Hepatic Gene Profile Analysis for Chronic Exposure of Clothianidin in Early Life Stage Sockeye Salmon (*Oncorhynchus nerka*)

by

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B.Sc., Simon Fraser University, 2014

Project Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Environmental Toxicology

in the
Department of Biological Sciences
Faculty of Science

SIMON FRASER UNIVERSITY
Summer 2018

Approval

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Degree: **Master of Environmental Toxicology**

Title: **Hepatic Gene Profile Analysis for Chronic Exposure of Clothianidin in Early Life Stage Sockeye Salmon (*Oncorhynchus nerka*)**

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Ethics Statement

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Abstract

This study investigated the effects of the neonicotinoid insecticide clothianidin on hepatic gene expression in juvenile sockeye salmon. Four genetically distinct pairs of wild sockeye salmon were collected and fertilized in clean water, and were subsequently exposed to 0.15, 1.5, 15, 150 µg/L clothianidin from 1 hour post-fertilization through to the swim-up fry developmental stage. Individual swim-up fry livers from all genetic crosses and each treatment were collected and various genes of interest were quantified using quantitative PCR. The genes of interest evaluated in this study were estrogen receptor alpha and beta 2, cytochrome P450 1A, suppressor of cytokine signaling 3, and glucocorticoid receptor 2. The glucocorticoid receptor 2 showed a significant 4-fold downregulation at 150 µg/L compared to the control treated fish ($p < 0.05$). This study indicates the utility of quantitative PCR in these early life-stage studies and potential impacts on the stress axis after prolonged exposure to clothianidin.

Keywords: Sockeye salmon; clothianidin; neonicotinoid; gene expression; glucocorticoid receptor; early development

Dedication

To my parents, Robert and Charmaine Calbick. Thank you for providing me with every opportunity that I could have ever asked for, and for always encouraging me every step of the way. Without you I'd be nowhere near the person I am today – and the person I am still becoming.

Acknowledgements

First and foremost I would like to thank my senior supervisor, Dr. Vicki Marlatt for her guidance, wisdom, and support throughout this degree. Thank you also to Dr. Chris Kennedy for his advice and encouragement as a member of my committee. This research was funded by a grant to Dr. Vicki Marlatt received from the National Contaminants Advisory Group within Fisheries and Ocean Canada.

Thank you to all my Marlatt lab mates, for their unwavering encouragement, friendship, and support. Thank you to my family for their continuous support and love throughout my entire education. Throughout this process I'm sure they've learned more about toxicology than they would've liked but never once complained.

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List of Acronyms

ºC	degrees Celsius
µg/L	Micrograms per litre
µL	Microlitre
AChE	Acetylcholine esterase
ANOVA	Analysis of variance
AOP	Adverse outcome pathway
BC	British Columbia
BC MOE	British Columbia Ministry of the Environment
CCME	Canadian Council of Ministers of the Environment
cDNA	Complimentary deoxyribonucleic acid
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
Cq	Quantification cycle
CYP	Cytochrome p450
DNA	Deoxyribonucleic acid
EC_{50}	Median effective concentration
ER	Estrogen receptor
g/L	Grams per litre
GOI	Gene of interest
GR	Glucocorticoid receptor
HPI	Hypothalamic-pituitary- interrenal axis
IUCN	International Union for Conservation of Nature
Kg	kilogram
Kow	Octanol – water partition coefficient
LC_{50}	Lethal concentration to 50% of test organisms
LD_{50}	Lethal dose to 50 % of test organisms
LOAEL	Lowest observed adverse effect level
mg/kg/day	Milligram per kilogram of body weight per day
mg/L	Milligram per litre
MIE	Molecular initializing event
MIQE	Minimum information for publication of quantitative PCR experiments
mRNA	Messenger rribonucleic acid

nAChR	Nicotinic acetylcholine receptor
NCAG	National Contaminants Advisory Group
ng/L	Nanograms per litre
NOAEL	No observed adverse effects level
PMRA	Pesticide Management Regulatory Agency
PPR	Prairie Pothole Region
QPCR	Quantitative polymerase chain reaction
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SFU	Simon Fraser University
SSD	Species sensitivity distribution
US EPA	United States Environmental Protection Agency
VTG	Vitellogenin
WSP	Wild Salmon Policy

Glossary

Acute Toxicity	Describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short period of time
Adverse Outcome Pathway	Defines a sequence of key events commencing with the interaction of a stressor (e.g., toxicant) on target cell or tissue and resulting in an adverse outcome for an organism
Alevin	Fish life stage after hatch with yolk sac still present
Chronic Toxicity	Describes the adverse effects as the result of long term exposure to a toxicant or other stressor
Downregulated	The process by which a cell decreases the quantity of a cellular component such as RNA or protein in response to an external stimulus
EC_{50}	The concentration of test substance which results in a 50 percent reduction in either algae growth or algae growth rate, or *Daphina* immobilization.
Ecotoxicology	the branch of science that deals with the nature, effects, and interactions of substances that are harmful to the environment
Gene	A distinct sequence of nucleotides forming part of a chromosome, the order of which determines the order of monomers in a polypeptide or nucleic acid molecule which a cell may synthesize
Gene expression	The process by which the genetic code - the nucleotide sequence - of a gene is used to direct protein synthesis and produce the structures of the cell.
in vitro	Performed or taking place in a test tube, culture dish, or elsewhere outside a living organism
in vivo	Performed or taking place in a living organism
Neuroimmunomodulation	Mechanisms of the interactions among the nervous, endocrine, and immune systems
Pesticide	A product that is manufactured and sold for means to directly or indirectly control, mitigate or destroy any pest
Proteome	The entire complement of proteins that is or can be expressed by a cell, tissue, or organism
Redd	Series of depressions dug into gravel substrate by a female salmonid in which eggs are deposited
Salmonid	A fish of the salmon family (Salmonidae)

Swim-up	Early-life event where by alevins swim-up through gravel into the water column for feeding, typically when yolk sac is nearly depleted.
Thesis	An extended research paper that is part of the final exam process for a graduate degree. The document may also be classified as a project or collection of extended essays.
Upregulated	The process by which a cell increases the quantity of a cellular component such as RNA or protein in response to an external stimulus

Chapter 1.

Introduction

1.1. Sockeye salmon (*Oncorhynchus nerka*)

Sockeye salmon (*Oncorhynchus nerka*) are a Pacific salmon species native to the western coast of North America in the Pacific Ocean (Fisheries and Oceans Canada, 2018). They can be located as far north as northern Alaska and as far south as northern California, with some sockeye species having been recorded in Russia and Japan. However it is likely the Japanese populations of sockeye were a result of introductions, and therefore not considered part of Sockeye's native range (Bickham, et al., 1995; Hasegawa, et al., 2004; Quinn, 2005; Rand et al., 2011). Sockeye salmon are born in lakes, rivers, or streams, then migrate to the Pacific Ocean where they spend most of their life (Fisheries and Oceans Canada, 2018). There are also populations of landlocked sockeye salmon, commonly referred to as kokanee, that spend their entire life in lakes (Rand et al., 2011). In 2008, approximately 7% of the historical range of sockeye salmon was estimated to have been lost due to localized extinction events, but the International Union for Conservation of Nature (IUCN) concludes that overall sockeye are not threatened on a global scale (Rand et al., 2011). At the species level, the population is considered to be relatively stable; however, some subpopulations are declining (Rand et al., 2011). An assessment in 2011 by the IUCN evaluated 98 subpopulations making up the global population of sockeye salmon, and identified five subpopulations in the Columbia River as extinct (Rand et al, 2011). Of the remaining subpopulations, nearly 31% are assessed as threatened (19 out of a total of 62 evaluated subpopulations), and an additional two are near threatened (Rand et al., 2011). Today, the IUCN notes the greatest number and concentration of threatened subpopulations are located in the Province of British Columbia (BC), Canada. Additionally, the Cultus lake and Sakinaw lake populations in BC are presently listed as endangered by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC).

Freshwater systems are vital to different salmon life stages, including early embryonic, fry and adult stages (British Columbia Ministry of Fisheries, 2018). Fraser

River sockeye salmon spend almost half of their lives in these freshwater systems, where critical events such as spawning, egg hatching and larval development take place (Fisheries and Oceans Canada, 2018). When adult Fraser River sockeye salmon are ready to spawn, male and female sockeye migrate from the Pacific Ocean up into the Fraser River to their natal stream or tributary (Fisheries and Oceans Canada, 2018). In the early life stage of sockeye salmon, embryos encased in eggs do not rely on parental protection but are instead released into the surrounding environment (British Columbia Ministry of Fisheries, 2018). Female sockeye will deposit and bury eggs in redds in fresh water tributary streams, lake outlets, or in some cases display lake-beach spawning where it is simultaneously fertilized by a male (Rand et al., 2011). Over months, the embryo develops within the gravel nest or redds until the embryo hatches out of the egg structure and becomes an alevin (or yolk sac fry) (British Columbia Ministry of Fisheries, 2018). The alevin possess a yolk sac that will provide food for two to three months (British Columbia Ministry of Fisheries, 2018). Once the nutrients in the yolk sac are absorbed, the free-swimming or swim-up fry may live in fresh water for a year or more (British Columbia Ministry of Fisheries, 2018). When the fry are ready to enter salt water they are referred to as smolts. Once in salt water, BC sockeye move north and north-westward along the coast (Rand 2011). Their maturing years find them covering a large area of the Pacific Ocean extending from the coast of Vancouver Island, north to the northern Gulf of Alaska and south to the Oregon-California border (Fisheries and Oceans Canada, 2018).

It is possible that since early life stage fish do not have fully developed livers and immune systems, embryos and swim-up fry may be more vulnerable to stressors in the environment (Castro et al., 2015; Tatner et al., 1996). In fish, innate immunity is particularly important in early phases of development, when adaptive immunity is still not fully established and functioning (Castro et al., 2015; Tatner et al., 1996; Lee et al., 2015). Teleost eggs and fry can come into contact with many pathogens or contaminants since they are so openly exposed and still developing immunological functions (Castro et al., 2015; Lee et al., 2015). These stressors could ultimately lead to mortality, developmental and reproductive problems, or a change in normal gene expression patterns (Hamdoun et al., 2007). During these early life stages sockeye can encounter both natural and anthropogenic stressors in the environment. Natural stressors include changing water temperature, predator attacks, and food restriction,

while anthropogenic stressors include pollution and habitat destruction (Cohen, 2012). Numerous stressors likely contributing to low sockeye salmon population numbers can be the result of human activities, both on land and in water bodies (Cohen, 2012). These anthropogenic activities can range from fishing practices, to resource extraction (mining), to agriculture, with the capacity to degrade habitat that are critically important for wild salmon health (McClure et al., 2008). While these anthropogenic stressors appear independent, their effects can culminate into larger consequences over long term (McClure et al., 2008). For example, gravel beds with clean, cold, oxygen-rich water are needed for eggs to hatch. However, activities such as water withdrawal for hydropower and flood control can manipulate river flows and eliminate spawning habitat critical for salmon reproduction (Angilletta et al., 2008; Franklin et al., 1995). Indeed, many anthropogenic activities fundamentally alter the ecology of freshwater systems, changing nutrient flow and food availability (Angilletta et al., 2008). Altering natural water conditions in rivers and streams via contaminants can also directly affect salmonids. Contaminants entering salmon bearing streams or rivers could have the ability to cause lethality or physiological changes such as gonadal development, adverse metabolic and growth effects, which can lead to severe downstream impacts on populations (Harding et al., 2016; Meador et al., 2018; Du Gas et al., 2017).

1.2. **Fraser Valley and Sockeye Salmon**

Located in the southwestern region of BC, Canada, the Lower Fraser Region of the Fraser River Basin encompasses a region approximately 223,000 km^2 downstream of the Fraser Canyon (Noakes, 2011). The Lower Fraser Valley extends from Hope, to the mouth of the Fraser River, which drains into the Strait of Georgia between Vancouver Island and the coast of BC (Cohen, 2012; Noakes, 2011). The entire Fraser River is 1375 km long with numerous tributaries flowing into the river, thus making it the longest river in BC (Noakes, 2011). One of the most valuable resources in BC comes from wild Fraser River sockeye salmon (*Oncorhynchus nerka*). Historically, approximately one-third of all five Pacific salmon species caught in the Strait of Georgia migrated from the Fraser River. There are about 151 sockeye spawning subpopulations in the Fraser River Basin. It's also been estimated that on average 250 million sockeye migrate to the Strait of Georgia every spring from the Fraser River (Labelle, 2009). Nevertheless, there continues to be conservation concerns for some of these Fraser

River sockeye populations (Labelle, 2009). Of the 22 subpopulation assessed in the Fraser River by the IUCN in 2011, 11 subpopulations were considered Least Concern, 7 were considered Endangered, and 4 subpopulations were Data Deficient (Rand et al., 2011).

BC's commercial fisheries are a vital natural resource to both the physical and material wealth of the province Sockeye salmon are a commonly fished species both commercial and recreationally (Iudicello, et al., 1999; Statistics Canada, 2012; Walters and Martell, 2004). In 2011, Canada exported around 69 thousand tonnes of salmon valued at $440 million (Statistics Canada, 2012). In 2013, BC alone produced approximately 98.8 thousand tonnes of salmon with a landed value $500.6 million in 2013 (Ministry of Agriculture, 2014). These wild sockeye populations are also of great cultural value to Canada's First Nations (Garner et al., 2006). First Nations people and salmon have maintained a complex relationship in the Pacific Northwest for more than 10,000 years (Garner et al., 2006). First Nations have also developed a profound spiritual relationship with salmon dating back thousands of years, with many First Nations communities being founded on traditional fishing grounds (Garner et al., 2006). The ecosystem services provided by salmon are numerous from their functioning as part of complex food web, to nutrient cycling, and maintaining ecosystem resilience; salmon are a central link between aquatic and terrestrial ecosystems (Holmlund and Hammer 1999).

