Effects of thiamethoxam and neonicotinoid mixtures on early life stages of wild sockeye salmon *(Oncorhynchus nerka)*

by

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Declaration of Committee

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Abstract

This is the first study to report the acute and chronic effects of environmentally relevant concentrations of thiamethoxam (ranging from 0.15-150 µg/L) and a mixture of clothianidin, imidacloprid, and thiamethoxam neonicotinoid pesticides (ranging from 0.045-450 µg/L) on individuals from one wild stock of British Columbia sockeye salmon. No effects were observed on growth, development, and a targeted suite of ten genes involved in reproduction, growth, stress response, nervous and immune system function after chronic thiamethoxam or neonicotinoid mixture exposures. However, acute thiamethoxam exposure during fertilization showed reproductive toxicity via a 25% reduction in fertilization success, and subsequent teratogenic effects via abnormal length, weight, and condition factor in swim-up fry in all thiamethoxam and some of the neonicotinoid mixture concentrations tested. These findings could have potential implications for wild salmon populations as pulse exposures are environmentally relevant, especially given the amount agriculture and rainfall in the lower Fraser River in British Columbia, Canada.

Keywords: neonicotinoid mixture; clothianidin; thiamethoxam; imidacloprid; sockeye salmon; growth; fertilization; development; teratogenic; endocrine axis; hepatic gene expression

Dedication

To my mom, Gayle, and sister, Darcy for their continued support and encouragement; to my daughter, Priscilla who makes me strive to be a better person every single day; and to me, for actually getting through this journey!

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List of Acronyms

ºC	Degrees Celsius
%CV	Percent coefficient of variability
µg/L	Micrograms per litre
µL	Microlitre
AChE	Acetylcholine esterase
ANOVA	Analysis of variance
BC	British Columbia
BC ENV	British Columbia Ministry of the Environment
CCME	Canadian Council of Ministers of the Environment
cDNA	Complimentary deoxyribonucleic acid
CRD	Complete Random Design
Cq	Quantification cycle
CYP	Cytochrome p450
dpf	Days post fertilization
DNA	Deoxyribonucleic acid
EC_{50}	Median effective concentration
Estradiol	Estradiol-17β
ELISA	Enzyme-linked immunoassay
g/L	Grams per litre
GOI	Gene of interest
IUCN	International Union for Conservation of Natural Resources
Kg	kilogram
M	Molar
MIQE	Minimum information for publication of quantitative PCR experiments
mg/L	Milligram per litre
mL	Millilitre
mm	Millimetre
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
nAChR	Nicotinic acetylcholine receptor
ng/L	Nanograms per litre
nm	Nanometre

NRT	no reverse transcriptase
NTC	No template control
$OD_{260/230;}$ $OD_{260/280}$	Optical density 260/230 or 260/280
RIN	RNA integrity value
PMRA	Pesticide Management Regulatory Agency
QAQC	Quality assurance quality control
qPCR	Quantitative polymerase chain reaction
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SE	Standard error
SFU	Simon Fraser University
US EPA	United States Environmental Protection Agency
VTG	Vitellogenin

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
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
EC_{50}	The concentration of test substance that results in a 50 percent reduction
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
Pesticide	A product that is manufactured and sold for means to directly or indirectly control, mitigate or destroy any pest
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)
SP-RCB	Split-plot randomized complete block (design)
Swim-up	An early-life stage where 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.

1 General Introduction

1.1 Sockeye Salmon

Sockeye salmon (*Oncorhynchus nerka*) are a Pacific salmonid species with populations found along the west coast of North America in the Pacific Ocean (Fisheries and Oceans Canada 2020), with a range that extends from northern California to northern Alaska and eastward into parts Russia (Rand 2011; Rand et al. 2012). The anadromous life history of sockeye salmon includes a freshwater stage where eggs are laid in redds in rivers, streams, and lakes and fertilized by males via external fertilization (Rand 2011; Rand et al. 2012; Fisheries and Oceans Canada 2020). These then hatch into alevins and grow and develop until complete yolk sac absorption, and then begin feeding on stream invertebrates and zooplankton, and generally rear in these freshwater conditions for one to three years before finally migrating at the smolt stage to the ocean (Rand 2011; Rand et al. 2012). Typically, sockeye salmon remain in the ocean two to three years until they reach 2.5 to 3 kg and 50 to 60 cm feeding on zooplankton, smaller fishes, and squids (Rand et al. 2012). These ocean dwelling sexually mature individuals then return to their natal stream in the late summer/fall and spawn, with both sexes competing for redds followed by external fertilization, and exhibit fecundity rates of approximately 2,000 to 5,000 eggs (Rand 2011). After spawning both sexes die, thus their carcasses are critical sources of nutrients and energy for the aquatic ecosystems they inhabit (Rand 2011; Rand et al. 2012).

Scale and geography are factors for the status of salmon populations; globally sockeye salmon populations appear stable but numerous sub-populations in Canada are in grave decline and have been for decades. The International Union for Conservation of Natural Resources (IUCN) is the international organization that is the global authority on the status of the world's natural resources (IUCN 2022). The IUCN has a *red list,* which is a ranking classification system for a species at risk of extinction. Salmon risk classification is determined both qualitatively and quantitatively. The quantitative classification is determined by the median rate of change at salmon spawning sites, with the following categories and criteria: *data deficient* = analysis not competed; *least concern* = populations increasing in abundance or stable; *near threatened* = close to being endangered; *vulnerable* species = 30 to 50% decline; *endangered* = 50 to 80%; and,

critically endangered = >80% or the species is threatened with global extinction. On the global species/population level, the IUCN reported that approximately 7% of the historical range of sockeye have been lost due to localized extinction events and that sockeye status is considered *least concern*, meaning not endangered and stable; however, some of the 48 British Columbia (BC) sub-populations are considered both *critically endangered*, *endangered*, *vulnerable*, *data deficient*, or *least concern*, depending on region (Rand 2011; Rand et al. 2012). The IUCN lists 3 main sources of threats to global Sockeye populations that include: (1) overfishing; (2) changes to ocean and river conditions, including temperature changes and prevalence of diseases; and (3) adverse effects from hatchery construction with other potential threats, including unknown threats to the BC sub-populations (Rand 2011). However, more recent reviews have further specified that salmon habitat continues to be depleted due to several stressors, including habitat loss, climate change, and pollutants (Hodgson et al. 2019). Indeed, anthropogenic contaminants have been identified as one of the planet's greatest threats to natural ecosystems (Landrigan et al. 2018), including to juvenile salmon health and survival (Hodgson et al. 2019)

1.2 Fraser River Sockeye Salmon

The Fraser River watershed, located in the Fraser basin, BC, consists of the Upper, Middle, and Lower Fraser watersheds (Figure 1-1; FBC 2013). The Lower Fraser watershed runs through highly populated areas and thus influenced by human activities. The Fraser River is the longest river in BC, spanning 1,600 km from the western side of Rockies at Mount Robson to the Georgia Strait, collecting 223,000 km^2 of water, annually. The Fraser River is one of the most abundant salmon rivers, globally, with approximately over 250 million sockeye that enter the Georgia Strait during the spring migration to the sea (Noakes 2011; Cohen 2012).

Along with intrinsic values of Fraser River salmonid species, salmon are not only ecologically important, but also economically and culturally important to Indigenous Peoples and Canadians. Ecologically, given the salmonid anadromous lifecycle, they are an important source of nutrients for the aquatic and terrestrial environments (Cederholm et al. 2000; Hildebrand et al. 2004). Decomposing carcasses and eggs provide carbon, nitrogen and phosphorus inputs that are important for ecosystem functioning (Cederholm et al. 2000; Hildebrand et al. 2004). Salmon are also a food source for numerous terrestrial

and aquatic wildlife (e.g., bears, raptors, marine birds; Cederholm et al. 2000; Hildebrand et al. 2004). Indeed, it has been estimated that in BC approximately 140 species rely on salmon as a food source (Hilderbrand et al. 1999; Cederholm et al. 2000; Hilderbrand et al. 2004). For example, Hilderbrand et al. (1999) reported that the salmon availability was positively correlated with productive grizzly populations, as demonstrated through carbon and nitrogen stable isotope analysis of grizzly bear hair. In the marine environment, Ayres et al. (2012) and Wasser et al. (2017) reported that the decline of salmon had directly impacted population crashes of the Southern resident killer whale (*Orcinus orca*) since the late 1990s via analyses of glucocorticoid and thyroid hormones metabolites present in feces that indicate nutritional deficiencies known to disrupt fecundity. These are just two of the many examples of the interconnectedness of the marine water, freshwater, and terrestrial environments, and reliance on salmon. With respect to the cultural and economic significance, Fraser River salmon historically and currently sustain Indigenous Peoples as both a food source and for cultural purposes (Garner and Parfitt 2006). In addition, a commercial Fraser River sockeye salmon fishery has existed for decades and is also important for both the provincial BC and national Canadian economy. In 2018, the annual sockeye harvest in BC accounted for 110,400 tonnes, which was 65% of all wild salmon harvested in the province (BC Gov 2020) This translated to a wholesale value of $151.7 million (also 65% of the share for wild salmon), and $56.1 million for international exports (BC Gov 2020). The bulk of sockeye exports were sold to Japan (BC Gov 2020). In 2016, it was estimated that the fisheries aquaculture sectors combined accounted for 15,000 jobs (BC Gov 2016). Ultimately, Fraser River salmonids are iconic species integral to the health of the Fraser River watershed and to humans relying on these fish for sustenance as well as cultural and economic purposes.

Given the significance of sockeye, it is important to understand the local conservation status along with threats to local populations. There are 22 sub-populations of Sockeye identified in the Fraser River basin (Rand 2011). Seven of those sub-populations are considered Endangered by the IUCN, eleven are considered least concern, and four are data deficient (Rand 2011). Cohen (2012) reported that Fraser River sockeye populations have been on a decline since the early 1990s as a result from the many likely threats described in several publications such as habitat destruction, overfishing, and potential exposure to environmental contaminants, such as agricultural pesticides (Rand 2011; Landrigan et al 2018; and Hodgson et al. 2019. With respect to

Fraser River sockeye salmon and the Lower Fraser River in particular, several of these threats related to habitat and contaminants are evident as this region of the river cuts through prime agricultural land. In the Fraser Valley alone, there are more than 2,500 farms that produce 50% of the provinces farm income (FVRD 2016), totalling $1.5 billion provincial farm receipts (Statistics Canada 2016). Given yield requirements to make farming economically viable, pesticide use is inevitable. However, the province (and Canada) does not regulate pesticide use, rather it monitors pesticide sales. During the last reporting period in 2015, BC ENV (2016) reported that BC vendors sold a total of 3,030,410 kg of commercial formulations of pesticides, which totaled 1,436,275 kg of active ingredient, with 1,212,512 kg sold to the agriculture sector, which represents 84% of all pesticide sales (it is important to note that BC ENV classifies aquaculture in with agriculture). Given the quantity of pesticides sold and the high rainfall in BC, equalling approximately 1,600 mm rainfall per year in Chilliwack (GoC 2022), pesticide runoff and pesticide leaching to groundwater discharge in surface waters is also inevitable and is documented (e.g., Main et al. 2014; Morrissey et al. 2015).

1.3 Neonicotinoids

The government of Canada defines pesticides as “any product, device, organism, substance or thing that is manufactured, represented, sold or used as a means for directly or indirectly controlling, preventing, destroying, mitigating, attracting or repelling any pest” (GoC 2007). While pesticides are beneficial for controlling pests in agricultural settings, many have been shown disrupt the environment by adversely impacting non-target species. Indeed, pesticides are managed and regulated in Canada by the Pesticide Management Regularity Agency (PMRA) that is governed by Health Canada under the Pest Control Products Act (PCPA) and the Canadian Environmental Protection Act (GoC 2009). Insecticides are one type of pesticide specifically designed to target insects, and currently the most frequently used class of insecticides in the world are the neonicotinoids. These insecticides were first developed in the 1980s and registered in the 1990s (Jeschke and Nauen 2008) and are now registered in over 120 countries and are routinely used worldwide on several agricultural crops (Jeschke et al. 2011; Van Dijk et al. 2013; Simon-Delso, 2014; Bonmatin et al. 2015). Neonicotinoids were developed in part to replace several classes of insecticides such as the organophosphorus and carbamate pesticides that were thought be causing a pest resistance (Anderson et al. 2015; Morrissey et al.

2015), childhood neural development issues (Morrissey et al. 2015; Eskenazi et al. 1999), and toxicity to non-target wildlife (Botha et al. 2015). In BC, neonicotinoids account for 1% or 1,511 kg active ingredient (AI) of pesticide sales (BC ENV 2016). Three common neonicotinoids used in Canada include the first generation, imidacloprid (accounting 1,156 active ingredient [AI] sales), and second generations clothianidin (accounting for 326 kg AI) and thiamethoxam (accounting for 29 kg AI sales) (BC ENV 2016). All three of these neonicotinoids are used on variety of crops such as potatoes (*Solanum tuberosum*), blueberries (*Vaccinium spp*.), raspberries (*Rubus spp*.), cranberries (*V. macrocarpon*), corn (*Zea mays*), alfalfa (*Medicago sativa*), an assortment of vegetables, hay, and other livestock crops (BC ENV 2016). Application of neonicotinoids are typically via seed coat and foliar spray in order to protect seedlings (Jeschke et a. 2011; Goulson 2013; Morrisey et al. 2015), with seed treatments thought to account for 60% of the global application rate (Bonmatin et al. 2015 and Jeschke et al. 2011).

Neonicotinoids structurally resemble nicotine and exhibit the same mode of action as nicotine by binding agonistically to nicotinic acetyl choline receptors (nAChRs) in the nervous system. These insecticides are classified in the N-nitroguanidine group and are considered broad spectrum, systemic, neurotoxicants developed for controlling piercing sucking insects (e.g., aphids, leafhoppers, phytophagous mites; Jeschke et al. 2011; Morrisey et al. 2015; Simon-Delso et al. 2015). When an invertebrate is exposed to a neonicotinoid via piercing/sucking/chewing a plant, the neonicotinoid competes with acetylcholine, a neurotransmitter and endogenous ligand of nAChRs (Nauen 1999). Neonicotinoids are more toxic to invertebrates relative to vertebrates because neonicotinoids have greater affinity for invertebrate nAChRs (Matsuda et al. 2001 and Matsuda et al. 2005). For insects, under normal conditions, acetylcholine binds to the postsynaptic nAChR in the central nervous system where a high density of nAChRs are located; whereas for vertebrates, acetylcholine binds to the postsynaptic nAChR in the central and peripheral nervous systems (Matsuda et al. 2001; Matsuda et al. 2005). Acetylcholinesterase then breakdowns the acetylcholine molecules after dissociation from nAChRs into acetyl CoA and choline; thus, nerve transmission/muscle contraction ceases (Simon-Delso et al. 2015). However, neonicotinoids agonistically bind to invertebrate postsynaptic nAChR in the central nervous system; therefore, resulting in competition with acetyl choline (Thany 2010 and Simon-Delso et al. 2015). Acetylcholinesterase is unable to breakdown neonicotinoids that results in contiguous activation of the nAChR (Thany

2010; Morrissey et al. 2015; and Simon-Delso et al. 2015). Thus, this continuous activation of the central nervous system by neonicotinoids results in paralysis and ultimately death for invertebrates (Goulson 2013; Simon-Delso et al. 2015).

Neonicotinoids can enter surface waters after applications to agricultural lands by many routes such as farm run-off, volatilization and deposition, leaching, etc. For example, thirty studies across nine countries (including Canada) found surface water neonicotinoid concentrations ranging from 0.001 to 320 µg/L, strongly suggesting the prevalence of this neurotoxic insecticide in numerous aquatic ecosystems (Bishop et al 2018; Crayton et al. 2020; Elbert et al. 2008; Main et al. 2014; Morrissey et al 2015; Schaafsma et al. 2015; Yamamoto et al. 2012). With detectable levels of neonicotinoids in the environment reported in several jurisdictions, concerns regarding the adverse effects on non-target wildlife of these neurotoxic insecticides have resulted in several government regulatory agencies worldwide re-evaluating their safety. In Canada, in 2012 the PMRA within Health Canada began an evaluation of the safety of neonicotinoids (GoC 2020). By 2019, the PMRA published decisions on risks of the three neonicotinoids registered in Canada (imidacloprid, clothianidin, and thiamethoxam) to pollinators resulting in some changes in use of these insecticides to minimize exposure to pollinators. The PMRA also recently announced that neonicotinoids are being measured in the environment at levels that are harmful to aquatic insects, and in 2018 proposed a phase-out of all agricultural and the majority of outdoor uses for imidacloprid however, re-evaluations of clothianidin and thiamethoxam are still under way as of 2022 (GoC 2020). Several studies have demonstrated adverse effects from neonicotinoids on honeybee populations (e.g., Straub et al. 2019 and Christen et al. 2016), aquatic invertebrate communities (e.g., Morrissey et al. 2015 and Cavallaro et al. 2019), and several vertebrate taxa including birds, rodents, fish, and amphibians (Gibbons et al. 2015; Morrissey et al. 2015; Marlatt et al. 2019; Danis and Marlatt 2021). For example, Danis and Marlatt (2021) demonstrated that larval salamanders chronically exposed to 100 µg/L of imidacloprid resulted in advanced development, which suggests these pesticides may be disrupting the thyroid endocrine axis. The US EPA (2010) showed a decrease in mean body weight and length of early life-stage fathead minnow after exposure to 0.02 µg/L of clothianidin, further supporting impacts on growth that are also influenced by the thyroid endocrine axis. Interestingly, Marlatt et al. (2019) demonstrated that growth was not affected in early life-stage sockeye salmon that were exposed to 0.15 µg/L of clothianidin, however, elevated whole body 17β-

estradiol occurred indicative of disruption of the reproductive endocrine axis (Marlatt et al. 2019). Several studies have also evaluated the effects of neonicotinoids in birds and demonstrated impairments on endpoints related to migration (behavior and body condition) and reproduction (i.e., fertilization success, egg size, eggshell thickness, embryo viability, hatching rate, and offspring survival; Eng et al. 2019, 2017; Pisa et al. 2017; reviewed by Gibbons et al. 2015). Collectively, these studies in vertebrates reveal sub-lethal impacts of neonicotinoids in several species beyond the known neurotoxic mode of action that warrant further investigation.

Research Objectives

Examining the direct adverse sub-lethal effects of individual and mixtures of the three neonicotinoids registered for use in Canada on early life stages of Fraser River sockeye salmon is necessary to better understand the impacts of these common environmental contaminants on salmonids native to the Fraser River. Currently no studies are available reporting the toxicity of thiamethoxam or mixtures of imidacloprid, clothianidin, and thiamethoxam in wild salmonids. Previous studies have demonstrated no effects of clothianidin alone on growth and development in early life stage wild collected sockeye, but a significant 4.7-fold increase in whole body 17β-estradiol levels in swim-up fry after exposure to 0.15 µg/L. To further investigate the impacts of the three main neonicotinoids used in Canada on a wild sockeye salmon, the present study examined the sublethal, chronic effects of thiamethoxam and a mixture of imidacloprid, clothianidin and thiamethoxam on critical early life stages of a wild salmon species, sockeye salmon (*Oncorhynchus nerka*). The study design entailed exposures during fertilization and exposures from 1-hour post-fertilization to swim-up or feeding fry stages. In this study, four to five concentrations of neonicotinoids (0.15, 1.5, 15 and 150 µg/L [2015 study] and 0.045, 0.45, 4.5, 45, and 450 µg/L [2016 study]) plus water controls were tested. These concentrations of neonicotinoids were selected based on the concentrations of neonicotinoids reported for surface waters globally and to incorporate the only neonicotinoid Canadian Water Quality Guideline of 0.23 µg/L for imidacloprid. Since wild salmon are not routinely studied, this research also examined the influence of parentage on toxicant responses by testing four unique offspring sets (crosses) in all experiments. The endpoints measured to assess the adverse effects of thiamethoxam and the mixture of neonicotinoids in developing wild sockeye salmon included survival and several sub-

lethal endpoints, specifically, growth, hatching, emergence and sex steroid hormone levels.

1.4 Figures

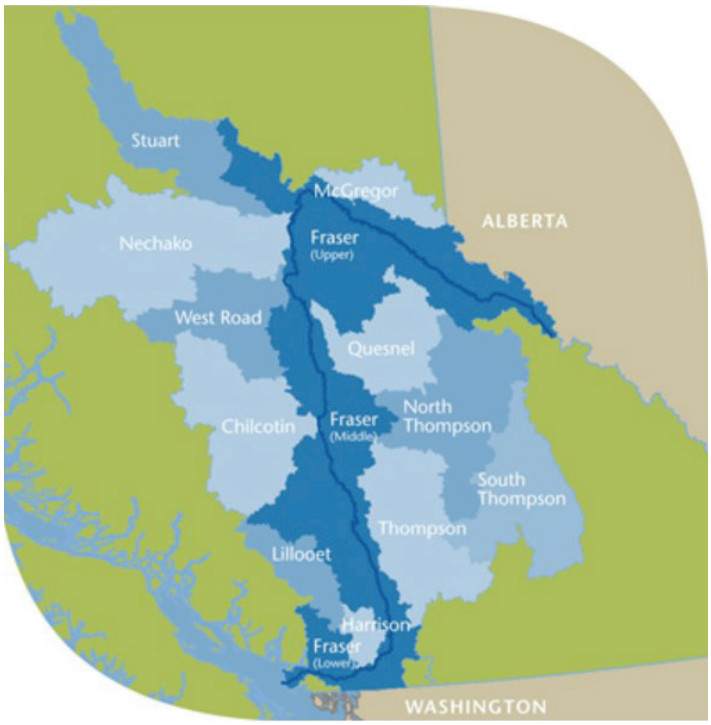


Figure 1-1. The twelve Fraser Basin watersheds that make up the Upper, Middle, and Lower Fraser basin. All water from the Fraser watershed drains into the Fraser River that begins at Mount Robson (Valemount, BC) and terminates in the Georgia Strait in Richmond, BC. Figure obtained from https://www.fraserbasin.bc.ca/basin_watersheds.html.

2 2015 Thiamethoxam Exposures

2.1 Introduction

The 2015 neonicotinoid study included two chronic exposures in glass tanks and gravel bed flumes. For both exposure scenarios four genetic crosses (crosses A through D) of Sockeye were exposed to thiamethoxam from one-hour post-fertilization to swim-up fry life stage. The objective of chronic exposures was to evaluate adverse effects of thiamethoxam, on sockeye salmon, at environmentally relevant concentrations and to determine if parentage or exposure scenario influenced these effects.

2.2 Methods

Cody Antos (technician) and Dr. Vicki Marlatt (principal investigator) conducted this experiment in 2015. In 2016, Debby Reeves assumed all data collected from this experiment, performed all data entry and statistical analyses. Detailed aquatic exposure methods are described in Leung (2018) and Marlatt et al. (2019) and summarized in the following sections.

2.2.1 Chemicals

Thiamethoxam stock solution was prepared using 98.0% pure thiamethoxam (CAS#: 153719-23-4, Sigma-Aldrich, Oakville, Ontario, Canada). Fish euthanasia was conducted using tricaine methanesulfonate (MS-222) and Ovadine was used for disinfection of tools in contact with fish, and were purchased from Syndel Laboratories LTD., Nanaimo, B.C., Canada. All other chemicals / reagents are identified in the respective sections and were sourced from commercial facilities and of analytical grade, as described below.

2.2.2 Gamete Collection and Fertilization

In the fall of 2015, four wild, sexually mature mating pairs of sockeye salmon (*Oncorhynchus* Nerka) were captured and donated by the Department of Fisheries and Oceans (DFO) from the Inch Creek Hatchery, located in Dewdney, BC. Approximately 2,000 to 3,000 eggs were harvested from each female and approximately 1 to 3 ml of milt

were collected from each male. The gametes were immediately stored and shipped at 6 to 10°C and delivered to Simon Fraser University, Burnaby, BC, on 8 September 2015. Dry fertilization was performed by Dr. Vicki Marlatt within six hours of collection where four unique offspring sets were created from the four mating pairs (referred to crosses A, B, C, D). For each of the four crosses, this was achieved by combining the eggs from one female and milt from one male in 1.5 L of dechlorinated water (10 ± 1 °C) followed by gentle swirling to ensure mixing. After 1 hour, the water hardened eggs were carefully transferred and subdivided into three separate PVC netted cylindrical egg containers, per genetic cross (labelled A, B, C, D), and totalled approximately 100 individuals per cross per tank.

2.2.3 Thiamethoxam Chronic Exposures in Glass Tanks and a Gravel Bed Flume System

This thiamethoxam exposure experiment was divided in to two exposure scenarios that included (1) a glass tank flow-through system and (2) a gravel-bed flume system that mimicked a natural streambed environment. In both exposure scenarios, all four genetic crosses were exposed in duplicate test vessels, to nominal concentrations of 0, 0.15, 1.5, 15, and 150 μg/L of thiamethoxam. A schematic of the design is shown in Figure 2-1. These concentrations were selected based on the concentrations of thiamethoxam and other neonicotinoids (imidacloprid and clothianidin) reported in global surface waters (e.g., 0.0035 to 320 μg/L; CCME 2007; Crayton et al. 2020; de Perre et al. 2015; Hladik et al. 2014; Main et al. 2014; Miles et al. 2017; Morrissey et al. 2015; Sánchez-bayo and Hyne 2014; Schaafsma et al. 2015; Starner and Goh 2012; Van Dijk et al. 2013), and to incorporate testing above and below the only neonicotinoid Canadian Water Quality Guideline of 0.23 μg/L for imidacloprid (CCME 2007).

Exposure methods outlined in the GoC (1998) Guidance, for Biological Toxicity Test Methods for Early Life Stages of Salmonids were followed with minor deviations, given Sockeye are not a typical test species in this guidance. The following outlines the methods and parameters (i.e., test conditions, water quality monitoring, termination, and test solution renewals) that were followed across both exposure scenarios, per the guidance. Both test vessel system exposures were initiated from one to three hours post-fertilization. Embryos were maintained in darkness, observed only with a red light, until 90 to 100% of the control fish in the glass tanks hatched. Approximately one week after

hatching, the alevins where then reared in a 16 hour/8-hour light/dark photoperiod. Water quality monitoring for temperature, dissolved oxygen concentration, pH, and conductivity was measured every 48 hours using an HQd Portable Meter (Hach® Company, Loveland, CO, USA) for most parameters. Ammonia concentrations were measured every two weeks using a Seachem® MultiTest Ammonia Test Kit (Seachem Laboratories, Madison, USA). Daily health monitoring included removal of dead embryos, alevins, and / or fry. Termination commenced upon fish reaching the swim up fry developmental stage (defined as 80 % of control animals exhibiting vertical swimming behavior and yolk sac resorption). This resulted in termination at 125 to 128 days post fertilization (dpf) for the glass tank exposure and 112 to 119 dpf for the flume exposure.

The thiamethoxam stock solution renewal deviated from the standard guidance outlined in GoC (1998). Stock solutions were freshly prepared every 48 hours based on Marlatt et al. (2019) previous laboratory-based studies indicating minimal degradation of neonicotinoids over 48 h flow through exposures. Thiamethoxam is soluble in water (4.1 g/L at 25^{o}C; MacBean 2010); therefore, stock solution was prepared by adding 0.2 g of Sigma-Aldrich thiamethoxam to 4 L of dechlorinated municipal water, which was mixed for 60 minutes to ensure the full solubility of thiamethoxam. The stock solution was then diluted with dechlorinated water to achieve the afore mentioned nominal concentrations, then distributed to the appropriate glass tanks or flume-system via a Masterflex® peristaltic pump using Masterflex® and food grade Tygon® silicone tubing at a rate of 2.0 ml/minute. The nominal concentrations of thiamethoxam were achieved using a water flow rate of 95 ml / minute and a pesticide stock solution flow rate of 2.0 ml / minute that were monitored (and adjusted if required) every 48 hours until termination. Measured concentrations of thiamethoxam were conducted by Dr. Chris Metcalfe (Trent University, Ontario, Canada) 70 dpf in one replicate per concentration of both glass tank and flume exposure scenarios as described by Liu (2018) and Marlatt et al. (2019).

The glass tank test apparatus included a flow-through system with ten glass tanks (four concentrations of thiamethoxam and a water control in duplicate) that contained a total volume of 28 L, with the dimensions of 22 cm x 26 cm x 52 cm (height, width, length, respectively), and a drainage hole at the 20 cm mark. Each glass tank contained three netted cylindrical egg containers (made up of food grade polyvinyl chloride [PVC]), per genetic cross (i.e., for 4 crosses there was a total of 12 egg containers per tank) with approximately 100 eggs evenly divided (i.e., 33 to 34 fertilized eggs per egg container).

Fish were separated into egg containers to maintain separation of the genetic crosses, monitor development, and for ease of removing dead individuals throughout the experiment. All tanks were randomly placed at opposite sides of a temperature-controlled room. Above the glass tanks included an overhead water storage tank to maintain consistent pressure of the system, which fed all the glass tanks with dechlorinated municipal water.

The gravel bed flume system deviated from the Government of Canada (1998) guidance by not utilizing standard glass aquaria, but still adheres the recommendations in terms of loading density and water quality. The aim of the gravel bed flume system was to mimic a natural stream, with the details as follows. The flume was a custom-built system adapted from a design from Pilgrim et al. (2013) and described in Du Gas et al. (2014). Briefly, the flume was 250 cm (l) x 40 cm (w) x 32 cm (h) and was divided evenly into 5 isolated chambers that could hold 64 L of water each. Each chamber was then subdivided into five more sections by stainless steel mesh that allowed the pesticide solution or water to flow throughout the system, while keeping the genetic crosses isolated from one another. Each chamber shared the same water; however, the outflow was in the middle chamber (that did not contain fish) and the outer two sub-sections housed the developing fish, with each of the 4 chambers housing a different genetic cross (i.e., four different genetic crosses). Gravel that consisted of rocks with a diameter of 10 mm and 25 mm were evenly distributed in each chamber with a 1:1 ratio (to mimic a natural environment per Kondolf and Wolman [1993]) to an initial depth of 5 cm. Similar to the glass tank exposure, 100 embryos from one genetic cross were evenly divided between three egg containers (n = 33 to 34 individuals per egg container) that were then placed in the gravel substrate. Once 92 to 100% of the embryos developed eyes in the control groups (i.e., on 28 dpf), the eyed embryos were gently poured out on top of the substrate and then buried with an additional 10 cm of gravel, totalling a height of 15 cm. Given these exposure conditions, fish stay submerged in the substrate until their yolk-sac was resorbed and they reach the swim-up stage; however, a consequence of this is that development and survival could not be determined with precise accuracy throughout the exposure and those parameters were determined on termination day.

2.2.4 Termination, Measurement of Survival, Morphometrics, and Deformities

Euthanasia was performed in compliance outlined in Canadian Council for Animal Care guidelines and with a permit issued by Simon Fraser University Animal Care Committee (Burnaby, BC, Canada). Briefly, individual swim-up fry were humanely euthanized in dechlorinated water and 0.4 g/L MS-222 and buffered with sodium bicarbonate (Sigma Aldrich, Oakville, ON, Canada) to pH 7.0 to 7.5. Upon termination, length, wet body weight, and presence of deformities were recorded. Craniofacial, skeletal and fin deformities were assessed according to Rudolph et al. (2008). At random, 20 whole bodies and 8 livers per tank or flume, per genetic cross, were harvested, placed in DNase/RNase free 2 ml Eppendorf tubes (whole bodies) or DNase/RNase free 1.5 ml Safe Lock Eppendorf tubes (livers), and flash-frozen on dry ice, prior to being transferred to a -80°C freezer for long-term storage. Stainless steel instruments used to harvest the liver tissue were cleaned between each animal, using an ultrapure water rinse then a 10% peroxide rinse followed by a final rinse with ultrapure water to ensure there was no cross contamination and the tools were RNase/DNase free.

Several endpoints to determine the effects of thiamethoxam were investigated in this study including fertilization, hatch, and survival success, growth, and frequency of deformities. Fertilization, hatch, and survival success were analyzed as percentages and determined by the number of live eyed embryos (i.e., fertilization success), followed by the number of eyed embryos that successfully hatched (i.e., hatch success), and by the number of fish that successfully hatched and survived until termination day (i.e., survival). Endpoints that required euthanasia / post-mortem analysis include morphometrics and presence of deformities. Methods for these endpoints include placing live fish in a clear observation vessel to briefly monitor for physical deformities immediately prior to euthanasia. After euthanasia deformities were identified using a graduated severity index (GSI) and stereomicroscope as outlined in Holm et al. (2005) and Rudolph et al. (2008). Deformity analysis included four main categories as follows: craniofacial (i.e., assessment of eyes, jaws, and head), edema (i.e., the presence of an accumulation of fluid around the head or pericardial cavity), finfold (i.e., size, shape, and presence of all fins), and skeletal (i.e., presence of a lordotic, kyphotic, scoliotic curvature to the spine). Deformities were ranked zero to three based on a severity scale, where zero was equal to no deformities / abnormalities and three was equal to severe deformities. Fish body weight (measured in

grams), length (snout to fork length, measured in mm) was recorded. Fulton's condition factor (K) was determined by multiplying the individual's wet weight (in grams) by 100, divided by the cubed length (in cm), which infers the overall body condition, and is simplified in Equation 2-1 as follows:

$$K = \frac{100W}{L^3} \qquad (2\text{-}1)$$

Where:

K = Fulton's Condition Factor

W = Wet weight (g)

L = Length (cm)

Quality assurance quality control measures were performed regularly through out the termination and included the following: Deformity analysis training was provided by Dr Marlatt for the technicians who were euthanizing the fish. The same technicians ranked the deformities throughout the entire termination. Validation for deformity ranking and length measurements was conducted by having the original technician record their results for an individual fish. The alternate technician would score the same induvial fish, and the results would be compared to confirm they arrived at the same conclusions. This process was repeated at regular intervals throughout the entire termination.

2.2.5 Statistical Analyses

To test the effects of the chronic thiamethoxam exposure, a split-plot randomized complete block (SP-RCB) design was used for all analysis. The exposure scenario included a complete block design with an n of 2 (i.e., 2 replicate tanks per treatment in the glass tank or gravel bed flume experiments). Details of the design include the thiamethoxam concentration that was considered the main plot with all four concentrations plus the control (i.e., 0, 0.15, 1.5, 15, and 150 µg/L), blocked by which side of the room the glass tanks were located (i.e., the left side and right side of the room). Within the main plot (the glass tanks or flumes), was the sub-plot that consisted of the different genetic crosses (i.e., crosses A, B, C and D; Figure 2-1). Given there were two blocks located on either side of the room, the block design can capture any effects regarding the side of the room the tanks were located (i.e.,

differing light or air ventilation). Also, given the flow-through exposure scenario, water parameters slightly varied between each tank/heath stack (the main plots); as such, the split plot design can capture any statistical differences in the main plot between the genetic crosses in the slightly different exposure scenarios between tanks (Figure 2-1).

All statistical analyses were performed using JMP®, Version *15* (SAS Institute Inc., Cary, NC). Given the experimental design for the chronic exposure, effects between genetic crosses from the different concentrations of neonicotinoids were examined to determine cross-specific effects on fertilization, hatching, survival, morphometrics, and deformities as well as thiamethoxam-specific effects on these endpoints. As such, a SP-RCB analysis of variance (ANOVA), followed by a Tukey's post hoc test or Student's T test (when all or two genetic crosses were considered, respectively; $p < 0.05$) was used for data that met parametric assumptions. For data that did not meet the assumptions of normality and homogeneity of variance, a Kruskal-Wallis test followed by a Mann-Whitney U-test for pairwise comparison was used.

2.3 Results

2.3.1 Water Chemistry and Water Quality

Thiamethoxam water concentrations were measured on day 70 within an hour after water renewal of the waterborne exposures of sockeye salmon to thiamethoxam at four concentrations (0.15, 1.5, 15, and 150 µg/L) and a water control in two replicate samples. Table 2-1 shows the difference between predicted nominal and the measured concentrations for each sample and demonstrates the average of two replicate samples achieved the range of concentrations desired. Generally, the measured concentrations for the nominal 0.15 µg/L concentration were slightly higher (0.25 and 0.35 µg/L; Table 2-1). Conversely, the measured concentrations for the nominal 1.5, 15, and 150 µg/L were slightly lower than the measured concentrations, but the average values approximated the desired concentration range (Table 2-1).

