The Impact of Restricted Ration and Chemical Stressors on Rainbow Trout (*Oncorhynchus mykiss*) and American Flagfish (*Jordanella floridae*)

by

Lindsay Alexandra Beyger

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Abstract

This research aimed to increase the understanding of the impact of multiple factors including both environmental and chemical stressors and their effects on fish survival, growth, and reproduction. Rainbow trout (*Oncorhynchus mykiss*) were used to assess the impact of restricted feed on acute toxicity and it was found that prior feeding regime is unlikely to affect short-term toxicity results. These findings are important when considering the potential implications in both setting regulatory guidelines, and in the natural environment. American flagfish (*Jordanella floridae*) were used to assess the impact of environmentally relevant concentrations of ibuprofen, naproxen, and 17 α-ethinylestradiol (EE2) alone and in mixture on both reproduction and subsequent sensitivity to offspring. Both a short-term reproduction test, and multi-generational study were used to assess a variety of endpoints. The partial life-cycle study noted a significant decrease in fertilization as a result of exposure to 0.1 µg/L naproxen, and 10 ng/L EE2, as well as a significant increase in egg production as a result of exposure to 0.1 µg/L ibuprofen. The multi-generational study demonstrated a significant decrease in fertilization after exposure to the highest concentration of mixtures of ibuprofen, naproxen, and EE2 for both generations. There were also significant changes in egg production. In both studies subsequent toxicity to offspring was not altered significantly. Overall, there appeared to be reproductive impacts related to pharmaceutical exposure either via either short-term exposure, or over multiple generations. Conducting studies that encompass both chemical and environmental stressors has always been challenging. In surface waters, wild fish may be exposed to numerous compounds over multiple generations with many different stressors and modifying factors. Thus, it is important to consider multiple factors together in order to understand the true scale of potential contaminant impacts on fish populations.

Keywords: Chemical Stressors, Environmental Stressors, Pharmaceuticals, Multi-generational, Reproduction, Restricted Ration, Rainbow Trout, American Flagfish

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Abbreviations

µg/L – microgram per liter
11-KT – 11-ketotestosterone
17, 20-DHP – 17α,20β-dihydroxypregn-4-en-3-one
AD – after death
ADME – absorption, distribution, metabolism, excretion
ASTM – American Society for Testing and Materials
B[a]P – benzo[a]pyrene
BDL – below detection limit
BW/d – body weight per day
COX – cyclooxygenase
$CuSO_4 \cdot 5H_20$ – Copper sulfate pentahydrate
Cyp – cytochrome
D – days
dph – days post hatch
E1 – estrone
E2 - 17β-estradiol
E3 – estriol
EC50 – effective concentration that causes 50% response in test organisms
EE2 – 17α-ethinylestradiol
ER – estrogen receptor
EREs – estrogen response elements
EROD – ethoxyresorufin-O-deethylase
FSH – follicle-stimulating hormone
GFAA – graphite furnace atomic absorption
GnRH – gonadotropin-releasing hormone
GSI – gonadosomatic index
GtH – gonadotropin
h – hour
HPG - hypothalamic-pituitary-gonadal
HPGL axis – hypothalamic-pituitary-gonadal-liver axis
Hsp – heat shock proteins
IBU – Ibuprofen
ILL – incipient lethal level
Kow – Octanol-water coefficient
LC50 – lethal concentration that causes 50% mortality in test organisms
LH – luteinising hormone
LSI – liver somatic index
MS-222 – Tricane methanesulfonate
MT – metallothioneins
NAP – Naproxen
ng/L – nanogram per liter
NSAID – non-steroidal anti-inflammatory drugs
OECD – Organization for Economic Cooperation and Development
OTC – over the counter

PG – prostaglandin
PGD2 – prostaglandin D2
PGE2 – prostaglandin E2
PGF2α – prostaglandin F2 alpha
PGG2 – prostaglandin G2
PGH2 – prostaglandin H2
PGHS – prostaglandin endoperoxide synthases
PGI2 – prostaglandin I2
PPCPs – pharmaceuticals and personal care products
Vtg – vitellogenin
WWTP – wastewater treatment plant
Zrp – zona radiata

1. Literature Review

1.1 Introduction

Over the past 10 to 15 years, a substantial effort has been made to study the effects of pharmaceuticals and personal care products (PPCPs) in the environment (Boxall *et al.,* 2012). Pharmaceuticals are used for both the prevention and treatment of illness whereas personal care products are used to better the quality of daily life (Boxall *et al.,* 2010; Corcoran *et al.,* 2010). Pharmaceuticals have been widely used by humans for centuries and it is estimated that several kilotons of pharmaceuticals are produced annually (Cleuvers, 2004; Hughes *et al.,* 2013). Two commonly consumed pharmaceutical groups include non-steroidal anti-inflammatory drugs (NSAIDs) and steroid hormones (Santos *et al.,* 2010). Ibuprofen and naproxen are common NSAIDs, and 17-alpha ethinylestradiol (EE2) is a common synthetic steroid hormone. The major routes of entry for pharmaceuticals into the environment are from sewage (treated and untreated), human consumption, personal use, and elimination (Santos *et al.,* 2010). All three of these pharmaceuticals have been detected in surface waters in the environment in either the nanogram per litre (ng/L) or microgram per litre (µg/L) range (Corcoran *et al.,* 2010; Santos *et al.,* 2010). Unless treatment processes are refined to better remove pharmaceuticals, levels will continue to increase with increasing population and use (Hughes *et al.,* 2013). The continual addition of these biologically active pharmaceuticals into the aquatic environment along with the high level of biological conservation among animals, makes them a potential risk to non-target organisms.

Many contaminants such as metals and PPCPs have been shown to have impacts on aquatic organisms at both the acute and chronic level (Overturf *et al.,* 2015; Rand *et al.,*

2008). Much of the research to date has been on single compounds and their potential toxicity to aquatic organisms. Although single compound exposures are important to understanding the causes and effects of pharmaceuticals on non-target organisms, it is also important to consider the real world environment in which fish would be exposed. In surface waters, wild fish could be exposed to numerous compounds over multiple generations with many different stressors and modifying factors. Conducting studies that encompass this has always been challenging, however, it is important to consider multiple factors together in order to get a clearer picture of the impact of contaminants on non-target organisms in the environment. This research aims to contribute a better understanding of the impact of multiple factors including both environmental and chemical stressors and their effects on fish survival, growth, and reproduction.

1.2 Pharmaceuticals in the environment

Pharmaceuticals are a very diverse group of bioactive compounds (Corcoran *et al.,* 2010). They are used for both the prevention and treatment of illness and there are numerous chemical classes, each having its own unique therapeutic purposes with specific physio-chemical properties and biological activities (Boxall *et al.,* 2012; Corcoran *et al.,* 2010). There are nearly 4000 different pharmaceutical compounds and in 2014, global spending on medicines was estimated to exceed one trillion U.S. dollars (IMS Health, 2013a; Monteiro & Boxall, 2010). Some of the main classes of pharmaceuticals are: antibiotics, beta-blockers, lipid regulators, selective serotonin reuptake inhibitors, non-steroidal anti-inflammatories, and steroid hormones (Santos *et al.,* 2010). Even though pharmaceuticals have been discharged into the environment for many years (unregulated for decades), only recently have concerns over the potential environmental effects on non-

target organisms become of interest (Daughton & Ternes, 1999; Monteiro & Boxall, 2010). With the advances of new analytical instrumentation and technology, pharmaceuticals are now more widely detected and studied (Santos *et al.,* 2010).

It is important to understand how pharmaceuticals enter the environment before discussing their potential toxic effects on non-target organisms. The two aspects affecting pharmaceutical presence in the environment are usage and disposal. Consumption of pharmaceuticals has been steadily rising over the years both globally and nationally (Corcoran *et al.,* 2010). In 2013, worldwide sales of pharmaceuticals were greater than 330 billion U.S. dollars for the United States, greater than 80 billion U.S. dollars for Japan, and greater than 20 billion U.S. dollars for Canada (IMS Health, 2013b). These numbers help to highlight the substantial levels of consumption on both the global and national scale. With an aging society, use and consumption of pharmaceuticals will continue to increase as will their levels within the environment (Hughes *et al.,* 2013).

After consumption pharmaceuticals typically enter the environment via wastewater discharge (treated or untreated) (Corcoran *et al.,* 2010). Pharmaceuticals reach wastewater treatment plants (WWTP) through elimination from the body via urine and feces either as the parent form or as a metabolite, through improper disposal techniques (many people flush expired medication down the toilet or sink), and via hospital/ manufacturer discharge (Corcoran *et al.,* 2010). Pharmaceuticals may also enter the environment through sewage sludge application to agricultural fields (Figure 1) (Fent *et al.,* 2006).

Once in the environment, the fate of pharmaceuticals can be influenced by factors such as seasonal conditions, receiving water chemistry, and chemical composition (Monteiro & Boxall, 2010). Seasonality, specifically temperature and light intensity, have

been documented to affect the fate of pharmaceuticals in surface waters; lower water temperatures and shorter daylight hours have the potential to decrease bio- and photo-degradation, respectively (Khetan & Collins, 2007). The chemical composition of water can also affect the fate of pharmaceuticals in the environment, changing their ability to adsorb onto solids or to remain in the aqueous phase, which is often controlled by the chemical properties of the specific compound (Corcoran *et al.,* 2010). Since WWTPs are the main source of pharmaceuticals, a more detailed look at WWTP processes is useful to better understand how pharmaceuticals are released into the environment.

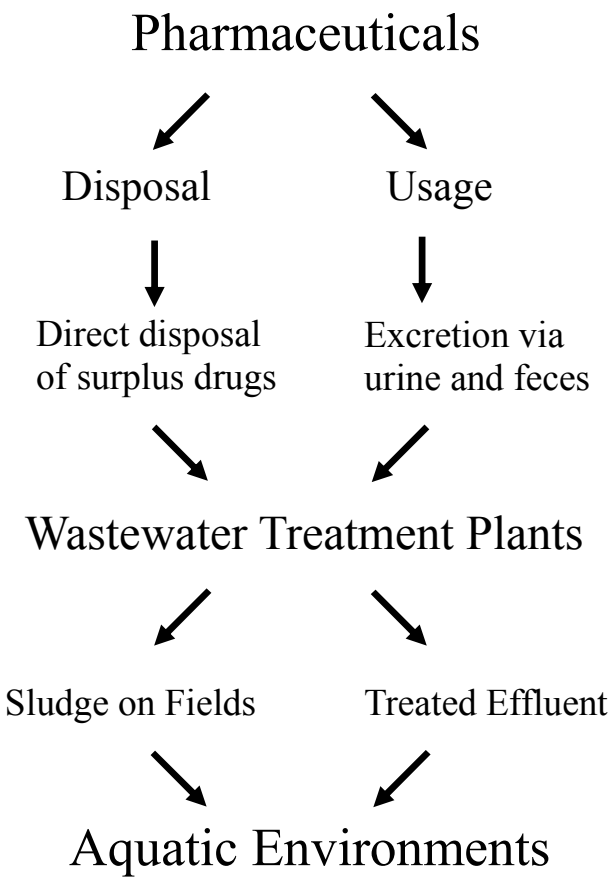

Figure 1: Simplified major route of human pharmaceuticals into the aquatic environment leading to potential exposure of non-target organisms in aquatic environments (adapted from Halling-Sørensen *et al.,* 1998).

1.3 Waste Water Treatment Plants (WWTPs)

1.3.1 Wastewater Treatment Processes

Wastewater treatment plant (WWTP) effluents are known as one of the major contributors of pharmaceuticals into the environment (Santos *et al.,* 2010). This is largely due to the fact that many WWTPs are not designed to remove such low level contaminants (Batt *et al.,* 2007). Pharmaceuticals enter the WWTP as result of household and consumer usage after excretion via urine and feces as either the active ingredient or its metabolites (Lishman *et al.,* 2006). Once discharge is received at the WWTP it generally undergoes five stages of treatment; pre-treatment, primary treatment, secondary treatment, tertiary treatment and sludge treatment (Batt *et al.,* 2007).

Pre-treatment is the first step of the treatment process where influent is received (Carballa *et al.,* 2004). The influent passes through coarse bar screens in order to remove garbage and other large debris that may interfere with downstream processes (CCME, 2006). This pre-treatment stage can also involve shredding and grinding to eliminate large debris (CCME, 2006). Once large debris has been removed the water moves into primary treatment.

Primary treatment involves influent waters being pumped into clarifiers to remove any remaining solid waste (Batt *et al.,* 2007; Carballa *et al.,* 2004). The flow is reduced and particulate matters are able to settle due to gravity (CCME, 2006). The sludge from primary treatment is collected to be further processed and the water is sent to secondary treatment (Carballa *et al.,* 2004).

Secondary treatment is specifically designed to remove biodegradable organic matter and suspended solids (CCME, 2006). The water moves from the clarifiers in

primary treatment to the biological reactor in secondary treatment (Carballa *et al.,* 2004). Secondary treatment at WWTPs can vary at this stage. Using either aerobic or anaerobic conditions, bacteria degrade material before it is moved to the secondary clarifier tank (Carballa *et al.,* 2004). Sludge and bacteria are able to settle out again, escaped bacteria are returned to the treatment system and sludge is collected and combined with sludge from the primary treatment phase (Carballa *et al.,* 2004). This sludge can be further treated and if approved used for application on agricultural land (Carballa *et al.,* 2004). Remaining water is either released into the environment as effluent or transferred to tertiary treatment (Carballa *et al.,* 2004).

Tertiary treatment is the final stage of wastewater treatment and is considered the final disinfection (CCME, 2006). The effluent can undergo a variety of treatments, however, the two most widely used are ozonation and chlorination (Carballa *et al.,* 2004). The effluent is released into the receiving environments after treatment is complete.

1.3.2 Removal of Pharmaceuticals in WWTPs

Pharmaceuticals are removed at varying rates and most conventional treatment technologies in WWTPs are not specifically designed to remove pharmaceuticals (Carballa *et al.,* 2004). The rates of removal for pharmaceuticals in WWTPs are highly dependent upon their chemical properties (Carballa *et al.,* 2004). During the wastewater treatment process pharmaceuticals may either undergo biological degradation, partition to solids, or remain unchanged in the effluent (Carballa *et al.,* 2004). The adsorption coefficient plays a major role in the compounds ability to partition to solids; compounds with low adsorption coefficients will tend to stay in the aqueous phase allowing them to pass through the

WWTPs (Carballa *et al.,* 2004). Compounds that more readily adsorb to the sludge may still enter the environment through agricultural practices (Corcoran *et al.,* 2010). At neutral pH, acidic pharmaceuticals (e.g. NSAIDs) occur as ions and don't readily adsorb to sludge and remain in the water column, favouring their mobility through the WWTP (Corcoran *et al.,* 2010). Steroid hormones tend to sorb to sludge due to their hydrophobic nature (Ying *et al.,* 2002).

It is important to note that microorganisms in the activated sludge are efficient at removing nitrogen, carbon, and other microbial contaminants and may also contribute to the metabolism of pharmaceuticals in WWTPs, however, this mechanism is not very efficient and can sometimes lead to more toxic metabolites than the parent compounds being released into the environment (Carballa *et al.,* 2004).

1.4 PPCP Environmental Relevance

Although WWTPs facilitate removal of some pharmaceuticals from influent, pharmaceuticals are still detected in effluent and receiving waters in the ng/L to µg/L concentration range (Corcoran *et al.,* 2010). Many pharmaceutical sources and modifying factors have been studied, including; consumption in an aging society, metabolism and excretion, removal efficiencies of WWTPs, point source entry and agricultural applications. As mentioned previously, where certain pharmaceuticals will be found in the environment is dependent on their specific chemical properties. Continual addition of pharmaceuticals into the environment has led pharmaceuticals to be considered "pseudo-persistent"; consequently research in this area has significantly increased.

A review paper completed by Santos and colleagues (2010) concluded that NSAIDs (16%) are the most widely detected PPCP while the fourth highest class detected are the sex hormones (9%). These two classes of pharmaceuticals are amongst the most frequently detected due to their prevalence and use in society. NSAIDs are available as over the counter drugs (OTC), and two of the most commonly detected drugs are ibuprofen and naproxen. Sex hormones encompass a variety of natural and synthetic varieties and estrogens are the most commonly detected (Santos *et al.,* 2010). 17α-ethinylestradiol (EE2) is commonly detected and is one of the main components found in oral contraceptives (Santos *et al.,* 2010). Although there are many different classes of pharmaceuticals present in the environment, the remainder of this work will focus on NSAIDs and sex hormones.

1.4.1 NSAIDs in the Environment

NSAIDs are amongst the most frequently detected pharmaceuticals in the environment due to their high level of consumption and incomplete removal from wastewater treatment plants (Bhandari & Venables, 2011). There are a wide variety of NSAIDs examples of which include diclofenac, ketoprofen, ibuprofen and naproxen, all of which have been detected in effluent and surface waters (Corcoran *et al.,* 2010). NSAIDs are typically detected in the ng/L to µg/L range and have been detected both globally and nationally (Corcoran *et al.,* 2010). Specifically, in recent years NSAIDS have been detected in surface waters ranging from 0.018 – 6 µg/L (Fent, 2008; Kolpin *et al.,* 2002; Overturf *et al.,* 2015). Ibuprofen and naproxen have been detected in Canadian effluent

with medians of 4.0 µg/L and 12.5 µg/L and maximums of 24.6 µg/L and 33.9 µg/L, respectively (Metcalfe *et al.,* 2003).

1.4.2 Sex Hormones in the Environment

As previously mentioned, a variety of estrogens, progestins, and androgens have been detected in the environment in the low ng/ L range (Overturf *et al.,* 2015). Sex hormones have been detected in surface waters ranging from approximately 0 – 5 ng/L (Overturf *et al.,* 2015), while EE2 specifically has been detected in surface waters ranging from 0 – 34 ng/L (Aris *et al.,* 2014).

1.5 NSAIDs

1.5.1 History/ Introduction to NSAIDs

One of the body's natural responses to injury is inflammation (Vane & Botting, 1998). Even before there was a clear understanding of the mechanisms behind them, people have been using chemicals to treat ailments and injuries. One such example of this dates back 3500 years ago when Egyptians would use dried myrtle leaves to treat pains from the womb (Vane & Botting, 1998). A 1000 years later, willow bark was used to relieve pain and reduce fevers, and by 30 AD physicians were using willow leaf to treat inflammation (Vane & Botting, 1998). It is now known that salicylic acid, a non-steroidal anti-inflammatory (NSAID) drug was the active component in these natural remedies (Vane & Botting, 1998). NSAIDs are a class of drugs that are used to treat pain and inflammation (Santos *et al.,* 2010). In 1971 Vane was able to demonstrate that NSAIDs

were capable of inhibiting cyclooxygenase (COX) enzymes and thus defined the mechanism of action for that class of drugs (Simmons *et al.,* 2004).

1.5.2 Prostaglandins and Cyclooxygenases

Prostaglandins (PG) and thromboxanes comprise the family of biologically active lipids known as prostanoids, which are part of a larger category of eicosanoids (Cha *et al.,* 2006). Eicosanoids are evolutionarily conserved, biologically active lipid molecules (Cha *et al.,* 2006). PGs produce a wide range of effects and are involved in almost every biological function (Botting, 2006). One of their main functions is to act as mediators of pain, fever, and swelling in inflammation, and they are also involved in regulating kidney blood flow, maintaining the gastric mucosa, and some reproductive mechanisms (Botting, 2006; Fent *et al.,* 2006). In mice, females with COX-1 knockouts fail to give birth, and female mice with COX-2 knockouts have reduced ovulation leading to fewer offspring, and often, infertility (Lim *et al.,* 1997; Reese *et al.,* 2000). Any alterations in the biosynthesis of PGs can cause major pathophysiological conditions (Botting, 2006).

PGs are present in almost all cells and are released via various mechanical and chemical stimuli (Botting, 2006). PGs are synthesized from arachidonic acid via the key enzyme cyclooxygenase (COX) (Botting, 2006). COX enzymes, also known as prostaglandin endoperoxide synthases (PGHS), are heme-containing bifunctional proteins that are responsible for the production of prostanoids (Knights *et al.,* 2010). The enzyme cascade starts with arachidonic acid being cleaved from the cell membrane by phospholipase A2 (Botting, 2006). Arachidonic acid is then converted into prostaglandin G_2 (PGG_2) by either COX-1 or COX-2, which is then reduced by peroxidase to

prostaglandin H_2 (PGH_2) (Botting, 2006). PGH_2 is further metabolized by synthases into tissue specific PGs such as PGD_2 (mast and immune cells), $PGF_{2\alpha}$ (reproductive tissue, brain), PGE_2 (kidney), and PGI_2 (smooth muscle) (Figure 2) (Botting, 2006; Cha *et al.,* 2006).

There are two different isoforms of COX; COX-1 and COX-2 (Santos *et al.,* 2010). COX-1 is constitutively expressed and maintains the baseline levels of prostaglandins to regulate normal cell activity in most tissues (Santos *et al.,* 2010; Vane & Botting, 1998). COX-2 is inducible and produces prostaglandins in response to stimulation at the site of inflammation (Knights *et al.,* 2010; Santos *et al.,* 2010; Vane & Botting, 1998). Both COX-1 and COX-2 consist of an epidermal growth factor-like domain, a membrane-binding domain, and an enzymatic domain (Botting, 2006). COX-1 and COX-2 structures are very similar, however, COX-2 has a slightly larger active site which allows for it to accommodate bigger structures (Botting, 2006). In humans both COX enzymes have a molecular weight of 71kDa and COX-2 has 60% homology with the amino acid sequence of COX-1 (Botting, 2006). COX homologues have also been identified in other vertebrates including fish. Zou *et al.,* 1999 successfully demonstrated that fish have an inducible form of COX (COX-2). COX-1 and COX-2 homologues have been identified in rainbow trout (Zou *et al.,* 1999), brook trout (Roberts *et al.,* 2000), and zebrafish (Grosser *et al.,* 2002). This conservation of COX genes amongst species may mean that their ability to be affected as a non-target organism by human designed NSAID drugs is likely to occur.

1.5.3 Mode of Action of NSAIDs

NSAIDS are weak acids that act by either reversibly or irreversibly inhibiting either one or both of the two COX isoforms (Fent *et al.,* 2006). There are three broad categories in which NSAIDs work to inhibit COX activity (Knights *et al.,* 2010). The first method is through rapid competitive reversible binding (i.e. ibuprofen), the second is through rapid low-affinity reversible binding followed by time-dependent binding (i.e. diclofenac), and the last is via rapid reversible binding followed by covalent modification (i.e. aspirin) (Knights *et al.,* 2010). These methods are dependent on how the drug interacts with the COX active site (Knights *et al.,* 2010). This is the mammalian mode of action, it has not yet been fully characterized in fish.

The COX active site is a long hydrophobic channel, tyrosine 385 and serine 530 act as the binding sites for arachidonic acid and NSAIDs, and are positioned at the apex of the long active site (Botting, 2006). Although the active sites of COX-1 and COX-2 are very similar, the COX-2 active site is larger than the COX-1 site due to a secondary internal side pocket (Vane & Botting, 1998). The larger active site of COX-2 is due to the substitution of isoleucine at position 523 with the smaller valine (Knights *et al.,* 2010). The central channel itself is also bigger by approximately 17% (Vane & Botting, 1998). This larger active site allows for the difference in selectivity amongst pharmaceuticals.

There are many over the counter (OTC) drugs that were developed after the mechanism of action was elucidated to act as COX inhibitors; two of the most commonly used being ibuprofen and naproxen (Botting, 2006). A brief review of both compounds is provided below.

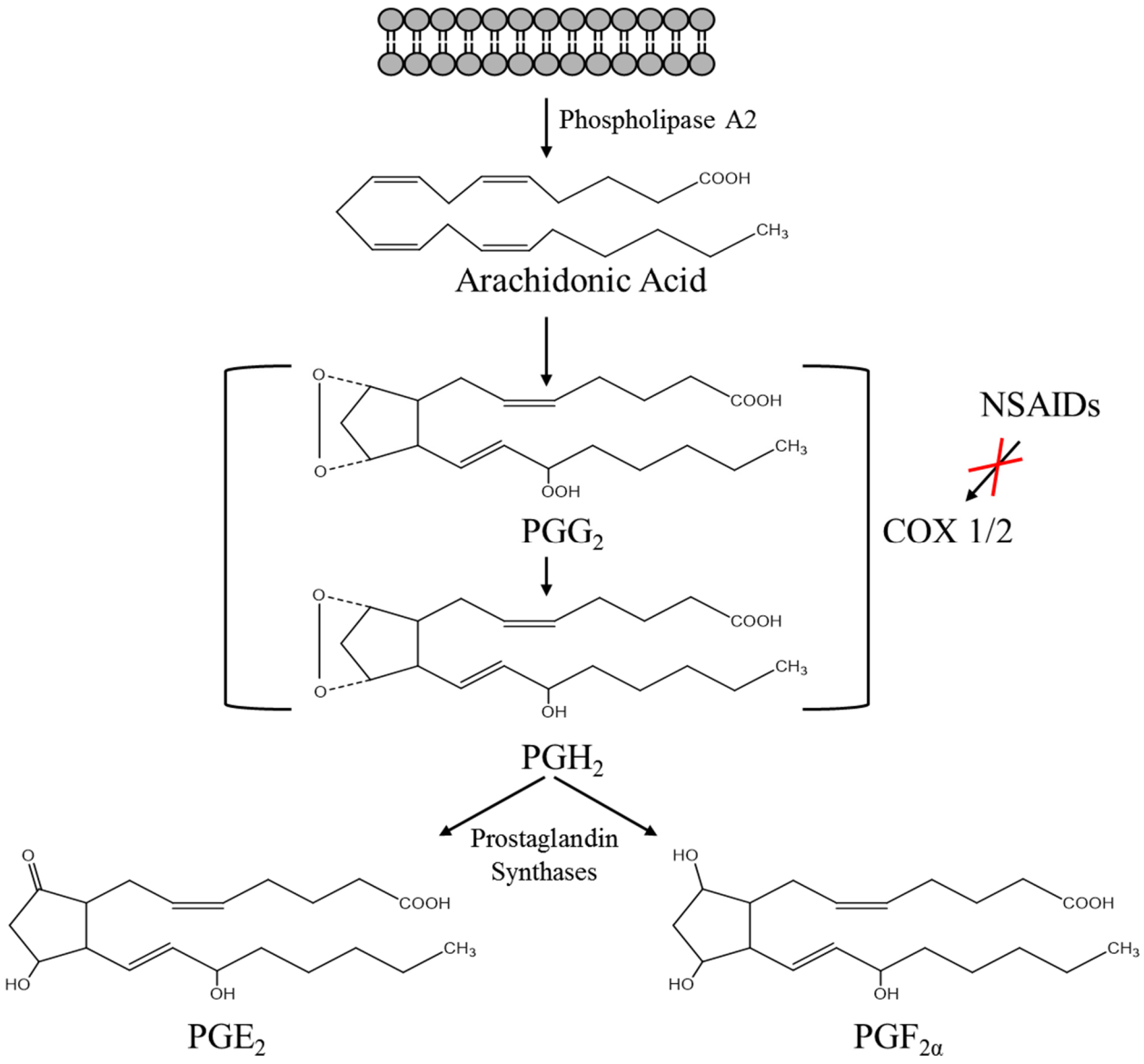

Figure 2: Mammalian mode of action of NSAIDs blocking COX enzymes which are involved in the conversion pathway of arachidonic acid to prostaglandins (Adapted from Knights *et al.,* 2010).

1.5.4 Ibuprofen

Ibuprofen is one of the top ten most frequently used over the counter drugs and its main uses are in the treatment of fever, pain, and inflammation. Ibuprofen is a white crystalline solid at room temperature and is readily soluble, it has a solubility of 21 mg/L in water at 25 ºC and a log Kow =3.97 (NCBI, CID=3672). Ibuprofen is a non-selective COX inhibitor and can reversibly bind to the active site of COX-1 or COX-2 by competing with arachidonic acid (Botting, 2006).

