SUB-LETHAL EFFECTS OF ENVIRONMENTALLY RELEVANT LEVELS OF AN ORGANO-PHOSPHATE INSECTICIDE ON ANURAN LARVAE

By

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By

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July, 2005
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CHAPTER I

PROLOGUE

Amphibian ecology and population declines.

Amphibians are a diverse class of vertebrates, with approximately 5800 species distributed worldwide, excluding the polar regions (AmphibiaWeb, 2005). Amphibians have persisted through more than 300 million years of evolutionary time, surviving multiple mass extinctions and drastic shifts in the topology of the earth (Stebbins and Cohen, 1995). The living members of the order Anura (frogs and toads) comprise a majority of the class with 5087 known species, followed by Caudata (salamanders) with 545 species and Gymnophiona (caecilians) with 170 species (AmphibiaWeb, 2005). However, the World Conservation Union currently lists 31% of the world’s amphibian species as threatened (1,770 out of the 5743 species evaluated) and state that this number will likely continue to increase if current circumstances creating these losses remain the same (IUCN, 2004).

Although often overlooked as critical members of the biotic community, amphibians have been reported to exceed other vertebrate members in moist woodland habitats both in terms of total numbers of individuals (Stebbins, 1954) and biomass.
(Burton and Likens, 1975). For example, one study in an aquatic habitat found the standing crop of lesser sirens (*Siren intermedia*), a type of salamander, to exceed the total standing crop of seven species of fish in that same pond (Gehlbach and Kennedy, 1978). This vital ecosystem position is particularly important as it relates to nutrient cycling and the transmission of energy upwards to other taxa, such as snakes, fish, birds and mammals (i.e., predators; Wake, 1991). The transfer of energy to higher trophic levels is particularly efficient in amphibians because, as ectotherms with low resting metabolic rates (compared to other ectotherms such as reptiles) and long periods of inactivity, they are highly efficient in biomass conversion (Pough, 1983). Furthermore, as adults, amphibians are the primary vertebrate consumers of invertebrate prey in many environments (Stebbins and Cohen, 1995). For example, a study in Iowa estimated that small pond populations of northern cricket frogs (*Acris crepitans*) could consume 4.8 million small arthropods per year (Bruce and Christiansen, 1976). Anuran tadpoles also play important roles in controlling algal growth in the aquatic environments they inhabit, particularly in small ephemeral wetland habitats, in addition to transforming tremendous amounts of aquatic plant biomass into an accessible energy source for invertebrate and vertebrate predators alike (Wassersug, 1975).

Because of their importance to communities, the loss of amphibian populations may have also indirect impacts on other members of the community (Boone and Semlitsch, 2001; Ranvestel et al., 2004; Relyea, 2005). For example, Ranvestel et al. (2004) found that when tadpoles were experimentally excluded from a neotropical stream, influences were clearly seen in sediment distribution, grazing insect abundance, diatom diversity, and algal abundance.
The First World Congress of Herpetology held in Canterbury, England, during the fall of 1989 is usually identified as the starting point of widespread recognition of amphibian population decline (Zug et al., 2001). Numerous anecdotes shared between attendees at the meeting prompted a National Research Council sponsored workshop specifically addressing the issue (Workshop for Declining Populations held in Irvine, CA, USA, February 1990; Wake, 1991). At the workshop, amphibian declines were identified as standing apart from declines in other taxa due to their apparent synchrony, their suddenness, instances occurring in areas presumed pristine, and declines occurring in some species but not in sympatrically occurring populations of other species (Wake, 1991).

Since 1989, there have been numerous hypotheses proposed and investigated as causes for the observed declines, including chemical pollution (Carey and Bryant, 1995), global warming (Crump et al., 1992; Pounds et al., 1999), habitat destruction/fragmentation (Fisher and Shaffer, 1996), introduction of non-native species (Bradford et al., 1993; Knapp et al., 2001), pathogens (Berger et al., 1998), and ultraviolet radiation (Blaustein et al., 1994). However, it is generally believed that the two major factors in most declines are habitat destruction/fragmentation and chemical pollution (Duellman and Trueb, 1994; Stebbins and Cohen, 1995; IUCN, 2004), which both occur extensively on a global scale (Zug et al., 2001).

An example in North America where chemical pollution in the form of agricultural pesticides is thought to play a significant role in population decline can be found in the Sierra Nevada Mountains of California, where declines have been documented in pristine regions (Drost and Fellers, 1996) and where habitat destruction
can be eliminated as a significant factor. An analysis of the spatial pattern of eight California declining species indicated that the presence of upwind agriculture was a significant predictor of where amphibian declines in the Sierra Nevada had occurred (Davidson et al., 2002). In a more detailed analysis of pesticide application data, Davidson (2004) found that, specifically, transport of carbamate and organophosphate (OP) pesticides was correlated with amphibian population absence in downwind areas. Additionally, studies have established that significant amounts of OPs originating from agricultural chemicals sprayed in the Great Central Valley are atmospherically transported to remote areas in the downwind mountain range (Zabik and Seiber, 1993; McConnell et al., 1998; Sparling et al., 2001). Alarmingly, agricultural chemicals have also been detected in tissues of larval and adult Pacific tree frogs, *Pseudacris regilla*; a species used as a surrogate for sympatric declining amphibians (Datta et al., 1998; Sparling et al., 2001; Angermann et al., 2002) as well as in tissues of the declining mountain yellow-legged frog, *Rana muscosa* (Fellers et al., 2004). Finally, work in this region has also indicated populations of *P. regilla* have significantly reduced activity of cholinesterase (ChE; Sparling et al., 2001), which is commonly used as a biomarker for recent exposure to these contaminants (Mayer et al., 1992; Walker et al., 2001).

*Amphibians in the agricultural landscape.*

Much of the agricultural landscape in the United States is dotted with small bodies of water, including ephemeral wetlands, small constructed wetlands, and farm retention ponds, that are utilized by many amphibians as breeding and larval habitat,
(Mazanti, 1999; Pough et al., 2001). These water bodies can be exposed to agricultural chemicals either indirectly from filling with contaminated water draining from the surrounding agricultural landscape, or directly from over-spraying of fields (Walker et al., 2001). Furthermore, the timing of many chemical applications in spring and early summer coincides with amphibian breeding periods and subsequent larval development (Harris et al., 1998; Sparling et al., 2001, Hayes et al., 2002).

The current understanding of the effects of agricultural chemicals on amphibians is cursory for several reasons. First, most currently published reports are based on tests completed in a laboratory setting with contaminant exposure for short periods of time (typically 24-96 hours) and mortality used as an endpoint (reviewed in Cowman and Mazanti, 2000). Second, these tests are usually completed on a single developmental stage (typically early stage larvae), despite differences in lethality known to occur within the same species exposed to the same concentration of a given chemical at different larval development stages (e.g., Howe et al., 1998; Richards and Kendall, 2002). Additionally, consideration of vast differences in ecology (e.g., diet, behavior, and habitat use) between adult and larval amphibians (Duellman and Trueb, 1994), which influence routes of exposure, uptake, and sensitivity, remains to be thoroughly addressed. Third, the current US EPA approach to risk assessments of amphibians is indirectly calculated by extrapolation from fish and invertebrate tests for the aquatic phases. This is despite the availability of standardized tests (ASTM, 1998, Birge et al., 2000) and results from simultaneous testing of fish and tadpoles indicating that amphibians are more sensitive to some types of contaminants (e.g., Howe et al., 1998; Birge et al., 2000). Terrestrial-phase amphibians are included in the terrestrial vertebrate definition for US EPA
assessments, but are rarely mentioned otherwise (e.g., US EPA, 1999; US EPA, 2000). Finally, and perhaps most importantly, laboratory toxicity tests have been conducted on an exceedingly limited number of anurans, such that the current toxicological literature reflects a strong bias towards the use of representatives of the genera *Rana* (especially *R. catesbeiana*, *R. clamitans*, *R. pipiens*, and *R. temporaria*), *Bufo*, and the African genus *Xenopus* (especially *X. laevis*; Cowman and Mazanti, 2000; Pauli et al., 2000), with many species tested so far indicating that sensitivity to contaminants is highly species-specific (Sanders, 1970; Bridges and Semlitsch, 2000; Cowman and Mazanti, 2000).

*Organophosphates and chlorpyrifos.*

Insecticides can be separated into four general classes based on chemical and physical properties: organochlorines; OPs; carbamates; and pyrethroids (Walker et al., 2001). OPs began to appear in agricultural markets in the 1940’s and gained in popularity when it became apparent that then widely used organochlorine pesticides were extremely persistent in the environment and subsequently bioaccumulated within non-target organisms, such as mammals and birds, with some occasional devastating population-wide effects (Laws, 1993). Generally, OPs are less persistent in the environment and do not accumulate in animal tissues, properties which were thought to make them less hazardous to wildlife and enhanced their popularity (Mayer et al. 1992). OPs act on the nervous system of target organisms by inhibiting normal activity of the neurological enzyme cholinesterase, ultimately resulting in respiratory failure and death (Walker et al., 2001). Normal nerve function involves the neurotransmitter
acetylcholine (ACh) transmitting a nerve impulse across the synapse from nerve to nerve or nerve to muscle (Walker et al., 2001). ChE catalyzes the breakdown of ACh in the synapse and thus ends local synaptic transmission of the nerve impulse (Walker et al., 2001). However, when ChE is inhibited, excess ACh builds up, which initially results in hyperactivity, then leads to uncontrolled muscular spasms, decreased activity, and eventually paralysis, resulting in respiratory failure and death (Walker et al., 2001). As broad spectrum insecticides, OPs are lethal to invertebrates, but because ChE is found in invertebrates and vertebrates alike, they can also be extremely toxic to vertebrates as well (Mayer et al., 1992).

Because the inhibition of ChE is the specific end result of exposure to OP and carbamate pesticides, measurement of this enzyme is a useful sub-organismal biomarker of exposure to these chemicals (Walker et al., 2001). Furthermore, ChE is a sensitive measure, generally responding to exposure well before signs of overt intoxication appear (Mayer et al., 1992). However, baseline information regarding normal levels of ChE must be developed for the taxa of interest (Mayer et al., 1992; Melancon, 1995). This characterization has been done and field validated for some birds, mammals and fish (Melancon, 1995), but, unfortunately, this vital knowledge is lacking for amphibians (Venturino et al., 2003). This gap of information leads to questions regarding what may be considered normal for field collected amphibians (except as compared to a reference site; Melancon, 1995; e.g., Sparling et al., 2001) and what the ecological relevance is of levels that deviate from “normal” or from a given reference (Venturino et al., 2003).

The OP chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) is most often applied on corn crops, but it is also used for controlling
pests on other crops, including wheat, alfalfa, hay, peanuts, cotton, tobacco, citrus and other fruit trees, and has been consistently one of the top two most commonly used insecticides in agriculture in the United States since 1987 (US EPA, 2004). Furthermore, average yearly household applications matched agricultural usage (~10 million lbs) until recent regulations withdrew nearly all previously approved registered domestic uses (US EPA, 2002). It is therefore not surprising that it is also one of the most frequently detected insecticides in the United States’ surface waters (USGS, 2003). The application rates for chlorpyrifos (based on current product labels) range from 0.25 lbs active ingredient (a.i.) per acre to a maximum of 8 lbs a.i. per acre with up to 10 applications per year (US EPA, 2002). Measured levels of chlorpyrifos in surface waters are often in the ng/l range (e.g., Lui et al., 2002; USGS, 2003); however, it is worth noting that water samples for these studies are not typically taken from ephemeral water bodies, but instead focus on permanent waters found in larger streams, lakes, and rivers. Ephemeral ponds and wetlands can be exposed to chlorpyrifos either directly from broadcast spraying (ground or air) of fields, or indirectly from leaching or runoff from the surrounding agricultural landscape, and may also occur by way of deposition of atmospheric chlorpyrifos (ATSDR, 1997).

Chlorpyrifos is reported to have a short half life (<1 – 13 d; Giddings et al., 1997; Moore et al., 2002; Mazanti et al., 2003), such that the presence of elevated levels of pesticide in surface waters would most likely be brief and therefore easily missed. However, chlorpyrifos has a strong tendency to sorb to sediment particles (K<sub>oc</sub> = 3680-36,000; US EPA, 2002), and as a result, expected environmental residues are strongly dependent on physical properties of the soil in the area (e.g., pH, proportions of sand, silt,
and clay) as well as other environmental properties, such as temperature, water turbidity, and amount of light exposure. Based on estimates from previous studies, the potential chlorpyrifos concentrations in runoff to ephemeral wetlands could range from 73 µg/l to over 700 µg/l (Moore et al., 2002; Mazanti et al., 2003). US EPA (2002) lists the expected environmental concentrations for a water body adjacent to a recently sprayed cornfield to be 5.5 to 8.6 µg/l; however, one field study reported levels of chlorpyrifos to be 486 µg/l in water adjacent to a recently sprayed citrus field (US EPA, 2002). Furthermore, short term, acute exposures to this chemical can have effects equivalent to those seen in continuous low level exposures (Jarvinen et al., 1988).

Even though chlorpyrifos has been heavily used in agricultural systems for more than 15 years, and native North American amphibian larvae are undoubtedly being exposed to at least small amounts as a result of this use, only a handful of studies have examined how this chemical impacts larval survival, and even fewer have examined sublethal endpoints. However, chlorpyrifos was one of the agricultural chemicals identified in the tissues of *P. regilla* adults and tadpoles in the previously mentioned study by Sparling et al. (2001) linking population declines in “pristine” areas to heavy agricultural land use upwind of the Sierra Nevada Mountain Range, indicating these studies are necessary.

*Tadpoles and contaminants.*

Tadpoles in particular may be excellent for indicating environmental stress due to contaminants. Cooke (1981) suggested this is true because tadpoles are easy to collect in
large numbers and are “fairly representative of freshwater life.” Birge et al. (1976) also recommended use of tadpoles in contaminant studies because of their availability, as well as being generally more sensitive than adults, confined to aquatic environments, and relatively easy to rear in the laboratory.

Tadpole life stage sensitivities are an important consideration for contaminant studies, as vastly different developmental and physiological processes are occurring at different stages of development. Complex physiological changes occur as the tadpole approaches metamorphosis, as the architecture of the tadpole (e.g., eye structure, skin and stomach anatomy) is transformed in preparation for the switch from an aquatic organism to a terrestrial one (Duellman and Trueb, 1994). An additional consideration of tadpole ecology in contaminant studies is species-specific larval duration time, which ranges from 8 days (in *Scaphiopus couchii*; Newman, 1992) to up to 3 years (in *Rana muscosa*; G. Fellers, pers. comm.). Contaminant exposure potentially has a greater significance for species that metamorphose quickly, because species with a longer larval period have a longer time to compensate for any detrimental effects resulting from the exposure. Alternatively, those with longer development periods may be at a greater total risk because individuals may have to face contamination over a longer period and/or for more than one episode. Sparling et al. (2001) recognized the role that species-specific time to metamorphosis may play in declining ranids in the Sierra Nevada Mountains of California, and Boone (2000) suggested that differences she found in species sensitivity might have been due to differences between species’ larval period. However, a comprehensive literature review revealed no study that directly addressed this potential determinant of differences observed in species sensitivities.
Contaminants can have a variety of sub-lethal effects on tadpoles. One endpoint often measured is growth (e.g., Berrill et al., 1993), as is time to metamorphosis (e.g., Boone, 2000) and teratogenicity (Venturino et al., 2003; Bonfanti et al., 2004). Impacts on any of these parameters can have a lasting indirect effect on adult fitness through delayed maturity (Smith, 1987; Berven, 1990), or possibly reduced clutch or egg size. However, these ecological parameters are difficult to relate to the population dynamics of frogs throughout their life cycle (Alford, 1999), even independent of the impact contaminants may pose.

