Mechanisms of reproductive inhibition in zebrafish (*Danio rerio*) exposed to ethinylestradiol, nitrate and ammonia

by

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ABSTRACT

MECHANISMS OF REPRODUCTIVE INHIBITION IN ZEBRAFISH (*Danio rerio*) EXPOSED TO ETHINYLESTRADIOL, NITRATE AND AMMONIA

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The objective of this thesis was to investigate the reproductive effects and mechanisms of action of three common MWWE constituents, 17α-ethinylestradiol (EE_2), nitrate and ammonia, using the zebrafish (*Danio rerio*) as the model. Fecundity assays indicated that ammonia and EE_2, but not nitrate, inhibited spawning. Further mechanistic investigations into reproductive processes such as steroidogenesis, maturation, and ovulation indicate that ammonia and EE_2 affect reproduction through different mechanisms. EE_2 acts by inhibiting steroidogenesis as shown by the reduction of sex steroid levels and expression of aromatase. Ammonia blocked the stimulatory effects of the gonadotropin analog human chorionic gonadotropin (hCG) on ovulation and spawning. Molecular analysis from these experiments indicated a potential role of prostaglandins in this response through the actions of the enzyme COX-2, but no effects on prostaglandin levels or receptor expression were found. Though the mechanism through which ammonia inhibits reproduction remains unknown, these findings help narrow the likely sites of action.

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LIST OF ABBREVIATIONS

Abbreviation	Proper Name
17,20β-P	17α, 20β-dihydroxy-4-pregnen-3-one
AA	Arachidonic acid
ANOVA	Analysis of variance
AO	Adverse outcome
AOP	Adverse outcome pathway
Arom	Aromatase
ARP	Acidic ribosomal protein
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
$cPLA_2$	Cytosolic phospholipase A_2
EDC	Endocrine disrupting chemicals
E_2	17β-Estradiol
EDTA	ethylenediaminetetraacetic acid
EE_2	17α-ethinylestradiol
EF1α	Elongation factor 1α
EIA	Enzyme Immunoassay
FSH	Follicle stimulating hormone
Gln	Glutamine
GnRH	Gonadotropin-releasing hormone
Glu	Glutamate
GSase	Glutamine synthetase
GSI	Gonadal somatic index
hCG	Human chorionic gonadotropin
HEA	High external ammonia
HPI	Hours post injection
LH	Luteinizing hormone
LHr	Luteinizing hormone receptor
MIE	Molecular initiating event
MIH	Maturation inducing hormone
MPF	Maturation promoting factor
mPR	Membrane progestin receptor
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
MWWE	Municipal wastewater effluent
nPR	Nuclear progestin receptor
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin F2α
PGH_2	Prostaglandin H_2
qPCR	Real-time quantitative polymerase chain reaction

StAR	Steroid acute regulatory protein
T	Testosterone
UIA	Unionized ammonia
WWTP	Wastewater treatment plant
YES	Yeast estrogen screen

INTRODUCTION

Reproductive responses are often used in the risk assessment of chemicals being released into the environment. There is a worldwide interest in the effects of anthropogenic wastes such as municipal wastewater effluent (MWWE) and its constituents on exposed organisms, and how these effects might affect populations and community structures of receiving environments. However, it is difficult to define effects of individual constituents in the context of mixtures. To address this, researchers have employed a mechanistic model known as an adverse outcome pathway (AOP) to separate and describe the responses to toxicants at different levels of biological organization. The objective of this thesis was to investigate the reproductive effects and mechanisms of action of three common MWWE constituents, 17α-ethinylestradiol (EE_2), nitrate and ammonia, using the zebrafish (*Danio rerio*) as the model. These findings will then be applied to an AOP framework to establish a better understanding of the effects of exposure.

This section will review the existing literature necessary to support the objective of this thesis. The usefulness of the zebrafish as a model for studies of reproductive toxicology will be outlined, and the pertinent information regarding their reproductive processes will be explained, with emphasis on oocyte maturation and ovulation, steroidogenesis, and the roles of prostaglandins. These concepts will be used to better understand the effects of exposure to the MWWE constituents of interest to this study. Lastly, the specific objectives and outline of the remaining sections will be discussed.

Zebrafish as a Model:

To determine the effects of MWWE constituents on fish reproduction and to investigate their mechanisms of action, I used the zebrafish (*Danio rerio*) as a model organism. Zebrafish are a tropical freshwater teleost belonging to the family Cyprinidae (Zhang et al., 2003). Their small size (2.5 – 4 cm) makes zebrafish very practical models as they can be housed in large numbers in relatively small facilities (Segner, 2009). The use of zebrafish in studies dates back to the 1930's and has resulted in the production of a vast collection of data, particularly from the fields of developmental biology and molecular genetics (Laale, 1977; Hill et al., 2005). With the advent of the fully sequenced genome, the zebrafish has become increasingly useful as a model for reproductive toxicology due to the conservation of zebrafish genes across other vertebrate taxa, their relatively high tolerance to environmental contaminants, and the nature of their reproductive behaviour (Hill et al., 2005; Segner, 2009). Zebrafish are asynchronous spawners, meaning that ovarian follicles of all developmental stages are present in the ovary (Niimi and LaHam, 1974). Usually, females can spawn once every two to five days throughout the year, and produce 50 – 200 eggs per spawning event (Wang and Ge, 2004; Hill et al., 2005; Segner, 2009). The reproductive endocrinology of zebrafish has been widely researched, and today is fairly well understood.

Reproductive pathways

To investigate the reproductive effects of exposure to environmental contaminants, it is important to understand the endocrine processes that regulate reproduction. In teleosts, the processes of follicle maturation, ovulation and spawning are fairly well understood (Clelland and Peng, 2009; Levavi-Sivan et al., 2010).

Reproduction in fish as in all vertebrates is regulated by a feedback system between the hypothalamus, pituitary, and gonads, known as the HPG axis. Beginning in the hypothalamus, the release of gonadotropin-releasing hormone (GnRH) from the preoptic area stimulates the synthesis and secretion of gonadotropins from the pituitary. In teleosts there are two distinct gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH is the primary regulator of early-stage follicular development. Nagahama (1994) proposed a two-cell ovarian follicle model, in which molecular signaling between the outer theca and inner granulosa cell layers of the follicles regulates follicular growth and maturation. According to this model, binding of FSH to G-protein coupled receptors in theca cell plasma membranes stimulates the secretion of testosterone (T), which then diffuses into granulosa cells and is converted to 17β-estradiol (E_2) by ovarian cytochrome P450 aromatase (Arom) (Nagahama, 1994; Nagahama et al., 1995). E_2 then travels via the blood to the liver where it initiates synthesis of the egg-yolk precursor protein vitellogenin (Okumura et al., 2002). Vitellogenesis, or the uptake of vitellogenin by the oocyte, results in an increase in follicle size.

During the initial growth stages of ovarian development, the oocyte is arrested in prophase I and it is not until the ovarian follicles reach their maximum size during late vitellogenesis that the oocytes are capable of resuming the meiotic division in preparation for ovulation, spawning and fertilization (Clelland and Peng, 2009). In late vitellogenesis, the fully grown follicles develop maturational competence, meaning that they are capable of maturing under the stimulation of a maturation inducing hormone (MIH) (Selman et al., 1993; Clelland and Peng, 2009). Oocyte maturation, which

involves resumption of the meiotic division in fish, is regulated by steroid hormones and proceeds in response to the second gonadotropin, LH. Following vitellogenesis, there is a shift in the steroid biosynthetic pathway away from the production of E_2 to the production of progestins that function as the MIH (Nagahama and Yamashita, 2008). Perception of LH by an LH receptor (LHr) begins a signaling cascade within the follicle resulting in the production of 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), which is a MIH in several fish species including zebrafish (Selman et al., 1994; Nagahama, 1997). 17,20β-P acts via a membrane bound progestin receptor (mPR) to activate a maturation promoting factor, which induces the resumption of meiosis leading to germinal vesicle breakdown (Clelland and Peng, 2009). The egg is now ready to be ovulated and spawned, processes that are regulated in part by a class of chemicals known as prostaglandins.

17,20β-P is thought to also play a role in ovulation and spawning through actions involving the nuclear progesterone receptor (nPR) which mediates the expression of cytosolic phospholipase A_2 ($cPLA_2$) leading to the release of arachidonic acid (AA) from the phospholipid membrane of granulosa cells that surround the oocyte (Lister and Van Der Kraak, 2008; Lister et al., 2009). AA and its metabolites, the prostaglandins, are important regulators of ovulation (Lister and Van Der Kraak, 2008; Knight and Van Der Kraak, 2015). AA is converted to the prostaglandin precursor prostaglandin H_2 (PGH_2) by cyclooxygenase 1 and 2 (COX-1 and COX-2), and is then further metabolized into prostaglandins $F_{2\alpha}$ ($PGF_{2\alpha}$) and E_2 (PGE_2) by prostaglandin synthases. $PGF_{2\alpha}$ and PGE_2 are the two major prostaglandins involved in teleost reproduction (Stacey and Pandey, 1975; Goetz and Theofan, 1979). PGs are thought to act via disctinct receptors and

one $PGF_{2\alpha}$ receptor (FP), and four PGE_2 receptors (EP1, EP2, EP3 and EP4), have been identified in vertebrates (Narumiya et al., 1999). $PGF_{2\alpha}$ has been identified as a trigger for the induction of ovulation in zebrafish and other teleost species (Yaron and Levavi-Sivan, 2006; Nagahama and Yamashita, 2008; Lister and Van Der Kraak, 2008). PGE_2 is primarily a mediator of sex steroid synthesis in goldfish (Wade and Van Der Kraak, 1993; Mercure and Van Der Kraak, 1996), and it has been shown capable of inducing ovulation in goldfish (*Carassius auratus*), yellow perch (*Perca flavescens*) and medaka (*Oryzias latipes*) (Stanley and Pandey, 1975; Goetz and Theofan 1979; Fujimori et al., 2012). However, the role of PGE_2 in zebrafish remains unclear; for example, reductions in whole-body PGE_2 levels caused by the administration of ibuprofen, a commonly used analgesic and known inhibitor of prostaglandin synthesis, has no consistent effects on reproductive endpoints (Morthorst et al., 2013). Aside from their role in ovulation, prostaglandins also serve as sex pheromones, stimulating male spawning behaviour and the spawning of the ovulated eggs (Clelland and Peng, 2009). Given the various roles of prostaglandins in oocyte maturation and ovulation, they are well suited as potential endpoints in the investigation of the effects of environmental contaminants on reproductive physiology in fish.

Municipal wastewater constituents and their effects

MWWE is a complex mixture of anthropogenic wastes containing a variety of chemicals capable of negatively affecting reproductive responses in fish (Mills and Chichester, 2005; Woodling et al., 2006; Vajda et al., 2008; Lister et al., 2009; Tetreault et al., 2011). One class of contaminants found in MWWE that is of particular concern is

endocrine disrupting chemicals (EDCs), which are classified as any exogenous compound that causes adverse health effects in an exposed organism, its offspring or later generations through alterations to the endocrine system (WHO-IPCS, 2002). EDCs can come in a variety of forms, including industrial chemicals (Kavanagh et al., 2011), natural and synthetic hormones (Servos et al., 2005; Aris et al., 2014), and pharmaceuticals (Metcalfe et al., 2003). Depending on the compound, an EDC can modulate hormone synthesis and metabolism, mimic or antagonize hormone action or alter levels of hormone receptors, resulting in impaired development or reproduction (Van Der Kraak et al., 2001; Mills and Chichester, 2005; Jukosky et al., 2008). EDCs are common constituents of MWWE (Heberer, 2002), and as such they pose a serious threat to exposed biota and their ecosystems.

One of the most common EDCs in MWWE and aquatic environments is 17α-ethinylestradiol (EE_2), a synthetic estrogen-receptor agonist used in oral contraceptives (Heberer, 2002; Aris et al., 2014). Exposure to EE_2 has been shown to have detrimental effects on reproductive function in fish, including altered sex ratios in juveniles, reduced fecundity, decreased sex steroid levels, and population collapse in chronic field studies (Örn et al., 2003; Kidd *et al*., 2007; Peters et al., 2007; Hogan et al., 2010; Cosme et al., 2015). EE_2 has been found worldwide in MWWE at concentrations reaching as high as 9 ng/L in North America and 17 ng/L in some parts of Europe (Stumpf et al., 1996; Ternes et al., 1999; Atkinson et al., 2012). The concentrations of EE_2 in the environment are normally lower than those of MWWE due to dilution and degradation, but are above the threshold of effects for multiple organisms exposed in laboratory studies (Grist et al., 2003; Williams et al., 2003; Parrott and Blunt, 2005; Atkinson et al., 2012; Aris et al.,

2014). In the Grand River, Ontario, the exact concentration of EE_2 is unknown; however, Tanna et al. (2013) reported 17 ng/L total estrogenicity using the yeast estrogen screen (YES), an assay which quantifies the binding affinity, bioavailability in plasma and persistence of EE_2 in the environment. Further work by Smith and Servos (unpublished) suggests that approximately half of the estrogenicity was due to EE_2.

Compared to the natural estrogen E_2, EE_2 is more resistant to biodegradation (Lust et al., 2012; Combalbert and Hernandez-Raquet, 2010), allowing it to potentially persist longer in aquatic environments. Additionally, recent studies on EE_2 uptake and clearance have shown that it bioaccumulates in shorthead redhorse suckers (Al-Ansari et al., 2010) and goldfish (Al-Ansari et al., 2013) through water-borne exposure at environmentally-relevant concentrations. These physicochemical properties along with the potentially physiological effects and widespread use of oral contraceptives place EE_2 among the most concerning environmental contaminants in aquatic environments.

Exposure to EE_2 can cause a variety of reproductive effects in aquatic organisms. One well-studied pathway for these effects is through steroidogenesis and the associated sex steroids. Sex steroid levels in juvenile fish are critical determinants of sexual differentiation, and perturbations to the natural levels can alter sex ratios in exposed populations (Scholz and Gutzeit, 2000; Xu et al., 2008). In adult fish, sex steroid levels regulate reproduction, and in cases of reduced sex steroid levels such as those caused by exposure to EE_2, gamete quality and fertility can be significantly reduced (Montgomery et al., 2011; Reyhanian et al., 2011). In male fish, exposure to EE_2 can induce an intersex phenotype, or ovo-testes, leading to male sterility (Nash et al., 2004; Örn et al., 2006; Kidd et al., 2007). Several studies have shown that exposure

to EE_2 can impact steroidogenesis by reducing the expression of critical steroidogenic enzymes, including steroidogenic acute regulatory protein (StAR), Arom, 17α-hydroxylase and 17β-hydroxysteroid dehydrogenase (Filby et al., 2007; Garcia-Reyero et al., 2009; Lister et al., 2009). Additionally, EE_2 exposure can reduce the production of the sex steroids E_2, T and 11-ketotestosterone (Flores-Valverde et al., 2010; Hogan et al., 2010). In extreme cases, these reproductive impairments can lead to population collapse as shown in Kidd et al. (2007).

Another reproductive pathway affected by exposure to EE_2 is that of follicular development. Some studies have shown that EE_2 acts on follicular development, as evident by an absence of mature follicles in exposed females, and a reduction of LHr expression (Van den Belt et al., 2001; Cosme et al., 2015). In addition to maturation, downregulation of COX-2, cPLA2 and nPR suggest that EE_2 also affects ovulation in exposed fish (Carnevali et al., 2010; Cosme et al., 2015). Though these responses to EE_2 have been well documented in single-contaminant exposures, the action of EE_2 in complex mixtures such as MWWE are not well understood.

Among the numerous chemical constituents of MWWE are the nitrogenous wastes, which include ammonia and nitrate. Nitrogenous wastes are metabolic waste products containing the element nitrogen, including ammonia, nitrite and nitrate. Ammonia is produced endogenously by all vertebrates through amino acid catabolism, while nitrite and nitrate are produced by nitrifying bacteria. Generally, concern regarding exposure to nitrogenous wastes stems from their ability to be toxic at high concentrations. Previous studies on the relative toxicities of these nitrogenous wastes have generally shown ammonia to be the more significant toxicant of the two (Alonso

and Camargo, 2003; Adelman et al., 2009). A preliminary toxicity study of ammonia in zebrafish indicates an LC_{50} of 6.1 mM NH_4Cl at pH 7.5, which equates to 84 mg/L TAN or 1.48 mg/L unionized ammonia (UIA) (Abdourahman, A. and Walsh, P.J., University of Ottawa, unpublished results). While there is no apparent information on the LC_{50} of nitrate in adult zebrafish, studies of nitrate toxicity in larval zebrafish suggest a recommended safety level of 200 mg/L NO_3-N, a level far above those found in most MWWE (Learmonth and Carvalho, 2015). Below toxic levels, there is some evidence of both ammonia and nitrate affecting reproductive physiology (Guillette and Edwards, 2005; Ip and Chew, 2010).

These wastes are of particular concern to aquatic ecosystems due to their introduction through both non-point and point sources (Fowler et al., 2013; de Vries et al., 2013; Wongsanit et al., 2015). Globally, ammonia is applied in massive quantities to agricultural operations to aid in crop production, but due to its high solubility in water it is easily mobilized, resulting in agricultural run-off and eutrophication of receiving waterways. Nitrate can also be found in MWWE, either due to the dissolution of chemicals containing nitrate or due to the biological oxidization of ammonia through a process called nitrification (Camargo et al., 2005).

In aqueous solutions, ammonia is present in both its unionized and ionized forms, NH_3 and NH_4^+, respectively; a relationship that is highly dependent on pH with a pKa of 9.2 at 25°C (Li et al., 2012). Above a pH of 9.2, the toxic unionized form becomes the dominant fraction. Temperature also influences the relative abundance of the two forms, though at environmentally-relevant temperatures pH is the primary determinant of this ratio (Figure 1). As such, a small increase in pH can drastically

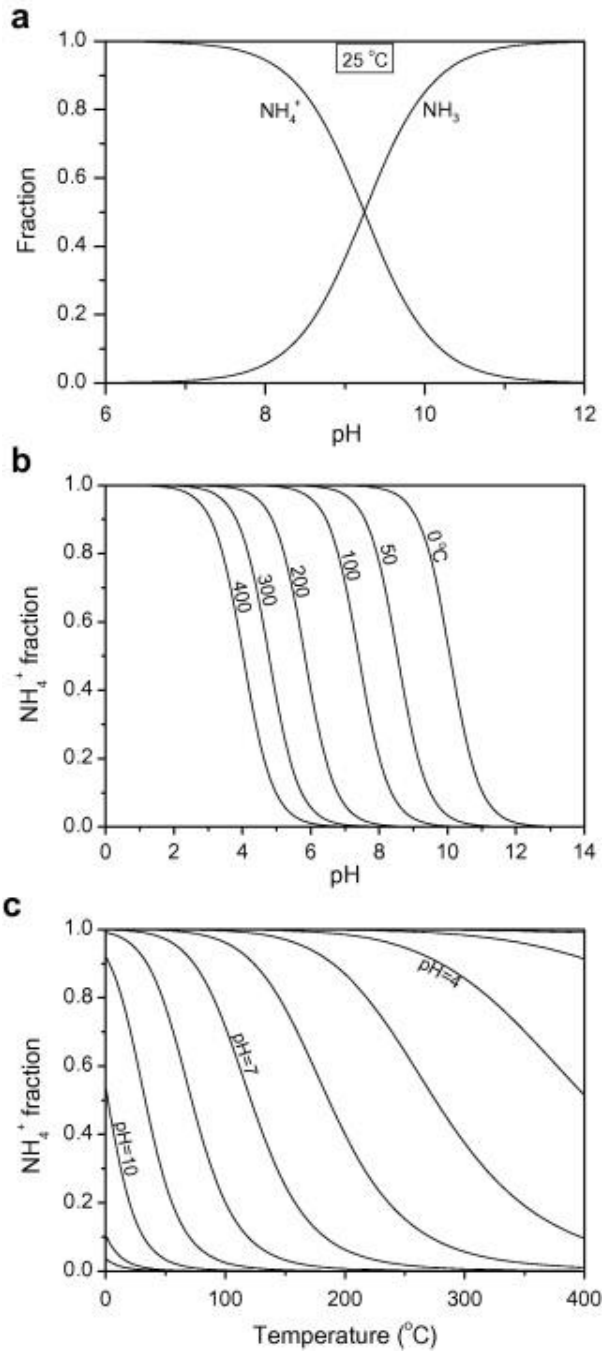


Figure 1. Taken from Li et al., 2012. Ammonia fractions in water at varying temperature and pH conditions. (a) proportional relationship of ammonia and ammonium at 25°C with varying pH; (b) changes in ammonia fractions with respect to pH at temperature ranges from 0 - 400°C; (c) changes in ammonia fractions with respect to temperature at pH ranges from 2 to 11.

increase toxicity without an increase in total ammonia. In studies of nitrogenous wastes, a common nomenclature for expressing concentration is expressed in terms of the total mass of nitrogen per volume of solution. For example, 1 mg/L TAN (total ammonia-nitrogen) indicates that the solution has 1 mg total of N in the form of both ammonia and ammonium per liter. In southern Ontario, ammonia levels in MWWE can become as high as 38.7 mg/L TAN, but average between 3 – 5 mg/L TAN (Lister et al., 2009; Anderson, 2012; Tetreault et al., 2012). However, in the Grand River watershed in Ontario, municipal wastewater treatment plants (WWTPs) are being upgraded in order to reduce ammonia levels in MWWE in favour of nitrate through the process of nitrification (Anderson 2012). As a result of these upgrades, nitrate levels are expected to rise above 30 mg/L NO_3-N in some effluents (Anderson 2012). So while ammonia may be the more concerning nitrogenous waste, changes to WWTPs and the resulting MWWE necessitate a better understanding of how these wastes will affect aquatic ecosystems and the communities therein.