In humans, ibuprofen is often ingested orally and > 98 % is protein bound, it is rapidly bio-transformed and has an approximate half-life of 2 hours (Bushra & Aslam, 2010; Davies, 1998). Ibuprofen is metabolized through phase I and phase II metabolism; during phase I metabolism ibuprofen is oxidized and then during phase II metabolism it is conjugated with glucuronic acid before it is excreted in the urine (Khetan & Collins, 2007). Greater than 90% of ibuprofen is eliminated as metabolites, leaving < 10 % unchanged in the urine (Corcoran *et al.,* 2010; Khetan & Collins, 2007). Even though very little active compound is excreted unchanged, it is the continual consumption and discharge into the environment that is causing what has been termed "pseudo-persistence" of ibuprofen (Daughton, 2002; Daughton & Ternes, 1999). Ibuprofen is commonly detected in effluents in the in the ng L^{-1} to µg L^{-1} range (Bhandari & Venables, 2011; Bushra & Aslam, 2010; Khetan & Collins, 2007; Metcalfe *et al.,* 2003), and there have been multiple detections of ibuprofen in surface waters in Canada, and around the world (Corcoran *et al.,* 2010).

Even at low concentrations (µg L^{-1}) ibuprofen has been shown to have effects in both invertebrates and vertebrates (Corcoran *et al.,* 2010). Toxic effects such as increased mortality, behavioural disruptions, limb and organ malformations at an acute level, and

reproductive disruptions at the chronic level have all been noted at varying concentrations (Bhandari & Venables, 2011; David & Pancharatna, 2009; Flippin *et al.,* 2007; Han *et al.,* 2010; Ji *et al.,* 2013). Some studies have been reported on the acute and chronic effects of ibuprofen on fish. The LC_{50} values are estimated to be > 100 mg/L on average for fish (Fent *et al.,* 2006). One study completed on developing zebrafish embryos (*Danio rerio*) exposed for 0 – 6 d found that 1 and 5 µg/L ibuprofen exposures resulted in increased embryo mortality and decreased larvae hatching (David & Pancharatna, 2009). Exposure to 10 µg/L ibupforen resulted in increased mortality, and a significant decrease in rate of hatch, body mass, and body length (David & Pancharatna, 2009). Pericardial edema, malformations, lower heart rate, and loss of pectoral fins were also noted for 10 – 100 µg/L ibuprofen exposure (David & Pancharatna, 2009). Zebrafish exposed to ibuprofen (21 – 506 µg/L) for 7 d saw no effect on egg production but a reduction in prostaglandin E2 was observed (Morthorst *et al.,* 2013). Similarly, a 48-h exposure of bluntnose minnows (*Pimephales notatus*) to 50 and 100 µg/L ibuprofen resulted in a decrease of gill tissue PGE2 (Bhandari & Venables, 2011). Medaka (*Oryzias latipes*) exposed to 10 µg/L ibuprofen showed a decrease in the frequency of spawning events and an increase in the amount of eggs produced per spawning event, but there were no significant changes in the total egg production (Flippin *et al.,* 2007; Han *et al.,* 2010). A delay in the time required for eggs to hatch was noted in exposure concentrations as low as 0.1 µg/L ibuprofen (Han *et al.,* 2010). Adult zebrafish pairs exposed for 21 d to ibuprofen showed a significant decrease in egg production and a delay in time to hatch at ≥ 1 µg/L, while 10 µg/L ibuprofen exposure significantly reduced egg hatchability (Ji *et al.,* 2013).

1.5.5 Naproxen

Naproxen has more recently become a widely used NSAID in Canada and is mainly used for the treatment of arthritis. In 2002, IMS Health Canada reported that Canadian physicians wrote approximately 2.5 million prescriptions for naproxen in 2001 and in 2009 it was made available over the counter which has led to even higher usage (DellaGreca *et al.,* 2004; IMS Health, 2002). Naproxen is a white crystalline powder at room temperature that is highly soluble in water (15.9 mg/L at 25 ºC) and a logKow = 3.18 (NCBI, CID=156391). Naproxen is a non-selctive COX inhibitor that has rapid low-affinity reversible binding followed by time-dependent binding (Knights *et al.,* 2010).

In humans, naproxen is typically ingested orally and > 98 % is protein bound; it has a half-life of approximately 12 – 17 hours (Davies & Anderson, 1997). The main mechanism of elimination of naproxen is via biotransformation to glucuroconjugated sulphate metabolites which can be excreted via urine. Photo-transformation also plays a role in naproxen breakdown and has been shown to produce compounds that are even more harmful than the parent compound (Brozinski *et al.,* 2011; Davies & Anderson, 1997). The increased accessibility to naproxen and subsequent increased usage has increased its presence in the environment. In Canada, median levels of naproxen (12.5 µg/L) up to maximum levels (33.9 µg/L) have been detected in surface waters (Metcalfe *et al.,* 2003).

Naproxen has been much less studied and as such reported toxic effects are relatively limited. Much of the non-target toxicity work completed to date has been on aquatic invertebrates. Acute tests on the water flea *Ceriodaphnia dubia*, rotifer *Brachionus calyciflorus*, and fairy shrimp *Thamnocephalus platyurus* determined a 24 h EC_{50} value of 66.37 (29.57 – 119.93) mg/L and 24 h LC_{50} values of 62.48 (53.80 – 72.56)

mg/L and 84.09 (39.83 – 137.55) mg/L, respectively, for naproxen (Isidori *et al.,* 2005). Naproxen sodium and the photoproducts were also tested for acute toxicity and values ranged from 1 – 100 mg/L (Isidori *et al.,* 2005). The photoproducts were significantly more toxic for all three organisms (*C. dubia, T. platyurus, and B. calyciflorus*) (Isidori *et al.,* 2005). Chronic testing has also been completed on *B. calyciflorus*, *C. dubia*, and the algae *Selenastrum capricornutum* (*P. subcapitata*) with EC_{50} values ranging from 0 – 40 mg/L, however algae was the least sensitive to naproxen and its photoproducts (Isidori *et al.,* 2005). The cnidarian *Hydra attenuata* exposed to naproxen resulted in a 96 h LC_{50} value of 22.36 mg/L and an EC_{50} value based on morphology of 2.62 mg/L (Quinn *et al.,* 2008). The chronic toxicity to *H. attenuata* resulted in a 96 h EC_{50} for feeding of 2.68 mg/L and hydranth number and attachment were reduced at 10 mg/L (Quinn *et al.,* 2008). To date two studies have been completed on fish; one was completed *in vitro* using carp liver subcellular fractions to determine the potential interactions of naproxen on the enzymatic system of fish and the other was completed on rainbow trout (*Oncrhynchus mykiss*) to study the uptake and metabolism of naproxen (Brozinski *et al.,* 2011; Thibaut *et al.,* 2006). No significant effect was found for naproxen on 7-Ethoxyresorufin O-Deethylase (EROD) activity, however it was an inhibitor of CYP2M-like activity which suggests CYP isoforms may be sensitive targets and it has the potential to act on the enzymatic system of fish (Thibaut *et al.,* 2006). It was found that fish can absorb and metabolize naproxen via the liver and as such the bile may be monitored for exposure to naproxen (Brozinski *et al.,* 2011). No specific studies on the toxicity of naproxen to fish have been reported to date.

1.6 Steroid Hormones

1.6.1 History/ Introduction to Steroid Hormones

As mentioned previously, chemicals have been used for many years for a variety of treatments, and for preventative measures. The first reported use of steroid hormones as a contraceptive method can be dated back 4000 years when Egyptians would grind up pomegranate seeds and mix them with wax to create a suppository to prevent ovulation (Bayer Health Care, 2017). It is now known that many of these natural remedies contained natural estrogen and other steroid hormones which were effective methods of contraception. Steroid hormones are an extremely active biological class of pharmaceuticals (Santos *et al.,* 2010). They are a major component of the endocrine system and they are synthesized from a cholesterol backbone (Villeneuve *et al.,* 2007). The endocrine system of fish involves a complex interaction between external stimuli, hypothalamic, pituitary, thyroid and gonadal hormones (Kime, 1999). Photoperiod and temperature, are two of the many external cues that often trigger the central nervous system in the reproductive process.

1.6.2 HPGL Axis

Steroid hormones are a major component of the endocrine system and there are five classes of steroids; estrogens, progestins, androgens, mineralocortocoids, and glucocorticoids all of which are synthesized from a cholesterol backbone (Villeneuve *et al.,* 2007). In fish, the three main classes of steroid hormones are estrogens, progestins,

and androgens and they are primarily controlled via the hypothalamus-pituitary-gonad-liver (HPGL) axis (Arukwe & Goksøyr, 2003).

The hypothalamic-pituitary-gonadal-liver (HPGL) axis is involved in controlling both sexual maturation and reproductive activity in teleost fish (Figure 3). Gonadotrophin-releasing hormone (GnRH) is secreted by the hypothalamus, which acts on the pituitary to stimulate the release of gonadotrophic hormones (GtHs) (Kime, 1999). The two GtHs released from the pituitary are leutenizing hormone (LH) and follicle stimulating hormone (FSH). FSH is involved in gametogenesis and steroidogenesis, and LH is involved in final maturation stages of gametogenesis (Arcand-Hoy & Benson, 1998; Arukwe & Goksøyr, 2003). LH and FSH activate a G-protein mediated signal transduction pathway within the gonads via plasma transport, prompting steroidogenesis and gonadal development (Kime, 1999). Steroidogenesis begins when cholesterol moves across the mitochondrial membrane via the steroidogenic acute regulatory protein (StAR) pathway followed by a series of conversions that lead to the production of androgens, estrogens, and progestins (Arukwe, 2008).

In female fish, the main steroids produced are 17β-estradiol (E2), estrone, and 17α,20β-dihydroxypregn-4-en-3-one (17, 20-DHP) (Kime, 1999). E2 enters the bloodstream and acts on its major target tissue, the liver, to stimulate the production of vitellogenin (Vtg) and zona radiata (Zrp). Vtg is a yolk precursor protein that is incorporated into the oocyte, and then produces lipovitellin and phosvitin (Kime, 1999). Zrp is also incorporated into the oocyte and is related to the eggshell hardening (Arukwe & Goksøyr, 2003). As the production of E2 ceases the progestogen 17, 20-DHP induces the final maturation of the oocytes.

In male fish, the main steroid produced is an androgen 11-ketotestoterone (11-KT), and the progestin, 17, 20-DHP. 11-KT regulates spermatozoa and spermiogenesis while 17, 20-DHP promotes the final maturation of sperm via capacitation and spermiation (Yaron & Levavi-Sevan, 2011). Each of these steroids in both females and males has the ability to activate a negative feedback loop by acting on either the hypothalamus or pituitary to prevent the release of GnRH which regulates steroid production within the gonad (Arcand-Hoy & Benson, 1998; Kime, 1999). For the remainder of this thesis the focus will be on estrogens.

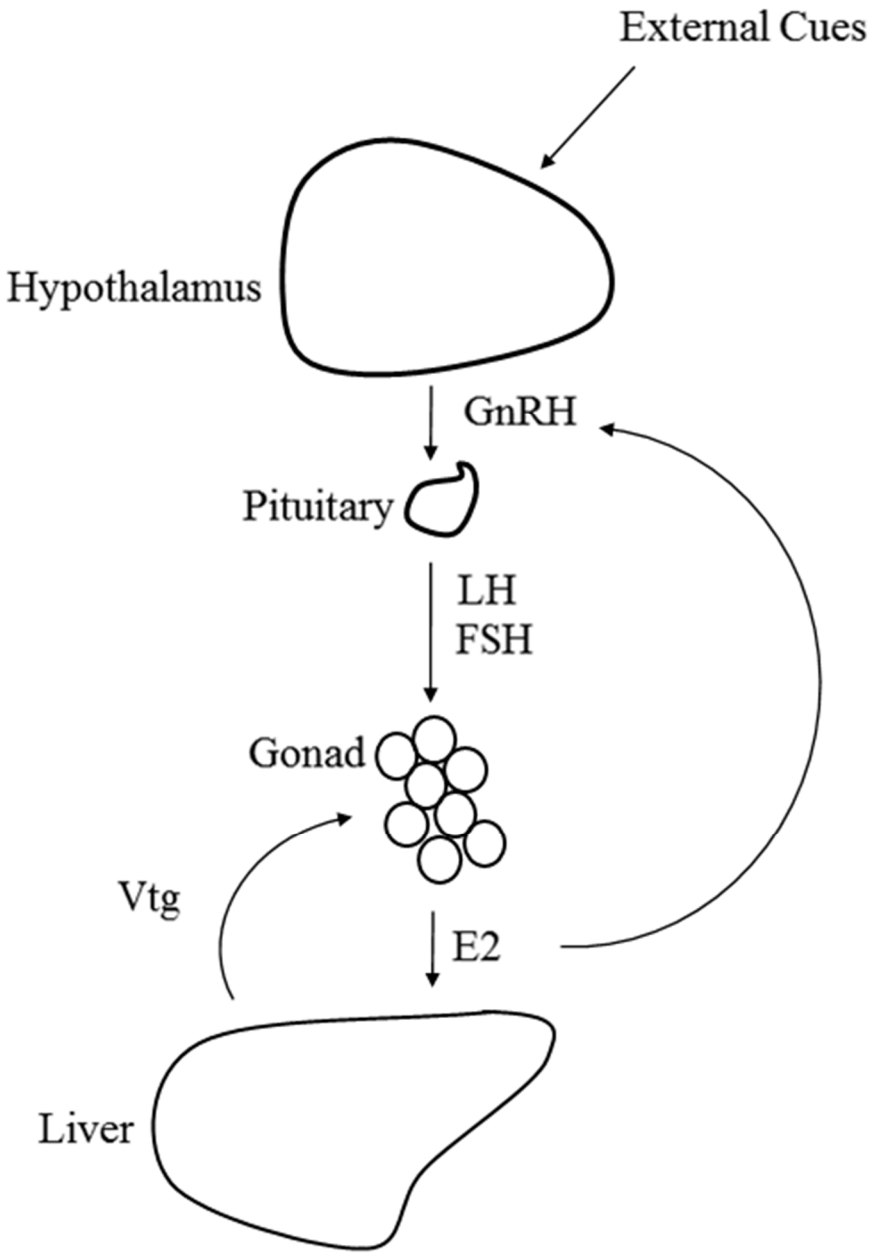

Figure 3: A schematic diagram showing the hypothalamus-pituitary-gonadal-liver (HPGL) axis in fish (Adapted from Arukwe & Goksøyr, 2003). E2 regulates the HPGL axis through negative feedback. GnRH = Gonadotropin relesasing hormone, LH = Luteinising hormone, FSH = follicle-stimulating hormone, E2 = 17β-estradiol, Vtg = Vitellogenin.

1.6.3 Estrogens and Mode of Action

Estrogens are steroid hormones that are ubiquitous and highly conserved among vertebrates (Pinto *et al.,* 2014). They are involved in a wide variety of physiological processes, specifically, they are an important regulator in reproduction and secondary sexual characteristics in both males and females (Pinto *et al.,* 2014). There are three main hormones that make up the family known as estrogens: estrone (E1), estradiol (E2), and estriol (E3) (Pinto *et al.,* 2014). In fish, E1 and E2 are the most common naturally occurring estrogens (Overturf *et al.,* 2015).

E2 produced by the ovaries is transported via the circulatory system and passively diffuses into the cell and then crosses the nuclear membrane (Pait & Nelson, 2002). The estrogen receptor (ER) is kept in an inactive conformation through interactions with a variety of proteins, mainly heat shock proteins (Hsp), until estrogen binds to the ligand binding domain, at which time the Hsp dissociates and the ER changes its conformation to the active form (Pait & Nelson, 2002). The ER then forms a homodimer complex which interacts with estrogen response elements (EREs) (Pait & Nelson, 2002). Binding of the homodimer to the ERE leads to the transcription of the gene and synthesis of proteins (Pait & Nelson, 2002). As mentioned above, estrogen is an important part of the HPGL axis and has the ability to influence gonad differentiation, behaviour, and the production of Vtg in fish (Arukwe & Goksøyr, 2003; Kime, 1999).

Estrogens are often classified as either naturally occurring or synthetic. The above mentioned estrogens are naturally occurring while one of the most common synthetic estrogens is 17α-ethinylestradiol (EE2). The remainder will focus on EE2 specifically.

1.6.4 17 α-ethinylestradiol (EE2)

Synthetic steroid hormones are another major class of pharmaceuticals. EE2 is a derivative of the natural hormone estradiol and it is found in almost all contraceptive pills (birth control) (Nagpal & Meays, 2009). EE2 is a fine white crystalline powder that has low water solubility and a moderately high octanol-water partitioning coefficient (Kow = 3.67 – 4.2) (Nagpal & Meays, 2009).

In humans, EE2 is typically ingested orally and > 97 % is protein bound, its half-life is approximately 36 ± 13 hours (Nagpal & Meays, 2009). The main mechanism of elimination of EE2 is via biotransformation to water-soluble sulfate or glucuroconjugated metabolites which can be excreted via urine (Aris *et al.,* 2014). In WWTP, there is little degradation of EE2, and bacterial deconjugation of EE2-glucuronides may release free EE2 (Andersen *et al.,* 2003; Aris *et al.,* 2014; Parrott & Blunt; 2005). Thus, EE2 contaminated effluent is often released into surface waters and has been detected in the low ng/L range (Kolpin *et al.,* 2002). The pseudo-persistence of EE2 in the aquatic environment and its high biological activity makes it a potent contaminant which poses potential risk to fish and other aquatic organisms (Colman *et al.,* 2009).

With regards to biological effects in fish EE2 is one of the most widely studied synthetic estrogens (Corcoran *et al.,* 2010). It is believed the main mechanism of action of EE2 in fish is to act on the HPGL axis (described above) as an estrogen mimic and compete with naturally occurring E2 to bind to the ER (described above) (Aris *et al.,* 2014). EE2 has been reported to have up to five x higher affinity for the ER than E2 in some fish species (Aris *et al.,* 2014). As such it has the ability to cause alterations in the HPGL axis and can elicit estrogenic responses (Aris *et al.,* 2014). EE2 has been extensively studied in various

organisms and it has been shown to have effects at the low ng/L range (Aris *et al.,* 2014). Much of the work done has demonstrated EE2s ability to impact reproduction and secondary sexual characteristics in a variety of fish species (Overturf *et al.,* 2015).

Exposure to 0.32 and 0.96 ng/L of EE2 over a complete life-cycle in fathead minnows resulted in an increase in egg production and a reduction in fertilization, while at 3.5 ng/L there was a complete cessation of egg production (Parrott & Blunt, 2005). Similarly, exposure of fathead minnows for 3 weeks to concentrations below 1 ng/L resulted in an increase in the mean number of spawned eggs per pair; at 10 ng/L there was a significant decrease in the number of spawned eggs per pair and 100 ng/L completely stopped spawning (Pawlowski *et al.,* 2004). Another study observed an increase in fecundity in Japanese medaka (*Orizias latipes*) exposed to 0.2 ng/L for 14 d while an exposure of 500 ng/L significantly decreased fecundity, spawning frequency, percent fertilization, and percent hatch (Tilton *et al.,* 2005). It has been suggested that concentrations below 1 ng/L may be stimulatory for ovulation, but may cause reproductive impairments in males (Overturf *et al.,* 2015; Parrott & Blunt, 2005; Pawlowski *et al.,* 2004).

Behavioural effects have also been studied with regards to EE2 exposure. It has been demonstrated that short-term exposure (48 h) to EE2 has the ability to alter male aggression of zebrafish (*Danio rerio*) exposed to 0.5, 5.0, and 50 ng/L (Colman *et al.,* 2009). Similarly, Sand goby (*Pomatoschistus minutus*) males exposed to 4 ng/L EE2 for 7 to 24 days were unable to acquire or defend a nest site and spent a reduced amount of time courting females, as a result females preferred to mate with control males (Saaristo *et*

al., 2009). Such behavioural effects have the potential to vastly disrupt the potential ability to reproduce and can be an early warning system of effects.

Generational effects of EE2 have also been noted in both a lab and field setting. In a 7 – year whole-lake study conducted in Ontario, Canada, the lake was dosed for three years with 5 – 6 ng/L EE2. This exposure resulted in induction of Vtg in both female and male fathead minnows, and intersex gonads in males (Kidd *et al.,* 2007). The fathead minnow population collapsed after the second year of exposure and the extirpation continued for the third year of exposure followed by an additional two years after the EE2 exposure had ceased (Kidd *et al.,* 2007). Fathead minnows were able to re-establish after four years post-exposure to EE2, and induced Vtg and intersex effects had disappeared (Blanchfield *et al.,* 2015). In a laboratory study, zebrafish were exposed to 0.5, 5, and 50 ng/L of EE2 for multiple generations. The F_0 generation had reduced fecundity at 50 ng/L, and the offspring F1 generation exposed (210 dpf) to 5 ng/L also had reduced fecundity (Nash *et al.,* 2004). Thus, life-long exposure to environmentally relevant concentrations of EE2 has the potential to have significant population reproductive impacts.

1.7 Aquatic Toxicology

1.7.1 Toxicity Testing

Aquatic toxicology is the study of adverse effects of chemical substances on aquatic organisms, alone and in mixtures, and the modifying factors affecting such toxicity (Wells, 2009). The concept and practice of toxicity testing became prevalent in the 1900s, with the major aim being to evaluate the toxicity of chemical contaminants and how they interact with aquatic species (Wells, 2009). In order to effectively study the toxicity of these

contaminants, standardized methodologies were developed by organizations such as the American Society for Testing and Materials (ASTM) and the Organisation for Economic Co-operation and Development (OECD); their protocols and guidelines are often used in aquatic testing (Rand, 2008). Standardization was necessary, to provide more experimental consistency and allows scientists to compare results and findings from different laboratories with confidence that most procedural differences have been eliminated. One of the main motivations for toxicity testing is to determine the relative toxicity of chemicals to non-target species, and to assess any potential biological effects (Rand, 2008). One significant limitation of standardization, however, is that standardized tests have the potential to lose some of their ecological relevance, since multiple factors are continuously fluctuating in a natural environment.

There are various toxicity testing methodologies that are followed depending on the research involved and the level of effect detection required. The most common methodologies used in toxicity testing are acute or chronic studies in which fish have been pulsed, intermittently, or continuously exposed (McKim, 1997; Rand, 2008; Sprague, 1969).

Another common standardized approach in toxicity testing involves the use of reference toxicants. Reference toxicants are used to assess the health and sensitivity of organisms (EPA, 2002), and help to evaluate and explain both intra- and inter-laboratory differences (EPA, 2002). One of the most commonly used reference toxicants in aquatic toxicity testing is copper sulfate ($CuSO_4$) (Environment Canada, 2007; EPA 2002). Copper sulfate has been deemed a good reference toxicant to use due to its high water solubility, stable shelf life, ease of analysis, and minimal hazard to user. Additionally,

copper is a very well-studied compound with regards to aquatic toxicity, and both the influence of pH and water hardness have been evaluated (Environment Canada, 2007).

1.7.2 Acute Toxicity

Acute toxicity, typically 24 – 96 h in duration for fish, can be defined as a stimulus that is severe enough to cause a response within a short period of time (Sprague, 1969). A LC_{50} is the most common acute toxicity endpoint which can be defined as, the lethal concentration that causes 50% mortality over a specified time period, generally 24 – 96 h in acute methodologies. Another common terminology used in acute toxicity is the effective concentration, EC_{50}, which is defined as the concentration at which a toxicant is able to elicit a biological response in 50% of the test organisms. The EC_{50} allows the ability to encompass a wide variety of endpoints as opposed to just lethality. LC_{50} and EC_{50} values allow reference points to be set for the acute toxicity of the compound, which is important when creating guidelines and regulations about safe thresholds in the environment.

1.7.3 Chronic Toxicity

Chronic toxicity studies are highly important in aquatic toxicology however they are not always completed as they can be lengthy in duration and costly. If the test encompasses more than one tenth of an organism's life cycle, it can be defined as a chronic toxicity test (Sprague, 1973). There are a variety of endpoints used to evaluate chronic toxicity such as reproductive endpoints (fertilization, hatch, time to spawn) and markers of

general health and condition of the organism (length, weight, condition factor, gonadosomatic index (GSI) and liversomatic index (LSI)). Studying organisms over a complete life-cycle is important as many developmental events occur throughout the transitional stages which may be critical periods in which fish may be vulnerable to disruption (McNabb *et al.,* 1999).

1.7.4 Life-cycle studies & Multi-generational studies

Full life-cycle studies are an important type of chronic toxicity test and are one of the most reliable ways to establish long-term environmentally safe concentrations of compounds (McKim, 1995). Life-cycle studies are important because they can give detailed information about delayed effects that may otherwise be missed, as they cover all of the developmental stages throughout the organism's life. They are also very comprehensive in terms of the endpoints studied; typically the effects of a constant exposure on the growth, survival, and reproduction of the species are monitored (McKim, 1995). A major limiting factor for undertaking life-cycle studies is that they are extremely resource and time intensive. Consequently, a variety of partial life-cycle studies have been developed to permit a less intensive and more practical toxicity tests (Miracle & Ankley, 2005). Life-cycle and partial life-cycle reproduction tests have been conducted using a variety of different fish species such as; fathead minnows (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and American flagfish (*Jordanella floridae*) (Anderson *et al.,* 2016; Beyger *et al.,* 2012; Holdway & Dixon, 1986; Lange *et al.,* 2001; McKim, 1995; Parrott & Blunt 2005).

Multi-generational studies are another important focus of aquatic toxicology. As with full life-cycle studies they help to provide important information that may otherwise be overlooked with traditional acute toxicity testing. Very few multi-generational studies have been completed with regards to pharmaceuticals, and currently no mixture multi-generational studies have been reported in the literature (Overturf *et al.,* 2015).

1.8 Fish Physiology

1.8.1 Toxicokinetics in Fishes

The toxicokinetics of contaminants involves four main processes once they enter the fish; absorption, distribution, biotransformation (metabolism) and excretion, often referred to as ADME (Kleinow *et al.,* 2008; Tierney *et al.,* 2014). Fish can absorb toxicants across the gills, skin, and gastrointestinal tract. Exposure routes are via sediment-borne, food-borne, or water-borne exposure (Kleinow *et al.,* 2008). Most often, contaminants are absorbed through oral consumption of food or sediment, or through direct absorption via the gills or skin (van der Oost *et al.,* 2003). Absorption rates are often toxicant dependant, and can take place through a variety of methods such as: active transport, endocytosis, facilitated diffusion, passive diffusion, and filtration through membrane channels (Tierney *et al.,* 2013).

Once a toxicant is present in the fish, it is distributed within the body. The contaminant is transported into the blood from across the epithelium. The aqueous portion of blood is the plasma and hydrophilic compounds are readily dissolved and transported via the plasma (Tierney *et al.,* 2013). Hydrophobic compounds, however, by their nature do not readily dissolve in plasma and instead associate with other constituents of the blood,

most often plasma proteins (Tierney *et al.,* 2013). Once in the blood, the contaminant is then transported to the site of action (organs, tissues) where it can elicit its effects (Kleinow *et al.,* 2008).

Biotransformation of the toxicant involves the enzymatic conversion of the chemical from its parent compound into a metabolite in order to allow for easier excretion (Tierney *et al.,* 2013; van der Oost *et al.,* 2003). Typically the liver is the most common organ involved in the biotransformation of xenobiotics, where the toxicant undergoes biotransformation via Phase I reactions which create a more reactive and often more hydrophilic compound, followed by Phase II reactions which conjugate the endogenous molecule and make it more bulky and hydrophilic (Tierney *et al.,* 2013; van der Oost *et al.,* 2003). Although, biotransformation occurs to assist with elimination, it can lead to the production of more toxic metabolites (van der Oost *et al.,* 2003). Following completion of biotransformation, the toxicant is now able to be excreted.

