Effects of Atrazine and Metolachlor on Snails, Tadpoles, and Their Trematode Parasites

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Science in Biological Sciences**

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Abstract:

The widespread use and subsequent release of pesticides into aquatic environments have sparked concerns about how organisms within these aquatic systems are affected by pesticide pollution. While many studies have examined the effects of pesticides on individual organisms, in a series of experiments, I investigated the effects of a pesticide mixture on members of a complex host-parasite system and on host susceptibility to infection. In my first experiment in the laboratory, I examined changes in survivorship when trematode parasites (*Echinostoma trivolvis)* and their first intermediate host, *Planorbella trivolvis* snails, were exposed to a low concentration (10 ppb: 15 ppb) and high concentration (85 ppb: 100 ppb) mixture of atrazine and metolachlor, respectively. There was a significant decline in parasite survivorship in the high concentration treatment at 14 hours, while snail survivorship was unaffected across all treatments. In my second experiment, prior to infection, I exposed the parasites and/or second intermediate hosts, *Rana clamitans* and *Rana sylvatica* tadpoles, to the pesticide mixtures and examined subsequent infection levels in the tadpoles. The atrazine and metolachlor mixtures had no significant effects on parasite load in the laboratory. Newly shed parasites were more likely than 10 hours old parasites to infect tadpoles, regardless of pesticide exposure. In my final experiment, I utilized outdoor mesocosms to expose parasites, snail hosts, and *Rana sylvatica* tadpoles to the pesticide mixture, and I examined differences in parasite load within the tadpoles after two weeks. The pesticides had no significant effect on parasite loads in the field. Overall, my findings suggest the atrazine and metolachlor mixtures used in this study had no significant effects on disease dynamics in a system involving Echinostome parasites, snails, and tadpoles.

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Research Objectives

My research addressed how disease dynamics in a complex aquatic system were influenced by two widely used agricultural herbicides - atrazine and metolachlor. In a series of laboratory and field experiments, I examined the impacts of individual and mixed concentrations of atrazine and metolachlor on a complex host- parasite system consisting of *Planorbella trivolvis* snails, *Rana* species tadpoles, and their *Echinostoma trivolvis* trematode parasites.

In my first laboratory experiments, I examined whether the herbicides affected the survivorship of non-infected snails and *Echinostoma* trematode parasites (Chapter I). In further laboratory experiments, I examined whether pesticide mixtures directly affected parasite load in two species of tadpoles under different exposure scenarios (e.g. tadpoles and /or parasites were exposed to low and high concentrations of pesticide mixtures prior to infection) (Chapter II). Successful disease transmission could have dire consequences for hosts, because high parasite load can result in host physiological stress, decreased immune response, and death.

Few studies have examined the indirect effects of pesticide mixtures on organisms in natural conditions. My final experiment investigated whether low and high concentrations of pesticide mixtures influenced parasite load in tadpoles in outdoor mesocosms (Chapter III). The mesocosm experiment addressed whether there are alterations in disease dynamics under simulated field conditions.

My thesis work provided insight into how anthropogenic chemical mixtures affected the success of disease transmission in amphibians and snails. Such insight is important because amphibians are sensitive organisms with permeable skin and they rely on water for survival. Amphibians are also hosts of trematode diseases. Any pesticide-induced alterations in the transmission of trematode disease to amphibian hosts can potentially affect transmission to other

successive hosts. In the case of trematode diseases, a variety of vertebrate and invertebrate hosts can fall victim to such alterations.

Chapter I: Introduction and Literature Review

Background

I. History of Pesticide Use and Regulation in the United States

Pesticides have been used all over the world for centuries to decrease the number of pests or to completely eradicate these pests. In 2002, 1.4 billion kilograms (kg) of pesticides were applied to crops all over the world (Kiely et al. 2004). The cost of these pesticides totaled $27.7 billion worldwide. The amount of money spent on pesticides in the United States accounted for 28% of the total cost of global pesticide application. Prior to regulation of pesticides by the United States government in the 1940's, pesticides were used with little regard to the effects they could induce in humans and wildlife. Because of this lack of pesticide regulation, health problems related to widespread pesticide exposures began to be recognized and cause alarm. For example, in the 1950's and 1960's populations of birds of prey began to decline due to exposures to the insecticide dichlorodiphenyltrichloroethane (DDT) and its degradate dichlorodiphenyldichloroethylene (DDE), which caused avian eggshells to thin and break (Bitman et al. 1969). During the time that DDT and DDE effects were becoming apparent in wildlife, other organochlorine pesticides such as hexachlorobenzene and hexachlorocyclohexane. For over 30 years, organochlorine residues were detected in the breast milk and adipose tissue of Americans (Lordo et al. 1996). Mice exposed to organochlorine compounds experienced increased incidences of liver neoplasms and estrogenic effects (Williams and Numoto 1984, Borgeest et al. 2002). Scientists have extrapolated these risks to human health based on the results of experiments involving mice. Therefore, cancer and estrogenic activity may be a reflection of potential effects in humans.

In response to existing and potential negative health effects found in humans and wildlife, the U.S. government developed laws that would help to maintain public health. In 1972,

Congress passed the Clean Water Act (formerly the Federal Water Pollution Control Act). The objective of the Clean Water Act was to ". . . restore and maintain the chemical, physical, and biological integrity of the nation's waters. . ." The Clean Water Act would accomplish this objective by eliminating the discharge of pollutants into navigable waters of the U.S. by 1985, to stop the release of toxic amounts of toxic substances, and to "provide for the protection and propagation of fish, shellfish, and wildlife [and to provide] for recreation in and on the water . . ." by 1983. The Federal Insecticide, Fungicide, Rodenticide Act (FIFRA) of 1947 imposed restrictions specifically on pesticides. The law required registration of pesticides and established storage, disposal, and transportation procedures for pesticides. In the 1975 amendments to FIFRA, a Scientific Advisory Panel was formed to review proposed regulations, and the Rebuttable Presumption Against Registration (RPAR) was practiced. RPAR required that all pesticides registered before August 1975 and shown to cause harm to humans and/or wildlife to be reviewed prior to re-registration. The RPAR required a panel to decide if the pesticides were relatively safe or too much of a risk to re-register. Ultimately, these laws encouraged the development and tightening of chemical restrictions and standards; especially on pesticides that had undergone extensive testing that revealed potentially hazardous properties of these pesticides.

II. Atrazine and Metolachlor Use in the United States

Approximately 490 million kilograms of pesticides are used on crops in the United States each year . Thousands of tons of a single pesticide are applied to crops annually. For example, the herbicides atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) and metolachlor [2 chloro-N-(2-ethyl-6-methyl-phenyl-N-(2-methoxy-1-methylethyl) acetamide] rank in the top ten

of the most widely used agricultural pesticides in the U.S. (Goolsby 1995). In 1993, 32,000-34,000 tons of atrazine were applied to crops in the U.S. (Solomon et al. 1996) (Fig.1). Atrazine is commonly applied to corn, cotton, sorghum, soybeans, potatoes, and peanuts. Atrazine prevents the growth of pre-emergent vegetation by inhibiting photosystem II by blocking electron transport which is responsible for photosynthesis, growth, biomass, and chlorophyll production (Roses et al. 1999). In 1997, 30,000-32,500 tons of metolachlor were applied to crops (Rivard 2003) (Fig. 2). Metolachlor is commonly applied to roadside vegetation, corn, sorghum, Christmas trees, and pineapples. Metolachlor is usually absorbed by the roots and shoots of plants; and the herbicide stops lipid synthesis and cell growth. These chemicals compose the most common pesticide mixture found in major freshwater environments because of the overlap in the type of plants to which these pesticides are applied (Goolsby 1995). For example, several studies have found (Solomon et al. 1996) atrazine and metolachlor residues in the Mississippi River, Chesapeake Bay, and streams, rivers, and tributaries in Iowa, New York, Louisiana, Missouri, Illinois, and other states (Goolsby 1995, Clark et al. 1999, Hall Jr et al. 1999, Rivard 2003, Hayes et al. 2006). Atrazine and metolachlor residues are especially abundant from May through August (Clark et al. 1999), because these pesticides are commonly applied to agricultural crops during these months; and spray applications, rainstorm events, or other sources of water wash the pesticides into ponds, streams, and rivers. Unfortunately, pesticides can be very persistent in these freshwater systems. Atrazine has a half-life of 3-12 days in estuarine water (Solomon et al. 1996) and 355 days in surface waters (Mazanti 1999). In surface waters, metolachlor has a half-life of 97-200 days depending on the pH of the water (Kamrin 1997). Ultimately, the distribution and properties of these pesticides could have a significant impact on freshwater organisms and the environment.

Figure 1: Amount of atrazine applied annually in the United States
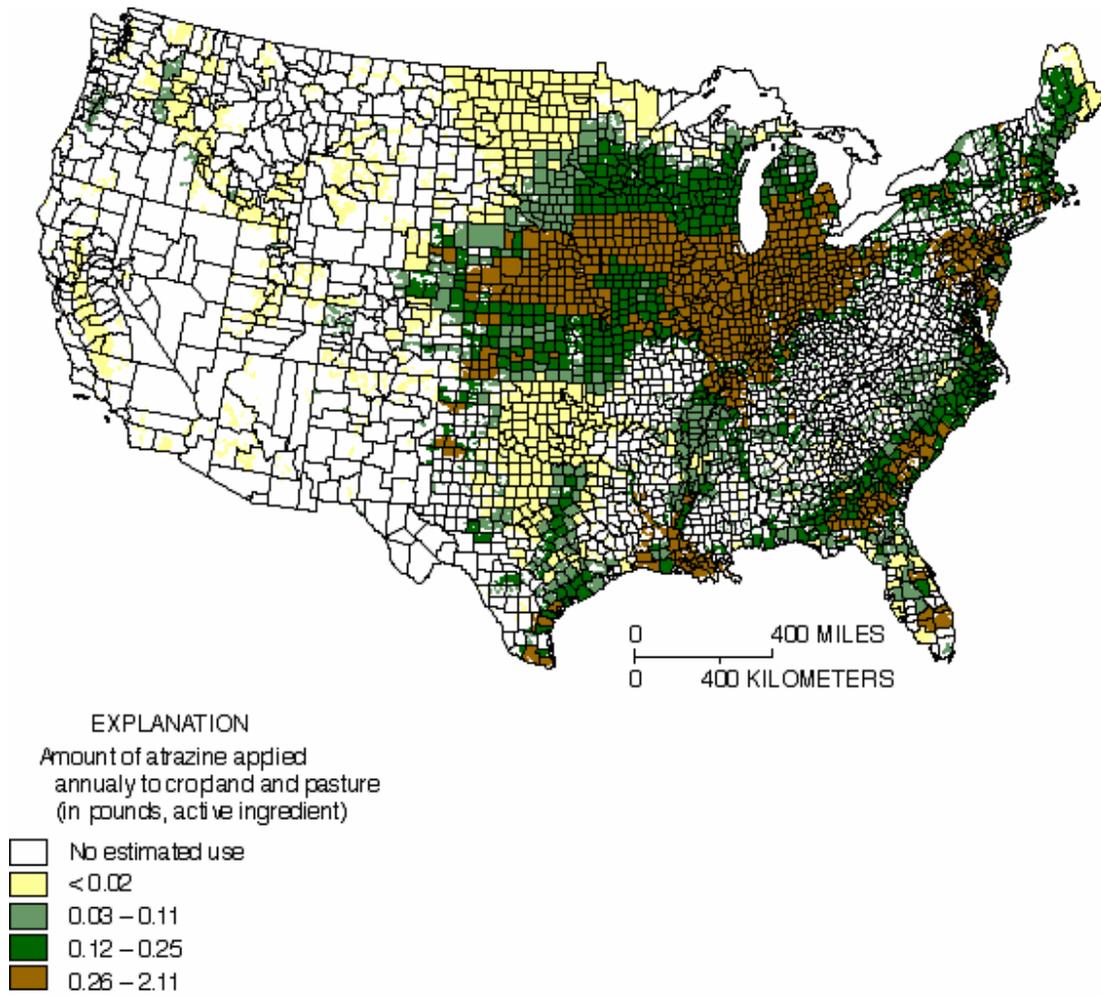
EXPLANATION

Amount of atrazine applied
  annualy to cropland and pasture
  (in pounds, active ingredient)

	No estimated use
	< 0.02
	0.03 – 0.11
	0.12 – 0.25
	0.26 – 2.11

Credit: U.S. Geological Survey
Department of the Interior/USGS
U.S. Geological Survey/photo By Gail P. Thelin, and Leonard P. Gianessi
(Thelin and Gianessi 2000b)

Figure 2: Amount of metolachlor applied annually in the United States
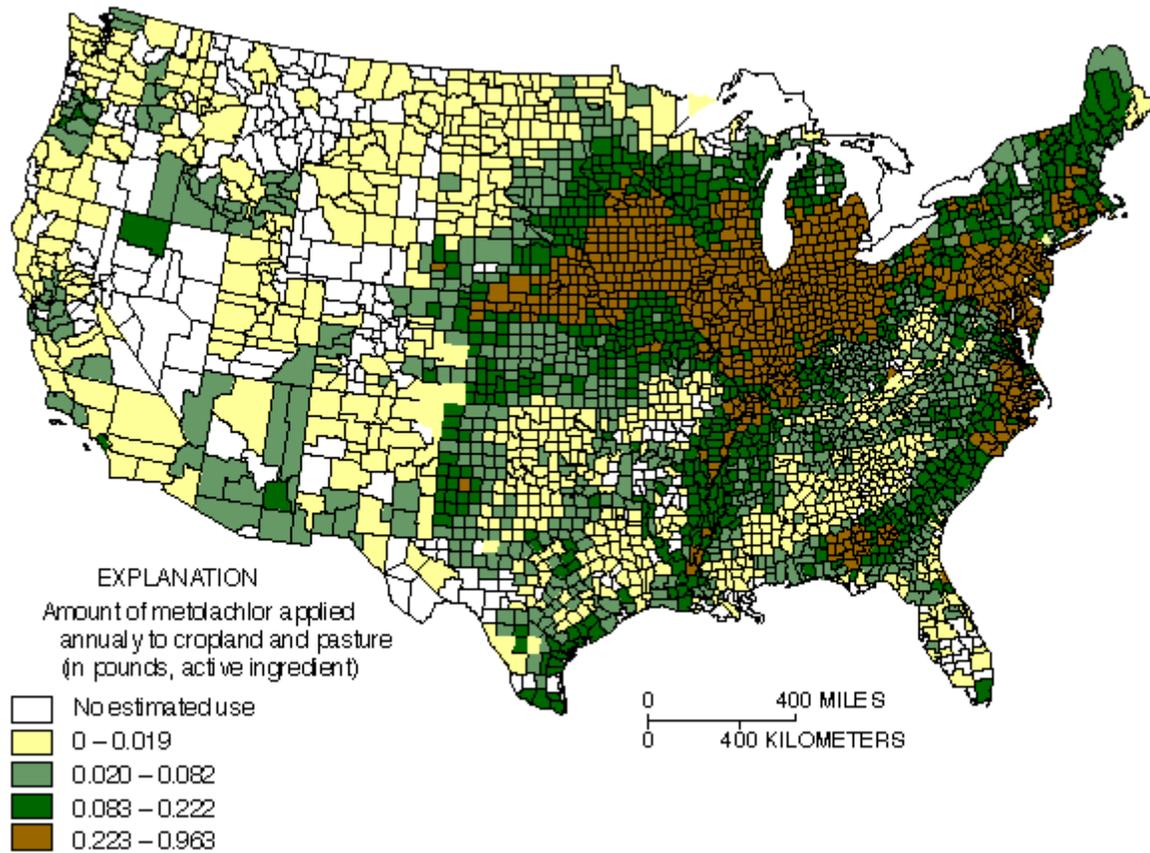
Credit: U.S. Geological Survey
Department of the Interior/USGS
U.S. Geological Survey/photo By Gail P. Thelin, and Leonard P. Gianessi
(Thelin and Gianessi 2000a)

III. Pesticides Effects on Aquatic Organisms

When substances are tested for toxicity, they are usually tested in simple systems (for example, using a single species of organism for bioassays, behavior tests, or developmental tests.) The results of these tests can yield important information about pesticide toxicity. For example in a behavior, survivorship, and development experiment, Hahn et al. (2001) concluded

that 17.4 parts per billion (ppb) and 30 ppb concentrations of the insecticide tebufenozide could negatively affect chironomid larvae in static and semi-static experimental setups. In static experiments, chironomid larvae emergence rates declined; there was a 70% increase in emergence accidents (emergence prior to complete molting), and mortality increased. Hahn et al. (2001) concluded the insecticide altered survivorship by interfering with detoxification and metabolic enzymes, disrupting hormone activity during critical stages of development, and modifying emergence behavior. Cold and Forbes (2004) examined the behavior and mortality of the amphipod *Gammarus pulex (G. pulex)* after pulse exposures to 0.1-0.6 ppb of the insecticide esfenvalerate. After a one-hour exposure to esfenvalerate, *G. pulex* larvae experienced delayed emergence, survivorship of pre-copulatory paired adults declined, and offspring survival declined.