Indeed, dozens of BC's salmon runs are now at their lowest numbers since industrial fisheries began in the late 1800's (Price et al., 2017). In the years 2007-2009, sockeye salmon returns were at a record lows, prompting the closure of both commercial and recreational Fraser River sockeye salmon fisheries for all three years (Cohen, 2012). Due to these historically low numbers, Canada's federal government launched the Cohen Commission which was an investigative working group set up by Canada's federal government, charged with collecting scientific evidence to determine the cause of the crisis (Cohen, 2012). The overarching goal of Cohen Commission was to improve the understanding of the Fraser River sockeye salmon, and provide recommendations for improving future sustainability of the sockeye salmon fishery in the Fraser River. Anthropogenic contamination including current-use pesticides was identified as a potential concern for negatively affecting sockeye salmon populations in the Fraser River Basin (Cohen, 2012; MacDonald et al., 2011). In August 2016, one of the 75

recommendations made by Justice Cohen was the regular testing and monitoring of fresh water for contaminants of emerging concern and for endocrine-disrupting chemicals affecting Fraser River sockeye salmon (Cohen, 2012). Even with the evidence and recommendations put forth by the Cohen report, the return of sockeye has still not fully recovered (Price et al., 2017). Between 1995 and 2005 commercial fishery catches were thought to be the lowest on record (Price, 2017). Since then, catches between 2006 and 2014 have further declined by nearly one-half (Price et al., 2017). In 2012, the total adult return was 2,065,300; however, in 2016 the total abundance of fish for the Fraser River sockeye run was only 858,000 making it one of the worst in BC's history. The Pacific Salmon Commission forecasted a return of 2.2 million Sockeye in the Fraser River, but with a total of only 858,000 fish, this run was 62% smaller than the median forecast of 2,271,000 fish (Pacific Salmon Commission, 2017).

In addition to fisheries exploitation, climate change, changing ocean conditions, and pollution, the question of whether the marine or freshwater systems or both water body types salmon inhabit are the main contributors to low population numbers is unknown (Price et al., 2017). Leading up to the adoption of the Wild Salmon Policy (WSP) in 2005, the monitoring effort for spawning streams had been in decline in BC where 70% of all streams on BC's north and central coasts were not monitored and did not have a single estimate of abundance (Price et al., 2017; Price et al. 2008). However, the streams that were monitored on BC's north and central coasts approximately 95% consistently failed to meet management escapement targets during 1950 to 2005 (Price et al. 2008). Escapement refers to the number of salmon that do not get caught by commercial or recreational fisheries and return to their freshwater natal stream or river to spawn (Pacific Salmon Commission, 2017). Therefore it is possible that these streams are changing in a way that negatively impacts sockeye populations, for example habitat loss or contamination.

By providing a valuable water resource, regions along the Fraser River have become some of the most populated and industrialized in BC. Approximately 83% of the people living within the Lower Fraser Region are concentrated near the drainage basin of the Fraser River (Lower Mainland) (Schreier et al. 1991). Water accessibility, fertile soils, and a moderate climate provide favorable conditions for agriculture in the lower Fraser Valley region. These agricultural crops range from a variety of fruits and vegetable, to field and cereal crops, nursery products, sod, pasture and other crops

(Fraser Valley Regional District, 2011). In 2005, farms in the lower Fraser Valley ranked number one in the province, generating nearly 1 billion dollars in Canadian revenue with nearly 8 million dollars being spent on the purchase of pesticides (Fraser Valley Regional District, 2011). Since 2008, a general trend has shown a small (3%) increase in the overall sales of pesticides in Canada with 68.7% of pesticide sales in Canada being agricultural sector products (Health Canada, 2011). According to the Pest Control Products Sales Report for 2011 (Health Canada, 2011), two neonicotinoids, thiamethoxam and clothianidin, were listed in the top 10 insecticides sold in Canada in 2011. In 2010, pesticide sales in BC totaled 1,289,933 kg of active ingredients, with the neonicotinoid imidacloprid increasing by 205% for quantity of active ingredient sold (kg) since 2003 (British Columiba Ministry of Environment, 2010). The lower Fraser Valley has been identified as one of the most intensive agricultural regions in Canada (Tuduri et al., 2006). Agricultural output or products from the lower Fraser Valley represents over one-half of the total agricultural production in BC (Woudneh et al., 2007). Indeed, according to a 2005 survey by the Ministry of Environment (British Columiba Ministry of Environment, 2005) this particular region consumed over 45% of all pesticides sold in BC in 2003 and several pesticides have been detected in salmon bearing streams (Woudneh et al., 2007).

1.3. **Neonicotinoids- A new class of insecticide**

Pesticides are defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest (Canadian Environmental Protection Act, 1996; US EPA, 2017). Pesticides accomplish this primarily by destroying, preventing growth, or repulsing pests (US EPA, 2017). While proven useful in agriculture, their extensive use disturbs the environment and, especially, non-target organisms (US EPA, 2017). One relatively recent group of insecticide that is known for high selectivity for insect pests is the neonicotinoids. Developed in the 1980's to replace organophosphate and carbamate insecticides, neonicotinoids were a new generation of nicotine-related insecticides (i.e. structurally similar to nicotine) possessing either a nitromethylene, nitroimine or cyanoimine group (Fulton et al., 2013). Over the last few decades neonicotinoids have become an important and quickly growing chemical class of insecticides on the global market largely for food crops and seed pre-treatment (Jeschke et al., 2008; Jeschke et al. 2011; Tomizawa et al., 2011; Casida et al., 2013).

One of the main neonicotinoids, imidacloprid, was released by Bayer CropScience in the early 1990s as the first commercially available product of its kind (Goulson, 2013; Jeschke et al., 2008). In Canada, neonicotinoids are approved for use as seed treatments, soil applications, and foliar sprays on a wide variety of agricultural crops such as oilseeds, grains, fruits, vegetables, greenhouse crops, and ornamental plants depending on jurisdiction (CCME, 2007; PMRA, 2004; Health Canada, 2017). Neonicotinoid pesticides have also been approved for uses on turf and trees in structures and outdoor residential areas and as pet care insecticidal products (Health Canada, 2011). Currently there are seven neonicotinoids available and registered in over 120 countries, and this accounts for more than 26% in total global insecticide sales (Jeschke et al, 2011; Simon-Delso et al., 2015). In the early 2000's, two second-generation neonicotinoid compounds, clothianidin and thiamethoxam, were released by Sumitomo Chemical/Bayer and Syngenta, respectively (Jeschke et al., 2011). These second-generation neonicotinoids differ from first generation neonicotinoid according to their heterocyclic group, and as such are grouped into these subclasses. First generation neonicotinoids have a chloropyridyl heterocycle, while second generation neonicotinoids possess a chlorothiazole heterocycle (Yamamoto et al., 2012). In 2012, thiamethoxam, imidacloprid and clothianidin accounted for almost 85% of the total neonicotinoid sales in crop protection based on total global insecticide sales (Bass et al., 2015).

1.4. **Neonicotinoid Mechanism of action**

Neonicotinoids are systemic insecticides, and as such, are taken up by plants by the roots or the leaves, and then transported along the phloem or the xylem to distal tissues different from those where the product was applied, including flowers, pollen (Krupke et al., 2012), and nectar (Paradis et al. 2014). The neonicotinoid that is taken up by the plant kills sucking and chewing insects by disrupting the nervous systems (US EPA, 2010). Neonicotinoids act on the nervous system as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs), present in invertebrates and vertebrates. The nAChRs of both vertebrate and invertebrates are pentameric membrane proteins that rapidly transduce the actions of the chemical neurotransmitter acetylcholine (ACh) and cause membrane depolarization at synapses, and are eventually released from the nAChR and degraded by the enzyme acetylcholinesterase (Sattelle, 2009; Crossthwaite,

2017; Taylor, 2012). Therefore, acetylcholine enables chemical signal transmission across a synapse from one neuron to another neuron, muscle cell, or gland cell (English et al., 2012; Norris et al., 2013). When neonicotinoids such as clothianidin bind to invertebrate nAChRs in a postsynaptic neuron it is irreversible, and there is continuous stimulation of neurotransmission which leads to various symptoms of neurotoxicity (Gibbons et al., 2014). This persistent activation leads to overstimulation of cholinergic synapses, resulting in hyperexcitation, desensitization, convulsion, paralysis and eventually death of the affected invertebrate (Tomizawa et al., 2004). Neonicotinoids exhibit greater affinity for nAChRs in invertebrates, particularly insects, which contributes to their higher toxicity in insects compared to vertebrates and efficacy as insecticides (Costa et al., 2009; Liu et al., 2010).

1.5. **Environmental Fate and Prevalence of Clothianidin**

According to the US EPA (2003), the data available on the environmental fate of clothianidin is based on lab and field studies and demonstrate that this insecticide is persistent, mobile, stable to hydrolysis, and has the potential to leach into ground water and be transported via runoff to surface waters. The half-lives of neonicotinoids in aerobic soil conditions can vary widely, but are measured in months or longer (for example 148–6,931 days for clothianidin; US EPA 2003). Due to their low volatility, neonicotinoids are present mostly as aerosols during or right after application (Bonmatin et al., 2015). Based on laboratory experiments alone, the main route of dissipation for clothianidin appears to be photolysis as it can also be rapidly photodegraded in water (< 24 hours) (Bonmatin et al., 2015; Fossen, 2006; US EPA, 2010). However, slow dissipation rates observed in field trials suggest that photolysis is probably not significant under most actual-use conditions (US EPA, 2010). The presence and fate of clothianidin in water depends mainly upon factors such as light, pH, temperature, and microbial action. In general, neonicotinoids are quite water soluble (0.33 g/L, 4.1 g/L and 0.61 g/L for clothianidin, thiamethoxam, and imidacloprid respectively, at 1 atm, 25 °C), allowing them to be readily absorbed into roots and leaves and transported systemically (Banerjee et al., 2008; Fossen, 2006). However, this also points towards a concern for the potential leaching and movement of neonicotinoids, including clothianidin, into surface waters and groundwater indicated by high soil organic carbon partition coefficient (Koc) values in laboratory studies (between 84-129 for clothianidin) (Banerjee

et al., 2008; Fossen, 2006; US EPA, 2010). Indeed, Sur and Stork (2003) investigated the uptake of imidacloprid in plants and found that depending on the application and crop type, approximately 1.6 – 28% the applied active ingredient for neonicotinoids in seed dressings is absorbed by the crop plant. Thus, there must be a larger portion of neonicotinoids partitioning into other environmental compartments, such as soil or water. Of the 80–98% of the active ingredient in seed dressings, which is not absorbed by the seed plant, a small proportion (>2%) is lost as dust during sowing (Tapparo et al., 2012). This areal dust can be found in sufficient quantities to have adverse effects on non-target insects, for example, direct mortality to honey bees flying nearby (Marzaro et al., 2011; Tapparo et al., 2012). Specifically, these airborne dusts are present at levels that approach both an oral and contact acute LD_{50} for honeybees which have been reported to be as low as 0.0037µg/bee and 0.044 µg/bee (Bonmatin et al., 2015). With the prevalent use of neonicotinoids and persistence in the environment, several studies have demonstrated the adverse population level effects in terrestrial and aquatic invertebrates after low level, environmentally relevant exposure to neonicotinoids (Beketov et al., 2008; Gibbons et al., 2015; Miles et al., 2017; Morrissey et al., 2015; Roessink et al., 2013; Van Dijk et al., 2013). However, whether these chronic low-level exposure scenarios are adversely affecting vertebrates is not known.

Pesticide entry into water bodies may be the result of direct applications scenarios, or indirectly via spray drift, leaching, run-off events, and even dry/wet deposition events (CCME, 2003; Topal et al., 2017). With the numerous tributaries proximal to agriculture in the Lower Fraser Valley all eventually draining into the Fraser River, pesticide contamination is hypothesized as a contributing factor to the decline or sockeye salmon (Cohen, 2012). Globally, neonicotinoids have been frequently detected in a variety of water bodies, typically at concentrations in the low µg/L range. In a review of 29 studies from nine countries, neonicotinoids were listed as one of the more common contaminants of surface water (Morrissey et al., 2015). Although there is little to no existing information on levels of clothianidin in the Fraser Valley, clothianidin has been detected frequently in aquatic environments across Canada. The Canadian Prairie Pothole Region (PPR) consists of 39 million hectares and accounts for 98% of the country's canola production (Main et al., 2014). A study by Main et al. (2014) collected water samples from 136 wetlands across four rural municipalities in Saskatchewan during the spring, summer and fall in 2012 as well as in the spring of 2013. This study

revealed that clothianidin and thiamethoxam were present in the majority of samples and were detected in all 4 sampling periods/seasons, with clothianidin being the most commonly detected neonicotinoid in the water samples (Main et al., 2014). Clothianidin also had the highest maximum (max) and mean concentrations during three of the sampling periods: spring 2012 (max: 144 ng/L), summer 2012 (max: 3110 ng/L), and spring 2013 (max: 173 ng/L). In a study conducted in southwestern Ontario, Canada, 76 agricultural surface water samples were collected within commercial maize farms, all of which had clothianidin at a mean concentration of 2.28 µg/L and a maximum concentration of 43.60 µg/L (Schaafsma et al., 2015). This study also reported that the total neonicotinoid concentration increased 6-fold after the planting season, suggesting the main source of pesticide pollution was from agricultural settings (Schaafsma et al., 2015). A study by Woudneh et al (2007) detected a total of 51 pesticides at varying levels in surface waters across various sites in the Lower Fraser Valley. Woudneh et al. (2007) results showed that surface waters close to agricultural land use were the most likely to be contaminated by pesticides based on higher concentrations and frequency of detections adjacent to agricultural sites. Pesticides have also been detected at low levels in groundwater samples collected from the lower Fraser Valley region. Individual pesticide concentrations for groundwater samples collected from agricultural sites and agricultural-urban mixed sites ranged from 0.006 to 90 ng/L, and 0.034 to 7.31 ng/L respectively (Woudneh et al., 2009). Contamination of these environments by pesticides could be the result of direct applications scenarios, or indirectly via spray drift, leaching, runoff events, and even dry/wet deposition events (CCME, 2003). Although the concentrations of most neonicotinoids have not been measured routinely across Canada, several studies have demonstrated ng/L to µg/L concentrations in surface waters. This is similar to the levels of several other types of pesticides in surface waters that have more frequently been measured in Canada.