\textit{ChE, behavioral endpoints, and chlorpyrifos.}

Behavior provides a link between an organism’s internal biochemistry and its external environment and allows an investigator to estimate ecological consequences of sublethal environmental contamination (Little, 1990; Rice et al., 1997). Behavioral endpoints are especially appropriate when analyzing sublethal exposures to OPs and carbamate pesticides because, as previously discussed, these chemicals have a direct impact on nervous systems (i.e., inhibition of ChE activity). Furthermore, many studies have found a positive relationship between reductions in ChE activity and impairment of ecologically relevant behavioral responses in a variety of different taxa (e.g., birds: Hart, 1993; Fryday et al., 1996; fish: Kumar and Chapman, 1998; Brewer et al., 2001; Beauvais et al., 2000; mammals: Dell’Omo et al., 1997; McDaniel and Moser, 2004).

The use of swimming activity as a behavioral endpoint is a well-established sensitive measure of sublethal contaminant exposure in fish (Little and Finger, 1990) and
has been used as a measure of toxic effect in some chlorpyrifos exposed fish larvae. For example, Rice et al. (1997) examined the effect of sublethal chlorpyrifos exposure (100 to 500 μg/l) on behavioral responses in 30 day old medaka (Oryzias lapites) and reported that these exposed juvenile fish exhibited loss of equilibrium, were lethargic and under reactive to startle stimuli, and had little or no movement of their pectoral fins. Furthermore, these alterations in behavior occurred within 24 hours, and were quicker to onset in higher concentrations, indicating a clear connection between exposure concentration and behavioral effects. Levin et al. (2003) exposed zebrafish (Danio rerio) as early embryos to 100 ng/l chlorpyrifos for 4 days and reported that when behavioral tests began 20 weeks later, exposure affected response latency (time from stimulus to onset of response) for at least six weeks and spatial discrimination up to 18 weeks later. Levin et al. (2004) also examined larval swimming activity using the same exposure scenario as Levin et al. (2003) and found that newly hatched larvae exposed to 100 ng/l chlorpyrifos had significantly impaired swimming activity at one and four days post exposure.

Carlson et al. (1998) directly measured electrophysiological responses of Mauthner cell initiated startle responses of juvenile medaka (Oryzias lapites) exposed to five concentrations of chlorpyrifos (from 30 μg/l to 270 μg/l). Mauthner cells are giant neuronal cells located within the brain with axons that extend the length of the spinal cord. These cells are known to initiate startle or ‘C-start’ responses in fish and aquatic amphibians (Eaton and Hackett, 1984; Lannoo, 1999). These authors measured the electric signals from Mauthner cells to the motoneurons, and from the motoneurons to actual muscle activity using an electrode chamber, and found that chlorpyrifos exposure
significantly increased the time a motoneuronal signal actually produced muscle activity. Furthermore, increasing chlorpyrifos exposure enhanced this delay.

In tadpoles, behavioral endpoints in contaminant studies are less common, but include swim speed (Jung and Jagoe, 1995; Britson and Threlkeld, 1998; Raimondo et al., 1998; Richards and Kendall, 2003), swim distance (Bridges, 1997), time spent feeding (Britson and Threlkeld, 1998; Broomhall, 2004), activity level (Bridges, 1997; Lefcort et al., 1998; Savage et al., 2002), and refuge use (Bridges, 1999). Impacts on endpoints such as time spent feeding indicate an alteration of ability to acquire required resources; whereas measures such as swim speed and swim distances are useful for indicating changes in predation risk (Watkins, 1996; Bridges, 1997).

Tadpoles and chlorpyrifos

Some previous work has examined the impact of chlorpyrifos exposure on amphibians. Johnson (1980) looked at temperature tolerance in *Pseudacris regilla* tadpoles exposed to chlorpyrifos for 24 h and found thermal tolerance to be significantly lowered at both concentrations tested (25 and 50 μg/l). Barron and Woodburn (1995), in their review of the ecotoxicology of chlorpyrifos, reported that, up to that time, few studies had been completed on the toxicity of chlorpyrifos to amphibians. The studies reported in Barron and Woodburn (1995) include a report from Whitney (1965) that tadpoles of the American toad (*Bufo americanus*) and the northern leopard frog (*Rana pipiens*) had LC50 (the concentration which is lethal to 50% of animals exposed) values of 1 and 3000 μg/l respectively (duration not given) and a study by Abasi and Soni
(1991) that indicate the 24 h LC50 and 144 h LC50 values for *Rana tigrina* were 177 and 10 μg/l, respectively. However, since the 1995 review several studies have examined the impact chlorpyrifos can have on amphibian embryos and larvae. Specifically, Moulton (1996) found that 7 and 14 day old Pinewoods treefrog (*Hyla femoralis*) tadpoles exposed to 265 μg/l exhibited 100% mortality within 12 hours. Mazanti (1999) looked at the effect of 100-μg/l and 1000-μg/l static exposure to chlorpyrifos on *Hyla versicolor* tadpole weight gain and made qualitative observations of feeding activity and responsiveness to prodding. Behavioral observations of tadpoles in the high dose indicated that, after three days, a third of the tadpoles failed to respond to prodding and over half were not observed feeding. These same tadpoles weighed significantly less than control animals 14 and 21 days after exposure, and froglets on average took 10 days longer to metamorphosis and weighed nearly 30% less than control animals (Mazanti, 1999). Gaizick et al. (2001) exposed *Rana pipiens* embryos to chlorpyrifos concentrations ranging from 10 to 200 ppb and found no effects on hatching time, hatching success, or occurrence of malformations. Conversely, Bonfanti et al. (2004) examined the teratogenicity of chlorpyrifos on early stage embryos (N/F stages 9-47, Nieuwkoop and Faber, 1967) of the African frog *Xenopus laevis* and found chlorpyrifos to cause abnormal tail flexures in half of the exposed animals at 161.5 μg/l.

A few studies have looked specifically at the relationship between chlorpyrifos exposure and ChE inhibition in larval amphibians. Bonfanti et al. (2004) examined the ChE inhibiting potential of chlorpyrifos on early stage embryos (N/F stages 9-47) of *X. laevis* and found that larvae exposed to 100 μg/l and 250μg/l had cholinesterase levels that were inhibited 76-80 percent from controls animals. Richards and Kendall (2002)
examined the relationship between chlorpyrifos dose and ChE activity in *X. laevis* at two development stages (N/F stage 14 and 46), and found that the chlorpyrifos concentration required to inhibit ChE activity more than 50% in the later stage was 10X lower than that required to inhibit ChE activity at the same level in the earlier development stage (10 μg/l vs 100 μg/l). These same authors then later expanded their look at chlorpyrifos effects on *X. laevis* tadpoles (Richards and Kendall, 2003) by measuring effects on tadpole body length, mass, and swimming ability. Again, considerable differences were seen in sensitivity between development stages, with effects on swimming ability observed at approximately the same concentrations that ChE was inhibited more than 50%. The authors hypothesized that development stage differences in sensitivity might be attributable to developmental differences in normal levels of cholinesterase, which have been previously shown to change throughout development in *X. laevis* (Gindi and Knowland, 1979). It is interesting to note that the LC50’s for the premetamorphs and postmetamorphs were calculated to be 14,600 μg/l and 560 μg/l, respectively, indicating that ChE activity is a greater than one order of magnitude more sensitive response than mortality in at least one amphibian species.

**Conclusions**

Amphibian population declines have been related to agricultural chemical pollution in a number of “hotspot” areas (Sparling et al., 2001; Davidson et al., 2002; Davidson, 2004), but the ecological mechanisms for these declines remain correlative. While methods exist to determine exposure of amphibians to agricultural chemicals
through the use of physiological biomarkers, the ecological relevance of changes in these parameters is largely unknown. Investigations of the potential ecological mechanisms of population decline via agricultural chemical exposure will provide greater insight towards understanding why these fascinating creatures are disappearing from pristine areas.

My research exposed native North American tadpoles to a common agricultural pesticide (chlorpyrifos), evaluated the response of a frequently used biomarker of pesticide exposure (ChE activity), and related this response to impacts on tadpole growth and swimming behavior. My first objective was to examine how, in tadpoles of the southern leopard frog (*Rana sphenocephala*), reductions of ChE activity due to sub-lethal exposure to chlorpyrifos would relate to swim speed, growth, and survival in the presence of a predator. I used tests that provided a gradient of exposure scenarios, ranging from controlled laboratory experiments with water-only exposure, to laboratory exposures that included water and pond sediment, and, finally, to semi-natural conditions that included sediment and plants in outdoor mesocosm experiments. I also conducted exposures for two different lengths of time to examine if longer duration of exposure increased effects on growth and swim speed responses. These experiments provide a greater understanding of how ChE inhibition relate to ecological endpoints in one species and demonstrate how results from laboratory experiments might be extrapolated to natural conditions.

My second objective was to examine how depressed ChE activity due to a short term chlorpyrifos exposure influenced measures of growth and swim speed in early stage tadpoles of four native Oklahoma anuran species (*Hyla chrysoscelis, Rana sphenocephala, Acris crepitans*, and *Gastrophryne olivacea*). I again examined how the
presence of pond sediment in test chambers influenced responses and examined
differences between species. Additionally, in two of these species I examined how
longer exposure (4 vs. 12 days) affected tadpole responses. These experiments provide a
greater understanding of differences in sensitivity between species.


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Chapter II:

Laboratory and Mesocosm Evaluations of Cholinesterase Activity and Behavior in Chlorpyrifos-Exposed Rana sphenocephala Tadpoles

Introduction

Small, often ephemeral, wetlands embedded throughout agricultural landscapes provide important breeding habitat for many amphibians. These systems may also receive inadvertent doses of insecticides and/or herbicides from run-off, over-spray, or atmospheric deposition (Mazanti 1999, LeNoir et al. 1999). Recent studies provide compelling correlative evidence linking agricultural chemicals with decline of some amphibian populations (Davidson et al. 2002, Sparling et al. 2001). Furthermore, pesticides that inhibit the enzyme cholinesterase (ChE) as a means of toxicity have been specifically linked with declines (Sparling et al. 2001, Davidson 2004). Understanding how these contaminants impact developing amphibian larvae through controlled laboratory and mesocosm experiments, however, will contribute vital information to understanding the underlying ecological mechanism for declines by means of chemical contamination. Therefore, in the present study we used laboratory and mesocosm
experiments to investigate the sublethal impacts of a ChE inhibiting pesticide, chlorpyrifos, on *Rana sphenocephala* tadpole growth, swim speed, and survival in the presence of a predator.

Organophosphorus (OP) compounds comprise the dominant class of insecticides currently used for both large-scale agriculture and domestic application (US EPA 2004). Within this group, chlorpyrifos is currently one of the two most commonly used in the United States, with an estimated 10 million pounds of active ingredient (a.i.) applied annually in agricultural landscapes (US EPA 2004). Additionally, chlorpyrifos has been identified in tissues of Pacific treefrog (*Pseudacris regilla*) adults and tadpoles collected in the Sierra Nevada Mountains of California (Sparling et al., 2001).

Measured levels of chlorpyrifos in United States surface waters are often in the ng/l range (e.g., USGS 2003, Lui et al. 2002), however, water samples for these studies are not typically taken from ephemeral wetlands, but instead focus on permanent waters found in larger streams, lakes, and rivers. Additionally, chlorpyrifos is reported to have a short half life (<1 – 13 d; Mazanti et al. 2003, Moore et al. 2002, Giddings et al. 1997), such that the presence of elevated levels of pesticide in surface waters would most likely be brief and therefore easily missed. Chlorpyrifos also has a strong tendency to sorb to sediment particles ($K_{oc} = 3680-36,000$; US EPA 2002), and as a result, expected environmental residues are strongly dependent on physical properties of the soil and/or sediment (e.g., pH, proportions of sand, silt, and clay) in addition to other environmental properties, such as temperature, water turbidity, and amount of light exposure. Furthermore, some tadpoles are known to consume sediments (Ranvestel et al. 2004, Flecker et al. 1999, personal obs. P. D. Widder), and because sediment is an integral part
of most natural aquatic systems, contaminated sediments could be an additional source of exposure for these animals.

OPs like chlorpyrifos act on the nervous system by inhibiting activity of the enzyme ChE, and dose-dependent reductions in ChE activity have been reported in a number of vertebrate and invertebrate species following exposure to these pesticides (vertebrates: Grue et al. 1997, and references therein; invertebrates: Fulton and Key 2001 and references therein). As such, measurement of ChE activity can be used as a biomarker to indicate exposure to this class of chemicals (Melancon 1995). For example, the previously mentioned study with *P. regilla* in the Sierra Nevada Mountains found not only significant levels of pesticide residues in these animals, but also reduced activity of ChE in tadpoles and adults living downwind of heavily farmed areas as compared to those with less upwind agriculture (Sparling et al. 2001). Furthermore, this reduced ChE activity was correlated with the declining population status of sympatric frog species in these same localities.

Behavioral studies can serve as a link between physiological biomarkers (such as ChE activity) and ecological processes (Little 1990, Mayer et al. 1992, Rice et al. 1997, Scott and Sloman 2004). Reductions in ChE activity have been associated with impacts on ecologically important behaviors such as feeding and activity in vertebrates other than anurans (e.g., [birds] Hart 1993, Fryday, et al. 1996; [fish] Kumar and Chapman 1998, Brewer et al. 2001, Beauvais et al. 2000; [mammals] Dell’Omo et al. 1997, McDaniel and Moser 2004). Additionally, an insecticide known to be a ChE inhibitor has been found to reduce tadpole activity levels and swim distances (Bridges 1997) and chlorpyrifos specifically has been shown to reduce swim speed in an African anuran.
species (*Xenopus laevis*; Richards and Kendall 2003). However, studies that directly link anuran ChE activity inhibition with quantified behavioral effects are otherwise lacking. An understanding of this relationship is important in order to determine how the effects of OP pesticides may be ecologically manifested in amphibians.

The current understanding of the effects of agricultural chemicals on amphibians is cursory for several reasons. First, the current EPA approach to risk assessment of amphibians is indirectly calculated by extrapolation from fish and invertebrate tests for the aquatic phases of their life cycle and extrapolation from birds and mammals for terrestrial phases. This is despite the availability of standardized tests (ASTM 1998, Birge et al. 2000) and some results from simultaneous testing of fish and tadpoles indicating that amphibians are more sensitive to some contaminants (e.g., Howe et al. 1998, Birge et al. 2000). Second, although research in the last few years is increasingly examining contaminant impacts on anurans in more complex exposure scenarios (e.g., Rohr and Palmer 2005, Relyea 2005, Boone and James 2003, Rowe et al. 2001), to date most currently published reports are based on tests completed in a laboratory setting with a single contaminant for short periods of time (typically 24-96 hours) with mortality typically used as an endpoint and behavioral aberrations anecdotally noted (Pauli et al. 2000, Cowman and Mazanti 2000). Finally, and perhaps most importantly, laboratory toxicity tests have been conducted on an exceedingly limited number of species, with those tested so far indicating that sensitivity to contaminants is highly species-specific (Sanders 1970, Cowman and Mazanti 2000).

The specific objective of our study was to determine if reductions in ChE activity due to exposure to environmentally-relevant levels of chlorpyrifos in *Rana*
sphenocephala tadpoles is associated with changes in mass, swim speed, or survival in the presence of an odonate predator. We sought to use a range of realistic exposure scenarios that used environmentally relevant exposure levels. Therefore, we used static renewal laboratory tests and evaluated responses using a range of exposure concentrations both with and without the presence of pond sediment in the exposure chamber. We also examined the potential effect of exposure duration by performing these tests for both 4 and 12 days, with renewals every fourth day. In a separate laboratory experiment, we examined the impact of chlorpyrifos on survival in the presence of an odonate predator. Finally, we assessed ChE, growth, and survival measures in constructed outdoor mesocosms, using two pesticide concentrations and examining changes in responses over time.

