THE EFFECTS OF THE ORGANOPHOSPHATE PESTICIDE DIAZINON ON TADPOLE GROWTH, DEVELOPMENT, AND FITNESS

A Report of Senior Thesis

by

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ABSTRACT

Research has provided evidence of dramatic declines in the size and number of amphibian populations globally over the last 30 years. Possible reasons for some of the declines are thought to be habitat destruction, drought and unusual rainfall patterns brought on by climate change, introduced invasive species, UV-B radiation, acid precipiation, disease and parasitism, and environmental contaminants, including heavy metals and pesticides. Organophosphate pesticides have been used increasingly in both agricultural and non-agricultural areas because of their relatively rapid environmental degradation. However, these pesticides are acutely toxic to a wide variety of organisms, and as this study has shown, they can have significant negative effects on amphibians at sub-lethal concentrations. This study investigated the effects of diazinon, the most commonly found insecticide in surface water nationally, at concentrations found in non-agricultural use areas on tadpole development. Three groups of Rana pipiens tadpoles were exposed to differing concentrations of diazinon (0.3 μg/L and 3.0 μg/L), and mean length and weight over time, liver mass, percent lipid, and stage were determined for each group. Mean weight over time differed significantly among the three groups, with individuals exposed to the highest levels of diazinon having the slowest rate of growth. Differences among the three groups in length over time, liver weight, and stage were not significant. Future studies should examine the mechanism of this growth disruption.

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CHAPTER I

INTRODUCTION

The California red-legged frog (Rana aurora draytonii) could once be seen in abundance in the California mountains and foothills. Today, however, these frogs can not be found in much of their historic range. Researchers report that this frog has lost 75% of known populations (P. Corn, 2000; Jennings, 1995). This is not an isolated event. Research has evidenced dramatic declines in the size and number of amphibian populations globally over the last 30 years (P. Corn, 2000), and this worldwide amphibian decline has captured the attention of scientists, media, and politicians (Buhlmann et al., 2000).

There has been controversy over what exactly is meant by "declining amphibian populations," as population studies themselves are very complex. Because of the great variability in population sizes and metapopulation dynamics, long-term data must be acquired in order to constitute a trend (P. Corn, 2000). Many of the studies that report these amphibian declines are short-term and focus on small geographical regions (Findlay, Houlahan, Kuzmin, Meyer, & Schmidt, 2000). Although most data may be anecdotal, the great accumulation of evidence has caused most scientists to accept that such a decline is occurring (Phillips, 1990).

In a study recently published in Nature, researchers compiled population data for 157 amphibian species from 37 countries and 8 regions of the world (Findlay et al., 2000). Using this data for 936 populations, they were able to consider population trends across multiple populations. They reported that while there is both geographical and temporal variability, on a global scale there have been significant amphibian declines from approximately 1960 to 1998, with the trends still continuing.

Causes of Amphibian Declines

The overwhelming evidence has researchers across many areas of science searching for causes. Some cases of declines have reasonably understood causes, such as habitat destruction. However some populations have declined or even gone extinct in areas that appear pristine, as in the case of Australia's gastric brooding frog (Rheobatrachus sulus), therefore lacking any distinguishable causes (Buhlmann et al., 2000).

In addition to habitat destruction, possible reasons for some of the declines are thought to be drought and unusual rainfall patterns brought on by global climate change, introduced invasive species, UV-B radiation, acid precipitation, disease and parasitism, and environmental contaminants, including heavy metals and pesticides (Buhlmann et al., 2000). Research has been done examining the relationships between each of these factors and declines of individual amphibian populations (Appendix A).

To add even more complexity to the medley of contributing factors, different species of amphibians do not appear to be affected in the same way by the factors responsible for the declines. In areas where some species have experienced dramatic population declines, others are still abundant (Beebee & Griffiths, 1992). This fact makes the suggestion of global climate change as a cause seem unlikely. In some instances in North America, some populations thrive while others decline even in the same lakes and ponds. A survey of populations in Wyoming's Laramie

Basin found that the Wyoming toad (*Bufo hemiophrys baxteri*) and the northern leopard frog (*Rana pipiens*) both declined to extinction in the 1980s while the boreal chorus frog (*Pseudacris triseriata*) and the tiger salamander (*Ambystoma tigrinum*) thrived (Beebee & Griffiths).

Habitat Destruction

In the endeavor to explain declining amphibian populations, anthropogenic habitat degradation is often cited as the most significant factor contributing to declines (Buhlmann et al., 2000). This degradation includes not only direct destruction of habitat through actions such as deforestation and draining of wetlands, but also fragmentation of habitat by man-made objects such as roads, channels, dams. Studies have shown that amphibian populations are absent or degraded in many urban and intensive agricultural landscapes (Delis, McCoy, & Mushinsky, 1996; Fisher & Shaffer, 1996). P. Corn (1994) suggested that timber harvest in the Pacific Northwest had reduced regional abundance of stream-dwelling amphibians by having long-term effects on their habitat, and Eldrige, Haley, and Petranka (1993) estimated that an annual loss of 14 million salamanders from the Appalachian Mountains could be attributed to the effects of timber harvest. Buhlmann et al. (2000) noted the relationship between Leja's finding that some regions of the United States retain less than 20% of their original wetland acreage (as cited in Buhlmann et al.) and documented declines in associated amphibian populations (Lang, Lannoo, Phillips, & Waltz, 1994 as cited in Buhlmann et al.). Many population declines can be confidently explained by habitat disturbances and destruction, but reports of declines in undisturbed habitats (Drost & Fellers, 1996) have warranted the need for other causes.

Climate Change

Climate change has been suggested as the cause of some cases of amphibian population declines or extinctions. It can be expected that climate change could result in conditions that eliminate or restrict species with limited distributions (Buhlmann et al., 2000; Root & Schneider, 1998). One example is the extinction of the golden toad (*Bufo periglenes*), a species that was once abundant in the Monteverde Cloud Forest Reserve in Costa Rica until 1988, when its observed population dropped to one single toad (Crump & Pounds, 1994). This extinction is thought to have been caused at least in part by global warming effects on montane dry-season mist frequencies (Cambell, Fogden, & Pounds, 1999). Because amphibian survival is intimately dependent upon water, either for breeding or water balance, any serious change in the availability of water could pose a serious challenge to population persistence (P. Corn, 2000). Drost and Fellers (1996) reported that a severe drought experienced by California between 1987 and 1992 undoubtedly had adverse effects on some frog populations in the area, but added that the drought was believed only to have intensified the effects of other factors rather than having been a primary cause of the observed declines.

Introduced Species

Some studies have found significant effects of introduced predatory fish species on amphibian populations. Intensive stocking of the originally fishless waters of the high elevations in the Yosemite area of the Sierra Nevada began in the 1920s, involving thousands of non-native salmonid fish (P. Corn, 2000; Drost & Fellers, 1996). Bradford (1989) reported that mountain yellow-legged frogs (Rana muscosa) and fishes did not coexist in any of 67 lakes sampled in the Sierra Nevada, and Bradford, Graber, and Tabatabai (1994) reported that this frog was absent from 26 of 27 lakes where it had once appeared. Apparently, several western U.S. frog species have been severely reduced by non-native fishes and bullfrogs (*Rana catesbeiana*) that have been and continue to be introduced to wetland habitats of low- and high-elevation lakes (Buhlmann et al., 2000; Fisher & Shaffer, 1996). While such species introductions have certainly been key factors in the fragmentation and decline of certain species of amphibians, frog populations have also disappeared from sites that either were never planted with fish or that are too small and ephemeral to support fish (Drost & Fellers, 1996).

