

The Effects of the Glyphosate-based Herbicide WeatherMax[®] on Sexual Differentiation
and Growth in the Wood Frog (*Lithobates sylvaticus*).

By

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Abstract

Glyphosate-based herbicides are the dominant pesticide on the market and are utilized worldwide in both the agricultural and forestry industries. Their prevalence comes at a time when concern over the potential effects of pesticide application in amphibian spawning grounds is growing. The primary goal of this thesis was to determine if the glyphosate-based herbicide WeatherMax[®] has the potential to disrupt sexual differentiation and growth in the wood frog (*Lithobates sylvaticus*) in a pulse exposure at the predicted maximal environmental concentration (PMEC) of 2.88 mg acid equivalent per liter. This was carried out in laboratory, mesocosm and *in-situ* field exposures, in an attempt to determine how a potential disruption might vary between experimental environments. In this study, tadpoles from three split-wetlands targeted at the PMEC for WeatherMax were found to display no significant change in survival or growth, however gene expression of several genes involved in steroidogenesis during sexual differentiation (*cyp19*, *cyp17*, *star*, *foxl2*) were found to be affected. The effects on these genes appeared to be dependant on the exposure concentration of WeatherMax in each wetland, which varied even though all three wetlands were meant to target the PMEC. The wetland that was measured as having the highest herbicide concentration (PMEC 13) was found to have a female biased sex ratio. The results found in the field varied from those found in the more artificial exposures. In the laboratory the PMEC of WeatherMax experienced complete mortality, whereas in the mesocosms survival was not significantly affected. Sex ratios were unaffected in the laboratory, however at the PMEC there was a significant male bias in the mesocosms. The discrepancies in the results obtained from the different exposure types highlights the importance of real world exposures. That the

same concentration that caused complete mortality in the laboratory caused sublethal effects in the field is of importance as it denotes that these endpoints may not be easily investigated in these synthetic exposures. This project is a part of the Long Term Experimental Wetlands Area (LEWA) and contributes to the body of information amassed therein on the impact of a glyphosate-based herbicide on amphibians in a wetland ecosystem.

Résumé

Les herbicides à base glyphosate sont les pesticides dominant le marché et sont utilisés mondialement dans les industries agricoles et forestière. Cette présence vient à un temps où l'application de ces pesticides dans les zones reproductives des amphibiens est une cause grandissante d'inquiétude. Cette inquiétude est dû en partie au fait que, récemment, il a été déterminé que la population globale des amphibiens décroît et des enquêtes de laboratoire et mesocosm démontrent que les herbicides à base de glyphosate ont le potentiels de modifier certain sujets cruciales à la survie de la population. L'objet principale de cette thèse était de déterminer si l'herbicide à base glyphosate WeatherMax a le potentiel d'interrompre la différenciation sexuel de la grenouille des bois (*Lithobates sylvaticus*) l'hors d'une exposition pulsé à une concentration prévisible maximale environnemental (PMEC) de 2.88mg a.e./L. Il y a une banque de preuve grandissante qui démontre que les herbicides à base glyphosate ont un effet sur le développement et métamorphose des amphibiens en laboratoire qui consiste généralement à des expositions brèves ou chronique à concentration spécifiques. Dû à ces expérimentations qui ne représentent pas des scenarios environnementaux réels un but secondaire est advenu pour cette enquête afin de déterminer si le développement somatique, le développement gonadal et métamorphiques, seraient affecté par une exposition réaliste à l'environnement au PME.C. Ceci a été accompli en laboratoire, mesocosm et dans des étangs expérimentaux, pour déterminer comment une interruption potentielle pourrait varier d'un environnement expérimental à l'autre. Cette information aiderait à déterminer la relevance des études conduites dans des

environnements artificiels. Dans cette étude, des têtards de 3 marais divisés exposés au PMEC pour WeatherMax n'ont pas démontré de changement majeur en survie ou développement par contre, l'expression génétique de plusieurs gènes impliqués durant la différenciation (*cyp19*, *cyp17*, *star*, *fox12*) sexuel s'avèrent avoir été affecté. Il semble que l'effet sur ces gènes dépend de la concentration de l'exposition de WeatherMax dans chaque marais qui a varié même si les trois marais étaient supposés cibler le PMEC. Le marais qui eu la mesure la plus importante de concentration d'herbicide (PMEC 13) a été trouvé comme ayant un plus grand quotient de femelle. Les résultats obtenus dans le domaine ont variés de ceux obtenus dans les expositions artificielles. En laboratoire le PMEC de WeatherMax ont souffert un taux de mortalité complet, hors dans les mesocosms la survie n'a pas été significativement affecté. Les quotients de sexe n'ont pas été affectés dans le laboratoire, par contre au PMEC il y avait un quotient male significatif dans les mesocosms. Les divergences dans les résultats obtenus dans les différents types d'expositions mets en valeur l'importance de l'exposition dans le monde réel. Que la même concentration qui, en laboratoire à causé un taux de mortalités total, a causé un effet subléthal dans les étangs est d'importance car cela démarque que certains sujets ne peuvent pas être simplement enquêté dans les expositions synthétiques. Ce projet forme partie du « Long Term Experimental Wetlands Area » (LEWA) et contribue au corpus de l'information accumulée au sujet de l'impact d'herbicide à base de glyphosate sur les amphibiens dans un écosystème de marais.

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Abbreviations

a.e.	Acid equivalent
a.i.	Active ingredient
AMPA	Aminomethylphosphonic acid
cyp17	Cytochrome 17-alpha hydroxylase/ 17,20
cyp19	Cytochrome p450 aromatase
DAC	Days after control
DAT	Days after treatment
EDC	Endocrine disrupting compound
foxl2	Forkhead box L2
GMC	Gonad mesonephros complex
POEA	Polyethoxylated tallowamine
star	Steroidogenic acute regulatory

Chapter 1- Introduction

1.1 Thesis Rational and Hypothesis

Pesticides have been shown to have the potential to detrimentally affect amphibian survival and development. This is of particular concern during a time when global amphibian populations are known to be declining due to undetermined causes. Glyphosate-based herbicides have been the dominant pesticide on the market since 2001 and thus merit particular investigation. Chapters in this thesis are organized in manuscript form with redundancies kept to a minimum. The first chapter is a general introduction presenting glyphosate-based herbicides and why studying them in relation to potential environmental impacts on amphibians is necessary. The second chapter of this manuscript is a comprehensive review of the available literature on the effect of glyphosate-based herbicides on anurans subdivided by exposure type. Overall, trends show that study designs and exposures need to become more environmentally relevant. This could be achieved by taking certain factors that are to be of particular importance into account such as pH, temperature and the inclusion of a sedimentary substrate. More comprehensive studies are required as results are highly species-specific and herbicide formulation dependent. My primary hypothesis was that a glyphosate-based herbicide would affect the survival, growth and normal sexual differentiation of tadpoles in an environmentally relevant exposure (Chapter 3). Secondary to this, I hypothesized that the results would vary quantitatively according to exposure type (lab, mesocosm or field). In order to test this, *L. sylvaticus* tadpoles were exposed to the herbicide WeatherMax and the components contained within a glyphosate-based herbicide and examined in a laboratory, mesocosm and *in-situ* field study. Exposure to the predicted maximal

environmental concentration (PMEC) of 2.88 mg a.e. /L of WeatherMax caused complete mortality in the laboratory, but no significant mortality in the mesocosms or field. Modifications were detected in sex ratios in both the mesocosms and the field. The dominant sex was contradictory between the exposures however, and no histological gonadal abnormalities were detected in the mesocosm samples, potentially indicating sex-preferential death. As it has previously been indicated that these herbicides may be affecting sexual differentiation in a laboratory setting (Howe et al. 2004), I further hypothesized that an environmentally relevant concentration of WeatherMax would affect the expression of genes involved in steroidogenesis during sexual differentiation in *L. sylvaticus* tadpoles (Chapter 4). In order to test this, a partial sequence for the steroidogenic acute regulatory (*star*) gene was cloned and characterized in *L. sylvaticus*. Tadpoles collected from *in-situ* enclosures placed in three split-wetlands were exposed to the glyphosate-based herbicide WeatherMax and exhibited changes in *star*, *cyp19*, *foxl2* and *cyp17* at or close to the PMEC for WeatherMax. The experimental exposures performed during this project were designed to mimic agricultural spray practices. The pulsatile exposures, application durations and the concentrations examined were all environmentally relevant. This is the first project of its kind to examine how a glyphosate-based herbicide may be affecting an anuran species under real world conditions.

1.2 Are Glyphosate-based Pesticides a Factor in Amphibian Population Declines?

In the year 2000, a study analyzing amphibian population trends in 936 populations from around the world indicated that amphibians are declining globally and have been

doing so since the late 1950s (Houlahan et al. 2000). Since then numerous studies have been published identifying a global trend of amphibian declines and extinctions (Stuart et al. 2004; Mccallum 2007; Nystrom et al. 2007; Alford & Richards 1999). The International Union for Conservation of Nature and Natural Resources reports 32.4% of amphibian species to be globally threatened or extinct (~2 030 species), while 42% of all species are declining in populations (IUCN 2008). It is expected that this list of globally threatened or extinct species will continue to grow. It is also important to note that 24.5% of the total 6260 species assessed are listed as “Data Deficient” and may potentially also be globally threatened (IUCN 2008).

While this global downturn in amphibian populations is now widely recognized, in many cases the reasons for this trend remain unclear. Much of this phenomenon can be explained due to habitat loss (IUCN 2008), however this factor alone does not wholly account for the declining populations. It is likely that the causal mechanisms are multifarious and potentially even synergistic, making them difficult to pinpoint. As such, many less obvious factors such as fungal diseases, UV-B radiation levels, introduced species and habitat contamination are currently also under investigation (Puglis & Boone 2011; Croteau et al. 2008; Gahl et al. 2011; Relyea 2009). The identification of causal mechanisms is important for the design and implementation of proper conservation strategies.

1.2.1 Pesticides as Endocrine Disrupting Compounds

A pesticide is defined as “any substance used for controlling, preventing, or destroying animal, microbiological or plant pests” (Nordberg et al. 2009). Pesticides have not only been shown to have the capacity to impact survival in many non-target

organisms at environmentally relevant concentrations, but also to have profound effects at sub-lethal concentrations due to their potential to act as endocrine disrupting compounds (EDC). Endocrine disrupting compounds are defined by The Canadian Wildlife Service as “an exogenous agent that directly interferes with the synthesis, secretion, transport, binding action, or elimination of endogenous hormones and neurohormones, resulting in physiological manifestations of the neuroendocrine, reproductive or immune systems in an intact organism” (Takacs et al. 2002). Examples of this would include the effects of dichlorodiphenyltrichloroethane (DDT) and its breakdown product dichlorodiphenyldichloroethylene (DDE) as well as other organochlorine pesticides, which have been well studied in avians. These chemicals have been shown to cause reproductive failures in many species including birds of prey such as ospreys, peregrine falcons and bald eagles (Grier 1982; Cade et al. 1971; Spitzer et al. 1978). DDT, along with its metabolites, were shown to cause eggshell thinning in seabirds and raptors (Abou-Donia & Menzel 1968). Persistent organochlorine pesticides including dieldrin, endrin, aldrin, mirex, kepone, chlordane, and lindane were all found to have adverse affects on avian reproduction (Fry 1995). Alligators from a lake accidentally contaminated with DDT and dicofol were found to have higher post-hatching mortality and modified steroid hormone levels, with females having high levels of plasma estradiol-17 β and males having low levels of plasma testosterone along with modified secondary sexual characteristics (Guillette et al. 1994; Milnes et al. 2008). The pesticide atrazine has been shown to affect pheromone mediated endocrine signaling in mature male Atlantic salmon parr decreasing their ability to detect and respond to the priming effects of the female pheromones (Moore & Waring 1998).

1.2.2 Pesticides and Anuran Sexual Development

While pesticides have been shown to have the potential to act as EDCs in many organisms, amphibians have been conjectured to be at particular risk of being affected by pesticides (Hayes et al. 2006; McDaniel et al. 2008). This is generally attributed to their semi-permeable skin and biphasic life strategy, which includes a larval aquatic phase and, most often, a terrestrial or semi-terrestrial adult phase (Milnes et al. 2008). Endpoints such as time to metamorphosis, growth and survival have all been demonstrated as being affected by exposure to these types of contaminants (Williams & Semlitsch 2009; Wojtaszek et al. 2004; Relyea 2005; Mann & Bidwell 1999; Howe et al. 2004; Cauble & Wagner 2005; Jones et al. 2010). Amphibian sexual differentiation is highly labile, and therefore these organisms are of great interest for studying sexual development and the potential effects of various EDCs. At sublethal treatment concentrations, these compounds have the potential to affect endpoints such as body weight, body length, developmental stage and gonadal development. Bernabò et al (2011) found that one-month post-metamorphosis *Rana dalmatina* froglets, which had been chronically exposed to the pesticide chlorpyrifos during their larval period, presented gonads with normal gross morphology of both testes and ovaries. While sex ratios in the exposed animals remained at the expected 1:1 male to female ratio, 20-25% of the males presented intratesticular oocytes (Bernabò et al. 2011). A similar result was found in a study examining *Lithobates pipiens* tadpoles chronically exposed to glyphosate based herbicides (Howe et al. 2004). While no treatments differed significantly in sex ratio, intersex animals were detected in the treatments containing the surfactant

polyethoxylated tallowamine, with no intersex animals observed in either the control or glyphosate active ingredient (a.i.) treatments (Howe et al. 2004). From these results, it can be concluded that pesticides have the ability to disrupt normal sexual development in amphibians. It is also worth noting that although a constant sex ratio was found in these sample populations, this result does not necessarily evidence a constant ratio of effective breeders. How these abnormally differentiated gonads affect the reproductive capacities of these animals is not, as of yet, well understood and merits further investigation.

1.3 Glyphosate-based Herbicides

1.3.1 Why Study Glyphosate-based Herbicides in Particular?

According to the U.S. Environmental Protection Agency, 682 million pounds of conventional pesticide active ingredient were used in the agricultural market sector in the year 2007 (Grube et al. 2011). Of the active ingredients examined, glyphosate (Fig. 1.1) was ranked as the most utilized in the U.S. with an estimated usage of 180 to 185 million pounds of active ingredient. This far overshadowed the second most used active ingredient Atrazine, which weighed-in with 73 to 78 million pounds in the same year. The utilization of glyphosate-based herbicides has been increasing steadily since the removal of patent protection in the year 2000, thus glyphosate has been ranked as the number one conventional pesticide used in the agricultural market sector since 2001. It's usage statistics show an increase of over two-fold from the 2001 estimate of 85-90 million pounds of a.i. used, to the 2007 estimate (180-185 million pounds a.i.). In Ontario alone, ~4,537,826 pounds of the glyphosate a.i. were utilized in the agricultural sector in 2008. This accounts for ~55% of all of the herbicide active ingredients and ~42% of all pesticides sprayed on major crops in Ontario during that year (Mcgee 2010).

Glyphosate-based herbicides are extensively used in both agriculture and forestry; two industries that differ in both their method and timing of herbicidal application. In an agricultural scenario, applications occur several times during a single year and generally take place early in the growing season. The risk of glyphosate exposure is due to factors such as spray drift and runoff. In forestry, application is done late in the season using aerial dispersion methods. Herbicide application is strictly regulated with respect to permanent bodies of water, which require a buffer zone between the intended spray target and the aquatic area. However spraying can often be carried out directly over ephemeral amphibian breeding sites, as these are often too small or transient to be accounted for or to require a buffer zone.

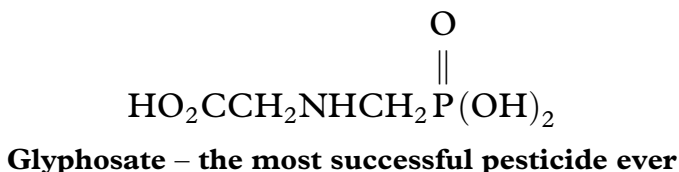


Figure 1.1 Structure of the active ingredient glyphosate, taken from Caseley (2000).

1.3.2 Glyphosate-based Herbicide Formulations – Diverse and Difficult to Study

Commercially available glyphosate based herbicides are a mixture of several ingredients, cooperatively known as a formulation. The glyphosate ingredient, which is responsible for the herbicidal effect of the formulation, is known as the “active ingredient” (a.i.). Most formulations also contain, or call for the addition of, a surfactant (a form of adjuvant), which heightens the effectiveness of the active ingredient by increasing its contact with and permeation into the target plants. The majority of the

formulations volume derives from the third ingredient, water (Foy 1987). Glyphosate-based formulations are currently being produced by many large companies including Monsanto, Syngenta, Cheminova and Dow AgroSciences. Each company generally produces numerous formulations with numerous patent protected inert ingredients, making the study of these products challenging.

1.3.2.1 Glyphosate -The Rise of a Titan

The herbicidal actions of glyphosate are due to its inhibition of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which is involved in the shikimate pathway. This pathway leads to the production of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan and is only active in plants and some microorganisms (Duke & Powles 2008). Glyphosate affects all higher plants and is considered a non-selective herbicide. To date, no other herbicide has been discovered that targets this enzyme (Duke & Powles 2008). This broad-spectrum characteristic, along with its high binding affinity to soil, makes glyphosate an ideal herbicide for pre-emergence crop spraying (spraying carried out before the crop emerges from the soil). With the advent of genetically modified glyphosate-resistant crops however, this chemical became a post-emergence herbicide as well. This allows agriculturalists to spray the herbicide directly on their genetically modified crops to kill undesired vegetation without negatively affecting their crop's health. This obviously desirable agricultural technology led to an increased use of this pesticide (Relyea 2011; Duke & Powles 2008).

The dissipation of glyphosate from soil is, for the greatest part, due to biodegradation by microbial or fungal vectors. The main biodegradation pathway in soil

is the breakdown of glyphosate to aminomethylphosphonic acid (AMPA), which is further metabolized to carbon dioxide. Abiotic degradation pathways contribute very little if at all to the active ingredients dissipation (Giesy et al. 2000). In a review of half life values (DT_{50}) encompassing forestry and agricultural soil types across Canada, Sweden, France and the USA, the arithmetic mean and 90th centile of the measured field DT_{50} values were calculated to be 32 and 95 days, respectively. In lentic waters, both glyphosate and its degradation product AMPA behave similarly. Their dissipation is highly dependent upon site-specific factors such as suspended organic matter in the water column, pH, alkalinity, etc (Goldsborough & Beck 1989) and half-life is estimated to fall within a 7-14 day range for both compounds (Giesy et al. 2000). Surprisingly, few studies have examined glyphosate residues in natural lentic environments and the majority of these have been conducted in Canada. Detection of glyphosate in the environment is generally low and environmental residues have been found to range from ~0.02 mg/L to ~2.8 mg/L when measured 1-1.5 hours post spray (spray rates varying from 1.5 to 1.8 kg a.i/ha) (Legris & Couture 1989). However higher spray rates lead to higher environmental detection as shown in a study by Edwards et al (1980) in which a concentration of ~5.2 mg a.e/L was found one day post spray after employing a spray rate of 8.96 kg/ha (Edwards et al. 1980).

1.3.2.2 Surfactants – Cheap and Toxic

While the number of studies indicating harmful effects of glyphosate formulations continues to grow, evidence is compounding that the toxicity of the end use product is greater than the toxicity accounted for by the glyphosate a.i. alone (Howe et al. 2004;

Perkins et al. 2000). This leads to the conclusion that other ingredients in the herbicide must be either compounding the toxic effects of the active ingredient or are in-and-of-themselves toxic.

Adjuvants, listed under “inert ingredients” on formulation MSDSs, can be broken down into several classifications according to their actions in the overall formulation. These chemicals can act as spreaders, stickers, humectants, penetration agents or herbicide modifiers (Hazen 2000). All of these have one goal in common: to cause the active ingredient to be more efficient in exterminating the intended target. Surfactants are a common subcategory of adjuvant, which cause droplets of the pesticide sprayed on the plant to overcome internal surface tension, allowing them to have a less spherical shape and cover a greater surface area on the plant. More importantly, they also aid the active ingredient in penetrating through the plant’s outer waxy epicuticle, thus increasing its efficiency and allowing for effective results with a smaller application of the more costly active ingredient. This raises the idea of profit, which is an obvious and definite motivator in the development and maintenance of surfactants. As \$2 worth of adjuvant can decrease the amount of herbicide needed by 20-50% and since a typical adjuvant may cost 1-3% the price of the herbicide, the addition of a surfactant not only facilitates agricultural practice, it also makes good pecuniary sense to utilize one (Hazen 2000).

Adjuvants are not regulated in the way that active ingredients are. In fact, herbicidal formulations are possessively guarded by the companies that patent them. Due to the expiration of the glyphosate patent, there has been a proliferation of glyphosate-based products released onto the market; each of these formulations having its own slightly different chemistry and surfactant mixture (Howe et al. 2004). This makes

evaluating new products extremely difficult and often makes it problematic to extrapolate research results between products that “appear” on the label to be very similar.

Commercial formulations of glyphosate have been on the market since 1974 and extensive studies have been carried out to determine their potential toxicities on non-target organisms (Giesy et al. 2000). As one of the first formulations, Roundup Original (Monsanto Company, St. Louis, MO), which is still in use today, is one of the most well known and best studied. It is also one of the few formulations for which the surfactant is known. This surfactant is named polyethoxylated tallowamine (POEA, CAS # 61791-26-2) and it is thought to be the surfactant, or a part of the surfactant mixture, used in many glyphosate-based products (Giesy et al. 2000). As no environmental detection method for POEA has yet been elucidated, real world concentration and dissipation is unknown. Its behaviour is assumed to be similar to that of glyphosate and its degradation product AMPA and, as in these two compounds, POEA is degraded through microbial processes. Much less research has been done on this subject due to the difficulties of studying surfactants, however a report by Banduhn and Frazier (1974) (unpublished, but cited in (Giesy et al. 2000)), estimates half-life to be approximately 3-4 weeks.

Studies comparing the toxicity of POEA to the overall toxicity of glyphosate formulations have indicated that both the surfactant and the overall formulations have comparable potential to cause amphibian mortality. On the other hand, the glyphosate a.i. contributes very little to the toxicity of the overall formulation (Folmar et al. 1979; Howe et al. 2004; Relyea & Jones 2009a). Further to this, the presence of intra-testicular oocytes in tadpoles of *L. pipiens* exposed to POEA demonstrated that this compound

could potentially be involved in the disruption of gonadal differentiation in amphibians (Howe et al. 2004).

1.3.3 The Long Term Experimental Wetland Area

The Long Term Experimental Wetland Area (LEWA) was established on a 6 km² area of the Gagetown Army base (Gagetown Nb) and was a cooperative effort between a large number of scientists from various backgrounds. This collaboration aimed at examining the effects of a glyphosate-based herbicide on wetland ecosystems and ephemeral amphibian breeding habitats.

1.4 The Wood Frog (*Lithobates sylvaticus*) as a Model Species

1.4.1 General Biology

The Wood Frog (*Lithobates sylvaticus*, formerly *Rana sylvatica*) was chosen as a target species for this study due to its abundance, ease of acquisition and its transition from egg to frog in a single season. Native to North America, the wood frog's habitat sweeps across a broad expanse of Canada and a portion of the United States and this species is considered to range further north than any other North American amphibian (Hammerson 2004). The status of *L. sylvaticus* is considered to be of "least concern" due to this wide distribution and presumed large populations (Hammerson 2004). These frogs commonly breed in the first warm spring rains and eggs are often laid in ephemeral bodies of water in wetlands or near forested areas (Berven 1990; Hammerson 2004). This species undergoes an aquatic larval stage as well as terrestrial juvenile and adult stages.

1.4.2 Gonadal Differentiation

In order to understand how exogenous chemicals may be affecting amphibian gonadal development, it is crucial to understand how sexual development occurs in these animals. Amphibians have been shown to employ a genetic sex determination mechanism (Hillis & Green 1990; Nakamura 2010), thus the genetic sex of each individual is determined at fertilization. However, most anuran species lack distinguishable sex chromosomes and no universal sex determination gene or sex marker has yet been found (Uno et al. 2008; Hayes 1998). Gonadal differentiation, on the other hand, can be defined as the development of an undifferentiated gonad into either an ovary or a testis and the emergence of the secondary sexual characteristics accompanying that sex (Devlin & Nagahama 2002). This process leads to the establishment of the phenotypic sex in each individual, which under normal conditions, coincides with the genetic sex. Gonadal differentiation is highly influenced by steroid hormones in many species including frogs (Urbatzka et al. 2007; Maruo et al. 2008), fish (Devlin & Nagahama 2002; Ijiri et al. 2008), reptiles (Bull et al. 1988) and birds (Freking et al. 2000). The regulation of endogenous steroids and steroidogenic enzymes in frogs is crucial for the differentiation of the male or female phenotype (Urbatzka et al. 2007) and sex steroids play a vital role in sexual differentiation and gonadal development. In amphibians, as in fish, phenotypic sex can be modified during certain early stages of development (Wallace et al. 1999; Shibata et al. 2002), and it is likely the balance of androgens and estrogens present in the gonads that influences phenotypic sexual differentiation. Exposing developing genotypic males to an estrogenic treatment has the potential to induce the development of female phenotypic characteristics, which, at the extreme, can result in a phenotypic sex change from male to female. This has been well examined in *Glandirana rugosa*, which presents

both male and female heterogametic genotypes in separate populations (Takase 1998)(sex reversal review (Wallace et al. 1999)).

1.4.3 Steroidogenesis and the Potential for EDCs to Affect the Genes Involved in Sexual Differentiation

The steroidogenic acute regulatory protein (*star*) has been identified as the rate-limiting step in steroidogenesis as it transports cholesterol from the outer to the inner mitochondrial membrane where steroidogenesis commences (Figure 1.2) (Clark et al. 1994). This important transport protein has been shown to have the potential to be affected by exogenous chemicals including 2, 4, 6-trinitrotoluene (TNT) (Paden et al. 2010) as well as pesticides such as Dimethoate (Walsh et al. 2000), Lindane (Walsh & Stocco 2000) and the glyphosate-based herbicide Roundup (Walsh et al. 2000).

The steroidogenic enzymes subsequent to *star* in the steroidogenic pathway are generally well conserved amongst vertebrates and this appears to be no exception in *L. sylvaticus*. A recently completed study cloned several key genes involved in this process of sexual differentiation including *cyp19*, *cyp17* and *foxl2* and characterized them in this species (Navarro-Martín et al. 2012).

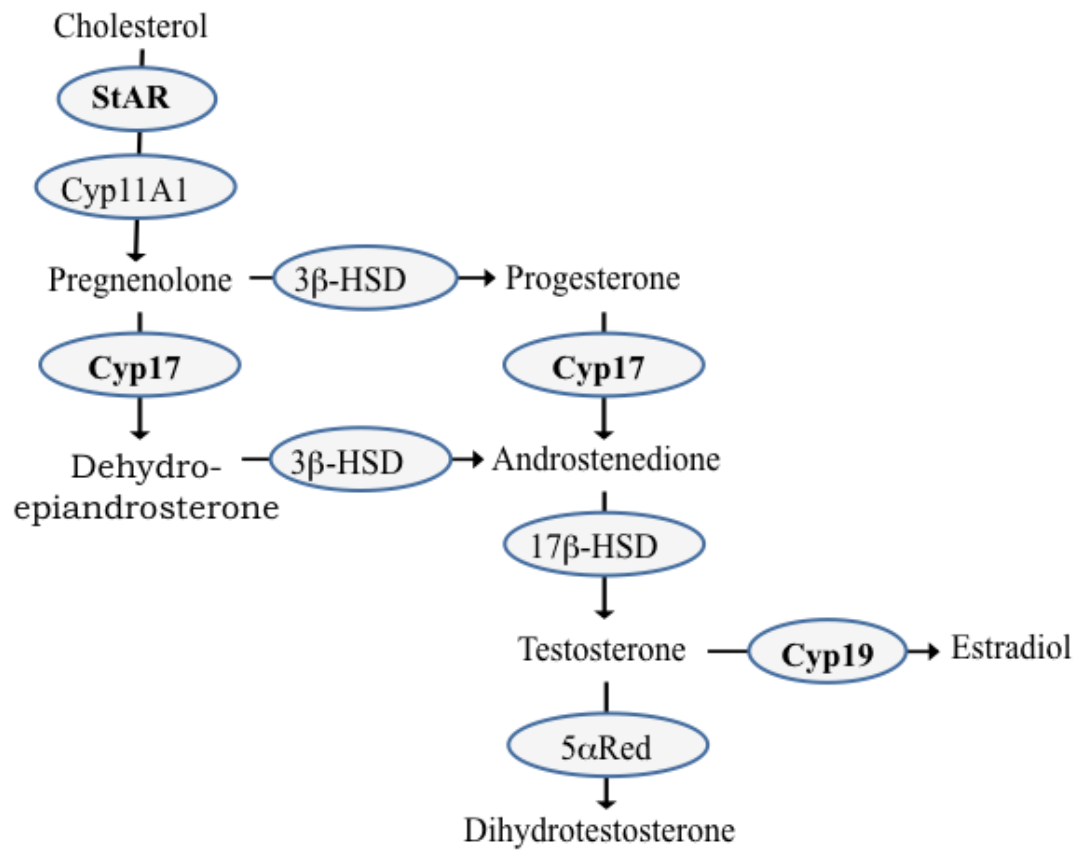


Figure 1.2 Steroidogenic pathway in vertebrates leading to the production of androgens and estrogens.

cyp19

In frogs, as in most vertebrates, the transformation of androgens to estrogens is catalyzed by the p450 aromatase, which is encoded by the gene *cyp19*. Aromatase (*cyp19*) is responsible for the conversions of testosterone to estradiol (Fig. 1.2) and androstenedione to estrone and its mRNA has been detected across many species in both gonadal and brain tissues of male and female vertebrates (Engel & Callard 2007). In the African frog *Xenopus laevis*, the sensitive window for sex reversal is considered to be between Gosner stages 24-34 (Urbatzka et al. 2007). In a study by Urbatzka et al (2007) *cyp19* mRNA is reported as being first detectable in whole body homogenates of *X. laevis* at Gosner stage 21/22. While highly expressed, aromatase in the brain is found to be similar in both males and females. This is in contrast to the lower expression in the gonads, which has been shown to be up-regulated in female *L. sylvaticus* tadpoles as early as Gosner stage 31 (Navarro-Martín et al. 2012). This female preferential up-regulation in the gonadal-mesonephros complex (GMC) is in accordance with previous studies on amphibians, which have demonstrated this dimorphism during gonadal sex differentiation in *X. laevis* (Urbatzka et al. 2007) and *G. rugosa* (Maruo et al. 2008). Modifications in the expression of *cyp19* have been shown to affect sex ratios in a variety of vertebrates. The pharmaceutical fadrozole has often been used as a tool for detecting the effects of decreases in expression as it acts as a competitive aromatase inhibitor (Olmstead et al. 2009). In some fish species, exposure to fadrozole results in complete masculinization (Liu et al. 2007), while female chickens exposed in ovo during the period of sexual differentiation developed as phenotypic males (Elbrecht & Smith 1992). This same phenomena has been observed in frogs as well. In *X. laevis*, masculinized gonadal tissues were detected in vitro after fadrozole exposure (Miyata & Kubo 2000). In *Rana*

catesbeiana tadpoles with intraperitoneal implants containing a steroid aromatase inhibitor (4-hydroxyandrostenedione) underwent a sex reversal after three months of exposure (Yu et al. 1993). Aromatase inhibition has also been reported in the field. Fish from contaminated sites have been found to have lower levels of *cyp19* than fish from non-contaminated sites (Lavado et al. 2004). Whether this decrease is from direct inhibition or the result of modifications upstream in the hypothalamic-pituitary-gonadal axis remains to be established.

foxl2

The forkhead box L2 gene (*foxl2*) is a member of the forkhead family containing a 100-amino acid domain known as the *forkheadbox*, which is known to be conserved in vertebrates (Oshima et al. 2008). This transcription factor is the earliest known marker for ovarian differentiation in mammals (Crisponi et al. 2001; Schmidt et al. 2004; Ottolenghi et al. 2005) and is a key player in ovarian development, folliculogenesis and follicular maintenance (Crisponi et al. 2001; Schmidt et al. 2004; Loffler et al. 2003; Ottolenghi et al. 2005). *Foxl2* is known to promote *cyp19* expression in mammals (Pannetier et al. 2006), fish (Wang et al. 2007) and likely in frogs as well (Oshima et al. 2008). It has also been shown to inhibit SF1 binding to the *Cyp17* promoter causing a decrease of SF1-mediated trans-activation of *Cyp17* expression (Park et al. 2010). Little information is as of yet available on *foxl2* expression in amphibians. In the anuran *R. rugosa*, *foxl2* was found to be expressed in both the testis and ovary as well as the brain. Gonadal expression was found to be preferentially upregulated in females and this increase in expression occurred early in development, well before sexual differentiation (Oshima et al. 2008; Navarro-Martín et al. 2012).

cyp17

Cyp17 is the endoplasmic reticulum membrane-bound enzyme responsible for the conversions of pregnenolone into dehydroepiandrosterone as well as that of progesterone into androstenedione (Maruo et al. 2008; Akhtar et al. 2011)(Fig. 1.2). It is considered to be a multifunctional enzyme as these conversions are carried out by the two enzymatic activities 17 α -hydroxylase and 17,20-lyase (Yang et al. 2003). The expression of *cyp17* has been found to increase significantly more in males prior to sexual differentiation and remain high after differentiation with respect to females (Iwade et al. 2008; Maruo et al. 2008; Sakurai et al. 2008).

