Viability, growth, development, and performance of juvenile sockeye salmon (*Oncorhynchus nerka*) exposed to neonicotinoid pesticides

by

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Ethics Statement

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Abstract

The effects of the neonicotinoid insecticide imidacloprid on sockeye salmon (*Oncorhynchus nerka*) exposed from 1 h post-fertilization to the swim-up fry developmental stage were evaluated using a gravel-bed flume incubator designed to simulate a natural streambed environment. This chronic exposure tested nominal imidacloprid concentrations of 0.15, 1.5, 15, and 150 µg/L to investigate the effects on hatching success and timing, deformity rates and growth. The effects of the neonicotinoid insecticides thiamethoxam, imidacloprid, and clothianidin, and mixtures of all three on burst swimming performance and routine metabolism in sockeye salmon (*Oncorhynchus nerka*) were also examined after acute 96-h exposures. There was no evidence that chronic exposures impacted growth, development, hatch timing and success or survival in sockeye salmon during the embryonic pre-hatch and post-hatch alevin developmental stages. There was also no evidence that acute exposures to environmentally relevant concentrations of neonicotinoids impacted swim performance or routine metabolism in swim-up fry.

Keywords: sockeye salmon; neonicotinoids; growth; development; performance

Dedication

To my parents, Murray and Carol Engelking. Thank you for providing me with every opportunity that I could have asked for and for encouraging me every step of the way.

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List of Acronyms and Abbreviations

%	percent
ºC	degrees Celsius
$	dollars
µg/L	micrograms per litre
µL	microliter
µS/cm	microSiemens per centimetre
ATU	accumulated thermal unit
Bl/s	body lengths per second
cm	centimetres
cm/s	centimetres per second
d	days
g	grams
g/ha	grams per hectare
g/L	grams per litre
h	hour
kg	kilograms
L	litres
mg/L	milligrams per litre
mg O2/g/h	milligrams of oxygen per gram per hour
min	minutes
mL	millilitres
mL/min	millilitres per minute
mm	millimetres
ng/L	nanograms per litre
Pa	pascals
ANOVA	analysis of variance
BC	British Columbia
BC MoE	British Columbia Ministry of the Environment
BCF	bioconcentration factor
CCME	Canadian Council of Ministers of the Environment
CI	confidence interval
DNA	deoxyribonucleic acid

GOT	glutamic oxaloacetic transaminase
GPT	glutamic pyruvic transaminase
K_{oc}	organic carbon – water partition coefficient
K_{ow}	octanol – water partition coefficient
LC_{50}	lethal concentration for 50% of test organisms
LOEC	lowest observed effects concentration
MATC	maximum acceptable toxicant concentration
MDA	malondialdehyde
MO_2	routine metabolism
MRID	Master Record Identification
nAChR	nicotinic acetylcholine receptor
NOEC	no observed effects concentration
OECD	Organization for Economic Cooperation and Development
PCB	polychlorinated biphenyl
PMRA	Pest Management Regulatory Agency
PVC	polyvinyl chloride
ROS	reactive oxygen species
SD	standard deviation
U_{max}	burst swimming speed
US EPA	United States Environmental Protection Agency
WSDA	Washington State Department of Agriculture

Chapter 1.

Introduction

1.1. Sockeye Salmon in the Fraser River Watershed

Oncorhynchus nerka, more commonly known as the sockeye salmon, is the third most abundant species of Pacific salmon belonging to the *Oncorhynchus* genus. Sockeye salmon inhabit a wide range of Pacific coastal waters and inland freshwaters along both the western coast of North America, and the eastern coast of Asia (Russia and Japan) (Groot 1991). The Fraser River supports the largest single river abundance of sockeye salmon in the world, and represents a site of both economic and cultural value to the region (Groot 1991; Cohen Commission 2012). The Fraser River flows for 1600 km from the Rocky Mountain headwaters along the Rocky Mountain Trench, eventually flowing south to the Coast Mountains and draining into the Strait of Georgia (Groot 1991). The watershed covers an area of 223 000 km^2, including a lake nursery area of 2500 km^2 (Groot 1991). Historically, the Fraser River sockeye fishery has played an important economic role in the industrial development of British Columbia (BC), and, with over 2000 commercial salmon licenses issued annually, continues to provide a source of employment in the catching, processing, and export of sockeye. Wild sockeye products are exported from BC to 63 countries, and in the 1990s, peak years yielded as much as 42.5 thousand tonnes of sockeye, valued at $195 million (Cohen Commission 2012). In addition to the economic value of the annual sockeye salmon runs, the sockeye are a source of cultural and spiritual well-being to the Aboriginal community in the Fraser River region (Cohen Commission 2012). The name sockeye itself is derived from an Anglicization of *suk-kegh* in Halkomelem, a language of indigenous populations that inhabit the southern reaches of the Fraser River watershed (Harris 1975). Beyond anthropocentric value, Pacific salmon populations provide an essential ecosystem service to riparian and estuarine systems (Naiman et al. 2002). Pacific salmon are semelparous, meaning that after the adults spawn, they typically die (Naiman et al. 2002). Since Pacific salmon spend the bulk of their adult lives in the ocean and do the majority of their mass accumulation there as well, when they return to their natal streams to spawn and die, the decay of their bodies provides an influx of marine-derived nutrients

to inland freshwater ecosystems (Naiman et al. 2002). This influx of the limiting nutrients nitrogen and phosphorous promote growth in the riparian ecosystem and helps to sustain the productivity of the nursery environments on which each season's newly hatched salmon depend (Janetski et al. 2009).

While 2010 was a dominant year and saw a resurgence in the sockeye salmon return, low returns in 2005, 2007, 2008, and 2009 offered little to no commercial fishing opportunities (Cohen Commission 2012). An observed trend of decreasing sockeye abundance and productivity by monitoring scientists and fisheries managers, including lower than predicted sockeye returns, and a record low return in 2009 prompted the Canadian federal government to initiate a judicial inquiry into potential causes for sockeye salmon population declines (Cohen Commission 2012). The Cohen Commission, led by Justice Bruce Cohen was established with the goals of encouraging cooperation between stakeholders, investigating potential causes of the decline, and improving the future sustainability of the sockeye fishery (Cohen Commission 2012). The life history of anadromous salmon, involving long periods spent in both nursery lakes and streams as well as the ocean, and long migrations between the two, provides opportunity for exposure to a wide variety of stressors at different life stages (Ross et al. 2013). In addition to the environmental changes that the salmon are exposed to throughout the course of their lives, they also undergo significant physiological changes during developmental stages, the smoltification process, and in preparation for spawning. Stressors can be biological in nature (e.g. pathogens or predators), physical (e.g. water temperatures, dissolved oxygen, and pH), or anthropogenic (e.g. landscape modification or introduction of contaminants) (Johnson et al. 2012). The final report that emerged from the Cohen Commission in 2012 identified a wide variety of potential stressors that may be responsible for declines in sockeye returns and productivity in the Fraser River, ranging from climate change, overfishing and transmission of disease and parasites, to aquaculture practices along important migratory routes and the presence of aquatic contaminants (Cohen Commission 2012). Low sockeye returns have continued in recent years, with another record low return observed in 2016 for the Fraser sockeye aggregate (Grant et al. 2017).

While the presence of a variety of anthropogenic contaminants has been identified as a potential contributing factor to the decline of sockeye salmon populations, information on the presence, concentration, and potential impacts of the contaminants is

limited (Cohen Commission 2012). Included in the list of potential point sources of contamination outlined in the report were pulp and paper mills, mines, contaminated sites, wastewater treatment facilities, aquaculture pens, and runoff from agricultural operations and forest management projects (Cohen Commission 2012). Similarly, Ross et al. (2013) examined the complexity of characterizing pollutant exposure to migratory salmonids over the course of their lives identified a variety of contaminant sources, including wastewater effluent, landfill leachate, pulp and paper mill effluent, wood preservation facilities, agricultural land, and deposition of atmospheric pollutants. Over 200 compounds were identified in the final Cohen report including metals, polycyclic aromatic hydrocarbons and other petroleum byproducts, flame retardants such as polybrominated diphenyl ethers, and metals. Although another major group of contaminants identified as a potential stressor for sockeye salmon populations is pesticides, there is limited information available pertaining to the presence and concentrations of pesticides in the Fraser River watershed, or BC freshwater in general, or the potential adverse effects of exposure to these chemicals in sockeye salmon and other teleost fish. However, a 2003-2004 study that sampled farm ditch water and sediments along fish bearing tributary streams in the Lower Fraser River basin found that 28 of 43 selected pesticide analytes were detected (Wan et al. 2006). In addition, a surface water sampling program was carried out by AXYS Analytical Services Ltd and Environment Canada from 2003 to 2005 in which 22 to 33 different pesticides were detected consistently in surface water adjacent to agricultural land in the Lower Fraser Valley (Woudneh et al. 2009). These findings indicate that it is likely that Pacific salmon are subject to exposure to a variety of pesticides throughout their life cycles.

1.2. Neonicotinoid insecticides

Synthetic and naturally-derived pesticides have been developed for diverse applications in urban, forest, and agricultural settings to minimize the impacts of identified target pest species. Target pest species range from plants considered to be weeds and moulds and fungi, to aquatic and terrestrial invertebrates and vertebrates (Environment Canada 2011). Due to the biocidal nature of pesticides, it is important that their use is carefully regulated. In Canada, regulation of pesticides occurs at the federal, provincial, and municipal level (Environment Canada 2011). At the federal level, the Health Canada Pest Management Regulatory Agency (PMRA) is responsible for

overseeing the import, registration, sale, manufacture, and application of pesticides under the Pest Control Products Act (Environment Canada 2011). The goal of the Pest Control Products Act is to “prevent unacceptable risks to human health and the environment from the use of pesticide products”. The pesticide market is continually evolving as researchers and manufacturers attempt to derive new chemicals that minimize the issues of persistence and long range transport in the environment, adverse effects to non-target organisms, and resistance in target pest species. As new pesticides continue to be developed, the challenge of regulation expands to include the evaluation of the newly introduced compounds in addition to assessing the impacts of past and current-use compounds on the environment.

Agricultural pesticides are a particularly important stressor to aquatic ecosystems due to their seasonal application and mobilization into aquatic environments via soil erosion, spray drift, atmospheric deposition, and surface runoff (Environment Canada 2011). Mobilization of agricultural pesticides into nearby aquatic systems is influenced by a variety of factors, including rainfall characteristics, the time interval between pesticide application and rainfall, the physical and chemical properties of the pesticide, the rate and mode of application, and the soil texture and topography (Willis and Mcdowell 1982). While aerial losses of the pesticide due to spray drift and post-application volatilization are typically greater than runoff losses in the water phase, pesticides that are highly soluble in water (10 mg/L or higher), tend to enter the aquatic environment mainly via runoff (Wauchope 1978; Willis and Mcdowell 1982). In cases where there is a rainfall event shortly after application, upwards of 10% of the total pesticide applied to a given area can be lost as runoff to the surrounding surface and groundwater (Wauchope 1978). A study on three urban, fish bearing waterways in BC found that concentrations of pesticides increased by an average of 8-fold following a rainfall event, further indicating that runoff can be an important source of entry for pesticides into the aquatic environment (Tierney et al. 2011a).

The first plant-derived insecticide used was nicotine, applied in the form of aqueous tobacco extracts, and it is still used as a minor insecticide in China (Tomizawa and Casida 2005). However, the toxicity of nicotine to mammals and its relatively low insecticidal activity prevented the development of a widely used insecticide with nicotine as the lead structure (Jeschke et al. 2011). Despite these limitations, the nicotinic acetylcholine receptor (nAChR), which is integral to the process of fast excitatory

synaptic transmission in the insect central nervous system, was an appealing target for the derivation of new insecticides (Jeschke et al. 2011). The neonicotinoids, named for their similarity in mode of action to that of nicotine, are the only major class of insecticides to be introduced to the market in the past 30 years, and are now registered for use in more than 120 countries (Tomizawa and Casida 2005; Jeschke et al. 2011). In 1990, prior to the introduction of neonicotinoids to the agrochemical market, insecticide sales were dominated by organophosphates (43%), pyrethroids (18%), and carbamates (16%). In 2008, the neonicotinoid market share had increased to 24%, and the share of organophosphates and carbamates had decreased to 14% and 11%, respectively (Jeschke et al. 2011).

Neonicotinoid insecticides currently on the market encompass a group of seven compounds including the N-nitroguanidines imidacloprid, thiamethoxam, clothianidin, and dinotefuran, the nitromethylene nitenpyram, and the N-cyanoamidines acetamiprid and thiacloprid (Jeschke et al. 2011; Figure 1). The most commonly used neonicotinoid insecticides are the N-nitroguanidines, which account for 85% of the neonicotinoid market share (Jeschke et al. 2011). Of these compounds, clothianidin, thiamethoxam, and imidacloprid, and their associated commercial formulations are currently under re-evaluation by the PMRA (Anderson et al. 2015). An important aspect of the re-evaluation process is the characterization of the potential for adverse effects in non-target aquatic organisms (Anderson et al. 2015).

One of the most attractive qualities of neonicotinoids as a pest control agent is that they are incorporated directly into a plant's tissues and act systemically. They are applied to crops in a variety of ways, including foliar, soil, and seed treatment, as well as stem injection and application via painting (Sur and Stork 2003). The compounds can be taken up in the roots, and translocated within the xylem to exert their toxicity on target insects. However, uptake is highly variable and can range from 1% to over 75%, indicating potential for losses to the surrounding environment (Sur and Stork 2003).

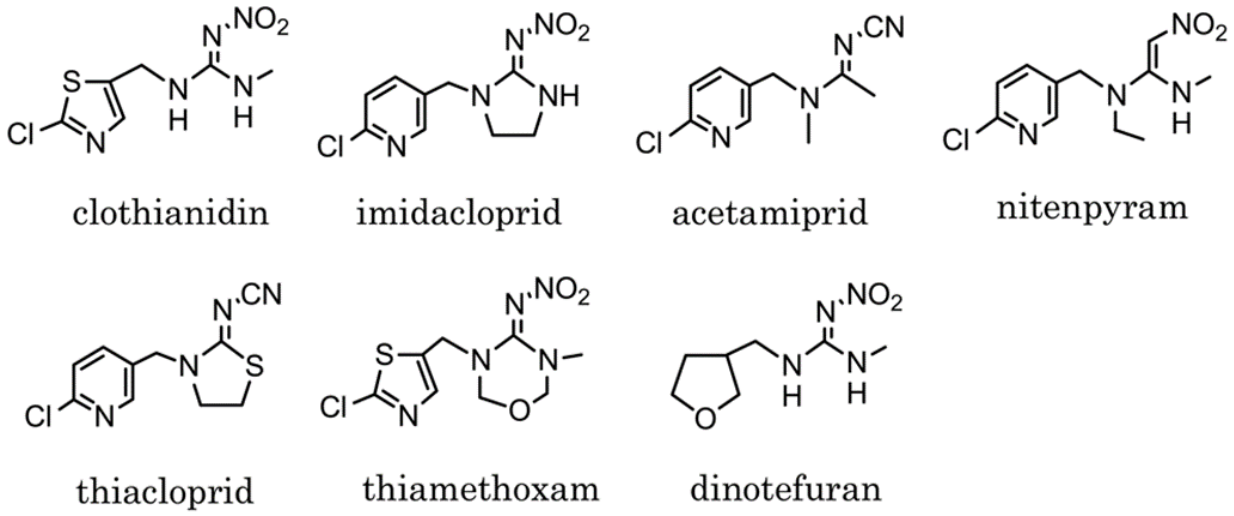

Figure 1. Chemical structures of the 7 neonicotinoid insecticides (Uchigashima et al. 2012).

1.2.1. Imidacloprid

Imidacloprid, produced by Bayer CropScience, was the first neonicotinoid made commercially available in the early 1990s (Jeschke and Nauen 2008). Both the active ingredient and the commercial formulation Admire® were approved for temporary registration in Canada in 1995 for control of the Colorado potato beetle (PMRA 2001). Other formulations that were registered in Canada include Merit®, Intercept®, and Concept® (Government of BC Ministry of Agriculture 2016). Imidacloprid is commonly used as a seed treatment or is directly applied to soil or foliage of crops, with application rates up to 312 g/ha active ingredient. Throughout Canada, imidacloprid is used in pest control on tomatoes, apples and field lettuce, as well as a seed pre-treatment in mustard, oilseed rape, canola, and corn (CCME 2007; PMRA 2001). Imidacloprid is a colourless, crystalline solid, with high water solubility (ranging from 510 to 610 mg/L at 20 °C), and low volatility (vapour pressure ranging from 4x10-10 to 2x10-7 Pa at 20 °C) (Macbean 2008; Tomlin 2000; Krohn 1989). In 2009, imidacloprid sales accounted for 41.5% of the total neonicotinoid market share and $1.09 billion (Simon-Delso et al. 2015).

1.2.2. Clothianidin

Clothianidin is a pesticide that was developed as a result of a collaboration between Takeda Chemical Industries and Bayer CropScience (Uneme 2011). The compound was originally developed to combat hemipteran pests in rice crops but has been found to be an effective method of pest control for a variety of pests in the orders Hemiptera, Thysanoptera, Coleoptera, Lepidoptera, and Diptera (Uneme 2011). Clothianidin was first registered for sale and use in 2004 in Canada as a seed treatment, as well as in the commercial formulations Clutch 50 WDGR® and Arena 50 WDGR® and has been used for insect control in crops such as grapes, pome fruits, stone fruits, potatoes, and turf grass (Government of British Columbia Ministry of Agriculture 2016; Health Canada 2011). Clothianidin is a colourless solid with high water solubility (327 mg/L at 20 °C) and low volatility (1.3 x 10-10 Pa at 25 °C) (Uneme 2011). In 2009, clothianidin sales accounted for 17% of the total neonicotinoid market share and $440 million (Simon-Delso et al. 2015).

1.2.3. Thiamethoxam

Thiamethoxam is a neonicotinoid that was developed by Ciba Crop Protection, which later became part of what is now Syngenta Crop Protection (Maienfisch et al. 2001). It has been marketed for use as a systemic insecticide since 1998 under the trademark Actara® for soil and foliar treatment, and the trademark Cruiser® for seed treatments (Maeinfisch et al. 2001). Thiamethoxam was first registered in Canada in 2001 and was approved for conditional registration and use in 2011 in the formulations Actara® and Veridian® for control of insect pests in turf grass (Health Canada 2011). Recent registrations in Canada include the registration of Actara® for control of blackvine weevil in strawberry crops in 2014 and the control of aphids and leafhoppers in outdoor ornamental plants in March 2014 (BC Ministry of Agriculture 2016). Thiamethoxam is a clear, crystalline compound with a high water solubility (4.1 g/L at 25 °C) and a low volatility (vapor pressure of 6.6 x 10-9 Pa at 25 °C) (Macbean 2010). In 2009, thiamethoxam sales accounted for 24% of the total neonicotinoid market share and $630 million (Simon-Delso et al. 2015). In addition to the introduction of thiamethoxam to the environment via direct application, a study on lepidopteran larvae and cotton plants indicated that thiamethoxam is a metabolic precursor to clothianidin and that this conversion can take place in some insects and plants (Nauen et al. 2003).

1.3. Neonicotinoid Contamination of Aquatic Environments

The neonicotinoid insecticides are a class of chemicals with high water solubility, which is conducive to their application as systemic insecticides. In addition to high water solubility, neonicotinoids can exhibit long half-lives in soil and water, with soil half-lives ranging from 7 to 72 d in the case of thiamethoxam to 104 to 228 d in the case of imidacloprid (PPDB 2017; HSDB 2017). Half-lives in water are more variable as neonicotinoids are resistant to degradation due to hydrolysis, but tend to be more sensitive to photolysis (Bonmatin et al. 2015). A major source of introduction of neonicotinoids to aquatic ecosystems is via agricultural runoff water following application (Ambrust and Peeler 2002). Loss of agriculturally applied neonicotinoids to the aquatic environment via runoff is augmented by rain events as well as their chemical properties such as low log K_{ow} and log K_{oc} values that limit soil adsorption and partitioning out of water once dissolved (Krohn 1989; Flores-Cespedes et al. 2002). In addition to runoff following irrigation and rainfall events, there are a variety of other common pathways for entry of neonicotinoids into aquatic ecosystems. Alternate transport pathways include introduction via snowmelt (Main et al. 2014), leaching into groundwater followed by subsurface movement into surface waters and wetlands (Lamers et al. 2011), and the decay of plants with systematically incorporated residues (Kreutzweiser et al. 2008). Neonicotinoids can also enter the aquatic environment via the deposition of treated seeds, soil, or dust associated with the seed drilling process, as well as via spray drift into water bodies (Krupke et al. 2012).

While many of the neonicotinoid transport routes are limited by integrated pest management techniques and following established application procedures such as treating seeds directly rather than spraying the active ingredients (Koch et al. 2005), depending on the application method and crop type approximately 1.6 to 28% of the total neonicotinoid insecticide applied to a crop will be taken up by the crop (Sur and Stork 2003). Any of the applied neonicotinoid that is not incorporated into plant tissue is susceptible to dispersal into other environmental compartments. Once neonicotinoids enter water, the persistence and fate of these compounds depends on a variety of factors including pH, light availability, temperature, the commercial formulation, and microbial action (Anderson et al. 2015). Upon entry into surface water, neonicotinoids tend to reach peak concentrations in the receiving environment within 24 h of application

or a rain or irrigation event, after which the concentrations tend to decrease according to first order kinetics, with a rapid initial breakdown followed by a slower second phase (Armbrust and Peeler 2002). Half-lives for thiamethoxam, clothianidin, and imidacloprid in surface waters are all on the order of hours to days, with estimates of 0.2 to 3.9 d for imidacloprid (US EPA 2008), 14.4 h for clothianidin (US EPA 2011a), and 2.3 to 3.1 d for thiamethoxam (US EPA 2011b) in the presence of sunlight and aerobic conditions due to susceptibility to photolysis, dilution, and the potential for microbial degradation (Morrissey et al. 2015). However, imidacloprid has been detected at concentrations of 0.1-0.2 µg/L up to a year after application (Kanrar et al. 2006; La et al. 2014)

Since neonicotinoids are the most recently introduced of the main insecticide classes, there is limited available environmental sampling data pertaining to surface water concentrations. In addition, there is no central database of pesticide use data in Canada and widespread sampling data in Canada is scarce (Environment Canada 2011). Most of the available sampling data focuses on imidacloprid due to it being the first neonicotinoid to be introduced to the Canadian market and, until recently, the most commonly used. A recent review compiled the findings from 29 studies performed in 9 countries from 2004 to 2014 and found that neonicotinoids were detected in the majority of surface water samples, including irrigation channels, streams, rivers, and wetlands in close proximity to or downstream from agricultural cropland (Morrissey et al. 2015). Detectable concentrations of imidacloprid, clothianidin, and thiamethoxam spanned a broad range, with imidacloprid ranging from 0.001 to 320 µg/L, thiamethoxam ranging from 0.001 to 225 µg/L, and clothianidin ranging from 0.003 to 3.1 µg/L. Maximum concentrations were observed for imidacloprid in Dutch agricultural surface waters and for thiamethoxam in wetlands in the Texas plains (Van Dijk et al. 2013; Anderson et al. 2013).

