Sub-Lethal Effects of Clothianidin on Early Life Stage Sockeye Salmon (*Oncorhynchus nerka*)

by

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Abstract

One of the contaminants possibly contributing to declining sockeye salmon (*Oncorhynchus nerka*) in the Fraser River is pesticides. In this 4-month study, the effects of environmentally relevant concentrations of waterborne clothianidin (0.15, 1.5, 15 and 150 μg/L) on embryonic, alevin and early swim-up fry sockeye salmon derived from four unique genetic crosses of the Pitt River, BC stock were investigated. There were no significant effects of clothianidin on survival, hatching, growth or deformities, although genetic variation significantly affected these endpoints. Clothianidin caused a significant 4.7-fold increase in whole body 17β-estradiol levels in swim-up fry after exposure to 0.15 μg/L, but no effects were observed on testosterone levels. These results indicate additional examination of clothianidin and its effects on salmonid gonad development and the reproductive endocrine axis in general, is warranted.

Keywords: sockeye salmon; clothianidin; growth; development; estradiol

Dedication

To my beloved family for all their unending love and encouragement. I appreciate their support throughout my entire life particularly through pursuing the master degree.

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List of Acronyms

%	Percent
^{o}C	Degrees Celsius
cm	Centimetres
pg	Pictograms
g	Grams
mL	Millilitres
L	Litre
mL/minute	Millilitres per minute
ng/L	Nanograms per litre
µg/L	Micrograms per litre
mg/L	Miligrams per litre
g/L	Grams per litre
µs/cm	Micro-Siemens per centimeter
µM	Micromolar solution
mM	Millimolar solution
ANOVA	Analysis of variance
ATU	Accumulated thermal unit
bw	Body weight
CCME	Canadian Council of Minsters of the Environment
CEPA	Canadian Environmental Protection Act
CNS	Central nervous system
CV	Coefficient of variability
DDT	Dichlorodiphenyltrichloroethane
DO	Dissolved oxygen
dpf	Post-fertilization day
EC_{50}	Median effective concentration
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-release hormone
GSI	Graduated severity index
H_{10}	10th percentile of hatched

H_{50}	50th percentile of hatched
H_{90}	90th percentile of hatched
IC_{50}	Median inhibitory concentration
K	Fulton's condition factor
kg	Kilograms
LD_{50}/ LC_{50}	Median lethal dose / median Lethal concentration
LH	Luteinizing hormone
LOAEL/LOAEC	Lowest observed adverse effect level/ concentration
nAChR	Nicotinic acetylcholine receptor
NOAEL/NOAEC	No observed adverse effect level/ concentration
PNS	Peripheral nervous system
SD	Standard deviation
SE	Standard error
SFU	Simon Fraser University
SOD	Superoxide dismutase
SS-RCB ANOVA	Split-plot randomized complete block analysis of variance
USEPA	United States Environmental Protection Agency

Chapter 1.

Introduction

1.1. The Lower Fraser Valley and Sockeye Salmon (*Oncorhynchus nerka*)

Pacific salmon are widely distributed in the North Pacific Rim, and there are five salmon species in Canada that migrate to Pacific Ocean: chinook, chum, coho, pink and sockeye [1], [2]. All of these salmon species have anadromous life cycles whereby salmon are born in freshwater rivers and streams and migrate to the ocean before returning to their natal freshwater source to spawn [3], [4]. Sockeye salmon are commonly referred as “red salmon” because their bodies turn varying shades of red during the spawning season [5]. The main sockeye spawning area ranges from the Fraser River to Alaska’s Bristol Bay and major sockeye runs in British Columbia (BC), Canada include the Fraser, Skeena, Nass, Stikine, Taku and Alsek watersheds [1], [5]. The Fraser watershed in the Fraser basin, BC, is divided into Upper, Middle and Lower Fraser watersheds, and the Lower Fraser watershed is highly populated and influenced by human activities (Figure 1). The Fraser watershed drains into the Fraser River, which is the longest river in BC. This 1,600-km long river flows from the western side of Rocky Mountains at Mount Robson to Strait of Georgia at the city of Vancouver collecting 223,000 km^2 of water. The Fraser River is one of the most abundant salmon rivers in the world and on average, over a billion juvenile salmon, including 250 million sockeye enter Strait of Georgia every spring to migrate out to the ocean [4], [6].

Fraser River sockeye salmon are culturally and economically important to Canadians. Fraser River salmon sustain many indigenous peoples as a food source and also for cultural purposes [7]. The commercial Fraser River sockeye fishery is also an influential contributor to the Canadian economy. In 2011, Canada exported around 69

thousand tonnes of salmon (based on all five Canadian species) valued at $440 million for international trading [8]. Canada was the 4th largest salmon producing country in the world and BC produced approximate 98.8 thousand tonnes of salmon with a landed value $500.6 million in 2013 [9]. As a result, the international trade of salmon products provides a remarkable amount of revenue for the Canadian economy.

Salmon is not only a resource for human consumption but also an important input of nutrients into freshwater, marine and terrestrial ecosystems. Salmon are born in freshwater, mature in oceans and return to their natal river to spawn, so salmon carry nutrients between freshwater and saltwater ecosystems [10], [11]. In particular, carbon, nitrogen and phosphorous in returning spawning adult salmon and unviable eggs decompose transporting significant amounts of nutrients into freshwater ecosystems from the marine environment [10]–[12]. Spawning salmon, their carcasses and juvenile salmon are also significant food resources for a wide variety of terrestrial wildlife such as eagles, waterfowl, grizzly bears and beavers [10]–[12]. In BC, approximately 137 species including terrestrial animals rely on salmon as their food sources [2]. For instance, the fish-eating bird, merganser, consume roughly 400 g/day (40% body mass) of juvenile salmon [11], [13]. Grizzly bears also consume large quantities of spawning adult salmon and carcasses in the fall (e.g. >1000 kg), and accumulate enough energy for winter hibernation and cub production [11], [13]. Furthermore, a study in brown bears demonstrated a positive relationship between salmon consumption, mean density and litter size of brown bears across North America [13]. With this dependence of numerous wildlife species on salmon, decreases in the abundance of salmon can have devastating effects on wildlife populations [14], [15]. For example, in addition to limited territoriality and increased contamination levels, reduced salmon populations in the 1980s and 2000s was suggested to be one of the key reasons for eagle population declines in south coastal B.C [15]. In the marine ecosystem, it is hypothesized that the cumulative effects of inadequate salmon availability and vessel disturbance on pregnancy failure contributed significantly to Southern resident killer whale (*Orcinus orca*) declines since the late 1990s [16]–[18]. Other studies have even demonstrated the significance of salmon-based nutrient inputs to plants in terrestrial ecosystems. For example, plants such as Sitka spruce (*Picea sitchensi*), ferns (broad buckler fern (*Dryopteris dilatata*) and devil's club (*Oplopanax horridus*)) were found to receive 22-24% of nitrogen from

spawning salmon near the spawning sites of salmon in Chichigof Island, Alaska, and this salmon-derived nitrogen increased the growth rate of Sitka spruce 3-fold compared to the growth in non-spawning grounds [19]. Thus, with the numerous ecosystem services provided by salmon for wildlife directly and indirectly, it is evident that conserving salmon populations is a key factor in protecting freshwater, marine and terrestrial ecosystems.

Due to a favorable climate and some of the most fertile soils in BC, the Lower Fraser Valley rapidly developed into a highly populated and high-density agricultural area and this has had adverse impacts on the Lower Fraser watershed. There are approximately 5,500 farms in the valley producing an average of $600 million of farm products annually, which is 50% of the total farm income in BC [20]. While agricultural activities were expanding, pesticides and fertilizers were introduced and are used routinely to increase crop yields. In 1988, around 100,000 kg of active ingredients of pesticides were applied in the Lower Fraser Valley [21]. By 2003, the use of pesticides in the Lower Fraser Valley increased to 2,146,686 kg of active ingredients, which accounted for 46% of the pesticide sold in BC in 2003 [22]. Excess irrigation and rainfall events can wash the pesticides into nearby streams, and these pesticides then have the potential to enter the Fraser River [21]. The human population growth in the Lower Fraser Valley increased 6.6% from 2011 to 2016 reaching 295,000 people [23]. This has also increased domestic and industrial waste production, and these anthropogenic wastes can either directly or indirectly enter surface water or groundwater in the Lower Fraser Watershed [21]. Daily ~1,181,546 m^3 of municipal and industrial wastewater are treated in the Lower Fraser Valley and ~875,000 m^3 is then discharged into the Lower Fraser River, along with several pesticides and other contaminants not removed by wastewater treatment processes [21], [24]. In 2009, a study revealed that a range of 22 to 33 pesticides were detected in surface water in 5 different sites in Lower Fraser Valley during 2003 to 2005 , 20.8 to 40.9% of those detected pesticides had 100% detection frequency [25]. For instance, garden and indoor insecticide, diazinon, was detected in surface water of both agricultural sites and urban sites in the Lower Fraser Valley with a mean of 12,500 ng/L and 5.39 ng/L, respectively [25]. With the rapid agricultural expansion and population growth, more pesticide contaminants from both the agricultural setting and municipal wastewater are produced and this could potentially increase the environmental impacts on aquatic life.

Historically, the Fraser River was the river that produced the highest numbers of sockeye salmon in the world, but the population of Fraser River sockeye has been declining since 1990s [4], [6]. Due to the low record of sockeye returns, salmon fisheries were forced into closure for 3 consecutive years (2007-2009), and all salmon sport fishing on Lower Fraser (from the mouth of the Fraser River to the Alexandra Bridge south of Hell's Gate) was shut down to protect sockeye in August, 2016 [4], [26]. The number of sockeye in 2016 was even lower than the average of 3.9 million over the past half century showing little sign of recovery [26], [27]. At the end of 2009, the Canadian federal government established the Cohen Commission of Inquiry to collect scientific evidence to investigate the causes of the sockeye salmon decline, and to develop an approach to restore the sustainability of salmon in the Fraser River. The possible causes identified in this inquiry included: climate change, such as increase in temperature; fishing pressures, caused by the growth in fishery industries; habitat destruction, such as landfills; disease or parasites; and, environmental contaminants. Anthropogenic contamination, including pesticides was identified as one of the main causes of sockeye population declines, but the fate of pesticides and potential effects on salmon are still largely unknown [4]. Hence, there is a need to examine the environmental impacts of pesticides in order to protect a Canadian national treasure— sockeye salmon.

1.2. Neonicotinoids– A New Class of Insecticides

Conventional agricultural practices use synthetic chemical pesticides and fertilizers to maximize crop yield, and it is thought by many jurisdictions that this yield benefit outweighs any potential health risks these chemicals pose to humans and wildlife [28]–[30]. The history of pesticides is lengthy and the first-generation of pesticides date back to the 15th century and was obtained from naturally occurring organic or inorganic chemicals. Indeed, by the 15th century, toxic inorganic compounds of sulfur, arsenic, mercury and lead were applied on agricultural crops for pest control [31]. In addition to these inorganic chemicals, secondary metabolites synthesized by some plant species were also discovered to naturally protect plants against pathogens and pests [32], [33]. There are over 2,500 plants that naturally produce these chemicals, or botanical pesticides, to protect plants against pests [32], [33]. For example, botanical pesticides

such as rotenone (extracted from roots of *Lonchocarpus*), pyrethrum (extracted from *Chrysanthemum* flower) and nicotine (extracted from tobacco (*Nicotiana rustica*)) were commonly used during 17th to 19th century [32], [33]. These were insecticides comprised of naturally occurring chemicals that, for example, interrupt the feeding mechanism, food seeking behavior, and/or development/metamorphosis of insects, and ultimately lead to death [32], [33]. For instance, tobacco was discovered in the 17th century as a natural insect repellent due to the presence of nicotine, an alkaloid extracted from the foliage of tobacco plants [34]. Nicotine was used as botanical pesticide since the 17th century, and in the 1940s, over 2,500 tonnes nicotine was used worldwide [34]. It is a non-persistent insecticide that mimics the neurotransmitter, acetylcholine, in insects by binding to a receptor in the nervous system causing convulsions and eventually death [33]. The use of nicotine had declined significantly to less than 200 tonnes by the early 1980s as a second generation of pesticides was developed in the 1940's and proved to be more efficient, cheaper and claimed to have lower risks to human [34]. This second generation of pesticides differed from the first-generation because they were synthesized by industrial methods [35], [36]. For example, synthetic pyrethroids (e.g. Allethrins), a household insecticide primarily for mosquito control, was derived from pyrethrin, which is a botanical pesticide extracted from chrysanthemum flowers [35], [36]. Other second-generation pesticides, organochlorine (e.g. Aldrin, Endrin), organophosphates (e.g. malathion, dimethoate) and carbamates (e.g. Carbaryl, Aldicarb) were introduced between 1950s to 1980s [35], [36]. However, some pesticides had unforeseeable environmental effects. For instance, Aldrin was later found to be too persistent, bioaccumulative and toxic and was banned in countries signatory to the Stockholm Convention in the 20th century [37]. In fact, several jurisdictions began to re-evaluate the risks of many second-generation pesticides in the mid-1950s, and many were subsequently banned in multiple countries, including one of the most infamous of the synthetic pesticides, dichlorodiphenyltrichloroethane (DDT) [38].

Currently, one of the most frequently used groups of synthetic pesticides of the 21st is the neonicotinoids, which are broad-spectrum, systemic insecticides [37]. There are 15 different neonicotinoids and some of the most popular include clothianidin, imidacloprid, thiamethoxam, acetamiprid, nitenpyram and thiacloprid [39]. Neonicotinoids are mainly used for agricultural purposes on corn, rice, potato, sunflower, soy and other

vegetable crops and also used in residential areas [39]. Due to the structural similarity with nicotine, neonicotinoids share the same mode of action as nicotine in invertebrates and vertebrates, binding agonistically to the nicotinic acetylcholine receptor (nAChRs) in the nervous system interfering with acetylcholine neurotransmitter signalling [39]. Neonicotinoids are more toxic to invertebrates because these insecticides have greater affinity for invertebrate nAChRs compared to vertebrate nAChRs [40][41]. In mammals, receptors are located in both the central nervous system (CNS) and peripheral nervous systems (PNS) (i.e. nervous system connecting the central nervous system to muscles or organs) [40]. In contrast, invertebrate, particularly insect, nAChRs are confined to CNS resulting in a high density of nAChRs in nervous tissues, and this contributes to invertebrates being more sensitive to the neurotoxic effects of neonicotinoids than vertebrates [40]. Furthermore, the general composition of nAChR subunits in invertebrates also contributes stabilizing electrostatic interactions and hydrogen bonds between nAChRs and neonicotinoid molecules, and more selective toxicity to invertebrates compared to vertebrates [40], [41]. Under the normal condition, the nerve transmission in CNS or PNS (the latter relevant to vertebrates only) relies on the neurotransmitter, acetylcholine [39]. When a nerve impulse is transmitted from a presynaptic cell to a postsynaptic cell, acetylcholine molecules will be released from an axon into the synaptic cleft and acetylcholine will bind to nAChRs on the postsynaptic cells [39]. The binding will then induce sodium ion channels to open allowing ions to enter to postsynaptic neurons causing signal transmission [39]. After the stimulation, acetylcholine has to be hydrolyzed by acetylcholinesterase to acetate and choline in order to terminate the stimulation [39]. When an invertebrate such as an insect is exposed to neonicotinoids, the neonicotinoid molecules irreversibly and selectively bind to nAChRs keeping ion channels open resulting in overstimulation, and eventually paralysis and death within minutes [39]. Therefore, this group of insecticides are efficient for controlling a wide range of economically important pests including aphids, leafhoppers and phytophagous mites, and as such, were rapidly adopted by both agricultural and urban pesticide users globally [39].

In addition to the fact that neonicotinoids are relatively less toxic to mammals than insects, other key characteristics make neonicotinoids successful on the global pesticide market. Neonicotinoids are persistent in soil, and therefore, protect the crop for

the long period. For instance, reports of clothianidin half-lives vary from 148 days in silt loam soil to 6,931 days in aerobic loamy sand in the laboratory, and in the field a half-life of 365 days in silty loam soils in Ontario was reported [39], [42]. These data for clothianidin deem it categorized as persistent in the soil environment according to the Canadian Environmental Protection Act (CEPA) (1999), which defines a substance as persistent in soil if its half-life is equal to or greater than 182 days. Moreover, neonicotinoids are able to translocate within plant tissues in the crop despite the method of application in agricultural fields [39]. This systemic property helps to distribute the insecticide throughout the plant making this pesticide efficacious against pests that damage both shoot and root systems [39]. As a result, neonicotinoids have a high efficiency for pest control and have become one of the most dominant groups of pesticides on the global the market and accounts for 27% of the insecticide marketed by 2010 [39].

1.3. Clothianidin– A New Generation of Neonicotinoids

There are two generations of neonicotinoids, imidacloprid is a first generation that is starting to be replaced by a second-generation of neonicotinoids such as clothianidin. Imidacloprid has been used for more than 140 crops globally and became the top selling pesticide worldwide in 2008 [39]. There was an estimated 20,000 tonnes of imidacloprid (US $1,091 million global value) produced in 2009 [39]. In the late 2000s, many target pests started to develop resistance to imidacloprid due to mutations in insect nAChRs and natural selection selecting for imidacloprid resistant strains, so second generation neonicotinoids (i.e. clothianidin, thiamethoxam) were developed and introduced to the market [39], [43]. For example, in United Kingdom (UK), the area that imidacloprid was used for as a seed treatment decreased dramatically from 76,8000 ha between 2004 and 2007 to 31,000 ha in 2012 [39]. Concurrently, the crop treated with clothianidin increased from 43,000 ha in 2007 to 806,000 ha in 2012 in the UK [39] . Similar trends were observed in Sweden, Japan and the U.S. [39]. According to the U.S. Geological Survey, approximately 2.0 million pounds of imidacloprid were used in the U.S. while the usage of clothianidin was estimated to be 3.75 million pounds in U.S. in 2014 [44], [45]. The movement towards replacement of imidacloprid with clothianidin is

being driven by the barrier to market growth of imidacloprid due to insect resistance, and has become more prevalent since the early 2010s [39].

Clothianidin is sold by Bayer Crop Science (Canada) and Sumitomo Chemical Takeda Agro Company (Japan) under the trade names as Poncho, Prosper and Votivo, and Dantosu, respectively [39]. According to the pest control products sales report conducted by Pest Management Regulatory agency, clothianidin was one of the top 10 insecticides sold in 2011, and >100,000 kg of clothianidin active ingredient was sold in 2011 [46]. Since many neonicotinoids, including clothianidin, are highly toxic to honeybees and aquatic invertebrates, clothianidin is suspended by European Union and some states in the U.S. are considering a ban [47], [48]. In Canada, clothianidin is still on the market and additional data will be reviewed by Pest Management Regulatory Agency to fully assess the potential effects of chronic exposure and consultations will take place by 2018 [49]–[51]. In particular, in the U.S., clothianidin is intended to treat corn and canola seeds with a proposed application rate of 0.25 to 1.25 mg active ingredient per kernel and 150 to 400 g active ingredient per 100 kg canola seeds [52]. It can also be directly applied as foliar sprays on crops such as fruits and rice [42], [52]. In addition to agricultural uses, this insecticide is registered in Canada to control cockroaches both indoors and outdoors [53].

Pesticides are frequently detected in various unintended environmental compartments due to agricultural run-off events, volatilization and atmospheric transport, spray drift etc., and neonicotinoids are no exception. However, since the most popular application of clothianidin and many other neonicotinoids for agricultural purposes is as a seed coating, an a priori treatment against pests, treated seeds eaten by birds can also be introduced into the food web [39], [54]. In addition, the active ingredient in the seed coating is absorbed by roots and translocated to all plant tissues [39], [54]. Around 1.6-20% of active substance will be absorbed by the crop and then translocated within plants [54]–[57]. Pollinators can then be exposed to clothianidin residues in nectar and pollen due to this systemic property of clothianidin [56], [57]. The remaining 80-98% will remain in the environment, rain and excess irrigation can wash the pesticides into nearby streams and rivers affecting aquatic life [39], [54], [56], [57]. As clothianidin is highly soluble in water (0.327 g/L at 20 °C [52]) and stable to hydrolysis (aerobic half-live of 148

days [58]), the dissolved clothianidin persists and can be carried to distant locations [54]. If clothianidin is released into the soil, it is expected to accumulate because it is persistent in soil with half-lives reported can exceed 1000 days (e.g. half-lives can be as long as 6,931 days in aerobic loamy sand in the laboratory studies) [42], [54]. Spray applications of clothianidin can also directly contaminate surface water near agricultural sites [54]. Also, because of very high leaching potential, clothianidin tends to contaminate ground water and surface water that will eventually enter nearby streams [54]. With several routes of entry into various environmental compartments and clothianidin's persistence in the environment, more closely examining the environmental fate and non-target adverse effects of clothianidin on wildlife and humans is prudent.

