EXAMINING THE IMPACTS OF PESTCIDE EXPOSURE ON THE SURVIVORSHIP AND DEVEOPMENT OF GREAT BASIN SPADEFOOT (*SPEA INTERMONTANA*) AND PACIFIC TREEFROG (*PSEUDACRIS REGILLA*) IN A LABORATORY ENVIRONMENT

by

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ABSTRACT

Amphibian populations are declining globally, and pesticides have been suggested as one of the contributing factors. Field experiments involving ponds immersed in agricultural environments have been observed to have dramatically lower biodiversity and amphibian abundance than ponds located in non-agricultural settings. There has been much work involving *in situ* pond experiments, and a plethora of laboratory pesticide experiments often involving test concentrations much higher than those observed in the field. To determine which pesticides impact amphibian embryo survivorship and tadpole development, three insecticides currently used in British Columbia were tested at their detected field concentrations in a laboratory environment. The commercial formulations of endosulfan, azinphos-methyl and diazinon were tested alone and in combination. Embryos of the Great Basin Spadefoot (*Spea intermontana* and Pacific Treefrog (*Pseudacris regilla*) were collected from reference sites in the South Okanagan of BC, and transported to a federal government laboratory facility in North Vancouver, BC. Here, 8-day LC20 experiments were conducted on the young embryos and young tadpoles with the following toxicological endpoints: acute mortality, behavioral abnormalities, morphological abnormalities and developmental abnormalities. Overall, endosulfan ($LC20_{8d}$ = 77.1 ng/L) was the most toxic pesticide to both species in the tadpole stage, causing acute mortality, behavioral abnormalities and morphological abnormalities. Embryos were observed to be very resilient to the low test concentrations of endosulfan, with the majority of mortalities occurring post-hatch ($LC20_{8d}$ = 2872.7 ng/L). The second most toxic insecticide was found to be azinphos-methyl ($LC20_{8d}$ > 50

000 ng/L); and lastly, diazinon was found to be the least toxic ($LC20_{8d}$ > 175 000 ng/L) to both life stages of amphibians. In addition to acute mortality, several behavioral abnormalities arose in the tadpoles exposed to endosulfan, including extreme agitation in both species of amphibians, tail kinking and melanophore aggregation in *P. regilla* tadpoles.

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LIST OF ABBREVIATIONS

A.I.	Active Ingredient
CETIS	Comprehensive Environmental Toxicological Information Software
CNS	Central Nervous System
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
DO	Dissolved Oxygen
EC	Emulsifiable Concentrate
EC20	Effective Concentration of 20%
EC50	Effective Concentration of 50%
HC	Hazardous Concentration
HC_5	Hazardous Concentration of 5%
HC_{10}	Hazardous Concentration of 10%
LC	Lethal Concentration
LC20	Lethal Concentration of 20%
$LC20_{8d}$	Eight-day LC20 experiment
LC50	Lethal Concentration of 50%
LCL	Lower Confidence Limit
MS222	Tricane Methyl-sulphanate
ng/L	nanograms per litre
OCL	Organochlorine
OP	Organophosphate
PESC	Pacific Environmental Science Centre

PSRE	*Pseudacris regilla*
SPIN	*Spea intermontana*
SSD	Species Sensitivity Distribution
UBCACC	University of British Columbia Animal Care Committee
UCL	Upper Confidence Limit
UV-B	Ultraviolet-B radiation
ug/L	micrograms per litre
WP	Wettable Powder

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STATEMENT OF CO-AUTHORSHIP

Alexandra de Jong Westman was responsible for egg collection, rearing of tadpoles, experiment conduction, data analysis and manuscript preparation. Initial experimental design was a joint-effort by Alexandra de Jong Westman, John Elliott, Christine Bishop and Graham van Aggelen. Assistance in data analysis was provided by Kim Cheng and Grant Schroeder. Assistance in manuscript preparation was provided by Christine Bishop.

CHAPTER 1

1.1 Introduction

There is evidence that many amphibian populations are declining in numbers and distribution, with a few unexplained extirpations throughout North America (Carey & Bryant 1995; Pauli et al. 2000; Wegoldt 1989; Welsh & Ollivier 1998; Wind 1999). Agricultural contaminants, such as pesticides, are thought to contribute to these declines (Allran & Karasove 2000; Aspengren et al. 2003; Berrill et al. 1998; Broomhall 2004; Carey & Bryant 1995; Pauli et al. 2000; Rohr et al. 2003; Sparling et al. 2001; Wind 1999). This is supported by reports that in agricultural areas, where pesticides are frequently used, amphibian species exhibit reduced richness and abundance than in adjacent non-agricultural sites (Bishop et al. 1999; Boone & Bridges 2003; Bridges & Semlitsch 2000; Carey & Bryant; Pauli et al. 2000; Rohr et al. 2003; Welsh & Ollivier 1998; Wind 1999). Thus, it is vital to the survival of many amphibian populations, that those responsible for pesticide regulation are informed about the effects of pesticides on amphibian species, their population numbers and their diversity (Bridges 2000; Pauli et al. 2000).

Breeding activity of amphibians typically occurs in early spring, coinciding with heavy rainfall and active pesticide application by agricultural growers (Richardson 2001; Wind 1999). Amphibian egg masses are typically laid as large clumps attached to sticks and aquatic vegetation, with their appearance and structure varying among species (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007). A typical amphibian egg consists of four distinct layers which protect the developing embryo from the

external environment and determine uptake rates, metabolism, distribution and excretion by the embryo (*see* Appendix A). The first jelly coat layer is used to anchor the egg masses to aquatic vegetation; as well as providing protection from mechanical disturbances, attenuating UV-B radiation, and decreasing the likelihood of polyspermy or cross-species fertilization (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974). Typically, amphibian eggs have a double-layered jellycoat, an outermost jellycoat, (J_2) and an innermost jellycoat (J_1), but the number and composition of these jellycoats does differ between amphibian species. Both jellycoats are made up of highly o-glycosylated mucin-type glycoproteins (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974). These jellycoats are synthesized by specific regions of the oviduct and sequentially deposited as the egg travels towards the cloacae of the female. Secretions from the o-glycosylated glycoproteins form components of the extracellular matrix, enabling one egg to adhere to another, and also give amphibian egg masses their slimy feel (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974). These glycoproteins are also what make the jelly coats structurally diverse and species-specific. Furthermore, the number and thickness of the jellycoats greatly affects water uptake clearance rates and elimination rates, which contributes to sensitivity differences between amphibian species (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Gilbert 2004; Iwamatsu et al. 1995; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974).

Inside the jelly layers, is the vitelline envelope, a thick glycoproteinaceous matrix which surrounds the egg plasma membrane (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Gilbert 2004; Iwamatsu et al. 1996; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974). This envelope functions to prevent and limit toxicants and sperm direct access to the egg plasma membrane, partly aided by a zona pelucida, all of which are species-specific, ensuring that no interspecific fertilization occurs (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Gilbert 2004; Iwamatsu et al. 1996; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974). Upon fertilization, this envelope becomes the fertilization membrane, or 'F-layer', when the cortical granules release their contents, transforming the membrane from a semipermeable membrane to a rigid and impermeable membrane, known as egg hardening. The 'F-layer' is typically thicker than the unfertilized, or vitelline envelope, due to the reorganization and growth of microvilli stimulated by the contents of cortical granules (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Gilbert 2004; Iwamatsu et al. 1995; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974).

All three of the aforementioned layers, (outer and inner jellycoats and the vitelline envelope) are wholly referred to as the extracellular matrix, (ECM). The space between the vitelline envelope and egg plasma membrane is known as the perivitelline space. Finally, the egg plasma membrane encloses the unfertilized egg or developing embryo, and its cytoplasm, yolk and cortical granules (Carroll et al. 1991; Duellman & Trueb 1994; Edington et al. 2007; Gilbert 2004; Iwamatsu et al. 1995; Sardet et al. 2002; Shibata et al. 2000; Wyrick et al. 1974).

All anuran species undergo roughly the same developmental changes, from embryo to free-swimming larvae, with the length of time for development and metamorphosis varying a great deal between species. The most widely-accepted and widely-used key to amphibian development is Gosner staging, developed by K. A. Gosner in 1960. The key outlines the changes that anuran embryos and tadpoles undergo en route to metamorphosis. Gosner stage 0 to roughly 10 includes blastulation, forming the vegetal and animal poles and early gastrulation. Stage 15 is indicated by the visible development of the dorsal lip of the blastopore, and extends to when the early neural structures become visible. Stage 17 has been affectionately deemed the "jelly-bean" stage, due to the fact that the developing embryo resembles a bean in shape. Visible twitching of the embryo begins roughly around stage 20, with hatching following in short order, at stage 22. Finally, stage 25 is defined by the tadpole's ability to swim freely and orientate its body upright when at rest (Gosner 1960). Stages 20 through to 25 are often lumped together, for easier identification and communication, to create Field Stage 1 (Gosner 1960).

Amphibians, because of their life-cycle strategies and overall sensitivity to environmental conditions can serve as indicators for ecosystem health. When an ecosystem is stressed, a number of changes can occur, some very obvious, and others more discrete. For example, a stressed ecosystem can undergo alterations in biotic community structure to favour smaller life forms, an overall reduction in species diversity, increased dominance by exotic species, shortened food-chain length, increased disease prevalence and reduced population stability (Carey & Bryant 1995; Khan et al. 2003; Richardson 2001; Sanders 1970; Seburn & Seburn 2000; Welsh & Ollivier 1998;

Wegoldt 1989; Wind 1999). Often the first to be impacted are sensitive species, also known as indicator species, such as amphibians. Amphibians make excellent indicator species because they are sensitive to both aquatic and terrestrial changes due to their biphasic lifecycle, their highly specialized physiological adaptations and their specific microhabitat requirements (Carey & Bryant 1995; Khan et al. 2003; Pauli et al. 2000; Richardson 2001; Sanders 1970; Seburn & Seburn 2000; Welsh & Ollivier 1998; Wegoldt 1989; Wind 1999). These specialized adaptations render amphibians susceptible to even the most minor environmental changes, which can alter their ability to seek cover, avoid predators and forage properly (Carey & Bryant 1995; Khan et al. 2003; Richardson 2001; Sanders 1970; Seburn & Seburn 2000; Welsh & Ollivier 1998; Wegoldt 1989; Wind 1999).

1.2 Literature Review

1.2.1. Aquatic Toxicology

Aquatic toxicology is the study of the effects of manufactured chemicals, natural materials and other activities on aquatic organisms at various levels of biological organization (Rand 1995). Studies in toxicology are centered on the study of cause-and-effect relationship that exists between an organism and the exposure element. In other words, an effect on or a response of an organism is either a direct or indirect result of an exposure to a toxic agent. These effects can be quantified and measured in a reproducible way that is relevant to the toxic processes under examination (Rand 1995). Causality is a critical principle, where there must be reasonable certainty that there is a causal

relationship between the observed effect and the presence of a toxin. The focus in toxicology remains on the adverse effects of the chemicals, and the ability of a system or organism to recover from the toxicant after the exposure has diminished (Landis & Yu 2004; Rand 1995). Common endpoints in aquatic toxicology testing include: acute or chronic mortality; morbidity; changes in development, growth, behavior or reproduction; rates of chemical uptake and detoxification; as well as changes in tissue structure and functioning (Landis & Yu 2004; Rand 1995).

Responses observed in toxicology studies are assessed on the basis of the amount of toxic agent an organism is exposed to; as well as how much of the toxicant actually reaches the site of toxic action (Rand 1995). This is known as a concentration-response relationship, which is a graded relationship between chemical concentration and the severity of the response elicited (Landis & Yu 2004; Rand 1995). Toxic effects occur at all levels of organization, both on a molecular level and on a developmental level, and can be quantified by a number of criteria. Some of these criteria include: the number of organisms killed or surviving; reproductive success, from egg production and hatchability to year-class recruitment; body length and weight changes as a result of exposure; as well as organ condition and physical abnormalities, including the development of tumors (Khan et al. 2003; Landis & Yu 2004; Rand 1995). Ideally there should be efforts to assess a chemical's impact on the overall population of the organism being studied, including population dynamics, species diversity and abundance within an area. In all, it is important to understand the chemical, physical and biological factors that affect environmental concentrations of chemicals to determine their toxicity, how the environment acts on these toxicants and to estimate the timing and duration an organism

is exposed (Boone & Bridges 2003; Bridges 2000). Assessments include determining acute toxicity, sublethal effects, mutagenicity and bioconcentration potential of chemicals on aquatic organisms (Environment Canada 1998; Landis & Yu 2004; Rand 1995).

The toxicity of a chemical is a relative property reflecting a chemical's potential to have a harmful effect on a living organism, and is determined, in part, by: the environmental availability, toxicological bioavailability and the residence time of the chemical in the water (Landis & Yu 2004; Rand 1995). The environmental availability of a toxicant is the portion of the total chemical present in all parts of the environment that could be involved in a particular process, and hence is subject to all chemical, physical and biological modifying factors (Landis & Yu 2004; Rand 1995). Environmental bioavailability refers to the fraction of the environmentally available material that an organism accumulates when processing or encountering it in its environment. A more specific term is the toxicological bioavailability, which is the fraction of the chemical concentration which is either absorbed or adsorbed by an organism, then distributed via systemic circulation and presented to the receptor sites of toxic action. Lastly, the average amount of time a chemical spends in the ecosystem, organism or tissue before being removed by any transport process, is referred to its' residence time (Landis & Yu 2004; Rand 1995). Both water and lipid soluble chemicals may persist and retain some of the parent chemicals' physical and chemical characteristics while being transported and distributed through an ecosystem or organism (Landis & Yu 2004; Rand 1995). This persistence can result in the accumulation of the chemical to toxic levels in both the environment and the exposed organism (Bridges 2000; Environmental Protection Agency 1986; Landis & Yu 2004; Rand 1995).

Chemical toxicity can be divided into two categories: direct toxicity and indirect toxicity. A pesticide's direct toxicity is determined by the reaction of an organism to the active ingredient, via direct contact, such as through the skin or gills. Indirect toxicity can be as a result of either chemical or physical changes to the toxicant, via environmental factors such as UV-radiation, temperature or other chemicals (Environment Canada 1998; Landis & Yu 2004; Rand 1995). An additional example of indirect toxicity is through the use of surfactants in chemical preparations, such as for pesticide application. These surfactants and other inert compounds present in commercial pesticide formulations, are often more toxic to wildlife than the active ingredients (Cox & Surgan 2006; Landis & Yu 2004; Mann & Bidwell 2001; Relyea 2004).

A chemical's direct toxicity can manifest either as acute mortality; chronically as behavioral or developmental abnormalities; or sub-lethally, where the abnormalities result in diminished fitness of the individual or population. Acute toxic effects typically occur rapidly as a result of short-term exposure to a chemical, with severe effects occurring typically within a few hours or days. A chemical that causes abnormalities as a result of a single, but persistent exposure, is said to produce chronic effects. These effects can be lethal or sublethal, with death not being the primary toxic endpoint (Environment Canada 1998; Landis & Yu 2004; Rand 1995). Changes in behaviour, physiology, histology or biochemistry resulting in mortality via the diminished ability to find food and avoid predators all fall under the category of sub-lethal effects (Bridges & Semlitsch 2000; Hatch & Blaustein 2000; Landis & Yu 2004; Rand 1995).

The aforementioned effects can also be reversible or irreversible, local or systemic, selective or non-selective. Reversible effects can typically be repaired by normal repair mechanisms, such as regeneration or recovery from narcosis; whereas irreversible effects often result in death (Landis & Yu 2004; Rand 1995). Some chemicals produce local toxic effects, where the effect is observed only at the site of exposure. Other chemicals may induce a systemic effect, where absorption and distribution of the chemical away from the primary site is required to produce a toxic reaction (Landis & Yu 2004; Rand 1995). Toxic effects can also be nonselective, such as tissue narcosis and have an undesirable effect on all cell or tissue types. Selective toxic effects affect one type of cell or tissue, and is determined by the presence or absence of a specific target site in the exposed cell system (Landis & Yu 2004; Rand 1995).

In every toxic action, there are three phases: the exposure phase, the toxicokinetic phase and the toxicodynamic phase (Landis & Yu 2004; Rand 1995). The exposure phase, as the name suggests, is the duration period an organism is exposed to the environmentally bioavailable portion of the chemical toxicant. Toxicokinetic phase involves the uptake, distribution, metabolism and elimination of the bioavailable portion of the toxic agent. The toxicodynamic phase is the time course of the biological response resulting from the agent reaching the site of toxic action in an organism and interacting with the receptors at the site to produce the observed effect (Landis & Yu 2004; Rand 1995).

There are many factors which can influence a chemical's toxicity, known as modifying factors. These factors can be related to the exposure regime, related to the organism or environment, or even related to the chemical itself (Landis & Yu 2004; Rand

1995). Within an exposure regime, factors such as chemical concentration, length of exposure and the mode of exposure can all impact a chemical's toxicity. In the case of an acute exposure, the chemical is delivered in a single or multiple event within a short period of time, and is rapidly absorbed and produces an immediate effect (Environment Canada 1998; Landis & Yu 2004; Rand 1995). In a chronic exposure, the organism or system is exposed to low concentrations of a chemical that is delivered continuously over a long period of time. Effects can be rapid and immediate, but can also manifest as effects that develop slowly over the course of the entire exposure (Environment Canada 1998; Landis & Yu 2004; Rand 1995). The physical and chemical properties of a chemical can also influence its toxicity by determining persistence, transformation, bioavailability and its fate in water. In solution, toxicity can be impacted by the solubility of a chemical, vapor pressure, pH and lipophilicity, or a chemical's ability to dissolve in fats, oils and lipids. Additionally, a chemical's composition and any impurities in the solution can alter the toxicity (Bridges & Semlitsch 2000; Gilbert 2004; Hatch & Blaustein 2000; Landis & Yu 2004; Rand 1995).

The toxicity of a chemical is also influenced by the sensitivity of the organism involved in the study or experiment. Species differ in susceptibility to a toxicant, due to physiological differences, rates and patterns of metabolism and biochemical functioning (Bridges & Semlitsch 2000; Boone & Semlitsch 2001; Gilbert 2004; Landis & Yu 2004; Rand 1995). Within a population, differences in susceptibility can arise due to dietary factors which can produce changes in body composition; as well, age classes appear to differ in sensitivity, where neonatal or immature individuals appear to be more sensitive in comparison with adults or embryos of the same species (Berrill et al. 1998; Bridges &

Semlitsch 2000; Boone & Semlitsch 2001; Gilbert 2004; Holcombe et al. 1987; Landis & Yu 2004; Ortiz-Santaliestra et al. 2006; Rand 1995; Rohr et al. 2003; Sparling et al. 2001). Those differences are thought to be due to the surface area to volume ratio differences between life stages; different rates of excretion resulting from these size differences; possession of external gills in the case of most amphibian larvae; and the presence of a physical barrier in the case of the jelly-coated embryos (Altig & Johnson 1989; Edginton et al. 2007; Gilbert 2004; Harris et al. 1998; Hatch & Blaustein 2000; Ortiz-Santaliestra et al. 2006).

On a larger scale, the vulnerability of an aquatic ecosystem to a chemical assault greatly depends on all the biological, physical and chemical properties of the ecosystem (Boone & Semlitsch 2000; Bridges 2000; Bridges & Semlitsch 2000; Hatch & Blaustein 2000; Landis & Yu 2004; Rand 1995; Relyea 2005). Each ecosystem has different properties which enable the system to be resistant to changes, or allow the system to revert back to its original state after an exposure. Physical and chemical properties of the system, such as: surface area-volume ratios, temperature, pH, salinity, flow, depth, amount of suspended material, sediment particle size and organic carbon content all impact the fate of a chemical in an aquatic environment (Bridges 2000; Broomhall 2004; Harris et al. 1998; Hatch & Blaustein 2000; Landis & Yu 2004; Rand 1995; Relyea 2005). The fate of the chemical greatly influences how the chemical will react when in contact with biological organisms within the ecosystem.