UV-B Radiation

Another factor believed to be contributing to declines is increased levels of UV-B radiation due to depletion of the ozone layer. Blaustein and colleagues suggested that UV-B radiation affects amphibians in ways such as reducing hatching success and decreasing survival to metamorphosis, which could have population level effects (Blaustein & Kiesecker, 1995; Blaustein et al., 1993). While increasing UV-B radiation is an inviting hypothesis to explain mysterious declines of species in seemingly pristine environments (P. Corn, 2000) and very well may be a factor in some species declines, the results of the aforementioned studies have been criticized for many reasons (Grant & Licht, 1997), and several other studies (P. Corn, 1998; 2000; Davis, Novales Flamarique, & Ovaska, 1997; Grant & Licht, 1995) contradict the results.

Acid Deposition

Acid deposition has been suggested as a factor involved in declines of some amphibian species. Wyman (1990) reported that ponds with pHs in the low 4s have been implicated in the declines of several *Ambystoma* species. He noted, however, that the temporary acidification of ephemeral ponds and streams early in the spring following snow melt may be only partially responsible for the declines (Wyman, 1990). He chose to focus on soil acidification, noting that most amphibians spend much of their time in terrestrial habitats and that forest soils are more acidic than are the freshwater aquatic habitats they surround (Wyman, 1988). Wyman (1988) found that adults of at least 10 species of amphibians in the northeastern U.S. show distributions and densities that are positively correlated with soil Ph, meaning that as Ph rises, so do the number of species and the densities of individuals belonging to those species. In a field study of lakes throughout the Sierra Nevada, however, Bradford and Gordon (1992) found no significant relationship between Ph and amphibian distribution. Similarly, S. Corn and Vertucci (1995) concluded that the declines of amphibian species in the Rocky Mountains were most likely due to factors other than acidic deposition.

Disease & Parasites

Parasites and disease are suspected as causes for declines of some amphibian species and may rival habitat destruction as the largest cause of declines (Buhlmann et al., 2000). Studies in Australia, the United Kingdom, and North and Central America have identified two diseases that are causing mass deaths globally (Berger et al., 1999). A new chytrid fungus is spreading like an "extinction wave" through Australia and is also causing mass deaths in the rain forests of Central and South America and some parts of North America. It has been reported from 38 amphibian species in 12 families. Ranaviral infections have caused mass deaths in the United States, the United Kingdom, and Canada. Both of these infectious diseases have the ability to infect a wide range of amphibian hosts, are globally distributed, and exhibit high virulence, clearly establishing them as global threats to amphibian populations (Berger et al.).

Contaminants

Many studies have been conducted concerning the effects of contaminants on amphibians. Most of these studies have concentrated on the direct toxic effects of contaminants such as metals and pesticides. Toxic chemicals could have a variety of lethal and sublethal effects on amphibians. Organochlorine pesticide residues were shown to cause reduced breeding success (Moore, 1999) and even sex reversal in reptiles (Crain, Guillette, Pickford, & Rooney, 1995). These nonlethal effects of endocrine-disrupting chemicals could result in demographic shifts with detrimental consequences for populations (Buhlmann et al., 2000). Carey (1993) hypothesized that contaminants could be the ultimate cause of disease in amphibians by acting to compromise the function of the immune system.

Combined Stresses

Some studies have suggested a combination of stresses as the reason for declines in amphibian populations. Crump and Pounds (1994) hypothesized that the disappearance of the golden toad was caused by climate change linked to either microparasites or atmospheric contaminants. Disease is the subject of much current discussion, and the relationships between contaminants and disease susceptibility is likely to get increased attention.

Pesticides

Despite the fact that research has been done on the many different possible factors that could be influencing amphibian populations, there remains a lack of clear answers for many of the declines (Drost & Fellers, 1996). Though amphibians are not frequently tested in published reports regarding the toxic effects of current use pesticides, they are known to have varying degrees of tolerance to pesticides among species (Bridges & Semlitsch, 2000), and their breeding season often coincides with the season of pesticide application (Alvarez, Herr‡ez, & Honrubia, 1994).

Organochlorine Pesticides

Paul Müller's discovery of the insecticidal properties of DDT (dichloro-diphenyl-trichloroethane) in 1939 was acclaimed as a great scientific achievement and led to his being awarded the Nobel Prize (Blus, 1995). DDT was found to be very successful in controlling human health pests and was therefore sprayed or dusted on crops, houses, people, and livestock all over the world in the attempt to combat insects. Cunningham and Saigo (1999) note that more than 50 million pounds of DDT had been sprayed on fields, forests, and cities in the U.S. by 1950. The effectiveness of DDT lead to the development of other similar organochlorine pesticides. In the 1960s, researchers saw a sharp decline in predatory bird populations (Cunningham & Saigo). Evidence accumulated that suggested widespread adverse effects of organochlorine pesticides on non-target organisms (Blus). Studies revealed the toxicity, persistence, and lipophilic characteristics of these chemicals, which were shown to result in accumulation of residues, mortality, lowered reproductive success, and decline—even extirpation—of certain wildlife populations (Blus). Not only was acute exposure to large concentrations of the pesticides shown to be lethal, but chronic as well as acute sub-lethal exposure to the chemicals was shown to cause eggshell thinning and to disrupt the endocrine systems of many animals, producing sub-lethal effects such as decreased reproductive success, metabolic and behavioral abnormalities, feminization of male species, and masculinization of female species (Moore, 1999).

To farmers and to countries combating malaria, organochlorines seemed to be a wonderful discovery, as they are cheap, stable, soluble in oil, and easily distributed. DDT is highly toxic to target insects but relatively non-toxic to mammals. For these reasons, DDT was widely used in the ’40s, ’50s, and ’60s throughout the world. However, as would be seen over time, widespread indiscriminate and excessive use of these chemicals would have enormous effects on non-target organisms, many of which are still yet to be fully understood or even discovered. Evidence of these effects led to the banning of DDT in most industrialized countries in the early 1970s (Cunningham & Saigo, 1999). Though virtually all uses of organochlorine pesticides have been discontinued in the U.S., some other countries still permit their use (Chambers, 1994). While overall concentrations have declined, DDT and other organochlorines along with their break-down products remain widespread throughout the environment (Bunck & Schmitt, 1995).

Organophosphate Pesticides

Because of the environmental longevity and toxic effects of organochlorines, the agriculture industry has increasingly relied upon organophosphate and carbamate pesticides (Ragnarsdottir, 2000). These pesticides are presumed to be safe due to their rapid environmental degradation (relative to organochlorines). The organophosphate pesticide diazinon, for example, has an aerobic soil half-life of approximately 38 days in sandy loam soil of neutral Ph and an abiotic hydrolysis half-life of 138 days under neutral conditions (Dye et al., 1999). However, the effects of such “new generation” pesticides on wildlife are a growing concern. Although organophosphates are generally less persistent and bioaccumulative than organochlorines, many have relatively high toxicities and are acutely toxic to a wide variety of non-target organisms (Cowman & Mazanti, 2000).

Organophosphate pesticides are neurotoxins that disrupt the central nervous system of animals by inhibiting the enzyme acetylcholinesterase (Ragnarsdottir, 2000). This enzyme is essential to the proper function of nerve impulses. Nerve impulses are transmitted across synapses from neuron to neuron or from neuron to muscle by neurotransmitters such as acetylcholine. In order to end transmission of the impulse, acetylcholine must be hydrolyzed. This hydrolysis is catalyzed by the enzyme acetylcholinesterase. Organophosphate compounds (and carbamates) bind to this enzyme covalently, thereby changing its form and inhibiting its ability to function. As a result, excessive acetylcholine accumulates at the synapses, and nerves are over-stimulated, causing muscular spasms (Cowman & Mazanti, 2000; Ragnarsdottir). Organophosphates do not only inhibit acetylcholinesterase but may also target other vital enzymes, such as neuropathy target esterase, serum cholinesterase, serum paroxonase, and serum arylesterase in humans (Ragnarsdottir).

