The Pennsylvania State University
The Graduate School
Eberly College of Science

CAUSES AND CONSEQUENCES OF SEASONAL DYNAMICS IN THE PARASITE COMMUNITY OF RED-SPOTTED NEWTS (*NOTOPHTHALMUS VIRIDESCENS*)

A Thesis in Biology
by
Thomas Richard Raffel

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2006

The thesis of Thomas R. Raffel was reviewed and approved* by the following:

Peter J. Hudson
Willamon Professor of Biology
Thesis Adviser
Chair of Committee

James H. Marden
Professor of Biology

Ottar N. Bjørnstad
Associate Professor of Entomology and Biology

Mary J. Kennett
Associate Professor of Veterinary & Biomedical Sciences

Joseph M. Kiesecker
Senior Ecologist of the Wyoming Field Office
The Nature Conservancy
Special Signatory

Douglas R. Cavener
Professor of Biology
Head of the Department of Biology

*Signatures are on file in the Graduate School.

ABSTRACT:

Recent implications of parasites as agents of worldwide amphibian decline make determining the drivers of parasitic infection in amphibians a priority for ecological research. Despite the apparently seasonal nature of these outbreaks, seasonal dynamics remain largely ignored in recent studies of amphibian parasites. In this thesis, I present results of field studies describing seasonal patterns for multiple parasitic infections and testing between potential drivers of parasite infection risk using red-spotted newts (*Notophthalmus viridescens*) as a model species. Most newt parasites had seasonal dynamics, with significantly more parasite species having peak infection rates during the early and late spring than in other seasons. These similar seasonal patterns appear to have been driven by multiple different factors, many of which may have been driven in turn by bottom-up effects of the spring bloom in pond productivity.

Seasonal patterns in white blood cell counts indicated that amphibian immunity has similar responses to temperature in the field as have been recorded in laboratory studies. Moreover, both temperature increases and decreases were associated with low levels of immunity relative to temperature, indicating a negative effect of temperature variability on amphibian immunity. This pattern is probably due to a lag in production of immune cells following temperature increases in spring, and to a lag in seasonal acclimation to winter temperatures causing low immune cell production rates in autumn.

The last three chapters focus on the ecology of *Ichthyophonus* sp., a protist parasite of newts which has caused mass morbidity events in North America. Based on data from the seasonal survey, an additional survey of sixteen populations, and a capture-mark-recapture study, several lines of evidence suggest that the amphibian leech *Placobdella picta* is the vector of *Ichthyophonus* sp. infection in newts. There was little indication of mortality due to infection despite high apparent morbidity, but *Ichthyophonus* sp. infection did appear to cause males to stop breeding earlier in the spring and to induce temporary emigration in female and possibly male newts. These results suggest that parasites may influence the the newt life history strategy by driving newts onto land during the summer.

TABLE OF CONTENTS:

LIST OF TABLES:

LIST OF FIGURES:

PREFACE:

Numerous students and researchers have contributed to the material presented in this thesis. Joseph M. Kiesecker, Peter J. Hudson, and Robert S. Huang coauthored Chapter 1. R. S. Huang performed many of the dissections and parasite identifications, J. M. Kiesecker helped conceive and design the study, and P. J. Hudson assisted with analyses and writing. Reehan S. Mirza, Lewis E. Grove, Dustin M. Weidner, Michael R. Faix, Angie D. Luis, Amy L. Raffel, and particularly Jack J. Falkenbach, Jennifer Snukis and James R. Dillard aided with field surveys and/or initial dissections of newts. J. Michael Kinsella and Tavis K. Anderson assisted with and confirmed parasite identifications. David Geiser assisted with the preliminary identification of the *Candida* sp. parasite. T. K. Anderson helped research anisakid taxonomy and assisted with measuring and identifying worm structures for Chapter 2. J. M. Kinsella confirmed the genus designation and important diagnostic characteristics of *Hysterothylacium burtti*.

J. M. Kiesecker, Jason R. Rohr, and P. J. Hudson helped write Chapter 3, which has been accepted for publication by the journal Functional Ecology. J. M. Kiesecker helped to conceive and design the study, J. R. Rohr and P. J. Hudson assisted with analyses and provided funding, and all three contributed to the writing of this chapter. Other contributors were Paul Buchter, who helped develop the lysozyme assay, and J. R. Dillard, who provided extensive help with fieldwork and dissections during the 2004 survey. Robert Paulson suggested the benzidine stain protocol for use with blood smears.

J. R. Dillard and P. J. Hudson coauthored Chapter 4, which has been accepted for publication by the Journal of Parasitology. J. R. Dillard assisted with much of the fieldwork and dissections for this chapter, and both coauthors assisted with writing the manuscript. Other contributors were Jeffrey W. Dodds, who assisted with initial identification of the *Ichthoyophonus* sp. parasite, and Ruth A. Haldeman and Robert P. LeGros, who conducted much of the histological sectioning and staining.

R. S. Huang coauthored Chapter 5, for which he helped with field collections and assisted with experiments. Eric W. Lukavsky aided with leech collection. P. J. Hudson coauthored Chapter 6, for which he helped conceive the project and assisted with fieldwork. J. R. Dillard, Dustin Weidner, Robin P. LeGros, R. S. Huang, Kristle Krichbaum, A. L. Raffel, and Kristin Tassone also assisted during marking sessions.

ACKNOWLEDGEMENTS:

I am immensely grateful to my two advisors, Joseph Kiesecker and Peter Hudson. Joe helped set the stage for my project, taught me how to think about and work with amphibians, and strongly influenced my approach to ecological research. Peter helped me to further develop my approach to disease ecology and writing. I cannot imagine a better combination of advisors.

From the Kiesecker lab I want to thank Michael Rubbo, Reehan Mirza, Sarah Storrs and Lisa Belden for our collaborations and for helping me to develop my study system. From the Hudson lab I want to thank Kurt Vandegrift, Sarah Perkins, Isabella Cattadori, Angie Luis, Vitor Rademaker, Eleca Dunham, Kristle Krichbaum and Nita Bharti for their friendship and for acting as frequent sounding boards for my ideas.

I also want to thank Jason Rohr for his extensive advice and collaboration and Matthew Ferrari for acting as my unofficial statistical consultant. Tavis Anderson and J. Mike Kinsella provided extensive help and training with helminth identification, and David Geiser and David E. Green provided assistance with identification of fungal and protist pathogens. From my committee, I want to thank James Marden for exposing me to his broad approach to biological research, Mary Kennett for for providing a pathologist's perspective, and Ottar Bjornstad for critiqueing my ideas and statistics.

I also want to give personal thanks to my sisters Amy and Susan Raffel, my father Gary Raffel, and especially my mother Amy Raffel for providing love and support. Sandra Cooke, Catriona McDonald and Joanna Castro also helped me to maintain my sanity at key time points. This thesis is based on work supported by a National Science Foundation predoctoral fellowship and a National Science Foundation Doctoral Dissertation Improvement Grant (#0508847), as well as a National Science Foundation Grant to Jason Rohr (#0516227).

DEDICATION:

This thesis is dedicated to the teachers who inspired me to pursue a career in science, Louise M. Temple (Drew University), A. John Gatz (Ohio Wesleyan University) and Edward H. Burtt (Ohio Wesleyan University), the namesake of the anisakid nematode *Hysterothylacium burtti*.

INTRODUCTION:

Several parasites including ranaviruses and a pathogenic chytrid fungus have been implicated as causative agents of amphibian declines and mass mortality events, spurring a recent surge in research on these organisms (Chinchar 2002, Green et al. 2002, Daszak et al. 2003, Kiesecker et al. 2004, Jancovich et al. 2005, Johnson and Lunde 2005, Lips et al. 2006, Pounds et al. 2006). Yet despite this recognition of the impact parasites can have on amphibians, relatively little attention has been paid to the potentially important regulatory effects of other parasites on amphibian populations (Tinsley 1995), and few studies have accounted for seasonal fluctuations in the force of infection (Wetzel and Esch 1996). Historically, research on amphibian parasites and particularly salamander parasites has focused on taxonomic relationships, life history descriptions and faunistic surveys (but see Aho 1990, Tinsley 1995). Furthermore, most studies of amphibian parasite dynamics have focused on only one or a few parasite species (e.g., Baker 1979, Jarroll 1979, Mock and Gill 1984, Vanderburgh and Anderson 1987, Wetzel and Esch 1996, Bolek 1997, Bolek and Coggins 2000, 2001), despite the diverse parasite communities of many amphibian hosts (Aho 1990, Muzzall 2005). Further knowledge of the factors driving parasite dynamics in amphibians will be needed to predict how human-induced environmental changes will influence population-level impacts of parasites on amphibians.

To fully understand parasite-induced amphibian declines, it is also important to study the effects parasites have on different age classes. Much of the recent literature on amphibian ecology focuses on larval stages (e.g., Wilbur 1997, Skelly et al. 2002), which are generally easier to obtain and manipulate than adults. However, amphibian population dynamics may be more sensitive to factors affecting juveniles and adults, since there can be density-mediated compensation in post-metamorphic stages for high larval mortality (Biek et al. 2002, Vonesh and De la Cruz 2002, Schmidt et al. 2005, Rohr et al. 2006). Therefore if we want to predict population-level impacts of parasites on amphibians, we must develop model systems for studying the impacts and drivers of parasitism in adult amphibians.

The red-spotted newt (*Notophthalmus viridescens*) is an excellent model species for studying the drivers and population-level consequences of parasitic infection in adult

amphibians. Newts are abundant in eastern North America and have a diverse, well-described parasite fauna (Rankin 1937, Petranka 1998, Muzzall 2005). Unlike most temperate amphibian species which breed in spring or summer and burrow in soil or sediment during the winter months, newts have aquatic adults that start breeding from early autumn until the following spring in permanent ponds (Duellman and Trueb 1986, Petranka 1998, Raffel et al. *in press-b*), making it possible to sample newts year-round from the same habitat and allowing comparisons of parasite burdens between seasons.

Despite the obvious difficulties of judging population-level impacts of parasites without accounting for temporal changes in the force of infection, most studies of amphibian parasites focus on a single time point during the year (Wetzel and Esch 1996). Seasonal dynamics are common for parasites in general (Altizer et al. 2006) and may be strong for parasites of amphibians, whose seasonal changes in body temperature, breeding activities, and recruitment of new susceptible individuals could drive seasonal changes in transmission and susceptibility (Duellman and Trueb 1986). In particular, the seasonal habitat use typical of many amphibian species (aquatic vs. terrestrial, Duellman and Trueb 1986) may drive more extreme seasonal patterns of infection risk than in other vertebrate groups such as birds and mammals.

Few recent studies of amphibian parasites have included a seasonal component, and fewer have assessed postulated drivers of seasonal parasite dynamics or sampled multiple years or populations, making it hard to assess whether the observed fluctuations are seasonally forced or random. Many of these studies have found evidence of high infection rates in late spring and summer. Wetzel and Esch (1996) found high prevalence of immature *Halipegus occigualis* and *Halipegus eccentricus* from May to July in a single *Rana clamitans* population in three years of sampling, corresponding to the seasonal pattern of infection in the odonate intermediate host. Bolek and Coggins (2001) found seasonal size variation in *Haematoloechus varioplexus* infecting *Rana clamitans* suggesting that new infections begin in late spring and continue throughout the summer. They also found that prevalence of *Glypthelmis quieta*, *Oswaldocruzia pipiens*, *Cosmocercoides* sp. and unidentified larval nematodes increased from late spring to summer in this host. Vanderburgh and Anderson (1987) found increased prevalence from April to May of *Cosmocercoides variabilis* infection in *Bufo americanus* but

sampled in different sites, so this increase could be attributed to site-specific differences in infection prevalence.

Similar patterns have been observed in analyses of parasite infracommunity diversity in amphibians. Bolek and Coggins (2001) found that the richness of the *Rana clamitans* helminth infracommunity increased in late spring. Muzzall (1991) found that the diversity of the red-spotted newt helminth infracommunity increased from early spring to summer in a Michigan pond, a pattern he attributed in part to ingestion of a larger number and wider range of intermediate hosts by the newts as they increased in size during this time period. While many of these authors have implicated intermediate host dynamics and seasonal recruitment as potential drivers of seasonal parasite dynamics in amphibians, none have tested between these and other potential drivers of seasonal parasite dynamics.

Parasites implicated in amphibian population declines also appear to have seasonal dynamics. Iridovirus infections and trematode-induced limb deformities seem to follow the general pattern of high spring and summer infection rates. Green et al. (2002) found that mortality events attributable to iridovirus outbreaks are most commonly reported in late spring and summer. Johnson et al. (2001b) found that the prevalence of amphibian deformities (associated with infection by *Riberoia* spp. infection, Johnson et al. 1999, Johnson et al. 2001a) increased from late spring to summer in metamorphosing pacific treefrogs and from early to late spring in california newts. These patterns may be due largely to the timing of amphibian metamorphosis, which is the age at which amphibian deformities and iridovirus infections most often occur (Johnson et al. 1999, Johnson et al. 2001b, Green et al. 2002). In contrast, chytrid fungus, the pathogen best documented to cause population declines (Lips et al. 2006), appears to infect amphibians primarily during colder months. Berger et al. (2004) found consistently high incidence of chytrid fungus infection in winter, based on numbers of dead or ill frogs submitted by government and private organizations in Australia over five years, whereas other diseases had high incidence in spring and summer. This high rate of infection at cold temperatures has been attributed to a combination of temperature-induced low immunity and a low optimal growth temperature for chytrid fungus (Woodhams et al. 2003, Berger et al. 2004).

Evidence of nonseasonality or high infection rates in the autumn and winter is less common in amphibians, at least for helminth parasites. In a 2-year study of *Rhabdias ranae* infection in *Rana sylvatica*, Baker (1979) found evidence that most transmission occurs in summer and early autumn and attributed low summer prevalence to an influx of uninfected juvenile recruits. Bolek (1997) found that *Cosmocercoides dukae* prevalence peaked in September for a population of blue-spotted salamanders, matching seasonal changes in abundance of the snail intermediate host, but had sample sizes too small to detect statistical patterns. Bolek and Coggins (2000) found no seasonal differences in prevalence or intensity of *Cosmocercoides variabilis*, *Oswaldocruzia pipiens* or *Rhabdias americanus* in a population of *Bufo americanus* over a single breeding season.

Seasonal changes in parasite infection rates can be driven by a variety of factors including changes in host contact rates, vector-host ratios, environmental variables which influence survival of free-living parasite stages, or host susceptibility (Altizer et al. 2006). Since seasonal factors tend to correlate with each other, it can be difficult to determine which factors are the most important drivers of a particular parasite's dynamics, since seasonal changes in ecosystems can be difficult to manipulate. Approaches used to study drivers of seasonal dynamics in ecological systems include laboratory simulation of seasonal conditions (Rani et al. 2005), comparisons of seasonal dynamics across latitudinal or altitudinal gradients (Rivera et al. 2002, Ahumada et al. 2004), and modeling parasite dynamics under different assumptions and comparing with field data (Roberts and Grenfell 1992, Marin et al. 1998). Another approach is to test which of several competing hypotheses best explain seasonal patterns, especially in multiple populations or years with differing levels of seasonal factors (e.g., Wikelski et al. 2000). I use the latter approach in chapter 1 to study drivers of parasite seasonality, measuring a variety of postulated drivers of parasite infection risk and using generalized linear models to test which of these factors are most likely to be drivers of seasonal parasite dynamics in newts.

To fully understand seasonal changes in the force of infection, it is important to determine how host susceptibility to infection changes seasonally. Seasonal immune suppression due to stress or breeding appears to be important for dynamics of some diseases, such as conjunctivitis in house finches (Altizer et al. 2004a, Altizer et al. 2006).

However, responses of ectotherm immune systems to changes in environmental temperature may be qualitatively different from those observed in birds and mammals. Amphibians experience drastic reductions in some immune parameters at cold temperatures (Maniero and Carey 1997), but since ectotherms allow their body temperatures to fluctuate with ambient temperature rather than expending energy to maintain constant high body temperature, they are less likely than endotherms to experience energetic stress at low temperatures. Instead, the low levels of immunity observed in amphibians at cold temperatures may be an adaptive response to slower within-host replication and growth rates of parasites at colder temperatures (Ratkowsky et al. 1982). Since the immune system is costly to maintain (Bonneaud et al. 2003, Ksiazek et al. 2003), there should be a trade-off between the metabolic cost of immunity and risk of parasitic infection that could lead to amphibians having lower optimal levels of immune cells and proteins at lower temperatures.

It may be difficult for amphibians to maintain optimal levels of immune cells and proteins during periods of temperature change. In Chapter 3 I propose two hypotheses for how changing temperature influences amphibian immunity. The first hypothesis is that temperature-dependent immune parameters remain at the same levels immediately following temperature changes until the amphibian has time to adjust to the new optimal levels by producing new immune cells and proteins (in the case of temperature increases) or removing excess cells and proteins (temperature decreases). This "lag effect" hypothesis predicts lower-than-optimal levels of immunity during temperature increases, an effect documented in the laboratory for complement activity in leopard frogs (Maniero and Carey 1997), and higher-than-optimal levels during temperature increases. The second hypothesis is based on laboratory observations that cold-acclimated (> 1 month at cold temperature) fish and amphibians produce higher levels of immune cells and proteins at cold temperatures than if they had been warm-acclimated (Bly and Clem 1991, Plytycz and Jozkowicz 1994). Based on these observations, I postulated that warm-acclimated amphibians have much reduced cell and protein production rates (i.e., cells per day) following long-term temperature decreases, until they can turn on additional metabolic processes to increase production rates at the new temperature. This "seasonal acclimation effect" predicts lower-than-expected levels of immune parameters

following seasonal temperature decreases and higher-than-expected levels following temperature increases.

Although these effects have been individually documented in laboratory studies, their consequences for amphibian immunity under more complex field conditions remain unknown. The lag effect and seasonal acclimation effect hypotheses have opposing predictions for how temperature changes should influence the amphibian immune system, and it is unclear which effect should dominate during temperature increases or decreases. In addition, amphibians are known to tune into other seasonal changes such as day length (Delgado et al. 1992), which might allow them to anticipate and counteract these changes. In chapter 3, I present field data regarding seasonal changes in red-spotted newt immunity and test for the effects of temperature and temperature change on their immune systems.

The ultimate goal of epidemiological studies is usually to determine the factors that drive the force of infection for a parasite, which requires data on the number of individuals becoming infected through time (i.e., incidence) as opposed to prevalence or abundance data at discrete time points. Whereas incidence data are often available for human pathogens and parasites, for which case mortality or case infection data are commonly recorded (e.g., Bjornstad et al. 2002), incidence data are difficult to obtain for most wildlife animal populations and are virtually unheard of for amphibians due to the need to follow individual hosts' infection status over time (but see Mock 1987, Wetzel and Esch 1997). In addition to diagnosing infection without killing hosts, tracking individual infection status through time (i.e., longitudinal data) for non-sessile animals requires confinement of focal sentinels (e.g., Mock and Gill 1984, Komar 2001) or the use of capture-mark-recapture techniques (e.g., Mock 1987, Wetzel and Esch 1997). *Ichthyophonus* sp. (Mesomycetozoa: Ichthyophonida) infection, which has been implicated as the cause of mortality and morbidity events in North America (Green et al. 2002), provides a good model system for obtaining incidence data in a wildlife population because it causes easily identifiable disease signs in infected red-spotted newts, allowing accurate diagnosis in the field without destructive sampling (Goodchild 1953, Herman 1984, Converse and Green 2005). This makes it possible to track the infection status of marked individuals through time.

Little is known about the life cycles of most mesomycetozoans, but culture studies of representative species suggest that organisms of the order Ichthyophonida release endospores following rupture of the outer wall of their encysted spores, and that these endospores typically develop into motile infectious amoeboid cells (Mendoza et al. 2002). *Ichthyophonus hoferi*, a parasite of freshwater and marine fishes found in the viscera and muscles of the gastrointestinal tract and heart, is possible unique in its ability to produce hyphae at acidic pH, which appears to aid its trophic transmission mechanism by allowing penetration of the gut wall (Spanggaard et al. 1995, Mendoza et al. 2002). Though ultrastructurally similar to *Ichthyophonus hoferi*, the *Ichthyophonus* sp. of amphibians infects only skeletal muscle (Mikaelian et al. 2000). Attempts to culture amphibian *Ichthophonus* sp. or to infect fish by feeding them have so far proven unsuccessful, and conspecific fish examined for evidence of infection during an epidemic in amphibians were apparently free of the infection (Mikaelian et al. 2000). It therefore seems probable that amphibian *Ichthyophonus* sp. is a different organism from *Ichthyophonus hoferi* and that it may use a different mode of transmission. Chapter 4 describes evidence that transmission of amphibian *Ichthyophonus* sp. may involve the amphibian leech (*Placobdella picta*) and tests between this and two hypotheses assuming trophic transmission. Chapter 5 describes preliminary attempts to observe leech-borne transmission under controlled laboratory conditions. Chapter 6 provides further evidence supporting the leech transmission hypothesis, describes seasonal changes in the force of infection of this parasite as measured in a capture-mark-recapture study, and tests for effects of *Ichthyophonus* sp. infection on mortality and emigration.

In general, this thesis focuses on seasonal patterns of parasitism in amphibians and the drivers of these patterns, using red-spotted newts as a model organism. The first chapter describes seasonal patterns of parasitism in red-spotted newts from a 2-year seasonal survey of four newt populations and a metanalysis of published seasonal data on red-spotted newt parasites, testing between hypothesized drivers of seasonal dynamics for parasites in the 2-year seasonal survey. The second chapter is a description of a new nematode species, *Hysterothylacium burtti*, discovered in newts collected for seasonal and spatial surveys of newt parasites. The third chapter describes seasonal changes in the immune system of wild-caught red-spotted newts and tests predictions of two hypotheses

for how changing temperature influences the amphibian immune system under complex field conditions. Chapters 4-6 focus on the ecology of *Ichthyophonus* sp. infection in red-spotted newts, providing evidence for leech-borne transmission, seasonal changes in the force of infection, and mortality/emigration of newts from ponds due to infection. I conclude that seasonal patterns of parasitism are remarkably consistent across parasite taxa in red-spotted newts despite being driven by different ecological and physiological factors, and I propose that spring increases in parasite abundance may ultimately be driven by bottom-up effects of the spring increase in temperate pond productivity on multiple drivers of infection risk.

CHAPTER 1:

General patterns and drivers of seasonal dynamics in the parasite community of a temperate amphibian species

T. R. Raffel[1], R. S. Huang[1], J. M. Kiesecker[2] and P. J. Hudson[1]

[1]Center for Infectious Disease Dynamics, Penn State University

[2]The Nature Conservancy, Wyoming Chapter, Lander, WY

Abstract:

Seasonality is a dominant force in most ecological systems but has been largely ignored in studies of amphibian parasites. Given reports of seasonality in the dynamics of parasites implicated in amphibian declines, gaining a general understanding of seasonal patterns and drivers of parasitism in amphibians might be crucial for amphibian conservation efforts. To examine the role of seasonality in amphibian parasite-host dynamics, we undertook a seasonal survey of four populations of red-spotted newts, tracking seasonal patterns of parasitism and associated environmental and immunological factors. We also conducted a meta-analysis of seasonal parasite data available from the primary literature seeking general patterns in the seasonal dynamics of newt parasites. Most parasites of red-spotted newts increased in abundance in the spring and early summer, but no single factor could account for this general pattern of high spring infection rates. Intermediate host abundance and environmental temperature were most often found to be significant predictors of parasite dynamics, but negative effects of host density, seasonal changes in host immunity, interactions with other parasites, and timing of parasite reproduction to coincide with peak densities of alternate hosts also appear to play important roles. We propose that similar seasonal dynamics could be driven by different ecological factors if seasonality in these factors were all driven by seasonal changes in pond temperature and productivity. Our results show that amphibian parasite dynamics are strongly seasonal and support the hypothesis that climate disruption will strongly influence amphibian parasite dynamics.

Introduction:

Seasonal environmental factors dominate the dynamics of many ecological systems, particularly in temperate regions. Seasonal forcing might be especially important for parasite-host systems, since infection rates depend on seasonal changes in host behavior, physiology and population density in addition to direct effects of seasonal temperature and precipitation (Altizer et al. 2004b, Altizer et al. 2006). Seasonal forcing of infection rates can strongly influence the severity and extent of epidemics and the long-term persistence of host-parasite interactions (Altizer et al. 2006). Since seasonal factors such as temperature and rainfall are closely linked to long-term climatic

fluctuations, understanding how seasonal drivers affect parasite communities will be important for predicting effects of global climate disruption on disease outbreaks in humans and wildlife (Harvell et al. 1999, Altizer et al. 2006, Pounds et al. 2006).

There is growing evidence that seasonal parasite dynamics might play an important role in the recent worldwide declines of amphibian species (Green et al. 2002, Berger et al. 2004). Emerging parasites have been implicated as a primary factor in large scale amphibian mortality events (Daszak et al. 1999, Lips et al. 2006), and many of the parasitic infections implicated (e.g., ranaviruses, chytrid fungus and trematode-induced limb deformities) appear to have seasonal dynamics (Johnson et al. 2001b, Green et al. 2002, Berger et al. 2004). Generally, amphibian parasite dynamics should have strong seasonal drivers given the seasonal changes in their use of aquatic and terrestrial habitats, breeding activities, and immunity (Duellman and Trueb 1986, Raffel et al. *in press-b*), any one of which may drive seasonal parasite dynamics (Altizer et al. 2006). Identifying the seasons during which amphibians are most at risk from infection and the factors driving infection rates will be important for predicting the long-term effects of these parasites on amphibian population dynamics.

One of the few temperate amphibian species for which seasonal parasite data have been obtained is the red-spotted newt (*Notophthalmus viridescens*). Unlike many other temperate amphibian species, newts have fully aquatic adults which can be sampled at any time of the year from permanent ponds (Raffel et al. *in press-b*). Nonetheless, newts share many ecological characteristics with other temperate amphibian species, such as seasonality in breeding activity, recruitment of new adults, population density and habitat use (Petranka 1998). Newts also have a diverse, well-described parasitic fauna (Rankin 1937, Muzzall 2005) and may have large population sizes (>2600 adults per pond, Gill 1978b), so that destructive sampling is unlikely to influence their population dynamics. These characteristics make newts ideal for studying seasonal dynamics of parasitism in temperate amphibians.

Seasonal host-parasite dynamics may be driven by a variety of factors (Altizer et al. 2006), but we will consider eight which are relevant to red-spotted newts. The infection rates of directly transmitted parasites generally increase with increased contact rates between hosts (Hudson et al. 2002), which may change due to (1) seasonal changes

in social behavior and aggregation (Altizer et al. 2006), leading to high rates of infection at times of peak newt density, or (2) seasonal changes in the density of alternate hosts such as larval amphibians and fish, which share parasites with newts (Hopkins 1933, Hedrick 1935, Chinchar 2002). Infection rates of vector-borne parasites, such as amphibian parasites transmitted by leeches, depend on (3) the ratio of vector to host abundance (van Riper et al. 1986, Hudson et al. 1995, Gubler et al. 2001, Smith et al. 2005). Infection rates of parasites requiring intermediate hosts, such as trematodes (which always have a mollusc intermediate host, Schnell 1985), are typically driven by (4) intermediate host abundance (e.g., Johnson and Chase 2004). In contrast to directly transmitted parasites, these parasites should have reduced infection rates per newt at high newt or alternate host densities, since there are a finite number of vectors or infectious particles available to infect newts and alternate hosts (Ostfeld and Keesing 2000). Four further factors which may apply to any parasite regardless of transmission mode are (5) seasonal changes in temperature and dissolved oxygen which may influence parasite pathogenicity and the growth and survival of parasite free-living stages (Saunders et al. 2000, 2002, Woodhams et al. 2003), (6) competitive or immune-mediated interactions between parasites in which seasonal dynamics of one parasite influence the seasonality of another parasite (Dobson 1985, Cox 2001, Lello et al. 2004), and seasonal increases in the proportion of susceptible hosts due to (7) recruitment of naïve hosts and (8) changes in host immunity (Altizer et al. 2004a, Altizer et al. 2006). Newts have been shown to have seasonal recruitment of new adults from the terrestrial juvenile population (Gill 1978b) and to experience seasonal periods of relatively low immunity, especially in autumn (Raffel et al. *in press-b*).

The aim of this study was to determine the degree and timing of seasonal infection dynamics in the newt parasitic community and to identify potential drivers of these patterns. Since seasonal drivers are difficult to manipulate, we conducted a natural experiment by monitoring newt parasite dynamics in four ponds which differed in the strength and seasonality of potential drivers of infection. We conducted a meta-analysis of these and previously published data in order to determine which parasites show seasonal dynamics, the times of year parasites increased in abundance, and the reproducibility of these seasonal patterns. To test which of our postulated mechanisms

are the most important drivers of host-parasite dynamics in newts, we regressed abundance of parasites collected in our seasonal survey against factors known or postulated to influence the dynamics of each parasite (Table 1.1). We will show that parasites of red-spotted newts have consistently high spring infection rates across taxa despite having different seasonal drivers, and we propose that these similar seasonal patterns may be caused by similar effects of high spring pond productivity on multiple drivers of parasite infection dynamics.

Methods:

Ponds Sampled:

Four ponds known to support newt populations were chosen in and around Centre County to represent a variety of suitable newt habitats. Mothersbaugh (N 40º 39' 12", W 77º 54' 9") is a flat-bottom beaver pond in the Penn State Experimental Forest which has decreased considerably in size and depth since the beavers left 5-10 years ago. Turtle Shell pond (40º 52' 26", W 78º 4' 36") is one of several beaver ponds along a stream system in Moshannon State Forest. Mystery Newt pond (40º 45' 53", 78º 0' 49") is a semi-permanent (drying some winters) landlocked woodland pond in the Scotia Barrens (PA State Game Lands #176), and Little Acre (N 40º 48' 6", W 77º 56' 37") is a landlocked permanent pond located in another part of the Scotia Barrens. Data from sweep-surveys conducted in 2002 were available for Mothersbaugh and Mystery Newt. Seasonal surveys and collections of newts for dissection were conducted in 2003 and 2004 in the early spring (20 March - 30 April; "Sp1"), summer (16 July – 17 August; "Sum"), autumn (25 September – 5 November; "Aut") and winter (14 January – 27 February; "Win"). Newts could not be obtained in winter from Mystery Newt or from Turtle Shell in 2004. A late spring (26 May – 18 June; "Sp2") survey was added in 2004 and replicated in 2005 following observations of differences in newt ecology between early and late spring. Sweep-survey data, but not dissection data, were also available for early spring 2005 from Mothersbaugh and Turtle Shell.

Survey Methods:

During each survey, meter-long sweeps of a dip net (12" x 24" aperture, 1/8" mesh) were taken at regular four-step intervals in a sinusoidal pattern going out to a depth of 0.5 m around the entire pond perimeter. Numbers of newts, amphibian larvae and several types of aquatic macroinvertebrates and fish were recorded for each sweep using a digital voice recorder. Sex, mass, snout-vent length, breeding status, visible signs of *Ichthyophonus* infection, the number of visible *Clinostomum* metacercaria, the number of attached leeches, and the presence of visible fungal cysts (thought to be caused by a pathogenic *Candida*) were recorded for each individual newt. Swelling of the axial musculature and visible subcutaneous spores in any body part were considered diagnostic signs of *Ichthyophonus* infection, as described by Raffel et al. (*in press-b*). After completion of each sweep-survey, ten newts were collected per pond for blood collection and dissection. Newts were transported to the lab in 250 mL Nalgene containers filled with pond water, anesthetized with a drop of 10% benzocaine (Orajel®) rubbed on the head, and euthanized by decapitation within three hours of initial capture.

Parasite identification and enumeration:

Trypanosoma diemyctyli (Tobey 1906) infection was determined from blood smear counts. Blood was collected from euthanized newts with a heparinized capillary tube and a drop was smeared on a glass microscope slide. Slides were air-dried for ten minutes, fixed in methanol for five minutes, and again allowed to dry. Slides were placed in 1% o-dianisidine (3,3'-dimethoxybenzidine, Sigma) in methanol for 90 s., destained in 1% hydrogen peroxide in 50% ethanol for 90 s, and rinsed twice in deionized water for 30 s. Slides were then stained in Giemsa stain for 30 min. and rinsed again in deionized water for 10 min. Cells were counted at 400x magnification starting in the upper left corner of the smear and working down the slide in a standardized search pattern, moving the objective 2 mm between fields and counting all cells in each field until the erythrocyte count reached 5000. Newts were considered infected if at least one trypanosome was observed per 5000 erythrocytes.

Following blood collection, newts were surface-sterilized briefly with 70% ethanol and a 20-30 mg liver sample removed using sterile technique. An equal volume

of sterile phosphate-buffered saline was added to the liver sample, which was then homogenized using a pellet pestle (Kontes Glass Co., Vineland, NJ), after which 25 μL of liver homogenate was spread onto 5% sheep's blood agar with a TSA base. Bacterial colonies were counted if they grew to a visible size within five days of incubation at room temperature. The remainder of the liver and the spleen were preserved in 10% buffered neutral formalin and the rest of the newt was preserved in 70% ethanol for further parasitological examination.

The pleuroperitoneal cavity, internal organs and digestive tract of each dissected newt were examined for helminth parasites, which were counted and stored in a solution of 10% glycerol in 70% ethanol. A subset of *Spiroxys* nematodes were excysted live in an acidic pepsin solution. Nematodes were gradually transferred to 100% glycerol by overnight evaporation of excess ethanol and identified according to Anderson et al. (1974) and original descriptions. Trematodes and cestodes were stained overnight in Ehrlich's Hematoxylin Stain, destained in acid alcohol if necessary, and gradually transferred to 100% ethanol by four 30-minute steps in 80, 90, 95 and 100% ethanol, followed by a final step in 100% ethanol. They were then cleared in methyl salicylate, mounted in balsam, and identified according to Schnell (1970), Schmidt (1970) and original descriptions. All macroparasites were recorded as numbers of worms per newt. *Plagitura* individuals were classified as adults or juveniles based on the presence or absence of eggs.

Leukocyte Counts:

Several immune system parameters were obtained from blood cell counts using procedures described by Raffel et al. (*in press-b*). Neutrophils are important phagocytic cells that rapidly respond to infections by a wide variety of parasites, and eosinophils and basophils help defend against larger parasites, such as parasitic helminths, which cannot be engulfed by phagocytes (Janeway et al. 2001). Lymphocytes are the primary cells of the host's adaptive immune response to infection (Janeway et al. 2001), and the effects of temperature on peripheral lymphocyte levels parallel effects on other measures of adaptive immunity, such as antibody responses and the size of the peripheral lymphoid tissues (Cooper et al. 1992). Blood was collected from each newt immediately after

euthanasia with a heparinized capillary tube and 3-5 µL smeared on a glass microscope slide. Slides were air dried for ten minutes, fixed in methanol for five minutes, and again allowed to dry. A variation of a benzidine staining procedure was used to aid differentiation of erythrocytes from other blood cell types (Beug et al. 1982). Slides were placed in 1% o-dianisidine (3,3'-dimethoxybenzidine, Sigma) in methanol for 90 s, destained in 1% hydrogen peroxide in 50% ethanol for 90 s, and rinsed twice in deionized water for 30 s. Slides were then counterstained in Giemsa stain for 30 min. and rinsed again in deionized water for 30 min. Blood cells were counted at 400x magnification starting in the upper left corner of the smear and working down the slide by moving the objective 2 mm between fields and counting all cells in each field until the erythrocyte count reached 5000. Leukocytes were identified as lymphocytes, thrombocytes, neutrophils, eosinophils, basophils or monocytes according to descriptions in Schermer (1967), Ussing and Rosenkilde (1995) and Hadji-Azimi et al. (1987), and were quantified as cells per 5000 erythrocytes. This method of quantifying leukocytes was possible due to high leukocyte/erythrocyte ratios in salamanders.

Stomach Lysozyme Activity:

Lysozyme activity in stomach tissue was assayed as a measurement of innate immunity as described by Raffel et al. (*in press-b*). Lysozyme breaks down bacterial cell walls and provides a first line of defense against bacteria, particularly in the gut mucosa (Lindsay 1986). Newt stomachs were dissected out, cut in half to remove the contents, and rinsed briefly in deionized water. An equivalent mass of phosphate-buffered saline (pH = 7.2) was added to each sample before samples were frozen at -20ºC. Stomachs were ground to a homogenate using a pellet pestle (Kontes Glass Co., Vineland, NJ). Lysozyme activity was assayed using a lysoplate assay originally described by Yousif et al. (1991). 15 µL of sample was put into wells (3.5 mm diameter x 4 mm deep) cut into 0.5% agarose in 100 mm diameter petri dishes. The agarose contained 0.06 M phosphate buffer (pH 6.5), 0.02 M NaCl, and 0.6 mg/mL freeze-dried *Micrococcus leisodeikticus* (Sigma). The diameters of zones of lysis were measured after 20 hr incubation at room temperature and 100% humidity. Activity levels were calculated by comparison with zones of lysis produced by hen egg-white lysozyme (Sigma) at standard concentrations

(5, 10, 15, 25, 50, 100, 250, 500 and 1000 µg/mL), the natural log of which relates linearly to the area of the zone of lysis (data not shown).

Analysis of parasite seasonality:

To determine which parasites underwent net seasonal changes in abundance, generalized linear models were run on data from the 2003 and 2004 surveys for each parasite including all ponds with sufficient data (i.e., at least four newts infected with that parasite from Sp1 2003 to Win 2005). *Trypanosoma diemyctyli*, *Ichthyophonus* sp., and *Candida*-cysts were analyzed using presence/absence data (binomial errors). Nematode larvae within pancreas cysts showed extreme aggregation, suggesting within-host replication, and were also analyzed using presence/absence data. Leech counts were poisson-distributed, and all other parasites fit the negative binomial distribution. Analyses of *Plagitura salamandra* abundance were run using only data for juveniles, which were assumed to be more indicative of recent infection rates. Pond was included as a blocking variable for analyses of parasites found in multiple newt populations, and effects of Year (defined as beginning in early spring) and Year by Pond interactions were tested for all parasites. Snout-vent length was included as a covariate in all models to control for seasonal demographic changes due to juvenile recruitment, as was sex. To confirm that significant seasonal effects were not artifacts of unbalanced designs resulting from seasons during which no data were collected, analyses were run again using only the 2003-2004 survey data (Sp1 2003 to Win 2005) and omitting data from late spring (Appendices 1, 2). Parasites for which the late spring data were crucial to their seasonal patterns (bacterial load and leeches) were instead analyzed using only data from the 2004 survey (Sp1 2004 to Win 2005, Appendices 1, 2).

To determine in which ponds and seasons the seasonal patterns occurred, parasites with significant seasonality in the previous analysis were analyzed separately for each pond (Appendices 3-6). For each pond with significant seasonality, differences between seasons were analyzed using one of the following two multiple comparisons procedures. Whenever possible, data from all years were combined and all possible comparisons between seasons were tested. Parasites with significant Pond by Season interactions, suggesting seasonal patterns differing between years, were analyzed as a time series and

only differences between sequential seasons and sequential minima and maxima were compared to reduce the total number of comparisons made. P-values were adjusted for a false discovery rate of 0.05 for each parasite in each pond using the procedure described by Benjamini and Hochberg (1995). This procedure is less prone to Type II errors than traditional multiple comparisons procedures and results in a family-wise error rate approximating the false discovery rate (Benjamini and Hochberg 1995).

Meta-analysis:

The degree and timing of seasonal dynamics of newt parasites was assessed by meta-analysis of seasonal patterns described in this and other studies of newt parasites. Only datasets including all of the five assigned seasons (winter, early spring, late spring, summer, and autumn) were included in the analysis, and prevalence data from published papers were used to test for seasonal changes when no tests for significance of seasonality were provided by the authors (Appendix A7). Following detection of significant seasonality, the time period of greatest increase in parasite prevalence or abundance was determined for each parasite in each population or year sampled. Next, the season most often showing an increase in parasite abundance in all the years and populations sampled was determined for each parasite, and numbers of parasites showing increases were compared between seasons using a Chi-square goodness of fit test. Parasites showing equally common increases in two seasons were assigned a value of 0.5 for each of these seasons. We defined parasites as consistently seasonal if data were available for at least two replicate years or ponds and the season of greatest increase was never more than one consecutive season away from the season when the parasite most commonly increased.

Testing postulated seasonal drivers:

For each parasite, the prevalence (angularly transformed) or mean log abundance ($\ln[n + 1]$) was calculated for each population at each sampling date, and this was regressed against predictors of parasite abundance in the current and last time step (previous season) using normal errors. Analyses were weighted for sample size (number of hosts sampled). The mean log abundance of newts, fish, larval amphibians and

potential intermediate hosts per dip-net sweep, water temperature and dissolved oxygen, the mean log abundance of leeches per newt, the average newt body size measured by snout-vent length (as a proxy for age, Caetano and Leclair 1996), levels of immune parameters, and current levels of other parasites were considered potential predictors of parasite prevalence or intensity. Since susceptibility is thought to be a function of both temperature and the immune system for amphibians, temperature-dependent immune parameters (lymphocyte, eosinophil and neutrophil counts) were corrected for temperature as described by Raffel et al. (*in press-b*) by generating residuals from a negative binomial generalized linear model with temperature as the only predictor. Since seasonal factors are likely to be highly correlated with each other, only factors thought to be biologically relevant to the dynamics of each parasite were included in this analysis to minimize the chance of including spurious factors. Potential predictors of the dynamics of each parasite are listed in Table 1.1, and additional information regarding the selection of these predictors can be found in the parasite notes (Appendix B).

Model parameters were selected using a forward selection procedure with Pond (but not Season) as a blocking variable in all models, starting with the predictor variable which explained the most variation in parasite abundance. At each subsequent step, the predictor explaining the most additional variation was added to the model until no more variables could be added. Due to the low sample size of ponds and sampling dates available for analysis, $P > 0.1$ was chosen for exclusion of model parameters. To reduce the probability of spurious results, only immune parameters with negative effects on parasite abundance were included in models (positive relationships were assumed to indicate effects of parasites on immune parameters), and effects of other parasites were only included when a significant interaction between those parasites could be demonstrated by addition into the individual-level model listed in Table 1.2 or 1.3. Parameters were also excluded if the direction of the effect was opposite the predicted effect and therefore not likely to be a causal relationship.

Results:

Seasonal patterns of parasite prevalence and intensity:

Out of thirteen parasite taxa examined from the seasonal survey, twelve showed evidence of seasonal changes in prevalence or intensity of infection (Tables 1.2, 1.3, Appendices A1, A2). The one parasite apparently lacking seasonality was *Amphibiocapillaria tritonispunctati* (Table 1.2). Most of these parasites showed evidence of significant increases prevalence or intensity of infection in spring and summer, with two showing increases from winter to early spring (*Candida* cysts and *Trypanosoma diemyctyli*), eight from early to late spring (unidentified metacercariae, *Neoechinorhynchus saginatus*, *Trypanosoma diemytyli*, *Spiroxys contortus*, *Clinostomum* sp., *Ichthyophonus* sp., *Placobdella picta*, and bacterial load), and six from late spring to summer (unidentified nematode larvae, unidentified metacercariae, *Spiroxys contortus*, bacterial load, *Clinostomum* sp., and *Ichthyophonus* sp.) (Fig. 1.1-1.3). Increases in abundance of juvenile *Plagitura salamandra* occurred variably in winter, spring or autumn depending on the year or population sampled (Fig. 1.2). *Brachycoelium* sp. showed different seasonal patterns in different ponds, ranging from no seasonality in Turtle Shell to increases in autumn, winter or early spring (Fig. 1.2). Mothersbaugh had the most consistent seasonal pattern in *Brachycoelium* sp. abundance, with a strong increase in winter followed by a strong decrease in late spring (Fig. 1.2). Findings of significant seasonal patterns of parasitism in both the overall and within-population analyses were robust despite the unbalanced experimental design caused by inclusion of the late spring survey data, which was not collected in 2003 (Tables 1.2-1.3, Appendices A1-A6). For most parasites, levels of parasitism increased during early and late spring, indicating increased transmission during this time period, and decreased during autumn and winter.

Six parasite taxa were detected in this study at levels too low to test for seasonality. These *Cosmocercoides dukae* (Holl 1928, N = 4) from Mothersbaugh and Little Acre, pseudophyllidian cestode larvae (N = 11) encysted in the liver and mesenteries of newts from Mothersbaugh, Turtle Shell and Little Acre, an undescribed *Hysterothylacium* species (Nematoda: Anisakidae, N = 6) from Little Acre, *Bothriocephalus rarus* (Thomas 1937a, N = 8) in Mothersbaugh and Little Acre,

Gorgoderina sp. (Trematoda, N = 2) individuals from Turtle Shell, and *Aegyptianella ranarum* infection (Desser 1987, N = 3) in Mothersbaugh and Turtle Shell.