Some of the more common toxicants studied in the field of aquatic toxicology are agricultural pesticides. Pesticides range from simple inorganic compounds, to complex organic substances, including both natural and synthetic derivatives of plant compounds

(Landis & Yu 2004; Rand 1995). These plant chemicals are selected and synthesized for their biocidal properties and are applied to kill or control pest organisms. Effective pesticides are designed to be selective in their effects, being extremely toxic to some forms of life, and relatively harmless to others. However, when introduced into an ecosystem, these chemicals may be resistant to abiotic or biotic degradation and can cause sublethal effects on a wide range of species (Landis & Yu 2004; Rand 1995; Rohr et al. 2003). Stagnant lentic aquatic ecosystems draining agricultural areas typically have a certain compilation of pesticides, often at low, but very persistent levels, which can yield unexpected affects on the organisms within the system. Often, these toxicants are introduced into aquatic ecosystems incidentally from manufacturing, agricultural runoff or aerial drift from application (Landis & Yu 2004; Nebeker et al. 1998; Rand 1995). Pesticide fate and transport in an environment are governed by subsurface and surface factors, including: hydrologic conditions, agricultural practices, plant cover type, soil type and soil quality (Health Canada 2004; Landis & Yu 2004; Morse et al. 2006; Rand 1995).

Much of the research on the impacts of agricultural pesticides on aquatic organisms has involved single-pesticide exposures. However, rarely is an ecosystem exposed to a single chemical (Boone & Bridges 2003; Boone & Semlitsch 2001; Gilbert 2004; Orton et al. 2006; Relyea 2004; Relyea 2005; Rohr et al. 2003). In actuality, most agricultural ponds have a cosmopolitan array of pesticides and other chemicals, which can have unexpected, and often magnified impacts on the system (Bishop et al. 1999; Boone & Bridges 2003; Boone & Semlitsch 2001; Gilbert 2004; Orton et al. 2006; Relyea 2004, 2005; Rohr et al. 2003). This biologically significant phenomena is known

as a toxicological interaction, where the exposure to two or more chemicals results in a biological response both quantitatively and qualitatively different result from the expected form of each chemical alone (Gilbert 2004; Landis & Yu 2004; Rand 1995). This interaction can cause alterations in absorption, protein binding, biotransformation and the excretion of interacting chemicals within an organism (Gilbert 2004; Goulet & Hontela 2003; Landis & Yu 2004; Park et al. 2001; Rand 1995; Sparling et al. 2001). These alterations can slow the rate of larval growth, having a significant and detrimental impact on later life stages by decreasing the chances for survival, decreasing size of adults, decreasing locomotion, decreasing rate of sexual maturation and lessening the capabilities for mate selection (Allran & Karasov 2000; Boone & Bridges 2003; Bridges 2000; Bridges & Semlitsch 2000; Gilbert 2004; Hatch & Blaustein 2000; Harris et al. 1998; Harris et al. 2000; Orton et al. 2006; Relyea 2004).

Chemicals in combination can produce the following effects: an additive effect, where two chemicals in combination produce a reaction equal to the sum of the individual chemicals; a synergistic effect, where the combined effects of two chemicals is much greater than the sum of the effects of the chemical applied alone; a potentiative effect, where one chemical has a toxic effect only when applied with another particular chemical; and an antagonistic effect, there the two chemicals actually interfere with each other's action (Landis & Yu 2004; Park et al. 2001; Rand 1995; Wan et al. 2005). Within the realm of antagonism, there are four different forms of chemical antagonism, namely: functional antagonism, chemical antagonism, dispositional antagonism and receptor antagonism. In the case of functional antagonism, the two chemicals counterbalance one another by eliciting opposite responses on the same physiological function. Chemical

antagonism involves a chemical reaction between the two chemicals to produce a less toxic product. If the absorption, biotransformation, distribution or excretion of a chemical is changed so that the concentration or duration of the chemical at the target site is decreased, the reaction is known as dispositional antagonism (Landis & Yu 2004; Rand 1995). Lastly, in the case of receptor antagonism, two chemicals bind to the same receptor site and produce less of an effect when given together than if they were delivered singularly (Landis & Yu 2004; Rand 1995).

1.2.2 Biological Effects of Pesticides

1.2.2.1 General

Examination of water samples taken from agricultural sites throughout the South Okanagan which have experienced high amphibian mortality revealed high levels of two organophosphates, (diazinon and azinphos-methyl) and an organochlorine (endosulfan) in particular (Bishop et al. 2006). Both organochlorines and organophosphorus pesticides have been implicated in the declines of several amphibian species in the Central Valley of California. These declines are most marked in downwind areas of the foothills, home to such amphibian species as the Cascades frog (*Rana cascadae*), California Red-legged frog (*R. daytonii*), Mountain Yellow-legged frog (*R. mucosa*) and the Foothill Yellow-legged frog (*R. boylii*) (Denton et al. 2003; Fellers et al. 2004; Scholz et al. 2006; Sparling et al. 2001; Sparling & Fellers 2007). More than three million kilograms of organophosphorus pesticides were used in California during 2004, the most recent year for which data are available (Agriculture Consumer Protection 2007; Scholz et al. 2006; Sparling et al. 2001). Many areas of the world have begun to phase-out the use of

organochlorines, substituting a combination of organophosphates and carbamates (Scholz et al. 2006), with Canada following suit in the next five to ten years (Pauli et al. 2000).

1.2.2.2 Impact on Cholinergic Pathways

Organophosphates have been implicated in altering acetylcholinesterase activity within the neuromuscular synapse in both invertebrates and vertebrates. Additionally, organochlorines have shown to alter acetylcholine concentrations within neuromuscular junctions in vertebrates. The neurotransmitter acetylcholine is associated with the cholinergic system of all developing organisms, which includes the entire parasympathetic system, neuromuscular junctions and preganglionic neurons of the sympathetic nervous system (Carr & Chambers 2001; Denton et al. 2003; Scholz et al. 2006; Sparling et al. 2001; Sparling & Fellers 2007). Upon release, acetylcholine stimulates a cholinergic receptor, and is thence broken down by acetylcholinesterase to choline and acetate (Aspengren et al. 2003; Carr & Chambers 2001; Sparling et al. 2001).

There are two primary cholinergic receptors, muscarinic (parasympathetic) and nicotinic (sympathetic). Stimulation of a muscarinic receptor then targets such organs as the heart, smooth muscle, secretory glands, autonomic ganglia and others throughout the Central Nervous System (CNS). Stimulation of these organs has resulted in altered behaviour and colour changes of the dermis, which can have serious ecological ramifications in amphibians (Allran & Karasov 2000; Aspengren et al. 2003; Carr & Chambers 2001; Boone & Bridges 2003; Bridges 2000; Bridges & Semlitsch 2000; Broomhall 2004; Broomhall & Shine 2003; Goulet & Hontela 2003; Park et al. 2001; Sparling et al. 2001). Reduced activity of acetylcholinesterase (characteristic of organophosphate exposure) and increased concentrations of acetylcholine (characteristic

of organochlorine exposure), cause neurological synapses to fire repeatedly and uncontrollably, leading to death, usually by asphyxiation as the animal loses respiratory control (Denton et al. 2003; Scholz et al. 2006; Sparling et al. 2001; Sparling & Fellers 2007).

1.2.2.3 Endosulfan

Endosulfan, an organochlorine, was developed in the early 1950s as an non-systemic insecticide, acaricide and miticide (Berrill et al. 1998; Landis & Yu 2004; Rand 1995). Primarily, endosulfan, also known as Thiodan®, is used in the control of sucking, chewing and boring insects and mites, including: aphids, leafhoppers, Colorado potato beetle, cabbage worms and other pests (Berrill et al. 1998; Health Canada 2004; Wan et al. 2005). Endosulfan is acutely neurotoxic to both insects and mammals, including humans; less is known regarding the toxicity of endosulfan to amphibians, as endosulfan residues can persist in organic matter within water bodies throughout the year, resulting in a continuous exposure of tadpoles and embryos to low levels (Boone & Bridges 2003; Bridges 2000; Bridges & Semlitsch 2000; Broomhall and Shine 2003; Harris et al. 2000; Relyea 2004; Wan et al. 2005). Endosulfan exists in two forms: alpha and beta isomers (*see* Appendix B). These isomers are metabolized by microbial activity to more persistent forms, endosulfan-sulphate and endosulfan-diol (Berrill et al. 1998; Broomhall & Shine 2003). Interestingly, a combination of the two isomers are the most potent to the widest range of organisms (Berrill et al. 1998; Broomhall & Shine 2003; Wan et al. 2005). Although endosulfan has low solubility in water, direct aerial application over water bodies has proven to be fatal to fish, and toxic to other aquatic organisms (Berrill et al. 1998; Health Canada 2004).

1.2.2.4 Azinphos-methyl

Azinphos-methyl, (*see* Appendix C) also known as Guthion®, is a broad-spectrum, non-systemic organophosphate insecticide and acaricide used on most fruit trees (Holcombe et al. 1987; Khan et al. 2003; Landis & Yu 2004; Rand 1995; Schulz et al. 2002; Schuytema et al. 1995). Fundamental research has determined that azinphos-methyl has high residual activity in both soil and water, and is toxic to most wildlife (Harris et al. 1998; Khan et al. 2003; Schulz et al. 2002; Schuytema et al. 1995). Direct contact or ingestion by an organism can result in the inhibition of acetylcholinesterase activity (Khan et al. 2003; Morton et al. 1997; World Health Organization 2001). Azinphos-methyl hydrolyses slowly in water, and has been observed to have a half-life of between 37 and 39 days (Morton et al. 1997). Overall, Guthion® is very mobile, with a high potential to reach surface water through spray drift and in a dissolved state in runoff (Erickson & Turner 2003; Khan et al. 2003; Rand 1995). As with many other pesticides, very little work has been conducted on the impacts of azinphos-methyl on the different life stages of amphibians (Holcombe et al. 1987; Khan et al. 2003; Morton et al. 1997; Nebeker et al. 1998; Schuytema et al. 1995).

1.2.2.5 Diazinon

Diazinon is an organophosphate (*see* Appendix D) largely used for the control of soil and household pests, as well as sucking and chewing insects on agricultural crops (Denton et al. 2003; Health Canada 2006; Scholz et al. 2006; Sunzenauer 1986). Less persistent than endosulfan, diazinon remains in the soil for between 21 and 80 days, and

can be hydrolyzed to a less toxic form, thiophosphoric acid, in under 185 days, depending on microorganism activity and soil water content (Denton et al. 2003; Environmental Protection Agency 1986). However, the two metabolites resulting from microbial activity, hydroxydiazinon and diazoxon, are both fat soluble and highly toxic to many avian and fish species (Denton et al. 2003; Environmental Protection Agency 1986; Scholz et al. 2006; Sparling & Fellers 2007). Both the parent compound and the metabolites inhibit acetylcholinesterase activity resulting in neurological dysfunction (Denton et al. 2003; Scholz et al. 2006; Sparling & Fellers 2007); however, the oxon metabolite binds directly to the acetylcholinesterase molecule and thus has a greater inhibitory effect (Denton et al. 2003; Sparling & Fellers 2007). Additionally, farmers often time the application of diazinon with the heavy spring rains to increase plant uptake of diazinon (Denton et al. 2003; Morse et al. 2006).

1.2.3 Methods for Evaluating the Toxicity of Chemicals

In aquatic toxicology, the test organisms are exposed to a pesticide or mixture of pesticides, by dissolving the chemical in water, producing a test concentration (Landis & Yu 2004; Rand 1995). Measuring lethality from this test concentration is a precise, important, unequivocal and useful method in the determination of the potency of a pesticide. Additionally, it is important to have sub-lethal effect criteria which would then indicate toxic stress at a stage before death, such as: number of normal embryos or any growth or morphological abnormalities which may lead to death. These endpoints tend to be more useful in applying to field observations, as mutations or abnormalities could significantly decrease the viability of a population (Allran & Karasov 2000; Boone &

Bridges 2003; Bridges 2000; Bridges & Semlitsch 2000; Broomhall 2004; Broomhall & Shine 2003; Gilbert 2004; Goulet & Hontela 2003; Hatch & Blaustein 2000; Harris et al. 1998, 2000; Orton et al. 2006; Park et al. 2001; Relyea 2004; Rohr et al. 2003; Sparling et al. 2001). However, the use of acute toxicity to evaluate the environmental impacts of pesticides has its limitations. The information garnered from bioassay testing is derived by exposing the test animals to "worst-case scenarios", rather than the prevailing ambient conditions, and often the focus remains on complete lethality as the primary endpoint (Boone & Bridges 2003; Bridges 2000; Bridges & Semlitsch 2000; Gilbert 2004; Hatch & Blaustein 2000; Relyea 2004; Wan et al. 2005).

To quantify both the lethal and sub-lethal effects observed within a bioassay, toxicologists use median effective concentration end points, such as Lethal Concentration 50% (LC50) and Effective Concentration 50% (EC50) tests (Environment Canada 1998; Ives 2005; Landis & Yu 2004; Rand 1995). LC50 tests estimate a typical chemical concentration that produces mortalities in 50% typical or average test organisms over a specific period of time, normally between 24 and 96 hours (Environment Canada 1998; Ives 2005; Landis & Yu 2004; Rand 1995). EC50 tests estimate the concentration of a chemical that produces a specific effect in 50% of the population, for example: immobility, developmental abnormalities, deformities, loss of equilibrium, and failure to respond to stimuli (Environment Canada 1998; Ives 2005; Landis & Yu 2004; Rand 1995). From the data collected throughout an experiment, a toxicity curve can be established. The shape of the curve can provide information about the mode of toxic action of the chemical, metabolic degradation or activation, and can also indicate the presence of more than one chemical agent in the water. Additionally, estimates of EC50s

from the toxicity curve can be made as the experiment progresses, and can be plotted as a toxicity curve on a logarithmic scale to determine when acute lethality ceases. However, EC50s and LC50s cannot be estimated by any method if there is not an effect greater than 50% in at least one concentration (Environment Canada 1998; Ives 2005; Landis & Yu 2004; Rand 1995). In cases where there is less than a 50% effect, then Effective Concentration 20% (EC20) tests, or other non-median endpoints are useful. These are more protective endpoints associated with lower proportional effects (Environment Canada 1998; Landis & Yu 2004; Rand 1995). However, anything less than a 20% effect can often be the result of circumstance, rather than the test environment itself (Environment Canada 1998; Landis & Yu 2004). Thus, one should never attempt to estimate an endpoint within the acceptable range of an effect within the control populations.

In the estimation of the LC50 and EC50 values, there are three primary methods of analysis, being: logit or probit regression, Spearman-Kärber analysis and the binomial method. The logit/probit regression model is an unbiased, effective statistical method for analyzing smooth and regular data (Environment Canada 1998; Landis & Yu 2004; Rand 1995). This model can only be used in cases where the highest concentration in the experiment yields one hundred percent mortalities, and partial mortalities in at least two of the lower test concentrations. This model produces 95% confidence limits, which are used to estimate any variation which may occur within the experiment. The Spearman-Kärber model is recommended for tests that produce only partial effects, and should be run with and without a 35% trim of data to compensate for outliers, and the lack of 0% and 100% effects (Environment Canada 1998; Landis & Yu 2004; Rand 1995). In order

for this model to be used, the data must be monotonic, where the toxic effects must increase with an increase in the dose concentration. This model also produces 95% confidence limits, typically of plus or minus two standard deviations, if there is at least one partial effect occurring within the experiment. The binomial method is a last-resort for data analysis, where neither of the other models mentioned can be used (Environment Canada 1998; Landis & Yu 2004; Rand 1995). For example, if the highest concentration does not produce a 100% effect, but the second to highest concentration does, and there are no other partial effects, then the binomial method is the best option. Unlike the other two methods, the binomial method does not produce confidence intervals within which the lethal or effective test concentration lies (Environment Canada 1998; Landis & Yu 2004; Rand 1995). All of these models are used with tests conducted on organisms exposed to a fixed test concentration for a fixed amount of time.

A commonly used software for conducting dose-response analysis is the Comprehensive Environmental Toxicological Information Software (CETIS™). This software has been developed by Tidepool Scientific Software (Ives 2005), and is a Microsoft® Access™ based application designed to analyze, maintain and organize complex and heterogeneous toxicity data (Environment Canada 1998; Ives 2005; Landis & Yu 2004; Rand 1995). This application stores and processes data derived from freshwater, estuarine and marine toxicity tests performed on various environmental media, such as: effluents, ambient water, reference toxicants and sediment (Environment Canada 1998; Ives 2005; Landis & Yu 2004; Rand 1995). It also provides statistical procedures with user-determined endpoints, such as: survival, growth or reproductive success or developmental abnormalities. The most commonly used model in the

CETIS™ program is a trimmed Spearman-Kärber, used to estimate for both LC50 and EC50. The CETIS program carries out steps to determine which model to use, determined partially by the chosen endpoints, it then calculates the EC50 or LC50 (Ives 2005). If the data is determined to be quantal data and possesses a lognormal distribution, then the program uses the probit or logit models. If the data is not lognormal in its distribution, then the program will run a trimmed Spearman-Kärber (Environment Canada 1998; Ives 2005).

1.2.4 Test Species

1.2.4.1 Importance of the test species

Two species of frogs, Great Basin Spadefoot (*Spea intermontana*) and the Pacific Treefrog (*Pseudacris regilla*), are of particular interest to toxicologists and ecologists throughout the South Okanagan Valley of British Columbia. In the dry sagebrush grasslands of the Okanagan, Kettle, Nicola, Thompson and Similkameen Valleys of BC, *S. intermontana* is at its most northerly distribution in North America (COSEWIC 2007; Hallock & McAllister 2005; NatureServe 2007). *P. regilla*, however, is the most common frog in BC, and can be found in a wide variety of habitats (Matsuda et al. 2006; NatureServe 2007; Sparling et al. 2001). Because of its apparent adaptability and broad-niche nature, *P. regilla* acts as an useful sentinel species for other amphibian species in BC, and throughout North America (Sparing et al. 2001).

1.2.4.2 *Spea intermontana*

Little is known about *S. intermontana*'s behavior and physiology, perhaps due to the unpredictable nature of their breeding congresses, which are restricted to a relatively short period of time and temporary habitats (COSEWIC 2007; Gilbert 2004; Hallock & McAllister 2005; Matsuda et al. 2006; Trowbridge & Trowbridge 1932). Breeding activity seems to be synchronous or dependent on the early heavy rainfalls during the late spring and early summer (Buseck et al. 2005; COSEWIC 2007; Gilbert 2004; Hallock & McAllister 2005; Trowbridge & Trowbridge 1932). Throughout the year, three distinct and accessible habitats are required by *S. intermontana*, namely: ephemeral pools for breeding, aquatic and grassland foraging areas; as well as hibernation sites, which must provide deep, loose soil for burrowing (Buseck et al. 2005; COSEWIC 2007; Gilbert 2004; Hallock & McAllister 2005; Matsuda et al. 2006). Unfortunately, these habitat characteristics also appeal to agriculturalists, as the soil is some of the most fertile in BC. As a result, there has been a significant loss of Spadefoot breeding areas and grassland foraging habitats due to urbanization and intensive agriculture (Buseck et al. 2005; COSEWIC 2007; Gilbert 2004; Hall et al. 1997; Hallock & McAllister 2005; Kellogg 1932; Matsuda et al. 2006; Trowbridge 1942; Voss 1961). Additionally, increased urban and agricultural use of water is thought to be the leading cause of a lowered water table, which significantly decreases the occurrence of the temporary ponds the Spadefoots require (Cannings 1999; COSEWIC 2007; NatureServe 2007). Furthermore, the temporary ponds that do appear are often in the middle of orchards, and thus receive a large amount of chemical runoff and drift from normal agricultural activities (Hall et al. 1997; Hallock & McAllister 2005; Kellogg 1932; Trowbridge 1942; Voss 1961).

Although *S. intermontana* is protected from capture and killing under the BC Wildlife Act (Ministry of Environment 2007), their habitat remains unprotected, as much is under private jurisdiction (COSEWIC 2007; NatureServe 2007), leaving the species extremely vulnerable.