While neonicotinoid monitoring data in BC is very limited, the BC Ministry of the Environment maintains detailed records of pesticide sales in the province that can be used as a proxy for use within the province. In 2010, a total of 2.96 million kilograms of commercially formulated pesticide products and a corresponding 1.29 million kilograms of active ingredients were sold in BC (BC MoE 2010). Insecticides accounted for 33% of the total active ingredient sales, or 426 thousand kilograms (BC MoE 2010). Total pesticide sales are lower in BC when compared to other provinces such as Alberta, with 12.5 million kilograms of active ingredient sold in 2008, and Quebec, with 4 million

kilograms of active ingredient sold in 2009 (BC MoE 2010). However, in BC 45% of all pesticides were sold in the Lower Mainland region and 89% of all pesticides were sold in southern BC, which encompasses the majority of the Fraser River watershed (BC MoE 2010). Of the total insecticide active ingredients sold in BC in 2010, 1297 kg was imidacloprid, all of which was sold in southern BC (BC MoE 2010). The total sales of 1297 kg of imidacloprid represents a 205% increase in sales compared to the 425 kg sold in BC in 2003 (BC MoE 2010). In addition, 113 kg of thiamethoxam and 86 kg of clothianidin were sold in BC in 2010, whereas neither of these ingredients were sold in BC in 2003 (BC MoE 2010). In 2015, both clothianidin and thiamethoxam were among the top 10 insecticide active ingredients sold in Canada, with over 100 000 kg of each active ingredient sold (Health Canada 2016). Over 50 000 kg of imidacloprid was sold in 2015 as well (Health Canada 2016). Clothianidin has consistently ranked in the top 10 insecticide active ingredients sold in Canada since the Pest Control Product Sales Information Reporting Regulations came into force in 2006 (Health Canada 2016). As the number of registrations of commercial formulations of neonicotinoids and their approved target crops increases, these numbers are expected to continue to increase.

An Environment Canada report in 2001 identified pesticides as one of 15 key threats to Canadian waters and emphasized the need for a national pesticide monitoring program (Environment Canada 2001). In addition, the PMRA identified water quality monitoring data as a top priority for support of their responsibility as a decision making regulatory body (Environment Canada 2011). In response, from 2003-2005 Environment Canada conducted the first nation-wide surveillance program focusing on sampling current use pesticides in vulnerable aquatic ecosystems and source waters (Environment Canada 2011). The sampling program focused primarily on surface water but samples of groundwater, precipitation, and runoff were also analyzed. The program focused on 5 major regions: BC, the prairies (Alberta, Saskatchewan, and Manitoba), Ontario, Quebec, and the Atlantic region (PEI, Nova Scotia, and New Brunswick). Due to the recent introduction of neonicotinoids to the market, imidacloprid was the only neonicotinoid that was monitored for in the program, and it was only monitored within the Atlantic and Ontario regions. Imidacloprid was only detected in 2 out of 57 samples in New Brunswick and was not detected in any of the other regions monitored (Environment Canada 2011).

More recent monitoring of neonicotinoids in groundwater wells in potato producing regions of Quebec from 2008 to 2009 resulted in the detection of imidacloprid in 61% of wells sampled, at concentrations up to 6.1 µg/L (Government of Quebec 2014). Thiamethoxam was detected in 8% of well samples at concentrations up to 0.83 µg/L, and clothianidin was detected in 4% of well samples at concentrations up to 0.059 µg/L (Government of Quebec 2014). Previous sampling in the same regions from 1999 to 2001 detected lower concentrations of imidacloprid less frequently and did not monitor clothianidin or thiamethoxam (Government of Quebec 2014).

In the fall of 2011, an Environment Canada monitoring program for neonicotinoids in freshwater Ontario streams detected clothianidin, imidacloprid, and thiamethoxam at sites adjacent to row crops, vineyards, and orchards. These findings indicate an increased aquatic presence of neonicotinoids corresponding with increased use when compared with the 2003-2005 monitoring program. All detections were below 1 µg/L, with maximum concentrations of 26.9 ng/L of imidacloprid, 34.8 ng/L of clothianidin, and 174 ng/L of thiamethoxam observed (Mineau and Palmer 2013).

A recent study by Main et al. (2014) in the prairie pothole region of Saskatchewan spanning from spring 2012 to spring 2013 sampled wetlands adjacent to grasslands and compared them to wetlands adjacent to agricultural fields. Water samples taken from wetlands located in close proximity to agricultural fields consistently contained greater neonicotinoid concentrations than those taken from wetlands adjacent to grasslands. Neonicotinoids were detected in 91% of samples in the spring 2013 monitoring, with clothianidin being detected the most frequently. Maximum concentrations measured in the water samples were 3.11 µg/L of clothianidin, 0.256 µg/L of imidacloprid, and 1.49 µg/L of thiamethoxam (Main et al. 2014).

In 2010 approximately 500 kg of imidacloprid was applied to an area in the Sacramento and Orange County region and 51% of samples taken from the region in 2010 and 2011 contained measurable concentrations of imidacloprid, although the highest measured concentration was 0.7 µg/L (Ensminger et al. 2013). Considering that in 2010 almost 1300 kg of imidacloprid was sold in southern BC, and that sales are expected to continue to increase, it is likely that there are detectable concentrations of imidacloprid throughout the Fraser River watershed (BC Ministry of the Environment 2010).

Another important consideration is that neonicotinoids are one of several classes of pesticides that affect activity at acetylcholine receptors, and it is common for multiple pesticides with a similar mode of action to be detected within the same environmental sample. Surface water samples commonly contain measurable quantities of organophosphates and carbamates, which inhibit acetylcholinesterase function, in addition to various neonicotinoids, which act as acetylcholine receptor agonists (Laetz et al. 2009). There is limited research available on the impacts of complex mixtures of pesticides on organisms, and typically risk assessments and regulatory guidelines are derived on an individual chemical basis.

1.4. Potential Impacts of Neonicotinoid Insecticides on Teleost Fish

Anthropogenic contaminants can have a wide variety of impacts in aquatic ecosystems, ranging from mass mortality of fish and other organisms, to more subtle sublethal effects. Sublethal effects are a broad category of adverse effects to physiological function at a sub cellular to population level, and can include alterations in an organism's ability to develop, reproduce, grow, find and consume prey, avoid predators, and cope with a variety of other environmental stressors (Environment Canada 2011). The effects of a given contaminant will vary in severity between different species as well as between different life stages within a given species (Hutchinson et al. 1998). Typically, the early life stages of fish development, including embryonic and larval stages, tend to be the most sensitive to contaminant exposure due to the extent of tissue differentiation during these stages (Hutchinson et al. 1998). A review of 56 life cycle toxicity tests with four species of fish and 34 different chemicals including metals, polychlorinated biphenyls (PCBs), and pesticides found that the embryo-larval and early juvenile life stages were frequently the most sensitive, or among the most sensitive life stages, with little difference between species of fish or type of contaminant (McKim 1977). A comparison of no observed effects concentrations (NOECs) and lowest observed effects concentrations (LOECs) between different life stages of the same species exposed to various contaminants indicated that fish embryos were the most sensitive life stage for the majority of contaminants with available information, followed by fish larvae, and then juvenile fish, followed by adult fish (Hutchinson et al. 1998).

There exists a large literature base describing studies on the effects of pesticides on salmonids at a variety of life stages and at levels of organization from the molecular to the population level. Exposure to different current use pesticides has been shown to have varying degrees of lethality in salmonids, with compounds like the widely used herbicide hexazinone having reported 96-h LC_{50} values ranging from 246 mg/L in juvenile coho salmon (*Oncorhynchus kisutch*) to 317 mg/L in juvenile chinook salmon (*Oncorhynchus tshawytscha*) and sockeye salmon (*Oncorhynchus nerka*; Wan et al.1988). This range in 96-h LC_{50} values indicates that there are differences in sensitivities to the same compound among different salmonid species. Salmonids are typically sensitive to pyrethroid pesticides, and 96-h LC_{50} values for exposure to permethrin as low as 0.62 µg/L for juvenile rainbow trout (*Oncorhynchus mykiss*) and 12 µg/L in Atlantic salmon (*Salmo salar*) have been reported (Kumaraguru and Beamish 1981; Mcleese et al.1980). Pesticides with a similar mode of action to the neonicotinoids, such as organophosphates and carbamates tend to exhibit moderate LC_{50}s in comparison to more potent compounds such as pyrethroids. 96-h LC_{50} values for juvenile rainbow trout exposed to carbaryl have been reported at 198 µg/L (Boran et al. 2007) and a similar exposure to malathion yielded a 96-h LC_{50} of 122 µg/L (Post and Schroeder 1971).

In addition to lethality, exposure to pesticides has been associated with a variety of sublethal effects. In Atlantic Canada, aerial spraying of carbamate pesticides has been correlated with a drop in returns of spawning Atlantic salmon adults (Fairchild et al.1999). Exposure to pesticides has also been demonstrated to impair olfactory capabilities in Coho salmon (Tierney et al. 2006; Tierney et al. 2008), and has resulted in an associated disruption of navigation and feeding behaviours (Scholz et al. 2000). Salmonid exposure to pesticides have also been associated with an induced stress response (Tierney et al. 2011b), and immune system suppression (Shelley et al. 2009). All of these findings indicate potential for ecologically relevant adverse effects in salmonids exposed to pesticides.

A large literature base exists regarding pesticide-induced toxicity in salmonid species performed on fish at the feeding fry, smolt, and juvenile stages, with limited available information on the impact of pesticide exposure to earlier life stages of salmonids. Few studies have focussed on the effects of current use pesticides on embryo and alevin stages, and even fewer on sockeye salmon specifically. However,

early life stage toxicity tests with rainbow trout are commonly included in applications for registration of new pesticides in the United States and Canada, but the extent of relevance to wild fishes and often environmentally relevant chronic exposure scenarios are poorly understood.

Established endpoints for chronic exposures of environmentally relevant contaminants to early life stages of salmonids include hatch success, survival, growth, incidence of developmental deformities, and timing of emergence. One study examined the effects of three different pesticides (dinoseb, diazinon, and esfenvalerate) at concentrations ranging from 1 µg/L to 100 mg/L on eyed eggs and alevins of Chinook salmon and demonstrated that a 96-h static renewal exposure was correlated with a decrease in salmon survival as well as metabolic changes in eggs and alevins at concentrations as low as 10 µg/L (Viant et al. 2006). A recent study involving the exposure of fertilized sockeye salmon eggs to chlorothalonil or atrazine throughout the developmental period until the swim up fry stage found that both pesticides resulted in reduced alevin condition factors and altered hatch and emergence times (Du Gas 2014). Another study that involved the exposure of fertilized coho salmon eggs to pulses of a pesticide mixture meant to mimic concentrations and pesticides reported in salmon bearing streams in Washington throughout development found no impact on hatching success, survival, deformities, or growth (King et al. 2013). The study included 8 herbicides including dicamba and 2,4-D, 2 insecticides (diazinon and carbaryl), and the fungicide pentachlorophenol, all at concentrations of approximately 0.5 mg/L. These studies indicate that there is variability in the susceptibility of early life stages of sockeye salmon to adverse developmental effects as a result of pesticide exposure.

The available published literature relating to neonicotinoid toxicity to fish are primarily laboratory studies on the direct effects to later life stages, such as adult LC_{50} exposures. Direct effects of contaminants are those that occur via direct contact of the organism with the active ingredient and can include ingestion of the active ingredient or formulated product, uptake via the skin or gills, or consumption of contaminated prey (Gibbons et al. 2015). In a natural environmental setting, it is impossible to ignore the potential for indirect effects, which can take the form of a loss in quantity or quality of habitat and/or prey as a result of pesticide use (Southerton and Holland 2002). However, these indirect effects are difficult to address effectively in laboratory-based studies. A field based microcosm experiment involving pulses of imidacloprid at concentrations

ranging from 0.6 to 40 µg/L to a lentic benthos assemblage in weekly intervals resulted in altered community structures (Colombo et al. 2013). Similarly, a mesocosm study involving exposure to imidacloprid found that at concentrations of 20 µg/L, there were observed decreases in overall phytoplankton density, as well as densities of mayflies, caddisflies, and the amphipod species *Hyalella azteca* (Moring et al.1992). A semi natural mesocosm experiment using clothianidin concentrations of 352 µg/L resulted in high invertebrate predator mortality and a resulting increase in prey survival, further indicating the potential for neonicotinoid exposure to alter aquatic invertebrate community structures (Miles et al. 2017). Rice paddy mesocosms exposed to 40 to 50 µg/L of imidacloprid effected zooplankton, benthic, and neuston communities, and resulted in reduced growth in medaka, which was hypothesized to be due to reduced food availability or feeding behaviour (Hayasaka et al. 2012). Declines in insectivorous bird populations have also been associated with regions containing high neonicotinoid concentrations, presumably due to reductions in food availability (Hallmann et al. 2014). Considering that during early life stages, Pacific salmon rely on invertebrate communities as a food source, altered community structures as a result of pesticide contamination may adversely impact the survival of juvenile sockeye salmon and therefore productivity of contaminated salmon bearing streams.

Neonicotinoid insecticides function by binding to nicotinic acetylcholine receptors (nAChRs), which are a family of ligand-gated ion channels responsible for post-synaptic neurotransmission in the central nervous system and at the neuromuscular junction (Jeschke et al. 2011). Neonicotinoids are classified as agonists of the nicotinic acetylcholine receptor and binding of neonicotinoids to the nAChR results in centrally-mediated toxicity with a variety of effects on the central nervous system and muscular function (Rodrigues et al. 2010). It has been demonstrated that neonicotinoids exhibit a high degree of selectivity for insect nAChRs as a result of electronegative interactions of the neonicotinoids with specific sub-sites that are unique to insect nAChRs and absent in the equivalent vertebrate receptors (Sanchez-Bayo 2012; Tomizawa and Casida 2005).

An experimental comparison of the binding affinity for neonicotinoids to insect nAChRs and the vertebrate α7 nAChR subtype demonstrated that clothianidin and imidacloprid have a much higher affinity for both the insect nAChR and the vertebrate α7 nAChR subtype than thiamethoxam, and that all of them exhibit a high degree of selectivity (i.e. more effectively bind to invertebrate receptors compared to vertebrate

receptors; Tomizawa and Casida 2005). These results highlight the importance of clothianidin as a metabolic product of thiamethoxam (Nauen et al. 2003). Despite a preference for insect nAChRs, it has been demonstrated that neonicotinoids can bind to vertebrate nAChRs. Zebrafish exposed to imidacloprid concentrations of 26 mg/L from the zygote to free swimming larvae (6 d past fertilization) exhibited a 4 fold reduction in acetylcholine levels after 5 d of exposure, potentially due to increased breakdown of acetylcholine in the synaptic cleft by acetylcholinesterase due to the agonistic action of imidacloprid competing with acetylcholine (Tufi et al. 2016). Due to the importance of acetylcholine in both central nervous system and muscle function, a proper understanding of the potential for neonicotinoid toxicity in non-target organisms such as fish is critical to ensuring adequate protection of aquatic ecosystems.

Acute toxicity studies in several fishes that have been tested in traditional laboratory-based LC_{50} studies have been shown to be relatively insensitive to imidacloprid and clothianidin, with limited available research on thiamethoxam (Gibbons et al. 2015). LC_{50} values for both imidacloprid and clothianidin for a variety of fish including bluegill sunfish (*Lepomis macrochirus*), Japanese carp (*Cyprinus rubrofuscus*), Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*), sheepshead minnows (*Cyprinidon variegatus*), orfe (*Leuciscus idus*) and zebrafish (*Danio rerio*) fall in the range of 100-300 mg/L (Gibbons et al. 2015; Smit et al. 2015). Two 96-h flow through thiamethoxam exposure tests of rainbow trout also included the endpoints swimming behaviour, loss of equilibrium, respiratory function, exophthalmos, and pigmentation in addition to lethality. In each of these studies, the no observed effects concentration (NOEC) was equivalent to the highest concentration tested at 100 mg/L and 125 mg/L for the two studies (Rufli 1996; Ruffli 1997a). A study performed using rainbow trout fry exposed to imidacloprid yielded a 96-h LC_{50} of 1.2 mg/L, indicating that they are much more sensitive than later stage fish of a variety of species (US EPA 1992). However, 1.2 mg/L is 4 times higher than the highest observed concentration of imidacloprid in any measured surface water of 320 µg/L (Van Dijk et al. 2013). In addition, a 96-h exposure of zebrafish embryos to pure imidacloprid indicated that no toxicity to embryogenesis and development was observed at concentrations up to 320 mg/L. However, the commercial formulation Confidor SL200® was found to be more toxic, with a 96-h LC_{50} of 214 mg/L (Tisler et al. 2009). The comparable LC_{50} values for zebrafish at adult and early life stages indicate that early life stages may not always be

more sensitive to acute imidacloprid toxicity. While direct acutely toxic effects may not be a concern for the environmental exposure of fish to neonicotinoids, there is some evidence that neonicotinoid exposure may result in a host of sublethal effects in fish that can lead to impaired performance or survival and potentially impact fish at the population level.

Sublethal effects, such as histopathic changes, induction of liver enzymes indicative of an oxidative stress response, and DNA damage have been observed in fish exposed to neonicotinoids. Adult Nile tilapia (*Oreochromis niloticus*) exposed acutely for 24 h to concentrations of imidacloprid >1.34 mg/L resulted in gonadal changes including extensive degradation of testicular tissue (Ocampo and Sagun 2007; Lauan and Ocampo 2013). Exposure of adult loach (*Misgurnus anguillicaudatus*) to concentrations of imidacloprid ranging from 43 to 115 mg/L for 6 d resulted in an increase in micronuclei and nuclear anomalies observed as well as a decrease in hepatic glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) activity (Xia et al. 2016). In the same experiment, histological examination of the loach testis also indicated that imidacloprid exposure resulted in disorganized lobules and the presence of cysts (Xia et al. 2016). Similarly, a study was conducted to assess the impacts of exposure to 0.3, 1.25, and 5 g/L concentrations of imidacloprid on zebrafish following 7, 14, 21, and 28 d of exposure. The 1.25 and 5 g/L treatments resulted in increased reactive oxygen species (ROS) production and increased malondialdehyde (MDA), an indicator of oxidative stress via lipid peroxidation, as well as increased DNA damage (Ge et al. 2015). A 24-h exposure of adult zebrafish to 27.5 mg/L of imidacloprid also resulted in elevated MDA production (Shukla et al. 2017). Another study involving zebrafish exposed to 0.3, 1.25, and 5 g/L concentrations of thiamethoxam over 28 d resulted in an increase in ROS production, MDA production, and DNA damage (Yan et al. 2016). These results are important given that lipid peroxidation has been identified as an important mechanism in pesticide induced toxicity (Nwani et al. 2010). However, all results were observed at concentrations much higher than have been measured in freshwater environments, and studies examining these sublethal effects at lower environmentally relevant concentrations are lacking.

There have been few studies, both published and unpublished that have examined the subchronic to chronic effects of exposure to neonicotinoids on the embryonic and alevin stages of freshwater fish. The most extensive literature base exists

for studies involving imidacloprid. A 60-d flow through exposure of rainbow trout (*Oncorhynchus mykiss*) to imidacloprid following fertilization resulted in a NOEC of 1.2 mg/L and a LOEC of 2.5 mg/L based on reductions to growth (Cohle and Bucksath 1991). A 7-d exposure of zebrafish to imidacloprid starting at fertilization and terminating at 168 h post fertilization found no effects on mortality or incidence of developmental deformities at concentrations up to 50 mg/L (Scheil and Kohler 2009). In another study by Crosby et al. (2015), zebrafish (*Danio rerio*) were exposed to 12 or 15 mg/L of imidacloprid from 4 h to 5 d post-fertilization. Following exposure, the zebrafish were either assessed for larval activity or reared to the adolescent and adult stages and then subjected to multiple neurobehavioural assays, including assessment of sensorimotor responses and habituation in a tactile startle test, novel tank swimming, and shoaling behaviour (Crosby et al. 2015). The results indicated that imidacloprid exposure during this developmental window resulted in decreased larval swimming activity, as well as decreased novel tank exploration and an increased sensorimotor response to startling stimuli (Crosby et al. 2015). The few studies to date suggest that zebrafish may be more sensitive to behavioural effects than to lethality or developmental deformities following early life stage exposure to imidacloprid, and that early life stages of salmonids such as the rainbow trout may be more sensitive to imidacloprid than zebrafish.

Only one early life stage toxicity test involving clothianidin exposure to fish was identified. A 28-d flow through exposure involving fathead minnows following fertilization resulted in a NOEC of 9.7 mg/L and a LOEC of 20 mg/L based on reduced growth (measured as length and dry weight; MRID 454224-13, as cited in US EPA 2011). A recent review of the available aquatic toxicity studies involving exposure to thiamethoxam identified two chronic studies using early life stages of rainbow trout (Finnegan et al. 2017). A 28-d flow through exposure initiated following fertilization that examined mortality, growth rate, food conversion efficiency, feeding activity, swimming behaviour, respiratory movement, pigmentation, exophthalmos, loss of equilibrium, and reaction to external stimuli found that the NOEC was equivalent to the highest concentration of thiamethoxam tested, which was 100 mg/L (Rufli 1997b). An 88-d flow through exposure initiated following fertilization investigated the endpoints hatching success, time to hatch, time to swim up, larvae and fry survival, and growth (Drottar et al. 1997). Similarly, no effects were observed at the highest thiamethoxam concentration tested of 20 mg/L (Drottar et al. 1997). The available information involving chronic

exposure of freshwater fish to neonicotinoids at early life stages indicates that the most sensitive whole body endpoint tends to be reductions to growth and that early life stages of fish seem to be more sensitive to exposure to imidacloprid and clothianidin than thiamethoxam.

While most of the available studies used concentrations of neonicotinoids much higher than might be expected in the environment, an experiment in which medaka (*Oryzias latipes*) were exposed to concentrations of imidacloprid as low as 0.03 mg/L resulted in the induction of a stress response and increased susceptibility to ectoparasite infection (Sanchez-Bayo and Goka 2005). Although a few unpublished studies have been performed, there are currently no published studies that have investigated the impact of neonicotinoid exposure on sublethal endpoints in salmonids. Given that rainbow trout (*Oncorhynchus mykiss*) fry have the most sensitive LC_{50} value of any fish exposed to neonicotinoids in a laboratory setting of 1.2 mg/L (US EPA 1992), and that zebrafish were approximately 20 times more sensitive to developmental sublethal effects than they were to lethality when exposed to imidacloprid, with swimming behaviour effects observed at 12-15 mg/L (Crosby et al. 2015) but LC_{50} values upwards of 200 mg/L (Tisler et al. 2009), a better understanding of the potential for sublethal effects in early life stage salmonids exposed to neonicotinoid insecticides is necessary.

1.5. Early Life Stages of Sockeye Salmon

A sockeye salmon's life cycle typically spans 4 years, and includes early developmental stages in nursery lakes and other freshwater bodies, a migration to the ocean, and finally a return of the adult to its natal stream, where it will spawn and die. Upon returning to spawning streams, female adult sockeye locate a suitable site based on stream flow, groundwater upwelling, and gravel size, where they create depressions in the gravel called redds, and deposit anywhere from 500 to 1000 eggs (Crisp and Carling 1989). Males simultaneously fertilize the eggs as they are being deposited into multiple redds, after which the female will cover the eggs with gravel from the streambed surrounding the red (Crisp and Carling 1989). Embryos will develop within the fertilized eggs in the redds for several months, protected from being washed downstream, damage from ice formation, and predatory threats (Crisp and Carling 1989). Embryos hatch from the eggs as alevins in the early spring but remain in the shelter of the gravel covered redds while they absorb the remaining nutrients from the yolk sac suspended

from their belly upon hatching (Groot and Margolis 1991). Approximately 8 months after fertilization, sockeye salmon will emerge from the gravel in the spring or early summer as fry and migrate up or downstream in pursuit of external food sources in a nursery lake (Groot and Margolis 1991). Fry will remain in nursery lakes feeding and developing for 1 to 2 years before undergoing a physiological alteration called smoltification. The smoltification process allows the juvenile salmon to migrate from the freshwater conditions of its nursery lake, through an estuary system and into the salt water of the Pacific Ocean (Groot and Margolis 1991). Sockeye salmon in the Fraser River watershed tend to migrate north through the Strait of Georgia to Queen Charlotte sound and beyond to the waters in the coastal region of Alaska, where they continue to grow and transition from feeding on plankton to fish and squid (Lebrasseur 1966). A typical sockeye will finish its maturation in its 4th year and undergo a final transition in preparation for return to its natal freshwater streams to spawn and continue the cycle.