Water quality parameters that were measured included water temperature, water pH, conductivity, dissolved oxygen, and ammonia concentration. All parameters in both the glass tank and gravel bed flume exposures fell within the guidelines outlined in Government of Canada (1998). The results are presented in Table 2-2. In summary, temperature from both exposure scenarios ranged from 9.6 to 14.7ºC with a mean of 11.9ºC; pH ranged from 6.9 to 8.6 with a

mean of 7.1; dissolved oxygen ranged from 8.2 to 12.0 mg/L; conductivity ranged from 22.1 to 40.4 µS/cm; and ammonia, measured in mg/L was not detected throughout the whole exposure.

2.3.2 Thiamethoxam Chronic Exposures in Glass Tanks

Endpoints analyzed for the chronic thiamethoxam glass tank exposure included fertilization, hatch, and survival success, body morphometrics, and deformities. Rationale for exclusion of some crosses and endpoints is described below.

Fertilization success was determined 24 dpf for cross A, B, C, and D. Fertilization success (± SE) in the control groups for crosses A, B, C, and D was 78 ±8%, 87 ±4%, 89 ±2%, and 94 ±1, respectively, and in the thiamethoxam treatment groups the fertilization success was 84 ±4%, 80 ±3%, 89 ±2%, and 86 ±1%, crosses A through D, respectively (Figure 2-2). No significant differences were observed between treatments or between crosses ($p>0.05$; RCB ANOVA followed by a Tukey's post hoc test; Figure 2-2).

Hatch success was determined approximately 67 dpf. The mean hatch success of the control fish in the glass tanks (±SE) for cross A and B was 71 ±21% and 57 ±4%, respectively, and ranged from 28 to 96% in the thiamethoxam treatments (Figure 2-3a). The mean hatch success of the control fish in the glass tanks (±SE) for cross C and D was 42 ±25% and 14 ±0.2%, respectively, and ranged from 12 to 60% in the thiamethoxam treatments (Figure 2-3a). Therefore, due to this low survival in the control fish upon hatching, crosses C and D were excluded from further analysis. No significant differences were observed between treatments or between crosses, for cross A and B ($p>0.05$; RCB ANOVA followed by a Tukey's and Student T post hoc tests).

The chronic exposure experiment in glass tanks was terminated commencing on 125 to 128 dpf. Survival (±SE) of the control fish in cross A and cross B was 63 ± 19% and 54 ± 5%, respectively. Mean survival (± SE) of the treatment groups exposed to 0.15 to 150 µg/L thiamethoxam ranged from 25 ±16% to 82 ±6% and 43 ±30% to 72 ±9%, for cross A and cross B, respectively. No significant differences were observed between treatments or between crosses ($p>0.05$; RCB ANOVA followed by a Tukey's and Student T post hoc tests; Figure 2-3b).

There was no statistical differences in mean lengths, weights, and condition factor across treatments; however, there was statistical differences between the two genetic crosses, where cross B was longer, heavier, and had a higher condition factor than cross A ($p < 0.001$, RCB ANOVA, followed by a Student's T tests). Mean length (±SE), weight (±SE), and condition factor (±SE) for cross B was 30.5 ±0.09 mm, 0.179 ±0.007 g, and 0.645 ±0.02 K, whereas the mean length (±SE), weight (±SE), and condition factor (±SE) for cross A was 29.5 ±0.09 mm, 0.160 ±0.007 g, and 0.627 ±0.02 g, respectively (Table 2-3, Figure 2-4, and Figure 2-5).

There was a total of 70 deformities out of 1,322 individual fish across all treatments. Of the 70 deformities, 92% were craniofacial, with the majority ranked as 1 on the severity index. There was no significant differences between presence of deformities for each type or pooled deformities across treatments of thiamethoxam, relative to the controls for both cross A and cross B ($p >0.05$, RCB ANOVA, followed by a Tukey's post hoc). Mean percent (±SE) of pooled deformities (i.e., craniofacial, edema, finfold, and skeletal) in the controls for cross A and cross B was 4.5 (±5)% and 15.5 (±10)% and the treatment groups ranged from 3.3 to 14.3 (±3)% and 10.1 to 18.4 (±4.2)% for cross A and cross B, respectively (Figure 2-6).

2.3.3 Thiamethoxam Chronic Exposures in the Gravel Bed Flume System to Mimic Natural Habitat

The endpoints analyzed for the chronic thiamethoxam gravel bed flume exposure included fertilization and survival success, body morphometrics, and deformities. Hatch success (emergence) of the fish reared in the gravel bed flumes could not be accurately determined due to individuals burrowing into the gravel upon hatching, resulting in inaccurate counts. Therefore, hatch success was excluded as an endpoint for all gravel bed flume analyses. Further details about endpoints are described below.

Fertilization success was determined approximately 23 dpf for cross A, B, C, and D. No significant differences were observed between treatments in mean fertilization success for sockeye ($p > 0.05$, RCB ANOVA, followed by a Tukey's test; Figure 2-7). However, there was a statistical difference in mean fertilization success between cross A and B and between cross B and D, where cross B had the lowest mean survival rate, but the greatest variability. Mean fertilization success (±SE) for cross A through D was 90 ±1.4%, 80.1 ±2.7%, 89.4% ±2.0%, and

92.2% ±1.8%, respectively ($p < 0.05$, RCB ANOVA, followed by a Tukey's post hoc test; n=2, with 3,046 eyed individuals; Figure 2-7).

Survival could not be determined with a high degree of accuracy based on a considerable number of individuals that could not be captured during termination because they burrowed into the gravel substrate. As such, statistics were not conducted to evaluate differences in survival at termination of this experiment between treatments. However, the mean percent survival is summarized in Figure 2-7. Briefly, mean survival for the control group ranged from 18% to 36% and 12% to 47% for the treatment groups (n=2, with 788 individuals).

There was no statistical differences in mean lengths, weights, and condition factor across treatments (Table 2-3 and Figure 2-7; $p > 0.05$ RCB ANOVA, followed by a Tukey's post hoc test; n=2); however, there was a significant difference in means length and weight between the genetic crosses. Specifically, all crosses were different and cross B was slightly longer and heavier than all the other crosses. The mean length (± SE) and weight (±SE) for crosses A through D were 30.4 ± 0.7 mm and 0.168 ±0.001 g (cross A), 30.8 ± 0.9 mm and 0.189 ±0.001 g (cross B), 29.3 ± 0.6 mm and 0.168 ±0.001 g (cross C), and 29.6 ± 0.4 mm and 0.1751 ±0.001g (cross D; $p < 0.05$, RCB ANOVA, followed by a Tukey's post hoc test; n=2 test vessels with 879 individuals;Table 2-3 and Figure 2-8). Lastly, there was also statistical differences in mean condition factor between crosses, where condition factor increased from cross A through D with cross A having the lowest condition factor (and different from the rest of the crosses). The mean (±SE) condition factor for cross A through D was 0.61 ±0.05 K, 0.65 ±0.06 K, 0.67 ±0.05 K, and 0.67 ±0.05 K, respectively ($p < 0.05$, RCB ANOVA, followed by a Tukey's post hoc; Table 2-3 and Figure 2-9)

A total of 879 individuals were captured during termination. Of the 879,19% had deformities, with 3% in the controls and 16% in the treatment groups. However, the proportion of the presence of deformities was not reliable because of the number of individuals that were not captured that could potentially skew the data (e.g., non-deformed fish could be faster and better able to escape capture). There was also a large range of individuals captured at termination (17 to 103 individuals per treatment) that could also potentially skew the proportions. As a result, statistical analysis was not performed.

2.4 Tables

Table 2-1.Thiamethoxam nominal and measured concentrations in two replicate glass tanks collected at 70 days post fertilization, within one hour after water renewal, during early life stage sockeye salmon chronic waterborne, flow-through exposure.

		Nominal Concentration				
	Replicate #	**0 µg/L**	**0.15 µg/L**	**1.5 µg/L**	**15 µg/L**	**150 µg/L**
Measured Concentration of Thiamethoxam (µg/L)	1	0	0.25	0.21	14.81	132.30
	2	0	0.35	1.57	13.26	145.52
	Mean	0	0.30	0.89	14.03	138.91

Notes: µg/L = micrograms per litre

Table 2-2. Water quality monitoring summary for the chronic glass tank and gravel bed flume thiamethoxam exposures, with sockeye salmon exposed for approximately 120 days, from one-hour post fertilization through to the swim-up fry life stage. Water quality monitoring occurred every 48 hours for all parameters except ammonia where monitoring was conducted weekly.

	Glass Tanks					**Gravel Bed Flumes**				
	Temp (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Ammonia (mg/L)	Temp (°C)	pH	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Ammonia (mg/L)
Mean	11.8	7.1	-	-	0	12.0	7.1	-	-	0
Minimum	9.6	6.9	8.24	22.1	0	9.7	7.0	8.18	22.7	0
Maximum	14.4	8.4	12.04	38	0	14.7	8.6	11.01	40.4	0
Standard Deviation	1.4	0.1	-	-	0	1.3	0.1	-	-	0
Count (n)	52	40	50	51	16	48	40	44	44	14

Notes: "-" = no data; °C = degrees Celsius; µg/L = micrograms per litre; µS/cm = micro siemens per centimetre; mg/L = milligram per litre.

Table 2-3. A summary of the mean parentage effects on body morphometrics for cross A and cross B (tanks) and cross A through cross D (flumes) irrespective of exposure conditions.

		Cross A	SE	Cross B	SE	Cross C	SE	Cross D	SE
Tanks	Length (mm)	30.5	0.09	29.5	0.09	NA	-	NA	-
	Weight (g)	0.18	0.007	0.16	0.007	NA	-	NA	-
	Condition Factor (K)	0.645	0.02	0.627	0.02	NA	-	NA	-
Flumes	Length (mm)	30.4	0.07	30.8	0.09	29.3	0.06	29.6	0.4
	Weight (g)	0.17	0.001	0.19	0.001	0.17	0.001	0.18	0.001
	Condition Factor (K)	0.607	0.008	0.653	0.007	0.671	0.007	0.677	0.007

Notes: "-" = no data; NA = not applicable

2.5 Figures

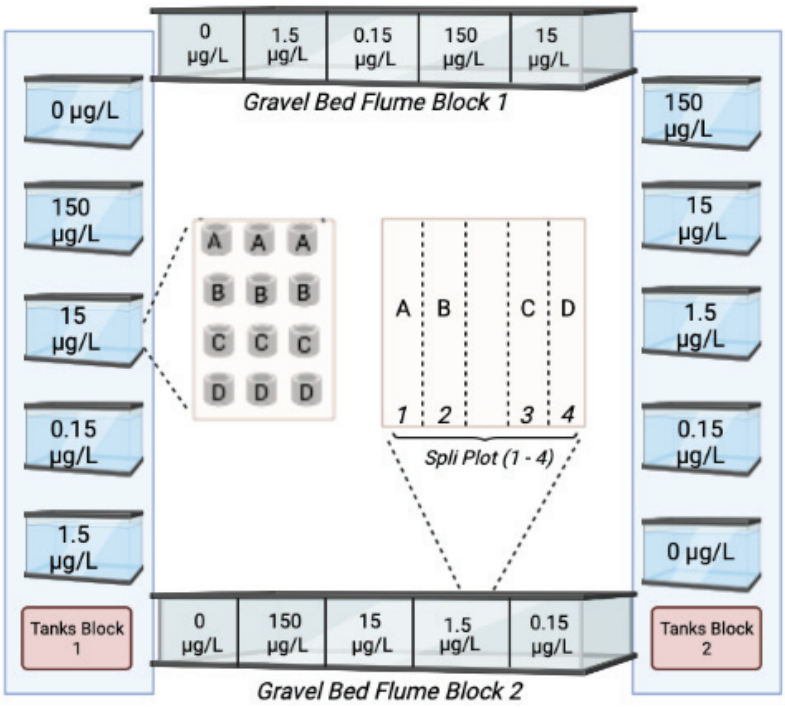


Figure 2-1. A schematic of the randomized split-plot complete block design of the glass tank and gravel bed flume exposure systems where sockeye salmon were exposed to nominal concentrations of 0, 0.15, 1.5, 15, and 150 µg/L thiamethoxam. In both systems, the test concentration (the main plot shown with the aquariums), assigned randomly, blocked in duplicate on either side of the room. Within each main plot was the split plot (the separation of genetic crosses, shown with the cylinders or dashed lines labelled A – D) and the genetic variation (the subplot, crosses A – D). In the tank exposure, the genetic crosses were separated with egg containers (shown above as grey cylinders labelled A, B, C, D) and in the gravel bed flume exposure, the crosses were separated by stainless steel dividers (shown above as dashed lines). The statistical model considered effects from the two different blocks, duplicate tanks, genetic cross, and an interactive effect of the crosses and exposure concentration.

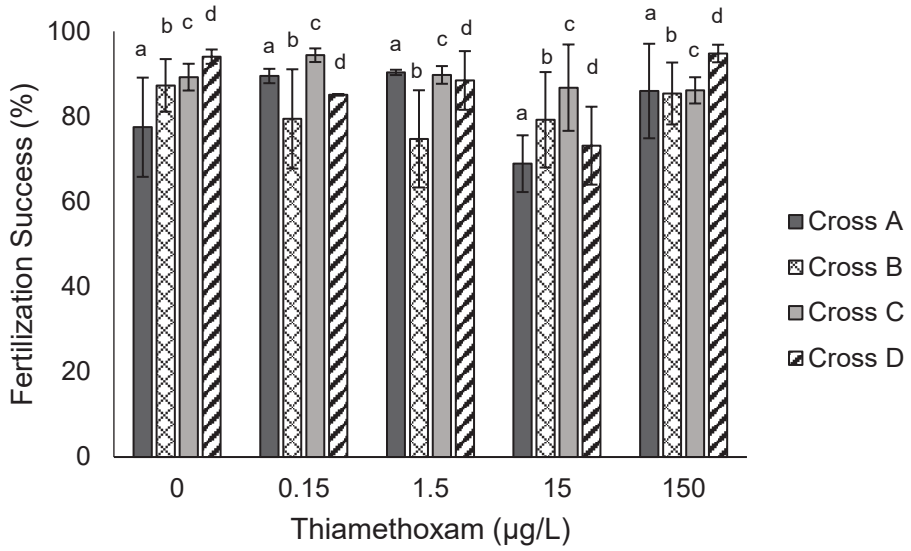


Figure 2-2 Fertilization success in crosses A, B, C, D sockeye salmon after chronic exposure to waterborne thiamethoxam in glass tanks from one-hour post fertilization. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels with ~100 individuals / vessel). Hatch and survival success were based on the mean survival of each replicate reared in the egg containers. Mean percent of fertilization success with errors bars indicating standard error. There were no significant differences between treatments ($p > 0.05$ RCB ANOVA, followed by a Tukey's post hoc test).

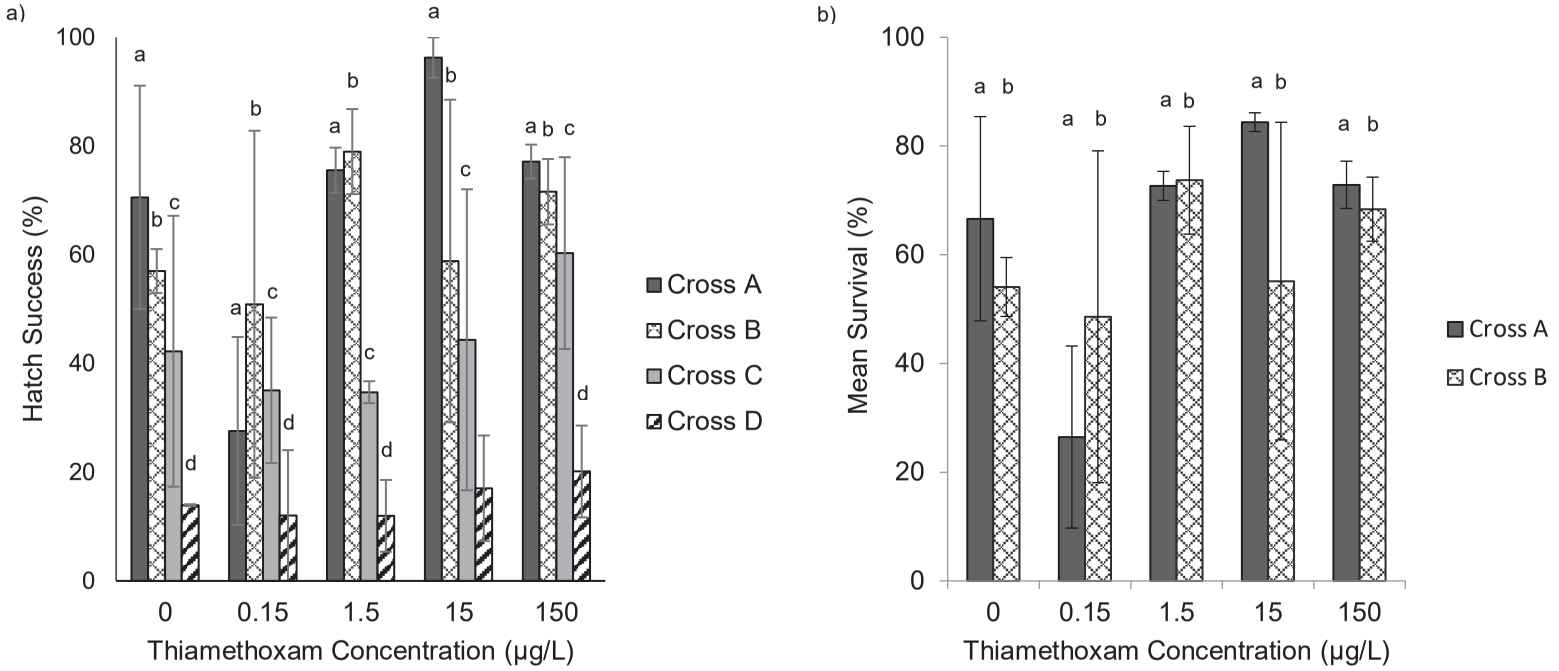


Figure 2-3. Hatch success in crosses A, B, C, and D and survival success in crosses A and B sockeye salmon after chronic exposure to waterborne thiamethoxam in glass tanks from one-hour post-hatch through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels with ~100 individuals). Hatch and survival success were based on the mean survival of each replicate reared in the egg containers. Mean percent of (a) hatch success and (b) survival, with errors bars indicating standard error. There were no significant differences between treatments ($p > 0.05$ RCB ANOVA, followed by a Tukey's post hoc test).

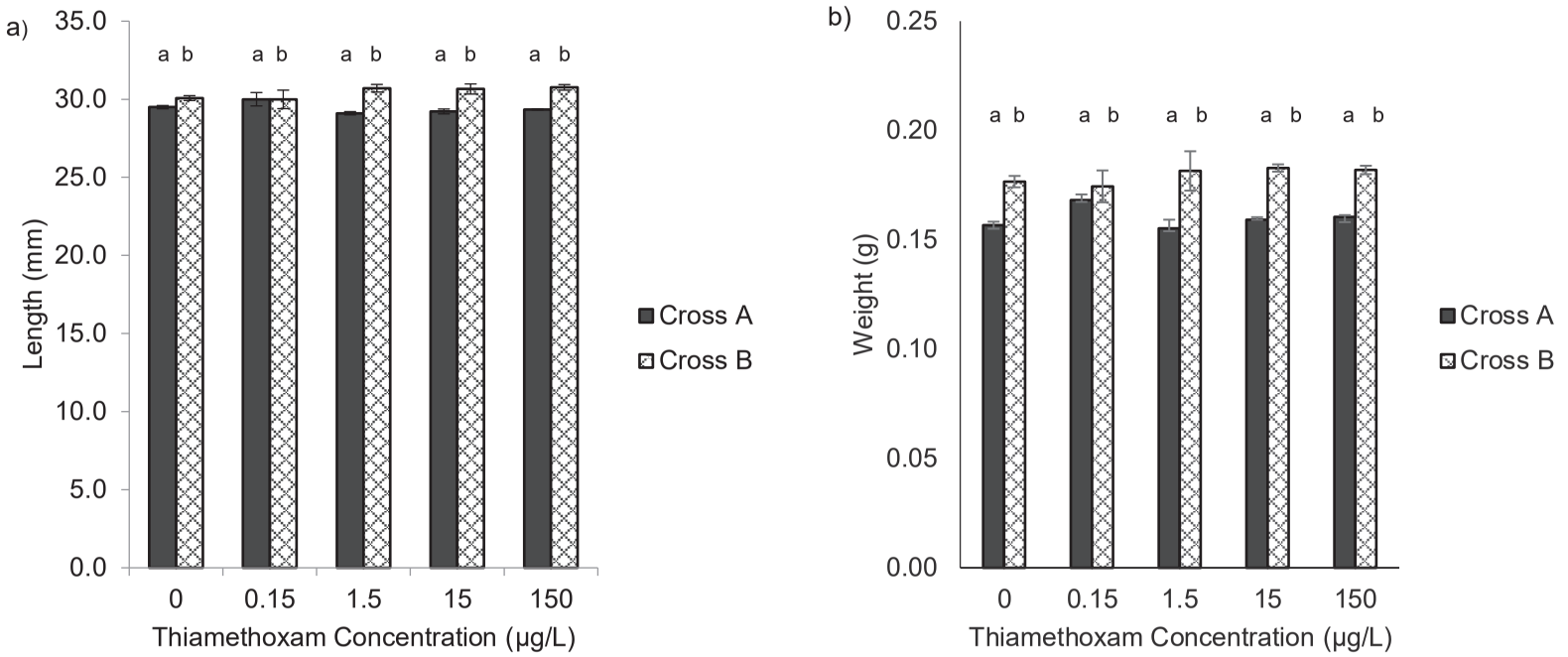


Figure 2-4. Mean length (mm) and weight (g) of cross A and B sockeye salmon after chronic exposure to waterborne thiamethoxam in glass tanks during from one-hour post fertilization through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels with 1322 individuals). Columns show (a) the mean length and (b) mean weight, with errors bars indicating standard error. There were no significant differences between treatments (P>0.05; RCB ANOVA, followed by a Student's T test). However, there was there was significant differences between the two genetic crosses, where Cross B was slightly longer and heavier than cross A ($p < 0.001$, RCB ANOVA, followed by a Student's T test).

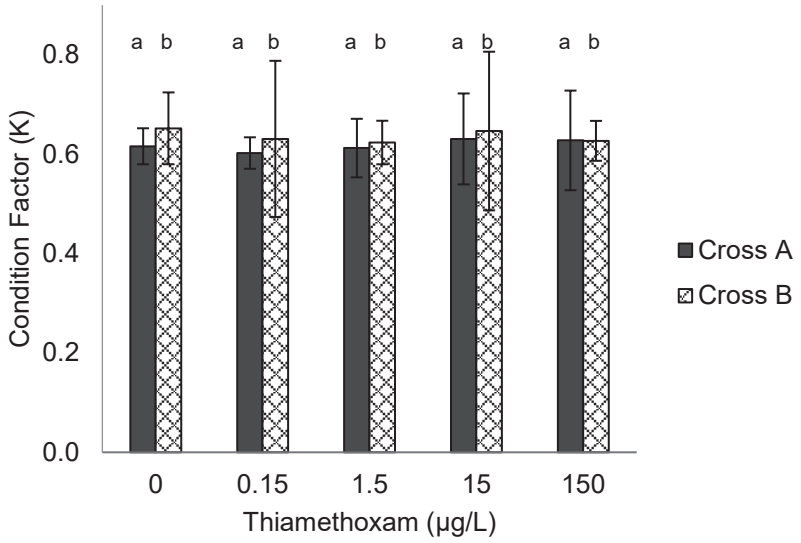


Figure 2-5 Mean condition factor (K) of Cross A and B sockeye salmon after chronic exposure to waterborne thiamethoxam in glass tanks from one hour post fertilization through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels with 1,322 individuals). Columns show the mean condition factor, with errors bars indicating standard error. There were no significant differences between treatments (P>0.05; RCB ANOVA, followed by a Student's T test). However, there was there was evidence of a difference between the two genetic crosses, where Cross B had a greater condition factor than cross A ($p < 0.001$, RCB ANOVA, followed by a Student's T test).

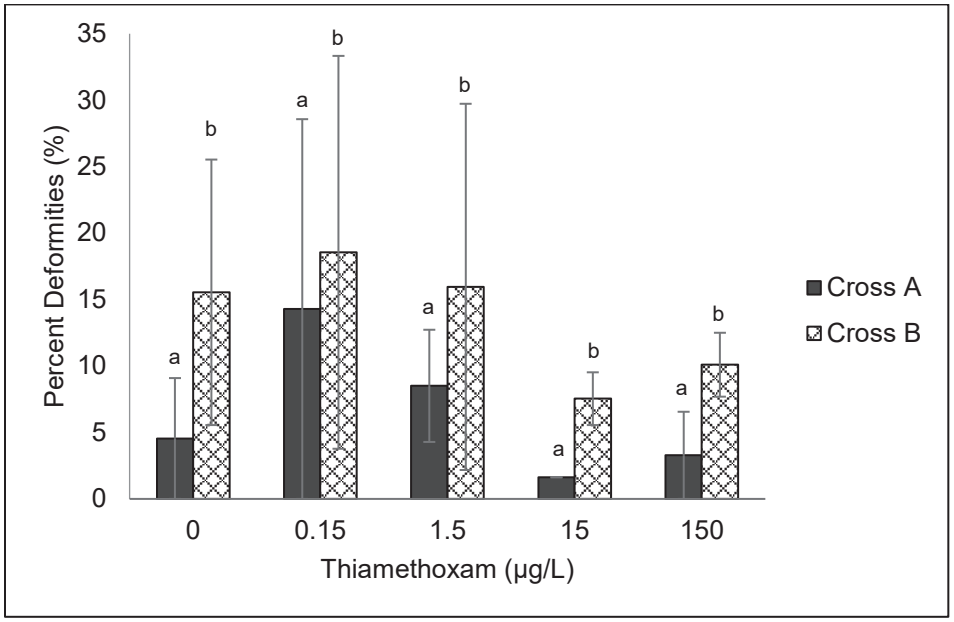


Figure 2-6 Proportion of the 70 / 1,322 fish with deformities of Cross A and B sockeye salmon after chronic exposure to waterborne thiamethoxam in glass tanks from one-hour post fertilization through to the swim up fry life stage.The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels with 1,322 individuals). Mean percent deformities of 2 replicate tanks are presented, and errors bars indicate standard error. There were no significant differences between treatments (P>0.05; RCB ANOVA, followed by a Tukey's post hoc test

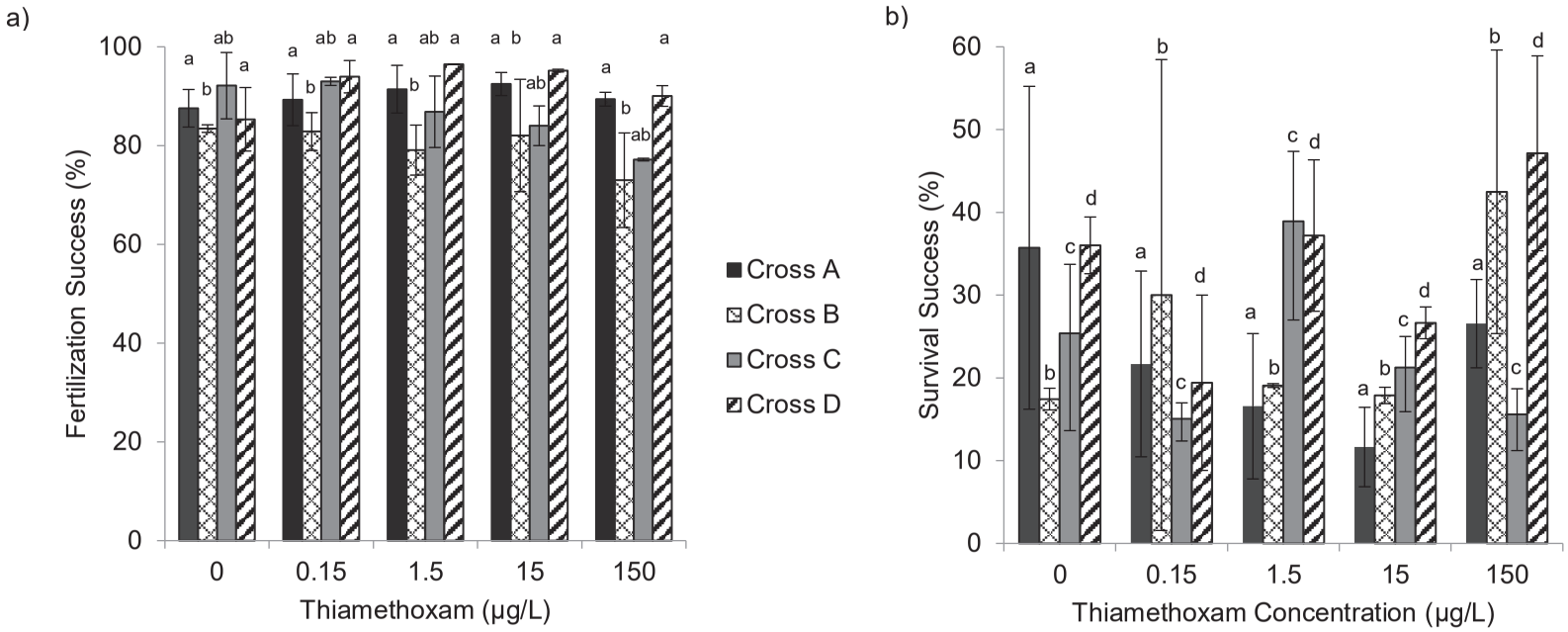


Figure 2-7. Fertilization and survival success in crosses A, B, C, and D sockeye salmon after chronic exposure to waterborne thiamethoxam in gravel bed flumes from one hour post fertilization through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels with approximately 50 to 100 individuals per cross/replicate). Fertilization and survival success was based on the mean eyed embryos and survival of each replicate reared in the egg containers. Columns show the mean percentage of (a) fertilization success and (b) survival success, with errors bars indicating standard error. There were no significant differences between treatments ($p > 0.05$ RCB ANOVA, followed by a Tukey's post hoc test); however, there was significant differences between crosses A and B and crosses B and D, where cross B had the lowest mean survival rate, but the greatest variability. Mean fertilization success (±SE) for cross A through D was 90 ± 1.4%, 80.1 ± 2.7%, 89.4% ± 2.0%, and 92.2% ± 1.8%, respectively ($p < 0.05$, RCB ANOVA, followed by a Tukey's post hoc test; n=2, with 3046 eyed individuals); mean differences are denoted by letters. All individuals were fertilized by 23 days post fertilization.

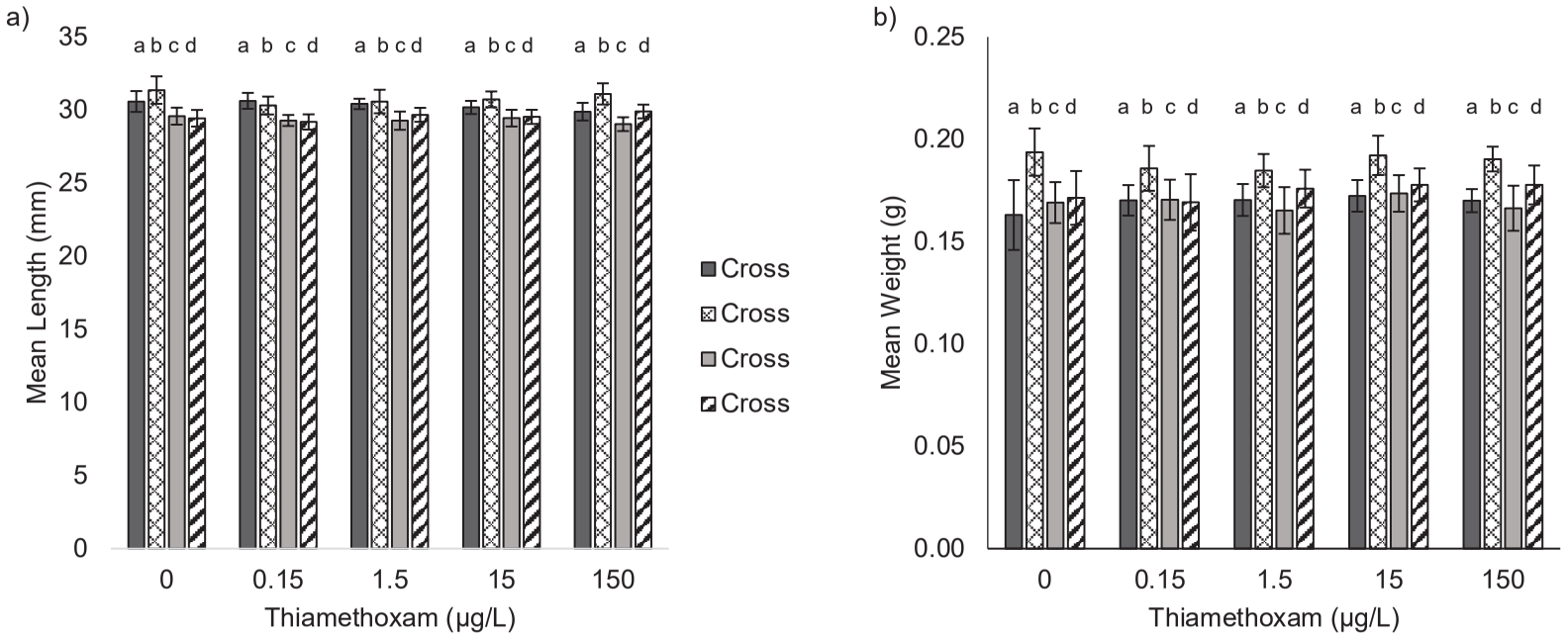


Figure 2-8.Mean length (mm) and weight (g) of Crosses A, B, C, D sockeye salmon after chronic exposure to waterborne thiamethoxam in the gravel bed flume from one hour post fertilization through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels; 1 to 45 individuals per cross/replicate). Columns show (a) the mean length and (b) mean weight, with errors bars indicating standard error. There were no significant differences between treatments ($P>0.05$; RCB ANOVA, followed by a Tukey's post-hoc test). However, there was there was significant differences of mean length and weight between the genetic crosses ($P<0.05$; RCB ANOVA, followed by a Tukey's post-hoc test). All crosses had different mean lengths (±SE), with Cross B, on average, being the longest at 30.8 ± 0.9mm. Mean weight (±SE) of cross B was also the greatest at 0.19 ±0.001g, followed by cross D (mean 0.1751 ±0.001g), and cross A and C (0.1683 ±0.001g). All differences are represented by letters.

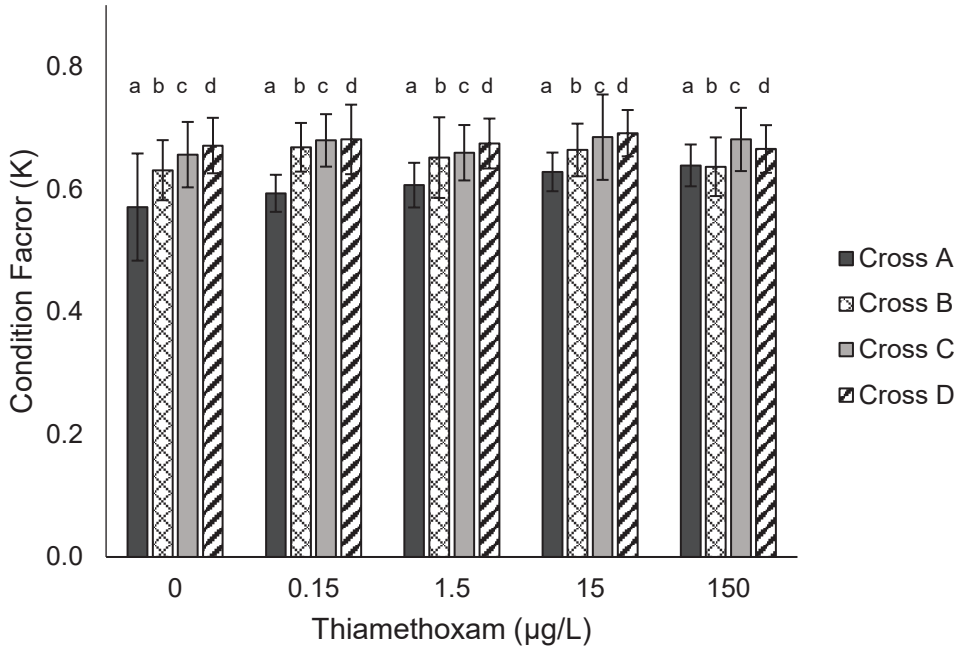


Figure 2-9 Mean Condition factor (K) of Crosses A, B, C, D sockeye salmon after chronic exposure to waterborne thiamethoxam in the gravel bed flume from one hour post fertilization through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 2 replicate test vessels; 1 to 45 individuals per cross/replicate). Columns show the mean condition factor, with errors bars indicating standard error. There were no significant differences between treatments (P>0.05; RCB ANOVA, followed by a Tukey's post-hoc test). However, there was there was evidence of a difference for condition factor between the genetic crosses, where cross A was different, with the lowest condition factor, relative to the rest of the crosses (P<0.05; RCB ANOVA, followed by a Tukey's post-hoc test). Mean (±SE) condition factor for cross A was 0.61 ±0.05, whereas the rest of the crosses mean (±SE) condition factor ranged from 0.65 (±0.06) to 0.67 (±0.05). Differences are represented by letters.