1.6. **Acute Toxicity of Clothianidin**

Many studies have examined the acute toxicity of neonicotinoids to both target and non-target organisms, including mammals, birds, fish, insects, crustacean, and molluscs. For aquatic life, aquatic insects are typically more sensitive to neonicotinoid exposure when compared to other aquatic species such as mollusks, crustaceans, and fish (Gibbons et al., 2015; Miles et al, 2017; Sánchez-Bayo et al., 2016). When

classifying the toxicity of a chemical, standard toxicological tests can involve exposing an organism to a high concentration or dose level to measure the median lethal dose or concentration. This is commonly termed LD50 or LC50. An LC50 refers to the concentration of a chemical that is lethal to 50% of the test organisms over a specific time interval (CCME, 2003). Another measure would be an EC50. An EC50 is the concentration of substance that will affect 50% of a test population during continuous exposure over a specified period of time. The endpoint that is measured by the US EPA is immobilization or lack of movement by the test organism (US EPA, 1996). The 48-hour EC50 for a freshwater invertebrate, *Chironomus riparius*, is determined to be 0.022 mg/L which the US EPA labels as highly toxic (US EPA, 2003). In addition, a study on mysid shrimp, a marine invertebrate, determined the LC50 for clothianidin to be 0.051 mg/L in a 96-hour toxicity study which is considered highly toxic (US EPA, 2003). However, while some aquatic invertebrates show increased sensitivity to acute clothianidin toxicity exposure, the 48-hour EC50 for the freshwater invertebrate *Daphnia magna*, a standard toxicity testing organisms, was calculated to be between 100.8 - 119 mg/L, which is considered to be practically non-toxic by the US EPA (US EPA, 2003). Most crustaceans are considered less susceptible than insects. A 96-hour acute toxicity test on the mollusk *Crassotrea virginica* (Eastern oyster), displayed an EC50 >129.1 mg/L.

Fish are markedly less susceptible than aquatic insects based on acute toxicity studies, with LC50 values ranging from 16 to 177 mg/L (US EPA, 2003). Based on current studies, the toxicity of clothiaindin for juvenile fish can range from virtually non-toxic to slightly toxic (US EPA, 2003). The 96-h LC50's for clothianidin are fairly consistent among the tests fish species, ranging from > 93.6 mg/L in *Cyprinodon variegatus* (sheepshead minnow) and 105 mg/L in *Oncorhynchus* mykiss (rainbow trout), to 117 mg/L in *Lepomis macrochirus* (bluegill sunfish) (US EPA, 2003). Toxicity to terrestrial vertebrates is also low compared to insects, but varies greatly among clothianidin; for example, the acute oral LD50 in young adult mice (*Mus musculus*) (between 8 -12 weeks old) via single dose by oral gavage is > 389-465 mg/kg/day (mg of active ingredient per kilogram body weight (bw)), while in rats (*Rattus norvegicus*), the LD50 is determined to be 5,000 mg/kg/day (US EPA, 2003). Birds appear to be generally more susceptible than rats, with LD50 values ranging from 200 mg/kg/bw in Japanese quail, up to 1333 mg/kg/bw in mallard ducks (US EPA, 2003). Although

neonicotinoids have exhibited relatively low toxicity in vertebrates, studies examining seed-eating vertebrates are only recently being conducted (Gibbons et al., 2015; Lopez-Anita et al., 2013; Lopez-Anita et al., 2015; Prosser et al., 2005). For example, Lopez-Anita et al. (2015) conducted two exposure scenarios on red-legged partridges (*Alectoris rufa*) to lethal doses through ingestion of imidacloprid treated seed, as well as a sub-lethal dose. To correspond to the duration of the two cereal sowing seasons, the first exposure lasted for 25 days, while the second one lasted 10 days. The estimated daily ingestion dose for the high and the low dose groups were calculated to be approximately 44 and 8.8 mg/kg/day, respectively for both exposures. The results revealed that imidacloprid exposure was not only lethal to red-legged partridges, but numerous deleterious sub-lethal effects on redox balance, secondary sexual traits, and reproduction were also observed. When the US EPA modelled the estimated daily intake of clothianidin for mammals and birds eating only a diet of treated seeds, the model showed that clothianidin could reduce the survival of small birds and mammals (DeCant and Barrett 2010; US EPA, 2003). The US EPA also estimated that ~1% of planted seeds remains accessible to granivorous vertebrates, not including spillage which may occur, for example, when transporting grain or loading hoppers (US EPA, 2003).

1.7. **Chronic Toxicity of Clothianidin**

Data demonstrating the toxic effects of clothianidin after chronic exposure is limited, but variety of adverse effects at the whole organisms and cellular level after chronic exposure in vertebrates has been reported. In mammals, clothianidin can cause detrimental effects on reproductive organs and gametes. Male rats exposed to 32 mg/kg/day of clothianidin for 90 days caused a significant decrease in epididymis and seminal vesicles weights and induced oxidative stress causing sperm DNA fragmentation (Bal et al., 2012). Similarly, oxidative induced DNA damage from clothianidin exposure was reported in birds. Quails orally administered sub-lethal doses (0.02- 50 mg/kg) of clothianidin showed increased DNA fragmentation in seminiferous tubules and inhibited embryonic growth in dose-response manner (Tokumoto et al., 2013). Several studies of neonicotinoids in zebrafish, fathead minnow, silver catfish (*Rhamdia quelen*) and medaka fish (*Oryzias latipes)* have reported changes in gene transcription, erythrocyte damage, degeneration of gonadal tissue, impaired swimming, and locomotor defects in embryos and larvae (Beggel et al., 2010; Beggel

et al., 2012; Ghisi et al., 2010; Sánchez-Bayo et al., 2005; Stehr et al., 2006). In one case, medaka in experimental rice fields became physiologically stressed following exposure to 215 g of active ingredient per hectare of imidacloprid, which corresponds to 1.5 times the commercially recommended rate of application. This was characterized by increased anaerobic metabolism leading to hyperglycemia, and subsequently fish became susceptible to infestation by the protozoan ectoparasite, *Trichodina* (Sánchez-Bayo et al., 2005). Another study using the phenylpyrazole insecticide fipronil, reported changes in gene expression for genes involved in endocrine function, energy metabolism and muscular and neuronal functions, as well as detoxification and general stress responses in larval fathead minnows (*Pimephales promelas)* (Beggel et al., 2012) Similar to clothianidin, fipronil is a potent disrupter of the insect central nervous system, however it acts via interference with the γ-aminobutyric acid (GABA)-gated Cl^- channel function (Gunasekara et al., 2007). GABA is a major inhibitory neurotransmitter in the vertebrate central nervous system, and in both insects and mammals the behavioral effects of GABA antagonists such as fipronil include hyperactivity, hyperexcitability, and convulsions (Gunasekara et al., 2007). Immediately after 24 hours of exposure to fipronil concentrations (≥ 31 µ/L), significant changes in gene transcription were observed for genes encoding aspartoacylase (muscular and neuronal function), metallothionein (stress response), glucocorticoid receptor (stress response), cytochrome P450 3A126 (Detoxification) and vitellogenin (endocrine function) (Beggel et al., 2012). This study also demonstrated multiphasic gene expression patterns (genes that were up-regulated at the lowest exposure concentration were down-regulated with increasing treatment concentration and again up-regulated at highest concentrations. A chronic toxicity study reported by the US EPA revealed significant difference in the dry body weight and body length (reduced growth) of newly hatched larval fathead minnow after exposure to 20 mg/L clothianidin for seven days in a static renewal test (NOAEC of 9.7 mg/L) (US EPA, 2010). With surfaces waters often the ultimate sinks for pesticides, future studies examining the impacts of low-level, chronic environmentally relevant exposure scenarios for clothianidin on wild aquatic vertebrates are needed to more accurately assess the risks these pesticides pose to non-target wildlife.

To date, aquatic invertebrates appear to be the most sensitive taxa studied with respect to the toxicity of several neonicotinoids, but indirect food chain effects of reduced invertebrate prey populations is a concern for many fish including salmon. Acute toxicity

concentrations lethal to aquatic invertebrates for clothianidin are in the range of 22 – 119, 000 µg/L (US EPA, 2003). A review by Morrissey et al. (2015) conducted a comprehensive species sensitivity distribution (SSD) analysis using 214 toxicity tests of 48 species to predict effect on sensitive aquatic communities at neonicotinoid water concentration ≥ 0.2 (max) or ≥ 0.035 µg/L (average). Results from the SSD conducted suggest that ecological thresholds for neonicotinoid water concentrations should be below 0.2 µg/L for acute exposure or 0.035 µg/L for chronic exposure to avoid deleterious effects on aquatic invertebrate communities. Morrissey et al. (2015) state that neonicotinoids can exert adverse effects on survival, growth, emergence, mobility, and behavior of many sensitive aquatic invertebrate taxa at concentrations at or below 1 µg/L under acute exposure and 0.1 µg/L for chronic exposure. This is of concern based on several studies reporting surface water concentrations of clothianidin ranging from < 0.0309 µg/L up to 43.6 µg/L in Canada (Main et al., 2014; Schaafsma et al., 2015). The impacts of clothianidin on aquatic invertebrates is concerning because benthic invertebrates play an important role in energy flow and nutrient cycling in aquatic systems (Schindler et al., 2003), and are the single most important food source for rearing juvenile salmon (Covich et al., 1999). Contaminants entering these aquatic systems, such as clothianidin, thus have the potential to alter or disturb the community structure and function of ecosystems by reducing the abundance and diversity of these organisms. Indeed, one study investigated annual successive applications of both imidacloprid and fipronil in aquatic rice paddy communities, adult and fry medaka and demonstrated reduced growth in both adults and juveniles (Hayasaka et al., 2012). It was hypothesized that this was most likely due a reduction in the abundance of medaka prey because the abundance of benthic organisms was significantly lower in both insecticide-treated fields than in the controls (Hayasaka et al., 2012). In this study, the concentrations of both insecticides appeared to be too low (~ 0.001 to 0.05 mg/L) to exert a direct toxic effect on medaka fry, but it was demonstrated that the concentration was sufficiently high enough to reduce the abundance of invertebrate prey. Others have shown indirect effects of insecticides via reductions in arthropod prey for juvenile salmon following applications of insecticides and, in turn, limit salmonid carrying capacity (Pimental, 2005; Elliott, 1980). This phenomenon of indirect effects of pesticides on salmon warrants careful consideration and future study to determine the extent it may be contributing to decreased salmon populations, such as those in BC, Canada.

1.8. Molecular Changes as Early Onset Indicators of Toxic Effects

Utilization of molecular endpoints such as gene expression can provide rapid and valuable information on immediate organismal responses to chemical stressors, and can offer a mechanistic interpretation of effects at higher levels of biological organization. Multiple molecular approaches are being employed in environmental toxicology for their application in *in situ* monitoring of biological effects in organisms; gene expression analyses being one of the more common approaches. Gene expression has been used in field studies to characterize transcriptome responses in fish inhabiting polluted environments (Bahamonde et al., 2014; Barjhoux et al., 2014; Garcia-Reyero et al., 2008; Ings et al., 2011; Marlatt et al., 2016). Evidence supporting correlations between gene expression patterns and morphological and physiological effects in complex, field-based exposures presents innovative applications for environmental monitoring programs, as molecular signatures or biomarkers may be useful in classifying and assessing responses of organisms to contaminants in natural environments (Ankley et al., 2010; Marlatt et al., 2016). These concepts are based on the paradigm that initial changes at the molecular level precede overall morphological and physiological effects. As such, these molecular endpoints are most useful when they are linked with responses at higher levels of biological organization. The idea being that these endpoints will contribute to the development of adverse outcome pathways (AOPs) (Ankley et al., 2010). An AOP provides a conceptual framework that intends to link existing knowledge concerning associations between molecular-level disruptions of a biological system from exposure to substances, with a series of events at different levels of relevant biological organization leading to an adverse health outcome (Bal-Price et al., 2017).

A biomarker can be defined as a measurable indicator of a biological state or condition in an organism. Gene expression as an early biomarker has the potential to indicate a molecular initiating event (MIE), whereby the binding of a toxicant to a receptor or enzyme responsible for a biological process can lead to a series of key events to adverse health outcome, including impaired reproduction, development, immunity, and altered metabolism. The MIE is defined as a specific type of interaction of a toxicant with a biological target, such as a receptor, enzyme, and/or DNA, which represents the first step in a directed cascade of dependent biological processes or key

events that lead to a defined adverse outcome (Bal-Price et al., 2017). Defining specific genes or gene sets that predict chemical-induced MIEs or key events would be valuable in building models of AOPs that predict adverse outcomes. An example of how gene expression was viewed as early onset change for adverse outcomes of organism health was by Russom et al. (2014) who described an AOP linking acetylcholinesterase (AChE) inhibition to acute toxicity in fish. This AOP is known to be applicable to dozens of organophosphate and carbamate insecticides. AChE plays an important role in the cholinergic system including nerve impulse transmission in synapses (Norris et al., 2013). The AChE enzyme in the brain can be a target for toxic chemicals (Schmidel et al., 2014), and these chemicals cause disruption of nerve function and excessive ACh accumulation by inhibiting the AChE enzyme (Battacharya, 1993). Therefore, AChE can be a biomarker in the evaluation of neurotoxic changes. Other examples of field-based studies have suggested an association between molecular responses to whole-organism level endpoints such as behavior (Garcia-Reyero et al., 2011) and fecundity (Kristensen et al., 2007).

Currently there is a wide assortment of molecular tools being adapted for environmental toxicology studies, with one being quantitative PCR (qPCR). Quantitative PCR provides a simple method for determining the amount of a target DNA sequence or cDNA template/gene (or reverse transcribed mRNA) that is present in a sample. In qPCR experiments there are two methods to analyze the amount of a target DNA or cDNA present in a sample, absolute and relative quantification (Bio-Rad Laboratories, 2006). Absolute quantification refers to the method that allows determination of the actual amount (i.e. number of copies, ng, etc.) of a target gene present in a particular sample based on a standard curve prepared from samples of known template concentration (Plaffl, 2004). The concentration of any unknown sample can then be determined by simple interpolation of its PCR signal (Cq) based on this standard curve (Plaffl, 2004). Relative quantification describes a qPCR in which the expression of a gene of interest in one sample is compared to expression of the same gene in another sample (e.g. control levels of beta-actin relative to toxicant exposed levels of beta-actin; Plaffl, 2004). Thus, relative quantification measures the relative change in mRNA expression levels. Unlike absolute quantification, relative quantification does not require a calibration curve or standards with known concentrations (Bustin, 2002). Relative RT-qPCR methods also entail standard mathematical models offered through a variety of

manufacturers to estimate target gene relative quantities. The relative method works optimally when target genes of interest are normalized to more than one stably expressed gene (also referred to as reference or housekeeping genes) to account for errors associated with different amounts of starting material.