1.2.4.3 *Pseudacris regilla*

P. regilla's range extends from the Yukon-BC border, to the tip of the Baja peninsula in California (Matsuda et al. 2006; NatureServe 2007). In BC, the Treefrog can be found in grasslands, woodlands, agricultural regions and residential areas. Typically, *P. regilla* is found among low vegetation near water, breeding in marshes, lakes, ponds, ditches, reservoirs and slow-moving streams. Throughout its range, *P. regilla* can breed as early as January, and as late as August, depending on rainfall and temperatures. Because the Treefrog inhabits so many different areas, it is a useful sentinel species for toxicology, as the results can, theoretically, be applied to other, more niche-specific amphibian species. The adaptability and broad habitat use of *P. regilla* implies that the species is not under the same threats as *S. intermontana* and/or may be physiologically less sensitive to environmental contaminants. For the purposes of our experiments, *P. regilla* acted as a reference species, as it and similar species have been used in a number of similar toxicological experiments, by Sparling et al. (2001), Relyea (2004), Schuytema et al. (1995) and Nebeker et al. (1998).

1.2.5 Motives for Conducting Present Research

Embryo mortalities and deformities of amphibians in agricultural ponds in South Okanagan orchards receiving pesticide runoff were observed in the field experiments conducted by Bishop et al. (2006) from 2004 to 2006. In 2007, I chose to test three pesticides commonly detected in those ponds, (endosulfan, azinphos-methyl and diazinon) in dose-response experiments in a controlled laboratory environment. Egg masses of *S. intermontana* and *P. regilla* were collected from a variety of locations along Highway 97 which serves the Osoyoos/Oliver area of the South Okanagan Valley, and transported to the Pacific Environmental Science Centre (PESC) in North Vancouver. The exposures were carried out with environmentally-relevant concentrations, and involved the same stages of anuran embryos as those exposed in early field experiments. Single pesticide and mixture exposures were conducted in the lab, in an effort to determine which pesticide caused effects in the two species of anurans.

1.3 Organization of the Thesis

Chapter 1 is an overview of basic ecotoxicological principles of relevant background information, and more specifically of dose response approaches measuring the effects of pesticides on amphibians; as well as the rational for conducting the presented research. Chapter 2 and 3 describe the dose-response studies of pesticide effects on the embryo and tadpole, respectively, of two species of amphibians native to Canada. Each chapter has a brief introduction to the importance of the using the particular lifestage in toxicology testing and a brief summary of the potential impacts of

the pesticides used on that lifestage. Additionally, each chapter outlines in detail, the materials and methods utilized in the experiment, and the results for each experiment. The individual discussions at the end each chapter reviews related research regarding methods used and results obtained.

Chapter 4 provides a synopsis of the results obtained from the two phases of experiments, including similar dose-response experiments conducted on other native amphibian species. Additionally, the implications of the findings on management and pesticide regulation in Canada are discussed. There are numerous hypothesized contributors to the global amphibian declines, and a few of these factors are discussed relating to potential synergism with pesticide application. Finally, directions of future research based on my findings, will be discussed.

1.4 Collaborators

Collaborators include: John Elliott and Christine Bishop of the Science and Technology Branch of Environment Canada, Delta, British Columbia; Sara Ashpole of the University of Waterloo, Waterloo, Ontario; Rachel Skirrow, Grant Schroeder and Brad McPherson of the Pacific Environmental Science Centre (PESC), North Vancouver, British Columbia.

Christine Bishop and John Elliott provided the amphibian population data collected in the four year field study conducted by Environment Canada from 2003 to 2006.

Rachel Skirrow, of the Ecotoxicology Lab at PESC, provided the analytical information used in the materials and methods section, pertaining to equipment type and model numbers used in the water quality analysis.

Grant Schroeder, of the Ecotoxicology Lab at PESC, provided the statistical analysis utilizing the Comprehensive Environmental Toxicological Information Software (CETIS) registered to PESC.

Brad McPherson, of the Analytical Chemistry lab at PESC, provided the resulting water chemistry data from the water samples taken from both the field study conducted by Bishop et al. (2006) from 2003-2006, and the experiments which I conducted in 2007.

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CHAPTER 2

Assessing the Impacts of Endosulfan, Azinphos-methyl and Diazinon on the Great Basin Spadefoot (*Spea intermontana*) and the Pacific Treefrog (*Pseudacris regilla*) Embryo Survivorship[†]

Introduction

Amphibian populations appear to be declining, both as a result of human activities and through natural changes in ecosystems (Allran & Karasov 2000; Bishop et al. 1999; Bridges 2000; Hayes et al. 2006; Hopkins et al. 2006; Pauli et al. 2000; Relyea 2005; Relyea et al. 2005; Sparling et al. 2001; Wind 1999). Reproductive success in amphibian populations can be impaired by environmental interference such as pollutants, that alter adult reproductive function, disrupt embryo development or disrupt tadpole growth (Bridges & Semlitsch 2000; Carey and Bryant 1995; Harris et al. 2000; Reeder et al. 1998; Relyea 2004; Sanders 1970; Seburn & Seburn 2000).

Environmental toxicants may interfere with amphibian growth and development in a number of ways. Sublethal concentrations may increase the susceptibility of eggs and larvae to pathogenic organisms and disease by compromising the immune system (Blaustein & Johnson 2003; Christin et al. 2004; Gendron et al. 2003; Gilbertson et al. 2003; Harris et al. 1998; Harris et al. 2000; Kiesecker 2002; Ouellet et al. 1997). Chronic exposure to sublethal concentrations may also retard growth and time to metamorphosis which can result in the larvae being unable to metamorphose and leave the pond at the

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appropriate time. Additionally, as many contaminants act as hormone mimics, endocrine disruptors and central nervous system stimulants, future reproduction and development can be completely inhibited (Bishop et al. 1999; Carey & Bryant 1995; Goulet & Hontela 2003; Hayes et al. 2006; Park et al. 2001; Rohr et al. 2003; Sparling et al. 2001). The biological ramifications include increased susceptibility to aquatic predation, increased vulnerability to pond-drying and overall reduction in population fitness (Boone & Bridges 2003; Bridges 2000; Bridges & Semlitsch 2000; Hatch & Blaustein 2000; Orton et al. 2006).

The Okanagan valley in British Columbia is an area of intensive agriculture where 40% of the natural wetlands and riparian zones have been drained or altered (Wind 1999), yet this valley historically contained a high abundance of amphibian species. In total, 64 ponds, including 23 agricultural ponds, were surveyed from 2003 to 2006, to determine adult breeding, larval productivity, and relative population densities of a variety of native amphibian species. To assess the risk of amphibian populations to multiple stressor effects of pesticides, Bishop et al. (2006) conducted *in situ* experiments on early native amphibian stages of development, with hatching success, tadpole survival, and abnormalities recorded. Enclosures with eggs were placed in ponds located in orchards and subjected to pesticide applications associated with conventional and organic orchards; as well as non-agricultural control ponds. Water samples were collected for analysis of current-use pesticide during the reproductive experiments and after known spray events. Azinphos-methyl (Guthion®), diazinon and endosulfan (Thiodan®) were found in some of the highest concentrations at the field sites experiencing substantial mortalities (Bishop et al. 2006).

This study used a controlled dose-response exposure to determine the specific effects of pesticides on development and survival of Great Basin Spadefoot (*Spea intermontana*) and the Pacific Treefrog (*Pseudacris regilla*) eggs to Gosner Stage 25. Tadpole mortality observed post-hatch was the only toxicological endpoint measured in this experiment.

These two species of amphibians are of particular interest to toxicologists and ecologists throughout British Columbia. In the dry sagebrush grasslands of the Okanagan, Kettle, Nicola, Thompson and Similkameen Valleys of BC, *S. intermontana* is at its most northern distribution in North America (Cannings 1999; COSEWIC 2007; Hallock & McAllister 2005; NatureServe 2007). To date, there are no published toxicology data on *S. intermontana*. *P. regilla*, however, is the most common frog in BC, and can be found in a wide variety of habitats (Matsuda et al. 2006; NatureServe 2007; Sparling et al. 2001); therefore, we predict that this species would be less sensitive in this experiment than *S. intermontana*.

Materials and Methods

Egg Collection

Egg masses for *S. intermontana* were collected from two separate locations in the Osoyoos/Oliver area of BC, approximately 3 km apart. Eggs of *P. regilla* were collected from three different locations within the same area of the South Okanagan, approximately 5-12 km between the sites. Egg masses were divided into individual containers, housing 15 eggs each, placed in temperature-controlled coolers, and transported to the Pacific

Environmental Science Centre (PESC) lab within 15 hours of laying. Eggs were held at the same temperature as their original environment, roughly 12-15ºC during transport, limiting egg mortality due to transport or temperature shock.

Pesticides

The pesticides used in the exposure regime were 50% active ingredient (A.I.) commercial formulas, as currently being sold by South Valley Sales (Oliver, BC) to orchardists in the South Okanagan valley (Joe Bunge *pers. comm.*). Guthion 50% Wettable powder (WP) was obtained from Bayer CropScience Inc., (Calgary, AB). Diazinon 50% Emulsifiable Concentrate (EC) was obtained from Nu-Grow Co. Inc. (Brantford, ON), and Thiodan® 50WP insecticide was obtained from FMC Co. (Philadelphia, PA). All formulations were prepared in 1 L volumetric flasks using deionized water to dissolve. The stock concentrations were Guthion®, 5 mg/L A.I., diazinon, 35 mg/L A.I.; and Thiodan®, 4 mg/L A.I.. The LC_{50} tests (LC_{50} tests are a standard acute toxicity metric used to determine the lethal concentration of a compound and its effects to fifty-percent of the test population [Landis & Yu 2004; Rand 1995]) followed a modification of the Environment Canada, Biological Test Methods: Acute Lethality Test Using Rainbow Trout, Report EPS 1/RM/9 July 1998. The Rainbow Trout Method was modified by using *S. intermontana* and *P. regilla* embryos rather than trout, by raising the temperature to 19ºC and by setting the loading density to roughly 0.8 g/L. The egg masses were exposed to the respective chemicals and dilutions for a total of 8 days. Eight-day LC_{50} parameters were entered into the CETIS™ program, and the acute toxicity of each pesticide established.

The toxicity of endosulfan, diazinon and azinphos-methyl commercial formulations on amphibian eggs were assessed using a dose-response experimental design. The dilution series (Table 2.1) for each chemical was determined from field data collected in the South Okanagan, from a single site which experienced significant egg mortality throughout the four year study (Bishop et al. 2006). These reference concentrations included all isomers and breakdown products, in an attempt to estimate total initial parent compound concentrations. Endosulfan had been detected at levels of approximately 60 ng/L; azinphos-methyl, at concentrations as high as 50 ng/L; and diazinon was detected at approximately 350 ng/L. The test concentrations were 10, 100 and 1000 times greater, and 10 times less than detected field concentrations (Table 2.1). Each chemical had 5 dilution concentrations, replicated 3 times, for a total of 45 glass aquaria. Additionally, three control aquaria, with well water, alone, used.

Laboratory Procedure

To normalize the clutch effect on hatching success, all 'A' replications of each dilution received eggs from the same egg mass. Fifteen eggs of one species were housed in a mesh sieve in a single 5.5 gallon (20 L) glass aquarium. Because the breeding cycles of *S. intermontana* and *P. regilla* varied so differently in 2007, the experiments were conducted on one species at a time.

All replicates were conducted with minimal aeration, as outlined by Environment Canada (Environment Canada 1998). Because these experiments were attempting to replicate field conditions, and none of the field sites possessed any form of aeration, a duplicate experiment was conducted without any aeration.

To better compare the results acquired in previous years' field work conducted by Bishop et al. (2006), egg masses of both *S. intermontana* and *P. regilla* were reared from Gosner stage 10 to two days post-Gosner stage 25, which included embryo development, hatching and swim-up (K.A. Gosner developed a table for staging amphibian embryos and larvae in 1960, revealing the intricate transformations that occur up to metamorphosis [*see* Appendix E]), which occurred over the eight day experiment. Survivorship was determined through daily checks, removing any dead embryos or tadpoles, and determined on an individual basis. Results from each replicate were then averaged, and transformed into a percentage of the total population. When the developing anurans reached Gosner stage 25, the surviving tadpoles were euthanized by an overdose of MS222 (tricane methyl sulphanate: an acceptable and commonly used fish, amphibian and invertebrate anesthetic [Western Chemical Inc. 2008]). This protocol was approved by the UBC Animal Care Committee (Certificate #A06-1532). For this experiment, acute mortality was the only toxicological endpoint, as no other abnormalities were observed.

Water chemistry and pesticide concentrations at the initiation and completion of the experiment were measured. On day 0 and day 8, composite water samples were generated from the replicates of three of the five concentrations tested (low, medium, high) and analyzed for nitrate, nitrite, ammonia and pesticide concentration (Table 2.2). Additionally, dissolved oxygen (D.O.), pH, conductivity and temperature were recorded at test start and end (Table 2.3). pH was measured using Oakton Waterproof pH Tester No. 2 (Oakton Instruments, Vernon Hills, IL, USA). Dissolved oxygen and temperature were measured with a YSI Model 59 (YSI Environmental, Yellow Springs, OH, USA).

Conductivity was measured using an Oakton Waterproof TDSTestr3 (Oakton Instruments, Vernon Hills, IL, USA). All of these factors were measured to ensure that there was no other possible chemical or physical interactions or stressors in the experiment.

Nitrate, nitrite and ammonia chemistry were analyzed by the Inorganic Chemistry Section of the Pacific Environmental Science Centre (PESC), using the Technicon AutoAnalyzer II Single-Channel Colorimeter, in accordance with the in-house automated colorimeter phenolhypochlorite method Version 2.2 (PESC, North Vancouver, BC).

Guthion®, Thiodan® and diazinon were measured by the Organic Chemistry Section of PESC using an Agilent 6890/5975 GC-MS (Gas Chromatography-Mass Spectrometry) in accordance with the in-house OP (Organophosphate) Pesticide method version 2.2 (Guthion® and diazinon) and OCL (organochlorine) Pesticides method version 2.3 (Thiodan®) (Environment Canada 2005, 2006). The OP method is applicable to the analysis of most organophosphate pesticides in tissue, effluent and sediment samples. Samples undergo a DCM (dichloromethane) extraction, dried and analyzed using a High Resolution Gas Chromatography with Electron Capture Detection (Environment Canada 2005, 2006). Positive identification of the various target compounds is made by a comparison of absolute retention times to those of standards combined with computer-based matching routines (Environment Canada 2005, 2006).

Statistical Analysis

Upon completion of the experiment, mortalities and water chemistry were entered into the CETIS software (CETIS v. 1.1.2.) to determine both the eight-day Lethal

Concentration of 50% ($LC50_{8d}$) and Lethal Concentration of 20% ($LC20_{20}$) values for each chemical. Results from all chemicals were analyzed using either a Spearman-Kärber model (running both an untrimmed and 35% trimmed to consider the impact of outliers), or a binomial interpolation for estimating median lethal concentrations in toxicity bioassays. These models were used because none of the test concentrations yielded 100% mortalities; therefore, utilizing a logit or probit regression was not possible. The nominal results were based on the theoretical pesticide concentration that would induce a response in twenty percent of the test population. The initial and final values were based on the initial (day 0) and final (day 8) water chemistry, respectively; with the mean LC20 values being based on the mean pesticide concentration over the 8 day period (Table 2.3).

The Least Squared Analysis of Variance (JMP IN v. 5.1.2.) was used to examine embryo survivorship rather than a regular Analysis of Variance, which fails to account for all variables and potential interactions. The following statistical model was utilized:

$$Y_{ijkl} = \mu + R_i + P_j + S_k + C_l + (PS)_{jk} + (PC)_{jl} + (SC)_{kl} + (PSC)_{jkl} + E_{ijkl}.$$

Where Y_{ijkl} is the embryo survivorship of the *kth* species in the *ith* replicate exposed to the *jth* pesticide at the *lth* level of concentration. μ is the population mean and *R* denotes any variation due to replication and *i* represents the replicate number (1, 2, 3). *P* denotes variation due to pesticide type and *j* indicates pesticide type (azinphos-methyl, endosulfan, diazinon). *S* denotes variation due to tadpole species and *k* represents the species (*S. intermontana* and *P. regilla*). *C* denotes variation due to pesticide dilution level (*l*) of 0 through 5. *PS*, *PC* and *SC* denote the 2-way interactions of the main effects

and *PSC* denotes the 3-way interaction. *E* is the error term for the model, differing from the standard error of the data.

All survivorship data were arcsine transformed before the analysis to normalize the data and minimize skewness.

Table 2.1. Test Concentration Series for $LC20_{8d}$ on *S. intermontana* and *P. regilla* embryos.

Sample Concentration	Azinphos-methyl (ng/L)	Endosulfan (ng/L)	Diazinon (ng/L)
Control	0	0	0
10 x < Field	5	6	35
Field	50	60	350
10 x > Field	500	600	3 500
100 x > Field	5 000	6 000	35 000
1 000 x > Field	50 000	60 000	350 000

Results

Water Chemistry

Composite water samples were collected on day 0 and day 8 (Table 2.2) and entered into the CETIS software to assess how the aquatic environment impacted embryo survivorship and development. Levels of pesticides on day 0 show experimenter accuracy in application; day 8 pesticide concentrations indicate breakdown rates throughout the experiment and the final aquatic environment.

Table 2.2. Initial and final water pesticide concentrations for the eight-day LC20 on *S. intermontana* and *P. regilla* tadpoles. Concentrations are average ranges from the two species experiments.

Sample	*Azinphos-methyl (ng/L)*		*Endosulfan (ng/L)*		*Diazinon (ng/L)*	
	Initial (d0)	*Final (d8)*	*Initial (d0)*	*Final (d8)*	*Initial (d0)*	*Final (d8)*
10x < field	5 – 50	< 10	< 10	< 10	20 – 170	< 10 – 28
100x > field	220 – 640	62 - 217	300 – 700	< 10 - 140	2 000 – 4 700	187 – 1 360
1 000x > field	48 000 – 53 000	12 000 – 14 000	57 430 – 64 400	14 334 – 17 300	310 000 – 460 000	10 000 – 34 300

Over the 8 day period, the following water parameters were measured: pH ranged from 7.8 to 8.2; dissolved oxygen ranged from 8.1 mg/L to 8.9 mg/L; temperature ranged from 18.1°C to 22.1°C; and conductivity ranged from 410 μS to 460 μS. Nitrate, nitrite and ammonia levels were also determined in the water chemistry analysis, and ranged from 0.060 mg/L to 0.124 mg/L nitrate; 0.001 mg/L to 0.107 mg/L nitrite and under 0.002 mg/L ammonia (Table 2.3). These measurements were deemed to be within normal parameter ranges (according to the Canadian Water Quality Guidelines for the Protection of Aquatic Life, Environment Canada 2004), and would not have influenced the pesticide-tadpole interactions.

Table 2.3. Water chemistry measurements for the $LC20_{8d}$ on *S. intermontana* and *P. regilla* tadpoles. Measurements are ranges over the two experiments and all tanks.

