EVALUATING THE SUB-LETHAL TOXICITY OF THE ORGANOPHOSPHATE PESTICIDE, CHLORPYRIFOS, ON THE AMPHIBIAN, *XENOPUS LAEVIS*

A Thesis Submitted to the College of
Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Toxicology Graduate Program
University of Saskatchewan
Saskatoon, Saskatchewan, Canada

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ABSTRACT

Chlorpyrifos (CPF) is an organophosphate pesticide used extensively in Canada and around the world. Due to its highly conserved mechanism of action involving inhibition of acetylcholinesterase (AChE), CPF has the ability to exert toxicity on non-target species in aquatic systems. In fish species, exposure to CPF has been associated with a range of adverse effects across physiological endpoints including abnormal development, inhibition of AChE, immunomodulation, and molecular level effects such as altered expression of specific genes and global transcriptomes. However, the literature on amphibians exposed to CPF is not as extensive despite the known global declines of amphibian species and the hypothesized links between these declines and anthropogenic pesticide contamination of aquatic systems worldwide. The overall objective of this thesis was to gain a better understanding of the sub-lethal effects of CPF exposure on the model amphibian, *Xenopus laevis*, across levels of biological organization from molecular to whole animal.

The first study (Chapter 2) examined the molecular toxicity pathways and mechanisms of toxicity after short-term exposure of early life-stage (ELS) *X. laevis* to CPF using whole body transcriptome analyses. The ELS transcriptomic responses were then compared to apical outcomes of chronic exposure to CPF to determine if identified dysregulated pathways could provide early indicators of these adverse outcomes. Post-hatch individuals were exposed to nominal CPF concentrations of 0.4, 2, or 10 μg L^{-1}. A subset of individuals were sampled at 96 hours (h) for whole-body transcriptomic analysis and remaining individuals were transferred to tanks for long-term exposure through to metamorphic climax (~ 75 days). Pathway analysis revealed dysregulated pathways that were related to outcomes known to be associated with exposure to CPF such as altered serine hydrolase activity, impacted metabolic processes, and immune-related outcomes. Other dysregulated pathways with less precedence in the literature included vasculature development and sensory perception of light stimulus. Apical outcomes of chronic CPF exposure included inhibition of AChE activity, increased relative liver weight, and a decrease in percentage of individuals that reached metamorphic climax. Dysregulation of serine hydrolase associated pathways after ELS CPF exposure is in agreeance with the decrease in AChE (a serine hydrolase enzyme) activity observed in the brains of individuals at metamorphic climax. Additionally, an increase in relative liver weight after chronic CPF

exposure could be related to dysregulation of ELS pathways associated with metabolic processes and immune function. In fact, several pathways related to immune function were depleted.

In Chapter 3, we more closely examined the potential immunotoxicity of sub-lethal CPF exposure. Post-metamorphic individuals were exposed 1 or 10 μg L^{-1} CPF (nominal) for 7 days (d), then administered a phosphate buffered sodium (PBS) control injection or a lipopolysaccharide (LPS) injection to stimulate an inflammatory response. At 24 h post-injection, morphometric indices were recorded and tissues were sampled for differential leukocyte counts (flow cytometry), liver pro-inflammatory cytokine expression (qPCR), and kidney histopathology. At 1 μg L^{-1} CPF, there was a decrease in circulating lymphocytes, an increase in circulating granulocytes, and an increase in the granulocyte:lymphocyte (GL) ratio regardless of immune state. Liver expression of pro-inflammatory cytokines TNF-α and CSF-1 was increased in individuals exposed to 10 μg L^{-1} CPF, independent of immune state. Exposure to 10 μg L^{-1} CPF increased kidney epithelial cell height (by 18 %) and decreased lumen space in the convoluted tubules of the kidney. This study provided evidence that exposure to CPF can lead to changes in key biomarkers of immune status in amphibians in both immune-rested (PBS-injected) and immune-stimulated (LPS-injected) states. Additionally, we found that LPS was an effective mitogen in our study, capable of inducing a robust and measurable stress response in *X. laevis*. This response included a decrease in circulating lymphocytes, and increase in circulating monocytes, and an increase in the GL ratio. In addition, increased liver expression of pro-inflammatory cytokines TNF-α, IL-1β, and CSF-1 was induced by LPS injection. We conclude that LPS is an appropriate immunostimulatory agent in an immune challenge assay using *X. laevis* and that exposure to CPF does not appear to impact the response to LPS exposure.

Overall, our findings show that exposure to environmentally relevant concentrations of CPF has the ability to impact amphibians at multiple levels of biological organization. A number of affected molecular pathways warrant further study in terms of the underlying mechanisms of CPF-mediated toxicity as well as the associated outcomes of CPF exposure in amphibians. This research provides novel data on the effects of CPF exposure to amphibians, which are generally overlooked and under-represented in the literature despite links between pesticide exposure and globally declining amphibian populations.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my graduate supervisor Dr. Natacha Hogan for her continuous leadership and guidance throughout the completion of this thesis. I have met very few people that come even close to the level of passion, caring, and support that Natacha offers to each of her students. For the 5 years that I worked with her, Natacha dedicated much of her time to teaching me about logical, rational, and honest science. More than that, however, I will be forever grateful for the lessons on curiosity, persistence, drive, balance, and hard work that Natacha teaches by exemplifying in everything that she does.

I would also like to thank my committee members, Dr. Markus Hecker and Dr. Lynn Weber for their direction and assistance during my Master's degree. I thank the staff and students of the Toxicology Centre and Aquatic Toxicology Research Facility at the University of Saskatchewan. In particular, I acknowledge the hard work and dedication of Yu Su, Darja Besermenji, Kaitlyn Baldwin, and Chelsea Grimard during exposures and sampling. I would like to thank Melanie Gallant and Zachary Currie for their expertise and leadership in all aspects of *Xenopus laevis* handling, care, sampling, and lab work. I would especially like to thank Alper James Alcaraz for his leadership, guidance, and constant willingness to help his fellow students out. I would like to thank my "Tox Fam" without whose friendship and support this degree absolutely would not have happened.

I would like to acknowledge the funding sources and partners that supported this research including: Genome Canada, Genome Quebec, Genome Prairie, Environment and Climate Change Canada, and colleagues at McGill University. I also acknowledge the support provided to me through the Toxicology Centre Devolved Scholarships.

Most importantly, I would like to thank my parents for their unconditional support throughout every life decision I've made and for the love that they show me at all times – even when they don't fully understand what acetylcholinesterase is or why I am crying about it on the phone again. I thank my little sisters, Kimberley and Kaitlyn, for being the most inspiring people I have ever met; Hayley, for celebrating every accomplishment and milestone in my life for the past 20 years; and Rylan, for his endless love and support.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
ATV	*Ambystoma tigrinum* virus
BCF	bioconcentration factor
CCME	Canadian Council of Ministers of the Environment
cDNA	complementary DNA
CPF	chlorpyrifos
CSF-1	colony stimulating factor 1
CYP	cytochrome P450
DEG	differentially expressed gene
DiOC5	3,3-dipentyloxacarbocyanine iodide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpi	days post injection
EC50	half maximal effective concentration
EF-1	elongation factor 1
ELS	early life stage
FDR	false discovery rate
FETAX	frog embryo teratogenesis assay – *Xenopus*
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
GL	granulocyte:leukocyte
GO	gene ontology
hCG	human chorionic gonadrotropin
H&E	hematoxylin and eosin

HSD	honest significant difference
IL-1β	interleukin 1β
IU	international unit
IUCN	International Union for Conservation of Nature
LC50	median lethal dose
log K_{ow}	octanol-water partition coefficient
LPS	lipopolysaccharide
LSD	least significant differences
mRNA	messenger RNA
MS-222	tricaine methanesulfonate
NF	Nieuwkoop and Faber
OP	organophosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMRA	Pest Management Regulatory Agency
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
SVL	snout-vent length
TCP	3,5,6trichloro-2-pyridinol
TNF-α	tumour necrosis factor α
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

PREFACE

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate Studies and Research guidelines for a manuscript-style thesis. Therefore, there is some repetition between the material presented in each chapter. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 4 is a general discussion and overall conclusion. Chapters 2 and 3 are organized as manuscripts for publication in peer-reviewed scientific journals and a description of author contributions is provided in the preface for these chapters. References cited in each chapter are combined and listed in the References section of the thesis. Supporting information associated with the research chapters are presented in the Appendix section at the end of this thesis

CHAPTER 1

INTRODUCTION

Preface

Chapter 1 is a review of the available literature regarding the topics of chlorpyrifos, it's physicochemical properties, fate in the environment, mechanism of toxic action, as well as toxicity to aquatic organisms, and the current state of knowledge regarding the effects of chlorpyrifos on various amphibian species. Chapter 1 also includes the overall goals and objectives of the research studies conducted as part of this thesis, as well as the null hypotheses.

1.1 Chlorpyrifos

1.1.1 Usage and application rates

Chlorpyrifos (CPF) [O, O-diethyl O-3,5,6-trichloro-2-pyridinyl phosphorothioate] is an organophosphate (OP) insecticide, acaricide, and miticide used in Canada and worldwide in both agricultural and commercial settings. It is one of the most widely used pesticides globally (Solomon et al., 2014). Chlorpyrifos was first manufactured and distributed by the Dow Chemical Company in 1965 and it's use increased significantly in the early 1970s with the banning of dichlorodiphenyltrichloroethane (DDT) (Lee, 2017). Currently, usage of CPF in Canada is being re-evaluated as a part of the Pest Management Regulatory Agency's (PMRA) initiative to re-evaluate active ingredients registered prior to 1995 with identified potential risk issues (PMRA, 2018). According to the manufacturer, CPF is authorized for use in almost 100 countries worldwide on more than 50 different types of crops (Dow AgroSciences, 2018). The global demand for CPF was projected to be 200 000 tonnes in 2015 and the production and consumption of the pesticide are increasing each year (John and Shaike, 2015). Application of CPF is done by ground or aerial equipment and the chemical is sold in a variety of forms including liquid, gel, granular, soluble, emulsifiable, and flowable concentrates, microencapsulated material, pellets, tablets, impregnated materials, baits, wettable powders, dusts, and ready-to-use formulations (Dow AgroSciences, 2018; John and Shaike, 2015). In Canada and the United States, homeowner use and commercial use around residential areas has been largely restricted or eliminated due to concerns surrounding human neurotoxicity after exposure (CCME, 2008; US EPA, 2018). In 2012, the United States Environmental Protection Agency (US EPA) limited the use of CPF by lowering the acceptable application rates and creating buffer zones around public spaces where the pesticide cannot be sprayed (US EPA, 2012). Regulations in Canada set forth by the PMRA dictate that spray buffer zones of 28-74 m, dependent on application method and rate, are to be maintained around aquatic ecosystems during agricultural use of CPF (PMRA, 2003).

1.1.2 Physicochemical properties and mechanism of action

The half-life of CPF is 30-50 days (d) in surface waters, 7-120 d in soil, and 39-200 d in sediment (Mackay et al., 2014; Racke, 1993). However, studies have shown that the aquatic half-

life of CPF can vary greatly with water quality parameters such as pH; for example, at pH 9, 7, and 5 the half-life of CPF ranges from 16, 72-81, and 73 d, respectively (Racke 1993). It is considered to have low to moderate water solubility with a range of 1.4-2 mg L^{-1} at 25°C (John and Shaike, 2015). The octanol-water partition coefficient (log K_{ow}) of CPF ranges from 3.31-5.27, indicating an affinity for lipids (CCME, 2008). The potential for bioconcentration in biota varies with species, exposure duration, and dose. The bioconcentration factor (BCF) of CPF ranges from 100-4667 under field conditions and 58-5100 in laboratory settings, indicating a moderate to very high potential for bioconcentration in fish (CCME, 2008; Franke et al., 1994; Racke, 1993). Key physicochemical properties of CPF are summarized in Table 1.1.

Table 1.1. Physicochemical properties of chlorpyrifos under laboratory and field conditions.

Property	Units	Value	Reference
Melting point	ºC	36-38	
Molar mass	g mol^{-1}	350.59	
Vapour pressure	mmHg	1.87×10^{-5}	(Gebremariam et al., 2012)
Water solubility	mg L^{-1}	1.4-2	(John and Shaike, 2015)
	mg L^{-1}	0.73	(Mackay et al., 2014)
Half-life in water	days	16-73*	(Racke, 1993)
	days	30-50*	(Mackay et al., 2014)
	days	<1-3†	(Racke, 1993)
Half-life in sediment	days	50-150*	(Mackay et al., 2014)
	days	39-200*	(Racke, 1993)
Half-life in soil	days	7-120*	(Mackay et al., 2014)
log K_{ow}		3.31-5.27	(CCME, 2008)
Bioconcentration Factor		100-4667†	(CCME, 2008)
		58-5100*	(Racke, 1993)

(*) indicates laboratory conditions; (†) indicates field conditions

Values measured at 25 ºC.

Organophosphate pesticides, such as CPF, target the enzyme acetylcholinesterase (AChE), which is responsible for hydrolysis of the neurotransmitter acetylcholine (ACh) in cholinergic synapses at neuromuscular junctions (Fukuto, 1990). Specifically, AChE inhibition occurs via a chemical reaction in which the serine hydroxyl moiety in the enzyme active site is phosphorylated. The phosphorylated serine hydroxyl group is no longer able to participate in the breakdown of ACh by hydrolysis (Fukuto, 1990). In target species, this inhibition leads to an accumulation of ACh at the neuromuscular junctions of nerve synapses, causing the desired effects through repeated and uncontrolled firing of neurons eventually leading to loss of respiratory control and death by asphyxiation (Costa, 2006; Sparling and Fellers, 2007). However, the neurotransmitter action of ACh and the role of AChE in the post-synaptic breakdown of ACh is conserved across species and as such, the mechanisms and adverse effects of CPF exposure is manifested in both target and non-target species (Fukuto, 1990; Giesy et al., 2014). The AChE enzyme is present in and has been isolated from numerous non-target species across various taxa including mammals, fish, birds, reptiles, amphibians, and insects (Fukuto, 1990). A decrease in AChE activity after CPF exposure has been reported across life stages in amphibian species including *Rhinella arenarum* (Liendro et al., 2015), *Xenopus laevis* (Richards and Kendall, 2002; Wacksman et al., 2006)*, Peudacris regilla, Rana boylii* (Sparling and Fellers, 2009)*, Rana sphenocephala* (Widder and Bidwell, 2006, 2008)*, Hyla chrysoscelis, Acris crepitans,* and *Gastrophryne olivacea* (Widder and Bidwell, 2008). These studies are evidence that CPF mechanism of action is conserved across amphibian species.

Although the phosphorylated enzyme is highly stable, recovery of AChE inhibited by CPF has also been demonstrated in a number of sub-lethal exposure scenarios in non-target organisms (Costa, 2006; Giddings et al., 2014). Additionally, chronic exposure to OPs generally results in tolerance to the cholinergic effects of these chemicals (Costa, 2006). Studies have shown that CPF exposure has the ability to elicit neurotoxicity prior to innervation of the cholinergic system, which suggests that there are multiple mechanisms of toxicity (Richards and Kendall, 2002). For example, a study where fetal rat brain cells were exposed to CPF reported altered cell numbers and packing densities in the brain that could not be explained by inhibition of AChE and subsequent cholinergic stimulation (Campbell et al., 1997). In humans, CPF is known to cause a syndrome unrelated to AChE inhibition (Organophosphate-Induced Delayed Polyneuropathy), which again demonstrates that toxic action of CPF can be unrelated to

inhibition of the enzyme (Costa, 2006; Lotti and Moretto, 2005). Additionally, multiple studies using AChE knockout mice exposed to OP pesticides showed the chemical has other potential targets of toxic action including butyrylcholinesterase, muscarinic receptors, adenylyl cyclase, acylpeptide hydrolase, and neuropathy target esterase (Duysen et al., 2001; Lockridge et al., 2005). These findings indicate that there are several important underlying mechanisms that can contribute to adverse effects of CPF exposure.

Metabolic activation and detoxification of CPF occurs primarily in the liver in fish species, although similar activity has been reported in other tissues including brain and intestine (ATSDR, 1997; Bonansea et al., 2017). Phase I biotransformation mediated by cytochrome P450 monooxygenase enzymes results in the oxon-analog of CPF, a more potent cholinesterase-inhibitor than the parent compound (Fukuto 1990; Wacksman et al., 2006). In fact, the formation of the oxon-analog from CPF is essential to the potency of the chemical, as the parent compound itself is not considered to be a strong inhibitor of AChE (Solomon et al., 2014). Other phase 1 biotransformation pathways such as hydrolysis via carboxylesterases produce degradation products that are less toxic than the parent compound and are eventually excreted (Wacksman et al., 2006). In addition to this biotransformation within the liver of the exposed individual, bacteria and other environmental factors can also convert CPF into its oxon-analog, making it available for uptake from the environment (Sparling and Fellers, 2007). A number of CPF degradation products are considered more toxic than the parent compound including the oxon-analog and the secondary metabolite 3,5,6trichloro-2-pyridinol (TCP) (Fig. 1.1; John and Shaike, 2015; Sparling and Fellers, 2007).

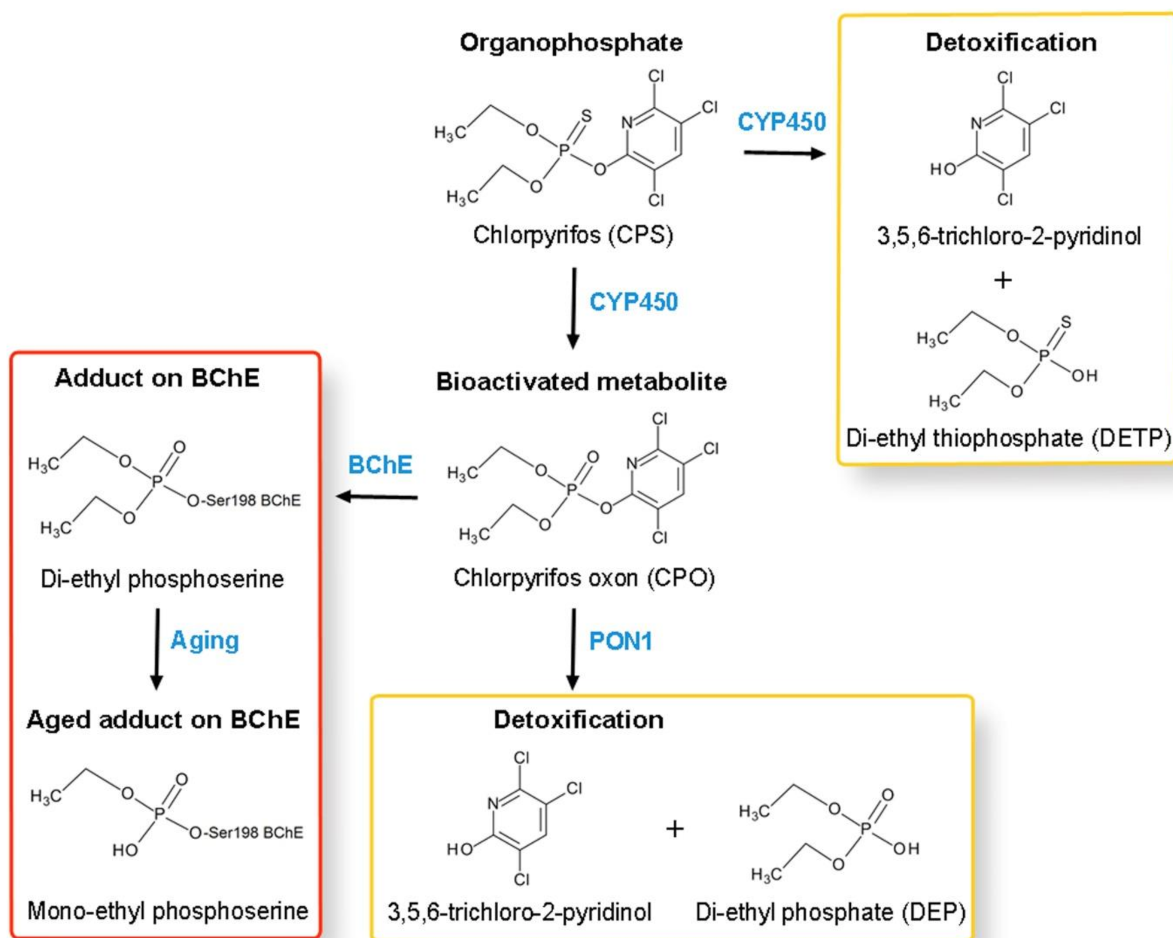


Figure 1.1. Biotransformation pathways of chlorpyrifos. Biotransformation occurs primarily in the liver, brain, and intestine in aquatic vertebrates. Structures in the yellow boxes are dialkylphosphate metabolites that are non-inhibitory and excreted in the urine. Structures in the red boxes are chlorpyrifos-adducted BchE, one example of a serine active-site enzyme that is inhibited by the oxon analog. Figure from Marsillach et al. (2016).

1.1.3 Environmental fate and occurrence in aquatic environments

Measurable levels of CPF have been reported in a variety of environmental mediums including marine sediments, streams, sumps, sloughs, rivers, urban storm drains, freshwater lakes, groundwater, fog, rain, and air around the world (Gebremariam et al., 2012). Chlorpyrifos is also involved in long range transport, and concentrations of the pesticide have been measured at locations distant from point sources (Mackay et al., 2014). In fact, samples taken from remote places including snow from Alaskan arctic estuaries and sea-ice from the Bering Sea have reported measurable CPF concentrations although there would be no CPF applications in the region (Muir et al., 2004).