Metanalysis:

Of twenty-seven parasite taxa included in the meta-analysis of seasonal parasitism in red-spotted newts, twenty-four showed some evidence of seasonality, although three of these showed variable seasonal patterns (*Brachycoelium*, *Plagitura* and *Amphibiocapillaria*, Table 1.4). Of the fifteen parasites for which data were available from two or more replicate ponds and/or years, eleven were consistently seasonal by our criteria (Table 1.4). We found significant differences in the number of parasite taxa most commonly increasing in each season ($X^2 = 15.1$, d.f. = 4, P = 0.0020), with more parasites increasing from early to late spring and from late spring to summer (Table 1.5). Nine of the eleven consistently seasonal parasites increased most commonly between early spring and summer (Table 1.4).

Effects of host density:

Newt density peaked during the early or late spring in all ponds except Mothersbaugh which peaked in autumn (Fig. 1.4d), but had no apparent positive effects on the temporal dynamics of any of the parasites analyzed, either as an individual predictor or in the final models (Table 1.1, Table 1.6). Current newt density had a negative effect on *Ichthyophonus* sp. and *Brachycoelium* sp. (Table 1.6) and was the best individual predictor of *Ichthyophonus* sp. prevalence (Table 1.1, F = 7.1, d.f. = [1, 19], P = 0.0151). No parasite except *Brachycoelium* sp. showed any effect of its own abundance or prevalence in the last time step (abundance last season, Table 1.1), either as an individual predictor or in the final model. The effect on *Brachycoelium* sp. was negative and only significant with metacercariae and lagged temperature as covariates (Table 1.1, Table 1.6).

Current fish density was the best single predictor of *Clinostomum* abundance (F = 55.8, d.f. = [1, 6], P = 0.0003), which excluded all other parameters from the model. However, fish had an apparently positive effect on *Clinostomum* (coefficient = 1.081), opposite the proposed effect, so this relationship was assumed not to be causal and fish

density was removed from the final model (Table 1.6). Lagged fish density also had a positive effect on abundance of unknown metacercariae but only with temperature as a covariate (Table 1.1, Table 1.6). Fish density consistently peaked in summer in Mothersbaugh and Turtle Shell, the only two ponds to contain fish (Fig. 1.4f).

Effects of intermediate host and vector densities:

Clinostomum sp. and *Plagitura salamandra* infection intensity were positively predicted by the densities of their respective *Helisoma* sp. and *Physa* sp. intermediate hosts (Table 1.6). *Helisoma* sp. snails increased in abundance in the spring or early summer (Fig. 1.3b and 1.3d), whereas *Physa* sp. snails, had less consistent seasonal population dynamics (Fig. 1.1c) which coincided with the inconsistently seasonal infection levels of *P. salamandra* (Fig. 1.1b). Odonate larvae, the second intermediate hosts of *P. salamandra*, had no significant effect on *P. salamandra* infection intensity despite consistent spring increases (Table 1.6, Fig. 1.4h). The unidentified trematode metacercariae showed no apparent effects of any potential mollusc intermediate hosts, either as individual predictors or in the final model (Table 1.1, 1.6), despite spring increases in *Helisoma* sp. and fingernail clam (Sphaeriidae) abundance (Fig. 1.3b, 1.4g).

Leeches per newt was the best individual predictor of *Trypanosoma diemyctyli* prevalence (coefficient = 0.964, F = 4.6, d.f. = [1, 25], P = 0.0409) (Table 1.1), but addition of lagged temperature caused it to be removed from the model, leading to the model in Table 1.6. If lagged temperature was excluded from the model, leeches per newt was the only significant predictor of *T. diemyctyli* prevalence. Leeches per newt was also a significant positive predictor of *Ichthyophonus* sp. prevalence (Table 1.6). Leeches peaked in abundance in late spring in all three ponds where leeches were detected (Fig. 1.3).

Effects of other seasonal drivers:

Temperature was a significant predictor of all parasites analyzed except for *Plagitura salamandra* (Table 1.6). Current temperature was a positive predictor of bacterial load, *Clinostomum* sp. and abundance of unknown metacercariae, and a negative predictor of *Candida*-cyst prevalence (Table 1.6). Lagged temperature (temperature in

the previous season) was a negative predictor for abundance of *Brachycoelium* spp., *Ichthyophonus* sp., and unknown metacercariae (Table 1.6). The only parasite showing a positive relationship with lagged temperature was *Trypanosoma diemyctyli* (Table 1.6). Temperature was highest in late spring and summer and lowest in winter and early spring (Fig. 1.4a).

Dissolved oxygen in the previous season added significantly to the model for *Clinostomum* sp. with *Helisoma* sp. abundance and temperature as covariates (coefficient = 0.007, F = 168.2, d.f. = [1, 1], P = 0.0490), but missing data for this variable resulted in an unacceptable loss of sample size (reduction of N = 7 to N = 4). Dissolved oxygen in the previous season was also significant in the final model for the unknown metacercariae but again caused a reduction of sample size (reduction of N = 10 to N = 9) (coefficient = -0.026, F = 12.8, d.f. = [1, 3], P = 0.0374). In neither case did addition of dissolved oxygen in the previous season change the significance of any of the other model parameters. Dissolved oxygen tended to be highest in winter and lowest in summer or autumn but showed no evidence of seasonality in Mothersbaugh, where these two parasites were most abundant (Fig. 1.4b).

Only two parasites showed evidence that seasonal changes in host immune parameters influenced their dynamics. As an individual predictor, lysozyme activity in the previous season had a highly significant negative effect on bacterial load (F = 8.8, d.f. = [1, 18], P = 0.0084) that was still marginally significant when temperature was added to the model (Table 1.6). Eosinophils in the previous season had a significant negative effect on *Trypanosoma diemyctyli* prevalence but only with lagged temperature as a covariate (Table 1.1, 1.6).

Two parasites showed evidence of a negative competitive interaction which could influence the dynamics of one or both parasites. Unknown metacercariae was the strongest individual predictor of *Brachycoelium* spp. abundance (Table 1.1, F = 10.3, d.f. = [1, 31], P = 0.0031) and remained a significant negative predictor in the final model (Table 1.6). *Brachycoelium* spp. was also a significant negative predictor of metacercaria abundance, both as an individual predictor (F = 8.9, d.f. = [1, 15], P = 0.0094) and in the final model (Table 1.6). Unknown metacercariae was also a significant predictor of *Brachycoelium* spp. abundance (X^2 = 25.7, d.f. = 1, P < 0.0001), and *Brachycoelium* spp.

of metacercaria abundance ($X^2 = 8.7$, d.f. = 1, P = 0.0031), when these predictors were added to the original models presented in Table 1.2 using individual newts as the replicate.

Discussion:

Nearly all parasites of red-spotted newts showed evidence of seasonal dynamics, both in this and previous studies of newt parasite dynamics. This held true when snout-vent length and sex were included in models, suggesting that these patterns are due to changes in infection rates rather than seasonal changes in demographic structure (i.e., older newts have had more time to obtain parasites, which could cause lower levels of infection in spring when many of the newts are new recruits). Moreover, the vast majority of these parasites had peak infection rates in the spring and early summer. Notably, most of these parasites infect newts in the aquatic environment. In contrast, the only two parasites which infect newts during terrestrial stages (*Brachycoelium* spp. and *Cosmocercoides dukae* both use land snails as intermediate hosts, Rankin 1945, Fischthal 1955, Anderson 1960, Jackson and Beaudoin 1967) showed high infection rates in autumn. Adult newts often leave ponds during the summer and return in autumn (Petranka 1998), which could help account for an autumn increase in these parasites. The intermediate host and transmission mechanisms for *Fessisentis*, which also had high autumn infection rates, are still unknown (Joy and Thomas 1997, McAlpine 1997).

Since the only characteristic shared by all newt parasites is their shared amphibian host and parasite transmission rates are often assumed to be proportional to host density due to high contact rates (Hudson et al. 2002), high newt densities during the spring breeding season might be hypothesized to drive the observed patterns of high spring infection rates. However, our results suggest that high newt density if anything slows transmission of the parasites analyzed in this study, contradicting this hypothesis. Negative density-dependence is a general prediction of theory for vector-borne diseases, whose transmission rates are predicted to be proportional to the vector-host ratio (Hudson et al. 1995). This explains the negative effect of newt density on *Ichthyophonus* sp. prevalence.

Intermediate host density and vector-host ratio were important drivers of parasites with complex life cycles. As predicted, *Physa* sp. and *Helisoma* sp. snail densities were positive predictors of *Plagitura salamandra* and *Clinostomum* sp. infection levels, respectively, and leeches per newt was a positive predictor of *Ichthyophonus* sp. and *Trypanosoma diemyctyli* prevalences. We suspect that intermediate host abundance may be the primary ecological factor driving seasonality for many newt parasites. The majority of parasites described from red-spotted newts have known or suspected intermediate hosts (Table 1.4), and many of these parasites have seasonal increases coinciding with increases in the population densities of these intermediate hosts. Jarroll (1979) showed that *Bothrocephalus rarus* (Thomas 1937a) has its highest infection rates at the time of peak copepod abundance, and *Spiroxys contortus*, which also uses a copepod intermediate host (Hedrick 1935, Thomas 1937b), showed similar patterns of seasonality in this and other studies (Table 1.4, Fig. 1.1, Rankin 1937). High spring infection rates in *Neoechinorhynchus saginatus* (Fig. 1.1) may be due to high densities of their ostracod intermediate hosts (Ferguson 1944, Uglem and Larson 1969). The spring and summer increases observed by Joy and Pennington (1998) and Russell (1951) in abundance of *Megalodiscus* sp. and *Plagitura parva*, which both use *Helisoma* sp. as intermediate hosts (Stunkard 1936, Williams and Esch 1991, Joy and Pennington 1998), are similar to the patterns observed for *Clinostomum* sp. in this study (Table 1.4, Fig. 1.3).

Our failure to find significant effects of any potential mollusc host on the dynamics of the unidentified trematode metacercariae might reflect a lack of effect of intermediate host density or the presence a nonlinear effect which was not picked up by our analysis. However, the finding of a significant positive effect of fish abundance is highly suggestive. Fish abundance was also the strongest single predictor of *Clinostomum* sp. metacercariae in newts, despite having the opposite effect from what we had predicted. Fish are known second intermediate hosts for *Clinostomum* sp. and possibly also act as alternate hosts for these unknown metacercariae. Peak levels of trematode infection in snails is itself seasonal, may not coincide with peaks in snail density, and can peak at different times depending on the seasonal presence of the definitive host (i.e., migrating brids for many trematodes) and the length of the prepatent

period for a given parasite species (Fernandez and Esch 1991, Williams and Esch 1991, Esch and Fernandez 1994, Sapp and Esch 1994, Schmidt and Fried 1997). Since it would be highly adaptive for the parasites to release cercariae when secondary intermediate host densities are high, perhaps seasonal patterns of infection for these two parasites are controlled in part by the parasites themselves, which could time the release of cercariae to coincide with peak fish densities.

If newts only become infected with *Brachycoelium* spp. on land (Fischthal 1955, Jackson and Beaudoin 1967), seasonality in this parasite must be due either to seasonal immigration of newts from land or to seasonal clearance from the gut. Notably, the negative effect of snout-vent length on individual worm burdens suggests that older newts clear the infections they had obtained as terrestrial juveniles. Temperature in the last time step may correlate with times when newts are likely to be moving into the pond from the terrestrial habitat (autumn for returning adults and early spring for new recruits, Brimley 1921, Morgan and Grierson 1932, Gill 1978b, Harris et al. 1988, Chapter 6). However, the strong negative effect of unidentified metacercariae on *Brachycoelium* abundance both in individual newts and in the overall analysis of hypothesized predictors suggests a competitive interaction between these parasites, probably immune-mediated since they reside in different compartments of the newt's body (the gut and the liver, respectively, Lello et al. 2004). This potential immune-mediated interaction with trematode metacercariae would explain why seasonality was only pronounced in Mothersbaugh, which was also the only pond with high abundance of the trematode metacercariae.

Both bacterial and *Candida* infections both showed evidence of a role for host susceptibility in driving their dynamics. Since length is a good proxy for age in newts (Caetano and Leclair 1996), the strong negative effect of snout-vent length on *Candida* infection in individual newts suggests that older newts may develop immunological memory to infection. Possibly the high prevalence levels observed in early spring are due primarily to the high recruitment rates of new adult newts at this time of year, as shown by the early spring decrease in the average snout-vent length of Mothersbaugh and Little Acre newts (Fig. 1.4), which would increase the proportion of susceptible individuals. This would fit the known biology of *Candida* sp. pathogens, which in

humans are opportunistic pathogens that normally grow on environmental substrates but occasionally infect immunocompromised hosts (Garber 2001). Alternately, *Candida* sp. may infect newts on land, so that infections are only observed in new recruits and returning adults.

Bacterial load in the liver was best predicted by environmental temperature and gut lysozyme levels in the last time step. Lysozyme is a class of enzymes that break down bacterial cell walls, and it plays an important role in the first line of defense against bacterial infection, especially in the gut (Lindsay 1986). Stomach lysozyme activity is highly seasonal in newts and may be influenced by breeding activity, with levels increasing in the summer and gradually decreasing throughout the extended breeding season until the late spring (Raffel et al. *in press-b*). Bacterial load was not apparently influenced by density of newts, amphibian larvae or fish, as would be predicted for directly transmitted parasites. Our results are more consistent with the hypothesis that bacterial parasites of red-spotted newts come primarily from environmental sources, and that high spring infection rates are caused by temperature-induced increases in environmental bacterial levels coupled with low immunity in newts due to breeding.

If sources of common bacterial infections are indeed environmental (i.e., saprophytic growth on decaying organic matter), exposure of amphibians to these pathogens may be largely independent of host density. This independence from host density may make effects of seasonal changes in host susceptibility more detectable than for parasites constrained by density of their definitive and intermediate hosts. Other parasites of amphibians which may grow on environmental substrates include the fungal pathogens *Saprolegnia* and *Batrachochytrium dendrobatidis* (Longcore et al. 1999, Czeczuga et al. 2005), both of which have been linked to amphibian population declines (Kiesecker et al. 2001a, Lips et al. 2006). Additionally, these fungi have low optimal temperatures for growth and high winter infection rates (Bly et al. 1993, Woodhams et al. 2003, Berger et al. 2004). Therefore these pathogens may be more likely than other parasites to show detectable influences of low levels of immunity relative to temperature in autumn, an effect described by Raffel et al. (*in press-b*) in red-spotted newts. A similar effect has been documented as a cause of winter *Saprolegnia* outbreaks in channel catfish (Bly et al. 1993).

The lack of seasonality for *Amphibiocapillaria tritonispunctati* may reflect a true absence of seasonal infection dynamics in adult newts, though perhaps not in the age class of newts being infected. Based on age-intensity relationships, this nematode appears to only infect larval newts (Jackson and Beaudoin 1967). The significant seasonal patterns seen in other studies (Holl 1932, Rankin 1937, Joy and Scott 1997) might have been due to newly recruited newts having different infection levels than older cohorts, as might happen if the force of infection varied between years in these populations.

The predominance of high spring infection rates in newt parasites is probably generalizable to parasites of many temperate amphibian species. Many of the parasites examined in this study are shared with other amphibian species (Rankin 1937, Muzzall 2005), and the intermediate host species important to newt parasites also act as intermediate hosts for other common amphibian parasites (Schmidt and Fried 1997, Johnson et al. 2004). Many temperate amphibian species in North America breed in spring and have shorter breeding seasons than newts (Conant and Collins 1998), which would if anything increase seasonal variation in their use of aquatic habitat and exposure to aquatic parasites. Since most parasitological studies of amphibians focus on a single time point in the year for comparisons between populations (Wetzel and Esch 1996), the potential for high spring infection rates should be considered when designing monitoring strategies for amphibian parasites, many of which may not be detectable if surveys are conducted too early in spring.

Our finding that most newt parasites have similar seasonal dynamics despite having different drivers suggests that a common underlying mechanism causes seasonality in different drivers of infection risk. One seasonal process that could have such an effect is the well-known spring peak in primary productivity that occurs in northern temperate ponds (Horne and Goldman 1994, Porter et al. 1996) (Figure 5). Increases in temperature and day length stimulate growth of phytoplankton and environmental bacteria in the spring (Horne and Goldman 1994, Felip et al. 1996), which have bottom-up effects on many of the factors that drive parasite infection in amphibians. Spring increases in growth of environmental bacteria and fungi in response to increasing temperature and high levels of detritus may lead to increased infection risk to amphibians

from opportunistic parasites (Hoff et al. 1984, Felip et al. 1996). Increases in pond productivitiy leads to increased densities of primary consumers such as zooplankton and snails (Pace 1986, Horne and Goldman 1994, Chase 2003), which in turn can drive increases in amphibian parasites that utilize these invertebrates as intermediate hosts. Fish and amphibians time their breeding activities in large part to ensure that hatchlings have sufficient food, though hydroperiod and terrestrial biology of the adults are also important deteminants of the timing of amphibian breeding (Wilbur 1997, Richardson et al. 1998, Chizinski and Pope 2003), leading to high fish and amphibian densities in spring and summer. The timing of *Placobdella picta* breeding coincides with and may depend upon peak spring densities of their amphibian prey (Gill et al. 1983), leading to high rates of vector-borne parasite transmission. In this way, seasonality in pond productivity could have cascading community-level effects leading to similar seasonal patterns in parasites with very different transmission mechanisms.

Our findings suggest that seasonality is a crucial part of the ecology of most amphibian parasites, at least for temperate amphibian populations, and that pond primary productivity may be the driving force behind much of this seasonality. Therefore, climate disruption and changes in the productivity of ponds due to anthropogenic eutrophication are likely to have substantial effects on the dynamics of amphibian parasites. Furthermore, pathogens like chytridiomycosis that have high infection rates in autumn and winter may be more the exception than the rule. Amphibian species with evolutionary histories of high spring infection rates may be ill-prepared immunologically and behaviorally to deal with pathogens that attack in autumn, which may be part of the reason chytrid fungus has had such an impact when introduced into naïve amphibian populations.

Table 1.1: Variables included in analysis of seasonal drivers of parasite dynamics. All variables except for parasite interactions and snout-vent length were analyzed for effects both in the current and last time step (previous season), as indicated by "(L)". Significant individual predictors of parasite abundance ($P < 0.1$) are indicated by bold or italicized type for current and time-lagged predictors, respectively.

	Brachycoelium	*Plagitura*	*Trypanosoma*	*Ichthyophonus*	*Clinostomum*	*Candida*	Metacercariae	*Bacteria*
Pond	X	X	X	X		X	X	X
Snout-vent length	X	X	X	X	X	X	X	X
Newt density (L)	*X*	X	X	**X***	X	X	*X*	X
Amphibian larvae (L)			X	*X*	X	X	X	X
Fish density (L)					**X***	X	X	X
Leech bite rate (L)			**X***	X				
Helisoma (L)					**X**		X	
Physa (L)		**X***					X	
Clams (L)							X	
All molluscs (L)							X	
Odonates (L)		X						
Temperature (L)	***X***	X	***X***	**X**	***X***	***X***	**X***	**X***
DO (L)	X	**X**	X	X	X	X	X	X
Lymphocytes (L)	X	X	X	X	X	X	X	X
Eosinophils (L)	*X*	X	X	X	X	X	X	X
Neutrophils (L)	*X*	*X*	X	X	X	**X***	X	X
Lysozyme (L)								*X*
Abundance last season**	X	X	X	X	X	X	X	X
Brachycoelium		X	**X**	X	**X**	**X**	**X**	X
Plagitura	X		X	**X**	X	X	X	X
Trypanosoma	**X**	X		**X**	X	X	X	X
Ichthyophonus	X	X	X		**X**	X	X	X
Clinostomum	**X**	X	X	**X**		X	**X**	X
Candida	X	X	X	X	X		X	X
Spiroxys	**X**	X	X	X	X	**X**	**X**	X
Unknown nematodes	X	X	X	**X**	**X**	X	**X**	X
Unknown metacercariae	**X***	X	X	X	X	X		X
Bacteria	X	X	X	X	X	X	X	

*Best individual predictor of parasite abundance or prevalence
**Abundance of target parasite in previous season

Table 1.2: Seasonal effects on the burden or prevalence of parasites detected in **dissected** newts, using **all available data** from populations with sufficient parasite infection levels. Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with $P < 0.05$ were excluded from models. Populations included in each analysis are indicated in parentheses.

Response	Predictor	Coef.	Δ Dev	d.f.	P
Spiroxys[1]	Pond		515.5	3	< 0.0001
N = 342	Snout-vent length	0.169	31.1	1	< 0.0001
(LA, MB, MN, TS)	**Season**		25.0	4	**< 0.0001**
	Pond:Season		29.0	11	**0.0023**
Amphibiocapillaria[1]	Pond		189.6	3	< 0.0001
N = 338	Snout-vent length	0.048	5.3	1	0.0214
(LA, MB, MN, TS)	Sex (M = 1, F = 0)	-0.208	12.4	1	0.0004
Brachycoelium[1]	Pond		23.7	3	< 0.0001
N = 343	Year		19.2	2	< 0.0001
(LA, MB, MN, TS)	Snout-vent length	-0.225	18.2	1	< 0.0001
	Season		41.4	4	**< 0.0001**
	Pond:Year		16.9	6	0.0095
	Pond:Season		29.5	11	**0.0019**
Plagitura[1]	Pond		67.3	2	< 0.0001
N = 276	Year		12.6	2	0.0018
(LA, MB, TS)	Snout-vent length	0.149	6.5	1	0.0108
	Season		9.3	4	**0.0543**
	Year:Pond		45.3	4	< 0.0001
	Season:Year		8.0	3	**0.0469**
	Pond:Season		44.0	8	**< 0.0001**
	Season:Year:Pond		32.6	3	**< 0.0001**
Trypanosoma[2]	Pond		32.0	1	< 0.0001
N = 190	**Season**		6.9	4	**0.1431**
(MB, TS)	**Pond:Season**		14.8	4	**0.0052**
Metacercariae[1]	Pond		93.7	1	< 0.0001
N = 182	Year		1.9	2	0.3951
(MB, TS)	Snout-vent length	0.046	4.0	1	0.0453
	Season		23.6	4	**< 0.0001**
	Pond:Year		8.2	2	0.0167
	Pond:SV length		13.2	1	0.0003
Neoechinorhynchus[1]	Year		6.8	2	0.0336
N = 115; (MB)	**Season**		23.0	4	**0.0001**
Spirurid cysts[2]	Snout-vent length	0.191	4.6	1	0.0314
N = 115; (MB)	**Season**		10.8	4	**0.0288**
Bacterial load[1]	Pond		6.7	3	0.0831
N = 342	Year		17.9	2	0.0001
(LA, MB, MN, TS)	**Season**		63.9	4	**< 0.0001**
	Pond:Year		28.7	6	< 0.0001
	Pond:Season		72.1	11	**< 0.0001**
	Year:Season		6.7	3	**0.0808**
	Pond:Year:Season		16.3	6	**0.0123**

[1]Negative binomial error distribution
[2]Binomial error distribution

Table 1.3: Seasonal effects on the burden or prevalence of parasites detected in **all observed** newts, including those collected for dissection, using **all available data** from populations with sufficient parasite infection levels. Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with $P < 0.05$ were excluded from models. Populations included in each analysis are indicated in parentheses.

Response	Predictor	Coef.	Δ Dev	d.f.	P
Ichthyophonus[2]	Pond		15.7	2	0.0004
N = 2440	Year		8.1	2	0.0174
(LA, MB, TS)	Snout-vent length	0.189	24.7	1	< 0.0001
	Season		8.3	4	**0.0805**
	Pond:Season		18.2	8	**0.0197**
	Year:Season		10.0	3	**0.0186**
Clinostomum[1]	Year		21.5	2	< 0.0001
N = 1860	Snout-vent length	0.089	5.1	1	0.0233
(MB)	**Season**		52.4	4	**< 0.0001**
	Year:Season		8.0	3	**0.0471**
Candida[2]	Pond		19.8	1	< 0.0001
N = 2105	Year		14.3	2	0.0008
(LA, MB)	Snout-vent length	-0.514	25.5	1	< 0.0001
	Season		20.9	4	**0.0003**
Placobdella[3]	Pond		283.7	2	< 0.0001
N = 5751	Year		65.4	3	< 0.0001
(LA, MB, TS)	**Season**		174.3	4	**< 0.0001**
	Year:Pond		16.0	4	0.0031

[1]Negative binomial error distribution
[2]Binomial error distribution
[3]Poisson error distribution

Table 1.4: Seasonal patterns of parasitism in red-spotted newts. The season immediately following the greatest increase in abundance or prevalence of each parasite (e.g., "Summer" indicates an increase from late spring to summer) is listed for all populations and years with sufficient data, followed in parentheses by the number of times this pattern has been observed. Multiple seasons (separated by "/") may be indicated if it could not be determined during which season the greatest increase occurred. The italicized season is when each parasite was most commonly observed to increase. Bold type indicates patterns observed in this study, and "NS" indicates lack of statistical significance in our analysis despite additional evidence for seasonal dynamics presented in the original study.

Parasite species	Seasonal Patterns	Intermediate host
Brachycoelium sp.	***Winter* (2)**; **Autumn(1)**; **Early Spring(1)**; **Not Seasonal (1,** 1[f]**)**	Land snails
Megalodiscus sp.	Late Spring/*Summer* (1[c]), Early Spring/*Summer* (1[f])	*Helisoma*
Pseudopisthodiscus americanus	*Not seasonal* (1[a])	unknown
Plagitura salamandra	***Autumn*/Winter (1)**; *Autumn* (2[a, f]), **Winter/Early Spring (1)**, **Early Spring (1)**	*Physa*/*Lymnaea* and
Plagitura parva	*Early Spring* (1[f])	*Helisoma* and Odonata
Clinostomum* sp. metacercariae	***Summer* (2); Late Spring (1)**	*Helisoma*
Unknown metacercariae*	***Late Spring* (2); Summer (1)**	unknown
Amphibiocapillaria tritonispunctati	**Not seasonal (6)**; *Summer* (1[e]); Autumn(1[a]); Late Spring/*Summer* (1[d])	Unknown
Cosmocercoides dukae	*Summer/Autumn* (1[a])	Land snails
Nematoda sp. encysted in pancreas*	***Late Spring*/*Summer* (2)**	Unknown
Philometra sp.	*Not seasonal* (1[a])	unknown
Spiroxys contortus*	***Summer* (2)**; **Late Spring (2)**; **Early Spring (1)**; *Summer*-NS (1[e])	Copepoda
Bothriocephalus rarus	*Summer*-NS (1[b])	Copepoda
*Fessisentis sp.**	*Autumn* (2[a, g])	Unknown
Neoechinorhynchus saginatus*	***Late Spring* (2)**	Ostracoda
Candida*-cysts	***Early Spring* (1); Winter/*Early Spring* (1)**	Environmental source?
Ichthyophonus*	***Late Spring* (1); Early/*Late Spring* (1); Summer (1)**	*Placobdella picta*
Cytamoeba bacterifera	*Late Spring* (1[e])	*Placobdella picta*?
Trypanosoma diemyctyli*	***Late Spring* (2); Early/*Late Spring* (1);** Summer (1[h]); Early Spring (1[e])	*Placobdella picta*
Placobdella picta*	***Late Spring* (5)**	Free-living
Bacterial load*	***Late Spring* (2); *Late Spring*/Summer (1); Summer (1)**	Environmental source?
Eutrichomastix batrachorum	*Late Spring* (1[e])	Direct transmission?
Hexamastix batrachorum	*Late Spring* (1[e])	Direct transmission
Hexamitus spp.	*Early/Late Spring* (1[e])	Direct transmission?
Karotomorpha swezi	*Late Spring* (1[e])	Direct transmission
Prowazekella longifilus	*Not seasonal* (1[e])	Direct transmission
Tritrichomonas augusta	*Summer* (1[e])	Direct transmission

[a]Holl 1932, [b]Jarroll 1979, [c]Joy & Pennington 1998, [d]Joy & Scott 1997, [e]Rankin 1937, [f]Russell 1951, [g]Joy & Thomas 1997, [h]Mock & Gill 1984

*Parasites with consistent seasonal patterns as defined in Methods

Table 1.5: Season(s) when each parasite most commonly increased. Parasites with equal numbers of observations in two seasons were assigned a value of 0.5 for each season (in parentheses), and total numbers of parasites were compared between seasons using a Chi-square goodness-of-fit test. Only parasites for which significant seasonal effects have been observed are included in this analysis.

Season	Parasites	Total
Early Spring	*Candida* (1), *Hexamitus* (0.5), *Plagitura parva* (1)	2.5
Late Spring	Bacterial load (1), *Cytamoeba* (1), *Eutrichomastix* (1), *Hexamastix* (1), *Hexamitus* (0.5), *Ichthyophonus* (1), *Karatomorpha* (1), *Neoechinorhynchus* (1), *Placobdella* (1), *Trypanosoma* (1), Unknown metacercariae (1), Unknown pancreas-nematodes (0.5)	11
Summer	*Amphibiocapillaria* (1), *Bothriocephalus* (1), *Clinostomum* (1), *Cosmocercoides* (1), *Megalodiscus* (1), *Spiroxys* (1), *Tritrichomonas* (1), Unknown pancreas-nematodes (0.5)	7.5
Autumn	*Plagitura salamandra* (1), *Cosmocercoides* (0.5), *Fessisentis* (1)	2.5
Winter	*Brachycoelium* (1)	1

Table 1.6: Effects of postulated seasonal drivers on parasite dynamics. Pond was included in all analyses as a blocking variable, and predictors with P < 0.1 were excluded from models. Populations included in each analysis are indicated in parentheses.

Response	Predictor	Coef.	F	d.f.	P
Bacterial load	Pond		1.2	3, 17	0.3518
(LA, MB, MN, TS)	Temperature*	0.043	6.5	1, 17	0.0208
	Lysozyme-L	-0.006	3.1	1, 17	0.0977
Brachycoelium	Pond		14.6	3, 10	0.0006
(LA, MB, MN, TS)	Metacercariae*	-0.580	5.4	1, 10	0.0432
	Temperature-L	-0.027	25.0	1, 10	0.0005
	Brachicoelium-L	-0.551	9.1	1, 10	0.0129
	Newt density	-0.464	3.8	1, 10	0.0787
Candida	Pond		4.7	1, 17	0.0439
(MB, LA)	Temperature	-0.003	4.3	1, 17	0.0541
Clinostomum	Temperature	0.006	8.9	1, 5	0.0308
(MB)	*Helisoma*	0.519	10.8	1, 5	0.0219
Ichthyophonus	Pond		25.7	2, 8	0.0003
(LA, MB, TS)	Newt density*	-0.120	22.3	1, 8	0.0015
	Leeches	0.821	18.6	1, 8	0.0026
	Temperature-L	0.003	11.0	1, 8	0.0105
Metacercariae	Pond		195.9	1, 5	< 0.0001
(MB, TS)	Temperature*	0.046	57.4	1, 5	0.0006
	Fish density-L	5.338	35.0	1, 5	0.0020
	Brachycoelium	-1.023	27.8	1, 5	0.0033
	Temperature-L	-0.025	7.5	1, 5	0.0407
Plagitura[1]	Pond		1.4	2, 19	0.2797
(LA, MB, TS)	Physid*	1.732	20.5	1, 19	0.0002
Trypanosoma	Pond		12.4	2, 15	0.0007
(MB, TS, LA)	Temperature-L	0.017	9.9	1, 15	0.0066
	Eosinophils-L	-0.217	5.9	1, 15	0.0284

*Best individual predictor of parasite abundance or prevalence

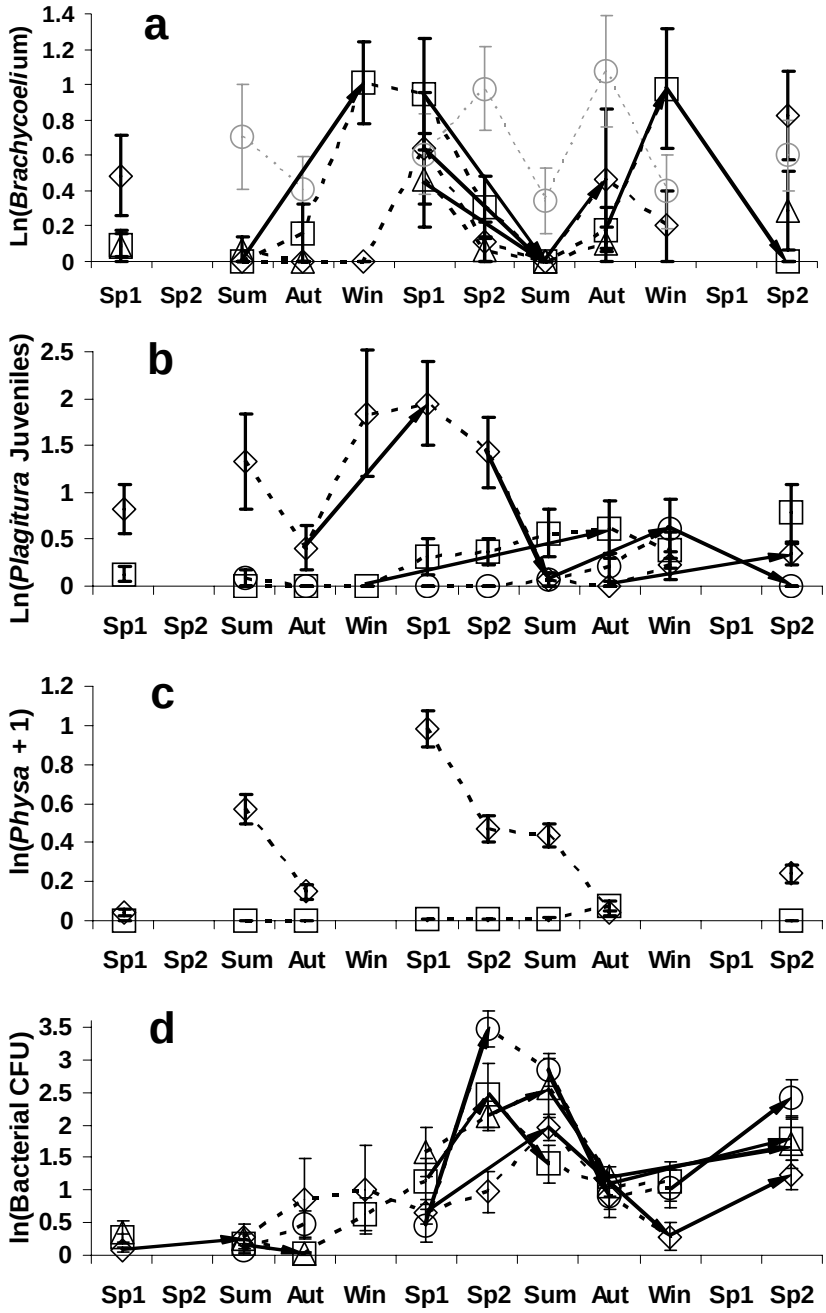

Figure 1.1. Seasonal patterns from early spring 2003 to late spring 2005 for parasites and one intermediate host with variable seasonal dynamics (significant year by season interactions): a. *Brachycoelium* sp., b. *Plagitura salamandra* juveniles, c. *Physa* sp. snails, and d. bacterial load (colony forming units). No samples were obtained in late spring 2003 or early spring 2005. Different symbols represent different populations of newts (△ Mystery Newt, ○ Turtle Shell, ◇ Little Acre, and □ Mothersbaugh), and error bars represent the standard error (standard error of a proportion for presence/absence data). All count data are represented by ln[N + 1]. Populations with significant differences between seasons have black symbols and lines; other populations' data are in gray. Significant increases and decreases are indicated by arrows.

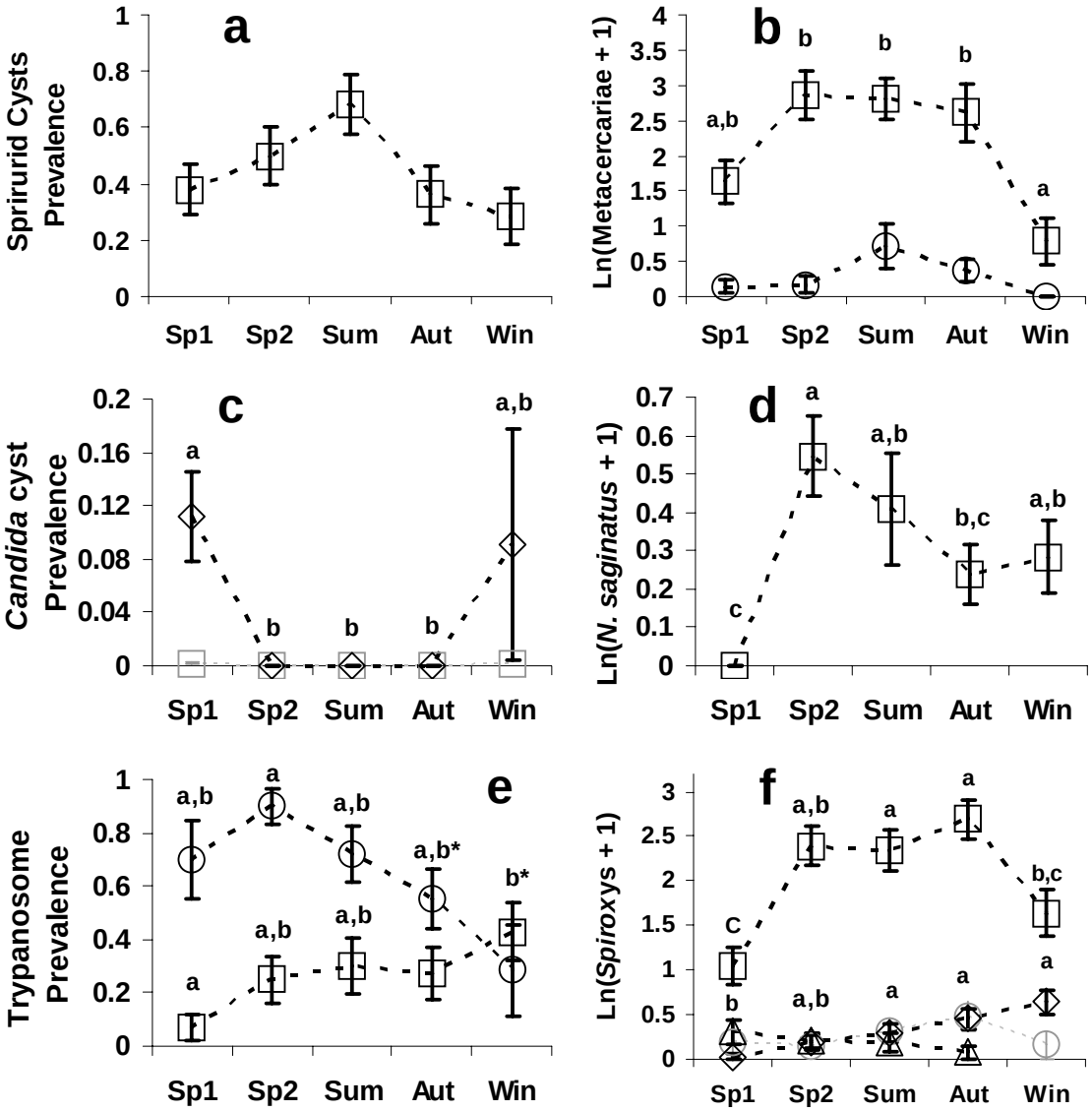

Figure 1.2. Seasonal patterns for parasites with consistent seasonal patterns (no significant year:season interactions): a. unidentified nematode larvae, b. unidentified trematode metacercariae, c. *Candida* cysts, d. *Neoechinorhynchus saginatus*, e. *Trypanosoma diemyctyli* and f. *Spiroxys contortus* larvae. Data were pooled from two years of sampling for each season. Different symbols represent different populations of newts (△ Mystery Newt, ○ Turtle Shell, ◇ Little Acre, and □ Mothersbaugh), and error bars represent the standard error (standard error of a proportion for presence/absence data). Populations with significant differences between seasons have black symbols and lines; other populations' data are in gray. All count data are represented by ln[N + 1]. Seasons with the same letters were not significantly different from each other in a multiple comparisons test (only done for ponds with overall significant seasonality). Starred letters apply to more than one pond.

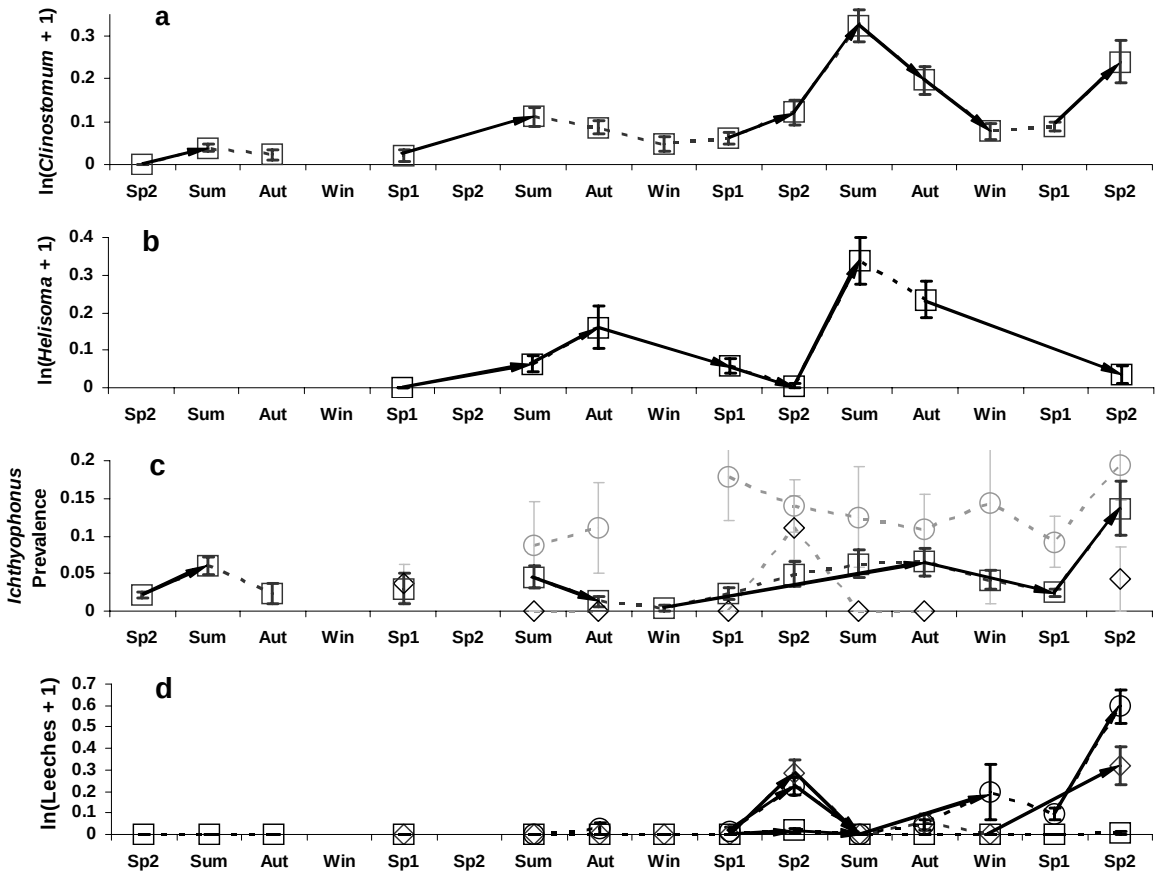

Figure 1.3. Seasonal patterns for parasites and intermediate hosts with variable seasonal dynamics (significant year:season interactions) and data available from late spring 2002 to late spring 2005: a. *Clinostomum* sp., b. *Helisoma* sp. (planorbid snails), c. *Ichthyophonus* sp., and d. *Placobdella picta* (the amphibian leech). No samples were obtained in winter or late spring 2003 or in early spring 2005. Different symbols represent different populations of newts (△ Mystery Newt, ○ Turtle Shell, ◇ Little Acre, and □ Mothersbaugh), and error bars represent the standard error (standard error of a proportion for presence/absence data). All count data are represented by ln[N + 1]. Populations with significant differences between seasons have black symbols and lines; other populations' data are in gray. Significant increases and decreases are indicated by arrows.