**MATERIALS AND METHODS**

*Animal collection and maintenance*

Small portions (approx. 10%) of fifteen egg masses of *R. sphenocephala* (southern leopard frog) were collected in late March 2004 from a single population located at the Oklahoma State University experimental pond facility near Lake Carl Blackwell in Payne County, Oklahoma, USA. Eggs were transported to a laboratory at Oklahoma State University in Stillwater, Oklahoma, where they were placed into individual 10-l plastic tubs and acclimated to aerated dechlorinated tap water for 24 h. Upon hatching, tadpoles were placed in 40-l plastic tubs filled with aerated dechlorinated...
tap water and fed boiled romaine lettuce *ad libitum* as well as rabbit food pellets that were first dissolved and then thoroughly rinsed with dechlorinated water. Water was replaced every 2-4 days as needed and maintained at 23 ± 2°C.

*Laboratory experimental design*

The laboratory experiment used a static renewal system with three factors (concentration, sediment, and duration) in a fully crossed randomized block design. We used five chlorpyrifos treatments (control, 1, 10, 100, and 200 μg/l chlorpyrifos formulated as Dursban) with four replicates per treatment. These were found to be sublethal to *R. sphenocephala* during preliminary tests (P.D. Widder, unpublished data). Test solutions were prepared by diluting a 100 mg a.i. chlorpyrifos/ l stock solution created at test initiation by adding 116.8 µl of commercially available Dursban TC into 500 ml of dechlorinated water. The stock solution was kept in a darkened glass container and refrigerated when not in use. Test solutions (including controls) were mixed in 10-l plastic containers and then immediately added to labeled replicate jars. Each replicate consisted of a single 950-ml wide-mouth mason jar containing 500 ml of test solution. All exposure jars were hexane and acid rinsed, followed by three sequential rinses with dechlorinated water and three rinses with reagent grade water prior to use.

To select tadpoles for use in the test, the organisms were temporarily aggregated into a single 40-l plastic tub, and individuals were chosen based on stage (Gosner stages 25-27; Gosner 1960) and absence of any injuries or abnormalities. Those tadpoles selected for use in tests were placed individually into 30-ml solo cups filled with
dechlorinated tap water. These cups were then haphazardly chosen to assign tadpoles to a concentration and replicate, with four individuals placed in each jar.

Every concentration had both a sediment and a non-sediment treatment (for a total of 40 jars and 160 tadpoles for one entire test). Sediment (sandy clay loam; 17.5% sand, 47.5% silt, 35.0% clay), taken from a dry pond adjacent to the natal pond, was homogenized, dried at 100 °C for 24 h, and 40 g added to each sediment treatment jar. Dechlorinated tap water was then added to each jar and allowed to sit for 48 h prior to use in the tests in order to allow the sediment to become fully saturated. Just prior to addition of experimental solution, the overlying water in the sediment jars was removed using a clean turkey baster. Treatment solutions were added slowly to sediment treatments and diverted away from the bottom sediment using a standard laboratory spatula as described.

Two complete tests were initiated on 26 April 2004 and allowed to run for four or 12 days at 20°C in an I-36LLVL incubator (Percival Scientific, Inc., Perry, IA, USA) equipped with plant grow lights maintained on a 14/10 light/dark cycle synchronized with true sunrise. Test chambers were arranged in alternating sediment and non-sediment rows, with concentration randomized within each row. Tadpoles were fed a piece (~16 cm²) of boiled romaine lettuce every other day and jars were cleaned of any solid waste on alternate days using small disposable pipettes. At the end of four days, one test was taken down, and swim speed measured (see behavior assessments below). Using a 5.0 megapixel C-5050 digital camera (Olympus America Inc., Melville, NY, USA), digital pictures were then taken from directly above tadpoles in 30-ml Solo cups, taking care that tadpoles were resting on the bottom of their respective cups and that the entire bottom of each cup was visible as the photo was taken. A metric ruler was included in each
photograph (placed in the center) and all tadpoles from a single jar were included in a single photograph. These photographs were then later used to determine snout-to-vent length (SVL) using SigmaScan® (Systat Software Inc., Richmond, CA, USA) software. Tadpoles were euthanized immediately after photographs were taken by emersion in liquid nitrogen.

The second test was renewed every four days until the twelfth day, when it was also terminated, swim speed assessed, and photographs taken. Renewals were 100 percent in nonsediment treatments, but were approximately 80 percent in sediment treatments due to the difficulty of removing tadpoles from exposure chambers without undue stress. Renewal treatment solutions were added as above. Water quality parameters (pH, dissolved oxygen (DO), conductivity, alkalinity and hardness) were recorded before and following renewal of the test solutions. DO was measured using a Model 50B Dissolved Oxygen Meter (YSI Incorporated, Yellow Springs, OH, USA), and pH was measured with an Accumet® portable AP62 pH/mV meter (Fisher Scientific, Pittsburgh, PA, USA). Conductivity was measured with a Hach® conductivity/TDS meter (Hach, Loveland, CO, USA), and alkalinity and hardness were measured by titration (APHA 1995).

Mesocosm experimental design

An array of 16 small polypropylene wading pools (150-l) was arranged in the bottom of an empty pond cell at the Oklahoma State University experimental pond facility, Lake Carl Blackwell, Payne County, Oklahoma, USA. Approximately two cm
of pond sediment were added to each pool, the pools were filled with water, covered with polyethylene-coated fiberglass screen lids, and allowed to settle for one week. Pond plants (*Potamogeton* sp.) were then collected from a nearby filled experimental pond and thoroughly rinsed and cleaned of predatory invertebrates. Plants were aggregated into clumps approximately 3.5 cm in diameter (at the stems) and held together with plastic cable ties. Each clump of plants, when placed in the water, covered approximately 1/8 of the total surface area of one pool. Four clumps of plants were added to each pool, for a total of 50 percent surface area coverage by plants. At the same time, two cylindrical plastic mesh containers (approximately 17 cm in height, 22 cm in diameter) were added to each pool. The first container was an open cylinder (without a top or bottom) and was used to secure a water resistant max-min recording thermometer (model # 3630; ThermoWorks, Alpine, UT, USA). The second cylinder had a secure mesh bottom, which served to keep a subset of tadpoles safe from predators. The pools were then left undisturbed for another 48 h.

The mesocosm experiment began on 26 April 2004 and used a static exposure with three factors (concentration, presence/absence of a predator, duration) examined in a fully crossed factorial design. Two concentration treatment levels were used (control and 200 μg/l chlorpyrifos) with eight replicates of each. Treatments that included a predator received two large free ranging *Anax* sp. naiads. The resulting design had four replicates of each factor combination.

Tadpoles were placed in each pool and allowed to acclimate for 20 min prior to addition of pesticide and the pesticide was added immediately prior to the addition of predators. Pesticide concentrations were added by using a watering can and gently
pouring either water (controls) or a pre-measured chlorpyrifos concentration (based on
the amount of pesticide needed to reach a 200 μg/L target in the mesocosms) uniformly
over the surface of the water, simulating a rainfall event. Tadpoles were assigned to a
pool in the same manner as the laboratory experiment and all tadpoles were supplied with
boiled romaine lettuce ad libitum every other day throughout the experiment. Each pool
received 120 tadpoles, 20 of which were placed inside the mesh container with a secure
bottom. These 20 tadpoles were used for cholinesterase (ChE) analysis as well as to
determine changes in mass over time. Four tadpoles were removed from each pool
container after 1, 2, 4, 8, and 12 d. These were immediately placed into Nalgene®
cryovials and euthanized by immersion in liquid nitrogen. The remaining 100 tadpoles
were left undisturbed for 12 d. At the end of twelve days, each pool was carefully
searched and all surviving tadpoles counted.

*Chlorpyrifos characterization*

Water samples in laboratory tests were taken initially, before and after each water
renewal, and at the conclusion of the second test. These samples were sealed in 950-ml
mason jars and stored in the dark at 4°C before being filtered and analyzed for
chlorpyrifos concentration using gas chromatography (see Gas Chromatography [GC]
Analysis section below). For samples taken from exposure jars, two replicates were
pooled into one sample to increase water sample volume to approximately 950 ml, except
for water control and 1 μg/l samples taken on day 8 and 12, where water from all four
replicates were pooled into one sample (approximately 1900 ml in volume). The two
950-ml initial water samples taken on renewal days (Day 4 and Day 8) for each concentration were also pooled.

In the mesocosm experiment, all chlorpyrifos dosed pools and two control ponds had 950-ml water samples taken initially, and then after one, two, four, eight, and 12 days to characterize initial concentrations and loss of pesticide through time.

To verify the percent a.i. in our Dursban TC source, three replicate 500-ml samples of dechlorinated water were spiked with 2.5 ml of the 100-mg a.i. chlorpyrifos/l stock solution, immediately extracted, and analyzed using the methods described below.

Behavior assessments

Tadpole behavior was assessed in two ways in laboratory tests. First, tadpole burst swim speed was measured using a 30-cm polyethylene channel, delineated at 1 cm intervals and filled to a depth of 1 cm with room temperature water (22 °C). A Sony DCR-TRV25 video camera (Sony Electronics, San Diego, CA, USA) situated directly above the center of the channel recorded all tests on MiniDV tapes at 30 frames per sec. At the end of an experiment, tadpoles were first removed from their respective jars (using one sediment row and one non-sediment row at a time) and then placed individually into labeled plastic Solo cups. Three tadpoles from each jar were sequentially placed in the channel and gently induced to swim (using a blunt probe) the length of the channel four times without rest. After each trial, the tadpole was returned to its labeled cup. Test tapes were later rerecorded on VHS tapes, with the VCR timer enumerating the frames. The VHS videotape was then reviewed and the time (to the nearest 0.03 sec) it took each
tadpole to swim two 10-cm intervals along each length of the channel was recorded (eight total measures). The fastest 10-cm interval was used in analysis. After all swim speed trials from a row were completed, tadpoles were placed into labeled Nalgene® cryovials and flash frozen by emersion in liquid nitrogen. Samples were stored in a -70 °C freezer until ChE was measured.

Behavior was also assessed in the laboratory by evaluating survival in direct predation encounters. Because ChE analysis destroys the tadpoles, a separate exposure set up and predation was measured based on the methods described by Skelly (1994). Groups of twelve tadpoles were exposed to three concentrations of chlorpyrifos (control, 10, 100 μg/l chlorpyrifos) for 24 or 48 h, with each concentration treatment and duration combination replicated five times. While shorter duration of exposure does not allow a direct comparison of ChE activity with survival, use of 96-hour ChE activity is a conservative estimate of ChE inhibition because chlorpyrifos is known to cause a peak in ChE inhibition within 24 hours, with slight recovery over time (Straus and Chambers 1995).

Group exposure chambers were 4-l glass fish bowls (hexane and acid rinsed as above) filled with 2 l of treatment solution. Each tadpole was assigned to a treatment and replicate as outlined above. Tadpoles were not fed and water temperature was maintained at 22 ± 2°C. At the conclusion of each 24- and 48-h exposure, ten of the twelve tadpoles from each replicate were gently removed from their exposure chambers using a small fish net and placed into predation arenas, where they were allowed to acclimate for 30 min. Predation arenas were 40-l plastic polypropylene rectangular boxes (approximately 50-cm length by 34-cm width), filled with 10 l of aerated dechlorinated
tap water (depth 5 cm) maintained at 23 ± 1°C. In each arena, four lengths (each approximately 35 cm) of plastic terrarium plants (“Malaysian Fern” and/or “Australian Maple,” Zoo Med Laboratories, Inc., Obispo, CA, USA) were arranged approximately in a square to provide naturalistic structural complexity. At the conclusion of the acclimation time, a single unexposed *Anax* sp. naiad (total length mean ± 1 SE: 40.6 mm ± 1.2, n=13) was released in the center of the predation arena. Each naiad had been previously fed *R. sphenocephala* tadpoles, but food was withheld for 48 h prior to tests to equalize odonate hunger levels. Each predation arena was visually isolated from the others and a blind was placed around the entire setup to prevent observer movements from disturbing tadpoles or odonates. After 2 h, all naiads were removed from arenas and remaining tadpoles counted. Survival was calculated as the proportion of individuals surviving.

Finally, we examined how exposed predators impacted exposed tadpole survival. We exposed 12 well-fed *Anax* sp. naiads in individual 600-ml glass beakers to 300 ml of a 1-μg/l chlorpyrifos solution for 24 h. Simultaneously, we exposed groups of 12 tadpoles to a 10-μg/l chlorpyrifos solution for 24 h in 4-l glass fish bowls as described above. Tadpoles and naiads were then placed in an uncontaminated predation arena constructed as above. Due to large predator mortality (see results section below), only three replicates of this experiment were possible.

*Mass and cholinesterase analysis*
Analysis of ChE generally followed procedures outlined by Ellman et al. (1961), with modifications for multiwell-plate readers. Briefly, each frozen tadpole was carefully removed from its cryovial, weighed to the nearest 0.1 mg, placed in a 2-ml Eppendorf tube containing 1 ml of chilled phosphate buffer (pH 8) and subsequently placed on ice. Whole tadpoles were then homogenized (still held on ice) for a minimum of 30 sec (longer for larger tadpoles) using a PowerGen 125 Homogenizer (Fisher Scientific International Inc., Hampton, NH, USA) equipped with a 5 mm X 95 mm generator. Samples were centrifuged at 14,000 rpm for 5 min with a Jouan CR3i refrigerated centrifuge (Jouan Inc., Winchester, VA, USA) maintained at -2°C. A 40-μl sample of the resulting supernatant was then added to three replicate wells of a 96-well plate (also held on ice) and a 300-μl aliquot of reagent solution [500 μl buffered Ellman's reagent (DNTB), 100 μl acetylthiocholine iodide and 15 ml phosphate buffer (pH 8)] was added to each well. The bottom of the well plate was carefully wiped dry and the plate was read on a SpectraMax® 190 Microplate Spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at 11-s intervals for 5 minutes. Data reduction was accomplished with a Softmax® PRO computer program (Molecular Devices Corporation, Sunnyvale, CA, USA), and ChE activity was expressed as μmol/min/g, using tadpole whole body mass corrected for the influence of tadpole body size on the final homogenate volume.

Gas chromatography analysis

Chlorpyrifos analysis from water samples followed the protocol outlined by Lui et al. (2002). Briefly, water samples were first filtered through a Whatman GF/F filter (0.7-
μm pore size) and then extracted using solid phase extraction cartridges (IST Isolute, ENV+ sorbent, 500-mg sorbent mass, 6-ml reservoir volume; Chromatographic Specialties Inc., Brockville, ON, CAN) attached to a Novagen® vacuum manifold (Merck Biosciences, Darmstadt, GER) set at 20-mmHg negative pressure. Prior to extraction, cartridges were preconditioned with 6 ml of dichloromethane, 6 ml of acetone, and 6 ml of deionized water. Following extraction, the cartridges were wrapped in aluminum foil and stored at -15°C until elution. For elutions, cartridges were first dried using the vacuum manifold (12 mmHg). The vacuum was then turned off and the captured chlorpyrifos on the cartridge medium was removed by sequential, gravity-fed elutions with 6 ml of dichloromethane and 9 ml of 3:1 acetone/acetonitrile. The resulting solution was analyzed on a Varian CP-3800 Gas Chromatograph (Varian Inc. Palo Alto, CA, USA) equipped with a CP-8400 auto-sampler either directly or following an appropriate dilution with hexane. GC oven conditions were as outlined in Lui et al. (2002).

Statistical analyses

All data analyses were conducted using the MIXED procedure in SAS/STAT software (SAS 2001). Prior to analysis, all ChE, swim speed, SVL, and mass data were first log transformed to obtain a normal distribution. Log transformed swim speed data was also regressed against log mass and log SVL to determine if speed scaled to body size. Deviations from the assumption of homogeneity of variance were checked across treatments according to model selection techniques based on fit statistics.
Separate three factor (concentration, sediment, duration) ANOVAs were performed for each of the laboratory response variables ChE, mass, SVL, and swim speed with each row included as a block. Tadpole survival values for unexposed predator laboratory tests were arcsine transformed and analyzed using a two factor ANOVA with concentration and duration of exposure as the two factors. Exposed predator survival tests were not analyzed.