Following soil or foliar application, CPF can be distributed to different environments. Degradation after application generally occurs through microbial processes, hydrolysis, and photolytic mechanisms (Gebremariam et al., 2012). Due to its relatively low water solubility and moderate vapour pressure, the pesticide exhibits a tendency to adsorb to soil, sediment, and organic matter (Gebremariam et al., 2012). The water-soil adsorption coefficient of CPF ranges between 973-31000 mL g^{-1} with a mean of 8216 mL g^{-1} indicating that it strongly adsorbs to soil (Solomon et al., 2014). Once adsorbed, the persistence of CPF is greatly increased as the chemical is less available for degradation (Bondarenko and Gan, 2004; John and Shaike, 2015) and adsorption to soil represents a major route by which the chemical can enter aquatic systems (Gebremariam et al., 2012; Williams et al., 2014). Once in these systems, CPF adsorbs to sediments where it can then be released into the water through erosion to interact with non-target species living in affected environments (Gebremariam et al., 2012). Chlorpyrifos is considered persistent in aquatic sediments (Bondarenko and Gan, 2004). Along with adsorption to soils and sediments, there are several other routes by which CPF can make its way into aquatic ecosystems directly. These include spray drift, foliar wash-off, and, in some cases, direct application (Díaz-Resendiz et al., 2015; Jin et al., 2015; John and Shaike, 2015). Numerous studies have measured CPF concentrations in various aquatic systems around the world, with concentrations as high as 3.7 µg L^{-1} being recently reported from agriculturally intensive areas in North America (Uniyal and Kumar Sharma, 2018). The Canadian Council of Ministers of the Environment (CCME) has set the maximum allowable concentration of CPF for protection of aquatic life in surface fresh water at 0.02 µg L^{-1} for short-term exposures and 0.002 µg L^{-1} for long-term exposures (CCME, 2008). In 2003, Environment Canada implemented the first nation-wide water surveillance

program for pesticides in Canada, which includes monitoring CPF levels. Detected concentrations of CPF in Canadian surface waters ranged from <0.0000005 to 0.205 μg L^{-1} with the highest concentrations reported in samples taken from the Ontario region (CCME, 2008).

1.2 Toxicity of chlorpyrifos

1.2.1 Toxicity to aquatic organisms

The World Health Organization (WHO) classifies CPF as R50/53, which means that it is considered very toxic to aquatic animals with the potential to cause long-term adverse effects in aquatic environments (WHO, 2015). Giddings et al. (2014) performed a comprehensive review of available CPF literature and an ecotoxicological risk assessment for aquatic taxa and found that crustaceans were the most sensitive, followed by insects and then fish. Literature values for 23 species of crustacea and 25 species of fish were evaluated. Median lethal dose (LC50) values at 96 hours (h) for crustacea ranged from 0.035 to 457μg L^{-1} CPF. In fish species, 96 h LC50 values ranged from 0.53 to >806 μg L^{-1} CPF (Giddings et al., 2014). In this same review it was noted that there was a lack of good quality data on CPF toxicity to amphibians and, as such, amphibians were not included in the ecotoxicological risk assessment. A study by (Sparling and Fellers, 2007) reported 96 h LC50 values for CPF as 1 μg L^{-1} in *Bufo americanus* and 3 mg L^{-1} in *Rana pipiens*. For the laboratory model amphibian *Xenopus laevis*, the CPF 96 h LC50 was 560 μg L^{-1} for metamorphs, 2410 μg L^{-1} for early embryos, and 14600 μg L^{-1} for premetamorphs (El-Merhibi et al., 2004; Richards and Kendall, 2002).

A wide range of sub-lethal effects have been reported in non-target aquatic species exposed to CPF including developmental delays and abnormalities (Jin et al., 2015; Kienle et al., 2009; Richards and Kendall, 2003), histological abnormalities across a range of organs (Colombo et al., 2005; Kunjamma et al., 2008; Scheil et al., 2009; Xing et al., 2012), inhibition of AChE activity (Colombo et al., 2005; Jin et al., 2015; Liendro et al., 2015; Richards and Kendall, 2002), immunologic effects (Adel et al., 2017; Jin et al., 2015; Kerby and Storfer, 2009; Maharajan et al., 2017), oxidative stress and reactive oxygen species (ROS) production (Liendro et al., 2015; Xing et al., 2012; Zhang et al., 2017), as well as molecular level changes including altered expression of specific genes and global transcriptomes (Wang et al., 2018; Zhang et al., 2017). There are a number of factors which ultimately influence the outcomes of exposure to

CPF in non-target organisms. Frequency, duration, and intervals between exposures to CPF will influence responses observed. The relationship between duration of exposure to CPF and toxicity observed is reciprocal – that is, shorter exposures at higher concentrations result in the same level of response as longer exposures at lower concentrations (Giddings et al., 2014). Additionally, inter- and intra-species differences can impact outcomes of CPF exposure. For example, a study by Watson et al. (2014) compared morphological abnormalities in zebrafish and *X. laevis* larvae after CPF exposure and concluded that the amphibian species was more sensitive based on that particular endpoint. Life stage also plays an important role in CPF-mediated toxicity, especially in amphibian species due to their unique life cycle. In studies with *X. laevis* exposed to CPF, metamorphs were the most sensitive life stage based on 96 h half-maximal effective concentration (EC50) for CPF-induced malformations, followed by embryos, and then premetamorphs (El-Merhibi et al., 2004; Richards and Kendall, 2002).

1.2.2 Immunomodulatory effects of chlorpyrifos

Immunomodulatory effects of CPF have been reported in a number of fish species (Chen et al., 2014; Díaz-Resendiz et al., 2015; Jin et al., 2015; Li et al., 2013; Ural, 2013; Wang et al., 2011; Zhang et al., 2017). The immunomodulatory effects of CPF on amphibians are not as extensively researched, although Kerby and Storfer (2009) reported an increase in susceptibility to viral infection and larval mortality after exposure of *Ambystoma tigrinum* to CPF. Due to the conserved nature of many components of the innate immune system across jawed vertebrates (Riera Romo et al., 2016), immunological assays have been adapted for use across taxa and it is proposed that immunomodulating effects of chemicals in one animal model could serve to predict the immunotoxicity in other animals (Zelikoff, 1998). However, there are a number of reasons why amphibian species should be considered separately. One of the main reasons is the unique amphibian life stage of metamorphosis, when the immune system undergoes widespread remodeling and the organism is particularly vulnerable to exposure to both environmental contaminants and biological stressors (Robert and Ohta, 2009; Rollins-Smith, 1998). Another important consideration is the potential disease-driven mechanisms of global amphibian declines. In a host-resistance study conducted by Kerby and Storfer (2009) on tiger salamanders, it was found that exposure to 200 $\mu g\ L^{-1}$ of CPF decreased larval survival rate by 20%. When combined with exposure to *Ambystoma tigrinum* virus (ATV) larval survival decreased by >60% at the

same concentration and the combination of ATV and CPF resulted in lower survival rates and higher infection rates at concentrations as low as 2 μg L^{-1} (Kerby and Storfer, 2009).

The mechanisms underlying CPF immunotoxicity in aquatic vertebrates, including amphibian species, are largely unknown. A putative mechanism of action suggests that the immunotoxicity of CPF is related to the well-characterized CPF-induced production of ROS, which can then induce cellular destruction via apoptosis of effector cells of innate immunity, such as leukocytes (Marchand et al., 2017). In a study by Ural (2013), common carp exposed to CPF demonstrated increased white blood cell count and altered expression and activity of antioxidant enzymes across a range of tissues; however, when an antioxidant agent, Lycopene, was administered concurrently with CPF exposure these toxic effects were neutralized. This supports the theory that ROS-mediated damage to effector cells of innate immunity, such as white blood cells, is an underlying mechanism of immunotoxicity (Ural, 2013).

1.3 Amphibians in the environment

1.3.1 Amphibian significance and status of decline

Often overlooked and underrepresented in the literature is the vast array of amphibian species that play a vital role in aquatic ecosystems in Canada, and around the world. In addition to being an important food source for higher trophic organisms, larval amphibians play vital roles as primary consumers, detritivores, as well as predators (Blaustein and Kiesecker, 2002; Hocking and Babbitt, 2014). Moreover, due to their unique ecothermic physiology, amphibians can exploit resources that are typically seen as “energy-poor”. This creates a valuable link between the lowest and highest trophic levels in a community (Hopkins, 2007).

Declines in amphibian populations have been noted from various locations worldwide since the early 1980s and were first recognized as a global issue in the early 1990s (Blaustein, 1994; Carey et al., 1999; Hayes et al., 2010). In 2004, a global assessment by the International Union for Conservation of Nature (IUCN) found that 32.5% of amphibian species are considered threatened and at least 43.2% of all amphibian species are known to be experiencing some form of population decrease (Stuart et al., 2004). In addition, the IUCN found that a much higher proportion of amphibian species (22.5%) compared to birds (0.8%), or mammals (5.3%) were considered data deficient, meaning not enough information was known about them to assign an

extinction threat category (Stuart et al., 2004). The causes of worldwide amphibian declines include habitat loss and degradation, environmental contamination, invasive species, emerging diseases, climate change, and overexploitation (Carey et al., 1999; Hocking and Babbitt, 2014). There is not likely to be a single cause for amphibian population declines, but rather a combination of factors. One proposed mechanism of globally occurring amphibian declines involves direct or indirect immunosuppression or immunomodulation by anthropogenic contaminants that would prevent the immune system from developing an adequate immune response against pathogen infection (Carey et al., 1999; Daszak et al., 1999). Amphibians are considered sensitive bioindicators of ecosystem health and, because of this, declining amphibian populations throughout the world and increasing rates of decline are potentially indicative of a much larger problem within our aquatic systems (Carey et al., 1999).

1.3.2 Amphibian immunity and disease

The two main components of the amphibian immune system are considered fundamentally similar to that of humans and immune defence mechanisms, in particular the molecules and cells involved in the innate response, maintain their functionality throughout vertebrates (Carey et al., 1999; Chen and Robert, 2011; Du Pasquier et al., 1989; Riera Romo et al., 2016). Resistance to pathogens encountered in the environment involves aspects of both the innate and adaptive immune responses in amphibian species. The innate immune system provides rapid and non-specific protection until the adaptive immune response has time to develop (Grogan et al., 2018). The innate immune response is of critical importance during larval life stages of the amphibian, a time when tadpoles are free swimming, developing externally, and confined to aquatic systems where contaminants and pathogens may be present concurrently. During the unique transitional life stage of metamorphosis, the immune system undergoes a substantial reorganization that includes, for example, a sharp decrease in lymphocyte populations and physical transformation of immune-related organs including the thymus and spleen (Du Pasquier et al., 1989; Rollins-Smith, 1998).This period of reorganization is accompanied by immunosuppression that persists for up to six months post-metamorphosis (Grogan et al., 2018). Effector cells of innate immunity that play vital roles in all amphibian species include leukocytes, macrophages, and natural killer type cells (Chen and Robert, 2011; Grogan et al., 2018). The actions of many of these classes of effector cells are mediated by the release of

signaling molecules known as pro-inflammatory cytokines (Chen and Robert, 2011). The role of the innate immune response does not end with metamorphic climax. In fact, the innate immune system continues to play a critical role into adulthood in initiation of adaptive responses to specific foreign antigens (Grogan et al., 2018; Robert and Ohta, 2009). The adaptive immune response requires time to be activated after exposure to a pathogen and provides a specific response to pathogen infection together with the formation of memory cells, which respond rapidly and specifically to subsequent pathogen exposure (Carey et al., 1999). The amphibian adaptive immune response is relatively slow to manifest, and responds with lesser magnitude and efficiency than that of mammals (Grogan et al., 2018). During early life stages and into metamorphosis, the tadpole immune response is competent but functionally less developed than in post-metamorphic amphibians. A study by Sifkarovski et al. (2014) highlights the impacts of compromised immune system during critical developmental stages on immune function in post-metamorphic amphibians. Following exposure to atrazine, a common herbicide, during metamorphosis (and even after a recovery period in clean water), researchers found that adult frogs displayed a persistent impaired ability to mount certain inflammatory responses to Ranavirus infection (Sifkarovski et al., 2014).

1.4 Integrating transcriptomics with adverse effects of chlorpyrifos in amphibians

Integrating 'omics technologies into the field of ecotoxicology has been a topic of much discussion in recent years as these technologies provide the unprecedented opportunity to link adverse outcomes of chemical exposure to a specific molecular perturbation pattern in an increasingly reliable and high-throughput manner (Zhang et al., 2018). The transcriptome is defined as the 'complete complement of mRNA molecules generated by a cell or population of cells' (Piétu et al., 1999). Looking at the transcriptome of an individual gives information on which genes are expressed in that individual and at what level (McGettigan, 2013). In the context of ecotoxicology, transcriptomic data can be utilized to determine which genes are differentially expressed in response to chemical exposure in both field and laboratory scenarios (Feswick et al., 2017). A list of these differentially expressed genes (DEGs) can then be mapped to biological pathways and processes to examine mechanisms of toxicity which, without this big data, would not be easily identifiable. When transcriptomic outcomes are integrated with other 'omic approaches within a systems biology framework, apical outcomes of exposure can be anchored

to molecular and cellular level responses, and better define the mechanism of action of specific chemicals (Basu et al., 2019; Kavlock et al., 2018). With the recent development and increasingly widespread adoption of RNA-Seq technologies based on next generation sequencing platforms, transcriptomic data can be used to evaluate chemicals for their ability to alter key molecular pathways that precede phenotypic adverse outcomes of chemical exposure. Thus, toxicogenomics, the combination of toxicology and genomics technologies, also shows great promise as an early screening tool to prioritize chemicals with potential risk for adverse effects.

In amphibians, there is a relative lack of mechanistic data regarding the toxicity of many contaminants of concern, including CPF. If predictive of apical outcomes, the larval transcriptomic profile has the potential to be employed as a rapid screening approach to assess the large number of contaminants threatening amphibian species globally. In addition, transcriptomic profiling after short-term, larval exposure has the potential to direct research into relevant outcomes of exposure to specific contaminants. To date, reference genomes for three anuran amphibian species, *Xenopus tropicalis* (Hellsten et al., 2010), *Nanorana parkeri* (Sun et al., 2015), and *X. laevis* (Session et al., 2016), have been published.

Studies in aquatic vertebrates other than amphibians have identified a number of gene pathways across a range of physiological processes and functions that respond to CPF exposure. Examining the transcriptome of the ciliate *Uronema marinum* following CPF exposure revealed changes in pathways related to cellular and metabolic processes, binding functions, and catalytic activities (Wang et al., 2018). Zhang et al. (2017) reported significant effects on the head kidney transcriptome of common carp exposed to CPF, specifically pathways involved in antioxidant systems and immune response as well as targeted effects on cytokine activity, oxidoreductase activity, and cell growth and death among others. Similarly, transcriptome and metabolome level analyses revealed that CPF exposure modulated pathways related to oxidative stress, energy and lipid metabolism, endocrine function, and proteolysis in Atlantic salmon hepatocytes (Olsvik et al., 2015). To date, there have been no studies examining CPF-mediated effects on the transcriptome of any amphibian species. However, in one study, exposure of *Rana chensinensi* to the OP pesticide trichlorfon led to significant transcriptome-level effects on cellular and metabolic processes, binding functions, and catalytic activities in the liver (Ma et al., 2018). Due to the widespread usage of CPF coupled with increased disease incidence and population

declines of some amphibian species, further research into the adverse effects and underlying mechanisms of CPF-mediated toxicity in amphibians is warranted.

1.5 Project rationale

There is limited data on the consequences of exposure to sub-lethal, environmentally relevant levels of CPF in amphibian species. Studies performed on fish species and other aquatic vertebrates are not always protective of sensitive amphibian species, especially when considering exposure during critical stages of development such as metamorphosis. To our knowledge, there is no literature regarding transcriptomic responses in amphibians after exposure to CPF. Additionally, there is no literature on the immune-related effects of short-term, sub-lethal exposure of amphibian species to CPF. For these reasons, and in the context of globally declining amphibian populations, it is important to address the lack of information currently available on the exposure of amphibian species to this common environmental contaminant.

The overall goal of this thesis research was to gain a better understanding of the effects of CPF exposure on amphibians and provide much needed data on responses across levels of biological organization, from transcriptome to whole animal. Research was first focused on evaluating adverse outcomes of chronic exposure to CPF and linking key molecular response patterns that may be altered during exposure to CPF to these outcomes. I then focused specifically on effects of short-term CPF exposure on immune parameters and the ability of amphibians to respond to immune stimulation. Studies were conducted with the laboratory model amphibian *X. laevis* because (1) of the abundance of publicly available information including a fully sequenced genome (Session et al., 2016) and standardized exposure protocols throughout life stages, (2) it has been used as a model for studies of immunity (Robert and Ohta, 2009) as well as of immunotoxicity (De Jesús Andino et al., 2017; Sifkarovski et al., 2014) in amphibians, (3) hormone-induced breeding can provide embryos year-round, and (4) the time it takes for development from early larval stages to metamorphic climax and early juvenile life stage is relatively short. The aim of this research was to investigate the effects on development, health, and immune status of *X. laevis* after exposure to the common and ubiquitous environmental contaminant, CPF. The first study (Chapter 2) assessed transcriptome level changes in larval *X. laevis* after acute (96 h) exposure to environmentally relevant concentrations of CPF. These transcriptional changes were then compared to apical outcomes observed after chronic exposure

throughout early life stages. The second study (Chapter 3) measured contaminant-induced changes in the immune system of juvenile amphibians, and ultimately the impacts on immunocompetence and ability to mount an appropriate immune response when challenged with a known immunostimulatory agent.

1.6 Research objectives and hypotheses

The overall objective of this thesis research was to characterize the sub-lethal toxicity of CPF in amphibians by first identifying biological pathways that are altered with acute exposure to CPF and relating these transcriptional changes to higher-level responses in following chronic exposure. This information was then used to inform a particular target system (i.e. immune system) and relevant endpoints for a more detailed assessment of CPF impacts on immune response in amphibians.

Chapter-specific objectives:

1. Determine if acute (96 h) exposure to CPF during embryo-larval life stage of *X. laevis* alters transcriptional response compared to unexposed individuals (Chapter 2)

   H0: There are no differences in transcriptomic responses of larval X. laevis *exposed to CPF compared to the control group*

2. Determine if chronic exposure (embryo to metamorphic climax) to CPF affects apical outcomes in *X. laevis* such as growth, development traits, incidence of deformities, liver histopathology, or AChE activity (Chapter 2)

   H0: CPF exposure has no effect on growth, development, liver histopathology, or AChE activity in X. laevis *when exposed from embryo to metamorphic climax.*

3. Determine if short-term (7 d) exposure to CPF results in immunomodulation in juvenile *X. laevis* as measured by differential leukocyte counts, changes in pro-inflammatory cytokine expression, and histopathology of immune organs (Chapter 3)

   H0: Exposure to CPF for 7 days does not alter basal levels of circulating leukocytes, expression of pro-inflammatory cytokines, or histopathology of immune organs in juvenile X. laevis

4. Determine if CPF exposure alters the ability of *X. laevis* to mount a response to the immunostimulatory agent, lipopolysaccharide (LPS) (Chapter 3)

    H0: Exposure to CPF for 7 days does not alter the response of juvenile X. laevis challenged with LPS

CHAPTER 2

INTEGRATING EARLY LIFE STAGE TRANSCRIPTOME ANALYSIS WITH APICAL OUTCOMES OF CHRONIC CHLORPYRIFOS EXPOSURE IN THE AMPHIBIAN, XENOPUS LAEVIS

Preface

This study assessed the toxicity of an emerging aquatic contaminant of concern, chlorpyrifos, in the model amphibian species *Xenopus laevis.* Transcriptomic responses were evaluated after 96 hour early life-stage exposure in an attempt to integrate observed transcriptomic changes with adverse outcomes in metamorphic individuals following chronic exposure to CPF. This chapter is organized as a manuscript for publication in a peer-reviewed scientific journal. Author contributions are as follows:

Nicole Baldwin (University of Saskatchewan): managed the study, conducted animal husbandry and exposures, collected and analysed the data and drafted the manuscript.

Brad Park (University of Saskatchewan): conducted the histological sample preparation and analysis.

Alper J. Alcaraz (University of Saskatchewan) and Othman Soufan (McGill University): provided input and guidance on analysis of transcriptomic data.

Dr. Nil Basu (McGill University) and Doug Crump (Environment and Climate Change Canada): provided scientific input and obtained funding for the project

Dr. Markus Hecker (University of Saskatchewan): provided scientific input, offered comments and edits to the manuscript, and obtained funding for the project.

Dr. Natacha Hogan (University of Saskatchewan): was the primary supervisor, provided scientific input, obtained funding for the project, and offered comments and edits to the manuscript.

2.1 Abstract

Chlorpyrifos (CPF) is one of the most widely used organophosphate pesticides worldwide with extensive occurrence in aquatic ecosystems. Exposure to CPF is associated with adverse effects across a wide range of physiological parameters in fish, most notably neurotoxicity through inhibition of the enzyme acetylcholinesterase (AChE). However, effects of exposure to CPF in amphibians are relatively poorly studied with little information on the underlying molecular toxicity pathways associated adverse outcomes. The main objective of this study was to identify key molecular response patterns that are altered in amphibians with early life stage exposure to CPF, which may enable prediction of apical outcomes of ecological relevance in amphibians. The model amphibian, *Xenopus laevis* was exposed to CPF (0.4, 2, 10 μg L^{-1}, nominal) from 24 hours (h) post-hatch through to metamorphosis (50-55 days post-hatch). Individuals were subsampled after 96 h and whole-body transcriptome profiles were assessed using high throughput sequencing (RNA-Seq). Pathway analysis revealed a number of significant dysregulated pathways including those associated with "classic" outcomes of CPF exposure, such as serine hydrolase activity (AChE is a serine hydrolase enzyme) and immune pathways, including immune function, inflammatory response, and cytokine receptor activity. Other affected pathways not typically linked to CPF were altered including vasculature development, sensory perception of light stimulus, and blood coagulation. Tadpoles exposed to CPF through to metamorphosis exhibited increased relative liver weight (30% increase in 10 μg L^{-1} treatment) and a dose-dependent decreased in brain AChE activity. Disruption of pathways associated with serine hydrolase following CPF exposure during early life stages is in agreement with decreased AChE activity at metamorphosis. Transcriptomic analysis also revealed a number of novel dysregulated pathways that could not be directly linked to apical outcomes measured in this study but suggests that CPF impacts a wide range of biological pathways in amphibians that warrant further study. This study is part of the EcoToxChip project (www.ecotoxchip.ca).

2.2 Introduction

Amphibian populations have been declining in locations worldwide since the 1980s with declines being first recognized as a global issue in the early 1990s (Blaustein, 1994; Carey et al., 1999; Hayes et al., 2010). Many hypotheses have been presented regarding the underlying factors driving dwindling amphibian populations including habitat loss and degradation, environmental contamination, invasive species, emerging diseases, climate change, and overexploitation (Carey et al., 1999; Hocking and Babbitt, 2014) although there is not likely to be a single driving factor for these declines. Either alone, or in combination with other environmental factors, agricultural pesticide use has been cited as a potential contributor to global amphibian declines and localized mass mortality events (Carey and Bryant, 1995; Hayes et al., 2006; Mann et al., 2009; Mason et al., 2013; Pounds and Crump, 1994).