Woin (1998) assessed the effects of 2 ppb and 20 ppb of esfenvalerate on the presence of macroinvertebrates in a pond community. There were 11-18 taxa per mesocosm prior to pesticide exposure. However, there were less than seven taxa per mesocosm after exposure to esfenvalerate. Woin concluded many sensitive taxa that were dominant prior to pesticide exposure were replaced by a higher abundance of tolerant taxa such as mollusks, oligochaetes, and leeches. Schulz and Liess (1999) also examined the presence of and behavioral changes in macroinvertebrates in a pond after pesticide exposure. They observed a decline in the number of macroinvertebrate species present (from 11 to 3) after exposure to the insecticides parathion-ethyl and fenvalerate (6 ppb and 302 ppb, respectively). The larvae of most of the sensitive species could not emerge from the stream due to the pesticides' impact on insect physiology. There was a decrease in the abundance of tolerant and sensitive species. Moreover, after

exposure to fenvalerate and parathion-ethyl, *G. pulex* displayed escape behavior via increased drift away from the impacted sites.

In a survivorship study conducted by Storrs and Keisecker (2004), the herbicide atrazine were found to decrease survivorship of early and late stage green frogs and late stage spring peepers at 3 ppb. As atrazine concentrations increased, survivorship of early stage spring peepers and early stage American toads increased (from 3 ppb to 30 ppb to 100 ppb). Storrs and Keisecker suggested that the data indicated a U-shaped response curve. Therefore, atrazine may decrease frog survivorship at low (3ppb) and high (100 ppb) concentrations.

Hayes examined the developmental and hormonal effects of pesticides on amphibians. In multiple studies, Hayes (2002a, 2002b) demonstrated low atrazine exposure (ranging from 0.1 ppb to 25 ppb) can induce gonadal malformations in larval amphibians in both the laboratory and natural populations. The malformations included multiple testes, multiple ovaries, voice box shrinkage in male amphibians, and an increase in the enzyme (aromatase) that converts estrogen into testosterone in male amphibians. In a separate study, Hayes (2006) exposed northern leopard frogs to individual or mixed pair concentrations of four herbicides (atrazine, metolachlor, alachlor, and nicosulfuron), three insecticides (cyfluthrin, cyhalothrin, and tebupirimphos), and two fungicides (metalaxyl and propiconizole). The pesticide mixtures delayed the time required for tadpoles to reach metamorphosis, as well as the time required for full tail absorption. When the tadpoles reached metamorphosis, they were significantly smaller than tadpoles that were not exposed to the pesticides. Exposed tadpoles also had high levels of the stress hormone, corticosterone. Tadpoles that were exposed to atrazine and metolachlor at 0.1 ppb and 10 ppb had damaged thymus glands (which regulate the immune system).

Unfortunately, some pesticides that are currently used and/or released have not been tested extensively and their toxicities and effects on organisms and system dynamics remain unknown. Although the aforementioned studies (Table 1) can provide valuable information about the toxicity of pesticides on aquatic organisms, these studies may not provide a true reflection of all environmental effects caused by lesser-studied pesticides.

Table 1: Examples of pesticides effects on aquatic organisms

Organism	Pesticide(s)	Pesticide Type		Effects at Specific Concentrations
		Insecticide	Herbicide	
Chironomids	Tebufenizide	X		**17.4 ppb:** Larvae emergence rates declined, and emergence accidents **30 ppb:** and larvae mortality increased (Hahn et al. 2001).
Gammarus pulex	Esfenvalerate	X		**0.1-0.6 ppb:** Mortality of adult's, offspring, and pre-copulatory paired adults as well as delayed emergence increased. (Cold and Forbes 2004).
Many Macro-invertebrate Taxa	Esfenvalerate	X		**2 ppb and 20 ppb:** Decreased abundance of sensitive taxa (mayflies and stoneflies). Increased abundance of tolerant taxa (mollusks, oligochaetes, and leeches) (Woin 1998).
Many Macroinvertebrate Taxa	Parathion-ethyl Fenvalerate	X		**6 ppb parathion and 302 ppb fenvalerate:** Sensitive species were unable to emerge from ponds. G. pulex displayed higher rate of drift behavior. Abundance of sensitive and tolerant species decreased (Schulz and Liess 1999).
Green Frogs, Spring Peepers, American Toads	Atrazine		X	**3 ppb:** Decreased survivorship of late stage green frogs and spring peepers. **3, 30, and 100 ppb:** Increased survivorship of early stage spring peepers and American toads (Storrs and Kiesecker 2004).
Leopard Frogs	Atrazine		X	**0.1 ppb to 25 ppb:** Amphibians had multiple testes, voice box shrinkage, multiple ovaries, and increased aromatase production (Hayes et al. 2002a, Hayes et al. 2002b).
Leopard frogs and Leopard frog tadpoles	Atrazine, metolachlor, alachlor, nicosulfuron, cyfluthrin, cyhalothrin, tebupirimphos, metalaxyl, propiconizole	X	X	**0.1 ppb of all pesticides:** Decreased survivorship and growth. Delayed time to metamorphosis and full tail absorption. High corticosterone levels. **0.1 ppb and 10 ppb of mixed atrazine and metolachlor:** Damage to thymus. (Hayes et al. 2006).

IV. Pesticides Effects on Complex Aquatic Systems

In the environment, pesticides typically occur in mixtures (for example atrazine and metolachlor mixtures) that can negatively affect the health of a single species. But, in reality, no system has only one organism living in it. If one organism is affected, then the pesticide mixture likely affects other species in that system as well, either directly or indirectly, by influencing species interactions. Unfortunately, few studies have been conducted on how individual and mixed pesticides influence more complex systems that involve multiple interacting species, especially in freshwater environments.

The few studies that have examined multiple species interactions after pesticide exposures have focused on complex interactions such as predator-prey interactions, overall community structure, and/or competition. For example, some experiments have examined the effects of pesticides on predation. Bridges (1999) established that the insecticide carbaryl altered behavior and predator-prey interactions between adult red-spotted newts and southern leopard frog tadpoles at 2.5 ppm. When prey (tadpoles) and not predators (adult newts) were exposed to carbaryl, prey activity levels decreased. This decrease made the tadpoles more susceptible to predation.

Other experiments have focused on pesticide effects on various forms of competition. For instance, Rohr and Crumrine (2005) looked at intraspecific competition in outdoor mesocosms and found that the herbicide atrazine caused a decrease in periphyton growth at 25 ppb. The resultant decline of this food source caused a marked decrease in the snail and tadpole populations that relied on this food source. Upon exposure to 10 ppb of the insecticide endosulfan, the abundance of insect competitors dropped. Tadpole populations were able to rebound because of competitor release. Effects on interspecific competition was the focus of a

separate study conducted by Rohr et al. (2006). Embryonic and larval salamanders that were exposed to environmentally realistic levels of atrazine (40, and 400 ppb) experienced decreased survivorship. While the reduction in salamander numbers decreased the negative effects associated with interspecific competition for food, salamanders continued to die well after exposure to atrazine. Schulz and Dabrowski (2001) examined the single and combined effects of predatory fish and sub-lethal insecticide exposure on mayfly activity, drift, and mortality. Mayfly activity and controlled voluntary drift downstream increased after exposure to 0.2 ppb of azinphos-methyl and 0.2 ppb of fenvalerate, because the insects tried to avoid the pollutants. Mortality increased because pesticide-impaired mayflies tended to drift involuntarily downstream, and this made the insects more susceptible to fish predation.

Scientists have also examined complex changes in some aspects of community structure after pesticide exposure. Changes in the presence, diversity, and abundance of animal and plant species is usually the focus of experiments observing changes in community structure. These experiments are particularly important because any effects on one species in a community can impact others that interact with the affected species. For example, in a study conducted by Hose et al. (2003), macroinvertebrate community structure and function were changed after exposure to the insecticide Endosulfan (6.87 ppb and 30.7 ppb). Endosulfan reduced the abundance of two mayfly taxa. These mayflies convert coarse particulate organic matter into fine particulate organic matter. Filterfeeders such as stoneflies rely on mayflies to produce this fine organic matter, because it is a major source of food for stoneflies. Therefore, as mayfly taxa declined, stonefly abundance declined, too. Endosulfan also caused a decline in tadpole abundance. As a result of this decline, grazing decreased and algal blooms surfaced. DeLorenzo et al. (1999) found that atrazine, and atrazine's degradate deethylatrazine eliminated six algal taxa. The

disappearance of the algae caused decreased photosynthetic biomass and low primary

productivity that resulted in decreased food availability for protozoan grazers. DeLorenzo

concluded the loss of the nutrient-rich algae would influence the quality and quantity of food

channeled to higher trophic levels.

In a study conducted by Woin (1998), fenvalerate exposures at a concentration of 2 ppb

eliminated sensitive predatory insects in an invertebrate pond community. Consequently, the

prey oligochaete population increased in abundance. Mollusks escaped the pressure of food

competition when other sensitive insects that competed with oligochaetes for food decreased.

These dead insects also served as an additional food source for mollusks. Woin observed a

change in the macrophyte community, too. Some specific insect species rely on specific

macrophytes for habitat. So, when the structure of the macrophyte community changed, the

structure of the insect community inhabiting those macrophytes changed.

Mills and Semlitsch (2004) also observed significant changes in community structure

after the community was exposed to the insecticide carbaryl at 2 ppm and 5 ppm. Carbaryl

caused cladoceran abundance to decrease. Cladocerans usually prey on phytoplankton that

competes with periphyton for food and is also a source of food for amphibians. When

cladocerans declined, phytoplankton could successfully compete with periphyton. And the size

of the leopard frog tadpoles declined, because of the decline in the once abundant food source

(periphyton). Prior to carbaryl exposure, dragonfly larvae *(Anax)* commonly preyed upon leopard

frogs' competitors, spring peeper tadpoles. However, because of carbaryl- induced *Anax*

mortality, spring peeper tadpoles were not preyed upon strongly and competitive pressure

between spring peeper and leopard frog tadpoles grew. All of these experiments (Table 2)

revealed a key effect of pesticide pollution in aquatic environments; they revealed pesticides that

are released into aquatic systems would not influence a single species. Direct and indirect effects

caused by pesticide exposure can have significant impacts on all interacting species in a system.

Table 2: Examples of pesticides' effects on complex systems

Pesticide(s)	Pesticide Type		Type of Interaction and Affected Organisms			Effects
	Insecticide	Herbicide	Predator-prey	Community structure	Competition	
Carbaryl	X		Prey = tadpoles Predator= newts			**2.5 ppm:** Increased prey activity made prey more susceptible to predation (Bridges 1999).
Azinphos-methyl Fenvalerate	X		Prey= mayflies Predator= fish			**0.2 ppb of both pesticides:** Involuntary drift of impaired mayflies made them more susceptible to predation (Schulz and Dabrowski 2001).
Endosulfan	X			Macroinvertebrates and algae		**6.87 ppb and 30.7 ppb:** Decline of mayfly taxa led to the decline of stonefly abundance. Decline in tadpole abundance led to decreased grazing and algal blooms (Hose et al. 2003).
Atrazine Deethylatrazine	X	X		Algae		**40 ppb and 160 ppb:** Six taxa went extinct locally. Photosynthetic biomass, low primary productivity, and food availability for protozoan grazers decreased (DeLorenzo et al. 1999).
Fenvalerate	X			Macroinvertebrates Macrophytes	Mollusks	**2 ppb:** Insect predator population declined and prey oligochaete population increased. Mollusks had additional food source, because of large abundance of dead insects. Changes in macrophyte community caused insect communities which inhabited those macrophytes to change (Woin 1998).
Carbaryl	X			Cladocerans Anax	Periphyton vs. Phytoplankton Spring peeper vs. Leopard Frog Tadpoles	**2 ppm and 5 ppm:** When cladoceran abundance declined, phytoplankton competed with periphyton for nutrients. Leopard frog size declined due to decreased food (periphyton) availability. Increased Anax mortality released leopard frog tadpoles from predation pressure. Competitive pressure between spring peepers and leopard frogs increased (Mills and Semlitsch 2004).
Atrazine Endosulfan	X	X		Periphyton	Snails vs. tadpoles	**25 ppb atrazine:** Periphyton growth decreased, so snails and tadpoles lost a food source. **10 ppb endosulfan:** Insect competitors declined (Rohr and Crumrine 2005).
Atrazine		X			Salamander (interspecific)	**40 ppb and 400 ppb:** Salamander survivorship declined. So interspecific competition decreased (Rohr et al. 2006)

V. Pesticides Effects on Host- Parasite Systems

Unfortunately, very few studies have assessed the direct and indirect impacts that pesticide exposures have on the dynamics of host- parasite systems. A study that ventured into this lesser studied area examined the direct effects of the insecticide malathion on parasitized Woodhouse toads. When Taylor et al. (1999) exposed non-infected Woodhouse toads to 0.011 and 0.0011 mg. of malathion per g. toad of malathion, the toads experienced a decrease in brain cholinesterase activity levels. The low cholinesterase activity levels can result in hyperexcitability and a single pre-synaptic stimulus can cause multiple post-synaptic impulses. When the effects of malathion were coupled with an *Aeromonas hydrophila* bacterial infection, these toads experienced a higher rate of mortality than infected toads that were not exposed to malathion and non-infected toads that were exposed to malathion.