In addition to their high toxicities, organophosphates are generally much more water-soluble than organochlorines, therefore allowing them to be easily transported through soils and into surface and groundwaters (Ragnarsdottir, 2000). Despite their supposed rapid degradation, they are commonly detected in soil, surface and groundwaters, river sediment, rain water, snow, fog, and air. Aston and Seiber (1996) even found organophosphates in pine needles in the Sierra Nevada mountains, miles from the agricultural area where they were sprayed. Such observations demonstrate that organophosphates can be transported long distances by water and by the atmosphere.

The presence of organophosphate pesticides in water may result from runoff, misapplication, drift, and disposal of cattle and sheep dip (Ragnarsdottir, 2000). Price, Szeto, and Wan (1994) reported that in a study of farm ditches flowing into important rivers in the Lower Fraser Valley of British Columbia, diazinon and dimethoate were consistently found at all sample locations, with average concentrations of 0.07 μg/L and 0.27 μg/L. Concentrations of diazinon detected in water samples collected at orchard pond sites in Ontario Canada ranged from 0.09 to 0.78 μg/L (Bishop, Bogart, Harris, Ripley, & Struger, 1997).

According to the United States Environmental Protection Agency (EPA), major and extremely significant environmental concerns are associated with the use of the organophosphate diazinon, over 6 million pounds of which are used each year in the U.S. (Dye et al., 1999). Up to 70% of the diazinon used each year in the U.S. is applied around residences and other buildings, as opposed to agricultural uses. The application rate is approximately 4 pounds of active ingredient per acre, and applications can be repeated "as necessary." Diazinon used in these areas is very prone to run off into creeks, streams, ponds, and other bodies of water. Diazinon is the most commonly found insecticide in surface water nationally, having been detected in the surface water of 24 states and the District of Columbia (Dye et al.). According to the EPA, 65.6% of surface-water samples in non-agricultural use-areas contained diazinon, with a 95th percentile concentration in streams of 0.28 μg/L and a peak concentration of 2.90 μg/L (p. 35). Diazinon is also one of the most commonly detected insecticides in air, rain, and fog. Diazinon is highly toxic to a wide variety of animals, and in recent years, has been the cause of more reported incidents of wildlife mortality than any other pesticide (Dye et al.).

Effects of Organophosphate Pesticides on Amphibians

Organophosphorus pesticides may be harmful to amphibian species through both lethal and sublethal effects. Most studies on the effects of pesticides on amphibians have focused on the toxicity of the chemicals to eggs or larvae in a laboratory environment. Many recent tests have used the clawed frog Xenopus laevis as the test species. This species is more tolerant to many environmental contaminants than are many other amphibian species (Cowman & Mazanti, 2000). This presents a problem for extrapolating such test information for use on determining the effects of the test pesticides on amphibians in the wild. Also, such tests do not provide information on the sublethal effects of the contaminants. Pesticides could have a variety of sublethal effects on amphibians, causing changes in behavior, growth, and development, that could decrease an individual’s likelihood of surviving in the wild and reproducing, thus affecting populations (P. Corn, 2000; Cowan & Mazanti).

Amphibian Metamorphosis

Following embryogenesis, during which cells are rapidly dividing and young have not yet developed a full complement of nonspecific humoral and cellular defenses or the metabolizing enzymes of a functioning liver, metamorphosis may be the period when amphibians are most sensitive to chemically and physically induced damage (Beasley, Murphy, & Phillips, 2000). Metamorphosis is a complex series of abrupt postembryonic changes, involving structural, physiological, biochemical, and behavioral transformations (Duellman & Trueb, 1986). Anurans convert from aquatic herbivores to air-breathing terrestrial carnivores through a complete restructuring of the respiratory system, digestive tract, cranium, jaw, and pelvic girdle. Immunological modifications occur during this time as well, in which the immune system is impaired in order to prevent autoimmune reactions to adult cells forming in the larval body (Beasley et al.). Thus, it could be expected that amphibians would be more susceptible to stresses produced by environmental disturbances during this period. In addition, the mobilization of energy reserves during this period could release stored contaminants into the blood.

All of the morphological and physiological changes that take place are controlled by an intricate integration of hormones. The thyroid is considered to be the key element exerting control over amphibian metamorphosis (Appendix B). The primary hormones produced by the thyroid glands are tetraiodothyronine (T_4, thyroxin) and triiodothyronine (T_3). These hormones are synthesized on the protein thyroglobulin by the iodination of thyrosine residues, and they will move into the bloodstream upon the stimulation of the thyroid gland by thyroid-stimulating hormone, which is produced by the pituitary gland. For production of this hormone to take place, the thyroid must be stimulated by thyrotropin-releasing hormone, which is produced by the hypothalamus. Thus, activation of thyrotropic activity ultimately depends upon stimulation by the hypothalamus. Other hormones, such as prolactin from the pituitary and corticosterone and cortisol from the interrenal glands also play roles in the direction of growth and development. Prolactin acts on peripheral endocrine organs and affects the activity of the thryroid gland. Corticosterone and cortisol are thought to facilitate thyroid induction of metamorphosis (Duellman & Trueb, 1986). Any disruptions to this complex system of hormone control (e.g., via altered synthesis, elimination, metabolism, interference at thyroxin receptors, or abnormal interactions with other endogenous hormones) may result in effects upon larval development (Beasley et al., 2000; Hayes & Wu, 1995).

Specific Effects of Organophosphates

As noted by Bryant and Carey (1995), environmental toxicants may affect amphibian young in a number of ways. Lethal concentrations can directly cause mortality of eggs, larvae or metamorphosing individuals. Many studies have determined the acute toxicities of organophosphorus pesticides for a variety of species (Bishop et al., 1997; Dutta & Mohanty-Hejmadi, 1981; Griffis, Nebeker, & Schuytema, 1994; Hall & Henry, 1992; Katdare & Pawar, 1982). As previously noted, however, acute toxicity determinations give no information about the effects of exposure to sublethal concentrations of pesticides, which may cause mortality indirectly by producing effects in individuals that reduce their ability to survive in their natural habitat. Sublethal concentrations of pesticides may indirectly affect survival of amphibian young by: (1) impairing the ability to reproduce by disrupting physiological, morphological, or behavioral processes; (2) inhibiting the ability of larvae to avoid predators; (3) increasing susceptibility to pathogenic organisms and disease; or (4) retarding growth and metamorphosis (Bryant & Carey, 1995).

Pesticides that have estrogenic, antiestrogenic, thyroid-disrupting, androgenic, or antiandrogenic properties may impair or inhibit reproduction by disrupting developmental processes. For example, studies have shown that the DDT metabolite DDE mimics the effects of estrogen and alters the balance of reproductive hormones, resulting in reproductive dysfunction in alligators (Crain, Guillette, Percival, Pickford & Rooney, 1996; Crain et al., 1995; Crain, Guillette, Pickford, Rooney & Woodward, 2000). It has also been noted that members of the last remaining population of the endangered Wyoming Toad (Bufo hemiophrys baxteri) may have been negatively impacted by endocrine-disrupting chemicals (Bryant & Carey, 1995).

Pesticides can also affect amphibian larvae in ways that make them more vulnerable to predation. Fish, snakes, birds, insect larvae, and other amphibians prey on amphibian larvae (Duellman & Trueb, 1986). Reduced swimming ability, an obvious consequence of the deformities that can be produced by organophosphorus pesticides, decreases the ability of larvae to avoid predation (Cooke, 1981). Abnormal pigmentation may also decrease the ability of larvae to use camouflage as a predator avoidance mechanism.