Chlorpyrifos (CPF; $C_9H_{11}C_{l3}NO_3PS$) is one of the most commonly used organophosphate (OP) pesticides throughout North America and worldwide (Adeyinka and Pierre, 2019). It is found ubiquitously within aquatic systems both in agriculturally intense areas and seemingly pristine locations far from anthropogenic sources (Muir et al., 2004; Zhang et al., 2012). In fact, samples taken from remote places including snow from Alaskan arctic estuaries and sea-ice from the Bering Sea have reported measurable CPF concentrations (Muir et al., 2004). Chlorpyrifos inhibits acetylcholinesterase (AChE), the enzyme responsible for hydrolysis of the neurotransmitter acetylcholine (ACh) in cholinergic synapses at neuromuscular junctions (Fukuto, 1990). In target species, this inhibition results in the desired toxic effects where ACh build up at neuromuscular junctions leads to loss of respiratory control and death by asphyxiation (Costa, 2006; Sparling and Fellers, 2007). However, AChE and the action of ACh are conserved across species and, as such, CPF exposure has the potential to cause toxicity in non-target species (Costa, 2006).

The World Health Organization (WHO) has classified CPF as R50/53 indicating that it is very toxic to aquatic animals with the potential to cause long-term adverse effects in aquatic ecosystems (WHO, 2015). Reported sub-lethal effects in aquatic vertebrates exposed to CPF include developmental delays and increased incidence of malformation (Jin et al., 2015; Kienle et al., 2009; Richards and Kendall, 2003), histological abnormalities across a range of tissues (Colombo et al., 2005; Kunjamma et al., 2008; Scheil et al., 2009; Xing et al., 2012), inhibition of AChE activity (Colombo et al., 2005; Jin et al., 2015; Liendro et al., 2015; Richards and

Kendall, 2002), immunomodulation (Adel et al., 2017; Jin et al., 2015; Kerby and Storfer, 2009; Maharajan et al., 2017), as well as changes at the molecular level, including changes in expression of specific genes and global transcriptome profiles in fish (Wang et al., 2018; Zhang et al., 2017).

In a recent comprehensive review of the available CPF literature that included an assessment of risk to aquatic organisms, the authors noted that there were few amphibian species with toxicity data for CPF (total of seven) and only three studies that met their criteria, which included data transparency and description of experimental procedures, among others (Giddings et al., 2014). For these reasons, the authors did not consider amphibians further in the risk assessment (Giddings et al., 2014). Indeed, amphibian species are often overlooked and underrepresented in toxicity studies despite the vital and unique roles they play in aquatic ecosystems. Weltje et al. (2013) reviewed and compared acute and chronic toxicity data between fish and amphibians and concluded that larval stages of amphibians are less sensitive than fish to a range of chemicals, and thus, the toxicity data for fish can be extrapolated to and be protective of amphibians. However, a study by Watson et al. (2014) assessed morphological and physiological abnormalities in both zebrafish (*Danio rerio*) and *Xenopus laevis* larvae after CPF exposure and concluded that the amphibian species was more sensitive than zebrafish as it displayed by decreased heart rate and increased incidence of morphological abnormalities at lower concentrations of CPF (Watson et al., 2014). Cholinesterase-inhibiting pesticides have also been correlated with a decline of amphibian species in California, a state with very intense pesticide use (Davidson, 2004). Early life-stage (ELS) amphibians and tadpoles are free-swimming, confined to aquatic systems where pesticides may be present, and lack fully functional detoxification pathways (Blaustein and Kiesecker, 2002; Ortiz-Santaliestra et al., 2006). Additionally, amphibian metamorphosis includes a substantial reorganization of nearly every physiological system which can be accompanied by increased sensitivity to chemical and biological stressors in their environment (Grogan et al., 2018). This unique transitional life-stage is often not considered when extrapolating data from other aquatic vertebrates or when examining outcomes of exposure in acute exposure scenarios.

The integration of ‘omics technologies into the field of ecotoxicology has been increasingly adopted in recent years due to the potential use of these technologies in both qualitative and quantitative measurement of changes from the molecular all the way to the

community level (Zhang et al., 2018). In the context of ecotoxicology, transcriptomic profiling can be used to determine which genes are differentially expressed in response to exposure to a contaminant (Feswick et al., 2017). This information can then be used to more clearly define previously unknown mechanisms of action of these contaminants of concern, such as CPF. These early toxicity pathways can be utilized to direct future research and inform on potential apical outcomes of relevance. Studies examining alterations in the transcriptomic profiles of aquatic vertebrates have identified a number of toxicity pathways across a wide range of physiological processes and functions after exposure to CPF. A study using the ciliate *Uronema marinum* reported effects on cellular and metabolic processes, binding functions, and catalytic activities as a result of CPF exposure (Wang et al., 2018). Another recent study by Zhang et al. (2017) reported alterations in the head kidney transcriptome of common carp (*Cyprinus carpio*) following exposure to CPF and included changes in transcripts related to cytokine activity, oxidoreductase activity, and cell growth and death among others. Similarly, Olsvik et al. (2015) reported effects on oxidative stress pathways, energy and lipid metabolism, endocrine function, and proteolysis in Atlantic salmon (*Salmo salar*) hepatocytes after exposure to CPF. To our knowledge, there have been no studies examining the effects of CPF exposure on the transcriptome of any amphibian species.

Despite the widespread use of CPF and its well-defined toxicity to fish species, there is a lack of amphibian data at multiple levels of biological organization regarding the outcomes of exposure to this ubiquitous aquatic contaminant. Therefore, the objectives of this study were to characterise the short-term, ELS transcriptomic response in *X. laevis* exposed to CPF and then identify effects of chronic exposure to sub-lethal levels of CPF from early life-stages to metamorphic climax. Differentially expressed genes were then identified and pathway analyses conducted in an attempt to establish key toxicity pathways for CPF in *X. laevis* and to determine if apical endpoints could be linked to CPF-induced changes at the molecular level.

2.3 Materials and methods

2.3.1 Obtaining Xenopus laevis *embryos for exposures*

An adult *X. laevis* colony was maintained at a 12 hours (h) light:12 h dark photoperiod at 16 ± 1 °C in the Aquatic Toxicology Research Facility at the Toxicology Centre, University of

Saskatchewan, Saskatoon, SK. Four sexually mature males and females were selected for breeding. Each was injected in the dorsal lymph sac with a 25 international unit (IU) priming dose of human chorionic gonadotropin (hCG, Sigma-Aldrich, St. Louis, MN, United States) dissolved in sterilized phosphate buffered saline (PBS;137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). After priming injections, males and females were housed in separate aquaria at 21 ± 1 °C overnight. A second dose of hCG was administered approximately 24 h after priming injections; males received a 250 IU dose and females received a 500 IU dose. Males and females were then randomly paired for breeding, placed into aquaria with water temperature maintained at 21 ± 1 °C, and left to spawn overnight.

After collection, selection and preparation of embryos followed protocols outlined in "Standard guide for conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)" (ASTM, 2012). A maximum of 50 normally cleaving embryos were placed in individual egg cups, a vertical PVC pipe with two holes cut below a 100 μm Teflon mesh insert, to incubate in facility water maintained at 22 ± 1 °C for 24 h. After this incubation period, individuals were moved into petri dishes to begin the 96 h exposure period. Animals used in this study were handled in accordance with the University of Saskatchewan's Animal Research Ethics Board (protocol #20160090) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.3.2 Test chemical preparation

Chlorpyrifos powder (CAS: 2921-88-2, purity: 98%) was purchased from TRC Canada (North York, ON, Canada). A CPF stock solution (1 mg ml^{-1}) was prepared by dissolving the powder in dimethyl sulfoxide (DMSO) in a 100 ml glass bottle and stored at -4 ºC. Working stock solutions (4, 20, and 100 µg L^{-1}) for each CPF exposure concentration were prepared in 250 mL glass bottles and stored at 4 ºC. Facility water control and 0.01% v/v DMSO solvent control treatments were included and nominal CPF exposure concentrations for both the 96 h and chronic exposure were 0.4, 2.0 and 10 µg L^{-1} CPF.

2.3.3 CPF exposures and sampling

2.3.3.1 96 h early life-stage exposure

Exposures were initiated when the collected embryos reached approximately Nieuwkoop and Faber (NF) stage 29 (Nieuwkoop and Faber, 1994). Individuals were transferred into 50 mL glass petri dishes (30 individuals per petri dish; 10 petri dishes per treatment). Mortality, malformation, and abnormal behaviour were recorded and dead individuals were removed daily. A 50% water renewal was performed daily during the exposure period. Subsampling for transcriptomics was performed at the completion of the 96 h exposure when individuals (NF stage 46) were collected, pooled in groups of five, weighed, and flash frozen in liquid nitrogen and stored at -80 ºC.

2.3.3.2 Long-term exposure through to metamorphic climax

After the 96 h subsampling was complete, remaining individuals were transferred into 7 L glass aquaria for the beginning of the chronic exposure period (30 individuals per tank; five tanks per treatment). During the chronic exposure 50 – 75% water changes were performed on each tank daily and mortality, malformation, and abnormal behaviour were recorded. Temperature was monitored daily and pH, dissolved oxygen, ammonia, nitrate, nitrite, hardness, alkalinity, and conductivity was monitored weekly for each tank (Appendix A). The chronic exposure was terminated at 75 days (d). Sampling occurred over five days, with one replicate tank per treatment sampled each day. Individuals were anesthetized by immersion in buffered 0.1% tricaine methanesulfonate (MS-222) and euthanized by cervical dislocation. Morphometric endpoints including NF stage, wet body weight (to the nearest 0.01 g), snout-vent length (SVL; to the nearest 0.01 cm), total length (to the nearest 0.01 cm), and liver weight (to the nearest 0.01 g) were measured and recorded for each individual. Brains from five individuals per tank were excised and flash frozen in liquid nitrogen before being stored at -80 ºC. A total of 25 whole-body individuals per treatment (five from each replicate tank) were submerged in CalEx-II (Fisher Scientific) for 48 h to fix and decalcify and then transferred to 70% ethanol for storage.

2.3.4 Analysis of CPF exposure concentrations

Water samples were collected from exposure tanks at five specific times over the exposure period and sent to SGS AXYS (Sidney, BC, Canada) for analyses of actual waterborne CPF concentrations. A 47 mm all glass vacuum filter holder (Wheaton, Millville, NJ, United States) and a 90 mm Macherey-Nagel glass fiber filter (Fisher Scientific, Hampton, NH, United States) were used for filtration of samples. 200 mL of water was collected from each tank (5 tanks/treatment) and poured into a 300 mL filtering cup where it passed through the glass fiber filter into a 1000 mL conical collection flask. The resultant 1 L composite sample was then transferred into a 1 L amber bottle for storage and shipment. An eyedropper was used to add 2 drops of chloroform to each composite sample. Samples were stored at 4 ºC for no longer than 5 days prior to shipment to SGS AXYS. Four time periods (exposure day 6, 40, 54, 75) throughout the chronic exposure period were selected for analysis of actual CPF concentrations. CPF concentrations were measured in each sample using high-resolution mass spectrometry.

2.3.5 RNA extraction and Illumina sequencing

Pooled, whole body NF stage 46 individuals were used for the transcriptomic analysis after 96 h exposure to CPF. RNA was extracted from five replicates each containing five individuals from three treatment groups: 0.01% DMSO solvent control, 2 μg $L^{-1}$and 10 μg L^{-1} CPF. Total mRNA was obtained using the QIAcube and the RNeasy Plus Universal Mini kit as directed by the manufacturer (Qiagen, Hilden, Germany). RNA concentration was measured using the QIAxpert (Qiagen). RNA integrity was assessed using the Bioanalyzer 2100 and associated RNA Nano 6000 Assay kit (Agilent Technologies, CA, USA). Samples with a minimum RNA Integrity Number of 7.0 were used for transcriptomic analysis. Samples were diluted using sterile RNAse-free water to a final volume and concentration of 25 µL of 100 ng μL^{-1} RNA and stored at -80 ºC until sequencing.

Transcriptome sequencing was performed at Genome Quebec Innovation Center (McGill University, Montreal, QC, Canada). Briefly, libraries of double-stranded DNA from total RNA were prepared using the Illumina TruSeq Stranded mRNA library prep kit (Illumina Inc., San Diego, CA, United States). Size selection of DNA fragments was performed using solid phase reversible immobilization beads. Quality of libraries were confirmed using the Bioanalyzer 2100 and associated DNA 1000 Nano Assay kit (Agilent Technologies). Libraries were multiplexed with TruSeq RNA Single Index kits and loaded onto HiSeq 3000/4000 PE Cluster kit for cluster

generation (Illumina Inc.). Clusters were loaded onto a HiSeq 4000 Sequence-by-Synthesis kit and run as 100 base pair paired-end reads over 200 cycles using a HiSeq 4000 system (Illumina Inc.).

2.3.6 Liver histology

Histological analysis of liver tissue was performed on NF stage 65 individuals from the chronic exposure. Twenty individuals, ten each from the solvent control and the 10 $\mu g\ L^{-1}$ CPF treatment groups, were randomly selected for histological analysis. Carcasses were trimmed to remove the limbs and head, and the body wall was cut away to fully expose the viscera. Each sample was then placed into a tissue embedding histocassette and rinsed twice with 70% ethanol to remove traces of fixative. Tissues were processed using a RVG1 vacuum tissue processor (Intelsint S.R.L., Villarbasse, TO, Italy). After processing, whole-body tissues were embedded in paraffin wax. Each sample was longitudinally step-sectioned at a thickness of 5 µm, mounted on slides, and dried at 37 °C for approximately 12 h. For each individual, two consecutive sections were taken at a minimum of 11 different levels at 50 µm intervals. Sections were stained with regressive hematoxylin and eosin and examined using an Axiostar Plus microscope (Carl Zeiss Microscopy, Thornwood, NY, United States). Photographs of each slide were taken using an attached INFINITY1-1M digital camera, and associated Infinity Analyze software (Lumenera Corporation, Ottawa, ON, Canada). Liver sections were screened qualitatively for signs of hepatoxicity and each individual was scored (0=absent; 1=mild; 2=moderate; 3=severe) for degree of hepatocyte vacuolization, incidence of hepatocyte necrosis, number and size of melanomacrophages, and degree of sinusoid dilation.

2.3.7 Acetylcholinesterease activity assay

Acetylcholinesterase activity in brain was measured using the Acetylcholinesterase Activity Assay kit (MAK119) in accordance with the manufacturer protocols (Sigma-Aldrich, St. Louis, MN, United States). Tissues were homogenized in 0.1 M phosphate buffer at pH 7.5 (75.4 mM $H_{15}Na_2O_{11}P$, 24.6 mM H_4NaO_5P) using a PowerGen 125 tissue homogenizer (Fisher Scientific, Hampton, NH, United States). Samples were then centrifuged at 14 000 rpm for 5 min at room temperature and the supernatant was collected to be used for the assay. Six brains from the 0.01% DMSO, and each CPF treatment group were analyzed in duplicate. A SpectraMax

microplate reader at 412 nm wavelength was used to determine the AChE activity of each sample (Molecular Devices LLC, San Jose, CA, United States). Data are presented as Units AChE per g tissue where units AChE is equal to µmol substrate hydrolyzed per minute.

2.3.8 Transcriptomic data preparation and statistical analysis

RNA-Seq reads were uploaded to the Galaxy platform https://usegalaxy.eu/ (Afgan et al., 2018). Quality control checks were performed using FastQC (Andrews, 2010) and two of the 2 ug L^{-1} CPF samples were found to have low per base sequence quality. Further analysis continued with the solvent control and 10 ug L^{-1} CPF concentration samples. Poor quality sequences were trimmed using the Trimmomatic tool (Bolger et al., 2014) with a phred quality score of 28 and a minimum sequence length of 35. Reads were mapped using HISAT2 (Kim et al., 2015) to the *X. laevis* reference genome (GCF_001663975.1) available online at the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/). SAMtools sort (Wysoker et al., 2009) was then used to sort the resultant alignments by read name. Finally, ht-seq count (Anders et al., 2015) was used to count the number of reads mapped to each gene feature.

Counts data to be used for gene-level differential expression analysis was then uploaded into EcoToxXplorer (https://www.ecotoxxplorer.ca). The edgeR tool built into the EcoToxXplorer platform was utilized for differential gene expression analysis (Robinson et al., 2009). The solvent control treatment was used as a common control and genes were considered significantly differentially expressed at false discovery rate (FDR) = 0.1. The ClueGo (v 2.5.3; Bindea et al., 2009) plugin on Cytoscape was used to identify functionally grouped networks (Shannon et al., 2003). ClueGo visualization of functional networks formed from significantly dysregulated pathways were based on Gene Ontology (GO) vocabularies biological processes, molecular functions, and cellular components (accessed on: 13/03/2019). Pathways were built using at least 5 genes from the differentially expressed genes (DEGs) after 96 h ELS exposure to 10 ug L^{-1} CPF ($p \leq 0.05$). Statistical test was set to a right-sided hypergeometrical test with a Bonferroni (step down) p-value correction and a kappa score of 0.4.

2.3.9 Statistical analysis

Data obtained from apical endpoints measured were tested for a normal distribution using the Shapiro-Wilk test and for homoscedasticity using Levene's test. In cases where the

assumption of normality was violated, data was analyzed using the non-parametric Kruskal-Wallis H test ($p \leq 0.05$). This was followed by pairwise comparisons using Dunn's procedure with a Bonferroni correction for multiple comparisons. When the assumptions of normality and homoscedasticity were met, data was analyzed using a one-way analysis of variance (ANOVA). Post-hoc tests (either Tukey's HSD or an LSD test) were employed in cases where ANOVA revealed a significant difference between groups ($p \leq 0.05$). Hypothesis testing was two-tailed. All analyses were conducted using SPSS Statistics 25 (IBM Corporation, Armonk, NY, United States).

2.4 Results

2.4.1 Chemical analysis of CPF

Recovery of CPF was 17% in the 0.4 ug L^{-1} concentration group (0.066 ug L^{-1}), 24% in the 2 ug L^{-1} concentration group (0.485 ug L^{-1}), and 29% in the 10 ug L^{-1} concentration group (2.890 ug L^{-1}) (Table 2.1). From this point forward, the concentrations are referred to as "low", "medium", and "high" treatment groups.

Table 2.1. Nominal and actual concentrations of chlorpyrifos (CPF) throughout the chronic exposure period as measured by high-resolution mass spectrometry.

	Exposure Day (D)	**0.01% DMSO**	**CPF Concentration (µg L^{-1})**		
			0.4	**2**	**10**
	D6	0.0029	0.143	0.864	4.200
	D40	0.0015	0.005	0.278	1.700
	D54	0.0038	0.044	0.342	1.750
	D75	0.0076	0.070	0.455	3.900
Average		**0.0039**	**0.066**	**0.485**	**2.890**
Percent of Nominal			17%	24%	29%

D6 = Exposure Day 6; D40 = Exposure Day 40; D54 = Exposure Day 54; D75 = Exposure Day 75

2.4.2 Differentially expressed gene and pathway analysis

Whole body transcriptome analysis examined 24205 genes total and revealed 50 DEGs after 96 h ELS exposure to the highest CPF concentration as compared to the solvent control (FDR = 0.1). Of these, 17 were upregulated and 33 were downregulated (Table 2.2). Key biological processes and pathways affected by CPF exposure were visualized as functional networks (Fig. 2.1) formed from significantly upregulated (Fig. 2.2) or depleted pathways (Fig. 2.3). These pathways were based on GO terms related to biological processes, molecular functions, and cellular components.

Table 2.2. Differentially expressed genes in *X. laevis* after 96 h early life-stage exposure to chlorpyrifos.