In Canada, there are more than 500 pesticides registered for use but only 141 pesticides were monitored by Environment Canada's National Pesticide Science Fund Water Quality Surveillance team according to the survey in 2011 March but clothianidin was not measured during any of these surveys [22]. Nonetheless, the Lower Fraser Valley was included in this study since it is one of highest pesticide use regions in BC [22]. Indeed, more than 50% of water samples were contaminated by pesticides such as permethrin, dicamba and metribuzin and high concentrations of pesticides were found in field runoff, surface water and groundwater [22]. Although there is little information on the levels of clothianidin in Fraser Valley, clothianidin has been detected frequently in aquatic environments across Canada, especially during planting season [55]. The average and maximum detected level of clothianidin in Canada are summarized in Table 1. In southwestern Ontario, Canada, all 76 water samples collected within and around commercial maize farms had detectable concentrations of clothianidin with a mean concentration of 2.28 μg/L and maximum concentration of 43.60 μg/L, and the total neonicotinoid concentration increased 6-fold after the planting season suggesting the main source of pesticide pollution is from agricultural settings [59]. Similarly, clothianidin was detected in 36-91% of samples collected four times a year in ponds in Prairie wetlands of central Saskatchewan, with a maximum concentration of 3.11 μg/L during the canola planting season (summer 2012) [60]. This high detection frequency reflects a high occurrence of clothianidin entering to the environment. Recent experiments in a model aquatic benthic invertebrate (*Chironomus dilutus*) demonstrated clothianidin exhibits similar acute and chronic toxicity compared to another neonicotinoid,

imidacloprid, thus it is likely that the current environmental quality guidelines for imidacloprid would apply to clothianidin as well [61]. Based on the mean detected concentration of clothianidin in Ontario and maximum value detected in Saskatchewan, clothianidin is present in concentrations at least 10-fold higher than water quality guideline established by Canadian Council of Ministers of the environments (CCME) for imidacloprid (0.23 μg/L), and exceeds the USEPA aquatic life benchmarks of 1.05 μg/L imidacloprid for invertebrates [62]. Exceeding these environmental quality guidelines for clothianidin translates into concentrations that may pose a risk to aquatic wildlife, especially aquatic invertebrates.

There are numerous reports of clothianidin contaminating various water bodies including ponds, river, groundwater, puddled water, soil water and run-off, and most frequently in proximity to agricultural areas [30]. The frequency and level of detection in several North American, Asian and Australian studies are summarized in Table 1. Detection data of clothianidin outside of the U.S. was limited, but this pesticide was frequently detected in the aquatic environment in 13 Australian rivers and 26 Japanese rivers, with a mean concentration of 0.06 and 0.0035 μg/L, respectively [63], [64]. Several studies reporting environmental contamination by clothianidin were conducted in North America. For example, in Illinois, U.S., several water samples were collected from 94-ha corn and soybean agricultural sites in 2011 to 2013 [65]. In this two-year study, the concentration was detected as high as 0.060 μg/L clothianidin in groundwater after corn planting in 2011 and the average concentration was 0.850 μg/L in run-off [65]. In this same study, soil water samplers were buried in 1 cm deep of the surface of water to collect infiltrated water near the runoff samplers and clothianidin concentration was up to 0.203 μg/L after corn planting in 2013 [65]. In addition, clothianidin was frequently found to contaminate streams at significant levels across the U.S. In 2012 and 2014, a nationwide study in U.S. revealed that at least one neonicotinoid was detected in 53% of 38 stream water samples across different states, and clothianidin was detected in 24% of those samples [66]. The detected clothianidin level ranged from 0.018 to 0.132 μg/L [66]. In Iowa, U.S., 79 water samples were collected from rivers and streams at 9 sites, which have 59 to 86% corn and soybean production between March and October 2013, and clothianidin was detected at a frequency of 75% in these 79 samples with a mean concentration of 0.0082 μg/L [67]. The concentration even increased 2-fold during the

planting season after pesticide applications in crops [67]. In the most extreme case in Noordwjjkerhout, Netherlands, the neonicotinoid, imidacloprid, in surface waters collected near agricultural area in 2005 had highest exceedance of 320 μg/L, which was over 4,700 times higher than 0.067 μg/L imidacloprid according to the Maximum Permissible Concentration, the environmental concentration used in the Netherlands to determine if human and species in an aquatic system are safe from an toxic substance in long-term exposure [68]. Collectively, global environmental surface water (i.e. rivers, streams, pond) concentrations of clothianidin appear to range from 0.0035 to 43.60 μg/L, and it is likely that this concentration range is similar in regions where neonicotinoid surveys have not been conducted, such as the Fraser watershed.

1.4. Potential Effects of Clothianidin

A common environmental concern in agricultural regions is pesticides contaminating water bodies, soil, sediments, food and air [54]. Pesticide contamination can directly or indirectly cause numerous effects on many types of non-target species by various routes of exposure [54]. Terrestrial organisms can be exposed by ingesting treated seeds, plant parts or residues in the soil, drinking contaminated water, dermal contact with contaminated soil and/or water in the treated area and/or inhaling pesticide vapors after application [54]. Aquatic organisms can be directly exposed by ingestion, dermal contact and inhalation of dissolved pesticide in the water [54]. Depending on the dose, route of exposure, duration, and inherent toxicity of the pesticide, direct toxic effects of pesticides may vary in whole organism adverse outcomes. In addition, the adverse effects of pesticide use on a non-target species may also be indirect due to changes in available resources (i.e. food, habitat, etc.) caused by a given pesticide.

Neonicotinoids have been widely used in the market due to their high selectivity on target pests and relatively low toxicity to non-target species. However, acute toxicity tests in wild aquatic invertebrates and pollinators have demonstrated high sensitivity in these taxa of paramount importance in aquatic and terrestrial ecosystems. To classify the toxicity of a chemical, most standard toxicological tests typically involve a high dose level with single administration to measure the median lethal dose or concentration, LD_{50} or LC_{50}, which estimates the dose or concentration of pesticide required to kill half of the

test animals. Clothianidin is considered to be highly toxic to insects, with acute LD_{50} as low as 3.28 ng/ honey bee, but it is moderately toxic in short-term to mammals with acute oral LD_{50} > 389 to 465 mg/kg and it is practically non-toxic for birds based on the high acute LD_{50} value of 423 to >5230 mg/kg (Apprendix B). For aquatic life, clothiandin is highly toxic to aquatic invertebrates, with a 96 hour LC_{50} of 0.051 mg/L clothianidin for mysid shrimp (*Mysidopsis bahia*) [52]. Acute toxicity of clothiaindin in fish ranges from practically non-toxic to slightly toxic. The 96 hour LC50's of 117 mg/L, 110 mg/L and 93.6 mg/L for blue gill fish, rainbow trout and sheepshead minnow, respectively, have been reported [52]. In terms of acute toxicity, clothianidin is most toxic to invertebrates.

Sub-lethal effects are more subtle and less well studied than acute effects. However, low-level, chronic exposure to clothianidin appear to be more environmentally relevant based on the reports of pesticide concentrations, including clothianidin, in global surface waters. Even though the exposure level is below the LD_{50} or LC_{50} to cause significant lethal effects, pesticides may still cause a range of problems on growth, development and reproduction in humans and wildlife. These detrimental impacts can be characterized by establishing the no observed adverse effect level/ concentration (NOAEL/NOAEC), lowest observed adverse effect level/concentration (LOAEL/LOAEC) and median effective or inhibitory concentration (EC_{50} or IC_{50}; concentration causing a 50% maximal/inhibitory response, such as enzyme induction, or reduction in growth; Table 2). Numerous studies have examined the sub-lethal effects of clothianidin long term in beneficial insects like honey bees. Clothianidin has been shown to suppress the honey bee's immune system making them vulnerable to disease & parasites, which can cause lethal effects on larvae and reduction in queen survival resulting in colony collapse disorder in honey bees [69]. In 2012, 29% bee loss reported by the Canadian beekeepers after the corn and soybean planting season [49]. The death of the bees was linked to the pesticide exposure, with 70% of the dead bees sampled containing clothianidin residues [49]. In mammals, clothianidin has been shown to cause detrimental effects on reproductive organs and gametes. For example, exposing rats to 32 mg/kg/day of clothianidin (oral administration; 90-day) caused significant decrease in the weight of epididymis and seminal vesicles [70]. This chronic exposure to this sub-lethal concentration also induced oxidative stress enhanced reactive oxygen species production causing sperm DNA fragmentation and reduced serum testosterone level in

these male rats [70]. In a 90-day study, the lowest observed effect level for reduction in Norway rat epididymis weight was as low as 2 mg/kg/day exposure (oral gavage) [71]. Similarly, oxidative induced DNA damage from clothianidin exposure was reported in birds. Quail orally administered sub-lethal doses (0.02- 50 mg/kg) of clothianidin increased DNA fragmentation in seminiferous tubules and inhibited embryonic growth in dose-response manner [72]. Unfortunately, chronic toxicity on aquatic vertebrates especially salmon are not fully studied. A chronic toxicity study reported by U.S. Environmental Protection Agency (EPA) revealed significant differences in the dry body weight and body length of fathead minnow after exposure to 20 mg/L clothianidin in a flow-through system [73]. Since the chronic, low-level, sub-lethal effects of clothianidin in various vertebrates are still largely unknown, environmentally relevant studies in these taxa are warranted, especially those inhabiting surface waters where contamination is prevalent.

1.5. Sockeye Salmon Developmental Stages and Critical Exposure Windows

In general, sockeye salmon has a 4-year life cycle. The life history of a salmon is intricate with time spent in both freshwater and saltwater and it can be roughly divided into 5 main developmental stages: embryo, alevin, fry, smolt and adult. The life cycle begins and ends in fresh water. Embryos remain in freshwater until they hatch and develop into alevins and then smolts [3], [4]. Smolts will then enter salt water, mature into adults in the ocean and return to freshwater to spawn [3], [4]. During the spawning season, mature sockeye salmon return to their natal river or lake and search for a suitable site for spawning. Female sockeye deposit eggs into redds, which are depressions they create in the gravel bed of a stream using their tail and fins [3], [4]. Approximately 3,000 to 4,000 eggs are laid by a female in each redd and the accompanying male simultaneously releases a cloud of milt to fertilize the eggs [4]. The redds are covered by gravel to prevent predation, ice condition and flooding, and the salmon eggs comprised of a protective outer membrane encasing an embryo that develop in the gravel during the winter [3], [4]. The rate of fish development depends on the water temperature and genetics but in the Fraser River, after ~5 months after

spawning, fertilized eggs develop into alevins and emerge from the gravel bed [4]. Yolk sacs attached below their bodies provide nourishment for alevins to remain in the gravel for 6 to 10 weeks, protected from predators. About 8 months after fertilization, alevins in Fraser River are around 3 cm long [4]. Once the yolk sacs are completely absorbed, the fish are referred as swim-up fry and emergence occurs as swim-up fry leave the gravel bed and migrate down the stream/nursery lake to search for food [3], [4]. Swim-up fry generally stay in the streams/nursery lake for a year to 2 years [3], [4]. Around 20 months after fertilization, in the Fraser River, smoltification occurs where the body develops silver pigmentation and changes physiologically for transition from freshwater life to seawater life before migrating downstream to Strait of Georgia [3], [4]. Smolts will then spend 2 to 3 years in the Gulf of Alaska and most of them become mature in their fourth or fifth year preparing to return to their natal streams or lakes in the Fraser Basin to spawn [4].

Sub-lethal effects of environmental stressor, including pesticides, on sockeye salmon can vary in severity at different developmental life stages. Typically, early life stages including embryonic and larval stages are the most sensitive window in fish development. Based on 3,000 eggs laid per a female sockeye, only 14%, which is 420 of eggs, survive and successfully develop into fry [4]. This high mortality rate could be the result of predation, physical disturbance due to high water flows and later spawners, dehydration or freezing due to low water levels, suffocation due to reduced oxygen levels or fine sediment, buildup of CO_2 due to high spawning density, parasitism, pathogens infection and pollution [3], [4]. During early life stages, salmon remain in their redds most of the time, and their immune systems are not fully developed as well as their protective egg chorion are not efficient to stop small molecules <500 g/mol like clothianidin passing through so embryo, alevin and swim-up fry life stages could be the most sensitive stage in their complete life cycle to any stressors in the surrounding environment, including contaminants [74], [75]. During these critical stages, any irreversible life-long impacts caused by pesticide exposure may affect their ability to feed or escape from predators, and ultimately, affect their survival. In general, standard toxicity tests focus on juvenile fish < 3.0 g or adult fish and acute testing using higher doses than environmental concentration to characterize the toxicity of a chemical [76].

These studies often overlook the long-term, low-level adverse effects on salmon embryos, alevins and an non-feeding early fry developmental stages.

1.6. Overview of this Research

Examining the direct adverse sub-lethal effects of clothianidin on early life stages of Fraser River sockeye salmon can provide a better understanding of the impacts of low-level exposure to this neonicotinoid on salmon in the Fraser River. Salmon are the key component of freshwater and marine aquatic ecosystem, so it often serves as a key indicator species in BC. The decline in the Fraser sockeye population over the past century has reflected the sockeye resource is vulnerable to anthropogenic stressors. Based on available data, it is not impossible that this decline directly or indirectly correlates with increased pesticides use in agricultural settings in the Fraser Valley. Therefore, studying the potential effects of clothianidin on sockeye salmon is imperative.

This study examined the effects of a neonicotinoid insecticide, clothianidin, following a chronic exposure, on critical early life stages of a wild salmon species, sockeye salmon (*Oncorhynchus nerka*). In this study, 4 concentrations of clothianidin (0.15, 1.5, 15 and 150 µg/L) plus a water control were tested in chronic exposure experiments that were initiated ~1 hour post-fertilization and the experiment continued through to the swim up fry developmental stage. These concentrations of clothianidin were selected based on the concentrations of clothianidin reported for surface waters globally and to incorporate the only neonicotinoid Canadian Water Quality Guideline of 0.23 µg/L for imidacloprid. Since wild salmon are not routinely studied to examine the influence of parentage on toxicant responses, four unique offspring sets (crosses) were tested. The endpoints measured to assess the adverse effects of clothianidin in developing wild sockeye salmon included survival and several sub-lethal endpoints, specifically, growth, hatching, emergence and sex steroid hormone levels.

Chapter 2. Methods

2.1. Sockeye Salmon Gamete Collection and Fertilization

Four sexually mature mating pairs of wild sockeye salmon (*Oncorhynchus nerka*) were captured in the Pitt River, BC, Canada during the fall 2015 spawning season. Fish were caught by Fisheries and Oceans Canada staff of the Inch Creek Hatchery (Dewdney, BC), and were generously donated for this project. Approximately 2,000 to 3000 eggs were collected from each female and 1 to 3 ml of milt were collected from each male. Gamete collections were performed by applying gentle pressure to the body in an anterior to posterior direction on the ventral surface into dry food grade plastic containers, and containers were transported at 6 - 10 °C to Simon Fraser University, Burnaby, BC on September 8, 2015.

Dry fertilizations were performed by Dr. Vicki Marlatt within 6 hours of the collection of gametes to create four unique offspring sets, referred to as cross A, B, C and D. As such, eggs and milt from each mating pair were fertilized independently and kept separate throughout all procedures and subsequent exposures to evaluate differences between different offspring sets/genetic crosses. Dry fertilizations were performed according to Patterson et al. (2004) whereby the eggs from one female were combined with the milt from one male in a 4 L food grade plastic container, followed by the addition of 1.5 L of dechlorinated water (10 ± 1 °C) and gentle swirling [77]. The fertilized eggs remained in these containers for a minimum of 60 minutes to allow for water hardening and then transferred into separate netted cylindrical egg containers (food grade polyvinyl chloride (PVC)) and placed in exposure vessels. Fertilization success was determined by the proportion of eyed embryos on 28 post-fertilization day (dpf) to the total number of eggs placed in the control exposure vessels at the onset of the experiment.

2.2. Aquatic Exposure Systems

This study was divided into two different aquatic exposure systems: 1) glass tank flow-through test vessels conducted in duplicate; and, 2) gravel-bed flume incubators that simulate a streambed environment conducted in duplicate. The fertilized eggs in the netted cylindrical egg containers placed in the glass tanks allowed for monitoring the survival and development of eggs throughout the experiment. However, the fish in the gravel-bed flume incubators mimicked a more natural incubation system whereby fish were buried in the gravel upon reaching the eyed embryo stage and allowed to emerge naturally from the gravel. As such, survival and development were monitored only at the end of the experiment in the gravel-bed flume incubators. Both aquatic exposures were conducted from 1-3 hours post-fertilization through to the swim-up fry developmental stage (duration: 118 days for glass tank exposures and 110 days for gravel-bed flume exposures).

The glass tank exposure system was monitored for survival, development and removal of dead embryos/alevins were daily throughout the experiment. For each mating pair, approximately 100 fertilized eggs were divided between three netted cylindrical PVC containers and placed in each of the duplicate tanks (i.e. ~100 eggs/glass tank divided among three netted cylindrical PVC containers). The total volume of each tank was 28 L and the dimensions of each glass tank were as follows: 22 cm height (with a 20 cm high drainage hole) by 26 cm wide and 52 cm long. Duplicate tanks for each test concentration and the control were placed in random order at two opposite sides of the temperature-controlled room. In order to maintain a uniform water pressure, an overhead tank was constructed to ensure consistency of water flow to the glass tanks. Dechlorinated municipal water was dispensed from the overhead tank through the food grade Tygon tubing connected with valves to adjust the flow rate into each glass tank. Flow rates were tested and adjusted every 48 hours throughout the exposures. Embryos were maintained in darkness until 90-100% hatching was achieved in the control glass tanks, and alevins were then reared under a 16 h light: 8 h dark photoperiod until termination of experiment [98]. Termination was performed when 87-98% of surviving alevins reached the swim-up fry developmental stage (100% yolk sac absorption) in the control glass tanks. The endpoints examined in the glass tank exposure experiment

included the following for each individual fish in all 4 genetic crosses: survival; hatching; body morphometrics and condition factor; % of deformities; and, whole body 17β-estradiol and testosterone concentrations in swim-up fry (hormones analyzed in 1 genetic cross only).

The gravel-bed flume exposure system was employed to simulate the natural environment in a stream, creek or river, and allowed the fish to naturally 'swim-up' or emerge from the gravel once their yolk sacs were fully resorbed. This is a key process and behavioral endpoint integral to the survival of a developing salmonid [78], thus monitoring the emergence of swim-up fry was included as a sub-lethal endpoint to examine adverse impacts of clothianidin on swim-up success. The design of gravel-bed flumes was adapted from Pilgrim et al. 2013 [78]. The dimension of each flume was 250 cm in length by 40 cm wide and 32 cm deep, and the flume was divided into five isolated sections with a total volume of 64 L each. Each of the five sections were sub-divided by stainless steel mesh to create five sub-compartments (Figure 2). Of these five sub-compartments that shared a single inflow and outflow, the middle compartment was used for drainage (outflow) while the other four compartments were used to house the developing sockeye salmon from each of the four genetic crosses. Similar to the glass tanks, approximately one hundred fertilized eggs from each of the four crosses were divided into three netted PVC cylindrical containers and placed in each of the four sub-compartments (i.e. one sub-compartment contained 3 PVC containers from a single cross). Stainless steel mesh dividers between 5 sub-compartments allowed movement of pesticide solution or water within one isolated section, while developing sockeye salmon from each of the four crosses remained separated. This five-section (each with a 5 sub-compartments) gravel-bed flume was assembled in duplicate, and these two replicates were located on opposite sides of a temperature-controlled room. The arrangement of each test concentration and the control was randomly assigned within each replicate of gravel-bed flume incubator. As described for the glass tank exposure system, dechlorinated municipal water was delivered from an overhead tank to the flumes through food grade Tygon tubing with adjustable valves to control the water flow into each isolated section of the flumes. Flow rates from the overhead tank into each of the five isolated sections of the duplicate gravel-bed flumes were tested and adjusted every 48 hours throughout the exposures. The inflow water line from the overhead tank

entered into each section via a PVC pipe, which was further spilt into five such that water inflow entered each of the five sub-compartments, as shown in section I in Figure 2. These PVC pipes in each of the five sub-compartments were located at the bottom of the flume with several holes such that water flow direction was from the bottom of the flume upwards through the gravel towards the surface of the water. Ultimately, the gravel-bed flume system allowed four genetic crosses to be kept separate yet exposed to a single test concentration in a single replicate.

Two sizes of gravel rock, 10 mm and 25 mm, were selected as substrate and to rear developing sockeye salmon in the gravel-bed flume system [79]. The two gravel rocks were mixed in a 1:1 ratio and disinfected using 1% Ovadine solution (Syndel Laboratories Ltd., Nanaimo, B.C., Canada) followed by a dechlorinated municipal water rinse before placement into the gravel-bed flumes. The flumes were filled with the gravel to a depth of 5 cm (Figure 2) and were flushed for a minimum of 24 hours with dechlorinated municipal water to remove any residual ovadine. The eyed embryos were housed in netted cylindrical egg containers on top of the gravel until 92-100% of the embryos developed eyes in the control. On 28 dpf, eyed embryos were gently deposited on top of the gravel and then buried with additional gravel to a height of 15 cm. The test volume of 28 L was maintained throughout the exposure by adjusting the height of the outflow pipe in each of the 5 sections in both of the replicate gravel-bed flumes (Figure 2). The photoperiod for the gravel-bed flume incubators was identical to that in the glass tank exposure system. Specifically, developing sockeye were kept in darkness until 90-100% of fish in the duplicate control glass tanks reached the hatching stage, followed by rearing alevins under a 16 h light: 8 h dark photoperiod. The experiment was terminated when 87-98% of fish in the duplicate controls in the glass tank exposures reached the swim-up fry developmental stage. The endpoints examined in the fish in the gravel-bed flume incubator exposure experiment included the following for each individual fish in all 4 genetic crosses: survival; hatching; emergence; body morphometrics and condition factor, and % of deformities.

