

THE SUBLETHAL EFFECTS OF 2,4-D DIMETHYLAMINE ON WOOD FROG TADPOLES
IN SASKATCHEWAN

A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in the Toxicology Graduate Program
University of Saskatchewan
Saskatoon, Saskatchewan
Canada

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ABSTRACT

Declining amphibian populations in association with an incidence of deformities have been observed globally. These observations have alarmed the scientific community as well as the general public. Potential causes include exposure to pesticides; therefore two experiments were performed to test the sublethal effects of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) on tadpoles of the wood frog (*Rana sylvatica*). Wood frog tadpoles in the first experiment were exposed to 2,4-D amine at 0.1, 1.0 and 100 µg/L in outdoor microcosms. Morphometric measures (total length, snout-vent length (SVL) and wet weight) were taken at metamorphic climax. Deformities and circulating hormone concentrations (corticosterone - CORT) were also assessed. Results showed that though tadpoles were exposed to various concentrations of 2,4-D throughout their aquatic life, there were no treatment differences associated with any of the endpoints except for total length ($p = 0.023$). Total length during metamorphosis was highly variable. Although statistically significant, biological significance was questionable. The second experiment was conducted in the field using natural ponds. The experimental groups included forested ponds (removed from pesticide exposure), agricultural ponds (potentially exposed) and treated agricultural ponds (intentionally treated with 2,4-D to achieve a concentration of 10 µg/L). Relatively rapid degradation of 2,4-D occurred in all treated ponds. The mean half-life was 8.0 ± 5.5 days. In spite of the degradation, 2,4-D was present in the ponds until tadpoles metamorphosed. Similar endpoints including morphometric measures, as well as deformities and plasma CORT hormone were determined. In addition, total lipid and total protein (of the carcass) were also measured. Unlike the microcosm study, statistically significant differences were observed in SVL, wet weight and total protein although the differences may have been unrelated to 2,4-D exposure. The metamorphs sampled from the forested ponds were smaller in

SVL (23% shorter) and wet weight (58% lighter) ($p < 0.029$) relative to the other two groups. Total protein in the metamorphs from the forested ponds was 22% lower than that of the agricultural ponds ($p = 0.020$). Reduced hormonal response to acute stress ($p = 0.001$) was found in metamorphs of the forested ponds compared to those of the other two groups (66% lower response). Herbicide exposure may have induced low level stimulation of growth (a positive impact on the exposed animals), as well as a possible elevation of baseline corticosterone, in the agricultural and treated metamorphs or there may have been a negative stressor present in the forested pond environment. It is not possible to identify the specific factors that were involved. It is unclear if 2,4-D affected the growth and development of wood frog tadpoles.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Doug Forsyth, for the opportunity to complete this project, which afforded me my Master of Science degree. I would also like to thank my committee members, Dr. Barry Blakley, Dr. Norm Rawlings and Dr. Mark Wickstrom as well as my external examiner (Dr. Tom Wolf) for their time and advice, above and beyond. Funding was received from Environment Canada (Pesticide Science Fund and Wildlife Toxicology & Disease Division).

I would like to acknowledge and sincerely thank Jon Bailey and Dr. Allan Cessna for the analytical work on the water residues, Gail Gray at National Hydrology Research Centre for water chemistry analyses as well as Susan Cook and Dr. Norm Rawlings for hormone analysis. My acknowledgements to the staff and students from CWS for their support, encouragement and guidance, especially Kevin Dufour and Shona Lawson. Egg collection sites and methods of blood collection were provided by Kerrie Serben and Lorne Doig; your prior experiences were invaluable to me. Assistance in the field was provided by Kaitlin Jaine-Waldram; thank you for the wild adventure. I would like to acknowledge the landowners who granted us permission to work in their wetlands and sample the tadpoles. I would also like to thank the faculty and graduate students in the Toxicology program who provided support and advice throughout my time in the program.

My sincerest thanks to Dr. Monique Dubé for providing me with the opportunity, support and encouragement to complete my thesis. Your guidance and concern were so greatly appreciated. Also, to the students of the Dubé lab for their advice and suggestions regarding statistics and all of your encouragement and support, thank you.

For their extra time, effort, support and encouragement, I would like to thank Sandra Kuchta and Allison Squires. Your involvement went above and beyond, and will be remembered always.

Finally, I would like to thank my family, especially my husband Bruce and children, Carter and Natalie. Your constant love and support throughout this process encouraged me immensely!

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATV	<i>Ambystoma tigrinum</i> virus
ChE	Cholinesterase
CORT	Corticosterone
CV	Coefficient of variation
DDT	Dichlor-diphenyl-trichlorethylene
DO	Dissolved oxygen
FV3	Frog virus - 3
GD	Gestation date
HD	Hodgkin's disease
HPI	Hypothalamic-pituitary-interrenal
K-W test	Kruskal-Wallis test
LC ₅₀	Median lethal concentration
LD ₅₀	Median lethal dose
LOD	Limit of detection
MC	Metamorphic climax
NHL	Non-Hodgkin's lymphoma
NHRC	National Hydrology Research Centre
NLET	National Laboratory for Environmental Testing
NSB	Non-specific binding
PCB	Polychlorinated biphenyls
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SD	Standard deviation
SE	Standard error
SGPT	Serum glutamic pyruvic transaminase
STS	Soft tissue sarcoma
SVL	Snout- to-vent length
T ₃	Triiodothyronine
T ₄	Thyroxine
TC	Total count
UV-B	Ultraviolet -B

Chapter 1

General Introduction

1.0 Introduction and Background

In 1990, nearly 10,000 tonnes of herbicide were applied to farms in Saskatchewan, which accounted for 46% of the total herbicide usage in the three prairie provinces (Waite *et al.* 2002). Of this total herbicide usage, 38% was 2,4-dichlorophenoxyacetic acid (2,4-D), a postemergent herbicide (Waite *et al.* 2002). In Saskatchewan, there is approximately 304,426 km² of land used for agricultural purposes, containing over a million small wetlands (Ducks Unlimited Canada 1993). Although not all of this land is directly sprayed with herbicides, the wetlands in these areas are at risk of chemical exposure from spray drift, runoff, atmospheric deposition and inadequate disposal. The rate of application in agricultural fields, forests and non-cropland ranges from 0.2 – 2.3 kg of active ingredient per hectare (Tomlin 2006). As a consequence of these management practices, 2,4-D can potentially contaminate wetlands directly adjacent to agriculture land as well as in areas distant from the original source.

1.1 2,4-Dichlorophenoxyacetic acid

2,4-dichlorophenoxyacetic acid (CAS Registry Number 94-75-7) is more commonly referred to as 2,4-D. Its physical state is a white, colorless powder and commercially it can be found in emulsion form, in aqueous solution or as a dry compound (Kamrin 1997). The synthesis of 2,4-D occurs from the condensation of 2,4-dichlorophenol with monochloroacetic acid in a strongly alkaline medium at moderate temperature (WHO 1984). Chemical and physical properties of 2,4-D are listed in Table 1.1.

1.1.1 Use of 2,4-D

2,4-D is registered for use as a pesticide in Canada. In the US, it is registered as a general use pesticide. The diethylamine salt has a toxicity class ranking between slightly toxic to highly toxic depending on exposures (WHO 1984). 2,4-D is used as a post-emergent herbicide on cereal crops and pastures in Saskatchewan (Saskatchewan Ministry of Agriculture 2009). It can also be used for lawn care, railways and control of aquatic plants (WHO 1984).

Table 1.1: The chemical and physical properties of 2,4-D.

Chemical Formula	$C_8H_6Cl_2O_3$ ^{1,2}
Molecular Weight	221.04 ¹
Melting Point (°C)	140.5 ¹
Vapour Pressure at 25°C (mPa)	0.0186 ²
Solubility in Water at pH 7 (mg/L)	23180 ²
Log Octanol/ Water Partition Coefficient	2.58 – 2.83 (pH 1) ² 0.04 – 0.33 (pH 5) ²

¹Kamrin 1997; ²Tomlin 2006

1.1.2 Mechanism of Action

2,4-D is a broadleaf herbicide that mimics the effects of indoleacetic acid, a plant growth hormone (auxin). Its mode of action is increased cellular division and activation of phosphate metabolism, which leads to increased ribonucleic acid (RNA) synthesis and protein synthesis. The result is prolonged abnormal elongation of the growing terminals without the changes needed for maturity and senescence. Distorted plant growth occurs and after 7-10 days, the plant withers, collapses and dies. The roots of plants readily absorb salt formulations, while the foliage absorbs esters of 2,4-D (Ware 2000).

1.1.3 Fate and Degradation

Drift and volatilization of 2,4-D can occur during and after application, depending on the formulation, particle size, spray technique, and climatic condition, which influence the movement of the chemical in the air that may be deposited in other areas, including wetlands. Movement of 2,4-D in soil depends on the concentration gradient of the pesticide, the soil characteristics, as well as temperature and pH. The three types of movement are diffusion, leaching (moving with percolating water) and surface movement (via wind or surface runoff). Pesticide availability is strongly influenced by adsorption and desorption in the soil. Increased adsorption of 2,4-D occurs in soil with increased organic matter. Increased adsorption will decrease movement and degradation of the chemical (WHO 1984). Studies have shown that 2,4-D does not persist or accumulate in the environment (Lavy *et al.* 1973; Birmingham and Colman 1985; Smith 1979). It is readily degraded by microbial action, hydrolysis, or photodecomposition (WHO 1984). The half-life of 2,4-D in soil is has been reported to be less than 7 days (Tomlin 2006).

1.1.4 2,4-D in the Environment

2,4-D is most commonly used as an herbicide in agricultural regions. Thus, there is the potential for 2,4-D to contaminate non-target wetlands and other water bodies via various pathways during or after application to cropland (Figure 1.1). 2,4-D has been detected throughout different media in the environment at varying frequencies. Due to its popular use in Saskatchewan, the frequency of detection of 2,4-D was relatively high, ranging from 10-100% of water samples examined (Waite *et al.* 1992; Grover *et al.* 1997). Donald *et al.* (2001) measured the frequency and concentrations of herbicides in wetlands both adjacent to and distant from areas where herbicide was applied as a land use practice. They found statistically significant similarities in frequency of detection of 2,4-D in wetland water collected from areas of all land use types. This suggested that 2,4-D was evenly distributed from the site of application to wetlands throughout the region (Donald *et al.* 2001).

Various processes may be at work to transport 2,4-D including atmospheric transport. 2,4-D has been detected in ambient air and bulk deposition samples even though the chemical was not applied in the immediate area that year (Waite *et al.* 2002). Detection of 2,4-D in air samples indicates that medium- to long-range transport of the chemical is occurring via atmospheric processes such as evapotransport or erosion (Waite *et al.* 2002). Waite *et al.* (2002) also reported that the maximum concentration of 2,4-D in ambient air was 3.90 ng/m³ with mean concentrations ranging between 0.49 ng/m³ and 0.77 ng/m³. It was also reported that the total bulk deposition of 2,4-D in the sampling area was 101 µg/m² in 1989 and 40 µg/m² in 1990 (Waite *et al.* 2002). Previously, Waite *et al.* (1995) measured the maximum weekly bulk deposition at 11.9-97.8 µg/m² over a 4-year period.

Maximum concentrations of 0.12-2.67 µg/L were reported in surface waters (ponds and dugouts) (Waite *et al.* 2002; Waite *et al.* 1992; Grover *et al.* 1997). The mean concentrations measured were 0.10 µg/L to 0.40 µg/L (Donald and Syrgiannis 1995; Donald *et al.* 2001). Precipitation can increase frequency of detection of 2,4-D in wetlands and may also lead to an increase in concentrations (Donald *et al.* 1999).

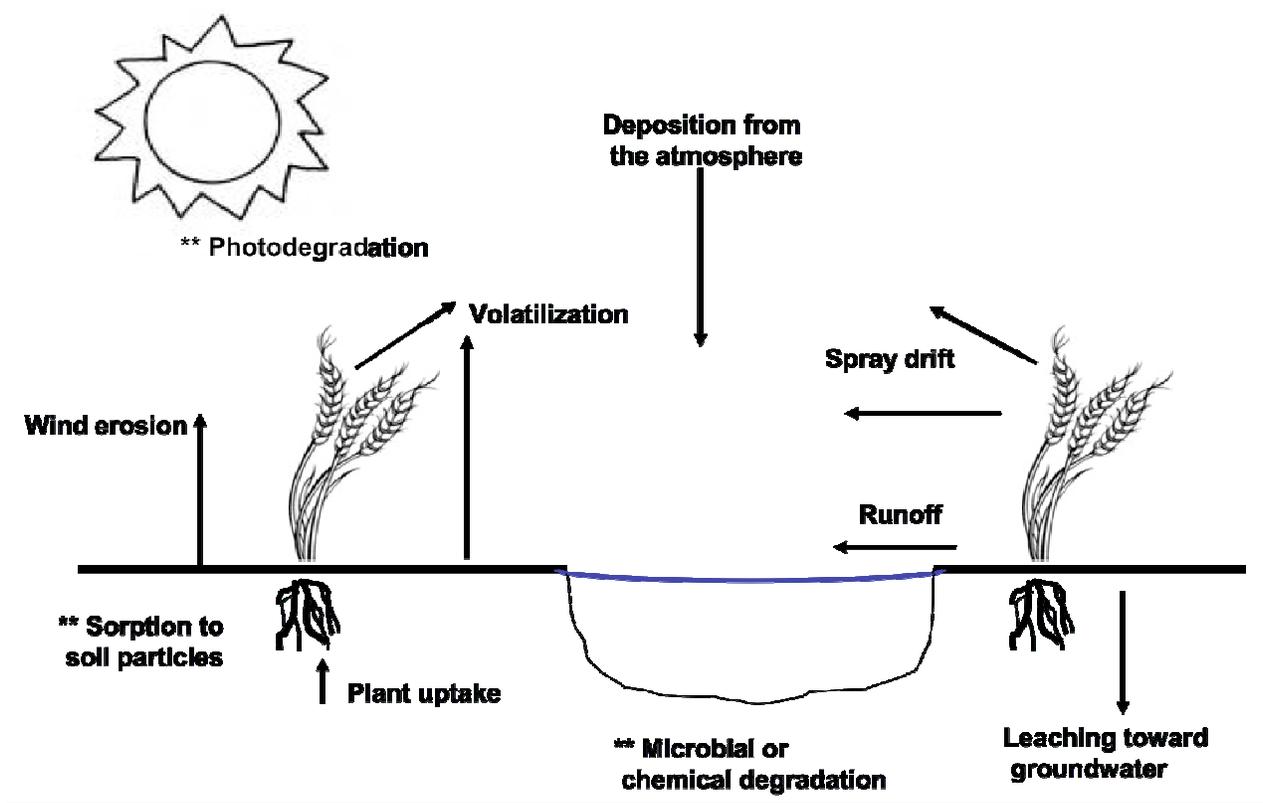


Figure 1.1: Fate of pesticides in the environment. As adapted from **Exttoxnet (2008)**.

** Represent methods of degradation or reduced bioavailability

2,4-D detected in runoff from treated cropland may also contribute to concentrations found in wetlands. White *et al.* (1976) determined that the highest concentrations of 2,4-D in surface runoff were present during the first runoff event after application. Waite *et al.* (1992) reported that the maximum spring runoff concentration of 2,4-D was 0.42 $\mu\text{g/L}$. Donald *et al.* (1999) found that the detection frequency of 2,4-D increased with increasing precipitation, suggesting the potential for chemical loss from the intended application site to nearby wetlands, if the chemical is applied before a significant rainfall event.

Surface film concentrations in wetlands are important to aquatic life, such as frogs, since they may live near the surface of the water. In surface film, maximum concentrations ranging between 308-714 ng/m^2 were reported by Waite *et al.* (2002). Additionally, Waite *et al.* (1992) reported the maximum concentration of 2,4-D detected in ground water to be 2.07 $\mu\text{g/L}$.

The water quality guideline for 2,4-D in Canada for protection of aquatic life in freshwater is 4 µg/L (CCME 2003). Though the concentrations in wetlands examined rarely exceed the guideline, consideration must be given to the potential chronic effects of mixed exposures as well as the possible synergistic effects with other contaminants found in the same wetlands (Donald *et al.* 2001; Forsyth *et al.* 1997).

1.1.5 Toxicity of 2,4-D

Toxicity of 2,4-D has been examined in various species but primarily in rats and mice (Blakley *et al.* 1998; Lee *et al.* 2001; Rawlings *et al.* 1998; Sarikaya and Yilmaz 2003). Toxicity is low to high in mammals, some birds, fish and reptile species, even taking into consideration species sensitivities (Tomlin 2006; Garabrant and Philbert 2002; Willemsen and Hailey 2001). Elimination from the body is rapid and the chemical remains mainly unchanged. Excretion, mostly through the urine, occurs within 24 – 48 hours (Tomlin 2006; WHO 1984). High acute doses may take longer, especially when the organic acid transport system is saturated, occurring at 50 mg/kg in rats (Gorzinski, *et al.* 1987).

Overt signs of toxicity in mammals and fishes include decreased locomotory activity with ataxia, central nervous system depression, muscle weakness and gasping for breath. The acute oral dose in rats was 600 mg/kg body weight, in which the overt signs were observed (Paulino *et al.* 1996). Behavioural changes began in the common carp (*Cyprinus carpio* L.) at 24 ppm and increased in severity with increased dosage in an acute toxicity test. Erratic swimming and colour changes were among the signs of toxicity (Sarikaya and Yilmaz 2003). Willemsen and Hailey (2001) assessed the effects of spraying 2,4-D and 2,4,5-T on olive groves and woody vegetation on tortoises (*Testudo hermanni*) in an area of Greece. They found a decrease in survival (average survival of 34%) in the area that was sprayed, which was 2-3 times higher than the natural mortality rate. Juveniles also had a higher mortality rate than the 10 cm tortoises. The tortoises also exhibited signs of poisoning such as swollen eyes, fluid discharge from the nose, and immobility. There was no effect on body mass. Other factors were consistent across all sites and therefore, ruled out. The physiological cause, though not known, points to acute rather than chronic processes. They concluded that increased mortality was not likely due to less ground cover as there was adequate cover and mortality was not increased when other herbicides were

applied. Tortoises were exposed to 2,4-D and 2,4,5-T via the diet. It was possible that the herbicides increased the nitrate accumulation in the vegetation and the tortoises converted it to nitrite, which caused tissue anoxia (Willemsen and Hailey 2001).

Lethal doses and concentrations of 2,4-D varies with species. The single-dose dermal LD₅₀ exceeded 2000 mg/kg in rabbits (Gorzinski *et al.* 1987). The LD₅₀ of a single oral dose in rats ranged from 553-1090 mg/kg (Gorzinski *et al.* 1987). Mice, pigs, sheep and monkeys have a similar range (Bovey and Young 1980). Dogs are more sensitive with an oral LD₅₀ of 100 mg/kg (Garabrant and Philbert 2002; Bovey and Young 1980). *Daphnia* (*Daphnia magna*) have an LC₅₀ of greater than 100 mg/L. The LC₅₀ ranged from 250 to greater than 600 mg/L for fathead minnows (*Pimephales promelas*), bluegills (*Lepomis macrochirus*) and rainbow trout (*Salmo gairdneri*). Bluegill fish appear to be the least sensitive (Alexander *et al.* 1985).

