The Effects of Glyphosate-based Herbicides on the Development of Wood Frogs,

Lithobates sylvaticus

by

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ABSTRACT

Amphibians develop in aquatic environments where they are very susceptible to the effects of pesticides and other environmental contaminants. Glyphosate-based herbicides are widely used and have been shown to affect survival and development of tadpoles under laboratory conditions. The goal my thesis is to determine if agriculturally relevant exposure to Roundup WeatherMax®, a herbicide formulation containing the potassium salt of glyphosate and an undisclosed surfactant, influences the survival and development of wood frogs tadpoles (*Lithobates sylvaticus*) under both laboratory and field conditions. In the field, experimental wetlands were divided in half using an impermeable curtain so that each wetland contained a treatment and control side. Tadpoles were exposed to two pulses of this herbicide at environmentally realistic concentration (ERC, 0.21 mg acid equivalent (a.e.)/L) and predicted environmental concentrations (PEC, 2.89 mg a.e./L), after which survival, growth, development, and expression of genes involved in metamorphosis were measured. Results indicate that exposure to the PEC is extremely toxic to tadpoles under laboratory conditions but not under field conditions. Results from both experimental conditions show sublethal effects on growth and development, and demonstrate that ERC of glyphosate-based herbicides have the potential to alter hormonal responses during metamorphosis. My secondary objectives were to compare the effects of Roundup WeatherMax® to the well-studied Vision® formulation (containing the isopropylamine (IPA) salt of glyphosate and POEA), and to determine which ingredient(s) are responsible for the sublethal effects on development. Survival, growth and gene expression results indicate that Roundup WeatherMax® has greater toxicity than Vision® formulation. Contrary to my prediction, results suggest that, under realistic exposure scenarios, POEA is not the sole ingredient

responsible for the observed developmental effects. However, my results demonstrate that chronic exposure to the POEA surfactant at the PEC (1.43 mg/L) is extremely toxic to wood frog tadpoles in laboratory. As part of the Long-term Experimental Wetlands Area (LEWA) project, this research contributes to overall knowledge of the impacts of glyphosate-based herbicides on aquatic communities.

RESUMÉ

Les amphibiens développent dans des milieux aquatiques où ils sont très vulnérables aux effets néfasts des pesticides et autres contaminants environnementaux. Les herbicides à base de glyphosate sont largement utilisés et ils ont été démontrés à affecter la survie et le développement des têtards sous des conditions de laboratoire. Le but de ma thèse est de déterminer si Roundup WeatherMax®, un herbicide qui contient le sel potassique de glyphosate ainsi qu'un surfactant inconnu, influence la survie et le développement des têtards de la grenouille des bois (*Lithobates sylvaticus*) sous des conditions de laboratoire et sous des conditions naturelles. Sur le terrain, des étangs expérimentaux ont été divisés en deux avec une barrière imperméable pour que chaque étang ait un côté témoin (control) et exposés. Les têtards ont été exposés deux fois pour une courte durée à cet herbicide à soit une concentration environnementale réelle (0.21 mg e.a./L) ou une concentration environnementale prédite (2.89 mg e.a./L). Nous avons mesuré la survie, la croissance, le développement, ainsi que l'expression des gênes impliquées dans le processus de la métamorphose. Nos résultats indiquent que 2.89 mg e.a./L de cet herbicide est extrêmement toxique aux têtards dans le laboratoire, mais pas dans les étangs expérimentaux. Nos résultats démontrent aussi des effets sur la croissance et le développement, et démontrent qu'une concentration réelle (0.21 mg e.a./L) a le potentiel d'alterner la réponse hormonale des têtards durant la métamorphose. Mes objectifs secondaires étaient de comparer les effets du Roundup WeatherMax® à une autre formule bien étudiée, le Vision® (qui contient le sel isopropylamine de glyphosate et POEA), ainsi que de déterminer quel(s) ingrédient(s) est responsable pour les effets développementaux. Nos résultats de survie, croissance et expression génétique indiquent que le Roundup WeatherMax® a des effets plus sévères que

Vision®. Contrairement à ce que prédit, nos résultats suggèrent que POEA n'est pas le seul ingrédient responsable pour les effets observés. Par contre, nos résultats démontrent qu'une exposition chronique au POEA (1.43 mg/L) est extrêmement toxique aux têtards dans le laboratoire. En collaboration avec LEWA (Long-Term Experimental Wetlands Area), cette recherche contribue aux connaissances de l'impact des herbicides à base de glyphosate sur les communautés aquatiques.

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Table of Contents

List of Tables

List of Figures

Abbreviations

28S	ribosomal RNA 28S
a.e.	acid equivalent
ACTH	adrenocorticotrophic hormone
AMPA	aminomethylphosphonic acid
ANCOVA	analyses of covariance
ANOVA	analysis of variance
BPA	bisphenol A
cDNA	complementary DNA
CRF	corticotropin-releasing factor
DDE	1,1-dichlo-2,2-bis(p-chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-bis ethane
DIO	deiodinase enzyme
DIO1	iodothyronine deiodinase type I
DIO2	iodothyronine deiodinase type II
DIO3	iodothyronine deiodinase type III
DNA	deoxyribonucleic acid
DO	dissolved oxygen
EDC	endocrine disrupting compound
EPSPS	5-enolpyruvyl-shikimate-3-phosphate synthase
ERC	environmentally realistic concentration
GAA	Global Amphibian Assessment
Gs	Gosner stage
GRII	glucocorticoid receptor
HAT	histone acetyltransferase
HDAC	histone deacetylase complex
HPI	hypothalamo-pituitary-interrenal
HPT	hypothalamic-pituitary-thyroid
IOP	iopanoic acid
IPA	isopropylamine
IUCN	International Union for Conservation of Nature
LC50	lethal concentration 50%
LEWA	long-term experimental wetlands area
mRNA	messenger ribonucleic acid
MS-222	3-aminobenzoic acid ethyl ester
N-CoR	nuclear co-repressor
NF	Nieuwkoop-Faber developmental stage
NH4	ammonium
NIS	sodium-iodide symporter
NO3	nitrate
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PEC	predicted environmental concentration
PGK1	phosphoglycerate kinase 1

POEA	polyethoxylated tallowamine
RA	retinoic acid
RIN	RNA integrity number
RNA	ribonucleic acid
rpl8	ribosomal protein L8
rT_3	reverse triiodothyronine
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
SD	standard deviation
SEM	standard error of the mean
SVL	snout-vent length
T_2	3,5-diiodo-l-thyronine
T_3	triiodothyronine
T_4	thyroxine
TDC	thyroid disrupting chemicals
TH	thyroid hormone
TKN	total Kjeldahl nitrogen
TL	tail length
TP	total phosphorus
TPO	thyroperoxidase
TR	thyroid hormone receptor
TRα	thyroid hormone receptor alpha
TRβ	thyroid hormone receptor beta
TRE	thyroid response element
TRH	thyrotropin-releasing hormone
TSH	thyroid stimulating hormone
TTR	transthyretin
TWAC	time weighted average concentration
UV	ultraviolet
W	weight

Thesis rationale and hypotheses

Amphibian larvae develop in aquatic environments where contaminants can be easily absorbed through their permeable skin, increasing their susceptibility to the effects of endocrine disrupting compounds. The International Union for Conservation of Nature (IUCN) Red list (2008) indicates that 30% of the 6347 amphibian species assessed are threatened globally and that 42.5% are undergoing population decline. Declines in amphibian populations around the world can be largely attributed to various environmental stressors, such as habitat destruction and degradation, increasing UV-B radiation, climate change, disease and pollution (Houlahan *et al.,* 2000). Pollutants, such as chemical contaminants, are listed as the second most important threat to amphibians after habitat loss on the IUCN Red List of Endangered Species. Therefore, the increased use of agricultural herbicides in the past decades may be a contributing factor to the global decline of amphibian populations, and it is important that we investigate effects and mechanisms of action of these contaminants on population survival and individual growth and development.

Glyphosate-based herbicides are currently the most used herbicide for agricultural purposes around the world, and there is an ongoing controversy regarding the potential impact of glyphosate, the active ingredient, and the various commercial formulations on amphibians (reviewed by Relyea, 2011). Studies exposing different species, life stages or using different methods, formulations and doses of glyphosate-based herbicides have highlighted different results suggesting that the effects on amphibians are strongly depend on these factors, thereby making results difficult to compare between studies. Laboratory and mesocosm studies generally suggest that glyphosate-based herbicides have significant effects on amphibian larvae, such as higher mortality and decreased developmental rates (Edginton

et al., 2003; Relyea, 2005a,b; Howe *et al.,* 2004; Lanctôt, 2010). However, there is a need to determine if the results from laboratory-based studies can be extrapolated to more complex situations in nature.

This thesis investigates the effects of glyphosate-based herbicides on the development of wood frog (*Lithobates sylvaticus*) tadpoles under both laboratory and field conditions. The general introduction (Chapter 1) includes a detailed review of the hormonal control of amphibian metamorphosis and the endocrine disruption of metamorphosis through thyroid hormone receptors and deiodinase enzymes. My first hypothesis is that agriculturally relevant exposure of Roundup WeatherMax® affects the development of wood frog tadpoles under laboratory conditions by disrupting the expression of genes involved in the control of metamorphosis (Chapter 2). To test this hypothesis I exposed tadpoles to two pulses at an environmentally realistic concentration (ERC) and a predicted environmental concentration (PEC) and measured survival, growth and development as well as the expression of thyroid- and stress-related genes. This relatively new formulation used in agriculture was chosen for this study due to its abundant use in North America (Woodburn, 2000; Thompson and Pitt, 2003) and because of the scarcity of knowledge on its potential effects on amphibians. My secondary hypotheses are that Roundup WeatherMax® and Vision® formulations will have similar effects on development and that the surfactant POEA is responsible these effects. To test this hypothesis I exposed tadpoles to two pulses of Vision® as well as the active ingredient isopropylamine (IPA) salt of glyphosate and the surfactant polyethoxylated tallowamine (POEA). Comparing the two formulations will allow us to elucidate if POEA or a similar surfactant is present in Roundup WeatherMax® and to determine which ingredient(s) in these formulations are responsible for the effects on development.

Furthermore, because laboratory studies do not capture the wide range of environmental parameters that can affect toxic responses, I conducted parallel field exposures to Roundup WeatherMax® to determine if developmental effects were observed under natural exposure conditions (Chapter 3). Finally, the general discussion (Chapter 4) compares results from laboratory (Chapter 2) and field (Chapter 3) exposures and highlights the differences between experimental conditions that can influence the effects on glyphosate-based herbicides on amphibians.

Determining the effects of commonly used herbicides on wood frogs aims to improve our understanding of the impact of commonly used contaminants on the environment. Delays in metamorphosis due to environmental contaminants can relate to decreased post-metamorphic growth rates (Semlitsch *et al.,* 1988), which could increase the vulnerability of tadpoles to predation, habitat desiccation and insufficient food supply, and reduce their reproductive success. This thesis helps to identify the mechanisms by which glyphosate-based herbicides are associated with changes in hormone production linked to metamorphosis. In addition, as part of the Long-term Experimental Wetlands Area (LEWA) project, this research contributes to understanding the impacts of glyphosate-based herbicides on aquatic communities and could be used for future herbicide risk assessments. Thus, this research provides a better understanding of endocrine disrupting properties of this herbicide and its impact on natural amphibian populations.

CHAPTER 1

General introduction

1.1. Amphibian development

Amphibian larvae develop in the aquatic environment, undergoing dramatic morphological and physiological transformations as they change from aquatic larvae to terrestrial adults. Amphibian metamorphosis can be divided into three phases: pre-metamorphosis (emergence of the hind limb buds), pro-metamorphosis (hind limb growth and digit differentiation) and metamorphic climax (emergence of forelimbs and tail regression) (Etkin, 1968). Each phase of metamorphosis corresponds with specific morphological characteristics, which are well described and understood for various anuran families (Gosner, 1960; Nieuwkoop and Faber, 1956). These developmental periods also correlate with profound physiological modifications, such as organogenesis of endocrine organs and the subsequent changes in circulating levels of different hormones (reviewed by Shi, 2000). Natural environmental factors (reviewed by Croteau *et al.,* 2008; Denver, 1997b) and environmental contaminants (reviewed by Jugan *et al.,* 2010; Kloas and Lutz*,* 2006; Crofton, 2008; Boas *et al*., 2006) can both stimulate and inhibit metamorphic changes via the endocrine systems and hormonal pathways, especially thyroid and stress hormones (Figure 1.1).

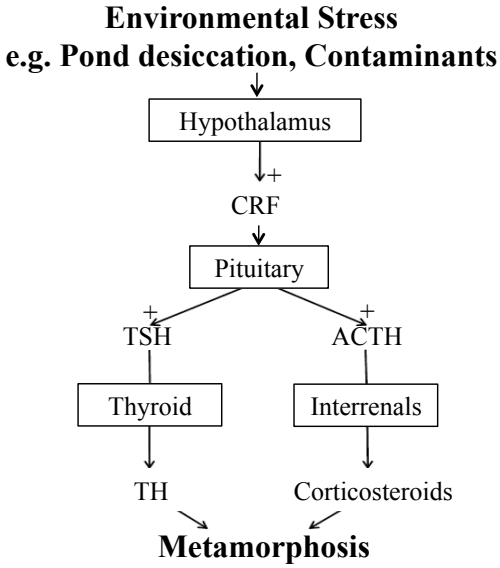


Figure 1.1 Hormonal pathways involved in amphibian metamorphosis (adapted from Denver, 1997b). CRF, corticotropin-releasing factor; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; TH, thyroid hormone.

1.1.1. Thyroid hormone axes

A variety of hormones are involved in the coordination of amphibian metamorphosis, particularly the thyroid hormones (THs), which are regulated by neuroendocrine control via the hypothalamic-pituitary-thyroid (HPT) axis. In amphibian tadpoles, corticotropin-releasing factor (CRF) functions as an equivalent to mammalian thyrotropin-releasing hormone (TRH). During tadpole development, the hypothalamus modulates the release of CRF, which stimulates the pituitary to secrete thyroid-stimulating hormone (TSH). TSH, in turn, acts on the thyroid gland to stimulate the release of TH (Figure 1.1). THs are present in two main forms: thyroxine (T_4) and triiodothyronine (T_3); T_3 is biologically active whereas T_4 is much less active because of its lower affinity for the thyroid hormone receptor. During tadpole development, endogenous TH is absent during pre-metamorphosis and increases gradually during pro-metamorphosis until metamorphic climax, when TH rapidly rises to maximal levels (reviewed by Shi, 2000) (Figure 1.2B).

1.1.1.1. Thyroid hormone receptors

Genes involved in metamorphosis are activated through the binding of TH to thyroid hormone receptors (TR) (reviewed by Shi, 2000; Yen and Chin, 1994). These receptors are encoded by two genes, *trα* and *trβ*, which mediate the genomic actions of TH (Yaoita *et al*., 1990). Thyroid hormone receptors act as transcriptional factors by binding to thyroid response elements (TREs), small DNA elements in the promoter region of TH-regulated genes. Initially, TRs heterodimerize with retinoid X receptor (RXR) and then bind to TREs,

which drives the transcription of several genes involved in metamorphosis (e.g. TRβ). The expression patterns of TRs vary at different stages of metamorphosis, depending on the tissue (Yaoita and Brown, 1990; Kawahara *et al.,* 1991)(Figure 1.2B). TRα is present in early stages (Yaoita and Brown, 1990) and remains in many tissues throughout development (Veldhoen *et al.,* 2002). Contrary to this, an increase in TH during metamorphic climax leads to an increase in TRβ (Yaoita and Brown, 1990). It has been shown that T_3 directly regulates TRβ gene expression (Wong and Shi, 1995). The temporal expression of TRα and TRβ throughout metamorphosis is the basis of the dual function model proposed by Buccholz *et al.* (2006). According to this model, unliganded TRs mediate the repression of transcription during pre-metamorphosis when TH is absent, and mediate the activation of transcription during metamorphosis when TH is present (Figure 1.2A). In the absence of TH early in development, unliganded RXR/TR (predominantly TRα) heterodimer binds to the TREs and recruits a co-repressor complex (histone deacetylase (HDAC) complex). Binding to the complex causes deacetylation of lysine residues on the histones, creating compact chromatin, thereby repressing genes involved in metamorphosis. Therefore, in early development, genes that are anti-growth are inhibited. Later in the developmental period, as T_3 levels increase, T_3 binds to the TR, which causes a conformational change triggering the switch from co-repressor to co-activator binding. RXR/TR complex recruits the co-activator complex (histone acetyltransferase (HAT) complex), resulting in changes in histone acetylation, unwinding of DNA around the histones and promoting transcription of metamorphosis genes. Thus, the activation of TRα by T_3 induces the high expression of TRβ, and TRβ

transcription is driven by an auto-induction mechanism. At the developmental level, unliganded TR mediates repression and is important for keeping metamorphic genes inactive, which allows tadpoles to grow and prevents metamorphosis from occurring too early. Gene activation in the presence of T_3 is essential to initiate the morphological and physiological changes of metamorphosis. Furthermore, TRs can also repress transcription when liganded to T_3 by binding to negative TREs (nTREs); however, this mechanism is not well understood (reviewed by Pascual and Aranda, 2012).

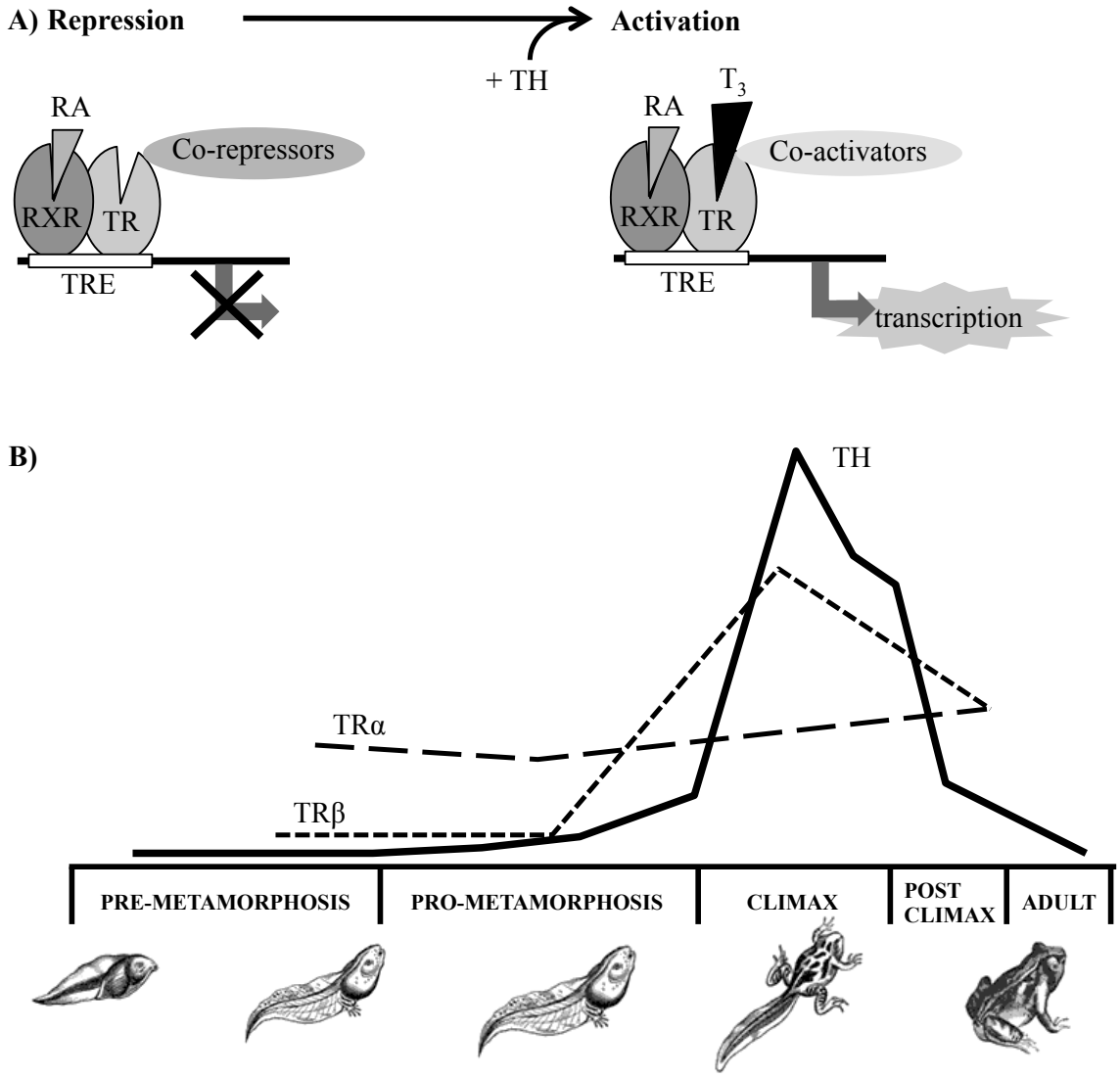


Figure 1.2 A) Dual function model of Buccholz *et al.* (2006). Early in development, in the absence of TH, co-repressors are recruited and TR-mediated gene transcription is repressed. During metamorphic climax, when TH is present, co-activators are recruited and TR-mediated gene transcription is activated (modified from Buchholz *et al.,* 2006). B) Developmental profile of thyroid hormone (solid line), thyroid hormone receptor α (long dash) and thyroid hormone receptor β (short dash) (modified from Hogan, 2006; Yaoita and Brown, 1990). TH, thyroid hormone; T_3, triiodothyronine; TR, thyroid hormone receptor; RA, retinoic acid; RXR, retinoid X receptor; TRE, thyroid response element.

1.1.1.2. Deiodinase enzymes

The THs are interconverted into active (T_3) and less active (T_4, T_2, and reverse T_3 (rT_3)) forms by deiodinase enzymes (DIOs; Figure 1.3). These enzymes act by removing iodide atoms from iodothyronine molecules, a process called deiodination (reviewed by Bianco and Kim, 2006). Three isoforms of deiodinase enzyme (DIO1, DIO2 and DIO3) coordinate the tissue-specific metabolism of TH during metamorphosis (Becker *et al*., 1997). DIO1 and DIO2 regulate T_3 production in specific tissues by converting T_4 into the active form T_3 or rT_3 into T_2. In addition, DIO1 and DIO3 convert T_4 into rT_3, or T_3 into T_2, both metabolites are less active than either T_3 or T_4 due to their lower affinities for the TRs. Thus, deiodinase enzymes are essential in regulating intracellular concentrations of THs and their availability to TRs.

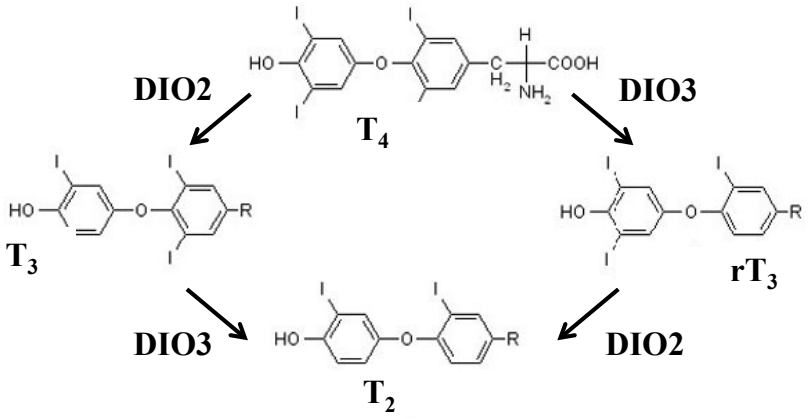


Figure 1.3 Metabolism of thyroid hormones by deiodinase enzymes. T_4, thyroxine; T_3, triiodothyronine; rT_3, reverse T_3; T_2, diiodothyronine; DIO2, deiodinase type II; DIO3, deiodinase type III (modified from Bianco and Kim, 2006).

In amphibians, DIO2 activity and expression are correlated with tissue susceptibility to metamorphic change and responsiveness to T_4 (Cai and Brown, 2004), since this enzyme is responsible for driving TH action and cellular metabolism (Croteau *et al.,* 1996). DIO2 plays an important role in regulating TH-dependent hind limb development and tail resorption. At metamorphic climax, circulating levels of T_4 are superior to those of T_3 (Huang *et al.,* 2001), but whole body T_3 levels are higher at this stage. This demonstrates the intracellular conversion of T_4 to T_3 by DIO2. Several studies have reported significant increases of DIO2 mRNA expression during metamorphic changes of specific tissues, such as hindlimb growth during pro-metamorphosis, and tail reabsorption during metamorphic climax (Becker *et al.*, 1997; Huang *et al.*, 2001). Furthermore, tissues that respond to low TH levels in early development have also been shown to constantly express DIO2 (Cai and Brown, 2004). During metamorphosis DIO3 regulates the availability of TH in specific tissue by inactivating T_4 and T_3 (Kawahara *et al.,* 1999). The high expression of DIO3 is associated with protection against TH-induced changes since DIO3 inactivates T_3. DIO3 is also involved with modulating local metamorphic change. For example, DIO3 has been shown to suppress tail T_3 concentration until metamorphic climax (Cai and Brown, 2004). The DIO1 gene has been identified in some amphibians, however, its role in metamorphosis is unclear (Kuiper *et al.,* 2006; Morvan-Dubois *et al.*, 2006).

To summarize, metamorphosis is regulated in a tissue- and stage-specific manner due to the ability of deiodinases to increase or decrease local TH levels (Cai and Brown, 2004; Huang *et al.,* 2001; Becker *et al.*, 1997). Both DIOs and TRs in amphibian larvae are

essential for the regulation of TH action within specific tissues, allowing successful coordination of metamorphic events. The modulation of intracellular TH action by DIOs and TRs allows for different spatial and temporal availability of T_3, independent of circulating TH levels (Bianco and Kim, 2006). This is critical for synchronizing specific metamorphic events during the asynchronous development of tadpoles. Since these changes correlate with differential expression of thyroid-related genes at different stages of development and in different tissues (Kawahara *et al.,* 1991), environmental chemicals are able to interfere with TH signaling by disrupting both TRs and DIOs, and therefore can also disrupt the entire metamorphic process.

1.1.2. Stress axis

Environmental stressors, such as pond desiccation, temperature change, diet and contaminants, act on the stress axis and are know to affect the timing of metamorphosis (reviewed by Shi, 2000; Denver, 2009). The physiological stress response consists of a series of responses that allow an organism to maintain homeostasis in stressful conditions (Selye, 1973). The physiological stress response in frogs, as well as other vertebrates, is mediated by the adrenal system. The hypothalamo-pituitary-interrenal (HPI) axis regulates the secretion of corticosteroids. A wide rage of stressors can activate the HPI axis, resulting in a hormonal response (Figure 1). Environmental stress stimulates specific neurons in the hypothalamus to secrete corticotrophin-releasing factor (CRF), which stimulates the pituitary gland to secrete adrenocorticotrophic hormone (ACTH). This hormone in turn stimulates the interrenal glands to synthesize and release corticosteroid hormones (Lacroix and Hontela, 2001; Bisson

and Hontela, 2002). A negative feedback loop controls the secretion of corticosteroids. Thus, high circulating ACTH and corticosteroids lead to a decrease in the production of corticosteroids by blocking the release of CRF and ACTH (Colby and Longhurst, 1992; Norris, 1997; Hontela, 1997). Although the main function of CRF is to stimulate the stress response, CRF also influences the thyroid axis during tadpole development (Denver, 1993; 1997a) by stimulating the pituitary to secrete TSH. Thus, both corticosteroids and thyroid hormone can be regulated via hormonal feedback to the hypothalamus and pituitary. Metamorphosis is therefore controlled by both the thyroid (HPT axis) and the stress (HPI axis) pathways through the release of CRF (Denver, 2009). The synergy of these two axes regulates the timing of metamorphosis in response to environmental stress by modulating the sensitivity of tissues to the actions of TH (Bonett *et al.,* 2010).