As opposed to looking at the effects caused by a single pesticide, Gendron et al., Hayes et al., and Morley et al. explored the direct effects of multiple pesticides on hosts. Gendron et al. (2003) exposed leopard frogs infected with lungworms to a mixture of atrazine, metribuzin, aldicarb, endosulfan, lindane, and dieldrin. When compared to frogs in low concentration (0.1x) exposures, leopard frogs in high concentration (10x) exposures had higher abundances of lungworms. Gendron et al. predicted the pesticide mixture might have suppressed lymphocyte production, thus impairing the frog's immune system and making the frogs more susceptible to parasite infection. Hayes et al. (2006) found that tadpoles exposed to individual or mixed pair concentrations of four herbicides (atrazine, metolachlor, alachlor, and nicosulfuron), three insecticides (cyfluthrin, cyhalothrin, and tebupirimphos), and two fungicides (metalaxyl and propiconizole) and then challenged with the water-borne bacteria *Chryseobacterium (Flavobacterium) menigosepticum* experienced a higher mortality rate than tadpoles that were

not exposed to the pesticides. Recent studies that examined the direct effects of multiple pesticides have shown that snail exposure to compounds in anti-fouling paints (which include the pesticide chemical tributyltin) can result in very high mortality rates in trematode infected snails when compared to non-infected snails (Morley et al. 2003, Morley et al. 2004).

Keisecker et al. took a different approach and examined the indirect effects (i.e. linking pesticide exposure to amphibian limb deformities) of a single pesticide on a disease system. Keisecker (2002) found that in the presence of 3 ppb and 30 ppb of atrazine, trematode infection rates increased in larval amphibians and there was a significant increase in amphibian malformations.

Tantawy took this approach one-step further and examined the direct and indirect effects of multiple pesticides on a disease system. Tantawy (2002) examined the direct effect of pesticides on an agent of disease and found that when trematode miracidia and cercariae were exposed to two herbicides (Butachlor and Fluazifop-p-butyl), the miracidia and cercariae had a higher mortality rate as compared to the control group. When Tantawy explored the indirect effects, Tantawy found that the two herbicides also caused a decline in the number of shed cercariae and a decline in the length of time that snails shed the cercariae. Tantawy attributed the decline in shed cercariae to the miracidia's continuous exposure to the pesticides upon penetrating snails' tissues (which had been exposed to the herbicides).

Ultimately, these studies are very important in that they investigated substances that are widespread and used continuously. These studies also provided a lot of insight into how pesticides directly and/or indirectly affect parasites, hosts, and infectivity. Scientists have predicted that pesticide-induced changes in host behavior and survivorship (for example, survival of snails and the subsequent tadpole hosts), parasite behavior and survivorship, and disease

transmission -from snails to tadpoles - may be a significant yet understudied cause of worldwide amphibian declines (Kiesecker 2002, Gendron et al. 2003, Lafferty and Kuris 2005). One could infer that other pesticides with similar characteristics and modes of action could elicit changes in systems similar to those caused by the aforementioned pesticides. Scientists strongly suggest that negative health effects are more likely elicited when there are environmentally realistic mixtures of pesticides as opposed to a single pesticide (Pietrock and Marcogliese 2003).

Table 3: Examples of pesticides' effects on host- parasite systems

Host and Parasite Species (respectively)	Pesticide(s)	Pesticide type		Effects
		Insecticide	Herbicide	
Woodhouse toads, *Aeromonas Hydrophila*	Malathion	X		**0.011 and 0.0011 mg. of malathion per g. toad:** High toad mortality (Taylor et al. 1999)
Snails, Trematodes	Tributyltin	X		**10 ppb:** High snail mortality (Morley et al. 2003, Morley et al. 2004)
Leopard frogs, lungworms	Atrazine (21 ppb), metribuzin (0.56 ppb), aldicarb (17 ppb), endosulfan (0.02 ppt), lindane (0.33 ppt), and dieldrin (0.33 ppt)	X	X	**0.1x, 1x, and 10x each pesticide concentration listed:** Increased lungworm abundance (Gendron et al. 2003)
Amphibians, Trematodes	Atrazine (3 ppb and 30 ppb), malathion (200 ppb and 2000 ppb), and esfenvalerate (180 ppb and 1800 ppb)		X	Increased trematode infection and amphibian limb deformities (Kiesecker 2002).
Leopard frogs, *Chryseobacterium (Flavobacterium) menigosepticum.*	Atrazine, metolachlor, alachlor, nicosulfuron, cyfluthrin, cyhalothrin, tebupirimphos, metalaxyl, propiconizole	X	X	**0.1 ppb of all pesticides:** High frog mortality rate after bacteria challenge (Hayes et al. 2006).
Snails, Trematodes	Butachlor and Fluazifop-p-butyl		X	**7 ppm and 12 ppm of butachlor, and 12 and 40 ppm of fluazifop-p-butyl:** Increased trematode mortality. Decreased rate of cercarial shedding and number of cercariae shed (Tantawy 2002).

VI. Generalized Trematode Life Cycle

The relatively few studies that have been conducted in host-parasite systems demonstrate that there may be significant changes in the dynamics of these systems after exposure to mixed pesticides. Thus, my thesis research addressed how pesticides influenced a host-parasite system involving trematodes that infect snails and tadpoles. I chose this system because of its complexity and because trematode diseases are very important biologically and economically. The generalized life cycle of *Echinostoma trivolvis* trematodes is unique from many other parasite life cycles in that it involves three successive hosts. Sexual reproduction of the trematode occurs within a definitive host (usually a vertebrate), which then releases trematode eggs into aquatic environments through feces. Trematode miracidia hatch out of the eggs and infect the first intermediate host, an aquatic snail. Once the snail becomes infected, the miracidia transform into rediae. The rediae produce cercariae. The cercariae are shed from the snail by emerging through the skin. The cercariae enter the second intermediate host (e.g. a tadpole, fish, or freshwater snail) and encyst there. The definitive hosts then consume the infected second intermediate host and the cycle starts over (Beckage 1997) (Fig.3).

Most trematodes are extremely host specific meaning that specific species of trematodes tend to infect specific host species, especially when it comes to selecting an intermediate snail host species. The distribution and abundance of these molluscan vectors can strongly influence the distribution and spread of trematode parasites; and these parasites can have devastating impacts on successive hosts. Schistosomiasis (a disease caused by trematode parasites) is the most common waterborne disease in the world ((WHO) 1997). In 1996, over 200 million people had schistosomiasis (Nithiuthai et al. 2004). This disease is endemic in 74 developing countries. Fasciolopsiasis is another trematode. This food-borne disease is especially prevalent in the

agricultural areas of underdeveloped countries where the disease is usually transmitted via food

crops that have been fertilized with the feces of infected humans and farm animals (Graczyk et

al. 2001). Another trematode species found in agricultural areas is *Ribeiroia*. Recent studies have

shown that the *Ribeiroia* trematode species may be contributing to limb deformities in amphibian

populations in mid-western agricultural areas (Hayes et al. 2002a, Kiesecker 2002, Hayes et al.

2006). Due to the existence and/or prevalence of trematode diseases in various species all over

the world there seems to be an apparent threat of trematodes to human, snail, and amphibian

health.

Figure 3: Generalized Trematode Life Cycle

Definitive Vertebrate Host

2nd Intermediate Host

Egg

Redia

Cercariae

Miracidia

1st Intermediate Host- Mollusk
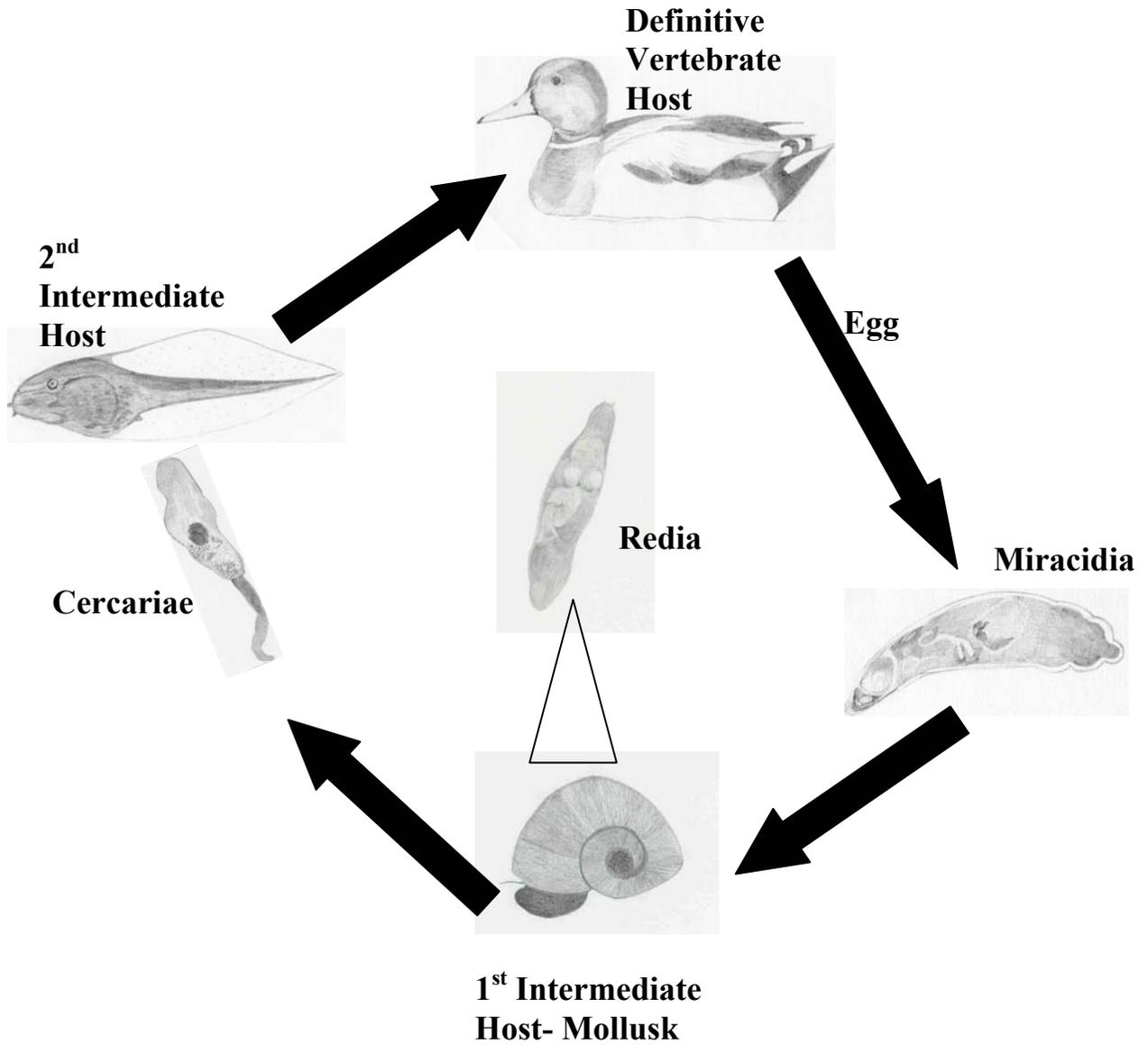
(Perkins 2006)

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Chapter II: The Effects of Atrazine and Metolachlor on the Survivorship of

Planorbella trivolvis **and** *Echinostoma trivolvis* **in the Laboratory**

Abstract:

Atrazine and metolachlor are two widely used agricultural herbicides that inhibit the growth of pre-emergent vegetation. Residues of these pesticides are commonly found in bodies of water near agricultural areas due to pesticide runoff. Most studies have focused primarily on the effects that individual pesticides have on individual species in aquatic systems. This study investigated the effects of environmentally realistic concentrations of atrazine and metolachlor on the first intermediate host of a trematode parasite, *Planorbella trivolvis* snails, and on the survival of *Echinostoma trivolvis* trematode parasites. A low concentration (10 ppb: 15 ppb) and high concentration (85 ppb: 100 ppb) mixture of metolachlor and atrazine, respectively, were used in the experiments. In the first experiment, non-infected snail hosts were exposed to the same four atrazine/metolachlor mixtures for two weeks. Regardless of exposure, all of the snails were alive at the end of the experiment. In the second experiment, the survivorship of *E. trivolvis* cercariae was monitored once every two hours for 20 hours after exposure to a water control, a solvent control, low concentration, or high concentration atrazine/metolachlor mixtures. The survivorship of cercariae exposed to the mixed pesticides was significantly lower only at 14 hours and only in the high concentration mixture when compared to control treatments. There were no significant differences in cercariae survivorship at any time in this experiment. Natural declines in cercariae survivorship were observed in both control and pesticide exposed cercariae. These results suggest that pesticide pollution may have variable impacts on members of host-parasite systems. In this case, the changes in parasite survival with exposure to atrazine and metolachlor mixtures could potentially affect disease dynamics by decreasing parasite loads in subsequent hosts and rates of disease transmission.

Introduction:

Digenetic trematodes are common parasites in many freshwater systems. The typical life cycle of these parasites (such as *Echinostoma trivolvis* used in this study) begins with sexual reproduction within a definitive host (usually a vertebrate), which releases trematode eggs into aquatic environments via host feces. Trematode miracidia (the first free-living stage) hatch and infect the first intermediate host, a freshwater mollusk. Once the mollusk becomes infected, the miracidia undergo repeated asexual reproduction to produce rediae. The rediae eventually produce cercariae (the second free-living stage). The mollusk sheds cercariae, which enter and encyst in the second intermediate host (i.e. vertebrate and invertebrate hosts). The definitive host consumes the infected second intermediate host and the cycle starts over again (Beckage 1997).

The free-living stages of trematode parasites can be sensitive bioindicators of environmental pollution. For example, free-living miracidia and cercariae stages are sensitive to heavy metals, sewage sludge, and various pesticides that are encountered by trematode parasites in the environment (Siddall and Clers 1994, Cross et al. 2001, Morley et al. 2003, Pietrock and Marcogliese 2003, Morley et al. 2004). In all of these studies, the survivorship of the free-living stages of the parasites decreased as pollution concentrations increased. Therefore, the parasites' decreased survivorship reflected the pollutant effects on a system.

One category of environmental toxicants of concern is pesticides. Over 1 billion kilograms of pesticides (Kiely et al. 2004) are applied to crops in the United States each year and these pesticides are transported into the nation's waters via runoff from rainstorm events, atmospheric deposition, and spray drift. Aquatic organisms are likely to encounter a mixture of atrazine and metolachlor in environments impacted by agricultural pesticide pollution. Snails and trematode parasites may frequently encounter this mixture, because these two pesticides rank in

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the top ten of the most widely used herbicides in the United States and they have been used in all 48 contiguous states (Goolsby 1995, Thelin and Gianessi 2000a, 2000b). Residues of atrazine and metolachlor ranging from 2 ppb to 500 ppb have been detected in measurable quantities in rivers, bays, and ponds in Virginia, and many other states (Goolsby 1995, Solomon et al. 1996, Clark et al. 1999, Hall Jr et al. 1999, Rivard 2003, Hayes et al. 2006).