While the life cycle of a sockeye salmon contains multiple stages with complex physiological transformations throughout, the most vulnerable stages of this process appear to be the embryo incubation, alevin, and fry stages (Bradford 1995). It is estimated that while a typical spawning sockeye female will lay approximately 3000 eggs, only 10-20% of these eggs will be successfully fertilized and develop to the fry stage (Bradford 1995). Multiple factors contribute to the low survival rate of early life stage salmon, including predation, disturbance of redds, desiccation or freezing as a result of low water levels, or suffocation or pathogen infection due to high density (Bradford 1995). Due to the immobility of the embryonic and larval alevin stages the salmon are vulnerable to the conditions of their surrounding environment and are unable to avoid environmental stressors or aquatic contaminants. Effects resulting from exposure to contaminants during these critical developmental stages can have lifelong consequences for the fitness and survival of sockeye salmon.

1.6. Swim Performance as a Behavioural Endpoint

Measurements of swimming ability in a laboratory setting provide a valuable means to assess an integrated physiological response in a controlled setting. Swimming assays are a useful tool for examining the impact of various contaminants, particularly those that act on the cardiovascular and neuromuscular systems (Scott and Sloman 2004). Neonicotinoids affect acetylcholine signalling and due to the importance of

acetylcholine in muscle coordination, (linking together the nervous and muscular systems at the neuromuscular junction) assays of an integrated swim response appear to be a useful indicator of toxicity. In addition to proper nervous and muscular function, well-coordinated swimming also requires the maintenance of ionic and energetic balance. Due to its integrative nature, swim performance can be considered to be an indicator of the general health and fitness of an organism, providing an ecologically relevant parameter for studying the effects of a toxicant on a fish species (Scott and Sloman 2004; Little and Finger 1990). Swim performance is a particularly valuable endpoint for active fish species such as salmonids exposed to chemicals that can impact the neuromuscular junction.

Swim performance is typically measured in three main categories of assay. These categories are sustained, prolonged, and burst swimming (Beamish 1978; Plaut 2001). Sustained swimming performance involves testing at low velocities for long periods of time and involves aerobic metabolism via mitochondria rich red muscle (Beamish 1978). Assays that measure sustained swimming performance typically do not result in muscle fatigue (Beamish 1978). Burst swimming involves mitochondria poor white muscle and anaerobic metabolism that occurs over shorter time scales and higher velocities, and typically ends in muscle fatigue (Beamish 1978). Assays for prolonged swimming performance fall somewhere in between burst and sustained swimming in terms of water velocity and test duration, and due to the involvement of both aerobic and anaerobic metabolism, the end result is typically muscle fatigue (Beamish 1978).

Optimal swim performance is important in salmonids for successful migration between the ocean and natal streams, maintenance of position in a current, predator avoidance, and capturing of prey. In juvenile salmonids in the slow moving waters of their nursery lakes the most important of these functions is predator avoidance. The best assessment of the ability to elude predators is burst swim assays, and decreased burst swim performance has been linked to increased predation in Atlantic salmon (Handeland et al. 1996). A common way to measure burst swimming capabilities is using constant acceleration tests that involve an acclimation period at a low water velocity followed by incremental increases in water velocity until the point of fatigue (Farrell 2008; Osachoff et al. 2014).

As previously mentioned, exposure to neonicotinoids in larval zebrafish resulted in impaired sensorimotor responses and swimming activity (Crosby et al. 2015). Assessment of the impacts of exposure to environmentally relevant concentrations of neonicotinoid insecticides on burst swimming performance in juvenile sockeye salmon can provide useful information regarding a coordinated whole body response that is relatable to survivability in the wild.

1.7. Oxygen Consumption as a Physiological Endpoint

Fish respirometry can provide information related to altered environmental conditions or physiological states. Impaired respiratory function has long been established as one of the earliest symptoms in acute pesticide poisoning (Holden 1973). Since aquatic animals have to move large quantities of water over their respiratory surfaces in order to extract oxygen, they are subject to a significant risk of exposure to toxic substances contained in that water (Shelke and Wani 2005). Fish exposed to pollutants have been demonstrated to develop a stress syndrome characterized by increasing anaerobic metabolism, which can result in elevated blood lactate concentrations, followed by hyperglycemia (Sancho et al.1997). This stress response can lead to protein catabolism to compensate for cellular energy losses associated with the increased anaerobic metabolism (Pfeifer and Weber 1979). A potential explanation for the stress response is that exposure to pollutants results in gill damage, which reduces the amount of oxygen taken up at the gills by fish (Evans 1987). Exposure to thiamethoxam has resulted in histopathological alterations in the gills of adult common carp as well as reduced oxygen consumption over time in a species of freshwater bivalve (Georgieva et al. 2014; Minakshi and Mahajan 2013).

The innervation and chemical modulation of gill circulation and respiration is a complex mechanism in which acetylcholine plays an integral role (Jonz and Zaccone 2009). Acetylcholine administration has experimentally been linked with constriction of the efferent vasculature, including arteries and arterioles in the gill filaments (Mauceri et al. 2005). A decrease in blood flow to the gills reduces the ability of a fish to extract oxygen from the water and decreases the oxygen available in the body for cellular respiration. Due to the agonistic action of neonicotinoids on the nicotinic acetylcholine receptor, there is potential for disruption of regular gill function as a result of exposure to neonicotinoids. Agonistic action of neonicotinoids could lead to constriction of the

efferent vasculature and a decrease in blood flow to the gills and therefore decreased oxygen uptake, as observed with acetylcholine administration. It is also possible that altered respiration could occur as a result of a combination of agonistic action on acetylcholine receptors and histopathological changes at the gill or a general physiological stress response as outlined above.

Aerobic metabolism in fishes can be categorized as standard, resting routine, routine, swimming, and active, although there is some overlap in the categorization (Cech and Brauner 2011). Standard metabolism is the minimal metabolic rate for healthy fish (i.e. the low point of metabolism in a period of inactivity (Cech and Brauner 2011). Resting routine metabolism typically represents a state of inactivity but not necessarily the lowest point in a cycle (Cech and Brauner 2011). Routine metabolism is defined as the metabolic rate occurring while a fish is quiescent to moderately active and includes spontaneous activity (Cech and Brauner 2011). Swimming metabolic rates are measured at a voluntary or forced level of swimming (Cech and Brauner 2011). Finally, active metabolism is measured as the maximum aerobic rate associated at the greatest sustainable velocity (Cech and Brauner 2011).

The most common way to measure oxygen consumption and associated metabolic rates is via an assay of routine metabolism, which is particularly suitable for naturally restless fish such as salmonids. Examining alterations in oxygen consumption in sockeye salmon following exposure to neonicotinoids may provide insight into potential impacts of neonicotinoids on gill function or whole organism metabolic demands. It is possible that an altered routine metabolism may have implications for survival in the face of additional environmental stressors such as hypoxic water. Taken together with information on swim performance, information on oxygen consumption rates can provide a better understanding of whole body sublethal effects of exposure to neonicotinoids in sockeye salmon.

1.8. The Importance of Early Life Stage Development in Toxicity Testing

Life cycle toxicity tests with fish, including all developmental stages, were first introduced by Mount and Stephan (1967) in a study involving fathead minnows (*Pimephales promelas*). During this pioneering study, the minnows were exposed to a

series of toxicant concentrations throughout their life cycles, and quantitative measures of survival, growth, and reproduction were taken. The quantitative data collected on these endpoints allowed for the calculation of a maximum acceptable toxicant concentration (MATC) that was defined as the threshold concentration between the highest concentration tested that would have no adverse effects, and the lowest concentration tested that would result in significant adverse effects (Mount and Stephan 1967). Following the introduction of life cycle toxicity testing in fish, methodologies were developed for a variety of fish species including salmonids (McKim and Benoit 1971). A 1977 review of 56 life cycle toxicity tests involving 4 species of fish exposed to 34 different organic and inorganic compounds showed that the embryo-larval and early juvenile (i.e. fry) stages were commonly the most sensitive or among the most sensitive life stages (McKim 1977).

Early life stage toxicity testing in fish is now widely used as a way of evaluating current use and prospective use compounds, and methodologies have been standardized by a variety of different organizations (OECD 1992; Environment Canada 1998; ASTM 2013). All of these methodologies have been adapted for use with salmonids. Endpoints common to each of the aforementioned protocols include observations of direct mortality to embryos, larvae and juveniles, hatch success, weight and length as a measure of growth, and abnormal physical and behavioural development (OECD 1992; Environment Canada 1998; ASTM 2013).

Reductions in growth or altered development may lead to increases in susceptibility to predation and disease, reduced ability to obtain food and compete for suitable habitats, and delays in maturation and onset of reproduction (Rosenthal and Alderice 1976). Juvenile growth has been identified as a critical predictor of freshwater and marine survival in salmon (Higgs et al. 1995). One commonly identified mechanism of developmental disruption in juvenile and adult salmonids involves the acetylcholine axis in the nervous system (Wheelock et al. 2005). Acetylcholinesterase inhibition as a result of 96-h exposures to organophosphate and carbamate pesticides has been modelled to predict reductions in growth and survival in juvenile salmonids based on the underlying assumption that acetylcholinesterase inhibition is associated with altered brain activity and feeding behaviour (Baldwin et al. 2009). Exposure to the carbamate pesticide carbaryl and the organophosphate pesticide chlorpyrifos in several species of freshwater fish has also resulted in reduced growth (Carlson 1971; Jarvinen and Tanner

1982), depleted glycogen in the liver and muscles (Sastry et al. 1988), and reduced swimming rate during feeding and total food strikes (Sandahl et al. 2005). Reduced growth and development may result from a generalized stress response to a pollutant that results in decreased energy reserves and protein catabolism, as outlined in the previous section. Altered growth and development may also result from altered acetylcholine expression as a result of agonistic action, as acetylcholine is an important neurotransmitter expressed during early fish development (Tufi et al. 2016). Measurements of growth and development such as weight/length ratios have been used as an indicator of health that may identify the presence of an environmental stressor such as water pollution (Van Gestel and Van Brummelen 1996).

A review of the available literature on pesticide induced teratogenicity and embryotoxicity in aquatic organisms indicated that many pesticides, including those that have a similar mechanism of action to neonicotinoids like carbamates and organophosphates, have been documented to induce embryotoxicity and teratogenicity in non-target aquatic organisms, including fish (Paskova et al. 2011). Exposure to the carbamate carbaryl has resulted in red blood cell accumulation, delayed hatching, pericardial edema, and bradycardia in zebrafish (Lin et al. 2007). Exposure to the organophosphate malathion has resulted in skeletal deformities and edema in the African sharptooth catfish (*Clarius gariepinus*; Lien et al. 1997). There is evidence to suggest that pesticide induced teratogenicity and embryotoxicity may be linked to oxidative stress endpoints such as lipid peroxidation, oxidative DNA damage, or modulation of antioxidant mechanisms (Paskova et al. 2011). Signs of oxidative stress and DNA damage have been observed in freshwater fish such as loach (*Misgurnus anguillicaudatus*) and zebrafish (*Danio rerio*) exposed to the neonicotinoids imidacloprid and thiamethoxam (Xia et al. 2016; Ge et al. 2015; Yan et al. 2016). These effects have typically been observed in exposure scenarios involving concentrations much higher than those measured in global surface waters for short periods of time. There is a lack of information regarding deformity analyses for fish chronically exposed to environmentally realistic concentrations of neonicotinoids.

1.9. Overview of Research

Understanding the potential impacts of current use pesticides in aquatic environments is important in order to adequately protect these ecosystems. Pacific salmon are an important species on the west coast of North America due to their cultural, economic, and ecological significance. The recent declines in Pacific salmon and sockeye salmon returns, in particular, highlights the importance of examining the potential sublethal effects of neonicotinoid insecticide contributions among a suite of environmental and anthropogenic stressors.

Based on the current information available, establishing a cause and effect relationship between the decline in sockeye salmon returns and exposure to any contaminant or group of contaminants is not possible. However, it is also not possible to rule out contaminants as a contributor to decreasing sockeye populations and it is important to characterize the potential adverse effects that contaminants such as current use pesticides may have on Sockeye salmon (Cohen Commission 2012; Johannessen and Ross 2002). Although neonicotinoids are just one class of contaminants that Sockeye salmon are exposed to in the Fraser River watershed, their increase in use in recent years and corresponding increase in prevalence in Canadian surface waters highlights the importance of understanding how neonicotinoids may contribute to the health of sockeye salmon (Macdonald et al. 2011).

A recent report assessed the current state of knowledge on neonicotinoid ecotoxicology and identified several knowledge gaps (van der Sluijs et al. 2014). Among the gaps identified were a lack of knowledge relating to chronic exposure, sublethal effects of exposure, and effects of exposure to a mixture of several neonicotinoids. The present study addresses many aspects of these knowledge gaps.

Three main experiments were performed in the present study. The first experiment included chronic exposures of sockeye salmon in a gravel-bed flume incubator system or traditional flow through glass tank exposure system to 4 environmentally relevant concentrations (0.15, 1.5, 15, or 150 µg/L) of imidacloprid from one hour post-fertilization through to emergence as swim-up fry; and an acute exposure to the same concentrations of imidacloprid (0.15, 1.5, 15, or 150 µg/L) during the fertilization process only followed by rearing to the swim-up fry developmental stage in

flow through clean water heath stack systems. Hatch success and timing, growth and development, and deformities were the main endpoints measured for these first experiments in the gravel-bed flume incubator, glass tank, and heath stack exposure systems. In the second set of experiments, feeding fry were exposed acutely for 96 h to 3 environmentally relevant concentrations (3, 30, or 300 µg/L) of imidacloprid, clothianidin, thiamethoxam, or a mixture of the three, and were then subjected to a burst swim assay to assess a coordinated physiological response critical for fish survival. In the third set of experiments, sockeye salmon feeding fry were exposed acutely for 96 h to the 3 concentrations (3, 30, or 300 µg/L) of imidacloprid, clothianidin, thiamethoxam, or a mixture of the three, and were then subjected to an oxygen consumption assay to assess potential impacts to metabolism and respiratory function.

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Chapter 2. Sub-lethal effects of the neonicotinoid insecticides imidacloprid, clothianidin, and thiamethoxam on developing sockeye salmon (*Oncorhynchus nerka*)

2.1. Abstract

The effects of the neonicotinoid insecticide imidacloprid on sockeye salmon (*Oncorhynchus nerka*) exposed from fertilization to emergence were evaluated using a gravel-bed flume incubator designed to simulate a natural streambed environment. Fertilized eggs were exposed to the pure insecticide imidacloprid at nominal concentrations of 0, 0.15, 1.5, 15, and 150 µg/L to investigate the effects on hatch success and timing, growth, and deformity incidence. The effects of the neonicotinoid insecticides thiamethoxam, imidacloprid, and clothianidin, and a mixture of all three on burst swimming performance and routine metabolism in fry were also examined in acute 96-h exposure scenarios. There was no consistent evidence that chronic exposure to environmentally relevant imidacloprid concentrations impacted growth, development, hatch timing and success, or survival. Acute exposures to concentrations ranging up to maximum levels reported in global surface waters did not affect either swim performance or routine metabolism. These results indicate that the current water quality guidelines for neonicotinoids are likely protective of sockeye salmon.

2.2. Introduction

In British Columbia (BC), Canada, 1.29 million kilograms of active pesticide ingredients were sold in 2010, and 33% of these sales were accounted for by insecticides (BC Ministry of the Environment 2010). Neonicotinoid insecticides are the fastest growing class of insecticides on the global market, and currently make up the largest percentage of the global market share of insecticides, at greater than 25% as of 2014 (Bass et al. 2015). A recent review of concentrations of neonicotinoids in surface waters found that detected concentrations averaged 0.13 µg/L, but in areas of high density neonicotinoid application, concentrations have been measured at levels as high as 320 µg/L (Morrissey et al. 2015). Neonicotinoid use in Canada is increasing, and

recently the detection of neonicotinoids in surface and groundwater systems has been noted (Government of Quebec 2014; Mineau and Palmer 2013; Main et al. 2014).

The most widely used neonicotinoids globally are imidacloprid, thiamethoxam, and clothianidin. All of these compounds are currently under review in Canada, and the Pest Management Regulatory Agency (PMRA) has proposed a phasing out of the agricultural use as well as the majority of other outdoor uses of imidacloprid over the next 3 to 5 years due to concern for the impact on aquatic insect populations and organisms that rely on them for food, as well as potential risks to birds and small mammals feeding on imidacloprid treated seeds. (Health Canada 2016). In addition, neonicotinoid use has been banned in several municipalities in recent years, including Montreal and Vancouver. However, imidacloprid, thiamethoxam, and clothianidin are all licensed for use in other regions of BC for the treatment of a variety of target insect pests and crops including strawberries, grapes, ornamental plants and turf grass (Government of British Columbia Ministry of Agriculture 2016). The Lower Mainland and southern interior regions of the province contain high density agricultural regions with associated pesticide application, which coincides with the Fraser River watershed, among other important salmon bearing watersheds in the region and provides avenues for pesticides to enter salmon bearing aquatic systems via runoff, deposition, spray drift, or leeching out of groundwater and other less likely routes (Krupke et al. 2011; Lamers et al. 2011).

All neonicotinoids exert their toxic effects on target pests via agonistic action on nicotinic acetylcholine receptors [nAChRs] (Tomizawa and Casida 2003). Binding to nAChRs on the post synaptic membrane leads to overstimulation of neurons and the neuromuscular junction, resulting in convulsions, paralysis, and death in insects (Goulson 2013). Although neonicotinoids have a higher affinity for invertebrate nAChRs due to electronegative interactions with specific regions of the receptor that are not present in their vertebrate counterparts, it has been demonstrated that they can bind to vertebrate nicotinic acetylcholine receptors as well (Tomizawa and Casida 2003). Zebrafish exposed to concentrations of 26 mg/L of imidacloprid from the zygote to free swimming larvae (6 d past fertilization) experience a 4 fold reduction in acetylcholine levels after 5 d of exposure, potentially as a result of breakdown of acetylcholine in the synaptic cleft due to competition at binding sites from the imidacloprid (Tufi et al. 2016).

Despite anthropogenic chemicals such as pesticides being identified as potential contributors to declining sockeye salmon populations in BC (Peterman et al. 2010), limited information relating to the presence, concentration, and effect of these chemicals in aquatic ecosystems, such as salmon bearing streams, is available (Cohen Commission 2012). A 2013 paper that examined the complexity of characterizing pollutant exposure to migratory salmonids over the course of their lives identified a variety of contaminant sources, including wastewater effluent, landfill leachate, pulp and paper mill effluent, wood preservation facilities, agricultural land, and deposition of atmospheric pollutants (Ross et al. 2013). A monitoring program undertaken from 2003 to 2005 in surface waters nearby agricultural lands in the lower Fraser Valley of BC detected mixtures of up to 33 individual pesticides at a single location, although this study did not include neonicotinoids (Woudneh et al. 2009). A monitoring program investigating pesticide occurrence in salmonid bearing streams during the typical pesticide use season in Washington State from 2003 to 2011 detected 74 individual pesticides and associated metabolites, including neonicotinoids, with imidacloprid detections increasing in frequency over time (WSDA 2013). There is a growing literature base regarding the potential for adverse effects to fish exposed to neonicotinoid insecticides.

Reviews of acute lethality of neonicotinoids indicate that freshwater fish that have been tested with imidacloprid and clothianidin are typically relatively insensitive. 96-h LC_{50} values for a variety of fish including bluegill sunfish (*Lepomis macrochirus*), Japanese carp (*Cyprinus rubrofuscus*), Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*), sheepshead minnows (*Cyprinidon variegatus*), orfe (*Leuciscus idus*) and zebrafish (*Danio rerio*) fall in the range of 100-300 mg/L (Gibbons et al. 2015; Smit et al. 2015). Two 96-h flow through thiamethoxam exposure tests of rainbow trout also included the endpoints swimming behaviour, loss of equilibrium, respiratory function, exophthalmos, and pigmentation in addition to lethality. In each of these studies, the observed NOEC was equivalent to the highest concentration tested at 100 mg/L and 125 mg/L for the two studies (Rufli 1996; Ruffli 1997a).

Sublethal effects, such as histopathic changes, induction of liver enzymes indicative of an oxidative stress response, and DNA damage, have been observed in fish exposed to neonicotinoids. Nile tilapia (*Oreochromis niloticus*) exposed acutely for 24 h to concentrations of imidacloprid >1.34 mg/L resulted in gonadal changes including

extensive degradation of testicular tissue (Ocampo and Sagun 2007; Lauan and Ocampo 2013). Exposure of adult loach (*Misgurnus anguillicaudatus*) to concentrations of imidacloprid ranging from 43 to 115 mg/L for 6 d resulted in an increase in micronuclei and nuclear anomalies observed as well as a decrease in hepatic glutamic pyruvic transaminase (GPT) and glutamic oxoalacetic transaminase (GOT) activity. Histological examination of the testes also indicated that imidacloprid exposure resulted in disorganized lobules and the presence of cysts (Xia et al. 2016). Similarly, a study was conducted to assess the impacts of exposure to 0.3, 1.25, and 5 g/L concentrations of imidacloprid on zebrafish following 7, 14, 21, and 28 d of exposure. The 1.25 and 5 g/L treatments resulted in increased reactive oxygen species (ROS) production and increased malondialdehyde (MDA), an indicator of oxidative stress via lipid peroxidation, as well as increased DNA damage (Ge et al. 2015). A 24-h exposure of adult zebrafish to 27.5 mg/L of imidacloprid also resulted in elevated MDA production (Shukla et al. 2017). Another study involving zebrafish exposed to 0.3, 1.25, and 5 g/L concentrations of thiamethoxam over 28 d resulted in an increase in ROS production, MDA production, and DNA damage (Yan et al. 2016). These results are important given that lipid peroxidation has been identified as an important mechanism in pesticide induced toxicity (Nwani et al. 2010). However, all results were observed at concentrations much higher than have been measured in freshwater environments.

The earliest life stages in fish tend to be the most vulnerable to xenobiotic toxicity (Hutchinson et al. 1998), and only approximately 10-20% of the roughly 3000 eggs laid by a spawning pacific salmon female will successfully develop to the fry stage (Bradford 1995). During embryonic and larval development stages, sockeye remain buried in gravel redds and are relatively immobile and susceptible to external stressors (Ross et al. 2013). While a large literature base exists regarding pesticide induced toxicity in salmonid species during fry, smolt, and juvenile stages, there is limited available published information on the impact of pesticide exposure to earlier life stages of salmonids. Early life stage toxicity tests with rainbow trout or fathead minnow are commonly included as supporting information in applications for registration of new pesticides in the United States and Canada, however, these studies are commonly proprietary information and often go unpublished. Common endpoints considered for chronic exposures to early life stages of salmonids and other fish include hatch success, survival, growth, incidence of developmental deformities, and timing of emergence.

A study performed using rainbow trout fry exposed to imidacloprid yielded a 96-h LC_{50} of 1.2 mg/L, indicating that they are much more sensitive than later stage fish of a variety of species (US EPA 1992). However, 1.2 mg/L is 4 times higher than the highest observed concentration of imidacloprid in any measured surface water of 320 µg/L (Van Dijk et al. 2013). In addition, a 96-h exposure of zebrafish embryos to imidacloprid indicated that no toxicity to embryogenesis and development was observed at concentrations up to 320 mg/L, although the commercial formulation Confidor SL 200® was found to be more toxic, with a 96-h LC_{50} of 214 mg/L (Tisler et al. 2009), indicating that early life stages may not always be more sensitive to acute imidacloprid toxicity. While direct lethality may not be a common concern for the environmental exposure of fish to neonicotinoids, there is evidence that neonicotinoid exposure may result in a host of sublethal effects in fish that can lead to impaired performance or survival and potentially impact fish at the population level.