In addition, studies have shown that CRF is expressed in several frog tissues, including brain, heart, liver, adipose tissue, skin, intestine, stomach, lungs, ovary and tail (Boorse and Denver, 2006; Boorse *et al*., 2006, Navarro-Martín *et al.,* 2012). Exogenous T_3 was shown to up-regulate *crf* mRNA expression (Denver *et al.,* 1997) in pro-metamorphic (NF stage 52-54; Nieuwkoop and Faber, 1956) *Xenopus laevis* tadpole. In specific brain regions, CRF production increases and peaks at metamorphic climax (Miranda and Dezi, 1997; Matsuda *et al*., 2010). Contrary to this, whole brain *crf* mRNA was shown to slightly decrease during development of *Lithobates* sylvaticus tadpoles (Navarro-Martín *et al.,* 2012), however, whole brain analysis may not reflect *crf* expression in specific brain regions. In peripheral tissues, CRF plays an important cytoprotective role by protecting diverse cell

types from apoptosis (reviewed by Denver, 2009). Developmental profiles show a slight decrease in *crf* mRNA expression prior to metamorphic climax in the tail of *L. sylvaticus* tadpoles (Navarro-Martín *et al.,* 2012). In addition, Boorse *et al.* (2006) demonstrated that environmental stressors cause an increase in the expression of CRF in *X. laevis* tail tissue *in vitro*, therefore, slowing the tail reabsorption process. These results suggest that, in the tail, CRF acts as a cytoprotective factor, preventing precocious tail regression.

1.2. Endocrine disrupting compounds disrupt amphibian metamorphosis

Amphibians develop in aquatic environments, where anthropogenic chemicals can be absorbed through their gills at early developmental stages, and through their highly permeable skin. This means that amphibian larvae are highly susceptible to the effects of these contaminants. Pollutants, such as chemical contaminants, have been listed as the second most important threat to amphibians after habitat loss, on the IUCN Red List of Endangered Species. Many chemicals, of both natural and anthropogenic origins have negative effects on the physiology of wildlife through effects on their endocrine system. These chemicals have come to be referred to as endocrine disrupting compounds (EDCs) and present a growing concern for the environment and human health. EDCs influence the endocrine system by affecting mechanisms such as hormone receptor binding, synthesis, metabolism and transport (reviewed by Patrick, 2009). Furthermore, delays in metamorphosis due to environmental contaminants can result in decreased growth rates, which could in turn increase the vulnerability of tadpoles to predation, habitat desiccation

and insufficient food supplies, and reduce their reproductive success. Although, several studies show that chemical compounds can disrupt the endocrine system, the mode of action of many EDCs remains unclear.

1.2.1. Thyroid disrupting chemicals

Thyroid disrupting chemicals (TDCs) are defined as "xenobiotics that alter the structure or function of the thyroid gland, alter regulatory enzymes associated with thyroid hormone homeostasis or change circulation or tissue concentrations of THs" (Crofton, 2008). Concerns about TDCs have increased greatly with the knowledge that amphibian populations are declining, because of the critical role that TH plays in development. TDCs have been shown to act at various levels of the thyroid axis (Figure 1.4), such as the pituitary where they can influence TRH or CRF synthesis or release (Patrick, 2009; Kloas and Lutz, 2006). TDCs can also interfere with the synthesis of THs at the level of the thyroid gland by affecting TSH receptors, by blocking the sodium-iodide symporter (NIS) inhibiting iodine uptake, or by inhibiting TH synthesis via thyroperoxidase (Opitz *et al.*, 2006a; Tietge *et al.,* 2005). Binding of TH to the transport protein transthyretin (TTR) in the bloodstream can be affected by certain TDCs (Yamauchi *et al.,* 2000). TDCs can also act at the cellular level, by disrupting TR expression and/or function (Heimeier *et al.*, 2009; Kashiwagi *et al.*, 2008; 2009; Iwamuro *et al.*, 2003; 2006; Goto *et al.*, 2006; Ishihara *et al.*, 2011; Iwasaki *et al.*, 2002; Mortensen *et al.,* 2006; Helbing *et al.*, 2006; Crump *et al.*, 2002; Veldhoen and Helbing, 2001; Howe *et al.,* 2004; Veldhoen *et al.,* 2006a; 2006b; Opitz *et al.,* 2009; Jagnytsch *et al.*, 2006; Schriks *et al.,* 2006; Sugiyama *et al.,* 2005; Davey *et al.,* 2008; Cheek

et al., 1999b; Zoeller, 2005; Miyazaki *et al.,* 2004; Moriyama *et al.*, 2002; Lanctôt, 2010; Langlois *et al*., 2010) as well as TH metabolism by affecting deiodinases (Becker *et al.,* 1997; Marsh-Armstrong *et al.,* 1999; Huang *et al.*, 2001; Cai and Brown, 2004; Havis *et al.,* 2006; Crump *et al.,* 2002; Lehigh Shirey *et al.,* 2006; ; Lanctôt, 2010; Langlois *et al* 2010).

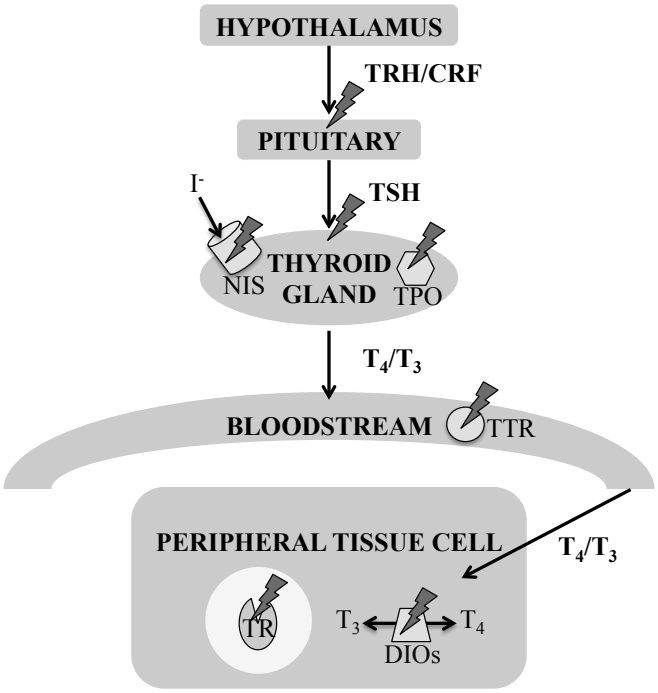


Figure 1.4 Disruption sites of thyroid disrupting chemicals (bolts) (modified from Patrick, 2009). TRH, thyrotropin-releasing hormone; CRF, corticotropin-releasing factor; TSH, thyroid-stimulating hormone; NIS, sodium-iodide symporter; TPO, thyroperoxidase; TTR, transthyretin, T_4, thyroxine; T_3, triiodothyronine; DIOs, deiodinase enzymes; TR, thyroid hormone receptor.

1.2.2. Amphibians: a model for thyroid disruption

Amphibian metamorphosis has been widely used as a model to detect disruption of the thyroid axis (OECD, 2004; OECD, 2009; Kloas and Lutz, 2006; Heimeier and Shi, 2010). Amphibians are excellent models to study endocrine disruption since they undergo complex, endocrine-mediated metamorphosis (reviewed by Shi, 2000). Aquatic development and permeable skin make amphibians particularly vulnerable to chemicals in the environment, and this is especially true because surface waters are the final destination for most anthropogenic EDCs. Since amphibian metamorphosis is influenced by environmental variables through hormonal changes, exposures to EDCs can induce morphological effects by either stimulating or inhibiting the thyroid system. Furthermore, because metamorphosis is dependent on TH, morphological changes due to EDCs provide a simple means of detecting disruption of the TH pathway. Simple and effective ways of measuring developmental effects of EDCs include the quantification of morphological changes, such as differences in gross morphology or hind limb development (Opitz *et al.*, 2005; Goleman *et al*., 2002), thyroid histopathology (Opitz *et al.*, 2006a; Opitz *et al*., 2009; Tietge *et al.,* 2005; Fort *et al.*, 2010a; Fort *et al.*, 2011) and expression of the TH-related genes such as TRβ (Opitz *et al.,* 2006b; Zhang *et al.,* 2006; Mortensen *et al.*, 2006; Langlois *et al*., 2010), TSH (Opitz *et al.,* 2006a; Mortensen *et al.*, 2006) and others (Zhang *et al.,* 2006).

1.2.3. Disruption of metamorphosis through thyroid hormone receptors and deiodinase enzymes

There are a number of anthropogenic chemicals in the environment with the capacity to influence amphibian development through specific effects on the endocrine system (e.g., TRs and DIOs). EDCs can alter hormonal action by binding to specific nuclear receptors, resulting in potential agonistic or antagonistic effects (Figure 1.5). For example, EDCs can interfere with hormone action by binding to their receptors and either interfering with co-factor recruitment (Figure 1.5A, antagonist) or preventing receptor activation (Figure 1.5B, antagonist), either of which results in the blocking of transcription. EDCs can also mimic hormones by binding to their receptor, inducing transcription and translation, and thus producing a normal hormonal response (Figure 1.5C, agonist). Some EDCs bind to multiple types of hormone receptors, however most EDCs have a lower affinity for the target receptor than the natural hormones. Very little is known about the ability of EDCs to bind to TRs in comparison to estrogen receptors. As such, only a few studies have described the effects of TDCs on TR mediated transcription during metamorphosis.

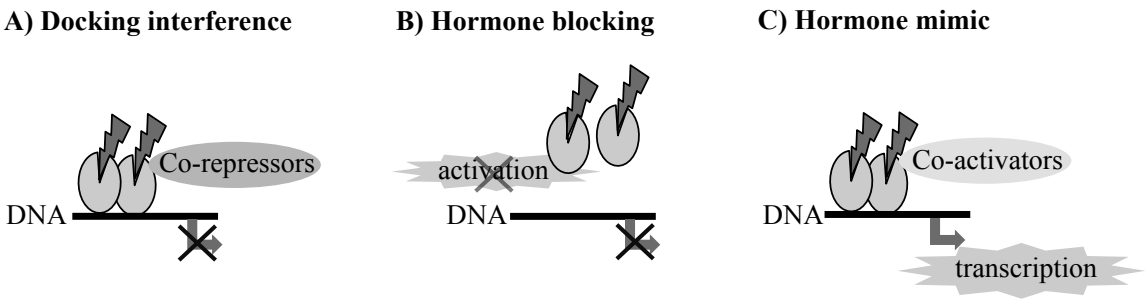


Figure 1.5 Potential antagonistic (A, B) and agonistic (C) effects of endocrine disrupting compounds (bolts) on nuclear hormone receptors (ovals).

Relatively little is known regarding compounds with direct affinity for TRs in anurans. Some EDCs influence transcription of TH related genes by interfering with up-stream signaling pathways without directly binding to the TR. Transcriptional effects of TDCs could occur through disruption of the recruitment/release of TR co-activators, as a result of interference with TR expression and their heterodimerization partner, or with the affinity between TR and TREs. Each of these potential mechanisms requires further investigation, as do their consequences for amphibian metamorphosis. What is clear is that TR disruption by chemical contaminants can affect physiological mechanisms that are pivotal for the normal development of amphibians and mammals (Jugan *et al.*, 2010).

Thyroid hormone receptor beta (*trβ*) mRNA expression has been used in numerous mammalian and amphibian studies as a marker for disruption of TH action. TRβ is a good biomarker for TH-disruption in amphibians (Crump *et al.,* 2002; Howe *et al.,* 2004; Veldhoen *et al.,* 2006b; Opitz *et al.*, 2006b; Zhang *et al.,* 2006) because it is ubiquitously expressed in tadpole tissues as well as directly induced by TH (Denver *et al.*, 1997; Eliceiri and Brown, 1994; Yaoita *et al.*, 1990). In addition, it is well known that TRβ gene expression correlates with increased levels of TH during metamorphosis (Figure 1.2 B). Furthermore, amphibians may be particularly sensitive to EDCs during developmental periods when TH levels are low but receptors are present.

In addition, numerous xenobiotics have the potential to alter thyroid hormone homeostasis by interfering with deiodinase enzymes (DIOs). Recent evidence indicates that these enzymes are essential for controlling tissue sensitivities to TH especially during

development. Despite this, few studies have investigated the ability of environmental contaminants to interfere with TH function through effects on DIOs. A variety of chemicals appear to influence deiodinases activity but it is still not known if these represent direct or indirect effects, secondary to changes in TH levels. It has been suggested that, in some instances, changes in deiodinase activity can act to compensate for the disruption of THs caused by EDCs (Morse *et al.*, 1993). For example, when EDCs leads to decreasing levels of THs, organisms could compensate by increasing DIO2 activity, thereby increasing T_3 levels. On the other hand, when EDCs lead to high levels of THs, organisms could compensate by increasing DIO3 activity, thereby decreasing T_3 levels.

In the last decade, many studies have reported the ability of various anthropogenic chemicals to adversely affect the thyroid system. Some of these chemicals, including bisphenol A, iopanoic acid, polychlorinated biphenyls (PCBs) and their hydroxylated metabolites, as well as various pesticides, have been shown to have adverse effects on amphibian metamorphosis and to act on TRs and DIOs. One of the dominant examples of EDCs disrupting TRs in the literature is bisphenol A (BPA), a chemical used primarily in the manufacturing of polycarbonate plastics and epoxy resins. This chemical is ubiquitously present in the environment and is a concern due to its slow breakdown and persistence. BPA is well known for its estrogenic properties, but has also been shown to affect the thyroid system. The chemical structure of BPA is similar to T_3 and consists of two benzoic rings linked by carbon with a hydroxyl group on each ring, whereas T_3 is also formed of two benzoic rings but is linked by oxygen with a hydroxyl and an alanine group. Studies have

shown that BPA can alter gene transcription via TRs. Receptor binding assays demonstrate that BPA binds TR and antagonizes T_3 action (Kitamura *et al.,* 2002; Moriyama *et al.*, 2002; Goto *et al.,* 2006; Kashiwagi *et al.*, 2008; Fini *et al.*, 2007; Heimeier *et al.,* 2009), inhibiting TR-mediated transcription and suppressing TH-regulated genes. For example, exposure of *X. laevis* to BPA resulted in the suppression of TRβ and TRα expression *in vivo* and *in vitro* (Iwamuro *et al.,* 2003). Exposure of *X. laevis* tail cultures to BPA also suppressed *trβ* and *trα* mRNA expression (Iwamuro *et al.,* 2006). Consequently, by antagonizing T_3, BPA inhibits T_3-induced metamorphosis. Specific effects of BPA exposure include inhibition of visceral organ remodeling (Heimeier *et al.,* 2009) as well as tail resorption (Iwamuro *et al.,* 2006) in *X. laevis*. The proposed mechanism of action is that BPA inhibits T_3-induced metamorphosis by blocking the TH-signaling pathway (Heimeier *et al.,* 2009). Mammalian two-hybrid assays have shown that exposure to BPA reduced the binding of T_3 to the TRs and recruited nuclear co-repressors (N-CoRs), resulting in a reduction of T_3-mediated gene transcription (Moriyama *et al.*, 2002). Based on these findings, we can hypothesize that BPA also blocks or displaces T_3 binding to TRs and recruits co-repressors (N-CoRs) in anurans, thereby blocking T_3-mediated gene transcription and consequently inhibiting T_3-induced metamorphosis (Figure 1.6), since the vertebrate thyroid system is relatively well conserved (Fort *et al.,* 2007). In addition, it has been suggested that the suppression of the main T_3-regulated genes is sufficient to causes the inhibition of metamorphosis since TR-mediated gene regulation is required for amphibian metamorphosis (Heimeier and Shi, 2010).

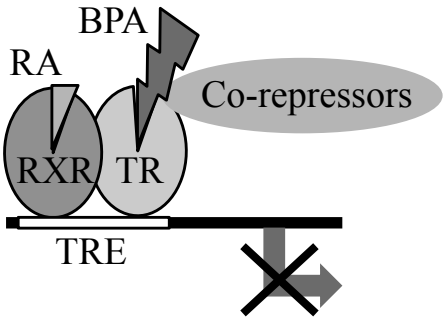


Figure 1.6 Hypothetical mechanism of BPA disruption in anurans based on mammalian cell culture studies. BPA blocks or displaces T_3, binds to nuclear TRs and recruits co-repressors, thus blocking T_3-mediated gene transcription and consequently inhibiting T_3-induced metamorphosis. BPA, bisphenol A; T_3, triiodothyronine; TR, thyroid hormone receptor; RA, retinoic acid; RXR, retinoid X receptor; TRE; thyroid response element.

Iopanoic acid (IOP), a compound used as X-ray contrast media in clinical applications, is another example of an anthropogenic chemical that impacts amphibian metamorphosis by disrupting the thyroid pathways. Contrary to bisphenol A, this chemical specifically targets the deiodinase enzymes, inhibiting both DIO2 and DIO3 (Galton, 1989; Becker *et al.,* 1997) and thus affecting both the activation and inactivation of TH (Figure 1.7). This leads to increased circulating T_4 levels (Cai and Brown, 2004; Huang *et al.*, 2001), which is less active than T_3. Studies have identified IOP as an inhibitor of metamorphic climax in *L. catesbeianus* (Becker *et al.,* 1997) and *X. laevis* (Havis *et al.,* 2006). Tadpoles exposed to IOP undergo early metamorphosis changes because of their high T_4 levels, however since T_3 is needed to complete metamorphosis and IOP interferes with the enzymatic conversion of T_4 to T_3, tadpoles exposed to IOP are unable to successfully reach metamorphic climax (*X. laevis,* Huang *et al.,* 2001).

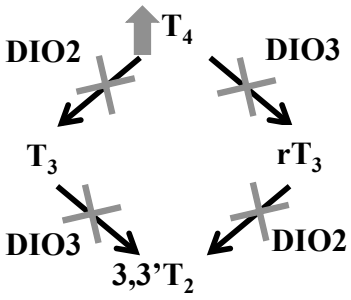


Figure 1.7 Iopanoic acid mechanism of action. Iopanoic acid blocks both DIO2 and DIO3 leading to an increase circulating T_4 levels. T_4, thyroxine; T_3, triiodothyronine; rT_3, reverse T_3; T_2, diiodothyronine; DIO2, deiodinase type II; DIO3, deiodinase type III.

A limited number of studies have investigated the effects of pesticides on TRs and DIOs in amphibians. DDT (1,1,1-trichloro-2,2-bis ethane) is a highly toxic and persistent organochlorine insecticide. Despite being banned in several North American and European countries, DDT is still widely used for mosquito control in countries where malaria is a concern and persists in the environment globally due to bioaccumulation and aerial dispersal. Traces of DDE (1,1-dichlo-2,2-bis(p-chlorophenyl)ethylene), a metabolite of DDT, have been identified in frog tissue (Fellers *et al.,* 2004), which poses concerns for amphibian development. DDE disrupts TR expression through indirect mechanisms since DDE does not bind to TR (Cheek *et al.*, 1999b). Exposure of pro-metamorphic *L. temporaria* tadpoles to DDE caused a decrease in *trβ* mRNA in tail tissue (Mortensen *et al.,* 2006). The authors also described a negative relationship between *trβ* mRNA expression and tail size after DDE exposure. This study suggests that reduced *trβ* levels resulting from DDE exposure can inhibit metamorphic re-absorption of the tail via TH-dependent mechanisms. Furthermore, these authors suggest that DDE can directly affect the thyroid hormone feedback system of the pituitary.

Acetochlor is the active ingredient found in many pre-emergent herbicides. Like DDE, studies have demonstrated that acetochlor does not bind to TR (Cheek *et al.*, 1999b). However, acetochlor has been observed to accelerate T_3-induced metamorphosis in *L. pipiens, L. catesbeianus* and *X. laevis* (Cheek *et al.,* 1999a; Crump *et al.*, 2002; Veldhoen and Helbing, 2001; Helbing *et al.,* 2006), suggesting non-TR related mechanism(s) of action (Cheek *et al.,* 1999a). Contrary to DDE, exposure of pre-metamorphic tadpoles to

environmental concentrations of acetochlor significantly increases T_3-induced *trβ* mRNA in both *X. Laevis* and *L. catesbeianus* tail tissue (Crump *et al.,* 2002; Veldhoen and Helbing, 2001) and *L. catesbeianus* brain tissue (Helbing *et al.*, 2006). The exact mechanisms by which the thyroid axis is affected are not known, but are likely related with increased expression of TH receptors (Veldhoen and Helbing, 2001; Helbing *et al.*, 2006). In addition to its non-specific effects on TR*β*, acetochlor has been shown to decrease T_3-induced *dio3* mRNA expression but has no effects on *dio2* mRNA expression (Crump *et al*., 2002). This suggests that the decrease in T_3 inactivation could lead to an increase in T_3 and result in the acceleration of T_3-induced metamorphosis. However, the relevance of this for influencing metamorphic timing is unknown, since it has yet to be demonstrated that changes in *dio3* mRNA directly translate to changes in enzyme activity (Crump *et al*., 2002). In general, research related to the action and disruption of deiodinase enzymes is quite limited, and so it is unclear if exposure to acetochlor directly alters *dio3* gene expression or if the changes are due to some type of indirect effect. The aforementioned studies suggest that both DDE and acetochlor disrupt amphibian metamorphosis by influencing gene transcription.

Glyphosate is a widely used chemical herbicide in both agriculture and forest management around the world (Woodburn, 2000; Thompson and Pitt, 2003). Currently, glyphosate is the most commonly used pesticide for agricultural purposes. Laboratory and mesocosm experiments have demonstrated that glyphosate-based herbicides have significant effects on amphibians such as higher mortality and decreased size and developmental rates of exposed tadpoles (Howe *et al.*, 2004; Relyea, 2005a, b). Similar to acetochlor, exposure of

pre-metamorphic *L. pipiens* tadpoles to glyphosate-based formulations (Roundup Original® and Roundup Transorb®) caused an increase in *trβ* mRNA expression in tail tissue (Howe *et al.*, 2004), indicating changes to gene expression as a potential mechanism of developmental effects on tadpoles. In addition, exposure of *L. sylvaticus* to VisionMax®, another glyphosate-based herbicide, increases both *dio2* mRNA in tail tissue and *dio3* mRNA in brain at metamorphic climax and delayed metamorphosis was also observed (Lanctôt, 2010). All together these studies suggest that glyphosate-based herbicides have the potential to affect tadpole development by disrupting genes involved in the control of metamorphosis, however more studies are require to confirm this hypothesis.

To summarize, there are a number of anthropogenic chemicals in the environment with the capacity to influence amphibian survival, growth and development. Understanding how these compounds exert their effects is important in order to potentially mitigate or eliminate harmful effects from occurring on natural wildlife populations. This is particularly critical for amphibians, because it has been shown that populations around the world are declining (Stuart *et al.,* 2004; Houlahan *et al.*, 2000), and because their unique larval developmental stages increase their susceptibility to waterborne contaminants, such as EDCs. Considering the pivotal role that TH signaling plays in amphibian development, understanding how different environmental contaminants interfere with this signaling is imperative. A commonly observed response resulting from compounds that interfere with TRs and DIOs is reduced growth and delayed development. Delays in metamorphosis due to environmental contaminants can relate to decreased post-metamorphic growth rates

(Semlitsch *et al.*, 1988), which could increase the vulnerability of tadpoles to predation, habitat desiccation and insufficient food supply, and reduce their reproductive success. The Wilbur-Collins model suggests that a threshold size must be reached for tadpoles to successfully undergo metamorphosis (Wilbur and Collins, 1973), therefore contaminants that prevent this critical size from being reached could lead to major devastating effects on populations, even if they are not acutely toxic. Relatively little information exists regarding TDCs exposure in amphibians at environmentally relevant concentrations, or with realistic durations of exposure. Furthermore, information pertaining to exposures in natural ecosystems is almost non-existent, despite the fact that this would provide the most accurate estimates of effects in natural populations. More research is needed to determine if results from laboratory-based studies of EDCs can be extrapolated to more complex situations in natural environments, where myriad interactions between biotic and abiotic factors exist. This is one of the major aspects of my thesis research.

1.3. The Long-term Experimental Wetlands Area project

The research for this thesis was carried out as part of the Long-term Experimental Wetlands Area (LEWA) project, in collaboration with the University of New Brunswick, Environment Canada, the Canadian Forest Service and the Canadian Forces Base-Gagetown. LEWA studies examine the effects of glyphosate-based herbicides on aquatic communities using laboratory, mesocosm, *in situ* enclosure and whole-ecosystem experiments (recent publications: Edge *et al.,* 2011; Gahl *et al.,* 2011; Edge *et al.,* 2012). This field site (6 km^2),

situated in Gagetown, New Brunswick, Canada, provides a rare opportunity for replicated, whole-ecosystem experiments. Twenty-four split-ponds have been set up to investigate the effects of glyphosate formulations on natural ecosystems, including amphibians, phytoplankton, macrophytes, and invertebrates. These wetlands are characterized by an average of 544 m^2 of spring surface area, 126141 L of spring volume and 46 cm of maximum depth. These wetlands are surrounded by mixed forest with *Scirpus cyperinus*, *Typha latifola*, *Potamogeton sp.* or *Eleocharis palustris* as dominant vegetation.

Pesticide risk assessments are generally based on laboratory dose-response experiments, but these are inadequate for estimating natural effects, as they do not consider the complete range of interactions that may intensify or reduce the effects of pesticides. Although laboratory studies provide a useful approach to understand interactive mechanisms and provide controlled environments for comparisons, these are often not carried out at naturally relevant spatial and temporal scales. For this reason, we have conducted a series of laboratory, mesocosm, *in situ* enclosure and field exposures in collaboration with our colleagues from LEWA to test the effects of Roundup WeatherMax®, a glyphosate-based herbicide, on wood frogs.

The collaborative nature of this project is unique in that it considers effects ranging from individual-level responses (such as gene expressional changes, growth, reproduction and survival) to population and community-level outcomes. We expect this to contribute substantially to policy and usage guidelines, by indicating potential mechanisms-of-action by

which effects of glyphosate on individual organisms could translate to effects on populations and communities in natural environments

1.3.1. Glyphosate-based herbicides

Glyphosate (see Figure 1.8 for molecular structure) is a widely used chemical herbicide for both agriculture and forest management across the world (Woodburn, 2000; Thompson and Pitt, 2003). It is a non-selective herbicide used to eliminate competing vegetation by affecting a specific metabolic pathway found in plants, bacteria and fungi (Woodburn, 2000). More specifically, glyphosate acts by inhibiting an enzyme called EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase) involved in the synthesis of specific amino acids (tyrosine, tryptophan, and phenylalanine) required by plants (Cox, 2004). The usage of glyphosate formulations has increased dramatically over the past decades. Currently, glyphosate is the most commonly used pesticide for agriculture. Glyphosate-based formulations are used in over 130 countries and on over 100 crops (Monsanto, 2009). It has been estimated that 4.6 million kg of glyphosate is used annually in Canada (Brimble *et al*., 2005). Most glyphosate-based herbicide formulations are manufactured by Monsanto under common trade names such as Roundup Original®, Roundup Transorb®, Roundup WeatherMax®, Vision®, VisionMax®, etc., which vary slightly in their formulations. The most common and well studied glyphosate-based formulation is Roundup Original® containing the isopropylamine salt of glyphosate and a polyethoxylated tallowamine (POEA) surfactant (Giesy *et al.,* 2000; Woodburn, 2000). Surfactants in the commercial formulations of these herbicides help spread glyphosate, the active ingredient, on the plant surface

(Thompson *et al.,* 2004), thereby allowing a more efficient penetration of the active ingredient through the leaf cuticle. Some surfactants (i.e., POEA of Roundup Original® and Vision®) have been found to be more toxic than glyphosate itself (Folmar *et al*., 1979; Perkins *et al.,* 2000; Howe *et al*., 2004; Tsui and Chu 2003). However, these chemical formulations are protected as proprietary information of the manufacturer and are not often disclosed. As a result, these chemicals are often not considered when investigating toxicity or establishing usage guidelines for glyphosate-based herbicides.