The major modes of excretion in fish are through fecal elimination, branchial elimination (gills), and renal elimination (kidneys) (Tierney *et al.,* 2013). Hydrophilic compounds are more readily excreted via the major methods. Hydrophobic compounds have the potential to partition into lipids, causing them to remain trapped until mobilization of the lipid occurs (van der Oost *et al.,* 2003). This mode of toxicant removal is known as lipid detoxification and is often only a temporary solution pending lipid metabolism.

1.9 Fish

Currently, there are approximately 32,000 species of fish with 3890 new species described since 2006 (Nelson *et al.,* 2016). Fish are ubiquitous and inhabit a wide variety

of aquatic ecosystems, being found in both marine and freshwater environments; including oceans, rivers, lakes, and streams (van der Oost *et al.,* 2003). As such, they are important species to study for assessing the effects of environmental contaminants at both the biochemical and biological level of response (van der Oost *et al.,* 2003). Fish are an important part of the aquatic food-web and play a large role in providing energy from lower to higher trophic levels (van der Oost *et al.,* 2003). The Cyprinodontidae family and Salmonidae family of fish have been found to be excellent test species for use in laboratory studies and will be discussed in more detail below.

1.10 American Flagfish (*Jordanella floridae*)

1.10.1 Habitat and Characteristics

The American flagfish (*Jordanella floridae)*, belongs to the *Cyprinodontidae* family, and is a warm-water killifish that is native to the central and southern areas of Florida (Bonnevier *et al.,* 2003; Foster *et al.,* 1969). They are commonly found in weedy, shallow, freshwater areas, but have also been observed in slightly brackish water (Foster *et al.,* 1969; St. Mary *et al.,* 2004).

Flagfish are an oviparous fish; male and female flagfish typically attain a maximum length of 50 mm, 45 mm, respectively (Foster *et al.,* 1969). Flagfish are highly sexually dimorphic with males displaying alternating red and yellowish-green stripes, and females displaying a black ocellus on the dorsal fin and a much paler appearance (yellowish-olive colour) (Foster *et al.,* 1969).

1.10.2 Behaviour and Breeding Patterns

The behaviour and breeding patterns of flagfish have been well studied and characterized (Bonnevier *et al.,* 2003; Foster *et al.,* 1969; Hale *et al.,* 2003; Mertz & Barlow 1966). Flagfish reproduce best under ideal conditions of 25 – 26 ºC water temperature and 16 h light and 8 h dark photoperiod (Foster *et al.,* 1969). The typical breeding behaviour begins with the male flagfish displaying his fins and guarding his nest (spawning substrate) (Foster *et al.,* 1969; Mertz & Barlow, 1966). The female then approaches (with a blanched appearance) and the "t-dance" begins, they will continue this until the female is ready to mate and they are oriented correctly to each other (Foster *et al.,* 1969; Mertz & Barlow, 1966). When spawning begins, the male and female move together in a coordinated clasp while she expels eggs and he externally fertilizes them as they are released (Foster *et al.,* 1969; Mertz & Barlow, 1966). The male flagfish will then clean, fan and guard the eggs (parental care) until they hatch, typically 5 – 7 d after fertilization (Klug *et al.,* 2005). It has been noted that the amount of eggs expelled by females can be influenced by the presence of food in the gut. The presence of more food can help to apply pressure on the ovary and can lead to the expulsion of eggs (Foster *et al.,* 1969).

1.10.3 American Flagfish as a Test Species

Flagfish are an excellent test species for reproductive toxicity studies. They have a short time to maturation, can be sexed within 60 – 90 d post hatch, and they are able to start reproducing within 3 – 4 months (Foster *et al.,* 1969; Hodway & Dixon, 1986). There

short life-cycle, ability to continuously reproduce (under ideal conditions), and their small size make them an ideal test species to work with in a laboratory setting.

Flagfish have been used for a variety of acute and chronic toxicity tests. Work has been completed using both pulse and continuous exposure to contaminants with a variety of endpoints assessed, mainly reproduction (Anderson *et al.,* 2016; Beyger *et al.,* 2012; Holdway & Dixon, 1986). It has been demonstrated that flagfish have similar responses to toxicants as many other fish species (Fogels & Sprague, 1977; Smith *et al.,* 1991).

1.11 Rainbow Trout (*Oncorhynchus mykiss*)

1.11.1 Habitat and Characteristics

Rainbow trout (*Oncorhynchus mykiss*) belong to the salmonidae family, and are a cold water fish that is native to western North America, but currently inhabits all Canadian provinces (Environment Canada, 2007). Rainbow trout typically reside in cool fresh water rivers, streams, and lakes but there is a subspecies of rainbow trout (steelhead) that are anadromous and go out to sea for a few years before returning to freshwater to spawn (Environment Canada, 2007). The ideal temperature for rainbow trout ranges from 10 – 16 ºC (EPA, 2002).

1.11.2 Growth and Feed

Rainbow trout growth is highly variable and is influenced by the habitat, life history, and type of food available to them (EPA, 2002). Rainbow trout are opportunistic feeders and in a natural habitat they typically eat plankton, crustaceans, snails, leeches, and smaller fish and fish eggs, as well as insects (NRCS, 2000). In a laboratory setting, rainbow

trout typically eat high quality commercial trout pellets that have been manufactured to include the necessary dietary components for optimal health for the varying size of fish (Hinshaw, 1999).

1.11.3 Rainbow Trout as a Test Species

Rainbow trout are an ideal test species and are a standard cold-water fish that have been used in aquatic toxicology (Environment Canada, 2007). They are easily maintained in the laboratory, they do not stress easily from handling, and they have proven sensitivity to a variety of contaminants (EPA, 2002). There are a series of Canadian regulations and guidelines created by Environment Canada which have helped to standardize the use of rainbow trout in toxicity testing. Much husbandry and nutritional research has been done using rainbow trout with regards to growth, feed, and reproduction, making them an ideal species to work with in a controlled laboratory setting.

1.12 Knowledge Gaps

Stress can play a large role in a fishes' ability to perform their necessary life functions, including growth, survivability, and reproduction (Schreck, 2010). Some of the main stressors for fish can either be chemical stress such as exposure to metals, pesticides, or pharmaceuticals, or environmental stress such as altered temperature, pH, oxygen, and food (Holmstrup *et al.,* 2010). The impacts of chemical stressors have dominated the field of aquatic toxicology while much less research has been done on the effects of environmental stressors.

As previously mentioned, limited research has been completed on the effects of nutritional conditions on the toxicity of chemicals (Holmstrup *et al.,* 2010). One area of concern involves the effects that food limitation may have on the overall life history of fish and the potential permanent effects on adults and offspring (Holmstrup *et al.,* 2010). Hatchery practices of maintaining desirable sized fish year round involve limiting feed rations ('holding back'), and may have the potential to influence toxicity results of fish that are used for regulatory testing. As such, it is important to investigate the impact that limited ration may have on toxicity thresholds.

Chemical stressors are also important regarding impacts on fish. Limited research has been completed on the impacts of both mixtures and multi-generational exposures of pharmaceuticals on fish. There are substantial knowledge gaps with regards to chronic long-term exposure of non-target aquatic organisms to pharmaceuticals (Hughes *et al.,* 2013). As the consumption of pharmaceuticals continues to increase, knowledge of the effects of long-term continual exposure to low levels of such contaminants on fish is critical. Pharmaceuticals are often thought to be low risk due to their therapeutic use in humans and extremely low environmental concentrations. However, many of the environmental and laboratory studies performed to date have been over simplified and of relatively short duration. Thus it is possible that the long term chronic effects of such contaminants may be underestimated since very little research regarding multi-generational impacts have been undertaken.

2 Rationale & Aims

This research was undertaken to assess the impact of various environmental and chemical stressors on both cold-water (rainbow trout) and warm-water (American flagfish) model species of fish. Little research has been completed on either the effect of feed manipulation and its impact on the toxicity of contaminants, or the impact of pharmaceuticals on fish over multiple generations. The overall goal of this research was to better understand how select stressors (both environmental and chemical) alter fish physiology and reproduction. This research is necessary in order to better understand the potential scenarios that wild fish may be experiencing in their natural habitat.

2.1 Aim 1

Assess the impact of restricted rations on subsequent acute lethal toxicity

The first objective was to determine if delayed growth would alter the acute lethal sensitivity of fish to toxicant exposure. More specifically, juvenile rainbow trout were held on restricted rations for 21 and 42 d. After restricted feeding for 21 and 42 d fish were exposed to the reference toxicant copper and 24 h LC_{50}s were determined. Results of these findings will be discussed with respect to current hatchery supply practices for regulatory toxicity experiments.

> *Null hypothesis: Restricted ration will not alter acute lethality of toxicants to rainbow trout*

2.2 Aim 2

Elucidate the impact of environmentally realistic chemical stressors on reproductive endpoints and subsequent toxicant exposure

The second objective of my research was to determine if exposure to pharmaceuticals at environmentally relevant concentrations would alter reproductive endpoints of fish and change the sensitivity of fish to subsequent toxicant exposure. More specifically, American flagfish were exposed to varying environmentally relevant concentrations of ibuprofen, naproxen, and 17α-ethinylestradiol and a mixture for 19 d. Reproductive endpoints of egg production, fertilization, and hatchability were assessed, along with subsequent toxicant challenges to the reference toxicant copper on larval offspring.

> *Null hypothesis: Short term exposure to environmentally relevant concentrations of pharmaceuticals will have no impact on flagfish reproduction and will not alter the sensitivity of offspring to contaminants*

2.3 Aim 3

Determine the impact of multi-generational and mixture exposure of environmentally realistic chemical stressors on reproductive endpoints and subsequent toxicant exposure

The final objective was to determine if multi-generational exposure to environmentally realistic pharmaceutical mixtures will alter any reproductive endpoints, or change the sensitivity of larval offspring to subsequent toxicant exposure. More specifically, American flagfish were exposed to varying environmentally relevant mixtures of ibuprofen, naproxen, and 17α-ethinylestradiol for multiple generations. Reproductive

endpoints of egg production, fertilization, and hatchability were assessed along with subsequent toxicant challenges of larval offspring to copper.

Null hypothesis: Multi-generational exposure to environmentally relevant concentrations of pharmaceuticals will have no impact on flagfish reproduction and will not alter the sensitivity of offspring to contaminants

3 The impact of restricted rations on subsequent acute lethal toxicity

3.1 Introduction

Every year tons of effluents are released into our waterways from both direct and indirect sources. These effluents can be complex mixtures of pharmaceuticals, metals, pesticides, and many other compounds. In order to establish guidelines and regulations for the compounds entering into our waterways, toxicity testing has been a common method used to determine safe thresholds and acceptable limits for chemicals being received by the environment. The standardization of traditional toxicity testing over the past few decades has helped to provide more streamlined and consistent testing methods, however, some variability is noted in the acute toxicity data available for specific contaminants in the scientific literature (Holmstrup *et al.,* 2010). Fogels and Sprague (1977) have noted a 5-fold difference in the sensitivity of rainbow trout to copper within the same laboratory, and when comparing between laboratories, an 8.6-fold difference was noted. Often, this variation in acute toxicity values has been attributed to species sensitivity, water chemistry, and body size (Howarth & Sprague, 1978). However, this variability poses a problem when trying to set reliable safe limits, as either an over-estimation, or an under-estimation, can be problematic for non-target organisms and the economy, and there may be other factors causing it (Holmstrup *et al.,* 2010).

Many organisms spend their lifespan in sub-optimal conditions with a variety of added environmental stressors (Holmstrup *et al.,* 2010). The interactions of important abiotic and biotic modifying factors such as temperature, water chemistry and pathogens have been

well studied and can significantly impact the toxicity of some chemicals and thus result in some of the reported variation (Holmstrup *et al.,* 2010).

One potential biotic modifying factor of toxicity that has been poorly studied is ration. The two main components of ration as an environmental stressor are via the quality or quantity of feed. Little work has been completed on both the impact of quality or quantity of feed on toxicity (Table 1). Variations in reported acute toxicity of different compounds due to variation in prior feeding ration have often not been considered. The prior feeding regime (life-history) of test fish prior to acute toxicity testing may play a large role in the sensitivity of the organism to a contaminant. Nutritional conditions during an organisms' key developmental stages may play an important role in its overall life-history (Metcalfe & Monaghan, 2001). Early food deprivation in fish may cause permanent effects on adult organisms, and potentially even offspring (Holmstrup *et al.,* 2010).

Nutritional status of an organism has been defined as 'both the quality and quantity of the organisms diet' with quality referring to the proximate composition of the feed, and quantity referring to the feeding regime (number and amount of feed per day) (Lanno *et al.,* 1989). It is known that an organism's metabolic rate will increase with feeding and therefore the uptake, metabolism, and excretion of a toxicant can be affected by its nutritional status (Lanno *et al.,* 1989).

Table 1: Overview of some nutritional studies demonstrating the interactions between quality or quantity of ration and toxicant sensitivity.

Nutritional Status	Toxicant	Fish Species	Life Stage	Impact Observed	Reference
Quality	Chlordane	Rainbow Trout[a]	Juvenile	Yes	Mehrle *et al.,* (1977)
Quality	Copper	Rainbow Trout[a]	Juvenile	Yes	Dixon and Hilton (1981)
Quality	11 Chemicals	Rainbow Trout[a]	Fry	No	Marking *et al.,* (1984)
Quality	NaPCP	Rainbow Trout[a]	Juvenile	No	Hickie and Dixon (1987)
Quantity	Copper	Carp[b]	Juvenile	Yes	Hashemi *et al.,* (2008)
Quantity	Ammonia	Rainbow Trout[a]	Juvenile	Yes	Wicks and Randall (2002)

[a] Rainbow trout (*Salmo gairdneri now Onchorhynchus mykiss*)
[b] Carp (*Cyprinus carpio*)

Fish used in standardized toxicity tests are often maintained on specific rations for varying lengths of time by hatcheries to ensure a constant supply of desired fish sizes are available for purchase. Test fish are also maintained at low rations in laboratories for significant amounts of time to maintain optimal (standardized) sized fish before executing acute toxicity tests. As reported by other researchers (Gourley & Kennedy, 2009; Holmstrup *et al.,* 2010), there is limited information on the potential for restricted rations to influence acute toxicity data. Thus it is important to assess whether or not restricted ration levels prior to conducting standard acute toxicity tests have the ability to significantly influence subsequent acute toxicity results.

Rainbow trout (*Oncorhynchus mykiss*) were selected as the test species for this study. They are a common freshwater species indigenous to Canada and they are often used for acute toxicity testing; much of the regulatory toxicity work is completed using hatchery-raised rainbow trout. The use of rainbow trout in this study increases the relevance of this research to standardized acute toxicity testing undertaken to protect Canadian freshwater environments. Rainbow trout are well-studied with regards to feed and nutritional requirements (Wurtsbaugh & Davis, 1977), and controlled ration size was utilized to restrict growth.

As mentioned above, there has been much study on proper feeding practices as hatcheries are always trying to improve the quality of their fish while decreasing the cost to feed them. Time and effort have been put into studying the proper timing, amounts, and composition of feed. The major nutrients necessary for fish are proteins, lipids, carbohydrates, vitamins, and minerals (Hilton & Slinger, 1981). Fish are capable of synthesizing most amino acids, however, there are a few essential amino acids that they

are unable to manufacture and thus must be provided (Hilton & Slinger, 1981). Previous work has established that the main factors which affect feed consumption in fish are ambient temperature, energy content of the feed, and water quality parameters (Hilton & Slinger, 1981). The present study was interested in the impact of restricted ration on acute toxicity and as such temperature, energy content, and water quality parameters were held constant through-out the duration of the study.

Specific feeding rates for salmonids have been well documented and are most commonly expressed as percentage of body weight per fed day (Hilton & Slinger, 1981). Larger fish require less feed percentage compared to smaller fish, and the percentage of body weight ranges anywhere from 0.5 – 10 % (Hilton & Slinger, 1981).

The toxicant copper sulphate pentahydrate ($CuSO_4 \cdot 5H_20$) was chosen as a reference toxicant to assess the effect of restricted rations on rainbow trout acute toxicity. Copper sulphate pentahydrate has been commonly used as a reference toxicant and has been well-studied in the literature (Environment Canada, 1990, 2007, 2014). Copper sulphate pentahydrate has a long stable shelf life, is easily available, highly water soluble, and safe to work with, thus making it an ideal reference toxicant for use in aquatic toxicity testing (Environment Canada, 1990, 2007).

The aim of this study was to determine if prior life-history (restricted ration) has the ability to significantly alter the acute toxicity of contaminants. Rainbow trout were maintained via restricted ration for various durations (0, 21, and 42 days) (Figure 4) prior to exposure to assess whether or not holding back fish for different periods of time through the use of restricted feed would affect their acute copper toxicity.

3.2 Materials & Methods

3.2.1 Test fish

Rainbow trout (~0.4 g wet weight) were obtained from Linwood Acres Trout Farm (Cambellcroft, ON, Canada) and were transported back to University of Ontario Institute of Technology Aquatic Toxicology lab in an oxygen-aerated insulated tank from which they were transferred to 70 L aquaria receiving continuous flow controlled temperature (12 º C) laboratory water, with 16:8 h (light: dark) photoperiod daily with 30 minutes of simulated dawn and dusk included in the light phase, and were fed using an automatic belt feeder to ensure a constant supply of food was available.

3.2.2 Feeding regime

During acclimation, fish were fed a 2.1% body weight per day (BW/ day) feeding regime which is in accordance with freshwater feeding guidelines. Once the study commenced, fish were fed a 1 % BW/ day feeding regime in order to maintain approximate zero growth for the duration of the experiment. Fish were weighed to check for growth 10 days into the experiment to assess if zero growth was being maintained. Upon measurement, the feeding regime was adjusted to 0.4 % BW/ day for the remainder of the study to try to maintain zero growth. A set of growth control fish were reared alongside the experimental fish and were fed a 3.9 % BW/ day ration in accordance with freshwater fish feeding guidelines (Figure 4).

3.2.3 Test chemicals

Copper sulphate pentahydrate ($CuSO_4 \cdot 5H_20$) was purchased from Fisher Scientific® and used to make stock solutions which were administered continuously via peristaltic pump. Copper sulphate stock solutions were prepared to ensure that desired test concentrations were attained upon mixing with laboratory water before entering the aquaria. A lab water control (municipal water treated and dechlorinated with charcoal and resins, put through a reverse osmosis process, and then buffered with calcium carbonate to a neutral pH) was also assessed. Fish were continuously exposed to copper concentrations for 24 h in 10 L aquaria in order to obtain a 24 h LC_{50} value. Although 96 h LC_{50}'s are the normal standard measure of acute toxicity, a 24 h LC_{50} was selected due to laboratory constraints and animal care compliance.

3.2.4 Experimental Set-up & Exposure

Upon arriving at the laboratory and prior to the start of the experiment, fish were housed in four 70 L glass aquaria, supplied with flow-through water (99% molecular replacement in 24 h) and fed an acclimation ration of 2.1% BW/d (Figure 5a). After two weeks of acclimation 120 fish (10 fish per treatment in duplicate) were removed from the 70 L aquaria and were placed into each of the 10 L test aquaria and exposed to copper via peristaltic pump (Figure 5b). For the first 24 h LC_{50} experiment, fish were exposed to five nominal copper concentrations plus a control (0, 10, 32, 56, 100, 180 µg/L) through water-borne exposure for 24 h, and mortalities were recorded. The remaining fish were maintained in 70 L tanks and either kept on a maintenance ration (1% BW/d) or an optimum

growth diet (3.9% BW/d) (Corey Aquafeeds, 2008) (Figure 4). However, at day 10 fish were weighed and the maintenance ration was adjusted down to 0.4% BW/d to better achieve zero growth. After the 21 d had elapsed, 140 of the now 0.4% BW/d ration fish were exposed under the same conditions as described above to six nominal copper concentrations plus a control (0, 10, 18, 32, 45, 56, 100 µg/L). Copper concentrations were adjusted following the first LC_{50} to allow for better accuracy. Alongside this second 24 h LC_{50} test, ten of the 3.9% BW/d fish (growth control) were put in a tank and exposed to the first LC_{50} value to see if their sensitivity to copper was the same; mortality was recorded for all treatments. The remaining population of fish continued to be housed in 70 L aquaria for another 21 d (42 d total) on a 0.4% BW/d ration and the optimum growth ration fish continued to be reared alongside (Figure 4). After a total of 42 d had elapsed, the final 140 fish were removed and again acutely exposed to copper using the same methodology as described above, and again with 10 growth control fish exposed to the initial LC_{50} value. All test vessels were maintained at 12 ºC (± 0.5), water hardness was maintained at 20 mg/L as $CaCO_3$, pH 7 (± 1), and 16:8 h (light:dark) photoperiod with one half our of simulated dawn and dusk included in the light phase.

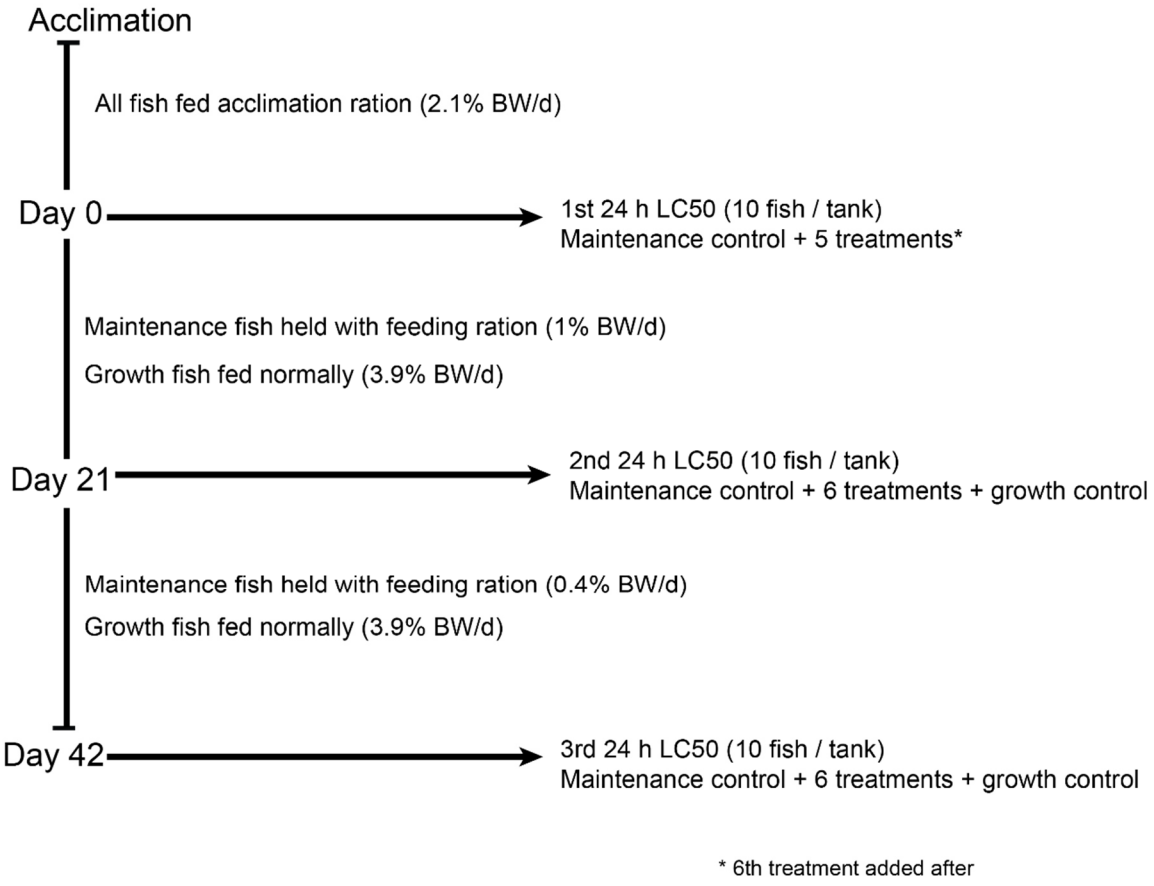

Figure 4: Study 1 schematic showing the timeline of activities and the feeding regime maintained for rainbow trout during the 42 d experiment. BW/d = body weight per day. Maintenance control was fed either 1% BW/d or 0.4 % BW/d. Growth control was fed 3.9% BW/d. **Ration changed at day 10 to 0.4% BW/d. Varying copper concentrations were used for the LC50 exposures (0 – 180 µg/L).

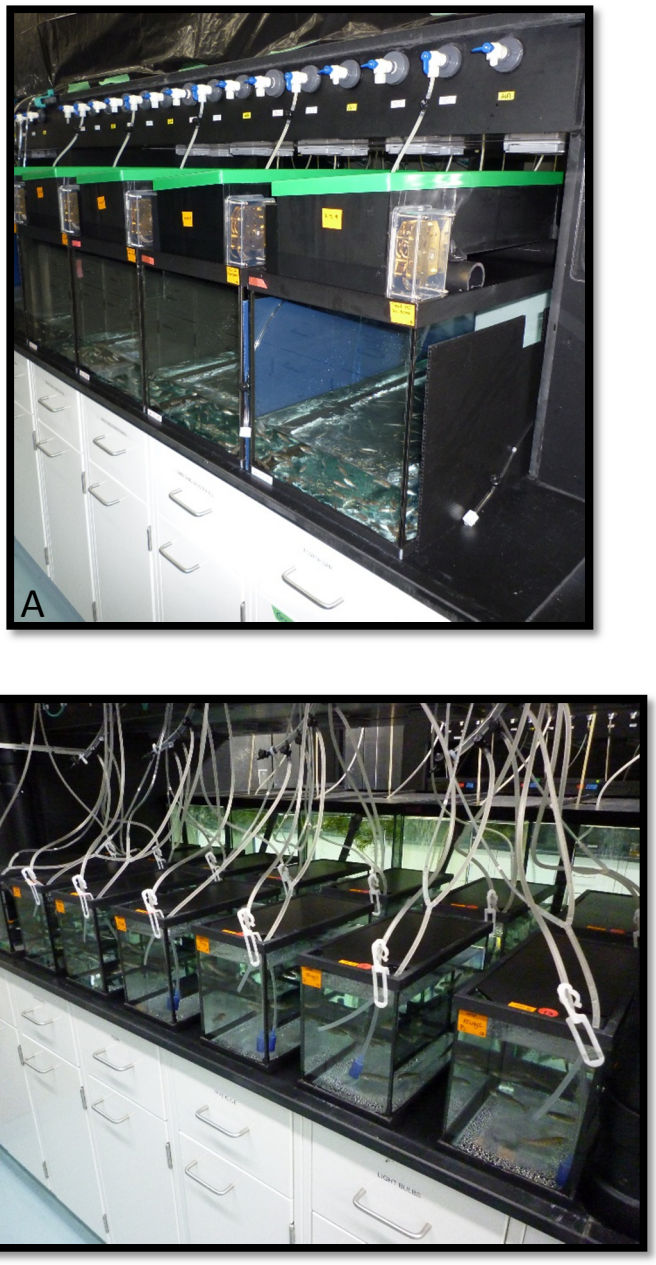

Figure 5: Set-up of flow-through aquaria. (A) 70 L aquaria for housing rainbow trout with automatic feeders installed for consistent delivery of feeding regime. (B) 10 L aquaria set-up with peristaltic pump delivery of various copper concertation's for LC_{50} trials.

3.2.5 Water analysis

Measured copper concentrations were determined for all nominal solutions from water samples that were collected during the flow-through exposure. The water samples were acidified and stored at 4 ˚C until they were analyzed. The samples were analyzed via graphite furnace atomic absorption (GFAA) in the University of Ontario Institute of Technology laboratories with assistance from Michael Allison (Table 2).

3.2.6 Statistical analysis

Data were analyzed with Biostat v5 (AnalystSoft Inc.). Acute lethal concentration (LC_{50}) data were analyzed by probit analysis. Significant differences ($p \leq 0.05$) were determined via overlapping confidence intervals.

