The reciprocal influence of trematode parasites and malathion on developing pickerel frogs (*Rana palustris*)

Sarah Ashcom Budischak

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Fisheries and Wildlife Sciences

Committee Chair: William A. Hopkins
Committee Member: Lisa K. Belden
Committee Member: Richard J. Neves

October 16, 2007
Blacksburg, Virginia

Keywords: acetylcholinesterase, malathion, trematode, latent, sublethal, multiple stressors, amphibian

The reciprocal influence of trematode parasites and malathion on developing pickerel frogs (*Rana palustris*)

Sarah Ashcom Budischak

ABSTRACT

To examine the interactions of disease and pollution on amphibian populations around the world, I investigated the effect of infection on contaminant susceptibility in pickerel frog, *Rana palustris*, larvae. I conducted standard 48-hr toxicity tests to examine effect of trematode parasite, *Echinostoma trivolvis*, infection (0, 10, or 30 cercaria) on the susceptibility of pickerel frog tadpoles to the widely used organophosphate insecticide malathion. LC_{50} values ranged from 16.5 – 17.4 mg/L, within the range reported for other amphibian species. I found no differences in susceptibility to malathion among parasite treatments. Nevertheless, this crucial question remains to be tested in other amphibian host-parasite systems. Second, I studied the reverse interaction, the effect of pesticide exposure on susceptibility to parasite infection. I exposed pickerel frog embryos to low doses of malathion, then subjected morphologically normal tadpoles to *E. trivolvis* later in development. Malathion significantly decreased hatching success and viability rates at concentrations lower than previously documented for anuran embryos. After 7 wk of development in water with no malathion, tadpoles previously exposed to malathion as embryos suffered increased parasite encystment rates compared to controls. My research identifies embryonic development as a sensitive window and the potential for increased susceptibility to infection long after pesticide exposure has ceased. With potential for increased parasite prevalence from eutrophication and climate change, my data underscore the importance of understanding the reciprocal influences of parasites and pesticides in amphibians.

GRANT RECOGNITION

Financial support was provided by Virginia Polytechnic Institute and State University in the form of new faculty start-up support to William Hopkins and as teaching assistantships from the Department of Fisheries and Wildlife Sciences.

ACKNOWLEDGEMENTS

I first want to thank my advisor, Bill Hopkins, for challenging and supporting me to achieve my best. I also need to thank Dr. Lisa Belden who shared her lab, expertise, graduate students (especially Jenn Griggs and Courtney Culp), and snails with me. Additionally, I thank Dr. Dick Neves for his input and support on my committee. I want to thank my lab mates Sarah DuRant, Christine Bergeron, Haruka Wada, and my personal parasite guru Sarah Orlofske for their moral support and help with all aspects of my graduate career. Jean Cobb and Elizabeth Watson went far beyond their required duties to help me mix the correct malathion concentrations and get my project off the ground. I thank Dr. Anne Zajac and fellow members of the Parasite Journal Club for introducing me to the diverse world of parasitology. My research would not have been possible without help from undergraduate assistants including Andrew Goodpasture, Adrian Roadman, and all those who came 'herping' with me on cold rainy nights. My chief technician and moral supporter was Tim Wade II who never denied any of my crazy requests including weighing leaves, hunting snails, and saving frogs from drowning. I would additionally like to thank my family and friends for their unwavering support. The faculty and graduate students in the Department of Fisheries and Wildlife Sciences have proven an invaluable asset, and I thank them for their support and assistance. My heart goes out to all those in the Virginia Tech community for standing together after tragedy befell us. We will prevail!

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

Chapter 2

LIST OF FIGURES

Chapter 1

Chapter 2

LITERATURE REVIEW

INTRODUCTION

The recent decline of some amphibian populations is a globally recognized problem (Baringa 1990, Blaustein and Wake 1990, Wake 1991, Blaustein et al. 1994, Alford and Richards 1999, Houlahan et al. 2000, Stuart et al. 2004), but the causes and their relative magnitudes are still debated (Collins and Storfer 2003). Although some of the declines have been explained by chytridiomycosis and ranaviral diseases, many remain enigmatic (Daszak et al. 2003). A combination of multiple stressors including overexploitation, habitat loss and fragmentation, invasive species, climate change, increased UV-B exposure, environmental contaminants, and disease have often been cited as the causes of declines (Blaustein and Wake 1990, Carey and Bryant 1995, Daszak et al. 1999, Blaustein et al. 2001, Davidson et al. 2001, Kiesecker et al. 2001, Sparling et al. 2001, Belden and Blaustein 2002, Davidson et al. 2002, Carey and Alexander 2003, Kats and Ferrer 2003, Muths et al. 2003, Davidson 2004, Kiesecker et al. 2004). My research examined the interaction between two of these factors, contaminants and infection. I investigated the reciprocal influences of a pesticide and a trematode parasite on larval amphibians. Specifically, I determined whether parasite load influenced pesticide susceptibility, and whether exposure to pesticides during early development influenced susceptibility to parasite infection later in development.

Sublethal Effects of Contaminants

Although the role of chemical contamination in amphibian declines is not well understood, regional amphibian declines, lower population densities, and decreased species diversity have been correlated to agricultural land use (Berger 1989, Bishop et al. 1999, Davidson et al. 2001, Davidson 2004, Johansson et al. 2005). Additionally, land use by humans has been linked with cases of amphibian malformation (Taylor et al. 2005, Hopkins et al. 2006), lower hatching success (Bishop et al. 1999, Hopkins et al. 2006), and decreased genetic diversity (Johansson et al. 2005).

Because environmental concentrations of pesticides are often below lethal levels, it is necessary to study the sublethal effects of pesticides to understand how they are affecting amphibian populations, to supplement the standard 96-hour teratogenity and lethal concentration tests used for U.S. regulations. Agricultural pesticides can affect amphibian life history characteristics, to include: altering behavior, causing smaller size at metamorphosis, and lengthening the time needed to reach metamorphosis (Bridges 1997, Cowman and Mazanti 2000, Linzey et al. 2003). Smaller size at metamorphosis has been linked to lower survival (Smith 1987, Semlitsch et al. 1988), increased disease susceptibility (Wilbur 1980), and decreased reproductive output (Berven 1982).

In addition to direct sublethal effects on amphibians, contaminants can affect survival and size at metamorphosis by altering community-level interactions such as predation and competition (Relyea et al. 2005, Rohr and Crumrine 2005). Contaminants may work synergistically with other stressors such as pond drying (Carey and Bryant 1995, Kiesecker and Skelly 2001, Boone and Semlitsch 2002, Rohr et al. 2004), predation (Cooke 1971, Knapp and Matthews 2000, Relyea and Mills 2001, Relyea 2003, 2004, Relyea et al. 2005), and disease (Carey 1999, Taylor et al. 1999, Kiesecker 2002,

Gilbertson et al. 2003, Linzey et al. 2003) by increasing larval period, affecting activity, or altering susceptibility. These sublethal and indirect factors cannot be measured in short-term laboratory studies, but are important to long-term survival and population persistence.

Contaminants and Disease Susceptibility

Concern for infectious diseases in wildlife conservation has been growing in recent years due to outbreaks of widespread, virulent diseases, including West Nile virus and chytridiomycosis (Daszak et al. 2001). Environmental contaminants have been correlated with increases in parasite infection prevalence in some wildlife populations (Whipple 1982, Kiesecker 2002, Christin et al. 2003, Lewis et al. 2003). Anthropogenic activities and environmental change are largely responsible for many of the underlying causes of wildlife disease outbreaks, to include: increased host density (due to habitat reduction), introduction of non-native wild and domestic animals, and increased contact with vectors (Daszak et al. 2001, Lafferty and Gerber 2002). In addition to facilitating the spread of disease, human actions may be responsible for increasing susceptibility by intensifying environmental stress to wildlife (Daszak et al. 2001, Lafferty and Gerber 2002, Lafferty and Holt 2003, Rachowicz et al. 2005).

The link between environmental stressors and infection risk has been studied in many animals (Esch et al. 1975, Daszak et al. 2001, Lafferty and Gerber 2002, Grove et al. 2003, Lafferty and Holt 2003), including amphibians (Kiesecker 2002, Daszak et al. 2003). Immune systems are costly to maintain and may not function at full efficiency during periods of stress (Rigby and Moret 2000). Chronic elevation of

glucocorticosteroid hormones, the vertebrate response to long-term stress, has been shown to reduce immune system function (Ottaviani and Franceschi 1996, Mastorakos et al. 1999). Amphibian larvae treated with glucocorticosteroids displayed decreased lymphocyte counts (Tournefier 1982, Rollins-Smith and Blair 1993), including circulating eosinophilic granulocytes, the type of white blood cell that fight macroparasites (Belden and Kiesecker 2005). Some environmental contaminants are known to induce the release of glucocorticosteroid hormones (Hopkins et al. 1997) and reduce immunocompetency in amphibians (Luebke et al. 1997, Christin et al. 2003, Gilbertson et al. 2003, Grove et al. 2003, Linzey et al. 2003). Sublethal injections of DDT, malathion, cyclophosphamide, and dieldrin lowered several types of antibody responses in adult northern leopard frogs, *R. pipiens,* for at least 8 wk after exposure. Immune responses were generally back to normal levels after 20 wk (Gilbertson et al. 2003). When exposed to a combination of six pesticides (atrazine, metribuzin, aldicarb, endosulfan, lindane, and dieldrin) for 21 days, larval northern leopard frogs showed significantly decreased lymphocyte proliferation and increased infection rates when exposed to the nematode parasite *Rhabdias rana*, although the difference was not significant (Christin et al. 2003). Some pesticides also have been linked to lower levels of circulating eosinophilic granulocytes (Kiesecker 2002).

In addition, pesticides may work indirectly to increase susceptibility to parasitic infection. Tadpoles can avoid free-swimming parasites by swimming away or in erratic patterns to resist skin penetration and fight internal cysts using immune defenses (Taylor et al. 2004). By remaining still to avoid predator detection, tadpoles can suffer increased trematode infection rates (Thiemann and Wassersug 2000, Taylor et al. 2004,

Koprivnikar et al. 2006). The decreased activity patterns caused by some pesticides (Bridges and Semlitsch 2000) could likewise make tadpoles more susceptible to trematode infection (Taylor et al. 2004).

Despite the large body of research on the effects of contaminants on immune systems of adults, their effects on the immune systems of animals exposed during very early development is not well studied. The developing immune system may be more susceptible to contaminants (Carey and Bryant 1995) and lead to long-term changes in immunocompetency (Luebke 2002, Milston et al. 2003). A study that exposed chinook salmon eggs to o,p'-DDE, a metabolite of DDT, found that although there was no effect on mortality, time to hatch, or gonadal development, the humoral response was significantly reduced even 1 yr after exposure (Milston et al. 2003), suggesting that immunosuppression due to early embryonic exposure may persist.

Dose Timing and Latent Effects

The long-term and latent effects of contaminant exposure are less well known than the direct lethal and sublethal effects (Carey and Bryant 1995). In one of the few studies of latent effects on amphibians, larval salamanders (*Ambystoma barbouri*) failed to show adaptive behavior and had decreased survival to 14 mo after exposure to the triazine herbicide atrazine (Rohr and Crumrine 2005, Rohr and Palmer 2005). Long-term behavioral changes such as this would not be detected by the typical short-term toxicity tests. Chemicals that affect the immune system also may lead to effects undetectable until after exposure (Luebke 2002).

A few studies have investigated latent effects of pesticides while examining the relative susceptibility of various amphibian life stages. This information is important because many of the modern, commonly used pesticides rapidly break down in the environment. Thus, a short, pulsed dose may be more representative of environmental exposure than constant renewal methods. However, there is conflicting evidence as to which developmental stages are most susceptible. Carbamate, pyrethroid, and organochlorine insecticides are most lethal to tadpoles, compared to eggs and hatchlings (Berrill et al. 1998, Bridges 2000, Greulich and Pflugmacher 2003). Although the 'egg' doses of these pesticides did not cause high mortality, in both the carbamate and pyrethroid cases, only individuals dosed as eggs were smaller at metamorphosis than their respective controls (Bridges 2000, Greulich and Pflugmacher 2003). In contrast to these studies, the toxicity of organophosphates follows the opposite trend in susceptibility among developmental stages, with egg-doses causing the highest mortality (Mohanty-Hejmadi and Dutta 1981). Interestingly, these developmental stage susceptibility trends for organophosphate and carbamate insecticides in amphibians are reversed in developing fish (Kaur and Dhawan 1993).

Although the different modes of action of the pesticides used in these life-stage studies may explain some of the life-stage susceptibility discrepancies, this variation also suggests the need for further research. Additionally, the only experiment that looked at the effects of organophosphates on amphibian larval stages had many major flaws. The high concentrations used, 5 to 350 mg/L, killed all egg-exposed and most feeding-stage-exposed tadpoles before they could reach metamorphosis (Mohanty-Hejmadi and Dutta 1981). Due to the high concentrations and high mortality rates, the sublethal effects of

these pesticides at environmentally realistic concentrations cannot be estimated from this study. Additionally, all the embryos came from one mating pair, making this an unreplicated study (Mohanty-Hejmadi and Dutta 1981), since sensitivity can vary by clutch (Bridges and Semlitsch 2001).

Parasites and Contaminant Susceptibility

Parasites have long been recognized to play a role in the regulation of some wild populations (Anderson and May 1979, May and Anderson 1979). Although the effects of environmental change on parasite dynamics have received attention, research has focused on vector-borne diseases of humans (Marcogliese 2001). It is difficult to predict the effects of environmental change on parasites with multiple hosts since each intermediate host may be differently affected by changes in temperature, precipitation, UV, and other abiotic factors (Marcogliese 2001). Additionally, cultural eutrophication has been linked to increased snail density, the primary host of many trematode species (Johnson and Chase 2004). Changes in host immune parameters because of environmental stressors or contaminants may compound the future impact of parasites on wildlife populations (Lafferty and Kuris 1999, Patz et al. 2000, Daszak et al. 2001, Lafferty and Holt 2003). Amphibian parasites have received more attention since the realization that the trematode *Ribeiroia ondatrae* can cause severe malformations (Johnson et al. 1999, Johnson et al. 2003, Schotthoefer et al. 2003b). However, even parasites that do not cause such obvious effects can be detrimental to amphibian populations (Beasley et al. 2003).