Glyphosate-based herbicides have been previously thought to be relatively harmless because of the specificity of the active ingredient for a pathway that is not found in animals. However, recent studies suggest that these herbicides might have greater negative impacts on the environment than suspected (reviewed by Relyea, 2011). Laboratory and mesocosm studies have shown that glyphosate-based herbicides have significant effects on amphibians, such as higher mortality, gonadal abnormalities, and decreased size and developmental rate of tadpoles (Howe *et al*., 2004; Relyea, 2004, 2005a, b, c; Relyea *et al.,* 2005; Relyea and Jones, 2009; Jones *et al.,* 2010, 2011; Williams and Semlitsch, 2009). Although some studies describe negative effects on amphibian development and reproduction (Mann and Bidwell, 1999; Howe *et al.,* 2004, Relyea*,* 2009), there exists ongoing controversy surrounding the potential impact of glyphosate and its various formulations on amphibians (Relyea, 2005a, b; Thompson *et al*., 2004; Thompson *et al.,* 2006; Wojteszek *et al.,* 2004; Relyea, 2011). One of the reasons for this controversy is that most of the glyphosate formulations being studied are not meant for aquatic use. Nevertheless, freshwater can be contaminated during or after

the application of these herbicides by drift, leaching and/or runoff. In addition, small ephemeral fish-less ponds, where many amphibian species breed and develop, are often not avoided or not avoidable during aerial applications. Currently, no-spray buffer zones are not required around these small wetlands. Because of this, studies are detecting significant concentrations of glyphosate in wetlands where glyphosate-based herbicides are oversprayed (Thompson *et al.,* 2004). Furthermore, glyphosate is highly soluble in water but can dissipate relatively fast in surface waters depending on the conditions (Giesy *et al.,* 2000). In aquatic environments, glyphosate is converted to the metabolite aminomethylphosphonic acid (AMPA) by microbial degradation and glyphosate binds to suspended particles or sediment. Thus, some researchers argue that laboratory exposure to glyphosate-based herbicides may not reflect realistic exposure conditions. In addition, most studies use acute or chronic exposures at relatively high doses in the lab to assess the toxicity of these chemicals but this type of exposure does not reflect realistic herbicide exposure in natural wetlands. In light of all this, it is evident that more work is required to assess the impacts of glyphosate-based herbicides on amphibians under realistic scenarios of exposure.

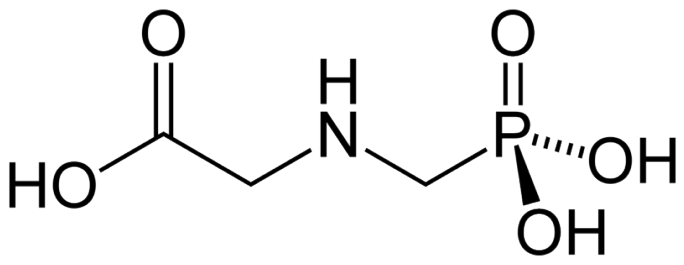


Figure 1.8 The molecular structure of glyphosate (*N*-(phosphonomethyl) glycine).

1.3.2. Lithobates sylvaticus as a model species

Lithobates sylvaticus (wood frog) was chosen as the model species because of its rapid development and because it is the most abundant amphibian species in the Long-term Experimental Wetlands Area (LEWA) where field studies were carried out (Gagetown, New Brunswick). This species is broadly distributed in northern North America, and can be found in or near moist wooded areas (Conant and Collins, 1998). Wood frogs are mostly terrestrial except for the breading period. They are considered as explosive breeders due to their short breeding season. Wood frogs lay their eggs in the course of a few days in early spring (Berven, 1981). Eggs are usually laid in small, temporary or permanent, fish-free wetlands in wooded areas. Egg and tadpole development is relatively rapid, varying from 60 to 120 days (Berven, 1981). Furthermore, although laboratory models species such as *Xenopus laevis* and *Silurana tropicalis* are typically used for molecular based studies, an increasing number of studies are using wood frogs as a model (Howe *et al.,* 2004; Relyea, 2005c; Comstock *et al.,* 2007; Floyd *et al.,* 2008; Relyea and Jones, 2009; Hersikorn *et al.,* 2010; Hersikorn and Smits, 2010; Krishnamurthy and Smith, 2011; Smith *et al.,* 2011; Lanctôt, 2010; Navarro-Martín *et al.,* 2012) and several genes involved in thyroid-, stress- and sex-pathways have recently been cloned and sequenced in this species (Navarro-Martín *et al.,* 2012).

CHAPTER 2

Effects of agriculturally relevant exposures of glyphosate-based herbicides on metamorphosis of wood frogs (*Lithobates sylvaticus*) under laboratory conditions

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

Main contributions of each co-author:

1. Contributed to the experimental design, helped with sampling and tissue collection, performed survival, growth, development and real-time RT-PCR data analysis and manuscript preparation.

2. Contributed to the experimental design, helped with sampling and tissue collection, revised the manuscript and supervised the research.

3. Contributed to the experimental design and helped with sampling and tissue collection.

4. Performed gonadal histology (This is part of Courtney Robertson's thesis and is not included in this chapter).

5. Conducted animal exposures, sampling and tissue collection.

6. Contributed to the exposures and provided financial support.

7. Provided financial support, and contributed to experimental design.

8. Contributed to the experimental design, revised the manuscript, provided financial support and supervised the research.

2.1. Introduction

Amphibian larvae develop in aquatic environments, making them extremely vulnerable to the absorption of contaminants through their permeable skin and gills. This, along with the fact that surface waters are often the final destination of contaminants released into the environment, increases the susceptibility of amphibian larvae to the effects of a wide range of environmental contaminants. Chemical contaminants have been listed as the second most important threat to amphibians after habitat loss (Vié *et al.,* 2009), so increased usage of agricultural herbicides over the past several decades may represent a contributing factor to the global decline of amphibian populations. This explanation is conceivable since metamorphosis is mediated through the regulation of complex hormonal pathways (Denver, 1997b), and many of these pathways are sensitive to environmental conditions and contaminants (reviewed by Mann *et al.,* 2009; Denver, 2009).

Both the thyroid and the stress pathways are important for metamorphosis and both corticosteroids and thyroid hormone can be regulated via hormonal feedback to the hypothalamus and pituitary. Thyroid hormones (TH) are the primary hormone involved in the regulation of metamorphosis, and are under neuroendocrine control via the hypothalamic-pituitary-thyroid (HPT) axis. Environmental factors act on the hypothalamus to release corticotropin-releasing factor (CRF), causing the pituitary to secrete thyroid-stimulating hormone (TSH). TSH, in turn, acts on the thyroid gland to stimulate its development as well as the synthesis and release of TH. The binding of TH to thyroid hormone receptors (TR, encoded by two genes: *trα* and *trβ* [Bassett *et al.*, 2003]) activates

transcription of genes involved in metamorphosis. Additionally, THs are present in two main forms: thyroxine (T_4) and 3,3', 5-triiodothyronine (T_3); T_3 is much more biologically active than T_4 because of its higher affinity for TR (reviewed by Cheng *et al*., 2010). Conversion of THs into active and less active forms is performed by deiodinase enzymes (DIO1, DIO2 and DIO3), which remove iodide atoms from iodothyronine molecules (reviewed by Bianco and Kim, 2006). Thus, these enzymes coordinate tissue-specific metabolism of TH during metamorphosis (Becker *et al*., 1997). Environmental stress, such as pond desiccation, temperature, low food and chemical contamination, act on the hypothalamo-pituitary-interrenal (HPI) axis and can affect the timing of metamorphosis through the release of corticotrophin-releasing factor (CRF) (reviewed by Shi, 2000; Mann *et al.,* 2009; Denver, 2009). Many different cell types in peripheral tissues benefit from CRF and related peptides as a protection from environmental stress (reviewed by Boorse and Denver, 2006). In the tail, CRF has been suggested to act as a cytoprotectant, preventing tail resorption until metamorphic climax (Boorse *et al.,* 2006; Navarro-Martín *et al.,* 2012). Environmental stress, through CRF and adrenocorticotropin hormone (ACTH), stimulates the release of corticosteroids, which act through the corticosteroid receptors (mineralocorticoid [GRI] and glucocorticoid [GRII] receptors) to activate transcription of genes involved in metamorphosis (e.g., *trβ* and *dio2*) (reviewed by Denver, 2009). Furthermore, with the increasing number of anthropogenic contaminants found in the environment, the potential for these chemicals to act as EDCs is a growing concern.

Glyphosate is a widely used chemical herbicide in both agriculture and forest management worldwide (Woodburn, 2000; Thompson and Pitt, 2003), and is currently being used in over 130 countries and on over 100 crops (Monsanto, 2009). The number of glyphosate-based products released onto the market has increased enormously since the removal of patent protection for the glyphosate active ingredient in the early 1990s. These formulations differ slightly based on the specific chemistry of the active herbicidal ingredient, as well as the properties of the 'inert' ingredients including surfactants, dyes and foaming agents. In these formulations, the glyphosate acid is converted to a salt in order to increase its water solubility and, for this reason, glyphosate concentrations are reported as acid equivalent (a.e.) per liter. Glyphosate in the form of the isopropylamine (IPA) salt is present in most formulations including Roundup Original®, however, some newer formulation including Roundup WeatherMax® contain a potassium salt which increases the amount of active ingredient per volume (Monsanto, 2009). Surfactants in these formulations help spread glyphosate across the plant surface, thereby, allowing a more efficient penetration of the active ingredient through the leaf cuticle. The chemical formulations of the inert ingredients are not often disclosed as they are protected as proprietary information of the manufacturer. As a result, these chemicals are often not considered when investigating toxicity or establishing usage guidelines for glyphosate-based herbicides. Roundup Original® (equivalent to Vision®) was one of the first glyphosate-based herbicides on the market and is one of the most studied to date. The surfactant used in this formulation is polyethoxylated tallowamine (POEA; CAS # 61791-26-2). POEA is one of the few identified

surfactants in these herbicide mixtures and is thought to be a principle surfactant used in many glyphosate-based herbicides (Giesy *et al*., 2000).

Although previously thought to be relatively benign to animals, recent studies suggest that glyphosate-based herbicides might have greater negative impacts on the environment than originally believed (reviewed by Relyea, 2011). For example, laboratory and mesocosm studies demonstrate that exposure to glyphosate-based herbicides increase mortality and decrease size and developmental rates of tadpoles (Howe *et al*., 2004; Relyea, 2004, 2005a, b, c; Relyea *et al.,* 2005; Relyea and Jones, 2009; Jones *et al.,* 2010, 2011; Williams and Semlitsch, 2009; Lanctôt*,* 2010). Previous studies have also demonstrated that Roundup® formulations and POEA each cause significantly higher mortality in amphibians than glyphosate alone (Edginton *et al.,* 2004; Perkins *et al.,* 2000; Howe *et al*., 2004), suggesting that, in at least some cases, the surfactant may be a more harmful constituent than the active herbicidal ingredient. However, despite evidence that chemical surfactants such as POEA may play a primary role in negative impacts associated with exposure of amphibians to glyphosate-based herbicides, there have been relatively few studies investigating this. Furthermore, previous studies have also suggested that chronic exposure to glyphosate-based herbicide formulations can disrupt the thyroid pathway involved in metamorphosis (Howe *et al.,* 2004; Lanctôt, 2010). However, since chronic exposures do not reflect realistic herbicide exposures, more work is required to assess the impacts of glyphosate-based herbicides on amphibians under realistic exposure scenarios. In agriculture, herbicides are generally applied several times a year at relatively high concentrations (pulse application), so

experiments reflecting this will provide the most accurate indication of effects on exposed tadpoles.

The purpose of this study is to determine if an agriculturally relevant exposure of wood frog tadpoles to Roundup WeatherMax®, a relatively new and understudied formulation, influences the development of wood frogs tadpoles (*Lithobates sylvaticus*), through effects on the expression of genes involved in the control of metamorphosis. Specific thyroid-related (*trβ, dio2,* and *dio3*) and stress-related (*crf* and *grII*) genes were selected because they have been shown to play important roles in mediating developmental processes (reviewed by Galton, 1992; Shi, 2000; Tata, 2006; Brown and Cai, 2007; Fort *et al.,* 2007; Denver, 2009). Despite their known importance for the orchestration of amphibian metamorphosis, and the knowledge that many environmental contaminants negatively influence metamorphosis, there have been few studies investigating the effects of exposure to glyphosate products on TH receptors, deiodinase enzymes and stress-related genes or how these may influence developmental processes (Howe *et al*., 2004; Lanctôt, 2010). This study will also allow us to compare results to the well-studied Vision® formulation (containing the IPA salt of glyphosate and POEA surfactant), as well as to determine which ingredient(s) in the formulations are responsible for the potential effects on development. Increasing our understanding of mechanisms through which specific compounds exert effects on amphibian development and metamorphosis is critical, as it may lead to insights regarding the mitigation or elimination of adverse health effects of environmental contaminants on natural populations.

2.2. Material and methods

2.2.1. Animals

Fertilized egg masses of *L. sylvaticus* were collected from natural wetlands in the Long-term Experimental Wetlands Area (LEWA) located in Gagetown, New Brunswick, Canada. When tadpoles reached Gosner stage (Gs; Gosner, 1960) 25, tadpoles (n = 50) were placed into nine 50-liter glass aquaria (1 tadpole/L) and divided into seven treatment groups (3 aquaria per treatment): control, Roundup WeatherMax® (0.21 and 2.89 mg a.e./L), Vision® (2.89 mg a.e./L), isopropylamine (IPA) salt of glyphosate (2.89 mg a.e./L), polyethoxylated tollowamine (POEA, 1.43 mg/L) and PEOA chronic (1.43 mg/L). Tadpoles were fed a combination of boiled Kale and Ward's dry tadpole food (4:1 Kale/Wards) daily *ad libitum*, supplemented with algal pellets weekly.

2.2.2. Roundup WeatherMax®, Vision®, IPA and POEA exposure

Roundup WeatherMax® (glyphosate 540 g a.e./L present as the potassium salt, Monsanto, Winnipeg, MB, CAN), a formulation used primarily for agricultural purposes, was chosen for this study due to its abundant use throughout North America and because of the lack of information pertaining to its potential effects on amphibians. Vision® formulation (glyphosate 356 g a.e./L present as the IPA salt, Monsanto, Winnipeg, MB, CAN), a formulation used for forestry, is the Canadian equivalent to the Roundup Original® formulation, and was chosen for this study because it one of the most studied glyphosate-

based herbicide to date and contains one of the few identified surfactants, POEA. The two concentrations (0.21 and 2.89 mg a.e./L) were chosen to match the target concentrations of parallel wetland exposures (Chapter 3). Our choice of the low concentration represents the environmentally realistic concentration (ERC) based on the upper 99th percentile concentration of aqueous glyphosate that has been measured in wetlands located in agricultural areas in Southern Ontario (Byer *et al.,* 2008; Struger *et al.,* 2008). The high concentration reflects the predicted environmental concentration (PEC) based on the maximum allowable application rates on the label (4.3 kg a.e./ha) for typical agricultural and forestry use patterns (Peterson *et al.,* 1994; Perkins *et al.,* 2000). The latter represents a "worst-case scenario" and is based on estimated concentrations measured in 15 cm deep wetlands with no intercepting vegetation, following direct over-spray of the maximum allowed rate. The concentration of IPA salt of glyphosate and POEA are equivalent to the amount found in 2.89 mg a.e./L of Vision® (356 g a.e./L glyphosate as IPA salt + 15% POEA by weight). Treatments were added to experimental aquaria twice (henceforth referred to as pulse exposure) to reflect standard application patterns used for agricultural practices. The first application took place when tadpoles reached Gs 25, which roughly reflects the developmental stage that wood frog tadpoles would reach when the first pulse application would be applied for agriculture, and the second application took place 2 weeks later (~ Gs 30). Each pulse exposure was carried out over four consecutive days with water renewal every day (during the 4 exposure days). Nominal concentrations (Table 2.1) were decrease by 25% each day over the course of 4 days to mimic natural degradation of the herbicide

(based on preliminary test from LEWA, unpublished data). The chronic POEA treatment was exposed to the PEC until tadpoles reach Gs 42 and water was renewed at the same time as the pulse treatments. Water samples (1 L) were taken on each day of the 4-day exposure as well as on day 5 for each pulse to confirm glyphosate concentrations. Samples were maintained at ~ 6 °C and analyzed within 11 days. Aqueous glyphosate concentrations were measured by ASL Laboratory Group (Waterloo, Canada), using a liquid chromatography/mass spectrometry method (MOE E3415, detection limit: 0.005 mg/L). In addition, temperature, dissolved oxygen and pH measurements were recorded regularly (~ 3 - 5 times/week).

2.2.3. Wood frog sampling

Tadpoles were staged and measured (snout-vent length (SVL), tail length and weight) weekly and prior to tissue sampling. Developmental stage was determined using the Gosner staging system (Gosner, 1960). SVL and weight measurements were used to calculate the condition factor of tadpoles [Condition factor (k) = $100(\text{weight}/\text{SVL}^3)$]. Tadpoles were sampled when the median developmental stage within a tank reached Gosner stage (Gs) 31 (pre-metamorphosis), 37 (pro-metamorphosis) and 42 (metamorphic climax). For each developmental stage, tadpoles were sampled from one aquarium tank per treatment (i.e., Gs 31 sampled from tank #1, Gs 37 sampled from tank #2, Gs 42 sampled from tank #3). After sampling from one tank the remaining tadpoles were sacrificed. This sampling design was necessary to maintain equal densities, thus ensuring comparable growth and development amongst treatments since it is well known that density strongly influences growth and

development of tadpoles (Newman 1987, 1989, 1994, 1998; Flecker *et al*., 1999; Griffiths, 1991; Semlitsch and Caldwell, 1982; Gromko *et al.,* 1973; Berven and Chadra, 1988). Anesthesia and euthanasia was performed by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma) in water. Subsequently, tadpoles were dissected and whole brain and tail tissues obtained were preserved in RNAlater (Ambion), a reagent used to stabilize RNA. Samples were first incubated in RNAlater overnight at 4 °C and then stored at -20 °C until further analysis. Sampling followed a protocol approved by the Animal Care Committee and according to the guidelines set by the Canadian Council on Animal Care.

2.2.4. RNA isolation and cDNA synthesis

Total RNA from whole brain and tail tissue (n = 7) was isolated using Qiagen RNeasy Micro Kit, or a combined protocol of TRIzol (Invitrogen) and Qiagen RNeasy Mini Kit, respectively (both including the RNase-free DNase set) from Gs 37 and 42 tadpoles. The extracted RNA was re-suspended in RNase-free water and concentrations of total RNA, the ratio of absorbance at 260 and 280 nm and the ratio of absorbance at 260 and 230 nm were determined using a spectrophotometer (NanoDrop-1000, Technologies, Inc). Samples were then stored at -80 °C. The RNA integrity (RIN) of selected samples was verified to confirm the quality of the samples using the Agilent 2100 Bioanalyzer. All quality parameters were optimal (ratios of absorbance of ~ 2 and RIN numbers ≥ 8). Total cDNA of brain and tail samples was synthesized from 1 and 3 μg of total RNA, respectively, using 200 ng random hexamer primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Each 20

μL reaction was diluted for real-time RT-PCR analysis to a final concentration of 6.25 ng. All procedures followed manufacturer protocols.

2.2.5. Gene expression analysis by real time RT-PCR

Gene cloning and real-time reverse transcriptase polymerase chain reaction (RT-PCR) of *trβ, dio2, dio3, crf,* and *rpl8* in *L. sylvaticus* have recently been sequenced and optimized (Navarro-Martín *et al*., 2012). Gene specific primers for *grII* were designed based on the *L. sylvaticus* sequence (Genebank HQ317703). The identity of the amplicon was confirmed by sequencing. Sequences of the primers, amplicon location, size and melting temperatures are presented in Table 2.2. Real-time RT-PCR were performed using SYBR Green I for *trβ* (tail and brain), *grII* (tail and brain), *crf* (tail) and *rpl8* (brain and tail) and fluorogenic 5' nuclease chemistry for *dio2* (tail and brain) and *dio3* (brain). Each sample was run in duplicate in optically clear 96-well plates in a final volume of 25 μl. Each plate contained all treatments for one developmental stage (2 plates per gene were performed on the same day using the same master mix to reduce variability). For every gene and real-time RT-PCR run, no template and no reverse transcriptase controls were included to verify that there is no genomic contamination and to confirm the specificity of target cDNA amplification. Each real-time RT-PCR reaction contained the following final concentrations: 6.25 ng first-strand cDNA template, 2.5 mM $MgCl_2$ (Qiagen), 1X PCR buffer (Qiagen), 0.2 mM dNTPs (Invitrogen), 0.1 μM ROX reference dye (Stratagene), and 1.25 U HotStarTaq (Qiagen). For the real-time RT-PCR reactions using the SYBR Green I chemistry, 0.25X SYBR green I dye (Invitrogen) and 200-300 nM gene specific primer were added to the

reaction. Alternatively, the reactions measured by fluorogenic 5' nuclease chemistry used PrimeTime PCR assays that contained 400 nM of primers and 200 nM of dual-labelled fluorescent probes (IDT). The thermal cycling parameters for SYBR Green reactions were: an initial cycle at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s, 60°C for 5 s, 72 °C for 1 min 6 s, 81 °C for 22 s. Amplification is followed by denaturation at 95 °C for 1 min. To confirm the specificity of the amplification products a dissociation curve was generated starting at 55 °C for 30 s and increasing 1 °C/30 s. The thermal cycling parameters for the PrimeTime PCR assay were: an initial cycle Taq activation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative mRNA abundance of target and reference genes was interpolated using the relative standard curve method (Applied Biosystems, 1997). Standard curves for each gene and tissue were generated using serial dilutions (four-fold dilutions starting at 50 ng) of cDNA pooled from all samples (control and treatments, n = 70). For each plate the standard curve was duplicated. Assays were performed using an Mx3000P real-time RT-PCR system (Stratagene, La Jolla, CA). The threshold for each gene was calculated using the MxPro-Mx3000P v4.10 software (Stratagene 2007). Efficiency of relative standard curve was 10 ± 10 %, slope between −3.1 and −3.6 and $r^2 > 0.985$ in all cases (Supplementary Table 2.1), in accordance with the acceptable conditions outlined in the manufacturer's instructions (Mx3000P system, Stratagene).

The ribosomal subunit L8 gene was chosen as an internal reference, however, significant changes in *rpl8* mRNA expression were caused by the treatment and

developmental stage (Supplementary Figure 2.1), making it an unsuitable reference gene for this experiment. For this reason, mRNA expression data in the present study was normalized using a data driven normalization algorithm (NORMA-Gene) developed by Heckmann *et al*. (2011). The algorithm, which runs in Excel (Microsoft Inc.), estimates a normalization factor by calculating mean expression values for each replicate of all target genes. This effectively reduces within treatment variation (e.g., that due to experimental bias) but has no effect on relative differences between treatments (Holmstrup *et al.,* 2011). Normalization was performed using 5 target genes for each tissue (brain *trβ, dio2, dio3, grII*, *rpl8;* tail *trβ, dio2, dio3, grII*, *crf, rpl8*), which was shown to be the minimum number of target genes required to achieve optimum results (i.e., reduced variance) using this technique (Heckmann *et al.,* 2011). Duplicate data obtained for each sample, considered technical replicates, were averaged prior to normalization. Fold change in normalized expression relative to control Gs 37 was then calculated for each sample. Biological replicates (n = 7) were averaged to obtain mean fold change in mRNA expression ± standard error of the mean (SEM).

2.2.6. Statistical analysis

All data were analyzed for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's test) and transformed to meet parametric assumptions when necessary. Differences in time to metamorphosis between treatments were determined using one-way analysis of variance (ANOVA). Analysis was performed using SPSS 15.0 (SPSS Inc., Chicago, IL). Differences in growth measurements (SVL, tail length, weight and condition factor) between treatments, from all tadpoles measured throughout the experiment at Gs 31

(tank 1, 2, 3), 36-38 (tank 2, 3) and 42 (tank 3), were also analyzed. This data did not meet parametric assumptions; therefore data were analyzed for significance using randomization tests. One thousand random permutations of the data were performed using Excel (Microsoft Inc.) to obtain the associated *p*-values (Motulsky, 1995). Randomizations were chosen since this test does not require normal distribution and random sampling and allows freedom in experimental design (e.g., unequal sample size) while being more powerful than non-parametric tests such as Kruskal-Wallis (Adams and Anthony, 1996; Gonzalez and Manly, 1998; Hooton, 1991). The effects of Gosner stage, treatments and the stage X treatment interactions on gene expression (mRNA) were analyzed using two-way ANOVAs followed by Bonferroni post-hoc comparisons. Analyses were performed using Prism® v5.0a (GraphPad Software Inc.). Gene expression data are presented as the mean (+ SEM) fold change in normalized mRNA expression relative to the control group of stage 37. For all analyses, differences were considered to be significant when $p < 0.05$.

2.3. Results

2.3.1. Water chemistry

The average aqueous glyphosate concentration measured was 91 and 110 % of the target nominal concentration in Roundup WeatherMax® ERC treatment, 115 % in Roundup WeatherMax® PEC, 66 and 103 % in Vision, and 99 and 88 % in IPA during the 5 days of the first and second pulse, respectively (Table 2.1). In general, aqueous glyphosate

concentrations measured were reasonably close to nominal target concentrations (80-120 % of target concentration). However, in some cases measured concentrations were much lower or higher than nominal concentrations (up to 43.3-159.5% of target concentrations, Table 2.1). Average temperature, pH and dissolved oxygen (± standard deviation) in all tanks were 21.4 °C (± 0.48), 7.1 (± 0.19), and 8.8 mg/L (±0.22), respectively (Supplementary Table 2.2).

2.3.2. Survival, growth and development

Survival was 5.4 % sixteen days after the first exposure to Roundup WeatherMax® at the PEC (> 20 times higher than control, Figure 2.1) and complete mortality was observed before the second pulse (day 18). Similarly, in the chronic POEA treatment, survival was 11.3 % sixteen days after the beginning of the exposure (19 times higher than control), and by day 23, we observed complete mortality. After the first pulse (day 6 - 16), survival in control, Roundup WeatherMax® (ERC), IPA (PEC), POEA pulse (PEC) and Vision® (PEC) was 92 - 100%. By the end of the experiment, survival was 78 % in control. At the end of the experiment survival was 8 % higher than control in the Roundup WeatherMax® (ERC) and IPA treatments, whereas survival was 8 and 10 % lower than control in the POEA pulse and Vision® treatment, respectively. In both Roundup WeatherMax (PEC) and POEA chronic (PEC) treatments the mean developmental stage was 2-4 Gosner stages behind all other treatments 9-16 days after the first exposure (data not shown). Differences in time to metamorphosis between remaining treatments was not statistically significant (Figure 2.2, $F_{(4,139)} = 1.53$, $p = 0.196$).