Water chemistry	**Levels detected**
pH	7.6 to 8.2
dissolved oxygen	8.3 mg/L to 9.1 mg/L
temperature	17.9°C to 22.3°C
conductivity	410 μS to 460 μS.
nitrate	0.260 mg/L to 0.324 mg/L
nitrite	0.003 mg/L to 0.203 mg/L
ammonia	under 0.002 mg/L

Embryonic Mortality

Endosulfan

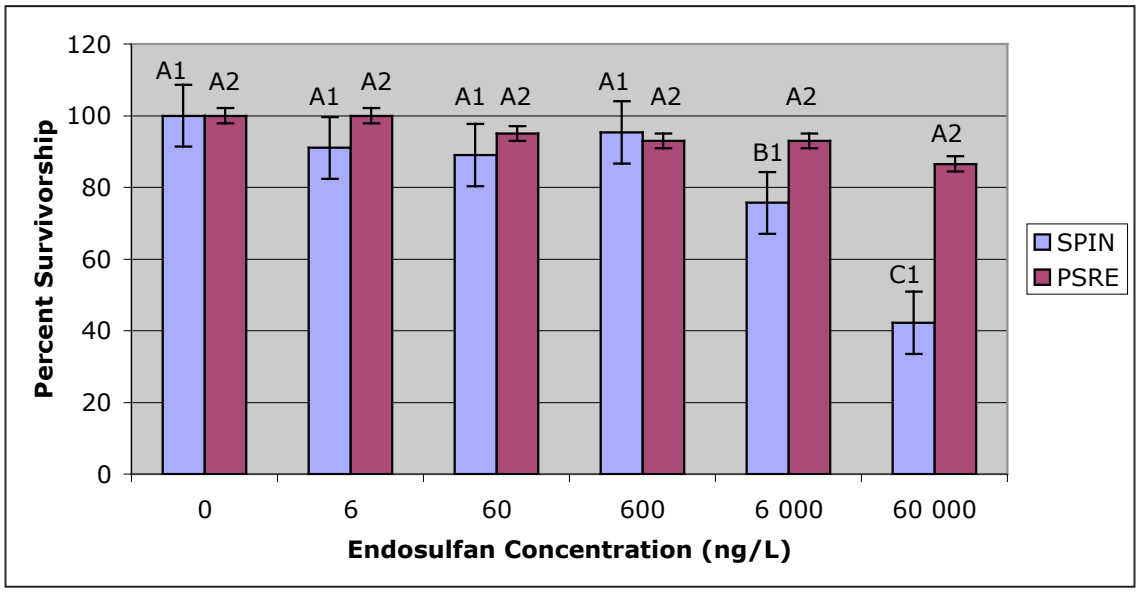

Figure 2.1. Eight-day survivorship for exposure of embryo to Gosner stage 25 *S. intermontana* (SPIN) and *P. regilla* (PSRE) tadpoles to commercial formulations of endosulfan. Data represents an average of 3 replicate exposures. A1 to C1 denotes significant differences in responses by SPIN when compared with PSRE in each concentration. PSRE received only A2, as the response was not significantly different from SPIN nor between different concentrations.

S. intermontana experienced a significant ($P<0.01$) reduction in survivorship in the two highest concentrations: 6 000 ng/L had 75.7% ± 4.0 survivorship and 60 000 ng/L of endosulfan had 42.4% ± 4.0 survivorship. *P. regilla* survivorship was not compromised by the increasing test concentration, with 86.6% ± 2.0 survivorship, but was significantly different than the response observed in *S. intermontana* at the same concentration.

Azinphos-methyl

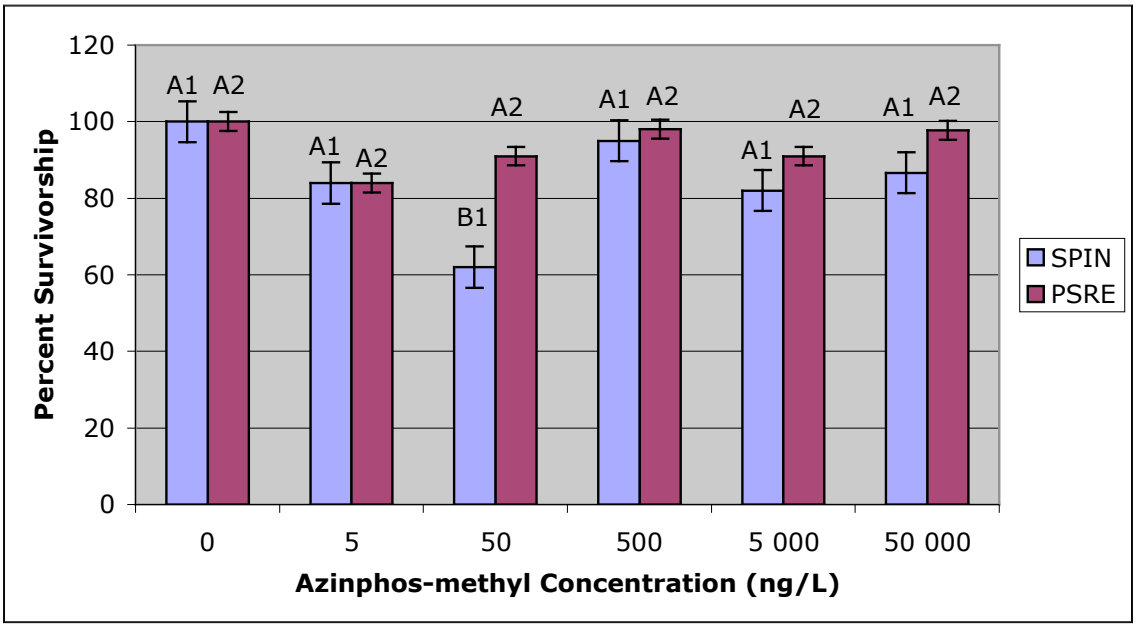

Figure 2.2. Eight-day survivorship for exposure of embryo to Gosner stage 25 *S. intermontana* (SPIN) and *P. regilla* (PSRE) tadpoles to commercial formulations of azinphos-methyl. Data represents an average of 3 replicate exposures. A1 to B1 denotes significant differences in responses by SPIN when compared with PSRE in each concentration. PSRE received only A2, as the response was not significantly different from SPIN nor between different concentrations.

Overall, *S. intermontana* experienced significantly ($P<0.01$) lower survivorship in the 50 ng/L (62% ± 6.0) test concentration of azinphos-methyl when compared with the other test concentrations, and with *P. regilla*. The response of *P. regilla* was variable, and did not indicate a dose-response to these dilutions of azinphos-methyl, as survivorship at 50 000 ng/L azinphos-methyl (97% ± 2.0) was higher than the lowest concentration of 5 ng/L azinphos-methyl (82% ± 2.0).

Diazinon

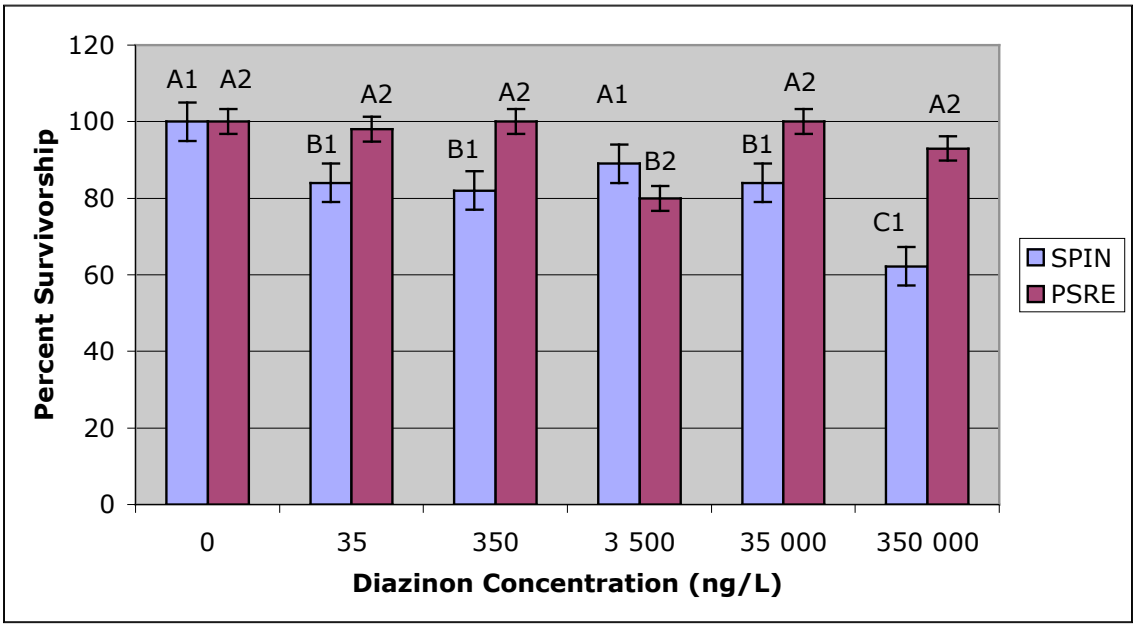

Figure 2.3. Eight-day survivorship for exposure of embryo to Gosner stage 25 *S. intermontana* (SPIN) and *P. regilla* (PSRE) tadpoles to commercial formulations of diazinon. Data represents an average of 3 replicate exposures. A1 to C1 denotes significant differences in responses by SPIN when compared with PSRE and each concentration. A2 to B2 denotes a significant difference in response by PSRE when compared with SPIN and other concentrations. PSRE received only A2, as the response was not significantly different from SPIN nor between different concentrations.

S. intermontana's overall survivorship decreased significantly ($P<0.01$) as diazinon concentration increased, seen in the response to the highest concentration of diazinon (as indicated by C1 above the last column). The response of *P. regilla* yielded a significant response in only the 3 500 ng/L concentration when compared with the other concentrations and the response by SPIN.

Interspecies Comparison

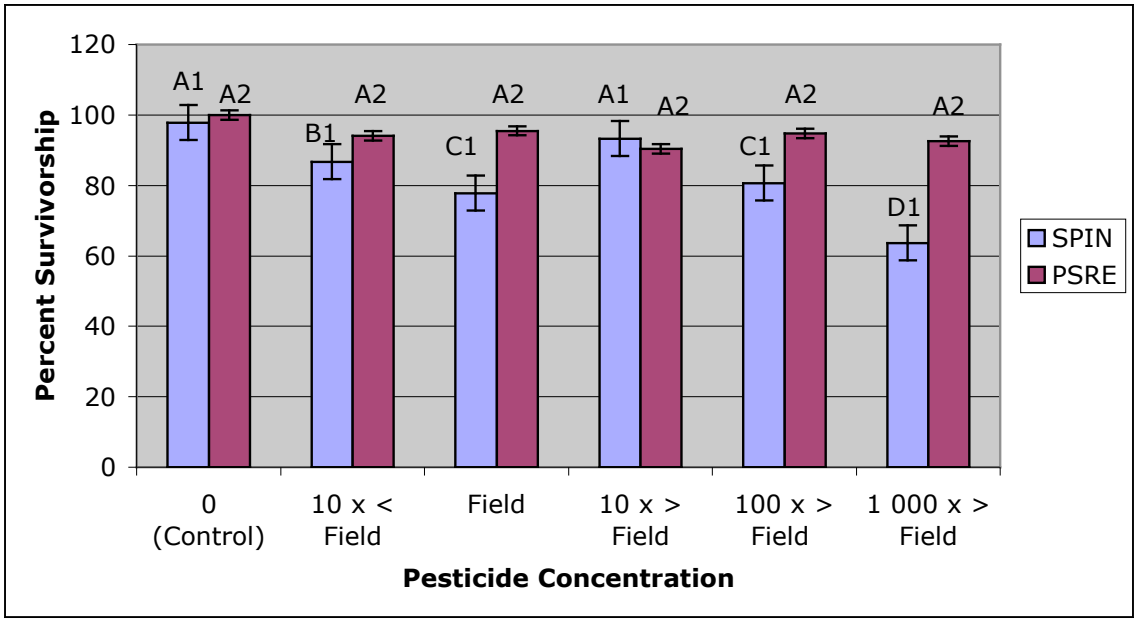

Figure 2.4. An interspecies comparison between *S. intermontana* (SPIN) and *P. regilla* (PSRE) of eight-day survivorship for all three pesticides. Columns labeled with different letters are significantly (P<0.01) different. A1 to D1 denotes significant differences in responses by SPIN when compared with PSRE and each concentration. PSRE received only A2, as the response was not significantly different from SPIN nor between different concentrations.

In comparing the overall response of the two amphibian species to all three pesticides, *S. intermontana* was more sensitive, and experienced significantly (P<0.01) lower embryo survivorship when exposed to the pesticides, (as indicated by different letters above each column).

$LC20_{8d}$ Values

Table 2.4. Eight day LC20 ($LC20_{8d}$) values for exposure of embryo to Gosner stage 25 *S. intermontana* tadpoles to commercial formulations of the pesticides azinphos-methyl, endosulfan and diazinon. Data represents an average of 3 replicate exposures.

Chemistry	Diazinon (ng/L) (95% LCL-95% UCL)	Endosulfan (ng/L) (95% LCL-95% UCL)	Azinphos-methyl (ng/L) (95% LCL-95% UCL)
Initial	> 350 000	723.3 (190.5-2289.2)	> 53 000
Final	1228.9 (NA-13530.1)	12.5 (0.2-69.2)	> 53 000
Average	>175 000	2872.7 (1267.3-5969.8)	> 53 000
Nominal	66691.6 (NA-208665.9)	5325.5 (922.4-10923.9)	12.6 (CL = NA)

For *S. intermontana*, endosulfan was the most toxic, at an average $LC20_{8d}$ value of 2872.7 ng/L. This is followed by azinphos-methyl, with a $LC20_{8d}$ of greater than 53 000 ng/L, and diazinon, with a $LC20_{8d}$ value of greater than 175 000 ng/L. Azinphos-methyl had a calculated nominal $LC20_{8d}$ of 12.6 ng/L, with unattainable confidence limits due to the fact that the levels of azinphos-methyl by the end of 8 days was almost undetectable. Because survivorship of the *P. regilla* test population was so high, $LC20_{8d}$ values could not be calculated.

Discussion

Embryos of *S. intermontana* and *P. regilla* reared in this variety of pesticide concentrations experienced no mortalities. Newly-hatched *S. intermontana* tadpoles, however, experienced significant mortalities in the endosulfan and diazinon experiments. *P. regilla* embryos and tadpoles were significantly more resilient, determined by no significant mortality or response to pesticide type or concentration.

Laboratory embryo and tadpole survivorship of *S. intermontana* and *P. regilla* surpassed survivorship of these two species experiencing agricultural runoff in the South Okanagan Valley, (Bishop et al. 2006). Due to the high survivorship, (more than fifty-percent), calculating the lethal concentration of the three pesticides for fifty-percent (LC50) of the population was not possible; therefore, upon completion of the experiment, it was decided that a lethal concentration of twenty-percent (LC20) could be calculated. Overall, exposure to these toxins produced a less lethal affect in *P. regilla* when compared with *S. intermontana*, which was consistent with our hypothesis as well as results from field experiments (Bishop et al. 2006).

Fick's Diffusion Equation states that the rate and amount of a material, anything from oxygen to pesticides, passing from the environment into an egg, is dependent upon the size of the egg sphere (Carroll et al. 1991; Wyrick et al. 1974), and more specifically, as the thickness of the jellycoat (and similarly the number) increases, the rate of diffusion decreases (Carroll et al. 1991; Edington et al. 2007; Shibata et al. 2000; Wyrick et al. 1974). This function could explain why embryos appear to be more resilient in toxicological experiments; and why different species showed differences in sensitivity in the field experiments conducted by Bishop et al. (2006) in 2004 to 2006.

An additional consideration for this difference in embryo sensitivity, is the fact that embryos utilized in the field were collected between 8 and 10 hours after oviposition; whereas, eggs utilized in the laboratory were roughly 15 hours old, due to travel time from the collection sites in the Okanagan to PESC in North Vancouver, BC. Though the developmental stages of embryos used in field experiments compared to lab experiments did not differ, the developing embryos were transported to the PESC laboratory in clean

water for many hours after laying, providing sufficient time for the fertilization envelope to develop, and the embryos to become close to impermeable to environmental contaminants. Embryological research has revealed that, upon fertilization, water uptake by the embryo is dramatically reduced, potentially altering the susceptibility of the embryo to pesticides (Carroll et al. 1991; Edington et al. 2007; Marquis et al. 2006; Sardet et al. 2002; Wyrick et al. 1974). In addition to the protection gained from the jellycoats, the vitelline envelope also appears to provide protection from environmental contaminants (Carroll et al. 1991; Edington et al. 2007; Marquis et al. 2006; Kadokami et al. 2004).

Mortalities observed in the lab occurred post-hatch, consistent with other laboratory results found in the literature review (Broomhall 2004; Carroll et al. 1991; Edington et al. 2007; Harris et al. 2000; Rohr et al. 2003; Shibata et al. 2000; Wyrick et al. 1974). Unhatched embryos seem to be less sensitive to xenobiotic chemicals, as numerous experiments have resulted in delayed mortalities in jellied embryos when compared with non-jellied embryos (Berrill et al. 1998; Bishop et al. 1999; Broomhall 2004; Edington et al. 2007; Hatch & Blaustein 2000; Hopkins et al. 2006; Ortiz-Santaliestra et al. 2006; Rohr et al. 2003). This is likely due to slower water uptake and lower clearance rate in the case of the jellied embryos (Edington et al. 2007; Shibata et al. 2000; Wyrick et al. 1974). These results are inconsistent with results observed in past field experiments, where the mortalities all occurred in the embryo stage. Hayes et al. (2006), Bishop et al. (1999) and Relyea et a. (2005) all observed significant embryo mortality in field conditions, but could not replicate those results under the same

conditions in subsequent laboratory exposures, which is consistent with our experiments conducted in 2007.

Field results are often difficult to replicate in the lab, as embryos in field conditions are exposed to a combination of pesticides, nitrates and other chemicals in a variety of water qualities, which appears to be relevant to egg mortality rates (Belden & Lydy 2000; Denton et al. 2003; Relyea 2004; Relyea et al. 2005; Scholz & Hopkins 2006; Storfer 2003). Variable dissolved oxygen, other pesticides, nutrients, surfactants, UV radiation, variable pH levels and temperatures and water levels can act synergistically or even additively with pesticides influencing the toxicity on wildlife (Blaustein et al. 2003; Burkhart et al. 1998, 2000; Cox & Surgan 2006; Davidson et al. 2001; Hatch and Blaustein 2000; Mann & Bidwell 2001). Toxicity tests conducted by Relyea (2004) and Relyea et al. (2005) under laboratory conditions showed pesticides in the lab break down at a faster rate than in the field; thus, developing amphibians in the field may be exposed to more persistent levels throughout their development than those exposed in a laboratory environment.

In the present study, the pesticide concentrations used were one hundred to one thousand times lower than those used in other laboratory experiments (Berrill et al. 1998; Broomhall 2004; Broomhall & Shine 2003; Denton et al. 2003; Goulet & Hontela 2003; Holcombe et al. 1987; Nebeker et al. 1998; Park et al. 2001; Rohr et al. 2003; Relyea 2004; Scholz et al. 2006; Schuytema et al. 1995; Sparling & Fellers 2007; Sparling et al. 2001; Wan et al. 2005). However, the resulting post-hatch mortalities were consistent with those from other lab studies. Water chemistry at the end of eight days showed negligible levels of pesticides remaining in solution, begging the question of whether the

levels at day four were sufficient to induce mortalities of newly-hatched tadpoles. However, the post-hatch mortalities could have been precipitated by the embryos being exposed, compromising, but not killing the embryos, and instead, resulting in a reduction in fitness of the tadpole (Bridges 2000; Boone & Semlitsch 2001; Bridges & Semlitsch 2000; Edington et al. 2004; Harris et al. 2000; Marquis et al. 2006; Relyea 2004; Sanders 1970; Sparling et al. 2001).

Conclusion

Although the levels of mortalities observed in field experiments could not be replicated in the lab, we have gained insight into the toxicity of low levels of these three pesticides. As pesticide regulation is based on acute toxicity, determined typically by 96 hour LC50 experiments, these significantly lower concentrations used in our experiment still yielded significant mortalities in both species of amphibians. Future research should be conducted using a multitude of pesticides, ideally a combination of insecticides and herbicides, in conjunction with nitrates. Amphibians can encounter all of these chemicals in their natural environments, and the additive and synergistic effects are still poorly understood.

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CHAPTER 3

Assessing the Impact of Endosulfan, Diazinon and Azinphos-methyl on Development and Survivorship of the Great Basin Spadefoot (*Spea intermontana*) and the Pacific Treefrog (*Pseudacris regilla*) Tadpoles[‡]

Introduction

Many amphibian species breed opportunistically in a range of aquatic habitats, many of which are embedded in agricultural landscapes (Allran & Karasov 2000; Altig & Johnston 1989; Bridges 2000; Duellman & Trueb 1994; Richardson 2001; Seburn & Seburn 2000; Wind 1999). Due to local adaptations to their aquatic environments, amphibian tadpoles can be very sensitive to changes both in the terrestrial and aquatic ecosystems within these agricultural landscapes, (Altig & Johnston 1989; Bridges 2000; Gilbert 2004; Harris et al. 2000; Hatch & Blaustein 2000; Orton et al. 2006; Relyea et al. 2005; Sanders 1970; Welsh & Ollivier 1998). Unfortunately, environmental contaminants are now ubiquitous in most environments, and the extent of their impact on aquatic communities is still poorly understood (Boone & Bridges 2003; Bridges & Semlitsch 2000; Relyea et al. 2005). There is increasing evidence that many amphibian populations are declining, and the impact of agricultural have been identified as a potential causative factor (Duellman & Trueb 1994; Relyea et al. 2005; Seburn & Seburn 2000; Wind 1999). In localized areas, declining density and occurrences of

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metapopulations have been correlated with heavier use (both direct and indirect application) of agricultural pesticides (Bishop et al. 1999; Relyea et al. 2005).