There have been a few studies, both published and unpublished, that have examined the subchronic to chronic effects of exposure to neonicotinoids on early life stages of freshwater fish. The most extensive literature base exists for studies involving imidacloprid. A 60-d flow through exposure of rainbow trout (*Oncorhynchus mykiss*) to imidacloprid resulted in a NOEC of 1.2 mg/L and a LOEC of 2.5 mg/L based on reductions to growth (Cohle and Bucksath 1991). A 7-d exposure of zebrafish (*Danio rerio*) to imidacloprid starting at fertilization and terminating at 168 h post fertilization found no effects on mortality or incidence of developmental deformities at concentrations up to 50 mg/L (Scheil and Kohler 2009). In another study, zebrafish were exposed to 12 or 15 mg/L of imidacloprid from 4 h to 5 d post-fertilization. Following exposure, the zebrafish were either assessed for larval activity or reared to the adolescent and adult stages and then subjected to multiple neurobehavioural assays, including assessment of sensorimotor responses and habituation in a tactile startle test, novel tank swimming, and shoaling behaviour (Crosby et al. 2015). The results indicated that imidacloprid exposure during this developmental window resulted in decreased larval swimming activity, as well as decreased novel tank exploration and an increased sensorimotor response to startling stimuli (Crosby et al. 2015). These results indicate that zebrafish may be more sensitive to behavioural effects than to lethality or developmental deformities following early life stage exposure to imidacloprid, and that early life stages

of salmonids such as the rainbow trout may be more sensitive to imidacloprid than zebrafish.

Only one early life stage toxicity test involving clothianidin was identified. A 28-d flow through exposure involving fathead minnows resulted in a NOEC of 9.7 mg/L and a LOEC of 20 mg/L based on reduced growth (measured as length and dry weight; MRID 454224-13, as cited in US EPA 2011). A recent review of the available aquatic toxicity studies involving exposure to thiamethoxam identified two chronic studies using early life stages of rainbow trout (Finnegan et al. 2017). A 28-d flow through exposure examining mortality, growth rate, food conversion efficiency, feeding activity, swimming behaviour, respiratory movement, pigmentation, exophthalamus, loss of equilibrium, and reaction to external stimuli found that the NOEC was equivalent to the highest concentration of thiamethoxam tested, which was 100 mg/L (Rufli 1997b). An 88-d flow through exposure investigated the endpoints hatching success, time to hatch, time to swim up, larvae and fry survival, and growth. Similarly, no effects were observed at the highest thiamethoxam concentration tested of 20 mg/L (Drottar et al. 1997).

While most of these studies used concentrations of neonicotinoids much higher than might be expected in the environment, an experiment in which medaka (*Oryzias latipes*) were exposed to concentrations of imidacloprid as low as 0.03 mg/L resulted in the induction of a stress response and increased susceptibility to ectoparasite infection (Sanchez-Bayo and Goka 2005). There are currently no published studies that have investigated the impact of neonicotinoid exposure on sublethal endpoints in salmonids. Given that rainbow trout (*Oncorhynchus mykiss*) fry have the most sensitive LC_{50} value of any fish exposed to neonicotinoids in a laboratory setting of 1.2 mg/L (US EPA 1992), and that zebrafish were approximately 20 times more sensitive to developmental sublethal effects than they were to lethality when exposed to imidacloprid, with swimming behaviour effects observed at 12-15 mg/L (Crosby et al. 2015) but LC_{50} values upwards of 200 mg/L (Tisler et al. 2009), a better understanding of the potential for sublethal effects in early life stage salmonids exposed to neonicotinoid insecticides is necessary.

As detailed above, the available literature base involving salmonid exposure to neonicotinoids primarily includes 96-h exposures focusing on lethality in adult rainbow trout. Due to the widespread use of neonicotinoids and their increasing presence in

salmon bearing streams, it is important to investigate the potential for toxicity to early life stages of salmonids, especially in chronic exposure scenarios involving environmentally realistic concentrations. Early life stage toxicity testing in fish was first introduced by Mount and Stephan in a study involving fathead minnows (1967) and is now widely used as a way of evaluating current use and prospective use compounds. Standard methodologies have been developed by a variety of organizations and these methodologies have been adapted for use with salmonids (OECD 1992; Environment Canada 1998; ASTM 2013). Endpoints common to established methodologies include observations of direct mortality to embryos, larvae, and juveniles, hatch success, weight and length as a measure of growth, and abnormal physical and behavioural development.

One of the most commonly identified mechanisms of developmental disruption in juvenile and adult salmonids involves the acetylcholine axis in the nervous system (Wheelock et al. 2005). Acetylcholine is expressed early on in zebrafish development, with activity increasing rapidly within 48 h of fertilization and imidacloprid has been demonstrated to result in a decrease in acetylcholine activity (Tufi et al. 2016). Exposure to other pesticides with similar methods of action to neonicotinoids has resulted in reduced growth (Carlson 1971; Jarvinen and Tanner 1982), and reduced energy reserves in the form of glycogen in the liver and muscles (Sastry et al. 1988). Acetylcholinesterase inhibition as a result of exposures to organophosphate and carbamate pesticides has also been modelled to predict reductions in growth and survival in juvenile salmonids based on the assumption that acetylcholinesterase inhibition (a similar net effect to the agonistic action of neonicotinoids) is associated with altered brain activity and feeding behaviour (Baldwin et al. 2009). Reductions in growth and abnormal development could occur due to disruption of the acetylcholine axis, or due to an increased metabolic demand associated with metabolism of the neonicotinoid, resulting in less energy reserves available for anabolic growth and development of tissues. Fish exposed to pollutants have been demonstrated to develop a stress response characterized by an increase in anaerobic metabolism, which can result in evaluated blood lactate concentrations followed by hyperglycemia (Sancho et al. 1997). This stress response can lead to protein catabolism to compensate for cellular energy losses associated with the increased anaerobic metabolism (Pfeifer and Weber 1979). The described stress response is consistent with observed reductions in glycogen in

liver and muscle tissue following exposure to acetylcholinesterase inhibiting pesticides (Sastry et al. 1988).

Another common way to assess toxicity to fish is via swimming performance assays. Coordinated swimming is an integrated physiological process that requires proper function of nervous and muscular systems, as well as maintenance of ionic and energetic balances. Due to its integrative nature, swim performance can be considered to be an indicator of the general health and fitness of an organism, providing an ecologically relevant endpoint for studying the effects of a toxicant on a fish species (Scott and Sloman 2004; Little and Finger 1990). Neonicotinoids affect acetylcholine signalling and due to the importance of acetylcholine in muscle coordination, linking together the nervous system and muscular systems at the neuromuscular junction, assays of an integrated swim response appear to be a useful indicator of toxicity. Acetylcholinesterase inhibition has previously been linked with reduced swimming stamina (Post and Leasure 1974; Van Dolah et al.1997), and exposure of zebrafish to imidacloprid during development resulted in altered swimming behaviour (Crosby et al. 2015).

Impaired respiratory function has long been established as one of the earliest symptoms in acute pesticide poisoning (Holden 1973). Acetylcholine plays an important role in gill circulation and respiration in fish (Jonz and Zaccone 2009), and acetylcholine administration has been experimentally linked with constriction of the efferent vasculature, including arteries and arterioles in the gill filaments (Mauceri et al. 2005). A decrease in blood flow to the gills reduces the ability of a fish to extract oxygen from the water and as result decreases the oxygen available in the body for cellular respiration. Agonistic action of neonicotinoids could potentially lead to constriction of the efferent vasculature and a decrease in blood flow to the gills and therefore decreased oxygen uptake. Alternatively, oxygen consumption could be altered as a consequence of the previously described toxicant induced stress response, in which energy stores are depleted due to increased anaerobic respiration (Sancho et al. 1997; Pfeifer and Weber 1979). One potential explanation for the stress response is that exposure to pollutants results in gill damage, which reduces the amount of oxygen taken up at the gills by fish (Evans 1987). Exposure to thiamethoxam has resulted in histopathological alterations in the gills of the common carp as well as reduced oxygen consumption over time in a species of freshwater bivalve (Georgieva et al. 2014; Minakshi and Mahajan 2013). Due

to acetylcholine's role in the sensorimotor system (including the innervation of the gills and muscles involved in coordinated swimming) there is potential for important sublethal effects to occur in sockeye salmon following exposure to neonicotinoids that may impact their survivability (Laetz et al. 2009).

The relatively recent introduction of neonicotinoids to the insecticide market and its subsequent growth in market share require that data be generated as to their potential for adverse effects on non-target organisms. Some of the gaps in the current knowledge base that were recently identified include sublethal effects associated with chronic exposures in non-target species including vertebrates, and a characterization of the effects of exposures to mixtures of several neonicotinoid compounds simultaneously (van der Sluijs et al. 2014). The present study addresses each of these areas through a chronic exposure of sockeye salmon embryos and alevins to environmentally relevant concentrations of imidacloprid as well as acute exposures to environmentally relevant concentrations of imidacloprid, clothianidin, thiamethoxam, and a mixture of the three neonicotinoids. Endpoints examined in the chronic exposure include measures of growth, deformities, and success and timing of hatch and development. Endpoints examined in the acute exposures include swim performance and oxygen consumption. Acute exposures were included to examine exposure scenarios that reflect short lived influxes of neonicotinoids such as following application or a rainfall event, and were considered to be more relevant to free swimming salmon fry due to their mobility in the water column. The chronic exposure scenario was considered to be more relevant to the developmental stages that include freshly fertilized eggs developing into alevins, because during these stages salmon are relatively immobile and may be exposed long term without the ability to avoid neonicotinoids like a fry may be capable of doing.

2.3. Methods

2.3.1. Fish

Sockeye gametes were collected by Dr. Vicki Marlatt in collaboration with the Department of Fisheries and Oceans on two separate occasions (November 7th and 14th, 2014). On the first collection, 4 male and 4 female spawning sockeye salmon from the Pitt River, BC were collected. On the second collection, 7 female and 5 male spawning sockeye salmon from the same Pitt River population were collected. During

both collections, fish were euthanized by cerebral concussion and eggs (~2000 to 2500 per female) and milt were extracted and stored in sealed containers filled with oxygenated air within coolers at 6-10 °C and transported to Simon Fraser University. Fertilization procedures took place within 24 h of gamete collection.

Before fertilization, milt from each of the males was assessed for motility under a microscope as an indicator of viability. In order to reduce variability in the response to exposure due to genetic variation as well as to investigate differences in magnitude or occurrence of adverse effects resulting from exposure, 4 unique sets of offspring were created by performing 4 independent fertilizations. The fertilization procedure used was a dry fertilization protocol adapted from Patterson (2004) that has previously been shown to be effective in incubation studies using sockeye salmon from Weaver Creek and Gates Creek (Patterson 2004, Burt et al. 2012). Eggs (16 g or ~100 eggs) were combined with 0.15 mL of milt and activated with 30 mL of either dechlorinated municipal water or an imidacloprid-water mixture. Following a 2-min activation period, an additional 200 mL of water or the imidacloprid-water mixture was added and the eggs were left undisturbed for a 10-min period to allow for water hardening of the fertilized eggs.

For fish incubated for the acute exposures and subsequent swim performance trials, after water hardening the fertilized eggs were gently deposited in trays in a flow through heath stack and raised until the swim-up fry stage. These swim-up fry were then transferred to flow through holding tanks and fed *ad libitum* three times daily until the acute exposures and swim performance trials as described in Sections 2.3.6 and 2.3.7. In the chronic exposure and acute fertilization exposure experiments, eggs were counted, divided, and gently poured into three cylindrical polyvinyl chloride baskets surrounded by a mesh netting and placed into an exposure chamber as described in Section 2.3.2. Each exposure chamber contained 3 baskets from each parental cross. Not all fertilizations were viable (details are provided in the results section) and therefore only two of four crosses were included in the analysis for each exposure.

For oxygen consumption experiments, sockeye fry were obtained from the University of British Columbia shortly after reabsorbing their yolk sacs. The sockeye were raised from eggs fertilized with milt that was obtained at the same time and location as the gametes used to raise the sockeye fry for the swim performance assay (i.e. the

Pitt River spawning population in November 2014). Fry were raised in holding tanks as described above.

2.3.2. Incubation of Embryos for Effects on Development

Two incubation systems were used to monitor egg development and survival from 1 h post-fertilization until the swim-up fry stage. Mesh covered cylindrical baskets containing approximately 33 eggs each were placed in either flow through glass tanks or a gravel-bed flume incubator designed to simulate a natural streambed environment. The glass tank system allowed for the visual monitoring of development throughout the experiment. However, the gravel in the gravel-bed flume system obscured visual daily monitoring of the hatching and development of fish so these endpoints could not be measured in this system, but emergence from the gravel and growth and development at the swim-up fry stage were measurable endpoints in this system.

The gravel-bed flume design used a modified design comprised of two 250 cm long by 40 cm wide by 30 cm deep compartments (Du Gas 2014; Pilgrim et al. 2013), where each compartment was subdivided into 5 large sections separated by plexiglass dividers to isolate each section and allow for exposures to multiple pesticide concentrations concurrently within the same flume (Figure 2). Each section is further divided into 5 subsections, separated by stainless steel mesh dividers. Drainage of the flow through system occurred through the central subsection, which was left unoccupied.

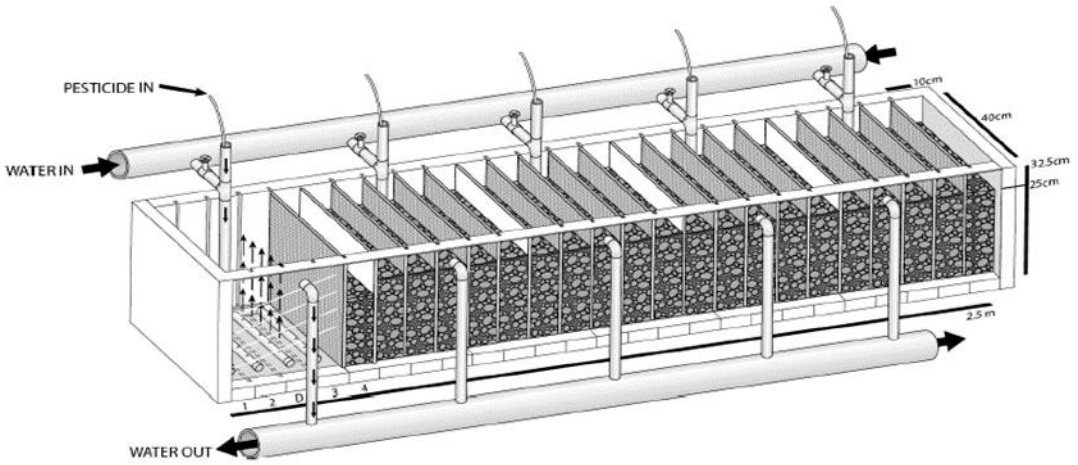


Figure 2. Flume incubator apparatus used to simulate natural gravel stream bed conditions for the chronic experiment investigating developmental effects of imidacloprid exposure on sockeye salmon at early life stages (Du Gas 2014).

The gravel-bed flume allowed water to flow up into the sections via several holes in polyvinyl chloride (PVC) pipes running along the base of each subsection. The gravel used to form the beds was composed of rocks of two sizes, 10 mm and 25 mm in diameter, based on reported gravel sizes at salmon redd sites (Crisp and Carling 1989; Pilgrim et al. 2013). The two sizes of gravel were combined in a 1:1 ratio, disinfected with a 1% Ovadine solution, and thoroughly rinsed with dechlorinated municipal water (Du Gas 2014). The gravel was then used to fill the four subsections of each section of the flume that would be holding eggs up to a height of 5 cm.

Eggs from each cross were separated into the subsections in the gravel-bed flumes by the stainless steel mesh dividers, but received the same treatment within each larger section. In the glass tanks, the fertilized eggs remained in their cylindrical, mesh-covered baskets throughout the exposure and were continuously monitored for signs of development. In the gravel-bed flumes, fertilized eggs in the cylindrical meshed baskets were placed on top of the gravel and the eggs were continuously monitored until the embryos reached the eyed stage. Once the embryos reached the eyed stage, a depression in the middle of the gravel within each subsection of the flume was formed to simulate the redds constructed in a natural streambed environment. Eggs from each cross were then gently poured into the artificially constructed redds and carefully covered with gravel by hand to a depth of 15 cm (Du Gas 2014: Pilgrim et al. 2013). The

flumes and glass tanks were covered with sheets of black plastic to prevent light exposure during the sensitive embryo and early alevin stages (Flamarique and Harrower 1999). One week after the eggs in the tanks and flumes had hatched, the plastic covering was removed and the photoperiod set to reflect the ambient natural photoperiod.

Fertilized eggs for acute exposures prior to swim performance and oxygen consumption assays remained in the heath stack until reabsorption of the hatched alevins' yolk sacs had occurred, at which point they were transported to large flow through tanks. Heath stacks received a continuous flow of dechlorinated municipal water at external ambient temperatures ranging from 6 to 11°C. Deceased eggs and alevins were removed daily to reduce the risk of infection and maintain optimal water quality. The flow through holding tanks were supplied with dechlorinated municipal water at 12°C receiving constant oxygenation. Fry in the holding tanks were fed three times daily *ad libitum* using EWOS Pacific complete feed for salmonids pellets (Cargill Inc, Surrey, Canada) ground into a fine powder. Lighting was regulated via an automated timer with a 12 h of light, 12 h of darkness (12:12 L:D) cycle.

2.3.3. Neonicotinoid Exposures

In experiments assessing the effects of neonicotinoids on development, exposure systems received dechlorinated municipal water at ambient external temperatures ranging from 5.2 to 11.6 °C over the course of the experiments. Water temperature, dissolved oxygen concentrations, and pH were measured 3 times a week for the duration of the experiment using an HQ40d multi-function Hach Probe (Hach Company, Loveland, CO, USA). Ammonia concentrations were monitored weekly using a Seachem free and total ammonia test (Seachem Laboratories Inc, Madison, GA, USA) with a detection limit of 0.05 mg/L.

Water flow rates to each flume section and glass tank were consistently monitored and adjusted to maintain a target flow rate of 95 mL/min. Gate valves were adjusted if necessary twice a week, and average flow rates varied from 91 to 97 mL/min for the exposure period for both the tanks and the flumes. These flow rates allowed for a complete replacement of the water in the flume section or tank to occur every 4 h.

Imidacloprid exposure solutions were prepared using 95% pure imidacloprid (Sigma-Aldrich, St. Louis, MO, USA). Imidacloprid was dissolved in dechlorinated municipal water (stock solution of 50 mg/L), gently warmed while monitoring with a thermometer to a maximum temperature of 30 °C and continuously stirred until the pesticide was fully dissolved. The stock solution was serially diluted to form 4 stock solutions that were delivered via Masterflex peristaltic pumps (Thermo Fisher Scientific, Waltham, MA, USA) into the flume section or glass tank at a target flow rate of 2 mL/min. The average flow rate for stock solutions ranged from 1.90 to 2.14 mL/min. The nominal exposure concentrations of imidacloprid in both the glass tanks and the gravel-bed flume systems were 0, 0.15, 1.5, 15, and 150 µg/L. These nominal concentrations were well below the water solubility of imidacloprid of 510 - 600 mg/L (Krohn 1989; Macbean 2008), thus the use of a vehicle was unnecessary. Exposure concentrations were chosen to represent a range of concentrations measured in surface waters as summarized by Morrisey et al. (2015), including concentrations below and above the CCME chronic water quality guideline for imidacloprid of 0.23 µg/L (CCME 2007). All exposures were performed in duplicate, with each flume containing one of each treatment section in a randomized complete block design. Water samples were taken from each treatment tank and flume section on November 25th, 2014 (approximately 2 weeks into the exposure) and samples were analyzed by ALS Environmental in Waterloo, Ontario for imidacloprid concentrations. Samples were analyzed via liquid chromatography followed by mass spectrometry. Further details related to the chemistry analysis are provided in the laboratory report in Appendix A.

The experiments assessing swim performance and oxygen consumption after 96 h exposures to imidacloprid adhered to the Environment Canada protocol for a 96-h rainbow trout LC_{50} test (Environment Canada 2000) for the acute exposure portion, with minor deviations. Specifically, a 50% water change was performed after 48 h (Environment Canada 2000). Unexposed sockeye feeding fry (n=130) with an average weight of 1.32 g (SD 0.18 g) and an average length of 5.4 cm (SD 0.37 cm) were used for the swim performance assay, and juveniles (n=78) with an average weight of 13.88 g (SD 0.3 g) and an average length of 10.9 cm (SD 0.07 cm) were used for the oxygen consumption assay. Fish were transferred from flow through holding tanks into duplicate 40 L aerated glass tanks. For the swim performance exposures, each tank contained 6 fish, with an average loading density of 0.26 g/L. For the oxygen consumption assay

exposure, each tank contained 3 fish, with an average loading density of 1.19 g/L. Tanks contained either 0 µg/L (control), 3, 30, or 300 µg/L concentrations of either imidacloprid, clothianidin, thiamethoxam, or an equal parts mixture (by mass) of the three pesticides. Treatment solutions were prepared fresh twice a week from 5 mg/L stock solutions dissolved in dechlorinated water and stored in darkness at ambient temperatures to prevent photodegradation. Similar to the developmental exposures, concentrations were chosen to represent a range of concentrations measured in surface waters as summarized by Morrisey et al. (2015). Fry were fed daily up until 24 h prior to the swim performance assay with finely ground pellets of EWOS Pacific complete feed for salmonids. Excess food and waste were removed daily. 48 h after the start of the exposure, a 50% water change was performed. Photoperiod was maintained at 12:12 L:D and measurements of temperature, pH, dissolved oxygen, and conductivity were taken daily. Ammonia was also measured in each tank using a Seachem free and total ammonia test with a detection limit of 0.05 mg/L.

2.3.4. Hatch Success and Timing

Eggs in both the glass tanks and the flumes were continuously monitored on a daily basis under red light for mortality until the embryos reached the eyed stage; dead eggs were removed using a pipette and counted throughout the exposure period. When embryos reached the eyed stage, eggs were thoroughly examined and those without clearly visible eyes were counted and removed (beginning at approximately 200 accumulated thermal units [ATUs], or 25 exposure days). The remaining eyed embryos in the flumes were then buried in gravel as described above. Eggs in the glass tanks were monitored every other day until the first embryos began to hatch and then monitoring of hatched embryo frequency increased to daily. Once the alevins had absorbed their yolk sacs, the glass tank swim-up fry were collected and euthanized in a solution of approximately 400 mg/L tricaine methane sulphonate (MS-222) buffered to pH 7 with sodium bicarbonate (beginning at approximately 800 ATUs, or 100 exposure days). Following euthanization, measurements of weights and fork lengths were taken and a deformity analysis was performed in accordance with standard methodology described below (Rudolph 2006).

Following the burial of the eyed embryos in the flume incubators, sections were monitored for emerging alevins that had completed the swim up process (i.e. present in

the uppermost region of the test vessel above the gravel). Emergence began at approximately 800 ATUs (or 100 exposure days) and sections were monitored daily until emerged salmon had reabsorbed their yolk sacs. Emerged salmon were then collected and gravel was removed from the beds carefully to collect and count any dead eggs and embryos as well as hatched fish that had not emerged from the gravel. All fish were euthanized in a solution of 400 mg/L MS-222 buffered to pH 7 with sodium bicarbonate and weight and fork length were measured, followed by a deformity analysis as described above according to Rudolph (2006). Alevins that were removed from the exposure prematurely due to death underwent a deformity analysis as well, and were preserved in Davidson's fixative solution for future reference.