At Gs 31, tadpoles exposed to one pulse of Roundup WeatherMax® at the ERC weighed 7.9 % more than control tadpoles (Figure 2.3c, $p = 0.001$), however no significant differences in SVL, tail length or condition factor were observed at this stage (Figure 2.3a, b, d, $p = 0.131$ and $p = 0.330$, $p = 0.500$, respectively). At Gs 36-38, tadpoles exposed to two pulses of Roundup WeatherMax® at the ERC had 11.4 % smaller SVL than control tadpoles (Figure 2.3a, $p < 0.001$) but had no differences in tail length (Figure 2.3b, $p = 0.634$) or weight (Figure 2.3c, $p = 0.174$). In addition, tadpole exposed to this treatment had 32.3% higher condition factor than controls at Gs 36-38 (Figure 2.3d, $p < 0.001$). At metamorphic climax (Gs 42), no significant differences in size (SVL, $p = 0.068$; tail length, $p = 0.623$; weight, $p = 0.612$; condition factor, $p = 0.299$) were observed (Figure 2.3). On the other hand, at Gs 31, tadpoles exposed to one pulse of Vision®, IPA and POEA weighted 7.10, 12.9 and 6.4 % more, respectively, than controls (Figure 2.3c, $p < 0.001$, $p = 0.048$, $p = 0.001$, respectively). In addition, tadpole exposed to one pulse of POEA had 10.8 % higher condition factor than controls at Gs 31 (Figure 2.3d, $p = 0.011$). SVL and tail length did not differ significantly between control, Vision®, IPA and POEA at this stage (Figure 2.3a, b, $p > 0.05$). At Gs 36-38, tadpoles exposed to two pulses of Vision®, IPA, and POEA had significantly lower (< 10 %) SVL (Figure 2.3a, $p = 0.002$, $p < 0.001$, $p = 0.001$, respectively), as well as 18.8, 34.7, 22.6 % higher condition factor than control, respectively (Figure 2.3d, $p = 0.041$, $p < 0.001$, $p = 0.011$, respectively). In addition, a 7.4 % increase in tail length at Gs 36-38 was observed in POEA treatment relative to control (Figure 2.3b, $p = 0.004$). At Gs 42, no significant differences in SVL ($p = 0.05$), tail length ($p = 0.623$) and

weight (p = 0.612) were observed between treatments (Figure 2.3a, b, c). However, tadpoles exposed to IPA and POEA had 28.0 and 22.2 % lower condition factor than control (Figure 2.3d, p = 0.003 and p = 0.004, respectively). Because complete mortality of Roundup WeatherMax® (PEC) and chronic POEA treatments occurred before the first sampling date we were unable to analyze growth and gene expression of tadpoles exposed to these treatments.

2.3.3. Gene expression

The effect of stage was significant for all genes except *grII* in brain (two-way ANOVA results are presented in supplementary Table 2.3). Specifically, we observed an increase in *trβ* (brain and tail), *grII* (tail), *dio2* (tail) and *dio3* (brain) mRNA expression between Gs 37 and 42. In contrast, we observed a decrease in *dio2* (brain) and *crf* (tail) mRNA expression between Gs 37 and 42. The effect of treatment was significant for all genes except brain *grII* (Figure 2.4c). Specifically, we observed significant changes in expression levels of thyroid- and stress-related genes after pulse exposures of wood frog tadpoles to Roundup WeatherMax® at ERC (The specific post-hoc comparions are presented in supplementary Table 2.4). At Gs 37, tadpoles exposed to this treatment had 1.8-fold higher tail *trβ* mRNA expression (Figure 2.4b) and 2.0-fold less brain *dio3* mRNA expression (Figure 2.4g), relative to controls. No significant differences were observed in the other genes at this stage. At metamorphic climax (Gs 42), exposed tadpoles had 1.7- and 1.5-fold less *trβ* mRNA expression in tail and brain tissue, respectively, compared to controls (Figure 2.4a, b). Similarly, exposed tadpoles had 1.9-fold less *grII* mRNA expression in tail

tissue at Gs 42 than control tadpoles (Figure 2.4d). No significant differences were observed in the other genes at this stage. Pulse exposures to Vision® formulation (2.89 mg a.e./L) and its individual components, isopropylamine (IPA) salt of glyphosate (2.89 mg a.e./L) and polyethoxylated tollowamine (POEA, 1.43 mg/L) also affected the levels of expression of thyroid-related genes of wood frog tadpoles. Specifically, at Gs 37, tadpoles exposed to the IPA salt of glyphosate had 1.9-fold less brain *trβ* mRNA expression relative to control (Figure 2.4a). Tadpoles exposed to the Vision® formulation had 2.0-fold higher tail *trβ* mRNA expression (Figure 2.4b) whereas tadpoles exposed to POEA had 2.47-fold higher tail *dio2* mRNA expression (Figure 2.4f) relative to control at this stage. At Gs 42, tadpoles exposed to the IPA salt of glyphosate had 1.6-fold less brain *trβ* mRNA expression relative to control (Figure 2.4a). Similarly, tadpoles exposed to the IPA salt of glyphosate and POEA had 2.0- and 1.6-fold less in tail *trβ* mRNA expression, respectively (Figure 2.4b). Tadpoles exposed to the IPA salt of glyphosate also had 1.43-fold less tail *grII* mRNA expression (Figure 2.4d) whereas tadpoles exposed to POEA had 2.2-fold decrease in brain *dio3* mRNA expression at Gs 42 (Figure 2.4g). In general, all treatments reduced the normal increase in *trβ* (brain and tail)*, grII* (tail) and *dio3* (brain) observed in control tadpoles between Gs37 and Gs 42 (Figure 2.4a, b, d, g). However, the interaction between Gosner stage and treatment was only significant for tail *trβ*, tail *grII,* and brain *dio2*.

2.4. Discussion

2.4.1. Effects of Roundup WeatherMax® on wood frog development

2.4.1.1. Survival, growth and development

This study demonstrates that agriculturally relevant 2-pulse exposure to predicted environmental concentrations (PEC) of Roundup WeatherMax® in the laboratory is extremely toxic to wood frog tadpoles, causing complete mortality 16 days after the first exposure. Consistent with this, a recent laboratory study found that chronic exposure to a concentration of Roundup WeatherMax® (0.57 mg a.e./L) that is closer to, but slightly higher than our environmentally realistic concentration (ERC) caused 80% mortality in *Pseudacris triseriata* tadpoles (Williams and Semlitsch, 2009). Chronic exposures to other glyphosate-based herbicide formulations (Roundup Original®, Roundup Transorb®, VisionMax®) ranging from 0.6 – 15 mg a.e. /L have also observed decreased survival in *Lithobates (Rana) pipiens, L. clamitans, L. catesbeianus, L. sylvaticus, Hyla versicolor* and *Bufo americanus* tadpoles (Howe *et al.,* 2004; Relyea, 2004; 2005b; Relyea *et al*., 2005, Lanctôt, 2010). In the present study, survival to metamorphic climax (Gs42) was not affected by pulse exposure to the ERC (0.21 mg a.e. /L), which is consistent with chronic (Relyea, 2005b; Lanctôt, 2010) and acute (Gahl *et al.,* 2011) exposures to glyphosate-based herbicides at equal or slightly lower concentrations. However, it is important to note that we did not measure survival to the end of metamorphosis (Gs 46, when tail regression is complete), thus we cannot conclude that tadpoles were able to complete normal metamorphosis.

External morphometric parameters (e.g., snout-vent length, tail length, weight and condition factor) offer estimates of the overall state of an animal. Energy storage can be correlated with the mass of an individual, and length relates well with growth and development. A concern then, is that contaminants that cause reductions in mass could have negative implications for overwintering survival and adult fitness (Smith, 1987; Semlitsch *et al.,* 1988; Berven, 1990; Scott, 1994), and thus could conceivably have a detrimental effect on population size. Pulse exposure to the ERC resulted in a slight decrease in SVL of pro-metamorphic tadpoles (Gs 36-38). However, differences were not significant at metamorphic climax (Gs 42), perhaps suggesting that tadpoles are able to compensate for decreases in size observed in early stages at this low exposure concentration. It is generally acknowledged that tadpoles must reach a threshold size in order to successfully complete metamorphosis (Wilbur and Collins, 1973), thus the ability of tadpoles to compensate for growth effects is extremely important.

In contrast to the lack of effect of treatments on tadpole size at metamorphic climax, exposure to ERC decreased condition factor at Gs 42 (9.2%, not significant). Condition factor is commonly used as an indicator of health, nutritional status or fitness of an animal (Le Cren, 1951; Gendron *et al*., 2003; Hoey and McCormick, 2004; Brodeur *et al*., 2011). Higher body condition translates into a greater weight for a given length. Animals with reduced body condition are at a disadvantage compared to conspecifics with greater condition with respect to foraging, reproduction, predator avoidance, and dehydration stress; each of these factors is extremely important for overall population fitness (Newman and

Dunham, 1994; Howard and Young, 1998; Newman, 1999; Beck and Congdon, 2000). Thus, as there was no difference in timing to metamorphic climax, reduced condition factor could indicate that tadpoles will have reduced fitness in terrestrial stages (Semlitsch *et al.,* 1988; Berven, 1990).

2.4.1.2. Roundup WeatherMax® alters the expression of genes involved in metamorphosis

Thyroid-dependent gene expression analysis is an indirect method of assessing thyroidal activity throughout development because tissue levels of mRNA in several genes (e.g., *trβ*, *grII, dio2, dio3*) are directly correlated with changes in TH concentrations (Yaoita and Brown, 1990; Krain and Denver, 2004; Opitz *et al.,* 2006b; Duarte-Guterman *et al*., 2012; Navarro-Martín *et al.,* 2012). Furthermore, blood samples are difficult to obtain from small tadpoles making measurement of circulating hormone levels very challenging. For this reason, gene expression analysis by real-time RT-PCR is an advantageous alternative. Our lab has recently published expression profiles of metamorphosis-related genes during natural metamorphosis of *L. sylvaticus* tadpoles and demonstrated that wild tadpoles showed similar trends in expression to laboratory-reared tadpoles from various species (Navarro-Martín *et al.,* 2012). The fold change in *trβ, dio2, dio3* and *crf* mRNA expression between Gs 37 and Gs 42 of control animals from the present study showed generally similar trends as described by Navarro-Martín and collaborators (2012). This indicates that control tadpoles had normal gene expression profiles throughout metamorphosis. Although the aforementioned study did

not measure developmental profiles for *grII* mRNA expression, Krain and Denver (2004) showed similar trends in *grII* mRNA expression in *X. laevis* as observed in the present study.

Our results present evidence of altered expression of TH- and stress-related genes after exposure to environmentally realistic concentration (ERC) of Roundup WeatherMax® (0.21 mg a.e. /L). Specifically, we observed altered *trβ* (brain and tail)*, grII* (tail) and *dio3* (brain) mRNA expression in exposed tadpoles, however the response was dependent on developmental stage. At Gs 37 we observed an up-regulation of *trβ* mRNA in tails, but not in brain of wood frog tadpoles. In addition, we observed a down-regulations in *dio3* mRNA in brain at this stage. Later in development, we observed a reduction in the normal increase in both *trβ* (brain and tail) and *grII* (tail) observed in control tadpoles between Gs37 and Gs 42. Similarly, in a different study, chronic exposure of pre-metamorphic *L. pipiens* tadpoles to two different glyphosate-based formulations (Roundup Original®, 1.8 mg a.e./L; and Roundup Transorb®, 0.6 and 1.8 mg a.e./L) also caused an increase of *trβ* mRNA expression in tail tissues (Howe *et al.*, 2004). Consistent with our results this study also observed a slight, non-significant, decrease in *trβ* mRNA expression at Gs 42 after chronic exposure of *L. pipiens* tadpoles to 0.6 mg a.e./L Roundup Original® but not 1.8 mg a.e./L. It is well established that transcription of *trβ* is directly regulated by THs and that *trβ* mRNA expression correlates with levels of TH during metamorphosis. Similar to *trβ*, tail *grII* and brain *dio3* mRNA are regulated by T_3 in a dose-dependent manner and have been shown to increase throughout metamorphosis (Krain and Denver, 2004; Morvan-Dubois *et al.,* 2006; Navarro-Martín *et al*., 2012). Decreases in both *trβ* and *grII* mRNA expression at

metamorphic climax suggest a decrease in THs in exposed tadpoles. Decreased THs at this stage in tadpole development could lead to slower or inhibited tail regression, since T_3 induces tail apoptosis (Beckingham Smith and Tata 1976; Tata, 1994). However, because animals were sacrificed before tail regression occurred, this could not be confirmed. Importantly, although the alternations in gene expression is difficult to link to clear phenotypic and developmental changes at low exposure concentrations (ERC) and with agriculturally relevant exposure conditions (pulses), gene expression results present evidence of disruption in both the thyroid- and stress-pathways involved in metamorphosis.

2.4.2. Effects of Roundup Vision®, IPA and POEA on wood frog development

2.4.2.1. Survival, growth and development

The second goal of this experiment was to compare the effects of Roundup WeatherMax®, a relatively new formulation containing the potassium salt of glyphosate and an unknown surfactant, to the well studied Vision® formulation (equivalent to Roundup Original®) as well as its individual components, isopropylamine (IPA) salt of glyphosate and polyethoxylated tallowamine (POEA) surfactant. This comparison aimed to elucidate if POEA (or a similar surfactant) is present in the Roundup WeatherMax® formulation and to determine which ingredient(s) in the glyphosate herbicide formulations are responsible for the effects on development. Previous studies suggest that POEA is primarily responsible for acute toxicity of many glyphosate-based formulations (e.g., Roundup Original® and Vision®), and not the active ingredient (Folmar *et al*., 1979; Perkins *et al.,* 2000; Tsui and

Chu, 2003; Howe *et al*., 2004; Carey *et al.,* 2008; Relyea and Jones, 2009). In our study, chronic exposure to POEA at PEC (1.43 mg/L) was extremely toxic to wood frog tadpoles in the laboratory, causing complete mortality after 23 days. Alternately, at the end of the experiment, we observed 30, 32 and 14 % mortality after pulse exposure to POEA, Vision® and IPA at the same concentration, respectively, whereas control had 22 % mortality. Previous studies (Relyea, 2004; Howe *et al.,* 2004) describing chronic exposure of tadpoles to lower concentrations of the same herbicidal formulation (Roundup Original®) observed greater mortality than the current study. This demonstrates the importance of considering exposure duration when analyzing toxicity. Comparison in survival between Roundup WeatherMax® and Vision® indicate that pulse expose to the former is more toxic to wood frog tadpoles at the PEC than the latter. However, it is important to note that measured glyphosate concentrations in Vision® treatment were lower than targeted (Table 2.1) which could explain the differences between the two formulations. Furthermore, in general, exposure to Vision® formulation and its components at the PEC caused similar growth and development as Roundup WeatherMax® at the ERC, a much lower concentration (Figure 2.3), which further suggests that the latter is more toxic to wood frog tadpoles.

2.4.2.2. Vision®, IPA and POEA alters the expression of genes involved in metamorphosis

Our results are indicative of sub-lethal thyroid disruption after pulse exposures to PEC of Vision®, IPA, and POEA. In general, pulse exposure to both IPA and POEA reduced the normal increase in *trβ* (brain and tail), *grII* (tail) as well as *dio3* (brain) observed in

control tadpoles between Gs37 and Gs 42. This response is similar to what we observed after exposure to Roundup WeatherMax® at the ERC. Interestingly, the overall trends in the gene expression results suggest that IPA and POEA at the PEC cause similar effects to Roundup WeatherMax® at the ERC, whereas Vision® (PEC) is more similar to control. These results further demonstrate that in tadpoles Roundup WeatherMax® is more biologically active than Vision®. Furthermore, Howe and collaborators (2004) also measured *trβ* mRNA expression in the tail of *L. pipiens* tadpoles after chronic exposure to 0.6 and 1.8 a.e./L of an equivalent formulation, Roundup Original® and its individual components, IPA and POEA. This study showed no significant differences in tail *trβ* mRNA at Gs 42 after exposure to lower concentrations of these treatments. However, significant increases in *trβ* mRNA expression were observed in Gs 25 tadpoles chronically exposed to 1.8 mg a.e./L Roundup Original® in that study, suggesting that earlier stages are more sensitive. This is largely consistent with the patterns we have observed. Very few studies have investigated the differences in sensitivity to pesticides between different developmental stages of amphibian larvae (Bridges, 2000; Howe *et al.,* 1998; Garcia-Munoz *et al.,* 2009; Berrill *et al.,* 1994; Edginton *et al.,* 2004), but this appears to be an important consideration for influencing responses to glyphosate herbicide formulations. Contrary to what we predicted, pulse exposure to POEA at the PEC did not cause a greater disruption to growth, development, or thyroid-dependent gene expression than the active ingredient (IPA). Thus, results suggest that POEA is not the only ingredient in glyphosate-based formulations responsible for the disruption of genes involved in metamorphosis.

2.5. Conclusion

Our results demonstrate that Roundup WeatherMax® at the ERC alters the expression of genes involved in the control of metamorphosis. Even though the disruption at low exposure concentrations (ERC) and with agriculturally relevant exposure conditions (pulses) was not high enough to translate in clear phenotypic and developmental changes, gene expression results demonstrated that glyphosate-based herbicides have the potential to alter the normal hormonal response during metamorphosis. It is possible that hormonal changes at this critical period in development could prevent the tadpoles from completing metamorphosis, since small changes in TH have been shown to affect metamorphosis. However, survival to metamorphosis (Gs 46) was not measured in the present study. Future studies should investigate if tadpoles exposed to these formulations at early stages of development are able to complete metamorphosis and if fitness of juvenile metamorphs is affected. Since time to metamorphic climax (Gs 42) was not affected, it is also possible that the disruption in mRNA levels in treated animals is indicative of a compensatory mechanism to regulate THs levels in order for tadpoles to undergo normal metamorphosis. Future studies should investigate if the effects of glyphosate-based herbicides are the results of direct disruption of the thyroid pathway or the result of indirect effects. Furthermore, we can conclude that agriculturally relevant exposure (i.e., two pulses) to Roundup WeatherMax® and chronic exposure to POEA at the PEC is extremely toxic to wood frog tadpoles under laboratory conditions. Survival, growth and mRNA expression results also indicate that Roundup WeatherMax® has greater toxicity than Vision® formulation containing the POEA

surfactant. As part of the Long-term Experimental Wetland Area (LEWA) project, this research contributes to overall knowledge of the impacts of glyphosate-based herbicides on aquatic communities. Further research is ultimately required to understand the endocrine disrupting properties of this herbicide, the individual constituents of the formulation, as well as their impacts on natural amphibian populations.

Table 2.1 Nominal and measured aqueous concentrations of glyphosate (mg a.e./L) and polyethoxylated tallowamine (POEA, mg/L) over the course of 5 days for each pulse. IPA, isopropylamine salt; TPT, time-post treatment; ERC, environmentally realistic concentration; PEC, predicted environmental concentration (PEC), [N], nominal concentration; [M], measured concentration; % of target=100([M]/[N]).

Pulse	TPT (h)	Control	Roundup WeatherMax®						Vision®			IPA			POEA*
			ERC		% of	PEC		% of	PEC		% of	PEC		% of	PEC
			[N]	[M]	target	[N]	[M]	target	[N]	[M]	target	[N]	[M]	target	[N]
1	0	< 0.005	0.21	0.12	58.1	2.89	2.72	94.1	2.89	1.25	43.3	2.89	2.47	85.5	1.43
1	24	< 0.005	0.16	0.17	105.4	2.17	2.80	129.0	2.17	1.15	53.0	2.17	2.02	93.1	1.07
1	48	< 0.005	0.11	0.11	103.8	1.45	1.92	132.4	1.45	1.15	79.3	1.45	1.44	99.3	0.71
1	72	< 0.005	0.05	0.05	96.2	0.72	0.75	104.1	0.72	0.65	89.8	0.72	0.85	117.2	0.36
1	96	< 0.005	0	0.01	-	0	0.16	-	0	0.11	-	0	0.12	-	0
2	0	< 0.005	0.21	0.34	159.5	2.89	†	†	2.89	2.17	75.1	2.89	1.72	59.5	1.43
2	24	< 0.005	0.16	0.14	89.5	2.17	†	†	2.17	2.19	100.9	2.17	1.74	80.2	1.07
2	48	< 0.005	0.11	0.11	104.8	1.45	†	†	1.45	1.57	108.3	1.45	1.37	94.5	0.71
2	72	< 0.005	0.05	0.05	89.5	0.72	†	†	0.72	0.92	126.7	0.72	0.86	119.2	0.36
2	96	< 0.005	0	0.01	-	0	†	†	0	0.12	-	0	0.12	-	0

- At 96 h nominal concentration was 0, thus calculation could not be done.

† Complete mortality was observed prior to second pulse.

* Aqueous POEA concentrations were not measured.

Table 2.2 Real-time RT-PCR primer sets and conditions.

Gene	Element	Primer sequence (5'-3')	Amplicon location and size (bp)	Melting (°C)
SYBR green				
trβ	Forward	AAGGAACCAGTGCCAAGAATGT	201-286 (86)	85
	Reverse	AACGCTTGCTGTCGTCCAAA		
rp18	Forward	GTGTAGAAGAGAAGCCAGGTGAT	1-79 (79)	86
	Reverse	GGATTGTGGGAGATGACGGTAG		
crf	Forward	TGAGAGAGCCCTGATCCAAC	14-132 (119)	85
	Reverse	ATGGTGCCAGAGACACAGAA		
grII	Forward	GACCTGATGTGAGTCCTTCTCC	178-340 (163)	86
	Reverse	TTGTGCTGACCTTCTACTGCTC		
Dual-labeled fluorescent probes				
dio2	Forward	CACCTTTTAGACTTTGCCAGC	291-368 (78)	n.a.
	Reverse	GCTTATAAAGGGAGGTCAGGTG		
	Probe	56-FAM/AGCGCCCTCTTGTCGTCAACTTT/3IABkFQ/		
dio3	Forward	CACCTTGGAGTCCCTGAAG	23-172 (150)	n.a.
	Reverse	CTGTGGCCCTTGGAGAAG		
	Probe	56-FAM/TCTGGTACGGACAGAAGCTCGACT/3IABkFQ/		

trβ, thyroid receptor beta; rpl8, ribosomal protein l8; crf, corticotropin-releasing factor; grII, glucocorticoid receptor; dio2, deiodinase type 2; dio3, deiodinase type 3.

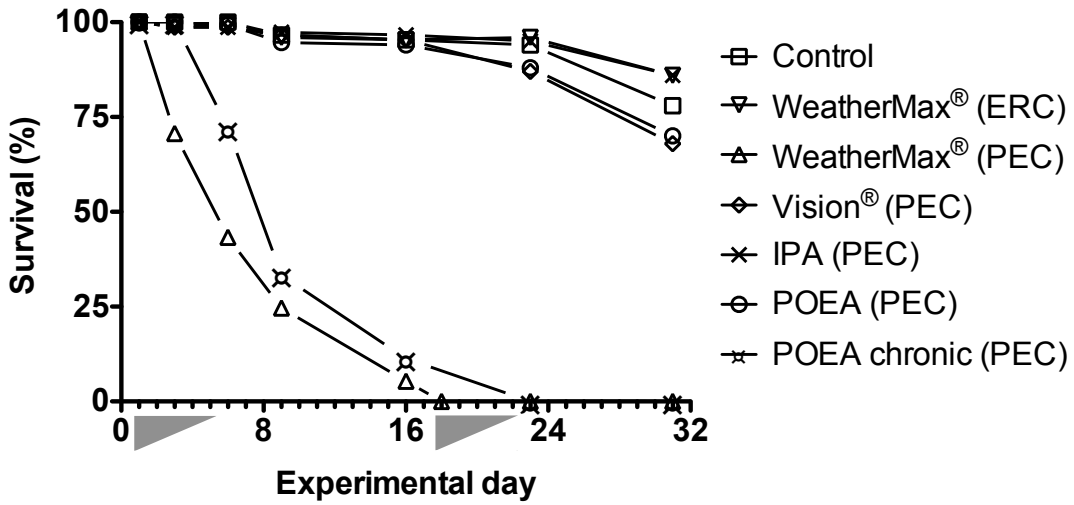


Figure 2.1 Percent survival of wood frog tadpoles (*L. sylvaticus*) exposed to two pulse treatments (grey triangles) of Roundup WeatherMax®, Vision®, isopropylamine salt of glyphosate (IPA), polyethoxylated tollowamine (POEA) as well as chronic exposure to POEA. ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

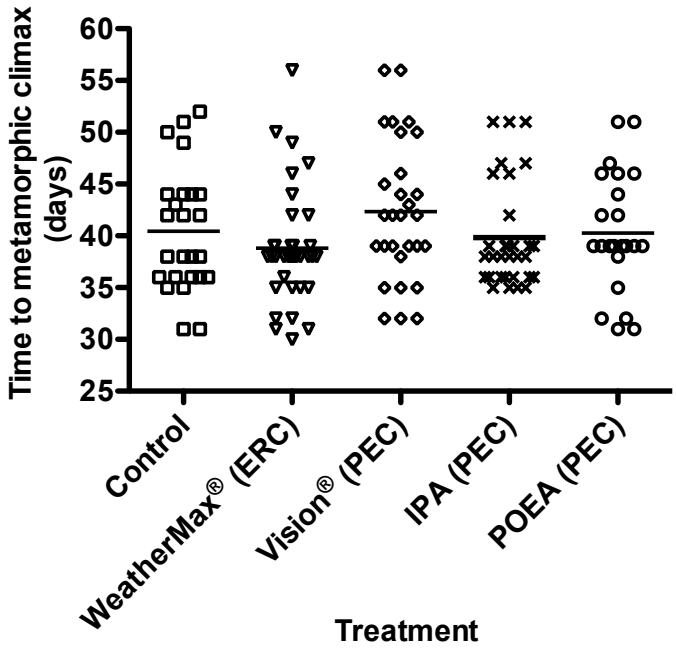


Figure 2.2 Time to metamorphic climax (days at which tadpoles reached Gosner stage 42) of wood frog tadpoles (*L. sylvaticus*) exposed to two pulse treatments of Roundup WeatherMax® (n = 36), Vision® (n = 29), isopropylamine salt of glyphosate (IPA, n = 31), polyethoxylated tollowamine (POEA, n = 23) and control (n = 25). Lines represent the mean. Data were analyzed by one-way ANOVA and significance is indicated if $p < 0.05$. ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