Amphibians have two ecologically distinct life phases: tadpole phase and a terrestrial-life phase (Duellman & Trueb 1994; Matsuda et al. 2006; Seburn & Seburn 2000; Wind 1999). The tadpole phase feeds primarily on detritus, zooplankton, phytoplankton, and in rare cases, other tadpoles (Altig & Johnson 1989; Duellman & Trueb 1994; Matsuda et al. 2006; Wind 1999). Tadpoles are thus prone to bioaccumulation of many aquatic contaminants, which can result in: decreased survivorship, decreased predator avoidance and decreased likelihood of metamorphosis leaving the animals more susceptible to pond-drying (Berrill et al. 1998; Besbelli & Szajewski 2000; Broomhall 2004; Harris et al. 2000; Park et al. 2001; Rohr et al. 2003). Due to their sensitivity to changes in the environment and their obligate aquatic lifestyle, the amphibian tadpole has been a useful model for toxicity testing (Bishop et al. 1999; Boone & Bridges 2003; Bridges 2000; Hatch & Blaustein 2000; Harris et al. 2000; Relyea et al. 2005).

The South Okanagan Valley in British Columbia is one of four endangered ecosystems in Canada (Cannings 1999; Environment Canada 2000), provides habitat to one-third of the provincially-listed wildlife species found in BC, including 3 species of at-risk amphibians. Additionally, the Canadian distributions of eight vertebrate species are restricted to the Okanagan Valley (Cannings 1999; Environment Canada 2000). Unfortunately, rapid urbanization and agricultural expansion have reduced and fragmented the already vulnerable habitat (Environment Canada 2000). Three of the most commonly used agricultural pesticides in the South Okanagan Valley of British

Columbia (Canada), are commercial formulations of endosulfan (50WP Thiodan®), azinphos-methyl (50 WP Guthion®) and diazinon. Endosulfan is a central nervous system stimulant, causing such symptoms as: excitability, melanophore aggregation, developmental abnormalities, depressed feeding and growth (Berrill et al. 1998; Besbelli & Szajewski 2000; Broomhall 2004; Harris et al. 2000; Park et al. 2001; Rohr et al. 2003). Azinphos-methyl and diazinon are both acetylcholinesterase inhibitors, disrupting the parasympathetic nervous system in the larval stages of many organisms and impairing reproductive functioning in adult amphibians manifesting as uncoordinated swimming, muscle twitching and impaired optomotor behaviour (Besbelli & Szajewski 2000; Denton et al. 2003; Nebeker et al. 1998; Schuytema et al. 1995; Sparling et al. 2001).

Toxicological experiments with these three pesticides and amphibian tadpoles have observed the effects of individual pesticides at concentrations in the parts-per-million range (Berrill et al. 1998; Broomhall 2004; Broomhall & Shine 2003; Denton et al. 2003; Goulet & Hontela 2003; Holcombe et al. 1987; Nebeker et al. 1998; Park et al. 2001; Rohr et al. 2003; Relyea 2004; Scholz et al. 2006; Schuytema et al. 1995; Sparling & Fellers 2007; Sparling et al. 2001; Wan et al. 2005). At those concentrations, complete mortalities usually occurred within 4 days of exposures, though these levels are rarely observed in field situations (Broomhall & Shine 2003; Relyea 2004; Sparling & Fellers 2007; Sparling et al. 2001; Wan et al. 2005). Tadpoles exposed chronically to low levels of a wide variety of contaminants, the potential sub-lethal effects of individual pesticides to amphibians at lower concentrations are currently unknown. Furthermore, the effects of simultaneous exposure of a mixture of those pesticides have not been examined.

The test species used in this experiment were the Great Basin Spadefoot (*Spea intermontana*) and the Pacific Treefrog (*Pseudacris regilla*). *S. intermontana* is currently listed as threatened in British Columbia, with much of the habitat currently threatened by industrial agriculture (COSEWIC 2007; Matsuda et al. 2006). *P. regilla* is the most common frog in BC (Matsuda et al. 2006), and has been used as a test species in several toxicological assessments, thus acting as a useful reference species for other amphibians native to BC (Sparling et al. 2001; Relyea 2004; Schuytema et al. 1995; Nebeker et al. 1998). Our hypotheses were: 1) Increasing pesticide concentrations will result in acute mortality, and or developmental/behavioral abnormalities in amphibian tadpoles, 2) *P. regilla* will be a more resilient species to the experiment, based on their adaptive physiology. Toxicological endpoints included, but were not limited to: acute mortality, behavioral abnormalities and morphological abnormalities.

Materials and Methods

Tadpoles of both *S. intermontana* and *P. regilla* were exposed for a period of 8 days to a variety of test concentrations of azinphos-methyl, diazinon and endosulfan, both as single pesticides and in combination. Water quality, breakdown rates of each pesticide and mortality rates at each concentration of the pesticides were determined during daily inspections. Additionally, sub-lethal effects, such as altered behaviour, physiological and morphological changes were also observed in animals exposed to endosulfan and noted.

Experimental Animals

Tadpoles of both *S. intermontana* and *P. regilla* used in this experiment were reared from field-collected embryos collected from a variety of reference sites (without any direct pesticide application) throughout the South Okanagan Valley, BC. Prior to this experiment, these individuals were not exposed to any pollutants, based on water source sampled by Bishop et al. in 2006 (Bishop et al. 2006). Embryos were reared in a lightly aerated (50 bubbles per second), 60 L glass aquarium filled with fresh well water. No water changes occurred within this timeframe, as embryos do not produce waste products, and the amount of waste produced post-hatch did not warrant a water change. The well water that supplies the Pacific Environmental Science Centre (PESC) is tested regularly to ensure that the water meets the Canadian Water Quality Guidelines for the Protection of Aquatic Life (Environment Canada 2004). Once hatched, *S. intermontana* tadpoles were fed *Xenopus laevis* food, and the *P. regilla* tadpoles were fed spirulina discs for four days prior to the start of the experiment.

Pesticides Tested

The pesticides used in the exposure regime were 50% active ingredient (A.I.) commercial formulas, consistent with those currently being sold by South Valley Sales (Oliver, BC) to orchardists in the South Okanagan valley (Joe Bunge *pers comm.*). Guthion® 50% Wettable powder (WP) was obtained from Bayer CropScience Inc. (Calgary, AB), Diazinon 50EC was obtained from Nu-Grow Co. Inc. (Brantford, ON), and Thiodan® 50WP Insecticide (endosulfan) was obtained from FMC Co. (Philadelphia, PA). All formulations were prepared in 1L volumetric flasks using deionized water to

dissolve. The stock concentrations were Guthion®, 5 mg/L A.I., Diazinon, 35 mg/L A.I.; and Thiodan®, 6 mg/L A.I..

Experiment 1

In Experiment 1, each of the three pesticides were tested individually, at 5 concentrations, replicated 3 times, for a total of 45-20L glass aquaria. Additionally, three control aquaria, with only well water, were used. The tadpoles were exposed to the respective chemicals and dilutions for a total of 8 days. Pesticide exposures involving *S. intermontana* were carried out first (April 2007) and tests using *P. regilla* commenced four weeks later.

For each species, 5 post-Gosner stage 25 tadpoles were chosen randomly from the holding tank, and placed into each of the 20L aquaria containing the pesticide solution. The LC_{20} tests followed a modification of Environment Canada, Biological Test Methods: Acute Lethality Test Using Rainbow Trout, Report EPS 1/RM/9 July 1990 (amended May 1996). The Rainbow Trout Method was modified by in the following manner: *S. intermontana* and *P. regilla* tadpoles were substituted for trout; the temperatures were raised to 19ºC to mimic spring daytime temperatures in the Okanagan (Bishop et al. 2006); the loading density was set to roughly 0.8 g/L.

Pesticide Test Concentrations

The dilution series for each chemical was determined from field data collected in the South Okanagan, from a single site where significant egg mortality occurred

throughout a four year period (Bishop et al. 2006). The concentrations tested were 10, 100 and 1000 times greater, and 10 times less than detected field concentrations (Table 3.1). Test concentrations were based on the field concentrations of the three pesticides detected from three separate sampling events, and include all isomers and breakdown products, in the attempt to estimate total initial parent compound concentrations.

Table 3.1. Pesticide Concentration Series for $LC20_{8d}$ on *S. intermontana* and *P. regilla* tadpoles.

Sample Concentration	Azinphos-methyl (ng/L)	Endosulfan (ng/L)	Diazinon (ng/L)
Control	0	0	0
10 x < Field	5	6	35
Field	50	60	350
10 x > Field	500	600	3 500
100 x > Field	5 000	6 000	35 000
1 000 x > Field	50 000	60 000	350 000

Water Quality Analysis

Throughout the duration of the experiment, water chemistry and water quality were determined. On day 0 and day 8, composite water samples were generated from the replicates of three of the five concentrations tested (10 x < field, 100 x > field and 1 000 x > field), to be analyzed for nitrate, nitrite, ammonia and pesticide concentration. Nitrate, nitrite and ammonia chemistry were analyzed by the Inorganic Chemistry Section of the Pacific Environmental Science Centre (PESC), using the Technicon AutoAnalyzer II Single-Channel Colorimeter, in accordance with the in-house automated colorimeter phenolhypochlorite method Version 2.2 (PESC, North Vancouver, BC). Dissolved oxygen and temperature were measured with a YSI Model 59 (YSI Environmental,

Yellow Springs, OH, USA). The dissolved oxygen (D.O.), pH, conductivity and temperature were recorded at test start and end. pH was measured using an Oakton Waterproof pH Tester No. 2 (Oakton Instruments, Vernon Hills, IL, USA). At the end of the experiment, water quality parameters were measured in every tank.

Guthion®, Thiodan® and Diazinon were measured by the Organic Chemistry Section of PESC using an Agilent 6890/5975 GC-MS (Gas Chromatography-Mass Spectrometry) in accordance with the in-house OP (Organophosphate) Pesticide method version 2.2 (Guthion® and diazinon) and OCL (chlorinated) Pesticides method version 2.3 (Thiodan®). The OP method is applicable to the analysis of most organophosphate pesticides in tissue, effluent and sediment samples. Samples undergo a DCM (dichloromethane) extraction, are then dried and analyzed using a High Resolution Gas Chromatography with Electron Capture Detection (Environment Canada 2005, 2006). Positive identification of the various target compounds is made by a comparison of absolute retention times to those of standards combined with computer-based matching routines (Environment Canada 2005, 2006).

Tadpole Morbidity and Behaviour Analysis

Tadpoles were observed for mortalities and any developmental or behavioural abnormalities were recorded. Abnormal behaviour in tadpoles exposed to endosulfan was noted during daily checks, and as endosulfan is a known central nervous system (CNS) stimulant (Goulet & Hontela 2003; Harris et al. 2000; Park et al. 2001; Sparling et al. 2001), we predicted that these animals would be more excitable. To test behavioral response, each tadpole was gently prodded with a glass rod, and the time to quiescence

was determined. Stimulation involved lightly touching the tip of a glass pipette to the tail of the tadpole, as recommended by the University of British Columbia Animal Care Committee (UBCACC) in assessing tadpole morbidity (Certificate # A06-1532). No one tadpole was stimulated more than once, to maintain an unbiased assessment of excitability. Time from stimulation to rest was determined on an individual basis by using a digital stopwatch, with the average time within each tank determined. At 50 000 ng/L, endosulfan is known to have a half-life of 4 days in a laboratory environment (Sunderam et al. 1994; Bayer CropScience 2004); therefore, the response observed by the tadpoles at 96 hours is representative of a response to the exposure to the initial parent compound concentration.

Experiment 2

A pesticide combination test was carried out using *S. intermontana* tadpoles. Environmental concentrations of all three pesticides were combined, resulting in three replicates of: 60 ng/L of Thiodan®, 50 ng/L Guthion® and 350 ng/L of diazinon. Five *S. intermontana* tadpoles were randomly selected from the holding tank, placed into each replicate 20L aquarium, and exposed for a period of 8 days. Daily checks of the experiment were conducted to measure all water quality parameters (*see* Experiment 1: Water Quality Analysis), and to assess mortality and any behavioural abnormalities (Table 3.2).

Statistical Analysis

Upon completion of the experiment, mortalities and water chemistry data were entered into the CETIS program (CETIS v.1.1.2.) for calculation of a lethal concentration of fifty-percent (LC_{50}) and twenty-percent (LC_{20}) of the test population. Results from all chemicals were analyzed using a binomial interpolation for estimating median lethal concentrations in toxicity bioassays. These models were used because none of the test concentrations yielded 100% mortalities; therefore, utilizing a logit or probit regression was not possible. The nominal results were based on the theoretical pesticide concentration that would induce a response (acute mortality, behavioural abnormalities, etc.) in 20% of the test population. The initial and final values are based on the initial (day 0) and final (day 8) water chemistry, respectively; with the average LC_{20} values being based on the average pesticide concentration based over the eight day period ($LC20_{8d}$) (Table 3.4).

The Least Squared Analysis of Variance (JMP IN v. 5.1.2.) was used to examine tadpole survivorship with the following statistical model:

$$Y_{ijkl} = \mu + R_i + P_j + S_k + C_l + (PS)_{jk} + (PC)_{jl} + (SC)_{kl} + (PSC)_{jkl} + E_{ijkl}.$$

The above variables include: Y_{ijkl}, the tadpole survivorship of the *kth* species in the *ith* replicate exposed to the *jth* pesticide at the *lth* level of concentration; μ being population mean; R denotes variation due to replication and $i = 1, 2, 3$; P denotes variation due to pesticide with j representing pesticide type (Guthion, Endosulfan, Diazinon); S denotes variation due to tadpole species; k is the test species (*S. intermontana*, *P. regilla*); C denotes variation due to pesticide dilution level, l, being 0, 10 times less than field concentration, field concentration etc.; PS, PC and SC denote 2-

way interactions of the main effects and *PSC* denotes the 3-way interaction; lastly, *E* is the error term. All survivorship percentage data were arcsine transformed before the analysis.

For testing tadpole excitability when exposed to endosulfan, the following statistical model was used:

$$Y_{ijkl} = \mu + R_i + S_j + C_k + (RS)_{ij} + (RC)_{ik} + (SC)_{jk} + (RSC)_{ijk} + E_{ijkl}.$$

Results

Water Chemistry

Table 3.2. Water chemistry measurements for the $LC20_{8d}$ on *S. intermontana* and *P. regilla* tadpoles. Measurements are ranges over the two experiments and all tanks.

Water chemistry	Levels detected
pH	7.6 to 8.2
dissolved oxygen	8.3 mg/L to 9.1 mg/L
temperature	17.9°C to 22.3°C
conductivity	410 μS to 460 μS.
nitrate	0.260 mg/L to 0.324 mg/L
nitrite	0.003 mg/L to 0.203 mg/L
ammonia	under 0.002 mg/L

The levels detected were deemed to be within normal parameter ranges under the Canadian Water Quality Guidelines for the Protection of Aquatic Life (Environment Canada 2004), and would not have influenced either the pesticide-tadpole interactions, nor tadpole survivorship. Initial and final pesticide levels are indicated in Table 3.3.

Table 3.3. Initial and final pesticide concentrations for the $LC20_{8d}$ on *S. intermontana* and *P. regilla* tadpoles. Concentrations are average ranges from the two separate experiments.

Sample	*Azinphos-methyl (ng/L)*		*Endosulfan (ng/L)*		*Diazinon (ng/L)*	
	Initial (D0)	*Final (D8)*	*Initial (D0)*	*Final (D8)*	*Initial (D0)*	*Final (D8)*
10x < field	5 – 50	< 10	< 10	< 10	20 – 170	< 10 – 28
100x > field	220 – 640	62 – 217	300 – 700	< 10 – 140	2 000 – 4 700	187 – 1 360
1000x > field	49 000 – 53 000	35 000 – 48 000	57 430 – 64 400	1 734 – 3 250	310 000 – 460 000	10 000 – 34 300

Tadpole Survivorship

For *P. regilla*, there was a significant ($P<0.02$) three-way interaction involving pesticide type, tadpole species, and pesticide concentration. Orthogonal comparisons indicated that none of the three pesticides significantly lowered survivorship; however, survivorship in the highest concentration of endosulfan was significantly ($P<0.05$) different than the other concentrations of the other pesticides (Figure 3.2). In this experiment, *P. regilla* tadpoles were more sensitive to the pesticide exposure (Figure 3.1) when compared with *S. intermontana* tadpoles.

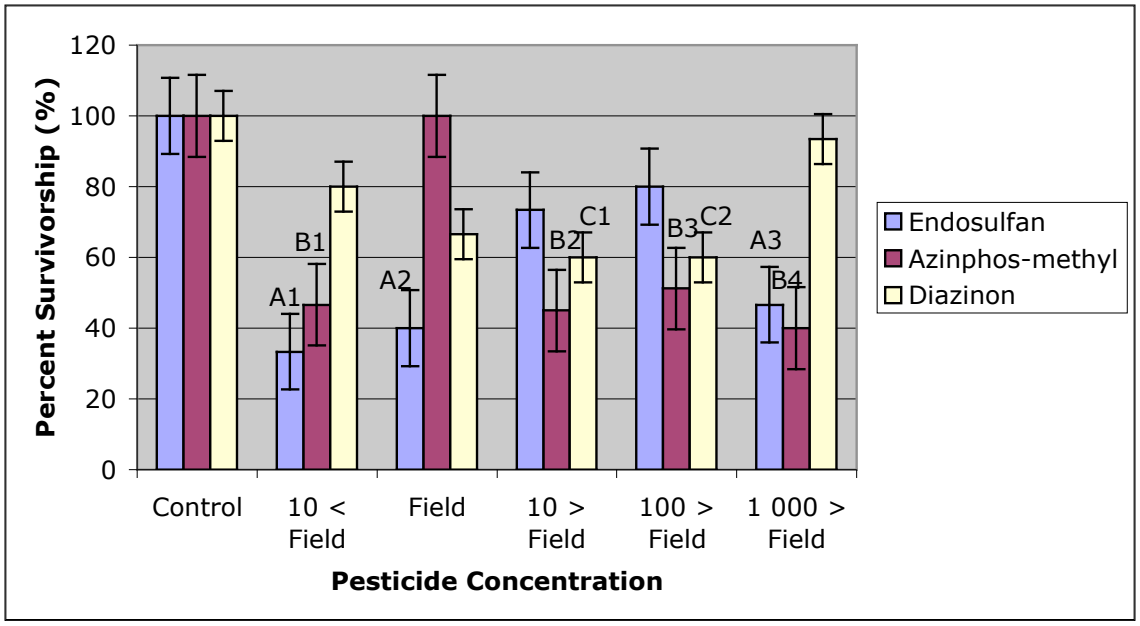

Figure 3.1 *P. regilla* (PSRE) tadpole survivorship after the eight day exposure to commercial formulations of endosulfan, azinphos-methyl, and diazinon, respectively. Significant ($P<0.05$) differences in response to concentration when compared with response to control and other concentrations are represented by different letters. Differences in response to endosulfan are represented by A1-A3; azinphos-methyl, B1-B3; and diazinon, C1-C2.

P. regilla exposed to diazinon showed a variable decline of tadpole survivorship as the concentration of diazinon increased. At 3,500 ng/L and 35,000 ng/L, tadpole survivorship became significantly ($P<0.05$) lower than control. However, unexpectedly, tadpoles exposed to 350,000 ng/L survived as well as control tadpoles. With the exception of tadpoles exposed to 50 ng/L of Guthion®, all other *P. regilla* tadpoles exposed to Guthion® had significantly ($P<0.05$) lower survivorship than control tadpoles. The survivorship of these tadpoles exposed to 3,500 ng/L and 35,000 ng/L of endosulfan did not differ significantly from control, but exposure to endosulfan concentration lower and higher than these resulted in significantly ($P<0.05$) lower tadpole survivorship.