In *R. rugosa*, *cyp17* mRNA expression is detectible as early in development as week two of Gosner stage 25 in the undifferentiated gonad of males and females. This expression becomes sexually dimorphic before sexual differentiation four weeks into Gosner stage 25, with males having significantly higher *cyp17* expression (Maruo et al. 2008). This sexual dimorphism in anurans is supported by findings in *L. sylvaticus* where sexual dimorphism was detected at Gosner stage 31 (Navarro-Martín et al. 2012). The biological relevance of the mRNA up-regulation in males is supported by the finding that the conversion rate of progesterone into androstenedione (Cyp17 action) is higher in undifferentiated male gonads in *R. rugosa* than in undifferentiated female gonads (Sakurai et al. 2008). Further evidence for the involvement of *cyp17* in sexual differentiation in frogs arises from sex reversal experiments, whereby a female (female genotype and female phenotype) frog is injected with an androgen to induce a sex reversal to male (female genotype with male phenotype). Changes in *cyp17* expression can be observed during this masculinization process. Iwade et al (2008) found that after

testosterone administration, female frogs underwent dramatic morphological changes whereby the immature oocytes, initially observable, were largely replaced 16 days post-injection by somatic cells and typical testis characteristics (Iwade et al. 2008). This was concurrent with an augmentation of *cyp17* mRNA, which increased after testosterone injection, becoming significantly higher than control on days 16 and 24 post injection.

1.4.4 Exogenous Chemicals Can Affect Steroidogenesis

Many studies have focused on the effects of exogenous chemicals on the expression of genes involved in this well conserved steroidogenic pathway. Pesticides such as Carbendazim, Benomyl, Bitertanol, Propazine, Cyproconazole, Fenarimol, Glyphosate, and Simazine have all been previously shown to modify *cyp19* expression in human cell lines (Morinaga et al. 2004; Trösken et al. 2004; Andersen et al. 2002; Richard et al. 2005; Sanderson et al. 2000). In amphibians, the fungicide clotrimazole has been shown to have the potential to increase *cyp19* activity in the gonads of *X. tropicalis* during development (Gyllenhammar et al. 2009). Estrogenic compounds have previously been shown to promote the expression of *foxl2*, while androgens repressed expression (Baron et al. 2004). Inhibition of *cyp17* has been reported due to the organochlorines polychlorinated biphenyls (PCB) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as well as the trialkyl organotins tributyltin and triphenyltin (see review by Papadopoulos 2007). Very little research has been done examining changes in *cyp17* expression in amphibians due to exogenous chemicals and, while no environmental contaminants have been studied in this context, the expression of *cyp17* in sex-reversed gonads has demonstrated an increase in the expression of *cyp17* 16-24 days after testosterone

administration in *G. rugosa* (Iwade et al. 2008). Taken together, these previous studies suggest that steroidogenic enzymes are susceptible to certain pesticides and exogenous chemicals and the lack of abundance in amphibian studies suggests that this is an area that requires further investigation.

Chapter 2 – The Effects of Glyphosate-based Herbicides and the Components Contained Therein on Anurans (Literature Review)

The studies on glyphosate herbicide formulations and the potential effects that these chemicals may have on anurans in particular and amphibians in general is environmentally significant research that requires further advancement. As these chemicals are widely used and amphibians are at risk of exposure, it is of importance that further studies focus on environmentally realistic exposures. These should target exposure concentrations along with application timing, frequency and durations that are prevalent in the habitat of the species studied. The 49 studies reviewed herein are composed of 39 laboratory studies, 6 mesocosm studies and 4 field-based studies (Table 2.4). The vast majority of these examine short-term acute or chronic exposures, both of which are useful for determining the potential of a treatment-based effect, but not necessarily in determining if these effects translate into a natural setting. The information within these papers has been summarized and divided according to exposure type (laboratory, mesocosm or field) in order to examine how effective synthetic exposures may be at predicting real world herbicide exposures on amphibian populations. The information was then further subdivided to examine lethal and sublethal endpoints separately.

Table 2.1 Summary of available studies examining anurans exposed to glyphosate-based herbicides and components contained therein.

Exposure	Number of anuran/ glyphosate-based herbicide studies	Herbicide formulations examined	Major endpoints examined
Laboratory	39	Glyphos (with Cosmo-flux), Roundup Original (Vision), Roundup weed and grass killer, WeatherMax, Rodeo, Roundup Ultra-Max, infosato, glifoglex, CK Yuyos Fav, Roundup Biactive, Touchdown, Roundup Original Max, Kleeraway grass and weed killer (as well as several unspecified formulations)	LC50, survival, behaviour, metamorphic rate, growth, development, sex ratios, gonadal morphology, malformations, DNA damage, gene expression, disease, external stressors
Mesocosm	6	Glyphos, Roundup Original Max, Roundup weed and grass killer	LC50 values, survival, mass, ovoposition site choice
Field	4	Vision and Roundup WeatherMax (as well as one unspecified formulation)	Capture rates, survival,

2.1. Laboratory Studies

2.1.1 Lethal Endpoints

LC₅₀ values (Acute Laboratory Studies)

Laboratory studies examining the lethal effects of glyphosate-based herbicides and/or the components therein have shown very clearly that lethality is highly dependant on both the formulation and the species studied (table 2.2). A relative order of toxicity can be established between certain formulations when considering LC50 values from multiple species.

The active ingredient, the isopropylamine (IPA) salt of glyphosate, when tested alone, has been found to have 24h LC50 values (Gosner stage 25) (Table 2.2) well over 100 mg/L, thus falling into the category of “practically nontoxic” according to the US Environmental Protection Agency (EPA; Mann & Bidwell, 1999) (Table 2.3). The glyphosate acid, has been shown to be of higher toxicity relative to its salt form, however it is also considered to be practically non-toxic by the EPA. The surfactant POEA, when tested on its own, is considered to be “moderately toxic” in an acute laboratory setting. This supports the hypothesis that the surfactants, or inert ingredients, are the most toxic component in glyphosate-based herbicides (Lajmanovich et al. 2011; Edginton et al. 2004; Mann & Bidwell 1999; Howe et al. 2004). We established a relative order of toxicity between the products and formulations evaluated (Tables 2.2 and 2.3) according to the lethal concentration values in the literature. This broad look at the LC50 values available gives an indication that some formulations of specific ingredients are more toxic than others regardless of the species being studied. A relative ranking of the

products studied in an acute laboratory setting from least toxic (1) to most toxic (6), according to the values presented in Table 2.2, was determined to be as follows:

- 1) Glyphosate isopropylamine salt, Roundup Biactive[®] (Monsanto)
- 2) Glyphosate Acid
- 3) Glyfos BIO[®] (Cheminova) (values for this compound are >17.9 with no upper bound, making it difficult to rank)
- 4) Touchdown[®] (Syngenta)
- 5) Glyfos AU[®] (Cheminova)
- 6) Roundup Original[®] (Monsanto), Roundup WeatherMax[®] (Monsanto), Glyfos[®] (Cheminova), Roundup Transorb[®] (Monsanto), Glyphos[®] (Cheminova) + surfactant Cosmo-flux[®] (Cosmoagro), POEA

Table 2.2 24 and 96 hour LC50 values (mg a.e./L) for various anuran species at Gosner stage 25 in laboratory exposures to glyphosate-based herbicide formulations and the components found therein.

	Gly. IPA	Gly. Acid	POEA	Roundup Original (Vision)	Weather- Max	Roundup Biactive	Roundup Transorb	Touchdown	Glyfos	Glyfos AU	Glyfos BIO	Glyfos & Cosmo- flux
POEA (% of formulation by weight)	0	0	n/a	~15	?	10-20 unspecified surfactant	15 surfactant blend with POEA	10-20 unspecified surfactant	~ 15	3-7 (in blend)	10-20 unspecified surfactant	?
Species	24 h LC 50 value^b (mg a.e./L)											
<i>Crinia insignifera</i>	>466					>494		13.1				
<i>Heleioporus eyrei</i>	>373			8.6		>427		16.6				
<i>Litoria moorei</i>	>343	107.8 ± 27.2 (2)^a		7.9 +6.8 (2)^a		333		10.4				
<i>Rana clamitans</i>	>17.9		2.4	2		>17.9	2.3	>17.9			>17.9	
<i>Rana pipiens</i>				3.7								
<i>Rana sylvatica</i>				5.6								
24 h LC50 Range	>17.9 – 466	107	2.4	2 – 8.6		>17.9 - >494	2.3	13.1 - >17.9			>17.9	
	96h LC50 value (mg a.e./L)											
<i>Bufo Americanus</i>				2.9 ± 1.2 (3)^a								
<i>Bufo fowleri</i>				4.21	1.96							
<i>Hyla chrysoscelis</i>				2.5	3.26							
<i>Rana catesbeiana</i>				2.77	1.97							
<i>Rana clamitans</i>	>17.9		2.2	2.8 ± 1.3 (4)^a	2.77	>17.9	2.2	>17.9		8.9	>17.9	
<i>Rana pipiens</i>				1.9 ± 0.7 (4)^a	2.27							
<i>Rana sphenocephala</i>				2.05	1.33							
<i>Rhinella typhonius</i>												1.5
<i>Rana sylvatica</i>				5.1								
<i>Scinax ruber</i>												1.6
<i>Scinax nasicus</i>									0.94			
<i>Xenopus laevis</i>				1.5 ± 0.9 (2)^a								
96 h LC50 Range	>17.9		2.2	1.49 – 5.1	1.33-3.3	>17.9	2.2	>17.9	0.94	8.9	>17.9	1.5 - 1.6

^a Values in bold represent the median ± standard deviation (N), several LC50 values were available for this species/chemical combination.

Sources: Mann and Bidwell 1999, Howe et al. 2004, Edginton et al 2004, Fuentes et al 2011, Relyea et al 2004, Bernal et al 2009, Relyea and Jones 2009, Lajmanovich et al 2003

Table 2.3 Glyphosate herbicide formulations and the components therein ranked by order of relative toxicity (1-least toxic, 6-most toxic). Glyphosate acid content, active ingredient (A.I) and surfactant included when known.

Rank	1	2	3	4	5	6						
	Gly. IPA	Roundup Biactive	Gly. Acid	Glyfos BIO	Touch- down	Glyfos AU	Roundup Original (Vision)	Weather- Max (Vision Max)	Roundup Transorb	Glyfos	Glyfos & Cosmo-flux	POEA
A.I. form	IPA	IPA	n/a			IPA	IPA	Potassium salt	IPA	IPA		n/a
Surfactant (% in formulation)	0	10-20% unspecified surfactant	0	10-20% unspecified surfactant	10-20% unspecified surfactant	Tallow alkylamine ethoxylate 3-7%	~15 POEA ^a	?	15% surfactant blend with POEA ^a	~ 15% POEA	10 to 15 POEA (with cosmo-flux surfactant)	n/a
U.S. EPA Toxicity Classification	Practica lly nontoxi c	Practically nontoxic	Practically nontoxic	Slightly Toxic	Slightly Toxic	Moderately toxic	Moderately toxic	Moderately toxic	Moderately toxic	Moderately toxic	Moderately toxic	Moderate toxic
European Toxicity Classification	n/a	n/a	n/a	Harmful	Harmful	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic

Survival (Chronic Laboratory Studies)

While LC50 values are useful for regulatory purposes, they are often not overly environmentally or biologically relevant beyond being a good starting point for further testing. Thus it is important to also be aware of survival estimates in order to make informed, case-by-case decisions. Chronic studies often examine survival in order to determine how a species may be affected by longer exposures to more environmentally relevant concentrations. For example, survival to metamorphosis in *Rana pipiens* tadpoles was shown to be significantly reduced when tadpoles were exposed to environmentally relevant concentration as low as 0.6 mg a.e./L of POEA and the POEA containing formulations Roundup Original and Roundup Transorb (Howe et al. 2004). Survival was not affected by exposure to the glyphosate active ingredient alone, even when the concentration was 3-fold higher (1.8 mg a.e./L) than that of the formulations. While the concentrations used in these exposures were environmentally relevant, the duration of the exposure was not. Tadpoles were treated once a week in a static renewal system for a total of 42 days of exposure followed by rearing in clean water to metamorphosis. This length of exposure is extremely unlikely in a natural environment. This is not uncommon and laboratory exposures utilizing compounds at environmentally relevant concentrations will often have exposures of extreme duration (Williams & Semlitsch 2009; Dinehart et al. 2010; Cauble & Wagner 2005).

Gahl and collaborators (2011) found no significant difference in survival due to a 10-day exposure (treated once and allowed to sit for 10 days, then reared in clean water) of Roundup WeatherMax at 0.21 and 2.89 mg a.e./L in *R. sylvatica* exposed at Gosner stage 32. This exposure stage is potentially outside of the “sensitivity window” where

tadpoles have been shown to exhibit the most severe reactions to contaminants (Hogan et al. 2008), however its environmental relevance would clearly depend on what stage of development this species would be at when spraying occurs in its habitat.

In an exposure to the glyphosate herbicide formulation Vision (10 day exposure, renewed treatment every two days, Gosner stage 25) an increase in the pH from 5.5 to 7.5 was shown to significantly increase mortality in *Rana pipiens* (Chen et al. 2004). Increased toxicity due to a change in pH from 6.0 to 7.5 was also found in an acute experiment (Vision, 96h exposure, Gosner 25). LC50 values increased 2.4 times for *Xenopus laevis*, 1.7 times for *Bufo americanus*, 2.5 times for *Rana clamitans* and 1.6 times for *Rana pipiens* (Edginton et al. 2004).

2.1.2 Sublethal Endpoints

Behaviour

Lithobates catesbeiana larvae exposed to the glyphosate formulation Roundup Original under laboratory conditions (24 hour exposure, Gosner stage ~25) have been shown to increase their swimming activity, leading to tachycardia, and thus increased energy expenditure at concentrations as low as 0.30 mg a.e./L (Costa et al. 2008). A transition from slow swimming and remaining near the bottom of the experimental enclosure to uncontrolled fast swimming and a vertical body positioning has been reported in *H. crepitans*, *R. granulosa*, *E. pustulosus*, *R. mariana*, *S. ruber*, *D. microcephalus*, *R. typhonius* and *C. prosoblepon*. These animals were exposed to the formulation Glyphos (contains IPA salt of glyphosate and POEA) mixed with the

surfactant Cosmo-flux at concentrations approaching their LC50 values (Gosner 25, 96 hour exposure)(Bernal et al. 2009b).

Metamorphic Rate

Glyphosate based formulations have been shown to delay metamorphosis in laboratory studies. This has been demonstrated in *R. pipiens* tadpoles exposed to POEA (0.6 mg a.e./L) and the POEA containing formulations Roundup Original (1.8 mg a.e./L) and Roundup Transorb (0.6 mg a.e./L) (42 day exposure starting at Gosner stage 25, treatment renewed weekly) (Howe et al., 2004). Utilizing a concentration of 0.572 mg a.e./L (Gosner 25 to metamorphosis, herbicide renewal every 3 days) *B. americanus* was found to metamorph 3.5 days later (14% later) when exposed to WeatherMax and ~2 days later (8% later) when exposed to RU Original Max (both K-salt formulations containing surfactant blends). In the same experimental setup, *H. versicolor* showed no change in time to metamorphosis for either formulation exposure, highlighting a species dependent sensitivity (Williams & Semlitsch 2009). This species dependence could potentially explain why *R. cascadae* tadpoles exposed to a 1 mg/L treatment of a Roundup formulation (50.2% IPA salt, exact formulation unspecified, potentially Roundup Weed and Grass killer super concentrate) were found to metamorph 4 days sooner than control tadpoles (Cauble & Wagner 2005). As it has been suggested that adult anurans can detect glyphosate herbicides in an aquatic environment (Takahashi 2007), the question arises of whether or not some species of anuran larvae can detect glyphosate-based formulations in their environment and react by increasing their metamorphic rate. This increase in metamorphic rate has been demonstrated in anurans in diminishing volumes of water (Wilbur 1987; Denver et al. 1998).

Growth, Development and Malformations

There is a predictable link between time to metamorphosis and mass at metamorphosis, with longer metamorphic times generally yielding larvae of greater mass as the larvae spend longer feeding and growing before undergoing this energetically demanding process. Supporting this assertion, *R. cascadae* tadpoles, which were found to metamorphose earlier due to a 1 mg/L Roundup treatment (43 day exposure with treatment renewal every 7 days, started 2 weeks post-hatch) were found to have a significantly lower mean body mass than control tadpoles. Conversely, *L. sylvaticus* tadpoles exposed to 2.89 mg a.e./L, that took longer to reach metamorphosis, exhibited a greater mass at metamorphosis than the animals exposed to the lesser treatment of 0.21 mg a.e./L (Gosner 32, chronic 10 day exposure, one initial treatment) (Gahl et al. 2011). Relyea (2004), reported a reduced growth rate (final mass-initial mass)/ days of exposure) in American toads, Green frogs, and Bullfrogs exposed to 1.48 mg a.e./L Roundup (unspecified formulation). No significant reduction in growth was recorded for these same species at a treatment concentration of 0.74 mg a.e./L and no reduction was found in Leopard frogs or Gray Tree frogs at either concentration (Gosner 25, 16 day exposure with pesticide reapplication every 4 days) (Relyea 2004). Two species, *B. americanus* and *H. versicolor*, were shown to be unaffected by an exposure of 0.572 mg a.e./L to either Roundup Original Max or Roundup Weather Max (Gosner 25 to metamorphosis, treatment renewed every 3 days) (Williams & Semlitsch 2009). This could indicate that the dosage was too low to have an effect or could indicate a species difference in sensitivity. Early metamorphosis and greater size are both beneficial attributes that lead to higher survival in anurans (Berven 1990) and it would appear that the balance between time to metamorphosis and mass at metamorphosis is a delicate one. Decreases in growth

or increases in time to metamorphosis have the potential to lead to decreased survival in a natural environment (Berven 1990).

Exposure to 1.8 mg a.e./L POEA, Roundup Original or Roundup, have been shown to reduce larval tail length and increase tail damage up to 94% (Howe et al. 2004). Malformations reported include intestinal malformations and edemas in *X. laevis* (~ Gosner stage 25, 48 hour exposure at 3.80 mg a.e./L, Roundup) (Lenkowski et al. 2010). In *S. nasificus*, the incidence of cranial deformities, mouth deformities, eye abnormalities and curved tails increased from a 4% after 24 hours to a 75% incidence after 4 days (Gosner 25/26, 1.09 mg a.e./L, Glyphos). Exposure to the higher concentration of 2.67 mg a.e./L caused a 70% incidence of malformations after 24 hours (Lajmanovich et al. 2003). Similar malformations have been reported in embryos (Gosner 8-25) exposed to the glyphosate-based formulation Vision. These malformations included, laterally bent tails in *R. pipiens* and abnormal face, eye and gut development in *R. clamitans*. The anurans *X. laevis* and *B. americanus*, exposed to the same treatments, did not exhibit a level of malformations above control (Edginton et al. 2004).

Considering that tadpole gills have been shown to be sensitive to pesticides (Bernabò et al. 2008; Honrubia et al. 1993), surprisingly little research has been conducted examining the effects of glyphosate-based herbicides on this endpoint. *S. nasificus* tadpoles exhibited a reduction in branchial cartilage (4.8mg Glyphosate formulation/L, 48 hour exposure) and at higher concentrations, partial destruction of the branchial arches and a general reduction of the structure of the branchial apparatus. It has been suggested that the surfactants present in the glyphosate herbicide formulations are likely responsible for this effect (Lajmanovich et al. 2003). Glyphosate-herbicide

formulations have been shown to alter normal gill morphology in fish (Jiraungkoorskul et al. 2002; Hued et al. 2012) and thus it is not surprising that similar effects may be detected in amphibians.

Gonadal Morphology and Sex Ratios

Sexual development can also be affected by glyphosate-based herbicides under laboratory conditions. Gonads in varying degrees of intersex (presence of both male and female gonadal characteristics) were detected in the presence of POEA and the POEA containing formulations Roundup Original and Roundup Transorb (0.6 mg a.e./L, exposure from Gosner 25 to metamorphosis, weekly treatment renewal). No intersex animals were detected in the treatment containing solely the glyphosate active ingredient. Sex ratios were not significantly affected in any of the treatments (Howe et al. 2004). Agriculture has been shown to affect anuran gonadal development (McCoy et al. 2008), however very little is known about the potential effects of glyphosate-based herbicides on gonadal morphology and sex ratios. More research is needed.

DNA Damage and Gene Expression

The molecular endpoints investigated with respect to anuran glyphosate exposures are numerous and varied. Roundup (unspecified) caused a significant increase of DNA damage in erythrocytes from *R. catesbeiana* tadpoles exposed to a 6.75 mg/L treatment, but not at the more environmentally relevant concentration of 1.687 mg/L (Clements et al. 1997). Exposure to Roundup Original has also been shown to significantly increase hepatic levels of lipid peroxidation in both liver and muscle tissue where the antioxidant enzymes superoxide dismutase and catalase significantly increased in the liver, but decreased in muscle tissue following Roundup exposure (Costa et al. 2008).

Glyphosate-based herbicides have been shown to affect thyroid hormone receptor β (*tr β*), one of the two genes encoding nuclear thyroid hormone receptors (Tsai & Malley 1994). An increase in the expression of *tr β* was detected in *R. pipiens* tails due to a 1.8 mg a.e./L treatment of Roundup Original and a 0.6 mg a.e./L Roundup Transorb treatment (Gosner 25). This increase was not detected for POEA exposed tadpoles or for tadpoles in any treatment at Gosner stage 42, however with a sample size of only five tadpoles and a relatively high variability further investigation is warranted (Howe et al. 2004). In a separate study examining thyroid hormones and stress related genes, *tr β* expression in *L. sylvaticus* exposed to the glyphosate-based herbicide WeatherMax (two 4-day exposures of decreasing concentration initiated at 0.21 mg a.e. /L, exposures at Gosner 25 and 30) was also found to increase in the tail (1.8 fold) compared to control at Gosner stage 37, however at Gosner stage 42, metamorphic climax, this expression was found to be 1.7 fold and 1.5 fold lower in the tail and brain respectively. Thus, effects of the herbicide on *tr β* expression may vary depending on the developmental stage observed. This same study found that the normal increases in the expression of *grII* in the tail and *dio3* in the brain during late tadpole development were impeded by glyphosate-based herbicides (Chantal Lanctôt 2012). Further research is needed to explore these changes in expression and to determine if they impact the metamorphic process.

Rana arenarum tadpoles (Gosner 36-38), were found to have significantly reduced activity levels of acetylcholinesterase, butyrylcholinesterase, carboxylesterase and glutathione S-transferase (whole body homogenates) after a 48 hour exposure. This effect was detected at an exposure concentration of 1.85 mg a.e./L for four different glyphosate-based herbicide formulations, Roundup Ultra-Max, Infosato, Glifoglex and C-K YUYOS

FAV, all of which contain ammonium salt N-(phosphonomethyl) glycine and an unknown surfactant (Lajmanovich et al. 2011). An exposure to the glyphosate active ingredient alone at 10^{-3} to 10^{-6} M however, was shown to have no effect on the activity levels of the steroid hormones testosterone and 17β -estradiol (Quassinti et al. 2009).

An unbuffered Roundup formulation was shown to inhibit melanosome aggregation by 50% in *X. laevis*, with the buffered Roundup requiring a concentration 2.4 times higher to cause the same effect. Unbuffered glyphosate acid alone caused the same effect at a concentration 2.7 times lower than the unbuffered Roundup, however this effect was completely lost when the glyphosate acid was buffered. The effects of Roundup were less dependent on pH than were those of the glyphosate acid itself. This could potentially be due to POEA causing the cell membrane to become permeable, allowing glyphosate to enter the cytoplasm (Hedberg & Wallin 2010).

Disease and Other External Stressors

While the glyphosate active ingredient is generally reported as having no significant effect on mortality, it has been shown that an exposure can lead to a significant increase in trematode infections and thus indirectly result in an increase in mortality (Rohr et al. 2008). This highlights the idea that multiple stressors can potentially aggravate the effect of a glyphosate-based contaminant. Combining a glyphosate-based herbicide exposure (2.89 mg a.e./L WeatherMax) with an exposure to the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), an amphibian disease which has been shown to decrease amphibian survival when larvae were exposed to the pathogen

alone, decreased mortality by approximately half in *L. sylvaticus* (Gahl et al. 2011). Thus demonstrating that having multiple stressors does not necessarily increase sensitivity.

2.2 Mesocosm/Microcosm

2.2.1 Lethal Endpoints

LC50

Comparing LC50 values calculated for both laboratory and microcosm exposures of four Columbian anurans exposed to a mixture of the herbicide Glyphos (POEA containing formulation) and the surfactant Cosmo-flux demonstrates that glyphosate formulations are often found to be less toxic in a more environmentally realistic exposure. In this comparison, the 2.2-4.2 fold change in toxicity could partially be attributed to the presence of a layer of sediment included in the microcosm exposures (Bernal et al. 2009a). However, it is important to note that the pH of the laboratory and mesocosm experiments being compared was different with the laboratory having a mean pH of 8.23 and the mesocosms having a more circumneutral pH (~6.8 to 7.2). As glyphosate formulation toxicity has been shown to be higher at a more alkaline pH, this has the potential to be at least partially responsible for the difference found in LC50 values between the two exposures. In a chronic mesocosm study (~22 days, Roundup Original Max), LC50 values of 2.29, 2.35 and 2.12 mg a.e./L were measured for the three anuran species *H. versicolor*, *R. clamitans*, *R. catesbeiana* respectively (Jones et al. 2011). These values are comparable to the values found in the laboratory study by Bernal et al (2009b). Even though these mesocosms contained 200 g of leaf litter, the occlusion of a

sedimentary substrate in the mesocosms as well as a pH of 8 are likely causal factors that led to the LC50 values being similar to those found in the laboratory setting.

Survival

L. sylvaticus and *Bufo americanus* were both found to have reduced survival in an early exposure (~Gosner 25) to 2 and 3 mg a.e./L of Roundup Original Max. When exposed 7 days after Gosner 25 (midway), *L. sylvaticus* still showed significant mortality to both 2 and 3 mg a.e./L treatments, but *B. americanus* showed sensitivity only to the 3 mg a.e./L treatment. When treatment application occurred later in development (14 days post Gosner stage 25), both amphibian species showed significant mortality only in the presence of the higher 3 mg a.e./L treatment. This supports species-specific differences in sensitivities and lends credence to the hypothesis that anuran sensitivity varies according to the application timing relative to the animal's development (Jones et al. 2010).

