ACUTE AND CHRONIC EFFECTS OF LINDANE ON FROG TADPOLES NATIVE TO SASKATCHEWAN

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ABSTRACT

The purpose of this research was to determine if exposure to the insecticide, lindane, during the aquatic stage of the amphibian life cycle had any deleterious effects on the normal development of native frog species. Acute high-level exposure produced effects on swimming behavior and malformations in wood frogs (*Rana sylvatica*) and boreal chorus frogs (*Pseudacris maculata*) that were similar to those caused by other organochlorine insecticides. Overall, these sublethal responses were most often quantified in hatchlings whereas mortality was most common in tadpoles. Wood frogs appeared to be more sensitive than boreal chorus frogs. For example, the 48-hour EC50s (median effective concentrations based on erratic swimming as the end point) were lower in wood frogs (1.82 and 2.13 mg/L) than in chorus frogs (3.37 and 4.04 mg/L) and significant mortality was only observed in the wood frogs. The lowest LC50 obtained was 1.90 mg/L at 72-h exposure with wood frog tadpoles. An outdoor microcosm study assessed the effect of chronic exposure to low concentrations of lindane on the survival, growth, and development of wood frog tadpoles. Groups of 40 tadpoles were exposed to three concentrations of lindane (0.1, 1.0, and 10 µg/L) and two control groups (negative and solvent) from 9-10 days post-hatch to metamorphic climax (MC). Survival was not affected ($p = 0.13$), and there were no significant treatment effects on time to MC ($p = 0.88$), fluctuating asymmetry ($p > 0.42$), or hepatic glycogen concentrations ($p = 0.06$). However, a possible hormetic effect of lindane was observed, as body condition of the metamorphs was highest at the lowest lindane concentration (0.1 µg/L), and decreased with increasing lindane concentration compared to control levels. Exposure to 0.1 µg/L lindane also affected sex ratios, resulting in 71% males in this treatment ($p < 0.005$). This effect was not dose-dependent, but, along with the alterations in corticosterone and thyroid hormones, and the possible hormetic effect, indicated a potential disruption of the endocrine system. For example, mean T4 (tetraiodothyronine) to T3 (triiodothyronine) ratio was 43% higher than in the 0.1 µg/L

treatment compared to the control group. Further investigation is necessary in order to confirm these results and to determine a mechanism of action.

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LIST OF ABBREVIATIONS

AGS	Amyloglucosidase
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
BAF	Bioaccumulation Factor
BCF	Bioconcentration Factor
BHC	Benzene Hexachloride
CAEAL	Canadian Association for Environmental Analytical Laboratories
CL	Copora Lutea
CNS	Central Nervous System
CORT	Corticosterone
CPM	Cycles Per Minute
CV	Coefficient of Variation
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
EC50	Median Effective Concentration
ECD	Electron Capture Detectors
EPA	Environmental Protection Agency
FA	Fluctuating Asymmetry
FV3	Frog Virus 3
GABA	Gamma-aminobutyric Acid
GC	Gas Chromatography
GPC	Gel Permeation Chromatography

GPS	Global Positioning System
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
L	Left
LC50	Median Lethal Concentration
LH	Luteinizing Hormone
LOEC	Lowest Observed Effect Concentration
LOEL	Lowest Observed Effect Level
MC	Metamorphic Climax
ME	Measurement Error
MSD	Mass Spectrometry Detection
NLET	National Laboratory for Environmental Testing
NO_2	Nitrite
NO_3	Nitrate
NOEL	No Observed Effect Level
NTU	Nephelometric Turbidity Units
NWA	National Wildlife Area
OC	Organochlorine
PFRA	Prairie Farm Rehabilitation Administration
PFU	Plaque Forming Units
PMRA	Pest Management Regulatory Agency
PNWA	Prairie National Wildlife Area
POC	Particulate Organic Carbon
R	Right
RED	Reregistration Eligibility Decision
RIA	Radioimmunoassay
RM ANOVA	Repeated Measures Analysis of Variance
SD	Standard Deviation
SE	Standard Error

SK	Saskatchewan
SVL	Snout-vent Length
TH	Thyroid Hormones
T4	Tetraiodothyronine
T3	Triiodothyronine

CHAPTER 1

GENERAL INTRODUCTION

1.1 Rationale for Studying Lindane

Lindane is an organochlorine insecticide still used in many countries around the world. As with other organochlorine chemicals, it is persistent, can accumulate in fatty tissues, and is a suspected endocrine disrupter (Kamrin 1997; Willett *et al.* 1998; U.S. EPA 2001). Annual global use of lindane was 5900 tonnes in 1980 and 4000 tonnes in 1990 (Li *et al.* 1996). When the γ-HCH component of technical hexachlorocyclohexane (HCH) is included, the annual global use of lindane was 11,900 tonnes in 1980 and 8,400 tonnes in 1990 (Li *et al.* 1996). Its cumulative global usage up to 1993 was 720,000 metric tonnes (Voldner and Li 1995). Lindane can contaminate areas distant from the original source of input to the environment because it is volatile and has a long half-life in the atmosphere (Brubaker and Hites 1998; Cortes *et al.* 1998; Willett *et al.* 1998; Walker *et al.* 1999). Lindane has been found in various environmental media throughout the world, including snow of the Canadian Rockies (Blais *et al.* 1998), and arctic air and seawater (Bidleman *et al.* 1995). While these media are sinks now, they are a potential source of continued lindane contamination in the environment (Bidleman *et al.* 1995; Blais *et al.* 1998). Lindane is also present in human breast milk and adipose tissue (WHO 1991), particularly in areas of heavy hexachlorocyclohexane use, such as India (Tanabe *et al.* 1990). Environmentalists have raised concerns over high levels of organochlorine insecticides, including lindane, in the native people of the arctic, and have pushed for the banning of all lindane products.

The ephemeral ponds of the Canadian prairies are highly likely to receive pesticide contamination from adjacent agricultural areas. Next to wheat, canola was the second

most important crop grown in the Prairie Provinces, the greatest amount being grown in Saskatchewan (Waite *et al.* 2001). Statistics Canada estimated the amount of land area planted to canola in Canada in 1998 at just over 5.4 million hectares (Waite *et al.* 2001). Most canola seed was treated before planting with lindane for control of flea beetles. The lindane content of the three seed treatment formulations that was registered in Canada for canola varied from 533 g/L to 680 g/L, and the amount of lindane added per kilogram of seed ranged from 12.0 to 15.6 g (Ali *et al.* 2001). The recommended seeding rates were 5-9 kg/ha (Thomas 1994); if canola were seeded at a rate of about 8 kg/ha, then the rate of lindane application was about 96-125 g/ha, depending upon the formulation used.

1.2 Characteristics of Lindane

Lindane (CAS Registry Number 58-89-9) is the gamma isomer of the compound, 1,2,3,4,5,6-hexachlorocyclohexane (HCH), and has the highest insecticidal activity of all the isomers. The seven other isomers consist of alpha (both enantiomers), beta, delta, epsilon, eta, and theta (Willett *et al.* 1998). Technical HCH (CAS Registry Number 608-73-1) is a mixture of isomers that typically includes 60-70% alpha, 5-12% beta, 10-15% gamma, 6-10% delta, and 3-4% epsilon. Technical HCH is produced by the photochemical chlorination of benzene, and is further refined to produce 99% lindane. It was first synthesized in 1825 by Michael Faraday, but its pesticidal properties were not identified until 1942 (Woolley 1995). It has been produced commercially since 1945 (Bidleman *et al.* 1995). Benzene hexachloride (BHC) is a common name for technical HCH or lindane, but is a misnomer, because HCH has a saturated, not an aromatic, ring (Woolley 1995). It also should not be confused with hexachlorobenzene (HCB), which is a fungicide. Chemical and physical properties of lindane are reported in Table 1.1.

Table 1.1. The chemical and physical properties of lindane.

Chemical Formula	$C_6H_6Cl_6$
Molecular Weight	290.85 [1]
Melting Point (°C)	112.9 [2]
Vapor Pressure at 20°C (mPa)	5.6 [1]
Solubility in Water at 25°C (mg/L)	6.8 to 7.8 [2]
Log Octanol/Water Partition Coefficient	3.72 [2]

References: [1] Kamrin 1997; [2] Saleh *et al.* 1982

1.2.1 Uses

Lindane is one of the few organochlorine insecticides to remain in widespread use in both industrialized and developing countries. Common uses of lindane include seed and wood treatments, and insecticidal sprays for a variety of food crops (ATSDR 1994). Until recently, lindane was registered in Canada as a seed treatment on wheat, barley, oats, rye, flax, corn, beans, soybeans, and peas for the control of wireworm, and on canola, mustard, cabbage, broccoli, Brussels sprouts, cauliflower and rutabaga for the control of flea beetles (PMRA 2002). In 1987, 95% of the lindane purchased in Canada was used in the three prairie provinces, mainly on canola (Crosley *et al.* 1998). In 1999, lindane was voluntarily removed as a seed treatment for canola, because the insecticide was not registered for use on canola in the United States. Lindane was only registered for use in the United States as a seed treatment on barley, corn, oats, rye, sorghum, and wheat. All use and sale of lindane-treated canola seed in Canada was to cease July 1, 2001, and its registration for use on other crops was to be reviewed by the Pest Management Regulatory Agency (PMRA). Replacement pesticides for the protection of canola from flea beetles include imidacloprid (Gaucho 75ST) and thiamethoxam (Helix). In April 2002, the PMRA announced that all uses of lindane that are subject to the *Pest Control Products Act* were to be phased out over the next two years (PMRA 2002). All use of lindane-treated seed is to be discontinued by December 31, 2004. In contrast, the United States' Environmental Protection Agency (EPA) has published a Reregistration Eligibility Decision (RED) document, in which they suggest that the reregistration of lindane seed treatments may be allowed pending the submission of more data such as acute toxicity studies (U.S. EPA 2002). Therefore, the future use of lindane in North America seems unclear. The European Commission ruled in December 2000 that its 15 European Union companies must remove lindane-containing plant protection products from the market within 18 months (Weinhold 2001).

Lindane is also in medicated lotions and shampoos for human and veterinary control of lice and scabies (ATSDR 1994; Woolley 1995). Many regulatory agencies have moved to ban lindane from lice shampoos, citing reported cases of neurotoxicity

and deaths in children relating to its use (Weinhold 2001). For example, the state of California has only recently banned lindane-based products used to kill lice and scabies (Weinhold 2001). Unfortunately, many of these incidences are the result of improper overuse of the shampoos (Rasmussen 1981), and some physicians consider the benefit of lindane to outweigh its risk (Shacter 1981).

1.2.2 Mechanism of Toxic Action

Lindane has the same mode of action as the chlorinated cyclodienes, although it is structurally different (Saunders *et al.* 1994). It binds to the picrotoxin site on the γ-aminobutyric acid (GABA) receptor, resulting in an inhibition of GABA-dependent chloride flux into the neuron. However, lindane does not fit into the active site as well as do the cyclodienes, and thus is a less potent inhibitor (Saunders *et al.* 1994). Lindane may also inhibit the sodium-potassium ATPase and the calcium-magnesium ATPase.

1.2.3 Levels in the Environment

Since lindane is only registered as a seed treatment in North America, it would mainly enter ponds via runoff from treated fields or by atmospheric deposition (Donald *et al.* 1999). A recent EPA risk assessment concluded that lindane concentrations in surface and ground water could reach environmentally significant levels even if lindane is used only as a seed treatment (U.S. EPA 2001). Donald *et al.* (1997) showed that surface waters that originated from surface runoff had higher detection frequencies of lindane than surface waters that originate from ground water (springs), which did not have detectable levels of HCH isomers. Lindane was detected in 53% of the 43 prairie lakes and ponds sampled between 1989 and 1992 with a maximum and median concentration of 0.011 and 0.004 μg/L, respectively (Donald *et al.* 1997). Lindane can also readily enter the atmosphere from treated canola seed. A recent study found that between 12 and 30% of lindane applied may volatilize from the fields and enter the atmosphere (Waite *et al.* 2001). Waite *et al.* (2001) also measured dry deposition rates of lindane: dry deposition rates were higher in the canola field (853 to 2203 $ng/m^2/day$), but

were below detection limit (20 ng/m^2/day) in the control site, which was 2 km away. Rainfall concentrations were similar between the canola field and control site, with values between <10 and 200 ng/L. Wash-out by rainfall and dry deposition of lindane may result in the contamination of areas where lindane is not applied. Donald *et al.* (1999) found that lindane concentration in prairie wetlands increased with increasing precipitation, with a maximum concentration of 0.4 µg/L. Lindane was detected in 74% of the samples, and exceeded Canadian water quality guidelines for the protection of aquatic life (0.01 µg/L; CCME 1999) in 17 out of 51 wetlands (Donald *et al.* 1999).

1.2.4 Fate and Degradation

Lindane is relatively persistent in the environment compared to other pesticides in use today, but not to the same degree as other organochlorine insecticides. Lindane can persist for over a year in agricultural soils, with estimates of half-lives ranging from 120 to 569 days (Crosley *et al.* 1998). Once in the soil, lindane is either degraded by microbes or enters the atmosphere by volatilization. Biodegradation occurs most rapidly under anaerobic conditions, such as in flooded soils (ATSDR 1994). Volatilization from the soil increases with increasing air temperature and soil moisture (Samuel and Pillai 1990).

Lindane is likely to accumulate in ponds because of its persistence in water. Lindane is water soluble up to 10 mg/L (Lichtenstein *et al.* 1966) and has a half-life of up to a year (Hamelink and Waybrant 1976). Lindane can be degraded by ultraviolet radiation to carbon dioxide and hydrochloric acid at pH 9 (half-life = 50 hours), but is very stable at pH < 5 (Hiskia *et al.* 1997). Sharom *et al.* (1980) showed that lindane is primarily degraded in water through microbial action. Hydrolysis occurred faster at higher pH than at neutral pH in experiments conducted with surface water, with half-lives ranging from 92 to 771 hours (Saleh *et al.* 1982). Photolysis was also enhanced at higher pH, but was slow with half-lives ranging from 169 to 1791 hours (Saleh *et al.* 1982). Lindane can also adsorb to sediments (Lotse *et al.* 1968), where it can accumulate.

Recent experiments indicate that HCH isomers can have long atmospheric lifetimes (Brubaker and Hites 1998; Cortes *et al.* 1998), which can explain why they are transported large distances and are found in the arctic (Willett *et al.* 1998). In fact, global sources of HCH and its atmospheric transport can be tracked by means of the ratio of α-HCH to γ-HCH. Areas with heavy lindane use tend to have lower ratios than more northern sites, which have higher ratios (Willett *et al.* 1998). While photoisomerization of γ-HCH to α-HCH has been demonstrated in laboratory experiments, it has not been shown to be significant under field conditions (Walker *et al.* 1999). Recent declines in atmospheric concentrations of α-HCH probably reflect the declining use of technical HCH in most parts of the world (Li *et al.* 1996; Walker *et al.* 1999). Atmospheric concentrations of γ-HCH have been shown to follow seasonal trends (Jantunen *et al.* 2000), especially in areas of high lindane use, such as in corn growing areas of Quebec (Poissant and Koprivnjak 1996). Waite *et al.* (2001) observed the highest atmospheric lindane concentrations the second week after seeding. Seasonal trends in concentrations of α-HCH and γ-HCH in the atmosphere are similar to those in Canadian surface waters, where the highest concentrations are observed during seeding (Crosley *et al.* 1998).

1.3 Common Prairie Frog Species

Among the seven species of amphibians found in Saskatchewan, the larvae of two were used in this research project: *Rana sylvatica* (wood frog) and *Pseudacris maculata* (boreal chorus frog). Wood frogs are widely distributed throughout Saskatchewan, except in the extreme northeast and southeast (Didiuk 1997). They are diurnal, cold tolerant amphibians that hibernate on land under litter and humus. In Saskatchewan, they breed between mid April and the first week of May in warmer years and as late as mid May in colder years (A. Didiuk, personal communication). The breeding season lasts only 1-2 weeks, and the population in a pond may only breed for one day (Berven 1981). Wood frogs prefer shallow, clear ponds or ephemeral water bodies when laying eggs. Eggs are laid near the water surface in globular masses, which may or may not be attached to plants. Females usually lay one clutch, which they tend to place near other

clutches (Howard 1980). The number of eggs per clutch can vary greatly, ranging from 2000 to 3000, with an egg diameter of 1.6 mm (Duellman and Trueb 1986). Hatching occurs after about 3 weeks; tadpoles emerge at about 7-10 mm in length. Development, from fertilization of the egg through resorption of the larval tail, has been described as 46 stages (Gosner 1960), a process that requires 6-12 weeks for *Rana sylvatica* (Russell and Bauer 1993). Adult frogs are 30-60 mm with the females 15% bigger than males on average (Berven 1981).

Boreal chorus frogs, *P. maculata*, are found throughout Saskatchewan, except in the extreme northeastern portion, and are common and abundant throughout their range (Didiuk 1997). It is a distinct species from the western chorus frog, *P. triseriata*, which is found in Eastern Canada and thought to be in decline there (Daigle 1997). Boreal chorus frogs are similar to wood frogs in that they are freeze-tolerant and hibernate beneath leaf litter. In general, chorus frogs are more abundant in drier areas of the province such as central and southern Saskatchewan, whereas wood frogs prefer more cooler, wetter habitats in central and northern Saskatchewan. Chorus frogs will also begin calling as soon as the snow melts (Russell and Bauer 2000), but typically will have a longer breeding period and lay their eggs later than wood frogs. From 150 to 1500 eggs are laid in small clumps attached to vegetation beneath the water surface over a period of a few days. Each egg is about 1 mm in diameter and will hatch in 10 to 14 days (Russell and Bauer 2000). Tadpoles hatch at about 4-7 mm in length and will reach up to 30 mm (Russell and Bauer 2000). The larval period lasts about 2 months. Metamorphs are 7-12 mm at metamorphic climax and adults will reach 20-40 mm in body length. Russell and Bauer (2000) state that chorus frogs reach maturity within a year and will only live to breed once, but field studies with *P. triseriata* in Michigan indicate that chorus frogs may take up to 2 years to reach sexual maturity and that they may reproduce more than once (Smith 1987). Breeding choruses of *P. triseriata* and *P. maculata* collected from South Dakota, Nebraska, and Kansas, were found to consist of 2 and 3 year old males according to skeletochronology of freeze-sectioned femurs (Platz and Lathrop 1993).

1.4 Use of Amphibians to Monitor Effects of Environmental Contaminants

The use of amphibians to study the effects of environmental contaminants has received much encouragement recently (Hall and Henry 1992; Pauli *et al.* 2000; Sparling *et al.* 2000). Amphibians are a useful animal model because of their presence in a variety of ecosystems around the world, their important role in the food web as both prey and predator, and their susceptibility to waterborne and terrestrial contaminants due to their biphasic life style. Indeed, effects of exposure during different stages of the embryonic and larval period can easily show future adverse effects. For example, Bridges (2000) showed that exposure of eggs to carbaryl can decrease size at metamorphic climax, and that the effects of exposure at different stages can differ. The correlation of size at metamorphic climax with future survival and reproductive success allows researchers to study a relatively short period of larval development and still make predictions of future consequences (Smith 1987). Tadpoles are also relatively easy to culture, and collection of eggs from the wild may not be detrimental to larval success in the wild, particularly in species that lays thousands of eggs per clutch, because although most eggs are viable, only a fraction are likely to survive to metamorphic climax in the wild due to interspecific competition and predation. Frog eggs have long been used as surrogates for the study of embryonic development because of their similarity to other vertebrate developmental systems (Rugh 1948; Murphy *et al.* 2000). Now frogs are being used as sentinels to determine if environmental pollution is causing ecological instability, and the recent declines may be interpreted as an early warning of the fate of other species (Murphy *et al.* 2000).

Regulatory agencies are developing standardized amphibian toxicity testing guidelines with the intent that amphibians will become part of required toxicity testing programs. It is interesting to note though, that amphibians may not provide the "overall" indication of the toxicity of a substance. Generally, amphibians tend to be less sensitive than other species to pesticides, especially to cholinesterase inhibitors (Hall and Henry 1992). Amphibians have a similar mixed function oxidase (MFO) system to mammals,

fish, and reptiles, but overall they have lower levels and activities of the components of the MFO system, and respond more slowly or not at all to enzyme inducers (Ertl and Winston 1998; Schwen and Mannering 1982a; Schwen and Mannering 1982b; Schwen and Mannering 1982c). Assessing overall toxicity of a particular chemical to amphibians may be difficult due to differences in sensitivity among species and among life stages. For example, working with one species, such as *R. pipiens*, may not give toxicity information that is representative of other amphibians. In fact, a recent study illustrated that different species of *Rana*, different populations of *R. sphenocephala* and different families of *R. sphenocephala* within one pond can show significantly different sensitivities to carbaryl (Bridges 2000). Species may also differ in sensitivity due to differential exposure to pesticides; they may develop resistance to commonly used pesticides after continuous use within their habitat. This resistance can allow some populations to be more tolerant (*i.e.*, produce higher LC50 estimates) than other populations of the same species (Boyd *et al.* 1963; Cooke 1972). These confounding variables can negate the usefulness of including native amphibian species into toxicity testing programs. On the other hand, amphibians may be more susceptible to pesticides, that is, more vulnerable to exposure, than other species. Amphibians are more likely to use ephemeral ponds or runoff ditches than fish. These waterbodies are also more likely to receive higher amounts of pesticides, and more often, from overspray or runoff than other, larger waterbodies. As well, larger, more permanent waterbodies, such as those inhabited by fish, are likely to dilute pesticide inputs to a greater extent than smaller waterbodies (Donald and Syrgiannis 1995) For example, Donald and Syrgiannis (1995) found higher pesticide concentrations in semipermanent lakes than in permanent lakes. They also found that detection frequencies of pesticides were higher in freshwater than saline lakes, and noted that semipermanent lakes tended to have lower salinity than permanent lakes. They suggested that freshwater lakes may have lower salinity because they are refilled with runoff from fields, which would also been laden with pesticides. Smaller waterbodies are also more likely to be sprayed in forest pesticide applications

(Berrill *et al.* 1994; Berrill *et al.* 1995). Therefore, amphibians are likely to be exposed to relatively high concentrations of pesticides at any stage in their life cycle.

1.5 Amphibian Declines and Malformations

Global amphibian population declines and the increased incidence of malformations in some populations have attracted the attention of researchers around the world. There are many possible causes of amphibian declines: habitat loss or degradation, agricultural and industrial chemicals, endocrine disrupting compounds, acid rain, introduction of exotic species, over-collection, disease, increased ultraviolet radiation, and global climate change (Carey 2000). A recent review described the controversy in assessing population declines, noted broad continental trends, and summarized the causes of declines into three categories: habitat destruction and alteration, global anthropogenic influences, and natural causes (Corn 2000). While the malformations observed in wild frogs are grotesque and are assumed to lead to lethality due to the inability to feed or avoid predators, it is unknown whether wild populations exhibiting high levels of malformations are declining in overall size (Carey 2000). Populations showing malformations differ in geographic location, habitat type, and taxonomic status from those experiencing high rates of mortality. In addition, populations showing rapid declines do not have the type or number of malformations seen in populations with high frequencies of malformations (Carey 2000). The history and potential causes of malformations in amphibians have been reviewed by Ouellet (2000) and include: abnormal regeneration after injury, presence of certain fish species, disease, high tadpole densities, exposure to extremes in water temperatures and the chemical composition of water in breeding ponds, genetic mutation, parasitic cysts, and developmental toxicity by agricultural pesticides and fertilizers, osteolathyrogenic agents, radioactive pollution, retinoids, trace metals, nutritional deficiencies, and ultraviolet radiation.

1.6 Lindane as an Endocrine Disrupter

Studies in mammals have indicated that lindane is possibly estrogenic. In studies with sheep, lindane-treated feed at 1 mg/kg per day was found to not affect reproductive or endocrine function in pregnant ewes (Beard *et al.* 1999b); to reduce reproductive behaviour (such as interest in oestrous ewes, signs of arousal or initiation of mating) in male offspring, perhaps related to decreased luteinizing hormone (LH), oestradiol and testosterone concentrations (Beard *et al.* 1999a); and to temporarily increase serum T4 concentrations in female offspring (Beard and Rawlings 1999). Detrimental effects on reproductive function (decreased number of copora lutea (CL), total CL volume, and increased LH pulse frequency) were only observed after estrous synchronization. Female mink, exposed to the same dose of lindane from before breeding until weaning, exhibited decreased fertility, primarily due to embyro mortality after implantation (Beard *et al.* 1997). Ewes given capsules containing 2.5 mg lindane per kg body weight twice a week for 43 days had increased serum insulin and estradiol concentrations and decreased basal LH concentrations (Rawlings *et al.* 1998). A single prenatal dose of lindane (30 mg/kg given by gavage to the pregnant rat on day 15 post conception) affected the sexual behaviour of male rat offspring by causing a transitory decrease in libido probably resulting from a reduction in testosterone concentration (Dalsenter *et al.* 1997).

1.7 Lindane and Amphibians

Few studies have been conducted on the effects of acute or chronic exposure to lindane on amphibians. Chronic exposure to lindane from egg to metamorphosis has been tested with *Xenopus laevis* at 0.5, 1, and 2 mg/L (Marchal-Segault and Ramade 1981). Hatching rate was decreased only at the highest concentration, probably due to disruption of the hatching mechanism. Significant mortality occurred after initiation of feeding, and increased with increasing exposure and concentration. Time to and weight at metamorphosis were lower in treated animals than in the control animals. The weight difference between control and treated tadpoles was noted 15 days after hatching. A temporary darkening of pigmentation was also noticed. The authors suggested that the

change in pigmentation, delayed development, and reduced growth may have been due to endocrine disruption by lindane. A follow-up study with 0.5 mg/L lindane indicated that lindane may have affected the proper development and function of the thyroid gland in hind limb bud *X. laevis* tadpoles exposed from eggs to 15 days post-hatch (Marchal-Segault 1982).

An analysis to detect patterns in the sensitivity of aquatic organisms to toxicants suggested that amphibians are likely to be as sensitive to lindane and dieldrin as fishes (Vaal *et al.* 1997). However, Thybaud (1990a) determined that the fish, *Gambusia affinis*, was 40 to 86 times more sensitive to lindane than were tadpoles of *R. temporaria*. He also compared his results to published LC50 values with eight other fish species and demonstrated that *R. temporaria* was the most tolerant by at least an order of magnitude (Thybaud 1990a).

1.8 Terminology

A brief discussion of the common terminology used in studies of amphibians and their larval development is essential in order for the reader to follow the discussions on pesticide effects later in this thesis. The typical temperate anuran follows a well-known life cycle: the adults return to waterbodies to lay eggs, which develop and hatch into tadpoles; these tadpoles grow and develop into young frogs; the young frogs leave the water, grow, hibernate, and reach sexual maturity within a few years; and the cycle begins again. The staging method of Gosner (1960) will be used in this thesis because it is simple, includes both embryonic and larval developmental periods, and is commonly used in the literature. Gosner (1960) divides the embryonic period into stages 1-25 and the larval period into stages 26-46.

The postembryonic, or tadpole, period as a whole is termed "metamorphosis". Metamorphosis can be divided into three distinct phases: premetamorphosis, prometamorphosis, and metamorphic climax. Rapid growth and little morphological change occurs during premetamorphosis, which lasts from stage 26 (free-swimming, feeding tadpoles) to stage 35 (prior to start of hind toe differentiation).

Prometamorphosis is characterized by slower growth but greater morphological change, especially in toe differentiation, and lasts from stage 36 to stage 41. Metamorphic climax begins with forelimb emergence (stage 42) and ends with complete tail resorption (stage 46). Mouth development and tail resorption characterize this period of metamorphosis. In this thesis, the term "metamorph" refers to the animal in any stage of metamorphic climax (McDiarmid and Altig 1999). After stage 46 and until the onset of hibernation, the animal will be referred to as a froglet.

Burggren and Just (1992) suggest defining these terms by thyroid-dependent phenomena. Premetamorphosis refers to stages that are independent of thyroid function and to development that will still occur after thyroidectomy. Prometamorphosis refers to stages dependent on thyroid hormones (TH). Metamorphic climax depends on a surge of thyroid hormones with a fully functional hypothalamus-pituitary-thyroid axis.

Other terms that need to be defined are those dealing with primary sexual development. Most amphibians do not have morphologically distinct sex chromosomes, but probably have genetic sex determination (Hayes 1998). Sex determination refers to mechanisms that direct sex or gonadal differentiation. Males are the heterogametic sex in ranids, (male = XY, female = XX), whereas females are the heterogametic sex in hylids (male = WW, female = ZW). Gonadal differentiation refers to the development of ovaries or testes from the undifferentiated (bipotential) gonad. It is believed that exogenous steroids probably affect gonadal differentiation, but not sex determination (Hayes 1998).

1.9 Research Objectives

The purpose of this research was to determine if exposure to lindane during the aquatic stage of the amphibian life cycle has any deleterious effects on the normal development of native frog species. This overall objective was divided into more detailed objectives: 1) to assess the acute toxicity of lindane to frog tadpoles indigenous to Saskatchewan, and 2) to expose the tadpoles of one common Saskatchewan frog to lindane throughout metamorphosis to evaluate effects on survival and various sublethal

endpoints. The second chapter of this thesis compares the lethal and sublethal responses of tadpoles of two species to acute lindane exposure and attempts to investigate differences in sensitivity due to developmental stage. The third chapter describes the design of an outdoor microcosm study used to assess the chronic toxicity of lindane to wood frog tadpoles. This chapter also describes issues related to the study design, such as how and when to collect blood from the metamorphs, when they begin insectivorous feeding, how shading from adjacent trees can affect time to metamorphic climax, and how ethanol can cause bacterial blooms in the microcosms. The fourth chapter describes and discusses the results of the microcosm study conducted in the year 2000. Endpoints for this study included survival, time to and size at metamorphic climax, incidence of malformations, blood hormone concentrations, sex ratios, fluctuating asymmetry estimates, and hepatic glycogen reserves at the onset of hibernation. The last chapter summarizes the implications of the research project in regards to protecting amphibians in the wild and suggests directions for future research.

CHAPTER 2

ACUTE TOXICITY OF LINDANE TO TWO SPECIES OF PRAIRIE FROGS AT TWO STAGES OF DEVELOPMENT

2.1 Introduction

Few studies have investigated the acute toxicity of lindane to amphibians (Pauli *et al.* 2000). Sanders (1970) determined the relative acute toxicities of 16 to 18 pesticides to 1-week-old *Pseudacris triseriata* tadpoles and 4- to 5-week old *Bufo woodhousii fowleri* tadpoles. Lindane was the least toxic insecticide to both species, with 48-hr LC50 values of 3.8 mg/L for the former and 5.4 mg/L for the latter. Thybaud (1990a) reported a 48-hr LC50 value of 5.9 mg/L for larval *Rana temporaria*. However, there are no data available for toxicity of lindane to frogs native to Saskatchewan, such as the wood frog (*R. sylvatica*) or the boreal chorus frog (*P. maculata*).

Sensitivity of frogs to pesticides may be influenced by developmental stage. For example, hatching success was unaffected by exposure of ranid eggs to endrin, toxaphene, endosulfan, or carbaryl (Hall and Swineford 1980; Berrill *et al.* 1998; Bridges 2000). Mortality of hatchlings exposed to lindane increased as feeding began after absorption of the yolk sac (Marchal-Segault 1976). Berrill *et al.* (1998) observed that endosulfan was more lethal to late-stage toad (*B. americanus*) tadpoles than to younger tadpoles. Therefore, lindane toxicity to native species may differ with developmental stage.

Although lindane may not be very toxic to amphibian larvae based on the findings of Sanders (1970) and Thybaud (1990a), acute toxicity testing with lindane was pursued to provide comparable data on native Saskatchewan frogs. The objectives of this research were: 1) to compare the acute toxicity of lindane to two native frog species, 2)

to investigate differences in sensitivity due to developmental stage, and 3) to assess both lethal and sublethal endpoints. The acute toxicity of lindane to larvae of two native frog species at two developmental stages is presented in this chapter.

2.2 Materials and Methods

2.2.1 Study Animals

Lindane toxicity was assessed with wood frog (*R. sylvatica*) larvae and boreal chorus frog (*P. maculata*) larvae at two stages of development: hatchlings (free-swimming tadpoles with external gills; Gosner (1960) stages 23-25) and mid-stage tadpoles (hindlimb bud development; Gosner stages 26-30).

Animals were collected in May and June 2000 as eggs or tadpoles from five ponds in northern Saskatchewan that were at least 80 km from agricultural cropland (locations of two of the ponds, Grassy Pond and Airport Side Pond, are given in Appendix A; the other ponds were located near Grassy Pond). The ponds were assumed not to be contaminated with pesticides from runoff or direct spray. The eggs and tadpoles were placed in coolers or pails with water from the pond, and transported back to the laboratory and kept at room temperature before use. Eggs generally hatched and were free-swimming within three or four days (hatchlings) of collection. Some of these hatchlings were kept in plastic pails with aeration and daily water changes for up to three weeks until use in two of the tests (wood frog tadpoles and chorus frog tadpoles). Wood frog tadpoles that were collected from the field were kept at room temperature for two to three days from the date of collection until use in the test. Gradual replacement of the pond water with dechlorinated tap water was conducted to acclimate the tadpoles to the water used in the tests.

2.2.2 Test Procedures

Five concentrations of lindane and two control groups (negative and solvent) were used in each 96-hr static test, following the protocol of ASTM (1996) with minor modifications. Dechlorinated municipal tap water was used for all tests. Gentle aeration

was provided in most of the tests. Treatment concentrations increased in a geometric series from 0.375 mg/L to 6.0 mg/L. One test on wood frog hatchlings (#1) had a range of concentrations of 0.125 mg/L to 4.0 mg/L. There were three replicates of each treatment. Test vessels were round 1-L, 2-L, or 2.5-L glass battery jars containing 1.0, 1.5, or 2.0 L of water with *R. sylvatica* or *P. maculata*. The size of container was kept constant within individual tests. Stock solutions of lindane were prepared in 95% or 100% ethanol (v/v). Dosing solutions were made by serial dilution from one stock solution for each test. The appropriate amount of lindane was added to the water before the tadpoles were added. The solvent concentration was kept constant throughout the treatments at 0.1% (v/v). The number of tadpoles per jar depended on the number of animals available at the desired stage and the volume of test solution per jar. Wood frog hatchling tests had 15 or 10 animals per jar, and wood frog tadpole tests had 5 animals per jar. Chorus frog hatchling tests had 15 or 7 animals per jar, and the chorus frog tadpole test had 5 animals per jar. Tadpoles were randomly selected and added to the individual jars one or a few at a time until the desired sample size was reached. The tadpoles were not fed during the tests.

The tests were conducted between May 9 and July 28, 2000. A total of seven tests were conducted: two tests with wood frog hatchlings, two tests with wood frog tadpoles, two tests with chorus frog hatchlings, and one test with chorus frog tadpoles. Mortality was the main endpoint in the acute tests and dead animals were removed every 24 hours. The number of individuals exhibiting erratic swimming and malformations in each replicate was also recorded. Almost all the EC50 estimates used erratic swimming as the endpoint because the incidence of malformations generally did not yield reliable estimates.

One water sample per treatment was collected at the start of each test for lindane analysis. Although more than one test was usually conducted with each species and life stage, due to fiscal constraints, water samples were only analyzed from tests that yielded the best dose response relationships. Therefore, water samples were analyzed from one wood frog hatchling test, one wood frog tadpole test, one chorus frog hatchling test, and

one chorus frog tadpole test. Residue analyses were conducted as described in Section 4.2.4 under *Water and Tissue Residues* except that the samples were kept for up to two months at 4°C before shipping. Recoveries of γ-HCH in two lab-spiked reference samples were 148% and 160%. The estimated method detection limit for lindane was 10 ng/L for the final extract volume of 10 ml. Generally, the measured lindane concentrations were within 57 to 100% of nominal concentrations with an overall mean of 75%.

Dissolved oxygen concentrations (YSI Model 55 Dissolved Oxygen meter from YSI, Yellow Springs, OH, USA) and pH (QuiKcheK Model 105 pH meter from Orion Research, Beverly, MA, USA) were measured in the test vessels every 48 hours in most tests. Mean (± SD) DO and pH were 6.86 ± 1.65 mg/L and 8.0 ± 0.2 in the wood frog and chorus frog tests. Water temperatures during the tests were measured either in a separate test vessel without animals, or in the test vessels using the DO meter. Water temperatures ranged from 16.7°C to 22.2°C, with an overall mean (± SD) of 20.5 ± 1.1°C. Water samples were taken at the end of each test for the measurement of alkalinity, hardness, and conductivity. Hardness and alkalinity were analyzed using a Hach Digital Titrator Model 16900 (Hach Company, Loveland, CO, USA) and conductivity was analyzed using an Orion model 170 conductivity meter (ATI Orion, Boston, MA, USA). Their overall means (± SD) were 86 ± 10 mg/L, 131 ± 14 mg/L, and 384 ± 32 μS/cm, respectively.