2.3. Pesticide Exposures

Clothianidin stock solutions were prepared fresh every 48 hours to prevent any degradation of the chemical. This was selected based on research indicating an aqueous photolysis half-life ranging from 0.35 to 3.31 days in freshwater mesocosm studies during 4 different seasons in Winnipeg, Canada [80], and 25.1 to 27.7 hour half-life in nonsterile river water under 9 h light: 15 h dark [58]. In the present study, fish were exposed from 1 hour post-fertilization through to the swim-up fry developmental stage in a water control and four concentrations of clothianidin: 0.15, 1.5, 15 and 150 µg/L. The stock was prepared using ≥ 98.0 % pure clothianidin (CAS#: 210880-92-5, Sigma-Aldrich, Oakville, Ontario, Canada). The concentrations were selected based on the CCME water quality guideline for imidacloprid of 0.23 µg/L, the USEPA aquatic life benchmarks of 35 (acute) and 1.05 µg/L (chronic) for imidacloprid for invertebrates, and the range of neonicotinoid concentrations reported in various surface waters in North American, Asian and Australian studies (0.0035 - 320 µg/L) [30], [59], [60], [62], [63], [65], [67], [68], [81]. Since clothianidin is soluble in water (0.327 g/L at 20 °C [82]), a solvent was not required to make the clothianidin stock solutions that were pumped into the test vessels containing sockeye salmon during the exposure period. The clothianidin stock solution was prepared by adding 0.200 g of Sigma-Aldrich clothianidin to 4 L of dechlorinated municipal water. This solution was allowed to mix for one hour until clothianidin fully dissolved. This solution was then further diluted with dechlorinated water and distributed into glass stock solution containers for each clothianidin test concentration. Clothianidin stock solutions were delivered to glass tanks and gravel-bed flumes housing the fish by a Masterflex peristaltic pump using Masterflex silicone tubing at a 2.0 ml/minute. The nominal concentrations of clothianidin in the treatment tanks/gravel-bed flumes were achieved by each tank/flume receiving a water flow rate of 95 ml/minute and a pesticide stock solution flow rate of 2.0 ml/minute. The pesticide and water inflow rates were monitored every 48 hours and adjusted if necessary throughout the entire duration of the exposure experiments. The actual clothianidin water concentrations were measured by Dr. Chris Metcalfe (Trent University, Ontario, Canada) in one of the replicate glass tanks and gravel-bed flumes per test concentration on 70 dpf.

Water temperature, dissolved oxygen concentration, pH and conductivity were measured every 48 hours using an HQd Portable Meter (Hach Company, Loveland, CO, USA). Ammonia concentrations were monitored every two weeks using Seachem MultiTest Ammonia Test Kit (Seachem Laboratories, Madison, USA), and the free ammonia level was below the detection limit (0.05 mg/L) in all of the glass tanks and gravel-bed flumes thoughout the experiment.

2.4. Measurement of Survival, Hatching and Emergence

The survival of embryos/alevins in both exposure systems was monitored every 48 hours post-fertilization until eyed embryo developmental stage. On 28 dpf, eyed eggs were counted to assess the fertilization rates and embryonic survival in both systems. The survival monitoring in the glass tank exposure system was daily until termination. However, survival monitoring in the gravel-bed flume system was not possible until termination since the eggs were buried in the gravel at the eyed egg stage. Eggs or eyed embryos that were opaque were considered dead and were removed daily. Daily survival in glass tanks was determined by calculating the proportion of surviving swim-up fry to the total eyed embryos buried on 28 dpf. Survival success in glass tanks was determined on 119 dpf when the swim-up fry developmental stage (i.e. complete yolk sac resorption) was reached in the control replicates.

The development of alevins was monitored daily in the glass tank system while eyed eggs were buried in gravel-bed flumes for fish development and no further observations could be made until emergence and swim-up out of the gravel. Hatching started on 49 dpf in glass tanks and emergence started on 88 dpf in gravel-bed flumes. Examination of hatching and emergence was daily during the hatching and swim-up stage. In the gravel-bed flume system, the number of emerged fry were recorded in each sub-compartment and the order of observations of each treatment was random minimizing the effect of disturbance, which could cause systematic error. Emerged fry were captured daily using a small fishing net and placed in covered/netted cylindrical egg containers and maintained on top of the gravel in corresponding sub-compartments until termination.

The daily percent of hatch was calculated by the number of hatched alevins divided by the total eyed embryos on 28 dpf. The hatching at the end of the experiment, on 119 dpf, was calculated in glass tanks to evaluate the hatching success. In addition, the 10th percentile (H_{10}), the 50th percentile or median (H_{50}) and 90th percentile (H_{90}) was determined based on the total emergence count per treatment according to Sternecker 2010 [83]. The average time required for alevins in replicates to reach the H_{50} and H_{90} and the duration between the H_{10} and H_{90} were examined in order to compare the timing of hatching across treatments and genetic crosses.

To examine the swim-up performance in fry, the daily emergence rate was calculated by the number of swim-up fry divided by the total eyed embryos buried on 28 dpf. The emergence rate before the termination was determined as the recovery rate in each treatment.

2.5. Morphometric and Deformity Analyses

Termination was conducted when the yolk sac was absorbed in 87-98% alevins in the control tank. Fish were placed in an observation container to examine skeletal and swim abnormalities and then individuals were euthanized with an overdose of tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd., Nanaimo, B.C., Canada, 0.4 g/L) buffered with sodium bicarbonate to pH 7.0-7.5 on 118-119 dpf and 110-119 dpf in the glass tanks and gravel-bed flumes, respectively. Fish were then weighed, snout to fork length was measured, external deformities were assessed followed by archiving livers or whole bodies by snap freezing on dry ice. The condition of the fry was determined by calculating Fulton’s condition factor (K): $K=100W/L^3$ where W= Wet weight of fry (g) and L=Length of fry (cm) [84], [85]. Some of the individual severely deformed fry showing significantly lower body length and body weight (outliers) were removed in the statistical analysis.

Four main categories of deformities (skeletal, craniofacial, finfold and edema) were assessed under a dissecting microscope immediately after euthanization for each individual fish according to the method described by Rudolph et al. (2006) [86]. Briefly, a skeletal deformity was defined as an abnormality of the backbone, and included lordosis

(inward curvature of backbone), kyphosis (outward curvature of backbone) and scoliosis (lateral curvature of backbone). A reduction in size or malformed eye or jaw were classified as a craniofacial deformity. Malformed fins, reduced sized of fins or missing fins were considered a finfold deformity. Edema was identified as fluid accumulation in the head or pericardial cavity. The severity of the deformities were scored using the graduated severity index (GSI) also described by Rudolph et al. (2006) [86]. This approach assigned a score for each of the deformity categories from 0 to 3 based on the severity of deformity (0 = absence for deformity, 1 = mild, 2 = moderate, and 3 = severe). A GSI of 3 represents a severe deformity that would adversely affect the survival or fitness of the fry. Each fry was assessed and given a GSI score from 0 to 3 for each category of deformities. The deformity rate for each category was determined by the proportion of deformed swim-up fry to total swim-up fry in the glass tanks or the section of gravel-bed flumes upon termination of the experiment.

2.6. Biochemical Analyses

Whole body concentrations of 17β-estradiol and testosterone per gram of body weight were quantified in fish from the glass tank exposure system in one genetic cross (cross A). Five fish from the two replicate glass tanks from each of the five treatments were included in this analysis (n = 2; 5 individual swim-up fry per tank).

Homogenization and extraction of hormones from swim-up fry whole bodies for both 17β-estradiol and testosterone hormone assays were performed according to Arukwe et al. (2008) [87]. Briefly, frozen swim-up fry were thawed on ice, homogenized in 1 mL of Na-phosphate buffer (100 mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol at pH 7.4), followed with 14,000 x g centrifuge for 15 min at 4°C. Although not included in this thesis, 150 μl of supernatant was removed after the centrifugation step and stored at -80 ^{o}C for future protein based analyses. The remaining supernatant was extracted by phase separation using diethyl ether on acetone/dry ice bath. First, the supernatant was mixed with 3 ml of diethyl ether by vortexing. After the phase separation, the aqueous phase was frozen in an acetone/dry ice bath, and the upper lipophilic ether phase was then transferred into a clean tube. This extraction procedure was repeated twice and then the ether was allowed to evaporate under a nitrogen gas

stream. The dry extracts were re-suspended in 350 μl of EIA Kit buffer (Cayman Chemical Company, Item No. 400060, Michigan, U.S.). Estradiol and testosterone levels of the five cross A whole body hormone extracts from two replicate glass tanks were measured using enzyme immunoassay kits: Estradiol ELISA Kit and Testosterone ELISA Kit (Cayman Chemical Company, Michigan, USA, Item No. 582251 and 582701, respectively) according to the manufacture's protocol. Briefly, multiple 96-well assay plates were used on the same day using all of the same reagents and standards, and each plate included the following: duplicate blank wells; duplicate non-specific binding wells; triplicate maximum binding wells; duplicate 8-point standard curve concentrations (6.6 pg/ml to 4,000 pg/ml estradiol or 3.9 to 500 pg/ml testosterone); and, a unique set of duplicate whole body samples. An EPOCH2 microplate reader (BioTek Instruments Inc., Winooski, Vermont, USA) and Gen 5.02 Software (BioTek Instruments Inc., Winooski, Vermont, USA) were used to read the absorbance of the assay in 96-well microplates at 70 and 65 min for estradiol and testosterone, respectively. Hormones concentrations in samples were quantified using the standard curve for estradiol and testosterone which was linearized by logit transformation (logit (Sample binding/maximum binding)) according to the manufacturer's protocol.

Several quality assurance/control measures were undertaken during the hormone ELISA procedures. The degree of difference between measurements was expressed by coefficient of variability (CV), which was calculated by the standard deviation divided by the mean. The intra-assay CV, the variability of sample measurements on different wells within the same plate, was the average of all individual CVs of duplicates on a microplate. In addition, a set of two known concentrations prepared from the hormone stock and another set of 2-fold dilution from the same batch of whole body samples of each tank were tested in duplicate on the same plate to analyze intra-assay variation. The mean intra-assay variation was 11.2% for estradiol and 11.0% for testosterone. As samples were run on multiple microplates and each microplate had its own calibration for the standard curve, plate-to-plate consistency was calculated by averaging the CVs of the same sample with known concentration (102.4 and 256 pg/ml for estradiol and 102.4 and 256 pg/ml for testosterone) on different plates. The overall inter-assay CV for estradiol and testosterone was 16.1% and 14.5%. The lower limit of detection was approximately 20 pg estradiol/ml and 6 pg testosterone/ml.

The extraction efficiency was determined by homogenizing, extracting and resuspending 1,600 pg/ml of testosterone in an identical procedure except no whole fish samples were added. Extraction of spiked samples was performed in triplicate and the concentration of spiked extracts were quantified in duplicate on the same microplate tested for whole body testosterone. The average recovery efficiency was 76 ± 5 % (mean ± standard error of 3 spiked samples).

2.7. Statistical Analyses

A split-plot design with two replicates was employed in a randomized complete block design to test the effect of pesticide concentration and genetic cross on the development of sockeye salmon in this study. The test concentration was the main plot, and genetic cross was the second factor applied to sub-plots within the whole main plots within each block (Figure 2). Each block (one replicate of glass tanks and gravel-bed flumes) contained all five clothianidin concentrations (0, 0.15, 1.5, 15 and 150 µg/L) assigned at random while the five main plots within a block (glass tanks or sections of gravel-bed flumes) for each test concentration were further divided to four sub-plots housing all four genetic crosses (A, B, C and D). Due to the isolated water system in the main plot, each main plot was slightly different exposure condition (pH, dissolved oxygen level, conductivity, ammonia level, water temperature and pesticide exposure) so using a split-plot design allows detection of any statistical difference of genetic effect on the fish under the same exposure condition. In the glass tank exposure system, each tank was tested with different test concentrations while all crosses housed separately within the tank receiving the same pesticide exposure allowed the sub-plot effect to be tested (Figure 3). Similarly, in gravel-bed flumes, the main plot effect of pesticide concentration on fish development was tested in five different isolated sections of the gravel-bed flume. The stainless steel dividers between compartments housing different crosses of alevins/fry allowed water and pesticide movement so the condition of pesticide exposure remained the same for all crosses of alevins/fry within a section allowing evaluation of sub-plot effect. In addition, the two replicates of glass tanks or gravel-bed flumes were located in opposite sides in the fish room (Figure 3) so blocking effect was also examined to assess if there was any disturbance by air ventilation and different light

level due to different location of the room. Moreover, since the fish embryos were randomly assigned to the set of glass tanks/ gravel-bed flumes and the glass tank/ section of gravel-bed flume, there were two random factors of egg arrangement to blocks and main plots applied in the statistical analyses. Although split-plot randomized complete block (SS-RCB) design requires more care in handling than a pooled test, it could minimize the noise of the variation between replicates at the same concentration and also detect any significant difference caused by genetic variations.

This SS-RCB design allowed to evaluate the effect of pesticide concentrations and genetic variations on survival, hatching, emergence, growth, deformity and steroids hormone levels. A 2-factor-split-plot-analysis of variance (ANOVA) followed by a Tukey's post hoc test ($p < 0.05$) on two replicate glass tanks/gravel bed flumes was performed on the following endpoints to analyze the interaction between the factors and two main fixed factors of the pesticide concentration and genetic cross: % survival of eyed embryos; % survival success; % emergence; timing and duration of hatching (the time at H_{50} and H_{90} and the duration between H_{10} and H_{90}); average body weight and mass; average condition factor; and, proportion of deformities. Since only one of the genetic crosses was used tested for whole body hormone concentrations, the difference in mean hormone level was analyzed by a randomized complete block (RCB) ANOVA with the fixed factor of treatment concentration and blocking effect followed by a Tukey's post hoc test ($p < 0.05$). Even though interactions between treatment and genetic cross were observed for some endpoints, only the effect of clothianidin concentration and genetic cross are presented. Cross D was not considered in any analyses due to low average survival rate (26% in control tanks). All statistical computations were performed by JMP 13.1 Statistical Discovery from SAS software package (SAS Institute Inc., Cary, North Carolina, United States).

Chapter 3. Results

3.1. Fertilization and Survival to Eyed Embryo Stage

The control treatment demonstrated high survival (mean= 88.4 ±SE 5.7% and 93.5 ±SE 1.6 % in glass tanks and gravel-bed flume, respectively) up to the eyed embryo developmental stage, indicating a high fertilization success rate for all crosses prior to the exposure period. Survival to the eyed embryo stage after exposures to clothianidin were initiated ~1 hour post-fertilization in all treatments was ~90% (Figure 4 and Figure 4B), and there was no effect of clothianidin on survival to the eyed embryo stage in the glass tanks or the gravel-bed flume systems (p= 0.521 and p=0.573, respectively). However, there were differences in survival between genetic crosses. The mean survival of cross A was significantly lower than the cross C and D in glass tanks (p= 0.0003; Figure 4A). Interestingly, in the gravel-bed flume system, there was no evidence of difference in survival to the eyed embryo stage in cross A between crosses C and D (p= 0.0125; Figure 4B). The accumulated thermal unit (ATU) on 28 dpf (92-100% eyes developed in the control) was 385.7 and 386.0 °C in glass tank and gravel-bed flume exposure system respectively (Table 5).

3.2. Clothianidin Exposures and Water Quality

Nominal concentrations of clothianidin were 0, 0.15, 1.5, 15 and 150 µg/L during this 119-day waterborne clothianidin exposure experiment, and the measured and predicted concentrations were similar to these nominal values. Although the flow of water and pesticide were adjusted every 48 hours, the flow did vary slightly and this is reported in Table 3. Throughout the entire experiment the water flow rate and pesticide flow rates were relatively consistent with an average of 91.5 (standard deviation (SD) 2.14) mL/min and 2.0 (SD 0.03) mL/min in each glass tank, and 92.0 (SD 0.594) mL/min

and 2.0 (SD 0.036) mL/min in each section of gravel-bed flume, respectively. The average predicted pesticide concentrations were calculated based on the measured water and pesticide flow rates every 48 hours during 119 days of exposure and are presented in Table 3. The predicted exposure concentrations were relatively close to the nominal concentrations, and the predicted concentrations consistently demonstrated a trend of increasing concentrations hovering near the targeted nominal concentrations. Likewise, the measured pesticide concentrations from a single sampling event from one replicate were close to the nominal concentrations. Indeed, many of the measured concentrations were within the range of the minimum and maximum predicted concentrations, and overall exhibited a trend of increasing concentration with increasing nominal concentrations.

Water quality including water temperature, dissolved oxygen concentration, pH and electrical conductivity were monitored and recorded every 48 hours (Table 4). The average temperature was 12.1 (SD 1.33) °C and 12.3 (SD 1.23) °C in the glass tank and gravel-bed flume systems, respectively (Table 4; Figure 5). Dissolved oxygen concentration was maintained at average of 9.90 (SD 0.70) and 10.10 (SD 0.42) mg/L in glass tank and gravel-bed flume systems, respectively. The pH was in a range of 6.90 - 8.42 and 7.00 - 8.16 in the glass tank and gravel-bed incubators. Electrical conductivity varied from 20.9 - 31.4 and 20.8 to 31.5 μS/cm in the glass tank and gravel-bed flume systems, respectively. The ammonia concentration was below 0.05 mg/L in all of the glass tanks and gravel-bed flumes.

3.3. Effects of Clothianidin on Survival from Eyed Embryo to Swim-up Fry Stage

Cross D had an extremely low survival rate of 11 and 41% in two replicate control glass tanks, and thus was excluded from further analyses. There were no significant differences in survival between any treatments ($p = 0.327$) or genetic crosses A, B and C ($p = 0.470$) upon termination at the swim-up fry developmental stage in the glass tanks. The survival of eyed embryos in the glass tanks was high, and although not significant, a dramatic decrease is clear during the hatching (Figure 6). On 51 dpf, major mortality occurred at the beginning of alevin stage, 51 dpf to 80 dpf and very few deaths were

observed during the rest of the exposure experiment. Mean survival from eyed egg to the alevin stage was 73.8% ± (standard error (SE)) 5.1% in control, while only 47.6% ± SE 4.7% survived in the clothianidin treatment groups in the glass tanks (Figure 7).

In the gravel bed flume systems only 34% of the swim-up fry at most were recovered at the end of the experiment due to the difficulty catching the fish in these systems (data not shown). The swim-up fry were hiding in the gravel and at the back of the flume most of the time during swim-up fry counts and collection attempts rendering netting unsuccessful. Therefore, with this low recovery rate, the survival rate was not reliably obtained and cannot be calculated.

3.4. Hatch and Emergence Success and Timing

Hatch success and duration was only visible in the glass tank exposure system and the daily, cumulative average % hatched is shown in Figure 8. Hatching was first observed on 49 dpf and completed by 80 dpf (Figure 8). Major hatching events occurred around the 49 dpf to 61 dpf (Figure 8). The ATU when the first hatched was observed in glass tank on 49 dpf was 658.9 and 661.4 °C in glass tanks and gravel-bed flume systems respectively (Table 5). Relatively higher average hatching success of 74.7 ± SE 5.5 % in control and 57.9 ± SE 10.2 % in 150 µg/L clothianidin treatment groups were observed, but no statistically significant effect of treatments ($p = 0.341$) or genetic crosses ($p = 0.412$) on hatching success were detected (Figure 9).

The average timing of hatching across treatments and genetic crosses is presented in Figure 10 to indicate the first hatch, H_{10}, H_{50}, H_{90} and the last hatch. In general, the H_{50} between cross A and B were similar, but the higher H_{50} in cross C suggests a possible delay in hatching for cross C. Cross C also appeared to require longer time of hatching as the length of boxes, which includes the mean time between H_{10} and H_{90}, are longer than in cross A and B. Effects on timing of hatching was further statistically evaluated by both the difference in average time to H_{50}, H_{90} and duration between H_{10} and H_{90}. Figure 11A demonstrates the mean time required for sockeye salmon to reach 50% of total hatched in each treatment. No significant effect of treatment was detected ($p = 0.886$), but there was a significant difference in H_{50} between

genetic crosses (p=0.0014; Figure 11A). Cross A and B fry reached H_{50} on 53 ± SE 0.45 dpf and 54 ± SE 0.62 dpf, while cross C was delayed 2 to 3 days to reach 50% of total hatched (H_{50} = 56 ± SE 0.88 dpf; Figure 10A). Similarly, no significant difference in the mean time to H_{90} was observed between treatments (p=0.440) (Figure 11B); however, cross C required, 67 ± SE 1.8 days, which was 9 and 6 days more than cross A and B, respectively, to reach 90% of total hatched (p=0.0003). On average, no significant effect of treatments on duration from 10% of total hatched to 90% of total hatched was observed (Figure 11C; p=0.498). However, significantly longer mean duration between H_{10} and H_{90} was detected in cross C (p= 0.0008). Cross A and B fry took an average of 7.1 ± SE 0.67 and 10 ± SE 1.5 days to reach H_{90} from H_{10}, while cross C required an average of 15-days in duration (SE= 1.8 days). In conclusion, the significant difference in average time at H_{50} and H_{90} and mean duration from H_{10} to H_{90} in cross C provided a statistical evidence of the delayed hatching in cross C as illustrated in Figure 10.