Previous studies have demonstrated that the presence of a sedimentary substrate decreases sensitivity to glyphosate-based herbicides (Tsui & Chu 2003; Wang et al. 2005b). Contrary to this, Relyea (2005) reported that, in a mesocosm experiment, the presence of sediment had no impact on survival in a 20-day larval exposure (from ~Gosner 25 to nearing metamorphosis, Roundup Weed and Grass Killer, 2.81 mg a.e./L, one application) of *R. pipiens*, *H. versicolor* or *B. americanus* (Relyea 2005). For all substrates (no soil control, loam or sand), exposure to the glyphosate-based herbicide caused nearly complete mortality. Therefore, it could be postulated that while the substrate may have absorbed a portion of the herbicide, the amount remaining in solution was still high enough to cause high mortality.

2.2.2 Sublethal Endpoints

Growth

While the window of sensitivity for survival appears to be early in tadpole development (Hogan et al. 2008), it would appear that the endpoint affected varies depending on the timing of the application relative to the anurans development. *L. sylvaticus* and *Bufo americanus*, when exposed to 3 concentrations of Roundup Original Max (1, 2, 3 mg a.e./L) at three different time points in their development (Gosner 25, 7 days post-Gosner 25, 14 days post-Gosner 25. All relatively early stages of tadpole development), were shown to have significant reductions in weight only when exposed to the higher concentrations at the later days of Gosner stage 25 observed. This decrease was significant for the *L. sylvaticus* only in the 14 days post-Gosner 25 application of 2 or 3 mg a.e./L and in the *Bufo americanus* for both the 7 days post-Gosner 25 and 14 days post-Gosner 25 application of 2 or 3 mg a.e./L (Jones et al. 2010). Exposure to Roundup (1 initial treatment at ~Gosner stage 25, duration ~22 days) led to a significant increase in mass in *Rana clamitans*, but not in *Hyla versicolor* or *Rana catesbeiana*. This experiment also highlighted the importance of density on tadpole growth, as all three species exhibited a density effect on growth with lower densities leading to larger tadpoles (Jones et al. 2011).

Avoidance Behavior

Adult frogs were shown to be able to both detect and avoid glyphosate herbicide formulations in an experiment examining ovoposition site choice. Adult *H. versicolor* frogs were placed in a milieu where they had the option of laying their eggs in mesocosms containing water contaminated with a glyphosate formulation (Roundup weed and grass killer) or in uncontaminated control water. Control ponds consistently

received the greatest number of eggs suggesting that adult frogs may be able to detect glyphosate-based chemicals in their environment and choose to avoid them if they have the option (Takahashi 2007). This is the only study on this endpoint in anurans and more research is needed. The environmental relevance of this finding would also require testing in a field scenario since glyphosate binds quickly to the soil. It remains to be seen if there would be a sufficient quantity of the chemical found in spawning areas at any given time to lead to an avoidance behavior?

2.3 Field (or *In-situ* Enclosures)

In the forestry industry, glyphosate based herbicides are often used to control the growth of deciduous woody plants and certain ferns, which could compete with newly planted trees for early succession. In an experiment comparing the capture rates of *R. aurora* in logged areas in the Oregon Coast Range, no significant difference was found between clearcut and broadcast burned sites and sites that had been clearcut, broadcast burned and treated with a glyphosate herbicide formulation (late summer spray, unspecified formulation, 1.3 kg/ha) (Cole et al. 1997).

Another study examining simulated forestry applications (average rate of 1.92 kg a.e./ha of Vision, single late season application) found no significant difference in 48 hours post spray survival between wetlands which were categorized as either being adjacent to a sprayed area, buffered from a sprayed area or as a directly over-sprayed area for either *R. pipiens* or *R. clamitans* (~Gosner 25) (Thompson et al. 2004). In this scenario, no concentration-dependent relationship was detected between mortality rates and the herbicide exposure concentration. A study examining juvenile *L. clamitans* 14

days post-spray found that there was no significant difference between the survival in the control treatment and the anurans oversprayed with Roundup WeatherMax (2.89 mg a.e./L) (Edge et al. 2011).

In comparing in-situ exposures in two ponds with varying physiochemical parameters, glyphosate was shown to have a much greater effect in the pond with higher pH and lower suspended sediment levels. This is unsurprising considering that the median dissipation time for Vision applied at the environmentally expected concentration (1.43 mg a.e./ L) was 4.2 days in the pond with higher suspended sediment and lower pH (Site A), while the alternate pond with lower suspended sediment levels and higher pH (Site B) was shown to have a median dissipation time of 26.4 days. While control mortality did not differ between the two sites, 96h LC50 values (Gosner 25) were 1.6 times greater for *R. clamitans* and 2.7 times greater for *R. pipiens* at site A compared to site B. At both sites *R. clamitans* appeared to be more sensitive than *R. pipiens*, which authors suggest may be due to a larval size difference between tadpoles (Wojtaszek et al. 2004). This difference in size was not detected in the laboratory (Edginton et al. 2004). At both exposure sites, maximal larval growth was found to be greater or equal to controls following exposure to the environmentally expected concentration (1.43 mg a.e./ L, Vision) for both species.

The effect of a glyphosate-based herbicide application on *L. sylvaticus* exposed to the chytrid fungus Bd was also investigated in the field. A marginally significant negative relationship was detected between the chemical application rate and the frequency of Bd detection (Edge et al. 2011). This follows the significant trend found in the laboratory of decreased infection in animals exposed to the herbicide (Gahl et al.

2011). This is an endpoint that merits further investigation examining other glyphosate herbicide formulations and other amphibian species.

2.4 Discussion

Abiotic Factors

pH

This parameter has been shown to affect the toxicity of a glyphosate herbicide, with higher pH leading to greater toxicity (Chen et al. 2004; Edginton et al. 2004). This holds true in the laboratory (Chen et al. 2004; Edginton et al. 2004) and likely in the mesocosm ((Bernal et al. 2009a) vs. (Bernal et al. 2009b)) and the field as well (Wojtaszek et al. 2004). When possible, the approximate pH of the amphibian's natural breeding habitat should be taken into consideration. The pH of experimental exposures is often not reported and greater care should be taken to provide this information.

Sedimentary Substrate

The inclusion of a substrate in a contrived experimental setting has been shown to decrease the sensitivity of amphibians to a glyphosate-based herbicide (Bernal et al. 2009a)(Tsui & Chu 2003) as well as the surfactant POEA (Wang et al. 2005). This is likely due to the glyphosate and surfactant binding to organic matter within the soil and an increased rate of microbial degradation (Giesy et al. 2000).

Temperature

Changes in temperature have also been indicated to have an effect on glyphosate toxicity, with higher temperatures leading to greater toxicity (Folmar et al. 1979). Therefore, just as the rigidly “room temperature” environment of a laboratory exposure may not necessarily reflect the temperature variations found outdoors, the toxicity of the glyphosate exposure may also be modified by these fluctuations in a natural environment. Amphibians spawning in shallow, poorly shaded ponds may be at particular risk due to high temperatures.

Pesticide Formulation

The specific formulation studied is shown herein to be of great importance as glyphosate-based formulations vary widely in their components and thus in their effects and toxicity levels. It is extremely important that authors specify the formulation utilized in their experiments and preferably provide exposure concentrations in acid equivalents to allow for easier comparison between exposures.

Biotic Factors

Species Specificity

Results also appear to be species specific, with certain species being more susceptible to certain endpoints. This is important, not only for regulation purposes (testing on more vulnerable species), but also for understanding how exposures could alter community composition. So far, 42 anuran species have been examined for glyphosate herbicide

effects with *Rana pipiens*, *Rana clamitans* and *Hyla versicolor* being the most frequently studied.

2.5 Conclusion

Glyphosate based herbicide formulations evidently have the potential to disrupt natural amphibian development. The question remains, is this potential realized in nature? Species-specific sensitivities and the variability in herbicide formulations make this a difficult question to answer. Further investigation is required on this subject. In order to allow for better extrapolation to natural systems, experimental details such as the precise glyphosate-based formulation tested, concentration (in mg a.e. /L) and duration of the exposure, stages and ages of the tadpoles exposed as well as parameters such as temperature, pH and tadpole density should all be taken into consideration and reported. Laboratory exposures have highlighted many endpoints of potential interest and narrowed the window of sensitivity to specific concentrations that should be further tested; now this information needs to be utilized in the creation of more environmentally relevant studies.

Chapter 3 – Can Artificial Exposures to Glyphosate-based Herbicides Truly Predict Real-world Effects in *Lithobates sylvaticus*?

Chapter adapted from **Robertson C.**¹, Navarro-Martin L.², Lanctot C.³, Edge C.⁴, Jackman P.⁵, Park B.⁶, Doe K.⁷, Pauli B.⁸ & Trudeau V.⁹
(manuscript in preparation).

Principal contributions of co-authors:

1. Contributed to experimental design and implementation including sample collection, monitoring survival, grow and development as well as performing RT-PCR analysis and manuscript preparation.
2. Contributed to experimental design and implementation as well as sampling and tissue collection. Supervised research and revised manuscript.
3. Contributed to experimental design and implementation including sampling and tissue collection.
4. Contributed to monitoring survival, growth and development as well as sample collection in the mesocosm and field exposures
5. Contributed to monitoring survival, growth and development as well as sampling and supervising the laboratory exposure.
6. Contributed by performing gonadal histology.
7. Contributed to laboratory exposures and provided financial support.
8. Contributed financial support and aided in experimental design.
9. Contributed as supervisor of research project, aided in experimental design, provided financial support and revised manuscript.

3.1. Introduction

3.1.1 Experimental Hypothesis and Rational

Previous research has indicated that glyphosate-based herbicides, and particularly the surfactant polyethoxylated tallowamine (POEA) contained in many formulations, may have the potential to disrupt sexual differentiation and normal growth in anuran larvae (Howe et al. 2004; Edginton et al. 2004; Comstock et al. 2007). We hypothesize that the glyphosate-based herbicide WeatherMax has the potential to disrupt natural growth and development in the anuran *L. sylvaticus* at an environmentally relevant exposure level. This led to the experiments elaborated herein and to the predictions that exposure to these herbicides and more particularly to the surfactant POEA would lead to changes in sex ratios and gonadal abnormalities as well as delays in metamorphosis and disruptions in natural growth patterns. Secondary to this, the predictive power of synthetic experimental environments was questioned. The exposure of *L. sylvaticus* to the glyphosate-based herbicide WeatherMax was expected to have qualitatively similar effects in the laboratory, mesocosms and field experiments. Quantitatively, these effects were expected to be highest in the lab, reduced in the mesocosms and lowest in the field.

3.1.2 Why Study Amphibians?

On a global scale, amphibian populations are now known to be declining. This trend is thought to have commenced in the late 1950s and early 1960s when a rapid and relatively wide scale decrease in amphibian populations occurred. This trend of dwindling populations continues at a decreased rate to the present day (Houlahan et al. 2000). While the cause of this decline is likely to be multifaceted, one of the potential

contributing factors is the widespread use of pesticides by an ever changing and growing agricultural industry.

3.1.3 Glyphosate-based Herbicides

Glyphosate is the active ingredient in a range of broad-spectrum herbicide formulations that are currently utilized internationally. First introduced by Monsanto (Monsanto Company, St. Louis, MO) in 1971, glyphosate was registered in Canada in 1976 (Takacs et al. 2002) and was originally developed as a pre-emergence herbicide. With the advent of Roundup Ready (glyphosate-resistant) crops in 1996, its usage has increased to post-emergence as well (Duke & Powles 2008). Since its debut, the usage of this chemical has proliferated enormously and, in 1996, 70 million hectares of land worldwide were estimated as having been treated with glyphosate-based products, most of which were Roundup formulations containing a polyethoxylated tallowamine (POEA) surfactant (Giesy et al. 2000; Woodburn 2000). In the year 2000, the patent on glyphosate expired and many generic forms began to appear on the market, making this chemical more accessible and more affordable (Woodburn 2000; Duke & Powles 2008). Global annual sales of glyphosate in that year approached 2 billion dollars (Caseley & Copping 2000). The most recent usage estimates show that in the year 2007 180-185 million pounds of glyphosate active ingredient were used in the United States. A more than 2 fold increase from the year 2001 estimates of 85-90 million pounds (Grube et al. 2011). Despite the increase in the usage of this herbicide, very little research has focused on environmental detection and the exposure concentrations to which non-target aquatic organisms may be subjected (further information on environmental detection in Chapter

1, section 2.2.1)

Potential environmental effects of glyphosate-based formulations remain controversial, as many of the studies previously conducted do not examine environmentally relevant exposures. These studies are however showing that these herbicide formulations have the potential to negatively impact amphibian growth, development and reproduction (Mann & Bidwell 1999; Howe et al. 2004; Relyea & Jones 2009a).

3.1.4 Three-tiered Approach to Investigating the Effects of the Glyphosate-based Herbicide WeatherMax on *L. sylvaticus*

In order to test the previously stated hypotheses, a three-tiered experiment was undertaken in which *L. sylvaticus* tadpoles were exposed to a glyphosate-based herbicide in a laboratory, mesocosm and in-situ field exposure. Contrary to the more popular approach, of commencing investigation in a laboratory and expanding out into the field, the experimental designs employed in all three tiers of experimentation undertaken in this project were based upon real world agricultural practices and previous field based projects. Thus the designs for both the mesocosm and laboratory experiments were constructed to mimic that of the field exposure. This led to more realistic exposure scenarios, referred to as “pulse” applications, which, unlike an acute or a chronic exposure, simulates the way glyphosate based herbicides have been shown to behave in a real world application setting. This pulse of pesticide exposes organisms to an initial peak concentration, which diminishes quickly over a number of days and is repeated several times during the course of an agricultural growth season.

3.2 Material and Methods

3.2.1 Overall Design for 3-tiered Exposure Approach

3.2.1.1 Experimental Animal: Egg Mass Collection and Handling

On April 13th 2010, *L. sylvaticus* egg masses were collected at the Long-term Experimental Wetland Area (LEWA) located on the Canadian Forces Base Gagetown (CFB Gagetown) in south eastern New Brunswick, Canada (45°40' N, 66°29' W). Collection was from both from the experimental wetlands and from surrounding undisturbed wetlands. These egg masses were gathered in a central site and randomly assigned to various experiments including the laboratory, mesocosm and in-situ enclosure exposures expounded upon herein. Eight egg masses were shipped to the Environment Canada laboratories in Moncton New Brunswick, Canada where they were kept in an environmental chamber for three weeks and gradually warmed from 7 degrees Celsius to 21°C for the laboratory exposure. Egg masses for the mesocosm and in-situ experiments were kept at the LEWA site outdoors in 5 gallon pails until the tadpoles reached Gosner stage 25 at which point the appropriate number of tadpoles were randomly selected and transferred to the appropriate experimental exposure.

3.2.1.3 Exposure Timing

Timing of the pulse applications was based on amphibian somatic development. For all three exposure types, the first application took place when tadpoles were at Gosner stage 25 (immediately post-hatch) and the second pulse application occurred when the median stage of the exposure was at Gosner stage 30. Sexual differentiation generally occurs between stages 25 and 30 (Hogan et al. 2008) and thus this spray design mimics an environmental exposure during the amphibian period of highest sensitivity. In

order to increase comparability between exposures samplings and developmental time-points are reported as both days after collection (DAC) and days after treatment (DAT).

3.2.1.4 Exposure Treatments

Tadpoles in the in-situ field exposures were solely exposed to the glyphosate-based herbicide formulation WeatherMax® (Monsanto), while laboratory and mesocosm tadpoles were also exposed to the components found within such a formulation. Treatments included (not all treatments performed in all exposures (Table 3.1)):

WeatherMax (VisionMax): a glyphosate based herbicide formulation composed (by weight) of 49% potassium salt of glyphosate and containing an unknown surfactant blend. Used in both agriculture and forestry. Two concentrations were tested, the lower environmentally relevant concentration (ERC) of 0.21 mg/L a.e. was based on the upper 99th percentile of aqueous glyphosate concentrations measured in wetlands located in agricultural areas in Southern Ontario (Struger et al. 2008). The higher concentration tested was the predicted maximal environmental concentration (PMEC) of 2.88 mg/L a.e., which was calculated for a wetland with an average depth of 15 cm and utilizing the maximal recommended spray rate.

Vision (Roundup Original): a glyphosate based herbicide formulation containing (by weight) 41% of isopropylamine salt of glyphosate. This is one of the first commercial formulations. As such, it has been well studied and the surfactant used within it has been identified as a polyethoxylated tallowamine (~15% by weight). The concentration of examined of this formulation was equivalent (in a.e.) to the PMEC WeatherMax

concentration.

Polyethoxylated tallowamine (POEA): a non-ionic surfactant added to herbicide formulations to promote penetration of the active ingredient into the target plants. Known to be included in Vision and thought to be found in various other formulations as part of a surfactant blend. As the surfactant blend is unknown for WeatherMax, the concentration of POEA examined is equivalent to what would be found in 2.88 mg a.e. /L Vision.

Isopropylamine salt of glyphosate: isopropylamine salt form of the active ingredient found in Roundup Original.

Table 3.1 Treatments performed for three different exposure types. All treatments are glyphosate based herbicides or components contained therein.

Treatment	Laboratory	Mesocosm	In-situ
Control	X	X	X
WeatherMax ERC* (0.21 mg/L a.e.)	X	X	
WeatherMax PMEC* (2.88 mg/L a.e.)	X died	X	X
POEA* (1.43 mg/L)	X	X died	
POEA chronic (1.43 mg/L)	X died	X died	
Isopropylamine salt of glyphosate* (2.88 mg/L a.e.)	X		
Vision* (2.88 mg/L a.e.)	X		
Sediment Control		X	
Sediment POEA chronic (1.43 mg/L)		X	
* pulse exposure			

3.2.2 Experimental Design

3.2.2.1 Laboratory Exposure

Laboratory methodology was carried out as reported in the thesis of Chantal Lanctôt (2012). In summary, laboratory exposures were conducted by Paula Jackman in the Environment Canada laboratories in Moncton New Brunswick, Canada. Six treatments were run with 3 replicates per treatment and two pulse exposures were performed during the course of the experiment. For each of the pulses, tadpoles were exposed to the target concentration on the first day of exposure. This concentration was decreased by $\frac{1}{4}$ of the original concentration daily until the fourth day when tadpoles were transferred to clean water. This decreasing concentration was implemented in order to mimic the pattern of decrease of aqueous glyphosate concentrations previously seen in the field (LEWA, unpublished data). The first pulse application occurred on May 4th 2010 or 21 days after egg mass collection (DAC) and the second application took place two weeks later. The six laboratory treatments were:

- 1) Control
- 2) WeatherMax ERC pulse (0.21 mg/L a.e.),
- 3) WeatherMax PMEC pulse (2.88 mg/L a.e.)
- 4) POEA pulse PMEC (1.43 mg/L)
- 5) Vision PMEC (2.88 mg/L a.e.)
- 6) Isopropylamine salt of glyphosate PMEC (2.88 mg/L a.e.)

Tadpoles were housed in 53 L aquaria (Hagen Allglass) with 50 tadpoles/tank (density ~1 tadpole/L). Each treatment was replicated three times. Tadpoles were fed boiled kale and Ward's dry tadpole food (4:1 Kale/Ward) ad libitum with a weekly addition of algal

pellets. Somatic staging was performed weekly and water changes were performed 3 times per week with water quality (temperature, dissolved oxygen, pH) verified before each change.

3.2.2.2 Mesocosm Exposure

The mesocosms consisted of 63L Get Sorted (#9426TWH, Zellers) storage bins with 3.2mm High Density Polyethylene mesh lids, that were placed in a shaded, treed area on the CFB Gagetown research site. Two distinct mesocosm experiments were run concurrently, one with a sedimentary substrate and one without. Mesocosms in each experiment received 35 tadpoles and ~35 L of water collected from an adjacent pond (1 tadpole per litre density). Mesocosms with sediment received 20 cups (~4536 g) of soil collected at the field site and thought to be representative of the soil underlying ephemeral ponds in the area. For all mesocosms, tadpoles were allowed to acclimate for 3 days before treatments were applied. The 6 treatments consisted of:

- 1) Control
- 2) WeatherMax ERC pulse (0.21 mg/L a.e.),
- 3) WeatherMax PMEC pulse (2.88 mg/L a.e.)
- 4) POEA pulse (1.43 mg/L)
- 5) Sediment Control
- 6) Sediment POEA chronic (1.43 mg/L)

Each treatment was replicated four times. The non-sediment mesocosms were treated (26 DAC) and allowed to sit for 5 days with the control treatment undergoing all manipulations and an addition of water instead of chemical treatment. The tadpoles in the mesocosms were then removed while their mesocosm container was scrubbed, rinsed and

refilled with 35L of untreated water from the same initial pond. The chronic POEA treatment was also subjected to the same manipulations (tadpoles removed, mesocosm cleaned, water changed) after which the treatment was reapplied. A second short-term (5 day pulse) treatment was applied 72 DAC, after the median of the treatments had reached Gosner stage 31. Subsequently, the water was once again changed, following the same procedure as the first water changes.

For the sediment treatments, the first pulse was applied at the same time as the non-sediment treatments. Sediment control underwent all of the same manipulations as the sediment POEA treatment with the addition of water instead of the chemical treatment. The sediment treatments were disturbed very little post-treatment as no water changes were performed and no second treatment was applied. Tadpoles were transferred to clean mesocosms without sediment 54 DAT.

Tadpoles from all treatments were fed rabbit pellets (Hagen) ad libitum and 30 g of local leaf litter was added to all treatments as a periphyton substrate 80 DAC to allow for extra nutrition.

3.2.2.3 In-situ Field Exposure

Three small (< 1 ha) semi-permanent wetlands were selected for analysis. These wetlands were located on the CFB Gagetown military installation (NB, Canada) and are part of the Long Term Experimental Wetland Area (LEWA). These wetlands had no permanent influx or efflux of waters and had relatively homogenous macrophyte cover. Each wetland was separated in two utilizing 0.76 mm high-density polyethylene plastic sheeting (Poly-Flex Inc. Geomembrane Lining Systems, Grand Prairie, Texas) (Fig. 3.1, A). The bottom of the sheeting was folded, filled with gravel and buried 10 cm into the

substrate. The edges of the plastic barrier extended 2 m above the high water mark for each wetland and rebar was placed every 3 m along the length to maintain structural integrity. A staff gauge was placed in the deepest point of each pond and, in order to calculate the water volume, water depth was measured every 2-4 m along transects (perpendicular to the plastic barrier) located 2-4 m apart.

This separation allowed for a split-wetland design, whereby one half was treated and the other half was monitored as a natural control. In order to contain tadpoles and expose them to a natural environment while excluding predators, in-situ enclosures were placed in each half of each wetland (Fig 3.1, B). The cages were left half immersed and remained anchored to the wetland bottom through the placement of a rock inside the enclosure. These in-situ enclosures were 42 X 42 X 76 cm mesh reptariums (Hagen Inc, Montréal, QC, Canada) composed of 1 mm mesh set in a plastic frame. Tadpoles were extremely small when first placed in the mesh enclosures at Gosner stage 25 and, in order to avoid escapees, 70 *L. sylvaticus* tadpoles were placed in a 1 L TupperwareTM container with a mosquito mesh lid held on by an elastic band within the larger mesh enclosures until sufficiently corpulent. Other than monitoring the somatic development of the tadpoles on an approximately weekly basis, the cages were disturbed very little until they reached the appropriate stage for sampling.

The treatment side of each pond was sprayed twice during the season; each spray consisted of a direct application of the glyphosate based herbicide formulation WeatherMax (540 g a.e./L, Monsanto, Winnipeg, MB, CAN). The target spray concentration was based on the PMEC of 2.88 mg a.e./L. In order to achieve this target concentration, wetland volumes were calculated immediately before spray and herbicide

volume applied was adjusted accordingly. The glyphosate herbicide formulation was diluted in ~ 3L of water drawn from the wetland and application was performed directly upon the wetland surface with a backpack sprayer (Flowmaster, Root-Lowell Manufacturing, Lowell, MI, USA). A separate backpack sprayer was utilised to spray an equivalent amount of wetland water, drawn from the uncontaminated side, on the control half of the ecosystem in order to ensure identical mechanical disturbance. Timing of the two herbicide applications on the wetlands was based upon the median developmental progression of the *L. sylvaticus* tadpoles within.



Figure 3.1 A) Split wetland design with mid-wetland divider at the LEWA site, summer 2010. Left side of pond has been treated with the glyphosate-based herbicide Weathermax and right side is control. B) In-situ enclosure placed in one half of an experimental wetland at the LEWA site.

3.2.3 Data and Sample Collection

3.2.3.1 Laboratory Exposure

Data for survival and development was collected, analyzed and discussed by Lanctot (2012). Additionally, gonadal samples were collected at Gosner stage 42 to determine sex ratios and histological analysis. Only data for days to metamorphosis gonadal histology and sex ratios are included in this thesis.

3.2.3.2 Mesocosm Exposure

The developmental progression of the tadpoles was monitored regularly by staging subsamples of replicates from each treatment. Early in development this was done every 5-7 days, becoming more frequent as development progressed and sped up. Three developmental stages were targeted: Gosner stages 31, 38 and 42. Stage 31 was targeted as it was after the first spray and immediately before the second spray, stage 38 was chosen as it was after the second spray and it was likely that the gonads would be fully differentiated by this stage and Gosner stage 42 was chosen as it is the peak of metamorphosis and the stage at which the tadpoles emerge from their aquatic habitat. Once the median stage of a treatment reached the target stage, a replicate was sampled, sacrificing only the tadpoles at that particular stage of development. Samples removed for histology were euthanized in tricaine methane sulphonate (MS222, AquaLife, Syndel Laboratories LTD) and measured for weight and snout-vent length.

Macroscopic sex of preserved samples was verified under a stereomicroscope (Olympus Model SZ2-ILST) connected to a camera (QImaging MicroPublisher 3.3 and gonadal features were photographed using Image J software.

3.2.3.3 In-situ Field Exposure

Water samples and aqueous glyphosate concentrations

Water samples were collected from the each side of the wetlands on the day of the herbicide application (day 0) and from the treatment side on days 1, 3 and 7 post-application. Water was pooled from five 50 ml samples collected from differing locations within the pond-half and a 50 ml aliquot was retained, placed in a sterile centrifuge tube and stored in a dark location until the end of the sampling day when it was frozen. Frozen samples were shipped to and processed at the Ontario Ministry of the Environment Laboratory. Both glyphosate and the metabolite AMPA were analyzed according to the method developed by Hao et al (Hao et al. 2011) utilizing liquid chromatography/tandem mass spectrometry. The instrumental detection limits were 1 µg a.e./L for glyphosate and 2 µg a.e./L for AMPA. Two ibutton thermochron f5 were installed in each wetland half and temperature was measured every 180 minutes. Wetland pH was measured weekly using a handheld probe (MPS 556, YSI, Yellow Springs, OHIO, USA).

Tadpole Collection

When the median of an in-situ enclosure reached the target median stage, tadpoles were removed from the enclosure, photographed on a 5 mm grid, euthanized in tricaine methane sulphonate (MS222, AquaLife, Syndel Laboratories LTD) and stored in RNAlater (Ambion) according to the protocol set optimized by Navarro-Martin (2012) (Navarro-Martín et al. 2012). The program Image J (<http://rsb.info.nih.gov/ij/>) was used to measure tadpole body size from the photographs taken during the collection process.

3.2.4 Determination of Sex Ratios and Histological Analysis of Gonads

In all exposures types, the gonad-mesonephros complex was preserved in Cal-Ex II

for a period of 48 hours and subsequently transferred to a 70% ethanol solution. These samples were shipped to and histologically analyzed for gonadal abnormalities (including the presence of testicular oocytes, inhibited development, oocyte atresia, etc) at the Freshwater Institute (Centre for Environmental Research on Pesticides, Fisheries and Oceans Canada). Samples were assessed macroscopically to determine gross morphological sex and subsequently a subset were processed for histological analysis. Tissues destined for histology were cleared in toluene, infiltrated with molten paraffin using a Tissue Tek VIP 5 tissue processor (Sakura), embedded in paraffin blocks for sectioning and prepared in serial transverse sections (6 microns) through the entire gonad. Sections were subsequently affixed to slides and stained with Harris' hematoxylin and eosin. From the laboratory exposure, the sex of the tadpoles collected from each treatment was determined macroscopically and a subset of 5 males and 3 females were selected from each treatment (control, isopropylamine salt of glyphosate, polyethoxylated tallowamine, Vision and WeatherMax low) for histological processing and sex verification. A much larger number of samples were analyzed from the mesocosm exposure (control N=58, WeatherMax low N=58, WeatherMax high N=29, sediment control N=81, sediment POEA N=34) for both gonadal abnormalities and sex determination. No samples were available for histology from the in-situ field exposure. The sex ratio of the wetland tadpoles was determined according to the method developed by Navarro-Martin et al (2012) whereby the expression pattern of the sexually dimorphic gene *cyp19* is utilized as a marker that is more highly expressed in female tadpoles than in males.

3.2.5 Statistical Analysis

All statistics were carried out utilizing the car, nlme or base packages in R (R foundation of Statistical Computing, Vienna, Austria). All data was tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's tests. Data was transformed (log or square root) as necessary when these assumptions were not met and subsequently retested for these assumptions. When data did not meet assumptions, it was ranked and then retested for assumptions. Once assumptions were met data was analyzed by t-test or ANOVA and a Tukey post-hoc test when applicable. Differences were considered significant at $p < 0.05$.

3.3 Results

3.3.1 Laboratory Exposure

3.3.1.1 Days to Metamorphosis

The number of days required to reach metamorphosis was analyzed in the laboratory setting for tadpoles exposed to glyphosate-based herbicide formulations and the components found therein (Fig. 3.2). Males in each treatment required the following average time to reach metamorphic climax (Gosner stage 42): 38 days for control (N=10), 59 days for isopropylamine salt of glyphosate (N=8), 58 days for POEA pulse to (N=11), 59 days for Vision (N=8) and 60 days for WeatherMax low (N=10). Female tadpoles in the control treatment took on average 61 days to reach metamorphosis (N=8), the isopropylamine salt of glyphosate took 58 days (N=12), the POEA pulse took 63 days (N=4), the Vision treatment took 67 days (N=5) and the WeatherMax low treatment took 57 days (N=9). No significant differences in time to metamorphosis were detected due to

treatment (2-way ANOVA, $F=1.4$, $Df= 2$, $p= 0.26$) or sex (2-way ANOVA, $F=0.15$, $Df= 1$, $p= 0.70$) with no significant interaction found between the two (2-way ANOVA, $F=89$, $Df= 2$, $p= 0.41$).