Gene ID	log Fold Change	Name
LOC108699634	5.5981	RNA polymerase II degradation factor 1-like, transcript variant X3
LOC108707016	4.6741	RING finger protein 212B-like
LOC108697970	2.76	taste receptor type 2 member 9-like
cyp2j2.L	1.4291	cytochrome P450 family 2 subfamily J member 2 L homeolog, transcript variant X1
MGC84235	1.0533	MGC84235 protein
LOC108716281	0.74148	fucolectin-4-like
arr3.L	0.70224	arrestin 3, retinal (X-arrestin) L homeolog
LOC108707450	0.53404	retinoschisin-like
hsd3b7.S	0.51542	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 S homeolog, transcript variant X1
masp2.S	0.39813	mannan-binding lectin serine peptidase 2 S homeolog, transcript variant X1
LOC108701136	0.39432	alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase 1-like
LOC108703002	0.34747	dual 3’,5’-cyclic-AMP and -GMP phosphodiesterase 11A-like
LOC108714924	0.26441	mucin-5AC-like
LOC108709438	0.14402	plakophilin-1-like
LOC108704439	0.13438	E3 ubiquitin-protein ligase TRIM39-like
xclca2	0.1117	calcium activated chloride channel
LOC108702356	0.068711	up-regulator of cell proliferation-like
LOC108711733	-0.04594	A.superbus venom factor 1-like
LOC108702434	-0.13085	NADPH oxidase organizer 1-like
MGC68455	-0.26697	Glucose-6-phosphatase-like
LOC108698286	-0.30285	NADPH oxidase 1-like
LOC108714562	-0.42	cytokine receptor common subunit beta-like
c3.L	-0.4549	complement component 3 L homeolog
LOC108717079	-0.52738	ceruloplasmin-like
LOC108715543	-0.54158	transmembrane protein 125-like
LOC108710049	-0.61908	glycerophosphodiester phosphodiesterase domain-containing protein 5-like, transcript variant X1
LOC398210.L	-0.62757	uncharacterized LOC398210 L homeolog
LOC494638	-0.68665	uncharacterized LOC494638, transcript variant X2
LOC108696196	-0.70299	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1-like, transcript variant X3

LOC108697412	-0.70506	probable carboxypeptidase X1
LOC108715761	-0.75516	protein FAM83F-like
LOC108710292	-0.90759	dual oxidase 1-like
LOC108719393	-1.3564	transient receptor potential cation channel subfamily V member 4-like
LOC108713225	-1.5865	protein DVR-1-like
LOC108719568	-1.786	thiosulfate sulfurtransferase-like
LOC108696752	-1.8144	uncharacterized LOC108696752, transcript variant X2
steap4.L	-1.9427	STEAP4 metalloreductase L homeolog
LOC108703485	-2.3044	nuclear factor interleukin-3-regulated protein-like
LOC108708918	-2.3482	alpha-tectorin-like
LOC108708107	-2.424	cell wall protein DAN4-like
LOC108709478	-2.5071	uromodulin-like
LOC100036845	-2.5134	uncharacterized LOC100036845
LOC108708109	-2.5619	pancreatic secretory granule membrane major glycoprotein GP2-like
LOC108706182	-2.6118	platelet glycoprotein Ib alpha chain-like
LOC108701668	-2.6226	NADPH oxidase organizer 1-like
thdl18.L	-2.7757	thyroid hormone down-regulated protein (gene 18) L homeolog
thdl20.S	-2.7968	thyroid hormone down-regulated protein (gene 20) S homeolog
LOC108709109	-2.851	uromodulin-like
lrg1.L	-3.011	leucine-rich alpha-2-glycoprotein 1 L homeolog

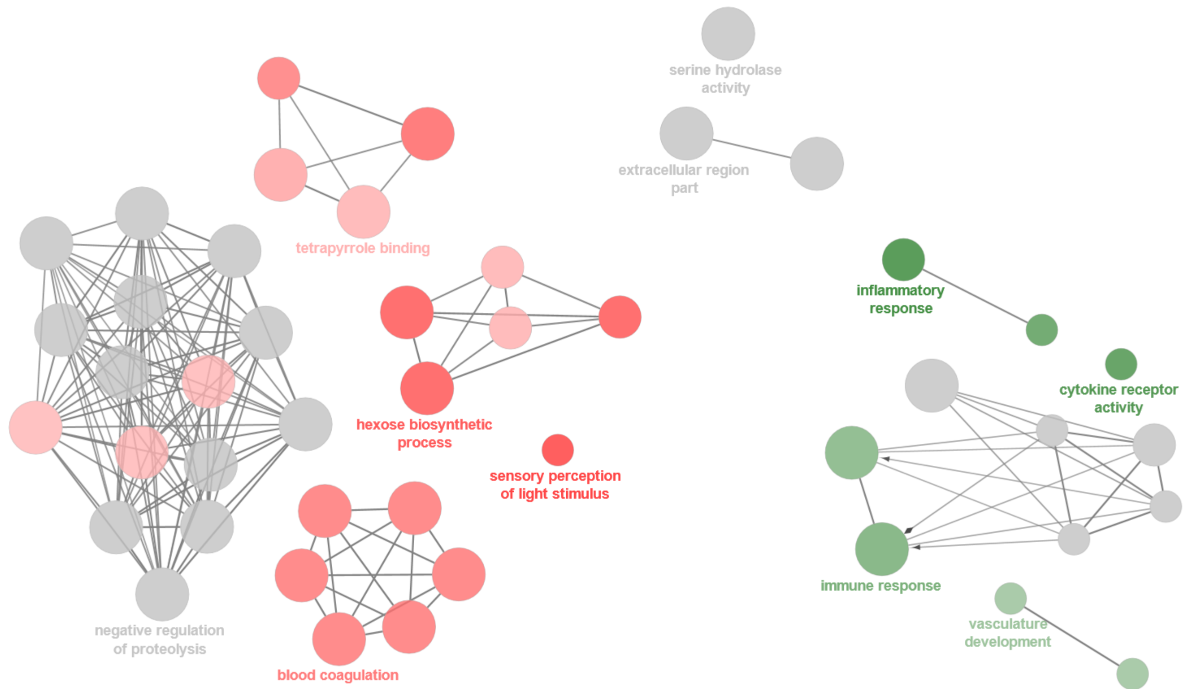


Figure 2.1. ClueGo visualization of functionally grouped networks formed from significantly dysregulated pathways based on Gene Ontology (GO) databases (13/03/2019). Pathways were built using at least five differentially expressed genes ($p \leq 0.05$) in early life-stage *X. laevis* after 96 h of chlorpyrifos exposure. Statistical test was set to a right-sided hypergeometrical test with a Bonferroni (step down) p-value correction and a kappa score of 0.4. Node size represents pathway significance and darker shades represent higher gene proportion associated with pathway. Green node = depleted pathway, gray node = un-specific pathway, red node = upregulated pathway. A list of all significantly dysregulated pathways as well as this figure with all terms labelled is provided in Appendix B.

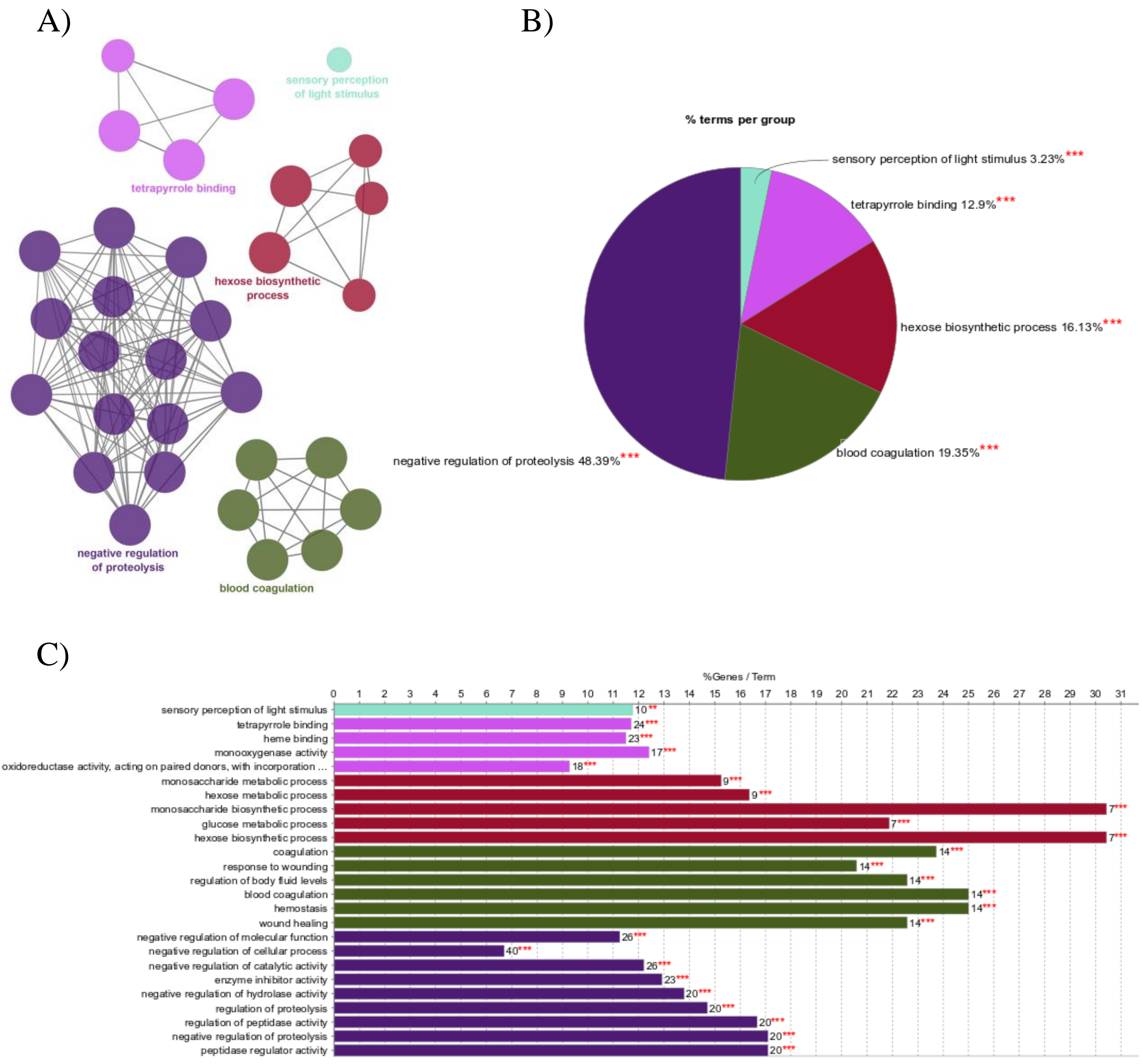


Figure 2.2. ClueGo output displaying pathways and biological processes associated with upregulated genes in *X. laevis* based on gene ontology (GO) vocabularies (13/03/2019) after 96 h early life-stage exposure to chlorpyrifos. **(A)** Networks formed from significantly upregulated pathways ($p \leq 0.05$). Node size represents pathway upregulation significance. **(B)** Overview chart of functional groups including specific pathways associated with upregulated genes. The proportion of each group (%) is based on the number of GO terms included within each group. **(C)** Bar chart displaying the percentage of total genes (top) and number of differentially expressed genes (end of bar) associated with each GO term colour coded by pathway or function.

Statistically significantly upregulated GO terms are represented by: (***) = $p \leq 0.001$, (**) = $0.001 < p < 0.05$, (*) = $p \leq 0.05$.

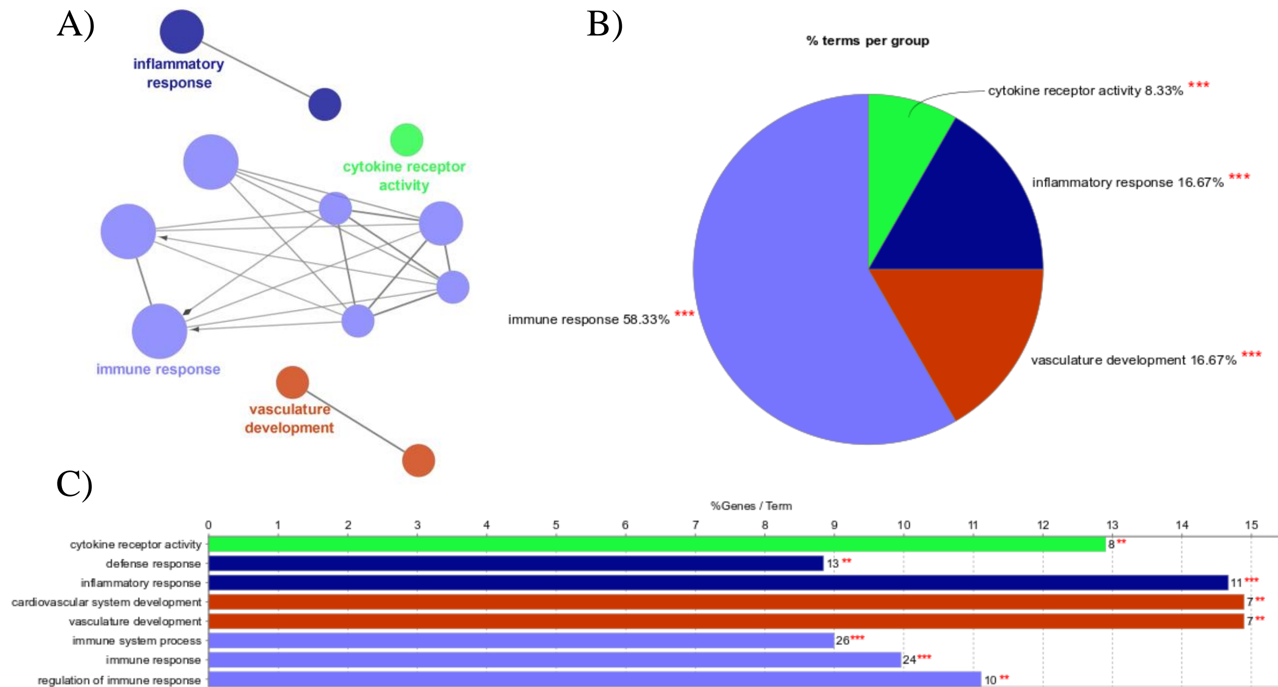


Figure 2.3. ClueGo output displaying pathways and biological processes associated with downregulated genes in *X. laevis* based on gene ontology (GO) vocabularies (13/03/2019) after 96 h early life-stage exposure to chlorpyrifos. **(A)** Networks formed from significantly depleted pathways ($p \leq 0.05$). Node size represents pathway depletion significance. **(B)** Overview chart of functional groups including specific pathways associated with downregulated genes. The proportion of each group (%) is based on the number of GO terms included within each group. **(C)** Bar chart displaying the percentage of total genes (top) and number of differentially expressed genes (end of bar) associated with each GO term colour coded by pathway or function. Statistically significantly depleted GO terms are represented by: (***) = $p \leq 0.001$, (**) = $0.001 < p < 0.05$, (*) = $p \leq 0.05$.

2.4.3 Morphometrics

There was no effect of treatment on survival after chronic CPF exposure (ANOVA: $F_{(4,24)} = 0.525$; $p = 0.718$). Overall survival in the water and solvent control treatments combined was 95%. No difference in SVL or wet body weight occurred after chronic exposure to CPF (Table 2.3). There was a statistically significant decrease in NF stage in the medium CPF treatment as compared to the low CPF treatment (data not shown, Kruskal-Wallis: $H_{(4)}=9.963$, $p=0.041$). A significant decrease in the percentage of tadpoles that reached metamorphic climax, NF stage 65, occurred in the medium and high treatment groups compared to the water control (Table 2.3, ANOVA: $F_{(2, 24)} = 3.393$, $p = 0.028$). Relative liver weight was 30% higher in individuals from the high CPF treatment group compared to the solvent control (Table 2.3**,** ANOVA: $F_{(4,122)} = 2.862$; $p = 0.026$).

2.4.4 Effects of CPF on liver histology

Individuals from the solvent control group displayed a typical liver structure, comprised of hepatocytes arranged in cords 1-2 cells thick with sinusoids between cords containing erythrocytes. Qualitative screening for histopathological indicators of hepatotoxicity revealed no apparent differences in the structure of high CPF exposed livers as compared to the solvent control (Table 2.4).

2.4.5 Effects of CPF on AChE activity

A concentration-dependent decrease in AChE activity was observed in brains of *X. laevis* at metamorphic climax following chronic exposure to CPF. Compared to the solvent control, median AChE activity was 2.1% lower in the low treatment group, 17.9% lower in the medium treatment group, and 24.0% lower in the high treatment group (Fig. 2.4). These differences were significant at the highest CPF treatment group (Kruskal-Wallis: $H_{(3)} = 9.647$, $p = 0.022$).

Table 2.3. Morphometric indices (mean ± SEM) including snout-vent length (SVL), wet body weight, relative liver weight, and % individuals at Nieuwkoop Faber stage 65 for *X. laevis* tadpoles exposed chronically to waterborne chlorpyrifos (CPF).

Treatment	SVL (mm)	Wet Weight (g)	Relative Liver Weight (mg g^{-1})	% at NF stage 65
Facility Water	15.74 ± 0.30	0.313 ± 0.019	0.0097 ± 0.0009	15^{a}
0.01% DMSO	15.86 ± 0.35	0.312 ± 0.020	0.0083 ± 0.0007	13^{ac}
0.4 µg L^{-1} CPF	14.97 ± 0.20	0.306 ± 0.018	0.0104 ± 0.0008	14^{a}
2 µg L^{-1} CPF	15.16 ± 0.24	0.340 ± 0.023	0.0089 ± 0.0007	5^{b}
10 µg L^{-1} CPF	15.47 ± 0.26	0.336 ± 0.021	0.0120 ± 0.0009*	7^{bc}

Asterisk (*) indicates significant difference from solvent control (ANOVA: $F_{(4,122)} = 2.862$; $p = 0.026$).
Letters indicate significant differences between groups (ANOVA: $F_{(2, 24)} = 3.393$, $p = 0.028$)
Sample size (n) = 62-74 for SVL, wet body weight, % at NF; n=25 for relative liver weight.

Table 2.4. Severity of histological parameters qualitatively examined in liver of *X. laevis* at metamorphic climax after chronic exposure to waterborne chlorpyrifos (CPF).

	Individual	Vacuolization	Necrosis	Melano-macrophages	Sinusoids
0.01% DMSO	1	--	--	--	--
	2	--	--	++	+
	3	--	--	+++	+
	4	--	--	+++	--
	5	--	--	++	++
	6	--	--	+	-
	7	--	--	+++	+
	8	--	--	+	--
	9	--	--	+	--
	10	--	+	+	++
10 µg L^{-1} CPF	1	--	--	+++	--
	2	++	--	++	--
	3	--	--	++	--
	4	--	--	+	--
	5	--	--	+++	+++
	6	--	--	++	--
	7	--	--	++	++
	8	--	--	+	--
	9	--	--	+++	++
	10	--	++	++	++

-- = absent; + = mild, ++ = moderate; +++ = severe

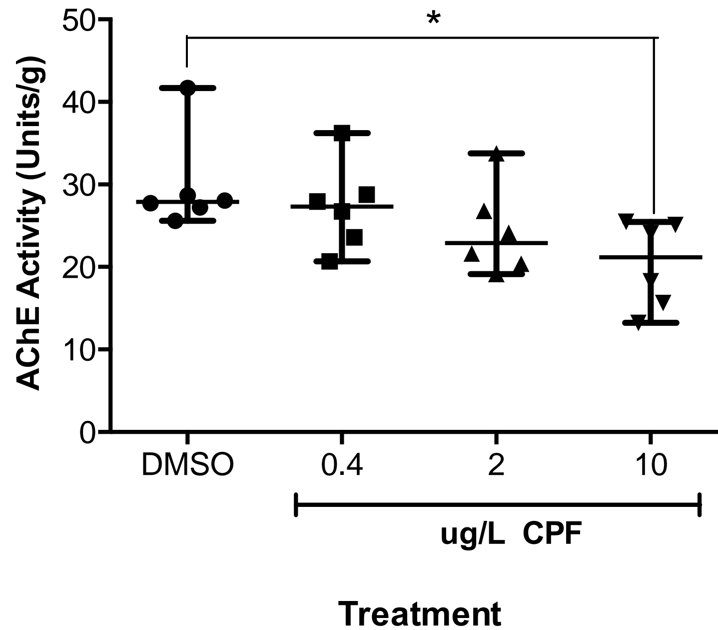


Figure 2.4. Median (± IQR) brain acetylcholinesterase (AChE) activity in NF stage 57 – 64 *X. laevis* after chronic exposure to chlorpyrifos (CPF). Data is presented in Units AChE per g tissue where units is equal to µmol substrate hydrolyzed per minute. Asterisk (*) indicates significant differences between groups (Kruskal-Wallis: $H_{(3)} = 9.647$, $p = 0.022$).

2.5 Discussion

In the present study, we analyzed the transcriptomic responses of *X. laevis* after 96 h of CPF exposure as well as apical outcomes of chronic CPF exposure on *X. laevis* at metamorphic climax. This was the first study to look at the short-term transcriptomic responses of ELS *X. laevis* after CPF exposure. Pathway analysis revealed impacts on the biological processes and molecular functions of ELS *X. laevis* after short-term exposure to environmentally relevant concentrations of CPF. Some of these significantly dysregulated pathways were associated with "classic" and well-documented outcomes of CPF exposure, such as serine hydrolase activity, and others were related to outcomes not generally associated with exposure to CPF or OP pesticides, such as altered vasculature development.

2.5.1 Developmental processes affected by CPF exposure

Pathways related to vasculature development (GO:0001944) were significantly depleted after 96 h exposure to CPF. Across vertebrate species, blood vessels are among the first organs to develop during embryogenesis (Carmeliet and Collen, 1998). Alterations of the developing vascular system in ELS organisms are associated with a range of pathologies from malformation and deformity to mortality (Katagiri, 1983; Zhong et al., 2018). However, evidence suggests that exposure to some compounds can cause vascular abnormalities without any external signs of malformation. In a study on zebrafish (*D. rerio*) embryos exposed to cadmium, Cheng et al. (2001) examined effects on development of the vasculature by injection of microbeads into the circulation and found vasculature defects which were not associated with any visible malformation. Components of the extracellular matrix, such as fibronectins and collagen, play a primary role in vasculature development (Carmeliet and Collen, 1998). In the present study, transcriptome profiling in ELS *X. laevis* revealed that the extracellular region (GO:0005576) was the most significantly impacted cellular component after 96 h CPF exposure. The extracellular region is specifically involved in the production of endothelial cells that line the vasculature. The impacts of CPF on the extracellular regions of cells, and the potential alteration of vasculature development as an outcome warrants further exploration.

The depletion of pathways associated with vasculature development included effects on the development of the cardiovascular system (GO:0072358) specifically. This is not surprising

as OP pesticides, including CPF, are known cardiotoxic agents in aquatic animals including *Carcinus maenas* and *Oreochromis niloticus* even after acute exposure (Lundebye et al., 1997; Thomaz et al., 2009). However, the literature regarding the cardiotoxic effects of CPF in amphibians is relatively scarce. A study by Watson et al. (2014) reported a dose-dependent decrease in heart rate in larval *X. laevis* after 96 h exposure to environmentally relevant concentrations of CPF. In the present study, DEG analysis revealed the most significantly differentially expressed gene after 96 h of CPF exposure in ELS *X. laevis* was an upregulated cytochrome p450 (CYP) isoform, CYP-2J2. In humans and zebrafish (*D. rerio*), this gene is an arachidonic acid epoxygenase with vasoprotective roles in the cardiovascular system (Goldstone et al., 2010). If the function of this gene is conserved across species, upregulation of CYP-2J2 agrees with the pathway analysis revealing cardiovascular effects of CPF exposure in early life stage tadpoles.

Body size of metamorphic individuals was not affected by CPF after chronic exposure despite the fact that ELS exposure to CPF clearly altered transcriptomic profiles related to vasculature development and other developmental processes. Studies on amphibian species *Rana dalmatina* and *Rana fernandezae* reported no effect on amphibian body size after chronic exposure to CPF concentrations ranging from 10 – 500 µg L^{-1} (Bernabò et al., 2011; Ruiz de Arcaute et al., 2012). Similarly, Sparling and Fellers (2009) reported no difference in SVL after exposure to CPF concentrations as high as 200 µg L^{-1} and a significant decrease in body weight only in the 200 µg L^{-1} treatment group in *Rana boylii* after a 56 d exposure period. Dimitrie and Sparling (2014) reported dose-dependent decreases in SVL and body weight after exposure to CPF from larval stages through to metamorphosis in *Pseudacris regilla*. However, this exposure was conducted at nominal concentrations ranging from 134 – 294 µg L^{-1} CPF, which is approximately 44 – 98 times higher than our highest measured CPF concentration. In contrast, Jayawardena et al. (2011) reported a dose-dependent increase in SVL and body weight in *Bufo melanostictus* after chronic CPF exposure at concentrations ranging from 50 – 500 µg L^{-1}. There was no increased incidence of malformations present in any CPF treatment group in this study. As with reported effects of CPF on body weight and SVL in across published studies, effects of CPF exposure on incidence of malformations is also highly variable across exposure concentrations and species (Dimitrie and Sparling, 2014; Jayawardena et al., 2011).