The swim-up behavior was first observed on 88 dpf in gravel-bed flume and the ATU on 88 dpf in glass tanks and gravel-bed flume systems are 1119.7 and 1125.2 ^{o}C respectively (Table 5). Unfortunately, this experiment failed to reliably assess the effect of clothianidin on the emergence of swim-up fry from the gravel because only 34% (at most) of the swim-up fry were collected at the end of the experiment due to the challenge of catching the fish (Table 6). Therefore, with this low recovery rate, the emergence success and timing of emergence endpoints were not obtained.

3.5. Morphometric Analysis

The average body weight in glass tanks and gravel-bed incubators was 0.172 ± SE 0.00221 g and 0.170 ± SE 0.00207 g, respectively (Figure 12). There was no effect of clothianidin concentration on the mean body weight in swim-up fry reared in the glass tanks and gravel-bed flume systems (p= 0.0700 and p= 0.320, respectively). However, genetic cross B swim-up fry exhibited a significantly higher body weight compared to cross A and C in both the glass tank and gravel-bed flume systems (p< 0.0001 in both systems). Similarly, the average body length of the fish in the glass tanks and gravel-bed flumes was 29.8 ± SE 0.132 mm and 29.8 ± SE 0.127 mm. There was no significant difference in mean body length between treatments in glass tank or gravel-bed systems

(p= 0.355 and p= 0.230), but the average body length in cross B was significantly higher than the other genetic crosses (p= 0.0069 and p< 0.0001).

The condition factor (K) was calculated based on the length and mass relationship of swim-up fry at the end of the clothianidin exposures to assess the effect of this pesticide on the growth of early life stage sockeye salmon. There was no significant effect of clothianidin concentration (p= 0.115 in glass tanks; p= 0.242 in gravel-bed incubators) or genetic cross (p= 0.143 in glass tanks; p= 0.765 in gravel-bed incubators) on K in sockeye salmon in both systems (Figure 13). The average condition factor in swim-up fry in the control glass tanks and gravel-bed incubators was 0.630 ± SE 0.00690 and 0.652 ± SE 0.00413 (Figure 13).

3.6. Deformities Analysis

The severity of the four main categories of deformities including skeletal, craniofacial, finfold and edema were scored from 0-3 GSI based on the angle or spinal column diversion, degree of eye or jaw malformed, degree of deformed finfold and volume of fluid accumulation immediately after euthanization, respectively, in both exposure systems. There was no deformity in finfold and edema at the swim-up fry developmental stage in both the glass and gravel-bed flume systems, and swim-up fry in gravel-bed flumes had no deformities. Only skeletal and craniofacial deformity were observed in the glass tanks. Out of 2382 swim-up fry in all experimental groups, a total of 85 swim-up fry exhibited skeletal deformities in all tanks and out of the 85 deformed fry the following deformities were observed: 39.3% kyphosis; 14.3% lordosis; 39.3% scoliosis; and, 7.14% 2-headed fish with a single body. The mean skeletal deformity rate in the glass tanks was 2.65 ± SE 1.18% in the control. There was no significant effect of clothianidin treatments (p= 0.1243) on the overall deformity rate, but the skeletal deformity rate in cross C was significantly lower than the other two crosses (p= 0.0149) (Figure 14A). The mean skeletal deformity rate in all groups in cross A and B was 4.23% ± SE 0.75% and 4.67 ± SE 0.78%, respectively, while cross C was 2 times lower than other crosses (2.12% ± SE 0.64%). The severity ranged from mild, moderate to severe at a similar ratio and fish that exhibited severe spinal deformities had obvious mobility impairment. For craniofacial deformity, a total of 103 deformed swim-up fry were

recorded with 86.2% of these fry having a reduced eye to head ratio, 9.23% had reduced pupil to eye ratio and 4.62% with a malformed head. The mean percent of craniofacial deformities in the control glass tanks was 5.02% ± SE 1.32%. There was no significant effect of clothianidin or cross on craniofacial deformities and all were assigned a severity score of 1, which is a minor malformation (Figure 14B). Thus overall, the degree of craniofacial deformities observed was mild and unlikely to impede swimming abilities.

3.7. Biochemical Analyses

To determine if sub-chronic clothianidin exposure could adversely affect hormones associated with the reproductive endocrine axis in early life stages of sockeye salmon, whole body 17β-estradiol and testosterone were measured in swim-up fry reared in the glass tank exposure system for cross A. There was no evidence of a difference in the testosterone levels ($p = 0.117$) in swim-up fry between treatments with a mean concentration of 153 ± SE 30 pg/ml/g body weight (bw) in the control and 289 ± SE 36 pg/ml/g bw in all treatments (0.15- 150 µg/L clothianidin; Figure 15A). However, swim-up fry exposed to 0.15 µg/L clothianidin had significantly higher concentrations of 17β-estradiol than any other treatments ($p < 0.0001$; $n = 2$; 5 fry/tank; Figure 15B). The mean 17β-estradiol level in the control was 1536 ± SE 368 pg/ml/g bw and it was 7312 ± SE 743 pg/ml/g bw for the clothianidin 0.15 µg/L exposed fish, which was 3 to 4.7-fold higher than other clothianidin treatments and the control.

Chapter 4. Discussion

Controlled laboratory studies examining the effects of environmentally relevant concentrations of pollutants on wild sockeye salmon are limited in the literature, and this is the first study to report the effects of the neonicotinoid clothianidin in this species. In this study, four unique genetic crosses were exposed to the clothianidin at concentrations of 0.15, 1.5, 15 and 150 µg/L initiated 1 hour post-fertilization through to the swim-up fry stage. There was high survival in the sockeye reared in the water control (mean survival in tanks= 73.8%), and no effects of clothianidin on survival were observed at any of the concentrations tested. Interestingly, several differences with respect to body size and development were evident between the four genetic crosses within the control treatments, but no adverse effects due to the clothianidin exposure concentrations tested were observed for hatch success/duration/timing, growth and deformity rates. However, the 0.15 µg/L clothianidin treatment significantly increased whole body 17β-estradiol levels in one of the genetic crosses, resulting in a non-monotonic concentration response curve. Whole body testosterone levels were unaffected by these environmentally relevant concentrations of clothianidin. It is well established that elevated circulating levels of 17β-estradiol feminize undifferentiated gonads in developing salmonids and many other teleosts. Although beyond the scope of the present study, these results indicate additional examination of clothianidin and its effects on salmonid gonad development, and the reproductive endocrine axis in general, is warranted. In addition, the significant differences observed in growth and development of the four unique genetic crosses of wild caught sockeye in this study underscores the influence of genetics on variation in apical endpoints in this species in toxicity study.

4.1. Genetic Cross Differences in Wild Sockeye Salmon

In this study, offspring from four distinct pairs of wild sockeye salmon were used to incorporate the influence of individual parentage on progeny response to clothianidin

in early life stage sockeye salmon. The results of the present study showed that at the sub-lethal concentrations of clothianidin tested, no adverse effects were evident with respect to apical endpoints (e.g. survival, hatch success, timing of hatching, morphometrics, condition factor and deformities). However, several endpoints were significantly different between crosses, specifically, embryonic survival for all four crosses, timing and duration of hatching, body length, body weight and skeletal deformity rate were significantly different between 3 of the crosses. Previous studies have demonstrated parental influence is a significant driver of offspring variation in fish populations [88]–[90], including in a BC sockeye salmon population [88]. For example, a study on the Weaver Creek, BC sockeye salmon population showed that the survival to hatch at 12 °C was 95 ± standard deviation (SD) 5%, which is comparable to our results survival (mean= 88.4 ±SE 5.7in glass tanks at average of 12.1 °C and 93.5 ±SE 1.6 % gravel-bed flume at average of 12.3 °C, respectively) [88]. Furthermore, in the Weaver Creek study, the survival to hatch with early incubation at 16 °C indicated a substantial degree of variation in responses of different genetic crosses of early life stage salmon to thermal stress [88]. In particular, the embryonic survival significantly decreased to an average of 60 ± SD 23% with a huge variation that ranged from 31.2 to 92.3% in crosses with different female and male spawners in the Weaver Creek study [88]. The early high temperature incubation in the Weaver Creek study showed a persistent high temperature effect on fry survival even if the thermal stress was removed after hatching, and the genetic cross variation in survival tended to increase as temperature increased from the optimal incubation temperature of 4 - 12.5 °C [88]. The mean fry survival at 14 °C between the 4 families varied from ≤ 75% to ≥ 90%, and at 16 °C, the mean fry survival varied even more from ≤ 25% to 80% [88]. This high variation may coincide with the present study whereby, one of the four crosses (Cross 'D') in the present study exhibited higher mortality than the other crosses and was omitted from subsequent analyses. Even though the average survival in the control for 3 out of 4 crosses was comparable to the 70% survival criteria for a the standardized toxicity test using early life stages of rainbow trout according to the Environment and Climate Change Canada [91]; indeed, the survival range of all four crosses was 19.5 to 76%, and at an early point during this study the average incubation temperature reached a maximum of 14.5 °C for ~24 hours in both systems and there were 48 hours (glass tanks) and 36 hours (gravel-bed incubators) that the temperature reached above 14 °C prior to hatching in the early

fall (Figure 4). Based on the Weaver Creek study demonstrating significantly different responses to temperature, it is also likely that significantly different responses to chemical stressors occurs in different sockeye salmon offspring sets as well. Although no significant differences were observed in average survival between treatments in this study, there was a large range in survival for all four crosses within a treatment. It is hypothesized that the different genetic compliment of the four crosses may have influenced control animal survival, and may also have contributed to the variability observed in survival between crosses within the clothianidin treatments. Future studies may be warranted with higher statistical power to verify no impacts on survival in wild sockeye salmon at the clothianidin concentrations tested.

In addition to effect on the embryonic survival, parentage appears to influence the timing and duration of hatching in sockeye salmon. In the present study, cross ‘C’ exhibited significantly delayed hatching based on the time to achieve 50% and 90% hatched in a replicate test vessel and an increased period for hatching compared to the two other crosses (‘A’ and ‘B’). This was also observed in another study on Weaver Creek sockeye salmon [88]. In particular, Weaver Creek, BC sockeye salmon exhibited genetic differences with respect to time 50% hatch and duration of hatching from 5% to 95% of hatch at both 14 and 16 ºC [88]. Furthermore, variation in fry wet mass and length between different families was also observed in the Weaver Creek study [88]. This is in line with the present study whereby cross ‘B’ exhibited a significantly larger body size based on average body length and weight, which could indicate different genetics underlying body size or a growth rate difference. Collectively, the numerous cross-specific differences in development, size and survival in the three crosses observed in the present study strongly support the hypothesis that there is considerable genetic variation due to parentage in wild sockeye salmon. In addition, it is hypothesized that this genetic variation due to parentage also influences sockeye salmon’s response to environmental stressors (i.e. thermal or chemical) and this should be considered when testing the toxicity of contaminants in this species.

4.2. Adverse Effects of Neonicotinoids in Sockeye Salmon During Early Development

The present study was designed to examine the adverse effects of clothianidin during chronic, environmentally relevant exposure scenarios. Although some variability between genetic crosses was evident, no significant differences in survival were observed during this chronic 4-month clothianidin exposure that ranged from 0.15 – 150 µg/L. These results coincide with shorter duration acute toxicity studies indicating that lethal concentrations in several fish are 3 orders of magnitude higher, with LC_{50} values of ~100 mg/L (Table 2). Additional studies in the literature also support no lethality due to sub-chronic or chronic exposure to the neonicotinoid imidacloprid at low mg/L concentrations in the fish species tested to date. For example, in a 60-day toxicity test reported no effect on survival in rainbow trout (*Oncorhynchus mykiss*) at 19,000 µg/L imidacloprid [92]. Similarly, no significant reduction in survival at low level of exposure was reported in a 98-day imidacloprid exposure (1,300 – 20,000 µg/L) on newly fertilized rainbow trout embryos in a flow-through system [93]. Furthermore, exposure to even a higher concentration, 320,000 µg/L of imidacloprid, did not induce any toxicity in zebrafish during larval development [94]. The results of the present study suggest that clothianidin is not lethal at concentrations equal to or below 150 µg/L during early life stage development of sockeye salmon, and the concentrations tested would be unlikely to cause high direct mortality in sockeye salmon.

In general, fish in pesticide-polluted waters may exhibit changes in biological processes, morphometrics, individual fitness and survival, and morphometrics are commonly used to indicate how a contaminated water affects the growth of an aquatic organism [95]. Growth parameters including body mass, length and condition factor reflect overall fish health, and are the culmination of several complicated and not fully understood molecular and biochemical processes that can be influenced by environmental contaminants [95]. For example, a xenobiotic can induce reactive oxygen species causing oxidative imbalance, and this triggers metabolically expensive detoxification processes in fish, ultimately depleting the limited energy reserve in the yolk sac and results in growth inhibition [96]. Although the present study did not show any clothianidin concentration related effects on salmon growth, a reduction in growth has

been observed in studies on another neonicotinoid, imidacloprid, on other fish species. For instance, a 98-day imidacloprid exposure to newly fertilized rainbow trout eggs (*Oncorhynchus mykiss*) in a flow-through system caused a significant decrease in body length at 36 and 60 days post-hatch, while significant reduction of body weight occurred at 60 days post-hatch [93]. The LOAEC and NOAEC were determined to be 19,000 µg/L and 9,800 µg/L imidacloprid respectively [93]. Also, a 60-day study reported a LOAEC of 2,300 µg/L imidacloprid for growth inhibition for rainbow trout from the fertilized egg development stage to the juvenile stage [92]. Another 7-day toxicity test, conducted for Canadian water quality guidelines for the protection of aquatic life determined the LOAEC for growth inhibition in larval inland silverside (*Menidia beryllina*) to be 34,000 µg/L [92]. In contrast to these aforementioned higher concentration imidacloprid studies in rainbow trout and silverside, in a 3-month field study on Japanese medaka effects on fish growth occurred at a lower concentration of imidacloprid and within the range of clothianidin tested in the present sockeye salmon study [97]. Specifically, Japanese medaka were exposed to imidacloprid treated rice paddy fields, whereby water concentrations measured in the first week in field water was 33-240 µg/L and an average of 0.75 µg/L imidacloprid was measured in the following months. Adult Japanese medaka were released and reproduced in the water collected from the rice paddy fields, hence developing embryos were exposed throughout spawning and development [97]. Imidacloprid was shown to significantly reduce the weight/length ratio (i.e. condition factor) in Japanese medaka fry compared to the control [97]. This reduction in condition factor after imidacloprid exposure may reflect decreased energy reserves which has been shown to be associated with this endpoint along with an inhibition of fish growth [95]–[97]. In present study, no significant effect of clothianidin on body mass and length was detected and the resulting condition factor showed no significant difference between all treatments. This contradicts the reported effects of the neonicotinoid imidacloprid in Japanese Medaka, but whether this is due to unique modes of action for these two different neonicotinoids, species specific effects, variation in the present study due to a lack of statistical power requires further study.

No significant difference in hatching success and delay in the timing and duration of hatching were observed between the clothianidin treatments in the present study. To date, there are few studies on the effects of neonicotinoids on fish hatching, except the

60-day imidacloprid exposure in rainbow trout reported in the imidacloprid water quality guideline by the CCME [92]. The CCME reported no effect on hatching at the highest nominal concentration of 19,000 µg/L imidacloprid conducted from newly fertilized eggs to juveniles [92]. Although in the present study on sockeye salmon the highest test concentration was considerably lower than that included in the CCME guideline study, no significant effects of clothianidin (up to 150 µg/L) on both the mean hatching success, timing and duration were observed for sockeye salmon. However, some evidence exists suggesting imidacloprid exposure during early-life stages can alter the rate and timing of developmental processes in other vertebrates [29]. A 21-day 2-generation exposure of 8,800 µg/kg/day of imidacloprid-treated seed in adult red-legged partridges (*Alectoris rufa*) resulted in an oxidative imbalance compensated with elevated activity of superoxide dismutase (SOD), an enzyme that protects against the damage from oxidative stress [98]. It was hypothesized that oxidative imbalance could reduce the body condition of the parents causing indirect effects on offspring such as reduction in clutch size and delay in the first egg lay date [98]. In addition, a delay in egg laying and hatching in mallards after a chronic feeding of 240,000 µg/L imidacloprid was reported by the US EPA [99]. Perhaps more convincing with respect to the effects of neonicotinoids on vertebrate development are evident in a recent study in an amphibian. In particular, sublethal effects on larval wood frogs (*Lithobates sylvaticus*) chronically exposed to two neonicotinoids showed different results. Imidacloprid was shown to inhibit metamorphosis, the key developmental response, in wood frogs (*Lithobates sylvaticus*) with a LOAEL of 10 µg/L in an outdoor mesocosm experiment (repeated dosing weekly for a total of 7 doses) [100]. However, exposure to another neonicotinoid, thiamethoxam, at 100 µg/L showed no significant delay in metamorphosis [100]. However, thiamethoxam can degrade into clothianidin (half-life in surface water photolysis at 25 °C = 2.7- 39.5 days, half-life in field soil at 20 °C = 7.1- 92.3 days [30], [101]) between weekly dosing, therefore, these results from the wood frog study may support the lack of effects on development observed in the present clothianidin sockeye salmon study. Taken together, although all neonicotinoids are purported to have the same mechanism of action in vertebrates (i.e. weak nAChR agonists), the studies on vertebrate development may suggest unique effects of imidacloprid or different binding affinities to different vertebrate nAChRs, and hence, different potencies and adverse effects compared to thiamethoxam. Since few studies have examined multiple

neonicotinoids thoroughly in individual vertebrate species aimed at sub-lethal effects such as development, future studies discerning species-specific sub-lethal effects and potential unique effects/modes of action of different neonicontinoids are needed.

The event and timing of emergence from a redd are crucial to salmonid fry survival, but the present study failed to recover enough swim-up fry to assess this sub-lethal endpoint. Once the energy reserve in yolk sac are almost depleted, swim-up fry have to leave the protection of the gravel and start searching for food [102]. For salmonids, swim-up fry start defending their feeding territories once emergence from the redd is complete, and the timing of emergence can vary by weeks within individual fry in the same redd [102]. Fry that are the first to emerge have prime access for feeding territories and have a competitive advantage over those that emerge later [102]. Thus, disruption to the timing of emergence due to chemical exposure could have fitness consequences and ultimately affect fish survival [102]. One study tested the effects of 96 hour exposures to 3, 30 and 300 µg/L of clothianidin, imidacloprid, thiamethoxam or a mixture of equal parts of these 3 neonicotinoids on short burst swimming behavior in juvenile sockeye salmon and reported no significant effects on swim behavior after these acute exposures [103]. However, whether adverse effects would be observed in earlier life stage larval sockeye salmon prior to and during emergence remains unknown. Interestingly, imidacloprid has been shown to impact swimming activity in larval zebrafish (*Danio rerio*). In the zebrafish study, Crosby et al. (2015) conducted neurobehavioral tests in larvae exposed to 11,200 or 15,000 µg/L imidacloprid from 4 hours to 5 days post-fertilization [104]. Both concentrations significantly reduced the distance travelled in the dark phases, and the effect was persistent through adolescence and into adulthood [104]. The exposed fish had decreased novel tank exploration behavior at both 1.5-month old adolescent and 3-month old adult stages, and the startle response increased in adolescent [104]. Therefore, these zebrafish exposed to imidacloprid might have altered behaviors, but the mechanism is not yet clear [104]. It was hypothesized in the present study that clothianidin exposed fish might engage more in the gravel bed and overreact to any stimuli during emergence, however the ability to reliably capture and count emerged fry in the present study was not achieved. For salmonid fry, light is a directive factor and lighting pattern can affect their diel vertical migration behavior [105]. Since visual acuity and capture efficacy of fry predators

improve as the light level increases, fry normally remain in the gravel or under shady area during daylight [3]. Due to this predator avoidance tactic, peak swim up generally occurs during the darkest hours of night [3]. As a result, this diel behavior made the daily count of swim-up fry difficult and the count was low in this study. In the future, fry count can be improved by increasing the number of counts per day and performing these collections solely in the dark using low intensity (5 lux) red lighting [105].

In the present study, the mean skeletal and craniofacial deformity rates in early life stage sockeye salmon were low after chronic clothianidin exposure and fell within the background rate observed in the control animals reared in clean water. Baseline deformity rates of 2 - 5% in salmonids spawned in a laboratory setting is typical, and deformities are influenced by many factors including genetic variation, parasite infections and elevated water temperatures [106]. Another study of a neonicotinoid, imidacloprid, in a field exposure scenario showed no significant increase in skeletal deformities in Japanese medaka [97]. This medaka study was a chronic exposure (3 months) with measured concentrations of 33-240 µg/L imidacloprid in the initial week and 0.75 µg/L imidacloprid measured in subsequent weeks in experimental rice fields [97]. However, imidacloprid was found to delay ocular development in some other aquatic species (fish and amphibian). For example, incomplete eye development was observed in zebrafish embryos exposed to imidacloprid at a concentration of 380 µg/L (inhibited 50% of eye development) [94]. In addition, in larval African clawed frog (*Xenopus laevis*) exposed to imidacloprid for 96 hours an increased frequency of craniofacial deformities was observed with a NOAEC of 10 µg/L [107]. In the same study, a 1000 µg/L imidacloprid significantly induced irregular retinal pigment epithelium and retina fracturing in larval frogs [107]. Similarly, 96-hour exposure of 5,000 µg/L of imidacloprid significantly increased the frequency of abnormal eye shape in the Northern leopard frog embryos (*Lithobates pipiens*) [107]. In contrast in the present study in gravel bed and glass tank systems, no significant eye deformities were observed in developing sockeye salmon. It is worth noting that no deformity was recorded in gravel-bed flume incubations in this study because fewer fish were recovered and those with deformity were unlikely to swim up. Overall, there is some evidence that craniofacial deformities are prevalent in fish and amphibians after imidacloprid exposure, and this is the first study to report clothianidin

exposure from 1 hour post-fertilization through to swim-up fry tested up to 150 μg/L does not appear to cause sockeye salmon deformities.