3.3 Results

3.3.1 Water Analysis

Water samples were taken from all control and treatment tanks, and were analyzed using graphite furnace atomic absorption (Table 2). The first LC_{50} experiment ran five treatments and a control; after analysis, treatments were adjusted accordingly and subsequently six treatments were run alongside a control (Table 2).

3.3.2 Acute Toxicity

A standard 24 h flow-through LC_{50} of 34.9 µg/L (20.3 – 49.5) was determined for larval rainbow trout (1.8 ± 0.07 g wet weight) continuously exposed to copper at day zero (Table 3). After 21 d of holding at restricted rations (0.4 % BW/d), the rainbow trout were 2.1 ± 0.08 g wet weight and a 24 h continuous flow-through LC_{50} value of 40.3 µg/L (33.5 – 47.1) was determined (Table 3). There was no significant difference seen between these two LC_{50} values. Following another 21 d of holding at restricted rations (0.4 % BW/d), day 42 fish were 2.8 ± 0.18 g wet weight and a 24 h continuous flow-through LC_{50} value of 48.5 µg/L (39.9 – 57.1) was calculated (Table 3). There were no statistically significant differences between any of the LC_{50} values. A set of the growth control fish were subjected to the 24 h LC_{50} value (~45 µg/L) at day 21 and day 42 and 100% mortality occurred between 24 – 48 h for both.

3.3.3 Growth

The fish run alongside the experiment as a growth control (3.9% BW/d ration) were able to attain a final weight of 7.7 g wet weight throughout the duration of the study (42 d) (Figure 6). The restricted ration fish grew to a modest 2.8 ± 0.18 g wet weight from an initial weight of 1.8 ± 0.07 g (Figure 6).

Table 2: Nominal and mean measured water concentrations of copper analyzed using graphite furnace atomic absorption (GFAA).

Sampling Time	**Nominal Values (µg/L)**	**Measured Copper (µg/L)**
1st LC_{50} (day 0)	0	BDL[a]
	10	1.38 (0.3)
	32	17.7 (0.8)
	56	41.1 (3.0)
	100	65.8 (2.1)
	180	138.4 (7)
2nd LC_{50} (day 21)	0	BDL[a]
	10	0.5 (0.1)
	18	6.6 (1.2)
	32	16.7 (0.2)
	45	25.9 (0.4)
	56	37.1 (1.1)
	100	65.8 (2.1)
3rd LC_{50} (day 42)	0	BDL[a]
	10	1.5 (0.5)
	18	7.7 (0.8)
	32	21.8 (0.7)
	45	25.3 (1.3)
	56	45.5 (0.1)
	100	64.4 (3.1)

Data expressed as mean ± standard deviation
[a]BDL (below detection limit)

Table 3: Lethal concentration values of copper for juvenile rainbow trout before and during restricted feeding regimes.

Time Period	Species	Contaminant	24 h LC_{50} Value (µg/L)	LCL	UCL	Slope
Day 0	Rainbow Trout	Copper	34.9	20.3	49.5	0.03
Day 21	Rainbow Trout	Copper	40.3	33.5	47.1	0.05
Day 42	Rainbow Trout	Copper	48.5	39.9	57.1	0.05

LCL: Lower confidence limit
UCL: Upper confidence limit

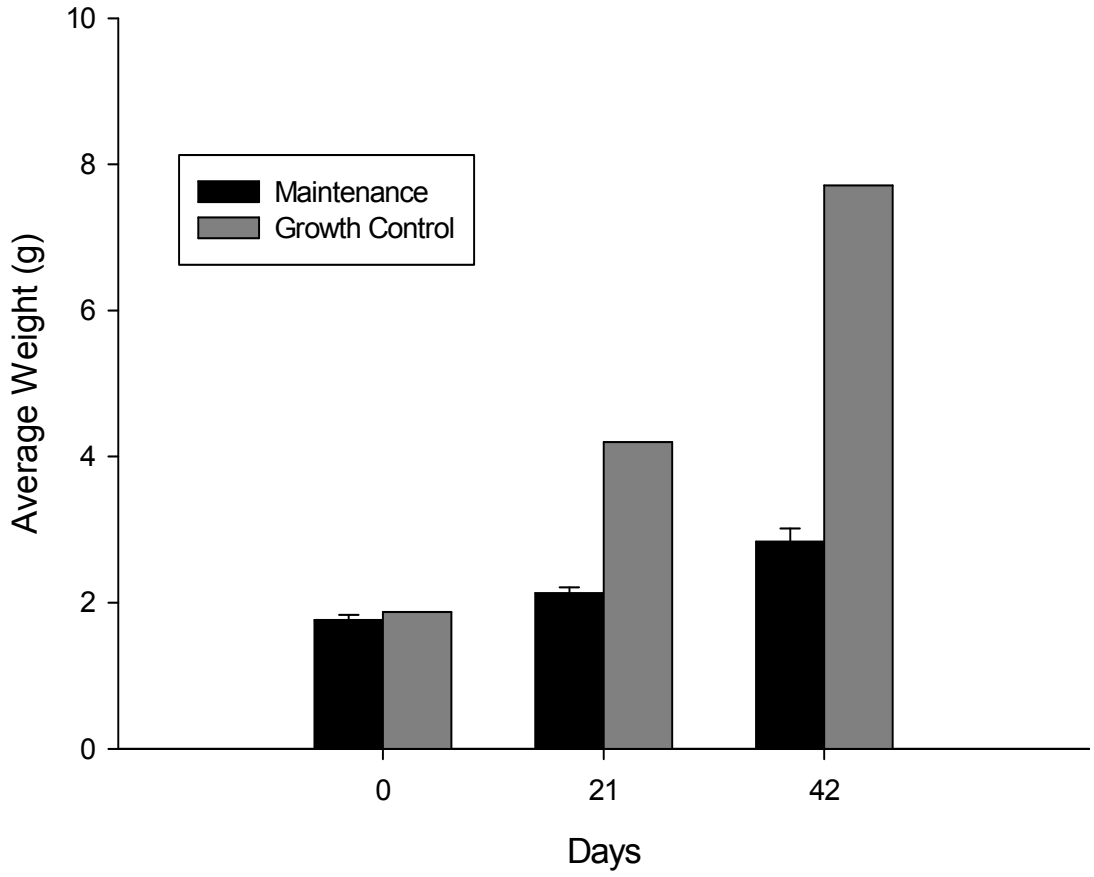

Figure 6: Average weight (g) of maintenance fish and growth control fish over the 42 day study.

3.4 Discussion

This study investigated whether or not prior restricted feeding regimes had the ability to alter acute toxicity of contaminants. The first aim of this study was to maintain approximate zero growth for rainbow trout over an extended period of time (42 d). The most important factor influencing growth in fish is ration (Diana, 2004). As such, ration was selected to be the mode of "maintaining" growth in this study. If a fish does not eat, it will ultimately lose weight which is considered negative growth, thus a zero ration has the potential to produce negative growth (Diana, 2004). In order to attain zero growth the fish actually requires some ration (Diana, 2004). Trout and their growth patterns based on feed have been well studied and trout were fed in accordance with published growth charts (Hinshaw, 1999). At day zero, rainbow trout weighed on average 1.8 g, and a rate of 1% BW/ day was selected based on previous research to try and maintain this weight throughout the duration of the study. Fish weight was reassessed after 10 d and on average fish weighed 2.0 g, therefore the ration was adjusted to 0.4% BW/ day to try and reduce any further growth. At 21 d fish weighed on average 2.1 g and by day 42 the fish weighed 2.8 g on average. Zero growth was not maintained on 0.4% BW/ day. The amount of ration required to truly maintain fish growth was not achieved. Due to animal care practices complete cessation of feed was not an option to try and maintain zero growth.

Growth in fish is very flexible and is often the last process to be performed; energy is first partitioned into metabolism and activity (Diana, 2004). A potential explanation for the weight gain mentioned above is that test fish were held in ideal conditions, so they were able to partition greater energy directly to growth. Being juvenile fish they were not concerned with reproductive growth and the laboratory setting removed the potential for

any predator-prey interactions reducing energy expenditure due to activity. Although true maintenance of weight was not achieved the fish were still restricted in growth when compared to the growth of control fish that were reared alongside at optimum ration levels.

The second aim of the study was to assess whether or not prior life-history of ration availability had the ability to alter the acute toxicity of contaminants for rainbow trout. Restricted ration for 21 and 42 d led to no significant differences in the acute lethal toxicity of copper. The 24 h LC_{50}'s were determined to be 34.9, 40.3, and 48.5 µg/L respectively for day 0, 21 and 42. Copper 96 h LC_{50}'s have been widely studied with a variety of organisms and values range from 10 µg/L to 10,000 µg/L (Taylor *et al.,* 2000). A study of rainbow trout ranging in weight from 1.1 – 3.2 g determined 96 h LC_{50}'s from 19.9 – 30.0 µg/L, respectively (Howarth & Sprague, 1978). These results are fairly similar to our observed LC_{50} values, with the slight difference in values likely due to the difference in timing of 24 h vs 96 h (Heath, 1995). Another potential explanation for the slight increase in the 24 h LC_{50} after 42 d could be due to the influence of soft water acclimation. Although all fish were acclimated for two weeks prior to the start of the experiment, research has shown that with a longer period of acclimation to soft water there is a reduced sensitivity to copper (Taylor *et al.,* 2000). One of the mechanisms of copper toxicity involves ionoregulation. Consequently, insufficient acclimation to an ion deficient (soft) water may lead to initial increased sensitivity to copper. Thus an acclimation of longer than two weeks may be necessary at very low hardness levels (Taylor *et al.,* 2000).

Nutrition as a modifying factor of toxicity has not been well studied and has often been overlooked (Lanno *et al.,* 1989). The nutritional status of an aquatic organism dictates whether or not the organism will be in a catabolic, anabolic, or maintenance physiological

state (Lanno *et al.,* 1989). A few studies have been completed on pre-experimental nutritional status and have noted an effect while others have not (Lanno *et al.,* 1989). A study found that the acute toxicity of chlordane to rainbow trout was altered depending on the quality of feed provided before toxicity testing occurred (Mehrle *et al.,* 1977). Rainbow trout were fed either a low or high protein diet for 42 d, and then 96 h LC_{50}'s were assessed. The high protein group had a statistically higher LC_{50} value than all other groups (Mehrle *et al.,* 1977). In contrast, another study investigated the effect of both diet and pre-exposure of sublethal levels of sodium pentacholorophenate (NaPCP) to rainbow trout. Fish were fed three different diets (low, intermediate, and high carbohydrate) for 12 weeks and then exposed to 0 or 50 µg/L NaPCP for 26 d as a pre-exposure (Hickie & Dixon, 1987). Rainbow trout were then exposed to 0 – 250 µg/L NaPCP for up to 216 h to determine the incipient lethal level (ILL) (Hickie & Dixon, 1987). The three different diets alone did not significantly impact the ILL, but the pre-exposure and dietary content together did affect the ILL. Low and intermediate carbohydrate NaPCP pre-exposed groups were significantly lower than their controls (Hickie & Dixon, 1987). Therefore, the diet alone did not directly influence the sensitivity of the fish to the toxicant.

Another study reared rainbow trout for 6-8 weeks on different diets (low and high carbohydrate) and then subjected the fish to bioassays assessing copper tolerance (0 – 250 µg/L) (Dixon & Hilton, 1981). Higher levels of dietary carbohydrate led to increased liver glycogen, and liver somatic indices, and lower liver protein content, ultimately leading to reduced copper tolerance (Dixon & Hilton, 1981). One possible explanation for this is that an increase in glycogen may be reducing the metabolism of copper by congesting hepatocytes (Dixon & Hilton, 1981). These findings demonstrate that the proximate

composition of diet has the ability to significantly modify toxicity when the feeding regime is kept constant (Lanno *et al.,* 1989). Although feed composition was not the focus of this study, it is still important to note that the quality of feed can play a role in the sensitivity of fish to toxicants.

As mentioned, few studies have been completed to determine the effect of fed or unfed fish, and different composition of diet, throughout the duration of the experiment but to the best of our knowledge no other studies have looked directly at the effect of prior feeding regime on acute lethal toxicity in fish. Although these results indicate that prior life-history involving restricted feeding regimes are not likely the source of variation in acute toxicity reported in the literature, there is the potential that feeding regimes may play a larger role in studies that are at a chronic or sub-lethal level. This has been documented in other studies that examined whether or not restricted feeding regimes throughout the experiment can alter the effects of sub-lethal toxicity.

One such study by Hashemi *et al.,* 2008 demonstrated that a low ration diet versus a high ration diet may have the ability to affect the acute toxicity of copper in fish. Fish fed a low ration diet were less sensitive to copper than fish fed a high ration diet which demonstrates that the quantity of ration during a study has the ability to affect the sensitivity of fish to contaminants (Hashemi *et al.,* 2008). Starved fish had 1.7-fold higher liver metallothioneins (MT) levels compared to fed fish. MT induction is likely species specific and is inducible by cortisol (stress) (Hashemi *et al.,* 2008). MT induction occurred in gills of starved fish only, providing better protection for the starved fish and thus a lower sensitivity (Hashemi *et al.,* 2008). Both induction of MT in the gills of starved fish and higher levels of MT in the liver of starved fish likely increased the tolerance of the starved

fish to copper exposure (Hashemi *et al.,* 2008). Other research has demonstrated that fish are fairly recalcitrant to the effects of restricted ration and have the ability to access other energy stores when experiencing reduced rations in order to combat toxicant exposures (Gourley & Kennedy, 2009).

Prior feeding regime is unlikely to affect short-term toxicity results; however, it is still important to consider including the feeding regime in the methodology when reporting on any toxicity data, as it could potentially be a factor in observed differences in the chronic or sub-lethal level toxicity. It is also important to note that both feed quality and quantity are likely to fluctuate in a wild setting. Periods of starvation and abundance are likely to occur as well as periods of poor or high nutritional quality of food. As such, seasonal variations and fluctuations of feed do need to be further researched as to their potential impact on the toxicity of contaminants to fish in the environment.

Overall these finding are important as they highlight that our current methodology for testing and setting safe limits/ thresholds for some chemicals are not likely influenced by prior feeding regime and thus support common hatchery practices of 'holding back' fish to maintain specific desired size classes. Future research should assess whether or not other specific classes of chemicals have the ability to be influenced by prior feeding regimes/life history.

4 The impact of environmentally realistic chemical stressors on reproductive endpoints and subsequent toxicant exposure

4.1 Introduction

Pharmaceuticals and personal care products (PPCPs) have been widely researched for the last 15 years. These chemicals are used for both the prevention and treatment of illness as well as general personal care and they contain numerous chemical classes, each having its own unique purpose with specific physio-chemical properties and biological activities (Boxall *et al.,* 2012; Corcoran *et al.,* 2010). Many pharmaceuticals are removed to some extent via wastewater treatment plants (WWTPs), however, their constant use by society allows these chemicals to be pseudo-persistent in the environment (Daughton & Ternes, 1999). Thus there has been significant interest and research into investigating the effects of pharmaceuticals on non-target organisms in the environment.

The most commonly detected pharmaceuticals in the environment are steroid hormones and NSAIDs (Santos *et al.,* 2010). Both ibuprofen and naproxen are classed as non-steroidal anti-inflammatory drugs (NSAIDs) and have been detected in surface waters in the ng/L to µg/L range (Fent, 2008; Kolpin *et al.,* 2002). Significant research has been completed on ibuprofen and its effects in fish, while much less has been done on naproxen. Ibuprofen has been shown to alter the pattern of breeding in Japanese medaka (*Oryzias latipes*) at relatively low concentrations (µg/L), while decreased fertility and hatchability have also been noted in zebrafish (*Danio rerio*) (Flippin *et al.,* 2007; Han *et al.,* 2010; Ji *et al.,* 2013). Much of the work available on naproxen is related to its acute lethality effects on aquatic invertebrates; furthermore it has also been demonstrated that the photo-degradation products of naproxen are more toxic than the parent compound (Cleuvers,

2003; Isidori *et al.,* 2005). 17 α-ethinylestradiol is a synthetic estrogen that is most commonly used in oral contraceptive pills. It is commonly detected in the low ng/L range in surface waters (Kolpin *et al.,* 2012). 17 α-ethinylestradiol has been widely studied; many of the findings reported are directly related to feminization in fish such as increases in vitellogenin (VTG) in males, induction of intersex, and other reproductive related endpoints (Jobling *et al.,* 1998; Shved *et al.,* 2008; Tyler *et al.,* 1999).

American flagfish (*Jordanella floridae*) are sexually dimorphic which allowed for easy selection of breeding harems. Under optimal conditions gender can be determined within 60-90 days, and a full life-cycle (spawning) experiment can be completed within 90- 120 days making them an ideal species for reproductive and multi-generational studies (Foster *et al.,* 1969; Overturf *et al.,* 2015).

Two studies were completed in order to elucidate the reproductive impact of pharmaceuticals on *Jordanella floridae* and as well as the potential impact of subsequent toxicity on offspring. Each study will be further discussed below.

4.2 Study 1

The first aim of the first study was to monitor the reproductive effects of exposure to environmentally relevant concentrations of ibuprofen (0.1 µg/L), naproxen (0.1 µg/L), and 17α-ethinylestradiol (10 ng/L) alone, and in a mixture with a pre-exposure (19 d) and exposure (19 d) phase. Of particular interest were the reproductive endpoints of fertilization, hatchability and egg production. The second aim was to assess if previous parental exposure could alter the sensitivity of offspring to a toxicant.

Null hypothesis: Short term exposure to environmentally relevant concentrations of pharmaceuticals will have no impact on flagfish reproduction and will not alter the sensitivity of offspring to contaminants

4.2.1 Study 1 Materials & Methods

4.2.1.1 Test Organisms

Sexually mature laboratory raised American Flagfish (*Jordanella floridae*) were used for this experiment. The fish were housed in 70 L glass flow-through aquaria which contained an air stone for aeration and circulation. A 16 hour light and 8 hour dark photoperiod with a thirty minute dawn and dusk was maintained for the duration of the experiment. The water temperature was also maintained at 25.0 ± 1.0 °C for the duration of the study. Mean (SE) characteristics of the water for chronic tests were as follows: dissolved oxygen 8.4 mg/L (0.08) and pH was 7.88 (0.04). All procedures involving animal handling were conducted in accordance with the Canadian Council on Animal Care Guidelines.

4.2.1.2 Chemicals & Flow-Through Dosing System

Ibuprofen sodium salt (α-Methyl-4-(isobutyl) phenylacetic acid), Naproxen sodium salt (S)-6-Methoxy-α-methyl-2-naphthaleneacetic acid sodium salt), and 17 α-Ethinylestradiol (17 α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol) were all purchased from Sigma-Aldrich Co. (*Oakville, Ontario*). Analytical grade acetone (>99%) was selected as the solvent carrier for EE2 treatments and carrier control. Acetone did not exceed 20 µl/L of dilution water and was equal in all treatments with EE2. A working stock solution was created for ibuprofen, naproxen, and EE2. Serial dilutions were then performed from the working stock solutions to obtain the desired stock solutions. A constant supply of ibuprofen, naproxen, and EE2 were delivered using a *Watson-Marlow* 200 Series 16

channel peristaltic pump (*Massachusetts, USA*). The flow of water into the 70 L aquaria was set to 5 turnovers per day to achieve a 99 % molecular turnover every 24 hours (Sprague, 1969). Stock solutions were delivered to aquaria at a rate of 80 µl/ minute. Each tank housed 67 L of water and the nominal concentration in the aquaria were as follows; Solvent Control (0), Ibuprofen (0.1 µg/L IBU), Naproxen (0.1 µg/L NAP), EE2 (10 ng/L EE2), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP), Mix 2 (0.1 µg/L IBU + 0.1 µg/L NAP + 10 ng/L EE_2).

Tricane methanesulfonate (MS-222) and sodium bicarbonate were used to anaesthetize the fish before dissections. A concentration of 350 µg/L of MS-222 was used by dissolving it into 25ºC laboratory water.

For copper challenges a working stock solution of copper sulphate pentahydrate (CuSO4·5H20) *(Thermo-Fisher Scientific)* was made by dissolving the desired amount of copper in laboratory water. The molecular weight was used for calculations so that the desired copper content was present rather than copper sulphate content. Serial dilutions were then completed to obtain the desired concentrations (0 – 25 µg/L).

4.2.1.3 Feed

Flagfish were fed three types of feed throughout the duration of the experiment which included flake food, frozen brine, and freshly hatched brine shrimp. Tetramin® Pro Flake food (Tetra United Pet Group), was composed of 46.0% minimum crude protein, 12.0% crude fat, 3.0% crude fibre, 1.1% phosphorus, 200 mg/kg ascorbic acid, and a maximum moisture of 8.0%. Bio-pure frozen brine contained 8.0 % minimum crude protein, 5.0% minimum crude fat, 2.0 % maximum crude fibre, and 86.0% maximum

moisture *Hikari Sales* (*Hayward, California*). The freshly hatched first instar nauplii were harvested from premium grade brine shrimp eggs purchased from Brine Shrimp Direct (*Ogden, Utah*).

4.2.1.4 Water Parameters

Nitrate, Nitrite, Alkalinity, Water Hardness

All Purpose 5-way Test Strips from Lifegard® Aquatics (*Cerritos, California*) were used to monitor nitrate, nitrite, alkalinity, and water hardness in each 70 L aquarium. The test strip was dipped into the water and swirled two times before being removed. The water hardness and alkalinity were immediately compared to the freshwater colour chart. After 30 seconds had elapsed the nitrate and nitrite were compared to the colour chart.

4.2.1.5 Temperature and pH

The pH was monitored daily for the duration of the experiment using a SevenEasy pH meter (*Mettler-Toledo*). Temperature was also monitored and recorded daily for each 70 L aquaria using a Traceable® infrared thermometer.

4.2.1.6 Water Sampling

A 500 mL sample was collected from each treatment, and carrier control as well as a composite sample from lab water control. Water samples were collected on day 11 of the exposure. Water samples were transported on ice on the day of collection to Trent Water Quality Centre (*Trent University*, *Peterborough, ON*) and were extracted and

analyzed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

4.2.1.7 Experimental Design

Breeding harems were selected (2 male: 4 female) and placed into an aerated 70 L flow-through aquaria. The male fish were selected according to size, one being slightly larger than the other, so that they could easily establish dominance. All females were selected to be slightly smaller than the males. One breeding substrate was assigned per aquaria. The substrate was composed of a glass plate wrapped with well washed green Orlon® wool in order to mimic an algae covered surface.

Once breeding harems were established a 19 d pre-exposure phase was conducted in order to assess reproductive capacity. Steady state was considered to be established once greater than 30 eggs / day were produced for four consecutive days. Egg production, fertilization, and hatch were assessed during the pre-exposure phase. Once reproductive capacity was determined, the peristaltic pump was turned on and the 19 d exposure began. Egg production, fertilization, and hatch were assessed during the exposure (Figure 7). Finally, a 1 week depuration period referred to as the 'post-exposure phase' was completed after the peristaltic pump was turned off.

For egg collection, breeding substrates were removed daily from each aquarium and eggs were dislodged into separate 1 L polypropylene containers filled with laboratory water. The substrate was then rinsed and placed back into the same tank it was removed from. The collection containers were placed in the temperature control room (27.0 °C). Once all eggs were collected from all aquaria the eggs were enumerated. As the eggs were

enumerated they were transferred into sterile petri dishes that contained rearing solution. Rearing solution consists of 10 % NaCl, 0.30 % KCl, 0.40 % $CaCl_2 \cdot 2H_2O$, 1.63 % $MgSO_4 \cdot 7H_2O$, 0.01 % methylene blue, and distilled water. Collected eggs were monitored daily for fertilization and hatch.

After the depuration period the adult flagfish were euthanized using Tricane methanesufonate (MS-222) and dissected. The fish were sexed and a variety of endpoints were assessed including total length, wet weight, LSI, GSI, and condition factor (Figure 7).

4.2.1.8 Larval Copper Challenges

Throughout the egg collection period some offspring were collected and kept to conduct larval copper challenges on. These copper challenges were conducted by doing a 96 h LC_{50} using copper as a reference toxicant to assess if the previous parental exposure to varying treatments would alter the LC_{50} value of the offspring compared to the control offspring. Once hatching began larval fish were pooled by treatment into crystallization dishes with laboratory water (25.0 °C). The fish were transferred into 6-well containers each containing a control (0), 2.5, 5, 7, 10, 25 µg/L of copper. There were 7 larval fish per well and the study was run in triplicate. A 95 % static renewal was performed daily along with a mortality check. Fish were unfed for the duration of the study (Figure 7).

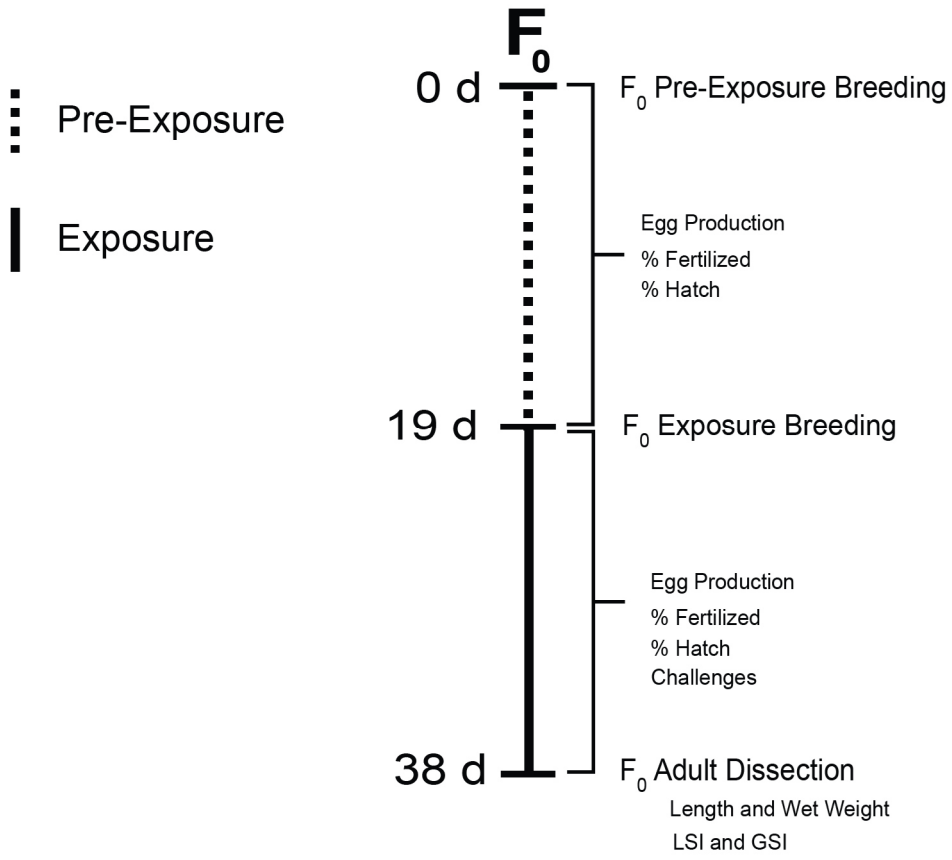

Figure 7: Experimental timeline for short term reproductive study exposing American flagfish to individual and mixtures of pharmaceuticals. Parental generation (F_0) were selected as adults and put into breeding harems. A 19 d pre-exposure and a 19 d exposure breeding period took place. Both periods assessed egg production, fertilization, and hatchability. A subset of offspring were kept and used for larval copper challenges throughout the 19 d exposure. At the end of the study the adult fish were dissected and length, wet weight, liver somatic index (LSI), and gonad somatic index (GSI) were assessed.

4.2.1.9 Statistical Analysis

Data were analyzed with STATISTICA 12.0 software (StatSoft, Tulsa, OK, USA). Data were checked for normality using Shapiro-Wilk's W test, and for homogeneity of variances using Brown and Forsythe's test. One-way and two-way analysis of variance (ANOVA) were performed to analyze overall differences for total length, wet weight, LSI, GSI, and reproductive endpoints. Cumulative egg production data was analyzed using daily intervals, with comparisons being made to controls and between treatments. Significant differences ($p \leq 0.05$) were then confirmed by Tukey's HSD post-hoc test. If the assumptions for ANOVA were not met and data could not be transformed, then significance was determined using a non-parametric Kruksal-Wallis test.