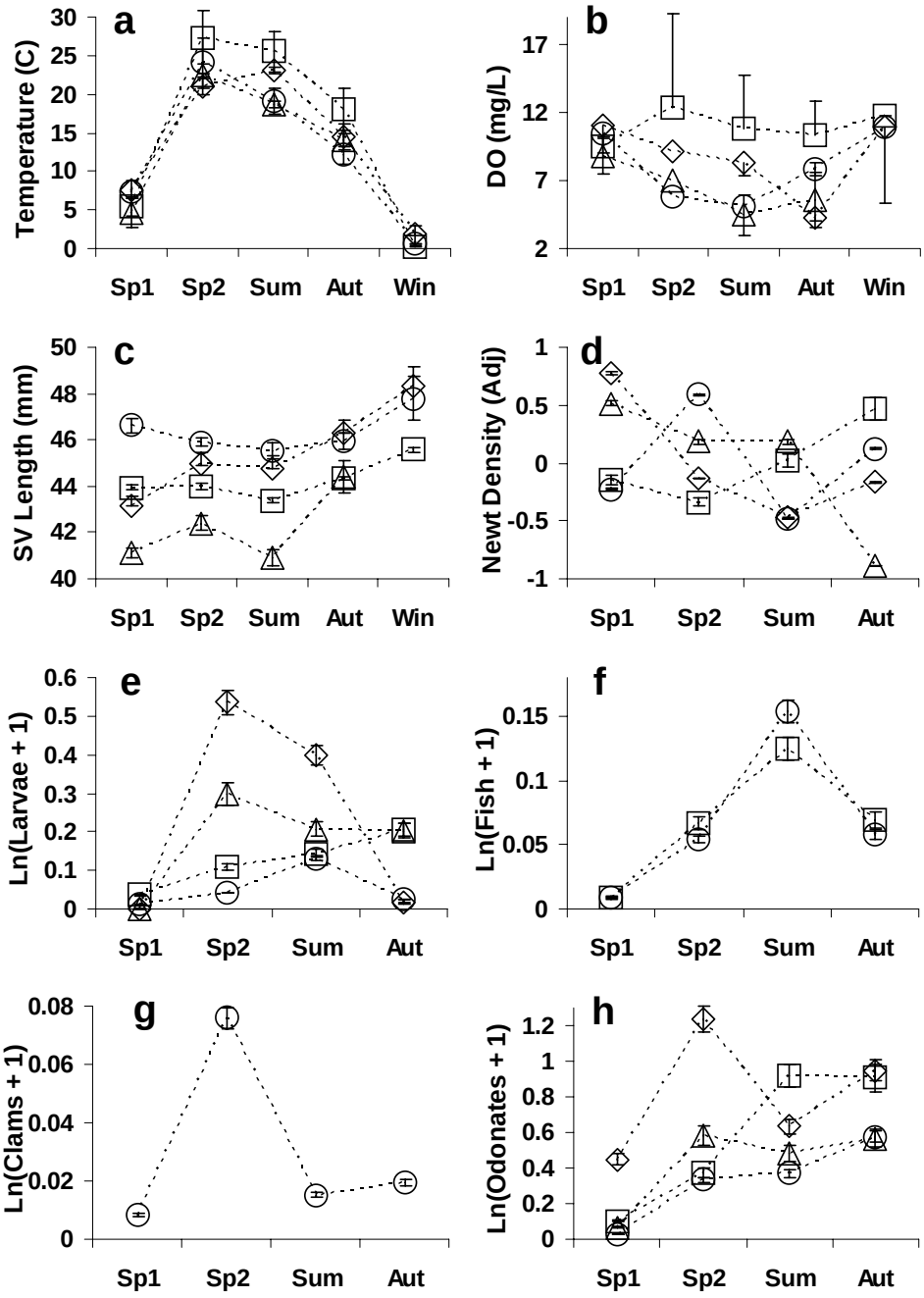

Figure 1.4. Seasonal patterns of postulated drivers of parasite dynamics: (a) temperature, (b) dissolved oxygen, (c) newt snout-vent length, (d) newt population density, (e) pooled density of all larval amphibians, (f) pooled density of all fish species, (g) fingernail clam density, and (h) pooled density of all odonate (dragonfly and damselfly) larvae. Newt density values were adjusted for mean density in each pond ([density-mean density]/mean density) to clarify seasonal patterns, due to major differences in newt density between ponds. Different symbols represent different populations of newts (△ Mystery Newt, ○ Turtle Shell, ◇ Little Acre, and □ Mothersbaugh), and error bars represent the standard error (standard error of a proportion for presence/absence data). All count data are represented by the mean ln[N + 1].

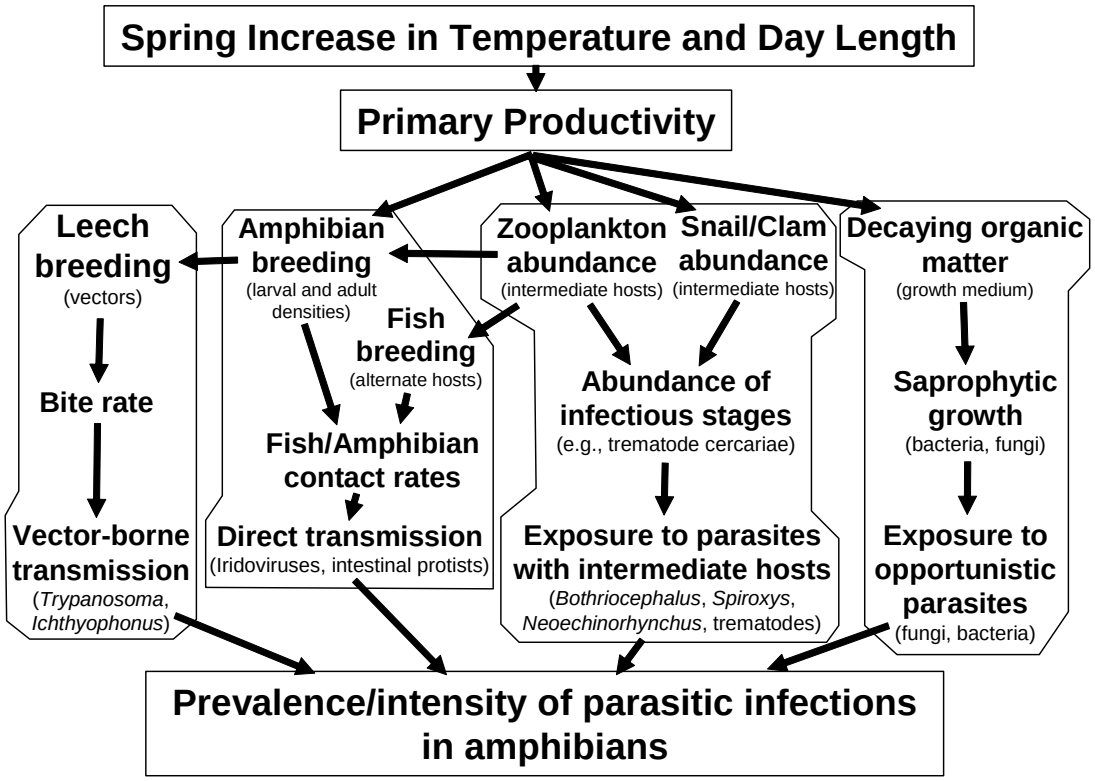

Figure 1.5: Proposed effects of pond productivity on parasite infection rates in amphibians. All arrows indicate positive effects. Increases in primary productivity due to increased temperature and day length may drive seasonal patterns of zooplankton and snail population densities, the timing of amphibian and fish breeding, and the growth of saprophytic organisms, in addition to direct effects of temperature on growth and development. These changes would drive high spring infection rates in amphibians by increasing availability of intermediate hosts for parasites with complex life cycles, transmission rates of directly transmitted parasites, and environmental levels of opportunistic parasites. Timing of leech breeding to coincide with peak amphibian density would lead to high spring transmission rates of vector-borne parasites.

CHAPTER 2:

Hysterothylacium burtti sp. nov. from red-spotted newts (*Notophthalmus viridescens*) in central Pennsylvania

T. R. Raffel[1] and T. K. Anderson[2]

[1]Center for Infectious Disease Dynamics, Penn State University

[2] Program in Ecology & Evolution, Rutgers University, New Brunswick, NJ

Abstract:

Helminth parasites of the anisakid genus *Hysterothylacium* Ward & Magath, 1917 (Nematoda: Anisakidae) have previously only been reported from marine and freshwater fishes. Here we describe a new species that infects red-spotted newts (*Notophthalmus viridescens*), a North American amphibian species with fully aquatic adults. The absence of fish in the ponds from which these specimens were obtained suggests that newts are the normal definitive host for this species. We speculate that this species may have diverged from *Hysterothylacium* species that infect fishes which often live in close proximity with newts.

Introduction:

The anisakid genus *Hysterothylacium* Ward & Magath, 1917 currently includes sixty-two species worldwide, all from marine, brackish or freshwater fishes (Bruce et al. 1994, Gopar-Merino et al. 2005). Three species of *Hysterothylacium* have been reported from freshwater fishes in North America: *H. brachyurum* Ward & Magath, 1917 from North American fishes (Ward and Magath 1917, Rye and Baker 1984), *H. dollfusi* in *Polyodon spathula* from Lake Sakajawea, North Dakota (Schmidt et al. 1974), and *H. analarum* Rye & Baker, 1984 in pumpkinseed sunfish *Lepomis gibbosus* from Canada and the USA (Rye and Baker 1984). No specimens of this genus have previously been described from an amphibian host, although larvae of *Contracaecum*, a similar genus, have been reported from four amphibian species (McAllister and McDaniel 1992, Kuperman et al. 2004). During a study of amphibian parasite ecology in central Pennsylvania, nine adult specimens of a previously unknown ascaridoid nematode species with characteristics of *Hysterothylacium* were obtained from the digestive tracts of red-spotted newts (*Notophthalmus viridescens*). This new species is described below.

Materials and Methods:

As part of a larger study of newt parasite ecology, 105 newts were collected and dissected from Little Acre (95 newts; N 40° 48' 5.8", W 77° 56' 36.5") and Greenbriar 1 (10 newts; N 40° 46' 41.3", W 78° 0' 27.4") ponds, both landlocked and fishless woodland ponds in the Scotia Barrens (PA State Game Lands #176), Centre County,

Pennsylvania. Little Acre newts were collected from March 2003 to June 2005; Greenbriar1 newts were all collected on 5/27/2004. Newts were euthanized within three hours of capture, and intestines and intestinal contents were fixed and preserved in 70% ethanol (30% water) until further dissections could be performed. Upon removal from the digestive tract, worms were transferred to 10% glycerol in 70% ethanol. Worms were cleared for study in 100% glycerol by evaporating the ethanol out overnight. Measurements were made using a light microscope.

Description:

Hysterothylacium burtti n. sp. (Fig. 2.1)

Medium-sized worms. Anterior end with 3 equal-sized labia, length similar to width. Interlabia medium-sized, each composed primarily of two hook-like protrusions and approximately half the length of the labia. Dorsal labium with 2 lateral double papillae. Subventral labia each with lateral papillae, 1 double and 1 single. Esophagus cylindrical, 8.8-14.6% body length. Ventricular appendix and caecum usually similar in length, but either can be longer than the other (ratio appendix:caecum: 0.63-2.52). Lateral alae along entire length of body but reduced towards anterior of the worm. Cuticle striated but lacking annulations. Nerve ring located one fifth down the esophagus. Single excretory pore immediately posterior to the nerve ring. Tail tip smooth, lacking spines or protuberances.

Male (Based on one mature specimen):

Body 12.0 mm long, maximum width 0.69 at second third of body. Head 130 μm long, 155 μm wide at base. Length of esophagus 1.40 mm, representing 11.7% of body length. Nerve ring and excretory pore 310 μm and 610 μm from base of head, respectively. Ventricular appendix 800 μm long, caecum 620 μm long (ratio 1:0.78). Spicules subequal, length 330 and 390 μm. Tail length 110 μm, tail tip lacking spines and protuberances. Ten pairs of precloacal caudal papillae; no adcloacal or postcloacal papillae located. Phasmid not located.

Female (based on eight mature specimens, holotype in parentheses):
Body 9.5-19.8 (11.7) mm long, maximum width 0.50-1.00 (0.85) mm at second third of body. Head 113-203 (165) µm long, 140-210 (193) µm wide at base. Length of esophagus 1.10-1.84 (1.29) mm, representing 8.8-14.6% (12.9%) of body length. Nerve ring and excretory pore 240-320 (280) µm and 260-520 (400) µm from base of head, respectively. Ventricular appendix 290-940 (920) µm long, caecum 540-730 (580) µm, with ratio 0.40-1.59 (1.59). Tail length 110-290 (245) µm; tail tip cylindrical with no spines or protuberances. Vulva position at 27.4-38.1% (27.4%) of body length. Uterus 3.8-4.9 (4.3) mm long. Eggs 43-68 (45) µm long and 50-80 (50) µm wide.

Discussion:

The new species possesses morphological characteristics of the genus *Hysterothylacium* despite its use of an amphibian host, something not otherwise known in this genus (Gopar-Merino et al. 2005). This nematode's triradiate head morphology, prominent labia and cylindrical esophagus place it in the order Ascaridida (Chabaud 1974), and the presence of interlabia coupled with both an intestinal caecum and an appendix limits the possible genera to *Contracacum*, *Hysterothylacium*, *Iheringascaris* or *Maricostula* (Hartwich 1974, Bruce and Cannon 1989). The unilateral excretory pore at the level of the nerve ring and relatively small interlabia are characteristic of *Hysterothylacium* and distinguish this worm from *Contracaecum* (Anderson 2000). The lack of cuticular annulations and well-defined posterior borders to the labia and interlabia distinguish it from *Iheringascaris* (Deardorff and Overstreet 1980). The lack of annulations and relatively short caecum relative to the esophagus (not always >50% length of esophagus) distinguish this species from the genus *Maricostula* (Bruce and Cannon 1989). The location of these worms in the stomach or between the stomach and duodenum is unusual for ascarid nematodes in general but not uncommon in the genus *Hysterothylacium* (Anderson 2000).

Only three species of *Hysterothylacium* have previously been reported from freshwater fish hosts in North America. The head morphology and relative dimensions of *H. burtti* closely resemble *H. analarum*, a parasite found in pumpkinseed sunfish (Rye

and Baker 1984). However, the presence of lateral alae, lack of tail tip spines, smaller body length (9.5-19.8 vs. 19.8-25.6 mm), shorter spicules (390 vs. 450-625 μm) and short caecum relative to the appendix (1:0.4–1.6 vs. 1:0.33) distinguish this species from *H. analarum* (Rye and Baker 1984). *H. burtti* can be distinguished from *H. brachyurum*, a common parasite of North American freshwater fishes, by smaller lateral alae in the anterior third of the body and lack of tail tip spines (Rye and Baker 1984). It can be distinguished from *H. dollfusi*, the only other *Hysterothylacium* found in N. American freshwater fishes, by its shorter spicule length (0.39 vs. 1.07-1.45), smaller size (9.5-9.8 vs. 45-65) and shorter ventricular appendix (0.29-0.94 vs. 4.5-6.0) (Schmidt et al. 1974).

The absence of minute spinous structures on the tail tip distinguish *H. burtti* from all but five of the 22 described *Hysterothylacium* species from North and South America and Hawaii, one of which is *H. dollfusi* (Gopar-Merino et al. 2005). *H. burtti* can be distinguished from *H. eurycheilum* by its smaller size (9.5–19.8 vs. 26.2–41 mm), larger ratio of head length to width (1:0.77–0.99 vs. 1:1.5–1.7), and shorter spicules (0.33–0.39 vs. 0.86) (Deardorff and Overstreet 1981). *H. burtti* can be distinguished from *H. ogcocephali* by its wider head, smaller length (9.5–19.8 vs. 25.5–48.1) and larger ratio of cecum to appendix length (1:0.4–1.6 vs. 1:2.7–7.3), from *H. incurvum* by the narrower shape of its interlabia, shorter spicule length (0.33–0.39 vs. 2.6-8.7) and smaller spicule length relative to body length (2.7–3.3% vs. 12–25%), and from *H. corrugatum* by its shorter total length (9.5-19.8 vs. 26-142), shorter spicule length (0.33-0.39 vs. 1.2-1.6) and the position of its nerve ring between the anterior 17-26% of the esophagus (6-12% in *H. corrugatum*)(Deardorff and Overstreet 1980)

The presence of a *Hysterothylacium* species in an amphibian is unusual, given that no worm of this genus, or indeed adult worms of the related genus *Contracaecum*, have ever been found in an amphibian host (Kuperman et al. 2004, Gopar-Merino et al. 2005). Moreover, these specimens were obtained from newts living in fishless ponds, reducing the probability that these worms represents spillover from conspecific fish and suggesting that newts are the normal definitive hosts for this parasite species. The presence of a *Hysterothylacium* species in red-spotted newts may be due to the close ecological association of newts with freshwater fishes during its recent evolutionary history. Red-spotted newts have mostly aquatic adults and often live in close proximity

to freshwater fishes, especially sunfish which share a similar niche and compete with newts for resources (Petranka 1998, Rohr and Raffel *in prep*). *Hysterothylacium* species typically use invertebrates as intermediate hosts which must then be ingested for infection of the definitive host to occur (Anderson 2000). Since sunfish and newts and share a variety of invertebrate prey species (Rohr and Raffel *in prep*), it seems plausible that an anisakid nematode parasite of freshwater fish such as *H. analarum* or *H. brachyurum* (Rye and Baker 1984) might spill over to newts and eventually diverge into a new species.

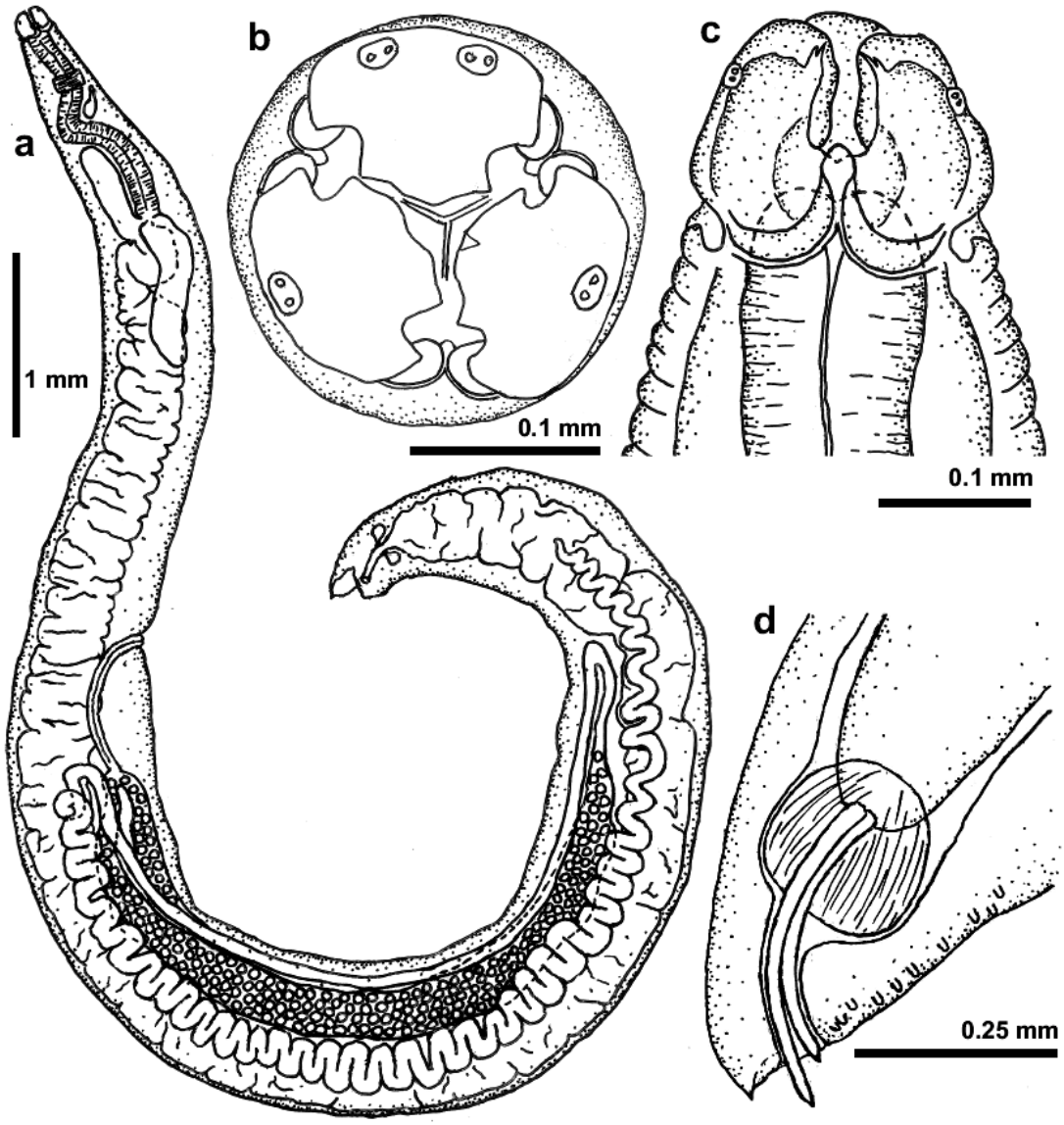

Figure 2.1: Line drawings of *Hysterothylacium burtti*, including (a) a lateral view of the holotype specimen, (b) an *en face* view of a female paratype, (c) a ventral view of the head of a female paratype, and (d) the posterior end of the male showing caudal papillae and spicules, lateral view.

CHAPTER 3:

Negative effects of changing temperature on amphibian immunity under field conditions

T. R. Raffel, J. R. Rohr, J. M. Kiesecker and P. J. Hudson

[1]Center for Infectious Disease Dynamics, Penn State University
[2]The Nature Conservancy, Wyoming Chapter, Lander, WY

Functional Ecology, in press

Abstract:

Recent evidence of the role of emerging diseases in amphibian population declines makes it increasingly important to understand how environmental changes affect amphibian immune systems. Temperature-dependent immunity may be particularly important to amphibian disease dynamics, especially in temperate regions. Changes in temperature are expected to cause deviations away from optimal levels of immunity until the immune system can respond. To test whether temperature changes cause deviations from optimal immunity under natural conditions, we conducted a seasonal survey of adult red-spotted newts and measured basal levels of several immunological variables. We then examined these findings in relation to: 1. the lag hypothesis, which predicts that changes in temperature-dependent immune parameters lag behind short-term temperature changes and 2. the seasonal acclimation hypothesis, which predicts that immune cell production declines during long-term temperature decreases until amphibians can fully acclimate to winter conditions. Our results supported both hypotheses, showing a spring lag effect on lymphocyte levels and an even stronger seasonal acclimation effect on lymphocytes, neutrophils and eosinophils in the autumn. Our findings suggest that temperature variability causes increased susceptibility of amphibians to infection, and they have implications for the emergence of disease and the potential for climate change to exacerbate amphibian decline.

Introduction:

One of the major questions in ecology is how environmental factors influence the dynamics of parasitism and disease in natural populations. A case in point is that environmental factors have been implicated in the emergence of new and more severe amphibian diseases (Kiesecker 2002, Blaustein et al. 2003), which in turn may be contributing to worldwide declines of amphibian populations (Daszak et al. 1999, Daszak et al. 2003, Kiesecker et al. 2004, Lips et al. 2006). In particular, changing climatic conditions have been suggested to be important for amphibian declines in both tropical and temperate regions (Pounds et al. 1999, Alexander and Eischeid 2001, Kiesecker et al. 2001a, Rohr and Madison 2003, Daszak et al. 2005), and increased infection risk due to warming trends has recently been implicated in the extinction of many tropical frog

species (Pounds et al. 2006). Environmental temperature has strong effects on the amphibian immune system and may be an important factor influencing susceptibility of amphibians to emerging pathogens (Maniero and Carey 1997, Carey et al. 1999, Rojas et al. 2005). Effects of temperature on susceptibility have been implicated in outbreaks of chytrid fungus infection, which causes higher mortality at lower temperatures (Woodhams et al. 2003, Berger et al. 2004).

Some components of the amphibian immune system are reduced during periods of low temperature (Maniero and Carey 1997), which may be an adaptive response to decreased infection risk during the winter. Host susceptibility is a function of both the strength of the immune response and the intrinsic growth rate of the parasite, an effect often ignored in mammals whose bodies remain at a constant temperature throughout the year (but see Prendergast et al. 2002). Unlike mammals, amphibians undergo major seasonal changes in body temperature, which should cause predictably slower pathogen growth rates within the body when the temperature is low (Ratkowsky et al. 1982). Even a reduced immune system may be sufficient to deal with pathogens in the seasonally cold environments experienced by temperate amphibians (Schmid 1982, Plytycz and Bigaj 1983, Wojtowicz and Plytycz 1997). Since the immune system is costly to maintain (Bonneaud et al. 2003, Ksiazek et al. 2003), temperature-dependent immunity could be an adaptive mechanism for ectotherms to save energy during the winter. Results from experimental studies suggest that cold-acclimated fish and amphibians up-regulate immune cell and protein production rates during the winter to counteract the direct effects of temperature on metabolic rate, implying that amphibians could maintain these immune parameters at higher levels in winter if that was adaptive (Bly and Clem 1991, Plytycz and Jozkowicz 1994). We therefore assume that amphibians regulate immune parameters to different levels at different temperatures to optimize fitness, due to a trade-off between the cost of immunity and temperature-dependent growth rates of amphibian parasites (we will refer to this as the optimal level of immunity for a given temperature).

However, not all amphibian immune parameters respond to temperature in the same way. Lymphocytes, eosinophils and complement activity remain at low basal levels at low temperatures even in winter-acclimated amphibians (held at 4°C for at least three weeks), despite the ability of ectotherms to up-regulate lymphocyte production in winter

(Green and Cohen 1977, Bly and Clem 1991, Maniero and Carey 1997). This observation suggests these immune parameters have temperature-dependent optimal levels, so we will refer to these as "temperature dependent" immune parameters. Neutrophils and phagocytic activity initially decrease when temperature drops but are brought back to high levels once amphibians acclimate to the lower temperature, suggesting temperature-independent optima for these immune parameters (Plytycz and Jozkowicz 1994, Maniero and Carey 1997).

When temperatures vary, maintaining optimal immune status may not be possible. Based on the results of laboratory studies, we have formulated two hypotheses for how temperature changes should influence the amphibian immune system, which we will call the "lag effect" and the "seasonal acclimation effect". **The lag effect is a hypothesized delay in the adjustment of immune parameters to their new optimal levels following a rapid temperature change, due to the length of time it takes to produce or remove a given immune cell or protein.** During periods of increasing temperature, the length of this delay should be limited by the length of time necessary to produce a given immune cell or protein (e.g., 7-11 days for development of stem cells into mature leukocytes, Bell and Hughes 1997). In support of this hypothesis, Maniero and Carey (1997) found that it took 7-9 days for complement activity in frogs to increase to its new level following an abrupt temperature increase. During periods of decreasing temperature, this delay should be determined by the rate of removal of immune cells or proteins from the blood (e.g., half-life of 3-8 hours for eosinophils, basophils and neutrophils and 3-8 weeks for lymphocytes, Bell and Hughes 1997, DeSantis and Strauss 1997, Janeway et al. 2001).

The seasonal acclimation effect is a hypothesized change in the rate of cell or protein production (i.e., number of cells or proteins produced per day) above or below optimal rates immediately following seasonal temperature increases or decreases, respectively, due to slow acclimation of amphibians to seasonal temperature extremes. Note that even if each of these cells still takes 7-11 days to complete development as with the lag effect, the rate of production will be higher if more cells are going through that process at any given moment. Cold-acclimated fish and amphibians maintain higher levels of phagocytic activity, antibody production and lymphocyte numbers at cold temperatures than warm-acclimated control animals recently

moved to cold temperatures (Bly and Clem 1991, Plytycz and Jozkowicz 1994). Conversely, cold-acclimated fish and amphibians moved to warm temperatures produce similar or slightly elevated levels of macrophage activity compared to warm-acclimated control animals (Plytycz and Jozkowicz 1994). These results suggest that ectotherms adjust metabolic pathways during the winter to accelerate the production of immune cells and proteins, particularly at low temperatures. Bly and Clem (1991) found that it takes four to six weeks for fish lymphocytes and antibody activity to return to stable levels following a rapid drop in temperature, suggesting this type of acclimation probably occurs only during long-term seasonal changes in temperature.

Although each of these effects involves a time delay and a type of acclimation, they are caused by different mechanisms acting on different time scales and predict different responses to temperature changes. The lag hypothesis predicts lower than optimal levels of temperature-dependent immune parameters following short-term (8-14 days) temperature increases and higher than optimal levels following short-term (1-2 days for eosinophils and neutrophils) temperature decreases (Fig. 3.1a). The seasonal acclimation hypothesis predicts lower than optimal levels of immune parameters relative to temperature following long-term (30-60 days) seasonal temperature decreases and slightly elevated levels following long-term temperature increases (Fig. 3.1b). The latter hypothesis applies to any immune cell or protein whose production rates are influenced by seasonal acclimation, including and perhaps especially those which are maintained at high levels during the winter (i.e., temperature-independent by our definition). These hypotheses are not mutually exclusive, and both may influence levels of immune parameters following temperature changes. The fine-scale adjustments to short-term temperature changes relevant to the lag effect might be considered analogous to changing speeds within gears in an automobile, whereas seasonal acclimation would be analogous to shifting gears, since different metabolic processes appear to be at work in the winter than in the summer.

Despite numerous laboratory studies of fish and amphibian immunity, the lack of published field data makes it difficult to assess the importance of temperature to amphibian immunity under complex natural conditions, such as seasonal cues which might allow amphibians to anticipate temperature changes (Delgado et al. 1992). The

goal of this study was to track seasonal changes in the immune system of free-living adult amphibians in order to address the following questions: (1) how do patterns of temperature-dependent immunity in wild amphibians compare to laboratory results, (2) do amphibians experience seasonal variation in immunity above and below temperature-dependent optima, and (3) is this variation consistent with the lag and seasonal acclimation hypotheses? We chose the red-spotted newt (*Notophothalmus viridescens*) as a model organism because adult newts are active in ponds throughout the year (Petranka 1998), allowing sampling from the same habitat in all seasons, and have a variety of responses that are strongly temperature- and season-dependent (Rohr et al. 2002, 2003). We used basal levels of peripheral neutrophils, eosinophils, basophils and lymphocytes, as well as stomach lysozyme activity, as measures of immune status.

Methods:

Survey:

Five ponds known to support newt populations were chosen in and around Centre County to represent a variety of adult newt habitats. Mothersbaugh (N 40º 39' 12", W 77º 54' 9") is a flat-bottom beaver pond in the Penn State Experimental Forest which has decreased considerably in size and depth since the beavers left several years ago. Turtle Shell Pond (40º 52' 26", W 78º 4' 36") is one of several beaver ponds along a stream system in Moshannon State Forest. Mystery Newt Pond (40º 45' 53", 78º 0' 49") and Twin Pond (N 40º 46' 49", W 78º 0' 14") are semi-permanent (drying some winters) landlocked woodland ponds in the Scotia Barrens area (PA State Game Lands #176), and Little Acre (N 40º 48' 6", W 77º 56' 37") is a landlocked permanent pond also located in the Scotia Barrens. Seasonal surveys were conducted in 2003 and 2004 in the spring (March-April), summer (July-August), autumn (September-November) and winter (January-February). A late spring (May-June) survey was added in 2004 and replicated in 2005 following observations of major differences in newt ecology between early and late spring.

Newt Collection:

At each sampling time point, dip nets were used to collect approximately ten newts per pond for blood collection and dissection (402 total newts). During the winter survey, newts were collected by drilling holes in the ice and setting minnow traps on the bottom of the pond overnight. Newts could not be obtained from Twin Pond in the autumn and winter or from Mystery Newt Pond during the winter. Similar numbers of male and female newts were collected when possible, leading to approximately constant male:female sex ratios across seasons except in winter (early spring 67:33, late spring 58:46, summer 53:35, autumn 45:26, winter 33:6). The strong male bias observed in winter may have been due to a higher trapping success of males, which have higher activity levels than females (Rohr et al. 2003). These male:female ratios reflect natural male biased sex ratios in newts (Harris et al. 1988, Rohr et al. 2002, 2003). For each pond at each time point, we recorded water temperature at a depth of 10 cm below the water surface using a YSI Model 95 meter (YSI Incorporated, Yellow Springs, OH). Newts were transported to the lab in 250 mL Nalgene containers filled with pond water, anesthetized with a drop of Oragel® on the head, and euthanized by decapitation within 3 hours of collection to minimize effects of transportation stress on the newt immune system.

Leukocyte Counts:

Several immune system parameters were obtained from blood cell counts. Neutrophils are important phagocytic cells that rapidly respond to infections by a wide variety of parasites, and eosinophils and basophils help defend against larger parasites, such as parasitic helminths, which cannot be engulfed by phagocytes (Janeway et al. 2001). Lymphocytes are the primary cells of the host's adaptive immune response to infection (Janeway et al. 2001), and the effects of temperature on peripheral lymphocyte levels parallel effects on other measures of adaptive immunity, such as antibody responses and the size of the peripheral lymphoid tissues (Cooper et al. 1992). Blood was collected from each newt immediately after euthanasia with a heparinized capillary tube and 3-5 μL smeared on a glass microscope slide. Slides were air dried for ten minutes, fixed in methanol for five minutes, and again allowed to dry. A variation of a

benzidine staining procedure was used to aid differentiation of erythrocytes from other blood cell types (Beug et al. 1982). Slides were placed in 1% o-dianisidine (3,3'-dimethoxybenzidine, Sigma, St. Louis, MO) in methanol for 90 s, destained in 1% hydrogen peroxide in 50% ethanol for 90 s, and rinsed twice in deionized water for 30 s. Slides were then counterstained in Giemsa stain for 30 min. and rinsed again in deionized water for 30 min. Blood cells were counted at 400x magnification starting in the upper left corner of the smear and working down the slide by moving the objective 2 mm between fields and counting all cells in each field until the erythrocyte count reached 5000. Leukocytes were identified as lymphocytes, thrombocytes, neutrophils, eosinophils, basophils or monocytes according to descriptions in Schermer (1967), Ussing and Rosenkilde (1995) and Hadji-Azimi et al. (1987), and were quantified as cells per 5000 erythrocytes. This method of quantifying leukocytes was possible due to high leukocyte/erythrocyte ratios in salamanders.

Stomach Lysozyme Activity:

Lysozyme activity in stomach tissue was assayed as a measurement of innate immunity. Lysozyme breaks down bacterial cell walls and provides a first line of defense against bacteria, particularly in the gut mucosa (Lindsay 1986). Newt stomachs were removed, cut in half to remove the contents, and rinsed briefly in deionized water. An equivalent mass of phosphate-buffered saline (pH = 7.2) was added to each sample before samples were frozen at -20ºC. Stomachs were ground to a homogenate using a pellet pestle (Kontes Glass Co., Vineland, NJ). Lysozyme activity was assayed using a lysoplate assay described by Yousif et al. (1991), and 15 µL of sample was put into wells (3.5 mm diameter x 4 mm deep) cut into 0.5% agarose in 100 mm diameter petri dishes. The agarose contained 0.06 M phosphate buffer (pH 6.5), 0.02 M NaCl, and 0.6 mg/mL freeze-dried *Micrococcus leisodeikticus* (Sigma). The diameters of zones of lysis were measured after 20 hr incubation at room temperature and 100% humidity. Activity levels were calculated by comparison with zones of lysis produced by hen egg-white lysozyme (Sigma) at standard concentrations (5, 10, 15, 25, 50, 100, 250, 500 and 1000 µg/mL), the natural log of which relates linearly to the area of the zone of lysis (data not shown).

Reconstruction of Pond Temperature Profiles:

Temperature data were collected from all five ponds every two hours from March 7 to June 1 in the spring of 2005, using temperature dataloggers (HOBO, Onset, Pocasset, MA) set at a depth of 20 cm to ensure they would remain submerged as water levels fluctuated. Hourly air temperature data for State College were obtained from a database maintained by the PA State Climatologist website (Bahrmann and Ayers 2005) and used to calculate average daily temperatures for the past three years. Average daily air temperature was correlated with average daily temperatures of each pond, providing that the presence of ice was taken into account ($r = 0.56$-0.75 for all ponds when free of ice; $r = 0.35$-0.55 for iced-over ponds with flowing water; temperature approximately constant for iced-over ponds with no water flow). The relationships between each pond's spring 2005 temperatures and air temperature, in addition to observations of the pond melting times in 2003 and 2004, were used to reconstruct estimated temperature profiles for each of the ponds from 2003 to 2005.

Statistical Analyses:

Generalized linear models were used for all analyses. All blood count data were found to fit the negative binomial error distribution, and lysozyme activity fit the gamma distribution. Between-population differences were not a focus of this study, so collection site ("Pond") was included in all models as a blocking variable.

To determine which immune parameters had temperature-dependent optima, the effects of temperature on immune parameters were first analyzed using Pond as a blocking variable. Temperature varied greatly by season, so to determine temperature-independent effects of season on immune parameters, temperature was included as a covariate and year and pond were included as blocking variables. Because data from late spring were recorded in 2004 and 2005 while data from all other seasons were recorded in 2003 and 2004, data from the late spring surveys were left out of this analysis to avoid an unbalanced design. To test for temperature-independent differences between individual seasons, residuals were calculated from models including pond and temperature and analyzed with multiple comparisons tests. Temperature was excluded

from analyses of seasonal effects for immune parameters not found to be temperature-dependent in the first analysis (i.e., basophils and lysozyme).

To test the lag and acclimation effect hypotheses, immune parameters with significant main effects of temperature were analyzed for evidence of effects of temperature change. Since different effects were expected depending on whether newts were acclimated to winter or summer temperatures, data were divided between warm-acclimated (Summer and Autumn) and cold-acclimated (Winter and Spring) newts for this analysis. The average rates of temperature change (°C/Day) over the previous 8, 14, 30 and 60 days were calculated for each sampling date in each pond using estimated average daily temperatures from the reconstructed pond temperature profiles. These time-scales were chosen to reflect the time-scales hypothesized to be important for the lag and acclimation effects (lag effect 1-2 weeks, acclimation effect 1-2 months). To control for the main effect of temperature on immune parameters, residuals were calculated from models including pond and temperature as predictor variables. These residuals, which represent deviations away from temperature-dependent optima, were regressed against rates of temperature change using normal errors. A backward selection procedure was used to determine which time scale(s) of temperature change were the best predictors of variation in immune parameters of cold-adapted and warm-adapted newts. Since four scales of temperature change were being compared simultaneously, Bonferroni-adjusted P-values were used to exclude model parameters (i.e., $P < 0.0125$ to include in model).

Results:

There were strong effects of temperature on circulating lymphocytes and eosinophils (Tables 1 and 2, Figs 2A and 2B). Neutrophils had a significant negative between-season relationship with temperature (Table 3.1, Fig. 3.2C). Neither basophils ($X^2 < 0.01$, d.f. = 1, $P = 0.955$) nor lysozyme activity ($X^2 = 0.01$, d.f. = 1, $P = 0.926$) showed significant effects of temperature.

Significant seasonal effects were still detected for lymphocytes, eosinophils, neutrophils and lysozyme activity after the direct effect of temperature had been removed (Table 3.2, Fig. 3.2). Basophils showed no significant seasonal effects ($X^2 = 7.35$, d.f. =

3, P = 0.061). Lymphocytes fell below expected levels in the autumn (i.e., lower than could be accounted for by temperature alone) and returned to higher-than-expected levels in the winter, a pattern which was consistent between years (Fig. 3.3A). Lymphocytes were also lower than expected in early spring, especially in 2003, and showed a similar pattern in late spring 2005 (Fig. 3.3A). Eosinophils had lower-than-expected levels in early spring and autumn of 2003 but showed no apparent seasonal pattern in 2004, leading to a significant year-by-season interaction (Fig. 3.3A, Table 3.2). Neutrophils decreased below expected levels in the autumn, especially in 2003, followed by an increase in the winter (Fig. 3.3C). Lysozyme activity followed a different seasonal pattern, with a strong increase in the middle of summer in both years followed by a gradual decrease during the rest of the year to very low levels in late spring (Fig. 3.3D).

For analyses examining how temperature change influenced immunity, only the 14 day time scale was significant for tests of the lag effect and only the 60 day time scale was significant for tests of the seasonal acclimation effect (Table 3.3), time scales that are consistent with the predictions for each hypothesis. Cold-acclimated newts, which were predicted to experience a lag but not an acclimation effect, showed a significant lag in lymphocyte production in response to short-term temperature changes. Numbers of lymphocytes, but no other immune parameters, were greater-than-expected with temperature declines and less-than-expected with temperature increases (Table 3.3, Fig. 3.4A). Warm-acclimated newts, which were predicted to experience an acclimation effect during seasonal temperature decreases and a lag effect during short-term temperature changes, exhibited only a strong acclimation effect for lymphocytes, neutrophils and eosinophils (Table 3.3, Fig. 3.4B-C). Newts had significantly lower-than-expected numbers of these cells if temperatures, on average, had been declining over the past 60 days. This effect accounted for some of the between-year and between-season variability in immune parameters for warm-acclimated newts (Fig. 3.4B-C), and was larger in magnitude than the lag effect on lymphocytes (as shown by larger coefficients for these models, Table 3.3).

Discussion:

The effects of temperature on immune parameters of wild newts were highly consistent with results from laboratory studies on anuran amphibians. The strong positive between-season temperature-dependence of circulating eosinophils and lymphocytes, the weak negative between-season relationship between temperature and neutrophil counts, and the lack of temperature-dependence in basophils were all consistent with the findings of Maniero and Carey (1997) in their laboratory study of Leopard frog immunity. These similarities suggest that overall effects of temperature on the amphibian immune system are robust to experimental conditions and may be generalized across amphibian taxonomic groups. The cause of the strong temperature-independent seasonal patterns for lysozyme activity remains unresolved.

Circulating lymphocyte levels showed patterns consistent with the lag effect hypothesis in the spring. As predicted, cold-acclimated newts had lower-than-expected lymphocyte levels following rapid, short-term (14-day) increases in temperature, which helps account for the lower-than-expected levels observed in early spring 2003 and late spring 2005. Despite low eosinophil levels in early spring 2003, the temperature-change analysis did not provide evidence of a spring lag effect for this immune parameter.

Lymphocytes, eosinophils and neutrophils all showed patterns consistent with predictions of the seasonal acclimation hypothesis. All three immune parameters fell below expected levels in the autumn, except in 2004 when eosinophils remained at expected levels. As predicted, these decreases could be accounted for by the rate of temperature decrease over the last 60 days, but not for shorter time scales. The seasonal acclimation effect in autumn appears to be greater in magnitude than the spring lag effect and probably affects newts for a larger proportion of the year due to the long time-scale over which it operates. This may lead to a period in the autumn during which amphibians predictably experience increased susceptibility to parasites and pathogens.

The absence of a seasonal acclimation effect in spring is unsurprising, given that cold-acclimated fish and amphibians produce similar levels of immune parameters at warm temperatures compared to warm-acclimated control animals (Plytycz and Jozkowicz 1994). The absence of a detectable reverse lag effect in autumn may be due to rapid turnover rates of most immune cells (Bell and Hughes 1997), which should

therefore closely match cell production rates as temperature decreases. However, lymphocytes have relatively long half-lives (3-8 weeks, Janeway et al. 2001) and might have been expected to show a detectable reverse lag effect in autumn. The apparent dominance of the seasonal acclimation effect in our results may be due to very slow acclimation of newts to winter conditions, as indicated by the long time-scale (60 days) of the effect we observed. Alternately, high levels of parasite antigens in newts may increase the proportion of rapidly cycling lymphocytes due to increased activation and removal of these cells (Tough and Sprent 1995).

The unfortunate need to use a different sampling technique during winter poses a problem for interpreting our results due to the potential effects of trapping stress. Handling stress causes a substantial decrease in the number of circulating lymphocytes in the hours following mist-net capture of wild birds (Davis 2005), and amphibians have been shown to experience elevated levels of stress hormones following prolonged capture stress (Coddington and Cree 1995). However, increased stress due to trapping is unlikely to have caused the patterns we observed. Acute stress generally leads to short-term decreases in circulating lymphocytes in amphibians (Maule and VanderKooi 1999), opposite the winter effect observed in this study. We know too little about context-dependent stress responses in amphibians to rule out the possibility that newts respond to handling stress differently in different seasons, but this effect is unlikely to have caused the seasonal patterns observed in this study. Amphibians have a slower glucocorticoid response to handling stress than do birds (3-12 hr. vs. 5-15 min., Coddington and Cree 1995, Romero and Romero 2002), making immune parameters unlikely to have substantially changed within the three-hour interval between capture and blood collection.