3 2016 Acute and Chronic Neonicotinoid Exposures

3.1 Introduction

The 2016 neonicotinoid study included acute and chronic exposures. The acute exposure involved fertilizing one genetic cross (cross B) of sockeye gametes in differing concentrations of a neonicotinoid mixture (i.e., equal parts of clothianidin, imidacloprid, and thiamethoxam) or fertilized in clothianidin or thiamethoxam, individually (all in triplicate) for one-hour (until egg hardening), followed by transfer to clean water. The sockeye were reared until the swim-up fry stage / 131 dpf, in flow through heath stacks. The objective of the acute exposures was to determine: 1) the acute toxicity of a mixture or individual select neonicotinoids during fertilization; and 2) if latent adverse effects ensued in embryos or larvae after acute to mixture or individual select neonicotinoids exposure during fertilization. These acute exposures were intended to represent a real-life exposure scenario whereby recently deposited / fertilized gametes could potentially be exposed to a pulse of neonicotinoids after a rainfall event during run-off.

The chronic study consisted of exposing four genetic crosses (crosses A through D) of sockeye to the neonicotinoid mixture (i.e., equal parts of clothianidin, imidacloprid, and thiamethoxam) from one-hour post-fertilization to swim-up fry or the feeding fry stage. The objective of chronic exposures was to evaluate adverse effects of a neonicotinoid mixture at environmentally relevant levels on multiple life stages of sockeye and to determine if parentage influenced these effects.

3.2 Methods

3.2.1 Chemicals

The neonicotinoid mixture stock solutions were prepared using equal parts of 98.0% pure clothianidin (CAS#: 210880-92-5), imidacloprid (CAS#: 138261-41-3), and thiamethoxam (CAS#: 153719-23-4), Sigma-Aldrich, Oakville, Ontario, Canada. The acute clothianidin and thiamethoxan exposures used the same chemicals to prepare the stock solutions. Fish euthanasia was conducted using tricaine methanesulfonate (MS-222) and Ovadine was used for disinfection of tools in contact with fish, and were

purchased from Syndel Laboratories LTD., Nanaimo, B.C., Canada. All other chemicals/reagents are identified in the respective sections and were sourced from commercial facilities and of analytical grade.

The environmentally relevant concentrations of the neonicotinoid mixture included equal parts of clothianidin, imidacloprid, and thiamethoxam, to make total nominal concentrations of 0.045, 0.45, 4.5, 45, and 450 µg/L (i.e., 0.015, 0.15, 1.5, 15, 150 µg/L of each neonicotinoid). The water solubility for each neonicotinoid used in this study were unique, but all concentrations tested did not exceed each neonicotinoid solubility limit, thus no solvents were required. Clothianidin solubility is 0.327 g/L at 20°C (US EPA 2003), imidacloprid is 0.61 g/L at 20°C (NPIC 2011), and thiamethoxam is 4.1 g/L at 25°C (MacBean 2010). Given these solubilities, a 50 mg/L stock solution was prepared by adding 50 mg of each ≥ 98% pure clothianidin, imidacloprid, and thiamethoxam to a combined total of 150 mg total neonicotinoid mass in 3 L of municipal dechlorinated water. The stock solution was heated to 25°C and stirred for 20 to 30 minutes to ensure full solubility. The stock solution was then diluted further with dechlorinated municipal water and added to glass aquaria to achieve the nominal exposure concentrations described below for the acute and chronic experiments (Table 3-1).

3.2.2 Gamete Collection and Fertilization

During the fall of 2016, four wild sexually mature mating pairs of sockeye salmon (*Oncorhynchus Nerka*) were captured and donated by the Department of Fisheries and Oceans (DFO) in the Pitt River at Inch Creek Hatchery, located in Dewdney, BC. Gamete collection and fertilization was performed in the same manner as the thiamethoxam exposure, as detailed in Leung (2018) and Marlatt et al. (2019). Approximately 2,000 to 3,000 eggs were harvested from each female and approximately1 to 3 ml of milt was collected from each male. For each of the four crosses the eggs from one female and milt from one male were combined in 1.5 L of dechlorinated water (10 ± 1 °C) followed by gentle swirling to ensure mixing. The gametes were immediately stored and shipped at 6 to 10°C and delivered to Simon Fraser University, Burnaby, BC, on 9 September 2016. Dry fertilization was performed by Dr. Vicki Marlatt within six hours of collection where four unique offspring sets were created from the four mating pairs (labelled crosses A, B, C, D). The eggs and milt were divided in half for the acute and chronic exposures such that

separate dry fertilization procedures were performed for use in the acute or chronic exposures as detailed below in Sections 3.2.3 and 3.2.4

3.2.3 Acute Neonicotinoid Exposure During Fertilization

The acute exposure conditions included fertilizing cross B gametes in (1) five concentrations, in triplicate, of an equal mass of clothianidin, thiamethoxam, and imidacloprid (the neonicotinoid mixture), totalling 0.045, 0.45, 4.5, 45, and 450 µg/L of the neonicotinoid mixture, and (2) four concentrations, in triplicate, of clothianidin and thiamethoxam, separately, totalling 0.15, 1.5, 15, and 150 µg/L of each neonicotinoid. Both scenarios included water controls, also conducted in triplicate. Specifically, 22 to 24 eggs from cross B were divided into three netted food grade PVC cylindrical egg containers (6 to 8 per egg container) and were fertilized with milt from one male while in a 1.5 L solution (4 L glass test vessel) of the appropriate concentration of pesticide for sixty minutes to ensure egg hardening. Each of the 3 egg containers per test concentration and control were then placed into clean water in flow-throw health stacks (i.e., a vertical tray incubator flow-through system 82 x 60 x 64 cm (height, width, depth) and housed 8 trays). Municipal dechlorinated water continuously flowed through the top tray and down into each of the seven lower trays prior to exiting via a discharge pipe on the lowest tray. Each shelf contained the egg containers that housed cross B fish, for each neonicotinoid fertilization exposure scenario (i.e., the neonicotinoid mixture, clothianidin only, or thiamethoxam only).

The experiment was terminated when the fish reached the swim-up fry developmental stage. The endpoints measured in this acute exposure consisted of fertilization and hatch success, survival, body morphometrics including length, wet weight, and condition factor. Further details regarding animal husbandry guidance, water quality parameters, termination, pesticide concentration rationale, and details are discussed in detail below in the chronic exposure, in Section 3.2.4.

3.2.4 Chronic Exposure to the Neonicotinoid Mixture

For the chronic exposure, four genetic crosses were created and tested (A, B, C and D). For each cross the eggs from one female and milt from one male were combined with 1.5 L of dechlorinated water (10 ± 1 °C) in a 4L vessel. The gametes were gently

swirled together (to ensure mixing), followed by a 60-minute resting period. Fertilized eggs were then carefully transferred and subdivided into three separate egg containers, per genetic cross (labelled A, B, C, D), which totalled 45 individuals per cross per tank (i.e., 15 individuals per egg container). As with the acute exposures, the egg containers were used to track the health and survival of each individual cross throughout the exposure until the feeding fry stage. Figure 3-1 shows a schematic of the exposure set-up.

The chronic exposures were conducted in a static system with 18 glass tanks and tested five concentrations of the neonicotinoid mixture, plus a water control in triplicate. The total test volume solutions in each of the tanks was 19.0 L with the dimensions of 22 cm x 26 cm x 52 cm (height, width, length, respectively). Each tank was placed in a water bath in order to maintain water temperature. Each glass tank contained 3 PVC netted cylindrical egg containers per genetic cross, and 15 eggs were placed in each container. All tanks were randomly placed at opposite sides of the room.

Similar to the thiamethoxam exposure, parameters outlined in GoC (1998), Biological Toxicity Test Methods for Early Life Stages of Salmonids, were followed with minor deviations. Measured and maintained water quality parameters included water pH, conductivity, and dissolved oxygen were measured daily. Daily monitoring of health and removal of dead embryos, alevins, and fry were conducted. Embryos were maintained in darkness, observed only with a red light, until 90 to 100% of the control animals were hatched. Upon hatching, the alevins where then reared in a 16-hour / 8-hour light/dark period, in order to mimic a natural photoperiod. Static renewal of water / test solutions occurred every 48 hours by gently siphoning out 80% of the old test solutions/control water and replacing with freshly mixed test solution/control water. Termination commenced once approximately 80% of the control fish resorbed their yolk-sac, entering the swim-up fry life stage (approximately 84 to 92 dpf). Cross C was then left to be reared for an additional 31 days of feeding to examine adverse effects of prolonged exposure during the feeding fry stage.

Termination was performed in compliance outlined in Canadian Council for Animal Care guidelines and with a permit issued by Simon Fraser University Animal Care Committee (Burnaby, BC, Canada). Briefly, individual swim-up fry and feeding fry were humanely euthanized in a dechlorinated solution consisting of 0.4 g/L MS-222 and buffered with 0.4 g/L sodium bicarbonate (Sigma Aldrich, Oakville, ON, Canada) to pH 7.0

to 7.5. Upon termination, length, wet body weight, and presence of deformities were recorded. At random, for the swim-up fry, ten whole bodies and ten livers per genetic cross were harvested, placed in DNase/RNase free 2 ml Eppendorf tubes (whole bodies) or DNase/RNase free 1.5 ml Safe Lock Eppendorf tubes (livers), and flash-frozen on dry ice, prior to being transferred to a -80°C freezer for long-term storage. In addition, three Cross C feeding fry were euthanized and livers, and whole bodies were dissected and placed in DNase/RNase free 1.5 ml Safe Lock Eppendorf tubes (livers) and 2 ml RNase/DNase Eppendorf tubes (whole bodies), and flash-frozen on dry ice, prior to being transferred to a -80°C freezer for long-term storage. Stainless steel instruments used to harvest the liver tissue were cleaned between each animal, using 10% peroxide followed by a double rinse with ultrapure water, to ensure there was no cross contamination and that the tools were RNase/DNase free. Swim-up fry endpoints for this exposure consisted of hatch success; survival; body morphometrics including length, wet weight, and condition factor, and percent deformities; and molecular endpoints included hepatic gene expression. Feeding fry endpoints consisted of the same endpoints as the swim-up fry, however, with addition of whole-body hormone concentrations.

The concentration of pesticides in one replicate glass tank was measured twice during this chronic exposure experiment. This entailed collection of a 20 ml grab-sample from one replicate fish tank per test concentration and control, immediately after water renewal, as outlined in Sultana et al. (2018). These water samples were collected 93 and 163 dpf. Samples were stored on ice and delivered to ALS Environmental (Burnaby, BC) for analysis within 30 minutes of collection.

3.2.5 Hatch Success, Survival, Condition Factor and Deformities Data Collection and Analyses

Endpoints in this study, to determine the effects of the neonicotinoid mixture include hatch success, survival, morphometrics, and presence of deformities. Hatch and survival success were analyzed as percentages and determined by the number of eyed embryos that successfully hatched (i.e., hatch success), and by the number of fish that successfully hatched and survived until termination day (i.e., survival). Methods for deformity analyses included placing live fish in a clear observation vessel to briefly monitor for physical deformities immediately prior to euthanasia. After euthanasia deformities were identified using a graduated severity index (GSI) and Stereomicroscope as outlined in

Holm et al. (2005) and Rudolph et al. (2008). Deformity analysis included four main categories as follows: craniofacial (i.e., assessment of eyes, jaws, and head); edema (i.e., the presence of an accumulation of fluid around the head or pericardial cavity); finfold (i.e., size, shape, and presence of all fins); and, skeletal (i.e., presence of a lordotic, kyphotic, scoliotic curvature to the spine). Deformities were ranked zero to three based on a severity scale, where zero was equal to no deformities/abnormalities and three was equal to severe deformities. Fish body weight (measured in grams), length (snout to fork length, measured in mm) were used to calculate Fulton's K. Fulton's K is determined by multiplying the individual's wet weight (in grams) by 100, divided by the cubed length (in cm), which infers the overall body condition, and is simplified in Equation 3-1 as follows:

$$K = \frac{100W}{L^3} \qquad (3\text{-}1)$$

Where:

K = Fulton's Condition Factor

W = Wet weight (g)

L = Length (cm)

Quality assurance quality control measures were performed regularly through out the termination and included the following: Deformity analysis training was provided by Dr Marlatt for the technicians who were euthanizing the fish. The same technicians ranked the deformities throughout the entire termination. Validation for deformity ranking and length measurements was conducted by having the original technician record their results for an individual fish. The alternate technician would score the same induvial fish, and the results would be compared to confirm they arrived at the same conclusions. This process was repeated at regular intervals throughout the entire termination.

3.2.6 Sex Steroid Hormone Measurements

Measurement of individual fish whole-body sex steroids (17β-estradiol and testosterone) were quantified in feeding fry sockeye from one genetic cross (cross C) from the chronic neonicotinoid mixture exposure. Specifically, nine individuals from each of the five treatments and the water control were used in this analysis (n = 2 to 3 individual fish

per replicate tank). Methods for homogenization and extraction of the whole bodies were adapted from Arukwe et al. (2008). Briefly, whole bodies of the feeding fry were thawed on ice, homogenized using two 3 mm stainless beads in a Retsh MM 400 mixer mill (Fisher Scientific, Ottawa, ON, Canada) at 30 Hz for a total of five minutes in a 1:4 0.1 M sodium phosphate buffer, and centrifuged at 14,000 g for 15 minutes (per Arukwe et al. 2008). Extraction of steroids from the supernatant was immediately conducted by isolating the steroids in 3 mL of diethyl ether (as opposed to 4 ml per Arukwe et al. [2008]), and vortexing in glass test tubes to allow for phase separation. The solution was then flash frozen in an ethanol / dry ice bath, where the aqueous phase froze, allowing the liquid lipophilic (steroid containing) phase to be decanted. This process was repeated 3 times. The ether phase was then evaporated in a fume hood for three days at room temperature and each sample was then reconstituted in 350 µL of the supplied 10x dilution of the ELISA Buffer Concentrate (made up of 1M phosphate containing 1% BSA [bovine serum album], 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide).

To calculate recovery efficiency of our extraction procedures, two spiked samples for 17β-estradiol and testosterone (i.e., one stock standard and one two-fold dilution of the stock standard, referred to as “spike high” and “spike low”, respectively) were run through the same hormone extraction protocols that was conducted on the whole-body homogenates. The percent recovery of the spiked was subsequently tested on one ELISA plate for each hormone analyzed and accounted for four samples (i.e., two spike high and two spike low), performed in duplicate, per the quantification methodologies, detailed below. Percent recovery was calculated by taking the average of the recovered hormone concentration divided by the known concertation multiplied by 100.

Hormones were quantified using enzyme-linked immunoassay (ELISA) kits according to the manufacturer’s instructions for 17β-estradiol and testosterone (Estradiol ELISA Kit, Item Number 582251; Testosterone ELISA Kit, Item Number 582701; Cayman Chemical Company, Ann Arbor, MI, USA). All procedures, including preparation and use of all reagents were conducted on the same day. The assays were performed on three of the supplied 96-well plates and each plate included the following: triplicate maximum binding wells (i.e., individual samples); duplicate blank and non-specific bunding wells; an eight-point standard curve; and 50 µl of whole-body homogenate samples per well tested in duplicate. The plates absorbance was measured using an EPOCH2 microplate reader and Gen 5.02 Software (BioTek Instruments Inc., Winooski, Vermont, USA) at 405 nm

with a development time of 75 and 60 minutes for 17β-estradiol and testosterone, respectively. As per the manufacturer's protocol, the standard curve was linearized via logit transformation (Logit = Sample Binding/Maximum Binding). The hormone concentrations were then interpolated using the standard curve. Where samples were below or exceeded detection limits, the minimum and maximum detection limit was assigned

3.2.7 Hepatic Gene Expression

3.2.7.1 Total RNA Extraction and cDNA Synthesis

Hepatic gene expression was conducted on cross C considering this cross was used for whole body hormone analysis, and tissue was available for two life-stages (i.e., the swim up fry and feeding fry life stages). For the swim-up fry life stage, in order to obtain enough RNA two livers were pooled due to insufficient RNA quantity, to make one sample, with a total of nine samples per treatment, from each of the six treatment groups (n = 9 per treatment obtained from 18 individuals). To confirm that swim-up fry liver pooling was necessary, and to rule-out any reagent or protocol issues, the RNA extraction protocol and reagents were used on ten-month-old wild sockeye salmon livers (collected from the Pitt River stock in 2015) that were reared in outdoor tanks under a natural photoperiod and municipal dechlorinated tap water. For the feeding-fry, three individual livers from cross C for each of the six treatment groups were used for hepatic gene expression analysis (n = 9 per treatment obtained from 9 individuals). Thus, one genetic cross across two early life-stages were tested for the neonicotinoid mixture waterborne exposures (i.e., the water control plus 0.045, 0.45, 4.5, 45, 450 µg/L in triplicate) and included for gene expression analyses in this study.

Methods used in this study were also described in Leung (2018), Calbick (2018), and Marlatt et al. (2019), including adhering to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for all qPCR experiments (Bustin et al. 2010; Taylor et al. 2010). Briefly, total RNA was isolated from the swim-up and feeding fry livers using TRIzol® Reagent as described by the manufacture (Invitrogen, Burlington, ON, Canada). Homogenization of either two pooled swim-up fry livers or one feeding fry liver was completed by using 1 mL of TRIzol reagent, and two 1 mm tungsten-carbide beads in a 1.5 mL safe-lock microcentrifuge Eppendorf tube, in a Retsch Mixer

Mill MM 400 (Fisher Scientific, Ottawa, ON, Canada) at 30 Hz for a total of six minutes. Halfway through the homogenization process, the mixer mill was stopped, and chambers were rotated 180 degrees, and homogenization was continued for the remainder three minutes.Total RNA obtained from the TRIzol® RNA isolation procedure was then reconstituted in 30 µL DNase/RNase-free water, and stored at -80°C.

RNA quantity and purity were quantified using an Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Quantity, measured in ng/µL, was determined by the optical density unit (OD_{260}) and the RNA purity was assessed measuring $OD_{260/280}$ and $OD_{260/230}$ ratios. After obtaining 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. The quality or integrity of RNA was then evaluated using a Bio-Rad Experion™ Automated Electrophoresis System and Experion software (version 3.20; Bio-Rad, Mississauga, ON, CAN). All RNA samples were stored at -80°C for long-term storage until subsequent cDNA synthesis.

Reverse transcription of 0.75 µg of DNase-treated total RNA into cDNA for each pooled swim-up fry liver and 0.7 µg of DNase-treated total RNA into cDNA for each feeding fry liver sample was performed using the Applied Biosystems™ High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems – Thermo Fisher Scientific, Ottawa, Ontario). As described in the manufacturer's protocol, 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 10 µL of master mix and DNase/RNase free water to make up a total volume of 20 µL and 0.70 µg (feeding fry) or 0.75 µg (swim-up fry) DNAse-treated total RNA. Reverse transcription was also performed on one sample for each life stage (i.e., two samples) and went through the manufacturer's protocol without the addition of the reverse transciptase enzyme that catalyzes the reaction (deionized distilled water was used a substitute). This was done for the use as a "no template control" in the subsequent qPCR experiments to ensure the DNase treatment effectively removed genomic DNA. The cDNA synthesis procedure resulted in a final concentration of 50 ng of cDNA/µL in water for each cDNA sample, which were stored at -20°C until subsequent qPCR experiments were performed.

3.2.7.2 Quantitative Real-Time PCR (qPCR) Methods

As described in Calbick (2018) and Marlatt et al. (2019), the relative quantification of each gene of interest in an individual hepatic cDNA sample measured in the qPCR experiments in this study, for each life-stage, was achieved via the ΔΔCq method, and by normalizing data to the expression level of two or three reference genes in each cDNA sample. A standard curve was included in each qPCR assay as it provides important information pertaining to the performance of the reaction (i.e., amplification efficiency), it is also a criterion to demonstrate quality qPCR experiments using novel primers (Bustin et al. 2009). In this study, the standard curves for all qPCR experiments were generated by performing a four-fold dilution series of a pooled cDNA sample that was composed of cDNA samples from all of the control and exposed fish liver samples. Specifically, a 50ng cDNA/μL of water standard was prepared by combining 1 μL of cDNA from all of the control and treatment samples (n = 54 individual cDNA liver samples) into a single 1.5 mL microcentrifuge tube; thus, totaling 54 μL of a 50ng cDNA/μL of water standard as the most concentrated standard in the qPCR experiments for both life-stages. A four-fold serial dilution of the 50 ng cDNA/μL of water standard was performed to create an 8-point standard curve in each qPCR experiment. As such, 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 of interest (GOI) standard curve, 2.5 μL of each dilution were then added as a cDNA template, in triplicate.

Standard curves were generated to test each primer set's specificity and designing optimal experimental conditions for subsequent qPCR assays with livers from the neonicotinoid exposures. Primer set optimization trials were conducted by adjusting the annealing temperature, template concentration, and primer concentration. Specifically, primer sets used to measure gene expression levels were tested for efficiency using a five to eight-point standard curve generated by the four-fold dilution of a 50ng cDNA/μL of water (comprised of the template/pooled liver samples from sockeye swim-up fry, and feeding fry, as mentioned above). Standard curve acceptance criteria for this study included in single peak melt curve, efficiencies between 90-110%, amplification in at least five concentrations of the standard curve, and an R^2 of the standard curve > 0.9 (Bustin et al. 2010). Primers that satisfied the aforementioned criteria and therefore included in this study are detailed in Table 3-2 and include product size, PCR efficiency, and correlation coefficient (R^2). Cycle quantification/threshold (Cq) values, regression slope,

PCR efficiency, Y-intercept, and the CV were calculated by Bio-Rad CFX software and checked considering MIQE guidelines of Bustin et al. (2010). Primer sets that did not meet these criteria and/or did not amplify a target GOI were compared to the literature and previous Marlatt Lab experiments to rule out primer design errors and were considered failed primer sets, and excluded in this study, and listed in Table 3-3. All primer sets used in this study were sequenced and previously published in Duarte-Gutterman et al. (in prep), Veldhoen et al. (2010) or Marlatt et al. (2019).

Several gene expression targets were examined that were related to various biological processes including reproduction, growth, stress responses, nervous and immune system function, and oxidative stress. Reference gene expression targets were used based on their stability and ubiquitous presence in the control and exposed fish. These genes are tabulated with details in Table 3-2.

The qPCR experiments were conducted using a Bio-Rad CFX384™ Real-Time PCR Detection System and the Bio-Rad CFX Manager™ Software ΔΔCq method for relative quantitation of target genes between treatments following the MIQE guidelines (Bustin et al. 2010; Taylor et al. 2010) on Bio-Rad Hard-Shell 384 well PCR plates. Fresh master mix was made immediately prior to each qPCR experiment and contained 0.38 to 0.75 µL of forward and reverse primers per sample (0.3 to 0.6 µMol/reaction), 6.25 µl of SsoFast™ EvaGreen® Supermix (Bio-Rad), and RNase free water to result in a total volume of 10 µL of master mix in each well. Also included in each well was 2.5 µL of 1:195 dilute cDNA template (non-dilute cDNA concentration = 50 ng/µL), for swim-up fry and feeding fry; thus, resulting in a total volume of 12.5 µL. Each plate contained three technical replicates per sample, with seven to nine individuals (considered as the biological replicates) as described in the figure caption in Section 3.5.2 and tabulated in Table 3-4.

Each qPCR experiment included a no template control (NTC) for each primer set tested in duplicate wells, where 2 µL of RNase-free water was used in place of cDNA. As previously discussed, a no reverse transcriptase control (NoRT) was also prepared for each primer set and tested in duplicate for each qPCR experiment, whereby 2 µL of DNase treated RNA was used in place of cDNA. The NTC was a negative control to confirm the absence of contamination of cDNA samples, and the NoRT was an additional negative control to confirm that the DNase treatment effectively removed genomic DNA.

As per Bio-Rad CFX settings, amplification reactions for each qPCR experiment included cycle 1 activation at 95 °C for 30 seconds, followed by 45 cycles at 95 °C for 5 seconds, and lastly primer annealing at 55-58 °C for 5 seconds. After 45 cycles, a melt curve analysis was performed following every run to confirm single amplicon amplification for each set of primers as indicated by a single peak. The instrument settings for melt curve analysis included an initial temperature of 65.0°C that increased by 0.5°C for 5 seconds to a maximum of 95.0°C. Amplicons that showed a single sharp peak between 82 and 87.5°C (indicating a single gene was amplified in each qPCR assay) were retained with results in Table 3-5. If the peak was not present, or multiple peaks were present, the gene was excluded from this study (summarized in Table 3-3).

3.2.8 Statistical Analyses

3.2.8.1 Acute Exposures

To test the effects of the acute neonicotinoid exposures, a complete randomized design (CRD) was used for all analysis (n=3). All statistical analyses were performed using JMP®, Version *15* (SAS Institute Inc., Cary, NC). Given the experimental design for the acute exposures, effects from the different concentrations of neonicotinoids allowed to determine if there were any adverse effects on fertilization, hatching, survival, morphometrics, and deformities. As such, one-way analysis of variance (ANOVA), followed by a Tukey's post hoc test ($p < 0.05$) was used for data that met parametric assumptions. For data that did not meet these assumptions, a Kruskal-Wallis test, followed by a Steel-Dwass all pairs test for pairwise comparison was used. All bar charts were created using Microsoft® Excel software.

3.2.8.2 Chronic Exposure

To test the effects of the chronic neonicotinoid mixture exposure, a split-plot randomized complete block (RCB) design was used for all analysis where the different genetic crosses were included. The exposure scenario included a complete block design with a statistical n=3; however, due to user error, one tank containing 4.5 µg/L of the neonicotinoid mixture was compromised and excluded from the experiment; therefore, for that concentration, the study was conducted in duplicate. The design is still considered a complete block with some missing values (per Ian Bercovitz, 2018, personal communication). Details of the design include the neonicotinoid mixture concentration that was considered the main plot with all five concentrations plus the control (i.e., 0, 0.045, 0.45, 4.5, 45, and 450 µg/L), blocked by which side of the room the glass tanks were located (i.e., two blocks that include side 1 and side 2, with 9 tanks in each block). Within the main plot (the glass tanks), was the sub-plot that consisted of the different genetic crosses (i.e., crosses A through D; Figure 3-1). Given there were two blocks located on either side of the room, the block design can capture any effects regarding the side of the room the tanks were located (i.e., differing light or air ventilation differences). Also, given the static exposure scenario, water parameters slightly varied between each tank (the main plots); as such, the split plot design can capture any statistical differences in the main plot between the genetic crosses in the slightly different exposure scenarios between tanks.

As with the acute exposures, all statistical analyses were performed using JMP®, Version *15* (SAS Institute Inc., Cary, NC). Given the experimental design for the chronic exposure, effects between genetic crosses from the different concentrations of neonicotinoids were tested to determine effects on hatching, survival, morphometrics, and deformities. As such, an RCB analysis of variance (ANOVA), followed by a Tukey's post hoc test ($p < 0.05$) was used for data that met parametric assumptions. For data that did not meet these assumptions, a Kruskal-Wallis test, followed by a Mann-Whitney U-test for pairwise comparison was used. For endpoints that only included one genetic cross (i.e., sex steroid levels, and gene expression) a one-way ANOVA, followed by a Tukey's post hoc test ($p < 0.05$) or a Kruskal-Wallis test, followed by a Mann-Whitney U-test on the two or three replicates were used. All bar charts were created using Microsoft® Excel software, whereas boxplots were created using JMP® software.

3.3 Results

3.3.1 Acute Neonicotinoid Exposure

3.3.1.1 Health Stack Water Quality

Water quality parameters that were measured included water temperature, water pH, conductivity, dissolved oxygen, and ammonia concentration. All parameters fell within the guidelines outlined in GoC (1998). The results are presented in (Table 3-6). In summary, temperature ranged from 1.3 to 13ºC with a mean of 8.6ºC; pH ranged from 6.9 to 8.4 with a mean of 7.1; dissolved oxygen ranged from 8.9 to 10.8 mg/L with a mean of 10.1 mg/L; conductivity ranged from 21.0 to 32 µS/cm, with a mean of 26.3 µS/cm; and ammonia, measured in mg/L was not detected throughout the whole exposure.

3.3.1.2 Hatch Success, Survival Success, and Morphometrics

For all three acute exposure scenarios the control group mean fertilization, hatch, and survival success (±SE) was >91 ±0 to 5%. Mean fertilization success (±SE) in the treatment groups ranged from 92 ±4% to 100 ±0%, 75.0 ±7% to 100 ±0%, and 83.3 ±4% to 96 ±4%, for clothianidin, thiamethoxam, and the neonicotinoid mixture, respectively (Figure 3-2a, Figure 3-3a, and Figure 3-4a). Mean hatch success (±SE) ranged from 88±7% to 100 ±0%, 84 ±10% to 100 ±0%, and 81±5%, to 100 ±0%, for clothianidin, thiamethoxam, and the neonicotinoid mixture, respectively (Figure 3-2b, Figure 3-3b, and Figure 3-4b). Lastly, survival success (±SE) ranged from 85 ±1 to 100 ±0%, 94 ±18%, and 100 ±0%, for clothianidin, thiamethoxam, and the neonicotinoid mixture, respectively (Figure 3-2c, Figure 3-3c, and Figure 3-4c). Survival for the group fertilized in 0.045 µg/L neonicotinoid mixture were excluded from the analysis based on suspect fungal overgrowth in the three egg containers (thus 100% mortality). The only statistical difference for all exposure scenarios and endpoints was mean fertilization success between the control and 15 µg/L thiamethoxam groups, where 75 ±7% (SE) of the treated eggs were successfully fertilized, relative to 100 ±0% of the control group ($p = < 0.05$, one-way ANOVA, $n = 3$ with 17 to 24 individuals per treatment; Figure 3-3a).

Analysis for body morphometrics included length (mm), weight (g), and condition factor (K), for all three acute exposures. There were no significant differences in the observed mean body morphometrics for the acute clothianidin exposure ($p>0.05$ n=3, one-way ANOVA; Figure 3-5a, Figure 3-5b, Figure 3-5c). However, there were significant differences in all three mean body morphometric parameters for the thiamethoxam and neonicotinoid mixture exposures between the control and treatment groups. Specifically, for thiamethoxam, there was a lower mean length (±SE) in the control group of 31.08 ±0.16 mm and all the treatment groups mean length ranged from 32.1 ±0.2% to 32.5 ±0.2mm ($p < 0.05$, one-way ANOVA followed by a Tukey's post-hoc test; n = 3; Figure 3-6a). There were significant differences in mean weights between the control group and the 150 µg/L thiamethoxam group, where the mean weight (±SE) for the control group was 0.20 ±0.003g and the thiamethoxam group was 0.21 ±0.001g ($p < 0.05$, non-parametric Kruskal-Wallis test, followed by a Steel-Dwass all pairs test, n = 3; Figure 3-6b). There was also a significant difference in the condition factor between the control group and the15 µg/L thiamethoxam group where the mean condition factor (±SE) was 0.68 ±0.7K and the thiamethoxam group was 0.62 ±0.6K ($p < 0.05$, non-parametric Kruskal-Wallis test, followed by a Steel-Dwass all pairs test; n = 3 Figure 3-6c).

For the acute neonicotinoid mixture exposures, significant differences in mean length, weight, and condition factor were observed between the control group and some of the treatments. Mean length (±SE) of the control group was 32.8 ±0.6mm. The mean length (±SE) of the 0.45 µg/L of the neonicotinoid mixture was 31.0 ±0.6mm, indicating these fish were slightly smaller ($p < 0.05$, non-parametric Kruskal-Wallis test, followed by a Steel-Dwass all pairs test; n = 3; Figure 3-7a); however, the rest of the mean lengths in the remainder of the treatments were similar to the control. Mean weight (±SE) of the control group was 0.24 ±0.003g; whereas the mean weight (±SE) of 4.5 and 45 µg/L of the neonicotinoid mixture was 0.26 ±0.002g, indicating these fish were slightly larger ($p < 0.05$, non-parametric Kruskal-Wallis test, followed by a Steel-Dwass all pairs test; n = 3; Figure 3-7b). Lastly, there was also evidence of a difference in mean condition factor (±SE) between the control group (0.73 ±0.07K) and the 0.45 µg/L of the neonicotinoid mixture (0.84 ±0.02K), indicating that these fish had a higher K-value ($p < 0.05$, non-parametric Kruskal-Wallis test, followed by a Steel-Dwass all pairs test; n = 3;Figure 3-7c).

Only four deformities were observed in the acute exposures. Two grade 3 fin fold deformities were identified in the control and 150 µg/L clothianidin exposures. One grade 2 and

one grade 3 fin fold deformities were identified in the 0.15 µg/L thiamethoxam and 0.15 µg/L neonicotinoid mixture exposures. No significances differences were observed for the proportion of deformities ($p < 0.05$, non-parametric Kruskal-Wallis test, followed by a Steel-Dwass all pairs test).

3.3.2 Chronic Neonicotinoid Mixture Exposure

3.3.2.1 Water Chemistry and Water Quality

The chronic mixture exposure included sockeye that were reared in an equal mass of waterborne clothianidin, imidacloprid, and thiamethoxam, from one hour post fertilization through to the swim up and feeding fry life stages. Water samples were submitted to ALS Environmental on day 93 and 163 of the waterborne exposure to an equal part mixture of neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) at 5 concentrations (0.045, 0.45, 4.5, 45, and 450 µg/L total neonicotinoids/L) and a water control. Table 3-7, shows the difference between predicted nominal concentration and the measured concentration. Generally, for predicted exposure concentrations of 0.045 and 0.45 µg/L, measured concentrations were slightly greater than equalling 0.078 and 0.47 µg/L. Conversely, predicted exposure concentrations of 4.5, 45, and 450 µg/L were slightly less than the measured concentrations equalling 3.84, 35.9, and 404 µg/L, respectively. A certificate of Analysis, including the detailed analytic table was provided by ALS Environmental, Burnaby BC and presented in Appendix A.

Water quality parameters that were measured included water temperature, water pH, conductivity, dissolved oxygen, and ammonia concentration. All parameters fell within the guidelines outlined in GoC (1998). The results are presented in (Table 3-8). In summary, temperature ranged from 1.3 to 14.4ºC with a mean of 10.1ºC; pH ranged from 6.9 to 8.2 with a mean of 7.1; dissolved oxygen ranged from 8.0 to 12.2 mg/L with a mean of 8.5 mg/L; conductivity ranged from 23.2 to 39.1 µS/cm, with a mean of 27.0 µS/cm; and ammonia, measured in mg/L was not detected throughout the whole exposure.

3.3.2.2 Hatch and Survival Success, Morphometrics, and Deformities Swim Up and Feeding Fry

The control group mean hatch success (±SE) for crosses A, B, C, and D ranged from 78 ±4.5% to 99 ± 0.7% to (Figure 3-8a). The mean hatch success (± SE) of the treatment groups for all crosses ranged from 83 ± 7.4% to 100 ± 0%. There were no significant differences across treatments, relative to the control groups; however, there was significant differences between crosses; where cross B mean hatch success (±SE) was lower at 85 ±2% relative to the rest of the crosses ranging from 92.2 ±2.0% to 96.5 ± 0.9 ($p < 0.5$, RCB ANOVA, followed by a Tukey's post hoc test). Survival success (±SE) ranged from 85 ±1 to 100 ±0%, 94 ±18%, and 100 ±0%, for clothianidin, thiamethoxam, and the neonicotinoid mixture, respectively ().

Swim up fry body morphometrics include length (mm), weight (g), and condition factor (K). The mean length and weight (±SE) of the control and the neonicotinoid mixture groups were 30.4 ±0.7mm (controls), 30.6 ±0.3mm (treatment) and 0.18 ±0.1g (controls and treatment), respectively (Figure 3-9a and Figure 3-9b). The mean condition factor (±SE) of all groups was 0.59 to 0.60 ±0.005K (Figure 3-10). There were no significant differences between the control and neonicotinoid mixture groups; however, there was significant differences between genetic crosses for the body morphometric parameters ($P > 0.5$, SP-RCB ANOVA). The mean length and weight (±SE) of the feeding fry control group were 34.8 ±0.5mm and 0.32 ±0.5g, respectively, and the neonicotinoid mixture groups ranged from 34.1 to 34.5 ±0.3mm and 0.28 ±0.5g, respectively (Figure 3-11a and Figure 3-11b). The mean condition factor (±SE) of the control group was 0.73 ±0.02 K and 0.69 ±0.01 K for the treatment group. No significant differences were observed between treatment groups ($P > 0.5$, RCB ANOVA, followed by a Tukey's post hoc test; Figure 3-12.