4.2.2 Study 1 Results

For this study ibuprofen and naproxen were compared back to control (CW) for significant differences, and 17-α-ethiynlestradiol, Mix 1 and Mix 2 were compared back to the carrier control (CCW).

4.2.2.1 Water Sampling

The nominal values for Ibuprofen, Naproxen, 17-α-Ethinylestradiol, Mix 1, and Mix 2 were as follows; Control (0), Control Carrier (0), IBU (0.1 µg/L), NAP (0.1 µg/L), EE2 (10 ng/L), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP), and Mix 2 (0.1 µg/L IBU + 0.1 µg/L NAP + 10 ng/L EE_2). The mean concentrations of measured water samples were relatively close to the expected nominal concentrations (Table 4). However, trace levels of ibuprofen and naproxen were found in both of the treatments.

Table 4: Nominal and mean measured water concentrations of pharmaceuticals

Treatment	**Nominal Values**			**Measured Ibuprofen (µg/L)**	**Measured Naproxen (µg/L)**	**Measured EE_2 (ng/L)**
	IBU (µg/L)	**NAP (µg/L)**	**EE_2 (ng/L)**			
Control (CW)	0	0	0	BDL [a]	BDL [a]	BDL [a]
Control Carrier (CCW)	0	0	0	BDL [a]	BDL [a]	BDL [a]
Ibuprofen	0.1	0	0	0.161 ± 0.002	0.015 ± 0.001	-
Naproxen	0	0.1	0	0.021 ± 0.002	0.067 ± 0.005	-
EE2	0	0	10	-	-	11.1 ± 0.002
Mix 1	0.1	0.1	0	0.176 ± 0.009	0.069 ± 0.005	-
Mix 2	0.1	0.1	10	0.173 ± 0.004	0.066 ± 0.002	10.0 ± 0.002

Data expressed as mean ± standard deviation
[a]BDL (below detection limit) < 0.8 ng/L

4.2.2.2 Percent Fertilization and Percent Hatch

There was a significant decrease ($p \leq 0.05$) in fertilization for flagfish exposed to 0.1 µg/L naproxen compared to its respective control (CW) (Figure 8). Fish exposed to EE2 also experienced a significant decrease ($p \leq 0.05$) in fertilization compared to its carrier control (CCW) (Figure 8). Along with a decrease in fertilization there was also a significant decrease in percent hatch for EE2 compared to the carrier control during the exposure. All controls and treatments had greater than 98% hatchability (data not shown).

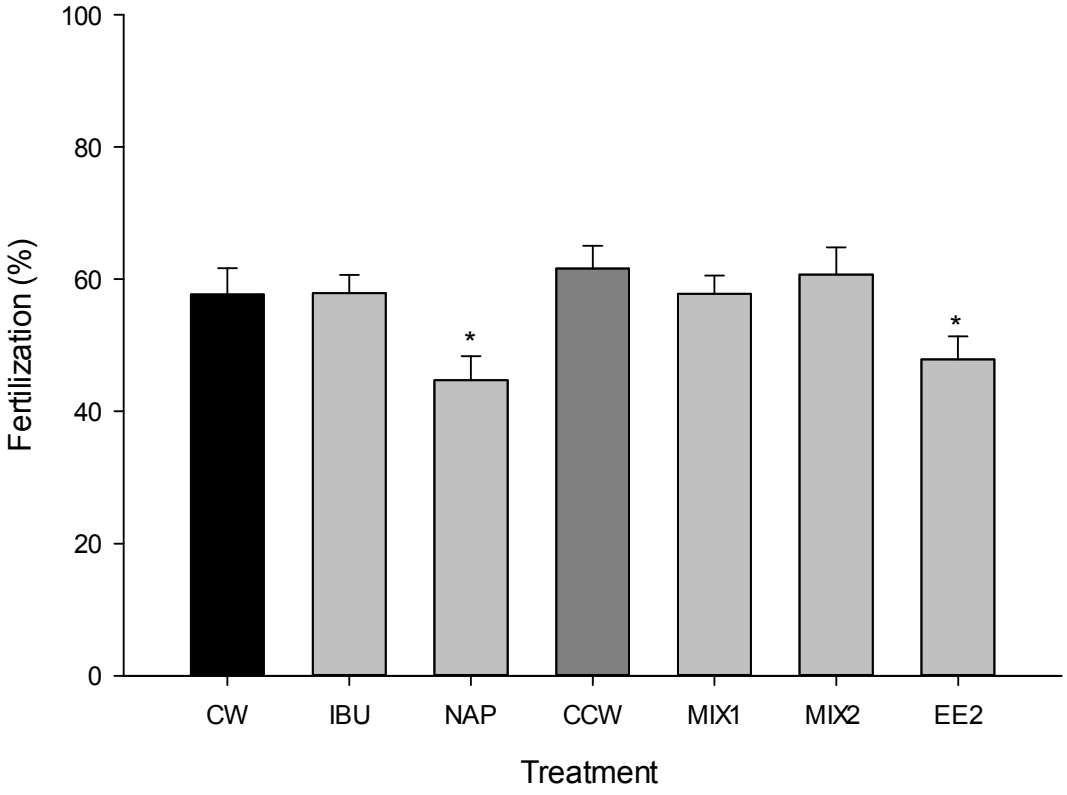

Figure 8: Fertilization success of flagfish eggs collected from adults exposed to varying to pharmaceuticals. Control (CW – 0), ibuprofen (IBU – 0.1 µg/L), naproxen (NAP – 0.1 µg/L), carrier control (CCW – 0), 17 α-etinylestradiol (EE2 – 10 ng/L), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP), and Mix 2 (0.1 µg/L IBU + 0.1 µg/L NAP + 10 ng/L EE2). Values given are means ± standard error. Treatments were run in triplicate and results were pooled. IBU and NAP were compared to CW, and Mix 1, Mix 2, and EE2 were compared to CCW. Asterisk (*) denotes significant differences ($p \leq 0.05$) compared to the respective control.

4.2.2.3 Egg Production

Egg production was monitored for 19 d during all viable phases of the experiment (Figure 9). A pre-exposure phase with adults was conducted in order to determine reproductive capability. There were no significant differences during this phase (data not shown).

There was no significant difference in mean daily egg production between treatments and controls for the pre-exposure or exposure phase (Figure 9 a,b). There was a significant increase ($p \leq 0.05$) in mean daily egg production for fish exposed to ibuprofen from the pre-exposure to the exposure phase (Figure 9a). There was also a significant increase in mean daily egg production for fish exposed to Mix 1 from the pre-exposure to the exposure phase (Figure 9b).

There were no significant differences in cumulative egg production between the treatments and controls for the pre-exposure phase (data not shown). There was a significant increase ($p \leq 0.05$) in the cumulative egg production of fish exposed to ibuprofen compared to its respective control (Figure 10a). There was no significant difference in cumulative egg production for fish exposed to Mix 1, Mix 2, and EE2 compared to its respective control (Figure 10b).

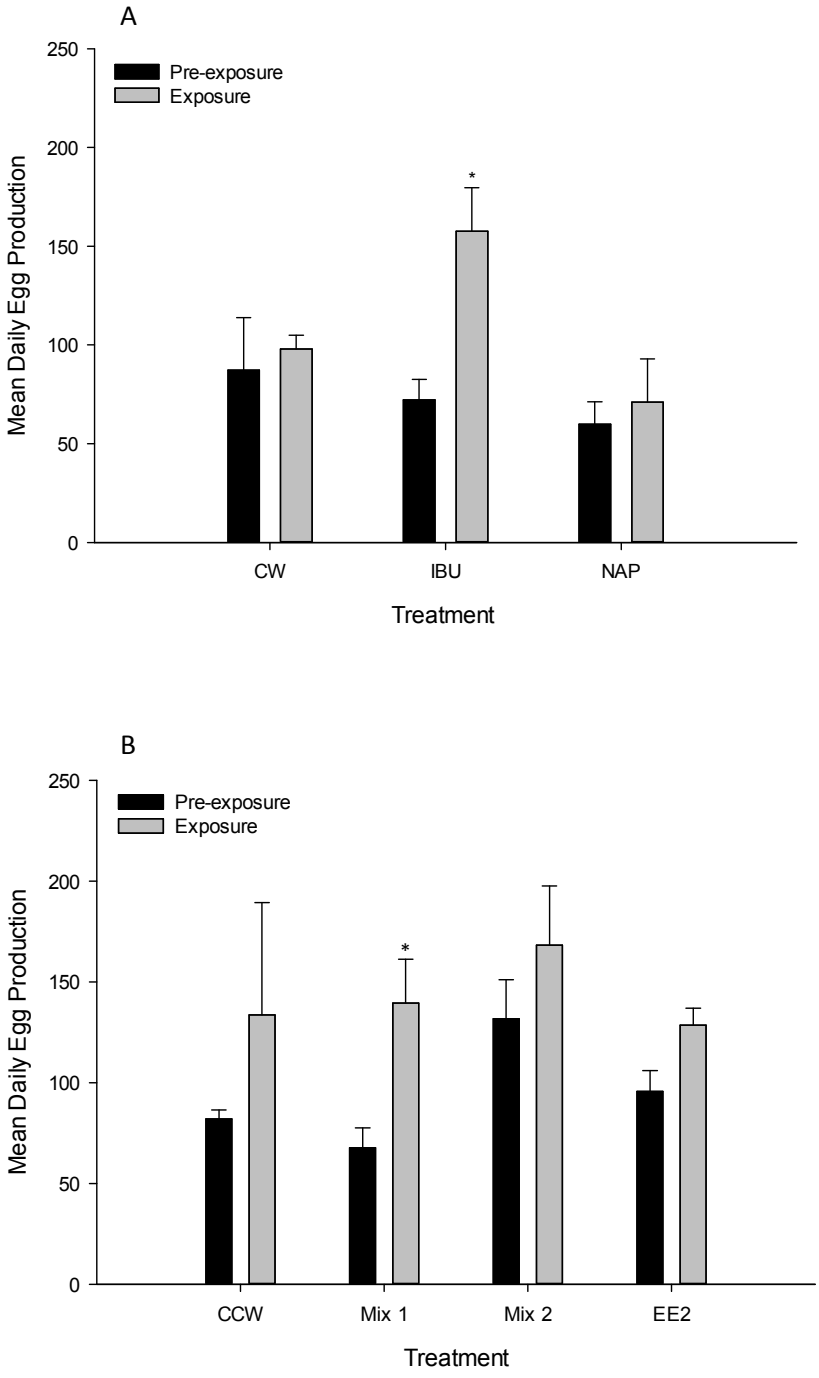

Figure 9: Mean daily egg production data for flagfish exposed to pharmaceuticals. Values given are means ± standard error. A) Mean daily egg production of adult parental generation flagfish for the pre-exposure and exposure phase, Control (CW – 0), Ibuprofen (IBU - 0.1 µg/L), Naproxen (NAP - 0.1 µg/L). B) Mean daily egg production of adult parental generation flagfish for the pre-exposure and exposure phase, Carrier Control (CCW - 0), 17-α-ethinylestradiol (EE2 - 10 ng/L), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP), and Mix 2 (0.1 µg/L IBU + 0.1 µg/L NAP + 10 ng/L EE_2). Asterisk (*) denotes significant differences ($p \leq 0.05$).

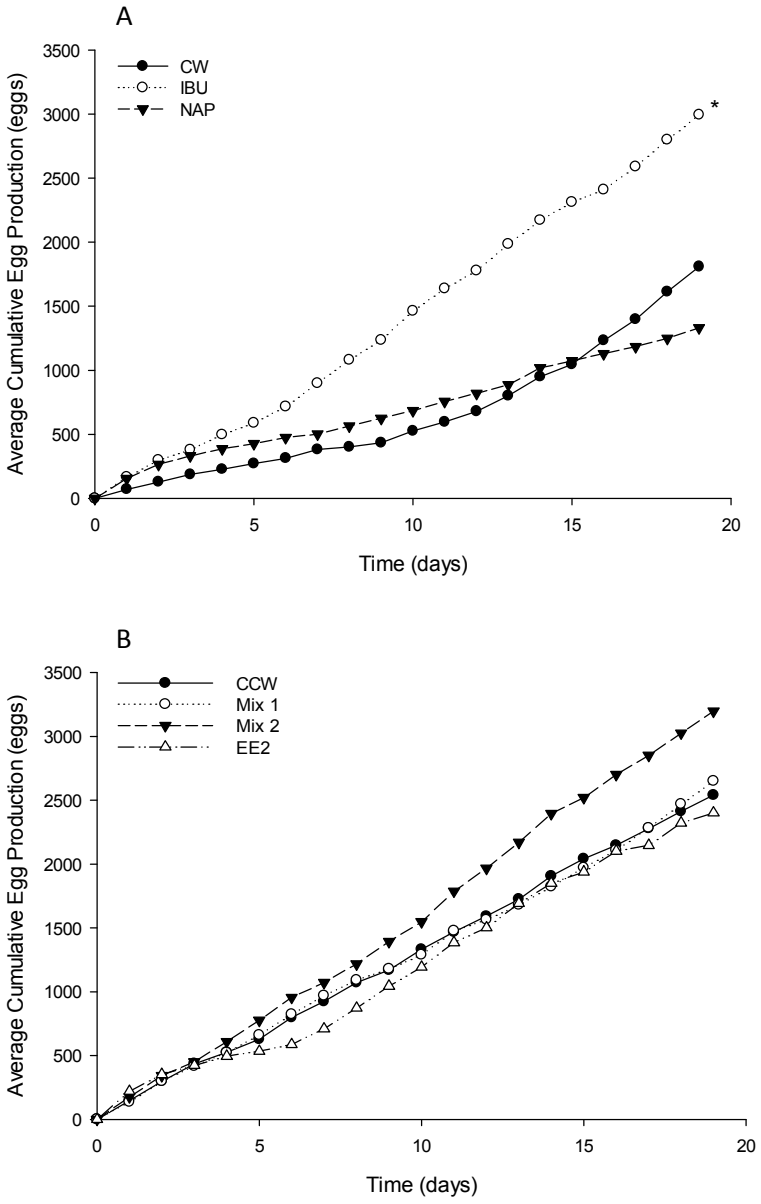

Figure 10: Cumulative egg production data for flagfish exposed to pharmaceuticals. Values given are means. A) Cumulative egg production of adult parental generation flagfish during the exposure phase, Control (CW – 0), Ibuprofen (IBU – 0.1 µg/L), Naproxen (NAP – 0.1 µg/L). B) Cumulative egg production of adult parental generation flagfish during the exposure phase, Carrier Control (CCW – 0), 17-α-ethinylestradiol (EE2 – 10 ng/L), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP), and Mix 2 (0.1 µg/L IBU + 0.1 µg/L NAP + 10 ng/L EE_2). Asterisk (*) denotes significant differences ($p \leq 0.05$).

4.2.2.4 Growth

Total length and wet weight were assessed for adult flagfish at the end of the experiment. There were no significant differences in total length or wet weight for male or female exposed flagfish compared to their respective controls (Table 5). On average male flagfish (control, ibuprofen, and naproxen) were 51.8 ± 0.64 mm in total length and 2.74 ± 0.10 g in wet weight (Table 5). On average female flagfish (control, ibuprofen, naproxen) were 46.19 ± 0.34 mm in total length and 1.94 ± 0.08 g in wet weight (Table 5). Male flagfish (carrier control, Mix 1, Mix 2, EE2) were 52.9 ± 0.46 mm total length on average and 2.90 ± 0.13 g wet weight on average (Table 5). Female flagfish (carrier control, Mix 1, Mix 2, EE2) were 44.9 ± 1.05 mm total length on average and 1.75 ± 0.08 g wet weight on average (Table 5).

Table 5: Effect of individual and mixture pharmaceuticals on total length and wet weight for adult flagfish exposed to varying pharmaceuticals

Parameter	**Stage**	**Control**	**Ibuprofen****	**Naproxen****	**Carrier Control**	**Mix 1*****	**Mix 2*****	**EE2*****
Male total length (mm)	Adult	51.8 (4, 0.88)	51.2 (6, 1.23)	52.5 (6, 0.62)	52.5 (6, 0.97)	52.8 (6, 1.00)	53.5 (6, 1.17)	52.5 (6, 0.33)
Female total length (mm)	Adult	46.3 (8, 0.73)	45.8 (12, 0.51)	46.5 (12, 0.90)	43.7 (12, 0.69)	46.2 (12, 0.80)	44.5 (12, 0.81)	45.2 (12, 0.75)
Male wet weight (g)	Adult	2.62 (4, 0.10)	2.81 (6, 0.13)	2.78 (6, 0.13)	2.87 (6, 0.20)	2.91 (6, 0.18)	3.08 (6, 0.16)	2.76 (6, 0.07)
Female wet weight (g)	Adult	2.00 (8, 0.10)	1.85 (12, 0.06)	1.97 (12, 0.14)	1.69 (12, 0.09)	1.85 (12, 0.09)	1.68 (12, 0.11)	1.78 (12, 0.08)

Data expressed as mean ± (n, standard error)

* Asterisk denotes significant difference from respective control ($p \leq 0.05$)

**Ibuprofen and Naproxen were compared to the control

***Mix 1, Mix 2, and EE2 were compared to the carrier control

4.2.2.5 Gonadosomatic Index (GSI), Liversomatic Index (LSI), Condition Factor (CF)

GSI, LSI, and Condition Factor were assessed at the end of the study when the adult flagfish were euthanized. There were no significant effects in male or female GSI or LSI of the adults, compared to their respective controls (Table 6). There was a significant decrease ($p \leq 0.05$) in condition factor in Mix 1 compared to the carrier control (Table 6).

Table 6: Effect of individual and pharmaceutical mixtures on Gonadosomatic Index (GSI), Liver Somatic Index (LSI), and Condition Factor (CF) for adult flagfish exposed to varying pharmaceuticals

Parameter	**Stage**	**Control**	**Ibuprofen****	**Naproxen****	**Carrier Control**	**Mix 1*****	**Mix 2*****	**EE2*****
Male GSI	Adult	1.27 (4, 0.33)	2.70 (6, 0.54)	1.82 (6, 0.35)	2.08 (6, 0.34)	2.94 (6, 0.46)	1.66 (6, 0.34)	2.22 (6, 0.39)
Female GSI	Adult	4.19 (8, 0.56)	4.35 (12, 0.45)	4.05 (12, 0.47)	4.23 (12, 0.33)	3.82 (12, 0.34)	3.48 (12, 0.36)	4.83 (12, 0.65)
Male LSI	Adult	1.28 (4, 0.14)	1.18 (6, 0.11)	1.12 (6, 0.17)	1.43 (6, 0.21)	1.41 (6, 0.09)	1.35 (6, 0.12)	1.14 (6, 0.14)
Female LSI	Adult	2.31 (8, 0.25)	2.30 (12, .19)	2.30 (12, 0.12)	2.36 (12, 0.12)	2.16 (12, 0.11)	2.07 (12, 0.11)	2.18 (12, 0.14)
Male CF	Adult	1.89 (4, 0.08)	2.11 (6, 0.13)	1.91 (6, 0.04)	1.96 (6, 0.06)	1.96 (6, 0.06)	2.01 (6, 0.06)	1.90 (6, 0.03)
Female CF	Adult	2.01 (8, 0.06)	1.92 (12, 0.04)	1.93 (12, 0.04)	2.00 (12, 0.04)	1.86 (12, 0.03)*	1.88 (12, 0.04)	1.92 (12, 0.03)

Data expressed as mean ± (n, standard error)

* Asterisk denotes significant difference from respective control ($p \leq 0.05$)

**Ibuprofen and Naproxen were compared to the control

***Mix 1, Mix 2, and EE2 were compared to the carrier control

4.2.2.6 Challenges

Acute toxicity challenges were run on larval offspring of previously exposed adult flagfish to determine the subsequent sensitivity of larval offspring to copper. All offspring from adult flagfish (both control and treated) demonstrated a shift to the right, a slight decreasing trend in sensitivity to copper (Figure 11 a-d). Offspring from parents exposed to varying pharmaceutical treatments for 5 – 7 d (challenge 1) had average LC_{50} values of ranging from 4.5 – 14.0 µg/L (Figure 12). Offspring from parents exposed for 13 – 14 d (challenge 2) had average LC_{50} values ranging from 7.3 – 16.0 µg/L (Figure 12). Offspring from parents exposed for 20 – 21 d (challenge 3) had average LC_{50} values ranging from 9.2 – 16.8 µg/L (Figure 12). Finally offspring collected during the depuration period had average LC_{50} values ranging from 9.0 – 21.4 µg/L (Figure 12). For all four challenges EE2 had a higher LC_{50} value compared to the respective control, and Mix 1 and Mix 2 (Figure 12).

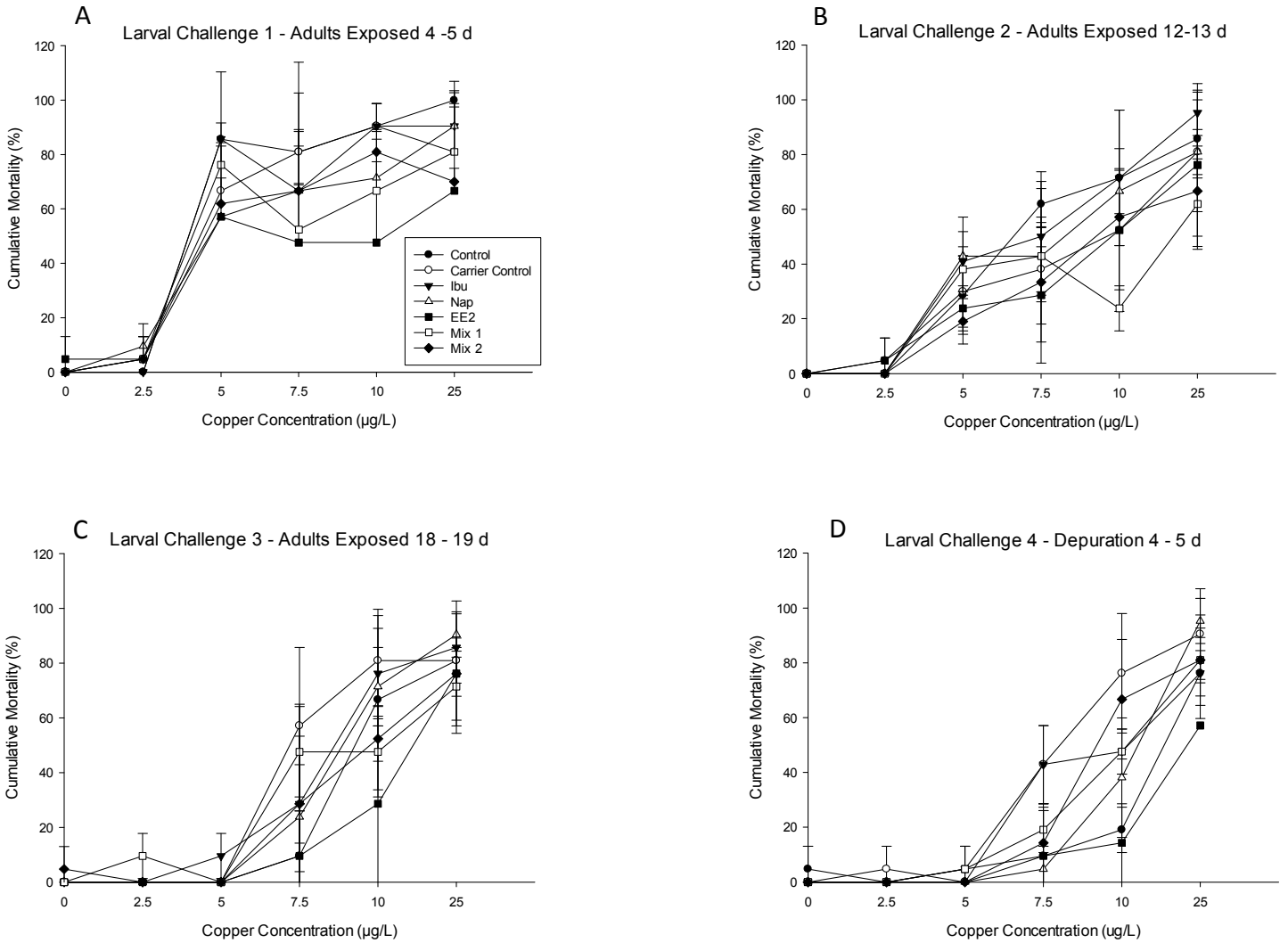

Figure 11: Cumulative mortality (%) at 96 h for larval American flagfish exposed to varying concentrations of copper (0 - 25 µg/L). A – D) Challenges 1 - 4 of copper exposed larval offspring of adult flagfish that were exposed to varying treatments. Adult flagfish were exposed to various treatments, Control (CW – 0), Ibuprofen (IBU - 0.1 µg/L), Naproxen (NAP - 0.1 µg/L), Carrier Control (CCW - 0), 17-α-ethinylestradiol (EE2 - 10 ng/L), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP), and Mix 2 (0.1 µg/L IBU + 0.1 µg/L NAP + 10 ng/L EE_2). Asterisk (*) denotes significant differences ($p \leq 0.05$).

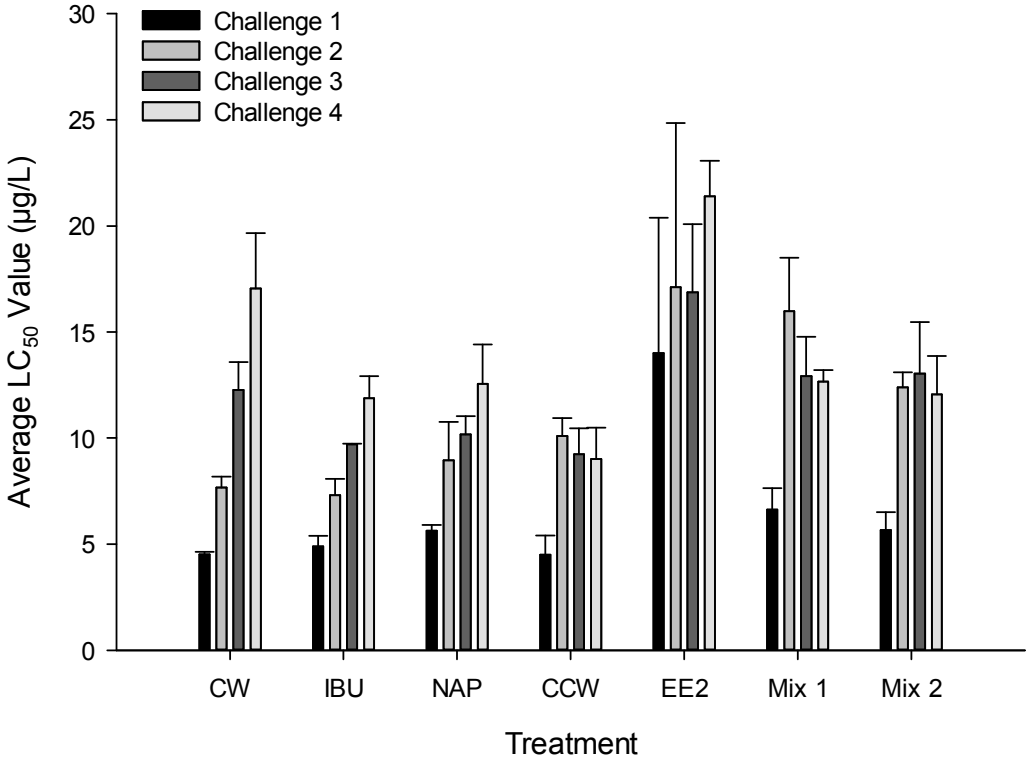

Figure 12: Average copper 96 h LC_{50} values for each respective challenge (1–4). Challenges were completed on larval offspring collected from adults exposed to varying pharmaceutical treatments, Control (CW – 0), Ibuprofen (IBU – 0.1 µg/L), Naproxen (NAP – 0.1 µg/L), Carrier Control (CCW – 0), 17-α-ethinylestradiol (EE2 – 10 ng/L), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP), and Mix 2 (0.1 µg/L IBU + 0.1 µg/L NAP + 10 ng/L EE_2). Asterisk (*) denotes significant differences ($p \leq 0.05$).