2.2.3 Statistical Analysis

The number of tadpoles that were dead or swimming erratically in each replicate was pooled in the statistical analysis. Therefore, the sample size per treatment varied from 15 to 45 depending on the test. The trimmed Spearman-Karber method (Hamilton *et al.* 1977; Hamilton *et al.* 1978; U.S. EPA 1994) was used to calculate the concentration at which 50% mortality is estimated to occur (LC50) and the concentration at which 50% sublethal response is estimated to occur (EC50). Occasionally the magnitude of the response at lower concentrations did not differ or did not increase with

concentration; therefore, some estimates had trim values above 10%. Only estimates with trim values below 20% were included in the results. The lack of overlap among 95% confidence intervals was used to assess differences between hatchlings and tadpoles, and wood frogs and chorus frogs.

The LC/EC50 estimates compared in this chapter are based on nominal lindane concentrations because this data set was larger as initial concentration of lindane was only measured in four out of the seven tests. Estimates with measured lindane concentrations are listed in Appendix B.

2.3 Results

2.3.1 Symptoms of Poisoning

Normal behaviour of control tadpoles consisted of spontaneous swimming activity and quick, straight, and relatively long bursts of swimming upon disturbance (tapping the jar or gentle prodding). In contrast, tadpoles at the lowest concentrations were excitable or irritable, that is, they were quick to respond to disturbance. However, they generally made very few spontaneous movements compared to control tadpoles. As concentrations increased, the swimming behaviour of treated tadpoles became more erratic, progressing from unsteady swimming to circular swimming, the degree of which was positively associated with lindane concentration. In some cases, erratic swimming was associated with obvious malformations, such as a lateral kink or downward curve in the tail. Bursts of swimming also became shorter at higher concentrations and often ended in twitching or quivering. At the highest concentrations, mild to severe convulsions (spastic, snapping motions) were observed; severity of the convulsions was positively related to lindane concentration. Occasionally, a tadpole would start swimming in circles and then enter into a brief convulsion, after which it would slowly sink to the bottom of the test chamber.

Malformations were observed in hatchlings, but not usually in older tadpoles. Most malformations were associated with the tail and spine such as kinking and lateral deflection of the tail. Hindlimb malformations were not observed. Malformed tadpoles

were common in the highest concentration tested, but the incidence of malformations varied at lower concentrations, so reliable EC50 estimates for this end point were not generated. Discolouration was noted in some tadpoles exposed to lindane; chorus frog hatchlings typically lightened within one day of treatment, whereas some wood frog tadpoles darkened. The number of discoloured individuals generally did not increase with increasing lindane concentration and could not be used to generate EC50 values.

2.3.2 Wood Frog

The 48-hr EC50 estimates from the two tests with wood frog hatchlings did not differ from each other (mean = 1.98 mg/L), and did not differ from the 72-hr LC50 (1.90 mg/L) (Table 2.1). Mortality was the only response of tadpoles. The 72-hr LC50 estimates from the two tests with wood frog tadpoles did not differ from each other and had a mean of 3.94 mg/L (Table 2.1). Thus, hatchlings appeared to be more likely to exhibit the sublethal effects of lindane, and to be the more sensitive stage for lethality.

Water residue analysis was conducted on one hatchling test (#2) and one tadpole test (#2). The measured concentrations were 69 to 93% of nominal concentrations in the hatchling test and 57 to 100% in the tadpole test. Estimates based on measured concentrations were, on average, 70% of those based on nominal concentrations (Appendix B).

2.3.3 Boreal Chorus Frog

Significant mortality was not observed in the chorus frog tests. The 72-hr EC50 estimates with hatchlings did not differ from each other and gave a mean of 3.46 mg/L (Table 2.2). The 48-hr or 72-hr EC50 estimates with the hatchlings and tadpoles did not differ (Table 2.2). Therefore, there were no differences in sensitivity between hatchlings and tadpoles. One test provided an EC50 estimate for the number of malformed hatchlings with edema and crooked tails, which were only noted in the two highest treatments. The EC50 estimate based on these malformation data was 3.97 mg/L with a 95% confidence interval of 3.63 to 4.34 mg/L (trim value = 0.0%). Some mortality

Table 2.1. Estimates of the median effective concentration (EC50) and the median lethal concentration (LC50) of lindane to hatchlings (Gosner stage 23-25) and tadpoles (Gosner stage 26-30) of wood frogs (*Rana sylvatica*) from acute toxicity tests. Confidence intervals (95%) as determined by the trimmed Spearman-Karber method are given in parentheses. Estimates are based on nominal concentrations.

			Duration of Exposure			
Median Estimate	Life Stage	Test Number[1]	24 hr	48 hr	72 hr	96 hr
EC50[2]	Hatchling	1	--[3]	2.13 (1.92 - 2.37)	--	--
EC50	Hatchling	2	2.27 (1.82 - 2.85)	1.82[4] (1.38 - 2.39)	--	--
LC50	Hatchling	2	--	--	1.90 (1.69 - 2.13)	--
LC50	Tadpole	1	--	2.91[4] (1.98 - 4.27)	2.91[4] (1.98 - 4.27)	3.25[4] (2.29 - 4.63)
LC50	Tadpole	2	--	4.06[4] (3.63 - 4.53)	4.06[4] (3.63 - 4.53)	3.94[4] (3.07 - 5.05)

[1] Each test was conducted twice.
[2] End point = erratic swimming.
[3] Estimates were not generated.
[4] Trim value was between 10 and 20%.

Table 2.2. Estimates of the median effect concentration (EC50) of lindane to hatchlings (Gosner stage 23-25) and tadpoles (Gosner stage 26-30) of boreal chorus frogs (*Pseudacris maculata*) from acute toxicity tests. Median lethal concentration estimates (LC50) could not be generated from the available data. Confidence intervals (95%) as determined by the trimmed Spearman-Karber method are given in parentheses. Estimates are based on nominal concentrations.

			Duration of Exposure			
Median Estimate	Life Stage	Test Number[1]	24 hr	48 hr	72 hr	96 hr
EC50[2]	Hatchling	1	--[3]	--	3.69 (3.38 - 4.03)	--
EC50	Hatchling	2	--	3.37 (2.90 - 3.92)	3.23 (2.62 - 3.98)	--
EC50	Tadpole	N/A	3.86 (3.39 - 4.40)	4.04 (3.68 - 4.44)	4.05 (3.69 - 4.44)	--

[1] Hatchlings were tested twice.
[2] End point = erratic swimming.
[3] Estimates were not generated.

occurred at the highest treatments in this test, but was insufficient to generate a LC50 estimate.

Water residue analysis was conducted on one hatchling test (#2) and the tadpole test. The measured concentrations were 75 to 91% of nominal concentrations in the hatchling test and 67 to 83% in the tadpole test. Estimates based on measured concentrations were, on average, 78% of those based on nominal concentrations (Appendix B).

2.4 Discussion

2.4.1 Symptoms of Poisoning

Lindane inhibits the gamma-aminobutyric acid (GABA) receptor, causing an overstimulation of the central nervous system (CNS). As such, the expected symptoms of acute lindane poisoning would be excitability, convulsions, and death. At high acute exposures, lindane may cause symptoms that are similar to other CNS stimulants. Sanders (1970) examined the acute toxicity of a number of organochlorine insecticides, including lindane, DDT, and dieldrin, and observed that the symptoms of poisoning followed a predictable pattern of irritability followed by loss of equilibrium and death. Cooke (1971) also stated that excitability is the general symptom of organochlorine poisoning.

The effects of lindane on swimming behaviour and malformations observed in this study were similar to effects of other organochlorine insecticides on tadpoles (Cooke 1970; Cooke 1972; Hall and Swineford 1980; Pawar and Katdare 1984; Licht 1985; Berrill *et al.* 1998). The excitability of tadpoles exposed to low concentrations of lindane was similar to the exaggerated or prolonged response to a disturbance produced in tadpoles of the southern leopard frog exposed to endrin or toxaphene (Hall and Swineford 1980). Tapping the side of the jar or touching the hyperactive tadpoles failed to produce any response in DDT-exposed tadpoles, unlike the control tadpoles, which swam violently (Cooke 1970). In contrast, treated tadpoles in this study were excitable, that is, more sensitive to disturbance and were not active prior to disturbance.

Convulsions observed in lindane-treated tadpoles were similar to the uncoordinated movements such as spasmodic lashing of the tail and twisting of the body that characterized acute DDT exposure in common frog (*R. temporaria*) (Cooke 1970) or to the intense whiplike convulsions observed in wood frog (*R. sylvatica*), green frog (*R. clamitans*), and American toad (*B. americanus*) tadpoles exposed to endosulfan at relatively high concentrations (Berrill *et al.* 1998). Abnormal twitching behaviour, loss of balance, and circular swimming was also reported in embryos and tadpoles of the frog *Microhyla ornata* exposed to fenitrothion, lindane, or carbofuran (Pawar and Katdare 1984). Erratic swimming and hyperactivity are important sublethal effects of poisoning because they can increase the risk of predation of tadpoles in the wild (Cooke 1971).

Treated tadpoles in this study exhibited tail malformations in response to pesticide exposure that were similar to those found in tadpoles with external gills exposed to 0.02 or 0.5 mg/L DDT (Cooke 1972). The effects on pigmentation were also similar to those caused by organochlorine insecticides in previous studies. For example, toad tadpoles exposed to DDT or dieldrin turned from black to pale brown (Cooke 1972). The author suggested that this effect was due to a decrease in the circulating level of melanocyte stimulating hormone (Cooke 1972). Chorus frog tadpoles exposed to lindane in this study also lightened in response to lindane exposure. On the other hand, wood frog tadpoles appeared darker with lindane exposure. This finding is similar to *Xenopus laevis* tadpoles exposed to lindane throughout the larval period; these tadpoles became darker, probably due to some dysfunction of the hypothalamo-pituitary axis since effects on growth and development were also observed (Marchal-Segault and Ramade 1981). Therefore, a common mechanism for effects of lindane on pigmentation may exist but may result in opposite effects in different species.

2.4.2 Sensitivity of Developmental Stage and Species

While there were no differences between hatchling and tadpole sensitivity in chorus frogs, hatchlings appeared to be the more sensitive developmental stage in wood frogs. In general, EC50 and LC50 estimates were lower for hatchlings than for tadpoles.

This finding contrasts with those of other organochlorine insecticides, such as the increasing sensitivity to DDT with age of toad tadpoles (Sanders 1970). In this study, a difference between the two species was evident in that chorus frog tadpoles were more resistant to the lethal effects of lindane than wood frog tadpoles. In fact, wood frog tadpoles did not exhibit sublethal effects, only lethal effects. Other studies have noted that older tadpoles are more likely to die from pesticide exposure than younger tadpoles. For example, common frog (*R. temporaria*) and common toad (*B. bufo*) tadpoles seemed more resistant to the lethal effects of DDT when they had external gills and became more susceptible as they developed (Cooke 1972). Greater mortality was observed in tadpoles treated with carbaryl at 0.40 and 1.0 mg/L throughout the larval period or only in the tadpole stage than among those treated at earlier stages (Bridges 2000). This difference in species response is not unexpected; species may differ in sensitivity at different developmental stages. For example, newly hatched tadpoles of the American toad were more tolerant to endosulfan than the other two species (green frog and wood frog), but this difference in sensitivity was not observed among 2-week old tadpoles (Berrill *et al.* 1998).

Differences in the ages of tadpoles used make it difficult to compare species sensitivity to lindane among published studies. The toad tadpoles used by Sanders (1970) were of similar age to the wood frog tadpoles in the present study, and a comparison of 48-hr LC50 estimates suggests that wood frogs are more sensitive to lindane than toads. However, toad tadpoles seem to be more tolerant to pesticides than most amphibians (Cooke 1972; Berrill *et al.* 1998). Sanders (1970) used a similar species of chorus frog (*P. triseriata*) at a similar age to the chorus frogs in this study, but Sanders (1970) could estimate LC50 values, whereas significant mortality did not occur in this study at similar concentrations. While median estimates allow us to rank the toxicity of lindane against other chemicals, the concentrations necessary to produce these sublethal and lethal effects (>1.82 mg/L) are higher than what would be expected in the breeding ponds of wood frogs and chorus frogs in Canada. For example, lindane has

been detected in prairie lakes and ponds of Saskatchewan at concentrations ranging from 0.011 to 0.4 µg/L (Donald *et al.* 1997; Donald *et al.* 1999).

2.5 Summary

Acute exposure to high concentrations of lindane produced effects on swimming behavior and malformations that were similar to those caused by other organochlorine insecticides. Overall, these sublethal responses were most often quantified in hatchlings whereas mortality was most common in tadpoles. Wood frogs appeared to be more sensitive than boreal chorus frogs to the sublethal and lethal effects of lindane as hatchlings and tadpoles. For example, the 48-hr EC50s were lower in wood frog hatchlings (1.82 and 2.13 mg/L) than in chorus frog hatchlings (3.37 mg/L) and significant mortality was only observed in the wood frogs. The lowest LC50 observed in this study was 1.90 mg/L at 72-hr exposure with wood frog hatchlings.

CHAPTER 3

MICROCOSM STUDIES: METHODS DEVELOPMENT AND PROBLEMS ENCOUNTERED

3.1 Introduction

Outdoor microcosms have several advantages over laboratory systems for testing the chronic effects of pesticides on amphibians. Laboratory tests are typically restricted in size, conducted with a single species, and lack the natural influences of photoperiod and climate that can only be obtained outdoors (Rowe and Dunson 1994). On the other hand, the use of outdoor microcosms provides a more complex ecological system that can be manipulated easily, allowing for greater control of abiotic and biotic factors as well as replication of treatments, while still adequately simulating natural ponds occupied by amphibians (Rowe and Dunson 1994). This increased realism, combined with the control of the background variables, improves the ability of the researcher to evaluate the direct and indirect effects of a toxicant and to model community-level responses (Rowe and Dunson 1994).

This project required the development and evaluation of methods for maintaining frog tadpoles and for exposing them to lindane in outdoor microcosms during 1998, the first field season. Three styles of microcosm designs were evaluated and methods to prevent avian predation and metamorph escape were developed. A microcosm study was undertaken in 1999 to investigate the toxicity of lindane to wood frog tadpoles, but problems with turbidity and low dissolved oxygen concentrations, apparently caused by bacterial blooms, confounded the results. Laboratory experiments were performed to determine the cause or causes of these problems and the microcosm study was repeated in the summer of 2000. Field studies were also undertaken in 1999 to corroborate

methods used in the microcosm studies, such as collection of blood during metamorphic climax. There was a question of when to sample blood in order to analyze for plasma corticosterone concentrations in metamorphs. In general, plasma corticosterone concentrations have been shown to be low at the beginning of the tadpole stage and increase before forelimb emergence, but when they peak during metamorphic climax is not well known (Burggren and Just 1992). In contrast, Krug *et al.* (1983) found that corticosterone concentrations in *R. catesbeiana* did not change between Gosner stages 41 and 45. As it is difficult to collect all the metamorphs at forelimb emergence, and therefore some metamorphs may be collected later in metamorphic climax, it was important to verify whether plasma corticosterone peaked during this developmental period, and therefore if it needed to be measured at a particular stage. Data from wild-caught wood frog metamorphs were used to verify whether a plateau in plasma corticosterone concentrations occurred between Gosner stages 42 and 45 by using progress in tail absorption as a rough indicator of development. Another aspect of the experiment design, whether the metamorphs required an insectivorous diet prior to removal from the microcosms, was also assessed in 1999. Anuran tadpoles usually stop feeding at metamorphic climax because the filtering apparatus degenerates (Hourdry *et al.* 1996). However, few studies have been conducted on the timing of the actual transition between larval feeding and postmetamorphic carnivorous feeding. *Xenopus laevis* stops filter-feeding at stage 41 at which point larval mouthparts break down, and does not begin adult-like feeding until at least stage 43 (Naitoh *et al.* 1989). It has been suggested that this species may be able to feed on insects because it acquires an adult-like alimentary tract sooner than other species (Hourdry *et al.* 1996). No information is available on the timing of carnivorous feeding in *Rana* metamorphs, although Naitoh *et al.* (1989) noted that *R. clamitans* at stage 45 pursued live prey, but did not ingest anything. The stomach contents of wild-caught metamorphs were examined to determine at what stage during metamorphic climax the microcosm metamorphs may require insect prey.

The objectives of this portion of the research were as follows:

1) to assess which of three microcosm styles yielded the healthiest metamorphs at the end of the exposure period (1998);

2) to develop a method for blood collection from *R. sylvatica* metamorphs (1998);

3) to verify whether plasma corticosterone remained constant during tail resorption (1999);

4) to investigate at what stage metamorphs began eating live food (1999);

5) to evaluate the toxicity of lindane at environmentally realistic concentrations to wood frog tadpoles using an outdoor microcosm design (1999), and

6) to elucidate the cause(s) of the high turbidity and low dissolved oxygen concentrations in the 1999 microcosms (2000).

The protocol for collecting and euthanizing frog eggs, tadpoles, and metamorphs was approved by the Animal Care Committee of the University of Saskatchewan (UCACS Protocol ID Number: 19980139).

3.2 Materials and Methods

3.2.1 Design and Testing of Microcosms

The study site and materials used in construction of the fenced enclosure and the microcosms therein are described in Section 4.2.1. In 1998, three microcosm styles were evaluated to determine the best style for optimal survival and growth of tadpoles. The basic style of microcosm measured 1.0 m x 1.0 m x 0.7 m (L x W x D), with a water depth of 50 cm to give a total water volume of 500 L. The second style was of the same dimensions, but had 25 cm of soil packed inside to give a final water depth of 25 cm and a total water volume of 250 L. The third style was designed to assess whether or not developing tadpoles required a shallow area as well as a deep area. It measured 2.0 m x 1.0 m x 0.7 m with soil added to form a slope from an area of 15 cm deep and 0.5 m long at one end to an area of 50 cm deep and 0.5 m long at the other end. The total water volume of this microcosm was 500 L. All microcosms were set 50 cm into the ground to

help control temperature fluctuations, and thus water level in the microcosms was at ground level.

Wood frog tadpoles (*Rana sylvatica*) with small hindlimb buds were collected from three ponds near Saskatoon, Saskatchewan. Numbers of tadpoles per microcosm were varied to assess possible effects of density on growth. Four of the basic microcosm style (500 L) received one tadpole per 50 L, and the other four received five tadpoles per 50 L. One of the 250-L microcosm style received one tadpole per 50 L and the other received five tadpoles per 50 L. The two long (sloping bottom style) microcosms received two tadpoles per 50 L. All tadpoles placed in the ten square microcosms were collected from two ponds; all those placed in the two long microcosms came from another pond. Whether the larvae preferred the shallow versus the deep end of the long microcosms was assessed seven times over 3 days. The number of tadpoles occupying either the shallow or the deep end was recorded every 10 to 15 minutes in both microcosms. Although abundant floating vegetation made it difficult to accurately count the numbers of animals, some results were obtained.

3.2.2 Sampling of Hormones and Stomach Contents

A total of 41 metamorphs was collected by dip-netting from three prairie ponds during July 1999. The metamorphs were at various stages of metamorphic climax between Gosner stages 42 and 45 and had tails of varying lengths. Tail length was used as an indication of developmental stage during metamorphic climax. A standardized acute stress protocol was followed that included placing the metamorphs into individual 500 ml plastic containers with 1-2 cm of water and waiting for one hour prior to blood collection. Metamorphs were anesthetized with Orajel® (7½% benzocaine); measured for body weight, snout-vent length (SVL), and total length; and sampled for blood from the midline abdominal vein as described in Section 4.2.5. The blood was kept cool on ice until it could be centrifuged for 2-3 minutes at ~3400 rpm (g-force = ~388). After centrifugation, the plasma was removed and frozen.

Plasma samples were analyzed for corticosterone (CORT) by radioimmunoassay (RIA), using the methods described in Section 4.2.6 except for the differences mentioned below. The volume of plasma extracted was 10 or 25 µl, depending on the original sample volume. Following evaporation of the ether, all samples were reconstituted with 100 µl assay buffer and frozen at -20°C for use in the RIA. Recovery of the ^{3}H-corticosterone from the spiked triplicates was high (mean ± SD; 90.5% ± 0.93) with a low variability (CV = 1.0%) within the extraction procedure. Consequently, final hormone levels were not corrected for extraction efficiency. The corticosterone RIA was conducted using routine steroid RIA procedures with slight modifications to obtain maximum RIA sensitivity. The antiserum was diluted so as to obtain specific binding of approximately 24%; non-specific binding was low at approximately 3%. All samples were assayed in duplicate on the same day. Assay variabilities were below the acceptable limit of 10% CV; between assay variability was 5.6% and within assay variability was 4.0 ± 1.3% (mean ± SD). The detection limit was 1.21 ng/ml.

After blood collection, the metamorphs were preserved in 10% formalin. Stomach contents of the preserved metamorphs were categorized as containing identifiable insects (whole body or parts), possible insect parts (inconclusive identification), possible plant matter (green matter), or nothing.

3.2.3 Microcosm Study, 1999

The study site, design and construction of microcosms, and the design of the lindane exposure experiment in 1999 are described in Section 4.2.1. The variables assessed in the metamorphs were: survival, size at metamorphic climax, days to metamorphic climax, plasma corticosterone concentrations, sex ratios, and hepatic glycogen concentrations at the onset of hibernation. Two steps were taken to avoid the potential introduction of disease, particularly frog virus 3 (FV3), to the experiment. Firstly, dechlorinated tap water was used in the microcosms instead of pond water. Secondly, wood frog eggs were collected from ponds in northern Saskatchewan where the possibility of collecting infected animals was assumed to be lower than it would have

been in the agricultural region where infected tadpoles were found in 1998 by the author. Locations of two of the ponds, Grassy Pond and Airport Side Pond, are given in Appendix A; Ralph Pond is located at the entrance to Little Bear Lake Resort, SK. Vegetation for the microcosms was obtained from either the same ponds or from plant supply companies outside the province.

Four concentrations of lindane and one control (negative) were tested: 0.1 μg/L, 1.0 μg/L, 10 μg/L, and 100 μg/L. The reasons for selecting the first three concentrations are discussed in Section 4.2.2. The highest concentration, 100 μg/L, was used for comparison purposes. Technical grade lindane (99% purity) was obtained from Uniroyal Chemical (Guelph, Ontario, Canada). The appropriate amount of lindane was dissolved in ethanol to create a stock solution for each of the 0.1 μg/L and 1.0 μg/L treatments. Aliquots of these stock solutions were added to the appropriate microcosms. Separate stock solutions were made for each of the microcosms of the two highest treatments (10 μg/L and 100 μg/L). As discussed in Section 4.2.2, lindane treatments were increased by 30% to compensate for possible adsorption to the plastic that was used to line the microcosms. The ethanol concentrations (% v/v) in the lindane treatments were: 0.0013% (0.1 μg/L), 0.013% (1.0 μg/L), 0.13% (10 μg/L), and 0.2% (100 μg/L).

3.2.4 Experiments with Bacterial Blooms

Problems with turbidity and low dissolved oxygen (DO) concentrations in the 1999 microcosms initiated further laboratory experiments to determine whether lindane, ethanol, or the dried leaf litter was the cause of the bacterial blooms. The water used in the laboratory experiments (pond water) was similar to that used in the microcosms. While the source of water in the microcosms was dechlorinated tap water, it had been inoculated with microbes from the leaf litter, tadpoles and living plant matter. Therefore, pond water that had been stored in an aquarium with snails and aquatic plants (and previously tadpoles) and diluted with dechlorinated tap water was used. Tap water was dechlorinated by adding 6 mg of sodium thiosulphate per litre of water and allowing it to stand for at least a day. Turbidity provided a fast and inexpensive estimate of

bacterial numbers (Lim 1989) and was measured as Nephelometric Turbidity Units (NTU) using a Model 2020 Turbidimeter, (LaMotte, Maryland, USA). DO was measured using a YSI Model 55 Dissolved Oxygen meter (YSI, Yellow Springs, Ohio, USA).

Two experiments were performed in the winter of 2000 to examine the effects of lindane, ethanol, and dried plant matter on bacterial populations in pond water. The first experiment examined the effects of 0.2% ethanol, the highest concentration used in the microcosms, on bacterial populations in pond versus dechlorinated tap water, with and without dried plant matter (*Sphagnum* moss or *Populus* leaves). The primary objective of this experiment was to determine if a bacterial bloom, caused by adding dry organic matter to water, was the primary cause of the persistent turbidity and low DO in the microcosms. The assumption in this experiment was that dried organic matter, when added to water, would induce a bacterial bloom. The type of plant matter, presence of ethanol, and the type of water were factors that could potentially affect the severity of the bloom, and were tested in this three-factor analysis of variance (ANOVA) experimental design. A total of 36 glass battery jars held 12 treatments with three replicates each and were arranged in a completely randomized block design. *Sphagnum* moss from a local gardening store was rinsed in tap water and re-dried in a drying oven at 90°C for at least 2 days. Fallen poplar leaves, collected from the campus at the University of Saskatchewan, were rinsed in tap water and oven-dried. The ratio of plant matter to water in the experiment was 0.1 g per litre, similar to the ratio of 50 g of leaves per 500 L of water as used in the 1999 microcosms. The ethanol was added to the jars after the plant matter.

The second experiment compared the effects of lindane and ethanol at various concentrations in pond water without dried plant matter. The main objective of this experiment was to determine whether ethanol or lindane encouraged bacterial growth in a dose-related manner and if lindane potentiated the effect of ethanol. The treatments included one control (without ethanol or lindane) and four concentrations of ethanol (0.01, 0.1, 0.2, and 1.0%), with and without 100 μg/L lindane. As well, three additional

concentrations of lindane were tested (0.1, 1.0, and 10 μg/L) with 0.01% ethanol. The control group for this test series consisted of the 0.01% ethanol treatment from the set of ethanol-only treatments. Technical grade lindane, purchased from Sigma-Aldrich Fine Chemicals (Oakville, Ontario, Canada), was dissolved in ethanol to form a stock solution. Serial dilutions were made from this stock solution, and aliquots of each were added to the jars to achieve the target concentrations. Appropriate volumes of ethanol were added to the jars to yield ethanol concentrations higher than 0.01%. The jars were arranged in a completely randomized block design.

3.2.5 Statistical Analysis

All statistical analyses were performed using SigmaStat® Statistical Software, version 2.03 (SPSS INC., Chicago, IL, USA) with a 95 % ($\alpha = 0.05$) level of confidence. When significant differences in plasma corticosterone concentrations were noted among different stages of metamorphs, the Tukey test was used to examine the differences among all groups. Hormone concentrations that were measured as below the detection limits as stated in Section 3.2.2 were assumed to be equivalent to the detection limit for the purposes of statistical analysis. When significant differences were noted among treatments in the experiments with bacterial blooms, or in the microcosm study, Dunnett's test was the multiple comparison procedure used to compare the control mean to each other group mean. Regression equations were derived using SigmaPlot®, version 3.06 (SPSS Inc., Chicago, IL, USA). The term "significant" refers to statistical significance, and "*p*" to the probability of Type I error.

3.3 Results

3.3.1 Design and Testing of Microcosms

Mortality due to disease in 1998 was high among treated and control microcosms. Affected tadpoles with swollen, bloody limbs exhibited odd swimming behavior. Post-mortem analysis by Dr. Trent Bollinger of the Canadian Cooperative Wildlife Health Centre, Western College of Veterinary Medicine, University of Saskatchewan, in

Saskatoon, SK, revealed the presence of frog virus 3 (FV3), the type species of the genus *Ranavirus*.

High mortality due to FV3 eliminated the possibility of comparing growth of tadpoles among microcosm styles and population densities, or of obtaining preliminary information on the effects of lindane. The surviving metamorphs were very similar in size among the microcosms at about 1 g, but true comparisons could not be made. Disease did not seem to be a problem in the sloped microcosms because dead tadpoles were not found. The tadpoles did not appear to prefer either the shallow or deep areas (paired t-tests, $p > 0.40$), and the mean (± SD) number of tadpoles observed in each area was the same (3 ± 2). However, the mean number of tadpoles observed represented only about 30% of the total number of tadpoles added, indicating that the majority of the tadpoles was not accounted for perhaps because the tadpoles were hidden in the floating vegetation.

3.3.2 Hormones and Stomach Contents

Plasma corticosterone concentrations (CORT) did not differ with tail length during metamorphic climax (Fig. 3.1), but tail length was not a good indicator of Gosner stage because it varied greatly in metamorphs of stages 43 and 44 (Table 3.1). Comparing CORT to developmental stage instead of tail length indicated that CORT was 53% lower in stage 42 metamorphs than in older metamorphs (Table 3.1). When the data were made to fit a normal distribution by square root and log10 transformation, stage 42 metamorphs had significantly lower CORT than older metamorphs (one-way ANOVA, $p = 0.035$; Tukey test).

Out of the 41 metamorphs caught, nine had definite insect bodies or parts, six had possible, but not definite insect parts, and 26 had nothing or green material in their stomachs. The longest tail length measured in a metamorph with possible or definite insect matter was 25 mm. Further examination of the 1999 metamorphs indicated that those with identified or possible insect matter in their stomachs were in Gosner stages 44 and 45 (Table 3.1).

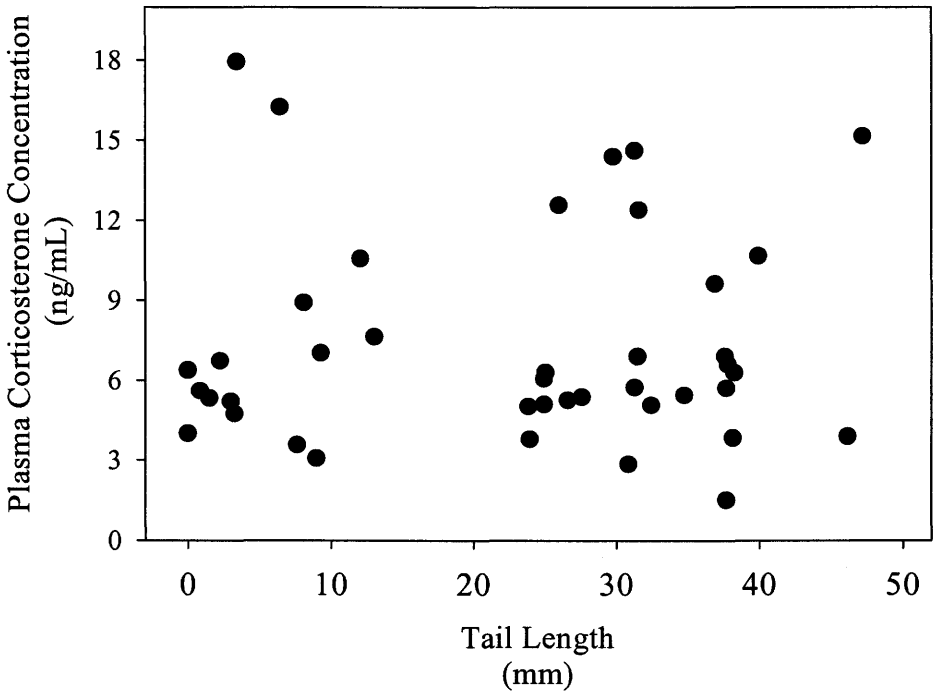

Figure 3.1. Plasma corticosterone concentration in wild-caught *Rana sylvatica* metamorphs collected from three ponds in 1999, as compared to tail length.

Table 3.1. Initiation of insectivorous feeding and mean plasma corticosterone (CORT) concentration in wild-caught *Rana sylvatica* metamorphs collected from three ponds in 1999. CORT and tail length values are means ± SD, with the ranges in parentheses. Different letters denote significant differences among groups ($p < 0.05$).

Stage [1]	*n*	Percent of Stomachs Containing Insect Matter	CORT (ng/ml)	Tail Length (mm)
42	4	0.0	3.63 ± 2.02 [a]	36.24 ± 3.60
			(1.51 - 6.30)	(30.85 - 38.25)
43	21	4.8	7.92 ± 3.78 [b]	32.57 ± 6.85
			(3.79 - 15.16)	(23.85 - 47.20)
44	9	77.8	7.56 ± 4.06 [b]	10.44 ± 6.20
			(3.07 - 16.24)	(3.25 - 25.05)
45	7	100.0	7.31 ± 4.77 [b]	1.57 ± 1.37
			(4.00 - 17.95)	(0.00 - 3.40)

[1] Gosner (1960) developmental stage.

3.3.3 Microcosm Study, 1999

White cloudiness was observed in the water of the microcosms shortly after the start of the experiment. This turbidity gradually cleared in a dose-related manner over the summer. Depressed dissolved oxygen (DO) concentration occurred concurrently with turbidity. Lower average pH was noted in the most affected microcosms (100 µg/L lindane with 0.2% ethanol). Water was collected from the control and 100 µg/L microcosms for enumeration of bacteria using a heterotrophic plate count analysis, which was conducted by BDS Laboratories, Qu'Appelle, SK. While this method is not ideal for the assessment of waterborne bacteria, the results did indicate that there were significantly more bacteria in the cloudy 100 µg/L microcosms than in the clear control microcosms (t-test, $p = 0.029$). At the time of collection, DO was between 3.0% and 24.8% saturation in the cloudy microcosms, and above 100% in the clear microcosms. Two main significant effects on the tadpoles of "treatment" were observed: high mortality in the highest treatment (one-way ANOVA, $p = 0.007$), and increased size at metamorphic climax (MC) with increasing magnitude of treatment (one-way ANOVA, $p < 0.007$) (Table 3.2). The highest treatment had the longest period with extremely low DO concentrations. Bacteria grown in ethanol-containing water were much larger in size than normal bacteria found in non-ethanol containing water, as observed during enumeration by epifluorescence microscopy. Whether lindane or ethanol caused the turbidity observed in the microcosms was investigated in experiments with bacterial blooms, as described in Section 3.3.4.

There was a significant correlation between mean microcosm temperature and days to MC (linear regression, $r^2 = 58.5\%$, $p = 0.001$). Mean days to MC decreased with increasing mean water temperature.

3.3.4 Experiments with Bacterial Blooms

The presence of plant matter of either type had no effect on DO or turbidity so it was removed as a factor in subsequent statistical analysis; therefore, only type of water

Table 3.2. Survival and size at metamorphic climax of *Rana sylvatica* metamorphs in relation to lindane concentration, dissolved oxygen (DO) concentration, and pH, in the 1999 microcosm study. Values are means ± SE. Different letters denote significant differences from the control group as determined by Dunnett's test ($p < 0.05$).

Nominal Lindane Concentration [1] (μg/L)		Survival [2] (%)	Snout-vent Length [3] (mm)	DO [4] (mg/L)	pH [5]
0.0	(0.0)	83.7 ± 2.9 [a]	18.08 ± 0.16 [a]	10.16 ± 0.39 [a]	8.5 ± 0.1 [a]
0.1	(0.0013)	71.5 ± 3.3 [a]	18.81 ± 0.17 [a]	9.33 ± 0.44 [a]	8.2 ± 0.1 [a]
1.0	(0.013)	74.4 ± 2.4 [a]	19.54 ± 0.13 [a]	7.87 ± 0.46 [a]	8.0 ± 0.1 [a]
10	(0.13)	78.8 ± 5.6 [a]	19.52 ± 0.14 [a]	7.96 ± 0.57 [a]	8.3 ± 0.1 [a]
100	(0.2)	26.4 ± 18.8 [b]	21.47 ± 0.24 [b]	2.51 ± 0.46 [b]	7.4 ± 0.1 [b]

[1] Nominal ethanol concentrations are in parentheses (% v/v).
[2] One-way ANOVA ($p = 0.007$).
[3] One-way ANOVA ($p = 0.005$).
[4] Friedman test ($p < 0.001$).
[5] One-way repeated measures ANOVA ($p < 0.001$).

and presence or absence of ethanol were considered. A slight decrease in DO occurred in all treatments within 2 days; however, the greatest response was observed in the pond water + 0.2% ethanol treatment, in which DO remained about 80% below that of the other treatments for about 3 weeks (Fig. 3.2). Turbidity was below 5 NTU in all treatments until day 12, when it increased sharply to a peak of 72 NTU in the pond water + 0.2% ethanol treatments on day 18 (Fig. 3.2).

The second laboratory experiment indicated that low concentrations of ethanol can cause bacterial blooms, as indicated by low DO and high turbidity. Ethanol significantly affected DO in pond water at the ethanol concentrations tested: 0.01%, 0.1%, 0.2%, and 1.0% (one-way repeated measures ANOVAs, $p < 0.023$), and DO declined in all ethanol treatments compared to the control (Fig. 3.3). The lowest ethanol treatment, 0.01%, initially dropped to below 4 mg/L DO, but rose to control levels by the end of the experiment. The control and 0.01% ethanol treatments had significantly lower turbidity than the other ethanol treatments (one-way repeated measures ANOVA, $p = 0.023$), but only 0.1% ethanol had significantly higher turbidity than the control group (Fig. 3.3). This experiment was repeated with the following ethanol concentrations: 0.01%, 0.05%, 0.1%, and 0.5%. Dissolved oxygen and turbidity in the ethanol-treated water diverged sharply from the control on day three (Fig. 3.4). The 0.01% ethanol treatment was similar to other treatments until day 3 (DO) and day 4 (turbidity), after which time DO and turbidity recovered to control levels. Overall, this treatment was not significantly different from the control treatment (Dunnett's test, $p < 0.05$). The other treatments recovered in a dose-related manner (Fig. 3.4). Interestingly, 0.1% ethanol affected bacterial bloom more than 0.5% ethanol (Fig. 3.4). On day 3, pH also decreased to approximately 7.2 in the ethanol-treated water compared to 7.8 in the control (Fig. 3.5). As with turbidity, pH in the 0.01% ethanol treatment returned to control levels by the end of the experiment; this treatment was not significantly different from the control treatment (Tukey test, $p > 0.05$).