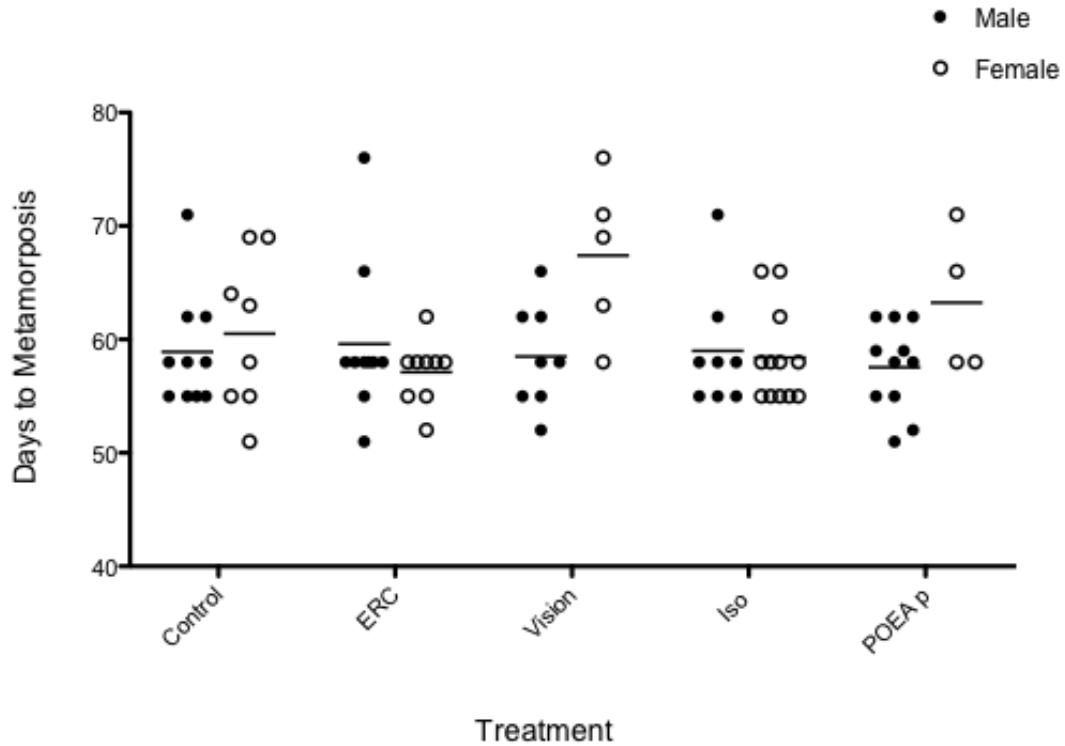


Figure 3.2 Days to metamorphosis of *L. sylvaticus* in a laboratory exposure to various treatments. Treatments include Control (N= 8 females, 10 males), ERC (WeatherMax Low, N= 9 females, 10 males), Vision (N= 5 females, 8 males), Iso (Isopropylamine salt of glyphosate, N= 12 females, 8 males) and POEA p (POEA pulse, N= 4 females, 11 males). Horizontal bars indicate the mean for each sex in each treatment. Significant differences are indicated by a star ($p < 0.05$).

3.3.1.2 Sex Ratios and Histology

Sex ratios at Gosner stage 42 did not vary significantly from the expected 50:50 male:female ratio across all laboratory treatments performed (Fig. 3.3). POEA pulse showed a pattern of an increasingly male population, however this difference was not found to be significant (compared to 50% $p = 0.71$, compared to control $p = 1.64$). Histological samples were analyzed for the presence of testicular oocytes, inhibited development, oocyte atresia as well as other potential abnormalities in gonadal development consistent with a treatment effect in either males (Fig. 3.4) or females (Fig. 3.5). Testes were composed of proliferating spermatogonia and somatic cells with preliminary organization into seminiferous tubules. Ovaries were composed of early perinucleolar oocytes surrounding an ovarian cavity. No anomalies were detected in the gonads that would indicate an obvious treatment effect.

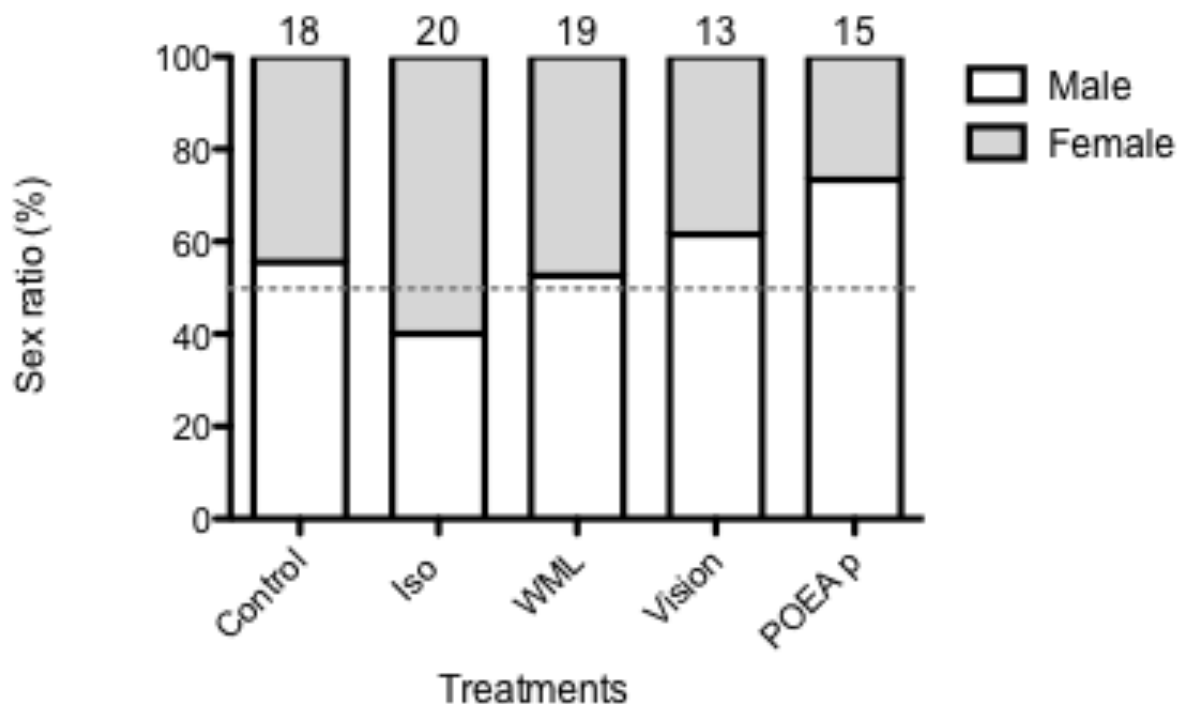


Figure 3.3 Sex ratios of *L. sylvaticus* in laboratory exposure to glyphosate formulations and formulation components. Treatments consisted of a control, isopropylamine salt of glyphosate, WeatherMax low, Vision and Polyethoxylated tallow amine pulse. Treatment sample sizes (N) indicated above bars. Dotted line indicates 50%. ($p < 0.05$).

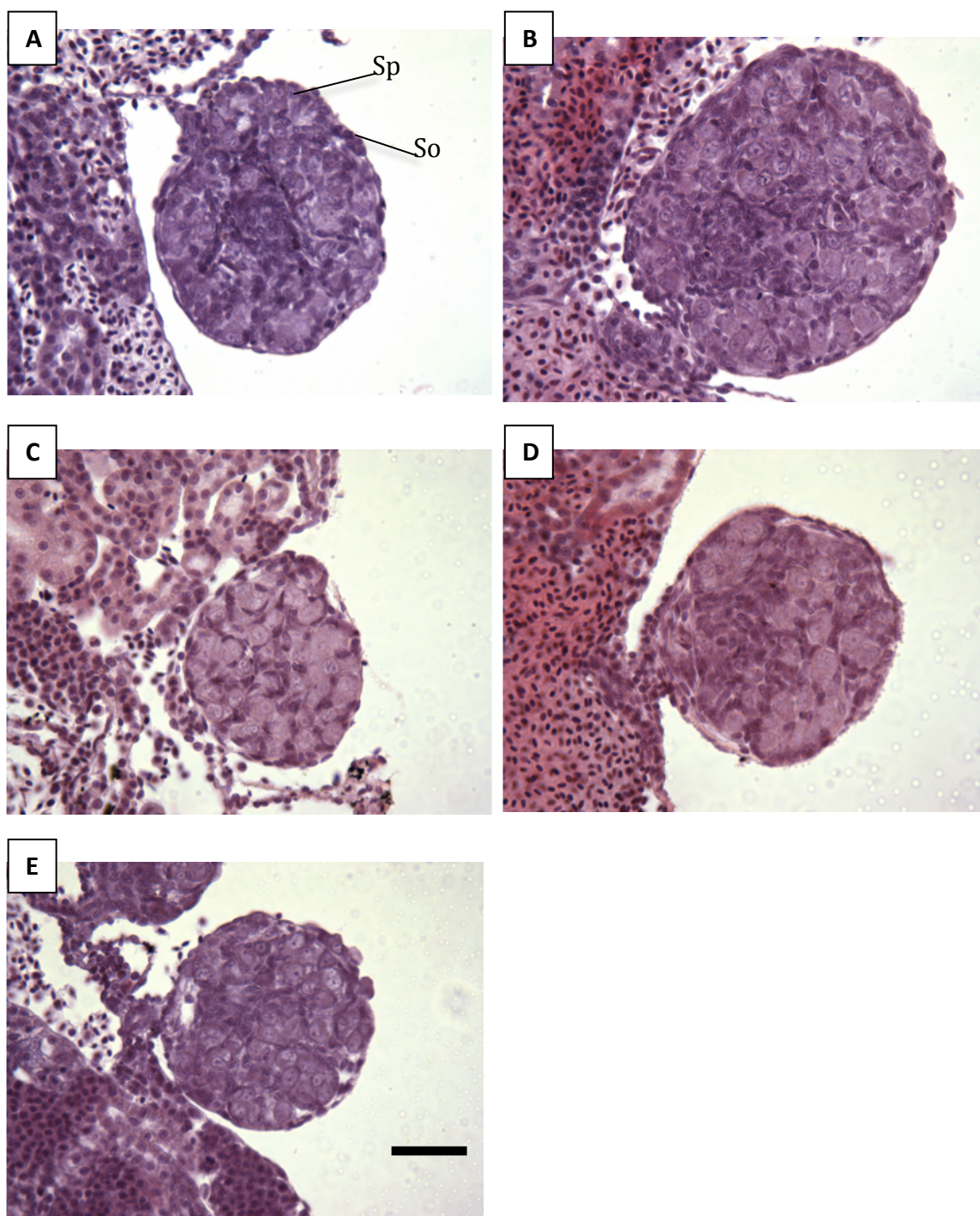


Figure 3.4 Photomicrographs of testes from *L. sylvaticus* metamorphs experimentally treated with pulse exposures to various Glyphosate formulations in a laboratory exposure. (A) Control (B) Isopropylamine salt of glyphosate (C) POEA (D) Vision (E) Weathermax ERC. Bar = 50 microns. Structures are only identified in control (A), but apply to all treatments shown (A-E). Sp- spermatogonia, So- somatic cells.

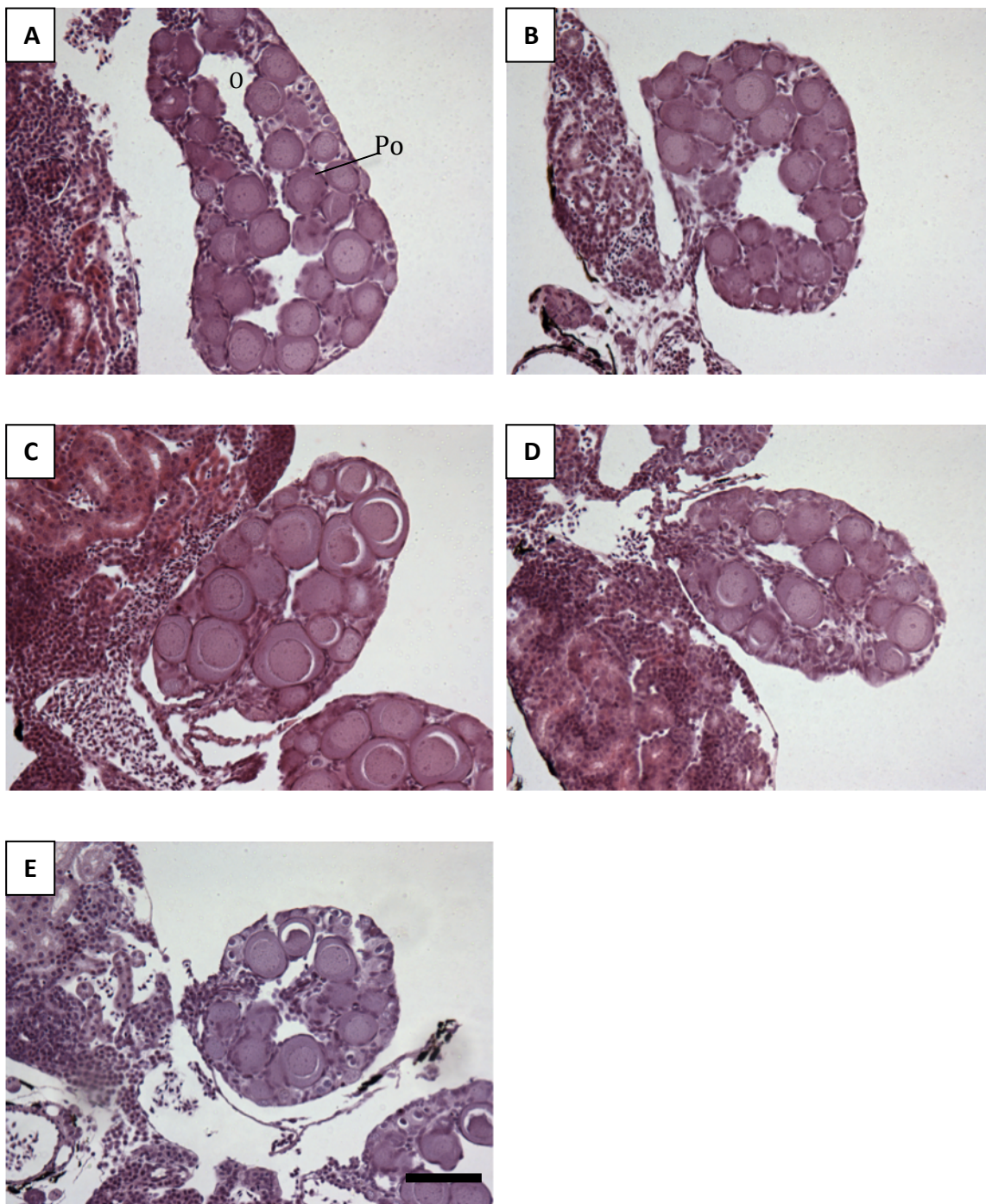


Figure 3.5 Photomicrographs of ovaries from *L. sylvaticus* metamorphs experimentally treated with pulse exposures to various Glyphosate formulations in a laboratory exposure. (A) Control (B) Isopropylamine salt of glyphosate (C) POEA (D) Vision (E) Weathermax ERC. Bar = 100 microns. Structures are only identified in control (A), but apply to all treatments shown (A-E). Po- perinuclear oocyte, O- ovarian cavity.

3.3.2 Mesocosm Exposure

3.3.2.1 Survival

Eight days after the first chemical application (34 DAC), survival in the POEA chronic treatment was $85.7 \pm 1.8\%$ SE (n=7). Ten days post-treatment (36 DAC), full mortality was observed in all POEA chronic replicates.). After the first pulse, but prior to the second pulse treatment application, 41 DAT (67 DAC), tadpole survival was above 90% in the remaining non-sediment treatments (Fig. 3.6, panel A). Survival 51 DAT (77 DAC), after the second pulse, remained above 90% for control, WeatherMax low and WeatherMax high treatments. Tadpoles in the POEA pulse treatment however, experienced full mortality (Fig. 3.6, panel B). Immediately after the second application, a large portion of the tadpoles in the POEA pulse treatment modified their swimming behaviour from sporadic swimming near the bottom of the mesocosms, to lethargic swimming near the surface. These tadpoles also ceased consuming the food that was introduced into all of the mesocosms (personal observation). Survival in the sediment control treatment was also above 90% on day 43 (Fig 3.6, panel C), however the sediment POEA chronic treatment had an average survival that was approximately 59% lower (t-test, $t=16.7$, $df= 3.86$, $p<0.001$)

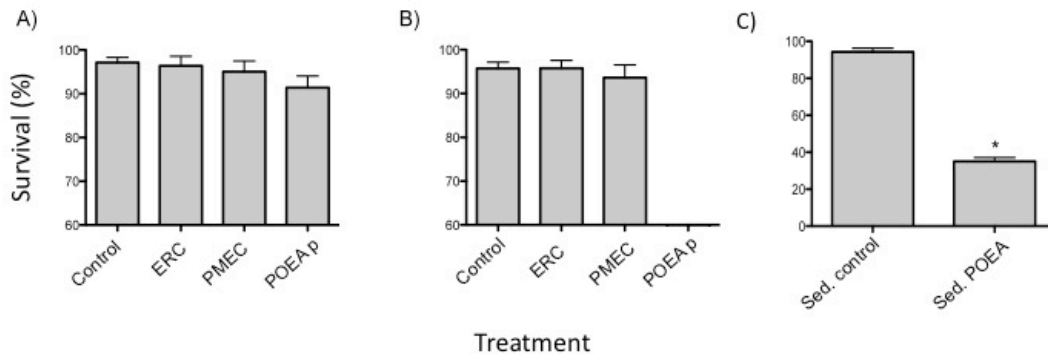


Figure 3.6 Survival of *L. sylvaticus* in mesocosm exposures A) Survival in mesocosms 67 DAC (41 DAT), between pulse treatment application 1 and 2 for the ERC and the PMEC as well as the POEA pulse treatment (POEA p). B) Survival on mesocosm 77 DAC (51 DAT), after the second pulse treatment application for the same treatments as in panel A. C) Survival 69 DAC of sediment mesocosm exposures. N= 4 replicates per treatment. Error bars represent the standard error of the mean. Significant difference indicated by a star ($p < 0.05$).

3.3.2.2 Sex Ratios and Histology

Tadpoles in the control treatment of the mesocosms had a 50:50 male: female sex ratio (n=58) (Fig. 3.7). The WeatherMax ERC tadpoles were 41.1% male to 58.6% female (compared to control: X-squared = 1.7, df = 1, p= 0.19) and WeatherMax PMEC tadpoles were 69.0% male to 31.0% female, a sex ratio that varied significantly from control (X-squared = 4.2, df = 1, p-value = 0.04). The sediment control treatment tadpoles exhibited 59.3% male to 40.7% female (compared to a 50% ratio: X-squared = 2.8, df = 1, p= 0.10) females and the sediment POEA tadpoles exhibited 50% male to 50% female (compared to control: X-squared = 1.2, df = 1, p= 0.27). Histological analysis of mesocosm samples showed no abnormalities in gonadal development consistent with a treatment effect in either males (Fig. 3.8) or females (Fig. 3.9). Testes were characterized by proliferating spermatogonia and somatic cells with preliminary organization into seminiferous tubules. Ovaries were made up of a low number of perinucleolar oocytes positioned around an ovarian cavity.

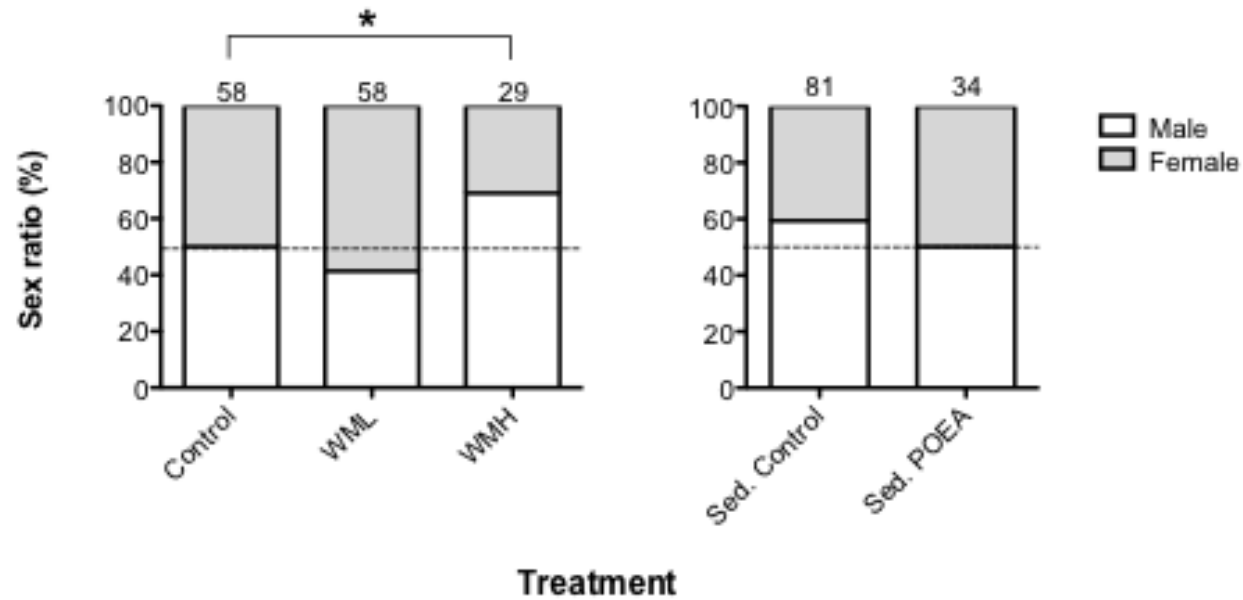


Figure 3.7 Sex ratios of Gosner stage 42 *L. sylvaticus* tadpoles in a mesocosm exposure to a glyphosate based herbicide formulation or a surfactant. Treatments include: Control, WML (WeatherMax low, ERC), WMH (WeatherMax high, PMEC), Sed. Control (control with sediment) and Sed. POEA (POEA with sediment). Sample size indicated above treatment bar. Significant difference (Chi-squared test) (from control/50%) are indicated by an asterisk ($\alpha < 0.05$). Dotted line indicates 50%.

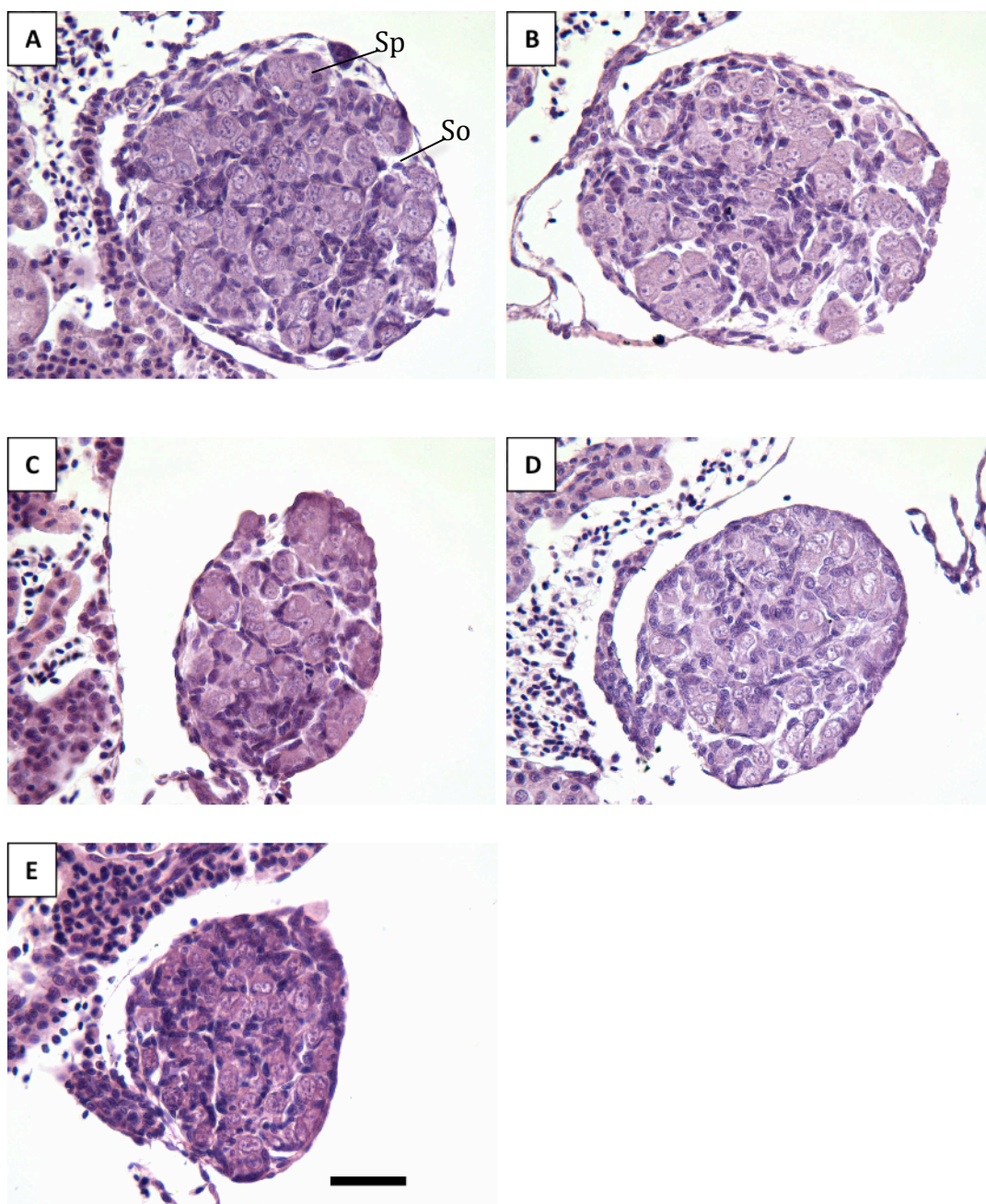


Figure 3.8 Photomicrographs of testes from *L. sylvaticus* metamorphs experimentally treated with pulse exposure to Glyphosate and POEA in a mesocosm exposure. (A) Control (B) WeatherMax ERC (C) WeatherMAX PMEC (D) Sediment Control (E) Sediment POEA. Bar = 50 microns. Structures are only identified in control (A), but apply to all treatments shown (A-E). Sp- spermatogonia, So- somatic cells.

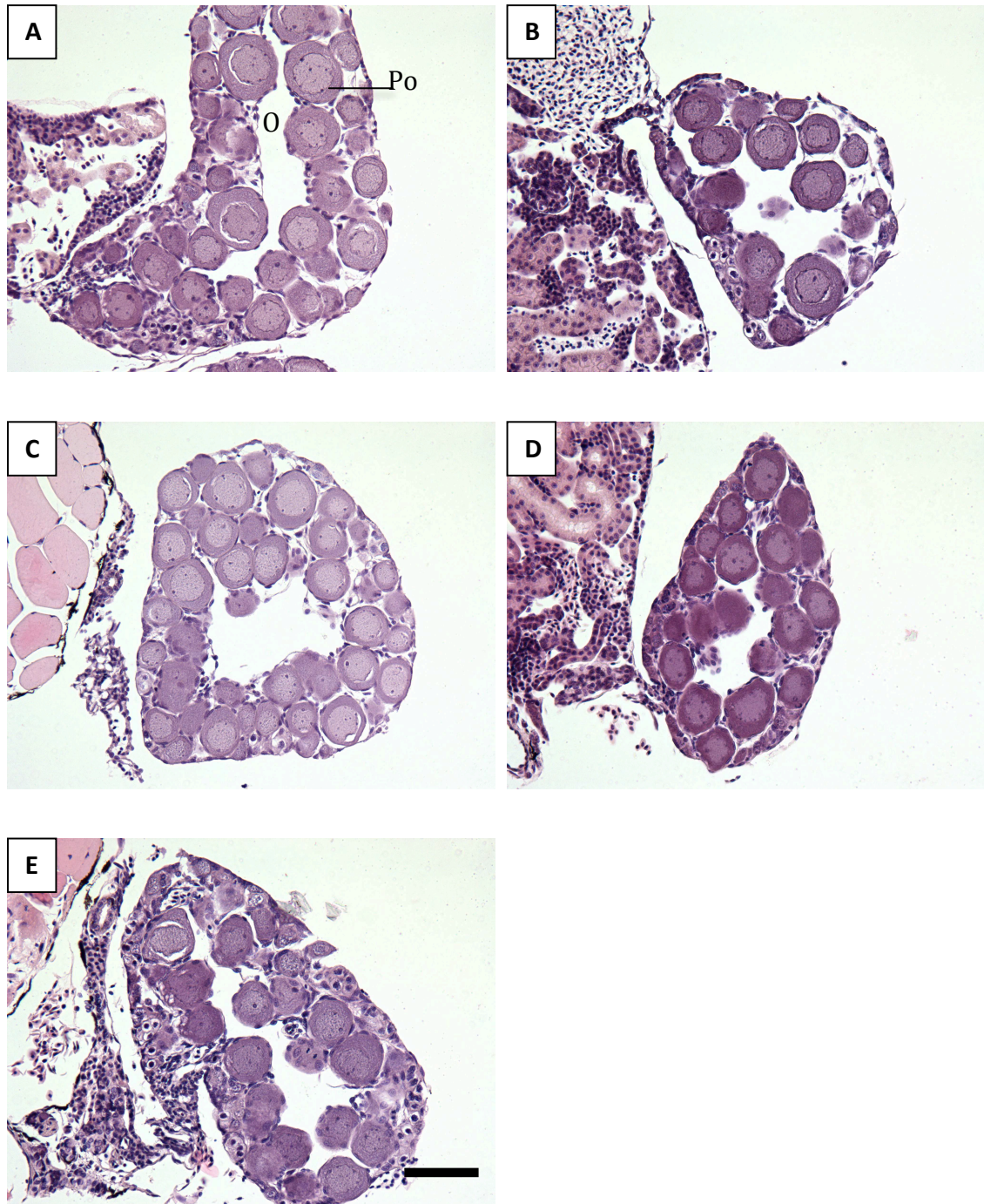


Figure 3.9 Photomicrographs of ovaries from *L. sylvaticus* metamorphs experimentally treated with pulse exposure to Glyphosate and POEA in a mesocosm exposure. (A) Control (B) WeatherMax ERC (C) WeatherMax PMEC (D) Sediment Control (E) Sediment POEA. Bar = 100 microns. Structures are only identified in control (A), but apply to all treatments shown (A-E). Po- perinuclear oocyte, O- ovarian cavity.