According to recent hypotheses, sublethal levels of environmental toxicants, acting singly or synergistically, could stress larval or postmetamorphic amphibians sufficiently that their immune systems become compromised, leading to infection by opportunistic pathogens followed by death (Bryant & Carey, 1995; Carey, 1993; Carey, Cohen & Rollins-Smith, 1999). One study found that severe immunodepression was induced by a rise in circulating corticosterone levels (el Deeb, el Ridi, Saad, & Soliman, 1986). Therefore, it is possible that toxicants causing a rise in corticosterone levels as a sublethal effect in amphibians could be, in effect, causing immunosuppression (Bryant & Carey). As noted by Bryant and Carey, because resistance to pathogens results from a complex, multifaceted immune system that gradually develops, amphibian eggs, larvae, and metamorphosing individuals may be more vulnerable to environmentally influenced disruption of immune function than adults in which the immune system is fully developed. Immunosuppression by environmental toxicants offers an attractive hypothesis for the cause of declines that occur in some amphibian species but not in others because of the possibility that variability in tolerance to environmental stressors may correlate with variation in vulnerability to disease (Bridges & Semlitsch, 1999).

Some studies have shown that sublethal concentrations of organophosphorus pesticides affect growth and development of amphibian young. Griffis et al. (1995) found that Guthion, a broad-spectrum organophosphate pesticide, impaired growth at 1.7 mg/L for _Xenopus laevis_ tadpoles and 9.67 mg/L for _Hyla regilla_ tadpoles (as cited in Cowman & Mazanti, 2000). Fenitrothion, an organophosphorus pesticide commonly used to manage insect pests in coniferous forests (Cowman & Mazanti), was found to cause retarded growth, abnormal curvature of body axis, poor body pigmentation, and feeble blood circulation at concentrations of 3 ppm and higher (Katdare & Pawar, 1982). Dutta and Mohanty-Hejmadi (1981) studied the effects of several organophosphorus pesticides on the development of the Indian bull frog *Rana tigerina*. They reported that nonlethal concentrations of the pesticides caused developmental arrest and that this occurred at three critical life stages—the feeding, limb bud, and well-developed hind limb stages. Diazinon and Basudin produced deformities at concentrations of 0.5-50 μg/L and 1-25 μg a.i./L in green frog tadpoles (Bishop et al., 1997). The deformities produced were characterized as abdominal and head edemas and blistering, ventral and lateral flexure of tail, stunting of tail, and underdevelopment of the gills. Chambers and Snawder (1989) reported that four organophosphorus pesticides caused dose-dependent developmental effects such as abnormal pigmentation, abnormal gut development, notochordal defects and reduced growth. Methyl parathion caused skeletal deformities in *Rana perezi* tadpoles exposed to low concentrations (0.25 mg/L) for 14 weeks beginning at the egg stage (Alvarez et al., 1994). Deformities occurred in 100% of treated tadpoles that survived to metamorphosis in the increased concentration group (1 mg/L).

Body size at metamorphosis in amphibians is extremely important because of the relation to risks associated with predation and inter- and intraspecific competition (Wilbur, 1984). The timing of metamorphosis is also important. In amphibian larvae that commonly complete metamorphosis in their first summer and overwinter as metamorphosed individuals, delayed growth may force them to overwinter as larvae (Bryant & Carey, 1995). These larvae may not survive the winter because their species lacks the specializations that promote winter survival in the larval form. Retardation of growth also has the effect of causing larvae to spend more time in smaller, more vulnerable life stages, causing them to suffer a higher cumulative risk of death than faster growing larvae.

Purpose

Many studies have concentrated on the toxic effects of acute exposure of amphibian larvae to pesticides. Fewer studies have shown that exposure to sublethal concentrations of organophosphorus pesticides can affect behavior, growth, and development of amphibian young in ways that would decrease their ability to survive in the wild. According to the U.S. EPA, the organophosphorus pesticide diazinon is the most commonly found insecticide in surface water nationally (Dye et al., 1999). In light of reports of widespread contamination of surface waters by diazinon and information reported about the effects of organophosphorus pesticides on amphibians and other wildlife, this thesis is intended to study the effects of the organophosphorus pesticide diazinon at concentrations found in agricultural runoff on the growth, development, and overall fitness of the young of the northern leopard frog (Rana pipiens) (Appendix C), which is widely distributed throughout the northern United States and southern Canada (Collins & Conant, 1991). It is hypothesized that chronic exposure to diazinon will affect behavior and will retard growth and development in Rana pipiens tadpoles.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

Northern leopard frog (Rana pipiens) tadpoles were ordered from Ward's Natural Science and, upon arrival, were on average about one week old. The tadpoles were divided into three groups of thirty to thirty-five individuals and were placed in three 10-gallon containers, each containing 6 gallons of dechlorinated tap water that had been allowed at least 24 hours for aeration. An equal measured amount of food (~ 0.6 g) was added to each container. Ground Nasco Xenopus frog brittle and Hartz Staple Flake Food were used. Exposure to the diazinon was not initiated until four days later so as to allow for any individuals that did not survive the transition.

On day 1, the pesticide exposure was initiated. Using a colander to collect the tadpoles, the water in each container was replaced with the water for the given experimental group. The three experimental groups were named Control, Low, and High. The control group was exposed only to dechlorinated tap water. The Low and High groups were exposed to de-chlorinated tap water with diazinon active ingredient concentrations of 0.3 μg/L and 3.0 μg/L. The pesticide used was No-Pest diazinon insect spray concentrate which was purchased from Lowe's®. The water temperature was measured. For each individual the weight, total body length, and snout-vent length were measured. Each individual was removed using a dip net, blotted with a paper towel to remove excess water, and weighed on a balance to the nearest milligram. The length and snout-vent length were then measured to the nearest millimeter using a ruler.

Every third and seventh day, the water temperature was recorded, the water in each container was changed, and an equal measured amount of food was added to each container. The lengths and weights of the tadpoles were measured on days 1, 4, 11, and 18. Appearance and behavior were also noted. When the tadpoles began to develop forelimbs, Styrofoam pieces were added to the containers to provide a surface upon which they could complete the final stages of metamorphosis.

Individuals that died during the study were removed when they were found and were preserved if they were not already decomposed. On day 25 all tadpoles were transferred into three smaller containers containing only dechlorinated water. After refrigerating for a 24-hour period to induce torpor, individuals were frozen in whirl packs at -70°C.

Endpoints Measured

Many endpoints were measured in order to determine how the experimental treatments affected certain aspects of the growth and development of the tadpoles. These endpoints were measured only for those individuals that were still alive at the conclusion of the study. Each individual was staged according to Gosner (1960). The mass of each individual was determined, as well as the mass of the liver of each individual. A percent lipid analysis was conducted for each individual. The lipid extraction method employed a 3:2 isopropanol/hexane solvent. Each individual tadpole was homogenized in 8 Ml of the solvent using a Glas-Col Stirrer with a ceramic tip on setting 3 for 1 minute. The homogenate was then poured into a glass filter funnel (Whatman No. 1 filter paper), and the retenate and ceramic bit were washed with 5 Ml and 2 Ml of the solvent, which were also poured into the funnel. The organic filtrate was then collected in a pre-weighed aluminum weighing boat and allowed to dry under a hood. The balance used was a Mettler Single Pan Analytical Balance. After approximately 22 hours when the solvent had evaporated, the boat was weighed again, and the weight difference was recorded as the total lipid in the individual. The percent lipid was calculated using the total lipid and the total wet weight of the individual.

Data Analysis

The stage, percent lipid, and liver mass data from the individuals in each of the three experimental groups were analyzed using Analysis of Variance (ANOVA) in Microsoft Excel to determine significance. ANOVA was also used to analyze the initial and final length and weight data. Mean weight and snout-vent length over time were compared among the groups, and the program StatView was used to perform an Analysis of Covariance (ANCOVA) on these data.

CHAPTER III

RESULTS

Length and Weight

As shown in Figures 1 and 2, the growth rates of the tadpoles did appear to be different among the three groups. This is depicted by differences in the slopes of the trendlines of each group's data when the data was log-transformed to obtain linearity (Fig. 3 and 4).

