Examining swim bladder inflation failure as a toxicological endpoint in Japanese medaka (*Oryzias latipes*)

By

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A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in

The Faculty of Science

Applied Bioscience Program

University of Ontario Institute of Technology

November 2017

Certificate of Approval

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Abstract

Inadequate swim bladder inflation, while not being lethal itself, can have serious long-term effects on fish populations. Many environmental contaminants can inhibit swim bladder inflation of larval fish. Understanding how swim bladder inflation failure due to pollutant exposure occurs and developing biomarkers for this effect is important. This study demonstrated that embryonic exposure to the pharmaceuticals 17α-ethinylestradiol, levonorgestrel, and diclofenac, impaired swim bladder inflation of Japanese medaka (*Oryzias latipes*). After exposure to individual compounds, mixture exposures to no-observed-effect concentrations of these compounds were conducted. Significant effects on swim bladder inflation were observed for a binary mixture of 17α-ethinylestradiol, and levonorgestrel, as well as in a mixture of all the compounds. These compounds thus have the potential for significant mixture effects below their individual no-observed-effect concentrations.

Many factors associated with swim bladder formation and inflation were investigated. The temporal requirement for an air-water interface for successful swim bladder inflation was determined to be ~5 days for Japanese medaka. Swim bladder inflation failure of medaka larvae was shown to be strongly correlated with decreased survival 12 days post-hatch. This implies that compounds that can disrupt swim bladder inflation of hatched larvae such as those studied in this experiment, may lead to increased mortality of larval fish

Embryonic exposure to 5 μM cyclopamine, a Hedgehog (Hh) signaling inhibitor, as well as 1 μM IWR-1, a Wnt signalling inhibitor, resulted in >95% inhibition of swim bladder inflation of Japanese medaka larvae. The effects of these inhibitors on the

expression of medaka genes related to the Hh and Wnt pathways, as well as genes involved in the formation of the three cell layers that make up the swim bladder (epithelial, mesenchyme, and outer mesothelium) were also determined at 80, 96, 101, 144, 180, and 216 hours post fertilization. Effects on expression of key genes related to swim bladder formation and inflation following exposure to 17α-ethinylestradiol, levonorgestrel, and diclofenac were determined. The effects on gene expression of these three pharmaceuticals when compared to those of select signalling pathway inhibitors were more closely related to effects on the wnt pathway than the Hh pathway. These pharmaceutical compounds significantly inhibited the expression of genes related to the formation of the three layers of the swim bladder, with levonorgestrel affecting the most genes tested. While all of the effects observed in this dissertation were caused by concentrations of pharmaceuticals generally above those found in the environment, this study demonstrated that pharmaceutical compounds have the potential to affect medaka embryo development as well as swim bladder inflation.

Acknowledgements

I would like to thank my supervisor Dr. Douglas Holdway, for his advice, suggestions, and guidance. I would also like to thank him for his endless confidence and support throughout this project.

Thanks to Dr. Matthew Overturf for his advice and friendship. I also really appreciated his supervision and teaching in the lab; he taught me many valuable laboratory techniques including RT-qPCR.

Thanks to John Guchardi for his continued support in lab.

I would like to thank Erin Ussery for her friendship and support with many of the experiments I conducted as well as her help with maintaining the lab population of medaka.

Thanks to Dr. Robert Bailey for fruitful discussion, his statistical expertise, and advice.

I would like to thank Lindsay Beyger, Jordan Anderson, and Jason Durante for their friendship and moral support.

I would like to thank my family, especially Mom and Dad, for their continuous support they have given me throughout my time in graduate school; I could not have done it without them

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List of Abbreviations

17,20βP	17 α,20β-Dihydroxy-4-prenen-3-on
acta2	Smooth muscle actin
ACTB	Beta-actin
ANOVA	Analysis of variance
anxa5	Annexin A10
AOP	Adverse outcome pathway
APC	*Adenomatous polyposis coli*
B2m	Beta-2-Microgobulin
bp	Base pairs
BS	Blood stasis
C	Control
CK1	Casein kinase 1
COX	Cyclooxygenase enzymes
Cyclo	Cyclopamine
°C	Degrees celcius
DEPC	Diethyl pyrocarbonate
D.I.	Deionized water
DIC	Diclofenac
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpf	Days post fertilization
Dunn's	Dunnet's

Dvl	Disheveled
E	Epithelium
E2	17β-Estradiol
EDTA	Ethylenediaminetetraacetic acid
EE2	17α-Ethinylestradiol
eEF1A	Eukaryotic elongation fact 1 alpha
elovl1a	Elongation of very long chain fatty acid protein 1-like
F1	First generation
FELS	Fish early life stage
fgf10a	Fibroblast growth factor 10
FLFII	Female leucophore-free
FSH	Follicle-stimulating hormone
Fz	Frizzled
g	Gram
g	Relative centrifugal force or G-force
GH	Growth hormone
Gli	Glioma-associated oncogene
GSK-3	Glycogen synthase kinase 3
GtH	Gonadotropin
h	Hour
has2	Hyaluronan synthase 2
hb9	Motor neuron and pancreas homeobox 1
Hh	Hedgehog

H_o	Null hypothesis
hpf	Hours post fertilization
hprt1l	Hypoxanthine-guanine phosphoribosyltransferase-like
IBU	Ibuprofen
Ihh	Indian hedgehog
Ihha	Indian hedgehog B protein
IWR-1	Inhibitor of Wnt response
KEGG	Kyoto encyclopedia of genes and genomes
KT	11-Ketotestosterone
L	Liter
LC_{50}	Lethal concentration that causes death of 50% of a group
LEF	Lymphoid enhancer factor
Lef1	Lymphoid enhancer-binding factor 1
LNG	Levonorgestrel
LRP	Lipoprotein receptor-related protein
LSH	Luteinizing hormone
M	Mesenchyme
m/z	Mass-to-charge ratio
min	Minute
µg	Microgram
µL	Microliter
µm	Micrometer
µM	Micromolar

mg	Milligram
mL	Milliliter
mm	Milimeter
mM	Milimolar
MMTV	Mouse mammary tumor virus
MO	Micropthalmia
NAP	Naproxen
NCBI	National center for biotechnology information
ng	Nanogram
NMDS	Non-metric multidimensional scaling
NOEC	No-observed-effect concentration
NSAID	Non-steroidal anti-inflammatory drugs
NSB	No swim bladder inflation
OD	Optical density, absorbance
OM	Outer mesothelium
%	Percent
Pbx1b	Pre-B-cell leukemia homeobox 1
PCB 126	3,3′,4,4′,5-Pentachlorobiphenyl
PCB 77	3,3'4,4'-Tetrachlorobiphenyl
PCR	Polymerase chain reaction
PKA	Protein kinase A
PE	Pericardial edema
PRL	Prolactin

Ptc	Patched
R^2	Coefficient of determination
RNA	Ribonucleic acid
rpl7	Ribosomal protein l7
rpl8	Ribosomal protein l8
RPM	Rotations per minute
RQI	RNA quality indicator
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SB	Normal swim bladder
SC	Solvent control
SD	Standard deviation
SE	Standard error
Shh	Sonic hedgehog
shha	Sonic hedgehog a
SL	Somatolactin
SL1	Sex-linked 1
SM	Spinal malformation
Smo	Smoothened
sox2	SRY (sex determining region Y)-box 2
SPF	Solid phase extraction
ssRNA	Single strand RNA
swb	Swim bladder
T	Testosterone

TALE	Three amino acid loop extension
tbp	TATA Box BiTATA Box Binding Protein Ending Protein
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TCF	T cell factor
tcf3	Transcription factor 3
tcf7l1	Transcription factor 7 like 1
TE	Tris-EDTA buffer
US EPA	United States Environmental Protection Agency
Vtg	Vitellogenin
Wif1	Wnt inhibitory factor-1
Wnt	Wingless-type MMTV integration site family
wnt5b	Wingless-type MMTV integration site family member 5B
WWTP	Wastewater treatment plant
Zrp	Zona radiata proteins

Chapter 1: Introduction and literature review

1.1 Introduction

Pharmaceuticals consist of a wide range of medicinal compounds used for the treatment, cure, mitigation, or prevention of diseases in both humans and animals. Pharmaceuticals are classified according to their therapeutic function i.e. analgesics, antibiotics, oral contraceptives, etc. A growing world population, increasing investment in the healthcare sector, as well as an aging population in industrialized societies, have led to a significant increase in the consumption of pharmaceuticals in the last few decades (Khetan and Collins, 2007). This is also demonstrated by the worldwide sale of pharmaceuticals rising annually, with an average increase of 6.2% from 2006-2014 (IMS Health, 2016).

Pharmaceuticals enter the environment as an unintended and unavoidable consequence of their medical and veterinary use. They have been measured in the environment of 71 countries across all continents (reviewed by: Aus der Beek *et al.,* 2016). Pharmaceuticals have been detected in wastewater treatment plant (WWTP) effluent, surface and ground water, and drinking water (Aus der Beek *et al.,* 2016; Fabbri and Franzellitti, 2016; Togola and Budzinski, 2008; Vulliet *et al.,* 2011). Numerous studies have measured pharmaceuticals in aquatic environments at low or very low concentrations (μg/L to ng/L) (Fent, 2006). They commonly enter the environment through human consumption, elimination, and disposal via wastewater systems (Nikolaou *et al.,* 2007). As the consumption of pharmaceuticals continues to increase, their discharge into the environment will as well.

The most significant characteristic of pharmaceuticals discharged into the environment is that they are designed to alter physiological function. Many of the

mechanisms through which pharmaceuticals act are conserved across animal phyla, potentially causing significant non-target effects (Gunnarsson *et al.,* 2008; Huggett *et al.,* 2003). As fish share many similarities in their physiological processes with mammals, they are likely to be susceptible to the effects of water-borne pharmaceuticals (Corcoran *et al.,* 2010). Thus, there is a concern regarding the potential impacts including effects on reproduction, growth, and survival, of pharmaceuticals on non-target aquatic species.

There are > 5000 human and veterinary pharmaceuticals in use or in production. Testing each pharmaceutical compound for adverse outcome effects in non-target organisms is not practical. Fish embryos are useful models for the environmental risk assessment of chemicals since their use permits small-scale high-throughput analyses. Monitoring developmental abnormalities caused by chemical exposure in fish embryos can allow for the prediction of long-term effects on populations.

This study investigated the effects of a few select pharmaceuticals that are known to cause impacts at low concentrations such as 17β-estradiol, 17α-ethinylestradiol (EE2) and levonorgestrel, or that are found in higher environmental concentrations like non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, on medaka (*Oryzias latipes*) embryo development. Medaka were used as a model species as their embryo has a clear chorion allowing the growth and development of the embryo to be viewed. They are also easy to rear in lab, and their genome is sequenced allowing the impact of compounds on gene expression to be assessed.

Inadequate swim bladder inflation, while not always being lethal itself, can have serious impacts on inidivdual fish such as impaired growth, or an inability to obtain food/avoid predators, that can lead to population level effects. Further more swim bladder

inflation failure has been linked with decreased survival of zebrafish (*Danio rerio*) larvae (Goolish and Okutake, 1999). This effect can be observed in medaka larvae shortly following hatch. Many environmental contaminants can inhibit swim bladder inflation of larval fish such as permethrin (González-Doncel *et al.,* 2003), PCB 126 (Jönsson *et al.,* 2012), urban storm water (Skinner *et al.,* 1999), thiobencarb (Villalobos *et al.,* 2000), and octylphenol (Gray and Metcalfe, 1999). However, how exposure to chemical compounds causes swim bladder inflation failure in larval fish is poorly understood, with very few studies being designed around this outcome. Understanding how exposure to compounds causes swim bladder inflation failure to occur in larval fish and developing biomarkers for this adverse effect is essential.

One way to explore swim bladder inflation failure is by assessing the effect of compounds on the expression of genes related to swim bladder inflation. Both the Wnt and hedgehog (Hh) signaling pathways are critical in mammalian early lung development (Bell *et al.,* 2008; Bitgood and McMahon, 1995), and have also been shown to be involved in fish swim bladder development (Winata *et al.,* 2009; Yin *et al.,* 2011). Linking the inhibition of cell signalling pathways and swim bladder inflation failure due to exposure to pollutant compounds would be novel and relevant for understanding this important adverse effect pathway.

1.2 Pharmaceuticals in the environment

Pharmaceutical use varies greatly across regions, and tends to be much higher in developed countries; with North America accounting for 40% of global sales in 2014 ($406.2 billion), Europe 23%, and Japan 8% (IMS Health, 2016). Due to their usage, as

well as a lack of funding for research in developing countries, they have been predominantly detected in developed nations: i.e. in their 2016 review Aus der Beek *et al.,* had nearly 100 times as many entries from Western Europe and others (including North America) when compared to Africa (96,000 vs 1000, respectively). The most commonly detected pharmaceutical compounds in surface/ground/tap water globally are analgesics such as ibuprofen, naproxen, and diclofenac, as well as natural and synthetic hormones such as 17α-ethinylestradiol (EE2), levonorgestrel, and 17β-estradiol (Aus der Beek *et al.,* 2016). As a result, their routes into the environment were explored in this section.

The many routes through which pharmaceuticals enter the environment are summarized in Figure 1.1. While there are many ways through which pharmaceuticals and their metabolites enter the environment, the main route is through human consumption, elimination, and disposal via wastewater systems; in the form of waste water treatment plant effluent (Fent, 2006; Nikolaou *et al.,* 2007).

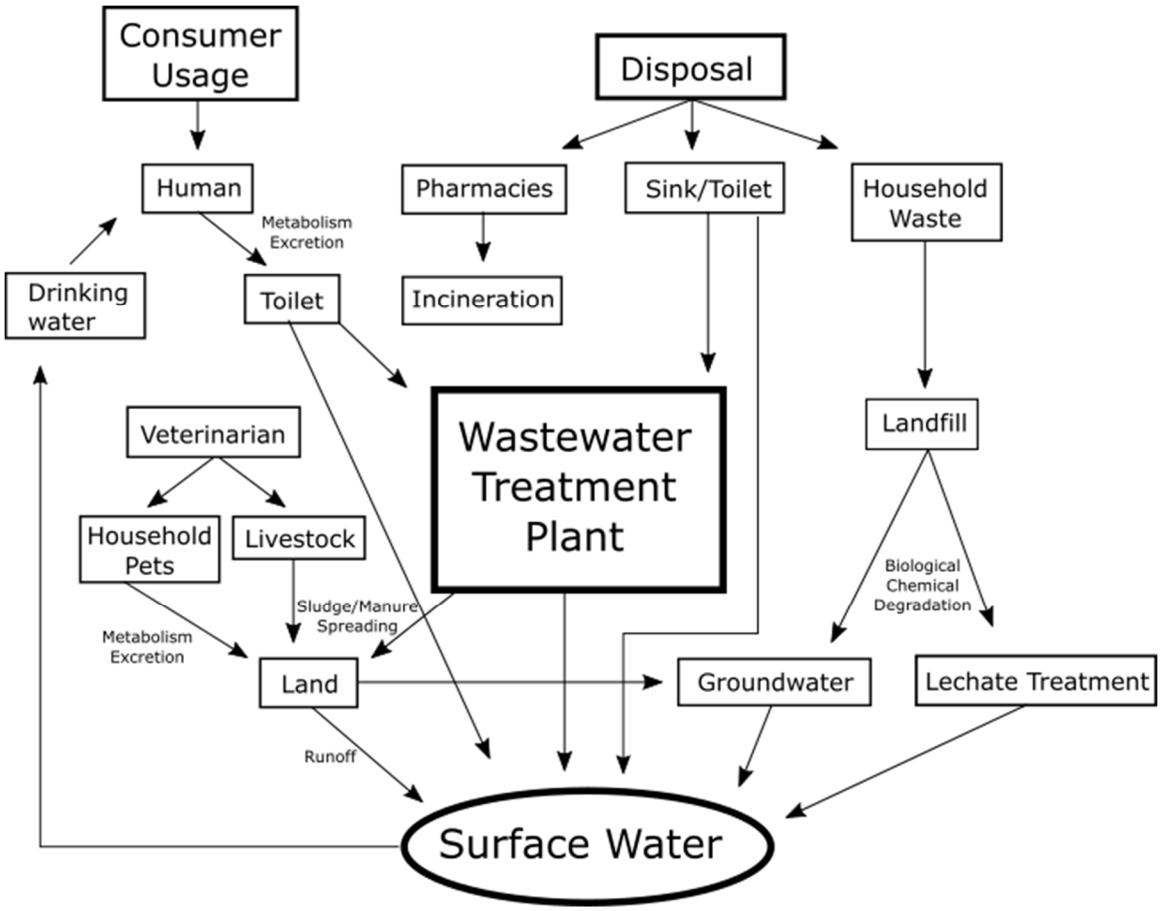


Figure 1.1: Major routes of entry of pharmaceuticals into the environment. The principal route that pharmaceuticals enter the environment is through human consumption, metabolism/excretion, and disposal into surface waters from wastewater treatment plants. Modified from (Overturf *et al.,* 2015).

After consumption, pharmaceuticals are excreted in urine and/or feces as either the parent compound, metabolites, or as conjugates (Kar and Roy, 2012). These excreted pharmaceuticals and their metabolites eventually reach a municipal wastewater treatment plant (WWTP). Generally, WWTP's are not equipped to break down complex pharmaceuticals, as they were mainly designed to remove pathogenic bacteria, and to aid in the removal of biodegradable carbon, nitrogen, and phosphorous compounds that usually arrive at the plant in mg/L concentrations (Verlicchi *et al.,* 2012). Verlicchi *et al.,* (2012) conducted an extensive review of the current literature pertaining to removal efficiencies of 118 pharmaceuticals, including many natural/synthetic hormones and analgesics, and found that WWTPs have two main mechanisms of removing/degrading pharmaceutical compounds: biological transformation and sorption onto sludge. Both the natural/synthetic hormones and analgesics do not readily adsorb onto sludge (< 5%) and their main mechanism of degradation is biological transformation (Joss *et al.,* 2004, 2005). The removal efficiencies vary drastically for different pharmaceuticals across different WWTPs, for example the removal efficiency for 17α-ethinylestradiol (EE2) and diclofenac, in some WWTP facilities high rates of removal were observed, and in others no or only slight removals were measured: -25 to 63% removal efficiency for EE2 and 0 to ~100% removal efficiency for diclofenac (Clara *et al.,* 2005). However, even with a 90% removal rate, residual amounts of the compound will still enter the environment.

After wastewater treatment, resultant effluent containing various pharmaceuticals is released into surface waters. There usually is a positive correlation between the most frequently used class of pharmaceutical and their detection in the environment (Corcoran *et al.,* 2010). Local prescription rates were found to correlate weakly (R^2 = 0.18 to 0.56),

with WWTP effluent concentrations (Saunders *et al.,* 2016). A few factors that may make it challenging to correlate prescription rates with environmental concentrations could include individuals who purchase a drug and do not consume it, the variance in dosages in prescribed drugs, or wastewater treatment processes. There are many types of wastewater treatment processes. Primary treatment generally involves settling and filtering to remove large solids and sediments (Santos *et al.,* 2009). Secondary treatment such as activated sludge involves degrading the organic matter in the wastewater, usually through microbial degradation (Santos *et al.,* 2009). The concentrations of pharmaceuticals tends to be highest in WWTP effluents where they are present in the µg/L to ng/L range, while they are found at significantly reduced concentrations following dilution in receiving waters (ng/L) (Fent, 2006).

Analgesics such as the non-steroidal anti-inflammatory drugs (NSAIDs) ibuprofen, diclofenac, and naproxen are among the most abundant pharmaceutical contaminants measured in the environment due to their high volume of consumption. They are found in surface waters with average global concentrations in µg/L (maximum) of 0.108 (303), 0.032 (18.74), and 0.05 (32), for ibuprofen, diclofenac and naproxen, respectively (Aus der Beek *et al.,* 2016). Natural/synthetic hormones including EE2 are generally found at lower environmental concentrations; a study sampling 139 streams across the United States found a median concentration of 0.073 µg/L EE2 (Kolpin *et al.,* 2002). The global average concentration of EE2 in surface waters is 0.043 µg/L with a maximum of 5.9 µg/L (Aus der Beek *et al.,* 2016). Pharmaceuticals have also been detected in sea water/sediment, ground water and drinking water (Togola and Budzinski, 2008) (reviewed: Aus der Beek *et al.,* 2016; Fabbri and Franzellitti, 2016).

The environmental concentrations of pharmaceuticals are influenced by degradation rates in the wastewater system and receiving waters, and how the compound partitions into the water column. Many compounds are readily degraded through abiotic/biotic transformation, or physical sequestration (Bendz *et al.,* 2005; Mathon *et al.,* 2016). However, their presence in the environment are usually a result of their rates of release being greater than their rates of degradation (Bendz *et al.,* 2005). Because of this, pharmaceuticals are often considered to be pseudo-persistent. However, not all natural degradation makes compounds less harmful. As some drugs such as EE2 pass through the body they will be metabolized into inactive conjugates (glucuronides and sulfates) prior to excretion (Tyler *et al.,* 1998). It is thought that the presence of the unconjugated EE2 in rivers and effluents is a result of deconjugation by bacteria such as *Escherichia coli* in WWTPs, which transforms the conjugated EE2 back to its original active form (Gomes *et al.,* 2009). Biotransformation of metabolites back to parent compounds may also explain why some WWTP's have negative elimination rates.

Despite the concentrations of pharmaceuticals in the environment being generally quite low (µg/L to ng/L) their continuous input into the environment can lead to chronic exposure, which has the potential to cause adverse effects on non-target organisms. One of the major conclusions of the review by Aus der Beers *et al.,* (2016) is that the more often pharmaceuticals are measured for in the environment, the more often they are detected. Thus, it is entirely possible that the reason certain pharmaceuticals are commonly detected is the result of frequently testing for them; conversely, it is entirely possible that some compounds may be commonly present in the environment but simply not tested for. This is very challenging for regulatory agencies as it is not feasible to test for the presence of all

pharmaceutical compounds, in all environments. What is clear is that most pharmaceuticals consumed will find their way into the environment at low concentrations. It is thus important to develop prioritization strategies to determine which if any, are a health concern for non-target organisms.

1.3 Effects of pharmaceuticals in fish

Many different types of pharmaceuticals are found in the environment including antibiotics, analgesics, blood lipid regulators, beta-blockers, selective serotonin reuptake inhibitors, and natural/synthetic hormones. Most pharmaceuticals are non-polar which allows them to easily cross biological membranes through diffusion in order to elicit their effects (Khetan and Collins, 2007). The majority of drug targets are proteins including, enzymes, receptors, carrier molecules, and ion channels; it is the diversity and complexity of these structures that allows pharmaceutical specificity (Williams and Cook, 2007). Their effects on non-target organisms can vary a great deal based on the mode of action of the drug. Since protein drug targets are relatively well conserved across vertebrates, it is likely that drugs would target similar systems in fish (Brown *et al.,* 2014). The conservation of drug targets in fish is further described in section 1.4. As analgesics and natural/synthetic hormones are found in the environment the most frequently (Aus der Beek et al., 2016), and have the most potential for causing effects, they will be the focus of the following section.

1.3.1 Analgesics

Analgesics or painkillers are a group of drugs used to treat or mitigate pain. One group of analgesics are nonsteroidal anti-inflammatory drugs (NSAIDs). There are many types of NSAIDs including diclofenac, naproxen, ibuprofen, ketoprofen, and indomethacin. Due to their prevalent use, NSAIDs are often detected in both WWTP effluent and surface waters at higher concentrations (usually in the µg/L range) when compared to other human pharmaceuticals (Aus der Beek *et al.,* 2016; Corcoran *et al.,* 2010; Fent, 2006; Helm *et al.,* 2012; Kolpin *et al.,* 2002; Stülten *et al.,* 2008; Ying *et al.,* 2009). Of all the NSAIDS diclofenac, ibuprofen, and naproxen are most often detected globally; with diclofenac being the most frequently detected pharmaceutical compound, and all three occupying the top 5 pharmaceutical compounds most frequently detected in the environment (Aus der Beek *et al.,* 2016).

In order to reduce inflammation and pain, NSAIDs function by inhibiting one or both of the two isoforms of the cyclooxygenase enzymes (COX-1 and COX-2), which are enzymes that catalyze the synthesis of prostaglandins from arachidonic acid, and are generally conserved (67%) across vertebrate species (Grosser *et al.,* 2002). Prostaglandins are involved in a large range of physiological processes including thermoregulation, water balance, glomerular filtration, homeostasis, and the control of ovulation (Fujimori *et al.,* 2011; Sugimoto and Narumiya, 2007; Takahashi *et al.,* 2013). Generally, COX-1 is constitutive and involved in the production of prostaglandins that are required for normal physiological function, such as protecting the stomach and kidney from damage (by promoting mucosal lining for example), whereas COX-2 is induced by inflammatory stimuli like cytokines or proteases and produces prostaglandins that cause undesirable side

effects such as pain and inflammation (Brideau *et al.,* 1996). Most NSAIDS are fairly non-specific in their binding to COX-1/COX-2, and their reported binding affinity varies greatly in the literature: i.e. the COX-2/COX-1 ratio of naproxen ranges from 0.5 to 100 between different studies (Vane and Botting, 1998). As NSAIDs are often used to treat inflammation, it is thought that those that favour the receptor of COX-2 over COX-1 will have reduced side effects in humans (such as irritation of the stomach lining, and toxic actions on the kidney) (Vane and Botting, 1998). This assumption, while relevant to therapeutic use in humans, is not always applicable when considering adverse effects in wildlife; i.e. an NSAID with preference for COX-2 inhibition may be more likely to cause reproductive effects, as prostaglandin E2 which is involved in ovulation, is thought to be regulated by COX-2 in fish (Fujimori *et al.,* 2011).

These COX enzymes are generally conserved across vertebrate species and have been identified in rainbow trout (*Oncorhynchus mykiss*) (Ishikawa and Herschman, 2007), Japanese medaka (*Oryzias latipes*) (Fujimori *et al.,* 2011), zebrafish (*Danio rerio*) (Lister and Van Der Kraak, 2008), and goldfish (*Carassius auratus*) (Zou *et al.,* 1999). While mammals have the two COX enzymes (COX-1/COX-2), teleosts have additional copies of COX-1 and/or COX-2 (fish may have two copies of genes, usually a result of gene duplication): for example the medaka genome contains two COX-1 genes (*ptgs1a* and *ptgs1b*) and one COX-2 gene (*ptgs2*) (Fujimori *et al.,* 2011). Aside from the functions mentioned previously, prostaglandins have also been shown in medaka and zebrafish to be involved with the function of reproductive organs such as controlling ovulation (Fujimori *et al.,* 2011; Lister and Van Der Kraak, 2008). Cultured zebrafish follicles are able to produce prostaglandin E2 in response to arachidonic acid, and arachidonic acid stimulates

the *in vitro* production of 17β-estradiol (Lister and Van Der Kraak, 2008). It has been determined that in medaka, much like in mammalian species, COX-2 is responsible for the generation of prostaglandin E2 in the pre-ovulatory follicles (Fujimori *et al.,* 2011). Thus, NSAIDs have the potential to impair reproduction by disrupting the production of prostaglandin E2 through inhibition of COX enzymes, such as COX-2 in medaka.

Despite their high measured environmental concentrations and potential for effects, there are relatively few studies determining the long-term effects of NSAIDs in fish. Exposure of medaka to ibuprofen did not affect total embryo production; however concentrations as low as 10 µg/L resulted in a decrease in frequency of spawning and an increase in the number of eggs per spawning event (Flippin *et al.,* 2007; Han *et al.,* 2010). A significant delay in time-to-hatch in embryos exposed to concentrations as low as 0.1 µg/L was also observed (Han *et al.,* 2010). Zebrafish exposed for 7 days to 21-506 µg/L ibuprofen had a reduction in prostaglandin E2, but no effect on egg production (Morthorst *et al.,* 2013). Another study of rainbow trout exposed to diclofenac found significantly reduced expression levels of COX-1 and COX-2 in liver, gills and kidney at 1 µg/L (Mehinto *et al.,* 2010). Furthermore, diclofenac exposure induced tubular necrosis in the kidney at 1 µg/L, indicating that diclofenac can cause alterations of biological function and tissue damage at low concentrations in fish (Mehinto *et al.,* 2010). Diclofenac at high concentrations (10 mg/L) significantly reduced fertility and hatchability of embryos produced by second-generation exposed medaka (Lee *et al.,* 2011). Medaka fed a diet containing either COX-1 or COX-2 inhibitors was demonstrated to alter reproductive parameters such as a decrease in the number of spawning females, lower sperm motility, and a decrease in embryo survival (Kowalska *et al.,* 2011).

In some rare cases, drugs may have an unpredictably severe effect on non-target organisms, due to the drug having an unexpected physiological response, or a species being much more sensitive to the effects of a drug compared to humans. An example of this is diclofenac, which has a severe effect on kidney function in some species of Asian vultures (Oaks *et al.,* 2004). This has led to a significant population decline (>95%) and even localized extinctions (Oaks *et al.,* 2004).

In summary, analgesics are pharmaceutical compounds commonly detected in the environment, are generally found at higher environmental concentrations relative to other pharmaceuticals, have the potential to cause chronic long-term toxicity in fish, and merit further study.

1.3.2 Natural and synthetic hormones

The endocrine system refers to a collection of glands of an organism that secretes hormones directly into the circulatory system and these hormones are carried toward target organs. The endocrine system of fish and mammals involves complex interactions between external stimuli, hypothalamic and pituitary hormones, thyroid hormones, gonadal hormones, and deactivation of hormones by the liver (Kime, 1999). Reproductive steroids are an important component of the endocrine system. They play a role in sexual differentiation, behaviour, and the development of secondary sex characteristics and gonads in fish (Kime, 1999, 1993). All of these factors are important to the reproductive success of a species. Thus, there is concern regarding the effects of natural and synthetic hormones in aquatic ecosystems, as disruption to reproductive systems may lead to deleterious population reproductive effects. There are many different classes of endocrine

active pharmaceuticals including estrogens, progestins, androgens, steroid receptor antagonists, aromatase inhibitors and others. This section will focus on two main classes: estrogens and progestins with particular focus on the effects of 17α-ethinylestradiol (EE2) and levonorgestrel.

1.3.2a Estrogens

Estrogenic compounds are commonly used in contraceptives and hormone therapy. Estrogens are naturally produced, such as 17β-estradiol, estrone, estriol, or are synthetic, such as EE2. Around 10-100 µg estrone, 17β-estradiol, and EE2 are commonly excreted daily by women taking the oral contraceptive pill (Baronti *et al.,* 2000). Whereas, estriol is commonly produced and excreted during pregnancy (Baronti *et al.,* 2000). All four of these estrogenic compounds are commonly detected in surface waters around the globe, at concentrations usually around >0.5-40 ng/L (Aus der Beek *et al.,* 2016; Baronti *et al.,* 2000; Nikolaou *et al.,* 2007; Vulliet *et al.,* 2011).

17β-Estradiol is the major reproductive estrogen in female teleosts (Arukwe and Goksøyr, 2003). Egg production in teleost fish is induced by species specific patterns of external cues such as photoperiod or temperature (Figure 1.2), which act via the hypothalamus to release gonadotropin releasing hormone, which induces the release of gonadotropin (GtH) from the pituitary (Kime, 1999). Fish usually produce two gonadotropins (GtH-I and GtH-II, or FSHβ and LHβ), which are analogous to human follicle-stimulating hormone (FSH) and luteinizing hormone (LSH), both of which stimulate the development of the gonad and the production of steroid hormones in fish (Yan *et al.,* 1992). These GtHs target G-protein-coupled receptors within the gonads,

which regulate gonadal steroidogenesis (Levavi-Sivan *et al.,* 2010; Villeneuve *et al.,* 2007). For normal reproduction in fish, 17β-estradiol acts along the hypothalamus-pituitary-gonadal (HPG) axis, influencing behavior, gonad differentiation, and the production of vitellogenin and *zona radiata* proteins in the liver (Arukwe and Goksøyr, 2003; Kime, 1999). A key difference between fish and mammals is that in fish an important target of 17β-estradiol is the liver. Excess 17β-estradiol can also activate a negative feedback loop suppressing the release of gonadotropin releasing hormone from the hypothalamus (Kime, 1999; Levavi-Sivan *et al.,* 2010).

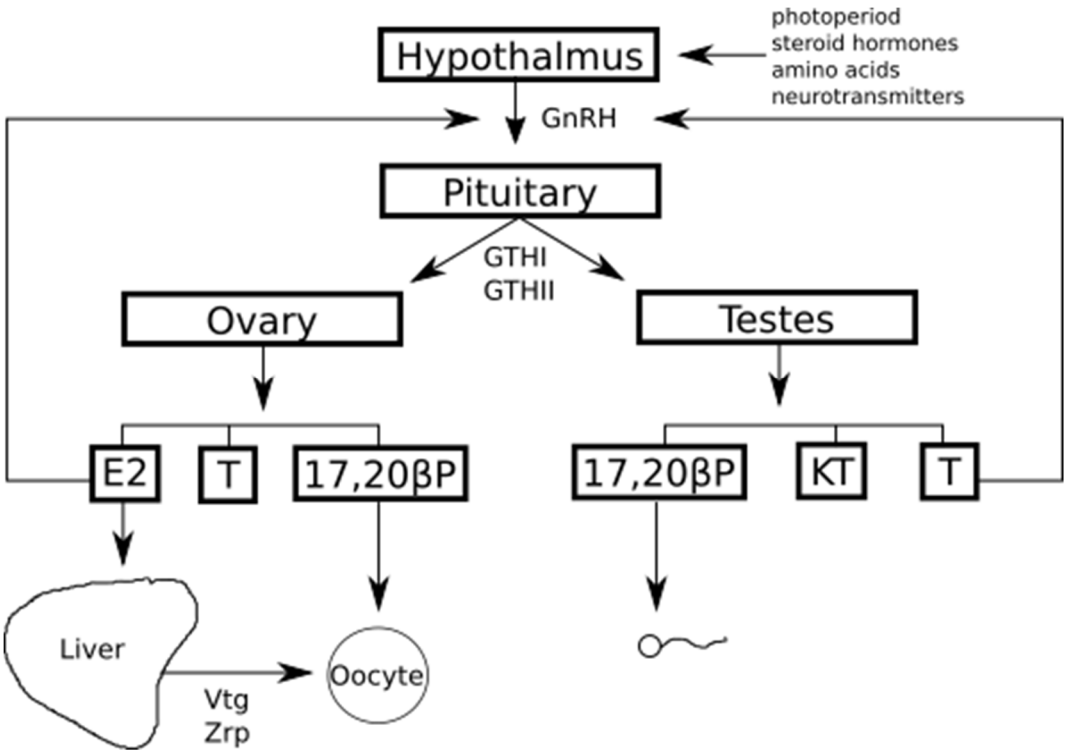


Figure 1.2: The reproductive system of teleost fish. External stimuli such as photoperiod etc. will signal the hypothalamus to release gonadotropin releasing hormone (GnRH) which will travel to the pituitary, which induces the release of gonadotropin (GtH) from the pituitary, which will stimulate the development of the gonads and the production of hormones in fish. If these hormones are produced in excess, they are able to activate a negative feedback loop suppressing the release of GnRH from the hypothalamus. 17β-Estradiol (E2), testosterone (T), 17 α,20β-dihydroxy-4-prenen-3-one (17,20βP), 11-ketotestosterone (KT), vitellogenin (Vtg), zona radiata proteins (Zrp), modified from Kime, 1999.

In humans, the main mode of action of EE2, the synthetic estrogen used in oral contraceptive pills, is by activating the negative feedback loop along the HPG axis. EE2 is thought to act in a similar manner in teleosts as estrogen receptors are well conserved among vertebrates; teleost fish have well conserved ligand binding domains (60-75% conserved in medaka) (Brown *et al.,* 2014). Synthetic pharmaceuticals can be extremely potent chemicals, and in some cases can be more potent than 17β-estradiol (Tyler *et al.,* 1998). For example, EE2 is more potent than 17β-estradiol for inducing vitellogenin in juvenile rainbow trout. Median effective concentrations for induction of vitellogenin expression following 14 days exposure in juvenile rainbow trout were found to be between 19 and 26 ng/L for 17β-estradiol, and 0.95-1.8 ng/L for EE2 (Thorpe *et al.,* 2003). Sex steroids play a critical role in the assignment of gonadal gender determination and differentiation (Devlin and Nagahama, 2002). If steroid-synthesizing capability is interfered with, sex determination can be disrupted. This has been demonstrated through the inhibition of estrogen synthesis using inhibitors of the enzyme aromatase during early development, which caused masculinization of rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis niloticus*) (Guiguen *et al.,* 1999).

Concentrations of the synthetic oestrogen EE2 as low as 5 ng/L have been shown to cause reproductive failure in zebrafish (*Danio rerio)* (Nash *et al.,* 2004). Exposure to 4 ng/L EE2 for two years resulted in complete feminization of a population of wild roach (*Rutilus rutilus*) (Lange *et al.,* 2009, 2008). Exposure of wild fathead minnows (*Pimephales promelas*) to 5-6 ng/L EE2, in the Experimental Lakes Area in northwestern Ontario, Canada, caused population collapse, due to feminization of males and altered oogenesis in females (Kidd *et al.,* 2007). EE2 has also been shown to have the potential

to bioaccumulate in fish (Gibson *et al.,* 2005). Exposure of fish to estrone and 17β-estradiol generally cause effects at higher concentrations when compared to EE2. Java medaka (*Oryzias javanicus*) exposed to estrone had reduced fertility, hatching rate, number of eggs per female per day, and an increase in time-to-hatch. These effects were generally observed at higher exposure concentrations, 484-1188 ng/L (Imai *et al.,* 2007). 17β-estradiol has been shown to also affect Java medaka by significantly lowering fertility at 16 ng/L and reducing growth at 9.5 ng/L (Imai *et al.,* 2005). The majority of studies on estrogenic compounds have focused either on acute toxicity, or on chronic reproductive effects. There is less knowledge about chronic effects that are not related to fecundity. Of all the pharmaceuticals studied, EE2 has the largest impact on fish, and is the only pharmaceutical to be directly linked to impacts on fish populations.

1.3.2b Progestins

Much like estrogens, there are both natural and synthetic progestins. Natural progestins include progesterone and 17α, 20β-dihydroxy-4-pregnen-3-one (17,20βP). Synthetic progestins such as levonorgestrel are commonly used in oral contraceptives and hormone replacement therapy. These compounds have all been detected in WWTP effluent, run off from animal agriculture facilities, and in surface waters in concentrations ranging from < 1 to 375 ng/L (Bartelt-Hunt *et al.,* 2012; Dequattro *et al.,* 2012; Orlando and Ellestad, 2014; Vulliet *et al.,* 2011).

One interesting difference between fish and mammals is that the mammalian pregnancy hormone, progesterone, is not considered to be a biologically active steroid in fish, and plasma concentrations in fish are usually below detection limits (Dequattro *et al.,*

2012; Kime, 1993). However, it is still an intermediate in the biosynthesis of steroids in fish including testosterone, 11-ketotestosterone, cortisol, 17β-estradiol, and 17,20βP (Nagahama, 1994). The natural progestin 17,20βP is important for oocyte maturation and ovulation in female fish, and may be related to spermatogenesis in male fish (Kime, 1993). Furthermore, the progesterone receptor in fish seem to have a different binding affinity for progestogens than those in other vertebrates; it has a much higher binding affinity for 17,20βP than progesterone for example (Pinter and Thomas, 1997; Thomas *et al.,* 2002).

Synthetic progestins are derived from three different parent compounds: 19-norprogesterone, 19-nortestosterone, and spirolactone (Schindler *et al.,* 2003). Synthetic progestins mainly function by suppressing the mid-cycle peaks of LSH and FSH, leading to an inhibition of ovulation, through negative feedback loops on the HPG axis (Erkkola and Landgren, 2005). Synthetic progestins have also been shown to have a binding affinity for the fish progestogen receptor (Pinter and Thomas, 1997). Progestins are often classified based on which parent compound they are synthesized from (Sitruk-Ware *et al.,* 2013). While there are many different types of progestins commercially available, this section will focus on those measured in the environment, with a focus on levonorgestrel.

Progesterone is usually measured in the environment in higher concentrations than the synthetic derivatives (Bartelt-Hunt *et al.,* 2012; Dequattro *et al.,* 2012; Orlando and Ellestad, 2014). Studies have measured that progesterone and its dirivatives can impact fish fecundity. Fathead minnows exposed to progesterone for 21 days experienced a decrease in fecundity at 100 ng/L (Dequattro *et al.,* 2012). However, fish seem more sensitive to synthetic progesterone. Japanese medaka and fathead minnow exposed to 22 and 1.2 ng/L

(respectively) of the oral contraceptive norethindrone for 28 days experienced significant decreases in egg production (Paulos *et al.,* 2010).

Runnalls *et al.* (2013) investigated the effects of levonorgestrel, gestodene, desogestrel, and drospirenone on adult fathead minnow; after a 21 day exposure to 100 ng/L of each progestin, significant reductions in egg production were observed following exposure to levonorgestrel and gestodene, but no effects were observed following desogestrel and drospirenone exposure (Runnalls *et al.,* 2013). They also found that exposure to levonorgestrel and gestodene significantly reduced plasma testosterone and 17β-estradiol levels in females, and that exposure to gestodene significantly increased testosterone and 11-ketotestosterone in males (Runnalls *et al.,* 2013). A follow up study showed that gestodene exposure significantly reduced the number of eggs produced by female fathead minnows at both 1 ng/L and 100 ng/L, but not at 10 ng/L (Runnalls *et al.,* 2013). The same study tested desogestrel, which was found to be much less potent and only caused effects at 10,000 ng/L (Runnalls *et al.,* 2013)

Levonorgestrel is of particular interest, as it bioaccumulates in juvenile rainbow trout. Juvenile trout exposed for 14 days to three different wastewater treatment plant effluents, significantly bioaccumulated levonorgestrel, with plasma concentrations ranging from 8.5-12 ng/mL (four times higher than human therapeutic levels) (Fick *et al.,* 2010). These plasma concentrations resulted from exposure to effluents containing only 61 ng/L levonorgestrel, thus demonstrating a bioconcentration factor of 8500-12000x (Fick *et al.,* 2010).

Exposure to fathead minnows to 0.8 ng/L levonorgestrel has been shown to significantly reduce egg production (Zeilinger *et al.,* 2009). Females exposed to

levonorgestrel also developed mature oocytes, whereas effects on the males included enlarged testes and advanced spermatogenesis relative to controls (Zeilinger *et al.,* 2009). Zebrafish embryos exposed to 2-200 ng/L levonorgestrel for up to 144 h exhibited altered expression levels for gene targets, including steroid hormone receptors such as the androgen receptor, as well as other genes like *vtg1* (Zucchi *et al.,* 2012). Progestins have the potential to affect fish, and like estrogens, most of their observed effects are on reproduction.

Both NSAIDs and natural/synthetic hormones compounds have the potential to cause effects at, or close to, their measured environmental concentrations in fish. Both of these classes of compounds have the potential to cause endocrine disruption due to their potential for reproductive side effects. However, these compounds and their effects were studied individually and over relatively short-term exposures (days/weeks). In the wild, mixtures of these compounds are constantly released into the environment. Despite being broken down naturally, their constant loading can cause pseudo-persistence, when combined with their ability to bioaccumulate, results in a higher likelihood for adverse effects in wildlife. Consequently, exposures of fish to pharmaceutical compounds are likely to be chronic in nature, to complex mixtures of compounds, and over multiple generations. Due to the large number of pharmaceuticals in use and in development, there is a need to develop ways to prioritize the risk of these compounds to non-target organisms, and to study their effects in mixtures.

1.4 Teleost fish as research organisms

Fish are considered to be the most diverse group of vertebrates, making up more than 40% of vertebrate species (Lagler *et al.,* 1962). Some species of fish are used as models to determining the effects of environmental pollutants, which can then be extrapolated to other species of fish, and fish are often considered to be the species which are most at risk to water pollution (Kime, 1999). As it is impossible to test every species found in the wild, scientists often use model species to extrapolate pollutant effects in other species of fish for risk assessment (Brown *et al.,* 2014).

Fish species belong to three super classes: Agnatha, jawless fish such as lampreys and hagfish; Chondrichthyes, cartilaginous fish such as sharks and rays; and Osteichthyes, bony fish. Osteichthyes are divided into two classes Sarcopterygii which contains lungfishes, and Actinopterygii or ray finned fish. The Actinopterygii class represents the majority of fish species, and Teleostei are the largest infraclass of Actinopterygii, making up 96% of all fish. Thus, it is not surprising that most fish used as model species for aquatic toxicology research are teleosts, as they represent the majority of fish species and some common examples include: zebrafish, medaka, rainbow trout, and fathead minnows (*Pimephales promelas*).

The response of fish to pharmaceutical exposures will depend on the level of conservation of the designated drug targets that cause the intended response in humans/animals. Generally, fish with well-conserved drug targets will be associated with an increased risk of adverse effects due to pharmaceutical pollution. Brown *et al.* (2014) reviewed the relative conservation of 459 human drug targets in 10 species of Actinopterygii. They determined that these ten species had very well conserved (81-86%)

human drug targets (Brown *et al.,* 2014). This study also determined that in the fish tested six mammalian nuclear steroid receptors were well conserved, with strongest conservation in the ligand binding domain (Brown *et al.,* 2014). Another study determined zebrafish had orthologues to 86% of the 1318 human drug targets tested, while only 61% were conserved in *Daphnia* and 35% in green algae (Gunnarsson *et al.,* 2008). Since fish tend to have the most conservation of drug targets of aquatic species, they are the most likely aquatic organisms to be affected by pharmaceutical pollution. While the majority of drug targets are conserved in fish, there are still some evolutionary divergences between species, physiology and behavior that make it challenging to predict individual and population level effects from a single model species. Therefore, scientists often apply a safety factor of 10 to measured effective concentrations (Brown *et al.,* 2014).

1.5 Fish early life stage testing

There are > 5000 human and veterinary pharmaceuticals in use or in production. Testing each pharmaceutical for adverse outcome effects in non-target organisms is not practical. An optimal study would involve testing the effects of pharmaceutical exposure on whole life cycles/preferably multiple life cycles. These are costly, require a large number of animals, and for aquatic toxicology, generate a large volume of contaminated water. Due to prohibitive costs, fish early life stage (FELS) tests were developed.

FELS tests were first introduced in the late 1970's as an alternative to long-term fish life cycle tests (McKim, 1977). Currently, the FELS test is the most frequently used bioassay for predicting chronic fish toxicity, and the most common freshwater species used include: rainbow trout, fathead minnow and medaka (Volz *et al.,* 2011). FELS testing is

advantageous because tests are shorter than a life cycle (usually 1-3 months), but they are still costly, and use a large number of fish (requiring a minimum of 180 fish, not including the stock fish used to breed up the fish used in the test) (Villeneuve *et al.,* 2014). FELS are usually focused on survival and growth, supplemented with behavioural and developmental abnormalities; they are not designed to provide information about the chemical mode of action. Thus, the development of alternative tests that have a reduced cost, reduced number of animals used, and that focus on the chemical mode of action are needed.

1.6 Tiered testing strategies

One way that scientists are moving toward the prioritization of chemicals is by developing tiered testing strategies. This could accomplished using short, inexpensive bioassays to help prioritize what compounds to utilize the FELS test. Volz *et al.* 2011 propose a three-tiered testing strategy (Figure 1.3). Tier 1 screening involves high throughput in vitro screening, which is generally limited to specific pathways or modes of action (Volz *et al.,* 2011). Tier 2 screening is considered medium-to-high-throughput in vivo screening, which can involve most non-protected lifestages including embryos, or the time-period immediately post-hatch until feeding (Volz *et al.,* 2011). Finally tier 3 screening would involve low-throughput FELS testing. While it is possible to use *in vitro* assays to help prioritizing which chemicals to test with tier 2 screening, the main challenge with tier 1 screening is that *in vitro* assays test for very specific effects. They are much more useful when screening compounds with a known mode of action.

In contrast with cell based assays, fish embryos provide the complexity and interactions of an intact organism, which enables the evaluation of chemically induced effects on multiple target organs. Fish embryo toxicity testing allows for potential adverse outcome pathway development, and the establishment of tier 1 screening methods/endpoints. Studies that characterize common chemically mediated mechanisms of toxicity for use in predicting adverse effects that relate to FELS toxicity are needed (Volz *et al.,* 2011). Thus, the development of adverse outcome pathways (AOPs) related to FELS testing is a critical step in the development of tiered testing approaches and fish embryos can play a role in this development.

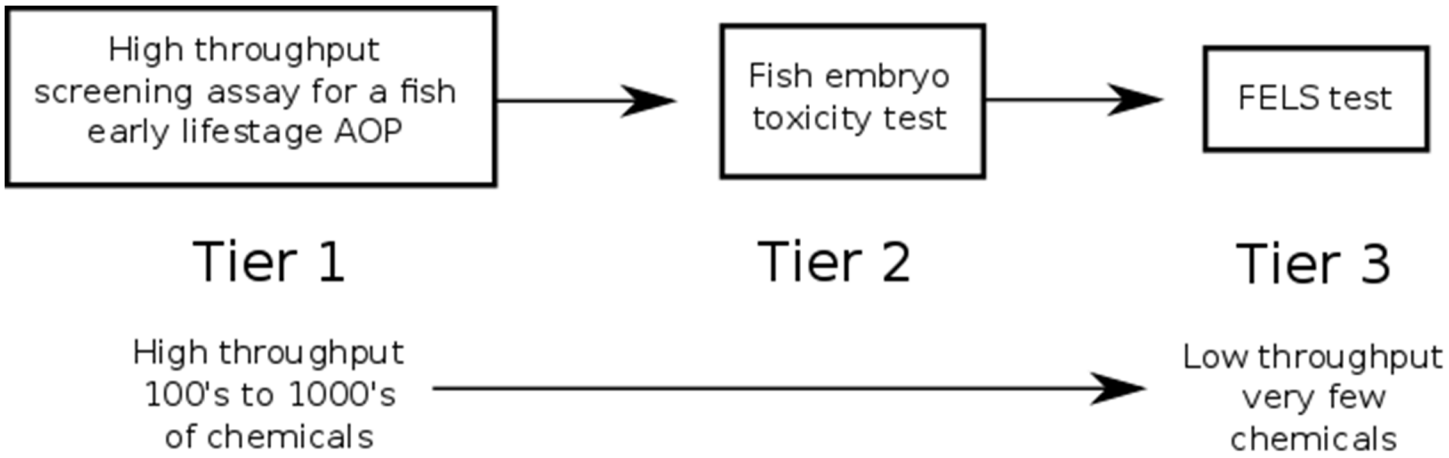


Figure 1.3: Three tiered testing strategy, proposed by Volz *et al.* 2011, for prioritizing chemicals to then be screened using FELS toxicity testing.

1.7 Adverse outcome pathways

An adverse outcome pathway (AOP) is a framework that links a direct molecular initiating event and an adverse outcome at a biological level of organization relevant to risk assessment (Ankley *et al.,* 2010). Each AOP begins with a molecular initiating event in which a chemical interacts with a biological target (such as a receptor etc.) which will lead to a sequential series of effects, ultimately resulting in a population level response (Figure 1.4) (Ankley *et al.,* 2010).

There are three major organism level effects that will have long-term effects on a population: lethality, impaired growth, and impaired reproduction. AOPs are a type of screening mechanism, where instead of utilizing whole organism tests, molecular effects are investigated that will lead to known organism level effects, and thus impact populations. AOP development is still a new field of toxicology, but there is an online database/wiki where scientists are currently collaborating on developing them. The development of AOPs will hopefully allow the eventual development of high-throughput predictive assays, and testing strategies, highly relevant for use in risk assessments (Villeneuve *et al.,* 2014).