Many studies have found the negative effects of pesticides on the survival of individual species living in aquatic environments. For example, the amphipod, *Gammarus pulex*, experienced decreased survivorship after exposure to the insecticide esfenvalerate at 0.1 ppb (Cold and Forbes 2004). When late stage green frogs (*Rana clamitans*) and spring peepers *(Pseudacris crucifer)* were exposed to 3 ppb of carbaryl, survivorship decreased (Storrs and Kiesecker 2004). Relyea et al. (2005) concluded exposure to 380 ppb of glyphosate (Roundup) resulted in a 40% decline in the total survivorship of American toad *(Bufo americanus)*, leopard frog *(Rana pipiens)*, and gray tree frog *(Hyla versicolor)* tadpoles. Other studies have concluded that some pesticides do not affect survivorship. For instance, Rohr and Crumrine (2005) concluded 25 ppb of an insecticide (endosulfan) or 10 ppb of an herbicide (atrazine) had no effect on the survivorship of *Planorbella trivolvis* snails. Diana et al. (2000) found atrazine had no significant effects on the survivorship of *Hyla versicolor*. The aforementioned studies examined the effects of individual pesticides on aquatic animals. Unfortunately, few studies have examined the impacts of pesticide mixtures on more than one member of a complex aquatic disease system.

In the present study, I investigated whether environmentally realistic low and high concentration mixtures of metolachlor and atrazine influenced the survivorships of (1) non-infected first intermediate hosts (*Planorbella trivolvis* snails) of the *Echinostoma* trematode, and

(2) *Echinostoma trivolvis* trematode cercariae. Based upon the results of the aforementioned Rohr and Crumrine (2005) study, I hypothesized that exposure to the pesticide mixtures would have no effect on the survivorship of *Planorbella trivolvis* snails. I also hypothesized the pesticides would cause a significant decline in parasite survivorship in a dose dependent manner.

Materials and Methods:

Snails and Cercariae Collection:

Planorbella trivolvis, first intermediate hosts in this interdependent system, were collected from a pond in Montgomery County, VA and screened for trematode infection as described by Belden (2006). The cercariae they were shedding were identified as *Echinostoma trivolvis*. Infected snails were moved into dechlorinated tap water and the snails were incubated at 16ºC to prevent further cercarial shedding. Snails were maintained in a 12:12 LD cycle and fed a diet of lettuce *ad libitum*.

Uninfected snails were maintained at room temperature (~24°C) in individual 120ml containers. They readily laid eggs under these conditions. Eggs from uninfected snails were placed outdoors in a 100-L plastic wading pool filled with 100L of wellwater and 100 g of dried leaf liter at Virginia Tech's Kentland Farm. The pools were covered with a plastic screen to keep out other organisms. The second-generation (= non-infected) snails used in the second experiment were reared in the wading pools.

Pesticides:

Stock solutions of the pesticides were prepared using a known mass of atrazine and volume of metolachlor (pestanal analytical standards, Sigma-Aldrich) dissolved in a known volume of 95% ethanol. Known volumes of stock solution were mixed with known volumes of dechlorinated tap water to achieve the desired pesticide and solvent concentrations. Two

environmentally relevant concentrations (low concentration and high concentration) were selected based on the results of studies which commonly found this range of concentrations in several bodies of water across the nation (Mazanti 1999, Allran and Karasov 2001). The amounts of atrazine and metolachlor in the low concentration treatment were 15 parts per billion (ppb) to 10 ppb, respectively. The amounts of atrazine and metolachlor in the high concentration treatment were 100 ppb to 85 ppb, respectively. A dechlorinated tap water control and a diluted solvent control (100 ppb) were used in each experiment. Water chemistry measurements (temperature, pH, alkalinity, hardness, and dissolved oxygen) were recorded at the beginning and end of each experiment. At the beginning of the experiment, the mean ($\pm$ SD) water chemistry measurements were: $23.6 \pm 0.07°C$ (temperature), 7.5 ± 0.05 (pH), 43.7 ± 0.5 ppm $CaCO_3$ (alkalinity), 48 ± 0.6 ppm $CaCO_3$ (hardness), and 11.4 ± 2.0 mg/L (dissolved oxygen). At the end of the experiment, the mean ($\pm$ SD) water chemistry measurements were: $23.6 \pm 0.09°C$ (temperature), 6.8 ± 0.3 (pH), 47.3 ± 7.0 ppm $CaCO_3$ (alkalinity), 57.3 ± 4.6 ppm $CaCO_3$ (hardness), and 12.0 ± 1.2 mg/L (dissolved oxygen).

Experimental Design:

Snail Experiment:

In November 2005, second generation snails were collected from the wading pool and their masses were recorded (mean mass ($\pm$SD) = 0.289 ± 0.126 g.). For the experiment, 60 ml of water, diluted solvent, low concentration, or high concentration pesticides were distributed to 8-60 ml glass jars. Each of the four treatments had eight snails with one snail per jar. Each glass jar was covered with plastic screen secured by a rim to prevent the snail from escaping the test container. Snails were fed boiled lettuce *ad libitum*. Snails were observed for signs of death once

every 24 hours for 14 days. A snail was recorded "dead" if it was immobile and failed to respond to prodding with a blunt needle. After 14 days, the masses of all surviving snails were recorded. Data were analyzed using an ANOVA where the proportion of snails surviving and mass were the dependant variables. A post-hoc Tukey-Kramer test was used to discern any differences between treatments.

Cercariae Experiment:

In August 2006, six infected snails were removed from the 16ºC incubator, weighed, and measured (mean mass (±SD) = 1.63 ± 0.65g., mean shell diameter (±SD) = 1.73 ± 0.42 cm). Each snail was placed in a 120 ml specimen cup containing 40 ml of dechlorinated water. The cups were placed under a 60-watt light bulb for forty minutes to stimulate cercarial shedding.

For the experiment, 3.1 ml of water, diluted solvent, low concentration mixture, or high concentration mixture were distributed to 3.5 ml wells of a 24-well microtiter wellplate (6 replicates per treatment). Ten cercariae were then transferred to each well. Each well received a mix of cercariae from three of the original six snails.

Cercariae were monitored for signs of death once every two hours until all parasites in the low concentration and high concentration pesticide treatments were recorded dead (20 hours). Parasites were recorded dead if they were immobile and failed to respond to prodding with a blunt needle, water agitation, and light.

Data were analyzed using a repeated measures ANOVA. Time was the within-subjects factor and pesticide concentration was the between-subjects factor. The proportion of surviving cercariae was the dependant variable. A post-hoc Tukey-Kramer test was used to discern any differences between treatments.

Results:

Snail Experiment:

All snails in all treatments were alive at the end of the experiment (mean mass ($\pm$ SD) = 0.387 $\pm$ 0.158 g.). Snail masses were not significantly different between treatments at the beginning and end of the experiment.

Cercariae Experiment:

Overall, there was a significant effect of treatment on survivorship ($F_{3,15}$ = 25.91, P< 0.0001). There was also a significant effect of time on survivorship for all treatments ($F_{9,45}$ = 294.85, P< 0.0001). Treatment and time had a significant interactive effect on cercariae survivorship ($F_{27,\ 135}$ =4.11, P<0.0001). There were no significant treatment effects until 14 hours post-exposure. When compared to the solvent control, a significant decline in survivorship occurred at 14 hours in the high concentration treatment (p< 0.0001) (Figure 1). There were no significant differences between the solvent controls and the pesticide treatments for hours 2-12 and hours 16-20. Survivorship in the solvent control was not significantly different from the water control for all hours.

Discussion:

The survivorship of non-infected *Planorbella trivolvis* snails, which are the only first intermediate hosts of *Echinostoma trivolvis* and can potentially serve as second intermediate hosts (Huffman and Fried 1990), was not affected by the pesticide mixtures used in this study. This result agreed with other field and laboratory studies that showed pesticides had no significant impact on snail survivorship (Woin 1998, Roses et al. 1999, Rohr and Crumrine

2005). Snail studies have not found changes in immune response or metabolism of toxicants after exposure to pesticides (Baturo and Lagadic 1996, Roses et al. 1999, Russo and Lagadic 2000). Therefore, the mechanism that allows snails to withstand exposure to pesticides remains unknown. My results suggest that first intermediate host snails are relatively resistant to atrazine and metolachlor pollution; and thus, would be poor organisms to use to assess ecological risk.

As observed in this experiment, *Echinostoma* parasites may be good organisms for use as bioindicators and to aid in assessing ecological risk, because cercariae respond to pesticide exposure within a short time. The survivorship of cercariae, which typically die within 24 hours after emerging from the first intermediate host, was significantly reduced by pesticide exposure at 14 hours post-emergence. My results on mixed pesticides differed from those on a single herbicide experiment in which *E. trivolvis* cercariae exposed to 20 ppb and 200 ppb of atrazine did not experience significant decreases in survivorship over the course of 24 hours (Koprivnikar et al. 2006). The effects of the two herbicides used in my experiment may have acted additively or synergistically when in combination, thus affecting parasite physiology and survivorship.

The differences in sensitivities in parasite and host survivorship observed in this experiment can potentially affect disease dynamics in aquatic systems. For example, if snails remain resistant to pesticide exposure, then these intermediate hosts will always be available in pesticide exposed environments. And non-affected hosts would allow the parasite to maintain its complex life cycle. If parasite numbers decrease with pesticide exposure, the likelihood of the parasites infecting subsequent hosts decreases and parasite transmission and persistence may decline.

Recommendations and Future Directions:

- Conduct range-finding tests to correlate significant changes in cercariae survivorship with varying pesticide concentrations over all 24 hours. This could allow scientists to estimate the concentration and toxicity levels of pesticide-polluted water based on how quickly the trematode parasites died.

- Repeat this experiment using different trematode genera and or species to determine if multiple kinds of trematodes exhibit a similar response to pesticide mixtures. Thus, scientists would not have to be restricted to using one trematode species in the future if parasite survivorship is used to examine ecological risk or as bioindicators.

- Examine the survivorship of infected snails and infected tadpoles. Infected host survivorship could potentially be a good bioindicator and can be used to assess ecological risk.

- Examine the effects of atrazine and metolachlor individually to determine whether combinations of the pesticides are synergistic, antagonistic, additive, multiplicative, etc.

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Figures Legends

Figure 1: Mean proportion of surviving cercariae in water, diluted solvent, low concentration, and high concentration treatments for the duration of the experiment. The treatment that is statistically different from the solvent control is circled.

Figure 1:
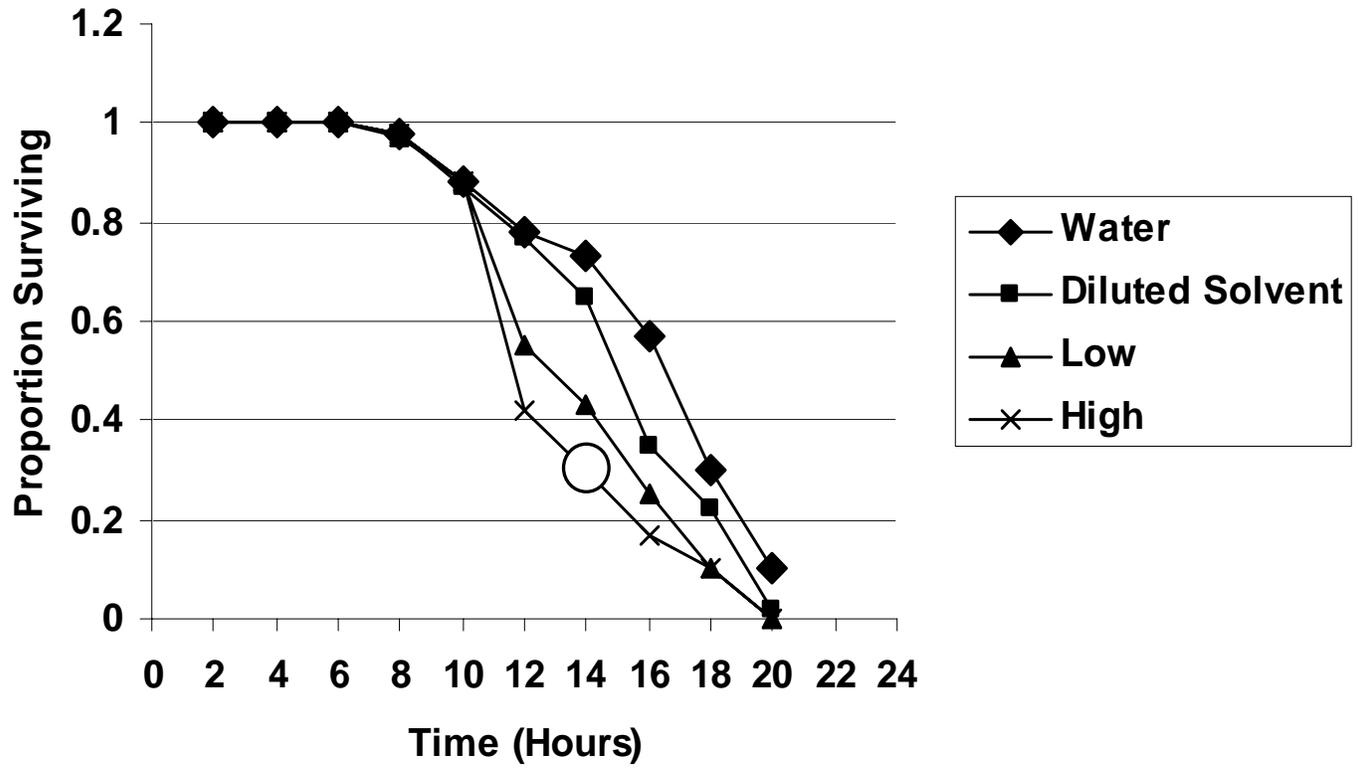
Chapter III: The Effects of Atrazine and Metolachlor on *Echinostoma trivolvis* Parasite Load in *Rana sylvatica* and *Rana clamitans* Tadpoles under Various Laboratory Exposure Conditions.

Abstract:

The release of pesticides into aquatic environments from agricultural areas can have very important effects on organisms within these environments. For example, hosts and parasites and the interaction between them are potentially affected by pesticide pollution. In this study, I examined the effects of a two-herbicide mixture – atrazine and metolachlor – on *Echinostoma trivolvis* trematode infection in *Rana sylvatica* and *Rana clamitans* tadpoles. A low concentration (15 parts per billion [ppb] to 10 ppb) and a high concentration (100 ppb to 85 ppb) of atrazine and metolachlor, respectively, were used. Three exposure conditions were used for the experiment with *R. sylvatica* tadpoles prior to exposing them to parasitic *E. trivolvis* cercariae for 72 hours: (1) tadpoles not treated with pesticides, cercariae treated with pesticides, (2) both tadpoles and cercariae treated with pesticides, and (3) tadpoles treated with pesticides, cercariae not treated with pesticides. Four exposure conditions were used for *R. clamitans* tadpoles prior to infecting them with cercariae: (1) tadpoles not treated with pesticides, cercariae treated with pesticides, (2) both tadpoles and cercariae treated with pesticides, (3) tadpoles treated with pesticides, 10 hours old cercariae not treated with pesticides, and (4) tadpoles not treated with pesticides, 10 hours old cercariae treated with pesticides. Parasite load was surveyed at the end of the experiments. The pesticides had no effect on parasite load. Parasite age was found to have an effect on parasite load in that new cercariae were more infective than 10-hours old cercariae. Atrazine and metolachlor pollutant mixtures may not influence disease dynamics, while the age of the cercariae plays an important role in the transmission of trematode disease.