Not only can contaminants increase host susceptibility to parasites (Kiesecker 2002, Taylor et al. 2004, Taylor et al. 2005), in some cases parasite load can alter the

susceptibility of hosts to contaminants. Invertebrates are first intermediate hosts to many parasites including echinostomes. Although some studies have found that parasites can increase invertebrate host susceptibility to heavy metals (Guth et al. 1977), many other studies found no effect of parasite load on toxicity (McCahon et al. 1988, Brown and Pascoe 1989, Heinonen et al. 1999) or a decrease in toxicity (Heinonen et al. 2001). In contrast to the invertebrates, fish species serving as the second intermediate hosts to parasites were more susceptible to the heavy metals cadmium (Pascoe and Cram 1977), copper (Ewing and Ewing 1982), and zinc (Boyce and Yamada 1977). Coho salmon fry, *Oncorhynchus kisutch*, were more susceptible to oil, naphthalene, and toluene when infected with the parasitic juveniles, glochidia, of the freshwater mussel *Anodonta oregonensis* (Moles 1980). Tadpoles generally play the role of second intermediate hosts, similar to many fish species, yet the effects of parasites on their susceptibility to contaminants has, to the best of my knowledge, never been studied.

Malathion

Malathion (diethyl (dimethoxy thiophosphorylthio) succinate) belongs to the organophosphate (OP) family of insecticides. Like carbamate insecticides, OP's work by inhibiting the acetylcholinesterase enzyme, leading to nervous system over-stimulation (NPIC 2001). OP's, however, cause irreversible acetylcholine inhibition, whereas carbamate inhibition is reversible (Ecobichon 1996, Kallander et al. 1997). Malathion's toxicity is classified as very low or low for mammals, moderate for birds, and moderate to very high for fish and aquatic invertebrates. It is slightly water soluble, 145 mg/L at

20°C. Malathion is rapidly degraded by microbes, and thus has a very short half-life in the soil, 1-25 d, and in natural, neutral pH water, 1-14 d (NPIC 2001).

Malathion is the single most heavily used agricultural insecticide and the 6th most used agricultural pesticide (including herbicides, insecticides, and fungicides) in the U.S. In 2001, 9.1-11.3 million kg of malathion were used for agriculture (Kiely et al. 2004). Malathion is primarily used on cotton crops for boll weevil eradication (41.6% - 60% of total use). It is also commonly used on alfalfa, sorghum, rice, and fruit crops (Larson et al. 1999). Malathion is also heavily utilized in the private and government sectors (Kiely et al. 2004). It is commercially available as an emulsifiable concentrate, dust, wettable powder, or 95% active ingredient (a.i.) ultra-low volume spray, under many alternate names including carbophos, mercaptothion, and Fyfanon. For cotton crops, ultra-low volume sprays (95% a.i.) are typically used at a rate of 33.6 kg of the active ingredient per km^2, but maximum spray rates of 280 kg/km^2 are permitted (EPA).

In addition to agricultural and home garden uses, malathion is used to combat mosquitoes. It is legal for direct application to mosquito-prone areas, including wetlands and other areas where amphibians breed, in many states including Virginia (VDH 2005). The EPA limits the malathion application rate for mosquito control at 70.7 kg/km^2 and estimates that 33,306 km^2 are treated with malathion annually as part of mosquito control programs (USEPA 2006a). Although this rate is lower than crop application rates, because it is applied directly to the water in which many amphibian species breed, mosquito control programs can cause much higher water concentrations. Therefore, the use of malathion and other pesticides for protecting humans and wildlife from mosquito-borne diseases may have unintended consequences for fish and wildlife.

Total insecticide use in the U.S. has decreased by nearly 45% since 1980, but the use of organophosphates has increased from 58% to 70% of total insecticide use over the past 20 years (Kiely et al. 2004). Although the EPA is pushing for the development of organophosphate alternatives (USEPA 2004), OP's are still legal and widely used (Kiely et al. 2004). Additionally, integrated pest management (IPM) strategies recommend rotating between pesticides with different modes of action to avoid evolution of resistance. Malathion continues to be the primary OP used in IPM plans to combat mosquitoes.

Malathion is commonly found in rivers, streams, and drinking water, but at very low concentrations (NPIC 2001). By quantity, more malathion is used on agricultural land than for urban use (59-61%) (USEPA 2006a), because of its short half-life in soil, the concentrations of malathion in urban runoff often exceed those in agricultural runoff. For example, the average malathion concentration in creeks downstream from urban areas was 177 µg/L and creeks downstream from agricultural fields had an average malathion concentration of only 15.5 µg/L (CDFG 1982). Thus, both the agricultural ULV and commercial 50% formulations have the potential to enter aquatic environments.

In a survey of eight streams from across the U.S., Hoffman et al. (2000) detected malathion in over 20% of urban surface water samples, but concentrations exceeded the aquatic life criterion, 0.43 µg/L (CADFG 1998), in very few samples. Other U.S. water quality surveys have found similar results; malathion is one of the top 5 insecticides detected but concentrations are generally below 0.1 µg/L, and in few instances were concentrations above 40 µg/L (Larson et al. 1999). Because of its extremely short half-life in water, surveys are likely to underestimate the maximum concentrations of

malathion in surface water. Under normal ULV spraying conditions, 21% of the spray concentration was found 100 m away, and 12 % of the original dose drifted 200 m from the spray site. Thus, spray drift could contribute significant quantities of malathion to nearby waters and lead to toxic concentrations depending upon depth of the water body and original spray concentration (Penn State 1993). Malathion sprayed for mosquito control may be applied directly to bodies of water, adding 100% of the spray concentration to the water.

Malathion is widely used because of its low mammalian and avian toxicity (NPIC 2001), but its toxicity to amphibians is higher. The teratogenic effects of malathion on anuran embryos have been studied using the model species African clawed frog, *Xenopus laevis* (Snawder and Chambers 1989, 1993, Bonfanti et al. 2004). At the high concentrations (1 mg/L to over 10 mg/L) used in these 96 h acute toxicity studies, there was lower hatching success, smaller size, increased incidence of malformations, aorta and notochord abnormalities, and decreased tadpole survival (Snawder and Chambers 1989). Published *Xenopus* TC50's for malathion are 5 mg/L (Snawder and Chambers 1989) and 2.394 mg/L (Bonfanti et al. 2004). Concentrations below 1 mg/L caused increased acetylcholinesterase inhibition in Hensel toad (*Bufo arenarum*) embryos compared to controls (40-90%), but survival remained high (de Llamas et al. 1985). At a much higher dose, 44 mg/L, surviving *B. arenarum* embryos had significantly decreased acetylcholinesterase, butyrylcholinesterase, and aliesterase activities than control embryos after 4 days (Rosenbaum et al. 1988).

Several studies have examined the chronic effects of malathion on free-swimming anuran larvae (stage 25, (Gosner 1960) and found slight decreases in survival at 1 mg/L

in some species (*Rana catesbeiana, Bufo americanus*) but not in others (*R. sylvatica, R. pipiens, R. clamitans, H. versicolor*) (Relyea 2004). Lower concentrations, 0.315 mg/L , caused few direct effects on the developing tadpoles (Relyea et al. 2005). *R. catesbeiana* tadpoles exposed to malathion in a 28 d static renewal test showed decreased survival at concentrations above 2.5 mg/L , slowed growth and development above 1 mg/L , and affected equilibrium posture maintenance at even the lowest concentration tested, 0.5 mg/L (Fordham et al. 2001).

Malathion has been shown to affect the immunocompetency of many amphibian species. In adult *R. pipiens*, an injection of 3 μg/g malathion severely decreased antibody responses and suppressed neutrophil activation for at least 8 wk (Gilbertson et al. 2003). After dermal exposure to 1.1 and 11 μg/g malathion, adult Woodhouse's toads, *Bufo woodhousi*, suffered higher mortality when injected with the bacterium *Aeromonas hydrophilia* than controls or those exposed to malathion after bacterial injection (Taylor et al. 1999). Sublethal doses of malathion (2 and 0.2 mg/L) for 4 wk caused increased trematode (*Ribeiroia sp.* and *Telorchis sp.*) infection in wood frog, *R. sylvatica*, larvae (Gosner stage 25) (Kiesecker 2002). This study demonstrated the immunosuppressive effects of long-term exposure to malathion to tadpoles and raises interesting questions for future research on the effects of exposure at different life stages and under different exposure durations.

Pond Mesocosms

Amphibian toxicology research, excluding federal regulatory testing, has expanded in recent years and has begun to switch from traditional 96-hour lethality tests

to incorporate longer-term laboratory studies and mesocosm experiments (Sparling et al. 2000, Boone and James 2005). Large (1000 L) pond mesocosms permit the study of sublethal and community-level effects (Wilbur 1989). Mesocosms may not completely mimic environmental conditions (Jaeger and Walls 1989), but can be truly replicated, incorporate greater realism than traditional laboratory experiments, and permit more sophisticated experimental designs than traditional field studies (Hairston 1989, Morin 1989, Wilbur 1989). Mesocosms are especially germane for toxicology research because contaminant tolerances vary by species and have the potential to impact community dynamics (Fleeger et al. 2003, Hopkins et al. 2004, Boone and James 2005).

Study Species

Pickerel frogs, *Rana palustris*, are found throughout all but the southernmost parts of the eastern United States. They commonly breed in permanent ponds with submerged aquatic vegetation in early spring. Females lay clutches of approximately 2,500 eggs (Conant and Collins 1998) which hatch in 7 -14 d, depending upon water temperature (Martof et al. 1980). The tadpoles metamorphose in one season, typically within 70-80 d. (Wright 1914, Moore 1939). *Planorbella trivolvis* snails are widely distributed throughout North America (Friesen 1981) and are commonly infected with the trematode *Echinostoma trivolvis*. *E. trivolvis* is a 37-collared-spined digenean trematode parasite in the family Echinostomatidae. It uses *P.trivolvis* snails as a first intermediate host and primarily uses ranid tadpoles as second intermediate hosts. Definitive hosts include semi-aquatic birds and mammals (Huffman and Fried 1990).

Summary and Predictions

There is a gap in our knowledge of the practical effects of organophosphate insecticides on amphibians because unnaturally high doses have been used in all previous studies of their effects on early embryonic stages. These short-lived pesticides may have sublethal, persistent effects on early stage embryos (eggs) exposed only during that sensitive developmental stage. Malathion is known to cause decreased immune system function in tadpoles and adults, but little is known of its immune system effects after embryonic exposure. I tested the hypothesis that an early embryonic exposure to malathion will, in a dose-dependent manner, cause decreased hatching success, increased susceptibility to parasite infection, and delayed and smaller size at metamorphosis compared to controls.

The reciprocal question, whether animals infected with parasites will be more susceptible to pesticides, also has not been adequately addressed in amphibians. Evidence from other taxa suggests parasitized individuals may be more susceptible to contaminants than non-infected conspecifics. I tested the hypothesis that the malathion LC_{50} of infected tadpoles would be lower, in an infection-dependent manner, compared to uninfected tadpoles.

CHAPTER 1

The relative toxicity of malathion to trematode-infected and non-infected *Rana palustris* tadpoles.

ABSTRACT

Amphibian populations around the world are facing threats including disease and pollution. Although the effect of environmental contaminants on susceptibility to infection has been demonstrated for several amphibian species, to our knowledge, the opposite interaction, infection status affecting contaminant susceptibility, has never been studied. I conducted standard 48-hr toxicity tests to compare the susceptibility of uninfected pickerel frog (*Rana palustris*) tadpoles to tadpoles infected with two levels (10, or 30 cercaria) of the trematode *Echinostoma trivolvis* to malathion, a widely used organophosphate insecticide. Encystment rates were high (> 90%) in both trematode treatment groups. LC_{50} values ranged from 16.5 – 17.4 mg/L, within the range reported for other amphibian species. However, I found no differences in susceptibility to malathion among parasite treatments. Although I detected no effect of parasites on susceptibility in this system, it is important to investigate this question using other pesticides, parasites, and amphibian hosts before dismissing this potentially threatening interaction.

INTRODUCTION

Amphibian populations are declining around the world (Wake 1991, Stuart et al. 2004), and both disease (Berger et al. 1998, Daszak et al. 1999, Daszak et al. 2003, Kiesecker et al. 2004) and environmental contaminants (Berger 1989, Bishop et al. 1999, Davidson 2004) have been implicated in some of the declines. Not only are contaminants and disease individually important, but they may act in combination to have a larger impact on amphibian populations (Kiesecker 2002, Beasley et al. 2003). Environmental

contaminants have been correlated with decreases in immune function (Kiesecker 2002, Christin et al. 2003, Gilbertson et al. 2003, Christin et al. 2004) and increases in prevalence of parasite infection (Christin et al. 2003, Lewis et al. 2003). Furthermore, pesticides may indirectly increase susceptibility to parasite infection by decreasing activity patterns (Bridges and Semlitsch 2000) since tadpoles can avoid free-swimming parasites by moving away or swimming in erratic patterns (Thiemann and Wassersug 2000).

Although the influence of pollution on disease susceptibility has been studied in amphibians, the reverse scenario has never been tested. However, the influence of parasite load on susceptibility to contaminants has been studied in other taxa. Although some studies reported that parasites can increase invertebrate host susceptibility to heavy metals (Guth et al. 1977), more studies found no effect of parasite load on toxicity (McCahon et al. 1988, Brown and Pascoe 1989, Heinonen et al. 1999). In contrast to invertebrates, fish species acting as second intermediate hosts to parasites were more susceptible to heavy metals than non-infected conspecifics (Boyce and Yamada 1977, Pascoe and Cram 1977, Ewing and Ewing 1982). Coho salmon fry were more susceptible to oil, naphthalene, and toluene when infected with parasitic glochidia of freshwater mussels (Moles 1980). Tadpoles generally play the role of second intermediate hosts to many parasite species, similar to many fish, yet the effects of parasites on their susceptibility to contaminants has never been studied.