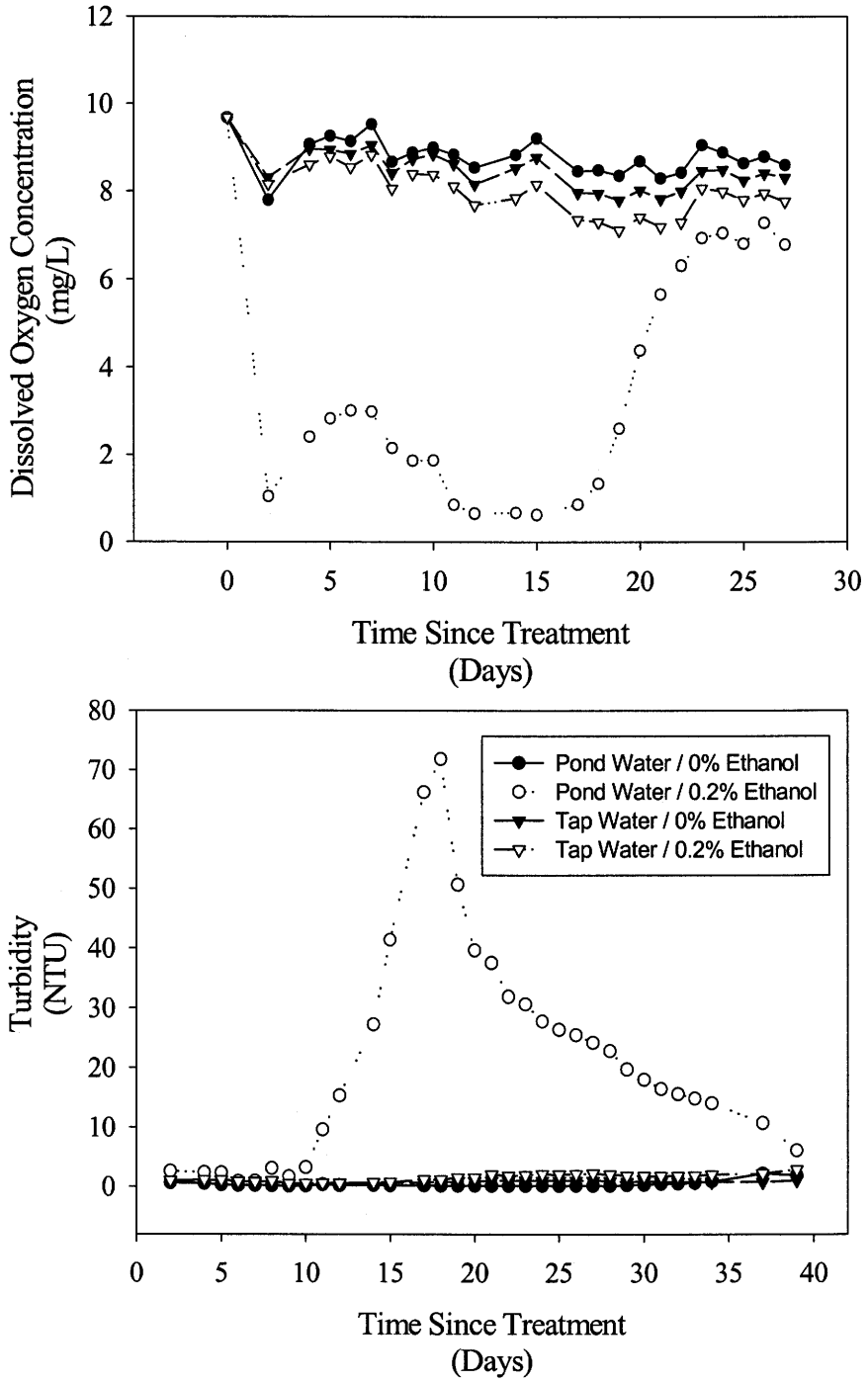

Figure 3.2. Dissolved oxygen (DO) concentrations and turbidity over time in pond water and dechlorinated tap water treated with and without 0.2% ethanol, in the presence or absence of dead plant matter (poplar leaves and *Sphagnum* moss). The presence or type of plant matter did not affect DO or turbidity; thus, each data point represents the mean of the three treatments (no plant matter, leaves, and moss; n = 9).

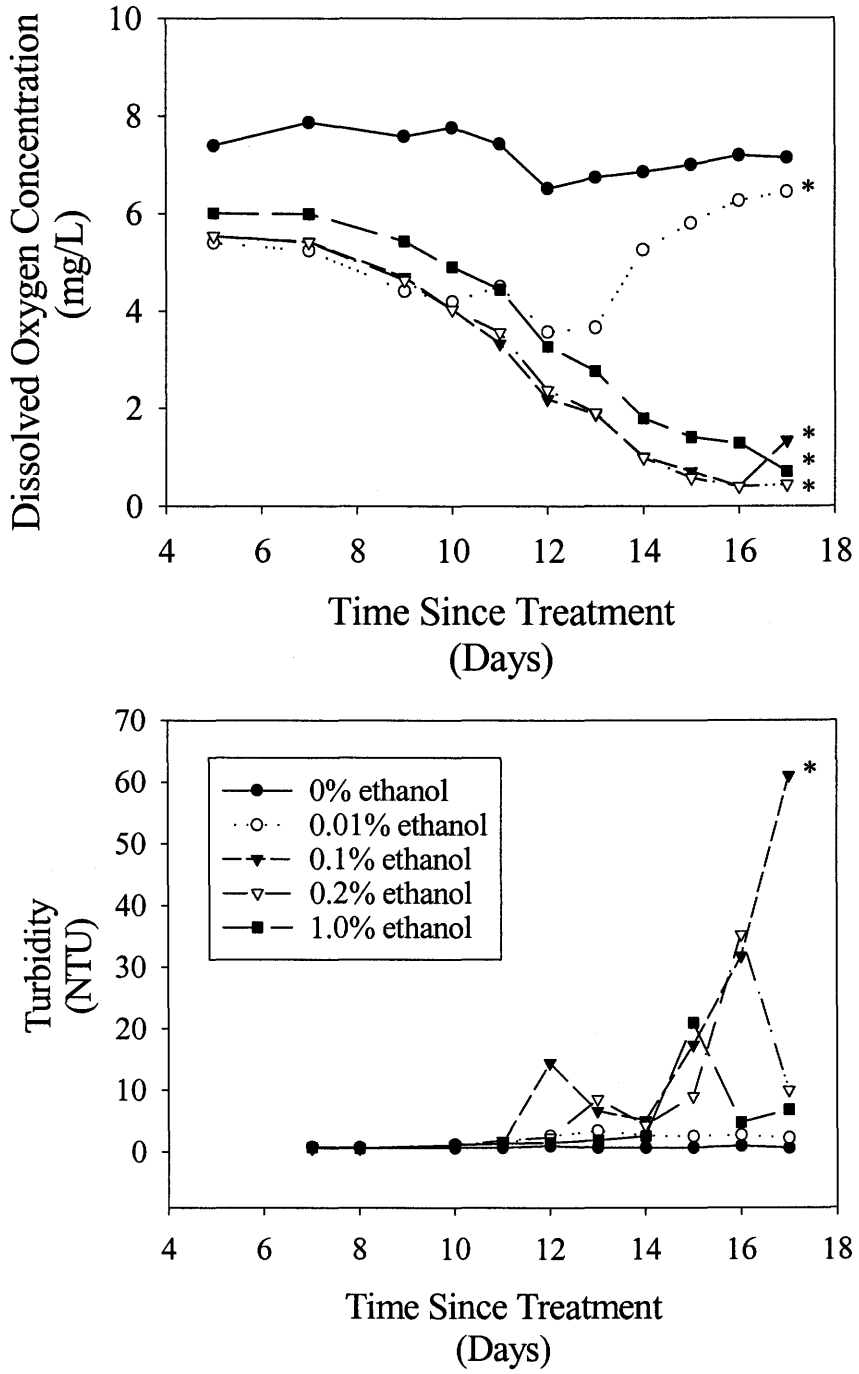

Figure 3.3. Dissolved oxygen (DO) concentrations and turbidity in pond water treated with various concentrations of ethanol. Data points are means of three replicates. Treatments were significantly different (one-way repeated measures ANOVAs; $p < 0.023$). Asterisks denote significant differences from the control group as determined by Dunnett's test ($p < 0.05$).

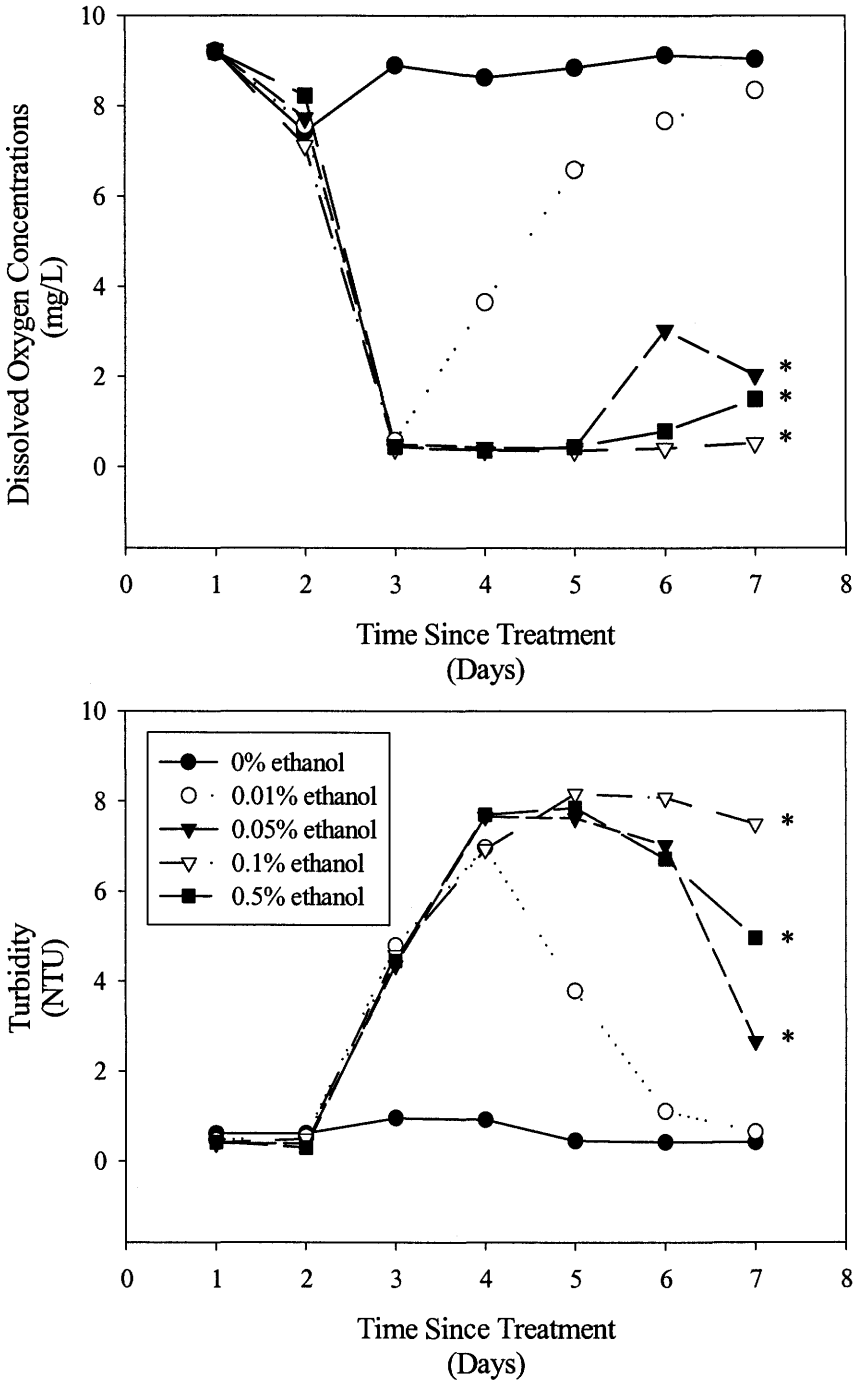

Figure 3.4. Dissolved oxygen concentrations and turbidity over time in ethanol-treated pond water. Values represent the mean of three replicates. Treatments were significantly different (one-way repeated measures ANOVAs; $p < 0.001$). Asterisks denote significant differences from the control treatment as determined by Dunnett's test ($p < 0.05$).

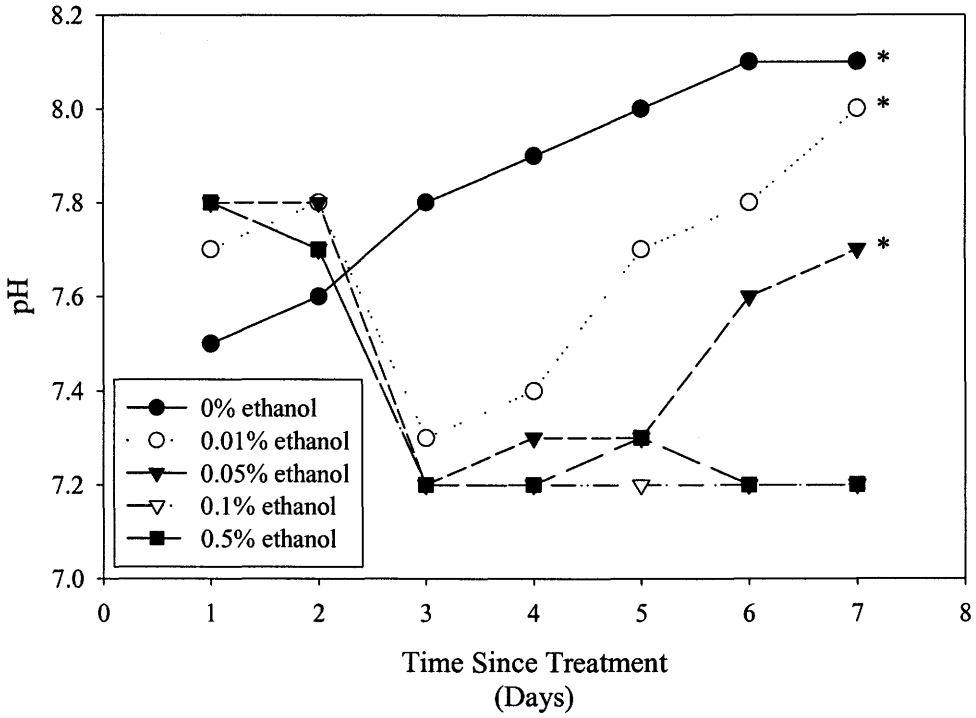

Figure 3.5. pH over time in ethanol-treated pond water. Values represent the means of three replicates. Treatments were significantly different (one-way repeated measures ANOVAs; $p = 0.002$). Asterisks denote significant differences from the control treatment as determined by Dunnett's test ($p < 0.05$).

Lindane did not significantly affect turbidity in the laboratory experiments (Friedman repeated measures ANOVA on Ranks, $p = 0.086$), which was below 4 NTU in all treatments throughout the experiment (Fig. 3.6). A significant effect of lindane was observed on DO (one-way repeated measures ANOVA, $p = 0.014$) but none of the treatments were significantly different from the control treatment (Dunnett's test; $p > 0.05$. It is possible that the 0.01% ethanol present in all lindane treatments caused this effect on DO. Ethanol significantly affected DO and turbidity in the treatments with 100 µg/L lindane (one-way repeated measures ANOVA, $p < 0.001$), but lindane had no effect on the response variables at day 17 of the experiment (two-way ANOVA, $p > 0.476$).

3.4 Discussion

3.4.1 Design and Testing of Microcosms

High mortality due to FV3 was observed in the 1998 microcosm study. It is a highly lethal virus; early-stage leopard frog tadpoles exposed to 2.7×10^6 to 4.5×10^6 plaque-forming units (PFU) died within 10 days with abdominal edema and dermal hemorrhages (Tweedell and Granoff 1968). While FV3 induces high rates of mortality in embryos, tadpoles, and recent metamorphs, it generally does not affect adult amphibians (Came *et al.* 1968; Tweedell and Granoff 1968). However, adults of certain amphibian species may be a possible source or reservoir and may infect susceptible young by shedding virus in urine during the breeding season (Mao *et al.* 1999). The source of the virus was unclear. Tadpoles were collected from three ponds and were added to three separate sets of microcosms, but dead tadpoles were removed from almost all of the microcosms. The design of future microcosm studies must take the potential of infection with FV3 into account when selecting the source of water, plants, and animals.

Although low survival of metamorphs made it difficult to gather meaningful data regarding the suitability of the microcosm style, the basic style (1.0 m x 1.0 m x 0.7 m) was chosen for use in the lindane experiments in 1999 and 2000 because it was easy to

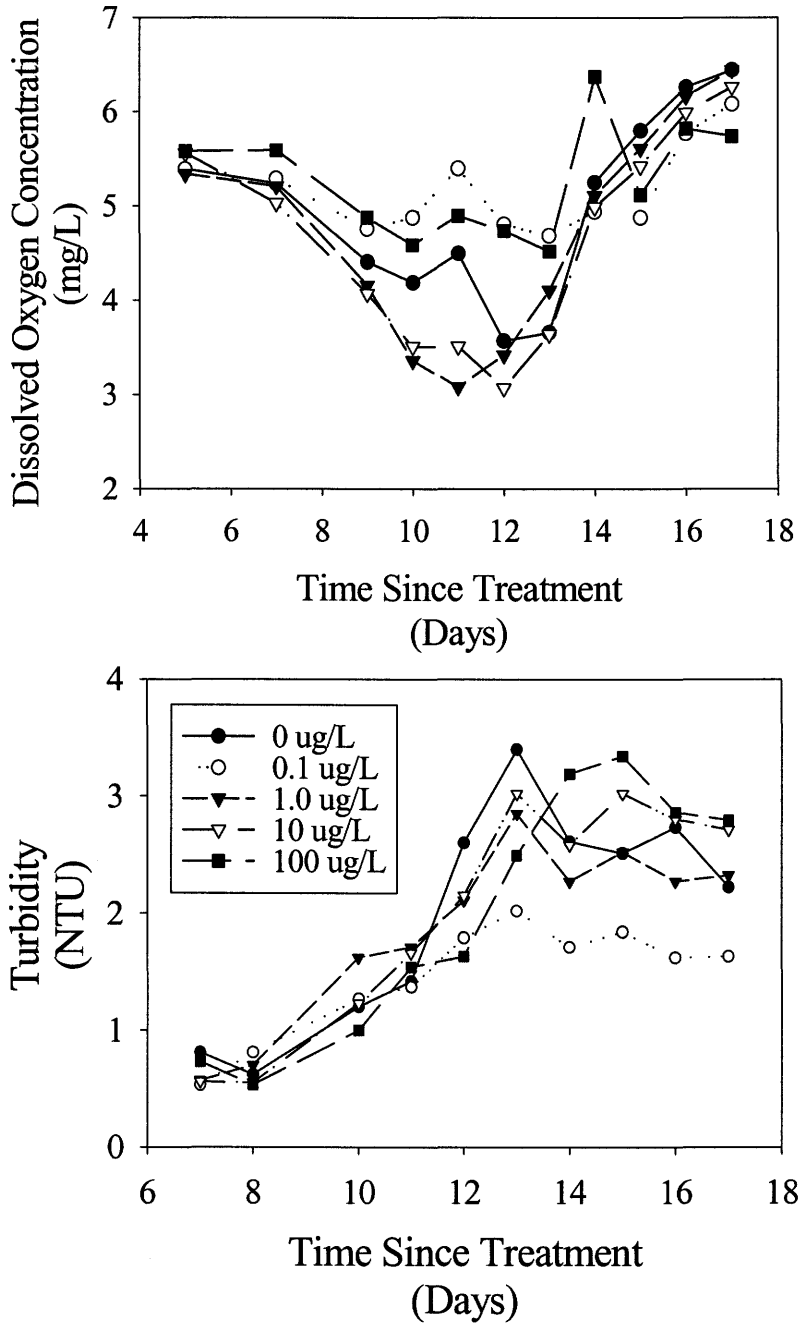

Figure 3.6. Dissolved oxygen concentrations and turbidity in pond water treated with various concentrations of lindane. Each treatment also contained 0.01% ethanol. Data points are means of three replicates. Dissolved oxygen significantly differed with treatment (one-way repeated measures ANOVAs; $p = 0.014$), but none of the treatments differed from the control treatment (Dunnett's test; $p < 0.05$). Effects on turbidity were not significantly different (one-way repeated measures ANOVAs; $p = 0.086$).

set up and because the surviving tadpoles seemed to grow as well in that style as in the others. Tadpoles of *R. sylvatica* were successfully reared in similar systems of outdoor plastic tanks that were as deep as 55 cm (Boone and Semlitsch 2001) and 95 cm (Goater and Vandenbos 1997).

The number of dead tadpoles removed from the microcosms did not account for the low number of metamorphs surviving to metamorphic climax. Metamorphs may have been captured by magpies that were observed sitting on the enclosure fence. As well, metamorphs, especially those with shorter tails, were observed in the folds in the plastic liners. They might have used these folds as ramps to climb up the sides of the microcosms. Steps that were taken in 2000 to correct these problems included adding a roof of netting to the enclosure, stapling the folds flat, and installing 15-25 cm wide strips of plastic as horizontal barriers around the top edges of the microcosms.

3.4.2 Hormones and Stomach Contents

Tail length has been used before as an indicator of development during metamorphic climax; Weil (1986) did not find a correlation between the ratio of tail length to SVL and thyroxine concentrations in *R. clamitans* metamorphs. The assumption that tail length was an accurate indicator of developmental stage was not supported by the data collected with wild-caught wood frog metamorphs, as the ranges of tail lengths in stages 43 and 44 overlapped. Therefore, while CORT concentrations did not vary with tail length, it did appear that they were lower in metamorphs at stage 42. However, the small sample size of metamorphs in stage 42 ($n = 4$) may not have given representative CORT values. As well, two of the values were inside the range of the stage 43 metamorphs. In the microcosm study of 2000 (Chapter Four), a trend of lower CORT in stage 42 metamorphs was not observed in the control microcosms, and mean CORT in stage 42 metamorphs (7.86 ± 4.19 ng/ml) was similar to mean CORT in stage 43 metamorphs (6.38 ± 2.52 ng/ml). However, there were only four metamorphs collected at stage 43. Mean CORT in control microcosm metamorphs of 2000 was also

slightly higher (18%) than that of the wild-caught metamorphs in 1999, perhaps reflecting the slight difference in stress protocols between the two years.

Examination of stomach contents indicated that metamorphs could have fed on insects before complete tail resorption, but that it was unlikely to occur in metamorphs before less than about one-third of the tail was reabsorbed. As well, the observation that metamorphs in stage 44 and 45 had insect matter in their stomachs indicates that metamorphs should be offered food after stage 44. However, microcosm metamorphs kept for glycogen analysis in 1999 and 2000 did not pursue live insects such as wingless fruit flies until after stage 45, which was similar to *Rana clamitans* metamorphs raised in the laboratory (Naitoh *et al.* 1989). The results of this study suggest that wild metamorphs may have been more likely to ingest insects prior to the end of metamorphic climax than microcosm metamorphs. Microcosm metamorphs may have been shy because of human disturbance during cage maintenance and feeding, and required time to become adjusted or required less energy than wild metamorphs.

3.4.3 Microcosm Study, 1999

The primary objective of the 1999 microcosm study was to investigate the effects of chronic, low-level exposure to lindane on developing wood frog tadpoles. This objective could not be assessed due to the confounding influence of the bacterial blooms on the survival and growth of the tadpoles. Low survival in the highest treatment was probably due to the extended period of low dissolved oxygen that was caused by the bacterial blooms. As well, the slight increase in size at MC with increasing treatment may have been due to the presence of bacteria as an alternate and possibly higher protein food item.

This effect of temperature on days to MC was most likely due to shading by the adjacent trees on the south side of the enclosure. Therefore, the microcosms were moved 3 m north of their original position for the 2000 microcosm study and treatments were assigned according to a randomized block design.

3.4.4 Ethanol as a Confounding Factor in the 1999 Microcosm Study

The experiment that compared the effects of ethanol, dried plant matter, and type of water on bacterial growth indicated that the bacterial blooms in the 1999 microcosms were not created by adding dried plant matter to the water. The strong growth of bacterial populations in the pond water + 0.2% ethanol treatment, and the fact that plant matter did not affect this response, indicated that ethanol alone encouraged bacterial growth in the pond water. It also suggested that the pond water, or water that has resident microbial communities, is more susceptible to the blooms than dechlorinated tap water, likely due to the near elimination of microbial communities by chlorination.

While ethanol affected DO and turbidity in the laboratory experiments, lindane had no effect on these variables. The experiments with bacterial blooms clearly indicated that ethanol, not lindane, can cause bacterial blooms in water that was very similar to the water used in the microcosms. As well, ethanol affected the growth of bacterial blooms, as measured by decreases in DO concentration and increases in turbidity, in a dose-related manner. It is possible that ethanol was used as a food source for the bacteria. The fact that 0.1% ethanol affected bacterial blooms the most in the laboratory experiment instead of the highest ethanol concentration tested, 0.5%, suggests perhaps that this higher ethanol concentration was slightly toxic to the bacteria.

The recovery of DO and clearing of the test vessels in the laboratory experiment with ethanol was similar to the apparent dose-related recovery observed in the microcosms. Low DO was first observed in the microcosms on the day after adding the lindane + ethanol stock solution. White cloudiness was observed within days, and gradually cleared from the microcosms over the course of the experiment. Only the highest treatment, with 0.2% ethanol, stayed turbid for the majority of the study. The DO in the second highest treatment, containing 0.13% ethanol, recovered to control levels within a month of lindane application, whereas the other treatments recovered sooner. The results of these laboratory experiments suggested that future microcosm studies should be conducted with the lowest concentration of ethanol necessary, such as 0.01% or lower.

Guidelines for conducting toxicity tests suggest that increasing solvent concentration with increasing treatment is acceptable (ASTM 1988; APHA 1992; Parrish 1995); however, the ethanol concentration exceeded the guideline of 0.05% in the two highest lindane treatments (ASTM 1988; APHA 1992). As well, the lack of a solvent control precluded the determination of the effects of the highest ethanol concentration on tadpoles.

Previous studies have shown that, at the concentrations used in the microcosms, ethanol is not acutely or chronically toxic to frog eggs or tadpoles. Using the FETAX assay, Dresser *et al.* (1992) calculated a NOEL for mortality as 1.25% and for malformation as 0.75 - 1.00%. They also calculated a NOEL for length as <0.25%, which is similar to the maximum ethanol concentration of 0.2% used in the microcosm study. Bullfrog tadpoles exposed to 0.75% ethanol for 2-4 months were arrested in their development, but were otherwise able to survive and function normally (Downes and Courogen 1996). A study of the acute effects of three organic solvents indicated that ethanol was less toxic than acetone, but recommended propylene glycol for use as a solvent in fish toxicity tests (Majewski *et al.* 1978). At 0.1%, acetone was found to delay development and reduce growth in premetamorphic tadpoles (Marchal-Segault and Ramade 1981).

The high mortality observed in the highest treatment (100 µg/L) of the 1999 microcosm study was unlikely to have been caused by lindane. Lindane has not been shown to be lethal at this concentration; acute toxicity tests with this species indicated that the median lethal concentration (LC50) of lindane to wood frog tadpoles was found to be greater than 1.90 mg/L (Chapter Two). Only one published study of chronic exposure of tadpoles to lindane exists; 79% of *Xenopus laevis* tadpoles exposed to 500 µg/L lindane throughout metamorphosis did not survive to forelimb emergence (Marchal-Segault and Ramade 1981). *X. laevis* tadpoles exposed to this concentration also experienced delayed development and decreased size at MC (Marchal-Segault and Ramade 1981). In contrast, metamorphs exposed to the 100 µg/L lindane treatment were larger at MC, and whereas one microcosm had no surviving tadpoles, another had

60% survive to MC. The cause of the mortality and larger size at MC may be explained by the occurrence of bacterial blooms, which significantly lowered DO past the tolerance limit of the tadpoles, and which may have provided a food source for the surviving animals and lessened the competition for available food. Experiments with bacterial blooms clearly indicated that the ethanol concentrations used in the microcosm study can cause bacterial blooms, and that these bacterial blooms can decrease DO concentration and increase turbidity. The lowest concentration of ethanol tested, 0.01%, initially caused a decrease in DO concentration and increase in turbidity, but the effect was not sustained. Therefore, future microcosm studies should be conducted with the lowest concentration of ethanol necessary, such as 0.01% or lower.

3.5 Summary

This chapter summarizes the development and evaluation of methods used in the microcosm study conducted in the summers of 1998 and 1999. In 1998, the unexpected and lethal nature of frog virus 3 showed the importance of carefully choosing the source of water, plants, and animals. A roof of netting on the enclosure and tactics to prevent metamorph escape maximized metamorph recapture in 1999. The influence of the differential shading by the adjacent trees on water temperature, and therefore on growth and development, was also noted. Observations with wild metamorphs in 1999 indicated that plasma corticosterone concentrations may not change over metamorphic climax and that metamorphs do not begin eating insects until the end of metamorphic climax. The effects of chronic low-level exposure to lindane could not be assessed with the data obtained from the 1999 microcosm study, due to the confounding influence of the bacterial blooms on survival and growth of tadpoles. The influence of ethanol on water quality was determined from laboratory experiments in 1999 and led to important improvements in experimental design for the 2000 microcosm study. These improvements included having a solvent control and using an ethanol concentration below that of the recommended guideline, 0.05% (ASTM 1988), to insure that the solvent does not promote bacterial blooms.

CHAPTER 4

THE EFFECT OF LINDANE ON TADPOLE SURVIVAL, GROWTH AND DEVELOPMENT: AN OUTDOOR MICROCOSM STUDY

4.1 Introduction

Recent reports of global declines and high numbers of malformed amphibians in some populations have alarmed scientists around the world. The causes of the declines and the malformations are as yet unknown, but it is likely that many factors contribute to both phenomena and that these factors vary among scenarios (Carey and Bryant 1995). Environmental toxicants such as pesticides may negatively impact amphibian populations by affecting disease susceptibility, larval growth and development, predator avoidance, endocrine homeostasis, or survival (Carey and Bryant 1995). Few studies have investigated the effect of environmentally representative concentrations of pesticides on tadpoles (Bishop 1992; Sparling *et al.* 2000).

Ouellet *et al.* (1997) reported hindlimb malformations in free-living anurans from agricultural habitats exposed to pesticide runoff in eastern Canada. More metamorphs exhibited severe ectromelia (absence of one or more limbs or parts of limbs) and ectrodactyly (absence of one or more digits or parts of digits) in farmland habitats (12.4%) than in unexposed habitats (0.7%) (Ouellet *et al.* 1997). Tadpoles exposed to organochlorine insecticides in laboratory and field studies developed axial skeleton and tail malformations (Cooke 1981).

Treatment of canola seed with Vitavax® for control of fungal diseases and flea beetles was commonly practiced by Saskatchewan farmers until 2002. The most likely component of Vitavax® to occur at significant concentrations in agricultural runoff is lindane, an organochlorine insecticide, that can persist for more than a year in prairie

ponds (Donald and Syrgiannis 1995). Lindane has the potential to accumulate in fatty tissues and has endocrine disrupting capabilities. Lindane has been shown to affect hormonal systems in mammals (Beard *et al.* 1997) and amphibians (Marchal-Segault and Ramade 1981). The lowest concentration (500 μg/L) tested by Marchal-Segault and Ramade (1981) on larval *X. laevis* caused bending of the tail, growth inhibition, darkened pigmentation, and a bump in the cranium. They suggested that endocrine disruption was taking place, perhaps due to inhibition of the thyroid. Lindane may also affect circulating corticosterone (CORT) concentrations. Chronic stress due to contaminants can keep CORT concentrations elevated and can reduce the ability of exposed organisms to elevate blood concentrations in response to acute stress (Hontela 1998).

Hepatic glycogen is the primary source of glucose, the cryoprotectant that wood frogs use to withstand freezing (Storey and Storey 1984) and is also an important source of energy during the winter (Pinder *et al.* 1992). Mudpuppies (*Necturus maculosus*) from sites contaminated with polychlorinated biphenyls and organochlorine pesticides had reduced corticosterone concentrations in response to a standardized acute stress treatment and depleted liver glycogen reserves relative to animals from reference areas (Gendron *et al.* 1997).

In amphibians, phenotypic sex is highly plastic because gonadal tissue is undifferentiated in early tadpole stages, and consists of an outer layer, the cortex, and an inner layer, the medulla (Foote 1964). In later stages, the cortex develops into the ovary, and the medulla develops into the testis (Foote 1964). Both the cortex and the medulla secrete substances which inhibit the activity and development of the other tissue, and thus phenotypic sex is determined by the dominance of one over the other (Foote 1964; Witschi 1971; Hayes 1998). These secretions are assumed to be steroidal compounds, and it is hypothesized that exogenous steroidal compounds may act on this undifferentiated gonadol tissue to influence sex differentiation and perhaps cause complete sex reversal (Hayes 1998). Recent research suggests that there may be an effect of contaminants on sex ratios in anurans, but no clear mechanism has been

elucidated (Reeder *et al.* 1998). Environmentally relevant concentrations of a herbicide, atrazine, has been shown to induce hermaphroditism and to demasculinize the larynges of male *X. laevis* frogs exposed as larvae (Hayes *et al.* 2002).

Stress during development, such as pesticide exposure, can affect an organism's developmental stability: its ability to reduce phenotypic variation resulting from genetic and environmental disturbances and to maintain developmental homeostasis (Clarke 1995). Departures from ideal symmetry can be used to assess the efficiency of developmental stability mechanisms. Fluctuating asymmetry (FA) is a common measure of developmental instability and is described by a normal distribution with a mean of zero. Measurement error (ME), relative to L-R differences, can also have a mean of zero and must be assessed in relation to fluctuating asymmetry in order to determine true FA.

Measurement of FA has been promoted as an early warning biomonitoring tool to detect effects of stressors on populations prior to the manifestation of more obvious effects, such as a decline in population size (Alford *et al.* 1999). In fact, two populations of frogs experienced large and prolonged increases in developmental instability two years before population declines were observed (as cited in Alford *et al.* 1999). The use of FA as an end point has been applied only to a few pesticide toxicity studies; a few studies with fish indicate that pesticide stress can increase levels of fluctuating asymmetry (Valentine and Soule 1973; Allenbach *et al.* 1999). Allenbach *et al.* (1999) examined the relationship between FA and pesticide stress in sand shiners (*Notropis ludibundus*) and mosquitofish (*Gambusia affinis*) exposed to lindane and parathion and found that adult fish with lower FA estimates took longer to die when exposed to a LC70 concentration, suggesting that they were more tolerant to the stress imposed by the pesticide (Allenbach *et al.* 1999). In another study, developing grunion (*Leuresthes tenuis*) fry that were exposed to concentrations of p,p'-DDT that ranged from 0.001 to 10.0 μg/L had FA estimates that increased with DDT concentration (Valentine and Soule 1973). There are currently no published studies known that use FA as an end point in investigating pesticide toxicity to amphibians.

The wood frog (*Rana sylvatica*) was chosen as the study animal because it is a common amphibian species on the prairies that breeds in semi-permanent ponds and whose larvae are likely to be exposed to agricultural pesticides in runoff. The effects of lindane on wood frog tadpoles were assessed with an outdoor microcosm study in the summer of 2000, using lindane in a range of concentrations that could occur in Saskatchewan ponds. The objectives of the study were to determine if chronic exposure to these low concentrations could lower survival; reduce size at and time to metamorphosis; affect other aspects of development, such as increased incidence of malformations, altered plasma hormone concentrations, skewed sex ratios, or higher fluctuating asymmetry; or affect hepatic glycogen concentrations at the onset of hibernation.

4.2 Methods and Materials

4.2.1 Microcosms

Tadpoles were exposed to lindane in outdoor microcosms that were set up on the campus of the University of Saskatchewan at a site that was relatively isolated from normal pedestrian traffic. The site, situated parallel to, and about 5 m south of, a row of trees, was enclosed by a secure six-foot chain-link fence (9 m x 6 m) to protect against vandalism and to exclude dogs, cats, and other large animals. A roof of plastic netting was installed to exclude predatory birds. Each microcosm consisted of four panels of oriented strand board, one cm thick, with 2 x 2 spruce nailed to the vertical and bottom edges. The panels were screwed together to form a bottomless box (1.0 m x 1.0 m x 0.7 m, L x W x H). A copper-based wood preservative and water repellent (Copper II, Recochem Inc., Montreal, Canada) was painted on the exterior surface of the walls that were in contact with soil. The 15 microcosms, arranged in a 3 x 5 grid pattern, were set 0.5 m into the ground to reduce temperature fluctuations. Each microcosm was lined with a sheet of clear reinforced polyethylene plastic (3 m x 3 m; 3.5 oz) that had been soaked in municipal tap water for at least 1 week to remove leachable plasticizers (Carmignani and Bennett 1976). They were filled with tap water to a depth of 0.5 m.