This study revealed that a concentration of 0.15 μg/L clothianidin significantly elevated whole body 17β-estradiol concentrations up to 4.7-fold in sockeye salmon swim-up fry, with no significant concomitant increase in testosterone. This is the first study to report a change in sex steroid hormones in a fish after a neonicotinoid exposure and these data show a non-monotonic concentration-response curve, which is commonly reported for chemicals impacting endocrine system endpoints. Although no studies in non-mammalian vertebrates on sex steroid levels after neonicotinoid exposure have been reported, studies in mammals in vitro and in vivo thus far show effects on reproductive endocrine axis hormones but a consistent pattern is not evident and may be due to various factors (i.e. tissue type, duration, exposure route, species, etc.). For example, a 24-hour exposure of imidacloprid (LOAEL= 10 μM), thiamethoxam (LOAEL= 0.1 μM) and thiacloprid (LOAEL= 0.1 μM) significantly increased aromatase activity and estradiol biosynthesis in co-culture of human adrenocortical carcinoma (H295R) cells and BeWo human choriocarcinoma cells [108]. Likewise, another 24-hour in vitro exposure of thiacloprid and thiamethoxam induced aromatase activity at concentrations of 0.1-1.0 μM with decreasing catalytic activity at a higher concentration in H295R cells and exhibited a non-monotonic response curve [109]. One in vivo study showed that imidacloprid reduced testosterone levels and epididymis mass in male rats exposed to 0.5 mg/kg for 90 days [110]. Bal *et al.* hypothesized that in these rats imidacloprid, an AchR agonist, somehow interacts with the gonadotropin-releasing hormone (GnRH) from the hypothalamus to reduce the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from anterior pituitary causing the inhibition of testosterone secretion in Leydig cells and spermatogenesis in testes [110]. In a female rat study sex steroids were not measured, however, oral administration of 20 mg/kg/day imidacloprid for 90 days significantly induced hormone imbalance (LH, FSH and progesterone) and ovarian oxidative damage, decreased weight and patho-morphological changes [111]. Although an absence in change in testosterone levels in fish this study, there are evident of the effect of neonicotinoids on aromatase activity in mammals, and additional studies are required to show that clothianidin is directly or indirectly influencing 17β-estradiol levels at the conversion of testosterone to 17β-estradiol via the enzyme aromatase or

decreased degradation pathways of this sex steroid hormone in fish. Regardless of the mechanism increasing 17β-estradiol observed in the present study in sockeye salmon, this combined with the evidence from mammalian studies suggests further inquiry is warranted to examine the adverse effects of clothianidin on the reproductive endocrine axis. Indeed, xenoestrogens in particular that mimic endogenous estrogens have been shown to cause population level outcomes in fish. One of the most definitive studies on a xenoestrogen involved chronic exposure of a fathead minnow population to 5-6 ng/L 17α-ethynylestraidol (a synthetic estrogenic hormone used in birth control pill) in an experimental lake and caused significantly increased intersex in males, ovarian follicle degeneration in females and ultimately a population level collapse and near extinction of this species from the lake [112]. Indeed, at least 105 pesticides, including atrazine (a herbicide in maize and sugarcane crops), and DDT (a banned insecticide used mainly in 1950s), are identified as endocrine disruptors or chemicals that interfere with the normal function of the endocrine system [113]. To more fully assess clothianidin for endocrine disrupting activity, future studies should include testing clothianidin in the suite of the standardized test methods available for the Testing and Assessment of Endocrine Disrupters listed in the Organization for Economic Cooperation and Development Conceptual Framework [114].

4.3. Conclusion

Pesticides are important inventions to agriculture aimed at increasing the yield of food production, but these chemicals tend to contaminate the environment and pose a risk to wildlife at the same time. Clothianidin is a new synthetic chemical evolved from the botanical pesticide, nicotine. It is used globally for a wide range of crops and it is mainly for corn and soybean seed treatment in Fraser Valley. High amounts of pesticides are used annually in the Fraser Valley due to the high density of agricultural lands in this region. The present study examined the adverse effects of clothianidin at low level, environmentally relevant concentrations on wild sockeye salmon and has several key findings. The first is that there is significant variation in size and development in wild caught salmon genetic crosses that should be taken into account when investigating adverse effects of contaminants in this species. One of the limitations

of the present study was a duplicate test vessel experimental design, and increasing replicates to improve statistical power in light of this significant natural variation between genetic crosses is recommended in the future to corroborate the results of this study. Nonetheless, although this variability between genetic crosses was evident, exposures up to 150 µg/L clothianidin did not affect the survival, growth or development of sockeye salmon in early life stages. This was unexpected in light of growth inhibition and reduction in body length, weight and condition factor that were previously reported for another neonicotinoid, imidacloprid in fish and frogs at similar test concentrations. However, different responses in metamorphosis in larval wood frogs after 2 different neonicotinoid exposures suggest different neonicotinoids may have unique potencies or modes of action and this warrants further examination. No significant deformities was observed in present study up to 150 µg/L clothianidin, although approximately 2-fold and 10-fold higher concentrations of a different neonicotinoid (imidacrloprid), ocular related craniofacial deformities in zebrafish embryos and larval African clawed has been reported. Lastly, the present study is the first to demonstrate an increase in 17β-estradiol levels in a developing teleost after chronic, low level clothianidin exposure, thus future studies examining the potential disruption of the reproductive endocrine axis function or development in teleosts are merited.

Tables

Table 1 The mean and maximum level of clothianidin detected in worldwide aquatic samples.

Location	Clothianidin Level (µg/L)		Sample Type	Frequency	Date	Reference
	Mean	Max				
Southwestern Ontario, Canada	2.28	43.60	Puddle water near maize farms	100% of 76 samples	2013	[59]
Prairie wetlands of central Saskatchewan, Canada	0.0424 (Summer); 0.0327 (Spring)	3.11 (Summer); 0.173 (Spring)	Ponds near canola crops	61% in summer; 91% in spring	2012-2013	[60]
Quebec, Canada (corn farms)	4.6	55.7	Puddle water in corn field	92% of 25 samples	2012-2013	[115]
Illinois, USA (corn and soybean farms)		0.850 (run-off); 0.060 (groundwater); 0.203 (soil water)	Run-off; groundwater; soil water		2011-2013	[65]
Iowa, USA (corn and soybean farm)	0.0082	0.257	River	75% in 79 samples (9 sites)	2013 (Mar-Oct)	[67]
Indiana, USA (corn and soybean crops)	0.10	0.67	Lentic water bodies	96% of 48 samples	2015	[81]
Omaha, NE, USA ; Fulton/ Iowa/	0.066	0.132	River stream	24% of 38 sites	2012-2014	[66]

Location	Clothianidin Level (µg/L)		Sample Type	Frequency	Date	Reference
	Mean	Max				
Wapello/ Sioux City/ Garber, IA, USA						
Sydney, Australia (Horticulture and vegetable fields)	0.06	0.42	River	53% in 13 rivers	2013 (Jan-Feb)	[63]
Osaka, Japan	0.0035	0.012	River	91% of 26 sites	2009-2010	[64]

Table 2 The summary of acute, subchronic and chronic toxicity studies reported by government agencies and in the peer-reviewed literature

	Species	Exposure route	Study Duration*	Effects	Toxicity Endpoint	Reference
Invertebrates	Honey bee (*Apis mellifera L.*)	Oral	Acute (24 h; 48 h; 72 h)	Mortality	LD_{50}= 3.53 (24 h); 3.35 (48 h); 3.28 (72 h) ng/bee	[116]
	Honey bee (*Apis mellifera*)	Oral	Acute	Mortality	LD_{50}= 3.7 ng/bee	[73]
	Honey bee (*Apis mellifera*)	Dermal	Acute	Mortality	LD_{50}= 4.43 ng/bee	[41]
	Italian honey bee (*Apis mellifera* ligustica)	Oral	Acute (72 hours)	Mortality	LD_{50}= 4.671 ng/bee	[117]
	Monarch Butterfly (*Danaus plexippus*)	Oral	Acute (1.5 days)	Mortality	LD_{50}= 15.63 µg/L	[118]
	Earthworm (*Eisenia fetida*)	Contact; treated soil	Acute (48 hours)	Mortality	LD_{50}= 0.23 $\mu g/cm^{-2}$ (contact); 6.06 mg/kg (Treated soil)	[119]
	Earthworm (*Eisenia fetida*)	Treated soil	Sub-chronic (14 days)	Mortality	LD_{50}= 15.5 mg/kg	[80]
	Earthworm (*Eisenia fetida*)	Treated soil	Chronic (28 days)	Oxidative stress (Increased reactive oxygen species level; upregulated HSP70 gene expression)	LOAEL= 0.5 mg/kg	[120]
Mammals	Mouse (*Mus musculus*)	Oral	Acute	Mortality	LD_{50}> 389 mg/kg	[43]
	Mouse (*Mus musculus*)	Oral	Acute	Mortality	LD_{50}= 465 mg/kg	[58]
	Norway Rat (*Rattus norvegicus*)	Gavage	Sub-chronic (90 days)	Oxidative stress (Reduce in glutathione level)	LOAEL= 2 mg/kg/bw/d	[70]
	Norway Rat (*Rattus norvegicus*)	Gavage	Sub-chronic (90 days)	Genotoxic (DNA fragmentation)	LOAEL= 32 mg/kg/bw/d	[70]

	Species	Exposure route	Study Duration*	Effects	Toxicity Endpoint	Reference
	Norway Rat (*Rattus norvegicus*)	Gavage	Sub-chronic (90 days)	Reproduction (Decrease in serum testosterone level)	LOAEL= 32 mg/kg/bw	[70]
	Norway rat (*Rattus norvegicus*)	Gavage	Sub-chronic (90 days)	Reproduction (decrease in weight of epididymis and seminal vesicles)	LOAEL= 32 mg/kg/d	[70]
	Norway Rat (*Rattus norvegicus*)	Gavage	Sub-chronic (90 days)	Reproduction (decrease in epididymis weight)	LOAEL= 2 mg/kg/bw/d	[71]
	Rabbit (*Sylvilagus sp.*)	Oral	Chronic	Development (increase in premature births, litter incidence of missing lung lobes); Reproduction (decrease in uterine weight)	LOAEL= 75 mg/kg/d	[43]
Birds	Japanese quail (*Coturnix japonica*)	Oral	Acute (14 days)	Mortality	LD_{50}= 423 mg/kg/bw	[121]
	Mallard (*Anas platyrhynchos*)	Oral	Acute	Mortality	LD_{50}>752 mg/kg/bw	[73]
	Bobwhite (*Colinus Virginianus*)	Subacute dietary	Acute (8 days)	Mortality	LC_{50}≥ 5230 mg/L	[73]
	Bobwhite (*Colinus Virginianus*)	Oral	Acute (14 days)	Mortality	LD_{50}> 2000 mg/kg/bw; NOEL= 500 mg/kg	[122]
	Bobwhite (*Colinus Virginianus*)	Oral	Chronic	Reproduction (decreased in eggshell thickness)	NOAEC= 205 mg/L, LOAEC= 525 mg/L	[73]

	Species	Exposure route	Study Duration*	Effects	Toxicity Endpoint	Reference
	Quail (*Coturnix japonica*)	Oral	Chronic (30 days)	Genotoxic (DNA fragmentation in seminiferous tubules); development (decrease in embryonic length)	LOAEL= 50 mg/kg/bw/d (Genotoxic); 1 mg/kg/bw/d (Development)	[72]
	Mallard (*Anas platyhynchos*)	Oral	Acute	Mortality	LD_{50}> 752 mg/kg/bw/d	[121]
Aquatic invertebrates	Mysid Shrimp (*Americamysis bahia*)	Waterborne	Acute (96 h)	Mortality	LC_{50}= 0.051 mg/L	[52]
	Midge (*Chironomus riparius*)	Waterborne	Acute (48 h)	Mortality	EC_{50}= 0.022 mg/L	[73]
	Waterflea (*Daphnia magna*)	Waterborne	Acute (48 h)	Mortality	LC_{50}> 119 mg/L	[73]
	Waterflea (*Daphnia magna*)	Waterborne	Chronic	Reproduction	NOAEC =0.042 mg/L; LOAEC= 0.12 mg/L	[73]
Fish	Rainbow trout (*Oncorhynchus mykiss*)	Waterborne	Acute (96 h)	Mortality	LC_{50} >105 mg/L	[52]
	Rainbow trout (*Oncorhynchus mykiss*)	Waterborne	Acute (96 h)	Mortality	LC_{50} >104.2 mg/L	[121]
	Rainbow trout (*Oncorhynchus mykiss*)	Waterborne	Acute (96 h)	Mortality	LC_{50} >110 mg/L	[73]
	Bluegill sunfish (*Lepomis macrochirus*)	Waterborne	Acute (96 h)	Mortality	LC_{50} >117 mg/L	[52]
	Fathead minnow (*Pimephales promelas*)	Waterborne	28 days	Development (decrease in length and dry weight)	NOAEC= 9.7 mg/L ; LOAEC= 20 mg/L	[45]
	Sheepshead minnow (*Cyprinodon variegatus*)	Waterborne	Acute (96 h)	Mortality	LD_{50} >93.6 mg/L	[52]

	Species	Exposure route	Study Duration*	Effects	Toxicity Endpoint	Reference
Aquatic plants	Green Algae (*Selenastrum capricornutum*)	Waterborne	5 days	Biomass	NOAEC= 3.5 mg/L; EC_{50}= 64 mg/L	[73]
	Duckweed (*Lemna gibba*)	Waterborne	14 days	Necrotic fronds	NOAEC= 59 mg/L; EC_{50}> 121 mg/L	[73]

*If the study duration was not specified, acute/sub-chronic/chronic was defined by the author of the literature/reports.

Table 3 Predicted and measured concentrations of clothianidin in glass tanks and gravel-bed flumes in flow through systems during chronic clothianidin exposures of sockeye salmon from 1 hour post-fertilization to the swim-up fry developmental stage.

Predicted values were based on measured pesticide and water flows into the flow through systems every 48 hours throughout the exposure period. Measured concentrations were based on 1 sampling event collected from 1 of 2 replicates, and submission to Dr. C. Metcalfe (Trent University, ON, Canada) for analysis.

Exposure System*	Nominal Concentration (µg/L)	Predicted concentration (µg/L)				Measured concentration (µg/L)
		Average±SE	Minimum	Maximum	N	
GBF	0	0.00±0.00	0.00	0.00	43	0
	150	158±2.25	136	227	43	114
	0.15	0.157±0.002	0.124	0.198	43	0.230
	15	15.8±0.42	0.766	20.8	43	28.9
	1.5	1.57±0.02	1.30	1.78	43	1.08
	15	15.6±0.27	11.3	18.7	43	
	0	0.00±0.00	0.00	0.00	43	
	150	154±1.75	132	185	43	
	0.15	0.151±0.002	0.125	0.172	43	
	1.50	1.53±0.023	1.17	1.90	43	
GT	0.15	0.156±0.0033	0.122	0.226	44	
	150	146±2.46	110	189	43	
	15	16.3±0.47	9.83	24.9	44	15.0
	1.5	1.65±0.06	0.950	3.21	44	
	0	0.00±0.00	0.00	0.00	44	
	15	15.2±0.29	11.2	19.8	44	5.36
	0	0.00±0.00	0.00	0.00	44	0.00
	150	161±4.35	99.7	245	44	165
	1.5	1.53±0.037	0.787	2.04	44	0.750
	0.15	0.159±0.0056	0.0712	0.269	44	0.140

*GBF, gravel-bed flume; GT, glass tank

Table 4 Water quality monitoring summary from 4-month waterborne clothianidin (0, 0.15, 1.5, 15 and 150 µg/L) exposure experiment of sockeye salmon 1 hour post-fertilization to swim-up fry developmental stage.

Two flow through systems were included involving test vessels housing fish that were comprised of glass tanks or flumes filled with gravel to mimic natural salmonid rearing substrate. Water quality was monitored every 48 hours for all parameters, except ammonia (monitored every 2 weeks).

	Glass tanks					Gravel-bed flumes				
	n	Mean	Min*	Max *	SD *	n	Mean*	Min	Max	SD *
Temp (°C) *	48	12.1	9.6	15.2	1.33	45	12.3	10.2	15.0	1.23
pH	40	7.14	6.90	8.42	0.13	39	7.14	7.00	8.16	0.14
Dissolved Oxygen mg/L)	48	9.9	8.00	11.08	0.70	45	10.10	8.83	10.74	0.42
Conductivity (µS/cm)	47	26.2	20.9	31.4	4.14	44	26.2	20.8	31.5	3.94
Ammonia (mg/L)	16	0.00	0.00	0.00	0.00	16	0.00	0.00	0.00	0.00

*SD, standard deviation; Min, minimum; Max, maximum; Temp, temperature

Table 5 The accumulated thermal units (ATUs) for sockeye salmon at eyed, hatch and swim-up stage during the chronic exposure of 0, 0.15, 1.5, 15 and 150 µg/L clothianidin from 1-hour post-fertilization to swim-up fry stage in glass tank and gravel-bed flume.

Temperature was recorded every 48 hours in all glass tanks and gravel-bed flume incubators averaged respectively. The average of this temperature was used to calculate ATU (average daily temperature x days post fertilization) in both systems.

Developmental Stage	Day post-fertilization	ATU (°C-days)	
		Glass tank	Gravel-bed Flume
Eyed embryo stage (92-100% developed eyes in the control/ Day of gravel burial)	28	340.8	346.2
Alvein (Day of 1st hatched observed in glass tanks)	49	596.4	605.8
Swim-up fry (Day of 1st swim-up observed in gravel-bed flume)	88	1071.2	1088.1

Table 6 Recovery rate of emergence for sockeye salmon under clothianidin exposure (0, 0.15, 1.5, 15 and 150 µg/L) from 1-hour post-fertilization to swim-up fry development stage in gravel-bed incubators.

The number of eyed eggs was counted and buried on dpf 28. Swim-up performance was monitored in every 24 hours, and emerged fry were captured and placed in covered/netted cylindrical egg containers on top of the gravel until termination.

Clothianidin conentration (µg/L)	0	0.15	1.5	15	150
Total Number of eggs buried	388	440	458	428	410
Total Number of alevins caught	130	119	75	124	67
% recovery	34%	27%	16%	29%	16%

Figures

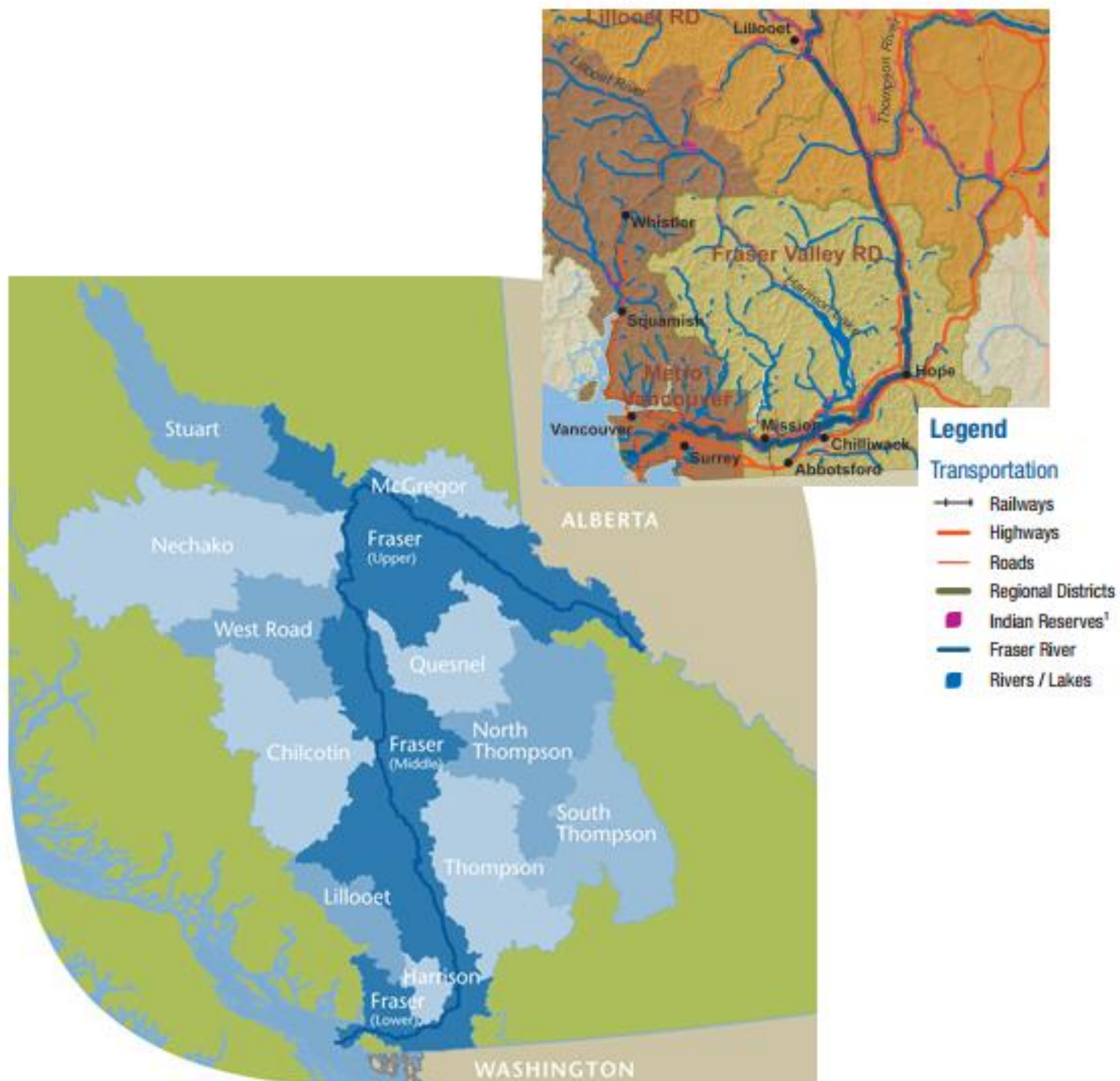


Figure 1 A Geographic map of Fraser Basin watersheds and Fraser Valley in British Columbia, Canada

The Fraser Basin contains 12 major watersheds including Fraser watershed, which is further divided into Upper, Middle and Lower Fraser watershed. Fraser watersheds drain into the Fraser River, which flows from Mount Robson near Valemount and ultimately into the Strait of Georgia at Vancouver. The Fraser Valley is located in the Southwestern region of the Fraser Basin, which is also located in the lower Fraser watershed. (Picture adapted by https://www.fraserbasin.bc.ca/resources_maps.html and https://www.fraserbasin.bc.ca/basin_watersheds.html.)