The lower than expected levels of circulating immune cells in the autumn could be attributed to low parasite abundance, breeding, seasonal changes in sex ratios of sampled newts, or stress due to high population density (Zerani and Gobbetti 1993, Rollins-Smith 2001, Kortet et al. 2003), but the seasonal acclimation effect seems like the parsimonious explanation for this pattern. Most parasites of red-spotted newts which have been found to have seasonal patterns infect them through the spring and summer, leading to high prevalence in summer and autumn and low in winter and early spring

(Holl 1932, Rankin 1937, Jarroll 1979, Joy and Pennington 1998). Newts would be predicted to have high levels of immune parameters in autumn (similar to those in summer) if seasonal patterns reflected a response to current infection. Similarly, densities of adult newts in ponds peak in the spring, dip to very low levels in the summer and increase again only slightly in the autumn (Gage 1891, Harris et al. 1988, Pers. obs. T. R. Raffel), offering a potential alternative explanation for low immunity in the spring but not in the autumn. Although breeding may influence newt immune parameters, breeding seems unlikely to have caused the observed seasonal patterns of immunity. The newt breeding season starts in the autumn and continues through winter to the following late spring (Gage 1891, Harris 1987, Rohr et al. 2002, Pers. obs. T.R. Raffel), predicting low immunity in the winter as well as in the autumn and spring. Likewise, seasonal differences in the sex ratio of sampled newts cannot explain the observed patterns, since winter was the only season when sex ratio was substantially different. Although a combination of these factors cannot be entirely ruled out, they fail to explain our results as well as the seasonal acclimation hypothesis does. Due to the presence of confounding variables in our study, experimental studies will be necessary to confirm whether the lag and seasonal acclimation effects are sufficient to explain the patterns we have observed.

The effects of short-term lags in immunity on infection rates have only been tested with a small number of parasites. Maniero and Carey (1997) found that Leopard frogs took 7-9 days to increase complement activity to expected levels following an abrupt temperature increase, but found no effect of increasing temperature on susceptibility to *Aeromonas* infection. Similarly, Jackson and Tinsley (2002) were unable to detect a lag effect of increasing temperature on amphibian susceptibility to infection by a monogenean parasite.

Effects of cold acclimation to the ectothermic immune system have been best studied in fish. Lymphocytes had much higher peripheral blood counts, proliferation potential and antibody responses at cold temperatures when fish were cold-acclimated, and it took between four and six weeks for warm-acclimated fish to raise immunity to the same levels as cold-acclimated fish following an abrupt temperature decrease (Bly and Clem 1991). This appears to be an important cause of outbreaks of the fungal disease saprolegniosis on fish farms, which often follow rapid drops in temperature (Bly et al.

1993). Cold-acclimation of the immune system has been less extensively studied in amphibians; however, Plytycz and Jozkowicz (1994) found that macrophages from cold-acclimated fish and amphibians had higher activity levels than macrophages from warm-acclimated amphibians when assayed at cold temperatures. Jackson and Tinsley (2002) found that frogs were more susceptible to helminth infection after temperature was lowered than when temperature was held constant or increased, a result consistent with the acclimation effect hypothesis. Further studies are needed to determine the length of time needed for amphibians to acclimate their immune systems to winter conditions, the magnitude of the acclimation effect in the absence of confounding variables, and whether or not other parasites show increased infectivity following temperature decreases.

Our results have implications for how temperature changes might affect disease dynamics in amphibians. Although the decrease in immunity during autumn may not strongly influence infection rates of parasites which peak in the spring and summer, there may be an impact on the ability of newts to clear these parasites, which have often built up to high levels by the end of summer (Holl 1932, Rankin 1937, Jarroll 1979, Joy and Pennington 1998). The lag and acclimation effects may also lead to outbreaks following unusual climatic events, or to the evolution of parasite life history strategies taking advantage of predictable periods of increased susceptibility. In addition, populations of amphibians having few cold-tolerant resident parasites might invest relatively little energy in immunity during colder seasons, making them more susceptible to invasion by parasites like chytrid fungus which grow well at low temperatures (Berger et al. 2004). Finally, the increased variability in climatic conditions predicted by some climate change scenarios might lead to longer or more frequent periods of immune suppression in amphibians, which could exacerbate amphibian declines (Hegerl et al. 2004, Schar et al. 2004).

Table 3.1. Regression statistics describing generalized linear models for the between-season effects of temperature on immune parameters (blocked by Pond). X^2 = change in deviance when predictor removed from full model.

Immune Parameter	Source of Variation	Coefficient	df	X^2	P
Lymphocytes	Pond		4	16.3	0.0027
	Temp*	**0.053**	**1**	**263.1**	**<0.0001**
Eosinophils	Pond		4	51.0	<0.0001
	Temp*	**0.116**	**1**	**241.9**	**<0.0001**
Neutrophils	Pond		4	34.1	<0.0001
	Temp*	**-0.012**	**1**	**6.5**	**0.0107**

* See Fig. 3.2 for plotted data

Table 3.2. Regression statistics describing minimal generalized linear models for effects of season and temperature on immune parameters (blocked by Pond). Only lysozyme showed a significant main effect of year, but eosinophils showed a significant year by season interaction. X^2 = change in deviance for each predictor when removed from the full model.

Immune Parameter	Source of Variation	df	X^2	P
Lymphocytes	Pond	4	11.2	0.0242
	Temp	**1**	**5.8**	**0.0157**
	Season*	**3**	**50.0**	**<0.0001**
Eosinophils	Pond	4	27.3	<0.0001
	Year	1	0.7	0.4115
	Temp	**1**	**6.6**	**0.0104**
	Season*	**3**	**10.0**	**0.0187**
	Year:Season*	3	26.2	<0.0001
Neutrophils	Pond	4	16.6	0.0024
	Temp	**1**	**5.8**	**0.3505**
	Season*	**3**	**40.9**	**<0.0001**
Lysozyme	Pond	4	7.2	0.0922
	Year	1	6.1	0.0054
	Season*	**3**	**24.6**	**<0.0001**

* See Fig. 3.3 for plotted data

Table 3.3. Regression statistics describing minimal models for the effects of temperature changes on deviation of immune parameters from their temperature-dependent optimal values (residuals from models described in Table 3.1). Residuals for cold-acclimated newts (sampled in winter or spring) and warm-acclimated newts (sampled in summer or autumn) were analyzed separately. Only a single time-scale of temperature change (8, 14, 30 or 60 days) was a significant predictor for any of the analyses.

Immune Parameter	Temp-change time scale‡	Coefficient	df	F	P
Cold-Acclimated Newts:					
Lymphocytes	14 Days*	-0.636	1	7.4	0.0070
Warm-Acclimated Newts:					
Lymphocytes	60 Days*	5.941	1	38.5	0.0000
Eosinophils	60 Days*	5.079	1	20.2	0.0000
Neutrophils	60 Days*	3.189	1	11.8	0.0007

‡ Number of days over which the rate of temperature change was estimated.

* See Fig. 3.4 for plotted data

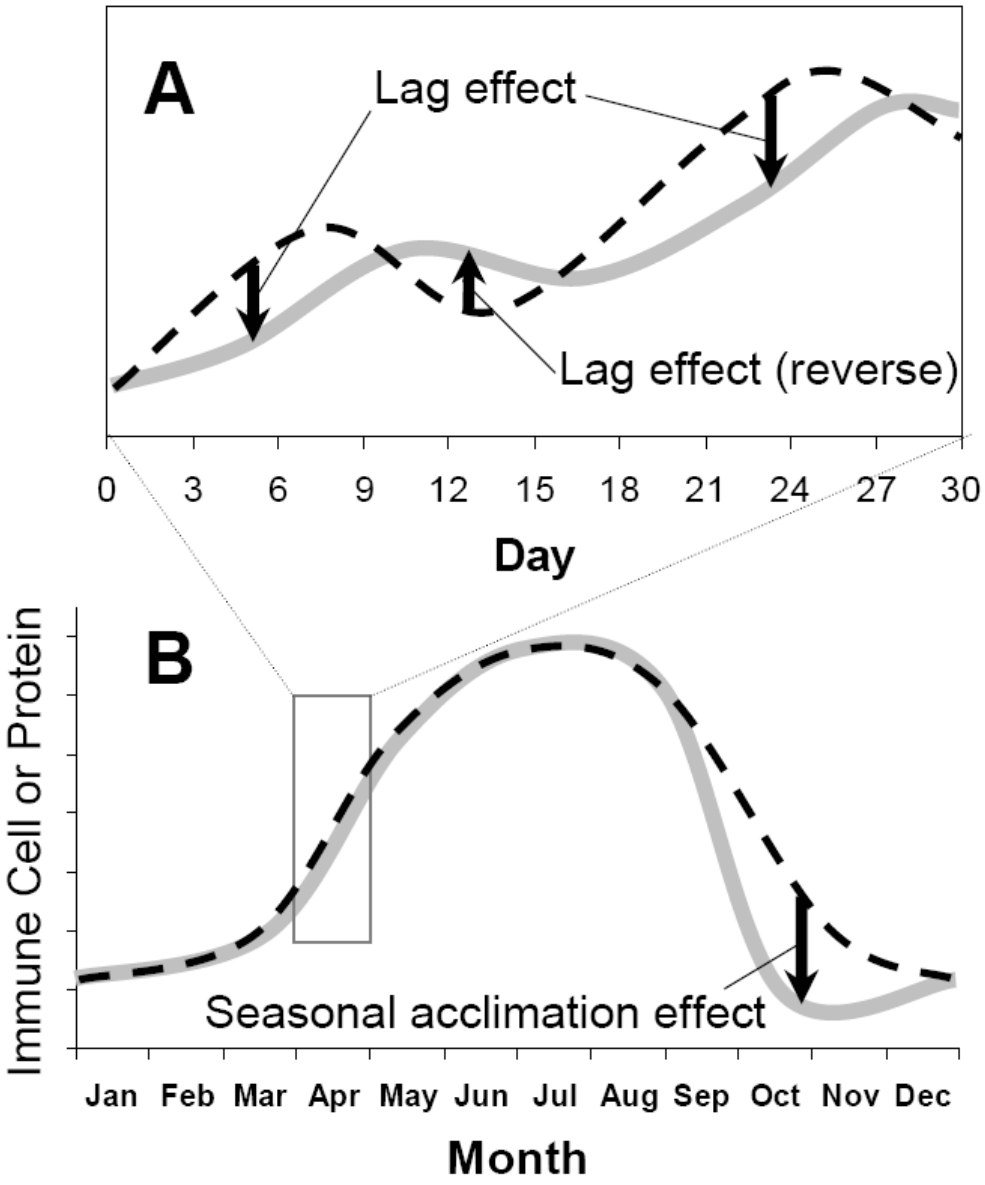

Figure 1: Hypothesized effects of changing temperature on amphibian immunity. The dotted curves indicate optimal levels of a hypothetical temperature-dependent immune parameter, and the grey curves indicate the actual levels of this immune parameter predicted by the lag and acclimation effect hypotheses at different time-scales. Temperature is assumed to exhibit both within and between-season fluctuations.

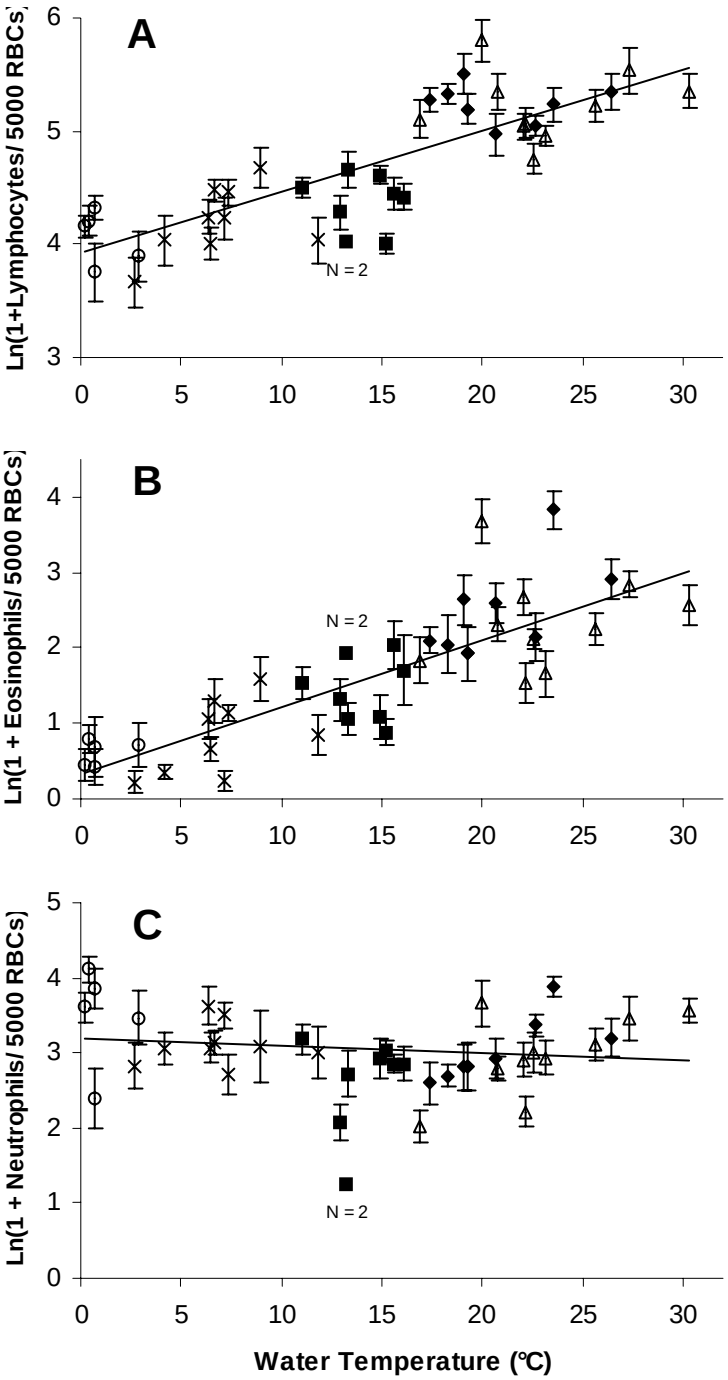

Figure 3.2: Between-season effects of temperature on immune parameters (log-transformed). Symbols represent the average and standard error for each pond at each sampling date (N = 10 for most points). Different symbols represent different seasons to illustrate seasonal deviations from expected values (✕ early spring, △ late spring**,** ◆ summer, ■ autumn, and ○ winter).

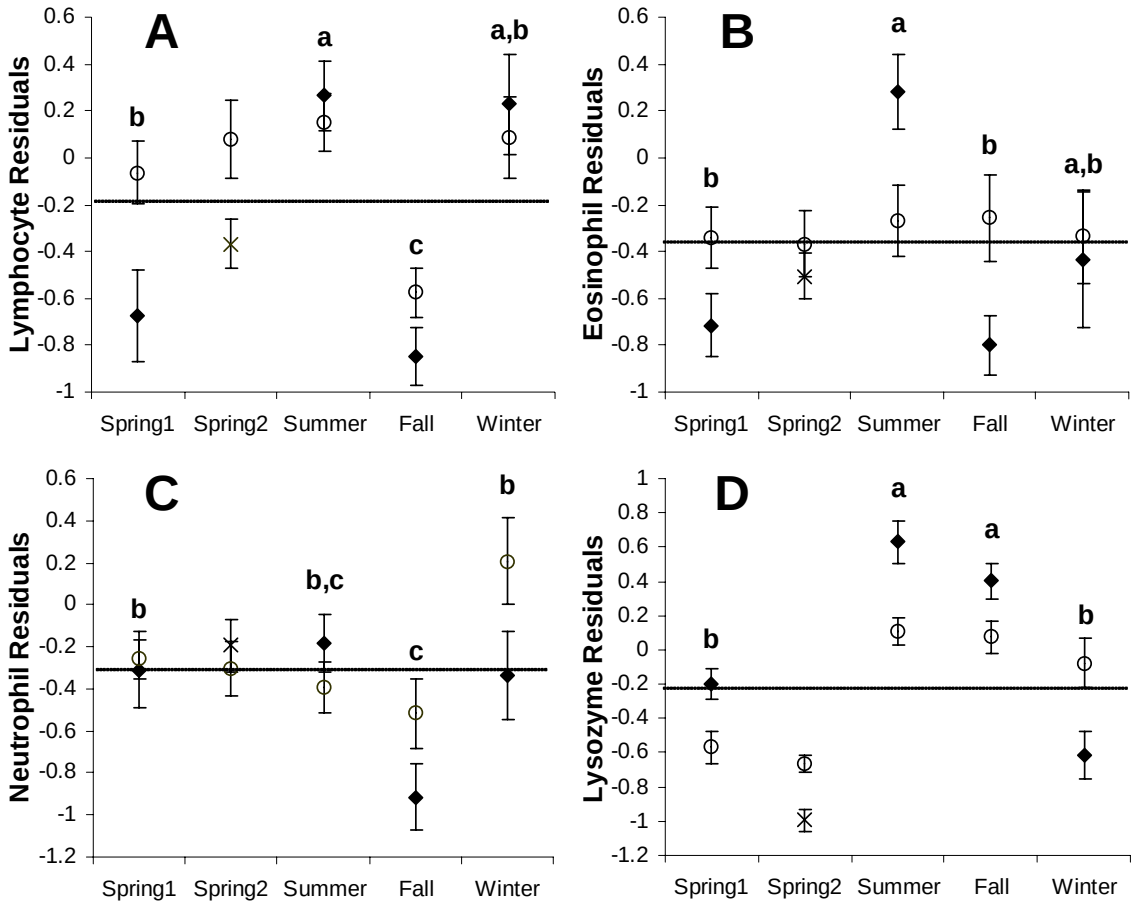

Figure 3.3: Seasonal effects on immune parameters, once the effects of temperature had been accounted for. Seasons which were not significantly different from each other ($P > 0.05$) by the multiple comparisons analysis are labeled with the same letter. Patterns differed between years for some immune parameters (◆ 2003 survey, O 2004 survey, ✕ 2005 spring survey). Residuals were calculated from generalized linear models including Pond and Temperature (only Pond for Lysozyme residuals, see Methods). Error bars represent standard errors, and dashed lines show expected values for immune parameters (residual means, which can be non-zero if error distributions are non-normal).

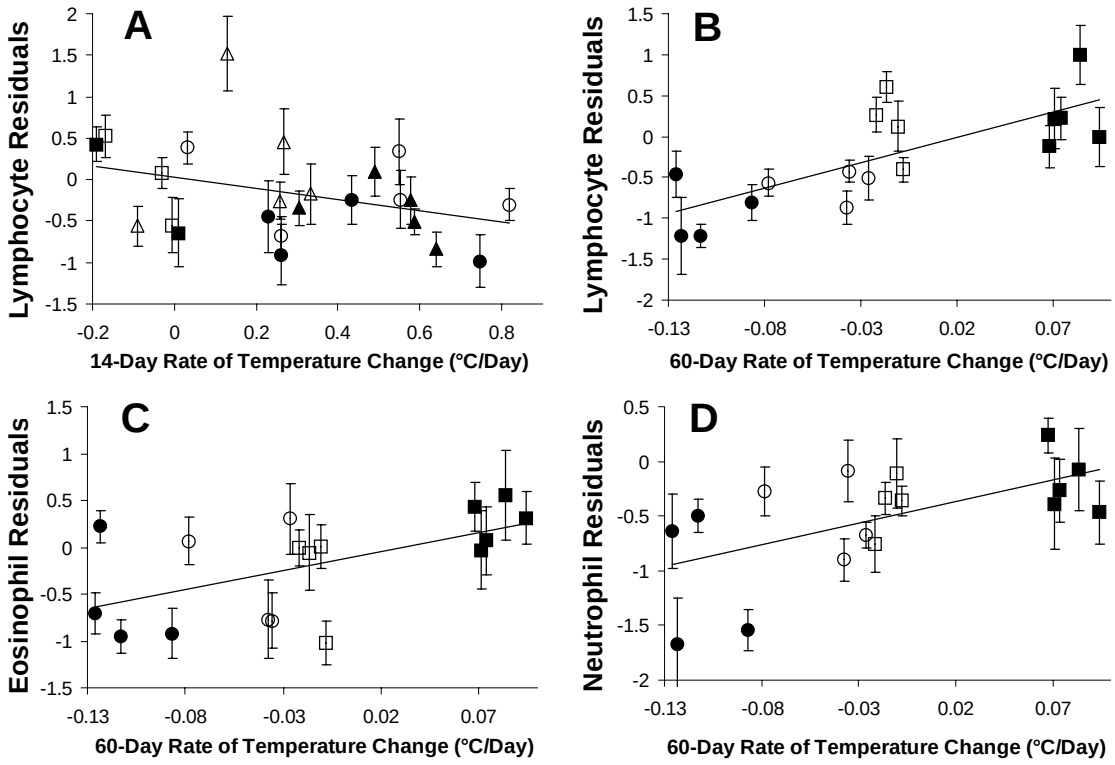

Figure 3.4: Effects of changing temperature on immune parameters for cold-acclimated (**A**) and warm-acclimated (**B-D**) newts. Different symbols represent different seasons (**A**: ■ winter, ● early spring, ▲ late spring; **B-D**: ■ summer, ● autumn). Rates of temperature change helped explain between-season and between-year (open symbols = 2004 survey) differences in residual values of immune parameters. Residuals were calculated from generalized linear models including Pond and Temperature, and symbols represent the average and standard error for each pond at each sampling date (N = 10 for most points).

CHAPTER 4:

Field evidence for leech-borne transmission of amphibian *Ichthyophonus* sp.

T. R. Raffel[1], J. R. Dillard[1] and P. J. Hudson[1]

[1]Center for Infectious Disease Dynamics, Penn State University

Journal of Parasitology, in press

Abstract:

Parasites have been implicated in mass mortality events and population declines of amphibians around the world. One pathogen associated with mortality events in North America is an *Ichthyophonus*. sp.-like organism which affects red-spotted newts (*Notophthalmus viridescens*) and several frog species, yet little is known about the distribution of this pathogen in wild populations or the mechanism of transmission. In an effort to identify factors influencing the distribution and abundance of this pathogen, we measured *Ichthyophonus* sp. prevalence and a series of factors that could contribute to transmission in 16 newt populations during the spring of 2004. In contrast to our initial hypotheses of trophic transmission, several lines of evidence suggested a role for the amphibian leech (*Placobdella picta*) in *Ichthyophonus* sp. transmission. We propose the mechanistic hypothesis that a leech acquires *Ichthyophonus* sp. infection when inserting its proboscis into the muscles beneath the skin of infected newts and transmits the infection to other newts in subsequent feeding bouts. We also found effects of host sex, body mass and breeding condition on *Ichthyophonus* sp. prevalence and the number of attached leeches. The number of leeches attached to newts was strongly related to the proportion of newt habitat containing emergent vegetation, suggesting that anthropogenic eutrophication might lead to more frequent or severe outbreaks of *Ichthyophonus* sp. infection in amphibians.

Introduction:

Amphibian populations throughout the world are declining, many without obvious causes (Stuart et al. 2004). Although a number of factors may contribute to these declines, new or more virulent pathogens have been identified as playing a central role (Kiesecker et al. 2001a, Kiesecker et al. 2001b, Collins and Storfer 2003, Daszak et al. 2003). Reports of parasite-induced mortality and morbidity events in amphibian populations have increased in recent decades, most attributed to ranaviruses, trematode metacercariae, and newly recognized fungal pathogens including a pathogenic chytrid fungus and an *Ichthyophonus*-like organism (Green et al. 2002, Johnson et al. 2004). In particular, the spread of this chytrid fungus has been implicated in declines of amphibian species in Central America, Australia, and California (Berger et al. 1998, Fellers et al.

2001). Disease emergence is often associated with anthropogenic environmental changes (Dobson and Foufopoulos 2001), and several anthropogenic factors have been implicated as causes of increased disease incidence in amphibians, including introductions of non-native species (Weldon et al. 2004, Jancovich et al. 2005), increased UV radiation (Kiesecker and Blaustein 1995), pesticide-induced immunosuppression (Kiesecker 2002, Gilbertson et al. 2003), and anthropogenic eutrophication (Johnson and Chase 2004)

Several mass mortality and morbidity events have been attributed to infection by an *Ichthyophonus*-like mesomycetozoan fungus first described in bullfrogs (*Rana catesbeiana*) and recorded in 5 additional North American amphibian species, including red-spotted newts (*Notophthalmus viridescens*) (Goodchild 1953, Herman 1984, Mikaelian et al. 2000, Green et al. 2002). However, we know little about its distribution, abundance, or impact in normal amphibian populations (Mikaelian et al. 2000). Since no other *Ichthyophonus* sp.-like organism has been described in amphibians, we will refer to this organism simply as *Ichthyophonus* sp., although its precise taxonomic status has yet to be determined. Amphibian *Ichthophonus* sp. is ultrastructurally similar to *Ichthyphonus hoferi*, a trophically transmitted pathogen of marine fish (McVicar 1982, Herman 1984, Spanggaard et al. 1995). However, the few studies of amphibian *Ichthyophonus* sp. have found no evidence of infection in sympatric fish, no evidence of infections in tissues other than the skeletal musculature, and no tendency for the organism to germinate after death of the host, and so far have not achieved successful transmission by oral or intraperitoneal administration of infected tissue (Herman 1984, Mikaelian et al. 2000). In red-spotted newts, macroscopic signs of infection range from visible spores to swelling of the axial musculature (Herman 1984). Severe infections are often associated with ulcerations which may lead to secondary infections with bacteria and fungi (Herman 1984). Amphibian *Ichthyophonus* sp. has been reported from widespread geographic locations in North America and from specimens collected as far back as 1953 (Goodchild 1953, Herman 1984, Mikaelian et al. 2000, Green et al. 2002). Since most reports have been in response to mass mortality or morbidity events, it has not been possible to evaluate how common this parasite is in amphibian populations or what factors contribute to between-population variation in infection prevalence.

Understanding the transmission process is essential if we expect to examine the consequences of parasitic infections to populations (Hudson et al. 2002). Previous researchers have assumed that amphibian *Ichthyophonus* sp. would be similar to ***I. hoferi***, which is transmitted through the ingestion of infected muscle tissue (McVicar 1982). Consequently, our initial hypotheses were that newts become infected by direct trophic transmission, either by ingestion of infected tissue through necrophagy or by ingestion of spherules dispersed from decaying dead individuals, or via indirect trophic transmission, i.e., ingestion of a carrier host which obtained the infection through predation or scavenging (Mikaelian et al. 2000). Assuming that contacts between individuals occur randomly, direct transmission via infected cadavers is generally predicted to increase with susceptible host density (newts, other amphibians, and possibly fish), although this prediction has not always been corroborated in empirical studies (McCallum et al. 2001). In contrast, direct trophic transmission may be independent of amphibian density and instead predicts a relationship between disease prevalence and the abundance of some prey species of red-spotted newts. In particular, benthic macroinvertebrates and tadpoles might pick up the infection while foraging. In either case, water quality or hydrological characteristics of ponds might influence parasite survival outside the host.

Preliminary 2002-2003 surveys of several red-spotted newt populations revealed striking between-pond variation in the prevalence of *Ichthyophonus* sp. disease signs. We therefore conducted an expanded survey in 2004 of 16 newt populations, measuring a wide range of ecological variables to identify the strongest predictors of infection prevalence. Based on results from these data, we propose the hypothesis that the amphibian leech (Placobdella picta, Siddall et al. 2005) is the vector of *Ichthyophonus* sp. infection in red-spotted newts. This hypothesis predicts a positive between-population relationship between disease prevalence and the ratio of leeches to newts, i.e., vector-host ratio (Hudson et al. 1995). In addition, *Ichthyophonus* sp. is predicted to have a similar distribution to other leech-vectored parasites such as *Trypanosoma diemyctili* (a blood-borne parasite known to be vectored by leeches, Mock 1987), to be found only in peripheral muscles (near the skin surface), to be more frequently observed on body parts regularly bitten by leeches, and to be found growing out from recent leech bites in new

infections. In this paper, we present results from field data testing predictions of the trophic transmission hypotheses and of the leech vector hypothesis.

Materials and Methods:

Study Sites

Sixteen lakes and ponds known to support newt populations were chosen in and around Centre County, Pennsylvania, to represent a variety of adult newt habitats including temporary woodland ponds, permanent landlocked ponds, beaver wetlands, and human impoundments (Table 4.1).

Survey

Sweep surveys were conducted in the late spring (May-June) of 2004 within a 24-day period to minimize any seasonal effects on disease prevalence. During each survey, m-long sweeps of a dip net (30 x 60 cm aperture, 3 mm mesh) were taken at regular 4-step intervals in a gridded pattern in which we sampled perpendicular to the shoreline to a depth of 0.5 m, took another sweep after 4 steps parallel to the shoreline, worked back to the shoreline taking sweeps every 4 steps, took another 4 steps parallel to the shoreline, and repeated the procedure until we had sampled the entire pond perimeter. Several ponds (BE, CR, IR, CO, PR) were too large to survey in their entirety, so we undertook a minimum of 40 sweeps along the shore. Numbers of newts, amphibian larvae, and several types of aquatic macroinvertebrates and fish were recorded for each sweep using a digital voice recorder. Substrate characteristics were also recorded for each sweep, including the approximate depth (< 15 cm, 15-30 cm, and 30-50 cm) and the presence of vegetation, leaves or rocks. The proportion of sweeps containing vegetation, leaves, rocks and only mud was calculated for each pond as well as the maximum depth of each pond, which was categorized as < 1 m., 1-2 m, and > 2 m. Sex, mass, snout-vent length and breeding status were recorded for each individual newt. In addition, the number and location of attached leeches and the location and extent (proportion of each body part affected) of *Ichthyophonus* sp. disease signs were also recorded. The location and extent of any skin ulcerations were also recorded for each newt. Dissolved oxygen and water

temperature were recorded at a depth of 10 cm for each pond at each time point using a YSI Model 95 dissolved oxygen meter; and pH was taken with a Corning Model 313 pH/temperature meter. Water chemistry analyses were conducted by the Penn State Institutes of the Environments Water Quality Laboratory, University Park, Pennsylvania (Ca^{+2}: Standard Methods 3500A-Ca AA Spectrometric; NO_3-N: Standard Methods 4500F Automated Cadmium Reduction City, State of source). After completion of each sweep-survey, 10 newts were collected per pond for blood collection and dissection. Newts were transported to the lab in 250-ml Nalgene containers filled with pond water, anesthetized with a drop of 15% benzocaine ointment (Orajel®, Del Laboratories, Inc., Uniondale, N.Y.) on the head, and killed by decapitation within 3 hr of initial capture.

Disease diagnoses

Swelling of the axial musculature (as described by Converse and Green, 2005) and visible spores (0.1-0.5 mm diameter) under the skin of any body part (generally under the transparent skin of the throat, abdomen, vent and limbs) were considered diagnostic signs of *Ichthyophonus* sp. infection during sweep surveys (Fig. 4.1). *Ichthyophonus* spp. spores are spherical or ovoid cells containing endospores which develop in infected tissues (Mendoza et al. 2002). All newts which had been collected for dissection and exhibited these disease signs (N = 22) were examined for the presence of *Ichthyophonus* sp. spores. Following fixation in 10% buffered neutral formalin for > 24 hours, muscle tissue containing disease signs was embedded in paraffin, sectioned at 10 µm thickness with a Shandon Finesse® Paraffin Microtome (Thermo Electron Corporation, Waltham, MA) and stained with hematoxylin and eosin. None of the samples were decalcified before sectioning. Additional newts collected from five of these ponds between March 2003 and June 2005 (Raffel et al. *in press-b*) that showed signs of *Ichthyophonus* sp. infection in gross dissection (N = 12) were also examined histologically. Identification of *Ichthyophonus* sp. in photos of histological sections was confirmed by David E. Green (United States Geological Survey National Wildlife Health Center, Madison, WI). Active and passive spores were identified according to Mikaelian et al. (2000).

Trypanosome infection was determined from blood smear counts. Blood was collected from newts with a heparinized capillary tube and a small drop was smeared on a

glass microscope slide. Slides were air dried for 10 min, fixed in methanol for 5 min, and again allowed to dry. Slides were placed in 1% o-dianisidine (3,3'-dimethoxybenzidine, Sigma, St. Louis, Missouri) in methanol for 90 sec, destained in 1% hydrogen peroxide in 50% ethanol for 90 sec, and rinsed twice in deionized water for 30 sec. Slides were then stained in Giemsa stain for 30 min. and rinsed again in deionized water for 10 min. Cells were counted at 400x magnification starting at the edge of the smear where the initial drop of blood had been placed and working down the slide in a gridded pattern, moving the objective 2 mm between fields and counting all cells in each field until the erythrocyte count reached 5,000. Trypanosomes were quantified as parasites per 5,000 erythrocytes.

Leeches were identified as *Placobdella picta* according to Klemm's (Klemm 1985) key. Voucher specimens of *Ichthyophonus* sp. (USNPC 98177-98181), *Trypanosoma diemyctyli* (USPNC 98171-98177), and *Placobdella picta* (USNPC 98182, 3 vials) have been submitted to the United States National Parasite Collection.

Statistical Analyses

Newts have high site fidelity to breeding ponds as adults (Gill 1978a), so newts from different ponds were treated as independent breeding populations in the analyses. The best between-population predictors of *Ichthyophonus* sp. prevalence, trypanosome prevalence, and numbers of leeches per newt were determined by backward selection of linear regression models with normal errors and weighted by sample size. Prevalence data were arcsine-transformed to correct for non-normality of proportional data, and count data, i.e., leeches per newt and individuals per sweep, were summarized for each pond by calculating the mean log of abundance (ln[count + 1]). Since there were more potential predictor variables than error degrees of freedom (Table 4.2), response variables were first regressed against each predictor individually and only predictor variables with $P < 0.1$ were included in the maximal model (Table 4.2). The minimum adequate model was selected as described by Crawley (2002), excluding variables which did not significantly add to the variance explained by the model (F-statistic, $P > 0.05$). Once the minimal model had been selected, all postulated predictors were tested for significance using the selected predictor(s) as covariates.

The significance of individual newt characteristics (sex, snout-vent length, mass, body condition, and breeding condition) for predicting *Ichthyophonus* sp. infection, trypanosome infection and numbers of leeches per newt was determined by backward selection of generalized linear mixed models including pond (population) as a random term. Effects of individual *Ichthyophonus* sp. infection status on the number of currently attached leeches and vice versa were also tested using these models. These models were estimated using restricted marginal quasi-likelihood, with binomial errors for presence/absence of infection and Poisson errors for the number of leeches per newt. Models were selected as described above for the between-population analyses, except that the significance of predictor variables were determined using submodel deviance tests (Chi-square statistic, $P < 0.05$). *Ichthyophonus* sp.-infected individuals were analyzed separately using generalized linear models to determine if intensity of infection (proportion of body surface covered by disease signs, arc-sine transformed) or the presence of swollen axial musculature (binomial errors) depended on the snout-vent length, sex, mass, breeding condition, or body condition of the affected newt.

One-way chi-square goodness of fit tests were used to test the null hypothesis that numbers of leech and *Ichthyophonus* sp. observations on each body part were proportional to the surface area of each body part. The number of *Ichthyophonus* sp. observations on each body part (corrected for surface area) was then regressed against the number of leeches on each part (also corrected for surface area) with normal errors, weighting for the percent surface area of each body part. All analyses were run using S-Plus statistical software.

Results:

Infection by amphibian *Ichthyophonus* sp. was recorded from 12 of the 16 newt populations surveyed (875 total newts observed, between 16 and 132 newts from a given pond). The only significant predictor of *Ichthyophonus* sp. prevalence in the between-population analysis was the number of leeches observed feeding on newts ($F = 4.75$, d.f. = [1, 14], $P = 0.0468$, Fig. 4.2). None of the variables predicted by the trophic transmission hypotheses showed any between-population relationships with *Ichthyophonus* sp. prevalence even before including leeches in the model (all $P > 0.1$).

Trypanosomes also showed a highly significant between-population effect of leeches (F = 17.56, d.f. = [1, 14], P = 0.0009), but not for other variables. For the 9 populations in which both *Ichthyophonus* sp. and trypanosomes were detected, trypanosome infection was approximately 6-fold more prevalent than *Ichthyophonus* sp. (mean ratio 5.66:1, SE = 1.09, Fig. 4.2).

Snout-vent length, mass, and breeding condition were all significant predictors in the within-population analysis of *Ichthyophonus* sp. infection risk (Table 4.3). Snout-vent length and mass were positive predictors of *Ichthyophonus* sp. infection, whereas newts in breeding condition were less likely to be infected (Table 4.3). There was also a significant sex by breeding interaction, with non-breeding males more likely to be infected than breeding males or females (Table 4.3). With breeding condition removed from the model, male newts had significantly higher *Ichthoyphonus* sp. prevalence than female newts (X^2 = 4.5, d.f. = 1, P = 0.0342, coef. = 0.239). The variance component for the random term Pond (SD = 0.589, 95% confidence interval between 0.292 and 1.188) significantly improved the deviance explained by the model (X^2 = 12.2, d.f. = 1, P = 0.0005). Newts were more likely to be infected with *Ichthyophonus* sp. if they had greater snout-vent lengths, had greater mass, or were male (Table 4.3). None of the variables measured for individual newts was significant within-population predictors of trypanosome prevalence ($P > 0.05$).

Both *Ichthyophonus* sp. disease signs and leeches were found to have a nonrandom distribution on the bodies of newts (*Ichthyophonus*: X^2 = 43.6, d.f. = 7, $P <$ 0.0001; leeches: X^2 = 114.2, d.f. = 7, $P < 0.0001$). Parts of the body on which leeches were observed more often (corrected for surface area of body part) were also more likely to exhibit *Ichthyophonus* disease signs (F = 20.1, d.f. = [1, 6], P = 0.0042), with both leeches and disease signs more likely to be found on the head and trunk of the body than on the extremities (Fig. 4.3).

Of 88 newts with visible signs of *Ichthyophonus* sp. infection, 65 (73.9%) had swollen axial muscles and 39 (44.3%) had visible spherules under the skin (16 had both). Of the 65 newts with swollen axial muscles, 14 (21.5%) had skin ulcerations over the affected areas. No other newts with skin ulcerations were observed during this study. The intensity of infection (proportion of body surface covered by disease signs) ranged

from approximately 0.35 to less than 0.01, with a mean intensity of 0.090 (SE = 0.008). The mean coverage of the head and trunk was 0.115 (SE = .0128). For newts which were infected, neither the presence of swollen axial muscles nor the intensity of infection (proportion of body surface covered) related significantly with a newt's snout-vent length, mass, body condition, breeding condition or sex (all $P > 0.05$).

The disease signs of all 22 newts collected for dissection and showing swollen axial muscles or visible spores (16 with swelling, 9 with visible spores, 3 with both) were confirmed by histological examination to be caused by *Ichthyophonus* sp.-like spores. We found no evidence of infection in any dissected newts lacking visible signs of infection, and even those newts with visible infections of the skeletal musculature showed no evidence of *Ichthyophonus* sp. infection in their internal organs or digestive tract. Most of the infected newts with swollen axial musculature had high proportions of active spores (13 of 16 with more than 50% of spores active), but all 6 newts lacking obvious axial swelling (visible spores only) contained predominantly (> 50%) passive spores. All 12 additional newts collected during the seasonal survey and having visible signs of infection at the time of dissection were also confirmed to be infected with *Ichthyohonus* sp. by histology. To find no false positives in 34 randomly selected infected newts provides the statistical power to conclude that the true false positive rate was less than 7.4% (based on a binomial test with alpha = 0.05), indicating that the presumptive field diagnoses were accurate indicators of *Ichthyophonus* sp. infection in red-spotted newts. Ten *Ichthyophonus*-infected newts were also found to be infected with trypanosomes, of 22 examined.

Significant predictors were found for leech attachment rates in both the within- and between-population analyses. The proportion of sweeps with emergent vegetation was the only significant between-population predictor of the number of leeches per newt ($F = 17.56355$, d.f. = [1, 14], $P = 0.0009$), with more leeches in ponds with more vegetation (Fig. 4.3). In the within-population analysis, newts had higher numbers of attached leeches if they were in breeding condition, had lower mass, or were female (Table 4.3). There was no significant effect of *Ichthyophonus* sp. infection on the current number of attached leeches ($X^2 = 0.6$, d.f. = 1, $P > 0.4$) or of the number of currently attached leeches on the probability of being infected with *Ichthyophonus* sp. ($X^2 < 0.1$,

d.f. = 1, $P > 0.8$). The variance due to the random term Pond (SD = 1.08, 95% confidence interval between 0.66 and 1.77) significantly improved the deviance explained by the model ($X^2 = 76.3$, d.f. = 1, $P < 0.0001$).

Discussion:

Several predictions of the leech transmission hypothesis were supported by our results. The number of leeches feeding on newts was the only significant between-population predictor of *Ichthyophonus* sp. prevalence in this study. This pattern is consistent with vector-borne disease models that predict that the basic reproductive number of the parasite (R_0) will be proportional to the ratio of vectors to hosts (Hudson et al. 1995, Randolph et al. 2002). Trypanosomes, known to be leech-transmitted, exhibited a similar between-pond relationship with leech abundance. An alternative hypothesis, that trypanosome-induced immunosuppression facilitates *Ichthyophonus* sp. infection from some source other than leeches, seems unlikely due to the absence of trypanosome infection in more than half of the *Ichthyophonus* sp.-infected newts examined. In addition, the distribution of *Ichthyophonus* sp. observations on newt bodies was nonrandom, with signs of infection concentrated on those body parts most likely to be bitten by leeches. Finally, during a capture-mark-recapture study of *Ichthyophonus* sp. infection dynamics in newts, Raffel (2006) observed new (less than 1-mo-old) infections growing out from distinctive subcutaneous haematomas. These haematomas frequently form during *Placobdella picta* feeding bouts (Barta and Sawyer 1990) and are common in ponds and seasons with high leech bite rates (Raffel 2006), suggesting that leeches are the most likely cause of these haematomas. It should be noted that the leech transmission hypothesis assumes that the prepatent period for *Ichthyophonus* sp. infection is longer than 5-48 hr. duration of leech feeding (Barta and Sawyer 1990), since recent *Ichthyophonus* sp. infections have not been observed growing out from sites of actively feeding leeches (T. R. Raffel, pers. obs.) and there was no effect of *Ichthyophonus* sp. infection status on the current numbers of leeches attached to newts. We found no support for either of the 2 trophic transmission hypotheses, with *Ichthyophonus* sp. prevalence showing no relationships with densities of newts, benthic macroinvertebrates, amphibian larvae, or fish.

The leech transmission hypothesis also helps explain three previously unexplained characteristics of this parasite. Amphibian *Ichthyophonus* sp. can be found in any of the skeletal musculature, including the tail and limbs, but not in the internal organs or digestive tract as predicted by the trophic transmission hypotheses (Mikaelian et al. 2000). Our results corroborated this observation. This distribution is better explained by the leech transmission hypothesis and suggests direct injection of the pathogen into the musculature by leeches. In addition, the absence of *Ichthyophonus* sp. in sympatric fish can be readily explained by the specialization of *P. picta* on amphibian hosts (Sawyer 1972, Mikaelian et al. 2000). Finally, amphibian *Ichthyophonus* sp. has proven impossible to culture using methods which have worked for *I. hoferi* (Mikaelian et al. 2000). This may be due to different conditions in the leech gut from that of the fish gut, where *I. hoferi* spores are induced by acidic pH to form hyphae (Spanggaard et al. 1995).

Most leech-vectored parasites are blood-borne (e.g., *Trypanosoma diemyctyli*, *Haemogregarina balli*, *Lankesterella minima*, and *Babesiosoma stableri*) and transmitted to leeches in blood meals from infected hosts (Tse et al. 1986, Mock 1987, Barta and Desser 1989, Siddall and Desser 1990). How then might a leech acquire an intramuscular parasite from its host? We think that the proboscis of *P. picta*, used to probe for blood vessels beneath the skin of their amphibian hosts (Barta and Sawyer 1990), may be crucial for transmission of *Ichthyophonus* sp. from infected amphibians to leeches. This proboscis is approximately 20% of the leech's body length (or 2.6-5 mm for adult leeches, Klemm 1985, Barta and Sawyer 1990), easily long enough to reach tissues containing *Ichthyophonus* sp. spores. We propose that a leech acquires *Ichthyophonus* sp. infection by inducing rupture of *Ichthyophonus* sp. spores as its proboscis probes into the muscles beneath the skin of an infected newt, either mechanically or by some chemical cue. If this organism is similar to other mesomycetozoeans, this rupture would induce the release of endospores which would develop into a motile infectious stage capable of developing into a new spore containing endospores (Mendoza et al. 2002). Members of the order Ichthyophonida with known transmission mechanisms (*Ichthyophonus hoferi*, *Amoebidium* spp. and *Psorospermium* spp.) have amoeboid infectious stages, unlike members of the related order Dermocystida, whose endospores

develop into flagellated zoospores (Mendoza et al. 2002). Following rupture, these amoebae or their endospore precursors could infect the leech proboscis, which would then inject infectious amoebae into host tissues during subsequent feeding bouts. This proposed mode of transmission by amoeboid cells attached to the proboscis is not altogether different from that of *Trypanosoma fallisi* in *P. picta*, which migrates to the proboscis following ingestion in a leech blood meal (Martin and Desser 1991).