The target organs appear to be the kidneys and liver (Gorzinski *et al.* 1987; Paulino *et al.* 1996; Lee *et al.* 2001). Paulino *et al.* (1996) observed toxicity in rats exposed to 2,4-D through the drinking water acutely (600 mg/kg once and observed 24 hours), subchronically (200 mg/kg daily for 30 days) and chronically (200 mg/kg for 180 days). They found no macroscopic or histopathological lesions in any of the groups. Biochemical and haematological data indicate there was an increase in specific enzymes, suggesting hepatic and muscular lesions were present but were not detectable with the methods used in this experiment (Paulino *et al.* 1996). Gorzinski *et al.* (1987) observed extensive changes in the kidneys especially after the organic acid transport system was saturated. The liver was also affected but the changes were minor and nonspecific in a subchronic rat study. The study used 0, 15, 60, 100 and 150 mg/kg of chemical administered daily in the diet for 13 weeks. This study also found changes in the thyroxine (T₄) concentrations, but there were no histological changes in the thyroid gland. Rawlings *et al.* (1998) also found a decrease in T₄ concentrations in sheep (*Ovis aries*) administered 10 mg/kg 2,4-D via gelatine capsule into the rumen three times a week for 43 days. But again, there were no histopathological changes associated with the endocrine changes. Some studies have also found haemorrhage of the digestive and excretory systems in fish (Sarıkaya and Yılmaz 2003); and decreased testicular size, as well as testicular damage (shrunken seminiferous tubules and depletion of germ cells), in rats exposed to 150 mg/kg for 5 days/ week for 9 weeks (Oakes *et al.*

2002). It is also worth noting, that the rats with the decreased testicular size had normal testosterone concentrations (Oakes *et al.* 2002).

In a review of 2,4-D toxicity conducted by Garabrant and Philbert (2002), it was determined that there was no teratogenicity unless clearance was inhibited or exceeded. Immune system activation or immunotoxicity was not evident. There was also no genotoxic/ mutagenic potential; carcinogenicity was unlikely; and neurotoxicity did not occur at concentrations below those required to induce significant systemic toxicity. In cohort studies, they concluded that there was no association between 2,4-D and NHL (non-Hodgkin's lymphoma), STS (soft tissue sarcoma) or HD (Hodgkin's disease). In the case controlled studies, the conclusions were mixed. There was a weak association in the occurrence of STS but exposure to other chemicals could not be ruled out. There was no association with the occurrence of HD. Their results were inconclusive with regards to NHL (Garabrant and Philbert 2002).

Pesticides are rarely found individually in the environment, and therefore, there is a potential for chemical interaction and enhanced toxicity. Studies using 2,4-D in a multichemical exposure have the potential to alter toxicity as compared to the toxicity of the chemical used singly (Kuntz *et al.* 1990; Chaturvedi *et al.* 1991). Kuntz *et al.* (1990) dosed mice via oral intubation to individual chemicals as well as various chemical mixtures for 14 days. They used parathion (1, 2.5, 5 or 10 mg/kg), toxaphene (50, 100, or 200 mg/kg) and 2,4-D (50, 100, or 200 mg/kg). For the chemical mixtures, they used parathion at 5 mg/kg, toxaphene at 50 mg/kg and 2,4-D at 50 mg/kg. The mixtures consisted of the chemicals paired with each other as well as all three at the stated concentrations. They found that weight gain was decreased in a dose-dependent manner. There was a recovery after the exposure had ended. They found changes to liver weight (decreased) and SGPT (serum glutamic pyruvic transaminase) activity (increased), which implies mild liver damage was present. The changes were reversible after discontinuation of exposure to individual chemicals, whereas recovery in the mixture groups was only partial. With regard to cholinesterase (ChE) activity, there was reduced activity observed in both the serum and brain ChE. Importantly, the effect appeared to be additive in the mixtures (Kuntz *et al.* 1990). Chaturvedi *et al.* (1991) exposed mice to mixtures of chemicals via drinking water for 90 days and by oral intubation for 14 days. The pesticides used were alachlor, aldrin, atrazine, 2,4-D, DDT, dieldrin, endosulfan, lindane, parathion and toxaphene. The concentrations of the

combined pesticides were 0.1, 1.0, 10 and 100 ppm in the drinking water and 25 and 50 mg/kg in the oral doses. They found a dose-dependent increased in liver: body weight ratio, as well as increased liver enzyme activity. Effects observed were additive in all cases; there were no interactions (Chaturvedi *et al.* 1991).

1.2 Wood Frogs

Seven species of amphibian are found in various locations throughout Saskatchewan. *Rana sylvatica* (wood frog) are abundant and widespread in Saskatchewan (Seburn 1992). They are diurnal, largely terrestrial and inhabit the boreal forest and open grassy areas with bordering thickets of willows and aspen. In northern Saskatchewan, spruce and other forest trees may be present (Stebbins 1996). Wood frogs are small to medium in size (approximately 30-60 mm) with females, on average, being larger than males (Berven 1981). They are brown, gray or green with a dark eye mask. Their coloration blends into the grasses and forest floors of their environment (Stebbins 1996). Breeding season lasts for 1-2 weeks and occurs from mid-April to late May, as soon as the ice melts and the temperature begins to increase (Russell and Bauer 1993). Adult males and females arrive together to breed in shallow, clear ponds (Russell and Bauer 1993; Berven 1981). Males begin calling and actively searching for females, then engage in amplexus. Eggs (1.6mm in diameter) are laid underwater, and are often attached to plants or debris in large globular masses of 2000-3000 eggs per a clutch (Russell and Bauer 1993). Females in the same pond tend to lay their eggs near the site of other clutches (Howard 1980). After fertilization, the adults leave the pond.

Tadpoles hatch after 3 weeks at about 7-10 mm in length (Russell and Bauer 1993). Hatchlings remain near and feed off the jelly egg mass. After a few days, the tadpoles disperse and move towards deeper water (Thurow 1997). In the species *R. sylvatica*, as the tadpole undergoes metamorphosis, tail resorption occurs. The process of development from fertilization through resorption of the larval tail was described by Gosner (1960). In wood frogs, this process requires 6-12 weeks (Russell and Bauer 1993). The new metamorphs may remain near the pond or disperse before winter. They will overwinter and return to the pond to breed the following year for males or in two years for females (Russell and Bauer 1993).

1.3 Amphibian Declines and Malformations

Amphibian populations have reportedly been declining over the last several decades. The decline has been observed globally, alarming both scientists and the general public (Houlahan *et al.* 2000; Collins and Storer 2003). Mass mortality of amphibians is a reflection of the poor state of the global ecosystem, which can negatively impact on all other organisms (Carey 2000). The causes for the decline are difficult to assess, as it appears to be site specific and not one global source. Many believe that ultimately human activity, in some form, is the primary cause (Carey 2000; Green 1997; Blaustein and Kiesecker 2002). Among the activities of interest are habitat destruction and fragmentation (Lehtinen *et al.* 1999); application of xenobiotics, such as agricultural fertilizers and pesticides (Berger 1989; Hayes *et al.* 2003); and introduction of predators (Collins and Storer 2003), infection and disease (Crawshaw 2000).

In addition to declining populations there has also been an increase in the occurrence of deformities in amphibian populations. Although determining a cause for this increase in deformities has also proven difficult (Kavlock 1998). Possible causes for deformities, including supernumerary limbs, ectrodactyly and ectromelia (absence of all or part of a digit or limb, respectively) being investigated are infestation of metacercariae (the encysted stage of trematode worms) (Sessions and Ruth 1990; Johnson and Sutherland 2003); exposure to pollutants, such as mill effluent and pesticides, combined with environmental factors (Harris *et al.* 2001; Burkhart *et al.* 1998); UV-B radiation (Schmidt 1997); or some combination of the above. One mechanism hypothesized was any or all of the above causes interfere with the retinoid-sensitive signaling pathway, which is responsible for limb growth (Johnson and Sutherland 2003; Schmidt 1997). The retinoid-sensitive signaling pathway is closely related to the thyroid system, which along with other hormones, play an important role in metamorphosis (Ouellet 2000). Ouellet *et al.* (1997) reported that the frequencies of developmental abnormalities in four species of frogs (*Rana clamitans*, *R. pipiens*, *R. catesbeiana*, and *Bufo americanus*) were 12% in exposed ponds and 0.7% in unexposed ponds. The exposed ponds were deemed those in agricultural areas, which were treated with herbicides, insecticides and/or fungicides (including but not limited to atrazine, bromoxynil, butylate, dicamba, azinophos-methyl, carbofluran and chlorothalonil). The unexposed ponds were in areas, such as pastures or old fields, without application or pollution in the previous years. Deformities are not only a concern because of the survival risk to

amphibians but because they may be indicative of a problem relevant to human health and the overall degradation of the ecosystem (Ouellet 2000).

1.4 Metamorphosis and Hormones

The hypothalamus and the pituitary gland control the metamorphosis from tadpole to frog by regulating the activity of the thyroid and interrenal glands (Beachy 2001). During metamorphosis, regulation of several cellular processes like apoptosis, proliferation and differentiation occurs. First, cell autonomous events (intracellular) are involved with upregulation of transcription factors and enzymes to maintain or alter the cells. Second, extracellular events occur, influencing the local cellular environment and the 3-D structure of tissues required to maintain the status and function of the cell. The extracellular matrix promotes transfer of information between cells. Gene regulation during metamorphosis is initiated by thyroid hormones. Proteins and the extracellular matrix are essential for tissue remodeling at this point. Specific roles depend on the tissue and the timing of expression (Stolow *et al.* 1997).

Several hormones that interact during this process are the thyroid hormones and corticosterone. Thyroid hormones are important in the development and metamorphosis of amphibians. Corticosterone, a corticosteroid, is important in the stress response as well as in growth and metamorphosis. Corticosterone can accelerate spontaneous or thyroid hormone-induced resorption of tadpole tails by increasing the conversion of thyroxine (T_4) to triiodothyronine (T_3) when T_4 is available, with T_3 being the physiologically important hormone (Galton 1990). If corticosterone is present prematurely or during cold temperatures, it prevents development of the thyroid axis thus inhibiting metamorphosis. In cold temperatures, T_4 does not reach levels necessary for metamorphosis to reach completion (Suzuki 1985; Hayes and Licht 1993). Corticosterone can increase due to stress. Increased corticosterone in the hypothalamus decreases plasma corticosterone concentrations affecting metamorphosis and development of amphibians (Laub *et al.* 1975; Hayes and Wu 1995).

1.5 Chemicals and Amphibians

Many amphibian species will occupy both the aquatic and terrestrial ecosystems at some point during their lifespan. This biphasic amphibian lifestyle is an important factor to consider during

studies evaluating the impact of chemicals within an ecosystem. There is the potential for amphibians to be exposed to chemical contaminants throughout development in both habitats. Amphibians also have highly permeable skin, which allows rapid transport of contaminants across membranes to the sensitive internal organs (Cowman and Mazanti 2000). For this reason, plus the increased occurrence of deformities and declining populations, concern has been growing over the use of pesticides and their effects on amphibian populations (Blaustein and Johnson 2003; Ouellet *et al.* 1997; Carey and Bryant 1995). A link between agricultural runoff and hindlimb deformities in amphibians has been reported, leading to fitness and survival issues in amphibians (Kiesecker 2002).

Few studies have been conducted to examine the effects of 2,4-D on amphibians. Many of these studies were conducted in laboratories with concentrations that exceed environmentally realistic values. Johnson (1976) reported that 2,4-D amine was not acutely toxic in *Limnodynastes peroni* or *Adelotus brevis* at 230 –340 ppm, but it did result in a reduction in temperature tolerance. It was also reported that sensitivity was species-dependent as well as age-dependent. Adults were more resistant to effects caused by 2,4-D, likely due to differences in ion permeability of the skin. Zaffaroni *et al.* (1986) studied a commercial formulation of 2,4-D and its effect on adult crested newts (*Triturus cristatus carnifex*). The highest concentrations (75, 100, 125, and 150 ppm) resulted in newts that displayed motionless periods, sinking to the bottom, decreased responsiveness and flaccid paralysis. There were no gross morphological abnormalities reported, but there were histological changes related to liver degeneration. Morgan *et al.* (1996) used FETAX (frog embryo teratogenic assay – *Xenopus*, an assay which uses the *Xenopus laevis* embryo) to determine the effects of 2,4-D and atrazine on development in buffered and natural water. They observed mild or severe (two or more abnormalities within the same embryo) abnormalities, which occurred in the gut (edema) and/ or tail. Tail abnormalities were found in the buffered water at low percentages, below approximately 250 mg/L. The occurrence of tail abnormalities and severe abnormalities increased at higher concentrations. Importantly, tail abnormalities, as well as some severe and gut abnormalities, occurred in the natural water at concentrations ranging from 180 mg/L to 270 mg/L.

Natural water plays an important role in the toxicity of herbicides to amphibians (Morgan *et al.* 1996). 2,4-D can also play a role in the response to stimulation at the membrane level in *Caudiverbera caudiverbera* (helmeted water toad) (Suwalsky *et al.* 1999). Although concentrations exceeding those expected in the environment were used in these studies, they demonstrated the potential toxicity of the chemicals on amphibians and the need for future research into the effects of 2,4-D at environmentally realistic concentrations.

More recently, there has been an interest to study the impact of chemicals on amphibians at environmentally realistic concentrations both in the laboratory and the field. Sower *et al.* (2000) proposed that endocrine disrupting chemicals were playing a role in malformed frogs of the species *Rana catesbeiana* (North American bullfrog) and *Rana clamitans* (green frog). Mackenzie *et al.* (2003) observed a female-biased shift and intersex animals in *Rana pipiens* (leopard frog) and *Rana sylvatica* (wood frog) frogs exposed to estrogenic and antiestrogenic compounds. Leopard frogs were more sensitive but both species were affected (Mackenzie *et al.* 2003). Hayes *et al.* (2003) examined the effects of atrazine (a frequently used herbicide in the USA) in the laboratory and compared the findings to field results. The authors reported that exposed males had underdeveloped testes and varying degrees of sex reversal (with a more pronounced effect at the lower dose [0.1 ppb]). They reported similar results from field studies. These findings were not restricted to a single species (*Xenopus laevis* and *Rana pipiens*).

Chemicals and other anthropogenic activities can also impact and alter the natural habitat that amphibians encounter. Chemicals, especially herbicides, can affect the nutrient levels, pH, oxygen levels and temperature both directly and indirectly (Rowe *et al.* 2003). Changes to the delicate balance of nutrients in the environment can adversely affect the populations of other species (primarily the flora) in the environment, indirectly negatively impacting on amphibians (Evans *et al.* 1995). Chemicals causing acidification of water can adversely affect the development of embryos. These changes created a vitelline membrane that did not allow for expansion resulting in embryo mortality or spinal defects (Dunson and Connell 1982). Hypoxia reduced the days to hatching in both *Rana* species and delayed hatching in *Ambystoma* species. *Ambystoma* species in hypoxic environments experienced delayed embryo development and poor post hatch survival (Mills and Barnhart 1999). Suzuki (1985) discusses the effects of low and

high temperatures on thyroid hormones with reference to metamorphosis. He found that low temperatures affected the uptake of thyroid hormones, but both low and high temperatures affects thyroid hormone synthesis. During metamorphosis, amphibians exposed to high temperatures had low T₄ concentrations (Suzuki 1985). As mentioned previously, this translates into potential delays in metamorphosis. All of these factors may work in conjunction together in nature. Therefore, it is important to consider both the chemicals and environmental factors when interpreting the observed toxic effects (Sparling *et al.* 2003).

1.6 Research Objectives

The objective of my research project was to assess the sublethal effects of 2,4-D (commonly found in wetlands) on the health of amphibians in wetlands located in prairie agricultural regions by determining whether wood frog (*Rana sylvatica*) tadpoles were adversely affected by 2,4-D at environmentally realistic concentrations. This was done with two experiments: a microcosm study on the University of Saskatchewan campus in 2004 and a field study in natural ponds in 2005. The objective of the microcosm study was to assess the effects of three concentrations of 2,4-D on survival, growth (size at metamorphosis), development (deformities) and plasma corticosterone concentrations of metamorphs in response to acute stress by raising recently hatched tadpoles through metamorphosis (Chapter 2). The objective of the field study was to assess the effects of a single relatively low (worst-case scenario) concentration of 2,4-D and other influences of agricultural activity on the health of tadpoles during metamorphosis in natural ponds by determining size at metamorphosis and plasma corticosterone concentrations in response to acute stress (Chapter 3). H_0 : There will be no direct effect of technical or formulated 2,4-D amine at environmentally realistic concentrations on the growth or development of wood frog larvae, in microcosms or in natural ponds. The predication if H_0 is false is that there will be negative effects of technical and formulated 2,4-D amine at environmentally realistic concentrations on the growth and development of wood frog larvae in microcosms and in natural ponds.

Chapter 2

The sublethal effects of 2,4-dichlorophenoxyacetic acid on tadpole survival, growth and development in outdoor microcosms

2.0 Introduction

Declining amphibian populations have been reported for decades on a global scale (Houlahan *et al.* 2000; Collins & Storfer 2003). An increased occurrence of deformities in amphibian populations (Ouellet *et al.* 1997; Kavlock 1998), have many within and outside the scientific community concerned (Houlahan *et al.* 2000; Collins & Storfer 2003). The causes of both phenomena are still not clear and are difficult to assess, but it is believed to be anthropogenic (Carey 2000; Green 1997; Blaustein and Kiesecker 2002). Agricultural pesticides have been identified as a cause for some population declines and deformities (Berger 1989; Hayes *et al.* 2003; Ouellet *et al.* 1997).

2,4-dichlorophenoxyacetic acid (2,4-D) is a post emergent herbicide commonly used in Saskatchewan (Donald *et al.* 1999; Waite *et al.* 2002). It is often used on cereal crops and pastures in Saskatchewan, but is also used for lawn care, railways and control of aquatic plants (WHO 1984). Movement of 2,4-D throughout the environment is often dependant on the application method, characteristics of the formulation and climatic conditions (WHO 1984). Although 2,4-D does not persist or accumulate in the environment (WHO 1984), low concentrations have been measured in wetlands throughout Saskatchewan (Waite *et al.* 1992; Grover *et al.* 1997; Donald *et al.* 2001). The concentrations rarely exceeded the water quality guideline for 2,4-D of 4 µg/L (CCME 2003), but chronic effects as well as possible synergistic effects with other contaminants should be considered (Donald *et al.* 2001; Forsyth *et al.* 1997).

2,4-D is relatively low in toxicity (Tomlin 2006). Studies with non-amphibians exposed to high concentrations of 2,4-D (beyond environmentally relevant levels) have resulted in behavioral changes (Sarıkaya & Yılmaz 2003), renal and hepatic lesions (Gorzinski *et al.* 1987; Paulino *et al.* 1996) as well as changes to endocrine function (reduced in T₄ concentrations) (Rawlings *et al.*

al. 1998). Toxic effects were seen in amphibians after exposure to 2,4-D but generally at concentrations in excess of 100 mg/L (Johnson 1976; Zaffaroni *et al.* 1986; Morgan *et al.* 1996).

Indirect exposure to low levels of 2,4-D may create a chronic stress environment. Stress affects the hypothalamic-pituitary-interrenal (HPI) axis by regulating corticosterone (CORT) release. Corticosterone is important in the stress response of amphibians influencing growth, development and metamorphosis (Galton 1990). Changes altering the release of CORT or the functioning of the interrenal (adrenal) gland can impact development and metamorphosis (Laub *et al.* 1975; Hayes & Wu 1995). For example, increased CORT early in development or during cold temperatures prevents development of the thyroid axis, thus inhibiting metamorphosis (Suzuki 1985; Hayes & Licht 1993).

Wood frogs (*Rana sylvatica*) are abundant and widespread in Saskatchewan (Seburn 1992). Tadpoles hatch three weeks after egg laying, remaining close to the jelly mass for 5 - 7 days, after which time they disperse into deeper water (Thurow 1997). They remain in the pond until completion of metamorphosis in 6-12 weeks (Russell & Bauer 1993). An abundant native species, with a short developmental period, the wood frog is an ideal species to study the toxic effects of agricultural chemicals in Saskatchewan.

The present study was conducted in outdoor microcosms, which allowed for direct observations of the causal nature of the effects observed while still adding an element of field realism (Rowe & Dunson 1994). Although it is difficult to replicate the conditions of the field in a laboratory experiment, microcosms allow natural temperatures and sunlight to be included in the study (Rowe & Dunson 1994). Predation and limited food resources, which may confound experimental results, are eliminated or reduced (Rowe & Dunson 1994).

The objective of this study was to assess the effects of environmentally realistic concentrations of 2,4-D on growth, development and survival of wood frog tadpoles from hatching to metamorphosis.

