abundance and intensity of breeding male Northern leopard frogs and male Wood frogs collected in early (n=18) and late April (n=66), 2007. Data reported are mean $\pm$ SE, however note that statistical analyses comparing parasitism between groups of male Wood frogs were done using non-parametric tests (see text for details). I have therefore also indicated median abundance and intensity in parentheses.

	Wood frogs (early April) (N=18)	Wood frogs (late April) (N=66)	Leopard frogs (N=54)
No. of worms	88	112	49
Prevalence (%) (CI)	33.3(13.34-59.00)	46.96(34.56-59.66)	27.8 (16.5-41.6)
Abundance	4.88 $\pm$ 2.38 (0)	1.69 $\pm$ 0.36 (0)	1.19 $\pm$ 0.36
Intensity	12.57 $\pm$ 5.01 (9)	3.61 $\pm$ 0.60 (2)	4.26 $\pm$ 0.93

APPENDIX III: GENERALIZED LIFE CYCLES OF HELMINTHS OF INTEREST

Here I outline more details on the life cycles of parasites that I studied to provide the context in which my studies were conducted. It is important to note that life history traits, such as growth in the definitive host, may also be traded off in a parasite, with the resultant effect of variation in fitness.

Trematodes

Trematodes, otherwise known as digeneans or flukes, are one of four classes in the Phylum Platyhelminthes (flatworms) containing approximately 25,000 species (Bush et al 2001; Esch et al 2002). Digeneans are endoparasites with complex lifecycles including up to four host species. However, all digeneans use an invertebrate (Mollusc) as their first intermediate host in which two generations of asexual reproduction occur, and typically, although not always, a vertebrate as their definitive host in which sexual reproduction occurs.

Although digeneans display very specific site fidelity within a host, they will demonstrate some limited flexibility in host species choice. Morphological traits unique to the adults of this taxon include the presence of prominent oral and ventral attachment organs (oral sucker and acetabulum, respectively), yolk-producing vitellaria and incomplete digestive systems with a diverticulate blind digestive tract (Gibson 1996, Bush et al 2001).

The life cycles of digeneans vary considerably in strategy and complexity between species and therefore one caveat to the generalized lifecycle presented

here is that there are many variations and exceptions at each stage. However, typically, eggs containing miracidia larvae (ciliated or unciliated) are shed into an aquatic environment along with the faeces of the definitive host. The eggs hatch and the miracidia actively swim towards and are ingested by the first intermediate molluscan host (usually a gastropod) and develop into either a sporocyst or redia. Beyond this point in the life cycle, much variation occurs in the pathway of the lifecycle.

Sporocysts are an asexual amplification stage of the parasite whereby many embryos are produced in a process known as polyembryony. Interspecies competition also occurs at this stage as larger rediae will consume smaller rediae and sporocysts of competing species that concurrently infect the host (Sapp and Esch 1994, Esch et al 2001). Thus, only rarely do multiple infections occur within a given snail host (Esch et al 2001). Rediae can produce from a few to several thousand cercariae (Esch et al 2001).

Cercariae are an infective stage that exits the aquatic gastropod in synchronized bursts forming clouds of free-swimming larvae. These clouds disperse and the cercariae either attach to vegetation and develop into non-motile metacercariae, or actively swim in search of a second definitive host with the whip-like action of their glycogen-rich tails. Their movements consist of highly active motile periods where they move through the water column, closely resembling mosquito larvae (perhaps to lure potential hosts into closer contact), followed by periods of quiescence where they settle to the bottom and move in an inch-worm fashion across the bottom substrate.

Not surprisingly, cercariae and miracidia are particularly sensitive to environmental conditions including temperature, photoperiod, pH and pollutants (Evans 1985, Poulin 1992, Pechenik and Fried 1995, Pietroock and Marcogliese 2003). Furthermore, as these are non-feeding stages, they survive for only a few hours (up to 36 hrs for miracidia and 72hrs for cercariae) before energetic resources are depleted (Esch et al 2001). Thus, the completion of the lifecycle is highly dependent on spatial and temporal overlap of cercariae with the second intermediate host, as well as the density of hosts to which the cercariae are exposed.

Second intermediate hosts are most often invertebrate hosts, but can also be aquatic larval stages of vertebrate hosts such as amphibians. Cercariae either actively penetrate into the host with the aid of a spear-like stylet or are accidentally ingested. Sometimes the cercariae are directly ingested by the definitive host and bypass the second intermediate host entirely (Schell 1970, Esch et al 2001). In other instances, cercariae may infect paratenic hosts where they encyst in the tissues until the host is ingested by a definitive host. Once inside the host, cercariae migrate quickly to target organs and develop into meso or metacercariae that encyst in the tissues. The second intermediate host is then predated by one of a large range of definitive vertebrate hosts including waterfowl and carnivorous mammals. Parasite manipulation of host behaviour to increase the potential for predation, and therefore transmission, has been documented widely at this stage of the life cycle.

During digestion of the second intermediate host by the final host, the metacercariae excyst and develop into non-ciliated adult digeneans. Adults of most species are usually found in the gut or body cavity, but can invade the vascular system and other tissues, where they consume epithelial or vascular tissues (Gibson, 1996).

Rhabditean nematodes

These nematodes are parasitic in all classes of vertebrates, including amphibians. Worms of the genus *Rhabdias* are protandrous hermaphrodites that first develop in the lung of hosts as functional males then later develop into functional females (Bush et al 2001). In amphibians, adult worms establish in the lungs. Adult female worms fertilize eggs with sperm stored from their preceding male sexual phase. Egg capsules released from the female are coughed up and swallowed. Once in the digestive tract, some of the eggs hatch into rhabditiform larvae (L1 stage) that are passed out along with eggs in the host's faeces. These larvae undergo four moults and develop into free-living male and female adults in about 3 days (Goater and Vandenbos 1997, Gendron et al 2003). This alternation of free-living and parasitic stages in the lifecycle is referred to as heterogonic development (Bush et al. 2001). In the free-living stage, females become reproductively mature in another 2 days and produce eggs that are fertilized by mature males. Some of the eggs are laid on host faeces, while a few eggs hatch within the female and consume her tissues (matricidal endotoky) (Bush et al 2001). These filariform, infective (L3) larvae develop and penetrate

the skin of amphibians. The larvae travel through various tissues, lymph and the blood towards the lungs. Only those that move into the lungs establish (Baker 1979). In Wood frogs, adult fecund worms can be found after 1 wk post-infection (Baker 1979, Goater and Vandenbos 1997).