If this hypothesis is correct, transmission of *Ichthyophonus* sp. to leeches should be less efficient than transmission of blood-borne parasites. Any blood meal may contain a small number of trypanosomes, but acquiring *Ichthyophonus* sp. infection would require feeding directly over a patch of infected muscle. The probability of transmission may be somewhat increased by the aggregation of leeches on the head and trunk of newts; however, the average coverage of these body parts was still only 11.5%. Therefore, a leech should be at least 8 times more likely to acquire a blood-borne parasite than *Ichthyophonus* sp. Our data show that trypanosome prevalence is approximately 6-fold higher than *Ichthyophonus* sp. prevalence in populations with both diseases, consistent with this prediction.

The increased prevalence of *Ichthyophonus* sp. in larger newts (greater snout-vent length) probably reflects an age prevalence relationship in newts. The higher prevalence of infection in non-breeding newts, however, is more likely reflect an effect of *Ichthoyphonus* sp. infection on breeding condition than vice versa. Breeding is generally predicted to increase susceptibility to parasitic infection (Rolff 2002), an effect unlikely to have led to higher prevalence in breeding newts. Since the breeding season of newts ends in late spring (Petranka 1998), about the time this survey was conducted, it seems more likely that newts come out of breeding condition sooner when infected. The greater prevalence in male newts and in those with greater mass is probably caused by differences in susceptibility or exposure to the parasite. These patterns were not apparently due to differences in leech bite rates, which showed the opposite trends. One possibility is that male newts spend more time in the water than females and are less likely to skip a yr of breeding, thereby increasing their lifetime exposure to leeches (Gill et al. 1983). This may also be true of individuals with greater mass, which may be more likely to breed in a given yr due to better nutritional status. Alternately, male newts may

be more susceptible due to suppression of the immune system by testosterone (Zuk and Stoehr 2002), although the lack of a difference in infection intensity between the sexes contradicts this hypothesis.

Our results suggest that *Ichthyophonus* sp. is a common infection in red-spotted newt populations. Twelve of 16 surveyed populations harbored the infection, and low prevalence has since been observed in 2 of the remaining ponds (data not shown). More than half of the infected newts showed severe swelling of the axial musculature, filled predominantly with active spores and often associated with ulcerations. These ulcerations may lead to secondary infections which act as the immediate cause of death, a phenomenon observed in our lab and by other researchers (Herman 1984, unpublished data). The replacement of muscle tissue by *Ichthyophonus* sp. spores may also increase mortality by lowering foraging ability and escape responses to predators. The near ubiquity and potentially severe consequences of *Ichthyophonus* sp. infection may make this parasite a significant source of mortality for red-spotted newts, but the results of this study are insufficient to draw substantive conclusions about the rate of mortality due to *Ichthophonus* sp. infection.

The leech transmission hypothesis appears to be the parsimonious explanation of patterns of *Ichthyophonus* sp. infection in red-spotted newts. The current evidence is correlational, however, and more definitive confirmation of this hypothesis will require experimental transmission of *Ichthyophonus* sp. between amphibians. If the leech transmission hypothesis is correct, predicting future outbreaks of *Ichthyophonus* sp. infection may be possible by considering the environmental factors that influence leech abundance. The strongest predictor of the number of leeches attached to newts in our study was the abundance of emergent vegetation. Aquatic vegetation provides the solid footing and structural complexity which glossophonid leeches need to reach their hosts, which are incapable of swimming and must crawl inchworm-like along a solid substrate (Sawyer 1986). *Placobdella picta* is also thought to be host-limited, with high leech attachment rates in years following peaks in amphibian population densities (Gill et al. 1983, Mock 1983). *Ichthyophonus* sp. outbreaks should therefore be most likely in wetlands with plenty of emergent vegetation and following years of high amphibian reproduction rates. Increased abundance of littoral vegetation is often associated with

anthropogenic eutrophication, which has also been linked to increasing rates of trematode-induced limb deformities in amphibians (Rast and Thornton 1996, Johnson and Chase 2004). If leeches are indeed the vector of *Ichthyophonus* sp. infection in amphibians, anthropogenic eutrophication might contribute to outbreaks of this parasite.

Table 4.1: Characteristics of the wetlands sampled

Pond/Lake	Latitude	Longitude	Wetland Type
Beaver 1 (BE)	40° 45' 52.6"	78° 0' 43.6"	Landlocked permanent lake
Clearcut Pond (CC)	40° 46' 27.7"	77° 57' 0.0"	Ephemeral pond
Catty Ninetails (CN)	40° 47' 45.5"	77° 57' 15.5"	Ephemeral pond
Colyer Lake (CO)	40° 46' 41.8"	77° 41' 9.2"	Human impoundment
Cranberry Lake (CR)	40° 46' 2.6"	78° 0' 15.5"	Landlocked permanent lake
Deep Woods (DW)	40° 52' 9.0"	78° 4' 54.6"	Beaver wetland
False Beaver (FB)	40° 42' 38.3"	77° 52' 54.3"	Human impoundment
Greenbriar 1 (GB)	40° 46' 41.3"	78° 0' 27.4"	Ephemeral pond
Irrigation Pond (IR)	40° 42' 18.4"	77° 56' 48.2"	Landlocked permanent pond
Little Acre (LA)	40° 48' 5.8"	77° 56' 36.5"	Landlocked permanent pond
Mothersbaugh (MB)	40° 39' 12.2"	77° 54' 9.6"	Beaver wetland
Mystery Newt (MN)	40° 45' 53.0"	78° 0' 49.2"	Ephemeral pond
Muskrat Pond (MP)	40° 53' 8.4"	78° 4' 3.8"	Beaver wetland
Penn Roosevelt (PR)	40° 43' 36.8"	77° 42' 8.3"	Human impoundment
Twin Pond (TP)	40° 46' 49.1"	78° 0' 13.9"	Ephemeral pond
Turtle Shell (TS)	40° 52' 26.1"	78° 4' 35.6"	Beaver wetland

Table 4.2: Variables included in between-population analyses of *Ichthyophonus* sp. prevalence and leech abundance (leeches per newt):

Hypothesized predictors of *Ichthyophonus* sp. prevalence	
Potential intermediate hosts:	*Host Population:*
- Tadpole density	- Newt density
- Salamander larva density	*Seasonality:*
- Snail density	- Date
- Fingernail clam density	*Water quality:*
- Dragonfly larva density	- Temperature (shallow)
- Damselfly larva density	- Ca^{+2} concentration
- Caddisfly larva density	- Nitrate concentration
- Leeches per newt*	- pH
Fish density:	- Dissolved Oxygen
- Total fish density	*Pond characteristics:*
- Sunfish density	- Water inflow to pond*
- Pickerel density	- Pond Depth
- Chub density	
Hypothesized predictors of leech abundance	
Host density:	*Substrate:*
- Adult newts	- Proportion vegetation*
- All amphibian larvae	- Proportion muddy
- Tadpole density	- Proportion rocks
- Salamander larva density	- Proportion leaves
Pond community:	*Seasonality:*
- Total fish density	- Date*
- Sunfish density	*Water Quality:*
- Pickerel density*	- Temperature (shallow)
- Chub density	- Ca^{+2} concentration
- Snail density	- pH
- Fingernail clam density*	- Dissolved oxygen
- Total mollusc density*	*Pond characteristics:*
Host behavior:	- Water inflow to pond
- Average newt depth	- Pond depth

* $P < 0.1$ in absence of other predictor variables

Table 4.3: Minimal adequate models for within-population variation in *Ichthyophonus* sp. prevalence and leech bite rates

Response	Predictor	Coefficient	df	X^2	P
Ichthyophonus (presence/absence)	Mass	0.514	1	11.4	0.0007
	SV Length	0.211	1	14.1	0.0001
	Breeding	-0.546	1	38.7	< 0.0001
	Sex*	0.199	1	2.3	0.1993
	Breeding:Sex	-0.297	1	5.2	0.0220
Leeches (number attached to newt)	Mass	-0.015	1	34.5	< 0.0001
	Sex*	-0.109	1	4.2	0.0400
	Breeding	0.205	1	85.0	< 0.0001

*F = 0, M = 1

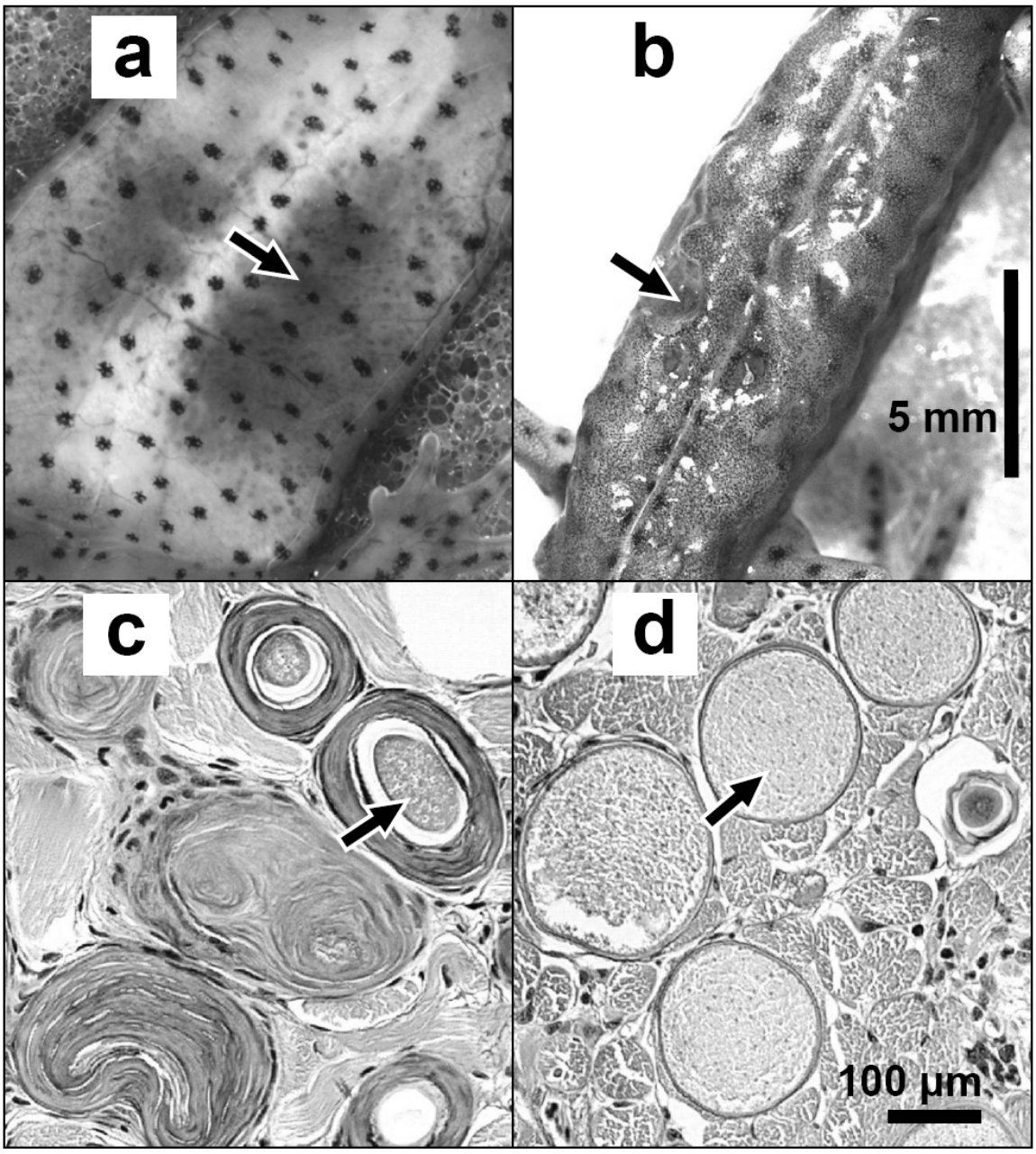

Figure 4.1. Macroscopic signs considered indicative of *Ichthyophonus* sp. infection: (a) visible intramuscular cysts, usually occurring in patches below the translucent skin of the throat, stomach, vent, or limbs (not to be confused with normal black spots on the skin surface), and (b) swelling of the axial musculature (the arrow indicates an ulceration). Histological examination of infected muscles: (c) resting spores (small, dense, with a wrinkled outer layer and often surrounded by a wall of host tissue) and (d) active spores (larger, with a smooth outer layer).

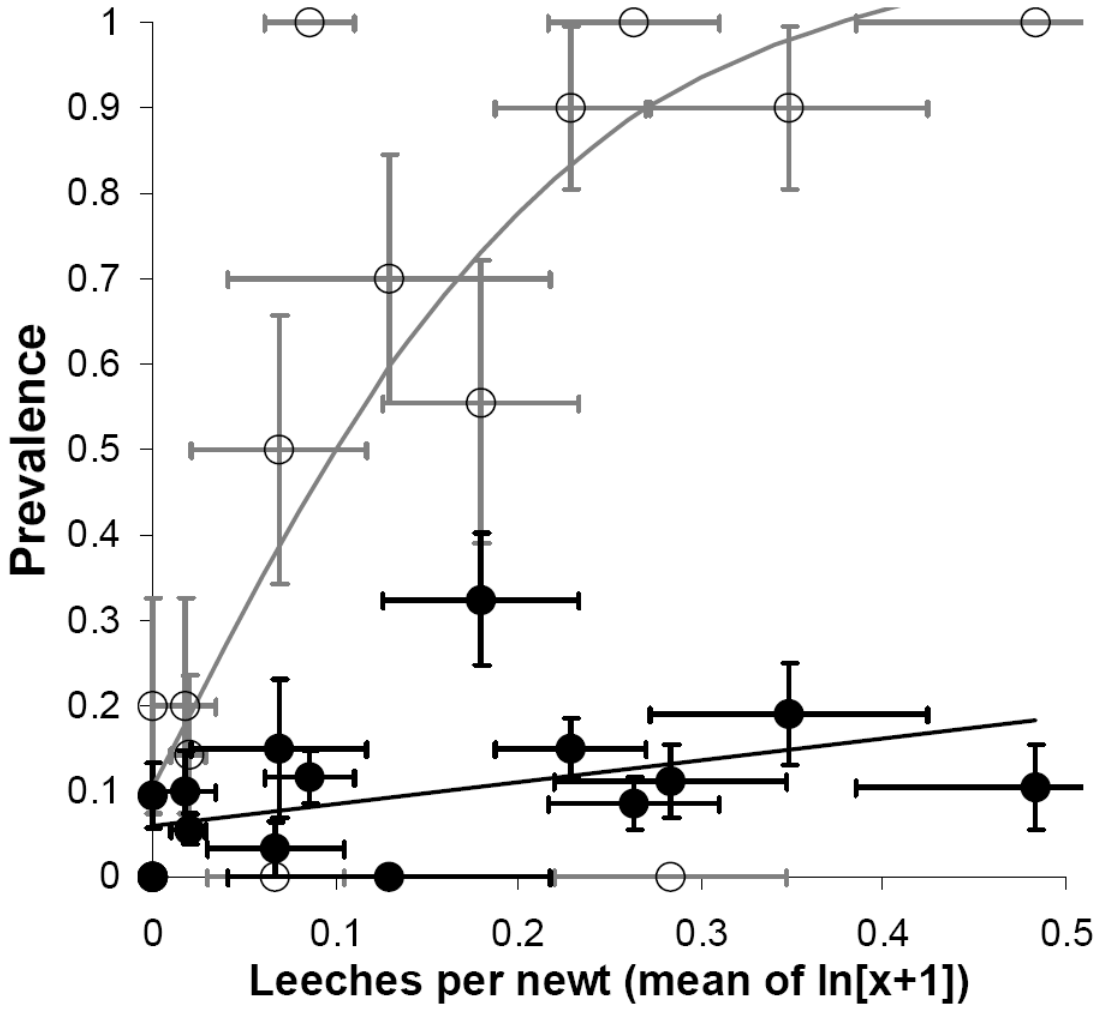

Figure 4.2. Between-population relationships between leech abundance and infection prevalence of *Ichthyophonus* sp. (closed circles and black error bars) and trypanosomes (open circles and gray error bars). The trypanosome data have been fitted with a spline curve (d.f. = 3 and omitting the outlying datapoint for LA [0.28, 0]). Leech counts were natural log-transformed. Error bars represent +/- standard errors.

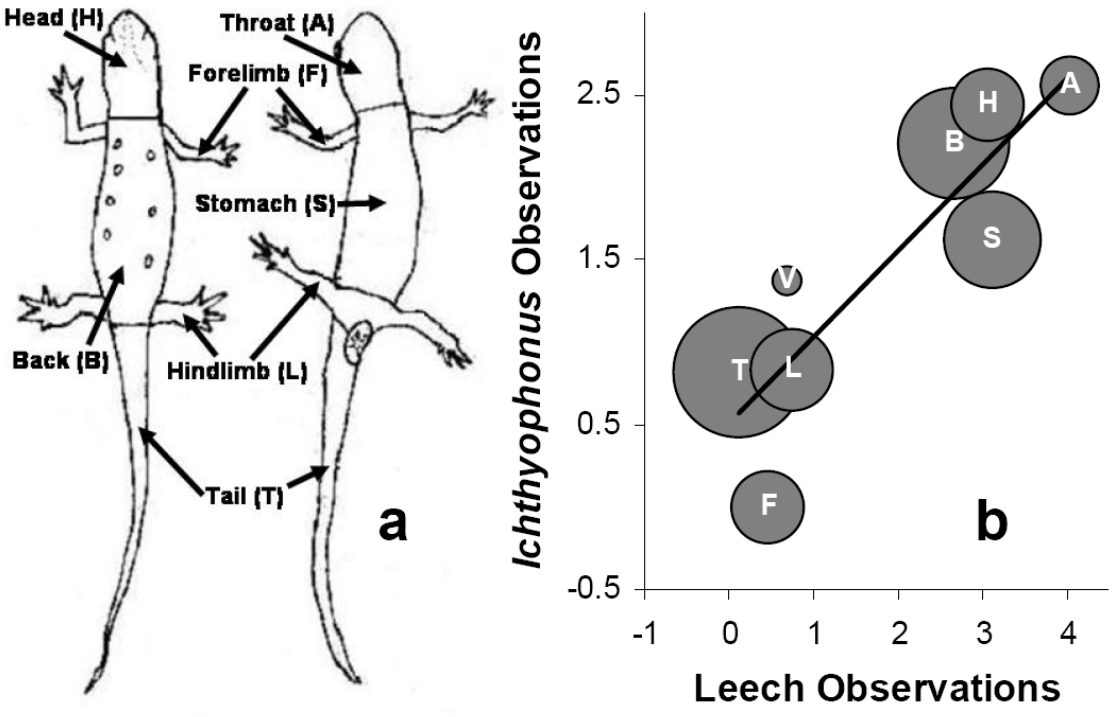

Figure 4.3. Relationship between the numbers of attached leeches and *Ichthyophonus* sp. disease signs observed on different newt body parts, shown in (a). Parts of newts' bodies with higher counts of leeches than expected were also more likely to have signs of *Ichthyophonus* sp. infection (b). Counts of leeches and *Ichthyophonus* sp. signs have been corrected for the surface area of the body part, which is indicated by the size of the circle. Data-point labels in (b) correspond to letters assigned to parts of the body in (a).

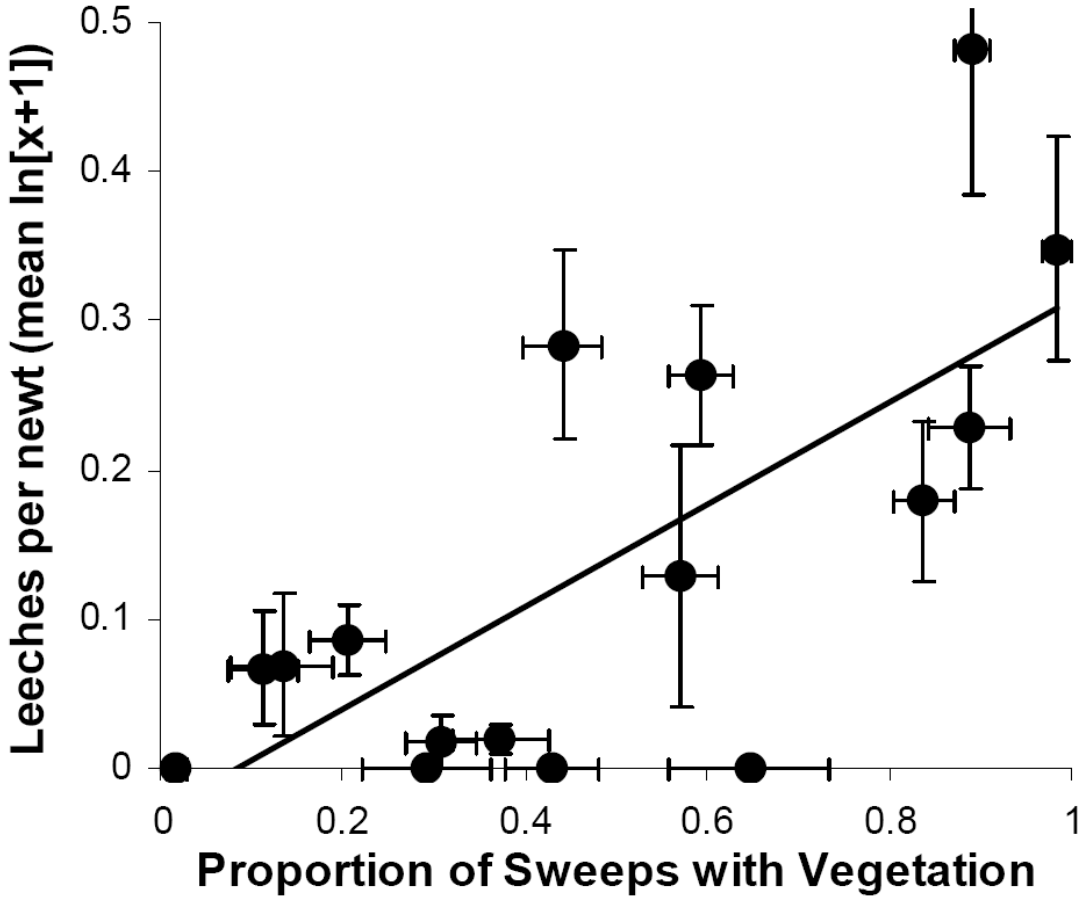

Figure 4.4. Relationship between emergent vegetation and leech attachment rates. Leech counts were log-transformed. Error bars represent +/- standard errors.

CHAPTER 5:

Preliminary experiments testing the leech transmission hypothesis for amphibian *Ichthyophonus* sp.

T. R. Raffel[1] and R. S. Huang[1]

[1]Center for Infectious Disease Dynamics, Penn State University

Abstract:

Two experiments were conducted to test the leech transmission hypothesis for *Ichthyophonus* sp. infection in red-spotted newts (*Notophthalmus viridescens*). Neither experiment resulted in new infections, a result that must be considered inconclusive due to insufficient statistical power and potentially important factors for transmission which were not incorporated into these experiments. The first experiment provides information about the rate and duration of leech-induced haematomas in red-spotted newts, providing a useful basis for comparison with field studies of *Ichthyophonus* sp. transmission which, if the leech transmission hypothesis is correct, allows estimation of the prepatent period of *Ichthyophonus* sp. infection.

Introduction:

Recent evidence suggests that the leech *Placobdella picta* may be the source and possibly the vector of an *Ichthyophonus* sp.-like organism that infects the skeletal musculature of amphibians (Raffel et al. *in press-a*). This would be a novel mode of transmission for a member of the class Mesomycetozoa, in which the few organisms with known or suspected transmission modes are thought to be directly transmitted either trophically (*I. hoferi*) or by motile spores (Mendoza et al. 2002). The evidence for leech-borne transmission of amphibian *Ichthyophonus* sp. is based on several field correlations but explains epidemiological and pathological patterns of infection better than either the trophic transmission hypothesis or direct transmission (Chapter 6, Raffel et al. *in press-a*). Confirmation of this hypothesis will require experimental transmission of the pathogen in controlled laboratory conditions using leeches. The leech transmission hypothesis predicts that an unknown proportion of leeches feeding on *Ichthyophonus* sp. infected newts will induce new infections in subsequent feeding bouts on uninfected newts, given that other conditions necessary for transmission are also met. Here we describe two preliminary experiments in which we tried to reproduce this transmission process in the laboratory in an attempt to determine whether leeches are capable of transmitting *Ichthyophonus* sp. infection.

Methods:

Experiment 1:

Uninfected newts (N = 72) were collected on 5/26/2005 from Parking Lot pond in the Scotia Barrens (N 40° 45' 51.4" W 78° 0' 58.6") known to have few leeches and low *Ichthyophonus* sp. prevalence (data not shown) and placed in a grid of 12 outdoor mesocosms (55 x 40 cm plastic tubs) at the PSU Agricultural Research Station, each with 24 L of water. Mesocosms were elevated 60 cm off the ground, covered with 3 mm mesh hardware cloth and shaded by shadecloth. Since conditioned tap water appears to kill these leeches (unpublished data), water was obtained from a pond known to support a large leech population (False Beaver Pond: N 40° 42' 38.3", W 77° 52' 54.3"; Rothrock State Forest, Huntingdon County, PA) and filtered through nitex (pore size 70 μm). Water changes were done once per week. Infected newts (N = 12) and leeches were collected in May 2005 from Turtle Shell pond (N 40° 52' 26.1", W 78° 4' 35.6"; Moshannon State Forest, Centre County, PA). All infected newts had a high precentage of the abdomen, throat and/or limbs showing visible *Ichthyophonus* sp. spores (>40% coverage), as described by Raffel et al. (in press), and two newts also had swollen neck muscles. A single 30 cm strand of *Elodea* sp. was placed in each tank to provide oviposition sites for female newts and substrate for leeches, and newts were fed blackworms (*Lumbriculus variegatus*) *ad libitum*. In response to high newt mortality possibly following a series of warm days, water temperature was measured for all mesocosms on June 8, 10 and 13 and the mesocosms were moved on 6/22/2005 to an indoor animal facility in Mueller Lab, University Park, PA, with a light/dark cycle of 12 hr light/12 hr dark and constant temperature of 25 °C.

Uninfected newts were randomly assigned to three spatial blocks each with four mesocosms randomly each assigned to one of four treatments: no leeches or infected newts, leeches only, two infected newts and no leeches, and both leeches and infected newts. Newts were individually marked in containers with a single injection of fluorescent elastomer dye (Northwest Marine Technology, Inc., Shaw Island, WA) in a unique location on the abdomen. Leeches were exposed to infected newts by placing them in a 250 mL water-filled plastic container with an infected newt and allowing them to feed on this newtfor up to 5 hours. Mesocosms with only leeches each received 21

adult leeches which had previously fed on one of the twelve infected newts. Mesocosms with both leeches and infected newts each received 20 adult and 20 juvenile (recently hatched and <5 mm. long) leeches, none of which had been experimentally exposed to infected newts. Leeches and infected newts were added to mesocosms on 5/27/2005 (day 1).

Newts were observed for evidence of *Ichthyophonus* sp. infection every 2 days for the first two weeks, after which they were observed twice per week. Data were also collected on the number of attached leeches and on the number and position of new subcutaneous haematomas which are frequently observed at attachment site following a *P. picta* feeding bout (Barta and Sawyer 1990). The time at which haematomas were no longer visible was recorded for all but seven of the observed haematomas. Mass and snout-vent length were recorded on day 1 of the experiment, and mass was again recorded on days 14 and 27.

Effects of spatial block, sex, average mass, snout-vent length, *Ichthyophonus* sp. infection and the presence of leeches or infected newts on newt survival were analyzed using a proportional hazards regression model with exponential errors and censoring, with the day on which a newt was observed to be dead as the response variable. Effects of these parameters on numbers of attached leeches and haematomas were tested with general linear models using newt as the replicate. For these analyses, the average log-transformed ($\ln[N + 1]$) numbers of leeches or new haematomas observed per day for each newt was used as the response variable. The analyses of leech attachment and haematoma formation were run twice, omitting the newts from tanks without leeches in the second analysis.

Haematoma duration was calculated as the time from first observation to the first day it was observed to have disappeared, and 95% confidence intervals (assuming a t-distibution) were calculated for mean haematoma duration and for the predicted duration of a new observation. For comparison with field data, the haematoma to leech ratio was estimated for each mesocosm by dividing the total number of haematoma observations (including repeated observations of the same haematomas) by the total number of attached leeches per day. The overall haematoma to leech ratio was also estimated for all mesocosms containing leeches. To make these estimations possible, the seven

haematomas lacking duration data were assumed to have lasted 6 days following the initial observation.

Experiment 2:

To test whether transmission would occur at a lower temperature, 6 newts held at 15°C were exposed to leeches which had previously fed upon infected newts. Twelve uninfected newts were collected from Penn Roosevelt lake (N 40°43'36.8", W 77°42'8.3"; Penn Roosevelt State Park, Centre County, PA) on 6/29/2005 and placed in individual 15 x 19 cm (2 L) plastic shoeboxes held in a refrigerator at 15°C with a 12:12 hr. light:dark cycle. Newts were held in filtered water from False Beaver pond and fed blackworms *ad libitum* as in the earlier experiment; water was changed once per week. Leeches were collected from Turtle Shell pond on 6/21/2005 (N = 50) and from Irrigation Pond (N 40°42'18.4", W 77°56'48.2"; Pennsylvania State University Agricultural Research Station) on 6/22/2005 (N = 70). Twenty leeches were placed in each of six 250 mL containers each containing one infected newt from Experiment 1 and allowed to feed at 15°C overnight from 6/29/2005 to 6/30/2005. Of these 120 leeches, approximately 90 (between 80 and 100; precise values not available due to destruction of one of the datasheets) fed on the infected newt during this time period based on the presence of blood in the gut. These leeches were transferred to new containers and not fed again until 7/18/2005, when the leeches fed on each of the infected newts were allowed to feed on each of six uninfected newts using the same procedure as before. Of the approximately 90 leeches which had fed on infected newts, approximately 65 (between 55 and 75) fed on the uninfected newts. Newts were held until termination of the experiment on 9/8/2005.

Power Analysis:

A binomial test statistic was used to estimate the sample size necessary to ensure at least one transmission event would occur given that laboratory conditions are conductive for transmission. Based on an experiment by Mock (1983), *Trypanosoma diemyctyli*, which utilizes *P. picta* as a vector, has a 3% probability of transmission given that a leech bites an uninfected newt after previously feeding on an infectious newt.

Based on the finding of Raffel et al. (*in press-a*) that *Ichthyophonus* sp. has approximately 1/6 the prevalence of *Trypanosoma diemyctlyi* across multiple populations and the prediction that prevalence should scale linearly with infection rate, we will assume that the probability of *Ichthyophonus* sp. transmission is 0.5%. Based on a two-tailed binomial test (alpha = 0.05), a sample size of 539 or more leeches feeding on uninfected newts following a previous feeding bout on an infectious newt would be necessary to conclude with 95% confidence that the transmission probability is less than 0.5%. The total number of leech bites with the potential for transmission was conservatively estimated (overestimated) for the first experiment assuming that all leeches survived and fed every 12 days, based on experimental measurements of leech bite frequency (unpublished data).

Results:

None of the focal uninfected newts became infected in either experiment. Average newt mass had a highly significant positive effect on survival in the first experiment ($X^2 = 24.7$, d.f. = 1, $P < 0.0001$), but none of the other variables had significant effects on survival (all $P > 0.1$). There were highly significant effects of leech addition on the number of attached leeches ($X^2 = 26.8$, d.f. = 1, $P < 0.0001$) and the number of new haematoma observations ($X^2 = 27.2$, d.f. = 1, $P < 0.0001$). A total of 45 attached leeches and 47 new haematoma observations were observed on newts in tanks with leeches, and none of the newts in tanks without leeches were ever observed to have haematomas. There were no significant effects of sex, average mass, snout-vent length, or individual *Ichthyophonus* sp. infection on either leech attachment or haematoma formation (all $P > 0.1$). When only tanks with leeches were considered, there were significant effects of spatial block ($F = 4.48$, d.f. = [1, 38], $P = 0.0180$) and the presence of infected newts ($F = 6.32$, d.f. = [1, 38], $P = 0.0163$) on the log number of attached leeches. Leech tanks with infected newts had significantly higher numbers of attached leeches than those without infected newts (Fig. 5.1a). There tended to be a greater rate of haematoma formation in tanks without infected newts (Fig. 5.1b), but this pattern was nonsignificant for each of the three blocks (all $P > 0.1$). The hematoma to leech ratios in the six leech tanks ranged from 1.35 to 12 and tended to be higher in tanks without

infected newts (Fig. 5.1c), with an overall ratio of 3.27 (147 haematoma observations and 45 leech observations). There were significant effects of sampling date ($F = 10.8$, d.f. = [2, 31], $P = 0.0003$) and block ($F = 7.9$, d.f. = [2, 31], $P = 0.0017$) on water temperature, with higher average temperature in Block C (Fig. 5.1d). There were no effects of the presence of leeches ($F = 1.1$, d.f. = [1, 30], $P > 0.5$) or infected newts ($F = 0.2$, d.f. = [1, 30], $P > 0.2$) on temperature.

Haematoma duration (time from first observation of a haematoma to the first day it was no longer present) ranged from 2 to 16 days (N = 29), with an average of 8.86 +/- 1.25 days (predicted 95% confidence interval for a new observation: 8.86 +/- 6.85 days). The average time from first to last observation of a haematoma was 5.93 days.

In experiment 1, the 63 leeches in the mesocosms without infected newts were known to have fed on infected newts, and if all lived and fed every 12 days they would have fed on newts approximately 252 times. The 120 leeches placed in mesocosms with infected newts (2 out of 8 infected) were not known to have previously fed on an infected newt, but if all of these lived and fed every 12 days there would have been approximately 114 bites with transmission potential in these mesocosms. There were a maximum of 85 bites with transmission potential in experiment 2, giving approximately 451 total bites with transmission potential in the two experiments. This number is lower than the 539 bites needed to reject the null hypothesis that the transmission rate was 0.5%.

Discussion:

These results provide no support for the leech transmission hypothesis but must be considered inconclusive until more specific predictions can be generated regarding the conditions necessary for transmission and the expected probability of transmission if conditions are ideal. While it is certainly possible that the leech transmission hypothesis is incorrect, the sample size of leech bites may have provided insufficient power to ensure detection of transmission. This infection has relatively low prevalence in most ponds compared to *Trypanosoma diemyctyli*, suggesting a relatively lower transmission rate, so that it may be necessary to have a very large sample size of leech bites before transmission will be detected. The estimated number of leech bites in this study with the potential for transmission was lower than that needed to conclude that the transmission

rate is lower than 0.5%, and actual number of leech bites was probably much lower since many of the leeches died during experiment 1.

In addition to a lack of statistical power, these experiments may lack some factor or factors necessary for transmission to occur, in addition to leech bites. One possibility is that none of the infected newts used in this study were contagious. All 12 infected newts had large numbers of visible sporse, but only 2 had any swelling of the axial musculature, a disease sign associated with the presence of active spores in wild-caught newts (Raffel et al. *in press-a*), and in neither case was this swelling very extensive. Based on this association, these newts were infected with predominantly passive spores, which are smaller in diameter than active spores, tend to fill up a lower proportion of the muscles, and are often surrounded by thick walls of host tissue (Mikaelian et al. 2000, Raffel et al. *in press-b*). These cyst walls may be greater than 70 μm in diameter (unpublished data). Raffel et al. (*in press-a*) proposed that leeches obtain *Ichthyophonus* sp. infection by inducing spore rupture with the proboscis during feeding bouts. If this hypothesis is correct, then active spores with their thinner cyst walls and greater cross-sectional surface area might be more infectious than passive spores. The lack of newts with abundant active spores may explain the failure to detect new infections in these experiments.

Another potentially missing factor is sufficient time. Field evidence suggests that transmission from leeches to newts occurs almost exclusively during the leech breeding season in April, May and June and is constant throughout this period, rather than peaking in June or July when with the influx of new juvenile leeches (Chapter 6). This result suggests that only adult leeches can transmit the infection, either because only adult leeches can become infected or because leeches do not become infectious until they mature. The latter would be the case if there was a very long latent period in leeches, so that leeches which become infected in the summer and autumn do not start infecting newts until the following spring breeding season. This hypothesis would explain the lack of transmission in these experiments, which lasted a maximum of 52 days, and would require much longer-term experiments to test. While a long prepatent period in newts would also explain this result, this is less likely since field evidence suggests a prepatent

period of less than 16 days based on observations of new *Ichthyophonus* sp. infections associated with leech haematomas (Chapter 6).

Although temperature has a strong influence on amphibian immunity and pathogen growth rates (Raffel et al. *in press-b*), it seems unlikely that high temperature was the only factor causing a lack of transmission in experiment 1. Transmission occurs in May and June in the field, at which time pond water temperatures can be comparable to the water temperatures observed in experiment 1 (Raffel et al. *in press-b*). In addition, no transmission was observed in experiment 2 when temperature was lower. Water quality is also unlikely to be a concern since the water for these experiments was taken from a pond with both leeches and *Ichthyophonus* sp. infected newts.

The frequent formation of small subcutaneous haematomas observed the mesocosms with leeches and complete absence of haematomas without leeches strongly supports Barta and Sawyer's (Barta and Sawyer 1990) observation that leeches frequently cause these haematomas during feeding bouts. The time to disappearance of these haematomas was approximately nine days on average and seldom lasted more than two weeks. These haematomas may therefore be useful for identifying recent sites of leech attachment in field studies.

The higher numbers of attached leeches in mesocosms containing infected newts is most likely due to twice as many leeches being added to these mesocosms than to the ones without infected newts. However, there was a trend toward lower numbers of leech-induced haematomas in these tanks despite the greater number of leeches. This pattern, as well as the lower haematoma to leech ratios in these mesocosms, may be due to a greater probability of haematoma formation from large adult leeches than from small juveniles. Adult leeches would have a larger proboscis which might be more likely to induce haematoma formation. An effect of leech size on the probability of haematoma formation may also explain the very high haematoma to leech ratios in some of the mesocosms compared to ratios seen in the field (Chapter 6), since the leeches in this experiment obtained larger sizes than commonly seen feeding on newts in field surveys (T. R. Raffel, personal observation). The lower number of attached leeches for mesocosms in Block C may have been caused by the significantly higher water

temperature in these mesocosms, which could have influenced leech survival or bite frequencies.

The finding that more massive newts had higher survival may be due to newts of greater mass having more energy stores and being better able to withstand stressful conditions. Previous studies have found effects of leeches on amphibian mortality (Brockelman 1969, Berven and Boltz 2001), but no effects were seen in this study, possibly due to insufficient sample size or fewer leeches per newt. There were also no effects of newt length, mass or sex on leech attachment, as was observed in natural ponds by Raffel et al (*in press-a*), which may be due to the much smaller sample size or to unnatural conditions influencing leech preferences or newt grooming behavior.

These experiments provide no evidence for leech-borne transmission of amphibian *Ichthyophonus* sp. but must be considered inconclusive in the absence of sufficient statistical power and factors which may be important for transmission. Future studies should focus on testing potentially important factors for transmission and refining predictions of the leech transmission hypothesis, so that future experiments can provide more definitive tests of whether leeches are the vector of *Ichthyophonus* sp. infection in amphibians.

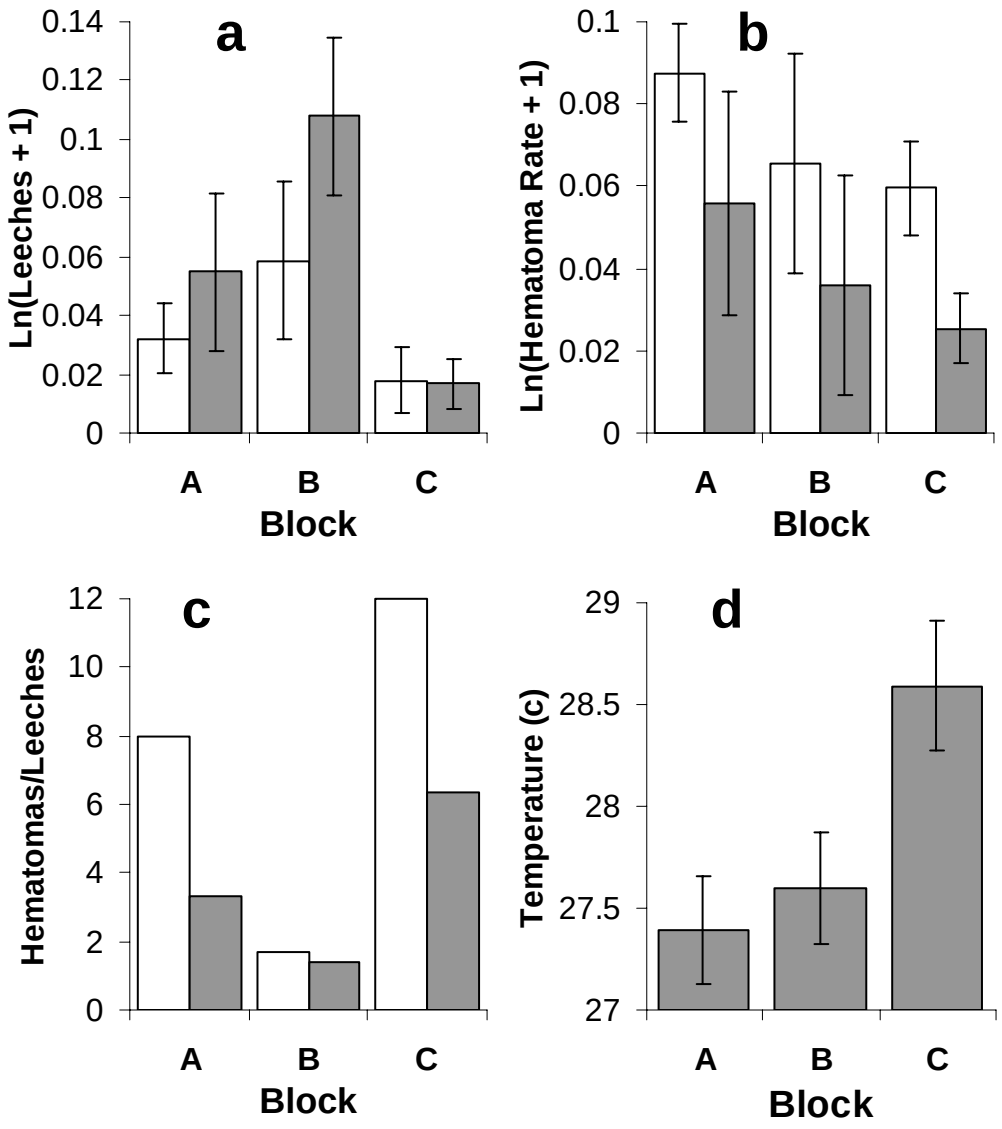

Figure 5.1. Differences in (a) numbers of attached leeches, (b) rate of haematoma formation (c) haematoma to leech ratios, and (d) temperature between spatial blocks and mesocosms with and without *Ichthyophonus* sp. infected newts. Filled and open bars represent mesocosms with and without infected leeches, respectively, except in (d) where data from all four tanks were pooled for each block. Leech attachment and haematoma formation were calculated as the mean log of numbers attached or haematomas formed per day for each newts and average for all newts in each mesocosm. Haematoma to leech ratio was estimated as the total number of haematoma observations divided by the total number of leech attachment observations for each mesocosm. Error bars represent the standard error of the mean for the 6 newts in each mesocosm, except in (d) where it represents the standard error for temperature measurements.

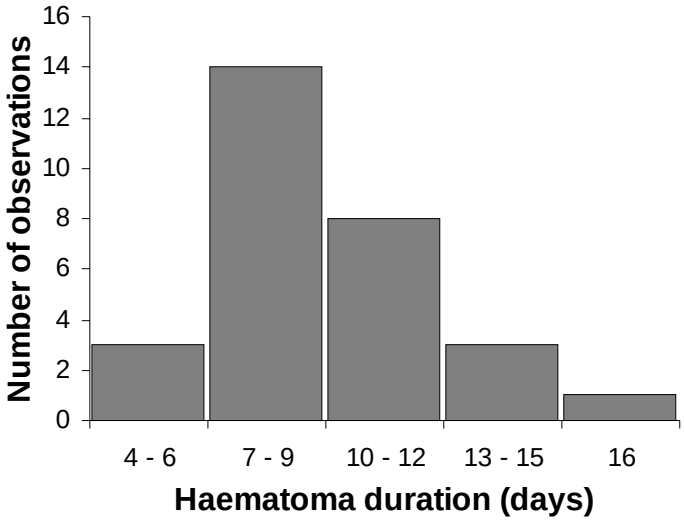

Figure 5.2. Distribution of haematoma duration times. Haematoma duration was calculated as the number of days from first observation of a haematoma to the first observation of its absence.