1.9. The Integrative Nature of Vertebrate Biological Systems and Toxicant Effects

Since the nervous system acts together with the endocrine system to integrate environmental information with bioregulation of physiology and behaviour, a sub-discipline called environmental endocrinology has emerged (Bradshaw, 2007). This area focuses not only on natural environmental factors such as salinity, light, and temperature, but has expanded to include effects of pesticides, heavy metals, and other organic compounds added into the environmental via human activity or anthropogenic means (Bradshaw, 2007). There are numerous ways in which disruption of the endocrine system could lead to adverse effects in fish biological systems; one major source being environmental contaminants. The interference of endocrine bioregulation by environmental contaminants or other natural chemicals is called endocrine disruption (Canadian Environmental Protection Act, 1996). Often the chemical that can alter the normal bioregulatory mechanism(s) of a system by modifying, mimicking or interfering with bioregulators is termed an “Endocrine Disrupting Compound” (EDC) (Norris et al., 2013). These EDC’s can prevent the synthesis or natural bioregulator, as well as their transport, normal mechanisms of action, or alter their rates of metabolism and/or excretion (Canadian Environmental Protection Act, 1996). Disruption of the vertebrate endocrine system can have many adverse effects on the organisms’ health and ability to properly function since it is involved in the regulation and coordination of various physiological functions including development, growth, reproduction, chemical balance, and chemical messaging. For example, the endocrine system regulated and maintains sexual development in vertebrate animals, and is often involved in the timing of changes in the body (Norris et al., 2013). EDC’s related to the disruption of sexual development have been studied and documented as causing effects such as feminization, masculinization, and the production of vitellogenin (a precursor of egg proteins) in male fish (Janz et al. 2011; Lange et al. 2001; Knudsen et al. 1997; Knudsen et al. 1998 Jobling et al. 1995; Rogers-Gray et al. 2000).

The immune system is responsible for resisting infectious agents, foreign substances and foreign or damaged cells (Wendelaar Bonga., 1997). There is also been wide recognition of the relationship between the endocrine system and the function of the immune system in vertebrates (Harris et al., 2000). Indeed, many studies have demonstrated that the functioning of the immune system depends on hormones provided by the endocrine system (Myers 2002; Warhurst, 1999; Johannessen et al., 2002). Hormones such as cortisol, androgens, estrogens and growth hormone have all been shown to alter the function of various components of the immune system (Currie and Woo, 2007; Harris et al., 2000; Khansari et al., 2017; Slater et al., 1995). Recent work has shown that exposure to pollutants can reduce the effectiveness of an organism's immune system thus making them more susceptible to disease and parasitism. Some studies have already demonstrated these effects in Pacific Salmonids (Arkoosh et al. 1998; Arkoosh et al. 1991). Studies have shown that anticholinergic substances (AChE activity inhibitors), such as organophosphorus pesticides, are able to modulate the immune response of fish (Toledo-Ibarra et al., 2013; Bols et al.,2001; Galloway et al., 2003;Dunier et al., 1991). For example an *in vivo* acute exposure of Nile tilapia (*O. niloticus*) to the organophosphorus pesticide diazinon (an AChE inhibitor) reduced the proliferative capacity of splenocytes and increased ACh concentration in the spleen (Girón-Pérez et al., 2007). From this the authors concluded that this pesticide presented immunotoxic properties which affected both the innate and cellular adaptive immune responses (Girón-Pérez et al., 2007). Ultimately these data suggest possible effects for pesticides can cause alterations in neuroimmunomodulation or immunosupression through cholinergic pathways. In vertebrates, ACh is widely distributed in the central and peripheral nervous systems and synthesized from choline and acetyl-Coenzyme A (acetyl-CoA) by the enzyme choline acetyltransferase (ChAT) (Albuquerque et al., 2009). ACh is stored in presynaptic vesicles until the cell is activated, and upon release into the nerve synapse, this neurotransmitter binds two distinct receptors on the postsynaptic cell: the ionotropic nAChR and the metabotropic muscarinic acetylcholine receptor (mAChR); both of which are bonded to G protein (Albuquerque et al., 2009). nAChR's are cholinergic receptors found in both the central nervous system and peripheral nervous systems, as well as skeletal muscles (Albuquerque et al., 2009). These receptors are ligand-gated ion channels with binding sites for acetylcholine and other molecules (Albuquerque et al., 2009). When ACh or ACh agonists bind to these receptors, it stabilizes the open state of the ion channel allowing influx of cations such as

potassium, calcium and sodium ions (Albuquerque et al., 2009). To stop the nerve from continuing to transmit the signal, the synaptic enzyme acetylcholinesterase (AChE) breaks down acetylcholine immediately after it has had its effect (Albuquerque et al., 2009; Norris et al., 2013). If AChE is inhibited in any way, the neuron becomes over stimulated (Zinkl et al. 1991; Murty 1986). The effects of AChE inhibition in vertebrates such as fish can widely vary. Some studies have shown that AChE inhibition as low as 8% can be lethal in some species of fish, while other species are able to survive inhibition of 70-90% (Fulton et al., 2001; Post et al., 1974; Coppage et al., 1972, 1974, 1975, 1976). Sub-lethal effects of AChE inhibition in fish can include: abnormal reproduction, reduced stamina, altered swimming and social interaction, hyperexcitability, and other behavioural changes (Gruber et al., 1998). A previous study by Post et al., (1974) reported that significant effects on swimming stamina in three species of salmonids were observed in conjunction with much lower levels of AChE inhibition. They reported that for the three species tested (brook trout, *Salvelinus fontinalis*; rainbow trout, *Oncorhynchus mykiss*; and coho salmon, *Oncorhynchus kitusch*), fish that had brain AChE activity reduced by 50% experienced stamina reductions of 23 to 44%. These findings on AChE inhibition due to pesticide exposure provide rational for studying neonicotinoids such as clothianidin that are known to weakly bind to vertebrate nAChRs and potentially impact the central and peripheral nervous system. It is hypothesized that chronic exposure to a neonicotinoid may cause prolonged low level occupation of nAChR that may have subsequent downstream, but unknown effects on the nervous system directly or other biological systems such as the immune and endocrine systems that work in concert in vertebrates.

1.10. **Objectives**

Fraser River sockeye salmon (Oncorhynchus nerka) are an integral part of freshwater and marine aquatic ecosystems in BC, and is one of BC's most important commercial fisheries. Records indicating decreasing numbers in sockeye salmon returns since the late 1980's is of concern for the future of maintaining sockeye salmon populations in the Fraser River. One of the proposed contributing factors to the salmon population decline is pesticide contamination (Cohen, 2012). With the introduction of a new generation of compounds, the neonicotinoids, understanding of the adverse effects of environmentally relevant concentration of neonicotinoids on wild sockeye salmon is

essential. The main focus of this research was to investigate gene expression in sockeye salmon following chronic exposure to low-level, waterborne clothianidin from fertilization to the swim-up fry developmental stage. This study was conducted in collaboration with Leung et al. (in prep) whereby offspring from four distinct genetic pairs of wild sockeye salmon from the Pitt River, BC and exposed to 0.15, 1.5, 15, 150 µg/L clothianidin in flow through glass tanks. The hepatic genes of interest evaluated in this study were related to various physiological processes including reproduction (estrogen receptors, ERβ1, ERβ2, ERα; androgen receptors, ARα, and ARβ; vitellogenin, VTG), growth (growth hormone receptors, GHR1, GHR2; thyroid hormone receptors, THα, THβ; deiodinases, Dio2), stress response (glucocorticoid receptor, GR1, GR2; mineralocorticoid receptor form A, MRfA; cytochrome P450 1A, CYP1A;) nervous system function (acetylcholine esterase receptor, AChE; nicotinic acetylcholine esterase receptor, nAChE), immune system function (interleukins, il-6, il-8; suppressor of cytokine signaling, SOCS2, SOCS3) and oxidative stress (catalase, CAT; heat-shock protein 27,hsp27).

Chapter 2.

Methods

2.1. Sockeye Salmon Clothianidin Exposure

Juvenile sockeye salmon livers collected in a previous study conducted at Simon Fraser University (Ginny Leung/Dr. Vicki Marlatt) were analyzed in the present study to examine gene expression changes associated with sub-chronic, waterborne clothianidin exposure during the early life stages of Sockeye salmon (*Oncorhynchus nerka)*. The exposure experiments entailed offspring from four mating pairs of wild sockeye salmon that were collected from the Pitt River, BC, Canada. Eggs and milt from these four mating pairs/genetic crosses of wild sockeye salmon were dry fertilized according to Patterson et al. (2004), allowed to water harden for approximately 1 hour, and then exposed to 0.15, 1.5, 15, 150 µg/L of waterborne clothianidin and a dechlorinated municipal water control in duplicate glass tanks for ~119 days until the swim-up fry developmental stage. Termination was performed when 87-98% of surviving alevins reached the swim-up fry developmental stage (100% yolk sac absorption) in the control glass tanks. Individual swim-up fry were euthanized with 0.4 g/L tricaine methanesulfonate (MS22; Sigma Aldrich, Oakville, Ontario, Canada; CAS# 886-86-2) buffered with 0.4 g/L sodium bicarbonate (Sigma Aldrich, Oakville, ON, Canada) to pH 7.0-7.5 on post-fertilization days 118-119. Body weight and length was recorded along with any developmental deformities. Livers were collected at random from individual swim-up fry from the five treatment groups for all four genetic crosses (n = 1-2/treatment/genetic cross), and placed in 1.5 ml safe-lock Eppendorf tubes (DNase/RNase free) and snap frozen immediately on dry ice, followed by transfer to -80°C for long term storage until further analyses. All instruments used in liver tissue collection were cleaned in between each animal using 10% peroxide and double rinsed with ultrapure water to ensure equipment was RNase/DNase free. All work with animals was performed in conformity with the principles for the use and care of laboratory animals in compliance with the Canadian Council for Animal Care guidelines and with a permit issued by Simon Fraser University Animal Care Committee (Burnaby, BC, Canada). The results of this experiment by Leung and Marlatt (in preparation) showed

that chronic exposure to all concentrations of clothianidin (0.15, 1.5, 15 and 150 µg/L) tested had no significant effect on fertilization success, survival, condition factor and deformity rates. However, exposure to 0.15 µg/L clothianidin significantly increased the average whole body concentrations of 17β-estradiol in swim-up fry ($p < 0.0001$) to 4 fold above the average control swim-up fry (n = 5-8 individual swim-up fry/treatment from 2 replicate tanks). The present study focuses on hepatic gene expression changes relevant to changes in reproductive endocrine axis based on the observed elevations in 17β-estradiol, as well as several other biological processes to investigate additional potential sub-lethal effects of clothianidin in a wild salmonid.

2.2. **Total RNA Isolation and cDNA Synthesis**

All RNA isolation and cDNA synthesis procedures for samples used in subsequent quantitative real-time PCR (qPCR) experiments adhered to the guidelines in Bustin et al. (2010) for accurate design, documentation and reporting of qPCR experiments. For each genetic cross, two livers from each of the five treatment groups (n = 2 per treatment/cross) were chosen for gene expression analysis in liver tissue at the swim-up fry stage. A genetic cross refers to the offspring from one of four mating pairs of wild sockeye salmon. Thus, all four genetic crosses tested in the clothianidin waterborne exposures (water control and 0.15, 1.5, 15, 150 µg/L) were included for gene expression analyses in this study.

Total RNA was isolated from the swim-up fry livers using TRIzol® Reagent as described by the manufacture (Invitrogen, Burlington, ON, Canada). Homogenization of one liver was performed utilizing 1 mL TRIzol reagent, and two 1 mm tungsten-carbide beads in a safe-lock Eppendorf 1.5 mL microcentrifuge tubes in a Retsch Mixer Mill MM 400 (Fisher Scientific, Ottawa, ON, Canada) at 30 Hz for a total of 8 min. During the homogenization procedure, chambers were stopped after 4 min and rotated 180°, then continued homogenized for another 4 min. Total RNA obtained from the TRIzol® RNA isolation procedure was subsequently reconstituted in 30 µL DNase/RNase-free water, and stored at -80°C. The quantity (ng/µL) of RNA was determined by measuring the optical density unit (OD_{260}) with an Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, VT, USA), and the RNA purity was assessed measuring $OD_{260/280}$ and $OD_{260/230}$ ratios. Following determination of RNA concentrations, total RNA samples were DNase treated using *TURBO DNA-free* kits™ (Ambion, Austin, TX) to remove any co-

extracted DNA according to the manufacturer's instructions. RNA integrity of DNase-treated RNA samples was evaluated using a Bio-Rad Experion™ Automated Electrophoresis System and Experion software (version 3.20; Bio-Rad, Mississauga, ON, CAN). RNA integrity values (RIN's) for all samples used in this study ranged from 7.9 to 9.7 (RIN average ± 9.0; standard deviation = ± 0.50, n = 38). In keeping with MIQE guidelines outlined in Bustin et al. (2010), total DNase-treated RNA samples devoid of significant contamination and RNA degradation with an RNA integrity ratio (RIN) score of > 8.0 and $OD_{260/280}$ and $OD_{260/230}$ ratios of 1.8–2.1 were included in subsequent qPCR experiments. The DNase-treated RNA samples were stored at -80°C for long-term storage until subsequent cDNA synthesis.

Reverse transcription of 1 µg of DNase-treated total RNA into cDNA for each liver sample was performed using qScript™ cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA) following supplier's instructions. Briefly, the DNAse-treated RNA samples were thawed on ice and depending on RNA concentration of each sample, the appropriate amount of DNAse-treated RNA sample was added to a master mix containing 4 µL of qScript Supermix and DNase/RNase free water to make up a total volume of 20 µL and 1 µg DNAse-treated total RNA. This cDNA synthesis procedure resulted in a final concentration of 50 ng of cDNA/µL of water for each cDNA sample, and these samples were maintained at -20°C until subsequent qPCR experiments were performed.