Separate three factor (concentration, predator presence/absence, duration) repeated measures ANOVAs were performed for the two mesocosm response variables, ChE and mass. Tadpole survival values for mesocosm tests were arcsine transformed and analyzed using a two factor ANOVA with concentration and presence/absence of a predator as the factors.

All post-hoc comparisons were conducted using a Tukey adjustment and individual mean comparisons developed a priori were conducted using the CONTRAST statement (SAS 2001). All statistical comparisons were conducted at $\alpha = 0.05$.

**RESULTS**

*Chlorpyrifos characterization*

Analysis of our Dursban commercial product source indicated that the amount of a.i. was 47.4%. Analyses of the initial water samples taken at Day 0 and Day 4 in laboratory experiments demonstrate that measured chlorpyrifos levels were generally slightly less than nominal concentrations (Table 2.1). Analysis of water samples taken immediately prior to water renewals illustrated that there was near complete loss of...
chlorpyrifos over the course of each four-day period (Table 2.1). However, sample contamination as a source of the observed nonzero levels in controls and the high level of variation could not be eliminated. Furthermore, initial concentrations for Day 8 were undoubtedly subject to significant levels of degradation prior to analysis (Table 2.1).

Several initial and Day 1 water samples from mesocosm experiments were accidentally destroyed (including all the control samples for Day 0), however the remaining samples indicated that initial concentrations in dosed ponds were close to nominal and these concentrations then dropped drastically over the course of the first 24 hours (Table 2.2). Chlorpyrifos levels then continued to decrease over the course of 4 days and leveled off near 6.5 μg/l. Again, cross contamination after water sample collection could not be eliminated a potential source for chlorpyrifos in control ponds, however, the consistency of the levels analyzed across days 2-12 lead us to conclude that contamination was more likely a result of movement of chlorpyrifos between ponds via volatilization.

Laboratory experiments

In laboratory tests, pH ranged from 7.4 to 7.6, DO ranged from 8.56 to 5.85 mg/l, conductivity ranged from 510.7 to 575.3 μS/cm, alkalinity ranged from 44 to 52 mg/l (as CaCo₃), and hardness ranged from 96 to 108 (as CaCo₃).

Throughout all experiments no pesticide induced tadpole mortality occurred. However, R. sphenocephala tadpole exposure to chlorpyrifos at concentrations of 100 and 200 μg/l resulted in significant inhibition of ChE activity (p < 0.0001) across both
duration and sediment treatments (Fig. 2.1). The greatest average jar enzyme inhibition level was a 43% reduction from control treatments and occurred in the Day 12 nonsediment treatment. Pairwise comparisons of concentrations revealed that while enzyme activity levels in tadpoles in the two highest concentrations were significantly lower than all others ($p < 0.0001$ for all), they were not significantly different from each other.

There was also an overall concentration effect on tadpole mass ($p = 0.0012$; Fig. 2.2) and pairwise comparisons across both days and sediment/nonsediment treatments found that tadpoles in 100- and 200-$\mu$g/l treatments had significantly lower mass than did the controls ($p<0.016$ for both). Aside from these treatment effects, all tadpoles exhibited a similar increase in mass during the course of the study, such that Day 12 individuals had significantly greater mass than Day 4 individuals ($p<0.0001$).

Exposure to chlorpyrifos had no significant effect on tadpole swim speed (Table 2.3) or survival (Table 2.4) in the presence of predators not exposed to the pesticide. A significant effect of day was noted in size-corrected swim speed, with Day 12 tadpoles swimming faster than Day 4 tadpoles regardless of pesticide treatment ($p = 0.013$). Individual swim speeds ranged from 7.7 to 37.5 cm/sec, while survival across both duration and concentration treatments ranged from 70 to 100 percent. Tests with exposed predators were not analyzed because just three of the twelve odonate naiads survived the 24-h exposure to 1-$\mu$g/l chlorpyrifos.

Mesocosm experiment
In the mesocosm experiments, exposure to 200-μg/l chlorpyrifos resulted in significant reductions in ChE activity ($p < 0.0001$) of *R. sphenocephala* tadpoles over the first four days of the study (Fig 2.3). On average, a 48% reduction in enzyme activity was observed in exposed tadpoles. However, we did not dilute tadpole ChE samples and tadpoles in the mesocosm tests grew considerably over the course of 12 days, resulting in kinetic swamping of the spectrometer in the day 8 and day 12 samples. Therefore, we do not report them here.

Tadpole mass was significantly influenced by concentration ($p = 0.003$), day ($p < 0.0001$) and presence/absence of a predator ($p = 0.014$; Fig. 2.4). Tadpole mass increased over each day sampled and this increase was generally enhanced by the presence of a predator, but reduced significantly by pesticide exposure. Individual tadpole mass across all treatment combinations ranged from 41 to 127 mg on Day 1, 37 to 241 mg on Day 2, 32 to 440 mg on Day 4, 45 to 713 mg on Day 8, and 115 to 696 mg on Day 12.

Exposure to chlorpyrifos had no significant effect on survival of tadpoles in mesocosms (Table 2.5). However, exposure to the pesticide did result in near complete mortality of the odonate predators. As a result, the only tadpole group that exhibited significantly reduced survival was that from the control mesocosms that included a predator ($p < 0.0001$). The percent survival for this group was 36 as compared to greater than 88 percent for the other treatments.

**DISCUSSION**

*Environmental concentrations and comparative sensitivity to chlorpyrifos*
Based on estimates of previous studies, the chlorpyrifos concentrations in runoff to ephemeral wetlands in which amphibians might occur could range from 73 μg/l to over 700 μg/l (Mazanti et al. 2003, Moore et al. 2002). US EPA (2002) lists the expected environmental concentrations for a water body adjacent to a recently sprayed cornfield to be 5.5 to 8.6 μg/l; however, one field study reported levels of chlorpyrifos to be 486 μg/l in water adjacent to a recently sprayed citrus field (US EPA 2002). As such, the nominal concentrations used in our work (1 μg/l to 200 μg/l) are well within this range of expected environmental values.

Previous studies of chlorpyrifos have found a range of lethalities among aquatic animals. Work completed on fish (an appropriate comparison group to amphibians because they share a common trait of being ectothermic) found bluegill sunfish (*Lepomis macrochirus*), channel catfish (*Ictalurus macrochirus*), and catfish (*Clarius lazera*) to have LC50 (the concentration which is lethal to half of animals exposed) values of 1.1-4.2 μg/l, 280 μg/l, and 5350 μg/l, respectively (reviewed in Barron and Woodburn 1995) in comparable static, 4 day tests. Fathead minnows (*Pimephales promelas*) and mosquitofish (*Gambusia yucatana*), both common toxicity test species, are reported to have LC50 values of 170 and 11 μg/l, respectively, in 4 day static toxicity tests (Jarvinen and Tanner 1982, Rendon-von Osten et al. 2005).

However, the non-target animals most sensitive to chlorpyrifos are usually aquatic crustaceans and insect larvae (Barron and Woodburn 1995). For example, nymphs of the mayfly, *Cloen dipterum*, were found to have a 96-h LC50 of 0.3 μg/l (van Wijngaarden et al. 1993, as referenced in Barron and Woodburn 1995) and *Daphnia magna* is reported in
(US EPA 2002) to have a 96-h LC50 of 0.1 μg/l and a reproductive no observed adverse effect concentration (NOAEC) of 0.04 μg/l.

The limited research examining the toxicity of chlorpyrifos to amphibians indicates thus far that sensitivity is highly species-specific. The LC50 values reported for chlorpyrifos range from 1 μg/l in the American toad (Bufo americanus) to 3000 μg/l in the northern leopard frog (Rana pipiens; Whitney 1965, as referenced in Barron and Woodburn 1995). Moulton (1996) found that 7- and 14-day old Pinewoods treefrog (Hyla femoralis) tadpoles exposed to 265 μg/l exhibited 100% mortality within 12 hours. Gaizick et al. (2001) exposed Rana pipiens early stage embryos (with jelly coat) to chlorpyrifos concentrations ranging from 10 to 200 ppb for four days without renewal and found no effects on hatching time, hatching success, or occurrence of malformations.

The R. sphenocephala tadpoles at Gosner stage 25-27 (Gosner 1960) used in this study were moderately tolerant to chlorpyrifos. Concentrations at or above 500 μg/l elicited high mortality in 48-h preliminary tests (P.D. Widder, unpublished data), however, concentrations of 200 μg/l elicited none, indicating that the 96-h LC50 value for these tadpoles is between these two concentrations. This demonstrates a sensitivity within the same order of magnitude as freshwater fish, but considerably less than the aquatic arthropods mentioned. Further, it is interesting to note that the drastic difference in mortality between 200 and 500 μg/l indicates an abrupt onset of toxic effects in this species.

Toxicity of chlorpyrifos is assumed to be the result of inhibition of the specific cholinesterase form acetylcholinesterase (AChE) by the metabolized product of the pesticide, chlorpyrifos oxon (Barron and Woodburn 1995). The large differences in
sensitivity observed between organisms could therefore be a result of inherent differences in cholinesterase forms (AChE versus butyryl-cholinesterase; BChE) or differences in activation mechanisms (e.g., inherent differences in P450 cytochromes). In the present study, we quantified total cholinesterase activity; however, a preliminary screen for BChE in *R. sphenoecephala* indicated exceedingly small amounts of the enzyme were detectable (P.D. Widder, unpublished data), suggesting the majority of that analyzed was AChE. Variation in toxicity between animals may also be the result of procedural differences, such as differences in exposure temperatures, use of technical grade versus commercial formulations (which may include additional stressors). Clearly, more work is needed to fully characterize the drivers of inherent differences in sensitivity between species and within animal groups. Whatever the mechanism, the considerably greater sensitivity to chlorpyrifos of aquatic arthropods versus anuran tadpoles combined with environmentally expected concentrations supports the suggestion by Saura-Mas et al. (2002) that negative indirect effects on food webs may be the primary mechanism by which natural amphibian communities are affected by insecticides.

**Mass**

Previous work with chlorpyrifos in fish and amphibians has examined sub-lethal effects on larval animal mass after short-term exposure. Jarvinen et al. (1988) found a reduction in mass of fathead minnow larvae (*Pimephales promelas*) after 5 hours of exposure to 155 μg/l. Richards and Kendall (2002) found a significant impact of static 4-
day chlorpyrifos exposure on mass at 100 μg/l for X. laevis at Neiuwkoop and Faber (1967; N/F) stages 14 and 46.

A decrease in tadpole growth may have lasting impacts on fitness components up to and after metamorphosis. Specifically, large size at metamorphosis has been correlated with increased survival to maturity, greater size at first reproduction, and greater fecundity (Berven and Gill 1983, Smith 1987, Semlitsch et al. 1988). In the laboratory, we found that tadpoles in higher concentrations weighed less. However, tadpole growth was not significantly affected because between 4- and 12-day treatments mass increased similarly across all treatments. The difference in mass between concentrations is therefore probably best explained by tadpoles in higher concentrations having less food within their gut coils (i.e., intestinal tract). Qualitative observations over the course of this experiment suggest that this was the case (P.D. Widder, unpublished data) and Mazanti (1999) recorded similar changes in feeding behavior in chlorpyrifos-exposed Hyla versicolor tadpoles.

We also found concentration to significantly impact tadpole mass in our mesocosm experiment; however, the effect on mass we found in the outdoor experiment was larger in magnitude than the laboratory experiment conducted over the same time interval. It is likely that the differences in temperature between laboratory and mesocosm conditions contributed to the differences seen in tadpole mass; however, differences in tadpole density and/or availability of food resources may also have been factors. Differences between animal condition in previous laboratory and field experiments with tadpoles have found differences between the two scenarios (e.g., Saura-Mas et al. 2002), and this discrepancy was therefore not unexpected. The impact of predators on tadpole
mass was also anticipated given that previous ecological investigations have found the presence of predators to increase tadpole mass and shorten the time to metamorphosis (e.g., Bridges 2002).

Cholinesterase

Studies examining the dose-dependent biochemical impact of chlorpyrifos on cholinesterase are considerably less common than standard toxicity tests with this chemical. However, some comparable studies with larval fishes have been conducted. Jarvinen et al. (1988) found that chlorpyrifos concentrations of 0.27 \( \mu \text{g/l} \) significantly inhibited brain ChE activity 10-15% in continuously exposed 60-day old fathead minnows (\( P. \text{promelas} \)), while enzyme levels in fish from 0.75 \( \mu \text{g/l} \) and 2.68 \( \mu \text{g/l} \) treatments were inhibited by 50-60% and 75-85% from control levels, respectively. van der Wel and Welling (1989) examined the sublethal effects of exposure to 3.25 and 1.13 \( \mu \text{g/l} \) chlorpyrifos in 17-18 week old guppies (\( P. \text{reticulata} \)) set up in a flow-through continuous exposure system and found that the higher dose significantly inhibited ChE after 24 h, while the lower dose had significantly inhibited ChE after 4 days (level of inhibition not reported). Rendon-von Osten et al. (2005) reported 50 and 80 percent reductions of ChE in tissues of the brain and muscle, respectively, for mosquitofish exposed to chlorpyrifos concentrations of 50 \( \mu \text{g/l} \) or higher in four-day static tests.

A few studies have looked specifically at the relationship between chlorpyrifos exposure and ChE inhibition in larval amphibians. Bonfanti et al. (2004) examined the ChE inhibiting potential of chlorpyrifos on early stage embryos (N/F stages 9-47) of \( X. \)
laevis and found that larvae continuously exposed to 100 μg/l and 250μg/l chlorpyrifos for 5 days had cholinesterase levels that were inhibited 76-80 percent from control animals. The previously mentioned Richards and Kendall (2003) study on X. laevis in two development stages also found ChE was significantly inhibited (defined as >50 percent of control values) at the 100-μg/l and 10-μg/l treatments for early and late developmental stages, respectively.

In our laboratory experiment, we found tadpole ChE activity decreased in a dose-dependent manner, and with increasing duration of exposure, both as we predicted at the outset. Exposure to 200 μg/l significantly inhibited ChE compared to controls, but this reduction was never more than 43% and occurred in the 12-day, nonsediment treatment. Similar work we have completed with other species (see Chapter III), as well as other comparative work with ChE inhibiting pesticides and anuran tadpoles (Bridges and Semlitsch 2000), lead us to conclude that early stage tadpoles of R. sphenocephala are not particularly sensitive to ChE inhibiting pesticides.

In the mesocosm experiment, ChE activity was significantly impacted by chlorpyrifos exposure across both predator treatments for the first four days. Interestingly, the absolute range of ChE activity values for tadpoles in the mesocosm experiment were larger than the range seen in the laboratory experiment (~1.5X). This may have been because laboratory tests included behavioral tests that involved considerable handling of the tadpoles prior to euthanization, whereas tadpole samples from mesocosms were immediately euthanized. It would be important to investigate if handling time influences ChE activity in amphibians, especially for contexts in which animals are collected from remote locations and immediate euthanization is not possible.
Alternatively, this difference could be due to unavoidable experimental changes in tadpole density. However, if differences in percent inhibition from control treatments are evaluated across directly comparable treatments (i.e., 4-day laboratory sediment treatment at 200 μg/l to the 4-day dosed, non-predator mesocosm ChE responses) we find very similar impacts on this response (both approximately 30% inhibition compared to controls). This finding underscores the importance of obtaining control or reference ChE activity, and illustrates how conclusions based on absolute ChE values obtained in the absence of a control/reference could be misinterpreted.