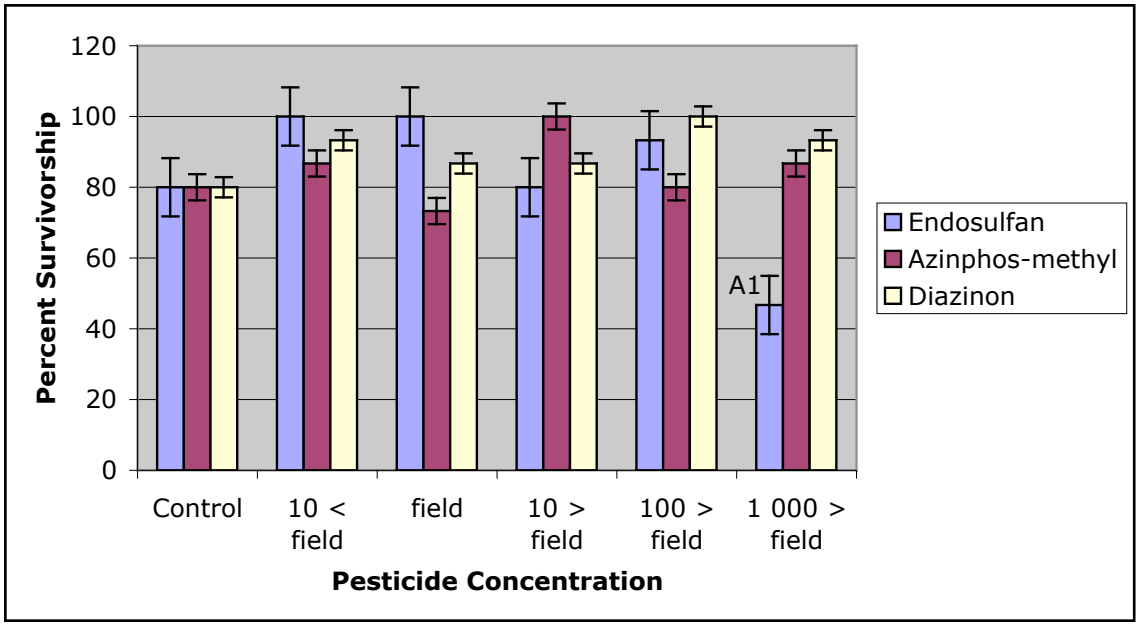

Figure 3.2 *S. intermontana* tadpole survivorship after the eight-day exposure to commercial formulations of diazinon, endosulfan and azinphos-methyl. Significant ($P<0.05$) differences in response to concentration when compared with response to control and other concentrations are represented by different letters.

S. intermontana tadpoles showed variable declines in survivorship in all pesticides, and a significant ($P<0.05$) reduction in survivorship in the highest concentration of endosulfan, indicated by the label A1 above column.

In Experiment 2, the exposure of *S. intermontana* tadpoles to all three pesticides (at field concentrations) simultaneously did not result in significantly lower survival. Tadpoles were exposed in three replicates of the pesticide mixture (total of 15 tadpoles), with a single tadpole mortality observed. No other sub-lethal effects were observed throughout the eight-day exposure.

$LC20_{8d}$ Values

Table 3.4. Eight-day LC20 values for exposure of Gosner stage 25 *S. intermontana* tadpoles to commercial formulations of the pesticides azinphos-methyl, endosulfan and diazinon. Data represents an average of 3 replicate exposures.

Chemistry	Diazinon (ng/L) (95% LCL-95% UCL)	Endosulfan (ng/L) (95% LCL-95% UCL)	Azinphos-methyl (ng/L) (95% LCL-95% UCL)
Initial	(insufficient mortalities)	2461 (CL = N/A)	(insufficient mortalities)
Final	(insufficient mortalities)	11.8 (CL = N/A)	(insufficient mortalities)
Average	(insufficient mortalities)	77.1 (CL = N/A)	(insufficient mortalities)
Nominal	(insufficient mortalities)	7982 (N/A – 36680)	> 50 000

$LC20_{8d}$ values for *S. intermontana* test population were calculated by a binomial interpolation, which averages survivorship between the highest concentrations which experience significant mortalities. Overall, tadpoles exposed to azinphos-methyl did not experience high mortality rates, so only a nominal $LC20_{8d}$ of greater than 50 000 ng/L could be calculated. Endosulfan was the most toxic to the tadpoles, with a $LC20_{8d}$ value of 77.1 ng/L. Overall, tadpoles exposed to diazinon did not experience sufficient mortalities to calculate a Lethal Concentration value.

Abnormal Tadpole Developments

Abnormal Behaviour

Upon being prodded with a glass rod, tadpoles reacted in a variety of ways, varying from swimming slowly away, to spinning in circles and swimming erratically for many seconds. Control tadpoles swam away from the glass rod and came to rest on average after 1.8 seconds: in contrast, both *S. intermontana* and *P. regilla* tadpoles took significantly ($P<0.01$) longer to resettle with each increased level of endosulfan concentration (Figures 3.3).

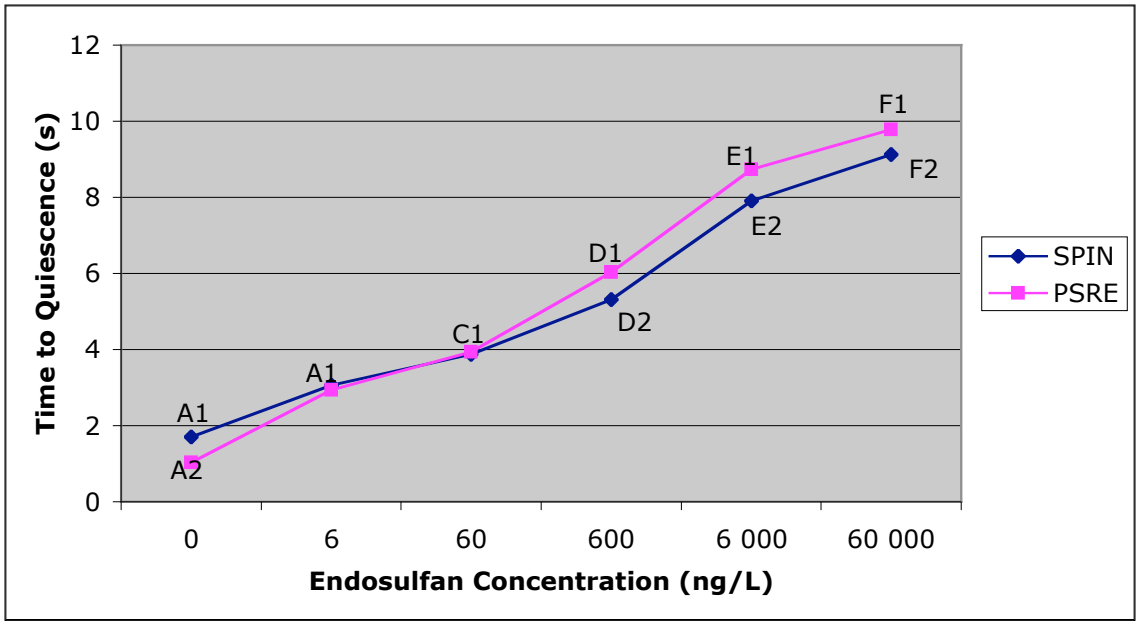

Figure 3.3 Time to quiescence (in seconds), for both *S. intermontana* (SPIN) and *P. regilla* (PSRE). Means followed by the same letter are not significantly different. Means followed by different letters are significantly (P<0.05) different.

In the control aquaria, *P. regilla* were less responsive to the glass rod and resettled significantly (P<0.05) faster than *S. intermontana* tadpoles. *P. regilla* tadpoles exposed to endosulfan took significantly (P<0.05) longer than *S. intermontana* to resettle. Additionally, they appeared to have more difficulty orientating themselves upright when they finally resumed a state of rest. At 6 000 ng/L endosulfan, tadpoles were clearly more agitated, and one *S. intermontana* tadpole swam in circles, likely due to the kink in its tails preventing normal swimming behaviour. At 60 000 ng/L, both species of tadpoles swam in circles when prodded with the glass rod, probably related to the tail kinking present in all individuals exposed to that concentration of endosulfan.

Abnormal morphology

One *S. intermontana* tadpole exposed to 6 000 ng/L endosulfan and all *P. regilla* and *S. intermontana* tadpoles exposed to 60 000 ng/L endosulfan developed kinks in their tails within the first 48 hours of exposure (this was not observed in tadpoles exposed to the other two pesticides). Additionally, *P. regilla* tadpoles exposed to 60 000 ng/L endosulfan lost pigmentation and became transparent to the human eye after roughly 4 days of exposure. Neither of these observations were made in the tadpoles exposed to azinphos-methyl or diazinon.

Discussion

The objective of this experiment was to determine the effects of three current-use pesticides on tadpoles of *S. intermontana* and *P. regilla* at environmentally relevant concentrations. By carrying out standardized eight-day LC20 experiments, those objectives were met. As laboratory mortalities were much less than the mortality rates observed in field experiments, it is likely that there are other factors influencing tadpole survivorship in their natural habitat.

In order for Lethal Concentration (LC) values to be calculated, 100% effects must be recorded in the highest test concentrations, or partial effects in the two highest concentrations must be recorded, ideally with a greater effect observed in the highest concentration. This requirement was fulfilled in the *S. intermontana* endosulfan test, yielding an average $LC20_{8d}$ value of 77.1 ng/L. However, neither of those requirements were fulfilled in the azinphos-methyl or diazinon experiments for *S. intermontana.* In the case of azinphos-methyl, overall survivorship was too high to calculate an effect. Thus, a

mortality rate of more than 20% was only observed in a test concentration of 50 ng/L. With the diazinon dilution series, survivorship was greater in the highest concentration when compared with the lower test concentrations, therefore, a LC value could not be calculated. Overall, a partial effect was observed in the lower concentrations; however, because survivorship increased in the highest concentration, this prevented any regression calculation from occurring.

A similar situation was observed with the *P. regilla* tadpoles, where survivorship was generally higher in the highest test concentration when compared with the lower test concentrations. Overall, no $LC20_{8d}$ values could be calculated for *P. regilla*. In the endosulfan test, percent survivorship was higher in the highest concentration, when compared with the two lowest concentrations. In the azinphos-methyl test, survivorship was adequate to calculate a $LC20_{8d}$ value, (i.e.: there was a 40% survivorship in the highest concentration); however, an error in water chemistry analysis yielded unusable water chemistry data, preventing any calculation from occurring. Lastly, similarly to the *S. intermontana*-diazinon experiment, survivorship of *P. regilla* in the highest concentration of diazinon was greater than in lower test concentrations. Therefore, no $LC20_{8d}$ value could be calculated.

Animals of both species at all concentrations of endosulfan exhibited varying degrees of excitability when stimulated by gentle prodding with a glass rod. This agitated behaviour is consistent with other experiments, which found that endosulfan was a central nervous system stimulant in many species of wildlife. Behaviours such as hyperactivity, tremors and seizures have all been reported in amphibian tadpoles exposed to endosulfan, due to endosulfan increasing synaptic concentrations of several

neurotransmitters, including acetylcholine noradrenalin (Berrill et al. 1998; Besbelli & Szajewski 2000; Harris et al. 2000; Park et al. 2001; Rohr et al. 2003). These behaviours are often followed by failure of pulmonary gas exchange and respiratory distress (Harris et al. 2000; Rohr et al. 2003).

Animals of both species at the highest concentration of endosulfan (60 000 ng/L), developed tail kinks within the first 48 hours of the experiment. In the case of *S. intermontana*, one individual in 6 000 ng/L endosulfan developed a tail kink. Berrill et al. (1998) also reported tail kinks at endosulfan levels of 200 ng/L up to 30 000 ng/L, along with prominently bloated heads. In experiments which tracked exposed tadpoles through metamorphosis, developmental abnormalities such as missing or deformed limbs, delayed metamorphosis and delayed growth were all reported (Berrill et al. 1998; Broomhall 2004; Rohr et al. 2003). However, there are no reports of melanin loss in endosulfan-exposed tadpoles of any species, nor were these observations made in field exposures (Bishop et al. 2006).

An additional experiment involving a mixture of the three pesticides at their respective environmental concentrations was carried out with *S. intermontana* tadpoles. As tadpoles in the wild tend to be exposed to a mixture of herbicides, insecticides and other contaminants, this pesticide mixture is representative of field conditions. However, the specific combination of azinphos-methyl, diazinon and endosulfan at these concentrations resulted in no observable effects. One tadpole in replicate A died, while every other tadpole survived. Additionally, none of the behavioural abnormalities observed in the endosulfan experiment manifested in the mixture experiment. These

results are partially explained by considering that the exposure to the pesticides singly at these concentrations also produced no observable effects.

Although no additive or synergistic effect was observed when exposed to the pesticide mixture; alternatively, combining an herbicide, insecticide and fungicide, with different modes-of-action, would yield results similar to those observed in field situations. In other toxicological tests, pesticides in combination with nitrates (known to reduce the oxygen-carrying capacity of hemoglobin) or heavy metals (known to block the active sites of many enzymes, inhibiting overall metabolism) have appeared to exacerbate the toxicity of the pesticides (Griffis-Kyle 2005; Harris et al. 1998; Johnson & Chase 2004; Marco et al. 1999; Natale et al. 2000; Ortiz-Santaliestra et al. 2006). Additionally, other biotic factors, such as the presence of predators or other competitors appear to increase the effect of a single pesticide (Bridges 2000; Broomhall 2004; Broomhall & Shine 2003).

Although no effects were observed in tadpoles exposed to the test concentrations of Guthion®, other experiments conducted by Schuytema et al. (1995) and Nebeker et al. (1998) revealed a number of effects of this pesticide at levels significantly higher than those tested in this experiment. Azinphos-methyl is a known acetylcholinesterase inhibitor and a potent endocrine disrupter in amphibian tadpoles (Nebeker et al. 1998). Furthermore, chronic exposure (over 10 days) to low levels (30 ng/L) of Guthion® appeared to be more detrimental than acute exposures to higher levels (1 000 000 ng/L) (Schuytema et al. 1995). Finally, Nebeker et al. (1998) observed impaired reproductive functioning in adult *P. regilla* exposed to up to 4 000 ng/L Guthion® in laboratory conditions.

Diazinon is a acetylcholinesterase inhibitor, (Denton et al. 2003; Sparling et al. 2001); depressed acetylcholinesterase activity often manifests as uncoordinated swimming and overall reduced activity, leaving the tadpole susceptible to predation (Sparling et al. 2001). Experiments by Denton et al. (2003) conducted on two-week old *P. regilla* tadpoles also showed muscle twitching, gyrating movements and impaired optomotor behaviour when exposed to concentrations ranging from 100 ng/L to 300 ng/L A.I. diazinon. However, none of the tadpoles exposed to diazinon in our experiment exhibited any of the previously reported behaviours. This is possibly due to the nominal test concentrations used in this experiment, compared to the 9 000 ng/L diazinon used in the experiments conducted by Sparling et al. (2001) and Denton et al. (2003).

Overall, *S. intermontana* experienced higher survivorship when compared with *P. regilla* in this experimental regime. This is contradictory to toxicity testing conducted on *P. regilla* exposed to Guthion® by Schuytema et al. (1995) and Nebeker et al. (1998), when compared to *Xenopus laevis* at higher concentrations of diazinon, where *P. regilla* was the more tolerant species. To date, *P. regilla* does not appear to have been used in toxicity testing of either azinphos-methyl or endosulfan, so a direct comparison of chemical sensitivity is not possible. Additionally, little work has been conducted on any *Spea* species, so no comparison of *S. intermontana*'s susceptibility to different agricultural pesticides is currently possible.

P. regilla animals also exhibited greater physiological responses to the endosulfan exposure, not only exhibiting greater agitation, but also experiencing melanophore aggregation for the duration of the experiment. It appears that aggregation (lightening of the dermis as observed in *P. regilla*) is under the control of acetylcholine, dopamine,

noradrenalin, serotonin and/or melatonin (Aspengren et al. 2003; Carr & Chambers 2001; Hadley & Goldman 1969; Hanumante et al. 1981; Natale et al. 2000; Novales & Davis 1969; Park et al. 2001; White et al. 1987). Endosulfan is known to disrupt neurotransmitter transmission in cholinergic neurotransmitter systems (systems which use acetylcholine as a neurotransmitter), altering the concentrations of melatonin, acetylcholine, serotonin, dopamine and norepinephrine (Goulet & Hontela 2003; Harris et al. 2000; Park et al. 2001; Sparling et al. 2001). There are a number of hypothesized biochemical pathways which are targeted by endosulfan which would lead to melanophore aggregation, and at present the affected pathway(s) of the afflicted *P. regilla* tadpoles of this experiment is not known.

As indicated by the results achieved here, when compared to field results in 2003 to 2006 (Bishop et al. 2006), there lies a major disconnect between toxicological results acquired from the field, and those achieved in laboratory environments (Bishop et al. 1999; Hayes et al. 2002, 2006; Relyea 2005). The widespread application of pesticides has attracted the attention of many ecologists, who recognize that management suggestions are based on laboratory-based, single-species, single-chemical exposures to determine the direct effects of a contaminant (Relyea et al. 2005). Field studies pose a much broader and ecologically-relevant stress regime, and may reveal indirect and direct pesticide toxicities that would have otherwise remained undetected in laboratory exposures (Bishop et al. 1999; Hayes et al. 2002, 2006; Johansson et al. 2006; Relyea 2005; Relyea & Hoverman 2006; Storfer 2003; Scholz & Hopkins 2006).

Laboratory tests conducted for the purpose of pesticide regulation determine the concentration of active ingredient lethal to 50% of the test population. These tests do not

consider the sublethal effects of the chemical, nor the ecological significance of results gained from lower concentrations. Hayes et al. (2002) found that levels of atrazine in the parts-per-billion range, (such as those found in field sites), resulted in feminized male *X. laevis*. Those levels were significantly less than the levels which elicit fifty percent acute mortality (*see* Appendix F). Furthermore, the levels of atrazine tested by Hayes et al. (2002) had no influence on survivorship, time to metamorphosis or size at metamorphosis, highlighting the importance of evaluating the sublethal, physiological effects of pesticides. Park et al. (2001) found that levels of endosulfan as low as 500 ng/L altered pheromonal gland alveoli growth in adult Red-Spotted Newts (*Notophthalmus viridescens*), thus altering pheromone production. Those test concentrations were significantly lower than the accepted LC_{50} values (*see* Appendix F), and although the endosulfan concentrations did not produce any mortalities, the implications on mating success have significant ecological ramifications.

Conclusion

In this phase of experimentation, *S. intermontana* was a more resilient species than *P. regilla*, where S. *intermontana* experienced lower mortality rates and a lower incidence of sub-lethal effects on physiology which is inconsistent with both our hypothesis and field results (Bishop et al. 2006). Other researchers attempting to replicate field results in a laboratory environment have also proven to be unsuccessful (Bishop et al. 1999; Hayes et al. 2006; Relyea et al. 2005). Though the three insecticides tested did not yield the same mortality rates at the same field concentrations, valuable observations were made on the neurological impacts of low levels of endosulfan. To

date, other toxicological research on endosulfan has occurred using concentrations in the parts-per-million range (up to one thousand times higher than detected in the field), with the same behavioral and morphological anomalies occurring. This not only has significant ecological ramifications, but also implications for pesticide regulation.

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CHAPTER 4

4.1 General Summary and Conclusions

Field studies conducted in the South Okanagan valley found embryos of Great Basin Spadefoot (*Spea intermontana*) and Pacific Treefrog (*Pseudacris regilla*) placed in ponds surrounded by agricultural operations suffered 80% to 100% mortality (Bishop et al. 2006). Embryos were previously unexposed to any environmental contaminants, though the history of the adults is unknown, and thus maternal transfer of contaminants cannot be ruled-out. The pesticide concentrations (of endosulfan, azinphos-methyl and diazinon) detected at the field sites experiencing the highest degree of mortality were used in the present laboratory study (Bishop et al. 2006). These concentrations were significantly lower (as little as 10 000 times less) than the published acute (96-hour) median lethal concentrations ($LC50_{96hr}$) values for those pesticides (*see* Appendix F), and lower than the calculated eight-day lethal concentration affecting twenty percent ($LC20_{8d}$) of the animals in this experiment. Other than appropriate daylight hours, no other environmental factors potentially experienced in the field, such as fluctuating temperature, predator cues, or dissolved oxygen levels, were replicated. Under single-pesticide exposure conditions, results were not consistent with those observed in the field.