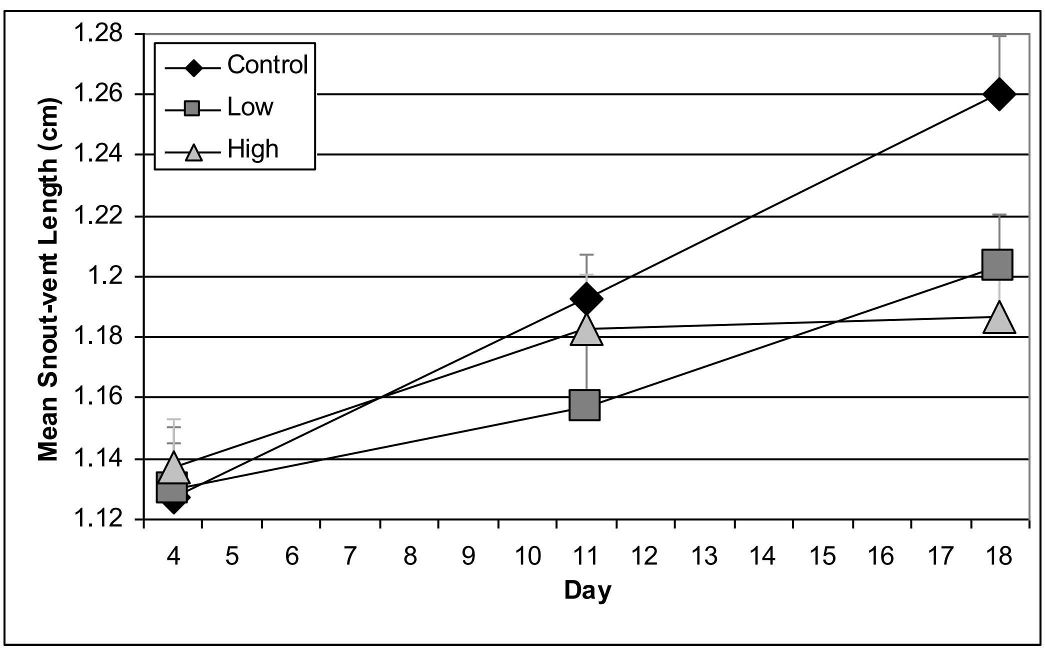

Fig. 1. Mean Snout-Vent Length (+ 1 SE) over Time for Tadpoles in the Three Experimental Groups.

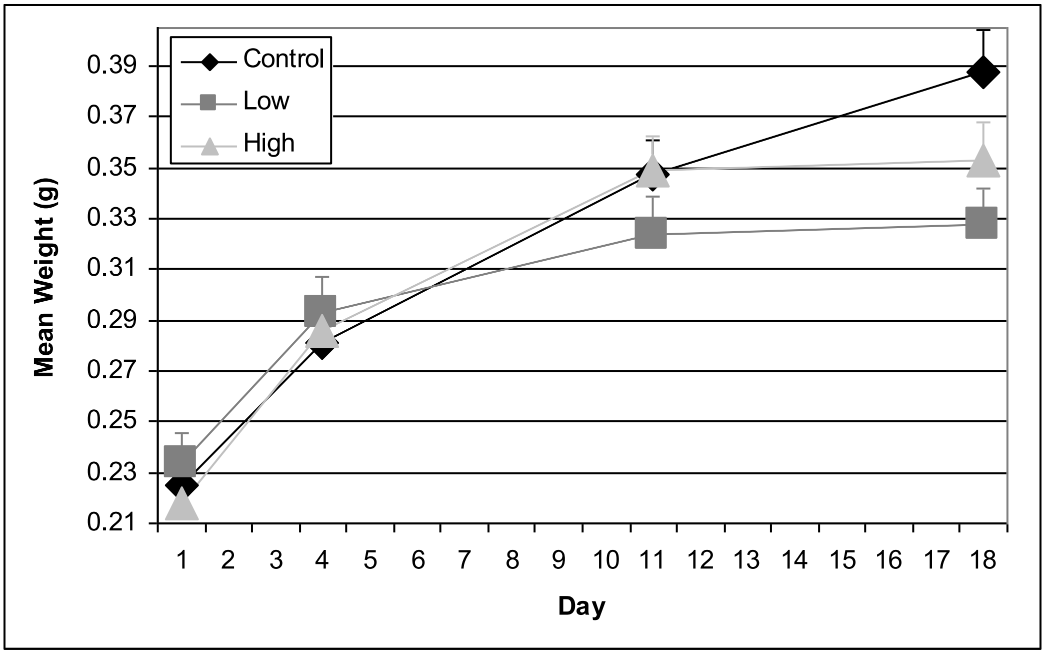

Fig. 2. Mean Weight (+ 1 SE) over Time for Tadpoles in the Three Experimental Groups.

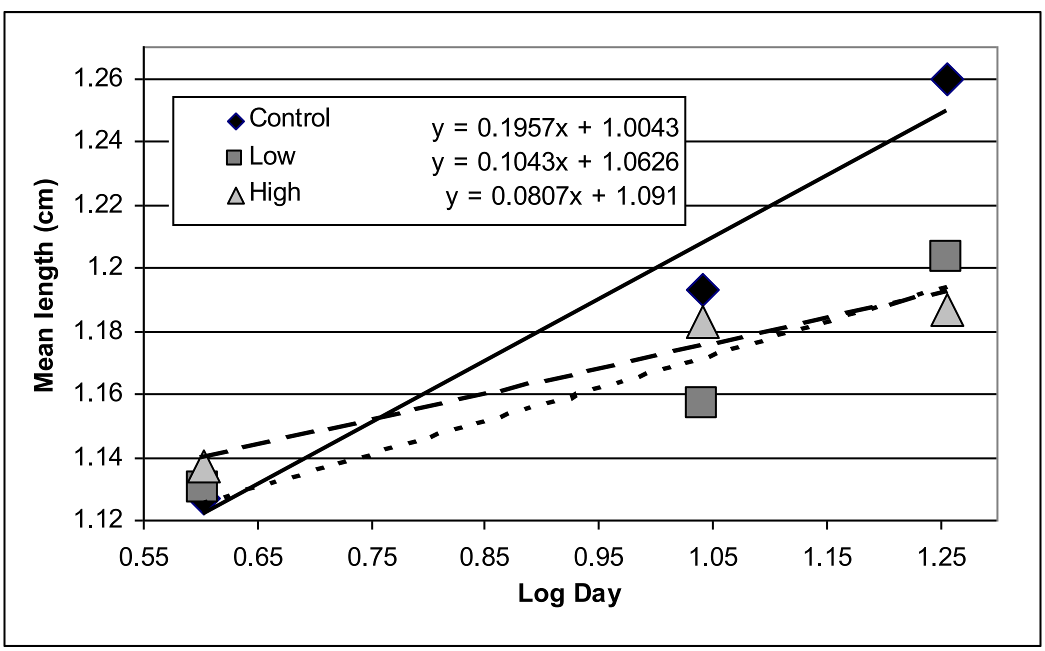

Fig. 3. Mean Length over Time (Log Day) for Tadpoles in the Three Experimental Groups.