2.3. Quantitative Real-Time PCR (qPCR) for Hepatic Gene Expression

The relative quantification of each gene target/gene of interest in an individual fish liver cDNA sample measured in qPCR experiments was achieved using the ΔΔCq method, and by normalizing data to the expression level of 3 reference genes in each cDNA sample. The inclusion of a standard curve in qPCR assays also provides valuable information regarding the performance of the reaction (e.g. amplification efficiency) and acceptable criteria for novel primers. In order to generate a standard curve during a qPCR assay, a dilution series of known cDNA template concentrations is tested in a series of wells in tandem with separate wells containing the experimental cDNA samples under investigation. The standard curve is then used to interpolate the quantity of the cDNA in the experimental cDNA samples (Bustin et al., 2009). The Bio-Rad CFX384™

Real-Time PCR Detection System and CFX Manager™ Software then plots the log of each concentration of the standard from the standard curve dilution series against the quantification cycle (Cq) value for that concentration. The Cq is the cycle number at which the fluorescent signal of a cDNA sample in a qPCR reaction crosses a threshold, and this threshold is the level of fluorescent signal that reflects a statistically significant increase over a calculated baseline (background) signal generated by the software. The baseline of the qPCR reaction refers to the signal level during the initial cycles of PCR in which there is little change in fluorescent signal (Life technologies, 2012). The standard curve for all qPCR experiments was generated by performing a dilution series of a pooled cDNA sample that was a mixture of cDNA samples from all of the control and clothianidin exposed fish liver samples. Specifically, a 50 ng cDNA /µL of water standard was prepared by combining equal amounts of cDNA from all of the control samples (8 individual cDNA liver samples) with 1 µL from one sample from each of the clothianidin treatment groups (30 individual cDNA liver samples) into a single 1.5 mL microcentrifuge tube; thus making 38 µL of a 50 ng cDNA/µL of water standard as the most concentrated standard in the qPCR experiments. Four-fold serial dilutions of the 50 ng cDNA/µL of water standard were performed to create an 8-point standard curve in each qPCR experiment. Therefore, the 8-point standard curve included the following: 50, 12.5, 3.125, 0.7813, 0.195, 0.049, 0.012 and 0.003 ng of cDNA/µL. For each gene standard curve, 2.5 µL of each dilution were then added as a cDNA template, in triplicate. Cq values were plotted in Bio-Rad CFX software against the log of each known dilution series concentrations of pooled cDNA template.

The gene expression targets examined in this study related to various biological processes including reproduction, growth, stress responses, nervous and immune system function and oxidative stress were examined after chronic exposure to clothianidin in qPCR experiments. A complete list of these genes is listed in Tables 4.1 – 4.3. Primers with optimal annealing temperature between 55-58°C were designed using Integrated DNA Technologies (IDT) OligoAnalyzer 3.1 (www.idtdna.com/calc/analyzer), and sequences obtained from GenBank National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) database for sockeye salmon when available, or rainbow trout (*Oncorhynchus mykiss*) and/or zebrafish (*Danio rerio*). Primers for estrogen receptors ERα, ERβ1, and ERβ2 (GenBank accession numbers: AJ242741, DQ177439 and DQ248229 respectively) were designed for this study based on the conserved

regions of the two genes in rainbow trout as reported by Nagler et al. (2007). Primers for the glucocorticoid receptor 2 (GR2) were designed based on conserved regions in rainbow trout. The primer set for suppressor of cytokine signaling 3 (SOCS3) was designed by aligning the zebrafish sequence for SOCS3 with a rainbow trout sequence for SOCS3 in order to determine most conserved regions. A primer set was then designed based on an area of most conserved nucleotides, and using the rainbow trout sequence for final primer sequence selection (GenBank accession number: NM001146168.1) By designing a primer set based on a highly conserved region of the SOCS3 gene, amplification of the target gene is further ensured.

To test the primer sets for suitability in qPCR experiments, primer optimization trials were conducted by modifying the annealing temperature, template concentration and primer concentration. Primer sets used to measure gene expression levels were tested for efficiency using a 4 to 8 point standard curve generated by a 4-fold dilution of a 50 ng cDNA/µL of water (comprised of pooled liver samples from sockeye swim-up fry and/or liver from a 10 month old sockeye salmon). The criteria for acceptance of the standard curve included in single peak melt curve, efficiencies between 90-110%, amplification in at least 4 concentrations of the standard curve, and an R^2 of the standard curve > 0.900 (Bustin et al., 2010). Primers that satisfied these criteria are shown in Table 4.1 and include product size, PCR efficiency, and correlation coefficient (R^2). Cycle threshold or cycle quantification (Cq) values and the regression slope, PCR efficiency, Y intercept and correlation coefficient was calculated by Bio-Rad CFX software and checked considering MIQE guidelines of Bustin et al. (2010). Primers that failed to amplify a target gene sequence or did not meet these criteria on swim-up fry cDNA samples were further evaluated using cDNA from 10 month old wild sockeye salmon that were collected from the Pitt River stock. These 10 month old wild sockeye salmon were reared in outdoor tanks under a natural photoperiod and municipal dechlorinated tap water. If primers for a particular gene displayed little to no amplification in the standard curves for swim-up fry, the primers were then tested once again using cDNA from the 10 month old sockeye salmon to rule out primer design errors and developmental stage specific-expression issues (i.e. demonstrate whether the primers could amplify target genes in juvenile sockeye salmon). Primers that amplified the genes of interest in the older 10 month old salmon livers, but were below detection limits for qPCR in the swim-up fry are listed in Table 4.2. Primers that were designed and did not

amplify a target gene in both swim-up fry and 10 month old sockeye salmon liver cDNA samples were considered non-specific/failed primer sets, and were not included in qPCR experiments (Table 4.3).

All primers sets that were designed and amplified targets in the swim-up fry cDNA samples in this study were also evaluated for specificity using PCR. This included GR2, ERα, ERβ2, CYP 1A, and SOCS3 in sockeye salmon liver tissue. These PCRs of sockeye salmon swim-up fry liver cDNA were conducted using a T100 Thermal Cycler (Bio-Rad) and Platinum® *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. PCR amplification was performed using 1µL of cDNA template (50 ng/µL) diluted either 20-fold or 80-fold using DNA/RNA free water, 39.3 µL DNA/RNA free water, 5 µL 10X PCR reaction buffer, 1.5 µL $MgCl_2$ (50 mM), 1 µL dNTPs (10 mM), 0.2 µL Platinum® *Taq* DNA Polymerase, and 1 µL each of gene specific primers (10 µM). A 40-cycle PCR was performed with each cycle consisting of 30 s at 95°C (denaturation), 1 min at 58°C (annealing) and 5 s at 72°C (extension). PCR products were electrophoresed on a 1% agarose gel containing SYBR Green for 90 min at 80 V. Bands were visualized using a Molecular Imager® ChemiDoc™ XRS+ system (Bio-Rad), and for specific genes of interest, the bands were excised on a FisherBiotech Transilluminator (Fisher Scientific, Pittsburg, PA, USA), and gel-purified using QIAquick™ Gel Extraction Kit according to the manufacturer's instructions (QIAgen, Mississauga, ON, Canada). SOCS3 for sockeye salmon was verified by Sanger Sequencing at the University of British Columbia Sequencing and Bioinformatics Consortium (Vancouver, BC, Canada). Sequencing results were then aligned using the European Bioinformatics Institute EMBOSS Needle nucleotide alignment tool (https://www.ebi.ac.uk) with the rainbow trout SOCS3 sequence from GenBank National Center for Biotechnology Information.

All qPCR experiments were carried out on a Bio-Rad CFX384™ Real-Time PCR Detection System, following the guidelines outlined for MIQE (Bustin et al., 2010; Taylor et al., 2010). All qPCR reactions for each gene were performed in triplicate on Hard-Shell 384 well PCR plates (Bio-Rad) according to the manufacturer's instructions. Each qPCR reaction contained the following master mix for each target gene or reference gene in total reaction volume of 10 µL: 0.29-0.44 µL forward and reverse primers (0.23-0.35 µMol/reaction), 6.25 µl of SsoFast™ EvaGreen® Supermix (Bio-Rad) and RNase-free water for the remaining volume. Each reaction well contained 10 µL of the qPCR

master mix followed by a 2.5 µL addition of cDNA template (50 ng/µL) diluted to either 1:20 or 1:80 to make up a 12.5 µL total reaction volume. The standard curve was performed in triplicate, for each gene on each plate. Each plate contained three technical replicates per sample, and biological replicates were as follows; control (*n* = 5-8), 0.15 µg/L (*n* = 5-8), 1.5 µg/L (*n* = 5-8), 15 µg/L (*n* = 5-7), and 150 µg/L (*n* = 5-7). A no template control (NTC) was prepared for each primer set and tested in triplicate, whereby 2 µL of RNase-free water was used in place of cDNA. A no reverse transcriptase control (NoRT) was also prepared for each primer set and tested in triplicate, whereby 2µL of DNase treated RNA was used in place of cDNA. Each qPCR experiment included three NTC replicate samples and three replicate NoRT samples. The NTC and NoRT were negative controls to confirm the absence of contamination of cDNA samples and that the DNase treatment sufficiently removed genomic DNA, respectively. Amplification reactions were performed using the following instrument settings: initial cycle 1 activation at 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s, and primer annealing at 55-58 °C for 5 s. After 45 cycles, a melt curve analysis was performed following every run to confirm a single amplicon was amplified for each set of primers as indicated by a single peak. Melt curve peaks occurred at 82.5°C (GR2), 83.5°C (ERβ2; CYP1A), 86°C (ERα), and 79.5°C (SOCS3). The purpose of the melt curve analysis was to ensure reaction specificity and to check for any primer-dimer artifacts. The melt curve analysis was conducted using the following instrument settings: initial temperature of 65.0°C and was increased by increment of 0.5°C for 5 s to a maximum of 95.0°C. All amplicons showed a single sharp peak at approximately, which indicated a single gene was amplified in each qPCR assay.

2.4. **Data Analysis for Quantification of Gene Expression**

For this study, the ΔΔCq method for relative quantitation of target genes between treatments was performed using Bio-Rad CFX Manager™ Software. The Cq values for GR2, ERα, ERβ2, CYP 1A, and SOCS3 measured in all swim-up fry cDNA samples range from 29.81–45.16. Normalized expression values were obtained for all technical replicates of each cDNA liver sample using Bio-Rad CFX Manager™ Software's Gene expression application. CFX Manager™ Software presents normalized expression as ΔΔCq, which is defined as the relative quantity of the target gene normalized to the quantities of the three reference genes for each sample. The ΔΔCq method compares

the results from experimental samples (i.e. clothianidin treatments) with both a calibrator, such as the water control treatment in this study, and a normalizer gene (reference gene) using the formula (equation 1):

Normalized target gene expression level = $2^{-\Delta\Delta Cq}$ (1)

With this method, the Cq value for the gene of interest in both the test samples and calibrator sample are adjusted in relation to a normalizer gene Cq value from the same two samples (Bio-Rad, 2010). A more simplistic representation of this is illustrated as follows (equation 2) (Bio-Rad, 2010):

$$Expression\ sample\ GOI = \frac{RQsample(GOI)}{[RQsample(Ref1)\times RQsample(Ref2)\times RQsample(Refn)]^{\frac{1}{n}}} \quad (2)$$

RQ = Relative Quantity of a sample (calculated using Cq)

Ref = Reference target in a run that includes one or more reference targets in each sample up to the nth reference

GOI = Gene of interest

Reference gene stability, also called the M-value, was determined by evaluation by CFX Manager™ Software Gene Expression Analysis. The M-value is a measure of reference gene expression stability and is calculated by the average pairwise variation of a single reference gene with all other reference genes (Vandesompele et al. 2002). Specifically, it is the mean standard deviation of the log-transformed expression ratios across samples for the particular gene relative to the reference genes and is illustrated in the equation below (equation 3) (Bio-Rad, 2010):

$$Mj = \frac{\sum_{K=1}^{n} Vjk}{n-1} \quad (3)$$

M_j = gene stability measure for control gene *j*;

V_{jk} = pairwise variation of gene j relative to gene k, and;

n = total number of number of genes of interest examined.

The most stable combination of reference genes will give the lowest M value, providing a stable baseline to normalize genes of interest. The criteria for an acceptable M value for three reference genes was < 1, and if the M value exceeded this threshold it was not considered for this study (Vandesompele et al., 2002).

2.5. **Statistical Analyses**

All statistical analyses were performed with JMP®, Version *13* (SAS Institute Inc., Cary, NC). Statistical comparisons were conducted on the normalized gene expression values of 4 to 8 biological replicates (i.e. individual fish livers; actual n is indicated in each figure caption for each gene) per treatment. A test for normality was determined on Log10 transformed normalized expression values (ΔΔCq values) using Sharpo-Wilk test, and a homogeneity of variance test was performed by Levene's test. Significant differences in normalized gene expression between treatments were determined by one-way analysis of variance (ANOVA; $p < 0.05$) followed by a Tukey's post-hoc. ERα and ERβ2 displayed non-normally distributed data, and were therefore analyzed using Wilcoxon (Kruskal-Wallis) Tests (Rank Sums).

Chapter 3.

Results

3.1. RNA Quality

All RNA sockeye salmon samples used in these gene expression studies were analysed by Experion™ Automated Electrophoresis System and Experion software (version 3.20) met the criteria outlined for high integrity and quality in the MIQE guidelines (Bustin et al., 2010). The average $OD_{260/280}$ ratio ± standard error mean for all 38 biological samples was 2.01 ± 0.06, and the RNA integrity number (RIN) values ranged from 7.9 - 9.8 (average RIN ± standard error mean = 9.06 ± 0.49. The virtual gel images from the Experion™ Automated Electrophoresis System results revealed the two dominant RNA fragments, the 18S (lower band) and 28S (upper band) rRNAs indicating intact RNA. The RNA concentration in ng/µL and the RIN values for all samples are shown in Table 4.4. RNA samples passing the RNA quality criteria ($OD_{260/280}$ ratio = 2.0 ± 0.2; RIN ≥ 7.9 as assessed using the Experion (Bustin et al., 2010) were utilized for qPCR gene expression analysis.

3.2. Primer Set Design and Evaluation

To explore gene expression in swim-up fry sockeye salmon liver, qPCR was used to obtain the relative quantitation of each GOI in detecting possible mRNA expression levels. A series of primers were designed for approximately 27 genes relating to either the endocrine, stress, immune, or nervous system. Out of the genes tested and evaluated using qPCR, only five meet the ideal parameters for all qPCR data outlined in the MIQE guideline (Bustin et al., 2010): acceptable efficiency (between 90-110%), one sharp melt peak, a 4 point standard curve minimum, and an $R^2 > 0.900$. While estrogen receptor alpha had an efficiency of 112%, it was still deemed acceptable as it's melt curve analysis showed only one sharp melt peak, and had an $R^2 = 0.997$. Target stability function using CFX Manager™ Software Gene Expression Analysis determined that the combined M-value for the three reference genes (CBA, GAPDH, and EF1α) was 0.56 with a coefficient of variation (CV) = 0.22.

The specificity of all primers that were previously tested and verified by sequencing in peer-reviewed scientific journals were also evaluated in this study by melt curve analysis using Bio-Rad CFX Manager™ Software. A single amplified product peak for all genes was desired, as this verifies that the primers did not generate any unspecific products. Single melt curve peaks occurred at 82.5°C (GR2), 83.5°C (ERβ2; CYP1A), 86°C (ERα), and 79.5°C (SOCS3). Primers displaying multiple peaks in the melt curve analysis were further analysed by testing amplification in 10 month old sockeye salmon liver cDNA, and if still not successful were then either re-designed or excluded from this study.