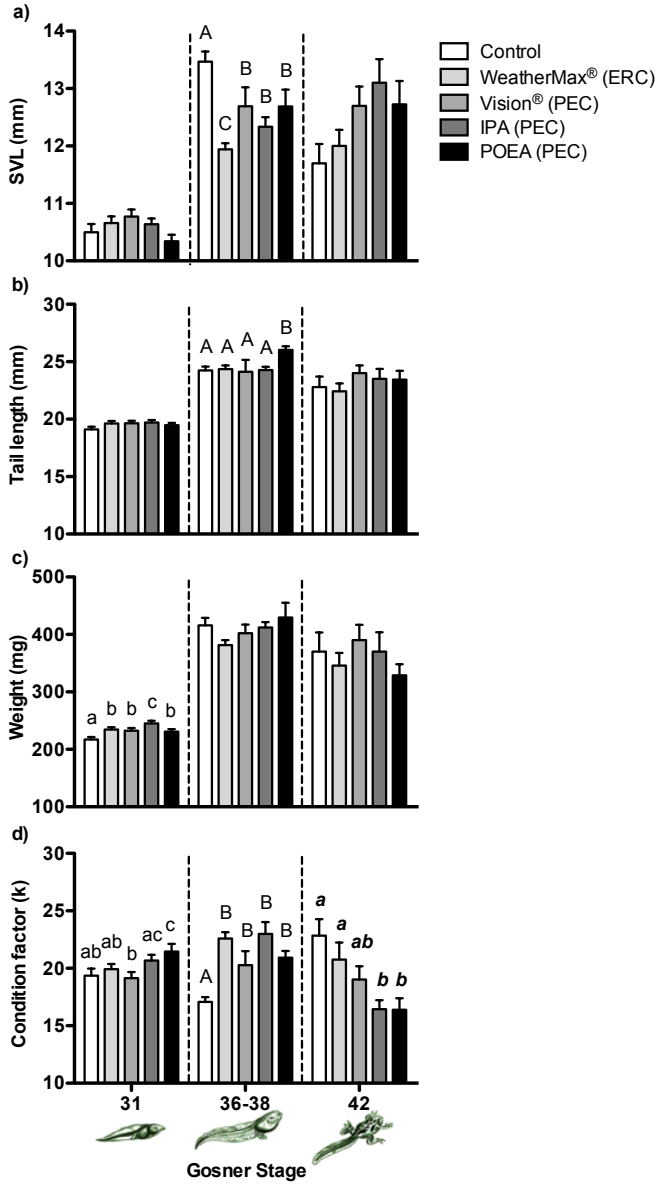


Figure 2.3 Snout-vent length (SVL, mm), tail length (TL, mm), weight (W, mg) and condition factor (k) of *L. sylvaticus* tadpoles at Gosner stage 31, 36-38 and 42 after exposures to Roundup WeatherMax®, Vision®, isopropylamine salt of glyphosate (IPA), polyethoxylated tollowamine (POEA) and control. Bars represent the mean + SEM (n = 15-87). Data from each stage were analyzed by randomization and significance is set at $p < 0.05$. Letters indicate significant differences between treatments of each stage. Condition factor = $100*(\text{weight}/\text{SVL}^3)$. ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

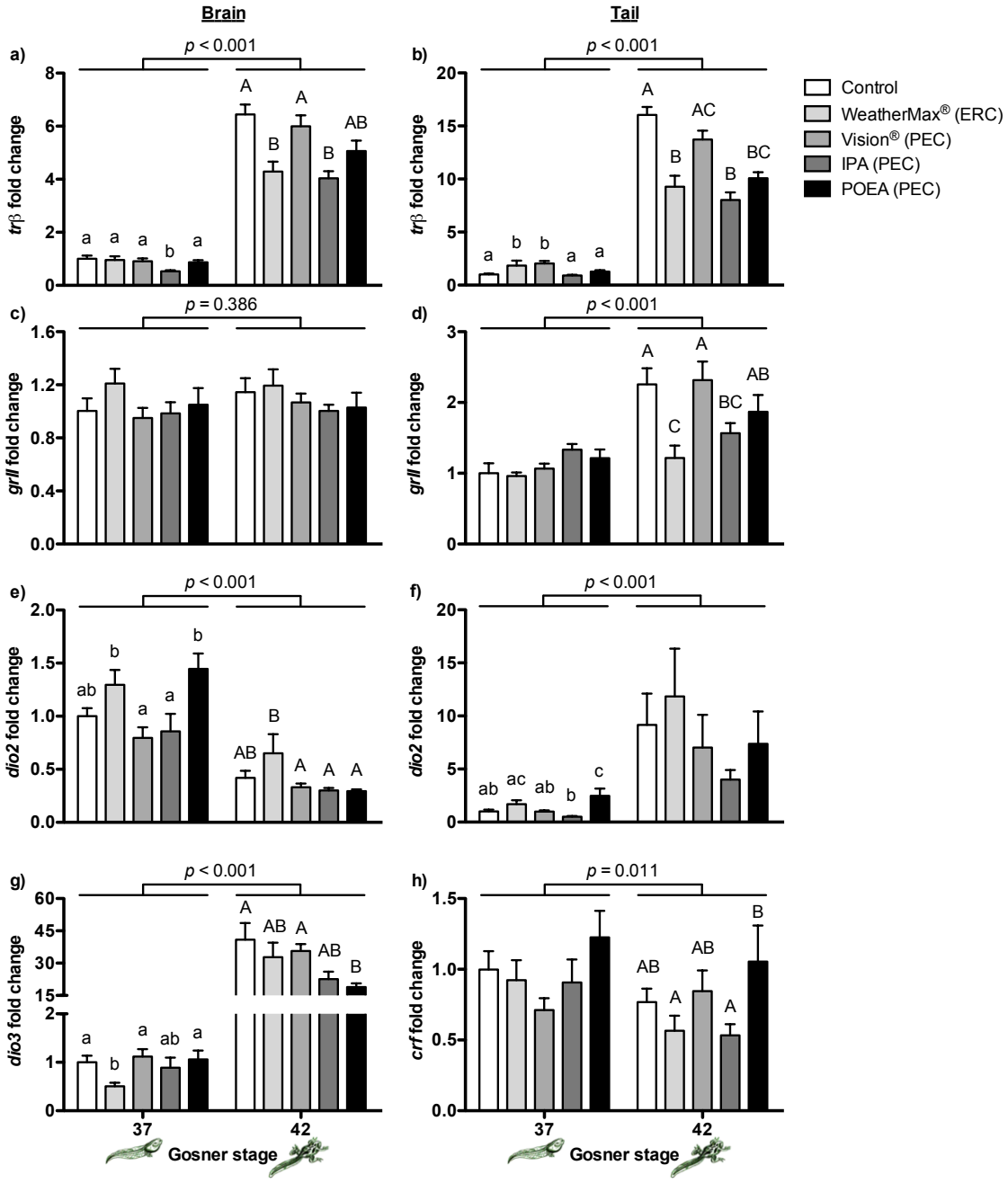

Brain
Tail
a)
b)
c)
d)
e)
f)
g)
h)
p < 0.001
p = 0.386
p = 0.011
trβ fold change
grll fold change
dio2 fold change
dio3 fold change
crf fold change
Control
WeatherMax® (ERC)
Vision® (PEC)
IPA (PEC)
POEA (PEC)
37
42
Gosner stage

Figure 2.4 Fold change in mRNA expression of *trβ* (a, b), *grII* (c, d), *dio2* (e, f), *dio3* (g) and *crf* (h) in the brain (a, c, e, g) and tail (b, d, f, h) of *L. sylvaticus* tadpoles at Gosner stage (Gs) 37 and 42 after exposure to two pulses of Roundup WeatherMax®, Vision®, isopropylamine salt of glyphosate (IPA), polyethoxylated tollowamine (POEA) and control as determined by real-time RT-PCR. Data are presented as the normalized fold change in mRNA expression relative to the mean of control of Gs 37. Bars represent the mean + SEM (n = 7). Data were analyzed by two-way ANOVA followed by Bonferroni post-hoc comparisons and the significance threshold is $p < 0.05$. Letters indicate significant differences between treatments of each stage. Brackets specify the Gosner stage effect, with corresponding *p*-values. See text for further description of the main effects of stage, treatment and the stage X treatment interactions. *trβ*, thyroid receptor beta; *grII,* glucocorticoid receptor; *dio2,* deiodinase type 2; *dio3,* deiodinase type 3, *crf,* corticotropin-releasing factor, ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

Supplementary material

Supplementary Table 2.1 Real time RT-PCR standard curve and assay performance characteristics per gene and tissue.

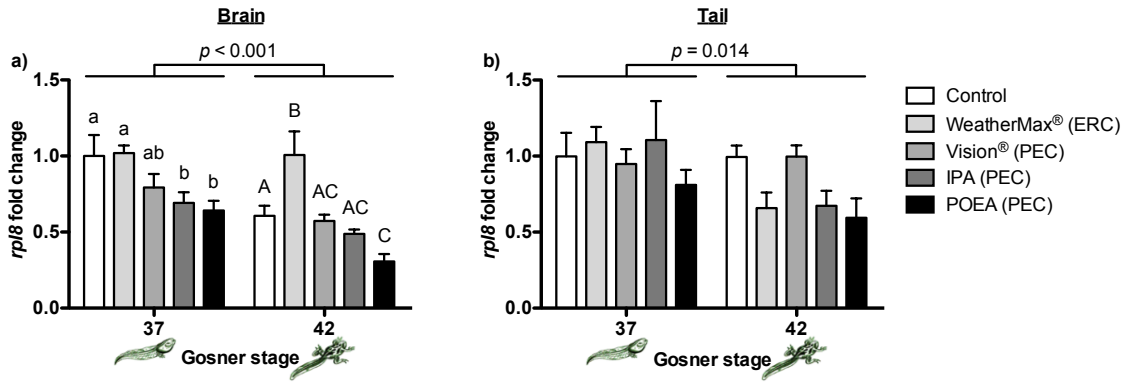
	trβ	***dio2***	***dio3***	***crf***	***grII***	***rpl8***
Brain						
E	102.3%*	98.2%	94.1%	n.a.	93.0%	93.2%
s	-3.271*	-3.366	-3.473		-3.501	-3.496
R^2	0.994*	0.997	0.990		0.996	0.996
Tail						
E	102.3%	100.7%	n.a.	94.0%	97.5%	98.6%
s	-3.269	-3.305		-3.475	-3.384	-3.357
R^2	0.997	0.999		0.993	0.992	0.995

E, efficiency; *s*, slope; R^2, regression coefficient; n.a., not analyzed

*Average value of 3 plates.

Supplementary Table 2.2 Average temperature (°C), pH, dissolved oxygen (DO, mg/L) in control, Roundup WeatherMax®, Vision®, isopropylamine salt of glyphosate (IPA), polyethoxylated tollowamine (POEA) and chronic POEA (POEAc) treatments. SD, standard deviation, ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

	Control	**WeatherMax®**		**Vision®**	**IPA**	**POEA**	**POEAc**	**Total**
		ERC	**PEC**	**PEC**	**PEC**	**PEC**	**PEC**	**Average**
Temperature	21.61	21.52	21.99	21.34	21.05	21.34	21.85	**21.43**
±SD	±0.39	±0.59	±0.22	±0.27	±0.41	±0.42	±0.24	**±0.48**
pH	7.14	7.12	6.96	7.11	7.13	7.11	6.97	**7.11**
±SD	±0.23	±0.17	±0.08	±0.22	±0.17	±0.14	±0.15	**±0.19**
DO	8.86	8.79	8.83	8.79	8.84	8.78	8.71	**8.81**
±SD	±0.10	±0.21	±0.17	±0.14	±0.25	±0.24	±0.45	**±0.22**



Supplementary Figure 2.1 Fold change in mRNA expression of ribosomal protein l8 (*rpl8*) in brain (a) and tail (b) of *L. sylvaticus* tadpoles at Gosner stage (Gs) 37 and 42 after exposure to two pulses of Roundup WeatherMax®, Vision®, isopropylamine salt of glyphosate (IPA, 2.89 mg a.e./L), polyethoxylated tollowamine (POEA, 1.43 mg/L) and control as determined by real-time RT-PCR. Data are presented as the fold change in mRNA expression relative to the mean of Gs37 control. Bars represent the mean + SEM (n=7). Data were analyzed by two-way ANOVA followed by Bonferroni post-hoc comparisons and significance is indicated if $p < 0.05$. Letters indicates significant differences between treatments of each stage. Brackets specify the Gosner stage effect, with corresponding *p*-values. ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

Supplementary Table 2.3 Two-way ANOVA results comparing mRNA expression in brain and tail tissues.

	Gosner stage (Gs)		**Treatment**		**Gs X Treatment**	
	$F_{(1,60)}$	***p* value**	**$F_{(4,60)}$**	***p* value**	**$F_{(4,60)}$**	***p* value**
Brain						
trβ	832.2	< 0.001	8.63	< 0.001	1.73	0.156
dio2	175.4	< 0.001	6.42	< 0.001	3.19	0.019
dio3	831.4	< 0.001	2.75	0.036	2.49	0.053
grII	0.387	0.364	1.22	0.311	0.31	0.868
Tail						
trβ	964.4	< 0.001	9.27	< 0.001	6.77	< 0.001
dio2	61.56	< 0.001	3.00	0.025	1.21	0.315
grII	50.62	< 0.001	4.26	0.004	4.63	0.003
crf	6.75	0.012	2.73	0.037	1.16	0.338

Supplementary Table 2.4 Results of Bonferroni post-hoc test comparing mRNA expression of target genes in brain and tail tissues of Gosner stage (Gs) 37 and 42 wood frog tadpoles (n=7/treatment) exposed to control, Roundup WeatherMax® (ERC, 0.21 mg a.e./L), Vision® (PEC, 2.89 mg a.e./L), isopropylamine (IPA) salt of glyphosate (PEC, 2.89 mg a.e./L), polyethoxylated tollowamine ([POEA] PEC, 1.43 mg/L) and control.

	Gs	**F**	***p* value**	**F**	***p* value**	**F**	***p* value**	**F**	***p* value**
Comparison		***trβ* brain**		***grII* brain**		***dio2* brain**		***dio3* brain**	
Control vs WeatherMax® (ERC)	37	0.47	n.s.	1.40	n.s.	1.39	n.s.	2.56	*
	42	3.03	**	0.25	n.s.	1.93	n.s.	0.78	n.s.
Control vs Vision® (PEC)	37	0.64	n.s.	0.37	n.s.	1.43	n.s.	0.42	n.s.
	42	0.55	n.s.	0.45	n.s.	1.22	n.s.	0.03	n.s.
Control vs IPA (PEC)	37	4.41	***	0.14	n.s.	1.31	n.s.	1.09	n.s.
	42	3.41	**	0.89	n.s.	1.71	n.s.	1.98	n.s.
Control vs POEA (PEC)	37	0.82	n.s.	0.18	n.s.	2.08	n.s.	0.03	n.s.
	42	1.79	n.s.	0.94	n.s.	1.79	n.s.	2.54	*
IPA (PEC) vs WeatherMax® (ERC)	37	3.77	***	1.54	n.s.	2.64	*	1.51	n.s.
	42	0.38	n.s.	1.15	n.s.	3.64	**	1.20	n.s.
IPA (PEC) vs Vision® (PEC)	37	3.77	***	0.23	n.s.	0.13	n.s.	1.51	n.s.
	42	2.86	*	0.44	n.s.	0.49	n.s.	1.95	n.s.
IPA (PEC) vs POEA (PEC)	37	3.59	**	0.32	n.s.	3.39	**	1.12	n.s.
	42	1.62	n.s.	0.05	n.s.	0.08	n.s.	0.57	n.s.
POEA (PEC) vs WeatherMax® (ERC)	37	0.32	n.s.	1.23	n.s.	0.62	n.s.	2.59	*
	42	1.24	n.s.	1.19	n.s.	3.72	***	1.77	n.s.
POEA (PEC) vs Vision® (PEC)	37	0.18	n.s.	0.55	n.s.	3.51	**	0.39	n.s.
	42	1.24	n.s.	0.49	n.s.	0.57	n.s.	2.51	*
Vision® (PEC) vs WeatherMax® (ERC)	37	0.15	n.s.	1.76	n.s.	2.76	*	2.96	**
	42	2.48	*	0.71	n.s.	3.15	**	0.75	n.s.
		***trβ* tail**		***grII* tail**		***dio2* tail**		***crf* tail**	
Control vs WeatherMax® (ERC)	37	3.28	**	0.03	n.s.	1.18	n.s.	0.52	n.s.
	42	3.73	***	4.42	***	0.28	n.s.	1.57	n.s.
Control vs Vision® (PEC)	37	4.37	***	0.63	n.s.	0.11	n.s.	1.38	n.s.
	42	1.03	n.s.	0.09	n.s.	1.18	n.s.	0.18	n.s.
Control vs IPA (PEC)	37	0.51	n.s.	2.18	n.s.	1.45	n.s.	0.58	n.s.
	42	4.60	***	2.49	*	1.84	n.s.	1.62	n.s.
Control vs POEA (PEC)	37	1.48	n.s.	1.39	n.s.	1.85	n.s.	0.81	n.s.
	42	3.03	**	1.50	n.s.	0.98	n.s.	0.96	n.s.
IPA (PEC) vs WeatherMax® (ERC)	37	3.79	***	2.21	n.s.	2.63	*	0.06	n.s.
	42	0.88	n.s.	1.94	n.s.	1.56	n.s.	0.05	n.s.
IPA (PEC) vs Vision®	37	4.89	***	1.55	n.s.	1.56	n.s.	0.80	n.s.

(PEC)	42	3.57	**	2.58	*	0.65	n.s.	1.81	n.s.
IPA (PEC) vs POEA (PEC)	37	1.99	n.s.	0.79	n.s.	3.30	**	1.39	n.s.
	42	1.57	n.s.	0.99	n.s.	0.85	n.s.	2.58	*
POEA (PEC) vs WeatherMax® (ERC)	37	1.80	n.s.	1.42	n.s.	0.67	n.s.	1.33	n.s.
	42	0.70	n.s.	2.93	**	0.70	n.s.	2.53	*
POEA (PEC) vs Vision® (PEC)	37	2.90	*	0.76	n.s.	1.74	n.s.	2.19	n.s.
	42	2.00	n.s.	1.59	n.s.	0.20	n.s.	0.77	n.s.
Vision® (PEC) vs WeatherMax® (ERC)	37	1.10	n.s.	0.66	n.s.	1.07	n.s.	0.86	n.s.
	42	2.69	*	4.52	***	0.90	n.s.	1.76	n.s.

trβ, thyroid receptor beta; *dio2*, deiodinase type 2; *dio3*, deiodinase type 3; *crf*, corticotropin-releasing factor; *grII*, glucocorticoid receptor.
ERC, environmentally realistic concentration; PEC, predicted environmental concentration.
n.s., non significant; *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

CHAPTER 3

Effects of the glyphosate-based herbicide Roundup WeatherMax® on metamorphosis of wood frogs (*Lithobates sylvaticus*) in natural wetlands

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Main contributions of each co-author:

1. Contributed to the experimental design, performed sampling and tissue collection, as well as abundance, growth, development and real-time RT-PCR data analysis, and manuscript preparation.

2. Contributed to the experimental design and performed sampling and tissue collection.

3. Contributed to the experimental design, performed sampling and tissue collection, revised the manuscript, and supervised the research.

4. Contributed to the experimental design, performed sampling and body length measurements.

5. Contributed to the experimental design and provided financial support.

6. Contributed to the experimental design, revised the manuscript, provided financial support, and supervised the research.

Note: Although the LEWA team member K. Kidd, B. Pauli, D. Thompson, M. Gahl, L. Baker, S. Melvin, J. Mudge and B. Reinhart are not co-authors on this manuscript; they have all contributed greatly to the experimental design and setup, herbicide exposure and water chemistry measurements and will be acknowledged in the manuscript.

3.1. Introduction

Glyphosate is a non-selective herbicide that is widely used in both agriculture and forest management across the world (Woodburn, 2000; Thompson and Pitt, 2003). The usage of glyphosate formulations has increased dramatically in recent decades and, as a result, glyphosate is currently the most commonly used pesticide for agricultural practices. Most glyphosate-based herbicide formulations are manufactured by Monsanto under common trade names such as Roundup Original®, Roundup Transorb®, Roundup WeatherMax®, Vision®, VisionMax®, etc., which vary slightly in their formulations. These formulations were previously thought to be relatively benign since glyphosate, the active ingredient, targets a metabolic pathway in plants that is not found in animals. However, recent studies suggest that common glyphosate formulations may have greater negative impacts on the environment than suspected (reviewed by Relyea, 2011). Laboratory and mesocosm studies have shown that glyphosate-based herbicides cause adverse effects on amphibians such as higher mortality, gonadal abnormalities, and decreased size and developmental rates of tadpoles (Howe *et al*., 2004; Relyea, 2004, 2005a,b,c; Relyea *et al.,* 2005; Relyea and Jones, 2009; Jones *et al.,* 2010, 2011; Williams and Semlitsch, 2009). Recent studies also suggest that these herbicides may exert their effects on growth and development through disruption of the thyroid pathway of exposed tadpoles (Howe *et al*., 2004; Lanctôt, 2010; Chapter 2). With the increasing use of glyphosate-based herbicides on a global scale, and the concerns regarding adverse effects on sensitive amphibian populations, the potential for these formulations to act as EDCs is a growing concern.

Most glyphosate-based herbicide formulations are designed for terrestrial use, but freshwater can become contaminated during or after application by mechanisms like drift, leaching and/or runoff. In addition, small ephemeral wetlands, where many amphibian species breed and develop, are often unintentionally oversprayed during aerial applications. Researchers have estimated "worst-case scenario" glyphosate concentrations of up to 7.6 mg acid equivalent (a.e.)/L in natural wetlands directly oversprayed with glyphosate-based herbicide formulations (reviewed by Relyea, 2011). A number of factors, such as interception by vegetation, depth, temperature, pH, and UV can influence glyphosate concentrations detected in water (Solomon and Thompson, 2003). Glyphosate can also dissipate relatively rapidly in aquatic environments; however, this depends highly on water quality parameters, sorption, microbial degradation and uptake by biota. Thus, many researchers argue that laboratory exposure to glyphosate-based herbicides does not reflect realistic exposure conditions and that experiments in natural environments are required.

Relatively little information exists regarding glyphosate exposure in natural ecosystems despite the fact that this would provide the most accurate estimates of effects in natural populations. Pesticide risk assessments are generally based on laboratory dose-response experiments, but these are inadequate for estimating natural effects, as they do not consider the complete range of interactions that may intensify or reduce the effects of herbicides. Although laboratory studies provide a useful approach to understand interactive mechanisms and provide controlled environments for comparisons, these are often not carried out on naturally relevant spatial and temporal scales. Laboratory studies generally

describe short-term acute or chronic exposures at relatively high doses that do not reflect realistic herbicide exposure. In addition, differences in experimental conditions (e.g., pH, food availability, predator cues, sediments) are likely to change not only the physiochemical properties of the receiving environment, but also the physiological state of the organism at the time of exposure. Since many factors are likely to affect results from toxicity studies, many scientists have indicated the need for more ecologically relevant studies for risk assessment (Schindler, 1987; Lemly and Richardson, 1997; Thompson *et al.,* 2004).

The Long-term Experimental Wetlands Area (LEWA) was established to examine the effects of glyphosate-based herbicides on aquatic communities using whole-ecosystem experiments. This field site provides a rare opportunity for replicated whole-ecosystem experiments. As part of this collaborative project, this study examines the effects of agriculturally relevant exposures to Roundup WeatherMax® on metamorphosis of wood frog (*Lithobates sylvaticus*) tadpoles exposed in natural wetlands. This formulation is a relatively new glyphosate-based herbicide used for agriculture and contains the potassium salt of glyphosate and an unknown surfactant. Specifically, we examine the effects of this herbicide on abundance, growth and development, and expression of genes involved in the control of metamorphosis.

3.2. Material and methods

3.2.1. Animals and experimental wetland setup

Field exposures were carried out at the LEWA site in Gagetown, New Brunswick, Canada, during the summer of 2009 and 2010. This field site contains numerous ephemeral wetlands that serve as breeding grounds for *L. sylvaticus* and developmental areas for tadpoles of this and other species. Of these, twelve wetlands were divided in half using an impermeable curtain so that each wetland contained a treatment and control side (Figure 3.1). Fertilized egg masses of *L. sylvaticus* were randomly collected from these natural experimental wetlands. Egg masses were then split up and randomly redistributed in each of the test wetlands based on the estimated volume (Supplementary Table 3.1) to reflect the natural range of densities observed in other wetlands at this study site (unpublished data).

3.2.2. Roundup WeatherMax® exposure

Roundup WeatherMax® (glyphosate present as the potassium salt, Monsanto, Winnipeg, MB, CAN) herbicide, at 0.21 or 2.89 mg a.e./L, was directly oversprayed across treatment sides of the wetlands (n = 6 wetlands per treatment). An equal volume of water was spayed onto control sides. Our choice of low concentration represents the environmentally realistic concentration (ERC) based on the upper 99th percentile concentration of aqueous glyphosate that has been measured in wetlands located in agricultural areas in Southern Ontario (Byer *et al.,* 2008; Struger *et al.,* 2008). The high concentration reflects the predicted environmental concentration (PEC) based on maximum

allowable application rates on the label (4.3 kg a.e./ha) for typical agricultural and forestry use patterns (Thompson *et al.,* 2004). The latter represents a "worst-case scenario" and is based on estimated concentrations measured in 15 cm deep wetlands with no intercepting vegetation, following direct over-spray of the maximum allowed rate. Wetland volumes were calculated immediately before each application. The volume of herbicide required to achieve the target concentration was mixed in ~3 L of water and applied directly to the surface of the wetland by Dr. D. Thompson, a licensed applicator, using a backpack sprayer (Flowmaster, Root-Lowell Manufacturing, Lowell, MI, USA). Wetlands were oversprayed twice (henceforth referred to as pulse exposure) to reflect standard application patterns used for agricultural practices. The first and second application coincided with median developmental stages of approximately Gs 25 and Gs 30, respectively, for tadpoles in all wetlands. Exposures and sampling of the same experimental wetlands were repeated in two consecutive years (2009 spray: [1] May 15-16, [2] June 9-10 and 2010 spray: [1] April 26-27, [2] May 24-25).

3.2.3. Water chemistry

Water samples were collected post-spray from control and treatment sides to determine aqueous glyphosate concentrations. Sampling time was modified after the first pulse in 2009 because preliminary tests of these samples showed faster dissipation than expected (unpublished data). Five 50 mL surface water samples were collected from different locations within each wetland half, and were pooled and mixed in a 1 L plastic bottle. Samples were stored on ice in the dark while on site and then transferred to -20°C

within 12 h. For samples collected in 2009, quantitative analysis of aqueous glyphosate concentrations was conducted using gas chromatography with nitrogen-phosphorous detection at the Great Lakes Forestry Laboratory in Sault Ste. Marie, Ontario (described by Thompson *et al.,* 2004). Detection limit for glyphosate analysis was 0.017 mg a.e./L. For samples collected in 2010, quantitative analysis of aqueous glyphosate and its metabolite aminomethylphosphonic acid (AMPA) was conducted using liquid chromatography/tandem mass spectrometry (Hao *et al.,* 2011) in the Ontario Ministry of the Environment Laboratory in Etobicoke, Ontario. The instrument detection limits for glyphosate and AMPA were 0.001 and 0.002 mg a.e./L, respectively. To ensure comparability between the two methods 24 samples were analyzed by both laboratories. A correlation analysis was performed. There was a strong relationship between the values obtained by both methods ($r^2 = 0.9228$), and the slope of the relationship was 1.046.

Temperature, specific conductance, pH and dissolved oxygen were measured weekly with a hand held probe (MPS 556, YSI, Yellow Springs, OHIO, USA). In 2010, water temperature was measured every 180 minute(s) using the ibutton thermochron f5 (2 on each side of wetland). Water depth was measured weekly using a meterstick in the deepest area of the wetlands. Ammonia, nitrate and total phosphorus (TP) concentrations were also measured throughout the experiment. Ammonia and nitrate analyses were performed at the University of Guelph (Guelph, ON, Canada) and TP analyses were performed at the Research and Productivity Council (Fredericton, NB, Canada). Detection limits for analysis of ammonia, nitrate, and TP were 0.005 mg/L, 0.002 mg/L, and 0.002 mg/L, respectively.