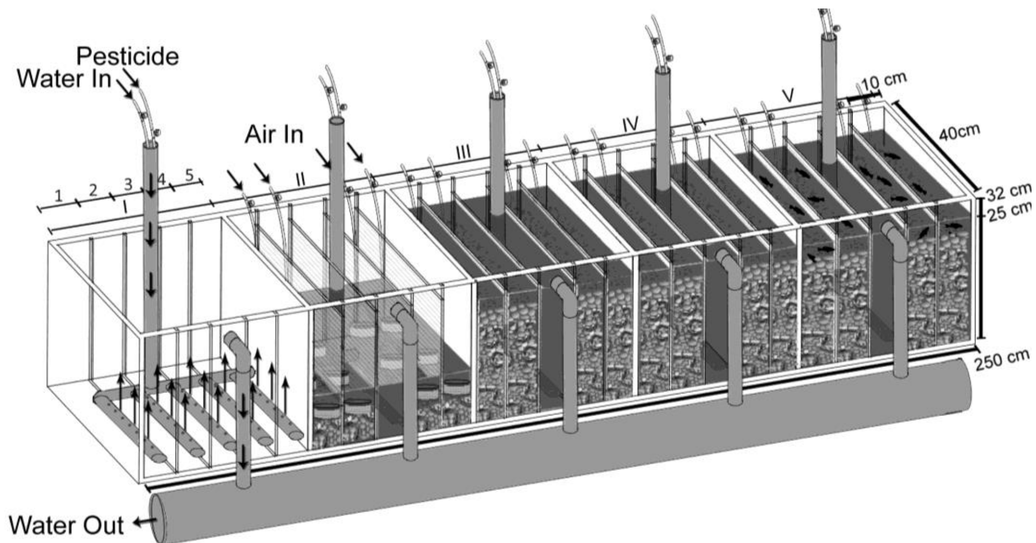


Figure 2 A schematic gravel-bed flume with the direction of water and pesticide flow.

A 320-L plexiglass tank was divided into five isolated sections (I, II, III, IV and V), with a total volume of 64 L in each section. Each section was subdivided into five sub-compartments (1, 2, 3, 4 and 5) by stainless steel mesh to individually house fertilized eyed embryos from four mating pairs of wild sockeye salmon, and sub-compartment 3 was used for test water drainage. Section I shows the direction of water movement in empty gravel-bed flume. Section II illustrates the 28 L of test water with 5 cm height of gravel filled in each sub-compartment except sub-compartment 3 (used for drainage) prior to egg burial. Each individual genetic cross was housed in 3 food graded PVC netted cylindrical egg containers and placed on top of the gravel bed in one sub-compartment. Each sub-compartment (1-5) were separated by stainless steel mesh allowing exchange of test solutions within each main sections (i.e. I-V) so that developing salmon from each of the 4 crosses within a main section received the same test solution. Once embryos reached the eyed embryo developmental stage, more gravel was added to bury the eyed embryos. Sections III, IV and V demonstrate the additional 15 cm height of gravel that was added and that test vessel volume always remained at 28 L. In Section V, emerged fry are shown above the gravel bed.

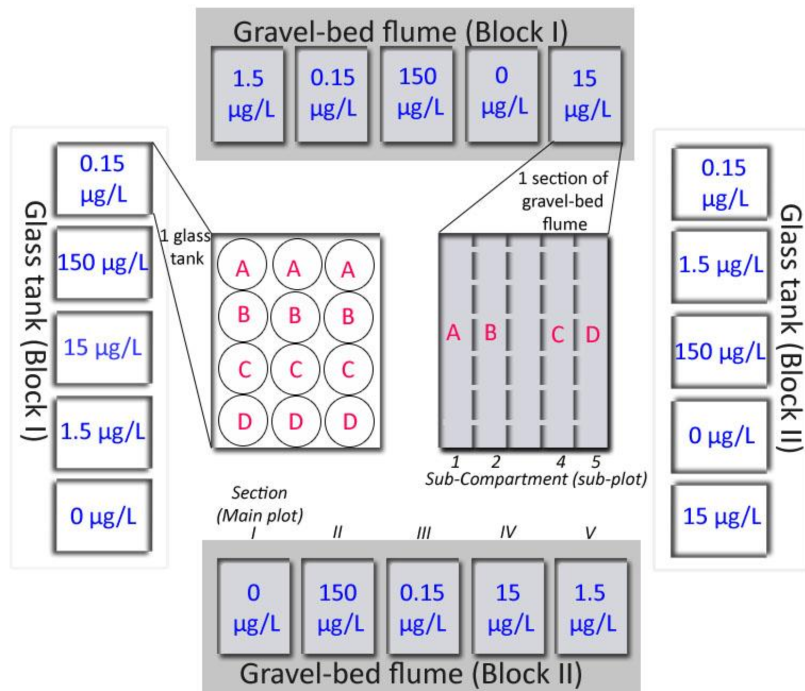
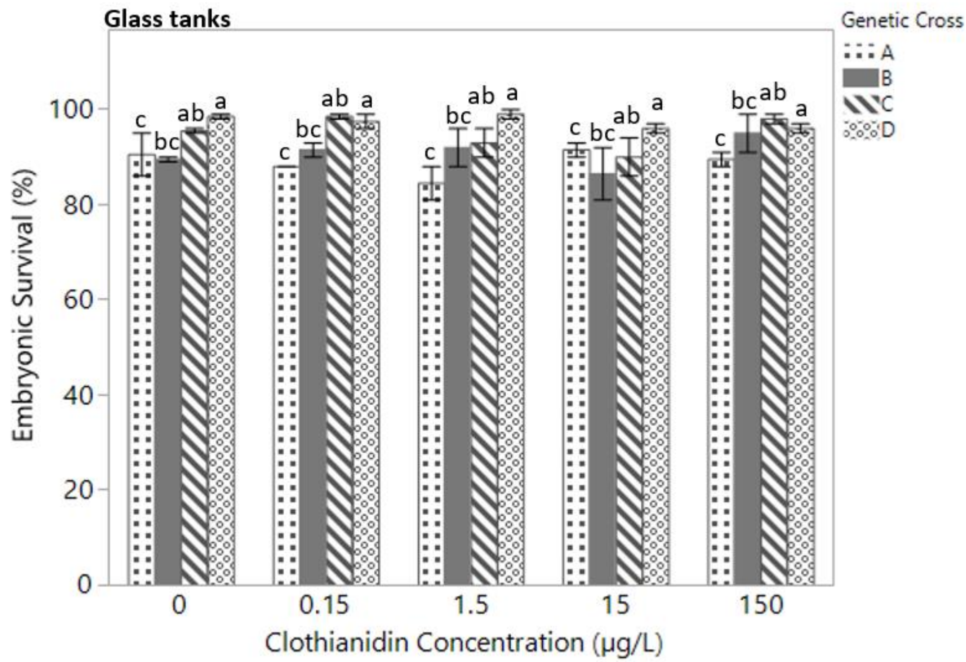


Figure 3 A split-plot randomized complete block design of glass tank and gravel-bed flume exposure system.

Sockeye salmon was exposed to 0, 0.15, 1.5, 15 and 150 µg/L clothianidin in glass tank and gravel-bed flume system with a split plot randomized complete block design to determine the main plot effect (test concentration) and the subplot effect (genetic variation) on salmon early development. Each set of glass tanks or sections of a gravel-bed flume representing as large light gray and gray rectangles acted as a block containing five main plots to test all five different test concentrations (main plot effect) in the two systems. The five main plots (glass tanks/sections in gravel-bed flume) representing as small rectangles within the large rectangle were arranged in randomized complete block design in the two systems, with all five test concentrations assigned to the plots at random. Each of the main plot was further split into four subplots (genetic cross). The glass tank in the center (left) showed all four crosses (A, B, C and D) of salmon embryo/alvien/fry were housed separately allowing the subplot effect, genetic variation, to be tested. The circle represents cylindrical egg containers and the letters A, B, C and D represents the genetic crosses of the housed eggs. A section of gravel-bed flume was shown in the center (right) to illustrate the four crosses of salmon housed individually in different sub-compartments. Stainless steel dividers inserted between sub-compartments allowed same exposure condition so the subplot effect could be tested in gravel-bed incubator system. Blocks in both systems were built in duplicated and the two blocks were located opposite sides of temperature-controlled room. Due to the different locations of the two blocks, blocking effect was assessed in statistical analyses.

A)



B)

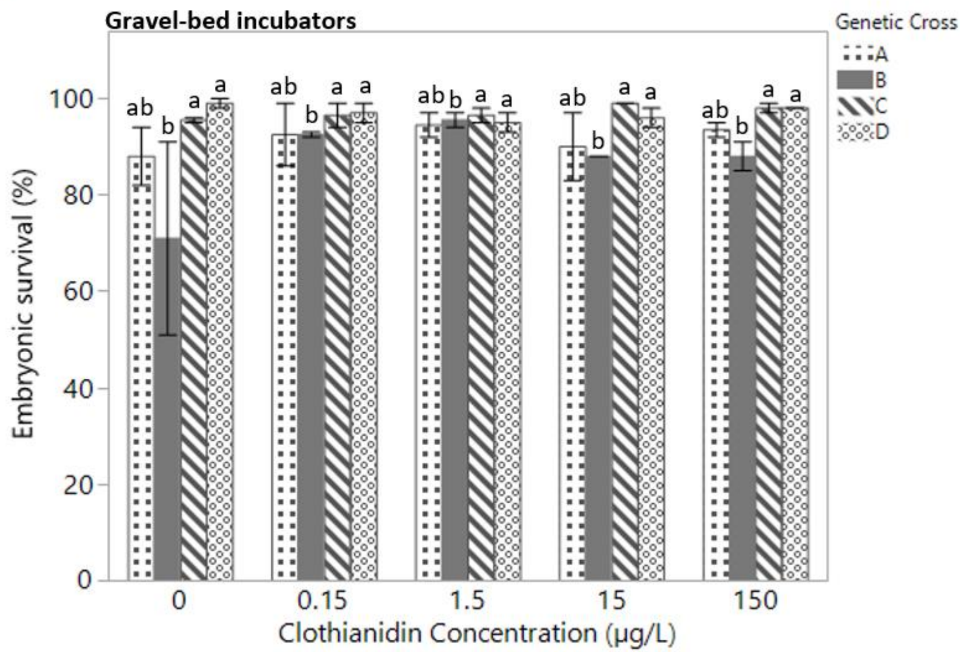


Figure 4 **The average embryonic survival of sockeye salmon after chronic clothianidin exposure in A) glass tank and B) gravel-bed incubation system.**

Salmon embryos were exposed by clothianidin from 1 hour post-fertilization to the eyed embryo stage. Percent survival to eyed embryonic developmental stage in each glass tank was calculated based on number of surviving salmon at 28 dpf/total number of eggs and the percent survival in the early development was then averaged between the duplicate. Means ± standard error are presented (n=2; ~100 fish/genetic cross in each tank). There is no significant difference between treatments but the survival of cross A was significantly lower than the other genetic crosses (SS-RCB ANOVA followed by a Tukey's post-hoc test). Different superscripts indicate significant differences.

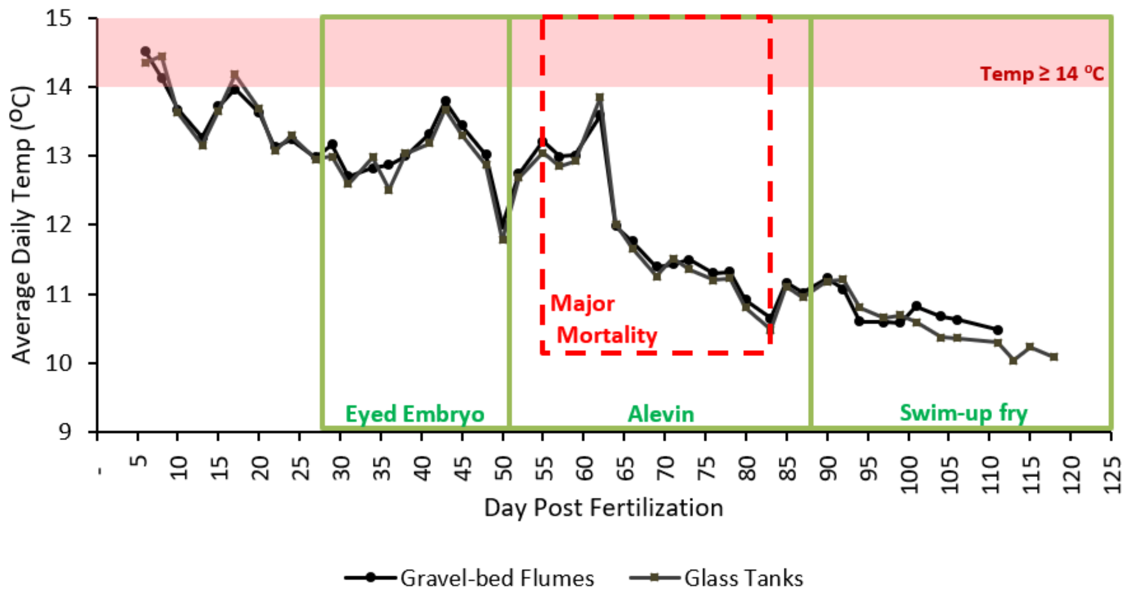


Figure 5 The average daily water temperature in the glass tank and gravel-bed incubation system.

Major mortality event during the chronic clothianidin exposure in sockeye salmon (n=10) was indicated by dashed box. Temperature of 14°C or above was shown in red shading. Several days prior to the major mortality exceeded 14°C was suspected to contribute the major death in combination with hatching stress and clothianidin pollution.

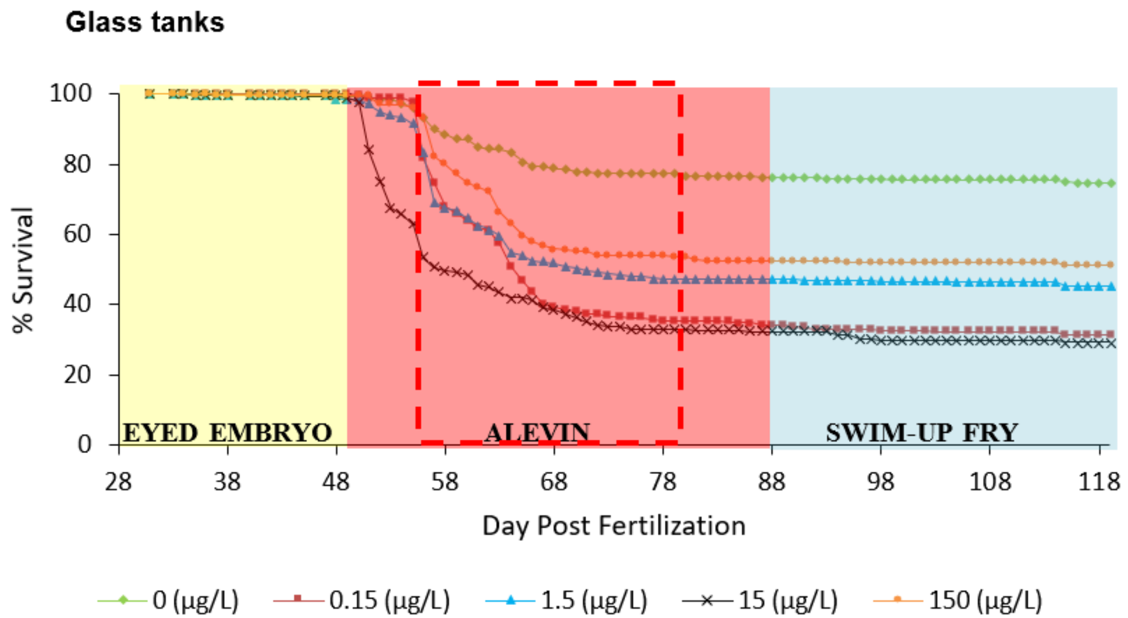


Figure 6 The daily % survival of sockeye salmon during chronic clothianidin exposure from eyed embryo to swim-up fry developmental stage in glass tank exposure system.

Percent survival was calculated based on number of surviving salmon /total number of eyed embryos counted on 28 dpf in duplicate glass tank incubators (~100 fish/genetic cross in each tank). The time of hatching of eyed embryos into free swimming alevins is indicated by the transition from yellow to pink shading (on 49 dpf), and the timing of the onset of the first swim-up fry performance (88 dpf) is estimated by the blue shading. Major morality was observed during the early alevin stage at 55 dpf – 80 dpf indicated in the dashed box.

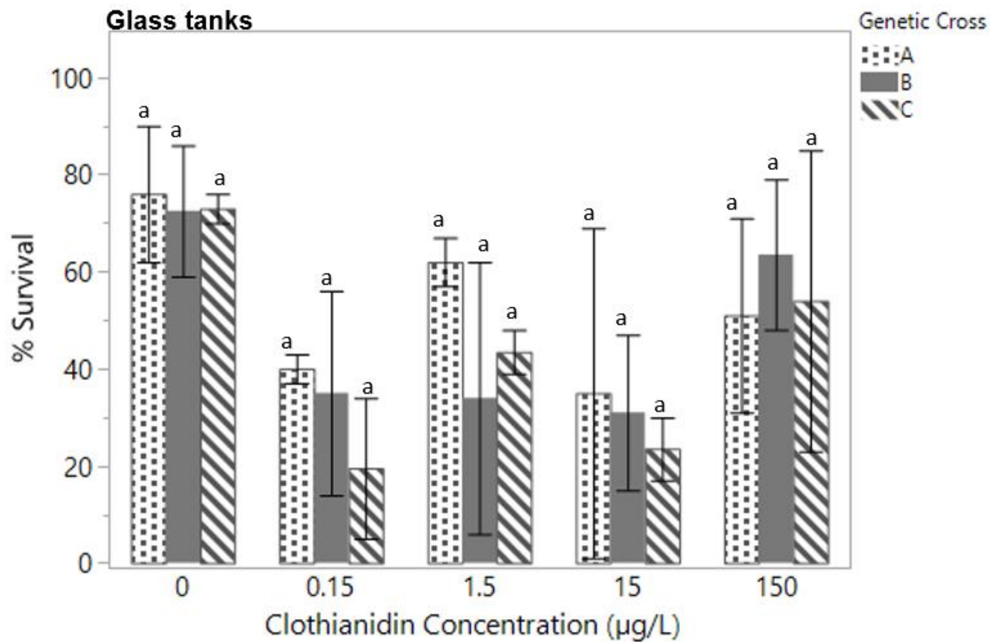


Figure 7 The average survival of sockeye salmon after varying concentrations of clothianidin chronic exposure in a flow through glass tank exposure system.

Exposure initiated from 1 hour post-fertilization to the swim-up fry developmental stage. Percent of survival in swim-up fry developmental stage in each glass tank was calculated based on number of surviving salmon on 119 dpf /total number of eyed embryos counted on 28 dpf. Means of 2 replicate tanks ± standard error are presented (n=2; ~100 fish/genetic cross in each tank). No evidence of difference in mean survival between any treatments or genetic crosses was observed (SS-RCB ANOVA followed by a Tukey's post-hoc test, $p < 0.05$).