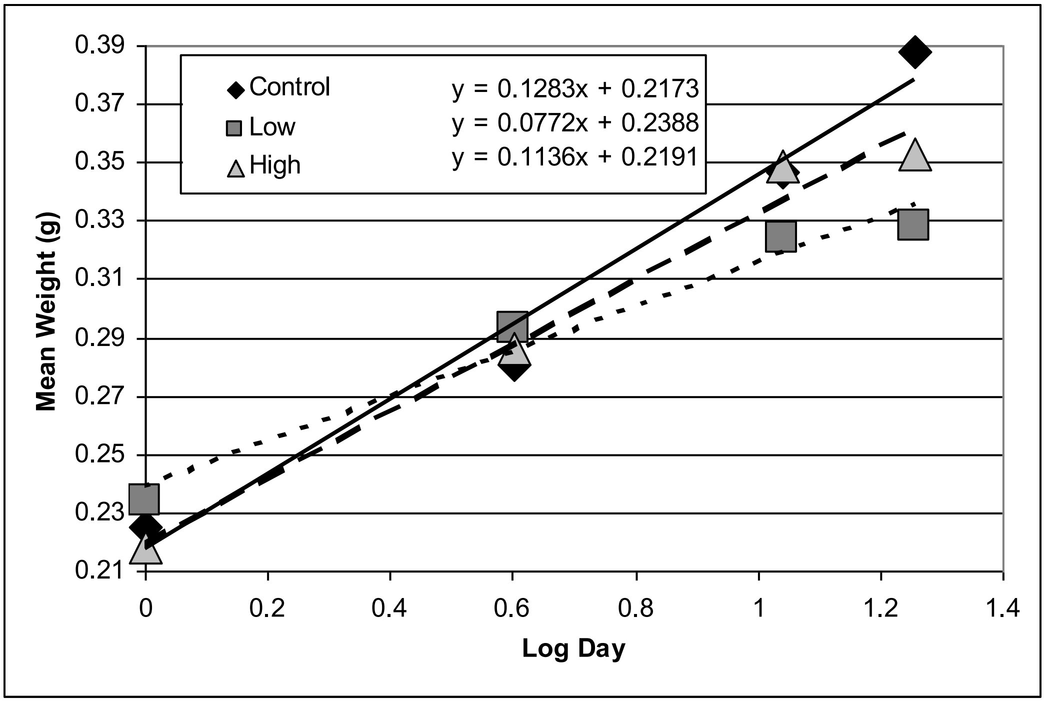

Fig. 4. Mean Weight over Time (Log Day) for Tadpoles in the Three Experimental Groups.

When analyzed using ANCOVA, weight over time (log day) was found to be significantly different among the three experimental groups ($p = 0.0280$). ANOVA analysis indicated that initial weights were not significantly different among groups ($p = 0.5706$), but final weights were significantly different among the three groups ($p = 0.0198$). ANCOVA indicated that differences among the three groups in length over time were not statistically significant ($p = 0.0821$).

However, ANOVA showed that difference in length among groups did become significant in the time between the initial and final measurements ($p_i = 0.9253$, $p_f = 0.0124$).

Liver

When mean liver mass was compared among the groups (Fig. 5), ANOVA showed significant differences among the three groups ($p = 0.0362$). When grouped according to stage (Fig. 6), ANOVA showed significant differences in mean liver mass among the three groups in individuals in stages 37-41 ($p = 0.01847$), but not in individuals in stages 42-45 ($p = 0.4533$). When liver mass was taken as a percentage of body weight (Fig. 7), ANOVA showed that there were no significant differences among the three groups in individuals in stages 37-41 ($p = 0.1847$) or 42-45 ($p = 0.8605$).

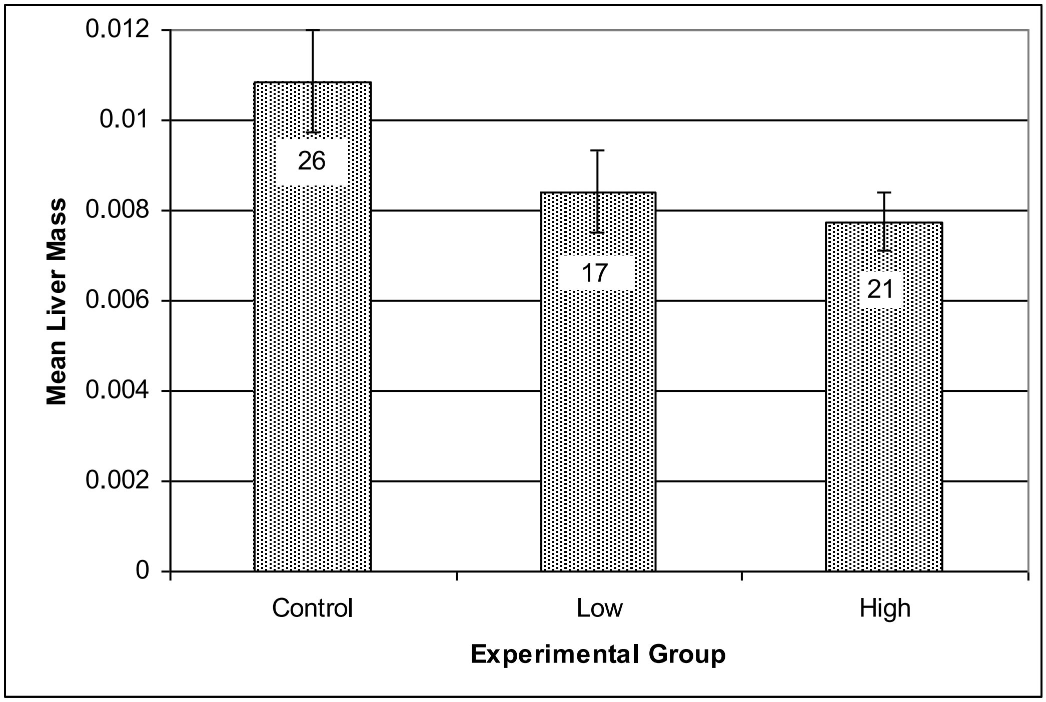

Fig. 5. Mean Liver Mass (± 1 SE) for Each Experimental Group at Day 25. Sample size is shown on each bar.

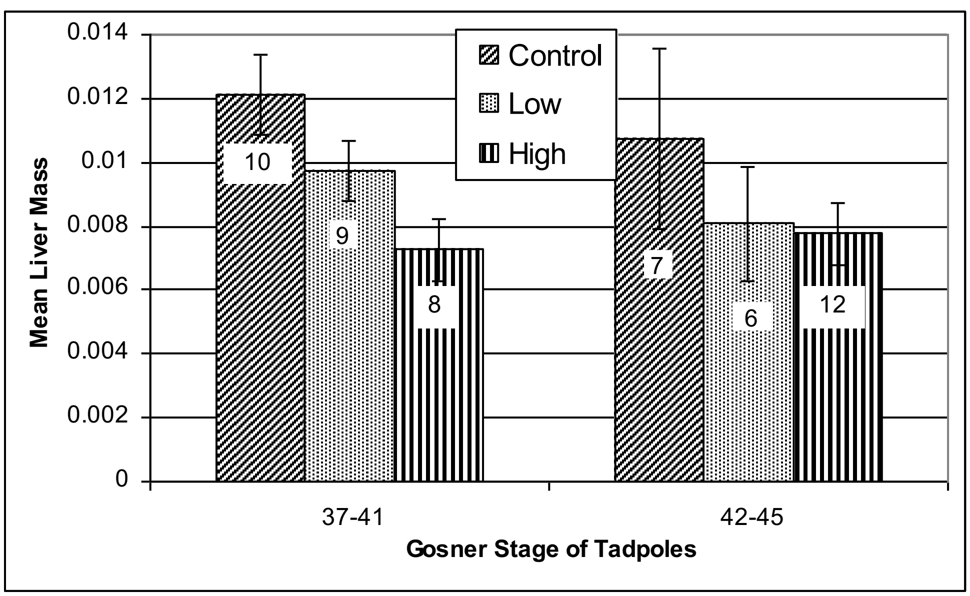

Fig. 6. Mean Liver Mass (+ 1 SE) for Tadpoles at Gosner Stages 37-41 and 42-45. Sample size is shown on each bar.