2.1 Materials and Methods

2.1.1 Microcosms

Twenty outdoor microcosms were set up at the University of Saskatchewan campus on a site that was isolated from normal pedestrian traffic, about 15 m south of a row of trees. The microcosms were enclosed by a secure 1.8-m high chain link fence (9 m x 9 m) with a roof of plastic bird netting to exclude predatory birds.

Microcosms were constructed with panels of oriented strand board, 1 cm thick, screwed to 2" x 2" spruce on the vertical and bottom edges. A 1.0 m x 1.0 m x 0.7 m (L x W x H) bottomless box was formed by screwing four panels together. The microcosms were placed in the ground at a depth of 0.5 m (to reduce temperature fluctuations) in a 4 x 5 grid pattern. Each microcosm was double lined with sheets of clear polyethylene plastic (3 m x 3 m; 3.5 oz). The plastic was soaked in municipal tap water for at least one week to remove leachable plasticizers (Carmignani and Bennett 1976). The plastic liners were stapled flush to the box, above the water line. Strips of plastic (about 15 cm wide) were hung over the inside edges and stapled to the top of each microcosm. These strips provided a non-shading lip to prevent the escape of metamorphs. The microcosms were filled to a depth of 0.5 m with municipal tap water (final volume of 500L). The water was treated with 25 mL of "Laguna" water treatment to eliminate chlorine and chloramines and neutralize harmful metals (Rolf C. Hagen Inc, Montreal, QC, Canada). Water depth was measured and water added periodically to maintain a level of 50 cm during the months of June and early July. Depth was not monitored regularly thereafter, but it had not fallen below 45 cm at the end of the experiment on August 18, 2004.

Microorganisms native to the original pond where the tadpoles were collected (Little Bear Lake, SK, Canada) were introduced by the addition of 130 mL of pond water to each microcosm. Leaf litter collected from the original pond was dried to prevent the introduction of tadpole predators. Leaf litter was added (40 g dry weight) to each microcosm to provide spatial complexity. Aquatic macrophytes, water milfoil (*Myriophyllum proserpinacoides*) and hornwort (*Ceratophyllum demersum*), were purchased from Moore Water Gardens (Port Stanley, ON, Canada). Water milfoil was individually (one stem) potted in ethanol-washed clay pots. Gravel, thoroughly rinsed in municipal tap water, was added in a layer on the bottom of the pot. The

roots of the plant were placed in dried garden soil in the pot, and a top layer of gravel was added to keep the soil out of direct contact with the overlying water. The hornwort was added to the water as an individual small floating bunch. The plants were added (one of each species per microcosm) to remove nitrogenous waste, and provide shade, hiding places and decomposing matter for tadpoles to eat.

2.1.2 Collection and Manipulation of Tadpoles

Recently hatched wood frog tadpoles were collected from a pond near Little Bear Lake, Saskatchewan on June 8, 2004. The closest cropland was at least 80 km south of the pond, so it was assumed to be uncontaminated by pesticides, as it may be beyond most atmospheric deposition (Hebert 2009). No 2,4-D (detection limit 50 ng/L) was detected in a sample of water from the pond. The tadpoles were transported to Saskatoon in a pail of pond water kept cool with ice. They were kept in clean plastic tubs containing the original pond water, and given gentle aeration with small airstones. Rinsed latex gloves were used when handling the tadpoles and the water in the tadpole tubs. The room was located in laboratory space at Canadian Wildlife Service in Saskatoon, SK. The room temperature was $16^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a seasonal natural light cycle. Water changes to dechlorinated tap water were made gradually before the tadpoles were moved to the microcosms. The tadpoles were fed mashed kale and powdered tropical fish flakes containing the alga, *Arthrospira platensis*, known as “Spirulina” (Northern Aquaselect Food & Supply, Lucan, ON, Canada). Examination of a sample of 20 tadpoles showed that the stages (Gosner 1960) ranged between 21 and 26, with the majority present at stages 24 and 25. Forty tadpoles were added to each microcosm on June 17, 2004; 2,4-D was added on June 22 at 3:00 pm, when the animals were approximately 15-16 days old. Crushed Spirulina flakes were added to the microcosms every 2-3 days. The Animal Care Committee of the University of Saskatchewan approved the collection and handling of the eggs, tadpoles and metamorphs (UCACS Protocol No. 20040037).

2.1.3 2,4-D Application

Technical grade 2,4-D (dimethylamine salt, 99% purity; Chem Service Inc., West Chester, PA, USA) was used to prepare a stock solution of 500 mg/L. Serial dilutions from this solution were added to the microcosms to obtain the desired concentrations. Mixing into the water column was

accomplished with the use of an individually marked 1-L pail for each microcosm. The treatment groups consisted of one negative control (water only) and three 2,4-D concentrations: 0.1 µg/L, 1.0 µg/L, and 100 µg/L. Maximum concentrations of 2,4-D in Saskatchewan ponds reported by Donald and Syrgiannis (1995) and Waite *et al.* (1992) were 0.43 and 0.51 µg/L. The range of the two lowest concentrations included these values. The concentration of 100 µg/L is a representative value of potential concentrations present in small, shallow ponds affected by runoff from a heavy rainfall event (Appendix A). There were five replicates of each treatment arranged in a randomized block design. There were five blocks (rows), running parallel to the trees; east to west, containing all four treatments in a row. Microcosms were numbered 1 to 20 from left to right in each row, beginning with number 1 in the northeast corner.

2.1.4 2,4-D Residues in Water

Water samples were collected from each microcosm for 2,4-D analysis at 0.75 days, 7 days, 17 days and 56 days post-treatment (the end of the experiment). Water was stirred gently before sampling. The pre-cleaned glass sample bottle (1-L or 100 mL, depending upon concentration) was uncapped and held 15 cm beneath the surface of the water during sampling. It was assumed that the 18-hour control sample would reflect any prior contamination. Samples were stored at 4°C in darkness until analyzed at the National Hydrology Research Center (NHRC) in Saskatoon. Analyses of water samples from the microcosm and pond studies were performed using a Waters 2695 Alliance high performance liquid chromatography (HPLC) system liquid chromatograph (Waters, Milliford, MA, USA) coupled with a Micromass Quattro Ultima mass spectrometer (Waters, Milliford, MA, USA) equipped with an electrospray interface. The HPLC separations were achieved using a Waters Xterra C18 chromatography column (2.1 mm x 100 mm, 3.5 µ particle size, Waters, Milliford, MA, USA). Injection volumes were 50 µl for 0.1 µg/L and 1.0 µg/L samples and 5 µl for 100 µl/L samples. The mobile phase was a 1:1 water/ acetonitrile mixture containing 0.1% ammonium hydroxide and the flow rate was 200 ml/min at 30°C. The acetonitrile was HPLC grade and the deionized water (18 MΩ) was obtained using a Millipore Milli-Q Gradient A10 (with total organic carbon detector) water purification system (Millipore, Billerica, MA). For mass spectrometry (MS) detection, the instrument was operated in the negative ion mode and the precursor and product ion mass monitored for 2,4-D was m/z 218.7>160.8 (Majzik *et al.* 2006). Concentrations in the samples were determined by comparing

the number of ions generated by the sample to the number of ions generated by the known calibration standards: 0.1 µg/L, 0.2 µg/L, 1.0 µg/L and 2.0 µg/L for low and mid concentrations and 50 µg/L, 100 µg/L, 250 µg/L, and 500 µg/L for high concentrations. Instrument variability was determined from the results of eight replicate injections of a 100 mg/L standard solution. The average was 99.57 mg/L with a standard deviation of 3.72 mg/L (Bailey 2004). The instrument limit of quantification was 100 ng/L and the instrument limit of detection was 50 ng/L. Analysis was performed in the laboratory of Dr. A.J. Cessna at the NHRC.

2.1.5 Water Quality Analysis

Water samples were collected the day before treatment from five randomly chosen microcosms. Twelve microcosms (three per treatment) were randomly sampled at 4 and 8 weeks post-treatment and analyzed for ammonia and nitrate/nitrite ($\text{NO}_3 + \text{NO}_2$) concentrations; hardness and alkalinity were also measured at 8 weeks. The ammonia and nitrate/nitrite analyses were performed at NHRC. Hardness and alkalinity were analyzed using a Hach Digital Titrator Model 16900 (Hach Company, Loveland, CO, USA). Dissolved oxygen concentrations and mid and bottom temperatures were measured two or three times a week (YSI Model 55 Dissolved Oxygen meter from Yellow Springs Inc. (YSI), Yellow Springs, OH, USA). Conductivity and pH were also measured two or three times a week (QuiKcheK Model 106 pH and automatic temperature compensation (ATC) meter from Orion Research, Beverly, MA, USA and Hanna Model HI 991301 Portable pH/electrical conductivity (EC)/ total dissolved solids (TDS)/Temperature Meter from Gasonic Instruments, Calgary, AB, Canada).

2.1.6 Survival, Growth and Development

The first developmental stage of MC (metamorphic climax: stages 42-46) is forelimb emergence (Gosner 42). The target stage for collection was Gosner 43, when corticosterone concentrations in metamorphs are maximal (Jaffe 1981; Krug *et al.* 1983). However, it was not possible to sample all the metamorphs at this stage because they developed through these stages within 3-5 days, and were able to hide in the vegetation and leaf litter. Therefore, metamorphs were sampled between Gosner 42 and 46 (complete tail resorption). Sample collection took place between 09:00 and 14:00. Time to metamorphosis in the microcosms was defined as the period between hatching to metamorph sampling (approximately stage 42). Metamorphic climax did

not occur at the same time for all tadpoles; thus, the first metamorphs were collected July 22 and the last on August 16, 2004. None of the tadpoles failed to metamorphose.

Metamorphs were captured using dip nets and were subjected to a standardized acute stress protocol (shaking) to measure the responsiveness of the hypothalamic-pituitary-interrenal (HPI) axis (Gendron *et al.* 1997; Brodeur *et al.* 1997). The standardized acute stress protocol with the metamorphs of the microcosms was similar to the methods described by Glennemeier & Denver (2002). The metamorphs from the microcosms were placed in a plastic container with 1-2 cm of water and vibrated on a shaded shaker table for five minutes. They were only shaken for five minutes based on the observation of extreme stress of the animals by the author during the protocol. This potentially high stress environment may lead to high mortality. A drop of Orajel (<1ml) (20% benzocaine) (Del Pharmaceuticals Inc.) was applied to the back of the head to anesthetize the specimen, causing it to become incapacitated (unable to right itself) and easy to manipulate within 1-2 minutes. Total length and wet weight were recorded. The presence of deformities was determined by a simple external examination. Blood was collected by cutting the midline abdominal vein near the posterior end of the abdomen with fine scissors. Blood was drawn into heparinized microhematocrit capillary tubes (Chase Scientific Glass Inc., Rockwood, TN, USA) and transferred into 1.5-ml polyethylene micro tubes (Sarstedt Inc., Montreal, PQ, Canada). Samples, which varied from a few μL to 75-100 μL per specimen, were kept on ice and centrifuged for five minutes at maximum speed (International Clinical Centrifuge, Model 98412H-3, International Equipment Co., Boston, MA, USA). The plasma was removed using insulin syringes and frozen at minus 21°C until hormone analysis. Euthanasia was completed with an overdose of Orajel (several drops to the inside of the mouth), although most metamorphs died following exsanguination. The specimens were preserved in 10% neutral buffered formalin, measured for snout-vent length (SVL) and dissected to determine the sex.

Plasma corticosterone (CORT) concentrations were analyzed using radioimmunoassay (RIA) by Prairie Diagnostic Services, Saskatoon. Standards were diluted 1:200. Due to their small size, plasma samples were diluted 1:20 (15 μl to 300 μl) and in some instances 1:30 (10 μl), 1:40 (7.5 μl), and 1:60 (5 μl). Glass assay tubes were numbered and 100- μl aliquots of samples and standards were added in duplicate. All tubes received 0.2 ml of ^{125}I corticosterone reagent (a

radioactive tracer) (MP Biomedicals, LLC; Orangeburg, NY). All tubes except the total count (TC) and the non-specific binding (NSB) tubes received 0.2 ml of anti-corticosterone (MP Biomedicals, LLC; Orangeburg, NY). All the assay tubes were vortically-mixed and incubated at room temperature (22°-25°C) overnight. Following incubation, 0.5 ml of precipitant solution (a mixture of polyethylene glycol [PEG] and goat anti-rabbit gamma globulins contained in trisamine [TRIS] buffer to precipitate all the antibody bound antigen) (MP Biomedicals, LLC; Orangeburg, NY) was added to all the tubes, which were vortically-mixed thoroughly and centrifuged for 20 minutes at 3000 rpm. The supernatant was decanted completely and the precipitate was analyzed in the Titertek plus series, model 28023 gamma counter (Titertek Instruments, Huntsville, AL, USA).

2.1.7 Statistical Analysis

Normality and homogeneity of variance were analyzed for all variables using the Shapiro-Wilk test and Levene test (Zar 1998), respectively. When the assumptions of normality and homogeneity were not met, the data were log transformed and retested. If transformation did not correct for non-normality and heterogeneity, nonparametric tests were used. Water residues were analyzed with a simple linear regression (Zar 1998) for each treatment to determine if 2,4-D degradation had occurred. Water quality variables were analyzed for significant treatment effects using one-way analysis of variance (one-way ANOVA) (Zar 1998). Dissolved oxygen and ammonia did not have homogeneity of variance; therefore, the Kruskal-Wallis (non-parametric) test was used (Zar 1998). Survival was analyzed using the Kruskal-Wallis test on percentage of survivors per microcosm. (It was not possible to differentiate between pre-treatment and treatment-related mortalities.) Frequency of deformities was analyzed with the Chi-Squared test. Metamorph variables (time to metamorphic climax, total length, snout-to-vent length, wet weight, hormone concentration) were averaged within each pond and the statistics determined using these averages. The metamorph variables and gender distribution in metamorphs at MC were analyzed using an ANOVA. Hormone concentrations that were measured below the detection limits were assumed to be equivalent to the detection limit for the purpose of statistical analysis. If significant differences among treatments were detected with the ANOVA, the Dunnett's test was used as a multiple (post-hoc) comparison.

Analyses were conducted using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Graphs were plotted with SigmaPlot Graphing Software, version 8.02 (SPSS Inc., Chicago, IL, USA). All tests were conducted using $\alpha = 0.05$. All results are presented as mean \pm standard error unless otherwise stated (i.e. water residues, percent survival and gender distribution). Standard deviation was used as a measure of variability when the means for these variables were derived from the raw data. The standard deviation was used to describe the variability of the population as opposed to standard error, which measures variability when analyzing the mean of population means.

2.2 Results

2.2.1 2,4-D Residues in Water

Concentrations of 2,4-D measured in water at 18 hours were 0.16 ± 0.04 , 0.91 ± 0.09 and 101.3 ± 4.50 $\mu\text{g/L}$, which closely matched the nominal values of 0.1, 1.0 and 100.0 $\mu\text{g/L}$. No 2,4-D was detected in the control treatment samples; 2,4-D residues were present in all the treatment water samples until the last day of the experiment (Fig. 2.1). Regression analysis demonstrated that the 0.1 and 1.0 $\mu\text{g/L}$ concentrations declined significantly over time ($p = 0.022$ and 0.001 , respectively). The percent decrease from day one to day 57 was 50% for 0.1 $\mu\text{g/L}$ and 14.2% for 1.0 $\mu\text{g/L}$. The 100 $\mu\text{g/L}$ treatment had no change in concentration over time ($p = 0.934$).

2.2.2 Water Quality

There were no differences among treatments regarding pH, water temperature, dissolved oxygen, or conductivity (one-way ANOVA or Kruskal-Wallis test, $p > 0.186$; Table 2.1). There was no difference in ammonia concentrations among treatments (Kruskal-Wallis test, $p = 0.588$). The mean concentrations of ammonia pre-treatment and post-experiment were 0.033 mg of nitrogen/L and 0.015 mg of nitrogen/L, respectively. The nitrate and nitrite concentrations were below the level of detection (< 0.010 mg of nitrogen/L) for all samples at all sampling periods. The pH ranged from 7.01 to 9.02. Differences between the warmest and coolest microcosms in mid and bottom temperatures were minimal, ranging between 0.7 and 1.9°C. The differences between the mid and bottom temperature were also minimal, ranging between 0.1 and 0.8°C. The temperature of the water fluctuated daily corresponding to variation in the air temperature (Appendix B). The mid temperature, taken at about 25cm, was below 20°C most days (10 of the

14 days measurements were taken). The bottom temperature (50 cm) was also below 20°C, 11 of the 20 days measurements were taken. The lowest temperatures recorded were 14.0-14.2, respectively. Conductivity ranged from 400 µS/cm early in the experiment to 530 µS/cm towards conclusion of the experiment. The dissolved oxygen ranged from 6.21 to 13.51mg/L over the duration of the experiment. The DO was higher initially, but declined about 2 weeks after treatment. A range between 7.56 and 10.46 mg/L was observed for the last 3 weeks of the experiment.

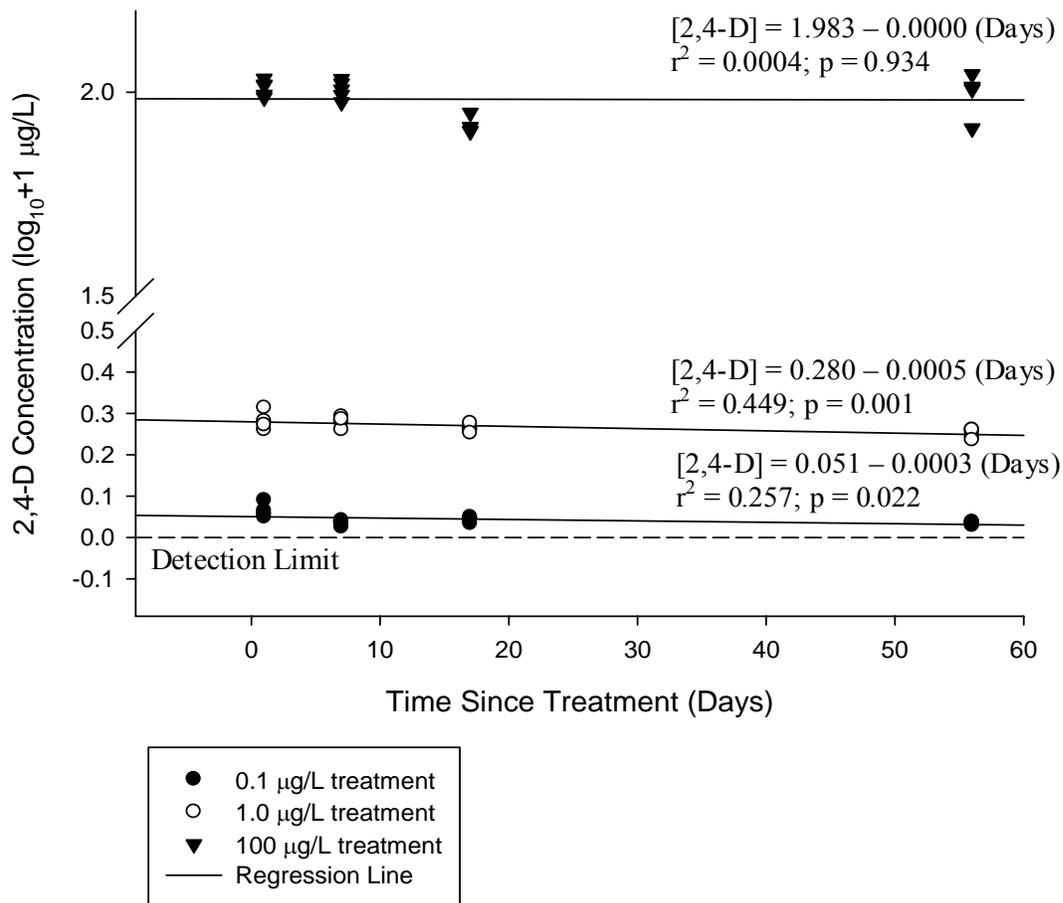


Figure 2.1: Regression analyses of 2,4-D from the microcosms. The solid lines are the regression lines fitted for the data of each treatment. Each point represents one microcosm. The dashed line represents the analytical detection limit for 2,4-D analysis (0.05 µg/L). Degradation of 2,4-D was observed in the 0.1 and the 1.0 µg/L treatment groups (simple linear regression, $p \leq 0.022$).