MRfA, THα, THβ, ERβ1, SOCS2, VTG, CAT, displayed low gene expression and poor efficiency in the swim-up fry qPCR assays (Table 4.2). The criteria for concluding low expression of a GOI included: standard curves with Cq values only above base-line for the 3 most concentrated standard curve points (50, 12.5, and 3.125 ng/µL) in swim-up fry; and, did amplify in the older, 10 month old sockeye salmon liver cDNA qPCR assays with a 4 or more point standard curve and efficiency within the range of 90-110%. AChE, HSP27, GHR1, GHR2, and nAChRα 9-1 also displayed low gene expression and poor efficiency in the swim-up fry qPCR assays, and, were amplified in the older, 10 month old sockeye salmon liver cDNA qPCR assays. However expression was still low for these genes and therefore the efficiencies fell outside of the acceptable 90-110% range (poor efficiency) (Table 4.2). Gene expression for TNF-α, Il-6, Il-8, Dio2, and GR1 were still not detected after multiple attempts to amplify and quantify mRNA expression levels in both swim-up fry stage and approximately 10 month old sockeye salmon. A full list of these genes that were tested at both development stages and failed (no amplification) is presented in Table 4.3.

Attempts to design unique primers for the nicotinic acetylcholine receptor and androgen receptors gene targets were not successful due to the high sequence similarity with the subtypes of these target genes. Comparison of the predicted amino acid sequence of AR-α to that of AR-β for rainbow trout revealed 85% identity in a study by Takeo et al., (1999). Upon further investigation, the EMBOSS Needle Pairwise Sequence Alignment tool (https://www.ebi.ac.uk/) was used to align the mRNA nucleotide sequences for AR-α and AR-β obtained from the GenBank National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) database for rainbow trout. Similar to the results of Takeo et al. (1999), a sequence similarity value was obtained

and revealed a 90.1% identity between the two gene sequences. Marlatt et al. (2010) also note a difficulty in designing primers for both rainbow trout ARs due to high sequence similarity. In the present study, both ARα and ARβ resulted in melt curves displaying multiple peaks. Melt curve analysis indicated that these multiple amplicons detected were mostly due to the presence of multiple subtypes being amplified or other non-specific products, rather than the presence of primer dimers. Primer-dimers exhibit a lower melting temperature than the amplicon, and most often form in the no-template controls (NTCs) due to an abundance of primer and no cDNA template being present (Real-Time qPCR handbook, Life Technologies, 2012). However all NTCs for these three genes displayed no amplification, and therefore indicated no primer-dimer formation or DNA contamination through procedural errors during the qPCR experimental set up.

3.3. **Hepatic Gene Expression**

For the five genes that meet the MIQE guidelines, individual normalized expression values were obtained for each biological replicate using Bio-Rad CFX Manager™ Software's Gene Expression Analysis module. No significant difference was detected between all treatments for CYP1A, ERα, ERβ2, and SOCS3 at any concentration ($p > 0.05$) (Figures 4.1- 4.4). However, a significant 4-fold decrease for GR2 was detected between the normalized expression in the liver of swim-up fry exposed to 150 µg/L of waterborne clothianidin compared to liver of the swim-up fry in the water control treatment group (Figure 4.5). The average normalized expression ± standard error mean for the 150 µg/L clothianidin was 1.01 ± 0.49, and for the water control was 4.29 ± 1.64,($P = 0.0281$).

Chapter 4.

Discussion

This is the first study to report the effects of environmentally relevant levels of a neonincotinioid pesticide, clothianidin (0.15-150 µg/L), on gene expression in wild sockeye salmon. This study was designed to evaluate gene expression relevant to various biological processes/systems, including the stress response, nervous, reproductive and immune system in the liver of sockeye salmon after chronic waterborne clothianidin exposures during the embryonic, alevin and onset of the swim-up fry developmental stages. As such, several RT-qPCR bioassays were designed and conducted to measure mRNA levels of select genes of interest, and several novel primers were developed. In total, 27 primer sets were designed, however only 8 were successful with 14 primer set tests indicating low levels of gene expression for particular genes of interest and 5 failed primer sets. One gene of interest, the glucocorticoid receptor 2, decreased ~4 fold in the highest clothianidin exposure concentration tested. The glucocorticoid receptor 2 is a key steroid hormone receptor that binds to glucocorticoids (i.e. cortisol) to regulate the stress response through modulation of metabolic, cardiovascular and immune function. Collectively, this study demonstrates that clothianidin potentially causes a reduced capacity to respond to stress, and that future work is needed to understand gene expression during early developmental stages of wild sockeye salmon

4.1. Effects of Parentage and Development on Gene Expression Profiles

The present study examining the effects of clothianidin on gene expression were samples of fish from Leung et al. (*in prep*) that demonstrated no adverse effects of 0.15-150 µg/L clothianidin on growth, development or survival, but some effects on a whole body sex steroid concentration. Specifically, Leung et al. (in prep) observed a non-monotonic concentration-response with a 3 to 4.7-fold higher whole body 17β-estradiol concentration in the 0.15 µg/L clothianidin treatment compared to the all other treatments and the control in one of the four genetic crosses tested. No changes in

whole body concentrations of testosterone were observed in these same fish in this experiment, suggesting a lack of effects on aromatase activity/expression levels and perhaps inhibitory effects on 17β-estradiol metabolism/degradation pathways. The results of the present study that examined a mix of genetic crosses showed no effects of chronic clothianidin exposure on 2 of the 4 nuclear ligand activated estrogen receptor subtypes known in salmonids. Since increased 17β-estradiol levels were observed by Leung et al. (*in prep*), it was hypothesized that some changes in estrogen receptor gene expression may also occur after clothianidin exposure. This hypothesis was based on previous studies demonstrating transcriptional regulation of some estrogen receptor subtypes by 17β-estradiol, particularly up-regulation of ERα in multiple teleosts (Filby et al., 2007; Fenske et al., 2009; Marlatt et al., 2010; Marlatt et al., 2014; Menuet et al., 2004; Nelson et al., 2007; Sabo-Attwood et al., 2004). However, estrogen receptor subtype auto-regulation by 17β-estradiol is dynamic with tissue-, physiological status- and sex-specific expression evident in many vertebrates. Indeed, several studies have shown that estrogen receptor subtypes in vertebrates, including fish, in the form of mRNA and protein concentrations vary between different tissues (Kuiper et al., 1997; Menuet et al., 2002; Nagler, 2007; Shughrue et al., 1998). For example, Nagler et al. (2007) demonstrated that transcription of all four ER subtypes occurs in almost every tissue in rainbow trout, but all are not equally transcribed (e.g. ERα1 and ERβ2 mRNA levels are higher in general). In particular, Nagler (2007) showed that in rainbow trout the highest levels for all subtypes was observed in liver and testes and that ERα1 is predominant in the liver, ERβ1 is predominant in the testes while ERβ2 is most predominant in the spleen. A limitation of the present study was only examining 2 of the 4 ER subtypes due to difficulty with successful primer sets, thus whether the untested ER subtypes (i.e. ERβ1 and the truncated ERα) transcript levels were affected by clothianidin treatment is unknown and requires further study. Specifically, further investigations are warranted to confirm the absence of effects of clothianidin on all ER subtypes in sockeye salmon swim-up fry, including exploring all 4 ER subtypes and additional tissues/organs.

Leung et al. (*in prep*) did observe cross specific effects on length and weight in the fish used to examine gene expression in the present study, thus is it possible that some of the variation in genes of interest was attributed to these cross-specific effects. In the present study, liver samples from all four genetic crosses were pooled and

analyzed together for each treatment, while Leung et al. (in prep) analyzed sex steroids in a single cross (cross A). This was a result of logistical issues surrounding collections, whereby livers were collected for some but not all fish due to time constraints. Regardless, the previous study by Leung et al. (*in prep*) noted one cross in particular (genetic cross B) having a significantly higher body weight and body length than the other 3 genetic crosses tested. Leung et al. (*in prep*) also noted significantly delayed hatching of the eyed-embryos for one genetic cross (cross C). Delayed hatching in genetic cross C could lead to delayed development in the developing swim-up fry compared to other crosses, and the extent of the impacts of slightly different developmental stage on the genes of interest in the present study is not known. Other studies have demonstrated that the transcription of ERs can change with developmental stage and shift the predominance of one ER subtype to another, and ultimately, an increased abundance of an ER subtype during ontogeny may play a role in hormone-driven developmental processes (Filby et al., 2005; Boyce-Derricott et al., 2010). Furthermore, White et al., (2012) linked body size to developmental stage in many fishes, and proposed that differences in mRNA levels are anticipated between such groups simply due to the fish being developmentally distinct, rather than due directly to genetic differences (White et al., 2012). Future studies examining gene expression, body size and developmental differences between genetic crosses are needed to understand the role parentage and developmental stage has in natural variation in gene expression in developing sockeye salmon, and in variation in response to toxicant exposure.

Evidence is mounting demonstrating that molecular changes, including gene expression changes, are linked to adverse whole organism outcomes after toxicant exposure, but this is not well established for feral species such as the sockeye salmon that were examined in the present study. Hook et al. (2007) evaluated temporal changes in rainbow trout gene expression exposed to the xenoestrogen ethynylestradiol and hypothesized that expression profiles for early life stages of fish would be dynamic with time, as well as change in ways that would reflect mode of toxic action. The results reported by Hook et al. (2007) indicated a large number of genes (e.g. ER, VTG, AR, CYP 1A3) exhibited different expression levels at individual time points, demonstrating that a single gene changes dramatically with time after xenoestrogen exposure (Hook et al., 2007). This was also reported by Marlatt et al. (2010) during 24 hour waterborne exposures of male sexually regressed goldfish to 1 nM 17b-estradiol that showed the

estrogen-mediated vitellogenin gene was significantly elevated after 12 h of exposure, and by 24 h returned to control levels (Marlatt et al., 2010). Developmental stage of the animal is also likely to influence basal and toxicant induced gene expression, in addition to the temporal effects aforementioned that may have influenced the results of the present chronic study. A review by Embry et al. (2010) notes a trend of differences in gene expression and protein/enzyme profiles between juvenile and adult fish, and hypothesizes that early life stages may not yet have the fully developed metabolic pathways to degrade xenobiotics. For example, a study by Wang Buhler et al., (1997) showed lower levels of some cytochrome P450 enzymes in trout embryos compared to adult fish. As suggested by Jin et al. (2010), one could argue that mRNA levels represent only a small glimpse of the cell activity at any given time, and how this correlates to protein or enzyme function is not well studied for most genes. For example, Jin et al. (2010) demonstrated that the mRNA induction patterns in zebrafish exposed to herbicide atrazine were not in accordance with the changes in levels of antioxidant enzyme activities. Such inconsistencies are not surprising based on the complex processes occurring during early development confounded by the myriad of factors influencing the regulation and interactions between transcription and translation (Glanemann et al., 2003). Post-translational modifications of proteins, for instance, are widely diverse, and catalyzed by enzymes that recognize specific target sequences in specific proteins (Bürkle, 2001). This idea is further supported in a study by Glanemann et al. (2003) who evaluated mRNA levels and protein activity for three genes encoding homoserine kinase, threonine dehydratase, and homoserine dehydrogenase in *Corynebacterium glutamicum*. Glanemann et al. (2003) demonstrated that responses at the mRNA level did not reflect responses at the protein level or at the level of active enzyme. Moreover, with the use of the more recent OMICs technologies, studies supporting the existence of large scale disparities between the cellular, global transcript and protein levels (i.e. transcriptome and proteome) in human liver, rainbow trout, and zebrafish have been reported (Anderson et al., 1997; Sathiyaa et al., 2003;Velki et al., 2017); ultimately suggesting that not all enzymes and their activities are controlled by transcriptional means, instead by other mechanisms at the enzymatic level. Nonetheless, changes at the mRNA level of a cell are still significant because these can be part of an important initial response of the organism to a toxicant that, in turn, affects protein levels, which are the ultimate physiological effectors of a whole organism response. Although the present study provides useful insights into the expression of

several genes in sockeye salmon at the onset of the swim-up fry stage, future studies measuring both protein and gene expression during multiple time points in control and clothianidin exposed fish is warranted. This would capture not only the natural temporal and toxicant induced transcriptional changes, but aid in understanding how the respective protein level changes correlate to transcriptional changes and whole organism outcomes.

4.2.

4.3. **Clothianidin Decreased Glucocorticoid Receptors**

The results of the present study showed an ~4 fold decrease in glucocorticoid receptor 2 (GR2) at the highest concentration tested for chronic clothianidin exposure on wild sockeye salmon. Since this was a waterborne exposure with foreign chemical administered via water, it was hypothesized that some changes in expression of genes related to stress response or the HPI axis would occur. This hypothesis was based on previous studies demonstrating HPI down-regulation resulting from negative feedback of cortisol mediated by GRs following either physical or chemical chronic stress (crowding, handling stress, water quality), or intraperitoneal implants of cortisol to mimic chronic stress in salmonids (Barton, 2002; Madaro et al., 2015; Moltesen et al., 2016; Vijayan et al., 1990). Recently, Madaro et al. (2015) conducted a study that investigated the effects of exposing Atlantic salmon smolts to an unpredictable chronic stressor (UCS) for three weeks (Madaro et al., 2015). Smolts in the UCS group were stressed three times per day using a total of eight types of stressors given in a random and unpredictable order (Madaro et al., 2015). These stressors included both physical and chemical changes such as temperature shock, brief hypoxia, chasing, and noise disturbance (Madaro et al., 2015), and all stressors induced a down-regulation of both GR1 and GR2 mRNA levels in the preoptic area of the hypothalamus. This reduced expression of both GRs also coincides with previous observations for UCS in zebrafish (Piato et al., 2011) and common carp (*Cyprinus carpio*) (Stolte et al., 2008). While both GRs are involved in the stress response pathway, the current dogma is that these two receptors have unique physiological functions that are yet to be fully elucidated (Bury et al., 2003; Bury et al., 2007; Stolte et al., 2006; Vijayan et al., 2003). Interestingly, in zebrafish and some salmonid species low levels of GRs may protect neurons from high levels of cortisol

during chronic stress, as cells containing GRs are more prone to apoptosis by elevated levels of glucocorticoids (Moltesen et al., 2016; Piato et al., 2011; Sapolsky et al., 2000). The suppressive effect of chronic stress in the form of toxicant exposure (i.e. clothiandin) in the present study on GR2 mRNA levels in sockeye swim-up fry is hypothesized to be due to negative feedback of cortisol on the HPI axis. However, cortisol levels were not measured in this study, and therefore future studies measuring cortisol levels are necessary to test this hypothesis. Given that clothianidin can potentially interfere with neuron function, more specifically nAChRs in the nervous system and at the neuromuscular junction of vertebrates and in light of studies linking reduced glucocorticoid receptor levels to reduced neuronal apoptotis, more focused studies on evaluating effects of clothianidin on the central and peripheral nervous system are recommended.

