ABSTRACT

Title of Dissertation: EXPOSURE EFFECTS OF ENVIRONMENTAL GESTAGENS ON THE REPRODUCTIVE BEHAVIOR AND FITNESS OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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Historically, endocrine disrupting chemical research has focused on environmental androgens, estrogens, thyroid hormones, and their antagonists. Recent efforts have begun to examine the effects of gestagens, which include endogenous progestogens and synthetic progestins, on aquatic organisms. Gestagens have been measured in wastewater treatment plant effluent, pulp mill effluent, and runoff from agricultural fields. While studies have documented profound effects on the reproduction of fish and amphibians, the effects of gestagen exposure on reproductive behavior and male gamete quality have been relatively unexplored. As such, a series of studies were conducted in which fathead minnows (*Pimephales promelas*) were exposed to either a water control, EtOH control, or one of two concentrations of gestodene (GES), levonorgestrel (LNG), or progesterone (P_4) using a flow-through

exposure system. Egg deposition was quantified daily, and alterations of reproductive behavior were examined on days 1, 2, and 8. After 8 days, fish were examined for the presence of secondary sexual characteristics (i.e. nuptial tubercle, fin spot, and dorsal fatpad formation). Egg deposition was affected by all treatments, with complete cessation observed at higher concentrations. Reproductive behaviors were affected after just one day in response to both treatments of LNG, GES, and P_4, with effects continuing through days 2 and 8. Exposure to both concentrations of LNG and GES also caused the physical masculinization of female fathead minnows. In a second set of studies using computer assisted sperm analysis, the same treatments were utilized to determine effects on sperm motility in males as a result of *in vivo* or *in vitro* exposure. LNG and GES showed no effect in either study, and P_4 caused decreases in sperm motility only as a result of *in vivo* exposure. Together, results from this study indicate that both GES and LNG function as environmental androgens in fishes, causing masculinization of secondary sex characteristics in females and disruption of reproduction over a short period of time. As altered reproductive behavior was observed after just one day of exposure for all treatments, this study underscores that behavior is an extremely sensitive endpoint that merits increased attention in future aquatic toxicology studies.

EXPOSURE EFFECTS OF ENVIRONMENTAL GESTAGENS ON THE REPRODUCTIVE BEHAVIOR AND FITNESS OF THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

by

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Dedication

Dedicated to my parents

Jack S. Frankel

and

Joan A. Frankel

for their unconditional love, support, dedication, and encouragement.

Familia mea, meum fundamentum

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Chapter 1: Literature Review

History of Endocrine Disrupting Chemical Research

The historical roots of research examining the effects of chemical exposure on wildlife can be traced back to the Federal Food, Drug, and Cosmetic Act (1938), regulating pesticides as food additives and sparking initial interest in their effects on animal health. The publication of Silent Spring (Carson 1962) which highlighted the impact of the insecticide DDT on wildlife, specifically avian species further increased interest. This focus was then turned towards aquatic ecosystems with the creation of the Clean Water Act (1972) and its goal of providing safe drinking water to the public. The Safe Drinking Water Act amendment (1974), which followed shortly thereafter, set standards of contaminant levels for certain substances in drinking water, along with the ability to enforce compliance. The term endocrine disrupting chemical (EDC) or "endocrine disruptor" first materialized during a meeting at the Wingspread Conference Center in 1991 (Markey et al. 2002), and was defined as chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife. During this meeting, general points of agreement included:

> *1) Large numbers of synthetic chemicals were being introduced into the wild, and that effects were already observable and correlated with laboratory studies.*
>
> *2) Mechanisms by which these compounds act vary but share general properties which disturb the balance of the endocrine system.*

3) Further research is required to examine the impact of EDCs on wildlife, and legislation is needed to ban those that are found to be persistent and harmful.

Early research involving EDCs focused on the effects of exposure on the development of the endocrine system and of the organs that respond to chemical signals during prenatal life, development, and post-natal life in birds, fish, invertebrates, and mammals (Colborn et al. 1993). These findings were again highlighted in the book Our Stolen Future (Colburn et al. 1996), which elevated public attention and placed pressure on the United States Congress to address the issue. The Endocrine Disruptor Screening and Testing Advisory Committee, formed in 1996, provided the EPA with recommendations for the development of a screening and testing program and formed the basis for the current EPA Endocrine Disruptor Screening Program.

Gestagens and Human Use

As one of the more recently examined classes of chemicals, the term gestagen refers to a collection of endogenous and synthetic progesterone receptor (PR) ligands. Progestogens are endogenous steroid hormones that act as important regulators of reproduction in vertebrates. Progestogens have been shown to play a vital role in the regulation of gamete maturation in a variety of fish species, including the Atlantic croaker (Tubbs et al. 2010), African clawed frog (Wasserman et al. 1982), spotted seatrout (Zhu et al. 2003), and several other teleost species (Boni et al. 2007). Progestogens have also been shown to serve as a method of signaling through

pheromonal interactions in goldfish (Sorensen et al. 1987), zebrafish (Kermen et al. 2013), and several other teleost species (Stacey et al. 2003).