Using a common parasite and pesticide, my study addressed the effects of parasite infection on the susceptibility of amphibians to contaminants. Malathion (Diethyl [(dimethoxyphosphinothioyl)-thio] butanedioate) is the primary organophosphate (OP)

used to combat mosquitoes, the most utilized agricultural insecticide, and the 6th most heavily used of all pesticides in the U.S. (Kiely et al. 2004). It is commonly found at low concentrations in rivers, streams, and drinking water (Larson et al. 1999, Hoffman et al. 2000, NPIC 2001). *Planorbella trivolvis* snails are widely distributed throughout North America (Friesen 1981), and are commonly infected with the trematode *Echinostoma trivolvis* which is known to infect a wide variety of *Ranid* frogs in North America (Huffman and Fried 1990). I used standard LC_{50} methods (ASTM 2005) to compare the toxicity of malathion among *E. trivolvis* infected and non-infected pickerel frog, *Rana palustris*, tadpoles.

MATERIALS AND METHODS

Species Information

Pickerel frogs are found throughout the eastern United States. In early spring, females lay clutches of approximately 2,500 eggs which hatch in 7 -14 d, depending upon temperature (Conant and Collins 1998). Metamorphosis typically occurs within 70-80 d of hatching (Wright 1914, Moore 1939). *Echinostoma trivolvis* is a 37-collared-spined digenean trematode parasite that encysts in the kidneys of *Ranid* tadpoles (McAlpine and Burt 1998). Definitive hosts include semi-aquatic birds and mammals (Huffman and Fried 1990).

Animal Husbandry

Pickerel frogs used in this experiment were collected from a pond in rural Botetourt County, VA adjacent to Jefferson National Forest lands. The pond did not

contain *H. trivolvis* snails. On March 25, 2007, I collected nine clutches of recently laid eggs and transported them in a cooler to a laboratory in Blacksburg, VA. Eggs were immediately separated by hand, keeping jelly coats intact. Eleven eggs from each clutch were combined to form 16 lots of 99 eggs and each lot was allowed to hatch in 1.5 L of water in the laboratory. The water used was a 75/25 mix of dechlorinated town water and well water, respectively. Previous research demonstrated the available well water was extremely hard (364 mg/L $CaCO_3$) and caused spinal malformations in developing wood frogs, *Rana sylvatica* (unpub. data). Town water (62 mg/L $CaCO_3$) was dechloraminated with a commercially available powder, ChlorAm-X (AquaScience). This mix was necessary to bring the hardness to acceptable levels (172 mg/L). Fifty percent water changes were carried out every 2 d prior to hatching.

Hatching success was assessed on April 4, 2007. Sixty-five well-formed hatchlings from each of the 16 lots were transferred to 16 corresponding mesocosms. The replicate aquatic communities were set up in 1,500 L polyethylene stock tanks in Blacksburg, VA. In early March, stock tanks were filled with approximately 475 L of well water and 475 L of dechlorinated city water. Because the mesocosms received natural precipitation as well as biological material (see below), this 50/50 mix was used, rather than the 75/25 mix used in the laboratory,. Each tank received 1 kg of air-dried deciduous leaf litter,17 g of finely ground Purina Rabbit Chow®, and two 1.5 L spikes of pond water on March 14 and April 2, 2007. The pond water, taken from a small permanent pond on the Virginia Tech property, was filtered through a 200 µm sieve to remove odonate eggs before addition to the tanks. To decrease the variability in initial phytoplankton and zooplankton communities, water was repeatedly exchanged among

mesocosms. The water hardness after addition of biological material was190 mg/L. To provide shade and exclude predators and competitors, mesocosms were covered with black mesh lids. In three randomly selected replicates, I measured conductivity, pH, temperature, and DO weekly at 7:30 am and 7:30 pm (approximate coolest and warmest daily water temperatures, respectively).

Parasite Exposure

The experiment was conducted in three separate 4-d runs over the course of 7 d, later combined to form three complete replicates as stipulated by ASTM (2005). The runs were overlapped to minimize tadpole size differences among runs. For each of the three runs, fourteen tadpoles were haphazardly selected from each of the 16 mesocosms. The tadpoles were 33, 36, and 38 d post hatch and at approximately Gosner stage 26 (Gosner 1960). The tadpoles were acclimated for 24 hr in bulk containers while their water was slowly changed to reconstituted water (ASTM 2005). Tadpoles were then placed individually in plastic cups with 90 ml of reconstituted water and randomly assigned to control, low (10 cercariae), or high (30 cercariae) parasite treatments. Four infected *P. trivolvis* snails were collected from a golf course pond in Riner, Virginia. The same four snails were induced to shed cercariae under a heat lamp for each run. Cercariae were immediately counted under a dissecting microscope, and then added to the tadpole cups with a glass pipette. Cercariae were given 24 hr to encyst in the tadpoles. After 24 hr, the tadpoles were removed from their parasite exposures and randomly assigned to pesticide concentrations.

Pesticide Exposure

I used a 48-hr static test rather than a 96-hr test because trial runs showed increased mortality across all treatments including controls towards the end of 96 hr tests. A stock solution was prepared by diluting pure malathion (Chem Service, West Chester, PA) with methanol. The pesticide treatments included five test concentrations ranging between 5.2 and 40 mg/L, each 60% of the next higher concentration, a water control, and a solvent control equal to the highest test concentration of methanol (0.4%). Concentrations were verified in duplicate by the Virginia Tech Pesticide Residue Laboratory (Table 1). The test containers consisted of glass 2L beakers, and were randomly assigned to a location within a temperature-controlled chamber. The tests were conducted at 17^{o}C so that ASTM temperature specific mass/volume limits were met. The tadpoles received 16 hr light and 8 hr dark.

Mortality was assessed every 8 hr for the duration of the 48-hr tests by gently stirring the jars and checking for movement. Unresponsive tadpoles were examined further for signs of life. Dead tadpoles were frozen in individual microcentrifuge tubes for later verification of parasite encystment levels. For all three parasite treatments (0, 10 and 30 cercaria), temperature, dissolved oxygen, and pH were measured at 0, 24, and 48 hr in the control, low, medium and high concentrations as stipulated by ASTM (2005). The ten tadpoles in the malathion control treatment (0 ppb) were weighed at the end of each run for each of the three parasite treatments. All surviving tadpoles were frozen for subsequent dissection to verify parasite encystment levels.

Parasite Dissections

Under a dissecting scope, the kidneys (pronephros and mesonephros) were removed from each tadpole with forceps and placed on a slide. A coverslip was gently pressed onto the tissue to produce a thin layer. Metacercarial cysts were counted at 100x using a compound microscope. Fifteen control (no parasite) tadpoles from each of the three runs were dissected to verify that they were infection-free. For each run, six parasite-infected tadpoles from each pesticide treatment were randomly subsampled to confirm infection levels. All parasite-infected tadpoles at the concentration (14.4 mg/L) with partial mortality were dissected. In total, 66% of the 420 low and high parasite tadpoles and 20% of the 210 non-infected tadpoles were dissected.

Statistical Analysis

Weight data were not normally distributed (Shapiro-Wilk p's < 0.05), so Kruskal-Wallis tests were used to compare tadpole weights among treatments and runs. To compare the mean number of cysts among runs, I conducted separate ANOVAs for each infection level, followed by Tukey's pairwise comparison tests. To compare encystment among pesticide concentrations within each run, I ran an ANOVA for each infection level. I compared the overall proportion of parasites encysting between the 10 and 30 cercariae exposure treatments using Wilcoxon's two-sample test because the proportions were not normally distributed. Because I only had one concentration with partial mortality, I used Spearman Karber methods to estimate an LC_{50} for each run followed by ANOVA to compare LC_{50}'s among runs. At the concentration with partial mortality, ANOVA was used to compare parasite encystment levels of surviving tadpoles to those that died for both parasite infection treatments. The experiment-wide α was set at 0.05.

RESULTS

All three runs met ASTM water quality standards. Mean dissolved oxygen (DO) was 96.8%, 89.4%, and 86.3% after 0, 24, and 48 hr, surpassing the ASTM guideline of minimum 60% DO. The minimum measured DO over all three runs was 79.3%. The average temperature (16.57 ± 0.04 ^{o}C) approximated the selected test temperature (17^{o}C). Temperatures met ASTM standards for allowable variation among replicates and runs. The average pH was 7.29, falling within ASTM guidelines and the range in which malathion is stable.

There were no differences in tadpole weight among parasite treatments within each run (p's = 0.25, 0.77, 0.71) although weight did differ significantly across the three runs ($F = 6.03$, $df = 2, 87$, $p = 0.004$). Mean tadpole weights (± 1 se) for the runs were 0.172 (0.009), 0.200 (0.009), and 0.215 (0.009) g. The consistency of toxicity results suggested this increase of 0.043 g between runs 1 and 3, although statistically significant, did not influence sensitivity to malathion. The overall proportion of cercariae encysting was high (over 91%) and did not vary between the 10 and 30 cercariae treatments (p = 0.83). Mean number of cysts varied significantly for the 10 cercariae treatment (Figure 1; $F = 3.67$, $df = 2, 135$, $p = 0.03$) but did not differ among runs for the 30 cercariae tadpoles ($p = 0.24$). The difference in cysts for the 10 cercaria treatment was due to an approximately 10% decrease in the number of cercariae encysting in the second run, which differed statistically from the third run. Mean number of cysts did not differ among malathion concentrations for either the 10 ($p = 0.78$) or 30 cercariae ($p = 0.73$) treatments.

The toxicity tests also met ASTM requirements for mortality. There were no mortalities in the control, solvent, and lowest malathion concentration during all three runs, and complete mortality occurred in the highest two concentrations (Figure 2). LC_{50} estimates were similar among runs and treatments (Table 2) and did not differ significantly by parasite treatment ($p = 0.59$). In the one concentration with partial mortality, 14.4 mg/L, parasite loads did not differ significantly between those that survived and died for both the 10 and 30 cercariae treatments (Figure 3, p's = 0.91 and 0.16, respectively).

DISCUSSION

I found no difference in the toxicity of malathion among parasite treatments but more amphibian studies are needed to assess this potential interaction. My results suggest that the results of toxicity tests on uninfected amphibians may be representative of similar sized amphibians carrying a low to moderate parasite burden. This lack of effect of parasite load on malathion toxicity was further supported by the similar parasite loads of tadpoles that survived and died at the 14.4 mg/L concentration. This latter comparison has not been reported in related fish literature (Boyce and Yamada 1977, Pascoe and Cram 1977, Moles 1980, Ewing and Ewing 1982), but could be a useful post-hoc test for understanding subtle differences in host susceptibility to contaminants attributable to parasite infection.

My LC_{50} results differ from previous studies with fish hosts that found large effects of parasite load on toxicity (Boyce and Yamada 1977, Pascoe and Cram 1977, Moles 1980, Ewing and Ewing 1982), although neither malathion nor any other OP was

previously tested with fish in this manner. Coho salmon, *Oncorhynchus kisutch*, fry artificially infected with *Anodonta oregonensis* glochidia were 4.2, 3.0, and 4.6 times more susceptible to naphthalene, toluene, and crude oil, respectively, than uninfected fry. LC_{50}'s for all three compounds decreased linearly with increasing parasite infection (Moles 1980). Sockeye salmon, *Oncorhynchus nerka*, smolts naturally infected with the cestode *Eubothrium salvelini* had a significantly shorter time to death (39.4 hr) when exposed to 1 mg/L zinc than uninfected conspecifics (52.8 hr)(Boyce and Yamada 1977). Similarly, three-spined stickleback, *Gasterosteus aculeatus*, infected with the cestode *Schistocephalus solidus* had decreased survival times (up to 555 hr) than uninfected fish when exposed to cadmium (Pascoe and Cram 1977). The mechanisms by which both internal (cestode) and external (glochidia) parasites affected the tolerances of these fish species to toxicants as diverse as heavy metals, aromatic hydrocarbons, and crude oil are unknown (Boyce and Yamada 1977, Pascoe and Cram 1977, Moles 1980).

Without further knowledge of the mechanisms explaining these susceptibility differences in infected fish, it is difficult to say why I failed to see a similar response in my study, but several possibilities exist. My infection period of only 36 hr may have been too short to affect contaminant susceptibility. Laboratory studies show chronic *Echinostoma trivolvis* infection can decrease growth and cause mortality of amphibian larvae (Fried et al. 1997, Schotthoefer et al. 2003a). Additionally, both high *E. trivolvis* infection (Fried et al. 1997, Schotthoefer et al. 2003a), and chronic malathion exposure are known to impair kidney function (Chakraborty et al. 1978, Bosco et al. 1997). Other OPs caused kidney pathology in fish (Gill et al. 1988, Srivastava et al. 1990), but not until 48 hr post exposure (Srivastava et al. 1990). This evidence suggests that my

exposures may have been too short to cause kidney damage. It is also possible that my infection level may have been below the level necessary to impair kidney function, e.g. Belden (2006), or compound damage due to malathion exposure. Finally, it is plausible that differences between my study and previous studies on fish may relate to species specific differences in the host-parasite systems studied. For example, glochidia cause significant damage to their hosts and mortality of fish infected with high loads (Moles 1983). The high cost of infection may explain why glochidia increased salmonid susceptibility to naphthalene, toluene, and crude oil (Moles 1980). This is unlikely the case for cestode parasites which, although known to increase the toxicity of zinc and cadmium to fish (Boyce and Yamada 1977, Brown and Pascoe 1989), cause little damage to their hosts (Hanzelova et al. 2005).

My LC_{50} values (16-17 mg/L) fell within the wide range of known values of malathion toxicity for other aquatic wildlife. The U.S. EPA lacks sufficient data to classify the toxicity of malathion for aquatic amphibians (USEPA 2006b), although preliminary data suggest aquatic invertebrates and fish are more susceptible than many, but not all, amphibian species. *Rana tigrina* has a LC_{50} of 40 ppm for tadpoles (Mohanty-Hejmadi and Dutta 1981), above my estimate for pickerel frogs. Other *Ranids* have LC_{50}'s as low as 2.2 mg/L for *Rana limnocharis* tadpoles (Pan and Liang 1993) and 0.6 µg/L for *Rana hexadactyla* hatchlings (Khangarot et al. 1985). Other anuran genera may be more susceptible to malathion; Fowler's toad, *Bufo woodhousei fowleri*, and western chorus frog, *Pseudacris triseriata triseria*, tadpoles have 96-hr LC_{50} values of 420 µg/L and 200 µg/L, respectively (Sanders 1970). Studies suggest that acetylcholinesterase inhibiting pesticides, a class that includes malathion, can decrease

the condition and survival of aquatic salamander larvae by decreasing food resources (Metts et al. 2005, Relyea 2005a), but more data on the toxicity of malathion to larval salamanders are needed. Malathion is classified as very highly toxic to fish, with a LC_{50} value of 30 µg/L for bluegill sunfish, *Lepomis macrochirus*. It is also highly toxic to aquatic invertebrates, with an EC_{50} of 1.0 µg/L (USEPA 2006b).