4.4. Conclusions

This is the first study to report the effects of environmentally relevant levels of a neonincotinioid pesticide, clothianidin (0.15-150 µg/L), on gene expression in wild sockeye salmon. It is increasingly apparent that vertebrate immune, nervous and endocrine systems are closely linked and that cross-talk or communication between these systems is necessary for the maintenance of homeostatic function (Cyr et al., 1996; Duarte-Gutterman et al., 2014; Habibi et al., 2012; Harris et al, 2000), thus studies examining these major systems after toxicant exposure is critical to provide a comprehensive assessment of the adverse effects of a toxicant. Although the present study was limited to 5 genes of interest, the genes did span the reproductive (ERα, ERβ2) and stress (GR2) endocrine axes, immune (SOCS3) system and a detoxification (CYP1A) pathway, and effects of chronic clothianidin were only evident on the stress endocrine axis. One of the challenges in the present study that contributed to the limited number of genes of interest measured was the low level of mRNA and inability to detect several genes of interest in swim-up fry liver that were detected in older juvenile liver using the same RT-qPCR assays. Future studies might include the use of qPCR technology with higher sensitivity (i.e. digital droplet PCR, or RNA sequencing) for enhanced detection of low expression gene targets and/or to overcome the challenges associated with extremely small organs (i.e. the transcript levels associated organs from small size, early life stage fish). It is also possible that gene expression changes due to

clothianidin exposure were missed by measuring at a single time point 4 months after chronic exposure and after the progression from embryo to swim-up fry. Therefore, it is recommended that multiple additional sub-sampling events throughout this chronic, multi-life stage exposure design are included in future gene expression studies. Furthermore, since differences between genetic crosses were prevalent with respect to body morphometrics and development, future experiments should consider using this data to conduct power analyses to determine sufficient sample size to evaluate gene expression profiles for individual genetic crosses. Indeed, it is hypothesized that differences between genetic crosses were confounding factors that added the variation to the gene expression data in the present study. Further studies aimed at investigating the role parentage and developmental stage has in natural and toxicant-induced changes in gene expression in sockeye salmon are necessary to test this hypothesis. In conclusion, this study demonstrates that clothianidin potentially attenuates the response the stress pathway after chronic, environmentally relevant exposure, and that future work is needed to characterize gene expression profiles during early developmental stages of wild sockeye salmon

4.5. **Tables**

Table 4-1. Successful primer sets used to examine the effects of clothianidin on various target genes in sockeye salmon swim-up fry. National Center for Biotechnology and Information accession identifiers, primer sequences (5' to 3'), annealing temperature (Tm), efficiency of primer pair (%E), and goodness of fit of linear regression for the relative standard curve (R^2) are provided for reference genes CBA, GAPDH, and EF1α, and target genes GR2, CYP1A, ERα, ERβ2, and SOCS3

Target Gene	Accession	Primer Sequence (5'-3')	Tm °C	Product size	%E	R^2	Reference
CBA (cytoplasmic Beta-actin)	FJ226383	Forward: GGACTTTGAGCAGGAGATGG Reverse: TCGTTGCCGATTGTGATG	57	96	99.8	0.984	Duarte-Guterman et al (in prep)
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)	FJ226385	Forward: CCATCACAGCCACACAGAAG Reverse: CCATTCAGCTCGGGGATAA	58	139	105.0	0.900	Duarte-Guterman et al (in prep)
*EF1*α (Elongation factor 1α)	NP001117811.1	Forward: GGTGGTGTGGGTGAGTTTGA Reverse: CCAGAGTGTAGGCGAGGAGA	58	79	99.5	0.976	This study
GR2 (Glucocorticoid receptor 2)	NM001124482	Forward: CTGGCTGATGACACTCTCCTG Reverse: CTGGCTTGGAGGTGGAGTTG	56	70	91.4	0.781	This study
CYP1A (Cytochrome P450 1A)	FJ226380	Forward: GCCTGTGGTTGTTCTGAGTG Reverse: TCTTGCCGTCGTTGATGA	55	116	104.1	0.718	Duarte-Guterman et al (in prep)
ERα (Estrogen receptor α)	AJ242741	Forward: CCT GGA GAT GCT GGA CGG T Reverse: CCT GTG GAG GTG GTA GTG GT	57	102	112.6	0.997	Duarte-Guterman et al (in prep)
SOCS3 (Suppressor of Cytokine Signaling 3)	NM001146168.1	Forward: TTCTCCTCCACTATGTCCAGCCT Reverse: TCCAGGTGTCCGTTGACTGTT	57	62	107.0	0.996	This study
ERβ2 (Estrogen receptor β2)	DQ248229	Forward: GAG CAT CCA AGG TCA CAA TG Reverse: CAC TTT GTC ATG CCC ACT TC	57	126	94.2	0.988	This study

Table 4-2. Primer sets used to amplify GOI in swim-up fry (118-119 dph) and approximately 10 month old sockeye salmon but excluded from qPCR experiment. National Center for Biotechnology and Information (https://www.ncbi.nlm.nih.gov/) accession identifiers, primer sequences (5' to 3'), and product size are provided for. Reference indicates if primers sets used were from published literature or designed specifically for this study

Gene of interest	Accession	Primer Sequence (5'-3')	Product size	Reason for exclusion	Reference
MRfA *(Mineralocorticoid receptor form A)*	NM001124483.1	Forward: GAGAGCCGTAGAAGGTAGGAAA Reverse: CGAGTCTTCGTTCACACCCTT	191	Low expression in swim-up fry (< 4 points on standard curve)	Becker et al., 2008
THRα *(Thyroid hormone receptor alpha)*	JX232610	Forward: CCTTCAGCGAGTTCACCA Reverse: TCTCCATACAGCAGCCTTTC	132	Low expression in swim-up fry (< 4 points on standard curve)	Duarte-Guterman et al (in prep)
THRβ *(Thyroid hormone receptor beta)*	JX232611	Forward: CTACGACCCCGAGAGTGAGA Reverse: GCGATAAGCCCAAATCAAAG	119	Low expression in swim-up fry (< 4 points on standard curve)	Duarte-Guterman et al (in prep)
ERβ1 *(Estrogen receptor beta 1)*	DQ177439	Forward: CCCAAGCGGGTCCTAGCT Reverse: TCCTCATGTCCTTCTGGAGGAA	133	Low expression in swim-up fry (< 4 points on standard curve)	This study
VTG *(Vitellogenin)*	FJ226375	Forward: ATCAGGGAAGATGCCAAGG Reverse: CTGTGTAAGCCAGACCAAAGTC	103	Low expression in swim-up fry (< 4 points on standard curve)	Duarte-Guterman et al (in prep)
SOCS2 *(Suppressor of Cytokine Signaling 2)*	NM001114550.1	Forward: TGTAGATTCCGACGAGACT Reverse: TCATGGCAGAGATGGTGAA	178	Low expression in swim-up fry (< 4 points on standard curve)	This study
GHR1 *(Growth hormone receptor 1)*	NM001124535.1	Forward: CTTCAGACAGGAGAGGCGTA Reverse: GGACCAGAGTCACAGGGAAC	142	Poor efficiency (> 110%)	Duarte-Guterman et al (in prep)
GHR2	AY573600.2	Forward: GCTCTGGCGATCAGGATAAT	115	Poor efficiency (> 110%)	Duarte-Guterman et al (in prep)

Gene of interest	Accession	Primer Sequence (5'-3')	Product size	Reason for exclusion	Reference
(Growth hormone receptor 2)		Reverse: CCGGTTAGACCCACATTCAG			
AChE *(Acetylcholine esterase receptor)*	NM131846.2	Forward: GACCGGAGTCACGTGA Reverse: CCT GTT GGG GTT CCA CAT C	113	Poor efficiency (> 110%)	This study
HSP27 *(Heat-shock protein 27)*	FJ226381	Forward: CTGACGCTGAGAAGGTGA Reverse: TAGGGCTTGGTCTTGCTG	136	Poor efficiency (> 110%)	Veldhoen et al., 2010
CAT *(Catalase)*	FJ226382	Forward: GCCAAGGTGTTTGAGCAT Reverse: GCGTCCCTGATAAAGAAGAT	185	Poor efficiency (> 110%)	Veldhoen et al., 2010
NAChRα9-1 (Nicotinic acetylcholine receptor alpha 9-I subunit)	NM001124439.1	Forward: CCACCACAGCACCCATCAAA Reverse: CTCCTCTCTCCCGATGTGGT	187	Poor efficiency (> 110%)	This study
ARα *(Androgen receptor alpha)*	NM001124184.1	Forward: GCACACTTGGCACACTTCAG Reverse: TCCGGCAGTTCATTATGCCT	185	Multiple peaks in melt curve analysis/ Poor efficiency (> 110%)	This study
ARβ *(Androgen receptor beta)*	NM001124185.1	Forward: ACTGGAGAAACAATGGAGGACAAA Reverse: ATACCCTGCCTCGGTCCAT	167	Multiple peaks in melt curve analysis/ Poor efficiency (> 110%)	This study

Table 4-3. Primer sets used to amplify GOIs in swim-up fry (118-119 dph) and approximately 10 month old sockeye salmon. National Center for Biotechnology and Information accession identifiers, primer sequences (5' to 3'), and product size are provided for target genes that were excluded due to no signal/ amplification for standard curve in both swim-up fry and approximately 10 month old sockeye salmon. Reference indicates if primers sets used were from published literature or designed specifically for this study.

Target Gene	Accession	Primer Sequence (5'-3')	Product size	Reference
TNF-α *(Tumour necrosis factor alpha)*	NM001124357.1	Forward: CTACGGTGATGCTGAGTCCGAAA Reverse: TGCCTCTCTCTCGTCATAGTGC	179	This study
Il-6 *(Interleukin 6)*	NM001124657.1	Forward: TCAGAAGCCCGTGGAAGAGA Reverse: TTGGAGGCGGAGCAAAGAG	93	This study
Il-8 *(Interleukin 8)*	AJ279069.1	Forward: TTGAGACGGAAAGCAGACGAAT Reverse: GACCCTCTTGACCCACGGA	158	This study
Dio2 *(Deiodinase 2)*	NM001124268.1	Forward: ATCACTGGAAGAAAGGGTGG Reverse: TCTCGTTGGACACACCGTAG	128	Quesada-Garcia et al., 2014
GR1 *(Glucocorticoid receptor 1)*	Z54210	Forward: TCAGCAGTCCAAAGGCAAGAC Reverse: ACGACGATGGAGCCGAAC	377	This study

Table 4-4. RNA integrity number (RIN) values and $OD_{260/280}$ values for all biological liver samples of sockeye salmon chronically exposed to waterborne clothianidin. Treatment refers to concentration of waterborne clothianidin: water control (0 µg/L), 0.15 µg/L, 1.5 µg/L, 15 µg/L, and 150 µg/L.

Treatment	Concentration (ng/µl)	Ratio 260/280	Ratio 260/230	Total Yield (µg)	RQI
Control	103.898	1.951	0.509	5.19	9.5
Control	95.634	1.868	0.352	4.78	9.1
Control	89.993	2.009	0.793	4.50	9.1
Control	171.163	1.928	0.614	8.56	8.6
Control	82.154	1.982	0.481	4.11	9.6
Control	113.882	2.033	1.244	5.69	9.7
Control	142.376	1.997	0.545	7.12	9.2
Control	107.776	2.013	0.684	5.39	9.2
0.15	103.368	2.044	1.331	5.17	8.8
0.15	94.258	2.038	0.876	4.71	9.2
0.15	89.673	2.073	1.111	4.48	9.6
0.15	82.459	1.997	1.009	4.12	8.2
0.15	108.526	1.922	0.689	5.43	8.6
0.15	93.334	1.966	0.694	4.67	8.3
0.15	77.219	2.050	1.068	3.86	7.9
0.15	173.572	2.036	1.213	8.68	8.6
1.5	120.605	2.046	1.325	6.03	9.5
1.5	76.944	2.012	1.030	3.85	8.3
1.5	310.361	2.149	1.621	15.52	9.3
1.5	78.185	1.892	0.441	3.91	9.1
1.5	100.472	1.944	0.912	5.02	9.2
1.5	211.854	1.995	0.726	10.59	9.4
1.5	202.607	2.011	1.084	10.13	8.6
1.5	95.419	2.021	1.130	4.77	8.4
15	184.320	2.056	1.116	9.22	9.0
15	112.053	2.002	0.678	5.60	9.7
15	179.403	2.028	1.322	8.97	9.6
15	113.087	1.943	0.712	5.65	9.3
15	423.625	2.068	1.092	21.18	9.5
15	68.507	2.052	1.487	3.43	9.8
15	147.363	1.976	0.799	7.37	9.0
150	139.224	1.916	0.282	6.96	9.2
150	114.060	2.074	1.373	5.70	8.7
150	143.110	2.070	1.363	7.16	9.7
150	100.807	2.040	0.641	5.04	9.1
150	513.172	2.125	1.609	25.66	9.5
150	91.068	2.025	0.868	4.55	8.8
150	245.602	2.065	1.416	12.28	8.3