Over time, a number of synthetic progesterone receptor ligands (progestins) have been manufactured, which mimic the biological effects of progesterone (Table 1.1). Due to their anti-gonadotropic properties, progestins have been used by humans primarily as a component of oral contraception and in hormone replacement therapy treatments (Laurell et al. 1968, McCann and Potter 1994, Petitti 2003, Shulman 2011). To date, four generations of progestins have been produced, and are generally classified based on the parent compound from which they are derived.(Kumar et al. 2015, Orlando and Ellestad 2014). Each successive generation of progestins bind more specifically to the human progesterone receptor (PR), thus producing fewer side effects.

Table 1.1

Common Name	Scientific Name	Age Class	Test	Effect	LOEC (ng/L)	Gestagen
Common carp	Cyprinus carpio	Juvenile	12 hr	Decreased phagocytic activity of head kidney derived macrophages	314	P4
		Juvenile	3 day	Decreased NO production by head kidney derived macrophages	314	P4
Fathead minnow	Pimephales promelas	Adult	7 day	Decreased male sperm motility	300	P4
		Adult	7 day in vivo and 24 hr ex vivo	Decreased 11-KT ex vivo	1	LNG
		Adult	7 day in vivo and 24 hr ex vivo	Decreased pregnenolone, 17,20 DHP, T, 11-KT in ovaries	100	LNG
		Adult	21 day	Female development of male secondary sex characteristics, decreased egg production	1	GES
		Adult	21 day	Decreased female egg production	0.8	LNG
		Adult	21-day	Decreased female egg production	1	NET
		Adult	21 day	Decreased female plasma E2	10	NET
		Adult	21 day	Decreased vtg mRNA in liver, decreased egg production, decreased fertilization	10	P4
		Egg to larvae	28 day	Decreased growth	740	NET
		Larvae	28 day	Decreased hsd3B, hsd20B, cyp17, ar, esra, and	16.3	LNG

				fshB mRNA expression		
		Larvae	28 day	Decreased growth, hsd3B, hsd20B, cyp1a, fshB mRNA expression	86.9	LNG
		Larvae	28 day	Decreased survival	462	LNG
Japanese medaka	Oryzias latipes	Adult	28 day	Decreased female egg production	25	NET
Mummichog	Fundulus heterolitus	Adult	14-day in vivo and 24 hr ex vivo	Decreased ovarian T and E2 production	1	CPA
Roach	Rutilus rutilus	Pubertal	28 day	Decreased male 11-KT plasma conc. And increased spgB in testes	31	LNG
		Pubertal	28 day	Decreased plasma 11-KT, increased spgB in testes, decreased fshB mRNA expression and plasma E2 concentrations in females	312	LNG
Three-Spined Stickleback	Gasterosterus aculeatus	Adult	21 day	Decreased female spiggin kidney mRNA expression	5.5	LNG
		Adult	21 day	Increased spiggin and decreased cyp1a mRNA expression in kidney, decreased vtg in liver	40	LNG
		Adult	45 day	Increased KEH in males	6.5	LNG
		Adult	45 day	Increased KEH, NSI, inhibition of spermatogenesis, increased	65	LNG

				spiggin and cyp1a mRNA expression in kidney		
Western mosquitofish	Gambusia affinis	Adult	8 day	Decreased esra, esrB, arB, and mt mRNA expression in testes and arB in liver	1000	P4
Zebrafish	Danio rerio	Adult	14 day	Decreased late VTG oocytes, vtg1 mRNA expression in liver, decreased nrld1 in brain	55	DRO
		Adult	14 day	Decreased vtg1 mRNA expression in the liver, decreased ccmb1, zp3 and increased arnt2, gr mRNA expression in the brain, decreased nrld1 in ovary	3.5	P4
		Adult	14 day	Increased female egg production	25	P4
		Adult	21 day	Decreased female GSI	25	P4
		Adult	21 day	Decreased scyp11b, hsd17b3, pgr mRNA expression in ovary and ar expression in brain	25	P4
		Embryo	48 hr	Decreased ar mRNA expression	2	LNG
		Embryo	48 hr	Increased esr1 and mr mRNA expression	2	NET

	Embryo	48 hr	Increased ar and gr mRNA expression	2	P4
	Embryo	96 hr	increased pgr and ar mRNA expression	2	P4
	Embryo	144 hr	Increased pgr mRNA expression	2	LNG
	Embryo	144 hr	Increased vtg1 mRNA expression	2	P4
	Embryo	120 hr	Decreased gr, pgr, cyp11b, and ar mRNA expression	254	P4

Commonly occurring gestagens (which include both synthetic progestins and endogenous progestogens), type, source, concentrations, and negative effects caused by each compound. From Orlando and Ellestad (2014) and Kumar et al. (2015).