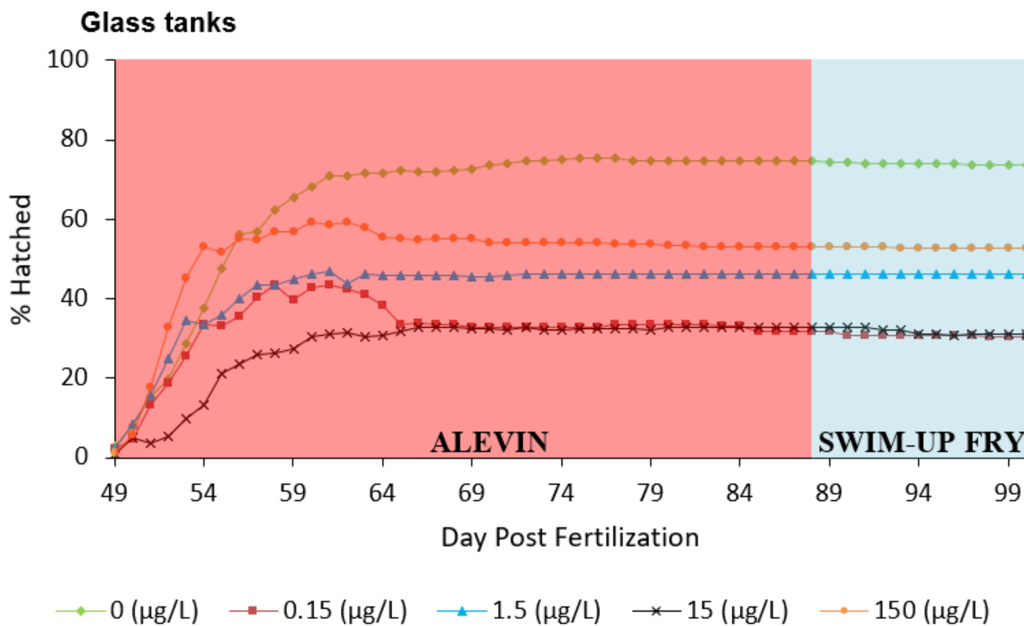


Figure 8 The cumulative, average % hatched of sockeye salmon during chronic clothianidin exposure from 1 hour post-fertilization to the swim-up fry developmental stage in glass tank exposure system.
Percent hatched in individual glass tank was calculated based on number of eyed embryos hatched each day/total number of eyed embryos counted on 28 dpf and the percent hatched was averaged between the duplicate (~100 fish/genetic cross in each tank). The time of hatching of eyed embryos into free swimming alevins is indicated by the pink shading, and the timing of the onset of the first swim-up fry event is estimated by the blue shading.

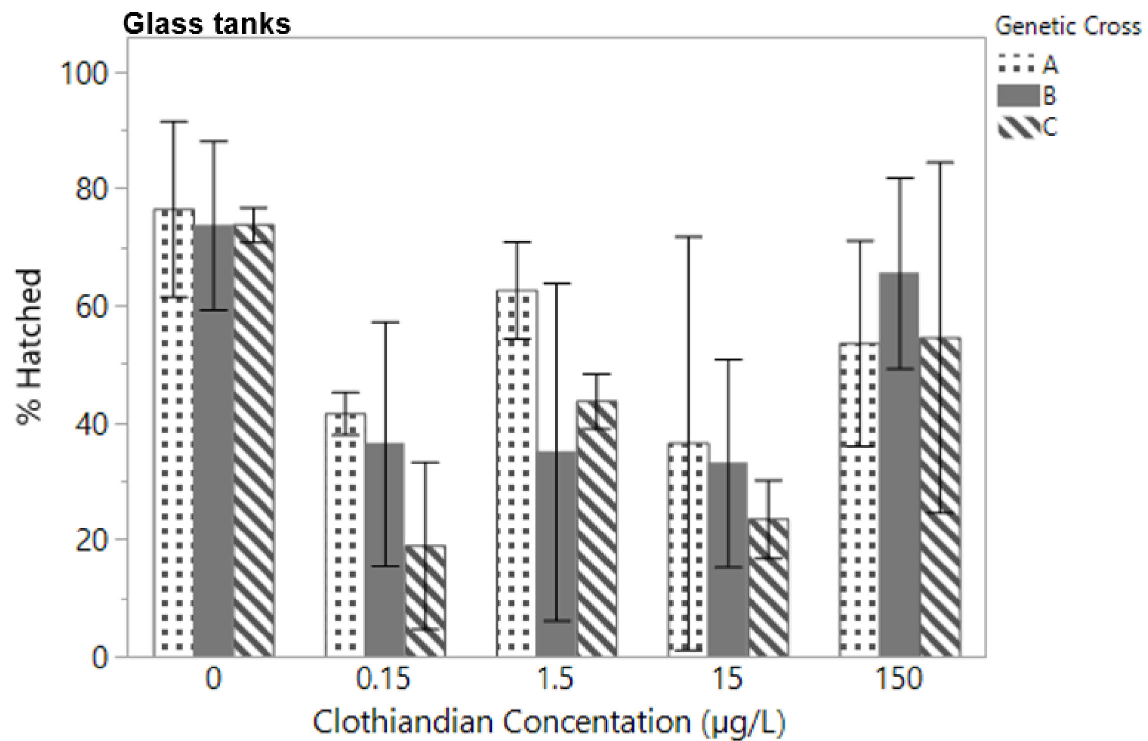


Figure 9 The hatch success of sockeye salmon after chronic exposure of clothianidin from 1 hour post-fertilization to the swim-up fry developmental stage.

Percent hatched success in individual glass tank was calculated based on number of eyed embryos hatched on 119 dpf/total number of eyed embryos counted on 28 dpf and the hatched success was averaged between the duplicate. Means ± standard error are presented (n=2; ~100 fish/genetic cross in each tank). No statistically significant effect of clothianidin concentration and genetic crosses were observed (SS-RCB ANOVA with Tukey's post hoc test, $p < 0.05$).

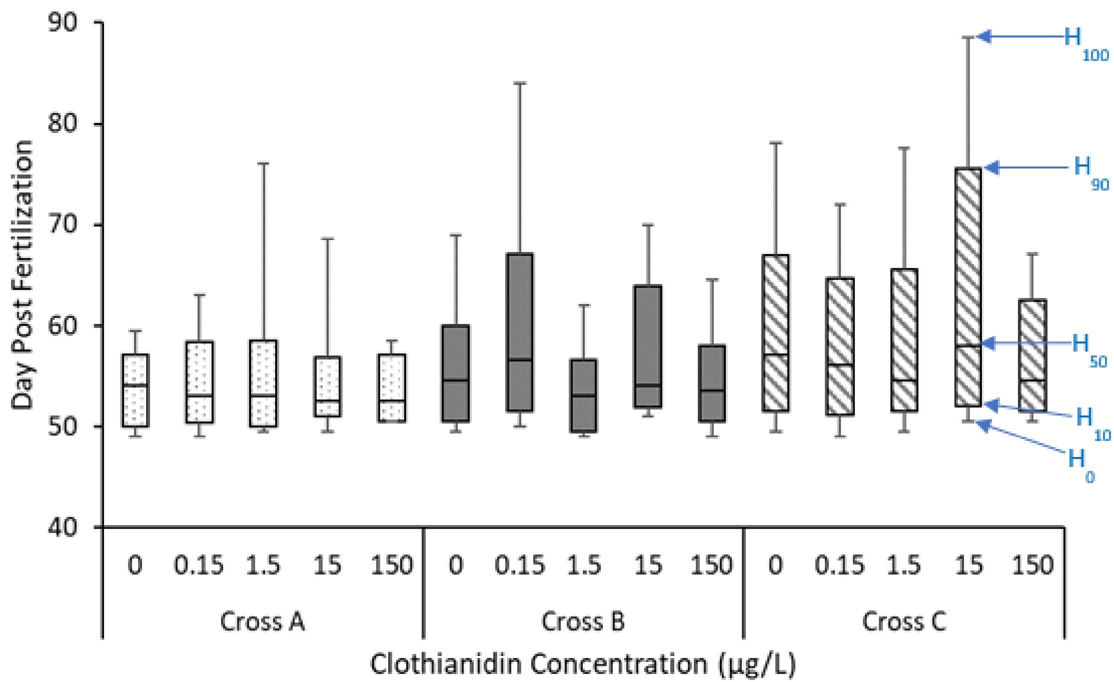
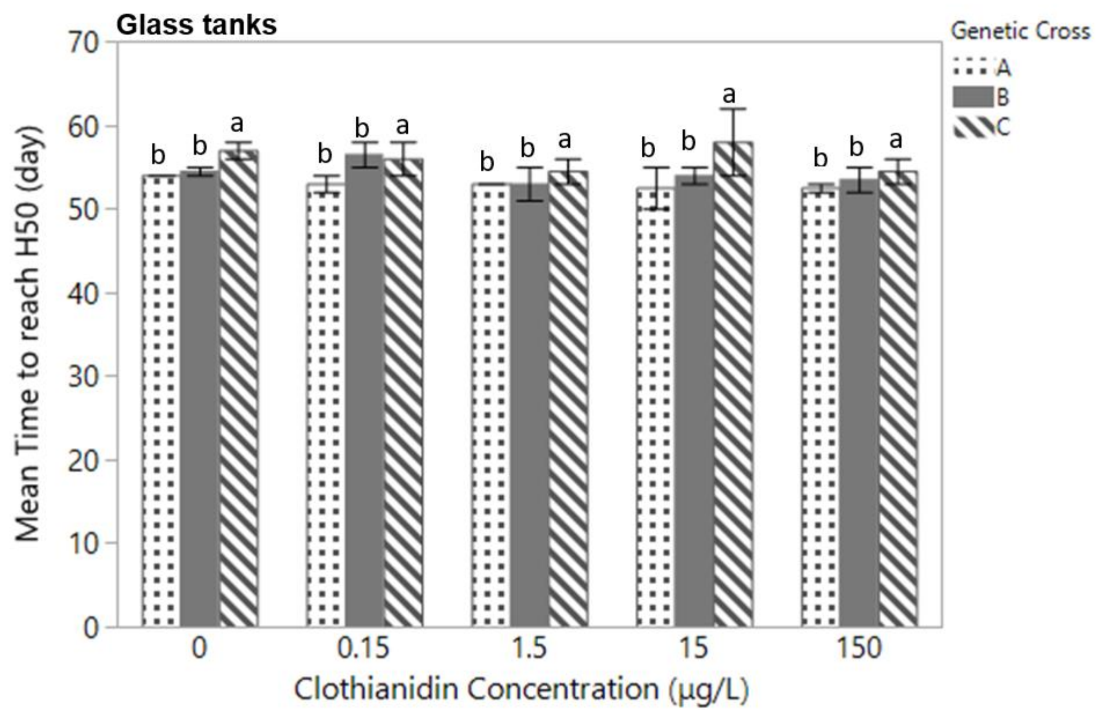


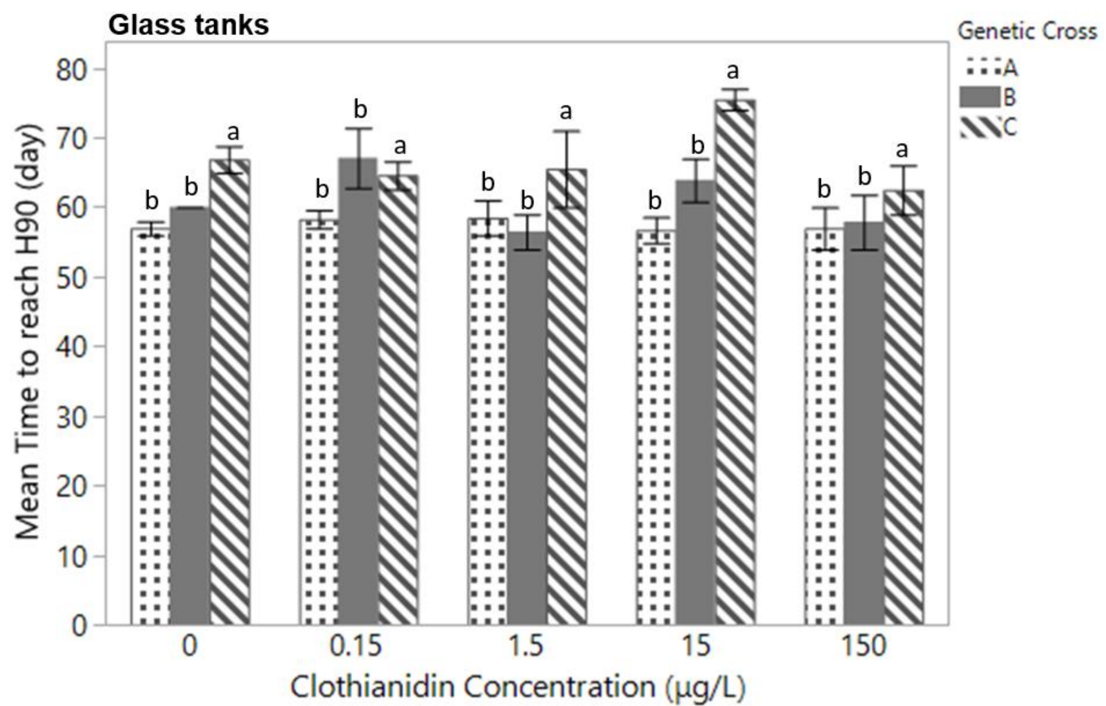
Figure 10 The percent hatched of sockeye salmon over time during clothianidin chronic exposure from 1 hour post-fertilization to the swim-up fry developmental stage.

Boxes represent the average H_{10} (day indicating when 10% of total number of embryos hatched), and H_{90} (day indicating when 90% of the total embryos hatched) in duplicate tanks (n=2; ~100 fish/genetic cross in each tank). Horizontal lines within each box represent the median (H_{50}, indicating when 50% of embryos hatched). The whiskers indicate the first (H_0) and last hatch (H_{100}) observed.

A)



B)



C)

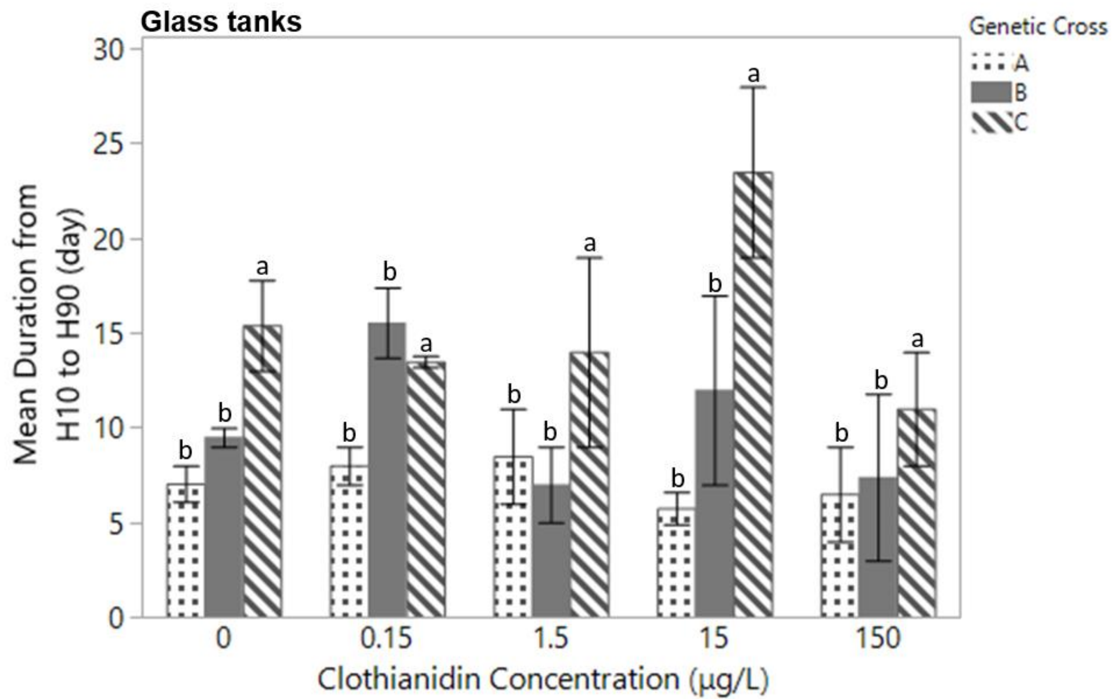
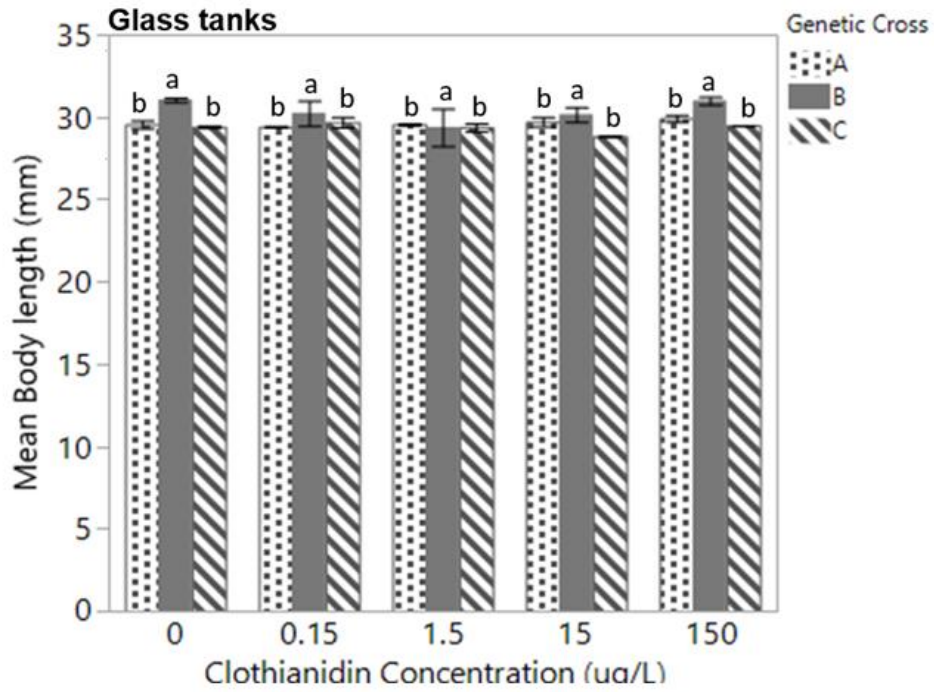


Figure 11 The average time required for sockeye salmon to reach A) H_{50}, B) H_{90} and C) H_{90} from H_{10} after clothianidin chronic exposure from fertilization to swim-up fry in glass tanks.

Means ± standard errors are presented (n=2; ~100 fish/genetic cross in each tank). No statistically significant difference in the mean time of H_{50} and H_{90} and the average duration between H_{10} and H_{90} between clothianidin concentrations, but cross C required significantly longer time to reach 50th and 90th percentile of hatching and duration to reach H_{90} from H_{10} on average (SS- RCB ANOVA with Tukey's post hoc test, $p < 0.05$). Different letters indicate significant differences. Different superscripts indicate significant differences between genetic.