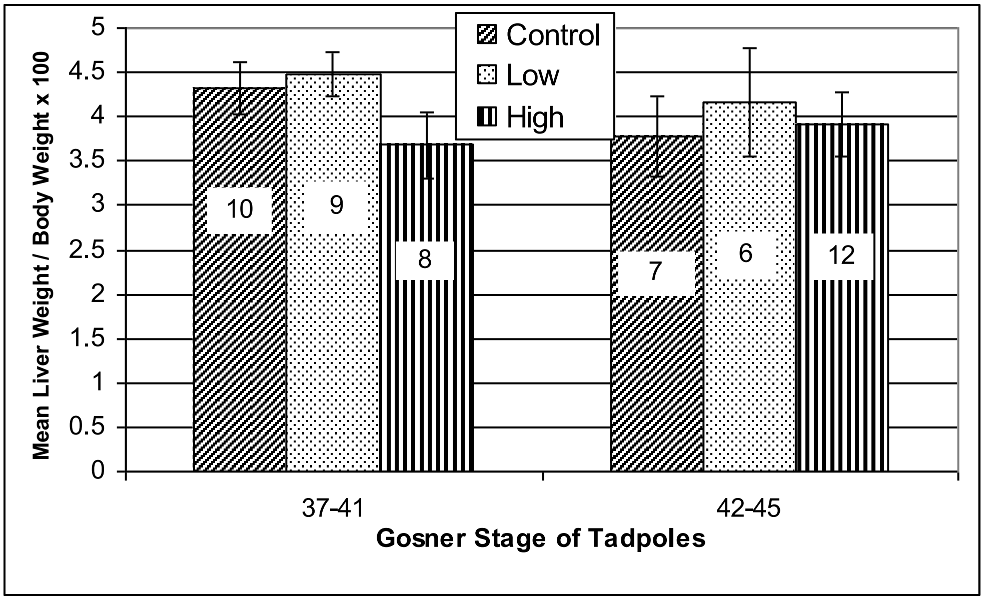

Fig. 7. Mean Liver Weight as a Percentage of Body Weight (+ 1 SE) for Tadpoles at Gosner Stages 37-41 and 42-45. Sample size is shown on each bar.

Stage and Percent Lipid

Differences in the mean stage of individuals in each group are depicted in Figure 8. These differences, however, were not significant ($p = 0.1107$). Differences in mean percent lipid between the three groups (Fig. 9) were also not significant ($p = 0.1026$).

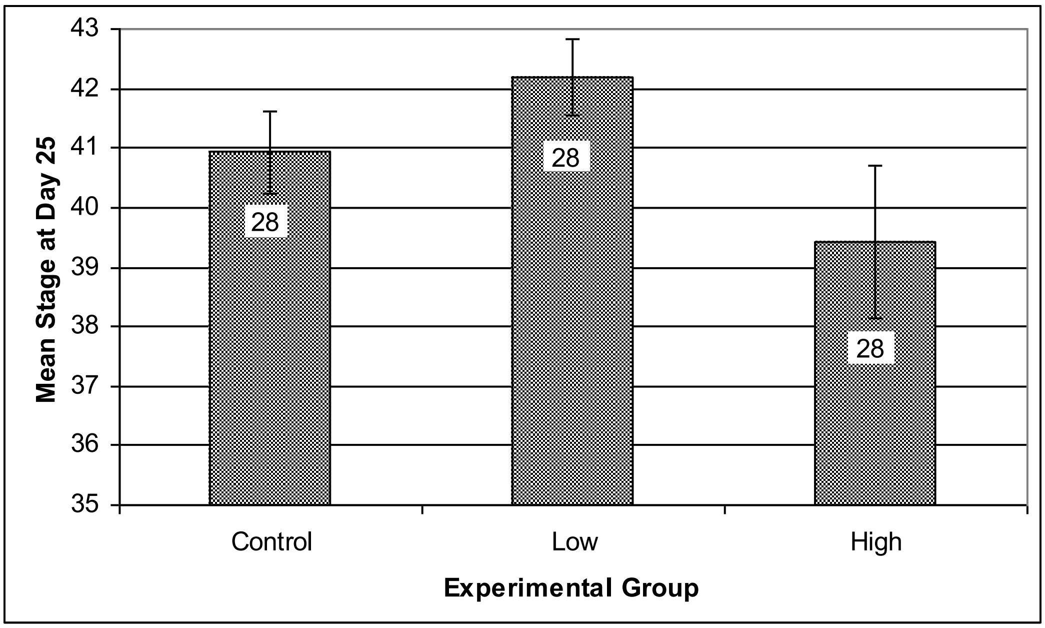

Fig. 8. Mean Stage of Each Experimental Group ($\pm$ 1 SE) at Day 25. Sample size is shown on each bar.

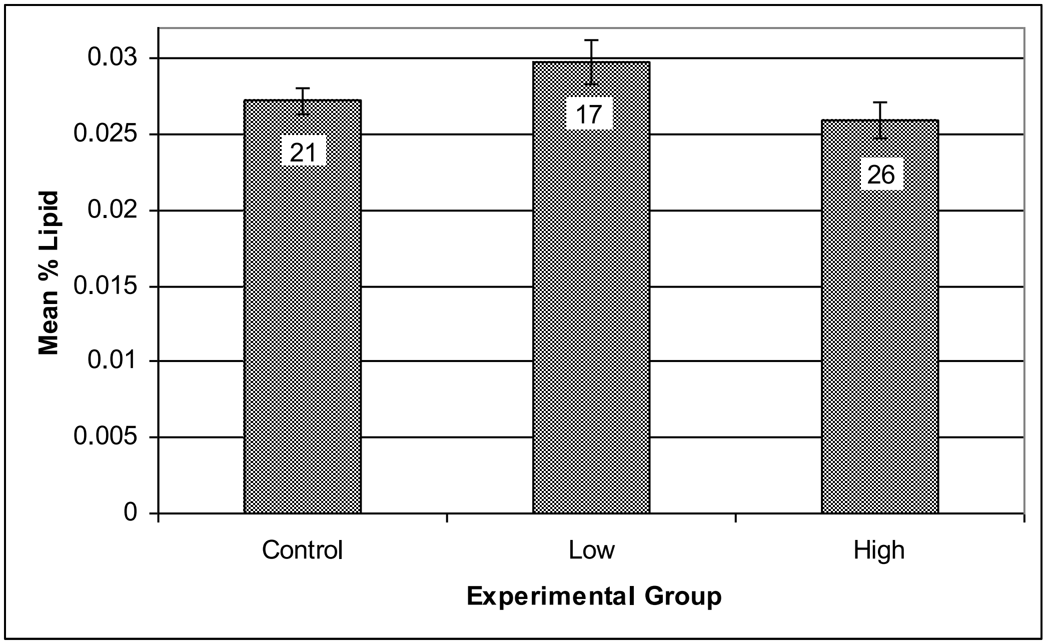

Fig. 9. Mean Percent Lipid (± 1 SE) at Day 25 for Each Experimental Group. Sample size is shown on each bar.

Appearance and Behavior

No differences in appearance or behavior in individuals were noticed among the three groups.

CHAPTER IV

DISCUSSION

This study shows that growth rates in *Rana pipiens* were significantly decreased by diazinon at concentrations that have been detected in nature (Dye et al., 1999). These concentrations were sub-lethal, having caused no mortality during the length of the study. They would therefore be perceived as non-dangerous by those researchers interested only in acute toxicity. As indicated by the present study, however, such concentrations can have significant effects.

Of all the response variables measured, growth rate was the most affected. Weight over time was significantly decreased as a result of diazinon exposure. While differences in length over time were not statistically significant among the three groups, a difference is apparent between the control group and the exposure groups, and dose-dependent effect is also noticeable. In addition, as previously noted, mean length was significantly different among the three groups on the final day of the study but was not significantly different on the first day of measurement.

Although the cause of decreased growth rate is unknown, there are several different mechanisms that could explain this effect. First, the effects of diazinon on growth rate may suggest that energy normally allocated to growth is instead being directed toward other processes, such as those involved in muscle contraction. This suggestion is consistent with the mechanism by which organophosphorus pesticides act—through the inhibition of acetylcholinesterase and consequent causation of continuous muscle contraction (Ragnarsdottir, 2000). Acetylcholinesterase activity could be measured to find if it was correlated with growth rate.