In most teleost fish, 60-95% of nitrogenous wastes are excreted as ammonia (Wood, 1993; Wright, 1995). Ammonia excretion is critical to prevent elevated and potentially harmful levels of endogenous ammonia, and therefore understanding the mechanisms of ammonia excretion is important to studies of ammonia toxicity. Recent studies of ammonia excretion have shown that Rhesus proteins, a family of integral membrane proteins found in the gill epithelium, facilitate ammonia transport in fish across a normally favourable blood-water gradient (Nakada et al., 2007; Braun et al., 2009). However, high environmental ammonia (HEA), such as that that could occur from eutrophication of waterways, cause plasma ammonia levels to rise as the diffusion

gradient is reversed (Wilson and Taylor, 1992; Braun et al., 2009). High endogenous ammonia, or hyperammonemia, can disrupt the central nervous system of fish, leading to coma and death by disruption of inner mitochondrial membranes resulting in the collapse of ionic gradients, which causes excessive calcium entry into astrocytes and astrocyte swelling (Hermenegildo et al., 2000; Randall and Tsui, 2002; Walsh et al., 2007; Ip and Chew, 2010). Exposure to HEA has also been shown to reduce cumulative fecundity in fathead minnows (*Pimephales promelas*) at concentrations as low as 2.2 mg/L TAN (Armstrong et al., 2012). It is clear that ammonia is a chemical of concern in MWWE in terms of both toxicity and physiological activity, and therefore understanding the methods by which fish deal with high endogenous ammonia may provide insight into the mechanisms of action.

Aside from increases in ammonia clearance, several ammonia detoxifying mechanisms have evolved in teleosts, and their enzymatic pathways have been shown to increase in activity when plasma ammonia levels become elevated (Randall and Tsui, 2002). These detoxifying mechanisms function by either decreasing the production of ammonia through reductions in amino acid breakdown, or by converting ammonia to a less toxic compound (Ip et al., 2001; Lim et al., 2001; Randall and Tsui, 2002). Synthesis of the amino acid glutamine (Gln) is one of the latter mechanisms, requiring ammonia and glutamate (Glu), and is catalyzed by the enzyme glutamine synthetase (GSase), also known as glutamine ligase (Felipo and Butterworth, 2002; Ip and Chew, 2010; Dhanasiri et al., 2012). In zebrafish, three gene paralogues of GSase (*glul*) have been identified, namely *glula, glulb, glulc*, along with several splice variants (Dhanasiri et al., 2012). Several other species of fishes, including goldfish, common carp,

mudskippers and rainbow trout exhibit increased glutamine levels in the brain during exposure to ammonia (Levi et al., 1974; Dabrowska and Wlasow, 1986; Mommsen and Walsh, 1992; Peng et al., 1998; Sanderson et al., 2010). Endpoints such as *glul* expression and GSase activity could serve as useful biomarkers of ammonia exposure, and could also provide insight into the mechanisms of reproductive inhibition shown by Armstrong et al. (2012).

Investigating the effects of single contaminant exposures are useful for building an understanding of the risks of MWWE exposure; however, given the complex nature of MWWE, the information generated by such investigations has limited usefulness when applied to natural environments. For example, although the effects of exposure to EE_2 are well documented, there is limited information regarding the potential for other MWWE constituents to influence the response of organisms to EE_2. There are a few studies that have considered how exposure to EE_2 is influenced by water quality parameters such as salinity, temperature, and dissolved oxygen (Gillio Meina et al., 2013; Blewitt et al., 2013), but to date none that have considered the potential interactive effects of dissolved nutrients or nitrogenous wastes. In order to better understand the physiological effects of MWWE exposure on organisms, it is necessary to study the effects of chemicals both individually and in combination with other constituents.

Adverse outcome pathways (AOP)

As new chemicals of interest emerge and increasing strict legislation is implemented regarding chemical management and water quality, risk assessors are under increasing pressure to quickly and efficiently evaluate the risks of toxicant

exposure, and to do so using fewer resources (Ankley et al., 2010). Historically, most risk assessments of environmental contaminants have focused on apical responses to exposure, while neglecting to investigate the underlying mechanisms of the observed responses (Ankley et al., 2009). However, to be useful to government regulators, predictive or causal links between quantitative biological changes and downstream outcomes need to be connected (Villeneuve et al., 2014).

While investigating the effects of environmental contaminants on reproduction, it is important to consider the mechanistic information generated through reproductive bioassays in the context of a complete biological system. To address this need, the AOP has been proposed as a framework for establishing a mechanistic understanding of a toxicant's pathway of effects, linking molecular, cellular, and organ-level effects to a whole-organism adverse outcome (AO) deemed relevant by regulators (Figure 2) (Ankley et al., 2010; Villeneuve et al., 2014). Based on this definition, spawning and fecundity make excellent reproductive AOs, as the inference of these effects on population-level effects is generally straight-forward. However, it is important to note that a single AOP is not meant to be a complete representation of all possible toxicological processes in exposed organisms (Knapen et al., 2015). A single chemical can have effects on numerous processes, or conversely may act on an individual process through multiple pathways. So, while a single AOP does not address the complete vulnerability of a system to perturbation, a combination of multiple AOPs, or an AOP Network, can be created to highlight common key events (Knapen et al., 2015). Figure 3 gives a basic example of an AOP network for reproductive inhibition in zebrafish exposed to an estrogenic compound. In this example, activation of an

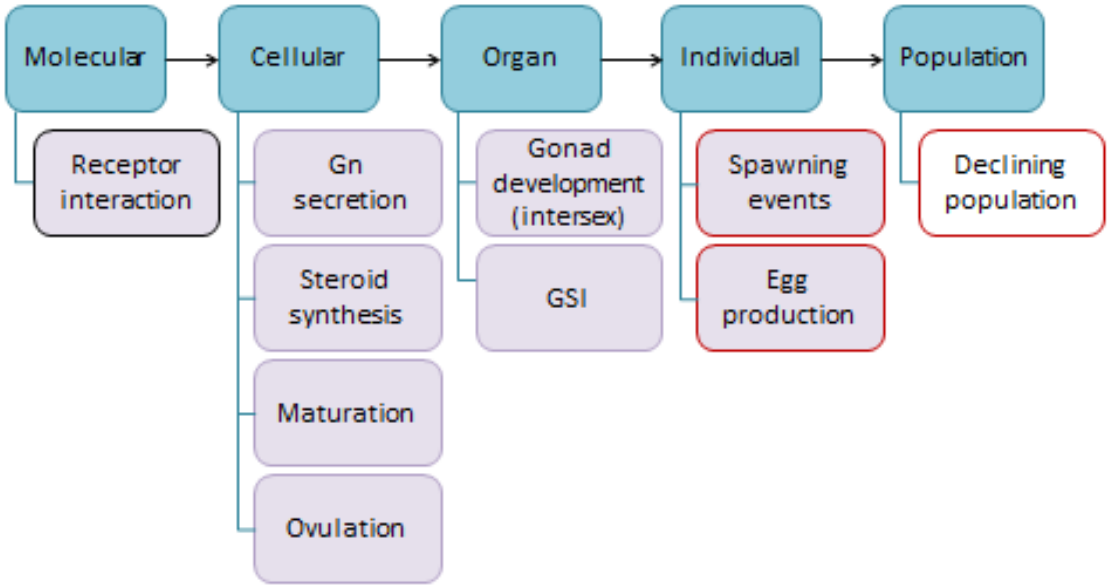


Figure 2. Example of a single, linear, reproductive AOP. In this example, the interaction of the toxicant with an unnamed receptor can influence multiple processes at the cellular level, and since receptor interaction is the initial point of the organism's interaction with the toxicant, it is classified as the molecular initiating event (MIE). These cellular responses then result in organ level responses, which ultimately affect reproduction in the individual, which in this case is the adverse outcome (AO). Once the AOP has been elucidated, inferences at the population level can be made. (Gn-gonadotropin, GSI – Gonadal somatic index).

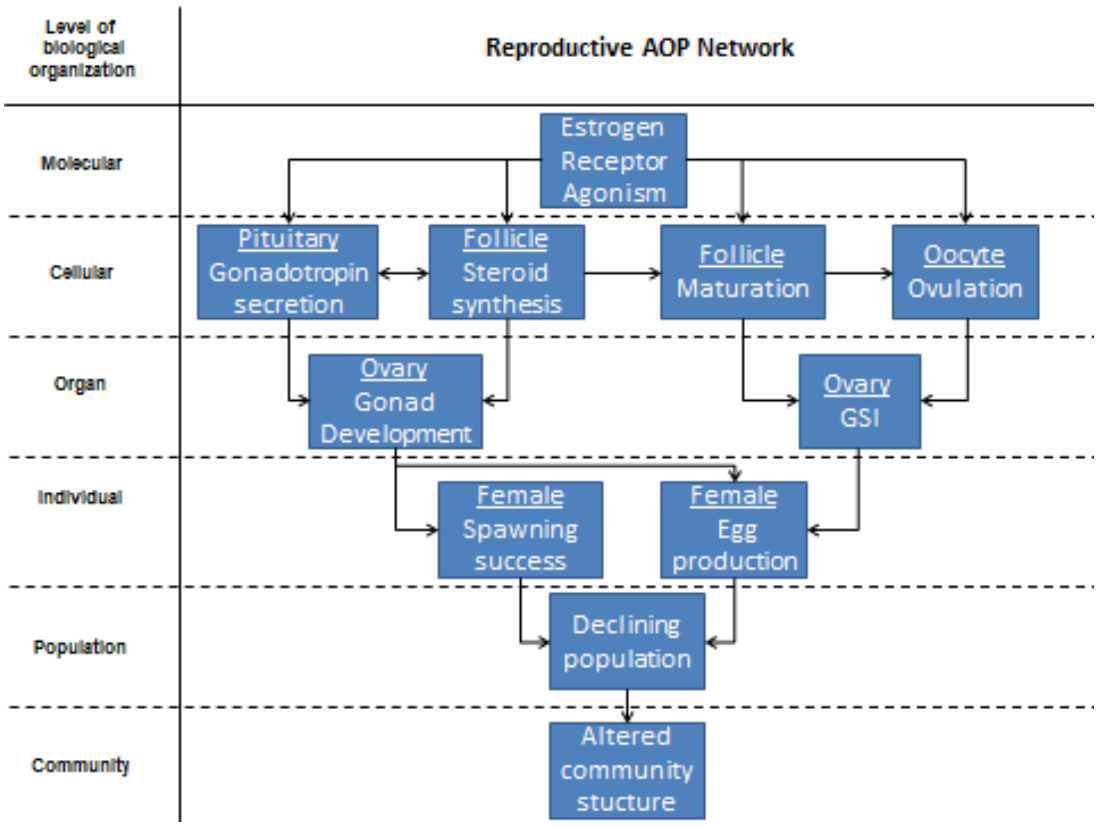


Figure 3. Example of an AOP network for an estrogenic compound. Activation of the estrogen receptor can affect gonadotropin secretion, sex steroid synthesis, follicle maturation and ovulation. All of these cellular effects can result in alterations at the organ level, which are causally linked to reduced egg production and spawning success in fish.

estrogen receptor is the molecular initiating event (MIE), which can then act on the AO of female spawning through steroid synthesis or follicle maturation and ovulation. By identifying common events among AOPs, AOP networks allow users to determine whether a particular toxicant has a unique and specific toxic mechanism, or that key events are shared among different MIEs. In the context of this study, AOPs could serve as useful tools to help understand the mechanisms by which EE_2 and the nitrogenous wastes affect reproduction, while AOP networks can help identify whether or not these toxicants act via similar processes.

Thesis Objectives and Outline

The goal of this thesis was to investigate the reproductive effects and mechanisms of action of three common MWWE constituents: EE_2, nitrate, and ammonia, both individually and in combination. The study focuses on testing the effects of these chemicals on adult zebrafish, by first investigating the effects of exposure on spawning and then focusing on the measurement of hormones and genes associated with reproductive processes such as steroidogenesis, maturation, and ovulation. The first experiments focus on nitrate and EE_2, using egg production as an apical endpoint followed by measurement of ovarian steroid content. Following the tests with nitrate, similar experiments were conducted using ammonia and EE_2, starting with similar endpoints as the previous experiments before expanding to gene expression analysis and an enzyme activity assay. The next experiments investigate the potential for ammonia to modify the normal gonadotropin response on reproduction through administration of an LHr agonist human chorionic gonadotropin (hCG). Given that hCG is known to induce oocyte maturation and ovulation leading to spawning in several species of fish (Mollah and Tan, 1983; Rowland, 1984; Hodson and Sullivan, 1993), its use will not only verify the effects of ammonia on the inhibition of spawning, but upon further molecular investigation may provide insight into the mechanisms of inhibition. In the final experiment, the role of male fish in reproduction is tested using both ammonia and hCG. Collectively, these results will be applied to an AOP in an effort to contribute to the existing knowledge regarding the toxicity of these MWWE constituents and general reproductive endocrinology by establishing a link between toxicant exposure and reproductive inhibition.

METHODS

Animals

Adult zebrafish were purchased from AQuality Tropical Fish Wholesale (Mississauga, ON) and kept in an environmental chamber in the Hagen Aqualab (University of Guelph, Guelph, ON). Fish were held in A-HAB fish containment units (Aquatic Habitats, Apopka, FL) in recirculating well water maintained at 28°C at a density of approximately 4 fish/L. The chamber had an artificial photoperiod of 12 hours light: 12 hours dark. Fish were fed daily with frozen brine shrimp (Hakari, Hayward, CA) and tropical fish flakes (Nutrafin Max, Baie d'Urfé, QC). All experiments were executed according to the animal care protocols approved by the University of Guelph Animal Care Committee on behalf of the Canadian Council on Animal Care.

Chemicals

EE_2, $NaNO_3$, NH_4Cl, and hCG were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol was purchased from Commercial Alcohols (Brampton, ON).

Experimental Protocol

Experimental fish were sexually mature, and efforts were made to use a 3:2 female to male sex ratio for fecundity experiments. Females were identified based on morphological features, primarily increased abdominal girth and presence of genital papilla. Water was maintained at 27°C, pH 8.05 throughout the duration of all experiments. During all experiments, fish were fed brine shrimp twice daily.

After sorting, fish were added to 4 L square glass beakers. A plastic mesh breeder was placed in the bottom of each tank in order to collect spawned eggs and prevent their consumption by the fish. Zebrafish generally spawn in the first hours of light; under the experimental conditions in the Hagen Aqualab, lights came on in the environmental chamber at 08:00.

Sampling Protocol

Fish were anesthetized in buffered tricaine methanesulfonate (MS-222) (Syndel Laboratories Inc, Vancouver, BC) and euthanized by spinal severance. The bodies and ovaries were weighed, snap frozen on dry ice then stored at -80°C pending further analysis. In experiments 3 and 6, a portion of the ovary from fish with ovaries larger than 20 mg were split and reweighed for use in both hormone measurement and gene expression assays.

Experiment 1- Nitrate fecundity study

The first experiment was performed to determine if exposure to nitrate would affect egg production. This experiment was conducted in a flow-through set up, with a flow rate of approximately 0.011 L/min, or 3.8 tank changeovers per day. Each 4 L glass aquaria contained 10 fish (six female, four male). Two treatments groups were used: control and nitrate (30 mg/L NO_3-N). Nitrate values were selected based on projected levels in the report by Anderson (Anderson, 2012). Water temperature was maintained by placing the aquaria in large heated water baths subject to constant mixing to prevent an unequal temperature distribution. Exposure solutions were prepared by adding 1

ml/L of a $NaNO_3$ stock solution prepared in ultrapure water, and held in 300 L glass reservoirs.

After a two-day acclimation period, eggs were collected and counted during a seven day pre-exposure period during which the fish were not exposed to any test compounds. These pre-exposure egg production data were used to establish a baseline of egg production for comparison to exposure egg production values. At the end of the pre-exposure period, tanks were sorted such that the number of eggs produced per group was similar. There were five tanks of fish per treatment. Treatment began the following morning, and eggs continued to be collected daily. Fish were sampled after seven days of exposure. Misidentification of sex resulted in a female to male ratio ranging from 7:3 to 2:3.

Experiment 2- Nitrate and steroids

The second experiment was designed to test the effects of nitrate, EE_2 and their combination on sex steroid levels in female zebrafish. This experiment was conducted using the flow-through setup described above. Each 4 L glass aquaria contained eight female fish. Four treatments groups were used: control, nitrate (30 mg/L NO_3-N), EE_2 (25 ng/L), and nitrate + EE_2 (30 mg/L NO_3 plus 25 ng/L EE_2). EE_2 values were selected based on values reported by Tanna et al. (2013), who reported total estrogenicity of Grand River effluents to be 17 ng/L. Exposure solutions were prepared as described above, with ethanol used as a carrier for EE_2.

Fish were exposed for seven days before sampling, with three tanks per treatment. Misidentification of sex resulted in some tanks having as few as four females.

Experiment 3- Ammonia, EE_2 exposure #1

This experiment was performed to determine if exposure to ammonia and EE_2 would reduce egg production, and to investigate the mechanisms of these effects. This experiment was conducted using the flow-through setup described previously. Each 4 L glass aquaria contained 10 fish (six female, four male). Four treatments groups were used: control, ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), and ammonia + EE_2 (4.5 mg/L TAN plus 25 ng/L EE_2). Ammonia values were selected based on projected levels in the report by Anderson (Anderson, 2012). Exposure solutions were prepared by adding 1mL/L NH_4Cl stock or 1µL/L EE_2 stock. Ethanol was also added to the control and ammonia groups to control for the solvents in the stock solutions.

After a two-day acclimation period, eggs were collected and counted during a seven day pre-exposure period to establish a baseline for egg production before being assigned to treatment groups, as previously described. Treatment began the following morning, and eggs continued to be collected daily. Fish were sampled after seven days of exposure. Misidentification of sex resulted in a female to male ratio ranging from 3:2 to 1:4.

Experiment 4- Ammonia, EE_2 exposure #2

Experiment 4 was conducted to verify the findings of experiment 3, and to investigate changes in ovarian gene expression after exposure to control, ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), and ammonia + EE_2 (4.5 mg/L TAN plus 25 ng/L EE_2). The experiment was conducted as previously described, except that groups contained five

tanks and fish were sampled after five days of exposure, by which time a significant reduction in egg production was observed in the ammonia, EE_2, and ammonia + EE_2 groups. Misidentification of sex resulted in a female to male ratio ranging from 7:3 to 3:7.

Experiment 5- Ammonia exposure and hCG injection assay #1

Experiment 5 was designed to further investigate the mechanism responsible for the reproductive inhibition caused by exposure to ammonia by testing the effects of ammonia on hCG-induced ovulation and spawning. This experiment was conducted in a static set-up, with daily water changes occurring at noon each day. Mixed-sex pairs were added to tanks containing either well water (control) or ammonia (4.5 mg/L TAN) at noon on day 0, with a total of 14 tanks per treatment. After 96 hours, females from half of each treatment group were injected with 50 IU hCG, and the other half injected with phosphate buffered saline (80 mM Na_2HPO_4, 20mM NaH_2PO_4, 100 mM NaCl; pH 7.4). Injections were performed using a ½ cc syringe with a 30G Ultra-Fine II needle (Becton Dickinson, Missisauga, ON), and were administered intraperitoneally through the abdomen. Given the short time frame from injection to sampling, injections were conducted without anesthesia to avoid confounding effects. Tanks were checked for the presence of eggs at four and eight hours post injection (HPI) with hCG. Fish were sampled at 8 HPI.

Experiment 6- Ammonia exposure and hCG injection assay #2

Experiment 6 was conducted to expand on the findings of experiment 5 by including data from an additional time point. The experiment was conducted as described in experiment 5, but with a total of 16 tanks per treatment at the experiment's onset. After four hours the tanks were checked for spawned eggs and fish were sampled.

Experiment 7 – Effects of ammonia on Males

Experiment 7 was designed to investigate how exposure of males to ammonia can affect spawning. This experiment was conducted in a static set-up over 96 hours, beginning at noon on day 0, with daily water changes occurring at noon each day. Groups of 12 male fish were exposed to one of three treatment groups; a well water control, an ammonia group (4.5 mg/L TAN) exposed for 24 hours, and an ammonia group (4.5 mg/L TAN) exposed for 96 hours. All groups were held in 20 L tanks for the duration of the experiment; however, the control and 96-hour ammonia groups were held in 20 L tanks containing their respective treatment for 96 hours, while the 24-hour ammonia group was held in well water for the first 72 hours before the addition of ammonia. Female fish were held in 20 L tanks in groups of 12 while the males were exposed to their respective treatments. After 96 hours, females were injected with 50 IU hCG as described above. Immediately after injection, females were placed in 4 L beakers containing well water, and then the males were introduced such that each beaker had one mixed-sex pair. After four hours tanks were checked for the presence of

eggs, and the females from tanks which did not spawn were squeezed to check for ovulated eggs.

Quantification of nitrate concentrations

Nitrate exposure concentrations were determined by using NitraVer® Nitrate Reagent Powder Pillows, 25 mL (HACH Company, London, ON) and a HACH DR 2800 spectrophotometer, which was calibrated for the test according to the measurement of a known nitrate nitrogen standard solution. For both experiments, water samples were taken from two tanks per treatment on days 1, 3, 5 and 7.

Quantification of EE_2 concentrations

EE_2 concentrations were only quantified from reservoir water samples. Duplicate water samples were taken from the reservoirs of the control, EE_2 and ammonia + EE_2 treatment groups during experiments 3 and 4 for the measurement of EE_2. In experiment 3 water samples were taken on the first, third and sixth day of treatment. In experiment 4 samples were taken on the first and fourth day of treatment. Water samples were collected in 1L amber glass bottles containing 5 mL of 200 g/L of sodium azide solution and 2.5 mL of 20 g/L ascorbic acid solution, ensuring there was no air at the top of each bottle. Samples were then refrigerated for no more than 6 days until further processing.