A)



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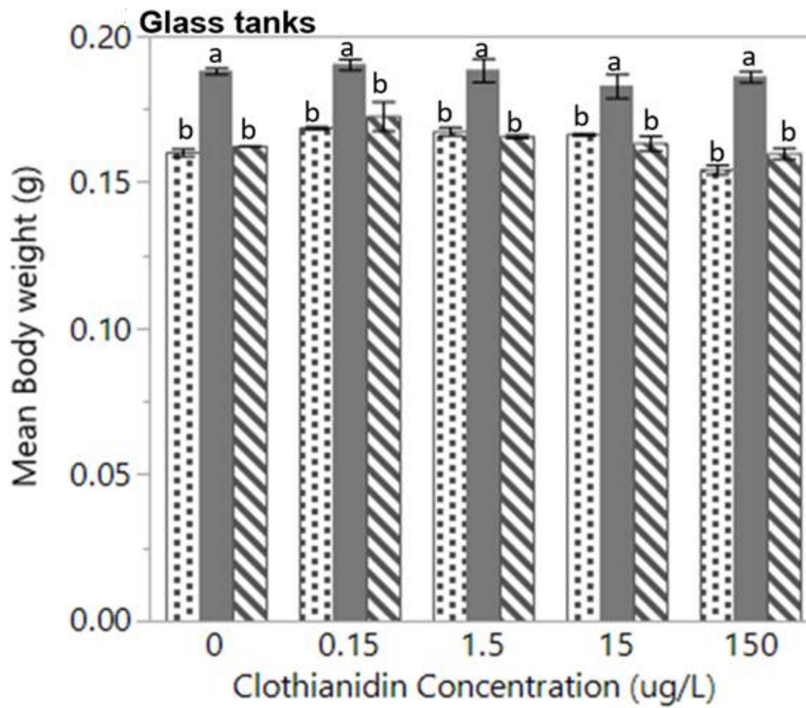
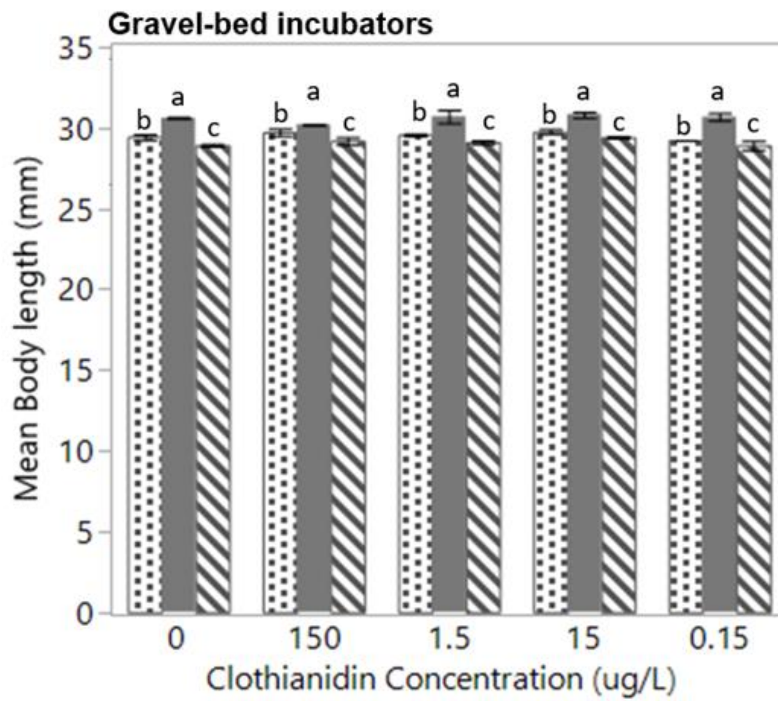


C)



D)

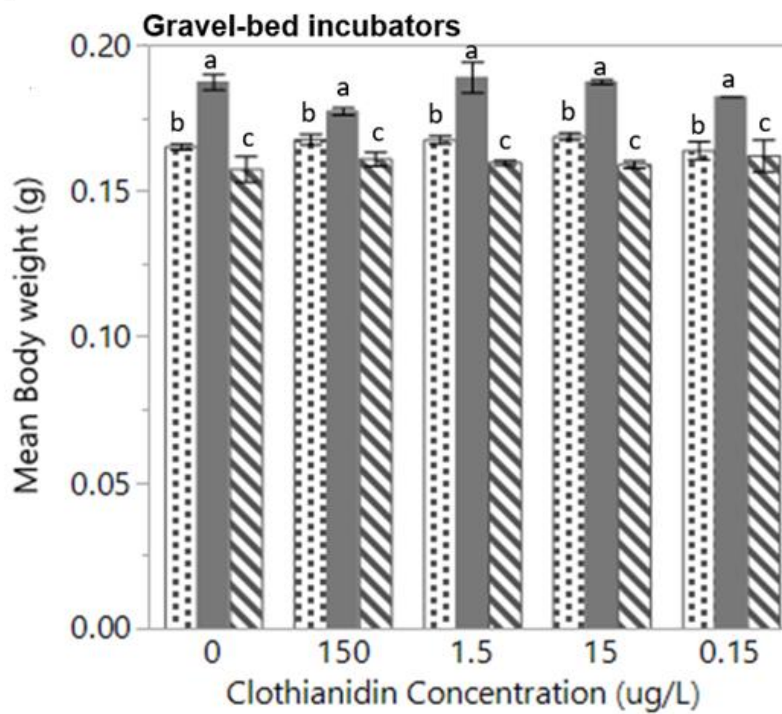
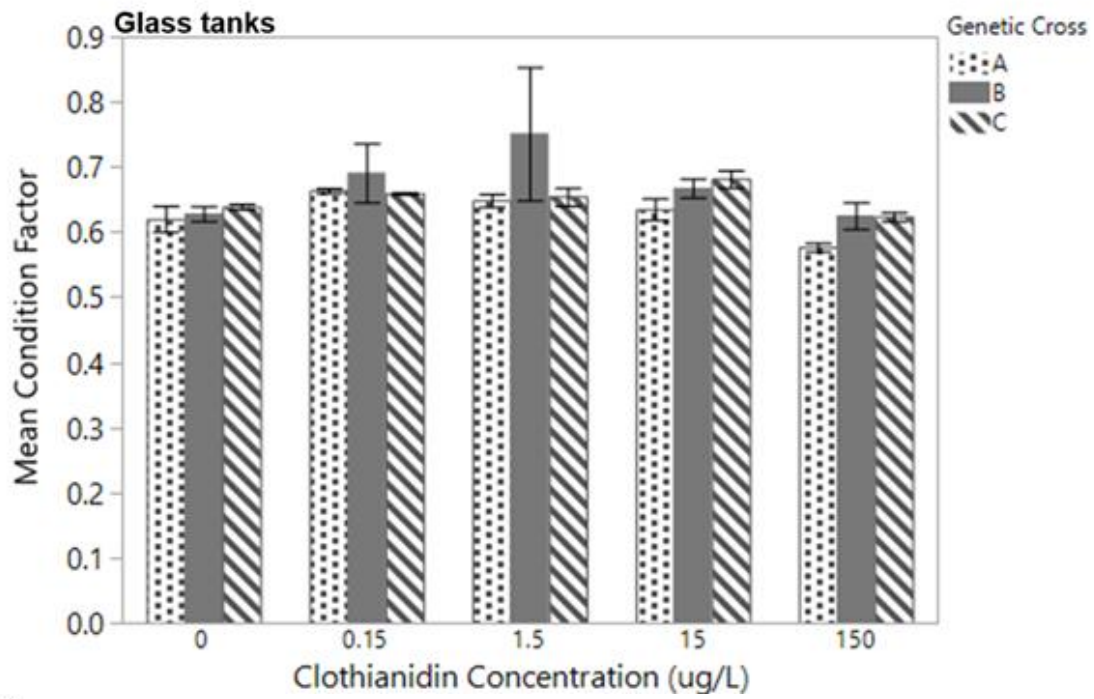


Figure 12 Comparison of the mean body length and weight of swim-up sockeye salmon in glass tank exposure (A and B) and in gravel (C and D) after chronic exposure to various concentration of clothianidin in glass tanks and gravel-bed incubators.

Means ± standard error are presented (n=2; ~100 fish/genetic cross in each tank). No significant difference in mean caused by pesticide concentration in both incubators was observed, but the mean body weight and length of Cross B are significantly higher than Cross A and C in both incubation systems (SS-RCB ANOVA with Tukey's multiple comparison test). Different superscripts indicate significant differences between crosses.

A)



B)

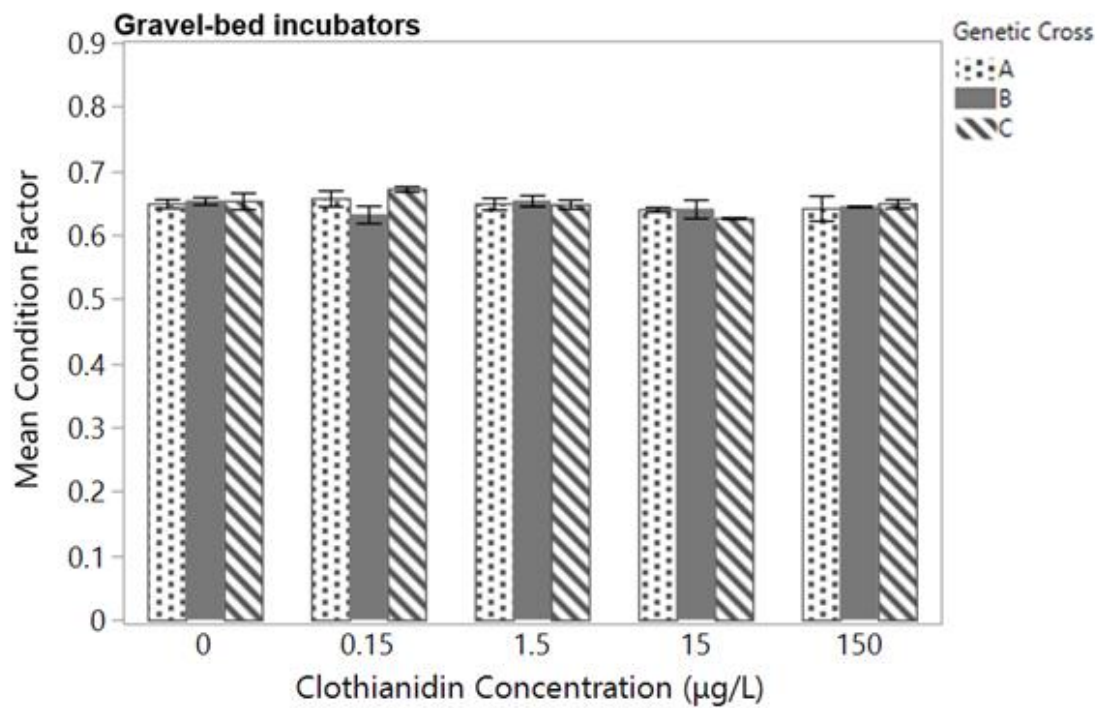
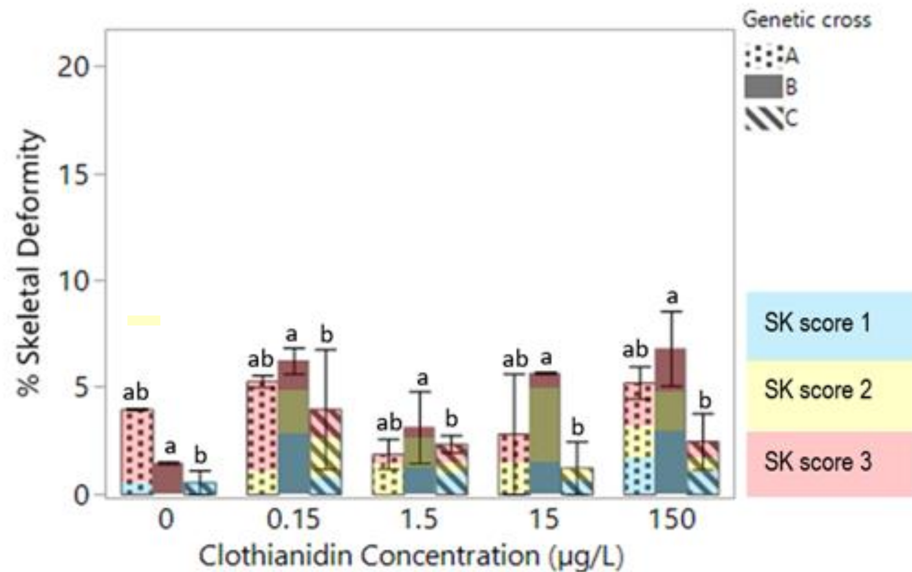


Figure 13 Comparison of the mean condition factor of swim-up sockeye salmon after chronic exposure from 1 hour post-fertilization to the swim-up fry developmental stage in A) glass tanks and B) gravel-bed incubators.

Means ± standard error are presented (n=2; ~100 fish/genetic cross in each tank). No significant effect of genetic crosses or pesticide concentration on condition factor were observed (SS- RCB ANOVA with Tukey's post hoc, $p > 0.05$).

A)



B)

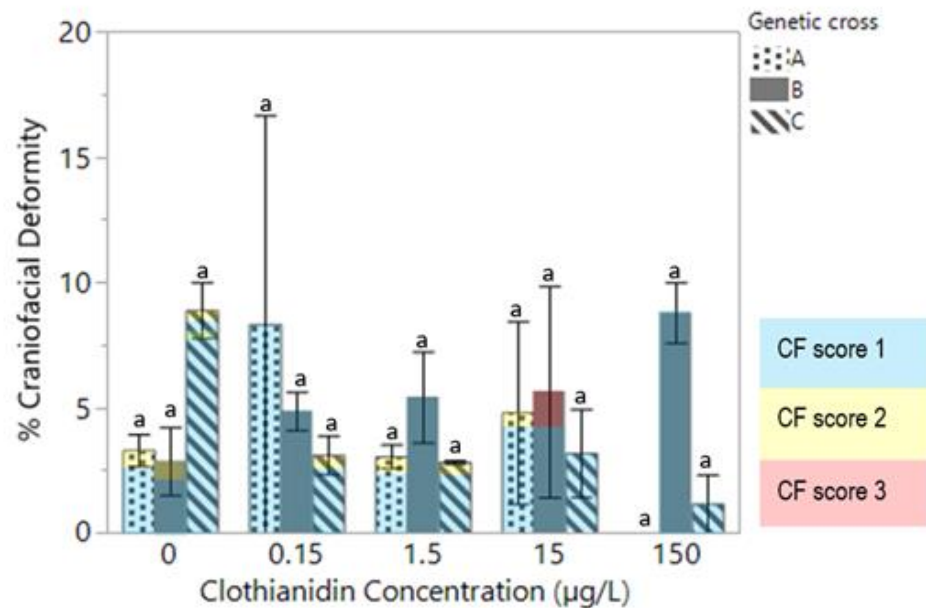


Figure 14 Average deformity rates of sockeye salmon after clothianidin chronic exposure from 1 hour post-fertilization to swim-up fry in glass tanks.

Means of 2 glass tanks ± standard error are presented (~100 fish/genetic in each tank). There was no significant effect of clothianidin concentration for A) skeletal deformity (SK) rate. The SK rate in Cross C is significantly lower than Cross A and B. No statistically significant difference in the mean B) craniofacial deformity rate between genetic crosses and clothianidin concentration (SS-RCB ANOVA followed by Tukey's post hoc, $p > 0.05$). Different superscripts indicate significant differences between crosses.

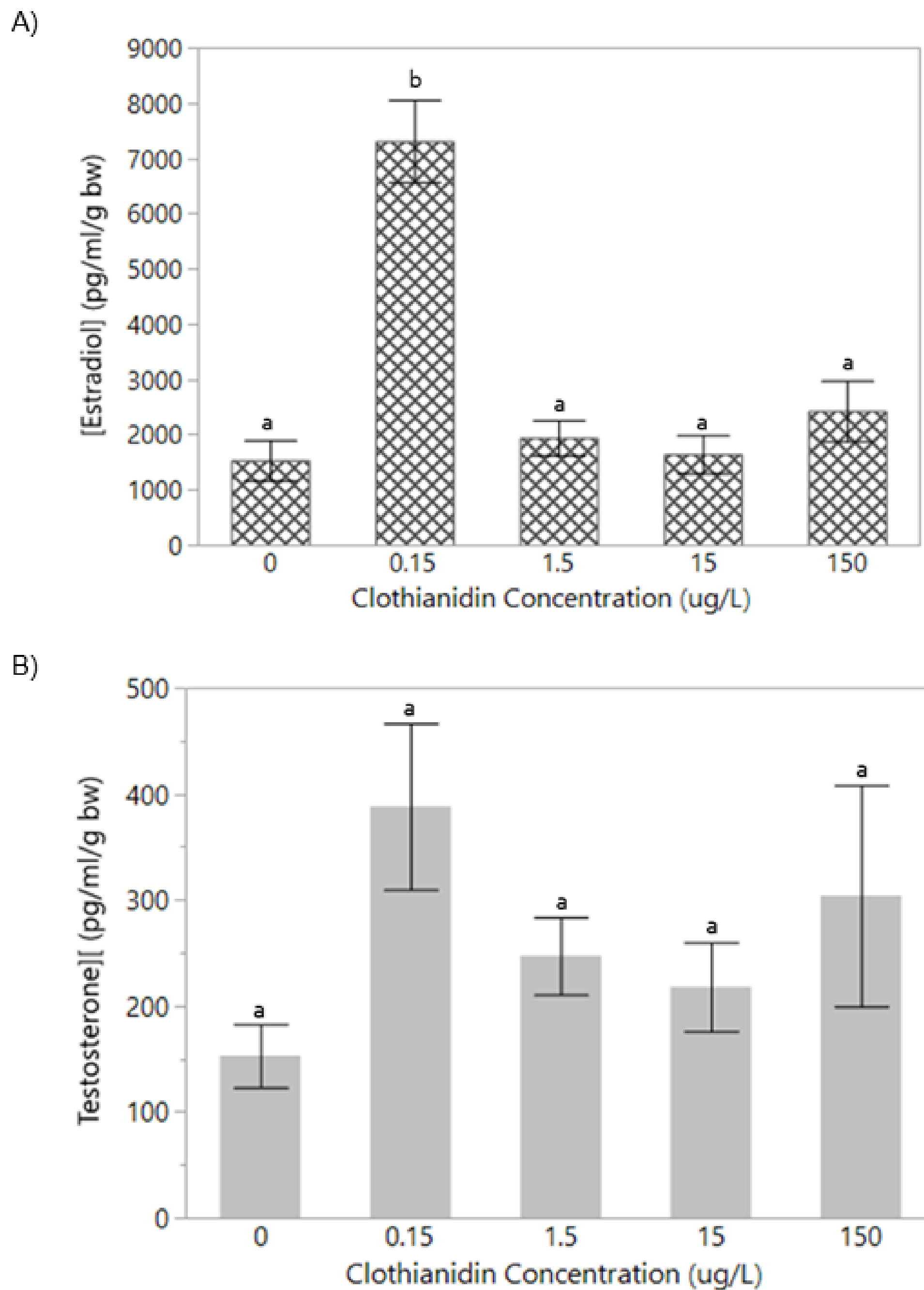


Figure 15 Mean whole body sex steroid hormone concentrations in swim-up sockeye salmon fry after chronic clothianidin exposure from 1 hour post-fertilization to swim-up fry developmental stage.

The mean level of A) 17β-estradiol and B) testosterone in whole body homogenates of swim-up fry collected at emergence from one genetic cross after clothianidin chronic exposure. The steroid hormone concentrations were measured by commercially available enzyme-linked immunosorbant assays. Means ± standard error from 1 genetic cross are presented (n=2; 5 fish per tank). Different superscripts indicate significant differences between treatments (RCB ANOVA, followed by Tukey's post-hoc test, $p<0.05$).

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Appendix.

Scoring scheme and examples of deformities analyses

Table A1 Graduated severity index (GSI) scoring scheme for assessing deformities during early fish development*

GSI	Skeletal	Craniofacial	Finfold	Edema
0	15° bent in backbone (lordosis, scoliosis or kyphosis)	Normal jaw, head and eye ratio	Normal fins	No fluid accumulated in eye or pericardial cavity
1	15-44° bent in backbone (lordosis, scoliosis or kyphosis)	<20% reduction in eye ratio or slightly malformed jaw, head or	Slightly malformed fins or reduced fins size and unlikely to impair swimming	<20% of fish or eye volume fluid accumulation
2	45-89° bent in backbone (lordosis, scoliosis or kyphosis)	20-49% reduction in eye ratio or moderately malformed jaw, head or	Moderately malformed fins or reduced fins size and unlikely to impair swimming	20-49% of fish or eye volume fluid accumulation
3	≥ 90° bent in backbone (lordosis, scoliosis or kyphosis)	≥50% reduction in eye ratio or severely malformed jaw, head or	Missing fins	fluid accumulation ≥50% of fish or eye volume

*Deformity assessment was adapted from Rudolph BL. 2006. The effects of selenium on westslope cutthroat trout reproduction and development. MET thesis. Simon Fraser University, Burnaby, BC, Canada.

Figure A1 Photographs of clothianidin-induced craniofacial deformities and associated scores in sockeye salmon swim-up fry. Craniofacial (eye) deformity scored as 3 in top fish. Fish with normal eyes shown as bottom.

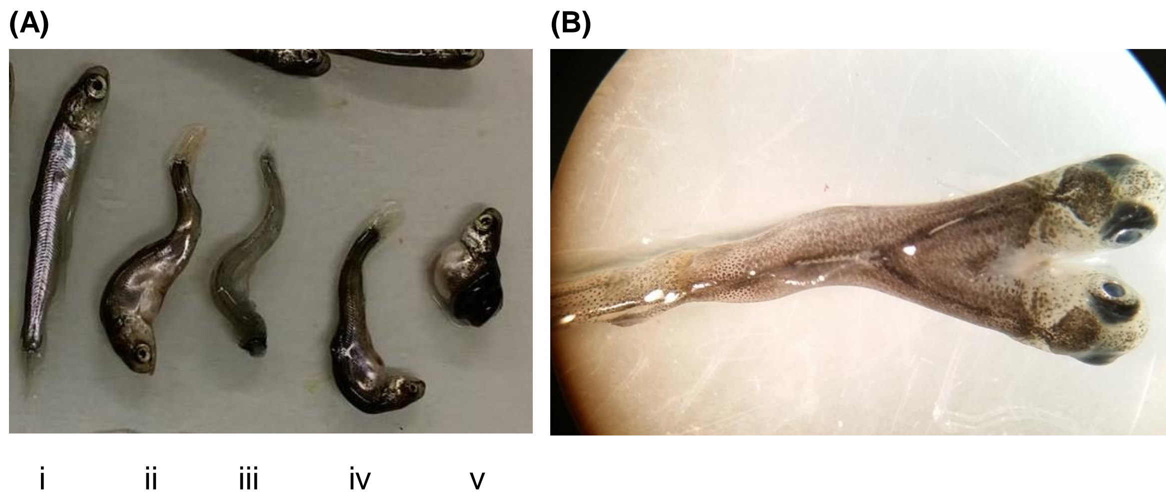

Figure A2 Photographs of clothianidin-induced skeletal deformities and associated scores in sockeye salmon swim-up fry. (A) Normal swim-up fry shown in far left (i). Fish *ii* to *v* demonstrating skeletal deformities. Kyphosis and lordosis scored as 2 (ii). Scoliosis scored as 2 (iii). Kyphosis scored as 2 (iv). Scoliosis scored as 3 (v). (B) Skeletal deformity (2 heads) scored as 3.