2.3.5. Deformity Analysis

Preserved alevins were examined under a dissecting microscope for evidence of skeletal, craniofacial, and finfold deformities, as well as for signs of edema (Rudolph 2006). The severity of any observed deformities was categorized according to a graduated severity index ranging from scores of 0 to 3: a score of 0 represents no apparent signs of deformity; a score of 1 represents a slight deformity that is unlikely to impair the organism's regular functions; a score of 2 represents a moderate deformity that is likely to impair fish functions such as movement, feeding, or sight; and a score of 3 represents a severe deformity that would significantly impair an organism's chances of survival (Rudolph 2006). A full summary of the graduated severity index applied in the deformity assessment is provided in Table 1 (Rudolph 2006). The graduated severity index was established prior to deformity analysis and the analysis was performed by two individuals independently during terminations. Any inconsistent assessments between individuals resulted in immediate re-assessment and discussion to arrive at a final deformity assessment.

Table 1. Deformity analysis criteria and probable outcomes for the graduated severity index applied to sockeye salmon from the chronic and acute fertilization exposures (Rudolph 2006).

Score	Skeletal	Craniofacial	Finfold	Edema
0	Normal backbone.	Normal eyes, jaw, and head.	All fins present, normal size and shape.	No fluid accumulation in head or pericardial cavity.
1	Sligh scoliosis, lordosis, or kephosis. Unlikely to significantly impair fish movement.	Slightly reduced or malformed eye or jaw. Unlikely to significantly reduce feeding ability or sight**.**	One or two fins slightly reduced in size or slightly malformed. Unlikely to significantly impair fish movement.	Slight fluid accumulation in eyes or pericardial cavity. Unlikely to significantly impair fish sight, movement, or feeding.
2	Moderate scoliosis, lordosis, or kephosis. Likely to impair fish movement.	Moderately reduced or malformed jaw or eyes. Likely to reduce feeding ability and sight.	More than two fins slightly reduced or malformed or one or two moderately deformed fins. Likely to impair fish movement.	Moderate fluid accumulation in eyes or pericardial cavity. Likely to impair fish sight, movement, or feeding.
3	Severe scoliosis, lordosis, or kephosis. Fish movement likely to cease or be greatly impaired.	Missing or severely malformed eyese or jaws. Sight and feeding severely impaired.	One or more missing fins or more than two deformed fins. Severely reduced swimming capacity.	Severe Fluid accumulation in eyes or pericardial cavity. Greatly reduced fish sight, movement, or feeding.

2.3.6. Swimming Performance

Sockeye feeding fry (n=78) with an average weight of 1.32 g (SD 0.18 g) and an average length of 5.4 cm (SD 0.37 cm) were tested in a Loligo mini swim tunnel (Loligo Systems, Viborg, Denmark) following a 96 h exposure to nominal 0, 3, 30, or 300 µg/L concentrations of imidacloprid, clothianidin, thiamethoxam, or an equal parts by mass mixture of the three as described in Section 2.3.3. The swim tunnel used was a modified

Blazka-type design (170 mL in volume; 100 mm length x 26.4 mm internal diameter [Figure 3]). Burst swimming speed (U_{max}) was measured using a constant acceleration test (Farrell 2008; Osachoff et al. 2014). Individual fry were allowed to acclimate in the chamber to an initial velocity of 7.4 cm/s, approximately 1.5 body lengths/s) for 15 min, which is similar to acclimation procedures for other short term assessments of swim performance (Kraskura and Nelson 2018; Nelson et al. 2015). Preliminary testing indicated no appreciable difference in burst swim performance with increasing acclimation times. Following the 15 min acclimation period, the water velocity was increased by 1.4 cm/s (approximately 0.25 body lengths/s) each min until the fish could no longer swim due to fatigue (Farrell 2008; Osachoff 2014). Fatigue was defined as the point at which fish can no longer maintain their position against the current in the swim chamber, and was operationalized as spending more than 5 consecutive seconds horizontally pressed against the honeycomb grating at the rear end of the chamber (Plaut 2001; Kolok 1991). At fatigue, fish were removed and euthanized in MS-222 buffered to pH 7 using sodium bicarbonate, and measurements of wet weight and fork length were collected.

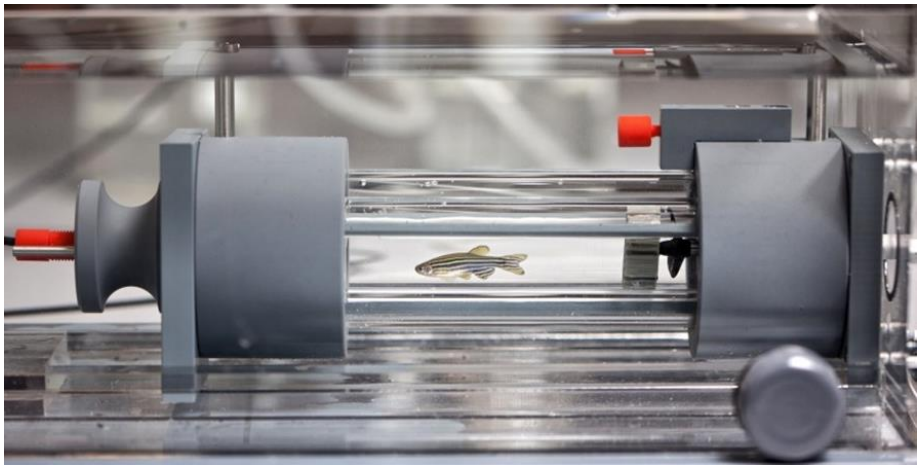


Figure 3. Swim tunnel apparatus used for the burst swimming speed assays (Loligo Systems, Viborg, Denmark)

2.3.7. Oxygen Consumption

Sockeye salmon juveniles with an average weight of 13.88 g and an average length of 10.9 cm were placed in a Loligo mini swim chamber equipped with a respirometer (Loligo Systems, Viborg, Denmark) following 96 h exposures to nominal 0, 3, 30, or 300 µg/L concentrations of imidacloprid, clothianidin, thiamethoxam, or an equal parts by mass mixture of the three as described in Section 2.3.3. The oxygen probe in the test chamber (2L; an appropriate volume based on the manufacturer's recommendations) measured the dissolved oxygen content in the chamber via a polymer optical fibre with a tip coated with a planar-oxygen sensitive foil. Sockeye salmon juveniles were acclimated one fish at a time to the test chamber which was supplied with a constant flushing and air supply for 30 min. While acclimation periods for metabolism tests tend to be longer, preliminary tests indicated that there was no appreciable difference in the rate of oxygen consumption over longer acclimation periods. In addition, fish from all treatment groups were subject to the same methodology, allowing for a focus on potential differences in oxygen consumption due to exposure concentrations of the various neonicotinoid treatments. After the acclimation period, the test chamber became a static respirometer (Cech and Brauner 2011) and oxygen concentrations were measured continuously for 1 h using Witrox software.

While oxygen consumption on a unit of mass basis does not typically follow a linear relationship, it is appropriate to compare the respiratory metabolic rates between groups of the same species if the mean body mass of the groups are statistically indistinguishable (Cech and Brauner 2011). There was no evidence of a difference in mean body mass between any of the treatment groups for the acute exposures prior to the oxygen consumption assay, and therefore comparison of respiratory metabolic rates were considered appropriate.

The assay was performed for all three fish in each exposure tank for a total of six fish per treatment. Following completion of the assay, juveniles were euthanized in a solution of MS-222 buffered to pH 7 with sodium bicarbonate and measurements of wet weight and fork length were taken and condition factor was calculated and compared between exposure groups.

2.3.8. Calculations and Statistical Analyses

Chronic exposures were performed using a two factor split plot design, with the factors being genetic cross (parentage) and imidacloprid treatment. The factor genetic cross initially had 4 levels due to the 4 different parental combinations involved in fertilization, however, parental cross A was determined inviable with a fertilization success of <10%, so the analysis was performed using the remaining 3 parental crosses. The factor of imidacloprid treatment had 5 levels, including the control, 0.15, 1.5, 15, and 150 µg/L concentrations. The factor of genetic cross operated on the level of individual fry, and was blocked by tank, flume section, or tray, with each tank, flume section, or tray containing a complete set of parental crosses of fry. The factor of imidacloprid concentration operated on the level of tanks or flume sections, and was blocked by location in the room, with each side of the room containing a complete set of the 5 levels of imidacloprid concentrations in the flume sections, and each row of the tanks containing a complete set as well. Within each row of tanks and side of the room, the imidacloprid concentrations were randomly assigned. The general statistical model used was as follows:

$$Y = \text{cross} + \text{pesticide} + \text{cross}*\text{pesticide} + \text{location}(r) + \text{tank}(r),$$

where Y represents the parameter of interest, the asterisk indicates an interaction effect, and r denotes a random blocking effect.

Using this general model, length and weight at termination of the chronic exposure was compared between the different treatment groups and parental crosses using a two-factor split plot analysis of variance (ANOVA). Length and weight were also used to calculate Fulton's condition factor K using the formula:

$$K = 100M/L^3,$$

where K is Fulton's condition factor, M is the fish's mass in grams, and L is the fish's length in cm. K values for swim-up fry were compared using the same method as length and weight.

Fertilization success was analyzed using the same two-factor split plot ANOVA approach, after comparing the number of eggs that successfully reached the visible eyed

stage to the total number of eggs in each cross (as a percentage). The same analysis was performed for survival by comparing the number of embryos that successfully reached the eyed stage to the number of fry that survived to reabsorb their yolk sacs. A two factor split plot ANOVA was used to compare mean time to 50% hatch (Sternecker and Geist 2010). Finally, deformity incidence rate was calculated for each replicate in each treatment as a percentage of total fish with observed deformities (Kennedy et al. 2000; Golder 2009). Due to limited observations of deformities, separate crosses were pooled for analyses and a single factor ANOVA was used to compare the mean incidence rate of deformities between treatment groups.

The acute fertilization exposure was performed in a two factor split plot design as well, with the two factors imidacloprid treatment and genetic cross. The factor of genetic cross (parentage) originally had 4 different levels based on the different combinations of parental gametes. However, parental crosses C and D were determined to be unsuccessful due to <10% fertilization success in one or more treatment. The factor of imidacloprid treatment used the same 5 treatment levels as in the chronic exposure, including the control, 0.15, 1.5, 15, and 150 µg/L concentrations. The factor of genetic cross operated on the level of individual swim-up fry, and was blocked by heath tray, with each tray containing a full set of parental crosses. The general model used for statistical analysis of the acute fertilization exposure experiment was as follows:

Y= cross + pesticide + cross*pesticide + tray(r),

where Y represents the parameter of interest, the asterisk indicates an interaction effect, and r denotes a random blocking effect. Similar to the analysis for the chronic exposures, two factor split plot ANOVAs were used to compare mean weights, lengths, Fulton's condition factors, fertilization success, survival, and time to 50% hatch for the sockeye in the acute fertilization exposures.

U_{max} was calculated using the equation:

$$U_{max} = U_f + U_i (T_f/T_i),$$

where U_f is the highest step velocity that was maintained for the full minute increment, T_f is the time swum at the the last velocity, T_i is the interval time (1 min in this case), and U_i is the velocity increment (1.4 $cm*s^{-1}$) (Brett 1964; Farrel 2008; Osachoff

2014). The maximum cross sectional area of all fish was below 10% of that of the swim chamber and therefore U_{max} was not corrected for the blocking effect (Bell and Terhune 1970).

The rate of oxygen consumption was calculated using the following equation:

$$MO_2=(CO_2(A)-CO_2(B))*(V_t-V_f)/T,$$

where MO_2 represents the oxygen consumption rate (mg/h), $CO_2(A)$ is the concentration of oxygen in the water at the beginning of the measurement period (mg/L), $CO_2(B)$ is the oxygen concentration at the end of the measurement period (mg/L), V_t is the volume of the test chamber (L), V_f is the estimated volume of the test fish (L), and T is the time (h) (Cech and Brauner 2011). Each measurement was normalized for body mass to yield an estimate of oxygen consumption (mg O_2/h/g). The oxygen probe was calibrated via a two point calibration using a beaker of water saturated with air using an air stone (considered to be 100% oxygenated) and a beaker of water saturated with sodium sulphate (considered to be anoxic), as recommended in the user manual for the respirometer. The probe was calibrated daily before use.

Acute exposures were performed in a randomized complete block design, blocked by week with each week containing an exposure tank of every treatment. Since the factor of treatment operated at the tank level, and measurements of swim performance and oxygen consumption were taken at the individual fish level, the U_{max} and MO_2 values for fish in each tank were pooled and the average U_{max} or MO_2 values were computed and compared between treatments at the tank level to avoid pseudoreplication (Hurlbert 1978). Differences in burst swim performance and oxygen consumption between control and exposed fish were compared using a randomized complete block one way ANOVA with an alpha level of $p>0.05$. An ANOVA was performed for each treatment compound or mixture comparing 3 µg/L, 30 µg/L, and 300 µg/L concentrations to the control fish. Following ANOVA tests, Tukey's post hoc test was performed to compare pairwise differences while correcting for the number of individual comparisons to avoid false positives. All statistical analysis was performed using JMP 12.

In order to facilitate comparisons of timing and duration of hatch, time elapsed from the beginning of exposures was converted to accumulated thermal units (ATUs),

also known as degree days (Sternecker and Geist 2010). ATUs were calculated by multiplying the time elapsed by the average temperature for each exposure compartment (i.e tank, flume section, or heath tray). In addition to statistical comparison of the mean time to 50% hatch, time series of cumulative hatch versus ATUs were plotted for each treatment group in the heath trays for the acute fertilization exposure and the tanks for the chronic exposure in order to visualize the timing and duration of the hatching period (Sternecker and Geist 2010).

2.4. Results

2.4.1. Water Quality and Actual Neonicotinoid Concentrations

For the chronic imidacloprid exposure and the acute fertilization exposure followed by rearing in clean water, the water temperature was allowed to fluctuate according to external ambient temperatures, and ranged from 5.2 to 11.6 °C. Dissolved oxygen levels ranged from 98.5 to 110.5%, pH ranged from 6.62 to 7.66, and conductivity ranged from 20.22 to 32.90 µS/cm (Table 2). Ammonia was not detected in any water sample.

Table 2. Summary of water quality parameters for the flow through chronic developmental assays.

Parameter	Test Chamber	Average	Min	Max	N
Temperature (ºC)	Tanks	8.6	5.4	11.5	66
	Flumes	8.6	5.2	11.6	66
	Heath Stack	8.2	6.0	11.5	64
pH	Tanks	7.2	6.8	7.7	66
	Flumes	7.2	6.6	7.5	66
	Heath Stack	7.1	6.8	7.4	64
Conductivity (µS/cm)	Tanks	26.0	20.5	31.5	66
	Flumes	26.4	21.1	32.9	66
	Heath Stack	25.7	20.2	31.3	64
Dissolved Oxygen (%)	Tanks	103.0	98.5	110.5	66
	Flumes	102.7	98.5	109.0	66
	Heath Stack	107.1	102.4	117.0	64

In tanks and the gravel bed flume incubators, water and pesticide flow rates were consistent based on flow rates measured throughout the exposures and on actual concentrations measured by an independent laboratory (ALS, Burnaby, BC). A summary of predicted and measured imidacloprid concentrations for the chronic exposure is provided in Tables 3 and 4. Predicted minimum, maximum, and average imidacloprid concentrations were calculated based on the imidacloprid stock solution and water flow rates. Control tanks and flumes were not expected to have any imidacloprid, and samples measured fell below the detection limit of 0.1 µg/L. The 0.15 µg/L exposure concentrations were predicted to fall between 0.12-0.19 µg/L and 0.13-0.16 µg/L in the measured tank and flume respectively, with measured concentrations of 0.17 µg/L and 0.14 µg/L, respectively. The 1.5 µg/L exposure concentrations were predicted to fall between 1.25-1.98 µg/L and 1.25-1.76 µg/L in the measured tanks and flumes respectively, with measured concentrations of 1.71 µg/L and 1.47 µg/L respectively. The

15 µg/L exposure concentrations were predicted to fall between 8.14-18.58 µg/L and 13.67-16.01 in the measured tanks and flumes respectively, with measured concentrations of 19.00 and 15.40 µg/L respectively. The 150 µg/L exposure concentrations were predicted to fall between 127-195 µg/L and 128-162 µg/L in the measured tanks and flumes respectively, with measured concentrations of 166 µg/L and 140 µg/L respectively.

Table 3. Predicted and measured imidacloprid concentrations based on water and imidacloprid stock solution intake flow rates in the glass tanks during chronic imidacloprid sockeye salmon exposures.

Tank Number	Imidacloprid Concentration (µg/L)				
	Nominal	Average Predicted	Minimum Predicted	Maximum Predicted	Measured
4	0.00	0.00	0.00	0.00	**-**
6	0.00	0.00	0.00	0.00	**<0.10**
5	0.15	0.17	0.13	0.21	**-**
10	0.15	0.15	0.12	0.19	**0.17**
3	1.50	1.57	1.32	1.96	**-**
9	1.50	1.53	1.25	1.98	**1.71**
2	15.0	15.5	11.5	18.8	**-**
7	15.0	15.3	8.1	18.6	**19.0**
1	150	163	134	194	**-**
8	150	163	127	196	**166**

Table 4. Predicted and measured imidacloprid concentrations based on water and imidacloprid stock solution intake flow rates during the chronic imidacloprid sockey salmon exposures in the gravel-bed flume system.

Flume Number	Imidacloprid Concentration (µg/L)				
	Nominal	Average Predicted	Minimum Predicted	Maximum Predicted	Measured
4	0.00	0.00	0.00	0.00	**-**
6	0.00	0.00	0.00	0.00	**<0.10**
5	0.15	0.14	0.13	0.16	**-**
10	0.15	0.15	0.13	0.16	**0.14**
3	1.50	1.50	1.32	1.68	**-**
9	1.50	1.45	1.25	1.76	**1.47**
2	15.0	15.0	13.5	17.0	**-**
7	15.0	14.9	13.7	16.0	**15.4**
1	150	146	128	171	**-**
8	150	146	128	162	**140**

The static acute exposures and the swim assays were performed using water acquired from the same water supply as the chronic exposures. Table 5 provides a summary of the water quality measurements in the exposure tanks. The water was chilled to 12 °C and over the course of the exposures the average temperature in the tanks was 11.6 °C and ranged from 11.1-12.2 °C. Dissolved oxygen was consistently high due to continuous aeration, and averaged 97.6% with a range of 95.0-100.8% over the course of the exposure period. Measured pH values were also consistently neutral and averaged 7.08, with a range of 6.94-7.16. Conductivity measurements averaged 31.4 µS/cm and ranged from 29.6-35.3 µS/cm. Ammonia was not detectable in any of the tanks during the exposure.

Table 5. Summary of water quality parameters for the 96-h static renewal exposures prior to burst swim performance assays.

Parameter	Average	Minimum	Maximum	N
Temperature (ºC)	11.6	11.1	12.2	14
Dissolved Oxygen (%)	97.6	95.0	100.8	14
pH	7.08	6.94	7.16	14
Conductivity (µS/cm)	31.4	29.6	35.3	14

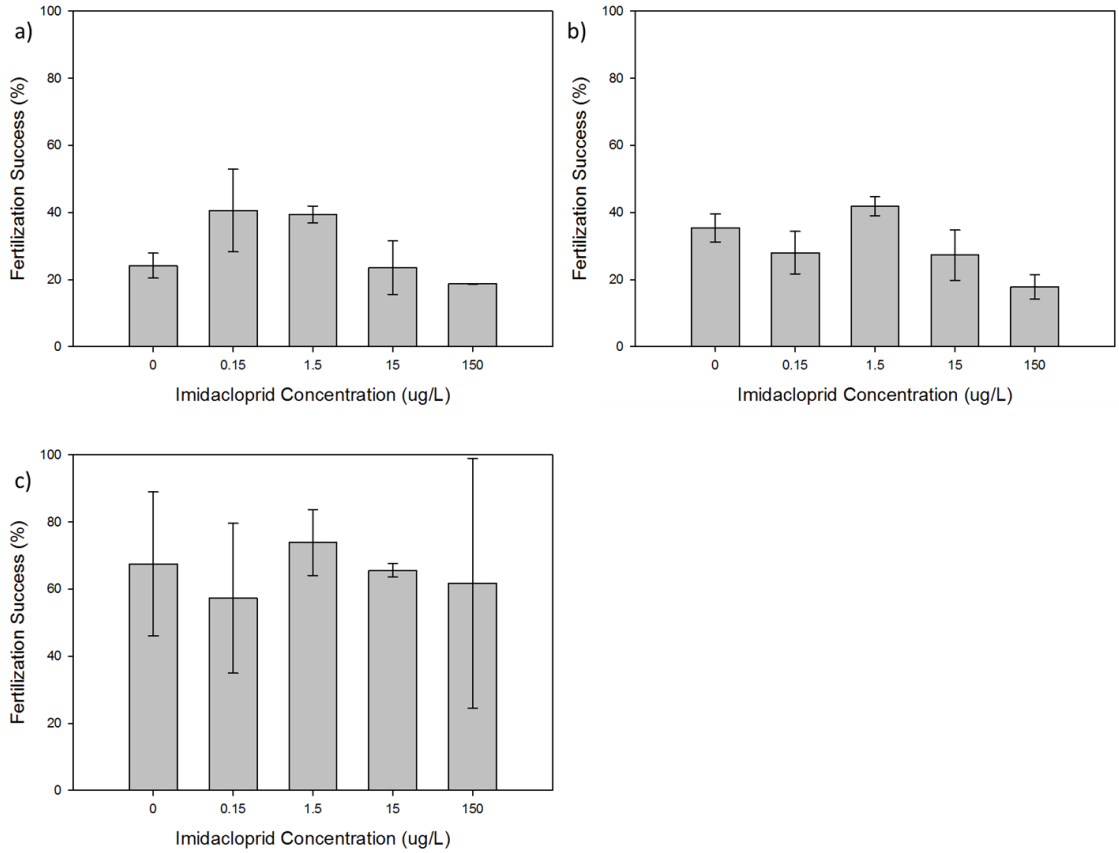
The static acute exposures and the oxygen consumption assays were also performed using the same water supply as the chronic exposure. A summary of the water quality data for the exposure is provided in Table 6. Since these exposures were performed in November, ambient temperatures were lower and the water supply temperature was below the 12 °C target. To counteract this, the room temperature was maintained at a target of 12 °C using overhead heating. The resulting average temperature in the exposure tanks was 12.0 °C with a range of 11.3-12.9 °C. Dissolved oxygen in the tanks was consistently high as a result of continuous aeration, averaging 98.8% with a range of 95.5-101.2%. The pH measurements in the exposure tanks were consistently neutral, with an average of 7.2 and a range of 7.1-7.3. Conductivity measurements averaged 30.8 µS/cm, with a range of 27.1-33.7 µS/cm. None of the exposure tanks contained detectable levels of ammonia.

Table 6. Summary of water quality parameters for the 96-h static renewal exposures of sockeye salmon juveniles to imidacloprid prior to oxygen consumption assays.