In spite of the observation that *R. sphenocephala* tadpoles have a 96-h LC50 that is similar to some larval fish, it appears that the concentration at which 50% ChE inhibition occurs is considerably greater than reported for larval fishes. This rather large difference may be related to differences in relative ChE levels during respective development periods. Previous work has found tadpole developmental stage to impact ChE activity in a non-linear manner (Sparling et al. 2001, Richards and Kendall 2002), and in this study we strove to examine concentration effect on ChE within similar tadpole stages. However, ChE has been shown to change throughout development in *X. laevis* (Gindi and Knowland 1979) and some investigations have found later stage tadpole ChE to be more sensitive to ChE inhibitors (Richards and Kendall 2002), supporting the idea that sensitivity to ChE inhibitors may be related to developmental differences in enzyme activity. Similar work characterizing ChE changes through development in fishes might facilitate a clearer understanding of these taxonomic differences in sensitivity. Conversely, differences between these two ecologically similar taxa may be related to inherent differences in the development of detoxification mechanisms between the two
groups. Concurrent with this idea, the liver of *X. laevis* tadpoles develops at N/F stage 35-36 (Viertel and Richter 1999), which is the exact stage at which Bonfanti et al. (2004) observed an abrupt change in the behavior of tadpoles exposed to chlorpyrifos and another ChE inhibiting OP, malathion.

Overall, we found the tadpole mass response to be less sensitive to chlorpyrifos exposure than ChE activity. This result generally agrees with previous work with chlorpyrifos and tadpoles (Richards and Kendall 2002). Furthermore, this difference in response sensitivity (with ChE being more sensitive than mass) has been found in similar investigations with other ChE inhibitors (e.g., Kumar and Chapman 1998, Callaghan et al. 2001), and may simply reflect the progression of effect that occurs across different levels of biological organization (Heath 1995).

**Behavior**

The impacts of chlorpyrifos on alterations in behavior have been relatively well established in juvenile aquatic vertebrates. Rice et al. (1997) examined the effect of sublethal chlorpyrifos exposure (100 to 500 μg/l) on behavioral responses in 30-day old medaka (*Oryzias lapites*) and reported that exposed juvenile fish exhibited loss of equilibrium, were lethargic and under reactive to startle stimuli, and had little or no movement of their pectoral fins. Furthermore, these alterations in behavior occurred within 24 hours, and were quicker to onset in higher concentrations, indicating a clear connection between exposure concentration and behavioral effects. Levin et al. (2003) exposed zebrafish (*Danio rerio*) as early embryos to 100-ng/l chlorpyrifos for 4 days and
report that when behavioral tests began 20 weeks later, exposure affected response latency (time from stimulus to onset of response) for at least six weeks and spatial discrimination up to 18 weeks later. Levin et al. (2004) also examined larval swimming activity using the same exposure scenario as Levin et al. (2003) and found that newly hatched larvae exposed to 100-ng/l chlorpyrifos had significantly impaired swimming activity at one and four days post exposure. One study with amphibians found significant changes in *X. laevis* swimming ability at 1 μg/l in two developmental stages immediately after a 96-h exposure; however, these effects were not evident three days later (Richards and Kendall 2003).

Carlson et al. (1998) directly measured electrophysiological responses of Mauthner cell (large neuronal cells in the brain that are associated with ‘C-start’ responses in fish and aquatic amphibians, Eaton and Hackett 1984, Lannoo 1999) initiated startle responses of juvenile medaka exposed to five concentrations of chlorpyrifos (from 30 μg/l to 270 μg/l). The authors found that chlorpyrifos exposure significantly increased the time it took for a motoneuronal signal to produce muscle activity.

Studies that examine the concurrent impact of chlorpyrifos on behavior and ChE in aquatic vertebrates are rare. Investigations across taxa generally indicate that a greater than 50 percent inhibition of ChE is associated with the onset of effects at the level of the whole organism. For example, Grue et al. (1997) reviewed studies of ChE inhibition in birds and mammals and concluded that reductions in body temperatures are frequently associated with decreases of greater than 50 percent in brain ChE activity. Beauvais et al. (2000) reported a correlation between reductions in brain ChE activity and swim speed in
larval rainbow trout (*Oncorhynchus mykiss*) acutely exposed to carbaryl, where maximum enzyme inhibition levels were approximately 50 percent.

We found no effect of chlorpyrifos concentration on *Rana sphenocephala* tadpole swim speed or survival in the presence of a predator. This lack of an effect could be due to an insufficient level of ChE inhibition, which never exceeded 50 percent in our tests. Additionally, other studies have found the correspondence between ChE and behavioral effects to occur only within the first 24-h of exposure to OP pesticides, despite continued inhibition of ChE (Grue et al. 1997, Richards and Kendall 2003). Potentially, if we had examined swim speed within this exposure timeframe, we may have seen detrimental impacts on tadpole swimming ability.

The absence of an impact on tadpole survival in the presence of a hungry, unexposed odonate predator is intriguing, but concurrent with the absence of a concentration effect on tadpole swim speed. More importantly, the extreme sensitivity of the odonate predators (with an 48-h LC50 of less than 1 µg/l) found in the current study implies that neither of our behavioral parameters is likely to be important in ephemeral wetlands where tadpoles and odonate predators are most likely to be exposed simultaneously to similar levels of a contaminant. Simply stated, invertebrate odonate predators would be eliminated at pesticide levels far less than would be expected to affect tadpole escape behavior. This difference in sensitivity underscores the importance of simultaneously exposing predator and prey in future investigations. However, considering our predators in a general sense (i.e., solely as tadpole consumers), the short timeframe used in assessing survival in our study may not have been sufficient to determine exposure-related differences. Overall, predators were able to catch 1-3
tadpoles over the course of the allotted 2 hours, regardless of tadpole chlorpyrifos
treatment. Potentially, leaving predators in predation arenas overnight (i.e., 12 hours) or
using another ecologically relevant predator (e.g., snakes [esp. *Thamnophis* spp., *Nerodia*
spp.] fish, or turtles; Duellman and Trueb 1994) would enhance ability to detect exposure
effects.

**Conclusions**

Sparling et al. (2001) investigated levels of ChE inhibition in *Pseudacris regilla*
tadpoles throughout the Sierra Nevada Mountain range and found a relationship between
inhibition of ChE and population status of sympatric declining frog species. The authors
hypothesized that the observed relationship could be due to uncoordinated swimming
and/or increased vulnerability to predators. In investigating these hypotheses we found
that, while ChE activity is reduced in a dose-dependent fashion by OPs in *R.*
*sphenocephala* tadpoles, these reductions were not associated with an impairment of the
behavioral endpoints we investigated. ChE activity may therefore be a more appropriate
biomarker of exposure in anuran tadpoles rather than an indicator of impaired ability to
avoid an odonate predator; however, more research on response differences between
species must be completed. Nevertheless, our data indicate that, due to the sensitivity of
invertebrate predators to OPs, effects on tadpole invertebrate predator avoidance is
probably not one of the factors driving the observed relationship between ChE inhibition
and amphibian population declines.
ACKNOWLEDGEMENTS

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Table 2.1. Gas chromatograph analysis results of chlorpyrifos concentrations used in *Rana sphenoecephala* laboratory tests.

Values are average μg/l ± 1 SE and values without SE are from a single sample.

<table>
<thead>
<tr>
<th>Chlorpyrifos Concentration</th>
<th>Day</th>
<th>Initial</th>
<th>Non</th>
<th>Sed</th>
<th>Initial</th>
<th>Non</th>
<th>Sed</th>
<th>Non</th>
<th>Sed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>2.7±2.7</td>
<td>2.1±2.0</td>
<td>0.03</td>
<td>—</td>
<td>0.01</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.2±1.2</td>
<td>0.5</td>
<td>0.03±0.01</td>
<td>1.7±1.6</td>
<td>6.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 µg/l</td>
<td>8</td>
<td>9.3±3.3</td>
<td>9.0</td>
<td>0.05±0.01</td>
<td>2.8±2.7</td>
<td>6.8</td>
<td>0</td>
<td>0</td>
<td>0.01±0.01</td>
</tr>
<tr>
<td>10 µg/l</td>
<td>12</td>
<td>74.3±1.0</td>
<td>73.3</td>
<td>1.7±0.1</td>
<td>0.5±0.1</td>
<td>23.1</td>
<td>0.5±0.1</td>
<td>0.2±0.01</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>100 µg/l</td>
<td></td>
<td>132.4±7.3</td>
<td>113.9</td>
<td>3.5±0.5</td>
<td>1.9±0.2</td>
<td>30.1</td>
<td>1.0±0.7</td>
<td>0.3±0.1</td>
<td>0.8±0.5</td>
</tr>
</tbody>
</table>
Table 2.2. Measured chlorpyrifos amounts in mesocosm water samples by day samples were taken. Values are average μg/l ± 1 SE.

<table>
<thead>
<tr>
<th></th>
<th>Day of Water Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial 1 2 4 8 12</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>* 4.25±2.8 3.94±2.4 3.19±0.2 2.65±1.8 1.76±0.9</td>
</tr>
<tr>
<td>200 μg/l</td>
<td>299.17±24.5 43.39±3.8 23.05±2.9 6.12±0.8 6.57±0.5 10.72±2.0</td>
</tr>
</tbody>
</table>
Table 2.3. Maximal swim speeds (cm/sec) of *Rana sphenoecephala* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in 4 and 12 day laboratory experiments with and without pond sediment in test chambers. Values represent means ± 1 SE.

<table>
<thead>
<tr>
<th>Chlorpyrifos Concentration</th>
<th>Day 4</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonsediment</td>
<td>Sediment</td>
</tr>
<tr>
<td>Control</td>
<td>15.0 ± 0.5</td>
<td>14.0 ± 0.9</td>
</tr>
<tr>
<td>1 µg/l</td>
<td>13.3 ± 2.2</td>
<td>16.9 ± 1.4</td>
</tr>
<tr>
<td>10 µg/l</td>
<td>14.2 ± 1.2</td>
<td>15.8 ± 1.5</td>
</tr>
<tr>
<td>100 µg/l</td>
<td>14.4 ± 2.1</td>
<td>13.8 ± 1.4</td>
</tr>
<tr>
<td>200 µg/l</td>
<td>13.5 ± 0.6</td>
<td>15.0 ± 2.3</td>
</tr>
</tbody>
</table>
Table 2.4. Percent survival of *Rana sphenoecephala* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in 24 and 48 h laboratory experiments both with predators that were not exposed to chlorpyrifos and 24 h laboratory experiments with predators that were exposed to 1 μg/l chlorpyrifos. Values represent means ± 1 SE.

<table>
<thead>
<tr>
<th>Chlorpyrifos Concentration</th>
<th>Non exposed predator</th>
<th>Exposed predator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Control</td>
<td>84 ± 2</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>10 μg/l</td>
<td>80 ± 4</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>100 μg/l</td>
<td>82 ± 2</td>
<td>86 ± 4</td>
</tr>
</tbody>
</table>
Table 2.5. Percent survival of *Rana sphenocephala* tadpoles exposed for 12 days to sub-lethal concentrations of chlorpyrifos in mesocosm experiments with and without invertebrates predator. Values represent means ± 1 SE.

<table>
<thead>
<tr>
<th>Chlorpyrifos Concentration</th>
<th>No predator</th>
<th>Predator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92 ± 2</td>
<td>36 ± 12</td>
</tr>
<tr>
<td>200 µg/</td>
<td>91 ± 2</td>
<td>88 ± 1</td>
</tr>
</tbody>
</table>
Figure 2.1. Whole body cholinesterase activity of *Rana sphenoecephala* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in 4 and 12 day laboratory experiments. Points represent means ± 1 SE. Refer to text for statistical differences.
Figure 2.2. Mass of *Rana sphenocephala* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in 4 and 12 day laboratory experiments. Bars represent means ± 1 SE.

Refer to text for statistical differences.
Figure 2.3. Whole body cholinesterase activity of *Rana sphenoecephala* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in mesocosm experiments with and without invertebrate predators. Points represent means ± 1 SE. Refer to text for statistical differences.
Figure 2.4. Mass of *Rana sphenoecephala* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in mesocosm experiments with and without invertebrate predators. Bars represent means ± 1 SE. Refer to text for statistical differences.
Ephemeral ponds and wetlands, where many amphibians breed and develop as larvae, can be exposed to agricultural pesticides either directly from broadcast spraying (ground or air) of fields, or indirectly as a product of leaching, runoff, or atmospheric deposition (ATSDR, 1997). Furthermore, due to their permeable skin and biphasic life cycle, amphibians may be more susceptible to some types of environmental contamination than other aquatic vertebrates (Cooke, 1981; Bishop, 1992; Cowman and Mazanti, 2000). Several recent studies have documented an apparent connection between the presence of pesticide residues and reductions in amphibian populations at both local (Burgess et al., 1992; Fellers et al., 2004) and landscape scales (Sparling et al., 2001; Davidson et al., 2002; Davidson, 2004; Hamer et al., 2004), with organophosphate [OP] and carbamate insecticides among the most commonly implicated (Sparling et al., 2001; Davidson, 2004).

OP and carbamate pesticides act on the nervous system of target organisms by inhibiting activity of the neurological enzyme cholinesterase (ChE), which facilitates the break down of the neurotransmitter acetylcholine (ACh) in muscle and nerve synapses.
and consequently ends local synaptic transmission of the impulse (Barron and Woodburn, 1995). When ChE is inhibited, excessive ACh builds up, resulting in hyperactivity, uncontrolled muscle spasms, and eventually paralysis, respiratory failure and death. Because the inhibition of ChE is the specific end result of exposure to OP and carbamate pesticides, measurement of this enzyme has proven to be a useful biomarker of exposure to these chemicals (Walker et al., 2001). Furthermore, ChE is a sensitive measure, responding to exposure well before signs of overt intoxication appear (Mayer et al., 1992; Richards and Kendall, 2002).

In the Sierra Nevada Mountains of California, a relationship has been found between reduced ChE activity in *Pseudacris regilla* tadpoles and adults and declining population status of sympatric ranid species (Sparling et al., 2001). Furthermore, Davidson (2004) found that, specifically, upwind use of carbamate and organophosphate (OP) pesticides was correlated with ranid population absence. However, these previous studies need to be supplemented by laboratory investigations that directly examine the relationship between environmentally realistic exposure levels and ecologically relevant endpoints in native anurans before a cause-effect relationship between agricultural chemicals and the loss of amphibian populations can be made.

Behavior provides a link between an organism’s internal biochemistry and its external environment that may allow an investigator to estimate ecological consequences of sublethal environmental contamination (Little, 1990; Rice et al., 1997). Behavioral endpoints are especially appropriate when analyzing sublethal exposures to OPs and carbamate pesticides because of their direct impact on nervous systems. Furthermore, many studies have found a positive relationship between reductions in ChE activity and
impairment of ecologically relevant behavioral responses in a variety of taxa (e.g., birds: Hart, 1993; Fryday et al., 1996; fish: Kumar and Chapman, 1998; Brewer et al., 2001; Beauvais et al. 2000; mammals: Dell’Omo et al., 1997; McDaniel and Moser, 2004). In fish, the use of swimming activity as a behavioral endpoint is a well established sensitive measure of sublethal contaminant exposure (Little and Finger, 1990), while in tadpoles, similar behavioral endpoints such as swim speed and swim distances have been used to indicate a sub-lethal effect of pesticide exposure (Jung and Jagoe, 1995; Bridges, 1997; Britson and Threlkeld, 1998; Raimondo et al., 1998; Richards and Kendall, 2003).