4.3 Study 2

Many studies have evaluated the effects of ibuprofen, naproxen, and EE2 individually on a number of fish species, however, to the best of our knowledge no studies have considered the multi-generational impact of these pharmaceuticals in mixtures. The first aim of the present study was to investigate the multi-generational effects of mixtures of ibuprofen, naproxen and EE2 at environmentally relevant concentrations on American flagfish (*Jordanella floridae*). The endpoints of interest in this multi-generational study were fertilization, egg production, hatching success, growth, gonadosomatic index (GSI) and liversomatic index (LSI) as well as second generation chemical tolerance.

Null hypothesis: Multi-generational exposure to environmentally relevant concentrations of pharmaceuticals will have no impact on flagfish reproduction and will not alter the sensitivity of offspring to contaminants

4.3.1 Study 2 Materials & Methods

4.3.1.1 Test fish

Sexually mature laboratory raised American flagfish (*Jordanella floridae*) were used to commence this experiment. Fish were housed in 70 L glass flow-through aquaria which contained an air stone for aeration and circulation. A 16 hour light: 8 hour dark photoperiod with 0.5 hour dawn and dusk were maintained for the duration of the experiment. The water temperature was maintained at 25.0 ± 1.0 °C for the duration of the study. Mean (SE) characteristics of the water for chronic tests were as follows: dissolved oxygen 8.5 mg/L (0.06) and pH was 7.82 (0.02). Fish were fed 3 times daily with a mix of flake food (*Tetramin®Pro Crisps*), frozen brine shrimp (*Artemia salina)* (*Hikari*) and freshly hatched brine shrimp nauplii larvae (Premium eggs, *Brine Shrimp Direct*). All procedures involving animal handling were conducted in accordance with the Canadian Council on Animal Care Guidelines.

4.3.1.2 Test Chemicals

Ibuprofen sodium salt (α-Methyl-4-(isobutyl) phenylacetic acid), Naproxen sodium salt (S)-6-Methoxy-α-methyl-2-naphthaleneacetic acid sodium salt), and 17 α-Ethinylestradiol (17 α-Ethinyl-1,3,5(10)-estratriene-3,17β-diol) were all purchased from Sigma-Aldrich Co. (*Oakville, Ontario*). Analytical grade acetone (>99%; *Sigma-Aldrich*) was used as the solvent (carrier) for EE2 and was used in equal amounts for all treatments and the control. Acetone did not exceed 20 µl/L of dilution water. Working stock solutions were created for ibuprofen, naproxen, and EE2. Serial dilutions were then performed from

the working stock solutions to obtain the desired stock concentrations. A constant supply of ibuprofen, naproxen, and EE2 were delivered using a *Watson-Marlow* 200 Series 16 channel peristaltic pump (*Massachusetts, USA*). The flow of water into the 70 L aquaria was set to 5 turnovers per day. Stock solutions were delivered to aquaria at a rate of 80 µl/ minute. Each tank housed 67 L of water and the nominal concentrations in the aquaria were as follows: Solvent Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE2).

4.3.1.3 Multi-generational Waterborne Exposure & Endpoints

Adult flagfish were separated into breeding harems (2 male: 4 female) and placed into aerated 70 L flow-through aquaria. Male fish were selected according to size, one being slightly smaller than the other so that dominance could easily be established. All females were selected to be slightly smaller than the males. Males and females can be easily distinguished using secondary sexual characteristics, with mature males display alternating red and yellowish-green banding, and mature females have a black ocellus present on their dorsal fin (Holdway & Dixon, 1986). One breeding substrate (a glass plate wrapped in green Orlon® wool) was assigned per aquaria. All treatments were run in triplicate (Mix 1 – Mix 5) and five solvent control tanks were run alongside for the duration (F_0, F_1, F_2) of the experiment. A pre-exposure internal control was done to assess reproductive viability prior to the start of F_0 exposure. Parental generation reproductive and biological endpoints were monitored and assessed. A set of F_1 offspring were collected, reared, and exposed

for a full life-cycle. At 30 dph larval growth was assessed and fish were transferred to 70 L aquaria. At 69 – 70 dph fish were thinned so that an even density was present in each aquaria, and total length and wet weight were assessed. At day 102 – 103 flagfish were thinned (total length, wet weight, LSI, and GSI were measured) and separated into breeding harems (same criteria as above) and reproductive and biological endpoints were once again monitored and assessed. At 129 dph F_1 adults were dissected and total length, wet weight, LSI and GSI were measured. A set of F_2 offspring were collected and reared for 30 days and wet weight and total length were assessed. Both the parental, F_1, and F_2 generations were continuously exposed to the desired treatments (Figure 13).

Growth was monitored over the duration of the study at different time points for the F_0, F_1, and F_2 generation. Depending on size and stage of the study, total length, wet weight, and condition factor were measured/calculated. Image J software® was used to determine the length of fish that were 30 dph. If dissections were performed, livers were removed and LSI was derived by expressing the liver weight as a percentage of the total body weight. If fish were mature when dissections occurred then gonads were also removed and GSI was expressed as the gonad weight as a percentage of the total body weight.

Reproduction was assessed over a multi-generational period (F_0, F_1). Eggs were collected and enumerated daily for 26 days for each reproductive period (F_0 pre-exposure, F_0 exposure, and F_1 exposure). Sixteen collections were kept and monitored for fertilization and hatch during each reproductive period. Fertilization was determined 24 h after collection using a microscope and eggs were monitored daily until hatch.

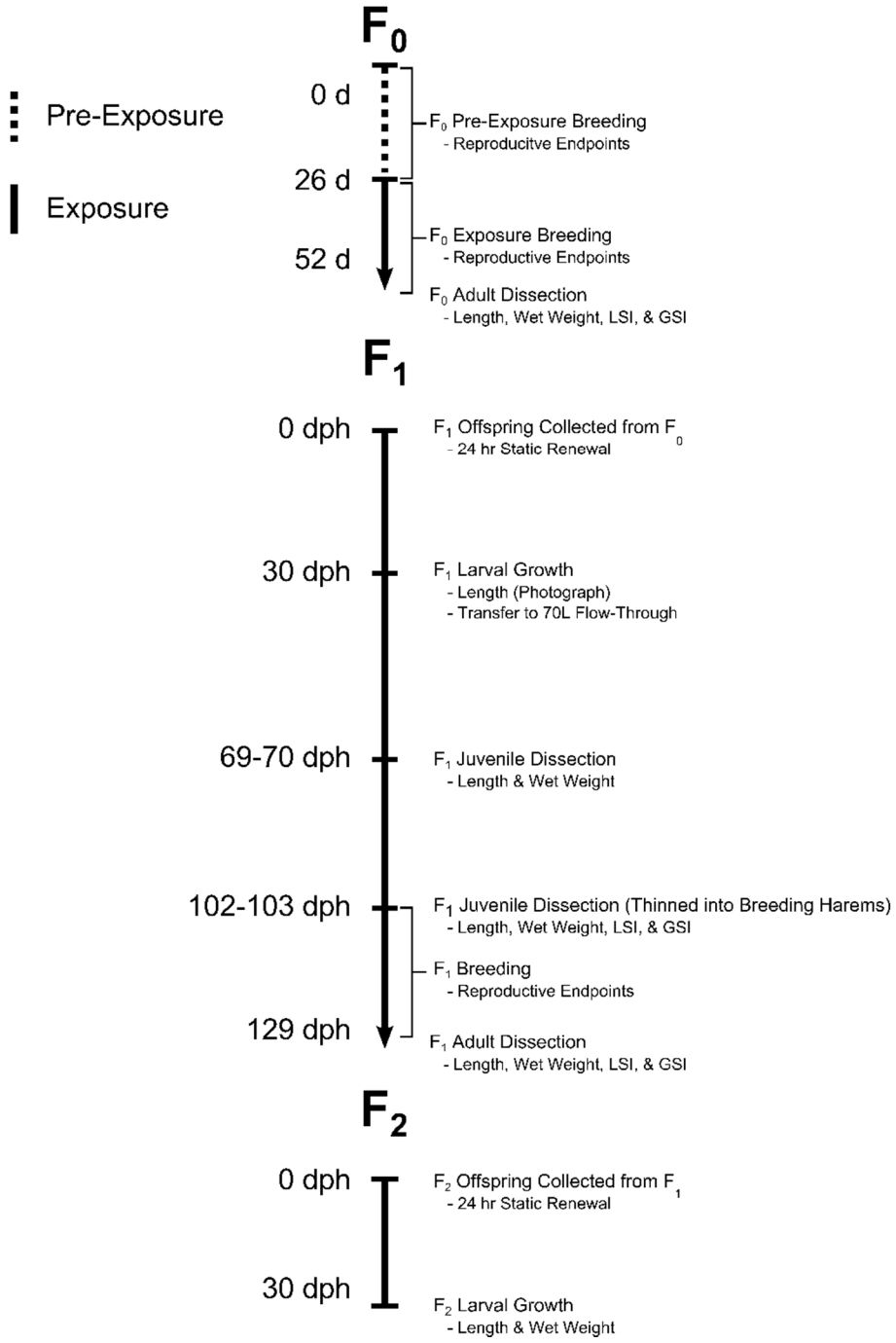

Figure 13: Experimental timeline for multi-generational study exposing flagfish to pharmaceutical mixtures. Parental generation (F_0) were selected as adults and put into breeding harems, the first generation (F_1) was collected and reared from the parental generation, followed by a second generation (F_2) collected and reared for 30 d. Endpoints observed for each portion of the study are noted. Age of fish is represented either by day (d) for adult fish taken from the general population or days post hatch (dph).

4.3.1.4 Water Analysis

Water samples for chemical analysis were tested twice in triplicate for each treatment over the duration of the study. Water samples were collected during the reproductive phase of the experiment. For each sampling period a 500 mL sample was collected from each treatment and a composite sample of the controls. Water samples were transported on ice on the day of collection to Trent Water Quality Centre (*Trent University*, *Peterborough, ON*) and were extracted and analyzed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), the measured values were relatively close to the nominal values (Table 7).

4.3.1.5 Statistical Analysis

Data were analyzed with STATISTICA 12.0 software (StatSoft, Tulsa, OK, USA). Data were checked for normality using Shapiro-Wilk's W test, and for homogeneity of variances using Brown and Forsythe's test. One-way and two-way analysis of variance (ANOVA) were performed to analyze overall differences for total length, wet weight, LSI, GSI, and reproductive endpoints. Cumulative egg production data was analyzed on daily intervals, with comparisons being made to controls and between treatments. Significant differences ($p \leq 0.05$) were then confirmed by Tukey's HSD post-hoc test. If the assumptions for ANOVA were not met and data could not be transformed, then significance was determined using non-parametric Kruksal-Wallis test.

4.3.2 Study 2 Results

4.3.2.1 Water Sampling

The nominal values for Mix 1 – Mix 5 were as follows; Solvent Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE2). The mean concentrations of measured water samples were relatively close to the expected nominal concentrations. The first three EE2 values were below the detection limit (BDL) < 0.8 ng/L (Table 7).

Table 7: Nominal and mean measured water concentrations of pharmaceutical mixtures

Treatment	Nominal Values			Sampling Time	Measured Ibuprofen (µg/L)	Measured Naproxen (µg/L)	Measured EE_2 (ng/L)
	IBU (µg/L)	NAP (µg/L)	EE_2 (ng/L)				
Control (CW)	0	0	0	F0	0.04 ± 0.00	0.01 ± 0.00	BDL [a]
				F1	0.01 ± 0.00	0.01 ± 0.00	BDL [a]
Mix 1	0.1	0.1	0.1	F0	0.13 ± 0.01	0.13 ± 0.04	BDL [a]
				F1	0.09 ± 0.01	0.15 ± 0.04	BDL [a]
Mix 2	0.32	0.32	0.32	F0	0.38 ± 0.04	0.36 ± 0.10	BDL [a]
				F1	0.35 ± 0.08	0.33 ± 0.04	BDL [a]
Mix 3	1.0	1.0	1.0	F0	1.01 ± 0.08	1.87 ± 0.06	1.00 ± 0.28
				F1	1.20 ± 0.17	0.89 ± 0.08	1.15 ± 0.23
Mix 4	3.2	3.2	3.2	F0	2.77 ± 0.06	3.63 ± 0.86	2.90 ± 0.44
				F1	3.50 ± 0.35	3.17 ± 0.35	3.00 ± 0.17
Mix 5	10	10	10	F0	4.40 ± 1.01	9.10 ± 0.95	8.53 ± 1.40
				F1	9.03 ± 1.19	9.17 ± 0.40	8.63 ± 0.45

Data expressed as mean ± standard deviation

[a]BDL (below detection limit) < 0.8 ng/L

4.3.2.2 Percent Fertilization & Percent Hatch

There were no significant differences between mixtures and controls for fertilization during the parental (F_0) pre-exposure phase (internal control). The Mix 5 parental generation (F_0) demonstrated a significant decrease in fertilization compared to the control during the exposure, and also when compared to its own internal control (baseline) from the pre-exposure phase (Figure 14). The F_1 generation demonstrated a significant decrease in fertilization for Mix 5 compared to the controls (Figure 14). The rate of decrease between the F_0 and F_1 generation is nearly proportional (Figure 14). There were no significant differences between mixtures and controls in hatchability. Eggs that were successfully fertilized had greater than 95 % hatching success (data not shown).

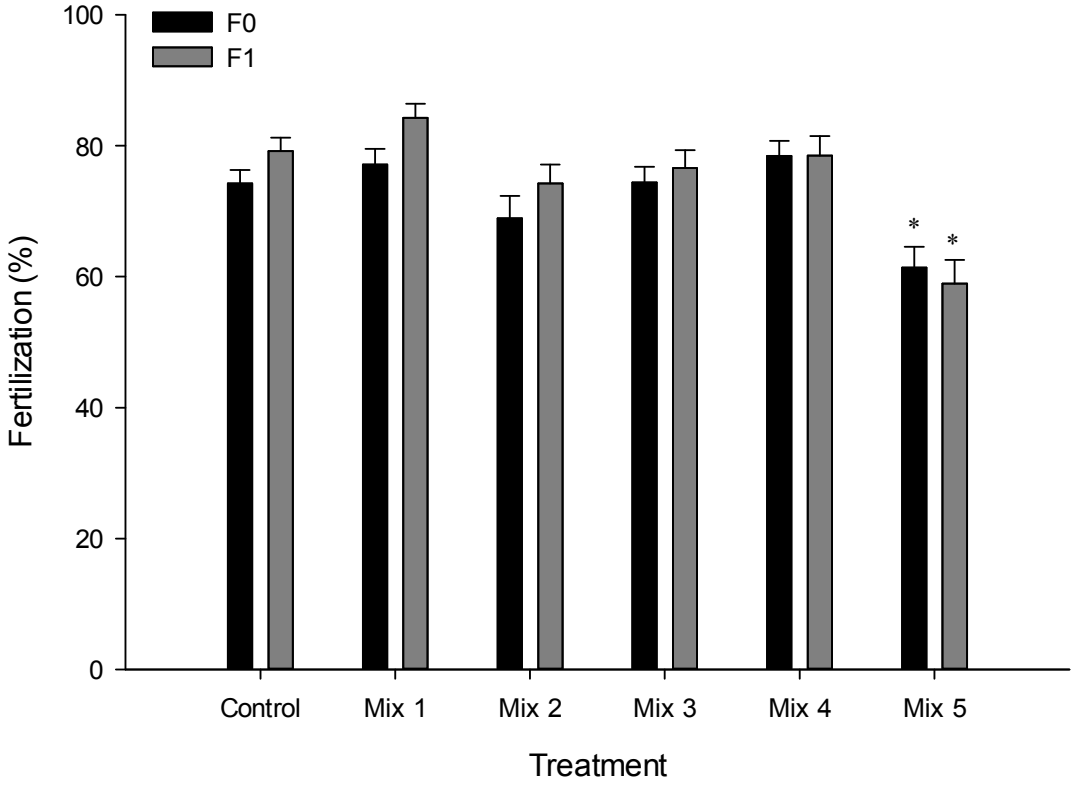

Figure 14: Fertilization success of flagfish eggs collected from the parental generation (F_0) (black) and F_1 generation (grey) exposed to varying to pharmaceutical mixtures. Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE_2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE_2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE_2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE_2). Values given are means ± SE. Mixtures were run in triplicate and results were pooled. Asterisk (*) denotes significant differences ($p \leq 0.05$) compared to the control. There were no significant differences between generations.

4.3.2.3 Egg Production

Egg production was monitored for 26 d during all viable phases of the experiment (Figure 15). A pre-exposure phase with the F_0 adults was conducted in order to determine reproductive capability. There were no significant differences in mean daily egg production during this phase (data not shown). F_0 adults and F_1 adults experienced no significant differences in mean daily egg production when compared to controls during the exposure to pharmaceutical mixtures (Figure 15a). The slope of the curve for Mix 3 cumulative mean egg production was slightly steeper compared to the controls in the pre-exposure phase (data not shown). The F_0 cumulative egg production displayed no significant differences during the first three days of exposure for any mixtures compared to control. Egg production of fish exposed to Mix 1 and Mix 3 was significantly increased compared to the controls from day 3 to day 26, with the exception of day 8, when egg production in Mix 3 was not different ($p \leq 0.05$) (Figure 15b). Egg production of fish exposed to Mix 2 and Mix 5 was significantly lower compared to the controls from day 8 to day 26 ($p \leq 0.05$) (Figure 15b). The F_1 cumulative egg production numbers had no significant differences for the first three days of exposure for any mixtures compared to control. Egg production of fish exposed to Mix 4 and Mix 5 was significantly lower compared to the controls from day 4 to day 26 of the exposure (Figure 15c). Fish exposed to Mix 1and Mix 2 experienced significantly lower egg production compared to the controls starting on day 12 and 10 respectively, with both effects lasting for the duration of the exposure (Figure 15c). Egg production of fish exposed to Mix 3 was significantly increased relative to controls starting on day 19 and continued for the duration of the experiment (Figure 15c).

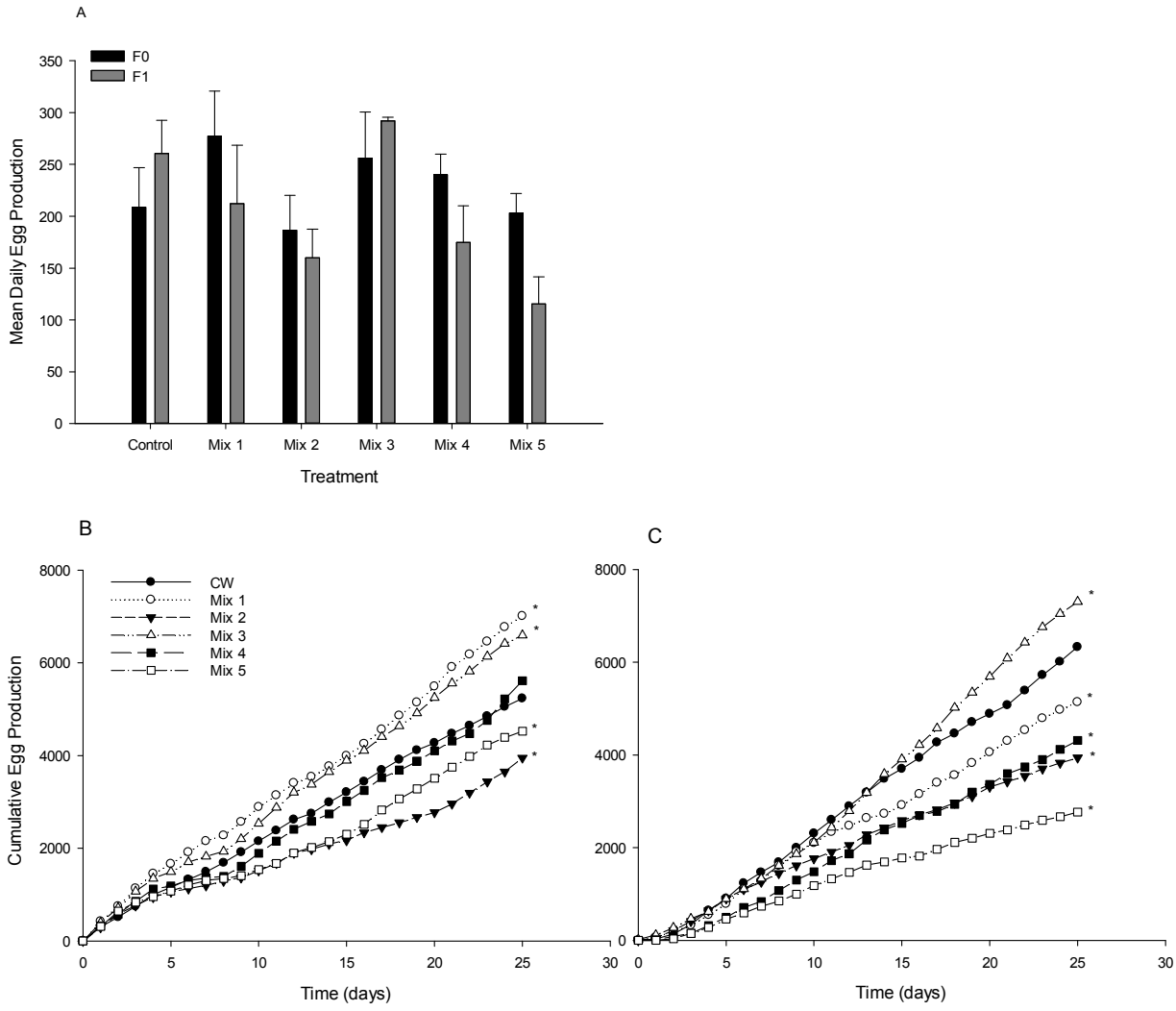

Figure 15: Egg production data for flagfish exposed to pharmaceutical mixtures. Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE_2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE_2). A) Mean daily egg production of adult parental generation (F_0) flagfish and first generation (F_1). B) Cumulative egg production data of adult parental generation (F_0) flagfish. Mix 1 and Mix 3 had a significant increase in cumulative egg production data for the majority of the exposure, while Mix 2 and Mix 5 were significantly deceased compared to the control. C) Cumulative egg production data of first generation (F_1) flagfish. Mix 1, 2, 4 and 5 had a significant decrease in egg production compared to the control. Values given are means ± SE. Mixtures were run in triplicate and results were pooled. Asterisk (*) denotes significant differences ($p \leq 0.05$)

4.3.2.4 Growth

Growth was monitored for the duration of the experiment at varying time points for the F_0, F_1, and F_2 generation (Table 8). There were no significant differences between mixtures and controls for growth (total length and wet weight) for the parental (F_0) generation. On average F_0 adult males were 55.9 ± 0.35 mm and F_0 adult females were 50.1 ± 0.34 mm in length. At 30 dph there were no significant differences between mixtures and controls; on average the 30 dph larval F_1 offspring were 10.9 ± 0.10 mm. Two thinning's of the population were conducted for the F_1 generation at 69 – 70 dph and 102 – 103 dph. There were no significant differences for growth (total length and wet weight) for either of the thinning periods. On average fish were 29.2 ± 0.10 mm total length, and 0.39 ± 0.01 g wet weight at 69 – 70 dph. At 102 – 103 dph on average total length of males were 40.8 ± 0.29 cm and females were 38.1 ± 0.29 cm and the male wet weight on average was 1.19 ± 0.03 g and females 0.98 ± 0.02 g. On day 148 the adult F_1 harems were sacrificed, the average male total length was 56.7 ± 0.25 mm with a wet weight of 3.77 ± 0.06 g and the average female total length was 49.6 ± 0.25 mm and a wet weight of 2.50 ± 0.04 g. The F_2 generation were sacrificed after 30 dph and Mix 1 and Mix 5 had a significant increase in total length ($p \leq 0.05$) compared to the controls (Table 8). Wet weight was significantly decreased for Mix 4, and Mix 5 was significantly increased ($p \leq 0.05$) compared to the controls at 30 dph for the F_2 generation (Table 8).

Table 8: Effect of pharmaceutical mixtures on total length and wet weight for flagfish during a multi-generational study

Parameter	Stage/ Age (days)	Control	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5
F0 male total length (mm)	Adult (N/A)	56.1 (10, 0.75)	55.3 (6, 0.53)	55.8 (6, 1.19)	55.4 (6, 0.62)	56.0 (6, 1.42)	56.9 (6, 0.30)
F0 female total length (mm)	Adult (N/A)	49.6 (20, 0.82)	50.4 (11, 0.81)	50.7 (12, 0.99)	48.9 (12, 0.90)	50.3 (12, 0.70)	51.0 (12, 0.63)
F0 male wet weight (g)	Adult (N/A)	3.55 (10, 0.20)	3.51 (6, 0.15)	3.60 (6, 0.21)	3.38 (6, 0.08)	3.59 (6, 0.32)	3.61 (6, 0.07)
F0 female wet weight (g)	Adult (N/A)	2.61 (20, 0.14)	2.72 (11, 0.16)	2.78 (12, 0.17)	2.55 (12, 0.15)	2.65 (12, 0.14)	2.75 (12, 0.11)
F1 total length (mm)	Larval (30)	10.9 (125, 0.10)	11.2 (75, 0.13)	10.9 (75, 0.11)	11.2 (75, 0.13)	10.7 (75, 0.13)	10.8 (75, 0.14)
F1 total length (mm)	Juvenile (69-70)	29.5 (88, 0.27)	29.5 (54, 0.31)	29.7 (53, 0.32)	28.5 (53, 0.28)	29.0 (52, 0.38)	28.5 (53, 0.32)
F1 wet weight (g)	Juvenile (69-70)	0.39 (88, 0.01)	0.41 (54, 0.01)	0.41 (53, 0.01)	0.37 (53, 0.01)	0.38 (52, 0.01)	0.36 (53, 0.01)
F1 male total length (mm)	Juvenile (102-103)	40.6 (20, 0.74)	40.8 (13, 0.73)	41.7 (18, 0.74)	41.3 (14, 0.90)	40.2 (18, 0.56)	40.3 (16, 0.62)
F1 female total length (mm)	Juvenile (102-103)	38.2 (20, 0.38)	39.8 (16, 0.79)	38.1 (11, 0.41)	36.8 (16, 0.80)	38.7 (11, 0.69)	37.2 (14, 0.76)
F1 male wet weight (g)	Juvenile (102-103)	1.17 (20, 0.07)	1.19 (13, 0.06)	1.22 (18, 0.07)	1.30 (14, 0.08)	1.13 (18, 0.05)	1.12 (16, 0.04)
F1 female wet weight (g)	Juvenile (102-103)	0.99 (20, 0.04)	1.13 (16, 0.07)	0.96 (11, 0.03)	0.89 (16, 0.06)	1.03 (11, 0.06)	0.89 (14, 0.07)
F1 male total length (mm)	Adult (148)	57.0 (10, 0.59)	56.7 (6, 0.47)	57.2 (6, 0.43)	57.1 (6, 0.64)	55.6 (6, 0.68)	56.2 (6, 0.77)
F1 female total length (mm)	Adult (148)	49.7 (20, 0.42)	50.3 (12, 0.56)	48.4 (12, 0.74)	50.2 (12, 0.67)	48.9 (12, 0.59)	50.0 (12, 0.67)
F1 male wet weight (g)	Adult (148)	3.84 (10, 0.16)	3.72 (6, 0.08)	3.91 (6, 0.14)	3.85 (6, 0.17)	3.61 (6, 0.16)	3.63 (6, 0.19)
F1 female wet weight (g)	Adult (148)	2.47 (20, 0.08)	2.68 (12, 0.10)	2.25 (12, 0.12)	2.58 (12, 0.11)	2.44 (12, 0.11)	2.58 (12, 0.11)
F2 total length (mm)	Larval (30)	13.2 (174, 0.07)	13.7 (105, 0.12)*	13.4 (104, 0.07)	13.2 (102, 0.10)	13.1 (103, 0.08)	14.1 (103, 0.09)*
F2 wet weight (mg)	Larval (30)	27.9 (174, 0.48)	32.0 (105, 1.06)	29.8 (104, 0.55)	25.9 (102, 0.69)	24.8 (103, 0.61)*	32.8 (103, 0.87)*

Data expressed as mean ± (n, standard error)

* Asterisk denotes significant difference from control ($p \leq 0.05$)

4.3.2.5 Gonadosomatic Index & Liver Somatic Index

GSI and LSI were monitored at various time points over the study. There were no significant effects in male or female GSI or LSI of the F_0 adults, compared to controls (Table 9). Sampled fish were too immature at the first thin to check GSI and LSI. At the second thin (102 – 103 dph) there were no significant differences in female GSI, but there was a significant increase in male GSI in Mix 5 ($p \leq 0.05$) compared to the controls (Table 9). At 102 – 103 dph, females again showed no significant differences in LSI, but male LSI was significantly increased in Mix 1, Mix 3, and Mix 4 compared to the control (Table 9). The F_1 adults had no significant difference in GSI for males or females compared to the controls. There was no effect observed in F_1 adult male LSI compared to controls. Female LSI had a significant increase in Mix 3 ($p \leq 0.05$) compared to the controls (Table 9). When comparing the F_0 adults and the F_1 adults there was a significant increase in F_1 male GSI for Mix 4 and Mix 5 (Figure 16). There were no significant generational differences in female GSI.