Although I found no effect of parasite load on toxicity to hosts, my results do not diminish the known individual and interactive effects of both disease and toxicants on amphibian populations. Additionally, *E. trivolvis* infection intensity in natural tadpole populations can exceed those used in this study by 10-fold (Fried and Bradford 1997), up to 1650 cysts in one individual (Skelly et al. 2006), and those higher parasite burdens may affect contaminant susceptibility. As tadpoles grow, susceptibility to *E. trivolvis* decreases and tolerance increases (Schotthoefer et al. 2003a). Thus, younger tadpoles may be more vulnerable to the interactive effects of parasites and pesticides. Also, the tadpoles used in this study were infected only 1 day prior to pesticide exposure. A longer infection period before exposure could weaken the tadpoles and potentially decrease their susceptibility to contaminants.

In ecological systems modified by human disturbances, the incidence and intensity of parasite infections in amphibians and other hosts may increase in the future. Increased snail density, the primary hosts of many parasite species, has been linked to eutrophication (Johnson and Chase 2004). As eutrophication expands, intensity and prevalence of parasite infection may rise, increasing the need for toxicity testing using infected organisms. Furthermore, global climate change may increase the range and infectivity of many parasite species (Marcogliese 2001). In the current era of climate

change, cultural eutrophication and pesticide use, it is important to investigate the effects of parasite load on toxicity using other pesticides, parasites, and amphibian hosts before dismissing this potentially threatening interaction.

Table 1. Nominal and actual malathion concentrations used in LC_{50} tests. Concentrations were verified in duplicate by the Virginia Tech Pesticide Residue Laboratory.

Nominal Concentration	Sample 1	Sample 2	Mean
5.2 mg/L	5.4	4.6	5.0
14.4 mg/L	14	15	14.5
40.0 mg/L	40	40	40

Table 2. LC_{50} values (mg/L) for each run and overall mean by parasite treatment (0 – 30 parasites).

Parasite Treatment	0	10	30
Run 1	17.67	17.67	17.67
Run 2	17.67	17.67	16.79
Run 3	15.95	16.79	15.16
Mean	17.09	17.37	16.54

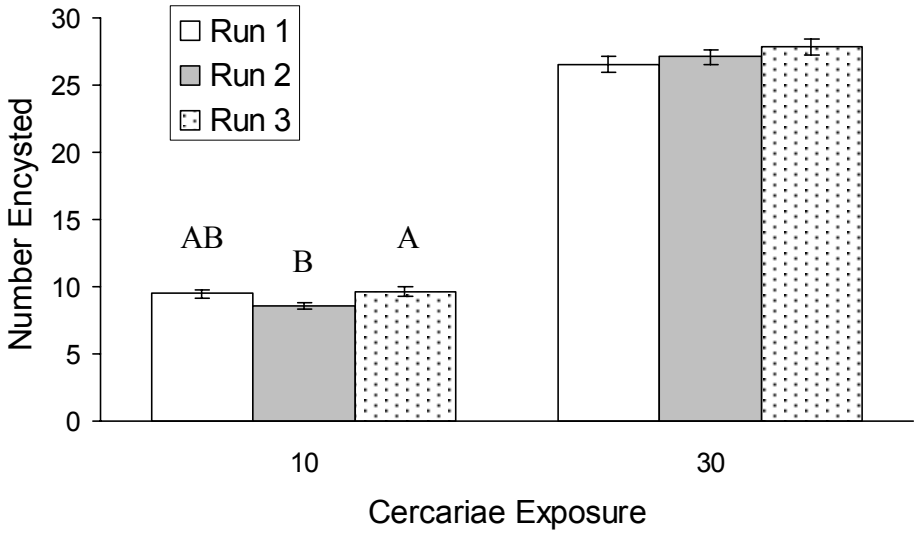

Fig. 1. Mean number of *E. trivolvis* metacercarial cysts (±1 se) in *R. palustris* tadpoles exposed to 10 and 30 cercariae in three sequential toxicity test runs (n = 10 tadpoles per concentration, n = 7 concentrations).

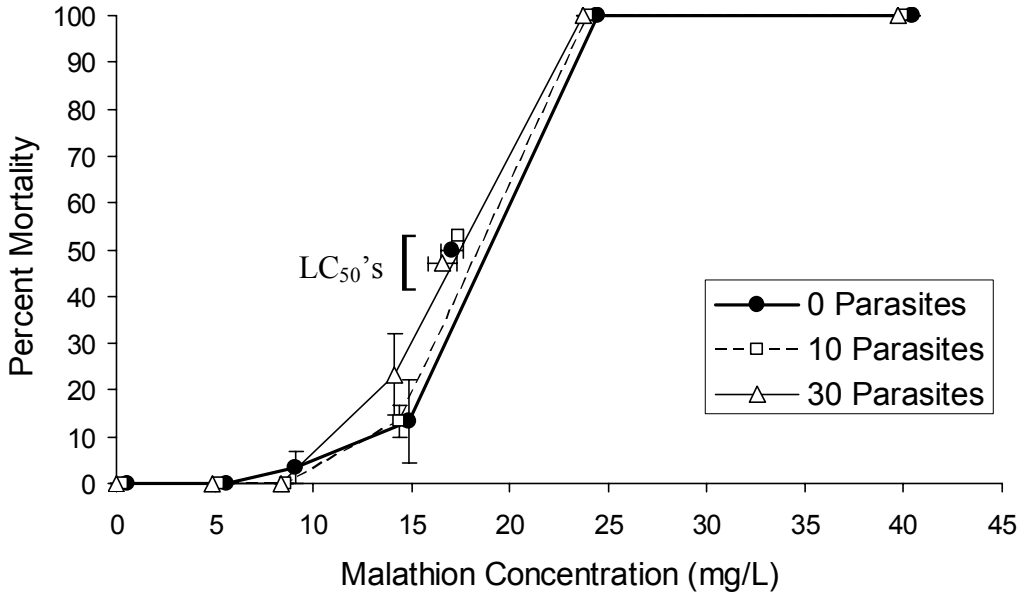

Fig. 2. Mean percent mortality (±1 se, vertical) across malathion concentrations and LC_{50} values (±1 se, horizontal) for the three parasite treatments. Points are staggered on both axes for clarity.

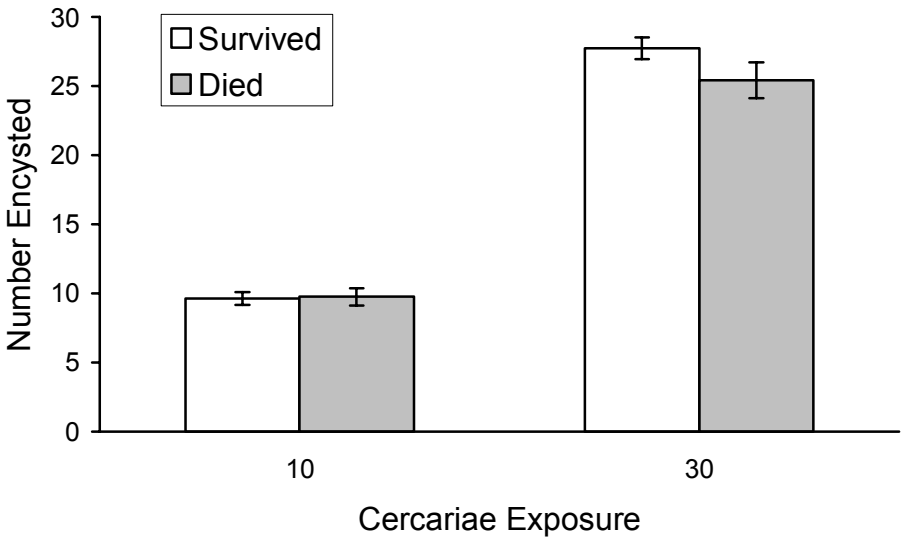

Fig. 3. Mean number of *E. trivolvis* metacercarial cysts (±1 se) in *R. palustris* tadpoles that survived or died during a 48 hr exposure to 14.4 mg/L malathion in two cercaria exposure treatments.

CHAPTER 2

The effects of malathion on embryonic development and latent susceptibility to trematode parasites in pickerel frog, *Rana palustris*, tadpoles.

ABSTRACT

I investigated the effects of embryonic exposure to low doses of the widely used organophosphate insecticide malathion on the early development and latent susceptibility of pickerel frog (*Rana palustris*) tadpoles to the trematode parasite *Echinostoma trivolvis*. Malathion decreased hatching success by 6.5% and viability rates by 17% at a concentration lower than previously documented for anuran embryos. Incidence of malformations increased from 0.5% in controls to 11.2% in the 600 µg/L malathion treatment. The primary malformations documented in the two highest pesticide doses were ventralization and axial shortening. After 7 wk of development in water with no malathion, tadpoles previously exposed as embryos for only 96 hr to 60 and 600 µg/L malathion suffered increased parasite encystment rates, when compared to controls. My research identifies embryonic development as a sensitive window for increased susceptibility to infection long after pesticide exposure has ceased. With potential for increased parasite prevalence from eutrophication and climate change, my data underscore the importance of understanding the role of pesticides in parasite susceptibility.

INTRODUCTION

The recent decline of some amphibian populations is a globally recognized crisis (Blaustein and Wake 1990, Wake 1991, Houlahan et al. 2000, Stuart et al. 2004), but the causes of declines and their relative magnitudes are still debated (Collins and Storfer 2003). Factors including overexploitation, habitat loss and fragmentation, invasive

species, climate change, increased UV-B exposure, environmental contaminants, and disease have often been cited as the causes of the declines (Carey and Bryant 1995, Kiesecker et al. 2001, Davidson et al. 2002, Blaustein et al. 2003a, Carey and Alexander 2003, Daszak et al. 2003, Kiesecker et al. 2004). These factors cannot only act alone, but in many cases they work in combination to have larger effects on amphibians. For example, increased UV-B exposure in dry summers increased the susceptibility of *Bufo boreas* eggs to a parasitic fungus (Kiesecker et al. 2001), and malathion exposure increased the susceptibility of adult *Bufo woodhousi* to a pathogenic bacteria (Taylor et al. 1999).

Although the role of chemical contamination in amphibian population declines is not well understood, regional amphibian declines, lower population densities, and decreased species diversity have been correlated with agricultural land use (Berger 1989, Bishop et al. 1999, Davidson 2004, Johansson et al. 2005). In addition, environmental contaminants are known to reduce immunocompetency in many species (Luebke et al. 1997, Grove et al. 2003, Linzey et al. 2003). Pesticides with several different modes of action decrease antibody responses (Gilbertson et al. 2003), lymphocyte proliferation (Christin et al. 2003, Christin et al. 2004), and levels of circulating eosinophilic granulocytes (Kiesecker 2002) in amphibians. Despite the growing body of research on the effects of contaminants on adult immune systems, their effects on the immune systems of animals exposed during very early development is not well studied. The developing immune system may be more susceptible to contaminants (Carey and Bryant 1995), and early exposure to foreign compounds could lead to long-term changes in immunocompetency (Luebke 2002, Milston et al. 2003).

The latent effects of contaminant exposure in amphibians are less well known than the direct lethal and sublethal effects (Carey and Bryant 1995). In one of the few studies of latent effects on amphibians, larval salamanders failed to show adaptive behavior and had decreased survival 14 mo after herbicide exposure (Rohr and Crumrine 2005, Rohr and Palmer 2005). Long-term behavioral changes such as this would not be detected by traditional short-term (e.g., 96 hr) toxicity tests. However, understanding latent effects of short-term exposure is important because many of the modern, commonly used pesticides rapidly break down in the environment.

I tested whether acute exposure to the organophosphate insecticide malathion during early embryonic development would affect embryonic survival, development, and latent susceptibility to parasitic infection. Malathion is the single most heavily used agricultural insecticide and the 6th most used agricultural pesticide in the U.S. (Kiely et al. 2004) and has been shown to affect the immunocompetency of several amphibian species (Rodgers and Xiong 1997, Taylor et al. 1999). I exposed embryos of pickerel frogs, *Rana palustris*, to three concentrations of malathion for 96 hr and then quantified hatchling viability. To investigate the latent effects of early embryonic exposure to malathion, I measured larval susceptibility to a common trematode 7 wk post-hatch.

MATERIALS AND METHODS

Species information

Pickerel frogs are found throughout all but the southernmost parts of the eastern United States. They commonly breed in permanent ponds with submerged aquatic

vegetation in early spring. Females lay clutches of approximately 2,500 eggs which hatch in 7 -14 d, depending upon temperature (Conant and Collins 1998). The tadpoles metamorphose in one season, typically within 70-80 d (Wright 1914). *Echinostoma trivolvis* is a 37-collared-spined digenean trematode parasite in the family Echinostomatidae. It uses the snail, *Planorbella trivolvis,* as a first intermediate host and often uses ranid tadpoles as second intermediate hosts, encysting in the kidneys. Definitive hosts include semi-aquatic birds and mammals (Huffman and Fried 1990).

Pickerel frogs used in this experiment were collected from one pond in rural Botetourt County, VA adjacent to Jefferson National Forest lands. The pond did not contain *P. trivolvis*. On March 25, 2007, I collected seven clutches of recently laid pickerel frog eggs and transported them in a cooler to a laboratory in Blacksburg, VA. Eggs were immediately separated by hand, keeping jelly coats intact. Eleven eggs from each clutch were combined to form 24 lots of 77 eggs each with similar genetic composition to determine hatching success. All eggs were initially raised in 1500 mL of 75/25 mix of dechloraminated city water (ChlorAm-X, AquaScience) and well water in the laboratory. The available well water was extremely hard (364 mg/L CaCO3), and previous research demonstrated it caused spinal malformations in developing wood frogs (unpub. data), so this mix was necessary to bring the hardness to acceptable levels (172 mg/L). Fifty percent water changes were carried out every 2 d.