Introduction:

Parasites play an important role in the functioning of an ecosystem; and parasites can have significant effects on hosts within these systems. For example, parasites regulate host fitness by influencing mortality, growth, and reproduction (Poulin 1999, Marcogliese 2004). Parasites with life cycles consisting of multiple hosts can also enhance transmission to successive hosts by influencing host behavior (Beckage 1997). Parasites utilizing multiple host species have co-evolved with all of their hosts by adapting each stage of their life cycle to enable consistent and successful transmission to, and survival in, hosts in healthy and stable environments (Poulin 1999, Lafferty and Kuris 2005). For example, in a typical trematode life cycle trematode eggs are released in the feces of a definitive vertebrate host. The eggs hatch and free-living miracidia infect the first intermediate host, typically an aquatic snail. Once inside the snail, the miracidia eventually produce cercariae. The snail sheds cercariae, which enter and encyst in the second intermediate host, such as a tadpole. The definitive hosts consume the infected tadpole and the cycle starts over (Beckage 1997). Any alterations in the transmission of trematodes to successive hosts can affect the parasites' fitness, host condition, and ecosystem function.

One factor that may alter trematode transmission is the release of chemicals (e.g. sewage sludge, anti-fouling agents, and pesticides) into aquatic environments (Kuntz 1946, Siddall and Clers 1994, Cross et al. 2001, Morley et al. 2003, Morley et al. 2004, Reddy et al. 2004). Pesticides are of special concern, because of the amount used (490 million kg of pesticides are used on crops in the United States every year) (Solomon et al. 1996) and the persistent residues that are often found in bodies of water. A portion of these pesticides is transported from crops to streams, rivers, ponds, and bays via runoff from rainstorm events and spray drift. Pesticide

residues can remain in these affected environments from a few hours to several months and they can potentially affect hosts and parasites living in these aquatic environments (Goolsby 1995, Solomon et al. 1996, Christin et al. 2003).

Studies that have examined how pesticides affect disease in amphibian populations reveal that infectivity is differentially affected after exposure to different pesticides. Keisecker (2002) exposed *Rana sylvatica* simultaneously to trematode cercariae and to one of three pesticides, esfenvalerate, malathion, or atrazine. *Rana sylvatica* tadpoles exposed to trematodes and a pesticide had higher numbers of cercariae than tadpoles that were exposed to water controls. Koprivnikar et al. (2006) exposed trematode cercariae to sequentially to atrazine and then the tadpoles were exposed to the pesticide-exposed cercariae. In contrast to Keisecker's experiment, tadpoles in Koprivnikar et al.'s study that were exposed to atrazine-treated cercariae had lower parasite loads than tadpoles in the control groups.

While many studies have examined the effects of individual pesticides on disease, combinations of pesticides are typically found in a single body of water. One common combination is atrazine and metolachlor. Each year over 30,000 tons of atrazine are used on corn, sorghum, soybeans, cotton, potatoes, and peanuts to stop the growth of pre-emergent vegetation via inhibiting photosynthesis (Solomon et al. 1996, Roses et al. 1999). Once atrazine enters freshwater environments, it can persist in water with a pH of 7 for 355 days in natural light (Solomon et al. 1996). Metolachlor is also an herbicide designed to stop the growth of pre-emergent vegetation. However, it differs from atrazine in that it inhibits growth by stopping cell growth and lipid synthesis (Goolsby 1995). Each year over 30,000 tons of metolachlor are applied to corn, sorghum, pineapple, and Christmas trees (Mazanti 1999). Once metolachlor enters freshwater environments, it can persist in water with a pH of 7 for 335 days in natural

light (Hayes and Laws 1991). Unfortunately, no studies have examined how atrazine and metolachlor in combination affect trematode infection in amphibians.

In the current study, I investigated whether pre-exposure of cercariae or tadpoles to high concentration or low concentration mixtures of atrazine and metolachlor affected parasite load in tadpoles as compared to controls (water versus diluted solvent). I also examined whether the age of cercariae would have an effect on parasite load in the presence and absence of pesticides. In my previous study *(see Chapter II),* I concluded that cercariae exposed to an atrazine-metolachlor mixture had a significantly higher rate of mortality than the controls following 14 hours of exposure. Based on the results of my previous experiment, I predicted that tadpoles exposed to pesticide-treated cercariae would have lower parasite infection rates. I also predicted that parasite load would be higher when tadpoles were treated with pesticides but cercariae were not treated with pesticides, because the pesticides could negatively affect tadpole host physiology.

Materials and Methods:

Parasites

Planorbella trivolvis snails were collected from a golf course pond in Montgomery County, VA. Snails were screened for parasite infection according to Belden (2006). Infected snails were placed into 120 ml specimen cups containing 60 ml of water, and incubated at 16°C to inhibit cercarial shedding. On the first day of each experiment, three snails were placed into their own 120-ml specimen cups containing 60-ml of water (1 snail/cup). Cups were placed under a 60-watt light bulb for one hour to stimulate cercarial shedding. Parasites were then transferred into a single cup and these were used in experiments as described below.

Tadpoles

Rana sylvatica and Rana clamitans egg clutches were collected from a pond on Virginia Tech's Kentland Farm in Blacksburg, VA in the spring and summer of 2006. Tadpoles of each species were raised in separate 1000-L cattle tanks (three clutches/tank, tanks = 2). At the time of the experiments, *R. sylvatica* frogs (n=78) were at an average developmental stage ± SD of 26.8 ± 0.9 (Gosner 1960) and had an average mass (± SD) of 0.253 ± 0.066 g. *Rana clamitans* (N=96) were at an average developmental stage (± SD) of 26.7 ± 0.9 (Gosner, 1960) and had a mass (± SD) of 0.230 ± 0.049 g.

Pesticides

Stock solutions of the pesticides were prepared using a known mass of atrazine and volume of metolachlor (pestanal analytical standards, Sigma-Aldrich) dissolved in a known volume of 95% ethanol. Known volumes of stock solution were mixed with known volumes of filtered, dechlorinated tap water to achieve the desired pesticide and solvent concentrations. Two environmentally relevant concentrations (a low concentration and a high concentration) were selected for this experiment (Mazanti 1999, Allran and Karasov 2001), because these approximate concentrations are often found in the nation's waters. The amount of atrazine and metolachlor in the low concentration treatment was 15 parts per billion (ppb) to 10 ppb. The amount of atrazine and metolachlor in the high concentration treatment was 100 ppb to 85 ppb (Mazanti 1999, Allran and Karasov 2001). There were two control treatments, solvent (100 ppb) and dechlorinated tap water.

Experiment 1: *Rana sylvatica*

Experimental tadpoles were divided into three groups: (1) tadpoles not treated with pesticides, cercariae treated with pesticides, (2) both tadpoles and cercariae treated with

pesticides, and (3) tadpoles treated with pesticides, cercariae not treated with pesticides. Within each of these groups, there were 4 treatments: (1) water, (2) solvent control, (3) low dose mixture of atrazine and metolachlor, and (4) high dose mixture of atrazine and metolachlor. This led to 12 treatment groups, with 6 replicate tadpoles in each group (Table 1).

For treatment groups 1 through 4 (tadpoles not treated, cercariae treated), one tadpole was distributed to each 120 ml specimen cup containing 60 ml of dechlorinated tap water. Forty cercariae were placed into each well of a 3.5 ml well of a microtiter wellplate. Each well contained 3.1 ml of water, diluted solvent, low concentration, or high concentration pesticide mixtures (6 wells/treatment). The parasites in each well were exposed to the solution for ten hours prior to being transferred to the individual 120 ml specimen cups containing dechlorinated tap water and the tadpoles. The tadpoles were exposed to the cercariae for 72 hours.

For treatments 5 through 8 (both tadpoles and cercariae treated), one tadpole was placed into a 60 ml glass jar containing 60 ml of water, diluted solvent, low dose, or high dose pesticide concentrations for ten hours (6 tadpoles/treatment). Snails were allowed to shed cercariae parasites for one hour. Forty shed cercariae were transferred to each 3.5 ml well in a 24-well microtiter wellplate containing 3.1 ml of water, diluted solvent, low dose, or high dose pesticide concentrations per well. The parasites were exposed to the solution for ten hours. After ten hours, the parasites from a single well and a single tadpole from the matching exposure conditions were transferred to a 120-ml specimen cup containing 60 ml of dechlorinated tap water for 72 hours.

For treatments 9 through 12 (tadpoles treated, cercariae not treated), one tadpole was placed into a 60 ml glass jar containing 60 ml of water, diluted solvent, low concentration, or high concentration pesticide mixtures for ten hours. After the tadpoles were exposed to the solution for ten hours, the snails were allowed to shed cercariae parasites for one hour. Forty

shed cercariae were transferred to 120-ml specimen cups containing 60 ml of dechlorinated water. A single tadpole was then transferred to each cup containing the parasites for 72 hours.

After 72 hours in the specimen cups with the cercariae, all 72 tadpoles were euthanized in MS-222, placed in plastic vials (one vial per tadpole), and frozen to preserve them for dissection. Later, each tadpole was thawed for two minutes, dissected, and cysts were counted.

During preliminary data analysis it was noted that the infection level in the group with only the tadpoles treated with pesticides was much higher than in the groups with both tadpoles and cercariae treated and with only cercariae treated. This did not appear to be due to the pesticide exposure because the number of cysts in the control treatments was also high. I concluded that it was likely due to the age of the cercariae. Previous research (Fried et al. 1997) has suggested that infectivity of *E. trivolvis* declines with age. In my experimental design, the tadpoles in the group with only the tadpoles exposed to pesticides were exposed to cercariae that were newly shed from snails, whereas in the other groups, the cercariae were exposed to pesticides for 10 hours prior to the addition of a tadpole host. Due to this confounding factor, cercariae age was included as an additional factor. I analyzed these data in two separate ANOVAs. One ANOVA compared the pesticide treatments within the group with only the tadpoles exposed to pesticides and newly shed, not treated cercariae. The pesticide treatment was the independent variable and the average number of cysts was the dependant variable. I used a 2-way ANOVA to compare the remaining groups that all utilized 10-hours old cercariae. In this factorial design, infection group (2 levels) and pesticide treatment (4 levels) were the independent variables and the average number of cysts was the dependent variable.

Experiment 2: *Rana clamitans*

The experimental design for *R. clamitans* was similar to that for *R. sylvatica*. However, it was modified based on the issue of cercariae age described above. The modification was to add in an additional treatment to specifically address the issue of cercarial age. Instead of having a single group of the tadpole treated and cercariae not treated group, I incorporated two of these groups. Individuals in one of these groups were exposed to newly shed cercariae, as in the *R. sylvatica* experiment. The other group was exposed to cercariae that sat for 10 hours prior to being exposed to tadpoles. With that exception, all methods were identical to those described above for the *R. sylvatica* experiment (Table 2).

For the tadpole treated, cercariae not treated groups, I used a 2-way ANOVA to examine the effects of cercariae age (2 levels) and pesticide treatment (4 levels) on the number of cysts in the tadpoles. I also used a 2-way ANOVA to compare infection levels for all 3 groups in which the tadpoles were exposed to 10 hours old cercariae. In this analysis, infection group (3 levels) and pesticide treatment (4 levels) were the independent variables and the number of cysts was the dependent variable. The alpha level was corrected from 0.05 to 0.025 because I used select data (tadpoles treated with pesticides and 10 hours old cercariae) in both analyses.

Results:

Pesticide pretreatment of tadpoles had no significant effect on parasite load for tadpoles exposed to 10-hours old cercariae (F= 2.539, P= 0.070). However, the experimental group had a significant effect (F= 4.609, P= 0.038) in that tadpoles had a significantly lower parasite load when only cercariae were treated with the pesticides than when both the tadpoles and the cercariae were exposed to the pesticides (mean number of cysts 4.75 ± 1.44 vs. 7.92 ± 1.14, respectively). There were no interactive effects of exposure group and pesticide treatment on

parasite load (F=1.930, P= 0.140). Pre-exposure of *R. sylvatica* to both a low concentration (15

parts ppb to 10 ppb) and a high concentration (100 ppb to 85 ppb) mixture of atrazine and

metolachlor, respectively, had no significant effects on parasite load when *R. sylvatica* tadpoles

were exposed to newly shed cercariae (Figure 1; F= 0.256, P= 0.856).

In treatments in which only 10 hours old cercariae were utilized, there were no significant

effects of pesticide exposure (F= 1.291, P= 0.286) or infection group (F= 1.106, P= 0.338) on *R.*

clamitans parasite load. There were no significant interactive effects of pesticide exposure and

infection group (F= 1.932, P= 0.090) on *R. clamitans* parasite load. In treatments in which only

newly shed cercariae were utilized, there were no significant effects of pesticide exposure (F=

1.241, P= 0.321) on *R. clamitans* parasite load. When *R. clamitans* tadpoles were exposed to a

low concentration (15 parts ppb to 10 ppb) and a high concentration (100 ppb to 85 ppb) mixture

of atrazine and metolachlor, respectively, and either 10-hours old or newly shed cercariae,

cercariae age had a significant effect on parasite load (Figure 2, F= 86.632, P< 0.005). Pesticide

exposure had no significant effect (F= 1.060, P= 0.377); and there were no significant interactive

effects of age and pesticide exposure (F= 1.507, P= 0.227) on parasite load.

Discussion:

In this experiment, I examined whether pesticides affected trematode infection of

tadpoles. I hypothesized that tadpoles exposed to pesticide-treated parasites would have lower

parasite loads than the controls. I also hypothesized that parasite load would be higher in

tadpoles when tadpoles and not cercariae were exposed to the pesticides. However, I did not find

support for either of these hypotheses. Pesticide treatment of either tadpoles or cercariae had no

significant individual or interactive effects on *R. sylvatica* and *R. clamitans* parasite load

regardless of whether hosts and/or cercariae were exposed. Fried et al. (1997) found that *E. trivolvis* are most infective between six and eight hours after emergence from snails. The results of my previous study (Chapter II) revealed pesticides do not affect parasite mortality until 14 hours after emergence. Thus, in this study, it seems likely that infectivity was so low by 10 hours, that any impact of the pesticides to infectivity was negligible (Figure 1-2). For *R. sylvatica* tadpoles, infection group had a significant effect on parasite load. When cercariae were treated and tadpoles were not treated, infection levels were significantly lower than when both cercariae and tadpoles were treated. Further study needs to be done to address this result, but it seems likely that this is somehow a result of the experimental design because the control and solvent treatment were also higher in the one group (as evidenced by the lack of a significant interaction term). This pattern was not seen for *R. clamitans*.