Only 25 individuals with deformities were observed in the chronic exposures for all crosses, out of a total of 2,523 fish. Fifteen of the 25 individuals with deformities were from the control groups. All types and ranks of deformities were observed, including two two-headed fish (from the 0 µg/L and 0.015 µg/L neonicotinoid mixture group). No significant differences were observed between the control and treatment groups ($p < 0.05$, non-parametric Kruskal-Wallis test, followed by a Steel-Dwass all pairs test).

3.3.2.3 Sex Steroid Hormones

Several inter and intra assay QA/QC methods were used to confirm the quality of the hormone analysis. The first method considered R^2 values and CVs to confirm detection limits of the assays. The detection limits of each plate were determined by a linear regression, by plotting the corrected absorbance with the eight standard curve points, while maintaining and R^2 of > 0.99 and CVs less than 28%. As such, mean CVs of the standard curves for 17β-estradiol (±SE) ranged from 0 to 28 (±1.2%) and testosterone ranged from 4.5 to 12.4 (±2.0%). Given this, detection limits for 17β-estradiol ranged from 6.6 to 4,000 pg/ml and testosterone ranged from 3.9 to 500 pg/ml. No samples exceeded the detection limits and no non-detect samples were observed. However, recovery efficiency (±SE) was low at 29.0 ±4.8% for 17β-estradiol and 11.6 ±5.0% for testosterone.

Another QA/QC method was the Intra plate CVs that shows the variability of the technical replicates within each plate. For all technical replicates, CVs greater than 28% were excluded for further analysis. The mean CV (±SE) of technical replicates for all the samples for the 17β-estradiol and testosterone assays were 9.7 ±4.9% and 11.6 ±6.0%, respectively. One fish exposed to 4.5 µg/L neonicotinoid mixture was excluded from the 17β-estradiol analysis based on a CV greater than 28%.

The final QA/QC method considered was the inter plate variability that was calculated by using a standard curve point from each plate to obtain a CV. The CV of technical replicates for all plate combinations from one point of the standard curve for 17β-estradiol and testosterone ranged from 5.6 to 17.8% and 1.1 to 6.4%, respectively. Therefore, all plates for both hormones were included for further analysis.

Two other samples were excluded from analysis. Both were excluded based on being identified as an outlier using a Grubb's test. The first exclusion was one fish exposed to 0.45 µg/L neonicotinoid mixture from the 17β-estradiol analysis and had an approximately 4 to 5-fold increase of whole-body 17β-estradiol relative to all other samples. The second exclusion was a fish exposed to 0.045 µg/L neonicotinoid mixture that had a 3 to 4-fold increase of whole-body testosterone, relative to all other samples.

After the QA/QC analysis, the remainder of fish were included for analysis of whole-body sex steroid hormone concentrations. Results of the ELISA assays showed no significant differences in mean whole-body 17β-estradiol or mean whole body testosterone concentrations across the treatments ranging from 0 to 450 µg/L neonicotinoid mixture ($p > 0.5$, Wilcoxn Test, followed by a Steel-Dwass all pairs test; n = 2 to 3 with 7 to 9 individuals per treatment; (Figure 3-13a and Figure 3-13b). Mean (±SE) 17β-estradiol concentration of the control group was 669.7 ±235.6 pg/ml/g body weight; whereas mean 17β-estradiol concentrations of the treatment groups ranged from 841.2 ±172.4 pg/ml/g body weight to 1039.9 ± 374.6 pg/ml/g body weight, with the greatest concentration found in the 45 µg/L neonicotinoid mixture concentration. The mean testosterone concentration (±SE) of the control group was 70.4 ±16.7 pg/ml/g body weight, whereas mean testosterone concentrations of the treatment groups ranged from 66.2 to 98.3 ±48.3 pg/ml/g body weight (Figure 3-13a and 3-13b).

3.3.2.4 Hepatic Gene Expression

3.3.2.4.1 RNA Quantity, Purity, and Integrity

The RNA integrity values (RINs) for all swim up fry used in this study ranged from 8.4 to 10 (RIN average ± 9.6; standard deviation = ± 0.2, n = 2-3) and the feeding fry ranged from 8.2 to 10 (RIN average ± 9.5; standard deviation = ± 0.3, n = 2-3. As per MIQE guidelines, outlined in Bustin et al. (2010), samples included for subsequent qPCR experiments had total DNase-treated RNA samples that were devoid of significant contamination and RNA degradation with an RNA RIN score of > 8.0 and $OD_{260/280}$ and $OD_{260/230}$ ratios of 1.8–2.1.

3.3.2.4.2 Primer Set Evaluation

Quantitative polymerase chain reaction (qPCR) was used to determine the relative quantitation of a selection of GOIs to evaluate any change of gene expression in swim up and feeding fry sockeye livers. A series of primers were designed for approximately 20 genes relating to either the endocrine, stress, immune, nervous systems, or general toxicity. Using qPCR for evaluation, only ten genes meet the ideal parameters for all qPCR data as outlined in the MIQE guidelines, and included an acceptable efficiency between 90-110%, one sharp melt peak, a 4-point standard curve minimum, and an $R^2 > 0.900$ (Bustin et al. 2010). These GOI used in the qPCR experiments, summarized in Table 3-2, and include estrogen receptor α, estrogen receptor β1, estrogen receptor β2 (relating to the reproduction system); thyroid receptor α, growth hormone

1, growth hormone 2 (relating to growth and development); glucocorticoid receptor 2 and suppressor of cytokine signaling 3 (relating to immune and stress); and cytochrome P450 family 1, subfamily A, polypeptide 1, and Catalase (relating to general toxicity). Primer sets that were excluded are summarized in Table 3-3. While estrogen receptor alpha had an efficiency of 116%, it was still considered acceptable because the melt curve analysis showed only one sharp melt peak and the $R^2 = 0.999$ (Table 3-5). Cq values of the technical replicates were determined using the CFX Manager™ Gene Expression Software (Bio-Rad, 2010). Mean Cq values for these GOIs for swim-up fry and feeding fry, ranged from 24.1 to 43.9 and 22.2 to 41.6, respectively. Target stability function/reference gene stability (i.e., M-values) were < 1 and also determined using the CFX Manager™ Software. Reference genes that met the M-value criteria for the swim up fry were beta-actin, glyceraldehyde 3-phosphate dehydrogenase, and 60S ribosomal protein L8; whereas for the feeding fry only two genes, beta-actin and elongation factor 1-alpha met the criteria.

The specificity of all primers used in the study were previously tested and verified by sequencing in either peer-reviewed scientific journals or publications in prep for submission. The primers were also evaluated again in this study by melt curve analysis (using Bio-Rad CFX Manager™ Software). This was done by confirming a single amplified product peak for all the genes, as this verifies the primers did not generate any unspecific products. These single melt curve peaks occurred at 82.0 to 87°C for all primers, including the reference gene primers, with the results summarized in Table 3-5.

Primer sets for GOIs that were excluded based on multiple melt peaks and poor efficiency include acetylcholine esterase receptor (AChE), androgen receptor-alpha and beta (ARα and ARβ), deiodinase 2, (Dio2), glucocorticoid receptor 1 (GR1), nicotinic acetylcholine receptor alpha 9-I subunit (NAChRα9-1), and vitellogenin (VTG). Poor efficiency was considered outside the optimal range of 90 to 110%, where these primer sets had an efficiency of >110%. Thyroid hormone receptor beta (THRβ) only had one melt peak but excluded based on an efficiency >110%. Mineralocorticoid receptor (MR) and suppressor of cytokine signaling 2 (SOCS2) were excluded based on multiple melt peaks and low expression. Low expression was determined when standard curves with Cq values were only above baseline for the 3 most concentrated standard curve points (50, 12.5, and 3.125 ng/µL) even after multiple attempts to amplify and quantify mRNA expression levels in both swim-up and feeding fry life stages. Lastly, elongation

factor 1-alpha (EF1α) and GAPDH were excluded as reference genes in the feeding fry and swim up fry qPCR experiments, respectively based on a high stability M-value for these life stages.

Attempts to design and run unique primers for the androgen receptors gene targets were not successful, likely based on the high sequence similarity with the subtypes of the androgen genes (ARα and ARβ), as also reported by Marlatt et al. (2019) and Calbick (2018). Calbick (2018) reported a study by Takeo et al. (1999) that showed the comparison of the predicted amino acid sequence of ARα to that of ARβ for rainbow trout showed an 85% identity. Calbick (2018) reported using the EMBOSS Needle Pairwise Sequence Alignment tool (https://www.ebi.ac.uk/) to align the mRNA nucleotide sequences for both androgen receptors that were obtained from the GenBank National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) database for rainbow trout and also showed a high identity of 90.1% between the two sequences. Similar to Marlatt et al. (2019) and Calbick (2018), both ARα and ARβ in this study resulted in multiple melt curve peaks. The melt curve analysis indicated that these peaks were detected likely due to the presence of non-specified products or multiple subtypes being amplified instead of the presence of primer dimers (primer-dimers occur when two primers bind to each other instead of the target). This conclusion was made because primer-dimers show a lower melting temperature relative to the amplicon, and often form in the no-template controls (NTCs), due to an abundance of primer and no cDNA template being present (Life Technologies 2012). However, the NTCs for the androgen genes displayed no amplification; therefore, indicating no primer-dimer formation or contamination and likely non-specific primers that were binding to multiple amplicons was the case.

3.3.2.4.3 Gene Expression

Due to a high M-Value, only two reference genes (Beta actin and elongation factor 1 alpha) were used for the feeding fry life stage (versus three for the swim up fry life stage; beta actin, glyceraldehyde 3-phosphate dehydrogenase, and 60S ribosomal protein L8). Reference genes with a high M-value include *EF1a* (swim up only); GAPDH and RPL8 (feeding fry).

For the ten genes of interest in swim up and feeding fry (excluding reference 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 differences were observed in any treatments for all the genes in either life stage ($p > 0.05$; Figure 3-14 to Figure 3-25).

3.4 Tables

Table 3-1. The volume of stock solution and dechlorinated municipal water that was combined to achieve the nominal, waterborne neonicotinoid mixture exposure concentrations used for chronic sockeye salmon experiments. The stock solution was comprised of a neonicotinoid mixture of equal mass of 150 mg per L.

Nominal Concentration (µg/L)	Stock Solution Volume (ml)	Dechlorinated Water (L)
0	0	19.0
0.045	0.257	19.0
0.45	2.565	19.0
4.5	25.65	19.0
45	256.5	18.7
450	2565	16.4

Notes: µg/L = microgram per litre; ml = millilitre; L = litre.

Table 3-2. Successful primer sets used to examine the effects of the neonicotinoid mixture, consisting of equal parts of clothianidin, imidacloprid, and thiamethoxam, on various genes of interest (GOI) in sockeye salmon swim-up and feeding 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 beta-actin (ACTβ), elongation factor 1-alpha (EF1α), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and, 60S ribosomal protein L8 (RPL8); and target genes catalase (CAT), Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A), Estrogen receptor alpha (ERα), Estrogen receptors beta 1 and 2 (ERβ1 and ERβ 2), Growth hormone receptors 1 and 2 (GHR1 and GHR2), Glucocorticoid receptor 2 (GR2), Suppressor of cytokine signaling 3 (SOCS3), and Thyroid hormone receptor alpha (THRα).

	Target Gene	Accession No.	Primer Sequences (5'-3')		Tm	Product Size (bp)	Swim-Up Fry		Feeding Fry		Reference
							%E	R^2	%E	R^2	
Reference genes	*ACTβ* Beta-Actin	AB481206	Forward	GGACTTTGAGCAGGAGATGG	57.96	96	103.9	0.990	106.4	0.998	Marlatt et al. (2019)
			Reverse	TCGTTGCCGATTGTGATG	55.81						
	EF1a Elongation factor 1-alpha	XM_029663596.1	Forward	GGTGGTGTGGGTGAGTTTGA	60.11	79	-	-	104.1	0.981	Marlatt et al. (2019)
			Reverse	CCAGAGTGTAGGCGAGGAGA	60.39						
	GAPDH Glyceraldehyde 3-phosphate dehydrogenase	XM_021562009	Forward	CCATCACAGCCACACAGAAG	58.84	139	102.6	0.996	-	-	Marlatt et al. (2019)
			Reverse	CCATTCAGCTCGGGGATAA	56.58						
	RPL8 60S ribosomal protein L8	AB889392.1	Forward	TTGGTAATGTTCTGCCTGTG	55.95	130	108.9	0.999	-	-	Veldhoen et al. 2010
			Reverse	GGGTTGTGGGAGATGACTG	57.44						
Genes of Interest	*CAT* Catalase	XM_021568213.2	Forward	GCCAAGGTGTTTGAGCAT	55.85	185	95.5	0.993	104.5	0.992	Veldhoen et al. 2010
			Reverse	GCGTCCCTGATAAAGAAGAT	54.94						
	CYP1A1 Cytochrome P450, family 1, subfamily A, polypeptide 1	XM_029641704.1	Forward	GCCTGTGGTTGTTCTGAGTG	56.60	317	103.9	0.990	106.0	0.994	Duarte-Guterman et al. (in prep)
			Reverse	TCTTGCCGTCGTTGATGA	56.21						
	ERα Estrogen receptor alpha	XM_029676443	Forward	CCTGGAGATGCTGGACGGT	58.36	142	116.9	0.999	104.2	0.991	Marlatt et al. (2019)
			Reverse	CCTGTGGAGGTGGTAGTGGT	58.86						
	ERβ1 Estrogen receptor beta 1	DQ177439	Forward	TGTAGATTCCGACGAGACT	61.08	133	104.9	0.994	104.7	0.976	Marlatt et al. (2019)
			Reverse	TCATGGCAGAGATGGTGAA	60.23						
	ERβ2 Estrogen receptor beta 2	DQ248229	Forward	GAGCATCCAAGGTCACAATG	59.38	125	103.8	0.989	105.8	0.992	Marlatt et al. (2019)
			Reverse	CACTTTGTCATGCCCACTTC	59.63						
	GHR1 Growth hormone receptor 1	JQ408978.2	Forward	CTTCAGACAGGAGAGGCGTA	58.54	142	108.0	0.994	104.0	0.978	Duarte-Guterman et al. (in prep)
			Reverse	GGACCAGAGTCACAGGGAAC	59.68						

Target Gene	Accession No.	Primer Sequences (5'-3')		Tm	Product Size (bp)	Swim-Up Fry		Feeding Fry		Reference
						%E	R^2	%E	R^2	
GHR2 Growth hormone receptor 2	AY573600.2	Forward	GCTCTGGCGATCAGGATAAT	57.24	115	108.0	0.994	104.2	0.997	Duarte-Guterman et al. (in prep)
		Reverse	CCGGTTAGACCCACATTCAG	57.98						
GR2 Glucocorticoid receptor 2	NM_001124482	Forward	CTGGCTGATGACACTCTCCTG	60.13	70	108.1	0.994	94.7	0.997	Marlatt et al. (2019)
		Reverse	CTGGCTTGGAGGTGGAGTTG	60.61						
SOCS3 Suppressor of cytokine signaling 3	NM_001146168.1	Forward	TTCTCCTCCACTATGTCCAGCCT	62.50	62	105.0	0.971	103.8	0.961	Marlatt et al. (2019)
		Reverse	TCCAGGTGTCCGTTGACTGTT	61.58						
THRα Thyroid hormone receptor alpha	JX232610	Forward	CCTTCAGCGAGTTCACCA	56.91	132	107.0	0.981	108.1	0.981	Duarte-Guterman et al. (in prep)
		Reverse	TCTCCATACAGCAGCCTTTC	57.30						

Notes: %E = efficiency percent; bp = base pair.

Table 3-3. Primer sets used to amplify genes of interest (GOI) and reference genes in sockeye salmon swim-up and feeding fry but excluded from qPCR experiment (with exclusion rationale provided). Accession identifiers, primer sequences (5' to 3'), and product size are provided by National Center for Biotechnology and Information (https://www.ncbi.nlm.nih.gov/).

	Target Gene	Accession No.	Primer Sequences (5'-3')		Product Size (bp)	Life Stage Excluded From	Rationale for Exclusion	Reference
Reference Genes	*EF1a* Elongation factor 1-alpha	XM_029663596.1	Forward	GGTGGTGTGGGTGAGTTTGA	79	Feeding Fry	High M-Value (feeding fry only)	Marlatt et al. (2019)
			Reverse	CCAGAGTGTAGGCGAGGAGA				
	GAPDH Glyceraldehyde 3-phosphate dehydrogenase	XM_021562009	Forward	CCATCACAGCCACACAGAAG	139	Swim-up Fry	High M-Value (swim-up fry only)	Marlatt et al. (2019)
			Reverse	CCATTCAGCTCGGGGATAA				
	RPL8 60S ribosomal protein L8	AB889392.1	Forward	TTGGTAATGTTCTGCCTGTG	130			Veldhoen et al. 2010
			Reverse	GGGTTGTGGGAGATGACTG				
Genes of Interest	AChE (Acetylcholine esterase receptor)	NM131846.2	Forward	GACCGGAGTCACGTGA	113	Both		
			Reverse	CCT GTT GGG GTT CCA CAT C				
	ARα (Androgen receptor-alpha)	NM001124184.1	Forward	GCACACTTGGCACACTTCAG	185			Calbick (2018)
			Reverse	TCCGGCAGTTCATTATGCCT				
	ARβ (Androgen receptor-beta)	NM001124185.1	Forward	ACTGGAGAAACAATGGAGGACAAA	167		Multiple melt peaks and poor efficiency (>110%)	
			Reverse	ATACCCTGCCTCGGTCCAT				
	Dio2 (Deiodinase 2)	NM131846.2	Forward	ATCACTGGAAGAAAGGGTGG	128			Quesada-Garcia et al., 2014
			Reverse	TCTCGTTGGACACACCGTAG				
	GR1 (Glucocorticoid receptor 1)	Z54210	Forward	TCAGCAGTCCAAAGGCAAGAC	377			Calbick 2018
			Reverse	ACGACGATGGAGCCGAAC				
	NAChRα9-1 (Nicotinic acetylcholine receptor alpha 9-I subunit)	NM001124439.1	Forward	CCACCACAGCACCCATCAAA	187			Calbick (2018)
			Reverse	CTCCTCTCTCCCGATGTGGT				
	MR Mineralocorticoid receptor	NM_001124483.1	Forward	GAGAGCCGTAGAAGGTAGGAAA	191		Multiple peaks and low expression	Becker et al., 2008
			Reverse	CGAGTCTTCGTTCACACCCTT				
	SOCS2 Suppressor of cytokine signaling 2	NM_001114550.1	Forward	TGTAGATTCCGACGAGACT	178			Calbick (2018)
			Reverse	TCATGGCAGAGATGGTGAA				
	THRβ (Thyroid hormone receptor beta)	JX232611	Forward	CTACGACCCCGAGAGTGAGA	119		Poor efficiency (>110%)	Duarte-Guterman et al. (in prep)
			Reverse	GCGATAAGCCCAAATCAAAG				
	VTG Vitellogenin	XM_036969074.1	Forward	CTGTGTAAGCCAGACCAAAGTC	103		Multiple melt peaks and poor efficiency (>110%)	
			Reverse	ATCAGGGAAGATGCCAAGG				

Table 3-4. A summary of the number of tanks and individual biological replicates used for the qPCR experiments.

Neonicotinoid Mixture Concentration (µg/L)	Life Stage	Number of Tanks	Number of Individual Animals/ Biological Replicates[1]
0	Swim-up fry	3	9
	Feeding fry		
0.045	Swim-up fry	3	9
	Feeding fry		
0.45	Swim-up fry	3	9
	Feeding fry		7
4.5	Swim-up fry	2	8
	Feeding fry		9
45	Swim-up fry	3	8
	Feeding fry		7
450	Swim-up fry	3	8
	Feeding fry		

Notes:
[1]Two individual swim up fry livers were pooled into one sample in order to acquire enough RNA

Table 3-5 Melt curve peak temperatures for genes used for further analysis in this study.

Target Gene	Life Stage	Melt Curve Peak Temperature (°C)
Beta Actin (ACTβ)	Swim-up fry / feeding fry	84.4 / 84.5
Elongation Factor 1-Alpha (EF1α)	Feeding fry	83.5
Glyceraldehyde 3-Phospahte Dehydrogenase (GAPDH)	Swim-up fry	87.0
60S Ribosomal Protein L8 (RPL8)	Swim-up fry	87.0
Catalase (CAT)	Swim-up fry / Feeding fry	86.0
Cytochrome P450 1A1, CYP1A	Swim-up fry / Feeding fry	83.5
Estrogen Receptor Alpha (ERα)	Swim-up fry / Feeding fry	86.0 / 86.5
Estrogen Receptor Beta 1 (ERβ1)	Swim-up fry / Feeding fry	86.5
Estrogen Receptor Beta (ERβ2)	Swim-up fry / Feeding fry	84
Growth Hormone Receptor 1 (GHR1)	Swim-up fry / Feeding fry	82.5 / 83.0
Growth Hormone Receptor 2 (GHR2)	Swim-up fry / Feeding fry	82.5 / 82.0
Glucocorticoid Receptor 2 (GR2)	Swim-up fry / Feeding fry	82.5
Suppressor of Cytokine Signaling 3 (SOCS3)	Swim-up fry / Feeding fry	87.5
Thyroid Hormone Receptor alpha (TRα)	Swim-up fry / Feeding fry	83.5

Table 3-6. The water quality monitoring summary for the acute neonicotinoid exposures with sockeye salmon exposed for one-hour (until egg hardening), followed by transfer to clean water and reared until for 131 days post fertilization (dpf). Water quality monitoring occurred every 48 hours for all parameters except ammonia where monitoring was conducted weekly.

	Heath Stacks				
	Temp (°C)	**pH**	**Dissolved Oxygen (mg/L)**	**Conductivity (µS/cm)**	**Ammonia (mg/L)**
Mean	8.6	7.1	10.1	26.3	0
Minimum	1.3	6.9	8.9	21.0	0
Maximum	13	8.4	10.8	32.0	0
Standard Deviation	3.3	0.1	0.4	3.9	0
Observations (n)	139	130	130	130	0

Notes: °C = degrees Celsius; µg/L = micrograms per litre; µS/cm = micro siemens per centimetre; mg/L = milligram per litre.

Table 3-7. The neonicotinoid mixture predicted and measured concentrations in glass tanks for sockeye salmon chronic exposure experiment.

	Nominal Concentration 0 µg/L	**Nominal Concentration 0.045 µg/L**	**Nominal Concentration 0.45 µg/L**	**Nominal Concentration 4.5 µg/L**	**Nominal Concentration 45 µg/L**	**Nominal Concentration 450 µg/L**
			Measured			
Clothianidin	<0.005	0.029	0.156	1.29	12.1	133
Imidacloprid	<0.005	0.025	0.152	1.21	11.2	127
Thiamethoxam	<0.005	0.024	0.166	1.34	12.6	144
Total Neonicotinoid Mixture	<0.005	0.078	0.47	3.84	35.9	404
Detection Limit	0.005	0.005	0.005	0.005	0.005	0.005

Notes: all units are in µg/L; µg/L = micrograms per litre.

Table 3-8. The water quality monitoring summary for the neonicotinoid mixture exposure with sockeye salmon exposed for approximately 131 to 180 days from one-hour post fertilization through to the swim-up and/or feeding-fry life stage. Water quality monitoring occurred every 48 hours for all parameters except ammonia where monitoring was conducted weekly.

	Chronic Glass Tank Exposure				
	Temp (°C)	**pH**	**Dissolved Oxygen (mg/L)**	**Conductivity (µS/cm)**	**Ammonia (mg/L)**
Mean	10.1	7.2	8.5	27.0	0
Minimum	1.3	6.9	8.0	23.2	0
Maximum	14.4	8.2	12.2	39.1	0
Standard Deviation	3.7	0.1	0.8	4.1	0
Observations (n)	770	730	756	766	26

Notes: °C = degrees Celsius; µg/L = micrograms per litre; µS/cm = micro siemens per centimetre; mg/L = milligram per litre.

3.5 Figures

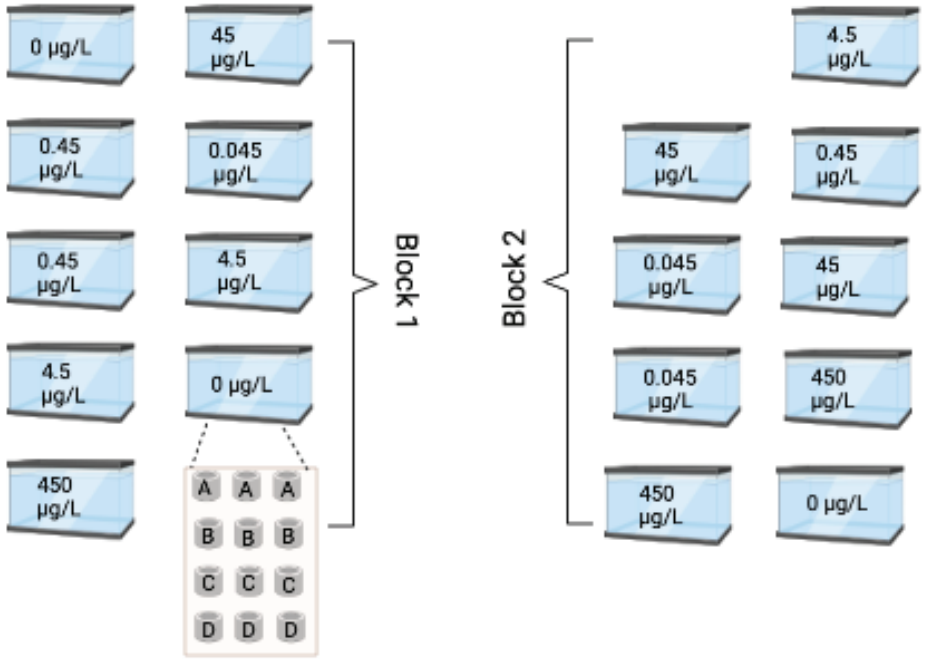


Figure 3-1. A schematic of the randomized split-plot complete block design of the chronic glass tank exposure system where sockeye were exposed to 0, 0.045, 0.45, 4.5, 45 and 450 µg/L of equal parts clothianidin, imidacloprid, and thiamethoxam. The test concentration (the main plot shown with the aquariums), assigned randomly, blocked in triplicate on either side of the room. Within each main plot was the split plot (the separation of genetic crosses, shown with the cylinders labelled A – D) and the genetic variation (the subplot, crosses A – D). In this exposure the genetic crosses were separated into individual egg containers (shown above as grey cylinders labelled A, B, C, D). The statistical model considered effects from the two different blocks, triplicate tanks, genetic cross, and an interactive effect of the crosses and exposure concentration.

3.5.1 Acute Exposures

Clothianidin Acute Exposure – Fertilization, Hatch, and Survival Success

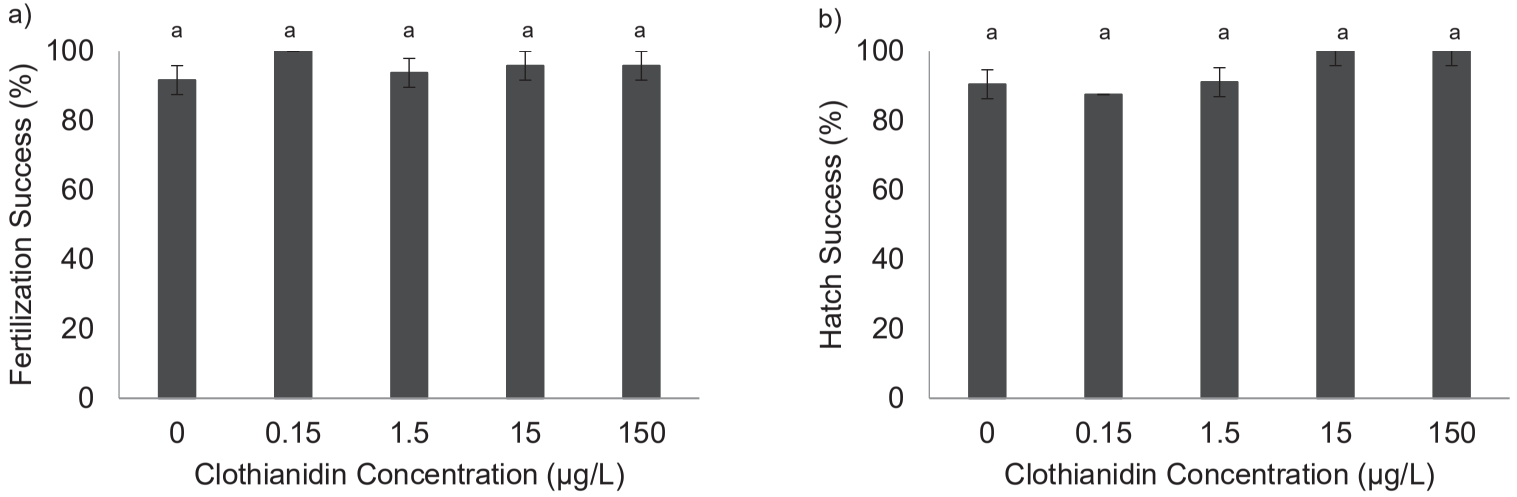


Figure 3-2a and Figure 3-2b. Fertilization and hatch success in Cross B sockeye salmon after acute exposure to waterborne clothianidin during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (n = 3). Fertilization and hatch success was based on the mean survival of each replicate reared in the egg containers (n = 6 to 8 individuals per container). Columns show the mean percentage of (a) fertilization success and (b) hatch success, with errors bars indicating standard error. There were no significant differences in mean fertilization and hatch success across treatments ($p > 0.05$ one-way ANOVA, followed by a Tukey's post hoc test).

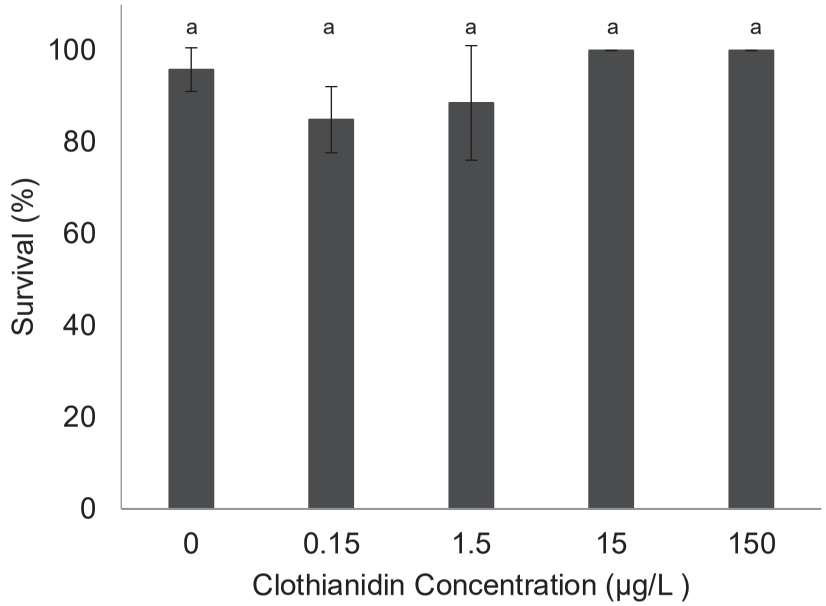


Figure 3-2c. Survival of Cross B sockeye salmon after acute exposure to waterborne clothianidin during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (*n* = 3). Survival, shown with the columns, was based on the mean survival of each replicate reared in the egg containers (n = 6 to 8 individuals per container), with error bars indicating standard error. There were no significant differences in mean survival success across treatments ($p > 0.05$ one-way ANOVA, followed by a Tukey's post hoc test).

Thiamethoxam Acute Exposure – Fertilization, Hatch, and Survival Success

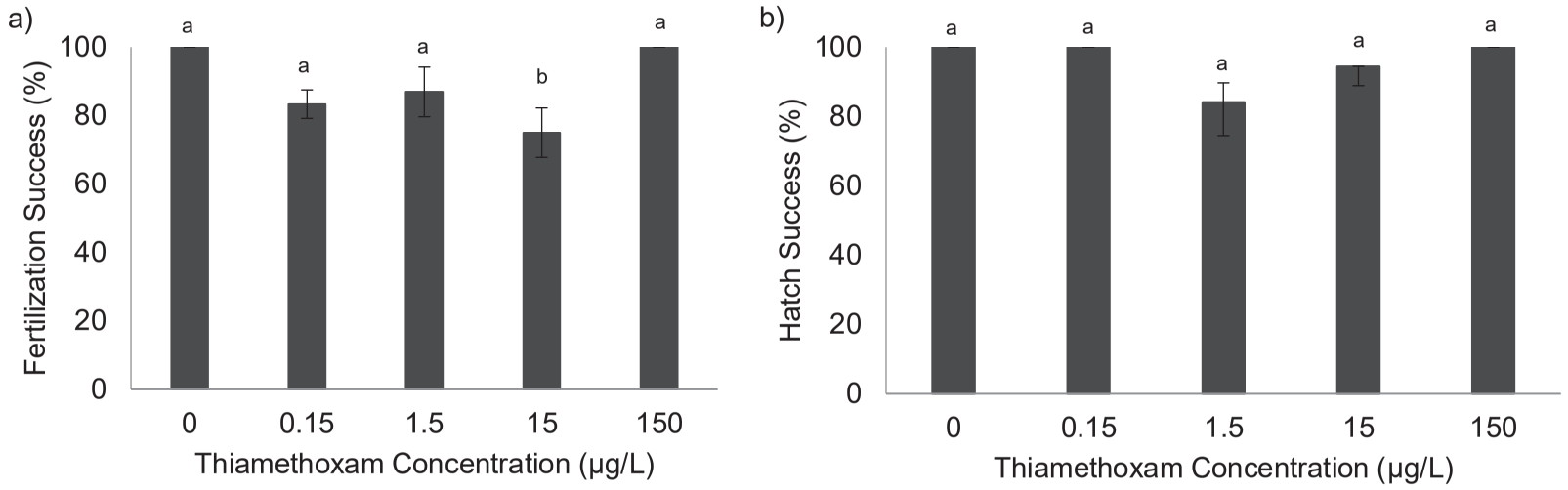


Figure 3-3a and Figure 3-3b. Fertilization and hatch success in Cross B sockeye salmon after acute exposure to waterborne thiamethoxam during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (*n* = 3). Fertilization and hatch success was based on the mean survival of each replicate reared in the egg containers (n = 6 to 8 individuals per container). Columns show the mean percentage of (a) fertilization success and (b) hatch success, with errors bars indicating standard error. There were significant differences in mean fertilization success between 0 µg/L and 15 µg/L thiamethoxam, where fertilization success decreased by 25 ±7% (SE; $p < 0.05$ one-way ANOVA, followed by a Tukey's post hoc test). Superscripts indicate the mean differences.

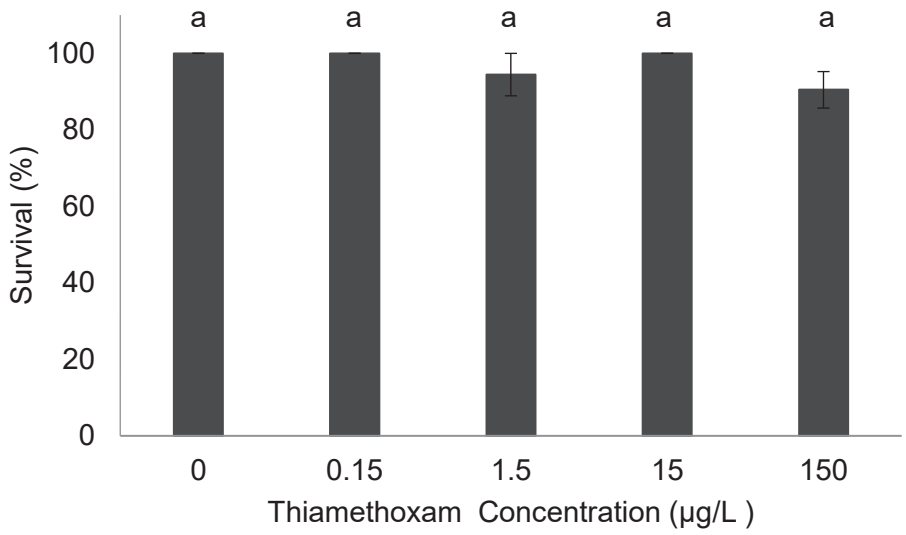


Figure 3-3c. Survival of Cross B sockeye salmon after acute exposure to waterborne thiamethoxam during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (*n* = 3). Survival, shown with the columns, was based on the mean survival of each replicate reared in the egg containers (n = 6 to 8 individuals per container), with error bars indicating standard error. There were no significant difference*s* in mean survival success across treatments (p > 0.05 one-way ANOVA, followed by a Tukey's post hoc test).