Tap water was used instead of pond water to minimize the probability of viral contamination in the microcosms. Folds in the plastic liners above the water level were stapled flush to the walls and strips of plastic (15 to 25 cm wide) were stapled along the top inside edge of the side of each microcosm to form a non-shading lip to prevent escape of metamorphs. The water was treated with 3 g of sodium thiosulphate (hypo-prismatic type, pentahydrated; Eastman Kodak Company, Rochester, USA) to neutralize chlorine and was left undisturbed for 24 - 48 hours before the addition of any plants or animals. Water depth was measured periodically to prevent low water levels from affecting tadpole development (Rowe and Dunson 1995; Laurila and Kujasalo 1999). It was necessary, however, to remove water from the microcosms once after rainfall to maintain the levels at 50-55 cm. Strips of oriented strand board, one cm thick and approximately 20 cm wide, were laid across the top of the microcosms in a north-south direction in order to provide shade for the tadpoles after mid-July.

Aquatic macrophytes and leaf litter were added to provide spatial complexity, a source of dormant stages of microorganisms, and a nutrient base for the aquatic community (Alford and Wilbur 1985; Wilbur and Alford 1985). The macrophytes were also expected to remove dissolved nitrogenous wastes of the tadpoles and provide shade and decomposing matter for tadpoles to eat. Filamentous green algae were added as a food source (Kupferberg *et al.* 1994). As a precaution against introducing FV3 into the microcosms, plants were either bought from out-of-province suppliers, or collected from areas with healthy tadpole populations. Algae, *Potamogeton crispus*, and *Hippuris vulgaris* were collected from wetlands and riverbanks associated with the Beaver River, near Beauval, Saskatchewan. Additional macrophytes cultivated in ponds outside the province were also purchased: *Myriophyllum prosperpinacoides* from Moore Water Gardens (Port Stanley, Ontario) and *Ceratophyllum demersum* from Dutch Growers Garden Centre (Saskatoon, SK). Plants were individually potted in ethanol-washed plastic pots, with the roots contained in a layer of garden soil sandwiched between layers of sand so that the garden soil was not in direct contact with the overlying water. Macrophytes, algae, and leaf litter were carefully inspected for predators such as beetles

and potential competitors such as snails. Fallen poplar leaves were collected in the boreal forest near Little Bear Lake, Saskatchewan, soaked in tap water, and dried before being added to the microcosms. The enclosure set-up and a typical microcosm are illustrated in Fig. 4.1.

4.2.2 Collection and Manipulation of Tadpoles

The experiment was initiated in late May 2000; however, an outbreak of frog virus 3 (FV3) occurred within 5 days and killed most of the tadpoles. The microcosms were therefore drained, washed with 10% bleach solution, scrubbed, rinsed, and re-filled with tap water, plants, and tadpoles for a second attempt. The treatments were assigned to the same microcosms used in the first attempt to avoid possible cross-contamination from the initial lindane treatment. Recently hatched tadpoles were collected in mid-June from two wood frog (*R. sylvatica*) egg masses, similar in stage of development, in a marsh adjacent to Cub Lake, a small lake located in the Cub Hills in the boreal forest area of northern Saskatchewan (Cub Lake Marsh; location given in Appendix A). The Cub Hills are at an elevation of about 730 metres and are easily accessible by the Hanson Lake Road. Relatively young tadpoles were found there late in the season, possibly because the high elevation and cool spring temperatures delayed wood frog breeding and egg development beyond that occurring in areas of lower elevation. There is no agricultural cultivation in the area and the closest agricultural cropland is located at least 80 km south. Thus, it was assumed that the marsh was relatively uncontaminated by pesticides, except for possible atmospheric transport (Donald *et al.* 1999; Waite *et al.* 2001). The tadpoles were between Gosner stages 23 and 25 (operculum development; Gosner 1960) when they were added to the microcosms. Forty wood frog tadpoles at the same stages, measured during the first study attempt, weighed 0.03 ± 0.001 g and were on average 16.1 ± 0.1 mm in length. Each microcosm contained 40 tadpoles, 30 from one egg mass, and 10 from the other. Lindane was added to the microcosms 7 days later, on June 19, 2000, when the tadpoles were 9-10 days old. Supplemental aquarium fish food (TetraMin®,

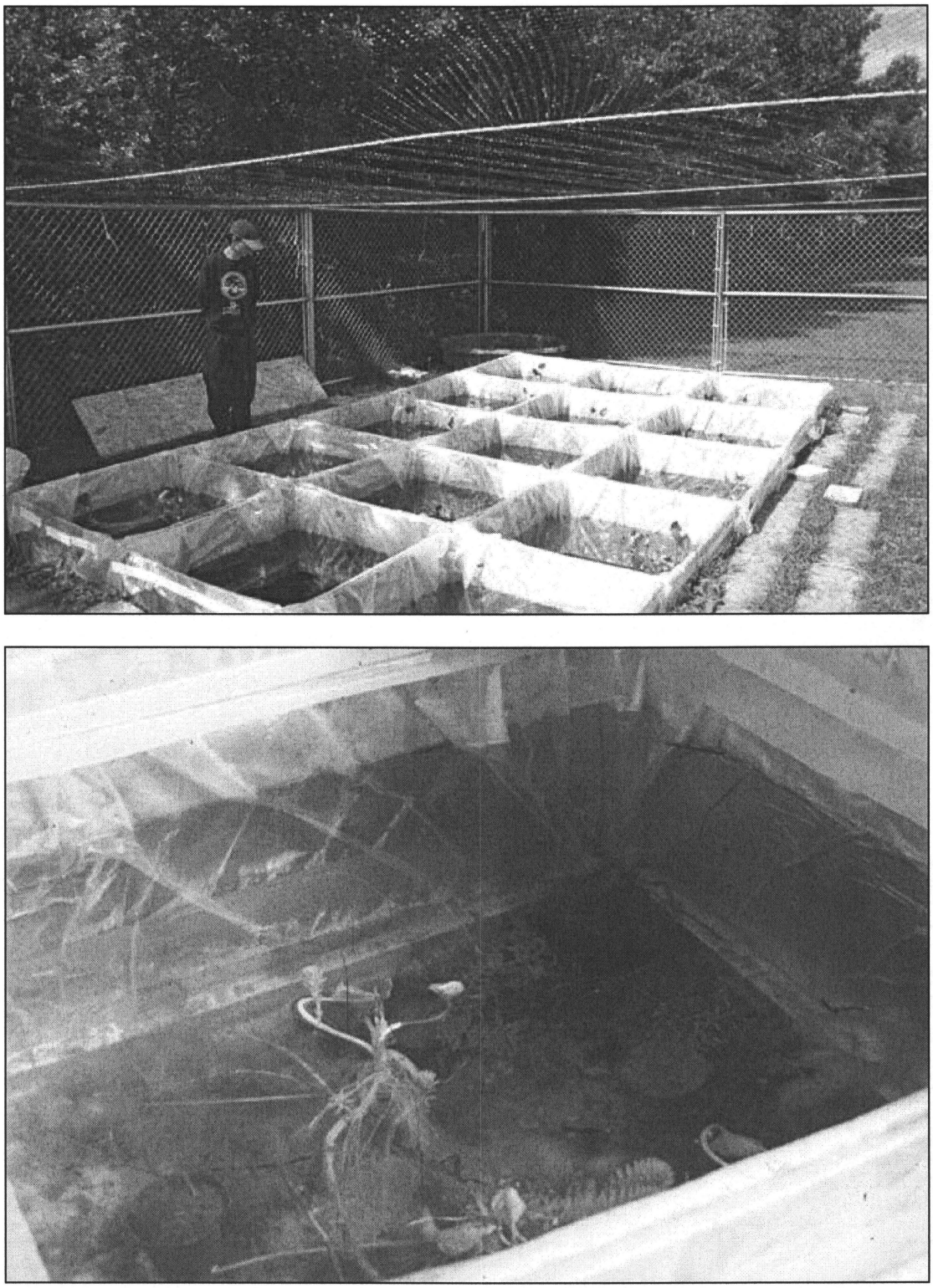

Figure 4.1. The enclosure set-up (top) and a typical microcosm (bottom) of the microcosm study. The photos were taken in the summer of 1999.

Tetra Werke, Germany) was provided for the tadpoles. This fish food was analyzed for contaminants such as organochlorine insecticides and herbicides and found to be uncontaminated. The protocol for collecting and euthanizing frog eggs, tadpoles, and metamorphs was approved by the Animal Care Committee of the University of Saskatchewan (UCACS Protocol ID Number: 19980139).

4.2.3 Lindane Application

Technical grade lindane (gamma-hexachlorocyclohexane, 99% purity; CAS # 58-89-9), purchased from Sigma-Aldrich Fine Chemicals (Oakville, Ontario, Canada), was dissolved in ethanol to form a stock solution. Serial dilutions were made from this stock solution and aliquots were added to the microcosms to achieve the target concentrations. Two control groups (negative and solvent) and three concentrations of lindane were tested: 0.1 μg/L, 1.0 μg/L, and 10 μg/L. The highest concentration found in Saskatchewan ponds by others was 0.4 μg/L (Donald *et al.* 1999). The two lowest concentrations bracket this value. The concentration of 10 μg/L is a representative value of potentially high concentrations present in small, shallow ponds receiving runoff from a large area of cropland (Appendix C). Adsorption of pesticide to plastic lining material has repeatedly reduced the concentration of pesticide in the water of aquatic enclosures by as much as 30% (Solomon and Liber 1988; Liber 1994). Therefore, in this study, 30% more lindane than the target concentration was added to each microcosm. Stock solution was poured into each microcosm, then gently and thoroughly mixed into the water column using a clean metre stick. The concentration of ethanol was kept constant at 0.001% throughout the treatments, including the solvent control. The negative control group did not contain either ethanol or lindane. The treatments were replicated three times and were assigned to the microcosms in a randomized block design to account for possible effects of shading from the trees along the south side of the enclosure (Section 3.3.3). Each of the three blocks, which were parallel to the trees, included all five treatments in a row from east to west. Blocks were numbered south to north (the block closest to the trees was block 1).

4.2.4 Water and Tissue Residues

Water samples were collected from the microcosms for lindane analysis in 1-L glass bottles at 24 hours, 7 days, 4 weeks, and 8 weeks post-treatment. Water was collected in an X-shaped pattern from five locations (at center and between center and corners) in each microcosm to produce one sample per microcosm. The sample bottle was uncapped below the water surface and held approximately 6 cm beneath the surface during sampling. The negative control treatment was sampled only at 24 hours; the solvent control treatment was sampled in the first three sampling periods. The low (0.10 μg/L) and mid (1.0 μg/L) treatments were sampled in all four sampling periods, and the high (10 μg/L) treatment was sampled at all times except 4 weeks. Pretreatment samples were not taken because of the cost of analysis. It was assumed that the 24-hr control samples would reflect any prior contamination. The samples were shipped to the analytical laboratory in Burlington, Ontario, on the day of collection or they were stored at 4°C for up to one day until shipping. Duplicate samples were also collected and stored at 4°C in Saskatoon in case of possible problems with shipping or analysis. One sample from the 0.10 μg/L treatment (collected at 7 days) was damaged during shipping, so its duplicate was analyzed instead.

The samples were analyzed by Environment Canada, National Water Research Institute, National Laboratory for Environmental Testing (NLET), Burlington, Ontario. Samples were preserved with dichloromethane (DCM) upon arrival. They were allowed to equilibrate to room temperature, spiked with 1,3 dibromobenzene and δ-HCH, and then liquid-liquid extracted with 3 x 100 ml of dichloromethane. Extracts were dehydrated with sodium sulphate and concentrated to 2 ml with hexane. Concentrated extracts were fractionated on 100% fully activated silica gel by elution with 30 ml of hexane followed by 60 ml of 1:1 DCM:hexane. Only the final fraction was retained, solvent-exchanged into iso-octane, and concentrated to 10 ml in iso-octane. The extracts were analyzed for alpha-hexachlorocyclohexane (α-HCH), beta-hexachlorocyclohexane (β-HCH), gamma-hexachlorocyclohexane (lindane; γ-HCH), and delta-hexachlorocyclohexane (δ-HCH) using a HP5973 gas chromatograph-mass spectrometer

fitted with a 30 m capillary column. Measurements were made by mass spectrometry using selected ion monitoring. One µl of final extract was automatically injected into a split/splitless injector (250°C). Chromatography conditions were 80°C isothermal for 1 minute, 5°C/minute to 250°C, 15°C/minute to a final temperature of 285°C, and isothermal for 5 minutes. Compounds were quantified using the slope from a five level calibration curve for standard concentrations that ranged between 2.5 to 500 pg injected. Calibration curves (quantification ion) were constructed using the m/z 183 ion response. Compound confirmation was based on a retention time match of ± 0.05 minutes and a relative fit of 80% between the ion abundance ratios for m/z 183, 181 and 109 (latter two being qualifier ions) of the analyte and standard material. Recovery of the δ-HCH surrogate ranged from 56% to 124% with a mean of 87%. Corrections were not made for extraction losses. Recovery of γ-HCH in lab-spiked reference samples ranged from 77% to 95% with a mean of 86.4%. The estimated method detection limit for lindane was 10 ng/L for the final extract volume of 10 ml.

Tadpoles were sampled prior to treatment and at 1, 4, and 6 weeks post-treatment for whole-body residue analysis. For the pretreatment sample, two tadpoles per microcosm were collected and pooled into one sample. Because the plastic was not replaced or washed with solvent to remove the adsorbed lindane resulting from the aborted experiment, the microcosms may have contained small amounts of lindane residue before treatment. Therefore, the pretreatment sample was not included in the analysis. Additional water and tissue samples were collected in the spring of 2001 from 10 ponds in the boreal forest of northern Saskatchewan in order to characterize the presence or absence of lindane in the area and particularly at Cub Lake Marsh, the collection site of the egg masses used in the microcosm study (see Section 4.2.9).

Post-treatment samples were pooled across replicates to create one sample (at least 1 g) per treatment at each sampling time. As the tadpoles grew, the numbers removed from each microcosm declined from five at 1 week post-treatment to two or three at 4 weeks post-treatment to zero to two by 6 weeks post-treatment. An extra tadpole was collected from one microcosm at the last two sampling times because these tadpoles

were smaller than those in other microcosms. At 6 weeks, one microcosm did not contain any tadpoles; an extra tadpole from another microcosm of the same treatment was collected in order to include three animals from this treatment.

One metamorph from each microcosm was kept outdoors in plastic containers as described in Section 4.2.7 until complete tail resorption (Gosner stage 46). They were then euthanized with Oragel® and specimens from the same treatment were pooled to create one sample to be analyzed for lindane concentration. A total of five samples was submitted and analyzed as described below. Four specimens were collected at the point of full tail resorption; all others were captured sooner and were maintained, without being fed, in plastic cages (as described in Section 4.2.7 for the glycogen study) until the tail was resorbed. Thus, except for four specimens, exposure to lindane ended with forelimb emergence. The time required for tail resorption varied among these metamorphs from 4 to 13 days (mean = 8.6 days). Frogs at the completion of the hepatic glycogen study were also analyzed for lindane residues. After removal of the liver, three carcasses from each treatment were pooled to make one sample per treatment.

Tissue samples were immediately frozen after collection and kept frozen at -20°C for 3 to 6 months until shipment for analysis at the National Wildlife Research Center, Canadian Wildlife Service, Hull, Quebec. Procedures for extraction, clean-up, and analysis are outlined in (Won *et al.* 2001). The samples were dehydrated with anhydrous sodium sulfate before lipid extraction with dichloromethane/hexane. Typically, the chemicals of interest are separated from lipids and biogenic compounds by gel permeation chromatography (GPC), but this step was not necessary due to the very low lipid content of the samples. Any residual lipids were removed by Florisil column chromatography. Quantitative analysis of lindane and DDE was determined via high resolution gas chromatography (GC) coupled to a mass spectrometry detection (MSD) system. Identification of target analytes was accomplished by comparing GC retention times and specific mass fragments known to be present in the spectra of authentic compounds. Quantitation was accomplished by comparing the intensity of targeted mass fragments in sample extracts to the same compounds in standard mixtures, injected

separately on the GC/MSD system. The detection limit was 0.0001 μg/g wet weight. Recovery of the ^{13}C-labelled internal standard of tetra-, penta-, and hexa-chlorobenzene ranged from 85.9% to 95.4% with a mean of 89.9%. The reported concentrations of lindane and DDE were not corrected for recovery. All concentrations are reported on a wet weight basis.

4.2.5 Water Quality

Water samples were collected an hour before the addition of lindane (pretreatment), and at 4 and 8 weeks post-treatment for analysis of ammonia and nitrate/nitrite ($NO_3 + NO_2$) concentrations, particulate and dissolved organic carbon, conductivity, hardness, and alkalinity. All analyses except hardness and alkalinity were performed by Environment Canada, Environmental Protection Branch, Water Quality Laboratory, Saskatoon, Saskatchewan. Hardness and alkalinity were analyzed using a Hach Digital Titrator Model 16900 (Hach Company, Loveland, CO, USA) at the Toxicology Centre, University of Saskatchewan. Dissolved oxygen concentrations (YSI Model 55 Dissolved Oxygen meter from YSI, Yellow Springs, OH, USA), pH (QuiKcheK Model 105 pH meter from Orion Research, Beverly, MA, USA), and mid and bottom water temperature were measured two or three times a week. Bacterial counts were performed on water samples collected at pretreatment and at 6 weeks post-treatment. Bacteria were fixed with 2% (v/v) formaldehyde and stored at 4°C for subsequent bacterial counts using epifluorescence microscopy (Fry 1988). The preserved bacteria were stained with 400 μl of 4',6-diamidino-2-phenylindole (DAPI), filtered onto a 0.2 μm pore-size, 25 mm Poretics Black Polycarbonate filter, and counted under 100x epifluoresence illumination. Total numbers of bacteria per unit of original sample were calculated from the sum count in the total field area used, the effective filtration area of the membrane, and the volume of sample filtered through the membrane (800 μl). Bacteria numbers were log-transformed prior to statistical analysis.

4.2.6 Survival, Growth, and Development

The exposure period ended at metamorphic climax (MC). Forelimb emergence (Gosner stage 42) is the first developmental stage of MC and is very easy to identify. However, it was difficult to sample all the metamorphs immediately following forelimb emergence because of the short duration of this stage (approximately one day), the ability of the metamorphs to hide in the vegetation and leaf litter, and the time limitations of the blood collection protocol: specimens were required every 5-10 minutes between noon and 6 p.m. Therefore, some metamorphs were collected after Gosner stage 42, but all were collected before Gosner stage 46 (complete tail resorption). Metamorphic climax did not occur at the same time in all metamorphs within the same microcosm, so the time to MC for each metamorph varied within microcosms. Metamorphs were collected from July 17 to September 13; the majority (88%) were collected between July 19 and August 18.

The microcosm study was terminated on September 13, 2000, when all remaining tadpoles were collected and euthanized. In total, 18 tadpoles did not complete the larval period; five of these had one arm emerged. The 0.1 µg/L treatment had one tadpole, the 1.0 µg/L treatment had five tadpoles, and all other treatments had four tadpoles. In terms of the block design, the numbers of tadpoles removed from block one (closest to the trees), two, and three were zero, eight, and 10, respectively. These tadpoles were not used in the analysis of survival, growth and development, blood hormone concentrations, or fluctuating asymmetry. Since the effect of lindane on sex determination would have occurred before MC, the tadpoles were included in the analysis of sex ratios. Survival was defined as the percentage of tadpoles surviving to MC and was estimated by dividing the total number of metamorphs removed by the number expected, which did not include tadpoles removed during the exposure period for lindane residue analysis.

The following endpoints were assessed for each metamorph: size at MC (total length, snout-vent length (SVL), wet weight), time to MC, and incidence of malformations. Malformations were classified according to (Meteyer 2000). Metamorphs were then divided among three groups for assessment of the other

endpoints: 1) plasma hormone concentrations (corticosterone and thyroid hormones) and fluctuating asymmetry (n = 280); 2) hepatic glycogen concentrations at the onset of hibernation (n = 36); and 3) lindane concentrations in tissues at the end of MC (n = 15; Section 4.2.4). The sex of the specimens in the first two groups was determined by identification of the gonads under a dissecting microscope.

If the metamorph was to be used for blood collection, it was subjected to a standardized acute stress protocol to minimize variance among samples. Acute stress, including handling and captivity, can affect corticosterone concentrations in plasma (Maser *et al.* 1980; Licht *et al.* 1983). Metamorphs were collected from the microcosms at 5-10 minute intervals. Once caught, individuals were placed in a 500 ml plastic container with 1-2 cm of microcosm water and vibrated on a shaded shaker table for 1 hour. The metamorph was then anesthetized with a drop of Orajel® (7½% benzocaine) rubbed onto the back of its head. Within 2-3 minutes, the subject lost its righting ability and could easily be turned onto its back and otherwise manipulated. During this time, the date of metamorphosis, body weight, SVL, and total length were recorded and any obvious external malformations noted. Using fine scissors, a cut was made through the midline abdominal vein near the posterior end of the abdomen. Heparinized microhematocrit capillary tubes were used to collect the blood that flowed from the vein. A syringe was not used to collect the blood because the vein was very small. One to two tubes of blood, each containing 50-75 µl, could be collected per specimen. The blood was kept cool until it was centrifuged for 2-3 minutes at ~3400 rpm (g-force = ~388); the plasma was removed and frozen until analysis. Ten samples (≥100 µl) for the thyroid hormones, triiodothyronine (T3) and tetraiodothyronine (T4), and seven to ten samples (≥50 µl) for corticosterone (CORT) were analyzed from each microcosm. Due to fiscal constraints, only three treatments were analyzed for the thyroid hormones: negative control, solvent control, and 0.1 µg/L.

Corticosterone (CORT) concentrations were quantified using radioimmunoassay (RIA) by Dr. T. Marchant, Department of Biology, University of Saskatchewan, according to the methods outlined in Kloepper-Sams *et al.* (1994) and Bortolotti *et al.*

(1996). Samples were prepared by extracting a measured volume of plasma with five volumes of diethyl ether. Each sample was extracted twice and the two ether extracts were pooled. The volume of plasma extracted ranged from 30 to 40 µl, depending on the original sample volume. Two samples had a lower extraction volume (15 and 25 µl) due to the limited volume of the original sample. Following evaporation of the ether, all samples were reconstituted with 125 µl of assay buffer and frozen at -20 C for use in the RIA. Extraction efficiency was estimated by extracting triplicate samples spiked with ^{3}H-corticosterone. Recovery of the ^{3}H-corticosterone from the spiked triplicates was high (89.2%, 91.1%, and 91.4%) with a low variability (CV = 1.3%) within the extraction procedure. Consequently, final hormone levels were not corrected for extraction efficiency. The corticosterone RIA was conducted using routine steroid RIA procedures with slight modifications to obtain maximum RIA sensitivity. Antiserum and purified corticosterone for the standards were purchased from Sigma Chemicals (St. Louis, Missouri), and the ^{3}H-corticosterone was purchased from New England Nuclear (Boston, Massachusetts). The antiserum was diluted so as to obtain specific binding of approximately 25%; non-specific binding was low at approximately 4%. Incubation of the diluted antiserum (200 µl) with sample extracts (diluted 1:4) or standards (final assay volume of 100 µl) and approximately 5000 CPM of ^{3}H-corticosterone (100 µl) was performed at room temperature for 24 hours. A dextran-coated charcoal stripping technique was then used to separate bound and free hormone. All samples were assayed in duplicate in a total of three separate assays, all performed on the same day. Assay variabilities were below the acceptable limit of 10% CV; between assay variability was 5.2%, and within assay variability was 3.5 ± 0.3% (mean ± SD). The detection limit was 0.86 ng/ml.

Thyroid hormone (T3 and T4) concentrations were quantified using RIAs by Dr. T. Marchant, Department of Biology, University of Saskatchewan, according to the methods outlined in Chopra (1972) and Kjeld *et al.* (1975). Samples were not extracted; therefore, the RIAs measured the total T3 and T4 concentrations. Antiserum and purified hormones for the standards were purchased from Sigma Chemicals. The

antisera were diluted so as to obtain specific binding of approximately 35% in each RIA; non-specific binding was low (6% or less). Incubation of the diluted antiserum (100 µl) with plasma samples (diluted 1:4 in the T4 RIA and 1:6.67 in the T3 RIA) or standards (100 µl) and approximately 12000 CPM of the appropriate radiolabelled hormone (300 µl) was performed at room temperature for 24 hours. A second antibody precipitation technique was then used to separate bound and free hormone. Most samples were assayed in duplicate in a single T3 or T4 RIA. However, some samples contained insufficient volume for a duplicate measurement. The lack of duplicate measurements will generally increase the variability of the experimental results and caution should be used when drawing overall conclusions. Ideally, the experiment should be repeated with larger sample volumes made available. Within assay variability was low for the T3 RIA at 7.9% (CV). However, within assay variability for the T4 RIA was high at 15.2%. Generally, an acceptable assay variability is a CV of <10%. The within assay variability for T4 TIA was higher than this because one of the internal standard measurements was higher than expected. Therefore, caution should be used in interpreting the results of this RIA. Ideally, this assay should be repeated. However, insufficient volume of the original plasma samples precludes this possibility. The detection limits were 773 pg/ml for T3 and 844 pg/ml for T4.

All metamorphs were preserved in 10% neutral buffered formalin for approximately 4 months before fluctuating asymmetry (FA) measurements were made. The metamorphs were removed from the formalin, soaked in tap water for a few minutes to 2 days, and blotted three times with paper towel before being measured. Four traits were measured six times on each specimen: femur length, tibiofibula length, radio-ulna length, and eye-naris length (Fig. 4.2). All six measurements for each trait were taken consecutively by the same person. The left (L) side was measured first, then the right (R), and the technician's thumb was flexed after each measurement. The six (L-R) estimates were averaged in the final analysis to reduce measurement error

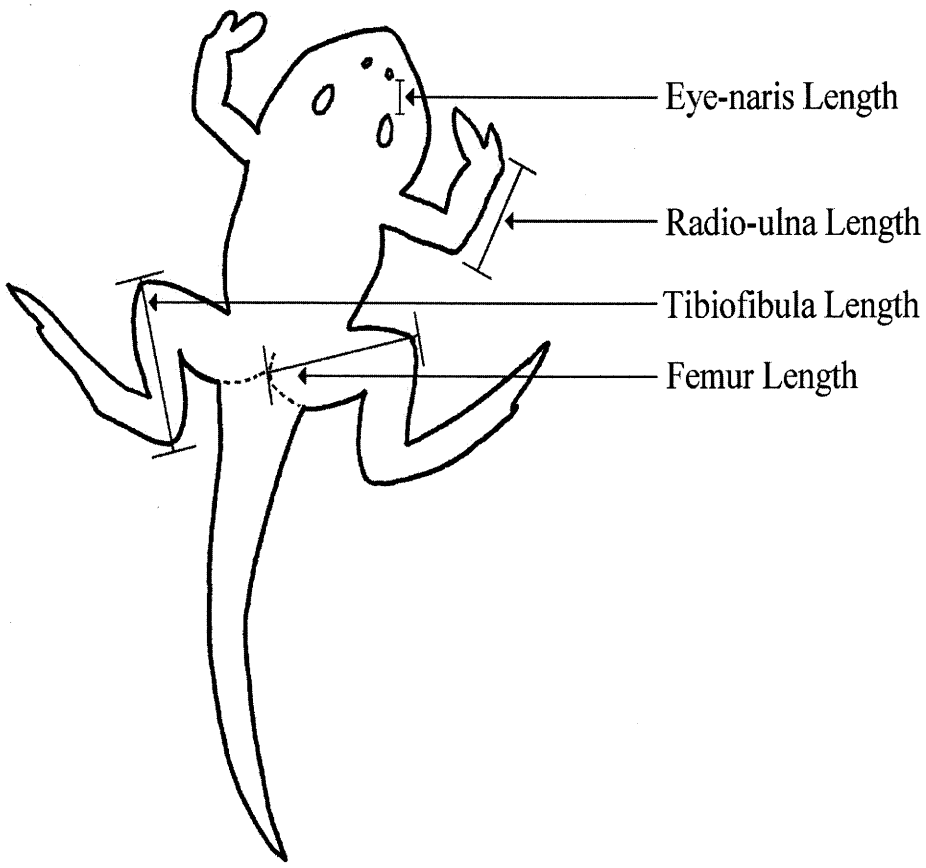

Figure 4.2. Illustration of the four external traits measured for fluctuating asymmetry analysis in the microcosm metamorphs.

(Yezerinac *et al.* 1992). Femur length was measured from the edge of the knee to the crease of the vent. This trait was most easily measured by laying the specimen on its back, and may have been affected by the sharpness of the vent crease. Tibiofibula length was measured from the knee to the ankle and had more obvious landmarks, but was difficult to measure if the legs were not fixed at a 90 degree angle. The radio-ulna length was measured from the elbow to the palm of the flexed hand. It was the most difficult to measure due to the lack of distinguishing landmarks, inflexibility of the limb, and small trait size. This trait was not measured on three metamorphs because the limb was missing or difficult to measure. The distance between the anterior edge of the eye and the corresponding posterior edge of the naris was termed the eye-naris length.

4.2.7 Hepatic Glycogen

Metamorphs intended for use in the glycogen study were not sampled for blood. Instead, they were raised individually in clear Sterilite® plastic containers outdoors from the day they were removed from the microcosms until 28-29 September 2000. During this time, they were fed wingless fruit flies, young grasshoppers, and Bertha armyworms. The containers measured 34 cm x 20 cm x 20 cm, and included plastic cups and wet newspaper to provide moisture and hiding places. The lid of each container had an approximately 10 cm x 24 cm hole covered with fabric mesh screening (sheer curtain material or fine mosquito netting) attached with hot glue. Each container also had a hole of approximately 6 cm x 1.5 cm at one end adjacent to the bottom to allow excess water to drain out. Mesh screening was also hot glued over the outside of this hole. A cooled strip of hot glue lined the top rim of the container so that the lid would fit snugly to prevent escape of frogs or uneaten insects. A total of 36 metamorphs from three treatments (negative control, solvent control, 10 µg/L treatment) was reared in cages for glycogen analysis. The animals were not exposed to lindane in the water during this period.

The onset of hibernation in native frogs was estimated to occur in late September. Therefore, the frogs were processed in groups of six between noon and 4:00 p.m. on 28-

29 September 2000 (18 frogs per day). They were anesthetized with Oragel® for 3 minutes, then decapitated. The livers, consisting of three to four lobes, were immediately removed, weighed, and all but one lobe placed in liquid nitrogen. One lobe of liver was placed in 10% neutral buffered formalin for possible future histopathology. Since hepatic glycogen reserves are related to body condition, body weight (before and after removal of liver), liver weight, SVL, weights of fat bodies, and sex were recorded. Fat-body and liver sizes are good indicators of the nutritional state of the organism (Jørgensen 1992). Fat bodies were located at the anterior end of each gonad.

Hepatic glycogen was analyzed by the procedure reported in (Wells and Bevier 1997), a modification of that given in (Keppler and Decker 1985). A sample (0.01 g to 0.04 g) of each liver tissue was weighed while still frozen, added to a 1.5 ml microcentrifuge tube, and homogenized with 100 parts cold 0.6 N perchloric acid (BDH Inc., Toronto, Canada) using a hand-held motorized pestle mixer. In order to determine total hepatic glucose content, 0.2 ml of homogenate was incubated with 1.0 ml of amyloglucosidase (EC 3.2.1.3; AGS) in acetate buffer (0.2 M; pH 4.8) in a 40°C shaking water bath for two hours. The AGS was purchased from Sigma-Aldrich Fine Chemicals (Sigma # A-3514). To prepare the AGS in acetate buffer, 1 ml of enzyme (13 mg protein per ml; 53 glycosyl units produced by each mg of protein) was dissolved in 9 ml of 0.2 M acetate buffer (4.8 ml of 96% acetic acid and 9.75 g of sodium acetate dissolved in Milli-Q water and made up to 1000 ml). Upon completion of the incubation period, the enzymatic reaction was stopped by adding 0.5 ml of 0.6 N perchloric acid to each sample. To neutralize the acid, 0.75 ml of 1.0 M potassium hydrogen carbonate (BDH Inc., Toronto, Canada) was added. The sample was then transferred to centrifuge tubes and centrifuged at ~4,000 rpm for 20 minutes. The supernatant was removed and frozen for use in the glucose assay. In order to determine free hepatic glucose content, the 1.0 ml of the remaining homogenate was centrifuged at 4°C at 4500 rpm for 20 minutes and the supernatant neutralized with 0.05 g of solid potassium hydrogen carbonate and frozen.

Glucose concentration was measured using a Sigma Glucose Diagnostic Kit (510-A) and a Cary100Bio UV-Visible Spectrophotometer at the wavelength of 450 nm. A mixture containing glucose oxidase, peroxidase, and o-dianisidine was added to aliquots of the thawed total and free glucose solutions from each liver sample and incubated in a 37°C shaking water bath for 30 minutes. This mixture is the basis of the coupled enzymatic, colourimetric assay as developed by (Raabo and Terkildsen 1960). Glucose oxidase catalyzes the oxidation of glucose and water to produce hydrogen peroxide. With the help of peroxidase, the hydrogen peroxide oxidizes the o-dianisidine, forming a brown colour. The final colour intensity is proportional to the glucose concentration. The colour intensity is assessed by measuring the absorbance of the solution at 450 nm. The glucose concentrations of the samples were determined by comparing their absorbances with that of known glucose concentrations (total and free glucose standards).

Each liver was assayed at least twice using different pieces of tissue. Concentrations of total and free glucose for each sample were analyzed in triplicate. Mass-specific tissue glycogen content, expressed as milligrams of glycogen per gram of wet tissue, was calculated for each sample by subtracting free glucose from total glucose and then converting from glycosyl units (molecular weight = 162 g/mol). Glycogen concentrations of samples for each liver were averaged and these averages were used in the statistical analysis. A range of standard concentrations was prepared with glycogen from rabbit liver (Sigma-Aldrich Fine Chemicals, Type III, G-8876). Frog glycogen is similar to rabbit glycogen with respect to molecular weight, attack by β-amylase, and type of bond at the branching point (Afanasyeva and Stepaneko 1956 as cited in Brown 1964). An aliquot of the highest standard concentration (hereafter called the manipulated standard) was subjected to the same manipulations and dilutions as the liver samples in order to determine recovery. As with the liver samples, all glycogen standards were hydrolyzed by incubation with AGS in acetate buffer and the resulting glucose production quantified enzymatically using the Sigma glucose diagnostic kit. Absorbances of the standards were assessed in duplicate (manipulated standard) and

triplicate (all other standards). Actual total glucose for the manipulated standard was calculated by interpolating from the standard curve and then compared with the known glucose content to calculate recovery. Recovery ranged from 80.4% to 97.3% with a mean recovery of 86.9% and a coefficient of variation of 6.1%.

4.2.8 Aberrant Microcosms

Data from two microcosms were not used for analysis of survival, growth, or development; potential confounding factors affected the health of the animals in these microcosms and limited the ability to determine the effects of lindane. One microcosm (1.0 µg/L, block 1, microcosm #7) had significantly lower water conductivity due to experimenter error during set-up. All microcosms were filled with equal volumes from two different faucets that yielded municipal water, however, microcosm #7 was drained (because the water was cloudy from the presence of mud) and refilled from only one of the original faucets. Metamorphs from this microcosm had significantly shorter larval periods and were significantly smaller in size at MC than in all other microcosms. As well, the period of time in which the metamorphs started MC was significantly shorter than for other microcosms. Metamorphs from one microcosm in the solvent control treatment (block 1, microcosm #1) exhibited symptoms of an unidentified disease. Symptoms included low survival, dwarfed hind-limbs at metamorphic climax, undersized and yellow liver, and lack of red colouring in the blood. Dead tadpoles were removed from this microcosm throughout the study. Pathological examination did not reveal any physical alteration or metacercariae in the limb, but did note an unusual ossification (T. Bollinger, personal communication). Data from these microcosms were used in the analyses of water quality, and water and tissue residues.

4.2.9 Comparison Between Microcosms and Ponds

Water quality and size of metamorphs at MC were compared between the microcosms and six semi-permanent or permanent ponds (Stewart and Kantrud 1971) that contained wood frog tadpoles. Six ponds near Saskatoon were selected in 1999:

three in croplands and three in uncultivated lands (pastures and designated wildlife areas) at least 1 km from areas of possible pesticide use. However, all of the ponds in the uncultivated lands were dry at the start of the 2000 field season. It was difficult to find ponds that contained tadpoles, so suitable replacements were not found until 1 to 4 weeks (23 June to 10 July) after the start of the second run of the experiment. Two of the cropland ponds were dry before the end of the larval period. Therefore, only the seven ponds from which data for water quality and size of metamorphs were obtained in both 1999 and 2000 were included in the analyses. The ponds were similar in size at approximately 0.1 - 0.5 ha and had an average maximum water depth of about 1.0 m (0.5 to 1.5 m, depending on time of year). Aquatic vegetation was also similar among the ponds, and included cattails, sedges, reeds, pondweed, and duckweed to varying degrees. Brief descriptions of the ponds are given in Appendix D.