The second experiment in my study also addressed the effects of parasite age on parasite load in tadpoles. I found that newly emerged cercariae were more infective than 10-hours old cercariae. This effect can be attributed to changes in parasite activity levels, physiology, or morbidity as time progressed. For example, *E. trivolvis* cercariae only have approximately 24 hours to locate and encyst in a second intermediate host after emergence from the first intermediate host. As time progresses after cercarial emergence, the activity levels of cercariae decrease (Kanev et al. 1995). In this experiment, it is likely that the high activity levels of the newly emerged cercariae allowed then to successfully seek out and encyst in *R. clamitans* tadpoles, while the 10-hours old cercariae had lower activity levels which decreased the parasites' ability to infect tadpoles. My result were comparable to a study in which Fried et al. (1997) also found parasite load was significantly affected by the age of the cercariae.

In my study, the tadpoles and cercariae were exposed to the pesticides for 10 hours prior

to the testing of infectivity. Pesticides can persist in aquatic environments for longer than 10 hours (Kamrin 1997); so the effects on parasite load may not be elicited until several days later. Even though the mixture I used did not have a significant effect on parasite load, this is only one of many pesticide combinations found in the environment. For example, Hayes et al. (2006) exposed *Rana pipiens* to a nine-pesticide mixture (consisting of four herbicides, three insecticides, and two fungicides). Such mixtures are typically applied to agricultural crops in the mid- western United States. Hayes concluded that pesticides increased the time it took for tadpoles to reach metamorphosis. While I did not find significant effects of two herbicides on parasite infectivity, my study shed light on how parasite age and differences in parasite load due to differences in exposure conditions can affect disease dynamics. And, the effects of parasite age and exposure conditions are just as important in disease systems as the physiological effects other scientists have seen in animals after exposure to pesticides. Ultimately, any factors that affect parasite condition will ultimately influence disease transmission, host condition, and ecosystem function.

Recommendations and future directions

- **Increase the number of pesticides in the pesticide mixtures.** Gendron et al. (2003) found that atrazine, metribuzin, aldicarb, endosulfan, lindane, and dieldrin caused an increase in lungworm abundance in leopard frogs. So, more complex mixtures involving a greater number of pesticides may elicit a significant change in trematode parasite loads. The complex mixtures used should be similar to the complex mixtures of which residues are detected in many bodies of water across the country.

- **Incorporate more tadpole species or amphibian genera.** In one experimental

treatment, there was a significant effect of exposure condition on parasite load in *R. sylvatica*, but not in *R. clamitans*. The experimental design in this experiment may have been inadequate in allowing me to evaluate my hypothesis, so this experiment can be repeated using *R. sylvatica* and *R. clamitans* to see if the results are reproducible. Other species and genera should be included to see if exposure conditions would affect parasite load in other frogs, because disease susceptibility may differ by genera and species.

- **Include other cercariae ages into the experiment.** In this experiment, I found that cercariae age had a significant effect on parasite load. Including more ages (e.g. 0, 2, 4, 6, 8, 10, 12, and 14 hours old cercariae) would tell me the age at which cercariae are least infective and most infective to tadpoles. And additional pesticide experiments would allow me to take into account the age of the cercariae when looking at the effects of pesticide exposure on parasite load.

- **Repeat this experiment using tadpoles at different developmental stages.** Schotthoefer et al. (2003) found that different developmental stages and ages of tadpoles result in differences in parasite load and infectivity in that late stage tadpoles were more susceptible to parasite infection and had higher parasite loads than earlier stages. Holland et al. (2006) found that trematode infectivity is influenced by the age of specific developmental stages in *R. clamitans* tadpoles in that tadpoles in the late part of a stage were more infective than tadpoles in the early part of a particular stage. Varying the age of the parasite may also reveal how tadpole age influences the pesticides effects on parasite load, too. I suggest using tadpoles in the range of developmental stages 25-39.

Acknowledgements:

Table Legends:

Table 1: Exposure groups for *Rana sylvatica* tadpoles exposed to water control, 100 ppb diluted solvent, 10 ppb metolachlor + 15 ppb atrazine, and 85 ppb metolachlor + 100 ppb atrazine. Treatment number corresponds to pesticide exposure + group exposure treatments.   page 56

Table 2: Exposure groups for *Rana clamitans* water control, 100 ppb diluted solvent, 10 ppb metolachlor + 15 ppb atrazine, and 85 ppb metolachlor + 100 ppb atrazine. Treatment number corresponds to pesticide exposure + group exposure treatments. page 57

Figure Legends

Figure 1: *Echinostoma trivolvis* metacercarial cysts in the kidneys of *Rana sylvatica* tadpoles in relation to exposure to an atrazine-metolachlor mixture. Values are mean (± SE) number of cysts per tadpole. Tadpoles were exposed to cercariae for 72 hours. Pesticide concentrations used were: water control, 100 ppb diluted ethanol, 10 ppb metolachlor + 15 ppb atrazine, and 85 ppb of metolachlor + 100 ppb atrazine.   page 58

Figure 2: *Echinostoma trivolvis* metacercarial cysts in the kidneys of *Rana clamitans* tadpoles in relation to exposure to an atrazine-metolachlor mixture. Values are mean (± SE) number of cysts per tadpole. Tadpoles were exposed to cercariae for 72 hours. Pesticide concentrations used were: water control, 100 ppb diluted ethanol, 10 ppb metolachlor + 15 ppb atrazine, and 85 ppb of metolachlor + 100 ppb atrazine.   page 59

Table 1: Exposure groups for *Rana sylvatica*

Treatment number	Water Control		100 ppb diluted ethanol		10 to 15 ppb		85 to 100 ppb	
	Tadpoles	**Parasites**	**Tadpoles**	**Parasites**	**Tadpoles**	**Parasites**	**Tadpoles**	**Parasites**
1-4	Not treated	Treated (10 hours old)	Not treated	Treated (10 hours old)	Not treated	Treated (10 hours old)	Not treated	Treated (10 hours old)
5-8	Treated	Treated (10 hours old)	Treated	Treated (10 hours old)	treated	Treated (10 hours old)	Treated	Treated (10 hours old)
9-12	Treated	Not treated (newly shed)	Treated	Not treated (newly shed)	Treated	Not treated (newly shed)	Treated	Not treated (newly shed)

Table 2: Exposure groups for *Rana clamitans*

Treatment numbers	Water Control		100 ppb diluted ethanol		10 to 15 ppb		85 to 100 ppb	
	Tadpoles	**Parasites**	**Tadpoles**	**Parasites**	**Tadpoles**	**Parasites**	**Tadpoles**	**Parasites**
1-4	Not treated	Treated (10 hours old)	Not treated	Treated (10 hours old)	Not treated	Treated (10 hours old)	Not treated	Treated (10 hours old)
5-8	Treated	Treated (10 hours old)	Treated	Treated (10 hours old)	treated	Treated (10 hours old)	Treated	Treated (10 hours old)
9-12	Treated	Not treated (newly shed)	Treated	Not treated (newly shed)	Treated	Not treated (newly shed)	Treated	Not treated (newly shed)
13-16	Treated	Not treated (10 hours old)	Treated	Not treated (10 hours old)	Treated	Not treated (10 hours old)	Treated	Not treated (10 hours old)

**Figure 1:**

Rana sylvatica
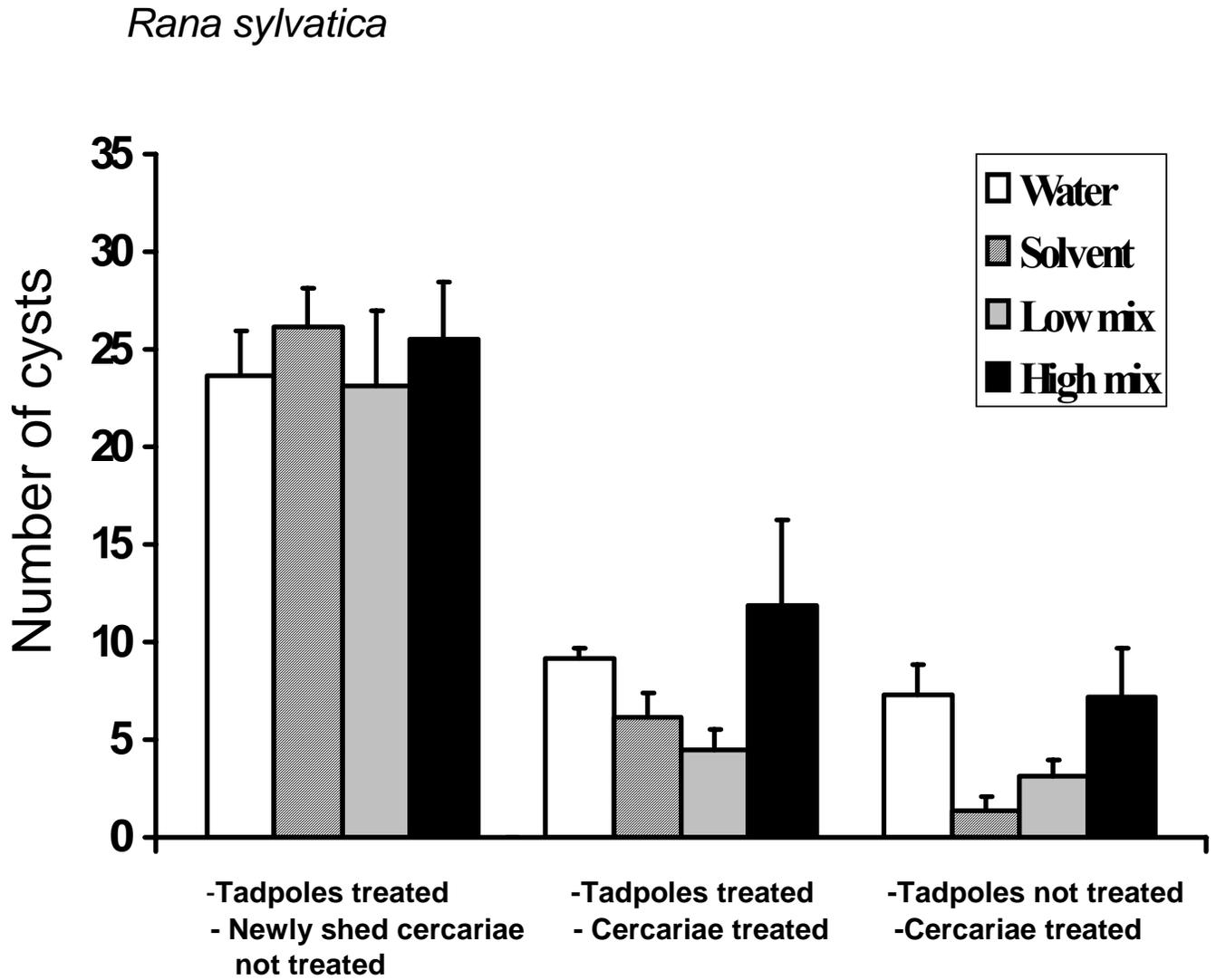
Figure 2:

Rana clamitans
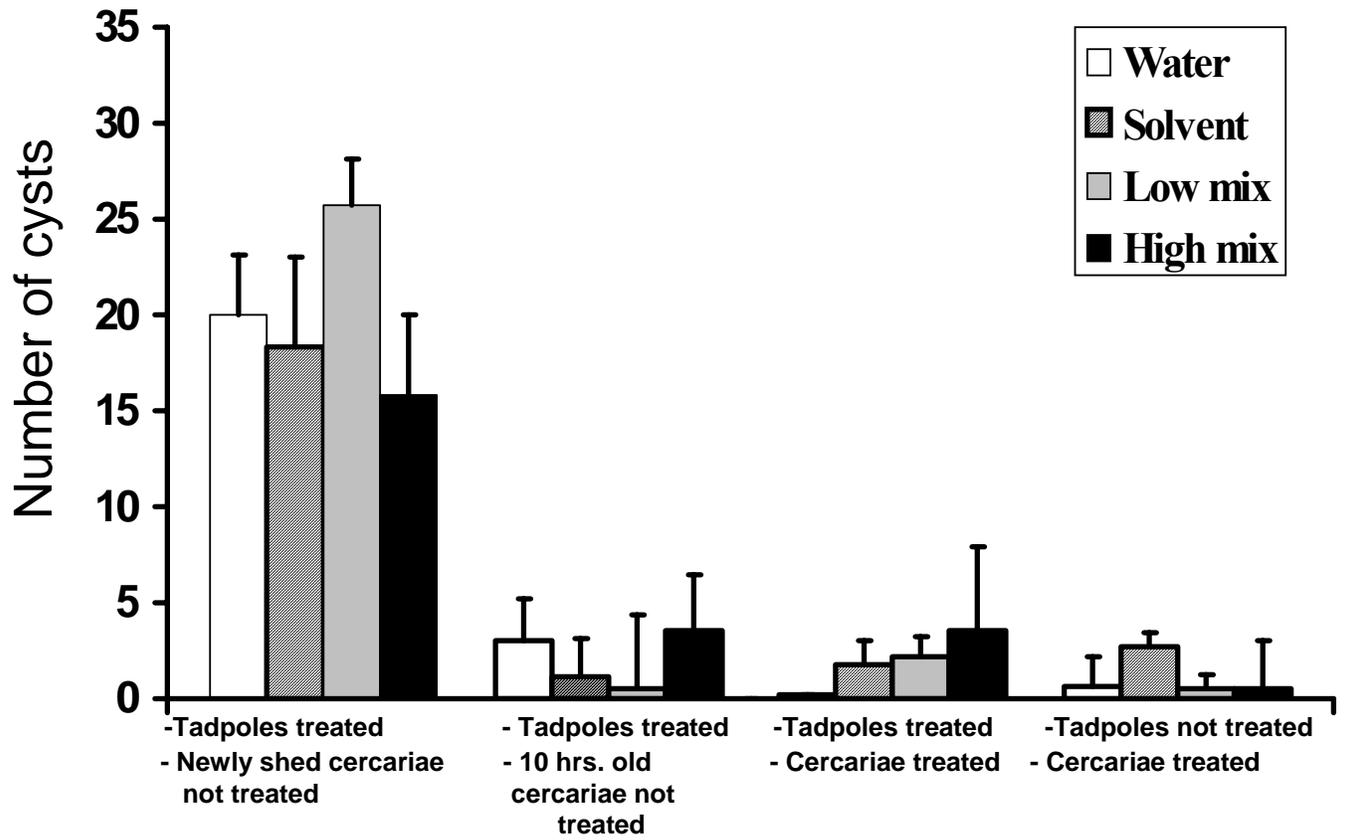
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Chapter IV: The Effects of Atrazine and Metolachlor on *Echinostoma trivolvis*

Parasite Load in *Rana Sylvatica* Tadpoles in Outdoor Mesocosms for 14 Days

Abstract:

Multiple stressor effects have been implicated in the decline of amphibian populations all over the world. Two stressors of recent concern are pesticide pollution and parasitism. In this study, I investigated the effect of two herbicides on parasite infectivity in *Rana sylvatica* tadpoles in outdoor mesocosms. *Rana sylvatica* tadpoles were simultaneously exposed to *Planorbella trivolvis* snails infected with *Echinostoma trivolvis* parasites and to a water control, diluted solvent control (100 ppb), low concentration mixture of metolachlor and atrazine (10 ppb: 15 ppb, respectively), or a high concentration mixture of metolachlor and atrazine (85 ppb: 100 ppb, respectively) in 100-L plastic wading pools located outdoors. After 14 days exposure, the masses of all snails were recorded and the number of cysts in each tadpole was surveyed. There were no significant differences in parasite load between tadpoles in the control treatments and tadpoles in the pesticide-exposed treatments. However, there was a positive correlation between the mass of the tadpoles and the number of cysts found in the tadpoles. The results suggest that metolachlor and atrazine mixtures do not have dramatic effects on trematode infection in amphibians under simulated field conditions. However, the size of the host influences trematode infection in that large tadpoles can support higher parasite loads.