Parameter	Average	Minimum	Maximum	N
Temperature (ºC)	12.0	11.3	12.9	14
Dissolved Oxygen (%)	98.8	95.5	101.2	14
pH	7.2	7.1	7.3	14
Conductivity (µS/cm)	30.8	27.1	33.7	14

2.4.2. Hatch Success and Timing

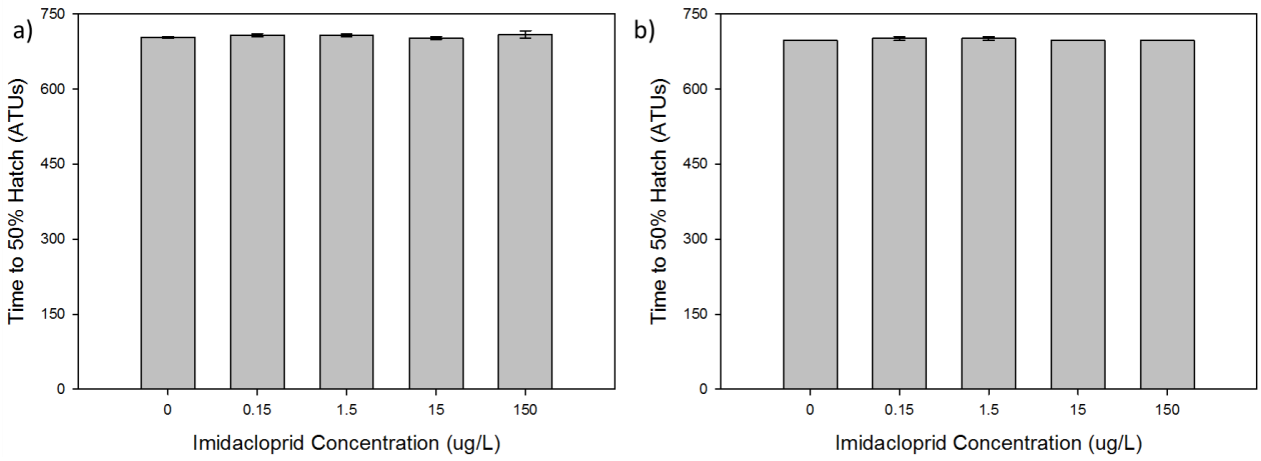
There was no observed effect of imidacloprid exposure on the percent of eggs successfully fertilized whether incubated in tanks (f=1.88; p=0.2777) or flumes (f=2.82; p=0.1693) in the chronic exposures, or in the heath trays (f=0.216; p=0.9168) in the acute fertilization exposures. However, differences in the percentage of eggs successfully fertilized between different genetic crosses of the sockeye were seen. In the chronic exposures, parental cross A was excluded from analysis and parental crosses C and D were excluded from the acute fertilization exposure analysis due to a fertilization success of <10% in at least one treatment. There was evidence of an estimated difference in the percentage of eggs successfully fertilized between offspring of parental crosses A and B in the acute fertilization exposure of 37.1% (95% C.I. 15.0-59.3%) (p=0.0055). There was also an observed difference in the percentage of eggs successfully fertilized between parental cross in the tanks in the chronic exposure (f=10.67; p=0.0033). Figure 4 provides a visualization of mean fertilization success by imidacloprid treatment.



Notes: Fertilization success was measured as a percentage of the total seeded eggs that reached the eyed embryo stage. Error bars represent the standard error of the mean. (n=2 exposure chambers per treatment; ~800 total eggs per treatment)

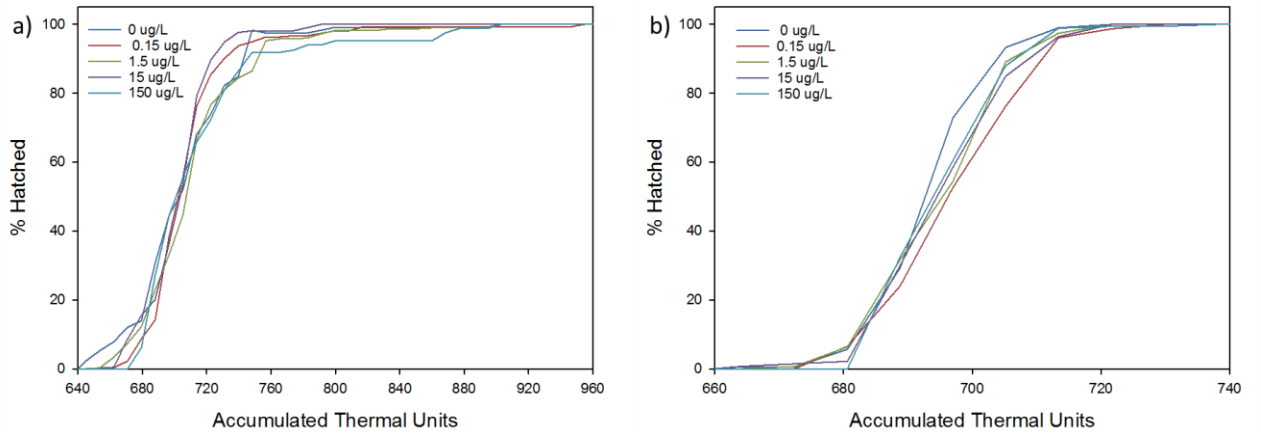
Figure 4. Mean fertilization success for sockeye salmon in tanks (a), flumes (b) and heath trays (c) by imidacloprid concentration.

There were no differences in the time to reach 50% hatch between any of the imidacloprid treatments in the tanks (f=1.8741;p=0.2834) in the chronic exposure, or in heath trays (f=0.667; p=0.6480) in the acute fertilization exposure. There were also no observed differences in mean ATUs to hatch between genetic crosses in either of the experiments. Figure 5 provides a visualization of the mean time taken to reach 50% hatch by imidacloprid treatment. Figure 6 provides a visualization of the cumulative percent hatched over time for the different imidacloprid treatments in the tanks and the heath trays.



Notes: Error bars represent the standard error of the mean. (n=2 exposure chambers per treatment; 86 – 227 hatched eggs per treatment)

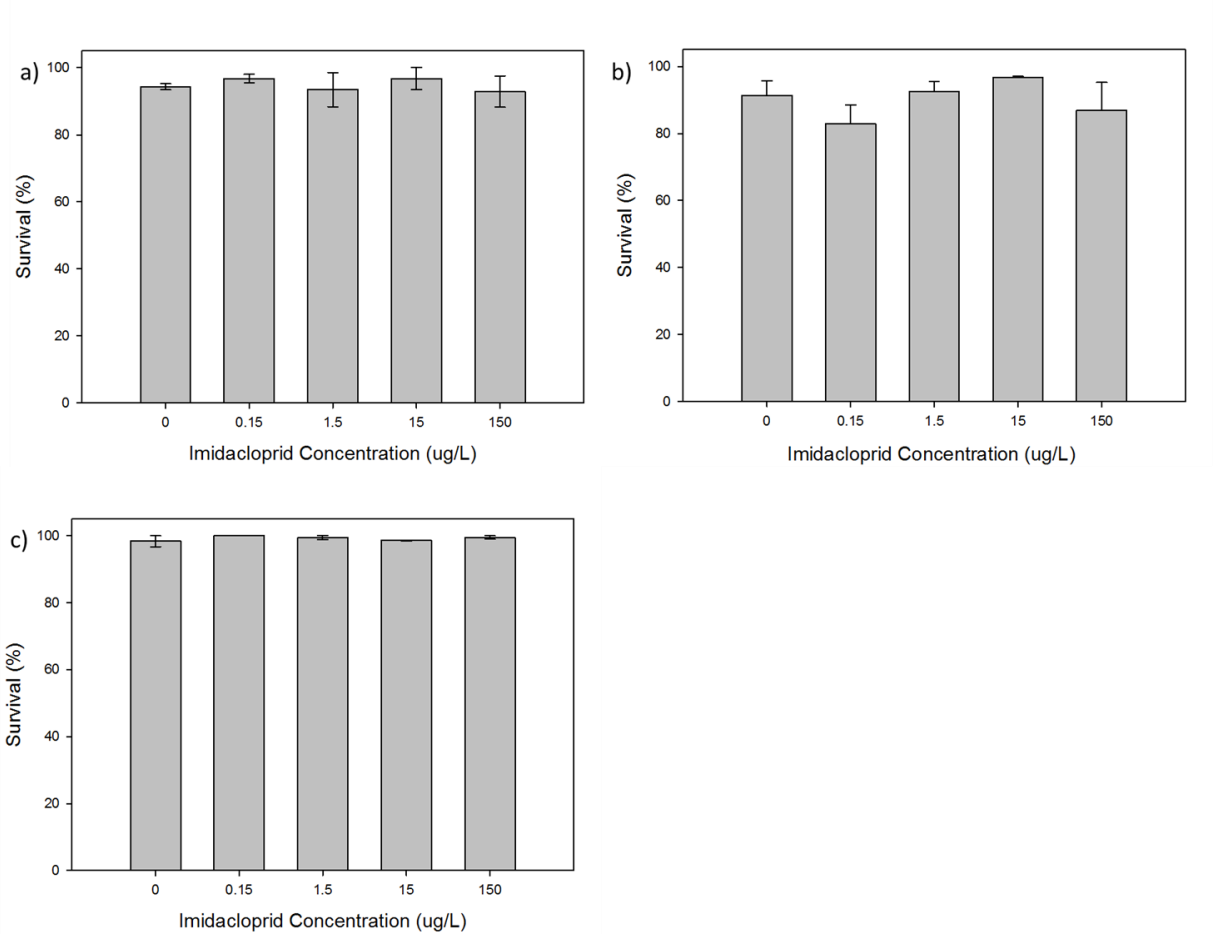
Figure 5. Mean time to 50% hatch in accumulated thermal units (ATUs) for successfully fertilized sockeye salmon eggs in tanks (a) and heath trays (b) by imidacloprid concentration.



Notes: Error bars represent the standard error of the mean. (n=2 exposure chambers per treatment; 86 – 227 hatched eggs per treatment)

Figure 6. Time series of cumulative percent hatch (%) versus accumulated thermal units (ATUs) for successfully fertilized sockeye salmon eggs in tanks (a) and heath trays (b) for the five different imidacloprid treatment concentrations.

No differences in the percent of fish surviving to the swim-up fry stage between any of the imidacloprid treatment groups was seen in the glass tanks (f=0.260; p=0.8901) or in the gravel-bed flumes (f=2.84; p=0.1678) in the chronic exposure, or in the heath stacks (f=1.03; p=0.4872) in the acute fertilization exposure. There was no effect of parental cross on the percent of fish that survived to the fry stage in either experiment. Mean survival by imidacloprid treatment is presented in Figure 7.

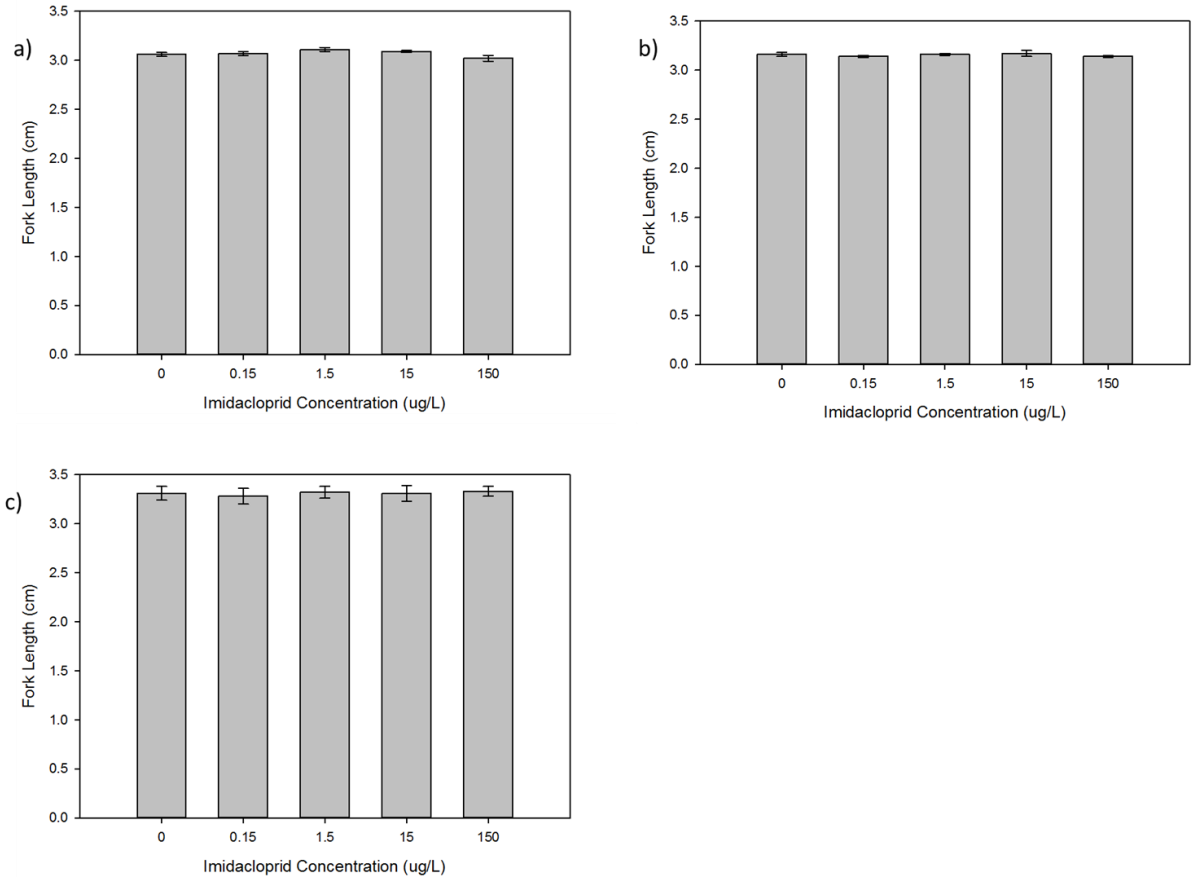


Survival was measured as the percentage of eyed embryos that successfully reached the swim-up fry stage. Error bars represent the standard error of the mean. (n=2 exposure chambers per treatment; 86 – 227 hatched eggs per treatment)

Figure 7. Mean survival (%) to the swim-up fry stage for successfully fertilized sockeye salmon eggs in tanks (a), flumes (b), and heath trays (c) by imidacloprid concentration.

2.4.3. Morphometrics

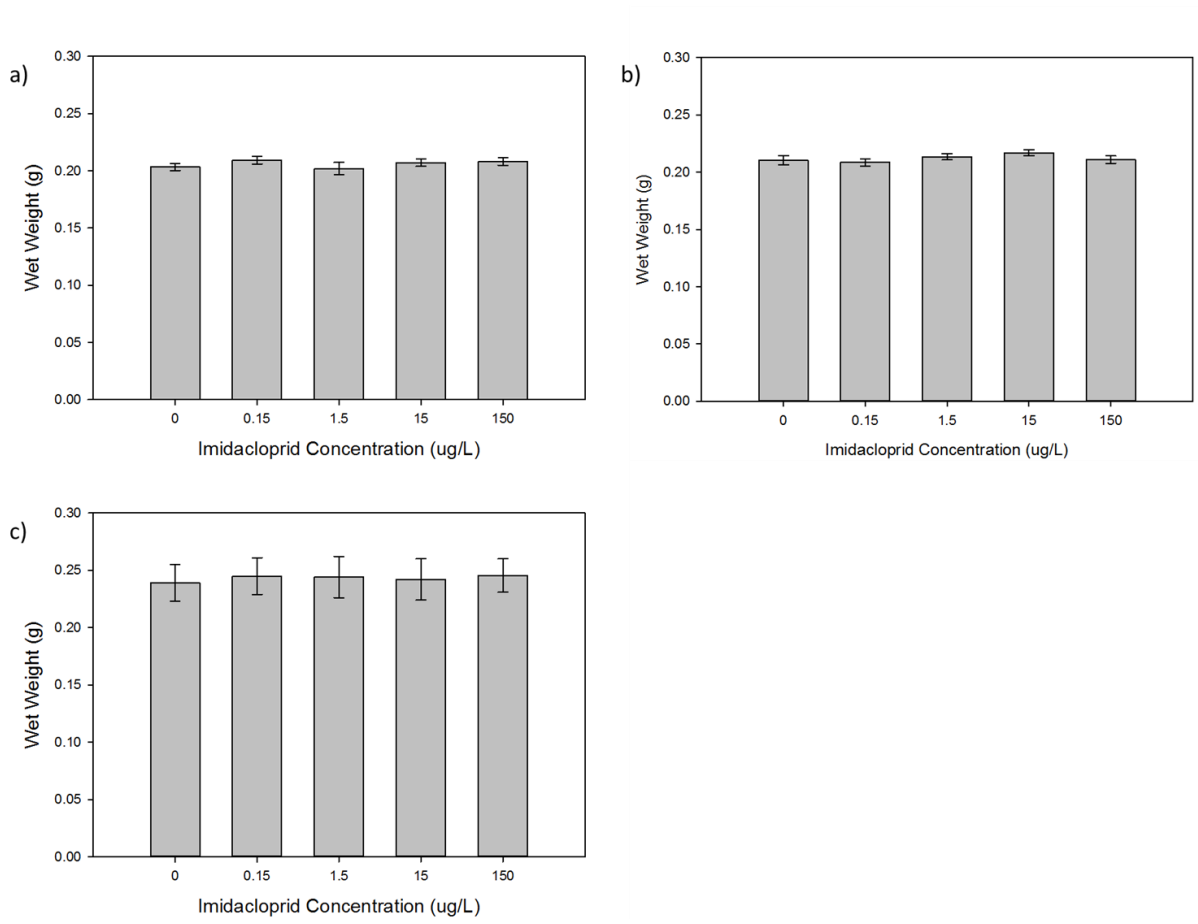
No differences were observed in mean swim-up fry length following yolk sac absorption between any of the imidacloprid treatment groups in the gravel-bed flumes (f=0.766; p=0.5647) in the chronic exposure, or the heath stacks in the acute exposure during fertilization (f=2.59; p=0.2899). However, in the glass tank exposures, a significant difference in mean fry length between the 1.5 µg/L imidacloprid and 150 µg/L imidacloprid of 0.91 (95% C.I. 0.16-1.66) mm (p=0.0262) and between the 15 µg/L imidacloprid fry and the 150 µg/L imidacloprid fry of 0.75 mm (95% C.I. 0.00-1.50 mm) was observed. The fry exposed chronically to the 150 µg/L imidacloprid treatment had the lowest mean fork length in the glass tanks at 30.16 mm (95% C.I. 29.87-30.46 mm). The fry exposed chronically to the high imidacloprid treatment in the flumes also had the lowest mean fork length at 31.41 mm (95% C.I. 31.07-31.75 mm). In addition, cross differences were observed in all 3 exposure conditions, with fry from cross C being the longest in the chronic exposure and fry from cross B being the longest in the acute fertilization exposure (Figure 8).



Notes: Error bars represent the standard error of the mean. (n=2 exposure chambers per treatment; 81 – 227 fish per treatment)

Figure 8. Mean fork lengths for sockeye salmon swim-up fry following reabsorption of yolk sacs in tanks (a), flumes (b), and heath trays (c) by imidacloprid concentration.

A significant difference in mean wet mass of fry in the flumes of the chronic exposure between the 15 µg/L treated fry and the 0.15 µg/L treated fry of 8.5 mg (95% C.I. 1.0-16.0 mg) (p=0.0224) was also observed. There was no difference in mean wet mass between any of the treatments in the glass tanks of the chronic exposure (f=1.44; p=0.3653) or the heath trays in the acute fertilization exposure (f=3.13; p=0.1473). Cross differences were observed in all 3 exposure conditions, with fry from cross C being the heaviest in the chronic exposures and fry from cross B being the heaviest in the acute fertilization exposure (Figure 9).

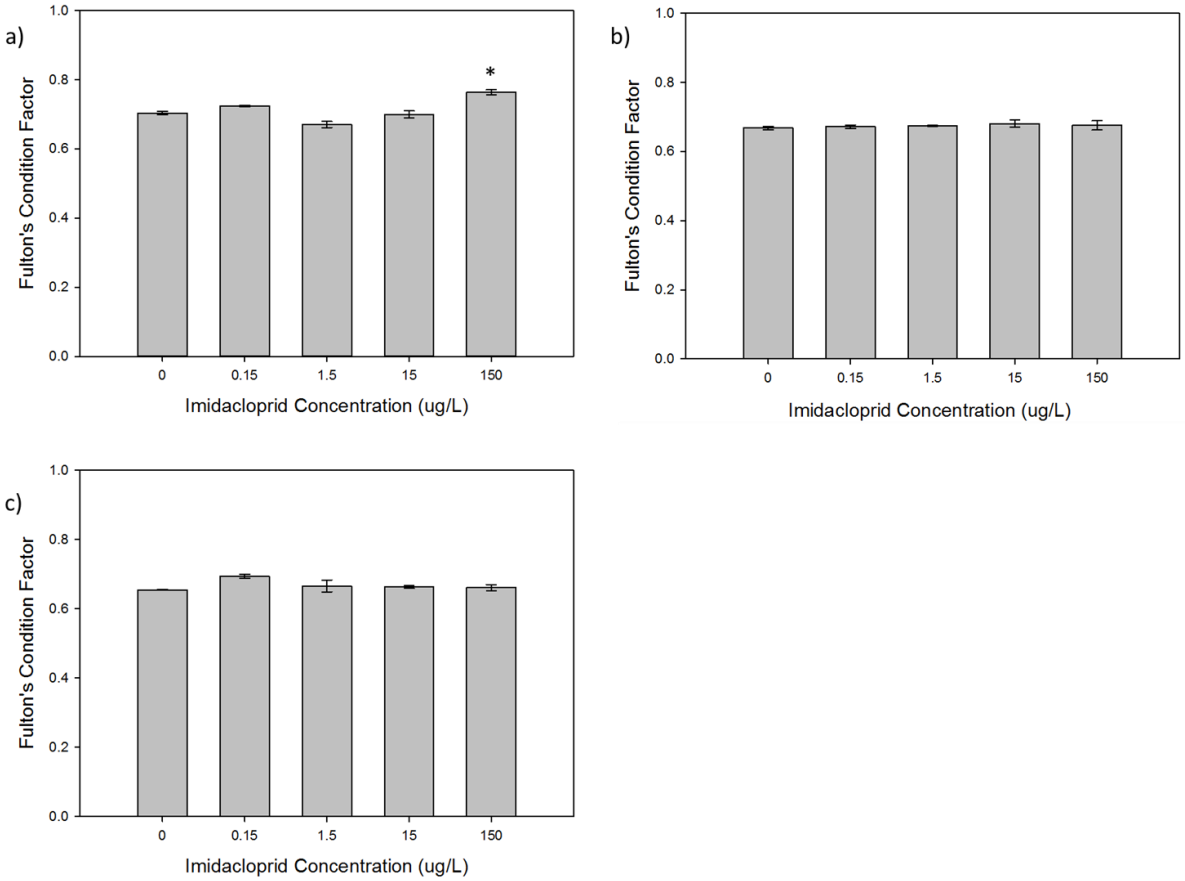


Notes: Error bars represent the standard error of the mean. (n=2 exposure chambers per treatment; 81 – 227 fish per treatment)

Figure 9. Mean wet weights for sockeye salmon swim-up fry following reabsorption of yolk sacs in tanks (a), flumes (b), and heath trays (c) by imidacloprid concentration.

Finally, there was evidence of a difference in mean Fulton's condition factor between several of the treatment groups in the chronic glass tank exposure. The largest estimated differences were observed between the 150 µg/L imidacloprid treated fry and the 1.5 µg/L imidacloprid treated fry. The estimated difference in Fulton's condition factor was 0.087 (95% C.I. 0.044-0.129; p=0.0040). There were also differences observed between the 150 µg/L treatment and the 15 µg/L treatment (95% C.I. 0.016-0.10; p=0.0173), the 150 µg/L treatment and the control treatment (95% C.I. 0.012-0.097; p=0.0213), and the 0.15 µg/L treatment and the 1.5 µg/L treatment (95% C.I. 0.011-0.096; p=0.0237). However, there was no evidence of a difference in the mean Fulton's

condition factor between any of the imidacloprid treatment groups in the gravel-bed flumes (f= 0.430; p=0.7846) in the chronic exposure or the heath trays (f=3.111; p=0.1487) in the acute fertilization exposure. There were differences in mean Fulton's condition factor observed between crosses in the tanks of the chronic exposure, but none observed between crosses in the flumes of the chronic exposure or the heath tray of the acute fertilization exposure. A visualization of mean Fulton's condition factor by imidacloprid treatment is provided in Figure 10.