3.2.4. Wood frog sampling

Abundance was estimated by measuring the sampling effort required to catch *L. sylvaticus* tadpoles using a D-Frame Aquatic Net (28 x 28 cm, Boreal Laboratories). Each wetland half was sampled by taking one sweep (~1 m) with the net every 3 m along transects spaced 3 m from one another. The abundance value was calculated by dividing the number of *L. sylvaticus* tadpoles caught by the number of sweeps performed with the dip net throughout each wetland half. Tadpoles were then staged using Gosner staging system (Gosner, 1960) and photographed on a 5 mm grid, from which body length was measured using ImageJ image analysis software (National Institute of Health, USA).

Tadpoles were sampled for gene expression analyses when the median developmental stage within a wetland reached Gs 36/37 (n = 10). Tadpoles were also photographed prior to sampling to measure body length. We could only sample sufficient tadpoles at this stage from control and treatment sides of ERC 10 and PEC 37 (2009 and 2010) as well as PEC 22 in 2009. The lack of Gs 36/37 tadpoles in the rest of the experimental wetlands was a result of drying, predation, or ranavirus infection, in one or both sides of wetlands. Although we intended to sample tadpoles at Gs 42, tadpoles at this developmental stage were scarce in most of the experimental wetlands and, therefore, were not sampled. Euthanasia was performed by immersion in 3-aminobenzoic acid ethyl ester (MS-222, Sigma) in water. Whole tadpoles with ventral incisions were preserved in RNAlater solution as validated and described previously (Navarro-Martín *et al.,* 2012). Samples were kept at ambient air temperature for < 12 h and then stored at 4 °C for < 15 days. Brains and tails were dissected

and stored at -80 °C until further analysis. Sampling followed a protocol approved by the University of New Brunswick Animal Care Committee and according to the guidelines set by the Canadian Council on Animal Care.

3.2.5. RNA isolation and cDNA synthesis

RNA was isolated from brain and tail tissue of Gs 36/37 tadpoles as described in section 2.2.4. (Chapter 2). Total cDNA of brain (sampled in 2009 and 2010) and tail (2010) samples was synthesized from 1 μg, whereas total cDNA from tail (2009) samples was synthesized from 3 μg of total RNA. This was done using 200 ng random hexamer primers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Each 20 μL reaction was diluted for real-time RT-PCR analysis to a final concentration of 6.25 ng. All procedures followed manufacturer protocols.

3.2.6. Gene expression analysis by real time RT-PCR

Real-time reverse transcriptase polymerase chain reactions (RT-PCR) were performed using SYBR Green I for *trβ* (brain and tail), *grII* (tail and brain), *crf* (tail), *28S* (brain and tail) and *pgk1* (brain and tail), and fluorogenic 5' nuclease chemistry for *dio2* (brain and tail) and *dio3* (brain), as described in section 2.2.5. These genes were selected because they have been shown to play important roles in mediating developmental processes (reviewed by Galton, 1992; Shi, 2000; Tata, 2006; Brown and Cai, 2007; Fort *et al.,* 2007; Denver, 2009). Sequences of the primers, amplicon location, size and melting temperatures are presented in Table 3.2. Efficiency of relative standard curve was 100 ± 10 %, slope

between −3.1 and −3.6 and $r^2 > 0.985$ in all cases (Supplementary Table 3.2) in accordance with the acceptable conditions outlined in the manufacturer's instructions (Mx3000P system, Stratagene).

The ribosomal RNA 28S and phosphoglycerate kinase 1 genes were tested as potential internal reference genes, however similar to *rpl8* mentioned in section 2.2.5, expression was significantly altered by treatment (Supplementary Figure 3.1). For this reason, gene expression data in the present study was normalized using a data driven normalization algorithm (NORMA-Gene) developed by Heckmann *et al.* (2011) as described in section 2.2.5. Duplicate data obtained for each sample, considered technical replicates, were averaged prior to normalization. Fold change in normalized expression relative to control for each wetland was then calculated for each sample. Biological replicates (n = 5 - 7) were averaged to obtain mean fold change gene expression ± standard error of the mean (SEM).

3.2.7. Statistical analysis

The relationships between body length and Gosner stage, and Gosner stage over time were examined using linear regressions. Analyses of covariance (ANCOVA) were used to test if the relationships differed significantly between control and treatment half of each wetland. Analyses were performed using Graph Pad Prism® v5.0a. Differences were considered to be significant when $p < 0.05$.

Differences in body lengths (n = 6 - 10) and gene expression (n = 5 - 7) of Gs 36/37 tadpoles between control and treatment sides of each wetland were determined using *t*-tests.

Data were first analyzed for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's test) and transformed to meet parametric assumptions when necessary. Body length data are presented as the mean (+ SEM). Gene expression data are presented as the mean (+ SEM) fold change in normalized mRNA levels relative to the control. Analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL). Differences were considered to be significant when $p < 0.05$.

3.3. Results

3.3.1. Water chemistry

The aqueous glyphosate concentrations (mg/L) measured in ERC 10, PEC 37 and PEC 22 wetlands are presented in Table 3.1. In general, aqueous glyphosate concentrations (mg/L) measured in the control after the first and second spray were less than 0.01 mg a.e./L. However, there were a few exceptions: the aqueous glyphosate concentrations measured in control sides was 0.03404 (day 0) and 0.06080 (day 3) mg a.e./L in PEC 22 (2009) after the second spray, 0.3560 (day 1) and 0.0729 (day 3) mg a.e./L in PEC 37 (2010) after the first spray, and 0.0922 (day 1) mg a.e./L in ERC 10 (2010) after the second spray. This is a result of unavoidable wind drift or leakage from the treated side to the control side. Aqueous glyphosate concentration measured 1, 3, and 7 days after the first spray in both the ERC and PEC treated sides of wetlands ERC 10, PEC 37 and PEC 22 in 2009 were lower than the target concentration. On the other hand, in 2010, the average aqueous glyphosate

concentration measured in the ERC 10 and PEC 37 treated halves immediately after the first spray was 4.2- and 2.9-times greater than the target concentration, respectively. Similarly, immediately after the second spray in 2009, the average aqueous glyphosate concentrations measured in the ERC 10 and PEC 22 treated halves were approximately 17.7- and 1.6-times higher than the target concentration. The average aqueous glyphosate concentrations measured immediately after the second spray in the treated half of PEC 22 was similar to the target concentration. Immediately after the second spray in 2010, average aqueous glyphosate concentrations measured in the ERC 10 and PEC 37 treated halves were approximately 15.3- and 2.4-times higher than target concentrations. Furthermore, in most wetlands, we observed a decrease in the aqueous glyphosate concentrations over time after both the first and second spray. In addition, concentrations of aminomethylphosphonic acid (AMPA) measured in control, ERC, and PEC treated halves of wetlands sampled for gene expression after the two sprays in 2010 was relatively lower, but followed similar trends as glyphosate concentrations measured in these wetlands (Supplementary Table 3.3).

Water temperature, pH, conductivity, DO and water depth between the first spray date and sampling of Gs 36/37 tadpoles ranged between 6.0 - 34.5 °C, 5.4 - 6.3, 12 - 53 S/m, 1.81 - 10.57 mg/L and from 1 to >100 cm, respectively (Supplementary Table 3.4, note: in 2010 only temperatures measured between 9:01-18:01 h were considered in order for data to be consistent with 2009). Average temperatures in PEC 22 were ~ 6 - 9 °C higher than ERC 10 and PEC 37. Average conductivity on the treatment side of PEC 37 was 9.83 S/m lower than control (2009). Average conductivity was ~10 - 11 S/m lower on the in both sides on

PEC 22 and the treatment side of PEC 37 than in ERC 10 and control of PEC 37. Nutrients (NH4, NO3, TKN and P) concentrations over time are presented in Supplementary Figure 3.2.

3.3.2. Abundance, growth and development

In general, abundance (estimated by tadpoles/sweep) of wood frog tadpoles was consistently lower on the treatment side than on the control side 12 - 16 days after the first spray (Figure 3.2). However, this trend did not persist throughout the experiment. Differences in abundance were also observed between wetlands and years. In ERC 10, abundance was approximately 3 times higher after the first spray in 2009 than in 2010. Additionally, in 2009, abundance in this wetland was very low 42-days after the first spray, whereas in 2010, abundance did not diminish until 70-days after the first spray. In PEC 22 (2009), abundance was very low (< 1 tadpole/sweep) throughout the experiment.

Tadpoles exposed to Roundup WeatherMax® at the environmentally realistic concentration in ERC 10 had a 3.6 % decrease in body length at Gs 36/37 relative to control tadpoles in 2010 ($T_{(1,18)} = 8.011$, $p = 0.011$), but not in 2009 ($T_{(1,15)} = 0.032$, $p = 0.860$, Figure 3.3). Tadpoles exposed to the predicted environmental concentration in PEC 37 had a 9.8 and 6.2 % increase in body length at Gs 36/37 relative to control tadpoles in 2009 ($T_{(1,14)} = 13.676$, $p = 0.002$) and 2010 ($T_{(1,18)} = 4.755$, $p = 0.043$), respectively. Similarly, exposed tadpoles in PEC 22 had a 4.5 % increase in body length but this was not significantly different from control ($T_{(1,13)} = 0.0889$, $p = 0.363$).

Linear regression of body length versus Gosner stages showed significant differences between control and treatment of PEC 22 in 2009 and ERC 10 in 2010 (Supplementary Figure 3.3). Specifically, we observed an increase in the slope of the relationship between body length and Gosner stage of exposed tadpoles in PEC 22 ($F_{(1,141)} = 4.22$, $p = 0.042$). However, in 2010, we observed a decrease in the slope of the relationship between body length and Gosner stage of exposed tadpoles in ERC 10 ($F_{(1,174)} = 12.99$, $p < 0.001$). No significant differences in body length at different developmental stages were observed between control and treatment halves of ERC 10 and PEC 37 in 2009, or PEC 37 in 2010. Furthermore, linear regression of Gosner stage versus time showed that development of treated tadpoles was accelerated in both PEC 37 in 2009 (Figure 3.4b, $F_{(1,159)} = 20.36$, $p < 0.0001$) and ERC 10 in 2010 (Figure 3.4d, $F_{(1,176)} = 7.31$, $p = 0.0075$). No significant differences in development were observed between control and treatment halves of ERC 10 and PEC 22 in 2009, or PEC 37 in 2010.

3.3.3. Gene expression

The mRNA expression of specific thyroid-related (*trβ, dio2,* and *dio3*) and stress-related (*crf* and *grII*) genes were measured to determine if Roundup WeatherMax® influences hormonal pathways involved in metamorphosis of wood frogs tadpoles (*Lithobates sylvaticus*). In whole brain samples, no significant differences in *trβ, grII, dio2* or *dio3* mRNA expression were observed between control and ERC 10, PEC 37 and PEC 22 exposed in 2009 (Figure 3.5a-d, *p*-values are presented in Supplementary Table 3.5). However, in 2010, we did observe significant differences in gene expression between control

and treatment groups (Figure 3.5i-l). Specifically, exposed tadpoles in ERC 10 had 1.3-fold more brain *trβ* mRNA expression relative to tadpoles from the control (Figure 3.5i). Contrary to this, exposed tadpoles in PEC 37 had 1.4- and 1.3-fold less brain *trβ* and *dio2* mRNA expression, respectively, relative to tadpoles from the control side (Figure 3.5i, k). No significant differences in brain *grII* and *dio3* mRNA expression were found between control and treatment halves in either wetland (Figure 3.5j, l).

In tail tissue, we observed a significant decrease in *trβ* (PEC 22, 1.9-fold), *grII* (PEC 37, 1.3-fold) and *crf* (ERC 10 and PEC 37, 1.8-fold) mRNA expression in treated wetland halves relative to control in 2009 (Figure 3.5e, f, h). No significant differences in tail *dio2* were observed between control and treatment of any wetland (Figure 3.5g). On the other hand, in 2010, we only observed a significant 1.4-fold decrease in tail *grII* mRNA expression in ERC 10 (Figure 3.5n). We did observe a similar trend in decrease *crf* mRNA expression as in 2009, however differences were not significant in 2010 (Figure 3.5p). No significant differences in tail *trβ* and *dio2* were observed between control and treatment of any wetlands (Figure 3.5m, o).

3.4. Discussion

In the present study, natural experimental wetlands were over sprayed with Roundup WeatherMax® herbicide, a formulation containing the potassium salt of glyphosate and an unknown surfactant. We used agriculturally relevant exposure regimes (i.e., two pulses) to further increase the relevance of responses to natural ecosystems. Similar exposure scenarios

were carried out with a parallel laboratory experiment that was designed to mimic the field exposure in terms of concentration, timing and duration of exposure (Chapter 2). In the laboratory, wood frog tadpoles from the same population as the field experiments were also exposed to two pulses of Roundup WeatherMax® at ERC (0.21 mg a.e./L) or PEC (2.89 mg a.e./L). In this laboratory experiment, exposure to the PEC resulted in complete mortality after a single pulse. Although survival was not directly measured in the field, abundance measurements after the second pulse do not show any major differences between control and treated halves of the experimental wetlands at either the ERC or PEC. Thus, contrary to the high mortality observed in the lab, exposure to PEC in the field did not result in high mortality relative to control. This supports the hypothesis that laboratory studies tend to overestimate effects of glyphosate-based herbicides compared to exposures in natural systems (Thompson *et al.,* 2004; Wojtaszek *et al.,* 2004).

In the present study, the average pH in experimental wetlands was 5.9 compared to 7.1 in the laboratory exposure. High pH has been found to increase the toxicity of tadpoles to glyphosate-based herbicide in both laboratory and field experiments (Edginton *et al.*, 2004; Chen *et al.,* 2004; Wojtaszek *et al*., 2004). Thus, one explanation for the differences in toxicity could be that effects in the lab are exacerbated by the higher pH. Many other explanations exist for differences in response across experimental conditions. For example, glyphosate and POEA might dissipate (e.g., sorption) or degrade (e.g., microbial break-down) more rapidly in the natural environment. The laboratory experiment used for comparison was designed to mimic the natural decrease of glyphosate in water, but measured

glyphosate concentration from these 3 natural wetlands showed faster dissipation/degradation than the 25% decrease per day implemented in the laboratory experiment (see Table 3.1). Thus, reduced toxicity observed in the field compared to the laboratory could also be attributed to faster dissipation and degradation processes that resulted in reduced exposure concentration in the field. Several researchers have suggested that the presence of soil in aquatic environments decreases the toxicity of glyphosate-based herbicides since glyphosate binds rapidly to soil, sediment and suspended particles (Giesy *et al.,* 2000; Solomon and Thompson, 2003). Furthermore, possible stresses associated with the un-natural environment in the laboratory could be another explanation for the differences observed amongst experimental scenarios. For example, in the lab study (Chapter 2) water changes were performed daily during the two pulses. This disturbance is possibly more stressful for tadpoles and could increase their vulnerability to the herbicide. What is clear from our results is that the experimental context, including environmental parameters and exposure conditions are important factors and should not be overlooked when assessing overt glyphosate toxicity in amphibians.

In the present study, thyroid-dependent gene expression analysis was used to assess the possibility that glyphosate exposure leads to endocrine disruption. This technique is recognized to serve as an indirect method for assessing thyroidal activity through development because there is a correlation between TH concentrations and the expression of key TH-dependent genes that regulate metamorphosis (Krain and Denver, 2004; Opitz *et al.,* 2006b; Duarte-Guterman *et al.,* 2012). In addition, as gene expression changes precede

morphological changes, gene expression analysis could allow us to interpret molecular mechanisms involved in the disruption of thyroid pathways. Pulse exposures to Roundup WeatherMax® at the PEC and ERC in natural wetlands significantly altered the expression of thyroid- and stress-related genes, which is indicative of a disruption in these pathways. Interestingly, in the two wetlands in which we observed a small but significant acceleration in development rate (PEC 37 in 2009 and ERC 10 in 2010), we also observed an up-regulation (not significant) in brain and tail *trβ* mRNA expression in Gs 36-37 tadpoles as well as a significant down-regulation in tail *grII* mRNA expression. It is well established that transcription of TRβ is directly regulated by THs and that *trβ* mRNA expression correlates with increased levels of THs during metamorphosis. Moreover, it is the tissue-specific developmental increase in TRβ that drives coordinated metamorphic transformations (Denver *et al.,* 2002). Thus, the increases in brain and tail *trβ* mRNA expression are consistent with the accelerated developmental rates observed in treated tadpoles. Similar to *trβ*, tail *grII* mRNA is regulated by T_3 in a dose-dependent manner and has been shown to increase throughout metamorphosis of *Xenopus laevis* tadpoles (Krain and Denver, 2004). Contrary to *trβ,* we observed a reduction in tail *grII* mRNA levels, which is not consistent with the acceleration in development observed, and suggest another level of disruption. An increase in THs in tail during pro-metamorphosis (Gs 36-37) could lead to precocious tail resorption, which could consequently affect tadpoles swimming. Thus, the decrease in tail *grII* could be a compensatory response to the observed changes in *trβ.* In addition, we observed a consistent decrease in tail *crf* mRNA expression in all experimental ponds. In

2009, we observed a 1.8-, 1.8- and 1.5-fold down-regulation of tail *crf* mRNA in exposed tadpoles relative to control in ERC 10, PEC 37 and PEC 22, respectively. A similar pattern was observed in 2010, however differences between control and exposed tadpoles were smaller (1.3- and 1.2-fold down-regulation in ERC 10 and PEC 37, respectively) and did not reach statistical significance. In the tail, the CRF peptide plays an important cytoprotective role and helps prevent the tail from regressing before metamorphic climax (Boorse *et al.,* 2006). A decrease in this peptide in the tail in some of the animals is suggestive of an endocrine-disrupting effect although it is presently difficult to link this observation to any morphological changes.

In general, differences observed in gene expression between control and treatment groups were not consistent between wetlands and years (2009 and 2010), nor were they consistent with parallel laboratory exposure (Chapter 2). Differences in abiotic and biotic stressors may explain disparity in results of gene expression analysis between experimental wetlands. For example, increases in temperature have been shown to increase developmental rates and growth of tadpoles (Collins, 1979). In addition, temperature variations between wetlands also have the potential to affect the toxicity of glyphosate by increasing absorption and chemical reaction rates. To our knowledge, no studies have examined the synergistic effects of temperature on the toxicity of glyphosate-based herbicides to tadpoles. Folmar (1979) showed that higher temperature correlate with increase toxicity of glyphosate in fish. In addition, Jones and colleagues (2010, 2011) demonstrated thermal stratification of glyphosate in mecososms. Thus, in deeper wetlands where thermal stratification occurs,

glyphosate concentration is expected to be higher at the surface where water is warmer, while shallow wetlands that have uniform temperature are expected to have a diluted concentration that is evenly distributed in the water column. Water depth in the aforementioned mesocosm studies was 40 cm, which corresponds to the average water depth in the present study. Consequently, stratification is expected to have occurred in deeper areas of our experimental wetlands. It is also important to note that wood frog tadpoles tend to congregate around the edge of wetlands (pers. observation) where water temperature is higher. Variations in density or intraspecific competition have also been shown to affect tadpole growth and development (Semlitsch and Caldwell*,* 1982; Collins, 1979) and have the potential to affect the response of tadpoles to the herbicide. Jones and colleagues (2010) showed that toxicity of Roundup Original MAX® increased as bullfrog density increased. In the present study, initial wood frog tadpole densities were equalized between experimental wetlands based on the volume. However, throughout the experiment abundance differed between wetlands, which could impact the toxicity of the herbicide. Other environmental stressors such as predator cues, UV-B radiation levels and food availability can also affect the response of tadpoles to the herbicide, however these have not been directly measured in the present study. Natural wetlands are dynamic ecosystems with a vast range of environmental condition, thus it is not surprising that the results obtained present a large amount of variability that can complicate interpretation.

3.5. Conclusion

This study investigated the effects of agriculturally relevant exposures of Roundup WeatherMax® formulation (containing an unknown surfactant) on the development of wood frog tadpoles. This is the first experiment of its kind to investigate endocrine responsiveness in tadpoles exposed to a contaminant in a natural experimental wetland. Pulse exposures to Roundup WeatherMax® at the PEC and ERC in these wetlands significantly affected the expression of thyroid- and stress-related genes of wood frog tadpoles, which is indicative of disruption to these pathways. However, more studies are needed to determine if the small differences in mRNA expression observed translate into biologically significant changes in functional protein levels. Furthermore, it is possible that responses observed represent indirect effects and not a direct consequence of herbicide uptake by the tadpoles. For example, the herbicide might affect important food resources (e.g., periphyton or algae), which could result in developmental effects in tadpoles (Jones *et al*., 2011). Based on our pulse exposure to environmentally realistic concentrations, Roundup WeatherMax® does not appear to pose a significant direct threat to wood frog development. However, as mentioned, a number of factors can influence toxicity of glyphosate-based herbicides and these should be considered when determining the potential impacts of these herbicides on organisms in the environment. Glyphosate-based herbicides cause a variety of effects in developing tadpoles in the laboratory, including gonadal abnormalities (Howe *et al.,* 2004) and DNA damage (Clements *et al*., 1997), but few of these responses have been considered under realistic exposure conditions in natural wetlands. More research is needed to determine if

results from laboratory-based studies of EDCs can be extrapolated to more complex situations in natural environments, where a multitude of interactions between biotic and abiotic factors exist.

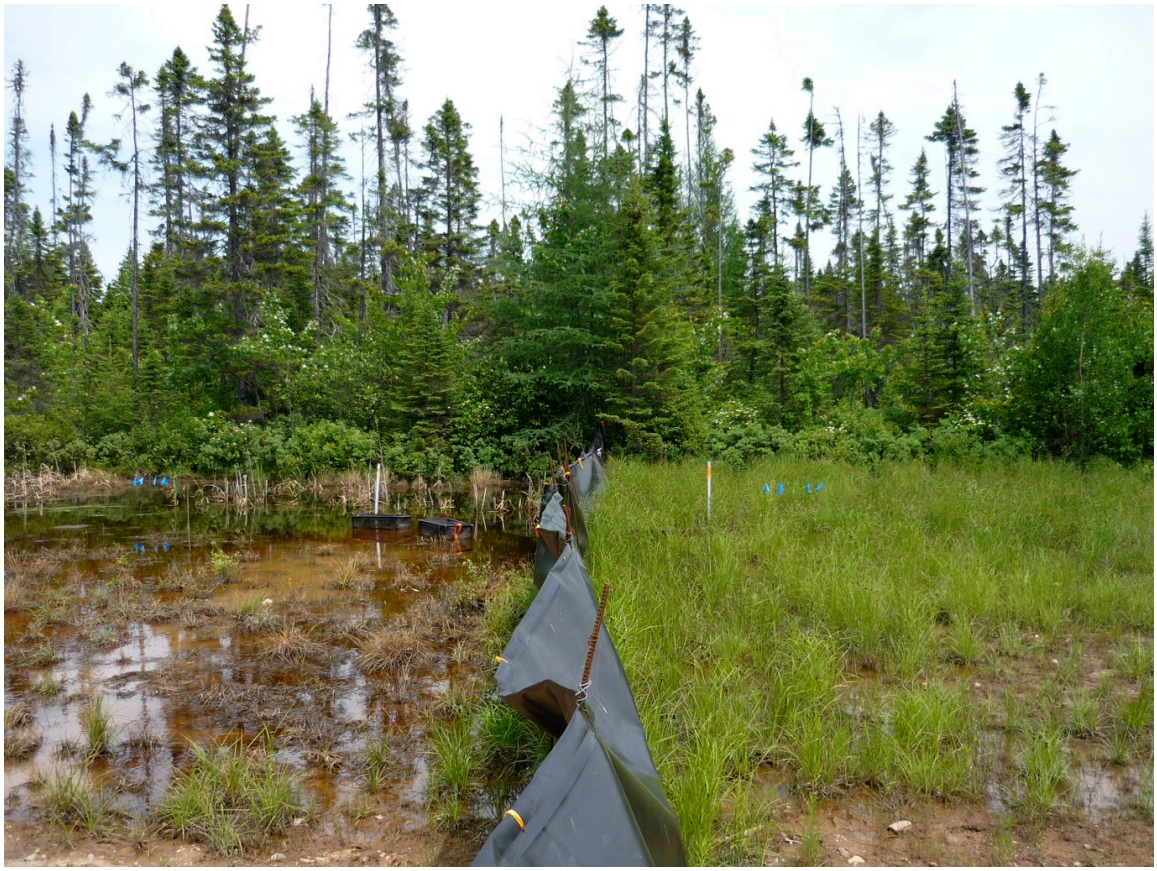


Figure 3.1 Photograph of experimental setup. Twelve wetlands were split in half using an impermeable curtain. One side of each wetland was oversprayed with Roundup WeatherMax® (0.21 or 2.89 mg a.e./L) and the other side was oversprayed with water (control). In this example (PEC 22), the left side of wetland was treated with 2.89 mg a.e./L Roundup WeatherMax® and the right side was treated with water (control).

Table 3.1 Aqueous glyphosate concentration (mg/L) measured in control and treatment sides of experimental wetlands. The treatment sides were oversprayed twice with Roundup Weathermax® at the environmentally realistic concentration (ERC, 0.21 mg a.e./L) or predicted environmental concentration (PEC, 2.89 mg a.e./L). Control sides were oversprayed with water.

		Wetland number									
		ERC 10				**PEC 37**				**PEC 22**	
		2009[a]		**2010[b]**		**2009[a]**		**2010[b]**		**2009[a]**	
Spray	**Days post-treatment**	**Control**	**ERC**	**Control**	**ERC**	**Control**	**PEC**	**Control**	**PEC**	**Control**	**PEC**
1	0				0.883			0.003	8.290		
	1	0.007	0.010	0	0.047	0.002	0.756	0.356	6.570	0	0.830
	3		0.011		0.001		0.025	0.073	0.004		0.005
	7		0.008		0		0.048		0.622		0.009
	TWAC[c]	**0.007**	**0.009**	**0**	**0.010**	**0.002**	**0.106**	**0.142**	**1.022**	**0**	**0.082**
2	0	0	3.717	0.004	3.220	0	2.302		6.940	0.034	4.619
	1			0.092	0.106			0.002	3.020		
	3	0.005	0.009	0	0.003	0.010	0.009		1.030	0.061	0.087
	7		0.010		0.001		0.010		0.096		0.004
	TWAC[c]	**0.005**	**0.025**	**0.023**	**0.023**	**0.010**	**0.019**	**0.002**	**0.640**	**0.060**	**0.048**

[a] Aqueous glyphosate concentrations measured using gas chromatography with nitrogen-phosphorous detection (Thompson *et al.,* 2004). Detection limit 0.017 mg a.e./L.

[b] Aqueous glyphosate concentrations measured using liquid chromatography/tandem mass spectrometry (Hao *et al.,* 2011). Detection limit 0.001 mg a.e./L.

[c] TWAC, time-weighted average concentration = glyphosate concentration at each measured time interval multiplied by that time interval and divided by the total time of observation.