Introduction:

For several decades, scientists have noticed significant declines in amphibian populations all over the world (Stuart et al. 2004). Many individual stressors such as UV-B radiation, habitat loss and destruction, acidification, pathogens, drought, introduced species, and toxic chemicals are believed to have brought about these negative changes (Keisecker et al. 2001, Blaustein and Kiesecker 2002, Christin et al. 2003, Krest et al. 2003, Hayes et al. 2006). Amphibians are often exposed to more than one of these stressors simultaneously and recently, studies have begun to address the effects of multiple stressors on amphibians. For example, in the Oregon Cascade Mountains, El Niño/Southern Oscillation cycles can cause changes in winter precipitation. As water depth decreases, *Bufo boreas* embryos are exposed to higher levels of UV-B radiation and become more susceptible to mortality when they become infected with *Saprolegnia ferax* (Keisecker et al. 2001). It is now widely acknowledged that multiple stressors can have synergistic effects and may be contributing to worldwide amphibian population declines.

Two stressors of recent concern are pesticides and disease. Studies have found that host exposure to pesticides, before the hosts are challenged with parasites, can alter mortality rates in response to infection. For instance, Hayes et al. (2002a, 2002b) examined the effects of a nine-ingredient pesticide mixture coupled with a *Chryseobacterium menigosepticum* infection on amphibians and found that disease rates increased when amphibians were exposed to the pesticide cocktail. Similarly, Christin et al. (2003) concluded that the herbicide atrazine increased lungworm infection in adult *Rana pipiens*; and Koprivnikar et al. (2006) found that atrazine caused an increase in the number of *Rana clamitans* tadpoles that were infected as well as an increase in the number of parasites infecting individual tadpoles. Studies have also found that host exposure to pesticides after the hosts are challenged with parasites, can alter mortality

rates in response to infection. For example, Woodhouse toads *(Bufo woodhousi)* infected with *Aeromonas hydrophila* and exposed to the insecticide malathion had a much higher rate of mortality than infected toads that were not exposed to malathion (Taylor et al. 1999). Pesticides have also been implicated in increased infection rates in amphibians. Furthermore, scientists have found that other sublethal effects that are more morphologically noticeable can also result from pesticide and parasite interactions. For example, increased trematode infection rates caused by pesticide exposure can increase the likelihood of limb deformities in frogs. Kiesecker (2002) exposed *Rana sylvatica* tadpoles to atrazine and the trematode parasite *Ribeiroia*. Pesticide-exposed amphibians weighed less and developed limb deformities when compared to amphibians that were not exposed to the pesticides (Kiesecker 2002).

In this study, I examined the effects of atrazine and metolachlor on tadpole development and trematode parasite load in tadpoles. Atrazine and metolachlor are the two most widely used herbicides that are found in the nation's waters during most of the year (Clark et al. 1999). Large amounts of metolachlor (over 56 million pounds in 1992) and atrazine (over 52 million pounds in 1992) are applied to crops annually (Mazanti 1999). Residues of both pesticides are typically detected in the environment, because the mixture is available as a single widely used commercial product (Bicep II Magnum); and this commercial mixture is applied on economically important crops such as corn and sorghum. Residues from the applications are relatively persistent once they enter the environment. For instance, atrazine has a half life of 355 days in surface waters (Mazanti 1999) and metolachlor has a half-life of 97-200 days in surface waters (Kamrin 1997). The parasite utilized in this study was *Echinostoma trivolvis*, a trematode parasite that is widely distributed across North America and has a complex life cycle with multiple free-living stages and hosts (Huffman and Fried 1990). Sexual reproduction of the trematode occurs within a

definitive host (usually a vertebrate), which releases trematode eggs into aquatic environments via feces. Trematode miracidia hatch out of the eggs and infect the first intermediate host, an aquatic snail. Once the snail becomes infected, the miracidia transform into mother sporocysts. The mother sporocysts produce daughter sporocysts, which produce cercariae. The snail sheds cercariae, which enter and encyst in the kidneys of the second intermediate host – a tadpole. The definitive hosts consume the infected second intermediate host and the cycle starts over (Beckage 1997).

I exposed *Rana sylvatica* tadpoles simultaneously to infected *Planorbella trivolvis* snails that were actively shedding *Echinostoma trivolvis* cercariae and to a low concentration mixture of metolachlor and atrazine (10 ppb: 15 ppb, respectively), or a high concentration mixture of metolachlor and atrazine (85 ppb: 100 ppb, respectively) in outdoor mesocosms for 28 days. Due to evidence of tadpole mortality and the low rate of re-capture of a sub-sample of the tadpole population at 14 days, I only surveyed parasite load in the tadpoles after 14 days of exposure rather than after 28 days (Table 1). In an earlier study (*see Chapter III*), I ascertained that, when compared to the control treatments, atrazine and metolachlor did not have a significant effect on parasite loads in tadpoles. Based on those results, I hypothesized that infection levels observed in tadpoles in the control treatments would not be significantly different from the infection levels of tadpoles in the pesticide treatments.

Experimental Design:

Pesticides

Stock solutions of the pesticides were prepared using a known mass of atrazine and volume of metolachlor (pestanal analytical standards, Sigma-Aldrich) dissolved in a known

volume of 95% ethanol. Each pesticide and solvent treatment was mixed with known volumes of filtered, treated tap water to achieve the desired nominal pesticide and solvent concentrations. Two environmentally relevant concentrations (a low concentration and a high concentration) were selected for this experiment, because these approximate concentrations are often found in the nation's waters (Mazanti 1999, Allran and Karasov 2001). The nominal amount of atrazine and metolachlor in the low concentration treatment was 15 parts per billion (ppb) to 10 ppb, in the high concentration treatment it was 100 ppb to 85 ppb (Mazanti 1999, Allran and Karasov 2001). There were two control treatments – solvent (which had a concentration of 100 ppb) and dechlorinated tap water.

Mesocosms:

Planorbella trivolvis snails were collected from a golf course pond in Montgomery County, VA. Snails were screened for *E. trivolvis* infection according to Belden (2006). Infected snails (n = 27) were weighed (mean mass (±SD) = 1.641 ± 0.443 g.), placed into 120 ml specimen cups containing 60 ml of dechlorinated water, and incubated at 16°C to inhibit cercarial shedding.

Four *Rana sylvatica* egg clutches were collected from a pond on Virginia Tech's Kentland Farm in Blacksburg, VA in Montgomery County, VA in 2006. The tadpoles were raised in 1000-L cattle tanks filled with well water, 300 g. of dried, mixed oak leaf litter, and 2 L of zooplankton. Sixteen 100-L plastic wading pools (diameter = 100.5 cm) were filled with approximately 90-L of well water or until the water depth was within the range of 90-100 mm. Sixty grams of dried, mixed oak leaf litter was distributed to each pool. Fiberglass mesh screen was clipped to the top of all pools to keep out animals and debris. After the pools were allowed to acclimate for two weeks, 20 tadpoles (average Gosner stage= 26; (Gosner 1960)) were

distributed to each pool. Water chemistry was measured for the well water two weeks before the start of the experiment (conductivity = 5.25 µs/cm, pH= 6.5, hardness= 76 ppm $CaCO_3$, alkalinity= 152 ppm $CaCO_3$, dissolved oxygen= 4.07 mg/L); and water chemistry was measured for the pool water on the day the experiment was started (conductivity = 6.72 µs/cm, pH= 7.0, hardness= 92 ppm CaCO, alkalinity= 92 ppm $CaCO_3$, and dissolved oxygen= 3.92 mg/L). One week after the addition of the tadpoles, pesticide treatments were added to the pools (N= 4 pools/treatment) and three infected snails were added to each pool. Twenty-one days (14 days of pesticide and cercariae exposure) after the addition of tadpoles, up to ten tadpoles surviving in each pool were euthanized in MS-222, placed in plastic vials (one tadpole per vial), and frozen to preserve them for later dissection (Table 1). Variations in the number of collected tadpoles may be due to tadpole mortality (I had planned to collect 10 tadpoles from each pool). I only analyzed the data from day 14, because very few tadpoles were collected from all pools on day 28. Mortality rates could not be confirmed, because some live individuals were found in the tanks when I went out to collect the remaining tadpoles at 28 days. Frozen tadpoles were later thawed for two minutes, and then dissected to count trematode cysts in the kidneys, which is where *Echinostoma trivolvis* metacercariae encyst in larval amphibians (Schotthoefer et al. 2003).

Water levels were maintained at a depth of 90-100 mm and water depth was monitored at day 7. On day 7, water depth had dropped below 90 mm for one pool containing a high concentration mixture and one pool containing diluted solvent. Well water was added to the pools until water levels were at a depth of 90-100 mm. Water chemistry measurements (temperature, pH, alkalinity, and hardness) were taken on day seven and day fourteen (Table 2). Daily weather conditions (minimum temperature [°C], maximum temperature, amount and type

of precipitation, wind speed, and wind direction) were obtained from the National Weather Service (http://www.weather.gov/climate/index.php?wfo=rnk) (Table 3).

The effect of treatment on mean cyst abundance between pools was analyzed using an ANCOVA with tadpole mass as a covariate. The mean numbers of cysts in tadpoles from individual pools were used in the analysis. I used an alpha level of 0.05. Due to potential bias introduced by the low number of tadpoles captured from some pools (ranging from 1-10 tadpoles) (Table 1), data were analyzed with and without the data from the 3 pools with two or fewer tadpole samples. There were no significant changes in the results of the experiment when replicates with low rates of tadpole recovery were excluded from the data analysis; so, I report only the results with all the samples included. A simple linear regression was used to determine if correlations existed between mass and the number of metacercarial cysts in individual tadpoles across all treatments.

Results:

There were no overall significant differences in parasite loads between treatments (F = 2.384, p = 0.125). The numbers of parasites encysting in tadpoles in the water (mean number of cysts ($\pm$ SD) = 574.89 $\pm$ 158.69) and solvent controls (510.89 $\pm$ 71.58) were not significantly different from the parasite loads of tadpoles in the high (443.05 $\pm$ 203.61) concentration and low (323.25 $\pm$ 167.50) concentration treatments (Figure 1). There was a trend for the number of cysts in tadpoles in the pesticide treatments to be lower than in the controls. There was a slight positive correlation between mass and the number of cysts across all individuals (Figure 2; R^2 = 0.0546).

Discussion:

There were no significant differences in tadpoles' parasite loads between all treatments. In my earlier experiment (*see Chapter II*), cercariae survivorship was negatively affected by pesticide exposure at 14 hours. In a study conducted by Fried et al. (1997), cercariae infectivity peaked at 6 to 8 hours after cercariae emerged from the first intermediate host. The results of my laboratory experiments in which I examined the effects of the pesticides on infectivity in newly emerged versus 10 hours old cercariae *(see Chapter III)* showed, the newly emerged cercariae were much more infective than the older cercariae regardless of pesticide exposure. These same effects may have occurred in the mesocosm experiment in that newly emerged cercariae were highly infective to the tadpoles regardless of pesticide exposure.

In this mesocosm experiment, the cercariae likely infected the second intermediate host tadpoles several hours before the pesticides would potentially affect cercariae survivorship. If survivorship was affected before the peak of infectivity, then parasite loads would have been significantly affected because the parasites would have died before having the chance to infect the tadpoles. For example, one study found that *Echinostoma trivolvis* cercariae exposed to copper sulfate experienced decreased survivorship (Reddy et al. 2004). Copper sulfate also decreased cercariae infectivity in *Biomphalaria glabrata* snails, because the cercariae died before they had a chance to infect the snails. In my mesocosm experiment, snails were likely shedding cercariae throughout the course of the experiment, because infected snails were still alive at the end of the experiment. Variations in the susceptibility of individual parasites would result in some individuals being very sensitive, and other individuals being very resistant, and most parasites would fall between those two extremes. Transmission to successive hosts would not be hindered in the individuals that are resistant to pesticides.

Many studies are conducted in the laboratory with standardized test methods and controlled environmental conditions. In these laboratory studies, factors such as weather conditions and natural temperature fluctuations are eliminated as factors that could potentially affect the results of laboratory experiments. These same factors may have altered the conditions of the environment in the mesocosm experiment thereby affecting the parasite and eliminating any changes in parasite infection that was a result of pesticide exposure. For instance, as temperatures increase, *Echinostomes* are reported to have a decrease in survivorship (Huffman and Fried 1990, Morley et al. 2003, Pietrock and Marcogliese 2003). Parasites that experience non-lethal physiological effects because of exposure to pesticides may experience increased mortality as the water temperatures fluctuated within the mesocosms. During the course of the experiment, the largest daily temperature range was 41°F-80°F. The 39°F increase in air temperature may have increased the water temperature in the pool, thus affecting the pesticide-exposed parasites. Various species of free-living trematodes are also sensitive to changes in pH (Morley et al. 2003, Pietrock and Marcogliese 2003). The pH of water used in the laboratory ranges from 6.5-7.0, whereas the water in the mesocosms ranged from 7.0-7.5. This 0-1.0 difference in pH between laboratory and mesocosm water may have affected the parasites' ability to infect the tadpoles. A total of 1.45 inches of rain accumulated in the pools over the course of the experiment. Additional water would dilute the pesticides in the pools and decrease the effects on disease caused by the pesticides.

One interesting feature observed in this experiment was the ability of most tadpoles to survive high levels of cercarial infections. These findings did not coincide with Fried et al. (1997) who found that when *R. pipiens* tadpoles (Gosner stages 24-25) were exposed to 100 cercariae at once, over 50% died within one week. When tadpoles were exposed to 250 cercariae

at once, all tadpoles died within 24 hours. In my experiment, tadpoles that were infected with over 600 cercariae were alive after two weeks. The differences in such tadpole survivorship between my experiment and Fried et al.'s experiment may be related to the differences in parasite exposure conditions. For example, Fried et al. performed pulse parasite exposures, while in my experiment tadpoles were presumably exposed to cercariae gradually over the course of two weeks. The high number of cysts within individuals that was encountered in my experiment is not uncommon in nature. For example, Skelly et al.(2006) surveyed parasite loads in tadpoles in urban and undeveloped sites, and they found that infection levels ranged from 1 to 1648 cysts per tadpole.