Alkalinity and hardness were measured pre-treatment and at 8 weeks after tadpole exposure began. Both alkalinity and hardness had increased in the last samples from the pre-treatment collection. The mean pre-treatment values were 93.2 mg/L of CaCO₃ for alkalinity and 150.6 mg/L of CaCO₃ for hardness. The values increased to mean post treatment values of 115.6 and 183.4 mg/L of CaCO₃ for alkalinity and hardness respectively.

Table 2.1: Water quality variables measured 2 - 3 times weekly in the microcosms. Values are means \pm SE, determined from averages/ microcosm across sampling times (one-way ANOVA or Kruskal-Wallis test, $p > 0.186$).

Nominal 2,4-D Conc. ¹ ($\mu\text{g/L}$)	n	pH	Mid Temp ² ($^{\circ}\text{C}$)	Bottom Temp ³ ($^{\circ}\text{C}$)	Dissolved Oxygen (mg/L)	Conductivity ($\mu\text{S/cm}$)
0.0 (Control)	4	8.20 \pm 0.05	18.33 \pm 0.12	18.9 \pm 0.17	8.55 \pm 0.02	470 \pm 2.4
0.1	5	8.14 \pm 0.05	18.23 \pm 0.15	18.9 \pm 0.17	8.68 \pm 0.07	471 \pm 2.9
1.0	5	8.19 \pm 0.05	18.21 \pm 0.15	18.9 \pm 0.16	8.61 \pm 0.09	470 \pm 1.7
100	5	8.22 \pm 0.05	18.33 \pm 0.13	19.0 \pm 0.16	8.74 \pm 0.02	475 \pm 2.7
No. Days sampled		15	14	20	20	11

¹ Nominal 2,4-D Concentrations ($\mu\text{g/L}$).

² Temperature measured at approximately 25 cm below the water surface.

³ Temperature measured at the bottom of the microcosm (50 cm).

2.2.3 Survival, Growth and Development

A total of 125 metamorphs (of the possible 800 tadpoles added) were collected and preserved in formalin from 19 of the microcosms. A die-off of undetermined cause occurred in the microcosms between June 19 and 20 (2-3 days pre-treatment); however, at the time, the extent of the mortality was not quantifiable because the tadpoles were very small (Gosner 21 – 26) and able to hide in the leaf litter. There were nonetheless sufficient numbers of surviving metamorphs for measurements of growth and development. One of the control microcosms did not have any animals present after the initial die-off. All specimens collected were between stages 42 and 46. There was no treatment effect on survival (K-W test, $p = 0.393$) (Figure 2.2).

There was no difference in time to metamorphic climax among treatments, with the average time being 50.2 days (ANOVA, $p = 0.424$) (Figure 2.3). There were no treatment effects on snout-vent length or wet weight (ANOVA, $p > 0.112$) (Table 2.2). There was a treatment effect on total length (ANOVA, $p = 0.023$). Post-hoc analysis determined that specimens the 0.1 $\mu\text{g/L}$ treatment (2.9 ± 0.26 cm) were significantly shorter than those in the control group (3.7 ± 0.08 cm) (Dunnett's t (<control), $p = 0.032$).

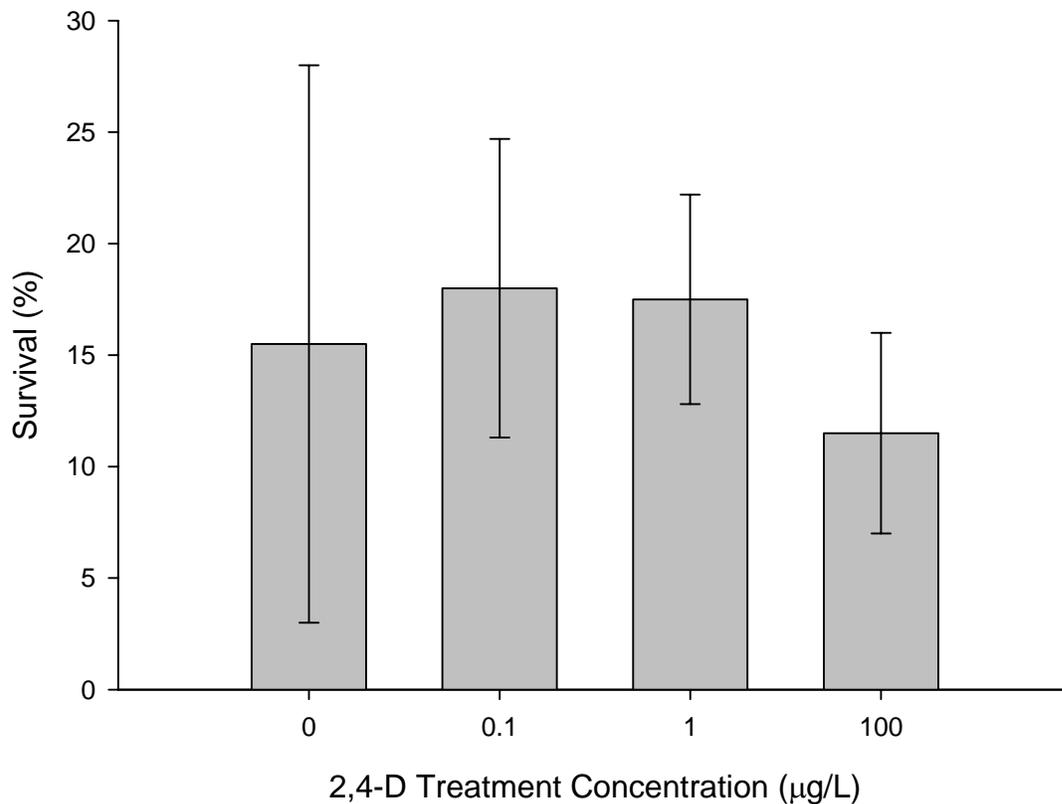


Figure 2.2: Percent survival of metamorphs for each treatment. Bars represent percent survival mean \pm SD, $n = 5$ except for control treatment where $n = 4$. There was no treatment effect on survival (K-W test, $p = 0.393$).

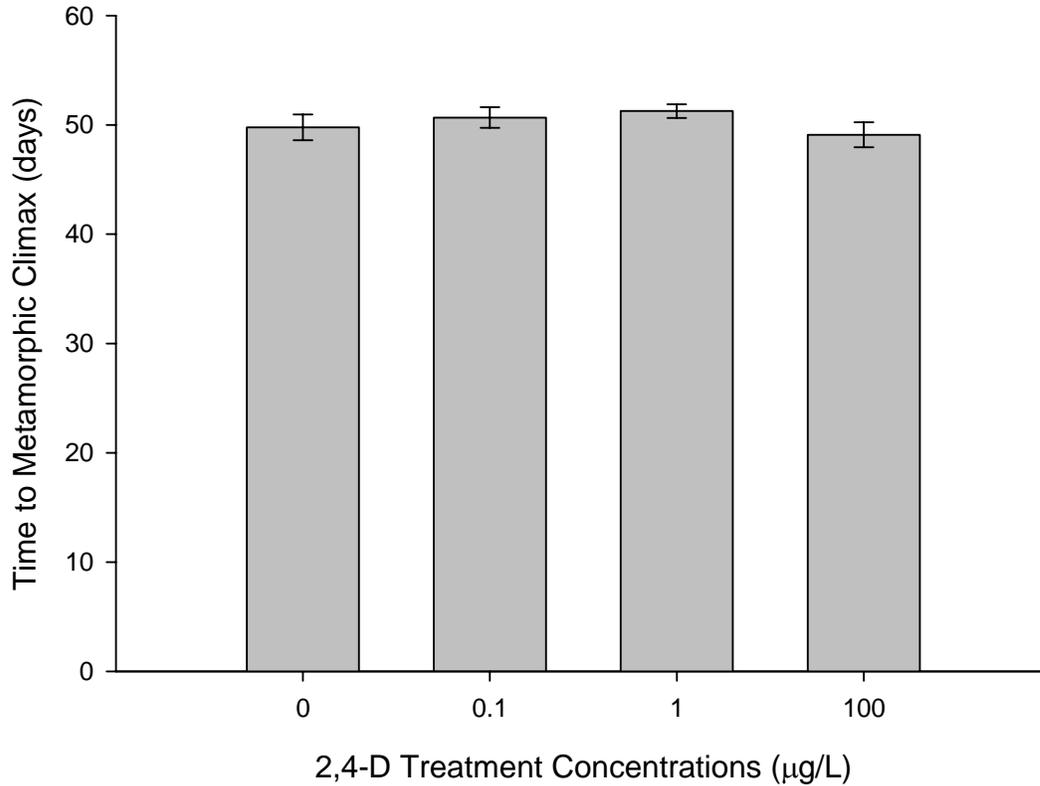


Figure 2.3: Days to metamorphic climax (determined as days from hatching to sampling) for each treatment. The bars represent days to metamorphic climax mean \pm SE, $n = 5$ except for control treatment where $n = 4$. There was no difference in time to metamorphic climax among treatments (ANOVA, $p = 0.424$).

Table 2.2: Growth and development endpoints of metamorphs measured at time of sampling for each treatment. The values represented are means \pm SE, $n = 5$ except for control treatment where $n = 4$. Different letters denote significant differences from control treatment regarding total length. There was a treatment effect on total length (one-way ANOVA, $p = 0.023$; Dunnett's t (<control), $p = 0.032$). There were no treatment effects on snout-vent length and wet weight (ANOVA, $p > 0.112$).

Nominal 2,4-D Conc. (µg/L)	Total Length (cm)	Snout-vent length (cm)	Wet weight (g)
Control	3.7 ± 0.08^a	$1.8 + 0.07$	0.8 ± 0.08
0.1 µg/L	2.9 ± 0.26^b	$1.8 + 0.08$	0.8 ± 0.08
1.0 µg/L	3.0 ± 0.13^a	$1.7 + 0.05$	0.7 ± 0.07
100 µg/L	$3.9 + 0.37^a$	$1.9 + 0.12$	$1.0 + 0.12$

2.2.4 Deformities

Scoliosis, defined as a lateral curvature of the spine was observed in three of the metamorphs, apparently only affecting the tails, two from the 0.1 µg/L treatment and one from the 100 µg/L treatment. There was no significant difference in the incidence of axial deformities (kinks in the tail or observable curves in the spine) in any of the treatments (Chi-squared test, $p = 0.317$). The overall occurrence of axial deformities was 2.4%. The occurrences for treatments with observed deformities were 5.6% for 0.1 µg/L treatment and 4.3% for 100 µg/L treatment.

Three specimens with deformed hind limbs were observed from three microcosms, one from each of the 2,4-D treatments. Ectromelia, the absence of all or part of a limb, was observed in two of the metamorphs (0.1 µg/L and 100 µg/L); ectrodactyly, absence of all or part of a digit, was observed in one from the 1.0 µg/L treatment. There was no difference in the incidence of limb deformities in any of the treatments (Chi-squared test, $p = 0.756$). The overall occurrence of limb deformities was 2.4%. No deformities were detected in the control treatment. The occurrences for treatments with observed deformities were 2.8% for 0.1 µg/L treatment, 2.9% for 1.0 µg/L treatment and 4.3% for 100 µg/L treatment.

2.2.5 Plasma Corticosterone Hormone

Corticosterone values were not obtained for 19 metamorphs due to lack of sufficient blood. The values of corticosterone concentrations ranged from 0.1 ng/mL of serum to 32.0 ng/mL of serum. The treatment means are presented in Figure 2.4. There were no differences in corticosterone concentrations among treatments (ANOVA, $p = 0.245$). The intra-assay coefficients of variation (CVs) for mean CORT concentrations of 66.8 or 517.4 ng/ml were 3.3% or 6.8%, respectively.

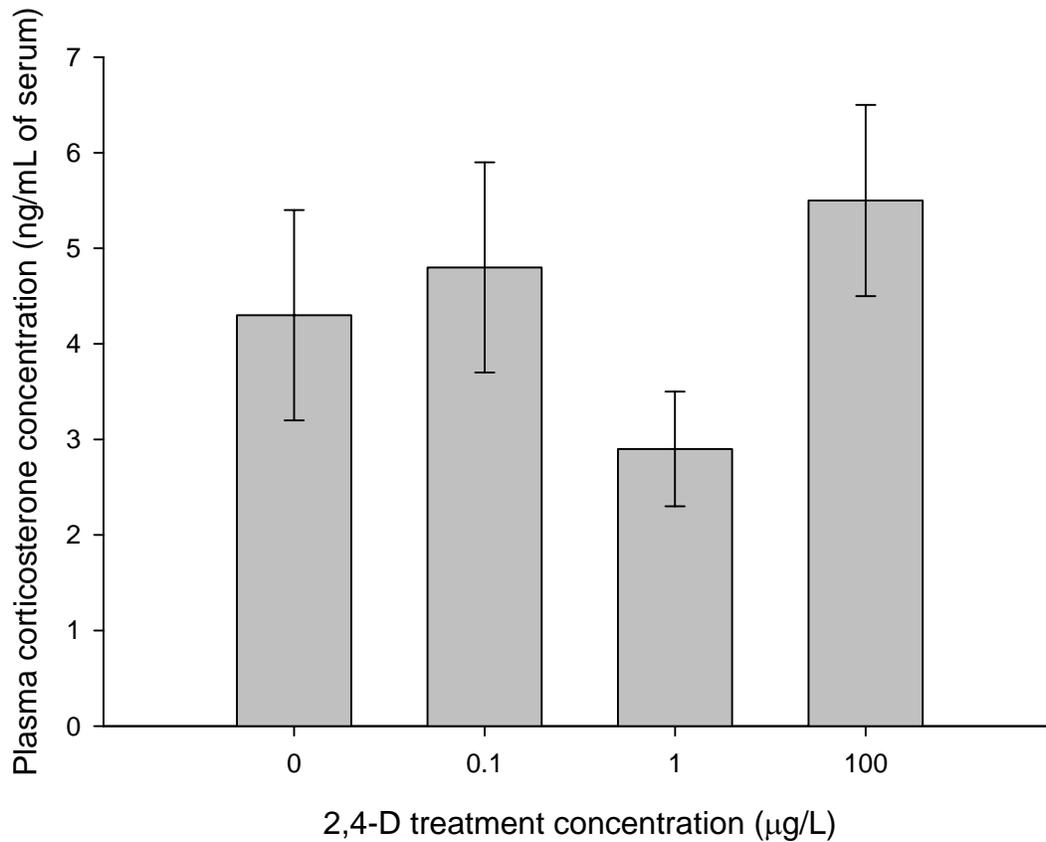


Figure 2.4: Plasma corticosterone concentration for each treatment. The bars represent plasma corticosterone concentration mean \pm SE, $n = 5$ except for control treatment where $n = 4$. There were no differences in corticosterone concentrations among treatments (ANOVA, $p = 0.245$).

2.2.6 Gender Distribution

Percentages of females were observed to be greater than those of males in control, 1.0 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ treatments. There was possibly a shift in percent gender to more males than females in the 0.1 $\mu\text{g/L}$ treatment (Fig. 2.5); however, it was not found to be statistically significant (ANOVA, $p = 0.807$). Therefore, the observed percentage of genders was probably normal population variability. Overall, the mean percentage of females in the microcosms was $53 \pm 21\%$.

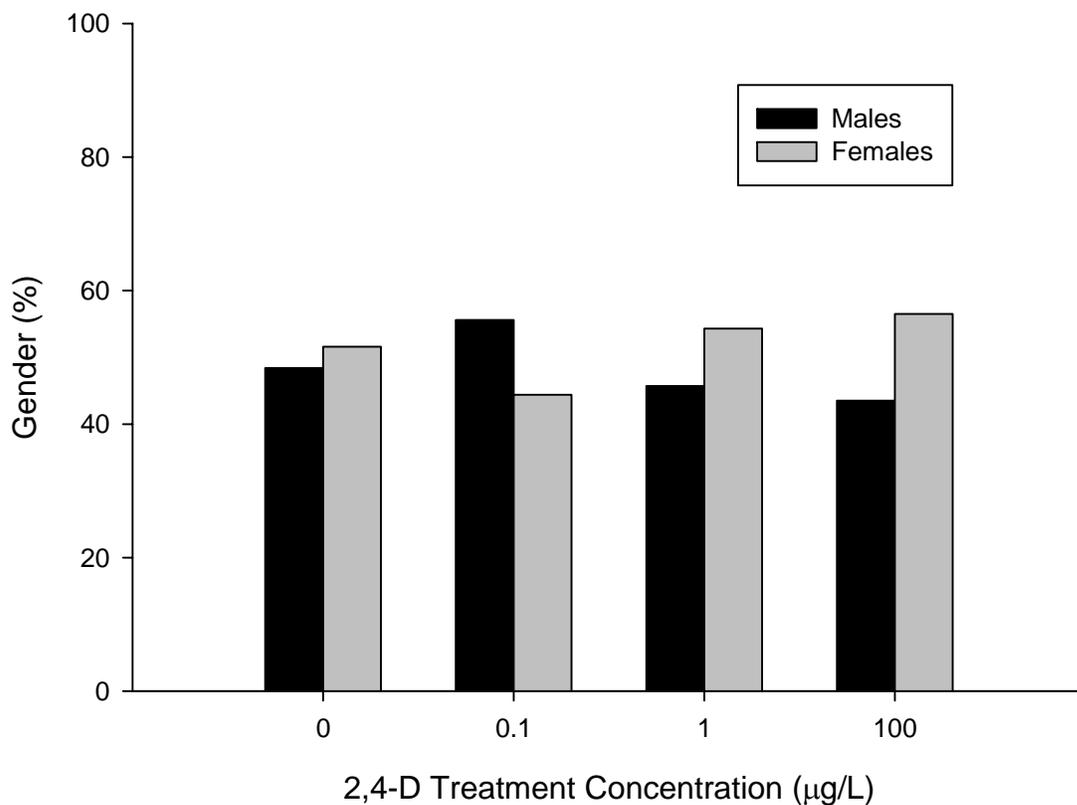


Figure 2.5: Percent of males and females in each treatment. All metamorphs collected from the microcosms are represented. There was no treatment effect regarding percent gender among treatments (ANOVA, $p = 0.807$).

2.3 Discussion

2.3.1 2,4-D Residues in Water

Degradation of 2,4-D from the water column is dependent on several factors such as temperature, pH, depth and other pond characteristics (Boyle 1980; Birmingham and Colman 1985; Aly and Faust 1964; Averitt and Gangstad 1976). Regression analyses indicate there was degradation in the 0.1 and the 1.0 µg/L treatment groups. Rate of degradation was not expected to be different among the treatments as Boyle (1980) found similar rates of degradation with two different concentrations of 2,4-D. Other studies have demonstrated significant degradation of 2,4-D within the time frame of this experiment with half-lives of 14-34 days (Boyle 1980; Birmingham and Colman 1985), although some half-lives were longer (> 56 days) (Wang *et al.* 1994).

Temperature and pH are important factors influencing the rate of degradation of 2,4-D (Birmingham and Colman 1985; Averitt and Gangstad 1976; Aly and Faust 1964). The rate was enhanced as temperature increased (Averitt and Gangstad 1976). Rate of photodegradation was slower in water with low pH (Aly and Faust 1964). In the microcosms, temperature and pH ranges (15 - 24°C; 7.01 – 9.02, respectively) were similar to ranges reported in other studies that observed a 90% loss of 2,4-D within 28 days at a temperature of 20°C (Averitt and Gangstad 1976) and 50% in an average 37.5 minutes at pH 7.0 and 9.0 when ultraviolet irradiation was used (Aly and Faust 1964). Therefore, temperature and pH did not likely impede degradation in the microcosms.

No data were collected regarding the microbial populations; therefore, it is not clear if microbes of the pond water played a role in degradation given the experimental time frame and the amount of water added. Wang *et al.* (1994) observed slow degradation of 2,4-D from river water that had very little microbial action. Microorganisms also seemed to adapt and degrade 2,4-D more efficiently subsequent to exposure to the chemical (Aly and Faust 1964). Any microorganisms present in the added pond water would have had no previous exposure to 2,4-D or other contaminants.