Pesticide exposure

Three days after collection, the majority of embryos completed neurulation, reaching Gosner stage 14 (Gosner 1960). Six lots were then randomly assigned to each

of the control and three malathion treatments. Previous studies suggest that exposure to acetylcholinesterase inhibiting pesticides during later embryonic development is more detrimental than exposure during the first few days of development (Rosenbaum et al. 1988, Snawder and Chambers 1990), possibly because natural expression of acetylcholinesterase increases exponentially between fertilization and hatching ((Rosenbaum et al. 1988). Thus, I exposed the pickerel frog embryos to malathion from Gosner stage 14 to 18-19, starting approximately 5 d before hatching and ending within a day of hatching.

Embryos were exposed to 0, 15, 60, or 600 µg/L malathion (Chem Service, West Chester, PA) for 96 hr with one complete solution change after 48 hr. Concentrations were confirmed in duplicate at the Virginia Tech Pesticide Residue Laboratory (means = 14.5, 62 and 600 µg/L). These concentrations are lower than most other amphibian studies (Mohanty-Hejmadi and Dutta 1981, Relyea 2004, 2005a), but they are higher than levels typically found in routine water quality surveys (Larson et al. 1999, Hoffman et al. 2000). Because malathion breaks down so rapidly, peak concentrations may be missed in routine water quality surveys. My concentrations represent realistic malathion concentrations in natural waters receiving urban runoff (CDFG 1982) and those exposed to spray drift or runoff from agricultural fields (CDFG 1982, Penn State 1993).

After 96 hr of pesticide exposure, embryos were transferred to a 75/25 mix of dechlorinated city water and well water until hatching. Hatching began the day after exposures ended, April 2, and continued until April 4. I calculated hatching rates for each replicate and checked all hatchlings for malformations (Bantle et al. 1998). Hatchling viability was defined as individuals hatching and lacking malformations

(Hopkins et al. 2006). Malformed individuals were separated and stored in ethanol. Forty properly-formed tadpoles from each replicate were then moved to 24 corresponding outdoor aquatic mesocosms to test for latent effects of embryonic exposure.

Aquatic mesocosms

Replicate aquatic community mesocosms were established in 1,500 L polyethylene stock tanks in Blacksburg, VA. Mesocosms were filled in early March with approximately 475 L of well water and 475 L of dechlorinated city water. A 50/50 mix was used, rather than the 75/25 laboratory mix, because the mesocosms received natural precipitation as well as biological material (see below). The resulting water hardness was 190 mg/L. Each mesocosm also received 1 kg of air-dried deciduous leaf litter and 17 g of finely ground Purina Rabbit Chow®. The mesocosms were spiked with 1.5 L of pond water, from a permanent pond on the Virginia Tech property, filtered through a 200 µ sieve on March 14 and again on April 2, 2007. To decrease the variability in initial phytoplankton and zooplankton communities, portions of water were repeatedly exchanged between mesocosms prior to the addition of tadpoles. Mesocosms were covered with black mesh lids to provide shade and exclude predators and competitors. Conductivity, pH, temperature, and DO were monitored weekly at 7:30 am and 7:30 pm (approximate coolest and warmest daily water temperatures, respectively) in five randomly selected replicates. No pesticides were added to mesocosms.

Parasite exposure

Forty-six days post-hatch, 11 tadpoles (~ Gosner stage 26) were haphazardly removed from each of the 24 mesocosms and moved into the laboratory. For unknown reasons, two mesocosms (one 0 µg/L and one 600 µg/L) failed to support tadpoles and were excluded from the remainder of the study, reducing sample size to n = 5 in these two treatments. The subsampled tadpoles, grouped by mesocosm, were acclimated to room temperature and reconstituted water (ASTM 2005) over 24 hr. Ten tadpoles from each mesocosm were randomly assigned to individual 120 ml plastic cups containing 90 ml of reconstituted water. The cups were coded so that pesticide treatments were unknown. The remaining tadpole from each mesocosm was assigned to a control treatment to verify initial absence of parasite exposure. These parasite-control tadpoles were placed in identical cups with 90 ml reconstituted water but did not receive any parasites.

Snails (*P. trivolvis*) were collected from a golf course pond in Riner, VA. Seven *E. trivolvis* infected snails were induced to shed cercaria under a heat lamp. Sixty freshly shed cercariae were transferred to each tadpole cup with a glass pipette. Due to the limited rate of parasite shedding by the snails, groups of approximately 30 tadpoles were exposed each hour until all received parasites. Subsequent measurements of tadpoles were adjusted accordingly so that all individuals were exposed to cercariae for the same amount of time.

After 48 hr of exposure to cercariae, all tadpoles were measured, weighed, and staged (Gosner 1960). Tadpoles were then euthanized with MS 222 and frozen in individual microcentrifuge tubes for subsequent dissection. The microcentrifuge tubes also were coded so that the pesticide treatment of each tadpole was unknown during

dissection. Because *E. trivolvis* encysts in the kidneys, the entire kidneys, both pronephros and mesonephros tissues, were removed using forceps under a dissecting scope and placed on a slide. A coverslip was gently pressed onto the tissue to produce a thin layer. Slides were scanned under a 100x magnification of a compound microscope and cysts were counted.

Statistical analysis

Hatching success and malformation rates were not normally distributed (Shapiro Wilk p's < 0.05), so were compared among treatments using Kruskal-Wallis tests. Viability was compared among pesticide concentrations using ANOVA with Tukey-Kramer pairwise comparisons. Because these three variables were not independent of each other, α was adjusted using a sequential Bonferoni procedure. The fraction of cysts, out of a potential 60, successfully encysted in each tadpole were treated as subsamples and averaged individually for each mesocosm. Encystment rates were compared among treatments using ANOVA with subsampling. The malathion treatment encystment rates were compared to the control using Dunnett's test. Tadpole size at the time of subsampling was compared among treatments using ANOVA. The relationship between tadpole mass and number of parasite cysts was examined using linear regression.

RESULTS

Malformation frequency increased and both hatching and viability decreased as malathion concentration increased (Figure 1). Proportion hatching ($p = 0.003$),

malformed ($p = 0.001$), and viable ($p < 0.0001$) varied significantly among treatments. Hatching rates were high for the control and two lowest malathion treatments, ranging from 96-98%, but were reduced to 91.7% in the highest malathion concentration (600 µg/L). Malformation frequency increased from 0.7%, 1.9%, and 2.2% in the control, 15 µg/L, 60 µg/L malathion treatments, respectively, all consistent with acceptable background levels (ASTM 1991), to 11.2% in the 600 µg/L treatment. The three malformed control tadpoles had edema, two tails, and craniofacial abnormalities with a axial flexure, respectively. The most common malformation in the lowest malathion concentration was axial flexures (Table 1). The two highest malathion concentrations were similar in their malformation profiles, both having ventralization with axial shortening as their most common malformation (Table 1). The proportion of viable tadpoles in the highest malathion treatment was significantly lower than the other three treatments. Viability was reduced by 17.3% in the 600 µg/l treatment, compared to controls.

Neither mass ($p = 0.96$) nor length ($p = 0.96$) differed significantly among malathion treatments in the tadpoles subsampled for parasite exposure. There was no relationship between tadpole mass ($p = 0.095$, $r^2 = 0.13$) or length ($p = 0.15$, $r^2 = 0.10$) and the number of parasites successfully encysting. The mean number of metacercarial cysts varied significantly among malathion treatments (Figure 2; $F = 2.95$, $df = 3, 18$, $p = 0.034$). Tadpoles in two highest malathion concentrations, 60 and 600 µg/L, had 11.7% and 10.8% more cysts than tadpoles in the control treatment (p's = 0.019 and 0.044, respectively). Although encystment in the low malathion concentration (15 µg/L) was also increased compared to controls, this difference was not significant ($p = 0.105$).

DISCUSSION

My study demonstrated that short-term exposure to environmentally realistic concentrations of malathion during a critical developmental window can have immediate, as well as long-term, effects on developing pickerel frogs. Overall hatchling viability was decreased by 17% in my highest concentration treatment, 600 µg/L, which resulted from a decrease in hatching success and an increased incidence of malformations. Pesticide exposure during early development caused long-term changes in susceptibility, evidenced by increased parasite infection 7 wk after exposure. I demonstrated potential for long-term effects after early exposure, even if most development occurs in uncontaminated water. My results underscore the necessity of alternate testing techniques to detect effects that standard toxicity testing (e.g., 96-hr) would overlook.

Exposure to malathion significantly increased the incidence of malformations, particularly ventralization, accounting for over 50% of malformations at the two highest malathion treatments. This type of malformation suggests that malathion is interfering in the development of the dorso-anterior axis (Kao and Elinson 1988). Xenobiotics including UV, lithium, and retinoic acid are known to cause ventralization (Kao and Elinson 1988), dorsalization (Kao and Elinson 1989), and posteriorization (Durston et al. 1989) in developing embryos, respectively. Although such developmental phenotypes are not listed in the malformation guide for embryo teratogenesis assays (Bantle et al. 1998) or noted in any previous studies for malathion (Rosenbaum et al. 1988, Snawder

and Chambers 1989, 1990, Bonfanti et al. 2004), they are widely utilized in developmental biology to study embryonic signaling pathways.

Malathion reduced hatching success to 80% in the 600 µg/L treatment, far below the 98% hatching success observed in the control treatment. Although my design precludes calculation of an EC_{50}, this concentration is far below the minimum concentration shown to decrease enzyme activity (4 mg/L) and the LC_{50} (19 mg/L) for *Bufo arenarum* larvae (Venturino et al. 1992). It is also orders of magnitude below LC_{50}s for embryos of *Rana tigrina* (30 mg/L)(Mohanty-Hejmadi and Dutta 1981) and *Microhyla ornata* (20 mg/L)(Pawar et al. 1983). Pickerel frog embryos appear comparatively vulnerable to malathion, with respect to other anuran larvae. This sensitivity trend may not necessarily continue though later developmental stages (unpub. data).

Although pickerel frog and other anuran embryos are vulnerable to malathion, evidence suggests that LC_{50} values for several anuran species decrease as larval development ensues (0.59 - 420 µg/L)(Sanders 1970, Khangarot et al. 1985). Carbamate, pyrethroid, and organochlorine insecticides are more lethal to tadpoles, compared to eggs and hatchlings (Berrill et al. 1998, Bridges 2000, Greulich and Pflugmacher 2003). Multiple hypotheses could explain this counter-intuitive ontogenetic pattern. AChE is expressed in exponentially increasing quantities throughout embryonic development (Rosenbaum et al. 1988). AChE inhibiting pesticides could have greater effects on older larvae and tadpoles if expression continues to increase throughout development. Alternatively, the increase in toxicity during development could be a result of increased cytochrome P450 conversion of malathion to its more toxic metabolite, malaoxon

(Bonfanti et al. 2004). Further research is necessary to differentiate which, if either, of these hypotheses correctly explains why embryos are less sensitive to AChE inhibiting pesticides than older larvae.

Although embryonic exposure to pesticides may cause less mortality than larval exposure, survivors of embryonic exposures may suffer lasting consequences. I found increased susceptibility to trematode infection 7 wk after embryonic exposure to malathion. I hypothesize that the most plausible explanation for increased susceptibility to malathion was an altered immune response. By exposing the tadpoles to parasites in a small volume of water, I diminished their ability to behaviorally avoid cercaria, forcing them to rely primarily on physiological defenses, such as immune responses (Koprivnikar et al. 2007). Malathion has been shown to negatively affect the immune system of amphibians (Taylor et al. 1999, Gilbertson et al. 2003), including lowering circulating eosinophilic granulocytes, the type of white blood cell known to fight parasitic infection (Kiesecker 2002). However, because I did not measure immune function, I can not exclude a number of other possible explanations. For example, I only examined encystment at one point in time (48 hr post-exposure), so it is possible that malathion-exposed tadpoles had a delayed, rather than decreased, immune response. The tadpoles that died as embryos during malathion exposure may have had stronger immune systems than those that survived. Therefore, I potentially selected for less immunocompetent tadpoles in my higher pesticide treatments, compared to those in the control treatment. Clearly, additional studies that address the mechanism by which enhanced latent susceptibility occurs are needed.

The long-term and latent effects of contaminant exposure are less well known than the direct lethal and sublethal effects (Carey and Bryant 1995). Chemicals that affect the immune system particularly have the potential to cause effects that do not manifest until after exposure (Luebke 2002). For example, a study that exposed chinook salmon eggs to o,p'-DDE, a metabolite of DDT, found that although there was no effect on mortality, time to hatch, or gonadal development, the humoral response was significantly reduced even one year after exposure (Milston et al. 2003). Similar long-term immunological would not be detected by typical short-term toxicity tests. Additionally, some pesticides are known to decrease cercariae survival (Griggs and Belden *In Press*) and infectivity (Koprivnikar et al. 2007). If hosts suffer pesticide-induced susceptibility that continues after exposure has ceased, they could encounter freshly shed parasites unhindered by pesticide exposure. Thus, latent susceptibility may lead to greater parasite transmission than cases where parasites and hosts are concurrently exposed to pesticides, although behavioral avoidance must also be considered.

E. trivolvis loads in individuals from naturally infected populations can exceed those used in this study by more than 25-fold (Skelly et al. 2006), and may become an increasing problem for many amphibian species. High nutrient conditions simulating cultural eutrophication increased snail biomass, prevalence of infection, and the cercaria output of infected snails. In those eutrophic conditions, amphibian larvae suffered significantly higher trematode infection than tadpoles in lower nutrient conditions (Johnson et al. 2007). Additionally parasite hosts may increase their ranges with global climate change. Preliminary research also suggests that many parasites proliferate more quickly in warmer waters and may benefit from longer reproductive seasons

(Marcogliese 2001). With potential for increased parasite prevalence from eutrophication and climate change, my data underscore the importance of understanding the role of pesticides in disease susceptibility and latent effects on the immune system.

Table 1. Comparison of the number (and relative percentages) of specific morphological abnormalities among abnormal *R. palustris* hatchlings after a 96 hr exposure to malathion. Total malformations exceeds the number of tadpoles malformed because some tadpoles had multiple malformations and are represented more than once.