Our objectives in this study were to examine 1) how depressed ChE activity due to a short term exposure to the OP chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) influenced measures of growth and swim speed in four native anuran species, 2) how the presence of sediment in exposure chambers influenced these tadpole responses, and 3) whether responses observed are general or differentially expressed among species. We chose to examine chlorpyrifos due to its relatively common agricultural use in the United States (US EPA, 2004) and because it is one of the most frequently detected insecticides in the United State’s surface waters (USGS, 2003). It is also one of the agricultural chemicals identified in the tissues of P. regilla adults and tadpoles at elevated levels in the previously mentioned study by Sparling et al. (2001) linking population declines in “pristine” areas downwind of heavy agricultural land use.

Although OPs have been on the commercial market for more than 30 years (Barron and Woodburn, 1995), most currently published reports are based on tests completed in a laboratory setting with contaminant exposure for short periods of time (typically 24 – 96 hours) and mortality used as an endpoint (reviewed in Cowman and Mazanti, 2000).
These investigations have used laboratory toxicity tests conducted on an exceedingly limited number of anuran species, such that the current toxicological literature reflects a strong bias towards the use of representatives of the genera *Rana, Bufo*, and the African genus *Xenopus* (especially *X. laevis*; Cowman and Mazanti, 2000; Pauli et al., 2000), with many species tested so far indicating that sensitivity to contaminants is highly species-specific (Sanders, 1970; Bridges and Semlitsch, 2000; Cowman and Mazanti, 2000).

Barron and Woodburn (1995) noted the need for tests that examined toxicity from dietary, water column, and/or sediment exposures. Chlorpyrifos specifically is moderately lipophilic, tends to partition into soil, sediment and organic material (Bondarenko and Gan, 2004; Wu and Laird, 2004), and, as a result, expected environmental concentrations are strongly dependent on physical properties of the soil in the area (e.g., pH, percent organic matter). Furthermore, an understanding of how sediment influences toxicity of a contaminant may be especially appropriate in amphibians because sediment is an integral part of most natural aquatic systems and some tadpoles are known to consume sediments (Ranvestel et al., 2004; Flecker et al., 1999; P. D. Widder, pers. obs.).

We used early stage tadpoles of *Hyla chrysoscelis, Rana sphenoecephala, Acris crepitans*, and *Gastrophryne olivacea* in exposure scenarios designed to mimic natural conditions in that we used a range of environmentally relevant levels of chlorpyrifos (in a commercially available formulation) and we used static exposures that imitated lentic conditions. We further examined how longer duration (twelve versus four days) influenced responses in two species (*H. chrysoscelis* and *R. sphenoecephala*).
MATERIALS AND METHODS

Animal collection and maintenance.—Newly hatched *H. chrysoscelis* tadpoles were collected from a single population located at the Oklahoma State University Experimental Pond Facility near Lake Carl Blackwell, Payne County, Oklahoma, USA, in late July of 2003. Small portions (approx. 10%) of fifteen egg masses of *R. sphenocephala* were collected in late March 2004 from the same facility. *A. crepitans* and *G. olivacea* tadpoles (Gosner stage 25; Gosner, 1960) were collected in July 2004 from a pond located 1 mi south of Lake Carl Blackwell, in Payne County, Oklahoma. Tadpoles and eggs were transported to a laboratory at Oklahoma State University in Stillwater, Oklahoma. Eggs were first placed into individual 10-l plastic tubs and acclimated to aerated dechlorinated tap water for 24 h, then moved to 40-l plastic tubs upon hatching. Collected tadpoles were acclimated to aerated dechlorinated tap water in 40-l plastic tubs and fed boiled romaine lettuce *ad libitum*. *R. sphenocephala* and *A. crepitans* tadpoles were additionally fed rabbit food pellets *ad libitum* that were first dissipated and repeatedly rinsed with dechlorinated water, while *G. olivacea* were fed the dissipated rabbit food exclusively. Water was replaced every 2-4 days as needed and maintained at 23 ± 2°C.

Experimental design.—The experiments with *R. sphenocephala* and *H. chrysoscelis* used a static renewal system with three factors (concentration, sediment, and duration) in a fully crossed randomized block design. The experiments with *A. crepitans* and *G. olivacea* used just two factors (concentration and sediment) because fewer individuals of these species were collected. We used five chlorpyrifos treatments (control, 1, 10, 100,
and 200 μg/l nominal concentration of chlorpyrifos formulated as Dursban) with four replicates per treatment in all experiments. These levels were found to be sublethal to *R. sphenocephala* in preliminary tests (P. D. Widder, unpublished data). Test solutions were prepared by diluting appropriate aliquots of a 100-mg/l chlorpyrifos stock solution in dechlorinated water. Stock solutions were made by adding 116.8 μl of Dursban into 500 ml of dechlorinated water, stored in covered glass jars, and kept refrigerated when not in use. No stock solutions were used more than 14 days after they were initially made. Test solutions (including controls) were mixed in 10-l plastic containers, then 500 ml of test solution was immediately added to one of the 40 950-ml wide-mouth mason jars, which served as exposure chambers. All exposure jars were first hexane and acid rinsed, followed by three sequential rinses with dechlorinated water and three rinses with reagent grade water prior to use.

To select tadpoles for use in tests, groups of animals were temporarily aggregated by species into a single 40-l plastic tub, and individuals chosen based on stage (Gosner stages 25-27) and absence of any injuries or abnormalities. Those tadpoles selected for use in tests were placed individually into 30-ml solo cups filled with dechlorinated tap water. These cups were then haphazardly chosen to assign tadpoles to a concentration and replicate, with three individuals placed in each jar for *H. chrysoscelis, A. crepitans*, and *G. olivacea* tests, and four individuals for *R. sphenocephala*.

Every concentration had both a sediment and a non-sediment treatment (for a total of 40 jars in one entire test). Sediment (sandy clay loam), taken from a dry pond adjacent to the natal pond, was homogenized, dried at 100°C for 24 h, and 40 g added to each sediment treatment jar. Dechlorinated tap water (500 ml) was then added to each jar and
allowed to sit for 48 h prior to use in the tests so that the sediment would become fully saturated. Just prior to addition of the experimental solution, the overlying water in the sediment jars was removed using a clean turkey baster. Treatment solutions were added slowly to sediment treatments and diverted away from the bottom sediment using a standard laboratory spatula.

Test durations for each species were four and twelve days for *H. chrysoscelis* and *R. sphenocephala* and four days for *A. crepitans* and *G. olivacea*. The twelve-day *H. chrysoscelis* test was initiated on 28 July 2003 and the four-day test began the following day. These tests were arranged on two shelves in a laboratory maintained at 22 ±1°C and illuminated by indirect sunlight and florescent lights set to a 14/10-light/dark cycle synchronized with the true sunset. Both the twelve- and four-day tests with *R. sphenocephala* were initiated on 26 April 2004. The *G. olivacea* test was started on 14 August 2004, and the *A. crepitans* test began on the following day. The *R. sphenocephala*, *A. crepitans*, and *G. olivacea* tests were each placed in an I-36LLVL incubator (Percival Scientific, Inc., Perry, IA, USA) equipped with plant grow lights maintained on a 14/10-light/dark cycle synchronized with true sunrise and with temperature held at 20 ±1°C.

All test jars were arranged in alternating sediment and non-sediment rows, with concentration randomized within each row. *H. chrysoscelis*, *R. sphenocephala*, and *A. crepitans* tadpoles were fed a piece (~16 cm²) of boiled romaine lettuce every other day and jars were cleaned of solid waste on alternate days using small disposable pipettes. *G. olivacea* tadpoles were given a 0.5-ml aliquot of dissipated rabbit food pellets every other day. At the conclusion of tests, tadpole swim speed was measured and individuals were
prepared for determination of mass and ChE activity as described below. The twelve-day
tests were renewed with freshly prepared test solutions every four days until the twelfth
day, when they were also terminated. Renewals were 100 percent in nonsediment
treatments, but were approximately 80 percent in sediment treatments due to the
difficulty of removing tadpoles from exposure chambers without undue stress.

Response measurements.—Tadpole burst swim speed was measured using a 30-cm
polyethylene channel, delineated at 1-cm intervals and filled to a depth of 1 cm with
water. A Sony DCR-TRV25 video camera (Sony Electronics, San Diego, CA, USA)
situated directly above the center of the channel recorded all tests on MiniDV tapes at 30
frames per sec. Tadpoles were first removed from their respective jars (using one
sediment row and one non-sediment row at a time) and then placed individually into
labeled plastic Solo cups. Three tadpoles from each jar were sequentially placed in the
channel and gently induced (using a blunt probe) to swim down the length of the channel
four times without rest. After each trial, the tadpole was returned to its labeled cup. Test
tapes were later rerecorded on VHS tapes, with the VCR timer enumerating frames. The
VHS videotape was then reviewed and the time (to the nearest 0.03 sec) it took each
tadpole to swim two 10-cm intervals along each length of the channel was recorded (eight
total measures). The fastest 10-cm interval was used in analysis.

After all swim speed trials from a row were completed, tadpoles were digitally
photographed using an Olympus 5.0 megapixel C-5050 digital camera (Olympus
America Inc., Melville, NY, USA). Pictures were taken from directly above tadpoles in
their Solo cups so that the entire bottom of each cup was visible and tadpoles were resting
on the bottom of their respective cups. All tadpoles from a single jar were included in a
single photograph. A ruler was placed in each photograph and used to calibrate distance for snout-to-vent length (SVL) measurements using SigmaScan Pro Image Analysis Software (version 5.0, SPSS Inc., Chicago, IL, USA). Immediately after photographs were taken, tadpoles were placed into labeled Nalgene® cryovials and flash frozen by emersion in liquid nitrogen. Samples were then stored in a -70°C freezer until ChE and mass were measured.

Analysis of ChE generally followed procedures outlined by Ellman et al. (1961), with modifications for multiwell-plate readers. Briefly, each frozen tadpole was carefully removed from its cryovial, weighed to the nearest 0.1 mg. This measurement of mass was recorded and used in growth analysis. Individuals were then placed in a 2-ml Eppendorf tube containing 1 ml of chilled phosphate buffer (pH 8) and subsequently placed on ice. Whole tadpoles were then homogenized (still held on ice) for a minimum of 30 s (longer for larger tadpoles) using a PowerGen 125 Homogenizer (Fisher Scientific International Inc., Hampton, NH, USA) equipped with a 5 or 7 mm X 95 mm generator. Samples were centrifuged at 14,000 rpm for 5 min with a Jouan CR3i refrigerated centrifuge (Jouan Inc., Winchester, VA, USA) set to -2°C. A 40-μl sample of the resulting supernatant was then added to three replicate wells of a 96-well plate (also held on ice) and a 300-μl aliquot of reagent solution [500-μl buffered Ellman's reagent (DNTB), 100-μl acetylthiocholine iodide and 15-ml phosphate buffer (pH 8)] was then added to each well. The bottom of the well plate was carefully wiped dry and the plate was read on a SpectraMax® 190 Microplate Spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) at 11-s intervals for 5 minutes. Data reduction was accomplished with a Softmax® PRO computer program (Molecular Devices Corporation,
Sunnyvale, CA, USA), and ChE activity was expressed as μmol/min/g, using tadpole whole body mass corrected for the influence of tadpole body size on the final homogenate volume.

Water chemistry.—Water quality parameters (pH, dissolved oxygen (DO), conductivity, alkalinity and hardness) were recorded before and following renewal of the test solutions in all tests. DO was measured using a Model 50B Dissolved Oxygen Meter (YSI Incorporated, Yellow Springs, OH, USA), and pH was measured with an Accumet® portable AP62 pH/mV meter (Fisher Scientific, Pittsburgh, PA, USA). Conductivity was measured with a Hach® conductivity/TDS meter (Hach, Loveland, CO, USA), and alkalinity and hardness were measured by titration (APHA, 1995).

Chlorpyrifos characterization.—To verify the % a.i. in our Dursban source, three replicate 500-ml samples of dechlorinated water were spiked with 2.5 ml of the 100-mg a.i. chlorpyrifos/l stock solution, immediately extracted, and analyzed using the methods described below.

Chlorpyrifos analysis from water samples followed the protocol outlined by Lui et al. (2002). Water samples were first filtered through a Whatman GF/F filter (0.7-μm pore size) and chlorpyrifos was then extracted using solid phase extraction cartridges (IST Isolute, ENV+ sorbent, 500-mg sorbent mass, 6-ml reservoir volume; Chromatographic Specialties Inc., Brockville, ON, CAN) attached to a Novagen® vacuum manifold (Merck Biosciences, Darmstadt, GER) set to 20-mmHg negative pressure. Prior to extraction, cartridges were preconditioned with 6 ml of dichloromethane, 6 ml of acetone, and 6 ml of deionized water. Following extraction, the cartridges were wrapped in aluminum foil and stored at -15°C until elution. For elutions, cartridges were first dried.
using the vacuum manifold (12-mmHg). The vacuum was then turned off and the captured chlorpyrifos on the cartridge medium was removed by sequential, gravity-fed elutions with 6 ml of dichloromethane and 9 ml of 3:1 acetone/acetonitrile. The resulting solution was analyzed on a Varian CP-3800 Gas Chromatograph (Varian Inc. Palo Alto, CA, USA) equipped with a CP-8400 auto-sampler either directly or following an appropriate dilution with hexane. GC oven conditions were as outlined in Lui et al. (2002).

**Statistical analyses.**—Prior to analysis, all ChE and mass data were first log transformed to obtain a normal distribution. Swim speed data were log transformed and regressed against log transformed snout-to-vent lengths to generate the residuals used for analysis.

All ANOVA analyses were conducted using the MIXED procedure in SAS/STAT software (SAS, 2001). To determine if there were significant differences in tadpole response to treatments, separate three factor (concentration, sediment, duration) ANOVAs were performed for each *H. chrysoscelis* and *R. sphenoecephala* response in ChE, mass, SVL, and swim speed with each tadpole included as a subsample. Similar separate two factor (concentration, sediment) ANOVAs were used for *A. crepitans* and *G. olivacea* tests. Individual mean comparisons developed *a priori* (i.e., comparison of controls to each concentration within sediment and day combinations, comparison of nonsediment and sediment within a concentration and day, and comparisons between days within a sediment and concentration combination) for each response were conducted using the CONTRAST statement (SAS, 2001).
Differences in the extent to which chlorpyrifos exposure inhibited ChE activity in each species were evaluated by calculating inhibition concentrations (ICx; concentration of pesticide that results in a designated percent reduction, x, in enzyme activity as compared to controls) and associated 95% confidence intervals. ICx values were generated using a linear interpolation method with CETIS statistical software (Comprehensive Environmental Toxicity Information System, Tidepool Software McKinleyville, CA, USA).

Log transformed ChE activity data for each species’ day and sediment combination were regressed against swim speed residuals using SYSTAT v. 10 (SPSS, 2000) to determine if swim speed correlated with ChE activity.

All statistical comparisons were conducted at $\alpha = 0.05$.

**RESULTS**

The only mortality observed in any of the bioassays occurred in the *H. chrysoscelis* 12-day, nonsediment treatments, where two individuals died in both the 100 and 200 $\mu$g/l concentrations.

*Chemical analysis.*—Across all tests, pH ranged from 7.2 to 8.0, DO ranged from 8.56 to 5.29 mg/l, conductivity ranged from 510.7 to 696.5 $\mu$S/cm, alkalinity ranged from 44 to 114 mg/l (as CaCO$_3$), and hardness ranged from 94 to 160 (as CaCO$_3$).

The % a.i. of our chlorpyrifos commercial product (Dursban TC) was 47.4 %.