Water samples were collected approximately at the start of the microcosm study and every 4 weeks thereafter. Ammonia and nitrate/nitrite ($NO_3 + NO_2$) concentrations, and conductivity were analyzed by Environment Canada, Environmental Protection Branch, Water Quality Laboratory, Saskatoon, Saskatchewan. Hardness and alkalinity were analyzed by the student as described in Section 4.2.5. Mid and bottom water temperatures in approximately 50-70 cm of water (or lower, if the water level was low) as well as pH just below the surface were measured at the site. It was not feasible to take water quality measurements in the ponds on the same days as in the microcosms. While water temperatures in the microcosms were consistently recorded in the mid-afternoon, temperatures in the ponds were recorded between late morning and late afternoon. Metamorphs were collected with dip-nets, measured for weight, total length, and SVL, then released at the site. Developmental stages of the wild metamorphs were not recorded.

4.2.10 Lindane in Boreal Ponds

Water samples were collected from four ponds in northern Saskatchewan in May-June 2000 for lindane residue analysis: Ralph Pond, Airport Side Pond, Grassy Pond,

and Cub Lake Marsh (locations of the ponds are given in Appendix A, except for Ralph Pond, which is located at the entrance to Little Bear Lake Resort, SK). Eggs and tadpoles from the first three ponds were used in the acute toxicity tests (Chapter Two) and in the 1999 microcosm study (Chapter Three). Water samples were stored at 4°C for 3 days, except the pond samples from Ralph Pond that was stored for 4 weeks before shipping. Water samples were analyzed according to the methods previously outlined in Section 4.2.4. Recovery of δ-HCH for the pond samples ranged from 71% to 106% with a mean of 84%.

Water and tissue samples were also collected in May 2001 in order to determine whether lindane was present in pond water and wood frog eggs in the boreal forest of Saskatchewan and therefore whether lindane could have been present in the microcosm tadpoles prior to treatment. Samples were collected over 3 days from 10 roadside ponds along a route that extended from Highway 155 north of Ile-à-la-Crosse to Highway 106 west of Deschambault Lake and spanned the entire length of Highway 165. All ponds except the two near Little Bear Lake were at least 30 km apart and were located between 54°-56° latitude and 103°-109° longitude (Appendix A).

Water samples were collected and preserved according to the methods described in Section 4.2.4 and analyzed according to the NLET CAEAL (Canadian Association for Environmental Analytical Laboratories) accredited method 03-3251 as described in Environment Canada (2000). A brief summary of the method is as follows: 1-L water samples were liquid/liquid extracted with dichloromethane, fractionated on 3% deactivated silica gel and quantified using dual capillary column gas chromatography with electron capture detectors (ECD). The method detection limits in water were 0.34 ng/L for α-HCH , 0.34 ng/L for β-HCH, and 0.25 ng/L for γ-HCH. Three to eleven halves or whole masses of wood frog eggs were collected from each pond and placed in ethanol-rinsed 10-L plastic pails with pond water. The pails were packed in ice or snow for the duration of the trip; upon return to the laboratory, air bubblers were added. Tadpoles were collected over 5 days because the egg masses hatched at different rates. Recently hatched tadpoles were gently pipetted into a clean nylon strainer to remove

excess water, placed in acetone-rinsed glass jars, then frozen with aluminum foil lined lids. Cool, ultra-pure Milli-Q water was added to each jar after freezing to prevent desiccation of the tissue. Tissue samples were immediately frozen after collection and held at -20°C for 10 days until shipping to the National Laboratory for Environmental Testing (NLET), Burlington, Ontario. NLET Method 03-3851 was used to determine the concentration of organochlorine (OC) pesticides in biota (OC1-B). The method involved extracting the homogenized tissue sample with dichloromethane using a polytron homogenizer. The concentrated extract was applied to a gel permeation column for the removal of lipids, concentrated, cleaned up, and fractionated on a 3% (w/w) deactivated silica gel column, and then reconcentrated to a final volume of 10 ml prior to analysis. Dual column capillary gas liquid chromatography with electron capture detector was used to quantify the concentrations of the following OCs (with detection limits in parentheses): α-HCH (0.34 ng/g), hexachlorobenzene (0.88 ng/g), γ-HCH (0.25 ng/g), heptachlor (1.81 ng/g), aldrin (0.71 ng/g), heptachlor epoxide (0.37 ng/g), γ-chlordane (0.45 ng/g), α-endosulfan (1.49 ng/g), α-chlordane (0.41 ng/g), dieldrin (0.67 ng/g), p,p'-DDD (2.32 ng/g), o,p'-DDT (0.82 ng/g), p,p'-DDT (3.02 ng/g), p,p'-methoxychlor (3.66 ng/g), and mirex (2.14 ng/g). Recoveries were assessed with 1,2,4,5-tetrabromobenzene (1%), δ-HCH (1%), and endrin ketone (1%), and were 84-102%, 77-87%, and 64-99%, respectively.

4.2.11 Statistical Analysis

The dissipation of lindane from the water column in each treatment was characterized by plotting the decline in lindane concentrations (C) on a logarithmic scale against time (t) and calculating the equation of the line:

$$\ln C = \ln C_0 - k_d t \qquad (4.1)$$

where C_0 is the initial lindane concentration. The slope of the line, k_d, was used to calculate the half-life ($t_{1/2}$) with the following equation:

$$t_{1/2} = (\ln 0.5) / k_d. \quad (4.2)$$

The slopes of the three lindane treatments were statistically compared using an analysis of covariance (ANCOVA). When the measured value of lindane was below detection limit (0.010 µg/L), the value was assumed to be 0.010 µg/L for the purposes of statistical analysis.

Significant differences in water quality parameters according to treatment were analyzed using one-way repeated measures analysis of variance (one-way RM ANOVA). Survival was analyzed using one-way ANOVA. Size at and time to MC, and plasma hormone concentrations in microcosm metamorphs were analyzed using two-way ANOVA with block and treatment as factors (n = 2 or 3). Hormone concentrations that were measured as below the detection limits as stated in Section 4.2.6 were assumed to be equivalent to the detection limit for the purposes of statistical analysis. Sex ratios among treatments were analyzed with the Chi-square test according to Zar (1984).

Estimates of FA were calculated by determining the mean left minus right (L-R) repeated measurements for each metamorph. Directional asymmetry was assessed on FA estimates with one sample t-tests (PROC MEANS, SAS). Departure from normality (antisymmetry) was assessed with the Kolmogorov-Smirnov test (Palmer 1994) using the SigmaStat program. The Sequential Bonferroni procedure was applied to correct for Type I error among comparisons (Palmer 1994). Data were not transformed to reduce positive skew or to force the distribution to be normal. Parametric statistics are robust at sample sizes greater than 25 despite some departures from normality or heterogeneity of variance (Gangestad and Thornhill 1998). Transforming the data may alter the nature of the relationship among the variables and reduce power without significant decrease in the probability of Type I error.

A dependence of asymmetry on trait size was assessed graphically on within and among samples with plots of |L-R| against mean trait size and (L-R) against SVL, and log variance (L-R) against mean trait size, respectively. No size dependence on FA was

observed.

A nested ANOVA (PROC NESTED, SAS) was used to partition out among and within variance in (L-R) measurements for each metamorph within a treatment and for all metamorphs combined for each trait. Percent measurement error (%ME) was calculated according to the following formula (Yezerinac et al. 1992):

$$\%ME = [s^2_W / (s^2_W + s^2_A)] \times 100 \quad (4.3)$$

where s^2_W is the within-individual component of variance and s^2_A is the among-individual component of variance. Percent measurement error was also calculated for each trait side (L and R).

Signed (L-R) differences were converted to absolute FA estimates (|L-R|) for analysis of lindane treatment effects on fluctuating asymmetry. By taking the absolute values of the FA estimates, the differences among treatments could be assessed by comparing means using a two-way ANOVA (treatment x block).

Statistical analyses were conducted using either SAS 6.12 (SAS Institute, Cary, NC, USA) or SigmaStat® Statistical Software, version 2.03 (SPSS Inc., Chicago, IL, USA). The Tukey test was used as a multiple comparison procedure if analysis of variance indicated significant differences among treatments. All tests were conducted using $\alpha = 0.05$. Graphs were plotted and linear regressions calculated with SigmaPlot® Graphing Software, version 3.06 (SPSS Inc., Chicago, IL, USA). Mean values in the text were expressed as ± one standard deviation (± SD), unless otherwise stated.

4.3 Results

4.3.1 Lindane Residues

Only the gamma isomer of HCH (lindane) was detected in the water samples and the concentrations measured at 24 hours closely matched the nominal values of 0.1, 1.0, and 10 µg/L (Table 4.1). Lindane was not detected in the control samples until 1 and 4 weeks post-treatment, when the solvent control samples contained lindane

Table 4.1. Lindane (γ-HCH) concentrations in the water of the microcosm study. Values are means ± SD (n = 3 unless otherwise stated).

Target Lindane Concentration (µg/L)	Measured Lindane Concentrations (µg/L)			
	24 hours	1 week	4 weeks	8 weeks
0.0 (Negative)	< 0.010 [1]	--- [2]	---	---
0.0 (Solvent)	< 0.010	0.017 ± 0.003	0.021 ± 0.003	---
0.13	0.10 ± 0.02	0.090 ± 0.009	0.050 ± 0.006	0.015 ± 0.008 [3]
1.3	0.81 ± 0.03	0.70 ± 0.03	0.31 ± 0.04	0.17 ± 0.04
13.0	9.9 ± 0.2	8.3 ± 1.1	3.9 [4]	2.0 ± 0.2

[1] The detection limit for γ-HCH was 0.010 µg/L.
[2] No samples were collected.
[3] Two out of three of the microcosms had lindane concentrations below the detection limit and were assumed to be 0.010 µg/L for the purposes of calculating mean and standard deviation (SD).
[4] Only one microcosm was sampled.

Table 4.2. Modeled first-order dissipation kinetics ($C_t = C_0e^{-kt}$) of lindane in the water of the three lindane treatments, where t is the time in days. There were no significant differences among the slopes (ANCOVA, p = 0.09).

Nominal Lindane Concentration (µg/L)	r^2	C_0 (µg/L)	k (/day)	95 % CI [1]	$t_{1/2}$ (days)
0.1	0.91	0.12	0.037	0.029 - 0.045	18.7
1.0	0.96	0.82	0.030	0.025 - 0.034	23.2
10.0	0.99	10.1	0.029	0.027 - 0.032	23.7

[1] 95 % Confidence Intervals for the slope ($k \pm [t_{0.05(2),n-2} \times SE]$). Standard error (SE) was estimated using PROC REG in SAS.

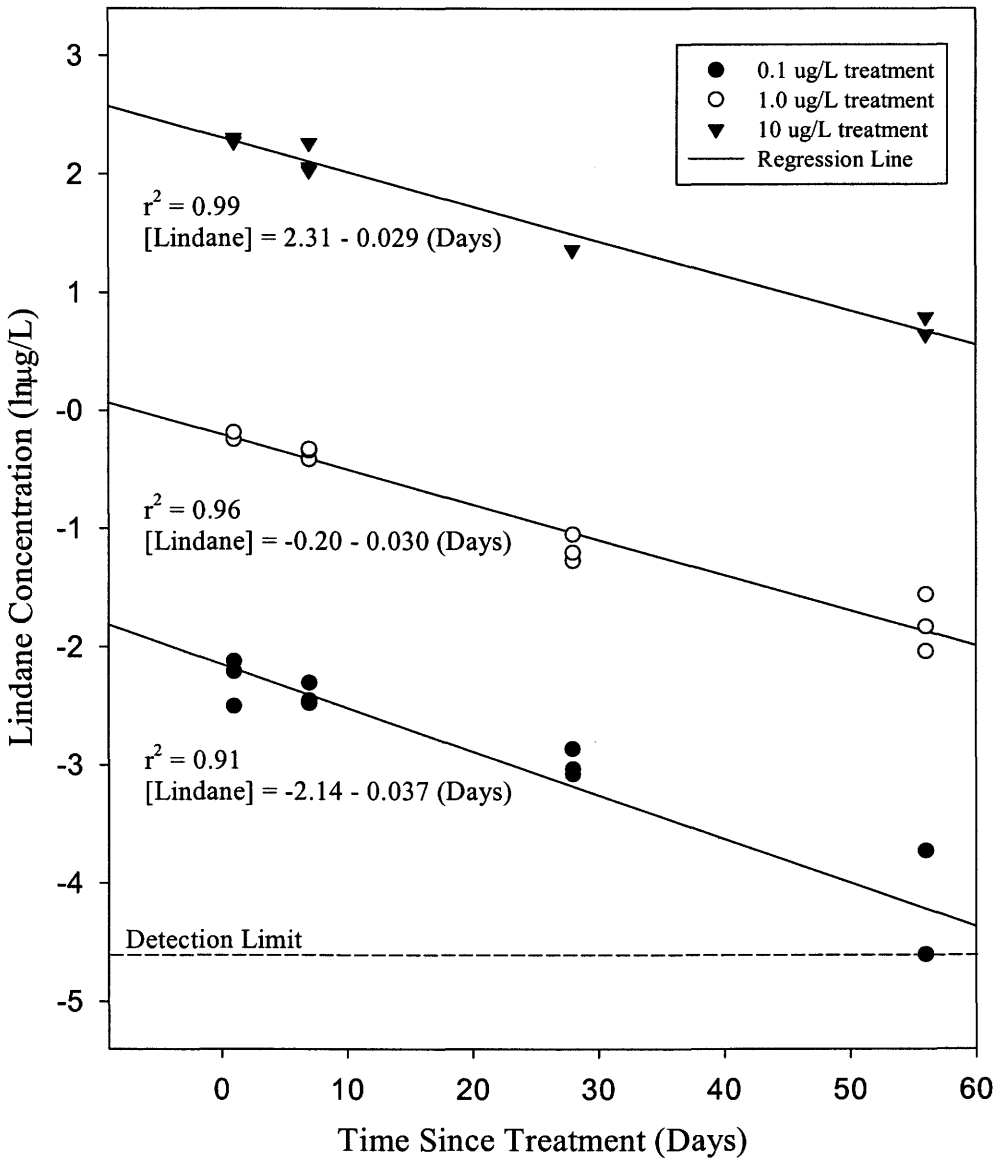

Figure 4.3. Dissipation of lindane from the water column in the microcosm study. Solid lines are first-order kinetic functions fitted to the lindane data. Each point represents one or two microcosms. Dashed line is the analytical detection limit for lindane analysis. There were no significant differences among the regression slopes (ANCOVA, p = 0.0858).

concentrations of approximately 0.02 µg/L. Dissipation of lindane from the water column was similar among treatments (Fig. 4.3). A first-order kinetic model ($C_t = C_0e^{-kt}$) was fitted to the lindane water residue data with the resulting equations having r^2 values of 0.91 to 0.99 (Table 4.2). The mean half-life was 21.9 ± 2.8 days. Two samples from the 0.1 µg/L treatment at week 8 were reported to be below detection limits (0.010 µg/L) and were considered to be 0.010 µg/L in the statistical analysis. There were no significant differences among the slopes of the regression lines (ANCOVA, $p = 0.0858$).

Lindane was present in the tadpoles from the control microcosms at 1, 4, and 6 weeks at concentrations ranging from 0.8 to 5.1 ng/g, and was higher at 6 weeks than at earlier sampling times (Table 4.3). Tissue concentrations in lindane-treated tadpoles at any sampling time were 35 ± 2 times nominal water concentrations (Table 4.3) and remained relatively constant over time. However, since water concentrations were decreasing over time, the calculated bioaccumulation factors (BAFs) in the lindane-treated tadpoles increased, from a mean of 43.6 ± 5.6 at 1 week, to 77.9 ± 9.8 at 4 weeks and 123.0 ± 48.9 at 6 weeks (Table 4.4). The mean weights of tadpoles increased over the sampling times, from 0.1 g at 1 week, to 1.1 g at 4 weeks and 2.8 g at 6 weeks. Therefore, calculated body burden also increased by a mean factor of 11 ± 3 from 1 week to 4 weeks, and by a mean factor of 2 ± 1 from 4 weeks to 6 weeks (Table 4.5). Lipid content also increased over time from a mean of 0.228 ± 0.103% at 4 weeks to 1.044 ± 0.195% at 6 weeks (Table 4.6). A significant relationship did not exist between lindane concentration and lipid content (PROC REG, $p = 0.3704$); therefore, lipid-normalization of the data was not appropriate (Hebert and Keenleyside 1995). However, lindane body burden did increase with increasing lipid content between the 4 and 6 week samples. Lipid content could not be calculated for the 1-week samples because of the lack of tissue available for analysis.

None of the 15 metamorphs kept until the end of tail resorption died prior to sampling for lindane analysis. The time required for tail resorption varied from 4 to 13 days except for four metamorphs, which were removed from the microcosms with nub

Table 4.3. Lindane concentrations in pooled tadpole and froglet samples of the microcosm study, expressed as µg/kg wet weight.

Nominal Lindane Concentration (µg/L)	Time Since Treatment				
	1 week	4 weeks	6 weeks	End of Metamorphic Climax [1]	Onset of Hibernation [2]
0.0 (Negative)	1.3	0.8	5.1	2.8	0.0
0.0 (Solvent)	0.8	0.8	2.3	5.6	0.0
0.1	3.7	4.0	4.2	0.9	---[3]
1.0	34.8	20.9	16.6	12.0	---
10.0	330.7	338.5	383.6	2.4	0.0

[1] Metamorphs were removed from the microcosms approximately 6 to 8 weeks post-treatment and allowed to complete metamorphic climax before tissue residue analysis.
[2] Froglets were held in outdoor containers until euthanized for hepatic glycogen analysis at approximately 14 weeks post-treatment.
[3] Only metamorphs from the controls and the 10 µg/L treatments were kept until the onset of hibernation (September 28-29, 2000) and used for glycogen analysis.

Table 4.4. Bioaccumulation Factor (BAF) for each treatment at each sampling time.

Nominal Lindane Concentration (µg/L)	Time Since Treatment		
	1 week	4 weeks	6 weeks [1]
0.0 (Solvent)	48	39	---[2]
0.1	41	80	169
1.0	50	67	72
10.0	40	87	129

[1] Lindane concentrations in water at 6 weeks were estimated from the regression equation of lindane dissipation from the water.
[2] No water samples were taken from the solvent control treatments at 6 weeks.

Table 4.5. Body burdens of lindane in tadpoles and froglets sampled from the microcosms. Each sample consisted of animals pooled from all three microcosms within the treatment. There were 3 to 15 animals per sample, depending on the time of sampling (fewer animals were collected near the end of the exposure period). Values given are nanograms lindane per animal.

Nominal Lindane Concentration (µg/L)	Time Since Treatment				
	1 week	4 weeks	6 weeks	End of Metamorphic Climax	Onset of Hibernation
0.0 (Negative)	0.2	0.8	17.3	5.5	0.0
0.0 (Solvent)	0.1	0.8	5.0	11.6	0.0
0.1	0.4	4.5	11.5	2.6	---[1]
1.0	3.7	27.9	42.1	23.2	---
10.0	28.7	366.7	1022.9	5.9	0.0

[1] Only metamorphs from the controls and the 10 µg/L treatments were kept until the onset of hibernation (September 28-29, 2000) and used for glycogen analysis.

Table 4.6. Percent lipid in pooled tissue samples of tadpoles and froglets from the microcosms. Each sample consisted of animals pooled from all three microcosms within the treatment. There were 3 to 15 animals per sample, depending on the time of sampling (fewer animals were collected near the end of the exposure period).

Nominal Lindane Concentration (µg/L)	Time Since Treatment				
	1 week	4 weeks	6 weeks	End of Metamorphic Climax	Onset of Hibernation
0.0 (Negative)	---[1]	0.124	0.890	1.926	0.824
0.0 (Solvent)	---	0.162	0.974	1.692	1.316
0.1	---	0.178	1.080	3.110	---[2]
1.0	---	0.338	1.366	1.176	---
10.0	---	0.340	0.908	2.542	0.972

[1] Percent lipid could not be calculated for the one week samples because of the lack of tissue available for analysis.

[2] Only metamorphs from the controls and the 10 µg/L treatments were kept until the onset of hibernation (September 28-29, 2000) and used for glycogen analysis.

tails and were immediately euthanized. Two of these specimens came from the 1.0 µg/L treatment, one from the negative control and one from the 0.1 µg/L treatment. The overall mean SVL and weight at complete tail resorption were 25.7 ± 2.2 mm and 1.73 ± 0.64 g, respectively. Concentrations in metamorphs at the end of MC varied among treatments, ranging from 0.9 to 12.0 µg/kg, and were not consistent with the trend of tissue concentrations increasing with treatment concentrations as observed in the tadpoles (Table 4.3). Lindane was not detected in carcasses at the onset of hibernation (Table 4.3).

4.3.2 Water Quality

There were no significant differences among treatments in ammonia and nitrate/nitrite concentrations, alkalinity, hardness, particulate nitrogen, dissolved organic carbon, particulate organic carbon, or conductivity (one-way RM ANOVAs, $p > 0.1535$). Therefore, the data were averaged across treatments for analysis of changes over time (Table 4.7). Ammonia and nitrate/nitrite concentrations were significantly higher at pretreatment (0.079 mg/L and 0.091 mg/L) than at subsequent sampling periods (0.024 mg/L and <0.010 mg/L). Hardness was significantly lower at pretreatment (119.6 mg/L) than at subsequent sampling periods (126.0 mg/L). There were no changes in these parameters post-treatment (Table 4.7). Alkalinity was slightly higher at 4 weeks (84 mg/L) than at pretreatment or 8 weeks post-treatment (81 mg/L). Particulate nitrogen, dissolved organic carbon (DOC), and particulate organic carbon (POC) increased over time (Table 4.7). Because tap water was used in the microcosms, POC would be expected to be low whereas DOC could start out as high as 10 mg/L, the normal concentration for prairie surface waters (M. Waiser, National Hydrology Research Centre, personal communication, 2003). The DOC reading taken at the Saskatoon water treatment plant in May 2000 was 2.9 mg/L (D. Forsyth, Canadian Wildlife Service, personal communication, 2003). By 8 weeks, the POC and DOC values were similar, indicating that the microbial or phytoplankton community was increasing the POC. Log bacteria count also did not differ among treatments, but

Table 4.7. Water quality parameters measured at pretreatment and at 4 and 8 weeks after lindane application in the microcosms. Pretreatment samples were taken from five microcosms prior to the addition of lindane. Subsequent samples were collected from all 15 microcosms. Values are means ± SE, averaged across all treatments ($n = 5$). Different letters denote significant differences among sampling periods (one-way ANOVAs, Tukey test, $p < 0.05$).

		Time Since Treatment	
Water Quality Parameter	Pretreatment	4 weeks	8 weeks
Alkalinity (mg/L as $CaCO_3$)	81 ± 1.0 [a]	86 ± 1.8 [b]	81 ± 1.0 [a]
Hardness (mg/L as $CaCO_3$)	120 ± 1.6 [a]	126 ± 0.6 [b]	126 ± 0.9 [b]
Ammonia (mg/L)	0.079 ± 0.040 [a]	0.024 ± 0.005 [b]	0.024 ± 0.002 [b]
Conductivity (µS/cm)	618 ± 67 [a]	705 ± 26 [a]	703 ± 26 [a]
Particulate Nitrogen (mg/L)	0.099 ± 0.011 [a]	0.180 ± 0.023 [b]	0.818 ± 0.102 [c]
Nitrate + Nitrite (mg/L)	0.091 ± 0.009 [a]	< 0.010 [b]	< 0.010 [b]
POC [1] (mg/L)	0.43 ± 0.04 [a]	1.11 ± 0.09 [b]	8.63 ± 1.18 [c]
DOC [2] (mg/L)	3.80 ± 0.57 [a]	5.15 ± 0.04 [b]	9.38 ± 0.22 [c]

[1] Particulate organic carbon.
[2] Dissolved organic carbon.

Table 4.8. Water quality parameters measured 2-3 times weekly in the microcosms. Values are means ± SE, averaged across sampling times. Different letters denote significant differences among treatments after the addition of lindane (one-way RM ANOVAs, Tukey test, $p < 0.05$).

Nominal Lindane Concentration (µg/L)	pH	DO [1] (mg/L)	Top Temp [2] (°C)	Mid Temp [3] (°C)	Bottom Temp [4] (°C)
Pre-treatment	7.8 ± 0.03	8.16 ± 0.03	20.5 ± 0.1	20.6 ± 0.1	20.5 ± 0.1
0.0 (Negative)	8.1 ± 0.1[ab]	9.86 ± 0.20	20.1 ± 0.5	19.2 ± 0.5	19.3 ± 0.5
0.0 (Solvent)	8.2 ± 0.1[a]	10.34 ± 0.22	20.3 ± 0.5	19.5 ± 0.5	19.7 ± 0.5
0.1	7.8 ± 0.1[c]	8.51 ± 0.13	20.1 ± 0.5	19.2 ± 0.5	19.4 ± 0.5
1.0	8.0 ± 0.1[bc]	9.79 ± 0.68	20.2 ± 0.5	19.3 ± 0.5	19.6 ± 0.5
10.0	8.4 ± 0.1[d]	10.76 ± 0.40	20.1 ± 0.5	19.1 ± 0.5	19.3 ± 0.5

[1] Dissolved oxygen concentration.
[2] Temperature measured at approximately 10 cm below the water surface.
[3] Water temperature at approximately 25 cm below the water surface.
[4] Water temperature at the bottom of the microcosm (50 cm).

increased over time from 3.1 ± 0.01 (mean ± SE) at pretreatment to 3.7 ± 0.03 at six weeks (paired t-test, $p < 0.0010$). Mean conductivity ranged from 635 to 859 µS/cm in most microcosms, except for one that had a significantly lower mean of 373 ± 8 µS/cm (Section 4.2.8). Overall mean conductivity of all microcosms did not change over time (Table 4.7). The differences between mid and bottom water temperatures within microcosms were minor throughout the study and differed by only 0.0 to 1.8°C (Fig. 4.4). Since both parameters were similar, further analysis of the data was conducted with bottom water temperature as the representative microcosm temperature. There were no significant differences in mean bottom temperature among treatments (one-way RM ANOVAs, $p = 0.3336$), and overall mean water temperatures ranged from 19.3 to 20.5°C (Table 4.8). Water temperatures declined during the study period (Fig. 4.5A), and likely because of declining air temperatures (Fig. 4.5B). Bottom water temperatures among the microcosms differed by 0.4 to 1.7°C until about the eighth week, when mean water temperatures began to drop and the difference between the warmest and coolest microcosms increased to between 1.5 and 3.5°C (Fig. 4.4). The mean bottom water temperatures then showed a trend of decreasing water temperatures in a northeast to southwest direction. Therefore, this pattern of decline in water temperatures among the microcosms was probably caused by shading on the south side of the enclosure.

Average microcosm pH varied throughout the exposure period, starting at pH 7.8, dropping slightly within days of treatment, then increasing to a peak of pH 8.8 at 51 days before falling back to around pH 8.0 (Fig. 4.6). As well, there were significant differences in pH among treatments (one-way RM ANOVA, $p < 0.001$, Tukey test). Mean pH was highest in the 10 µg/L treatment, followed by the controls, and then the two lowest lindane treatments (Table 4.8).

4.3.3 Survival, Growth and Development

A total of 387 metamorphs was removed from all 15 microcosms. Of these, 61 were not preserved in formalin, but were used for analyses of lindane residue or glycogen. The majority (67%) of the metamorphs was at stage 42, as estimated by

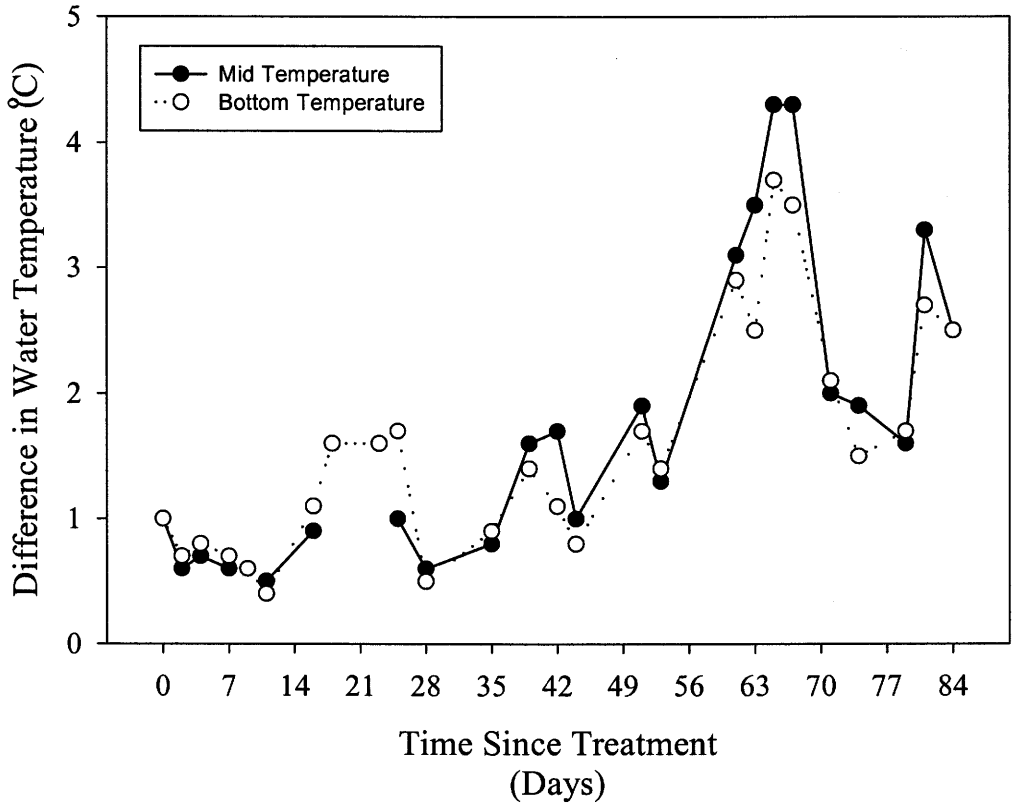

Figure 4.4. Differences in water temperatures among microcosms. Each data point represents the difference in water temperature between the warmest and coolest microcosm on each sampling day (not the same two microcosms throughout).

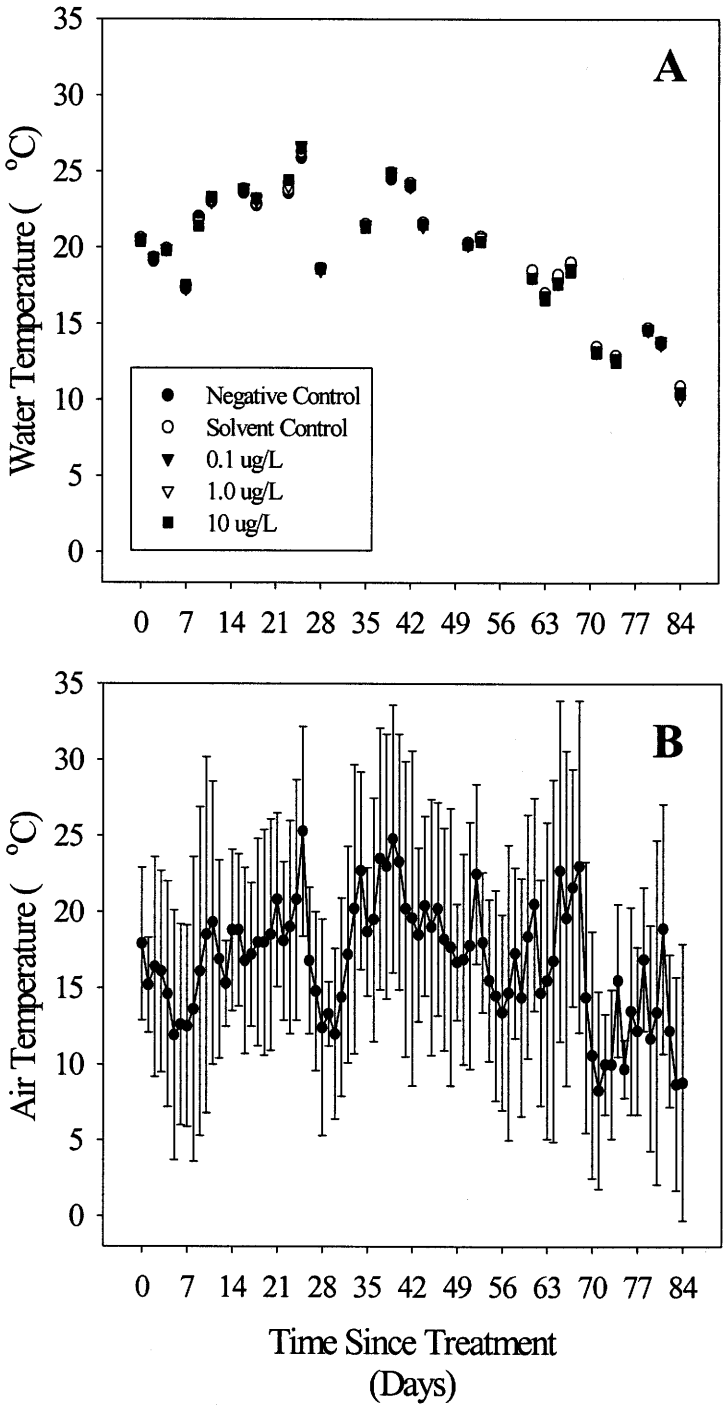

Figure 4.5. A) Water temperature in microcosms at a depth of 50 cm. Each data point represents the mean of three microcosms for each treatment. B) Mean air temperature at the Saskatoon airport during the microcosm study. Each data point represents daily mean air temperature, calculated from the minimum and maximum values, represented by the vertical lines.

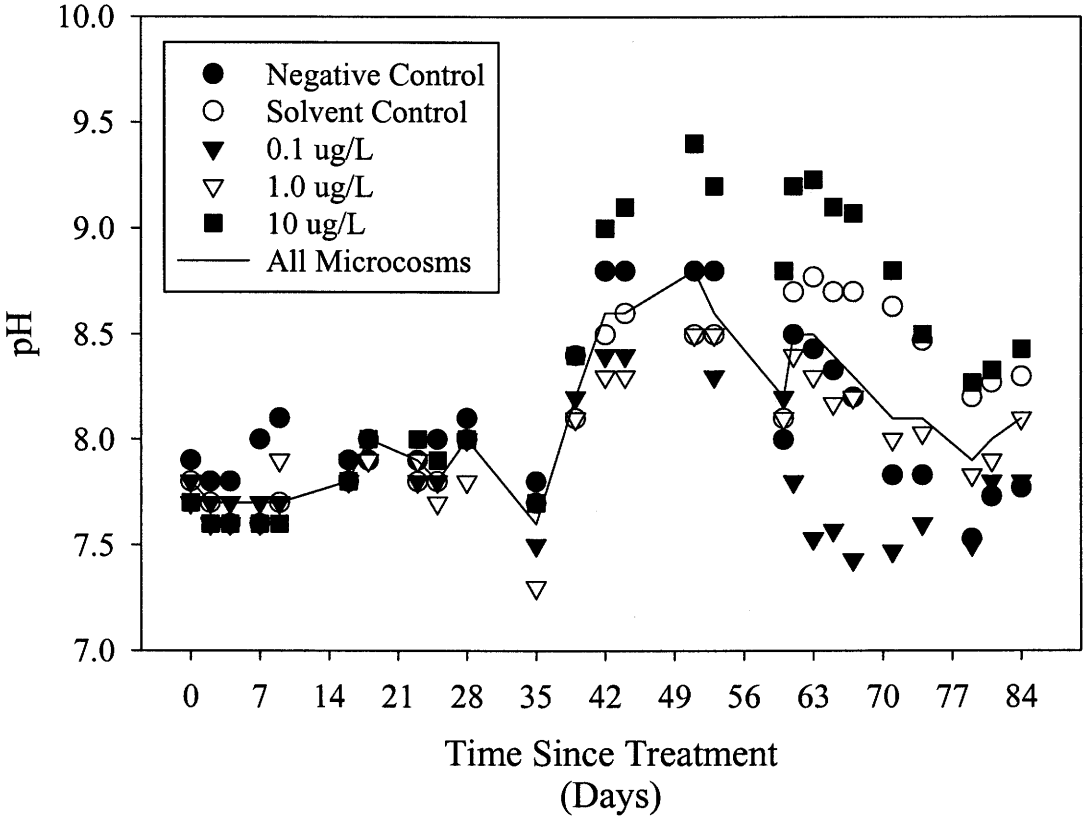

Figure 4.6. Mean pH in the microcosms. Each data point represents the mean value for a treatment (n = 3). The line represents the mean for all 15 microcosms combined.

examining 282 preserved specimens (not including those from microcosms #1 and #7). The remaining specimens were at stage 43 (13%) stage 44 (15%), and stage 45 (5%). Survival to MC ranged from 73 to 93% among the microcosms with a mean of 87 ± 6% (Table 4.9). There were no significant differences in survival among treatments (one-way ANOVA, $p = 0.134$).