CHAPTER 6:

Longitudinal patterns of *Ichthyophonus* sp. infection in red-spotted newts: population-level effects and further evidence for leech-borne transmission

T. R. Raffel[1] and P. J. Hudson[1]

[1]Center for Infectious Disease Dynamics, Penn State University

Abstract:

We conducted a mark-recapture study in three newt populations, tracking *Ichthyophonus* sp. infection status of individual newts through time in order to directly measure seasonal changes in the infection rate and to estimate mortality and emigration due to infection. *Ichthyophonus* sp. is a parasite of the peripheral skeletal muscles which has been associated with amphibian mass morbidity events in North America and is thought to be transmitted by the amphibian leech, *Placobdella picta*. Results from this study corroborated predictions of the leech transmission hypothesis and showed that high vector-host ratios were associated with increased prevalence of infection. Female newts had higher numbers of attached leeches than males and this was associated with high short-term infection rates while they are in ponds. Overall males had higher prevalence of *Icthyophononus* sp. but they remained in the pond for longer with greater exposure. In general individuals infected with *Ichthyophonus* sp. appear to be more likely to emigrate from the pond than uninfected newts. These findings lead us to speculate that the summer exodus of newts from ponds may be parasite mediated.

Introduction:

The ultimate goal of epidemiological studies is often to determine which factors drive the risk of parasitic infection, which requires data on the number of individuals becoming infected through time (i.e., incidence) as opposed to prevalence or abundance data at discrete time points. Incidence data are common for many human pathogens and parasites, for which case mortality or case infection data are commonly recorded (e.g., Bjornstad et al. 2002), but are difficult to obtain for studies of wildlife animal populations due to the need to follow the infection status of individual hosts through time, i.e., longitudinal data (but see Cattadori et al. 2005). In addition to the ability to diagnose infection without killing hosts, collection of longitudinal data for non-sessile animals requires confinement of focal sentinels (e.g., Mock and Gill 1984, Komar 2001) or the use of capture-mark-recapture techniques (e.g., Mock 1987, Wetzel and Esch 1997). Determining the factors which influence the rate of infection may be especially important for amphibian parasites, given the potentially important role of parasites in the decline of amphibians worldwide (Daszak et al. 2003, Kiesecker et al. 2004, Lips et al. 2006).

Nevertheless, direct measurements of the infection rate in natural amphibian populations remain rare (but see Mock 1987, Wetzel and Esch 1996).

Ichthyophonus sp. infection in red-spotted newts (*Notophthalmus viridescens*) provides an excellent model system for the study of amphibian disease dynamics, since infection can be accurately diagnosed in live newts (Raffel et al. *in press-a*), making it possible to track infection status through time. This protist parasite of the skeletal musculature has been associated with mass morbidity events in North American amphibian populations (Herman 1984, Mikaelian et al. 2000, Green et al. 2002). Based on evidence from field studies, Raffel et al. (*in press-a*) suggested that the amphibian leech *Placobdella picta* may act as a vector for *Ichthyophonus* sp. infection in red-spotted newts, but further evidence will be necessary to confirm this hypothesis since experimental demonstration of *Ichthyophonus* sp. transmission by leeches has not yet been possible (Chapter 5).

In apparent contradiction of this hypothesis, female newts had lower *Ichthyophonus* sp. prevalence than male newts despite having a higher leech attachment rate (Raffel et al. *in press-a*). Since female newts are more likely to skip a year of breeding than male newts (Gill et al. 1983), Raffel et al. (*in press-a*) suggested females spend less time in the aquatic habitat, leading to lower long-term exposure over multiple breeding seasons despite high leech attachment rates. This hypothesis predicts higher leech bite frequency, force of infection, and emigration rates for female newts, in addition to male-biased sex ratios outside of the breeding season. The leech transmission hypothesis predicts high force of infection in ponds and months with high leech bite rates, a higher infection rate for female newts during periods when they have higher leech attachment rates, and an association between new infections and recent leech bites.

Ichthyophonus sp. exhibits a seasonal pattern of infection prevalence in newts, with infection levels increasing in late spring and decreasing in autumn and winter (Chapter 1). Leech bite rate was a significant positive predictor of seasonal changes in *Ichthyophonus* sp. prevalence, suggesting that the spring breeding season of *P. picta* is indeed an important driver of *Ichthyophonus* sp. dynamics (Chapter 1). However, this study could not confirm that seasonal changes in prevalence reflected true changes in infection rate rather than demographic changes in the newt population, and it lacked the

temporal resolution to determine whether the spring increase in prevalence was driven by increased bite rates of adult leeches or recruitment of new juveniles. Leech age may also be important for transmission, since the walls of tissue surrounding *Ichthyophonus* sp. spores can be thick relative to the size of a juvenile leech proboscis (some greater than 70 μm, unpublished data), or there may be an extended latent period such that leeches infected in summer and autumn cannot transmit the infection for several months (Chapter 5). These hypotheses predict high force of infection during the leech breeding season when larger adult leeches are thought to increase their biting frequency (Gill et al. 1983), rather than coinciding with the later peak in the total number of attached leeches due to recruitment of juvenile leeches.

The high morbidity sometimes caused by this infection and anecdotal observations of lethal secondary infections by *Saprolegnia* sp. in *Ichthyophonus*-induced skin ulcerations (Herman 1984, Raffel et al. *in press-a*) suggest that *Ichthyophonus* sp. may be a significant source of increased mortality for red-spotted newts. *Ichthyophonus* sp. infection might also induce newts to leave ponds temporarily, which would allow them to escape further infection by this and other aquatic parasites. Infection of female newts with *Trypanosoma diemyctyli* has been shown to induce them to skip a year of breeding (Gill et al. 1983), showing that newts may respond to parasitic infection by temporary emigration from ponds.

The aim of this study was to directly measure the force of infection and mortality/emigration due to infection for *Ichthyophonus* sp. in natural populations of newts, using capture-mark-recapture data to test predictions of the following hypotheses: (1) leeches transmit *Ichthyophonus* sp. infection between newts, (2) female newts have a greater rate of exposure to *Ichthyophonus* sp. due to having a higher leech attachment rate but lower long-term exposure due to spending more time on land, and (3) *Ichthyophonus* sp. infections lead to increased mortality and/or emigration rates of red-spotted newts.

Methods:

Study sites:

Three permanent ponds within 20 km of State College, Pennsylvania were chosen for the presence of large newt populations harboring *Ichthyophonus* sp. infection and for

having variable leech attachment rates. Mothersbaugh (N 40° 39' 12.2", W 77° 54' 9.6") is an abandoned beaver wetland in the Penn State Experimental Forest (Huntingdon County, Pennsylvania) which has decreased in size since the beavers left 5-10 years ago and contains an extremely dense newt population. Turtle Shell Pond (N 40° 52' 26.1", W 78° 4' 35.6") is a more recently abandoned beaver wetland in Moshannon State Forest (Centre County, Pennsylvania) which still has high water levels. False Beaver Pond (40° 42' 38.3", 77° 52' 54.3"), called "Beaver Pond" on maps, is a human impoundment in Rothrock State Forest (Huntingdon County, Pennsylvania) where the original dam has been replaced with piled rocks.

Diagnosis of *Ichthyophonus* sp. infection:

Ichthyophonus sp. infection was diagnosed by disease signs described by Raffel et al (*in press-a*), for which they found a 0% false positive rate in a random sample of 34 newts examined histologically. Newts were considered infected if spores were visible underneath the transparent skin of the throat, abdomen or limbs, or if the neck, back or tail had distinctive swelling of the axial musculature as described by Converse and Green (2005) and Raffel et al. (*in press-a*).

Diagnosis of leech haematomas:

Data were collected on the number of small (5-10 mm) subcutaneous haematomas visible on newts in False Beaver pond in the April marking session, the May recapture session, and for all newts starting in August 2006. These haematomas are frequently caused by biting *P. picta* leeches (Barta and Sawyer 1990) and may be accurate indicators of recent leech bites, especially by larger leeches (Chapter 5).

Marking Sessions:

Newts were captured using dip-nets (30 x 60 cm aperture, 3 mm mesh), except for a few winter sessions when newts were collected from under the ice by drilling holes and setting minnow traps on the bottom of the pond overnight. Marking sessions were conducted monthly (except in winter for Mothersbuagh and Turtle Shell ponds, where only a single winter marking session was conducted) and lasted for 1-4 consecutive days,

with a goal of marking at least 15% of the resident newt population in any given marking session. Mothersbaugh and Turtle Shell ponds were sampled from April 2004 to June 2005, and False Beaver pond was sampled from April 2005 to May 2006.

Newts were batch marked according to infection status and the date of marking with fluorescent elastamer dye injections (Northwest Marine Technology, Inc., Shaw Island, WA), using different colors to indicate *Ichthyophonus* sp. infection status at the time of marking (green = uninfected; orange = infected) and location of the mark on the abdomen to indicate the date of marking. Elastamer was injected subcutaneously using a 27-gauge needle by first inserting the needle through the body wall and pushing the tip of the needle back out between skin and muscle before injecting. This procedure results in a low rate of lost marks (< 1%, unpublished data), many of which can still be identified by traces of elastamer at the injection site. Newts were remarked at each capture, so that the capture history of each individual newt can be determined from its marks. Capture histories could therefore be reconstructed for all newts according to sex and infection status, but not for other variables that change from one marking session to the next such as the number of attached leeches. Sex, *Ichthyophonus* sp. infection status, the presence of any marks, and the number of attached leeches were recorded upon the capture of all newts.

Recapture Surveys:

A sweep survey was conducted on the day following the last day of each marking session to obtain an estimate of the proportion of newts marked, so long as the pond was free of ice. During each recapture survey, meter-long sweeps of a dip net (30 x 60 cm aperture, 3 mm mesh) were taken at regular three-step intervals in a gridded pattern throughout the entire pond out to a specified distance up the stream channel which was held constant for each pond. Four steps were taken between sweeps for the April, June, August and October recapture surveys in Mothersbaugh and Turtle Shell, which were part of a larger two-year seasonal survey of newt parasites (Chapter 1). Regular sweeps ensured that marked and unmarked newts in the pond would have equal capture probabilities regardless of the location at which marked newts had been released. Using a voice recorder, the sex, number of attached leeches, and *Ichthyophonus* sp. infection

status and intensity (i.e., proportion of body with disease signs) were recorded for each newt, and the number of newts was recorded for each sweep. Newts were not marked during recapture surveys.

Linear relationships between the total number of newts caught in each recapture sweep-survey and the size of the active newt population in the pond (see Results) were used to estimate the population size when mark-recapture estimates were unavailable.

Statistical Analyses:

The effects of pond and sex on the overall infection rate were tested using proportional hazards regression with exponential errors as described by Crawley (2002), in which the days to infection (the time from first observation of an uninfected newt to the first observation of this newt with an infection) was used as the response variable and newts which were never observed to become infected were censored. This is analogous to a survival analysis in which infection is substituted for mortality, and the daily hazard of infection is an estimate of the force of infection, defined as the rate of infection per susceptible (i.e., the number of new infections per susceptible individual per day, Hudson et al. 2002). Only newts observed in marking sessions were included in this analysis.

The effect of season on the infection rate was tested using generalized linear regression with binomial errors, in which we determined whether the probability of becoming infected in any given capture interval (the time between one capture and the next, N = 1694) depended upon whether that capture interval included a given month. Only newts observed in marking sessions were included in this analysis.

The effects of pond, sampling date and sex on *Ichthyophonus* sp. prevalence were tested using generalized linear regression with binomial errors, including only the first capture of each newt for a given sampling period (including both marking sessions and recapture surveys, N = 18, 270). The effects of pond, sampling date and sex on the leech bite frequency (number of attached leeches per newt) were tested using a generalized linear regression with poisson errors. Since *P. picta* leeches only remain attached for a few hours, all newt captures (N = 19,243) were included in this analysis. Differences in the haematoma to leech ratio between months were tested for using a chi-square contingency table.

Relative differences in mortality/emigration due to infection status or sex were analyzed using a proportional hazards regression model, with the total number of days known alive (one plus the difference between the dates of first and last capture) for each individual newt as the response variable (N = 6465). Weibull errors were found to substantially improve the fit of the model compared to exponential errors ($AIC_{Weibull}$ = 57,306 vs. $AIC_{Exponential}$ = 74051). Using the Weibull distribution relaxes the assumption of constant hazard (Crawley 2002), allowing for a change in the rate of mortality or emigration through time. Since only one value could be given to each newt for infection status, newts which became infected during the study were treated as two separate newts with different infection status. Newts which became infected or which were captured on the last sampling date were censored, since there was no opportunity for them to be recaptured. Date of first capture was included in the model as a covariate, since newts marked later in the study have less potential for being recaptured.

Results:

Seasonal changes in newt activity:

In all three ponds, the size of the active aquatic newt populations peaked in May, decreased to very low levels during summer, and had a second smaller peak in the autumn (Fig. 6.1). The May peak in adult newt density was driven largely by an influx of female newts, which caused a decrease in the proportion of male newts fom about 80% male to 35-60% male from May to July (Fig. 6.2). The proportion of male newts subsequently rose to 70-80% in August for all three ponds and remained high until the following spring (Fig. 6.2). In 2005, the influx of females occurred earlier in False Beaver, with the sex ratio dropping below 60% in March (Fig. 6.2). The total count of newts caught in each recapture survey was a strong predictor of the mark-recapture population estimate when blocked by pond ($F = 137.4$, d.f. = [1, 19], $P < 0.0001$, $R^2 = 0.9492$, AIC = 430.3693). This relationship was even stronger ($R^2 = 0.9543$, AIC = 427.9443) when the number of newts caught was corrected for differences in the sweep interval (multiplied by 4/3 when the sweep interval was every fourth step instead of every third). There was a highly significant main effect of pond in this relationship ($F = 20.3445$, d.f. = [2, 19], $P < 0.0001$), with fewer newts caught in Mothersbaugh sweep-

surveys relative to population size compared to False Beaver and Turtle Shell. Based on a sweep-survey estimate of population size, the active newt population in False Beaver decreased again to a low level in January (Fig. 6.1a).

Effects of pond and sex on *Ichthyophonus* sp. infection and leech attachment rates:

The force of infection differed significantly between ponds (Table 6.1), with high daily hazards of *Ichthyophonous* sp. infection in False Beaver ($1.59 * 10^{-4}$ infections per susceptible per day) and Turtle Shell ($1.90 * 10^{-4}$), and a lower daily hazard of infection in Mothersbaugh ($8.45 * 10^{-5}$; Fig. 6.3a). This pattern was consistent both with significant between-pond differences in *Ichthyophonus* sp. prevalence and with numbers of attached leeches (Table 6.2), with False Beaver and Turtle Shell having higher prevalences and numbers of leeches per newt than Mothersbaugh (Fig. 6.4).

Sex had a significant interaction effect with pond on the force of infection but no significant main effect, showing that the effect of sex on infection rate differed between ponds (Table 6.1). Females had a significantly higher force of infection than males (hazard = 2.26×10^{-4} vs. 1.21×10^{-4}) in False Beaver ($X^2 = 10.3$, d.f. = 1, P = 0.0013) and a significantly lower force of infection than males (hazard = 4.64×10^{-5} vs. 2.26×10^{-4}) in Mothersbaugh ($X^2 = 4.1$, d.f. = 1, P = 0.0441; Fig. 6.3b, c). There was no significant effect of sex on force of infection in Turtle Shell ($X^2 = 0.682$, d.f. = 1, $P > 0.4$, N = 115). These results were consistent with differences in *Ichthyophonus* sp. prevalence by sex in different ponds, for which there was also a significant interaction with pond in addition to overall higher prevalence in male newts (Table 6.2, Fig. 6.4). Males had higher prevalence than females in Mothersbaugh but approximately equal prevalence in False Beaver and Turtle Shell (Fig. 6.4). In False Beaver, prevalence started out somewhat higher for male newts (albeit not significantly, $X^2 = 2.2$, d.f. = 1, P = 0.1363) in April 2005 (16.0% of 832 males infected vs. 12.9% of 542 females) but increased more rapidly for female newts, so that prevalence was approximately equal in May (18.2% of 391 males vs. 18.8% of 532 females) and greater for female newts in June (19.0% of 348 males vs. 26.8% of 544 females; $X^2 = 7.3$, d.f. = 1, P = 0.0071) There was also a very highly significant effect of sex on the number of attached leeches (Table 6.2), with female newts having higher numbers of attached leeches than males in all three ponds

(Fig. 6.4). There was no effect of current *Ichthyophonus* sp. infection on the number of attached leeches ($X^2 < 0.1$, d.f. = 1, $P > 0.9$).

The force of infection appears to have declined with time since first capture in all three ponds, with the observed proportion of infected newts dropping off afer the first 100 days for all three ponds (Fig. 6.3). However, this proportion increased again in False Beaver after approximately 350 days to a level similar to the proportion infected at 50-100 days (Fig. 6.3). This pattern was especially strong of female newts, which dropped to zero newts infected since first capture observed at intermediate times since first capture in Mothersbaugh and Turtle Shell (Fig. 6.3b, c)

The proportion of infected newts in recapture surveys with swollen axial musculature also differed between ponds ($X^2 = 30.9$, d.f. = 2, $P < 0.0001$), with a higher percentage in Mothersbaugh (54.8%; 62/113) than in Turtle Shell (27.3%; 24/88) or False Beaver (14.1%; 29/205).

Seasonal changes in the force of infection:

There was a significant effect of the date of first capture on infection rate in the proportional hazard model, with newts caught earlier in the study more likely to become infected (Table 6.1). In the binomial analysis of seasonal effects on the force of infection, the only months with significant individual effects ($P < 0.05$) on the probability of becoming infected during a capture interval were April ($X^2 = 17.0$, d.f. = 1, $P < 0.0001$) and May ($X^2 = 20.3$, d.f. = 1, $P < 0.0001$). Both months remained significant when the other month was added to the model (April: $X^2 = 7.9$, d.f. = 1, $P = 0.0050$; May: $X^2 = 11.2$, d.f. = 1, $P = 0.0008$). This pattern was consistent across the three ponds but only significant for False Beaver (April: $X^2 = 4.7$, d.f. = 1, $P = 0.0294$; May: $X^2 = 10.0$, d.f. = 1, $P = 0.0015$), which may have been due to smaller sample sizes in the other two ponds ($N_{FB} = 1104$; $N_{MB} = 464$; $N_{FB} = 123$).

All observations of newts becoming infected within a known 1-month time interval occurred in April, May and June, and most were observed in False Beaver (26/537 infected vs. 3/157 in Mothersbaugh and 5/68 in Turtle Shell; Fig. 6.5). Thirteen of these new infections occurred from April to May 2005 in False Beaver, at which time it was observed that the new patch of *Ichthyophonus* sp. infection for six newly infected

newts was directly below a haematoma. This was a significantly higher proportion of newts with haematomas than was recorded in the subsequent recapture survey, during which haematomas were recorded for 38 out of 266 newts ($X^2 = 4.03$, d.f. = 1, P = 0.0447). Even given the fact that leeches and *Ichthyophonus* sp. signs are both more likely to be observed on the stomach and throat than on the limbs (Raffel et al. *in press-a*), the odds that all six new infections should have been within the same quadrat of the stomach or side of the throat as a haematoma was less than 1 in 46,000 ($P < 0.0001$). The haematoma to leech ratio varied between months in False Beaver ($X^2 = 76.9$, d.f. = 6, $P < 0.0001$, Table 6.3), with the highest ratio observed in May.

There were very highly significant effects of sampling date (month) on *Ichthyophonus* sp. prevalence (Table 6.2), which increased in April and May of 2004 in False Beaver, May of 2003 and 2004 in Mothersbaugh, and May of 2004 in Turtle Shell (Fig. 6.1). There were also significant seasonal differences in the numbers of attached leeches (Table 6.2), which peaked peaked in June in all three ponds and gradually decreased throughout the rest of the year, except in December when the number of leeches per newt transiently increased in False Beaver (Fig. 6.1).

Mortality/emigration and recovery from infection:

The number of days known alive was significantly higher in False Beaver than in Mothersbaugh or Turtle Shell (Table 6.1, Fig. 6.6), reflecting the proportion of each population which was marked during the study (average proportion marked in each session: False Beaver 0.244; Mothersbaugh 0.076; Turtle Shell 0.053). There was also a significant negative effect of the date of first capture (Table 6.1), probably because there were fewer opportunities for newts caught late in the survey to be recaptured. There was a significant interaction between sex and pond, but the main effect of sex was the same in all three ponds with females having fewer days known alive than males in all three ponds (Table 6.1, Fig. 6.6).

Although the main effect of *Ichthyophonus* sp. infection on days known alive was not significant, there were significant interactions between infection status and both sex and pond (Table 6.1). Female newts had fewer days known alive compared with male newts ($X^2 = 11.2$, d.f. = 1, P = 0.0008), with no significant interaction between

Ichthyophonus sp. infection and pond for female newts ($X^2 = 1.6$, d.f. = 2, $P = 0.4367$; Fig. 6.6). There was no main effect of *Ichthyophonus* sp. infection on days known alive for male newts ($X^2 = 0.2$, d.f. = 1, $P > 0.6$), but there was a significant interaction between pond and *Ichthyophonus* sp. infection ($X^2 = 13.2$, d.f. = 1, $P = 0.0014$). Newts infected with *Ichthyophonus* sp. had significantly fewer days known alive in Turtle Shell for both sexes ($X^2 = 9.9$, d.f. = 1, $P = 0.0016$). There was no significant main effect in False Beaver ($X^2 = 1.8$, d.f. = 1, $P > 0.1$), but there was a significant interaction between sex and infection status ($X^2 = 5.7$, d.f. = 1, $P = 0.0172$), where male newts had higher mean days known alive if infected (Fig. 6.6). This pattern was similar to that in Mothersbaugh, where there was no significant sex by infection interaction ($X^2 = 2.6$, d.f. = 1, $P > 0.1$) but where the greater mean days known alive for infected males (Fig. 6.6) led to a significant positive main effect of *Ichthyophonus* sp. infection ($X^2 = 4.5$, d.f. = 1, $P = 0.0339$; Fig. 6.6).

Out of 267 recaptures of 204 newts marked as infected with Ichthyophonus sp. in all three ponds, only one newt in Mothersbaugh no longer had detectable signs of infection, 280 days since this newt had last been observed.

Discussion:

The results from this study corroborated predictions of the leech transmission hypothesis and of the hypothesis that low prevalence in female newts is due to females spending less time in ponds than males. As predicted by the leech transmission hypothesis, False Beaver and Turtle Shell had higher rates of infection the Mothersbaugh, which had the lowest leech attachment rate. The relatively constant high infection rates in April, May and June coincide with the breeding season of adult leeches, rather than the June peak in leech abundance caused by an influx of new juvenile leeches. If leech transmission hypothesis is correct, this result indicates that leech age or maturity may be important factors for transmission of this infection, as suggested in Chapter 5. Possibly leech size is important for obtaining infection from infected newts, particularly if a larger proboscis is more likely to lead to rupture of *Ichyhophonus* sp. spores. Alternately, the infection may have a long latent period in leeches so that they are unable to transmit the infection until the season following parasite acquisition. Seasonal changes in prevalence

corresponded to changes in the rate of infection, showing that prevalence data can be used to reconstruct changes in infection rate for *Ichthyophonus* sp. infection in newts.

The higher prevalence of *Ichthyophonus* sp. infection in male newts was consistent with the results of Raffel et al. (*in press-a*), as were the higher numbers of attached leeches on females in all three ponds. False Beaver had overall higher prevalence in female newts, but males had higher prevalence in April 2005, before many newts would have contracted new infections. As predicted by the leech transmission hypothesis, female newts in False Beaver had higher infection rates than males, as shown by a proportional hazards analysis and a greater rate of increase in prevalence throughout the spring. The opposite effect of sex was observed in Mothersbaugh for the infection rate over the entire time period, but initial infection rates of male and female newts were similar (Fig. 6.1). The observation of higher infection rates for male newts might be due to the low recapture rate in this pond, which could have led to many of the females leaving the pond for summer before many new infections had been detected.

Perhaps the most compelling evidence for the leech transmission hypothesis is the observations of new patches of *Ichthyophonus* sp. infection associated with small subcutaneous haematomas. These haematomas are frequently formed when *P. picta* leeches feed (Barta and Sawyer 1990). Since thousands of attached leeches were observed in this study and no other potential sources of these haematomas are known (e.g., biting insects), it is probable that all such haematomas were induced by leech bites. In support of this assertion, the haematoma to leech ratios observed throughout this study and during May 2005 in particular were within the range recorded in the lab when leeches were the only potential source of these haematomas (Chapter 5). Indeed the ratios observed in the field were low relative to those observed in the lab especially later in the year. This might be due to the relatively large leeches used in the lab experiment, which seem more likely than small leeches to induce formation of detectable haematomas (Chapter 5). An association between recent leech bites and new infections seems unlikely to be due to an attraction of leeches to *Ichthyophonus* sp. disease signs, since leeches were not found to be more likely to feed on infected newts despite a sample size of >18,000 observations. Therefore the parsimonious explanation of this pattern is that leeches were the source of infection, though fulfilling Koch's postulates will still be

necessary to confirm whether leeches act as a vector for *Ichthyophonus* sp. infection in amphibians.

The higher short-term infection rate of female newts in False Beaver despite a higher prevalence in April lends support to the hypothesis that male newts have greater long-term exposure to *Ichthyophonus* sp. infection despite greater short-term exposure of female newts in the spring, as proposed by Raffel et al. (*in press-a*). Furthermore, the observations of male-biased sex ratios except in the spring suggest that females are more likely to leave the pond outside the breeding season. Female newts may also have a higher rate of mortality than males as in populations studied by Gill (1978b, 1983), but it seems unlikely that the difference in mortality between sexes would be so great as to cause the rapid change in sex ratios in July and August. Females also had lower recapture rates (shorter days-known-alive) than males in all three populations, which may be driven by females leaving ponds in the summer and/or a greater trendency for females to remain on land during the second breeding season, as observed by Gill (1983). Further analyses using robust design capture-mark-recapture models will be necessary to determine the degree to which each of these two factors drive the lower recapture rates for female newts.

Assuming that the leeches which caused formation of these haematomas were the same leeches that infected the newts, it is possible to estimate prepatent period of *Ichthoyophonus* sp. infection in newts, i.e., the time from initial infection till the parasite becomes detectable. Laboratory data have indicated that leech-induced haematomas last from 2 to 16 days (Chapter 5). Since leeches generally remain attached for less than a day (Barta and Sawyer 1990) and new infections have never been observed associated with currently attached leeches (T. R. Raffel, personal observation), the prepatent should be between 1 and 16 days.

Despite reports of morbidity due to *Ichthyophonus* sp. infection and suggestions that it may be a significant mortality factor for amphibians (Herman 1984, Green et al. 2002, Raffel et al. *in press-a*), the only pond where infection had a significant effect on the number of days known alive was Turtle Shell, making this the only pond which might have detectable mortality due to infection. This result was unexpected, since Turtle Shell had the lowest sample size out of the three ponds sampled and Mothersbaugh had a much

higher proportion of newts with swollen axial muscles, suggesting higher morbidity due to infection in this pond. The significant effect of infection status on days-known-alive for female newts, which was consistent between populations, suggests mortality due to infection in female newts, whereas the effect of infection on male newts varied by pond and appears to have been positive in False Beaver and Mothersbaugh.

Rather than indicating mortality due to infection, these results may be more consistent with the hypothesis that *Ichthyophonus* sp. infection causes increased rates of temporary emigration from ponds. Mortality and permanent emigration are impossible to distinguish using capture-mark-recapture methods, and the single year of sampling in each of these ponds is insufficient to distinguish between permanent emigration and temporary emigration that lasts for more than a few months. Since older adult newts are unlikely to stop breeding permanently and have high site fidelity to ponds once they start breeding (Gill 1978b, 1979), permanent emigration of adult newts due to infection seems unlikely. It is simpler to suppose that the shorter days-known-alive for infected female newts is caused by infected females skipping the second year of breeding, as has been shown to occur for female newts infected with *Trypanosoma diemyctyli* (Gill et al. 1983). Although the seasonal pattern of adult emigration from ponds varies by location, newts commonly emigrate in the summer and in some locations have been observed returning to ponds in autumn (Brimley 1921, Noble 1929, Morgan and Grierson 1932, Chadwick 1944, Hurlbert 1969, Healy 1974, Gill 1978b, Harris et al. 1988, Johnson 2002). The seasonal abundance patterns observed in this study suggest that adult newts in this area follow this seasonal breeding strategy of leaving ponds in summer and returning in autumn, at least in permanent ponds.

The decreased proportion of newly infected newts (infected since first capture) at intermediate time scales in False Beaver and Mothersbaugh, especially for female newts (Fig. 6.1), suggests that recently infected newts have an increased probability of temporarily emigrating from the pond during the summer. If the decrease had been caused by mortality due to infection, the proportion of infected individuals would not have increased again at the end of the study, a pattern presumably caused by the return of these infected newts for the next breeding season. Temporary emigration in summer has been documented for red-spotted newts by Gill (1978b), who suggested that newts left

ponds to avoid extreme high temperatures and to remove attached leeches. Perhaps temporary emigration of *Ichthyophonus* sp. infected newts and a higher survival rate on land can explain the apparently higher survival rates of infected male newts in Mothersbaugh and False Beaver.

The reasons for the decrease in *Ichthyophonus* sp. prevalence throughout the summer and autumn remain unclear, though mortality and/or emigration due to infection probably play a role. Recruitment of new adults has been observed in the autumn and spring (Hurlbert 1969), and this influx of new susceptible individuals may explain why prevalence does not return to higher levels in autumn as infected adults return to the pond after spending the summer on land. Certainly this decrease cannot be explained by recovery from infection, since nearly every *Ichthyophonus* sp. infected newts retained detectable disease signs when recaptured. The one newt which had apparently cleared the infection after nearly a year suggests that some newts can eventually clear *Ichthyophonus* sp. infection, but this newt might also have been mistakenly marked as infected. The very low rate of recovery observed in this study does not necessarily imply that newts are incapable of controlling *Ichthyophonus* sp. infection, since histological examination often reveals host-derived granulomas surrounding passive spores, especially in mild infections lacking swelling of the axial musculature (Raffel et al. *in press-a*). Full recovery might be detected more often if newts were followed for multiple years, as was the case for *T. diecyctlyi* infections (Mock 1987), but *Ichthyophonus* sp. seems to generally cause chronic granulomatous infections which might increase in severity when the host becomes immunosuppressed, similar to tuberculosis infections in mammals (Manabe and Bishai 2000).

Qualitatively, the lower infection rate in Mothersbaugh is consistent with the lower leech attachment rates in this pond. However, quantitatively the infection rate in this pond seems high for the very small number of attached leeches observed. One potential explanation is that newts are more susceptible to infection due to high population density in Mothersbaugh, which may cause stress-induced immune suppression (Rollins-Smith 2001, Tella et al. 2001, Moore and Jessop 2003). Infected newts had more severe disease signs in Mothersbaugh than in the other ponds, with a much higher proportion having swelling of the axial musculature. Since these newts tend

to contain more active spores than those with milder infections visible in the throat, abdomen or limbs (Raffel et al. *in press-a*), they may be more infectious to leeches (as suggested in Chapter 5). Turtle Shell and False Beaver might also have had higher rates of false negative diagnoses if newts in these ponds were more likely to have subclinical infections underneath the opaque skin of the neck, back and tail. More sophisticated models will be necessary to determine whether increased infection severity can account for the infection rate observed in Mothersbaugh.

Based on these results and those of Raffel et al. (*in press-a*), it is possible to generate a working hypothesis for the overall life cycle of *Ichthyophonus* sp. infection in red-spotted newts. Leeches induce rupture of *Ichthyophonus* sp. spores during feeding bouts, either by chemical cues or mechanical action of the leech proboscis, causing the release of endospores which could infect the proboscis (Raffel et al. *in press-a*). Once they infect the leech, these endospores develop into infectious amoebae, ready for injection into amphibian muscle tissues during future feeding bouts (Raffel et al. *in press-a*). The amoebae then develop into spores in the muscle, probably reaching a visible size 2 – 16 days following infection (based on the predicted 95% confidence interval of 2.01 to 15.71 days from Chapter 5).

In conclusion, longitudinal patterns of *Ichthoyphonus* sp. infection corroborate predictions of the leech transmission hypothesis and lead to the additional prediction that leech age is an important factor for transmission, possibly due to a long latent period in leeches. Female newts have higher short-term infection rates due to higher rates of leech attachment but lower long-term exposure due to spending more time on land. Finally, parasites such as leeches, trypanosomes and *Ichthyophonus* sp. may induce temporary emigration of newts from ponds and may therefore be an important factor in the seasonal reproductive strategy of red-spotted newts.

Table 6.1: Proportional hazards model outputs measuring the force of infection and relative rates of mortality/emigration (days known alive) for newts in all three ponds. "Days to infection" of susceptible individuals is the reciprocal of the force of infection, defined as the infection rate of susceptible individuals. Interactions between pond, sex (M = 1), and *Ichthyophonus* sp. infection ("Ich") were included if they added significantly to the model ($P < 0.05$ for addition to model). The date of first capture for each newt relative to the first date of marking in that pond (Date 1st) was included as a covariate in both models. The total sample sizes are shown in parentheses.

Response	Predictor	Coef.	Δ Dev	d.f.	P
Days to infection[1]	Date 1st	0.010	16.3	1	< 0.0001
(N = 1367)	Pond		6.5	2	0.0378
	Sex (M)	-0.238	3.3	1	0.0712
	Pond:Sex		11.7	2	0.0029
Days known alive[2]	Date 1st	-0.003	430.6	1	< 0.0001
(N = 11,450)	Pond		2155.4	2	< 0.0001
	Sex (M)	1.438	302.6	1	< 0.0001
	Ich	-0.357	3.2	1	0.0743
	Pond:Sex		120.2	2	< 0.0001
	(M)		11.1	2	0.0038
	Ich:Pond	0.297	4.2	1	0.0416
	Ich:Sex (M)				

[1]Proportional hazards model, exponential errors
[2]Proportional hazards model, weibull errors

Table 6.2: Model outputs from generalized linear models testing for significant predictors of *Ichthyophonus* sp. infection prevalence and number of leeches per newt. Interactions between pond and sex (M = 1) were included if they added significantly to the model ($P < 0.05$ for addition to model). The month during which each observation was made was included in both models. The total sample sizes are shown in parentheses.

Response	Predictor	Coef.	Δ Dev	d.f.	P
Ichthyophonus[1]	Pond		807.4	2	< 0.0001
(N = 18,290)	Month		117.7	11	< 0.0001
	Sex (M)	0.101	4.0	1	0.0446
	Pond:Sex		19.9	2	< 0.0001
Leeches[2]	Pond		3473.1	2	< 0.0001
(N = 19,244)	Month		2966.1	11	< 0.0001
	Sex (M)	-0.088	16.5	1	< 0.0001

[1]Binomial error distribution
[2]Poisson error distribution

Table 6.3. Average numbers of leeches and haematomas observed per newt, and ratios of total haematoma observations to total numbers of attached leeches, for different months in False Beaver pond. Total numbers of haematomas and leeches are shown in parentheses (haematomas/leeches).

Month	Leeches	Haematomas	Haematoma/leech ratio
Apr	0.068	0.127	1.86 (203/109)
May	0.053	0.150	2.86 (40/14)
Aug	0.280	0.080	0.29 (2/7)
Jan	0.067	0.167	2.50 (5/2)
Feb	0.273	0.273	1.00 (3/3)
Mar	0.332	0.145	0.44 (61/140)
Apr	0.196	0.168	0.86 (108/126)

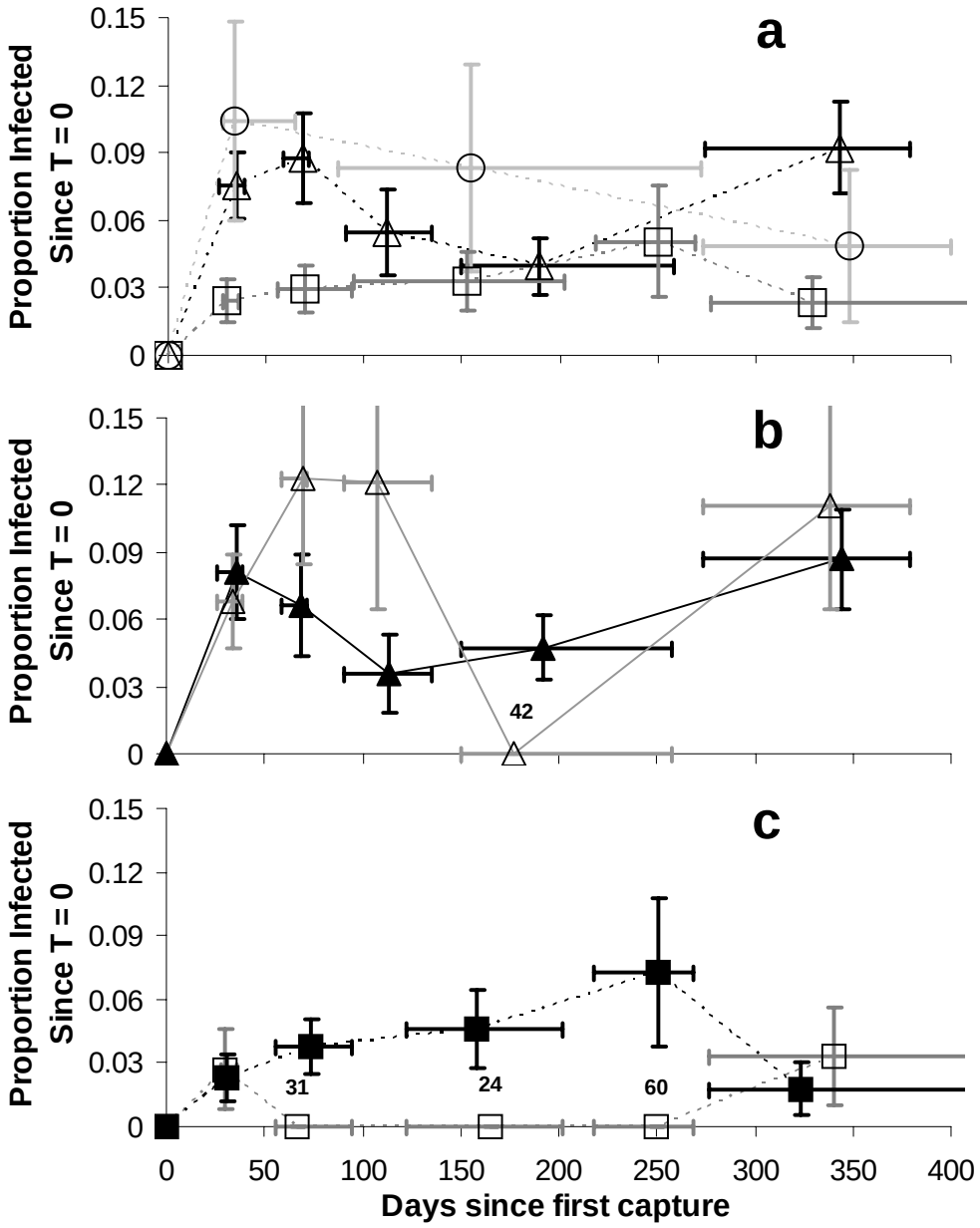

Figure 6.1: Proportion of originally uninfected newts which had become infected at various times since first capture in (a) different populations (△ False Beaver, □ Mothersbaugh and ○ Turtle Shell), (b) different sexes in False Beaver (closed symbols male; open symbols female), and (c) different sexes in Mothersbaugh. The rate of change in the proportion infected indicates the force of infection, assuming no mortality or immigration/emigration due to infection. Displayed are proportions of uninfected newts which have become infected for ranges days since first first capture (point = average of days of capture, horizontal error bar = range). Vertical error bars indicate the standard error of a proportion.

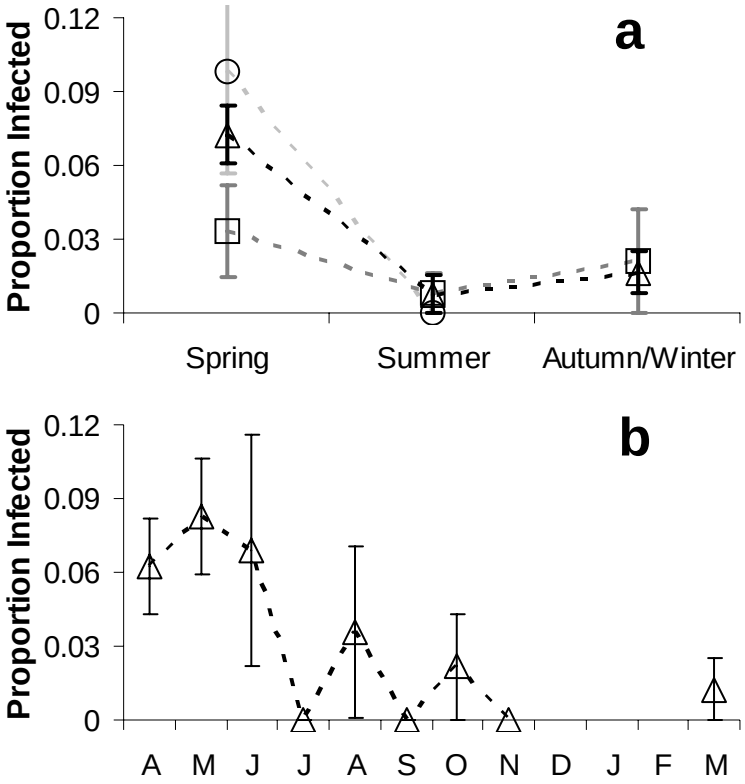

Figure 6.2: Proportion of originally uninfected newts which became infected in (a) different seasons for all three ponds (△ False Beaver, □ Mothersbaugh and ○ Turtle Shell) and (b) different months for False Beaver. The proportion infected in (b) is the monthly force of infection for False Beaver (i.e., infections per susceptible per month). Error bars indicate the standard error of a proportion. Symbols represent the proportion of newts becoming infected which had a capture interval (interval between one capture and the next) within the indicated month or season (i.e., known to have become infected during that month or season).

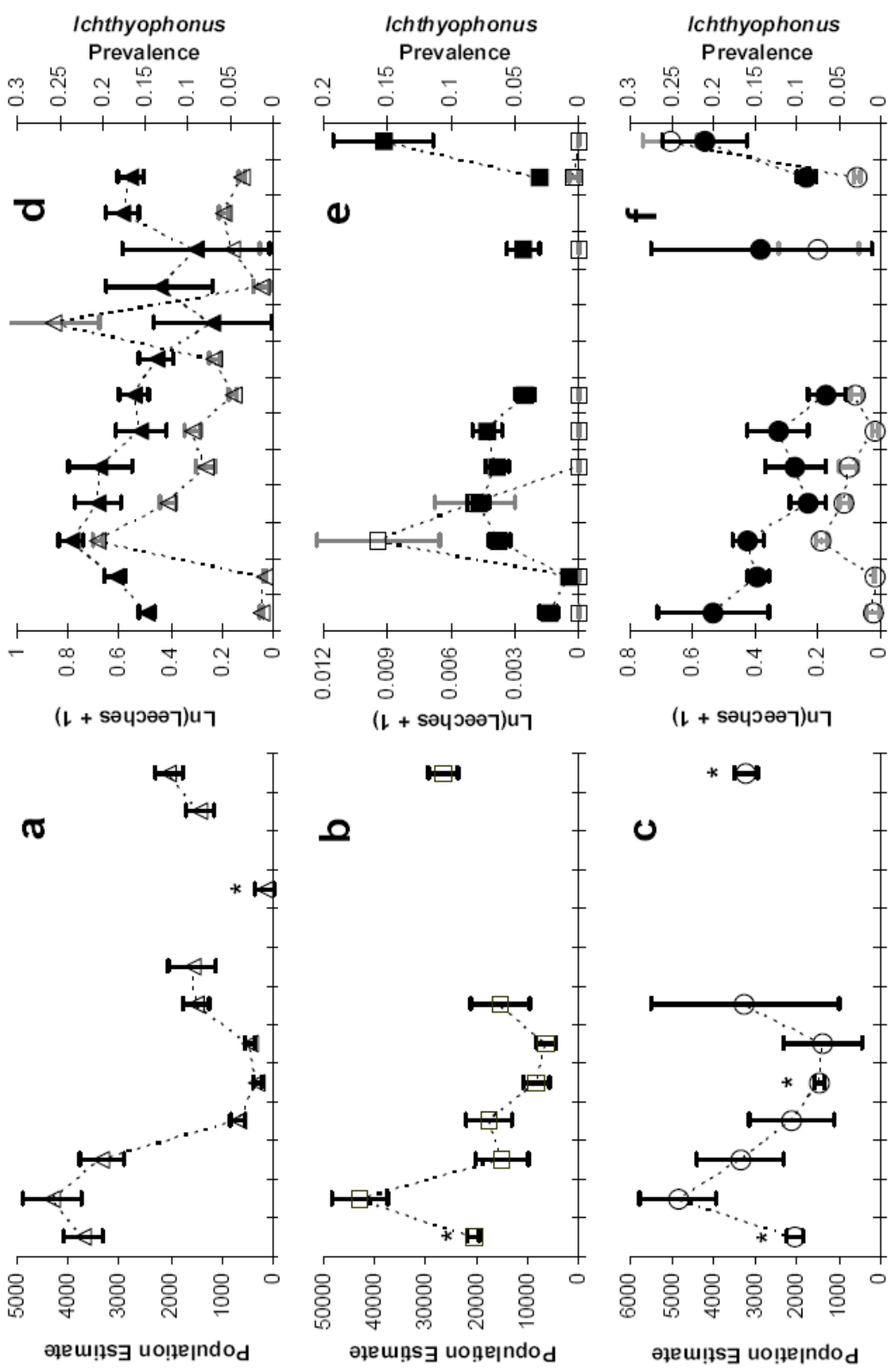

Figure 6.3: Monthly changes in newt population size (a, b, c), leech bite rate (open symbols; d, e, f), and *Ichthyophonus* sp. prevalence (d, e, f: closed symbols) for the False Beaver (a, d), Mothersbaugh (b, e) and Turtle Shell (c, f) populations. Population sizes are estimates based on closed-population capture-mark-recature estimates at each time point; five population sizes were estimated from sweep survey density esimates (*). Leech bite rate is presented as the mean log abundance of leeches per newt. Error bars represent standard deviations of population sizes (a, b, c) and the standard errors of leech bite rates (d, e, f) and *Ichthyophonus* sp. prevalence.