Immediately prior to column purification, each water sample was spiked with 100 μL of 1 mg/L *d*-EE2 (CDN Isotopes, Pointe-Claire, QC) in methanol. Water samples were passed through 6cc 500mg Oasis HLB cartridges (Waters, Milford, MA) according

to the manufacturer's instructions using 12-port visiprep vacuum manifold (Sigma Aldrich) and Gast oil-less diaphragm-tube pressure vacuum pump (Fisher Scientific). The final eluate was collected with 5 mL of methanol (Fisher Scientific) and 5 mL of 10/90 (v/v) of methanol/tert-butyl methyl ether (Sigma-Aldrich). Samples were then dried under a nitrogen gas stream. Samples were reconstituted in 500 μL of methanol (Fisher Scientific), shaken on a vortex mixer and transferred to a 2 mL vial and stored at -80°C until analysis.

EE_2 levels in water extracts were quantified using liquid chromatography-mass spectrometry by Dr. Mark Servos' lab at the University of Waterloo, Waterloo, ON.

Quantification of ammonia concentrations

For experiment 3, ammonia concentrations were determined by two methods. The first was an assay based on the work of Verdouw et al. (1978). All reagents were prepared immediately before conducting the assay. Each water sample was diluted 2x to ensure ammonia levels fell within the standard curve. Two hundred µL of each sample and the standards were added to a 96-well plate before the addition of salicylate (40% sodium salicylate), sodium nitroprusside (0.2 g/L kept in a light-tight bottle) and alkaline hypochlorite (1:1 6% sodium hypochlorite:alkaline citrate (350 g/L sodium citrate in 1.0 N NaOH)). The entire plate was vortexed for 5 seconds between the addition of each reagent. The plate was then incubated in dark at room temperature for 15 to 30 minutes before being read in a SpectraMax Plus 384 microplate reader (Molecular Devices, California, USA).

Ammonia levels in experiment 3 were also quantified by using Ammonia TNTplus, LR test vials (HACH Company) and a HACH DR 2800 spectrophotometer, which was calibrated for this test using 0.5 ml of a 10mg/L ammonia nitrogen standard (HACH Company). Results from the Verdouw et al. (1978) method agreed with those of the HACH method, therefore for the remainder of my work the HACH method was used.

For experiment 3, samples from two tanks per treatment were taken on days 0, 2, 4 and 6 and also from each reservoir on days 0 and 4. For experiment 4, samples from two tanks per treatment were taken on days 0, 2 and 4, and also from each reservoir on days 0 and 4. For experiments 5 and 6, one tank per treatment was tested daily, as well as each reservoir on days 2 and 4.

Ovarian steroid and prostaglandin levels

Ovarian homogenates from fish in experiments 3 and 6 were prepared by sonicating approximately 20 mg of ovarian tissue in a 1.5 mL centrifuge tube with100 µL PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl; pH 7.4) containing 1mM EDTA. After sonicating, 400 µL of methanol was added. Samples were then incubated at 4°C for an hour and vortexed at 20 minute intervals. After the incubation, the samples were centrifuged at 3000 x *g* for 5 minutes at 4°C. The resulting pellets were snap-frozen on dry ice, and then the methanol layer was decanted into 7 mL glass scintillation vials. The pellets were then thawed, an additional 400 µL of methanol was added, and then the samples were vortexed and incubated again at 4°C for 30 minutes. Afterwards the pellets were snap-frozen on dry ice, the methanol layer decanted into the

scintillation vials, and the process repeated once more. All three methanol phases were combined in a single vial and then dried under a stream of N_2.

Dried ovarian extracts were reconstituted in 300 µL of 50 mM acetate buffer (2.35 mL glacial acetic acid, 1.23 g sodium acetate trihydrate in 1 L; pH 4.0). Samples were passed through Amprep c-18 octadecyl mini columns (Amersham Biosciences, Little Chalfont, UK), according to the manufacturer's instructions for non-polar analytes. The final eluate was collected using 2 mL of ethyl acetate + 1% methanol. This fraction was dried under an N_2 stream, and then reconstituted in 200 µL EIA buffer (Cayman Chemical, Ann Arbour, MI) and stored at -20°C. T, E_2, $PGF_{2\alpha}$ and PGE_2 were analyzed by EIA (Cayman Chemical) as per the manufacturer's instructions. The analytes of the extracted tissue demonstrated parallelism in the EIA, which was an indication that samples did not contain compounds that interfere with the assays.

Brain and pituitary excision protocol

In order to isolate the pituitaries and remaining brain tissue for analysis of gonadotropin expression and GSase activity, female ovarioectomized carcasses were removed from storage at -80°C and immediately placed on ice. With the aid of a dissecting microscope, the skull cap was removed, the brain excised and then placed into a sterile petri dish. The pituitary was identified as the region ventral to the hypothalamus (Figure 4), and was regionally dissected and immediately snap-frozen on dry ice before proceeding with RNA extraction. The remaining brain tissue was weighed before being snap-frozen and stored at -80°C for later use in a GSase activity assay.

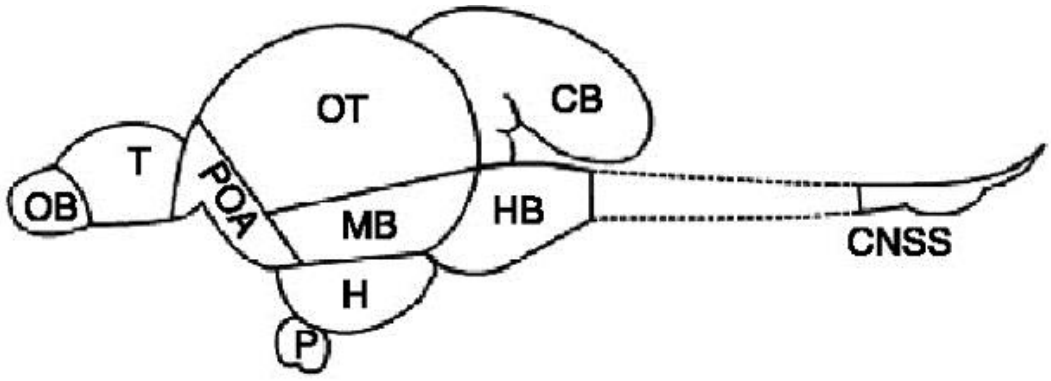


Figure 4. Taken from Bernier et al., 2008. Schematic of zebrafish brain regions: pituitary (P), olfactory bulb (OB), telencephalon (T), preoptic area (POA), optic tectum (OT), midbrain (MB), hypothalamus (H), cerebellum (CB), hindbrain (HB), and caudal neurosecretory system (CNSS).

Glutamine synthetase (GSase) activity

GSase is a critical enzyme in the detoxification of endogenous NH_3 in many teleost fish. In order to test the effects of NH_3 exposure on this detoxification mechanism, GSase activity was quantified by measuring the production of ɣ-glutamyl hydroxymate in brain tissue extracts using methods adapted from Shankar and Anderson (1985). Extraction of the enzyme GSase was adapted from Steele et al. (2001). Samples were sonicated in 750 µL of ice-cold extraction buffer (0.05 mol/L Hepes buffer, pH 7.5, 0.05 mol/L KCl, 0.5 mmol/L EDTA, 1mmol/L DL-dithiothreitol), and then centrifuged at 14000 x *g* for five minutes at 4°C. Twenty microliters of tissue extract was added to 80 µL reaction buffer (100 mM imidazole-HCl, pH 6.8, 60 mM L-glutamine, freshly prepared 15 mM hydroxylamine-HCl, pH 7.0, 3 mM $MnCl_2$, 20 mM sodium arsenate, freshly prepared 0.4 mM ADP) in 0.2 mL microcentrifuge tubes and allowed to react for five minutes at room temperature. The reaction was terminated by adding 100 µL of stop solution (0.2 M TCA, 0.37 M $FeCl_3$, 0.67 M HCl). Fifty microliters were plated in duplicate in addition to a standard curve (5.0 mM L-glutamic acid ɣ-monohydroxymate diluted with the reaction buffer) and tissue blank in a 96-well plate. Absorbance was then measured at 500 nm using a SPECTRAmax 384 Plus spectrophotometer and analyzed with SoftMax Pro (v6.2.2) (Molecular Devices, US)

In order to ensure that measurements of the reaction are taken during the initial rate period before product formation begins to plateau, a pool of enzyme extract was created and then apportioned into 15 aliquots. Each aliquot was allowed to react for an increasingly large increment of time, starting at every 30 seconds for five minutes, and every minute after up to 10 minutes, for a total of 15 time points. The resulting data

were plotted as a time series and ensured that product formation occurred at a constant rate by indicating a linear reaction rate.

Total RNA Extraction and reverse transcription

To extract RNA from liver tissue, 10-20 mg of tissue was placed in a 1.5 mL centrifuge tube containing 800 µL Trizol reagent (Life Technologies, Burlington, ON) at room temperature, and then homogenized by repeated and vigorous extraction and expulsion with a syringe equipped with a 3 mL 21G needle (Becton Dickinson). Samples were then incubated for five minutes at room temperature, then 160 µL of chloroform were added and the sample mixed by inversion for 15 seconds. Samples were allowed to incubate for 3 minutes at room temperature, then centrifuged at 12000 x *g* for 15 minutes at 4°C.The aqueous phase was transferred to a new tube. Four-hundred microliters isopropyl alcohol was added and the samples pulse-vortexed to precipitate the RNA. Samples incubated at room temperature for 10 minutes then were centrifuged at 12000 x *g* for 10 minutes at 4°C. For the extraction of RNA from pituitaries, 0.02 mg glycogen was added to the aqueous phase prior to the addition of isopropanol to act as a carrier. If glycogen was used, samples were allowed to incubate overnight at -20°C before proceeding with centrifugation.

After centrifugation, the supernatant was discarded and the pellet washed with 800 µL 75% ethanol. Samples were vortexed and centrifuged at 7500 x *g* for five minutes at 4°C. Supernatant was discarded and the excess removed by pipette. Tubes were then left open to allow samples to air dry for 3-5 minutes. Ultrapure DNase/RNase-free water was added depending on the size of the pellet, from 10-100 µL, and then the

samples were incubated at 60°C for 10 minutes and mixed by vortexing. A Nanodrop8000 (Thermo Scientific, Waltham, MA) was then used to quantify RNA. In separate tubes, RNA was diluted to 500 ng/µL. DNase treatment and reverse transcription were conducted according to Nelson and Van Der Kraak (2010). After reverse transcription, cDNA samples were held at -20°C until used for gene expression analysis.

Quantitative real-time PCR

Relative gene expression was measured using real-time quantitative polymerase chain reaction (qPCR). The primer sequences used for qPCR are shown in Table 1. cDNA was diluted 30x for ovarian tissue and 4x for neural tissue. For the analysis of experiments 1 and 2, 2.5 µL of diluted sample was added to a 96-well PCR plate with 1.25 µL each of forward and reverse primers (2.4 µM, Sigma-Aldrich) and 5 µL SsoFast EvaGreen Supermix (Bio-Rad, Mississauga, ON).

Samples were run in duplicate on a CFX96 Real-Time System (Bio-Rad Laboratories, Mississauga, ON). Samples were incubated at 95°C for 30 seconds, then a cycle of one second at 95°C and five seconds at 60°C was repeated 40 times.

For experiments 3 and 4, 3.75 uL of diluted sample was added to a 96-well PCR plate with 1.875 µL of forward and 1.875 uL of reverse primers (1.6uM, Sigma-Aldrich) and 7.5 uL Perfecta SYBR Green Fastmix (Quanta Biosciences, Gaithersburg, MD). Samples were run in duplicate on a CFX96 Real-Time System (Bio-Rad Laboratories, Mississauga, ON). Samples were incubated at 50°C for two minutes and 95°C for five

Table 1. List of primers and their sequences used in this study

Gene	Sequence (5'-3')	Database ID
EF1α	Forward GATCACTGGTACTTCTCAGGCTGA Reverse GGTGAAAGCCAGGAGGGC	NM_131263
cCPA2	Forward TGCTCTTGGAAGTTTGCGC Reverse AGCAGTCCAACTCGATGCATC	NM_131295.1
LHr	Forward ACCTGACCTCCATCTTCTCCC Reverse TGGATGGTACTGAGCGCAGA	NM_205625
COX-2	Forward GTTAAAAGATGGAAAGCTTAAATACCAGG Reverse CAAGAGAATCTCCATAAATGTGTCCA	NM_153657.1
Arom	Forward AGTTCAACTGGCACACGCAG Reverse AGCTCTCCATGGCTCTGAGC	NM_131154
mPRβ	Forward GGCCATCAGATCTTCCATGTG Reverse TCTCTCTGTGTAATCCAGTCGAACA	XM_005158330.2
nPR	Forward GGATCACCTTTCTGCGCT Reverse GACAACCAGAAGCCTC	NM_001166335.1
ARP	Forward CCAGAACACCGGGCTCG Reverse TTGATAAGCTGAACGTCACTCAAGA	BC_049058
StAR	Forward ACCTGTTTTCTGGCTGGGATG Reverse GGGTCCATTCTCAGCCCTTAC	NM_131663
FSH	Forward GTTTTTCATGCATCCCACACAC Reverse AGTACCAACTATAGAATTAACACAACAGATTAATT	XM_009303208.1
LH	Forward CGAAACGCCTGTCAAGATG Reverse TCACAGCGTGGAAAAACCAAG	NM_205622.2
glula-002	Forward GGTACATTCGAGAGGGGGTAAA Reverse TCTGTGGTTAAAATTGGAGTAGCA	ENSDART00000146819

glula-003	Forward	CCTCTGCCCCAGTCTGCTCT	ENSDART00000147472
	Reverse	CTTCACACGGGCCAACCTG	
glulb-002	Forward	GTATAAACTCCATTTCTATCCTAACAG	ENSDART00000150194
	Reverse	GACCTGAACTTTTTCTCCCTG	
EP4α	Forward	TGCTCAATCCCGCTTGTTGTCC	NM_001039629.1
	Reverse	CGAAGCGGATGGCCAGAAGAT	
EP4β	Forward	ATCGTTCTCATAGCCACGTCCAC	NM_001128367.1
	Reverse	CCGGGTTTGGTCTTGCTGATGAA	
FP	Forward	CTGTCAGCTTTTGGCAATCA	NM_001185071.1
	Reverse	AACAGCCTTGCGTAGAAGGA	

minutes**.** Then a cycle of one second at 95°C and 30 seconds at 60°C was repeated 40 times.

The housekeeping genes used in these experiments were chosen based on their stability of expression across treatments within the particular tissue being tested. Samples of ovarian and brain/hypothalamus cDNA from experiments 1 and 2 were normalized to elongation factor 1α (EF1α). Samples of ovarian cDNA from experiments 3 and 4 were normalized to both EF1α and acidic ribosomal protein (ARP) using the reference residual normalization method developed by Edmunds et al. (2014). A six-point standard curve for each gene was run with a pooled sample containing all the treatment groups to quantify gene expression.

Statistical Analyses

Statistical analyses were performed using SAS v9.4 (SAS Institute, North Carolina, USA) with significance set at α=0.05. Egg production, gene expression and hormone data were tested for homogeneity of variance using Levene's test, and log transformed where necessary to meet assumptions of normality. In cases where normality could not be achieved, a Kruskal-Wallis non-parametric test was used. For gene expression and hormone data from experiments 2, 3 and 4, a nested one-way ANOVA and Tukey's multiple comparison test was conducted to test for significance between treatment groups. GSI data was analyzed for statistical significance using an ANCOVA. Differences in egg production between pre-exposure and exposure were compared using a t-test. Spawning success and ovulation rate (experiments 5 and 6) were tested against control treatments using Fisher's exact probability test.

RESULTS

Experiment 1- Nitrate fecundity study

Experiment 1 was designed to test the effects of nitrate on zebrafish egg production using a flow-through exposure. The concentration of nitrate during the experiment was 31 ± 0.6 mg/L NO_3-N. No significant changes in egg production were observed by the end of the seven day exposure period (Figure 5, Figure 6).

Experiment 2- Nitrate and steroids

To determine the effects of nitrate, EE_2 and their combination on sex steroids in zebrafish, another flow-through experiment was conducted. The concentration of NO_3 during the exposure was 31 ± 0.4 mg/L NO_3-N and 31 ± 0.3 mg/L NO_3-N in the nitrate and nitrate + EE_2 groups, respectively. Ovarian EE_2 levels in the EE_2 and nitrate + EE_2 groups were not quantified. There was a significant reduction in both testosterone and estradiol in female ovaries from fish in the EE_2 and nitrate + EE_2 groups compared to the control group; no effects of nitrate alone were found (Figure 7).

Experiment 3- Ammonia, EE_2 exposure

This experiment was designed to determine if exposure to ammonia, EE_2 and their combination would affect reproduction in zebrafish, and to investigate the mechanisms of these effects. The concentrations of EE_2 in the EE_2 and ammonia + EE_2 groups were 39 ±1.7 ng/L and 40 ± 1.8 ng/L, respectively, while ammonia concentrations in the ammonia and ammonia + EE_2 groups were 4.4 ± 0.02 mg/L and 4.6 ± 0.03 mg/L, respectively, with pH values of 8.05 ± 0.03 and 8.06 ± 0.03

respectively. After seven days of exposure, egg production was significantly reduced in the ammonia (46%), EE_2 (75%) and ammonia + EE_2 (74%) treatments when compared to pre-exposure production levels (Figure 8, Figure 9). Females from the EE_2 treatment had significantly reduced GSI compared to all other treatment groups (Figure 10). Ovarian levels of T and E_2 were significantly reduced in both EE_2 and ammonia + EE_2 treatment groups to less than half that of the control group, while ammonia alone had no effect on sex steroids (Figure 11).

Gene expression analysis of LH and FSH in the isolated pituitaries of females showed no significant changes among any of the treatment groups (Appendix I). Expression of Gla-2 in the ammonia + EE_2 group was significantly higher than that of fish in the control and ammonia groups, and expression of Gla-3 was significantly increased in fish exposed to EE_2 and ammonia + EE_2 (Figure 12). Glutamine synthetase activity did not differ significantly among any of the treatment groups (Appendix I).

Experiment 4- Ammonia, EE_2 exposure

Experiment 4 was designed to verify the effects of ammonia and EE_2, both individually and combined, on zebrafish egg production, and to investigate further the mechanisms of these effects. The concentrations of EE_2 in the EE_2 and ammonia + EE_2 groups were 37 ± 0.23 ng/L and 34 ± 1.0 ng/L, respectively, while ammonia concentrations in the ammonia and ammonia + EE_2 groups were 4.5 ± 0.02 mg/L and 4.6 ± 0.02 mg/L, respectively, with pH values of 8.04 ± 0.03 and 8.06 ± 0.02 respectively. After five days of exposure, egg production was significantly reduced in the ammonia (51%), EE_2 (74%) and ammonia + EE_2 (95%) treatments when compared to

pre-exposure production values (Figure 13, Figure 14). Female GSI values were significantly higher in the ammonia group compared to the EE_2 and ammonia + EE_2 exposed fish, though none differed significantly from the control (Figure 15). Ovarian expression of nPR and LHr was significantly reduced in the ammonia + EE_2 exposed fish compared to expression levels of fish exposed to control and ammonia, while expression in the EE_2 group did not differ from any other (Figure 16). Also, Arom expression was significantly lower in fish exposed to EE_2 and ammonia + EE_2 compared to both control and ammonia treatment groups (Figure 16).

Experiment 5- NH_3 exposure and hCG injection assay

This experiment was designed to test whether ammonia blocked the stimulatory effects of hCG ovulation and spawning. Ammonia concentrations in the ammonia group were 4.5 ± 0.02 mg/L with a pH of 8.05 ± 0.01. During the four day exposure prior to injection with hCG, spawning in the ammonia group was significantly reduced compared to the control (Figure 17). After the injections with hCG, measurements of spawning at four and eight HPI indicated a significant increase in spawning success in the control + hCG group compared to the control; at 4 HPI 57% of control + hCG tanks spawned, and by 8 HPI 86% had spawned while none of the control spawned at either time point. None of the ammonia exposed fish spawned regardless of whether or not they were injected with hCG (Figure 18). Although none spawned, 40% of females injected with hCG after exposure to ammonia ovulated (Figure 19).

Analysis of ovarian gene expression showed significant increases in the expression of StAR in hCG-injected females (Figure 20). COX2 expression was

significantly increased in the ammonia + hCG groups compared to all other treatments, and cPLA2 expression was significantly higher in the ammonia + hCG group compared to the control (Figure 20).

Experiment 6- Ammonia exposure and hCG injection assay

Experiment 6 was conducted to verify the findings of experiment 5, but also to investigate the hCG-induced spawning behaviour in a shorter timeframe and generate ovarian tissue for analysis of prostaglandins by EIA. Ammonia concentrations in the ammonia group were 4.5 ± 0.03 mg/L with a pH of 8.06 ± 0.01. During the four day exposure prior to injection with hCG, spawning in the ammonia group was significantly reduced compared to the control group (Figure 21). After the injections with hCG, measurements of spawning at 4 HPI showed a significant increase in spawning success in the control + hCG group (43%) compared to the control (0%) (Figure 22). In the ammonia and ammonia + hCG groups, 0% and 13% of the mixed pairs spawned, respectively, and did not differ significantly from the control (Figure 22). Quantification of ovulated females indicated that 13% of the control, 43% of the control + hCG group, 38% of the ammonia group and 50% of the ammonia + hCG group ovulated, though the differences were not statistically significant (Figure 23).

Levels of PGE_2 were significantly reduced in the control + hCG treatment group compared to the ammonia group, but were not significantly different than control or ammonia + hCG groups (Figure 24). Additionally there were no significant changes in $PGF_{2\alpha}$ among any of the treatment groups (Figure 24).