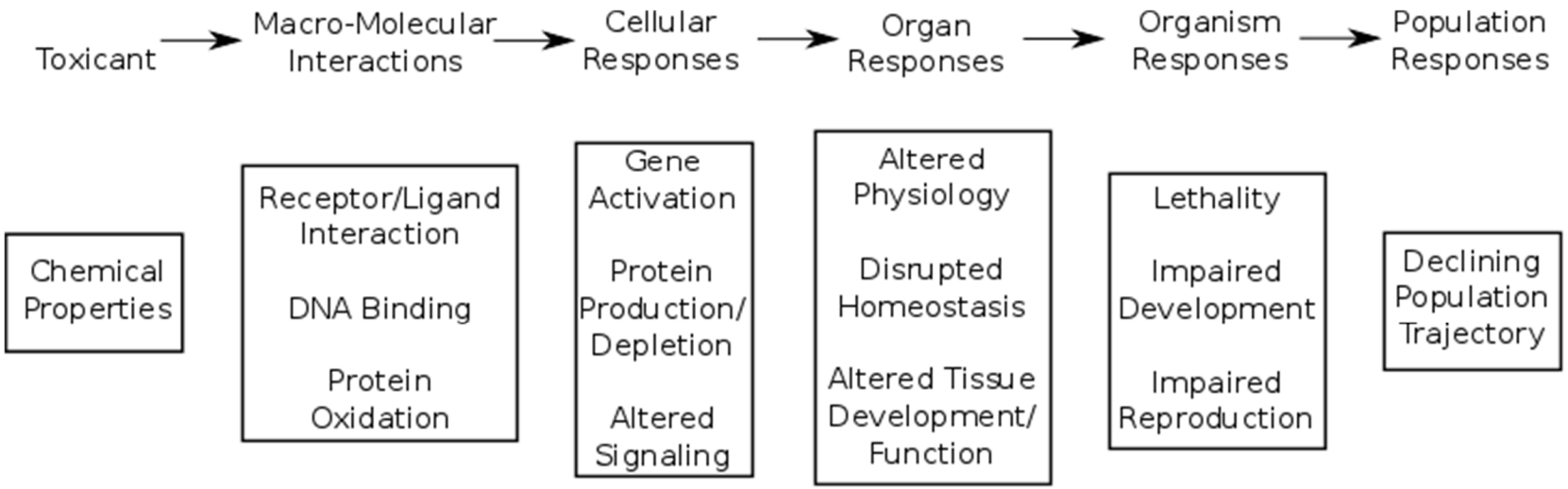


Figure 1.4: Diagram depicting the sequential events of an adverse outcome pathway (AOP), proposed by Ankley *et al.* 2010. Each AOP begins with a macro-molecular interaction such as a drug binding to a receptor, this will then lead to a cascade of responses that will end in a population level effect.

1.8 Japanese medaka as a model organism

The medaka (*Oryzias latipes*) is a small, egg-laying, freshwater, bony fish that is native primarily to Japan, Korea, and China (Shima and Mitani, 2004). At sexual maturity their body length is about 2.5 to 3 cm, which is usually reached within 2 months post-hatch (Shima and Mitani, 2004). The medaka has been a widely used vertebrate species for experimental biology due to its many ideal features for laboratory use: small size, external sexual dimorphism, large and clear embryos, longer development time (8-10 days compared to 3 days for zebrafish), ease to maintain, wide availability and strains, and the adult fish spawn daily (Parenti, 2008). There are many different strains of Japanese medaka: the female leucophore-free (FLFII) strain was used in this study, and the strain was obtained from the National Institute for Basic Biology (Okazaki, Japan). The FLFII strain introduced by Wakamatsu *et al.,* (2003) has three observable sexual dimorphisms: two markers for body color and a male/female specific DNA marker (*SL1*). Consequently, the FLFII strain has several advantages. Females do not produce the leucophore pigment cells while the males are wild type for leucophores; this allows gender identification as early as 3 days post fertilization. Juveniles can be identified by the presence of orange-red xanthopores which are present in the males while absent in females. Finally, definitive gender confirmation can be determined via polymerase chain reaction (PCR), using the *SL1* marker.

Early dimorphism is novel since in normal populations of fish, sexual identification is generally only possible after maturation which requires months/years. The PCR marker is particularly useful for identification of intersex individuals, which normally are difficult to identify; for example, distinguishing if an individual fish is a partially masculinized

female or a partially feminized male (Balch *et al.,* 2004). Furthermore, Balch *et al.* (2004) determined there is no significant difference in sensitivity to estradiol between the FLFII strain and wild type medaka (measured by feminization of males to the female gonadal phenotype, confirmed by genotype analysis).

Medaka are very valuable for studying embryo development. The developmental biology of medaka embryos is well studied and a detailed staging of normal development was published by Iwamatsu (2004). The embryos of medaka are clear which allows monitoring as they develop, permitting both the determination of gender and the ability to see developmental abnormalities. Medaka embryos normally hatch within 8-10 days of fertilization which also allows a longer window of development when compared to an alternate model species like the zebrafish, which generally hatch in 96 hours (Braunbeck and Lammer, 2006). A previous embryo-toxicity study comparing three different species of fish concluded that medaka was the most sensitive to exposure to pulp mill extracts, measured by effects on mortality, hatchability, time to hatch, malformations, and sex-ratio to female (Orrego *et al.,* 2011). As the medaka genome is completely sequenced, they also make good candidates for genetics, experimental embryology and molecular biology investigations (Furutani-Seiki and Wittbrodt, 2004). Additional favorable characteristics of medaka include daily spawning, as well as their short time of maturation (allows for multigenerational exposures). This species is thus an excellent model species when studying embryo development and compounds that may disrupt the endocrine system.

1.9 Teleost swim bladder overview

Teleost fish have been the focal point of most research on fish as ~96% of modern fishes belong to this class (Lagler *et al.,* 1962). These fish are made up of a variety of tissues including muscle, cartilage, and bone which are much denser than the aqueous environment (Lindsey *et al.,* 2010). As a result, without some form of compensation method they would sink. Many teleost fish have a swim bladder which is a gas-filled sac positioned at the dorso-anterior part of the body cavity (Alexander, 1966; Finney *et al.,* 2006). The swim bladder is a structure that maintains buoyancy in the fish allowing the fish to hold its vertical position in the water column with a minimal amount of energy expenditure (Alexander, 1966; Georgijevic *et al.,* 2007). Fish can maintain neutral buoyancy as they move through the water column by regulating the volume of gas in their swim bladder (Finney *et al.,* 2006). Whereas, a fish without a swim bladder would need to constantly swim in an upward direction to counter their negative buoyancy, expending much more energy (Czesny *et al.,* 2011).

In teleost fish the swim bladder develops from the anterior gut endoderm (Field *et al.,* 2003; Winata *et al.,* 2009). Organogenesis of the swim bladder will begin by the evagination of the anlagen connected to the digestive tract via the pneumatic duct (Woolley and Qin, 2010). The swim bladder will then develop over three phases: budding, formation of the three swim bladder layers (epithelial, mesenchyme, and outer mesothelium) and growth, and finally inflation (Winata *et al.,* 2009). The initial inflation of the swim bladder commonly occurs when the larvae gulps air at the water surface (Woolley and Qin, 2010). The connection via the pneumatic duct is crucial for this inflation process and occurs during a finite time period in the larval stage just after hatching (Woolley and Qin, 2010).

Soft rayed fish (Malacopterygii) are predominantly physostomous (fish with a connection between the swim bladder and the pneumatic duct) and spiny rayed fishes (Actinopterygii) are predominantly physoclistous (fish with a closed swim bladder) (Lagler *et al.,* 1962). Teleost fish display both types of swim bladder morphologies, for example Japanese medaka are physoclistous and zebrafish are physostomous (Egloff, 1996; Woolley and Qin, 2010). Physostomous fish have an 'open' swim bladder in that they maintain their embryonic connection between the gut and the gas bladder via a pneumatic duct that connects the swim bladder to the digestive track (Lagler *et al.,* 1962; Woolley and Qin, 2010). In adult physoclistous fish, this connection degenerates in resulting in a 'closed' swim bladder (Jones and Marshall, 1952). It is common in physoclistous fish to still have the connection to the pneumatic duct during their larval life, and such fish must gulp air for the first filling of their swim bladder (Bailey and Doroshov, 1995; Lagler *et al.,* 1962). In these physoclistous larval fish, the pneumatic duct only exists for a short period of time before it regresses and inflation must occur during this critical period of time (Czesny *et al.,* 2011).

Once the initial inflation has occurred, physoclistous fish can regulate the volume of their swim bladder by the secretion and absorption of gasses from the blood into the swim bladder through highly vascular regions on the wall of the swim bladder (Lagler *et al.,* 1962). Parts of the swim bladder wall along with specific blood vessels make up the two parts of this gas secreting complex: the gas gland and the rete mirabile (Lagler *et al.,* 1962). The gas gland is a patch of glandular epithelium supplied with blood from the coeliac artery and drained by the hepatic portal vein (Alexander, 1966). The rete mirabile is composed of many capillaries and venules placed perpendicularly to the swim bladder

wall that functions as a countercurrent exchange system bringing blood to the gas gland (Alexander, 1966). The rete mirabile essentially allows gasses from the blood to be condensed on one side of the organ, in this case the gas glands.

The swim bladder is regulated by populations of autonomic neurons which govern gas secretion and reabsorption (Finney *et al.,* 2006). As a general rule the anterior part of the swim bladder is specialized for gas secretion and the posterior half is specialized for diffusion (Jones and Marshall, 1952). Gas resorption usually occurs across the oval, a thinned region of the swim bladder where a complex of capillaries is only separated from the swim bladder lumen by a one-cell layer of epithelium, located on the dorso-posterior wall of the bladder (Lagler *et al.,* 1962). This area of blood vessels, which come from the intercostal arteries and drain into the cardinal veins is called the oval organ because it is usually surrounded with a circular sphincter muscle that controls the rate of gas resorption by expanding or contracting (Lagler *et al.,* 1962). Relaxing the sphincter muscle and contracting a set of longitudinal muscles will expose the oval, allowing gas to diffuse from the swim bladder into the blood (Jones and Marshall, 1952).

While having both functional and anatomical differences, the teleost swim bladder shares a common evolutionary history to the tetrapod lung (Perry *et al.,* 2001). Even though it is a much more complicated structure than the swim bladder, the molecular mechanisms regulating lung development have been extensively studied and are better understood (Reviewed by: Cardoso and Lü 2006). In contrast, there is much less known about the molecular events and mechanisms regulating swim bladder development in fish (Winata *et al.,* 2009). The teleost swim bladder, much like the mammalian lung, arises from an outgrowth of the foregut endoderm in close proximity to the liver and pancreas

(Field *et al.,* 2003). The pattern of smooth muscle development of the gut and swim bladder are closely linked, with their molecular markers being expressed in a similar order but temporally delayed in the swim bladder (Georgijevic *et al.,* 2007). Like the gut, the swim bladder epithelium is surrounded by a smooth muscle layer, which is used to regulate the amount of air inside (Finney *et al.,* 2006).

Embryonic development is characterized by specific regulation of cellular behaviours so that cells form tissues at the correct time and place. Cell signaling pathways such as Fgf, Hedgehog, Wnt, TGFβ, Notch etc., operate during embryo development to help control and specify cell growth and proliferation. The Wnt signaling pathway is critical in mammalian lung development (Bell *et al.*, 2008), and has also been shown to be involved in fish swim bladder development *(*Yin *et al*., 2011). The hedgehog (Hh) pathway is involved in many developmental events in vertebrates ranging from angiogenesis to limb development (Ingham and Mcmahon, 2001). It has been shown to play a role in the early development of the mammalian lung (Bellusci *et al.,* 1997) and also has been implicated in the specification and organization of the zebrafish swim bladder (Winata *et al.,* 2009). The development of the teleost swim bladder first begins during embryogenesis, and there have been certain genes that have been associated with its early differentiation and growth as described below.

1.10 Genetic markers for teleost swim bladder formation

There are several genes that have been found to be expressed in the developing swim bladder; Hh signaling, Wnt signaling, and pre-b-cell leukemia transcription factor 1 (*pbx1*) genes have been linked to its development (Georgijevic *et al.,* 2007; Teoh *et al.,*

2010; Winata *et al.,* 2009; Yin *et al.,* 2011). Gene markers (Table 1.1) have been identified for all three tissue layers of the developing zebrafish swim bladder including *hb9* and *sox2* in the epithelial layer, *has2*, *fgf10a*, and *acta2* in the mesenchyme, as well as *elovl1a, hprt1l*, and *anxa5* in the outer mesothelium (Korzh *et al.,* 2011; Winata *et al.,* 2009; Yin *et al.,* 2011). Markers for Wnt and Hh signaling ligands have been identified, as well as their receptors. The TALE (three amino acid loop extension) proteins pbx1a and pbx1b have also been identified and shown to play a crucial role in late swim bladder development (Teoh *et al.,* 2010). As this process has been well studied in zebrafish it will be described below. While swim bladder development has not been as well established in Medaka, many of the physiological processes may be conserved across species.

Table 1.1: Genetic markers involved in swim bladder formation/differentiation in zebrafish. Epithelium (E), mesenchyme (M), outer mesothelium (OM), hours post fertilization (hpf).

Marker	Tissue layer	Time of Expression	Function	Citation
Hh markers				
shha	E	48-72 hpf	Growth, epithelial bud elongation	Winata *et al.,* 2009
ihha	E	48-72 hpf	Growth	Korzh *et al.,* 2011
Hh receptors				
ptc1	M	48-60 hpf	Receptor for Hh ligands	Winata *et al.,* 2009
ptc2	M	48-60 hpf	Receptor for Hh ligands	"
Wnt markers				
wnt5b	M	36-72 hpf	Development, growth, differentiation	Yin *et al.,* 2011
tcf3	All 3, strong OM	36-72 hpf	Development, growth, differentiation	"
lef1	M & OM	36-72 hpf	Development, growth, differentiation	"
Wnt receptors				
fz2	M & OM	36-72 hpf	Receptor for wnt ligands	Yin *et al.,* 2011
fz7b	M & OM	36-72 hpf	Receptor for wnt ligands	"
Tissue markers				
hb9	E	36-72 hpf	Formation of epithelial bud, growth,	Winata *et al.,* 2009
sox2	E	36-72 hpf	Organization & growth	Yin *et al.,* 2011
has2	M	48-72 hpf	Organization & growth	"

Table 1.1. Continued.

Marker	Tissue layer	Time of Expression	Function	Citation
Tissue markers				
fgf10a	M	48-72 hpf	M differentiation & growth	Korzh *et al.,* 2011
acta2	M	65-96 hpf	Marker for SM	Winata *et al.* 2009
hprt1l	OM	48-72 hpf	Organization of OM, growth	Yin *et al.,* 2011
elovl1a	OM	48-72 hpf	Organization of OM, growth	Yin *et al.,* 2011
anxa5	OM	60-96 hpf	Organization of OM	Winata *et al.,* 2009
TALE proteins				
Pbx-1 (a & b)	All 3	28-72 hpf	Swim bladder inflation, surfactant production	Teoh *et al.,* 2010
Pbx-4	"	"	development	"

1.10.1 Budding phase

In the teleost zebrafish, swim bladder development has been first observed at 36 hours post fertilization (hpf) as a small outgrowth of the epithelial bud, growing dorsally from the anterior gut endoderm, located in line with the second somite, anterior to the pancreatic bud and posterior to the liver bud (Field *et al.,* 2003; Winata *et al.,* 2009). At this stage (36 hpf), the bud is found to be expressing *hb9*, *pbx*, and *sox2* (Winata *et al.,* 2009; Yin *et al.,* 2011). The swim bladder continues to enlarge up to 48 hpf without major changes in morphology under *hb9* and *foxa2* expression (Winata *et al.,* 2009). By 48 hpf, the swim bladder consists of a ductal structure that connects to the esophagus, formed by 8-10 cells organized around a small lumen (Winata *et al.,* 2009). These cells are all made up of just epithelial cells of endodermal origin and by 48 hpf the bud extends to 2 somites in length and at this point is located from the 3rd and 4th somite (Winata *et al.,* 2009). Once this structure forms, the budding phase of the swim bladder is complete.

1.10.2 Growth and formation of the three layers

Following the budding phase, the swim bladder enters a phase of growth. At the onset of growth the *fgf10a* positive mesenchyme is surrounded by the epithelial bud but remains undifferentiated (Winata *et al.,* 2009). At approximately 60 hpf a third outer layer of mesothelium expressing *anxa5* is incorporated into the growing swim bladder; thus by this time point the swim bladder consists of three distinct tissue layers (Winata *et al.,* 2009). An initiation of *acta2* expression at 65 hpf marks the mesenchymal layers differentiation into smooth muscle, indicating that from that moment onward the mesenchyme consists of mostly smooth muscle cells (Georgijevic *et al.,* 2007; Winata *et al.,* 2009).

By 77 hpf, the lumen of the developing swim bladder has expanded and its ventral wall has thickened (Georgijevic *et al.,* 2007). Interestingly, at 96 hpf, when smooth muscle genes are down-regulated in the intestine, they are still expressed in the swim bladder, suggesting that swim bladder smooth muscle cells are still differentiating prior to inflation (Georgijevic *et al.,* 2007).

Up to 96 hpf, the swim bladder is observed as a sac-like structure in line with the second to fifth somite extending up to 250 μm in length, and is located directly ventral to the notochord and dorsal to the gut (Winata *et al.,* 2009). At 96 hpf or 4 days post fertilization (dpf), the swim bladder interior exhibits a less compact organization compared to earlier stages, with an expanded lumen prepared for inflation, this marks the end of the second stage (Winata *et al.,* 2009).

1.10.3 Inflation

By 4.5-5 dpf in zebrafish, the swim bladder is inflated by air gulped from the water surface (Winata *et al.,* 2009). This swim-up behavior is crucial for the zebrafish larvae to inflate their swim bladder (Lindsey *et al.,* 2010). If zebrafish are denied the air-water interface to initially fill their swim bladder, they will not do so successfully (Goolish and Okutake, 1999). This swim-up behavior can occur in both larval physoclistous and physostomous fish. Following this initial inflation, the swim bladder appears as a thin layer of cells surrounding a large lumen and three distinct tissue layers can be morphologically distinguished under magnification (Winata *et al.,* 2009).

Cyprinids like zebrafish are a large group of teleosts in which the swim bladder consists of two chambers separated by a narrow ductus communicans allowing for gas

exchange between the two (Evans, 1924). In this case the swim bladder consists of two chambers the posterior chamber used for buoyancy and the anterior used as an acoustic resonator which aids in echolocation (Finney *et al.,* 2006; Popper, 1974). The second anterior chamber develops as an outgrowth of the first chamber as soon as the first chamber has completed the budding stage around 64 hpf (Winata *et al.,* 2009). At around 20 dpf, the second anterior chamber will start to inflate, after this event the swim bladder appears as a two chambered structure (Winata *et al.,* 2009). The inflation of the second chamber marks the completion of development of a fully functional swim bladder in zebrafish.

1.11 Swim bladder development in Japanese medaka

The Japanese medaka is similar to zebrafish in many ways; they are both teleosts and are thus part of the same infraclass (Teleostei) and super order (Acanthopterygii). However, they are part of different orders with zebrafish being cypriniformes and medaka being beloniformes. As a result, while sharing similar embryonic development, there are some key differences.

Timing is the largest difference, with embryonic development in medaka taking about twice as long from fertilization to hatch (9 days in medaka compared to only 4.5 days in zebrafish) (Iwamatsu, 2004; Winata *et al.,* 2009). Adult zebrafish are physostomous and still maintain a connection to their gut via a pneumatic duct, while adult medaka are physoclistous and do not. However, physoclistous medaka larvae still maintain that connection to allow the initial inflation of their swim bladders, so this difference is not as pronounced just after hatching. A third difference is that zebrafish (and most cyprinids)

also have a second chamber of their air bladder used for echolocation that inflates later in development (Evans, 1924).

Swim bladder development in medaka is not as well understood as it is in zebrafish, and a comparison of various time points in development of the air bladder of zebrafish compared to medaka are described in Table 1.2. In medaka the presumptive swim bladder is discernible at the ventral side of the 3rd somite at 3 days 2 hours (Iwamatsu, 2004). By 4 days 5 hours the three layers of the swim bladder are formed and viewable as a transparent vacuolar body beneath the third somite (Iwamatsu, 2004). The medaka will hatch by 9 days and inflate their swim bladder in the first 24 hours after hatching (González-Doncel. *et al.,* 2004; Iwamatsu, 2004).

Studies have demonstrated that completely denying medaka access to air water interface (by enclosing them in a sealed vessel) results in medaka being unable to inflate their swim bladder (Marty *et al.,* 1990a; Marty and Hinton, 1995). These results suggest that the air water interface is required for successful swim bladder inflation by medaka.

While medaka embryo development follows a similar pattern, it is temporarily delayed compared to the zebrafish. For example the swim bladder bud becomes visible in the zebrafish from 36-48 hpf, while in the medaka it is visible starting at 74 hpf (Iwamatsu, 2004; Kimmel *et al.,* 1995; Winata *et al.,* 2009). Based on a comparison of the stages of development of medaka and zebrafish the developmental window of the swim bladder can be estimated as follows: budding 74-87 hpf, formation of 3 layers 87-101 hpf, growth 101-216 hpf, and inflation shortly after hatching at 216 hpf.

Table 1.2: Time of development (hours post fertilization (hpf)) of key developmental stages related to swim bladder (swb) formation and inflation, a comparison of medaka and zebrafish embryo development (Kimmel *et al.,* 1995, Iwamatsu, 2004).

Zebrafish Stage	Time (hpf)	Medaka Stage	Time (hpf)
10-Somite	14	9-Somite	38
14-Somite	16	12-Somite	41
		16-Somite	44
30-Somite	24	30-Somite	64
Prim 25 stage, swb bud formed	36	Stage 29, swb bud formed	74
Long-pec	48	Stage 31-32	87
Swb formed 3 layers	72	Stage 32, swb formed	101
Hatch/inflation	96	Hatch/inflation	216

1.12 Embryogenesis

The growth and patterning of multicellular embryos is directly linked to events at a cellular level, which mediate intercellular communication, function and interaction of a cell with its extracellular environment (Simpson *et al.,* 2009). These events are controlled by the coordinated action of a few developmental signaling pathways whose actions are mediated by secreted factors (Simpson *et al.,* 2009). These factors are grouped into a handful of families of secreted peptide factors including the hedgehog (Hh), Wnt, Notch, platelet derived growth factor, fibroblast growth factor, and the transforming growth factor-β superfamilies (Ingham and Mcmahon, 2001; Simpson *et al.,* 2009).

Studies in many different species have shown that there is substantial conservation in the expression of signaling families to regulate the development of organisms (Ingham and Mcmahon, 2001). The action of these pathways are linked to a host of cellular functions that allow the coordinated uptake, secretion and movement of proteins to specific locations within the cell (Simpson *et al.,* 2009). Disrupting the action of these signaling pathways can lead to developmental abnormalities and improper tissue formation/organization. For example, disrupting the regulation and transduction of Wnt and Hh signaling pathways have been linked to causing birth defects, tissue regeneration and even cancerous growth (Beachy *et al.,* 2004). As both Wnt and Hh pathways are involved in the formation of the swim bladder in teleost fish during embryogenesis, they will be further discussed.

1.13 Hedgehog signaling pathway

The hedgehog (Hh) signaling pathway is a major signaling network that regulates key events during the growth and patterning of multicellular embryos (Simpson *et al.,* 2009). In mammals the *Hh*-genes encode for three unique proteins including sonic hedgehog (Shh), indian hedgehog (Ihh), and desert hedgehog (Heretsch *et al.,* 2010). Orthologues of all three these proteins have been found in zebrafish including two for Ihh and Shh (Avaron *et al.,* 2006; Currie and Ingham, 1996; Krauss *et al.,* 1993). *Desert hedgehog* is normally expressed in the Sertoli cells of the testes (Bitgood *et al.,* 1996), and has not been linked to lung or swim bladder formation. *Ihh* is generally expressed in the gut and cartilage (Bitgood and McMahon, 1995). As the teleost fish swim bladder develops as an evagination of the gut tissue, it follows that Ihh is involved the formation of the swim bladder, however, it should be noted that Ihh has not been found to be involved in mammalian lung formation (Korzh *et al.,* 2011). Shh has the largest number of functions of the hedgehog homologues (patterning of the neural tube, somites, and limbs, and has also been found to be expressed during the development of the tooth, gut, and lungs of mice embryos), and the gut is one of the few areas where Shh and Ihh signaling overlap (Bitgood and McMahon, 1995). These Hh proteins are ligands of the membrane bound receptors patched1 (Ptc1) and patched2 (Ptc2) (Heretsch *et al.,* 2010; Stone *et al.,* 1996). Homologues of these two receptors have also been identified in zebrafish (Concordet *et al.,* 1996; Lewis *et al.,* 1999).

Ptc normally functions by inhibiting Smoothened (Smo), which is a seven-transmembrane protein that resembles a G-protein coupled receptor, and functions downstream of Ptc (Heretsch *et al.,* 2010). Smo has three forms, SmoA which is an

internalized inactive form which exists in equilibrium with an inactive cilium-bound form SmoB and an active form SmoC that is generated from SmoB (Heretsch *et al.,* 2010; Kim *et al.,* 2009; Rohatgi *et al.,* 2009). In the absence of Hh ligands, Ptc inhibits the activation of SmoB into SmoC (Choudhry *et al.,* 2014; Heretsch *et al.,* 2010).

The activation of the Hh signaling pathway is accomplished by the binding of an Hh ligand to Ptc. When bound, Ptc will translocate out of the primary cilium and be degraded by lysomes (Heretsch *et al.,* 2010). Without the inhibition from Ptc, SmoC is now able to be generated and can now initiate the Shh downstream signaling cascade (Choudhry *et al.,* 2014). For a summary the Hh pathway with and without a bound ligand, see Figure 1.5.

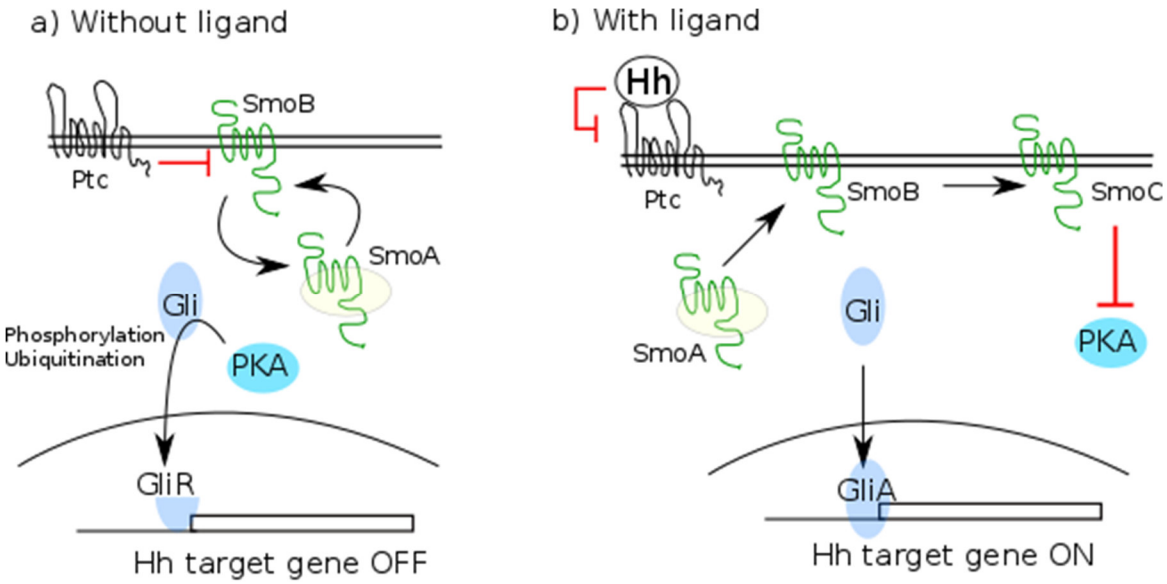


Figure 1.5: Simplified Hh signaling pathway. A) Without a ligand bound, patched (Ptc) is located in the cellular membrane and inhibits the cilium bound SmoB (which exists in equilibrium with an internalized inactive form SmoA) from forming its active form SmoC. Without the presence of SmoC, the Gli transcription factors are labelled for phosphorylation and ubiquitination, which result in a truncated repressor form GliR. B) In the presence of a ligand Ptc is inhibited, and SmoC is now able to be formed. SmoC will now inhibit kinases that destroy Gli such as PKA. Gli transcription factors can now be processed into active forms GliA that can be transported to the nucleus and activate gene transcription.

1.13.1 Hedgehog signaling in swim bladder formation

Hedgehog signaling plays a crucial role in the development of the zebrafish swim bladder. Winata *et al.,* (2009) found strong expression of sonic hedgehog (*shha*) in the swim bladder epithelium and pneumatic duct between 48-72 hpf. The Indian hedgehog gene of zebrafish, *ihha*, was expressed in the gut and swim bladder primordia between 48-72 hpf (Winata *et al.,* 2009). Indian hedgehog is not involved in the development of the tetrapod lung, and its role in the specification and development of the swim bladder may be unique to fishes (Korzh *et al.,* 2011). *Ihha* was found to be strongly expressed in the swim bladder between 48-72 hpf (Korzh *et al.,* 2011; Winata *et al.,* 2009). The expression of both *shha* and *ihha* were restricted to the epithelial lining of the swim bladder much like *hb9* (Korzh *et al.,* 2011; Winata *et al.,* 2009).

The receptor genes *ptc1* and *ptc2* are two homologues for the patched genes in zebrafish, which are receptors for Hh signaling. The transcription of *ptc* is a sensitive indicator of the level of Hh signal transduction occurring within cells (Hollway *et al.,* 2006). Strong expression of *ptc1* and *ptc2* in the swim bladder were observed starting from 48-60 hpf (Winata *et al.,* 2009). The expression was reduced and disappeared from the swim bladder by 72 hpf. *Ptc1* and *ptc2* were found to be expressed in a domain adjacent to the epithelial domains of *shha* and *ihha*, which overlaps the *fgf10a* mesenchymal domain (Winata *et al.,* 2009). The presence of these receptors in the mesenchyme cell layer suggests that the mesenchymal layer is targeted by Hh signaling. This is expected as Shh is involved in smooth muscle development (Georgijevic *et al.,* 2007), which occurs in the mesenchymal layers.

In the zebrafish mutant *syu*^{t4}, which is partially deficient in Hh signaling due to a mutation in *shha*, the expression of *ptc1* & *ptc2* is reduced (Hollway *et al.,* 2006; Winata *et al.,* 2009). In this mutant strain, the swim bladder was observed to be delayed in growth, reduced in size, and had a delay in inflation (Winata *et al.,* 2009). The study by Winata *et al.,* (2009) also observed severe defects in all three tissue layers of the swim bladder in the *syu*^{t4} mutants. They observed a reduction in the epithelium marked by a reduction in *hb9* expression, a reduction in *fgf10* and *acta2* expression in the mesenchyme which was disorganized and reduced in size and lacked smooth muscle differentiation, and a reduction in *anxa5* expression in the outer mesothelial layer which was also reduced and disorganized (Winata *et al.,* 2009).

In zebrafish morpholino experiments knocking down *ihha* expression, a reduction in the expression of *ptc1* and *ptc2* was also observed (Winata *et al.,* 2009). The epithelium was also reduced, but not as significantly as in the *syu*^{t4} mutant, and a reduction in *hb9* expression was observed (Winata *et al.,* 2009). The mesenchyme layer was reduced but was still organized, yet much like the *syu*^{t4} mutants, there was an absence of *acta2* expression which resulted in a lack of differentiation into smooth muscle (Winata *et al.,* 2009). Mesothelial expression of *anxa5* was also affected but not as much in the *syu*^{t4} mutants (Winata *et al.,* 2009). Overall the effects of *ihha* knockdown was not as significant as inhibiting *shha* signaling, but still resulted in a mild reduction of the epithelium, mesenchyme and outer mesothelium as well as an absence of smooth muscle differentiation (Winata *et al.,* 2009).

1.13.2 Modulators of the hedgehog signaling pathway

There are many compounds that can modulate the Hh signaling pathway (reviewed by: Heretsch *et al.,* 2010). Cellular responses to Hh signaling are controlled by two transmembrane proteins Smo and ptc (Taipale *et al.,* 2000). The majority of drugs that inhibit the Hh pathway do so by inhibiting the protein Smo (Heretsch *et al.,* 2010). Cyclopamine is a plant steroidal alkaloid and inhibits the Hh pathway by interacting with Smo, causing it to accumulate in the primary cilium, and causing Smo to change into its closed form even in the presence of Hh ligands (Cooper *et al.,* 1998; Heretsch *et al.,* 2010; Taipale *et al.,* 2000).

In a study by Winata *et al.,* (2009), zebrafish embryos were exposed to 20 μM cyclopamine at various stages of development. It was found that exposing embryos at 10, 12, and 14 hpf caused an absence of swim bladder epithelium, while exposing from 16 hpf onward did not disrupt the specification of epithelium measured at 40 hpf, but still caused a smaller swim bladder bud being formed by 72 hpf (Winata *et al.,* 2009). The swim bladder bud is first visible at 36 hpf, so the Hh inhibition by cyclopamine demonstrates that specification of the cells occurs much earlier (Winata *et al.,* 2009). This suggests that Hh signaling during segmentation may be required for the differentiation of presumptive cells which later become the swim bladder (Winata *et al.,* 2009). Another study treating zebrafish embryos with 50 μM cyclopamine at 5.5 hpf resulted in a delay in smooth muscle development (Barresi *et al.,* 2001).

1.14 Wnt signaling

Much like the hedgehog signaling pathway, the Wingless-type MMTV integration site family (Wnt) family of proteins are a critical mediator of cell-cell signaling events during embryogenesis (Reviewed by: Logan and Nusse, 2004). The canonical Wnt pathway is a critical and heavily studied Wnt pathway that functions by regulating the amount of β-catenin (a transcriptional coactivator), which controls important developmental gene expression (MacDonald *et al.,* 2009). Canonical Wnt signaling is one of the main regulators of mammalian lung development (Volckaert and De Langhe, 2014). It has also been implicated in zebrafish swim bladder development, and as a result, will be the focus of this section (Yin *et al.,* 2011).

In the absence of Wnt, cytoplasmic β-catenin levels are kept low through the continuous proteasome-mediated degradation by the Axin complex, which is composed of glycogen synthase kinase 3, the tumor suppressor *adenomatous polyposis coli* gene product, casein kinase 1, and the scaffolding protein Axin (Liu *et al.,* 2002; Logan and Nusse, 2004). The destruction of β-catenin is achieved by sequentially mediated phosphorylation by a priming kinase (casein kinase 1), followed by phosphorylation by glycogen synthase kinase 3 of the amino terminal region of β-catenin; this targets β-catenin for β-Trcp (an E3 ubiquitin ligase subunit) mediated ubiquitination and its subsequent degradation by the proteasome (Liu *et al.,* 2002). This continual breakdown of β-catenin prevents it from reaching the nucleus, and Wnt target genes are thus repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins (MacDonald *et al.,* 2009).

The Wnt pathway is activated when a Wnt ligand binds to the cysteine-rich domain of the trans-membrane Frizzled (Fz) receptor at the cell surface (Logan and Nusse, 2004). When a Wnt ligand binds to the Fz receptor (as well as its co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6) or LRP5, a Wnt-Fz-LRP5/6 complex is formed (MacDonald *et al.,* 2009). This binding to frizzled results in the recruitment/activation of the disheveled protein. The Wnt-Fz-LRP5/6 complex along with disheveled will result in LRP6 phosphorylation and recruitment of the Axin complex to the receptors (MacDonald *et al.,* 2009). These events will lead to a disassembly of the Axin complex (Rao and Kühl, 2010). Without the Axin complex degrading it, β-catenin will begin to accumulate in the cytosol and nucleus (Logan and Nusse, 2004). Once in the nucleus, it will form complexes with TCF/LEF to activate Wnt gene transcription/expression (Logan and Nusse, 2004). An outline of the model for Wnt canonical signal transduction is shown in Figure 1.6.

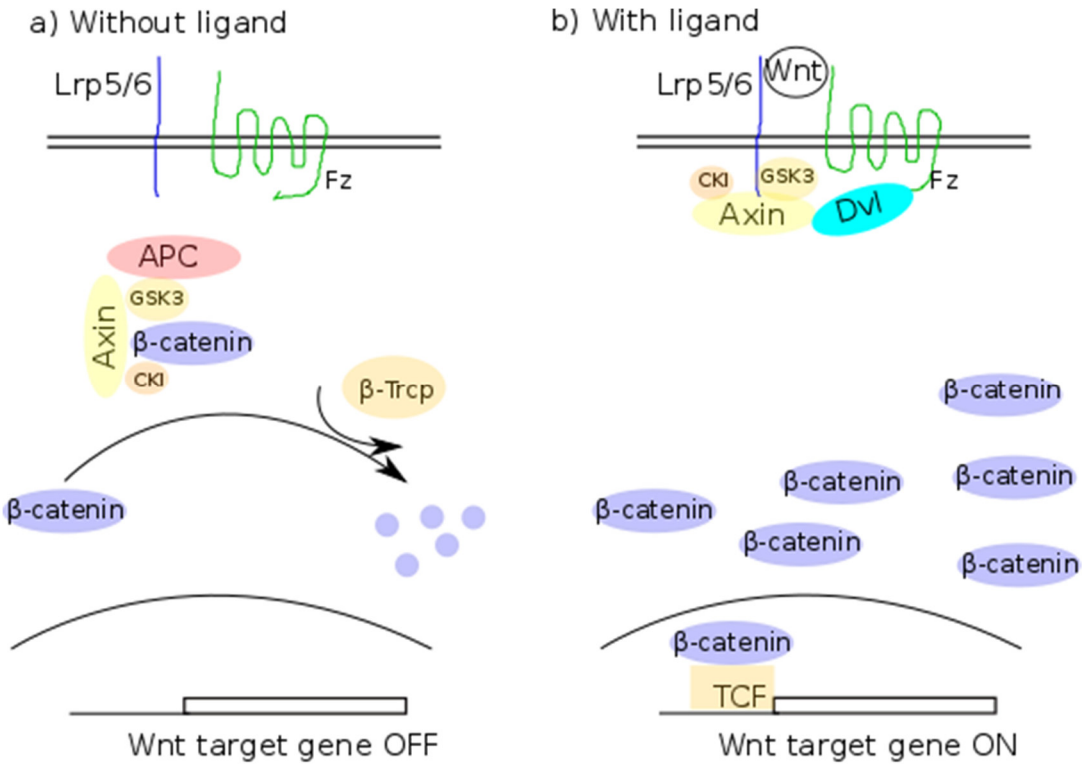


Figure 1.6: Canonical Wnt signaling. A) Without a ligand bound, β-catenin is bound and phosphorylated by the Axin complex, which is composed of glycogen synthase kinase 3 (GSK3), the tumor suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1), and the scaffolding protein Axin. β-catenin is then ubiquitinated by β-Trcp (an E3 ubiquitin ligase subunit) and then degraded by the proteasome. This continual breakdown of β-catenin prevents it from reaching the nucleus. B) An appropriate ligand will bind to the Frizzled (Fz) receptor and its co-receptor, low-density lipoprotein receptor 5/6 (LRP5/6), to form a complex. This complex results in the recruitment and activation of the disheveled protein (Dvl). This will result in the recruitment of the Axin complex to the receptors, thus inhibiting its normal intracellular destruction of β-catenin. This will allow β-catenin to accumulate, and once in the nucleus, it will form complexes with TCF to activate Wnt gene transcription/expression.

1.14.1 Wnt signaling in swim bladder formation

Canonical Wnt signaling is one of the main regulators of mammalian lung development (Volckaert and De Langhe, 2014). The target of Wnt signaling, β-catenin, has been shown to be essential to mesenchymal development of the lungs (De Langhe *et al.,* 2008). Wnt signaling has also been shown to be crucial for zebrafish swim bladder development (Yin *et al.,* 2012, 2011). Yin *et al.,* (2011) found expression of *wnt5b* in the zebrafish swim bladder mesenchyme from 36-72 hpf, but did not find any expression of *wnt5a*. This is interesting as *wnt5a* has been reported to be expressed in mouse lung (Li *et al.,* 2002) and *Xenopus* lung (Yin *et al.,* 2010), but there are no studies that have reported *wnt5b* in the lungs other species of vertebrates. It may be unique to fish that *wnt5b* is involved in swb development instead of *wnt5a*, and might be a result of divergence. The genes for the Fz receptor for Wnt ligands (*fz2* and *fz7b*) have been shown to be expressed in the mesenchyme and outer mesothelium from 36-72 hpf (Yin *et al.,* 2011). The expression of two targets of Wnt signaling (*LEF* & *TCF*) have been measured from 36-72 hpf: *lef1* in the mesenchyme and outer mesothelium and *tcf3* expressed strongly in outer mesothelium, weak in the epithelium and very weak in the mesenchyme (Yin *et al.,* 2011). The expression of the ligand, receptors, and targets of Wnt signaling, strongly supports the role of Wnt in the developing swim bladder.

The role of Wnt was further confirmed by using two heat shock transgenic lines hs:Dkk1-GFP, and hs:ΔTcf-GFP (Yin *et al.,* 2011). These lines were used to block Wnt signaling at various developmental stages and found that precursor cells specification, organization and patterning were all disrupted in the three tissue layers (Yin *et al.,* 2011). This study also demonstrated blocking Wnt signaling led to decreased proliferation of

swim bladder cells at 66 hpf and increased apoptosis, both of which contributed to a disturbance of swim bladder growth (Yin *et al.,* 2011). Thus, much like in mammalian lung, Wnt signaling is crucial to the early development and organization of the zebrafish swim bladder. The roles that Wnt signaling plays in growth and formation of swim bladders of other species of fish have yet to be established.

1.14.2 Epithelial/mesenchyme/outer mesothelium crosstalk

Epithelial, mesenchyme, and outer mesothelium cell layers do not develop in isolation or separately to each other. Interactions between the endoderm and the mesenchyme are a common theme throughout embryonic development (Volckaert and De Langhe, 2014). It has been demonstrated that epithelial-mesenchymal interactions are vital to proper lung morphogenesis, and the Wnt and Hh signaling pathways have been shown to be crucial for this cross talk during lung development (Shannon and Hyatt, 2004). These interactions are important during embryonic development, and the cell layers will not form properly without them.

Such interactions exist for both Hh and Wnt signaling in zebrafish swim bladder development. Hh signaling has been shown to originate in the epithelium and signal to receptors found in the mesenchyme (Winata *et al.,* 2009). Wnt ligands were found to be expressed in the mesenchyme with receptors found in the mesenchyme and outer mesothelium (Yin *et al.,* 2011). Both Hh and Wnt signaling is crucial to the development, organization and proliferation of all three layers of the swim bladder. When either pathway is blocked, a time dependent inhibition of swim bladder formation occurs.

1.14.3 Wnt/Hh interactions

In zebrafish, Wnt signaling is crucial for the specification of the epithelium, and it is generally expressed before Hh signaling, which implies Wnt signaling may act upstream of Hh signaling in the development of the swim bladder (Winata *et al.,* 2009; Yin *et al.,* 2011). Wnt signaling is important to maintaining Hh signaling, but Hh signaling appears to inhibit Wnt through the Wnt inhibitory factor-1 (Wif1) (Yin *et al.,* 2012). Thus, it is important to understand how these signaling pathways interact when studying the effects of chemical exposure on them.

1.14.4 Modulators of the Wnt signaling pathway

Due to uncontrolled Wnt signaling playing a role in cancer, there are many modulators of the Wnt pathway that are currently used in therapeutics or in development (Rey and Ellies, 2010). A large number of them function by inhibiting GSK3, which normally is part of the destruction complex for β-catenin (Rey and Ellies, 2010). Inhibition of GSK3 can lead to an over production of β-catenin and mimic over-expression of Wnt signaling. Lithium can also act as a modulator of Wnt signaling by inhibiting GSK3 (Bajoghli *et al.,* 2009). Zebrafish embryos exposed to 0.3 M LiCl for 10 minutes experienced perturbations in development; depending on the age of exposure the results ranged from complete mortality to an inhibition of eye development (Stachel *et al.,* 1993). IWR-1 is a potent and specific inhibitor of Wnt signaling. It blocks β-catenin accumulation by stabilizing Axin2, allowing it to function in the absence of an *adenomatous polyposis coli* gene complex (Chen *et al.,* 2009). Zebrafish embryos treated with 10 μM IWR-1 resulted in a time dependent inhibition of the specification of the swim bladder epithelium

(Yin *et al.,* 2011). It was demonstrated that swim bladder epithelium was missing when embryos were exposed from 12 hpf, but when exposed from 14-30 hpf, a small epithelium bud was observed (Yin *et al.,* 2011). This establishes that canonical Wnt signaling pathway is required for swim bladder specification in zebrafish.

1.15 Pbx and its role in teleost swim bladder

The Pbx family belongs to the PBC group of the TALE (three amino acid loop extension) class of homeodomain proteins (Teoh *et al.,* 2010). Pbx1 has been shown to have a role in mammalian lung organogenesis (Schnabel *et al.,* 2001) and is involved in the growth of the zebrafish swim bladder (Teoh *et al.,* 2010). Pbx1 has two isoforms: a longer (Pbx1a) and a shorter (Pbx1b), both of these have been identified in zebrafish (Monica *et al.,* 1991; Teoh *et al.,* 2010). A loss of *pbx1* function was found to not affect swim bladder budding, but did result in the larvae being unable to inflate their swim bladders once hatched (Teoh *et al.,* 2010). *Pbx1* may play a role in regulating genes related to surfactant production, which are crucial for swim bladder inflation (Teoh *et al.,* 2010).

Fish embryonic swim bladder development is a complex process that involves many different genes and signaling pathways. Zebrafish swim bladder development genes and a proposed timeline of development for medaka are summarized in Figure 1.7. While zebrafish have been studied quite extensively, medaka have not received nearly as much attention and would provide a novel species to study effects on swim bladder inflation.

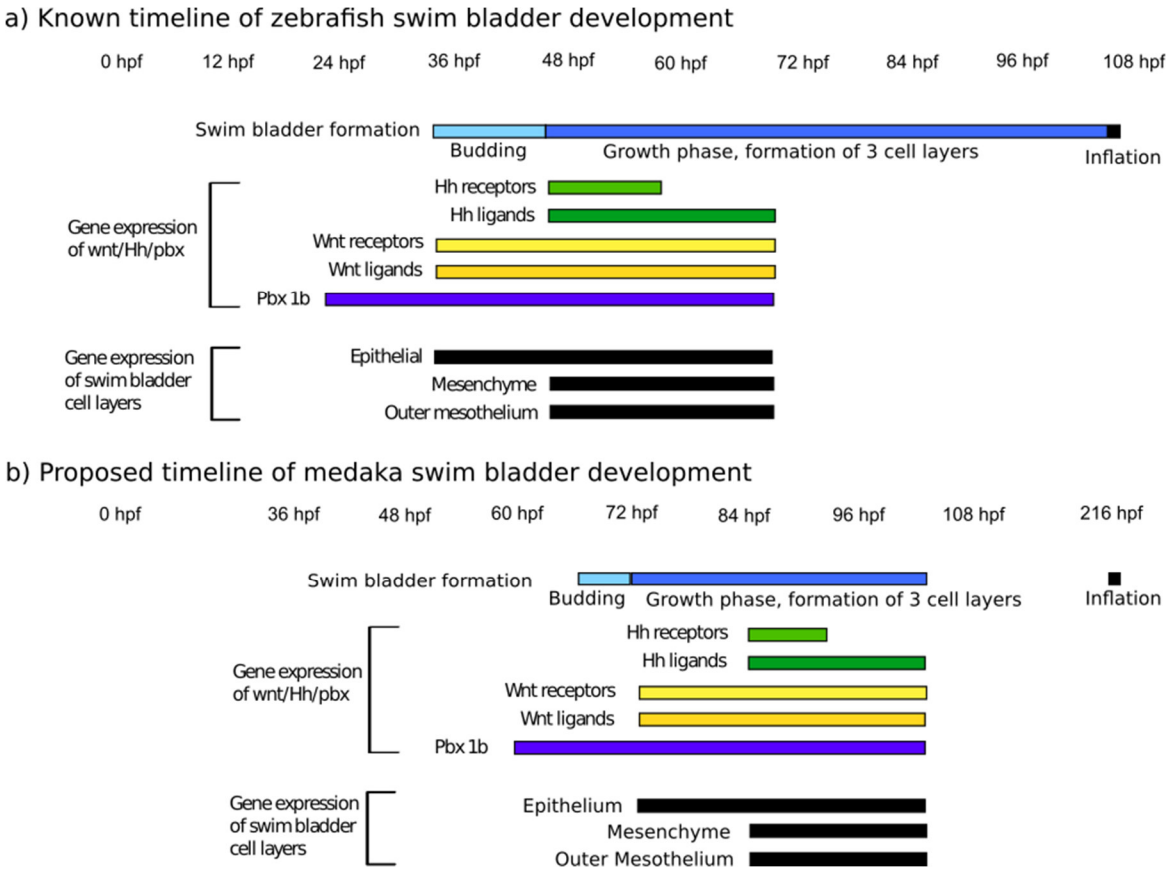


Figure 1.7: A) Summary of the timeline of genetic expression in hours post fertilization (hpf) of *Hh*, *Wnt*, and *Pbx1b* during zebrafish swim bladder development. The time expression (hpf) of genes related to the three cell layers: epithelium, mesenchyme and outer mesothelium are also displayed. B) A proposed timeline of medaka swim bladder development and potential gene expression, based on that of the zebrafish.

1.16 Effects of environmental contaminants on swim bladder inflation

Failure of swim bladder inflation occurs following exposure to many different types of pollutants including antibiotics, coatings, chemical surfactants, aryl hydrocarbon receptor agonists, pyrethroids, pesticides, herbicides, and thyroid peroxidase inhibitors (Table 1.3.). Swim bladder inflation failure is typically observed in hatched fry following embryonic exposure to a chemical. Despite reports in the literature, failure of swim bladder inflation is not a commonly used toxicological endpoint. Generally, studies are not designed with swim bladder inflation failure in mind, and often report it as an unexpected outcome of exposure. In some cases, such as following exposure to 5.6 ng/L 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 0.17 µg/L 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) and 0.21 mg/L 3,3'4,4'-tetrachlorobiphenyl (PCB 77), inhibition of swim bladder inflation was the most sensitive endpoint measured (they measured mortality, formation of lesions/malformations, swim bladder inflation success, and hatchability) (Kim and Cooper, 1999). Medaka exposed to selenomethionine during stages 9 to 25 had a significantly higher percentage of non-inflated swim bladders than those exposed at stage 34 (Kupsco and Schlenk, 2016). This result is not unexpected, since the stage of development that embryos are exposed can be quite crucial to effects on organogenesis.

Few studies mechanistically explain why swim bladder inflation is not occurring. Most hypothesize that the effect is a result of either disrupted air water interface, disrupted production of mucus surfactants, or a disruption in signaling during swim bladder development. The most comprehensive study to date examined how 2-mercaptobenzothiazole (compound used in the vulcanization of rubber) caused failure of anterior swim bladder inflation in both zebrafish and fathead minnows (Nelson *et al.,* 2016;

Stinckens *et al.,* 2016), which ultimately attributed the effect to the thyroid system. However, 2-mercaptobenzothiazole mostly affected anterior swim bladder inflation, which does not inflate until much later in development. It did not seem to affect posterior swim bladder inflation, which was attributed to thyroid levels not being depleted until later in development (as the posterior swim bladder forms earlier). The applicability of this study to medaka is not very strong as medaka only have one swim bladder that develops during embryogenesis.

There is no doubt that many pollutants can cause failure of swim bladder inflation in fish. Understanding how embryonic exposure to chemicals can influence swim bladder inflation could allow the further development of adverse outcome pathways. This is important to aquatic toxicology, as the mechanisms through which many chemical compounds cause this effect are poorly understood.

Table 1.3: Summary of studies that have measured impaired swim bladder (swb) formation and/or inflation due to exposure to various chemicals.