3.3.2.3 Days to Metamorphosis

The number of days required for the tadpoles to reach metamorphosis did not vary in non-sediment mesocosms by treatment (2 way ANOVA, $F=1.4$, $Df= 2$, $p= 0.26$) or by sex ($F=0.15$, $Df=1$, $p=0.70$) and the interaction between the two factors was not significant ($F=0.89$, $Df= 2$, $p=0.41$). The average time to reach metamorphosis was 93.5 days for control (94.3 for males, 92.7 for females), 93.6 days for WeatherMax low (93.4 days for males, 93.6 days for females) and 91.7 days for WeatherMax high (91.3 days for males, 92.7 days for females) (Fig 10, panel a). The sediment POEA treatment required 96.5 days to reach metamorphosis (98 days for males, 95.1 days for females), which was not significantly different from the sediment control treatment (2-way ANOVA, $F\text{-value}= 3.8$, $Df= 1$ $p= 0.053$), which required an average of 93.8 days (93.6 days for males, 94.3 days for females) (Fig. 10, panel b). Time to metamorphosis in the sediment treatments was not significantly different by sex ($F\text{-value}= 0.09$, $Df= 1$ $p = 0.76$) and no significant interaction was detected ($F\text{-value}= 1.8$, $Df= 1$ $p = 0.19$).

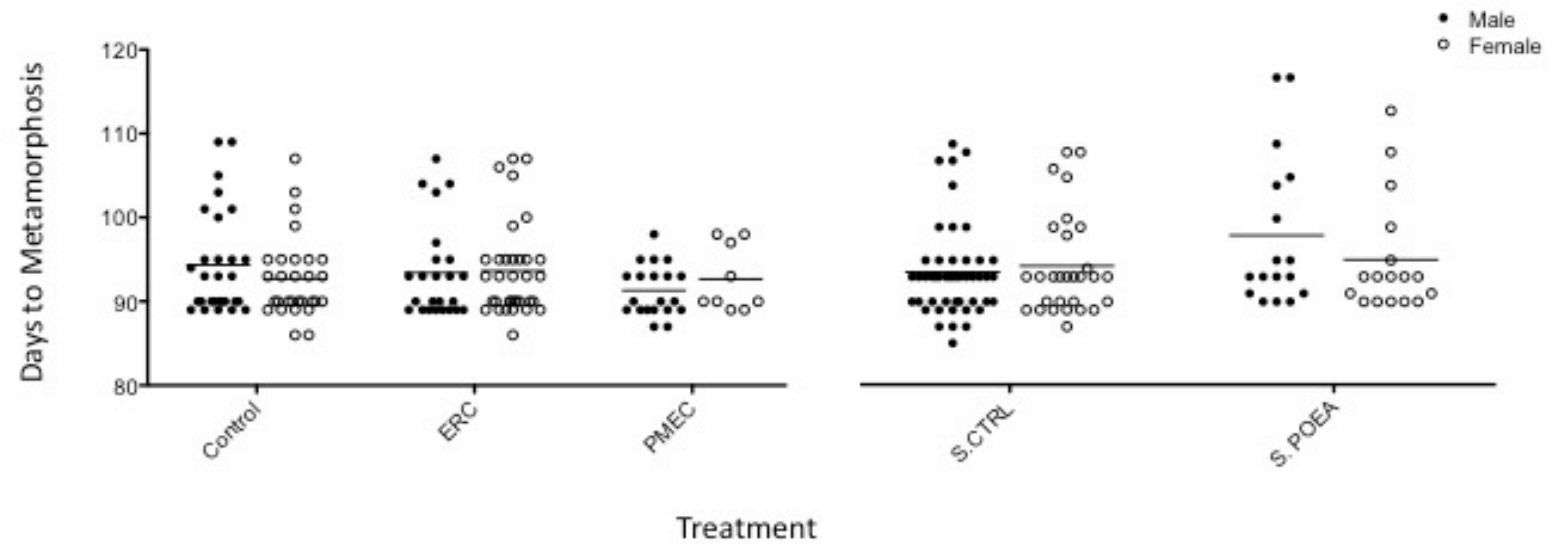


Figure 3.10 Days to metamorphosis (Gosner stage 42) of *L. sylvaticus* in a mesocosm exposure to WeatherMax or POEA. Treatments include: a) Control (N =29 females, 29 males), ERC (WeatherMax low, N= 34 females, 24 males), PMEC (WeatherMax high, N = 9 females, 20 males) and b) Sed. Control (control with sediment, N = 29 females, 48 males) and Sed. POEA (POEA with sediment, N = 17 females, 17 males). Horizontal bars indicate the mean value for each sex within each treatment.

3.3.2.4 Growth

At Gosner stage 30-31, both snout-vent length (ANOVA, $F = 0.96$, $Df = 3$, $p = 0.42$) and weight (ANOVA, $F = 0.65$, $Df = 3$, $p = 0.58$) remained unaffected for non-sediment treatments (Fig. 3.11 & Fig. 3.12, panel A), yet at stage 36-38 a significant reduction (ANOVA, $F = 6.0$, $Df = 2$, $p < 0.001$) was seen in mass in the WeatherMax PMEC treatment ($p = 0.04$) and a trend of decreased snout-vent length is observable, but not significant with respect to control ($p = 0.09$) (Fig. 3.11 & Fig. 3.12, panel B). As this decrease occurred in both endpoints, it did not translate into a decrease in overall fitness (K) (ANOVA, $F = 0.11$, $Df = 2$, $p = 0.90$) (Fig. 3.13, panel B). At stage 42, the difference between treatments was lost and once again animals from all three treatments did not vary significantly in mass (ANOVA, $F = 0.51$, $Df = 2$, $p = 0.60$), snout-vent length (ANOVA, $F = 2.0$, $Df = 2$, $p = 0.14$), or condition factor (ANOVA, $F = 1.9$, $Df = 2$, $p = 0.15$). Animals in the sediment treatments exhibited an increased mass in all three Gosner staging groups (t-test. Gosner 30-31: $t = -3.6$, $Df = 13$, $p = 0.003$, Gosner 36-38: $t = -2.4$, $Df = 7$, $p = 0.049$, Gosner 42: $t = -4.7$, $Df = 40$, $p < 0.001$) as well as an increase in snout-vent length at Gosner stages 36-38 (t-test, $t = -4.0$, $Df = 40$, $p < 0.001$) and 42 (t-test, $t = -7.8$, $Df = 125$, $p < 0.001$). At Gosner stage 42 this translates into a 37.3% increase in mass and an 11.2% increase in snout-vent length. This gives rise to a 17.4% higher condition factor for sediment POEA samples with respect to their control at stage 42 (t-test, $t = -3.1$, $Df = 33$, $p = 0.004$).

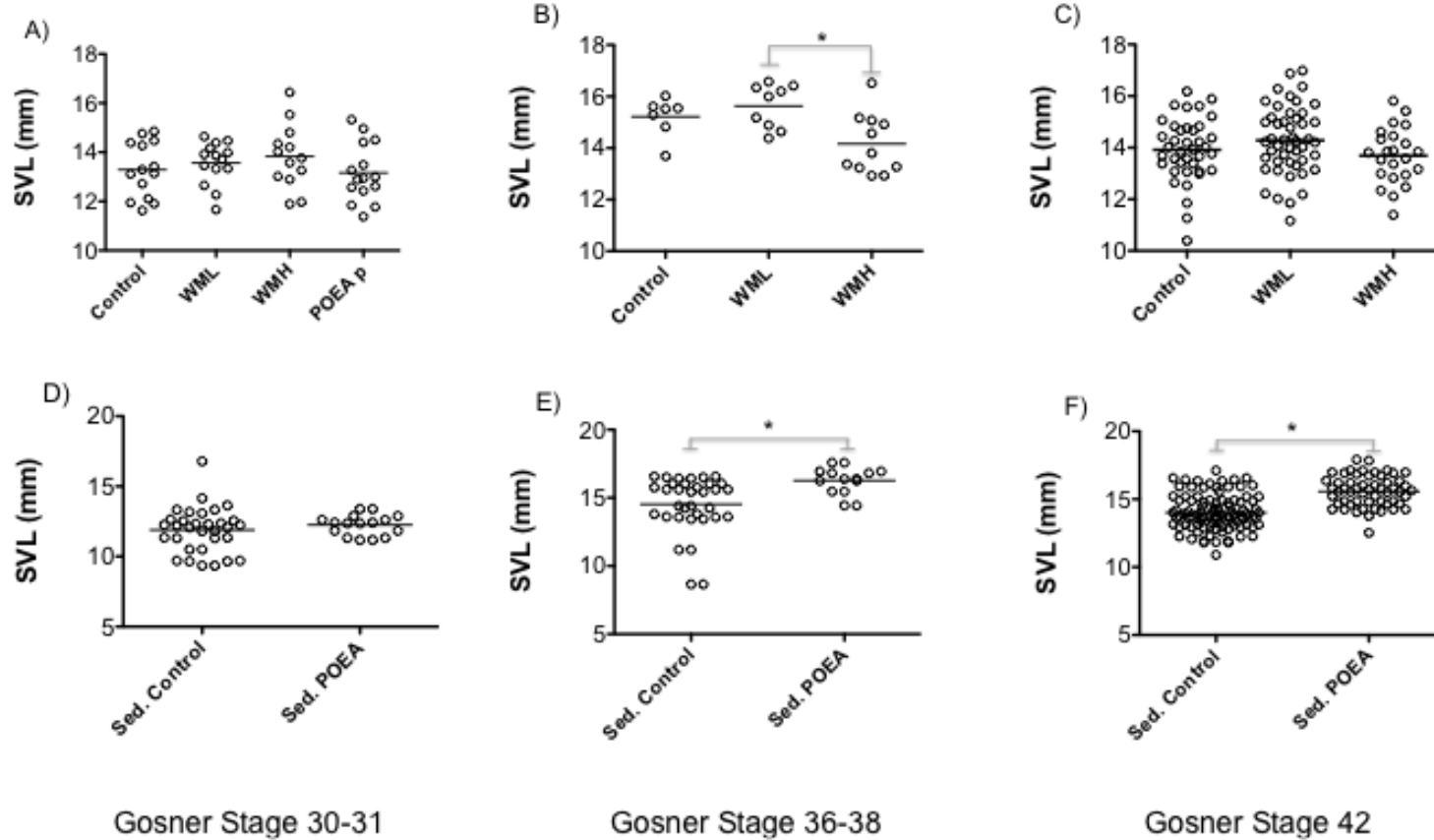


Figure 3.11. Snout-vent length (mm) of *L. sylvaticus* tadpoles in mesocosm exposure to the herbicide WeatherMax and the surfactant POEA. Treatments include Control, WML (WeatherMax low (ERC)), WMH (WeatherMax high, (PMEC)), POEA p (POEA pulse), Sed. Control (control with sediment) and Sed. POEA (POEA with sediment). Panels A and D are Gosner stage 30-31, B and E are stage 36-38 and C and F are stage 42. Significant difference indicated by a star ($p < 0.05$)

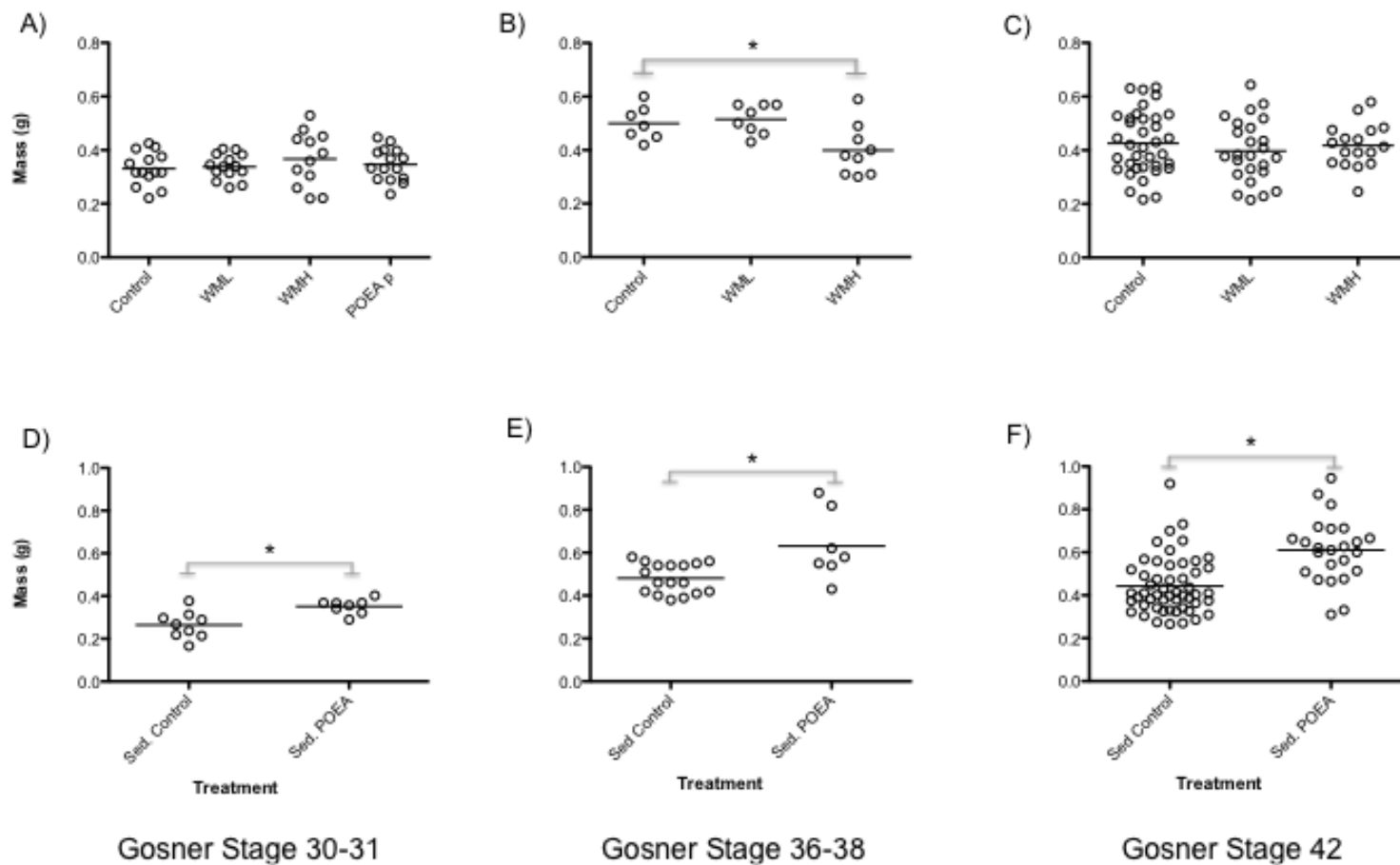


Figure 3.12. Mass (g) of *L. sylvaticus* tadpoles in mesocosm exposure to the herbicide WeatherMax and the surfactant polyethoxylated tallowamine (POEA). Treatments include Control, WML (WeatherMax low (ERC)), WMH (WeatherMax high, (PMEC)), POEA p (POEA pulse), Sed. Control (control with sediment) and Sed. POEA (POEA with sediment). Panels A and D are Gosner stage 30-31, B and E are stage 36-38 and C and F are stage 42. Significant difference indicated by a star ($p < 0.05$).

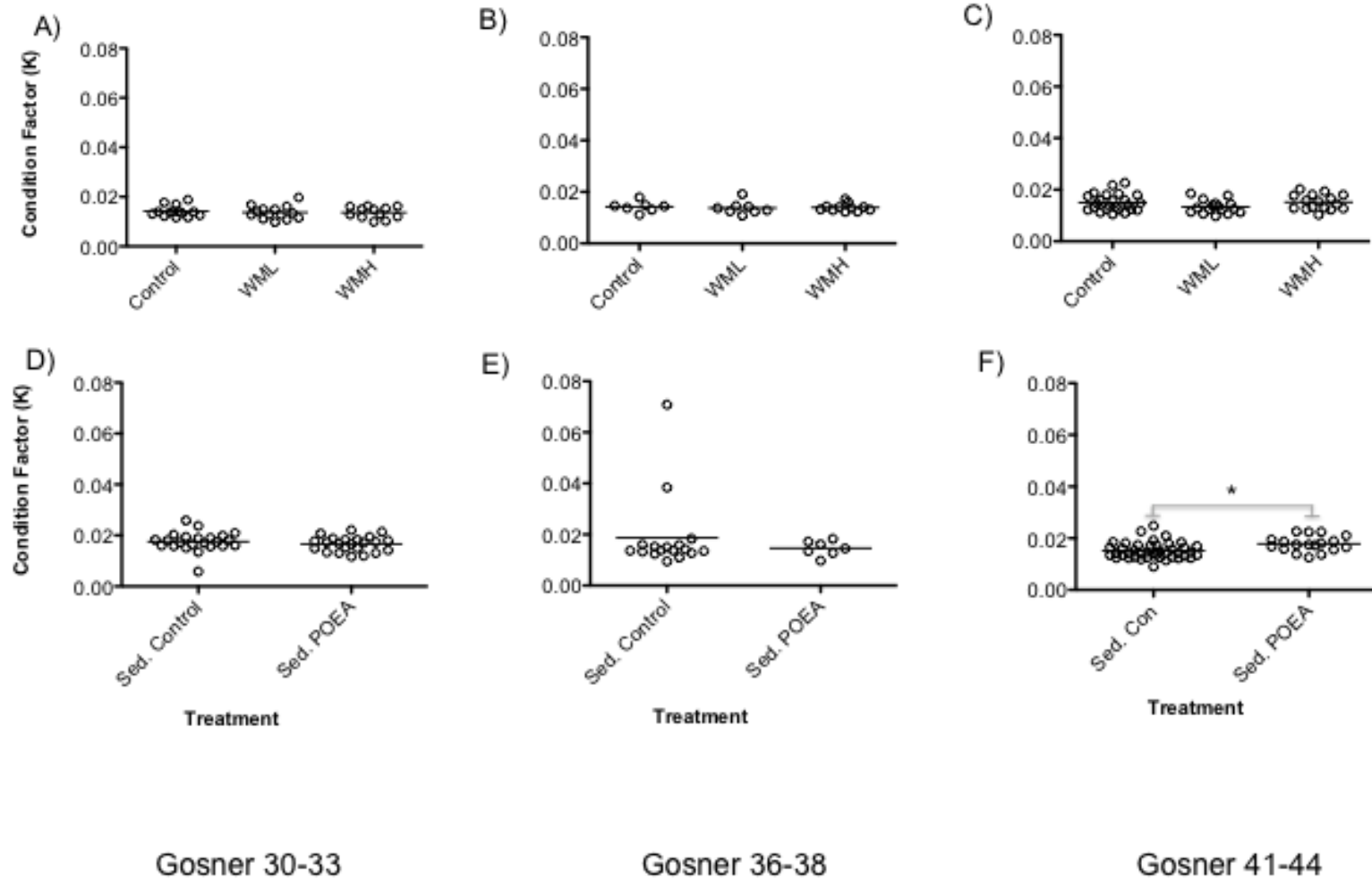


Figure 3.13 Condition factor (K) of *L. sylvaticus* tadpoles in mesocosm exposure to the herbicide WeatherMax and the surfactant polyethoxylated tallowamine. Treatments include Control, WML (WeatherMax low (ERC)), WMH (WeatherMax high, (PMEC)), Sed. Control (control with sediment) and Sed. POEA (POEA with sediment). Panels A and D are Gosner stage 30-31, B and E are stage 36-38 and C and F are stage 42. Significant difference indicated by a star ($p < 0.05$).

3.3.3 In-situ Field Exposure

3.3.3.1 Exposure Concentrations

Aqueous glyphosate concentrations were measured in the three experimental wetlands (PMEC 13, PMEC 22, PMEC 24), which were oversprayed twice in order to mimic a potential agricultural spraying scenario. The measured concentrations of glyphosate after each spray varied by wetland as did the dissipation (Table 3.2). In all three experimental systems, the treatment concentration values measured on day 0 of the first spray approximated the target concentration, varying from 0.95 to 1.3 times the PMEC of 2.88 mg/L a.e. The second pulse treatment overshot the target concentration somewhat for two of the ponds, thus the measured values on day 0 were 1.63 times for PMEC 22, 2.5 times for PMEC 13 and 1.05 times for PMEC 24. Glyphosate dissipation occurs more rapidly after the second spray for all three ponds, with PMEC 24 having the fastest dissipation rate. Taking into consideration both of the initial measured spray concentrations as well as the dissipation shown for each pond, the three wetlands can potentially be ranked by the overall WeatherMax exposure that the tadpoles within them would have received over the course of the experimental season. Therefore, even though all three ponds had the same application target, tadpoles in PMEC 13 are ranked as having the highest overall exposure (high), PMEC 22 are considered as having the intermediate exposure (medium), and PMEC 24 had the lowest overall exposure (low). Control aqueous glyphosate concentrations were zero for PMEC 24 and negligibly low, varying between 1.4-3 µg/L, in PMEC 13 and PMEC 22. This detection is likely due to slight spray drift during application.

Table 3.2 Glyphosate concentrations (mg a.e./ L) in three treated experimental wetlands. Target concentration was the PMEC 2.88 mg a.e./ L. Glyphosate concentrations remaining post spray are the percentage remaining of the initial concentration.

Pond	Spray	Measured	Measured	Concentration remaining		
		(mg a.e./ L)	concentration/target concentration (%)	from Day 0 (%)		
		Day 0		Day 1	Day 3	Day 7
PMEC 22	1	2.73	94.79	45.79	20.22	1.21
PMEC 13	1	3.77	130.90	61.01	14.59	1.06
PMEC 24	1	3.59	124.65	24.62	7.60	0.38
PMEC 22	2	4.69	162.85	33.26	5.48	1.55
PMEC 13	2	7.20	250.00	12.50	3.31	3.86
PMEC 24	2	3.01	104.51	na	0.95	0.04

na: no glyphosate detected in water from this sampling.

3.3.3.2 Wetland Characterization

While an attempt was made to choose wetlands that were similar, discrepancies always exist when dealing with “natural” experimental units. In this case, all three ponds were permanent and did not experience a full dry down at any point during the study period (Table 3.3). all three ponds were slightly acidic, P MEC 22 had a pH of 5.0 while P MEC 13 and P MEC 24 had a pH of 4.5. Wetlands P MEC 22 and P MEC 13 were considered mesotrophic due to measured wetland nutrient data (not shown), while P MEC 24 fell into the category of oligotrophic. The temperatures of the wetlands varied as would be expected of natural systems, increasing over the course of the growing season. The control and treated sides of the wetlands had highly similar average monthly temperatures in P MEC 22 and P MEC 24, however the average monthly temperatures in P MEC 13 were consistently several degrees warmer on the treated side of the wetland with averages ranging from 2.4-3.9°C higher (Fig 3.14).

Table 3.3 Characterization of the three P MEC wetlands analyzed at the LEWA site.

Wetland names	Trophic status	pH	Max volume (L)	Min volume (L)
P MEC 22	Mesotrophic	5.0	29858	21158
P MEC 13	Mesotrophic	4.5	202653	147090
P MEC 24	Oligotrophic	4.5	181483	67080

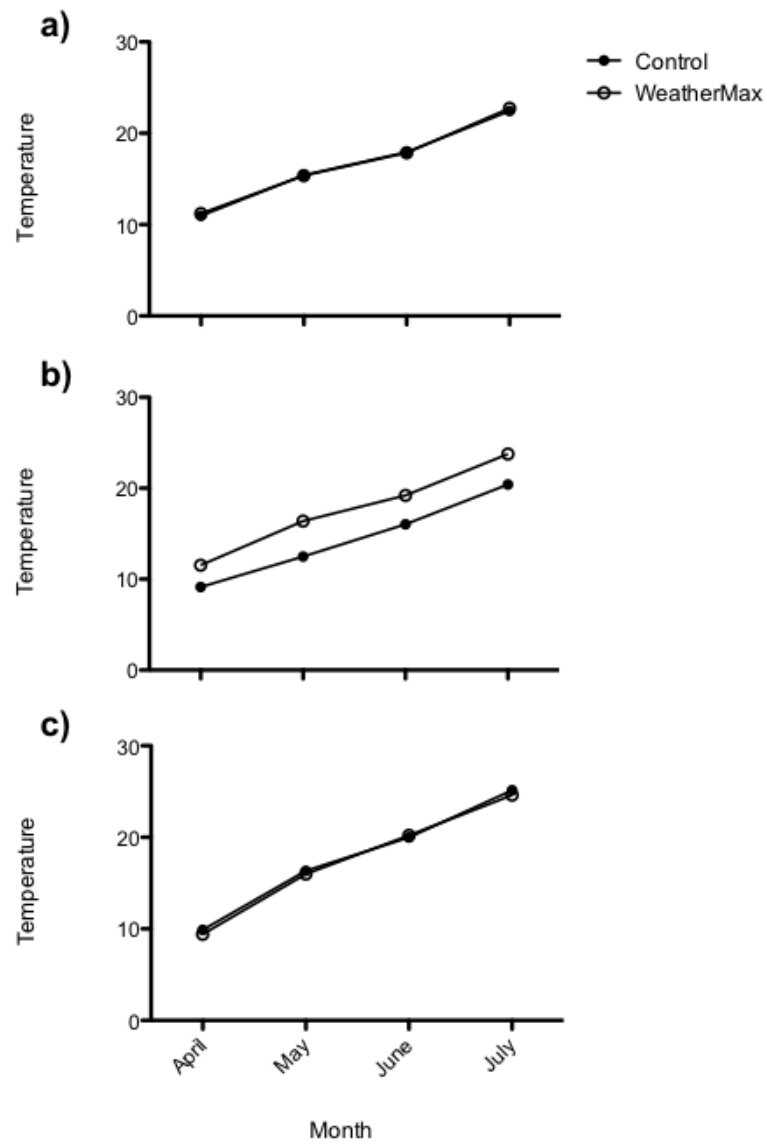


Figure 3.14 Average monthly temperatures for control and WeatherMax treated wetland halves during the summer of 2010. a) PMEC 22, b) PMEC 13, c) PMEC 24

3.3.3.3 Sex Ratios

The tadpoles in PMEC 13 (high) were found to have a significantly higher amount of females than would be expected with a 1:1 male:female ratio (X-squared = 4.6, $df = 1$, $p = 0.033$) and the sex ratio in the WeatherMax treated side of the wetland varied significantly from the control side (X-squared = 12.7, $df = 1$, $p < 0.001$) (Fig 3.15). Neither PMEC 22 (compared to control and 50%: X-squared = 0.18, $df = 1$, $p = 0.670$) nor PMEC 24 (compared to control: X-squared = 0.21, $df = 1$, $p = 0.643$. compared to 50%: X-squared = 2.3, $df = 1$, $p = 0.131$) varied from the expected 1:1 ratio or from the control.

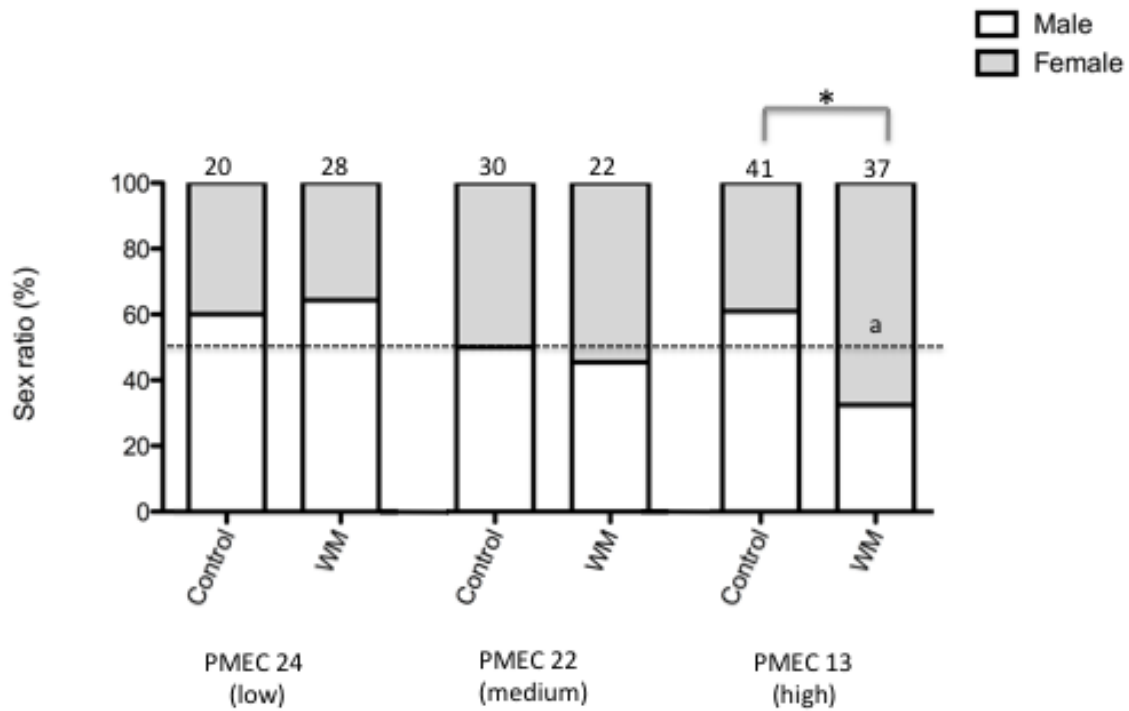


Figure 3.14 Sex ratios of *L. sylvaticus* tadpoles from *in-situ* enclosures in PMEC 22, PMEC 13 and PMEC 24. Sample size (N) indicated above bar. Significant difference between WeatherMax PMEC (WM) and Control indicated by an asterisk and significant difference between measured sex ratio and expected 50% ratio indicated by a letter ($p < 0.05$). Dotted line indicates 50%.