Analysis of ovarian gene expression showed no changes associated with exposure to ammonia alone, but did show a significant increase in the expression of

StAR in hCG-injected females (Figure 25). COX-2 expression was significantly increased in the ammonia + hCG groups compared to all other treatments, and LHr expression was significantly increased in the ammonia + hCG group compared to the control (Figure 25).

Experiment 7 - Effects of ammonia on males

Experiment 7 was conducted to investigate whether the male zebrafish are responsible for the reduced spawning success associated with ammonia exposure. Ammonia concentrations in the 24-hour and 96-hour ammonia groups were 4.6 ± 0.02 mg/L TAN and 4.6 ± 0.03 mg/L TAN, respectively, with pH values of 8.05 ± 0.01 and 8.05 ± 0.01, respectively. At 1 HPI, 33%, 13% and 0% of the control, 24-hour ammonia and 96-hour ammonia treatments had spawned, respectively, and at 2 HPI, 44%, 13% and 25% of tanks had spawned (Figure 26). By 4 HPI, spawning had climbed to 67%, 38% and 63% for the control, 24-hour ammonia and 96-hour ammonia groups, respectively, with no further spawning occurring by 6 HPI. No statistically significant differences were found at any of the time points.

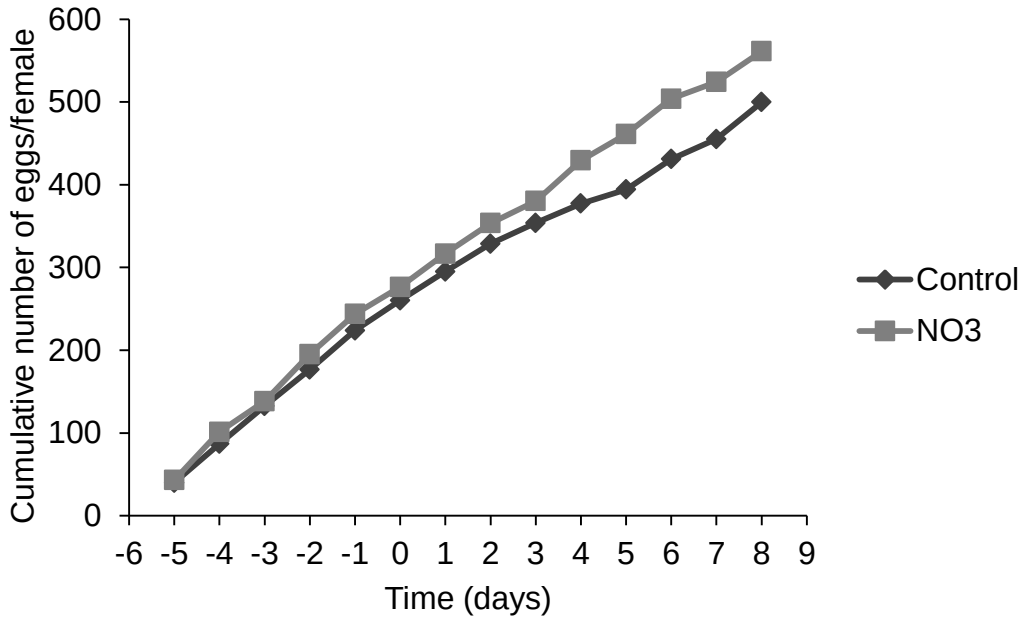


Figure 5. Cumulative egg production per female zebrafish during 7 day well water pre-exposure and 7 day exposure to nitrate (30 mg/L NO_3-N) or well water (control). Data were generated from experiment 1 and represent the cumulative number of eggs spawned in five tanks per treatment averaged over the number of females per treatment.

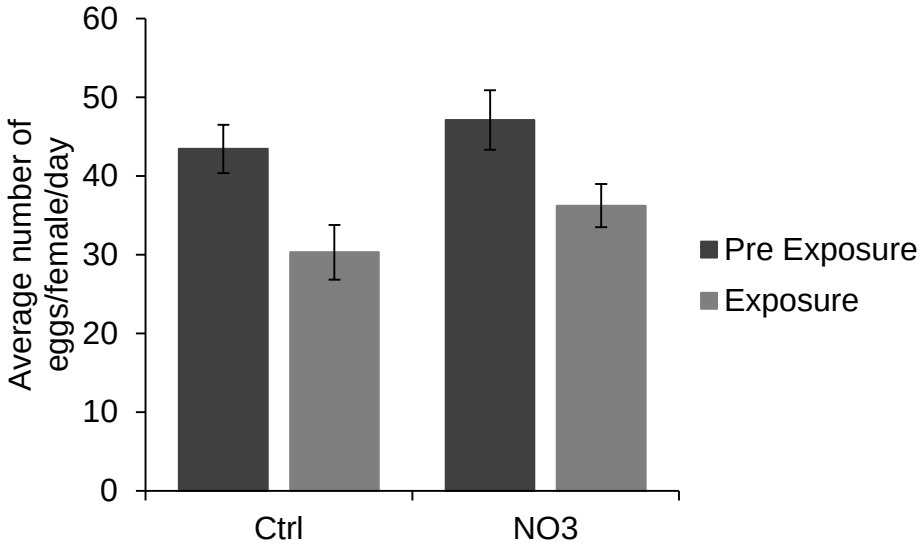


Figure 6. Average number of eggs produced per female averaged over the course of the 7 day well water pre-exposure and 7 day exposure periods to nitrate (30 mg/L NO_3-N) or well water (control; Ctrl). Data were generated from experiment 1 and represent mean ± S.E.M of five tanks per treatment. No significant changes in egg production were found between pre-exposure and exposure values ($p>0.05$; t-test), or in pre-exposure egg production among treatment groups ($p>0.05$; ANOVA, Tukey's).

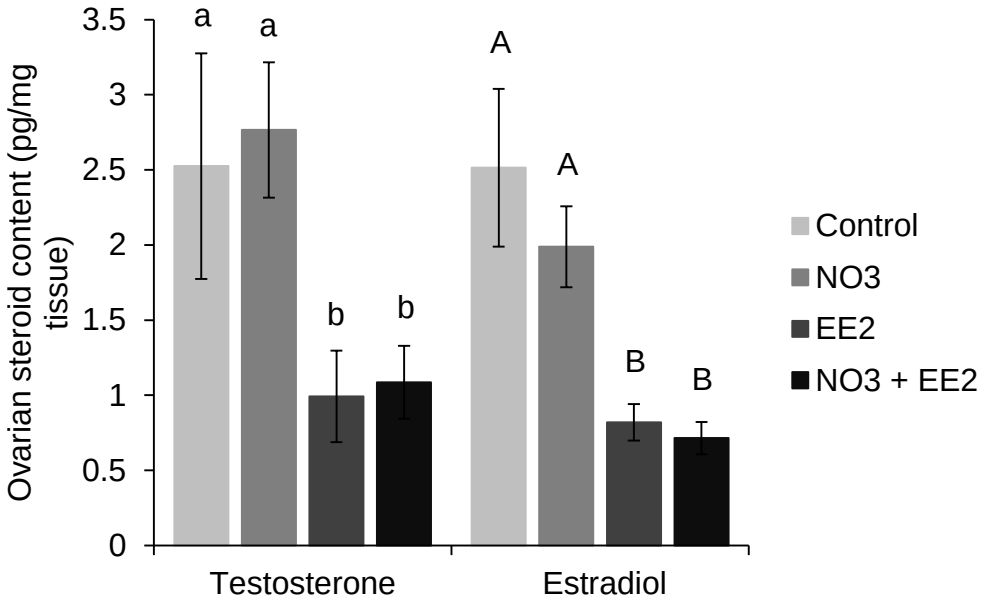


Figure 7. Average ovarian steroid content after a 7 day exposure period to nitrate (30 mg/L NO_3-N), EE_2 (25 ng/L), nitrate + EE_2 (30 mg/L NO_3-N and 25 ng/L EE_2) or well water (control). Data were generated from experiment 2 and represent mean ± S.E.M of three tanks per treatment. Different letters indicate a significant difference in testosterone (lower-case) and estradiol (upper-case) among treatments ($p<0.05$; Nested ANOVA, Tukey's).

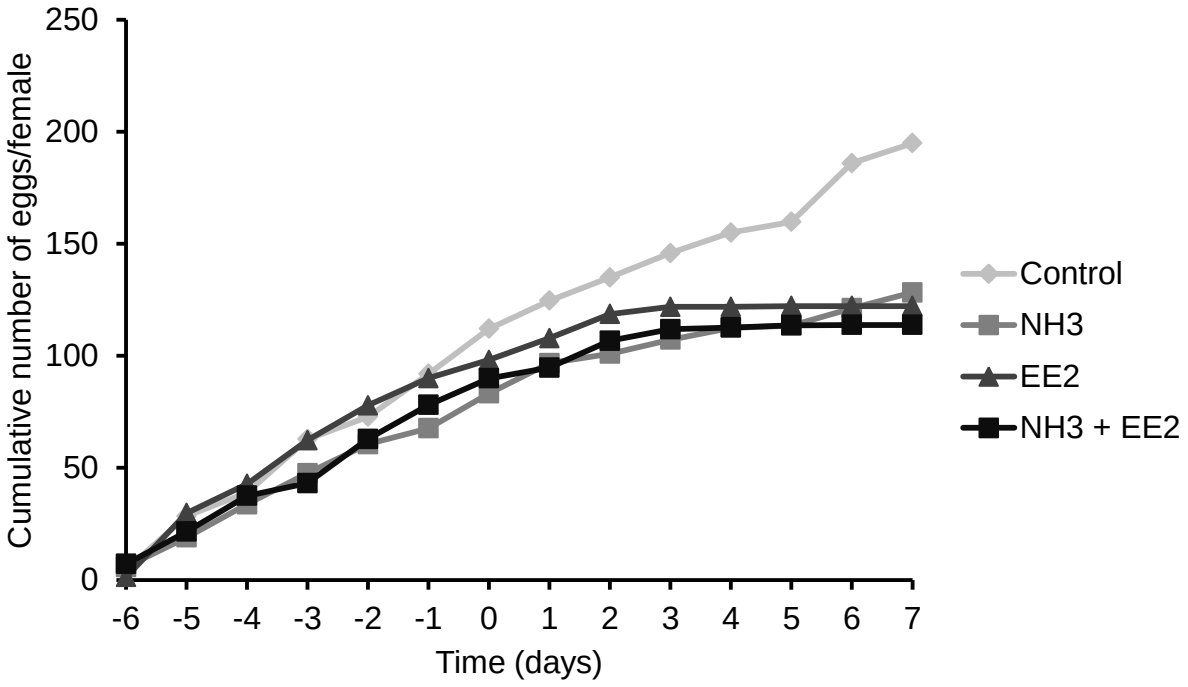


Figure 8. Cumulative egg production per female zebrafish during 7 day well-water pre-exposure and 7 day exposure to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control). Data were generated from experiment 3 and represent the cumulative number of eggs spawned in six tanks per treatment averaged over the number of females per treatment.

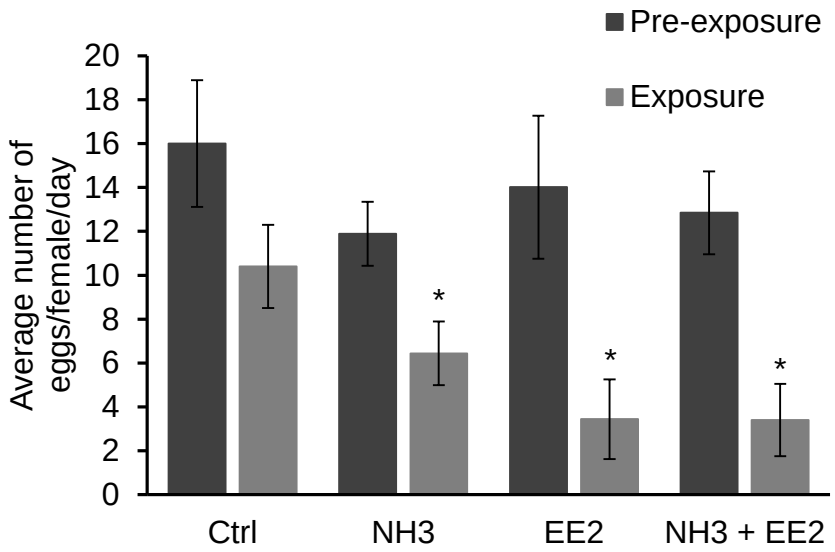


Figure 9. Average number of eggs produced per female averaged over both the 7 day well water pre-exposure and 7 day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control; Ctrl). Data were generated from experiment 3 and represent mean ± S.E.M of six tanks per treatment. * indicates a significant reduction in egg production during the exposure period compared to the pre-exposure period ($p<0.05$; t-test). No significant difference in pre-exposure egg production was found cross treatment groups ($p>0.05$; ANOVA).

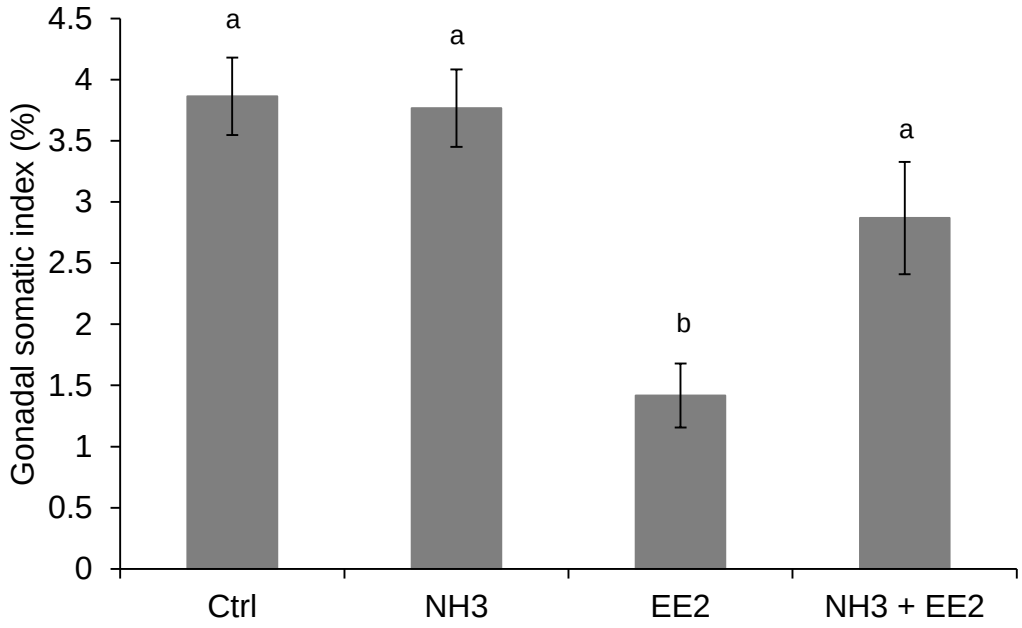


Figure 10. Average gonadal somatic indices after 7 day well water pre-exposure and 7 day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control; Ctrl). Data were generated from experiment 3 and represent mean ± S.E.M of six tanks per treatment. Different letters indicate a significant difference among treatments ($p<0.05$; Nested ANOVA, Tukey's).

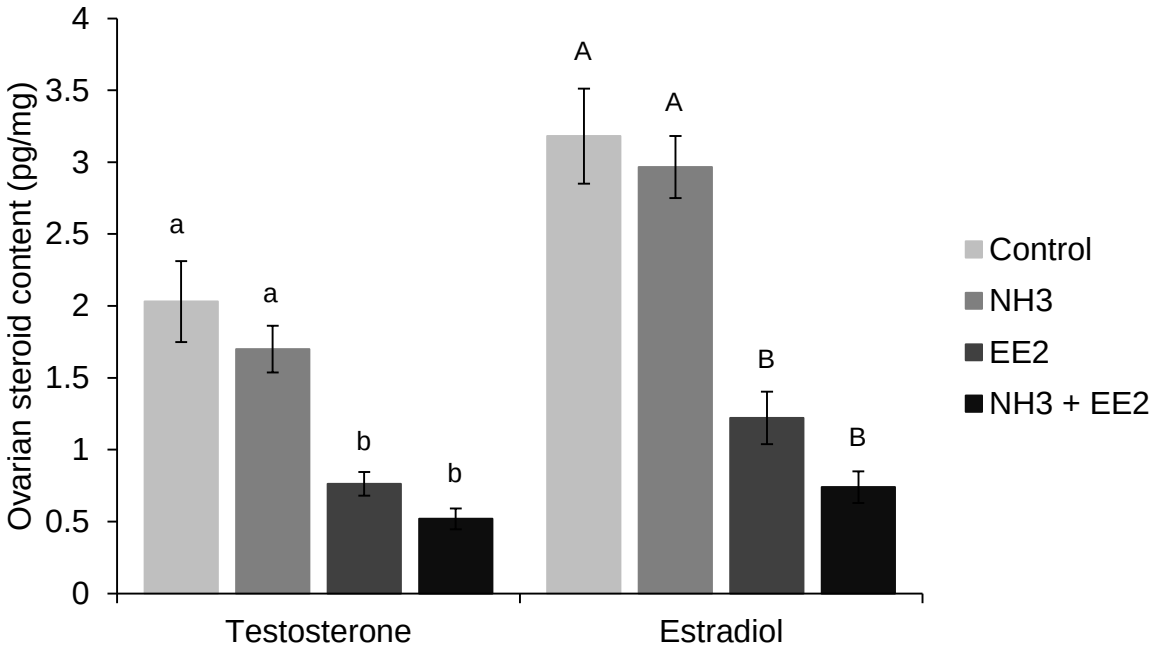


Figure 11. Average ovarian steroid content after 7 day well-water pre-exposure and 7 day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control). Data were generated from experiment 3 and represent mean ± S.E.M of six tanks per treatment. Different letters indicate a significant difference in testosterone (lower-case) and estradiol (upper-case) among treatments ($p<0.05$; Nested ANOVA, Tukey's).

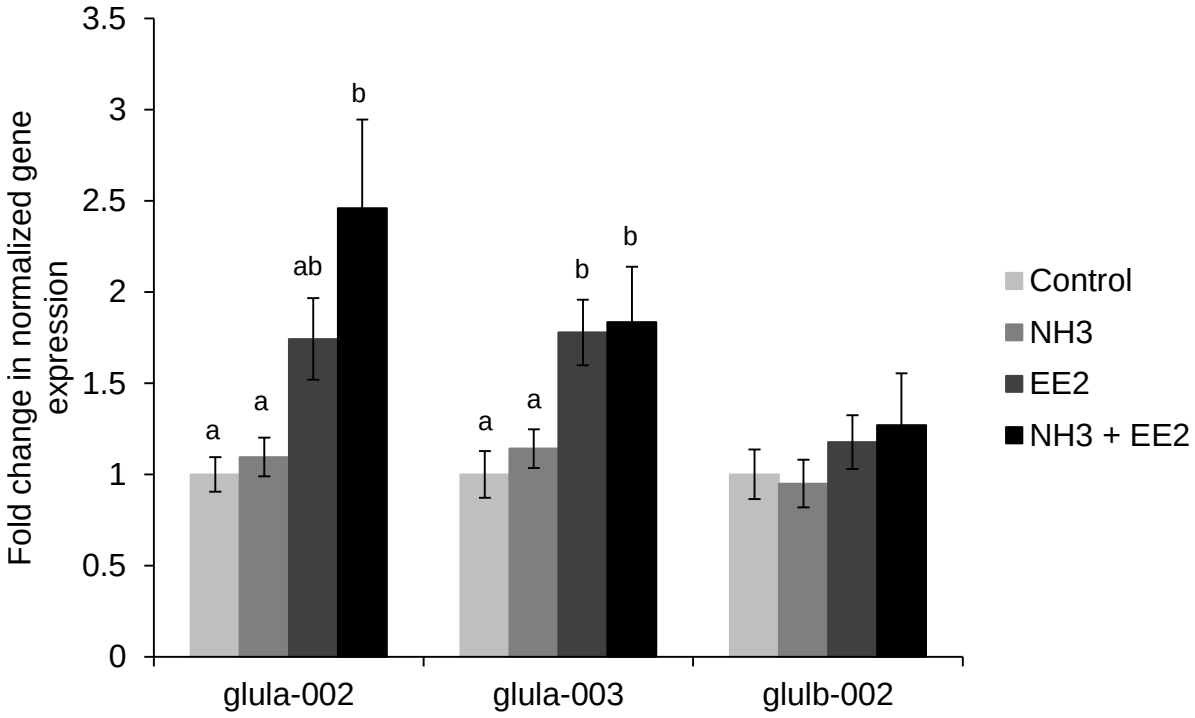


Figure 12. Pituitary expression of glutamine synthetase genes (glula-002, glula-003, glulb-002) in fish after 7 day well water pre-exposure and 7 day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control). Data were generated from experiment 3 and represents mean ± S.E.M of six tanks per treatment. Expression data was normalized to elongation factor-1α and expressed as a fold change relative to gene expression of the controls. Different letters indicate a significant difference in expression among treatments ($p<0.05$; Nested ANOVA, Tukey's).