Notes: Error bars represent the standard error of the mean. Asterisk (*) denotes evidence of a difference from the control treatment. (n=2 exposure chambers per treatment; 81 – 227 fish per treatment)

Figure 10. Mean Fulton's condition factor for sockeye salmon fry following reabsorption of yolk sacs in tanks (a), flumes (b), and heath trays (c) by imidacloprid concentration.

2.4.4. Deformity Analysis

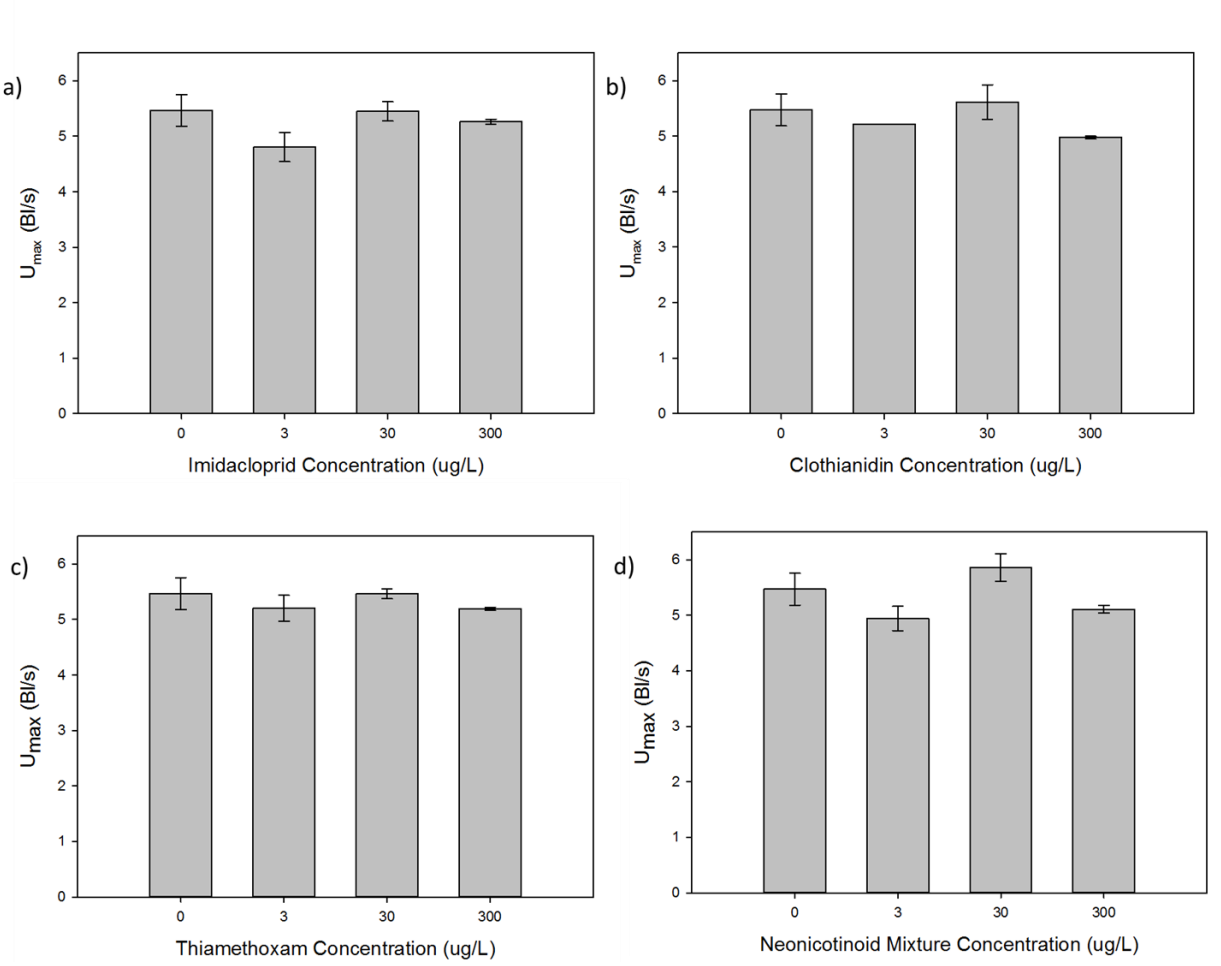
Deformities were recorded and rates based on treatment, cross, and severity were calculated. There was no evidence of a difference in mean incidence rates of deformities between any of the treatment groups. A summary of the mean deformity incidence rates for each treatment group is provided in Table 7 below. There were a total of 13 deformities observed in the chronic exposure between the tanks and flumes combined and the prevalence of the observed deformities was evenly distributed among the 5 imidacloprid treatment groups. The mean incidence rate for total observed deformities ranged from 0% to 2.6%, with no evidence of a difference in the mean incidence rate of deformities between imidacloprid treatment groups in the tanks (f=1.039; p=0.471) or the flumes (f=1.00; p=0.486) The average severity of the deformities was highest in the high imidacloprid treatment, with both observed deformities scoring a full 3 on the scale. There were also a total of 6 deformities observed in the heath trays from the acute fertilization exposure. While these deformities were not evenly distributed, there were as many deformities observed in the control group as there were in any other treatment group, and the highest average severity of the observed deformities was in the control treatment.

Table 7. Summary of developmental deformities observed in sockeye salmon during the heath stack acute fertilization, chronic tank, and chronic flume imidacloprid exposures.

Exposure Chamber	Nominal Imidacloprid Concentration (µg/L)	Average Severity	Total Deformities Observed	Mean Deformity Incidence Rate (%)
Tanks	0	1.67	3	1.30
	0.15	1.67	3	1.25
	1.5	2.00	2	0.91
	15	1.50	2	1.09
	150	3.00	2	2.56
Flumes	0	0	0	0
	0.15	2.33	3	0.5
	1.5	0	0	0
	15	0	0	0
	150	0	0	0
Heath Trays	0	2.50	2	1.39
	0.15	0	0	0
	1.50	2.00	2	1.32
	15	1.50	2	1.39
	150	0	0	0

2.4.5. Burst Swim Assay

Table 5 provides a summary of the water quality measurements in the exposure tanks for the swim performance exposures. There was no evidence of a difference in mean burst swimming performance (U_{max}) between any of the treatment groups (Figure 11).

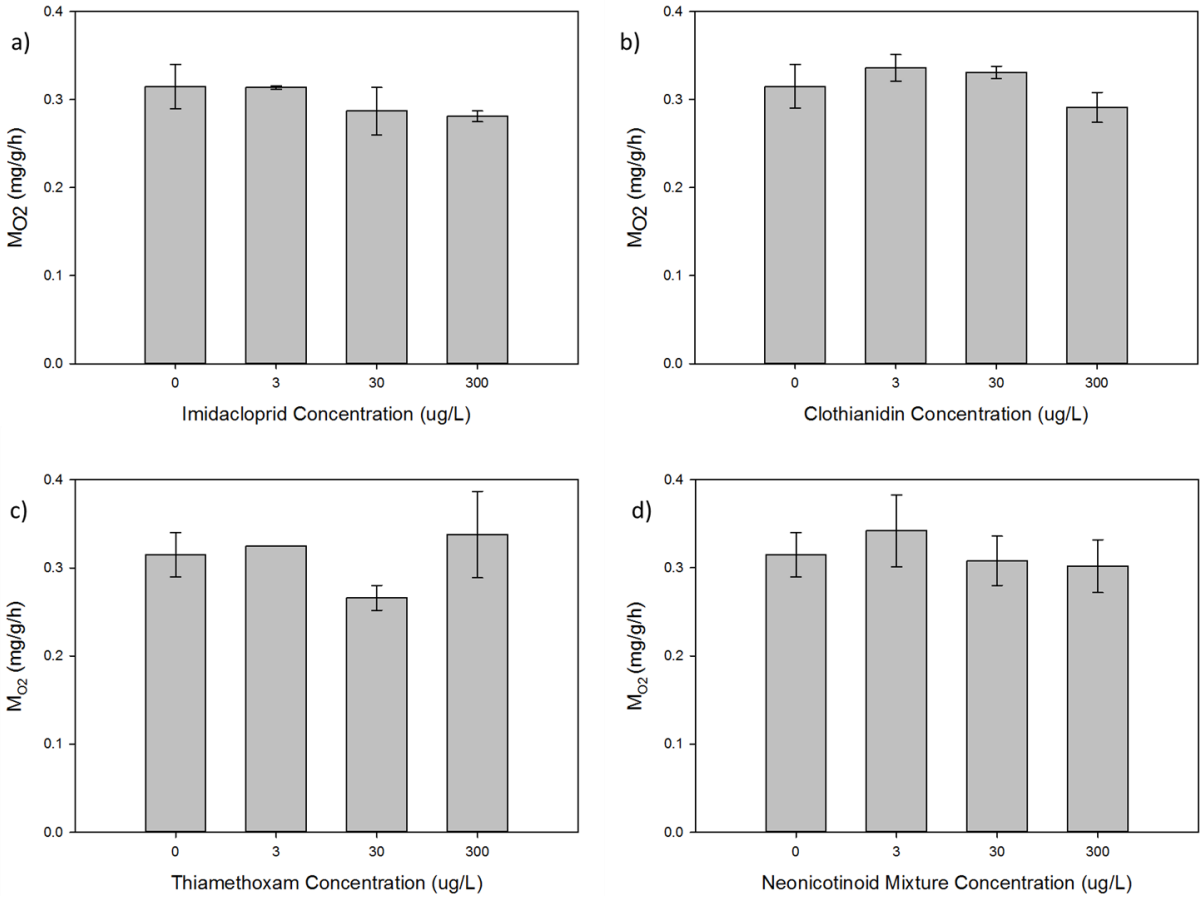


Notes: Error bars represent the standard error of the mean. (n=2 exposure tanks per treatment; 10 fish per treatment)

Figure 11. Mean burst swimming performance (U_{max}) for juvenile sockeye salmon exposed for 96 h to 0, 3, 30, or 300 µg/L of imidacloprid (a), clothianidin (b), thiamethoxam (c), or an equal parts by mass mixture of the three (d).

2.4.6. Oxygen Consumption Assay

A summary of the water quality data for the exposure is provided in Table 6. There was no evidence of a difference in mean routine metabolism (MO_2) between any of the treatment groups (Figure 12).



Notes: Error bars represent the standard error of the mean. (n=2 exposure tanks per treatment; 6 fish per treatment)

Figure 12 Routine metabolism (MO_2) for juvenile sockeye salmon exposed for 96 h to 0, 3, 30, or 300 µg/L of imidacloprid (a), clothianidin (b), thiamethoxam (c), or an equal parts by mass mixture of the three (d).

2.5. Discussion

2.5.1. Chronic Exposures and Acute Fertilization Exposure

The nominal imidacloprid concentrations used in the present study were selected based on recently reported imidacloprid concentrations in surface waters. The lowest concentrations selected (0.15 and 1.5 µg/L) fall within the probability distributions derived for average imidacloprid concentrations based on the data from 29 studies in 9 countries (Morrisey et al. 2015). In addition, the higher concentrations used (15 and 150 µg/L) fall below maximum measured concentrations reported for surface waters in close proximity to agricultural land in the Netherlands (up to 320 µg/L [Van Dijk et al. 2013]). Imidacloprid has been detected with increasing frequency in surface and groundwater in agricultural regions in the Canadian prairies and eastern Canada at concentrations up to 6.1 µg/L (Main et al. 2014; Government of Quebec 2014) In addition, imidacloprid has been detected with increasing frequency in salmon bearing streams in Washington State over the period of 2003 to 2011, at concentrations up to 0.962 µg/L (WSDA 2013). Therefore the concentrations chosen appear to be well within the range of environmentally relevant values for Canadian freshwater systems and salmon bearing streams in the Pacific Northwest.

While imidacloprid is typically applied in agricultural regions in the form of commercial formulations, the imidacloprid used in this study was the pure compound. Therefore, any synergistic, additive or antagonistic interactions of imidacloprid with chemicals in commercial formulations on sockeye salmon during typical agricultural applications was beyond the scope of this study and remain unknown at this time. In the present study, the analysis of water samples from both the glass tanks and gravel-bed flumes in the chronic exposures indicated that measured imidacloprid concentrations were very close to the nominal concentrations and to the predicted concentrations based on measured flow rates of the imidacloprid stock solution and dechlorinated water intake pumps entering the test vessels (Tables 2 and 3). These results indicate that imidacloprid behaved as expected in these waterborne exposures and was stable during stock solution storage and the experimental exposure conditions.

The objective of the acute fertilization exposures and chronic embryo/alevin exposures was to examine the impacts of varying timing, duration, and concentration of

imidacloprid exposure to sockeye salmon during critical developmental stages. The majority of fish studied are particularly susceptible to poor environmental conditions at early life stages (Mckim 1977; Hutchinson et al.1998). In addition to environmental factors such as temperature, pH, dissolved oxygen, and ammonia concentrations, another common source of altered development in salmonid and other fish species is exposure to xenobiotics (Finn 2007). Exposure to various xenobiotics in the early stages of salmonid development have been observed, including teratogenesis (Leatherland 1994; Lower and Moor 2003; Honkanen et al. 2005; Viant et al. 2006), altered sex ratios (Matta et al. 1998; Carlson et al. 2000; Hutchinson et al. 2003), depleted energy reserves (Maenpaa et al. 2004; Viant et al. 2006), delayed development and altered hatching times (Lower and Moore 2003; Honkanen et al. 2005), reduced hatch success (Ankley et al. 1991; Mac and Schwartz 1992; Matta et al. 1997), and yolk sac edema and early mortality (Spitsbergen et al. 1991; Giesy et al. 2002). This information indicates that xenobiotic exposure may be as significant as the other aforementioned environmental factors in determining whether the successful development of salmonids occurs. Established protocols for toxicity to early life stages of fish commonly focus on the potential for adverse outcomes including direct mortality, altered hatch timing, impaired growth, the induction of developmental deformities, and decreased fertilization success (OECD 1992; Environment Canada 1998; ASTM 2013). In the present study, these endpoints were examined in sockeye following exposures to imidacloprid.

One of the most commonly identified mechanisms of developmental disruption in juvenile and adult salmonids involves the acetylcholine axis in the nervous system (Wheelock et al. 2005). Since the normal mode of action of neonicotinoid compounds in their target species is to act as an acetylcholine agonist on nicotinic acetylcholine receptors, there is reason to suspect that neonicotinoid exposure during early life stages of sockeye salmon may result in developmental disruptions.

There was no evidence of a difference in fertilization success between the different imidacloprid concentrations in the heath trays. However, fertilization success in control treated fish was lower in the tanks and flumes than it was in the heath stack, indicating that heath stacks may be a more suitable arrangement for incubation of sockeye salmon embryos. In addition, several of the genetic crosses experienced very little fertilization success and even the more successful of the crosses had fertilization successes as low as 20% in some treatments. Since the fertilization methodology that

was used is one that has previously been applied successfully (Burt et al. 2012; Du Gas 2014), and the parental gametes came from wild caught spawning sockeye rather than a hatchery source, these low fertilization rates may be symptomatic of existing problems in the wild sockeye population. However, laboratory fertilization success rates using gametes acquired from wild caught sockeye have previously been shown to be highly variable, with success rates in one experiment ranging from 8.3 to 97.5%, albeit at a lower concentration of sperm of 5 µL per 500mL of water (Hoysak and Liley 2000). Variation in success rates was found to be related to highly variable quality of eggs (Hoysak and Liley 2000). Fertilization success in the current experiment was measured as the percentage of eyed embryos counted out of the total eggs initially used during the fertilization procedure. It is possible that fertilization success was underestimated due to mortality of embryos prior to reaching the eyed stage. However, there is no reason to suspect that this would systematically bias the results and therefore the process still allows for a comparison of fertilization success between treatments.

There was no evidence of a difference in survival between any of the different imidacloprid treatments in the exposures during the fertilization process only nor in the embryo/alevin continuous chronic exposures in the present study. In general, once embryos had developed to the eyed embryo stage, the probability was high that they would successfully hatch and survive to the swim up fry stage and mean survival greater than 80% in all exposure groups was observed. While there have been no published studies done that examine survival of any fish species chronically exposed to imidacloprid during early developmental stages, the US EPA problem formulation for the registration of imidacloprid includes reference of an unpublished study examining the effects of a 60-d post hatch flow through exposure to rainbow trout. In this study, no effects on survival were observed, however a LOEC and NOEC of 2.5 mg/L and 1.2 mg/L respectively were reported for effects on growth (Cohle and Backsath 1991). Similarly, an unpublished 60-d post hatch flow through exposure of rainbow trout to thiamethoxam reported a NOEC for growth and reproduction at the highest concentration used, which was 20 mg/L (Drotter et al. 1997), and an unpublished 28-d flow through exposure of fathead minnow following fertilization to clothianidin reported a LOEC and NOEC of 20 mg/L and 9.7 mg/L, respectively for effects to length and dry weight, with no effects on survival observed (US EPA MRID 454224-13 as cited in US EPA 2011). A 7-d flow through exposure of zebrafish to imidacloprid starting at

fertilization and terminating at 168 h post fertilization found no effects on mortality at concentrations up to 50 mg/L (Scheil and Kohler 2009).

However, a recent study indicated that sockeye salmon hatch success was reduced after exposure to environmentally relevant concentrations of chlorothalonil (Du Gas 2014). Reduced hatch success has also been observed in several salmonid species following exposure to PCBs including Chinook salmon (Ankley et al. 1991) and lake trout (Mac and Schwartz 1992). Exposure to selenium has also resulted in reduced hatch success in Dolly Varden trout (Golder 2009). These results indicate that it's likely that fertilized sockeye salmon eggs are not particularly sensitive to direct mortality as a result of imidacloprid exposure.

There was no evidence of an effect of imidacloprid concentration on hatch timing, which was defined as the number of exposure days taken to reach 50% hatch in a given treatment. Patterns in duration and timing of the hatching period were similar across treatments and experiments. The timing of first hatch in all treatment groups in both the acute fertilization exposure and the chronic exposure occurred at around 680 ATUs, and all treatment groups in both experiments reached 80% hatch by approximately 720 ATUs. Hatch timing is a trait that is often affected by exposure to xenobiotics; for example, premature hatch has been observed in Atlantic herring following exposure to a bitumen emulsion fuel (Williams et al. 2003) and Pacific herring exposed to petroleum hydrocarbons (McGurk et al. 1993). In addition, pesticide exposure has previously been associated with delayed hatching in Japanese medaka exposed to endosulfan (Gormley and Teather 2003) and zebrafish exposed to a carbaryl insecticide (Todd and Van Leeuwen 2002). A recent study by Du Gas (2014) demonstrated that normally timed development of sockeye salmon embryos can be altered by pesticide exposure. Specifically, sockeye salmon exposed to environmentally relevant concentrations of the herbicide atrazine experienced premature hatching, whereas sockeye exposed to environmentally relevant concentrations of chlorothalonil experienced delayed hatching times (Du Gas 2014). The results of the current experiment are consistent with the available unpublished studies on chronic flow through exposures of neonicotinoids which have not reported any effects to hatch timing (Cohle and Backsath 1991; Drotter et al.1997; Rufli et al.1997b; US EPA MRID 454224-13).

Growth parameters such as mass, length, and condition factor, are one of the most commonly used indicators of fish health, as growth rates are symptomatic of a variety of underlying issues (Smolders et al. 2002). One hypothesis for impeded growth in fish embryos and larvae exposed to contaminants is that embryos/larvae are reliant on a finite energy reserve in the form of the yolk, any additional energy spent detoxifying contaminants would reduce the energy available for anabolism and growth (Wiegand et al. 2001). At the swim up fry stage, fish in the 150 µg/L imidacloprid concentration exposure tanks had the lowest fork length measurements, and there was evidence of a difference in mean fork lengths between the 150 µg/L exposure sockeye and the 1.5 µg/L and 15 µg/L concentration exposed sockeye. While the relationship was not found to be statistically significant at an alpha level of 0.05 in the flumes, the fry exposed to the 150 µg/L treatment of imidacloprid exhibited the shortest fork lengths in the flumes as well. However, there was no evidence of a clear concentration-response relationship between imidacloprid concentrations and mean fork length at the swim up fry stage. In the heath stacks the fry exposed to the 150 µg/L concentration of imidacloprid at fertilization had the longest fork lengths, indicating that the relationship was not consistent and may depend on timing and duration of exposure, or that observed effects were due to chance. There were cross differences observed in the tanks, flumes, and heath stacks in the long term development experiments, and these cross differences tended to outweigh the treatment differences in terms of magnitude, indicating a greater contribution of genetics compared to neonicotinoid exposure to growth during early life stages.

Sockeye chronically exposed to 15 µg/L concentrations of imidacloprid in the flumes had the greatest mean wet weights, with evidence of a statistically significant difference when compared to the fry in the 0.15 µg/L exposure treatment. However, there was no evidence of a consistent relationship with the tank exposures or the heath trays, which showed no differences between mean wet weights of any treatment groups, and no evidence of a dose-response relationship. Once again, cross differences were observed in all 3 exposure conditions, indicating that the genetic lineage of the sockeye fry was more important than exposure to the chosen concentrations of imidacloprid and that it is unlikely that imidacloprid exposure influenced the wet weights of the fry.

Condition factors are a way of integrating both body length and weight measurements into a ratio that provides an indication of a fish's overall body shape and

is a better representation than length or weight on their own. Condition factors are commonly used as an indicator of health when doing stock assessment for recreational fisheries (State of Victoria 2003). A lower condition factor is thought to be representative of a decreased availability of energy reserves (Smolders et al. 2002). At the swim up fry stage, the sockeye exposed to the 150 µg/L treatments of imidacloprid had the highest condition factors in the tanks. There was evidence for a statistically significant different at an alpha level of 0.05 between the fry in the 150 µg/L exposure and the fry in the 1.5 µg/L, 15 µg/L, and control exposed fry. In addition there was evidence of a difference between the 0.15 µg/L exposed fry and the 1.5 µg/L exposed fry, with the 0.15 µg/L exposed fry exhibiting higher mean condition factors in the tanks. There was no evidence of a consistent concentration-response relationship in the tanks, and no evidence of any differences between treatments in the flume or acute fertilization exposure scenarios. There were cross related differences observed in the fry in the exposure tanks but not in the flumes or the heath trays. The cross differences that were present in length and weight were reduced when looking at the condition factor due to the fact that certain genetic crosses tended to be both longer and heavier, but exhibited similar body shapes to the other crosses.

An unpublished 60-d flow through exposure of rainbow trout to imidacloprid resulted in a NOEC of 1.2 mg/L and a LOEC of 2.5 mg/L based on reductions to growth (Cohle and Bucksath 1991). Similarly an unpublished 28-d flow through exposure to clothianidin involving early life stage fathead minnows resulted in a NOEC of 9.7 mg/L and a LOEC of 20 mg/L based on reduced growth (measured as length and dry weight; MRID 454224-13 as cited in US EPA 2011). Two unpublished studies involving chronic flow through exposures to thiamethoxam in rainbow trout showed no reductions in growth compared to control groups at concentrations up to 20 mg/L over 88 d (Drottar et al.1997) and 100 mg/L over 28 d (Rufli 1997b). These results indicate that there is potential for neonicotinoid exposure to impact growth in freshwater fish and that there is variation in sensitivity to the different neonicotinoid compounds. However, the concentrations used in the aforementioned studies as well as derived NOEC values were all higher than expected environmental concentrations for chronic exposure scenarios, as well as the concentrations used in this study. Decreased condition factors as well as alteration of weight following exposure to the pesticides atrazine and chlorothalonil has previously been demonstrated in sockeye salmon fry following

exposure throughout the early developmental stages at concentrations similar to those used in this experiment (Du Gas 2014). Alterations in salmonid swim up fry size can impact swimming ability (Bams 1967), downstream migration rate (Connor et al. 2002), and future growth (Connor et al. 2003). In the present study, there was no evidence of a consistent relationship between imidacloprid concentrations and mean swim-up fry condition factors. However, the evidence in favour of the ability of neonicotinoid exposure to alter growth at higher concentrations than those used in this experiment suggests that it is possible that sockeye salmon would be susceptible to reduced growth at higher concentrations as well.