Chemical	Type	Fish	Concentration	Effect	Citation
Urban storm water	Suspected metals	Medaka	Effluent	Significant reduction in swb inflation	(Skinner *et al.,* 1999)
Thiobencarb	Herbicide	Medaka	5, 11 mg/L	5 mg/L resulted in 72% inhibition, 11 mg/L 100%	(Villalobos *et al.,* 2000)
b-diketone antibiotics	Antibiotic	Zebrafish	37.5-75 mg/L	Significant reduction in swb inflation	(Wang *et al.,* 2012)
Three sulfonamides	Antibacterial	Zebrafish	10-1000 µg/L	Reduction in swb inflation	(Lin *et al.,* 2013)
Permethrin	Pyrethroid	Medaka	50 µg/L	Permethrin may inhibiting the production of surfactants	(González-Doncel *et al.,* 2003)
Cypermethrin	Pyrethroid	Medaka	12.5 µg/L	Significant reduction in swb inflation	(González-Doncel *et al.,* 2004)
AgNO3	Metal	Zebrafish	0.1 and 0.3µM	Significant reduction in swb inflation	(Powers *et al.,* 2010)
PCB 126	AHR agonist	Zebrafish	EC50 2.5 nM	Impaired swb inflation. Effect is AHR2 dependent (increased cyp1 and cox 2 expression)	(Jönsson *et al.,* 2012)
TCDD	PCDD	Medaka	5.6 ng/L	All inhibited swb inflation, the most sensitive endpoint measured	(Kim and Cooper, 1999)
PCB 126	AHR agonist	Medaka	100 ng/L	"	“
PCB 77		Medaka	200 µg/L	"	“
Octylphenol	Surfactant	Medaka	EC50 400-800 µg/L	Inhibition of swb inflation	(Gray and Metcalfe, 1999)
Perfluorooctane sulphonate	Coating	Zebrafish	EC10/80 = 1/3mg/L	Inhibition of swb inflation	(Hagenaars *et al.,* 2014)
Intersleek 425	Coating	Medaka	Coated surface	100% reduction in swb inflation	(Feng *et al.,* 2012)
Intersleek 970	Coating	Medaka	Coated surface	Similar effects to intersleek 425	“
TBC	Flame retardant	Zebrafish	2.5 mg/L	80% lack of inflation of swb, no effect on Shh signaling (*shha*, *ihha*)	(Li *et al.,* 2011)
n-nitroso compounds		Medaka	3 Compounds	Ability to inflate swb most sensitive endpoint of exposure	(Marty *et al.,* 1990b)
Microcystin-LR	Canobacteria toxin	Medaka		Lack of swb inflation	(Huynh-Delerme *et al.,* 2005)

Table 1.3 continued.

Chemical	Type	Fish	Concentration	Effect	Citation
2-Mercapto-benzothiazole	Thyroid peroxidase inhibitor	Fathead minnow	1 mg/L	Failure to inflate anterior swb	(Nelson *et al.,* 2016)
2-Mercapto-benzothiazole	Thyroid peroxidase inhibitor	Zebrafish	0.35 mg/L	Failure to inflate anterior swb	(Stinckens *et al.,* 2016)
Selenomethoinine	Selenium	Medaka	5 µM	Failure to inflate swb	(Kupsco and Schlenk, 2016)
Bitumen	Oil	Medaka	Dilutions	Malformed swb or delayed in development	(Madison *et al.,* 2015)
Methoxychlor	Pesticide	White sucker	0.1 mg/L for 2h at 2days post-hatch	Failure to inflate swb	(Holdway *et al.,* 1987)
Iopanoic Acid	Deiodinase inhibitor	Fathead minnow	6.0 mg/L	Reduced incidence and length of swb	(Cavallin *et al.,* 2017)

1.17 Long-term effects of failure in swim bladder inflation

A larger question that arises using swim bladder inflation failure as an endpoint is, what are the long-term effects? Does it affect the fish at a population level? While failure to inflate the swim bladder by itself is not lethal to the organism, there are studies that have associated it with a decreased chance of survival zebrafish in the lab (Goolish and Okutake, 1999). Wild fish lacking a swim bladder would be at a significant disadvantage, and face challenges with metabolic demand, obtaining food, and avoiding predators (Czesny *et al.,* 2011). Studies have also linked failed swim bladder inflation with a reduction in growth due to increased metabolic demand, of both zebrafish and perch (Czesny *et al.,* 2011; Goolish and Okutake, 1999; Jacquemond, 2004). There are examples of wild caught fish without inflated swim bladders, but they are considered to be relatively rare (Egloff, 1996). Wild caught fish with uninflated swim bladders tend to have two developmental abnormalities: a dorso-ventral curvature of the spine (due to pressure on the vertebrae from having to swim in an upward direction to maintain their position in the water column), and mutilated/abrased/contorted pelvic fins (most likely due to making frequent contact with the lake bottom) (Egloff, 1996). Commercially, failure of swim bladder inflation is considered a serious problem, and is a concern for aquaculture due to increased mortality and decreased growth (Reviewed: Woolley and Qin, 2010). Thus, impaired swim bladder inflation can lead to individual effects that have the potential to impact fish populations including: mortality, reduced growth, increased risk of predation, increased metabolic demand, and developmental abnormalities.

1.18 Combinatorial toxicity of pharmaceuticals

Most studies focus on single compounds ignoring the fact that pharmaceuticals are normally present in the environment in complex mixtures. Chemical mixtures can be tested directly as an environmental sample (whole-mixture, or whole-effluent), or as a mixture prepared in the laboratory (component-based approach). Whole-mixture samples are useful for determining the environmental impact/relevance of an effluent, and allow exploratory studies to determine which compounds in the mixture may be causing these effects. The results of whole-mixture tests have limitations as they are only applicable to an environment under the exact same concentration range and component ratio (Backhaus *et al.,* 2007). Whole mixture results are very hard to apply to environmental conditions, as the number and concentration of pollutants vary greatly both spatially and temporally. Whole-mixtures also are not designed to determine the contribution of individual components to the overall effects.

Mixtures can produce a variety of interactions such as synergistic such as less than additive, additive, or potentiated (interaction between two or more compounds resulting in a response greater than the sum of indiual responses to each comopound), or antagonistic effects (Barata *et al.,* 2006). The toxicity of mixtures can be at concentrations where a single substance shows no or very little effect, which is very important for environmental risk assessment (Cleuvers, 2004). NSAIDs have been demonstrated to act additively for acute diclofenac and ibuprofen exposure to *Daphnia* (48 hour acute immobilization toxicity test) (Cleuvers, 2004, 2003; Parolini and Binelli, 2012). In some cases potentiation can occur, with the percentage of immobilized daphia greater than would be expected by the ibuprofen and diclofenac acting through independent action or through concentration

addition (Cleuvers, 2003). EE2 has been shown to act additively with estradiol at environmentally relevant concentrations of 0.6 ng/L EE2 and 14.4 ng/L 17β-estradiol to induce vitellogenin concentrations in juvenile rainbow trout after 14 days of waterborne exposure (Thorpe *et al.,* 2003).

In WWTP effluent there are many different classes of pharmaceutical compounds, some of which cause opposite effects. This poses interesting questions regarding their interactions e.g. do the presence of anti-estrogenic compounds negate the effects of estrogenic pharmaceuticals? A co-exposure of medaka to 17β-estradiol and antiestrogens resulted in a reduction in some of the effects of 17β-estradiol alone, but did not prevent reproductive impairment, and in some cases, made it more severe (Sun *et al.,* 2009).

Most mixture studies tend to focus on trying to elucidate additive effects of compounds that share similar modes of action. Few studies have examined the effects of a mixture of compounds with unrelated modes of action. One such study was a life-cycle exposure of fathead minnows to a mixture of water-borne pharmaceutical and personal care products at exposure concentrations ranging from 10-1000 ng/L (nominal), of six common pharmaceutical and personal care products including naproxen, gemfibrozil, diclofenac, ibuprofen, salicylic acid, acetaminophen, and triclosan (Parrott and Bennie, 2009). This study is unique since the chemicals tested have very different modes of action but are ubiquitous in surface water. They found an increase in larval deformities in the first (F1) generation (Parrott and Bennie, 2009). A short, 6-week exposure of zebrafish to a mixture of 0.5 and 10 mg/L acetaminophen, carbamazepine, gemfibrozil, and venlafaxine significantly reduced embryo production and increased the incidence of developmental abnormalities (Galus *et al.,* 2013).

However, there are no published studies that compare mixtures of NSAIDs and natural/synthetic hormones compounds. One reason that so few mixture studies are done in fish is the large cost and time it takes to establish effective concentration curves in order to be able to conduct concentration addition experiments. For example, first each compound would have to be assessed individually to determine their impacts. Then combinatorial studies can be designed using a component-based approach, which would then have to be conducted. Due to this, component-based studies require a large number of fish and time to conduct. One potential solution to this issue would be the use of embryo-toxicity test to model mixture effects. Embryos are advantageous over whole fish because they require much less space and materials, which greatly reduces the cost to conduct the multiple experiments required for assessing mixture toxicity. Thus, fish embryos may be useful for mixture modeling the impacts of pharmaceutical compounds on fish development.

1.19 Aims and research objectives

Using the higher throughput embryo-toxicity model, many compounds can be screened and their effects on fish development can be assessed. As there is a need to develop highthroughput alternative testing methods, this thesis will focus on using embryotoxicity to predict effects on Japanese medaka larvae of pharmaceutical compounds. The main objective of this thesis was to assess the impact of exposure to various human pharmaceuticals and natural hormones including non-steroidal anti-inflammatory drugs and natural/synthetic hormones on the development of Japanese

medaka embryos. Specifically, the effects of embryonic exposure to diclofenac, ibuprofen, naproxen, 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel were assessed on embryo development, hatchability, malformations, and time to hatch. Single compound experimental effects were determined along with mixture effects of these compounds where possible, using embryo-toxicity tests.

By using embryotoxicity as a model, potential adverse outcomes related to pharmaceutical exposure were assessed. The effects of these compounds on embryo development were observed, and the impacts of these compounds on whole embryo gene expression was assessed to determine if there were any genetic markers that could be used to predict embryo malformations. Linking these short-term effects of pharmaceutical exposure on gene expression to adverse outcomes in the hatched larvae is crucial to AOP development.

Chapter 2: General methodology

2.1 General fish conditions

Initial stocks of medaka (FLFII strain) were obtained from the National Institute for Basic Biology (Okazaki, Japan). Stock medaka fish were housed in 70-L glass aquaria under flow-through conditions with 25°C water (see Table 2.1 for general characteristics) and a 16h:8h (light:dark) period including 30 minutes of dawn and dusk included in the light period. All medaka fish were fed twice daily using flake food (Tetramin® Pro) and fresh brine shrimp (Premium grade brine shrimp eggs). Tanks were cleaned once daily following the morning feed by vacuuming out any uneaten food/waste at the bottom of the tanks.

Table 2.1. Chemical characteristics of the wet-lab water used to house the adult breeding medaka. All values expressed in mg/L.

Cations			Anions		Total nitrogen	pH	Hardness
Calcium	Magnesium	Sodium	Chloride	Sulfate			
28.9	7.2	19	24.7	24.9	0.232	8.2	102

2.1.1 Fish breeder selection and breeding conditions

Once the stocks had reached maturity (3-4 months post-hatch) they were set up into breeding harems for optimal embryo production. In order to select spawners, fish would be netted from the stock tank and transferred to a 500 mL glass beaker allowing them to be viewed from the side. Sexually mature medaka exhibit dimorphism which is most easily seen in their dorsal and anal fins (Denny *et al.,* 1991). Males have a notch in their dorsal fin, and their anal fin appears elongated and pointed at the distal-posterior edge. Females have no notch in the dorsal fin and have a more compact anal fin that has a rounded groove in the middle. If separated early in the day, females may also be carrying a clutch of embryos. The breeding harems were set up in a ratio of 3 females to 2 males as outlined in the United States Environmental Protection Agency (US EPA) guidelines (Denny *et al.,* 1991). Briefly, the brood stock from one collection time point would be separated into males and females and then redistributed to 70-L tanks in groups of 24 females to 16 males. Usually there would be enough fish to set up three 70-L breeding tanks per cohort of stock fish.

The breeding harems would produce 200-400 eggs per tank. These fish would produce eggs for 2-3 months once set up into harems, after which egg quality would drop. A minimum of two groups of fish produced eggs at any given time, which allowed the collection of up to 2000 viable eggs daily. This permited experiments to proceed even if the egg quality in one group was poor (fertilization < 60% is considered poor). To accomplish this, the fish were cultured every 1.5 months by collecting 600 viable embryos, which provided for the replacement of a group of breeders every few months (allowing the set up of harems every ~2 months).

2.1.2 Egg collection/rearing conditions

Medaka embryos were obtained from adult fish (FLFII strain) by gently netting carrying females into a shallow white plastic tray with 2 cm of water. Embryos were gently stripped from each female with a gloved finger and the female was returned to the breeding harem. Embryos can be collected daily from the breeding harems in this manner. The embryos were rolled out on a piece of paper towel to remove any attached fibers or debris and were then separated into 200 mL glass dishes (200 embryos per dish max) containing rearing solution for viability evaluation. Rearing solution consisted of 0.1% NaCl, 0.0163% $MgSO_4 \cdot 7H_2O$, 0.004% $CaCl_2 \cdot H_2O$, 0.003% KCl, and 0.0001% Methylene blue, all made up in D.I. water. Unfertilized embryos or embryos with irregularities were discarded 24 hours after placing them in rearing solution. Embryos in glass dishes were kept on a plate shaker set to 60 RPM, in order to narrow down the time-to-hatch to 8-10 days, based on a paper by Farwell *et al.,* (2006). Embryos were kept in a 25°C temperature controlled room, rearing solution was renewed daily, and the embryos usually hatched within 8-10 days post fertilization.

2.2. Embryonic staging and end points

Select embryonic developmental stages of medaka were examined twice daily, using a dissecting stereomicroscope LEICA EZ4D (20x magnification) following the Iwamatsu (2004) fate map. Developmental abnormalities, mortality (lack of heartbeat), hatchability and time-to-hatch were all recorded daily. The endpoints evaluated and the earliest point they could be observed are summarized in Table 2.2. The majority of the

sub-lethal endpoints measured were developmental malformations, such as improper eye formation, spinal formation, or improper formation/inflation of the swim bladder.

Table 2.2 Japanese medaka embryo-toxicity endpoints used in this study. Hours post fertilization (hpf), days post fertilization (dpf).

	Time observable			
	24 hpf	48 hpf	96 hpf	8-10 dpf
Lethal endpoints (mortality)				
No heart beat		x	x	x
Lack of hatching (hatchability)				x
Sub-lethal developmental endpoints				
Development of eyes (microphthalmia)		x	x	x
Heartbeat/blood circulation (stasis)		x	x	x
Formation of edema/pericardial edema	x	x	x	x
Swim bladder inflation				x
Time-to-hatch				x
Overall Endpoints				
Mortality/hatchability				
Time-to-hatch				
Malformations				

2.3 Gene expression techniques

All Quantitative Reverse Transcription PCR (RT-qPCR) was performed in order to meet the specifications outlined by Bustin *et al.,* 2009, and Taylor and Mrkusich, 2014.

2.3.1 Sample extraction and RNA isolation through phenol-chloroform extraction

Embryos at a specific sampling time point were removed from treatment, rinsed three times in rearing solution, transferred to a 1.5 mL RNAse/DNAse free Eppendorf tube, any residual rearing solution was drawn off, and put on ice. Once all samples were collected, 250 µL of TRI reagent (PureZOL™ RNA Isolation Reagent, Bio-Rad) was added to each sample. After homogenization with a hand held homogenizer, 250 µL of TRI reagent was added, the samples were vortexed, and then centrifuged at 12,000 *g* for 10 minutes (min) at 4°C. After centrifugation the samples had three layers, a thin fatty layer on top, a clear layer in the middle, and an organic layer on the bottom. The middle layer was transferred to a new 1.5 mL RNAse/DNAse free Eppendorf tube, and let stand for 5 minutes at room temperature. Afterward 150 µL of chloroform was added to each sample and then they were vortexed. The samples were left to stand at room temperature until the layers separate, afterward they were centrifuged at 12,000 *g* for 15 min at 4°C. This resulted in three phases: a red bottom layer containing protein, an interface with DNA and a colorless upper aqueous phase containing RNA. The upper aqueous phase was transferred to a fresh 1.5 mL tube and 200 µL of isopropanol is added. The samples are then vortexed, left to stand for 5 min, and then centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was discarded and the RNA pellet is washed with 500 µL of 75% EtOH. The samples were vortexed then centrifuged at 7600 *g* for 5 min at 4°C after which the

excess EtOH was removed without disturbing the pellet of RNA. The RNA pellet was then dissolved into 50 µL of Tris-EDTA (TE) buffer and RNA extracts were stored at -20°C until use.

2.3.2 RNA purity and concentration using a TrayCell

RNA purity and concentration was determined using a 0.2 mm TrayCell and a Cary 50 Bio UV-Visible Spectrophotometer. Samples were first removed from the -20°C fridge, and thawed on ice. Samples were analyzed at the 260 nm and a 2000 factor, with factor = sample specific factor x virtual dilution factor (ssRNA = 40 x 50, respectively). Before a sample was run the Cary was zeroed using 2 µL TE buffer pipetted directly onto the mirror. Samples were run in duplicate by pipetting 2 µL sample onto the mirror. The TrayCell was cleaned between each sample/replicate by swabbing it with EtOH. A sample was determined to be acceptable if the purity ratio ($OD_{260/280}$) was > 1.7.

2.3.3 RNA quality using the Experion automated electrophoresis station

Samples were removed from the -20°C fridge and thawed on ice. Quality was assessed using the Experion RNA StdSens kit, following the protocol supplied by Biorad. Briefly, the electrodes were first cleaned by pipetting 800 µL of electrode cleaner into any middle well of the electrode cleaner chip, and placed into the Experion for 2 min. The chip was then removed, and 800 µL of diethyl pyrocarbonate (DEPC)-H_2O was pipetted into the DEPC chip, and left in the Experion for 5 min. After removing the DEPC chip, the Experion was allowed to air dry for a minute. This procedure was then repeated once. Both chips were knocked out on paper towel, allowed to air dry, and returned to storage after use. The gel and gel stain were prepared by pipetting 600 µL of gel stain into the filter

tube, which was then centrifuged at 1500 *g* for 10 min. This filtered gel is stable for up to 1 month. To prepare the gel stain, 65 µL of filtered gel and 1 µL of stain, were added to a 1.5 mL tube and vortexed. Samples and the RNA ladder were prepared by pipetting 2 µL of sample/ladder into 0.2 mL PCR tubes. These were then denatured for 2 minutes at 70°C, and left for 5 minutes on ice. Samples were within a specific concentration range of 25-50 ng/µL (min) – 500 ng/µL (max). Each chip ran 12 samples and one ladder. The RNA StdSens chip was then primed with 9 µL of gel stain into the gel priming well. The chip was inserted into the Experion priming station, and primed using setting B1. After priming the microchannels at the back of the chip were no longer visible. The chip was then loaded in the following order: 9 µL of filtered gel, 5 µL of loading butter into the 13 wells well, and 1 µL of samples + ladder into the wells. The chip was then placed in the Experion vortex station II for 1 minute. Afterward the chip was run on the Experion using the Experion RNA StdSens Assay. Following a run the Experion was cleaned with two rinses of DEPC H_2O. Quality was reported in RNA quality indicator (RQI), samples with a value of > 7 were deemed acceptable (not degraded).

2.3.4 Primer selection

Medaka genome sequences were found in the national center for biotechnology information (NCBI) database, and sequences were taken using fasta format. Primers were then designed using Primer3 with a product size range of 75-200 bp. Primers were checked by blasting them in the Kyoto encyclopedia of genes and genomes (KEGG), to make sure they did not have any cross over with other genes.

Primers were purchased from Invitrogen and arrived as a lyophilized powder. They were immediately suspended in Tris-EDTA (TE) buffer to a concentration of 100 µM/L.

Briefly, the primers were first centrifuged for a few seconds to collect them at the bottom of the tubes, TE buffer was added, samples were vortexed and spun down. Primers were split into 15 µL aliquots and stored at -20°C until use.

2.3.5 Primer validation (temperature gradient, concentration curve)

Primers were validated through an 8-point temperature gradient in order to verify that they anneal at 60°C. Briefly, a template RNA sample was prepared by pooling RNA extracted from embryos at multiple time points in development. After adjusting the RNA sample concentration to 40 ng/µL, the temperature gradient was run in duplicate, allowing 6 genes to be run per 96 well plate. Quantitative RT-PCR was run as per section 2.3.7 using the temperature gradient previously mentioned. Melt peaks were also checked to ensure only one product was created.

A 4-point primer concentration curve was also run using a 10 fold dilution. For each gene the following concentrations were tested in triplicate: 100, 10, 1, and 0.1 ng/µL. This allowed for 8 genes to be run per 96 well plate.

2.3.6 Reference gene validation

For every experiment the following reference genes were tested: ribosomal protein l7 (*rpl7*), ribosomal protein l8 (*rpl8*), eukaryotic elongation fact 1 alpha (*eEF1A*), TATA Box BiTATA Box Binding Protein Ending Protein (*tbp*), Beta-2-Microgobulin (*B2m*), and Beta-actin (*ACTB*). Primers for these proteins were validated in the same fashion as primers for experimental genes. For each experiment a subset of the samples were tested against all the reference genes. Data was analyzed using the NormFinder Microsoft excel

add-in, and the reference gene with the best stabilization value was used. Once selected the reference gene was run for all samples using the procedure in 2.3.7 (for the gene expression conducted in Chapter 6, *rpl7* was used).

2.3.7 RT-Quantitative PCR

RNA samples were aliquoted to a concentration of 40 ng/µL in TE buffer and stored at -20°C prior to use. All samples were run in triplicate, using the iTaq Universal SYBR Green One-Step Kit.

Primers were prepared by pipetting 10 µL of each primer aliquot into 90 µL TE buffer, and kept on ice until use. Afterward, a master mix was made up per gene tested using the following volumes per 20 µL reaction. The master mix was calculated for the total number of wells used + a 10% margin of error.

Compound	Per 20 µL Reaction
iTAQ SYBR	10 µL
iScrip RT	0.25 µL
Forward primer	0.2 µL
Reverse primer	0.2 µL
H_2O	6.85 µL

The SYBR Green One-Step Kits are stored at -20°C until use, and all reagents were thawed on ice. The master mix was prepared as follows, first the H_2O was added to a 2 mL Eppendorf tube, and then the SYBR green was slowly and carefully pipetted into the liquid. Afterward the primers were pipetted into the mix, and lastly the iScript RT was added and the solution was vortexed. 17.5 µL per well of Master Mix was then added to a Multiplate 96-Well Unskirted PCR Plate, Thin-Wall Polypropylene, while the plate was

kept on ice. Following this, 2.5 µL of sample was added to each well in triplicate, and the plate was sealed using a Microseal 'B' Adhesive Seal for PCR plates. The plate was then spun for ~5 seconds using a plate spinner. Finally, the plate was then run on the Biorad CFX using the following settings: setting, SYBR/FM; Reverse Transcription Reaction, 10 min at 50°C; Polymerase Activation and DNA Denaturation, 1 min at 95°C; Amplication: Denaturation at 95°C, 10 sec; Annealing/Extension + plate read at 60°C, 10-30 sec; Cycles, 45; Melt-Curve Analysis, 65-95°C with 0.5°C increments 2-5 sec/step.

Data was analyzed using the Bio-Rad CFX manager. Relative quantification was expressed using the $2^{-\Delta\Delta C_T}$ (relative change of change) method (Livak and Schmittgen, 2001).

Chapter 3: Embryo-toxicity pilot

3.1 Abstract

Japanese medaka (*Oryzias latipes*) embryos were exposed to ibuprofen, naproxen, and diclofenac, as well as 17β-estradiol, 17α-ethinylestradiol (EE2), and levonorgestrel, and their sub-lethal effects were determined. Medaka embryos were exposed 24 hours post fertilization until hatch to a range of concentrations (0.005 to 50 mg/L) of ibuprofen, naproxen, and diclofenac, as well as 0.05 to 500 µg/L 17β-estradiol, EE2, and levonorgestrel. There was no effect by any compound on hatchability, with > 80% of fry hatching across all treatments and controls.

There were effects on time-to-hatch, consisting of a decrease in the median time-to-hatch (by one day) for embryos exposed to 500 µg/L EE2 and levonorgestrel, 50 mg/L ibuprofen, and an increase in the median time-to-hatch by one day of embryos exposed to 50 mg/L diclofenac. Malformations including blood stasis, microphthalmia, pericardial edema, development of the spine, as well as inflation of the swim bladder were assessed following exposure. Failure to inflate swim bladder post-hatch was the most frequently detected malformation, as well as the most sensitive for embryos exposed to EE2, 17β-estradiol, levonorgestrel, and diclofenac. There was a trend of increasing failure of swim bladder inflation with increased concentrations of pharmaceuticals. This study demonstrates that embryonic exposure to high concentrations of pharmaceuticals have the potential to disrupt the formation/inflation of the swim bladder in Japanese medaka.

3.2 Introduction

Fish early life stages are known to be sensitive to the effects of exposure to pollutants and medaka (*Oryzias latipes*) are commonly used for this assay e.g. the US EPA 28 day toxicity test (Benoit *et al.,* 1991). Fish early life stage toxicity tests are usually focused on survival and growth, supplemented with behavioural and developmental abnormalities; however, they are not designed to provide information about the chemical mode of action. Fish early life stage toxicity testing is also both time consuming and animal intensive, which can be prohibitive if many compounds are needed to be screened. Thus, the development of alternative tests that have a reduced cost, reduced number of animals used, and that focus on the chemical mode of action are needed.

Fish embryos provide both the complexity and interactions of an intact organism, which enables the evaluation of chemically induced effects on multiple target organs. Fish embryo-toxicity tests are much shorter (3-9 days) than a fish early life stage toxicity test (1-3 months) and require fewer resources. Studies that characterize common chemically mediated mechanisms of toxicity for use in predicting adverse effects that relate to fish early life stage toxicity are in need (Volz *et al.,* 2011), and embryo-toxicity may be a useful tool to accomplish this. Common endpoints of embryo-toxicity tests include effects on time-to-hatch, malformations (skeletal deformities, pericardial edemas, blood stasis, malformed organs, etc.), and hatchability.

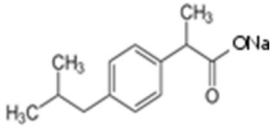
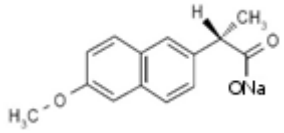
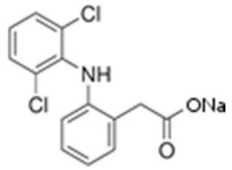
Pharmaceuticals consist of a wide range of medicinal compounds used for the treatment, cure, mitigation, or prevention of diseases in both humans and animals. Increases in health care investment, as well as a growing and aging population in society has led to a significant increase in consumption of pharmaceuticals in the last few decades

(Khetan and Collins, 2007). Pharmaceuticals find their way into aquatic ecosystems primarily through human consumption, elimination, and disposal via wastewater systems (Nikolaou *et al.,* 2007). These compounds, some of which may be highly potent, have the potential to cause detrimental impacts on many non-target aquatic organisms, which may in turn have broader repercussions on the structure and function of ecosystems.

Non-steroidal anti-inflammatory drugs NSAIDs are commonly found in the environment, being detected in both wastewater treatment plant effluent and surface waters at higher concentrations (usually in the µg/L range) when compared to other human pharmaceuticals (Aus der Beek *et al.,* 2016; Corcoran *et al.,* 2010; Fent, 2006; Helm *et al.,* 2012; Kolpin *et al.,* 2002; Stülten *et al.,* 2008; Ying *et al.,* 2009). Of all the NSAIDS, diclofenac, ibuprofen, and naproxen are most often detected globally; with diclofenac being the most commonly detected pharmaceutical compound (Aus der Beek *et al.,* 2016). A summary of the general structure and characteristics of the three NSAIDs used in this study can be seen in Table 3.1.

There have been some studies showing effects of NSAIDs on adult fish: exposure to ibuprofen has been linked to a decrease in frequency of spawning (Flippin *et al.,* 2007), an increase in plasma vitellogenin (Han *et al.,* 2010), decreased prostaglandin E2 (Morthorst *et al.,* 2013), similarly, exposure to diclofenac has been linked to a reduction in prostaglandin E2 synthesis, increased monocyte infiltration in the liver and mild tubular necrosis on the trunk kidney (Hoeger *et al.,* 2005). However, limited information is available considering the effects of NSAIDs on embryo development.

Table 3.1: Chemical structure, molecular formula and weight, and pKa of the NSAIDs ibuprofen, naproxen, and diclofenac.

	Ibuprofen Sodium	Naproxen Sodium	Diclofenac Sodium
Chemical Structure			
Molecular formula	$C_{13}H_{17}O_2Na$	$C_{14}H_{13}O_3$ Na	$C_{14}H_{10}Cl_2NO_2Na$
Molecular Weight (g/mol)	264.297	252.24	318.129
pKa	4.52[a]	4.15[b]	4.15[a]
log Kow	2.48[a]	3.18[b]	1.90[a]
Water solubility mg/mL (@ 25˚C)	100[c]	100[c]	50[c]

a). Scheytt *et al.,* 2005. b). drugbank.ca. c) Sigma-Aldrich. All other information in table gathered from pubchem.ncbi.nlm.nih.gov.

Reproductive steroids are an important component of the endocrine system as they are involved in sexual differentiation, behavior, and the development of secondary sex characteristics and gonads in fish (Kime, 1999, 1993). All of these factors are important to the reproductive success of a species. This study focused on two main classes: estrogens and progestins, specifically assessing the effects of embryonic exposure to 17α-ethinylestradiol (EE2), 17β-estradiol, and levonorgestrel (Table 3.2.).

17β-Estradiol is the major reproductive estrogen in female teleosts (Arukwe and Goksøyr, 2003). For normal reproduction in fish, 17β-estradiol acts along the hypothalamus-pituitary-gonadal axis, influencing behavior, gonad differentiation, and the production of vitellogenin and *zona radiata* proteins in the liver (Arukwe and Goksøyr, 2003; Kime, 1999). Exposure to 17β-estradiol has been shown to affect Java medaka (*Oryzias javanicus*) by significantly lowering fertility and reducing growth (Imai *et al.,* 2005).

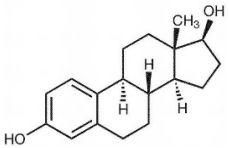
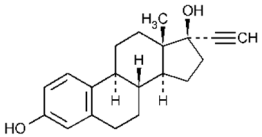
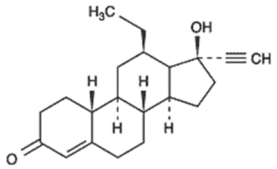
Synthetic pharmaceuticals can be extremely potent chemicals, and in the case of EE2, commonly used in oral contraceptives, are actually more potent than 17β-estradiol (Tyler *et al.,* 1998). Exogenous sex steroids administered during the embryonic development period of sex determination can strongly influence the course of sex differentiation in fish, suggesting they play a critical role in assignment of gonadal gender determination as well as subsequent differentiation (Devlin and Nagahama, 2002).

Synthetic progestins such as levonorgestrel are commonly used in oral contraceptives and hormone replacement therapy. Synthetic progestins mainly function by suppressing the mid-cycle peaks of luteinizing hormone, leading to an inhibition of ovulation, through negative feedback loops on the hypothalamus-pituitary-gonadal axis

(Erkkola and Landgren, 2005). Synthetic progestins have also been shown to have a binding affinity for the fish progestogen receptor (Pinter and Thomas, 1997). Levonorgestrel is of particular interest, as it has been shown to bioaccumulate in juvenile rainbow trout (*Oncorhynchus mykiss*) (Fick *et al.,* 2010). Exposure to levonorgestrel has also been shown to reduce embryo production and cause enlarged ovaries/testes (Zeilinger *et al.,* 2009). Zebrafish (*Danio rerio*) embryos exposed to 2-200 ng/L levonorgestrel for up to 144 hours exhibited altered expression levels for gene targets, such as the androgen receptor (Zucchi *et al.,* 2012), or vitellogenin mRNA (Notch and Mayer, 2013). Progestins have the potential to impact wildlife, and like estrogens, most of their observed effects have been mainly focused on reproduction.

The Japanese medaka (*Oryzias latipes*) female leucophore-free (FLFII) strain (Wakamatsu *et al.,* 2003) was used as a model species in this study. Japanese medaka embryos are excellent models for studying the effects of aquatic pollutants. The rapid development and clear chorion allows easy observation of embryogenesis and tracking of developmental malformations. One challenge with medaka is a wide variation observed in time to hatch for the embryos taking from 7 to 30 days (Appendices C1). Previous studies have demonstrated that housing medaka embryos on a plate shaker can narrow down the time-to-hatch Farwell *et al.,* 2006. This current study will determine if this improves the time-to-hatch for the FLFII strain. In this current study, Japanese medaka embryos were exposed to ibuprofen, naproxen, diclofenac, 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel from 24 hours post fertilization until hatch and effects on fish development and survival were recorded.

Table 3.2: Chemical structure, molecular formula and weight, and pKa of the 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel.

	17β-Estradiol	17α-Ethinylestradiol	Levonorgestrel
Chemical Structure			
Molecular Formula	$C_{18}H_{24}O_2$	$C_{20}H_{24}O_2$	$C_{21}H_{28}O_2$
Molecular Weight (g/mol)	272.388	296.41	312.453
pKa	-0.88 and 10.33[b]	-1.7 and 10.33[b]	-1.5 and 17.91[b]
log Kow	4.01	3.67	3.48
Water Solubility mg/L (@ 25˚C)	3.6[b]	11.3[b]	2.05[b]

a). Scheytt *et al.,* 2005. b). drugbank.ca; pKa for 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel are all predicted properties. All other information in table gathered from pubchem.ncbi.nlm.nih.gov.

3.2.1 Objectives

A pilot experiment was undertaken to follow up on the initial range finder performed in 2013 (Results in Appendix A1-D1). This pilot implemented new techniques and had more end points. The objectives of this experiment were to:

- determine if ibuprofen, naproxen, diclofenac, 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel cause sub-lethal effects when medaka embryos are exposed to these compounds;
- determine appropriate concentration ranges and endpoints for future experiments; and
- verify the use of a plate shaker to reduce variation in the time-to-hatch for the FLFII strain of medaka, and eliminate the mortality observed in the previous range finder controls.

3.2.2 Hypotheses:

H_{o1}: embryonic exposure to ibuprofen, naproxen, diclofenac, 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel does not result in any sub-lethal effects including: malformations, effects on time-to-hatch, and hatchability.

H_{o2}: use of a plate shaker to rear embryos will not affect the time to hatch or hatchability of the Japanese medaka embryos

3.3 Materials and methods

3.3.1 Pilot methodology

The experiment was conducted using Japanese medaka embryos to determine what concentrations of pharmaceuticals cause significant effects on embryo development (experiment started July 7th 2014, concluded end of August 2014). Initial stocks of medaka were obtained from the National Institute for Basic Biology (Okazaki, Japan). Medaka embryos were obtained from adult fish (FLFII strain) bred under standardized conditions (section 2.1). Embryos were collected as described in section 2.1.2 and assessed for viability. This experiment was run in triplicate with 10 embryos used per replicate. Ten embryos per treatment (180 total embryos) were randomly distributed across three replicate 6-well microplates, containing five treatments and one control. Ten embryos were placed in each well with 10 mL of test solution renewed daily.

3.3.2 Pilot conditions of embryo exposure

Embryos were assessed for viability 24 hours post fertilization, and exposure to treatments started following viability assessment. A semi-static exposure was conducted until hatch (95% exchange every 24 hours until hatch) using plastic 6-well plates. Pharmaceutical stocks were prepared before commencing the experiment and renewal solutions were prepared daily. Medaka embryos were maintained in a temperature controlled room maintaining a constant temperature of 25°C and a 16:8h (light: dark) photoperiod with 30 minutes of dawn and dusk included in the light period. Plates were kept on a plate shaker set to 60 RPM, in order to narrow down the time-to-hatch, based on a paper by Farwell *et al.,* 2006. Five treatment concentrations and a control were tested

per compound and are described in Table 3.3. Concentrations sub acute concentrations based on the results from the range finder (Appendices A-C).

Table 3.3 Concentrations of three NSAIDs: ibuprofen, naproxen, diclofenac, as well as three hormones/Synthetic hormones: 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel, tested for the pilot experiment.

Treatment	Concentration (Nominal)				
NSAIDs	50 mg/L	5 mg/L	500 µg/L	50 µg/L	5 µg/L
Hormones/synthetic hormones	500 µg/L	50 µg/L	5 µg/L	0.5 µg/L	0.05 µg/L

3.3.3 Solution preparation

NSAID stocks were purchased from Sigma-Aldrich, and prepared in 500 mL of rearing solution. For each NSAID stock 27.7 mg of ibuprofen sodium salt (lot#BCBJ1499V), 27.4 mg of naproxen sodium salt (lot#118k1353) and 26.9 mg of diclofenac sodium salt (lot#BCBG3484V) were each dissolved into 500 mL of rearing solution in 1000 mL glass Pyrex® bottles. NSAID solutions were prepared daily by performing serial dilutions using the stock solution into 50 mL Falcon tubes. For example, 36 mL of 500 mg/L ibuprofen stock was transferred to a 50 mL Falcon tube. Then 3.6 mL of this solution was transferred to a new tube along with 32.4 mL of rearing solution to achieve a final concentration of 50 mg/L in the new tube. This was repeated for the 5, 0.5 and 0.05 mg/L concentrations. Finally, 10 mL of solution was transferred to a 6-well plate in triplicate for each concentration as well as the control. These solutions were renewed every 24 hours in the same manner

EE2 (lot#0.96k1686), and 17β-estradiol (lot#010M0142), were also purchased from Sigma-Aldrich, while levonorgestrel (lot#4-ARP-37-1) was purchased from Toronto Rearch Chemicals. For EE2, 17β-estradiol, and levonorgestrel treatments, 2.5 mg of compound was first dissolved in 500 µL of acetone in a 2 mL glass sampler vial creating a 5000 mg/L super stock. This stock was used to make up sampler vials of 5000, 500, 50, 5, and 0.5 mg/L in acetone through serial dilution. To prepare working solutions 5 µL of each respective concentration was diluted in 50 mL of rearing solution using a glass 50 mL volumetric flask.

All stock solutions were stored at 4°C until use. These solutions were renewed every 24 hours in the same manner as described previously. Three replicate wells were

used per treatment, each well contained 10 embryos.Embryos and larvae were observed daily using a dissection microscope until hatched (7-16 days).

3.3.4 End points

Select embryonic developmental stages of medaka were examined twice daily, using a dissecting stereomicroscope LEICA EZ4D (20x magnification) following the Iwamatsu (2004) fate map. Developmental abnormalities, mortality/hatchability and time-to-hatch were recorded daily. The endpoints were evaluated and the earliest point they could be observed is summarized in section 2.2, Table 2.2.

3.3.5 Statistical analysis

As the sample size was small and had high variance, data were analyzed with $\alpha = 0.1$ instead of the traditional 0.05 to increase the power of the analysis. This approach guarded against excessive type II error, which in the case of this experimental design would be concluding that the pharmaceuticals caused no effect, when in fact they did. Data were first assessed for normality using Shapiro-Wilk W test, as well as for homogeneity of variance using a Brown-Forsythe test. If the data passed, one-way analysis of variance (ANOVA, $p \leq 0.1$) were performed, confirmed by Dunnet Post – hoc Test, $p \leq 0.1$. If the data failed normality, Kruskal-Wallis one-way analysis of variance on ranks were performed (ANOVA on ranks, $p \leq 0.1$), confirmed by multiple comparisons versus their respective control group (Dunn's method $p \leq 0.1$). For endpoints, malformations and hatchability were calculated as a percent of malformations/hatched per well (n=3), time to hatch was recorded for individual larvae (10 per well, n=30). All statistical analyses and graphs were performed/created using SigmaPlot 13.0 software.

3.4 Results

The small sample size of the range finder experiment (n = 5, 2 replicates) made it challenging to determine significance at many of the concentrations tested (full results of range finder: Appendices A1-C1). There also was a very large range in time-to-hatch (up to 28 days). The subsequent pilot embryo-toxicity experiment improved on the previous design by using a plate shaker to narrow down the time-to-hatch and improve hatchability (Figure 3.1). Due to a larger population size, the pilot study was also conducted with a larger sample size (n = 10, 3 replicates) and consequently, results were greatly improved compared to the range finder results.

3.4.1 NSAIDs

Hatchability was > 80% for all concentrations of NSAIDs tested, and there was no significant decrease in hatchability due to exposure observed ($p > 0.1$, Figure 3.2a).

Many types of malformations were observed in the developing embryos of treatment fish including: blood stasis, pericardial edema, spinal malformation, failed swim bladder inflation, and microphthalmia (Figure 3.3a). However, many of these malformations were also observed in the control, with controls exhibiting ~15% malformed embryos on average. The most common malformation observed in NSAID treated embryos was a failure of swim bladder inflation after hatching (Figure 3.5). This was significantly increased ($p \leq 0.001 \leq 0.1$) in the 50 mg/L diclofenac treatment. Of the three NSAIDs tested diclofenac caused the highest proportion of malformations. Ibuprofen and naproxen did not cause any significant increase in malformations when compared to their respective controls.

Exposure to 0.05 mg/L naproxen caused a significant delay in time-to-hatch; with a median time-to-hatch of 10 days post fertilization (dpf) compared to the controls of 9 dpf ($p = 0.057 \leq 0.1$, Table 3.2). Ibuprofen caused a significant decrease in time-to-hatch ($p = 0.025 \leq 0.1$) in the 50 mg/L treatment with a median of 8 dpf compared to 9 dpf for the controls. Diclofenac caused a significant increase in time-to-hatch in the 50 mg/L treatment with a median of 10 dpf compared to 9 dpf for the controls ($p \leq 0.001 \leq 0.1$). Diclofenac also had the highest observed time-to-hatch of 16 dpf in the 50 mg/L treatment (all other concentrations and compounds tested had 9-10 dpf as the maximum time-to-hatch). With the exception of the 50 mg/L diclofenac treatment, all embryos hatched between 7 and 10 dpf.

3.4.2 Natural/synthetic hormones

Hatchability was > 80% for all concentrations of EE2, 17β-estradiol, and levonorgestrel tested, and there was no significant reduction in hatchability due to exposure observed ($p > 0.1$, Figure 3.2b). These results were quite similar to those observed for the NSAIDs tested.

Similar to the NSAIDs treatments, many types of malformations were observed in the developing embryos including: blood stasis, pericardial edema, spinal malformation, failed swim bladder inflation, and microphthalmia (Figure 3.3a). Images of these malformations in the embryos treated with 17β-estradiol and levonorgestrel are shown in Figure 3.4. However, blood stasis, pericardial edema and failed swim bladder inflation on hatch, were also observed in the controls. While observed blood stasis, pericardial edema and micropthalmia were not significantly elevated when compared to the controls in any

treatment ($p > 0.1$). The most common malformation observed in embryos treated with 17β-estradiol, EE2, and levonorgestrel was a failure of swim bladder inflation after hatching. There was elevated swim bladder inflation failure observed in most of the treatments relative to the controls but this was only significant at the higher concentrations. Impaired swim bladder inflation was observed in 3-76% (averages of concentrations tested) of embryos exposed to levonorgestrel, 32-74% of embryos exposed to EE2, and 10-37% of embryos exposed to 17β-estradiol. However, failure of swim bladder inflation was significant ($p = 0.034 \leq 0.1$, $p = 0.004 \leq 0.1$,) in the 500 µg/L 17β-estradiol and levonorgestrel treatments, respectively. Exposure to 0.5 and 50 µg/L 17β-estradiol also resulted in a significant increase ($p = 0.058 > 0.1$). Furthermore, controls had up to 20% of the hatched fry unable to inflate by 24 hours post-hatch. Overall, all compounds caused a high proportion of malformations.

17β-Estradiol caused no significant effect on time-to-hatch, typically taking between 8-9 dpf to hatch (Table 3.4). Levonorgestrel exposure caused a significant decrease in time-to-hatch ($p = <0.001 \leq 0.1$) in the 500 µg/L treatment despite having the same median time-to-hatch of 8 days. Levonorgestrel had a trend of embryos hatching out earlier in all concentrations tested (at 7 dpf, compared to the control hatching time of 8 dpf). EE2 also caused a significant decrease in time-to-hatch in the 500 µg/L treatment (median of 8 dpf compared to 9 dpf for controls) ($p = <0.001 \leq 0.1$).

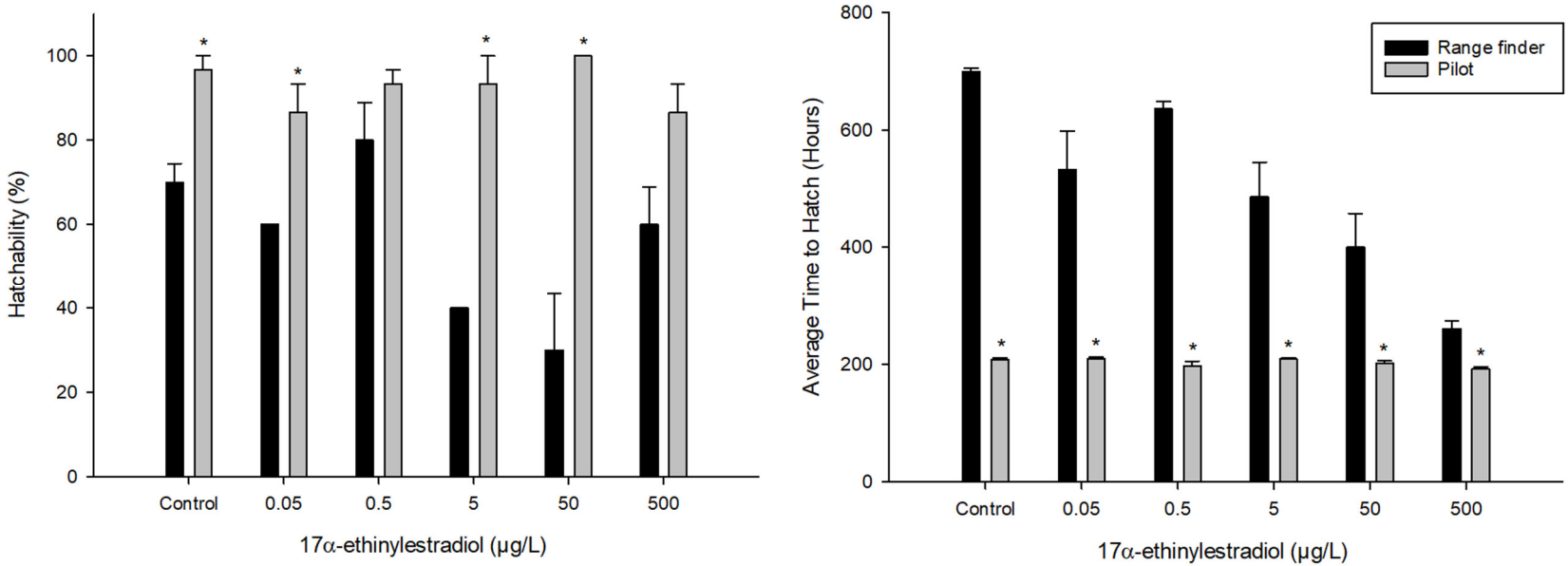


Figure 3.1: Comparison between the range finder (Appendices A-C) (black) and the current pilot experiment (Chapter 3) (grey) results following exposure to 17α-ethinylestradiol. The use of a plate shaker at 60 RPM and increasing sample size from 5 to 10 improved the time-to-hatch and hatchability. Bars represent mean values ± standard error (n = 10 for the range finder, n = 30 for the pilot). *Indicates significant difference (between pilot/range finder at that concentration) hatchability was tested using a 2-tailed t-test ($p < 0.1$), and time-to-hatch was determined using Mann-Whitney rank sum test ($p < 0.1$).

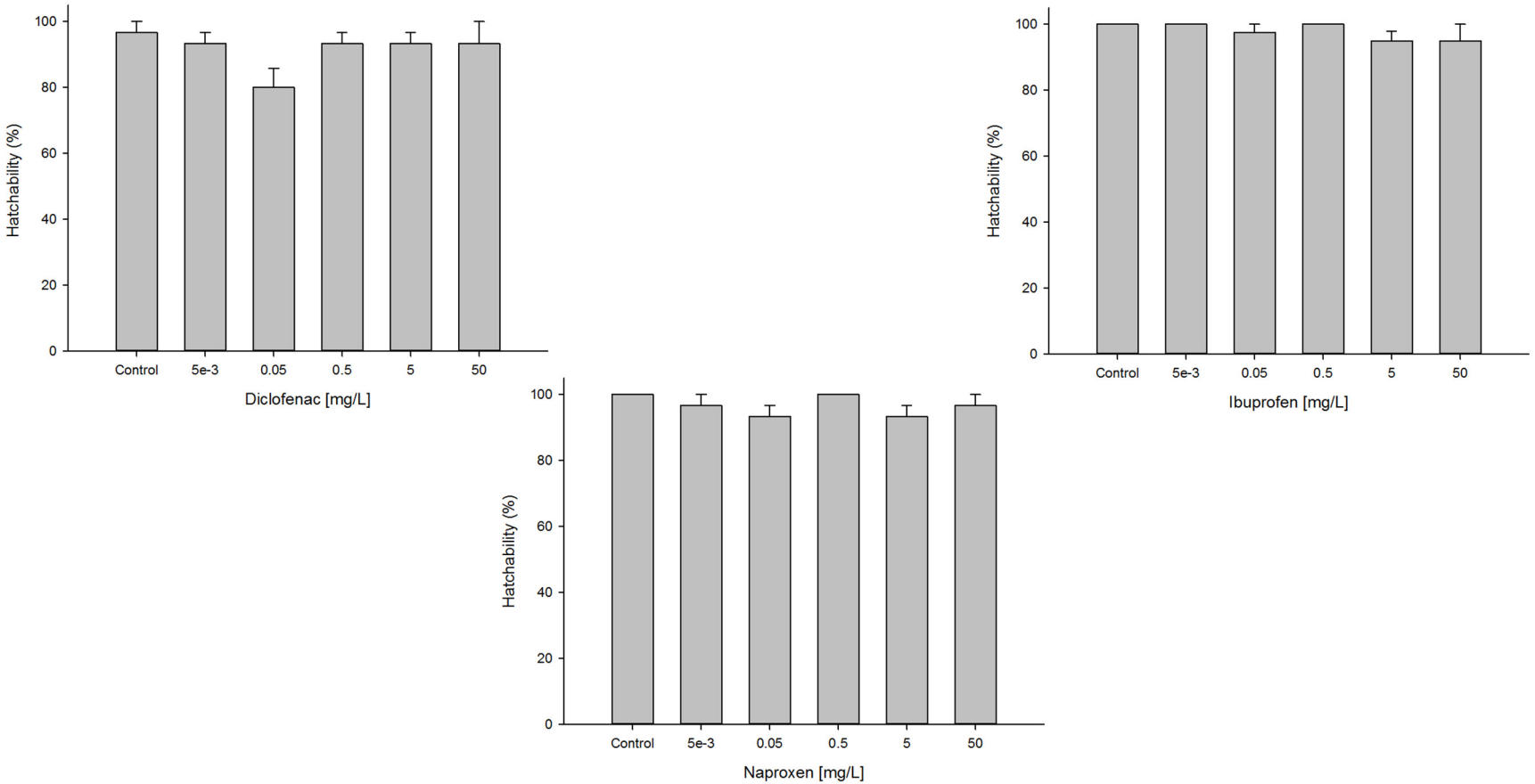


Figure 3.2a: The hatchability (%) of medaka embryos exposed to nominal concentrations of ibuprofen, naproxen, and diclofenac until hatch. Bars represent mean ± standard error (SE), three replicates of 10 embryos. No significant difference between treatments and controls (ANOVA, confirmed by Dunn's post-hoc test ($p > 0.1$)).