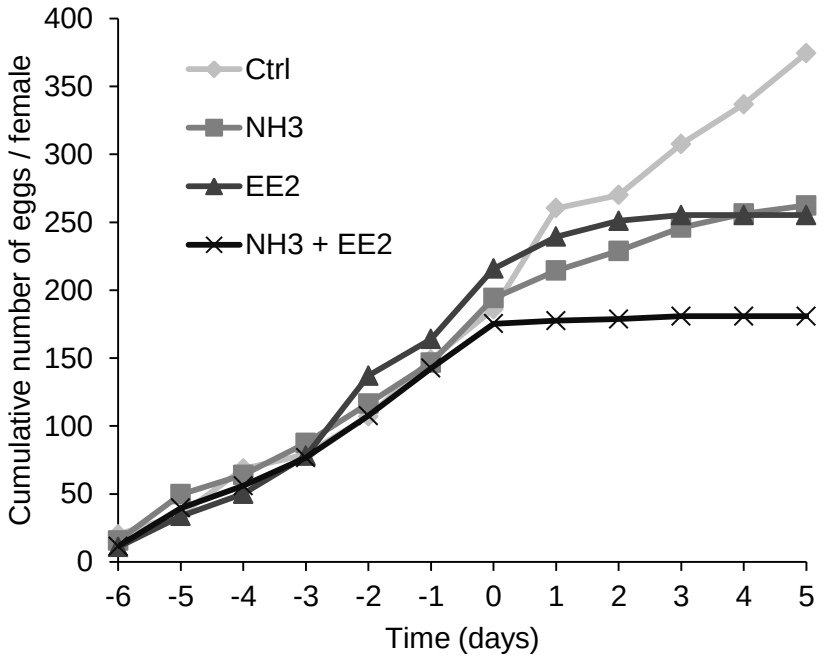


Figure 13. Cumulative egg production per female zebrafish during 7 day well-water pre-exposure and 5 day exposure to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control; Ctrl). Data were generated from experiment 4 and represent the cumulative number of eggs spawned in five tanks per treatment averaged over the number of females per treatment.

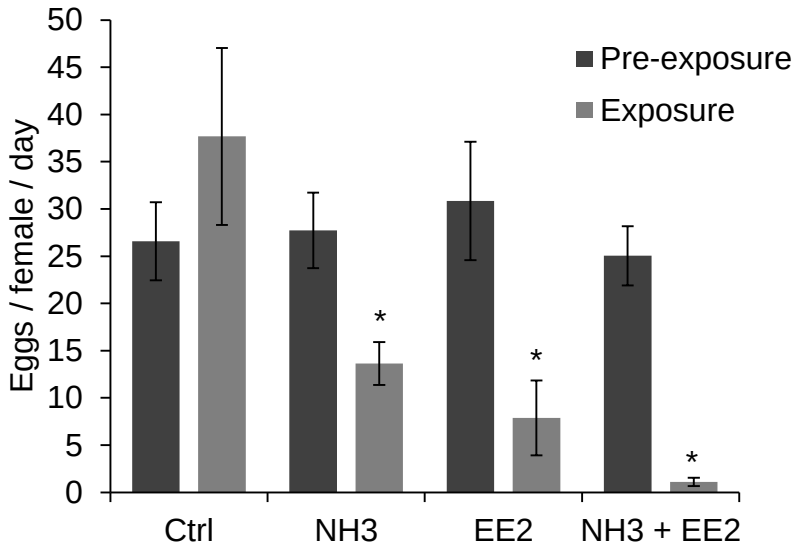


Figure 14. Average number of eggs produced per female averaged over the course of the 7 day well-water pre-exposure and 5 day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control; Ctrl). Data were generated from experiment 4 and represents mean ± S.E.M of five tanks per treatment. * indicates a significant reduction in egg production during the exposure period compared to the pre-exposure period ($p<0.05$; t-test). No significant difference in pre-exposure egg production was found across treatment groups.

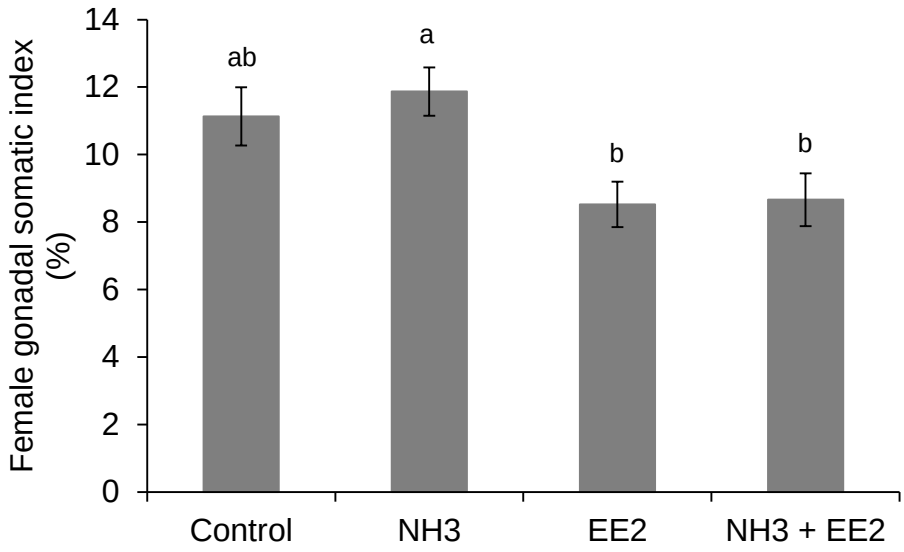


Figure 15. Average female gonadal somatic indices after 7 day pre-exposure and 5 day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control). Data were generated from experiment 4 and represent mean ± S.E.M of five tanks per treatment. Different letters indicate a significant difference among treatments ($p<0.05$; Nested ANOVA, Tukey's).

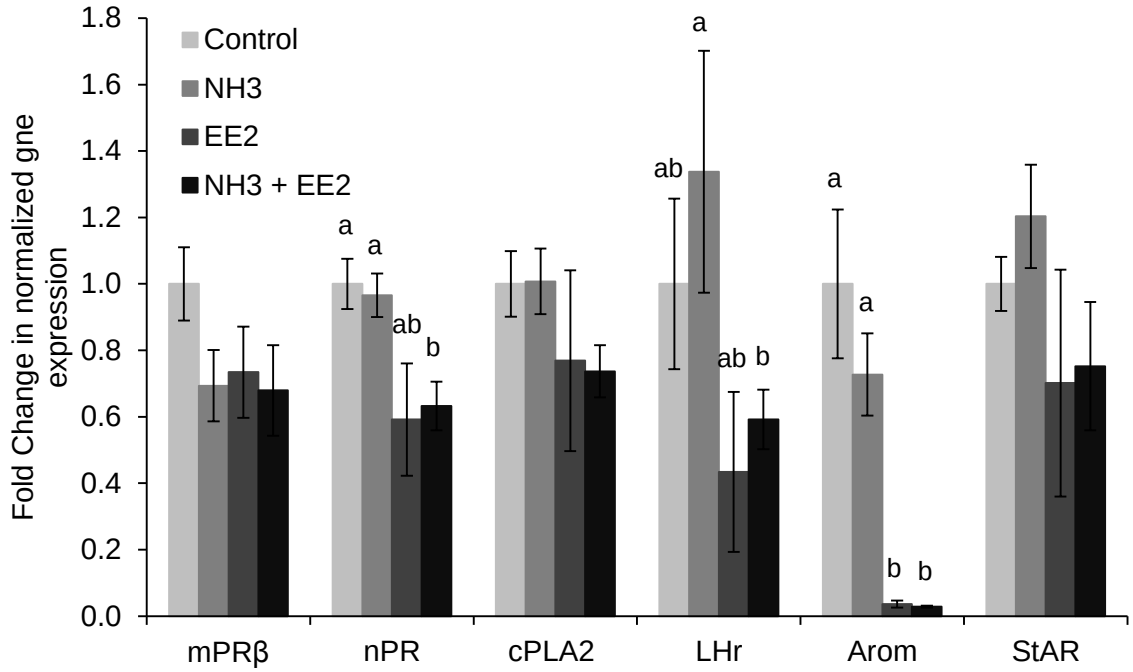


Figure 16. Ovarian expression of membrane progesterone receptor-β (mPRb), nuclear progesterone receptor (nPR), cytosolic phospholipase A2 (cPLA2), luteinizing hormone receptor (LHr), aromatase (Arom) and steroidogenic acute regulatory protein (StAR) in fish after 7 day well-water pre-exposure and 5 day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control). Data were generated from experiment 4 and represent mean ± S.E.M of five tanks per treatment. Expression data was normalized to elongation factor-1α and expressed as a fold change relative to gene expression of the controls. Different letters indicate a significant difference in gene expression among treatments ($p<0.05$; Nested ANOVA, Tukey's).

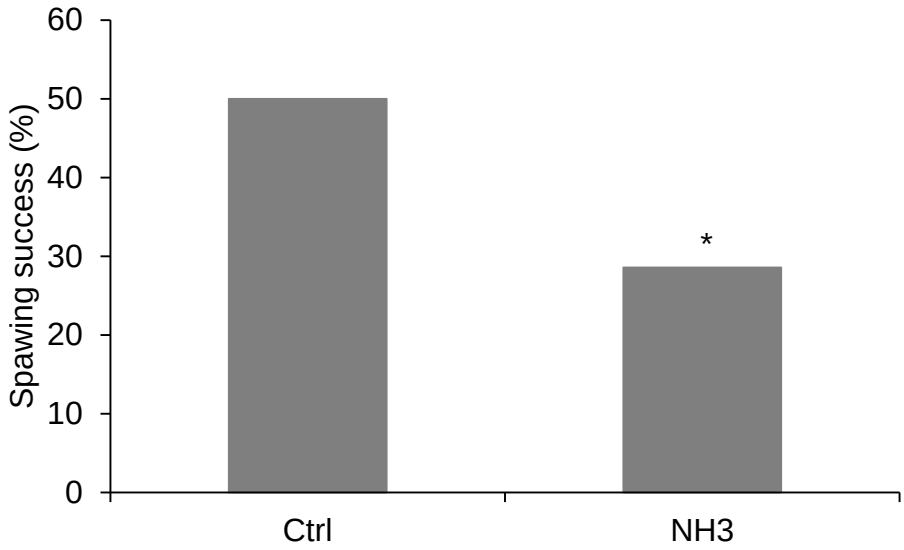


Figure 17. Proportion of mixed pairs that spawned during 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control; Ctrl) prior to injection with hCG or PBS. Data were generated from experiment 5 and represent the total number of spawning events during the 4 day exposure expressed as a proportion of total potential spawning events in 14 tanks per treatment. * indicates a significant reduction in spawning success between treatment groups ($p<0.05$; Fisher's exact test).

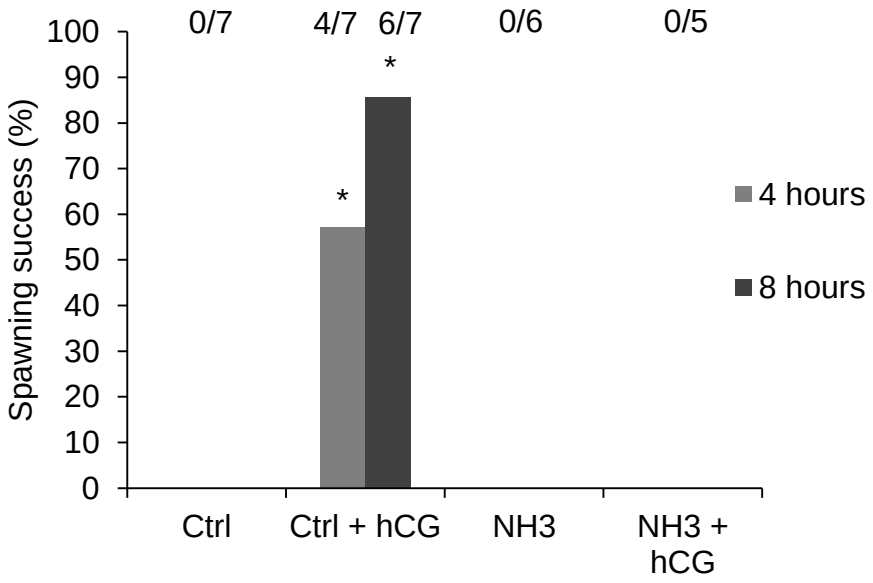


Figure 18. Proportions of mixed pairs that spawned at 4 and 8 hours after an injection of hCG (50 IU) or phosphate buffered saline following a four day exposure to ammonia (4.5 mg/L TAN) or well water (control; Ctrl). Data were generated from experiment 5 and represent the percentage of mixed pairs that spawned in five to seven pairs per treatment. Fractions above bars indicate the exact numerical data expressed as successful events over the number of tanks. * indicates a significant increase in spawning success compared to the control groups ($p<0.05$; Fisher's exact test).

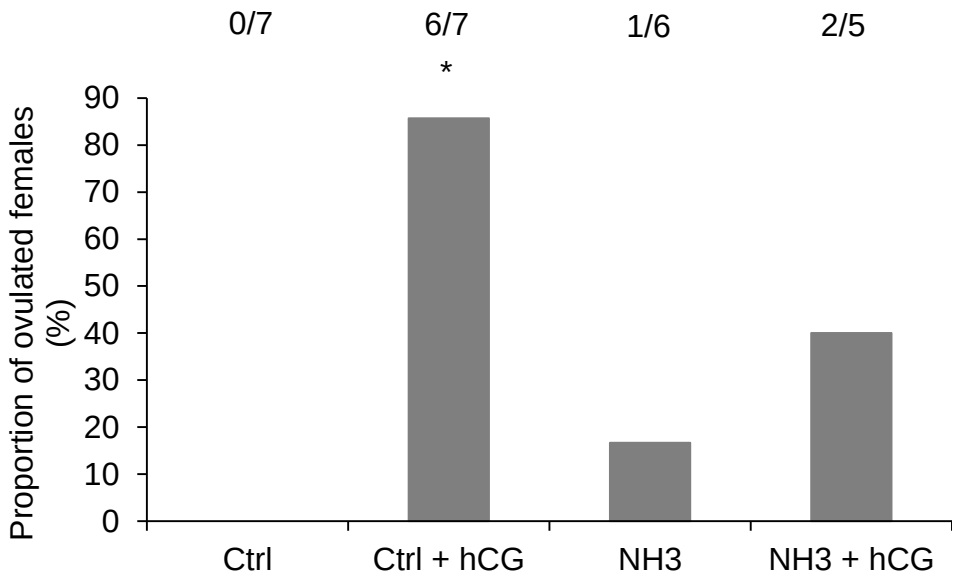


Figure 19. Proportions of females that ovulated 8 hours after an injection of hCG (50 IU) or PBS following a 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control; Ctrl). Data were generated from experiment 5 and represent the proportion of females that ovulated in five to seven tanks per treatment. Fractions above bars indicate the exact numerical data expressed as successful events over the number of tanks. * indicates a significant increase in ovulation compared to the control groups($p<0.05$; Fisher's test).

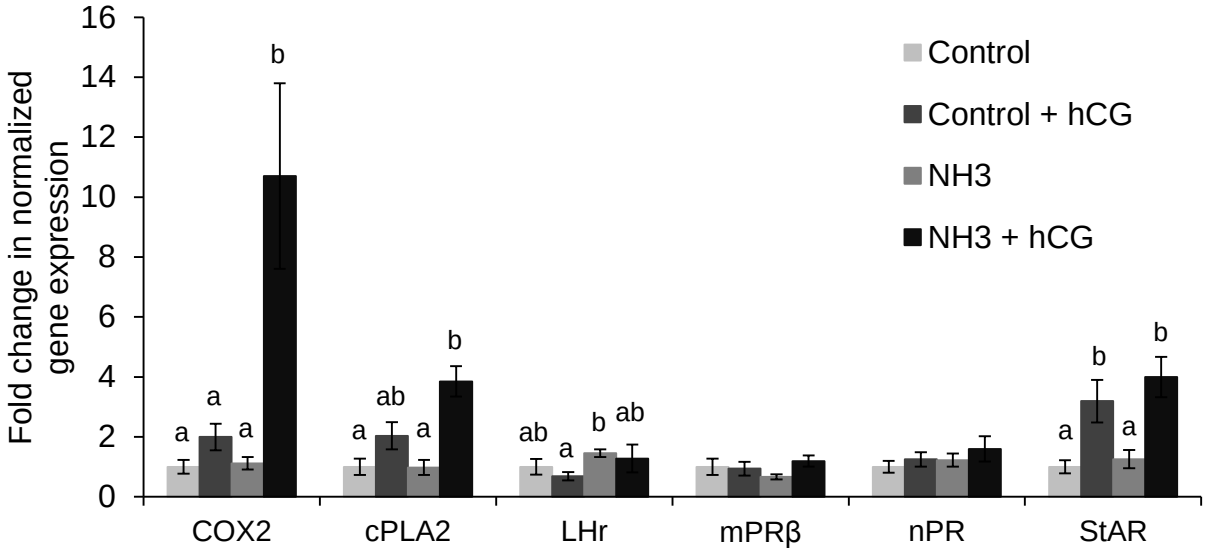


Figure 20. Ovarian expression of cyclooxegenase-2 (COX2), cytostolic phospholipase A2 (cPLA2), luteinizing hormone receptor (LHr), membrane progesterone receptor-β (mPRβ), nuclear progesterone receptor (nPR), aromatase (Arom) and steroidogenic acute regulatory protein (StAR) in fish after a 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control) and 8 hours after an injection of hCG (50 IU) or phosphate buffered saline. Data were generated from experiment 5 and represent mean ± S.E.M of seven tanks per treatment. Expression data were normalized to elongation factor-1α and acidic ribosomal protein and expressed as a fold change relative to gene expression of the controls. Different letters indicate a significant difference in gene expression among treatments ($p<0.05$; ANOVA, Tukey's).

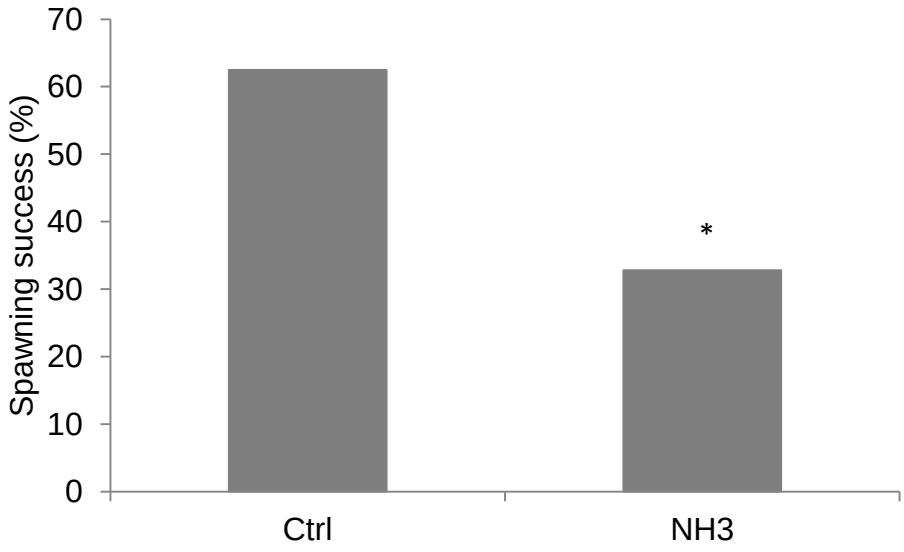


Figure 21. Proportion of mixed pairs that spawned during 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control; Ctrl) prior to injection with hCG or phosphate buffered saline. Data were generated from experiment 6 and represent the total number of spawning events during the 4 day exposure expressed as a proportion of total potential spawning events with 16 tanks per treatment. * indicates a significant reduction in spawning success between treatment groups ($p<0.05$; Fisher's exact test).

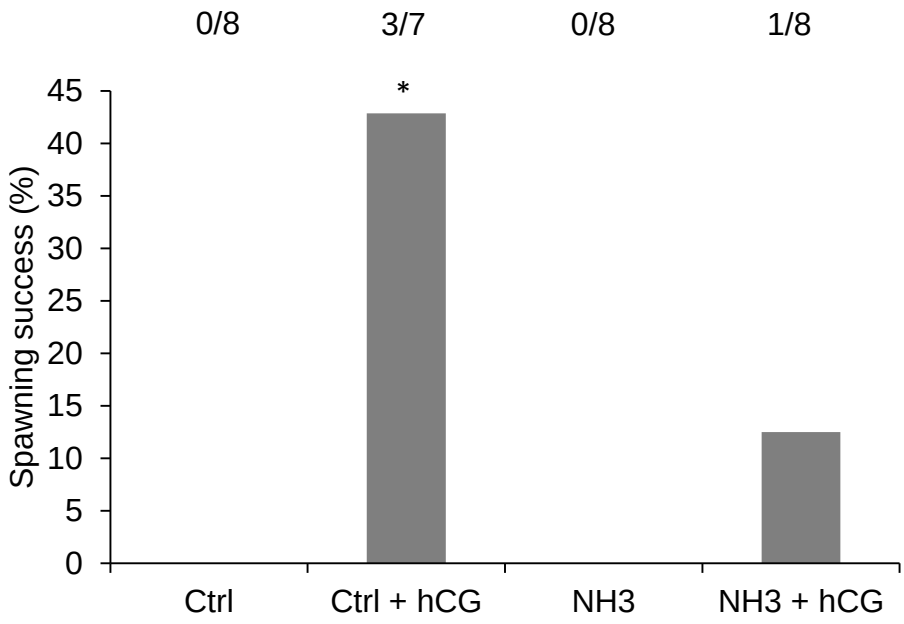


Figure 22. Proportions of mixed pairs that spawned 4 hours after an injection of hCG (50 IU) or phosphate buffered saline following a 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control; Ctrl). Data were generated from experiment 6 and represent the proportion of mixed pairs that spawned in seven to eight tanks per treatment. Fractions above bars indicate the exact numerical data expressed as successful events over the number of tanks. * indicates a significant increase in spawning success among treatment groups ($p<0.05$; Fisher's exact test).