Disruption of the endocrine system is another likely mechanism by which diazinon could have caused a decrease in growth rate. Many environmental chemicals have been shown to alter reproduction, growth, and survival by disrupting the normal functioning of the immune system (Crain, Guillette, Orlando, & Rooney, 2000). The thyroid is the key element exerting control over all of the morphological and physiological changes that take place during amphibian metamorphosis (see Appendix B) (Duellman & Trueb, 1986). This is demonstrated by the fact that metamorphosis can be induced by the administration of exogenous thyroid hormone to amphibian larvae and can be prevented by thyroidectomy (Leatherland, 2000). While various hormones and interactions comprise the complex pathways that are ultimately responsible for causing the actual changes to take place in the animal, the primary hormones involved are the thyroid hormones tetraiodothyronine and triiodothyronine (T_4 and T_3). Any disruptions to the production, bioavailability, or action of these hormones would have the potential to elicit significant effects in tadpole growth, development, and survival (Crain, Guillette, Orlando, & Rooney, 2000).

Malathion, another organophosphate pesticide, was found to disrupt thyroid function in fish (Lal, Singh, & Sinha, 1991) and to cause a significant decrease in the amount of circulating hormones T_4 and T_3 in rats (Ahmad, Akhtar, Kayani, & Shahab, 1996). The concentrations of these hormones could have been measured for individuals in this study as well to determine if exposure to diazinon affected thyroid function. If the diazinon acted via this mechanism, however, one would expect to see developmental abnormalities or delays in addition to delayed rate of growth.

While differences in mean stage at day 25 were not significant among the three exposure groups in this study, differences were apparent between the low and high exposure groups. In a 28-day static renewal test, development of bullfrog (Rana catesbeiana) tadpoles was delayed significantly by exposure to malathion (Fordham, Keefe, Ramsdell, & Tessari, 2001). In another study, bullfrog tadpoles were also smaller and had developed more slowly following exposure to fenitrothion, another common AchE inhibiting pesticide (Berrill, Bertram, Kolohon, McGillivray, & Pauli, 1994). Developmental abnormalities and delays in the present study would probably have been more evident if higher concentrations of diazinon had been used. However, this study was not concerned with the effects of concentrations to which tadpoles are not likely to be exposed in the wild. Also, because tadpoles were staged only at the conclusion of the experiment and not during the duration, the stage data do not present good indicies of development.

Negative effects on growth and/or development can hinder an individual's ability to survive and reproduce in the wild. Body size is related to reproductive capacity and to risks associated with predation and interspecific and intraspecific competition (Wilbur, 1984). Size at metamorphosis may affect body size at first reproduction, age at first reproduction, and fecundity (Moeur & Istock, 1980 & Prout & McChesney, 1985 as cited in Pechmann & Semlitsch, 1988). In a study of adult recruitment in chorus frogs (Pseudacris triseriata) long larval period and small body size at metamorphosis delayed maturity, thus reducing the chance that the frogs would return to breed in the next breeding season (Smith, 1987). The first reproduction of the frogs would be an entire year later than that of the tadpoles that metamorphosed more quickly and at a larger size. Therefore, their chance of surviving to first reproduction would be decreased. In a study of the salamander Ambystoma talpoideum, larger body size at metamorphosis lead to larger size at first reproduction (Pechmann & Semlitsch, 1988). Due to the body-size advantage being maintained for the entire lifetime of the individual and to the strong positive relationship of body size with egg number and potentially with mating success, these advantages would affect the lifetime fitness of the individual.

Growth and development are not the only factors important to the survival of amphibian larvae that may be affected by endocrine disrupting chemicals. The immune system of vertebrates is also highly sensitive to endocrine control (Brousseau, Cyr, Fournier, & Tryphonas, 2000). By directly or indirectly influencing endocrine-immune interactions, endocrine disruptors can exert considerable effects on immune function. Immunosuppression, either via this mechanism or through alteration of immune system development, would most certainly lead to decreased survivability and hence decreased fecundity. Further studies should be conducted to determine if immune system capabilities are compromised during or after development by prolonged exposure to sub-lethal concentrations of various pesticides.

While differences in percent lipid were not significant among the three groups, differences were apparent between the low and high exposure groups.

Differences were also not significant when the lipid data was grouped by stage.

Mean liver mass was found to be significantly different among the three groups. However, when liver mass was taken as a percent of body weight, there were no significant differences among the groups. Therefore, it can be assumed that the differences in liver masses among the groups is attributable to the significant differences in body mass among the groups. This suggests that the mechanism of action of diazinon did not act preferentially on the liver in retarding growth.

In conclusion, this study has clearly indicated that the pesticide diazinon, at concentrations found in surface waters of non-agricultural use-areas, can decrease growth rate in _Rana pipiens_ tadpoles. Mean weight over time differed significantly among the three experimental groups and was the most affected of the variables measured. This finding is supported by other similar studies in which organophosphorus pesticides were found to delay growth and development in amphibian young (Berrill et al., 1994; Dutta & Mohanty-Hejmadi, 1981; Katdare & Pawer, 1982). While differences were apparent among the three groups in length over time, liver weight, and stage, these differences were not significant. Further studies should investigate the mechanisms of diazinon-induced growth inhibition via altered acetylcholinesterase and thyroid hormone levels.

APPENDICES

APPENDIX A

References Citing Specific Factors Involved in Amphibian Declines

Factor(s)	References
Habitat destruction	P. Corn, 1994
	Delis, McCoy, & Mushinsky, 1996
	Eldrige, Haley, & Petranka, 1993
	B. Johnson, 1992
Climate change	Caldwell et al., 1991
	Crump & Pounds, 1994
	Drost & Fellers, 1996
Introduced species	Bradford, 1989
	Fisher & Shaffer, 1996
UV Radiation	Blaustein & Kiesecker, 1995
	Blaustein, Sousa, & Wake, 1994

Acid Deposition	Bradford & Gordon, 1992
	S. Corn & Vertucci, 1995
	Clark, 1992
	Wyman, 1988
Disease and parasitism	Berger et al., 1999
	Carey, 1993
	Crawshaw, 1992
	P. Johnson, Lunde, & Ritchie, 1999
Environmental contaminants	Bishop, 1992
	Herman & Scott, 1992
Combined stresses	Blaustein & Kiesecker, 1995
	Crump & Pounds, 1994
	Wake, 1991

APPENDIX B

Major Morphological and Functional Changes Induced by Thyroid Hormones During Amphibian Metamorphosis

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Skin

- Formation of dermal glands
- Degeneration of skin on tail
- Proliferation of skin on limbs
- Formation of skin for forelimb
- Degeneration of operculum
- Formation of nictitating membrane
- Differentiation of Leydig cells
- Sodium transport
- Changes in skin pigments

Muscle

- Degeneration of caudal muscle
- Growth of limb muscles
- Growth of extrinsic eye muscles

Respiratory system

- Regression of gills

Connective and supportive tissues

- Degeneration of tail
- Degeneration of gill arches
- Restructuring of mouth and head

	Calcification of skeleton
Kidney	
	Resorption of pronephros
	Induction of prolactin receptors
Nervous and sensory systems	
	Reduction of Mauthener cells
	Growth of cerebellum
	Increase in retinal rhodopsin
	Growth of dorsal root ganglia
	Growth of hypothalamic nucleus preoticus
	Degeneration of Rohon-Beard cells
	Fusion of internal and external retinas
	Growth of lateral motor column cells
	Development of hypophysial portal system and median eminence
	Growth of mesencephalic V nucleus
Gastrointestinal tract and associated structures	
	Regression and reorganization of intestinal tract
	Reduction and restructuring of pancreas
	Induction of urea-cycle and other enzymes in liver

Source: (based on Nicoll & White, 1981, pp. 363-396)

APPENDIX C

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