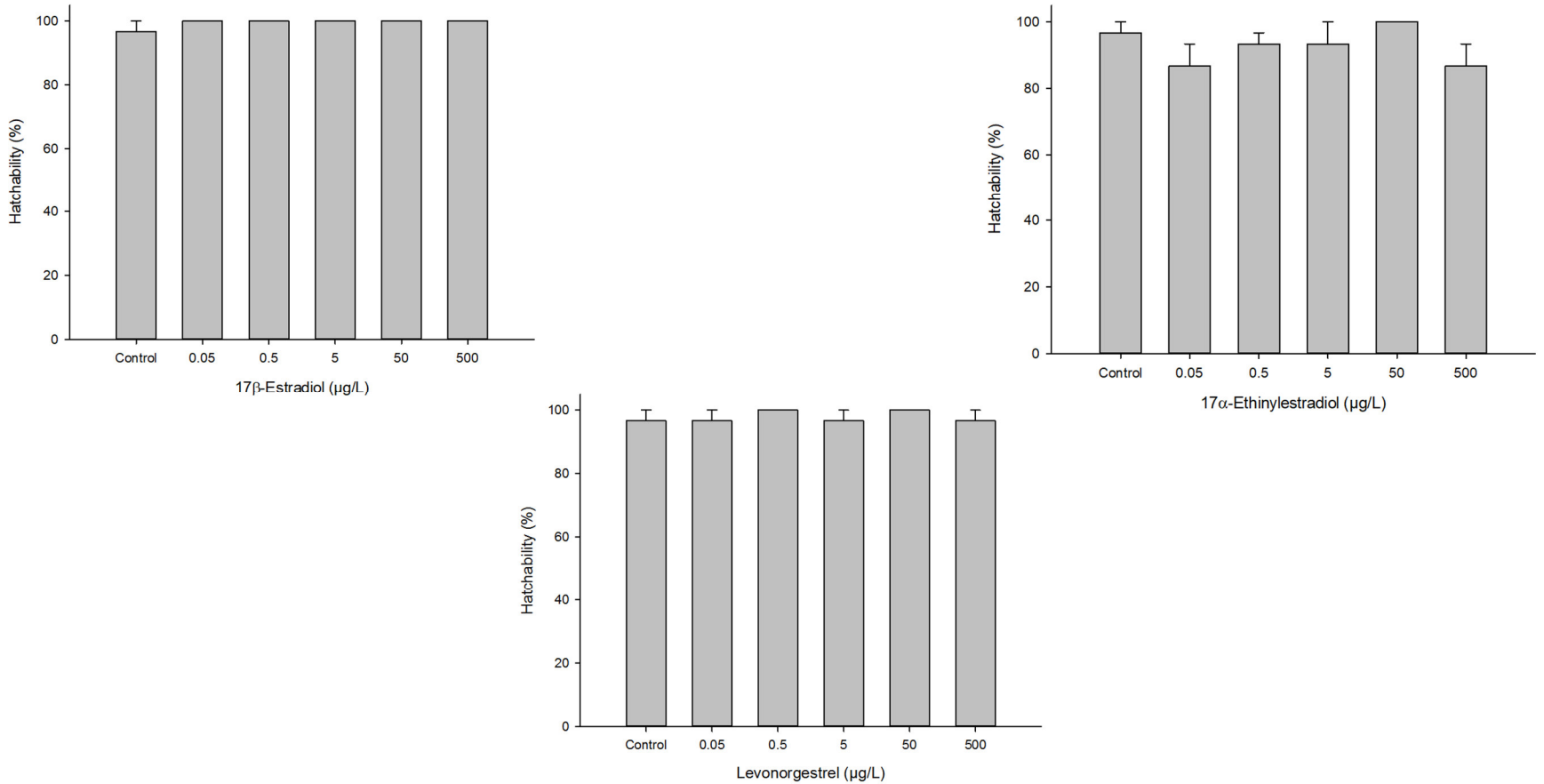


Figure 3.2b: The hatchability (%) of medaka embryos exposed to nominal concentrations of 17α-ethinylestradiol, 17β-estradiol, levonorgestrel, until hatch. Bars represent mean ± SE, three replicates of 10 embryos. No significant difference between treatments and controls (ANOVA, confirmed by Dunn's post-hoc test ($p > 0.1$)).

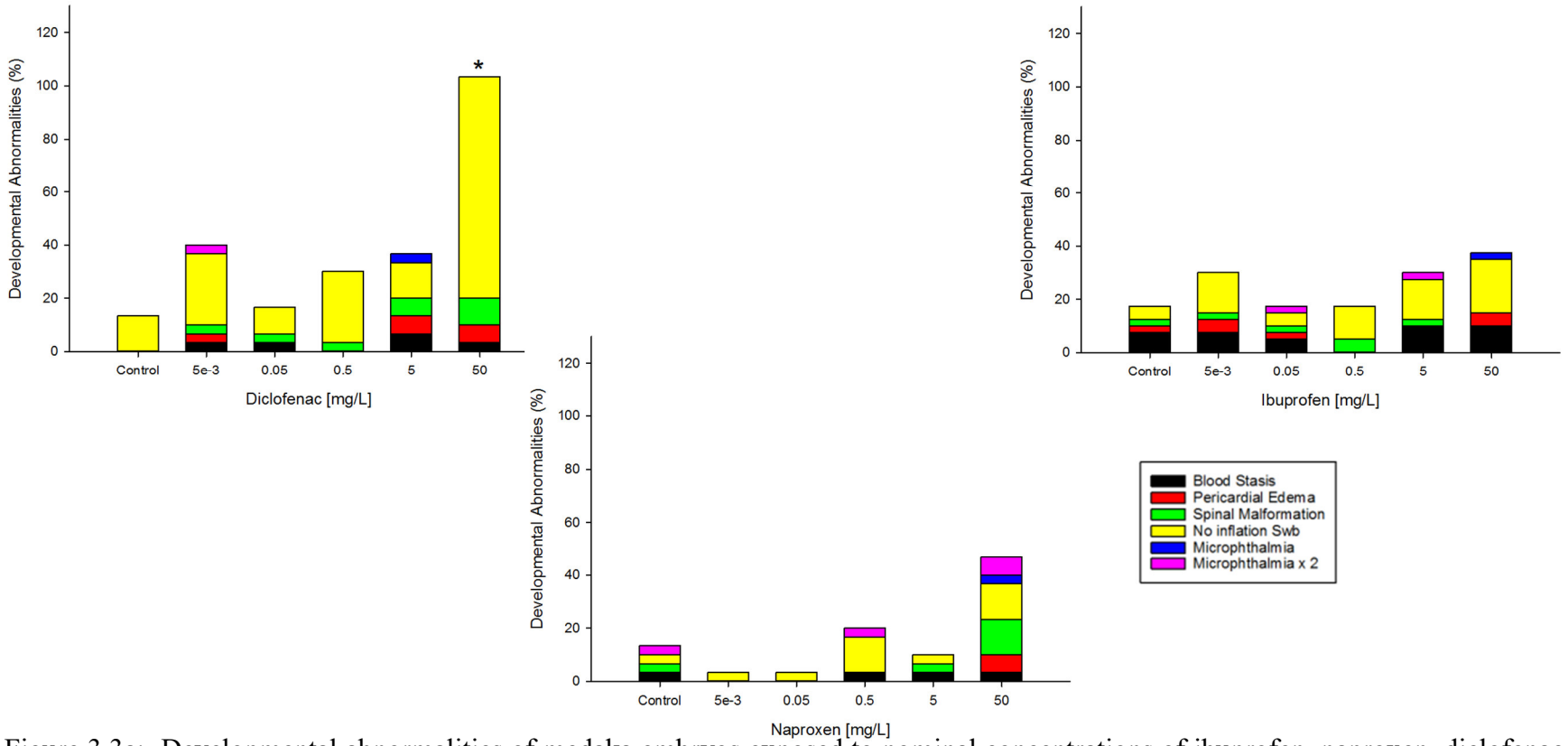


Figure 3.3a: Developmental abnormalities of medaka embryos exposed to nominal concentrations of ibuprofen, naproxen, diclofenac until hatch. Bars represent the relative amount of each type of malformation observed by treatment (embryos can exhibit more than one type of malformation; bars represent the mean of each type of malformation of three replicates (n = 3) consisting of 10 embryos). *Indicates significant difference from control (ANOVA, confirmed by Dunn's post-hoc test ($p < 0.1$)), swim bladder (swb).

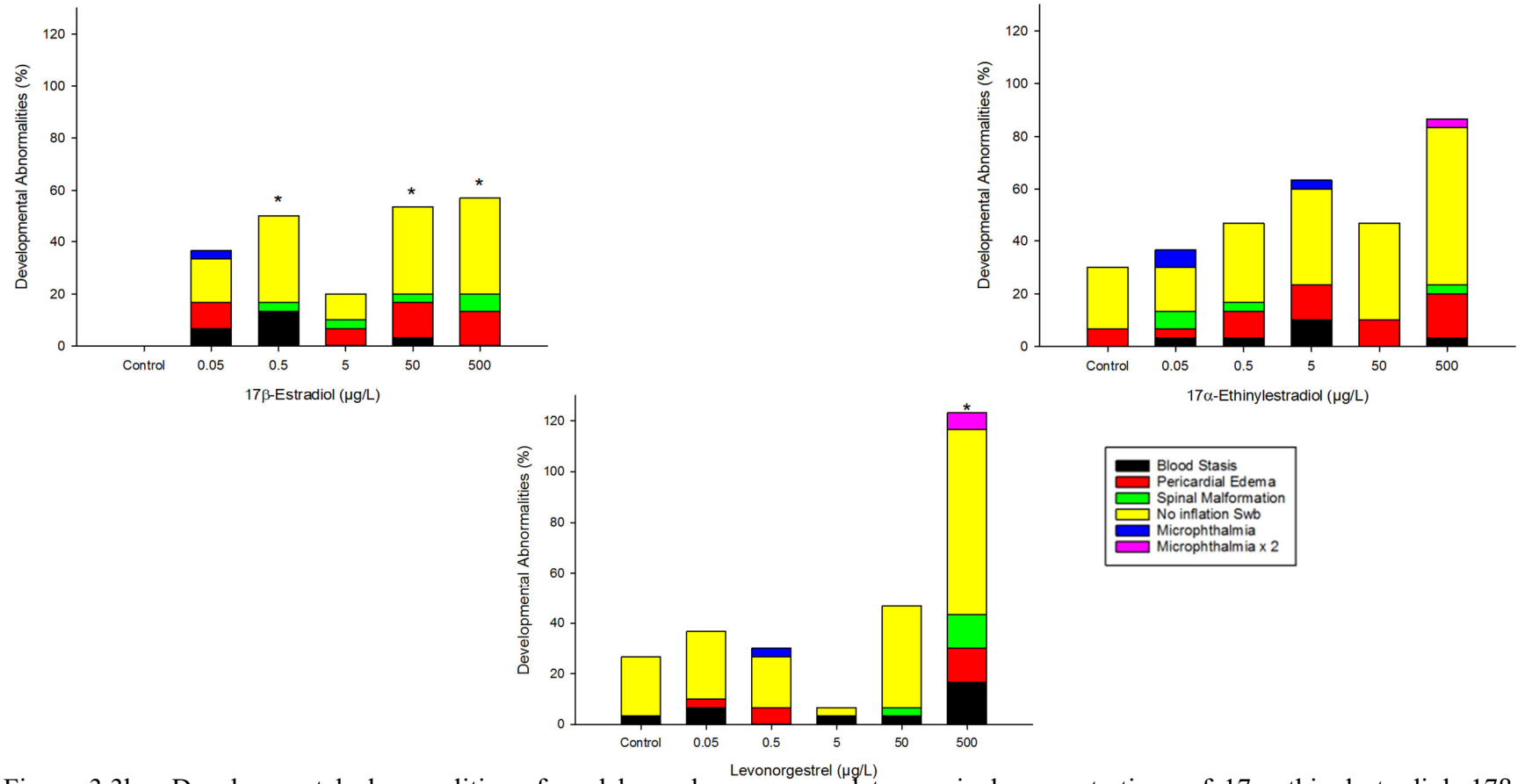


Figure 3.3b: Developmental abnormalities of medaka embryos exposed to nominal concentrations of 17α-ethinylestradiol, 17β-estradiol, levonorgestrel, until hatch. Bars represent the relative amount of each type of malformation observed by treatment (embryos can exhibit more than one type of malformation; bars represent the mean of each type of malformation of three replicates (n = 3) consisting of 10 embryos). *Indicates significant difference from control (ANOVA, confirmed by Dunn's post-hoc test ($p < 0.1$)).

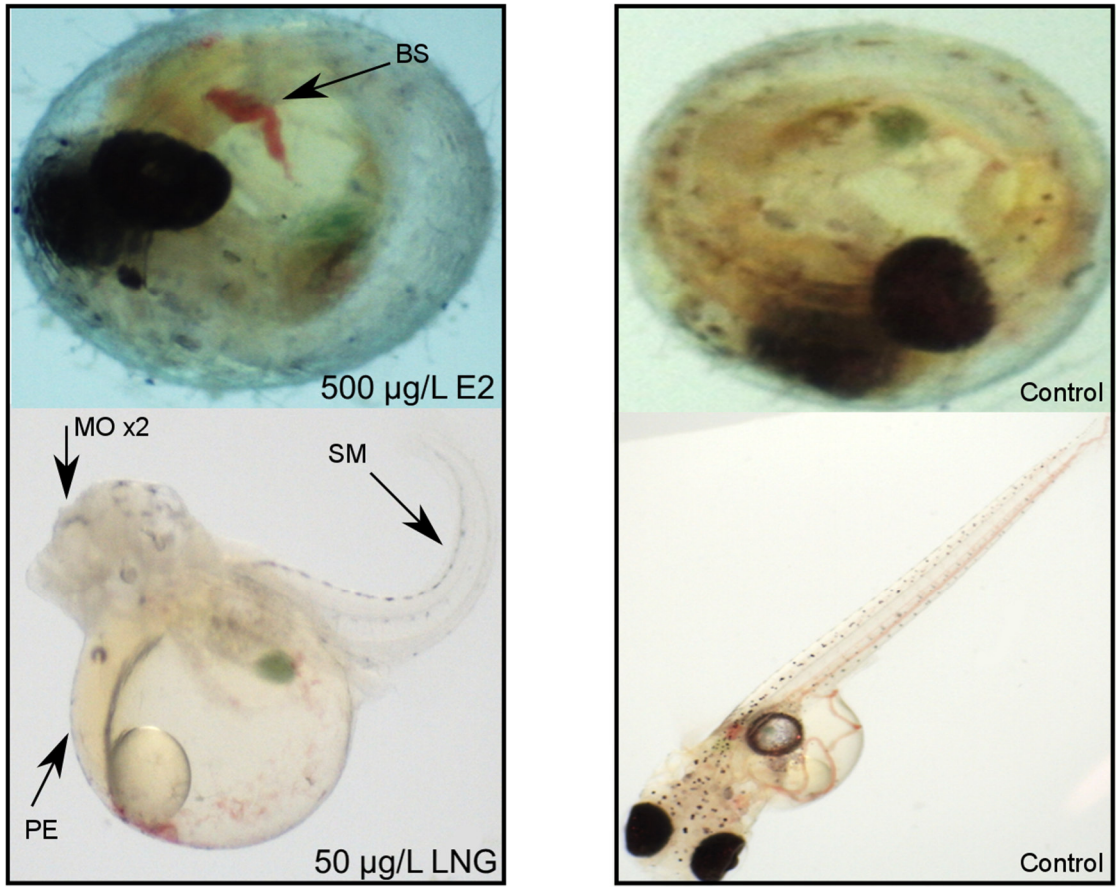


Figure 3.4: Examples of malformations observed following exposure to 500 µg/L 17β-estradiol (E2) showing blood stasis (BS), as well as 50 µg/L levonorgestrel (LNG) demonstrating microphthalmia in both eyes (MO x2), a spinal malformation (SM) and pericardial edema (PE). Controls of the same age (5 days post-fertilization above, and 1 day post-hatch below) are shown opposite.

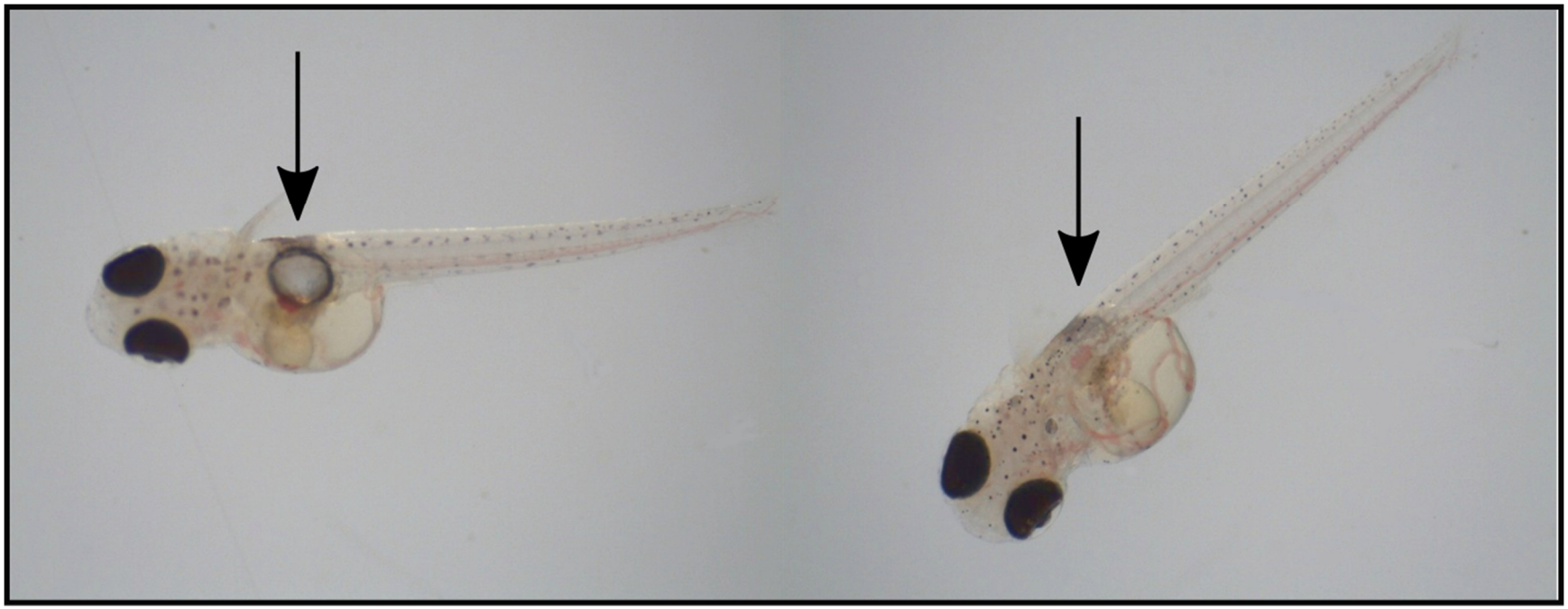


Figure 3.5: Medaka larvae 24 hours post-hatch with (leftmost) and without (rightmost) an inflated swim bladder.

Table 3.4: Median time-to-hatch (days (min, max)) for ibuprofen (IBU), naproxen (NAP), diclofenac (DIC), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and levonorgestrel (LNG), n=30. Concentrations for EE2, 17β-estradiol and LNG displayed to the left in µg/L and concentrations for DIC, IBU and NAP displayed in mg/L. *Indicates a significant difference from control (ANOVA on ranks, $p \leq 0.1$, Dunn's post hoc $p \leq 0.1$).

[µg/L]	EE2	E2	LNG	[mg/L]	DIC	IBU	NAP
Control	9 (7,10)	9 (8,9)	8 (8,9)	Control	9 (8,9)	9 (8,9)	9 (8,10)
0.05	9 (8,9)	9 (8,9)	8 (7,9)	0.005	9 (8,10)	9 (8,9)	9 (8,10)
0.5	8 (7,10)	9 (8,9)	8 (7,9)	0.05	9 (8,10)	8 (8,9)	10 (8,10)*
5	9 (7,9)	9 (8,9)	8 (7,9)	0.5	9 (8,9)	9 (8,9)	9 (8,10)
50	9 (7,9)	9 (8,9)	8 (7,9)	5	9 (8,10)	8 (8,9)	9 (8,10)
500	8 (7,9)*	9 (9,9)	8 (7,9)*	50	10 (8,16)*	8 (8,9)*	8 (8,10)

3.5 Discussion

The range finder conducted previously (Appendices A-C) had a small sample size (n = 5, 2 replicates) which made it difficult to determine significance at many of the concentrations tested. Of particular concern was the large variation in time-to-hatch (taking anywhere from 7 to 33 days), as well as the high mortality (in many cases due to the larvae not hatching at all). The variation in time-to-hatch was completely negated by using an agitation method (Farwell *et al.,* 2006), consisting of a placing the embryo containing plates on plate shaker set to 60 RPM. By using this method almost all of the medaka embryos in this experiment hatched within 11 days post fertilization. One potential issue with narrowing the time-to-hatch, is reducing the time of exposure to the chemicals. However, previous studies have shown little difference in toxicity for Japanese medaka embryos exposed to mixtures of polycyclic aromatic hydrocarbons, as well as mixtures of naphthenic acids when comparing an 18 day standard toxicity test to a modified agitation protocol with fish hatching between 7 and 10 days (Farwell *et al.,* 2006).

None of the treatments of both NSAIDs and natural/synthetic hormones caused significant mortality. All treatments and controls had > 80% hatchability. This is in contrast with the previous range finder experiment that measured > 30% mortality in some of the controls, and high mortality (up to 80%) in some of the treatments. One factor that may have contributed to the mortality measured in both tested and control embryos was a failure to hatch. After switching to a narrowed time-to-hatch window, there was a significant reduction in the mortality observed. As the goal of this experiment was to establish sub-lethal effects, the use of the plate shaker permitted success. These results are in contrast with those of Versonnen and Janssen, (2004) who observed a significant

reduction in hatching success by zebrafish exposed to 100 ng/L EE2 (67% compared to 95% in controls), as well as Ortiz-Zarragoitia *et al.,* (2006) who found that exposure to 5 µg/L EE2 caused a 50% reduction hatchability compared to controls. However, both these studies exposed embryos from hours after fertilization until hatch, whereas this experiment exposed day old embryos until hatch. It is possible that the early time period of embryo development of is more sensitive to EE2, and/or that the embryos are more sensitive to chemical exposure prior to the chorion hardening.

Hatching is an essential step in the survival of larval fish. Delayed hatch will result in an organism being more susceptible to predation, and if the hatch is completely inhibited will result in death. Aquatic pollutants have been shown to significantly impact the time-to-hatch: a delay in time-to-hatch of Japanese medaka embryos has been observed following exposure to cypermethrin (an insecticide), thiobencarb (a herbicide), as well as a bisphenol A metabolite (4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene, an endocrine disrupting compound) (González-Doncel *et al.,* 2004; Ishibashi *et al.,* 2005; Villalobos *et al.,* 2000). Exposure of parental Japanese medaka to 0.1 µg/L ibuprofen resulted in significant delayed hatching of embryos (Han *et al.,* 2010). Nano-injection of diclofenac in medaka embryos has also resulted in a delayed time-to-hatch (Nassef *et al.,* 2010). This study detected a significant delay in time-to-hatch following exposure to 50 mg/L diclofenac and 0.05 mg/L naproxen. A significant reduction in time-to-hatch was found following exposure to 500 µg/L levonorgestrel and EE2, as well as 50 mg/L ibuprofen. Environmental pollutants could affect hatching enzymes by either inducing early hatch through stimulation or delay through their inhibition (Gray and Metcalfe, 1999). Early

hatching can also be a result of the larvae trying to escape from an endangered/unsuitable environment (Korwin-Kossakowski, 2012).

The main and most sensitive malformation following chemical exposure in this experiment was a failure for the hatched larvae to inflate their swim bladder. Other developmental malformations observed included microphthalmia, pericardial edemas, blood stasis, and spinal malformations. However, none of the malformations aside from swim bladder inflation faiure were significantly elevated in treated fish compared to the controls.

Of the three NSAIDs tested, diclofenac caused significant malformations, while both naproxen and ibuprofen did not cause any significant abnormalities compared to the controls. Diclofenac has been shown to affect the development of medaka embryos, with nano-injection of diclofenac causing a reduction survival rate, a delay in development, and hemorrhages in the developing embryos (Nassef *et al.,* 2010). Zebrafish embryos exposed to 5-10 mg/L diclofenac displayed a disruption in actin organization and muscle fiber alignment, leading to malformations in the somites of the developing embryos (Chen *et al.,* 2011). Finally, exposure to diclofenac has been recently linked to teratogenicity in the form of developmental malformations in *Xenopus laevis* embryos (Cardoso-Vera *et al.,* 2017; Chae *et al.,* 2015). However, both of the experiments using zebrafish and medaka did not mention examining swim bladder inflation following exposure, nor did the *Xenopus* study note any effects on lung development. Although, there have been some reports of maternal exposure to diclofenac resulting in adverse effects on human fetal lungs such as pulmonary hypertension (Siu and Lee, 2004). Diclofenac may act as a teratogen due to its inhibition of voltage-gated sodium channels in myoblasts (Fei *et al.,* 2006). Diclofenac

has also been shown to have estrogenic effects (Gröner *et al.,* 2017). Due to its multimodal mechanisms of action, diclofenac may cause a wide range of physiological effects (Chae *et al.,* 2015).

All three natural/synthetic hormones, levonorgestrel, EE2, and 17β-estradiol, had a trend of increased swim bladder inflation failure with increasing concentration. These results support the findings of Santos *et al.,* (2014), who also found that the most prominent malformation observed was a failure of swim bladder inflation following zebrafish embryonic exposure to endocrine disrupting compounds such as EE2. The Santos *et al.,* 2014 study demonstrated that endocrine disrupting compounds interfere with the estrogen receptor/androgen receptor signaling pathways to influence cell apoptosis related pathways, leading to an impairment of physiological processes essential to normal development during embryogenesis. It is possible that diclofenac causes swim bladder inflation failure due to its effects on a similar pathway to 17β-estradiol/EE2/levonorgestrel. This could explain why it was the only NSAID that had similar effects to these compounds. Other studies have also found swim bladder inflation failure to be a sensitive endpoint following embryo exposure to bitumen, cypermethrin, and urban storm water to name a few (González-Doncel. *et al.,* 2004; Madison *et al.,* 2015; Skinner *et al.,* 1999), refer to Table 1.3 for a complete summary.

One confounding factor was that up to 20% of the controls were unable to inflate their swim bladders at hatch. Previous work by González-Doncel *et al.,* (2003), who found medaka embryo exposure to permethrin results in swim bladder inflation failure, also measured up to 20% non-inflation in their controls. However, they demonstrated that the majority (98-99%) of unexposed medaka inflated their swim bladders within 3 days post-

hatch. One problem with this current study design was that the swim bladder inflation was only evaluated shortly after hatching. It is not possible to know if this effect was simply a delay in inflation or if it was permanent. Future studies using Japanese medaka embryos should be extended to 3 days post-hatch if swim bladder inflation failure is a major endpoint of interest.

Swim bladder inflation failure is a significant endpoint that may directly affect the survival of individual fish. It is likely that the longer larvae are affected, the less chance they have to capture food, which could result in reduced growth. Even if this delay in inflation is temporary in terms of hours or days, a few hours of greater vulnerability to predation may prove crucial to the survival of larvae. While the effects of embryonic exposure to ibuprofen and naproxen were relatively minor, exposure to diclofenac, 17β-estradiol, EE2, and levonorgestrel warrant further exploration. Of particular concern was their effects on swim bladder inflation. Thus the impacts of embryonic exposure to EE2, levonorgestrel, and diclofenac on larval swim bladder inflation will be assessed, and the effects up to three days post hatching will be determined.

Chapter 4: Embryo-toxicity and mixture toxicity experiments

4.1 Abstract

Fish embryos are useful models for the environmental risk assessment of chemicals since their small size and quick development permits small-scale high-throughput analyses, and monitoring developmental abnormalities caused by pollutant exposure in fish embryos can allow for the prediction of long-term effects on populations. The objective of the first part of this study was to determine the effects of embryonic exposure to diclofenac, 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel on swim bladder inflation failure of Japanese medaka (*Oryzias latipes*), and to determine effective concentration-response curves. Fish embryos were exposed 24 hours post fertilization until hatch to nominal concentrations of 1, 10, 32, 320, 1000 µg/L 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel, as well as to 0.32, 1, 3.2, 32, and 100 mg/L diclofenac, and were monitored up to 72 hours post-hatch. The main effect observed across all four compounds was a linear increase (24 hour R^2 varied from 0.80 to 0.99) in the inhibition of swim bladder inflation success with increasing exposure concentration (24, 48 and 72 hours post-hatch). Based on these concentration-response curves, combinatorial experiments were designed using no-observed-effect concentratrions obtained from the first study. The main effect observed was a significant decrease in inflation success 24 hours post-hatch following a binary mixture of levonorgestrel and 17α-ethinylestradiol, as well as a significant decrease in swim bladder inflation success at all times for the quaternary mixture of all four compounds. This study demonstrates that embryonic exposure to pharmaceutical compounds can result in swim bladder inflation failure of the hatched larval medaka up to 72 hours post hatch both alone and in combination.

4.2 Introduction

Swim bladder inflation is a biologically critical endpoint as fish that are unable to inflate their swim bladders are unlikely to survive long in the wild. Embryo testing also facilitates mixture toxicity investigations, as it allows multiple experiments to be undertaken in a short period of time. A number of studies have shown chemical induced delay or inhibition of swim bladder inflation of newly hatched medaka using permethrin (González-Doncel *et al.,* 2003), PCB 126 (Jönsson *et al.,* 2012), urban storm water (Skinner *et al.,* 1999), thiobencarb (Villalobos *et al.,* 2000) and octylphenol (Gray and Metcalfe, 1999). This effect was observed (in Chapter 3) in newly hatched medaka fry following exposure to 17β-estradiol, 17α-ethinylestradiol (EE2), levonorgestrel, and diclofenac, but this effect was only monitored 24 hours post hatch. However, one major question from the previous study was whether exposure caused inflation failure or a delay in swim bladder inflation. Based on the work of González-Doncel *et al.* (2003), this current study was extended to 72 hours post-hatch to determine if the effects on swim bladder inflation success were permanent or simply a delay in inflation.

While most studies on the effects of pharmaceuticals have focused on singular compounds, the toxicity of pharmaceutical mixtures has been shown in some cases to be additive or even potentiated, as for diclofenac and ibuprofen exposure to daphnids (Cleuvers, 2003; 2004). A significant factor about mixture toxicity is that it can occur at concentrations below those which single substances have effects (Cleuvers, 2004). The 24 hour no-observed-effect concentrations established in the first component of this study were used to conduct simple binary and quaternary mixture experiments in order to

determine if these compounds in combination have the potential to cause swim bladder inflation failure.

4.2.1 Objectives

This study is a follow up of the work conducted in the previous chapter (Chapter 3). Prior to conducting this current study, it was determined that untreated medaka embryos inflate their swim bladders within 48 hours of hatch (Appendix D1). A follow up exposure experiment using levonorgestrel was conducted to determine if swim bladder inflation failure was observable up to 72 hours post-hatch and demonstrated that there is potential for swim bladder inflation failure to be observed up to 72 hours post hatch (Appendix D2).

The objectives of this experiment were to:

- determine if exposure to 17α-ethinylestradiol, 17β-estradiol, levonorgestrel, and diclofenac affects swim bladder inflation of Japanese medaka larvae up to 72 hours post-hatch;
- establish no-observed-effect concentrations on swim bladder inflation failure for each of these compounds; and
- determine if these compounds cause effects in combination at their individual no-observed-effect concentrations (NOECs).

4.2.2 Hypotheses

H_{o1}: embryonic exposure to diclofenac, 17β-estradiol, 17α-ethinylestradiol, and levonorgestrel does not result in a significant decrease in swim bladder inflation at any of the time points measured (0, 24, 48 and 72 hours post-hatch).

H_{o2}: Binary and quaternary mixtures of individual no-observed-effect concentrations does not result in a significant decrease in swim bladder inflation of hatched medaka larvae.

4.3 Materials and methods

4.3.1 Study investigating the temporal nature of swim bladder inflation

Prior to conducting this experiment a few follow up studies were conducted to confirm that there was a temporal effect on swim bladder inflation. First it was established that there was no difference between the control (rearing solution) and carrier control (0.01% acetone), as well that the majority (> 95%) of larval medaka inflated their swim bladder within 24 hours of hatching (Appendix D1). Next a pilot experiment was conducted to determine if one of the compounds tested in Chapter 3, levonorgestrel, caused a delay in inflation or if the lack of inflation was permanent (Appendix D2). It was determined that swim bladder inflation was significantly delayed at 50 and 500 µg/L exposure concentrations. While only 8.1% of fry were able to inflate their swim bladders shortly after hatch following exposure to 500 µg/L of levonorgestrel, 43.3% were able to inflate their swim bladders by 72 hours post-hatch. This percentage inflation was significantly lower than for controls, with 80% of control larvae being able to inflate shortly after hatch and 100% by 72 hours post-hatch. Based on these results the following experiment was designed and conducted. Chemical concentrations used were altered in an attempt to observe 0 to 100% inhibition of swim bladder inflation to permit accurate modelling and mixture studies. To this end, nominal exposure concentrations were changed from 0.05, 0.5, 5, 50, and 500 µg/L used in Chapter 3 to: 1, 10, 32, 320, and 1000 µg/L for the natural/synthetic hormones. The nominal exposure concentrations of diclofenac were also changed from 0.005, 0.05, 0.5, 5, and 50 mg/L to: 0.32, 1, 3.2, 32, and 100 mg/L.

4.3.2 Breeding, embryo collection and embryo exposure

Initial stocks of medaka were obtained from the National Institute for Basic Biology (Okazaki, Japan). The experiment was conducted from January 26th 2015, and concluded by mid-February 2015. The follow up mixture experiments were conducted July-August 2015. Medaka embryos were obtained from adult fish (FLFII strain) bred under standardized conditions (section 2.1). Embryos were collected as described in section 2.1.2 and assessed for viability. This experiment was run in quadruplicate with 10 embryos used per replicate well. Forty embryos per treatment (240 total embryos per compound) were distributed across four replicate 6-well microplates, containing five treatments and one control. Ten embryos were placed in each well with 10 mL of test solution renewed daily.

4.3.3 Conditions of embryo exposure

Embryos were assessed for viability 24 hours post fertilization, and exposure to treatments started following viability assessment. A semi-static exposure was conducted until hatch (95% exchange every 24 h until hatch) using plastic 6-well plates. Pharmaceutical stocks were prepared before the experiment was begun and renewal solutions were prepared daily. Diclofenac (purchased from Sigma-Aldrich) was prepared the day before an exposure would start in a 500 mL glass Pyrex® bottle; daily renewals were prepared in a 50 mL volumetric flask. The treatments and stocks were prepared in, and diluted with, rearing solution (see section 2.1.2). 17β-estradiol, 17α-ethinylestradiol (EE2), and levonorgestrel stocks (purchased from Sigma-Aldrich) were prepared in 2 mL glass sampler vials in acetone the day before an experiment was begun. Dilutions were also prepared in acetone (HPLC grade), 5 µL of each concentration was diluted in a 50 mL

volumetric flask by filling it to 50 mL with rearing solution (achieving a 0.01% solvent concentration). Medaka embryos were maintained in a temperature controlled room maintaining a constant temperature of 25°C and a 16:8h (light: dark) photoperiod with 30 minutes of dawn and dusk included in the light period. Plates were kept on a plate shaker set to 60 RPM, in order to narrow down the time-to-hatch, based on a paper by Farwell *et al.,* 2006. Five treatment concentrations and a control (a solvent control for EE2, 17β-estradiol, and levonorgestrel), as well as a rearing solution control for diclofenac) were tested per compound and are described in the section below.

4.3.4 Solution preparation

To prepare diclofenac stock 53.7 mg of diclofenac sodium salt was dissolved into 500 mL of rearing solution in a 500 mL glass Pyrex® bottle. Diclofenac solutions were prepared daily by performing dilutions using the stock solution in a glass 50 mL volumetric flask. Finally, 10 mL of solution was transferred to a 6-well plate in quadruplicate for each concentration as well as the control. These solutions were renewed every 24 hours in the same manner

For EE2, 17β-estradiol and levonorgestrel stocks, 5 mg of compound was first dissolved in 500 µL of acetone in a 2 mL glass sampler vial creating a 10 000 mg/L stock. This stock was used to make up through dilution 10 000, 3200, 320, 100 and 10 mg/L in acetone stocks in sampler vials. To prepare working solutions, 5 µL of each respective concentration was diluted in 50 mL of rearing solution using a glass 50 mL volumetric flask. Levonorgestrel was not able to be dissolved in 500 µL of acetone but was able to be dissolved into 1000 µL acetone. This resulted in a stock of 5000 mg/L and the highest

concentration was obtained by pipetting 10 µL of stock into 50 mL of rearing solution achieving a slighter higher solvent concentration of 0.02% (all other lower concentrations of levonorgestrel were diluted so that they were still 5 µL into 50 mL rearing solution (0.01% solvent concentration)). To account for this, the carrier control for levonorgestrel was prepared using 10 µL of acetone into 50 mL of rearing solution. All stock solutions were stored in the 4°C fridge until use. These solutions were renewed every 24 hours in the same manner.

4.3.5 Mixture exposure

Mixture studies including six binary mixtures and one quaternary mixture were conducted using the 24h NOECs established in the above experiment. Medaka embryos were exposed to these using the same methods as above. The stocks used were prepared in the same way and at the same concentrations (10 000 mg/L for 17β-estradiol and EE2, 5 000 mg/L for levonorgestrel). Mixture stocks were prepared using 500 µL of acetone in 2 mL glass sampler vials. For example, the 17β-estradiol & EE2 binary mixture stock was created by mixing 16 µL of 10 000 mg/L EE2 stock, with 160 µL of 10 000 mg/L 17β-estradiol stock, and adding 324 µL of acetone. To expose the embryos, 5 µL of this solution was diluted into 50 mL of rearing solution using a glass 100 mL volumetric flask, to achieve a final concentration of 32 µg/L EE2 & 320 µg/L 17β-estradiol mixture. Diclofenac was prepared by mixing 1.72 mg of diclofenac sodium salt into 500 mL of rearing solution to achieve a stock concentration of 3.2 mg/L. Diclofenac mixtures were prepared by pipetting 5 µL of hormone stock into 50 mL of diclofenac / rearing solution.

Mixture exposures including six binary mixtures and one quaternary mixture were conducted using the 24h NOECs established in the above experiment. Medaka embryos were exposed to these using the same methods as above (forty embryos per treatment, 4 replicates of 10 embryos). Embryos and larvae were observed daily for mortality, hatching, and malformations using a dissection microscope. Once hatched, swim bladder inflation was observed; if the fish did not inflate their swim bladder, they were kept and checked daily for up to 72 hours post-hatch.

4.3.6 Water quality analysis

Refer to Appendix E: *Water sampling methodology* for complete details on the methodology of the water quality analysis performed by Naomi Stock at the Water Quality Center at Trent University, Peterborough, Ontario. A 100 mL aliquot of representative water samples (for 17β-estradiol, EE2, and levonorgestrel) and 10 mL of diclofenac were collected at 0 hours as well as 24 hours post exposure. Measured concentrations relative to nominal values are listed in Table 4.2.

As there was little degradation within 24 hours seen at the concentrations used in the single compound experiment, only a time 0 representative sample was prepared and analyzed for the mixture experiment (Table 4.3).

Table 4.1 Nominal and measured values of single compound exposure of the following compounds 17α-ethinylestradiol, 17β-estradiol, levonorgestrel, and diclofenac, sampled at 0 and 24 hours (h).

Levonorgestrel (µg/L)

Nominal	Measured		
	0 h	24 h	[average]
C	< 0.1	< 0.1	N/A
SC	< 0.1	< 0.1	N/A
1.00	0.80	0.70	0.80
10.0	6.60	6.80	6.70
32.0	23.0	23.0	23.0
320.0	236	198	217
1000	611	850	731

Diclofenac (mg/L)

Nominal	Measured		
	0 h	24 h	[average]
C	< 0.1	< 0.1	N/A
SC	< 0.1	< 0.1	N/A
0.32	0.48	0.36	0.42
1.00	1.20	0.80	1.00
3.2	3.90	3.40	3.70
32.0	27.0	31.0	29.0
100	117	104	111

17α-Ethinylestradiol (µg/L)

Nominal	Measured		
	0 h	24 h	[average]
C	< 0.5	< 0.5	N/A
SC	< 0.5	< 0.5	N/A
1.00	1.30	1.10	1.20
10.0	12.9	8.4	10.7
32.0	34.8	30.8	32.8
320.0	256	255	256
1000	478	390	434

17β-Estradiol (µg/L)

Nominal	Measured		
	0 h	24 h	[average]
C	< 0.5	< 0.5	N/A
SC	< 0.5	< 0.5	N/A
1.00	1.40	1.10	1.30
10.0	9.40	8.90	9.20
32.0	31.2	30.4	30.8
320.0	212	221	217
1000	413	438	426

Table 4.2 Summary of measured vs nominal values for mixture experiments using binary as well as a quaternary mixture of 17α-ethinylestradiol (EE2), 17β-estradiol, levonorgestrel, and diclofenac.

Nominal	Measured (0h)			
	Diclofenac (mg/L)	Levonorgestrel (µg/L)	EE2 (µg/L)	17 β-estradiol (µg/L)
(32 µg/L LNG) & (3.2 mg/L DIC)	3.7	39	< 0.5	< 0.5
(32 µg/L EE2) & (320 µg/L E2)	<0.0001	< 0.1	29	320
(32 µg/L EE2) & (32 µg/L LNG)	<0.0001	< 0.1*	28	< 0.5
(32 µg/L EE2) & (3.2 mg/L DIC)	4.5	< 0.1	30	< 0.5
(32 µg/L LNG) & (320 µg/L E2)	<0.0001	25	< 0.5	257
(3.2 mg/L DIC) & (320 µg/L E2)	5.2	< 0.1	< 0.5	264
(32 µg/L EE2) & (32 µg/L LNG) & (3.2 mg/L DIC) & (320 µg/L E2)	3.6	38	27	336
Control	<0.0001	< 0.1	< 0.5	< 0.5
Solvent control	<0.0001	< 0.1	< 0.5	< 0.5

*Indicates an error in sample preparation, most likely LNG was not added to the representative mixture sample, as the average concentration in samples prepared correctly was 35 µg/L LNG. Analysis assumed that the actual lab exposures were prepared correctly with a concentration close to nominal.

4.3.7 End points

Select embryonic developmental stages of medaka were examined daily, using a dissecting stereomicroscope LEICA EZ4D (20x magnification) following the Iwamatsu (2004) fate map. Developmental abnormalities, mortality/hatchability and time-to-hatch were recorded daily. The main endpoint observed was if the larvae was able to inflate its swim bladder. This was monitored daily, for up to 72 hours post-hatch. Fish were separated based on their treatment and day hatched, and monitored for swim bladder inflation up to 3 days post-hatch. Where possible measured concentrations are reported in the results section.

4.3.8 Statistical analysis:

Data was analyzed with $\alpha = 0.1$ as well as $\alpha = 0.05$. Considering effects at $\alpha = 0.1$ guards against excessive type II error in experiments with relatively small sample size such as these. However, for mixture experiments minimizing type I error was considered to be more important for determining exposure concentrations, so the no-observed-effect concentrations used were based on $\alpha = 0.05$ for significant effects. Replicate wells were analysed for % malformations (swim bladder inflation failure), as well as % hatched (n=4), replicate fish were analyzed for time to hatch (n=40).

Data were first assessed for normality using Shapiro-Wilk W test, as well as for homogeneity of variance using a Brown-Forsythe test. If the data passed, one-way analysis of variance (ANOVA, $p \leq 0.05$ and $p \leq 0.10$) were conducted, confirmed by Dunnet Post-hoc test, $p \leq 0.05$ and $p \leq 0.10$. If the data failed normality Kruskal-Wallis one-way analysis of variance on ranks were performed (ANOVA on ranks, $p \leq 0.05$ and $p \leq 0.10$),

confirmed by multiple comparisons versus their respective control group (Dunn's method $p \leq 0.05$ and $p \leq 0.10$). All statistical analyses and graphs were performed/created using SigmaPlot 13.0 software unless otherwise noted.

Regression analysis on swim bladder inflation failure was performed, and the best resulting fit (linear) along with the coefficient of determination (R^2) were displayed. Due to the data being non-monotonic in nature, probit analysis was not performed, instead the no observed effect concentrations (NOEC) (highest concentration tested that did not show a significant difference compared to the control) were used for designing the mixture experiments.

4.4 Results:

4.4.1 Single compound study

There were few effects of drug exposure on hatchability with two notable exceptions. Exposure to 111 mg/L diclofenac caused acute mortality with > 97% of the embryos failing to hatch (Table 4.3). Exposure to 434 µg/L 17β-estradiol caused a non-significant decrease in hatchability 62 ± 2.5% compared to the control of 80 ± 10.5%. Aside from these two concentrations, all other treatments resulted in > 80% hatchability and were not significantly different from controls ($p > 0.1$).

Exposure to 17α-ethinylestradiol (EE2) and 17β-estradiol did not significantly affect time-to-hatch (Table 4.5). Exposure to 0.42, 1.00, and 3.70 mg/L diclofenac significantly reduced the median time-to-hatch by 1 day. Exposure to 23.0, 217 and 731 µg/L levonorgestrel also significantly reduced median time-to-hatch by 1 day.

On average ~80% of control fish inflated their swim bladder shortly after hatch with > 99% inflating by 24 hours post-hatch. Exposure to all compounds generally resulted in high inhibition of swim bladder inflation shortly after hatching, with a general trend of increasing swim bladder inflation failure with increasing concentration (Figure 4.1-4.4). However, it should be noted that often these effects were not strictly monotonic.

Exposure to 23.0, 217, and 731 µg/L levonorgestrel significantly reduced swim bladder inflation to 60.0 ± 7.1% ($p = 0.082$), 17.5 ± 4.7% ($p < 0.001$), and 5.8 ± 2.9% ($p < 0.001$) respectively 0h after hatch (Figure 4.1). This effect was significant for the two highest concentrations used, from 24-72 hours post-hatch, but appeared to be somewhat recoverable (still significantly lower), with 85 and 52% at 217 and 731 µg/L respectively, of medaka inflating their swim bladders by 72 hours post-hatch. Exposure to EE2, resulted in similar effects to levonorgestrel with 256 and 434 µg/L EE2 significantly reducing swim bladder inflation at all the time points tested (Figure 4.2). However, exposure to 1.20 and 10.7 µg/L also resulted in a significant ($p = 0.032 < 0.1$) reduction in swim bladder inflation 0 hours post-hatch. Exposure to 10.7 and 32.8 µg/L resulted in a significant reduction ($p = 0.076$ and 0.053, respectively) in inflation at 24 hours post-hatch as well, but exposure to 1.20 µg/L did not ($p = 0.108$). Similar to levonorgestrel, the effects recovered over time, but still were significantly reduced at 256 and 434 µg/L exposures even at 72 hours post-hatch. Exposure to 1.30, 9.20, 217, and 426 µg/L 17β-estradiol caused a significant reduction in swim bladder inflation at 0 hours post-hatch ($p = 0.024$, 0.063, 0.080, and < 0.001, respectively, Figure 4.3). These effects were significant following exposure to 217 and 426 µg/L at each of 24, 48 and 72 hours post-hatch. Finally, exposure to 3.70 and 29.0 mg/L diclofenac resulted in a significant inhibition of swim bladder inflation 0 hours post-

hatch ($p = 0.034$ and < 0.001, respectively, Figure 4.4). Like all compounds tested there was some recovery of swim bladder inflation, but 29.0 mg/L diclofenac significantly reduced inflation compared to the controls up to 72 hours post-hatch. Based on these results, no-observed-effect concentrations for swim bladder inflation inhibition were determined at 0 and 24 hours and summarized in Table 4.4.

There was a trend seen across all times tested of decreasing swim bladder inflation with levonorgestrel concentration, with linear regression being the strongest fit and coefficients of determination (R^2) ranging from 0.78 to 0.99 (Figure 4.7). Linear regression was significant ($p < 0.1$) for all treatment/times with the exception of 0 h post hatch E2 treatment ($p = 0.1183 > 0.1$). Linear regression of swim bladder inflation to EE2 concentration can be observed in Figure 4.8; the relationship was weakest at 0 hours post-hatch ($R^2 = 0.76$) and strongest 24 and 72 hours post-hatch ($R^2 = 0.98$). Swim bladder inflation linear regression of 17β-estradiol exposure was the weakest of all compounds tested at 0 hours post-hatch with $R^2 = 0.61$, and regressed well at all other time points with $R^2 = 0.80$ to 0.82 (Figure 4.9). Diclofenac exposure had the strongest correlation of concentration to swim bladder inflation failure, with $R^2 = 0.75$ at 0 hours post-hatch (Figure 4.10). The linear regression was improved at the other three timepoints ranging from 0.97 to 0.999.

4.4.2 Mixture results

Binary mixtures and a quaternary mixture were created using the 24 hour no-observed-effect concentrations from the single mixture study. The binary mixture of 32 µg/L EE2 and 32 µg/L levonorgestrel caused a significant reduction in swim bladder

inflation 24 hours post-hatch ($p = 0.013 < 0.05$, Figure 4.5). The other binary mixtures appeared to cause a trend of decreased swim bladder inflation compared to the controls but these effects were not significant ($p > 0.05$). The quaternary mixture of 17β-estradiol, EE2, levonorgestrel and diclofenac caused a significant decrease in swim bladder inflation at all times (Figure 4.6).

The binary mixture of levonorgestrel and diclofenac and the quaternary mixture of all four compounds caused a significant reduction in median time-to-hatch by 1 day (Table 4.6). All other binary mixtures did not cause any significant effects on time-to-hatch.