Malathion Treatment (μg/L)	0	15	60	600
Axial flexure	1(25)	7(70)	5(36)	20(35)
Craniofacial abnormality	1(25)	1(10)	2(14)	7(11)
Ventralization	0	2(20)	7(50)	34(54)
Other	2(50)	0	0	2(3)
Total malformations	4	10	14	63
Number malformed	3(0.7)	9(1.9)	10(2.2)	50(11)

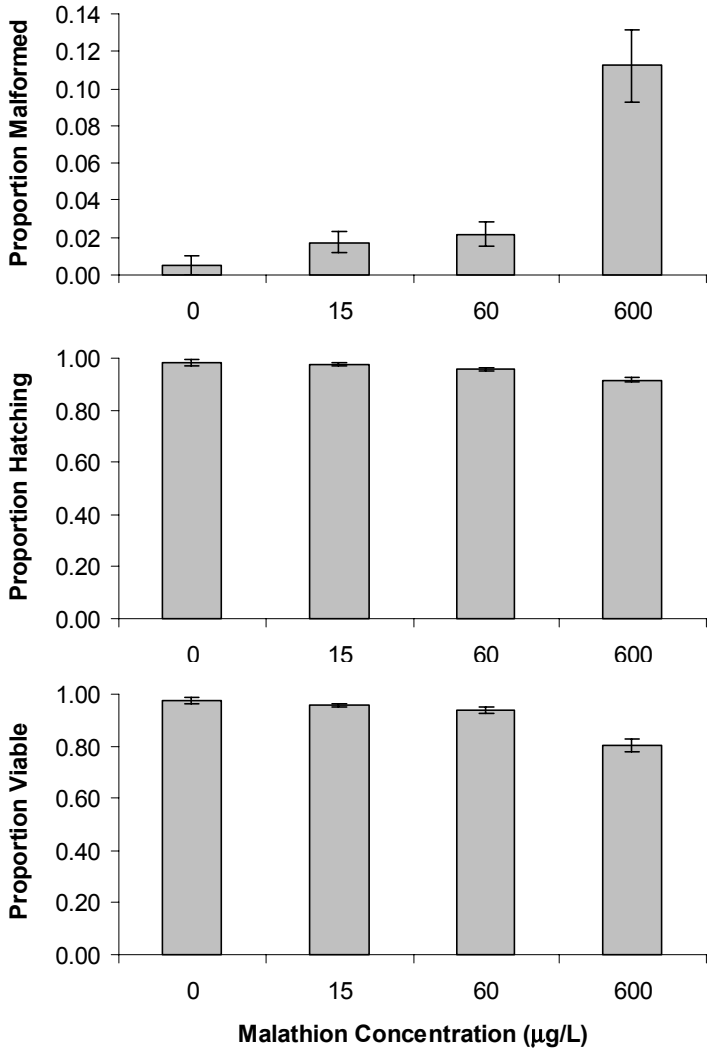

Figure 1. Comparison of hatching success, malformation rate, and viability in *R. palustris* tadpoles exposed to a range of malathion concentrations for 96 hr.

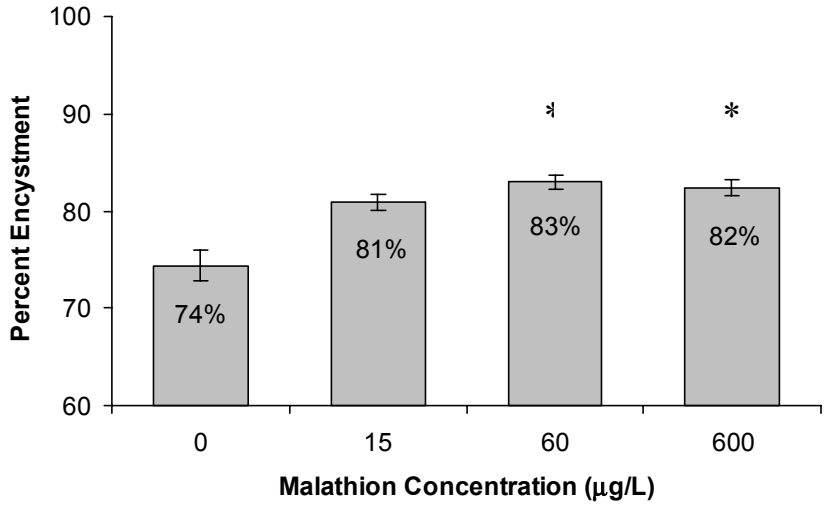

Figure 2. Percent of *E. trivolvis* cercaria successfully encysting in *R. palustris* tadpoles 7 wk after a 96 hr exposure to a range of malathion concentrations. Asterisks denote significant differences from the control.

CONCLUSIONS

Several causative factors have been implicated in the recent, global decline of some amphibian populations (Baringa 1990, Blaustein and Wake 1990, Wake 1991, Blaustein et al. 1994, Alford and Richards 1999, Houlahan et al. 2000, Stuart et al. 2004), but their relative magnitudes are still debated (Collins and Storfer 2003). These factors include: overexploitation, habitat loss and fragmentation, invasive species, climate change, increased UV-B exposure, environmental contaminants, and disease (Blaustein and Wake 1990, Pounds and Crump 1994, Carey and Bryant 1995, LeNoir et al. 1998, Daszak et al. 1999, Blaustein et al. 2001, Davidson et al. 2001, Kiesecker et al. 2001, Sparling et al. 2001, Belden and Blaustein 2002, Blaustein et al. 2003b, Carey and Alexander 2003, Daszak et al. 2003, Kats and Ferrer 2003, Muths et al. 2003, Davidson 2004, Kiesecker et al. 2004). In many cases, individual factors cannot explain population declines. Consequently, research into the interactions of these multiple stressors has grown (Kiesecker and Blaustein 1995, Davidson et al. 2002, Kiesecker 2002, Blaustein et al. 2003a), although many gaps still remain. I investigated two new angles with respect to contaminants and disease, the effect of parasite infection on susceptibility to contaminants and latent susceptibility to parasite because of early exposure to contaminants.

The effects of pathogen infection on the toxicity of environmental contaminants has been studied in several taxa, but, to my knowledge, my research is the first to examine their reciprocal effects on amphibians. After pickerel frog larvae were infected with moderate burdens of trematodes, I found no difference in the acute toxicity of

malathion among individuals carrying these different parasite loads. LC_{50} values for tadpoles exposed to 0, 10, and 30 cercaria did not differ. Furthermore, there was no difference in parasite loads among those that survived and died at the only concentration (14.4 µg/L) where I observed partial mortality. My data suggest that moderate parasite loads will not increase the susceptibility of pickerel frog larvae to malathion. I also was the first to calculate a malathion LC_{50} for pickerel frogs, 17.1 mg/L. Although I found no effects of parasite infection on toxicity in this system, more amphibian studies are needed to assess this potentially serious interaction. Research priorities in this area include reexamining this system with higher *E. trivolvis* loads and studying sublethal parasite loads in other amphibian species.

In my second experiment, I investigated the effects of pesticide exposure on parasite susceptibility. I found that a 96-hr exposure to malathion during early development decreased hatching success by 18% and increased incidence of malformations by 11%, particularly axial flexures and ventralization, in my highest malathion treatment, 600 µg/L, compared to the control. Furthermore, tadpoles exposed to 60 and 600 µg/L malathion were over 11% more susceptible to parasite infection 7 wk after exposure than the control. My research builds upon previous studies that investigated susceptibility to parasite infection in tadpoles during or immediately following pesticide exposure. A month exposure to each of three pesticides, atrazine (3, 30 µg/L), esfenvalerate (180, 1800 µg/L), and malathion (2000 µg/L), increased wood frog, *R. sylvatica*, susceptibility to *Ribeiroia sp*. and *Telorchis sp.* trematodes (Kiesecker 2002). Atrazine (30 µg/L) also increased wood frog susceptibility to *E. trivolvis*, however when the cercaria also were exposed to atrazine, the increased infection in

tadpoles exposed to 30 µg/L disappeared (Koprivnikar et al. 2007). Similarly, after 14 hr exposure to a mixture of metolachlor (85 µg/L) and atrazine (100 µg/L), *E. trivolvis* survivorship was decreased compared to controls (Griggs and Belden *In Press*). My findings are also consistent with previous studies that found pesticides may increase susceptibility to or incidence of *Ribeiroia* infection (Kiesecker 2002, Blaustein and Johnson 2003, Taylor et al. 2005). Additional research is needed to understand the complex effects of contaminants on these host-parasite systems.

In contrast to previous research, I exposed embryos before hatching and reared them in clean water until parasite exposure. Low, environmentally realistic concentrations of malathion decreased hatching success and increased susceptibility to trematode infection 7 wk after exposure. My results demonstrate that embryonic development is a sensitive life stage, and exposure to contaminants at low levels can have long lasting effects. This finding is important because LC_{50} tests suggest that eggs are less sensitive to acetylcholinesterase-inhibiting pesticides than tadpoles. My data illustrate that seemingly normal hatchlings may suffer consequences of early exposure later in ontogeny. Clearly, endpoints other than immediate mortality will better inform regulators, wildlife managers, and the public of the true consequences of contaminant exposure on non-target species.

Because my experiments were conducted without many of the potential stressors wild animals experience, it is likely that my results underestimate the negative impacts of pesticides, parasites, and their interactions. Although I used mesocosms to rear my tadpoles in a more natural, competitive setting than the laboratory, they were sheltered from several factors that could increase the interactive effects of parasites and pesticides.

Amphibians may experience chronic exposure or multiple acute exposures to mixtures of pesticides. Pesticides may further affect amphibians by altering community dynamics (Boone and Semlitsch 2003, Mills and Semlitsch 2004, Metts et al. 2005). Both competition and predation can alter the magnitude of pesticide impacts on amphibians (Boone and Semlitsch 2001, Relyea and Mills 2001, Relyea 2003, Boone 2005, Relyea 2005b). Reduced body size and condition due to limited food availability could increase susceptibility to both parasites and pesticides (Wobeser 2006). Furthermore, pesticides themselves may decrease food availability (Mills and Semlitsch 2004, Metts et al. 2005). The indirect effects of exposure may exceed direct effects for some species (Boone and Semlitsch 2003, Fleeger et al. 2003, Relyea et al. 2005).

My results may also represent conservative scenarios because natural parasite loads may greatly exceed those used in this study. Research has shown that the anti-predator behavior of tadpoles, seeking shelter in the benthos, can increase their exposure to parasites (Thiemann and Wassersug 2000). Natural infections of over 1600 Echinostome metacercariae per tadpole have been documented (Skelly et al. 2006), and preliminary evidence suggests larger green and bullfrog tadpoles can endure over 3,000 metacercariae (Courtney Culp, unpub. data). Differences in pesticide susceptibility because of parasite infection may be more pronounced at near-lethal levels. If higher parasite infections are considered, a small percent increase in parasite susceptibility because of contaminant exposure could mean the difference between a sublethal and lethal burden.

With the potential for increased parasite prevalence from eutrophication (Johnson and Chase 2004) and climate change (Marcogliese 2001), my data may underestimate the

interactive effects of parasites and pesticides. Increased snail density, the primary hosts of many Echinostome species, has been linked to eutrophication (Johnson and Chase 2004). As eutrophication expands, intensity and prevalence of parasite infection may rise, increasing the need for toxicity testing using infected organisms. Furthermore, global climate change may increase the range and infectivity of many parasite species (Marcogliese 2001). If agricultural pesticides decrease resistance to parasites and other agricultural and land use practices lead to excess nitrogen and eutrophic conditions that support parasite hosts, amphibian populations may be doubly affected.

Pesticides play an important, possibly irreplaceable, role in modern society. They aid in the production of food and reduce of disease vectors such as mosquitoes. To make informed decisions about their use, we must understand not only how they directly affect wildlife but also how they interact with other stressors. Emerging infectious diseases in wildlife are a growing problem (Daszak et al. 1999, Daszak et al. 2000, 2001, 2003, Kiesecker et al. 2004), and it is important to understand the role that chemical contamination may play in susceptibility.

BIBLIOGRAPHY

Alford, R. A., and S. J. Richards. 1999. Global amphibian declines: A problem in applied ecology. Annual Review of Ecology and Systematics **30**:133-165.

Anderson, R. M., and R. M. May. 1979. Population biology of infectious diseases: Part I. Nature **280**:361-367.

ASTM. 1991. Standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (FETAX). E 1439-91. Annual book of ASTM Standards. Philadelphia, PA.

ASTM. 2005. Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E 729-96 (2002). Pages 135-156 *in* Annual Book of ASTM Standards. ASTM International, West Conshohocken, PA.

Bantle, J. A., J. N. Dumont, R. A. Finch, and G. Linder. 1998. Atlas of abnormalities, a guide for the performance of FETAX, 2nd edition. Oklahoma State University, Stillwater, OK.

Baringa, M. 1990. Where have all the froggies gone? Science **247**:1033-1034.

Beasley, V. R., S. A. Faeh, B. Wikoff, J. Eisold, D. Nichols, R. Cole, A. M. Schotthoefer, C. Staehle, M. Greenwell, and L. E. Brown. 2003. Risk factors and the decline of the cricket frog, *Acris crepitans*: evidence for involvement of herbicides, parasitism, and habitat modifications. Pages 75-92 *in* M. J. Lannoo, editor. Amphibian declines: Status and conservation of U.S. amphibians. University of Chicago Press, Chicago.

Belden, L. K. 2006. Impact of eutrophication on wood frog, *Rana sylvatica*, tadpoles infected with *Echinostoma trivolvis* cercariae. Canadian Journal of Zoology **84**:1315-1321.

Belden, L. K., and A. R. Blaustein. 2002. Exposure of red-legged frog embryos to ambient UV-B radiation in the field negatively affects larval growth and development. Oecologia **130**:551-554.

Belden, L. K., and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria sp*. trematode cercariae. Journal of Parasitology **91**:686-688.

Berger, L. 1989. Disappearance of amphibian larvae in the agricultural landscape. Ecology International Bulletin **17**:65-73.

Berger, L., R. Speare, P. Daszak, D. E. Green, A. A. Cunningham, L. Goggin, R. Slocombe, M. A. Ragan, A. D. Hyati, 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 of the United States of America **95**:9031-9036.

Berrill, M., D. Coulson, L. McGillivray, and B. Pauli. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. Environmental Toxicology and Chemistry **17**:1738-1744.

Berven, K. A. 1982. The genetic basis of altitudinal variation in the wood frog *Rana sylvatica*, an experimental analysis of life history traits. Evolution **36**:962-983.