3.3.3.4 *Survival and Growth*

No significant differences in survival were detected in any of the PMEC wetlands between the control and WeatherMax treated halves (t-test, PMEC 24: $t = 0.56$, $Df = 7.7$, $p = 0.59$. PMEC 22: $t = -0.22$, $Df = 12$, $p = 0.83$. PMEC 13: $t = 0.78$, $Df = 8.0$, $p = 0.50$)(Fig. 3.17). No significant differences in body size were found between control and WeatherMax exposed pond halves at either the early Gosner stages 26-29 (Fig 3.16, panel A) (t-test. PMEC 13: $t = -0.15$, $Df = 40$, $p = 0.885$. PMEC 22: $t = -0.18$, $Df = 40$, $p = 0.858$. PMEC 24: $t = -1.3$, $Df = 50$, $p = 0.21$) or the later stages 36-38 (Fig. 3.16, panel B) for in any of the three experimental wetlands (t-test. PMEC 13: $t = -2.5$, $Df = 2.5$, $p = 0.103$. PMEC 22: $t = -1.1$, $Df = 21$, $p = 0.247$. PMEC 24: $t = -0.85$, $Df = 20$, $p = 0.405$). There was a trend of increased body size in the tadpoles from WeatherMax exposed side of PMEC 13, however the sample size is small and no significant difference was detected.

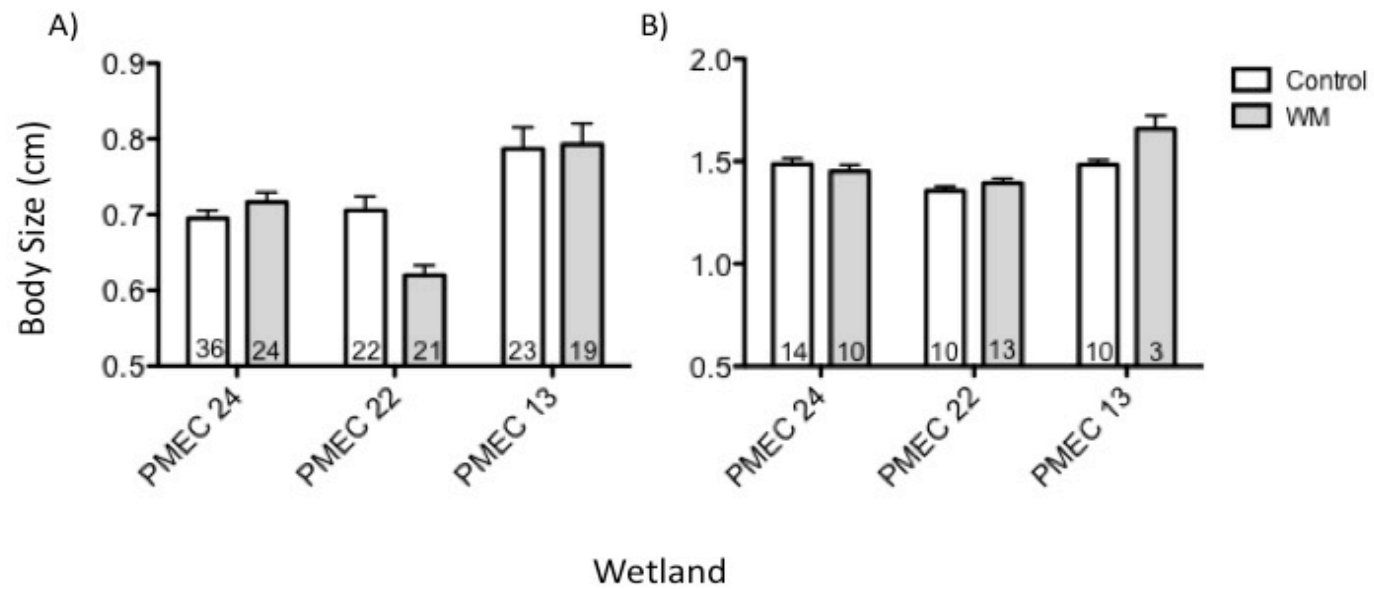


Figure 3.16 Body size (cm) of *Lithobates sylvaticus* tadpoles A) Gosner stages 26-28 B) Gosner stages 36-38. Samples size is indicated on bar. Error presented is SEM.

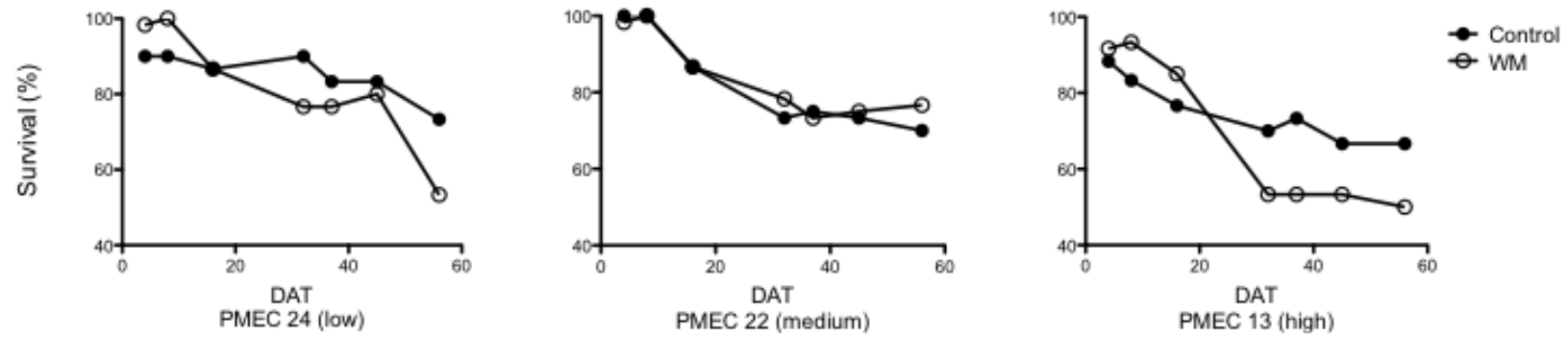


Figure 3.17 Survival (%) of tadpoles in in-situ field exposure in split PMEC wetlands according to days after treatment (DAT)

3.4. Discussion

3.4.1 POEA Affects Wood Frog Survival and Growth

The chronic exposure to POEA at a concentration expected to be found in 2.88 mg a.e./L. of the herbicide formulation Vision caused complete early mortality in both the laboratory and mesocosm exposures. While the POEA pulse treatment in the laboratory experienced high survival, which did not vary significantly from control (Lanctôt 2012), complete mortality occurred in this treatment in the mesocosms after the second pulse exposure. Tadpoles from the sediment POEA chronic treatment in the mesocosm exposure did not experience full mortality, however survival was significantly reduced with respect to the sediment control. Tadpoles in the control treatment of the sediment mesocosms, experienced survival above 90% as seen in the non-sediment control treatment. POEA concentration is known to decline rapidly when a sedimentary substrate is present (Wang et al. 2005a), a dissipation that occurs due to the chemical binding of POEA to organic matter present in the substrate (Carlisle et al. 1988). Early during tadpole development in the sediment POEA treatment, it became apparent that the larvae were not growing at the same rate as those in the sediment control, eventually two distinct groups arose with some tadpoles beginning to increase in size and others remaining extremely small (personal observation)(Fig. 3.18). Prior to the first collection of measurements and samples at Gosner stage 30-31, a decrease in survival occurred in which the smaller contingent of tadpoles in the treatment died. Measurements taken subsequent to this die-off show sediment POEA tadpoles to have a significantly greater mass, longer snout-vent length and an increased fitness at stage 42. The surviving tadpoles could be hypothesized as being the “stronger” tadpoles as they were able to withstand the chemical stressor. These surviving tadpoles were also at a much reduced

density, known to allow for greater growth (Semlitsch & Caldwell 1982), which likely accounts for the increases in growth observed in this treatment. From these results it can be surmised that the presence of sediment is a major variable in glyphosate-based herbicide exposure. This supports past research in which it was found that when included in an experimental exposure, sediment causes glyphosate based herbicides and their components to decrease at a rate similar to that found in treated ponds (Goldsborough & Beck 1989). This argues strongly for the inclusion of sediment in future studies.

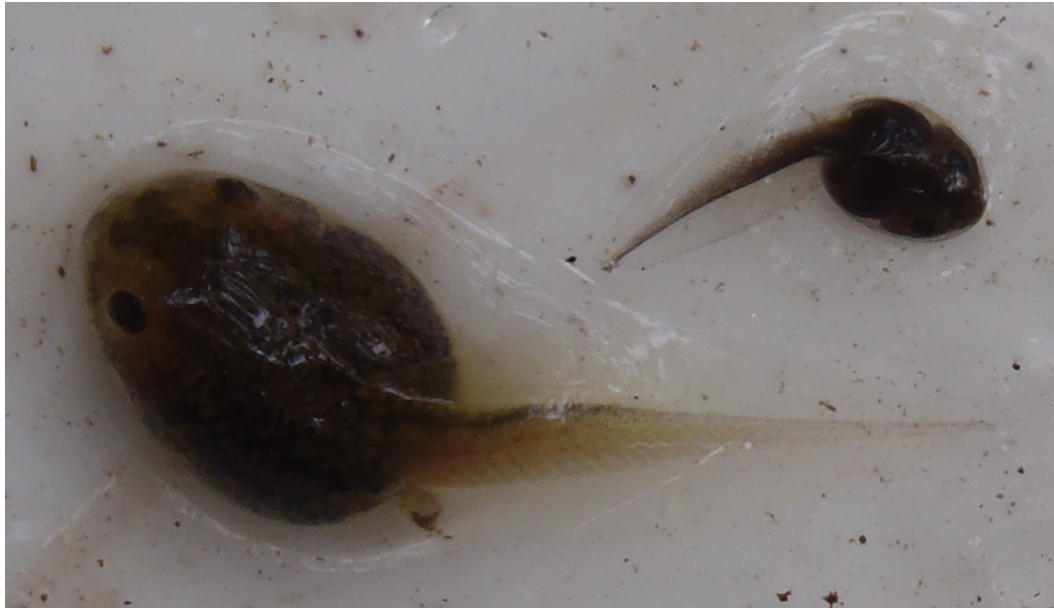


Figure 3.18 *L. sylvaticus* tadpoles from mesocosm exposure 54 DAC. The tadpole on the left is representative of the approximate average size of tadpoles in the sediment control treatment and the tadpole on the right is representative of the approximate average size of tadpoles in sediment POEA.

3.4.2 WeatherMax Causes Changes in Sex Ratios in L. sylvaticus

Tadpoles exposed to the ERC of WeatherMax did not exhibit modified sex ratios in either the laboratory or mesocosm exposures. As complete mortality occurred in the PMEC treatment in the laboratory exposure, no sex ratio data is available. In the mesocosm exposure however, a significantly higher number of males were detected in the PMEC treatment with respect to the control in which a 1:1 ratio of males to females was observed. If the change in sex ratio is truly due to the glyphosate-based herbicide in the mesocosms it is unlikely in this case that this effect was directly caused by the action of the herbicide on gonadal differentiation as no abnormalities were detected in gonadal histology. This could potentially indicate preferential death in one sex as has previously been demonstrated in goldfish exposed to 4-tert-octylphenol (Blázquez et al. 1998).

In the in-situ wetland exposure PMEC 13, a female skewed sex ratio was observed in the treated half of the experimental unit. No tadpoles were available for histological analysis from the field and thus no conclusions can be drawn as to the development of the gonads. This female preference was not detected in either of the other two wetlands. Tadpoles in PMEC 13, designated “high”, were estimated as having the highest exposure level to the WeatherMax herbicide in the wetlands. Also of pertinence is the fact that the treated side of the PMEC 13 wetland was consistently several degrees warmer than the control side. This temperature difference is unlikely to be affecting sexual phenotype directly as it has previously been shown in *L. sylvaticus* that extended exposure to a very high temperature of 32 °C is required to modify phenotypic sex and this extreme temperature leads to a masculinization, which is opposite to these findings (Witschi 1929). However, glyphosate herbicides are known to become more toxic to fish species when exposed at higher temperatures (Folmar et al. 1979). Therefore it could be a

combination of higher glyphosate concentration and higher temperature causing the changes seen in this wetland. If so, this would indicate that tadpoles in warmer wetlands could potentially be at risk to glyphosate concentrations that appeared less harmful in a milder temperature laboratory setting. It is also important to note that tadpoles in our laboratory experiment experienced full mortality due to a pulse exposure with a peak concentration of 2.88 mg a.e. /L, which is 2.5 times lower than the highest pulse concentration detected in the field exposure, which inflicted no significant reduction in survival. Thus, if the higher exposure concentrations and temperature are responsible for the change in sex ratio observed in PMEC 13, it is unlikely that this phenomenon would be detectable in a laboratory exposure.

Thus in three exposure types, a target PMEC concentration was found to be lethal in the laboratory, cause masculinization in the mesocosms and, when the concentration was increased and combined with an elevated temperature, to cause feminization in the field. To the best of our knowledge this is the first time skewed sex ratios have been demonstrated in an anuran species due to a glyphosate-based herbicide exposure. These findings are counter to a previous chronic laboratory study in which *R. pipiens* tadpoles were found to have no changes in sex ratio, but presented gonadal abnormalities (Howe et al. 2004). This discrepancy could be caused by various factors including species-specific sensitivities as well as differences in exposure methodology. Evidently this is an endpoint meriting further investigation utilizing environmentally relevant exposure scenarios.

3.4.3 Defining an Environmentally Relevant Exposure

The differences in initial spray concentrations on the treated half of the PMEC wetlands exemplify the challenges as a pesticide applicator in determining the exact amount of herbicide applied to an intended target. As shown (Table 2), glyphosate concentrations decrease extremely quickly in a natural system. In the three wetlands examined, concentration values from 1 day post-spray ranged from 12.5 to 61.01% of the originally detected concentration (with one sampling showing no detection). Values 7 days post-spray ranged from 0.04 to 3.86% the initially detected concentration, thus indicating a rapid decrease in aqueous bioavailability. This data raises questions regarding concentrations detected in natural environments. If a pulse application has the potential to affect an amphibian population, studies reporting glyphosate concentrations in nature need to time sample collections meticulously with spray dates in order to determine the actual peak pulse concentration that amphibians and other unintended spray targets may be exposed to.

3.4.4 Survival and Growth

Tadpole survival in the non-sediment mesocosm treatments (41 DAT, 67 DAC) after the first pulse exposure was extremely high across control, the WeatherMax ERC and the WeatherMax PMEC treatments as well as the POEA pulse treatment. After the second pulse treatment, survival remained high (51 DAT, 77 DAC) in control and in both WeatherMax treatments, however complete mortality was observed in the POEA pulse animals. This is not a completely unexpected result as most studies indicate that POEA, or the surfactant in general, is the toxic component in herbicide formulations (Howe et al. 2004; Edginton et al. 2004; Comstock et al. 2007). However the POEA concentration utilized in this experiment was based on the amount (by weight) contained in 2.88 mg/L

a.e. of the formulation Vision, the acid equivalent to the WeatherMax formulation PMEC studied in this experiment. In the laboratory, the POEA chronic treatment exhibited similar mortality trends to the WeatherMax PMEC, however the POEA pulse survival remained high for the duration of the experiment (Supplementary Fig. 3.1). In the mesocosm exposure, the tadpoles in the POEA pulse treatment experienced complete mortality, while those in the WeatherMax PMEC treatment had a survival above 90% after the two pulses. As pond water was used in the mesocosms while dechlorinated water was used in the lab, the higher survival of the tadpoles exposed to the PMEC of WeatherMax in the mesocosms relative to the lab could potentially be attributed to the higher quantity of organic matter presumably found in the mesocosm water.

3.4.5 Days to Metamorphosis was Unaffected by Treatment

In this study, tadpoles in both the laboratory and mesocosm exposures exhibit no sex specific difference in time to metamorphosis in *L. sylvaticus*. In the laboratory exposure, neither the surfactant-containing glyphosate formulations, the glyphosate salt nor the POEA pulse exposure showed any changes in days to metamorphosis. This was also true in the mesocosms for both concentrations of the glyphosate formulation WeatherMax as well as the sediment POEA treatment. Time to metamorphosis is an endpoint, which has previously been shown to be species-specific in its sensitivity to disruption (Williams & Semlitsch 2009; Howe et al. 2004). Therefore, while this lack of sensitivity may be due to the more environmentally relevant nature of the exposures conducted herein, it may also reflect a particular robustness in the *L. sylvaticus* with regards to this endpoint. Previous results indicate that *L. sylvaticus* may be a species that is particularly resilient relative in (Relyea & Jones 2009) in shorter exposures to a

glyphosate-based herbicide, however it may be particularly sensitive to longer exposures (Relyea 2005). Thus these results may not necessarily reflect the sensitivity of other species. As time to metamorphosis is crucial to survival in the wild, particularly for amphibians breeding in ephemeral bodies of water, future studies should include this as an endpoint meriting further investigation.

3.4.6 Critical Experimental Considerations

In the laboratory exposure, the temperature was maintained at a relatively stable 21°C for the course of the experiment while both the mesocosm and wetland temperatures varied during the experimental season. As the mesocosms were set up in a shaded area, their temperatures remained lower than those of the natural wetlands and while temperature fluctuations did occur both daily and during the season, afternoon temperatures generally remained between 13-15 °C. The experimental wetlands experienced the greatest variations in temperature as they were subject to extended periods of direct sunlight. The monthly average water temperature for the wetlands in which the in-situ enclosures were placed varied from 9-25 °C over the course of the experimental season. Temperature is an important factor in both tadpole growth (Harkey & Semlitsch 1988; Blouin & Brown 2000) and glyphosate toxicity (Folmar et al. 1979) and this factor aids in explaining the discrepancies in developmental times between exposures. Tadpoles reached Gosner stage 30-31 earliest in the laboratory at 35 DAC likely due to a consistently warm temperature. This stage was reached 41 DAC in the field potentially due to its low early season temperatures and higher peak temperatures on sunny summer days. The mesocosm exposure took the longest with 72 DAC potentially due to their consistently shaded and cooler environment. How these temperature

differences may affect endpoints other than rate of somatic development is unclear and merits further investigation.

pH is another factor that merits consideration. Laboratory exposures had a circumneutral pH, while mesocosms had a more alkaline pH between 7.5 and 8.5. The wetlands studied were much more acidic with a pH of 4.5 for PMEC 13 as well as PMEC 24 and a pH of 5 for PMEC 22 (Table 3.3). In certain species of fish, an increase in pH is known to increase the toxicity of glyphosate formulations and particularly POEA (Folmar et al. 1979). This increase in the toxicity of a glyphosate-based herbicide formulation due to increased pH has also been demonstrated in a variety of anuran species including *Xenopus laevis*, *Bufo americanus*, *Rana clamitans* and *Rana pipiens* (Edginton et al. 2004; Chen et al. 2004). The increased pH in the mesocosms relative to the laboratory exposure could be a factor in the lower survival found in the POEA pulse treatment in the mesocosms. If so, this could potentially indicate that the surfactant in the WeatherMax formulation is less impacted by a change in pH than POEA is as higher survival was found in the mesocosms relative to the lab. To our knowledge, the possibility of a differential response to a change in pH by various glyphosate-based herbicides has not previously been examined and such a study may lead to better characterizing the type of surfactant contained in the WeatherMax herbicide.

3.5 Conclusions

The glyphosate-based herbicide WeatherMax or the surfactants contained therein were found to affect survival, sex ratios and growth in *L. sylvaticus* tadpoles under environmentally relevant exposure conditions. The effects of this herbicide in the more

synthetic laboratory and mesocosm exposures are not necessarily reflective of the consequences of a more realistic field exposure. This is clearly exemplified by the PMEC for WeatherMax, which caused complete mortality in the laboratory, no significant mortality in the mesocosms and no mortality in the field even when the exposure concentration was increased. The change in sex ratio detected at the higher exposure concentration in the field, a concentration that cannot be studied in the lab due to mortality, demonstrates that important sub-lethal effects may be overlooked in these artificial experimental environments. The increased survival of POEA treated tadpoles due the inclusion of a sedimentary substrate, causing the exposure environment to more closely approximate a natural wetland, demonstrates that modifying the experimental setup in order to mimic a natural exposure as closely as possible may lead to more environmentally relevant results. As field exposure are not always feasible, the contrasting results between exposure types in this project demonstrates that mimicking a field environment through the consideration of variables including pH, the inclusion of a sedimentary substrate and the choice of experimental species, may have a great impact on the outcome of a study examining glyphosate-based herbicides.

Chapter 4 - Steroidogenic Acute Regulatory in *Lithobates sylvaticus* and the Effects of a Field Based WeatherMax Exposure on Genes Involved in Sexual Differentiation

Chapter adapted from **Robertson C.**¹, **Lancot C.**², **Navarro-Martin L.**³, **Edge C.**⁴, **Pauli B.**⁵ & **Trudeau V.**⁶
(manuscript in preparation).

Principal contributions of co-authors:

1. Contributed to experimental design and implementation including sample collection, monitoring survival, grow and development as well as performing RT-PCR analysis and manuscript preparation.
2. Contributed to experimental design and implementation as well as sampling and tissue collection. Supervised research and revised manuscript.
3. Contributed to experimental design and implementation including sampling and tissue collection.
4. Contributed to monitoring survival, growth and development as well as sample collection in the mesocosm and field exposures
5. Contributed financial support and aided in experimental design.
6. Contributed as supervisor of research project, aided in experimental design, provided financial support and revised manuscript.

4.1. Introduction

4.1.1 *Steroidogenesis and the Role of Steroidogenic Acute Regulatory (star) Protein*

The synthesis of steroid hormones is a process known as steroidogenesis and the first step in this process, in any steroidogenic tissue, is the conversion of cholesterol to pregnenolone, a transformation that is carried out by the cytochrome P450 side-chain cleavage enzyme (P450_{scc} or Cyp11A1) (Stocco 2001). In order for this conversion to occur, cholesterol must be present within the mitochondria and, as steroid hormones cannot be stored within cells and must be synthesized on demand, this process is acutely regulated. Thus the rate-limiting step in steroidogenesis has been identified as the active transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where CYP11A1 is located (Stocco 2001). Steroidogenic acute regulatory (star) protein is responsible for this movement of cholesterol across the mitochondrial membranes where it interacts with CYP11A1, initiating the steroidogenic pathway. The production of star is therefore a key factor in regulating timing and rate of steroidogenesis.

Full length cDNA encoding *star* has been isolated in an array of mammals (Ariyoshi et al. 1998; Fleury et al. 1996; West et al. 2001; Pilon et al. 1997; Hartung et al. 1995; Kerban et al. 1999) including humans (Sugawara et al. 1995) and has been shown to have a reasonably high degree of sequence similarity in these species (85-88%) (Kusakabe et al. 2002). More recently, the role and expression of *star* has come under great scrutiny in non-mammalian vertebrates leading to its isolation in a variety of species (Li et al. 2003; Kocerha et al. 2010; Evans & Nunez 2010; Bauer et al. 2000; London et al. 2006) including several anuran species such as *R. catesbeiana* (Kim et al.

2009), *X. laevis* (Gohin et al. 2010) and *R. rugosa* (Maruo et al. 2008). Across both mammalian and non-mammalian vertebrates, star appears to be conserved, supporting its crucial role in steroid hormone production. Star is localized in the mitochondria of steroidogenic tissues. Its expression has been detected in tissues including both adult and fetal testes, ovary and adrenals as well as the ovarian theca, granulosa, ovarian corpora lutea cells, testicular leydig cells and the adrenal cortex (Pollack et al. 1997; Nishikawa et al. 1996; Pilon et al. 1997; Kiriakidou et al. 1996).

Abnormalities in *star* expression have been shown to result in dire consequences. As an extreme example of this, mutations in the *star* gene have been linked to congenital lipoid adrenal hyperplasia (lipoid CAH) in humans; an autosomal recessive disorder characterized by detrimentally low levels of adrenal and gonadal steroid hormones as well as an accumulation of cholesterol in the enlarged adrenals (Lin et al. 1995; Saenger 1997). This condition is fatal in infants unless treated with steroid hormone replacement therapy and leads to genetic males being born with female secondary sexual characteristics due to an absence of testicular testosterone synthesis (Lin et al. 1995). Mice with a null mutation for *Star* also display a phenotype similar to human lipoid CAH patients (Caron et al. 1997).

Disruptions or changes in the modulation of a non-mutant *star* gene likely have the potential to impact similar endpoints in a more subtle fashion and *star* has been shown to have the potential to be influenced by exogenous factors including certain environmental contaminants. Nonylphenol, a degradation product of an important group of non-ionic surfactants, has been shown to increase *star* mRNA in Atlantic Salmon subjected to a 15 µg/L exposure (Arukwe 2005). The organophosphate insecticide

Dimethoate and the organochlorine insecticide Lindane were found to inhibit steroid hormone production in the mouse MA-10 Leydig tumor cell line through a reduction in *star* transcription (Walsh & Stocco 2000; Walsh, Webster, et al. 2000; Walsh, McCormick, et al. 2000). *Star* expression has also been shown to be decreased by exposure to 2, 4, 6-trinitrotoluene (TNT) in *L. catesbeiana* (Paden et al. 2010). This demonstrates that while a mutation in the *star* gene can be a death sentence, changes in the modulation of the gene may also lead to more subtle and yet potentially severe disruptions in normal hormonal signalling. The glyphosate-based herbicide Roundup has also been shown to decrease star protein in the MA-10 Leydig tumor cell line and has also been shown to affect steroidogenic enzyme levels (Walsh, McCormick, et al. 2000).

Amphibians are known to be particularly sensitive to steroidogenic hormone imbalances and their phenotypic sex can be modified by these changes, particularly if exposure occurs early in their larval development (Mikamo & Witschi 1963; Witschi 1927). Interest in these potential effects on reproduction is growing as amphibian populations are now known to be decreasing worldwide (Houlahan et al. 2000; Alford & Richards 1999). As the transfer of cholesterol by star is the rate-limiting step in the production of steroidogenic hormones (review: Stocco 2001), modulations in this transport protein could conceivably lead to severe disruption in the natural development and functioning of these animals. Therefore, as further investigation of *star* as a potential endpoint for EDC is envisaged, it is important to understand how its expression varies in the target species under natural conditions. The wood frog was chosen as a target species in this case due to its broad habitat distribution, ease of availability and the fact that it progresses from an egg to a fully developed frog in a single season (Berven 1990). All of

these characteristics make it an ideal model species. In this study, *star* was cloned and characterized in the wood frog (*L. sylvaticus*) and its expression, as well as that of three other genes involved in sexual differentiation *cyp19*, *foxl2* and *cyp17*, were examined in an in-situ field exposure to the glyphosate based herbicide Roundup WeatherMax.

4.2. Materials and Methods

4.2.1 Cloning of *star* in *L. sylvaticus*

Total RNA was isolated from the gonad-mesonephros complex (GMC) of *L. sylvaticus* tadpoles. Total RNA extraction was carried out using the RNeasy Mini Kit (including RNase-free DNase step) following the manufacturer's instructions (Qiagen). The extracted RNA was suspended in RNase-free water and stored at -80°C . 0.5 μg micrograms of total RNA were reverse transcribed to cDNA using Superscript II (Invitrogen) and 200 ng of random hexamer primers following the manufacturer's instructions. The resulting cDNA was used to amplify the desired genes using native Taq DNA polymerase (Invitrogen) and the respective primers. Primers were designed based on conserved regions of known sequences from other anuran species (*Lithobates catesbeianus*, ACJ53927; *Glandirana rugosa* BAH09112) using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The reverse transcriptase (RT) polymerase chain reaction (PCR) was carried out in a final volume of 25 μL containing 2.5 mM MgCl_2 (Qiagen), 1X PCR buffer (Qiagen), 0.20 mM dNTPs (Invitrogen), 0.1 μM ROX reference dye (Stratagene), 1.25 U HotStarTaq (Quiagen) and 6.25 ng first-strand cDNA. RT-PCR amplification was achieved using a Mastercycler gradient thermalcycler (Eppendorf; Mississauga, On, Canada). The amplification product was run on a 1% agarose gel, excised and purified utilizing a QIAQuick Gel Extraction

Kit (Qiagen). The purified product was then ligated into the PCR 2.1 TOPO[®] vector (TOPA TA cloning kit; Invitrogen), and transformed into *Escherichia coli* competent cells (One Shot TOPO 10 chemically competent cells; Invitrogen). These cells were then plated on ampicillin and X-gal containing LB-agar plates from which, positive colonies were collected and incubated overnight in LB broth containing ampicillin. Plasmids were purified with a QIAprep Spin Miniprep (QIAGEN). All of the above procedures were carried out following the manufacturers protocols. A portion of the purified plasmid was sent to the Ottawa Health Research Institute (OHRI/IRSO; On, Canada) for sequencing. Nucleotide identities with other vertebrates sequences was determined using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>).

Table 4.1 Real time RT-PCR primers utilized to amplify the steroidogenic acute regulatory (*star*) and ribosomal protein l8 (*rpl8*).

Gene	Element	Primer Sequence (5'→ 3')
<i>star</i>	Forward	GAAATACCGCCTAACCACCA
	Reverse	GGAAATGCCCGAGTAGAGGAA
<i>rpl8</i>	Forward	GTGTAGAAGAGAAGCCAGGTGAT
	Reverse	GGATTGTGGGAGATGACGGTAG

4.2.2 Amino Acid Sequence Analysis, Alignment and Phylogenetic Tree

The predicted *L. sylvaticus star* protein sequence was obtained using The Expert Protein Analysis System (EXPASY, <http://www.expasy.ch/tools/dna.html>). Available amphibian *star* protein sequences were aligned using the ClustalW multiple sequence alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) according to the default settings.