Between 34 and 45 metamorphs in each treatment were at stage 42 (mean ± SD = 38 ± 4). There were no significant effects of treatment on SVL or total length in stage 42 metamorphs (Table 4.10; two-way ANOVAs, $p > 0.063$), although SVL and total length was highest in the lowest treatment. It was obvious from graphs of weight versus total length of stage 42 metamorphs that there was a minimum weight and length among all treatments corresponding to approximately 0.6 g and 48 mm (Fig. 4.7). As well, maximum weight and length were greater in the lindane treatments than in the controls. Although parametric statistical tests indicated that weight and weight/total length ratio was significantly higher in the lowest treatment (0.1 µg/L) and decreased with increasing lindane concentration (two-way ANOVAs, $p < 0.013$), these two end points exhibited homogeneity of variance, and were therefore analyzed with less powerful Kruskal-Wallis one-way ANOVAs. This non-parametric test did not detect significant differences in weight among treatments ($p = 0.148$), but did detect slightly significant differences in weight/total length ($p = 0.04$) although the multiple comparison test (Dunn's method) did not isolate significantly different groups ($p \geq 0.05$). Graphs of weight versus total length indicated that the slopes of the lindane treatments were different from those of the controls, as indicated by lack of overlap among the confidence intervals (Table 4.11). There was no difference among treatments in days to metamorphic climax (two-way ANOVA, $p = 0.818$). Neither mean water temperature nor conductivity was significantly correlated with days to MC (PROC CORR, $r^2 < 0.1784$, $p > 0.1505$). Snout-vent length did not change during metamorphic climax (see Appendix E). No significant effect of lindane treatment was found on the SVL of metamorphs (at various stages during MC) (Table 4.9; two-way ANOVA, $p = 0.3550$).

Table 4.9. Mean survival and snout-vent length (SVL) of metamorphs in the microcosms (regardless of stage of development). Values are means ± SE ($n = 3$ except for the solvent control and 1.0 µg/L, which only had two microcosms per treatment). *N* refers to the total number of metamorphs per treatment. There were no significant differences among treatments (one-way ANOVA, $p > 0.355$).

Nominal Lindane Concentration (µg/L)	Survival (%)	*N*	SVL (mm)
0.0 (Negative)	81.1 ± 4.0	73	22.9 ± 0.2
0.0 (Solvent)	90.2 ± 0.2	55	23.2 ± 0.1
0.1	87.8 ± 4.4	79	23.6 ± 0.5
1.0	84.7 ± 2.0	50	23.5 ± 0.5
10.0	93.3 ± 0.0	84	23.2 ± 0.2

Table 4.10. The effect of lindane exposure on larval growth and development of Gosner stage 42 metamorphs in the microcosm study. Values are means ± SE (n = 3 except for the solvent control and 1.0 µg/L, which only had two microcosms per treatment). N refers to the total number of metamorphs per treatment. There were no significant differences among treatments on days to metamorphic climax among treatments (two-way ANOVA, $p = 0.818$) or on total length, snout-vent length, or tail length (two-way ANOVAs, $p > 0.057$). There were no significant differences among treatments on wet weight (Kruskal-Wallis one-way ANOVA on ranks, $p = 0.148$) or weight/total length (Kruskal-Wallis one-way ANOVA on ranks, $p = 0.040$; Dunn's method, $p \geq 0.05$).

Nominal Lindane Concentration (µg/L)	N	Days to Metamorphic Climax [1]	Wet Weight (g)	Total Length (mm)	Snout-vent Length (mm)	Tail Length (mm)	Weight/Length (g/mm)
0.0 (Negative)	39	48 ± 2.2	1.87 ± 0.10	59.6 ± 1.7	22.9 ± 0.4	36.7 ± 1.4	0.0309 ± 0.0009
0.0 (Solvent)	34	47 ± 0.4	1.87 ± 0.001	63.1 ± 0.01	23.2 ± 0.2	39.9 ± 0.2	0.0295 ± 0.00004
0.1	43	46 ± 0.6	2.29 ± 0.22	63.6 ± 2.0	24.0 ± 0.8	39.6 ± 1.2	0.0347 ± 0.0024
1.0	35	49 ± 1.1	2.06 ± 0.29	61.2 ± 3.3	23.8 ± 0.7	37.4 ± 2.6	0.0329 ± 0.0028
10.0	37	46 ± 1.9	1.91 ± 0.06	60.8 ± 0.8	23.2 ± 0.2	37.6 ± 0.7	0.0307 ± 0.0006

[1] The number of days from the start of exposure until removal from the microcosms at Gosner stage 42. The tadpoles were 9-10 days old at the start of the exposure period.

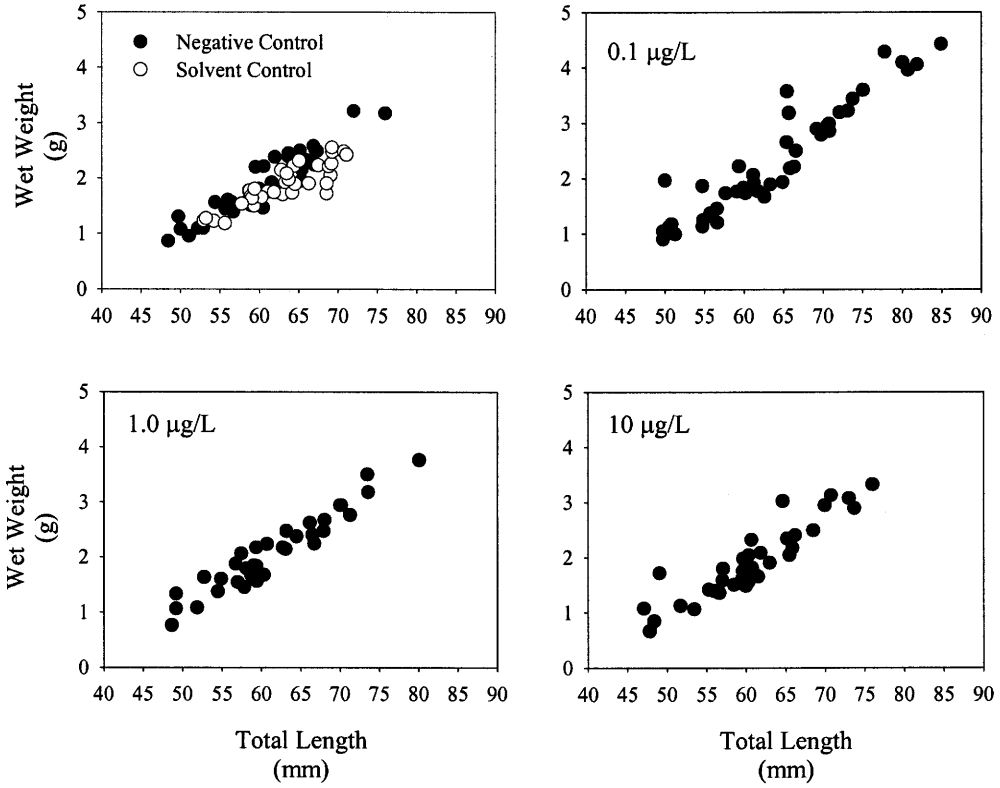

Figure 4.7. The relationship between wet weight and total length of metamorphs at Gosner stage 42 in the microcosm study.

Table 4.11. Components of the regressions describing the linear weight/total length relationship for Gosner stage 42 metamorphs of each treatment of the microcosm study.

Nominal γ-HCH Concentration (µg/L)	r^2	y-intercept	slope [1]	95% CI [2]
0.0 (Negative)	0.6856	-1.636	0.059	0.046 to 0.072
0.0 (Solvent)	0.7677	-2.147	0.064	0.051 to 0.076
0.1	0.8818	-4.017	0.099	0.088 to 0.111
1.0	0.9164	-3.329	0.088	0.079 to 0.097
10.0	0.8250	-3.256	0.085	0.072 to 0.098

[1] The slope was significantly different from zero in all treatments (PROC REG, $p < 0.0001$).

[2] 95% Confidence Intervals for the slope (slope ± [$t_{0.05(2),n-2}$ x SE]). Standard error (SE) was estimated using PROC REG in SAS.

4.3.4 Malformations

Scoliosis, defined as a lateral curvature of the spine, was the most frequently observed malformation, with an overall mean occurrence of 22 ± 11% in the microcosms (exempting the aberrant microcosms). Three of the microcosms, two from the 0.1 µg/L treatment and one from the 1.0 µg/L treatment, had low occurrences (0 to 8.0%), whereas the remaining microcosms had high occurrences (18.2 to 34.5%). Usually only the tail appeared affected, and these metamorphs, if allowed to develop to the end of MC, showed no signs of malformation. There were no significant differences among treatments (one-way ANOVA, $p = 0.134$).

Two metamorphs with limb malformations were collected from two microcosms of the 0.1 µg/L treatment. One metamorph was missing its left forearm and associated rib cage (amelia). The other metamorph had a complete right hindlimb with primary rotation (no associated bone bridging or skin webbing). The overall occurrence of limb malformations was 0.3%.

4.3.5 Plasma Corticosterone and Thyroid Hormones

Concentrations of T3 were very similar among treatments, with an overall mean of 1.27 ± 0.01 ng/ml (Table 4.12). Concentrations of T4 were more variable among treatments, with an overall mean of 1.41 ± 0.15 ng/ml (Table 4.12). There were no significant differences in log-transformed T3 or log-transformed T4 concentrations among treatments (two-way ANOVAs, $p > 0.3160$). The mean T4 to T3 ratio was 1.12 ± 0.11 ng/ml (Table 4.12). The log-transformed T4 to T3 ratios were significantly higher in the 0.1 µg/L than in the solvent control treatment, but were not significantly different from the 1.0 µg/L treatment (two-way ANOVA, $p = 0.0306$). Corticosterone concentrations ranged from 4.82 ng/ml (solvent control) to 8.53 ng/ml (0.1 µg/L), with an overall mean of 6.98 ± 0.80 ng/ml (Table 4.12). There were significant differences in log-transformed corticosterone concentrations among lindane treatments (two-way ANOVA, $p = 0.0026$). The solvent control treatment was significantly different from all treatments except the 1.0 µg/L treatment.

Table 4.12. Plasma thyroid hormone (triiodothyronine (T3) and tetraiodothyronine (T4)) and corticosterone (CORT) concentrations in metamorphs from the microcosm study. Values are means ± SE (n = 3 except for the solvent control and 1.0 µg/L treatments, which only had two microcosms per treatment). N refers to the total number of metamorphs per treatment. Different letters denote significant differences among treatments (two-way ANOVAs, Tukey test, $p < 0.05$).

Nominal Lindane Concentration (µg/L)	N	T3 (ng/ml)	T4 (ng/ml)	T4 to T3 ratio	CORT (ng/ml)
0.0 (Negative)	21	--[1]	--	--	7.79 ± 0.62[a]
0.0 (Solvent)	20	1.30 ± 0.04	1.14 ± 0.15	0.92 ± 0.10[a]	4.82 ± 0.57[b]
0.1	30	1.25 ± 0.08	1.66 ± 0.25	1.32 ± 0.09[b]	8.53 ± 0.72[a]
1.0	20	1.26 ± 0.06	1.42 ± 0.02	1.12 ± 0.00[ab]	5.28 ± 0.72[ab]
10.0	25	--	--	--	8.46 ± 1.04[a]

[1] Plasma samples from metamorphs of the negative control and 10 µg/L treatments were not analyzed for thyroid hormone concentrations.

4.3.6 Fluctuating Asymmetry

The magnitude of the percent measurement error (%ME) was high in all four traits, with femur length exhibiting the lowest mean %ME of 46.4% (Table 4.13). Tibiofibula and radio-ulna lengths had the next lowest mean %ME of 53.5% and 62.3%, respectively (Table 4.13). Eye-naris length exhibited the highest %ME, with the mean %ME of 82.6%. Percent ME was also calculated for each trait side (L and R); see Appendix F. Despite the high amount of %ME, there was more among-individuals variation in signed (L-R) differences than expected based on measurement error alone (within-individual variation); taking six measurements on each individual would also have reduced the measurement error in the final analysis.

Six out of 13 t-tests on the FA estimates for femur length indicated significant directional asymmetry (PROC MEANS, one-sample t-test, $p < 0.0098$). All mean values of (L-R) were negative, indicating a possible right-handed bias due to using a right-handed measurement tool (Dufour and Weatherhead 1996). FA estimates for femur measurements were therefore corrected for bias according to the formula (Palmer 1994):

$$(L\text{-}R)' = (L\text{-}R) - \text{mean}(L\text{-}R) \tag{4.4}$$

where (L-R) is the FA estimate for a metamorph, mean(L-R) is the mean FA estimate for the microcosm, and (L-R)' is the adjusted FA estimate for the individual metamorph. Re-analysis of the corrected femur FA estimates indicated no significant directional asymmetry.

Comparisons among traits with all metamorphs combined indicated significant directional asymmetry in tibiofibula length (PROC MEANS, one sample t-test, $p = 0.004$). However, t-tests performed on each microcosm within that trait did not reflect that result (PROC MEANS, one-sample t-test, $p > 0.5610$). Significant directional asymmetry was not observed in radio-ulna length with pooled data, but was observed in two microcosms within this trait. Significant directional asymmetry was not observed in eye-naris length.

Table 4.13. Percent measurement error (%ME) for unsigned fluctuating asymmetry (L-R) of four external traits as measured in the microcosm metamorphs. The mean of six repeated measurements for each metamorph was used to calculate %ME per microcosm. Values are means ± SE ($n = 3$ except for the solvent control and the 1.0 µg/L treatments, which only had two microcosms per treatment). *N* refers to the total number of metamorphs per treatment.

Nominal Lindane Concentration (µg/L)	N^1	Radio-ulna Length	N^2	Eye-naris Length	Femur Length	Tibiofibula Length
all[3]	277	62.3	280	82.6	46.4	53.5
0.0 (Negative)	53	73.7 ± 21.7	54	85.5 ± 10.3	43.7 ± 6.2	59.1 ± 8.9
0.0 (Solvent)	45	67.8 ± 15.3	45	83.9 ± 10.8	39.9 ± 13.2	51.2 ± 31.2
0.10	72	68.8 ± 8.6	73	74.4 ± 6.5	57.1 ± 20.3	60.8 ± 28.3
1.0	48	71.2 ± 15.9	48	89.7 ± 0.9	44.6 ± 1.2	77.5 ± 4.8
10.0	59	60.7 ± 13.7	60	86.3 ± 11.5	58.2 ± 17.0	54.7 ± 16.3

[1] Sample size for radio-ulna length differed from other traits because of difficulties in measuring some individuals.
[2] Sample size for all traits except radio-ulna length.
[3] Values given are for all metamorphs combined regardless of treatment.

Tibiofibula length and radio-ulna length with pooled data showed significant departures from normality (Kolmogorov-Smirnov tests, $p > 0.0200$), but none of the microcosms within radio-ulna length did, and only one of the microcosms within tibiofibula length did ($p = 0.0260$). No other traits exhibited significant departures from normality in the pooled data or among microcosms.

A possible dependence of asymmetry on trait size was assessed graphically on within samples with plots of unsigned (L-R) differences (absolute L-R or |L-R|) against mean trait size and (L-R) against SVL, and among samples with plots of log variance (L-R) against mean trait size. No size dependence on FA was observed (Appendix F). Descriptive statistics for the trait size were calculated using the mean of repeated measurements for each frog and are reported in Appendix F.

Tables 4.14 and 4.15 list the mean values for two types of FA estimates: signed differences (L-R) and unsigned differences (absolute L-R or |L-R|). Two-way ANOVAs were conducted on absolute FA estimates for each trait and on the sum of absolute FA estimates for each metamorph. No significant differences among lindane treatments were observed ($p > 0.4229$).

4.3.7 Hepatic Glycogen Reserves

Hepatic glycogen concentrations in froglets ranged from 175.0 to 185.6 mg/g, with a mean of 179.5 ± 3.2 mg/g (Table 4.16). Froglets from the solvent control treatment had significantly higher hepatic glycogen concentrations than froglets from either the negative control or 10 µg/L treatment (two-way ANOVA, $p = 0.0246$). However, the solvent control froglets had smaller livers (143.32 mg) than froglets of the two other treatments (164.36 mg and 158.40 mg, negative control and 10 µg/L treatment, respectively), perhaps indicating that the livers of the solvent control frogs were more densely packed with glycogen (Table 4.16). Using liver weight as a covariate, there were no significant differences in glycogen among treatments (ANCOVA, $p = 0.0619$). However, the model statistic was not significant ($p = 0.1155$) indicating that the differences in liver weight did not explain all the variation in glycogen concentration.

Table 4.14. Signed fluctuating asymmetry (FA) estimates (L-R) for four external traits measured on metamorphs in the microcosm study. Values are means ± SE (n = 3 except for the solvent control and the 1.0 µg/L treatments, which only had two microcosms per treatment). N refers to the total number of metamorphs per treatment.

Nominal Lindane Concentration (µg/L)	N^1	Radio-ulna Length (mm)	N^2	Eye-naris Length (mm)	Femur Length (mm)	Tibiofibula Length (mm)
all	277	-0.02 ± 0.02	280	0.01 ± 0.01	0.00 ± 0.02	-0.09 ± 0.02
0.0 (Negative)	53	-0.03 ± 0.08	54	0.04 ± 0.02	-0.03 ± 0.05	-0.13 ± 0.01
0.0 (Solvent)	45	0.00 ± 0.04	45	-0.01 ± 0.003	0.08 ± 0.03	-0.02 ± 0.03
0.10	72	-0.04 ± 0.06	73	-0.01 ± 0.01	-0.03 ± 0.11	-0.09 ± 0.04
1.0	48	-0.09 ± 0.03	48	0.01 ± 0.02	-0.02 ± 0.15	-0.07 ± 0.02
10.0	59	0.09 ± 0.10	60	0.01 ± 0.01	0.01 ± 0.01	-0.12 ± 0.02

[1] Sample size for radio-ulna length differed from other traits because of difficulties in measuring some individuals

[2] Sample size for all traits except radio-ulna length.

Table 4.15. Unsigned fluctuating asymmetry (FA) estimates (|L-R|) for four external traits measured on metamorphs in the microcosm study. Values are means ± SE (n = 3 except for the solvent control and the 1.0 µg/L treatments, which only had two microcosms per treatment). N refers to the total number of metamorphs per treatment. There were no significant differences among treatments (two-way ANOVAs, $p > 0.43$).

Nominal Lindane Concentration (µg/L)	n^1	Radio-ulna Length (mm)	n^2	Eye-naris Length (mm)	Femur Length (mm)	Tibiofibula Length (mm)
all	277	0.21 ± 0.01	280	0.10 ± 0.00	0.24 ± 0.01	0.20 ± 0.01
0.0 (Negative)	53	0.23 ± 0.05	54	0.10 ± 0.01	0.24 ± 0.02	0.20 ± 0.00
0.0 (Solvent)	45	0.18 ± 0.05	45	0.10 ± 0.01	0.26 ± 0.01	0.21 ± 0.08
0.10	72	0.21 ± 0.01	73	0.11 ± 0.01	0.24 ± 0.04	0.20 ± 0.04
1.0	48	0.19 ± 0.02	48	0.09 ± 0.01	0.26 ± 0.01	0.18 ± 0.02
10.0	59	0.23 ± 0.05	60	0.09 ± 0.01	0.21 ± 0.03	0.22 ± 0.01

[1] Sample size for radio-ulna length differed from other traits because of difficulties in measuring some individuals.

[2] Sample size for all traits except radio-ulna length.

Table 4.16. Summary of parameters measured in froglets of the hepatic glycogen experiment at the onset of hibernation (September 28-29, 2000). Values are means ± SE (n = 3 except for the solvent control treatment, which only had two microcosms per treatment). N refers to the total number of metamorphs per treatment. Different letters denote significant differences among treatments for hepatic glycogen concentration (ANCOVA with liver weight as a covariate, p = 0.0619, Tukey test). Significant treatment effects were not observed in the other variables (two-way ANOVA, $p > 0.592$)

Nominal Lindane Concentration (µg/L)	N	Percent Males [1] (%)	Body Weight (g)	Snout-vent Length (mm)	Hepatic Glycogen (mg/g)	Liver Weight (mg)	Absolute Glycogen [2] (mg)	Fat Body Weight (mg)
0.0 (Negative)	12	67	1.43 ± 0.02	23.6 ± 0.2	175.0 ± 1.0 [a]	164.36 ± 5.06	28.72 ± 0.72	21.36 ± 0.83
0.0 (Solvent)	7	43	1.41 ± 0.06	24.0 ± 0.5	185.6 ± 1.9 [b]	143.32 ± 6.20	26.61 ± 0.88	16.18 ± 4.21
10.0	17	59	1.47 ± 0.08	24.0 ± 0.3	177.9 ± 4.4 [a]	158.40 ± 17.20	28.16 ± 3.44	21.69 ± 3.95

[1] Based on the total number of males in each treatment.

[2] Absolute glycogen is the amount of glycogen present in the liver. It was calculated for each froglet by multiplying hepatic glycogen concentration by the liver weight.

Another factor may have influenced the higher glycogen concentration in the solvent control. There were no significant differences among treatments in other parameters such as SVL at, or time to, MC; body weight or SVL at the onset of hibernation; or weight of fat bodies. There was no difference in mean glycogen concentration between male and female froglets (t-test, $p = 0.8375$).

4.3.8 Sex Ratios

Sex ratios differed significantly among treatments (Chi-square test, $p < 0.005$), due to the altered sex ratio of the 0.1 µg/L treatment (Chi-square test, without this treatment, $p > 0.975$). There were 71% males in this treatment compared to 39 to 49% males in the other treatments (Fig. 4.8). There were more males than females present in all microcosms comprising the 0.1 µg/L treatment.

4.3.9 Comparison Between Microcosms and Ponds

Water temperatures at a depth of approximately 50 cm in the ponds (12.9°C to 24.2°C) were lower compared to water temperatures in the microcosms during the same time period (17.4°C to 26.4°C; Fig. 4.9). The Denis Pond became as warm as the microcosms in mid-July; this pond was relatively large and permanent, with a depth of approximately 1-1.5 m (Appendix D).

Unlike the microcosms, pH generally decreased over time in the ponds during the summer months (Fig. 4.10). With few exceptions, conductivity, alkalinity, and hardness did not vary in the ponds over time, and thus mean values were calculated for comparison to the microcosms (Table 4.17). Mean conductivity in the microcosms (675 ± 28 µS/cm) was within the range seen in the ponds (157 to 2810 µS/cm), but alkalinity and hardness were lower in the microcosms (83 mg/L and 124 mg/L, respectively) than in the ponds (89 to 318 mg/L and 108 to 1728 mg/L, respectively) (Table 4.17). Ammonia concentrations did not change over time in the ponds except for Duckweed Pond. Ammonia concentrations increased over time in Duckweed Pond, which was

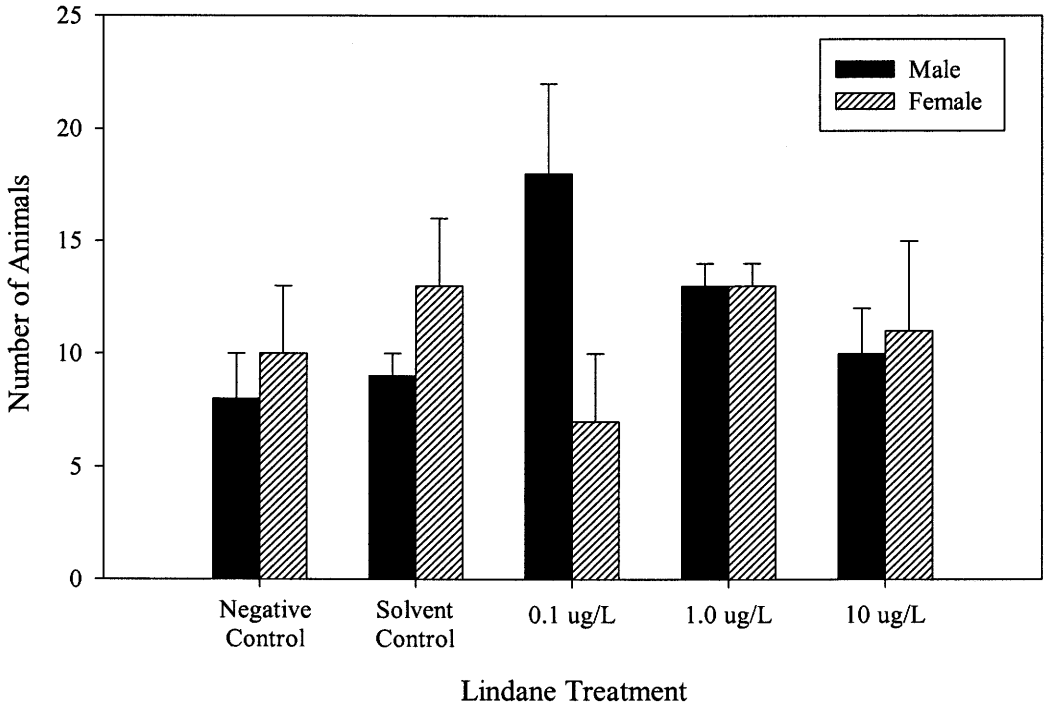

Figure 4.8. Mean sex ratios among lindane treatments of the microcosm study. Error bars represent standard deviation ($n = 3$). The mean sex ratio of the 0.1 µg/L treatment was significantly different from the ratios of the other treatments (Chi-square analysis, $p < 0.005$).

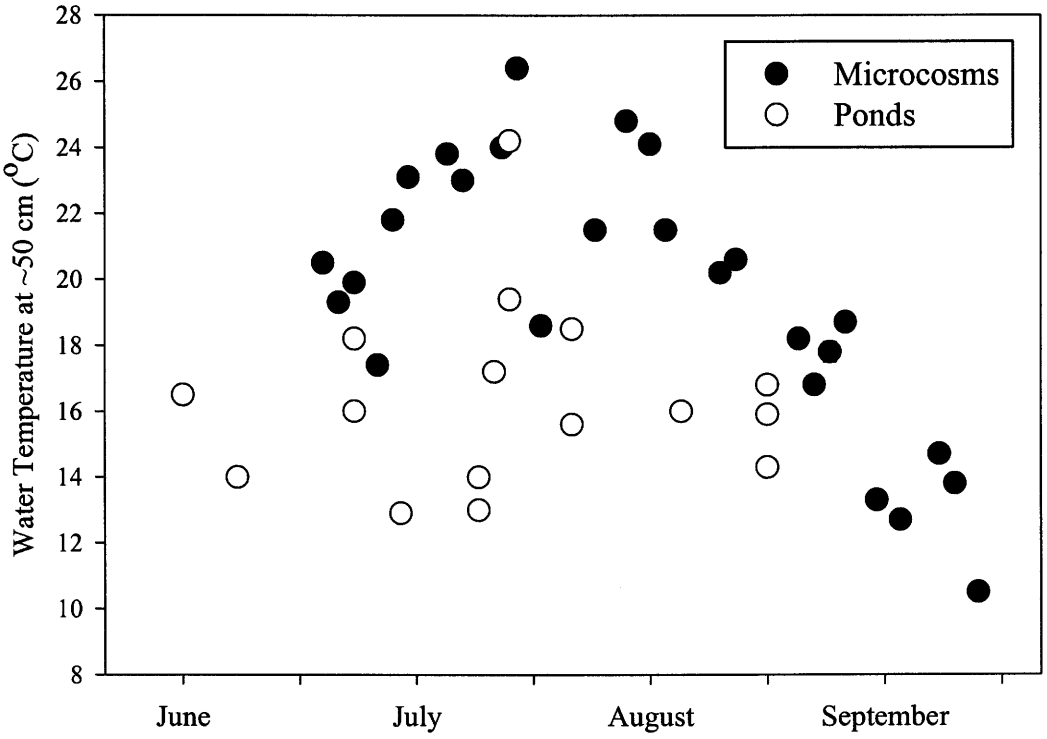

Figure 4.9. Water temperature at a depth of approximately 50 cm in five ponds compared to the microcosms. Each microcosm data point is the mean ± SE of all microcosms per day. Each pond data point is the water temperature of a pond measured on various days over the summers of 1999 and 2000. Some pond data points are superimposed on others.

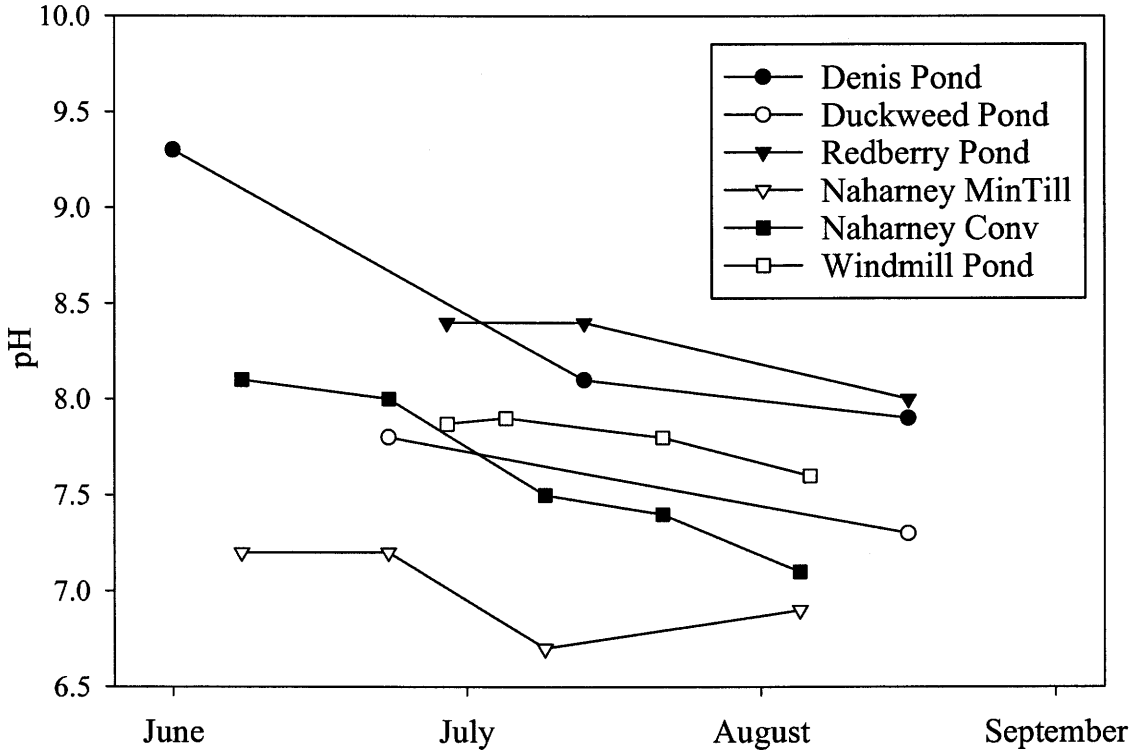

Figure 4.10. pH of water samples collected from prairie ponds in 1999 and 2000. Samples were collected during the same time period as the microcosm study.

Table 4.17. Comparison of water quality parameters measured in the microcosms in 2000 and the ponds in 1999 and 2000. Values are means ± SE, where *n* is the number of times sampled between early June and mid-August.

Site	Year	*n*	Conductivity (µS/cm)	Alkalinity (mg/L as $CaCO_3$)	Hardness (mg/L as $CaCO_3$)	Ammonia (mg/L)
Microcosms [1]	2000	3	675 ± 28	83 ± 2	124 ± 2	0.042 ± 0.018
Denis Pond	2000	2	1104 ± 28	310 ± 12	597 ± 12	0.254 ± 0.021
Duckweed Pond	2000	3	554 ± 4	264 ± 3	296 ± 4	0.775 ± 0.456
Naharney Conv	1999	5	767 ± 228	277 ± 25	583 ± 111	0.073 ± 0.016
Naharney MinTill	1999	5	157 ± 35	89 ± 5	108 ± 31	0.062 ± 0.012
Redberry Pond	2000	3	2287 ± 580	305 ± 63	1728 ± 104	0.187 ± 0.034
Round Prairie Pond	2000	2	699 ± 77	318 ± 36	243 ± 32	0.064 ± 0.023
Windmill Pond	1999	4	1336 ± 96	289 ± 17	326 ± 9	0.084 ± 0.023

[1] Mean values calculated from mean values for all microcosms at each sampling time.

adjacent to a cattle grazing area. The highest ammonia concentration measured in this pond was 1.65 mg/L on July 11th, 2000. Mean ammonia concentrations throughout the study in the microcosms (0.042 ± 0.018 mg/L) were lower or similar to those observed in the field ponds (0.062 to 0.775 mg/L) (Table 4.17). Whereas nitrate/nitrite concentrations were high at pretreatment in the microcosms, but decreased to below detection limits post-treatment (Table 4.7), nitrate/nitrite concentrations in the ponds were either near or below the detection limit throughout the sampling period, except in the case of the July sample from the Denis Pond, which had a concentration of 0.041 mg/L. Appendix G lists the range of values for the water quality parameters of all Saskatchewan ponds monitored in this project.

The wild-caught metamorphs ranged in mean SVL from 20.2 to 25.4 mm, with an overall mean of 23.2 ± 0.6 mm (Table 4.18). The mean SVL of the microcosm metamorphs (23.3 mm) was within the range of mean sizes of metamorphs from wild populations (Table 4.18). The microcosm metamorphs were significantly larger in mean SVL than metamorphs from Round Prairie, Duckweed, and Redberry ponds (Kruskal-Wallis one-way ANOVAs, $p < 0.001$). Appendix H lists the mean sizes at metamorphic climax of populations of wild-caught wood frog metamorphs that were measured in 1999 and 2000.

4.3.10 Lindane in Boreal Ponds

Lindane was not detected in any of the ponds sampled in May-June 2000. Lindane was also not detected in any of the water samples collected from ponds in 2001 (Table 4.19). Lindane concentrations detected in the recently hatched tadpoles collected as egg masses in 2001 ranged from 0.81 to 3.55 ng/g with a mean of 1.63 ± 1.02 ng/g (Table 4.20).

Table 4.18. Size at metamorphic climax of wood frog metamorphs: comparing microcosm and wild-caught metamorphs that were at Gosner stages 42-45 when measured. Values are means ± SE. Only snout-vent length (SVL) was tested for significant differences among treatments. Different letters denote significant differences (Krustal-Wallis one-way ANOVA on ranks, $p < 0.001$, Tukey test).

Site	Year	*n*	Wet Weight (g)	Total Length (mm)	SVL (mm)
Microcosms	2000	341	1.90 ± 0.04	55.2 ± 0.8	23.3 ± 0.1 [a]
Denis	2000	20	2.14 ± 0.06	61.6 ± 1.0	23.8 ± 0.2 [a]
Duckweed	2000	22	1.87 ± 0.06	59.7 ± 1.0	21.7 ± 0.2 [bc]
Naharney Conv	1999	42	1.67 ± 0.05	48.4 ± 2.0	23.7 ± 0.2 [a]
Naharney Mintill	1999	10	2.19 ± 0.15	53.9 ± 5.2	25.4 ± 0.3 [a]
Redberry	2000	26	1.17 ± 0.06	42.8 ± 2.0	20.2 ± 0.2 [c]
Round Prairie	2000	20	1.48 ± 0.07	51.8 ± 1.2	21.7 ± 0.3 [bc]
Windmill	1999	14	1.81 ± 0.05	57.9 ± 1.1	22.8 ± 0.3 [ab]

Table 4.19. Concentrations (μg/L) of hexachlorocyclohexane (HCH) in boreal ponds of northern Saskatchewan, as sampled in May and June 2000 and May 2001.

Site	Year	α-HCH	β-HCH	γ-HCH	Total HCH
Ralph Pond	2000	< DL [a]	< DL	< DL	< DL
Airport Side Pond	2000	< DL	< DL	< DL	< DL
Grassy Pond	2000	< DL	< DL	< DL	< DL
Cub Lake Marsh	2000	< DL	< DL	< DL	< DL
Airport Side Pond	2001	< DL	< DL	< DL	< DL
Pond #2	2001	< DL	< DL	< DL	< DL
Pond #3	2001	< DL	< DL	< DL	< DL
Pond #4	2001	< DL	< DL	< DL	< DL
Pond #5	2001	< DL	< DL	< DL	< DL
Pond #6	2001	< DL	< DL	< DL	< DL
Pond #7	2001	< DL	< DL	< DL	< DL
Grassy Pond	2001	< DL	< DL	< DL	< DL
Cub Lake Marsh	2001	< DL	< DL	< DL	< DL
Pond #10	2001	< DL	< DL	< DL	< DL

[a] Detection limits (DL) were 0.010 μg/L (all HCHs) for the 2000 data and 0.00034 μg/L (α-HCH and β-HCH) and 0.00025 μg/L (γ-HCH) for the 2001 data.

Table 4.20. Concentrations (ng/g) of organochlorine insecticides[1], including lindane, detected in recently hatched tadpoles collected as wood frog egg masses in 10 ponds in Northern Saskatchewan. The ponds were located at least 80 km from agricultural cropland. All ponds except #8 (Grassy Pond) and #9 (Cub Lake Marsh) were at least 30 km from each other.

Pond	Lindane	HCB [2]	Heptachlor Epoxide	Dieldrin	p,p'-DDE
1	1.33	<DL [3]	<DL	<DL	<DL
2	3.50	<DL	<DL	<DL	<DL
3	0.81	<DL	<DL	<DL	<DL
4	1.12	<DL	<DL	1.18	6.91
5	1.29	<DL	0.38	<DL	<DL
6	3.55	0.94	<DL	0.77	<DL
7	1.24	<DL	0.95	<DL	<DL
8	1.12	0.96	<DL	<DL	<DL
9	0.89	<DL	0.57	<DL	<DL
10	1.46	<DL	<DL	<DL	<DL

[1] Only organochlorine insecticides concentrations above the detection limit are reported
[2] Hexachlorobenzene
[3] Detection limits (DL) were 0.25 ng/g for lindane, 0.88 ng/g for HCB, 0.37 ng/g for heptachlor epoxide, 0.67 ng/g for dieldrin, and 2.45 ng/g for p,p'-DDE.