*Growth.*—Average tadpole mass and SVL are summarized in Table 3.1 and Table 3.2 respectively. In *H. chrysoscelis*, mass of tadpoles in the non-sediment, 200-$\mu$g/l treatment was significantly lower than that of their respective controls for both the 4- and
12-day exposures. At the end of the 12-day treatment, tadpoles in the nonsediment 200-ug/l treatment also weighed significantly less than those in the 200-ug/l treatment that contained sediment (p=0.0093). The presence of sediment also influenced the mass of the control *H. chrysoscelis* tadpoles after 12 days, although in this case, the mass of the organisms was significantly less than that of their non-sediment counterparts. *R. sphenocephala* tadpoles grew significantly more in 12-day treatments than in 4-day treatments (p<0.0001), with mass significantly decreased by pesticide concentration whether sediment was present or not (p=0.012). *A. crepitans* tadpole mass was not influenced by chlorpyrifos concentration, but tadpoles exposed in sediment treatments were larger overall than nonsediment treatment tadpoles (p=0.0097). Within concentrations, this sediment effect was evident in the 200-μg/l exposure level (p=0.0121). Interestingly, the reverse was true of *G. olivacea*: across all concentrations tadpole mass was unaffected by sediment treatment, but significantly reduced by concentration (p=0.0148) and specifically within concentrations, tadpole mass was reduced by an average of 27 to 30 mg from control values in the 200-μg/l nonsediment (p=0.0207) and sediment (p=0.0497) treatment combinations.

SVL results followed these same general patterns, however, because of the greater variability in results, the only overall statistically significant difference noted was across days in *R. sphenocephala* (p<0.0001).

*Cholinesterase.*—Tadpole ChE activity generally decreased with increasing concentration of chlorpyrifos in all species (Fig 3.1), however, the pattern and degree of inhibition varied between species. Overall, control activity ranged from 2.5 to 4.5 μmol/min/g.
The duration of exposure had a significant influence on ChE activity across both concentration and sediment treatments in *H. chrysoscelis* tadpoles (p<0.0001; Fig 3.1A) and specifically in the 1-μg/l concentration of the pesticide, individuals in the Day 12 treatments had significantly lower enzyme activity levels than those at four days (p<0.0007). Interestingly, in the chlorpyrifos treatments of 10 μg/l and higher, the *H. chrysoscelis* tadpoles experienced similar levels of enzyme inhibition regardless of exposure duration. However, the presence of sediment did ameliorate the degree of pesticide-induced inhibition. At the end of the twelve-day exposure period, ChE activity levels at the 200-μg/l treatments were reduced to 86 and 73 percent of control values in the nonsediment and sediment treatments, respectively.

In *R. sphenocephala*, concentration also significantly decreased tadpole ChE activity, however, there were differences in response to sediment treatments across days (p=0.0029; Figure 3.1B). Specifically, individuals in the Day 4 nonsediment treatments had significantly higher activity than their counterparts on Day 12 (p<0.0104), although this difference between days was not evident in the presence of sediment. The highest overall degree of inhibition was observed in the twelve day 200-μg/l concentrations, where ChE activity levels of the tadpoles was reduced to 43 and 33 percent of control values in nonsediment and sediment treatments, respectively.

*A. crepitans* tadpole ChE activity decreased with increasing concentration (p<0.0001) and was higher overall in nonsediment treatments (p<0.0001; Figure 3.1C). However, enzyme activity in organisms from the control and 1- and 10-μg/l sediment treatments ranged between 23 and 37 percent lower than the corresponding treatments without sediment, although this effect was diminished at 100- and 200-μg/l treatments. In the
highest concentration, nonsediment treatment combination, enzyme activity levels were inhibited 60 percent from corresponding control animals.

The presence of sediment influenced ChE activity levels in both control and pesticide-exposed *G. olivacea* tadpoles, however the response was not consistent across concentrations (*p*<0.0001; Figure 3.1D). In particular, individuals in control, nonsediment treatments had ChE activity that was 10 percent higher than controls that included pond sediment. This increased to 24 percent in the 1-μg/l concentration, however the relationship was reversed at the 10-μg/l concentration, and was absent in the highest two concentrations. In the highest concentration, tadpole ChE activity was reduced from controls by 92 and 89 percent in the nonsediment and sediment treatments, respectively.

Inhibition concentrations for parameters such as growth or biomarker activity are commonly reported at the 50% level (IC50- that concentration of test material causing a 50% reduction in response as compared to controls) for statistical purposes. However, calculations of the chlorpyrifos IC50 for *R. sphenocephala* tadpole ChE activity was not possible since inhibition of enzyme activity levels did not reach 50%. As such, IC25 values are reported so comparisons between species can be made. These values ranged from a low of 0.59-μg/l chlorpyrifos for *H. chrysoscelis* tadpoles exposed to the pesticide for 12 days without sediment to 115-μg/l for *R. sphenocephala* tadpoles exposed for 12 days with sediment present (Table 3.3). Generally, IC25 values were considerably lower for *H. chrysoscelis* and *G. olivacea* than for the other two species. For all species tested, the presence of sediment reduced toxicity of the pesticide (resulting in an increase in the
IC25 value) although based on overlap of the 95% confidence intervals, the effect for this endpoint was not statistically significant.

**Swim Speed.**—In all species tested, qualitative differences in tadpole swimming behavior were evident in the 200-μg/l nonsediment treatments. With few exceptions, tadpoles in these treatments took longer to swim the prescribed four lengths of track (including turn around time) and became desensitized to prodding over a shorter course of time.

Tadpole swim speed was negatively impacted by chlorpyrifos exposure in *H. chrysoscelis* (Figure 3.2A), but these impacts were different across sediment treatments (p=0.0005). Specifically, tadpoles from the 200-μg/l nonsediment treatment swam, on average, 3.5 cm/sec slower than the corresponding sediment treatments on Day 4 (p=0.0065). Furthermore, after twelve days of exposure to the highest two concentrations, tadpole from nonsediment tests swam 3.0 and 5.9 cm/sec slower than their sediment counterparts.

*G. olivacea* tadpole swim speed was also significantly different across concentrations (p=0.0357), however pairwise comparisons between control and all concentrations within sediment treatments revealed that none were significantly different from controls. However, the fastest swim speeds for this species were seen in the 1-μg/l concentration, with tadpoles at this level swimming 5 to 15 percent faster than controls. Accordingly, the largest concentration-related difference in swim speed was observed between the 1- and 200-μg/l treatments. Tadpoles in the 200-μg/l concentration swam 4.3 to 3.6 cm/sec slower than those at 1-μg/l and 1.4 to 3.7 cm/sec slower than control animals.
*Acris crepitans* and *R. sphenoecephala* tadpole swim speed was not significantly impacted by concentration or presence of sediment. However, *R. sphenoecephala* tadpole swim speed was marginally significantly different between days (p=0.0563) in that older tadpoles swam faster.

Individual *R. sphenoecephala* tadpole swim speed did not correlate with the measured ChE activity (p>0.245 for all, Figure 3.3A,B). A similar result was seen in *H. chrysoscelis* tadpoles in sediment treatments, however, for *H. chrysoscelis* tadpoles that were exposed to chlorpyrifos without sediment, there was a significant correlation between swim speed and ChE in both days (Day 4: r=0.536, p<0.001; Day 12: r=0.467, p<0.001, Figure 3.3C,D). In *G. olivacea*, there was a correlation between swim speed and ChE activity in both sediment treatments (Non-sediment: r=0.342, p=0.007; Sediment r=0.364, p=0.004, Figure 3.3E). *Acris crepitans* tadpole swim speed did not correlate with ChE activity, however in the sediment treatment the relationship was nearly significant (r=0.241, p=0.064).

**DISCUSSION**

*Chlorpyrifos concentrations.*—Within agricultural systems, environmental concentrations of chlorpyrifos in water bodies containing larval amphibians are certain to vary considerably. The determinants of these concentrations include physical parameters such as distance to and amount of cropland, application concentrations, soil properties, and size of the water body, as well as temporal factors such as season, frequency of application, and rainfall events. Previous work examining chlorpyrifos concentrations in small water bodies adjacent to cropland has found concentrations to range from 73 μg/l to
over 700 μg/l (US EPA, 2002; Moore et al., 2002; Mazanti et al., 2003), demonstrating that the nominal concentrations used in our work (1 μg/l to 200 μg/l) are realistic.

*Growth.*—Exposure to pesticides, specifically OPs and carbamates, has previously been shown to have sub-lethal effects on tadpoles (Richards and Kendall, 2003; Venturino et al., 2003; Relyea, 2004). For instance, Richards and Kendall (2003) found a significant impact of static 4-day chlorpyrifos exposure at 100 μg/l on mass of *X. laevis* at N/F stages 14 and 46. Reductions in tadpole growth have been reported to have impacts on adult frog fitness measures such as reduced survival to, and smaller size at first reproduction and less fecundity (Berven and Gill, 1983; Smith, 1987; Semlitsch et al., 1988). For example, Smith (1987) found that newly metamorphosed *Pseudacris triseriata* males which were 1 mm or larger than the average metamorph returned the following year as significantly larger adults. Those frogs that were smaller at metamorphosis and metamorphosed later took two years to return to breed and were among the smallest adults breeding.

The *H. chrysoscelis*, *R. sphenoecephala*, and *G. olivacea* tadpoles examined in this study weighed 20–35 percent less than controls after 4 days in the 200-μg/l concentration. However, within *H. chrysoscelis*, change in tadpole mass over the course of 12 days was not discernable within controls, and within *R. sphenoecephala* tests, tadpole mass increased similarly across concentrations over the same time period. It is possible that the significant differences in mass between concentrations is the result of tadpoles having less food within their gut coils (i.e., intestinal tract) and qualitative observations of less food being eaten by individuals in the higher concentrations over the course of this experiment (P. D. Widder, unpublished data) corroborate this conclusion. Similarly,
Mazanti (1999) documented behavioral disinterest in feeding in chlorpyrifos exposed *Hyla versicolor* tadpoles. Because analysis of ChE necessitates euthanizing the tadpole, we were not able to examine the long term effects of chlorpyrifos exposure on tadpole mass or size, and further work will be needed to determine if the observed changes in feeding have discernable impacts on size at metamorphosis in these species.

*Cholinesterase.*—Compared to other vertebrate groups, few studies have quantified the relationship between organophosphate exposure and ChE inhibition in larval amphibians (reviewed in Cowman and Mazani, 2000; Venturino et al., 2003). However, studies examining effects of chlorpyrifos on larval fishes are perhaps the most comparable to the current one in that both groups are aquatic ectothermic vertebrates. Jarvinen et al. (1988) found that chlorpyrifos concentrations of 0.27 μg/l reduced brain ChE activity 10-15% in 60-day old fathead minnows (*P. promelas*) that had been exposed continuously from hatching, while enzyme levels were inhibited by 75-85% from control levels in fish from 2.68-μg/l treatments. van der Wel and Welling (1989) examined the sublethal effects of exposure to 3.25 μg/l in 17-18 week old guppies (*Poecilia reticulata*) set up in a flow-through continuous exposure system and found it to significantly inhibit ChE after 24 h. However, it should be noted that ChE activity has been shown to change with development in fish (Phillip et al., 2002).

In tadpoles, ChE has also been found to fluctuate with developmental stage (Gindi and Knowland, 1979) and season (Venturino et al., 2003), however differences between species at the same development stage have not been characterized (Henry, 2000). To date, two studies have examined the impact of chlorpyrifos exposure on larval amphibians. In their study on *X. laevis* using static exposures to chlorpyrifos, Richards
and Kendall (2002) found ChE was significantly inhibited (defined as >50 percent of control values) at the 100-μg/l and 10-μg/l treatments at N/F stages 14 and 46, respectively. Bonfanti et al. (2004) examined the ChE inhibiting potential of chlorpyrifos on early stage embryos (N/F stages 9-47) of *X. laevis* and found that larvae continuously exposed to 100 μg/l and 250 μg/l chlorpyrifos for 5 days had cholinesterase levels that were inhibited 76-80 percent from control animals. Taken together, this past work indicates that the concentrations of pesticide in which we saw effects on ChE are generally in agreement (within an order of magnitude) with these past investigations. Furthermore, the sensitivity of tadpole ChE appears to be less than corresponding larval fishes.

Toxicity of chlorpyrifos is assumed to be the result of inhibition of the specific cholinesterase form acetylcholinesterase (AChE) by the metabolized product of the pesticide, chlorpyrifos oxon (Barron and Woodburn, 1995). Differences in sensitivity between and within taxonomic groups (Barron and Woodburn, 1995; Grue et al., 1997) may be a result of inherent differences in cholinesterase forms (AChE versus butyrylcholinesterase; BChE) or differences in activation mechanisms (e.g., inherent differences in P450 cytochromes; Grue et al., 1997). In the present study, we report total cholinesterase activity; however, a preliminary screen for BChE in *R. sphenocephala* indicated that only minute amounts of the enzyme were present (P. D. Widder, unpublished data).

In the bioassays with both *H. chrysoscelis* and *R. sphenocephala*, the duration of exposure significantly influenced the level of enzyme inhibition for tadpoles exposed to 1-μg/l chlorpyrifos, with the degree of inhibition greater at 12 versus 4 d. Interestingly,
at pesticide levels of 10 µg/l and higher, the level of enzyme inhibition was similar for organisms from the 4 and 12 d exposures. Since dose and duration of exposure determine the degree of response to any compound (Heath, 1995), this is not a surprising result. At higher chlorpyrifos concentrations, a maximum level of enzyme inhibition is probably attained before day 4 and was further maintained by renewal of the test solutions. In the 1-µg/l exposure, maximum inhibition levels had not been reached by day 4 and a response difference based on exposure time was therefore observed.

Swim speed.—Using control tadpole swim speeds (not corrected for size), we can examine general differences in linear swimming ability among the species we examined. *G. olivacea* tadpoles were the quickest, averaging 15.1 cm/sec across both controls, and *R. sphenocephala* and *A. crepitans* tadpoles were similar, with both averaging 14.8 cm/sec. *H. chrysoscelis* tadpoles were the slowest at 11.0 cm/sec, which also corresponds to that species being the smallest among those we examined. These results generally agree with a past investigation examining tadpole swim speed in *Rana sphenocephala* and *Hyla versicolor* (Richardson, 2002).

Studies in a variety of other taxa have reported changes in behavior associated with OP exposure (e.g., birds: Hart, 1993; Fryday et al., 1996; fish: Kumar and Chapman, 1998; Beauvais et al. 2000; Brewer et al., 2001; mammals: Dell’Omo et al., 1997; McDaniel and Moser, 2004). Effects of chlorpyrifos exposure on larval fish swimming ability have been quantified in more than one species (Carlson et al., 1998; Levin et al., 2003; Levin et al., 2004) and qualitative descriptions of altered behavior in chlorpyrifos exposed fish are similar to those of exposed larval amphibians (Rice et al., 1997). For example, Levin et al. (2004) examined larval swimming activity in zebrafish (*Danio*
and found that newly hatched larvae exposed to 100-ng/l chlorpyrifos for 4 days had significantly impaired swimming activity at one and four days post exposure, however ChE was not measured. Carlson et al. (1998) found that chlorpyrifos exposure (from 30 μg/l to 270 μg/l) significantly increased the time it took for the motoneuronal signal to produce muscle activity in juvenile *O. lapites* by directly measuring the neuroelectrical activity of ‘C-start’ startle responses. Additionally, increasing exposure concentrations increased the number of times an individual needed to be prodded to elicit a response and decreased the time it took a predator to consume exposed animals (Carlson et al., 1998). Amphibians are among the aquatic organisms that exhibit this stereotypic ‘C-start’ startle response (Eaton and Hackett, 1984; Lannoo, 1999) and this latter study provides a clear mechanism by which tadpole response to predators might be impaired by chlorpyrifos exposure.