In the present study, chronic exposure to CPF resulted in fewer individuals reaching NF stage 65 (metamorphic climax) at termination of the exposure. This finding is supported by a number of studies with amphibians that report increased time to metamorphosis after CPF exposure (Dimitrie and Sparling, 2014; Jayawardena et al., 2011; Sparling and Fellers, 2009). Exposure to CPF led to dysregulation of a number of pathways related to energy metabolism and various metabolic processes, which will be discussed in further detail in following sections. Disruption of these metabolic functions could be tied to the observed decrease in successful completion of metamorphosis as more energy is used to keep up with increased metabolic demand and less is available for the process of metamorphosis. The range of reported effects on morphometric indices in amphibian species after exposure to CPF highlights the importance of considering species differences in amphibian research. At our exposure concentrations, significant dysregulation of pathways specific to vasculature development and development of the cardiovascular system occurred after short-term exposure. This, combined with the lack of significant alteration of classic morphometric indices such as wet body weight and SVL after long-term exposure, suggests that future research into the developmental effects of CPF exposure in amphibians should consider morphological changes in the vasculature and cardiovascular system.

2.5.2 CPF inhibits AChE and pathways associated with serine hydrolase activity

One of the classic biomarkers of exposure to OP pesticides is inhibition of AChE, a serine hydrolase enzyme, and this is the mechanism of toxic action of CPF. Specifically, enzyme inhibition occurs when the serine hydroxyl moiety in the enzyme active site is phosphorylated leaving the enzyme unable to participate in the breakdown of ACh (Fukuto, 1990). Short-term exposure to CPF can inhibit AChE activity in amphibian species (including *X. laevis*) at concentrations as low as 8.87 µg L^{-1} (Colombo et al., 2005; El-Merhibi et al., 2004; Liendro et al., 2015; Wacksman et al., 2006; Widder and Bidwell, 2006). It is important to note, however, that these studies are limited in scope in that they use only short-term exposures with CPF concentrations higher than environmentally relevant and consider early larval life-stages only, despite evidence that metamorphs are more sensitive to AChE inhibition by CPF than premetamorphs (Richards and Kendall, 2002). The present study is the first to show that chronic exposure to CPF at environmentally relevant concentrations results in AChE inhibition in the

brain of metamorphic *X. laevis.* This observed characteristic response to CPF also confirms waterborne CPF exposure in our experimental system, despite the fact that our analysed concentrations were 20% of our nominal concentrations. There was also a significant and non-specific dysregulation in pathways associated with serine hydrolase activity after short-term exposure in ELS individuals. Acetylcholinesterase is a serine hydrolase enzyme. Thus, dysregulation of this pathway as indicated in the ELS transcriptome may be associated with the apical outcome of AChE inhibition in metamorphic individuals.

2.5.3 CPF-induced changes in metabolic processes and liver weight

Pathways associated with metabolic processes were significantly upregulated in ELS *X. laevis* after 96 h exposure to CPF. These included pathways related to the regulation of proteolysis as well as biosynthesis of carbohydrates. Within the pathways associated with proteolysis and protein metabolism, negative regulation of proteolysis (GO: 0045861) was the most significantly upregulated. This indicates that CPF interfered with the frequency, rate, or extent of the hydrolysis of a peptide bond or bonds within a protein (MGI, 2019). A study by Olsvik et al. (2015) examined the transcriptomic and metabolomic profiles of Atlantic salmon (*S. salar*) after exposure to CPF and reported a significant increase in multiple free amino acids in hepatocytes. They concluded that the accumulation of these compounds was indicative of a decrease in protein synthesis, or an increase in proteolysis (Olsvik et al., 2015). Other studies have reported significant activation of protease, a hydrolytic peptidase enzyme, after CPF exposure in rats and mice with the highest level of activation found within liver tissues (Muniya Naik et al., 2018; Savithri et al., 2016). This activation is indicative of altered protein metabolism and increased proteolysis. A number of GO terms in the present study that were related to protease activity were significantly upregulated including: peptidase regulator activity (GO:0061134), regulation of peptidase activity (GO:0052547), and negative regulation of hydrolase activity (GO:0051346). In addition to protein metabolism, CPF exposure in ELS *X. laevis* also altered carbohydrate metabolism with the most significantly upregulated pathway being the hexose biosynthetic process (GO:0019319). This indicates that CPF exposure interfered with the chemical reactions and pathways resulting in the formation of hexose (MGI, 2019). Metabolomic profiles in Atlantic salmon (*S. salar*) liver after CPF exposure also revealed dysregulation of carbohydrate metabolism, specifically a reduction in multiple hexoses as well as

their metabolic pro- and pre-cursors (Olsvik et al., 2015). Although the present study analysed whole-body transcriptomic profile while the study by Olsvik et al. (2015) focused on fish hepatocytes specifically, similar pathways related to protein and carbohydrate metabolism and metabolic processes were affected, suggesting metabolic consequences of CPF exposure can be applied across taxa.

The liver is a key metabolic organ across vertebrate species, including amphibians, and the site of many metabolic processes, such as carbohydrate and protein synthesis (Rui, 2014) (Akiyoshi and Inoue, 2012). Despite dysregulation of key metabolic pathways after the ELS exposure to CPF, there were limited apical effects of chronic CPF exposure in the liver of *X. laevis* at metamorphic climax. Relative liver weight was higher in individuals chronically exposed to the highest concentration of CPF. This could be associated with the observed dysregulation of metabolic processes at the early life stages, specifically upregulation of processes associated with proteolysis and carbohydrate biosynthesis, as hepatocytes expand to compensate for increased metabolic demand. Endpoints for qualitative histopathological assessment were selected to lend further insight into the impacts on metabolism and metabolic processes after CPF exposure. Qualitative histopathological assessment of liver tissue from metamorphic *X. laevis* revealed no obvious tissue-level effects of chronic CPF exposure despite overall increase in liver weight relative to body size and an upregulation of metabolic pathways in the transcriptome of ELS individuals.

2.5.4 CPF exposure depletes pathways related to immune function and response

Exposure to CPF has been linked to immunosuppression and immunomodulation in fish following both short-term (Adel et al., 2017; Jin et al., 2015; Marchand et al., 2017) and long-term (Ural, 2013; Wang et al., 2011, 2013; Zhang et al., 2017) exposures. A recent study examined transcriptomic profiles in *C. carpio* exposed to CPF and reported significant dysregulation in pathways related to immune response and immune system function (Zhang et al., 2017). In the present study, short-term exposure to CPF significantly affected pathways involved in immune function in ELS *X. laevis*. Gene ontology terms immune function (GO:0006955), inflammatory response (GO:0006954), and cytokine receptor activity (GO:0004896) were all significantly depleted after 96 h exposure to CPF. In the only other study examining the impact of CPF on immune-related responses in an amphibian, Kerby and Storfer

(2009) reported a >60 % decrease in survival in tiger salamanders (*Ambystoma tigrinum*) when exposed concurrently to CPF and *Ambystoma tigrinum* virus as compared to individuals exposed to the same concentration of CPF alone. The depletion immune-related pathways after ELS exposure to CPF observed in our study provides some insight into which pathways are modulated by CPF, potentially leading to immunomodulation/immunosuppression and pathogen susceptibility. The present study did not measure apical endpoints associated with immunotoxicity after chronic CPF exposure; however, given the established immunotoxicity of CPF in fish species and the alteration of immune-related pathways in the ELS *X. laevis* transcriptome, further examination of the immunotoxic effects of CPF in amphibian species is warranted.

2.5.5 ELS exposure to CPF modulates other biological pathways in X. laevis

There were several other pathways dysregulated with ELS exposure to CPF and included pathways associated with blood coagulation, sensory perception of light stimulus, and molecular function and binding. Blood coagulation (GO:0007596) falls under the umbrella of response to stress (GO:0006950) and, more specifically, wound healing (GO:0042060), which is the series of events that restores integrity to damaged tissue following an injury (MGI, 2019). Blood coagulation is an integral part of the immune system, and is considered a sentinel for the immune response when considered in conjunction with activation of the complement system (Bougas et al., 2013). In fact, when visualized in Cytoscape the upregulated pathways involving blood coagulation were overlapping with the depleted pathways involving immune response, suggesting that the two responses are highly related. Dysregulation of blood coagulation pathways has been reported in a number of aquatic vertebrates after exposure to biological and contaminant stressors including Chytrid fungus in *Xenopus tropicalis* (Rosenblum et al., 2009) and heavy metals in *Perca flavescens* (Bougas et al., 2013).

There were two categories of significantly dysregulated GO terms related to molecular function in the present study – those to do with enzyme activity, which were both upregulated and depleted, and those associated with cellular binding, which were specifically upregulated. The most significantly upregulated GO term associated with binding was tetrapyrrole binding (GO:0046906). Tetrapyrrole binding is the parent term for heme binding, which was also significantly upregulated (GO:0020037). Heme binding is defined as a selective and non-

covalent interaction with heme, any compound of iron complexed in a tetrapyrrole ring (MGI, 2019). When taken up by an organism, CPF undergoes phase I biotransformation mediated by CYP monooxygenase enzymes to produce the oxon-analog, a more potent anticholinesterase (Fukuto 1990; Wacksman et al., 2006). In fact, the formation of the oxon-analog from CPF is essential to the mode of action as the parent compound itself is not considered to be a strong inhibitor of AChE (Solomon et al., 2014). More specifically, the mechanism of action involves an irreversible binding of CPF to the heme iron of CYP enzymes to produce the phosphate oxon active metabolite (Rajpoot et al., 2013). Monooxygenase activity (GO:0004497) was significantly upregulated in the same network as upregulation of tetrapyrrole binding after CPF exposure. From these results, it appears that in amphibians, as in other vertebrates, the mechanism of toxic action for CPF is mediated by phase I biotransformation enzymes.

Another significantly upregulated pathway in ELS *X. laevis* after CPF exposure was sensory perception of light stimulus (GO:0050953), neurological process that falls under the umbrella of nervous system process (GO:0050877). The primary mechanism of toxic action of OP pesticides in general, including CPF, is neurotoxicity, as such, effects on the nervous system are an expected outcome of CPF exposure (Galloway and Handy, 2003). Altered swimming behaviour in response to stimulation of light to dark photoperiod transitions have been reported in zebrafish after 96 h exposure to CPF (Jin et al., 2015). There are no studies on behavioural responses to light stimulus following CPF exposure in amphibians but based on the transcriptomic response in the present study and response in zebrafish, altered behavioural responses may be a relevant adverse outcome to CPF in amphibians as well.

2.6 Conclusion

This work demonstrated that short-term (96 h) exposure to CPF during early stages of larval development has the potential to disrupt many important pathways and processes in the model amphibian species, *X. laevis*. Physiological systems are often interdependent with both obvious and subtle overlap, and it is likely that the pathways found to be dysregulated in this study are linked to one another as well as to apical outcomes of exposure. Pathway analysis of the transcriptomic data attests that various metabolic pathways and enzymatic processes are disturbed by CPF exposure and may contribute to the observed adverse outcomes when chronic

CPF exposure was carried through to metamorphic climax. In terms of novel transcriptomic pathways identified in this study, immune function, cardiovascular development, metabolic processes, and behavioural responses to light stimulus are biological processes that could be a target for CPF in amphibians, with consequences for apical outcomes that should be explored. This study contributes to a better understanding of the potential consequences of chronic exposure of amphibians to environmental concentrations of CPF, suggesting that this widespread and heavily-used pesticide could impact a wider range of biological pathways.

CHAPTER 3

EFFECT OF CHLORPYRIFOS, ALONE AND COMBINED WITH A LIPOPOLYSACCHARIDE CHALLENGE, ON IMMUNE PARAMETERS IN THE AMPHIBIAN *XENOPUS LAEVIS*

Preface

Chlorpyrifos (CPF) has known immunotoxic effects in fish species, but the research regarding the immunotoxicity of the pesticide in amphibians is relatively scarce. In the previous chapter, we used whole transcriptome analysis to demonstrate that short-term, early life stage exposure to CPF in *Xenopus laevis* leads to disruption of a number of biological pathways related to immunotoxicity. This aided in design of the second study that focused on the immune system for a system-specific assessment of CPF impacts in amphibians. In this study, we further examined the potential immunotoxicity of CPF in the model amphibian species, *X. laevis*, using an immune challenge exposure model. The immune challenge assay allows for evaluation of a host's ability to mount an appropriate immune response when challenged with a known immunostimulatory agent after exposure to a potential immunotoxic chemical. The objectives of the present study were to: (1) evaluate the effects of short-term exposure (seven days) to sub-lethal concentrations of CPF on immune-related parameters in the amphibian *X. laevis,* and (2) determine whether exposure to CPF alters their ability to mount an immune response when challenged with lipopolysaccharide (LPS), a known immunostimulatory agent. Specific endpoints measured were histopathology of kidney tissues, circulating blood leukocyte populations, and expression of key pro-inflammatory cytokines in the liver. This chapter is being prepared as a manuscript for submission to a scientific journal. Author contributions are as follows:

Nicole Baldwin (University of Saskatchewan): conducted the animal husbandry, managed the study, collected and analysed the data, and drafted the manuscript.

Melanie Gallant (University of Saskatchewan): assisted with and provided expertise on all aspects of *X. laevis* husbandry and care, helped with data collection and analysis, and offered comments and edits to the manuscript.

Kimberly Hamonic (University of Saskatchewan): provided input on the experimental design, assisted with sampling, and conducted the qPCR analysis.

Dr. Natacha Hogan (University of Saskatchewan): was the research supervisor, provided scientific input, funding, guidance, and assistance throughout the exposures, data collection and analyses, and manuscript preparation.

3.1 Abstract

Numerous contaminants present in the aquatic environment have the potential to disrupt immune defences and potentially increase disease susceptibility. Amphibians are known to inhabit contaminated environments, and there is growing concern that known immunotoxic chemicals may magnify the impacts of globally emerging infectious diseases. Chlorpyrifos (CPF) is one of the most commonly used organophosphate pesticides in Canada and worldwide. This pesticide can induce a variety of adverse effects in non-target aquatic vertebrates, including immunomodulation; however, studies specifically on amphibians are lacking. The objectives of this study were to (1) determine if short-term exposure to CPF results in immunomodulation in juvenile *Xenopus laevis*, the model amphibian species and, (2) determine if this immunomodulation results in impaired ability to mount a response to a simulated pathogen, lipopolysaccharide (LPS). Individuals were aqueously exposed to CPF (1 or 10 $\mu g\ L^{-1}$) for seven days, then injected with either phosphate buffered saline (PBS; immune-rested) or 10 $\mu g\ g^{-1}$ LPS (immune-stimulated) and sampled one day post-injection. Differential leukocyte profiles (by flow cytometry), expression of cytokines in the liver (by qPCR), and histopathology of the kidney were assessed in immune-rested and immune-stimulated individuals. Exposure to 10 $\mu g\ L^{-1}$ CPF resulted in an increase in kidney epithelial cell height (18% compared to control). Individuals exposed to CPF also displayed an overall reduction in circulating lymphocytes, an increase in circulating granulocytes, and increased expression pro-inflammatory cytokines, TNF-α and CSF-1, in the liver. This study demonstrates that CPF can modulate the inflammatory immune parameters, providing some of the first evidence of the immunotoxic potential of CPF in amphibians. Given the fundamental role of inflammation in immune responses, these findings suggest that exposure to CPF may have consequences for amphibian susceptibility to ecologically relevant pathogens.

3.2 Introduction

Chlorpyrifos (CPF; $C_9H_{11}Cl_3NO_3PS$) is one of the most commonly used organophosphate (OP) pesticides in Canada and worldwide (Adeyinka and Pierre, 2019). Numerous studies have assessed CPF concentrations in various aquatic systems around the world, with concentrations as high as 3.7 µg L^{-1} reported in agriculturally intense areas in North America (Zhang et al., 2012). Chlorpyrifos contamination of aquatic ecosystems poses a threat to the non-target species inhabiting these systems. The main mechanism of toxic action of CPF is inhibition of acetylcholinesterase (AChE), the enzyme responsible for the breakdown of acetylcholine (ACh) at nerve synapses (Fukuto, 1990). The accumulation of ACh at nerve junctions causes uncontrolled firing of neurons resulting in secondary effects that can lead to the death of fish and other aquatic animals (Fukuto, 1990; Kienle et al., 2009; Watson et al., 2014). Exposure of aquatic vertebrates to CPF can also alter a wide range of physiological parameters including growth and development (Kharkongor et al., 2018; McClelland et al., 2018; Tussellino et al., 2016), oxidative stress (Jin et al., 2015; Zahran et al., 2018), cellular integrity (Ali et al., 2009; Altun et al., 2017), and immune response (Chen et al., 2014; Li et al., 2013; Wang et al., 2011). To date, most of the detailed studies specifically regarding CPF immunomodulation have been conducted in rats with clear effects on a variety of immune parameters, including pathology of immune organs, changes in immune cell populations, and reduced responsiveness to immune stimulating factors (Mokarizadeh et al., 2015; Oostingh et al., 2009).

Studies on immune-related outcomes of CPF exposure in aquatic species are largely biased towards fish, with reported effects such as histopathological changes in key immune organs, altered immune parameters (e.g. leukocyte populations, cytokine expression) and decreased resistance to pathogen infection. In both fish and amphibians, the kidney is a key immune organ (Chen and Robert, 2011; Zhang et al., 2017) and an infection target for some pathogens such as Ranavirus, a prevalent viral agent plaguing wild amphibian populations (De Jesús Andino et al., 2012; Wendel et al., 2018). Changes in kidney structures such as tubules, glomeruli and interstitial connective tissues in fish following CPF exposure have been observed in various species, including *Danio rerio* (Scheil et al., 2009), *Oreochromis niloticus* (Zahran et al., 2018), *Cyprinus carpio* (Altun et al., 2017; Wang et al., 2013; Xing et al., 2012) and this tissue damaged is often attributed to oxidative stress. In a study by Xing et al. (2015), the kidney was a site of accumulation for CPF and its metabolites in *C. carpio* and this accumulation was

detectable after up to 40 days of depuration. Leukocytes (or white blood cells) play diverse and complex roles in the inflammatory response to pathogens. Fish exposed to CPF have displayed altered circulating leukocyte populations including lymphocytes (Adewumi et al., 2018; Ali et al., 2009; Zahran et al., 2018), granulocytes (Maharajan et al., 2017), and monocytes (Adewumi et al., 2018; Raibeemol and Chitra, 2018). Changes in cell populations are sometimes observed together with altered expression of cytokines (Zahran et al., 2018), effector molecules that are synthesized and secreted by immune cells and are vital in modulating the amplitude of an immune response. Specific roles of cytokines identified in vertebrates (including amphibians) include initiation of pro-inflammatory cascades (De Jesús Andino et al., 2012), macrophage activation and recruitment (Wang et al., 2011; Zahran et al., 2018), and development, differentiation, and proliferation of phagocytes (Grayfer and Robert, 2016). Across many fish species, exposure to CPF is reported to upregulate expression of tumor necrosis factor alpha (TNF-α) (Chen et al., 2014; Jin et al., 2015; Zahran et al., 2018) as well as interleukin 1-beta (IL-1β) (Jin et al., 2015; Wang et al., 2011; Zahran et al., 2018). While there is clear evidence that CPF exposure can have immunomodulatory effects in fish, similar studies with amphibians are scarce despite the hypothesized links between pesticide contamination of aquatic environments and globally declining amphibian populations (Davidson, 2004; Hayes et al., 2006; Sparling and Fellers, 2009).

The impact of pesticides, such as CPF, entering the aquatic environment and their potential immunomodulatory effects in amphibians are of particular concern. Amphibian populations have been declining globally since the 1980s (Hayes et al., 2010; Rollins-Smith, 1998; Stuart et al., 2004). The reasons for this decline are complex with a number of potential contributing factors including climate change, habitat loss, anthropogenic chemical contamination, and presence of opportunistic pathogens (Carey et al., 1999; Miller et al., 2011). Environmental stressors rarely occur in isolation and it is likely that a combination of these factors play a role in localized mass mortality events and global declines. Exposure to environmental contaminants may alter components of the immune system, resulting in a level of immunomodulation that impairs an individual's ability to mount an effective immune response to pathogens encountered in their environment thus leading to infection, disease, and mortality (Buck et al., 2015). Resistance to pathogens encountered in the environment involves aspects of both the innate and adaptive portion of the amphibian immune system. Beginning at the late

tadpole stages (pro-metamorphosis) and continuing into early metamorphosis, the amphibian immune system undergoes a substantial reorganization, which is accompanied by immunosuppression that persists for up to six months post-metamorphosis (Grogan et al., 2018). Exposure to environmental contaminants during these sensitive early life-stages may lead to long-term impacts on the organism's ability to defend against pathogen infection. Although it is difficult to make causative links, immunomodulation due to exposure to anthropogenic contaminants and increased susceptibility to disease have been implicated in localized mass mortality events of amphibian populations, as well as widespread declines (Carey et al., 1999; Hayes et al., 2010).

The objective of the present study was to determine the effects of short-term exposure to sub-lethal concentrations of CPF on immune-related parameters in the amphibian *Xenopus laevis* and to evaluate whether exposure to CPF alters their ability to mount an immune response when challenged with lipopolysaccharide (LPS), a known immunostimulatory agent. A major component of the bacterial outer cell wall membrane, LPS is often used in immunotoxicology studies to stimulate the inflammatory response and simulate a pathogen infection (Swain et al., 2008). We conducted an immune challenge study and examined the effects on immune-related parameters in both non-stimulated and immune-stimulated (LPS-injected) individuals. Specific endpoints evaluated included histopathology of kidney tissues, circulating blood leukocyte populations, and expression of key pro-inflammatory cytokines in the liver. Concentrations of CPF chosen for the short-term exposure were within range of those found in aquatic systems; for example, concentrations of CPF have been recently reported as high as 3.7 µg L^{-1} in agriculturally intensive areas in North America (Uniyal and Kumar Sharma, 2018). The CPF concentrations chosen are also considered sub-lethal for amphibians (based on previous literature

3.3 Materials and Methods

3.3.1 Obtaining Xenopus laevis *for exposures*

An adult *X. laevis* colony was maintained under a 12 hours (h) light:12 h dark photoperiod at 16 ± 1 °C in the Aquatic Toxicology Research Facility at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK. Four sexually mature males and females were selected for breeding. Each was injected in the dorsal lymph sac with a 25 international unit (IU)

priming dose of human chorionic gonadotropin (hCG, Sigma-Aldrich, St. Louis, MN, United States) dissolved in sterilized phosphate buffered saline (PBS;137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). After priming injections males and females were housed in separate aquaria at 21 ± 1 °C overnight. A second dose of hCG was administered approximately 24 h after priming injections; males received a 250 IU dose and females received a 500 IU dose. Breeding pairs were then selected at random, placed into aquaria with water temperature maintained at 21 ± 1 °C, and left to spawn overnight.