Neonicotinoid Mixture Acute Exposure – Fertilization, Hatch, and Survival Success

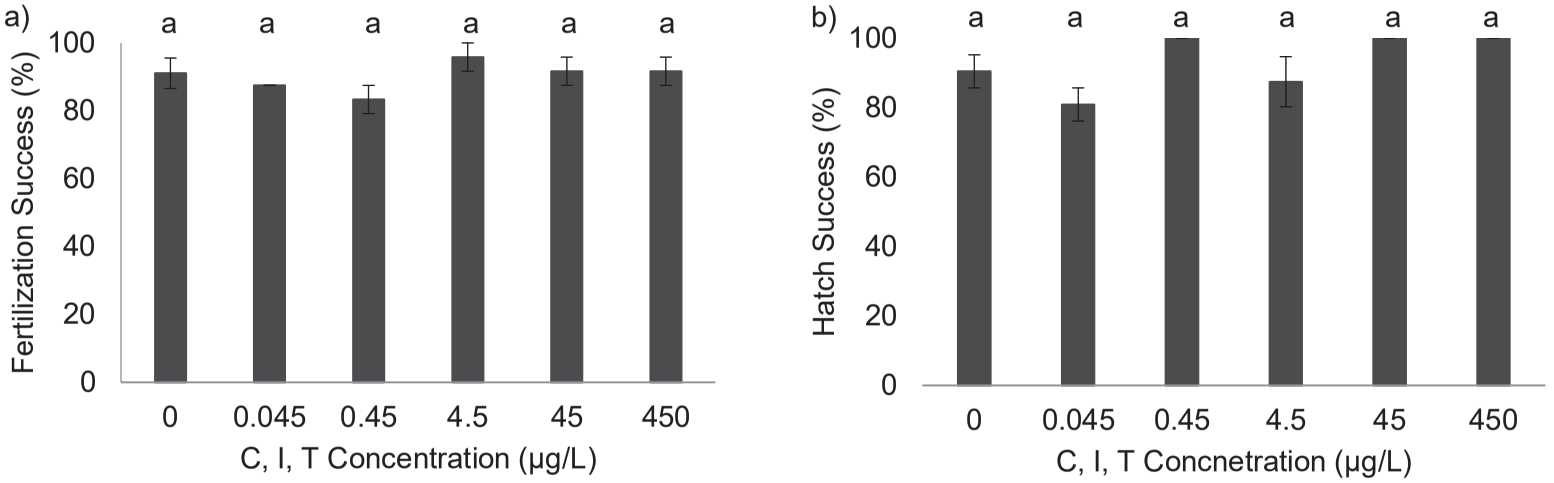


Figure 3-4a and Figure 3-4b. Fertilization and hatch success in Cross B sockeye salmon after acute exposure to waterborne neonicotinoid mixtures during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Fertilization and hatch success was based on the mean survival of each replicate reared in the egg containers (n = 6 to 8 individuals per container). Columns show the mean percentage of (a) fertilization success and (b) hatch success, with errors bars indicating standard error. There were no significant differences in mean fertilization or hatch success across treatments ($p > 0.05$ one-way ANOVA, followed by a Tukey's post hoc test).

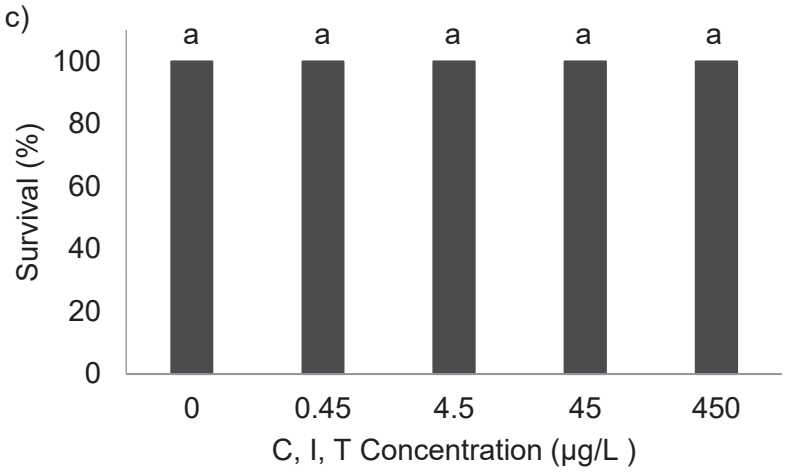


Figure 3-4c. Survival of Cross B sockeye salmon after acute exposure to waterborne neonicotinoid mixtures during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Survival, shown with the columns, was based on the mean survival of each replicate reared in the egg containers (n = 6 to 8 individuals per container), with error bars indicating standard error. There were no significant differences in mean survival success across treatments ($p > 0.05$ one-way ANOVA, followed by a Tukey's post hoc test).

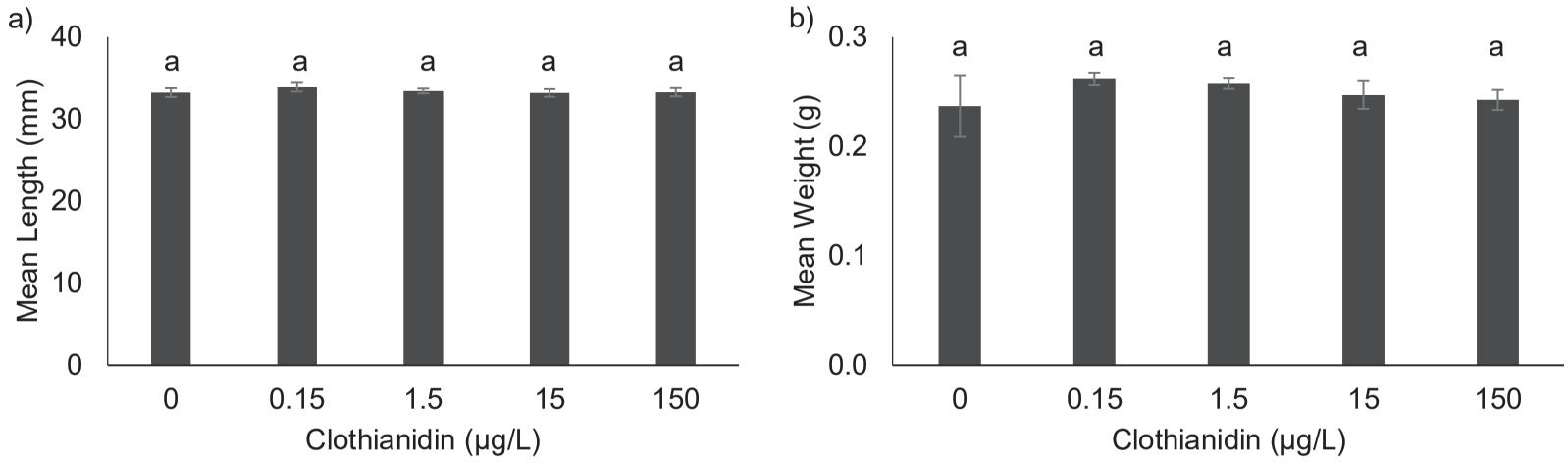


Figure 3-5a and Figure 3-5b. Mean length (mm) and weight (g) of Cross B sockeye salmon after acute exposure to the waterborne clothianidin during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (*n* = 3 with 6 to 8 individuals). Columns show (a) the mean length and (b) mean weight, with errors bars indicating standard error. There were no significant differences between the control and treatments mean lengths or weights (P>0.05; one-way ANOVA).

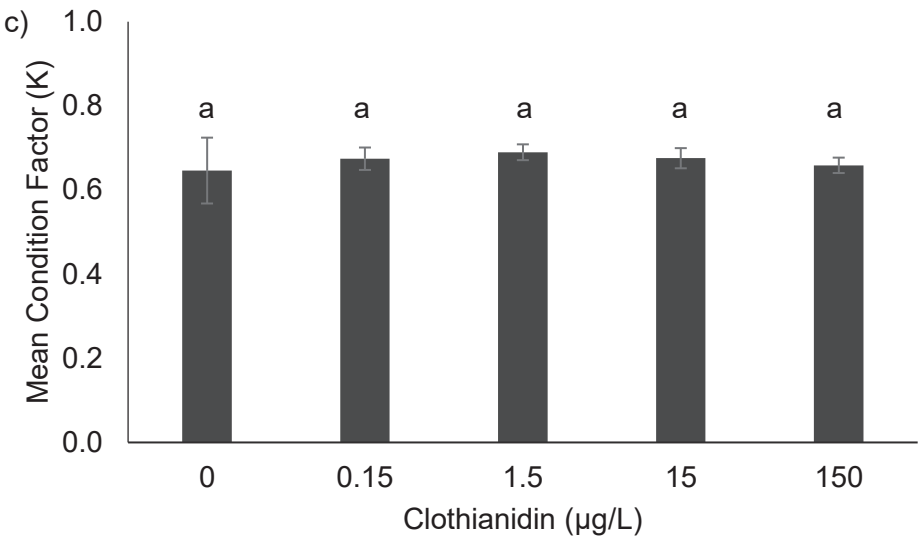


Figure 3-5c. Mean Condition Factor of Cross B sockeye salmon after acute exposure to waterborne clothianidin during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (*n* = 3 with 15 to 24 individuals per treatment). Columns show the condition factor, with errors bars indicating standard error. There were no significant difference*s* between the control and treatment mean condition factors (P>0.05; one-way ANOVA, followed by a Tukey's post hoc test).

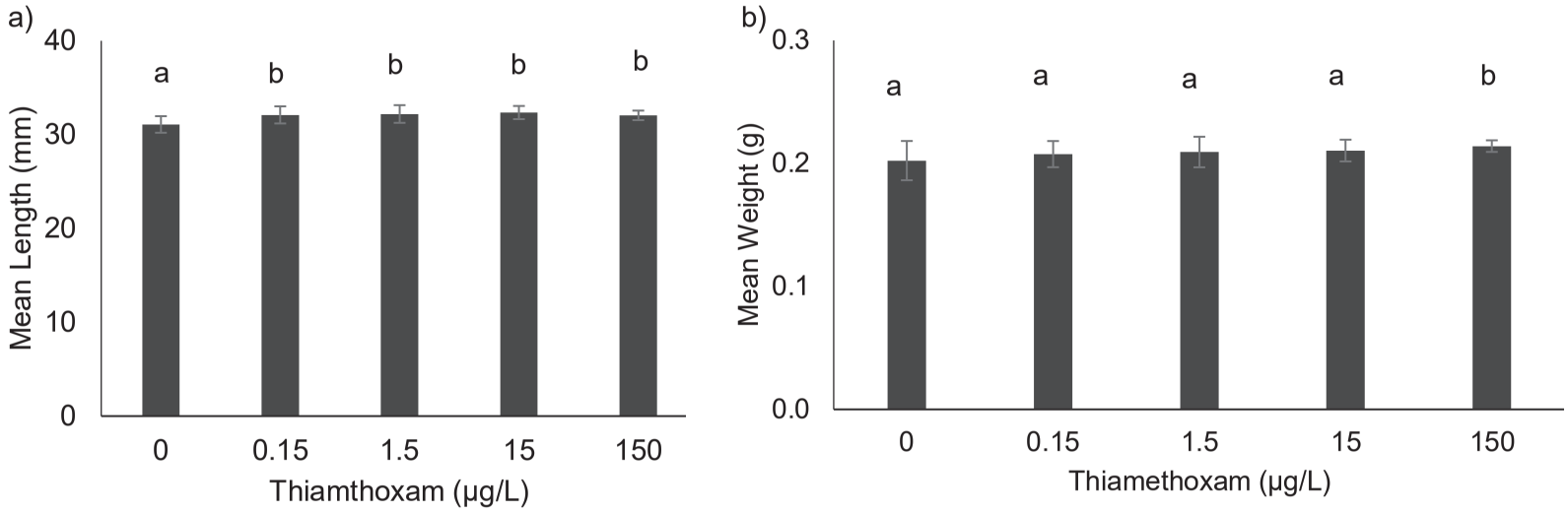


Figure 3-6a and Figure 3-6b. Mean length (mm) and weight (g) of Cross B sockeye salmon after acute exposure to waterborne thiamethoxam during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (*n* = 3 with 15 to 24 individuals per treatment). Columns show (a) the mean length and (b) mean weight, with errors bars indicating standard error. There were significant differences for (a) mean length ($p < 0.05$, one-way ANOVA, followed by a Tukey's post hoc test) and (b) mean weight ($p < 0.05$, Kruskal-Wallis test followed by a Steel-Dwass all pairs test). Differences are indicated by superscripts.

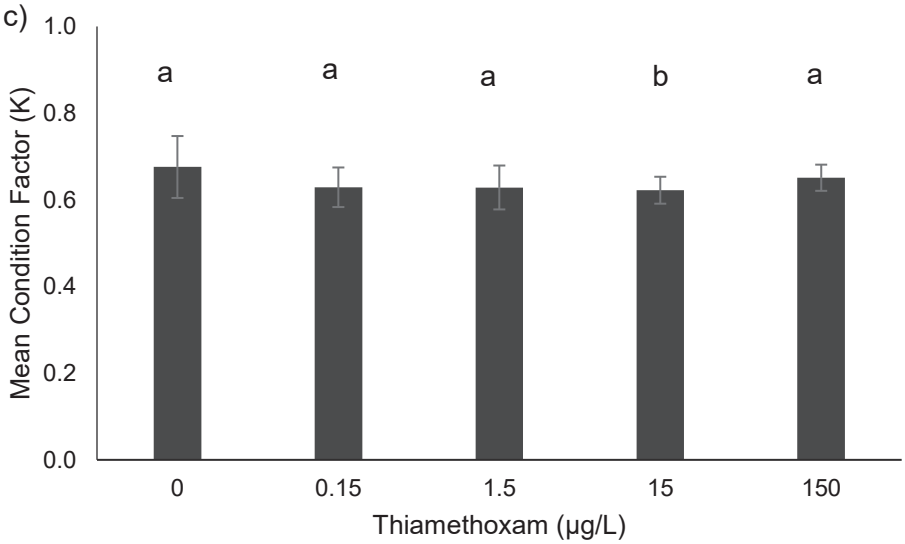


Figure 3-6c. Mean Condition Factor of Cross B sockeye salmon after acute exposure to waterborne thiamethoxam during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. The treatments include a water control, 0 µg/L; 0.15 µg/L; 1.5 µg/L; 15 µg/L; and 150 µg/L (*n* = 3 with 15 to 24 individuals per treatment). Columns show the condition factor, with errors bars indicating standard error. There were significant differences in mean condition factor ($p < 0.05$, Kruskal-Wallis test, followed by a Steel-Dwass all pairs test; n =3). Differences are indicated by superscripts.

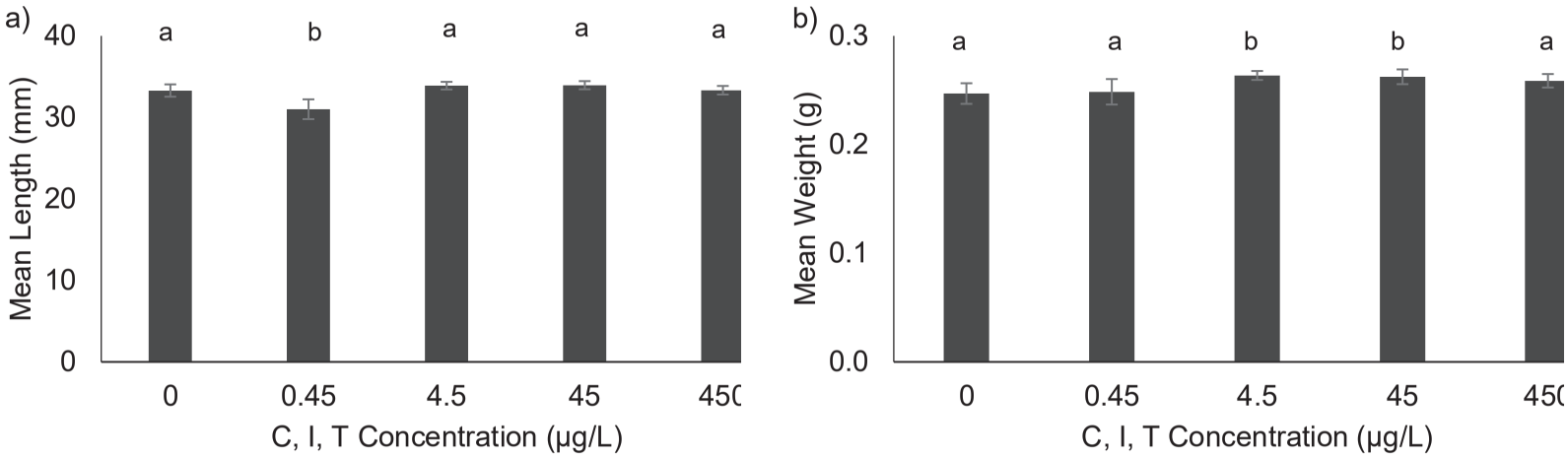


Figure 3-7a and Figure 3-7b. Mean length (mm) and weight (g) of Cross B sockeye salmon after acute exposure to waterborne neonicotinoid mixtures during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Columns show the (a) mean length and (b) mean weight with errors bars indicating standard error. There were significant differences in mean length and weight ($p < 0.05$, Kruskal-Wallis test, followed by a Steel-Dwass all pairs test; n =3). Differences are indicated by superscripts.

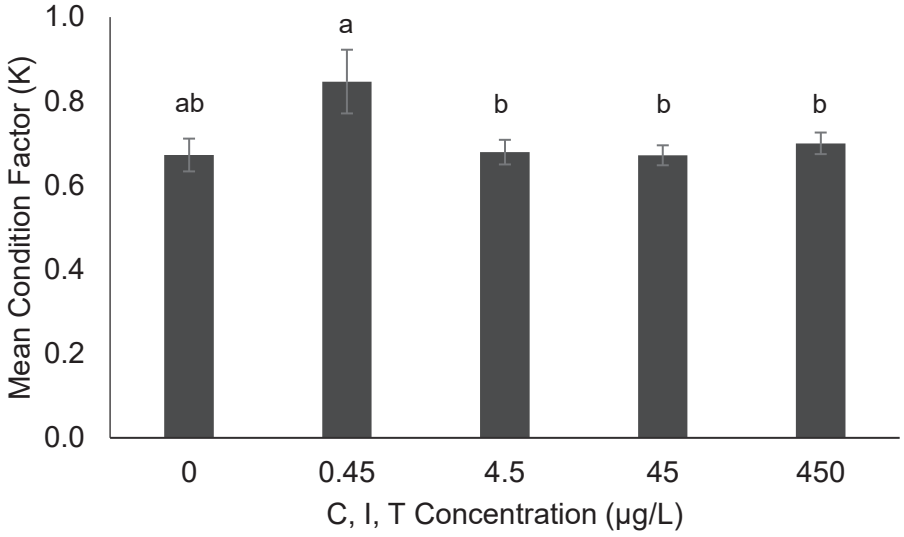


Figure 3-7c. Mean condition factor (K) of Cross B sockeye salmon after acute exposure to waterborne neonicotinoid mixtures during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Columns show the mean condition factor with errors bars indicating standard error. There were significant differences in mean condition factor ($p < 0.05$, Kruskal-Wallis test, followed by a Steel-Dwass all pairs test; n =3). Differences are indicated by superscripts.

3.5.2 Chronic Neonicotinoid Mixture Exposure

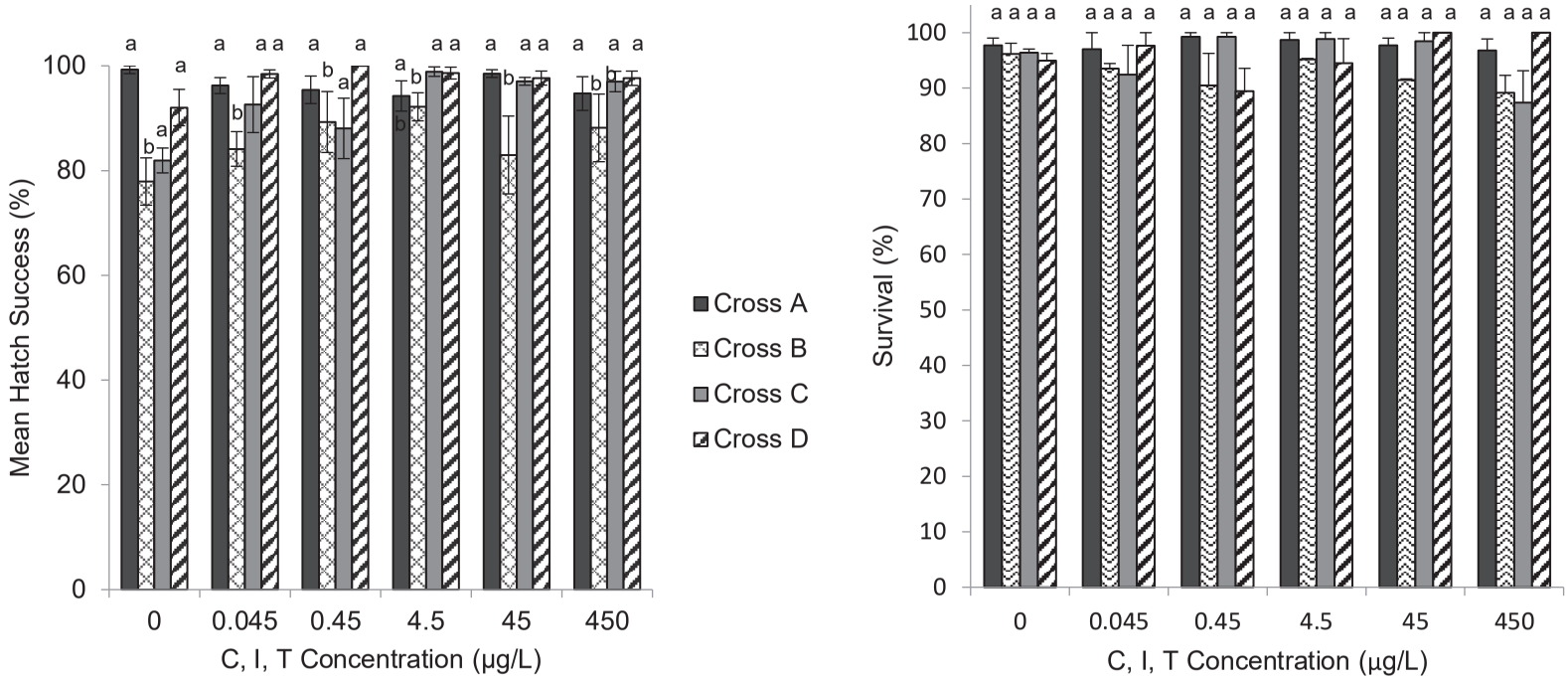


Figure 3-8. Hatch and Survival success in crosses A, B, C, and D sockeye salmon after chronic exposure to waterborne neonicotinoid mixtures during fertilization for one hour, followed by rearing in fresh water through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Hatch success was based on the mean survival of each replicate reared in the egg containers (n = 6 to 8 individuals per container). Columns show the mean percentage of (a) hatch success and (b) survival success, with errors bars indicating standard error. There were no significant differences in mean hatch or survival success across treatments ($P > 0.5$; RCB ANOVA), only significant differences between cross B hatch success and the rest of the crosses ($P < 0.5$, RCB ANOVA followed by a Tukey's post hoc test). Differences are indicated by superscripts.

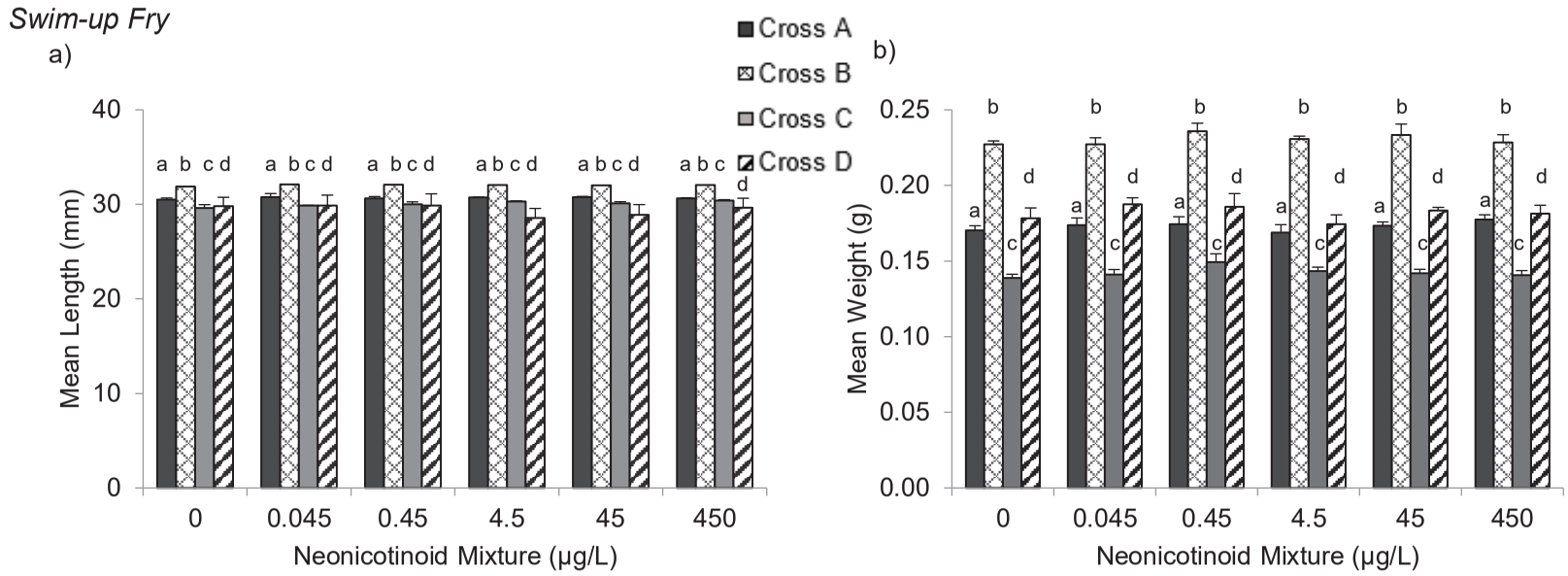


Figure 3-9. Mean length (mm) and weight (g) of Crosses A, B, C, and D sockeye salmon after chronic exposure to waterborne neonicotinoid mixtures from one hour through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Columns show the (a) mean length and (b) mean weight with errors bars indicating standard error. There were no significant differences across treatments (P > 0.5; SP-RCB ANOVA), only significant differences between all crosses. Differences are indicated by superscripts.

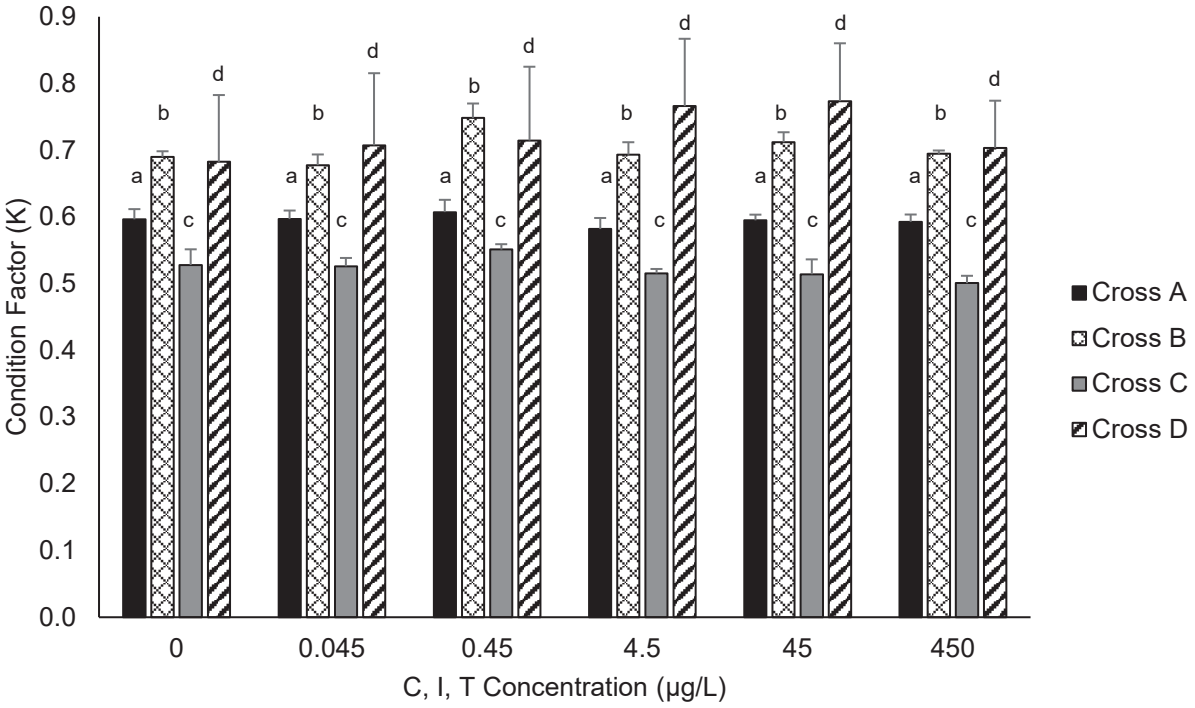


Figure 3-10. Mean condition factor (K) of crosses A, B, C, D sockeye salmon after chronic exposure to waterborne neonicotinoid mixtures from one-hour post fertilization through to the swim up fry. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Columns show the mean condition factor with errors bars indicating standard error. There were no significant differences across treatments (P > 0.5; RCB ANOVA), only significant differences between all crosses of the swim up fry. Differences are indicated by superscripts.

Feeding Fry

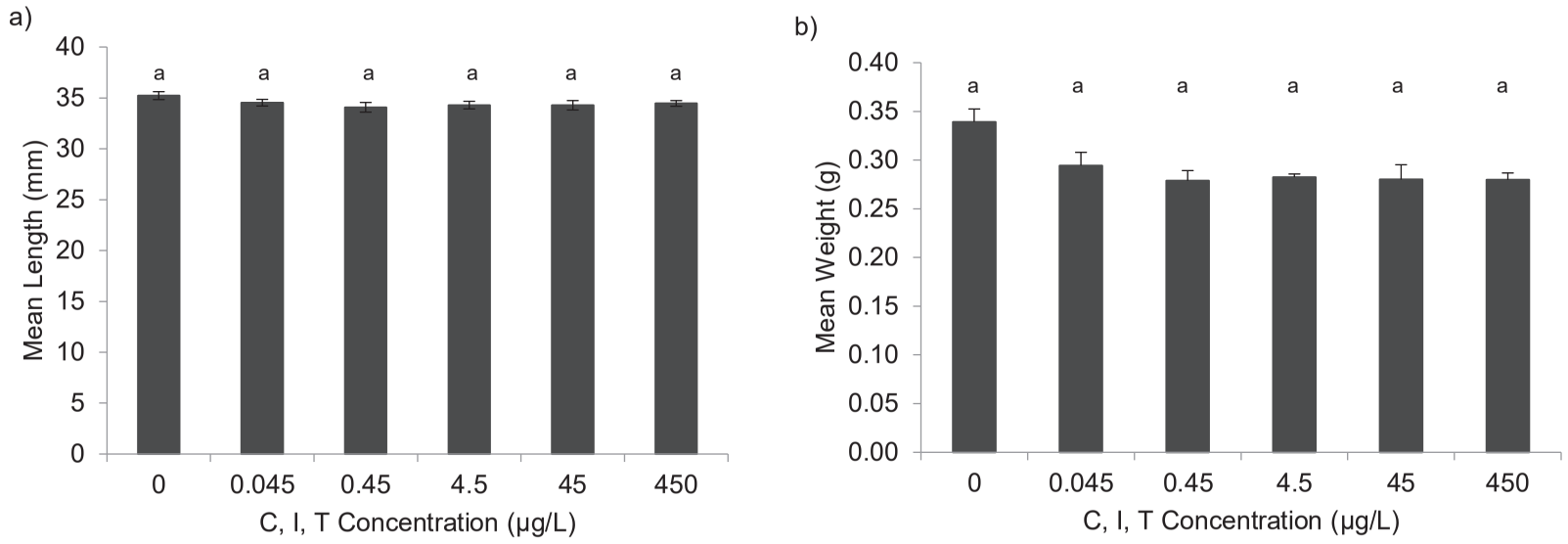


Figure 3-11. Mean length (mm) and weight (g) of Cross C sockeye salmon after chronic exposure to waterborne neonicotinoid mixtures for one hour through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Columns show the (a) mean length and (b) mean weight with errors bars indicating standard error. There were no significant differences across treatments (P > 0.5; RCB ANOVA).

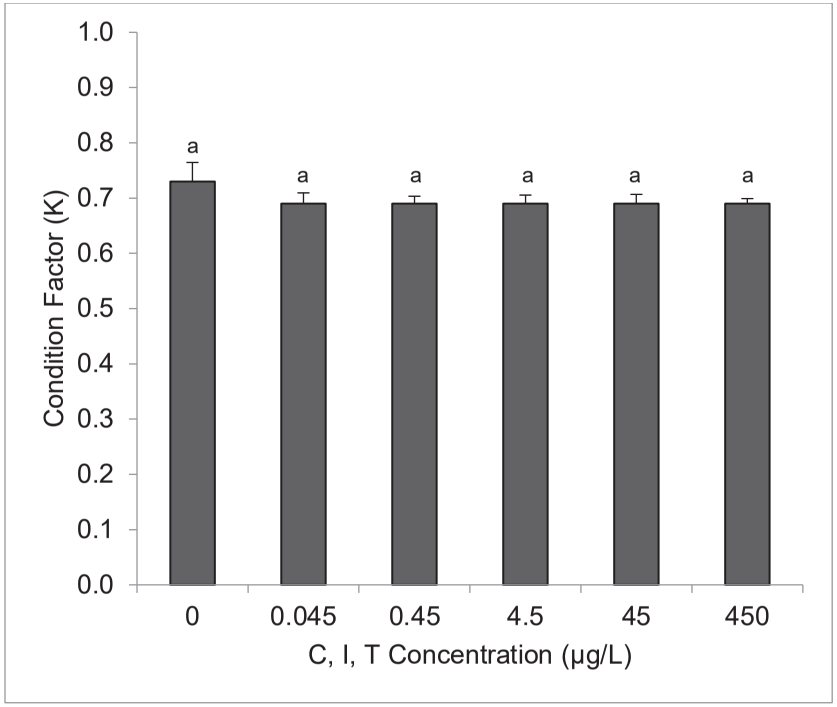


Figure 3-12. Mean condition factor (K) of cross C sockeye salmon after chronic exposure to waterborne neonicotinoid mixtures from one-hour post fertilization through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3). Columns show the mean condition factor with errors bars indicating standard error. There were no significant differences across treatments ($P > 0.5$; RCB ANOVA). Differences are indicated by superscripts.