4.4 Discussion

4.4.1 Lindane Residues

Lindane was not found in the water of either control group at 24 hours post-treatment, suggesting that pretreatment water did not contain lindane. Subsequent detection of lindane may have been due to aerial deposition from lindane-treated canola fields in the area (Waite *et al.* 1995). Waite *et al.* (1999) developed a unique wet/dry deposition sampler to simulate the deposition of pesticides from the atmosphere onto pond surfaces and to measure both wet and dry deposition. It should be noted that the data from the deposition sampler is not directly similar to the microcosms, because the sampler preserves the material collected over time and the extrapolation does not take into account the natural loss of lindane through adsorption, volatilization, degradation, or biological uptake. However data collected using the deposition sampler does allow for conservative estimates of the input of atmospheric lindane to the microcosms. Dry deposition of lindane in canola-growing areas in May and June of 2000 and 2001 averaged 438 ng/m^2/day, as estimated using a deposition sampler (D. Waite, Environment Canada, personal communication, 2001). During the 3-week period prior to the collection of the 1-week samples, the total possible deposition to a 500-L microcosm could have given a final lindane concentration of 0.018 µg/L, very similar to the mean 0.019 µg/L that was measured in the solvent control. Rainfall might have also contributed to the lindane detected in the controls, although probably very little. While the concentration of lindane in two rainfall samples collected in May of 1998 was 50 and 200 ng/L, lindane concentrations in the June rainfall samples were <10 to 10 ng/L (Waite *et al.* 2001). Higher rainfall concentrations in May coincided with relatively high atmospheric concentrations (Waite *et al.* 2001). Assuming rainfall during the 3 weeks prior to the 1-week sample collection contained 10 ng/L lindane, and that the total amount of rainfall that fell in June 2001 (38.3 mm; Environment Canada) fell during this time, then the final lindane concentration due to rainfall would have been 0.008 ng/L. Lindane may also have entered the controls from deposition of volatilized lindane from adjacent treatments. While this is possible, lindane would not be expected to evaporate

from water quickly, due to its low Henry's law constant of 4.93×10^{-7} atm m^3/mol, which predicts a half-life of 191 days for lindane in 1 m of water (Mackay and Leinonen 1975).

Water concentrations at 24 hours confirmed that target concentrations were achieved and the additional 30% lindane disappeared before this sampling time. Subsequent samples indicated that the lindane dissipated from the water column according to a first order kinetic model ($C_t = C_0 e^{-kt}$). The half-lives of lindane in microcosm water (18.7 - 23.7 days) were similar to values published in the literature for water with similar water chemistry, such as 27 days at pH 7.8 in nature surface water (Saleh *et al.* 1982) and 16.7 days in outdoor microcosms (10 m^3) filled with tap water (Caquet *et al.* 1992). In the latter example, the presence of sediment may have hastened the disappearance of lindane from the water; laboratory studies indicate that sediment will adsorb lindane and encourage decomposition (Lichtenstein *et al.* 1966; Thybaud 1990a). The half-lives in this study were very similar at 18.7 (0.1 µg/L), 23.2 (1.0 µg/L), and 23.7 (10 µg/L) days; the half-life from the 0.1 µg/L treatment was lower than the others, but had a wider confidence interval. It is possible that there was a lack of accuracy in measuring lindane concentrations so close to the detection limit of 0.010 µg/L at the final sampling time in this treatment, which may have contributed to the estimate of slope being less precise than for the other treatments.

Tadpoles from the control treatments contained low lindane concentrations, ranging from a mean of 1.1 µg/kg at 1 week to a mean of 3.7 µg/kg at 6 weeks, which verifies the presence of lindane in the water at those sampling times. It is also possible that the tadpoles contained lindane before the start of the experiment. The presence of lindane in the tissue samples, but not the water samples, from the boreal ponds in 2001 suggests that the newly-hatched tadpoles from those ponds obtained the lindane from maternal transfer. Since the closest agricultural cropland was at least 80 km away, and most of the pond sites were at least 30 km apart, the most likely source of lindane entering the northern ecosystem was through atmospheric transport from southern canola fields. Lindane has been detected in dry deposition samplers at Waskesiu in Prince

Albert National Park (D. Waite, personal communication, 2001). Female frogs may have accumulated lindane from their insectivorous diet and have passed on their residues to their lipid-rich eggs (Jung and Walker 1997). Although no data exist to document the concentration of lindane in wood frog eggs, adult wood frogs in the Yukon contained lindane concentrations of up to 12 µg/kg (Russell and Haffner 1997). Contaminants in the fat and liver of adult female frogs may be passed to offspring perhaps bound to vitellogenin (Hayes *et al.* 2002). The lindane concentrations in the control tadpoles at 1 week were similar to the concentrations found in recently hatched tadpoles from egg masses collected in May 2001 from 10 ponds in the boreal forest (Table 4.20). The lack of detection of lindane in pond water may reflect the low water solubility of lindane and its potential to adsorb to the sediment and to accumulate in lipids.

Tissue concentrations did not decrease during the larval period despite declining water concentrations. Since the animals increased in size during this period, it is possible that growth diluted the amount of lindane in the body, thereby resulting in unchanged tissue concentrations (Spacie *et al.* 1995). Body burdens in the microcosm tadpoles, calculated to adjust for this growth dilution, increased over time, and the increase in body burden with increasing lipid content suggests that the amount of lindane accumulated by the tadpoles increased due to the increase in lipid content during the development of the tadpoles. Increasing tissue concentrations over time in the control tadpoles may have been due to exposure to relatively constant lindane concentrations in the water, unlike the declining water concentrations that the treated tadpoles were exposed to.

The bioaccumulation factors (BAFs) measured in this study at 1 week (mean = 44) are similar to those reported previously. Thybaud (1990b) exposed *Rana temporaria* tadpoles to lindane and calculated Bioconcentration Factors (BCFs) of 30 with static (2 days) exposure and 32 with continuous (6 days) exposure. Thybaud (1990a) found that, under static conditions (without daily water renewal), a maximum BCF of ~30 was reached by the second day of exposure, and did not change over the 6-day experiment.

Lindane concentrations were variable in metamorphs sampled at the end of MC. This variability could have been partly due to variation in lindane exposure; the treatment with the highest tissue residues had the shortest exposure to clean water, because they were collected from the microcosms with nub tails prior to euthanasia for lindane analysis. In general, metamorphs from the other treatments were collected with full or partial tails and therefore spent more time in clean water during the period of tail resorption before euthanasia and tissue analysis. However, concentrations were generally lower in metamorphs sampled at the end of MC than in tadpoles, indicating that lindane residues in the tail were likely metabolized or excreted during tail resorption. As well, lindane was not detected in metamorphs at the onset of hibernation. Once the metamorphs were removed from the source of lindane in the environment, depuration would be expected to be rapid (Schimmel *et al.* 1977; Yamato *et al.* 1983; Marcelle and Thome 1983). Organic contaminants accumulated during the larval period might affect the survival of metamorphs during tail resorption, when the lipid-stored contaminant is metabolized and becomes bioavailable (Cooke 1970; Fioramonti *et al.* 1997), but no mortality occurred in this study, possibly because the body burden was not high enough to be lethal. Removal of the livers of the metamorphs kept until the onset of hibernation may also have reduced their body burden. Nevertheless some lindane should have been detected at the onset of hibernation, if it was present, since the fat bodies were included in the carcasses. The lack of even trace concentrations at the onset of hibernation indicate that lindane was eliminated from the young froglets during the 4 to 6 weeks after removal from the microcosms.

4.4.2 Possible Evidence of Endocrine Disruption

Corticosterone and Thyroid Hormones

Corticosterone (CORT) concentrations did not follow an expected dose-dependent response pattern with increasing lindane concentrations. Instead, CORT was highest in the 0.1 µg/L treatment and was significantly different from the solvent control. The T4 to T3 ratio was also significantly higher in the 0.1 µg/L treatment than in the solvent

control. The similar responses of these two hormones are not surprising; CORT and thyroid hormones (TH) may work together throughout the larval period to regulate development (Hayes and Wu 1995; Hayes 1997; Shi 2000). CORT accelerates TH-induced metamorphosis by increasing 5'-monodeiondinase activity (converts T4 to T3), decreasing 5-monodeiondinase activity (converts T4 to reverse T3, and T3 to T2), and decreasing T3 turnover (Galton 1990). Proper functioning of T4 may depend on CORT and T4 may even stimulate increased CORT production in order to down-regulate itself by increasing T4 to T3 conversion and by the negative feedback of CORT on the hypothalamus or pituitary (Hayes and Wu 1995). Increased T3 concentrations would exert negative feedback onto the hypothalamo-pituitary-thyroid axis, thereby decreasing T4 production. In this study, exposure to 0.1 μg/L may have reduced the conversion of T4 to T3 or increased the secretion or production of T4. It is also possible that elevated T4 induced higher CORT, but the down-regulating effects on T4 were not yet obvious.

The T4 to T3 ratio was significantly higher in the 0.1 μg/L treatment and could have been due to elevated T4, although there were no significant differences in T4. Short-term and long-term exposure to organochlorines can alter T3, T4, and their ratio. Sinha *et al.* (1991) observed increased T4 and decreased T3 and T3 to T4 ratio in serum and pharyngeal thyroid follicles in fish (*Clarias batrachus*) that were exposed to 8 μg/L endosulfan for 96 hr or to 1.5 μg/L endosulfan for 16 days. Thyroid peroxidase activity, a key enzyme in iodine oxidation, was also increased. They suggested that the pesticide either stimulated T4 secretion or blocked the conversion of T4 to T3. Extra-thyroidal conversion of T4 to T3 was blocked by endosulfan treatment in thyroidectomized fish (Sinha *et al.* 1991). Similar results with another fish (*Heteropneustes fossilis*) were observed in response to exposure to lindane for 4 days (Yadav and Singh 1987a) or 28 days (Yadav and Singh 1987b). Fish exposed to 8 mg/L lindane for 4 days had increased thyroid peroxidase activity, increased T3 and T4 concentrations in plasma and thyroid tissue preparations, and decreased extra-thyroidal T4 to T3 conversion (Yadav and Singh 1987a). Similarly, exposure to high concentrations of lindane (8 and 16 mg/L) for 28 days affected TH concentrations, but these effects varied with reproductive phase (Yadav

and Singh 1987b). The authors suggested that their results indicated that lindane inhibited circulating T3 concentrations, possibly through inhibition of extra-thyroidal conversion of T4 to T3 or the increased consumption of T3. In this study, no difference was noted in T3, but T4 was slightly elevated in the 0.1 μg/L, which resulted in a higher T4 to T3 ratio.

The altered sex ratio at the lowest treatment coincided with increased T4 to T3 ratio. It is possible that lindane may have affected the thyroid axis, thus interfering with normal gonadal differentiation. The role of thyroid hormones in gonadal differentiation is currently debated. While the thyroid-dependent developmental events are similarly ordered among amphibian species, the timing of gonadal development differs even within species (Hayes 1998). This phenomenon may be interpreted as an indication that TH do not affect gonadal differentiation, or that TH may regulate this process differently in different species (Hayes 1998). For example, species that differentiate when TH are low (e.g. some *Rana* species), may be more sensitive to THs than species that differentiate when TH are high (e.g. bufonids) (Hayes 1998). Administration of thiourea to block TH production resulted in 100% females in *Xenopus laevis* and 100% males in *Hyperolius viridiflavus* (Hayes 1997). The author suggested that normal sex ratios could not occur in the absence of TH and that exposure of the tissues to TH was required prior to sex steroid exposure for normal sexual development.

The fact that a typical monotonic dose-response was not observed is not unusual in cases of endocrine disruption. Non-monotonic dose-responses have been observed in a variety of animal models including mice, rats, fruit flies, *Daphnia*, and human cell cultures, and the dose response is in the form of a U-shaped or inverted U-shaped curve, where low doses exert effects opposite to those of slightly higher doses (Calabrese and Baldwin 2001). For example, vom Saal *et al.* 1997) showed that mice fed low doses of diethylstilbestrol (DES) developed larger prostate glands, whereas mice fed high doses developed smaller prostate glands than control mice. The lack of response in blood hormone concentration or sex ratio at lindane concentrations above 0.1 μg/L in this study may indicate the initiation of mechanisms to compensate for the interference. Since

exogenous steroids can affect the sex ratio differently at different concentrations in amphibians, it is possible that lindane may enhance male differentiation at low concentrations but cause increased female production at higher concentrations that were not tested in this study. The effect of lindane on thyroid hormones may have been the underlying cause of the altered sex ratio, but further research needs to be done to verify this hypothesis and to determine possible mechanisms.

It must be cautioned that the values obtained only represent a snapshot of plasma concentrations at the time of collection. Caution must be used in interpreting the absolute concentrations of hormones in the blood because these values do not reflect changes in hormonal activity at the target organ(s) (Wingfield and Farner 1993). Changes in metabolic clearance rate, secretion rate, protein binding, and concentration and distribution of target receptors may affect hormone activity or bioavailability without altering its concentration.

Hormesis: Effects on Size at Metamorphic Climax

Some toxicologists suggest that the fundamental nature of the dose-response curve is neither linear nor threshold, but is instead U-shaped (Calabrese and Baldwin 2001). Apparent low dose stimulatory effects, or hormesis, may be due to overcompensation by the homeostatic mechanisms to contaminants (Calabrese and Baldwin 2001). In this study, increased mean size at MC was observed at the lowest concentration, with size decreasing to control levels with increasing lindane concentration although it was not statistically significant. However, the same trend was observed in the slopes of the wet weight to total length relationships; these slopes are an indication of body condition (Bailey 1968). By comparing the 95% confidence intervals of the slopes, it can be noted that metamorphs from the lowest lindane treatment (0.1 μg/L) were more stout than metamorphs from the controls, and that the body condition decreased to control levels with increasing lindane concentration. It is possible that lindane had an hormetic effect on growth at the lowest treatment, perhaps through some stimulation of the thyroid

gland. No other published literature on hormetic effects of organochlorines in frogs has been found.

Sex Ratio

Since survival in the 0.1 μg/L treatment did not differ from the other treatments, it is unlikely that lindane exposure caused differential survival in this treatment. Instead it is possible that lindane may have affected sexual differentiation. Lindane has been shown to be estrogenic in mammals (Kamrin 1997); thus, it is possible that lindane could alter gonadal sex development in amphibians. However, how lindane, or other pesticides, may alter gonadal sex development is unknown. To date, a clear pattern of the effects of sex steroids has not been determined for species even with a similar genetic sex-determining system (Hayes 1998). Exogenous reproductive steroids have been shown to cause partial to complete sex reversal, but the response depends on the species, type of steroid compound tested, and its concentration (Witschi 1971; Hayes and Wu 1995; Hayes 1998). In species where the female is the heterogametic sex, such as *Xenopus laevis*, estrogen is the dominant hormone, and treatment with anti-estrogenic or androgenic compounds will produce intersexual gonads and occasionally complete sex reversal. This trend would seem to be the opposite in species like ranids or hylids, where the male is the heterogametic sex (Witschi 1971; Gallien 1974; Hayes and Wu 1995). In their case, testosterone and other androgens increase the production of males, but estrogen can have varying success in creating all females, intersexes, or even more males such as in the case for *R. sylvatica* (Hayes 1998).

Lindane may have had a direct action on the gonadal tissue during differentiation, acting as an androgenic or anti-estrogenic compound. Lindane may have also blocked key receptors, down-regulated estrogen receptors, or up-regulated androgen receptors. As an organochlorine chemical, lindane has the potential to cause enzyme induction: it is possible that the lowest concentration induced sex steroid-metabolizing enzymes, whereas higher concentrations had no effect or induced toxicity. Lindane has the potential to affect key pathways in the production of estrogen. Lindane inhibited

cholesterol side-chain cleavage in mice as judged by the decreased conversion of cholesterol to pregnenolone and progesterone (Sircar and Lahiri 1990). Inhibition of aromatase activity or an increase in its metabolism can also affect the production of estrogen and thereby alter sex differentiation (Hayes 1998). A recent study illustrates the effect of low, environmentally-relevant concentrations of atrazine on sexual differentiation (Hayes *et al.* 2002).

Glycogen

Chronic exposure to lindane during larval development did not affect hepatic glycogen concentration at the onset of hibernation. This finding is contrary to what was observed in mudpuppies chronically exposed to organochlorine chemicals in the St. Lawrence River. Gendron *et al.* (1997) found low glycogen with concurrent lower CORT in response to acute stress at contaminated compared to reference sites. Chronic exposure to contaminants can impair the ability of fish and mudpuppies to raise CORT in response to acute stress and can perhaps reduce its capacity to mobilize glycogen reserves (Hontela 1998). Since CORT was measured at metamorphic climax whereas hepatic glycogen was measured several weeks later in the juvenile frog, it is possible that the treated frogs recovered from the effects of chronic exposure and were able to accumulate glycogen reserves similar to those of control frogs. Frogs exposed to lindane as tadpoles in the wild would also be removed from lindane exposure upon dispersal and hence would recover.

4.4.3 Use of Fluctuating Asymmetry as a Biomarker of Exposure

The levels of FA in my study, after factoring out measurement error, were too low to detect any differences due to lindane treatment. Despite the large number of repeated measurements and the large sample size, the measurement error was higher in this study than in another study measuring similar external traits on frogs (Alford *et al.* 1999). Evidently, this type of measurement was not sensitive enough as a biomarker of exposure to lindane in the wood frog.

4.4.4 Evaluating the Microcosm Design

To my knowledge, there have been no other microcosm studies that compared habitat quality between wild and microcosm amphibian populations. Typically, examples of outdoor microcosm or mesocosm studies from the literature contained water from local ponds, lakes, or wells, and were assumed to be acceptable environments for the species being tested. In this study, local pond water was not used because of the risk of an outbreak of FV3. However, other studies have used aged tap water to successfully raise and test the effects of contaminants on frog tadpoles (Cooke 1971; Bauer-Dial and Dial 1995; Semlitsch *et al.* 1995; Fioramonti *et al.* 1997; Jung and Walker 1997; Ingermann *et al.* 1999). Aged tap water was also used to raise wood frog tadpoles outdoors in large tanks to examine the effect of size at metamorphosis on size at hibernation and susceptibility to lungworm infection (Goater and Vandenbos 1997). Despite lower densities (4, 8, and 16 tadpoles per 250 L), their metamorphs took longer to reach metamorphic climax (52 to 55 days compared to 46 to 48 days) and were smaller at complete tail resorption (0.80 to 1.35 g) than 15 metamorphs at the same stage in this microcosm study (1.73 ± 0.64 g).

The occurrence of malformations, excluding scoliosis, observed in this study was 0.3% for all treatments combined, which is within published background levels (0-2%) for wild populations (Ouellet 2000). While malformations in the axial skeleton, the vertebral column, and the notochord have been experimentally induced in tadpoles by toxicants (Cooke 1970; Cooke 1972; Cooke 1981; Schuytema *et al.* 1991; Alvarez *et al.* 1995), the frequency of scoliosis in my study was high and was similar among treatments. Skeletal malformations such as scoliosis have been observed at frequencies of up to 95% in larvae reared in the laboratory and are most likely due to nutritional deficiencies. Leibovitz *et al.* (1982) reported decreasing frequencies of malformations with increasing amounts of vitamin C in the tadpole diet. Marshall *et al.* (1980) argued that increasing total calcium levels, not vitamin C levels, decreased the frequency of skeletal malformations in bullfrog larvae. An investigation of 10 artificially

compounded and fresh diets revealed lower frequencies of skeletal malformations in tadpoles fed fresh diets or compounded diets complemented with fresh food (Martinez *et al.* 1992). Leibovitz *et al.* (1982) also mentioned that tadpoles fed only artificial diets had a greater frequency of skeletal malformations than tadpoles that also had access to microbial growth on the tank walls. Although there were other food sources in the microcosms, such as the periphyton on the plastic liner, the leaf litter, and the potted plants, the supplementation of the tadpole diet with Tetramin® Fish Food may have contributed to the nutrient imbalance that caused scoliosis as observed in this study. As well, most of the curvature in the tails of the microcosm metamorphs appeared as distinct S-shaped kinks starting at the base of the tail. This phenomenon is similar to that found by Martinez *et al.* (1992), who hypothesized that nutritional deficiencies obstruct the correct formation of collagen, leading to weaknesses in the notochord and spine that become damaged during the S-shaped movement of swimming.

The occurrence of scoliosis illustrates a potential flaw in the experimental procedure. Efforts to collect hair algae from ponds considered free of FV3 were not always successful, and therefore small amounts of hair algae were added to the microcosms. The problem with scoliosis could be avoided in the microcosms by feeding the tadpoles a more balanced diet. Algae could have been cultured in clean water in the laboratory and more extensive periphyton growth encouraged by adding more plants and leaving the microcosms set up for a longer time before starting the experiment. The latter recommendation was not possible in this study due to the short time period available to re-start the study after the occurrence of FV3 in the first run.

Temperature is a factor that can affect growth and development of tadpoles. The trees on the south side of the enclosure shaded some of the study area from mid-August to the end of the study. Metamorphs that were kept outside in cages in the shade took longer than the expected four days to complete metamorphic climax (complete tail resorption). As well, there were tadpoles remaining in the microcosms at the end of the study period, when all the microcosms were cooler in water temperature. This slower development may have been caused by increased shading by the trees which lowered air

and water temperatures. Most of the metamorphs were removed from the microcosms before the shading began to differentially affect water temperatures. While the effect of shading was accounted for in the experimental design by blocking for distance from the trees, the fact that shading from the trees appeared to have a negative effect on development for these remaining tadpoles suggests that the microcosms should not be set up near the trees. However, some shading was necessary, particularly in mid-July, when the water temperatures were high. In fact, water temperatures appeared to be slightly higher in the microcosms than in the ponds. Instead of placing the microcosms near the trees, it would be better to develop artificial shade that provides equal shading to each microcosm. Such shading was in fact provided by placing oriented strand board planks from north to south across the top edges of the microcosms. As the sun moved overhead, the shade created by the planks moved similarly within the microcosms, thereby providing each microcosm with approximately the same amount of shade. Water temperature did not appear to affect development in the majority of microcosm metamorphs because there were no differences in time to MC. Growth, although significantly affected by treatment, was not related to temperature. Although the water temperatures in the ponds were generally lower than in the microcosms, the microcosm metamorphs were within the range of mean sizes seen in wild populations.

The purpose of gathering water quality and size at MC data from ponds with wood frog tadpoles was to determine if the microcosms adequately simulated the habitat of wild tadpoles. In this study, the microcosms had mean values for water quality parameters that were within the range of the mean values obtained in the 1999 and 2000 ponds. Ammonia concentrations were lower or similar in the microcosms than in the ponds, despite the potential for build-up from tadpole excretion. The microcosms most closely resembled Naharney MinTill in terms of alkalinity, hardness, and ammonia, and the mean sizes at MC were similar. Metamorphs in the microcosms were significantly larger in SVL than those measured in three of the seven ponds.

4.5 Summary

Lindane, at the concentrations tested, did not adversely affect survival, size at or days to metamorphic climax of wood frog tadpoles. However, a possible hormetic effect of lindane was demonstrated, as body condition of the metamorphs was highest at the lowest lindane concentration, and decreased with increasing lindane concentration to control levels. Exposure to 0.1 μg/L lindane during the larval period of this study also affected sex ratios, resulting in significantly more males than females. This effect was not dose-dependent, but, along with the alterations in corticosterone and thyroid hormones, and the possible hormetic effect, does indicate a potential disruption of the endocrine system. Further investigation is necessary in order to confirm these results and to determine a mechanism of action.

CHAPTER 5

GENERAL DISCUSSION

The purpose of this research was to determine if exposure to lindane during the aquatic stage of the amphibian life cycle has any deleterious effects on the normal development of native frog species. This goal was achieved with acute toxicity tests in the laboratory and a chronic exposure scenario using outdoor microcosms. The specific objectives of the acute toxicity tests were to compare the acute toxicity of lindane to native frog species, to investigate differences in sensitivity due to developmental stage, and to assess both lethal and sublethal endpoints. The specific objectives of the microcosm study was to examine the effects of low concentrations of lindane on the survival, growth and development of wood frog tadpoles.

The results of the acute toxicity tests allowed me to put my work into context with other studies on organochlorines and tadpoles, on lindane and tadpoles, and on lindane and other species. The acute toxicity tests conducted in this study used native frog tadpoles (*Rana sylvatica*, *Pseudacris maculata* and *R. pipiens*) whose distribution overlapped that of lindane use on the Canadian prairies. Acute lindane poisoning caused symptoms in tadpoles that were similar to that observed with other organochlorines (Cooke 1970; Cooke 1972; Hall and Swineford 1980; Pawar and Katdare 1984; Licht 1985; Berrill *et al.* 1998) indicating a common response to exposure. However, LC50s obtained in this study indicated that lindane was not as acutely toxic to tadpoles as some other organochlorines (Sparling 2000). Comparing lethality of lindane to wood frogs (*R. sylvatica*) in this study to toads (*Bufo woodhousii fowleri*) and western chorus frogs (*P. triseriata*) in Sanders (1970) indicated that wood frogs were somewhat less tolerant. However, differences in sensitivity among studies may be due to differences in test

protocols or ages of the tadpoles. Species of the same age may differ in sensitivity as well, due to some unknown mechanism. Unlike results from other published studies, recently hatched tadpoles (hatchlings) in this study were more sensitive to lindane than older tadpoles, as shown by the lower EC50s and LC50s. Boreal chorus frog (*P. maculata*) tadpoles were also more tolerant than wood frog tadpoles, as LC50s could not be generated with the former species. The frog species tested in this study support the trend shown in the literature that tadpoles are more tolerant to lindane than fish or aquatic invertebrates (Thybaud 1990a).

The concentrations of lindane that caused acute toxicity in tadpoles were too high to occur in Canada under current patterns of lindane use. In other countries where lindane is still used as a spray or where higher amounts are likely to enter water bodies due to higher use (such as India; Voldner and Li 1995), the information from this study may be useful. Although lethality is an important endpoint to consider, sublethal effects of lindane are just as useful in countries with high lindane use. Hyperactivity, erratic swimming, and increased malformations can have serious consequences for predator avoidance and feeding activity (Berrill *et al.* 1993; Cooke 1970; Bridges 1997). Even short periods of abnormal swimming can make tadpoles more vulnerable to predation by attracting predators or by prolonging the larval period due to decreased feeding and therefore growth.

The microcosm study provided a more realistic exposure scenario for tadpoles on the Canadian prairies. Amphibian fitness is largely determined in the larval period (Smith 1987), and thus exposure to environmental contaminants during this period may have serious consequences. For example, exposure to environmental contaminants can affect swimming performance and activity, thereby affecting predator avoidance and tadpole growth (Semlitsch *et al.* 1995; Bridges 1997; Rowe *et al.* 2001). Developmental abnormalities induced during larval exposure can also have a negative effect on future growth and survival (Rowe *et al.* 1996; Rowe *et al* 2001; Harris *et al.* 2001). Size at and time to metamorphic climax have serious implications for future survival and reproductive fecundity, by affecting the size of insect prey that can be eaten, amount of

energy reserves prior to overwintering, survival during hibernation and breeding, time to reproductive maturity, size at first reproduction, and the number of eggs produced by a female (Berven and Gill 1983; Smith 1987).

Exposure of wood frog tadpoles to lindane throughout their larval period did not adversely affect survival or size at metamorphic climax at the concentrations tested in this study. In fact, an apparent hormetic effect on growth was observed at the lowest lindane concentration. Whether this effect would occur in the wild is unknown. While the microcosms were intended to mimic natural ponds, the influences of predation, predator avoidance, interspecific competition, food quality and quantity, and hydroperiod were controlled or eliminated. As well, the microcosm design is limited in its ability to completely mimic the natural complexity and dynamics that are found in a natural pond environment. On the other hand, effects on sex ratio and T4 to T3 ratio were also observed at this lowest lindane concentration. It is possible that lindane at this low concentration could have affected the endocrine system by having a direct action on the gonadal tissue during differentiation; blocking key endocrine receptors; down-regulating estrogen receptors, or up-regulated androgen receptors; or inducing metabolizing enzymes. Further study is required to confirm the results of this study as well as to elucidate the mechanism by which lindane affects the endocrine system.

Microcosm studies of pesticide effects offer the advantages of increased realism over laboratory systems, while maintaining the degree of control and manipulative ability necessary when testing hypotheses. I found the advantages of the outdoor microcosms to be: 1) the ability to provide a pond-like environment with exposure to natural conditions of light and weather, 2) the requirement of less daily maintenance (no water changes or cleaning of tanks), 3) the low cost of construction, and 4) the ability to raise healthier animals than in the laboratory. However, the microcosm design is laborious to set-up and it can be difficult to find the necessary components (plants, animals, leaf litter), especially in regards to finding sources uncontaminated by pesticides or FV3. A disadvantage to the use of outdoor microcosms is the potential for atmospheric contamination. In this microcosm study, low concentrations of lindane

were observed in the water and tadpoles of the control microcosms at 1 week post-treatment. The presence of trace contaminants may affect the results when testing the effects of low concentrations, but may in fact be unavoidable due to the ubiquitous nature of environmentally persistent contaminants.

The real application of this research will be in assessing the risk of lindane exposure to tadpoles in the wild. Although the Pest Management Regulatory Agency (PMRA) has recently decided to phase out all uses of lindane in Canada (PMRA 2002), lindane is still in use in the United States, Mexico, and other parts of the world. A recent U.S. EPA risk assessment determined that lindane, even if applied as a seed treatment, was likely to leach into groundwater and to enter surface waters via runoff (U.S. EPA 2001). As well, its volatility and long half-life in the atmosphere allows lindane to be transported long distances from its original source (Walker *et al.* 1999). Therefore, the potential still exists for lindane to enter breeding ponds via runoff, dry deposition and wet deposition. This research study showed that lindane can bioaccumulate in tadpoles, but that it can be eliminated after the metamorph leaves the contaminated water. The lowest observed effect concentration (LOEC) in this study was 0.1 μg/L, which led to a tissue concentration in the tadpoles of 3.7 μg/kg after 1 week of exposure. This tissue concentration is similar to the concentrations found in newly-hatched tadpoles from some boreal ponds monitored in this study. It is possible that sex ratios may become skewed in these wild populations of wood frogs, which would significantly affect future fecundity and survival of those populations. The presence of lindane in the tadpoles from these ponds, but not in the water, suggests that lindane was maternally transferred. As well, the lack of α-HCH in the tissues suggests that the lindane came from the treated croplands in the south. There are no data on effects of lindane on field populations of tadpoles. Extrapolation is not possible if there are no data to show that the effects observed in the microcosms, such as skewed sex ratios, occurred in wild frog populations after lindane was introduced. Extrapolation is the process of evaluating causal relationships by integrating laboratory experience with field observation, and applying the weight of evidence criteria (strength, consistency, specificity, temporality,

presence of a biological gradient, plausible mechanism of action, coherence, experimental evidence, and analogy) (Chapman 1995). The microcosm study should be repeated to confirm the results and to elucidate a possible dose-response relationship including concentrations below 0.1 μg/L. Field studies need to be conducted that compare tissue residues to sex ratios and concentrations of circulating hormones in wild populations of wood frogs in order to demonstrate if such effects are occurring in the wild. The usefulness of these data in extrapolating to other native frog species, such as the boreal chorus frog or leopard frog, also needs to be evaluated by determining their relative sensitivity to lindane.

The Canadian water quality guidelines for the protection of aquatic life sets 0.01 μg/L as the highest concentration of lindane allowable in surface water (CCME 1999). This guideline has been exceeded in many prairie ponds (Donald *et al.* 1999). Since effects on growth and development were only seen at the lowest concentration of lindane tested (0.1 μg/L), and some of these effects (particularly the hormetic effects on growth) decreased with increasing lindane concentration, it would be interesting to determine what effects and body burdens would occur at lindane exposures of 0.01 μg/L and lower.

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APPENDIX A
PONDS OF THE BOREAL FOREST

Table A.1. Location, size, and general water quality characteristics of the boreal ponds sampled in 2000 and 2001 for lindane contamination. Data were collected during May 10-12, 2001.

Pond	GPS [1] Co-ordinates	Size [2]	pH	DO [3] (%)	Water Temp [4] (°C)	Conductivity (µS/cm)
1 [5]	55° 06.690' 107° 43.643'	35 x 20 x 1	5.4	109.7	15.9	28.38
2	55° 28.677' 108° 02.844'	100 x 30 x 1	5.4	103.0	14.5	51.8
3	55° 14.396' 106° 51.116'	150 x 7 x 1	7.3	92.7	11.3	34.5
4	55° 08.080' 106° 01.843'	50 x 20 x 0.5	5.9	83.4	15.8	90.9
5	54° 53.258' 105° 45.016'	50 x 35 x 1.5	8.6	133.3	16.6	90.1
6	54° 16.808' 104° 34.920'	30 x 8 x 0.3	7.2	64.3	14.6	251.7
7	54° 28.945' 104° 17.464'	60 x 35 x 1	6.4	57.8	14.4	272.9
8 [6]	54° 16.808' 104° 34.920'	30 x 30 x 1	7.3	69.0	9.2	48.7
9 [7]	54° 16.669' 104° 34.414'	30 x 10 x 0.5	5.8	84.5	8.5	53.7
10	54° 32.924' 103° 40.166'	35 x 40 x 1	7.6	107.9	16.1	350

[1] Global Positioning System
[2] Approximate size of the pond in metres as Length x Width x Depth (at deepest point) at the time of sample collection
[3] Dissolved oxygen concentration
[4] As recorded within the egg masses in the water
[5] Airport Side Pond
[6] Grassy Pond
[7] Cub Lake Marsh; GPS co-ordinates were taken on the roadside by the walk-in entrance. The marsh was approximately 800 m from the road.

APPENDIX B
LC50 AND EC50 ESTIMATES BASED ON MEASURED LINDANE CONCENTRATIONS

Table B.1. Estimates of the median effective concentration (EC50) and the median lethal concentration (LC50) of lindane to hatchlings (Gosner stage 23-25) and tadpoles (Gosner stage 26-30) of wood frogs (*Rana sylvatica*) from acute toxicity tests. Confidence intervals (95%) as determined by the trimmed Spearman-Karber method are given in parentheses. Estimates are based on measured lindane concentrations.

			Duration of Exposure			
Median Estimate	Life Stage	Test Number [1]	24 hr	48 hr	72 hr	96 hr
EC50 [2]	Hatchling	2	1.78 (1.38 - 2.30)	1.41 [3] (1.04 - 1.91)	-- [4]	--
LC50	Hatchling	2	--	--	1.50 (1.32 - 1.71)	--
LC50	Tadpole	2	--	2.49 [3] (2.21 - 2.81)	2.49 [3] (2.21 - 2.81)	2.44 [3] (2.08 - 2.85)

[1] Each test was conducted twice, but only one test of each life stage was analyzed for lindane concentrations in water at the start of the test.
[2] End point = erratic swimming
[3] Trim value was between 10 and 20%.
[4] Estimates were not generated.

Table B.2. Estimates of the median effect concentration (EC50) of lindane to hatchlings (Gosner stage 23-25) and tadpoles (Gosner stage 26-30) of boreal chorus frogs (*Pseudacris maculata*) from acute toxicity tests. Median lethal concentration estimates (LC50) could not be generated from the available data. Confidence intervals (95%) as determined by the trimmed Spearman-Karber method are given in parentheses. Estimates are based on measured lindane concentrations.

			Duration of Exposure			
Median Estimate	Life Stage	Test Number [1]	24 hr	48 hr	72 hr	96 hr
EC50 [2]	Hatchling	2	-- [3]	2.63 2.25 - 3.08	2.53 2.04 - 3.13	--
EC50	Tadpole	N/A	3.01 (2.63 - 3.45)	3.15 (2.84 - 3.49)	3.16 (2.87 - 3.48)	--

[1] Tests with hatchlings were conducted twice. Only one test was analyzed for lindane concentrations in water at the start of the test.
[2] End point = erratic swimming
[3] Estimates were not generated.

APPENDIX C
CALCULATION OF POTENTIAL LINDANE TRANSFER (RUN-OFF) TO PONDS UNDER HEAVY RAINFALL CONDITIONS (WORST CASE SCENARIO)

1. Rate of Application to the field:

The maximum recommended rate of seeding is 7 lb per acre (Thomas 1994). This rate converts to 3.18 kg per acre (1 lb = 0.45359 kg) or 7.85 kg of seed per ha (1 acre = 0.40468 ha).

According to the Alberta Crop Protection guide, (Evans 1995), the seed treatment formulation Vitavax rs Flowable contains 45 g/L carbathiin, 90 g/L thiram, and 680 g/L lindane. The rate of mixing is 562 ml per 25 kg of seed.

Vitavax rs Flowable contains lindane at 680 g/L, or 0.680 kg/L. This concentration multiplied by the rate of mixing gives:
(0.680 kg $_{\text{of lindane}}$/L) x (0.562 L / 25 kg $_{\text{of seed}}$) = 0.01529 kg $_{\text{of lindane}}$/kg $_{\text{of seed}}$.
At the seeding rate of 7.85 kg of seed/ha, the rate of lindane treatment is:
(7.85 kg $_{\text{of seed}}$/ha) x (0.01529 kg $_{\text{of lindane}}$/kg $_{\text{of seed}}$) = 120 g $_{\text{of lindane}}$/ha.