After collection, selection and preparation of embryos followed protocols outlined in “Standard guide for conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)” (ASTM, 2012). A maximum of 50 normally cleaving embryos were placed in individual egg cups, a vertical PVC pipe with two holes cut below a 100 µm Teflon mesh insert, to incubate in facility water maintained at 22 ± 1 °C for 24 h. After this incubation period tadpoles were reared in a Min-O-Cool tank (Frigid Units Inc., Toledo, OH, United States) under a 12 h light:12 h dark photoperiod at 21 ± 1 °C until they reached Nieuwkoop Faber (NF) stage 65 (Nieuwkoop and Faber, 1994). Tadpoles were fed SeraMicron (Sera, Heinsberg, Germany) *ad libitum* daily and then transitioned to Arcadia EarthPro Amphibigold pellets (Arcadia Reptile, Mepal, Cambridgeshire, England) at metamorphosis. Animals used in this study were handled in accordance with the University of Saskatchewan’s Animal Research Ethics Board (protocol no. 20130045) and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

3.3.2 Short-term chlorpyrifos exposure with LPS challenge

Chlorpyrifos powder (CAS: 2921-88-2, purity: 98%) was purchased from TRC Canada (North York, ON, Canada). Stock concentrations of 10 and 100 µg L^{-1} CPF were prepared in dimethyl sulfoxide (DMSO) and added to exposure tanks for a final solvent concentration of 0.01% v/v and nominal CPF exposure concentrations of 1 and 10 µg L^{-1}, respectively.

Sixty individuals at NF stage 65 were transferred from the general population into 7 L tanks (5 individuals/tank) in a temperature controlled environmental chamber for a one-week acclimation period under a 16 h light:8 h dark photoperiod at 21 ± 1 °C. Frogs were fed crushed Arcadia EarthPro Amphibigold pellets *ad libitum* daily throughout the acclimation and the exposure period. One day prior to exposure, individuals were weighed and sorted to ensure an

even distribution of sizes across treatments. Individuals were exposed to CPF for seven days under static renewal conditions with four tanks per treatment and five individuals per tank (n=20 individuals/CPF treatment). Water parameters were recorded daily (pH 7-7.5, dissolved oxygen > 90%, temperature = 20 ± 0.5°C) and a 75% water change was conducted where tanks were re-dosed with CPF to maintain the nominal target concentrations. During the exposure period water quality was monitored daily (Appendix C) and 75% water renewal was conducted where tanks were re-dosed with CPF to maintain the nominal target concentrations.

After seven days of CPF exposure, each CPF treatment was divided so that ten individuals received PBS injection and ten received the LPS injection. This resulted in a 2 x 3 factorial design, with six unique treatments and 10 individuals per treatment. LPS (*E. coli* 055:B5; Sigma-Aldrich, St. Louis, MN, USA) was solubilized in sterile PBS on the day of injections. Animals were lightly anesthetized by immersion in buffered 0.2 g L^{-1} tricaine methanesulfate (MS-222) prior to injection and then injected intraperitoneally on the lower left portion of the abdomen with sterilized PBS or with 10 µg LPS g^{-1} body weight using a 29-gauge needle. Following injections, PBS-injected and LPS-injected individuals of each CPF treatment group were housed separately in clean facility water.

Individuals were sampled one day post-injection (1 dpi). This time frame was chosen based on data from previous work with LPS showing maximal immune stimulatory response (Gallant and Hogan, unpublished). Individuals were euthanized by immersion in buffered 0.1% MS-222 and morphometric endpoints such as wet body weight (to the nearest 0.01 g), total body length (to the nearest 0.01 cm), and liver weight (to the nearest 0.01 g) were measured and recorded (n = 9 for solvent groups; n = 10 for 1 and 10 µg L^{-1} CPF treatment groups). Whole blood (10 µl) was collected from each individual by cardiac puncture with a heparinized hematocrit tube. Livers were excised, weighed (to the nearest 0.01 g), and immediately flash frozen for targeted gene expression analysis. The whole carcasses with all other organs intact were then submerged in CalEx-II (Fisher Scientific, Hampton, NH, United States) for 48 h of fixing and decalcifying prior to transfer into 70% ethanol for preservation.

3.3.3 Kidney histology

Kidneys from each individual were excised, placed into a tissue embedding histocassette, and fixed using a RVG1 vacuum tissue processor (Intelsint S.R.L., Villarbasse, TO, Italy). After

processing, kidneys were embedded in paraffin wax. Each kidney was longitudinally sectioned at a thickness of 6 µm, mounted on slides, and dried at 37 °C for approximately 12 h. Sections were stained with regressive hematoxylin and eosin (H&E) and examined using an Olympus BX41 microscope (Olympus, Center Valley, PA, United States). Photographs of each slide were taken using an attached Olympus DP71 camera and the associate DP Manager software (Olympus). One section at the same plane of view per kidney was chosen for measurement of tubule epithelial cell height. Ten tubules were chosen at random from each section and four random epithelial cells within each tubule were measured. Measurement of kidney tubule epithelial cells was performed blinded using the open source ImageJ 1.x software (Bethesda, Rockville, MD, United States).

3.3.4 Leukocyte profiles using DiOC$_5$(3) staining and flow cytometry

Blood collected during the sampling period was transferred from the hematocrit tube to a heparinized vacutainer (32 IU sodium heparin; BD Bioscience, Franklin Lakes, NJ, USA) containing 1 mL cold PBS to prevent coagulation. Samples were taken in duplicate per individual and stored on ice for a maximum of 1 h before staining and preparation for flow cytometry analysis as described in Burraco et al. (2017). Briefly, 1 µL of 2 mM 3,3'-dipentyloxacarbocyanine iodide (DiOC$_5$(3)) dye was added to the blood and PBS solution, for a final concentration of 2 µM DiOC$_5$(3). Samples were mixed and incubated at room temperature for 5 min, centrifuged at 4°C for 5 min at 1000 rpm, and then supernatant was aspirated prior to re-suspension of the cells in 1 mL PBS. Stained samples were analyzed by flow cytometry on the Accuri C6 (BD Bioscience), where 100,000 events were recorded per sample. In each sample, erythrocytes, lymphocytes, monocytes, and granulocytes were identified based on side scatter measuring the granularity or complexity of the cell and the fluorescent intensity of the cell in the FL1 channel. Each homogeneous cell population was gated, and event rate and population percentage were calculated using the BD Accuri C6 Plus software (BD Biosciences).

3.3.5 Gene expression analysis of pro-inflammatory cytokines

Liver tissues were disrupted using an Omni Bead Ruptor (Omni International, Kennesaw, GA, USA) for two 45 second (s) cycles. Total liver mRNA was obtained using TRI reagent as directed by the manufacturer (Invitrogen, Carlsbad, CA, United States) and re-

suspended in RNase-free water. Concentration of RNA was measured using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, United States) and RNA quality was confirmed on a 1% agarose gel. Genomic DNA (gDNA) cleanup and complementary DNA (cDNA) synthesis of 1 μg total RNA was performed using the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada) as described by the manufacturer.

Gene expression analysis was performed by qPCR using the CFX96 Real-time C1000 Thermal Cycler (Bio-Rad Laboratories) and SsoFast EvaGreen Supermix (Bio-Rad Laboratories) using previously described methods (Gallant and Hogan, 2018). Gene-specific primer sets (Invitrogen, Carlsbad, CA, United States) for target genes IL-1β, TNF-α, and CSF-1 as well as GAPDH and EF1α (reference genes) are detailed in Table 3.1. Briefly, each 20 µL qPCR reaction volume contained 1x SsoFast EvaGreen Supermix (Bio-Rad Laboratories), optimized concentrations of each primer set, and 2 µL of diluted cDNA template. Samples were run in duplicate and a no template and no reverse transcriptase control were included on each plate. A standard curve made from a serially diluted pool of cDNA was also included on each plate for interpolation of relative mRNA abundance of targeted genes in each sample. Each standard curve had an $R^2 > 98\%$. The thermal cycle program was comprised of an enzyme activation at 95 °C for 30 s, 40 amplification cycles at 95 °C for 5 s and 5 s at the gene specific annealing temperature (Table 3.1), denaturation at 95 °C for 1 min, and melt curve analysis from 55 °C to 95 °C (increasing 1 °C every 30 s). The mean starting quantity of each sample was normalized to the geometric mean of the reference genes, EF-1 and GAPDH. Data are presented as fold-change relative to the 0.01% DMSO control + PBS-injected group.

Table 3.1 List of target genes and associated forward and reverse primers used for transcript expression analysis by qPCR in *Xenopus laevis* liver tissues.

Target	Primer	Sequence (5' → 3')	Accession Number	Amplicon Length (bp)	Annealing Temp (°C)	References
IL-1β	FWD	GGCCTCAATGAAACCTCCAC	NM_001085605.1	232	60	(Gallant and Hogan, 2018)
	REV	AGGCAGATATCTCCCAGCAC				
TNF-α	FWD	TGTCAGGCAGGAAAGAAGCA	AB298595.1	203	62	(Sifkarovski et al., 2014)
	REV	CAGCAGAGAGCAAAGAGGATGGT				
CSF-1	FWD	ATCGAACTCTGTCCAAGCTGGATG	NM_001280600.1	123	60	(Sifkarovski et al., 2014)
	REV	GGACGAAGCAAGCATCTGCCTTAT				
GADPH	FWD	GACATCAAGGCCGCCATTAAGACT	AF549496.1	130	58.4	(Sifkarovski et al., 2014)
	REV	AGATGGAGGAGTGAGTGTCACCAT				
EF-1	FWD	GAGGGTAGTCTGAGAAGCTCTCCACG	NM_001086133.1	221	60	(Gallant and Hogan, 2018)
	REV	CCTGAATCACCCAGGCCAGATTGGTG				

Fwd = forward; Rev = reverse.

3.3.6 Statistical analysis

Data were tested for a normal distribution using the Shapiro-Wilk test and for homoscedasticity using Levene's test. In cases where data failed the assumptions of parametric statistics, a log transformation was applied and data was reanalyzed. Data were analyzed using a two-way ANOVA (CPF exposure × LPS challenge). In cases where a CPF × LPS interaction was significant (interaction $p \leq 0.05$), pairwise comparisons were applied with a Bonferroni-adjusted p value. In cases where there was no significant interaction detected (interaction $p > 0.05$), each main factor (CPF exposure and LPS challenge) was interpreted and if found significant ($p \leq 0.05$), groups were distinguished using the appropriate post-hoc test (Tukey's HSD for CPF exposure and multiple pair-wise comparisons test for LPS challenge). Hypothesis testing was two-tailed. All analyses were conducted using SPSS Statistics 25 (IBM Corporation, Armonk, NY, United States) and graphs were generated using Prism 8 (GraphPad Software, La Jolla, CA, USA).

3.4 Results

3.4.1 Morphometrics

Two individuals died with undetermined cause in the 0.01% DMSO treatment during the 7 day (d) CPF exposure period and prior to injection. There was no effect of CPF exposure on body weight (CPF: $F_{(2,58)} = 0.246$, $p = 0.783$; LPS: $F_{(1,58)} = 0.731$, $p = 0.396$; CPF × LPS: $F_{(2,58)} = 0.190$, $p = 0.828$) or total length (CPF: $F_{(2,58)} = 0.403$, $p = 0.671$; LPS: $F_{(1,58)} = 0.270$, $p = 0.606$; CPF × LPS: $F_{(2,58)} = 0.304$, $p = 0.739$) following CPF exposure with LPS challenge (Appendix D). There was no difference in relative liver weight across treatments (CPF: $F_{(2,57)} = 0.814$, $p = 0.449$; LPS: $F_{(1,57)} = 3.727$, $p = 0.059$; CPF × LPS: $F_{(2,57)} = 0.265$, $p = 0.768$); however, there was a trend towards higher relative liver weights in individuals injected with LPS.

3.4.2 Effects of CPF on kidney histology

There was no significant interaction between CPF × LPS ($F_{(2,36)} = 2.667$, $p = 0.086$) on kidney epithelial cell height (Fig. 3.1) but there were main effects of both CPF exposure ($F_{(2,36)} = 10.702$, $p \leq 0.01$) and LPS challenge ($F_{(1,36)} = 17.454$, $p \leq 0.01$). Regardless of challenge state, epithelial cells in individuals exposed to 10 $\mu g\ L^{-1}$ CPF were 18% larger than in individuals from

the 0.01% DMSO control group (mean ± SEM; 7.126 ± 0.424 μm vs 8.689 ± 0.217 μm; $p \leq 0.001$). Upon observation, the tubules appeared swollen with a reduction in lumen size (Fig. 3.2). Kidney epithelial cell height also increased with LPS injection when compared with PBS-injected individuals independently of CPF exposure ($p \leq 0.001$).

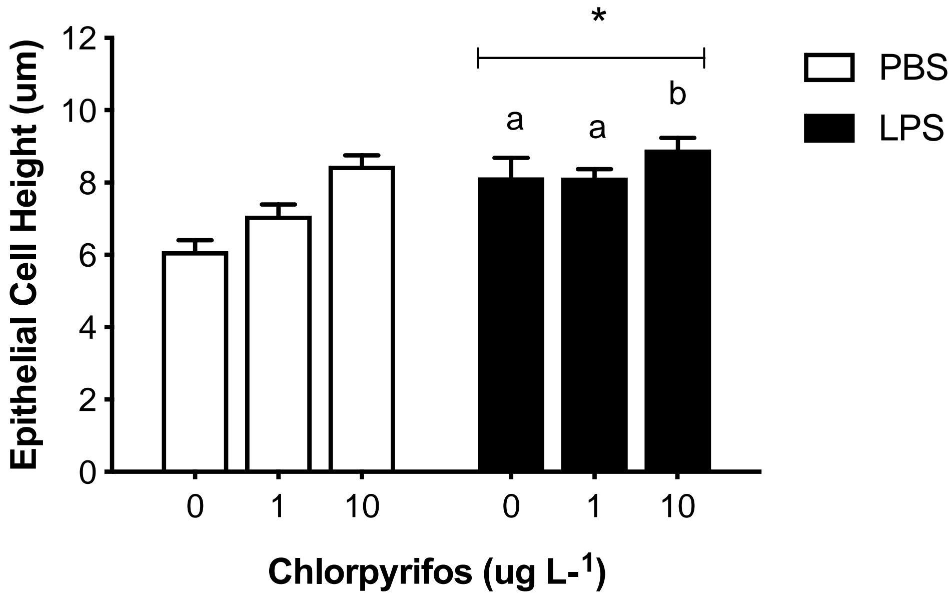


Figure 3.1. Effects of chlorpyrifos (CPF) exposure and lipopolysaccharide (LPS) challenge, alone and in combination, on mean (± SEM) kidney tubule epithelial cell height in metamorphic *X. laevis*. Individuals were exposed to CPF for 7 days and then injected with phosphate buffered saline (PBS) or LPS (10 µg g^{-1}) and sampled 1 dpi. Ten tubules from each kidney (n=6 individuals/treatment group) were randomly selected and four epithelial cells per tubule were measured. Data were analyzed using two-way ANOVA with CPF exposure and LPS challenge as factors. Letters indicate significant differences among CPF exposure ($p \leq 0.05$) and * indicates significant effect of LPS injection ($p \leq 0.05$).

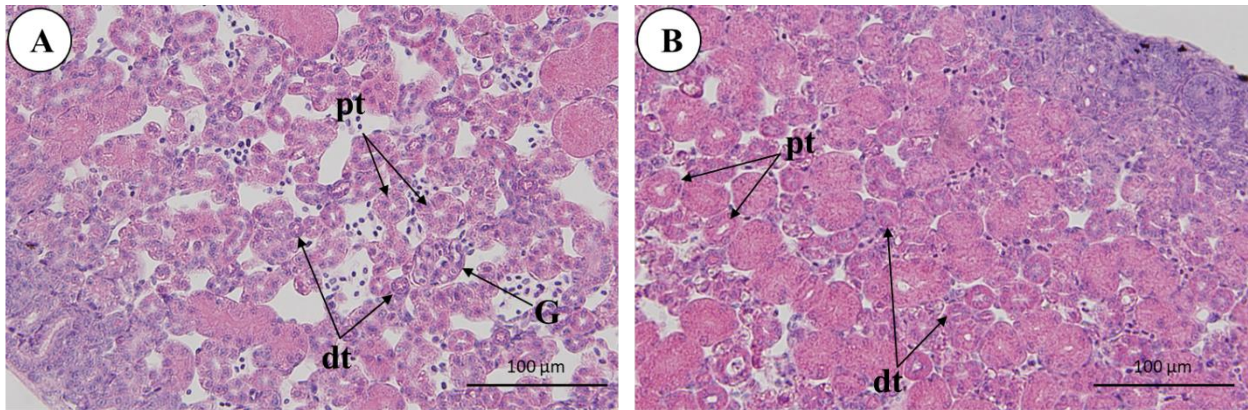


Figure 3.2. Photomicrographs of hematoxylin and eosin stained kidney sections from metamorphic *X. laevis* after 7 day exposure to chlorpyrifos (CPF). (A) Representative section from kidney of individual exposed to 0.01% DMSO control + PBS-injected showing normal organization of structures. (B) Representative section of kidney from an individual exposed to 10 µg L^{-1} CPF + PBS-injected showing epithelial cell hypertrophy and decreased lumen size in convoluted tubules. G = glomerulus, dt = distal convoluted tubule, pt = proximal convoluted tubule. Bar = 100.0 µm.

3.4.3 Effects of CPF on basal and LPS-stimulated leukocyte populations

For the proportion of lymphocytes in blood (Fig. 3.3A), there were main effects of both CPF exposure ($F_{(2,58)}$= 3.973, p = 0.025) and LPS challenge ($F_{(1,58)}$= 95.664, p ≤ 0.001) but no interaction between factors ($F_{(2,58)}$= 0.061, p = 0.941). Individuals exposed to 1 µg L^{-1} CPF had lower proportion of circulating lymphocytes compared to those exposed to 0.01% DMSO (p = 0.020), but this was not observed in the 10 µg L^{-1} CPF treatment group (p = 0.218). There was also an overall decrease in circulating lymphocytes in the LPS-injected animals compared to those injected with PBS (p ≤ 0.001). For blood monocyte populations (Fig. 3.3B), there was a main effect of LPS challenge ($F_{(1,58)}$= 107.763, p ≤ 0.001) but no main effect of CPF exposure ($F_{(2,58)}$= 0.053, p = 0.949) or interaction between CPF exposure and LPS challenge ($F_{(2,58)}$= 1.115, p = 0.336). Circulating monocyte populations increased after LPS injection compared to PBS-injected individuals (p ≤ 0.001).There was a main effect of CPF exposure ($F_{(2,58)}$= 5.299, p = 0.008) on circulating granulocytes (Fig. 3.3C) where individuals exposed to 1 µg L^{-1} CPF had higher circulating granulocytes when compared to the 0.01% DMSO control (p = 0.007) but this increase was not present in the 10 µg L^{-1} CPF exposure group (p = 0.071). There was no main effect of LPS challenge ($F_{(1,58)}$= 1.830, p = 0.182) on proportion of circulating granulocytes and no interaction between CPF exposure and LPS challenge ($F_{(2,58)}$= 1.557, p = 0.220). There were main effects of both CPF exposure ($F_{(2,58)}$= 4.725, p = 0.013) and LPS challenge ($F_{(1,58)}$= 11.540, p = 0.001) on the granulocyte:lymphocyte (GL) ratio (Fig. 3.3D) but no interaction between main factors ($F_{(2,58)}$= 0.455, p = 0.637). When compared to individuals exposed to 0.01% DMSO, there was an increase in the GL ratio with exposure to 1 µg L^{-1} CPF (p = 0.002) and 10 µg L^{-1} CPF (p = 0.028). There was also an increase in the GL ratio in individuals injected with LPS as compared to those injected with PBS (p = 0.001).

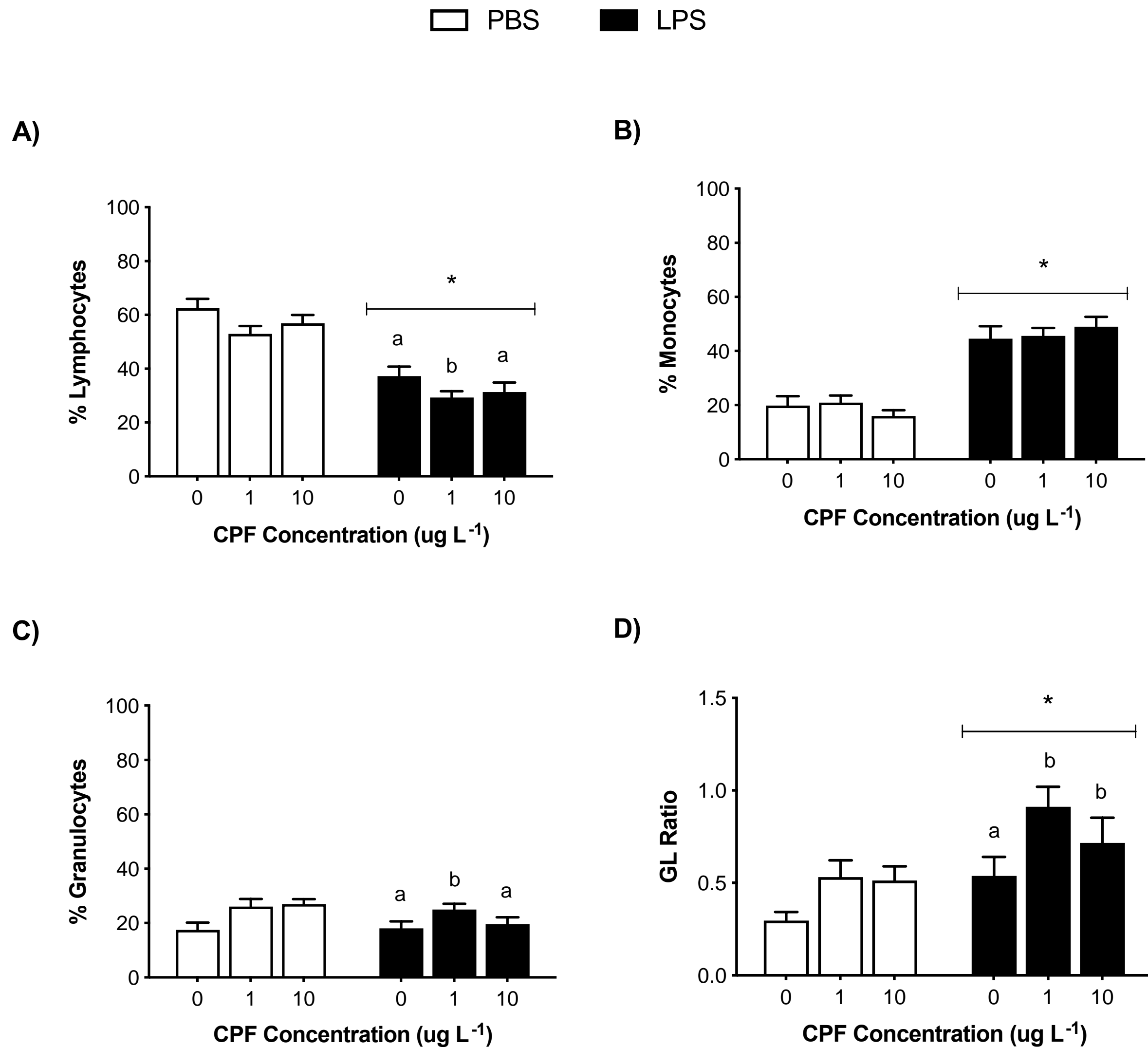


Figure 3.3. Effects of chlorpyrifos (CPF) exposure and lipopolysaccharide (LPS) challenge on the proportion of (A) lymphocytes, (B) granulocytes, (C) monocytes, and (D) granulocyte:lymphocyte (GL) ratio in whole blood of *X. laevis* as measured by DiOC5(3) staining and flow cytometry. Individuals were exposed to CPF for 7 days and then injected with phosphate buffered saline (PBS) or LPS (10 µg/g) and sampled 1 dpi. Bars represent mean ± SEM (n = 9-10). Data were analyzed using two-way ANOVA with CPF exposure and LPS challenge as factors. Letters indicate significant differences across CPF exposure and * indicates significant effect of LPS injection ($p \leq 0.05$).