Photolysis and hydrolysis are also important reactions involved in the loss of 2,4-D from water (Zepp *et al.* 1975). But similar to temperature and microbial activity, all treatments were exposed to the same factors that influence photolysis and hydrolysis. If these reactions were important to the loss observed in the microcosms, there should have been some loss at all concentrations of 2,4-D.

The percent decrease of 2,4-D from the microcosms was inversely related to the concentration of the treatments. This trend indicates that microorganisms were not likely the cause for the degradation in the microcosms as there should have been some degradation in all of the treatments. The differences in degradation rates may be related to the metabolism of aquatic plants. 2,4-D may have affected the plants so as to stimulate metabolism of 2,4-D absorbed from the water in the 0.1 µg/L treatment group as seen by Forsyth *et al.* (1997). Conversely, in the

high concentration treatment (100 µg/L), the plants may have been incapable of metabolizing chemicals from the water, due to injury caused by 2,4-D (Forsyth *et al.* 1997) although plant injury was not monitored.

2.3.2 Water Quality

Chemical application did not alter the water quality in the microcosms, as there were no significant differences among treatments. Measurements of ammonia, nitrate/ nitrite and dissolved oxygen fell below the toxic limits or within the acceptable range for each variable (CCME 2003; NRC 1974). The pH fell within the guidelines (pH 6.5 -9) set out by the CCME (2003) but was slightly high according to the values recommended by the NRC (1974) for *Rana* species (pH 6.5). In addition, the range of pH measured in this study was similar to the range of 5.4 to 9.3 recorded by Serben (2003) for nine agricultural and boreal ponds in Saskatchewan. Mean conductivity in the microcosms (471 µS/cm) was similar to the mean values of about 300 mS/cm found in agricultural and boreal ponds (Table 3.1). The temperature of the water did not appear to be a factor, since there were no significant differences among treatments and the values did not surpass the extreme ranges that may inhibit growth, development or metamorphosis (Suzuki 1985; Hayes and Licht 1993). Alkalinity was lower than the level recommended by the NRC (1974) but was within the normal range reported in agricultural ponds (Serben 2003). The hardness was within both the recommended range and the range found in the environment (NRC 1974; Serben 2003).

2.3.3 Deformities

All 2,4-D treatment groups were associated with the occurrence of deformities (scoliosis, ectrodactyly or ectromelia). Cooke (1981) determined that axial deformities (simple curvatures and kinking of the tail) were the most common types occurring in tadpoles, whether exposed to pollutants or not and simple curvatures occurred at the rate of 3 – 8 % in laboratory control groups. The background benchmark for deformities in metamorphs and frogs in the wild is less than 5% (Blaustein and Johnson 2003), although some consider the background occurrence to be less than 1% (Ouellet 2000; Gardiner & Hoppe 1999). Assuming that these control values can be used as a background benchmark, the frequency of kinks in the microcosm study was well below the benchmark. It is worth noting that due to low survivorship (mean of $15.6 \pm 7.6\%$), the

frequency of deformities (2.4%) may have been underestimated. The mortalities that occurred may have been weaker and deformed animals. Also, the early developmental stages of the larvae (egg and embryo) were not exposed to 2,4-D treatment, as the tadpoles were exposed after they were free from dependence on their egg sacs. Sensitive developmental stages may not have been exposed (Bridges 2000).

Tadpoles exhibiting kinking occasionally recover with no detrimental effect (Cooke 1981), but deformities may reduce swimming performance as seen by Hopkins *et al.* (2000). The result could be reduced efficiency of evading and/ or detecting predators, which in turn, could decrease recruitment to the terrestrial environment, thereby reducing populations (Hopkins *et al.* 2000). The causes for axial deformities are still unknown. Cooke (1981) suggested that some species are more susceptible to the development of specific deformities. An external stressor, such as freezing temperatures, could therefore potentially increase the incidence of an already common deformity (Cooke 1981).

The results of this study suggest that 2,4-D did not cause the deformities observed. As mentioned, however, due to low survivorship, the frequency of deformities may have been underestimated. The effect of 2,4-D with regard to deformities warrants further investigation.

2.3.4 Survival, Growth and Development

There are several potential causes for mortality in tadpoles unrelated to exposure. Viruses that affect amphibians, specifically tadpoles have been detected in Saskatchewan ponds (Bollinger *et al.* 1999; Schock *et al.* 2008). Frog virus-3 (FV3) and to a lesser extent, the *Ambystoma tigrinum* virus (ATV) have been implicated in wood frog mortalities (Schock *et al.* 2008). These viruses can affect the eggs, tadpoles and adults of the species (Duffus *et al.* 2008). The microcosms may have been infected by the tadpoles or by the pond water (130 mL) that was added; however, no attempt was made to test for virus.

Another potential cause of the microcosm mortalities is exposure to latex gloves. Gutleb *et al.* (2001) investigated the potential impact latex exposure can have on the mortality of tadpoles. This was discovered by the inadvertent exposure through cleaning of aquaria. Exposing *X. laevis*

and *R. temporaria* tadpoles to dilutions of latex-exposed water, Gutleb *et al.* (2001) found that both species were highly sensitive to latex manifested by high mortality. Cashins *et al.* (2008) also found that both latex and nitrile gloves were toxic to tadpoles (*Litoria genimaculata* and *Litoria nannotis*) even with short exposures. Thoroughly washed/ rinsed vinyl gloves are recommended but it should be noted that the toxicity could vary between brands, types and even boxes (Cashins *et al.* 2008). The use of latex gloves is still recommended in some references (de la Navarre 2006). In the current study latex gloves were used in the holding tubs for the tadpoles prior to acclimation in the microcosms. Therefore, they were held in latex-exposed water prior to introduction to the microcosms. The significance of this exposure in the current study is unknown. Further work is required to determine the extent of latex toxicity. For future studies with tadpoles, it is recommended that latex gloves should not be used or if unavoidable, they should be thoroughly rinsed before contact with the tadpoles or their environment.

There was no significant difference in time to metamorphosis among treatments, with the average time being 50.2 days, which was 1-4 days longer than occurred in a similar study (Serben 2003). Temperature and hydroperiod can impact the larval period (Riha & Berven 1991; Rowe & Dunson 1995). Riha and Berven (1991) found that larval period increases with decreased temperature, which may be a result of the slowed growth. Although density alone does not affect larval period, it may interact with temperature to change the time to metamorphosis, thereby increasing the larval period in tadpoles reared in cool, low density waters (Riha & Berven 1991). The temperature effect may be related to a perceived hydroperiod effect. Rowe and Dunson (1995) observed the effects of decreasing water volume on the larval period of tadpoles. They found tadpoles were unable to metamorphose before 56 days (the method to determine this time was not mentioned) but were able to metamorphose at day 84 and 158 of treatment. Evidence indicates a minimum size of the tadpoles is required to complete metamorphosis, even if the pond is drying (Rowe & Dunson 1995). The average temperature overall recorded by Serben (2003) was approximately 1°C higher with some daily maximum values in excess of those in the present study (27°C versus 24°C). In addition, due to the mortalities in the microcosms, tadpole densities were also low.

The only endpoint with a significant treatment effect was total length in the 0.1 µg/L treatment (ANOVA, $p = 0.018$; Dunnett's test, $p = 0.024$). The significant difference seen in total length may be an artifact of stage of development or could be treatment related. Collection of metamorphs was targeted at stage 43. The metamorphs of the 0.1 and 1.0 µg/L treatment groups appear to have shorter tails than the other treatment groups (Table 2.2), suggesting that resorption of the tail may have been enhanced by 2,4-D at the lower concentrations. Future studies could investigate this possible effect further. Metamorphs were approximately 23 % smaller in SVL than those in the similar microcosm study by Serben (2003). It may have taken metamorphs in the present study longer to reach the minimum size requirement for recruitment into the terrestrial environment. Although there was no significant treatment effect on SVL, an investigation into the cause of prolonged aquatic stage without an increase in size may be warranted. As mentioned earlier, sensitive developmental stages may not have been exposed to 2,4-D treatment. Therefore, potential differences in SVL and wet weight may not have been observed as the effects of 2,4-D on those endpoints may have missed the critical period (Bridges 2000).

2.3.5 Plasma Corticosterone Hormone

Although there was no significant difference in CORT concentrations among treatments in the microcosms, there was an interesting pattern observed. The mean CORT concentration of the 1.0 µg/L treatment was much lower than the other three treatment groups (Figure 2.4). Glennemeier & Denver (2002) found that the response to acute stress (shaking) by tadpoles and metamorphosing frogs peaked at 30 minutes. The 5-minute period of shaking used in the present study may not have been sufficient to attain a maximum response. Also, due to the undetermined cause of mortality, the tadpoles may have been exposed to a stressful environment for the duration of their aquatic life, resulting in a chronic stress response unrelated to the treatment. The metamorphs were sampled during metamorphic climax (within 4 stage levels), therefore the lower concentration of CORT observed in the 1.0 µg/L treatment group was unlikely due to differences in stage. In this case, the possible decreased ability to respond to acute stress may have been due to impairment or exhaustion of the HPI axis, due to prolonged exposure to another chronic stressor (low level 2,4-D concentrations) as seen in fish and mudpuppies exposed to heavy metals, bleached kraft mill effluent or chlorinated hydrocarbons

(Norris *et al.* 1999; Hontela *et al.* 1995; Hontela *et al.* 1997; Gendron *et al.* 1997). Another possible explanation was that the baseline CORT was reduced as observed in birds (Love *et al.* 2003; Marra & Holberton 1998; Walker *et al.* 2005). If there was a change in the baseline response, the response to acute stress may or may not have been impaired. Regardless, there may be a possible impact on a group of animals. The 1.0 µg/L group may have experience hormesis in response to chronic stress. The added low-level concentration of 2,4-D produced a decrease in response to acute stress or a decrease in baseline CORT concentrations that translated into the appearance of reduced acute stress response (Calabrese 2008; Calabrese & Baldwin 2001). Due to the limitations in blood sample collection (only one per animal), it was not possible to obtain samples to test for baseline CORT concentrations, which might have demonstrated chronic stress in the microcosms. The possible difference in response to acute stress warrants some investigation. Also, investigation into the circadian effects on tadpoles' CORT concentrations and the variability of CORT concentrations through metamorphosis is needed to determine those confounding factors to hormone analyses. In metamorphosing amphibians (e.g. bullfrogs *Rana catesbeiana*), it has been observed that the CORT concentration changed throughout the day. It peaked in early morning and began to decline in late afternoon through the evening to its lowest level during the night (Wright *et al.* 2003). It was believed little fluctuation occurred during the sampling periods of the microcosm study but may warrant further study for this species of frog. Corticosterone concentrations rise and fall through premetamorphosis to post-metamorphic climax (Wright *et al.* 2003). Although stage 43 (peak CORT concentrations) was targeted, there should be little fluctuation during stages 42 to 46. It is possible that the rise and fall of CORT in wood frogs throughout metamorphic climax occurred during those stages.

2.3.6 Gender Distributions

In the microcosms, it is the 0.1 µg/L treatment that appears to have shifted its distribution, but towards more males (rather than the expected 50:50 ratio or more females as seen in the other treatments). Although these results were not statistically significant, further investigation into 2,4-D as a contaminant that may affect gender distributions may be warranted (Hayes *et al.* 2002).

2.4 Summary

Size at and days to metamorphic climax, as well as corticosterone of Saskatchewan wood frog tadpoles were not adversely affected by exposure to environmentally relevant concentrations of 2,4-D. The possibility of decreased CORT hormone in the 1.0 µg/L treatment, although not statistically significant, combined with the non-significant shift in gender distribution suggest that further investigation is needed to determine if there is potential for endocrine disruption by 2,4-D.

Chapter 3

The sublethal effects of 2,4-dichlorophenoxyacetic acid on tadpole growth and development in ponds located throughout Saskatchewan.

3.0 Introduction

Scientists along with the general public have developed a growing concern for the health of amphibian communities (Houlahan *et al.* 2000; Collins & Storfer 2003). These concerns are driven by the reported global declining of amphibian populations as well as the increasing numbers of deformities (Houlahan *et al.* 2000; Collins & Storfer 2003; Ouellet *et al.* 1997; Kavlock 1998). The causes for the negative impact on the amphibian populations are not clear although anthropogenic activities are believed to be a likely source (Carey 2000; Green 1997; Blaustein & Kiesecker 2002), especially agricultural pesticides (Berger 1989; Hayes *et al.* 2003; Ouellet *et al.* 1997).

A large proportion of land-use in Saskatchewan is devoted to agriculture. As a result, there is relatively high usage of pesticides, particularly herbicides. 2,4-dichlorophenoxyacetic acid (2,4-D) is a commonly used post-emergent herbicide. Nearly 3800 tonnes of 2,4-D were applied to approximately 304,426 km² of agricultural land in Saskatchewan in 1990 (Waite *et al.* 2002; Ducks Unlimited Canada 1993). Although it does not persist in the environment, it has been reported to be easily transported from the fields of application to adjacent wetlands via surface runoff (WHO 1984). 2,4-D has been detected in various wetlands throughout Saskatchewan with maximum concentrations ranging from 0.12-2.67 µg/L (Waite *et al.*, 2002; Waite *et al.* 1992; Grover *et al.* 1997; Donald *et al.* 2001).

2,4-D is rarely used alone, thus, the possibility of synergistic interactions with other chemicals and contaminants (such as metals) is also of concern (Donald *et al.* 2001; Forsyth *et al.* 1997; Perez-Coll & Herkovits 2006). Along with other pesticides being used in conjunction, surfactants are often added to agricultural formulations to aid in ease of treatment but these surfactants alone may Along with its intended effect, there is also the potential that 2,4-D will indirectly alter the environment causing an impact on non-target species (WHO 1989; Boyle 1980). The manifestation of adverse 2,4-D effects (both direct and indirect) in animals such as

rats and carp can range from mild to severe and may include behavioral changes, hepatic damage or changes in corticosterone responses (via indirect environmental stresses) (Sarıkaya & Yilmaz 2003; Paulino *et al.* 1996; Hayes & Wu 1995). Agricultural formulations of herbicides containing “inert” ingredients, such as surfactants, may have detrimental effects on non-target species. Surfactants that may be used in 2,4-D formulations have alone been observed to create changes in endocrine function (vitellogenin-induction studies) in fish, inhibition of oxidative functions in mitochondria and increased toxicity in fish (Siemering *et al.* 2008; Oakes & Pollak 1999; Abdelghani *et al.* 1997). There is also the potential for these surfactants to act as weak pseudoestrogens and genotoxins, as observed in *in vitro* studies using human lymphocytes (Burroughs *et al.* 1999). Other effects of 2,4-D, which may be beneficial, include increased productivity and growth of phytoplankton in ponds (Boyle 1980). These changes, in one way or another, will affect the survival and fitness of amphibians in general.

Wood frogs (*Rana sylvatica*) are a common species of amphibian found throughout Saskatchewan (Seburn 1992). Their development begins as eggs in an aquatic environment and proceeds quickly through metamorphosis and emergence into the terrestrial environment (Thurow 1997; Russell & Bauer 1993). In Saskatchewan, this usually occurs in one summer, making it an ideal species for study. Adults also return to the same pond every year to lay eggs as long as the pond conditions remain ideal (Russell & Bauer 1993).

Lipids can be a good measure of the condition of an animal as an indication of energy stores that can be mobilized (Tocher 2003). The amount of lipids in newly metamorphosed frogs is minimal, however (Alvarez & Nicieza 2002). In amphibians, the primary storage site for lipids is in the abdominal fat bodies, followed by the liver and muscle (Fitzpatrick 1976; Girish & Saidapur 2000). The fat bodies are conspicuous finger-like deposits of fat located in the abdominal area specifically around the gonads (Fitzpatrick 1976). Fat bodies increase through the fall in preparation for overwintering (Fitzpatrick 1976).

A study of 2,4-D was conducted in natural ponds so as to include environmental factors (Rowe & Dunson 1994), such as temperature, depth of water, predators, parasites and food sources, that affect the degradation of chemicals as well as the contribution of environmental stressors in

wood frog habitat (Boyle 1980; Aly and Faust 1964; Averitt and Gangstad 1976; Riha & Berven 1991). Conducting the experiment in the field also allowed for the inclusion of non-chemical stressors (e.g. metals) and other pesticides, especially herbicides, that may be present and act synergistically with 2,4-D (Perez-Coll & Herkovits 2006; Hayes *et al.* 2006). The ability to determine a causal relationship is complicated by the inclusion of many factors but the results will aid in the planning of future studies with amphibians (Rowe & Dunson 1994).

The objective of this study was to assess the effects of environmentally realistic concentrations of 2,4-D in natural ponds on the health of amphibians by determining whether the growth and development of wood frog tadpoles would be adversely affected by agricultural activity combined with direct application of 2,4-D to selected ponds.

3.1 Materials and Methods

3.1.1 Field Sites

Three experimental groups were chosen based on geography and treatment: likely unexposed, potentially exposed and treated. The ultimate selection of ponds in each group was determined by the presence of eggs and tadpoles of sufficient quantity and quality. Three ponds from the boreal forest were selected as likely unexposed based on their distance from active agricultural practices (> 80km). The ponds in the boreal forest were in northern Saskatchewan and are referred to as the “forested ponds” (Appendix C). All forested ponds were surrounded to varying degrees by willows and grasses. Some were surrounded by aspen, spruce and pine trees. Horsetails (*Equisetum sp.*) were present in some of these ponds. The potentially exposed group (referred to as the “agricultural ponds”; n = 4) was located in the agricultural area of Saskatchewan where conventional farming practices occur, including pesticide application. The treated group (referred to as the “treated ponds”; n = 4) was also selected from ponds in the agricultural region of Saskatchewan. These ponds were specifically treated with 2,4-D, as described in Section 3.2.2. Both groups were located near Saskatoon and Humboldt (Appendix C). All of the ponds were surrounded to varying degrees by cropland with borders of aspen, willows and grasses and were monitored throughout the summer to ensure that metamorphs were collected at the optimal time. Tadpoles did not develop into metamorphs in all ponds, however, resulting in a reduced sample size.

3.1.2 2,4-D Application

The agricultural formulation, “2,4-D Amine 600” (560 g/L of dimethylamine salt, Registration No. 17511, Interprovincial Cooperative Limited, Inc. (IPCO), Saskatoon) was used to treat the four ponds, with landowner permission. Application rates were calculated for each pond to yield a target concentration of 10 µg/L (Appendix D). The ponds were sprayed on June 15. The calculated dose for a pond was first diluted with pond water and the diluted solution was sprayed using a 1-gallon (3.8 L) polyethylene compressed air hand sprayer (Chapin International, Inc., Batavia, NY, USA) over the entire surface. The spray was applied in front of the person spraying in a sweeping motion while backing across the pond, followed by mixing with a long stick recovered from each pond.

3.1.3 Herbicide Residue Analysis

Water samples were collected from all ponds at the time of selection (agricultural: May 12 - 26; forested: May 30 through June 6) and again at the time of metamorph collection (June 30 through August 11). Samples were also collected from the four agricultural ponds and the four treated ponds on June 16-17, when local farmers were applying herbicides to cereal crops. The treated ponds were sampled post treatment at 24 hours, 2 days, 4 days, 2 weeks, 4 weeks, 6 weeks and 8 weeks. A glass sample bottle (1-L pre-cleaned) was held 15 cm beneath the surface of the water during sampling. All samples were kept on ice in darkness in the field. The samples were transported to the National Hydrology Research Centre (NHRC) in Saskatoon and stored at 4°C until analysis. They were analysed for 2,4-D at NHRC using LC-MS-MS (method described in Section 2.2.4). The limit of detection (LOD) for this method was 0.5 µg/L. Select samples were stored at 4°C and shipped to the National Laboratory for Environmental Testing (NLET), Environment Canada, Burlington, Ontario, for analysis of 2,4-D, MCPA, dicamba, bromoxynil and clopyralid after analysis was completed at NWRI. Some breakdown of the samples during storage may have occurred between collection and shipment as the samples were not stabilized with acid in the field and were held beyond the holding period recommended by NLET. The samples were stabilized with 2 mL of sulfuric acid just prior to being packed for shipment to NLET on November 30, 2005.