Table 9: Effect of pharmaceutical mixtures on Gonadosomatic Index (GSI) and Liver Somatic Index (LSI) for flagfish during a multi-generational study

Parameter	Stage/ Age (days)	Control	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5
F0 male GSI	Adult (N/A)	2.46 (10, 0.44)	1.93 (6, 0.27)	2.92 (6, 0.48)	2.00 (6, 0.23)	2.21 (6, 0.21)	2.17 (6, 0.32)
F0 female GSI	Adult (N/A)	7.38 (20, 0.48)	7.51 (11, 0.55)	7.31 (12, 0.68)	6.86 (12, 0.46)	5.99 (12, 0.29)	6.92 (12, 0.51)
F0 male LSI	Adult (N/A)	2.07 (10, 0.22)	1.93 (6, 0.14)	2.33 (6, 0.26)	1.92 (6, 0.10)	1.96 (6, 0.16)	2.11 (6, 0.15)
F0 female LSI	Adult (N/A)	3.28 (20, 0.08)	3.29 (11, 0.09)	3.29 (12, 0.11)	3.38 (12, 0.12)	3.39 (12, 0.15)	3.35 (12, 0.13)
F1 male GSI	Juvenile (102- 103)	1.08 (20, 0.10)	1.06 (13, 0.08)	1.12 (18, 0.12)	1.38 (14, 0.15)	1.34 (18, 0.09)	1.57 (16, 0.14)*
F1 female GSI	Juvenile (102- 103)	2.66 (20, 0.15)	3.20 (16, 0.27)	2.69 (11, 0.21)	2.68 (16, 0.17)	2.58 (11, 0.16)	2.63 (14, 0.24)
F1 male LSI	Juvenile (102- 103)	1.19 (20, 0.05)	1.47 (13, 0.08)*	1.40 (18, 0.10)	1.56 (14, 0.08)*	1.57 (18, 0.07)*	1.42 (16, 0.07)
F1 female LSI	Juvenile (102- 103)	2.22 (20, 0.11)	2.63 (16, 0.10)	2.36 (11, 0.12)	2.55 (16, 0.15)	2.46 (11, 0.12)	2.02 (14, 0.14)
F1 male GSI	Adult (148)	2.76 (10, 0.34)	2.58 (6, 0.28)	3.31 (6, 0.46)	2.45 (6, 0.31)	3.48 6, (0.20)	3.26 (6, 0.34)
F1 female GSI	Adult (148)	7.46 (20, 0.41)	7.62 (12, 0.64)	6.22 (12, 0.36)	6.32 (12, 0.38)	6.80 (12, 0.57)	6.78 (12, 0.34)
F1 male LSI	Adult (148)	2.00 (10, 0.17)	2.24 (6, 0.18)	2.41 (6, 0.24)	2.07 (6, 0.08)	1.95 (6, 0.16)	2.37 (6, 0.11)
F1 female LSI	Adult (148)	3.18 (20, 0.09)	3.67 (12, 0.18)	3.28 (12, 0.12)	3.77 (12, 0.18)*	3.21 (12, 0.13)	3.52 (12, 0.16)

Data expressed as mean ± (n, standard error) * Asterisk denotes significant difference from control ($p \leq 0.05$)

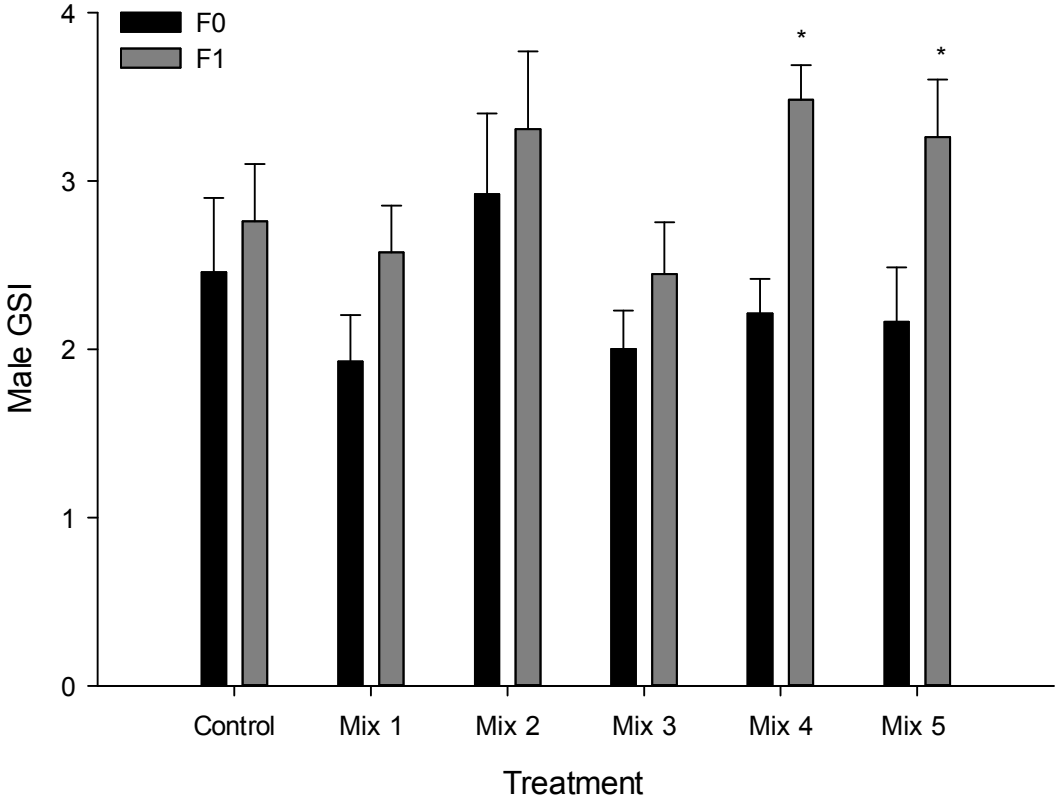

Figure 16: Male GSI of flagfish collected from the parental generation (F_0) (black) and F_1 generation (grey) exposed to varying pharmaceutical mixtures. Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE2). Values given are means ± SE. Mixtures were run triplicate results were pooled. Asterisk (*) denotes significant differences ($p \leq 0.05$) between generations.

4.3.2.6 Challenges

Acute toxicity challenges were run on larval offspring of previously exposed adult flagfish to determine the subsequent sensitivity of larval offspring to copper. These challenges were first completed on offspring that had not been exposed to make sure they were viable, challenge 1, 2, and 3 (pre-exposure). Challenge 1, 2, and 3 had LC_{50} values ranging from 3.4 – 7.1 µg/L (Figure 17a-c). Offspring from parents exposed to varying pharmaceutical treatments for 1 – 2 d (challenge 4) had average LC_{50} values ranging from 2.8 – 10.8 µg/L (Figure 17d). Offspring from parents exposed for 9 – 10 d (challenge 5) had average LC_{50} values ranging from 7.0 – 9.2 µg/L (Figure 18e). Offspring from parents exposed for 17 d (challenge 6) had average LC_{50} values ranging from 5.4 – 7.0 µg/L (Figure 18f). Offspring collected during the 1 – 2 d depuration period (challenge 7) had average LC_{50} values ranging from 5.1 – 7.7 µg/L (Figure 18g). Finally offspring collected during the 8 – 9 d depuration period had average LC_{50} values ranging from 5.9 – 8.3 µg/L (Figure 18h). Of interest the highest LC_{50} value was for Mix 5 during challenge 4 (10.9 µg/L), and the lowest was for Mix 4 during challenge 4 (2.9 µg/L).

F_2 generation underwent three challenges. F_2 offpsring from adults exposed 127 d (challenge 1) had average LC_{50} values ranging from 5.4 – 10.3 µg/L (Figure 19a). F2 offspring from adults exposed 133 – 134 d (challenge 2) had average LC_{50} values ranging from 4.5 – 7.7 µg/L (Figure 19b). Finally, F_2 offspring from adults exposed for 139 – 140 d (challenge 3) had average LC_{50} values ranging from 7.3 – 17.4 µg/L (Figure 19c).

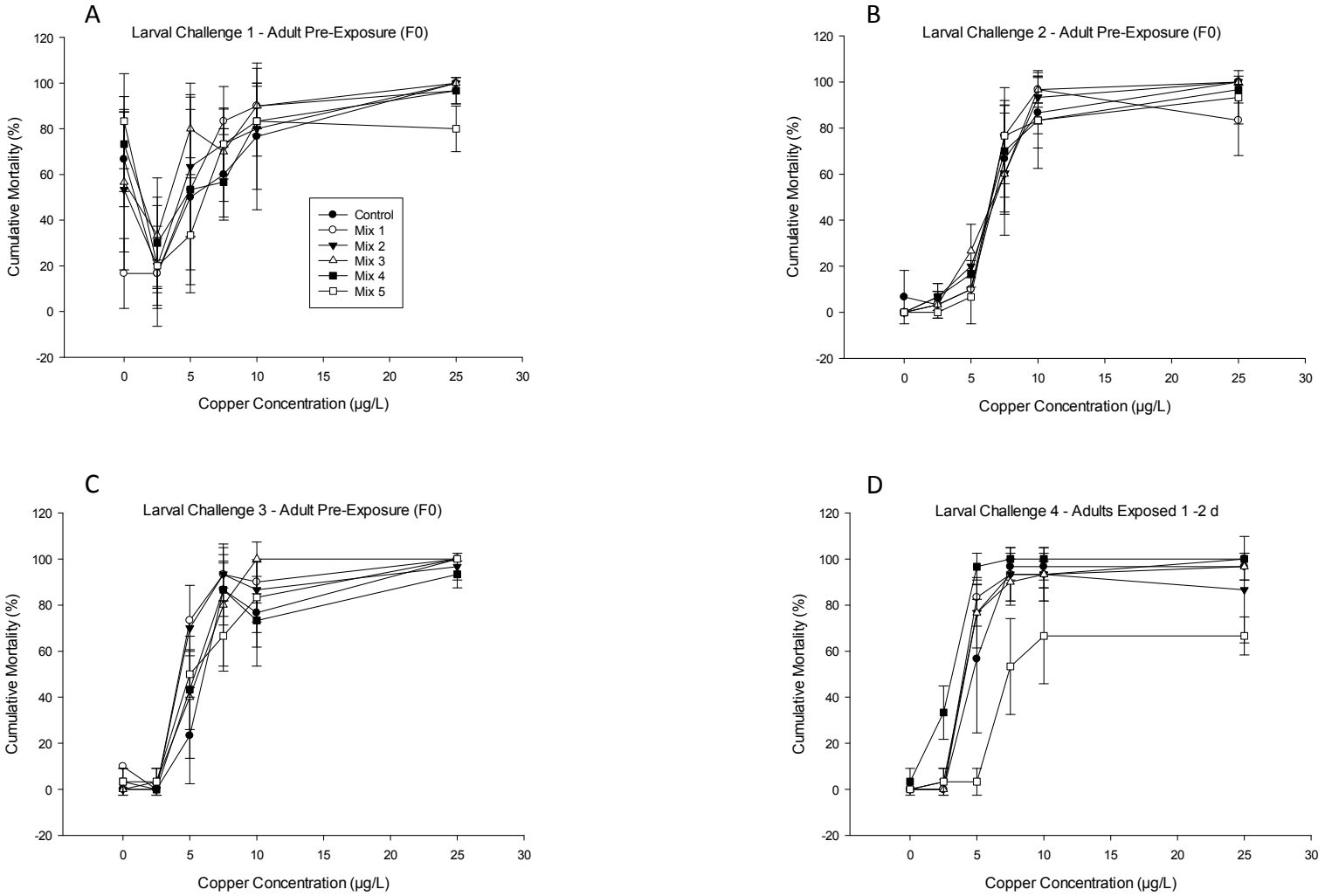

Figure 17: Cumulative mortality (%) at 96 h for larval American flagfish exposed to varying concentrations of copper (0 - 25 µg/L). A – C) Challenge 1 -3 (pre-exposure) larval offspring of adult flagfish without prior exposure to pharmaceuticals. D) Larval offspring of adult flagfish with prior exposure to various treatments, Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE_2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE_2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE_2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE_2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE_2). Asterisk (*) denotes significant differences ($p \leq 0.05$).

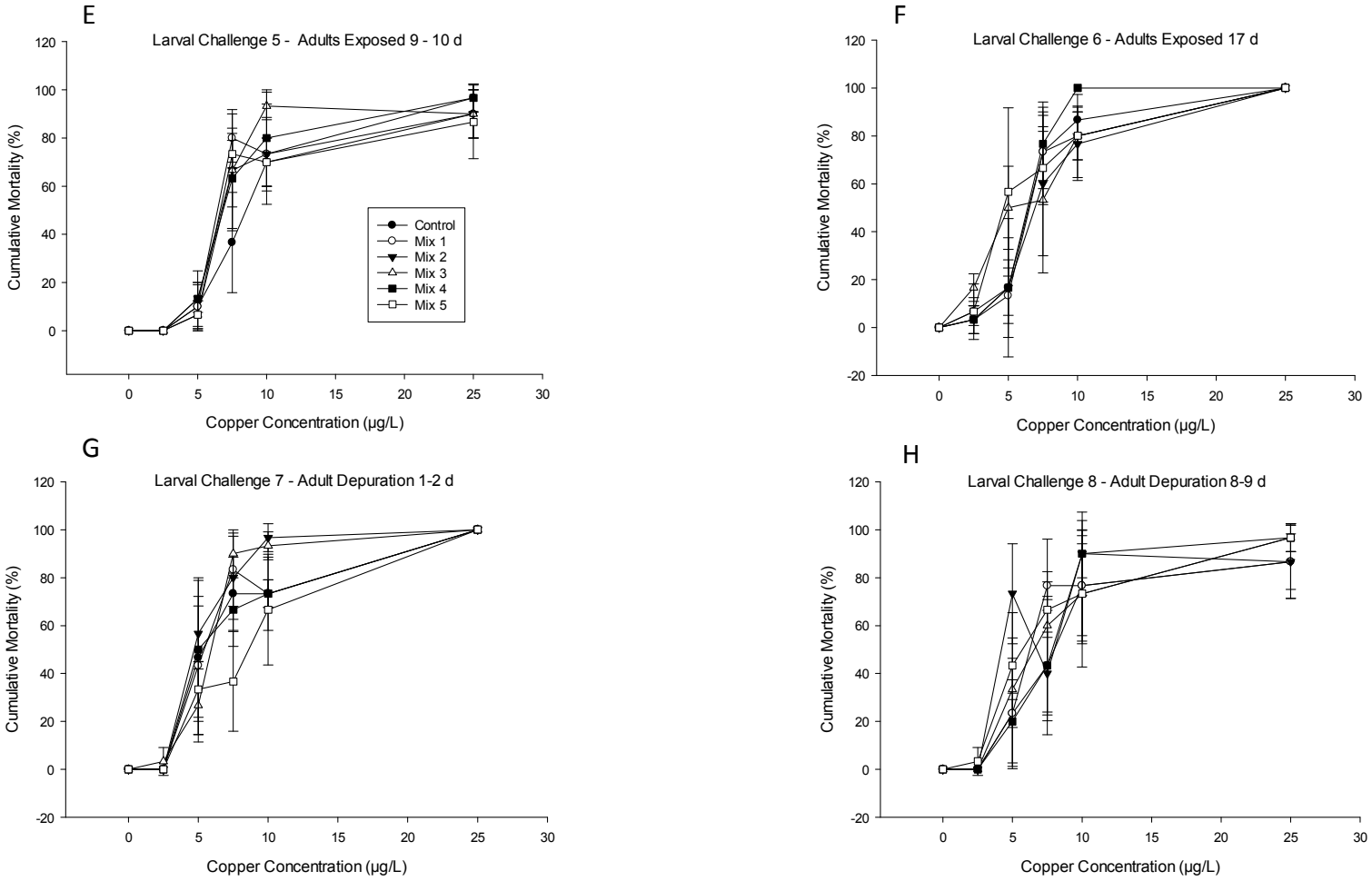

Figure 18: Cumulative mortality (%) at 96 h for larval American flagfish exposed to varying concentrations of copper (0 - 25 µg/L). E – F) Challenge 5 – 6 larval offspring of adult flagfish with prior exposure to various treatments, Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE_2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE_2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE_2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE_2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE_2). G – H) Larval offspring of adult flagfish that had undergone a depuration period after exposure to varying pharmaceuticals. Asterisk (*) denotes significant differences ($p \leq 0.05$).

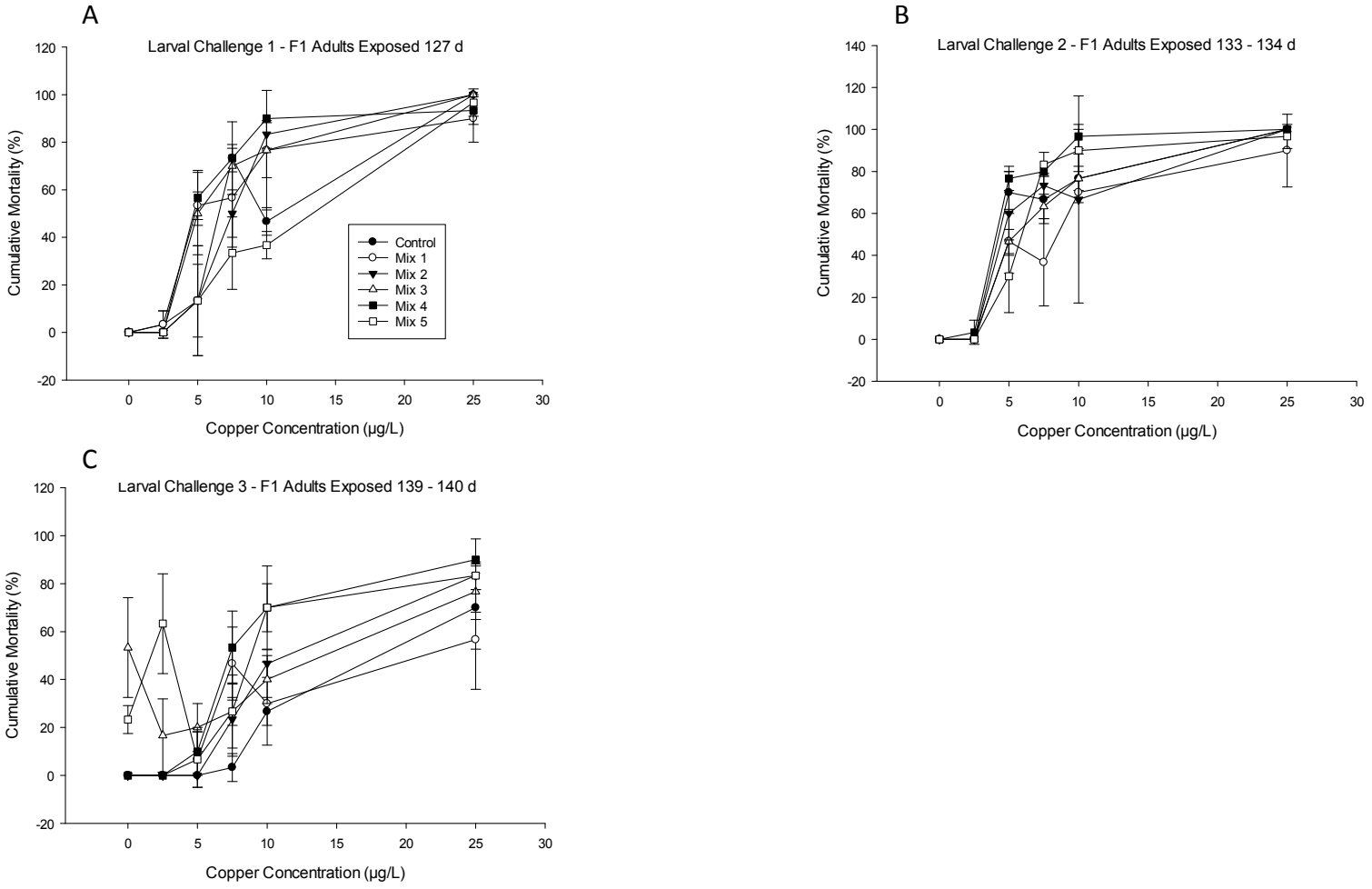

Figure 19: Cumulative mortality (%) at 96 h for F_2 larval American flagfish exposed to varying concentrations of copper (0 – 25 µg/L). A – C) Challenge 1 – 3 larval offspring of adult flagfish with prior exposure to various treatments, Control (0), Mix 1 (0.1 µg/L IBU + 0.1 µg/L NAP + 0.1 ng/L EE_2), Mix 2 (0.32 µg/L IBU + 0.32 µg/L NAP + 0.32 ng/L EE_2), Mix 3 (1.0 µg/L IBU + 1.0 µg/L NAP + 1.0 ng/L EE_2), Mix 4 (3.2 µg/L IBU + 3.2 µg/L NAP + 3.2 ng/L EE_2) and Mix 5 (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE_2). Asterisk (*) denotes significant differences ($p \leq 0.05$).

4.4 Study 1 & Study 2 Discussion

Pharmaceuticals have been a topic of interest for over a decade and much research has investigated the potential effects of individual compounds on non-target organisms. The present studies investigated the chronic effects of individual and mixtures of a commonly used sex hormone (EE2) and two NSAIDs (ibuprofen and naproxen). NSAIDs are a class of drugs that are used to treat pain and inflammation, through the inhibition of cyclooxygenase (COX) enzymes (Simmons *et al.,* 2004; Vane & Botting, 1998). EE2 is a sex hormone that is commonly used in contraceptive pills; it binds and activates membrane bound estrogen receptors (Arukwe & Goksøyr, 2003). As mentioned previously, many studies have been performed highlighting the effects of EE2, ibuprofen, and naproxen individually on non-target organisms.

Study 1 was completed to assess the short-term reproductive impact of ibuprofen, naproxen, and EE2, alone and in mixtures on American flagfish. Study 2 was completed to assess the chronic multi-generational impact of pharmaceutical mixtures (ibuprofen, naproxen, and EE2) on American flagfish. Both studies assessed the impact of prior parental exposure of contaminants to larval offspring chemical tolerance. Since study 1 and study 2 examined the effects of ibuprofen, naproxen, and EE2 either alone or in mixtures, and assessed similar endpoints for both a short-term and multi-generational study the results of both studies will be discussed together.

4.4.1 Percent Fertilization & Percent Hatch

Fertilization and hatch are important endpoints to assess as they potentially impact fish populations if altered. Fertilization impacts may indicate reproductive impairments in either gender, and effects on hatch may lead to reduced offspring survival. Study 1 noted a significant decrease in percent fertilization for American flagfish exposed to 0.1 µg/L naproxen and 10 ng/L EE2 compared to their respective controls. In study 2, exposure of American flagfish to mixtures of pharmaceuticals led to a significant decrease in fertilization at the highest concentration mixture (10 µg/L IBU + 10 µg/L NAP + 10 ng/L EE2) for both the parental and F_1 generation. To the best of our knowledge no studies to date on ibuprofen or naproxen have previously demonstrated fertilization effects in fish. One possible explanation for changes in fertilization may be due to reproductive impairment of the quality of male or female gametes (Lahnsteiner & Leitner, 2013). Another potential cause of the observed changes in fertilization in this study could be due to behavioural changes; both of these potential explanations will be further discussed with regards to the individual compounds.

Although naproxen has not been well studied in fish it has been examined in other organisms. A study completed by Uzun *et al.,* (2015) studied naproxen sodium exposure on male Wistar rats to assess its potential effects on male reproduction. The rats were orally treated for 35 days with a dose of 10 mg/kg naproxen sodium. A significant decrease in sperm count and motility was observed compared to controls (Uzun *et al.,* 2015). Study 1 did not specifically examine sperm count or motility in male flagfish however this could be one potential explanation for the observed decrease in fertilization. It has been shown that the decrease in sperm count and motility was caused by decreased prostaglandin levels

(Uzun *et al.,* 2015). Prostaglandin levels were not monitored in study 1 but naproxen sodium is a known COX inhibitor which has the potential to influence prostaglandin levels.

Effects on fertilization have been demonstrated before with respect to EE2 in fish. Parrott and Blunt (2005) demonstrated that exposure of fathead minnows (*Pimephales promelas*) for 150 days to EE2 resulted in decreased fertilization at concentrations of 0.32 and 0.96 ng/L (Parrott & Blunt, 2005). Similarly, Pawlowski and colleagues (2004) demonstrated a significant decrease in fertilization after a 3 week exposure of adult fish to 10 ng/L of EE2 (Pawlowski *et al.,* 2004). It has been observed that EE2 at 10 ng/L has increased spermatocyte and spermatid cell death, which may potentially explain the decrease in fertilization (Weber *et al.,* 2004).

Another possible explanation for decreased fertilization for both study 1 and study 2 may be due to the fact that flagfish undergo external fertilization and have a distinct breeding behaviour (Fogels & Sprague, 1977). Flagfish have a specific "T-dance" ritual they perform when mating, and a disruption of this behaviour, either by incorrect alignment or by disruptive behaviour of other fish in the tank, may lead to reductions in successful fertilization. As behaviour was not specifically evaluated for this study this hypothetical mechanism would need to be experimentally tested. More specific research into the mechanistic effects of NSAIDs on fertilization should be assessed. A decrease in fertilization may pose a biologically significant problem in the wild as less viable eggs may influence the abundance of flagfish.

The above mentioned effects of naproxen and EE2 on male gametes is likely the contributing factor to the decrease in fertilization that was noted for the study 2 mixture of ibuprofen, naproxen, and EE2.

In study 1 a significant decrease in percent hatch of fertilized eggs from parents treated with 10 ng/L EE2 was observed. Although there was a significant decrease in hatch compared to the control, hatch for EE2 treated eggs was still greater than 98%. Thus, it is not likely that the effect on hatch would cause any significant biological effect in the wild. In study 2, percent hatch was not affected by exposure, and as long as the eggs were successfully fertilized they were able to develop normally and hatch successfully.

While reproductive effects are well known for 17α-ethinylestradiol, to the best of our knowledge the effects of naproxen on fertilization at the environmentally relevant concentration of 0.1 µg/L has not previously been noted. This finding indicates that more research is necessary into the potential reproductive effects of naproxen on fish and other aquatic organisms.