Table 4.3 Hatchability (mean % hatch ± SE) of Japanese medaka embryos following nominal exposures to diclofenac, 17β-estradiol, 17α-ethinylestradiol (EE2), and levonorgestrel, four replicates of 10 embryos. **Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

Treatment (µg/L) (nominal)	17β-Estradiol	EE2	Levonorgestrel	Treatment (mg/L) (nominal)	Diclofenac
Control	82.5 ± 10.3	97.5 ± 2.5	95.0 ± 2.9	Control	92.5 ± 4.8
1	85.0 ± 15.0	100 ± 0.0	100 ± 0.0	0.32	97.5 ± 2.5
10	95.0 ± 5.0	100 ± 0.0	100 ± 0.0	1	100 ± 0.0
32	87.5 ± 9.5	97.5 ± 2.5	97.5 ± 2.5	3.2	100 ± 0.0
320	100 ± 0.0	95.0 ± 2.9	95.0 ± 2.9	32	95.0 ± 5.0
1000	62.5 ± 2.5	87.5 ± 2.5	100 ± 0.0	100	2.5 ± 2.5**

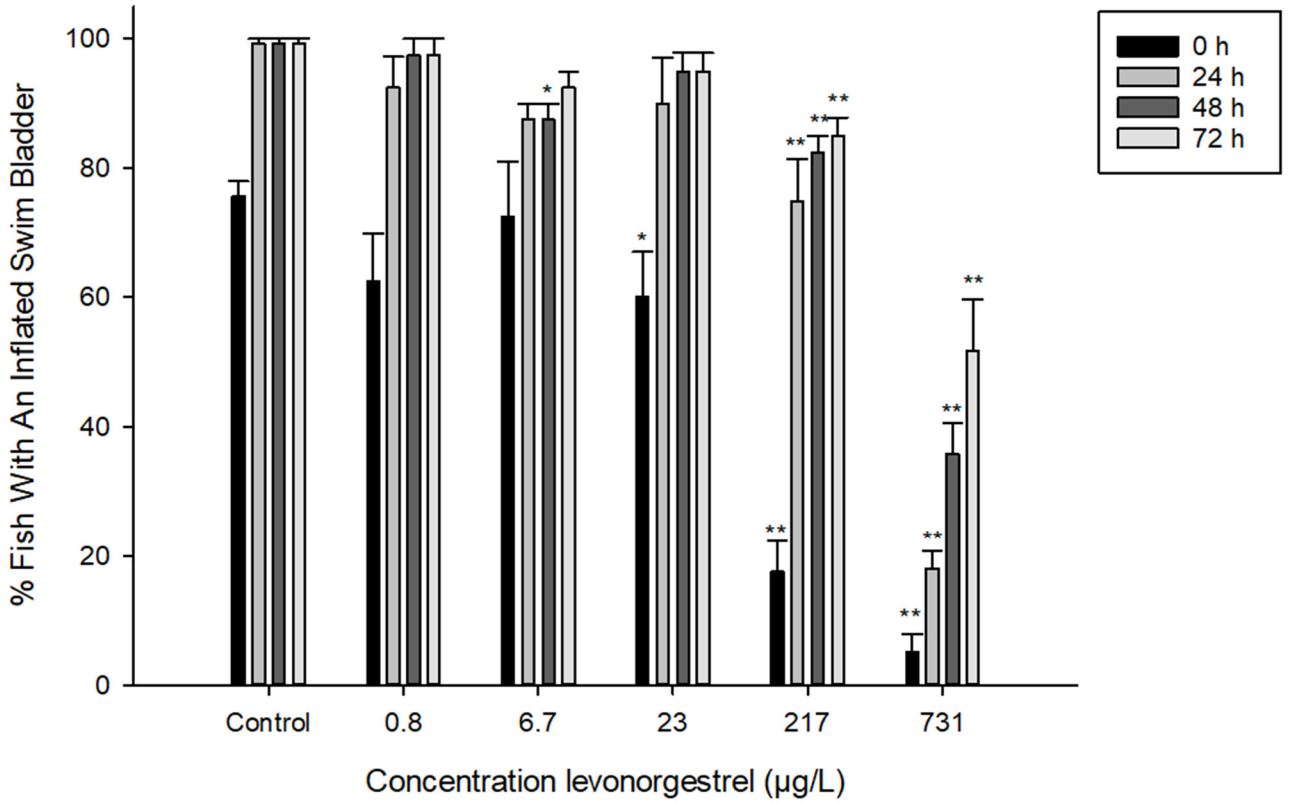


Figure 4.1: Swim bladder inflation (%) (mean ± SE) for medaka embryos exposed to levonorgestrel, assessed at 0, 24, 48, and 72 hours post hatch, bars are an average of four replicates (n=4), each of 10 embryos. **Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

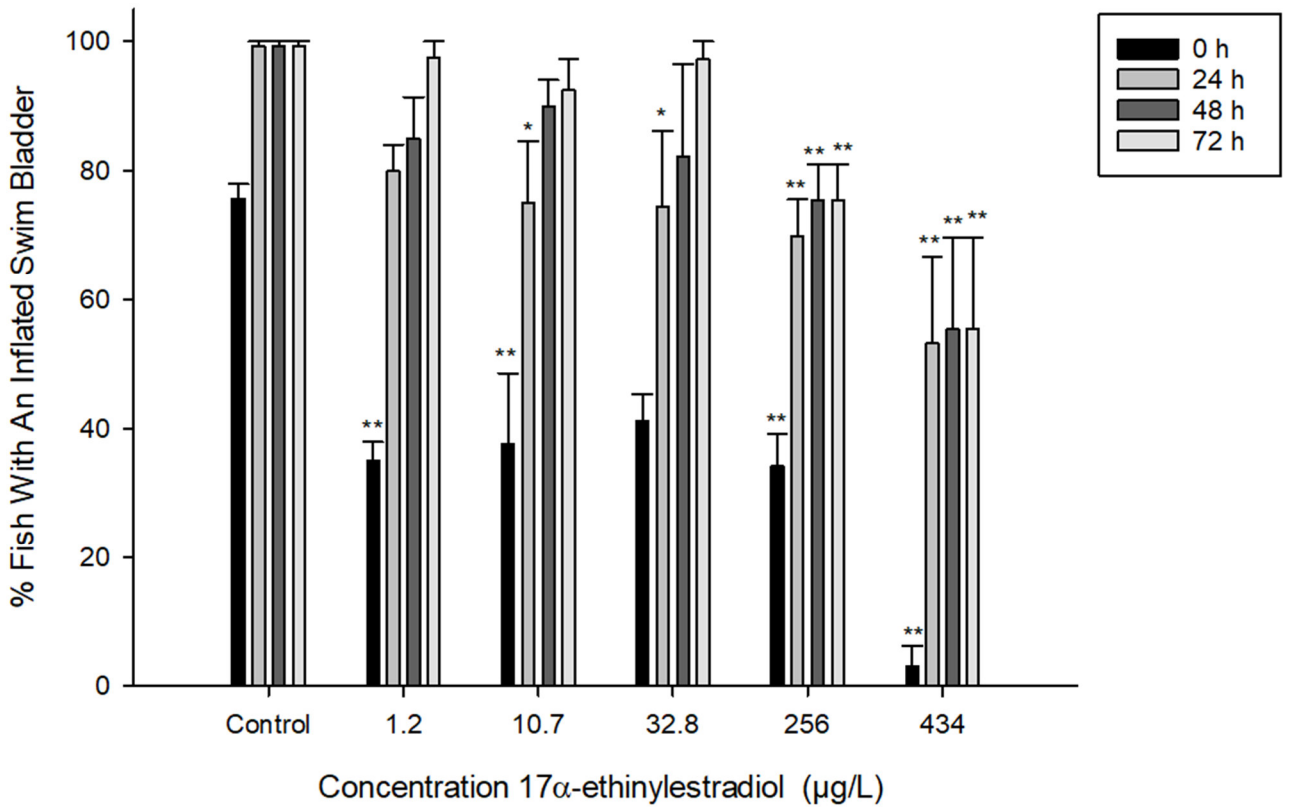


Figure 4.2: Swim bladder inflation (%) (mean ± SE) for medaka embryos exposed to 17α-ethinylestradiol, assessed at 0, 24, 48, and 72 hours post hatch, bars are an average of 4 replicates (n=4), each of 10 embryos. **Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

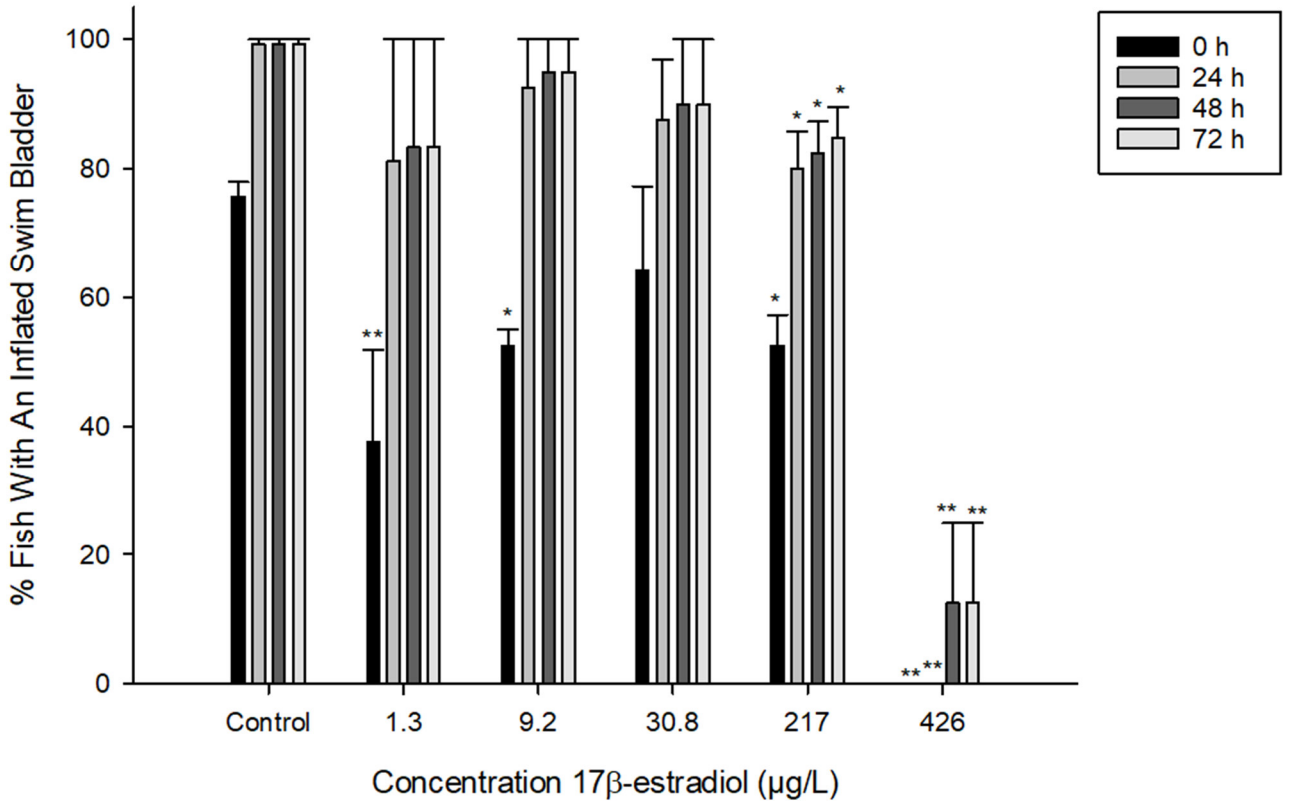


Figure 4.3: Swim bladder inflation (%) (mean ± SE) for medaka embryos exposed to 17β-estradiol, assessed at 0, 24, 48, and 72 hours post hatch, bars are an average of 4 replicates (n=4), each of 10 embryos. **Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

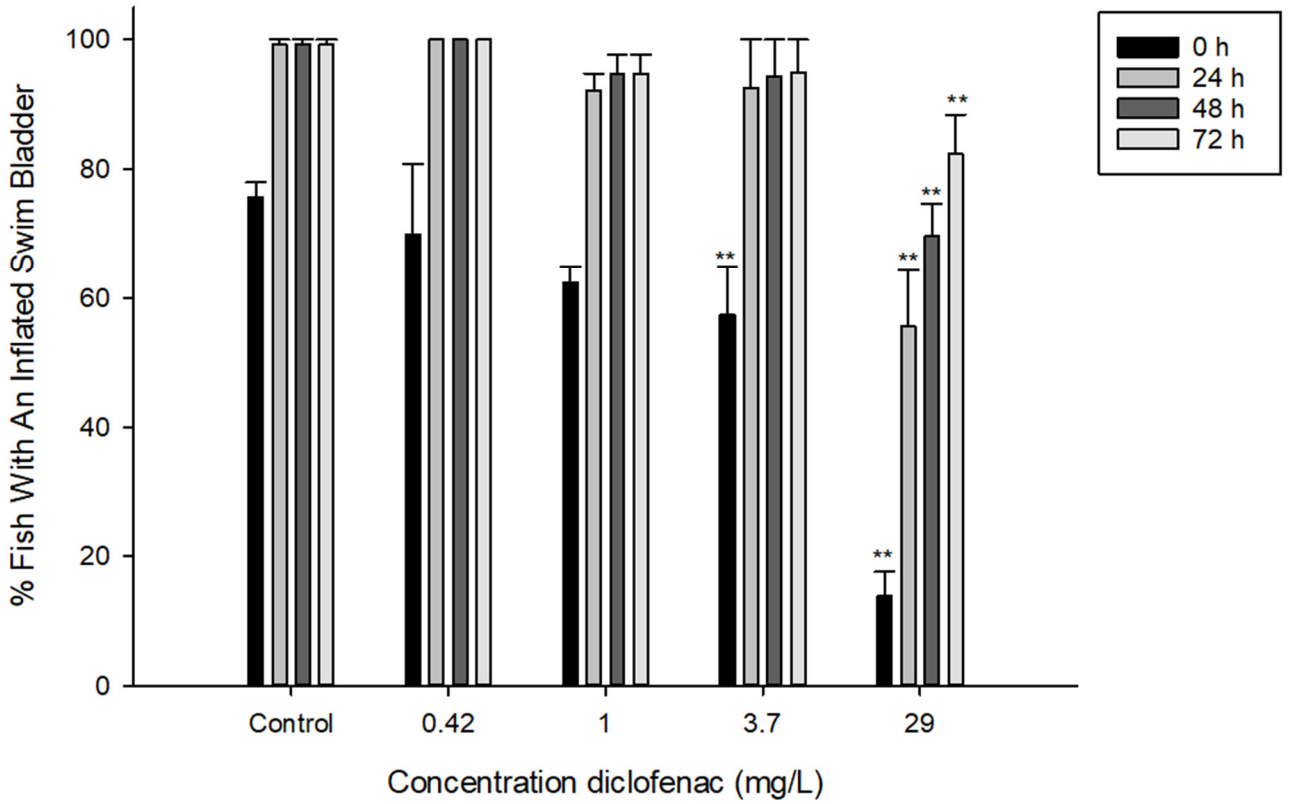


Figure 4.4: Swim bladder inflation (%) (mean ± SE) for medaka embryos exposed to diclofenac, assessed at 0, 24, 48, and 72 hours post hatch, bars are an average of 4 replicates (n=4), each of 10 embryos. **Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

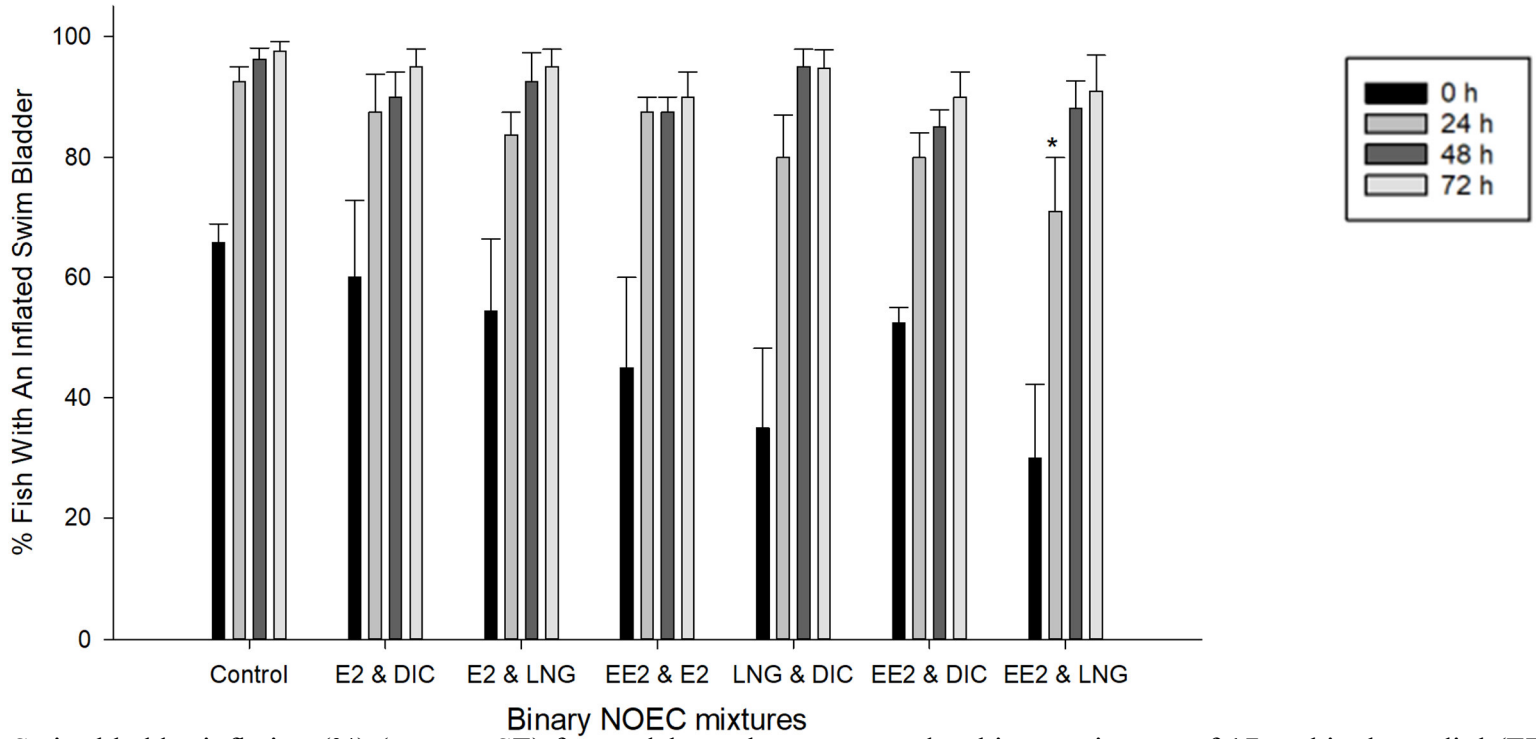


Figure 4.5: Swim bladder inflation (%) (mean ± SE) for medaka embryos exposed to binary mixtures of 17α-ethinylestradiol (EE2), 17β-estradiol (E2), levonorgestrel (LNG), and diclofenac (DIC) until hatch, assessed at 0, 24, 48, and 72 hours post hatch,, bars are an average of 4 replicates (n=4), each of 10 embryos. *Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$).

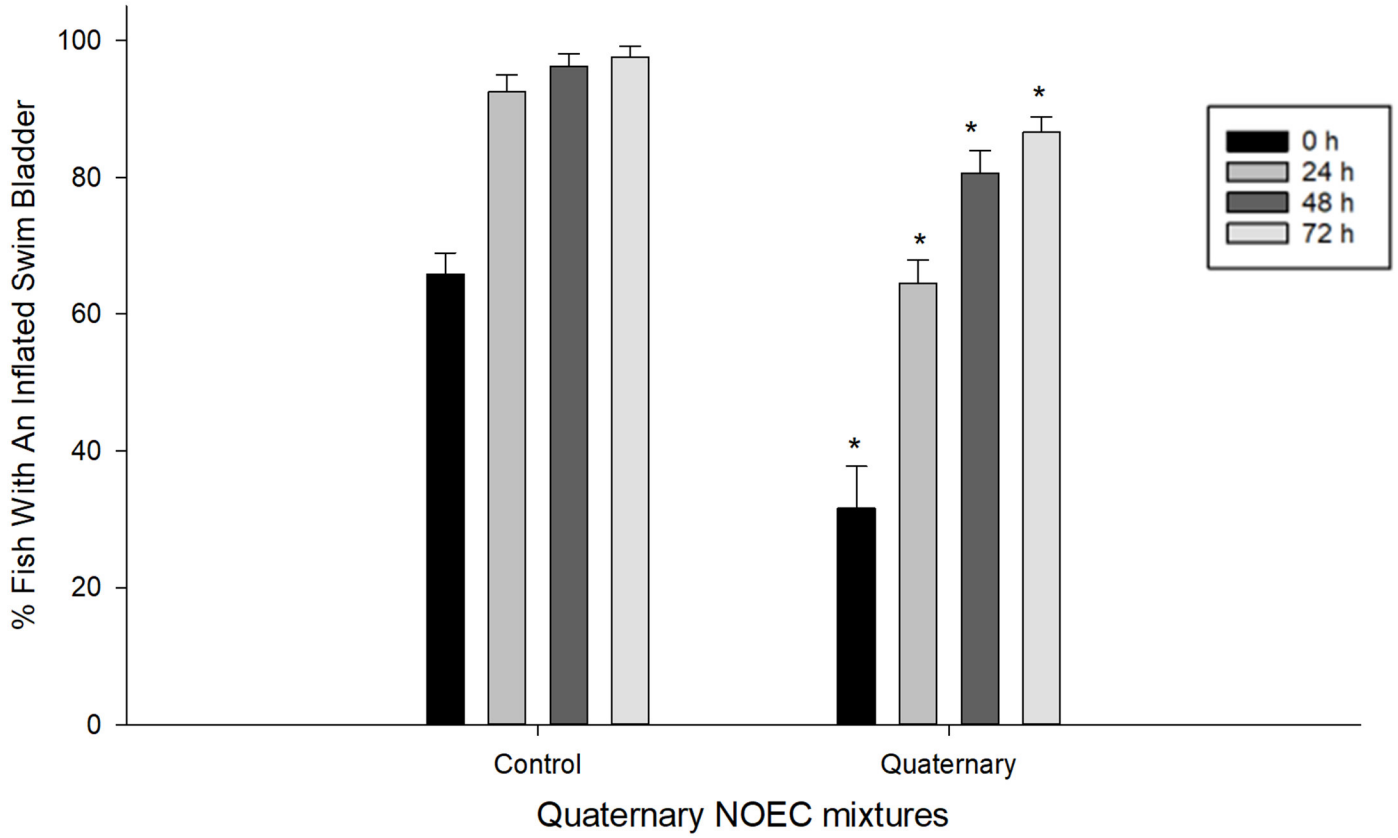


Figure 4.6: Swim bladder inflation (%) (mean ± SE) for medaka embryos exposed quaternary mixtures of 17α-ethinylestradiol, 17β-estradiol, levonorgestrel, and diclofenac until hatch, assessed at 0, 24, 48, and 72 hours post hatch, bars are an average of 4 replicates (n=4), each of 10 embryos. *Indicates significant difference from control (t-test, $p \leq 0.05$).

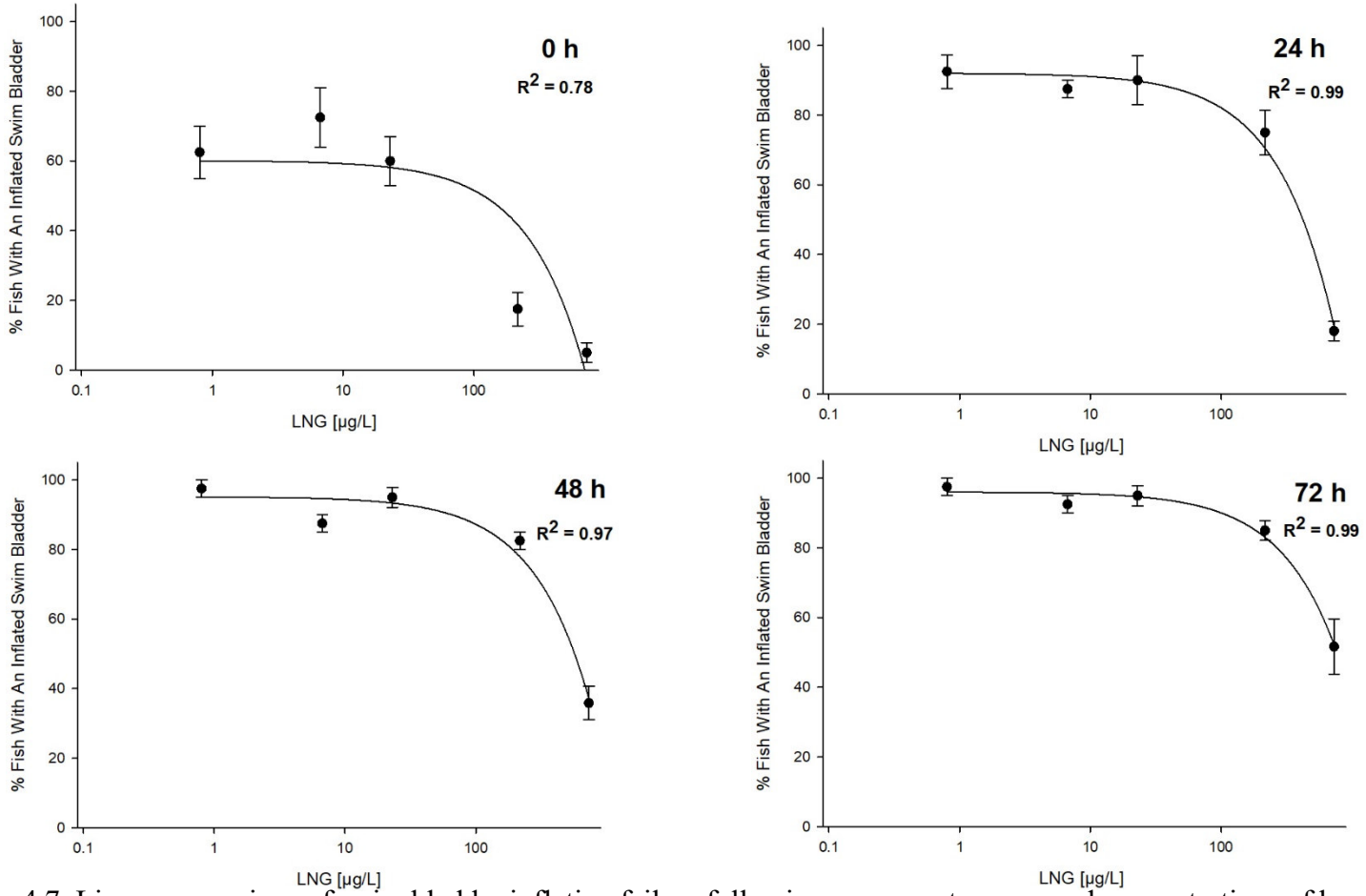


Figure 4.7: Linear regressions of swim bladder inflation failure following exposure to measured concentrations of levonorgestrel (LNG) split up by time post-hatch (hours).

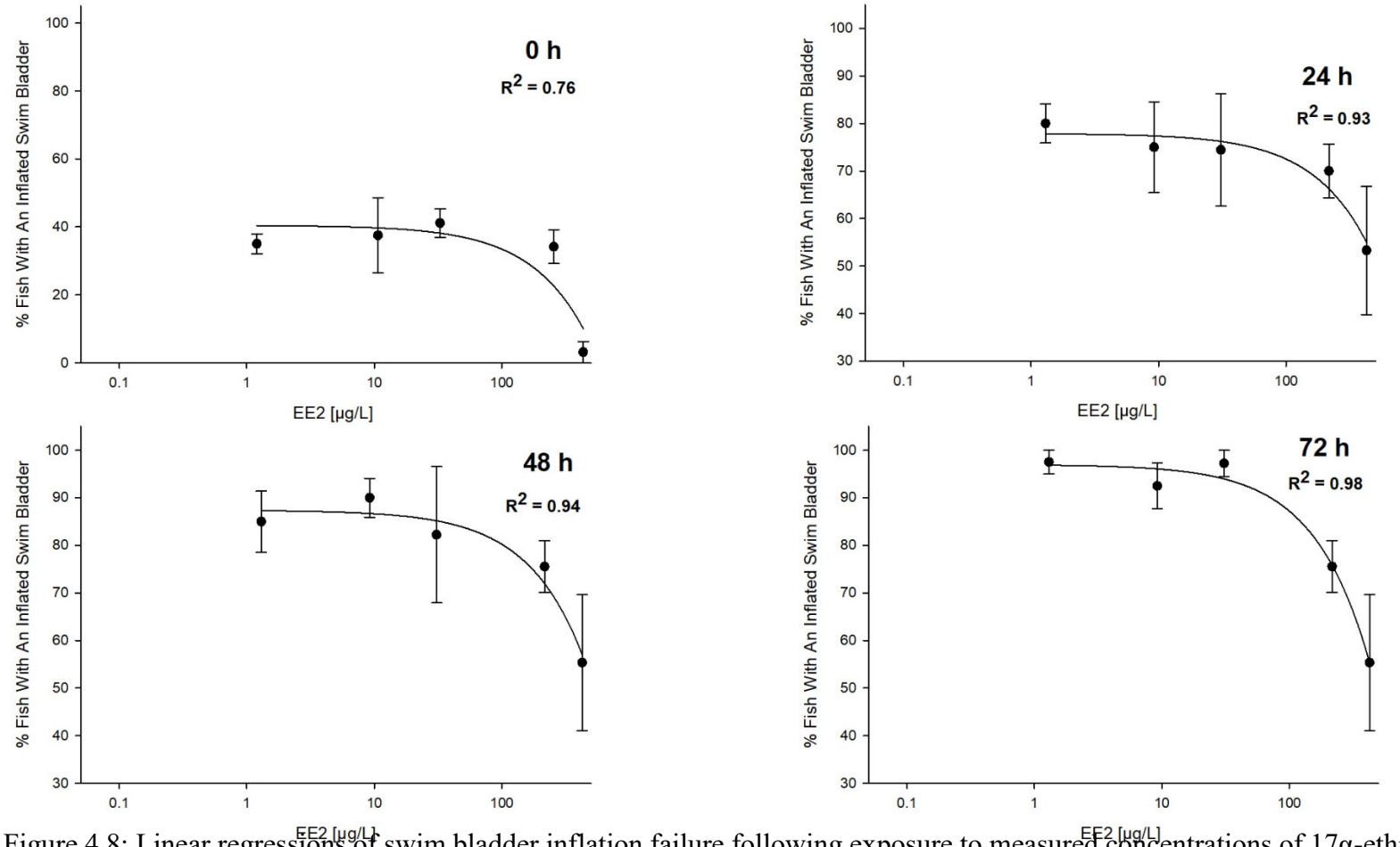


Figure 4.8: Linear regressions of swim bladder inflation failure following exposure to measured concentrations of 17α-ethinylestradiol (EE2) split up by time post-hatch (hours).

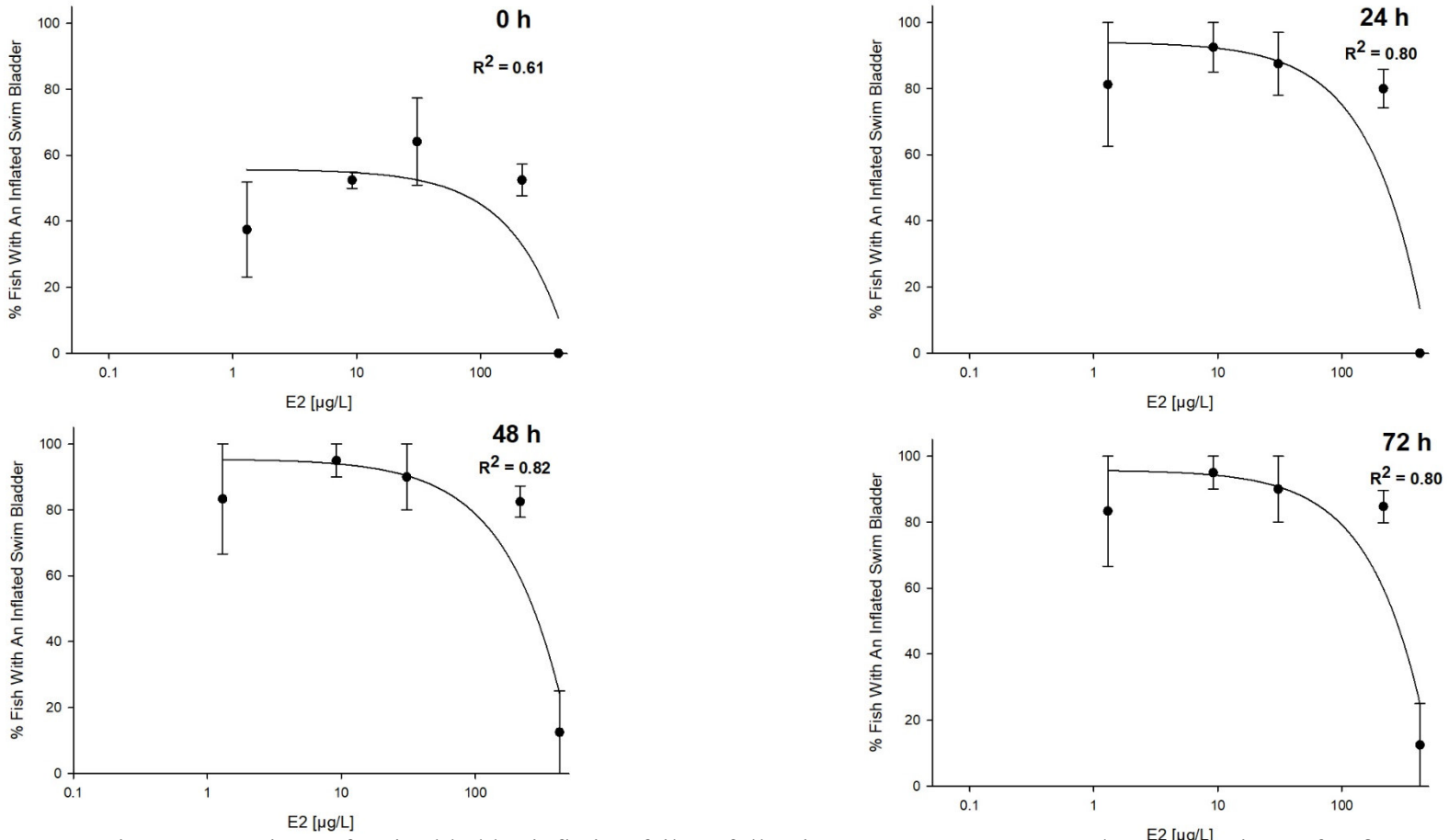


Figure 4.9: Linear regressions of swim bladder inflation failure following exposure to measured concentrations of 17β-estradiol (E2) split up by time post-hatch (hours).

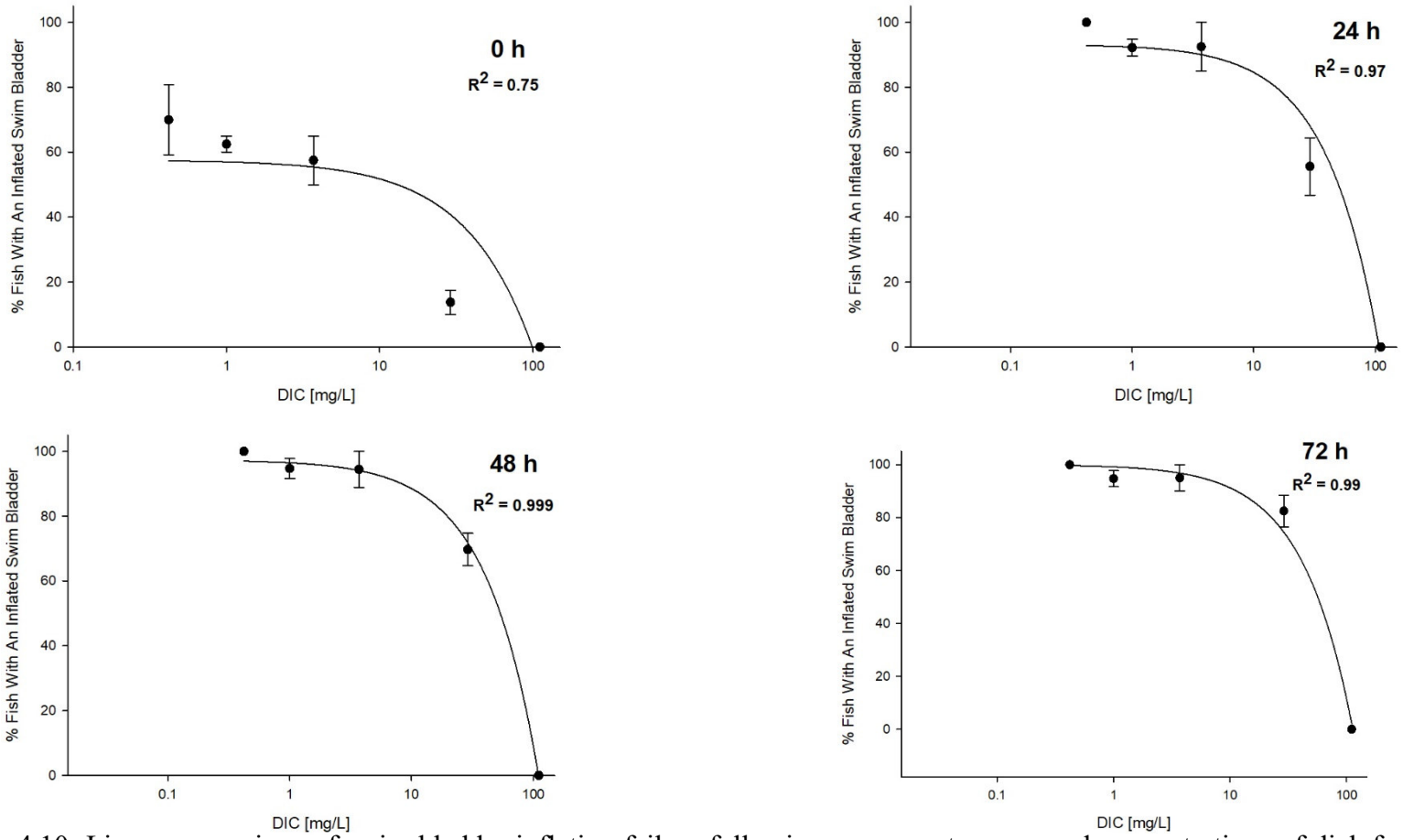


Figure 4.10: Linear regressions of swim bladder inflation failure following exposure to measured concentrations of diclofenac (DIC) split up by time post-hatch (hours).

Table 4.4: Summary table of no-observed-effect concentrations (NOECs) for swim bladder inflation inhibition of the individual compounds 17α-ethinylestradiol, 17β-estradiol, levonorgestrel, and diclofenac, determined by One-Way ANOVA $p \leq 0.05$.

	Diclofenac	17β-Estradiol	17α-Ethinylestradiol	Levonorgestrel
0h NOEC	1.00 mg/L	< 1.30 µg/L	< 1.20 µg/L	23 µg/L
24h NOEC	3.70 mg/L	217 µg/L	32.8 µg/L	23 µg/L

Table 4.5: Median time-to-hatch (days (min, max) following nominal exposures to control (rearing solution only), solvent control (SC), 17α-ethinylestradiol (EE2), 17β-estradiol (E2), levonorgestrel (LNG), and diclofenac (DIC). **Indicates a significant difference from pooled controls (ANOVA on ranks ($p \leq 0.05$), confirmed by Dunn's post-hoc test, $p \leq 0.05$), *indicates a significant difference from pooled controls $p \leq 0.10$)

Treatment (Nominal) [µg/L]	EE2	E2	LNG	Treatment (Nominal) [mg/L]	DIC
SC	10 (9,12)	11 (9,13)	9 (8,12)	Control	11 (9,14)
1	10 (9, 13)	11 (9,14)	9 (8,10)	0.32	10** (8,12)
10	9 (9,11)	11 (9,14)	9 (8,11)	1	10** (9,11)
32	10 (9,12)	11 (9,14)	8** (7,11)	3.2	10** (9,12)
320	10 (9,14)	10 (9,14)	8** (5,13)	32	12 (9,14)
1000	10 (8,14)	11 (9,14)	7** (5,11)		

Table 4.6: Median time-to-hatch (days (min, max) for binary and quaternary mixtures of 24h no-observed-effect concentrations of 17α-ethinylestradiol (EE2), 17β-estradiol (E2), levonorgestrel (LNG), and diclofenac (DIC). **Indicates a significant difference from pooled control and solvent control (SC) (ANOVA on ranks ($p \leq 0.05$), confirmed by Dunn's post-hoc test, $p \leq 0.05$).

	Control	SC	Quaternary	LNG & DIC	EE2 & E2	EE2 & LNG	EE2 & DIC	E2 & LNG	E2 & DIC
Time-to-hatch	9 (8,10)	9 (7,10)	8.5** (7,10)	8** (8,10)	9 (8,10)	9 (7,10)	9 (8,10)	9 (7,11)	9 (8,10)

4.5 Discussion

Exposure to the majority of the concentrations tested resulted in > 80% hatchability, which agrees with previous results detailed in Chapter 3. However, exposure to 111 mg/L diclofenac caused significant acute mortality with > 97% of the embryos failing to hatch. Diclofenac has been shown to cause acute effects in algae at 71.9 mg/L and daphnia at 68 mg/L, and typically shows effect concentrations below 100 mg/L (Cleuvers, 2004; Fent, 2006). Since acute effects were not observed at 50 mg/L in Chapter 3, the range for acute toxicity is somewhere between 50 and 111 mg/L.

Early hatching can be a result of the larvae trying to escape from an endangered/unsuitable environment, which may be a result of pollutants influencing hatching enzyme production (Korwin-Kossakowski, 2012). Similar to results previously observed in Chapter 3, exposure to levonorgestrel significantly reduced median time-to-hatch. Diclofenac also reduced the time-to-hatch in this current study, while exposure to 0.005-5 mg/L (nominal) caused no significant effect, and exposure to 50 mg/L (nominal) significantly increased time to hatch in Chapter 3. Inconsistency in these results for diclofenac could be a result of a different cohort of fish, or a different sample size (n=30 in Chapter 3, n=40 in this current study), but in either case suggests further experiments should be performed to clairify diclofenacs' effect on time to hatch. It is possible that the embryos were stressed by the presence of diclofenac and levonorgestrel in their environment and dedicated more energy to developing quicker in order to escape their unfavorable environmental conditions.

It has been previously established by Marty *et al.,* (1990a) that swim bladder inflation generally occurs in healthy medaka fry within 24 hours of hatching. The

unexposed fish used in this experiment follow a similar trend, with the majority (> 99%) of them inflating their swim bladder 24 hours post-hatch While many of the lowest concentrations of 17β-estradiol, 17α-ethinylestradiol (EE2), levonorgestrel, and diclofenac tested caused a delay in inflation that was recoverable within 72 hours of hatching, exposure to the two highest concentrations of all compounds tested appeared to significantly impair inflation up to 72 hours post-hatch. Japanese medaka embryos exposed to a range of concentrations of permethrin (25 – 300 µg/L) experienced inhibited inflation ranging from 40% failure at their lowest concentration tested to 95% at their highest; these effects recovered either completely at their lowest concentration, to 60% being able to inflate 72 hours post-hatch at their highest (González-Doncel *et al.,* 2003). The effects observed by the aforementioned study are quite similar to those observed for the four pharmaceutical compounds tested in this experiment. Since control fish could inflate their swim bladder within 24 hours post hatch, this study used 72 hours as the cut off, assuming if a fish could not inflate their swim bladder within 72 hours then it was likely a permanent effect. However, future studies should carry measurements further to confirm this assumption. Despite the effects observed, the concentrations required to cause significant swim bladder inflaton impairment are higher than those generally found in the environment. For example the global average concentration of EE2 in surface waters is 0.043 µg/L with a maximum of 5.9 µg/L (Aus der Beek *et al.,* 2016). This experiment observed a significant delay in inflation as low as 1 µg/L EE2, but the effect was only permanent at 256 µg/L. Thus, while this research shows these pharmaceutical compounds have the potential to cause significant effects, they are unlikely to be occurring at the concentrations generally found in surface waters.

Other studies have shown swim bladder inflation failure in Japanese medaka following embryonic exposure to cypermethrin, 4-tert-octylphenol, selenomethionine, and diluted bitumen (González-Doncel. *et al.,* 2004; Gray and Metcalfe, 1999; Kupsco and Schlenk, 2016; Madison *et al.,* 2015). It is not clear how these compounds are causing swim bladder inflation failure. Diclofenac is a known cyclooxygenase inhibitor, but previous work by Jönsson *et al.,* (2012) failed to correlate exposure NS-398 (a cyclooxygenase inhibitor) with swim bladder inflation failure in medaka. Work by Santos *et al.,* (2014) have linked swim bladder inflation failure in zebrafish larvae following exposure to endocrine disrupting compounds, but the mechanism through which they exert this effect on the developing embryo is not known. However, diclofenac has also been linked to endocrine disruption in tilapia (*Oreochromis niloticus*) (Gröner *et al.,* 2017).

It is possible that endocrine disrupting compounds may dysregulate normal cell signaling and progression, leading to adverse impacts in early development. The formation of the swim bladder during embryogenesis is dependent on both Wnt and hedgehog signaling pathways which help coordinate the growth and development of the cell layers making up the swim bladder (Winata *et al.,* 2009; Yin *et al.,* 2011). If these compounds are able to impair either hedgehog or Wnt signaling during the development it is plausible that they will cause a disorganization in the development of the swim bladder. The swim bladder is also dependent on the presence of surfactant lipid/proteins in order to facilitate the opening and inflation of the swim bladder (Prem *et al.,* 2000). Should exposure to these pharmaceutical compounds alter the secretion or production of surfactants this may also explain the delayed/inhibited inflation.

The percentage of larvae which successfully inflated their swim bladders was negatively correlated to the concentration of chemical in treatments. However, this relationship was not strictly monotonic, which prevents the use of probit analysis. In fact, other studies have also observed swim bladder inflation failure to not follow a strict dose response relationship, usually at the lower concentrations tested (González-Doncel. *et al.,* 2004; González-Doncel *et al.,* 2003; Gray and Metcalfe, 1999; Kupsco and Schlenk, 2016). Non-monotonic responses makes for difficult mixture modeling, which may suggest that swim bladder inflation failure may not be the most ideal endpoint for mixture modeling.

Much like Cleuvers, (2003) observed with mixtures NSAIDs, this study also measured significant effects in combination at individual no-observed-effect concentrations despite these compounds not sharing a common mode of action. The binary mixture of the no-observed-effect concentrations of EE2 and levonorgestrel caused significant reduction in swim bladder inflation 24 hours post-hatch, as the concentrations of these compounds were the lowest used of the compounds tested in this study, this suggests they were the most potent. When all four compounds were combined, a significant reduction in swim bladder inflation was observed at all time points tested. However, these NOEC concentrations were still elevated compared to those found in the environment. The ability of the compounds to act synergistically at individual no-observed-effect concentrations is of particular concern since these compounds would not be individually expected to cause adverse outcomes at these concentrations. Despite these compounds not sharing a principle mode of action, the results from this study suggests that they may be able to exert their effects in combination. This is possibly due to actions on similar targets, such as cell signaling disruption etc.

This study demonstrated that embryonic exposures to 17β-estradiol, 17α-ethinylestradiol, levonorgestrel, and diclofenac were able to cause swim bladder inflation failure of Japanese medaka larvae. In some cases this effect was only a delay in inflation, and at higher concentrations, was maintained up to 72 hours post-hatch. How these compounds disrupt swim bladder inflation is still not known. Investigating the effects of these compounds on select gene expression in medaka might further elucidate the mechanism of toxicity and assess if any of these genes are useful biomarkers of swim bladder inflation inhibition. Longer term effects of swim bladder inflation inhibition on growth and survival of larval medaka should also be investigated.

Chapter 5: Access requirement for an air-water interface to enable successful swim bladder inflation in Japanese medaka

5.1 Abstract

Japanese medaka (*Oryzias latipes*) are physoclistous fish and were used to assess the time of access to the air-water interface needed to inflate the swim bladder before the closure of the pneumatic duct. This was determined by denying medaka access to the air-water interface for 4-9 days, uncovering them and recording how many larvae had inflated their swim bladders daily. Control Japanese medaka larvae have a swim bladder inflation rate of 99.6 ± 1.4% within three days of hatching. Covered larvae, denied access to an air-water interface, were unable to inflate their swim bladders post-hatch until they were exposed to the air-water interface. Furthermore, the amount of time post-hatch which the larvae were denied access to an air-water interface significantly reduced their ability to inflate their swim bladders upon eventual exposed to an air-water interface. This effect was time-dependent, the longer the larvae were denied an air-water interface the less larvae were able to inflate their swim bladders when uncovered. It was determined that the ET_{50} (time to inhibit swim bladder inflation of 50% of the larvae) was 5 days post-hatch. By twelve days post-hatch, only larvae restricted from accessing an air-water interface for five days or longer had a significant reduction in swim bladder inflation success compared to the control. A significant increase in mortality was observed in all treatments (days covered) that were covered for eight or more days. These results indicate that access to an air-water interface is crucial for the initial swim bladder inflation of Japanese medaka. Inhibition of swim bladder inflation of larval Japanese medaka has the potential to cause population level effects by reducing both growth and survival.

5.2 Introduction

The swim bladder is an important organ within fish, allowing them to maintain a neutral position in the water column. In teleost fish the swim bladder develops from the anterior gut endoderm (Field *et al.,* 2003; Winata *et al.,* 2009). Organogenesis of the swim bladder will begin by the evagination of the anlagen connected to the digestive tract via the pneumatic duct (Woolley and Qin, 2010). In physostomous fish, this connection is retained, allowing the fish to initially inflate their swim bladder throughout development. However, in physoclistous species there is a critical period of time during early larval development in which the larvae must access the air-water interface in order to initially inflate their swim bladder before the pneumatic duct connecting the gut to the air bladder degrades (Goolish and Okutake, 1999).

The Japanese medaka (*Oryzias latipes*) is a physoclistous fish, and studies have demonstrated that completely denying medaka access to air-water interface (by enclosing them in a sealed vessel) results in medaka being unable to inflate their swim bladder (Marty *et al.,* 1990a; Marty and Hinton, 1995). These results suggest that the air-water interface is required for successful swim bladder inflation by medaka. However, these studies did not determine if there is a crucial time window for inflation of the air bladder by the larvae before the pneumatic duct degrades. Therefore, this study investigated if there is a critical time period for swim bladder inflation by larval Japanese medaka. Under normal conditions, larval medaka typically inflate their swim bladders within 24 hours of hatch. This study denied medaka access to the air-water interface for 3 to 9 days post-hatch, after which they were allowed access, and the number of fish still able to inflate their swim

bladders was recorded. Effects on survival were also established, as swim bladder inflation failure has been linked to decreased survival in zebrafish (Goolish and Okutake, 1999).

5.2.1 Objectives

This study was conducted to determine:

- Confirm Japanese medaka larvae require access to the air-water interface to initially inflate their swim bladder;
- to determine if there is a critical time window in which the larvae must have access to the surface in order to successfully inflate their swim bladders before the connection to the pneumatic duct degrades; and
- establish if swim bladder inflation failure directly reduces the survival of medaka larvae.

5.2.2 Hypotheses

H_{o1}: medaka denied access to the air-water interface will still be able to inflate their swim bladders.

H_{o2}: when medaka are denied access to the air-water interface for 3-9 days, they are still capable of inflating their swim bladders when they are given access to the air-water interface.

H_{o3}: survival of medaka larvae is not affected or correlated with swim bladder inflation success.

5.3 Materials and methods

5.3.1 Breeding, embryo collection and embryo exposure

Initial stocks of medaka were obtained from the National Institute for Basic Biology (Okazaki, Japan). Preliminary proof of concept experiments were run from January to February 2016. After this, a series of replicated experiments (three replicates per treatment, 20 embryos per replicate) with container surfaces covered preventing surface access by larval fish for 4, 5, 6, 7, and 8 days (treatments), were conducted from March to May of 2016 (Appendix F). The experiment below was subsequently conducted to follow up the initial experimental findings.

The follow-up experiment was conducted starting May 9th 2016, and concluded by July 4th 2016. Medaka embryos were obtained from adult fish (FLFII strain) bred under standardized conditions (Section 2.1). Embryos were collected as described in section 2.1.2 and assessed for viability. This experiment was run in triplicate, with 30 embryos used per replicate. Embryos were kept on a plate shaker until 8 days post fertilization when they were transferred to their respective treatments.

5.3.2 Conditions of embryo exposure

Embryos were assessed for viability 24 hours post fertilization and exposure to treatments started 8 days post fertilization. Ninety embryos per treatment were distributed across three replicate 250 mL beakers, which were placed in 10 L glass aquaria. Thirty embryos were placed in each beaker filled with lab water. Upon hatch (9 days post fertilization) replicates were thinned to 20 larvae, with extra larvae and unhatched embryos being discarded (20 larvae per replicate, 60 larvae per treatment/control). The controls

were kept in a 10 L glass aquaria with the water level set to just below the top of the 250 mL beaker, the treatment groups were placed in a 250 mL beaker and then covered with a mesh netting held in place by an elastic band. The treatment beakers were submerged below the water line so as to not allow the hatching larvae access to the surface, but to still allow gas exchange of the water. When control fish hatched, they were able to reach the surface of the water, while the treated fish were prevented due to the mesh. Medaka embryos and larvae were maintained in a temperature-controlled room maintaining a constant temperature of 25°C and a 16:8h (light: dark) photoperiod with 30 minutes of dawn and dusk included in the light period.