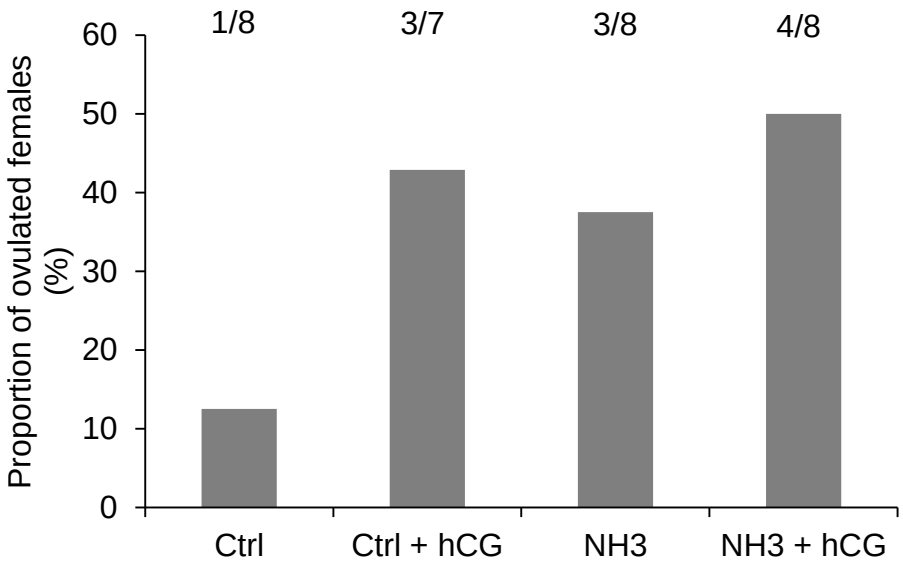


Figure 23. Proportions of females that ovulated 4 hours after an injection of hCG (50 IU) or phosphate buffered saline following a 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control; Ctrl). Data were generated from experiment 6 and represent the proportion of females that ovulated in seven to eight tanks per treatment. Fractions above bars indicate the exact numerical data expressed as successful events over the number of tanks. No significant changes in ovulation were found ($p>0.05$; Fisher's exact test).

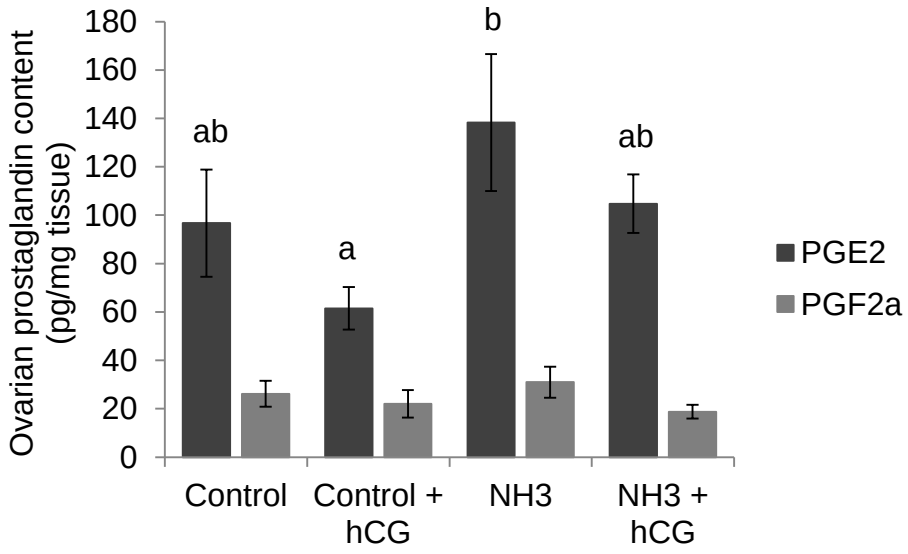


Figure 24. Average ovarian prostaglandin E_2 and $F_{2\alpha}$ content 4 hours after an injection of hCG (50 IU) or phosphate buffered saline following a 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control). Data were generated from experiment 6 and represent mean ± S.E.M of eight tanks per treatment. Different letters indicate a significant difference in ovarian steroid content, though no differences were found in PGF2α content ($p<0.05$; ANOVA, Tukey's).

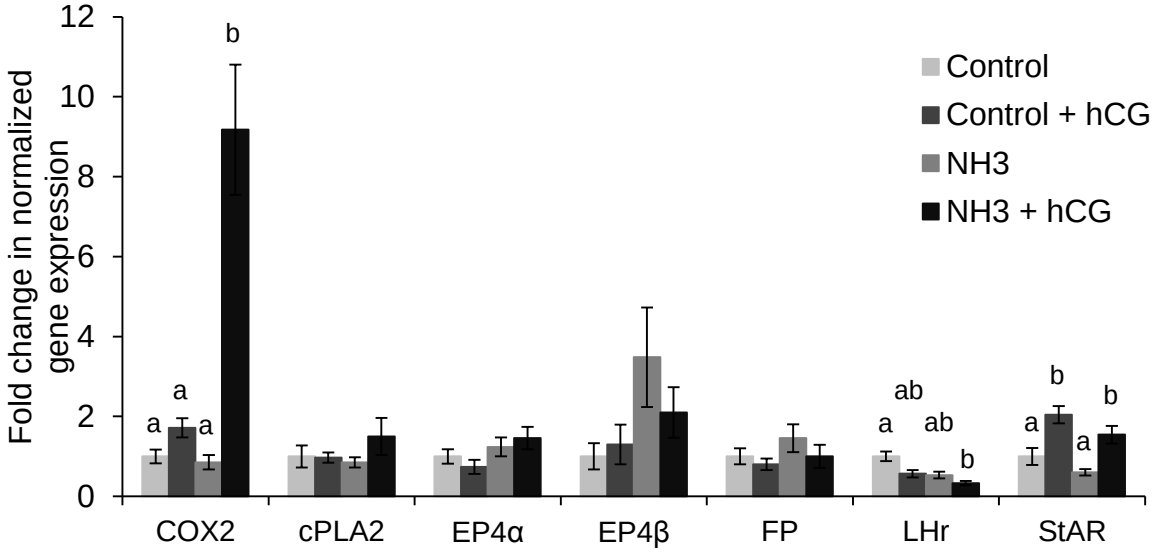


Figure 25. Ovarian expression of cyclooxygenase-2 (COX2), cytostolic phospholipase A2 (cPLA2), prostaglandin E receptor 4-α (EP4α), prostaglandin E receptor 4-β (EP4β), luteinizing hormone receptor (LHr), and steroidogenic acute regulatory protein (StAR) in fish 4 hours after an injection of hCG (50 IU) or phosphate buffered saline following a 4 day exposure to ammonia (4.5 mg/L TAN) or well water (control). Data were generated from experiment 6 and represent mean ± S.E.M of eight tanks per treatment. Expression data was normalized to elongation factor-1α and acidic ribosomal protein and expressed as a fold change relative to gene expression of the controls. Different letters indicate a significant difference in gene expression among treatments ($p<0.05$; ANOVA, Tukey's).

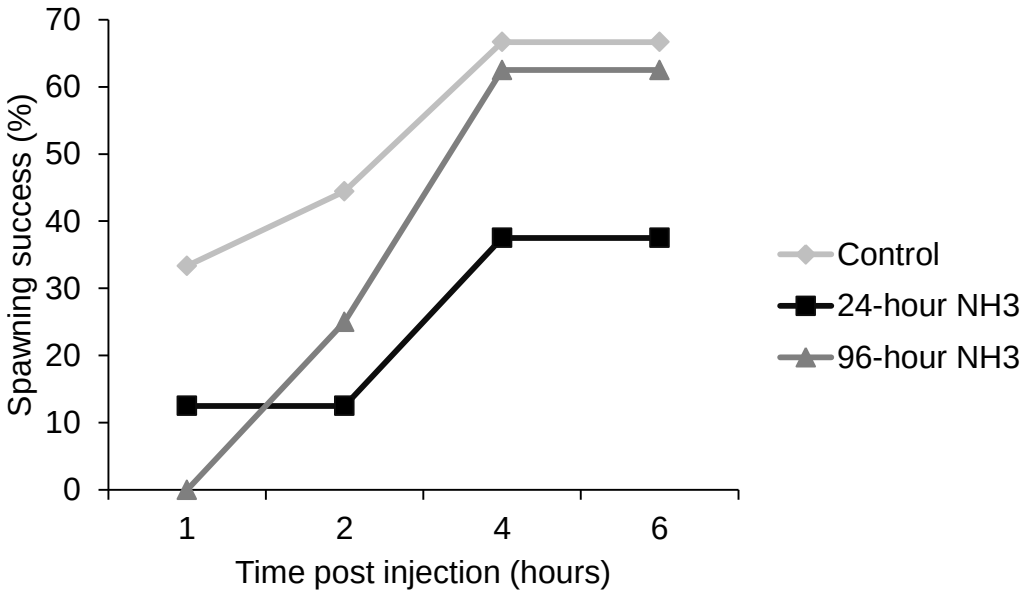


Figure 26. Proportion of females that spawned following an injection of hCG (50 IU) and the introduction of a male that had been exposed to well water for four days, or ammonia for 24 or 96 hours. Data were generated from experiment 7 and represent the proportion of females that spawned in eight to nine tanks per treatment. No significant changes in spawning success were found ($p>0.05$; Fisher's exact test).

DISCUSSION

EE_2

In both fecundity experiments performed for this study, exposure to EE_2 alone caused a rapid inhibition of egg production, resulting in the complete cessation of egg production after 3 – 4 days and a significant reduction in average daily egg production. Similar reductions in fecundity have been found in several other EE_2 exposure studies using zebrafish (Nash et al., 2004; Schäfers et al., 2007; Cosme et al., 2015), as well as studies with fathead minnow (Parrott and Blunt, 2005; Brian et al., 2012) and medaka (Scholz and Gutzeit, 2000; Balch et al., 2004). Since the effects of exposure to EE_2 are well documented, I will focus on developing an understanding of the mechanisms by which EE_2 acts on reproduction.

My research into the mechanisms of reproductive inhibition associated with EE_2 exposure show reductions in ovarian sex steroid levels and GSI, which agree with previous work on the effects of EE_2 (Hogan et al., 2010; Aris et al., 2014; Cosme et al., 2015). In my studies, the reductions in ovarian T and E_2 levels were correlated with reductions in the mRNA expression of the steroidogenic enzyme Arom, and indicate that alterations to steroidogenesis in zebrafish likely play a role in the observed reduction of fecundity. Analysis of the mRNA expression of genes involved in maturation and ovulation, namely nPR, cPLA2 and LHr showed no effects of exposure to EE_2 in my work; however, Cosme et al. (2015) found a downregulation of these genes correlated with an absence of mature follicles in exposed females. The experiments by Cosme et al. (2015) differed in sampling time: by sampling between 00:00 and 06:00, the gene expression profile differs from that at 09:00, given that the hormonal cascade that prepares oocytes for spawning at dawn causes dynamic

changes in the genes analyzed. Therefore, while these findings regarding maturation and ovulation do not indicate an effect of EE_2 after spawning has occurred, the complete study shows that EE_2 alone impacts reproduction through both steroidogenesis and follicular development.

Nitrogenous Wastes

Nitrification of MWWE in the Grand River is a focal point for the upgrades to the WWTPs, and will result in the conversion of ammonia to nitrate. Ammonia has been shown to be the more toxic of the two nitrogenous wastes; however, there is a paucity of information regarding the potential physiological effects of nitrate and ammonia exposure. My work provided evidence that nitrate has little effect on reproductive physiology of zebrafish. Specifically, exposure to 30 mg/L NO_3-N had no significant effects on egg production after seven days. Investigation into sex steroids was prompted by a review by Guillette and Edwards (2005) which proposed that nitrate exposure could alter steroidogenesis, an idea substantiated by Hamlin et al. (2008) when they showed elevated T, E_2 and 11-ketotestosterone in Siberian sturgeon exposed to 57 mg/L NO_3-N for 30 days. Experiment 2 attempted to verify these findings in zebrafish at an environmentally-relevant concentration (30 mg/L NO_3-N); however, after seven days of exposure there were no significant changes in ovarian levels of the sex steroids E_2 and T. These findings suggest that nitrate at environmentally-relevant concentrations does not interfere with the reproductive physiology of zebrafish.

The level of ammonia used in my experiments was 0.3 mg/L unionized ammonia (UIA) (4.5 mg/L TAN, pH 8.05, 27°C), which is above the federal guidelines in UIA content but is not high in terms of expected total ammonia levels in the Grand River

(Anderson 2012). My experiments with ammonia showed a rapid and significant reduction in egg production, through reductions in both the number of spawning events and the number of eggs produced per spawn. Other work by Armstrong et al. (2012) in which fathead minnow were exposed to several concentrations of ammonia found reductions in cumulative egg production at 0.17 mg/L UIA (15 mg/L TAN, pH 7.3, 25°C) and 0.34 mg/L (30 mg/L TAN, pH 7.3, 25°C) UIA, while a study by Spencer et al. (2008) that exposed slimy sculpin to several levels of UIA found an increase in GSI in both males and females, and an increase in T in female gonads when exposed to 1.7 mg/L UIA. These studies show that ammonia is capable of affecting at least three reproductive processes, gonadal development, steroidogenesis, and egg production, at concentrations that are not lethal to the exposed organisms.

The experimental ammonia levels in these experiments also highlight an interesting aspect of ammonia toxicity; that is, the sensitivity of ammonia speciation to pH. At 4.5 mg/L TAN and a pH of 8.05, my experiments had a comparable level of UIA to the levels used in the studies by Armstrong et al. (2012), even though they used 3 – 6 times the amount of total ammonia. This highlights a critical factor for ammonia toxicity in MWWE; while the removal of ammonia is crucial, even relatively low ammonia levels can still be dangerous if the pH of the effluent or receiving environment remains high. So, while the conversion of ammonia to nitrate in Grand River WWTPs will certainly reduce the risk of ammonia exposure, there is still potential for effects of ammonia on aquatic wildlife.

My studies and the work highlighted above provide evidence that ammonia is a significant reproductive toxicant in zebrafish, whereas nitrate has little effect. In terms of

nitrogen content, the nitrate group contains over six times the amount of elemental nitrogen in the form of nitrate compared to the ammonia group, meaning that at equivalent levels of nitrogen content nitrate is far less toxic than ammonia. These findings agree with previous studies on nitrogenous wastes that report ammonia to be a far more significant toxicant than nitrate (Handy and Poxton, 1993). Given these findings, the decision in the Grand River watershed to nitrify MWWE to remove ammonia in favour of nitrate is well founded given the relative toxicities of the two nitrogenous wastes.

Ammonia's mechanism of reproductive inhibition

Ammonia was shown to inhibit egg production in my initial fecundity studies. However, the mechanism by which this inhibition occurs is not yet understood. Analysis of molecular endpoints involved in steroidogenesis and follicular development, which were affected by EE_2, were not affected by ammonia (Table 2), suggesting that EE_2 and ammonia inhibit reproduction through different AOPs. Experiments 5 and 6 were designed to investigate whether ammonia exposure affected the responsiveness of the zebrafish to hCG. hCG is a LHr agonist that has long been used in aquaculture as a means of inducing final maturation and spawning for a variety of fish species (reviewed in Zohar and Mylonas, 2001). Both experiments indicated that pre-exposure to ammonia blunted the inductive effects of hCG by blocking spawning. Although they did not spawn, some of the NH_3 + hCG fish did ovulate. The only molecular endpoint from these experiments that showed a differential response to ammonia + hCG was COX-2; there were no differences in COX-2 expression between the control, control + hCG or

Table 2. Endpoints from experiments 3 and 4.

	NH3	EE2	EE2 + NH3
Spawning			
# of eggs produced/female/day	↓	↓	↓
Steroidogenesis			
E2 levels	No change	↓	↓
T levels	No change	↓	↓
GSI	No change	↓	No Change/↓
StAR expression	No change	No change	No change
Arom expression	No change	↓	↓
Maturation			
mPR expression	No change	No change	No change
LHr expression	No change	No change	↓
Ovulation			
cPLA2 expression	No change	No change	No change
nPR expression	No change	No change	↓
Gonadotropins			
FSH expression	No change	No change	No change
LH expression	No change	No change	No change

ammonia groups, while the ammonia + hCG group experienced a significant increase in COX-2 expression. COX-2 is the final enzyme in the synthesis of the prostaglandins, which are important regulators of ovulation and steroidogenesis. Therefore an increase in COX-2 expression could correlate with an increase in COX-2 activity in the ovaries, leading to elevated prostaglandin levels. Alternatively, an elevation in COX-2 expression could be a compensatory mechanism; if the ammonia + hCG group experienced a reduction in prostaglandin levels, the appropriate response may be to upregulate COX-2 expression as a means of promoting prostaglandin synthesis. However, measurement of ovarian $PGF_{2\alpha}$ and PGE_2 indicated no significant changes in these hormones compared to the control. So while ammonia may be acting on reproduction via actions on prostaglandins, the levels of prostaglandins appear to be unaffected.

With no effects on ovarian prostaglandin content, it is still unknown how ammonia affects reproduction in zebrafish. My studies have shown that ammonia partially inhibits both ovulation and spawning, while previous unpublished work by Alkema and Van Der Kraak showed that an *in vivo* exposure to 5 mg/L TAN suppressed the maturation of follicles when incubated *in vitro* with 17,20β-P. Although this does identify that ammonia is likely acting at the level of follicular development, the processes of follicle maturation, ovulation and spawning are too complex to allow for conclusions to be drawn regarding specific mechanisms. Additionally, these findings do not preclude the ability of ammonia to act on mechanisms upstream of follicular development, or on behavioural responses. Although the specific mechanism by which ammonia inhibits reproduction remains unknown, these findings do help narrow the window of potential mechanisms of action, and contribute to the AOP by allowing for the exclusion of some pathways.

All of my studies thus far have focused on effects in female fish. However, there is potential for ammonia to be affecting spawning through effects on the males. To investigate this, in experiment 7 male fish were exposed to ammonia for 24 or 96 hours before being introduced to a clean tank containing an hCG-injected female. By one hour post injection (HPI), 33% of the control group spawned, while 12.5% of the 24-hour ammonia group and none of the 96-hour ammonia group spawned. At this time point, these data suggest that males may have an important role in the inhibition associated with ammonia exposure, as their pre-exposure to ammonia correlates with an inhibition of spawning. However by 4 HPI, 67% of the control, 37.5% of the 24-hour ammonia group and 62.5% of the 96-hour ammonia group had spawned, and the apparent effect of male exposure to ammonia was lost. One explanation for this could be that the males had a chance to clear the ammonia from their systems sometime after the 1 HPI time point, which allowed them to resume normal spawning behaviour resulting in the recurrence of spawning success levels comparable to the control. Therefore, males may have a role in the reproductive inhibition caused by ammonia exposure; however, more work needs to be done to quantify their response to ammonia.

The mechanism by which ammonia inhibits reproduction remains unclear. Data from these experiments are still helpful in pinpointing where ammonia acts in the reproductive pathway of zebrafish, which is useful in describing its AOP. Although none of the molecular endpoints measured gave positive results, they did indicate that steroidogenesis, prostaglandin levels and receptor expression, and gonadotropin expression were not affected. This will be helpful in future studies by allowing the focus of studies to be on other pathways that have not yet been investigated.

Interactive effects of MWWE constituents

Given the chemical complexity of MWWE, the responses of organisms exposed to whole MWWE will not be as simple as the responses of laboratory animals to single-chemical exposures. If there are multiple physiologically-active chemicals in this waste, it is reasonable to assume that there may be interplay between the effects of the individual chemicals, causing an interactive effect on the organism. Developing an understanding of the effects of MWWE exposure requires the study of not only the effects of individual constituents, but necessitates the study of chemical mixtures as well. To this end, my experiments tested the effects of EE_2 in combination with nitrate and ammonia. In the second nitrate experiment, female fish were exposed to nitrate, EE_2 and their combination, and then ovarian steroid content was quantified. This experiment indicated a significant reduction in the sex steroids E_2 and T in both the EE_2 and nitrate + EE_2 groups compared to the control and nitrate groups, but between the EE_2 and nitrate + EE_2 groups there was no difference. Therefore in the case of nitrate and EE_2, there is no evidence of an interactive effect on sex steroid content.

In the ammonia and EE_2 fecundity studies, exposure to EE_2 and ammonia individually and in combination caused reductions in fecundity. In the first of these studies, the reduction in egg production occurred in the same time frame and at a similar magnitude as EE_2 alone (Figure 13). After four days of exposure, the EE_2 and ammonia + EE_2 groups experienced a complete cessation of spawning. Interestingly, in the second of these studies, egg production was arrested in the ammonia + EE_2 group almost immediately (Day 1), while the EE_2 group continued producing eggs until day 3

(Figure 13). The findings of the second experiment suggest that the combination of ammonia and EE_2 may exacerbate the inhibition of egg production compared to the single-chemical exposure groups. Future studies should consider whether the observed effects of these two chemicals are additive. However, other studies by Deeming (2014) and Armstrong et al. (2012) which exposed fathead minnow to both EE_2 and ammonia individually and in combination showed no differential effects on egg production between the treatment groups. These studies show that there is potential for interactive effects of exposure to multiple toxicants on organism-level responses, although the effects may be inconsistent. Further investigation into the underlying mechanisms of these inhibitions is necessary to determine if there are interactive effects at the molecular or cellular level.

When exposed to ammonia + EE_2, most of the measured reproductive responses were statistically similar to those of EE_2, including ovarian sex steroid content and the expression of nPR, LHr and Arom. The only endpoint that differed significantly was GSI from experiment 3, but not from experiment 4, suggesting that the result could be due to individual biological variability. However, given that there was no effect of ammonia treatment on any measured molecular endpoint it is not surprising the responses in the ammonia + EE_2 treatment group matched those of the EE_2 group. This shows that at the molecular level in the endpoints measured, there does not appear to be any interactive effect of the addition of ammonia to the EE_2 treatment. Therefore no conclusions can be drawn regarding the potential for these two chemicals to interact.