3.1.4 Water Quality Analysis

Samples for water quality analysis were collected along with those taken for herbicide analysis at the start of the experiment in May and early June (referred to as Collection Day 1) and at the time of metamorph sampling (referred to as Collection Day 2). Due to the absence of viable metamorph specimens in many of the forested ponds, an additional pond was added and therefore was sampled only at the time of metamorph collection. Water was analyzed for ammonia, nitrate/nitrite ($\text{NO}_3^- + \text{NO}_2^-$) concentrations and specific conductivity at NHRC, Saskatoon. Temperature and pH were measured using a hand-held meter (Hanna Model HI 991301 Portable pH/EC/TDS/Temperature Meter from Gasonic Instruments, Calgary, AB, Canada).

3.1.5 Growth and Development

Metamorphic climax, which begins with emergence of the forelimbs and ends with complete absorption of the tail, signaled the end of the exposure period. The target stage for collection was Gosner 43, when corticosterone concentrations in metamorphs are maximal (Jaffe 1981; Krug *et al.* 1983). However, it was difficult to sample all the metamorphs at this stage because each pond was sampled in a single day, whereas metamorphs progress through MC at varying rates. Therefore, samples of metamorphs included Gosner 42-45. The target number of metamorphs per pond was 20.

Metamorphs were captured using small and large dip nets, and large, long-handled nets. Nets and boots were disinfected between ponds by rinsing in a 20% chlorine bleach solution. Metamorphs were subjected to a standardized acute stress protocol for 30 min, anesthetized and blood collected as described in Section 2.2.6 (Glennemeier & Denver, 2002). Total length, wet weight and external deformities were recorded. Blood samples collected from the field were centrifuged for five minutes at 3000 rpm (SilentSpin microcentrifuge, Continental Lab Products, San Diego, CA, USA). Euthanasia was completed with an overdose of Orajel, although most metamorphs died of exsanguination. The specimens were preserved in 10% neutral buffered formalin, then measured for snout-vent length (SVL) and dissected to determine sex and to remove the liver. Once all dissections were complete, the metamorphs were shipped to the Avian Energetics Laboratory in Port Rowan, Ontario for analysis of total lipid reserves in the

carcass (body condition) by the method described by Weatherhead and Brown (1996). The metamorphs were weighed, oven-dried, homogenized (extracted in petroleum ether) and reweighed. This procedure determined the carcass wet, dry and lean dry weights. The lean dry samples were placed in a muffle furnace (550°C) for 12 hours to burn the protein. The remaining ash was weighed and protein content was calculated as lean dry weight minus ash.

Corticosterone (CORT) concentrations in plasma were analyzed using radioimmunoassay (RIA) by Prairie Diagnostic Services, Saskatoon, using methods described in Section 2.2.6. The intra-assay coefficients of variation (CVs) for mean CORT concentrations performed in duplicate of 68.4 ng/ml or 433.1 ng/ml are 3.7% or 3.9%, respectively.

3.1.6 Statistical Analysis

Normality and homogeneity of variance were analyzed for all variables using the Shapiro-Wilk test and Levene test (Zar 1998), respectively. When the assumptions of normality and homogeneity were not met, the data for that variable were log or arcsine transformed and retested. If transformation did not correct for non-normality and heterogeneity, nonparametric tests were used. Linear regression analyses were run on the water residues from the treated ponds to determine the rate of 2,4-D degradation. The slopes were compared with a parallelism test (Chi-Squared test) in ordinal regression. This analysis was conducted for sample days for which all ponds had measurements, therefore, not all data points were used.

The half-life of 2,4-D was determined using the slopes of the degradation graph in the following equation:

$$t_{1/2} = (\log_{10} (1/2 [2,4-D])) - (y\text{-intercept}) / \text{slope} \quad (\text{Equation 1}).$$

Water quality variables were analyzed for experimental group differences using one-way analysis of variance (ANOVA) or Kruskal-Wallis Test (Zar 1998).

Metamorph variables (SVL, wet weight, liver weight, CORT, total fat and total protein) were averaged within each pond and the statistics determined using these averages. The ponds were treated as replicates. SVL and gender distribution were analyzed using Kruskal-Wallis test, with

Mann-Whitney tests for post-hoc analysis. Wet weight, CORT, percent fat and percent protein were analyzed using a one-way ANOVA. The Tukey test was used for post-hoc analysis of variables analyzed with ANOVA indicating significant differences among treatments. Hormone concentrations that were measured as below the detection limits were assumed to be equivalent to the detection limit for the purpose of statistical analysis. The liver weights were analyzed using an analysis of co-variance (ANCOVA), with wet weight as the covariant. All tests were conducted using $\alpha = 0.05$. Metamorph endpoints were presented as mean \pm standard error of the mean. For the mean half-life of 2,4-D residues, the water quality data and mean percent females results were presented as mean \pm standard deviation of the mean. Standard deviation was used as a measure of variability when the means for these variables were derived from the raw data. The standard deviation was used to describe the variability of the population as opposed to standard error, which measures variability when analyzing the mean of population means.

Statistical analyses were conducted using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). Graphs were plotted with SigmaPlot Graphing Software, version 10.0 (SPSS Inc., Chicago, IL, USA).

3.2 Results

3.2.1 2,4-D Residues in Pond Water

Concentrations of 2,4-D measured in the treated pond water collected at 24 hours after treatment were above the target value of 10 $\mu\text{g/L}$ in three of the four treated ponds. The mean for the three ponds was 35 $\mu\text{g/L}$, ranging from 29.3 to 39.3 $\mu\text{g/L}$. The fourth pond had a concentration of 4.7 $\mu\text{g/L}$ at 24 hours, which increased to 17.1 $\mu\text{g/L}$ by day 4. The loss of 2,4-D from all four ponds followed a similar pattern from day 4 until the end of the experiment (Figure 3.1). Mean (\pm SD) residues declined from 14.2 ± 2.1 $\mu\text{g/L}$ on day 4 to 7.5 ± 1.5 $\mu\text{g/L}$ at 2 weeks and 1.0 ± 0.1 $\mu\text{g/L}$ at 4-5 weeks post-treatment, when metamorphs were collected (July 14-21). Linear regression analyses indicated degradation in the four treated ponds ($p \leq 0.001$ for ponds 2, 3, and 4; $p = 0.059$ for pond 1). The parallelism test (conducted only on common sampling dates) determined that the linear regression lines were parallel ($p > 0.050$). The mean half-life for all four treated ponds was 8.0 ± 5.5 days. Residues of 2,4-D were detected until metamorph sampling in all treated ponds. Residues were detected until the last scheduled water sampling period (8 weeks)

in two of the treated ponds, while the other two treated ponds had desiccated before 6 weeks (Figure 3.1). 2,4-D was detected in one of the samples from an untreated agricultural pond (0.6 µg/L) at the time of metamorph sampling. No forested ponds had detectable 2,4-D residues. The results from NLET were somewhat different (Table 3.1). As mentioned in Section 3.1.3, the samples were held beyond the recommended holding period and were not stabilized until shipped to the laboratories in Burlington, ON. Residues of 2,4-D, clopyralid, dicamba and MCPA were detected in all of the experimental groups (Table 3.1). Bromoxynil was detected in the agricultural and treated ponds but not in the forested ponds.

Table 3.1: Water residue results from analyses completed by the National Laboratory for Environmental Testing (Burlington, ON) for the five major-use herbicides in Saskatchewan. Values are means \pm SD for each experimental group. Only one sample/ pond is represented within the mean. The three forested ponds were sampled between June 30 and August 11, during collection of metamorphs. The four agricultural and four treated ponds were sampled June 16, when application by local farmers was occurring.

Herbicide (µg/L)	Forested	Agricultural	Treated
2,4-D	0.0779 \pm 0.0498	0.142 \pm 0.0763	8.04 \pm 10.3
Clopyralid	0.00352 \pm 0.00306	0.00812 \pm 0.00834	0.00608 \pm 0.00195
Dicamba	0.00253 \pm 0.00438	0.00752 \pm 0.00600	0.00748 \pm 0.00309
MCPA	0.0175 \pm 0.0304	0.142 \pm 0.129	0.0938 \pm 0.0759
Bromoxynil	N/D ¹	0.0234 \pm 0.0187	0.0132 \pm 0.0115

¹ N/D = non-detection in all of the samples for that experimental group.

3.2.2 Water Quality

There were no significant differences among experimental groups with respect to ammonia, nitrate, nitrite, pH or specific conductivity for day 1 (ANOVA, $p \geq 0.210$) or day 2 (ANOVA and K-W test, $p \geq 0.296$). The mean concentration of ammonia was 0.057 \pm 0.082 mg of nitrogen/L on day 1 and 0.068 \pm 0.047 mg of nitrogen/L on day 2. The nitrate and nitrite concentrations were below the level of detection (<0.010 mg of nitrogen/L) for all samples at all sampling

periods. The pH ranged from 6.15 to 8.56, with a mean pH of $6.95 \pm .042$ on day 1 and 7.24 ± 1.08 on day 2. The water temperature was measured at each sampling period and varied according to air temperature. Because the sampling periods were on different days among ponds, the water temperatures were not compared. The conductivity ranged from 197 $\mu\text{S}/\text{cm}$ to 477 $\mu\text{S}/\text{cm}$ throughout the course of the experiment. Two values were higher than 477 $\mu\text{S}/\text{cm}$, likely due to poor the quality of the water sample (inclusion of sediment) so they were excluded from the analysis, as they would not be representative of conductivity for those ponds. The mean specific conductivity for day 1 was $290 \pm 75 \mu\text{S}/\text{cm}$ and for day 2 was $334 \pm 81 \mu\text{S}/\text{cm}$.

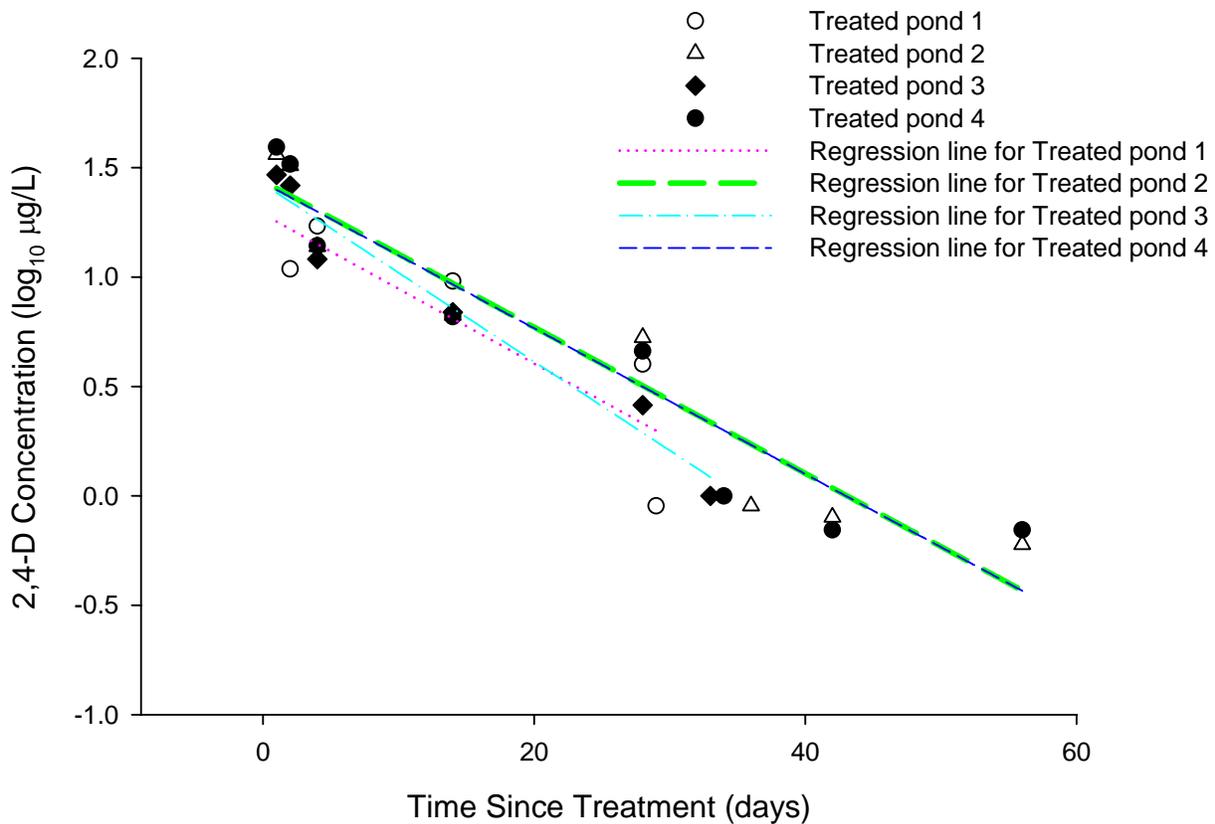


Figure 3.1: Regression analyses of 2,4-D from the treated ponds. The lines are the regression lines fitted for the data of each treatment. Each point represents one water sample per pond. (The detection limit for 2,4-D analysis was 0.5 $\mu\text{g}/\text{L}$). Linear regression analyses indicated degradation in the four treated ponds ($p \leq 0.001$ for ponds 2, 3, and 4; $p = 0.059$ for pond 1). The parallelism test (conducted only on common sampling dates) determined that the linear regression lines were parallel ($p > 0.050$).

Table 3.2: Water quality variables measured during pond selection (early summer) and metamorph sampling (late summer) in all ponds. Values are means \pm SD for each day for each experimental group.

Exp.	n	pH		Ammonia (mg of N/L) ³		Conductivity (μ S/cm)	
		C Day 1 ¹	C Day 2 ²	C Day 1 ¹	C Day 2 ²	C Day 1 ¹	C Day 2 ²
Forested	3	7.11 \pm 0.61	7.43 \pm 0.99	0.01 \pm 0.00	0.04 \pm 0.02	232 \pm 0.7	299 \pm 2.8
Agricultural	4	6.97 \pm 0.29	6.94 \pm 0.47	0.04 \pm 0.03	0.08 \pm 0.06	295 \pm 74	322 \pm 35
Treated	4	6.86 \pm 0.55	6.65 \pm 0.32	0.09 \pm 0.12	0.08 \pm 0.05	315 \pm 91	373 \pm 143

¹ Collection Day 1 -- Water samples taken during pond selection. May 12 through June 6, 2005.

² Collection Day 2 -- Water samples taken during metamorph sampling. June 30 through August 11, 2005.

³ Ammonia is measured in mg of nitrogen per liter of water.

3.2.3 Growth and Development

A total of 211 metamorphs were collected and preserved in formalin from the three experimental groups. All specimens collected were between stages 42 and 45.

There were significant differences among exposure groups for SVL and wet weight (K-W test, $p = 0.029$; and ANOVA, $p < 0.001$, respectively). The metamorphs were shorter and lighter in the forested ponds than in the agricultural and treated ponds (LSD Mann-Whitney test, $p < 0.034$; and Tukey HSD, $p \leq 0.001$, respectively) (Figure 3.2). The liver weights of the metamorphs were examined with wet weight as a covariate; there were no differences among experimental groups (ANCOVA, $p = 0.289$). Liver weight was highly dependent upon wet weight.

The condition of the metamorphs was measured by examining the percent fat and percent protein of each animal (Figure 3.3). There were no differences among experimental groups in percent fat (ANOVA, $p = 0.494$). There were differences among experimental groups in percent protein (ANOVA, $p = 0.020$). Post-hoc analyses determined that metamorphs from the forested ponds had a smaller percentage of protein than the metamorphs from the agricultural ponds (Tukey HSD, $p = 0.018$), but not from the treated ponds although that comparison approached

significance (Tukey HSD, $p = 0.061$). There was no significant difference between the agricultural and the treated ponds in percent fat and percent protein.

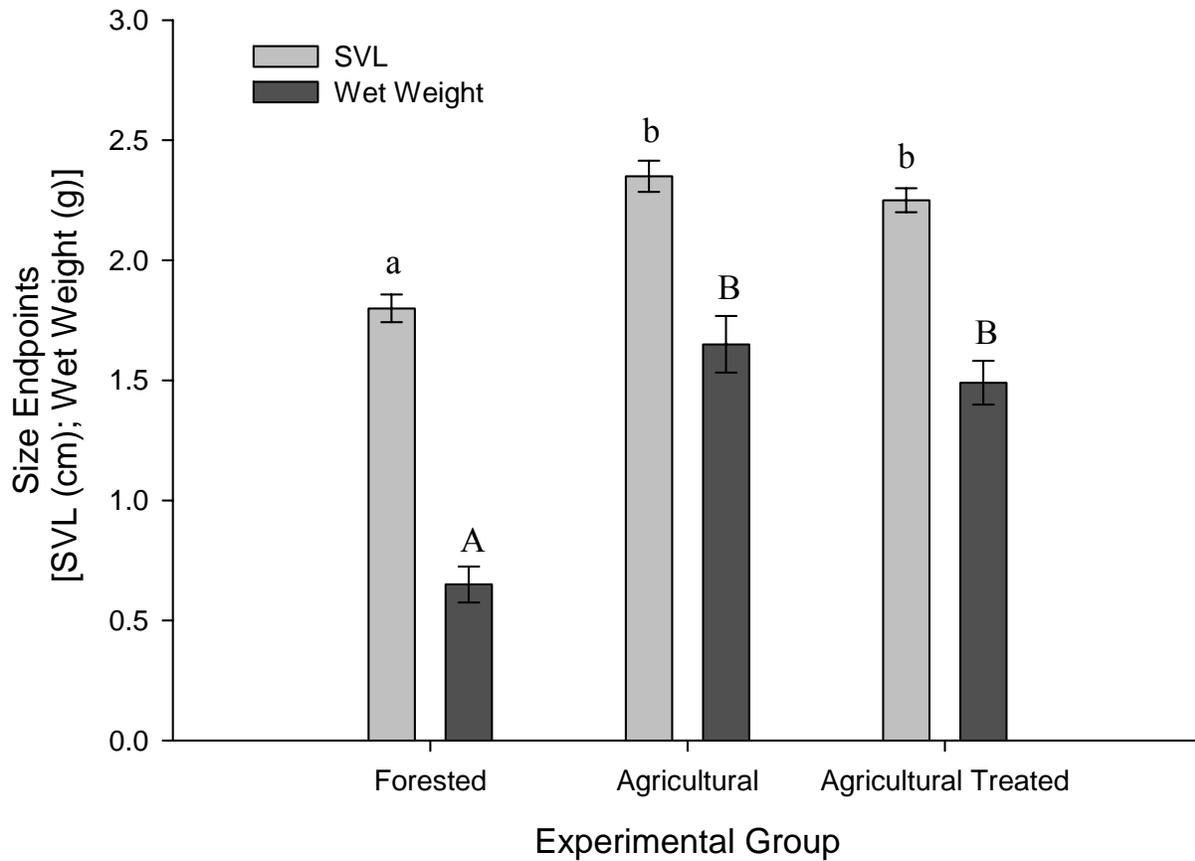


Figure 3.2: Growth and development endpoints of metamorphs measured at the time of sampling for each experimental group. Sample sizes were four agricultural, four treated and three forested ponds. Bars represent means \pm SE. Different lower case letters denote significant differences in SVL among experimental groups (K-W test, $p = 0.029$). Different upper case letters denote significant differences in wet weight among experimental groups (ANOVA, $p < 0.001$).