Water was changed twice daily, and larvae were fed fresh brine shrimp once daily in the morning, with waste / uneaten brine shrimp being removed in the afternoon. Due to time and space limitations, generally two treatments and one control were conducted at a time. Larvae were maintained in a covered beaker until the following points of time (3, 4, 5, 6, 7, 8, 9 days post-hatch), following which the beaker was uncovered and larvae were placed in 250 mL beakers in 10 L glass tanks with the same water level and conditions as the controls.

5.3.3 End points

Medaka larvae were examined daily, using a dissecting stereomicroscope LEICA EZ4D (20x magnification). Developmental abnormalities, and mortality was observed daily. The main endpoint observed was if the larvae was able to inflate its swim bladder. This was monitored daily, upon uncovering, up to 11 days post-hatch.

5.3.4 Statistical analysis

The data were analyzed with $\alpha = 0.1$. Data were first assessed for normality using Shapiro-Wilk W test, as well as for homogeneity of variance using a Brown-Forsythe test. If the data passed, one-way analysis of variance (ANOVA, $p \leq 0.1$) was performed, confirmed by Dunnet Post-hoc test, $p \leq 0.1$. If the data failed normality, a Kruskal-Wallis one-way analysis of variance on ranks was performed (ANOVA on ranks, $p \leq 0.1$), confirmed by multiple comparisons versus their respective control group (Dunn's method $p \leq 0.1$). All statistical analyses and graphs were performed using SigmaPlot 13.0 software unless otherwise noted.

5.4 Results

The majority of control fish (> 97%) were able to inflate their swim bladder within one day of hatching. When denied the air-water interface, medaka larvae were unable to inflate their swim bladder (Table 5.1), and a group of larvae denied the air-water interface 24 hours post-hatch with uninflated swim bladders is depicted in Figure 5.4. Covering a beaker with mesh netting and submerging it below the waterline successfully prevented medaka from inflating their swim bladders in all treatments except for 1.9% (1 larvae) of the 5 day covered medaka ($p < 0.001$).. It is possible that a small pocket of air was present under the mesh at some point during the 5 days this beaker was covered, allowing a larvae to inflate its air bladder.

After 1 day of being exposed to the air-water interface the larvae showed some recovery, with 81.7 ± 7.6% being able to inflate after the interface was denied for 3 days, and a comparison between a control larvae and a treated larvae with an uninflated swim bladder 24 hours post-hatch is shown in Figure 5.5. This recovery generally showed a linear decrease the longer the air-water interface was denied, with only 5.7 ± 5.1% of medaka larvae being able to inflate their swim bladders 1 day following exposure to the air-water interface, after they were denied an air-water interface for 9 days. For all time points (3 to 9 days) there was still a significant decrease in inflation 1 day post uncovering when compared to the controls ($p < 0.10$). However, swim bladder inflation of larvae denied an air-water interface for 3 and 4 days was not significantly lower than the controls after 2 days of being uncovered ($p > 0.10$). Medaka larvae denied the air-water interface for 5 days or longer were significantly less successful at inflating their swim bladders (ranging from 63.4% to 2.5%, $p < 0.01$) when compared to the controls (non-covered fish)

up to 12 days post-hatch. A comparison between a control larvae with an inflated swim bladder and a treated larvae without an inflated swim bladder 11 days post-hatch is shown in Figure 5.6. When the percentage of larvae with an inflated swim bladder 12 days post-hatch are correlated to the time the air-water interface was denied, there is a strong negative linear relationship between them ($R^2 = 0.82$, Figure 5.1). Using this fit, it was determined that denying the larvae access to the air-water interface for 5.3 days inhibited 50% of the population from inflating their swim bladders (ET_{50}). Over time, the percentage of medaka larvae with an inflated swim bladder in treatment groups tended to continue to increase, which may be related to mortality of larvae with uninflated swim bladders. As medaka can generally inflate their swim bladders within 1 day of being exposed to the surface, this observation was unexpected.

There was a general trend of increased mortality the longer the air-water interface was denied. However, mortality was not significantly increased until 8 days post-hatch. Increased mortality was significant generally in the larvae denied the air-water interface for 7, 8, and 9 days post-hatch ($p < 0.05$, Table 5.2). There is a linear relationship between the days denied an air-water interface and mortality ($R^2 = 0.41$, Figure 5.2). It was observed that larvae with an inflated swim bladder tended to be immobile with empty stomachs. Increased mortality was generally observed in larvae with uninflated swim bladders. Since fish with uninflated swim bladders were dying and those with inflated swim bladders were not, the percentage of medaka with inflated swim bladders would keep increasing over time, as observed in the results. In fact, the percentage of medaka with an inflated swim bladder correlates more strongly with mortality than with the days denied the air-water interface (Figure 5.3, $R^2 = 0.59$).

Table 5.1: Percentage of medaka with an inflated swim bladder following denial of air-water interface for no time (controls) or 3-9 days post-hatch (dph) (treatments), mean of 3 replicates of 20 larvae each ± standard deviation (SD). Significant differences are shown with p < *0.1, **0.05, ***< 0.01, ****< 0.001.

	Days Post-Hatch (mean ± SD)												
Treatment	0 dph	1 dph	2 dph	3 dph	4 dph	5 dph	6 dph	7 dph	8 dph	9 dph	10 dph	11 dph	12 dph
Control	70.8 ± 17.3	97.4 ± 3.5	97.9 ± 3.5	98.7 ± 2.3	99.1 ± 2.0	99.1 ± 2.1	99.6 ± 1.4	99.6 ± 1.5	99.6 ± 1.5	99.6 ± 1.5	99.5 ± 1.6	98.4 ± 8.2	99 ± 2.4
3 Days				0.0 ± 0.0 ****	81.7 ± 7.6 ***	86.7 ± 7.6	91.5 ±7.7	91.5 ± 7.7	92.8 ± 6.3	94.1 ± 5.9	93.5 ± 6.7	92.4 ± 8.4	94.4 ± 9.6
4 Days					0.0 ± 0.0 ****	73.3 ± 22.5 *	78.3 ± 20.2	83.3 ± 15.3	83.0 ± 10.6	86.4 ± 7.7	88.0 ± 7.5	89.4 ± 5.4	89.4 ± 5.4
5 Days						1.9 ± 3.2 ****	33.4 ± 12.2 ****	44.6 ± 12.3 ****	49.5 ± 16.0 ****	58.2 ± 17.4 ***	58.8 ± 19.7 ****	62.6 ± 22.9 ***	63.8 ± 20.8 ***
6 Days							0.0 ± 0.0 ****	20.7 ± 5.7 ****	32.6 ± 7.9 ****	38.5 ± 26.0 ****	43.5 ± 11.2 ****	42.5 ± 11.6 ****	43.5 ± 11.2 ****
7 Days								0.0 ± 0.0 ****	16.3 ± 5.4 ****	21.8 ± 6.1 ****	21.7 ± 6.1 ****	25.3 ± 5.4 ****	25.3 ± 5.4 ****
8 Days									0.0 ± 0.0 ****	10.0 ± 3.9 ****	20.6 ± 8.8 ****	26.2 ± 2.0 ****	22.6 ± 7.4 ****
9 Days										0.0 ± 0.0 ****	5.7 ± 5.1 ****	2.6 ± 4.4 ****	2.6 ± 4.4 ****

Table 5.2: Percentage mortality of medaka following denial of air-water interface for no time (controls) or 3-9 days post-hatch (dph) (treatments), mean of 3 replicates of 20 larvae each ± standard deviation (SD). Significant differences are shown with p < *0.1, **0.05, ***< 0.01, ****< 0.001.

	Days Post-Hatch (mean ± SD)												
Treatment	0 dph	1 dph	2 dph	3 dph	4 dph	5 dph	6 dph	7 dph	8 dph	9 dph	10 dph	11 dph	12 dph
Control	0.0 ± 0.0	1.3 ± 2.3	1.7 ± 2.5	2.1 ± 2.6	2.5 ± 2.6	3.3 ± 3.9	4.2 ± 5.6	5.4 ± 5.4	7.9 ± 6.6	9.2 ± 6.3	14.6 ± 6.9	20.8 ± 6.7	24.2 ± 10.0
3 Days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	1.7 ± 2.9	5.0 ± 5.0	13.3 ± 2.9	18.3 ± 7.6	23.3 ± 15.3	28.3 ± 12.6
4 Days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	1.7 ± 2.9	3.3 ± 5.8	5.0 ± 5.0	5.0 ± 5.0
5 Days	0.0 ± 0.0	3.3 ± 2.9	3.3 ± 2.9	3.3 ± 2.9	3.3 ± 2.9	10.0 ± 5.0	11.7 ± 7.6	15.0 ± 5.0	16.7 ± 5.8	26.7 ± 10.4	36.7 ± 10.4	40.0 ± 8.7	41.7 ± 10.4
6 Days	0.0 ± 0.0	5.0 ± 5.0	5.0 ± 5.0	13.3 ± 12.6	16.7 ± 15.3	16.7 ± 15.3	16.7 ± 15.3	20.0 ± 18.0	26.7 ± 10.4	33.3 ± 7.6	40.0 ± 15.0	43.3 ± 10.4	45.0 ± 8.7
7 Days	0.0 ± 0.0	1.7 ± 2.9	1.7 ± 2.9	1.7 ± 2.9	10.0 ± 10.0	15.0 ± 8.7	15.0 ± 8.7	21.7 ± 12.6	31.7 ± 16.1 **	41.7 ± 24.7 **	41.7 ± 24.7	50.0 ± 21.8 *	50.0 ± 21.8
8 Days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	1.7 ± 2.9	8.3 ± 2.9	11.7 ± 7.6	13.3 ± 10.4	23.3 ± 12.6	45.0 ± 20.0 **	58.3 ± 5.8 ***	61.7 ± 2.9 ***	63.3 ± 2.9 ***
9 Days	0.0 ± 0.0	1.7 ± 2.9	5.0 ± 5.0	5.0 ± 5.0	8.3 ± 7.6	16.7 ± 11.5	16.7 ± 11.5	26.7 ± 16.1	35.0 ± 17.3 **	43.3 ± 12.6 **	51.7 ± 22.5 **	53.3 ± 20.2 **	53.3 ± 20.2 *

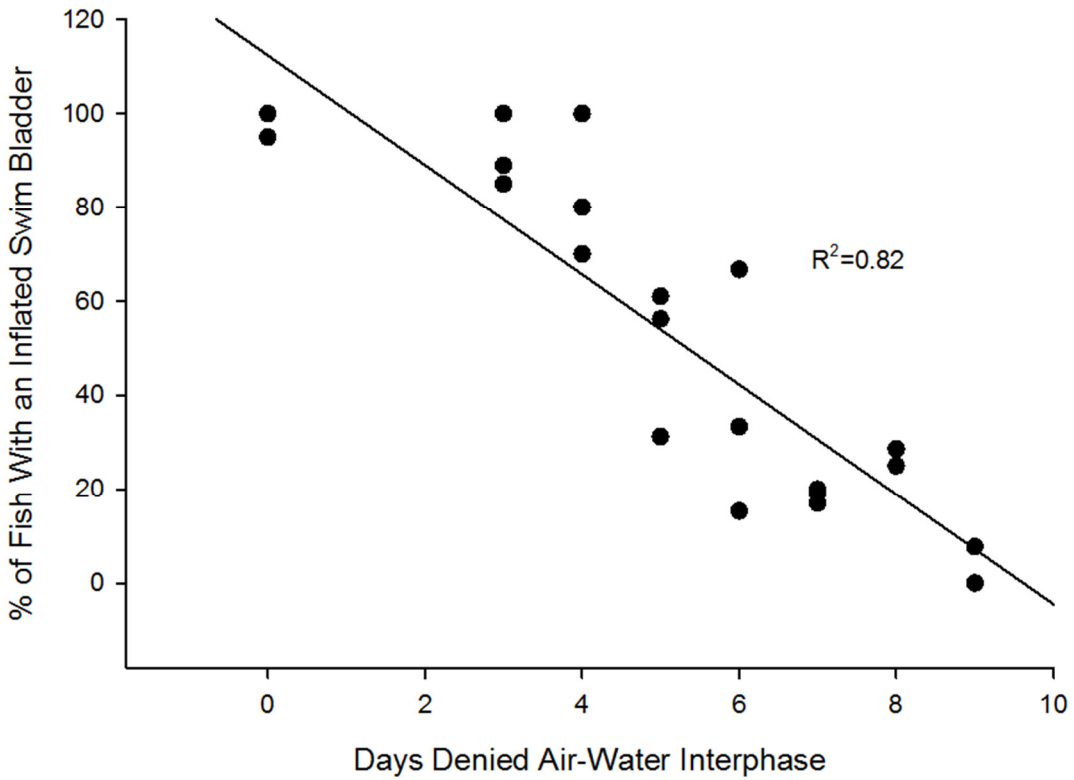


Figure 5.1: Days access to air-water interface was denied with a linear regression to the percentage of larval medaka with an inflated swim bladder 12 days post-hatch. Three replicates of 20 larval fish for each group are plotted along with a linear regression and R^2 are displayed.

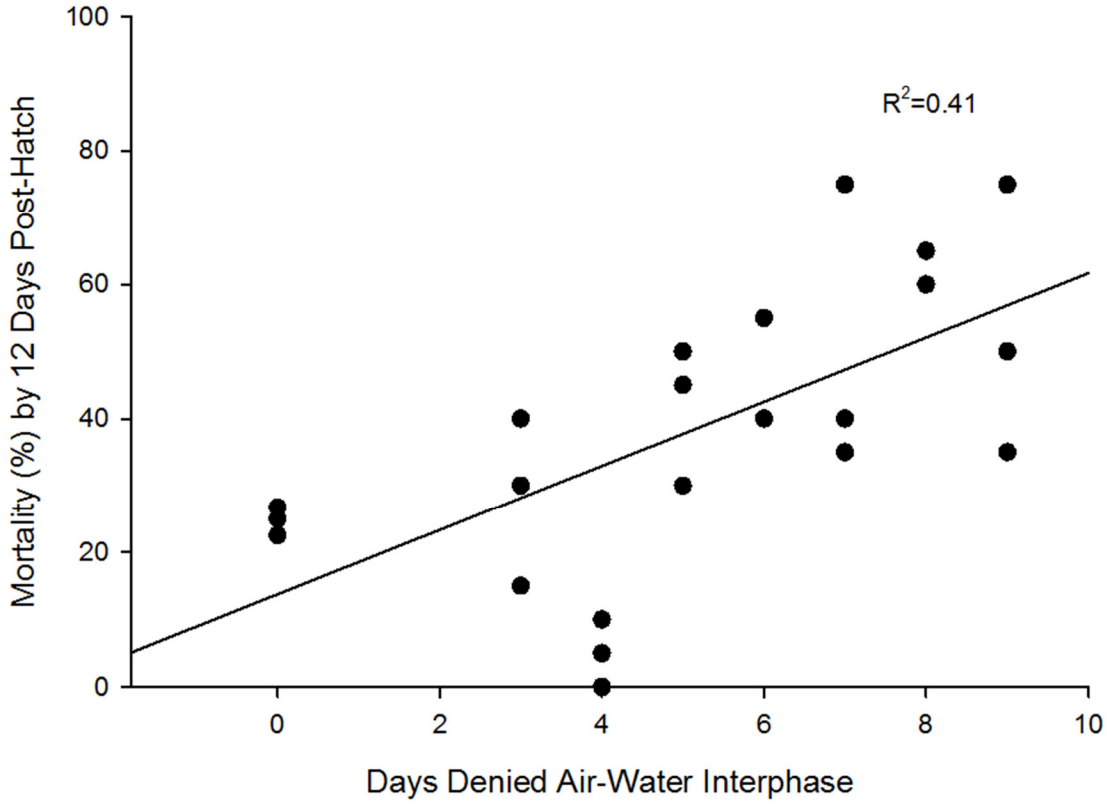


Figure 5.2: Days denied air-water interface regressed to the percentage mortality 12 days post-hatch. Three replicates of 20 larval fish for each group are plotted along with a linear regression and R^2 are displayed.

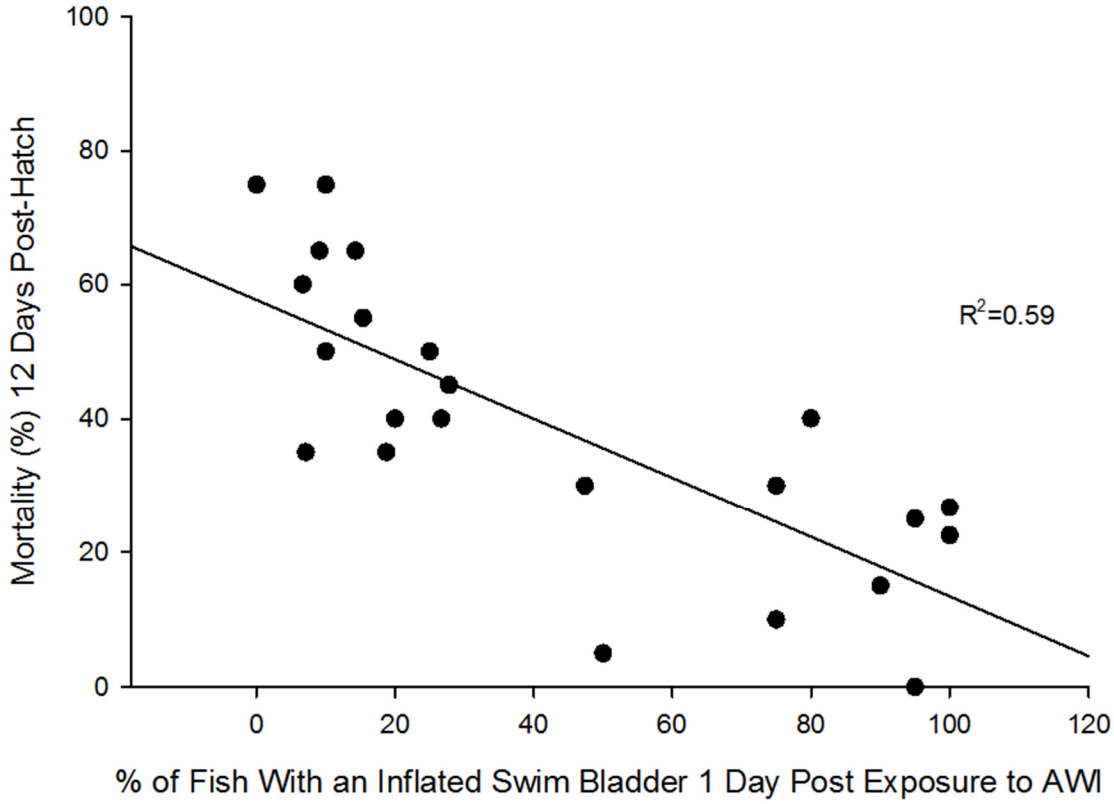


Figure 5.3: Percentage of larval medaka with an inflated swim bladder regressed to the percentage mortality 12 days post-hatch. Three replicates of 20 larval fish for each group are plotted along with a linear regression and R^2 are displayed.

Figure 5.4: Larval medaka without inflated swim bladders 24 hours post-hatch from a covered beaker denied the air-water interface.

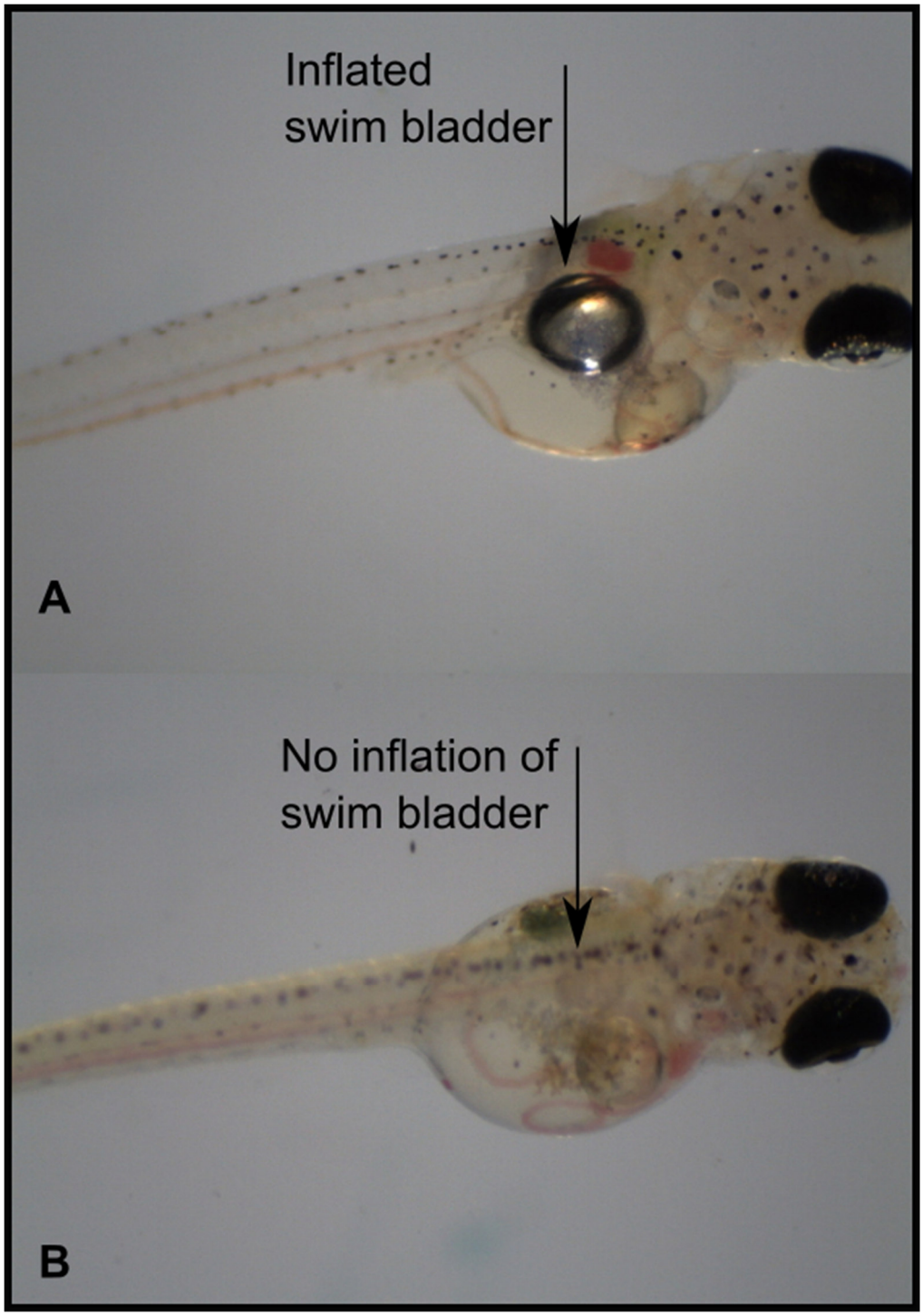


Figure 5.5: Medaka larvae 24 hours post-hatch A) from a control beaker allowed an air-water interface with an inflated swim bladder, and B) from a covered beaker denied the air-water interface without an inflated swim bladder

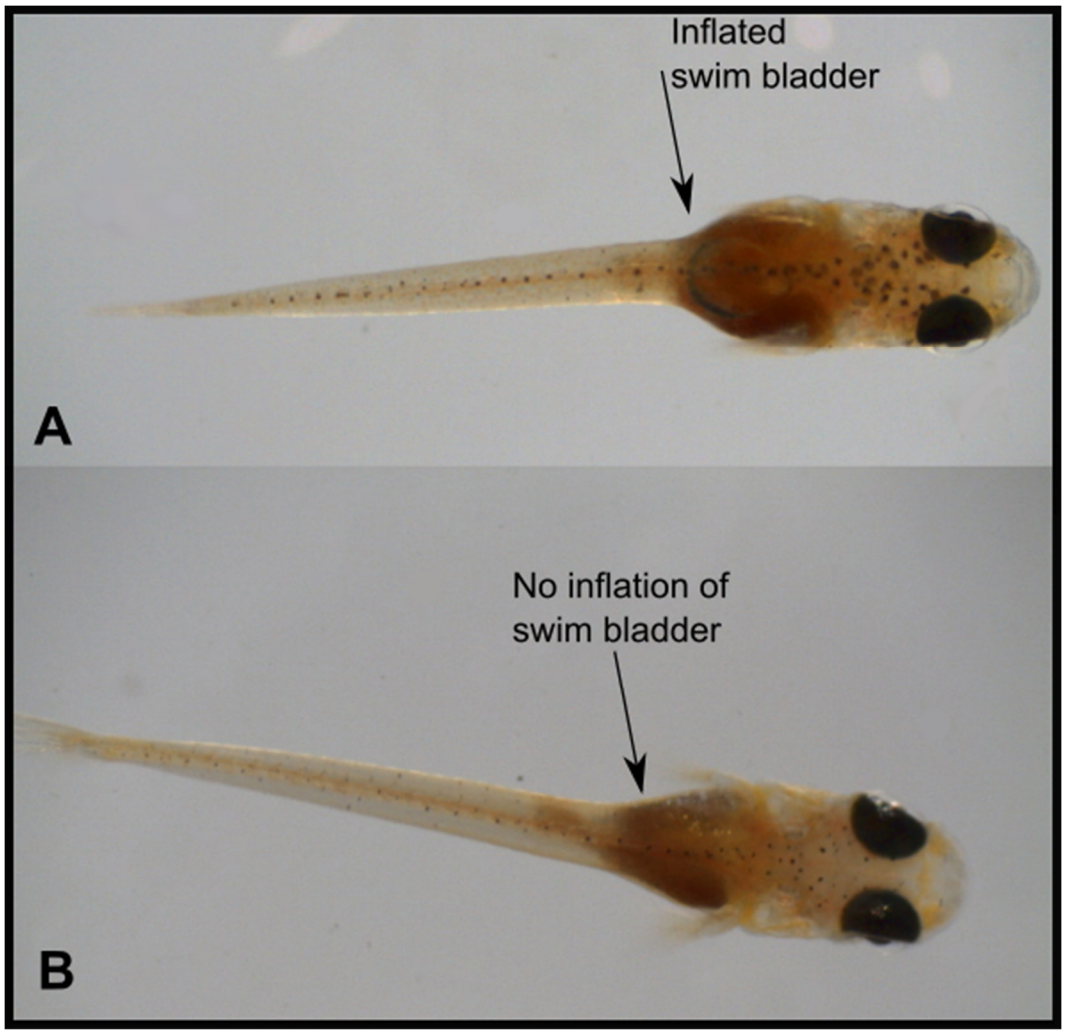


Figure 5.6: Medaka larvae 11 days post-hatch A) from a control beaker immediately allowed access to the air-water interface after hatch with an inflated swim bladder, and B) from a covered beaker denied the air-water interface for 5 days without an inflated swim bladder.

5.6 Discussion

The results of the present study confirm that the larvae of medaka require access to an air-water interface in order to successfully inflate their swim bladders. These findings support those of Marty *et al.,* (1990a) who observed medaka in a sealed chamber with no air-water interface are unable to inflate their swim bladders. The single case of a medaka larvae inflating its swim bladder before uncovering could have been a result of a small air pocket being trapped under the mesh during uncovering/recovering after feeding or cleaning. The cheap and easy method of covering beakers of larval fish with a mesh netting appears to be very successful at inducing swim bladder inflation failure of medaka larvae. This method was more effective than complex experimental set ups; such as the sealed test chambers used by Goolish *et al.,* (1999) which had 20% inflation of sealed zebrafish larvae.

The longer medaka were denied access to the air-water interface, the fewer larvae were able to inflate their swim bladders following hatch. Based on these results, it appears that there is a crucial time window for swim bladder inflation to occur in medaka larvae, with a significant portion of them being unable to inflate their swim bladders if access to the air-water interface is denied for 5 days or longer. The existence of a finite temporal window for swim bladder inflation has been observed in other physoclistous species and typically occurs during between 1 and 11 days post-hatch (reviewed: Woolley and Qin, 2010). For example, the swim bladder inflation of the striped trumpeter (*Latris lineata*) must occur within 9-10 days post-hatch (Trotter *et al.,* 2005). Should fish not inflate their swim bladder by these critical time windows the pneumatic duct will degrade and they will not be able to inflate their swim bladder.

This study also linked swim bladder inflation failure to an increase in mortality. Swim bladder inflation failure has been linked to mortality in zebrafish (32% survial after 10 days being restricted access to the surface) (Goolish and Okutake, 1999), and Japanese medaka (Gray and Metcalfe, 1999). In both species of fish, larvae without inflated swim bladders exhibited reduced activity, similar to the results observed in this study. The majority of the zebrafish mortality were observed 7-10 days post-hatch (Goolish and Okutake, 1999), which is similar to the results observed in this study, where mortality became significantly higher than the controls after 7 days post-hatch. These zebrafish larvae were generally found at the bottom of the experimental chambers, and did not have evidence of stomach contents. The majority of medaka larvae in this current study with uninflated swim bladders were also found at the bottom of the experimental chambers and showed little mobility, with empty stomachs. It is possible that the elevated mortality in the medaka with uninflated swim bladders was due to a lack of feeding, which would account for the increase in mortality around 7 days post-hatch when all their energy reserves from the embryo have been used up.

Based the observations of this experiment, swim bladder inflation failure may be associated with lower swim-up behavior in medaka larvae. Studies that have measured a reduced swim-up behavior due to pollutant exposure in larval fish often do not examine or mention whether or not the larvae had inflated their swim bladder, such as embryonic exposure 10 ng/L EE2 causing a significant reduction in swim-up behavior of larval Japanese medaka (Versonnen and Janssen, 2004). While not the only reason a fish may be unable to swim up, it is possible that swim bladder inflation failure may be a contributing

factor to this behaviour, and future studies that measure swim-up should consider measuring swim bladder inflation failure as well.

However, it was interesting to find that some larvae with uninflated swim bladders exhibited behavior similar to medaka with inflated swim bladders, and survived up to 12 days post-hatch. Other studies have demonstrated that fish with uninflated swim bladders expend more energy to maintain their position in the water column (Czesny *et al.,* 2011), as well as consume more oxygen (Marty and Hinton, 1995). Presumably, surviving exposed larvae with uninflated swim bladders would spend more energy than control fish and thus have reduced growth and success. They would also may be more vulnerable to predation due to having lower energy stores.

Previous studies have suggested that low inflation rates of *Sparus auratus* larvae be due to the oily surface film that arises from the feed (Chatain and Ounais-Guschemann, 1990). This is particularly relevant to aquaculture, since these results would imply using feeding regimes of some dry foods could potentially cause unexpected harm to larvae by disrupting the air-water interfacewith oily compounds, and that such foods should be avoided until the larvae are older. It is also relevant to compounds which directly disrupt the air-water interface, such as oil. Recently, embryonic exposure to diluted bitumen (crude oil) has been shown to disrupt swim bladder inflation of larval medaka (Madison *et al.,* 2015).

This study contributes to the understanding of swim bladder inflation in Japanese medaka by demonstrating the importance of access to the air-water interface within 4 days of hatch. This study also demonstrates that swim bladder inflation failure caused by compounds will likely cause mortality in the larvae 7 days following hatch. Finally, swim

bladder inflation failure correlated with mortality, which implies that any compounds that disrupt swim bladder inflation are likely to negatively affect survival as well. Future experiments could attempt to link swim bladder inflation failure with reduced growth of the surviving larvae.

This study supports the previous study with pharmaceutical compounds showing that groups of larval fish that are unable to inflate their swim bladder will be more likely to suffer higher mortality within twelve days of hatching. However, exactly how these pharmaceutical compounds are causing swim bladder inflation failure is still not understood. The next study will attempt to form linkages between swim bladder inflation failure and a disruption of cell signaling pathways.

Chapter 6: Linking changes in gene expression caused by pharmaceutical exposure of Japanese medaka embryos to swim bladder inflation failure.

6.1 Abstract

Inadequate swim bladder inflation can have serious long-term effects on many larval fish populations. Many different environmental contaminants including pharmaceuticals inhibit swim bladder inflation. Understanding how this occurs and developing biomarkers for this effect is critical. This study first established that embryonic exposure to 5 μM cyclopamine, a hedgehog (Hh) pathway signaling inhibitor, as well as 1 μM IWR-1, a Wnt pathway signaling inhibitor, resulted in > 95% inhibition of swim bladder inflation of Japanese medaka (*Oryzias latipes*) larvae. The effects of these inhibitors on the expression of medaka genes related to the Hh and Wnt pathways, as well as genes involved in the formation of the three cell layers that make up the swim bladder (epithelial, mesenchyme, and outer mesothelium) were determined at 80, 96, 101, 144, 180 and 216 hours post fertilization.

The main effect of exposure to IWR-1 was a down regulation of the expression of genes related to swim bladder cell layers, such as *fgf10a*, while exposure cyclopamine tended to cause up regulation of genes related to the formation of the three cell layers that make up the swim bladder, such as *fgf10a*, *sox2*, *hprt1l*. The effects on expression of the same genes related to swim bladder formation and inflation were measured following exposure to 17α-ethinylestradiol, levonorgestrel, and diclofenac. The effects of pharmaceutical exposure on gene expression was conducted to determine if exposure to these compounds causes swim bladder inflation failure by acting on similar targets or pathways related to early Hh or Wnt signaling. It was found that the exposure to these three pharmaceutical compounds generally caused down regulation of gene expression, such as the expression of *has2*, *hb9*, *anxa5*, etc., and of the three compounds, levonorgestrel

caused the most significant effects on the expression of the thirteen genes tested. Overall, the pharmaceutical compounds were more similar to each other in terms of how they altered gene expression, although they were closer to the effects of IWR-1 than cyclopamine. These results imply that embryonic exposure to pharmaceutical compounds may be affecting swim bladder inflation by potentially affecting the expression of surfactants and/or affecting the wnt signaling pathway.

6.2 Introduction

Monitoring developmental abnormalities caused by pollutant exposure in fish embryos can allow for the prediction of long-term effects on populations. Many environmental contaminants such as permethrin (González-Doncel *et al.,* 2003), PCB 126 (Jönsson *et al.,* 2012), urban storm water (Skinner *et al.,* 1999), Thiobencarb (Villalobos *et al.,* 2000) and octylphenol (Gray and Metcalfe, 1999) can inhibit swim bladder inflation of larval fish. Previous studies in Chapter 3 & 4 have demonstrated embryonic exposure of Japanese medaka (*Oryzias latipes*) to pharmaceuticals compounds such as ethinylestradiol (EE2), levonorgestrel, and diclofenac can cause swim bladder inflation failure of hatched larvae, and swim bladder inflation failure has been correlated to increased mortality in Chapter 5. Understanding how swim bladder inflation failure occurs in larval fish and developing biomarkers for this effect are thus important.

Both the Wingless-type MMTV integration site family (Wnt) and hedgehog (Hh) signaling pathways are critical in mammalian early lung development (Bell *et al.,* 2008; Bitgood and McMahon, 1995), and have also been shown to be involved in zebrafish (*Danio rerio*) swim bladder development (Winata *et al.,* 2009; Yin *et al.,* 2011). To explore embryonic effects due to pollutant exposure on swim bladder development of Japanese medaka, this study first established the effects of exposure to modulators of Wnt and Hh signalling pathways. Potential adverse outcome pathways for swim bladder inflation failure in larval medaka are varied (Figure 6.1). As shown, a modulation of Wnt/Hh signalling could lead to an improper organization of the three cell layers which make up the swim bladder.

During embryogenesis, specific cell signaling pathways guide cell development, specification, and organization. As Hh and Wnt signaling pathways are involved in swim bladder development during embryogenesis, their disruption by chemical exposure may lead to a malformed swim bladder. An inhibitor of Wnt response (IWR-1) is a potent and specific inhibitor of Wnt signaling. It blocks β-catenin accumulation by stabilizing Axin2, allowing it to function in the absence of an *adenomatous polyposis coli* gene complex (Chen *et al.,* 2009). Zebrafish embryos treated with 10 μM IWR-1 had a time dependent inhibition of the specification of the swim bladder epithelium (Yin *et al.,* 2011). Cyclopamine is a plant steroidal alkaloid and it inhibits the Hh pathway by interacting with Smo, causing it to accumulate in the primary cilium, and causing Smo to change into its closed form even in the presence of Hh ligands (Cooper *et al.,* 1998; Heretsch *et al.,* 2010; Taipale *et al.,* 2000). Zebrafish embryos exposed to 20 μM cyclopamine at various stages of development had disrupted swim bladder formation (Winata *et al.,* 2009). Lithium is known to modulate Wnt signaling by inhibiting GSK3 (Bajoghli *et al.,* 2009).

The effects of these inhibitors on the expression of medaka genes related to the Hh and Wnt pathways, as well as on the genes involved in the formation of the three cell layers that make up the swim bladder (epithelial, mesenchyme and outer mesothelium) were determined in this current study at 80, 96, 101, 144, 180 and 216 hours post fertilization. The 13 genes tested include Hh markers *shha* and *ihha*, Wnt markers *wnt5b*, *tcf3*, and *lef1*, a marker for surfactant production *pbx1b*, as well as tissue markers for the epithelium *sox2*, *hb9*, the mesenchyme fgf10a, *has2*, and the outer mesothelium, *hprt1l*, *elovl1a*, and *anxa5*. These genes have been shown to be crucial during embryonic zebrafish swim bladder development (Korzh *et al.,* 2011; Teoh *et al.,* 2010; Winata *et al.,* 2009; Yin *et al.,* 2011).

Subsequently, the effects on expression of key genes related to swim bladder formation and inflation were measured following exposure to 256 µg/L EE2, 217 µg/L levonorgestrel, and 3.7 mg/L diclofenac using the lowest concentrations that significantly inhibited swim bladder inflation 24 hours post hatch (Chapter 4). The effects of select pharmaceuticals on gene expression were compared to those of the signaling pathway inhibitors to determine if the effects of pharmaceutical exposure on gene expression are related to a modulation of Hh or wnt signaling pathways. An alternate pathway related to a gene (*pbx1b*) associated with the production of surfactants, the production of which is required to inflate the swim bladder post-hatch, was also investigated.

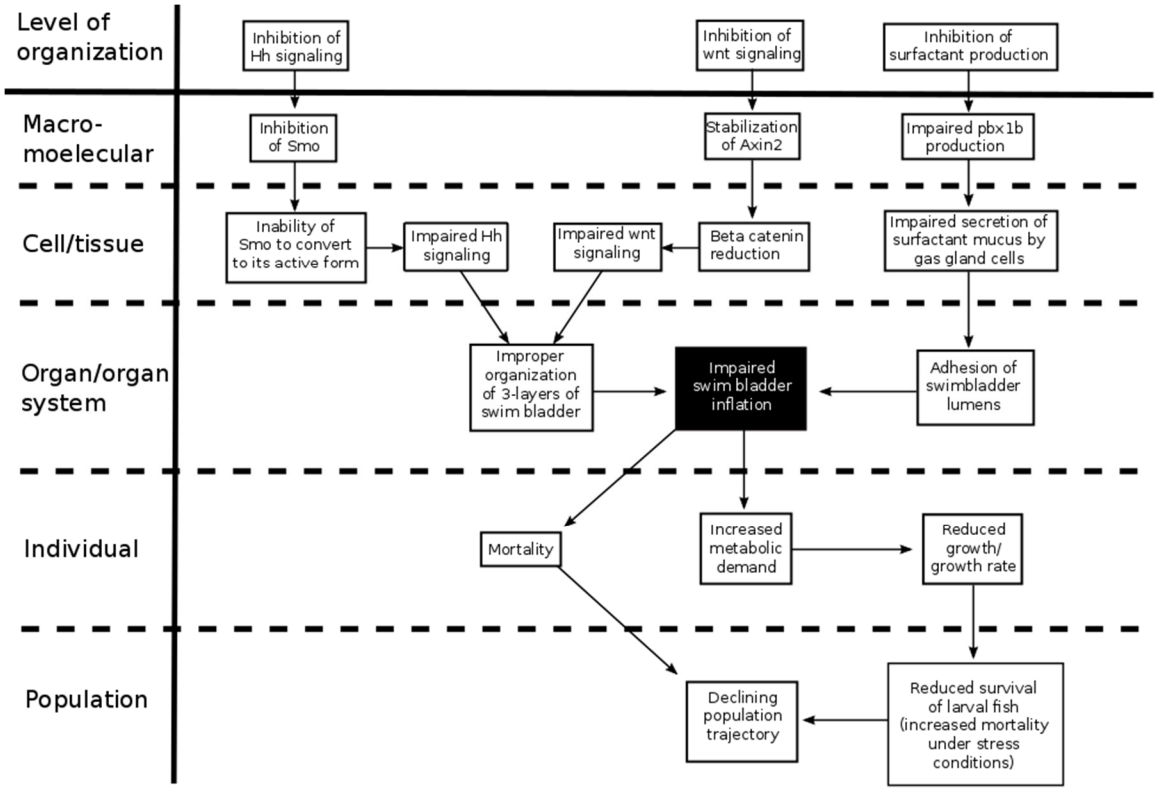


Figure 6.1 Adverse outcome pathway demonstrating potential ways a compound could inhibit Hh signaling, Wnt signaling, or surfactant production at the molecular level can lead to swim bladder inflation failure at the organ level and ultimately impact the population. Based off the pathway proposed in (Villeneuve *et al.,* 2014).

6.2.1 Objectives

This study was conducted to:

- determine if swim bladder development of Japanese medaka larvae can be impaired following embryonic exposure to cyclopamine, lithium, or IWR-1;
- determine their effects on whole embryo gene expression of 13 genes related to swim bladder formation/inflation; and
- establish if embryonic exposure to known concentrations of diclofenac, 17α-ethinylestradiol (EE2), and levonorgestrel that cause swim bladder inflation failure do so by affecting the gene expression in a similar manner to the inhibitors.

6.2.2 Hypotheses

H_{o1}: The inflation of Japanese medaka swim bladders will not be impaired following exposure to IWR-1, cyclopamine, and lithium.

H_{o2}: Exposure to IWR-1, cyclopamine, and lithium will not affect whole embryo gene expression of genes related to swim bladder formation/inflation

H_{o3}: Exposure to diclofenac, EE2, and levonorgestrel at concentrations known to cause swim bladder inflation failure will not affect whole embryo gene expression.

6.3 Materials and methods

6.3.1 Breeding, and embryo collection

Initial stocks of medaka were obtained from the National Institute for Basic Biology (Okazaki, Japan). The experiment assessing the effects of IWR-1, lithium, and cyclopamine were conducted from January 27th 2016, and concluded by April 27th 2016. The follow up experiments using diclofenac, levonorgestrel, and 17α-ethinylestradiol (EE2) were conducted from May 2nd to the 18th 2016. Gene expression analysis was conducted from June-August 2016. Medaka embryos were obtained from adult fish (FLFII strain) bred under standardized conditions (Section 2.1). Embryos were collected as described in section 2.1.2 and assessed for viability.

Embryos were collected from female medaka and separated into 200 mL glass dishes containing rearing solution for viability evaluation. Unfertilized embryos or embryos with irregularities were discarded. Embryos were kept on a plate shaker until either 24 or 36 hours post fertilization where they were transferred to their respective treatments.

6.3.2 Conditions of embryo exposure for cyclopamine and IWR-1 experiments

Embryos were assessed for viability 24 hours post fertilization (hpf) and staged following Iwamatsu (2004) medaka embryo development maps; embryos that had not yet developed an embryonic body (stage 16-17) were discarded. Embryos were then exposed to IWR-1 and cyclopamine at 36 hpf (stage 21-22). A semi-static exposure was conducted until hatch (95% exchange every 24 h until 101 hpf) using plastic 6-well plates. Chemical stocks were prepared before the experiment was begun and renewal solutions were

prepared daily for both inhibitors and pharmaceutical compounds. The treatments and stocks were prepared in, and diluted with, rearing solution (see section 2.1.2). Chemical stocks were prepared in 2 mL glass sampler vials in their respective carrier the day before an experiment was begun. Dilutions were also prepared in their carrier, 50 µL of each concentration was diluted in a 50 mL volumetric flask by filling it to 50 mL with rearing solution (achieving a 0.1% solvent concentration). Medaka embryos were maintained in a temperature-controlled room maintaining a constant temperature of 25°C and a 16:8h (light: dark) photoperiod with 30 minutes of dawn and dusk included in the light period. Plates were kept on a plate shaker set to 60 RPM, in order to narrow down the time-to-hatch (Farwell *et al.,* 2006).

6.3.3 Determining cyclopamine and IWR-1 effective concentrations

IWR-1 was purchased from Sigma-Aldrich., the 25 mg powder was dissolved into 3.06 mL of dimethyl sulfoxide (DMSO) to create a 20 mM stock solution. The solution was stored at -20°C in the dark. Concentrations first tested were 20, 10, 5 mM. To prepare a working solution 50 µL of each respective concentration was diluted in 50 mL of rearing solution using a glass 50 mL volumetric flask, resulting in final concentrations of 20, 10 and 5 µM. A control of just rearing solution and a solvent control containing 50 mL of rearing solution and 50 µL DMSO were both used. Ten embryos per replicate, with four replicates per concentration were exposed in 6 well plates (40 embryos per compound, 200 embryos total). A second experiment was performed using IWR-1 with final concentrations of 1, 0.5 0.1, and 0.05 µM being used. This was also conducted with ten

embryos per replicate, with four replicates per concentration were exposed in 6 well plates (40 embryos per compound, 240 embryos total).

Cyclopamine was purchased from Toronto Research Chemicals Inc., and the 25 mg powder was dissolved into 12.14 mL of ethanol to create a 5 mM stock solution. The solution was stored at -20°C in the dark. Concentrations tested were 5, 1, 0.5, and 0.1 mM. To prepare a working solutions 50 µL of each respective concentration was diluted in 50 mL of rearing solution using a glass 50 mL volumetric flask. Final concentrations were 5, 1, 0.5, and 0.1 µM. A control of just rearing solution and a solvent control containing 50 mL of rearing solution and 50 µL ethanol were both used. This was conducted in 6 well plates with 10 embryos per replicate and 4 replicates per treatment (40 embryos per compound, 240 embryos total). Lithium methodology and results can be found in Appendix G: *Lithium experiments*.

Exposure solutions were renewed every 24 hours from 36 to 101 hpf (during swim bladder development, Figure 6.2). At 101 hpf the exposure was stopped, the embryos were rinsed three times with rearing solution and then transferred to a new 6 well plate. From 101 hpf until hatch rearing solution was renewed daily. Embryos and larvae were observed daily using a dissection microscope until hatched. Upon hatch swim bladder inflation was measured daily up to 3 days post-hatch.

6.3.4 Cyclopamine and IWR-1 effects on gene expression

Embryos were kept on a plate shaker until 36 hours post fertilization (hpf) where they were transferred to their respective treatments. Embryos were exposed to 1 µM IWR-1, a solvent control (0.1% DMSO) or a rearing solution control. Fifteen embryos were

exposed per replicate, three replicates were conducted and the embryos were sampled at six different time points: 80, 96, 101, 144, 180, and 216 hpf (810 total embryos). The first three time points were selected as they occur during the development of the medaka swim bladder, 144, 180 and 216 were selected as they occur after the exposure up until hatch at 216 hpf (Figure 6.2). The cyclopamine exposure was conducted in the same manner as the IWR-1 exposure, but with exposure to 5 µM cyclopamine and a solvent control of 0.1% EtOH being used instead, the sampling times and replicate size was identical to the IWR-1 experiment. Fifteen embryos were placed in each well with 10 mL of test solution. Embryos were exposed either to 1 µM IWR-1, 5 µM cyclopamine (based on the results from 6.3.3), 0.1% DMSO, 0.1% EtOH, or a rearing solution control. Embryos were exposed from 36-101 hpf after which they were rinsed and transferred to normal rearing solution until hatch/sampled. Fifteen embryos were sampled in triplicate at each time point, RNA gene expression was analyzed using RT-qPCR.

Samples were transferred from the -80°C to the -20°C the day prior to use to thaw. Phenol-chloroform extraction was performed to extract and isolate RNA, as outlined in section 2.3.1. RNA purity and concentration were determined using a TrayCell and a Cary 50 Bio UV-Visible Spectrophotometer, outlined in section 2.3.2. RNA quality was assessed using Experion RNA StdSens kits, outlined in section 2.3.3. The method for primer selection and validation is outlined in 2.3.4 and 2.3.5 respectively, and Table 6.1 summarizes the primers used. The following reference genes were expressed for 10 samples across two time points: ribosomal protein l7, ribosomal protein l8, Beta-2-Microglobulin, beta-actin, eukaryotic elongation factor 1 alpha. NormFinder software was used to determine which reference gene was the most stable for the samples in this

experiment (ribosomal protein l7). RT-quantitative PCR were run using the iTaq Universal SYBR Green One-Step Kits, and the methodology is outlined in 2.3.7. Data were analyzed using the Bio-Rad CFX manager. Relative quantification was expressed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), and data was analyzed using the log(2) of the $2^{-\Delta\Delta C_T}$ values.

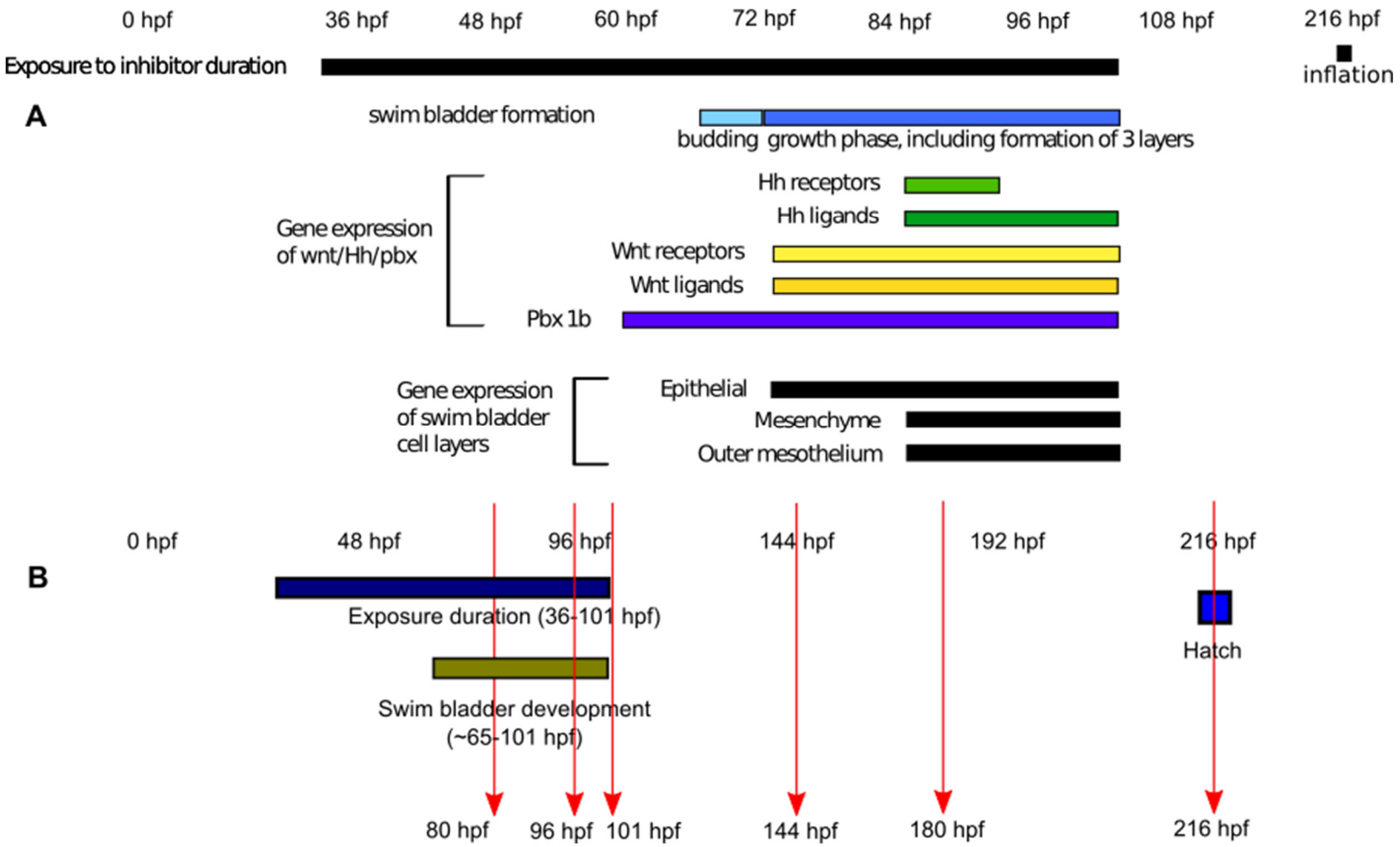


Figure 6.2: Gene expression sampling time line, A) displays the normal gene expression and swim bladder development of the Japanese medaka at various times in hours post fertilization (hpf), B) displays from 0 hpf to hatch (216 hpf) and the six sampling times are denoted by red arrows.