While there is potential for interactive effects in these types of exposures, it is very difficult to distinguish the roles each constituent plays in the molecular and apical

responses. Without any significant changes in the measured endpoints between the EE_2 and ammonia + EE_2 groups, it would seem that any interactive effect, if present, acts through some process other than steroidogenesis and follicle maturation. However, these findings do show the potential for mixtures to produce interactive effects, which in order to be understood must be predicated on a strong understanding of how the individual contaminants function.

FUTURE DIRECTIONS

Although my studies were unsuccessful in identifying the AOP for reduced fecundity associated with ammonia exposure, there are several avenues for further investigation. Another important consideration when investigating dynamic processes such as reproduction is the time of sampling. Leading up to spawning, there is a complex series of gene expression and hormonal changes that control folliculogenesis, oocyte maturation and ovulation (Ge, 2005; Lister and Van Der Kraak, 2009; Clelland and Peng, 2009). For example, gonadotropin expression peaks shortly after midnight, initiating the signaling cascade eventually resulting in ovulation, and then gradually decreases as the oocyte progresses through maturation (Zohar et al., 1986; So et al., 2005). With the exception of the hCG injection bioassays, the fish used in my studies were sampled between 9 – 11 am, by which time gonadotropin expression has decreased significantly (So et al., 2005). Had these fish been sampled early in this cycle, gene expression data would likely have been different from the data I generated. In the hCG bioassays, fish were sampled at noon in an effort to avoid the natural elevations in gonadotropins. A drawback to this approach is that it only provides a narrow window for the analysis of changes in the reproductive cycle. Elaborating on the time points chosen in this study by sampling at additional timepoints may provide further insight into the mechanisms under investigation.

Further investigation of the role of males in spawning behaviour during ammonia exposures may provide insight into the mechanisms of reproductive inhibition. A drawback of experiment 7 was that males had the opportunity to clear ammonia upon introduction to the tank containing the female. It may be useful to quantify the rate of

ammonia clarance under the experimental conditions. With these data, it would be possible to compare the different time points in terms of endogenous ammonia levels of the males. Another modification to the experimental protocol could be to add the males at multiple time points post injection of the females, to allow more time for hCG to act prior to the introduction of the males. Though an effect on male reproductive behaviour would not explain the effects of ammonia on follicle maturation, it could be a significant factor in the reduced probability of spawning events.

A third aspect of ammonia exposure that could be investigated is the stress response; it has been well established that exposure to ammonia causes increased plasma cortisol in a variety of fish (Spotte and Anderson, 1989; Knoph and Olsen, 1994; Carballo et al., 1995). And although the physiological response to stressors can vary considerably between species, the generalized response to stressors is the reallocation of energy for the purpose for coping (Schreck et al., 2001). This reallocation could draw necessary resources away from follicular development during ammonia exposure, leading to the reduced capacity for egg production. Quantification of whole-body cortisol levels in these studies could prove useful in identifying the AOP for ammonia; however, in the injection bioassays, which is a relatively stressful assay given the necessity to remove fish from water followed by injection, it would be difficult to draw conclusions regarding the response to ammonia. Still, an investigation into the stress response of zebrafish the less-stressful flow-through exposures may be useful.

CONCLUSIONS

This thesis aimed to investigate the reproductive effects of exposure to nitrate, ammonia and EE2, and to determine the mechanisms of their reproductive effects through use of an AOP. Examination of the effects of nitrate and ammonia on egg production and ovarian sex steroid levels provided evidence that ammonia is a far more significant toxicant than nitrate at the concentrations used in this study. These experiments confirmed previous findings regarding the AOP of EE_2, in which reductions in egg production were associated with changes in steroidogenesis and the expression of genes critical to follicle development. Though the AOP of ammonia could not be fully defined, the likely sites of action have been narrowed, and seem to be located at or downstream of follicle maturation.

REFERENCES

Adelman, I. R., Kusilek, L. I., Koehle, J., & Hess, J. (2009). Acute and chronic toxicity of ammonia, nitrite, and nitrate to the endangered Topeka shiner (*Notropis topeka*) and fathead minnows (*Pimephales promelas*). *Environmental Toxicology and Chemistry / SETAC*, *28*(10), 2216–2223. http://doi.org/10.1897/08-619.1

Al-Ansari, A. M., Atkinson, S. K., Doyle, J. R., Trudeau, V. L., & Blais, J. M. (2013). Dynamics of uptake and elimination of 17α-ethinylestradiol in male goldfish (*Carassius auratus*). *Aquatic Toxicology (Amsterdam, Netherlands)*, *132-133*, 134–40. http://doi.org/10.1016/j.aquatox.2013.02.006

Al-Ansari, A. M., Saleem, A., Kimpe, L. E., Sherry, J. P., McMaster, M. E., Trudeau, V. L., & Blais, J. M. (2010). Bioaccumulation of the pharmaceutical 17α-ethinylestradiol in shorthead redhorse suckers (*Moxostoma macrolepidotum*) from the St. Clair River, Canada. *Environmental Pollution (Barking, Essex : 1987)*, *158*(8), 2566–71. http://doi.org/10.1016/j.envpol.2010.05.020

Alonso, A, & Camargo, J. A. (2003). Short-term toxicity of ammonia, nitrite, and nitrate to the aquatic snail *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca). *Bulletin of Environmental Contamination and Toxicology*, *70*(5), 1006–1012. http://doi.org/10.1007/s00128-003-0082-5

Anderson, M. (2012). Assessment of future water quality conditions in the grand and speed rivers. *Water Management Plant Assimilative Capcity Working Group Report, Grand River Conservation Authority, Cambridge, ON*.

Ankley, G. T., Bencic, D. C., Breen, M. S., Collette, T. W., Conolly, R. B., Denslow, N. D., Edwards, S. W., Ekman, D. R., Garcia-Reyero, N., Jensen, K. M., Lazochak, J. M., Martinović, D., Miller, D. H., Perkins, E. J., Orlando, E. F., Villeneuve, D. L., Wang, R-L. & Watanabe, K. H. (2009). Endocrine disrupting chemicals in fish: developing exposure indicators and predictive models of effects based on mechanism of action. *Aquatic Toxicology (Amsterdam, Netherlands)*, *92*(3), 168–78. http://doi.org/10.1016/j.aquatox.2009.01.013

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., Mount, D. R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrrano, J. A., Tietge, J. E. & Villeneuve, D. L. (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry*, *29*(3), 730–741. http://doi.org/10.1002/etc.34

Aris, A. Z., Shamsuddin, A. S., & Praveena, S. M. (2014). Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: A review. *Environment International*, *69*, 104–119. http://doi.org/10.1016/j.envint.2014.04.011

Armstrong, B. M., Lazorchak, J. M., Murphy, C. a, Haring, H. J., Jensen, K. M., & Smith, M. E. (2012). Determining the effects of ammonia on fathead minnow (*Pimephales promelas*) reproduction. *The Science of the Total Environment*, *420*, 127–33. http://doi.org/10.1016/j.scitotenv.2012.01.005

Atkinson, S. K., Marlatt, V. L., Kimpe, L. E., Lean, D. R. S., Trudeau, V. L., & Blais, J. M. (2012). The occurrence of steroidal estrogens in south-eastern Ontario wastewater treatment plants. *Science of the Total Environment*, *430*, 119–125. http://doi.org/10.1016/j.scitotenv.2012.04.069

Balch, G. C., Mackenzie, C. A, & Metcalfe, C. D. (2004). Alterations to gonadal development and reproductive success in Japanese medaka (*Oryzias latipes*) exposed to 17alpha-ethinylestradiol. *Environmental Toxicology and Chemistry / SETAC*, *23*(3), 782–791.

Bernier, N. J., Alderman, S. L., & Bristow, E. N. (2008). Heads or tails? Stressor-specific expression of corticotropin-releasing factor and urotensin I in the preoptic area and caudal neurosecretory system of rainbow trout. *Journal of Endocrinology*, *196*(3), 637–648. http://doi.org/10.1677/JOE-07-0568

Blewett, T., MacLatchy, D. L., & Wood, C. M. (2013). The effects of temperature and salinity on 17-α-ethynylestradiol uptake and its relationship to oxygen consumption in the model euryhaline teleost (*Fundulus heteroclitus*). *Aquatic Toxicology*, *127*, 61–71. http://doi.org/10.1016/j.aquatox.2012.04.009

Braun, M. H., Steele, S. L., & Perry, S. F. (2009). The responses of zebrafish (Danio rerio) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. *The Journal of Experimental Biology*, *212*(23), 3846–3856. http://doi.org/10.1242/jeb.034157

Brian, J. V., Harris, C. A., Scholze, M., Kortenkamp, A., Booy, P., Lamoree, M., … Sumpter, J. P. (2007). Evidence of estrogenic mixture effects on the reproductive performance of fish. *Environmental Science and Technology*, *41*(1), 337–344. http://doi.org/10.1021/es0617439

Camargo, J. A., Alonso, A., & Salamanca, A. (2005). Nitrate toxicity to aquatic animals: A review with new data for freshwater invertebrates. *Chemosphere*, *58*(9), 1255–1267. http://doi.org/10.1016/j.chemosphere.2004.10.044

Canadian Council of Ministers of the Environment.(2010). Canadian water quality guidelines for the protection of aquatic life: Ammonia. In: Canadian environmental quality guidelines, 1999, Canadian Council of Ministers of the Environment, Winnipeg.

Carballo, M., Munoz, M. J., Cuellar, M., & Tarazona, J. V. (1995). Effects of waterborne copper, cyanide, ammonia, and nitrite on stress parameters and changes in

susceptibility to saprolegniosis in rainbow trout (*Oncorhynchus mykiss*). *Applied and Environmental Microbiology*, *61*(6), 2108–2112.

Carnevali, O., Tosti, L., Speciale, C., Peng, C., Zhu, Y., & Maradonna, F. (2010). DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. *PLoS ONE*, *5*(4), 1–7. http://doi.org/10.1371/journal.pone.0010201

Clelland, E., & Peng, C. (2009). Endocrine/paracrine control of zebrafish ovarian development. *Molecular and Cellular Endocrinology*, *312*(1-2), 42–52. http://doi.org/10.1016/j.mce.2009.04.009

Combalbert, S., & Hernandez-Raquet, G. (2010). Occurrence, fate, and biodegradation of estrogens in sewage and manure. *Applied Microbiology and Biotechnology*, *86*(6), 1671–1692. http://doi.org/10.1007/s00253-010-2547-x

Cosme, M. M., Lister, A. L., & Van Der Kraak, G. (2015). Inhibition of spawning in zebrafish (*Danio rerio*): Adverse outcome pathways of quinacrine and ethinylestradiol. *General and Comparative Endocrinology*. http://doi.org/10.1016/j.ygcen.2015.01.013

Dabrowska, H., & Własow, T. (1986). Sublethal effect of ammonia on certain biochemical and haematological indicators in common carp (*Cyprinus carpio* L.). *Comparative Biochemistry and Physiology. C, Comparative Pharmacology and Toxicology*, *83*(1), 179–184. http://doi.org/10.1016/0742-8413(86)90033-2

Deeming, S. (2014). The combined effects of 17α-ethynylestradiol and ammonia on the reproductive status of fathead minnow (*Pimephales promelas*) (Undergraduate thesis). Wilfrid Laurier University, Waterloo, Ontario.

De Vries, W., Kros, J., Kroeze, C., & Seitzinger, S. P. (2013). Assessing planetary and regional nitrogen boundaries related to food security and adverse environmental impacts. *Current Opinion in Environmental Sustainability*, *5*(3-4), 392–402. http://doi.org/10.1016/j.cosust.2013.07.004

Dhanasiri, A. K. S., Fernandes, J. M. O., & Kiron, V. (2012). Glutamine synthetase activity and the expression of three glul paralogues in zebrafish during transport. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *163*(3-4), 274–284. http://doi.org/10.1016/j.cbpb.2012.06.003

Edmunds, R. C., McIntyre, J. K., Adam Luckenbach, J., Baldwin, D. H., & Incardona, J. P. (2014). Toward enhanced MIQE compliance: Reference residual normalization of qPCR gene expression data. *Journal of Biomolecular Techniques*, *25*(2), 54–60. http://doi.org/10.7171/jbt.14-2502-003

Felipo, V., & Butterworth, R. F. (2002). Neurobiology of ammonia. *Progress in Neurobiology*, *67*(4), 259–279. http://doi.org/10.1016/S0301-0082(02)00019-9

Filby, A. L., Thorpe, K. L., Maack, G., & Tyler, C. R. (2007). Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. *Aquatic Toxicology*, *81*(2), 219–231. http://doi.org/10.1016/j.aquatox.2006.12.003

Flores-Valverde, A. M., Horwood, J., & Hill, E. M. (2010). Disruption of the steroid metabolome in fish caused by exposure to the environmental estrogen 17α-ethinylestradiol. *Environmental Science and Technology*, *44*(9), 3552–3558. http://doi.org/10.1021/es9039049

Fowler, D., Pyle, J. A., Raven, J. A., Sutton, M. A., Cape, J. N., Reis, S, Sheppard, L. J., Jenkins A., Grizzetti, B., Galloway, J. N., Vitousek, P., Leach, A., Bouwman, A. F., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M., Voss M. (2013). The global nitrogen cycle in the twenty-first century. *Philosophical transactions of the Royal Society B*, 368, 2013–2015.

Fujimori, C., Ogiwara, K., Hagiwara, A., & Takahashi, T. (2012). New evidence for the involvement of prostaglandin receptor EP4b in ovulation of the medaka, *Oryzias latipes*. *Molecular and Cellular Endocrinology*, *362*(1-2), 76–84. http://doi.org/10.1016/j.mce.2012.05.013

Garcia-Reyero, N., Lavelle, C. M., Escalon, B. L., Martinović, D., Kroll, K. J., Sorensen, P. W., & Denslow, N. D. (2011). Behavioral and genomic impacts of a wastewater effluent on the fathead minnow. *Aquatic Toxicology*, *101*(1), 38–48. http://doi.org/10.1016/j.aquatox.2010.08.014

Ge, W. (2005). Intrafollicular paracrine communication in the zebrafish ovary: The state of the art of an emerging model for the study of vertebrate folliculogenesis. *Molecular and Cellular Endocrinology*, *237*(1-2), 1–10. http://doi.org/10.1016/j.mce.2005.03.012

Gillio Meina, E., Lister, A., Bosker, T., Servos, M., Munkittrick, K., & MacLatchy, D. (2013). Effects of 17α-ethinylestradiol (EE2) on reproductive endocrine status in mummichog (*Fundulus heteroclitus*) under differing salinity and temperature conditions. *Aquatic Toxicology*, *134-135*, 92–103. http://doi.org/10.1016/j.aquatox.2013.03.014

Goetz, F. W., & Theofan, G. (1979). In Vitro Stimulation of Germinal Vesicle Yellow Perch (*Perca flavescens*) Breakdown and Ovulation Oocytes . Effects of and Prostaglandins. *General and Comparative Endocrinology*, *285*, 273–285.

Grist, E. P. M., Wells, N. C., Whitehouse, P., Brighty, G., & Crane, M. (2003). Estimating the effects of 17alpha-ethinylestradiol on populations of the fathead minnow *Pimephales promelas*: are conventional toxicological endpoints adequate? *Environmental Science & Technology*, *37*(8), 1609–1616. http://doi.org/10.1021/es020086r

Guillette, L. J., & Edwards, T. M. (2005). Is nitrate an ecologically relevant endocrine disruptor in vertebrates? *Integrative and Comparative Biology*, *45*(1), 19–27. http://doi.org/10.1093/icb/45.1.19

Hamlin, H. J., Moore, B. C., Edwards, T. M., Larkin, I. L. V., Boggs, A., High, W. J., … Guillette, L. J. (2008). Nitrate-induced elevations in circulating sex steroid concentrations in female Siberian sturgeon (*Acipenser baeri*) in commercial aquaculture. *Aquaculture*, *281*(1-4), 118–125. http://doi.org/10.1016/j.aquaculture.2008.05.030

Handy, R. D., & Poxton, M. G. (1993). Nitrogen pollution in mariculture: toxicity and excretion of nitrogenous compounds by marine fish. *Reviews in Fish Biology and Fisheries*, *3*(3), 205–241. http://doi.org/10.1007/BF00043929

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters*, *131*, 5–17. http://doi.org/10.1016/S0378-4274(02)00041-3

Hermenegildo, C., Monfort, P., & Felipo, V. (2000). Activation of N-methyl-D-aspartate receptors in rat brain in vivo following acute ammonia intoxication: characterization by in vivo brain microdialysis. *Hepatology 31*(3), 709–715. http://doi.org/10.1002/hep.510310322

Hill, A. J., Teraoka, H., Heideman, W., & Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences*, *86*(1), 6–19. http://doi.org/10.1093/toxsci/kfi110

Hodson, R. G., & Sullivan, C. V. (1993). Induced maturation and spawning of domestic and wild striped bass, *Morone saxatilis* (Walbaum), broodstock with implanted GnRH analogue and injected hCG. *Aquaculture and Fisheries Management*, *24*, 389–398. http://doi.org/10.1111/j.1365-2109.1993.tb00562.x

Hogan, N. S., Currie, S., LeBlanc, S., Hewitt, L. M., & MacLatchy, D. L. (2010). Modulation of steroidogenesis and estrogen signalling in the estuarine killifish (*Fundulus heteroclitus)* exposed to ethinylestradiol. *Aquatic Toxicology 98*(2), 148–56. http://doi.org/10.1016/j.aquatox.2010.02.002

Ip, Y. K., Chew, S. F., & Randall, D. J. (2001). Ammonia toxicity, tolerance, and excretion. *Fish Physiology*, *20*(C), 109–148. http://doi.org/10.1016/S1546-5098(01)20005-3

Ip, Y. K., & Chew, S. F. (2010). Ammonia production, excretion, toxicity, and defense in fish: A review. *Frontiers in Physiology*, *1*(134), 1–20. http://doi.org/10.3389/fphys.2010.00134

Jukosky, J. A., Watzin, M. C., & Leiter, J. C. (2008). The effects of environmentally relevant mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction. *Aquatic Toxicology*, *86*(2), 323–331. http://doi.org/10.1016/j.aquatox.2007.11.012

Kavanagh, R. J., Frank, R. a, Oakes, K. D., Servos, M. R., Young, R. F., Fedorak, P. M., MacKinnon M. D., Soloman, K. R., Dixon, D. G., Van Der Kraak, G. (2011). Fathead minnow (*Pimephales promelas*) reproduction is impaired in aged oil sands process-affected waters. *Aquatic Toxicology (Amsterdam, Netherlands)*, *101*(1), 214–20. http://doi.org/10.1016/j.aquatox.2010.09.021

Kidd, K. A, Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., & Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(21), 8897–901. http://doi.org/10.1073/pnas.0609568104

Knapen, D., Vergauwen, L., Villeneuve, D. L., & Ankley, G. T. (2015). The potential of AOP networks for reproductive and early developmental toxicity assay development. *Reproductive Toxicology*. http://doi.org/10.1016/j.reprotox.2015.04.003

Knight, O. M., & Van Der Kraak, G. (2015). The role of eicosanoids in 17α, 20β-hihydroxy-4-pregnen-3-one-induced ovulation and spawning in *Danio rerio*. *General and Comparative Endocrinology, 213*, 50-58. http://doi.org/10.1016/j.ygcen.2014.12.014

Knoph, M. B., & Olsen, Y. A. (1994). Subacute toxicity of ammonia to Atlantic salmon (*Salmo salar* L.) in seawater: effects on water and salt balance, plasma cortisol and plams ammonia levels, *Aquatic Toxicology, 30*, 295–310.

Laale, H. W. (1977). The biology and use of zebrafish, *Bracbydanio rerio*, in fisheries research. A literature review. *Journal of Fish Biology*, *10*, 121–173.

Learmonth, C., Carvalho, A. P. (2015). Acute and chronic toxicity of nitrate to early life stages of zebrafish – setting nitrate safety levels for zebrafish rearing. *Zebrafish,12(4)*, 305-311. http://doi:10.1089/zeb.2015.1098

Levavi-Sivan, B., Bogerd, J., Mañanós, E. L., Gómez, A., & Lareyre, J. J. (2010). Perspectives on fish gonadotropins and their receptors. *General and Comparative Endocrinology*, *165*(3), 412–437. http://doi.org/10.1016/j.ygcen.2009.07.019

Levi, G., Morisi, G., Coletti, A., & Catanzaro, R. (1974). Free amino acids in fish brain: normal levels and changes upon exposure to high ammonia concentrations in vivo, and upon incubation of brain slices. *Comparative Biochemistry and Physiology*, *49A*, 623–636.

Li, L., Lollar, B. S., Li, H., Wortmann, U. G., & Lacrampe-Couloume, G. (2012). Ammonium stability and nitrogen isotope fractionations for NH4+-NH 3(aq)-NH 3(gas) systems at 20-70°C and pH of 2-13: Applications to habitability and nitrogen cycling in low-temperature hydrothermal systems. *Geochimica et Cosmochimica Acta*, *84*, 280–296. http://doi.org/10.1016/j.gca.2012.01.040

Lim, C. B., Chew, S. F., Anderson, P. M., & Ip, Y. K. (2001). Reduction in the rates of protein and amino acid catabolism to slow down the accumulation of endogenous ammonia: a strategy potentially adopted by mudskippers (*Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*) during aerial exposure in constant darkness. *The Journal of Experimental Biology*, *204*, 1605–1614.