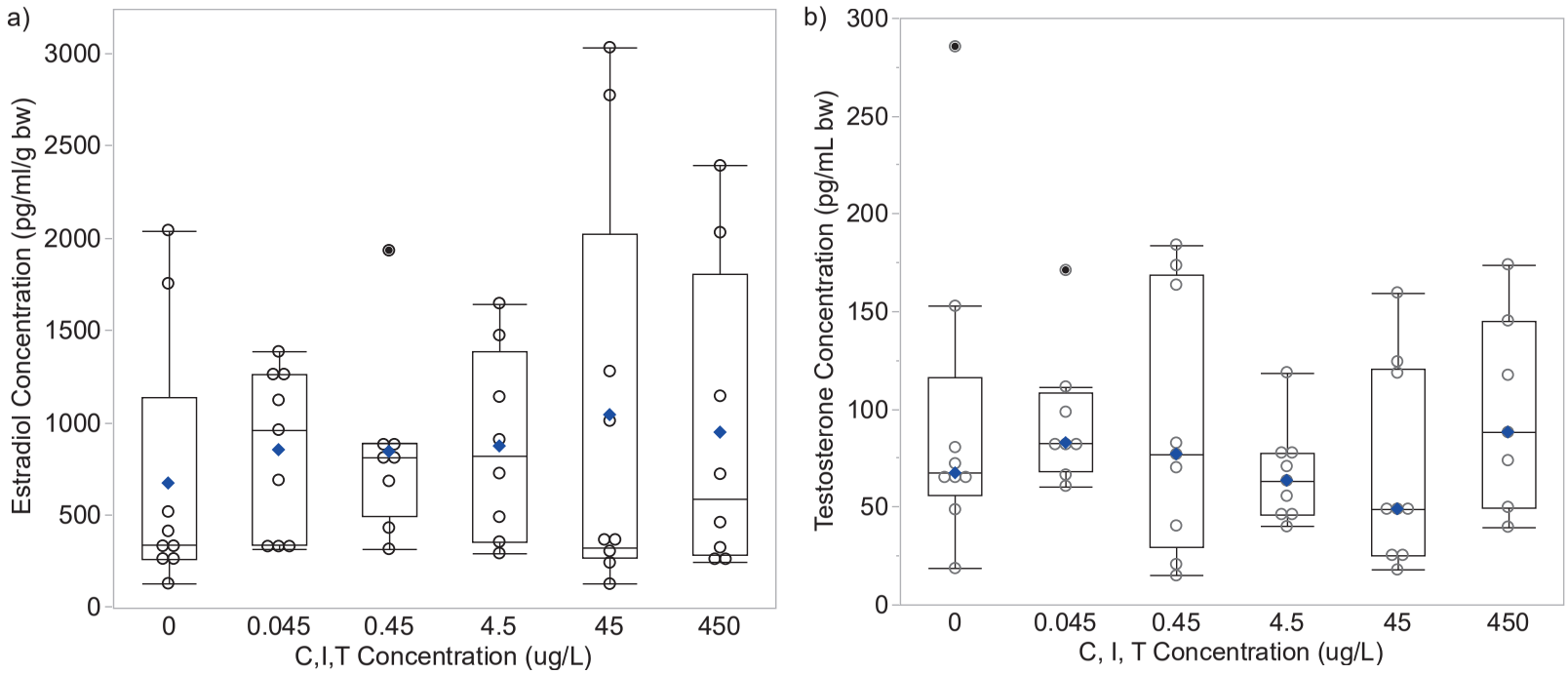


Figure 3-13a and Figure 13b. Mean whole-body sex steroid hormone concentrations, adjusted based on weight, in cross C feeding-fry sockeye salmon following the chronic neonicotinoid mixture exposure from one-hour post-fertilization through to the feeding-fry developmental stage. The mean concentration of (a) 17β-estradiol and (b) testosterone in whole body homogenates of feeding-fry terminated 5 weeks into feeding, post emergence, from one genetic cross (cross C) after the chronic exposure. The steroid hormone concentrations were measured by the enzyme-linked immunosorbant assays (ELISA). Means (denoted by diamonds) ± standard error, are presented (n = 2 to 3; 7 to 9 individuals per treatment). There were no significance differences across treatments for mean 17β-estradiol or testosterone (Wilcoxon Test, followed by Steel-Dwass post-hoc test, $p<0.05$).

Normalized Hepatic Gene Expression - Swim Up Fry

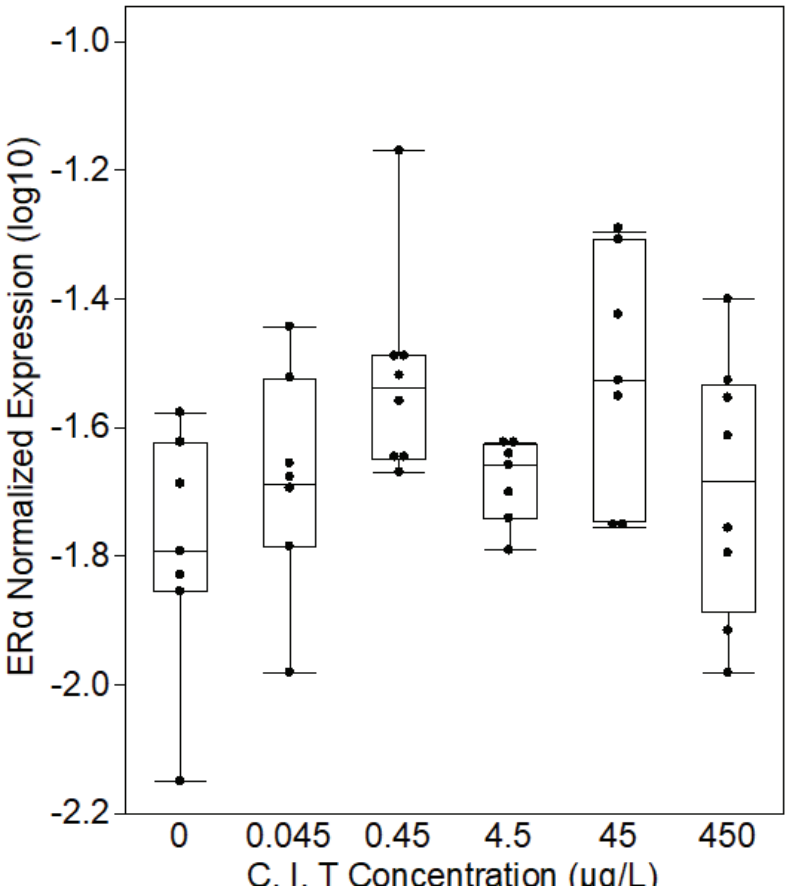


Figure 3-14. Hepatic gene expression in cross C sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (estrogen receptor alpha (ERα) was calculated based on the ΔΔCq method for relative quantitation of a target gene using three reference genes, beta action (ACTβ), glyceraldehyde 3-phosphae dehydrogenase (GAPDH), and 60S ribosomal protein L8 (RPL8). A pool of two fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

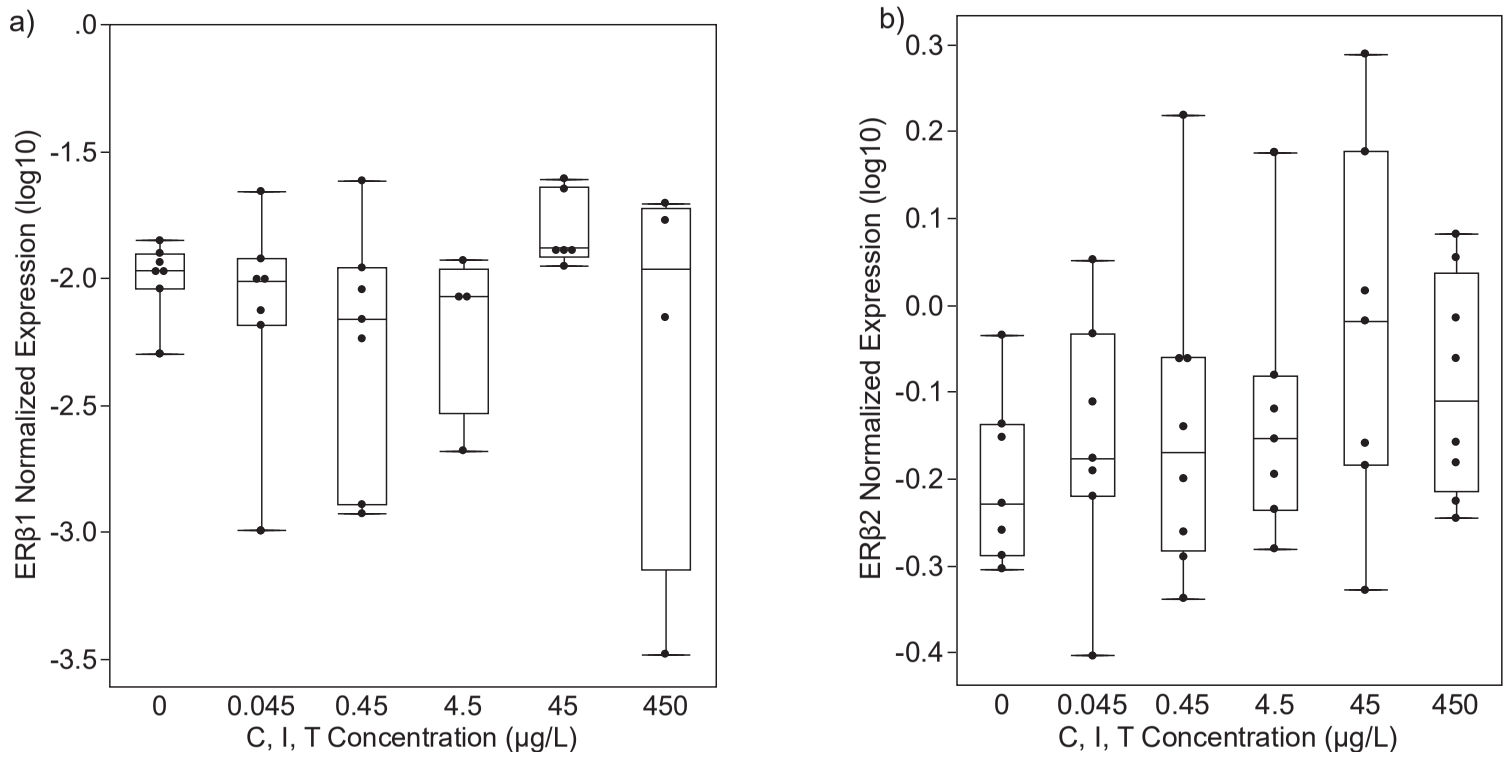


Figure 3-15a and Figure 3-15b. Hepatic gene expression in cross C sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) estrogen receptor beta one (ERβ1) and (b) estrogen receptor beta two (ERβ2) were calculated based on the ΔΔCq method for relative quantitation of a target gene using three reference genes, beta action (ACTβ), glyceraldehyde 3-phosphae dehydrogenase (GAPDH), and 60S ribosomal protein L8 (RPL8). A pool of two fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

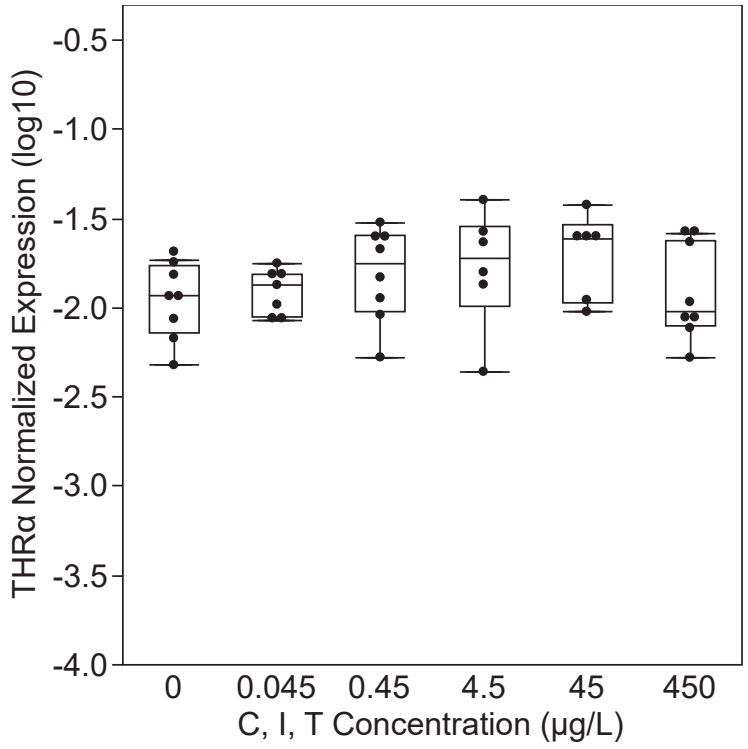


Figure 3-16. Hepatic gene expression in cross C sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for thyroid receptor alpha (THRα) was calculated based on the ΔΔCq method for relative quantitation of a target gene using three reference genes, beta action (ACTβ), glyceraldehyde 3-phosphae dehydrogenase (GAPDH), and 60S ribosomal protein L8 (RPL8). A pool of twofish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

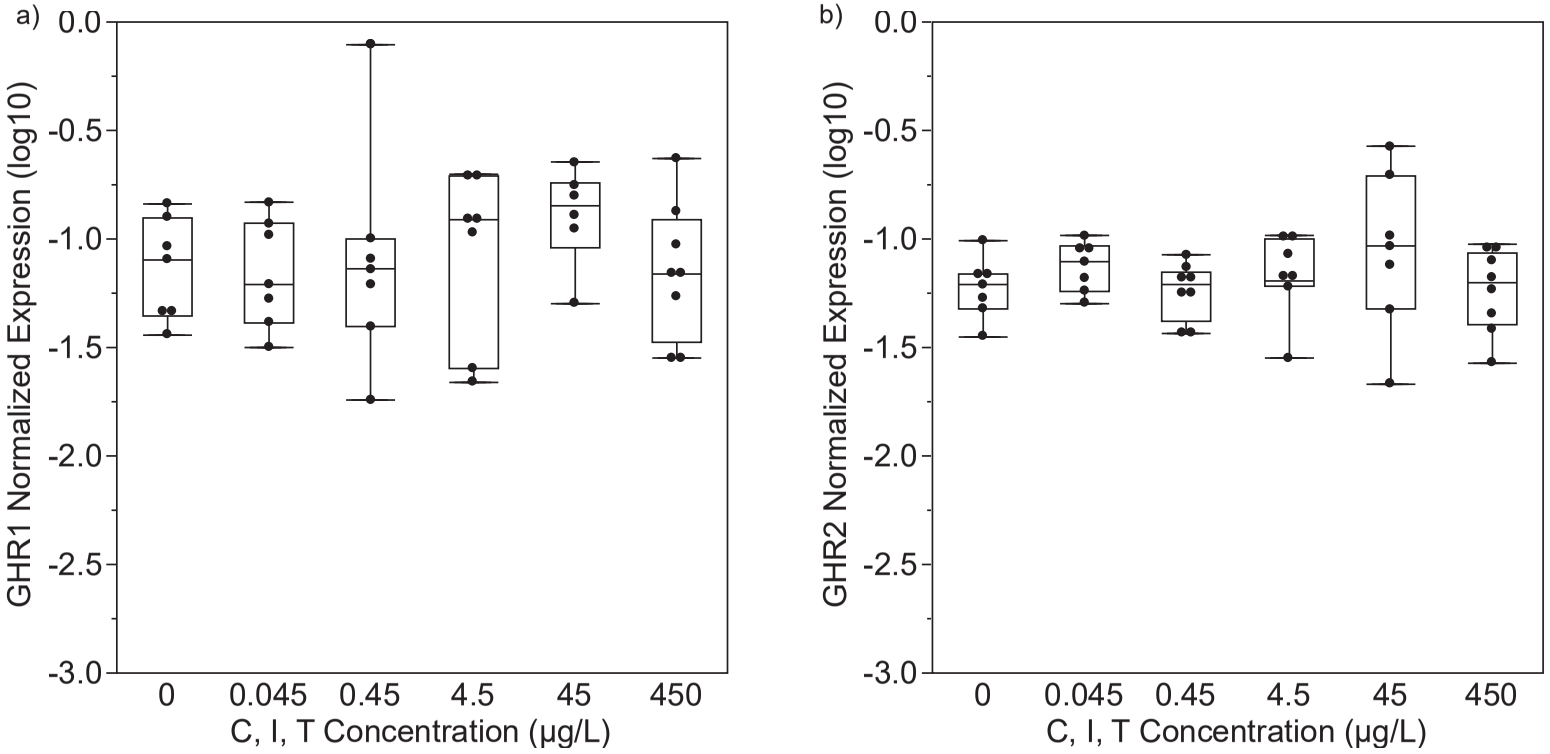


Figure 3-17a and Figure 3-17b. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) growth hormone receptor one (GHR1) and (b) growth hormone receptor two (GHR2) were calculated based on the ΔΔCq method for relative quantitation of a target gene using three reference genes, beta action (ACTβ), glyceraldehyde 3-phosphae dehydrogenase (GAPDH), and 60S ribosomal protein L8 (RPL8). A pool of two fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

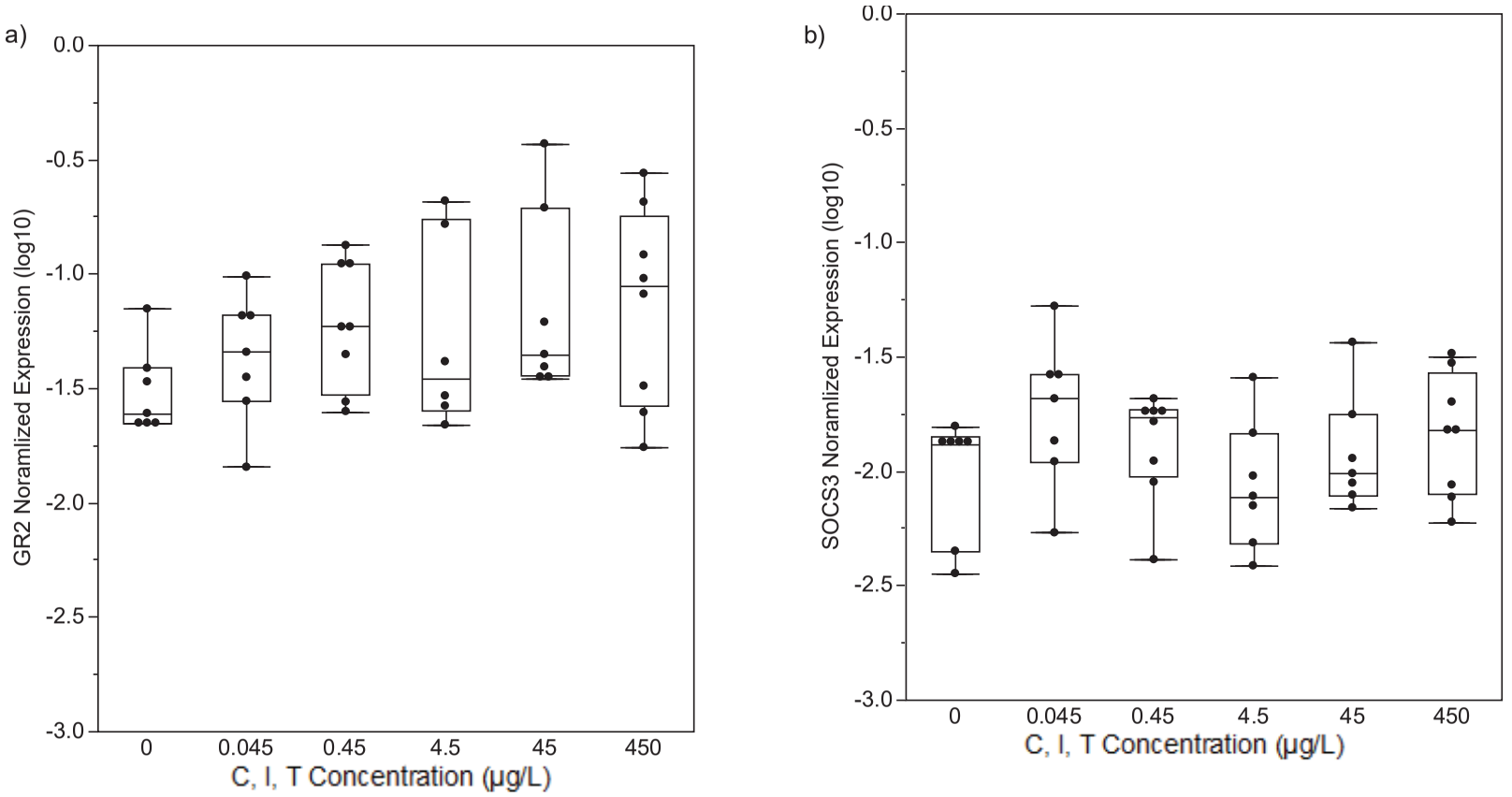


Figure 3-18a and Figure 3-18b. Hepatic gene expression in cross C sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) glucocorticoid receptor one (GR2) and (b) suppressor of cytokine signaling 3 (SOCS3) were calculated based on the ΔΔCq method for relative quantitation of a target gene using three reference genes, beta action (ACTβ), glyceraldehyde 3-phosphae dehydrogenase (GAPDH), and 60S ribosomal protein L8 (RPL8). A pool of two fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

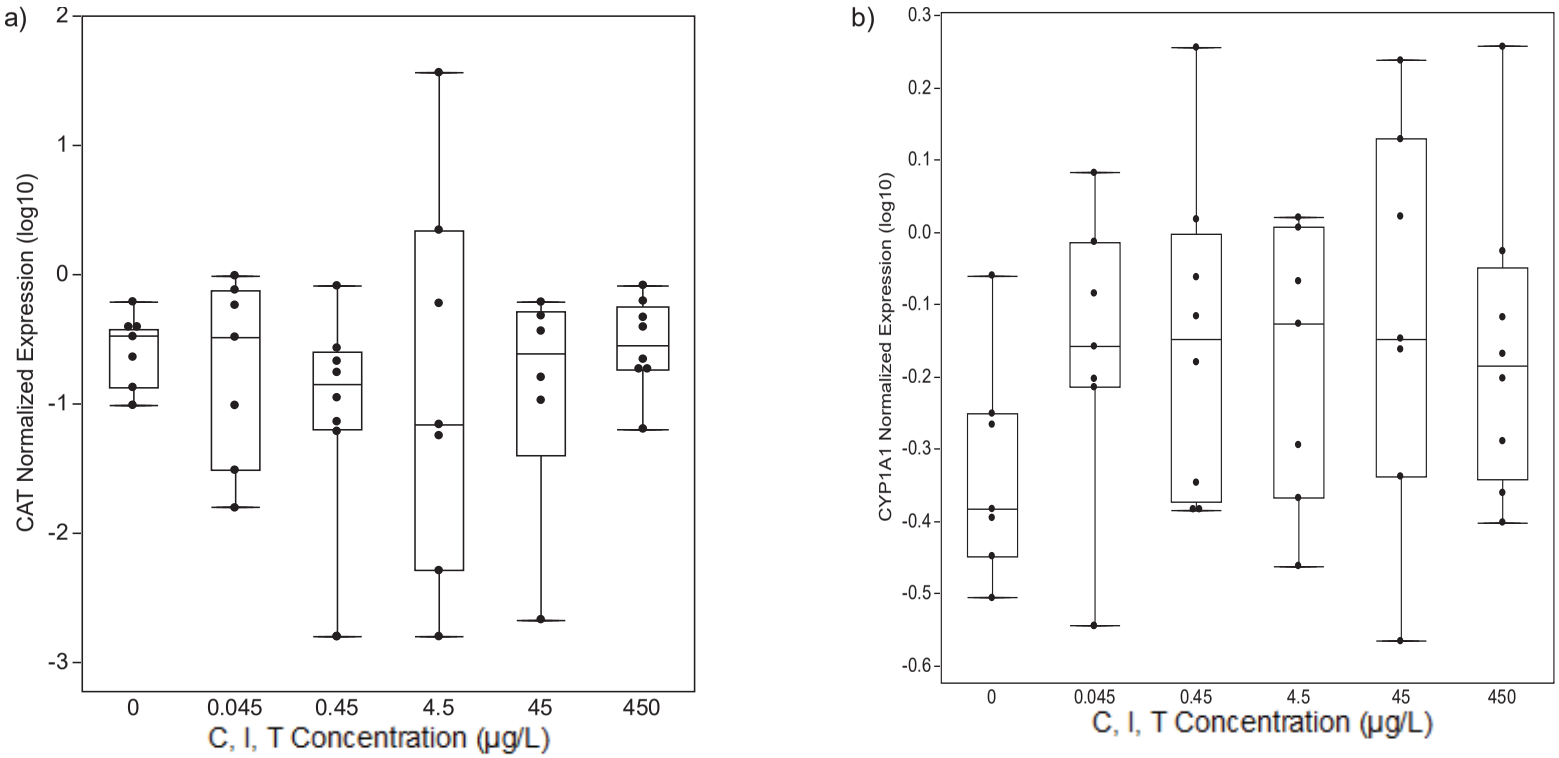


Figure 3-19a and Figure 3-19b. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the swim up fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) catalase (CAT) and (b) *CYP1A1* Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) were calculated based on the ΔΔCq method for relative quantitation of a target gene using three reference genes, beta action (ACTβ), glyceraldehyde 3-phosphae dehydrogenase (GAPDH), and 60S ribosomal protein L8 (RPL8). Each black circle represents an individual sample comprised of a pool of two livers (n = 6 to 8 individual livers randomly collected from 2 -3 replicate glass tanks per treatment). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

Normailized hepptic gene expression – Feeding Fry

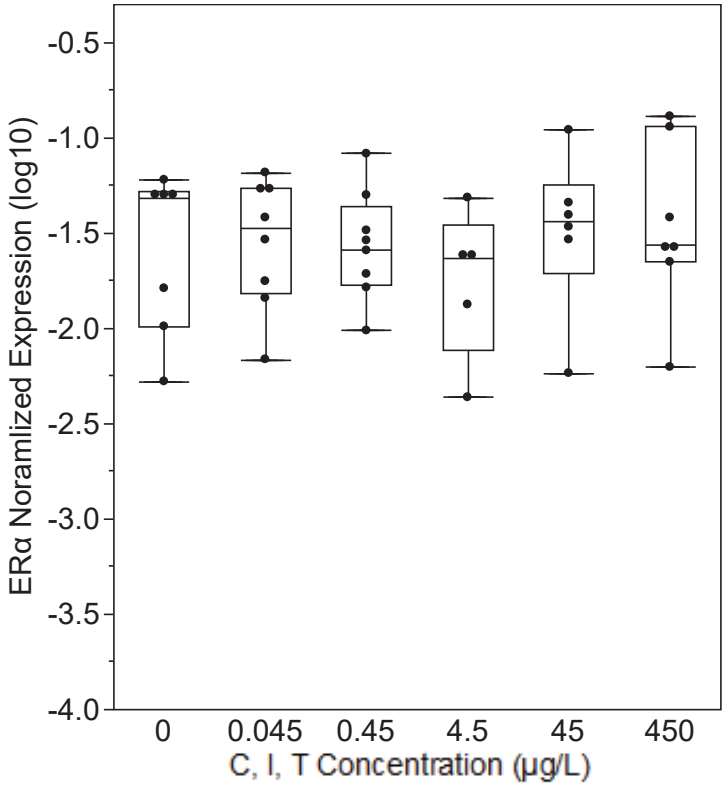


Figure 3-20. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (estrogen receptor alpha (ERα) was calculated based on the ΔΔCq method for relative quantitation of a target gene using two reference genes, beta action (ACTβ) and elongation factor 1-alpha (EF1α). Individual fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

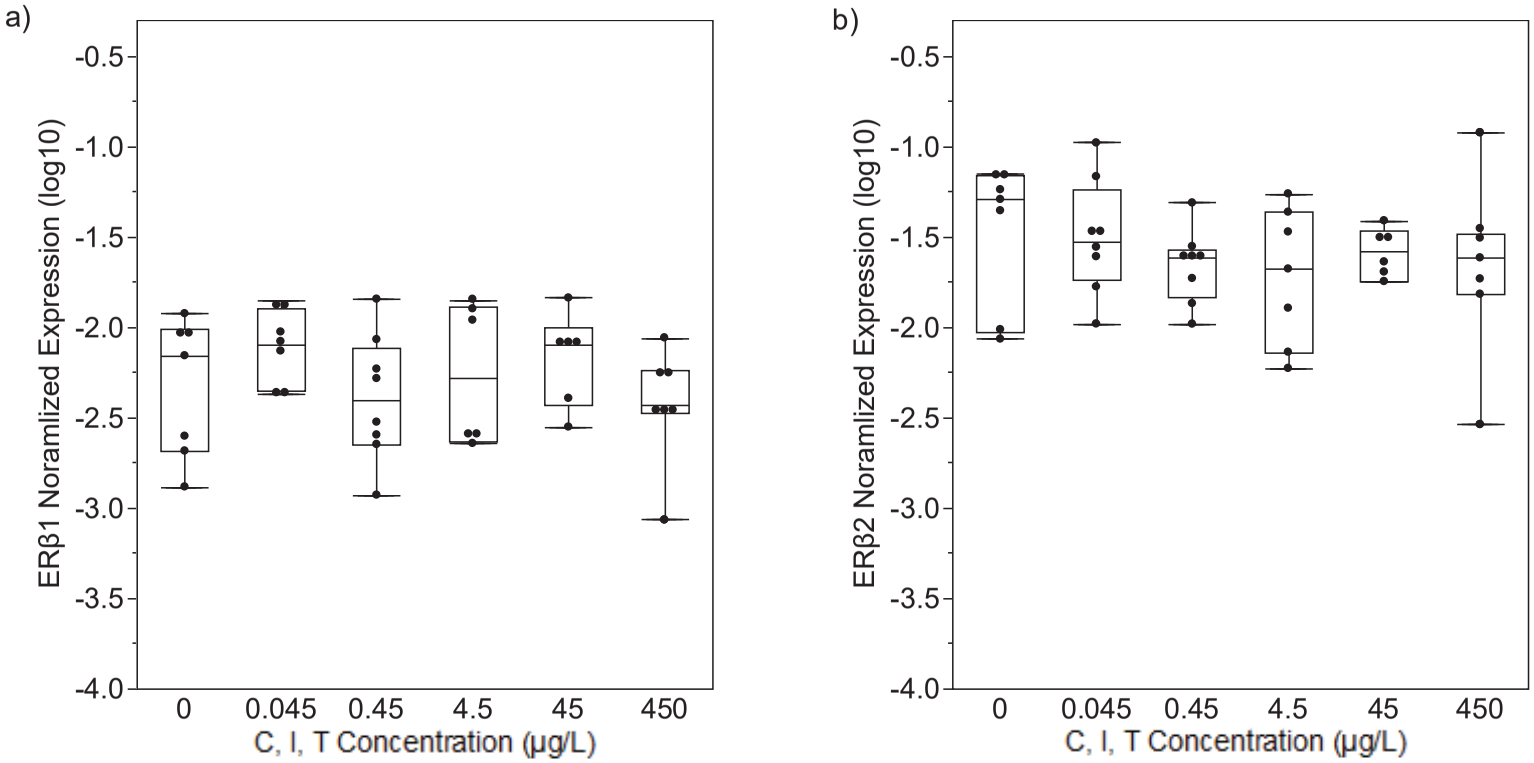


Figure 3-21a and Figure 3-21b. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) estrogen receptor beta one (ERβ1) and (b) estrogen receptor beta two (ERβ2) were calculated based on the ΔΔCq method for relative quantitation of a target gene using two reference genes, beta action (ACTβ) and elongation factor 1-alpha (EF1α). Individual fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

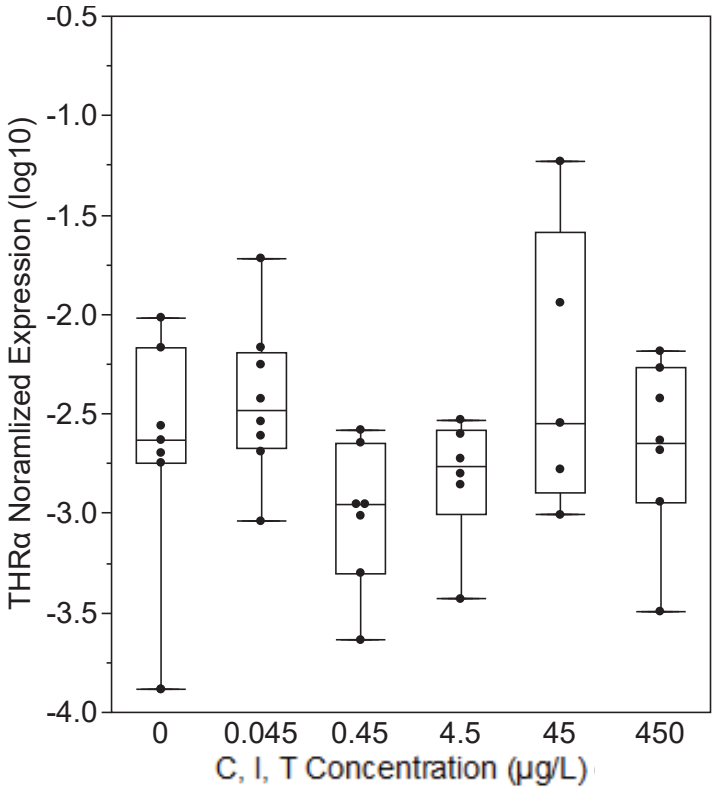


Figure 3-22. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for thyroid receptor alpha (THRα) was calculated based on the ΔΔCq method for relative quantitation of a target gene using two reference genes, beta action (ACTβ) and elongation factor 1-alpha (EF1α). Individual fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

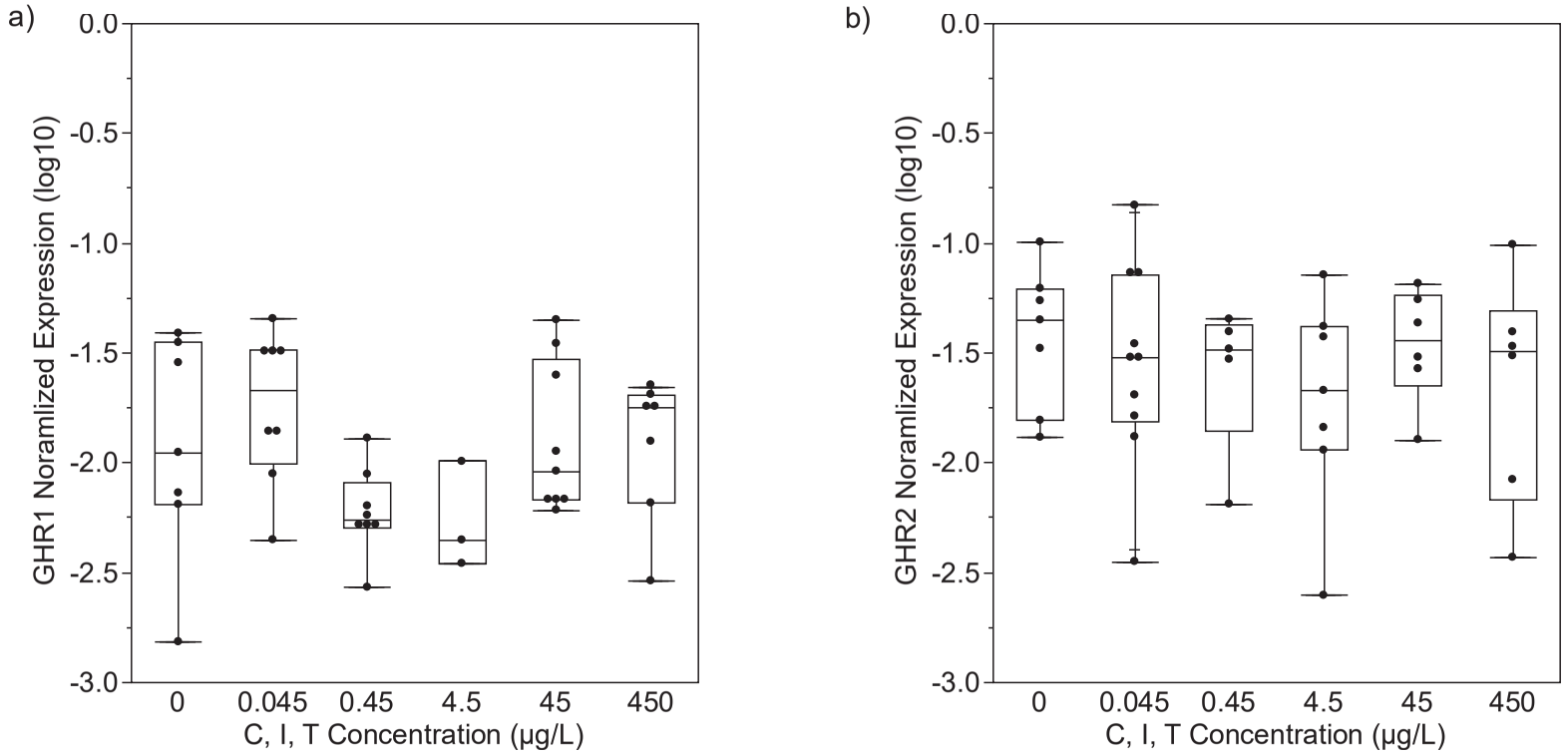


Figure 3-23a and Figure 3-23b. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) growth hormone receptor one (GHR1) and (b) growth hormone receptor two (GHR2) were calculated based on the ΔΔCq method for relative quantitation of a target gene using two reference genes, beta action (ACTβ) and elongation factor 1-alpha (EF1α). Individual fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