As tadpoles develop, their kidneys grow larger in relation to the increase in body size and mass (Schotthoefer et al. 2003). The increase in renal tissue growth may have allowed more cercariae to encyst in the tadpoles' kidneys. The average stage of tadpoles at the end of the experiment across all treatments was (31.66 ± 3.2). Schotthoefer et al. (2003) found that early stage tadpoles (stage = 25) were more likely to die after parasite exposure, because the cysts hindered osmotic regulation and excretory functions. The later stage tadpoles (stage = 27) had a lower rate of mortality, because they were larger and more tolerant of cercarial infection. In my experiment, the higher average stage of the tadpoles may have made them more tolerant of parasite infection, even in the presence of pesticide mixtures. Eventually, increased parasite load may enhance and maximize successful transmission of many *Echinostoma trivolvis* to successive hosts.

This experiment revealed that there was a positive correlation between mass and the number of cysts in the second intermediate host tadpoles. Host mortality was also observed in this experiment; and the occurrence of this event may be a negative reflection of the effects of

pesticide mixtures on tadpole populations. Such effects have broad implications for hosts within disease systems. For example, if a disease or the number of metacercarial cysts significantly increases in hosts, then hosts can become very ill or in the case of tadpoles infected with *E. trivolvis*, develop severe edema, thereby increasing the risk of amphibian mortality (Fried et al. 1997, Schotthoefer et al. 2003). Increases in the number of metacercarial cysts may also increase the abundance of parasites that are transmitted from one host to the next. Any changes in disease dynamics can potentially have negative effects on amphibian populations that are at risk of decline and extinction.

Recommendations and Future Directions

- Use younger tadpoles (stage 24 and/or 25) and record cyst abundance and mortality to determine whether there are pesticide effects on this system at different tadpole developmental stages. And, use older tadpoles (stage 27- 29) because they are less likely to succumb to high parasite infection and thus will survive to the end of the experiment.

- Use a different species of tadpole (e.g. *Rana pipiens*), that is accustomed to living in stagnant water with lower levels of dissolved oxygen.

- Put a total of 30 (instead of 20) tadpoles in each pool to obtain a larger and equal sub-sampling size when 10 tadpoles are collected from each pool after two weeks.

- I suggest conducting the mesocosm experiment a maximum of 14 days, because the tadpoles may have been stressed by changes in air temperature fluctuations (e.g. 39°F increase) or parasite infection thus making them more susceptible to mortality when exposures exceeded 14 days.

- Conduct the experiment in a shaded environment to limit the pools exposure to drastic increases in air temperature or conduct the experiment during a different season or time of year.

- Include a tadpole exposed to pesticides and not exposed to infected snails treatment. This treatment would allow me to see if parasite infection was the source of tadpole mortality observed before the end of 28 days.

- Include a treatment where tadpoles are exposed to neither pesticides nor parasites. This treatment would allow me to observe tadpole mortality in the absence of two stressors – pesticide exposure and parasite exposure.

- Give the tadpoles an alternate food source (e.g. alfalfa pellets or spinach) instead of leaf litter. Decaying leaf litter can consume much oxygen, thus depriving tadpoles of available oxygen in the aquatic environment.

- Monitor dissolved oxygen levels everyday at the field site, starting from the day pools are filled with water. Any fluctuations in dissolved oxygen may have affected the tadpoles throughout the experiment.

- Aerate the water to increase the amount of dissolved oxygen in the pools, because dissolved oxygen was very low (~ 4 mg/L) at the start of the experiment.

- Measure the pesticide levels in each pool on days 0, 7, and 14 of the test.

- Have the tissues of all collected tadpoles analyzed for the presence of atrazine and metolachlor residues and for the amounts of these pesticides in the tissue samples.

Acknowledgements

Table Legends:

Table 1: Number of *Rana sylvatica* tadpoles recovered from each pool for each treatment after 14 days. Tadpoles were exposed to infected snails shedding cercariae for 14 days. Pesticide concentrations used were: water control, 100 ppb diluted solvent, 10 ppb metolachlor + 15 ppb atrazine, and 85 ppb metolachlor + 100 ppb atrazine. page 78

Table 2: Water chemistry measurements for each treatment taken on day 7 and day 14.   page 79

Table 3: Daily weather conditions (as obtained from the National Weather Service website for Blacksburg, VA) during the course of the experiment.   page 80

Figure Legends:

Figure 1: *Echinostoma trivolvis* metacercarial cysts in the kidneys of *Rana sylvatica* tadpoles in relation to exposure to an atrazine-metolachlor mixture in outdoor mesocosms. Values are mean (± SE) number of cysts per tadpole. Tadpoles were exposed to infected snails shedding cercariae for 14 days. Pesticide concentrations used were: water control, 100 ppb diluted solvent, 10 ppb metolachlor + 15 ppb atrazine, and 85 ppb metolachlor + 100 ppb atrazine.   page 81

Figure 2: Simple linear regression correlation between mass and number of *Echinostoma trivolvis* metacercarial cysts in the kidneys of *Rana sylvatica* tadpoles for all 16 pools used in the experiment.   page 82

Table 1: Number of tadpoles recovered from each pool for each treatment after 14 days.

	Water	Diluted solvent	Low	High
Replicate 1	8	6	8	4
Replicate 2	2	8	10	4
Replicate 3	10	9	10	2
Replicate 4	1	9	4	10

Table 2: Water chemistry measurements for each treatment taken on day 7

and day 14.

Day 7	pH(Δ pH at day 14)	Hardness (Δ Hardness at day 14) (ppm CaCO$_3$)	Alkalinity (Δ Alkalinity at day 14)(ppm CaCO$_3$)	~ Temperature (^{o}F) (Δ Temperature at day 14)
Water	7.5	184	164	62
Diluted solvent	7.5	180	176	62
Low	7.9	152	160	60
High	7.0	168	186	62
Day 14				
Water	7.5 (0)	156 (-28)	184 (+20)	70 (+8)
Diluted solvent	7.5 (0)	172 (-8)	176 (0)	68 (+6)
Low	7.5 (-0.4)	152 (0)	168 (+8)	68 (+8)
High	7.5 (+0.5)	172 (-4)	164 (-22)	72 (+10)

Table 3: Daily weather conditions (as obtained from the National Weather Service website for Blacksburg, VA) during the course of the experiment.

Day	Minimum temperature (ºF)	Maximum Temperature (ºF)	Precipitation (rain in inches)	Average wind speed (mph)
1	42	71	0	2.6
2	37	74	0.05	3.6
3	48	65	0	8.6
4	41	80	0	3.2
5	54	65	0.13	1.9
6	46	69	0	5.9
7	43	50	0.48	4.3
8	44	52	0	4.6
9	46	62	0	3.1
10	49	75	0	3.2
11	41	70	0.07	6.2
12	39	59	0	3.9
13	40	69	0.02	3.3
14	39	62	0.70	4.4

Figure 1:
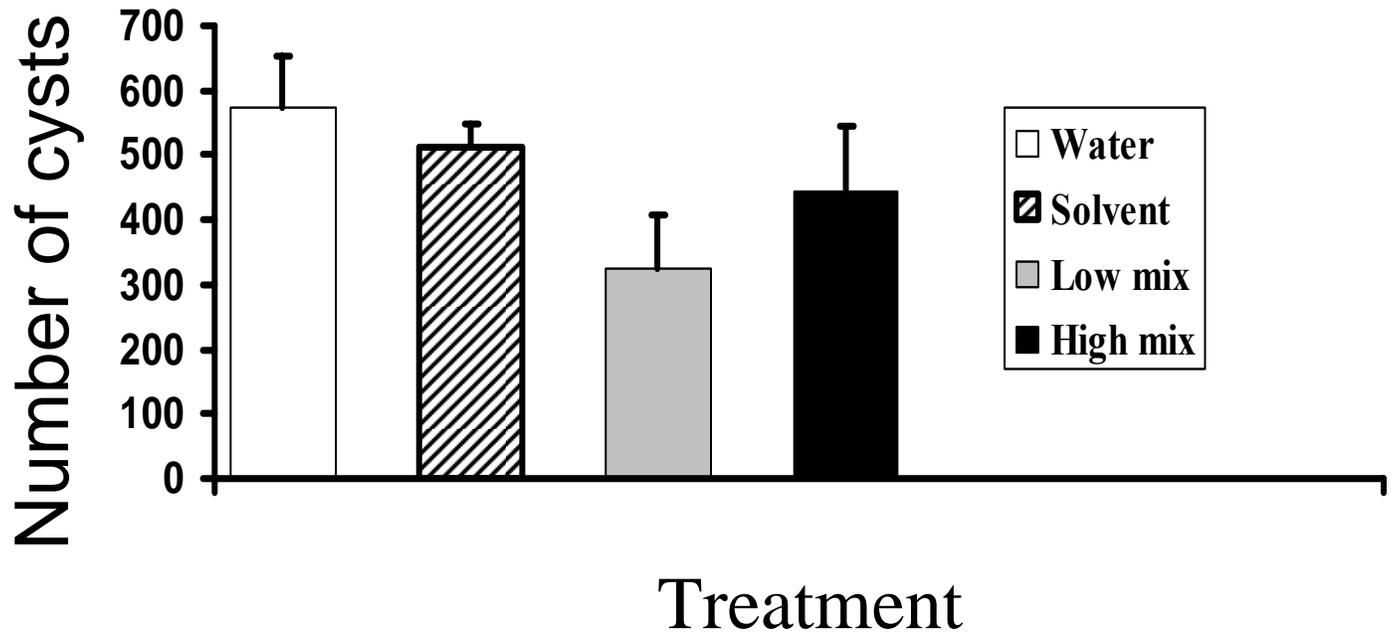
Figure 2:
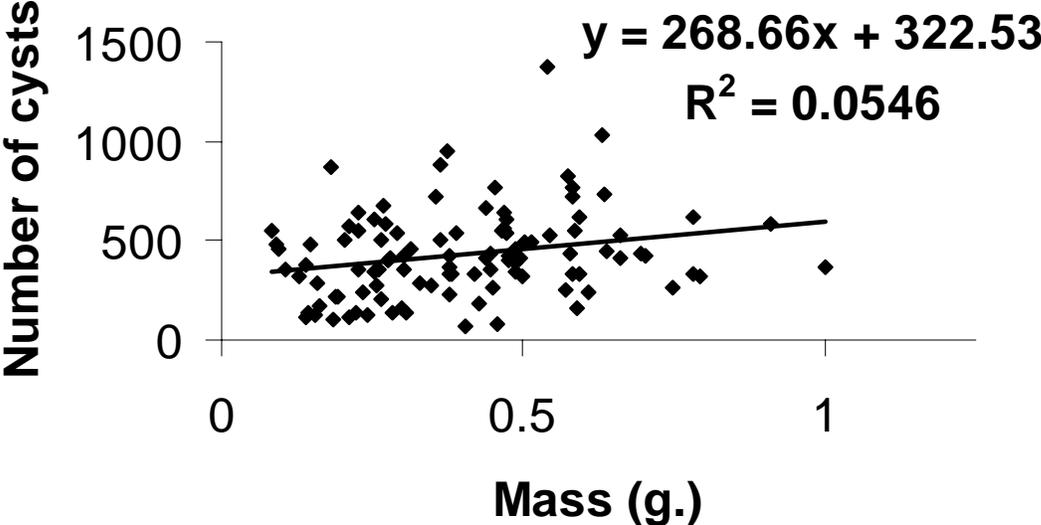
The scatter plot shows Number of cysts (y-axis, ranging 0 to 1500) versus Mass (g.) (x-axis, ranging 0 to 1). The regression equation is $y = 268.66x + 322.53$ and $R^2 = 0.0546$.

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Chapter V: General Conclusions:

My research addressed whether mixtures of two herbicides – atrazine and metolachlor– influenced host- parasite survivorship and infectivity in an aquatic disease system consisting of snails, tadpoles, and trematode parasites. Based on the results of my studies, I have concluded that the atrazine and metolachlor mixture at concentrations of 85 ppb and 100 ppb, respectively, significantly affects survivorship of the free-living cercarial stage of the parasite; however, pesticides do not affect parasite load under laboratory conditions. The result of my mesocosm study further supported this premise.

In my first experiment, I investigated the effects of 10 to 15 ppb of metolachlor and atrazine, respectively, and 85 to 100 ppb of metolachlor and atrazine, respectively, on *Echinostoma trivolvis* and their first intermediate host snails, *Planorbella trivolvis*. I found that the parasites experienced decreased survivorship at 14 hours post-exposure. The survivorship of the intermediate hosts was not affected by pesticide exposure. The results of this experiment could have important impacts on disease persistence in an aquatic environment affected by atrazine and metolachlor. Snails will persist in environments exposed to atrazine and metolachlor. As long as the snails are present, the trematodes will have a first intermediate host to infect.

In my second experiment, I examined the effects of the same concentration of pesticides on parasite load in tadpole hosts under different exposure conditions, because in the survivorship experiments, pesticides negatively affected cercariae survivorship. This experiment allowed me to address whether the declines observed in cercariae survivorship in the first experiments affected the number of cercariae that successfully encyst in second intermediate host *Rana* species tadpoles. I examined responses in two different species of tadpoles, *Rana clamitans* and

Rana sylvatica and also addressed whether the age of the cercariae affected parasite load after pesticide exposure. I ascertained that pesticide exposure had no significant effect on parasite load in either tadpole species. However, the age of the cercariae significantly influenced parasite load among all pesticide exposure treatments in that younger cercariae caused an increase in parasite load. Cercariae only have 24 hours to find a suitable host before they die (Huffman and Fried 1990), and parasite infectivity likely decreased as time progressed towards 24 hours. These results showed the pesticides have no significant effects on parasite load, even though the parasites were shown to be susceptible to pesticide-induced mortality in an earlier experiment. Trematode infection has the potential to persist in tadpoles after exposure to pesticides.

In my third experiment, I examined whether the results of the laboratory experiments are valid when the infection of tadpoles was allowed to occur naturally in outdoor mesocosms for two weeks. Parasite load was surveyed in mesocosms containing *R. sylvatica* tadpoles and snails (which were shedding *E. trivolvis* cercariae). The results of this experiment coincided with the results of the laboratory experiments. The pesticides had no significant effect on parasite load in tadpoles under natural environmental conditions.

Overall, atrazine and metolachlor appeared to have no significant effects on trematode disease transmission via altering parasite load. However, one should not assume the release of pesticides is harmless to the members of an aquatic disease system. Studies have shown individual concentrations of pesticides can elicit effects on disease systems. For example, Koprivnikar et al. (2006) established that atrazine increased trematode parasite load in tadpoles and Taylor et al. (1999) concluded that malathion increased toads' susceptibility to *Aeromonas hydrophila* infections. Pesticides still pose potential risks to amphibian disease systems that should not be ignored.

One explanation for the results of my experiments is that the combination of pesticides used might be antagonistic, with each herbicide impeding the effects of the other herbicide. The differences in sensitivity of the hosts and parasites to the pesticides could also be a factor in the lack of effects on parasite loads. Pesticide residues are often found as complex combinations in the environment. A realistic concentration of pesticide mixtures composed of three or more pesticides might elicit significant alterations in parasite loads, because all of the pesticides have the potential to interact with each other differently. These pesticide interactions can provide a greater challenge to the health and fitness of hosts and parasites thereby influencing how members of a disease system are affected. While this research addressed concerns as to how atrazine and metolachlor influenced a trematode disease system, future studies can address the effects of more complex pesticide combinations and the effects of host age and condition on disease infectivity and abundance in host-parasite systems.

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