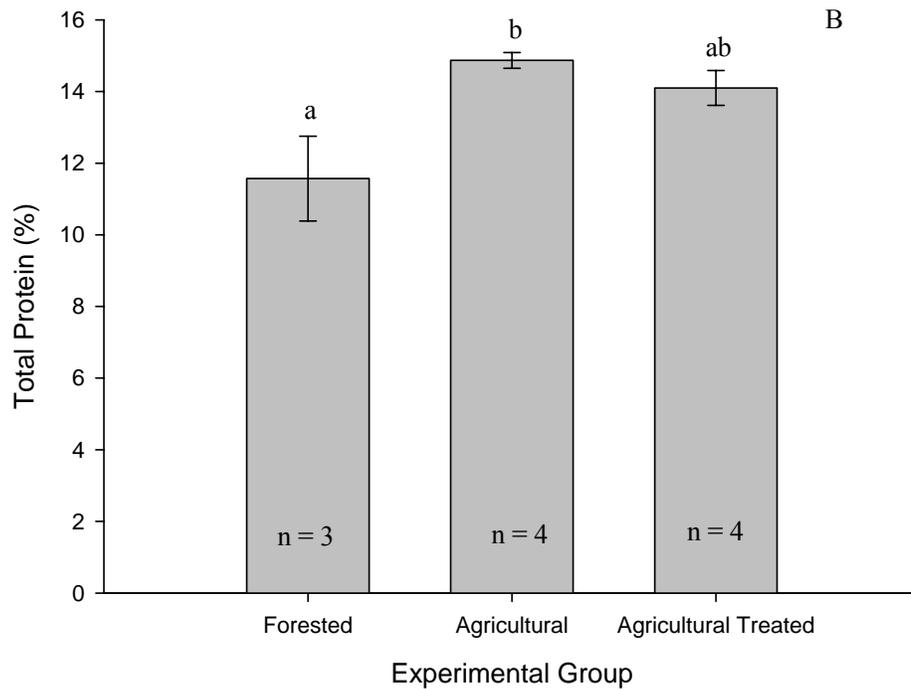
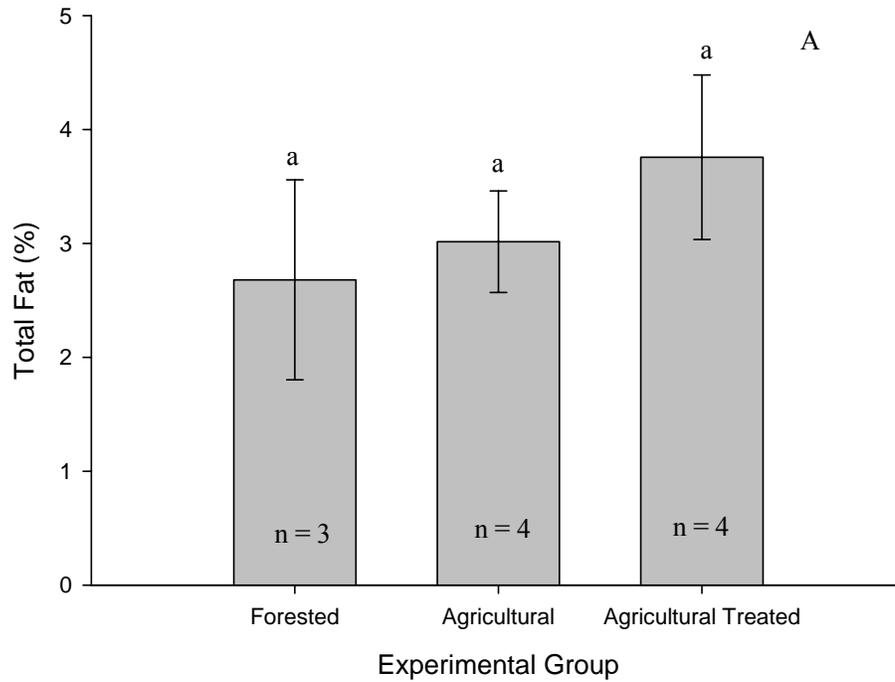


Figure 3.3: Percent fat (A) and percent protein (B) in metamorphs of the three experimental groups. Sample sizes were four agricultural, four treated and three forested ponds. Bars represent means \pm SE. Different lower case letters denote significant differences among experimental groups for each endpoint (3.3B ANOVA, $p = 0.020$).

3.2.4 Deformities

Scoliosis, an axial deformity defined as a lateral curvature of the spine was observed in a total of 14 metamorphs, apparently only affecting the tails (three from the agricultural ponds, six from the treated ponds, and five from the forested ponds). There was no difference in the incidence of axial deformities among experimental groups (χ^2 , $p = 0.594$). The overall occurrence of axial deformities in all ponds was 6.6%. The occurrences for experimental groups were 8.3% for the forested pond, 4.2% for the agricultural ponds and 7.5% for the treated ponds.

A total of five specimens had a malformation of the hind limb. Ectromelia, the absence of all or part of the limb, was observed in one metamorph collected from the agricultural ponds and in one metamorph collected from the treated ponds. Two ectromelic metamorphs were observed from the forested ponds. Ectrodactyly was observed in one metamorph collected from the forested ponds. There was no significant difference in the incidence of hind limb deformities among the treatments (χ^2 , $p = 0.285$). The overall occurrence of limb malformations in all ponds was 2.4%. The occurrences for experimental groups were 3.3% for the forested pond, 1.4% for the agricultural ponds and 1.3% for the treated ponds.

3.2.5 Plasma Corticosterone Hormone

Corticosterone values were not obtained from 13 of the 211 metamorphs collected from the three experimental groups due to lack of sufficient blood. Corticosterone concentrations ranged from 0.3 ng/mL of serum to 37.1 ng/mL of serum. The treatment means are presented in Figure 3.4. There were differences in corticosterone concentrations among experimental groups (ANOVA, $p = 0.001$). The metamorphs from the agricultural and treated ponds had about 3 times higher plasma corticosterone concentrations than those from the forested ponds (Tukey HSD, $p = 0.001$). The inter-assay coefficients of variation (CVs) for mean CORT concentrations of 68.4 or 433.1 ng/ml were 4.6% or 4.0%, respectively.

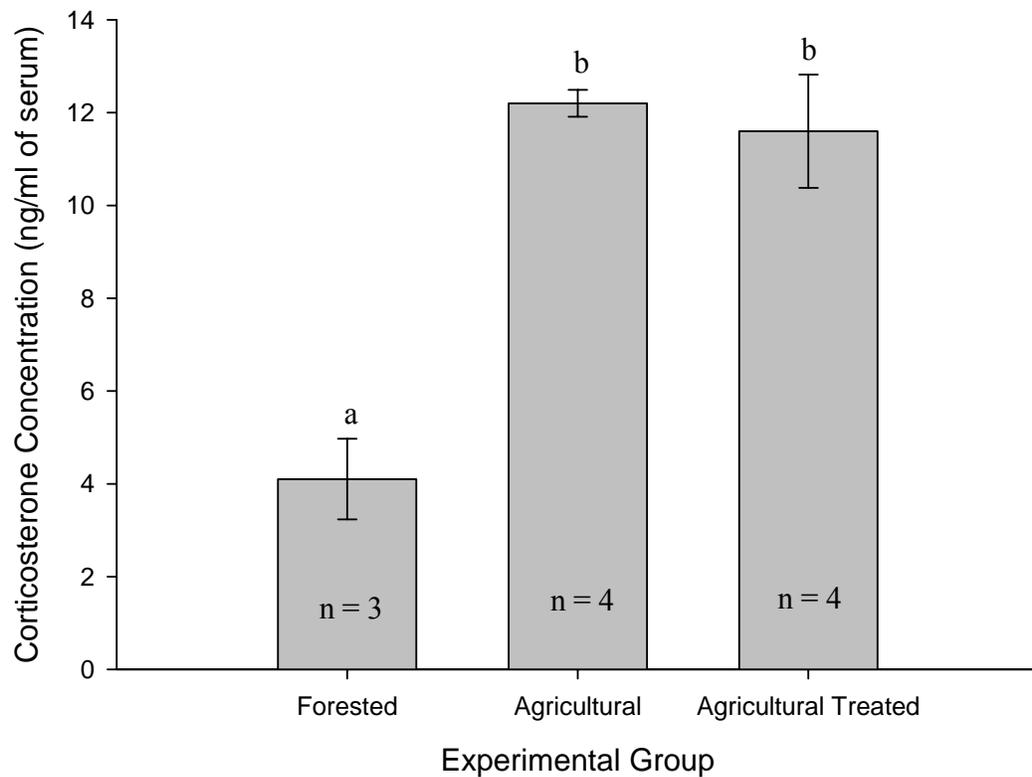


Figure 3.4: Plasma corticosterone concentration for each experimental group. The bars represent plasma CORT concentration means \pm SE, $n = 4$ except for metamorphs from the forested ponds where $n = 3$. Different letters denote significant differences among experimental groups (ANOVA, $p = 0.001$).

3.2.6 Gender Distribution

There was no significant difference in percent females among experimental groups (K-W test, $p = 0.687$). All experimental groups had more females than males. The distribution among the experimental groups was also not different from a 50% female population (K-W test, $p = 0.086$). Overall, the mean percentage of females in the ponds was $59 \pm 7\%$.

3.3 Discussion

3.3.1 2,4-D Residues

The concentrations of 2,4-D measured in the water collected from the treated ponds were higher than the mean concentrations of 0.1-0.4 $\mu\text{g/L}$ reported for Saskatchewan wetlands in other studies (Donald & Syrgiannis 1995; Donald *et al.* 2001). Treatment of the ponds succeeded in creating a worst-case scenario of 2,4-D exceeding the guideline for the protection of aquatic life

(4.0 µg/L) for at least the first 28 days of exposure in the treated ponds (CCME 2003). The tadpoles were, therefore, exposed to 2,4-D at environmentally relevant concentrations (Appendix A) for a significant portion of their aquatic life. The degradation pattern was very similar to that reported by Boyle (1980). In treated pond 1, there were aberrant concentrations measured at the first sampling dates, which may be from lack of adequate mixing prior to sampling. The parallelism test indicated similar degradation in all four treated ponds. Degradation probably would have proceeded in a similar manner across all ponds, as many of the determining factors should have been similar.

The low LOD of the NLET analyses (e.g. 0.00047 µg/L for 2,4-D) allowed for detection of background concentrations of the five major-use herbicides in the agricultural and forested ponds. There was overlap in concentrations (with standard deviations) for the forested, agricultural and treated ponds for the herbicides other than 2,4-D. Nonetheless, the forested ponds contained the lowest concentrations (Table 3.1). The concentrations in the agricultural ponds may have been sufficient to produce a source of low-level stress that was not present in the forested ponds.

3.3.2 Water Quality

2,4-D treatment had no impact on the water quality of the ponds. There were no significant differences among experimental groups. The pH, nitrate, nitrite and ammonia values were within the guidelines established by the CCME (2003). Specific conductivity in 12 Saskatchewan ponds ranged from 312 to 33,493 µS/cm (Waiser 2006). The specific conductivity values observed in the ponds of the current study were similar to the low value (freshwater) of the aforementioned study.

3.3.3 Growth and Development

There were differences among experimental groups for both SVL and wet weight with the forested pond animals being smaller than the agricultural pond and the treated pond animals. Size of metamorphs can vary geographically as reported by Riha and Berven (1991) and Berven and Gill (1983). A portion of variation can be attributed to a genetic component, but the majority of the variation is attributed to varying environmental factors of the different sites

(Berven & Gill 1983). Animals exposed to chronic stressors tend to be smaller in size, as growth and development are slowed (Hontela *et al.* 1995). Environmental factors that may act as stressors include exposure to contamination, population density, predators, pond temperature and food availability (Wilbur 1977; Riha & Berven 1991; Goater & Vandebos 1997). Boyle (1980) determined there was an increase in productivity translating into greater growth in fish in ponds exposed to 2,4-D. This effect may have been observed in the agricultural ponds associated with 2,4-D or other chemicals that were present. Low-level stimulation (hormesis) induced by exposure to 2,4-D in the agricultural and treated ponds (Table 3.1) may have occurred as suggested by the larger SVL and wet weight (Calabrese 2008). Further investigation into the effects of 2,4-D or other chemicals on aquatic habitats would elucidate the potential effect on amphibians. Due to the distance between the agricultural sites and the forested ponds, it should not be assumed that these populations were similar genotypically.

Fat stores are important for successful overwintering by the new metamorphs (Fitzpatrick 1976). No differences among exposure groups were observed; this may be due to the importance of fat stores for overwintering. In other words, although the frogs were different in aspects of growth and hormone response, diversion of energy to fat accumulation was important enough to result in similar fat stores, regardless of stress or stimulation. Analysis of body condition in frogs can be done by various methods, including whole body triglycerides, liver glycogen and plasma triglycerides (Gupta 2009; Zabelinskii *et al.* 2006). In this study, the decision to examine lipids and proteins came after sampling the metamorphs, therefore, there were limitations in the methods available. Blood samples were very small, which did not allow for additional analyses beyond corticosterone. The specimens were preserved in formalin, whereas triglycerides are examined on frozen samples (Gupta 2009). The livers were not available at the time of analysis for lipids. The analysis chosen also allowed for the examination of percent protein of the metamorphs, which may be affected by stress (exposure).

The metamorphs from the forested ponds had less protein than did the metamorphs from the agricultural ponds. Since the protein measure is a percentage accounting for size, there may be an adverse impact on the animals from the forested ponds. The lower percent protein may be an

indication of a stressful environment (Wingfield *et al.* 1997), although it is not clear what environmental stressor may be present in the forested ponds.

3.3.4 Deformities

The overall frequency of axial deformities in the 11 ponds of this study was 6.6%, which falls within the range of incidence observed by Cooke (1981). As there were no significant differences among the experimental groups, it is likely the incidence of deformities in the present study is representative of background levels. The overall occurrence of hind limb deformities in the 11 ponds of my study was 2.4%. This percentage is somewhat higher than the background frequency of less than 1% reported by Ouellet (2000) and Gardiner and Hoppe (1999), but within the baseline maximum of 5% determined by Blaustein and Johnson (2003).

3.3.5 Plasma Corticosterone Concentration

Concentrations of corticosterone in this study were lower in the forested ponds than in the agricultural and treated ponds. These values represent the metamorphs' response to acute stress (Glennemeier and Denver 2002) indicating that there may have been an impaired response to stress in the forested metamorphs or a site-specific difference in response. Other studies have reported increased basal (baseline) CORT concentrations in toads and yellow perch exposed to intermediate levels of contamination (Hopkins *et al.* 1997; Gravel *et al.* 2005; Levesque *et al.* 2003). Although baseline concentrations were not measured in this study, baseline CORT concentrations may have been chronically elevated in metamorphs of the agricultural ponds, creating the illusion of a decreased response to acute stress among the metamorphs of the forested ponds. The addition of 2,4-D to the treated ponds did not adversely affect the CORT response in these tadpoles. Determination of the CORT values for *Rana sylvatica*, both baseline and response, would add more to our understanding of the species as well as allow comparisons to evaluate the health status on the environment related to stress response.

3.4 Summary

Significant differences were observed in metamorphs between the forested ponds and the agricultural and treated ponds. Metamorphs from the forested ponds tended to be smaller both in SVL and wet weight. They also had a reduced stress response compared to both agricultural groups. There was the possibility that baseline CORT concentrations were raised by chronic low-level herbicide exposure (Hopkins *et al.* 1997; Gravel *et al.* 2005; Levesque *et al.* 2003). Along with the increase in baseline CORT concentrations, Levesque *et al.* (2003) also observed a decrease in condition of the fish exposed to intermediate levels of metal contamination. Baseline CORT concentrations may have been elevated (although not measured) in metamorphs of the agricultural and treated ponds. Unlike Levesque *et al.* (2003), metamorphs with potentially increased baseline CORT concentrations did not have decreased size. This may be associated with the differences in contamination (herbicides vs. metals). In addition, hormesis may have acted upon the metamorphs to stimulate growth. It is not clear if environmentally realistic concentrations of 2,4-D in natural ponds have adverse effects or stimulatory effects on the growth and development of wood frog tadpoles in the agricultural community. Further investigation is required to determine the effects of environmentally realistic concentrations of 2,4-D and herbicide exposure on baseline CORT concentrations and size (SVL and wet weight). Studies into the potential geographic differences, both in the amphibian populations (i.e. genetics) as well as environmental differences (i.e. temperature, population density, food availability) should also be conducted, as these factors may also play a role in the CORT concentrations and the size of metamorphs.

Chapter 4

General Discussion

4.0 Discussion

The objective of my research project was to examine the sublethal effects of the chemical 2,4-dichlorophenoxyacetic acid (2,4-D) exposure on native Saskatchewan wood frog tadpoles from hatching to metamorphosis as a potential contributing factor in the decline of amphibian populations and the increase in amphibian deformities. This was achieved through two experiments. Firstly, tadpoles were exposed to low level, environmentally relevant concentrations of 2,4-D for the duration of their aquatic life in microcosms. Secondly, metamorphs were sampled from three experimental groups; potentially unexposed (forested ponds), potentially exposed (agricultural ponds) and treated ponds (agricultural ponds fortified with 2,4-D) throughout Saskatchewan. The tadpoles in the treated ponds were exposed to low levels of 2,4-D (target concentration 10 ug/L). These experiments provided useful information regarding the toxicity of 2,4-D on non-target species in the aquatic environment while illuminating issues for future research. To get a clear (yet simplistic) picture of the results, a comparison of the microcosms to the field ponds will be discussed.

2,4-D residues degraded throughout the duration of the experiment in the microcosms at rates inversely proportional to the treatment concentration. Despite degradation, 2,4-D residues were present in all treatments until the end of the experiment. This indicates that the tadpoles were exposed to 2,4-D throughout their aquatic life. Although, it is not clear why there was a difference in degradation rates in the microcosms, it could be an effect of the 2,4-D on the metabolism of the aquatic plants. The degradation of 2,4-D that was observed in the four treated ponds is in agreement with findings reported by Boyle (1980). Measured water quality variables were different in the microcosms than in the field (Table 4.1), although all variables fall within the water quality guidelines established by CCME (2003). Conductivity and pH were somewhat higher in the microcosms, also less variability was evident. This is important because particular water quality variables can influence the degradation of chemicals or create fluctuations in the toxicity (Birmingham and Colman 1985; Ho *et al.* 1999).

As mentioned in Section 2.2.3, the mortality in the microcosm study was significant and therefore impacted the results of that experiment. Comparisons between the microcosm and the field studies should be done with caution since the survival rate in the microcosm study was very low and could bias the results. The measurements of SVL and wet weight were interesting. For both variables, the measurements of animals from the microcosms tended to be more similar to those of the metamorphs from the forested ponds (Table 4.1). Corticosterone concentrations appeared to follow the same trend. Factors contributing to these similarities were not clear. The tadpoles used in the microcosm study were collected as eggs from a pond located in the forested area (northern Saskatchewan), which could indicate a site-specific difference in the populations from the forested ponds (including the tadpoles used in the microcosm study) compared to the agricultural ponds. These site-specific differences may include population genotype differences. As mentioned earlier, a similar microcosm study conducted with tadpoles from a forested pond and reared on the University campus resulted in metamorphs that were larger than those reported in the current microcosm study or the forested area (Serben 2003). This observation supported the conclusion of Berven and Gill (1983) that the influence of the environment contributed more to the differences than genotype. Since the microcosms were located in central Saskatchewan (exposed to similar geographic influences as the agricultural ponds) other environmental influences would have to be present to explain the different results. The influences may be a condition of the location (i.e. food availability or predator presence). The microcosms should have been similar to the agricultural ponds with respect to temperature and food availability (assuming food was not limited in the agricultural ponds demonstrated by the increased growth). There were no predators introduced into the microcosms. Low-level exposure to herbicide contaminants may also be present. The observed increase in metamorph size from the agricultural and treated ponds may be low level stimulation from exposure to 2,4-D and/ or other major-use pesticides (Table 3.1) (Calabrese 2008). While the increase in CORT concentration in the metamorphs from the agricultural and treated ponds may be associated with an increase in baseline concentrations following chronic low level exposure to contaminants (Hopkins et al. 1997; Gravel et al. 2005; Levesque et al. 2003). Genotype may have contributed to the similarity in measured size between microcosms and forested ponds, but it is not clear the role genotype

played in plasma CORT response to acute stress or in baseline levels of CORT in *Rana sylvatica*. Further research into the CORT values, both baseline and acute responses are needed.