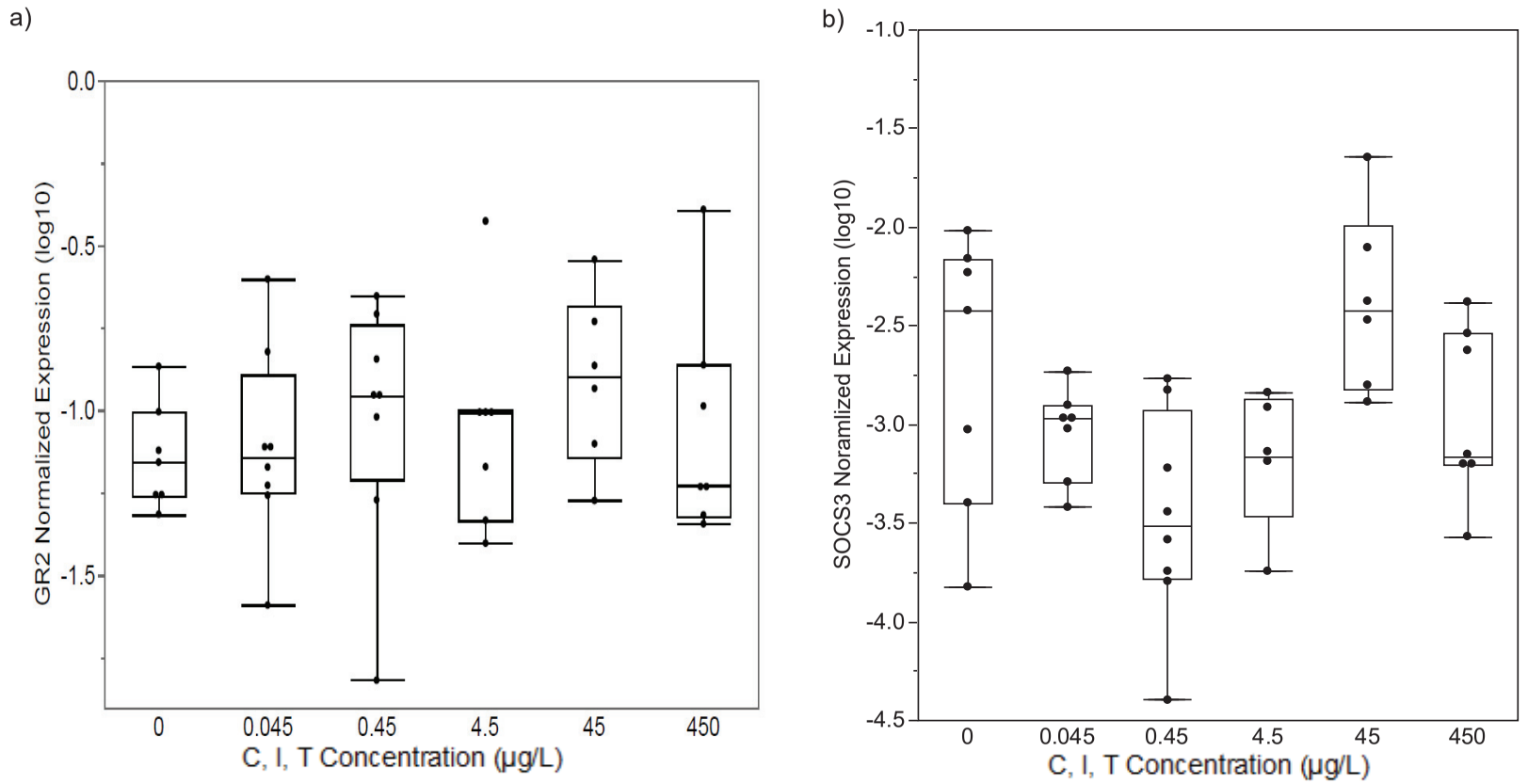


Figure 3-24a and Figure 3-24b. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per liter of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) glucocorticoid receptor one (GR2) and (b) suppressor of cytokine signaling 3 (SOCS3) were calculated based on the ΔΔCq method for relative quantitation of a target gene using two reference genes, beta action (ACTβ) and elongation factor 1-alpha (EF1α). Individual fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

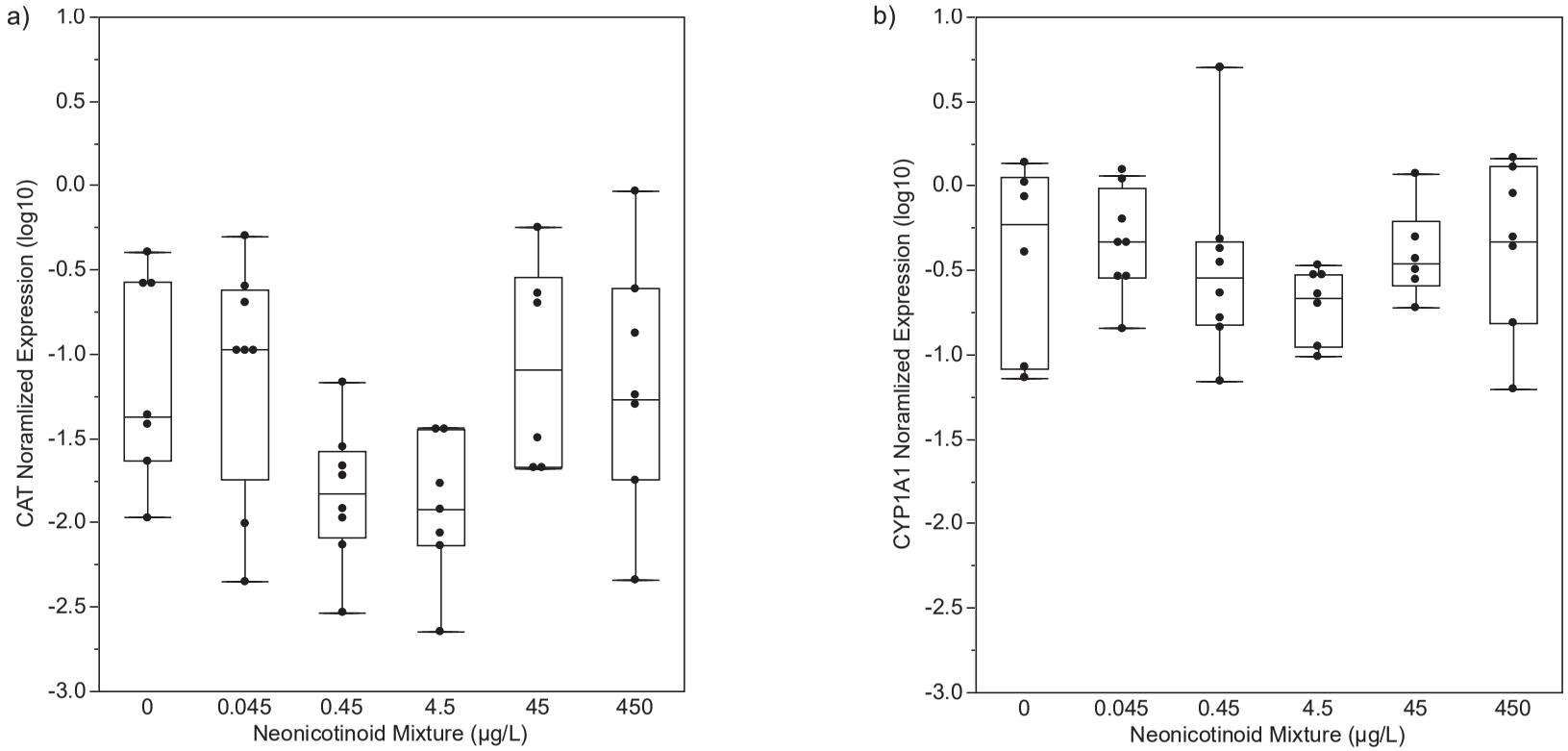


Figure 3-25a and Figure 3-23b. Hepatic gene expression in sockeye salmon after exposure to waterborne neonicotinoid mixtures from fertilization through to the feeding fry life stage. Each test concentration consisted of a mixture of an equal mass of clothianidin (C), imidacloprid (I), and thiamethoxam (T) per litre of test water. The treatments include a water control, 0 µg/L; 0.045 µg/L; 0.45 µg/L; 4.5 µg/L; 45 µg/L; and 450 µg/L (n = 3 with the exception of 4.5 µg/L where n = 2). Normalized gene expression for (a) catalase (CAT) and (b) *CYP1A1* Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) were calculated based on the ΔΔCq method for relative quantitation of a target gene using two reference genes, beta action (ACTβ) and elongation factor 1-alpha (EF1α). Individual fish liver samples are represented by black circles (n = 6 to 8 individual livers per tank). Box plots show the minimum and maximum log10 expression values indicated by whiskers, the upper and lower quartiles are indicated by the box, and median values represented by the horizontal line. No significant differences between the means were identified using a one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test.

4 Discussion

4.1 Chronic Thiamethoxam Exposure

This study evaluated the effects of thiamethoxam on wild early life stage sockeye salmon growth and development in traditional glass tank exposures and in gravel bed flume exposure systems. The endpoints to test the potential effects of thiamethoxam included fertilization success, hatch success (glass tanks only), survival (glass tanks only), body morphometrics and deformities on four unique genetic crosses to account for the influence or parentage in this wild Pacific salmonid. The findings of this study showed no adverse effects on fertilization, hatching (glass tanks only), survival (glass tanks only), body size, and deformities during chronic exposures to 0.15 to 150 µg/L thiamethoxam from 1-3 hours post-fertilization through to the swim up fry developmental stage.

The influence of parentage on growth and survival in wild sockeye salmon was significant in this study, and strongly supports the incorporation of multiple genetic crosses when examining growth and development during toxicity testing in this wild salmonid. For example, in the present study in the glass tank exposure, cross B was 3.3% (or 1 mm) longer, 10% heavier (or 0.02 g), and exhibited a 2.6% (or 0.02 K) higher condition factor than cross A. Parentage effects on progeny have previously been reported as a significant factor causing variation in BC sockeye populations (Burt et al. 2012; Marlatt et al. 2019) and fish populations in general (Marshall et al. 2008 and Green 2008). Burt et al. (2012) demonstrated that when different genetic crosses of sockeye embryos were reared under different temperature regimes followed by fry rearing in a single cool temperature after hatching, the resultant significant variation in body weight was attributed to parentage (both maternal and paternal identity). Furthermore, parentage effects on growth were also observed in a similar sockeye salmon study entailing a chronic exposure to another neonicotinoid, clothianidin (Marlatt et al. 2019). As in the present study, Marlatt et al. (2019) reported that one genetic cross was heavier and longer than the other two crosses tested in both the tank and gravel bed flume exposure scenarios. Collectively, the studies in early life stage sockeye salmon strongly support the hypothesis that parentage affects body size and survival under control conditions, and these must be taken into consideration when evaluating the toxicity of contaminants in this species.

The hypothesis that individuals reared in the more natural habitat of the gravel bed flumes would be larger relative to individuals reared in the glass tanks was supported in this study. This hypothesis was based on previous studies indicating reduced stress, and a more efficient use of energy for growth (Weil et al. 2001) when rearing conditions mimicked the natural environment more closely for salmonids (e.g., Hansen and Torrissen 1984; Hansen et al. 1990; McCormik et al. 1998; Bamberger 2009). For example, Bamberger (2009) developed a "Bamberger-Box" that mimicked a natural environment and compared three different breeding stocks of Atlantic salmon hatched in the Box to fish reared in traditional hatchery troughs. Bamberger (2009) found that in each of the four years tested, salmon fry reared in the Bamberger-Box were 4% longer and 12% heavier than fry reared in troughs. Hansen et al. (1990) described the reasoning for these increases because fish have substrate to hide in and can use more energy reserves for growth. McCormik et al. (1998) and Bamberger (2009) reported that there is species variation in terms of the organism's ability to cope with stress while efficiently growing (i.e., domesticated vs wild stock) and suggested that when in captivity, wild populations inefficiently convert food energy to growth, as shown temporally in both studies with wild Atlantic salmon. Interestingly, in the present study, cross A in the gravel bed flume exposure, mean length was 4% greater, weight was 15% greater, and condition factor was 3% greater than cross A in the tank exposure; however, for cross B body morphometrics were nearly identical under both exposure scenarios. Another sockeye salmon study using the same genetic crosses and conducted concurrently by Marlatt et al. (2019) showed that body length and weight for cross A and B did not differ between glass tank or gravel bed flume exposure scenarios. Noteworthy is that in the present study and Marlatt et al. (2019), issues recapturing individuals during termination from the gravel bed flumes resulted in a lower number of fish measured compared to the glass tank number of fish measured. Thus, these smaller test populations in the gravel bed flumes likely presents a less accurate estimate of fish size and future studies with more comparable numbers of individual fish measured are needed to ascertain trends in growth between these two rearing systems. In terms of fish welfare and optimizing fish health, there is evidence that more natural rearing conditions are ideal; however, this study also highlights the challenges with successful recovery of fish in such scenarios that must be addressed.

4.2 2016 Neonicotinoid Exposures

4.2.1 Acute Exposure During Fertilization

This is the first study to report a neonicotinoid as a reproductive toxicant and teratogen in a wild salmonid species. In this study acute exposure to waterborne treatments of clothianidin, or thiamethoxam, and a neonicotinoid mixture (clothianidin, thiamethoxam and imidacloprid) were performed at the onset of fertilization and continued for one hour, at which point fertilized eggs were transferred to clean water and reared until the swim-up fry stage. Thiamethoxam exhibited potential as a reproductive toxicant by causing a 25% decrease in fertilization success in the 15 µg/L treatment relative to the control group. Teratogenic effects of thiamethoxam were evident based on an approximate 4% increase in mean length at all concentrations tested (i.e., 0.15, 1.5, 15 and 150 µg/L), a 5% increase in weight in the 150 µg/L thiamethoxam treatment, and an approximate 9% decrease in the mean condition factor of the 15 µg/L thiamethoxam exposed fish relative to the control fish**.** Interestingly, the neonicotinoid mixture did not affect fertilization but did exhibit teratogenic effects based on abnormal weight and length compared to control fish (i.e., 0.45 µg/L caused a 5% decrease in length and a 13% increase of condition factor; 4.5 and 45 µg/L caused 8% increase in weight). These data also suggest that additive effects of neonicotinoids were not observed since the thiamethoxam growth response profiles were different compared to those observed in the mixture. Furthermore, the concentrations of neonicotinoids in the present study were sublethal with >80% survival in all controls and neonicotinoid exposures, thus this study provides strong evidence that sublethal, environmentally relevant neonicotinoid exposure may impede fertilization and cause abnormal growth in surviving fish.

Arukwe and Anders (2003) and Berois et al (2011) provides a potential mechanism that explains how a toxicant can be taken up during fertilization. In brief, teleost oocytes are surrounded by a vitelline envelope / zona radiata ([synonym]; Arukwe and Anders 2003; Berois et al 2011). Teleost ooyctes contain a unique micropyle, which is formed during egg deposition, and is a funnel shaped channel that sperm interact with. As a single sperm fuses with the oocyte, the micropyle closes within minutes of activation (i.e., egg and sperm fusion [Arukwe and Anders 2003; Berois et al. 2011]). After activation the zona radiata takes up water to gain resistance to mechanical force, which results in an egg that can support up to 100 times more weight than oviductal eggs (Arukwe and Anders 2003).

Given the acute exposures were conducted prior to the micropyle closing and activation, it is likely that dissolved thiamethoxam was taken up during water hardening. If this is the case, then the exposure duration was greater than one hour. Furthermore, fertilization success was defined as the number “eyed” embryos that survived up until the hatching stage. If an egg was determined dead, it was removed; however, it was not confirmed if embryogenesis had begun or not, which could potentially provide valuable information. While uptake of neonicotinoids is an un-tested hypothesis, *in-vitro* testing to confirm (or not) the uptake is warranted, while confirming the presence of a developing embryo.

Typically, acute toxicity fish studies evaluating the effects of neonicotinoids have exposure durations of 48 to 96-hours post-fertilization during larval and later life stages (e.g., Bowman and Bucksath 1990; Finnegan et al. 2017; Malhotra et al. 2021). In a recent review, Anderson et al. (2015) reported that the majority of acute toxicity studies in fish are limited in terms of the species examined. For example, as of 2015, Anderson et al. (2015) reported that only ten fish species had undergone acute toxicity testing for the effects of neonicotinoids, and of those, six were with imidacloprid, four were with clothianidin, and one was with thiamethoxam. Of those studies, only one tested the effects on the embryo life-stage (Zebrafish; Tisler et al. 2009) with imidacloprid for 48-hours and reported an EC_{50} of 408 to 1,160 mg/L. Other studies have also reported acute effects in the $\geq$ 1 mg/L range; however, exposure was not during fertilization or embryonic life stages. For example, Sanchez-Bayo and Goka (2012) reported that the 96-hour the lethal concentration to 50% of the test animals (LC_{50}) based on neurotoxicity was 60,800 µg/L. Ma et al. (2019) reported that an acute exposure of 120-hour duration with zebrafish embryos and Acetamiprid (a neonicotinoid), whereby embryos were exposed 6-hours after fertilization resulted in significant mortality at 374 mg/L and complete mortality at 760 mg/L. These latter toxic concentrations of a neonicotinoid are a factor of at least 100x greater than the highest concertation used in the present sublethal study. Indeed, the present study is the first to report acute fish embryo “pulse” exposures in fish, for neonicotinoids.

In addition to the findings in the present study, adverse effects after of neonicotinoids on various aspects of the reproductive endocrine axis have been shown in other fish. Ma et al. (2019) reported endocrine and teratogenic effects on zebrafish embryos and larvae after acute exposure to Acetamiprid, from 6 hours through to 120 hours post fertilization. Adverse effects included embryonic deformities, a decrease in hatchability, and

decreased larval length, albeit at concentrations 1,000 times greater than the current study. Effects on the embryos included spinal and soft tissue deformities, pericardial and yolk sac edemas, with the EC50 concentration at 263 mg/L after 120 hours post fertilization (Ma et al. 2019). Hatchability was significantly decreased 72 hours post fertilization in concentrations greater that 537 mg/L, and larval length was significantly decreased in all test concentrations of Acetamiprid as low as 54 mg/L. Kocamaz and Oruc (2020) showed endocrine disruption in adult male tilapia (*Oreochromis niloticus*) after acute pulse exposures to 24 to 48 mg/L thiamethoxam (i.e., two "pulse" exposures on day 7 and day 15, followed by a week of clean water exposure / depuration). After the second "pulse" of thiamethoxam and after depuration, blood plasma estradiol levels were significantly lower than the controls. Similarly, after depuration, plasma levels of testosterone were also significantly lower than the controls. As with Ma et al. (2019), these concentrations were 1,000 times greater than the current study. To date, this is the only acute exposure that supports the hypothesis that neonicotinoids disrupt the reproductive endocrine axis at environmentally relevant concentrations. Given the limited data on acute "pulse" exposures of neonicotinoids and sublethal endpoints in fish and the findings in the present study reporting decreased fertilization success and abnormal growth, further investigations examining the disruption of various aspect(s) of the endocrine system are warranted.

4.2.2 Chronic Exposure

Hatch Fertilization, and Survival Success

This is the first chronic study to evaluate effects of a neonicotinoid mixture on fertilization, hatch, and survival success, body morphometrics, and deformities on multiple genetic crosses in a fish species. In this study exposure to waterborne exposure to mixtures of equal parts clothianidin, imidacloprid and thiamethoxam (ranging from 0.15 to 150 µg/L) was performed for one-hour post-fertilization and continued through to the swim-up and feeding fry life stages (i.e., approximately 90 to 120 dpf). The neonicotinoid mixture did not affect growth and development, however, parentage significantly influenced weight, length, and condition factor based on the inclusion of four unique genetic crosses (referred to as A, B, C and D) in this experiment. Interestingly, one cross (B) exhibited reducing hatching success (12% decrease compared to the control). These data suggests that multiple genetic crosses are necessary when examining survival, growth, and

development during toxicity testing in this wild salmonid, which is consistent with the 2015 thiamethoxam results in the present study and for similar clothianidin experiments by Marlatt et al. (2019).

Several studies have reported that chronic exposure to neonicotinoids is not lethal below approximately 1 mg/L, which concurs with the findings of the present study. For example, data used to derive the CCME interim imidacloprid guideline for freshwater aquatic life, showed no effect on rainbow trout (*Oncorhynchus mykiss*) survival after a 60 day exposure at concentrations up to 19 mg/L. Likewise, Tišler et al. (2009) reported that toxicity was not evident during zebrafish larval development after exposure to 320,000 µg/L of imidacloprid; nor was survival reduced for rainbow trout embryos exposed for 98 days to imidacloprid to concentrations ranging from 1.3 to 20 mg/L (Anatra-Cordone and Durkin 2005). However, the only known study that showed significant effects on rainbow trout survival was one of the two unpublished studies used by the US EPA to derive the chronic imidacloprid benchmark for freshwater aquatic life (Gries 2002). In this US EPA study swim-up fry survival was significantly reduced at 1.2 to 19 mg/L, relative to the water controls; however, survival was also greater in the solvent-control relative to the water control as well (Gries 2002). Interestingly, the two (unpublished) studies used to derive the chronic US EPA imidacloprid benchmark for freshwater aquatic life did not have typical dose-response relationships nor consistency of sub-lethal effects. Gries 2002 found a 13% reduction in weight of rainbow trout swim-up fry at the lowest test concertation of 1.2 mg/L but lacked statistical significance in the highest concertation (2 mg/L), where a 10% reduction in weight was observed. In the second study (Cohle and Bucksath 1991), no morphological effects (with similar exposure concentrations as above) were observed for rainbow trout, with imidacloprid concentrations ranging from 0.1 to 9 mg/L; however, percent hatch success significantly enhanced in the highest concertation of 26.9 mg/L. Collectively, these results also support the need for genetic variation during toxicity testing for salmonid species, and importantly, to consider sublethal effects at environmentally relevant concentrations as well as lethal concentrations.

4.2.3 Feeding Fry Sex Steroid Hormones

This is the second study to measure sex steroid hormones in fish after chronic exposure to neonicotinoids. The first was a study published in Marlatt et al. (2019) showed a non-monotonic concentration-response curve whereby chronic exposure to clothianidin

resulted in an increase of estradiol only at the lowest concentration tested (i.e., 0.15 µg/L). This was not the case in the current study where mean estradiol and testosterone concentrations measured in one genetic cross (C) were not statistically different between neonicotinoid mixture and control fish. Although this may be evidence that a neonicotinoid mixture is not affecting whole body sex steroid concentrations, a critical quality assurance/quality control issue in the present study confounds the ability to make a sound conclusion. Specifically, the spiked hormone samples in the present study during the whole-body hormone extraction phase of this experiment were unacceptably low. For 17β-estradiol, the present study reports an average recovery efficiency (±SE) of 29±5%, whereas Marlatt et al. (2019) had an average recovery efficiency (±SE) of 76 ±5%. Similarly, the recovery efficiency of testosterone in this study was also low at 11.6%, while Marlatt et al. (2019) mean recovery efficiency (±SE) was 76 ±5% for testosterone. Possible explanations for the low recovery in the present study for both the estradiol and testosterone could be a result of human error or different homogenization methods. Regarding the latter, Marlatt et al. (2019) used a hand operated homogenizer and individually homogenized each fish, whereas the present study employed a multi sample automated mixer mill that may not have homogenized the whole bodies as thoroughly. In any case, extraction efficiencies in the present study were very low and unacceptable, with typical sex steroid extraction efficiencies for fish blood or tissues approximately ≥70% (McMaster et al. 1992). Collectively, it is difficult to make conclusions based on the present study regarding the effects of chronic waterborne sublethal neonicotinoid mixtures on sex steroid hormone levels in wild sockeye salmon due to quality assurance and/or sample size short comings.

4.2.4 Molecular Analysis Gene Expression

This study was designed to assess effects on gene expression, in the liver of sockeye salmon, after chronic waterborne neonicotinoid mixture exposures during the embryonic, alevin, swim-up fry, and the onset of the feeding up fry developmental stages. The genes investigated in this study were related to biological processes / systems including reproduction, growth, stress response, and nervous and immune function. Several RT-qPCR bioassays were designed (and conducted) to measure mRNA levels of select genes of interest, including two novel primers that were developed (but excluded). In total, 24 primer sets were used (including references genes) and of these ten were

successful while 14 primer set tests did not meet quality assurance/quality control criteria for various reasons (i.e., low levels of gene expression, poor efficiency, or multiple peaks from melt curve analysis, etc.). Nonetheless, of the successfully conducted RT-qPCR assays, no statistical differences between treatments for each of the 10 target genes were observed. These findings are similar to those reported by Marlatt et al. (2019) where these same genes were examined in wild sockeye salmon after exposure to 0.15 to 150 µg/L clothianidin, and only glucocorticoid receptor 2 transcript levels decreased approximately 4-fold in the highest clothianidin treatment. In general, this targeted gene expression approach after continuous chronic exposure post-fertilization through to the fry stage to neonicotinoids supports the lack of sublethal effects observed on growth and development after both the thiamethoxam and the neonicotinoid mixture exposure in the present study. However, more thorough investigations of changes in transcript abundance by inclusion of multiple time points, given the dynamic nature of hormones and non-monotonic responses, along with global gene expression analysis using RNAseq, would provide more definitive evidence of whether (or not) neonicotinoids influence gene expression under such chronic exposure scenarios during early life stages in sockeye salmon.

5 Future Recommendations and Conclusions

There were a few notable limitations of the experiments presented in this research that could be rectified in future works. The first limitation was the duplicate replicate test vessel exposure design in 2015, that was employed to accommodate multiple genetic crosses. A greater number of replicate test vessels would provide more statistical power given the genetic variation between crosses. Also, for enhanced statistical power, given the acquired 2016 data, a priori power analyses would also be beneficial for the biochemical and molecular endpoints. There was also some unreliability for survivorship in the gravel bed flume exposure, due to the inability to capture individuals, which could be modified to rectify the issues. There was also unacceptable recovery of hormone extractions in the 2016 experiment. Validation of the mixer mill is recommended to determine if full body homogenization (followed by hormone extraction) is different than the manual homogenizer. Furthermore, the evaluation of histopathology of feeding fry gonads in order to support or reject the hypothesis that neonicotinoids disrupt the development of the gonads, and thus, the reproductive endocrine axis, is recommended.

Nonetheless, despite the limitations of this study, valuable insights on the impacts of neonicotinoid pesticides on a wild Pacific salmonid have been gained. Indeed, this is the first study to report the acute and chronic effects of environmentally relevant concentrations of (1) thiamethoxam, ranging from 0.15-150 µg/L and (2) a mixture of clothianidin, imidacloprid, and thiamethoxam neonicotinoid pesticides, ranging from 0.045-450 µg/L on wild sockeye salmon. The 2015 thiamethoxam chronic exposures resulted in no adverse effects; however, given the low n (n=2) and the considerable variation between replicates in survival, including the control replicates, further investigation into survival at the lowest test concentrations is recommended. Parentage did influence growth in both the 2015 thiamethoxam and 2016 neonicotinoid mixture studies, which strongly suggests that multiple genetic crosses are necessary when examining survival, growth, and development during toxicity testing in this wild salmonid. Interestingly, despite no effects on growth and development during chronic thiamethoxam or neonicotinoid mixture exposures, acute thiamethoxam exposure during fertilization showed reproductive toxicity via decreased fertilization success and subsequent teratogenic effects with abnormal length, weight, and condition factor in swim-up fry in both the thiamethoxam and the neonicotinoid mixture exposures. These findings could

have potential implications for wild salmon populations as pulse exposures are environmentally relevant, especially given the amount of rainfall in the Fraser Valley. During the wet season and rainfall events, recently deposited or fertilized salmon eggs could be acutely exposed to low levels of thiamethoxam in surface water. A decrease of 25% in fertilization success would be detrimental to sockeye populations, especially those designated as endangered of concern by the IUCN. Finally, this study also demonstrates adverse effects of neonicotinoids on growth ensue after acute exposure during fertilization in wild sockeye salmon, but further studies to examine the underlying molecular mechanisms of action in this species and if such reproductive toxicity and teratogenicity are observed in other fish species are warranted. These additional studies are recommended particularly in light of the reported adverse effects of noenicitinoids on endocrine system measures observed in sockeye that some exhibit evidence of a non-monotonic dose-response, thus adding a layer of complexity that merits further investigation.

References

Anderson JC, Dubetz C, Palace VP. 2015. Neonicotinoids in the Canadian aquatic environment: A literature review on current use products with a focus on fate, exposure, and biological effects. Science of the Total Environment, 505:409-422.

Anatra-Cordone M and Durkin P. 2005. Imidacloprid - human health and ecological risk assessment. New York: Syracuse Environmental Research Associates Inc.Arukwe, A., Nordtug, t., Kortner, T.M., Mortensen, A.S., and Brakstad, O.G. (2008). Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (Danio rerio) exposed to water-soluble fraction of crude oil. Environ Res, 107(3).

Arukwe A and Anders G. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comparative Hepatology 2:4.

Arukwe A, Nordtug T, Kortner TM, Mortensen AS, and Brakstad OG. 2008. Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (Danio rerio) exposed to water-soluble fraction of crude oil. Environ. Res. 107(3):362–370.

Ayres KL, Booth RK, Hempelmann JA, Koski KL, Emmons CK, Baird RW, Balcomb-Bartok K, Hanson BM, Ford MJ, Wasser SK. 2012. Distinguishing the impacts of inadequate prey and vessel traffic on an endangered killer whale (Orcinus orca) population. PLoS One, 7(6). Available online at: https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0036842&type=printable.

Bamberger A. (2009). Evaluation of a new semi-natural incubation technique for Atlantic salmon Salmo salar fry. Journal of Fish Biology 74.

Bercovits, I. 2018. Experimental design, personal communication 16 February 2018.

Berois N, Arezo MJ, Papa NG. 2011. Gamete interactions in teleost fish: the egg envelope. Basic studies and perspectives as environmental biomonitor. Biol Res 44:119-124.

Bio-Rad laboratories.(2010) CFX96 and CFX384 Real-Time PCR Detection System: Instruction Manual. Available online at: http://www.bio rad.com/webroot/web/pdf/lsr/literature/10010424.pdf.

Bishop CA, Moran AJ, Toshack MC, Elle E, Maisonneuve F, Elliott JE. 2018. Hummingbirds and bumble bees exposed to neonicotinoid and organophosphate insecticides in the Fraser Valley, British Columbia, Canada: hummingbirds, bees, and neonicotinoids in Canada. Environ Toxicol Chem 37(8):2143–2152.

Botha CJ, Coester H, Labuschagne L, and Basson A. 2015. Confirmed organophosphorus and carbamate pesticide poisonings in South African wildlife (2009–2014). Journal of South African Vetrinary Association, 86(1):1329.

Bowman J and Bucksath J. 1990. Acute toxicity of NTN 33893 to rainbow trout (Oncorhynchus mykiss). Analytical Bio-Chemistry Laboratories, Inc., Columbia, Missouri (performing laboratory). Mobay Corporation, Kansas City, Missouri (submitting laboratory).

BC ENV (BC Ministry of Environment and Climate Change Strategy). 2016. 2015 Pesticide sales in British Columbia. Integrated Pest Management Program. Available online at: https://www2.gov.bc.ca/assets/gov/environment/pesticides-and-pest-management/publications-and-guides/surveys/2015_pesticide_sales_in_british_columbia_-_integrated_pest_management_program_-_final.pdf.

BC Gov (BC ministry of Agriculture). 2016. British Columbia's Fisheries and Aquaculture sector, 2016 edition.

BC Gov (BC Agriculture Service). 2020. British Columbia seafood industry in review 2018. Available online at: http://www2.gov.bc.ca/gov/content/industry/agriculture-seafood/statistics/industry-and-sector-profiles.

Brown B. (2010). Books: Sustainable and biodegradable reading technology. New York, NY: Hydraulic Press. doi:10.1026/0022-005X.52.6.803.

Bonmatin JM, Moineau I, Charvet R, Colin ME, Fleche C, Bengsch ER. 2015. Behaviour of imidacloprid in fields. Toxicity for honeybees. In: Lichtfouse E, Schwarzbauer J, Robert D, editors. Environmental chemistry. Berlin: Springer; pp. 483–494.

Burt JM, Hinch SG, and Patterson DA. (2012). Parental identity influences progeny responses to incubation thermal stress in sockeye salmon Oncorhynchus nerka. J. Fish Biol. 80: 444–462.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, and Wittwer CT. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time qPCR Experiments. Clinical Chemistry, 55(4), 611.

Calbick, Sarah. 2018. Hepatic Gene Profile Analysis for chronic exposure of clothianidin in early life stage sockeye salmon (*Oncorhynchus nerka*). MET Thesis, Simon Fraser University, Burnaby, BC.

CCME (Canadian Council of the Ministers of the Environment). 2007. Imidacloprid Canadian water quality guideline for the protection for aquatic life.

Cavallaro MC, Main AR, Liber K, Phillips ID, Headley JV, Peru KM, Morrissey CA. 2019. Neonicotinoids and other agricultural stressors collectively modify aquatic insect communities. Chemosphere 226:945–955.

Cederholm CJ, Johnson D, Bilby RE, Dominguez LG, Garrett AM, Graeber WH, Greda EL, Kunze MD, Marcot BG, Palmisano JF, Plotnikoff RW, Pearcy WG, Simenstad CA, Trotter PC. 2000. Pacific salmon and wildlife - ecological contexts, relationships, and implications for management. Washington Department of Fish and Wildlife, Olympia, Washington, 2nd ed. Available online at: https://wdfw.wa.gov/publications/00063.

Christen V, Mittner F, Fent K. 2016. Molecular effects of neonicotinoids in honeybees (Apis mellifera). Environ Sci Technol 50(7):4071–4081.

Cohen B. 2012. The uncertain future of Fraser River sockeye - causes of the decline, Volume 2. Final Report. Bruce Cohen, commissioner. Canada Privy Council Office. Commission of Inquiry into Decline of Sockeye Salmon in the Fraser River.

Cohle P, Bucksath J. 1991. Early Life Stage Toxicity of NTN 33893. Technical to rainbow trout (Oncorhynchus mykiss) in a flow-through system: Lab Project Number: 38347: 101214. Unpublished study prepared by Analytical Bio-Chemistry Labs Inc. 8 p.

Crayton SM, Wood PB, Brown DJ, Millikin AR, McManus TJ, Simpson TJ, Ku KM, Park YL. 2020. Bioaccumulation of the pesticide imidacloprid in stream organisms and sublethal effects on salamanders. Glob Ecol. Conserv. 24.

Danis BEG and Marlatt VL. 2021. Investigating Acute and Sub-chronic Effects of neonicotinoids on northwestern salamander larvae. Archives of Environmental Contamination and Toxicology. 80:691–707.

de Perre C, Murphy TM, Lydy MJ (2015) Fate and effects of clothianidin in fields using conservation practices. Environ Toxicol Chem34(2):258–265.

Eng ML, Stutchbury BJ, Morrissey CA. 2017. Imidacloprid and chlorpyrifos insecticides impair migratory ability in a seed eating songbird. Sci Rep 7(1):1–9.

Eng ML, Stutchbury BJ, and Morrissey CA. 2019. A neonicotinoid insecticide reduces fueling and delays migration in songbirds. Science, 365(6458): 1177-1180.

Elbert A, Haas M, Springer B, Thielert W, Nauen R. 2008. Applied aspects of neonicotinoid uses in crop protection. Pest Manag Sci 64:1099–1105.

Eskenazi B. Bradman A. and Castorina R. 1999. Exposures of children to organophosphate pesticides and their potential adverse health effects. Environ. Health Perspect. 107 (Suppl. 3): 409–419.

Finnegan MC, Baxter LR, Maul JD, Hanson ML, and Hoekstra PF. 2017. Comprehensive characterization of the acute and chronic toxicity of the neonicotinoid insecticide thiamethoxam to a suite of aquatic primary producers, invertebrates, and fish. Environmental Toxicology and Chemistry, 36 (10):2838–2848.

Fisheries and Oceans Canada. (2020). Information about Pacific Salmon. Available online at: http://www.pac.dfo-mpo.gc.ca/fm-gp/species-especes/salmon-saumon/facts- infos-eng.html.

FBC (Fraser Basin Council). 2013. Fraser Basin Watersheds. Available online at: https://www.fraserbasin.bc.ca/basin_watersheds.html. Accessed March 2022.

FVRD (Fraser Valley Regional District) 2016. Regional Snapshot Series: Agriculture. Agriculture Economy in the Fraser Valley Regional District.