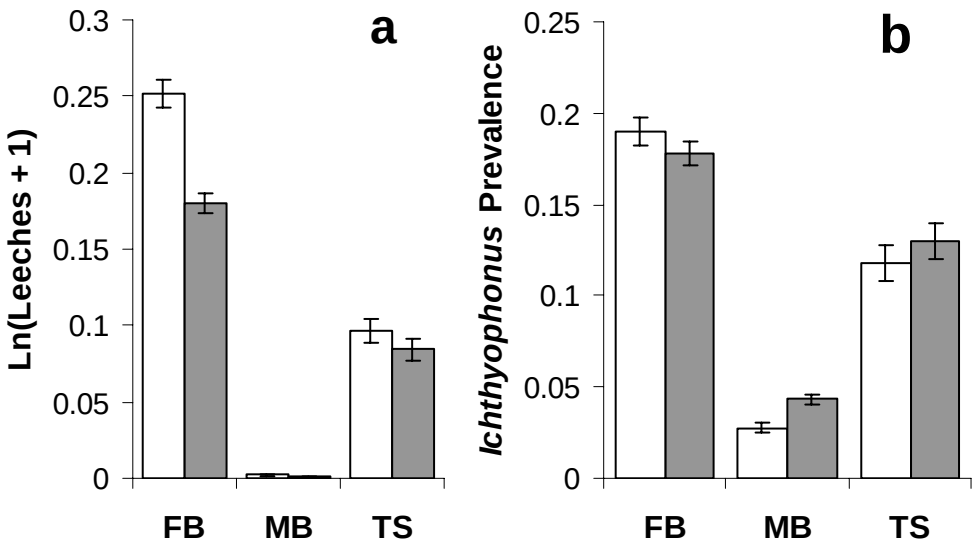

Figure 6.4: Effects of sex on (a) leech attachment frequency (mean log abundance of leeches per newt, ln[N + 1]) and (b) *Ichthyophonus* prevalence ("FB" = False Beaver, "MB" = Mothersbaugh, "TS" = Turtle Shell). Open and grey bars represent females and males, respectively. Error bars indicate standard errors.

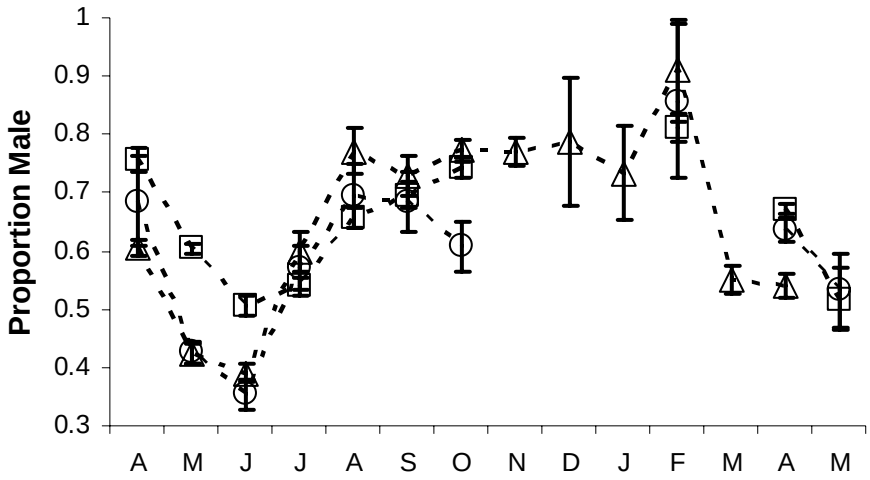

Figure 6.5: Monthly changes in sex ratio for False Beaver (△), Mothersbaugh (□) and Turtle Shell (○), presented as the proportion of the population which is male. Error bars represent the standard error of each proportion.

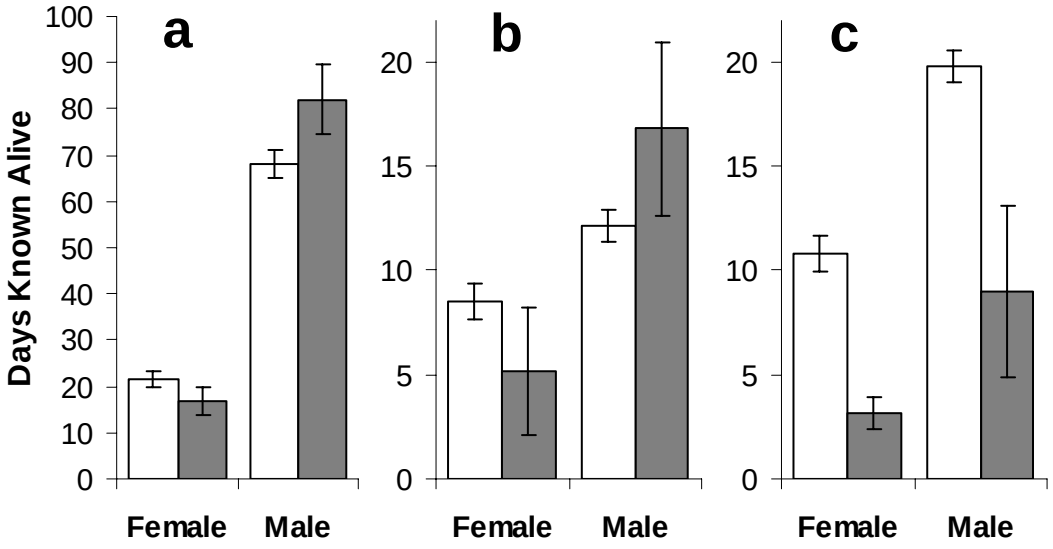

Figure 6.6: Effects of sex and infection status on the number of days known alive for newts in (a) False Beaver, (b) Mothersbaugh, and (c) Turtle Shell. Open and grey bars represent uninfected and infected individuals respectively. Bars indicate means +/- standard errors.

FINAL DISCUSSION:

Parasites of red-spotted newts generally have high infection rates in early and late spring, but no single factor can account for this general pattern of high spring infection rates for newt parasites, which appear to be driven by many different seasonal factors (Chapter 1). These similarities may instead be driven by bottom-up effects of high spring productivity on the abundance of intermediate and alternate hosts, both of which appear to be important driving factors of parasite dynamics in newts (Chapter 1). Productivity may be an ultimate rather than proximate cause of seasonality in many cases, with organisms timing seasonal reproductive bouts to coincide with periods of high food abundance. Most recent reports of seasonal parasite dynamics in other amphibian species have also found high infection rates in the spring (Introduction), suggesting that this may be a general pattern for parasites of amphibians, at least in temperate regions.

Although temperature-driven seasonal changes in immunity do occur in red-spotted newts, immune parameters were consistently low relative to temperature in autumn rather than in spring (Chapter 3), and seasonal changes in temperature-dependent immune parameters such as lymphocytes and eosinophils had little apparent effect on parasite dynamics (Chapter 1). The finding that levels of immunity are lower than optimal during times of both increasing and decreasing temperature suggests that temperature variability causes increased susceptibility to infection, despite the presence of additional seasonal cues which newts could use to anticipate and counteract these effects. Possibly there has been little selection pressure for newts and other amphibians to develop mechanisms to counteract an autumn depression of immunity, since autumn infection rates appear to be low for most newt parasites (Chapter 1). However, the effects of temperature variability on immunity might increase in importance following the introduction of cold-tolerant pathogens such as chytrid fungus or the increased temperature variability predicted to result from human-induced climatic warming.

The discovery of adult *Hysterothylacium* sp. nematodes in red-spotted newts is highly unusual since no other adult worm of this or any closely related genus has ever before been described from an amphibian. Especially surprising is the fact that these nematodes came from newts living in ponds without sympatric fish. *Hysterothylacium burtti* resembles *H. analarum* and *H. brachyurum*, parasites of North American

freshwater fishes, and might have recently diverged from one of these species following repeated spillover from a fish host (Chapter 2).

Taken together, several independent correlations presented in Chapters 4 and 6 provide compelling support for the hypothesis that the amphibian leech *Placobdella picta* is the vector of *Ichthyophonus* sp. infection in red-spotted newts. Alternate hypotheses for transmission and to explain correlations between leeches and *Ichthyophonus* sp. infection have so far not been supported by the data. This conclusion is based on correlational evidence, however, and cannot be considered conclusive until leech-borne transmission can be confirmed experimentally (Chapter 5).

Assuming that *Ichthyophonus* sp. is vectored by leeches, it is possible to create a general picture of the life cycle and general ecology of this infection in red-spotted newts. Spore capsules of members of the order Ichthyophonida generally release endospores which develop into infectious amoebae (Mendoza et al. 2002). Leeches may induce rupture of *Ichthyophonus* sp. spores during feeding bouts, either by chemical cues or mechanical action of the leech proboscis, causing the release of endospores which could infect the proboscis. Once they infect the leech, these endospores could develop into infectious amoebae, ready for injection into muscle tissues of uninfected amphibians during future feeding bouts. The amoebae would then develop into spores in the muscle, probably reaching a visible size 2 – 16 days following infection (Chapter 6).

Several factors might influence *Ichthyophonus* sp. transmission if this mechanism is correct. Pond eutrophication may lead to higher infection rates, since leech abundance correlates with availability of emergent vegetation (Chapter 4). The extent of infection in newts should influence the probability of transmission, since leeches would have to bite an affected area to become infected (Chapter 3). Transmission to feeding leeches may also be more likely if the infection is composed of active spores rather than predominantly passive spores, which tend to take up less muscle volume and to be encysted by host tissue which might prevent spore rupture (Chapter 5). Based on the temporal association of new infections with the spring breeding season of leeches rather than total leech abundance, leech size or age may influence transmission, possibly due to an extended latent period such that leeches infected in the summer and autumn do not become infectious until the following spring (Chapter 6).

Perhaps the most exciting implications of this study are for the evolution of the red-spotted newt life history strategy. Parasites have been shown to influence the timing of reproduction in several species, for which high levels of parasitism have often been found to induce earlier reproduction (Lafferty 1993, Michalakis and Hochberg 1994). The predicted response to parasites is reversed in organisms for which parasites are specific to adults, leading to extension of the juvenile stage and a greater size at first reproduction (Michalakis and Hochberg 1994). This would allow increased annual reproductive output during their adult lives, which may be shortened by high parasite burdens.

Newt populations in much of eastern North America have terrestrial juveniles that return to ponds after several years to reproduce (Petranka 1998). Gill (1978b, Gill 1978a) suggested that the terrestrial juvenile stage is an adaptation for increased dispersal in landscapes with ephemeral aquatic habitats, leading to an increased need for metapopulation dynamics. However, terrestrial newts would also have reduced infection risk, since most of their parasites have aquatic stages (Chapter 1). Several patterns suggest that parasites drive newts to temporarily leave ponds. Gill et al. (1983) found that female newts which skipped years of breeding had unusually high trypanosome burdens and suggested that females with high burdens were more likely to skip a year of breeding, though subsequent studies have failed to confirm this effect (Gill and Mock 1985). Patterns of recapture rates presented in Chapter 6 may indicate a similar effect of *Ichthyophonus* sp. infection on female and possibly even male newts. *Ichthyophonus* sp. infection also appears to induce temporary emigration from ponds during the summer (Chapter 6). Newts have also been observed leaving ponds in an apparent effort to remove attached leeches (Gill 1978b). Since the effects of other parasites have yet to be investigated, emigration to land may be a general response to parasitism in red-spotted newts.

Adding to the complexity of the newt reproductive schedule is their unique seasonal breeding strategy. Newts living in permanent ponds have an extended breeding season lasting from early autumn until the following spring, when female newts oviposit (Petranka 1998). Newt breeding is interrupted for only a few months during the summer, when both male and female newts lose the horny excrescences characteristic associated

with breeding (Gage 1891, T. R. Raffel unpublished data), and many leave the pond until autumn breeding (Chapter 6). This temporary exodus seems unlikely to be driven by a need to disperse, since adult newts have strong site fidelity (Gill 1978a), by risk of predation, since newts are unpalatable to most predators (Hurbert 1970), or by better foraging opportunities on land, since juvenile newts grow faster in aquatic habitats (Healy 1973). Gill (1978b) and Hurlbert (1969) suggested that adults leave ponds in summer in response to extreme temperature conditions, but this seems an inadequate explanation for emigration from permanent ponds with relatively cool water during summer. Gill (1983) also suggested that parasites may cause temporary emigration from ponds. High parasite burdens in summer due to high spring infection rates, and continued risk of infection by some parasite species, may provide strong selection pressures favoring temporary emigration during the summer (Chapter 1).

For the few newts which remain in ponds during the summer, cessation of breeding might allow more effective immune defenses against new infections and for the clearance of infections acquired in springtime. In support of this hypothesis, field patterns suggest that male newts lose their secondary sexual characteristics sooner in the spring if infected with *Ichthyophonus* sp. (Chapter 4), and preliminary results suggest that breeding has a negative effect on immunity (unpublished data). Temporary emigration and reduced summer breeding may therefore both be adaptive mechanisms to increase the probability of successful future reproduction by reducing the current risk of mortality or morbidity due to infection. Since newts are excellent model organisms for experimental ecology, these patterns provide a rare opportunity to test how parasites influence the evolution of complex life history strategies in vertebrates.

APPENDIX A: SUPPLEMENTARY STATISTICS

Appendix A1: Seasonal effects on the burden or prevalence of parasites detected only in **dissected** newts, with **data from some years or seasons omitted to balance statistical designs.** Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with $P < 0.05$ were excluded from models. Populations included in each analysis are indicated in parentheses.

Response	Predictor	Coef.	Δ Dev	d.f.	P
Spiroxys[1]	Pond		353.2	3	0.0000
N = 258	SV	0.193	31.9	1	0.0000
(MB, TS, LA, MN)	**Season**		23.4	3	**0.0000**
	Season:Pond		25.0	8	**0.0016**
Amphibiocapillaria[1]	Pond		146.9	3	0.0000
N = 255	SV	0.062	8.0	1	0.0048
(MB, TS, LA, MN)	Sex	-0.259	13.8	1	0.0002
Brachycoelium[1]	Pond		20.2	3	0.0002
N = 259	Year		20.1	1	0.0000
(MB, TS, LA, MN)	SV	-0.323	23.2	1	0.0000
	Season		37.0	3	**0.0000**
	Season:Year		8.6	3	**0.0347**
	Pond:Season		24.5	8	**0.0019**
Plagitura[1]	Pond		61.3	2	0.0000
N = 221	Year		11.9	1	0.0006
(MB, TS, LA)	**Season**		2.8	3	**0.4218**
	Year:Pond		33.8	2	0.0000
	Season:Year		9.1	3	**0.0285**
	Pond:Season		25.5	6	**0.0003**
	Season:Year:Pond		29.2	4	**0.0000**
Trypanosoma[1]	Pond		12.1	1	0.0005
N = 147	Year		3.5	1	0.0606
(MB, TS)	**Season**		4.5	3	**0.2135**
	Pond:Year		4.1	1	0.0420
	Pond:Season		10.22	3	**0.0167**
Metacercaria[1]	Pond		49.7	1	0.0000
N = 138	**Season**		17.4	3	**0.0006**
(MB, TS)	SV	0.063	3.5	1	0.0609
	Pond:SV		12.5	1	0.0004
Neoechinorhynchus[1]	Year		5.4	1	0.0197
N = 91; (MB)	**Season**		20.2	3	**0.0002**
Spirurid cysts[1]	SV	0.226	5.3	1	0.0207
N = 91; (MB)	**Season**		8.3	3	**0.0398**
Bacterial load[2]	Pond		21.4	3	0.0001
N = 178	**Season**		90.5	4	**0.0000**
(MB, TS, LA, MN)	**Pond:Season**		50.5	11	**0.0000**

[1]Late spring removed

[2]Only 2004 Survey included (Early spring 2004 to Winter 2005)

Appendix A2: Seasonal effects on the burden or prevalence of parasites detected in **all observed** newts, including those collected for dissection, with **data from some years or seasons omitted to balance statistical designs.** Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with $P < 0.05$ were excluded from models. Populations included in each analysis are indicated in parentheses.

Response	Predictor	Coef.	Δ Dev	d.f.	P
Ichthyophonus[2]	Pond		15.8	2	0.0003
N = 1956	Year		2.7	1	0.1021
(MB, TS, LA)	SV	0.232	19.0	1	< 0.0001
	Season		7.0	3	**0.0704**
	Pond:Season		14.4	6	**0.0258**
	Year:Season		10.1	3	**0.0177**
Clinostomum[1]	Year		17.8	1	< 0.0001
N = 1603	SV	0.091	5.8	1	0.0161
(MB)	**Season**		52.8	3	**< 0.0001**
	Year:Season		8.1	3	**0.0431**
Yeast-cysts[1]	Pond		19.8	1	< 0.0001
N = 1793	Year		14.3	1	0.0002
(MB, LA)	SV	-0.514	25.5	1	< 0.0001
	Season		14.2	3	**0.0027**
Leeches[2]; N = 1579	Pond		89.7	2	< 0.0001
(MB, LA, TS)	**Season**		96.1	4	**< 0.0001**

[1]Late spring removed

[2]Only 2004 Survey included (Early spring 2004 to Winter 2005)

Appendix A3: Seasonal effects **within each newt population** on the burden or prevalence of parasites only detected in **dissected** newts, using **all available data** from populations with sufficient parasite infection levels. Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with P > 0.05 were excluded from models. Sample sizes are indicated in parentheses.

Response	Pond	Predictor	Δ Dev	d.f.	P
Spiroxys[1]	MB (114)	SV	7.8	1	0.0053
		Season	33.4	4	**< 0.0001**
	TS (74)	Season	5.7	4	0.2243
	LA (93)	SV	7.0	1	0.0084
		Season	12.9	4	**0.0118**
	MN (66)	SV	7.8	1	< 0.0001
		Season	17.6	3	**0.0497**
Amphibiocapillaria[1]	MB (115)	Season	9.2	4	0.0569
	TS (74)	Season	5.7	4	0.2259
	LA (92)	Season	7.1	4	0.1317
	MN (63)	Sex	9.8	1	0.0018
		Season	0.4	3	0.9401
Brachycoelium[1]	MB (115)	Year	10.9	2	0.0042
		Season	31.6	4	**< 0.0001**
		Year:Season	8.0	3	**0.0466**
	TS (74)	Year	0.5	1	0.4985
		Season	2.8	3	0.4273
		Year:Season	5.1	1	**0.0240**
	LA (93)	Year	11.6	2	0.0030
		SV	20.8	1	< 0.0001
		Season	15.7	4	**0.0035**
	MN (66)	Season	5.4	3	0.1431
Plagitura[1]	MB (115)	Year	29.1	2	< 0.0001
		Season	2.8	4	0.5948
	TS (74)	**Season**	12.1	3	**0.0070**
	LA (94)	Year	16.3	2	.0003
		Season	27.8	4	< 0.0001
		Year:Season	33.9	3	< 0.0001
Trypanosoma[2]	MB (115)	Year	7.4	1	0.0065
		Season	8.9	3	**0.0310**
	TS (74)	**Season**	11.6	4	**0.0203**
Metacercariae[1]	MB (115)	SV	17.7	1	< 0.0001
(MB, TS)		**Season**	17.0	4	**0.0019**
	TS (74)	**Season**	14.4	4	**0.0061**
Bacterial load[1]	MB (109)	Year	10.6	2	0.0050
		Season	64.8	4	**< 0.0001**
		Year:Season	7.8	1	**0.0053**
	TS (73)	Year	10.6	2	0.0050
		Season	64.8	4	**< 0.0001**
		Season:Year	7.8	1	**0.0053**
	LA (92)	Year	10.6	2	0.0049
		Season	36.1	4	**< 0.0001**
	MN (65)	**Season**	15.9	3	**0.0012**

[1]Negative binomial error distribution
[2]Binomial error distribution

Appendix A4: Seasonal effects **within each newt population** on the burden or prevalence of parasites detected in **all observed** newts, using **all available data** from populations with sufficient parasite infection levels. Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with $P < 0.05$ were excluded from models. Sample sizes are indicated in parentheses.

Response	Pond	Predictor	Δ Dev	d.f.	P
Candida[2]	MB (5063)	Season	7.1	4	0.1319
	LA (244)	Year	11.6	2	0.0030
		SV	21.6	1	< 0.0001
		Season	15.2	4	0.0044
Ichthyophonus[2]	MB (1860)	Year	12.2	2	0.0023
		SV	4.6	1	0.0326
		Season	11.1	4	**0.0253**
		Year:Season	8.4	3	**0.0391**
	TS (334)	SV	24.1	1	< 0.0001
		Season	2.3	4	**0.6841**
	LA (267)	**Season**	15.3	4	**0.0041**
Placobdella[1]	MB (5063)	Year	21.8	3	< 0.0001
		Season	21.4	4	**0.0003**
	LA (267)	**Season**	90.4	4	**< 0.0001**
	TS (419)	Year	54.9	2	< 0.0001
		Season	124.1	4	**< 0.0001**

[1]Poisson error distribution
[2]Binomial error distribution

Appendix A5: Seasonal effects **within each newt population** on the burden or prevalence of parasites only detected in **dissected newts**, with **data from some years or seasons omitted to balance statistical designs.** Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with P < 0.05 were excluded from models. Sample sizes are indicated in parentheses.

Response	Pond	Predictor	Δ Dev	d.f.	P
Spiroxys[1]	MB (90)	SV	8.5	1	0.0036
		Season	30.1	3	**< 0.0001**
	TS (54)	Season	3.3	3	0.3528
	LA (73)	SV	9.6	1	0.0020
		Season	12.0	3	**0.0075**
	MN (43)	SV	29.8	1	< 0.0001
		Season	15.5	2	**0.0004**
Amphibiocapillaria[1]	MB (91)	Year	4.8	1	0.0278
		Season	8.1	3	**0.0443**
	TS (54)	Season	5.2	3	0.1597
	LA (73)	Season	6.0	3	0.1117
	MN (43)	Sex	15.5	1	< 0.0001
		Season	0.1	2	0.9455
Brachycoelium[1]	MB (91)	Year	4.9	1	0.0273
		Season	33.5	3	**< 0.0001**
		Year:Season	8.9	3	**0.0307**
	TS (54)	Year	0.5	1	0.4985
		Season	2.8	3	**0.4273**
		Year:Season	5.1	3	**0.0240**
	LA (73)	SV	11.3	1	0.0008
		Season	11.9	3	**0.0079**
	MN (46)	**Season**	6.2	2	**0.0456**
Plagitura[1]	MB (91)	Year	21.7	1	< 0.0001
		Season	1.7	3	0.6428
	TS (54)	**Season**	12.1	3	**0.0070**
	LA (74)	Year	7.5	1	0.0063
		Season	23.8	3	**< 0.0001**
		Season:Year	29.1	3	**< 0.0001**
Trypanosoma[1]	MB (91)	Year	7.4	1	0.0065
		Season	8.9	3	**0.0310**
	TS (54)	Season	4.6	3	0.2000
Metacercariae[1] (MB, TS)	MB (91)	SV	13.8	1	0.0002
		Season	12.6	3	**0.0057**
	TS (54)	**Season**	13.0	3	**0.0046**
Bacterial load[2]	MB (47)	**Season**	29.5	4	**< 0.0001**
	TS (46)	**Season**	99.5	4	**< 0.0001**
	LA (46)	**Season**	16.6	4	**0.0023**
	MN (36)	**Season**	22.4	3	**< 0.0001**

[1]Late spring removed
[2]Only 2004 Survey included (Early spring 2004 to Winter 2005)

Appendix A6: Seasonal effects **within each newt population** on the burden or prevalence of parasites detected in **observed** newts, including those collected for dissection, with **data from some years or seasons omitted to balance statistical designs**. Pond, snout-vent length and sex were included as covariates in all analyses, and predictors with $P < 0.05$ were excluded from models. Sample sizes are indicated in parentheses.

Response	Pond	Predictor	Δ Dev	d.f.	P
Candida[1]	MB (1874)	**Season**	4.2	3	**0.2450**
	LA (189)	Year	11.6	1	0.0007
		SV	21.6	1	< 0.0001
		Season	9.7	3	0.0209
Ichthyophonus[1]	MB (1603)	Year	5.8	1	0.0160
		SV	10.0	1	0.0015
		Season	12.5	3	**0.0058**
		Year:Season	8.4	3	**0.0379**
	TS (162)	SV	9.1	1	0.0026
		Season	1.1	3	**0.7655**
	LA (190)	**Season**	9.3	3	**0.0258**
Placobdella[2]	MB (1579)	**Season**	20.2	4	**0.0005**
	LA (139)	**Season**	45.7	4	**< 0.0001**
	TS (221)	**Season**	39.9	4	**< 0.0001**

[1]Late spring removed

[2]Only 2004 Survey included (Early spring 2004 to Winter 2005)

Appendix A7: Tests for seasonality in parasitism with data from previously published studies of newt parasites. All analyses were run using a binomial error distribution due to the absence of published abundance data for individual newts. Data were categorized into five seasons (early spring, late spring, summer, autumn, and winter), and datasets lacking data in two or more seasons were excluded from this analysis. All datasets were from a single year of sampling from a single population except for the Russell 1951 dataset. In this case, data from several populations had been pooled by the author and could not be distinguished based on data presented in the published paper, so these were treated as a single replicate population in this analysis.

Reference	N	Parasite	Δ Dev	d.f	P
Holl 1932	123	*Amphibiocapillaria*[1]	23.4	4	0.0001
		Cosmocercoides dukae	14.4	4	0.0062
		Fessisentis acutulus[2]	14.2	4	0.0067
		Opisthodiscus americanus	3.3	4	0.5030
		Philometra	3.8	4	0.4285
		Plagitura salamandra	12.6	4	0.0136
Jarroll 1979*	249	*Bothriocephalus rarus*	8.0	4	*0.0922
Joy & Pennington 1998[5]	124	*Megalodiscus temperatus*[5]	22.7	4	0.0001
Joy & Scott 1997[5]	124	*Amphibiocapillaria*	12.4	4	0.0146
Joy and Thomas 1997[5]	124	*Fessisentis necturorum*	40.0	4	< 0.0001
Mock 1984*		*Trypanosoma diemyctyli*	-	-	***
Rankin 1937	111	*Amphibiocapillaria*[1]	14.6	4	0.0057
		Brachycoelium	6.1	4	0.1946
		Cosmocercoides dukae	5.4	4	0.2531
		Aegytianella ranarum[6]	18.1	4	0.0012
		Entamoeba	10.6	4	0.0314
		Eutrichomastix batrachorum	12.7	4	0.0128
		Hexamastix batrachorum	21.7	4	0.0002
		Hexamitus batrachorum	21.9	4	0.0002
		Hexamitus intestinalis	31.3	4	< 0.0001
		Karotomorpha swezi	20.9	4	0.0003
		Proteocephalid cysts	4.7	4	0.3162
		Prowazekella longifilus	3.2	4	0.5300
		Spiroxys contortus[3]	7.6	4	0.1056
		Tritrichomonas augusta	13.9	4	0.0077
		Trypanosoma diemyctyli[4]	41.3	4	< 0.0001
Russell 1951†	1358	*Brachycoelium*	6.0	4	0.1997
		Megalodiscus rankini[5]	97.5	4	< 0.0001
		Plagitura parva	54.0	4	< 0.0001
		Plagitura salamandra	12.7	4	0.0127

[1]Called "*Capillaria*" by author, redescribed by Moravec (Moravec 1986)
[2]Called "*Acanthocephalus acutulus*" Van Cleave 1931 by author, redescribed by McAlpine (1997)
[3]Called "Spirurid cysts" by author, fits descriptions of *Spiroxys contortus* (Hedrick 1935)
[4]Called "*Cryptobia borreli*" by author; probably *Trypanosoma diemyctyli* (Appendix B)
[5]Same dataset
[6]Called "*Cytamoeba bacterifera*" by author, redescribed by Desser (1987)
*Statistical tests of seasonality presented in reference

APPENDIX B: PARASITE NOTES

Amphibiocapillaria tritonispunctati (syn. *Capillaria tenua*, *Capillaria brevicollis*, *Capillaria brachyauchenia*, *Capillaria inequalis*):

The capillariid nematodes seen in this study resembled description of *C. tenua* (Mueller 1932); however, the four described species of *Capillaria* in newts have been synonymized by Moravec (Moravec 1986) and placed in the genus *Amphibiocapillaria*. We accepted the conclusions of Moravec (Moravec 1986) and (Joy and Scott 1997) that these species are synonymous.

Brachycoelium sp. (Cheng 1958)

Despite Rankin's (1938) suggestion that all described species of *Brachycoelium* in North American amphibians are synonymous with *B. salamandrae*, we observed two distinct types. The more common species (506 specimens examined) fits previous descriptions of *B. salamandrae* but lacks obvious spines in its tegument (Cheng 1958); the other type (79 specimens) is clearly spinous, shorter relative to its width, smaller as mature adults, and contains an enlarged cirrus sac, resembling descriptions of *B. obesum*, which Cheng (1960) believed was a distinct species from *B. salamandrae*. This parasite uses land snails (*Zonatoides* sp.) and slugs (*Agriolimax* sp.) as intermediate hosts (Cheng 1960). For the purposes of this study, we assumed that both species have similar life cycles (i.e., transmission only on land) and therefore pooled the data for statistical analyses.

Candida sp. cysts:

White subcutaneous cysts were observed in several newts during this survey, especially in Little Acre pond in the early spring. To the authors' knowledge this infection has not been previously described in amphibians. These cysts are generally elongate (appx. 0.5-1 by 1-2 mm) and often curved, resembling tiny encysted maggots. Microscopic examination of dissected cysts and preparations of histological sections revealed that these cysts were filled with white pus made up of single spherical cells, 6.0 μm in diameter. Culturing this pus on cornmeal agar was unsuccessful in most cases but did on one occasion result in an unusual *Candida* sp. colony with pseudohyphae which may be the etiological agent of the disease (unpublished data). Researchers in West Virginia have found similar cysts from which they also managed to obtain *Candida* sp. cultures (personal communication, Deborah S. Merritt, Marshall University, West Virginia). In preliminary attempts to fulfill Koch's postulates, one out of fourteen newts developed a small white cyst at the inoculation site 24 hours after inoculation (unpublished data). This cyst faded after 16 days and could not be confirmed as matching observed infections in wild newts (unpublished data). Newts appear capable of clearing this infection, since the cysts from an infected newt which was brought into the lab was virtually invisible when examined approximately two weeks following collection (Personal observation, T. R. Raffel). The negative effect of snout-vent length on infection probability suggests that this parasite induces immunological memory to infection, which would account for the difficulties in fulfilling Koch's postulates. For the purposes of this paper we will refer to this parasite as "*Candida* sp. cysts".

Clinostomum sp. metacercariae:

Clinostomum sp. are common trematode parasites of fish and amphibians, in which metacercariae encyst in the subcutaneous musculature and form large yellow cysts that are generally visible upon external examination of the host (Hopkins 1933, Muzzall 1991). Adult worms are found in the gular pouches of wading birds, and *Helisoma* sp. snails act as first intermediate hosts (Edney 1950). These parasites were common in Mothersbaugh newts, as might be expected given the abundance of *Helisoma* sp. in this pond. Since trematodes that infect fish or other amphibians would no longer be available for infecting newts, densities of these hosts would not drive parasite dynamics but should instead act as a sink for the parasite and cause a decrease in the infection rate of newts.

Ichthyophonus sp. (syn. *Hystocystidium ranae*, Goodchild 1953)

The precise taxonomic status of this protist parasite of the peripheral skeletal musculature has yet to be determined, but recent evidence strongly suggests that the amphibian leech plays an important role in transmitting the parasite between amphibians, probably as an intermediate host or vector (Raffel et al. *in review*). *P. picta* bite rate was the strongest between-population predictor of *Ichthyophonus* prevalence, as for *T. diemyctyli* (Raffel et al. *in review*). Amphibian *Ichthyophonus* infects several amphibian species but is not found in freshwater fishes, and it has not been observed in amphibian larvae (Mikaelian et al. 2000). Spores of this parasite are often visible in the subcutaneous musculature and may cause severe swelling of skeletal muscles sometimes accompanied by ulceration of the skin (Raffel et al. *in review*).

Neoechinorhynchus saginatus (Van Cleave and Bangham 1949): New host record

Acanthocephalans from the intestines of Mothersbaugh newts were identified as *Neoechinorhynchus saginatus* based on the keys of Amin (1987) and Amin (2002) and the description of Van Cleave and Bangham (1949). To the authors' knowledge, this species has not previously been reported from red-spotted newts. *N. saginatus* uses an ostracod intermediate host (*Cypridopsis vidua*) and uses creek chubs (*Semotilus atromaculatus*) and fallfish (*S. corporalis*) as definitive hosts (Uglem and Larson 1969, Muzzall and Bullock 1978). Creek chubs were by far the most abundant fish in Mothersbaugh and ostracods were common food items in the intestines of Mothersbaugh newts (observation, T. R. Raffel), suggesting that the presence of *N. saginatus* in newts may be due to spillover from the normal definitive host.

Placobdella picta (Verrill, 1872), syn. *Batrachobdella picta*, *Desserobdella picta* (Siddall et al. 2005)

The amphibian leech *P. picta* is a common ectoparasite of newts in North America. This species feeds only on amphibians but is not specific to any particular amphibian species (Sawyer 1972). Like other leeches, this *P. picta* is thought to breed in the spring, with young leeches taking their first blood meals from amphibian larvae (Gill et al. 1983, Sawyer 1986). In support of this assertion, many of the leeches observed on newts in the late spring survey of this study were very small compared to leeches observed in other seasons (personal observation, T. R. Raffel). Obtaining this

first blood meal appears to help determine leech survival to the following year, so that high leech densities may be determined by densities of amphibian larvae in the preceding year (Gill et al. 1983). Leeches are an important source of mortality for amphibian larvae (Brockelman 1969) and act as important vectors of many diseases in aquatic systems. *P. picta* acts as a vector for *Trypanosoma diemyctyli* and amphibian *Ichthyophonus* (Barrow 1953, Raffel et al. *in review*). *P. picta* is also the vector for *Aegyptianella ranarum*, an intraerythrocytic bacterial pathogen of amphibians which has been found in red-spotted newts (Rankin 1937, Desser 1987, Zhang and Rikihisa 2004). Related leech species have been found to transmit *Haemogregarina balli* in turtles (Siddall and Desser 2001) and *Trypanosoma mukasai*, *Babesiosoma mariae* and *Cyrilia nili* in fishes (Negm-Eldin and Davies 1999)

Plagitura salamandra (Holl 1928):

Plagitura from the Little Acre and Turtle Shell populations were identified as *P. salamandra* based on morphology and the absence of *Helisoma* sp. snails (the intermediate host for *Plagitura parva*) in these ponds (Stunkard 1936). *P. salamandra* has been shown to use *Pseudosuccinea columella* (a lymnaeid snail) as its first intermediate host and is unable to infect *Helisoma anceps* (Owen 1946). The overall correlation of *P. salamandra* with *Physa* sp. abundance in this study and the absence of *P. columella* suggest that physid snails can also act as intermediate hosts for this parasite. Some of the worms from Mothersbaugh, where both *Helisoma* sp. and *Physa* sp. snails occur, had morphological characteristics consistent with either *P. parva* or *P. salamandra* (Stunkard 1936), but the correlation of trematode abundance with *Physa* sp. abundance within Mothersbaugh suggests that these worms were all *P. salamandra*. *Plagitura salamandra* uses a variety of second intermediate hosts but its metacercariae are primarily found in dragonfly larvae and adult aquatic insects (Owen 1946).

Spiroxys contortus:

Larval nematodes encysted in the lining of the stomach wall were identified as *Spiroxys contortus* based on descriptions in (Hedrick 1935) and (Bartlett and Anderson 1985). S. *contortus* uses turtles as definitive hosts and copepods (*Cyclops*) as first intermediate hosts (Hedrick 1935). The larval stage found in newts (second intermediate host) has also been reported from minnows, tadpoles (Hedrick 1935) and some snails (Bartlett and Anderson 1985).

Trypanosoma diemyctyli (Tobey 1906)

This flagellate is a common blood parasite of red-spotted newts and other amphibians which uses *Placobdella picta*, the amphibian leech, as an intermediate host or vector (Barrow 1953). Leech bite rate is a strong predictor of *T. diemyctyli* prevalence between populations of newts (Raffel et al. *in review*). The ecology of this parasite has been studied in detail by Mock (1983), who later reported seasonal dynamics for this parasite (Mock and Gill 1984). Rankin (1937) reported seasonal dynamics of "*Cryptobia borreli*", which we assumed to have been *T. diemyctyli* based on morphological similarities between the two genera (Tobey 1906, Woo 2003), the commonness of *T. diemyctyli* in red-spotted newts (Barrow 1953, Mackiewicz 1954,

Gill and Mock 1985, Siddall and Desser 1992), and the absence of *Cryptobia* sp. reports in subsequent literature on newt parasites.

Unidentified metacercariae:

Metacercariae of an unknown trematode species were common in newts from Mothersbaugh and Turtle Shell ponds. This parasite was similar to unidentified metacercariae in three previous studies, which described metacercariae in the liver, kidney and coelomic mesenteries of newts surrounded by thick hyaline cysts (Kelley 1934, Fischthal 1955, Muzzall et al. 2003). All known digenetic trematodes have a mollusc host at some stage in their life cycle (Schnell 1985), but the mollusc intermediate host for this species is undetermined. Although *Physa* sp. snails were found at very low abundance in both ponds, *Helisoma* sp. snails were the most abundant mollusc in Mothersbaugh, and fingernail clams (Sphaeriidae) were the only molluscs found in Turtle Shell throughout most of the survey. *Helisoma* sp. snails from Mothersbaugh have high shedding rates of echinostome cercariae, but experimental exposure of three newts with 80 cercariae each led to no apparent infections upon examination three weeks later (unpublished data).

Unidentified nematode larvae

Numerous nematode larvae with bilateral cephalic symmetry were found encysted in the pancreas of many newts in Mothersbaugh. Although we were not able to identify this nematode to species or even family, we feel confident that this species has not been previously described in red-spotted newts. The most unusual characteristic of this nematode is its extreme aggregation within the pancreas, where multiple larvae were nearly always found in single cysts, ranging from one to well over one hundred larvae per cyst. The distribution of this parasite was so aggregated within hosts as to require analysis using binomial errors and presence-absence data, because models with negative binomial errors using count data would usually not even run, much less fit the data. These patterns suggest within-host replication of some type, despite the lack of eggs in any of the worms we found and the lack, so far as we know, of asexual reproduction (aside from parthenogenesis) in any known nematode species (Anderson 2000).

LITERATURE CITED:

Aho, J. M. 1990. Helminth communities of amphibians and reptiles: comparative approaches to understanding patterns and processes. Pages 157-195 *in* G. E. Esch, A. O. Bush, and J. M. Aho, editors. Parasite Communities: Patterns and Processes. Chapman and Hall, New York.

Ahumada, J. A., D. Lapointe, and M. D. Samuel. 2004. Modeling the population dynamics of *Culex quinquefasciatus* (Diptera : Culicidae) along an elevational gradient in Hawaii. Journal of Medical Entomology **41**:1157-1170.

Alexander, M. A., and J. K. Eischeid. 2001. Climate variability in regions of amphibian declines. Conservation Biology **15**:930-942.

Altizer, S., A. K. Davis, K. C. Cook, and J. J. Cherry. 2004a. Age, sex, and season affect the risk of mycoplasmal conjunctivitis in a southeastern house finch population. Canadian Journal of Zoology **82**:755-763.

Altizer, S., A. Dobson, P. Hosseini, P. J. Hudson, M. Pascual, and P. Rohani. 2006. Seasonality and the dynamics of infectious diseases. Ecology Letters **9**:467-484.

Altizer, S., W. M. Hochachka, and A. A. Dhondt. 2004b. Seasonal dynamics of mycoplasmal conjunctivitis in eastern North American house finches. Journal of Animal Ecology **73**:309-322.

Amin, O. M. 1987. Key to the families and subfamilies of Acanthocephala, with the erection of a new class (Polyacanthocephala) and a new order (Polyacanthorhynchida). Journal of Parasitology **73**:1216-1219.

Amin, O. M. 2002. Revision of *Neoechinorhynchus* Stiles & Hassall, 1905 (Acanthocephala : Neoechinorhynchidae) with keys to 88 species in two subgenera. Systematic Parasitology **53**:1-18.

Anderson, R. C. 1960. On the development and transmission of *Cosmocercoides dukae* of terrestrial molluscs in Ontario. Canadian Journal of Zoology **38**:801-825.

Anderson, R. C. 2000. Nematode parasites of vertebrates: Their development and transmission., 2nd edition. CABI Publishing, New York.

Anderson, R. C., A. G. Chabaud, and S. Willmott, editors. 1974. CIH keys to the nematode parasites of vertebrates. Commonwealth Agricultural Bureaux, Bucks, England.

Bahrmann, C. & Ayers, B. 2005. State College Hourly Weather Data 6/27/2001-9/1/2005, collected by Pennsylvania Department of Environmental Protection. Retrieved September 1, 2005 from PA State Climatologist website (DEPSTC, State College): http://climate.psu.edu/data/mesonet/datainv.php

Baker, M. R. 1979. Seasonal population changes in *Rhabdias ranae* Walton, 1929 (Nematoda, Rhabdiasidae) in *Rana sylvatica* of Ontario. Canadian Journal of Zoology **57**:179-183.

Barrow, J. H. 1953. The biology of Trypanosoma diemyctyli (Tobey). I. Trypanosoma diemyctyli in the leech, Batrachobdella picta (Verrill). Transactions of the American Microscopical Society **72**:197-216.

Barta, J. R., and S. S. Desser. 1989. Development of *Babesiosoma stableri* (Dactylosomatidae, Adeleida, Apicomplexa) in its leech vector (*Batracobdella picta*) and the relationship of the dactylosomatids to the piroplasms of higher vertebrates. Journal of Protozoology **36**:241-253.

Barta, J. R., and R. T. Sawyer. 1990. Definition of a new genus of glossiphoniid leech and a redescription of the type species, *Clepsine picta* Verrill, 1872. Canadian Journal of Zoology **68**:1942-1950.

Bartlett, C. M., and R. C. Anderson. 1985. Larval nematodes (Ascarida and Spirurida) in the aquatic snail, *Lymnaea stagnalis*. Journal of Invertebrate Pathology. **46**:153-159.

Bell, A., and V. Hughes. 1997. Hematopoiesis: Morphology of human blood and bone marrow cells. Pages 3-39 *in* D. M. Harmening, editor. Clinical Hematology and Fundamentals of Hemostasis. F. A. Davis Company, Philadelphia.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B-Methodological **57**:289-300.

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

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

Berven, K. A., and R. S. Boltz. 2001. Interactive effects of leech (*Desserobdella picta*) infection on wood frog (*Rana sylvatica*) tadpole fitness traits. Copeia:907-915.

Beug, H., G. Doederlein, C. Freudenstein, and T. Graf. 1982. Erythroblast cell lines transformed by a temperature-sensitive mutant of avian erythroblastosis virus: a model system to study erythroid differentiation in vitro. J Cell Physiol Suppl **1**:195-207.