6.3.5 Pharmaceutical exposure and effects on gene expression

Medaka embryos were obtained from adult fish (FLFII strain) bred under standardized conditions. They were suspended in rearing solution for 24 hours and checked for fertilization. Forty five embryos per time point were distributed across a 6 well microplate in triplicate (15 embryos per replicate, 135 embryos per compound). Fertilized embryos were exposed to 217 µg/L levonorgestrel, 256 µg/L 17α-ethinylestradiol (EE2), or 3.7 mg/L diclofenac (24 hour lowest observed effect concentrations from chapter 4), renewed daily and prepared in the same manner as Chapter 4. A solvent control (0.01% acetone) and a rearing solution control were also used in triplicate. Embryos were sampled at 80, 96 and 101 hpf. RNA gene expression was analyzed using RT-qPCR using the same methods as section 6.3.4.

6.3.6 Sampling time points

Fifteen embryos were sampled in triplicate at each of the following time points 80, 96, 101, 144, 180, 216 hpf for IWR-1 and cyclopamine, and at 80, 96, and 101 hpf for diclofenac, EE2, and levonorgestrel. The embryos were placed in a 2 mL cryogenic tube, all excess water was pipetted out, and the embryos were flash frozen in liquid nitrogen. The tubes were stored at -80°C until use.

Table 6.1: Primers used for RT-qPCR analysis, see Table 1.1 for more information on individual markers

Target Gene	Marker For	Role in swb Formation	GenBank Number	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)
hb9	Epithelial	Growth	NC_019878.1	CTGCGGGTTGATTCCTAAGC	GTGTGATGGTGATGTGAGCC	195
sox2	Epithelial	Organization & growth	NM_001278881.1	GAGGCCAGAGGAGGAAGATG	CCTCTTAGCCTCGTCGATGA	131
fgf10a	Mesenchyme	Differentiation & growth	XM_004072263.2	GAAGGGGACATACGGAGGAG	GTTGCTGTTCAGCCCTTTGA	168
has2	Mesenchyme	Organization & growth	XM_004086643.2	CAGAAGTGGGGAGGGAAGAG	AACTGATGATGCTGGGTCCA	117
anxa5	Outer mesothelium	Organization & growth	NM_001104875.1	GTTGTGAAGTGTGCCAGGAG	CCACCAGTGTCTCCCTTGAT	200
hprt1l	Outer mesothelium	Organization & growth	XM_011476251.1	CCGGGGCAAGAATGTTTTGA	TCGGGGTCCTCTTCACTAGA	134
elovl1a	Outer mesothelium	Organization & growth	XM_004079253.2	TTCGAGTTGCTTGGCTGTTC	TGTCCAGGGCATGAAAGAGT	131
shh	Hh	Growth	XM_004081111.2	CAGAAAACTCAGTCGCTGCA	GAGATCTTTGACGGGCCTCT	95
ihha	Hh	Growth	XM_011489656.1	ACATCAGTCTCACAGCTGCT	GCTGAACTTGACTGGCGAAA	156
wnt5b	Wnt	Development, growth, differentiation	NM_001104659.1	GTGTATTACGGCAAGCGGTT	GTCTTCCGGCCTCATTGTTG	127
lef1	Wnt	Development, growth, differentiation	XM_011476166.1	CATGTACGGTAAAGGCCACG	GGGGACTTTGTTGGACGTTC	127
tcf7l1	Wnt	Development, growth, differentiation	NM_001252248.1	CACACCCTTTGAGCTGGTTC	TGGACACTAGGCTGGACATG	123
pbx1b	Surfactant	Swb inflation and surfactant production	XM_011474409.1	AGAGGAGCTGGCAAAGAAGT	TTCTCGCAGCGTACATGTTG	128

(swb) swim bladder, (*hb9*) motor neuron and pancreas homeobox 1, (*sox2*) SRY (sex determining region Y)-box 2, (*fgf10a*) fibroblast growth factor 10, (*has2*) hyaluronan synthase 2, (*anxa5*) Annexin A10, (*hprt1l*) hypoxanthine-guanine phosphoribosyltransferase-like, (*elovl1a*) elongation of very long chain fatty acid protein 1-like, (*shh*) sonic hedgehog, (*ihha*) indian hedgehog B protein, (*wnt5b*) wingless-type MMTV integration site family member 5B, (*lef1*) lymphoid enhancer-binding factor 1, (*tcf7l1*) transcription factor 7 like 1, (*pbx1b*) pre-B-cell leukemia homeobox 1

6.3.8 End points

Select embryonic developmental stages of medaka were examined daily, using a dissecting stereomicroscope LEICA EZ4D (20x magnification) following the Iwamatsu (2004) fate map. Developmental abnormalities, mortality/hatchability and time-to-hatch were recorded daily for determining cyclopamine and IWR-1 effective concentrations. Swim bladder inflation was monitored up to 3 days post-hatch.

6.3.9 Statistical analysis

For determining the effective concentrations of IWR-1 and cyclopamine, data were first assessed for normality using Shapiro-Wilk W test, as well as for homogeneity of variance using a Brown-Forsythe test. If the data passed, a one-way analysis of variance (ANOVA, $p \leq 0.05$ and $p \leq 0.10$) was performed, confirmed by a Dunnet Post – hoc Test, $p \leq 0.05$ and $p \leq 0.10$. If the data failed normality, a Kruskal-Wallis one-way analysis of variance on ranks was performed (ANOVA on ranks, $p \leq 0.05$ and $p \leq 0.10$), confirmed by multiple comparisons versus their respective control group (Dunn's method $p \leq 0.05$ and $p \leq 0.10$). All statistical analyses and graphs were performed/created using SigmaPlot 13.0 software.

For assessing the effects on gene expression, paired t-tests, $p \leq 0.05$ and $p \leq 0.10$, were conducted between IWR-1/cyclopamine and their respective solvent control (DMSO and ethanol, respectively). For the effects of diclofenac, EE2, and levonorgestrel, paired t-tests were conducted between the pooled controls and the respective treatment. These were conducted using Microsoft Excel.

Normality of residuals of the data was assessed for normality using Shapiro-Wilk W test, as well as for homogeneity of variance using a Brown-Forsythe test. To describe the multivariate response in expression to the treatment groups, non-metric multidimensional scaling (NMDS) plots was carried out with the expression of each gene in a replicate as one point in the ordination using R-studio using the vegan package, and stress values are displayed on each corresponding Figure. A NMDS plot with a stress value of > 0.3 is considered highly suspect, > 0.2 should be interpreted with caution, ≤ 0.1 is considered a fair fit, and ≤ 0.05 indicates a good fit. NMDS is a indirect gradient analysis approach, and produces an ordination based on distance or dissimilarity; labels similar to one another will be ordinated closer together on the plots.

6.4 Results

6.4.1 Determining cyclopamine and IWR-1 effective concentrations

There was no significant effect of medaka embryonic exposure to concentrations of cyclopamine ranging from 0.1-5 μM on median time-to-hatch, or hatchability from 36 hours post fertilization (hpf) until 101 hpf (Table 6.2). There also was no significant effect of embryonic exposure to 0.05-1 μM IWR-1 from 36-101 hpf on hatchability (Table 6.3). However, exposure to 5, 10, and 20 μM IWR-1 caused a significant reduction in hatchability with only 20-10% of embryos surviving until hatch ($p < 0.001$). There was a difference in time-to-hatch between the two batches off embryos collected; the embryos in the second group took ~1 day longer to hatch than the earlier group, and as a result the controls are shown separately (Table 6.4). There was no significant effect on median time-to-hatch of embryos exposed to 0.05-10 μM IWR-1 however, there was a significant delay in median time-to-hatch by 1 day of IWR-1 exposed fish at the highest concentration tested of 20 μM IWR-1 ($p = 0.010$).

Exposure to 5 μM cyclopamine resulted in medaka larvae being completely unable to inflate their swim bladders at hatch and up to 3 days post-hatch ($p < 0.001$, Table 6.5), but none of the other lower concentrations tested had any significant effect on swim bladder inflation when compared to the control. Swim bladder inflation was completely inhibited at 5 μM IWR-1 and higher ($p < 0.001$, Table 6.5). Following exposure to 1 μM IWR-1, only 3.6% of larvae were able to inflate their swim bladders, and after 72 hours post-hatch only 7.3% were successful; in both cases these were significantly lower than controls with 65.7% of controls inflating at hatch, and 98.8% inflating by 72 hours post-hatch ($p < 0.001$, $p = 0.016$, at 0 and 72 hours post-hatch, respectively). Exposure to 0.5 μM IWR-1 caused

a significant inhibition of inflation at 0 ($p = 0.001$) and 24 hours post-hatch ($p = 0.002$), but this effect was recovered by 48 hours post-hatch.

Exposure of medaka embryos to 5-20 μM IWR-1 caused significant abnormalities in the growth of the medaka (Figure 6.3). Generally the higher the concentration of IWR-1 the higher the mortality and severity of abnormalities. Exposure of medaka embryos to 5 μM cyclopamine caused significant effects on the tail formation of the larvae, with many having a curved tail (Figure 6.4). Concentrations lower than 5 μM cyclopamine or 1 μM IWR-1 did not cause any observable effects on development.

Based on these results 5 μM cyclopamine and 1 μM IWR-1 were chosen for the gene expression experiments as they cause a significant inhibition of swim bladder inflation in the hatched larvae without causing a significant increase in mortality.

6.4.2 Effects of compounds on gene expression

IWR-1 generally cause a down regulation of expression of the 13 genes tested (Table 6.6a). However, these effects were only significant for a few of the genes tested (*fgf10a*, *sox2*, *hb9*, and *shh*). Most of the effects on gene expression were low, with the majority of effects being a decrease in gene expression of 15-50%. However, exposure to 1 μM IWR-1 did cause a significant increase in expression of hprt1l by 74% at 144 hours post fertilization. A Wnt signaling pathway inhibitor, exposure to 1 μM IWR-1 generally resulted in down regulation of the expression of *lef1*, *tcf7l1*, or *wnt5b*, which are all associated with the Wnt signaling pathway, however, none of these effects were significantly different compared to the controls.

The effects of cyclopamine on gene expression were much more varied than those of IWR-1 (Table 6.6b). Generally, exposure to 5 µM cyclopamine caused more significant upregulation of gene expression during the 96-144 hour post fertilization (hpf) and caused significant down regulation of *has2* at 80 hpf and *hb9* at 216 hpf (Table 6.8). Exposure to cyclopamine did not significantly affect the expression of *shh* or *ihha*, which are both involved in the Hh pathway. Neither cyclopamine nor IWR-1 caused a significant effect on the expression of *pbx1b*, which is associated with surfactant production.

Exposure to 217 µg/L levonorgestrel, 256 µg/L 17α-ethinylestradiol (EE2), or 3.7 mg/L diclofenac significantly affected gene expression (Table 6.7). Exposure to these pharmaceutical compounds generally caused down regulation of many of the genes tested (Table 6.9). All three compounds significantly altered the expression of *pbx1b*. Exposure to EE2 and levonorgestrel also significantly down regulated the expression of genes involved in the Wnt pathway such as *lef1* or *tcf7l1*. The expression of *has2*, *hb9*, and *anxa5* were significantly affected by all three natural/synthetic hormones, with most of the significant downregulation occurring at 101 hpf. These compounds generally caused more significant effects on gene expression than IWR-1 or cyclopamine, with levonorgestrel causing the most significant effects observed.

Non-metric multidimensional scaling (NMDS) showed that cyclopamine exposure does not appear to be related to the effects of any of the other compounds, whereas the effects of the three pharmaceutical compounds seem clustered at each of their sampling time points (Figure 6.5). The pharmaceuticals also follow a similar temporal pattern, and the effects of IWR-1 appear to be more related to those of the three pharmaceutical compounds at 101 hours post fertilization. When split into separate replicates, the variance

of the replicates tended to increase with time for cyclopamine, did not seem time dependent for IWR-1 or diclofenac, and tended to decrease with time for EE2 and levonorgestrel (Figure 6.6).

Table 6.2: Median time-to-hatch (days (min, max) and hatchability (mean ± SE) following embryonic exposure to cyclopamine (nominal) from 36 hours post fertilization (hpf) until 101 hpf, 4 replicates of 10 embryos per concentration, the control was embryos in rearing solution control, and the solvent control (SC) was 0.01% ethanol. Significant levels shown as *0.1, **0.05, ***< 0.01, ****< 0.001.

Treatment (μM) (nominal)	Median (days (min,max))	Hatchability (%)
Control	9 (8,10)	95.0 ± 5.0
Solvent Control	10 (9,10)	95.0 ± 5.0
0.1 μM	10 (8,10)	95.0 ± 2.9
0.5 μM	10 (9,11)	97.5 ± 2.5
1 μM	9 (8,10)	97.5 ± 2.5
5 μM	9 (8,10)	95.0 ± 2.9

Table 6.3: Hatchability (mean ± SE) following embryonic exposure to IWR-1 (nominal) from 36 hours post fertilization (hpf) until 101 hpf, 4 replicates of 10 embryos per concentration, the control was embryos in rearing solution control, and the solvent control (SC) was 0.01% DMSO. Significant levels shown as *0.1, **0.05, ***< 0.01, ****< 0.001.

Treatment (μM) (nominal)	Hatchability (%)
Control	97.5 ± 2.5
SC	98.8 ± 1.3
0.05 μM	90.0 ± 7.07
0.1 μM	100 ± 0.0
0.5 μM	97.5 ± 2.5
1 μM	87.5 ± 9.5
5 μM	20 ± 7.1 ****
10 μM	10 ± 4.1 ****
20 μM	10 ± 4.1 ****

Table 6.4: Median time-to-hatch (days (min, max) following embryonic exposure to IWR-1 (nominal) from 36 hours post fertilization (hpf) until 101 hpf, 4 replicates of 10 embryos per concentration, the control was embryos in rearing solution control, and the solvent control (SC) was 0.01% DMSO. Note: there was a difference in the time-to-hatch between the two experiments so the controls are shown separately. Significant levels shown as *0.1, **0.05, ***< 0.01, ****< 0.001.

Treatment (µM) (nominal)	Median (days (min,max))	Treatment (µM) (nominal)	Median (days (min,max))
Control	8 (7,9)	Control	9 (8,11)
SC	8 (7,9)	SC	9 (8,10)
0.05 µM	8 (7,9)	5 µM	9 (9,9)
0.1 µM	8 (7,9)	10 µM	9 (9,10)
0.5 µM	8 (7,8)	20 µM	10 (9,11) **
1 µM	8 (7,9)		

Table 6.5: Effects of exposure to nominal concentrations of cyclopamine and IWR-1 on swim bladder inflation at 0, 24, 48, and 72 hours post-hatch. Values are percent (%) of fish able to inflate their swim bladder (n=10/replicate, 4 replicates per concentration). Significant levels shown as *0.1, **0.05, ***< 0.01, ****< 0.001 when compared to their respective solvent control (ethanol & DMSO).

Cyclopamine	Swim Bladder Inflation (%, mean ± SE)			
	0-h	24-h	48- h	72-h
C	72.5 ± 5.5	91.9 ± 5.6	91.9 ± 2.8	94.4 ± 3.3
SC	79.4 ± 10.6	95.0 ± 2.9	97.5 ± 2.5	97.5 ± 2.5
0.1 µM	83.6 ± 7.2	97.5 ± 2.5	97.5 ± 2.5	97.5 ± 2.5
0.5 µM	73.9 ± 10.2	94.4 ± 5.6	94.4 ± 5.6	94.4 ± 5.6
1 µM	66.7 ± 6.2	89.4 ± 4.1	92.2 ± 4.8	92.2 ± 4.8
5 µM	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****

IWR-1	Swim Bladder Inflation (%, mean ± SE)			
	0-h	24-h	48- h	72-h
C	65.7 ± 4.3	95 ± 2.5	95 ± 2.5	98.8 ± 1.3
SC	71.3 ± 8.8	93.8 ± 1.25	95.7 ± 0.8	97.5 ± 2.5
0.05 µM	51.2 ± 10.5	84.4 ± 6.6	91.9 ± 5.3	94.4 ± 5.6
0.1 µM	57.5 ± 16	90.0 ± 7.1	95.0 ± 5.0	97.5 ± 2.5
0.5 µM	12.8 ± 7.3 ***	66.4 ± 8.9 ***	94.7 ± 3.1	94.7 ± 3.1
1 µM	3.6 ± 3.6 ****	6.7 ± 4.1 ****	6.9 ± 4.2 **	7.3 ± 4.3 **
5 µM	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****
10 µM	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****
20 µM	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****	0.0 ± 0.0 ****

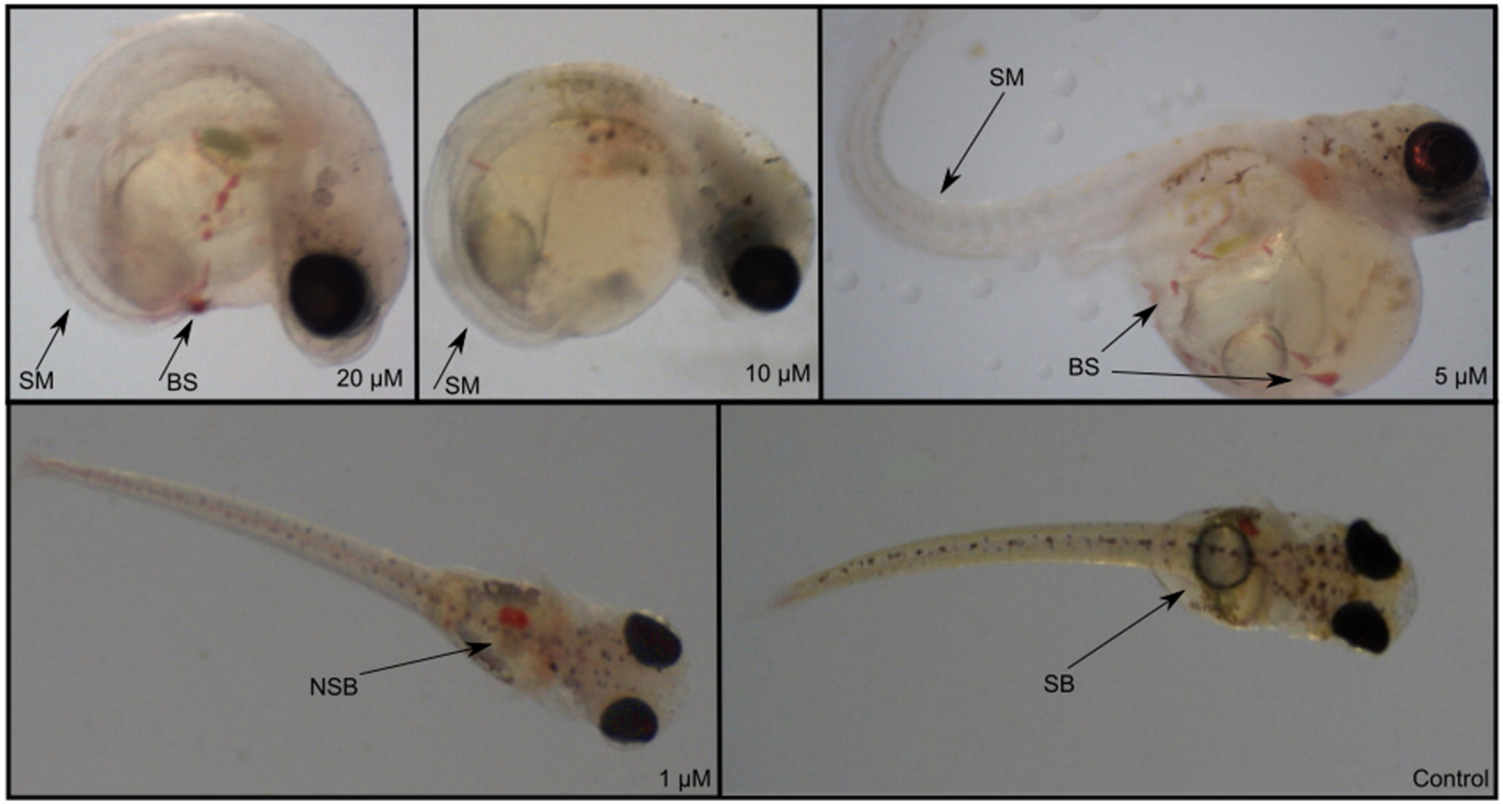


Figure 6.3: IWR-1 exposed medaka 72 hours post-hatch. It can be observed that the larvae is increasingly malformed with higher exposure concentration of IWR-1, with the larvae not developing fully at higher concentrations of IWR-1 (5-20 µM), the control (solvent control 0.01% DMSO) is also displayed. Spinal malformation (SM), blood stasis (BS), no swim bladder inflation (NSB) and normal swim bladder (SB).

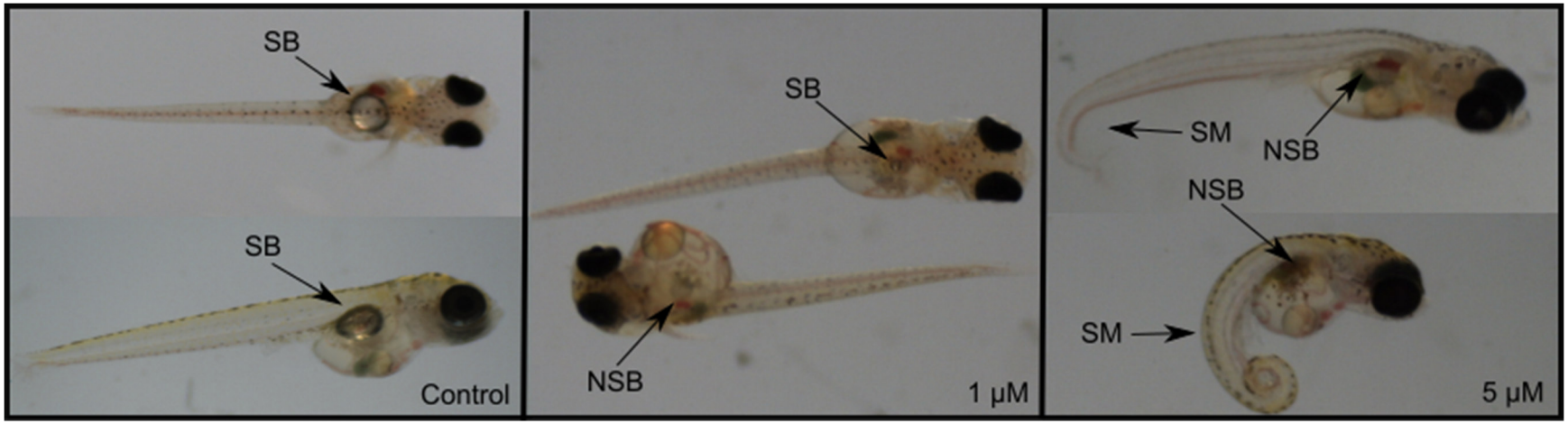


Figure 6.4 Cyclopamine exposed medaka 24 hours post-hatch. At the highest concentration of cyclopamine tested (5 µM) it can be observed that larvae were unable to inflate their swim bladder and had a malformed spine, control (Solvent control, 0.01% ethanol). Concentrations < 5 µM did not have a significant effect on swim bladder inflation (or presence of other malformations), with the majority of larvae inflating their swim bladder within 24 hours of hatching. Spinal malformation (SM), no swim bladder inflation (NSB) and normal swim bladder (SB).

Table 6.6a: Summary of the expression of 13 genes sampled at various hours post fertilization (hpf) after exposure to 1 µM (nominal) IWR-1, expression values are presented as mean log(2) ± SE (of the calculated $2^{-\Delta\Delta C_T}$ values), this was used to display effects in an intuitive manner (a value of -1 is twofold down and 1 is twofold upregulated). Paired t-tests were conducted between the solvent control (0.01% DMSO) and the respective treatments and the resulting p-values (p) are listed and significant differences are displayed **bolded**.

	80 hpf			96 hpf			101 hpf			144 hpf			180 hpf			216 hpf		
Gene	Control	IWR-1	p	Control	IWR-1	p	Control	IWR-1	p	Control	IWR-1	p	Control	IWR-1	p	Control	IWR-1	p
shh	0.00 ± 0.26	-0.14 ± 0.18	0.68	0.00 ± 0.07	-0.19 ± 0.45	0.70	0.00 ± 0.27	-0.39 ± 0.33	0.41	0.00 ± 0.41	0.10 ± 0.32	0.85	0.00 ± 0.11	-0.53 ± 51	0.37	0.00 ± 0.40	**-0.87 ± 0.05**	**0.095**
ihha	0.00 ± 0.28	-0.16 ± 0.22	0.67	0.00 ± 0.02	-0.21 ± 0.47	0.68	0.00 ± 0.40	-0.60 ± 0.33	0.32	0.00 ± 0.41	0.18 ± 0.37	0.76	0.00 ± 0.42	-0.31 ± 0.24	0.55	0.00 ± 0.46	-0.56 ± 0.10	0.31
lef1	0.00 ± 0.26	-0.61 ± 0.17	0.12	0.00 ± 0.03	-0.49 ± 0.26	0.13	0.00 ± 0.38	-0.85 ± 0.19	0.12	0.00 ± 0.36	0.17 ± 0.31	0.74	0.00 ± 0.47	-0.21 ± 0.11	0.68	0.00 ± 0.22	0.21 ± 0.08	0.42
tcf7l1	0.00 ± 0.20	-0.45 ± 0.29	0.28	0.00 ± 0.14	0.04 ± 0.19	0.87	0.00 ± 0.16	-0.21 ± 0.38	0.64	0.00 ± 0.23	0.17 ± 0.03	0.50	0.00 ± 0.28	0.12 ± 0.43	0.83	0.00 ± 0.03	-0.04 ± 0.21	0.85
wnt5b	0.00 ± 0.24	-0.39 ± 0.24	0.32	0.00 ± 0.04	-0.14 ± 0.23	0.58	0.00 ± 0.29	-0.49 ± 0.20	0.24	0.00 ± 0.29	-0.09 ± 0.30	0.85	0.00 ± 0.45	-0.40 ± 0.20	0.46	0.00 ± 0.43	-0.22 ± 0.13	0.64
fgf10a	0.00 ± 0.02	-0.73 ± 0.20	0.022	0.00 ± 0.08	**-0.62 ± 0.11**	**0.012**	0.00 ± 0.31	**-0.91 ± 0.23**	**0.08**	0.00 ± 0.35	-0.26 ± 0.37	0.63	0.00 ± 0.37	-0.41 ± 0.10	0.35	0.00 ± 0.19	-0.41 ± 0.05	0.11
has2	0.00 ± 0.10	-0.04 ± 0.54	0.94	0.00 ± 0.17	-0.38 ± 0.23	0.26	0.00 ± 0.20	-0.31 ± 0.16	0.29	0.00 ± 0.43	-0.27 ± 0.23	0.61	0.00 ± 0.32	0.52 ± 0.32	0.31	0.00 ± 0.23	-0.63 ± 0.37	0.22
sox2	0.00 ± 0.14	-0.34 ± 0.27	0.32	0.00 ± 0.05	0.04 ± 0.20	0.86	0.00 ± 0.15	-0.16 ± 0.16	0.51	0.00 ± 0.26	0.39 ± 0.11	0.24	0.00 ± 0.09	-0.04 ± 0.27	0.89	0.00 ± 0.12	**-0.82 ± 0.13**	**0.01**
hb9	0.00 ± 0.27	-0.21 ± 0.39	0.68	0.00 ± 0.09	-0.54 ± 0.50	0.35	0.00 ± 0.20	-0.24 ± 0.32	0.56	0.00 ± 0.19	0.09 ± 0.19	0.84	0.00 ± 0.19	-0.33 ± 0.49	0.56	0.00 ± 0.48	**-1.26 ± 0.20**	**0.07**
hprt1l	0.00 ± 0.26	-0.17 ± 0.27	0.68	0.00 ± 0.08	-0.09 ± 0.13	0.57	0.00 ± 0.12	0.18 ± 0.08	0.28	0.00 ± 0.26	**0.74 ± 0.04**	**0.050**	0.00 ± 0.53	-0.25 ± 0.30	0.70	0.00 ± 0.14	0.27 ± 0.52	0.72
elovl1a	0.00 ± 0.12	0.03 ± 0.34	0.94	0.00 ± 0.07	0.21 ± 0.40	0.63	0.00 ± 0.42	0.08 ± 0.10	0.86	0.00 ± 0.30	0.14 ± 0.46	0.81	0.00 ± 0.36	-0.74 ± 0.39	0.24	0.00 ± 0.42	-0.50 ± 0.14	0.32
anxa5	0.00 ± 0.17	-0.82 ± 0.37	0.11	0.00 ± 0.17	0.01 ± 0.14	0.98	0.00 ± 0.38	-0.85 ± 0.19	0.12	0.00 ± 0.36	0.17 ± 0.31	0.74	0.00 ± 0.39	-0.40 ± 0.07	0.37	0.00 ± 0.13	0.07 ± 0.07	0.67
pbx1b	0.00 ± 0.20	-0.15 ± 0.22	0.65	0.00 ± 0.04	0.26 ± 0.15	0.18	0.00 ± 0.14	-0.09 ± 0.44	0.85	0.00 ± 0.26	0.38 ± 0.16	0.28	0.00 ± 0.20	0.32 ± 0.44	0.54	0.00 ± 0.77	0.81 ± 0.07	0.29

Table 6.6b: Expression of 13 genes sampled at various hours post fertilization (hpf) after exposure to 5 µM (nominal) cyclopamine (Cyclo), expression values are presented as mean log(2) (of the calculated $2^{-\Delta\Delta C_T}$ values) ± SE. Paired t-tests were conducted between the solvent control (0.01% EtOH) and the respective treatments and the resulting p-values (p) are listed and significant differences are displayed **bolded**.

	80 hpf			96 hpf			101 hpf			144 hpf			180 hpf			216 hpf		
Gene	Control	Cyclo	p	Control	Cyclo	p	Control	Cyclo	p	Control	Cyclo	p	Control	Cyclo	p	Control	Cyclo	p
shh	0.00 ± 0.83	-0.41 ± 0.19	0.66	0.00 ± 0.58	0.08 ± 0.09	0.89	0.00 ± 0.14	0.60 ± 0.26	0.12	0.00 ± 0.21	0.03 ± 0.17	0.93	0.00 ± 0.31	0.26 ± 0.30	0.58	0.00 ± 0.37	-0.57 ± 0.05	0.20
ihha	0.00 ± 0.95	-0.04 ± 0.35	0.97	0.00 ± 0.56	0.49 ± 0.29	0.48	0.00 ± 0.36	0.86 ±0.34	0.16	0.00 ± 0.41	0.18 ± 0.16	0.76	0.00 ± 0.33	0.15 ± 0.45	0.80	0.00 ± 0.28	-0.32 ± 0.47	0.59
lef1	0.00 ± 0.37	0.50 ± 0.11	0.26	0.00 ± 0.06	0.42 ± 0.24	0.17	0.00 ± 0.24	0.60 ± 0.19	0.12	0.00 ± 0.36	0.69 ± 0.16	0.16	0.00 ± 0.14	0.47 ± 0.40	0.34	0.00 ± 0.08	0.21 ± 0.08	0.87
tcf7l1	0.00 ± 0.17	0.37 ± 0.25	0.29	0.00 ± 0.60	0.60 ± 0.11	0.38	0.00 ± 0.18	0.41 ± 0.16	0.17	0.00 ± 0.06	**0.19 ± 0.02**	**0.03**	0.00 ± 0.20	0.54 ± 0.20	0.13	0.00 ± 0.02	-0.72 ± 0.56	0.27
wnt5b	0.00 ± 0.53	0.19 ± 0.23	0.76	0.00 ± 0.19	0.46 ± 0.16	0.13	0.00 ± 0.29	0.65 ± 0.21	0.15	0.00 ± 0.37	0.39 ± 0.12	0.37	0.00 ± 0.12	0.37 ± 0.41	0.44	0.00 ± 0.19	-0.15 ± 0.44	0.76
fgf10a	0.00 ± 0.32	0.68 ± 0.10	0.22	0.00 ± 0.07	**0.55 ± 0.14**	**0.026**	0.00 ± 0.23	0.10 ± 0.27	0.79	0.00 ± 0.28	0.47 ± 0.05	0.17	0.00 ± 0.18	0.63 ± 0.30	0.15	0.00 ± 0.08	0.11 ± 0.41	0.81
has2	0.00 ± 0.28	**-0.68 ± 0.15**	**0.099**	0.00 ± 0.38	-0.53 ± 0.16	0.27	0.00 ± 0.22	-0.04 ± 0.36	0.92	0.00 ± 0.04	-0.58 ± 0.32	0.14	0.00 ± 0.15	0.60 ± 0.24	0.102	0.00 ± 0.39	0.09 ± 0.46	0.89
sox2	0.00 ± 0.29	-0.36 ± 0.08	0.31	0.00 ± 0.27	0.04 ± 0.10	0.89	0.00 ± 0.04	**0.78 ± 0.09**	**0.00031**	0.00 ± 0.10	-0.21 ± 0.08	0.18	0.00 ± 0.13	0.05 ± 0.19	0.85	0.00 ± 0.09	-0.01 ± 0.18	0.95
hb9	0.00 ± 0.72	-0.33 ± 0.31	0.60	0.00 ± 0.44	-0.25 ± 0.01	0.60	0.00 ± 0.31	0.61 ± 0.27	0.21	0.00 ± 0.18	-0.48 ± 0.38	0.32	0.00 ± 0.28	-0.05 ± 0.36	0.92	0.00 ± 0.14	**-0.69 ± 0.30**	**0.10**
hprt1l	0.00 ± 0.20	-0.20 ± 0.39	0.67	0.00 ± 0.03	**0.57 ± 0.23**	**0.07**	0.00 ± 0.14	0.09 ± 0.16	0.71	0.00 ± 0.04	**0.22 ± 0.01**	**0.008**	0.00 ± 0.37	0.01 ± 0.55	0.99	0.00 ± 0.42	-0.45± 0.77	0.64
elovl1a	0.00 ± 0.75	0.23 ± 0.34	0.79	0.00 ± 0.44	0.56 ± 0.28	0.34	0.00 ± 0.30	0.86 ± 0.37	0.14	0.00 ± 0.38	0.26 ± 0.07	0.54	0.00 ± 0.24	0.40 ± 0.43	0.47	0.00 ± 0.24	-0.22 ± 0.40	0.67
anxa5	0.00 ± 0.53	0.64 ± 0.09	0.30	0.00 ± 0.12	0.70 ± 0.40	0.17	0.00 ± 0.24	0.60 ± 0.19	0.12	0.00 ± 0.36	0.69 ± 0.16	0.16	0.00 ± 0.20	0.47 ± 0.36	0.32	0.00 ± 0.19	-0.03 ± 0.46	0.96
pbx1b	0.00 ± 0.08	-0.01 ± 0.22	0.98	0.00 ± 0.09	**0.27 ± 0.08**	**0.09**	0.00 ± 0.27	0.29 ± 0.05	0.35	0.00 ± 0.13	-0.01 ± 0.08	0.94	0.00 ± 0.17	0.25 ± 0.20	0.40	0.00 ± 0.07	0.09 ± 0.20	0.70

Table 6.7: Expression of 13 genes sampled at various hours post fertilization (hpf) after exposure to 217 µg/L levonorgestrel (LNG), 256 µg/L 17α-ethinylestradiol (EE2), or 3.7 mg/L diclofenac (DIC), expression values are presented as mean log(2) ± SE. Paired t-tests were conducted between the pooled control and the respective treatments and the resulting p-values (p) are listed and significant differences are displayed **bolded**.

	80hpf							96hpf							101hpf						
Gene	Control	DIC	p	LNG	p	EE2	p	Control	DIC	p	LNG	p	EE2	p	Control	DIC	p	LNG	p	EE2	p
shh	0.00 ± 0.14	0.14 ± 0.05	0.67	0.03 ± 0.16	0.99	0.26 ± 0.20	0.42	0.00 ± 0.32	-0.03 ± 0.05	0.95	-0.10 ± 0.21	0.85	0.02 ± 0.08	0.97	0.00 ± 0.08	-0.27 ± 0.19	0.19	**-0.35 ± 0.08**	**0.041**	-0.10 ± 0.03	0.44
ihha	0.00 ± 0.18	0.14 ± 0.05	0.62	0.07 ± 0.14	0.80	0.43 ± 0.13	0.17	0.00 ± 0.39	0.06 ± 0.14	0.91	-0.02 ± 0.20	0.98	0.23 ± 0.03	0.70	0.00 ± 0.12	-0.21 ± 0.06	0.28	-0.22 ± 0.05	0.25	-0.11 ± 0.10	0.57
lef1	0.00 ± 0.11	-0.14 ± 0.18	0.51	**-0.67 ± 0.16**	**0.0091**	-0.16 ± 0.15	0.43	0.00 ± 0.23	-0.37 ± 0.05	0.31	**-0.89 ± 0.12**	**0.036**	-0.49 ± 0.15	0.20	0.00 ± 0.11	-0.28 ± 0.15	0.20	-0.29 ± 0.07	0.14	-0.26 ± 0.16	0.23
tcf7l1	0.00 ± 0.07	0.00 ± 0.12	0.99	0.17 ± 0.03	0.12	0.00 ± 0.00	0.96	0.00 ± 0.03	-0.08 ± 0.00	0.11	**-0.11 ± 0.02**	**0.062**	**-0.51 ± 0.37**	**0.082**	0.00 ± 0.08	-0.16 ± 0.16	0.35	**-0.29 ± 0.03**	**0.044**	**-0.33 ± 0.16**	**0.090**
wnt5b	0.00 ± 0.05	-0.03 ± 0.05	0.74	0.13 ± 0.07	0.14	0.13 ± 0.10	0.21	0.00 ± 0.17	-0.01 ± 0.08	0.96	0.01 ± 0.07	0.97	-0.11 ± 0.08	0.68	0.00 ± 0.05	-0.06 ± 0.03	0.51	0.07 ± 0.02	0.42	0.04 ± 0.03	0.63
fgf10a	0.00 ± 0.05	-0.01 ± 0.04	0.87	**0.22 ± 0.06**	**0.039**	0.09 ± 0.09	0.40	0.00 ± 0.07	-0.13 ± 0.05	0.27	0.10 ± 0.06	0.40	0.36 ± 0.29	0.14	0.00 ± 0.05	-0.16 ± 0.11	0.21	-0.12 ± 0.06	0.23	0.06 ± 0.07	0.55
has2	0.00 ± 0.38	-0.99 ± 0.23	0.13	**-1.23 ± 0.25**	**0.07**	**-1.47 ± 0.12**	**0.036**	0.00 ± 0.09	**-0.61 ± 0.17**	**0.0084**	0.13 ± 0.17	0.466	**-0.40 ± 0.06**	**0.02**	0.00 ± 0.11	-0.36 ± 0.13	0.11	**-0.81 ± 0.08**	**0.0039**	**-0.56 ± 0.09**	**0.021**
sox2	0.00 ± 0.09	0.13 ± 0.03	0.39	0.25 ± 0.14	0.17	0.13 ± 0.17	0.47	0.00 ± 0.11	-0.21 ± 0.07	0.24	-0.28 ± 0.14	0.17	-0.07 ± 0.07	0.70	0.00 ± 0.13	-0.06 ± 0.10	0.79	-0.03 ± 0.20	0.91	0.24 ± 0.04	0.26
hb9	0.00 ± 0.13	0.23 ± 0.01	0.25	0.12 ± 0.12	0.58	**0.46 ± 0.11**	**0.056**	0.00 ± 0.16	0.25 ± 0.06	0.32	-0.01 ± 0.22	0.98	0.32 ± 0.14	0.25	0.00 ± 0.03	**-0.29 ± 0.12**	**0.025**	**-0.46 ± 0.13**	**0.0051**	**-0.32 ± 0.04**	**0.0011**
hprt1l	0.00 ± 0.09	0.01 ± 0.15	0.97	-0.02 ± 0.16	0.91	0.02 ± 0.35	0.96	0.00 ± 0.17	-0.46 ± 0.17	0.13	0.19 ± 0.28	0.56	-0.23 ± 0.14	0.40	0.00 ± 0.14	**-0.46 ± 0.03**	**0.060**	0.24 ± 0.07	0.29	0.00 ± 0.09	0.99
elovl1a	0.00 ± 0.16	-0.20 ± 0.18	0.48	**-0.73 ± 0.16**	**0.024**	-0.21 ± 0.15	0.42	0.00 ± 0.23	-0.37 ± 0.05	0.31	**-0.89 ± 0.12**	**0.036**	-0.49 ± 0.15	0.21	0.00 ± 0.16	-0.32 ± 0.10	0.25	**-0.67 ± 0.05**	**0.031**	-0.14 ± 0.04	0.58
anxa5	0.00 ± 0.04	0.10 ± 0.13	0.38	**0.16 ± 0.07**	**0.085**	-0.10 ± 0.10	0.29	0.00 ± 0.04	**0.24 ± 0.02**	**0.0075**	**0.14 ± 0.04**	**0.087**	-0.22 ± 0.19	0.16	0.00 ± 0.09	**-0.32 ± 0.09**	**0.080**	-0.37 ± 0.07	0.046	**-0.68 ± 0.06**	**0.0031**
pbx1b	0.00 ± 0.06	0.30 ± 0.22	0.12	**0.26 ± 0.12**	**0.063**	**0.61 ± 0.07**	**0.00040**	0.00 ± 0.06	**-0.59 ± 0.14**	**0.0021**	0.03 ± 0.01	0.71	**-0.70 ± 0.35**	**0.024**	0.00 ± 0.11	-0.11 ± 0.14	0.58	0.01 ± 0.10	0.96	0.11 ± 0.10	0.56

Table 6.8: Summary table of significant effects on gene expression following nominal exposure to 1 µM IWR-1 or 5 µM cyclopamine from 36 hours post fertilization (hpf) until various time points. Confidence intervals, paired t-tests, $p \leq 0.05$ and $p \leq 0.10$, and ranges of fold induction (log(2)) are displayed, if no arrow is displayed this indicates induction is < 0.25, which while statistically significant, may not be biologically relevant.

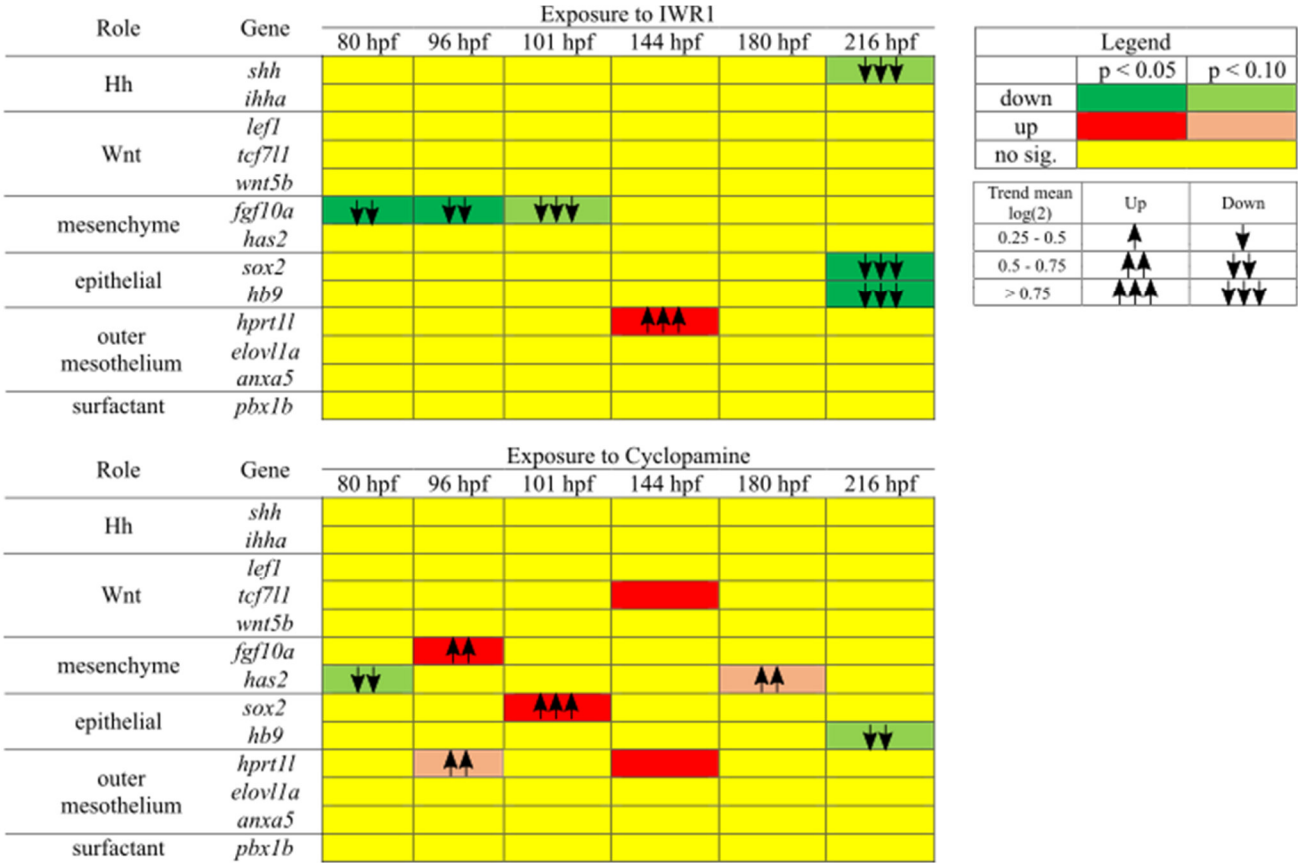
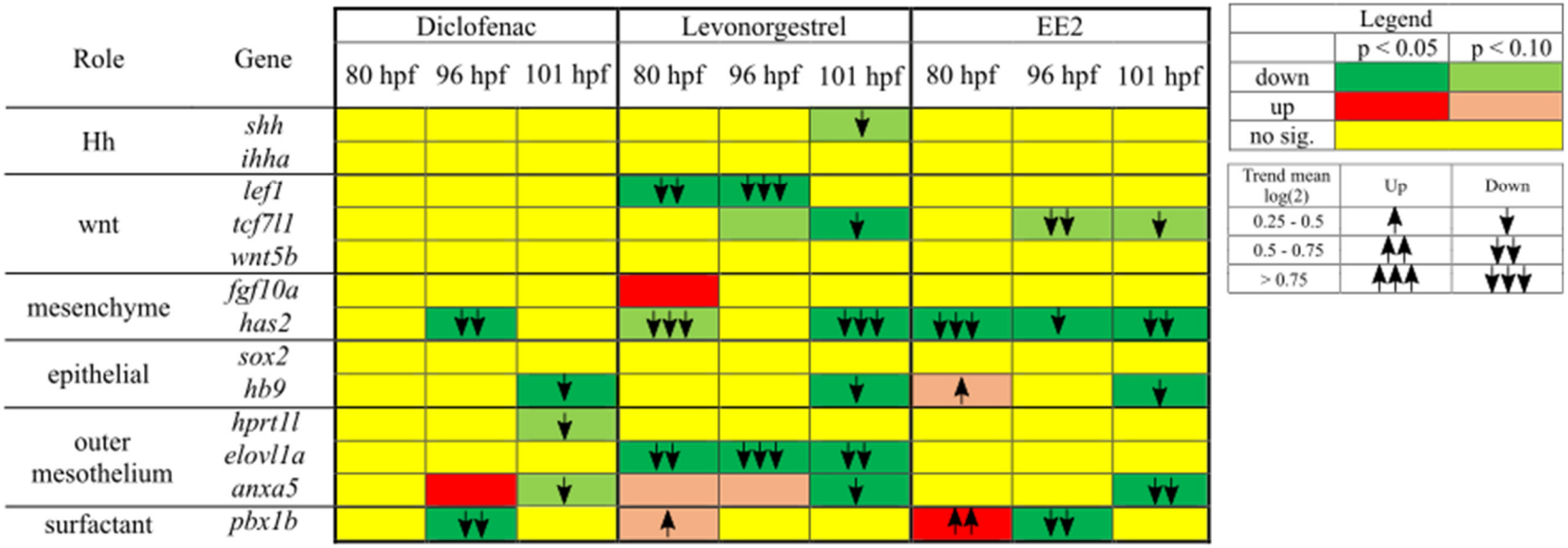


Table 6.9: Summary table of significant effects on gene expression following exposure to 217 µg/L levonorgestrel, 256 µg/L 17α-ethinylestradiol (EE2), or 3.7 mg/L diclofenac. Confidence intervals, paired t-tests, $p \leq 0.05$ and $p \leq 0.10$, and ranges of fold induction (log(2)) are displayed, if no arrow is displayed this indicates induction is < 0.25, which while statistically significant, may not be biologically relevant.



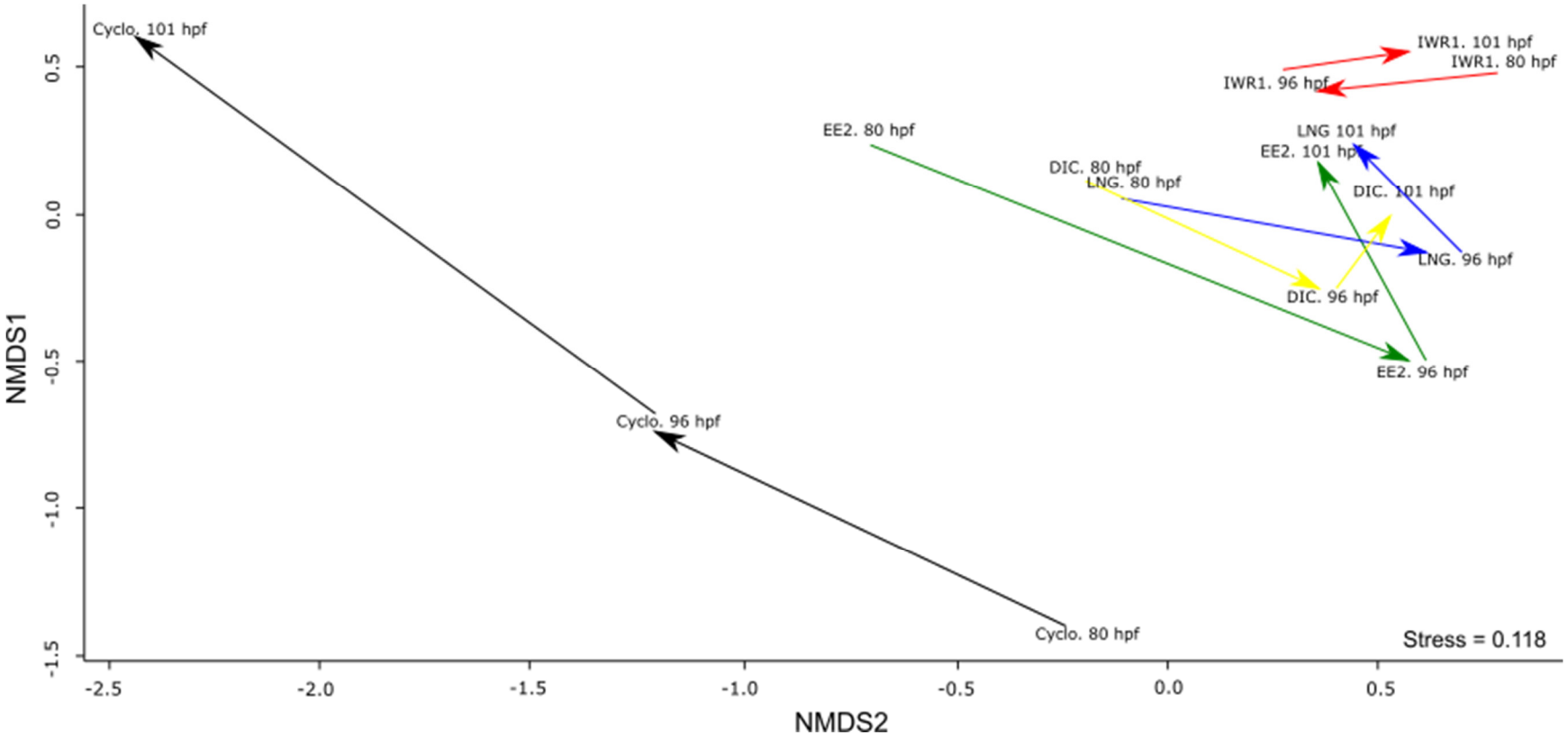


Figure 6.5: Non-metric multidimensional scaling (NMDS) plot of cyclopamine (Cyclo), IWR-1, 17α-ehtinylestradiol (EE2), 17β-estradiol (E2), and levonorgestrel (LNG) gene expression sampled at 80, 96, and 101 hours post fertilization (hpf). Each label represents the average z-scores of all 13 genes expressed and ordinated such that labels similar to one another are closer together. The axes are arbitrary as is the ordination of the plot.