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

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

Blaustein, A. R., and P. T. J. Johnson. 2003. The complexity of deformed amphibians. Frontiers in Ecology and the Environment **1**:87-94.

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

Blaustein, A. R., T. L. Root, J. M. Kiesecker, L. K. Belden, D. H. Olson, and D. M. Green. 2003b. Amphibian breeding and climate change: Reply to Corn. Conservation Biology **17**:626-627.

Blaustein, A. R., and D. B. Wake. 1990. Declining amphibian populations - a global phenomenon. Trends in Ecology & Evolution **5**:203-204.

Blaustein, A. R., D. B. Wake, and W. P. Sousa. 1994. Amphibian declines - judging stability, persistence, and susceptibility of populations to local and global extinctions. Conservation Biology **8**:60-71.

Bonfanti, P., A. Colombo, F. Orsi, I. Nizzetto, M. Andrioletti, R. Bacchetta, P. Mantecca, U. Fascio, G. Vailati, and C. Vismara. 2004. Comparative teratogenicity of chlorpyrifos and malathion on *Xenopus laevis* development. Aquatic Toxicology **70**:189-200.

Boone, M. D. 2005. Juvenile frogs compensate for small metamorph size with terrestrial growth: Overcoming the effects of larval density and insecticide exposure. Journal of Herpetology **39**:416-423.

Boone, M. D., and S. M. James. 2005. Aquatic and terrestrial mesocosms in amphibian ecotoxicology. Applied Herpetology **2**:231-257.

Boone, M. D., and R. D. Semlitsch. 2001. Interactions of an insecticide with larval density and predation in experimental amphibian communities. Conservation Biology **15**:228-238.

Boone, M. D., and R. D. Semlitsch. 2002. Interactions of an insecticide with competition and pond drying in amphibian communities. Ecological Applications **12**:307-316.

Boone, M. D., and R. D. Semlitsch. 2003. Interactions of bullfrog tadpole predators and an insecticide: predation release and facilitation. Oecologia **137**:610-616.

Bosco, C., R. Rodrigo, S. Diaz, and S. Borax. 1997. Renal effects of chronic exposure to malathion in *Octodon degus*. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology **118**:247-253.

Boyce, N. P., and S. B. Yamada. 1977. Effects of the parasite *Eubothrium salvelini* (Cestoda: Pseudophyllidea), on the resistance of juvenile sockeye salmon, *Oncorhynchus nerka*, to zinc. Journal of the Fisheries Research Board of Canada **34**:706-709.

Bridges, C. M. 1997. Tadpole swimming performance and activity affected by acute exposure to sublethal levels of carbaryl. Environmental Toxicology and Chemistry **16**:1935-1939.

Bridges, C. M. 2000. Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). Archives of Environmental Contamination and Toxicology **39**:91-96.

Bridges, C. M., and R. D. Semlitsch. 2000. Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. Conservation Biology **14**:1490-1499.

Bridges, C. M., and R. D. Semlitsch. 2001. Genetic variation in insecticide tolerance in a population of southern leopard frogs (*Rana sphenocephala*): implications for amphibian conservation. Copeia **2001**:7-13.

Brown, A. F., and D. Pascoe. 1989. Parasitism and host sensitivity to cadmium: an acanthocephalan infection of the freshwater amphipod *Gammarus pulex*. Journal of Applied Ecology **26**:473-487.

CADFG. 1998. Hazard assessment of the insecticide malathion to aquatic life in the Sacramento-San Joaquin River system. Office of Spill Prevention and Response, California Department of Fish and Game. Administrative Report 98-2.

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

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

Carey, C., and C. J. Bryant. 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environmental Health Perspectives **103**:13-17.

CDFG. 1982. Monitored aquatic incidents during broadscale aerial application over San Francisco Bay area, 1981. California Department of Fish and Game. Sacramento, CA. California Administrative Report 82-2.

Chakraborty, D., A. Bhattacharyya, K. Majumdar, K. Chatterjee, S. Chatterjee, A. Sen, and G. C. Chatterjee. 1978. Studies on L-ascorbic acid metabolism in rats under chronic toxicity due to organophosphorus insecticides: effects of supplementation of L-ascorbic acid in high doses. Journal of Nutrition **108**:973-980.

Christin, M. S., A. D. Gendron, P. Brousseau, L. Menard, D. J. Marcogliese, D. Cyr, S. Ruby, and M. Fournier. 2003. Effects of agricultural pesticides on the immune system of *Rana pipiens* and on its resistance to parasitic infection. Environmental Toxicology and Chemistry **22**:1127-1133.

Christin, M. S., L. Menard, A. D. Gendron, S. Ruby, D. Cyr, D. J. Marcogliese, L. Rollins-Smith, and M. Fournier. 2004. Effects of agricultural pesticides on the immune system of *Xenopus laevis* and *Rana pipiens*. Aquatic Toxicology **67**:33-43.

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

Conant, R., and J. P. Collins. 1998. A Field Guide to Reptiles and Amphibians: Eastern and Central North America. Third edition. Houghton Mifflin Company, New York.

Cooke, A. S. 1971. Selective predation by newts on frog tadpoles treated with DDT. Nature **229**:275-276.

Cowman, D. F., and L. E. Mazanti. 2000. Ecotoxicology of new generation pesticides to amphibians. Pages 233-268 *in* D. W. Sparling, G. Linder, and C. A. Bishop, editors. Ecotoxicology of Amphibians and Reptiles. SETAC Press, Pensacola, FL.

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. 2000. Emerging infectious diseases of wildlife - threats to biodiversity and human health. Science **287**:443-449.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica **78**:103-116.

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

Davidson, C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. Ecological Applications **14**:1892-1902.

Davidson, C., H. B. Shaffer, and M. R. Jennings. 2001. Declines of the California red-legged frog: Climate, UV-B, habitat, and pesticides hypotheses. Ecological Applications **11**:464-479.

Davidson, C., H. B. Shaffer, and M. R. Jennings. 2002. Spatial tests of the pesticide drift, habitat destruction, UV-B, and climate-change hypotheses for California amphibian declines. Conservation Biology **16**:1588-1601.

de Llamas, M. C., A. C. de Castro, and A. M. P. De D'Angelo. 1985. Cholinesterase activities in developing amphibian embryos following exposure to the insecticides dieldrin and malathion. Archives of Environmental Contamination and Toxicology **14**:161-166.

Durston, A. J., J. P. Timmermans, W. J. Hage, H. F. Hendriks, N. J. de Vries, M. Heideveld, and P. D. Nieuwkoop. 1989. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature **340**:140-144.

Ecobichon, D. J. 1996. Toxic effects of pesticides. Pages 643-689 *in* C. D. Klaassen, editor. Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw-Hill, New York City.

Esch, G. W., J. W. Gibbons, and J. E. Bourque. 1975. Analysis of relationship between stress and parasitism. American Midland Naturalist **93**:339-353.

Ewing, M. S., and S. A. Ewing. 1982. Susceptibility to *Ichthyophthirius multifiliis* of channel catfish exposed to sublethal concentrations of copper. Pages 401 *in* M. Muller, W. Gutteridge, and P. Kohler, editors. Molecular and Biochemical Parasitology: Parasites - Their World and Ours. Elsevier Biomedical Press, Amsterdam.

Fleeger, J. W., K. R. Carman, and R. M. Nisbet. 2003. Indirect effects of contaminants in aquatic ecosystems. Science of the Total Environment **317**:207-233.

Fordham, C. L., J. D. Tessari, H. S. Ramsdell, and T. J. Keefe. 2001. Effects of malathion on survival, growth, development, and equilibrium posture on bullfrog tadpoles (*Rana catesbeiana*). Environmental Toxicology and Chemistry **20**:179-184.

Fried, B., and J. D. Bradford. 1997. *In vitro* excystation of metacercarial cysts of *Echinostoma trivolvis* from *Rana* species tadpoles. The Korean Journal of Parasitology **35**:75-77.

Fried, B., P. L. Pane, and A. Reddy. 1997. Experimental infection of *Rana pipiens* tadpoles with *Echinostoma trivolvis* cercariae. Parasitology Research **83**:666-669.
Friesen, M. K. 1981. *Helisoma trivolvis* (Say). Pages 23-30 *in* S. G. Lawrence, editor. Manual for the culture of selected freshwater invertebrates. Canadian Special Publication in Fish and Aquatic Sciences, Ottawa, Canada.
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, T. S., J. C. Pant, and J. Pant. 1988. Gill, liver, and kidney lesions associated with experimental exposures to carbaryl and dimethoate in the fish (*Puntius conchonius* Ham). Bulletin of Environmental Contamination and Toxicology **41**:71-78.
Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**:183-190.
Greulich, K., and S. Pflugmacher. 2003. Differences in susceptibility of various life stages of amphibians to pesticide exposure. Aquatic Toxicology **65**:329-336.
Griggs, J. L., and L. K. Belden. 2007. Effects of atrazine and metolachlor on the survivorship and infectivity of *Echinostoma trivolvis* trematode cercaria. Archives of Environmental Contamination and Toxicology (I*n Press*).
Grove, L. E., L. K. Belden, M. J. Rubbo, and J. M. Kiesecker. 2003. Effects of atrazine exposure on a physid snail species and its trematode parasites. Integrative and Comparative Biology **43**:1020.
Guth, D. J., H. D. Blankespoor, and J. Cairns. 1977. Potentiation of zinc stress caused by parasitic infection of snails. Hydrobiologica **55**:225-229.
Hairston, N. G. 1989. Hard choices in ecological experimentation. Herpetologica **45**:119-122.
Hanzelova, V., R. Kuchta, T. Scholz, and A. P. Shinn. 2005. Morphometric analysis of four species of *Eubothrium* (Cestoda: Pseudophyllidea) parasites of salmonid fish: An interspecific and intraspecific comparison. Parasitology International **54**:207-214.
Heinonen, J., J. V. K. Kukkonen, and I. J. Holopainen. 1999. The effects of parasites and temperature on the accumulation of xenobiotics in a freshwater clam. Ecological Applications **9**:475-481.
Heinonen, J., J. V. K. Kukkonen, and I. J. Holopainen. 2001. Temperature and parasite-induced changes in toxicity and lethal body burdens of pentachlorophenol in the freshwater clam *Pisidium amnicum*. Environmental Toxicology and Chemistry **20**:2778-2784.
Hoffman, R. S., P. D. Capel, and S. J. Larson. 2000. Comparison of pesticides in eight U.S. urban streams. Environmental Toxicology and Chemistry **19**:2249-2258.
Hopkins, W. A., S. E. DuRant, B. P. Staub, and C. L. Rowe. 2006. Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. Environmental Health Perspectives **114**:661-666.
Hopkins, W. A., M. T. Mendonca, and J. D. Congdon. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. General and Comparative Endocrinology **108**:237-246.

Hopkins, W. A., B. P. Staub, J. W. Snodgrass, B. E. Taylor, A. E. DeBiase, J. H. Roe, B. P. Jackson, and J. D. Congdon. 2004. Responses of benthic fish exposed to contaminants in outdoor microcosms - examining the ecological relevance of previous laboratory toxicity tests. Aquatic Toxicology **68**:1-12.

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

Huffman, J. E., and B. Fried. 1990. *Echinostoma* and Echinostomiasis. Pages 215-269 *in* J. R. Baker and R. Muller, editors. Advances in Parasitology. Academic Press, New York.

Jaeger, R. G., and S. C. Walls. 1989. On salamander guilds and ecological methodology. Herpetologica **45**:111-119.

Johansson, M., C. R. Primmer, J. Sahlsten, and J. Merila. 2005. The influence of landscape structure on occurrence, abundance and genetic diversity of the common frog, *Rana temporaria*. Global Change Biology **11**:1664-1679.

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., J. M. Chase, K. L. Dosch, R. B. Hartson, J. A. Gross, D. J. Larson, D. R. Sutherland, and S. R. Carpenter. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proceedings of the National Academy of Sciences of the United States of America **104**:15781-15786.

Johnson, P. T. J., 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., K. B. Lunde, D. A. Zelmer, and J. K. Werner. 2003. Limb deformities as an emerging parasitic disease in amphibians: evidence from museum specimens and resurvey data. Conservation Biology **17**:1724-1737.

Kallander, D. B., S. W. Fisher, and M. J. Lydy. 1997. Recovery following pulsed exposure to organophosphorus and carbamate insecticides in the midge, *Chironomus riparius*. Archives of Environmental Contamination and Toxicology **33**:29-33.

Kao, K. R., and R. P. Elinson. 1988. The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. Developmental Biology **127**:64-77.

Kao, K. R., and R. P. Elinson. 1989. Dorsalization of mesoderm induction by lithium. Developmental Biology **132**:81-90.

Kats, L. B., and R. P. Ferrer. 2003. Alien predators and amphibian declines: review of two decades of science and the transition to conservation. Diversity and Distributions **9**:99-110.

Kaur, K., and A. Dhawan. 1993. Variable sensitivity of *Cyprinus carpio* eggs, larvae, and fry to pesticides. Bulletin of Environmental Contamination and Toxicology **50**:593-599.

Khangarot, B. S., A. Sehgal, and M. K. Bhasin. 1985. Man and Biosphere-Studies on the Sikkim Himalayas. Part 6: Toxicity of selected pesticides to frog tadpole *Rana hexadactyla*. Acta Hydrochimica et Hydrobiologica **13**:391-394.

Kiely, T., D. Donaldson, and A. Grube. 2004. Pesticides Industry Sales and Usage, 2001-2002 Market Estimates. Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, Biological and Economic Analysis Division. U.S. Environmental Protection Agency. Washington, DC.

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 of the United States of America **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 of the United States of America **92**:11049-11052.

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

Kiesecker, J. M., and D. K. Skelly. 2001. Effects of disease and pond drying on gray tree frog growth, development, and survival. Ecology **82**:1956-1963.

Knapp, R. A., and K. R. Matthews. 2000. Non-native fish introductions and the decline of the mountain yellow-legged frog from within protected areas. Conservation Biology **14**:428-438.

Koprivnikar, J., M. R. Forbes, and R. L. Baker. 2006. On the efficiency of anti-parasite behaviour: a case study of tadpole susceptibility to cercariae of *Echinostoma trivolvis*. Canadian Journal of Zoology **84**:1623-1629.