Garner K and Parfitt B. 2006. First Nations, salmon fisheries and the rising importance of conservation. Pacific Fisheries Resource Conservation Council.

Gibbons D, Morrissey C, and Mineau P, 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environmental Science and Pollution Research, 22(1): 103-118.

Goulson D. 2013. An overview of the environmental risks posed by neonicotinoid insecticides. Journal of Applied Ecology, 50: 977–987.

GoC (Government of Canada). 1998. Biological test method: toxicity tests using early life stages of salmonid fish (rainbow trout). Method development and application section, environmental protection series, second edition, Environment Canada, Ottawa, Ontario.

GoC. 2007. Pesticides and health. Her Majesty the Queen in Right of Canada, represented by the Minister of Health Canada. Available online at: https://www.canada.ca/en/health-canada/services/environmental-workplace-health/reports-publications/environmental-contaminants/pesticides-health.html.

GoC. 2009. Pest Management Agency (PMRA). Her Majesty the Queen in Right of Canada, represented by the Minister of Health Canada. Available online at: https://www.canada.ca/en/health-canada/corporate/about-health-canada/branches-agencies/pest-management-regulatory-agency.html.

GoC. 2020. Update on the neonicotinoid pesticides (January 2020). Her Majesty the Queen in Right of Canada, represented by the Minister of Health Canada. Available online at: https://www.canada.ca/en/health-canada/services/consumer-product-safety/reports-publications/pesticides-pest-management/fact-sheets-other-resources/update-neonicotinoid-pesticides-january-2020.html.

GoC. 2022. Canadian climate normal 1981-2010 station data. Chilliwack, BC. Her Majesty the Queen in Right of Canada. Available online at: https://climate.weather.gc.ca/climate_normals/. Green BS. 2008. Maternal Effects in Fish Populations. Advances in Marine Biology, Tasmanian Fisheries and Aquaculture Institute, University of Tasmania 54: 1 – 105.

Green BS. 2008. Maternal effects in fish populations. Advances in Marine Biology, 54. Tasmanian Fisheries and Aquaculture Institute, University of Tasmania.

Gries T. 2002. Imidacloprid: early life-stage toxicity test with rainbow trout (Oncorhynchus mykiss) under flow-through conditions. Project Number: 1022/016/321. Unpublished study prepared by Springborn Smithers Laboratories (Europe) AG. 102p.

Hansen T, Christiansen R, Nortvedt, R, Stefansson, S, and Taranger GL. 1990. Artificial hatching substrates improve growth and yolk absorption of salmonids. Canadian Technical Reports of Fisheries and Aquatic Sciences 1761.

Hilderbrand GV. Schwartz CC, Robbins CT, Jacoby ME, Hanley TA, Arthur SM, and Servheen C. 1999. The importance of meat, particularly salmon, to body size, population productivity, and conservation of North American brown bears. Canadian Journal of Zoology, 77(1).

Hilderbrand GV, Farley SD, Schwartz CC, and Robbins CT. 2004. Importance of salmon to wildlife: Implications for integrated management. Ursus, 15(1):1–9.

Hladik ML, Kolpin DW, Kuivila KM. 2014. Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. Environ Pollut 193:189–196.

Hodgson EE, Wilson SM, Moore JW. 2019. Changing estuaries and impacts on juvenile salmon: A systemic review. Global Change Biology.

Holm J, Palace V, Siwik P, Sterling, G, Evans R, Baron C, Werner J, and Wautier K. 2005. Developmental effects of bioaccumulated selenium in eggs and larvae of two salmonid species.

IUCN (International union for conservation of nature). 2022. IUCN, International Union for Conservation of Nature. Available online at: https://www.iucn.org/.

Jeschke P and Nauen R. 2008. Neonicotinoids – from zero to hero in insecticide chemistry. PubMed 64(11):1084-1098.

Jeschke P, Nauen R, Schindler M, Elbert A. 2011. Overview of the status and global strategy for neonicotinoids. J Agric Food Chem. 59(7):2897–2908.

Kocamaz D and Oruc E. 2020. Effect of thiamethoxam and λ cyhalothrin, administered individually and in mixture on the endocrine function and antioxidant defense of gonads of *Oreochromis niloticus*. Pakistan Jouranl if Zoology, 52(3).

Kondolf, G.M. and Wolman, M.G. 1993. The sizes of salmonid spawning gravels, Water Resources Research, 29(7):2275–2285.

Landriga PJ, Fuller R, Acosta NJR, Adeyi O, Arnold R, Basu NN, Baldé AB, Bertollini R, Bose-O'Reilly S, Boufford JI, Breysse PN, Chiles T, Mahidol C, Coll-Seck AM, Cropper ML, Fobil J, Fuster V, Greenstone M, Haines A, Hanrahan D, Hunter D, Khare M, Krupnick A, Lanphear B, Lohani B, Martin K, Mathiasen KV, McTeer MA, Murray CJL, Ndahimananjara JD, Perera F, Potocnik J, Preker AS, Ramesh J, Rockstr€om, J, Salinas C, Samson LD, Sandilya K, Sly PD, Smith KR, Steiner A, Stewart RB, Suk WA, van Schayck OCP, Yadama GN, Yumkella K, Zhong M. 2018. The Lancet Commission on pollution and health. Lancet, 391: 462-512.

Leung TYG. 2018. Sub-lethal effects of clothianidin on early life stage sockeye salmon (*Oncorhynchus nerka*). Master of Environmental Toxicology thesis, Simon Fraser University.

Life Technologies. 2012. Real-time PCR handbook.

Liu TYG. 2015. Sub-lethal effects of clothianidin on early life stage sockeye salmon (Oncorhynchus nerka). MSc thesis, Simon Fraser University, Burnaby BC.

Ma X, Li H, Xiong J, Mehler WT, and You J. 2019. Developmental Toxicity of a Neonictinoid Insectivide, Acetamiprid to Zebrafish. J. Agric. Food Chem. 67(9): 2429-2436.

MacBean, C (ed). 2010. The e-pesticide manual, 15th ed., version 5.0.1, Surrey, UK, British Corp Protection Council. Thiamethoxam (153719-23-4).

Main AR, Headley JV, Peru KM, Michel NL, Cessna AJ, Morrissey CA. 2014. Widespread use and frequent detection of neonicotinoid insecticides in wetlands of Canada's prairie pothole region. PLoS ONE 9(3).

Malhotra N, Chen KH-C, Huang J-C, Lai H-T, Uapipatanakul B, Roldan MJM, Macabeo APG, Ger T-R, Hsiao C-D. 2021. Physiological effects of neonicotinoid insecticides on non-target aquatic animals—an updated review. International Journal of Molecular Sciences, 22(17):9591.

Marshall DJ, Allen RM, and Crean AJ. 2008.The ecological and evolutionary importance of maternal effects in the sea. Oceanogr. Mar. Biol. An Annu. Rev. 46: 203–250.

Marlatt VL, Leung TYG, Calbik S, Metcalfe C, and Kennedy C. 2019. Sub-lethal effects of a neonicotinoid, clothianidin, on wild early life stage sockeye salmon (Oncorhynchus nerka). Aquatic Toxicology, vol 217.

Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M., and Sattelle DB. 2001. Neonicotinoids : insecticides acting on insect nicotinic acetylcholine receptors. Pharmacol. 2(11).

Matsuda K, Shimomura M, Ihara M, Akamatsu M, and SattelleDB. 2005. Neonicotinoids show selective and diverse actions on their nicotinic receptor targets: electrophysiology, molecular Biology, and receptor modeling Studies. Biosci. Biotechnol. Biochem. 69 (8).

McCormick SD, Hansen LP, Quinn TP, Saunders RL. 1998. Movement, migration, and smolting of Atlantic salmon (Salmo salar). Can. J. Fish. Aquat. Sci. 55 (Suppl. 1), 77–92.

McMaster ME, Munkittrick KR, Van Der Kraak GJ. 1992. Protocol for measuring circulating levels of gonadal sex steroids in fish. Department of Fisheries and Oceans, Ecotoxicology Division, Bayfield Institute. Burlington, Ontario.

Miles JC, Hua J, Sepulveda MS, Krupke CH, Hoverman JT. 2017. Effects of clothianidin on aquatic communities: evaluating the impacts of lethal and sublethal exposure to neonicotinoids. PLoS ONE 12(3).

Morrissey CA, Mineau P, Devries JH, Sanchez-Bayo F, Liess M, Cavallaro MC, Liber K. 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ Int 74:291–303.

National Centre for Biotechnology Information [NCBI]. (2020). PubChem compound summary for CID 107646. Available online at: https://pubchem.ncbi.nlm.nih.gov/compound/Thiamethoxam.

Nauen R. 1999. Ligands of the nicotinic acetylcholine receptor as insecticides. Bayer AG, Agrochemicals Division Research, D-51368, Leverkusen, Germany.

National Pesticide Information Centre (NPIC). 2011. Imidacloprid technical factsheet. Oregon State University. Available online at: http://npic.orst.edu/factsheets/archive/imidacloprid.html.

Noakes D. 2011. Impacts of salmon farms on Fraser River sockeye salmon. Cohen Commission Tech. Report 5C.

Pilgrim NL, Royer R, Ripley L , Underwood J , Rasmussen JB, and Hontela A. 2013. Multicompartment gavel bed flume to evaluate swim- up success of salmonid larvae. North American Journal of Fisheries Management, 33(1).

Pisa L, Goulson D, Yang EC, Gibbons D, Sánchez-Bayo F, Mitchell E, Aebi A van der Sluijs J, MacQuarrie CJ, Giorio C, and Long EY. 2017. An update of the worldwide integrated assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. Environmental Science and Pollution Research, 1-49.

Rand, P.S. 2011. Oncorhynchus nerka. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.2.

Rand PS, Goslin M, Gross MR, Irvine JR, Augerot X, McHugh PA, and Bugaev VF. 2012. Global assessment of extinction risk to populations of sockeye salmon *Oncorhynchus nerka.* PLoS ONE 7(4). Available online at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0034065.

Rudolph, B., Andreller, I, Kennedy, C.J. (2008). Reproductive success, early life stage development, and survival of westslope Cutthroat Trout (Oncorhynchus clarki lewisi) exposed to elevated selenium in an area of active coal mining.

Sánchez-Bayo F and Goka K. 2005. Unexpected effects of zinc pyrithione and imidacloprid on Japanese medaka fish (Oryzias latipes). Aquat Toxicol 74:285–293.

Sánchez-Bayo F, Hyne RV. 2014. Detection and analysis of neonicotinoids in river waters–development of a passive sampler for three commonly used insecticides. Chemosphere 99:143–151.

Schaafsma A, Limay-Rios V, Baute T, Smith J, Xue Y. 2015. Neonicotinoid insecticide residues in surface water and soil associated with commercial maize (Corn) fields in Southwestern Ontario. PLoS ONE 10(2).

Simon-Delso N, Amaral-Rogers, Belzunces LP, Bonmatin JM, Chagnon M, Downs C, Furlan L, Gibbons DW, Giorio C, Girolami V, Goulson D, Kreutzweiser DP, Krupke CH, Liess, E Long M, McField M, Mineau P, Mitchell EAD, Morrissey CA, Noome DA, Pisa L, Settele J, Stark JD, Tapparo A, Van Dyck H, Van Praagh J, Van der Sluijs JP, PR Whitehorn, Wiemers M. 2014. Systemic insecticides (Neonicotinoids and fipronil): Trends, uses, mode of action and metabolites. Environ. Sci. Pollut. Res.22(1): 5–34.

Statistics Canada. 2016. 2015 provincial gross farm receipts for the Fraser Valley.

Straub L, Williams GR, Vidondo B, Khongphinitbunjong K, Retschnig G, Schneeberger A, Chantawannakul P, Dietemann V, Neumann P. 2019. Neonicotinoids and ectoparasitic mites synergistically impact honeybees. Sci Rep 9(1):1–1.

Starner K and Goh KS. 2012. Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010–2011. Bull Environ Contam Toxicol 88(3):316–321.

Sulatan, T., Murray, C., Kleywegt, S., Metcalfe, C.D., 2018. Neonicotinoid pesticides in drinking water in agricultural regions of southern Ontario, Canada. Chemosphere, 202, 506-513.

Takeo J, and Yamashita S. 1999. Two Distinct Isoforms of cDNA Encoding Rainbow Trout Androgen Receptors. Journal of Biological Chemistry, 274(9):5674–5680.

Taylor, S. Wakem, M., Dijkman, G., Alsarraj, M., and Nguyen, M. (2010). A Pratical Approach to RT-qPCR – Publishing Data That Conform to The MIQUE Guidelines. Methods, 50.

Thany SH. 2010. Neonicotinoid insecticides. Historical evolution and resistance mechanisms. In: Thany SH (Ed.) Insect nicotinic acetylcholine receptors. Adv Exp Med Biol 683:75–84.

Tisler T, Jemec A, Branks M, and Trebse P. 2009. Hazard Identification of Imidacloprid to Aquatic Environment. Chemosphere, 76: 907-914.

US EPA (United States Environmental Protection Agency). 2003. US EPA. Office of pesticide programs, factsheet clothiandin. EPA Publication 7501C.

US EPA. 2010. Clothianidin registration of prosper T400 seed treatment on mustard seed (oilseed and condiment) and poncho/votivo seed treatment on cotton. United States Environmental Protection Agency, Washington, DC.

Wasser SK, Lundin JI, Ayres K, Seely E, Giles D, Balcomb K, Hempelmann, Parsons K, Booth R. 2017. Population growth is limited by nutritional impacts on pregnancy success in endangered Southern Resident killer whales (Orcinus orca). PLoS One, 12(6). Available online at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0179824.

Weil LS, Barry TP, Madison JA. 2001. Fast growth in rainbow trout is correlated with a rapid decrease in post-stress cortisol concentrations. Aquaculture,193: 373-380.

Van Dijk TC, Van Staalduinen MA, Van der Sluijs JP (2013) Macroinvertebrate decline in surface water polluted with imidacloprid PLoS ONE 8(5).

Yamamoto A, Terao T, Hisatomi H, Kawasaki H, Arakawa R. 2012. Evaluation of river pollution of neonicotinoids in Osaka City (Japan) by LC/MS with dopant-assisted photoionisation. J Environ Monit 14(8):2189–2194.

Appendix A.

Water Chemistry Results

SIMON FRASER UNIVERSITY
ATTN: Vicki Marlatt | Michael Moreton
Department of Biological Sciences-SFU
8888 University Drive
Burnaby BC V5A 1S6

Date Received: 07-FEB-17
Report Date: 01-MAR-17 10:46 (MT)
Version: FINAL

Client Phone: 778-782-4107

Certificate of Analysis

Lab Work Order #: L1888035
Project P.O. #: NOT SUBMITTED
Job Reference:
C of C Numbers: 15-589645
Legal Site Desc:

Amber Springer, B.Sc.
Account Manager

ADDRESS: 8081 Lougheed Hwy, Suite 100, Burnaby, BC V5A 1W9 Canada | Phone: +1 604 253 4188 | Fax: +1 604 253 6700
ALS CANADA LTD Part of the ALS Group A Campbell Brothers Limited Company

ALS ENVIRONMENTAL ANALYTICAL REPORT

	Sample ID	L1888035-1				
	Description	Water				
	Sampled Date	06-FEB-17				
	Sampled Time	17:02				
	Client ID	NEONC WL #2				
Grouping	Analyte					
WATER						
Pesticides	Acetamiprid (ug/L)	<0.0050				
	Clothianidin (ug/L)	0.0290				
	Imidacloprid (ug/L)	0.0248				
	Nitenpyram (ug/L)	<0.0050				
	Sulfoxaflor (ug/L)	<0.0050				
	Thiacloprid (ug/L)	<0.0050				
	Thiamethoxam (ug/L)	0.0295				

Reference Information

Test Method References:

ALS Test Code	Matrix	Test Description	Method Reference**
NEONICOTINOID-SPE-WT	Water	Neonicotinoids in Water by LC-MS/MS	Env. Canada NWRI

This analysis is carried out using procedures adapted from Environment Canada. Neonicotinoids is determined on a water sample that is evaporated near dryness, reconstituted and then analyzed on a LC-MS/MS

** ALS test methods may incorporate modifications from specified reference methods to improve performance.

The last two letters of the above test code(s) indicate the laboratory that performed analytical analysis for that test. Refer to the list below:

Laboratory Definition Code	Laboratory Location
WT	ALS ENVIRONMENTAL - WATERLOO, ONTARIO, CANADA

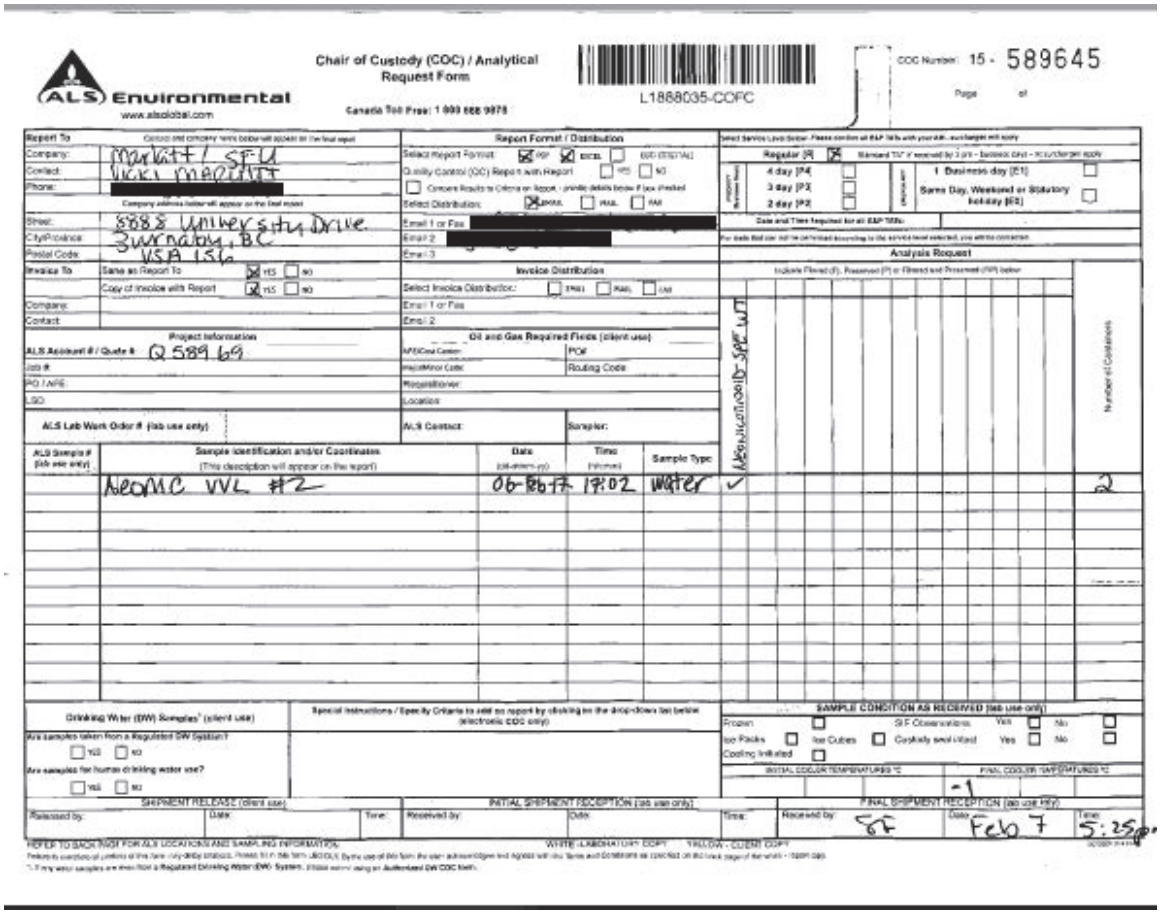
Chain of Custody Numbers:

15-580645

GLOSSARY OF REPORT TERMS

Surrogate - A compound that is similar in behaviour to target analyte(s), but that does not occur naturally in environmental samples. For applicable tests, surrogates are added to samples prior to analysis as a check on recovery.
mg/kg - milligrams per kilogram based on dry weight of sample.
mg/kg wwt - milligrams per kilogram based on wet weight of sample.
mg/kg lwt - milligrams per kilogram based on lipid-adjusted weight of sample.
mg/L - milligrams per litre.
< - Less than.
D.L. - The reported Detection Limit, also known as the Limit of Reporting (LOR).
N/A - Result not available. Refer to qualifier code and definition for explanation.

Test results reported relate only to the samples as received by the laboratory.
UNLESS OTHERWISE STATED, ALL SAMPLES WERE RECEIVED IN ACCEPTABLE CONDITION.
Analytical results in unsigned test reports with the DRAFT watermark are subject to change, pending final QC review.



ALS Environmental
www.alsglobal.com

Chain of Custody (COC) / Analytical Request Form

Canada Toll Free: 1 800 668 9878

L1888035-COFC

COC Number: 15 - 589645

Page of

Report To	Markit / SFU
Company:	Nicki Markit
Contact:	[redacted]
Phone:	8888 University Drive
Street:	Burnaby, BC
City/Province:	V5A 1S6
Postal Code:	
Invoice To	Same as Report To: YES NO
	Copy of Invoice with Report: YES NO
Company:	
Contact:	

Report Format / Distribution: Select Report Format: PDF, EXCEL, EDD (DIGITAL); Quality Control (QC) Report with Report: NO; Select Distribution: EMAIL, MAIL, FAX; Email 1 or Fax: [redacted]; Email 2: [redacted]; Email 3:

Invoice Distribution: Select Invoice Distribution: EMAIL, MAIL, FAX; Email 1 or Fax; Email 2

Project Information: ALS Account # / Quote #: Q 589 69; Job #; PO / AFE; LSD:

Oil and Gas Required Fields (client use): AFE/Cost Center; PO#; Major/Minor Code; Routing Code; Requisitioner; Location

ALS Lab Work Order # (lab use only):

ALS Contact:

Sampler:

Select Service Level Below - Please confirm all EAP Tests with your AM - surcharges will apply

Regular [R]: Standard TAT if received by 3 pm - business days - no surcharges apply

Priority (Surcharges Apply)	4 day [P4]		
	3 day [P3]		
	2 day [P2]		
Emergency (Surcharges Apply)	1 Business day [E1]		
	Same Day, Weekend or Statutory holiday [E2]		

Date and Time Required for all E&P TATs:

For tests that can not be performed according to the service level selected, you will be contacted.

Analysis Request

Indicate Filtered (F), Preserved (P) or Filtered and Preserved (FP) below

ALS Sample # (lab use only)	Sample Identification and/or Coordinates (This description will appear on the report)	Date (dd-mmm-yy)	Time (hh:mm)	Sample Type	Number of Containers
	Aconic VVL #2	06-Feb-17	19:02	water	2

Drinking Water (DW) Samples? (client use)

Are samples taken from a Regulated DW System? YES NO

Are samples for human drinking water use? YES NO

Special Instructions / Specify Criteria to add on report by clicking on the drop-down list below (electronic COC only)

SAMPLE CONDITION AS RECEIVED (lab use only)

Frozen; SIF Observations: Yes No

Ice Packs; Ice Cubes; Custody seal intact: Yes No

Cooling Initiated

INITIAL COOLER TEMPERATURES °C: -1

FINAL COOLER TEMPERATURES °C

SHIPMENT RELEASE (client use)			INITIAL SHIPMENT RECEPTION (lab use only)			FINAL SHIPMENT RECEPTION (lab use only)		
Released by:	Date:	Time:	Received by:	Date:	Time:	Received by: SF	Date: Feb 7	Time: 5:25 pm

REFER TO BACK PAGE FOR ALS LOCATIONS AND SAMPLING INFORMATION

WHITE - LABORATORY COPY YELLOW - CLIENT COPY

SIMON FRASER UNIVERSITY
ATTN: Vicki Marlatt
Department of Biological Sciences-SFU
8888 University Drive
Burnaby BC V5A 1S6

Date Received: 12-DEC-16
Report Date: 28-DEC-16 17:01 (MT)
Version: FINAL

Client Phone: 778-782-4107

Certificate of Analysis

Lab Work Order #: L1869013
Project P.O. #: NOT SUBMITTED
Job Reference:
C of C Numbers: 15-589722
Legal Site Desc:

Amber Springer, B.Sc
Account Manager

ADDRESS: 8081 Lougheed Hwy, Suite 100, Burnaby, BC V5A 1W9 Canada | Phone: +1 604 253 4188 | Fax: +1 604 253 6700

ALS ENVIRONMENTAL ANALYTICAL REPORT

Sample ID	L1869013-1	L1869013-2	L1869013-3	L1869013-4	L1869013-5
Description	WATER	WATER	WATER	WATER	WATER
Sampled Date	12-DEC-16	12-DEC-16	12-DEC-16	12-DEC-16	12-DEC-16
Sampled Time	13:05	13:15	13:18	13:19	13:22
Client ID	NEONICOTINOID COCKTAIL #1	NEONICOTINOID COCKTAIL #2	NEONICOTINOID COCKTAIL #3	NEONICOTINOID COCKTAIL #4	NEONICOTINOID COCKTAIL #5
Grouping **Analyte**					
WATER					
Pesticides Acetamiprid (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
Clothianidin (ug/L)	<0.0050	0.142	0.156	1.29	12.1
Imidacloprid (ug/L)	<0.0050	0.148	0.152	1.21	11.2
Nitenpyram (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
Sulfoxaflor (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
Thiacloprid (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
Thiamethoxam (ug/L)	<0.0040	0.161	0.166	1.34	12.6

SIMON FRASER UNIVERSITY
ATTN: Vicki Marlatt
Department of Biological Sciences-SFU
8888 University Drive
Burnaby BC V5A 1S6

Date Received: 12-DEC-16
Report Date: 28-DEC-16 17:01 (MT)
Version: FINAL

Client Phone: 778-782-4107

Certificate of Analysis

Lab Work Order #: L1869013
Project P.O. #: NOT SUBMITTED
Job Reference:
C of C Numbers: 15-589722
Legal Site Desc:

Amber Springer, B.Sc
Account Manager

ADDRESS: 8081 Lougheed Hwy, Suite 100, Burnaby, BC V5A 1W9 Canada | Phone: +1 604 253 4188 | Fax: +1 604 253 6700
ALS CANADA LTD Part of the ALS Group A Campbell Brothers Limited Company

ALS ENVIRONMENTAL ANALYTICAL REPORT

Sample ID		L1869013-1	L1869013-2	L1869013-3	L1869013-4	L1869013-5
Description		WATER	WATER	WATER	WATER	WATER
Sampled Date		12-DEC-16	12-DEC-16	12-DEC-16	12-DEC-16	12-DEC-16
Sampled Time		13:05	13:15	13:16	13:19	13:22
Client ID		NEONICOTINOID COCKTAIL #1	NEONICOTINOID COCKTAIL #2	NEONICOTINOID COCKTAIL #3	NEONICOTINOID COCKTAIL #4	NEONICOTINOID COCKTAIL #5
Grouping	Analyte					
WATER						
Pesticides	Acetamiprid (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
	Clothianidin (ug/L)	<0.0050	0.142	0.156	1.29	12.1
	Imidacloprid (ug/L)	<0.0050	0.148	0.152	1.21	11.2
	Nitenpyram (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
	Sulfoxaflor (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
	Thiacloprid (ug/L)	<0.0050	<0.0050	<0.0050	<0.0050	<0.0050
	Thiamethoxam (ug/L)	<0.0040	0.161	0.166	1.34	12.6

ALS ENVIRONMENTAL ANALYTICAL REPORT

		Sample ID	L1869013-6				
		Description	WATER				
		Sampled Date	12-DEC-16				
		Sampled Time	13:24				
		Client ID	NEONICOTINOID COCKTAIL #6				
Grouping	Analyte						
WATER							
Pesticides	Acetamiprid (ug/L)		<0.0050				
	Clothianidin (ug/L)		133				
	Imidacloprid (ug/L)		127				
	Nitenpyram (ug/L)		<0.0050				
	Sulfoxaflor (ug/L)		<0.0050				
	Thiacloprid (ug/L)		<0.0050				
	Thiamethoxam (ug/L)		144				

Reference Information

Test Method References:

ALS Test Code	Matrix	Test Description	Method Reference**
NEONICOTINOID-SPE-WT	Water	Neonicotinoids in Water by LC-MS/MS	Env. Canada NWRI

This analysis is carried out using procedures adapted from Environment Canada. Neonicotinoids is determined on a water sample that is evaporated near dryness, reconstituted and then analyzed on a LC-MS/MS

** ALS test methods may incorporate modifications from specified reference methods to improve performance.

The last two letters of the above test code(s) indicate the laboratory that performed analytical analysis for that test. Refer to the list below:

Laboratory Definition Code	Laboratory Location
WT	ALS ENVIRONMENTAL - WATERLOO, ONTARIO, CANADA

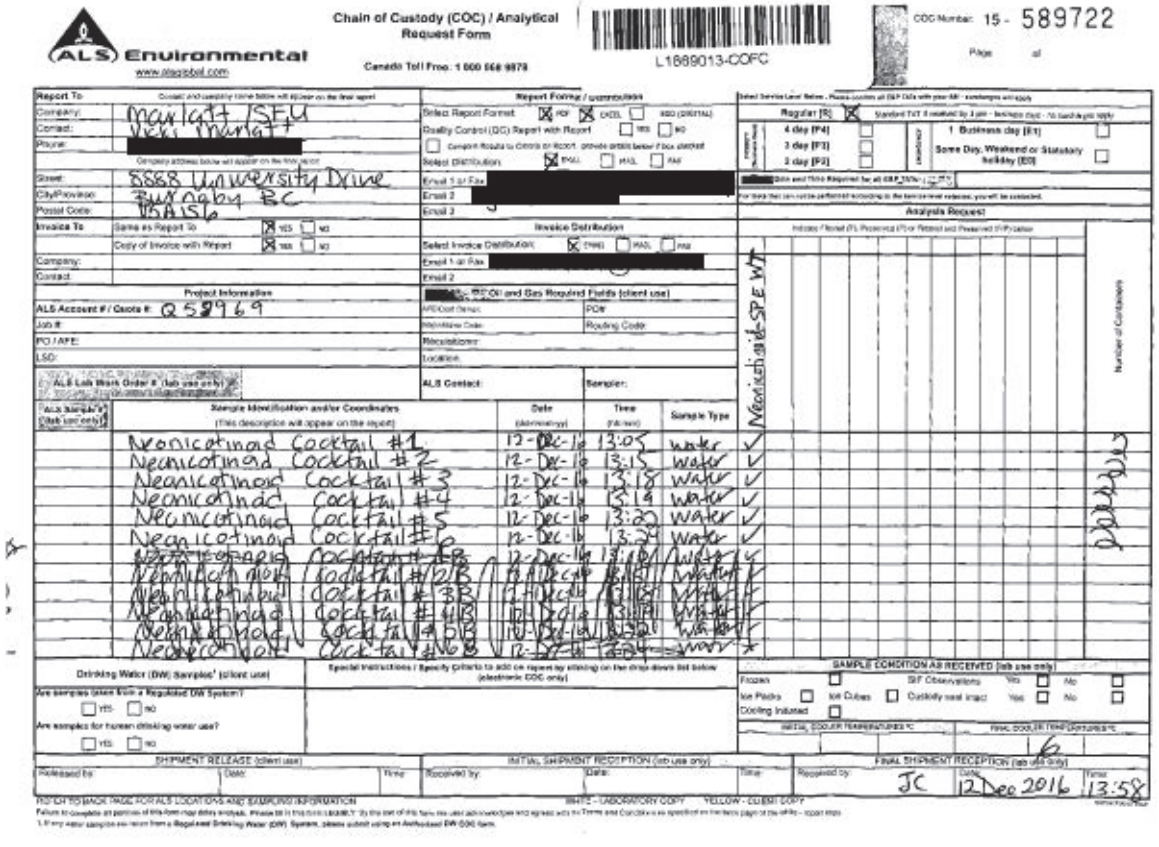
Chain of Custody Numbers:

15-589722

GLOSSARY OF REPORT TERMS

Surrogate - A compound that is similar in behaviour to target analyte(s), but that does not occur naturally in environmental samples. For applicable tests, surrogates are added to samples prior to analysis as a check on recovery.
mg/kg - milligrams per kilogram based on dry weight of sample.
mg/kg wet - milligrams per kilogram based on wet weight of sample.
mg/kg lwt - milligrams per kilogram based on lipid-adjusted weight of sample.
mg/L - milligrams per litre.
< - Less than.
D.L. - The reported Detection Limit, also known as the Limit of Reporting (LOR).
N/A - Result not available. Refer to qualifier code and definition for explanation.

Test results reported relate only to the samples as received by the laboratory.
UNLESS OTHERWISE STATED, ALL SAMPLES WERE RECEIVED IN ACCEPTABLE CONDITION.
Analytical results in unsigned test reports with the DRAFT watermark are subject to change, pending final QC review.



Chain of Custody (COC) / Analytical Request Form

ALS Environmental
www.alsglobal.com
Canada Toll Free: 1 800 668 9878

L1669013-COFC

COC Number: 15 - 589722
Page of

Report To: Contact and company name below will appear on the final report
Company: Maxlift / SFU
Contact: Ken Maxlift
Phone: [redacted]
Company address below will appear on the final report
Street: 8888 University Drive
City/Province: Burnaby BC
Postal Code: V5A1S6

Report Format / Distribution
Select Report Format: PDF, EXCEL, EDD (DIGITAL)
Quality Control (QC) Report with Report: YES, NO
Compare Results to Criteria on Report - provide details below if box checked
Select Distribution: EMAIL, MAIL, FAX
Email 1 or Fax: [redacted]
Email 2
Email 3

Invoice To: Same as Report To: YES, NO
Copy of Invoice with Report: YES, NO
Company:
Contact:

Invoice Distribution
Select Invoice Distribution: EMAIL, MAIL, FAX
Email 1 or Fax: [redacted]
Email 2

Project Information
ALS Account # / Quote #: Q58969
Job #:
PO / AFE:
LSD:
ALS Lab Work Order # (lab use only):
ALS Contact:
Sampler:

[redacted] EDD (DIGITAL) and Gas Required Fields (client use)
AFE/Cost Center: PO#
Major/Minor Code: Routing Code
Requisitioner:
Location:

Regular [R]: X
Standard TAT if received by 3 pm - business days - no surcharges apply
4 day [P4]
3 day [P3]
2 day [P2]
1 Business day [E1]
Same Day, Weekend or Statutory holiday [E0]
Date and Time Required for all E&P TATs:
For tests that can not be performed according to the service level selected, you will be contacted.

Analysis Request
Indicate Filtered (F), Preserved (P) or Filtered and Preserved (F/P) below
Neonicotinoid-SPE WT
Number of Containers

ALS Sample # (ALS use only)	Sample Identification and/or Coordinates (This description will appear on the report)	Date (dd-mmm-yy)	Time (hh:mm)	Sample Type
	Neonicotinoid Cocktail #1	12-Dec-16	13:05	water
	Neonicotinoid Cocktail #2	12-Dec-16	13:15	water
	Neonicotinoid Cocktail #3	12-Dec-16	13:18	water
	Neonicotinoid Cocktail #4	12-Dec-16	13:19	water
	Neonicotinoid Cocktail #5	12-Dec-16	13:20	water
	Neonicotinoid Cocktail #6	12-Dec-16	13:24	water
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]

Drinking Water (DW) Samples* (client use)
Are samples taken from a Regulated DW System? YES, NO
Are samples for human drinking water use? YES, NO

Special Instructions / Specify criteria to add on report by clicking on the drop-down list below (electronic COC only)

SAMPLE CONDITION AS RECEIVED (lab use only)
Frozen, SIF Observations Yes, No
Ice Packs, Ice Cubes, Custody seal intact Yes, No
Cooling Initiated
INITIAL COOLER TEMPERATURES °C
FINAL COOLER TEMPERATURES °C
6

SHIPMENT RELEASE (client use)
Released by: Date: Time:

INITIAL SHIPMENT RECEPTION (lab use only)
Received by: Date: Time:

FINAL SHIPMENT RECEPTION (lab use only)
Received by: JC
Date: 12 Dec 2016
Time: 13:58

REFER TO BACK PAGE FOR ALS LOCATIONS AND SAMPLING INFORMATION
WHITE - LABORATORY COPY YELLOW - CLIENT COPY

Failure to complete all portions of this form may delay analysis. Please fill in this form LEGIBLY. By the use of this form the user acknowledges and agrees with the Terms and Conditions as specified on the back page of the white - report copy.
1. If any water samples are taken from a Regulated Drinking Water (DW) System, please submit using an Authorized DW COC form.