4.6. **Figures**

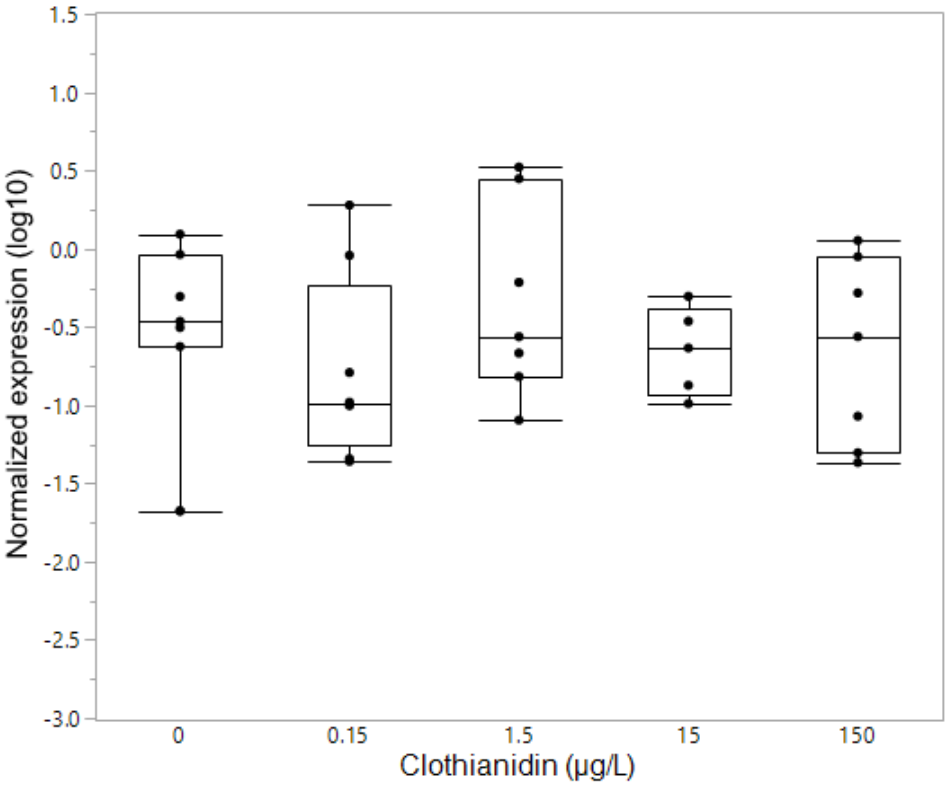

Figure 4-1. **Effects of waterborne clothianidin exposures in sockeye salmon from fertilization to swim-up fry on CYP1A hepatic gene expression. Box plots demonstrating the upper and lower quartiles (25%; whiskers) and the median values (horizontal black line within box) for each treatment group are presented: water control (n = 7), 0.15 µg/L (n = 8), 1.5 µg/L (n = 7), 15 µg/L (n = 5), and 150 µg/L (n = 7). Expression of the target gene was analyzed together with the three reference genes CBA, GAPDH, and EF1α that were used to normalize the expression level of the target gene. No significant differences were detected using a one-way ANOVA.**

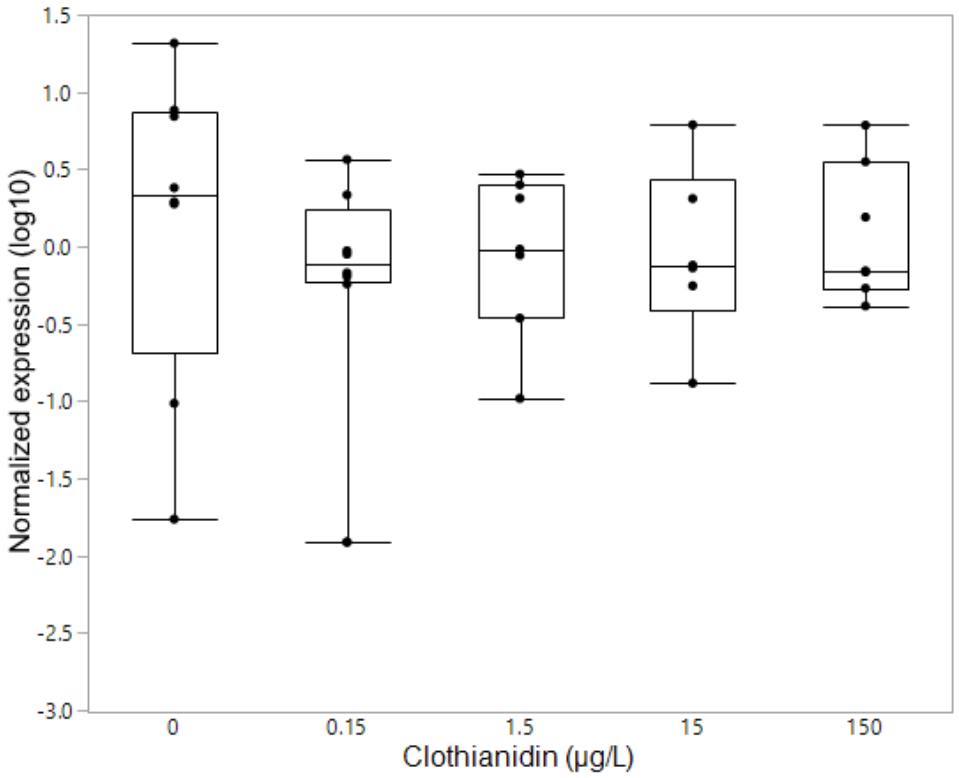

Figure 4-2. **Effects of waterborne clothianidin exposures in sockeye salmon from fertilization to swim-up fry on ERα hepatic gene expression. Box plots demonstrating the upper and lower quartiles (25%; whiskers) and the median values (horizontal black line within box) for each treatment group are presented: water control (n = 7), 0.15 µg/L (n = 8), 1.5 µg/L (n = 7), 15 µg/L (n = 6), and 150 µg/L (n = 7). Expression of the target gene was analyzed together with the three reference genes CBA, GAPDH, and EF1α that were used to normalize the expression level of the target gene. No significant differences were detected using a non-parametric analysis of variance (Wilcoxon Rank-Sums test).**

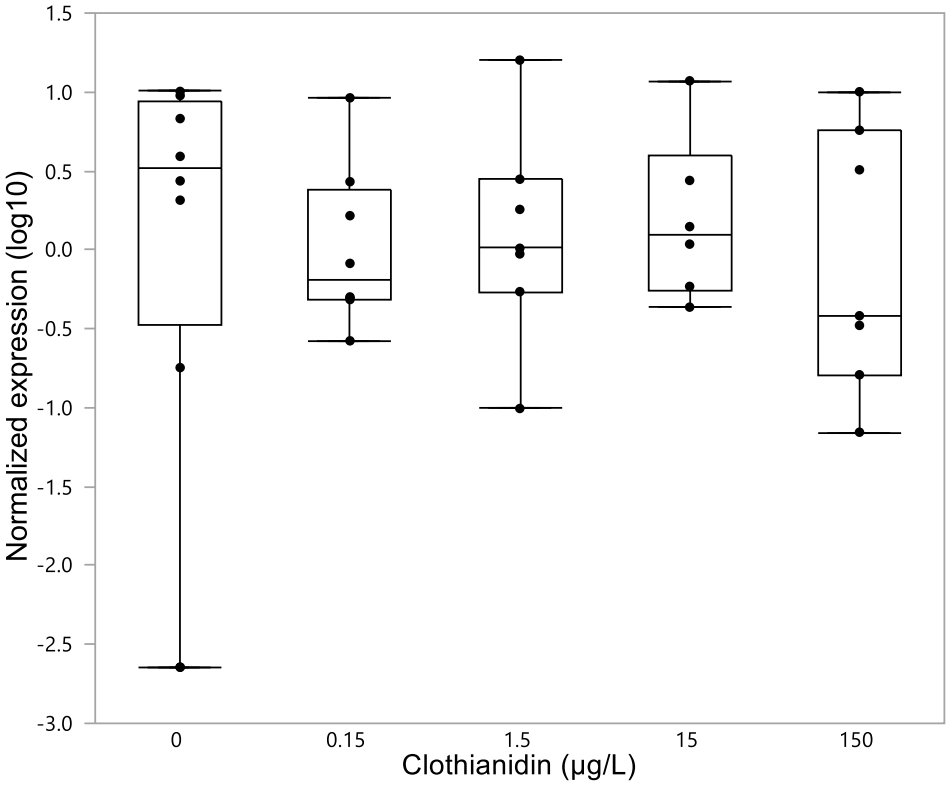

Figure 4-3. **Effects of waterborne clothianidin in sockeye salmon from fertilization to swim-up fry on ERβ2 hepatic gene expression. Box plots demonstrating the upper and lower quartiles (25%; whiskers) and the median values (horizontal black line within box) for each treatment group are presented: water control (n = 8), 0.15 µg/L (n = 8), 1.5 µg/L (n = 7), 15 µg/L (n = 6), and 150 µg/L (n = 7).. Expression of the target gene was analyzed together with the three reference genes CBA, GAPDH, and EF1α that were used to normalize the expression level of the target gene. No significant difference were detected using a non-parametric analysis of variance (Wilcoxon Rank-Sums test).**

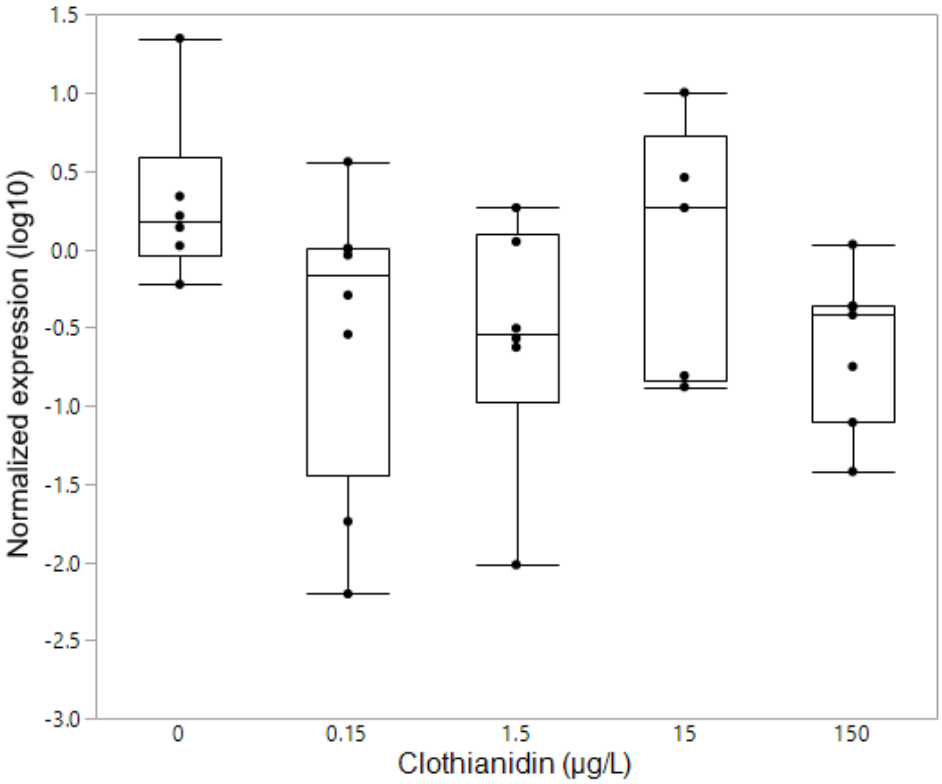

Figure 4-4. **Effects of waterborne clothianidin exposures in sockeye salmon from fertilization to swim-up fry on SOCS3 hepatic gene expression. Box plots demonstrating the upper and lower quartiles (25%; whiskers) and the median values (horizontal black line within box) for each treatment group are presented: water control (n = 6), 0.15 µg/L (n = 8), 1.5 µg/L (n = 6), 15 µg/L (n = 5), and 150 µg/L (n = 7). Expression of the target gene was analyzed together with the three reference genes CBA, GAPDH, and EF1α in order to normalize the expression level of the target gene (normalized gene expression). No significant difference were detected using one-way ANOVA.**

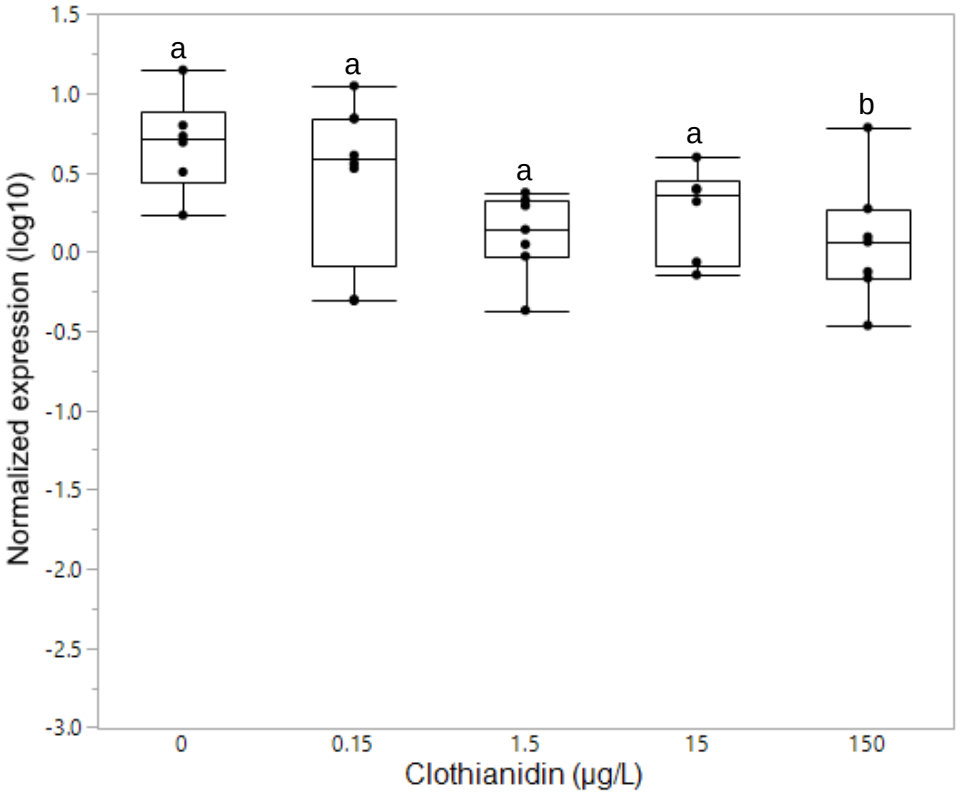

Figure 4-5. **Effects of waterborne clothianidin exposures in sockeye salmon from fertilization to swim-up fry on GR2 hepatic gene expression. Box plots demonstrating the upper and lower quartiles (25%; whiskers) and the median values (horizontal black line within box) for each treatment group are presented: water control (n = 6), 0.15 µg/L (n = 8), 1.5 µg/L (n = 7), 15 µg/L (n = 6), and 150 µg/L (n = 7). Expression of the target gene was analyzed together with the three reference genes CBA, GAPDH, and EF1α in order to normalize the expression level of the target gene (normalized gene expression). Different letters indicate significant differences between treatments (one-way ANOVA; P < 0.05).**

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