4.4.2 Egg Production

In study 1 a significant increase from the pre-exposure to the exposure phase was noted in mean daily egg production for fish exposed to ibuprofen, as well as a significant increase in cumulative egg production compared to its respective control. Although it was not significant, a decrease in cumulative egg production was noted for naproxen treated individuals.

Ibuprofen has been studied with respect to fish and reproduction. Han and colleagues (2010) demonstrated exposures of 10 and 100 µg/L ibuprofen for 144 days to Japanese medaka resulted in a decrease in the number of broods, but an increase in the number of eggs per brood (Han *et al.,* 2010). A similar finding was elucidated after a six

week exposure of medaka at concentrations of 100 µg/L ibuprofen, noting a significant increase in the number of eggs produced per day, but with a decrease in the reproductive events (Flippin *et al.,* 2007). These findings are similar to the findings of study 1 as a significant increase in egg production was noted for flagfish exposed to 0.1 µg/L ibuprofen. In contrast exposure of adult zebrafish for 7 d to ibuprofen (0 – 500 µg/L) resulted in no significant changes in cumulative egg production (Morthorst *et al.,* 2013). The shorter duration of exposure, and difficulty in assessing egg production in zebrafish may be the cause of no noted change in spawning (Morthorst *et al.,* 2013). As mentioned previously, little research has been done on naproxen, and to the best of our knowledge no research has investigate the effects of naproxen on egg production in fish. More research into the specific mechanisms causing increased reproduction should be completed, as well as further research on naproxen and its impact on reproduction.

Study 2 observed a significant increase in egg production for the parental generation (F_0), Mix 1, and Mix 3; whereas fish exposed to Mix 2 and Mix 5 noted significantly lower egg production. The F_1 generation cumulative egg production for Mix 1, Mix 2, Mix 4 and Mix 5 was significantly lower compared to the controls. All mixtures had varying concentrations of ibuprofen, naproxen, and EE2. The known effects of ibuprofen and naproxen individually on egg production have been discussed above. EE2 effects will now be discussed.

Fathead minnows exposed to 0.32 and 0.96 µg/L EE2 for 150 days demonstrated a 1.5 – 2 times increase in egg production compared to the controls (Parrott & Blunt, 2005). Levels of EE2 below 1 ng/L have been reported to cause low level stimulation of egg production (Parrott & Blunt, 2005). In contrast, fish exposed to low levels (ng/L) of EE2

have also observed decreases in egg production. Exposure of zebrafish to EE2 for a full life-cycle demonstrated that F_0 had reduced egg production at 50 ng/L, while the F_1 generation had a reduction at 5 ng/L, and it was suggested that decreased egg production was due to a lack of expressible sperm, and feminization of males (Nash *et al.,* 2004). Schäfers *et al.,* (2007) demonstrated that impairments to zebrafish egg production were reversible, with a clean water depuration, after a chronic exposure to ≤ 1.1 ng/L EE_2, but became irreversible at 9.3 ng/L (Schäfers *et al.,* 2007).

There is a lack of data available in the literature with regards to naproxen and its potential impact on egg production. Studies of fish exposure to ibuprofen often report an increase in the amount of eggs produced, and studies of exposure of fish to EE2 have noted both increases and decreases in egg production. Such differences may help to explain why there was no general trend observed in the effects on egg production from study 2. The parent generation egg production data was variable with Mix 1 and 3 having an increase and Mix 2 and 5 having a decrease. A few studies have been completed on pharmaceutical mixtures for a variety of species. Mixture toxicity of ibuprofen, diclofenac, naproxen, and acetylsalicyclic acid was evaluated on Daphnia (*D. magna*) using acute studies (Cleuvers, 2004). Mixture effects were demonstrated to take place for the EC_{50} and EC_{80} doses, these higher doses followed the predicted mixture toxicity (Cleuvers, 2004).

Although effects were noted and followed the predicted mixture toxicity, it is believed that acute studies of pharmaceutical effects are not likely to be observed in the field as values were higher (~ 68–166 mg/L) than those detected in surface waters. Accordingly, pharmaceutical mixture effects should be studied at the chronic level (Cleuvers, 2004). Mixture toxicity of acetaminophen, carbamazepine, gemfibrozil, and

venlafaxine was assessed for adult zebrafish (*Danio rerio*) over 6 weeks (Galus *et al.,* 2013). They noted a significant decrease in embryo production for both the low MIX (0.5 µg/L) and high MIX (10 µg/L) demonstrating that pharmaceutical mixtures have the potential to cause adverse effects on fish populations (Galus *et al.,* 2013).

A full life cycle study exposing fathead minnows to six common pharmaceuticals (naproxen, gemfibrozil, diclofenac, ibuprofen, triclosan, salicyclic acid, and acetaminophen), assessed the chronic effects of mixture toxicity (Parrott & Bennie, 2009). The experiment exposed fathead minnows to concentrations ranging from 10 – 1000 ng/L of each pharmaceutical in a mixture. The study observed a wide range of egg production data and no significant effects were seen (Parrott & Bennie, 2009). These studies highlight the necessity for more research on mixture studies as no consistent effects have been noted. The findings of study 2 elucidate the idea that in a real world scenario mixtures of contaminants may influence effects on egg production which may result in either increases or decreases.

The second component of this study was the multi-generational aspect of reproductive effects. Of interest in the F_1 generation, all mixtures except Mix 3 saw a reduction in egg production from the control at some point during the experiment. Also, it is interesting to note that while ibuprofen has been shown to increase egg production, there was a non-significant trend for the mixture of ibuprofen, naproxen, and EE2 to decrease egg production in the second generation of this study at the highest treatments.

One important study that investigated the chronic multi-generational impact of exposure to low concentrations of EE2 (5 – 6 ng/L) was the Kidd *et al.,* 2007 whole lake study which took place over a 7 year period. The study demonstrated the ability of EE2 to

feminize males, alter oogenesis in females, and nearly caused the extirpation of fathead minnows in the lake (Kidd *et al.,* 2007). This massive study demonstrated the importance of chronic low level exposures and the impact that some contaminants can have at the population level.

There have been very few studies that have focused on the impacts of pharmaceutical mixtures and to the best of our knowledge no previous multi-generational mixture studies have been completed to date on fish. A study completed by Dietrich and colleagues (2010) examined the impact of carbamazepine (~0.49 µg/L), diclofenac (~0.36 µg/L), EE2 (~0.10 ng/L) and metoprolol (~1.17 µg/L) alone and as a mixture at environmentally relevant concentrations over six generations of *Daphnia magna*. It was determined that the multi-generational effects were present in some generations but not all and without a consistent pattern (Dietrich *et al.,* 2010). Exposure to sub lethal levels of the contaminants prior, may be a possible explanation for the multi-generational patterning leading to a developed resistance (Dietrich *et al.,* 2010).

4.4.3 Growth (Total Length & Wet Weight)

Study 1 assessed both total length and wet weight for adult flagfish and noted no significant differences in either for male or female exposed flagfish compared to their respective controls.

Study 2 assessed both total length and wet weight at a variety of time points over the multi-generational study and no significant effects on growth for the parental or F_1 generation were demonstrated.

Much of the data previously available on growth has been produced using individual compounds or mixtures of short exposure duration. Similarly to study 1, no effect on growth has been reported for ibuprofen. Medaka were exposed to varying concentrations of ibuprofen from 0.01 – 1,000 μg/L and there were no significant differences in length, and wet weight observed at 90, 120, and 132 dph (Han *et al.,* 2010). Fathead minnows exposed to varying concentrations of ibuprofen (43 – 680 μg/L) during a 28 day early life stage test also demonstrated no significant differences in growth (Overturf *et al.,* 2012). No fish growth studies have been completed on naproxen to our knowledge. In contrast to our findings in study 1 of no effect, Parrott and Blunt (2005) exposed fathead minnows to EE2 at concentrations ranging from 0.32 – 23 ng/L and found a significant effect of EE2 on growth (length) at 60 dph; fish treated with 23 ng/L were shorter than control fish (Parrott & Blunt, 2005). Again at 150 dph, females exhibited growth effects with the lowest EE_2 treatment (0.32 ng/L) being slightly longer and the highest treatment (23 ng/L) being shorter and weighing less compared to controls (Parrott & Blunt, 2005). American flagfish appear less sensitive to growth impacts from exposure to low levels (10 ng/L) of EE2.

Our multi-generational study demonstrated that some of the mixture treatments had significant effects on total length and wet weight of the F_2 generation. Total length was significantly increased for Mix 1 followed by a decrease for Mix 2, 3, and 4 (not significant), and a significant increase for Mix 5. A similar trend was noted for wet weight, where Mix 4 was significantly decreased and Mix 5 was significantly increased. To the best of our knowledge no multi-generational growth data for pharmaceutical mixtures is

available for fish. A study exposing fathead minnows to pharmaceutical mixtures over one generation found no significant effects on growth (Parrott & Bennie, 2009).

There has been some discussion around the potential role of environmental stressors and their impact on maternal transfer. The hypothesis that mothers in fluctuating environments may help to induce adaptive changes in offspring so that they are better prepared to handle the potential environment they are entering has recently been tested (Dantzer *et al.,* 2013). This may explain why the highest mixture concentration (Mix 5) had a significant increase in growth; perhaps it was above a threshold and maternal transfer helped to better prepare the offspring to cope with the environment they were entering.

4.4.4 Gonadosomatic Index, Liversomatic Index, Condition Factor

One way of assessing the impact of contaminants is to do a first screening by determining morphological parameters such as gonadosomatic index (GSI), liver somatic index (LSI) and condition factor (CF) (van der Oost *et al.,* 2003). All three of these parameters were assessed in both Study 1 and 2. GSI is a ratio of gonad weight to total body weight, and LSI is a ratio of liver weight to total body weight (van der Oost *et al.,* 2003). Both are used as a potential way to assess the impact of toxicants.

In study 1, no significant effects were obseved in male or female GSI or LSI of the adults, compared to their respective controls, but a significant decrease ($p \leq 0.05$) in condition factor in Mix 1 compared to the carrier control was noted for females. Although not significant, Mix 2 and EE2 also had a smaller condition factor than the carrier control. Condition factor is based on a relationship between the weight and length of a fish and can be used to give an overall indication of fish health (van der Oost *et al.,* 2003). It is possible

that exposure to low level mixtures of NSAIDs and EE2 impacted the condition of female fish. Possible explanations may be reduced feeding behaviour, energy allocation for detoxification, or altered water retention. Also, since study 1 was a relatively short reproductive study that started with adult flagfish, initial weights and lengths were not taken and condition factor was only assessed at the cessation of the experiment. Without being able to compare back to the initial starting size of the adult flagfish, it is entirely possible that the Mix 1 female adult flagfish were slightly smaller at the start and thus just maintained this slight difference throughout the duration of the study. It is more important to have breeding harems matched in size accordingly for successful breeding than it is to ensure that all adult fish are the exact same size at the beginning of the study.

In study 2 at 102 – 103 dph, a significant increase was detected in juvenile males GSI for Mix 5, and an increase in juvenile males LSI for Mix 1, Mix 3, and Mix 4 compared to controls. Exposure to Mix 5 may have led to an early maturation of the male gonads during their juveniles phase, hence the increase in GSI, however this effect was temporary and there were no significant differences in male GSI by the time the fish were reproducing adults. Similarly the increased LSI in juvenile males disappeared by the time they were reproducing adults. The increase in LSI in juveniles may have been due to an increased need to detoxify the toxicants present in the mixtures (van der Oost *et al.,* 2003).

Female adult flagfish (148 d) had a significant increase in LSI for Mix 3 when compared to the controls. All other mixtures had non-significant increases in LSI as well. The increase in LSI may either be a result of increased volume of liver cells (hypertrophy), or an increase in the actual number of liver cells (hyperplasia) (van der Oost *et al.,* 2003).

A significant generational difference was also noted in adult (148 d) male GSI for Mix 4 and Mix 5 when comparing the F_0 adults and the F_1 adults. In contrast to our findings a full life-cycle exposure of fathead minnows to pharmaceutical mixtures demonstrated no significant changes in LSI or GSI for male or female fish (Parrott & Bennie, 2009). One possible explanations for the generational increase in male GSI for Mix 4 and Mix 5 may be due to better nutrition allowing for more energy to partition to larger gonads during the second generation. The F_0 fish were originally part of the main lab population which is fed daily by many people in the lab, whereas, the F_1 adults in this study were only feed by me and followed a much stricter regime. Another possibility for the generational difference could be due to feminization of males due to the increased length of exposure to pharmaceuticals. Since histology was not performed this cannot be confirmed and it is speculation as to a potential cause. Some discrepancy over the reliability of GSI as an endpoint in multi-spawning fish has been noted before and the timing of the last spawning event must be taken into consideration as it may have the ability to vary the GSI value that is obtained (Rinchard & Kestemont, 1996).

4.4.5 Challenges

Challenges were completed in both studies in order to determine if subsequent exposure to reference toxicants would alter the sensitivity of the fish based on their previous exposure/ life-history. Study 1 demonstrated no significant changes but did show a slight decreasing trend in sensitivity to copper amongst all treatments and the control. Study 2 predominantly had consistent LC_{50}s for the F_1 and F_2 offspring challenges, however, the final F_2 challenge experienced a higher LC_{50} value (less sensitivity). One

potential explanation for the change in sensitivity seen across the board for study 1 and noted at the end for study 2 may be due to experimental stress from the daily routine. As mentioned earlier, maternal stress may lead to changes in the quality of offspring produced (Dantzer *et al.,* 2013).

The increased stress from the daily routine to the female flagfish may have led to a better quality of offspring being produced. Alternatively, female flagfish may have acclimated to the daily routine therefore causing a decreased level of stress and leading to a better quality of offspring being produced. Either explanation identifies the potential role of maternal stress (whether increased or decreased) in producing a better quality of offspring that is better prepared to deal with the environment with which it is entering. An increase in egg quality would potentially increase offspring tolerance to copper.

In fish, the strategies for coping with stress can be species specific (Schrek *et al.,* 2001). Studies on fish have demonstrated that stress encountered during different reproductive stages may lead to reduced reproductive endocrinology (Schrek *et al.,* 2001). Increased levels of cortisol as a result of stress have usually been accompanied by decreased levels of sex steroids and vitellogenin levels in females, and a decrease in plasma testosterone in male fish, resulting in smaller eggs and sperm counts, as well as altered ovulation times (Eriksen *et al.,* 2006; Schreck *et al.,* 2001). These impacts demonstrate the concept that parental stress can lead to impacts on offspring. In contrast to our findings, a study completed on farmed Atlantic salmon (*Salmo salar*) demonstrated that embryos exposed to prenatal stress have a reduced ability to handle a secondary stressor (temperature) (Eriksen *et al.,* 2006).

A study completed by Dantzer and colleagues (2013) on North American red squirrels demonstrated that in fluctuating environments mothers are able to enhance the fitness of their offspring via an adaptive hormone-mediated effect. The elevated maternal glucocorticoid levels led to a quicker offspring growth (Dantzer *et al.,* 2013). The quicker growth allowed offspring to better match the environment to which they were entering (Dantzer *et al.,* 2013). These finding are similar our findings and the potential explanation that the stress of parental female flagfish led to a better quality of offspring that was more well prepared for the environment into which is was entering.

A final potential explanation is that the fish population just naturally maturated irrespective of their surrounding environmental stresses and as such were able to produce better quality offspring. More research on the specific impact of stress on fish and their potential to maternally transfer/ better prepare offspring for their surrounding environment should be investigated.

5 Overall Discussion

The purpose of this research was to investigate the impacts of multiple stressors, both environmental and chemical, on cold-water and warm-water fish. This research expanded the experimental evidence of the impacts of pharmaceuticals on fish at environmentally relevant concentrations and provided new knowledge about interactions that may be present in a natural environment.

Our first study assessed the multiple stressors of feeding regime / prior life-history and toxicants on the cold water fish, rainbow trout. The impact of ration on subsequent acute toxicity was an important area to investigate because acute toxicity findings are often used to establish regulatory guidelines for compounds entering our waterways. Many researchers, regulatory agencies and hatcheries do not report on the feeding regimes used to produce specific sized fish required for acute toxicity testing, and there is the potential that either the quality or quantity of ration could impact the results. Either an over-estimation, or an under-estimation, of acute toxicity could be a problem for non-target organisms and the economy (Holmstrup *et al.,* 2010). Our findings highlighted that it is unlikely that a reduced quantity of feed such as hatcheries holding back food to maintain specific size classes of fish for regulatory testing will significantly impact the acute toxicity thresholds of contaminants. This is very reassuring to know, however a better standardization of reporting feed quantity is needed, as it has been shown to affect sub-lethal toxicity. Following these findings, the focus of our research was shifted to assess other multiple-stressors in the environment.

With increasing consumption of pharmaceuticals over the years, and an increasingly aging population, the load of pharmaceuticals present in the waterways is

likely to continue over the years. Consequently, non-target organisms, specifically fish, are being continuously exposed to low levels of pharmaceuticals and occupy a wide variety of aquatic habitats where pharmaceuticals have been detected. To date, much of the work on pharmaceuticals has focused on acute or chronic studies involving the impact of one or two pharmaceuticals on fish, without considering the environmental relevance of additional stressors.

There are over 4,000 pharmaceuticals currently in use and the decision of which compounds to investigate was given consideration. The decision to study ibuprofen and naproxen (NSAIDs); and 17 α-ethinylestradiol (sex steroid) was made. A major factor in the selection of studying NSAIDs was their consistent detection in the ng/l - µg/L range in surface waters (Corcoran *et al.,* 2010; Santos *et al.,* 2010). Additionally, with an aging society the use of NSAIDs is increasing due to their general mode of action of reducing pain and inflammation, and their ease of access through over-the-counter purchasing. Some studies had been completed on ibuprofen but very little to no research had been completed on naproxen. As such, these two NSAIDs were selected for further investigation. Although EE2 has been well studied in fish, the continued use of it by women in the form of the birth control pill, and its ability to exert effects at low ng/L ranges makes it of interest to consider when looking at natural systems and the different mixtures of compounds that may be present.

The experiments studied pharmaceuticals both alone, and in mixtures and using a variety of different methodologies. Since the effects of both environmental and chemical stressors were of interest, it was important to consider which methodology would best help to investigate our aims. Acute toxicity was used as a way to study subsequent exposure to

toxicants (tolerance), and was important in assessing if any short term impacts were present after either environmental or chemical stressors. Both chronic and multi-generational studies were completed to investigate long-term and transgenerational impacts as a consequence of continued exposure. In a natural setting pharmaceuticals are present with a "pseudo-persistence" so the use of multi-generational studies along with environmentally relevant concentrations was necessary. Although understanding the potential effects and mechanisms of individual compounds on non-target organisms is important, the need for both mixture and multi-generational studies is critical. Fish are often exposed to multiple contaminants over an extended duration and the potential effects from extended exposures need to be further investigated. Multi-generational studies are important as they have the ability to address the potential issues of bioaccumulative substances, endocrine-mediated trans-generational effects, and maternal transfer effects (Crane *et al.,* 2010).

A significant decrease in fertilization was observed in the partial life-cycle study as a result of exposure to 0.1 µg/L naproxen, and 10 ng/L EE2, as well as a significant increase in egg production as a result of exposure to 0.1 µg/L ibuprofen. A significant decrease in fertilization was demonstrated in the multi-generational study after exposure to the highest concentration of mixtures of ibuprofen, naproxen, and EE2 for both generations. There were also significant changes in egg production. These endpoints have the potential to influence population level dynamics, such as decreased fertilization leading to fewer viable offspring. The decreased amount of offspring may potentially lead to community level impacts.

In both the partial life-cycle study and multi-generational study there was no significant impact of subsequent acute toxicity to offspring. It is probable that any impacts

of parental exposure on subsequent offspring sensitivity would be happening at the sub-lethal level instead.

Conducting studies that encompass both chemical and environmental stressors has always been challenging. In surface waters, wild fish may be exposed to numerous compounds over multiple generations with many different stressors and modifying factors. Thus, it is important to consider multiple factors together in order to understand the true scale of potential contaminant impacts on fish populations. Overall, there were some important findings when considering the potential implications in both setting regulatory guidelines, and in the natural environment.

5.1 Limitations and Sources of Error

Each component of research investigated for this dissertation was not without its limitations and sources of error:

Aim 1

Initially getting CCAC approval for the experiment proved to be difficult. The main limitation of this study was not being able to completely starve the fish (CCAC requirements). Rainbow trout were put on a limited diet that was aimed at maintaining their weight for the duration of the experiment but they were able to partition even the small amount of food that was given to them into somatic growth. This small amount of growth made it difficult to consistently select the concentration range for copper exposures, and thus making it difficult to ascertain the LC_{50} value as accurately as possible. Due to

the inaccuracy of the concentration range a 24 h LC_{50} was determined as opposed to the typical 96 h LC_{50} value, this presents a challenge when trying to compare and contrast to the literature in the field.

Aim 2 & 3

Much of the work was similar for the second and third experiment of this research and as such many of the same limitations and challenges were encountered. One of the main limitations to this research was the lack of biochemical endpoints that are available for American flagfish. The ability to assess things such as VTG and gene expression on flagfish was not available and as such the studies were limited to more general endpoints like reproduction and morphometric measures. With that said another limitation was only using one species (American flagfish) to study the endpoints of interest. Results observed for one species may not always be present when encountered with a different species and this must be kept in mind when evaluating findings and relating them to other species. Another limiting factor of this research was both time and cost constraints. The ability to replicate and test a certain number of concentrations was limited by my ability to complete all of the work each day. With 20 tanks in the multi-generational study the entire day was busy with lab work and no more additional replicates or concentrations would have been feasible to include. Another source of error that was present from the reproductive aspect was consistency of egg production. There is a high amount of variability in egg production and as such it made it hard to determine some effects and at times made it difficult to get enough offspring to run subsequent toxicity challenges.

5.2 Conclusion

The main aims were; to assess the impact of restricted rations on subsequent acute lethal toxicity; elucidate the impact of environmentally realistic chemical stressors on reproductive endpoints and subsequent toxicant exposure; and to determine the impact of multi-generational and mixture exposure of environmentally realistic chemical stressors on reproductive endpoints and subsequent toxicant exposure. By researching these three aims I was able to expand the literature as follows:

Aim 1

The first objective investigated the impacts of restricted rations on subsequent acute lethality and found that prior feeding regime is unlikely to affect short-term toxicity results. These findings are important news when considering the potential implications in both setting regulatory guidelines, and in the natural environment. Fish that may have restricted access to food via hatchery/ laboratory practices will not have their toxicant thresholds affected and as such our common laboratory practices for acute studies are not likely to be impacting our reported results.

Aim 2

The second objective investigated exposure to pharmaceuticals at environmentally relevant concentrations and their potential effects on reproduction and subsequent toxicant exposure. It was demonstrated that short-term partial life-cycle exposure to naproxen, ibuprofen, and EE2 affected reproduction, mainly fertilization and fecundity. However, prior exposure to contaminants did not impact subsequent toxicant exposure significantly.

Aim 3

The third objective investigated whether multi-generational exposure to environmentally realistic pharmaceutical mixtures would impact any reproductive endpoints, or change the sensitivity of larval offspring to subsequent toxicant exposure. It was demonstrated that the highest mixture of ibuprofen, naproxen, and EE2 caused a decrease in fertilization over multiple generations. Exposure to pharmaceutical mixtures also had an impact on egg production, and some growth and morphometric endpoints as well. Subsequent toxicant exposure was not altered.

Overall, there appears to be some potential reproductive impacts related to pharmaceutical exposure either via a short-term exposure or over multiple generations.

5.3 Future Directions

There are a variety of future considerations for this work that has been completed. Our findings demonstrated that there is likely no impact of prior ration restriction on acute toxicity. With that being said it is important to further investigate the role that ration plays as a modifying factor when it comes to toxicity as this was only a short-term study and effects may be present under different conditions. Further investigation into the impact that different durations of either depleted or excess rations may play in altering toxicity should be assessed. It is important to assess the sub-lethal and potential chronic toxicity effects that may be impacted due to varied amounts of ration. There may also be a difference amongst species, specifically cold-water vs warm-water species; life histories and life-strategies may also play a role in the susceptibility of a species to ration as a modifying

factor. Further research into the area of ration as a modifying factor should be completed and the standardization of reporting ration in toxicity testing would greatly help to increase the knowledge and literature available with regards to ration as a modifying factor.

Our studies on pharmaceutical exposures demonstrated that there are some reproductive impacts present and as such further studies should be completed to investigate the specific mode of action and potential sites of effect. Further research exploring whether or not it is the male, female, or both fish being affected by the exposures should be examined. Also the potential that the effects are being cause by behavioural changes rather than biochemical changes should be further studied. More studies into the multi-generational impact of mixture pharmaceuticals is necessary to elucidate more of the potential effects that may be observed in the wild.

Determining if prior life histories and multiple stressors play a role in subsequent sensitivity to toxicants is an area of research that deserves more investigation. With the constant exposure of fish and other non-target organisms to pollutants in the environment it is an important question to consider whether or not sensitivity to a contaminant will be influenced by prior life history or multiple stressors. The potential effects of maternal stress and thereby adaptation of offspring to environments should be further investigated.

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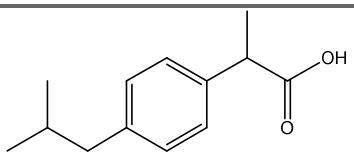
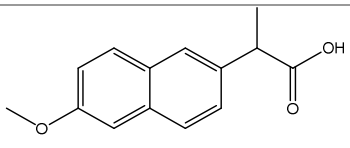
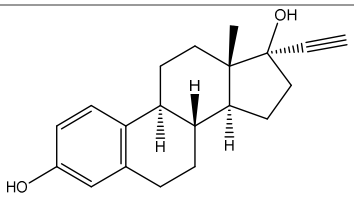
Appendix 1: Feed amounts for rainbow trout during 42 d experiment.

Tank #	Average weight (g)	Body weight (%)	Total (g)	Fish/ tank (#)	Total Amount of feed (g)
LC_{50} 1					
w-70-41	1.67	0.01	0.0167	95	1.6
w-70-42	1.76	0.01	0.0176	95	1.7
w-70-43	1.8	0.01	0.018	94	1.7
w-70-44	1.82	0.01	0.0182	95	1.7
w-70-45	1.87	0.039	0.07293	81	5.9
LC_{50} 2					
w-70-41	2.01	0.004	0.00804	62	0.5
w-70-42	2.17	0.004	0.00868	62	0.5
w-70-43	2.18	0.004	0.00872	62	0.5
w-70-44	2.16	0.004	0.00864	61	0.5
w-70-45	4.2	0.032	0.1344	71	9.5
LC_{50} 3					
w-70-41	2.57	0.004	0.01028	29	0.3
w-70-42	2.94	0.004	0.01176	28	0.3
w-70-43	2.87	0.004	0.01148	29	0.3
w-70-44	2.96	0.004	0.01184	29	0.3
w-70-45	7.71	0.032	0.24672	61	15.0

Appendix 2: Pharmaceutical structures and some chemical properties

Chemical Name	**Chemical Structure**	**Molecular Formula**	**Molecular Weight (g/mol)**	**pKa**
Ibuprofen		$C_{13}H_{18}O_2$	206.285 g/mol	4.91
Naproxen		$C_{14}H_{14}O_3$	230.263 g/mol	4.15
EE2		$C_{20}H_{24}O_2$	296.41 g/ mol	10.33

Appendix 3: Timeline of activities and daily assessments during a multi-generational study

Duration	Daily Activities
6:30 am – 7:30 am	Vacuum tanks
7:30 am – 8:30 am	Harvest brine & first feeding
9:00 am – 11:00 am	Check eggs from previous day (fertilization, hatchability, malformations)
11:30 am	Second feed
12:00 pm – 1:30 pm	Egg Collection
1:30 pm – 4:30 pm	Egg Enumeration
4:30 pm	End of day feed
6:00 pm – 8:00 pm	Challenge set-up if it was running