Ours is the first deformity assessment performed following exposure to imidacloprid in aquatic organisms. However, a recent study did investigate deformities in sockeye salmon following exposure to atrazine or chlorothalonil, finding that the presence of finfold deformities increased following exposure to chlorothalonil (Du Gas 2014). In the present study, very few deformities were observed and there were no differences in prevalence of deformities between any of the imidacloprid treatment groups within the tanks, flumes, or heath trays. The most commonly observed deformities in the present study were edema of the yolk sac followed by craniofacial and then skeletal deformities, and the majority of deformities observed were of minor (score of 1; Table 6) to medium (score of 2; Table 6) severity. Nonetheless, the observed mean incidence rates of deformities was low overall and ranged from 0% to 2.6% in all treatment groups. Similar incidence rates of deformities in sockeye salmon for all deformity types except for skeletal (which were observed in approximately 25% of fish across all treatments) were observed in control fish following the same protocol (Du Gas 2014). In another study, incidence rates of total deformities for cutthroat trout (*Oncorhynchus clarkii*) raised from gametes collected in reference streams ranged from 0% to 2.4% (Kennedy et al. 2000). Similarly, incidence rates of total deformities in Dolly Varden trout (*Salvelinus malma*) raised from gametes collected in reference streams ranged from 0% to 9% (Golder 2009). In the present study, it is possible that the prevalence of deformities was underestimated in the gravel-bed flumes due to deformed fish being unable to swim up out of the gravel beds and not being recovered, as mentioned in a previous study using a similar apparatus (Pilgrim et al. 2013). Overall, the observed mean incidence rates of deformities in all imidacloprid treatment groups in

the present study were within the range of those observed in salmonids raised from gametes from wild caught fish.

A review of the available literature on pesticide induced teratogenicity and embryotoxicity in aquatic organisms indicated that many pesticides, including those that have a similar mechanism of action to neonicotinoids like carbamates and organophosphates, have been documented to induce embryotoxicity and teratogenicity in non-target aquatic organisms including fish (Paskova et al. 2011). There is evidence to suggest that pesticide induced teratogenicity and embryotoxicity may be linked to oxidative stress endpoints such as lipid peroxidation, oxidative DNA damage, or modulation of antioxidant mechanisms (Paskova et al. 2011). Signs of oxidative stress and DNA damage have been observed in freshwater fish such as loach (*Misgurnus anguillicaudatus*) and zebrafish (*Danio rerio*) exposed to the neonicotinoids imidacloprid and thiamethoxam (Xia et al. 2016; Ge et al. 2015; Yan et al. 2016). These effects have typically been observed in exposure scenarios involving concentrations much higher than those measured in global surface waters for short periods of time. There is a lack of information regarding deformity analyses for fish chronically exposed to environmentally realistic concentrations of neonicotinoids. However, the results from the current experiment indicate that exposure to environmentally realistic concentrations of imidacloprid to sockeye salmon during early life stages does not result in an increase in developmental deformities.

It was initially intended that the gravel bed flume system would be used to measure emergence of sockeye swim-up fry as had been done in previous experiments with similar systems (Du Gas 2014; Pilgrim et al. 2013). The flume system was designed to facilitate the measurement of timing and success of emergence of salmonid fry in order to provide information on an important early life event in developing salmonids. However, in the current experiment there were several difficulties that prevented accurate and reliable measurements of sockeye fry swim-up. Sockeye swim-up fry were free to swim throughout the gravel bed and therefore swim-up fry that had been previously counted as emerging may have returned to the gravel bed before the next count. In addition, it was discovered that sockeye fry could move between subsections of the flumes within a given treatment section, further contributing to unreliability of counts. As a result, the timing and success of emergence were not included as endpoints for analysis in the current study.

One limitation of the chronic and acute fertilization exposure scenarios was that due to the fact that exposure tanks, flume sections, and heath trays each received a single source of water and pesticide stock solution intakes the number of replicate test vessels was low (i.e. 2 replicates, each with ~90-100 fish), and the acute fertilization exposure scenario lacked true replicates during the rearing in clean water. While there were no observed effects at any concentrations tested with the exception of increased condition factor in the highest concentration in one of the chronic exposure systems, with a higher number of replicates the power to detect these trends would have increased. In addition, the low fertilization success using the wild sourced gametes resulted in the unviability of two crosses, decreasing the number of crosses and overall fish that remained for the duration of the experiment. The concentrations used in this study were chosen to represent environmentally relevant exposure scenarios, and higher concentrations appear to be required to establish NOECs and LOECs on growth and development in sockeye salmon embryos/alevins exposure to imidacloprid. Higher concentrations were not tested in this study in part due to cost constraints. Another potential limitation of this study is that tissue concentrations of imidacloprid and its degradation products were not measured and therefore there was no confirmation that the compound was being taken up from the water by the fish. Tissue concentrations were not measured for several reasons. One such reason was that there is a lack of analytical laboratories capable of measuring imidacloprid and its degradation products in fish tissue. This is likely due to the fact that imidacloprid (and other neonicotinoids) have low log K_{ow} values, and correspondingly low estimated bioconcentration factors (BCFs) for fish tissue (Macbean 2008). A high BCF is indicative of a compound's tendency to partition into tissue preferentially from other environmental media (Arnot and Gobas 2006), whereas a low BCF indicates the opposite and it's likely that imidacloprid (and other neonicotinoids) exhibit limited partitioning into tissue from water, which would make tissue concentrations difficult to analyze. It was assumed that verification of water concentrations of imidacloprid was sufficient to represent the potential for exposure.

Due to the decreased selectivity of imidacloprid and other neonicotinoids for the nicotinic acetylcholine receptors in vertebrates compared to analogous receptors in invertebrates, and the corresponding lower toxicity, the more important concern related to imidacloprid contamination of salmonid bearing freshwater systems may be indirect

toxicity, which can occur a result of a loss in quantity or quality of prey as a result of pesticide use (Southerton and Holland 2002). A field based microcosm experiment involving pulses of imidacloprid at concentrations ranging from 0.6 to 40 µg/L to a lentic benthos assemblage in weekly intervals resulted in altered community structures (Colombo et al. 2013). Similarly, a mesocosm study involving exposure to imidacloprid found that at concentrations of 20 µg/L, there were observed decreases in overall phytoplankton density, as well as densities of mayflies, caddisflies, and the amphipod species *Hyalella azteca* (Moring et al. 1992). A semi natural mesocosm experiment using clothianidin using concentrations of 352 µg/L resulted in high invertebrate predator mortality and a resulting increasing prey survival, further indicating the potential for neonicotinoid exposure to alter aquatic invertebrate community structures (Miles et al. 2017). A mesocosm study focusing on Japanese medaka fish in a simulated rice paddy ecosystem exposed to imidacloprid at concentrations of 40 to 50 µg/L found that imidacloprid exposures resulted in decreased growth in the medaka due to lower availability of food sources (Hayasaka et al. 2012). Similar studies have been performed finding decreased food availability for insectivorous birds in regions of imidacloprid use (Hallmann et al. 2014). These altered aquatic invertebrate community structures and food availability have been observed at concentrations significantly lower than concentrations that have been associated with direct whole body toxicity. Studies with imidacloprid involved concentrations from 0.6 to 50 µg/L, which are within the range of surface water concentrations reported globally as summarized by Morrissey et al. (2015). Since sockeye salmon depend primarily on insects as a food source in critical early developmental stages, imidacloprid toxicity to insects could have a greater impact on their survival than direct adverse effects at ecologically relevant concentrations (Baldwin et al. 2009).

2.5.2. Acute Exposures for Swim Performance and Oxygen Consumption

For the 96-h exposures, stock solutions of imidacloprid, clothianidin, and thiamethoxam were prepared in a similar fashion to the imidacloprid solutions used in the chronic exposures. While nominal concentrations in the exposure tanks were not verified via chemical analysis, they were expected to be accurate based on the analysis in the chronic exposures as well as measures taken to limit photodegradation such as a 12 h light cycle and frequent preparation of stock solutions as well as storage of stock

solutions in a cool, dark environment. The target concentrations for the acute exposures were derived based on similar data to the chronic exposures. The 0.15 µg/L treatment group in the chronic study was removed and the remaining concentrations were doubled so that the highest concentration in the exposure (300 µg/L) would be comparable to the maximum concentration observed in agricultural surface waters of 320 µg/L (Van Dijk et al. 2013). The acute exposures were intended to represent scenarios such as runoff following agricultural application or a heavy rain event, in which concentrations of water soluble contaminants reach a peak in nearby freshwater systems (Tierney et al. 2011).

The targets for water quality parameters were similar to those set for the chronic exposures. However, rather than allowing temperature to fluctuate with ambient temperatures of the municipal water supply, water temperatures were maintained over a limited range since temperature can have a significant impact on both swim performance and metabolic rates in salmonids and other fish (Lee et al. 2003). In order to reduce variation in swim performance and routine metabolic rates due to temperature fluctuations, the temperatures were regulated at approximately 12°C, to reflect conditions that are common in natural sockeye bearing streams and to keep them consistent with the holding tanks that the sockeye fry were raised in prior to the exposure.

There was no evidence of a difference in mean burst swimming performance between any of the acute exposure treatment groups and control fish, and the salmon fry were not kept separate by genetic lineage so cross effects were not analyzed. Previously, imidacloprid exposure in juvenile zebrafish was found to decrease swimming activity and novel tank exploration, and alter sensorimotor responses to startling stimuli (Crosby et al. 2015). These results indicate that imidacloprid can have sublethal effects on the neuromuscular system in aquatic vertebrates. However, the concentrations used were 12-15 mg/L, which are several orders of magnitude higher than the highest concentrations (300 µg/L) used in the current study, and those measured in the environment. In addition, no adverse impact on swimming behaviour was observed in rainbow trout exposed to 20 mg/L of thiamethoxam for 88 d (Drottar et al. 1997) or 100 mg/L of thiamethoxam for 28 d (Rufli 1997b). The LC_{50} for imidacloprid in rainbow trout has been experimentally measured as 1.2 mg/L, indicating a high degree of variability in species sensitivity (US EPA 1992).

There was no evidence of a difference in mean routine metabolism between any of the treatment groups. Routine metabolism can indicate efficiency of energy usage. Allocating energy reserves to metabolize contaminants may alter energetic demands (Wiegand et al. 2001), or vasoconstriction in the gills via acetylcholine activity may decrease the oxygen available for energy production in the tissues (Minakshi and Mahajan 2013). Alternatively, exposure to pollutants can result in a stress syndrome characterized by increased anaerobic metabolism, resulting in depleted cellular energy resources (Sancho et al. 1997) and protein catabolism to compensate for increased energy demands (Pfeifer and Weber 1979). It has been hypothesized that gill damage reducing the amount of oxygen taken up at the gills may contribute to this stress response (Evans 1987). Metabolic alterations such as increased heat dissipation in Atlantic salmon (*Salmo salar*) exposed to pentachlorophenol (Maenpaa et al. 2004) and depleted cellular ATP in Chinook salmon exposed to the pesticides dinoseb, diazinon, and esfenvalerate (Viant et al. 2006) have been observed.

Exposure to thiamethoxam has resulted in histopathological alterations in the gills of the common carp, but no associated investigation of potential impacts to metabolic rate or respiration was performed (Georgieva et al. 2014). The effects of imidacloprid on oxygen consumption and metabolic rates in aquatic vertebrates have not previously been investigated. However, no adverse impact on respiratory movement (frequency of gill opercula opening and closing) was observed in rainbow trout exposed to 20 mg/L of thiamethoxam for 88 d (Drottar et al.1997) or 100 mg/L of thiamethoxam for 28 d (Rufli 1997b). It has been suggested that measuring oxygen consumption in a space constrained respirometer as well as the process of capturing fish for the assessment may lead fishes to be stressed and could impact derived metabolic impacts based on measurements of oxygen consumption (Clark et al. 2013). However, the respirometry procedure followed was the same for both exposed and control fish to limit the likelihood that any differences in estimated routine metabolism would be due to difference in handling procedures rather than exposure concentrations. In addition, mean measurements of routine metabolism in the current experiment ranged from 266 to 342 mg/kg/h, which fell between values of mean standard metabolism of 60 to 71 mg/kg/h and mean active metabolism of 627 to 895 mg/kg/h for yearling sockeye salmon acclimated at similar temperatures (Brett 1964). One limitation of the current study is that sockeye fry were exposed in groups in exposure tanks, and since each group of fish in a

single tank was subject to the same water, the number of true replicates for each treatment was limited to two, reducing the statistical power of the analysis to detect smaller differences in burst swimming performance and routine metabolism.

Although neonicotinoid use has been banned in cities such as Vancouver and Montreal, and the compounds used in this study are all under review in Canada, the current US EPA aquatic benchmarks for acute exposure in fish for imidacloprid, clothianidin, and thiamethoxam are 41.5, 50.8, and 50 mg/L, respectively, due to the high LC_{50}s observed in a variety of fish species (US EPA 2017). However, the lowest aquatic benchmarks for neonicotinoids set by the US EPA are set for chronic exposure in invertebrates. These values for imidacloprid, clothianidin, and thiamethoxam are 1.1, 1.1, and 17.5 µg/L, respectively (US EPA 2017). In addition, the current water quality guideline for the protection of all freshwater aquatic life for imidacloprid in Canada is 0.23 µg/L (CCME 2007). Currently there are no corresponding values for clothianidin and thiamethoxam in Canada. Based on the endpoints examined in this study, the guidelines set in the United States and Canada appear to be protective of exposures to single chemical exposures of neonicotinoids in sockeye salmon during sensitive life stages. However, due to the action of neonicotinoids on the commonly exploited acetylcholine-acetylcholinesterase axis, more research into the impacts of exposure to more realistic pesticide mixtures including pesticides with similar modes of action such as organophosphates and carbamates should be performed.

2.6. Conclusion

The present study aimed to characterize the effects of the recently introduced and rapidly expanding neonicotinoid class of insecticides on sensitive life stages of the declining species *Oncorhynchus nerka*. Several life stages and endpoints were investigated including development during embryonic and larval stages as well as physiological performance during the fry stage. Exposures were carried out in a realistic manner both by using concentrations of neonicotinoids comparable to those previously measured in the environment and by simulating a stream bed environment with the flume system. These experiments provide evidence that direct lethal and sub-lethal effects on growth and development in early life stages of wild sockeye salmon are unlikely based on the majority of studies reporting environmental concentrations to date under 150 µg/L and that current water quality guidelines for neonicotinoids are protective

of sockeye salmon. Furthermore, based on the available ecotoxicity data on neonicotinoids to date, the higher sensitivity of aquatic invertebrates to neonicotinoids, a major food source of salmonids, likely presents a more significant risk as an indirect adverse effect in pesticide contaminated areas.

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Chapter 3. Conclusions and Future Research

This research represents the first investigation of sublethal toxicity of the rapidly growing neonicotinoid class of insecticides on sockeye salmon. The study is also one of few related to neonicotinoid exposure in aquatic vertebrates that used concentrations of neonicotinoids comparable to those typically found in the environment, and the only chronic flow through exposure to early life stages of fish to do so. Use of the isolated active ingredients imidacloprid, clothianidin, and thiamethoxam facilitated focusing specifically on adverse effects related to neonicotinoid exposure rather than potentially being confounded by additives in commercial formulations. The concentrations used in this experiment yielded no evidence of impairment of sockeye growth, development, or physiological performance in early life stages. Additionally, the present study investigate exposure to a mixture of the three neonicotinoids in order to assess whether or not there are interactions between the neonicotinoids following exposure. Since neonicotinoids are acetylcholine agonists and other pesticides such as organophosphates and carbamates are cholinesterase inhibitors, it is possible that the different classes could interact at the receptor level and lead to synergistic adverse effects. Pesticides and other environmental contaminants are typically present in complex mixtures rather than as individual entities. Therefore, single chemical studies, while convenient for laboratory implementation and analysis and informative, are not typically representative of environmental exposures.

One of the current information gaps in the literature is the lack of information regarding the concentration of neonicotinoids in Pacific salmon bearing freshwater systems such as the Fraser River watershed. Monitoring in salmon bearing streams has indicated increasing detection frequency and concentrations of imidacloprid in Washington State (WSDA 2013). A more extensive monitoring program that characterized the pesticide contamination in these systems would allow for more accurate laboratory studies using realistic mixtures of pesticides. These studies could incorporate different classes of pesticides that affect acetylcholine function and responses such as organophosphates and carbamates in concert with neonicotinoids. By temporally varying the sampling program based on growing seasons, pesticide application periods, and rainfall events, the variation of pesticide concentrations could be

better characterized and more realistic laboratory exposure experiments could be performed.

Given the regional importance of sockeye and other Pacific salmon species, and the recent declines in productivity and returns of Pacific salmon populations as well as complete loss of stream specific populations, it is important to gather as much information as possible related to potential contributing factors. It is common to use rainbow trout as a representative species for all salmonid species when it comes to laboratory toxicity testing. However, given the demonstrated variability in species sensitivity to different contaminants, it would be useful to use a broader variety of species in toxicity testing.

Almost all laboratory based aquatic toxicology studies focus on the direct effects of contaminant exposure to a single species, whether the species is a target or non-target species. However, in the case of neonicotinoids, there is a vast difference in sensitivity between invertebrates and vertebrates due to the affinity of neonicotinoids for invertebrate nicotinic acetylcholine receptors. Since sockeye and other anadromous salmon populations rely primarily on invertebrates as a food source in the early developmental period following yolk sac absorption, there is high potential for indirect effects on sockeye growth and development if their food supply is limited. Neonicotinoids have already been implicated in indirect adverse effects to insectivorous bird species in field studies and the Japanese medaka in laboratory mesocosms (Hayasaka et al. 2012; Hallmann et al. 2014). Similar field and mesocosm studies investigating the juvenile stages of sockeye salmon in ecosystems with neonicotinoids present could provide a better characterization of the potential for indirect effects. Field studies that determine the common invertebrate components of wild juvenile sockeye salmon diets followed by laboratory toxicity testing using these invertebrate species may also provide useful information related to the potential for indirect effects in sockeye salmon bearing freshwater systems. While the present study indicates that the current US EPA and CCME water quality guidelines are likely to be adequately protective for direct effects of neonicotinoid exposure in sockeye salmon, it is possible that indirect effects and combination of neonicotinoid exposure with other environmental contaminants may be more significant for sockeye salmon survival.

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Appendix

SIMON FRASER UNIVERSITY
ATTN: Vicki Marlatt
Dept. of Biological Sciences
8888 University Drive
Burnaby BC V5A 1S6

Date Received: 26-NOV-14
Report Date: 10-DEC-14 16:09 (MT)
Version: FINAL

Client Phone:

Certificate of Analysis

Lab Work Order #: L1551546
Project P.O. #: NOT SUBMITTED
Job Reference:
C of C Numbers: 14-428210
Legal Site Desc:

Amber Springer
Account Manager

ALS ENVIRONMENTAL ANALYTICAL REPORT

Sample ID	L1551546-1	L1551546-2	L1551546-3	L1551546-4	L1551546-5
Description	IMIDACLOPRID	IMIDACLOPRID	IMIDACLOPRID	IMIDACLOPRID	IMIDACLOPRID
Sampled Date	25-NOV-14	25-NOV-14	25-NOV-14	25-NOV-14	25-NOV-14
Sampled Time					
Client ID	FLUME-CTRL	FLUME-VERY LOW	FLUME-LOW	FLUME-MED	FLUME-HI
Grouping **Analyte**					
WATER					
Pesticides Imidacloprid (ug/L)	<0.10	0.14	1.47	15.4	140

ALS ENVIRONMENTAL ANALYTICAL REPORT

Sample ID	L1551546-6	L1551546-7	L1551546-8	L1551546-9	L1551546-10
Description	IMIDACLOPRID	IMIDACLOPRID	IMIDACLOPRID	IMIDACLOPRID	IMIDACLOPRID
Sampled Date	25-NOV-14	25-NOV-14	25-NOV-14	25-NOV-14	25-NOV-14
Sampled Time					
Client ID	T-CTRL	T-VL	T-L	T-MED	T-HI
Grouping **Analyte**					
WATER					
Pesticides Imidacloprid (ug/L)	<0.10	0.17	1.71	19.0	166

Reference Information

Test Method References:

ALS Test Code	Matrix	Test Description	Method Reference**
PEST-POS1-DI-LCMS-WT	Water	PPCP by LC/MS	EPA 549.2

** 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:

14-428210

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

L1551546-COFC

COC Number: 14 - 428210

Page 1 of 1

Report To: VICKI MARLATT	Report Format / Distribution: Select Report Format: ☐ PDF ☒ EXCEL ☐ EDD (DIGITAL); Quality Control (QC) Report with Report ☒ Yes ☐ No; ☐ Criteria on Report - provide details below if box checked; Select Distribution: ☒ EMAIL ☐ MAIL ☐ FAX; Email 1 or Fax [redacted]@sfu.ca; Email 2
Company: Biological Sciences Simon Fraser University	
Contact: VICKY MARLATT	
Address: 8888 University Dr, Burnaby, BC, V5A 1S6	
Phone:	
Invoice To: Same as Report To ☑ Yes ☐ No; Copy of Invoice with Report ☑ Yes ☐ No	Invoice Distribution: Select Invoice Distribution: ☒ EMAIL ☐ MAIL ☐ FAX; Email 1 or Fax [redacted]@sfu.ca; Email 2
Company: Simon Fraser University, Biol. Sciences	
Contact: VICKI MARLATT	
Project Information	Oil and Gas Required Fields (client use)
ALS Quote #: Jenny Poon #300/sample	Approver ID: Cost Center:
Job #:	GL Account: Routing Code:
PO / AFE:	Activity Code:
LSD:	Location:

Select Service Level Below (Rush Turnaround Time (TAT) is not available for all tests)

- R ☒ Regular (Standard TAT if received by 3pm)
- P ☐ Priority (2-4 business days if received by 3pm)
- E ☐ Emergency (1-2 business days if received by 3pm)
- E2 ☐ Same day or weekend emergency if received by 10am - contact ALS for surcharge.

Specify Date Required for E2,E or P:

Analysis Request

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

ALS Lab Work Order # (lab use only): ALS Contact: Sampler:

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
	FLUME - ctrl	25/11/14		Imidacloprid	
	FLUME - VERY LOW				
	FLUME - LOW				
	FLUME - MED				
	FLUME - HI				
	T-Ctrl				
	T- VL				
	T- L				
	T-MED				
	T-HI	"	"		

Drinking Water (DW) Samples[1] (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 (client Use)

SAMPLE CONDITION AS RECEIVED (lab use only)

Frozen	☐	SIF Observations	Yes ☐	No ☐	
Ice packs	Yes ☐	No ☐	Custody seal intact	Yes ☐	No ☐
Cooling Initiated	☐				

INITIAL COOLER TEMPERATURES °C	FINAL COOLER TEMPERATURES °C
9.8	

SHIPMENT RELEASE (client use)			INITIAL SHIPMENT RECEPTION (lab use only)			FINAL SHIPMENT RECEPTION (lab use only)		
Released by:	Date:	Time:	Received by: Shayen	Date: Nov. 26	Time: 17:35	Received by:	Date:	Time:

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.