Blanks = not measured

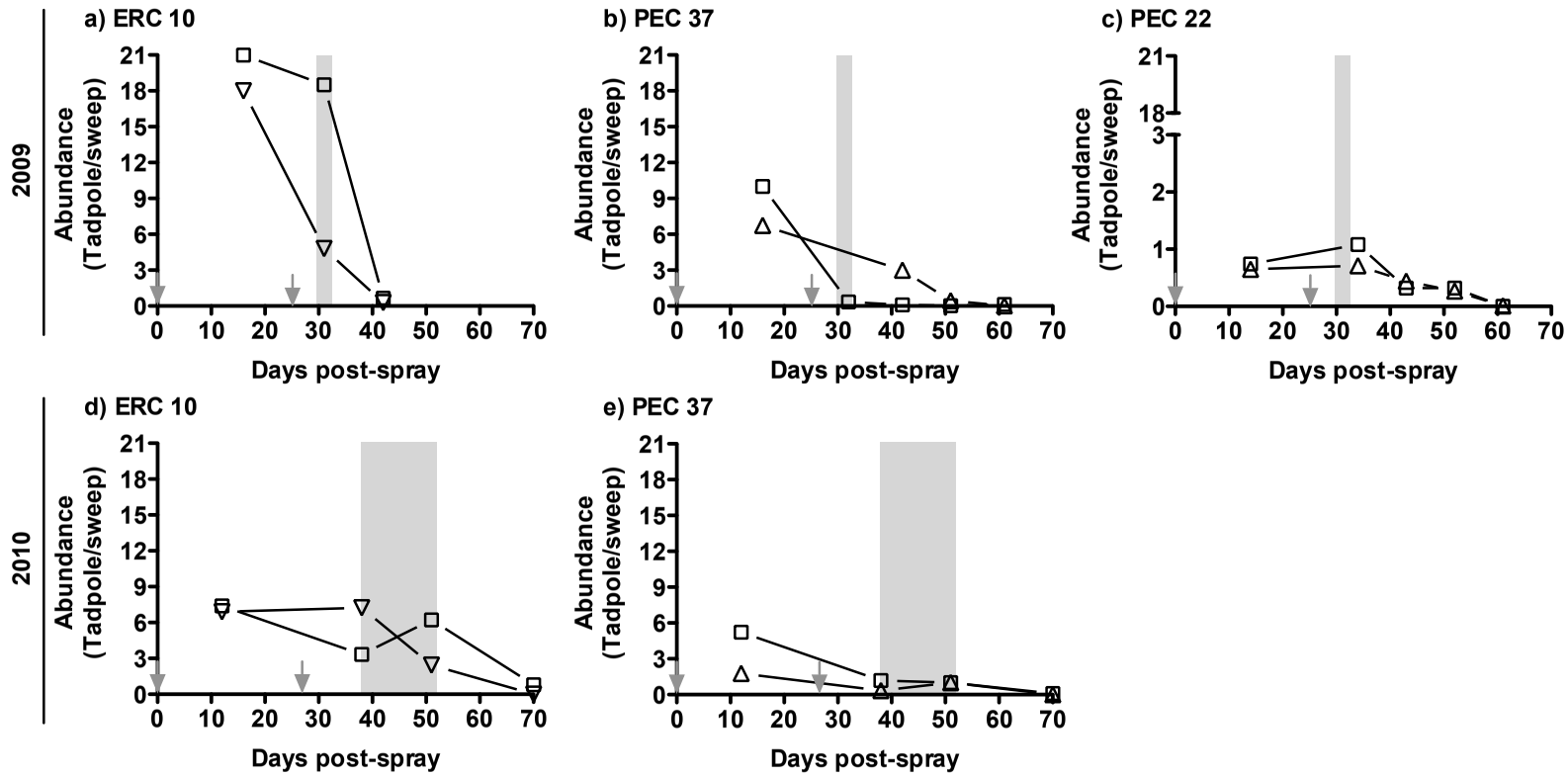


Figure 3.2 Abundance (tadpole/sweep) of *L. sylvaticus* tadpoles in experimental wetlands (ERC 10, PEC 37 and PEC 22) in 2009 (a - c) and 2010 (d, e). Treatment sides were oversprayed twice each year (represented by ⬇) with either 0.21 mg a.e./L (ERC 10, ▽) or 2.89 mg a.e./L (PEC 37 and PEC 22, △) Roundup WeatherMax®. Control sides were oversprayed with water (□). ERC, environmentally realistic concentration; PEC, predicted environmental concentration; Gs 36/37 sampling (shaded area).

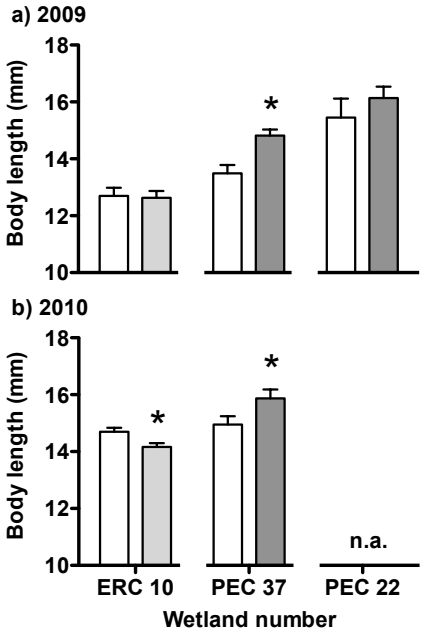


Figure 3.3 Body length (mm) of Gosner stage 36/37 *L. sylvaticus* tadpoles collected from 3 wetlands (ERC 10, PEC 37 and PEC 22) in 2009 (a) and 2010 (b). Treatment sides were oversprayed twice each year with either 0.21 mg a.e./L (ERC 10, light grey bars) or 2.89 mg a.e./L (PEC 37 and PEC 22, dark grey bars) Roundup WeatherMax®. Control sides were oversprayed with water (white bars). Bars represent the mean + SEM (n = 6 - 10). Data were analyzed using *t*-tests and significance is indicated if $p < 0.05$. Asterisk (*) indicates significant differences between treatment and control of each wetland. ERC, environmentally realistic concentration; PEC, predicted environmental concentration. n.a., not available.

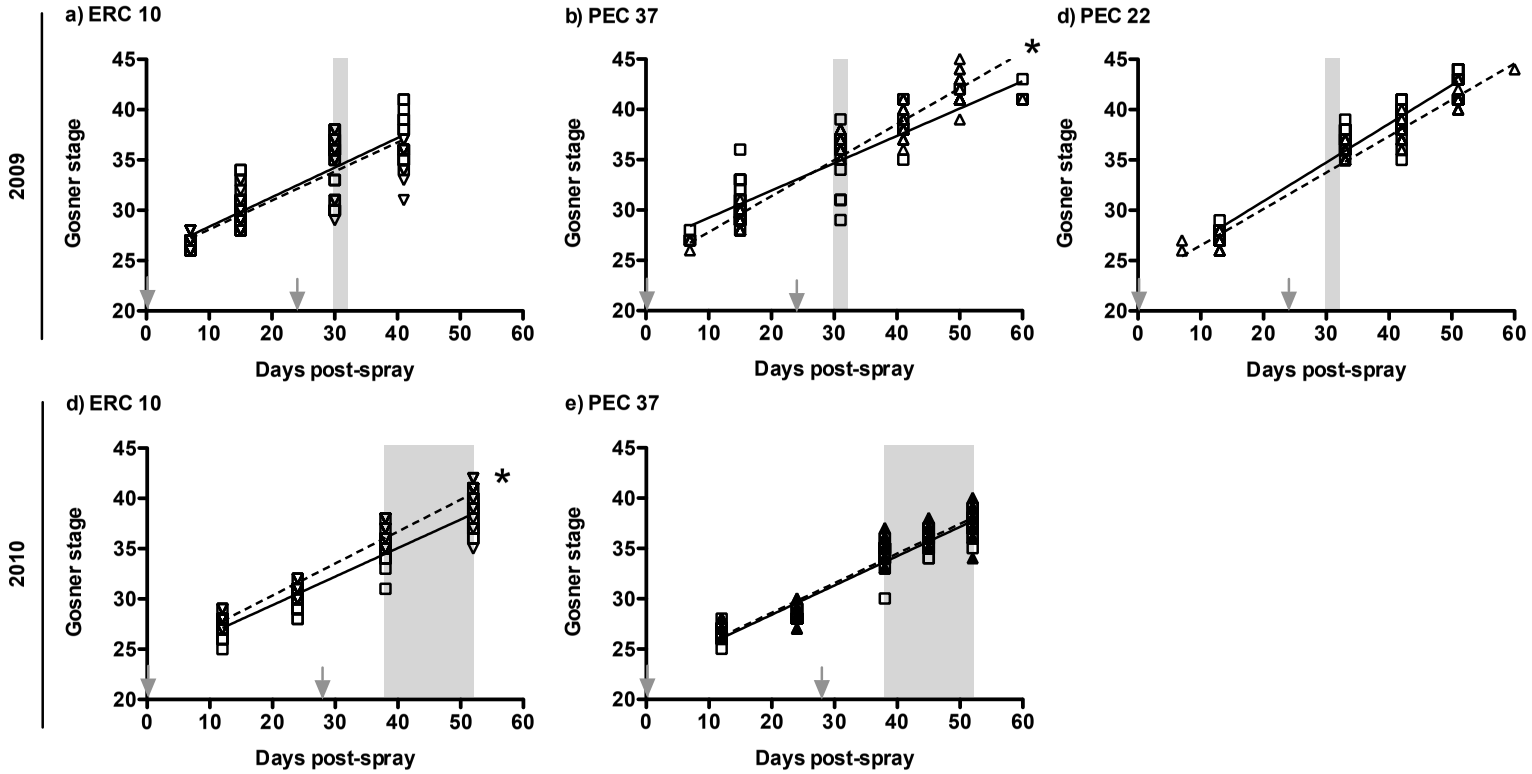


Figure 3.4 Development (Gosner stage) of *L. sylvaticus* tadpoles from experimental wetlands (ERC 10, PEC 37 and PEC 22) in 2009 (a - c) and 2010 (d, e). Treatment halves were oversprayed twice each year (represented by ↓) with either 0.21 mg a.e./L (ERC 10, ▽) or 2.89 mg a.e./L (PEC 37 and PEC 22, △) Roundup WeatherMax®. Control halves were oversprayed with water (□). Data were analyzed by linear regression. Asterisk (*) indicates significant differences between control (solid line) and treatment (dashed line) slope (significance is indicated if $p < 0.05$). ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

Table 3.2 Real-time RT-PCR primer sets and conditions.

Gene	Accession number	Element	Primer sequence (5'-3')	Amplicon location and size (bp)	Melting (°C)
SYBR green					
trβ	HQ630606	Forward	AAGGAACCAGTGCCAAGAATGT	201-286 (86)	85
		Reverse	AACGCTTGCTGTCGTCCAAA		
28S	DQ283702	Forward	GAGATTCCCACTGTCCCTACCT	33-181 (149)	87
		Reverse	GCCTCCCACTTATCCTACACCT		
pgk1	AF175978	Forward	AGGAGGGTAAAGGCAAAGATG	383-465 (83)	84
		Reverse	AGACAGAGAGGCACGGAAAG		
crf	HQ630608	Forward	TGAGAGAGCCCTGATCCAAC	14-132 (119)	85
		Reverse	ATGGTGCCAGAGACACAGAA		
grII	HQ317703	Forward	GACCTGATGTGAGTCCTTCTCC	178-340 (163)	86
		Reverse	TTGTGCTGACCTTCTACTGCTC		
Dual-labeled fluorescent probes					
dio2	HQ630604	Forward	CACCTTTTAGACTTTGCCAGC	291-368 (78)	n.a.
		Reverse	GCTTATAAAGGGAGGTCAGGTG		
		Probe	56-FAM/AGCGCCCTCTTGTCGTCAACTTT/3IABkFQ/		
dio3	HQ630605	Forward	CACCTTGGAGTCCCTGAAG	23-172 (150)	n.a.
		Reverse	CTGTGGCCCTTGGAGAAG		
		Probe	56-FAM/TCTGGTACGGACAGAAGCTCGACT/3IABkFQ/		

trβ, thyroid receptor beta; *28s*, 28S ribosomal RNA; *pgk1*, phosphoglycerate kinase 1; *crf*, corticotropin-releasing factor; *grII*, glucocorticoid receptor; *dio2*, deiodinase type 2; *dio3*, deiodinase type 3.

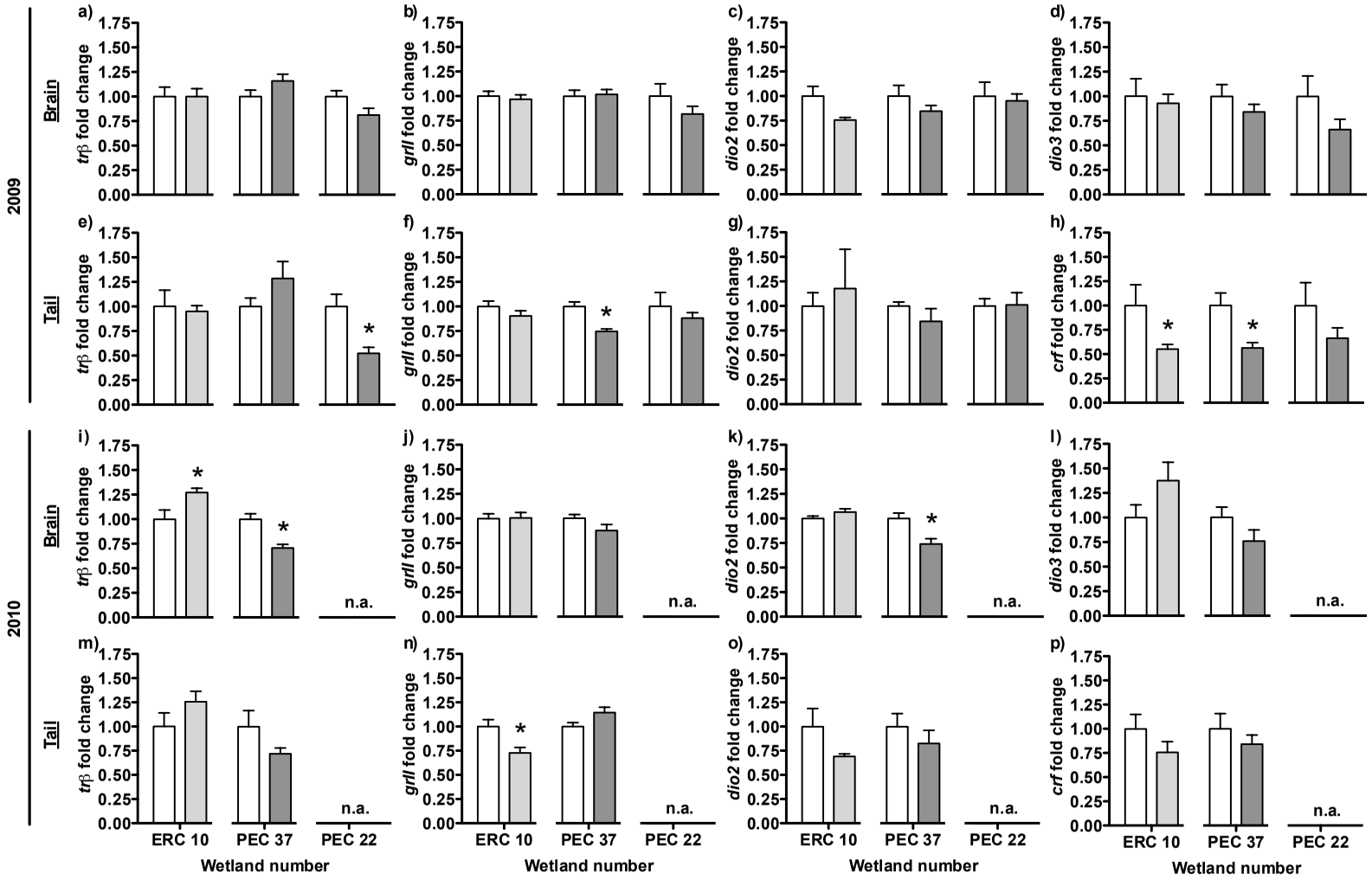

2009
2010
Brain
Tail
a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
k)
l)
m)
n)
o)
p)
trβ fold change
grll fold change
dio2 fold change
dio3 fold change
crf fold change
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
*
n.a.
ERC 10
PEC 37
PEC 22
Wetland number

Figure 3.5 Fold change in mRNA expression of *trβ* (a, e, i, m), *grII* (b, f, j, n), *dio2* (c, g, k, o), *dio3* (d, l) and *crf* (h, p) in the brain (a - d, i - l) and tail (e - h, m - p) of Gosner stage 36/37 *L. sylvaticus* tadpoles collected from 3 wetlands (ERC 10, PEC 37 and PEC 22) in 2009 (a - h) and 2010 (i-p) as determined by real-time RT-PCR. Treatment sides were oversprayed twice each year with either 0.21 mg a.e./L (ERC 10, light grey bars) or 2.89 mg a.e./L (PEC 37 and PEC 22, dark grey bars) Roundup WeatherMax®. Control sides were oversprayed with water (white bars). Data are presented as the normalized fold change in mRNA expression relative to the mean of control side for each wetland. Bars represent the mean + SEM (n = 5 - 7). Data were analyzed using *t*-tests and significance is indicated if $p < 0.05$. Asterisk (*) indicates significant differences between treatment and control of each wetland. *trβ*, thyroid receptor beta; *grII,* glucocorticoid receptor; *dio 2,* deiodinase type 2; *dio 3,* deiodinase type 3, *crf,* corticotropin-releasing factor. ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

Supplementary material

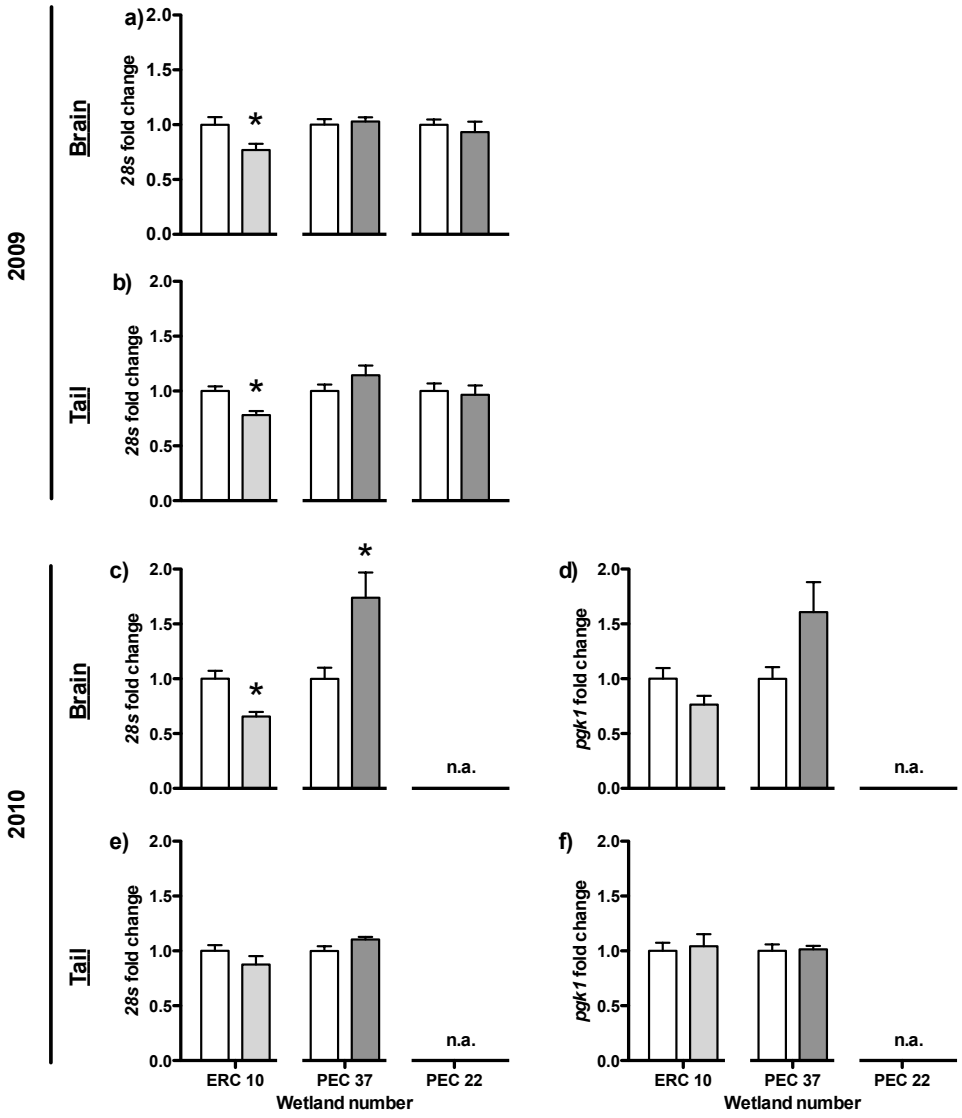
Supplementary Table 3.1 Number of egg masses added to each wetland halves based on volume. ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

Wetland	Egg masses/side	
number	2009	2010
ERC 03	50	17
ERC 05	13	13
ERC 10	25	26
ERC 23	50	13
ERC 39	13	0
ERC 42	13	13
PEC 02	13	17
PEC 13	13	0
PEC 22	13	13
PEC 24	50	6
PEC 37	13	15
PEC 43	25	15

Supplementary Table 3.2 Real time RT-PCR standard curve and assay performance characteristics for each gene and tissue.

		trβ	***dio2***	***dio3***	***crf***	***grII***	***28s***	***pgk1***
2009	**Brain**							
	E	101.1%	98.6%	100.8%	n.a.	99.8%	94.1%	n.a.
	s	-3.295	-3.355	-3.304		-3.327	-3.472	
	R^2	1.000	0.999	0.997		0.991	0.999	
	Tail							
	E	98.0%	100.2%	n.a.	98.0%	90.1%	93.1%	n.a.
	s	-3.371	-3.316		-3.370	-3.583	-3.498	
	R^2	0.995	0.997		0.993	0.995	0.997	
2010	**Brain**							
	E	102.3%	93.7%	97.2%	n.a.	99.2%	99.5%	90.6%
	s	-3.267	-3.483	-3.390		-3.342	-3.333	-3.570
	R^2	0.992	0.999	1.000		0.999	0.995	0.996
	Tail							
	E	93.9%	98.5%	n.a.	92.3%	90.0%	99.4%	90.3%
	s	-3.478	-3.358		-3.520	-3.587	-3.337	-3.580
	R^2	0.995	0.999		0.994	0.996	0.998	0.999

E, efficiency; *s*, slope; R^2, regression coefficient; n.a., not analyzed



Supplementary Figure 3.1 Fold change in mRNA expression of ribosomal RNA 28S (*28s*) and phosphoglycerate kinase 1 (*pgk1*) in brain (a, c, d) and tail (b, e, f) of Gosner stage 36/37 *L. sylvaticus* tadpoles collected from experimental wetlands (ERC 10, PEC 37 and PEC 22) in 2009 (a - b) and 2010 (c - f) as determined by real-time RT-PCR. Treatment sides were oversprayed twice each year with either 0.21 mg a.e./L (ERC 10, light grey bars) or 2.89 mg a.e./L (PEC 37 and 22, dark grey bars) Roundup WeatherMax®. Control sides were oversprayed with water (white bars). Data are presented as the fold change in mRNA expression relative to the mean of control side for each wetland. Bars represent the mean + SEM (n = 5 - 7). Data were analyzed using *t*-tests and significance is indicated if $p < 0.05$. Asterisk (*) indicates significant differences between treatment and control of each wetland. n.a., not available.

Supplementary Table 3.3 Aqueous aminomethylphosphonic acid (AMPA) concentration (mg/L) measured in control and treatment sides of experimental wetlands after the two sprays in 2010. The treatment sides were oversprayed with Roundup Weathermax® at the environmentally realistic concentration (ERC, 0.21 mg a.e./L) or predicted environmental concentration (PEC, 2.89 mg a.e./L). Control sides were oversprayed with water.