Several studies have reported inactivity and sluggish responses to prodding in tadpoles exposed to chlorpyrifos similar to those observed here (Moulton, 1996; Britson and Threlkeld, 1998; Mazanti, 1999; Bonfanti et al., 2004). Furthermore, tadpoles exposed to another ChE inhibitor (carbaryl) were found to swim shorter distances and exhibit less activity with increasing concentration levels (Bridges, 1997). However, behavioral effects of exposure to chlorpyrifos appear to have only been quantified in one study with amphibian larvae (Richards and Kendall, 2003). These authors examined the relationship between chlorpyrifos dose and swim speed in *X. laevis* at two development stages (N/F stage 14 and 46), and found effects on swimming ability at 100 μg/l and 10 μg/l respectively. These concentrations were approximately the same concentrations that ChE was inhibited more than 50% (Richards and Kendall, 2002).
In our study, while qualitative changes in tadpole swimming behavior were evident in all species, the only significant impacts of chlorpyrifos exposure on tadpole swim speed over a given 10-cm interval were observed for *H. chrysoscelis* exposed for four days to 200 μg/l and twelve days to 100 and 200 μg/l of the pesticide without sediment present and, to a lesser degree, for *G. olivacea* in nonsediment 200-μg/l treatments. Individuals within these treatment combinations had ChE activity levels that were inhibited more than 80 percent from control tadpoles and it was only within these species treatment combinations that a correlation was apparent between ChE activity and swim speed within individuals. This leads us to conclude that the levels of ChE inhibition in the concentrations we examined were generally not sufficient to exert an effect on swim speed, as we measured it. This conclusion is consistent with the idea that contaminants begin exerting an effect at the biochemical level (measurable at low concentrations), progress to effects in organ or organ system functions at moderate levels of contaminants, and, at the highest concentrations, effects become evident in the individual (e.g., growth, reproduction effects) or even at the population level (Heath, 1995).

While the design of our swim speed channel attempted to minimize swimming in lateral directions, some species’ response to prodding was consistently nonlinear, thus increasing individual variation in our measurement of swim speed and probably reducing our ability to detect differences. We used identical experimental methods because similar systems have been used successfully in past investigations (e.g., Raimondo et al., 1998) and to facilitate direct comparisons between species. In retrospect, using an apparatus suited to the specific swimming preferences of individual species may have provided a more ecologically relevant measure of effect. For instance, many *A. crepitans* tadpoles
attempted to swim in a downward direction rather than forward, often swimming in place
as a result, and all but a few *G. olivacea* tadpoles continuously zigzagged the length of
the channel. However, it should be noted that a system that allows for movement in
three-dimensional space would likely be paired with a trade off in measuring fine scale
distances or require use of specialized equipment.

*Influence of sediment.*—Sediment can be an integral component of the environment in
which larval amphibians naturally occur, with this phase often acting as a sink for
contaminants that enter the system. Binding of contaminants to sediments and associated
organic material can significantly alter the potential for uptake and toxicity (Power and
Chapman, 1992). Since chlorpyrifos is among those chemicals with a tendency to bind to
colloidal material and sediments (Bondarenko and Gan, 2004; Wu and Laird, 2004), we
expected some degree of ameliorative effect of including pond sediment in test chambers.

The influence of sediment on tadpole response to chlorpyrifos was inconsistent
between species. *H. chrysoscelis* and *A. crepitans* tadpoles exposed with sediment were
heavier than their nonsediment counterparts in 200 μg/l concentrations after 12 d and 4
day time intervals, respectively. ChE activity was lower for *R. sphenocephala* and *A.
crepitans* tadpoles after 4 d in the control and 1-and 10-μg/l concentration sediment
treatments compared to nonsediment treatments, however, for *R. sphenocephala* this
difference was no longer apparent at the end of 12 d. *G. olivacea* tadpoles displayed also
this effect in the 1-μg/l treatment, however, *H. chrysoscelis* tadpoles showed a reverse
effect after 12 d of exposure, not only at the 1-μg/l concentration but also the higher
exposure levels.
Differences between species responses were seen in swim speeds as well. For example, on average, *G. olivacea* tadpoles swam faster in swim trials when they were exposed in sediment treatment jars across all concentrations. *H. chrysoscelis* tadpole swim speed was similar to control swim speeds when tadpoles were exposed with sediment, however, this ameliorative effect was not seen in the other species.

The basis for the observed differences between species in the presence of sediment is not entirely clear, but may be related to differences in microhabitat preferences, including where tadpoles are typically found within the water column and the extent to which they ingest sediment particles, which could serve as a source for the pesticide if desorption occurs in the intestinal tract.

*Response differences between species.*—A comparison of responses to a stressor within an given group of closely related animals can facilitate not only an understanding of how the stressor affects each of those species individually, but also allows for generalizations to be made regarding the relationship between the stressor and the group as a whole. For example, Chuiko (2000) examined the differential sensitivity of the specific types of cholinesterase enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) to an OP among representatives of four different teleost fish families and found BChE was more sensitive to OP exposure than AChE and that significant BChE activity occurred in only one of the fish families evaluated.

Generally, the order of tadpole sensitivity in the present study was *H. chrysoscelis* = *G. olivacea* > *A. crepitans* > *R. sphenoecephala*. Species sensitivity to contaminants can vary based on differences in rates of uptake and elimination from the body which, as discussed for the influence of sediment, can be affected by the microhabitat in which the organism
spends most of its time. In the case of OP insecticides such as chlorpyrifos, differences in levels of metabolic enzymes that transform the parent compound into the more toxic oxidized form can also form the basis for differences in response (Hill, 2003). Recent work with birds and mammals also indicates that differential sensitivity to OP compounds may be due to differences in levels of the enzymes involved in hydrolyzing the active metabolites themselves (Costa et al., 2003).

Conclusions.—The presence of sediment in tadpole test systems can clearly influence the response of the organisms to chemicals such as chlorpyrifos, and given the prevalence of sediment as a component of the average developing tadpole’s environment, these differences probably more accurately reflect responses to contamination in the natural environment. Perhaps more importantly, we found species-specific differences in response to the pesticide which underscores the need for caution when attempting to make generalizations regarding anurans for the purposes of risk assessments and when using “standard” or surrogate test species to predict responses across different anuran taxa.


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Table 3.1. Tadpole mass (mg) ± 1 SE averaged within exposure chambers then across replicates (n=4) by species at conclusion of chlorpyrifos exposure tests, separated by nonsediment (Non) and sediment (Sed) treatments. Significant pairwise differences from controls in concentrations within a sediment treatment are indicated in bold, differences between nonsediment and sediment within a concentration are indicated by *, and differences between days within a concentration and sediment treatment are indicated by †.

<table>
<thead>
<tr>
<th>Species/Day</th>
<th>Control</th>
<th>1 μg/l</th>
<th>10 μg/l</th>
<th>100 μg/l</th>
<th>200 μg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hyla chrysoscelis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>84±10</td>
<td>86±2</td>
<td>80±6</td>
<td>85±3</td>
<td>78±3</td>
</tr>
<tr>
<td>Day 12</td>
<td>98±9</td>
<td>79±8*</td>
<td>82±3</td>
<td>83±7</td>
<td>81±2</td>
</tr>
</tbody>
</table>

*Rana sphenoecephala*

| Day 4       | 166±11  | 166±19 | 153±15  | 155±12   | 131±7    | 147±15   | 134±16   | 135±3    | 129±11   | 122±5    |          |          |          |          |          |          |          |          |          |          |          |          |
| Day 12      | 194±15  | 203±11 | 185±9   | 211±15†  | 217±14†  | 235±10†  | 180±6†   | 170±14†  | 183±19†  | 175±8†   |          |          |          |          |          |          |          |          |          |          |          |          |

*Acris crepitans*

| Day 4       | 169±10  | 189±5  | 158±19  | 173±8    | 172±6    | 169±6    | 177±6    | 186±13   | 161±4    | 198±5*   |          |          |          |          |          |          |          |          |          |          |          |          |

*Gastrophryne olivacea*

| Day 4       | 175±10  | 167±9  | 162±5   | 157±10   | 178±12   | 170±7    | 162±13   | 157±7    | 144±7    | 139±5    |          |          |          |          |          |          |          |          |          |          |          |          |
Table 3.2. Tadpole snout-to-vent length (mm) ± 1 SE averaged within exposure chambers then across replicates (n=4) by species at conclusion of chlorpyrifos exposure tests, separated by non-sediment (Non) and sediment (Sed) treatments. Significant pairwise differences from controls in concentrations within a sediment treatment are in bold, between non-sediment and sediment treatments within a concentration are denoted by *, and between days within a concentration and sediment treatment are denoted by †.

<table>
<thead>
<tr>
<th>Species/Day</th>
<th>Nominal Concentration of active ingredient Chlorpyrifos (formulated as Dursban)</th>
<th>Control</th>
<th>1 μg/l</th>
<th>10 μg/l</th>
<th>100 μg/l</th>
<th>200 μg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
</tr>
<tr>
<td>Hyla chrysoscelis</td>
<td></td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>7.4±0.3</td>
<td>7.5±0.2</td>
<td>7.3±0.4</td>
<td>7.5±0.1</td>
<td>7.2±0.2</td>
</tr>
<tr>
<td>Day 12</td>
<td></td>
<td>7.2±0.2</td>
<td>6.8±0.3†</td>
<td>7.0±0.1</td>
<td>7.3±0.2</td>
<td>7.1±0.1</td>
</tr>
<tr>
<td>Rana sphenoecephala</td>
<td></td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>9.4±0.2</td>
<td>9.8±0.5</td>
<td>9.0±0.4</td>
<td>9.1±0.2</td>
<td>8.8±0.3</td>
</tr>
<tr>
<td>Day 12</td>
<td></td>
<td>10.4±0.3</td>
<td>10.7±0.2</td>
<td>10.0±0.2</td>
<td>10.8±0.4†</td>
<td>10.9±0.2†</td>
</tr>
<tr>
<td>Acris crepitans</td>
<td></td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>9.7±0.2</td>
<td>9.8±0.3</td>
<td>9.6±0.3</td>
<td>9.1±0.2</td>
<td>9.5±0.1</td>
</tr>
<tr>
<td>Gastrophryne olivacea</td>
<td></td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
<td>Sed</td>
<td>Non</td>
</tr>
<tr>
<td>Day 4</td>
<td></td>
<td>10.1±0.3</td>
<td>10.0±0.3</td>
<td>9.9±0.4</td>
<td>9.6±0.3</td>
<td>9.7±0.4</td>
</tr>
</tbody>
</table>

Note: Significant differences from controls in concentrations within a sediment treatment are in bold, between non-sediment and sediment treatments within a concentration are denoted by *, and between days within a concentration and sediment treatment are denoted by †.
Table 3.3 The predicted 25% inhibition concentrations for 4 day or 12 day chlorpyrifos exposure tests organized by species with 95% confidence intervals indicated. Values are separated by sediment treatment. * indicates the upper confidence interval could not be calculated.

<table>
<thead>
<tr>
<th>Sediment Treatment</th>
<th>Species/Day</th>
<th>Nonsediment</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyla chrysoscelis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>4.34 (2.75 – 5.74)</td>
<td>7.66 (6.12 – 9.55)</td>
<td></td>
</tr>
<tr>
<td>Day 12</td>
<td>0.59 (0.52 – 0.75)</td>
<td>0.81 (0.64 – 3.61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rana sphenoecephala</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>71.74 (41.18 – 97.27)</td>
<td>77.71 (38.08 – *)</td>
<td></td>
</tr>
<tr>
<td>Day 12</td>
<td>54.64 (33.70 – 85.62)</td>
<td>114.79 (58.22 – 178.06)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acris crepitans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>36.99 (12.44 – 55.75)</td>
<td>56.29 (6.33 – 97.49)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gastrophryne olivacea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>4.44 (3.90 – 4.71)</td>
<td>4.49 (2.40 – 6.12)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 Whole body cholinesterase (ChE) activity of A) *Hyla chrysoscelis* B) *Rana sphenocephala* C) *Acris crepitans* and D) *Gastrophryne olivacea* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in 4 and 12 day laboratory experiments. Points are averages within exposure chambers then across replicates (n=4) and bars represent standard errors. Refer to text for statistical differences.
Figure 3.2 Maximal swim speeds of A) *Hyla chrysoscelis* B) *Rana sphenocephala* C) *Acris crepitans* and D) *Gastrophryne olivacea* tadpoles exposed to sub-lethal concentrations of chlorpyrifos in 4 and 12 day laboratory experiments with and without pond sediment in test chambers. Points are averages within exposure chambers then across replicates (n=4) and bars represent standard errors. Refer to text for statistical differences.
Figure 3.3 Log transformed individual tadpole cholinesterase activity exposed to concentrations of chlorpyrifos ranging from 0 to 200 μg/l plotted against swim speeds (size corrected), separated by sediment treatments for A) *Rana sphenoecephala* Day 4 B) *R. sphenoecephala* Day 12, C) *Hyla chrysoscelis* Day 4, D) *H. chrysoscelis* Day 12, E) *Gastrophryne olivacea*, and F) *Acris crepitans*. Solid and dashed lines are linear regression lines for sediment and nonsediment treatments, respectively.
VITA

Pamela Dawn Widder

Candidate for the Degree of

Master of Science

Thesis: SUB-LETHAL EFFECTS OF ENVIRONMENTALLY RELEVANT LEVELS OF AN ORGANOPHOSPHATE INSECTICIDE ON ANURAN LARVAE

Major Field: Zoology

Biographical:

Personal Data: Born in Lancaster, Pennsylvania, on March 1, 1975, the fifth child and third daughter of Christopher T. Widder and Patricia L. Widder, fifth granddaughter of Dr. Donald and Ruth Lynch and seventh granddaughter of George and Alice Widder.

Education: Graduated from Sheboygan High School South, Sheboygan, Wisconsin, in May 1993; received an Associate of Science from William Rainey Harper College, Palatine, IL in May 1997; and received a Bachelor of Science from the University of Wisconsin – Stevens Point, Stevens Point, Wisconsin, in May 2001. Completed the Requirements for the Masters of Science at Oklahoma State University in July, 2005.

Experience: Wrote and successfully completed an Undergraduate Research Grant entitled “Daily Variations in Early Spring Anuran Populations and Related Implications to Anuran Monitoring Programs;” completed undergraduate research project on microhabitat parameters associated with herpetofaunal encounters; assisted in a Wisconsin Department of Endangered Resources extensive field survey of two river basins for rare and endangered herpetofauna; and maintained a summer position for two consecutive years as a field research technician in Yosemite National Park in a USGS translocation study assessing impacts of atmospherically transported pesticides on amphibian larvae within “pristine” areas.

Scope and Method of Study: While methods exist to verify exposure of amphibians in natural populations to agricultural chemicals through the use of physiological biomarkers, the ecological relevance of changes in these parameters is unknown in amphibians. My research examined the impact of exposure to a commonly used organophosphate pesticide (OP), chlorpyrifos, on tadpole cholinesterase (ChE; a frequently used enzymatic biomarker of exposure to OP pesticides), growth, and swim speed after four days in four native North American species of anurans (\textit{Hyla chrysoscelis}, \textit{Rana sphenocephala}, \textit{Acris crepitans}, and \textit{Gastrophryne olivacea}) using laboratory tests with an environmentally realistic experimental design. This design included five environmentally relevant concentrations of chlorpyrifos (formulated as the commercially available Dursban). I also examined if the presence or absence of pond sediment influenced tadpole responses. In two species, I further examined how a longer exposure (twelve days) influenced these same responses. Furthermore, in one species (\textit{R. sphenocephala}) I examined differences in ChE, growth, and survival in the presence of a predator between tadpoles at the same development stage exposed in the laboratory (with and without pond sediment) as well as in outdoor mesocosms.

Findings and Conclusions: First, invertebrate predators such as odonate naiads appear to be considerably more sensitive to chlorpyrifos than any tadpole of the species examined here. Second, the presence of sediment in tadpole test systems can clearly influence response to chemicals such as chlorpyrifos, and given the prevalence of sediment as a component in an average developing tadpole’s environment, these differences probably more accurately reflect responses to contamination in the natural environment. Perhaps most importantly, I found species-specific differences in response to the pesticide, underscoring the need for caution when attempting to make generalizations regarding anurans for the purposes of risk assessments and when using “standard” or surrogate test species to predict responses across different anuran taxa.