Laboratory results attained from exposing *S. intermontana* and *P. regilla* embryos and tadpoles to single pesticides appears to be consistent with other single-species, single-chemical exposures conducted in other amphibian toxicity assays. Overall, embryos appeared to be more resilient than tadpoles, with mortalities occurring post-hatch. This is consistent with other experiments involving other organophosphates at

varying concentrations (Bridges 2000; Edington et al. 2004; Edington et al. 2007; Harris et al. 2000; Marquis et al. 2006; Welsh & Olivier 1998), with the exception of experiments involving pyrethroids and pyrethroid/organophosphate combinations. Pyrethroids appear to be able to permeate not only the jelly coat of amphibian embryos, but the fertilization membrane as well (Denton et al. 2003; Greulich & Pflugmacher 2003).

In the embryo experiment, *S. intermontana* embryos and young tadpoles were more sensitive to all three pesticide exposures when compared with *P. regilla* embryos and young tadpoles, (with mortalities occurring post-hatch in both species). However, in the tadpole experiment, *S. intermontana* experienced lower mortalities and fewer physiological abnormalities than *P. regilla* tadpoles. Although both species of tadpoles exhibited agitated behavior in the higher concentrations of endosulfan; *P. regilla* experienced slightly greater agitation, as well as complete melanophore-aggregation, manifesting as a complete loss of pigmentation in the highest concentration of endosulfan (60 000 ng/L). Additionally, tadpoles of both species exposed to the highest concentration of endosulfan, developed tails that kinked to the left. This greater sensitivity in the tadpole life-stage is consistent with other amphibian early-lifestage experiments (Bridges 2000; Bridges & Semlitsch 2000; Edington et al. 2004; Harris et al. 2000; Marquis et al. 2006; Relyea 2004; Sanders 1970).

The loss of pigmentation observed in the *P. regilla* tadpoles exposed to the highest concentration of endosulfan (60 000 ng/L in this experiment) has likely some connection to a disruption in biochemical control of the melanophores (a cell type found in the skin of lower vertebrates that contain granules of melanin) beneath the tadpole's

dermis. Skin chromatophores (also termed melanocytes or melanophores in amphibians) are exposed directly to the water, and hence to any contaminants that may be present. Therefore, changes in skin colour (either lightening brought on by melanin aggregation, or darkening brought on by melanin dispersion) can be a response to pollutants in the water (Carr & Chambers 2001; Tomar & Pandey 1988).

Dispersion of the melanophores (resulting in the darkening of the dermis) is under the control of the Melanocyte-Stimulating Hormone (MSH) released by the intermediate lobe of the pituitary gland (Aspengren et al. 2003; Carr & Chambers 2001; Krisfaluis et al. 1998; Tomar & Pandey 1988; White et al. 1987); it appears that aggregation (lightening of the dermis) can be under the control of acetylcholine, dopamine, noradrenalin, serotonin or melatonin (Aspengren et al. 2003; Carr & Chambers 2001; Hadley & Goldman 1969; Hanumante et al. 1981; Natale et al. 2000; Novales & Davis 1969; Park et al. 2001; White et al. 1987). Additionally, exposure to any beta-blocking agents or pollutants mimicking the involved hormones could also give rise to melanophore aggregation (Aspengren et al. 2003; Hadley & Goldman 1969).

The primary pathway for melanophore dispersion includes MSH (acts as a first messenger) targeting dermal melanophores, resulting in the formation of cAMP (cyclic adenosine monophosphate found in the melanophores acts as a second messenger) facilitated by the enzyme adenyl-cyclase (Aspengren et al. 2003; Hadley & Goldman 1969; Novales & Davis 1969; White et al. 1987). The cAMP acts as a beta-adrenergic stimulator, which favours the increase of cAMP in the melanophores, resulting in the dispersion of melanin granules within the melanophores, indicated by an observable darkening of the dermis.

In *Anolis* lizards, melanophore aggregation is a response to sympathomimetric stimulation (Hadley & Goldman 1969), which is regulated by alpha-adrenergic receptors. In times of stress or excitement (such as an eight-day exposure to endosulfan), these alpha-receptors are stimulated by catecholamines (i.e.: norepinephrine and dopamine), resulting in what is known as "excitement-pallor". This is often followed by "excitement-darkening", resulting from beta-receptor stimulation (Aspengren et al. 2003; Hadley & Goldman 1969; Novales & Davis 1969; White et al. 1987).

Endosulfan is known to disrupt neurotransmitter transmission in cholinergic neurotransmitter systems (systems which use acetylcholine as a neurotransmitter), altering the concentrations of melatonin, acetylcholine, serotonin, dopamine and norepinephrine (Goulet & Hontela 2003; Harris et al. 2000; Park et al. 2001; Sparling et al. 2001). There are a number of hypothesized biochemical pathways which are targeted by endosulfan, which would lead to melanophore aggregation. Catecholamines and melatonin have shown to increase in animals exposed to endosulfan due to endosulfan stimulating the sympathetic nervous system (Park et al. 2001; Rohr et al. 2003; Scholz & Hopkins 2006). These neurotransmitters act as antagonists to MSH, and favor a decrease in melanophoric cAMP levels, and thus facilitate melanophore aggregation (Aspengren et al. 2003; Hadley & Goldman 1969; Novales & Davis 1969; White et al. 1987).

Melanophore aggregation has been observed in a number of toxicological experiments conducted on amphibians, teleosts and crustaceans. Tomar & Pandey (1988) exposed anuran tadpoles to Dichlorvos® insecticide (a synthetic organophosphate commonly used in India), and observed significant aggregation of melanophores in all concentrations (25 000 ng/L – 100 000 ng/L A.I.). Tadpoles appeared to turn grey or

beige during the fifteen-day experiment. There arose two hypothesis as to why this was observed: the inhibition of MSH-regulating mechanism within the central nervous system; or changes in the biochemical properties of MSH by a direct toxic effect on the melanophores. Additionally, the melanophores appeared to have suffered irreversible damage, as the recovery of pigmentation did not occur.

Pawar et al. (1983) exposed de-jellied embryos of the Ornate Narrow-Mouthed Frog (*Microhyla ornata*) to varying concentrations of Malthion® (organophosphate used in mosquito control), from 1 000 000 ng/L – 20 000 000 ng/L A.I.. Embryos in every dilution of the exposure experienced delayed melanogenesis, suggesting that the insecticide interfered with the enzymatic synthesis of melanin.

Furthermore, Krisfaluis et al. (1998) exposed alevin Rainbow Trout (*Oncorhynchus mykiss*) in a 96 hour acute toxicity test involving methoxynclor (MXC is a broad-spectrum organochlorine insecticide). At 6-days post-hatch, the dermis of exposed animals was visibly lighter than the control animals. Interestingly, the melanophore number in both exposed and control animals was the same; however, the melanin within appeared to be significantly more dispersed in the control animals. This was thought to be due to a disruption of MSH secretion from the pituitary. Unlike in the observations reported by Pawar et al. (1983), pigmentation returned after 21-days post-hatch. It was proposed that MXC modifies but does not permanently damage the neuroendocrine system of trout.

Hanumante et al. (1981) exposed Fiddler Crab (*Uca pugilator*) to poly-chlorinated biphenyls (PCBs), and observed a marked aggregation of melanophores. The hypothesis was that PCBs affected the release of MSH, stimulated by the

neurotransmitter, norepinephrine. A normal increase in norepinephrine levels mediates the release of MSH, and thence melanin dispersion within the melanophores. However, PCBs appeared to actively decrease the amount of norepinephrine available at the appropriate synapses in order trigger sufficient release of MSH to induce dispersion, thus causing the melanophores to aggregate instead.

The aggregation of melanophores can have serious ecological implications, as melanin acts to protect organisms against ultra-violet radiation. Exposure to ultra-violet radiation has shown to adversely impact all stages of amphibians, including suppresssion of the immune system, altered behavior, slowed rate of metamorphosis and has shown to act synergistically when combined with some agricultural pesticides (Blaustein et al. 2003; Burkhart et al. 1998, 2000; Davidson et al. 2001; Hatch & Blaustein 2000; Kiesecker 2002). Additionally, tadpoles exhibiting melanophore aggregation would potentially be more visible and thus more susceptible to predation (Carr & Chambers 2001).

Tail kinking has been observed in a number of toxicological experiments involving a variety of toxicants. Cooke (1973) observed tail kinking (also to the left) in Common Frog (*R. temporia*) tadpoles exposed to chronic doses of 200 ng/L DDT. Materna et al. (2005) observed tail kinking in developing *R. sphenocephala* tadpoles exposed to 3 400 ng/L A.I. of the synthetic pyrethroid, esphenvalerate. Bridges (2000) observed tail kinking in tadpoles exposed as embryos to 16 000 ng/L of carbaryl. Furthermore, Greulich and Pflugmacher (2003) observed tail kinking in developing tadpoles exposed to a pyrethroid insecticide, cypermethrin. It is hypothesized that exposure to a variety of contaminants known to impact the CNS inhibit either the

development of the spinal cord, or the closure of the dorsal hollow nerve cord in embryo development. Unfortunately, little is known of the direct pathway targeted by organochlorines (in the case of our experiment), pyrethroids or some heavy metals (Bridges 2000; Cooke 1973; Greulich & Pflugmacher; Materna et al. 2005).

Following the exposure to a potentially toxic chemical, any modification in behavior in an organism frequently suggests that the nervous system is the target of the chemical (Carr & Chambers 2001). The observed agitated behavior in the tadpoles exposed to increasing concentrations of endosulfan is likely because endosulfan has shown to increase acetylcholine synaptic concentrations significantly, acts as a GABA (g-aminobutyric acid)-gated chlorine channel antagonist; as well as a calcium- and magnesium-ATPase inhibitor (both of these enzymes are involved in nerve impulse transmission) (Goulet & Hontela 2003; Harris et al. 2000; Park et al. 2001; Sparling et al. 2001). GABA is an inhibitory neurotransmitter which operates through membrane polarization as mediated by taking up chlorine ions into the nerve cells. By impairing this inhibitory complex, there is a constant chlorine ion influx, demonstrated by hyperactivity observed in the tadpoles exposed to the increasing concentrations of endosulfan in this experiment. This increase in agitated behavior often facilitates increases in norepinephrine, dopamine and acetylcholine synaptic concentrations (Carr & Chambers 2001; Park et al. 2001; Sparling et al. 2001). High acetylcholine concentrations potentially result in the continual stimulation of cholinergic receptors, resulting in not only agitated behavior, but could also precipitate the aggregation of melanocytes seen in the *P. regilla* tadpoles.

The mechanisms underlying global amphibian declines are complex, and require multi-factorial studies to understand and predict amphibian population trends (Relyea 2004; Storfer 2003). The majority of amphibian studies investigate single factors, such as pesticides, pH, UV-B radiation, heavy metals or nitrates. However, amphibians are exposed to several "stressors" simultaneously, and exposure occurs at every lifestage. In BC agriculture, insecticides are often applied in conjunction with other insecticides, herbicides and fertilizers, resulting in the exposure of the amphibians to a complex mixture of pollutants (Richardson 2001; Rohr et al. 2003; Wind 1999). Often the response of developing amphibians to environmental stressors depends on other abiotic conditions or stressors present in the environment (Hatch & Blaustein 2000).

Naturally occurring "stressors" such as competition, predation or pathogens can act as additional stressors, overwhelming an amphibian's defenses (Blaustein et al. 2003; Hatch & Blaustein 2000; Scholz & Hopkins 2006). For example, Relyea and Mills (2001) tested the pesticide carbaryl in combination with predator chemical cues, and found that mortality in developing amphibians was significantly higher when the two factors were combined. Relyea (2003) tested the effects of malthion and glyphosate in combination with predator chemical cues. In this experiment, malthion alone had very little indirect or direct effect on either Northern Leopard Frog (*Rana pipiens*) or Grey Treefrog (*Hyla versicolor*). However, when tadpoles were exposed to a combination of malthion and predator chemical cues, there was a significant direct effect. Furthermore, glyphosate in conjunction with predator cues, completely eliminated the test populations of both *R. pipiens* and *H. versicolor* at 320 000 ng/L AI of glyphosate. Relyea et al. (2005) deduced that the chemical cues of predators (resulting in the release of adrenaline)

appeared to cause synergistic interactions with the pesticides (Relyea & Mills 2001; Relyea 2003, 2004).

As amphibians are often exposed to a multitude of pesticides in aquatic environments, it is important to test the effects of pesticide mixtures when considering pesticide toxicities (Belden & Lydy 2000; Denton et al. 2003; Relyea 2004; Relyea et al. 2005; Scholz & Hopkins 2006; Storfer 2003). Scholz et al. (2006) observed the impacts of the organophosphate diazinon in conjunction with carbamates on Chinook salmon smelts. In this particular species, the combination appears to have an additive effect, inhibiting acetylcholinesterase-mediated synaptic transmission. Relyea (2004) tested the combined effects of diazinon, malthion, glyphosate and carbaryl on four species of amphibians. When the pesticides were presented alone, there was rarely any negative impact, at test concentrations much lower than the accepted LC_{50}. The combination of pesticides caused mortalities between 30% and 70%, which was found to be the equivalent effect in exposures twice the concentration of the most toxic pesticide (Relyea 2004). Denton et al. (2003) exposed Flathead Minnows (*Pimephales promelas*) to a combination of esfenvalerate and diazinon. This combination had a marked synergy, reducing carboxylesterase (enzymes such as acetylcholinesterase) activity by upwards of 50% in all test concentrations. Because there was no dose-response, Denton et al. (2003) determined that the affect was not additive. These synergistic properties of pesticide combinations are often used to the benefit of the farmer, enhancing the toxicity of a pesticide to its target organisms (Belden & Lydy 2000; Denton et al. 2003; Relyea 2004). Given that the mentioned pesticide combinations are encountered in the field, it is imperative to test the effects on non-target organisms.

This thesis focused on the impacts of single-pesticide exposures on embryos and tadpoles; however, adults are often subjected to the same contaminants. Maternal and paternal transfer of contaminants, ranging from pesticides to heavy metals is an emerging field of study in amphibian toxicology. Kadokami et al. (2004) observed polychlorinated biphenyls (PCBs), dibenzofurane and polychlorinated-dibenzo-dioxin levels in Montane Brown Frog (*R. ornativentris*) equal to triple the levels detected in adult female livers. Fontenot et al. (2000) observed bioaccumulation of PCBs in *R. catesbeiana* adults, up to 2 260 000 ng/L; with embryo levels reaching 10 000 000 ng/L in embryos laid by the tested females. Interestingly, the PCB levels in females who recently laid eggs had an observable decrease in PCB levels. Hopkins et al. (2006) observed maternal transfer of significant levels of selenium and strontium to Narrow-Mouthed Toad (*Gastrophryne carolinesis*) embryos. Decreased success at hatching and an increase in abnormalities at hatching and throughout development were all observed in embryos laid by contaminated adults despite being transferred to uncontaminated sites upon oviposition.

Many of the pesticides used in North America are applied prior to the growing season, and often are applied in conjunction with fertilizers. As many species of amphibians breed in ponds within agricultural settings, they are often exposed to a combination of pesticides and nitrates. In the presence of nitrogen-based fertilizers, amphibians appear to be significantly more sensitive to other chemicals, such as pesticides (Allran & Karasov 2000; Blaustein et al. 2003**;** Harris et al. 1998; Marco et al. 1999; Ortiz-Santaliestra et al. 2006; Orton et al. 2006). Behaviors such as reduced feeding, reduced swimming, disequilibrium and physiological malformations have all been observed in exposed tadpoles and tadpoles exposed as embryos (Allran & Karasov

2000; Blaustein et al. 2003**;** Harris et al. 1998; Marco et al. 1999; Ortiz-Santaliestra et al. 2006; Orton et al. 2006). Overall, these results appear to be a result of a decreased ability to cope with environmental stressors in the presence of other stressors, rather than a mechanistic action of either the pesticides or nitrates (Allran & Karasov 2000; Hatch & Blaustein 2000; Orton et al. 2006). Orton et al. (2006) found that nitrates combined with atrazine affected gonad differentiation and resulted in digestive system abnormalities in *R. pipiens* tadpoles. Allran and Karasov (2000) observed similar abnormalities, with the addition of significantly slower growth and development times to metamorphosis.

A growing concern with the reduction in the ozone layer, is the increasing exposure of amphibians to UV-B radiation (Davidson et al. 2001). Alone, UV-B has shown to produce a number of sub-lethal effects, including: altered behavior, slowed growth and development, physiological and developmental abnormalities (Blaustein et al. 2003; Burkhart et al. 1998; Davidson et al. 2001). Davidson et al. (2001) observed species sensitivity differences to UV-B radiation, as well as life-stage sensitivity differences. Overall, two-week old tadpoles exposed to UV-B exhibited significantly higher abnormality and mortality rates (greater than 80%), over a seven day test. Burkhart et al. (1998, 2000) observed significant limb abnormalities associated with UV-B radiation exposure. UV-B radiation as an additional stressor in a contaminated environment seems to overwhelm amphibian defenses, increasing their sensitivity to low levels of pesticides, nitrates and pathogens. There is an apparent synergy between contaminants and UV-B radiation, specifically those contaminants that are known to be phototoxic (Blaustein et al. 2003). Hatch and Blaustein (2000) observed significantly reduced survivorship in tadpoles exposed to UV-B radiation and nitrate in a low-pH

environment. In addition, UV-B radiation and nitrates in a neutral environment were found to significantly reduced amphibian tadpole activity levels. Kiesecker (2002) determined that Western Toad (*Bufo boreas*) embryos exposed to UV-B had an increased susceptibility to infection by a naturally occurring oomycete fungus.

Not all physiological changes brought on by chemical exposures are internal, however. Several incidences of additional or lack of limbs, bloated heads, tail kinks have all been observed in exposed amphibians (Berrill et al. 1998; Bridges 2000; Burkhart et al. 1998, 2000; Materna et al. 1995; Rohr et al. 2003). These abnormalities are not always observed in laboratory conditions, as the test concentrations are often too high, and the experimental timeframe too short, with mortality occurring before any abnormalities can be observed. Berrill et al. (1998) observed bent tails and bloated heads in American Toad (*B. americanus*) tadpoles exposed to 20 000 ng/L of endosulfan in field sites. Rohr et al. (2003) observed missing limbs and digits and developmental abnormalities in Streamside Salamander (*Ambystoma barbourii*) larvae exposed to a mixture of insecticides and herbicides, including 100 000 ng/L endosulfan. These abnormalities greatly depress the ecological fitness of the animal and the overall population.

Sub-lethal effects can also impact amphibians at the community-level. Boone and Semlitsch (2002) examined the effect of carbaryl on Woodhouse's Toad (*B. woodhousii*) tadpole survivorship, and discovered that survivorship was higher in exposed tadpoles than in the controls. Increased survivorship has the potential to result in greater numbers of sub-adults and adults, resulting in an increase in competition for resources, and ultimately the reduction of adult survivorship. The same study observed increased time

to metamorphosis, which not only could result in the increased competition for pond resources, but also a greater risk of mortality in ephemeral pools.

Many behavioral changes have been observed in amphibians exposed to a combination of stressors. An overall reduction in activity has been observed in amphibian tadpoles exposed to an array of stressors, alone in combination, including: pesticides, UV-B radiation, extreme temperatures, low pH, pesticides and fertilizer. An overall reduction in activity can have several ecological implications, including: decreased predator avoidance, decreased feeding, an increase in disease susceptibility and a decrease in breeding success (Berrill et al. 1998; Broomhall 2004; Broomhall & Shine 2003; Nebeker et al. 1998; Park et al. 2001). These sub-lethal effects are often not observed in laboratory tests, as acute mortality is the most commonly-used toxicological endpoint. Furthermore, Schuytema et al. (1995) observed that chronic exposures to pesticides at lower levels are more detrimental than acute exposures to higher levels. Therefore, it is important to not only test chemicals at environmentally-relevant concentrations in laboratory environments, but to also test stressors in combination to achieve a better understanding of the changes observed in field situations.