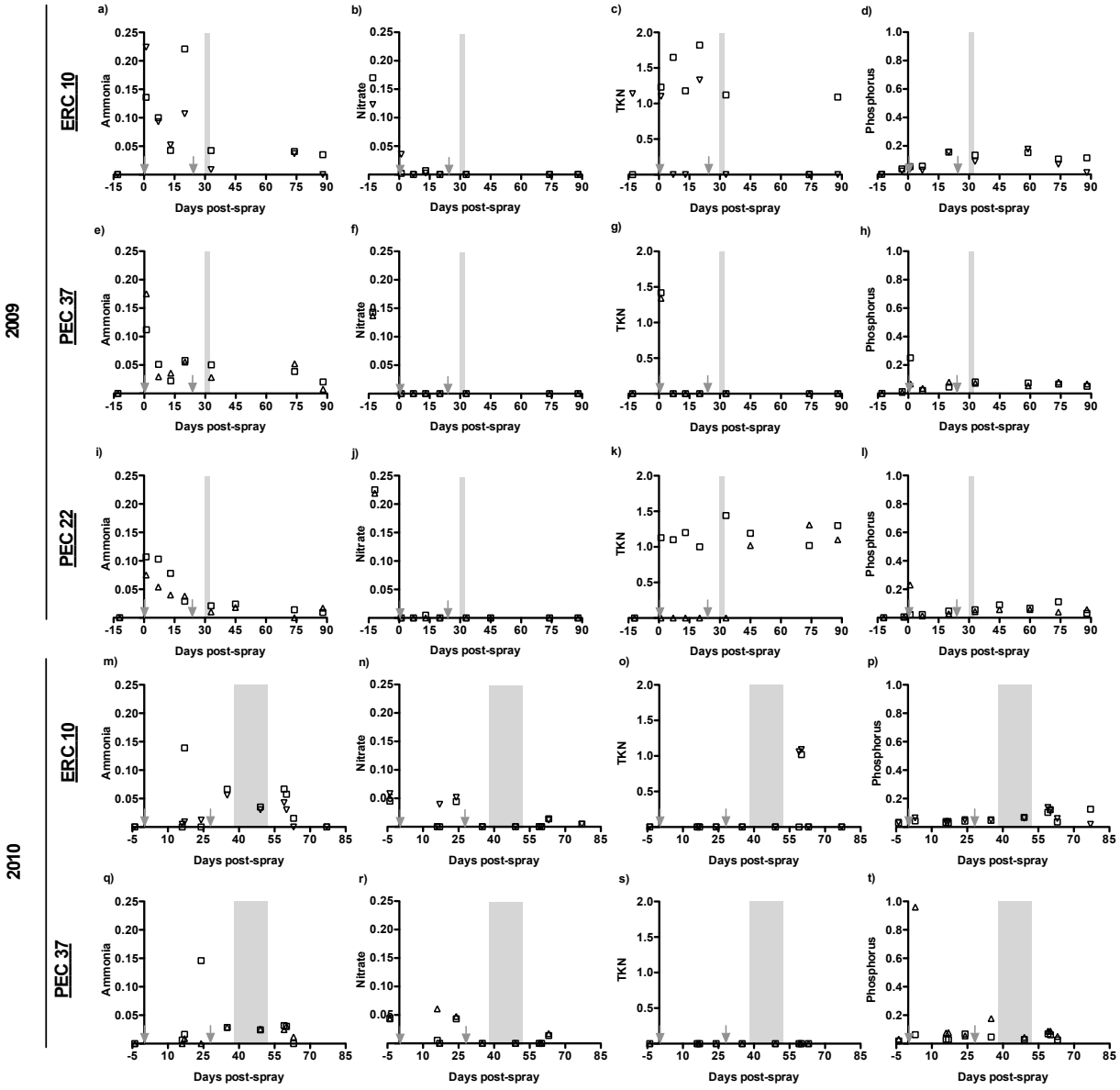
		Wetland number			
		ERC 10		PEC 37	
Spray	**Days post-treatment**	**Control**	**ERC**	**Control**	**PEC**
1	0		0.013	0	0.118
	1	0	0.003	0.005	0.126
	3		0.001	0.009	0
	7		0		0.392
2	0	0	0.061		0.126
	1	0.013	0.015	0	0.193
	3	0	0.003		0.362
	7		0		0.194

Blanks = not measured

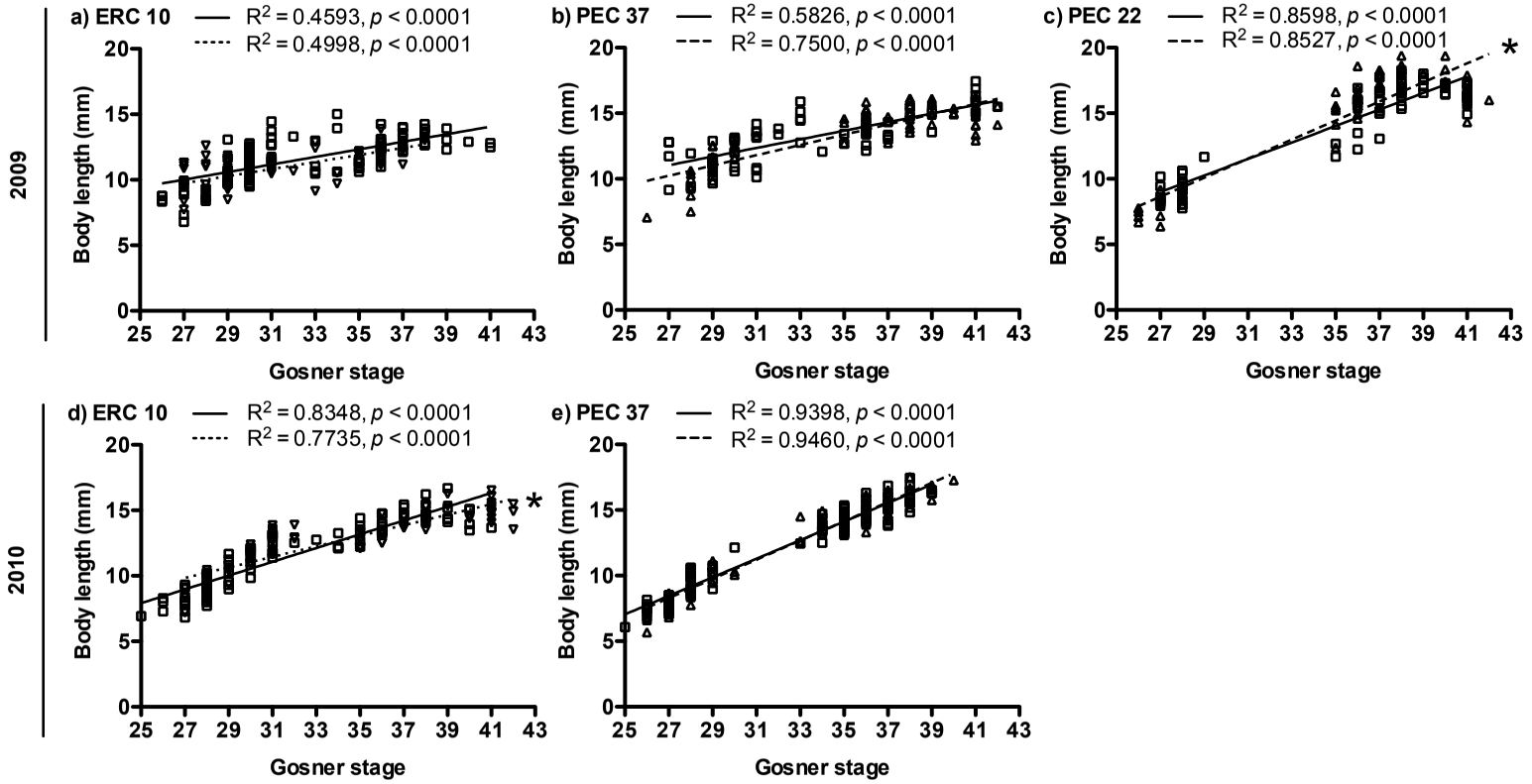
Supplementary Table 3.4 Average pH, conductivity (S/m), dissolved oxygen (DO, mg/L), and depth (cm) ± standard deviation (SD) in control and treatment sides of experimental wetlands between the first spray date and sampling of Gs 37 tadpoles. The treatment sides were oversprayed with Roundup Weathermax® at the environmentally realistic concentration (ERC, 0.21 mg a.e./L) or predicted environmental concentration (PEC, 2.89 mg a.e./L). Control sides were oversprayed with water.

	Wetland number									
	ERC 10				**PEC 37**				**PEC 22**	
	2009		**2010**		**2009**		**2010**		**2009**	
	Control	**ERC**	**Control**	**ERC**	**Control**	**PEC**	**Control**	**PEC**	**Control**	**PEC**
Temperature ± SD (°C)	15.18 ± 3.71	17.97 ± 7.88	13.11 ± 3.34	15.72 ± 4.99	16.00 ± 2.86	15.54 ± 2.32	11.54 ± 2.87	15.79 ± 4.85	23.57 ± 6.45	21.74 ± 4.56
pH ± SD	5.98 ± 0.10	6.09 ± 0.03	5.70 ± 0.13	6.17 ± 0.04	6.00 ± 0.29	6.11 ± 0.23	5.76 ± 0.06	5.84 ± 0.09	5.81 ± 0.13	5.46 ± 0.08
Conductivity ± SD	26.7 ± 4.3	25.23 ± 11.3	n.a.	n.a.	25.73 ± 18.23	15.90 ± 1.6	n.a.	n.a.	15.33 ± 0.83	14.50 ± 1.92
DO ± SD	4.76 ± 2.70	6.19 ± 6.19	n.a.	n.a.	5.73 ± 2.20	4.33 ± 0.75	n.a.	n.a.	9.42 ± 0.39	9.12 ± 0.50
Depth ± SD	31.1 ± 16.73	29.1 ± 18.19	27.00 ± 11.40	24.58 ± 11.87	40.68 ± 34.58	45.70 ± 28.81	76.83 ± 14.44	79.25 ± 13.52	17.00 ± 4.06	23.75 ± 4.19

n.a., not available.



Supplementary Figure 3.2 Ammonia, nitrate, total Kjeldahl nitrogen (TKN) and phosphorus concentrations (mg/L) in ERC 10 (a - d, m - p), PEC 37 (e - h, q - t) and PEC 22 (i - l) in 2009 (a - l) and 2010 (n - t). Detection limits for analysis of ammonia, nitrate and TP were 0.005, 0.002 and 0.002 mg/L, respectively. Control (□); WeatherMax® ERC (▽); WeatherMax® PEC (△); Spray dates (⬇); Gs 36/37 sampling (shaded area); TKN = ammonia-nitrogen + organic nitrogen.



Supplementary Figure 3.3 Body length (cm) at different Gosner stages of tadpoles from experimental wetlands (ERC 10, PEC 37 and PEC 22) in 2009(a - c) and 2010(d, e). Treatment halves were oversprayed twice each year with either 0.21 mg a.e./L (ERC 10, ▽) or 2.89 mg a.e./L (PEC 37 and PEC 22, △) Roundup WeatherMax®. Control halves were oversprayed with water (□). Data were analyzed by linear regression. R^2 values are indicated and p values indicate if slope is significantly different from 0. Asterisk (*) indicates significant differences between control (solid line) and treatment (dashed line) slope (significance is indicated if $p < 0.05$). ERC, environmentally realistic concentration; PEC, predicted environmental concentration.

Supplementary Table 3.5 T-test results comparing mRNA expression in brain and tail tissues of Gosner stage 36/37 *L. sylvaticus* tadpoles between control and treatment side of experimental wetlands (ERC 10, PEC 37 and PEC 22). The treatment sides were oversprayed with Roundup Weathermax® at the environmentally realistic concentration (ERC, 0.21 mg a.e./L) or predicted environmental concentration (PEC, 2.89 mg a.e./L). Control sides were oversprayed with water.

Year	Wetland	Tissue	Gene[a]	T	*p* value
2009	ERC 10	Brain	*trβ*	0.000	1.000
			dio2	3.819	0.074
			dio3	0.001	0.971
			grII	0.238	0.634
2009	PEC 37	Brain	*trβ*	2.840	0.118
			dio2	1.401	0.259
			dio3	0.798	0.389
			grII	0.057	0.815
2009	PEC 22	Brain	*trβ*	4.315	0.060
			dio2	0.000	0.998
			dio3	1.336	0.270
			grII	1.516	0.242
2009	ERC 10	Tail	*trβ*	0.087	0.773
			dio2	0.000	0.994
			crf	5.580	0.036
			grII	1.627	0.226
2009	PEC 37	Tail	*trβ*	2.213	0.163
			dio2	2.367	0.150
			crf	7.108	0.021
			grII	23.217	< .001
2009	PEC 22	Tail	*trβ*	12.056	0.005
			dio2	0.029	0.868
			crf	0.889	0.364
			grII	0.627	0.444
2010	ERC 10	Brain	*trβ*	7.582	0.019
			dio2	2.407	0.149
			dio3	2.269	0.163
			grII	0.010	0.923
2010	PEC 37	Brain	*trβ*	20.731	0.001
			dio2	10.821	0.008
			dio3	2.220	0.167
			grII	2.450	0.149
2010	ERC 10	Tail	*trβ*	2.074	0.175
			dio2	3.425	0.089
			crf	1.686	0.219
			grII	8.961	0.011
2010	PEC 37	Tail	*trβ*	2.516	0.139
			dio2	0.878	0.367
			crf	0.751	0.403
			grII	4.494	0.056

CHAPTER 4

General discussion

4.1. Thesis result summary

The first objective of this thesis was to determine if exposure to Roundup WeatherMax® at agriculturally relevant concentrations and application rates influences the survival and development of wood frogs tadpoles (*Lithobates sylvaticus*) under laboratory conditions (Chapter 2). Tadpoles were exposed to two pulses of Roundup WeatherMax® at an ERC and PEC. Results indicate that exposure to the PEC is extremely toxic to wood frog tadpoles, and show changes in the expression of thyroid- and stress-related genes after exposure to ERC. Even though the changes in mRNA, at low exposure concentrations (ERC) and with agriculturally relevant exposure conditions (pulses), were not high enough to translate in major phenotypic and developmental changes, gene expression results demonstrated that glyphosate-based herbicides have the potential to alter normal hormonal responses during metamorphosis. It is possible that hormonal changes at this critical period in development could prevent (or upset) metamorphosis, since small changes in the thyroid axis have been shown to affect metamorphic outcome (Shi, 2000). The second objective was to compare results from Roundup WeatherMax® exposure to Vision®, and to determine which ingredient(s) in the formulations are responsible for effects on development. Survival, growth and gene expression results indicated that Roundup WeatherMax®, which contains an unknown surfactant, has greater toxicity than the Vision® formulation containing the POEA surfactant. Contrary to what we hypothesized, results suggest

that under realistic exposure scenario (i.e., pulse exposure) POEA is not the sole ingredient responsible for the observed effects on growth and development (Chapter 2). However, we did find that chronic exposure to the POEA surfactant at PEC (1.43 mg/L) is extremely toxic to wood frog tadpoles under laboratory conditions. Because laboratory studies do not capture the wide range of environmental parameter that can influence toxic responses, it is important to determine to what extent lab based studies of environmental contaminants can be extrapolated to more complex situations in nature. For this reason, we conducted parallel field exposures to Roundup WeatherMax® to determine if developmental effects were observed under natural exposure conditions at the LEWA site (Chapter 3). Pulse exposures to Roundup WeatherMax® at the ERC and PEC in these wetlands significantly affected growth, development and the expression of thyroid- and stress-related genes of wood frog tadpoles, which is indicative of disruption to these pathways. However, differences observed between control and treatment groups were generally not consistent between wetlands and years (2009 and 2010), nor were they consistent with the more severe results obtained in the parallel laboratory exposure.

4.2. Comparison between laboratory and field experiments

Laboratory studies provide a useful approach to understand interactive mechanisms and provide controlled environments for comparisons. However, laboratory studies investigating pesticide risk assessments generally use short-term acute or chronic exposures designs, which may be poor representations of real-world herbicide exposure scenarios. Differences in experimental conditions (e.g., pH, temperature, food availability, predator cues, sediments, water

chemistry) between laboratory and field may influence the physiological state of the organism at the time of exposure. These factors may have contributed to the different responses across experimental approaches. Larger effects sizes tend to be observed in laboratory experiments compared to effect sizes in natural populations (Thompson *et al.,* 2004). Therefore, comparisons between results obtained from laboratory and whole-ecosystems experiments are required to understand the relationship between these effects sizes and the reliability of laboratory results as predictors of effects in the ecosystem. The experiments described in this thesis allow us to compare for the first time developmental responses to glyphosate in laboratory populations with populations in natural ecosystems, using individuals collected from the same natural populations. Toxicological effects of exposures to glyphosate-based herbicides in amphibian larvae have been a topic of hot debate in recent years (reviewed by Relyea*,* 2011). Our results indicate that differences in response amongst studies may be largely attributed to differences in sensitivity between tadpoles raised under laboratory versus field conditions, or between exposure conditions within these different experimental settings.

When exposed to environmentally realistic concentrations at relevant application rates, we found differences in responses between the two exposure scenarios. Although survival was not directly measured in the field, abundance measurements after the second pulse did not show any major differences between control and treated halves of the experimental wetlands at either the ERC or PEC. Thus, contrary to the high mortality observed in the lab, exposure to PEC in the field did not result in high mortality relative to control. This supports the hypothesis that laboratory studies tend to overestimate effects of glyphosate-based herbicides in comparison to

natural exposures (Thompson *et al.,* 2004; Wojtaszek *et al.,* 2004). Furthermore, we observed a slight but significant acceleration in development rate and decrease in body size of pro-metamorphic tadpoles exposed to the environmentally realistic concentration in natural wetlands (ERC 10, 2010). The decrease in body size of pro-metamorphic tadpoles is consistent with that observed in the laboratory exposure. However, we observed a decrease in developmental rate in tadpoles exposed to the PEC in the laboratory.

The current findings also indicate that glyphosate-based herbicides have the potential to alter endocrine systems that are directly related to development, which explains how effects could be mediated. However, gene expression results from the field experiment (Chapter 3) were generally not consistent with the parallel laboratory exposure (Chapter 2), which suggests that glyphosate-based herbicides do not directly affect thyroid pathways related to metamorphosis. Rather, the response appears to be indirect and is suggestive of compensatory mechanisms, which we think occurs as a means to regulate metamorphosis-related endocrine homeostasis. Alternately, it is possible that variations in the observed effects are the results of interactive effects and not a direct consequence of the actual herbicide. Interactions with biotic and abiotic conditions (pH, temperature, UV, substrate, competitor and predatory stress, food availability, etc.) have the potential to either increase or reduce the impact of the herbicide. Thus, differences in abiotic and biotic stressors between laboratory and field experiments may explain variation in responses amongst these experimental settings. Our laboratory recently compared developmental profiles of several genes involved in metamorphosis in wood frogs maintained in the laboratory and those collected directly from natural populations. We determined that tadpoles raised in the

laboratory were good representations of those in the field under normal circumstances (i.e., no exposure) (Navarro-Martin *et al.,* 2012). However, given the range of environmental factors occurring in natural wetlands compared to stable conditions in laboratory aquaria, it would not be surprising to find that this causes individuals in field settings to react differently to contaminants than laboratory-reared individuals.

The pH of water in natural wetlands is often different from that of de-chlorinated tap water used for laboratory experiments, which may be one explanation for differences in the effects of glyphosate-based herbicides in the lab versus the field. Average pH in our experimental wetlands (Chapter 3) was 5.9 whereas pH in the lab exposure (Chapter 2) was 7.1. Higher pH has been found to increase the toxicity of tadpoles to glyphosate-based herbicide in both lab and field experiments (Edginton *et al.*, 2004; Chen *et al.*, 2004; Wojtaszek *et al.*, 2004). One hypothesis for the increased toxicity is that variations in pH can affect the molecular structure on the gill membrane and influence the toxicity of the active ingredient (Chen *et al*., 2004). The Canadian environment is quite heterogeneous and variable, and the pH of wetlands is also highly variable ranging from 4.5 to 9.1, therefore it is extremely important to consider pH when assessing potential impacts of these herbicides in aquatic environments.

It is generally assumed that the toxicity of chemicals increases with increasing temperature due to increased absorption and chemical reaction rates. Folmar (1979) showed that higher temperatures correlate with increased toxicity of glyphosate in fish. However, to our knowledge, no studies have examined the effects of temperature on the toxicity of glyphosate herbicide to tadpoles. In addition, increases in temperature have been shown to increase developmental rates

and growth of tadpoles (Collins, 1979). Differences in temperature between lab and field experiments could be another explanation for the different results obtained between these scenarios, but this requires further investigation. In our laboratory study (Chapter 2) the average temperature was 21 °C with very little deviation, whereas temperatures in the experimental wetlands (Chapter 3) were generally lower but fluctuated much more (5 – 35 °C over the course of a day and throughout the entire season). In the field, the average water temperature was approximately 16 °C when the herbicide was being applied, so higher temperatures in the lab could have contributed to the variability in responses observed.

Ultraviolet (UV) radiation is another abiotic stressor that has the potential to act synergistically with chemical contaminants to alter toxicity. Exposure to UV radiation can either increase or decrease the toxicity of contaminants by accelerating breakdown into more (e.g., carbaryl) or less (e.g., permethrin) toxic forms, respectively (Zaga *et al.,* 1998; Puglis and Boone, 2011). Lund-Høie and Friestad (1986) demonstrated that UV-light degrades glyphosate, and that impurities in the water decreased the degradation rates. Although UV-B radiation has been shown to slow and even prevent the development of tadpoles (Croteau *et al.,* 2008), very few studies have examined the synergistic effect of UV and pesticides such as glyphosate-based herbicides. To our knowledge, only one study has examined the combined effects of UV and these herbicides on amphibians and they found that UV slightly increased the effects of Roundup® on the mortality of green frog tadpoles (Puglis and Boone, 2011). Thus, degradation by UV radiation in natural environments could be yet another explanation for the reduced toxicity of the herbicide in the field. However, depending on the amount of impurities and dissolved organic carbon in the

water, which reduces the amount of UV penetration (Puglis and Boone, 2011; Morris *et al.,* 1995), the amount of photo-degradation in wetlands might be reduced.

Various researchers suggest that the presence of soil in aquatic environment should reduce the toxicity of amphibians to glyphosate-based herbicide since glyphosate and POEA bind rapidly to soil, sediment and suspended particles, and are degraded by microbes (Giesy *et al.,* 2000; Malone *et al.,* 2004). Therefore, another explanation for the differences between laboratory and field exposures is that glyphosate and POEA might dissipate (e.g., sorption) and degrade (e.g., microbial break-down) faster in the field. The laboratory experiment (Chapter 2) was designed to mimic the natural decrease of glyphosate in water. However, measured glyphosate concentration from the 3 experimental wetlands indicated even faster dissipation/degradation than the 25% decrease per day that was used for the laboratory experiment. Thus, the reduced toxicity observed in the field compared to the laboratory could also be attributed to faster dissipation and degradation processes that reduce the exposure concentration in the field.

Biotic variables, which are largely absent from laboratory settings, may also influence the toxicity of chemicals in the environment. For example, variations in density or intraspecific competition have the potential to influence how tadpoles respond to the herbicide. High tadpole density leads to increased intraspecific competition, which has been shown to negatively impact survival, growth, and development (Relyea, 2002). Jones and colleagues (2010) showed that toxicity of Roundup Original MAX® increased as bullfrog density increased. In the present study, initial wood frog tadpole densities in both laboratory and field experiment was ~1 tadpole/liter. Throughout the experiments densities were altered due to uneven mortality between

the laboratory treatments and experimental wetlands. Although survival was not directly measured in the field, abundance estimates decreased over time in all experimental wetlands regardless of the treatment (ERC or PEC) whereas survival in the lab was ≥ 78% in both control and treatment (ERC). This suggests that wood frog tadpole density was lower in the field than in the lab. However, in the field, wood frog tadpoles are under additional stress caused by interspecific competition, which can also influence the effects of contaminants in the environments (Jones *et al.,* 2011). Moreover, predatory stress has been shown to have detrimental effects on tadpole growth and survival, and can also act synergistically with chemical contaminants to influence toxicity (Relyea, 2005b; Relyea, 2003; Relyea and Mills, 2001). Relyea (2005b) showed that predator cues in the water increased the toxicity of Roundup Original® for *L. sylvaticus* tadpoles. Thus, predator cues in natural habitat can be another contributing factor to the inconsistency between laboratory and field studies.

Contaminants in the environment can influence the availability of various food sources for amphibian larvae. It is not surprising that herbicides have the potential to negatively affect periphyton growth, a main food source for tadpoles, but is it less clear how this might subsequently lead to decreased growth and development. Food deprivation has the potential to affect growth and development of tadpoles as well as the toxicity of glyphosate-based herbicides. For example, Chen *et al.* (2004) suggested that the interactive effects of high pH and reduced food could increase the toxicity of Vision® herbicide to tadpoles. In the present study, tadpoles appeared to have ample food in both exposure scenarios; however, tadpoles in the laboratory might lack nutrients that they would normally have in nature, which could affect growth and

developmental responses (Kupferberg, 1997). Furthermore, the way that differences in biotic and abiotic environmental factors influence the response to glyphosate-based herbicides may depend on which components of the food web are affected.

A final possible explanation for difference between the laboratory and whole-ecosystem experiments may relate to the different levels of tadpole survival. Survival of control animals in the laboratory was greater than 78% throughout the experiment, whereas overall tadpole abundance clearly decreased over time in the field. Importantly, Melvin and Houlahan (2012) recently demonstrated that survival is consistently much higher in laboratory than natural populations, and hypothesized that this fundamental difference causes laboratory populations to exaggerate effect sizes. Thus, animals that do survive and that were sampled in the field might be more fit than tadpoles in the lab.

4.3. Concluding remarks and future direction

As part of the Long-term Experimental Wetland Area project, this research contributes to overall knowledge of the impacts of glyphosate-based herbicides on aquatic communities. This is the first experiment of its kind to investigate endocrine responsiveness in tadpoles exposed to a contaminant in a natural experimental wetland. Similar whole ecosystem system studies, such as those carried out at the Canadian Experimental lakes Area (ELA), have provided some of the most beneficial environmental research in the past several decades (Carpenter *et al.* 1995). It is widely recognized that these types of whole ecosystems studies provide the most relevant, direct measurements of the effects of anthropogenic stressors on wildlife and wildlife communities. It is

for this reason that we chose to emulate ELA research with our investigation of glyphosate-based herbicides at the LEWA.

There has been quite a controversy about the effects of glyphosate on amphibians due to the variability of responses reported in the literature (reviewed by Relyea, 2011). Studies using different species, life stages, formulations, doses, timing, frequency, and experimental conditions are reporting different results. This indicates that the effects of this herbicide on tadpoles strongly depend on these factors. Based on the results of the present study, pulse exposure to environmentally realistic concentrations of Roundup WeatherMax® in natural wetlands does not appear to pose a significant direct threat to wood frog development. However, as mentioned, a number of factors can influence toxicity of glyphosate-based herbicides and these should be considered when determining the potential impacts of these herbicides on organisms in the natural environment. In natural situations of high environmental stress, tadpoles may not be able to cope adequately with the additional stress caused by contaminants even at these low environmental concentrations. Natural wetlands are dynamic ecosystems with a vast range of environmental conditions, thus it is not surprising that the results obtained from the field present a large amount of variability that can complicate interpretation. However, it is clear that laboratory studies investigating a single stressor and single species do not reflect the complex interactions that can influence toxicity in natural environments; therefore results should be interpreted with caution. It is important to keep in mind that even though we do not show major effects on development at the ERC, sub-lethal effects can have long-term effects on populations

(e.g., Kidd *et al.*, 2007). Thus, long-term monitoring is needed for accurate assessment of these herbicides.

In addition, an important question that needs to be considered as researchers advance in their understanding of how EDCs affect metamorphosis at the genetic level, is whether or not changes in mRNA levels translate into biologically significant changes in functional protein levels, and whether these differences actually cause significant effects on the organism. This type of comprehensive analysis is often difficult with amphibian larvae, because tadpoles are generally quite small, and blood and tissues for analysis are often limited. However, new methods in proteomics are highly sensitive (Martyniuk *et al.,* 2012), and may offer the possibility of such studies on tadpoles. Furthermore, glyphosate-based herbicides have been found to cause a variety of effects in developing tadpoles in the laboratory, including gonadal abnormalities (Howe *et al.,* 2004) and DNA damage (Clements *et al*., 1997), but few of these responses have been considered under realistic exposure conditions in natural wetlands. Future investigations of these effects under realistic exposure conditions would contribute substantially to answering questions regarding the toxicity of glyphosate formulations on populations of tadpoles in natural environments.

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Appendix 1. Other contributions to research

a. Other articles published in refereed journals

Navarro-Martín L., **Lanctôt C**., Edge, C., Houlahan J. & Trudeau V. (2012) Expression profiles of metamorphosis-related genes during transformations in wild wood frog (*Lithobates sylvaticus*) tadpoles. *Canadian Journal of Zoology* **90:** 1059-1071.

Melvin S. D., **Lanctôt C.**, Craig P. M., Moon T. W., Peru K. M., Headley J. V. & Trudeau V. L. Effects of naphthenic acid exposure on development and liver metabolic processes in anuran tadpoles. *Environmental Pollution* (Submitted).

Navarro-Martín L., Velasco-Santamaría Y. M., Duarte P., **Lanctôt C.** & Trudeau V. Sexing frogs by real-time PCR: Using *cyp19* aromatase as an early ovarian differentiation marker in both laboratory and wild animals. *Sexual Development* (Submitted).

b. Conference proceedings (*Presenting author)

*Melvin S., **Lanctôt C.**, Craig P., Moon T., Peru K., Headley J. & Trudeau V. (2012) Naphthenic acid concentrations found in groundwater in the Canadian oil sands region affect anuran larval development and metabolic processes. Oral presentation in the SETAC Laurentian Conference, Peterborough, Canada; 22 June 2012.

*Bulaeva E., Erdman L., **Lanctôt C.**, Navarro-Martin L. & Trudeau V. (2012) Consequences of Chemical Disruption on Growth and Sexual Development Pathways in Wood Frog Tadpoles. Poster presentation in the Ottawa Carleton Institute for Biology Symposium, Ottawa, Canada; 25-26 April 2012.

*Navarro-Martín L., Velasco-Santamaría, Y.M., Duarte-Guterman, P., **Lanctôt C.** & Trudeau V. (2011) Sexing frogs by real-time pcr: Using cyp19 aromatase as an early ovarian differentiation marker. Oral presentation in the ISAREN Conference and poster presentation in the NASCE Conference, Ann Arbour, Denver, USA; 11-16 July 2011.

***Lanctôt C.**, Navarro-Martín L., Robertson C., Jackman P., Pauli B. & Trudeau V. (2011) Glyphosate-based formulations are toxic and disrupt wood frog (*Lithobates sylvaticus*) development. Poster presentation in the Canadian Society of Zoologists Conference, Ottawa, Canada; 16-20 May 2011.

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