2. Quantity of lindane lost to runoff:

Assume that 0.5% of the quantity applied to the field is lost as runoff (Wauchope 1978). If the amount of rainfall required to cause runoff is at least 25 mm (Woo and Rowsell 1993), and the amount that runs off the cropland is 12% (Forsyth *et al.* 1997), then the amount of runoff from an area of cropland is calculated by multiplying the amount of rainfall by the area of cropland by 12%. An arbitrary rainfall of 30 mm will be used for the following calculations.

3. Quantity lost in runoff from possible ratios of cropland to pond area:

a) If we use a conservative ratio of 10:1 (4 ha of cropland draining into a 0.4 ha pond), then the volume of runoff entering the pond would be:
Volume of rainfall = 0.03 m x [(4 ha) x (10,000 m^2/ha)] = 1200 m^3
Volume of runoff = 12% of 1200 m^3 = 144 m^3

The amount of lindane in the runoff would be:
Lindane applied to cropland = (120 g/ha) x (4 ha) = 480 g $_{\text{of lindane}}$
Lindane lost in runoff = 0.5% of 480.12 g $_{\text{of lindane}}$ = 2.40 g $_{\text{of lindane}}$

Assuming uniform depth of 45 cm, the pond volume before influx of rainfall would be:
Pond volume = [(0.4 ha) x (10,000 m^2/ha)] x (0.45 m) = 1800 m^3
New pond volume = 1800 m^3 + 144 m^3 = 1944 m^3

Concentration of lindane in pond = 2.40 g / [1944 m^3 x 1000 L/m^3] = 1.23 µg/L

Assuming a water depth of only 15 cm (according to Freemark and Boutin (1994) criteria for Estimated Environmental Concentrations), the lindane concentration would be:
Pond volume = [(0.4 ha) x (10,000 m^2/ha)] x 0.15 m = 600 m^3
Pond volume with runoff = 600 m^3 + 144 m^3 = 744 m^3
Concentration of lindane in pond = 2.40 g / [744 m^3 x 1000 L/m^3] = 3.23 µg/L

b) Assume that a relatively large area of cropland planted to canola drains into a small pond. For example, consider 57 ha draining into a 0.5 ha pond (i.e., a ratio of 100:1) as described by Hall (1968). The volume of runoff entering the ponds would be:
Volume of rainfall = 0.03 m x [(57 ha) x (10,000 m^2/ha)] = 17,100 m^3
Volume of runoff = 12% x 17,100 m^3 = 2052 m^3

The amount of lindane in the runoff would be:
Lindane applied to cropland = (120 g/ha) x (57 ha) = 6842 $g_{\text{of lindane}}$
Lindane lost in runoff = 0.5% of 6842 $g_{\text{of lindane}}$ = 34.2 $g_{\text{of lindane}}$

The concentration of lindane in the pond with a water depth of 45 cm would be:
Pond volume = (0.5 ha) x (10,000 m^2/ha) x (0.45 m) = 2250 m^3
Pond volume with runoff = 2250 m^3 + 2052 m^3 = 4302 m^3
Concentration of lindane in pond = 34.21 g / [4302 m^3 x 1000 L/m^3] = 7.95 µg/L

Assuming a water depth of 15 cm, the lindane concentration would be:
Pond volume = [(0.5 ha) x (10,000 m^2/ha)] x 0.15 m = 750 m^3
Pond volume with runoff = 750 m^3 + 2052 m^3 = 2802 m^3
Concentration of lindane in pond = 34.2 g / [2802 m^3 x 1000 L/ m^3] = 12.2 µg/L

APPENDIX D
DESCRIPTION OF PONDS

Monitoring data from four ponds in 2000 and three in 1999 were used to compare the microcosms to the ponds in terms of water quality and size of metamorphs. The 2000 ponds include two in cropland (Redberry and Denis) and two adjacent to pastureland (Duckweed and Round Prairie). The 1999 ponds include two in cropland (Naharney Conv and Naharney MinTill) and one in pastureland (Windmill). These three ponds from 1999 could not be used in 2000 because they went dry before the end of the study.

Redberry Pond is located on the west side of a dead-end cart trail approximately 10 km southeast of Hafford, 3 km east of Redberry Lake. Bordered with cropland to the west and southeast, it is situated in a well-established agricultural area. Denis Pond is also accessible by a cart trail and is situated in an agricultural field which was seeded to canola in 2000. It is located approximately 16 km north of St. Denis and was named for the farmer who owns the land.

Duckweed Pond is situated in the Prairie National Wildlife Area Unit 7, which is 33 km northeast of Hafford. The surrounding area is used generally for cattle grazing. The pond is surrounded by aspen and willows and is named for its heavy cover of duckweed. Round Prairie Pond is found on a side road off Highway 219, approximately 45 km from Saskatoon. On the southeast edge of the Dundurn Community Pasture, the pond has the South Saskatchewan River to the west, a defunct gravel quarry and active bee farm to the north, and cattle-grazing land to the east and south. It is named for the Metis graveyard located further west along the road.

Naharney Conv and Naharney MinTill were two ponds used in another study that examined the influence of cultivation practices on biodiversity on the prairies. In 1999, metamorphs from these ponds were used to determine when insectivorous feeding began and when and if corticosterone concentrations peaked during metamorphic climax. These ponds are located approximately 2 km southwest and northeast of Redberry Pond, respectively, and are surrounded by agricultural land.

Windmill Pond is situated on the Prairie Farm Rehabilitation Administration Area (PFRA) Pasture #280, which is located near the north end of Last Mountain Lake. This pond is at least 2 km from any roads and is directly impacted by cattle grazing.

APPENDIX E
EVALUATING THE WET WEIGHT/TOTAL LENGTH RATIO

As the tail is resorbed during metamorphic climax (MC), body weight and total length decrease. To determine whether a weight/total length ratio would be suitable to account for varying tail lengths, a separate group of wood frog metamorphs ($n = 30$) was maintained in small plastic containers indoors during tail resorption. Wet weight, total length, and snout-vent length (SVL) were measured regularly during MC until tail resorption was complete. The total variance for each parameter (SVL and weight/total length) was partitioned into within- and among-individual components using the PROC GLM procedure in SAS (SAS Institute 1990). Repeatability values for SVL and weight/total length were calculated according to the equations listed in Lessells and Boag (1987). Repeatability, r, was given as

$$r = s^2_A/(s^2 + s^2_A), \tag{E.1}$$

where s^2_A is the among-groups variance component and s^2 is the within-group variance component. The variance components were calculated from the mean squares as:

$$s^2 = MS_W \tag{E.2}$$

and

$$s^2_A = (MS_A - MS_W)/n_0, \tag{E.3}$$

where n_0 is the coefficient related to the sample size per group in the analysis. Because sample sizes were not equal among individuals (number of measurements ranged from three to five), n_0 was also calculated according to Lessells and Boag (1987) from the equation

$$n_0 = [1/(a-1)] \times [\, \textstyle\sum_{a,i=1} n_i - (\sum_{a,i=1} n_i^2 / \sum_{a,i=1} n_i) \,], \tag{E.4}$$

where a is the number of groups and n_i is the sample size in the ith group. Repeatability of SVL and weight/total length was 0.80 and 0.03, respectively.

As well, weight/total length was significantly correlated with measurement date (PROC CORR, r = 0.59521, $p = 0.001$) whereas SVL was not (r = 0.13829, $p = 0.1195$). The slopes of curves fitted to the weight/length data were significantly different from zero, indicating that the ratio of weight/total length increases over the period of tail resorption in the metamorphs analyzed (one-sample t-test, $p < 0.001$). Therefore, this ratio could not be considered a feasible parameter to compare metamorphs of differing tail lengths. However, SVL did not change over time (one-sample t-test, $p > 0.50$) and was a consistent measurement of metamorph size at metamorphosis regardless of tail length. Thus, only data from stage 42 metamorphs (the stage immediately before tail resorption begins) were used to analyze treatment effects on wet weight and total length.

APPENDIX F
ADDITIONAL FLUCTUATING ASYMMETRY TABLES

This appendix contains additional information that complements, but is not critical to the understanding of, the analysis of fluctuating asymmetry in metamorphs of the 2000 microcosm study. Table F.1 lists the percent measurement error (%ME) for each side (left and right) of the traits measured for fluctuating asymmetry. Table F.2 gives descriptive statistics on the size of each trait. The dependence of asymmetry on trait size was assessed graphically within microcosms with plots of |L-R| against mean trait size and (L-R) against SVL (Fig. F.1), and among microcosms with plots of log variance(L-R) against mean trait size (Fig. F.2). Tables F.3 to F.6 lists the mean values (± SE) of signed (L-R) differences as well as two other types of fluctuating asymmetry (FA) indexes as described by Palmer (1994): FA1 and FA10. Index FA1 is the unsigned difference calculated as the absolute value of (L-R). Index FA10 describes the magnitude of the non-directional asymmetry after partitioning out measurement error. The principal advantage of this index is that, by partitioning out measurement error variance, it yields a more accurate estimate of true non-directional asymmetry {Palmer and Strobeck 1986; Palmer 1994). FA10 is calculated from the mean square values from a sides x individuals ANOVA using the equation:

$$\sigma_i^2 = (MS_{sj} - MS_m)/M \qquad \text{(F.1)}$$

where MS_{sj} is the mean square value of the interaction term, MS_m is the mean square value of the error term, and M is the number of repeated measurements (Palmer and Strobeck 1986, Palmer 1994). Also included in the tables are the approximate degrees of freedom (df) associated with each value of FA10. See Palmer (1994) for the equation.

Table F.1. Percent measurement error (%ME) for left (L) and right (R) measurements of four external traits as measured in the microcosm metamorphs. Values are given as L & R. The mean of six repeated measurements for each metamorph was used to calculate the statistics below. Data were not collected from two microcosms, one of the solvent control and one of the 1.0 µg/L treatment.

Treatment	Block	n^1	Radio-ulna Length	n^2	Eye-naris Length	Femur Length	Tibiofibula Length
all	--	277	17.53 & 19.22	280	28.24 & 26.88	3.81 & 3.22	2.40 & 2.14
Negative Control	1	17	17.22 & 27.87	17	46.96 & 27.57	3.17 & 2.66	1.56 & 1.34
Negative Control	2	18	74.58 & 72.25	19	39.39 & 45.49	4.25 & 5.14	3.70 & 3.66
Negative Control	3	18	34.55 & 29.16	18	43.83 & 44.50	3.33 & 2.61	2.51 & 1.46
Solvent Control	2	23	25.77 & 40.70	23	31.71 & 30.31	7.38 & 5.31	5.84 & 4.28
Solvent Control	3	22	18.02 & 16.20	22	49.33 & 35.96	6.56 & 4.98	4.71 & 2.82
0.1 µg/L	1	24	16.76 & 18.38	24	15.82 & 18.55	2.46 & 2.67	1.40 & 1.74
0.1 µg/L	2	23	29.65 & 28.57	24	19.13 & 17.93	2.46 & 2.64	1.50 & 1.78
0.1 µg/L	3	25	11.57 & 18.87	25	20.99 & 24.98	3.76 & 3.07	2.15 & 2.90
1.0 µg/L	2	23	15.63 & 16.35	23	42.27 & 37.73	3.82 & 2.79	1.89 & 1.85
1.0 µg/L	3	25	36.69 & 25.28	25	25.21 & 29.27	5.31 & 3.14	3.68 & 2.61
10 µg/L	1	22	36.56 & 34.78	22	21.82 & 29.43	3.08 & 3.00	1.56 & 1.05
10 µg/L	2	16	29.62 & 40.77	17	29.18 & 31.06	14.15 & 8.21	5.88 & 5.57
10 µg/L	3	21	28.90 & 29.51	21	33.16 & 34.05	7.09 & 5.76	4.89 & 3.94

[1] Sample size for radio-ulna length differed from other traits because of difficulties in measuring some individuals.

[2] Sample size for all traits except radio-ulna length.

Table F.2. Descriptive statistics for trait size (L+R/2) for four external traits as measured in the microcosm metamorphs. The mean of six repeated measurements for each metamorph was used to calculate the statistics below. Values are means ± SE. Data were not collected from two microcosms, one of the solvent control and one of the 1.0 µg/L treatment.

Treatment	Block	n [1]	Radio-ulna Length (mm)	n [2]	Eye-naris Length (mm)	Femur Length (mm)	Tibiofibula Length (mm)
all	--	277	3.79 ± 0.03	280	1.77 ± 0.02	9.51 ± 0.06	9.63 ± 0.08
Negative Control	1	17	3.98 ± 0.10	17	1.70 ± 0.06	9.07 ± 0.28	9.05 ± 0.36
Negative Control	2	18	3.34 ± 0.04	19	1.70 ± 0.04	9.65 ± 0.19	9.92 ± 0.22
Negative Control	3	18	3.84 ± 0.09	18	1.76 ± 0.04	9.25 ± 0.25	9.28 ± 0.28
Solvent Control	2	23	3.89 ± 0.06	23	1.85 ± 0.05	9.34 ± 0.15	9.38 ± 0.19
Solvent Control	3	22	3.23 ± 0.09	22	1.68 ± 0.04	9.35 ± 0.15	9.50 ± 0.19
0.1 µg/L	1	24	3.92 ± 0.12	24	1.82 ± 0.07	9.72 ± 0.25	10.07 ± 0.33
0.1 µg/L	2	23	3.79 ± 0.08	24	1.68 ± 0.05	9.39 ± 0.22	9.44 ± 0.25
0.1 µg/L	3	25	4.25 ± 0.11	25	1.90 ± 0.07	9.52 ± 0.26	9.69 ± 0.36
1.0 µg/L	2	23	3.71 ± 0.10	23	1.76 ± 0.04	9.61 ± 0.02	9.74 ± 0.27
1.0 µg/L	3	25	3.99 ± 0.08	25	1.91 ± 0.05	10.08 ± 0.19	10.15 ± 0.24
10 µg/L	1	22	3.64 ± 0.08	22	1.79 ± 0.05	9.35 ± 0.25	9.54 ± 0.34
10 µg/L	2	16	4.25 ± 0.08	17	1.76 ± 0.06	9.91 ± 0.13	9.88 ± 0.17
10 µg/L	3	21	3.42 ± 0.07	21	1.69 ± 0.05	9.30 ± 0.16	9.37 ± 0.21

1 Sample size for radio-ulna length differed from other traits because of difficulties in measuring some individuals.

[2] Sample size for all traits except radio-ulna length.

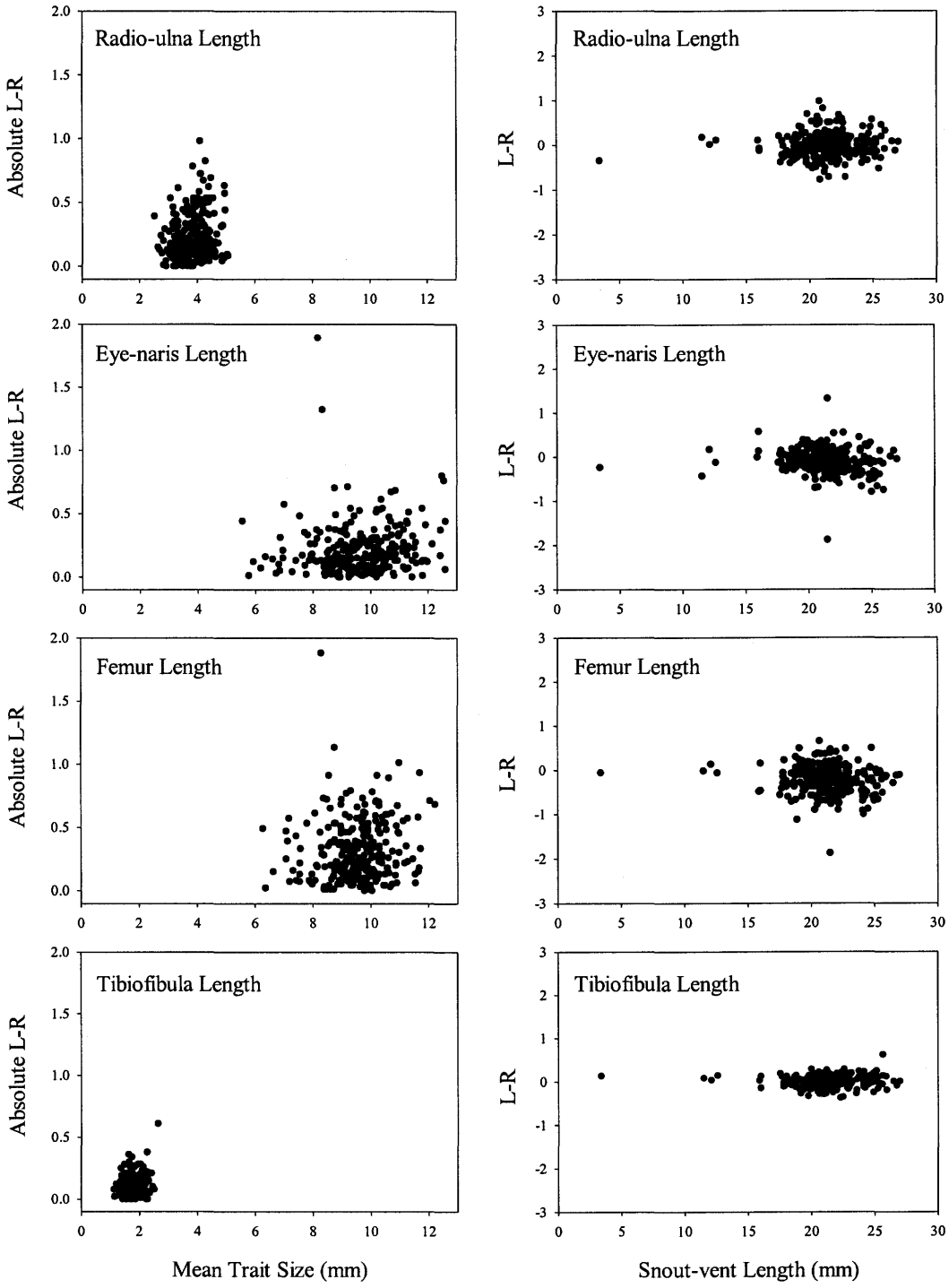

Figure F.1. Graphic tests for size dependence of fluctuating asymmetry within microcosms ($n = 277$ for radio-ulna length, and $n = 280$ for eye-naris length, femur length, and tibiofibula length).

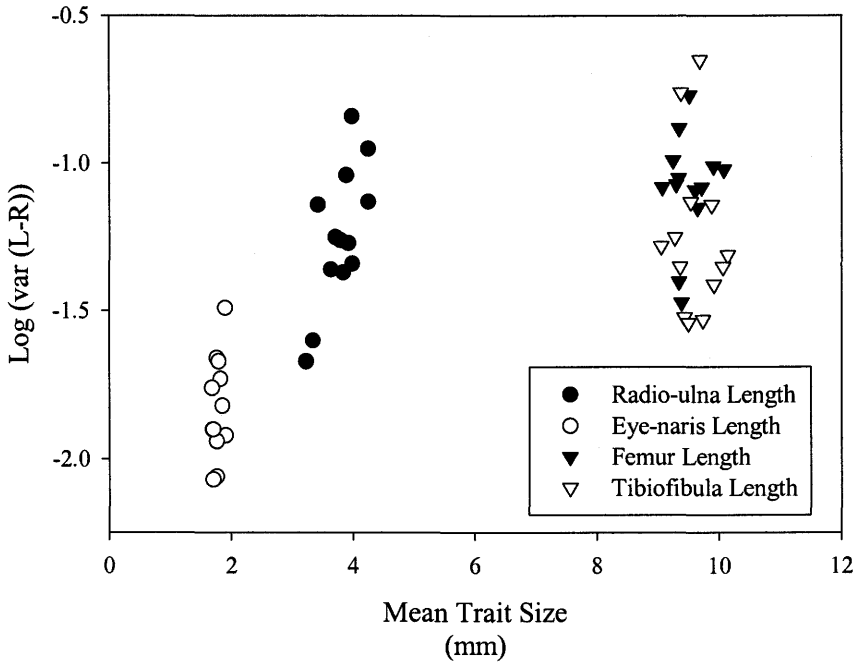

Figure F.2. Graphic tests of size dependence of fluctuating symmetry among microcosms (n =13).

Table F.3. Descriptive statistics of fluctuating asymmetry estimates for radio-ulna length as measured in the metamorphs of the microcosm study. Data were not collected from two microcosms, one of the solvent control and one of the 1.0 µg/L treatment.

			(L-R) [1]			|L-R| = FA1 [2]	FA10 [3]		
Treatment	Block	*n*	Mean ± SE (mm)	Skewness	Kurtosis	Mean ± SE (mm)	MS_m	σ_i^2	σ_i^2 df
all	--	277	-0.02 ± 0.02	0.37	0.85	0.21 ± 0.01	0.059	0.61	145.25
Negative Control	1	17	0.08 ± 0.09	-0.54	-0.12	0.32 ± 0.05	0.059	1.23	11.93
Negative Control	2	18	0.02 ± 0.04	0.08	-0.59	0.13 ± 0.02	0.063	0.20	0.38
Negative Control	3	18	-0.20 ± 0.05	0.12	-1.03	0.23 ± 0.04	0.063	0.34	4.27
Solvent Control	2	23	0.05 ± 0.06	0.33	-0.06	0.23 ± 0.04	0.050	0.91	14.65
Solvent Control	3	22	-0.04 ± 0.03	0.53	-0.15	0.12 ± 0.02	0.041	0.27	2.82
0.1 µg/L	1	24	-0.05 ± 0.05	0.70	1.30	0.18 ± 0.03	0.068	0.40	7.59
0.1 µg/L	2	23	-0.14 ± 0.05	0.23	-0.89	0.22 ± 0.03	0.060	0.46	8.78
0.1 µg/L	3	25	0.07 ± 0.05	-0.59	1.63	0.21 ± 0.04	0.051	0.73	14.18
1.0 µg/L	2	23	-0.12 ± 0.05	-0.34	0.36	0.21 ± 0.03	0.049	0.58	11.10
1.0 µg/L	3	25	-0.06 ± 0.04	-0.40	-0.56	0.17 ± 0.03	0.070	0.32	5.54
10 µg/L	1	22	0.03 ± 0.04	0.23	-0.85	0.17 ± 0.02	0.082	0.27	2.83
10 µg/L	2	16	0.29 ± 0.08	0.81	-0.54	0.32 ± 0.08	0.059	0.96	10.20
10 µg/L	3	21	-0.05 ± 0.06	0.01	0.29	0.21 ± 0.04	0.048	0.75	12.05

[1] Descriptive statistics were performed using the mean (L-R) per metamorph. FA4 = n * (SE of mean L-R)^2.

[2] Index FA1 was calculated by taking the absolute value of mean (L-R) value for each metamorph.

[3] Index FA10 (σ_i^2) describes the magnitude of non-directional asymmetry after partitioning out measurement error (Palmer and Strobeck 1986, Palmer 1994). See text for calculations. Also listed are measurement error mean square and approximate degrees of freedom for σ_i^2.

Table F.4. Descriptive statistics of fluctuating asymmetry estimates for eye-naris length as measured in the metamorphs of the microcosm study. Data were not collected from two microcosms, one of the solvent control and one of the 1.0 µg/L treatment.

			(L-R) [1]			|L-R| = FA1 [2]	FA10 [3]		
Treatment	Block	*n*	Mean ± SE (mm)	Skewness	Kurtosis	Mean ± SE (mm)	MS_m	σ_i^2	σ_i^2 df
all	--	280	0.01 ± 0.01	-0.02	1.54	0.10 ± 0.00	0.025	0.33	67.18
Negative Control	1	17	0.04 ± 0.02	0.01	0.13	0.08 ± 0.02	0.029	0.15	0.25
Negative Control	2	19	0.06 ± 0.03	-0.53	-0.16	0.10 ± 0.02	0.021	0.30	3.54
Negative Control	3	18	0.01 ± 0.03	-0.35	0.60	0.12 ± 0.02	0.028	0.39	5.57
Solvent Control	2	23	-0.01 ± 0.03	-1.37	3.01	0.09 ± 0.02	0.023	0.33	5.35
Solvent Control	3	22	-0.01 ± 0.03	-0.17	-0.57	0.11 ± 0.02	0.034	0.26	2.61
0.1 µg/L	1	24	-0.01 ± 0.03	-0.62	0.27	0.10 ± 0.02	0.022	0.42	8.21
0.1 µg/L	2	24	0.01 ± 0.03	-0.26	-0.64	0.11 ± 0.02	0.020	0.44	8.66
0.1 µg/L	3	25	-0.02 ± 0.04	1.74	5.65	0.13 ± 0.03	0.026	0.62	12.76
1.0 µg/L	2	23	0.03 ± 0.02	-0.76	1.81	0.08 ± 0.02	0.023	0.25	2.33
1.0 µg/L	3	25	-0.02 ± 0.02	0.41	-0.53	0.09 ± 0.01	0.022	0.27	3.36
10 µg/L	1	22	-0.01 ± 0.03	-0.76	0.28	0.11 ± 0.02	0.022	0.48	8.82
10 µg/L	2	17	0.01 ± 0.02	-0.15	-0.30	0.07 ± 0.01	0.027	0.16	0.04
10 µg/L	3	21	0.02 ± 0.02	0.78	-0.08	0.09 ± 0.01	0.026	0.24	1.86

[1] Descriptive statistics were performed using the mean (L-R) per metamorph. FA4 = n * (SE of mean L-R)^2.

[2] Index FA1 was calculated by taking the absolute value of mean (L-R) value for each metamorph.

[3] Index FA10 (σ_i^2) describes the magnitude of non-directional asymmetry after partitioning out measurement error (Palmer and Strobeck 1986, Palmer 1994). See text for calculations. Also listed are measurement error mean square and approximate degrees of freedom for σ_i^2.

Table F.5. Descriptive statistics of fluctuating asymmetry estimates for femur length as measured in the metamorphs of the microcosm study. Data were not collected from two microcosms, one of the solvent control and one of the 1.0 µg/L treatment.

			(L-R) [1]			|L-R| = FA1 [2]	FA10 [3]		
Treatment	Block	*n*	Mean ± SE (mm)	Skewness	Kurtosis	Mean ± SE (mm)	MS_m	σ_i^2	σ_i^2 df
all	--	280	0.00 ± 0.02	-0.45	2.55	0.24 ± 0.01	0.038	1.25	209.04
Negative Control	1	17	0.05 ± 0.07	0.38	-1.41	0.24 ± 0.04	0.040	1.05	11.28
Negative Control	2	19	0.00 ± 0.06	0.23	0.11	0.20 ± 0.04	0.033	1.06	12.74
Negative Control	3	18	-0.13 ± 0.08	-0.20	-0.02	0.27 ± 0.05	0.035	1.44	13.28
Solvent Control	2	23	0.11 ± 0.06	-0.23	-1.23	0.27 ± 0.03	0.037	1.19	16.24
Solvent Control	3	22	0.05 ± 0.08	0.93	0.38	0.25 ± 0.06	0.032	2.05	17.71
0.1 µg/L	1	24	0.18 ± 0.06	-0.04	-0.08	0.28 ± 0.04	0.041	1.03	16.14
0.1 µg/L	2	24	-0.07 ± 0.04	-0.33	0.21	0.15 ± 0.03	0.043	0.40	7.69
0.1 µg/L	3	25	-0.20 ± 0.08	-2.14	6.07	0.28 ± 0.07	0.047	1.84	19.84
1.0 µg/L	2	23	0.13 ± 0.06	0.42	0.25	0.25 ± 0.04	0.033	1.24	16.47
1.0 µg/L	3	25	-0.16 ± 0.06	-0.22	-0.60	0.27 0.04	0.037	1.28	18.13
10 µg/L	1	22	0.00 ± 0.04	0.11	0.02	0.15 ± 0.03	0.043	0.46	8.51
10 µg/L	2	17	0.00 ± 0.08	-0.50	-0.16	0.23 ± 0.05	0.036	1.38	12.35
10 µg/L	3	21	0.04 ± 0.06	0.09	-0.84	0.24 ± 0.04	0.036	1.20	14.81

[1] Descriptive statistics were performed using the mean (L-R) per metamorph. FA4 = n * (SE of mean L-R)^2.

[2] Index FA1 was calculated by taking the absolute value of mean (L-R) value for each metamoph.

[3] Index FA10 (σ_i^2) describes the magnitude of non-directional asymmetry after partitioning out measurement error (Palmer and Strobeck 1986, Palmer 1994). See text for calculations. Also listed are measurement error mean square and approximate degrees of freedom for σ_i^2.

Table F.6. Descriptive statistics of fluctuating asymmetry estimates for tibiofibula length as measured in the metamorphs of the microcosm study. Data were not collected from two microcosms, one of the solvent control and one of the 1.0 µg/L treatment.

			(L-R) [1]			|L-R| = FA1 [2]	FA10 [3]		
Treatment	Block	*n*	Mean ± SE (mm)	Kurtosis	Skewness	Mean ± SE (mm)	MS_m	σ_i^2	σ_i^2 df
all	--	280	-0.09 ± 0.02	-0.66	8.89	0.20 ± 0.01	0.039	0.94	188.56
Negative Control	1	17	-0.12 ± 0.06	0.34	-0.04	0.20 ± 0.04	0.032	0.81	10.04
Negative Control	2	19	-0.12 ± 0.05	0.75	-0.23	0.20 ± 0.03	0.035	0.56	8.84
Negative Control	3	18	-0.16 ± 0.06	-0.77	-0.22	0.20 ± 0.05	0.029	0.97	11.63
Solvent Control	2	23	0.01 ± 0.09	1.37	3.35	0.29 ± 0.06	0.044	1.96	18.40
Solvent Control	3	22	-0.05 ± 0.04	0.15	1.12	0.13 ± 0.02	0.030	0.49	8.95
0.1 µg/L	1	24	-0.11 ± 0.04	0.10	-0.22	0.19 ± 0.03	0.043	0.52	10.61
0.1 µg/L	2	24	-0.02 ± 0.04	0.60	0.69	0.14 ± 0.02	0.040	0.38	7.20
0.1 µg/L	3	25	-0.15 ± 0.09	-2.26	7.09	0.28 ± 0.08	0.054	2.07	20.27
1.0 µg/L	2	23	-0.08 ± 0.04	0.62	-0.07	0.16 ± 0.02	0.033	0.45	8.65
1.0 µg/L	3	25	-0.05 ± 0.04	0.10	-0.96	0.20 ± 0.02	0.046	0.54	11.31
10 µg/L	1	22	-0.15 ± 0.06	-0.50	-0.86	0.24 ± 0.04	0.032	1.13	15.24
10 µg/L	2	17	-0.07 ± 0.07	-0.88	0.51	0.21 ± 0.04	0.030	1.21	11.87
10 µg/L	3	21	-0.13 ± 0.05	0.54	0.02	0.21 ± 0.03	0.043	0.52	9.07

[1] Descriptive statistics were performed using the mean (L-R) per metamorph. FA4 = n * (SE of mean L-R)^2.

[2] Index FA1 was calculated by taking the absolute value of mean (L-R) value for each metamorph.

[3] Index FA10 (σ_i^2) describes the magnitude of non-directional asymmetry after partitioning out measurement error (Palmer and Strobeck 1986, Palmer 1994). See text for calculations. Also listed are measurement error mean square and approximate degrees of freedom for σ_i^2.

APPENDIX G
WATER QUALITY PARAMETERS IN SASKATCHEWAN PONDS

Table G.1. The range of values for water quality parameters measured in Saskatchewan ponds in 1999 and 2000. The ponds were sampled between June 1 and August 27 at various times of the day.

Year	Pond	Approximate Location	*n*	pH	Bottom Temperature (°C)	Conductivity (µS/cm)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	Ammonia (mg/L)	Nitrate/Nitrite (mg/L)
1999	Little Cattail	Last Mountain Lake NWA [1]	4	8.0 - 9.2	14.0 - 21.0	119 - 1373	190 - 523	215 - 283	0.088 - 0.145	0.010 - 0.021
1999	Naharney Conv	Hafford	5	7.1 - 8.1 [3]	14.0 - 18.2	112 - 1308	282 - 840	209 - 356	0.054 - 0.138	< 0.010 [2]
1999	Naharney MinTill	Hafford	5	6.7 - 7.2	13.0 - 16.0	20.56 - 219.6	73 - 250	77 - 107	0.036 - 0.099	< 0.010
1999	Sally	Last Mountain Lake NWA [1]	5	7.2 - 8.6	13.0 - 21.0	26 - 311	75 - 126	89 - 135	0.039 - 0.115	< 0.010
1999	Windmill	PFRA [4] Area #280	4	7.6 - 7.9	14.0 - 21.0	1213 - 1622	301 - 345	247 - 320	0.052 - 0.151	< 0.010
1999	Denis	Vonda	5	8.4 - 9.1	18.0 - 21.5	82 - 999	420 - 570	231 - 330	0.074 - 0.149	0.010 - 0.298 [3]
2000	Denis	Vonda	3	7.9 - 9.3	14.1 - 21.3	1050 - 1143	585 & 608 [3]	298 & 322 [3]	0.219 - 0.291	0.012 & 0.041 [3]
2000	Duckweed	PNWA [5] Unit #7	3	7.3 - 7.8 [3]	16.4 - 19.0	549 - 561	288 - 301	259 - 268	0.116 - 1.65	0.010 - 0.011
2000	Redberry	Hafford	3	8.0 - 8.4	12.9 - 19.4	1140 - 3010	1540 - 1900	232 - 430	0.141 - 0.253	< 0.010
2000	Round Prairie	Dundurn Community Pasture	2	7.4 [3]	17.8 [3]	622 & 776	211 & 275	282 & 353	0.041 & 0.086	0.010 & 0.015

[1] NWA = National Wildlife Area.
[2] Detection limit was 0.010 mg/L.
[3] Sample size = *n* - 1.
[4] PFRA = Prairie Farm Rehabilitation Administration.
[5] PNWA = Prairie National Wildlife Area.

APPENDIX H
SIZE OF WOOD FROG METAMORPHS IN SASKATCHEWAN PONDS

Table H.1. Size at metamorphic climax of wood frog metamorphs caught in Saskatchewan ponds in 1999 and 2000. Values are means ± SE with range in parentheses.

Year	Pond	Approximate Location	*n*	Wet Weight (g)	Total Length (mm)	Snout-vent Length (mm)	Species Found (as larvae)
1999	Little Cattail	Last Mountain Lake NWA[1]	4	2.17 ± 0.17 (1.80 - 2.62)	57.26 ± 4.30 (48.30 - 68.95)	24.66 ± 0.66 (23.35 - 26.50)	*Rana sylvatica*, *Ambystoma tigrinum*
1999	Naharney Conv	Hafford	42	1.67 ± 0.05 (1.02 - 2.28)	48.40 ± 2.00 (24.30 - 63.10)	23.72 ± 0.21 (20.00 - 26.95)	*R. sylvatica*
1999	Naharney Mintill	Hafford	10	2.19 ± 0.15 (1.51 - 3.18)	53.88 ± 5.15 (25.15 - 74.15)	25.37 ± 0.33 (23.90 - 26.95)	*R. sylvatica*
1999	Ralph	Little Bear Lake	9	1.54 ± 0.13 (1.02 - 2.34)	52.89 ± 3.79 (24.30 - 65.30)	22.69 ± 0.26 (21.70 - 24.15)	*R. sylvatica*
1999	Windmill	PFRA[2] Area #280	14	1.81 ± 0.05 (1.57 - 2.19)	57.89 ± 1.10 (51.15 - 68.10)	22.84 ± 0.28 (21.00 - 25.10)	*R. sylvatica*, *A. tigrinum*, *Pseudacris maculata*
2000	Denis	Vonda	20	2.14 ± 0.06 (1.64 - 2.66)	61.63 ± 1.03 (50.25 - 69.95)	23.80 ± 0.21 (21.75 - 25.55)	*R. sylvatica*, *A. tigrinum*
2000	Doug's	Vonda	25	2.05 ± 0.05 (1.52 - 2.68)	59.59 ± 1.05 (51.55 - 71.25)	24.21 ± 0.18 (22.15 - 25.55)	*R. sylvatica*
2000	Duckweed	PNWA[3] Unit #7	22	1.87 ± 0.06 (1.35 - 2.32)	59.70 ± 0.99 (42.80 - 65.20)	21.68 ± 0.15 (20.35 - 22.85)	*R. sylvatica*
2000	Redberry	Hafford	26	1.17 ± 0.06 (0.71 - 1.88)	42.81 ± 1.96 (25.15 - 58.80)	20.20 ± 0.23 (18.05 - 22.70)	*R. sylvatica*
2000	Round Prairie	Dundurn Community Pasture	20	1.48 ± 0.07 (0.90 - 2.07)	51.84 ± 1.20 (36.80 - 58.50)	21.74 ± 0.34 (19.00 - 24.40)	*R. pipiens*, *R. sylvatica*
2000	Wheat Field	Vonda	25	2.47 ± 0.07 (1.90 - 3.49)	65.24 ± 1.31 (53.90 - 78.10)	25.41 ± 0.18 (23.80 - 27.50)	*R. sylvatica*

[1] NWA = National Wildlife Area, [2] PFRA = Prairie Farm Rehabilitation Administration, [3] PNWA = Prairie National Wildlife Area.