In addition, a phylogenetic analysis of *star* protein sequences was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved full-length amino acid sequences from 24 species and the *L. sylvaticus* sequence cloned in this study. Accession numbers are listed in figure 4.3. All positions containing gaps and missing data were eliminated. There were a total of 127 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

4.2.3 Tissue Distribution of star Gene Expression in L. sylvaticus

4.2.3.1 Animals and Sample Collection

Wild *L. sylvaticus* egg masses were collected at the Long-term Experimental Wetlands Area (LEWA) at the Canadian Forces Base Gagetown (New Brunswick, Canada) and subsequently hatched and raised under laboratory conditions at the University of Ottawa in dechlorinated water at ~26 °C. These tadpoles were untreated and utilized to determine a natural profile for gene expression in various tissues. Tadpoles were fed a daily diet of rabbit pellets supplemented with wards tadpole food and waste was removed twice weekly. Metamorphs were collected at Gosner stage 42, anesthetized utilizing MS222 (Sigma) and decapitated. Excised tissues included hind limb, forelimb, tail, skin, stomach, liver, eyes, gall bladder, lung, intestines, brain, interrenals, ovary and testes. These tissues were removed, placed on dry ice and subsequently stored at -80°C.

Wild untreated adult *L. sylvaticus* frogs (3 males and 3 females) were collected at the LEWA experimental site. Animals were anesthetized utilizing MS222 (Sigma) and decapitated. Interrenals and gonads were dissected separately, frozen and stored at -80°C until further analysis. All procedures followed approved animal care protocols of the University of Ottawa and the Canadian Council on Animal Care.

4.2.3.2 RNA Extractions, cDNA Synthesis and RT-PCR

Tissues from laboratory raised Gosner stage 42 tadpoles were pooled (n=2-10, depending on tissue) from several animals until an adequate amount of material was amassed in order to obtain sufficient RNA. Total RNA was extracted using the RNeasy micro kit (including RNase-free DNase step) following the manufacturer's instructions (Qiagen). After extractions, RNA quality was verified by determining the absorbance

ratios of 260/230 nm and 280/260 nm utilising a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies Inc.) and the RNA integrity numbers (RIN) using the Agilent 2100 Bioanalyzer. The absorbance ratios were all approximately 2 and all samples with RIN numbers above 5 were acceptable for PCR. Half a microgram (0.5 µg) of total RNA was reverse transcribed to cDNA following the QuantiTect Reverse Transcription protocol (QIAGEN). For the wild caught *L. sylvaticus* adults, total RNA was isolated from the gonad and interrenals samples using RNeasy Mini Kit (including RNase-free DNase step) following the manufacturer's instructions. The extracted RNA was then re-suspended in RNase-free water and RNA quality was verified (as explained above). RIN numbers were all above 7 and the absorbance ratios were all approximately 2.

Gene specific primers (Table 4.1) based on the *L. sylvaticus star* sequence obtained in this study were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers for *rpl8* were taken from Navarro-Martin et al 2012a. The resulting cDNA was utilized to amplify *star* and *rpl8* by RT-PCR using native Taq DNA polymerase (Invitrogen). The RT-PCR was performed using a Mastercycler gradient thermalcycler (Eppendorf; Mississauga, On, Canada). The RT-PCR cycling conditions for both genes were as follows: 2 min at 95°C, 35 cycles of 45 sec at 95°C, 30 sec at 60°C and 1 minute at 72°C followed by a final 10 min step at 72°C. The amplified RT-PCR products were resolved on 1% agarose gel stained with ethidium bromide. Each lane was loaded with 7.5 µg of *star* PCR product and 7.5 µg *rpl8* PCR product.

4.2.4 Developmental Profile of star Gene Expression in the GMC of Wild Metamorphosing Tadpoles

4.2.4.1 Animals: Sample Collection and Preparation

Wild *L. sylvaticus* tadpoles were collected from an untreated and undisturbed wetland at four different sampling time points from the LEWA experimental site during the spring of 2009 (see Navarro-Martin et al 2012a). A subset of the samples from the four sampling time-points were analyzed for gene expression within this study. The first 3 samplings were carried out when the median Gosner stage of the tadpole population within the wetland reached a predetermined stage (stage 26 (n=12), stage 28 (n=12), stage 30 (n=12)) and only tadpoles at these stages were collected. The fourth sampling did not target a specific stage, but instead a range of stages were collected on the same day (stages 31-42 (n=36)). This was in part due to the wider spread of stages present later in tadpole development and partly to allow for varying stages to be analyzed for tadpoles of the same age. Thus for the fourth sampling, the Gosner stage (Gosner 1960) of the tadpoles varied, but they were all of approximately the same age. All collection and sample preparation procedures followed approved animal care protocols of the University of Ottawa and the Canadian Council on Animal Care.

4.2.4.2 Real-time RT-PCR Reactions and Conditions

Real-time RT-PCR assays were used to analyse *star* expression at the different developmental stages. SYBR Green I chemistry was employed and a dissociation curve indicating the presences of a single amplification product was established in order to verify primer specificity. A primer concentration of 300 nM was found to be optimal in order to obtain maximal amplification. Relative standard curves were generated using a serial dilution of a mixture of cDNA from all stages and optimized in order to obtain an

efficiency of $100 \pm 10\%$, a slope between -3.1 and -3.6 and an r^2 value greater than 0.985. Real-time RT-PCR cycling conditions were 15 min at 95°C succeeded by 40 cycles of 15 sec at 95°C, 22 sec at 62°C and 30 sec at 72°C followed by cycles of 1 min at 95°C, 30 sec at 55°C and a final step of 30 sec at 95°C. Samples analyzed were run in duplicate along with non-template controls in optically clear 96-well plates with a final volume of 25 μ l. Each PCR reaction contained 3.91 ng of RNA transformed to cDNA, 1X Q-PCR buffer (Qiagen), 2.5 mM $MgCl_2$ (Qiagen), 200 μ M dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), 1.25 U HotStarTaq (Qiagen) and 0.25X SYBR Green I Dye (Molecular Probes). The gene expression data for *star* was normalized to *rpl8*.

4.2.5 GMC Gene Expression Analysis of Premetamorphic L. sylvaticus Tadpoles Exposed to the Pesticide WeatherMax Under Seminatural Conditions (in-situ Field)

4.2.5.1 Experimental Design

In-situ enclosures were set up in split wetlands as described in Chapter 3 section “4.2.4 In-situ enclosure field exposure”

4.2.5.2 Animals and Exposures

L. sylvaticus tadpoles were exposed to two pulse treatments of the glyphosate-based herbicide WeatherMax in an attempt to mimic the agricultural spraying practices of this type of herbicide. Exposures, herbicide applications and water chemistry were performed as described in the thesis by Edge (2012) for the “High” treatment of 2010. The High treatment represents the predicted maximal environmental concentration (PMEC) calculated for a wetland with an average depth of 15 cm and utilizing the maximal recommended spray rate. The split wetland design separated each experimental wetland into halves, allowing one half to be treated with the herbicide and the other half

to serve as a control. The in-situ enclosures were 42 X 42 X 76 cm mesh reptariums (Hagen Inc, Montreal, Quebec, CAN) composed of 1 mm mesh set in a plastic frame. Two enclosures were placed in each wetland half. One enclosure contained 70 tadpoles sampled for gene expression analysis and the second contained 30 tadpoles sampled for developmental endpoints. Tadpoles were extremely small when first placed in the mesh enclosures at Gosner stage 25 and, in order to avoid escapees, tadpoles were placed in a 1 L TupperwareTM container with a mosquito mesh lid held on by an elastic band within the larger mesh enclosures until sufficiently large. Samples from three ponds designated as PMEC 13, 22 and 24 from the High treatment (2.88 mg a.e./ L), were chosen for analysis.

4.2.5.3 Sample Collections

Tadpoles (Gosner stages 36-38) were collected when the median Gosner stage was ~38, after both the first and the second wetland sprays had taken place. Animals were anaesthetised with MS222 (Sigma), cut ventrally and preserved in RNAlater solution at ambient air temperature for <12h. Preserved tadpoles were then stored at 4°C for 3 days, at which point the gonad-mesonephros complex was dissected and stored at -80°C until further analysis. This sampling protocol followed previous experiments performed in our lab and was optimised to ensure high RNA quality (Navarro-Martín et al. 2012). All procedures followed approved animal care protocols of the University of Ottawa and the Canadian Council on Animal Care.

4.2.5.4 RNA Extractions, cDNA Synthesis and RT-PCR

Total RNA was extracted using the RNeasy micro kit (including RNase-free DNase step) following the manufacturer's instructions (Qiagen). After extractions, RNA quality was verified as explained above. The absorbance ratios were all approximately 2 and RIN

numbers above 5 were considered acceptable. Half a microgram of total RNA was reverse transcribed to cDNA following the QuantiTect Reverse Transcription protocol. This cDNA was utilized to amplify *star*, *cyp19*, *cyp17*, *foxl2* and *rpl8* using native Taq DNA polymerase (Invitrogen). Designed primers for *star* are described above (section 4.2.3.2, Table 4.1). Primers for *cyp19*, *foxl2*, *cyp17* and *rpl8* were taken from Navarro-Martin 2012a,b. SYBR Green I chemistry was employed and a dissociation curve indicating the presences of a single amplification product in order to verify primer specificity. Primer concentrations of 450 nM for *cyp19*, *cyp17* and *rpl8* and 300 nM for *star* and *foxl2* were found to be optimal in order to obtain maximal amplification. Relative standard curves were generated using a 4X serial dilution of 50 ng and optimized in order to obtain an efficiency of $100 \pm 10\%$, a slope between -3.1 and -3.6 and an r^2 value greater than 0.985. RT-PCR cycling conditions for the genes analyzed were as follows for *star*: activation step at 95°C for 15 min, succeeded by 40 cycles of 15 sec at 95°C, 22 sec at 62°C and 30 sec at 72°C followed by cycles of 1 min at 95°C, 30 sec at 55°C and a final step of 30 sec at 95°C). For *foxl2*, *cyp17* and *rpl8*: activation step at 95°C for 15 min, succeeded by 40 cycles of 15 sec at 95°C, 22 sec at 60°C and 30 sec at 72°C followed by cycles of 1 min at 95°C, 30 sec at 55°C and a final step of 30 sec at 95°C). For *cyp19*: activation step at 95°C for 10 min, succeeded by 40 cycles of 30 sec at 95°C, 1 min at 62°C and 1 min sec at 72°C followed by cycles of 1 min at 95°C, 30 sec at 55°C and a final step of 30 sec at 95°C). Samples analyzed were run in duplicate along with non-template controls and Non-reverse transcriptase controls in optically clear 96-well plates with a final volume of 25 μ l. Each PCR reaction contained (3.91 ng) of RNA transformed to cDNA, 1X Q-PCR buffer (Qiagen), 2.5 mM MgCl₂ (Qiagen), 200 μ M

dNTPs (Invitrogen), 100 nM passive reference dye (Stratagene), 1.25 U HotStarTaq (Qiagen) and 0.25X SYBR Green I Dye (Molecular Probes). Gene expression data for this set of samples was normalized using a data-driven normalization algorithm (NORMA-Gene). The NORMA-gene approach calculates the mean expression values for each replicate across the target genes, then determines a normalization factor, which will estimate and reduce the among replicate variation across all genes. (Heckmann et al. 2011).

*4.2.5.5 Determining Phenotypic Sex using GMC *cyp19* Gene Expression*

According to the method validated by Navarro-Martin (2012a), gene expression analysis of the GMC utilising *cyp19* as the sexually dimorphic marker was utilized to determine the phenotypic sex of the tadpoles examined. In tadpoles that had surpassed a certain stage of development, in the case of this experiment: Gosner stage 30 or further, females have been shown to display a distinctly higher expression pattern of *cyp19* than do the males. Animals that were collected at later developmental stages were also assessed macroscopically for apparent morphological differences between the sexes in order to support the gene expression findings.

4.2.6 Data Analysis

All statistics were carried out utilizing the car, nlme or base packages in R (R foundation of Statistical Computing, Vienna, Austria). All data was tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's tests. Data was transformed (log or square root) as necessary when these assumptions were not met and subsequently retested for these assumptions. To determine differences in *star* gene expression at the different developmental stages a one-way ANOVA with Gosner stage

as the fixed factor was carried out followed by Tukey's multiple comparisons test. Differences in gene expression for *star*, *cyp19*, *foxl2*, and *cyp17* in the experimental pond PMEC 22 were determined by a two-way ANOVA with sex and treatment as the fixed factors, followed by Tukey's multiple comparisons test. If the interaction term was found to be not-significant, then the two-way ANOVA was retested excluding it. Unfortunately, for wetlands PMEC 13 and PMEC 24, sample size for presumptive males in the treatment side (n=2) did not allow for statistical comparisons. For that reason, only the comparison between sexes in the control side and between females from control and treatment sides were included in this statistical analysis. In these cases, differences in gene expression were examined using a Student's t-test. Differences were considered significant at $p < 0.05$.

Pooling of the gene expression data for the three wetlands (PMEC 13, PMEC 22 and PMEC 24) was done by normalizing the data within each pond separately (dividing each data point by the pond average) and averaging the data from the three normalized ponds. The ponds were treated as replicates and two-way ANOVAs were performed once the assumptions were met (as described above).

4.3. Results

4.3.1 Characterization of Star in L. sylvaticus

A 1021 base-pair partial nucleotide sequence for *star* in *L. sylvaticus* was cloned and translated. The resulting predicted *star* protein was 282 amino acids long. Multiple amino-acid (aa) alignments of *L. sylvaticus* against other vertebrate *star* proteins showed moderate aa identities (67-78%) (Fig 4.1) and high aa identity with other anurans in particular (89-99%) (Fig 4.2). Published vertebrate sequences had the following

percentages of identity and query coverage respectively with *L. sylvaticus star*: *Homo sapiens* (72%, 97%), *Macaca mulatta* (71%, 97%), *Canis lupus familiaris* (70%, 98%), *Rattus norvegicus* (67%, 98%), *Acipenser transmontanus* (78%, 97%), *Anguilla japonica* (77%, 97%), *Gallus gallus* (77%, 98%), *Taeniopygia guttata* (76%, 97%), *Lithobates catesbeianus* (99%, 100%), *Glandirana rugosa* (99%, 100%) and *Xenopus laevis* (88%, 99%). The *L. sylvaticus star* sequence has been submitted to Genbank and the accession number assigned was JX512958.

A phylogenetic tree was constructed based on the amino acid sequences of star from 25 vertebrate and invertebrate species. The neighbor-joining method was used and the unrelated foxl2 amino acid sequence of *X. laevis* was utilized as an outgroup. The star aa sequence for *L. sylvaticus* groups most closely with the other species of frogs on the tree as expected (Fig. 4.3).

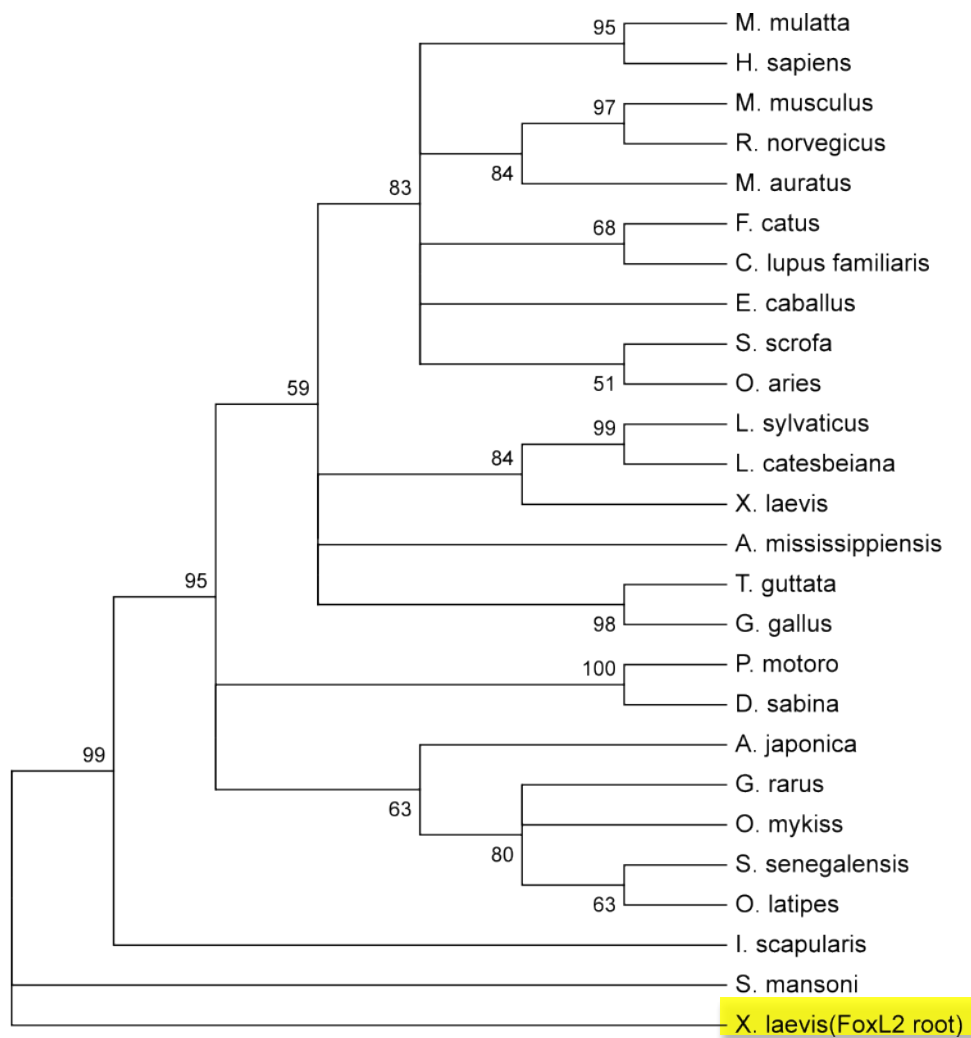


Figure 4.3 Phylogenetic analysis of StAR proteins. the Scientific names of the organisms used as sources for the sequences analyzed are represented on the figure. the StAR protein sequences were aligned using X and the phylogenetic tree was constructed with the program Mega 5. the neighbor-joining method and bootstrap analysis with 1000 replicates was used and support values higher than 50% are shown next to their nodes in the tree. Accession numbers in alphabetical order were as follows: *Alligator mississippiensis* (BAF43707.1), *Anguilla japonica* (BAC66210.1), *Canis lupus familiaris* (NP_001091011.1), *Dasyatis Sabina* (ACX31686.1), *Equus caballus* (AAC04704.1), *Felis catus* (NP_001233125.1), *Gallus gallus* (NP_990017.1), *Gobiocypris rarus* (AEV91663.1), *Homo sapiens* (AAH10550.1), *Ixodes scapularis* (XP_002413856.1), *Macaca mulatta* (NP_001252698.1), *Mesocricetus auratus* (AAB06763.1), *Mus musculus* (AAH82283.1), *Oncorhynchus mykiss* (AAH82283.1), *Oryzias latipes* (NP_001098380.1), *Ovis aries* (NP_001009243.1), *Potamotrygon motoro* (AAS64369.1), *Rana catesbeiana* (ACJ53927.1), *Rattus norvegicus* (BAA19245.1), *Schistosoma mansoni* (XP_002574353.1), *Solea senegalensis* (ADW08748.1), *Sus scrofa* (AAQ76091.1), *Taeniopygia guttata* (AAR91038.1), *Xenopus laevis* (NP_001167502.1). The outgroup was the forkheadbox L2 in *Xenopus laevis* (NP_001128256.1).

4.3.2 Tissue Distribution of star

Fourteen tissues were isolated from Gosner stage 42 *L. sylvaticus* frogs and examined for *star* expression (front and hind legs, tail, skin, stomach, liver, eyes, gall bladder, lung, intestines, brain, interrenals, ovary and testes). Of these, only the interrenals and gonadal tissues showed any detectable *star* gene expression (Fig 4.4). A visual appraisal of this data would indicate that *star* may be more highly expressed in adults than Gosner stage 42 tadpoles. It would also appear that this expression may be higher in the interrenals tissue than in the gonadal tissues. There does not appear to be a difference in expression between the two sexes at either developmental stage. Further analysis through real time RT-PCR would confirm these preliminary results.

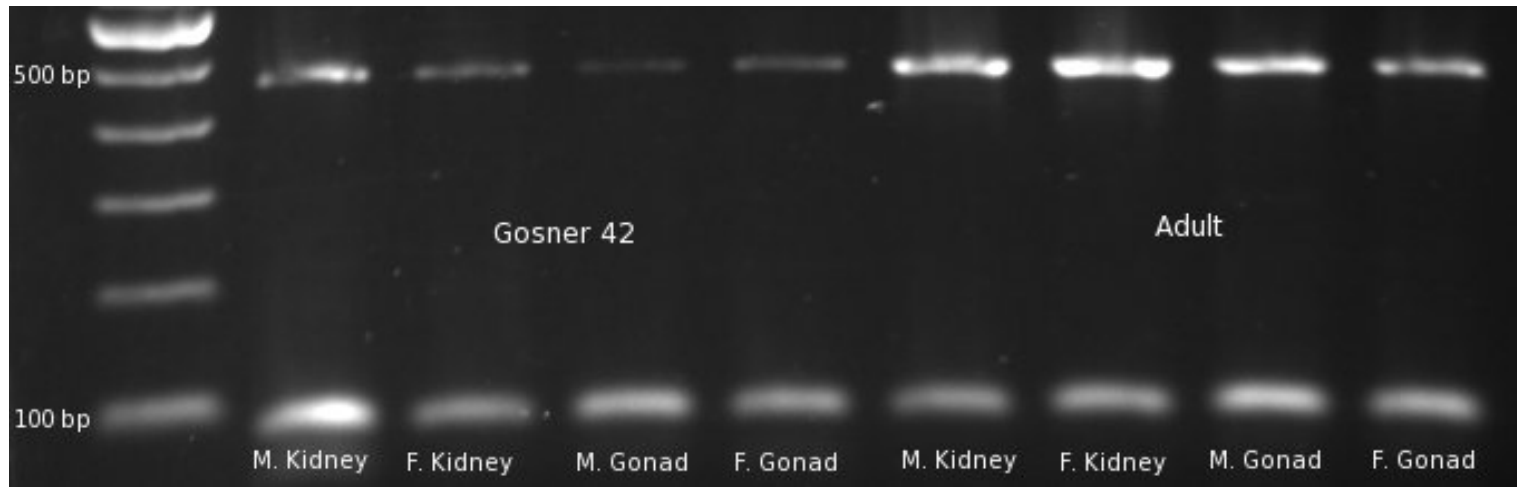


Figure 4.4. Expression of *star* 444 bp in kidney and gonad tissue for males (M) and females (F) for *L. sylvaticus* at Gosner stage 42 and Adults. L8 serves as an indicator of equal loading. PCR product resolved on 1% agarose gel.

4.3.3 Developmental Profile of *star* Expression

Expression of *star* was examined at various developmental stages over four sampling time-points (four different ages) and was found to increase in the GMC of *L. sylvaticus* tadpoles during their development from sexually undifferentiated tadpoles to metamorphosing froglets (Fig 4.5) (ANOVA, Df= 3, F=74.1, $p < 0.001$). *Star* expression, which was detectable at all stages analyzed, is at its lowest at Gosner stage 26 (first sampling time-point), the earliest stage examined. It increased ~2.5 fold from Gosner stages 26 to 28 (second and third sampling time-points) ($p < 0.001$) and increased again by ~ 3.8 fold from Gosner stage 30 to the Gosner stage group 32-41 (third and fourth sampling time-points) ($p < 0.001$) (Fig 4.5). Animals collected during the fourth sampling time point (Gosner stage 32-41) were all of approximately the same age, but of varying Gosner stages. When subdivided by stages, these animals showed a decrease in *star* expression from Gosner stage group 36-38 to Gosner stage group 40-41 ($p = 0.005$) (Fig 4.6). *Star* expression levels in the GMC of *L. sylvaticus* at all developmental stages were found to be indistinguishable between males and females during larval development ($p = 0.1932$) (Fig 4.5 and 4.6).

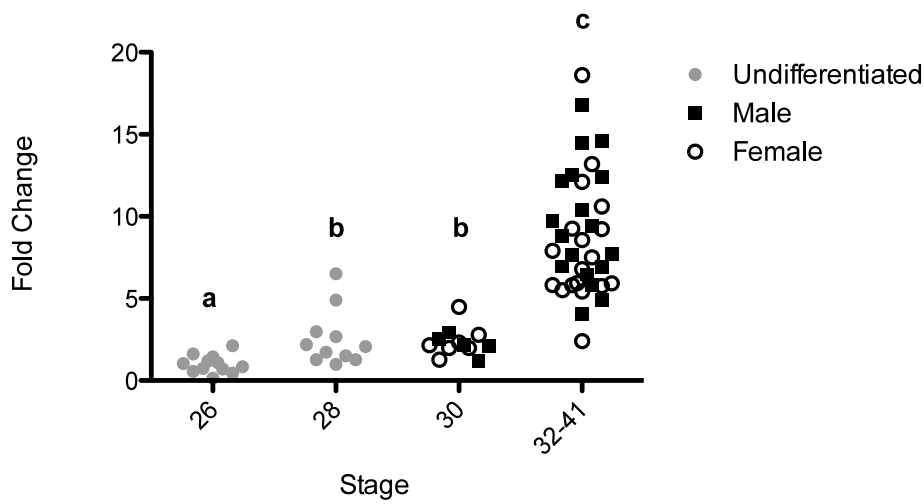


Figure 4.5. *star* gene expression in the gonad/mesonephros complex of *L. sylvaticus* at various Gosner stages at four sampling time-points. Data are presented as fold change in expression relative to the lowest sample value. Expression not sexually dimorphic, N=12 for Gosner stages 26 and 30, N=11 for Gosner stage 28 and N= 36 for Gosner stages 32-41. Letters indicate statistically significant differences ($p < 0.05$).

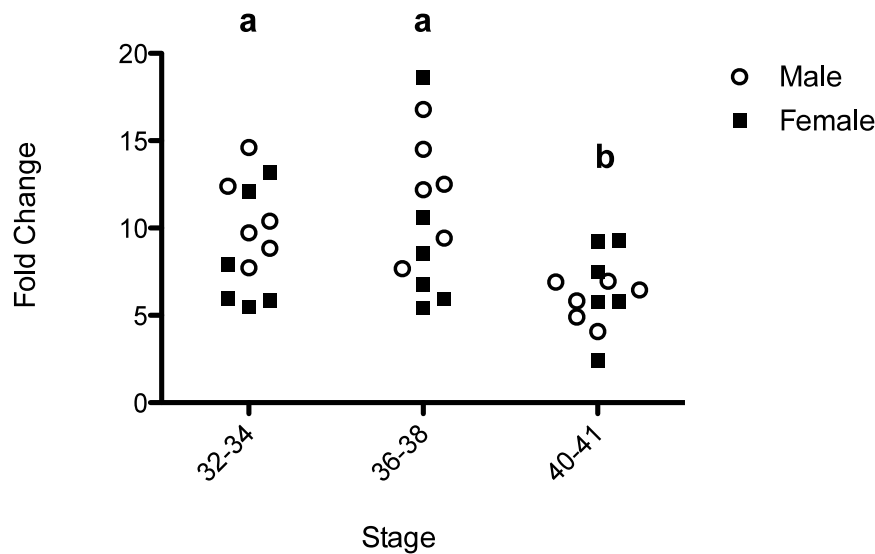


Fig 4.6 *star* expression levels at various Gosner stages in tadpoles of the same age. N= 12. Letters indicate statistically significant differences ($p < 0.05$).

4.3.4 WeatherMax Affects the Genes Involved in Steroidogenesis in L. sylvaticus in In-situ Wetland Exposures.

The newly characterized partial sequence of *L. sylvaticus star* as well as *cyp19*, *cyp17* and *foxl2* were analyzed in Gosner stage 36-38 animals exposed in-situ to Roundup WeatherMax. Tadpoles (Table 4.2) from three experimental wetlands were analyzed and separated according to sex.

Table 4.2 Number of tadpoles analyzed for gene expression from each of the wetlands examined. Categorized according to treatment and sex.

Wetland	Control		WeatherMax	
	Male	Female	Male	Female
Ag 22	7	3	3	6
Ag 13	8	5	2*	8
Ag 24	8	3	2*	7

* Sample size insufficient for statistical analysis of males from this wetland

star

(Fig 4.7, panel a, e, i)

In PMEC 22, *star* expression was found to be sexually dimorphic (ANOVA, $df=1$, $F=35.6$, $p < 0.001$), with control males having a 2.9 fold higher expression than control females and treatment males having 3.2 fold higher than treatment females. PMEC 13 control samples follow this trend with control males having significantly higher expression than control females (t-test, $t = -3.701$, $df = 10.454$, $p = 0.004$), but PMEC 24 did not ($t = -0.9387$, $df = 3.887$, $p = 0.403$).

The expression of *star* was found to increase due to treatment in PMEC 22 for both males and females (ANOVA, $df=1$, $F=18.8$, $p < 0.001$) with treatment males exhibiting 3.0 fold higher expression than control males and treatment females having 2.7 fold higher than control females. PMEC 13 females showed a significant 4.9 fold increase in expression due to treatment (t-test, $t = -4.7323$, $df = 7.424$, $p = 0.002$), but again, this increase was not found in PMEC 24 (t-test, $t = 2.1145$, $df = 2.191$, $p = 0.158$).

As only two treatment exposed male samples were collected for both PMEC 13 and PMEC 24, no statistical analysis can be performed including these samples. In PMEC 13 the *star* expression of these two male samples suggests sexual dimorphism in the WeatherMax treatment and potentially an increased *star* expression due to treatment. These trends concur with the findings from PMEC 22. PMEC 24, on the other hand, shows no trend towards sexual dimorphism or a treatment-based increase in expression.

When examined across the three wetlands, *star* gene expression was sexually dimorphic (2-way ANOVA, $F = 6.1$, $Df = 1$, $p = 0.04$), but no treatment effects were detected ($F = 4.4$, $Df = 1$, $p = 0.07$) with no significant interaction ($F = 1.3$, $Df = 1$, $p = 0.28$).

cyp19 & *foxl2*

(Fig 4.7, panel b, f, j)

In all three wetlands, females had a higher expression of both *cyp19* and *foxl2* than males. PMEC 22 control females exhibited 14 fold higher *cyp19* (ANOVA, $df=1$, $F=114.4$, $p<0.001$) and 9.6 fold higher *foxl2* expression than males (ANOVA, $df=1$, $F=188.8$, $p<0.001$). Control females in PMEC 13 had a 2.0 fold higher *cyp19* expression (Wilcoxon with continuity correction, $W=45$, $p<0.001$) and a 12.8 fold higher *foxl2* expression than control males (t-test, $t=8.7618$, $df=4.05$, $p\text{-value}<0.001$). PMEC 24 control females had 4.2 fold higher *cyp19* (Wilcoxon with continuity correction, $W=24$, $p=0.003$) and 21.1 fold higher *foxl2* (t-test, $t=7.20$, $df=2.01$, $p\text{-value}=0.019$) expression than control males.