Lister, A. L., & Van Der Kraak, G. (2008). An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. *General and Comparative Endocrinology*, *159*(1), 46–57. http://doi.org/10.1016/j.ygcen.2008.07.017

Lister, A. L., & Van Der Kraak, G. J. (2009). Regulation of prostaglandin synthesis in ovaries of sexually-mature zebrafish (*Danio rerio*). *Molecular Reproduction and Development*, *76*(11), 1064–75. http://doi.org/10.1002/mrd.21072

Lister, A., Regan, C., Van Zwol, J., & Van Der Kraak, G. (2009). Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: a mechanistic evaluation. *Aquatic Toxicology*, *95*(4), 320–9. http://doi.org/10.1016/j.aquatox.2009.04.011

Lust, M., Makinia, J., & Stensel, H. D. (2012). A mechanistic model for fate and removal of estrogens in biological nutrient removal activated sludge systems. *Water Science and Technology*, *65*(6), 1130–1136. http://doi.org/10.2166/wst.2012.958

Mercure, F., & Van Der Kraak, G. (1996). Mechanisms of action of free arachidonic acid on ovarian steroid production in the goldfish. *General and Comparative Endocrinology*, *102*(1), 130–140. http://doi.org/10.1006/gcen.1996.0054

Metcalfe, C. D., Miao, X.-S., Koenig, B. G., & Struger, J. (2003). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environmental Toxicology and Chemistry / SETAC*, *22*(12), 2881–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14713027

Mills, L. J., & Chichester, C. (2005). Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of the Total Environment*, *343*(1-3), 1–34. http://doi.org/10.1016/j.scitotenv.2004.12.070

Mollah, M. F. A., & Tan, E. S. P. (1983). HCG-induced spawning of the catfish, *Clarias macrocephalus* (Gunther). *Aquaculture*, *35*, 239–247. http://doi.org/10.1016/0044-8486(83)90094-7

Mommsen, T. P., & Walsh, P. J. (1992). Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia*, *48*(6), 583–593. http://doi.org/10.1007/BF01920243

Morthorst, J. E., Lister, A., Bjerregaard, P., & Der Kraak, G. Van. (2013). Ibuprofen reduces zebrafish PGE2 levels but steroid hormone levels and reproductive parameters are not affected. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *157*(2), 251–257. http://doi.org/10.1016/j.cbpc.2012.12.001

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tanaka, M. (1994). Regulation of oocyte maturation in fish In: Sherwood N.M. Hew, C.L. (Eds), *Fish physiology*. Academic Press Inc., New York, pp. 393-439

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tokomoto, T., Katsu, Y.(1995). Regulation of oocyte growth and maturation in fish. *Current Topics in Developmental Biology*, 30, 103-145.

Nagahama, Y. (1997). 17α,20β-Dihydroxy-4-pregnen-3-one, a maturation-inducing hormone in fish oocytes: Mechanisms of synthesis and action. *Steroids*, *62*(1), 190–196. http://doi.org/10.1016/S0039-128X(96)00180-8

Nagahama, Y., & Yamashita, M. (2008). Regulation of oocyte maturation in fish. *Development Growth and Differentiation*, *50*, S195–S219. http://doi.org/10.1111/j.1440-169X.2008.01019.x

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K., Hirose, S. (2007). Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *American Journal of Physiology: Regulator, Integrative and Comparative Physiology, 293,* R1743 – R1753. http://doi:10.1152/ajpregu.00248.2007

Narumiya, S., Sugimoto, Y., Ushikubi, F., & Conclusions, V. I. (1999). Prostanoid Receptors : Structures , Properties , and Functions. *Physiological Reviews*, *79*(4), 1193–1227.

Nash, J. P., Kime, D. E., Van der Ven, L. T. M., Wester, P. W., Brion, F., Maack, G., … Tyler, C. R. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental Health Perspectives*, *112*(17), 1725–1733. http://doi.org/10.1289/ehp.7209

Nelson, S. N., & Van Der Kraak, G. (2010). Characterization and regulation of the insulin-like growth factor (IGF) system in the zebrafish (*Danio rerio*) ovary. *General and Comparative Endocrinology*, *168*(1), 111–20. http://doi.org/10.1016/j.ygcen.2010.04.020

Niimi, A. J., & LaHam, Q. N. (1974). Influence of breeding time interval on egg number, mortality, and hatching of the zebra fish *Brachydanio rerio*. *Canadian Journal of Zoology*, *52*(4), 515–517.

Okumura, H., Todo, T., Adachi, S., & Yamauchi, K. (2002). Changes in hepatic vitellogenin mRNA levels during oocyte development in the Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology*, *125*(1), 9–16. http://doi.org/10.1006/gcen.2001.7716

Örn, S., Holbech, H., Madsen, T. H., Norrgren, L., & Petersen, G. I. (2003). Gonad development and vitellogenin production in zebrafish (*Danio rerio*) exposed to ethinylestradiol and methyltestosterone. *Aquatic Toxicology*, *65*(4), 397–411. http://doi.org/10.1016/S0166-445X(03)00177-2

Örn, S., Yamani, S., & Norrgren, L. (2006). Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17α- ethinylestradiol and 17β-trenbolone. *Archives of Environmental Contamination and Toxicology*, *51*(2), 237–243. http://doi.org/10.1007/s00244-005-0103-y

Parrott, J. L., & Blunt, B. R. (2005). Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Environmental Toxicology*, *20*(2), 131–141. http://doi.org/10.1002/tox.20087

Peng, K. W., Chew, S. F., Lim, C. B., Kuah, S. S. L., Kok, W. K., & Ip, Y. K. (1998). The mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* can tolerate environmental NH3 concentrations of 446 and 36 μM, respectively. *Fish Physiology and Biochemistry*, *19*(1), 59–69. http://doi.org/10.1023/A:1007745003948

Peters, R. E. M., Courtenay, S. C., Cagampan, S., Hewitt, M. L., & MacLatchy, D. L. (2007). Effects on reproductive potential and endocrine status in the mummichog (*Fundulus heteroclitus*) after exposure to 17α-ethynylestradiol in a short-term reproductive bioassay. *Aquatic Toxicology*, *85*(2), 154–166. http://doi.org/10.1016/j.aquatox.2007.08.010

Randall, D. J., & Tsui, T. K. N. (2002). Ammonia toxicity in fish. *Marine Pollution Bulletin*, *45*(1-12), 17–23. http://doi.org/10.1016/S0025-326X(02)00227-8

Reyhanian, N., Volkova, K., Hallgren, S., Bollner, T., Olsson, P. E., Olsén, H., & Hadllstrm, I. P. (2011). 17α-Ethinyl estradiol affects anxiety and shoaling behavior in adult male zebra fish (*Danio rerio*). *Aquatic Toxicology*, *105*(1-2), 41–48. http://doi.org/10.1016/j.aquatox.2011.05.009

Rowland, S. J. (1984). The hormone-inducing spawning of silver perch, *Bidyanus bidyanus* (Mitchell) (Teraponidae). *Aquaculture*, *42*, 83–86.

Schäfers, C., Teigeler, M., Wenzel, a, Maack, G., Fenske, M., & Segner, H. (2007). Concentration- and time-dependent effects of the synthetic estrogen, 17α-ethinylestradiol, on reproductive capabilities of the zebrafish, *Danio rerio*. *Journal of Toxicology and Environmental Health. Part A*, *70*(9), 768–779. http://doi.org/10.1080/15287390701236470

Sanderson, L. A., Wright, P. A., Robinson, J. W., Ballantyne, J. S., Bernier, N. J. (2010). Inhibition of glutamine synthetase during ammonia exposure in rainbow trout indicates a high reserve capacity to prevent brain ammonia toxicity. *The Journal of Experimental Biology, 213*, 2343 – 2353. http://doi:10.1242/jeb.039156

Scholz, S., & Gutzeit, H. O. (2000). 17-α-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquatic Toxicology*, *50*(4), 363–373. http://doi.org/10.1016/S0166-445X(00)00090-4

Schreck, C. B., Contreras-Sanchez, W., & Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture*, *197*(1-4), 3–24. http://doi.org/10.1016/S0044-8486(01)00580-4

Segner, H., Caroll, K., Fenske, M., Janssen, C. R., Maack, G., Pascoe, D., Schäfers, C., Vandenbergh, G. G., Watts, M., Wenzel, A. (2003). Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates : report from the European IDEA project, *54*, 302–314.

Segner, H. (2009). Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology : CBP*, *149*(2), 187–95. http://doi.org/10.1016/j.cbpc.2008.10.099

Selman, K., Petrino, T. R., & Wallace, R. A. (1994). Experimental Conditions for Oocyte Maturation in the Zebrafish , Brachydanio rerio, *550*, 538–550.

Selman, K., Wallace, R. a, & Sarka, A. (1993). Stages of Oocyte Development in the Zebrafish , *Brachydanio rerio*. *Journal of Morphology*, *224*, 203–224. http://doi.org/10.1002/jmor.1052180209

Servos, M. R., Bennie, D. T., Burnison, B. K., Jurkovic, a, McInnis, R., Neheli, T., … Ternes, T. A. (2005). Distribution of estrogens, 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. *The Science of the Total Environment*, *336*(1-3), 155–70. http://doi.org/10.1016/j.scitotenv.2004.05.025

Shankar, R. A, & Anderson, P. M. (1985). Purification and properties of glutamine synthetase from liver of *Squalus acanthias*. *Archives of Biochemistry and Biophysics*, *239*(1), 248–259. http://doi.org/10.1016/0003-9861(85)90833-1

So, W.-K., Kwok, H.-F., & Ge, W. (2005). Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits--their spatial-temporal expression patterns and receptor specificity. *Biology of Reproduction*, *72*(6), 1382–1396. http://doi.org/10.1095/biolreprod.104.038216

Spencer, P., Pollock, R., & Dubé, M. (2008). Effects of un-ionized ammonia on histological, endocrine, and whole organism endpoints in slimy sculpin (*Cottus cognatus*). *Aquatic Toxicology*, *90*(4), 300–309. http://doi.org/10.1016/j.aquatox.2008.08.017

Spotte, S., & Anderson, G. (1989). Plasma Cortiso Changes in Seawater-Adapted Mummichogs (*Fundulus heteroclitus*) Exposed to Ammonia. *Canadian Journal of Fisheries and Aquatic Sciences*, *46*, 2065–2069.

Stacey, N. E., & Pandey, S. (1975). Effects of indomethacin and prostaglandins on ovulation of goldfish. *Prostaglandins*, *9*(4), 597–607.

Steele, S. L., Chadwick, T. D., & Wright, P. A. (2001). Ammonia detoxification and localization of urea cycle enzyme activity in embryos of the rainbow trout (*Oncorhynchus mykiss*) in relation to early tolerance to high environmental ammonia levels. *The Journal of Experimental Biology*, *204*, 2145–2154.

Stumpf, M., Ternes, T. A., Haberer, K., Seel, P., & Baumann, W. (1996). Determination of pharmaceutics in sewage plants and river water. *Vom Wasser*,*86*, 291-304.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R. D., & Servos, M. (1999). Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil. *Science of the Total Environment*, *225*(1-2), 81–90. http://doi.org/10.1016/S0048-9697(98)00334-9

Tetreault, G. R., Bennett, C. J., Cheng, C., Servos, M. R., & McMaster, M. E. (2012). Reproductive and histopathological effects in wild fish inhabiting an effluent-dominated stream, Wascana Creek, SK, Canada. *Aquatic Toxicology, 110-111*, 149–61. http://doi.org/10.1016/j.aquatox.2012.01.004

Tetreault, G. R., Bennett, C. J., Shires, K., Knight, B., Servos, M. R., & McMaster, M. E. (2011). Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. *Aquatic Toxicology*, *104*(3-4), 278–90. http://doi.org/10.1016/j.aquatox.2011.05.008

Vajda, A. M., Barber, L. B., Gray, J. L., Lopez, E. M., Woodling, J. D., & Norris, D. O. (2008). Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environmental Science & Technology*, *42*(9), 3407–14. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18522126

Van den Belt, K., Verheyen, R., & Witters, H. (2001). Reproductive effects of ethynylestradiol and 4t-octylphenol on the zebrafish (*Danio rerio*). *Archives of Environmental Contamination and Toxicology*, *41*(4), 458–467. http://doi.org/10.1007/s002440010272

Van Der Kraak, G., Hewitt, M., Lister, A., McMaster, M. E., & Munkittrick, K. R. (2001). Endocrine Toxicants and Reproductive Success in Fish. *Human and Ecological Risk Assessment: An International Journal*, *7*(5), 1017–1025. http://doi.org/10.1080/20018091094826

Villeneuve, D., Volz, D. C., Embry, M. R., Ankley, G. T., Belanger, S. E., Léonard, M., … Wehmas, L. (2014). Investigating Alternatives to the fish early-life stage test: A strategy for discovering and annotating adverse outcome pathways for early fish development. *Environmental Toxicology and Chemistry*, *33*(1), 158–169. http://doi.org/10.1002/etc.2403

Wade, M. G., & Van Der Kraak, G. (1993). Arachidonic acid and prostagandin E_2 stimulate testosterone production in goldfish testis in vitro. *General and Comparative Endocrinology*, *90*, 109–118.

Walsh, P. J., Veauvy, C. M., McDonald, M. D., Pamenter, M. E., Buck, L. T., & Wilkie, M. P. (2007). Piscine insights into comparisons of anoxia tolerance, ammonia toxicity, stroke and hepatic encephalopathy. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *147*(2), 332–343. http://doi.org/10.1016/j.cbpa.2006.09.001

Wang, L.-S., Wang, L., Wang, L., Wang, G., Li, Z.-H., & Wang, J.-J. (2009). Effect of 1-butyl-3-methylimidazolium tetrafluoroborate on the wheat (*Triticum aestivum* L.) seedlings. *Environmental Toxicology*, *24*(3), 296–303. http://doi.org/10.1002/tox

Wang, Y., & Ge, W. (2004). Cloning of zebrafish ovarian P450c17 (CYP17, 17α-hydroxylase/17, 20-lyase) and characterization of its expression in gonadal and extra-gonadal tissues. *General and Comparative Endocrinology*, *135*(2), 241–249. http://doi.org/10.1016/j.ygcen.2003.09.015

Weihrauch, D., Wilkie, M. P., & Walsh, P. J. (2009). Ammonia and urea transporters in gills of fish and aquatic crustaceans. *The Journal of Experimental Biology*, *212*(Pt 11), 1716–1730. http://doi.org/10.1242/jeb.036103

Wilkie, M. P. (2002). Ammonia excretion and urea handling by fish gills: Present understanding and future research challenges. *Journal of Experimental Zoology*, *293*(3), 284–301. http://doi.org/10.1002/jez.10123

Williams, R. J., Johnson, A. C., Smith, J. J. L., & Kanda, R. (2003). Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. *Environmental Science and Technology*, *37*(9), 1744–1750. http://doi.org/10.1021/es0202107

Wilson, R. W., & Taylor, E. W. (1992). Transbranchial ammonia gradients and acid-base responses to high external ammonia concentration in rainbow trout (*Oncorhynchus mykiss*) acclimated to different salinities. *The Journal of Experimental Biology*, *166*, 95–112.

Wongsanit, J., Teartisup, P., Kerdsueb, P., Tharnpoophasiam, P., & Worakhunpiset, S. (2015). Contamination of nitrate in groundwater and its potential human health: a case study of lower Mae Klong river basin, Thailand. *Environmental Science and Pollution Research*. http://doi.org/10.1007/s11356-015-4347-4

Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In The Physiology of Fishes (ed. D. Evans), pp. 379-425. Boca Raton, FL: CRC Press.

Woodling, J. D., Lopez, E. M., Maldonado, T. a., Norris, D. O., & Vajda, A. M. (2006). Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *144*(1), 10–15. http://doi.org/10.1016/j.cbpc.2006.04.019

World Health Organization, International Programme on Chemical Safety (WHO-IPCS). Global Assessment of the State-of-the-Science of Endocrine Disrupters. Damstra, T., Barlow, S., Bergman, A., Kavlock, R., Van Der Kraak, G., eds. 2002.

Wright, P. A. (1995). Review Nitrogen Excretion : Three End Products , Many Physiological Roles. *Journal of Experimental Biology*, *281*(2), 273–281. Retrieved from http://jeb.biologists.org/content/198/2/273.short

Wright, P. A, & Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *The Journal of Experimental Biology*, *212*(Pt 15), 2303–2312. http://doi.org/10.1242/jeb.023085

Xu, H., Yang, J., Wang, Y., Jiang, Q., Chen, H., & Song, H. (2008). Exposure to 17α-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*). *Aquatic Toxicology (Amsterdam, Netherlands)*, *88*(1), 1–8. http://doi.org/10.1016/j.aquatox.2008.01.020

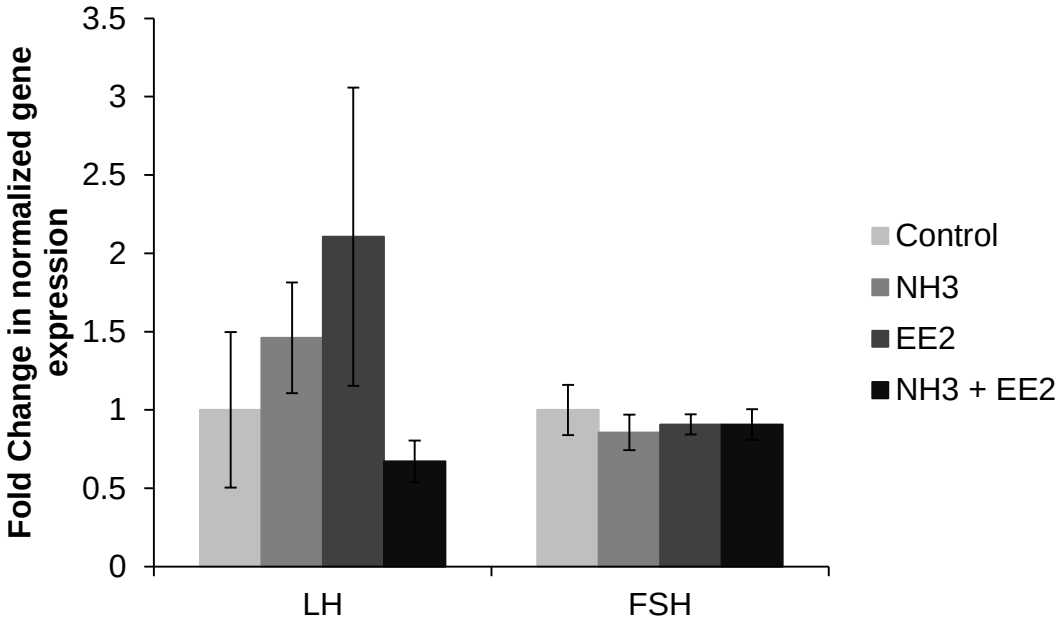
Yaron, Z., & Levavi-Sivan, B. (2006). Fish reproduction. *Physiology of Fishes*, 345-388.

Zhang, C., Willett, C., & Fremgen, T. (2001). Zebrafish: An Animal Model for Toxicological Studies. In *Current Protocols in Toxicology*. John Wiley & Sons, Inc. http://doi.org/10.1002/0471140856.tx0107s17

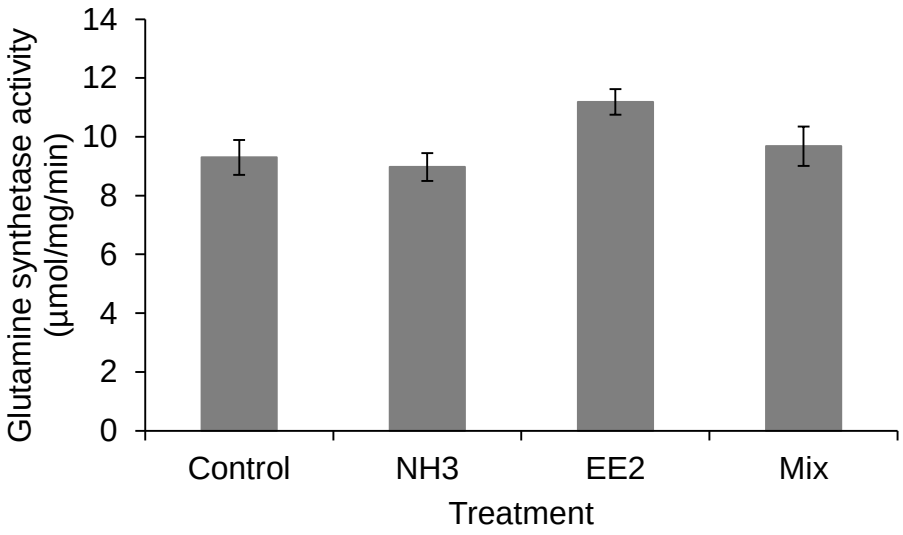
Zohar, Y., Breton, B., & Fostier, a. (1986). Short-term profiles of plasma gonadotropin and 17α-hydroxy, 20 β-dihydroprogesterone levels in the female rainbow trout at the periovulatory period. *General and Comparative Endocrinology*, *64*(2), 189–198. http://doi.org/10.1016/0016-6480(86)90003-1

Zohar, Y., & Mylonas, C. C. (2001). Endocrine manipulations of spawning in cultured fish: From hormones to genes. *Aquaculture*, *197*(1-4), 99–136. http://doi.org/10.1016/S0044-8486(01)00584-1

APPENDIX I



Ovarian expression in luteinizing hormone (LH) and follicle stimulating hormone (FSH) in fish after 7-day pre-exposure and 7-day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control). Data were generated in experiment 3 and represent mean ± S.E.M of six tanks per treatment. Expression data was normalized to elongation factor-1α and expressed as a fold change relative to gene expression of the controls. No significant change in expression was found ($p>0.05$; ANOVA, Tukey's).



Enzyme activity from whole-brains extracts of female zebrafish after 7-day pre-exposure and 7-day exposure periods to ammonia (4.5 mg/L TAN), EE_2 (25 ng/L), ammonia + EE_2 (4.5 mg/L TAN and 25 ng/L EE_2) or well water (control). Data were generated in experiment 3 and represent mean ± S.E.M of six tanks per treatment. Activity is measured by the production of ɣ-glutamyl hydroxymate per mg brain tissue per minute. No significant differences were found ($p>0.05$; Nested ANOVA, Tukey's).