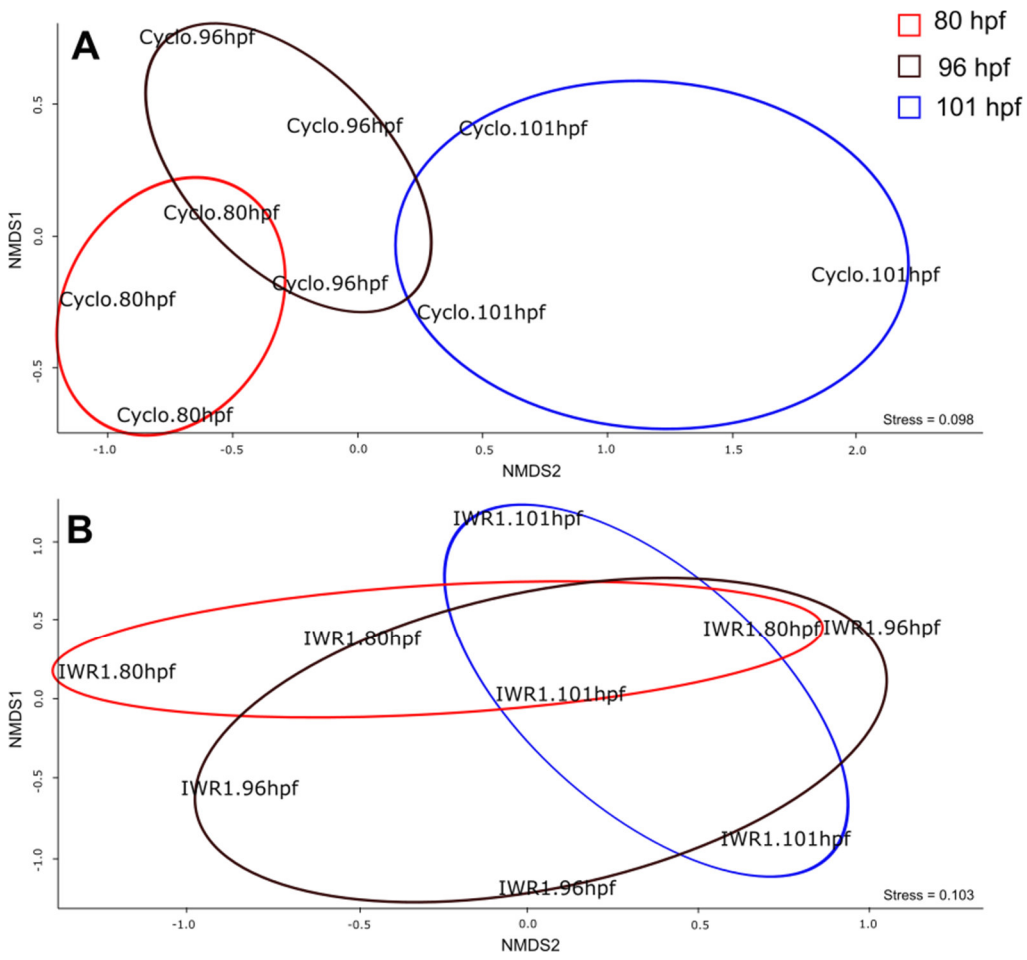


Figure 6.6a: NMDS plots of the three replicates sampled at 80, 96 and 101 hours post fertilization (hpf), A cyclopamine, B IWR-1.

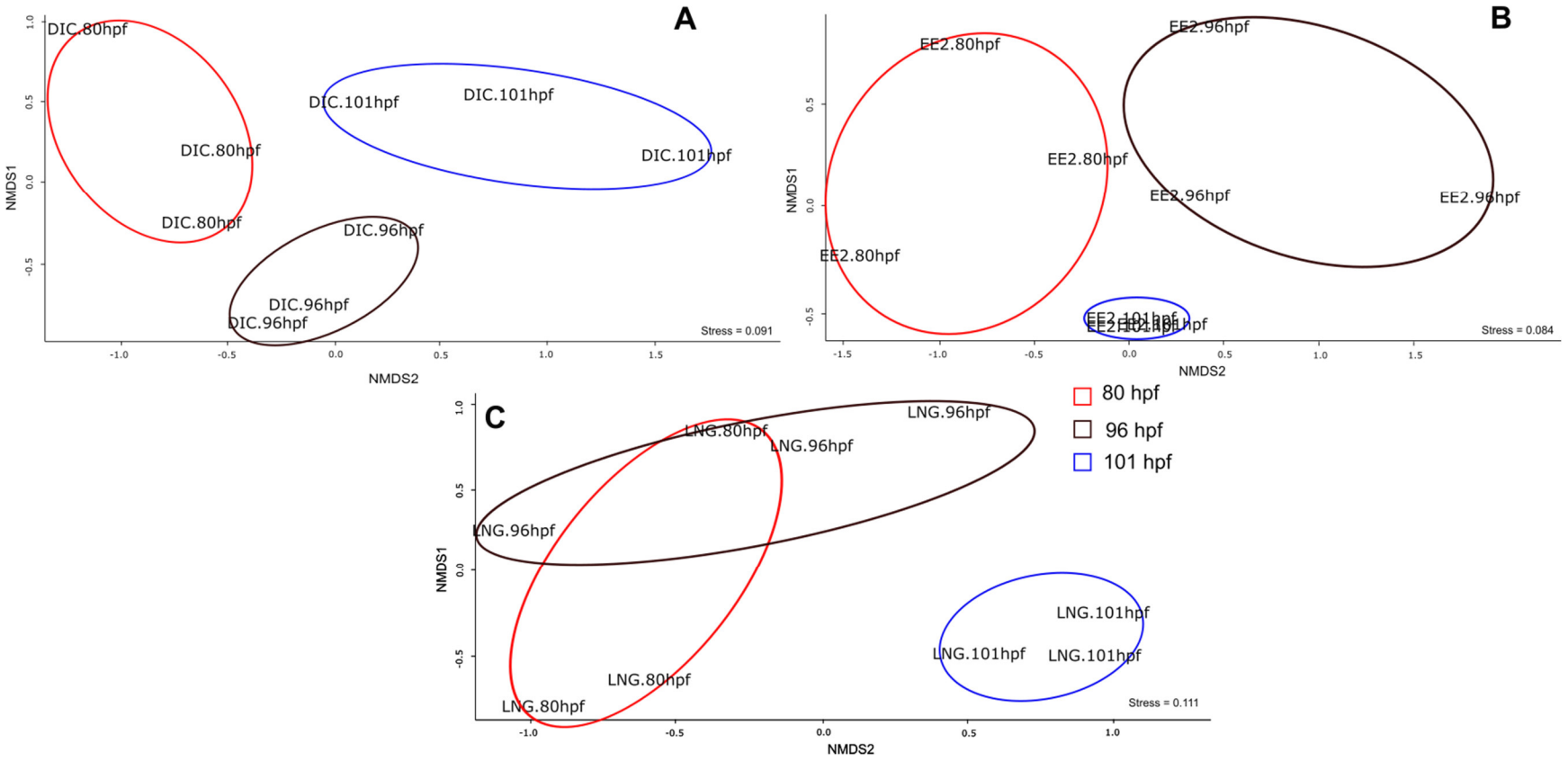


Figure 6.6b: NMDS plots of the three replicates sampled at 80, 96 and 101 hours post fertilization (hpf), A diclofenac, B 17α-ethinylestradiol, and C levonorgestrel.

6.6 Discussion

Exposure to 5 μM cyclopamine and 1 μM IWR-1 caused significant inhibition of swim bladder inflation. This inhibition was permanent, with > 95% of larval fish being unable to inflate post-hatch. These results are similar to studies in zebrafish that showed embryonic exposure to 20 μM cyclopamine or 10 μM IWR-1 caused a failure of swim bladder development in developing zebrafish (Winata *et al.,* 2009; Yin *et al.,* 2011). These findings suggest that medaka embryos may be more sensitive to embryonic exposure of cyclopamine and IWR-1, as the effective concentrations in this study are lower for both compounds. A significant delay in inflation was observed at 0.5 μM IWR-1, but the larvae were able to inflate their swim bladders by 48 hours post-hatch (hpf), while cyclopamine exposure did not delay swim bladder inflation at any concentration tested. This effect of delayed inflation is similar to the delayed inflations following exposure to lower concentrations of pharmaceutical compounds detailed in Chapter 4.

Embryonic exposure to IWR-1 caused a general trend of down regulation of genes at 80-101 hpf, but this was only significant for *fgf10a* which is involved in the formation of the mesenchyme. As Wnt ligands and receptors are found in swim bladder mesenchyme and outer mesothelium, an inhibitor of this signaling pathway would be expected to disrupt the expression of genes involved in their formation *(*Yin *et al.,* 2011). There appears to be a brief period of recovery to normal baseline levels of expression following exposure to IWR-1 (from 36-101 hpf) by 180 hpf, however, there was significant down regulation of genes measured at 216 hpf. In normal medaka the swim bladder has fully developed by 101 hpf (Iwamatsu, 2004), so effects past this time may not be related to swim bladder inflation. Cyclopamine caused a significant up regulation of gene expression in many of

the tissue layers and both signaling pathways. The upregulation of genes related to Wnt expression is expected following exposure to a Hh inhibitor as normally the Hh pathway negatively regulates Wnt signaling through wif1 (Yin *et al.,* 2012). The upregulation seen in genes related to the three swim bladder layers is more difficult to explain. However, in some cases the significant upregulation observed may not be biologically relevant, such as *tcf7l1*and *hprt1l* at 144 hpf, as their fold change is very small < 0.25.

An unexpected result of cyclopamine exposure was an up regulation of gene expression. Previous studies showing significant down regulation of gene expression (generally < 1 fold) following cyclopamine in zebrafish was caused by exposure to a much higher concentrations 100 µM (Xu *et al.,* 2006). It is likely that down regulation of genes in medaka following cyclopamine exposure could occur at higher concentrations. Neither cyclopamine nor IWR-1 influenced the expression of *pbx1b* at any of the time points sampled which suggests that surfactant production is not related to Wnt or Hh signaling. Overall exposure of Japanese medaka embryos to 5µM cyclopamine and 1 µM IWR-1 resulted in small changes (generally less than 1 fold) in gene expression. This is expected as the organism is dependent on these signaling pathways to fully develop, and large changes in expression would likely result in the organism failing to develop. However, these findings may suggest that minor changes in gene expression during embryo development can result in significant organ level effects such as failure of swim bladder inflation.

Of the pharmaceuticals tested, levonorgestrel significantly affected the expression of the largest number of genes tested. It is possible that exposure to these three pharmaceutical compounds results in a disruption of cell signaling in the embryo that leads

to a disruption of swim bladder formation. Embryonic exposure to pharmaceutical compounds such as diclofenac have been linked to changes in gene expression in previous studies (Chae *et al.,* 2015). Levonorgestrel, EE2, and diclofenac significantly affected *pbx1b* expression, which may indicate these compounds affect swim bladder inflation through a disruption of surfactant production (Teoh *et al.,* 2010). When this gene was inhibited in zebrafish embryos, the swim bladder still developed normally but was unable to be inflated (Teoh *et al.,* 2010). A delay in the secretion of surfactants could explain why some fish experienced a delay in swim bladder inflation.

None of the pharmaceutical compounds affected the expression of *shh* or *ihha*, which suggests these compounds may not disrupt the hedgehog pathway, but levonorgestrel and EE2 significantly downregulated the genes for the Wnt/beta catenin transcription factors LEF/TCF. This suggests their effects on swim bladder inflation may involve the Wnt pathway. This hypothesis is supported by the NMDS that showed that the effects of the pharmaceutical compounds on gene expression were closer to that of the wnt inhibitor IWR-1 than the hedgehog inhibitor cyclopamine. Similar to exposure to IWR-1 and cyclopamine, the changes in gene expression following exposure to levonorgestrel, EE2, and diclofenac were small, generally less than 1 fold.

The three pharmaceutical compounds significantly affected gene expression of markers for all three tissue layers, indicating they may cause swim bladder inflation failure by disrupting the formation and organization of the cells that make up the swim bladder. It is not known how these compounds are able to alter cell signaling, but endogenous steroids may substitute for cholesterol, which is involved in the maturation of signaling pathway proteins such as those for the Hh family (Heretsch *et al.,* 2010). Of the genes

has2, *hb9*, and *anxa5* were all significantly disrupted by embryonic exposure, with the down regulation being most significant at 101 hpf.

Histological examination of the swim bladder in future experiments could determine if these pharmaceutical compounds are affecting the organization and formation of the swim bladder or simply the ability to inflate. Further experimentation could determine if other compounds known to cause a disruption of swim bladder inflation in larval fish are acting on similar genes such as *pbx1b*, *hb9*, *has2*, or *anxa5*. Recent studies have linked impaired anterior swim bladder inflation of zebrafish with exposure to a thyroid peroxidase inhibitor (Stinckens *et al.,* 2016). As thyroid hormones are regulated through the hypothalamus, and the hypothalamic-pituitary-thyroidal axis plays an important role in fish swim bladder inflation, it would be interesting to see if any of these natural/synthetic hormones disrupt swim bladder inflation through a disruption of normal thyroid regulation. A potential adverse outcome pathway summarizing how embryonic exposure to pharmaceutical compounds may lead to swim bladder inflation failure is outlined in Figure 6.7. These results support the hypothesized adverse outcome pathway; while the changes in gene expression are minor, the results still suggest some potential ways through which these pharmaceutical compounds are causing their effects.

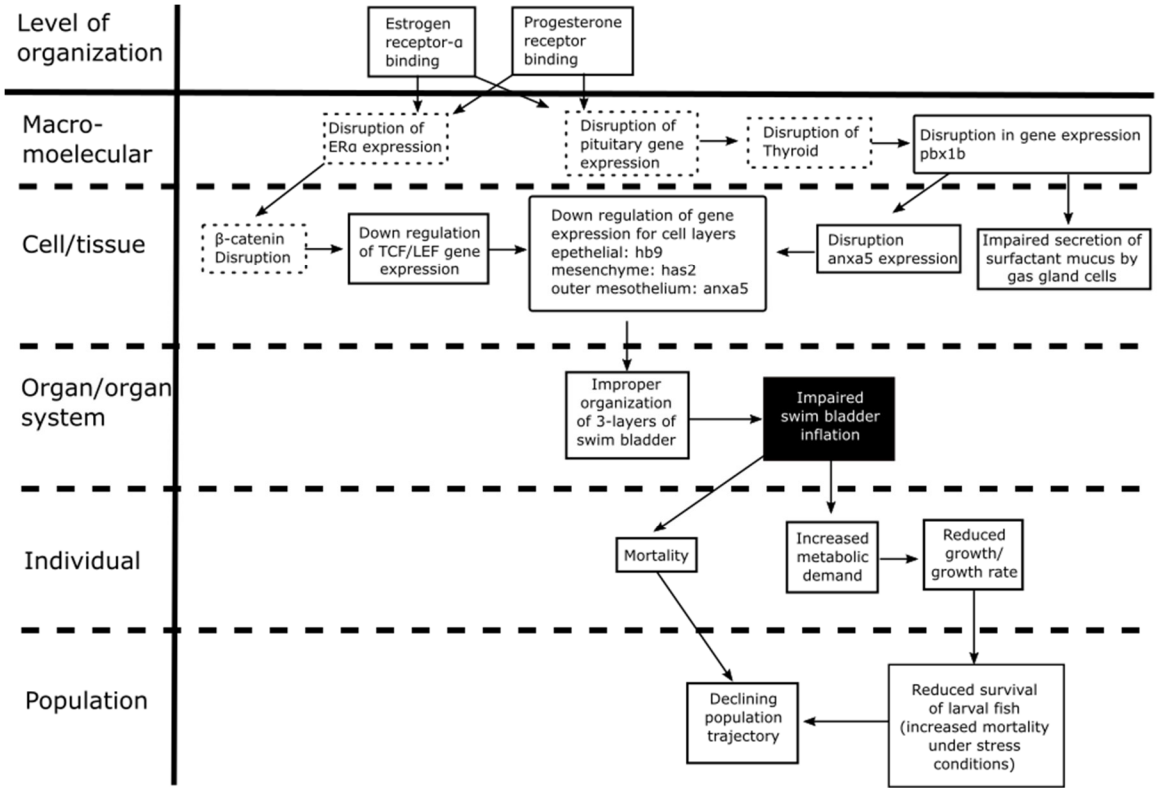


Figure 6.7: Possible adverse outcome pathway (AOP) for swim bladder inflation failure following exposure to pharmaceutical compounds

Chapter 7: General conclusions

7.1 General conclusions

The most sensitive non-lethal effect observed following embryonic exposure to diclofenac, levonorgestrel, 17β-estradiol, and 17α-ethinylestradiol (EE2) was a failure of larvae to inflate their swim bladders following hatch. Swim bladder inflation failure following embryonic exposure to compounds is often reported in the literature, but experiments are not usually designed with this endpoint in mind. Thus, understanding the mechanisms behind which environmental pollutants can affect swim bladder inflation is an important knowledge gap. Most of the recent research examining swim bladder inflation failure has been typically conducted using physostomous fish with a two-chambered swim bladder such as zebrafish. As the majority of fish species are physoclistous and have a single chambered swim bladder, Japanese medaka may be a more suitable model species to study the effect of chemical compounds on swim bladders.

This dissertation investigated many factors associated with swim bladder formation and inflation. The temporal requirement for an air-water interface was determined to be ~5 days for Japanese medaka, which has not been previously described in the literature. This is particularily relevant to Japanese medaka aquaculture, as the results suggest that reared larvae should be exposed to an undisrupted airwater interface. In addition, this study showed that swim bladder inflation failure of medaka larvae is regressed strongly with decreased survival. This has implications for the pharmaceutical results, as it implies that exposure to pharmaceuticals that significantly decrease swim bladder inflation may lead to decreased survival of the larvae.

Furthermore, it was established that these compounds have the potential to act in combination at individual no-observed-effect concentrations. This demonstrated that

embryo-toxicity experiments may be well suited to evaluate mixture effects, as they allow high-throughput which would not be possible when using adult fish.

Embryonic exposure to both Hh and Wnt signaling pathway inhibitors such as cyclopamine and IWR-1 were able to prevent swim bladder inflation of hatched medaka larvae. The effects of cyclopamine and IWR-1 were compared to the effects of diclofenac, EE2, and levonorgestrel on whole embryo gene expression. Based on observed effects, the pharmaceutical compounds were more closely related to each other in terms of their effects on gene expression, and more closely related to the Wnt inhibitor IWR-1 than the Hh inhibitor cyclopamine. The pharmaceutical compounds did cause significant disruption (generally < 1 fold) of whole embryo gene expression of markers related to the three cell layers, surfactant production, and wnt markers. These results are novel, as there have been no previous studies linking pollutant exposure to effects on gene expression related to swim bladder inflation in Japanese medaka. Future studies using other compounds known to cause swim bladder inflation failure of larval medaka could assess the impact on gene expression to determine if any of the genes tested are a predictor for swim bladder inflation failure. However, it should be noted that gene expression is just a snap shot of the expression at the hours sampled. It is possible these compounds altered the expression of genes during critical time-periods that were not measured. Future experiments wishing to futher explore these results could use knock out mutants to show the relation of these genes to proper swim bladder morphogenesis in Japanese medaka. Experiments could also measure the proteins these genes code for to in order to establish a stronger relationship between effects on gene expression and effects on swim bladder development.

Despite the effects by pharmaceuticals observed, the concentrations required to cause significant effects are higher than those generally found in the environment. In order to make embryo exposure more relevant to environmental conditions, exposures could be conducted from fertilization, before the chorion hardens, until hatch, or medaka embryos could be microinjected in order to simulate maternal transfer of contaminants. Additionally, some recent studies have linked swim bladder inflation failure to effects on the thyroid. As the compounds studied in this thesis are all able to act on the endocrine system, it would be interesting to determine if their effects are able to be linked to modulation of the hypothalamic-pituitary-gonadal-axis.

Swim bladder inflation failure is a sensitive endpoint that has the potential to lead to the ecological elimination of species. Swim bladder inflation failure directly causes increased mortality of larvae. Even in the case of larvae that are able to initially survive, they would presumably experience reduced growth, decreased ability to obtain food, and increased chances of predation. All of this could eventually lead to a population collapse, indicating that any chemical that inhibits swim bladder inflation would affect fish populations in multiple ways. The use of swim bladder inflation failure as an adverse outcome pathway is thus of high relevance to the field of aquatic toxicology. Swim bladder inflation failure is a sensitive endpoint that should be measured by studies determining the impact of embryonic exposure to chemical compounds on hatched larval fish. While the exposure concentrations of pharmaceutical compounds to elicit effects were much higher than those generally found in the environment, this study demonstrates these compounds have the potential to cause adverse effects in Japanese medaka, and helps increase the understanding of swim bladder inflation failure as an adverse outcome.

Overall, this thesis has contributed to the understanding of swim bladder inflation failure following exposure to common pharmaceutical compounds. The findings on gene expression are a significant contribution to adverse outcome pathway establishment and provides insight for future studies aimed at determining potential molecular initiating events for swim bladder inflation failure. This study also demonstrated that swim bladder inflation failure is a critical endpoint that leads to increased larval mortality.

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Chapter 9: Appendices

Appendix A: Range finder results hatchability (%)

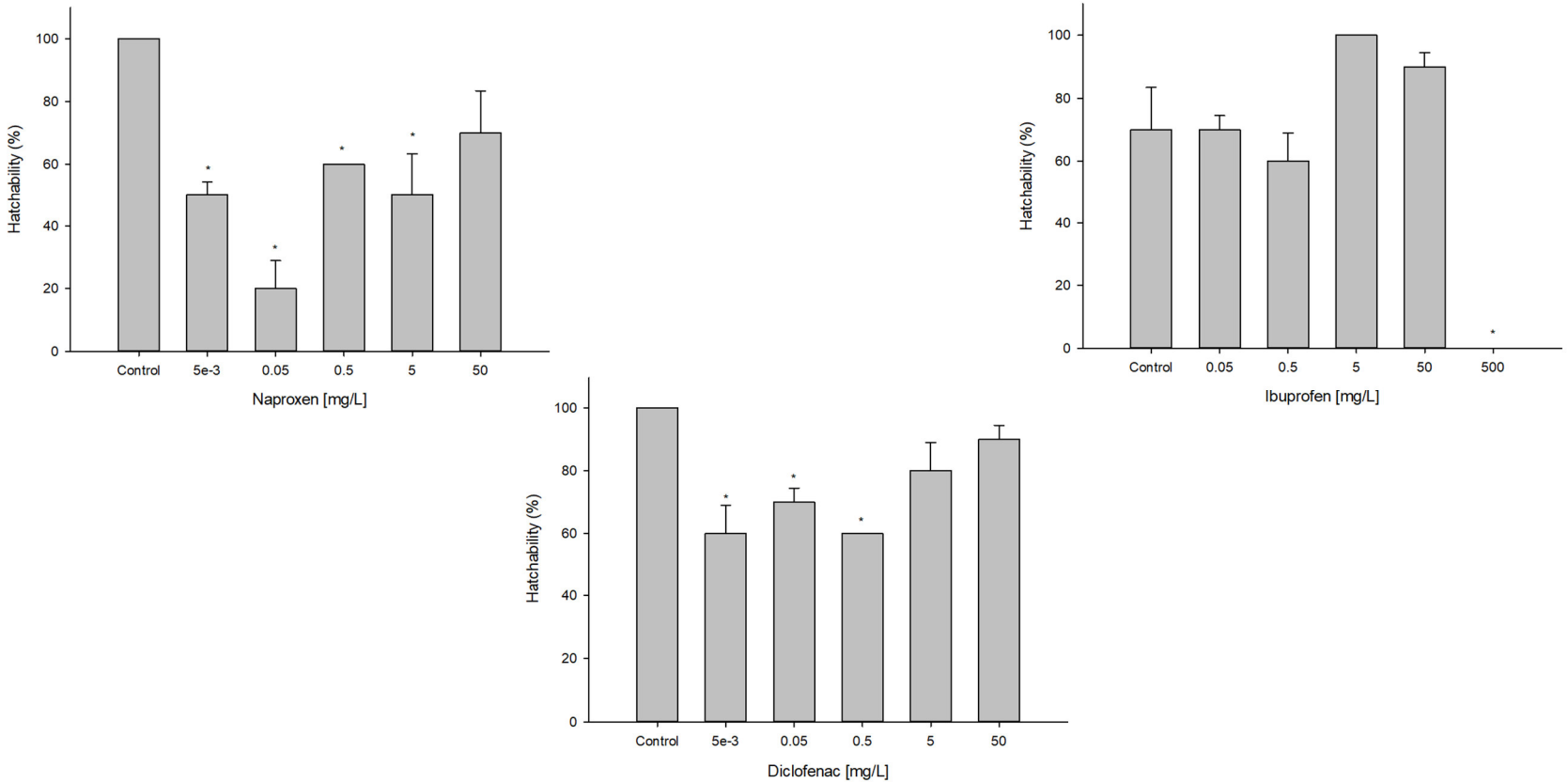

Figure A1: The hatchability (%) of medaka embryos exposed to nominal concentrations of ibuprofen, naproxen, and diclofenac until hatch. Bars represent mean ± SE, n = 10. *Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$).

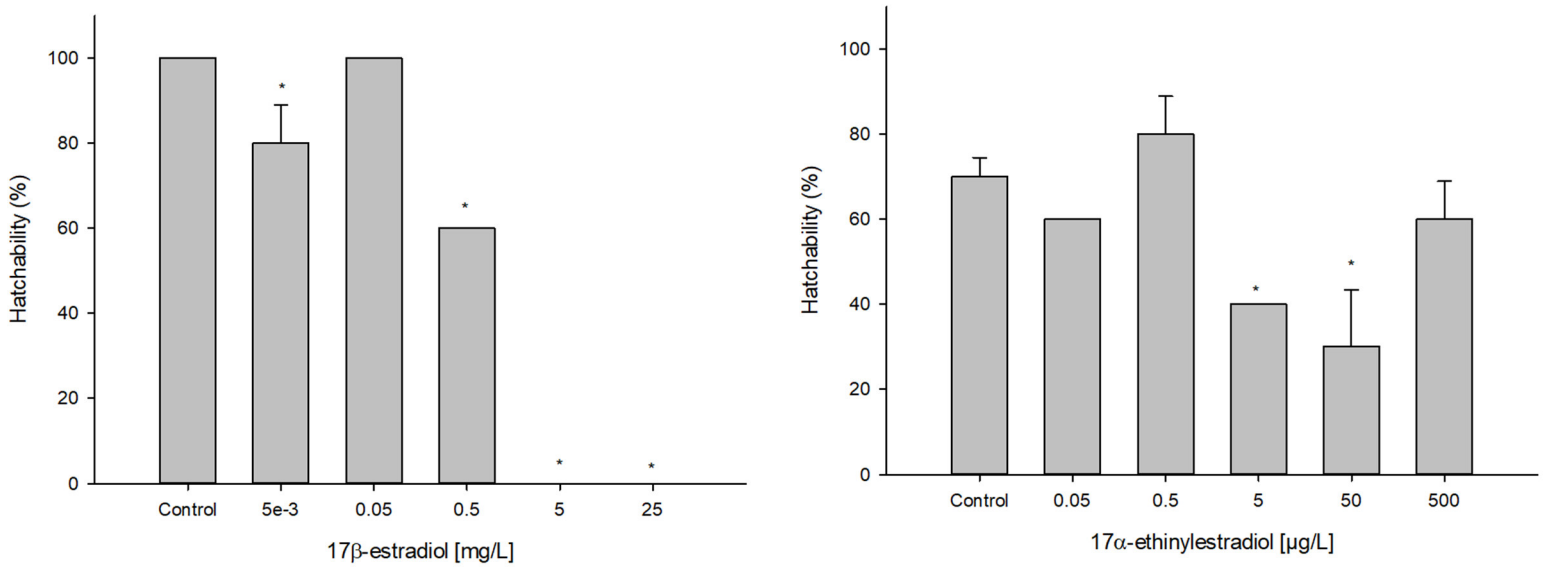

Figure A2: The hatchability (%) of medaka embryos exposed to nominal concentrations of estradiol and 17α-ethinylestradiol until hatch. Bars represent mean ± SE, n = 10. *Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$).

Appendix B: Range finder results developmental abnormalities

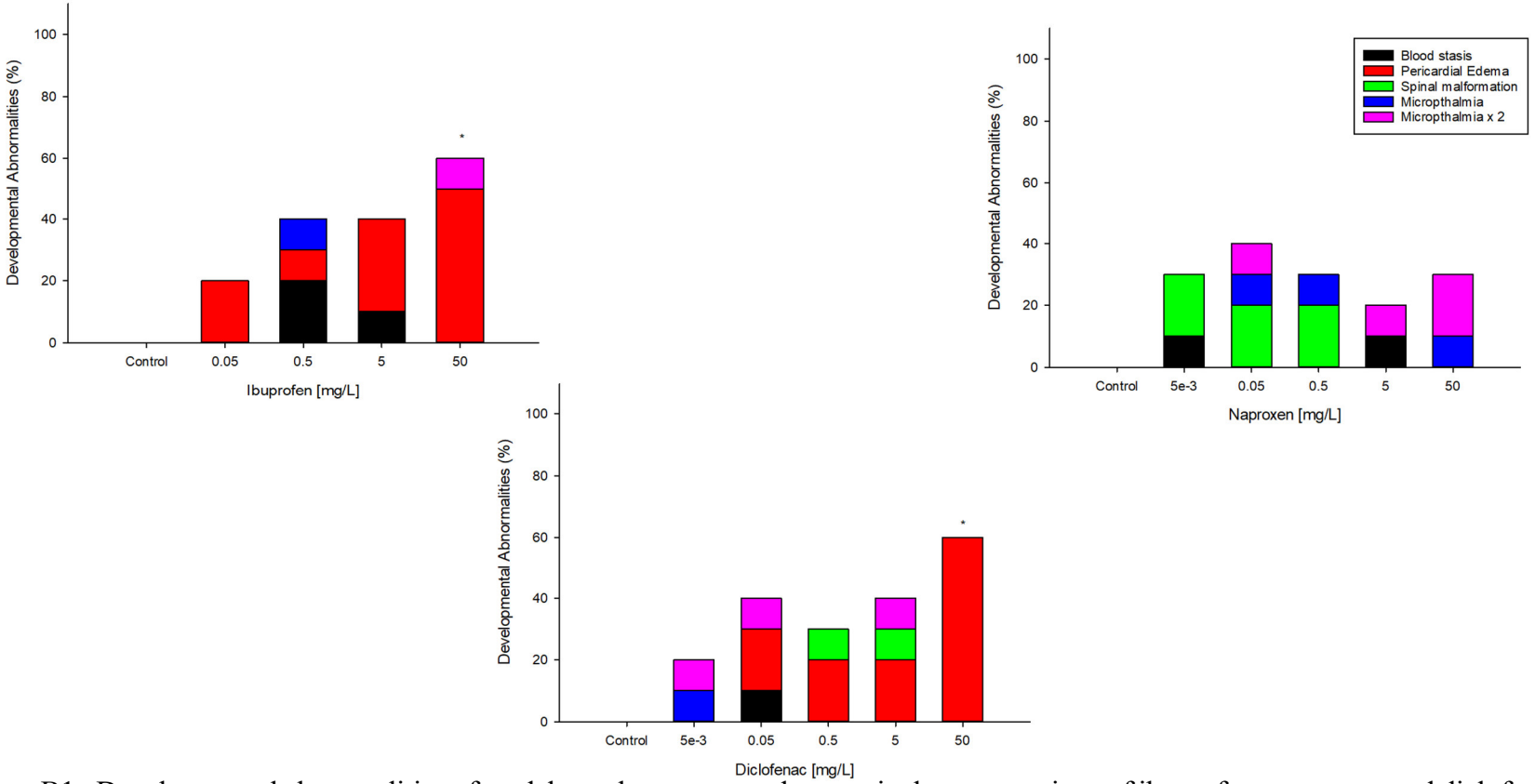

Figure B1: Developmental abnormalities of medaka embryos exposed to nominal concentrations of ibuprofen, naproxen, and diclofenac until hatch. Bars represent the relative amount of each type of malformation observed by treatment. *Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$).

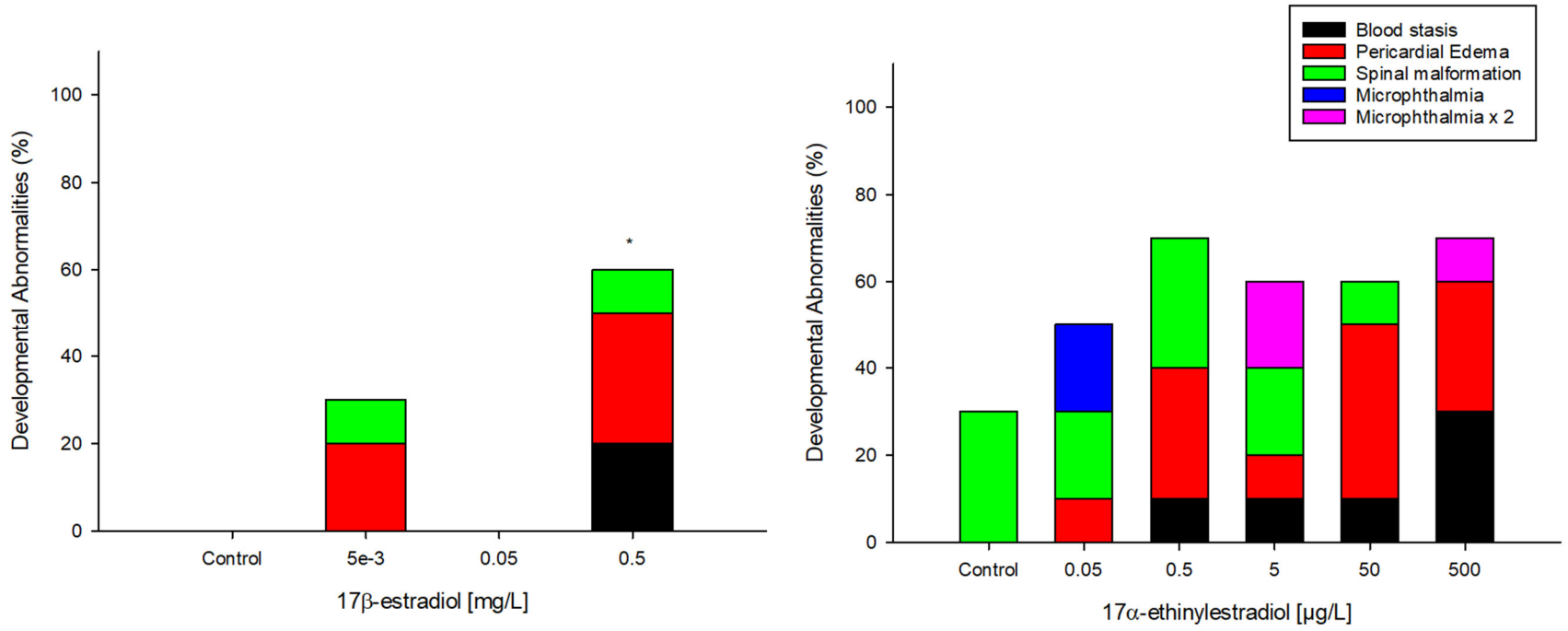

Figure B2: Developmental abnormalities of medaka embryos exposed to nominal concentrations of estradiol and 17α-ethinylestradiol until hatch. Bars represent the relative amount of each type of malformation observed by treatment. *Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$).

Appendix C: Range finder time-to-hatch

Table C1: Median time-to-hatch (days (max, min)) for EE2, E2, IBU, NAP, and DIC. *Indicates a significant difference from control (ANOVA on ranks, $p \leq 0.05$, Dunn's post-hoc $p \leq 0.05$).

Concentration [µg/L]	EE2	E2	Concentration [mg/L]	DIC	IBU	NAP
control	29.5 (30,28)	8.5 (10,7)	C	14 (25,10)	11 (11,11)	11 (27,10)
0.05	26 (29,11)*		0.005	10 (25,5)		18 (33,8)
0.5	26 (29,25)		0.05	8.5 (12,7)*	10 (16,10)	26 (33,7)
5	22.5 (30,9)*	14 (16,9)*	0.5	9 (14,5)*	14 (16,11)	29 (32,9)
50	23 (26,13)*	10 (10,7)	5	9 (18,8)	10.5 (14,10)	18.5 (27,10)
500	11 (12,8)*	9.5 (10,7)	50	10 (11,9)	10 (10,9)*	13 (25,8)

Appendix D: Swim bladder follow up 3 days post-hatch

Table D1: Normal rearing solution and rearing solution with 0.01% acetone (carrier control), showing percentage of hatched larvae able to inflate their swim bladder at 0, 24, and 48 hours post-hatch (h). Values are percent (%) of fish able to inflate their swim bladder (n = 15/rep, 3 reps).

	n	Swim Bladder Inflation (%) (mean ± SE)		
		0 h	24 h	48 h
Control	45	48.9 ± 8.9	93.6 ± 2.2	97.8 ± 2.2
Carrier control	45	71.1 ± 5.9	97.8 ± 2.2	97.8 ± 2.2

Table D2: Effects of exposure to nominal concentrations of levonorgestrel on inhibition of swim bladder inflation at 0, 24 and 48 hours post-hatch. Values are percent (%) of fish able to inflate their swim bladder (n=10/rep, 4 reps). Indicates significant difference from control ANOVA, $p \leq 0.05$, Dunn's post hoc $p \leq 0.05$.

[Levonorgestrel] (µg/L)	Swim Bladder Inflation (%, mean ± SE)			
	0 h	24 h	48 h	72 h
Carrier control	80.0 ± 6.7	95.0 ± 3.3	100 ± 0	100 ± 0
0.05 µg/L	55.6 ± 8.0	90.0 ± 5.8	96.7 ± 3.3	96.7 ± 3.3
0.5 µg/L	52.4 ± 9.9	90.0 ± 5.8	90.0 ± 5.8	90.0 ± 5.8
5 µg/L	55.3 ± 18.6	80.0 ± 11.5	80.0 ± 11.5	80.0 ± 11.5
50 µg/L	42.2 ± 16.8	62.2 ± 3.3*	76.7 ± 3.3	80.0 ± 5.8
500 µg/L	8.1 ± 4.2*	20.0 ± 5.8*	30.0 ± 5.8*	43.3 ± 3.3*

Appendix E: Water sampling methodology

Water sampling was performed by Naomi Stock at the Water Quality Center at Trent University. Below are the methodologies based on information she provided.

Summary of Methodology for the Analysis of E2 and EE2 in Water Samples

Samples containing E2 or >10 µg/L of EE2 were diluted with methanol and spiked with 10 µL of 1 ppm isotopically labelled standard (E2-D4 or EE2-D4). Samples containing <10 µg/L of EE2 were extracted using a modified version of EPA 1694 solid phase extraction (SPE) method that employed Oasis HLB cartridges. Sample were analyzed on an AB Sciex Qtrap 5500 mass spectrometer coupled with a Shimadzu 10A liquid chromatograph instrument. Analytes were separated using a Thermo Acclaim RSLC120 C18 column (2.2 µm, 4.6 x 150 mm) with 20 mM ammonium acetate in milli-Q water and methanol as mobile phases. Injection volume was 20 µL. Samples were analyzed in duplicate, using negative ion and multiple reaction monitoring modes. The transitions monitored were 271 > 145 and 295 > 145 for E2 and EE2 respectively. Samples were quantified with internal calibration of the isotopically labelled standard. The method limits of quantitation for both E2 and EE2 were 0.5 µg/L (dilution only). For samples extracted using SPE, the method limit of quantitation for EE2 was 2 ng/L.

Summary of Methodology for the Analysis of Levonorgestrel in Water Samples

Samples were diluted 1:1 with methanol and spiked with 10 µL of 1 ppm isotopically labelled standard (levonrgestrel-D6). Sample were analyzed on an AB Sciex Qtrap 5500 mass spectrometer coupled with a Shimadzu 10A liquid chromatograph instrument.

Analytes were separated using a Thermo Acclaim RSLC120 C18 column (2.2 µm, 4.6 x 150 mm) with 0.1% formic acid in acetonitrile and 0.1% formic acid in milli-Q water as mobile phases. Injection volume was 20 µL. Samples were analyzed in duplicate, using positive ion and multiple reaction monitoring modes. The transitions monitored for levonorgestrel were m/z 313 > 245 and 313 > 109. Samples were quantified with internal calibration of the isotopically labelled standard. The method limits of quantitation were 0.1 µg/L.

Summary of Methodology for the Analysis of Diclofenac in Water Samples

Samples were diluted with methanol and spiked with 10 µL of 1 ppm isotopically labelled standard (ibuprofen-D3). Sample were analyzed on an AB Sciex Qtrap 5500 mass spectrometer coupled with a Shimadzu 10A liquid chromatograph instrument. Analytes were separated using a Thermo Acclaim RSLC120 C18 column (2.2 µm, 4.6 x 150 mm) with 20 mM ammonium acetate in milli-Q water and methanol as mobile phases. Injection volume was 20 µL. Samples were analyzed in duplicate, using negative ion and multiple reaction monitoring modes. The transitions monitored for diclofenac were *m*/z 294 > 250 and 294> 35. Samples were quantified with internal calibration of the isotopically labelled standard. The method limits of quantitation were 0.0001 mg/L.

(Naomi Stock, 2017 unpublished data).

Appendix F: Air-water interface n=20 initial experiment.

Table F1: Percentage of medaka larvae with an inflated swim bladder following denial of air-water interface for no time (controls) or 3-9 days post-hatch (treatments), mean of 3 replicates of 20 embryos/larvae each ± standard deviation (SD). Significant differences are shown with p < *0.1, **0.05, ***< 0.01, ****< 0.001.

	Days Post-Hatch (mean ± SD)											
	0 dph	1 dph	2 dph	3 dph	4 dph	5 dph	6 dph	7 dph	8 dph	9 dph	10 dph	11 dph
Control	92.6 ± 5.6	96.6 ± 2.2	97.9 ± 1.6	99.0 ± 0.9	99.7 ± 0.7	99.7 ± 0.7	99.7 ± 0.7	99.7 ± 0.7	99.7 ± 0.7	99.7 ± 0.7	100 ± 0.0	100 ± 0.0
4 Days					0.0 ± 0.0 ****	44.7 ± 11.5 ****	59.6 ± 12.2 ****	62.7 ± 9.8	66.3 ± 11.9 ****	70.8 ± 19.6 **	73.7 ± 17.2 **	75.3 ± 16.5 **
5 Days						0.0 ± 0.0 ****	26.5 ± 8.7 ****	33.9 ± 19.9 ****	41.7 ± 8.3 ****	50.3 ± 14.9 ****	65.2 ± 10.7 ***	71.2 ± 6.6 **
6 Days							0.0 ± 0.0 ****	17.3 ± 11.3 ****	25.2 ± 9.5 ****	25.2 ± 9.5 ****	23.3 ± 9.6 ****	22.4 ± 8.2 ****
7 Days								0.0 ± 0.0 ****	19 ± 10.2 ****	25.5 ± 11.2 ****	22.8 ± 14.4 ****	26.2 ± 14.6 ****
8 Days									0.0 ± 0.0 ****	4.8 ± 8.2 ****	5.3 ± 4.6 ****	7.9 ± 7.7 ****

Table F2: Percentage mortality of medaka larvae following denial of air-water interface for no time (controls) or 3-9 days post-hatch (treatments), mean of 3 replicates of 20 embryos/larvae each ± standard deviation (SD). Significant differences are shown with p < *0.1, **0.05, ***< 0.01, ****< 0.001.

	Days Post-Hatch (mean ± SD)											
Treatment	0 dph	1 dph	2 dph	3 dph	4 dph	5 dph	6 dph	7 dph	8 dph	9 dph	10 dph	11 dph
Control	1.7 ± 1.9	2.9 ± 2.5	4.6 ± 3.4	5.0 ± 4.1	6.3 ± 3.7	6.7 ± 3.0	7.5 ± 3.2	7.5 ± 3.2	7.5 ± 3.2	8.8 ± 4.6	10.8 ± 2.2	12.5 ± 5.2
4 Days	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 2.9	3.3 ± 2.9	6.7 ± 2.9	5.0 ± 0.0	6.7 ± 2.9	13.3 ± 10.4	13.3 ± 10.4	20.0 ± 8.7	21.7 ± 10.4
5 Days	1.7 ± 2.9	3.3 ± 2.9	3.3 ± 2.9	3.3 ± 2.9	3.3 ± 2.9	11.7 ± 2.9	11.7 ± 2.9	11.7 ± 2.9	23.3 ± 15.3	46.7 ± 18.9 ***	55.0 ± 18.0 ***	61.7 ± 17.6 ****
6 Days	1.7 ± 2.9	1.7 ± 2.9	1.7 ± 2.9	1.7 ± 2.9	8.3 ± 2.9	15.0 ± 0.0	21.7 ± 7.6 *	26.7 ± 10.4 **	33.3 ± 15.3 *	30.0 ± 17.3	33.3 ± 18.9 *	36.6 ± 17.6 *
7 Days	0.0 ± 0.0	13.3 ± 5.8 **	30.0 ± 18.0 ***	30.0 ± 18.0 ***	30.0 ± 18.0 ***	33.3 ± 17.6 ***	40.0 ± 10.0 ****	40.0 ± 10.0 ****	40.0 ± 10.0 **	43.3 ± 7.6 ***	45.0 ± 8.7 ***	45.0 ± 8.7 **
8 Days	3.3 ± 5.8	5.0 ± 8.7	8.3 ± 7.6	8.3 ± 7.6	8.3 ± 7.6	15.0 ± 13.2	21.7 ± 10.4 *	30.0 ± 15.0 **	30.0 ± 15.0	38.3 ± 7.6 **	40.0 ± 5.0 **	40.0 ± 5.0 **

Appendix G: Lithium experiments

Lithium experimental methodology

LiCl stocks were prepared from an 8.0 M stock solution (1.31 M Li). A carrier was not needed and all dilutions were conducted using D.I. water. All dilutions were prepared in 2 mL glass sampler vial creating a 10 mM stock. For example 7.6 µL of LiCl was added to 992.4 µL of D.I. H_2O to prepare a 10 mM Li stock. This stock was used to make up the following stocks in sampler vials 10, 5, 1, 0.5 and 0.1 mM in through dilution. To prepare a working solutions 50 µL of each respective concentration was diluted in 50 mL of rearing solution using a glass 50 mL volumetric flask. Final concentrations were 10, 5, 1, 0.5 and 0.1 µM. Four replicates of 10 medaka embryos were exposed (40 per replicate, 240 total embryos), along with a rearing solution control (as no solvent was used for lithium). No effects were found following these initial concentrations and a second experiment was performed using LiCl with the following concentrations: 1000, 320, 100, 32 and 10 mM. These were made up in the same manner as the first experiment. This follow up experiment also found no significant effects. A third experiment was conducted using lab water instead of rearing solution to house the eggs, using similar methodology and final concentrations of 1000, 320, 100, 32 and 10 µM lithium. This was conducted as there is a high concentration of Na in rearing solution, which has been shown to act antagonistically with lithium (Kszos *et al.,* 2003). Results of exposure to 10-1000 µM lithium on hatchability, time-to-hatch, and swim bladder inflation success are displayed in Tables H1-H4. After finding no effect in lab water at concentrations as high as 1000 µM lithium the study of this compound was discontinued.

Table G1: Hatchability (mean ± SE) of Japanese medaka embryos following nominal exposures to lithium in lab water and rearing solution, (4 replicates of 10 embryos per concentration). **Indicates significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

Treatment (µM)	Hatchability (%)	
(nominal)	In Rearing Solution	In Lab Water
C	100 ± 0.0	97.5 ± 2.5
10 µM	95.0 ± 1.6	95 ± 2.9
32 µM	97.5 ± 0.8	97.5 ± 2.5
100 µM	100 ± 0.0	95 ± 2.9
320 µM	100 ± 0.0	97.5 ± 2.5
1000 µM	100 ± 0.0	97.5 ± 2.5

Table G2: Median time-to-hatch (days (min, max) following nominal exposures to lithium in lab water and rearing solution, (4 replicates of 10 embryos per concentration). **Indicates a significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

Treatment (μM)	Median (days (min,max))	
(nominal)	In Rearing Solution	In Lab Water
C	9 (9,10)	8 (8,11)
10 μM	9 (9,11)	8 (8,12)
32 μM	9 (9,10)	9 (8,10)
100 μM	9 (9,10)	9 (8,10)
320 μM	9 (9,9)	9 (8,10)
1000 μM	9 (9,10)	9 (8,11)

Table G3: Effects of exposure to nominal concentrations of lithium on swim bladder inflation at 0, 24, 48, and 72 hours post-hatch. Values are percent (%) of fish able to inflate their swim bladder (4 replicates of 10 embryos per concentration). **Indicates a significant difference from control (One-Way ANOVA, $p \leq 0.05$ confirmed by Dunn's post-hoc test, $p \leq 0.05$, *indicates a significant difference from control $p \leq 0.10$).

Lithium in Rearing Solution	Swim Bladder Inflation (%, mean ± SE)			
	0 h	24 h	48 h	72 h
C	60.0 ± 8.2	90.0 ± 0.0	95.0 ± 2.9	100 ± 0.0
10 µM	38.8 ± 5.2	89.4 ± 4.1	89.4 ± 4.1	91.9 ± 2.8
32 µM	53.1 ± 13.3	94.7 ± 3.1	94.7 ± 4.1	94.7 ± 3.1
100 µM	65.0 ± 9.6	85.0 ± 5.0	90.0 ± 2.5	97.5 ± 2.5
320 µM	42.5 ± 8.5	87.5 ± 2.5	92.5 ± 2.5	95.0 ± 2.9
1000 µM	52.5 ± 11.1	92.5 ± 2.5	97.5 ± 2.5	97.5 ± 2.5

Lithium in lab Water	Swim Bladder Inflation (%, mean ± SE)			
	0 h	24 h	48 h	72 h
C	51.7 ± 6.1	94.7 ± 0.0	97.5 ± 2.5	100 ± 0.0
10 µM	48.1 ± 10.0	79.4 ± 5.5	87.2 ± 4.7	92.2 ± 4.8
32 µM	61.1 ± 6	85 ± 6.5	92.2 ± 4.8	97.5 ± 2.5
100 µM	39.4 ± 4.7	84.2 ± 9.2	89.7 ± 7.1	95 ± 5.0
320 µM	48.3 ± 11.4	76.9 ± 4.7	89.4 ± 7.1	91.9 ± 4.9
1000 µM	58.6 ± 6.7	84.7 ± 8.6	87.2 ± 6.3	87.2 ± 6.3