Biek, R., W. C. Funk, B. A. Maxell, and L. S. Mills. 2002. What is missing in amphibian decline research: Insights from ecological sensitivity analysis. Conservation Biology **16**:728-734.

Bjornstad, O. N., B. F. Finkenstadt, and B. T. Grenfell. 2002. Dynamics of measles epidemics: Estimating scaling of transmission rates using a time series SIR model. Ecological Monographs **72**:169-184.

Blaustein, A. R., J. M. Romansic, J. Kiesecker, and A. C. Hatch. 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. Diversity and Distributions **9**:123-140.

Bly, J. E., and L. W. Clem. 1991. Temperature-mediated processes in teleost immunity: *In vitro* immunosuppression induced by *in vivo* low-temperature in channel catfish. Veterinary Immunology and Immunopathology **28**:365-377.

Bly, J. E., L. A. Lawson, A. J. Szalai, and L. W. Clem. 1993. Environmental factors affecting outbreaks of winter saprolegniosis in Channel catfish, *Ictalurus Punctatus* (Rafinesque). Journal of Fish Diseases **16**:541-549.

Bolek, M. G. 1997. Seasonal occurrence of *Cosmocercoides dukae* and prey analysis in the blue-spotted salamander, *Ambystoma laterale*, in southeastern Wisconsin. Journal of the Helminthological Society of Washington **64**:292-295.

Bolek, M. G., and J. R. Coggins. 2000. Seasonal occurrence and community structure of helminth parasites from the eastern American toad, *Bufo americanus americanus*, from southeastern Wisconsin, USA. Comparative Parasitology **67**:202-209.

Bolek, M. G., and J. R. Coggins. 2001. Seasonal occurrence and community structure of helminth parasites in green frogs, *Rana clamitans melanota*, from southeastern Wisconsin, USA. Comparative Parasitology **68**:164-172.

Bonneaud, C., J. Mazuc, G. Gonzalez, C. Haussy, O. Chastel, B. Faivre, and G. Sorci. 2003. Assessing the cost of mounting an immune response. American Naturalist **161**:367-379.

Brimley, C. S. 1921. The life history of the American newt. Copeia **94**:31-32.

Brockelman, W. Y. 1969. An analysis of density effects and predation in *Bufo americanus* tadpoles. Ecology **50**:632-644.

Bruce, N. L., R. D. Adlard, and L. R. G. Cannon. 1994. Synoptic checklist of ascaridoid parasites (Nematoda) from fish hosts. Invertebrate Taxonomy **8**:583-674.

Bruce, N. L., and L. R. G. Cannon. 1989. *Hysterothylacium*, *Iheringascaris* and *Maricostula* new genus, nematodes (Ascaridoidea) from Australian pelagic marine fishes. Journal of Natural History **23**:1397-1441.

Caetano, M. H., and R. Leclair. 1996. Growth and population structure of red-spotted newts (*Notophthalmus viridescens*) in permanent lakes of the Laurentian shield, Quebec. Copeia:866-874.

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

Cattadori, I. M., B. Boag, O. N. Bjornstad, S. J. Cornell, and P. J. Hudson. 2005. Peak shift and epidemiology in a seasonal host-nematode system. Proceedings of the Royal Society B-Biological Sciences **272**:1163-1169.

Chabaud, A. G. 1974. Keys to subclasses, orders and families. Pages 6-17 *in* R. M. Anderson, A. G. Chabaud, and S. Willmott, editors. CIH Keys to the Nematode Parasites of Vertebrates. Commonwealth Agricultural Bureaux, Bucks, England.

Chadwick, C. S. 1944. Observations on the life cycle of the common newt in western North Carolina. American Midland Naturalist **32**:491-494.

Chase, J. M. 2003. Strong and weak trophic cascades along a productivity gradient. Oikos **101**:187-195.

Cheng, T. C. 1958. Studies on the trematode family Dicrocoeliidae. I. The genera *Brachycoelium* (Dujardin, 1845) and *Leptophallus* Luhe, 1909, (Brachyceoliinae). American Midland Naturalist **59**:67-81.

Cheng, T. C. 1960. The life history of *Brachycoelium obesum* Nicoll, 1914, with a discussion of the systematic status of the trematode family Brachycoellidae Johnston 1912. Journal of Parasitology **46**:464-474.

Chinchar, V. G. 2002. Ranaviruses (family Iridoviridae): emerging cold-blooded killers. Archives of Virology **147**:447-470.

Chizinski, C. J., and K. L. Pope. 2003. Importance of food ration and water temperature on growth of juvenile green sunfish (*Lepomis cyanellus*). Texas Journal of Science **55**:263-270.

Coddington, E. J., and A. Cree. 1995. Effect of acute captivity stress on plasma concentrations of corticosterone and sex steroids in female Whistling frogs *Litoria ewingi*. General and Comparative Endocrinology **100**:33-38.

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

Conant, R., and J. T. Collins. 1998. A field guide to reptiles and amphibians: Eastern and central North America, 3rd edition. Houghton Mifflin, New York.

Converse, K. A., and D. E. Green. 2005. Diseases of salamanders. Pages 118-130 *in* S. K. Majumdar, J. E. Huffman, F. J. Brenner, and A. I. Panah, editors. Wildlife diseases: landscape epidemiology, spatial distribution and utilization of remote sensing technology. The Pennsylvania Academy of Science, Easton, P.A.

Cooper, E. L., R. K. Wright, A. E. Klempau, and C. T. Smith. 1992. Hibernation alters the frog's immune system. Cryobiology **29**:616-631.

Cox, F. E. G. 2001. Concomitant infections, parasites and immune responses. Parasitology **122**:S23-S38.

Crawley, M. J. 2002. Statistical computing: An introduction to statistical analysis using S-Plus. John Wiley & Sons Ltd., Chichester, U.K.

Czeczuga, B., B. Mazalska, A. Godlewska, and E. Muszynska. 2005. Aquatic fungi growing on dead fragments of submerged plants. Limnologica **35**:283-297.

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

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

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

Davis, A. K. 2005. Effect of handling time and repeated sampling on avian white blood cell counts. Journal of Field Ornithology **76**:334-338.

Deardorff, T. L., and R. M. Overstreet. 1980. Review of *Hysterothylacium* and *Iheringascaris* (both previously *Thynnascaris*) (Nematoda: Anisakidae) from the northern Gulf of Mexico. Proceedings of the Biological Society of Washington **93**:1035-1079.

Deardorff, T. L., and R. M. Overstreet. 1981. *Raphidascaris camura* sp. n., *Hysterothylacium eurycheilum* (Olsen) comb. n., and comments on *Heterotyphlum* Spaul (Nematoda, Ascaridoidea) in marine fishes. Journal of Parasitology **67**:426-432.

Delgado, M. J., A. L. Alonsogomez, and M. Alonsobedate. 1992. Role of environmental temperature and photoperiod in regulation of seasonal testicular activity in the frog, *Rana perezi.* Canadian Journal of Physiology and Pharmacology **70**:1348-1352.

DeSantis, D. E., and R. G. Strauss. 1997. Cell biology and disorders of neutrophils. Pages 265-282 *in* D. M. Harmening, editor. Clinical Hematology and Fundamentals of Hemostasis. F. A. Davis Company, Philadelphia.

Desser, S. S. 1987. *Aegyptianella ranarum* sp. n (Rickettsiales, Anaplasmataceae): ultrastructure and prevalence in frogs from Ontario. Journal of Wildlife Diseases **23**:52-59.

Dobson, A., and J. Foufopoulos. 2001. Emerging infectious pathogens of wildlife. Philosophical Transactions of the Royal Society of London, Series B **356**:1001-1012.

Dobson, A. P. 1985. The population dynamics of competition between parasites. Parasitology **91**:317-347.

Duellman, W. E., and L. Trueb. 1986. Biology of Amphibians. The Johns Hopkins University Press, Baltimore.

Edney, J. M. 1950. Productivity in *Clinostomum marginatum* (Trematoda: Clinostomadidae). Transactions of the American Microscopical Society **69**:186-188.

Esch, G. E., and J. C. Fernandez. 1994. Snail-trematode interactions and parasite community dynamics in aquatic systems: a review. American Midland Naturalist **131**:209-237.

Felip, M., M. L. Pace, and J. J. Cole. 1996. Regulation of planktonic bacterial growth rates: The effects of temperature and resources. Microbial Ecology **31**:15-28.

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

Ferguson, E. 1944. Studies on the seasonal life history of three species of freshwater Ostracoda. American Midland Naturalist **32**:713-727.

Fernandez, J., and G. W. Esch. 1991. The component community structure of larval trematodes in the pulmonate snail *Helisoma anceps*. Journal of Parasitology **77**:540-550.

Fischthal, J. H. 1955. Ecology of worm parasites in south-central New York salamanders. American Midland Naturalist **53**:176-183.

Gage, S. H. 1891. Life-history of the vermilion-spotted newt (*Diemyctylus viridescens* Raf.). American Naturalist **25**:1084-1110.

Garber, G. 2001. An overview of fungal infections. Drugs **61 Suppl 1**:1-12.

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

Gill, D. E. 1978a. Effective population size and interdemic migration rates in a metapopulation of the red-spotted newt, *Notophthalmus viridescens* (Rafinesque). Evolution **32**:839-849.

Gill, D. E. 1978b. The metapopulation ecology of the red-spotted newt, *Notophthalmus viridescens* (Rafinesque). Ecological Monographs **48**:145-166.

Gill, D. E. 1979. Density dependence and homing behavior in adult red-spotted newts, *Notophthalmus viridescens* (Rafinesque). Ecology **60**:800-813.

Gill, D. E., K. A. Berven, and B. A. Mock. 1983. The environmental component of evolutionary biology. Pages 1-36 *in* C. E. King and P. S. Dawson, editors.

Population biology: retrospect and prospect. Columbia University Press, New York, New York.
Gill, D. E., and B. A. Mock. 1985. Ecological and evolutionary dynamics of parasites: The case of *Trypanosoma diemyctyli* in the red-spotted newt *Notophthalmus viridescens*. Pages 157-183 *in* D. Rollinson and R. M. Anderson, editors. Ecology and genetics of host-parasite interactions. Academic Press, New York.
Goodchild, C. G. 1953. A subcutaneous, cyst-parasite of Bullfrogs: *Histocystidium ranae*, n. g., n. sp. Journal of Parasitology **39**:395-405.
Gopar-Merino, L., D. Osorio-Sarabia, and L. Garcia-Prieto. 2005. A new species of *Hysterothylacium* (Nematoda : Anisakidae) parasite of *Ariopsis guatemalensis* (Osteichthyes : Ariidae) from Tres Palos lagoon, Mexico. Journal of Parasitology **91**:909-914.
Green, D. E., K. A. Converse, and A. K. Schrader. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Annals of the New York Academy of Sciences **969**:323-339.
Green, N., and N. Cohen. 1977. Effect of temperature on serum complement levels in the Leopard frog, *Rana pipiens*. Developmental and Comparative Immunology **1**:59-64.
Gubler, D. J., P. Reiter, K. L. Ebi, W. Yap, R. Nasci, and J. A. Patz. 2001. Climate variability and change in the United States: Potential impacts on vector- and rodent-borne diseases. Environmental Health Perspectives **109**:223-233.
Hadji-Azimi, I., V. Coosemans, and C. Canicatti. 1987. Atlas of adult *Xenopus laevis laevis* hematology. Developmental and Comparative Immunology **11**:807-835.
Harris, R. N. 1987. An experimental study of population regulation in the salamander, *Notophthalmus viridescens dorsalis* (Urodela, Salamandridae). Oecologia **71**:280-285.
Harris, R. N., R. A. Alford, and H. M. Wilbur. 1988. Density and phenology of *Notophthalmus viridescens dorsalis* in a natural pond. Herpetologica **44**:234-242.
Hartwich, G. 1974. Keys to the genera of the Ascaridoidea. Commonwealth Agricultural Bureaux, Bucks, England.
Harvell, C. D., K. Kim, J. M. Burkholder, R. R. Colwell, P. R. Epstein, D. J. Grimes, E. E. Hofmann, E. K. Lipp, A. D. M. E. Osterhaus, R. M. Overstreet, J. W. Porter, G. W. Smith, and G. R. Vasta. 1999. Emerging marine diseases: Climate links and anthropogenic factors. Science **285**:1505-1510.
Healy, W. R. 1973. Life history variation and growth of juvenile *Notophthalmus viridescens* from Massachusetts. Copeia **1973**:641-647.
Healy, W. R. 1974. Population consequences of alternative life histories in *Notophthalmus viridescens*. Copeia **1974**:221-229.
Hedrick, L. R. 1935. The life history and morphology of *Spiroxys contortus* (Rudolphi); Nematoda: Spiruridae. Transactions of the American Microscopical Society **54**:307-335.
Hegerl, G. C., F. W. Zwiers, P. A. Stott, and V. V. Kharin. 2004. Detectability of anthropogenic changes in annual temperature and precipitation extremes. Journal of Climate **17**:3683-3700.

Herman, R. L. 1984. *Ichthyophonus*-like infection in newts. Journal of Wildlife Diseases **20**:55-56.
Hoff, G. L., F. L. Frye, and E. R. Jacobson, eds., editors. 1984. Diseases of amphibians and reptiles. Plenum Press, New York.
Holl, F. J. 1928. Two new nematode parasites. Journal of the Elisha Mitchell Scientific Society **43**:184-186, 181 pl.
Holl, F. J. 1932. The ecology of certain fishes and amphibians with special reference to their helminth and linguatulid parasites. Ecological Monographs **2**:83-107.
Hopkins, S. H. 1933. Note on the life history of *Clinostomum marginatum* (Trematoda). Transactions of the American Microscopical Society **52**:147-149.
Horne, A. J., and C. R. Goldman. 1994. Limnology, 2nd edition. McGraw-Hill, New York.
Hudson, P. J., R. Norman, M. K. Laurenson, D. Newborn, M. Gaunt, L. Jones, H. Reid, E. Gould, R. Bowers, and A. Dobson. 1995. Persistence and transmission of tick-borne viruses: *Ixodes ricinus* and louping-ill virus in red grouse populations. Parasitology **111 (Suppl)**:S49-58.
Hudson, P. J., A. Rizzoli, B. T. Grenfell, H. Heesterbeek, and A. P. Dobson. 2002. The ecology of wildlife diseases. Oxford University Press Inc., New York, New York.
Hurbert, S. H. 1970. Predator responses to the vermillion-spotted newt (*Notophthalmus viridescens*). Journal of Herpetology **4**:47-55.
Hurlbert, S. H. 1969. The breeding migrations and interhabitat wandering of the vermilion-spotted newt *Notophthalmus viridescens* (Rafinesque). Ecological Monographs **39**:465-488.
Jackson, J. A., and R. C. Tinsley. 2002. Effects of environmental temperature on the susceptibility of *Xenopus laevis* and *X. wittei* (Anura) to *Protopolystoma xenopodis* (Monogenea). Parasitology Research **88**:632-638.
Jackson, T., and R. L. Beaudoin. 1967. Comparison of the parasite fauna in two metamorphic stages of the red-spotted newt, *Notophthalmus viridescens viridescens*. Proceedings of the Pennsylvania Academy of Science **40**:70-75.
Jancovich, J. K., E. W. Davidson, N. Parameswaran, J. Mao, V. G. Chinchar, J. P. Collins, B. L. Jacobs, and A. Storfer. 2005. Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread. Molecular Ecology **14**:213-224.
Janeway, C. A., P. Travers, M. Walport, and M. J. Schlomchik. 2001. Immunobiology: The immune system in health and disease, 5th edition. Garland Publishing, New York.
Jarroll, E. L. 1979. Population biology of *Bothriocephalus rarus* (Thomas, 1937) in the red-spotted newt *Notopthalmus viridescens* Raf. Parasitology **79**:183-193.
Johnson, P. T., and K. B. Lunde. 2005. Parasite infection and limb malformations: A growing problem in amphibian conservation. Pages 124-138 *in* Amphibian Declines: the Conservation Status of United States Species. University of California Press, Lannoo, M. J.
Johnson, P. T., K. B. Lunde, E. G. Ritchie, and A. E. Launer. 1999. The effect of trematode infection on amphibian limb development and survivorship. Science **284**:802-804.

Johnson, P. T. J., and J. M. Chase. 2004. Parasites in the food web: linking amphibian malformations and aquatic eutrophication. Ecology Letters **7**:521-526.

Johnson, P. T. J., K. B. Lunde, R. W. Haight, J. Bowerman, and A. R. Blaustein. 2001a. Ribeiroia ondatrae (Trematoda : Digenea) infection induces severe limb malformations in western toads (Bufo boreas). Canadian Journal of Zoology **79**:370-379.

Johnson, P. T. J., K. B. Lunde, E. G. Ritchie, J. K. Reaser, and A. E. Launer. 2001b. Morphological abnormality patterns in a California amphibian community. Herpetologica **57**:336-352.

Johnson, P. T. J., D. R. Sutherland, J. M. Kinsella, and K. B. Lunde. 2004. Review of the trematode genus *Ribeiroia* (Psilostomidae): Ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. Advances in Parasitology **57**:191-253.

Johnson, S. A. 2002. Life history of the striped newt at a north-central Florida breeding pond. Southeastern Naturalist **1**:381-402.

Joy, J. E., and J. L. Pennington. 1998. Ecology of *Megalodiscus temperatus* (Digenea: Paramphistomatidae) in red-spotted newts, *Notophthalmus v. viridescens*, from West Virginia. Journal of the Helminthological Society of Washington **65**:205-211.

Joy, J. E., and J. B. Scott. 1997. *Amphibiocapillaria tritonispunctati* (Nematoda: Trichuridae) infections in the red-spotted newt *Notophthalmus v. viridescens* from western West Virginia. American Midland Naturalist **138**:408-411.

Joy, J. E., and S. W. Thomas. 1997. *Fessisentis necturorum* (Acanthocephala: Fessisentidae) from the red-spotted newts, *Notophthalmus v viridescens*, in West Virginia. Journal of the Helminthological Society of Washington **64**:281-283.

Kelley, R. S. 1934. The trematode parasites of *Triturus viridescens viridescens* (Rafinesque). University of Pittsburgh Bulletin **31**:201-210.

Kiesecker, J. M. 2002. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? Proceedings of the National Academy of Sciences USA **99**:9900-9904.

Kiesecker, J. M., L. K. Belden, K. Shea, and M. J. Rubbo. 2004. Amphibian decline and emerging disease. American Scientist **92**:138-147.

Kiesecker, J. M., and A. R. Blaustein. 1995. Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. Proceedings of the National Academy of Sciences USA **92**:11049-11052.

Kiesecker, J. M., A. R. Blaustein, and L. K. Belden. 2001a. Complex causes of amphibian population declines. Nature **410**:681-684.

Kiesecker, J. M., A. R. Blaustein, and C. L. Miller. 2001b. Potential mechanisms underlying the displacement of native red-legged frogs by introduced bullfrogs. Ecology **82**:1964-1970.

Klemm, D. J. 1985. Freshwater leeches (Annelida: Hirudinea). Pages 70-173 *in* D. J. Klemm, editor. A guide to the freshwater Annelida (Polychaeta, naidid and tubificid Oligochaeta, and Hirudinea) of North America. Kendall-Hunt Publishing Co., Dubuque, Iowa.

Komar, N. 2001. West Nile virus surveillance using sentinel birds. West Nile Virus: Detection, Surveillance, and Control **951**:58-73.
Kortet, R., J. Taskinen, T. Sinisalo, and I. Jokinen. 2003. Breeding-related seasonal changes in immunocompetence, health state and condition of the cyprinid fish, *Rutilus rutilus*. Biological Journal of the Linnean Society **78**:117-127.
Ksiazek, A., M. Konarzewski, M. Chadzinska, and M. Cichon. 2003. Costs of immune response in cold-stressed laboratory mice selected for high and low basal metabolism rates. Proceedings of the Royal Society of London Series B: Biological Sciences **270**:2025-2031.
Kuperman, B. I., V. E. Matey, R. N. Fisher, E. L. Ervin, M. L. Warburton, L. Bakhireva, and C. A. Lehman. 2004. Parasites of the African clawed frog, *Xenopus laevis*, in Southern California, USA. Comparative Parasitology **71**:229-232.
Lafferty, K. D. 1993. The marine snail, *Cerithidea californica*, matures at smaller sizes where parasitism is high. Oikos **68**:3-11.
Lello, J., B. Boag, A. Fenton, I. R. Stevenson, and P. J. Hudson. 2004. Competition and mutualism among the gut helminths of a mammalian host. Nature **428**:840-844.
Lindsay, G. J. H. 1986. The significance of chitinolytic enzymes and lysozyme in Rainbow trout (*Salmo gairdneri*) defence. Aquaculture **51**:169-173.
Lips, K. R., F. Brem, R. Brenes, J. D. Reeve, R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America **103**:3165-3170.
Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. Mycologia **91**:219-227.
Mackiewicz, J. S. 1954. Observations on the incidence if Trypanosoma diemyctyli (Tobey, 1906) in the common newt, Diemyctylus v. viridescens (Raf.). shelf. Cornell University, Ithaca, New York.
Manabe, Y. C., and W. R. Bishai. 2000. Latent *Mycobacterium tuberculosis*: persistence, patience, and winning by waiting. Nature Medicine **6**:1327-1329.
Maniero, G. D., and C. Carey. 1997. Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. Journal of Comparative Physiology B **167**:256-263.
Marin, S. L., W. E. Grant, and N. O. Dronen. 1998. Simulation of population dynamics of the parasite *Haematoloechus coloradensis* in its three host species: effects of environmental temperature and precipitation. Ecological Modelling **105**:185-211.
Martin, D. S., and S. S. Desser. 1991. Development of *Trypanosoma fallisi* in the leech, *Desserobdella picta*, in toads (*Bufo americanus*), and *in vitro*: a light and electron microscopic study. Parasitology Research **77**:18-26.
Maule, A. G., and S. P. VanderKooi. 1999. Stress-induced immune-endocrine interaction. Pages 205-245 *in* P. H. M. Balm, editor. Stress Physiology in Animals. Sheffield Academic Press, Sheffield, England.
McAllister, C. T., and V. R. McDaniel. 1992. Occurrence of larval *Contracaecum* sp. (Ascaridida: Anisakidae) in Rio Grande lesser sirens, *Siren intermedia texana*

(Amphibia: Caudata), from south Texas. Journal of the Helminthological Society of Washington **59**:239-240.
McAlpine, D. F. 1997. *Fessisentis acutulus* (Van Cleave, 1931) comb. n. (Acanthocephala: Fessisentidae): A parasite of caudate amphibians in North America, with comments on the single North American report of *A. ranae* (Schrank, 1788). Journal of the Helminthological Society of Washington **64**:142-147.
McCallum, H., N. Barlow, and J. Hone. 2001. How should pathogen transmission be modelled? Trends in Ecology & Evolution **16**:295-300.
McVicar, A. H. 1982. *Ichthyophonus* infections of fish. Pages 243-269 *in* R. J. Roberts, editor. Microbial diseases of fish. Academic Press, London, U.K.
Mendoza, L., J. W. Taylor, and L. Ajello. 2002. The class mesomycetozoea: a heterogeneous group of microorganisms at the animal-fungal boundary. Annual Review of Microbiology **56**:315-344.
Michalakis, Y., and M. E. Hochberg. 1994. Parasitic effects on host life-history traits: a review of recent studies. Parasite **1**:291-294.
Mikaelian, I., M. Ouellet, B. Pauli, J. Rodrigue, J. C. Harshbarger, and D. M. Green. 2000. *Ichthyophonus*-like infection in wild amphibians from Quebec, Canada. Diseases of Aquatic Organisms **40**:195-201.
Mock, B. A. 1983. The population biology of *Trypanosoma diemyctyli*. Ph. D. Thesis. University of Maryland, College Park, Maryland.
Mock, B. A. 1987. Longitudinal patterns of trypanosome infections in red-spotted newts. Journal of Parasitology **73**:730-737.
Mock, B. A., and D. E. Gill. 1984. The infrapopulation dynamics of trypanosomes in red-spotted newts. Parasitology **88**:267-282.
Moore, I. T., and T. S. Jessop. 2003. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Hormones and Behavior **43**:39-47.
Moravec, F. 1986. Review of capillariid nematodes (Capillariinae) parasitic in amphibians and reptiles. Part 2. Genus *Amphibiocapillaria*. Vestnik Ceskoslovenske Spolecnosti Zoologicke **50**:217-230.
Morgan, A. H., and M. C. Grierson. 1932. Winter habits and yearly food consumption of adult spotted newts, *Triturus viridescens*. Ecology **13**:54-62.
Mueller, J. F. 1932. *Capillaria tenua*, a new species of nematode parasitic in the newt, *Triturus viridescens*. Transactions of the American Microscopical Society **51**:263-266.
Muzzall, P. M. 1991. Helminth infracommunities of the newt, *Notophthalmus viridescens*, from Turkey Marsh, Michigan. Journal of Parasitology **77**:87-91.
Muzzall, P. M. 2005. Parasites of amphibians and reptiles from Michigan: a review of the literature 1916–2003. Michigan Department of Natural Resources Fisheries Division.
Muzzall, P. M., and W. L. Bullock. 1978. Seasonal occurrence and host-parasite relationships of *Neoechinorhynchus saginatus* (Van Cleave and Bangham 1949) in the fallfish, *Semotilus corporalis* (Mitchill). Journal of Parasitology **64**:860-865.

Muzzall, P. M., J. D. Peterson, and M. G. Gillilland. 2003. Helminths of *Notophthalmus viridescens* (Caudata: salamandridae) from 118th Pond, Michigan, USA. Comparative Parasitology **70**:214-217.

Negm-Eldin, M. M., and R. W. Davies. 1999. Simultaneous transmission of *Trypanosoma mukasai*, *Babesiosoma mariae* and *Cyrilia nili* to fish by the leech *Batracobdelloides tricarinata*. Deutsche Tierarztliche Wochenschrift **106**:526-527.

Noble, G. K. 1929. Further observations on the life-history of the newt, *Triturus viridescens*. American Museum Novitiates **348**:1-22.

Ostfeld, R. S., and F. Keesing. 2000. Biodiversity and disease risk: The case of lyme disease. Conservation Biology **14**:722-728.

Owen, M. 1946. The life history of *Plagitura salamandra* Holl 1928 (Trematoda: Plagiorchiidae). Journal of Parasitology **32**:553-562.

Pace, M. L. 1986. An empirical analysis of zooplankton community size structure across lake trophic gradients. Limnology and Oceanography **31**:45-55.

Petranka, J. W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, D. C.

Plytycz, B., and J. Bigaj. 1983. Seasonal cyclic changes in the thymus gland of the adult frog *Rana temporaria*. Thymus **5**:327-344.

Plytycz, B., and A. Jozkowicz. 1994. Differential effects of temperature on macrophages of ectothermic vertebrates. Journal of Leukocyte Biology **56**:729-731.

Porter, K. G., P. A. Saunders, K. A. Haberyan, A. E. Macubbin, T. R. Jacobsen, and R. E. Hodson. 1996. Annual cycle of autotrophic and heterotrophic production in a small, monomictic Piedmont lake (Lake Oglethorpe): Analog for the effects of climatic warming on dimictic lakes. Limnology and Oceanography **41**:1041-1051.

Pounds, J. A., M. R. Bustamante, L. A. Coloma, J. A. Consuegra, M. P. L. Fogden, P. N. Foster, E. La Marca, K. L. Masters, A. Merino-Viteri, R. Puschendorf, S. R. Ron, G. A. Sanchez-Azofeifa, C. J. Still, and B. E. Young. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Nature **439**:161-167.

Pounds, J. A., M. P. L. Fogden, and J. H. Campbell. 1999. Biological response to climate change on a tropical mountain. Nature **398**:611-615.

Prendergast, B. J., D. A. Freeman, I. Zucker, and R. J. Nelson. 2002. Periodic arousal from hibernation is necessary for initiation of immune responses in ground squirrels. American Journal of Physiology **282**:R1054-R1062.

Raffel, T. R. 2006. Drivers of seasonal infection dynamics in the parasite community of red-spotted newts (*Notophthalmus viridescens*). Ph. D. Thesis. The Pennsylvania State University, University Park.

Raffel, T. R., J. R. Dillard, and P. J. Hudson. *in press-a*. Field evidence for leech-borne transmission of amphibian *Ichthyophonus*. Journal of Parasitology **XX**:XX.

Raffel, T. R., J. R. Dillard, and P. J. Hudson. *in review*. Field evidence for leech-borne transmission of amphibian *Ichthyophonus*. Journal of Parasitology **XX**:XX.

Raffel, T. R., J. R. Rohr, J. M. Kiesecker, and P. J. Hudson. *in press-b*. Negative effects of changing temperature on amphibian immunity under field conditions. Functional Ecology **XX**:XX.

Randolph, S. E., C. Chemini, C. Furlanello, C. Genchi, R. S. Hails, P. J. Hudson, L. D. Jones, G. Medley, R. A. Norman, A. P. Rizzoli, G. Smith, and M. E. J. Woolhouse. 2002. The ecology of tick-borne infections in wildlife reservoirs. Pages 119-138 *in* P. J. Hudson, A. Rizzoli, B. T. Grenfell, H. Heesterbeek, and A. Dobson, editors. The ecology of wildlife diseases. Oxford University Press, New York, New York.

Rani, S., S. Singh, M. Misra, S. Malik, B. P. Singh, and V. Kumar. 2005. Daily light regulates seasonal responses in the migratory male redheaded bunting (*Emberiza bruniceps*). Journal of Experimental Zoology A: Comparative Experimental Biology **303A**:541-550.

Rankin, J. S., Jr. 1937. An ecological study of parasites of some North Carolina salamanders. Ecological Monographs **7**:169-270.

Rankin, J. S., Jr. 1938. Studies on the trematode genus *Brachycoelium*. Transactions of the American Microscopical Society **57**:358-375.

Rankin, J. S., Jr. 1945. An ecological study of the helminth parasites of amphibians and reptiles of western Massachusetts and vicinity. Journal of Parasitology **31**:142-150.

Rast, W., and J. A. Thornton. 1996. Trends in eutrophication research and control. Hydrological Processes **10**:295-313.

Ratkowsky, D. A., J. Olley, T. A. McMeekin, and A. Ball. 1982. Relationship between temperature and growth rate of bacterial cultures. Journal of Bacteriology **149**:1-5.

Richardson, W. B., S. J. Zigler, and M. R. Dewey. 1998. Bioenergetic relations in submerged aquatic vegetation: an experimental test of prey use by juvenile bluegills. Ecology of Freshwater Fish **7**:1-12.

Rivera, G., S. Elliott, L. S. Caldas, G. Nicolossi, V. T. R. Coradin, and R. Borchert. 2002. Increasing day-length induces spring flushing of tropical dry forest trees in the absence of rain. Trees-Structure and Function **16**:445-456.

Roberts, M. G., and B. T. Grenfell. 1992. The population dynamics of nematode infections of ruminants: the effect of seasonality in the free-living stages. Ima Journal of Mathematics Applied in Medicine and Biology **9**:29-41.

Rohr, J. R., and D. M. Madison. 2003. Dryness increases predation risk in efts: support for an amphibian decline hypothesis. Oecologia **135**:657-664.

Rohr, J. R., D. M. Madison, and A. M. Sullivan. 2002. Sex differences and seasonal trade-offs in response to injured and non-injured conspecifics in red-spotted newts, *Notophthalmus viridescens*. Behavioral Ecology and Sociobiology **52**:385-393.

Rohr, J. R., D. M. Madison, and A. M. Sullivan. 2003. On temporal variation and conflicting selection pressures: A test of theory using newts. Ecology **84**:1816-1826.

Rohr, J. R., and T. R. Raffel. in prep. Spatiotemporal dynamics in the parasite community of a temperate amphibian species: general patterns and probable causes. target journal: Ecology.

Rohr, J. R., T. Sager, T. M. Sesterhenn, and B. D. Palmer. 2006. Exposure, postexposure, and density-mediated effects of atrazine on amphibians: breaking down net effects into their parts. Environ Health Perspect **114**:46-50.
Rojas, S., K. Richards, J. K. Jancovich, and E. W. Davidson. 2005. Influence of temperature on *Ranavirus* infection in larval salamanders *Ambystoma tigrinum*. Diseases of Aquatic Organisms **63**:95-100.
Rolff, J. 2002. Bateman's principle and immunity. Proceedings of the Royal Society of London Series B-Biological Sciences **269**:867-872.
Rollins-Smith, L. A. 2001. Neuroendocrine-immune system interactions in amphibians: Implications for understanding global amphibian declines. Immunologic Research **23**:273-280.
Romero, L. M., and R. C. Romero. 2002. Corticosterone responses in wild birds: The importance of rapid initial sampling. Condor **104**:129-135.
Russell, C. M. 1951. Survey of the intestinal helminths of *Triturus v. viridescens* in the vicinity of Charlottesville, Virginia. Virginia Journal of Science **2**:215-219.
Rye, L. A., and M. R. Baker. 1984. *Hysterothylacium analarum* n. sp. (Nematoda: Anisakidae) from pumpkinseed, *Lepomis gibbosus* (Linnaeus), in southern Ontario. Canadian Journal of Zoology **62**:2307-2312.
Sapp, K. K., and G. W. Esch. 1994. The effects of spatial and temporal heterogeneity as structuring forces for parasite communities in *Helisoma anceps* and *Physa gyrina*. American Midland Naturalist **132**:91-103.
Saunders, L. M., D. M. Tompkins, and P. J. Hudson. 2000. The role of oxygen availability in the embryonation of Heterakis gallinarum eggs. International Journal for Parasitology **30**:1481-1485.
Saunders, L. M., D. M. Tompkins, and P. J. Hudson. 2002. Stochasticity accelerates nematode egg development. Journal of Parasitology **88**:1271-1272.
Sawyer, R. T. 1972. North American freshwater leeches exclusive of the Piscicolidae, with a key to all species. University of Illinois Press, Urbana, Illinois.
Sawyer, R. T. 1986. Leech biology and behaviour II: Feeding biology, ecology and systematics. Clarendon Press, Oxford, U.K.
Schar, C., P. L. Vidale, D. Luthi, C. Frei, C. Haberli, M. A. Liniger, and C. Appenzeller. 2004. The role of increasing temperature variability in European summer heatwaves. Nature **427**:332-336.
Schermer, S. 1967. The Blood Morphology of Laboratory Animals. F. A. Davis Company, Philadelphia, PA.
Schmid, W. D. 1982. Survival of frogs in low temperature. Science **215**:697-698.
Schmidt, B. R., R. Feldmann, and M. Schaub. 2005. Demographic processes underlying population growth and decline in *Salamandra salamandra*. Conservation Biology **19**:1149-1156.
Schmidt, G. D. 1970. How to know the cestodes. William C. Brown Company Publishers, Dubuque, Iowa.
Schmidt, G. D., P. D. Leiby, and D. C. Kritsky. 1974. Studies on helminths of North Dakota. VIII. Nematodes from the paddlefish, *Polyodon spathula* (Walbaum), including *Thynnascaris dollfusi* sp. nov. (Nematoda: Stomachidae). Canadian Journal of Zoology **52**:261-263.

Schmidt, K. A., and B. Fried. 1997. Prevalence of larval trematodes in *Helisoma trivolvis* (Gastropoda) from a farm pond in Northampton County, PA with special emphasis on *Echinostoma trivolvis* (Trematoda) cercariae. Journal of the Helminthological Society of Washington **64**:157-159.

Schnell, S. C. 1970. How to know the trematodes. William. C. Brown Company Publishers, Dubuque, Iowa.

Schnell, S. C. 1985. Handbook of trematodes of North America, North of Mexico. University Press of Idaho, Moscow, Idaho.

Siddall, M. E., R. B. Budinoff, and E. Borda. 2005. Phylogenetic evaluation of systematics and biogeography of the leech family Glossiphoniidae. Invertebrate Systematics **19**:105-112.

Siddall, M. E., and S. S. Desser. 1990. Gametogenesis and sporogonic development of *Haemogregarina balli* (Apicomplexa, Adeleina, Haemogregarinidae) in the leech *Placobdella ornata*. Journal of Protozoology **37**:511-520.

Siddall, M. E., and S. S. Desser. 1992. Alternative leech vectors for frog and turtle trypanosomes. Journal of Parasitology **78**:562-563.

Siddall, M. E., and S. S. Desser. 2001. Transmission of *Haemogregarina balli* from painted turtles to snapping turtles through the leech *Placobdella ornata*. Journal of Parasitology **87**:1217-1218.

Skelly, D. K., L. K. Freidenburg, and J. M. Kiesecker. 2002. Forest canopy and the performance of larval amphibians. Ecology **83**:983-992.

Smith, A., S. Telfer, S. Burthe, M. Bennett, and M. Begon. 2005. Trypanosomes, fleas and field voles: ecological dynamics of a host-vector-parasite interaction. Parasitology **131**:355-365.

Spanggaard, B., H. H. Huss, and J. Bresciani. 1995. Morphology of *Ichthyophonus hoferi* assessed by light and scanning electron microscopy. Journal of Fish Diseases **18**:567-577.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science **306**:1783-1786.

Stunkard, H. W. 1936. The morphology and life history of *Plagitura parva* Stunkard 1933 (Trematoda). Journal of Parasitology **22**:354-374.

Tella, J. L., M. G. Forero, M. Bertellotti, J. A. Donazar, G. Blanco, and O. Ceballos. 2001. Offspring body condition and immunocompetence are negatively affected by high breeding densities in a colonial seabird: a multiscale approach. Proceedings of the Royal Society of London Series B-Biological Sciences **268**:1455-1461.

Thomas, L. J. 1937a. *Bothriocephalus rarus* n. sp.: A cestode from the newt, *Triturus viridescens* Raf. Journal of Parasitology **23**:119-132.

Thomas, L. J. 1937b. Environmental relations and life history of the tapeworm *Bothriocephalus rarus* Thomas. Journal of Parasitology **23**:133-152.

Tinsley, R. C. 1995. Parasitic disease in amphibians: control by the regulation of worm burdens. Parasitology **111 Suppl**:S153-178.

Tobey, E. N. 1906. Trypanosomiasis of the newt. Journal of Medical Research **15**:147-148.

Tough, D. F., and J. Sprent. 1995. Lifespan of lymphocytes. Immunological Research **14**:1-12.

Tse, B. K., J. R. Barta, and S. S. Desser. 1986. Comparative ultrastructural features of the sporozoite of *Lankesterella minima* (Apicomplexa) in its anuran host and leech vector. Canadian Journal of Zoology **64**:2344-2347.

Uglem, G. L., and O. R. Larson. 1969. Life history and larval development of *Neoechinorhynchus saginatus* Van-Cleave and Bangham, 1949 (Acanthocephala . Neoechinorhynchidae). Journal of Parasitology **55**:1212-&.

Ussing, A. P., and P. Rosenkilde. 1995. Effect of induced metamorphosis on the immune system of the Axolotl, *Ambystoma mexicanum*. General and Comparative Endocrinology **97**:308-319.

Van Cleave, H. J., and R. V. Bangham. 1949. Four new species of the acanthocephalan family Neoechinorhynchidae from fresh-water fishes of North America, one representing a new genus. Journal of the Washington Academy of Sciences **39**:398-409.

van Riper, C., S. G. van Riper, M. L. Goff, and M. Laird. 1986. The epizootiology and ecological significance of malaria in Hawaiian land birds. Ecological Monographs **56**:327-344.

Vanderburgh, D. J., and R. C. Anderson. 1987. Preliminary observations on seasonal changes in prevalence and intensity of *Cosmocercoides variabilis* (Nematoda, Cosmocercoidea) in *Bufo americanus* (Amphibia). Canadian Journal of Zoology **65**:1666-1667.

Vonesh, J. R., and O. De la Cruz. 2002. Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. Oecologia **133**:325-333.

Ward, H. B., and T. B. Magath. 1917. Notes on some nematodes from fresh-water fishes. Journal of Parasitology **3**:57-65.

Weldon, C., L. H. du Preez, A. D. Hyatt, R. Muller, and R. Speare. 2004. Origin of the amphibian chytrid fungus. Emerging Infectious Diseases **10**:2100-2105.

Wetzel, E. J., and G. W. Esch. 1996. Seasonal population dynamics of *Halipegus occidualis* and *Halipegus eccentricus* (Digenea: Hemiuridae) in their amphibian host, *Rana clamitans*. Journal of Parasitology **82**:414-422.

Wetzel, E. J., and G. W. Esch. 1997. Infrapopulation dynamics of *Halipegus occidualis* and *Halipegus eccentricus* (Digenea: Hemiuridae): temporal changes within individual hosts. Journal of Parasitology **83**:1019-1024.

Wikelski, M., M. Hau, and J. C. Wingfield. 2000. Seasonality of reproduction in a neotropical rain forest bird. Ecology **81**:2458-2472.

Wilbur, H. M. 1997. Experimental ecology of food webs: Complex systems in temporary ponds - The Robert H. MacArthur Award Lecture - Presented 31 July 1995 Snowbird, Utah. Ecology **78**:2279-2302.

Williams, J. A., and G. W. Esch. 1991. Infra- and component community dynamics in the pulmonate snail *Helisoma anceps*, with special emphasis on the hemiurid trematode *Halipegus occidualis*. Journal of Parasitology **77**:246-253.

Wojtowicz, A., and B. Plytycz. 1997. Seasonal changes of gut-associated lymphoid tissue in the common toad, *Bufo bufo*. Journal of Nutritional Immunology **5**:57-64.

Woo, P. T. K. 2003. *Cryptobia* (*Trypanoplasma*) *salmositica* and salmonid cryptobiosis. Journal of Fish Diseases **26**:627-646.

Woodhams, D. C., R. A. Alford, and G. Marantelli. 2003. Emerging disease of amphibians cured by elevated body temperature. Diseases of Aquatic Organisms **55**:65-67.

Yousif, A. N., L. J. Albright, and T. P. T. Evelyn. 1991. Occurrence of lysozyme in the eggs of Coho salmon *Oncorhynchus kisutch*. Diseases of Aquatic Organisms **10**:45-49.

Zerani, M., and A. Gobbetti. 1993. Corticosterone during the annual reproductive cycle and in sexual behavior in the crested newt, *Triturus carnifex*. Hormones and Behavior **27**:29-37.

Zhang, C. B., and Y. Rikihisa. 2004. Proposal to transfer '*Aegyptianella ranarum*', an intracellular bacterium of frog red blood cells, to the family Flavobacteriaceae as 'candidatus *Hemobacterium ranarum*' comb. nov. Environmental Microbiology **6**:568-573.

Zuk, M., and A. M. Stoehr. 2002. Immune defense and host life history. American Naturalist **160**:S9-S22.

VITA

Thomas Richard Raffel

EDUCATION:

August 2001-August 2006: Ph.D. student in Biology, Penn State University, University Park, PA. Advisors: Dr. Joseph M. Kiesecker and Dr. Peter J. Hudson

August 1997-May 2001: B.A. Pre-Professional Zoology, Summa Cum Laude, Ohio Wesleyan University, Delaware, OH.

Raffel, T. R., K. B. Register, S. A. Marks, L. M. Temple. 2002. Prevalence of *Bordetella avium* in wild and domestic bird species of eastern USA. *Wildlife Diseases*. 38(1): 40-46.

PUBLICATIONS:

Raffel, T. R., J. R. Dillard, and P. J. Hudson. (*in press*). Field evidence for leech-borne transmission of amphibian *Ichthyophonus* sp. The Journal of Parasitology.

Raffel, T. R., J. R. Rohr, J. M. Kiesecker, and P. J. Hudson. (*in press*). Negative effects of changing temperature on amphibian immunity under field conditions. Functional Ecology.

Raffel, T. R. and A. J. Gatz. 2003. The orientation of beavers (*Castor canadensis*) when cutting trees. The Ohio Journal of Science. 103(5): 143-146.

Raffel, T. R., K. B. Register, S. A. Marks, L. M. Temple. 2002. Prevalence of *Bordetella avium* in wild and domestic bird species of eastern USA. The Journal of Wildlife Diseases. 38(1): 40-46.

FELLOWSHIPS AND AWARDS:

2005: Graduate Excellence in Teaching Award (Penn State Biology Dept.)

2005-2006: National Science Foundation Doctoral Dissertation Improvement Grant

2002-2005: National Science Foundation Predoctoral Fellowship

2001-2002: Penn State University Fellowship

2001-2002: Braddock Fellowship

TEACHING EXPERIENCE:

Penn State University, University Park, PA:

Fall 2003: Teaching assistant. Biology 421: Comparative Vertebrate Anatomy.

Fall 2005: Teaching assistant. Biology 421: Comparative Vertebrate Anatomy

Spring 2006: Teaching assistant. Biology 473: Vertebrate Physiology

PROFESSIONAL SOCIETIES:

Wildlife Disease Association

Ecological Society of America

Sigma Xi