3.4.4 Effect of CPF on LPS-stimulated expression of pro-inflammatory cytokines

Levels of TNF-α mRNA were affected by both CPF exposure ($F_{(2,33)}$= 3.726, p = 0.037) and LPS challenge ($F_{(1,33)}$= 34.607, p ≤ 0.001) but there was no interaction between the two factors ($F_{(2,33)}$= 0.503, p = 0.610; Fig. 3.4A). Individuals exposed to 10 μg L^{-1} CPF had increased liver TNF-α mRNA expression when compared with the 0.01% DMSO treatment group (p = 0.044). Liver TNF-α mRNA levels were also increased in LPS-injected individuals when compared to those injected with PBS (p ≤ 0.001). Similarly, CSF-1 expression was altered with exposure to CPF ($F_{(2,35)}$= 3.723, p = 0.036) and there was also a main effect of LPS challenge ($F_{(1,35)}$= 15.747, p ≤ 0.001) but there was no interaction between factors ($F_{(2,35)}$= 0.639, p = 0.535; Fig. 3.4B). Expression of CSF-1 in the liver was increased with exposure to 10 μg L^{-1} CPF compared to the 0.01% DMSO control (p = 0.044) and after LPS injection compared to PBS-injected individuals (p ≤ 0.001). There was a main stimulatory effect of LPS challenge on expression of IL-1β ($F_{(1,34)}$= 36.562, p ≤ 0.001) but no effects of CPF exposure ($F_{(2,34)}$ = 1.213, p = 0.312) or interaction between factors ($F_{(2,34)}$= 2.021, p = 0.151; Fig. 3.4C). Expression of IL-1β in liver was significantly increased in individuals injected with LPS as compared to the PBS-injected group (p ≤ 0.001).

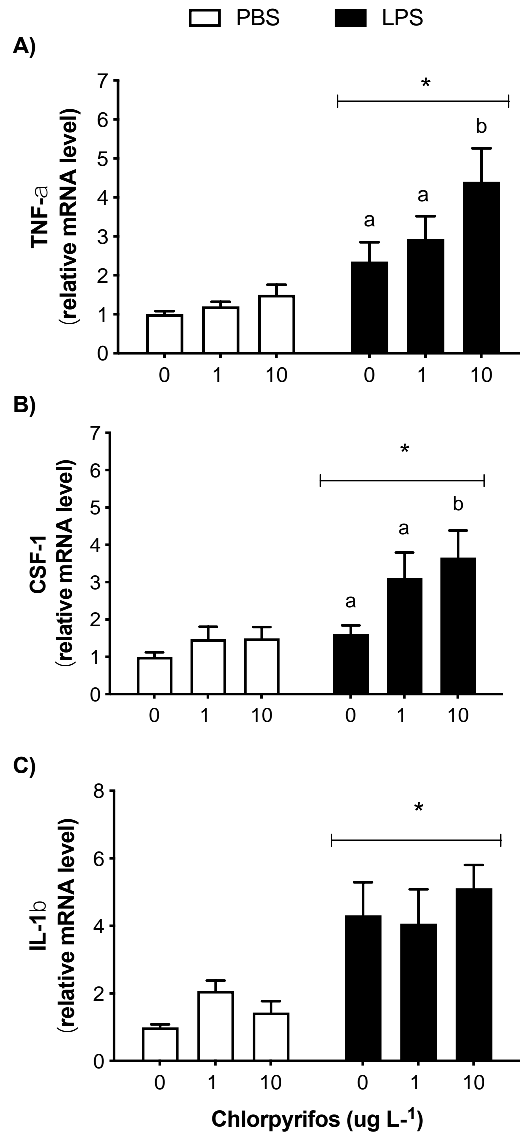


Figure 3.4. Effects of chlorpyrifos (CPF) exposure and lipopolysaccharide (LPS) challenge on mRNA expression of (A) tumor necrosis factor α (TNF-α), (B) colony stimulating factor 1 (CSF-1), and (C) interleukin 1β (IL-1β) in liver of *X. laevis* as measured by qPCR. Individuals were exposed to CPF for 7 days and then injected with phosphate buffered saline (PBS) or LPS (10 µg/g) and sampled 1 dpi. Data were normalized to the geometric mean of GAPDH and EF1α and expressed relative to the 0.001% DMSO + PBS-injected group. Bars represent the mean ± SEM (n = 6). Data were analyzed using two-way ANOVA with CPF exposure and LPS challenge as factors. Different letters indicate significant differences between CPF concentrations and * indicates significant effect of LPS injection ($p \leq 0.05$).

3.5 Discussion

The objective of this study was to assess the immunomodulatory effects of CPF exposure in the laboratory model amphibian species, *X. laevis*, using an immune challenge approach. We found that short-term (7 d) exposure to 1 and 10 μg L^{-1} CPF resulted in structural changes in the kidney, altered specific circulating leukocyte populations, and increased expression of pro-inflammatory cytokines. This study provides some of the first evidence of the immunotoxic effects of CPF in amphibians and indicates that exposure to CPF may impair the ability of amphibians to resist pathogen infection with consequences for disease occurrence.

When analyzed by flow cytometry, the proportion of different leukocyte populations in peripheral blood changed with both CPF exposure and with LPS-stimulated immune challenge. Exposure to 1 μg L^{-1} CPF resulted in a decrease in circulating lymphocytes and an increase in circulating granulocytes and this effect was independent of the LPS challenge. A decrease in circulating lymphocytes and an increase in circulating granulocytes is characteristic of a chemical or biological stress response in amphibians (reviewed in Davis et al., 2008). Exposure to CPF has been associated with decreased circulating lymphocytes in a variety of vertebrates including rats (*Rattus norvegicus*) (Elelaimy et al., 2012; Goel et al., 2006), chickens (*Gallus domesticus*) (Begum et al., 2015), and catfish (*Clarias gariepinus*) (Adewumi et al., 2018). Increased circulating granulocyte populations after CPF exposure have been reported in species across taxa, including silk worm (*Philosamia ricini*) (Kankana Kalita and Devi, 2016), rats (*R. norvegicus*) (Elelaimy et al., 2012), and fresh water crab (*Paratelphusa jacquemontii*) (Maharajan et al., 2017). Interestingly, the effect of CPF exposure at 1 μg L^{-1} was not observed at 10 μg L^{-1} CPF where both lymphocyte and granulocyte populations were the same as control group. However, exposure to both 1 and 10 μg L^{-1} CPF increased the GL ratio, which is a composite measure of the stress response and is considered a more relevant parameter than looking at either leukocyte population alone (Davis et al., 2008; Uchendu et al., 2018). In birds, an elevated GL ratio has been linked to increased susceptibility to infection (Al-Murrani et al., 2002) and an increase in GL ratio is positively related to both the magnitude of the stressor and circulating glucocorticoids in most vertebrates (reviewed in Davis et al., 2008). Based on this composite measure of inflammatory state, it appears that exposure to 1 and 10 μg L^{-1} CPF can induce an inflammatory response in metamorphic *X. laevis.* It is also important to note that for this immune-challenge study, we targeted nominal CPF concentrations that were sub-lethal for

amphibians (based on previous studies) and that were environmentally relevant. These concentrations were not confirmed analytically and are likely to fall below the nominal values, based on what is known about CPF physicochemical properties including adsorption to glass (Thomas and Mansingh, 2002) and algae (Giddings et al., 2014), and a high escaping tendency in laboratory exposure systems (Manzanti et al., 2003). Therefore, we would hypothesize that CPF exposure could have effects on immune parameters in amphibians at actual concentrations lower than those nominally tested in the present study.

Expression of key pro-inflammatory cytokines, which are effector molecules that allow communication between components of the immune system, was also evaluated as an indicator of immune status. Pro-inflammatory cytokines have many important roles during the immune response to chemical or biological stress, including leukocyte production and migration (Nourshargh and Alon, 2014). We observed that exposure to 10 $\mu g\ L^{-1}$ CPF increased liver mRNA levels of pro-inflammatory cytokines TNF-α and CSF-1. Increased TNF-α expression has been reported after CPF exposure in various fish species including *C. carpio* (Chen et al., 2014), *O. niloticus* (Zahran et al., 2018), and *D. rerio* (Jin et al., 2015). Upregulation of TNF-α is indicative of an inflammatory response and expression of TNF-α is rapidly induced after viral and bacterial infection in fish and amphibians (Morales et al., 2010; Teles et al., 2011). During the innate immune response, TNF-α induces apoptosis and enhances granulocyte migration (Erger and Casale, 1998; MacKenzie et al., 2003). A potential explanation for the apparent return to basal levels of granulocytes observed in the 10 $\mu g\ L^{-1}$ CPF group may be due, in part, to the increased expression of TNF-α enhancing migration of granulocytes out of circulation and into tissues, such as the kidney. CSF-1 is one of the principal mediators of the development, differentiation, proliferation, and survival of macrophages across vertebrates, including amphibians (Grayfer and Robert, 2014; Pixley and Stanley, 2004). Currently, there are no studies characterizing the response of CSF-1 to CPF or any OP pesticide exposure in aquatic vertebrates, although our results indicate that exposure to CPF can induce CSF-1 expression in *X. laevis*. Monocytes are the activated precursors to macrophages, and in the present study there was no apparent effect of CPF exposure on circulating monocyte populations. CSF-1 is critically involved in proliferation and differentiation of progenitor cells into macrophages, so it is possible that increased CSF-1 led to increased activated macrophage development from circulating

monocytes and may explain, in part, the apparent return to basal monocyte levels at the highest CPF concentration.

Histological examination of kidney tissues from CPF-exposed *X. laevis* revealed an increase in kidney epithelial cell size in individuals exposed to 10 µg L^{-1} CPF. Hypertrophy of kidney epithelial cells and decreased lumen size with exposure CPF has been reported in a number of species including rats (*R. norvegicus*) and common carp (*C. carpio*) (Raina and Hamid, 2013; Xing et al., 2015). The kidney plays a dual role in amphibians, both as a key component of body fluid homeostasis and an immune organ (Carey and Bryant, 1995), and is the known target organ for Ranavirus, an environmentally relevant pathogen of concern that has been implicated in globally declining amphibian populations (Daszak et al., 1999; Miller et al., 2011). Kidney epithelial cells also play a role in the innate immune response and act as both the site of production and cellular target for inflammatory mediators such as pro-inflammatory cytokines and leukocytes (Cantaluppi et al., 2014). In a recent study by Zhang et al. (2017), transcriptomic responses were assessed in head kidney of common carp (*C. carpio*) after CPF exposure and pathways related to immune response, immune system processes, and cytokine activity and function were significantly upregulated. As kidney epithelial cells are an active site of cytokine production, CPF-induced inflammation resulting in increased production and release of pro-inflammatory cytokines, such as TNF-α and CSF-1, may be contributing to the observed cellular hypertrophy, along with tissue damage associated with this inflammatory response. In this study, histopathological examination of kidney tissue did not include lymphoid tissue which would lend further insight into the immunotoxicological effects of CPF exposure in *X. laevis*.

In our study, *X. laevis* mounted an appropriate molecular and cellular response to LPS although this characteristic response did not appear to be impacted by CPF. When compared to the PBS-injected group, LPS-injected individuals had lower circulating lymphocytes, increased monocytes, and increased GL ratio in whole blood. The observed changes in lymphocytes and GL ratio are characteristic of amphibians in stressed states, such as after exposure to corticosterone (Falso et al., 2015) and hydrocortisone (Bennett et al., 1972). In fish, LPS exposure is reported to increase circulating monocyte populations (Swain et al., 2008), which is consistent with the results of our study. There is a lack published data on changes in blood monocyte population in response to stress; however, in a previous study by our lab, there was a similar an increase in monocytes in *X. laevis* after LPS exposure (Gallant and Hogan,

unpublished). In contrast, Falso et al. (2015) reported no change in circulating monocyte populations in *X. laevis* or *Lithobates catesbeianus* with long-term corticosterone exposure. Interestingly, an increase in circulating granulocytes is also a characteristic response in amphibians in stressed conditions (reviewed in Davis et al., 2008) and this was not observed in our study. However, in a recent study in three-spined stickleback (*Gasterosteus aculeatus*), the authors reported no change in proportion of granulocytes after 4 d exposure to CPF concentrations up to 1.75 μg L^{-1} followed by intraperitoneal LPS injection (Marchand et al., 2017). In a separate immune-challenge study by our group, there was also no change in granulocyte populations after LPS injection in *X. laevis* and it was hypothesized that contrasting responses of different types of granulocytes (neutrophils and eosinophils), which are indistinguishable using the DiOC5(3) assay, may contribute to apparent lack of overall change in granulocytes (Gallant and Hogan, unpublished).

The roles of leukocytes and cytokines in the inflammatory response are interdependent and so it was not surprising to find expression of pro-inflammatory cytokines TNF-α, IL-1β, and CSF-1 increased in liver of LPS-injected frogs. Both lymphocytes and activated macrophages (derived from monocytes) play critical roles in the synthesis and release of pro-inflammatory cytokines including TNF-α and IL-1β. (Arango Duque and Descoteaux, 2014; Swain et al., 2008). Overall, it appears that LPS is an effective mitogen in amphibians, affecting leukocyte populations and pro-inflammatory cytokine expression as well as inducing hypertrophy of kidney epithelial cells.

3.6 Conclusion

In this study, short-term (7 d) exposure to environmentally relevant concentrations of CPF resulted in changes to markers of immune status as well as histopathological changes of the kidney in metamorphic *X. laevis*. In general, exposure to CPF appeared to induce an inflammatory response independent of the LPS challenge but individuals were still able to mount an appropriate immune response to LPS exposure, specifically changes in targeted leukocyte populations and increased expression of pro-inflammatory cytokines. This research contributes to a better understanding of the potential immunotoxicity of CPF in amphibians in light of recent concern regarding pesticide contamination of aquatic systems, immunotoxicity and disease-driven amphibian declines. Future research using this immune-challenge approach with an

environmentally relevant pathogen, such as Ranavirus, could provide further insight into the potential consequence of CPF contamination and exposure on immunocompetence in amphibians.

CHAPTER 4

GENERAL DISCUSSION

4.1 Project objective and focus

As the global demand for pesticide use increases, so too does the amount of these chemicals occurring within aquatic systems in Canada, North America, and worldwide. Chlorpyrifos (CPF), is one of the most commonly used organophosphate (OP) pesticides and has been measured in aquatic systems close to point sources as well as in remote and seemingly pristine locations (Adeyinka and Pierre, 2019; Muir et al., 2004). This ubiquitous contamination poses a threat to non-target species interacting with or inhabiting affected aquatic environments. Amphibian species are of particular concern due to potential links between pesticide contamination of aquatic ecosystems leading to increased susceptibility to pathogen infection and disease outbreaks (Carey et al., 1999). Exposure to CPF has been associated with a range of sub-lethal effects in aquatic vertebrates including morphometric abnormalities (Jin et al., 2015; Kleinhenz et al., 2012; Richards and Kendall, 2003), histopathological alteration across a range of tissues (Colombo et al., 2005; Scheil et al., 2009; Xing et al., 2012), acetylcholinesterase (AChE) inhibition (Colombo et al., 2005; Liendro et al., 2015; Richards and Kendall, 2002), immunotoxicity (Adel et al., 2017; Harford et al., 2005; Kerby and Storfer, 2009), oxidative stress (Liendro et al., 2015; Xing et al., 2012; Zhang et al., 2017), and changes in expression of target genes and transcriptomic profiles (Wang et al., 2018; Zahran et al., 2018; Zhang et al., 2017). The overall objective of this thesis was to gain a better understanding of the sub-lethal effects of exposure to CPF on the model amphibian species, *Xenopus laevis*, and to provide novel amphibian data on responses across levels of biological organization from molecular to whole animal.

The research conducted in Chapter 2 of this thesis aimed to characterize and compare the transcriptome responses of short-term early life stage (ELS) exposure to CPF in *X. laevis* and apical outcomes after chronic CPF exposure through to metamorphic climax. There were several reasons for choosing amphibians as the test animal and CPF as the chemical of interest. Firstly, amphibians are generally underrepresented in the toxicological literature and little is known related to sub-lethal effects of CPF exposure at environmentally relevant concentrations. Secondly, there is a complete lack of data regarding the effects of CPF on the transcriptome of amphibians which, if known, could help us understand underlying toxicity pathways leading to negative apical outcomes of exposure. Finally, this work was conducted as part of the EcoToxChip project, which aims to to develop and validate quantitative PCR arrays and a data evaluation tool (EcoToxXplorer.ca) for characterization, prioritization, and management of environmental contaminants in model laboratory species and native species of concern (www.ecotoxchip.ca; Basu et al., 2019). The pathway analysis in Chapter 2 revealed a number of pathways specifically related to immune functions and processes, suggesting that the developing immune system may be a target of CPF toxicity in ELS *X. laevis*. To determine whether this CPF-mediated ELS pathway dysregulation led to a functional impact, the immune system was selected as the focus for further examination. The research conducted in Chapter 3 of this thesis focused on immunotoxicity as a potential mode of action for adverse effects of CPF exposure in amphibians. The aim of this chapter was to determine if CPF was able to alter biomarkers of innate immunity and whether this translated to impairment in the ability to mount an effective immune response to a simulated pathogen, lipopolysaccharide (LPS). Again, there is a lack of literature regarding the effects of short-term, sub-lethal CPF exposure on the amphibian immune system despite evidence of immunotoxicity in various fish species. The limited studies that do exist in this context do not use an immune-challenge approach that actually evaluates the capacity for an individual to mount an immune response following exposure. Previous studies have also not examined impacts during the metamorphic life-stage. Amphibians undergo a widespread remodeling of many physiological systems, including the immune system, during metamorphosis and there is evidence that metamorphs are more sensitive to CPF exposure than pre-metamorphs in some cases (Richards and Kendall, 2003; Rollins-Smith, 1998). Thus, this research focussed on the effects of CPF exposure during the unique transitional life-stage of metamorphosis.

4.2 Major findings and conclusions of research

The research conducted as part of this thesis evaluated the effect of sub-lethal exposure to CPF in *X. laevis* across levels of biological organization from transcriptome to whole animal. Overall, exposure to CPF caused significant changes in several endpoints measured across various levels of biological organization. Effects of CPF exposure included dysregulation of pathways and expression of specific genes at the molecular level, changes in circulating leukocytes at the cellular level, histopathological alterations at the tissue/organ level, and morphometric changes at the level of the whole animal.

In Chapter 2, 96 hour (h) exposure to CPF in ELS *X. laevis* resulted in upregulation of pathways associated with protein and carbohydrate metabolism, blood coagulation, tetrapyrrole binding, and sensory perception of light stimulus. We also observed depletion of pathways associated with immune function and vasculature development after CPF exposure. Additionally, non-specific dysregulation of pathways involving serine hydrolase activity and the extracellular region of cells was induced by short-term CPF exposure. Due to the interdependency of many physiological systems when exposed to a contaminant such as CPF, it is likely that these pathways are linked to one another. In some cases, the links between pathways are obvious. For example, pathways involved in serine hydrolase activity, tetrapyrrole and heme binding, and monooxygenase activity are all related to the inhibition of AChE activity which is an expected outcome of exposure to OP pesticides, including CPF. In other cases, the links between pathways may be more subtle. For example, it is possible that the multitude of effects on energy metabolism and metabolic processes were a more general response to the changes observed in more specific outcomes, such as immune function and neurological processes. Apical outcomes of chronic CPF exposure were also observed, including inhibition of AChE activity, increased liver weight relative to body weight, and a decrease in percentage of individuals that reached Nieuwkoop and Faber (NF) stage 65 (Nieuwkoop and Faber, 1994). In some cases, the altered transcriptomic pathways following ELS exposure could be related to the observed apical outcomes. For example, pathways related to serine hydrolase activity were dysregulated after 96 h ELS exposure and AChE, a serine hydrolase enzyme, was inhibited in the brains of *X. laevis* at metamorphic climax after chronic exposure to 10 $\mu g\ L^{-1}$ CPF. Additionally, dysregulation of pathways associated with immune function as well as protein and carbohydrate metabolism occurred, which could potentially be associated to the observed increase in liver weight relative

to body weight after chronic exposure to 10 μg L^{-1} CPF. Pathways associated with immunotoxicity, cardiovascular toxicity, tetrapyrrole and heme binding, and blood coagulation were unsurprisingly dysregulated after CPF exposure. Outcomes of CPF exposure related to these pathways have precedence in the literature and have been noted in amphibians or other aquatic vertebrates. These results strengthen the known mechanisms of action and endpoints associated with CPF exposure in aquatic species and provide novel data for amphibians to add to the relatively large amount of data for fish species. Some of the transcriptomic pathways that were dysregulated by CPF exposure had little or no precedence in the literature, regardless of taxa. These included effects on vasculature development, response to light stimulus, and effects on the extracellular region of cells specifically. These findings have the potential to direct new research into the outcomes of CPF exposure in amphibians. In Chapter 2, we demonstrated that short-term, ELS exposure to CPF has the potential to disrupt a range of important pathways and processes in *X. laevis.* Pathway analysis revealed disruption of biological processes and molecular functions which may be associated with adverse outcomes after chronic exposure through metamorphic climax. To our knowledge, this is the first research to assess changes in the ELS transcriptomic profile after CPF exposure in amphibian species.