Koprivnikar, J., M. R. Forbes, and R. L. Baker. 2007. Contaminant effects on host-parasite interactions: atrazine, frogs, and trematodes. Environmental Toxicology and Chemistry **26**:2166-2170.

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

Lafferty, K. D., and R. D. Holt. 2003. How should environmental stress affect the population dynamics of disease? Ecology Letters **6**:654-664.

Lafferty, K. D., and A. M. Kuris. 1999. How environmental stress affects the impacts of parasites. Limnology and Oceanography **44**:925-931.

Larson, S. J., R. J. Gilliom, and P. D. Capel. 1999. Pesticides in streams of the United States—Initial results from the National Water-Quality Assessment Program. Washington, DC.

LeNoir, J., L. Aston, S. Datta, G. Fellers, L. McConnell, and J. Seiber. 1998. Pesticides and PCBs in Sierra Nevada ecosystems: Potential relationship to decline of amphibians. Abstracts of Papers of the American Chemical Society **216**:U794-U794.

Lewis, J., D. Hoole, and L. H. Chappell. 2003. Parasitism and environmental pollution: parasites and hosts as indicators of water quality. Parasitology **126**:S1-S3.

Linzey, D. W., J. Burroughs, L. Hudson, M. Marini, J. Robertson, J. P. Bacon, M. Nagarkatti, and P. S. Nagarkatti. 2003. Role of environmental pollutants on immune functions, parasitic infections and limb malformations in marine toads and whistling frogs from Bermuda. International Journal of Environmental Health Research **13**:125-148.

Luebke, B. 2002. Pesticide-induced immunotoxicity: Are humans at risk? Human and Ecological Risk Assessment **8**:293-303.
Luebke, R. W., P. V. Hodson, M. Faisal, P. S. Ross, K. A. Grasman, and J. Zelikoff. 1997. Aquatic pollution-induced immunotoxicity in wildlife species. Fundamental and Applied Toxicology **37**:1-15.
Marcogliese, D. J. 2001. Implications of climate change for parasitism of animals in the aquatic environment. Canadian Journal of Zoology **79**:1331-1352.
Martof, B. S., W. M. Palmer, J. R. Bailey, J. R. I. Harrison, and J. Dermid. 1980. Amphibians and Reptiles of the Carolinas and Virginia. The University of North Carolina Press, Chapel Hill, NC.
Mastorakos, G., C. Bamburger, and G. P. Chrousos. 1999. Neuroendocrine regulation of the immune process. Pages 17-37 *in* N. P. Plotnikoff, R. E. Faith, A. J. Murgo, and R. A. Good, editors. Cytokines: Stress and Immunity. CRC Press, Boca Raton, FL.
May, R. M., and R. M. Anderson. 1979. Population biology of infectious diseases: Part II. Nature **280**:455-461.
McAlpine, D. F., and D. B. Burt. 1998. Helminths of bullfrogs, *Rana catesbeiana*, green frogs, *R. clamitans*, and leopard frogs, *R. pipiens* in New Brunswick. Canadian Field-Naturalist **112**:50-68.
McCahon, C. P., A. F. Brown, and D. Pascoe. 1988. The effect of the acanthocephalan *Pomphorhynchus laevis* (Muller 1776) on the acute toxicity of cadmium to its intermediate host, the amphipod *Gammarus pulex* (L.). Archives of Environmental Contamination and Toxicology **17**:239-243.
Metts, B. S., W. A. Hopkins, and J. P. Nestor. 2005. Interaction of an insecticide with larval density in pond-breeding salamanders (*Ambystoma*). Freshwater Biology **50**:685-696.
Mills, N. E., and R. D. Semlitsch. 2004. Competition and predation mediate the indirect effects of an insecticide on southern leopard frogs. Ecological Applications **14**:1041-1054.
Milston, R. H., M. S. Fitzpatrick, A. T. Vella, S. Clements, D. Gundersen, G. Feist, T. L. Crippen, J. Leong, and C. B. Schreck. 2003. Short-term exposure of chinook salmon (*Oncoryhnchus tshawytscha*) to o,p '-DDE or DMSO during early life-history stages causes long-term humoral immunosuppression. Environmental Health Perspectives **111**:1601-1607.
Mohanty-Hejmadi, P., and S. K. Dutta. 1981. Effects of some pesticides on the development of the Indian bull frog *Rana tigerina*. Environmental Pollution. Series A **24**:145-161.
Moles, A. 1980. Sensitivity of parasitized coho salmon fry to crude oil, toluene and naphthalene. Transactions of the American Fisheries Society **109**:293-297.
Moles, A. 1983. Effect of parasitism by mussel glochidia on growth of coho salmon. Transactions of the American Fisheries Society **112**:201-204.
Moore, J. A. 1939. Temperature tolerance and rates of development in eggs of amphibia. Ecology **20**:459-478.
Morin, P. J. 1989. New directions in amphibian community ecology. Herpetologica **45**:124-128.

Muths, E., P. S. Corn, A. P. Pessier, and D. E. Green. 2003. Evidence for disease-related amphibian decline in Colorado. Biological Conservation **110**:357-365.
NPIC. 2001. Malathion (Technical Fact Sheet). National Pesticide Information Center. Oregon State University, Corvallis, OR.
Ottaviani, E., and C. Franceschi. 1996. The neuroimmunology of stress from invertebrates to man. Progress in Neurobiology **48**:421-440.
Pascoe, D., and P. Cram. 1977. The effect of parasitism on the toxicity of cadmium to the three-spined stickleback, *Gasterosteus aculeatus* L. Journal of Fish Biology **10**:467-472.
Patz, J. A., T. K. Graczyk, N. Geller, and A. Y. Vittor. 2000. Effects of environmental change on emerging parasitic diseases. International Journal for Parasitology **30**:1395-1405.
Pawar, K. R., H. V. Ghate, and M. Katdare. 1983. Effect of malathion on embryonic development of the frog *Microhyla ornata* (Dumeril and Bibron). Bulletin of Environmental Contamination and Toxicology **31**:170-176.
Penn State. 1993. Study of off-site deposition of malathion using operational procedures for the southeastern cotton boll weevil eradication program. Aerial Application Technology Laboratory, Department of Entomology, Penn State University, State College, PA.
Pounds, J. A., and M. L. Crump. 1994. Amphibian declines and climate disturbance - the case of the golden toad and the harlequin frog. Conservation Biology **8**:72-85.
Rachowicz, L. J., J. M. Hero, R. A. Alford, J. W. Taylor, J. A. T. Morgan, V. T. Vredenburg, J. P. Collins, and C. J. Briggs. 2005. The novel and endemic pathogen hypotheses: Competing explanations for the origin of emerging infectious diseases of wildlife. Conservation Biology **19**:1441-1448.
Relyea, R. A. 2003. Predator cues and pesticides: A double dose of danger for amphibians. Ecological Applications **13**:1515-1521.
Relyea, R. A. 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. Environmental Toxicology and Chemistry **23**:1080-1084.
Relyea, R. A. 2005a. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications **15**:618-627.
Relyea, R. A. 2005b. The lethal impacts of roundup and predatory stress on six species of North American tadpoles. Archives of Environmental Contamination and Toxicology **48**:351-357.
Relyea, R. A., and N. E. Mills. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proceedings of the National Academy of Sciences of the United States of America **98**:2491-2496.
Relyea, R. A., N. M. Schoeppner, and J. T. Hoverman. 2005. Pesticides and amphibians: The importance of community context. Ecological Applications **15**:1125-1134.
Rigby, M. C., and Y. Moret. 2000. Life history trade-offs with immune defenses. Pages 129-142 *in* R. Poulin, S. Morand, and A. Skorping, editors. Evolutionary biology of host-parasite relationships: theory meets reality. Elsevier Science, Amsterdam.
Rodgers, K. E., and S. Xiong. 1997. Effect of administration of malathion for 14 days on macrophage function and mast cell degranulation. Fundamental and Applied Toxicology **37**:95-99.

Rohr, J. R., and P. W. Crumrine. 2005. Effects of an herbicide and an insecticide on pond community structure and processes. Ecological Applications **15**:1135-1147.

Rohr, J. R., A. A. Elskus, B. S. Shepherd, P. H. Crowley, T. M. McCarthy, J. H. Niedzwiecki, T. Sager, A. Sih, and B. D. Palmer. 2004. Multiple stressors and salamanders: Effects of an herbicide, food limitation, and hydroperiod. Ecological Applications **14**:1028-1040.

Rohr, J. R., and B. D. Palmer. 2005. Aquatic herbicide exposure increases salamander desiccation risk eight months later in a terrestrial environment. Environmental Toxicology and Chemistry **24**:1253-1258.

Rollins-Smith, L., and P. J. Blair. 1993. The effects of corticosteroid hormones and thyroid hormones on lymphocyte viability and proliferation during development and metamorphosis of *Xenopus laevis*. Differentiation **54**:155-160.

Rosenbaum, E. A., A. C. de Castro, L. Guana, and A. M. P. de D'Angelo. 1988. Early biochemical changes produced by malathion on toad embryos. Archives of Environmental Contamination and Toxicology **17**:831-835.

Sanders, H. O. 1970. Pesticide toxicities to tadpoles of the western chorus frog *Pseudacris triseriata* and Fowler's toad *Bufo woodhousii Fowleri* Copeia **2**:246-251.

Schotthoefer, A. M., R. A. Cole, and V. R. Beasley. 2003a. Relationship of tadpole stage to location of echinostome cercariae encystment and the consequences for tadpole survival. Journal of Parasitology **89**:475-482.

Schotthoefer, A. M., A. V. Koehler, C. U. Meteyer, and R. A. Cole. 2003b. Influence of *Ribeiroia odatrae* (Trematode: Digenea) infection on limb development and survival of northern leopard frogs (*Rana pipiens*): effects of host stage and parasite-exposure level. Canadian Journal of Zoology **81**:1144-1153.

Semlitsch, R. D., D. E. Scott, and J. H. K. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology **69**:184-192.

Skelly, D. K., S. R. Bolden, M. P. Holland, L. K. Freidenburg, N. A. Freidenfelds, and T. R. Malcolm. 2006. Urbanization and disease in amphibians. Pages 153-167 *in* S. K. Collinge and C. Ray, editors. Disease ecology: community structure and pathogen dynamics. Oxford University Press, New York.

Smith, D. C. 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. Ecology **68**:344-350.

Snawder, J. E., and J. E. Chambers. 1989. Toxic and developmental effects of organophosphorus insecticides in embryos of the South African clawed frog. Journal of Environmental Science and Health **24**:205-218.

Snawder, J. E., and J. E. Chambers. 1990. Critical time periods and the effects of tryptophan in malathion-induced developmental defects in *Xenopus* embryos. Life Sciences **46**:1635-1642.

Snawder, J. E., and J. E. Chambers. 1993. Osteolathyrogenic effects of malathion in *Xenopus* embryos. Toxicology and Applied Pharmacology **121**:210-216.

Sparling, D. W., C. A. Bishop, and G. Linder. 2000. The current status of amphibian and reptile ecotoxicological research. Pages 1-13 *in* D. W. Sparling, G. Linder, and C. A. Bishop, editors. Ecotoxicology of Amphibians and Reptiles. SETAC Press, Pensacola, FL.

Sparling, D. W., G. M. Fellers, and L. L. McConnell. 2001. Pesticides and amphibian declines in California, USA. Environmental Toxicology and Chemistry **20**:1591-1595.

Srivastava, S. K., P. R. Tiwari, and A. K. Sirvastav. 1990. Effects of chlorpyrifos on the kidney of freshwater catfish, *Heteropneustes fossilis*. Bulletin of Environmental Contamination and Toxicology **45**:748-751.

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

Taylor, B., D. Skelly, L. K. Demarchis, M. D. Slade, D. Galusha, and P. M. Rabinowitz. 2005. Proximity to pollution sources and risk of amphibian limb malformation. Environmental Health Perspectives **113**:1497-1501.

Taylor, C. N., K. L. Oseen, and R. J. Wassersug. 2004. On the behavioural response of *Rana* and *Bufo* tadpoles to echinostomatoid cercariae: implications to synergistic factors influencing trematode infections in anurans. Canadian Journal of Zoology **82**:701-706.

Taylor, S. K., E. S. Williams, and K. W. Mills. 1999. Effects of malathion on disease susceptibility in Woodhouse's toads. Journal of Wildlife Diseases **35**:536-541.

Thiemann, G. W., and R. J. Wassersug. 2000. Patterns and consequences of behavioral responses to predators and parasites in *Rana* tadpoles. Biological Journal of the Linnean Society **71**:513-528.

Tournefier, A. 1982. Corticosteroid action on lymphocyte subpopulations and humoral immune responses of axolotl (urodele amphibian). Immunology **46**:155-162.

USEPA. 2004. Taking care of business: protecting public health and the environment, EPA's pesticide program FY 2004 Annual Report. U.S. Environmental Protection Agency, EPA-735-R-705-001.

USEPA. 2006a. Malthion preliminary risk assessments: Environmental fate and effects. U.S. Environmental Protection Agency, Washington, DC.

USEPA. 2006b. Reregistration eligibility decision (RED) for malathion. Office of Prevention, Pesticides and Toxic Substances, U.S. Environmental Protection Agency. Washington, DC.

VDH. 2005. Virginia Arbovirus Plan. Virginia Department of Health.

Venturino, A., L. E. Guana, R. M. Bergoc, and A. M. P. D'Angelo. 1992. Effect of exogenously applied polyamines on malathion toxicity in the toad Bufo arenarum Hensel. Archives of Environmental Contamination and Toxicology **22**:135-139.

Wake, D. B. 1991. Declining amphibian populations. Science **253**:860.

Whipple, J. A. 1982. Impacts of pollutants on striped bass in the San Francisco Bay-Delta. National Marine Fisheries Service, Tiburon Laboratory. Project Summary.

Wilbur, H. M. 1980. Complex life cycles. Annual Review of Ecology and Systematics **11**:67-93.

Wilbur, H. M. 1989. In defense of tanks. Herpetologica **45**:122-123.

Wobeser, G. A. 2006. Essentials of Disease in Wild Animals. Blackwell Publishers, Ames, IA.

Wright, A. H. 1914. North American Anura: Life Histories of the Anura of Ithaca, New York. The Carnegie Institution of Washington, Washington.