Another possible explanation for the differences between the microcosm study and the field study was that there were unrelated stressors impacting both populations (microcosms and forested ponds) in a similar manner. If this was the case, it would indicate that the values obtained in both the microcosm study and the forested ponds represent size and hormone endpoints of chronically stressed animals (Norris *et al.* 1999; Hontela *et al.* 1995; Hontela *et al.* 1997; Gendron *et al.* 1997) perhaps exacerbated by decreased genetic variability (Lebarreres *et al.* 2007). Therefore, it would indicate that the tadpoles in the microcosms (including the control treatment) were exposed to an outside stressor, which may have been the cause of the pre-treatment mortality (virus or latex-exposed water). When comparing the results obtained from the microcosm study, where the tadpoles were exposed to 2,4-D, and the forested ponds of the field study, the similar results create doubt in using metamorphs of the forested ponds as unexposed (reference) animals for future studies using *R. sylvatica* tadpoles. The potential stressor(s) in the forested ponds were not clear. Temperature, population density, predator presence and food availability were all factors that may influence the development of frogs, but they were also factors that, when outside the optimal range, may be perceived as stressors. These factors were not monitored or observed closely in the field during this study, although it should be noted that leeches were observed in the forested ponds, but not examined further than general observation. Berven and Boltz (2001) reported decreased growth and survival in *R. sylvatica* exposed to leech parasites. Further research is needed to investigate the environmental factors that wood frog tadpoles may perceive as stress.

Deformities present in the microcosms versus field study were also compared. There were no differences among the experimental groups in the field, but there appeared to be an interesting trend. The occurrence of axial deformities was higher in the field than in the microcosms, although the values fall below the background frequency of 3 - 8% determined by Cooke (1981). The metamorphs of the forested ponds experienced a frequency of axial deformities on the upper limit of the background level, but this may be exaggerated by the small sample of metamorphs collected in the field compared to the agricultural ponds or the microcosms. The occurrence of

deformities in the microcosms may have been underestimated due to the pretreatment mortality. Animals predisposed to deformities may have died prior to sampling. The latter explanation is supported by the occurrence rate of hind limb deformities. The occurrence of deformities in the forested pond metamorphs was on the upper limit of the background level, perhaps indicating an underestimation of deformities in the microcosm metamorphs. Although there was no statistical difference in the occurrence of deformities between the field and microcosm studies, the trend of higher incidence in the forested ponds may be noteworthy. It could indicate the potential stressor that caused the decrease in size and acute stress response may also cause deformities. Other potential causes for deformities have been investigated including increases in retinoic acid, radiation by UV-B and exposure to parasitic trematodes (Ankley *et al.* 2004; Sessions & Ruth 1990). The parasitic trematodes should be highlighted as snails play an important role in the parasite life cycle and snails were observed in several ponds in the present study, although this was just a general observation. Nonetheless, it would be useful to investigate the possibilities of external stressors causing both deformities and chronic stress effects or perhaps the role that chronic stress plays in the development of deformities.

4.1 Summary

Exposure to 2,4-D may have detrimental effects on wood frog tadpoles found in ponds located in forested and agricultural areas of Saskatchewan. In the current study, the analyses of the results of the microcosm study demonstrated that tadpoles exposed to consistent low levels of 2,4-D throughout their entire aquatic stage were not biologically significantly different from control tadpoles. In the field study, the concentrations of 2,4-D decreased over time in the treated agricultural ponds. Nonetheless, the exposure time was representative of potential exposure under environmentally realistic field conditions. Analyses of the results demonstrated that the metamorphs reared in ponds located in the agricultural region of Saskatchewan appeared to be “healthier”, regardless of intentional 2,4-D exposure compared to the metamorphs from unexposed forested ponds. That is, they were larger than those from the forested ponds in SVL and wet weight, as well as percent protein. The results from the metamorphs sampled from the microcosms appeared to be more similar to the metamorphs of the forested ponds. There are many knowledge gaps in our understanding of *R. sylvatica* and the endpoints examined. Further

research is necessary to help interpret these results and expand the knowledge base for these endpoints.

In the current study, several variables or factors need to be considered to replicate the research or in this area in the future. The use of latex or nitrile gloves should be removed from research with amphibians, especially tadpoles. Blood sampling time needs to be included both for time of day and stage effects. Blood sampling required substantial time with each specimen therefore it was difficult to narrow the time span, if several specimens need to be sampled in one day. Nonetheless, possible effects of circadian rhythms and variability in CORT concentrations throughout metamorphosis need to be examined further with the species *R. sylvatica*. In the microcosm study, potentially sensitive development stages (egg and embryo) may not have been exposed to 2,4-D. Adding egg sacs to treated water would allow the specimens to be exposed throughout all aquatic development stages, and perhaps the results would provide a different profile of toxicity. To eliminate some of the possible geographic factors, eggs of wood frogs from forested ponds could be transplanted to agricultural ponds and vice versa. It would be important to investigate the impact of these factors on the measured size, CORT and deformities. An examination of fat and protein levels in *R. sylvatica* throughout metamorphosis could also provide some clarity as to what is considered normal, the impact of metamorphosis and additional stressors, as well as the requirements for survival and fitness in the terrestrial environment. Although 2,4-D exposure may not be harmful to amphibians in the aquatic environment at these low concentrations, potential interactions with other chemicals should also be considered. As the population of amphibians continue to decline with increasing evidence of deformities, the improved understanding of the biology of the frog species and the factors affecting development will become more relevant.

Table 4.1: Summary table of water quality variables and metamorph endpoints for microcosms and field ponds. Note that agricultural and treated ponds were pooled, as there were no statistical differences between the groups. Variables not common to both experiments were excluded from the table. The water quality values for the microcosms represent the means \pm SE across all treatments. The water quality values for the field ponds represent the mean \pm SD for each sampling day. The metamorph endpoints represent the range of the means \pm SD for the experiment or the experimental group, except for the deformities where the percent deformities is the occurrence of deformities in all animals sampled from that group. In the microcosms, n = 5 per treatment except in the control treatment where n = 4. In the field ponds, the agricultural/ treated ponds n = 8 (n = 4 for each experimental group) and the forested ponds where n = 3.

Variables/ Endpoints	Microcosm		Field			
			Forested		Agricultural/ Treated	
			C Day 1 ²	C Day 2 ³	C Day 1 ²	C Day 2 ³
Water Quality						
pH	8.19 \pm 0.02		7.11 \pm 0.61	7.43 \pm 0.99	6.91 \pm 0.41	7.17 \pm 1.17
ammonia (mg of N/L) ¹	0.02 \pm 0.001		0.01 \pm 0.00	0.04 \pm 0.02	0.07 \pm 0.09	0.08 \pm 0.05
conductivity (μ S/cm)	471 \pm 1.2		232 \pm 0.7	299 \pm 2.8	305 \pm 77.5	344 \pm 90.9
Metamorphs	125 (all treatments)		60 forested		151 (71 agricultural + 80 treated)	
Total Length (cm)	2.90 \pm 0.26 – 3.90 \pm 0.37		1.80 \pm 0.10 – 2.60 \pm 0.48		2.70 \pm 0.70 – 3.50 \pm 0.86	
SVL (cm)	1.70 \pm 0.05 – 1.90 \pm 0.12		1.70 \pm 0.11 – 1.90 \pm 0.11		2.20 \pm 0.09 – 2.50 \pm 0.09	
Wet Weight (g)	0.70 \pm 0.07 – 1.00 \pm 0.12		0.55 \pm 0.09 – 0.80 \pm 0.11		1.33 \pm 0.14 – 1.91 \pm 0.27	
Deformities						
-- axial(%)	2.4		8.3		6.0	
-- hind limb(%)	2.4		5.0		1.3	
CORT (ng/mL) ⁴	2.90 \pm 0.63 – 5.5 \pm 1.04		2.40 \pm 2.70 – 5.1 \pm 4.42		8.90 \pm 2.78 – 14.8 \pm 9.0	

¹ Ammonia is measured in mg of nitrogen per liter of water sample.

² Collection Day 1 - Water samples taken during pond selection.

³ Collection Day 2 - Water samples taken during metamorph sampling.

⁴ CORT refers to the plasma corticosterone concentrations sampled from the metamorphs

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APPENDIX A
JUSTIFICATION FOR HIGH CONCENTRATION IN MICROCOSMS
(CALCULATION OF POTENTIAL 2,4-D TRANSFERRED TO PONDS VIA RUNOFF)
(as per Serben 2003)

Registered rates of application of 2,4-D amine (2004 Guide to Crop Protection Saskatchewan):

1. Wheat, barley, spring rye: 0.45 L/acre @ 500 g/L for susceptible weeds.
2. Wheat, barley, spring rye: 0.74L/acre @ 500 g/L for harder to kill weeds.

Using the higher application rate:

$$\begin{aligned} 0.74 \text{ L/acre} &= 0.74 \text{ litre} / 0.4047 \text{ ha} = 1.1 \text{ litres/ha} = 1.8285 \times 500 \text{ g/ha} \\ &= 914.26 \text{ g a.i. per ha} \end{aligned}$$

Quantity of 2,4-D lost to runoff – Assumptions (Forsyth *et al.* 1997):

- Quantity of herbicide applied to the field lost to runoff = 0.5% (Wauchope 1978)
- Rainfall required to cause runoff = 25mm (Woo and Rowsell 1993)
- Amount of rainfall that is runoff from cropland = 12%. The amount of runoff from an area of cropland is calculated by multiplying the amount of rainfall by the area of cropland by 12%.
- Arbitrary rainfall amount = 30mm
- Conservative ratio of cropland to pond = 10:1 (i.e. 4 ha of cropland draining into a 0.4 ha pond)
- Water depth on pond = 15 cm (according to Freemark and Boutin (1994) criteria for Estimated Environmental Concentrations)
- Relatively large “Worst-Case Scenario” ratio of cropland to pond = 100:1 (i.e. 57 ha draining into a 0.5 ha pond) as described by Hall (1968).

Quantity lost in runoff from possible ratios of cropland to pond area (Forsyth *et al.* 1997):

- a) Conservative ratio of 10:1, then the volume of runoff entering the pond would be:

$$\text{Volume of rainfall} = 0.03 \text{ m} \times [(4 \text{ ha}) \times (10,000 \text{ m}^2/\text{ha})] = 1200 \text{ m}^3$$

$$\text{Volume of runoff} = 12\% \text{ of } 1200 \text{ m}^3 = 144 \text{ m}^3$$

The amount of 2,4-D in the runoff would be:

$$2,4\text{-D applied to cropland} = (914.26 \text{ g/ha}) \times (4 \text{ ha}) = 3,657.0 \text{ g}$$

$$2,4\text{-D lost in runoff} = 0.5\% \text{ of } 3,657.0 \text{ g} = 18.285 \text{ g}$$

Assuming a water depth of only 15 cm, the 2,4-D concentration would be:

$$\text{Pond volume} = [(0.4 \text{ ha}) \times (10,000 \text{ m}^2/\text{ha})] \times 0.15 \text{ m} = 600 \text{ m}^3$$

$$\text{Pond volume with runoff} = 600 \text{ m}^3 + 144 \text{ m}^3 = 744 \text{ m}^3$$

$$\begin{aligned} \text{Concentration of 2,4-D in pond} &= 18.285 \text{ g} / [744 \text{ m}^3 \times 1000 \text{ L/m}^3] \\ &= 0.0000245 \text{ g/L} = \mathbf{24.5 \mu\text{g/L}}. \end{aligned}$$

b) Large area ratio of 100:1, the volume of runoff entering the ponds would be:

$$\text{Volume of rainfall} = 0.03 \text{ m} \times [(57 \text{ ha}) \times (10,000 \text{ m}^2/\text{ha})] = 17,100 \text{ m}^3$$

$$\text{Volume of runoff} = 12\% \text{ of } 17,100 \text{ m}^3 = 2052 \text{ m}^3$$

The amount of 2,4-D in the runoff would be:

$$2,4\text{-D applied to cropland} = (914.26 \text{ g/ha}) \times (57 \text{ ha}) = 52,112.8 \text{ g}$$

$$2,4\text{-D lost in runoff} = 0.5\% \text{ of } 52,112.8 \text{ g} = 260.56 \text{ g}$$

Assuming a water depth of only 15 cm, the 2,4-D concentration would be:

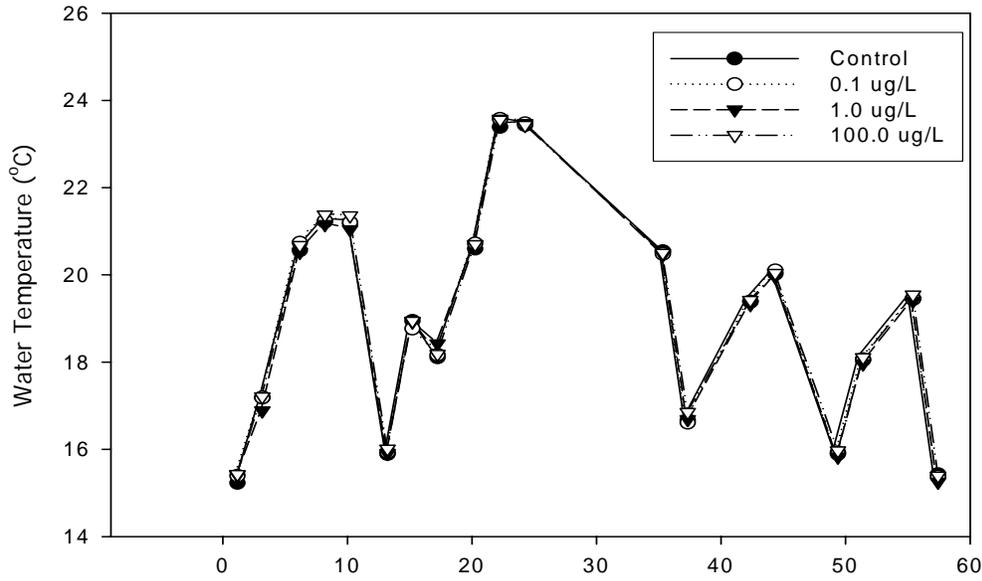
$$\text{Pond volume} = [(0.5 \text{ ha}) \times (10,000 \text{ m}^2/\text{ha})] \times 0.15 \text{ m} = 750 \text{ m}^3$$

$$\text{Pond volume with runoff} = 750 \text{ m}^3 + 2052 \text{ m}^3 = 2802 \text{ m}^3$$

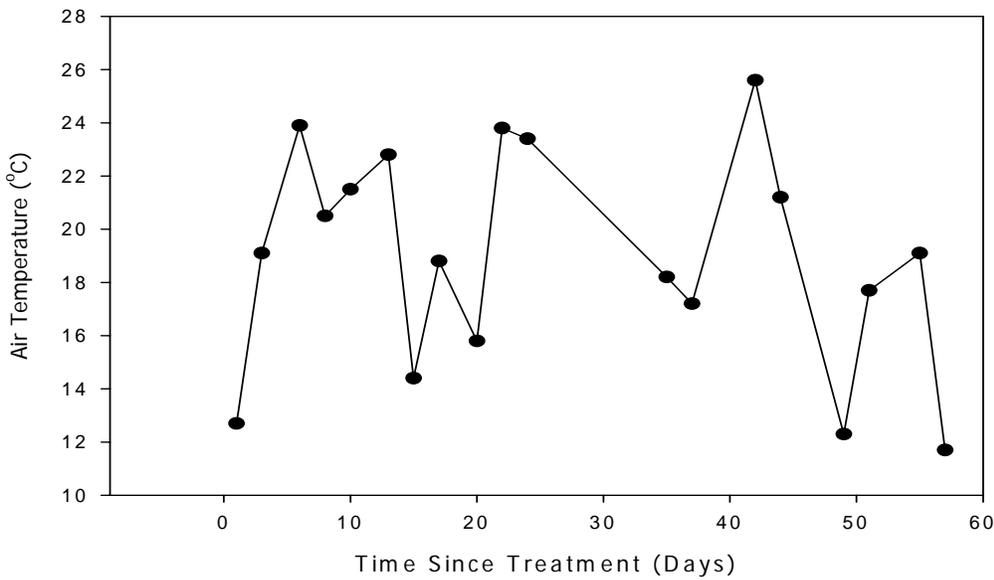
$$\begin{aligned} \text{Concentration of 2,4-D in pond} &= 260.56 \text{ g} / [2802 \text{ m}^3 \times 1000 \text{ L/m}^3] \\ &= 0.0000929 \text{ g/L} = \mathbf{92.9 \mu\text{g/L}}. \end{aligned}$$

Thus, the highest concentration that was added to the microcosms (**100 $\mu\text{g/L}$**) is close to the estimated amount that could enter ponds in runoff under worst-case scenario conditions. However, the lower concentration of 24.5 mg/L is more realistic. But for logistical purposes, the worst-case scenario concentration was chosen as well as two low concentrations (**0.1 and 1.0 $\mu\text{g/L}$**) that bracket concentrations measured in Saskatchewan ponds (Donald & Syrgiannis 1995; Waite *et al.* 1992).

APPENDIX B
GRAPHS OF WATER TEMPERATURE AND AIR TEMPERATURE DURING
MICROCOSM EXPERIMENT



A



B

Figure B.1: A) Water temperature in microcosms at a depth of 50cm. Each data point represents the mean of 5 microcosms for each treatment. B) Air temperature measured above the microcosms at time of sampling.

APPENDIX C

LOCATION OF PONDS SELECTED FOR THE FIELD STUDY

Table C.1. Location, size, and general water quality characteristics of the ponds sampled in the field study (2005). Data represented were collected during pond selection in May and June, 2005, except one forested pond¹.

Pond	Experimental Group	GPS ¹ Coordinates	Size ²	pH	Water Temp ³ (°C)	Conductivity (µS/cm)
1	Forested	54°11'06.0 105°57'48.6	70 x 40 x 0.5	7.54	15.7	232
2	Forested	54°55'29.3 105°34'57.6	20 x 20 x 1	6.68	12.8	231
3	Forested ⁴	52°13'22.5 101°42'15.6	N/A ⁵	8.56	21.1	N/A ⁵
4	Agricultural	52°16'35.5 106°03'14.7	114 x 50 x 1.5	7.01	13.6	238
5	Agricultural	52°32'27.9 105°37'36.1	90 x 15 x 0.6	6.66	13.1	402
6	Agricultural	52°05'16.5 106°04.31.7	100 x 90 x 1	7.35	9.1	279
7	Agricultural	52°26'56.7 105°06'09.0	70 x 45 x 1	6.85	13.8	259
8	Treated 1	55°53'46.7 106°14'26.0	70 x 20 x 1.5	7.48	11.8	310
9	Treated 2	52°06'54.3 106°04'37.1	100 x 40 x 1.5	6.95	12.7	400
10	Treated 3	52°01'39.1 105°55'14.8	45 x 20 x 0.6	6.84	13.2	309
11	Treated 4	52°40'55.3 105°28'11.9	90 x 45 x 0.6	6.15	9.4	197

¹ Global Positioning System

² Approximate size of the pond in metres as Length (longest point) x Width (widest point) x Depth (at deepest point) at time of pond selection

³ Water temperature measured approximately 25 cm below the surface at time of pond selection

⁴ Forested pond measurements only taken at time of metamorph sampling

⁵ Measurements were not taken.

APPENDIX D

APPLICATION RATES OF 2,4-D TO SELECTED AGRICULTURAL PONDS

Ponds selected to be treated with 2,4-D were based on size, access and permission of the landowners. Pond volume was determined using the method described by Kehmeier (2005) for irregular-shaped ponds. The volume of stock solution required was determined using the following equation:

$$C_1V_1 = C_2V_2 \quad \text{(Equation 2).}$$

Where C1 and C2 are the concentrations of the pond and the stock solution, respectively and V1 and V2 are the volumes of the pond and the stock solution, respectively.

The concentration of the stock solution was 560 g/L. The target concentration for each treated pond was **10 µg/L**. This concentration was chosen as it was in the same order of magnitude as the more realistic runoff calculation (**24.5 µg/L**) (Appendix A), thereby complementing the series of treatments from the microcosm study.

The estimated volumes of the treated ponds ranged from 142,630 to 2,331,710 L.