4.2 Management Implications

One of the biggest challenges faced by ecological risk assessors, is to derive threshold concentrations for environmental contaminants that protect both species diversity and the functional attributes of natural ecosystems (Maltby et al. 2005). Species vary markedly in their sensitivity to environmental contaminants; thus, toxicological data derived from tests conducted on *Daphnia* or a salmonids cannot necessarily be applied to

avians or amphibians (Maltby et al. 2005; Scholz & Hopkins 2006; Storfer 2003). In ecological risk assessments, a species sensitivity distribution (SSD) is established, based on the concentration of chemical at which a specified percentage of the population will be affected, known as the hazardous concentration (HC). Most commonly a HC_5 (five percent of the population) or HC_{10} (ten percent of the population) is used (Maltby et al. 2005). However, these concentrations are based on acute toxicity data, which is restricted in the responses measured and the time allowed; thus, is not representative of field conditions (Maltby et al. 2005; Relyea 2005; Scholz & Hopkins 2006; Storfer 2003). Additionally, these examinations are often conducted on the simplest life-form found in the ecosystem (arthropods), and applied to the entire ecosystem, as indicated by studies conducted by Maltby et al. (2005).

Recent toxicological research has revealed that many of the current-use pesticides are more toxic to amphibians than initially realized. This discovery comes in the wake of field observations rarely being supported or explained by observations made in laboratory experiments under similar conditions. As discussed previously, wildlife in aquatic environments are chronically exposed to low levels of a litany of contaminants, undergoing changes that are not observed in lab tests involving single chemical testing. Laboratory experiments involving agricultural pesticides in combination with other stressors, such as: predation, competition, low dissolved oxygen or ultraviolet radiation, observed higher amphibian mortalities than in tests with only one factor. With this in mind, using field data or results of pesticide-mixture experiments in management or regulation recommendations for amphibian conservation would be more effective. However, as there are currently over 80 000 pesticide formulations currently in use across

North America (Relyea 2004), testing all possible combinations of pesticides is not feasible. However, if the issue of current amphibian declines is to be addressed, more investigation is required.

Amphibians appear to be more resilient to a chemical onslaught if there are no other stressors present. In the South Okanagan Valley, breeding ponds of *S. intermontana* and *P. regilla* often lie within agricultural settings. The water within these ponds is a product of surface and groundwater runoff, carrying sediment, pesticides, fertilizers and other chemicals into the pond. Although the detected levels of individual pesticides within these ponds may not be lethal to amphibians, significant mortalities can occur in the embryo stage. However, when combined with low oxygen levels, high nitrate levels, fluctuating temperatures and ultraviolet radiation, these low levels become lethal. To that end, improving the water quality of these breeding ponds by monitoring inputs, and limiting polluted run-off could greatly improve embryo and larval survival.

To have the greatest relevance for the management of at-risk species, the spatial and temporal scales of pesticide exposures, interactions between pesticides and other stressors (chemical and non-chemical), and the environmental realism of exposure concentrations should be considered when applying current toxicological studies to ecological risk assessments (Scholz & Hopkins 2006). Future management and regulation of pesticides would benefit from studies that address novel taxa, mixture toxicities, sublethal effects affecting individual and population fitness, and the indirect ecological effects of pesticides in complex communities (Scholz & Hopkins 2006).

4.3 Future Research

The aim of this study was to determine the effects of three current-use pesticides on *S. intermontana* and *P. regilla* embryo and tadpole survivorship and development. Initially, the purpose was to answer some of the questions that arose from previous field studies, namely which pesticide was causing the 80% to 100% mortalities in ponds surrounded by traditional agriculture. The three pesticides examined (endosulfan, azinphos-methyl and diazinon) alone had little impact on embryo survivorship or development, and only minor impacts on tadpole survivorship in the laboratory. However, as these two species are exposed to a litany of pesticides in field environments, it is necessary to determine which combinations are having the most impact. In addition, other abiotic factors such as temperature fluctuations, low pH, low dissolved oxygen and ultra-violet radiation encountered in the field, must be taken into consideration in future experiments.

Subsequent toxicity tests on *S. intermontana* and *P. regilla* will involve exposures to combinations of the commercial formulations of the following current-use pesticides: endosulfan, atrazine, diazinon, glyphosate, carbamate and azinphos-methyl. In addition to the pesticides, varying temperature, pH and dissolved oxygen levels is recommended, in order to mimic field conditions more accurately. Furthermore, exposing some of the treatments to UV-B radiation could provide a better understanding of how the pesticides under examination behave in field environments. As mentioned in numerous other toxicological research, to better understand the reason for the current amphibian declines, exposing the test organisms to multiple stressors in laboratory environments will provide a more accurate picture of what is occurring in the wild.

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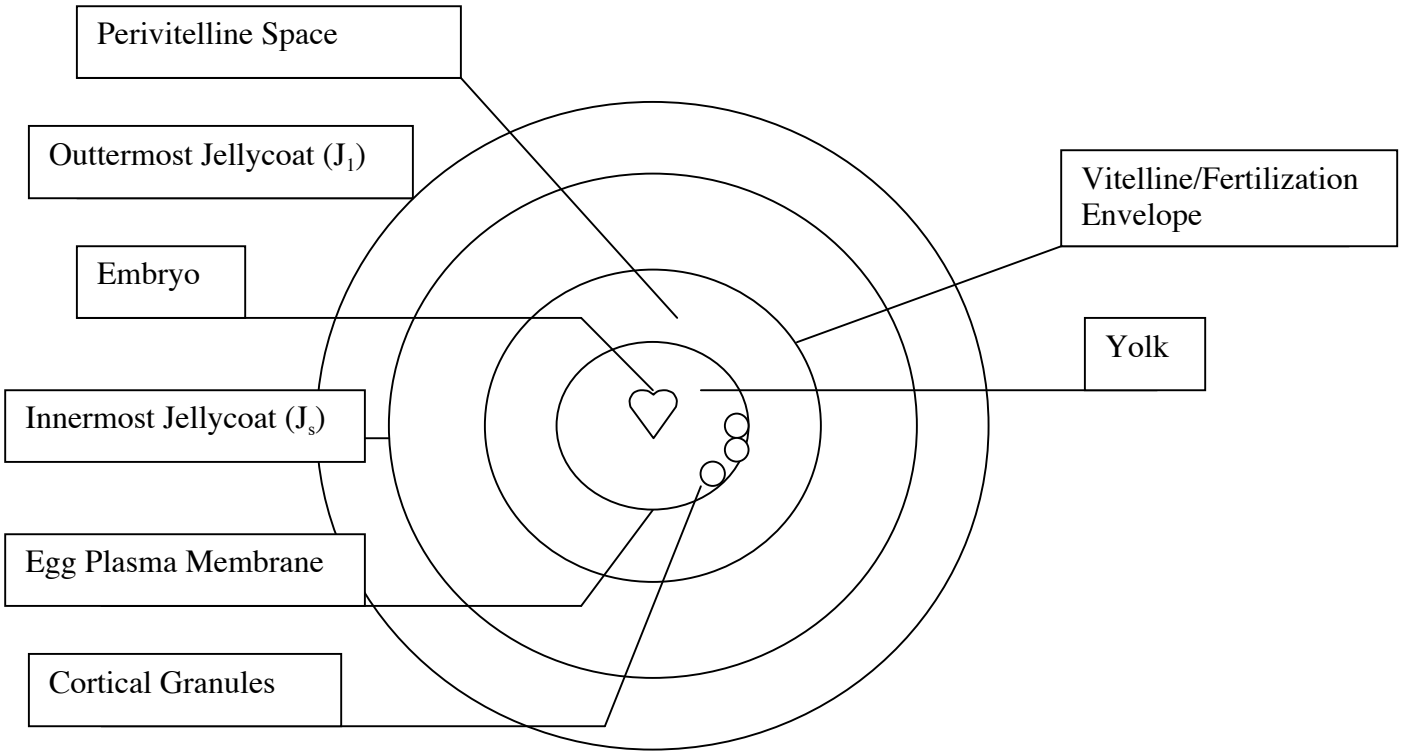
APPENDIX A

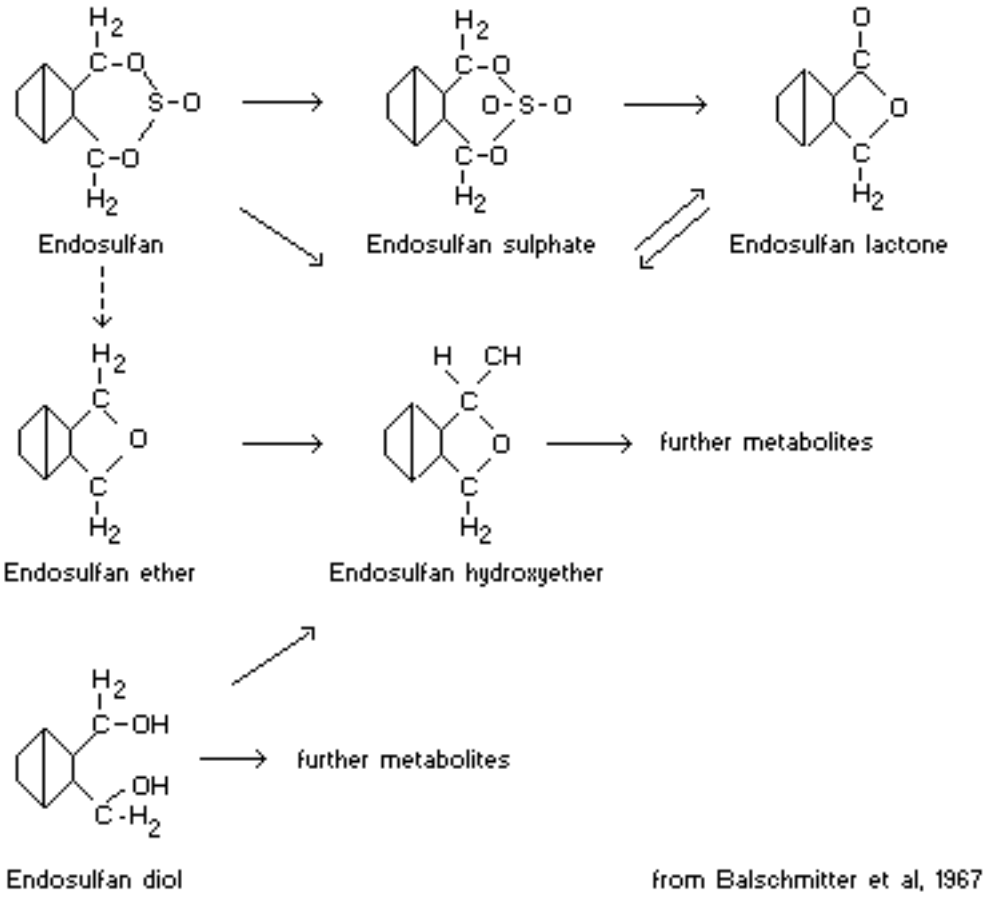
CELLULAR STRUCTURE OF AN AMPHIBIAN EMBRYO



APPENDIX B

CHEMICAL STRUCTURE OF ENDOSULFAN

INSECTICIDE



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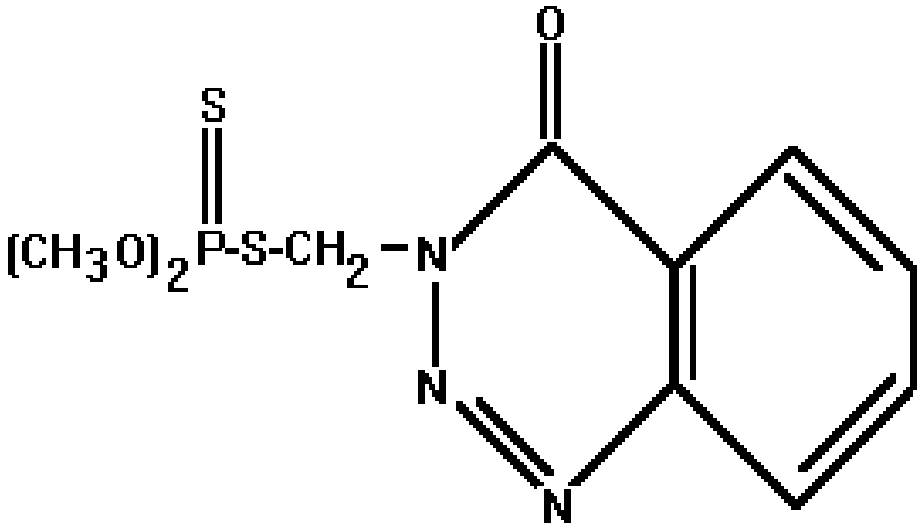
APPENDIX C

CHEMICAL STRUCTURE OF AZINPHOS-METHYL

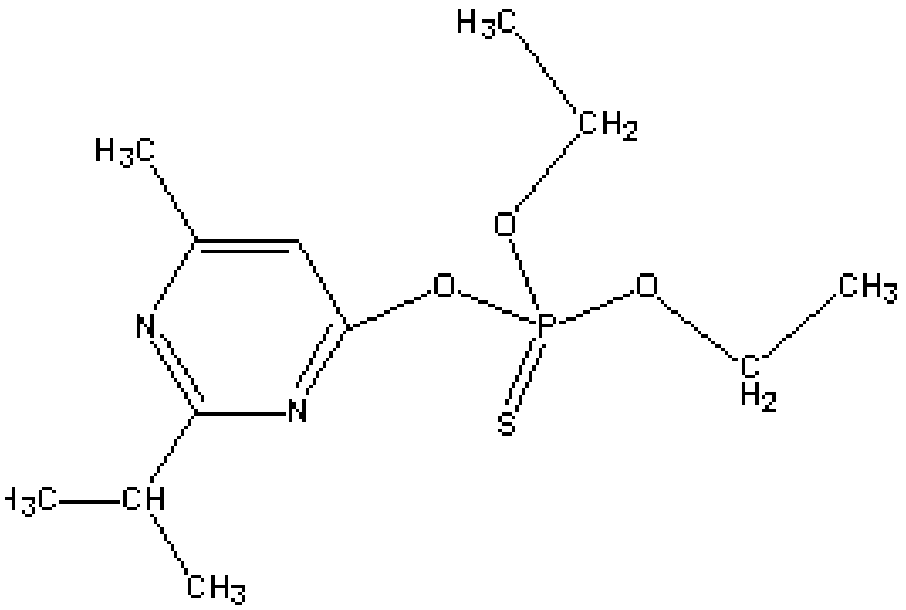
INSECTICIDE



Wikipedia. 2008. Organophosphate: Azinphos-methyl. Accessed January 14, 2008 from **HTTP://EN.WIKIPEDIA.ORG/WIKI/IMAGE:AZINPHOS-METHYL-2D-SKELETAL.PNG**

APPENDIX D

CHEMICAL STRUCTURE OF DIAZINON INSECTICIDE



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APPENDIX E

GOSNER STAGING OF AMPHIBIAN DEVELOPMENT

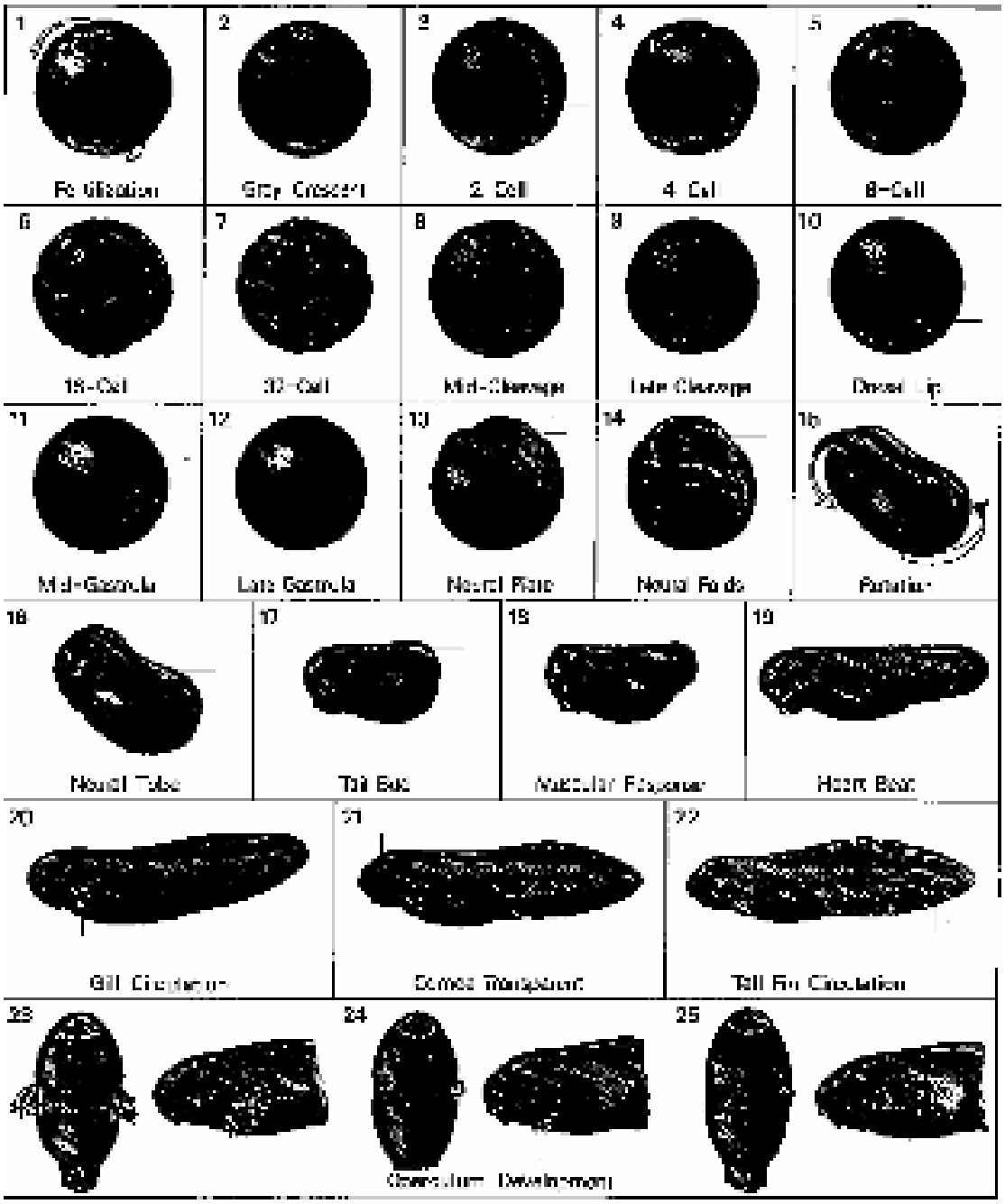

Figure A.1 Standard early stages of development of anurans. Stages are according to Gosner (1960).

APPENDIX F

96-HOUR LETHAL CONCENTRATIONS OF 50%

($LC50_{96HR}$) FOR VARIOUS PESTICIDES:

Pesticide	Species	$LC50_{96hr}$
Atrazine	Rainbow Trout	4 500 ug/L
Atrazine	Daphnia sp.	6 900 ug/L
Azinphos-methyl	Large Mouth Bass	25 ug/L
Azinphos-methyl	Daphnia sp.	0.26 ug/L
Azinphos-methyl	American Bullfrog	5983.6 ug/L
Diazinon	Rainbow Trout	6 350 ug/L
Diazinon	Daphnia sp.	0.522 ug/L
Diazinon	American Bullfrog	3505 ug/L
Endosulfan	Rainbow Trout	1.5 ug/L
Endosulfan	American Bullfrog	7.8 ug/L
Endosulfan	Stoneflies	3.3 ug/L
Glyphosate	Rainbow Trout	86 000 ug/L
Glyphosate	Daphnia sp.	780 000 ug/L
Malthion	Rainbow Trout	60 ug/L
Malthion	Mudminnow	240 ug/L