Immunotoxicity is a known outcome of CPF exposure in fish species; however, the literature regarding CPF-mediated immunotoxicity of amphibians is relatively scarce. In Chapter 2, depletion of immune-related pathways including cytokine receptor activity, immune function, and inflammatory response occurred after 96 h ELS exposure to CPF. Further examination of CPF-induced immunotoxicity was warranted due to the potential links between sub-lethal pesticide exposure, immunomodulation, and increased susceptibility to disease. In some cases, pesticide exposure in the environment has been linked to both localized mass mortality events, as well as widespread declines of amphibian populations globally (Carey et al., 1999; Hayes et al., 2010). Therefore, research presented in Chapter 3 aimed to determine whether impacts on immune-related pathways after ELS exposure translated to disrupted immune function and to examine the potential immunomodulatory effects of CPF exposure on amphibians at the critical and unique transitional life stage of metamorphosis when challenged with a the known immunostimulatory agent, LPS. Exposure to CPF for 7 days (d) resulted in immunomodulation in *X. laevis* metamorphs as demonstrated by changes in endpoints related to immune function. Exposure to 10 μg L^{-1} CPF increased epithelial cell height as well as decreased lumen space in

convoluted tubules of the kidney. Circulating leukocyte populations were also affected with decreased lymphocytes and increased granulocytes observed after exposure to 1 μg L^{-1} CPF along with a higher granulocyte:lymphocyte (GL) ratio at 1 and 10 μg L^{-1} CPF. Lastly, the expression of pro-inflammatory cytokines TNF-α and CSF-1 in the liver increased with exposure to 10 μg L^{-1} CPF. To our knowledge, this is the first study to examine the immune-related effects of short-term exposure to CPF in amphibians and considering responses in an immune-stimulated state. However, despite evidence that CPF exposure alone can alter immune parameters, this exposure did not appear to hinder the animal's ability to mount an appropriate response to the immunostimulatory agent LPS. Following LPS injection we observed the characteristic responses reported in aquatic vertebrates, namely decreased circulating lymphocytes, increased circulating monocytes, and an increased GL ratio (Swain et al., 2008). We did not observe an increase in proportion of circulating granulocytes which is an expected response to stress in amphibians (reviewed in Davis et al., 2008). Injection of LPS also induced expression of pro-inflammatory cytokines, TNF-α, IL-1β, and CSF-1. Several *in vitro* studies with fish cells report that LPS stimulates macrophages to produce pro-inflammatory cytokines (reviewed in Swain et al., 2008) and this appears to be conserved in amphibians as well (Robert and Ohta, 2009). We conclude that LPS is an effective mitogen in *X. laevis* and is appropriate for usage in an immune-challenge assay as it induces a robust and measurable inflammatory response. Overall, we conclude that CPF exposure altered biomarkers of immune status and histopathological changes in *X. laevis* kidneys but that these observed outcomes did not necessarily translate into immunosuppression or impaired ability to mount an appropriate response to LPS exposure.

4.3 Limitations of research and future directions

The nominal waterborne concentrations of CPF used in Chapter 2 (0.4, 2, and 10 μg L^{-1}) were chosen based on a literature review to determine sub-lethal and environmentally relevant concentrations in amphibians as well as a 21-d pilot exposure to identify sub-lethal concentrations in *X. laevis*. Water samples were collected from each tank at five time points throughout the exposure period, and composite samples from each treatment group were analysed to determine actual water CPF concentrations throughout the chronic exposure. The average recovery throughout the exposure ranged from 17 – 29% of nominal, with the highest

recovery in the sample taken at the earliest time point in the exposure period. In the aquatic environment, CPF is reported to adsorb to algae (Giddings et al., 2014) and glass (Thomas and Mansingh, 2002). In the present study, it is possible that some of the waterborne CPF was being adsorbed to the algae and that this adsorption proportionally increased throughout the exposure period with increasing algal growth. Additionally, adsorption of the chemical to the glass aquaria, the glass sampling equipment, and the glass bottles used to store and ship the composite samples could account for further losses of measured CPF. A study by Mazanti et al. (2003) examined the aqueous-phase disappearance of CPF from both laboratory and field exposure systems. They found that CPF was rapidly lost from these systems initially, and concluded that, based on its physicochemical properties, CPF had a very high escaping tendency in aquatic systems (Mazanti et al., 2003). Measuring actual body burden of CPF within exposed individuals may provide a more accurate picture of the actual exposure concentrations. In addition, future studies using waterborne CPF over a chronic exposure period would benefit from measuring chemical concentration within the stock solutions as well as water samples to ensure degradation is not occurring in the stock solutions themselves.

The endpoints measured in Chapter 2 at the end of chronic exposure to CPF (at metamorphic climax) were pre-determined based on scientific literature and selected prior to knowledge of the changes in the transcriptome profiles of ELS individuals. To ensure that the most relevant outcomes of chronic CPF exposure were measured, apical endpoints could have been selected after completion of pathway analysis. This would have allowed us to target pathways and processes that were shown to be dysregulated after ELS CPF exposure and to determine if these ELS disturbances translated to apical outcomes of chronic exposure. For example, the immune-related effects of exposure to OP pesticides, including CPF, in aquatic vertebrates are becoming more widely discussed in the literature. The ELS transcriptomic profile showed dysregulation of a number of pathways associated with immune function after short-term CPF exposure. An immune challenge study measuring infection rates, morbidity, and mortality after chronic CPF exposure would help determine if immune-related ELS pathway dysregulation led to functional alteration of the immune system later in life.

In Chapter 3, LPS injection was used as an immunostimulant and to determine whether CPF-exposed individuals were able to mount an appropriate immune response. Lipopolysaccharide is commonly used in the context of immune challenge exposures and, as

such, the expected immune response to LPS exposure is noted in the literature (Swain et al., 2008). Although effective in simulating a pathogen infection, LPS is not a pathogen of concern in an environmentally relevant scenario. Use of an environmentally relevant amphibian pathogen of concern, such as Ranavirus, would better reflect the immune-related outcomes of amphibians in a CPF-contaminated environment. In the wild, Ranavirus has been implicated in both localized mass mortality events as well as globally declining amphibian populations. Amphibian pathogen and contaminant exposure occurs simultaneously in the environment. Recent studies have explored the links between amphibian exposure to pesticides leading to immunosuppression and increased incidence of disease. Some have discussed the possibility that Ranavirus, and other pathogens of concern, are not emergent but rather the inability to mount an appropriate immune response because of pesticide exposure is what is emergent (Hayes et al., 2006). A follow up study examining the effects of CPF exposure on the immune response to an environmentally relevant pathogen, such as Ranavirus, could provide insight on the potential links between ubiquitous CPF contamination and increased opportunistic pathogen infection.

4.4 Applicability of research findings

In the research conducted in Chapter 2 of this thesis, we examined the transcriptomic responses to CPF exposure in ELS *X. laevis*. This study was a part of a large, collaborative research project (the EcoToxChip project) that aims to to develop and validate quantitative PCR arrays and a data evaluation tool (EcoToxXplorer.ca) for characterization, prioritization, and management of environmental contaminants in model laboratory species and native species of concern (www.ecotoxchip.ca; Basu et al., 2019). Specifically, the EcoToxChip project aims to link multi-omics endpoints (transcriptomic, metabolomic, and proteomic) after ELS exposure to apical outcomes of chronic exposure to contaminants of concern. The findings of this study will be combined with other ‘omic and apical endpoint analyses in both *X. laevis* and a native North American Ranid amphibian, *Lithobates pipiens* (Northern leopard frog), as well as fish and avian species. The observed transcriptome-level responses to CPF in ELS individuals, especially identification of novel dysregulated pathways not identified in previous studies, have the potential to drive future research into the underlying mechanisms of CPF-mediated toxicity in amphibians. Over the chronic exposure period, there were a number of apical outcomes that were altered at environmentally relevant concentrations. However, some endpoints that are normally

associated with CPF exposure in aquatic vertebrates, such as changes in morphometric indices and incidence of malformation, were largely unaffected in our study. This could be attributed to both species and life stage differences and highlights the importance of considering amphibians separately when conducting toxicological research. Some of the dysregulated pathways identified in this study after ELS exposure to CPF could direct future research on relevant and novel apical outcomes of chronic CPF exposure in amphibians. These outcomes may, in some cases, differ from the more widely examined endpoints of CPF exposure in aquatic vertebrates which are largely based on studies performed in fish species.

In Chapter 3 of this thesis, we used an exposure approach optimized in our lab where a short-term chemical exposure using the model amphibian species, *X. laevis*, is followed by an immune challenge using LPS as an immunostimulant. Our work shows that this exposure model could be effective for rapid screening of environmental contaminants for potential immunotoxicity and specifically their capacity to compromise an appropriate immune response. This approach could be used in a variety of exposure scenarios with different classes of chemicals, different species of amphibians, and different immunostimulatory agents or pathogens including environmentally relevant pathogens of concern.

4.5 Concluding statement

This thesis research was conducted to gain a better understanding of the effects of CPF exposure on amphibians and to provide novel data on responses across levels of biological organization, from molecular to whole animal. The results indicate that exposure to CPF can affect endpoints across a range of physiological parameters at all levels of biological organization, suggesting that CPF exposure in aquatic environments poses a threat to amphibian species. These findings add to the growing body of evidence regarding the potential links between pesticide contamination, opportunistic infection leading to disease, and global amphibian declines and overall highlight the importance of considering amphibian species when examining the impacts of contaminants on aquatic vertebrates.

APPENDICES

Appendix A: Mean (± SEM) for water quality parameters measured over the chronic chlorpyrifos (CPF) exposure period. Parameters were measured from each tank a minimum of twice weekly over 75 days of exposure.

			CPF Concentration (µg L^{-1})		
	Facility Water	**0.01% DMSO**	**0.4**	**2**	**10**
Temperature (ºC)	21.72 ± 0.04	21.60 ± 0.05	21.48 ± 0.05	21.62 ± 0.04	21.56 ± 0.05
pH	7.96 ± 0.03	7.97 ± 0.04	7.99 ± 0.04	7 .97 ± 0.04	7.95 ± 0.04
Dissolved Oxygen (%)	75.87 ± 1.40	77.13 ± 1.41	78.88 ± 1.13	77.66 ± 1.04	77.94 ± 1.17
Ammonia (ppm)	0.53 ± 0.04	0.50 ± 0.04	0.54 ± 0.04	0.54 ± 0.04	0.53 ±0.04
Nitrate (ppm)	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01
Nitrate (ppm)	0.05 ± 0.00	0.05 ± 0.00	0.07 ± 0.02	0.05 ± 0.00	0.05 ± 0.00
Hardness (ppm)	179.88 ± 0.22	180.12 ± 0.19	180.08 ± 0.38	179.80 ± 0.12	179.96 ± 0.04
Alkalinity (mg/L)	122.33 ± 0.55	122.08 ± 0.48	122.32 ± 0.48	122.24 ± 0.53	122.49 ± 0.56
Conductivity (µS/cm)	472.13 ± 1.25	471.64 ± 1.05	471.94 ± 1.10	472.28 ± 1.15	472.40 ± 1.14

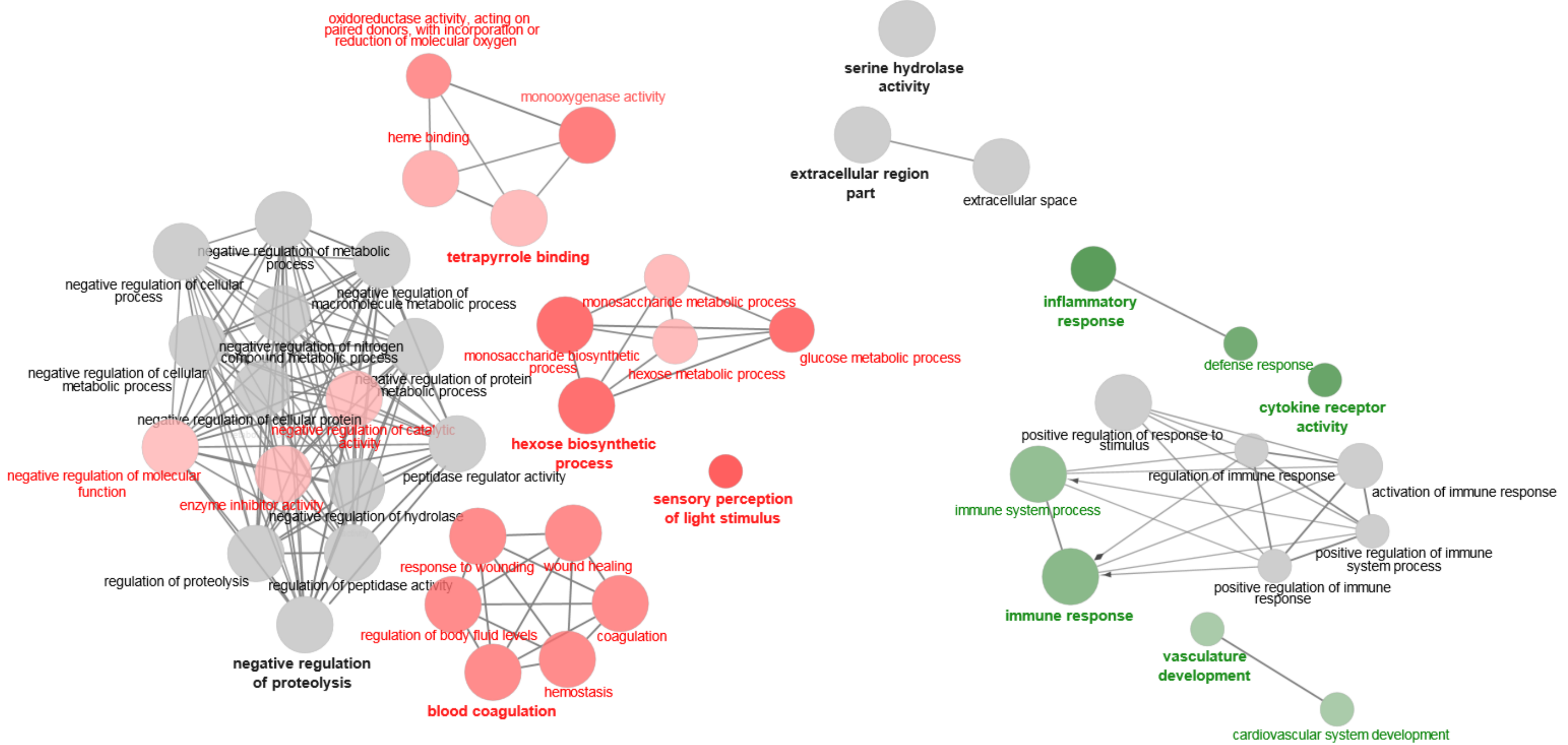
Appendix B: **(A)** Table displaying all significantly altered ($p \leq 0.05$) Gene Ontology (GO) terms after 96 h chlorpyrifos (CPF) exposure in early life-stage *X. laevis*. The term p values were corrected with a Bonferroni step down. **(B)** ClueGo visualization of functionally grouped networks formed from significantly ($p \leq 0.05$) altered pathways based on GO databases. Pathways were built using at least 5 differentially expressed genes ($p \leq 0.05$) in early life-stage *X. laevis* after 96 h of CPF exposure. Statistical test was set to a right-sided hypergeometrical test with a Bonferroni (step down) p-value correction and a kappa score of 0.4. Node size represents pathway significance and darker shades represent higher gene proportion associated with pathway. Green node = depleted pathway, gray node = un-specific pathway, red node = upregulated pathway. Text colour has been changed for clarity.

(A)

GO Term	GO ID	Term P Value	GO Levels	% Associated Genes	# Associated Genes	Upregulated/Depleted
sensory perception of light stimulus	GO:0050953	0.01	[5]	11.76	10.00	Upregulated
tetrapyrrole binding	GO:0046906	0.00	[3]	11.71	24.00	Upregulated
heme binding	GO:0020037	0.00	[3,4]	11.50	23.00	Upregulated
monooxygenase activity	GO:0004497	0.00	[4]	12.41	17.00	Upregulated
oxidoreductase activity, acting on paired donors	GO:0016705	0.00	[4]	9.28	18.00	Upregulated
monosaccharide metabolic process	GO:0005996	0.00	[3,4]	15.25	9.00	Upregulated
hexose metabolic process	GO:0019318	0.00	[4,5]	16.36	9.00	Upregulated
monosaccharide biosynthetic process	GO:0046364	0.00	[4,5]	30.43	7.00	Upregulated
glucose metabolic process	GO:0006006	0.00	[5,6]	21.88	7.00	Upregulated
hexose biosynthetic process	GO:0019319	0.00	[5,6]	30.43	7.00	Upregulated
blood coagulation	GO:0007596	0.00	[3,5]	25.00	14.00	Upregulated
response to wounding	GO:0009611	0.00	[3]	20.59	14.00	Upregulated
regulation of body fluid levels	GO:0050878	0.00	[3]	22.58	14.00	Upregulated
hemostasis	GO:0007599	0.00	[4]	25.00	14.00	Upregulated
wound healing	GO:0042060	0.00	[4]	22.58	14.00	Upregulated
negative regulation of metabolic process	GO:0009892	0.00	[2,3,4]	7.79	31.00	None Specific

negative regulation of macromolecule metabolic process	GO:0010605	0.00	[3,4,5]	8.05	31.00	None Specific
negative regulation of cellular metabolic process	GO:0031324	0.00	[3,4,5]	8.96	30.00	None Specific
negative regulation of molecular function	GO:0044092	0.00	[3]	11.26	26.00	Upregulated
negative regulation of nitrogen compound metabolic process	GO:0051172	0.00	[3,4,5]	9.23	30.00	None Specific
negative regulation of protein metabolic process	GO:0051248	0.00	[4,5,6]	12.44	27.00	None Specific
negative regulation of cellular process	GO:0048523	0.00	[2,3,4]	6.70	40.00	Upregulated
negative regulation of catalytic activity	GO:0043086	0.00	[4]	12.21	26.00	Upregulated
enzyme inhibitor activity	GO:0004857	0.00	[5]	12.92	23.00	Upregulated
negative regulation of hydrolase activity	GO:0051346	0.00	[5]	13.79	20.00	Upregulated
regulation of proteolysis	GO:0030162	0.00	[5,6]	14.71	20.00	Upregulated
regulation of peptidase activity	GO:0052547	0.00	[5,6,7]	16.67	20.00	Upregulated
negative regulation of proteolysis	GO:0045861	0.00	[5,6,7,8]	17.09	20.00	Upregulated
peptidase regulator activity	GO:0061134	0.00	[5,6,7,8]	17.09	20.00	Upregulated
cytokine receptor activity	GO:0004896	0.03	[5,6,7]	12.90	8.00	Depleted
defense response	GO:0006952	0.03	[3]	8.84	13.00	Depleted
inflammatory response	GO:0006954	0.00	[4]	14.67	11.00	Depleted
positive regulation of response to stimulus	GO:0048584	0.00	[2,3,4]	10.16	19.00	None Specific
activation of immune response	GO:0002253	0.00	[2,4,5,6]	15.52	9.00	None Specific
positive regulation of immune system process	GO:0002684	0.02	[2,3,4]	12.16	9.00	None Specific
positive regulation of immune response	GO:0050778	0.01	[3,4,5]	13.43	9.00	None Specific
cardiovascular system development	GO:0072358	0.02	[4,5,6]	14.89	7.00	Depleted
vasculature development	GO:0001944	0.02	[4,5,6,7]	14.89	7.00	Depleted
regulation of immune response	GO:0050776	0.02	[3,4]	11.11	10.00	Depleted
Serine hydrolase activity	GO:0017171	0.00	[3]	9.09	25.00	None Specific
extracellular space	GO:0005615	0.00	[2,3]	12.83	24.00	None Specific

(B)



Appendix C: Water quality parameters measured over a 7 day exposure of metamorphic *Xenopus laevis* to chlorpyrifos (CPF).

	0.01% DMSO	CPF Concentration ($\mu g\ L^{-1}$) 1	10
Temperature (°C)	21.50 ± 0.10	21.35 ± 0.08	21.48 ± 0.11
pH	7.91 ± 0.23	7.97 ± 0.22	7.98 ± 0.22
Dissolved Oxygen (%)	87.54 ± 2.21	90.44 ± 1.53	88.16 ± 1.98
Ammonia (ppm)	0.78 ± 0.11	0.84 ± 0.10	0.78 ±0.11
Nitrate (ppm)	0.92 ± 0.08	1.00 ± 0.00	0.92 ± 0.08
Nitrate (ppm)	0.07 ± 0.02	0.05 ± 0.00	0.05 ± 0.00
Hardness (ppm)	180.00 ± 0.00	180.00 ± 0.00	180.00 ± 0.00
Alkalinity (mg/L)	120.00 ± 0.00	120.00 ± 0.00	120.00 ± 0.00
Conductivity (µS/cm)	464.46 ± 2.80	464.16 ± 2.77	465.08 ± 2.52

Parameters were measured from each tank every two days over the exposure period (4 tanks/concentration; 5 individuals/tank) and are provided as the mean (± SEM) for each CPF treatment.

Appendix D: Morphometric parameters following 7 day exposure to chlorpyrifos (CPF) and PBS or LPS injection in metamorphic *Xenopus laevis.*

Exposure	Injection	Body weight (g)	Body length (mm)	Relative Liver Weight (mg g^{-1})
0.01% DMSO	PBS	1.665 ± 0.444	24.87 ± 2.08	0.032 ± 0.005
	LPS	1.501 ± 0.479	24.24 ± 2.09	0.037 ± 0.006
1 μg L^{-1} CPF	PBS	1.485 ± 0.582	23.71 ± 3.62	0.037 ± 0.009
	LPS	1.486 ± 0.509	24.10 ± 2.51	0.038 ± 0.008
10 μg L^{-1} CPF	PBS	1.568 ± 0.549	24.27 ± 2.71	0.034 ± 0.005
	LPS	1.386 ± 0.489	23.42 ± 2.70	0.039 ± 0.007

Data are presented as mean ± SEM (n=9-10 individuals/treatment).
Data were analyzed using a two-way ANOVA with Exposure and Injection as factors.

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