PMEC 22 WeatherMax exposed females had 8.9-fold higher *cyp19* and 10-fold higher *foxl2* expression than WeatherMax exposed males. No significant differences were detected in either sex for *cyp19* (ANOVA, $df=1$, $F=0.034$, $p=0.856$) or *foxl2* (ANOVA, $df=1$, $F=0.046$, $p=0.834$) due to treatment in PMEC 24. Only PMEC 13 females showed a significant increase in both *cyp19* (t-test, $t=-3.18$, $df=9.92$, $p\text{-value}=0.010$) and *foxl2* (t-test, $t=-5.70$, $df=9.68$, $p\text{-value}<0.001$) due to treatment.

The low expression for both *cyp19* and *foxl2* in the only two male treatment samples suggests that it is likely that this gene is sexually dimorphic in treatment samples in PMEC 13 and PMEC 24 as was found in PMEC 22. This trend is more apparent in *foxl2* than in *cyp19*. No trend towards a treatment-based effect is apparent in males from either of these two wetlands, which corresponds to the findings from PMEC 22.

For both genes, when examined across the three wetlands, gene expression was sexually dimorphic (2-way ANOVA, *cyp19*: $F=26.3$, $Df=1$, $p<0.001$)(*foxl2*: $F=64$, $Df=1$, $p<0.001$), but no treatment effects were detected (*cyp19*: $F=0.12$, $Df=1$, $p=0.74$)(*foxl2*: $F=0.39$, $Df=1$, $p=0.55$) with no significant interaction (*cyp19*: $F=0.2$, $Df=1$, $p=0.67$)(*foxl2*: $F=0.35$, $Df=1$, $p=0.57$).

cyp17

(Fig 4.7, panel d, h, l)

The expression of *cyp17* was higher in control males than in control females in all three wetlands. Control males showed a significant increase in *cyp17* expression compared to control females. In PMEC 22, male exhibited 11.4-fold higher expression (ANOVA, $df=1$, $F=125.03$, $p<0.001$), in PMEC 13 male expression was 21.4 times higher (t-test, $t=-7.64$, $df=8.17$, $p<0.001$) and 8.9 times higher in PMEC 24 (t-test, $t=-6.46$, $df=4.10$, $p=0.003$) than the females from their respective controls. A significant treatment effect was shown in PMEC 22 with males increasing 1.5-fold and females increasing 1.3-fold due to WeatherMax treatment (ANOVA, $df=1$, $F=7.20$, $p=0.016$). This significant increase in expression due to treatment was also found in PMEC 13 in which females increased by 2.2-fold ($t=-3.12$, $df=7.71$, $p=0.015$). PMEC 24 females did not show any significant difference due to treatment (t-test, $t=2.65$, $df=4.19$, $p=0.055$).

The two treatment male samples collected in both PMEC 13 and PMEC 24 showed a trend of higher expression than the treatment females from the same wetlands.

This trend would suggest that sexual dimorphism carries through to the WeatherMax treated samples in these wetlands.

Examined the three wetlands together, *star* gene expression was sexually dimorphic (2-way ANOVA, $F = 10.4$, $Df = 1$, $p = 0.01$), but no treatment effects were detected ($F = 0.03$, $Df = 1$, $p = 0.87$) with no significant interaction ($F = 0.02$, $Df = 1$, $p = 0.97$).

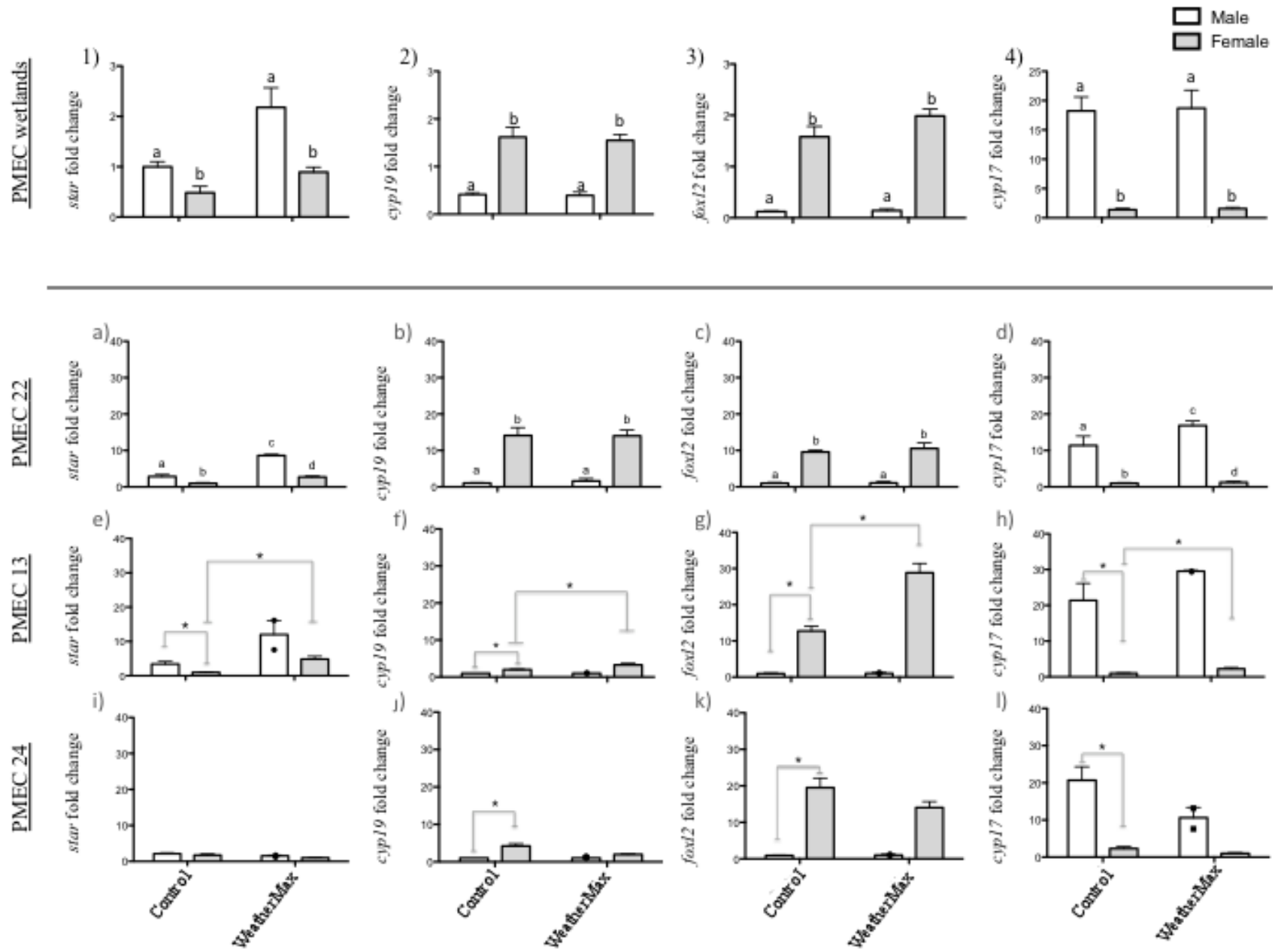


Figure 4.7 Gene expression of *star* (1. a, e, i), *cyp19* (2. b, f, j), *foxl2* (3. c, g, k) and *cyp17* (4. d, h, l) from Gosner stage 36-38 *L. sylvaticus* tadpoles exposed *in-situ* to Roundup WeatherMax. The gene expression fold change is presented for three wetlands, PMEC 22 (medium)(a, b, c, d), PMEC 13 (high)(e, f, g, h) and PMEC 24 (low)(I, j, k, l) as well as the fold change across the three wetlands (1, 2, 3, 4). Statistical differences are indicated by letters above bars (alpha=0.5). Sample sizes presented in Table 4.2.

4.4. Discussion

4.4.1 Predicted Protein and Molecular Conservation

In the present study a partial cDNA sequence for *L. sylvaticus star* gene was cloned and sequenced. The multiple alignments of *star*, including the predicted amino acid sequence for *L. sylvaticus*, support previous research showing it to be well conserved in vertebrates in general (Bauer et al. 2000) and very highly conserved in anurans in particular (Kim et al. 2009; Maruo et al. 2008). Since its discovery as a cholesterol transport protein and as the rate-limiting step in steroidogenesis in vertebrates (Clark et al. 1994), *star* has also been identified in several invertebrate species including a trematode and a tick species (accession numbers in Fig. 4.3). That *star* is present in both vertebrates and invertebrates, indicates that it evolved early and has been relatively well conserved during evolution. This high degree of molecular conservation supports a vital role for *star* in steroidogenesis.

4.4.2 Tissue Distribution of *star*

In this study analyzed a variety of tissues from both male and female Gosner stage 42 *L. sylvaticus* tadpoles were analyzed including front and hind legs, tail, skin, stomach, liver, eyes, gall bladder, lung, intestines, brain, kidney, ovary and testes. Of the fourteen tissues analyzed, only the interrenal and gonadal tissues showed any detectable *star* gene expression (Fig 4.4). It is possible that *star* is expressed in some of the other tissues analyzed, but that the expression levels are much lower than those of the interrenals and gonads and thus were not detected during this experiment. A similar tissue distribution of *star* expression was found in a study on *R. catesbeiana* whereby, utilising RT-PCR, the gene product was only found in the adrenals, testis and follicles (Kim et al. 2009). A

subsequent study on male *R. catesbeiana* employing Q-PCR, detected *star* expression not only in testis and kidney, but also in skin and brain of both juveniles (Gosner 48) and adults (Paden et al. 2010). The majority of studies focusing on tissue distribution have found *star* to be expressed mainly in steroidogenic tissues. In mammals, *star* has been isolated in the adrenal cortex, brain, kidney, testis and ovary (use citations in Kusakabe 2002) as well as liver (Arukwe 2008). In non-mammalian species, *star* has also been detected in the skin (Paden et al. 2010), intestine, pyloric caeca, trunk kidney and spleen (Kusakabe 2002). The tissue distribution of *star* in *L. sylvaticus* evidently merits further investigation potentially using a more sensitive and quantitative method.

4.4.3 *Star* Developmental Profile

The expression of *star* in *L. sylvaticus* appears to be higher in adult frogs relative to Gosner stage 42 metamorphs in both the gonadal and internal tissues of males and females (Fig 4.4). Examining all sampling time-points together, this trend of increase over development holds true (Fig. 4.5). The increase in *star* expression in *L. sylvaticus* during the course of development is in accordance with the literature, as, under normal circumstances, sexual differentiation is expected to commence for most anurans between the Gosner stages 28-31 (Ogielska & Kotusz 2004) and *star* is an essential component in the production of the hormones involved in this process. This increase in expression would be expected to continue into adulthood, as it has previously been demonstrated in *R. catesbeiana* that adult anurans show higher *star* expression levels than do their juvenile counterparts (Paden et al. 2010).

If the fourth sampling time-point of *L. sylvaticus* (range of Gosner stages sampled on same day) is examined alone and subdivided into smaller groups according to Gosner

stage (Fig. 4.6), there is a significant drop in *star* expression from Gosner stage group 36-38 to Gosner stage group 40-41. This is interesting as we expect expression to continue to rise over the course of development in order to reach its highest levels at adulthood. Gosner stage 42 is often considered to be the climax of metamorphosis; a benchmark in amphibian development marked by extreme transformations of nearly all physiological systems (see review (Brown & Cai 2007)). From the literature, it does not appear as though any experiments examining the gonadal expression of *star* during metamorphosis have been undertaken in amphibians. The expression of *star* in the gonadal tissues appears to increase before and/or during sexual differentiation and then continues to rise, to be at its maximum during adulthood, however the expression profile of the period during metamorphosis requires further investigation.

Star expression levels in *L. sylvaticus* were also found to be indistinguishable between males and females during larval development. If *star* were expressed in a sexually dimorphic manner, groupings by expression levels according to presumptive phenotypic sex (estimated using *cyp19* levels) would be expected to commence around the same time, or shortly before, sexual differentiation and the trend could be expected to increase into adulthood. This is not the case for *star* in *L. sylvaticus* and a similar lack of dimorphism was found in *L. rugosa*, wherein *star* was similarly expressed in male and female tadpoles between Gosner stages 25-30 (Maruo et al. 2008). While the majority of the Gosner stages examined by Maruo et al (2008) would be considered pre-sexual differentiation, a sexually dimorphic gene would be expected to become apparent towards the later stages examined.

Understanding the expression patterns of a gene that regulates the production of

steroidogenic hormones is important as these hormones perform a vast array of vital functions in the body (review: Stocco 2001). This is particularly true in the sexual development of amphibians as changes in the ratio of sex steroid hormones can have drastic consequences such as incomplete or abnormal sexual differentiation and, in extreme cases, complete phenotypic sex reversal (Mikamo & Witschi 1963; Witschi 1927).

4.4.4 Genes Examined in an In-situ Field Exposure to WeatherMax

In this study we examined the effects of an environmentally relevant in-situ exposure based on agricultural practices and examined the expression of *star*, the gene responsible for the rate-limiting step of steroidogenesis. The genes *cyp19*, the gene responsible for the conversion of androgens into estrogens, *foxl2*, a transcription factor driving *cyp19* expression and *cyp17*, a gene crucially involved in the steroidogenic pathway were also analyzed. Very few studies have previously examined the effects of glyphosate-based herbicides on the genes involved in steroidogenesis, particularly in amphibians. This is the first study to do this in a field-based exposure, because sampling for gene expression endpoints in an outdoor environment is extremely challenging.

4.4.4.1 Sexually Dimorphic Expression Found in all Genes Examined

The expression of *star* was found to be sexually dimorphic in PMEC 22 and PMEC 13, but not in PMEC 24. This sexual dimorphism is surprising as previous results have indicated that *star* is equally expressed in males and females in the amphibians *L. sylvaticus* (this study), *R. rugosa* (Maruo et al. 2008) and *X. laevis* (Lorenz et al. 2011), the fish *Micropogonias undulates* (Nunez & Evans 2007) and in Swiss CD1 mice

(Bastida et al. 2007). This merits further investigation as samples from two of the three wetlands demonstrate the same pattern of increased expression in males with respect to females. Expression levels from animals in these two wetlands also appear to increase with treatment. This could be due to the higher concentrations of glyphosate detected in these two wetlands. Very little is known about the effects that a glyphosate-based herbicide may have upon *star* in general and no experiment has previously examined this endpoint in amphibians. A previous study examining both *star* expression and *star* levels in the MA-10 Leydig tumour cell line exposed to the glyphosate based herbicide Roundup found a decrease in *star* protein, but not mRNA levels, which led them to conclude that this disruption occurred post-transcriptionally (Walsh, McCormick, et al. 2000). Expression of *star* in amphibians exposed to a glyphosate formulation merits further investigation. Concurrent with previous studies (Navarro-Martín et al. 2012; Maruo et al. 2008; Oshima et al. 2008), *cyp19* and *foxl2* both showed sexual dimorphism with females exhibiting higher expression and *cyp17* showing this dual expression pattern with males having the higher expression in all three wetlands.

4.4.4.2 Gene Expression in Tadpoles Exposed in the Field Exhibit a Potential Dose Dependent Change.

While all three PMEC wetlands had the same target concentration, water chemistry results indicate that, out of the three wetlands examined, PMEC 13 reached the highest glyphosate concentration after each of the two spray applications with nominal concentrations reaching 2.5 times the target after the second spray. This, combined with the slower decrease in glyphosate concentration, could potentially indicate why females from this wetland had increased *star*, *cyp19*, *foxl2* and *cyp17* expression while females in

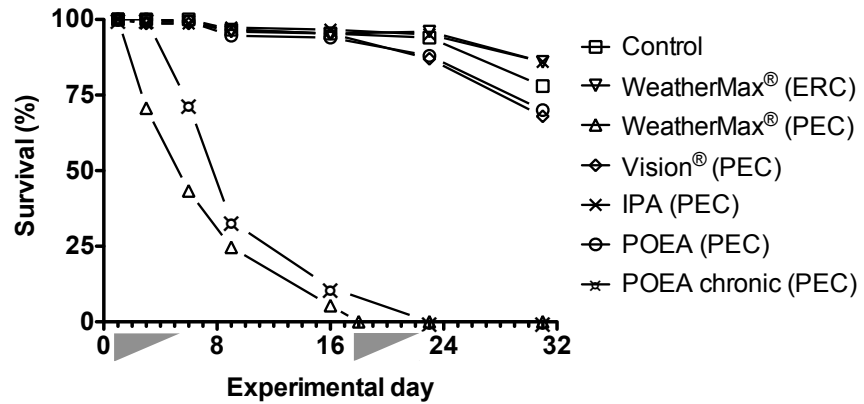
PMEC 24, with lower treatment exposure, showed no such significant increase. The temperature on the treatment side of the wetland in PMEC 13 was also found to be several degrees warmer than the control half of the wetland and higher temperatures have been shown to increase glyphosate toxicity in aquatic organisms (Folmar et al. 1979). PMEC 22 had a lower initial nominal concentration than PMEC 13, however it also had the slowest dissipation out of the three wetlands. According to the peak concentrations as well as the dissipation time for each pulse, the three wetlands were classified as having been exposed to a “low, medium or high” exposure of the herbicide. This is a relative categorization based on the measured concentrations, as all three wetlands had the same PMEC target concentration. Animals in PMEC 24, designated “low”, showed no treatment effect on any of the four genes analyzed. Tadpoles in PMEC 22, designated “medium”, exhibited changes in *star* and *cyp17* gene expression. In the wetland designated as having the “high” exposure, PMEC 13, all four genes analyzed increased significantly in females due to treatment. PMEC 13 was also the only wetland in which a change in sex ratio was detected (see Chapter 3).

Analyzing all three wetlands together, no significant treatment effects are detected in any of the genes. This is unsurprising as treatment based effect appear to be dependent on the herbicide exposure to each wetland. As differing exposure concentrations were measured in the three wetlands, the effects varied across the three wetlands. All four genes (*star*, *cyp19*, *foxl2*, *cyp17*) are sexually dimorphic in *L. sylvaticus* at Gosner stage 36-38.

4.5. Conclusions

In this study, the cDNA encoding *star* was partially cloned in *L. sylvaticus* and was determined to be expressed in both the interrenal and gonadal tissues. Laboratory results indicate that expression levels may be higher in the interrenals relative to the gonads and that gonadal expression likely does not differ between sexes. This expression of *star* in the GMC increased during development and was at its highest level in adulthood. In an in-situ exposure designed to mimic agricultural practices, *L. sylvaticus* tadpoles exposed to two pulses of WeatherMax were found to exhibit increased expression of *star*, *cyp19*, *foxl2* and *cyp17* in what would appear to be an exposure concentration dependant manner. The expression of *star* in two of the wetlands examined showed higher expression in males than in females, an occurrence not previously reported in the literature. As the production and action of *star* is the rate limiting step in steroidogenesis in vertebrates and it has been shown to have the potential to be disrupted by exogenous endocrine disrupting compounds, this endpoint requires further investigation for both WeatherMax induced expression changes as well as a possible pattern of sexual dimorphism in the anuran *L. sylvaticus*.

Supplementary Figures



Supplementary Figure 3.1 From the thesis of Chantal Lanctôt (2012). Percent survival of wood frog tadpoles (*L. sylvaticus*) exposed to two pulse treatments (grey triangles) of Roundup WeatherMax, Vision, isopropylamine salt of glyphosate (IPA), polyethoxylated tallowamine (POEA) as well as chronic exposure to POEA. ERC, environmentally realistic concentration; PEC, predicted environmental concentration (identical to PMEC)

Chapter 5 - Thesis Summary, Conclusions and Future Directions

5.1 Glyphosate Affects the Expression of Genes Involved in Sexual Differentiation and Sex Ratios of *L. sylvaticus* Tadpoles in Experimental Wetlands

The primary objective of this thesis was to determine if the glyphosate-based herbicide WeatherMax can detrimentally affect sexual differentiation in the anuran *L. sylvaticus* in a real world herbicide application scenario. I hypothesized that the contaminant would disrupt normal gene expression patterns of genes involved in the steroidogenic pathway during sexual differentiation. In order to test this hypothesis, a field study mimicking agricultural practices was designed in which wetlands were split into halves and one half of each wetland was oversprayed targeting the PMEC (2.88 mg a.e. /L) of the herbicide WeatherMax. This treatment was carried out twice during a spring season, as multiple sprays are standard in agricultural practices. As glyphosate and its breakdown products dissipate rapidly (Chapter 3), tadpoles *in-situ* in the wetlands were exposed to two diminishing pulses of the herbicide formulation. These pulses were administered according to the somatic development of the tadpoles with Gosner stages 25 and 30 targeted. Gene expression of *cyp19*, *foxl2*, *cyp17* and *star* was measured in the GMC of Gosner stage 36-38 tadpoles. At these developmental stages tadpoles had been exposed to both treatment pulses and their gonads were expected to be fully differentiated (Hogan et al. 2008). When the gene expression of the tadpoles was normalized and averaged across the three wetlands, gene expression in the GMC was not affected by treatment for any of the four genes examined (Chapter 4). While *cyp19*, *foxl2* and *cyp17*

are known to have a sexually dimorphic expression pattern (Navarro-Martín et al. 2012; Maruo et al. 2008; Oshima et al. 2008), *star* has previously been shown to be expressed equally in both males and females (Maruo et al. 2008; Lorenz et al. 2011; Nunez & Evans 2007; Bastida et al. 2007). In this experiment, *star* expression was higher in males than in females in both control and WeatherMax *L. sylvaticus* at Gosner stages 36-38. No indication of sexual dimorphism has previously been demonstrated in the literature and the natural profile elaborated for this gene (see Chapter 4) indicated an equal expression in both sexes. No previous studies have looked at the expression of this gene during the anuran stages 36-38 examined herein. This requires further investigation that should focus on the prometamorphic stages of field-collected tadpoles.

When analyzed singularly, gene expression was shown to vary between the three wetlands examined and these differences appeared to be due to variations in the actual exposure concentration and duration experienced by tadpoles in each wetland. The wetland with the lowest herbicide exposure level PMEC 24, designated “low”, showed no treatment based effects on expression for any of the genes examined. The wetland PMEC 22, designated “medium”, showed a treatment-induced increase in expression of *star* and *cyp17*. Neither of these two wetlands showed any changes to sex ratios after WeatherMax treatment. The tadpoles in PMEC 13, the wetland designated as “high”, had the highest exposure to the herbicide and also experienced a temperature difference between wetland halves with the WeatherMax side consistently measuring approximately 3 degrees higher than the control side (Chapter 3). Tadpoles from the treatment side of this wetland had significant changes in gene expression for all four genes tested (Chapter 4). This was the only wetland to show a treatment-related increase in *cyp19* and *foxl2*, both genes up-

regulated in females, and it was also the only wetland in which a significant female preference in sex ratios was detected (Fig 3.15). That the concentration to which these tadpoles were exposed was higher than the intended target (Table 3.2) for both sprays is important. The highest pulse for this wetland peaked at 7.2 mg a.e. /L (spray 2, PMEC 13), a concentration that is 2.5 times higher than the PMEC target concentration that was found to cause complete mortality in the corresponding laboratory study (Supplementary table 3.1). This change in gene expression along with the corresponding increase in females suggests that exposure to the glyphosate-based herbicide WeatherMax may cause feminization. The environmental relevance of these findings can be argued, as the concentration to which these tadpoles were exposed is higher than what has been predicted as the PMEC. However, the fact that the target was environmentally relevant may be an indication of the variation in the intended spray versus actual sprays concentrations. There is little research to date that shows concentrations measured shortly after a herbicide spray in lentic bodies of water (see Chapter 1) and it is distinctly possible that these concentrations fluctuate from the intended spray concentration. Further research needs to be undertaken to explore this, taking careful note of peak concentrations. That the toxicity of the concentration of herbicide present may also be compounded by other biotic factors such as pH and in this case temperature should also be taken into account. In this study, a pulse exposure to a glyphosate-based herbicide at a concentration which would likely cause complete mortality in a laboratory study, and thus could not easily be investigated in this synthetic system, was found to have no significant impact on survival in a field study. In the field environment however,

WeatherMax treatment was shown to have the potential to modify the expression of genes involved in steroidogenesis and affect sex ratios.

We have demonstrated that changes in expression are induced in genes involved in steroidogenesis during sexual differentiation and that sex ratios may also be altered in an environmentally relevant exposure. Future studies should examine potential changes to both gene expression as well as protein levels and potentially examine other genes involved in this pathway. Further investigation is also required to better understand the potential for modifications of sex ratios under environmentally-relevant conditions.

5.2 Laboratory and Mesocosm Exposures Do Not Reflect the Results of a Field-based Exposure of Anurans to a Glyphosate-based Herbicide.

The second major objective was to determine if synthetic experimental designs could be predictive of a real world field exposure. I hypothesized that if the design of the mesocosm and laboratory studies mimicked the field study, the consequences of an exposure to WeatherMax would be qualitatively similar across exposures, but the magnitude of the effects would be greater in the more synthetic environments. In order to test this, a laboratory and a mesocosm study were designed and run parallel with the field study. Tadpoles in these two exposures were treated with the same two pulse applications of the WeatherMax herbicide at the same two stages of development as the *in-situ* field exposed tadpoles. Tadpoles were collected from all three exposures and examined for endpoints including survival, sex ratios, gonadal histology, growth, and days to metamorphosis. It was not possible to analyze all endpoints in all exposures due to experimental limitations, however comparisons were made where possible.

The findings in the *in-situ* field exposed tadpoles showed no treatment-based effects on survival at the PMEC (2.88 mg a.e./ L) to the herbicide WeatherMax. This same target concentration led to no significant reduction in survival in the mesocosm treatment (Chapter 3). However, in the laboratory, the PMEC of WeatherMax led to complete mortality. As this was the only treatment performed in the experimental wetlands tested, it can be concluded that relying on the laboratory findings alone would lead to an overestimation in the lethality of the herbicide in a real world environment.

The surfactant POEA was tested alone in both the mesocosm and laboratory exposures and several variations in treatment application were examined. In both exposures, the chronic POEA treatment at the PMEC target experienced full early mortality. Tadpoles in the POEA pulse treatment in the laboratory experienced high survival up to experimental completion, whereas in the mesocosms full mortality occurred shortly after the second pulse application. An additional POEA treatment was included in the mesocosm exposure that incorporated a sedimentary substrate. While survival was still significantly lower than the sediment control treatment, full mortality was not observed. This indicates quite clearly that surfactants have the potential to affect amphibian larval survival and that stricter regulation needs to be enforced for these proprietary chemicals. These results also confirm that the inclusion of one element found in the natural environment, in this case sediment, can drastically change the outcome for an endpoint such as survival in an artificial experimental exposure. Knowing this, there is very little reason, other than convenience, to not include such a simple experimental factor in the more easily performed artificial experiments.

Characterizing the effects of the surfactant is important as it is often reported as being the most toxic constituent in glyphosate-based herbicide formulations (Howe et al. 2004; Giesy et al. 2000; Tsui & Chu 2003). Understanding how it affects non-target organisms may aid in convincing or pressuring manufacturers to include “inert” ingredients that would be less harmful. It is important to note however, that for environmentally relevant studies it is the effects of the formulations as a whole that are of the greatest significance, as this is what amphibians are being exposed to.

As the tadpoles in the laboratory WeatherMax PMEC treatment perished, it was not possible to establish sex ratios in this exposure. In the mesocosms, a significant male bias was detected at the PMEC treatment level (Chapter 3). This is contradictory to the findings in the PMEC 13 wetland in which a female bias was detected. That this is due to differences in exposure type cannot be established. It can only be noted that there is disagreement between the two exposures and that, as an important endpoint, the potential changes in sex ratios due to a glyphosate-based herbicide should be further investigated in the most environmentally relevant exposure possible.

As hypothesized, it would appear that the more synthetic the environment, the less likely that the results obtained will be true predictors of real world exposures. For an endpoint such as survival, there did appear to be a gradient as predicted with the most severe results being detected in the laboratory and less severe results found in the field study. However, for sex ratios, results were not a quantitatively different, but contradictory. This confirms the essentiality of field studies. As these are not always feasible, several key experimental conditions have been pinpointed in this thesis, which

should be included and accounted for in synthetic experiments in order to make the results as relevant as possible to the real world (Chapter 2).

5.3 Conclusions and Future Directions

My research focused on the potential for the herbicide WeatherMax to act as an endocrine disrupting compound targeting reproductive and fitness endpoints in the anuran *L. sylvaticus*. As discussed in Chapter 2 it is important to keep in mind that the results of a glyphosate-based herbicide on anurans have previously been found to be highly species-specific as well as being specific to the particular herbicide formulation studied. This is made more complex by the importance of factors such as pH, temperature as well as the potential for sorption of the herbicides to sedimentary substrates. In order to successfully extrapolate these results to other situations, it would be important to establish where *L. sylvaticus* falls with respect to sensitivity in the gamut of potentially exposed amphibians. How WeatherMax compares to other glyphosate-based herbicides for the various endpoints examined would also be critical information.

As is evidenced by the results of my research, as well as that of many other scientists (Howe et al. 2004; Jones et al. 2011; Gahl et al. 2011; Williams & Semlitsch 2009; Lenkowski et al. 2010; Lajmanovich et al. 2003), glyphosate-based herbicides have the potential to affect endpoints crucial to the survival of amphibian populations. An essential next step in this research is to establish whether current agricultural application, timing and concentrations pose a risk of exposure with peak concentrations high enough to induce harmful affects. Many of the lentic aquatic detection studies in existence postulate that glyphosate, when detected, is present only in minute concentrations (see Chapter 1). As it is known that glyphosate binds rapidly to organic matter and dissipates

quickly from the water column, my research was designed to mimic short pulses of exposure. Even at these rapidly diminishing concentrations, survival, sex ratios and gene expression patterns of genes involved in sexual differentiation were affected. Thus it is of great importance to assess the peak exposure concentrations that wild amphibians experience.

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