Desirable effects of progestins are produced as the result of ligand binding to progesterone receptors. However, earlier generations of progestins derived from either 19-nortestosterone or 17-OH-progesterone were relatively non-specific, and often showed cross-reactivity with other steroid receptors, such as the estrogen, androgen, glucocorticoid, and mineralocorticoid receptor (Davtyan 2012). As a result, women taking these compounds as oral contraceptives often exhibit several undesirable side effects including uncontrolled bleeding, mood swings, and masculinizing traits, such as excess acne and hirsutism (Bair 1998, Oddens 1999). The most recent generation progestins, such as drospirenone (derived from spironolactone) and nestorone (derived from 19-norpregnane), were designed to be the most specific for human nuclear PR. As a result, they are thought to produce little to no side effects in humans when used at therapeutic doses. This specificity has also allowed for the overall decrease of progestin concentrations used in contraceptives, which has declined from 500 µg/dose in the 1960s to less than 100 µg/dose today (Davtyan 2012). One of the unfortunate environmental side effects of many of these contraceptives is that they are only partially metabolized in the human body, resulting in the excretion of quantifiable amounts of steroids and their associated conjugates in human urine (Braselton et al. 1977, Carballa et al. 2004, Johnson and Williams 2004). These then enter the environment through wastewater treatment plants, and in some cases have been shown to persist in the environment (Chang et al. 2009, DeQuattro et al. 2012, León-Olea et al. 2014, Orlando and Ellestad 2014, Runnalls et al. 2013, Vulliet et al. 2007, Zeilinger et al. 2009).

Sources of Environmental Gestagen Contamination and Relevant Concentrations

Gestagens, which include endogenous progestogens as well as synthetic progestins, enter the environment from three main sources traceable to humans; wastewater treatment plant effluent, runoff from land used for animal agriculture, and paper mill plant effluent.

Wastewater Treatment Facility Effluent

In the United States, there are over 21,000 wastewater treatment facilities (USEPA 2008). Flow rates for each plant vary greatly, and range from 1 to 200 million gallons per day (Partnership 2011). Previous studies have shown that many of these facilities are ineffective at removing gestagens during processing. Various levels of gestagen contamination in WWTF effluent have been found, typically ranging from less than 1 ng/L to 375ng/L (Table 1.1). As a result, there is a continual introduction of various gestagens into natural water bodies worldwide, including areas of the United States (Chang et al. 2009, DeQuattro et al. 2012, Kolodziej et al. 2003, Runnalls et al. 2013, Vulliet et al. 2007), China (Chang et al. 2009, Qiao et al. 2009), France (Vulliet et al. 2007), and elsewhere. Given the large number of WWTFs combined with the relatively few studies examining the exposure effects of gestagens, further research examining the impacts on fish and wildlife is required.

Agricultural Runoff

In agricultural settings, gestagens are often utilized to control and manipulate reproduction and growth rates of the animals being raised for food production. These

gestagens include progesterone, melengestrol acetate, and medroxyprogesterone acetate (Table 1.1). Similar to progestins in humans, many of these compounds are excreted from livestock, and can be transported into natural water bodies by runoff due to heavy rain events and/or poor management of animal waste. Unlike contaminants found in WWTP effluent, the amount of gestagens released from agricultural runoff is more variable, depending on the frequency and amount of rainfall (Mansell et al. 2011), the fertilization of fields with manure from treated animal and the density of animals being housed in a specific area (Kolodziej et al. 2003, Lorenzen et al. 2004).

Paper Mill Plant Effluent

Phytogestagens, substances that are produced by plants that have biological properties mimicking progestogens, have been measured as a component of paper mill effluent. Most of these compounds consist of either phytosterol or flavonoid metabolites, which are produced by plants in response to various environmental changes including photoperiod, temperature, atmospheric conditions, and physical damage as a result of herbivory (Weston and Mathesius 2013). These compounds have been shown to bind and activate estrogen, androgen, and progesterone receptors in various species (Adlercreutz et al. 1993, Durhan et al. 2002, Jenkins et al. 2003, MacLatchy and Van Der Kraak 1995, Patisaul and Jefferson 2010).

Fathead Minnow in Aquatic Toxicology

As a member of the Cyprinidae family, the fathead minnow (FHM) has long been a popular model for aquatic toxicology in the United States. Due to its

widespread distribution in the United States, a comparatively short time to reach sexual maturity, overall hardiness, and ability to live in a wide range of water conditions (AE 2008). Also, FHMs exhibit sexual dimorphism that allows for easy identification of sex and signs of disruption (Cole and Smith 1987a, Nico et al. 2014). Further, the reproductive physiology of this species has been well studied (Ankley and Villeneuve 2006, Jensen et al. 2001) and, as such, the FHM has been included as a component of the USEPA's endocrine disruptor screening program (Ankley and Villeneuve 2006, Jensen et al. 2001). As a result of this program, there is a wealth of studies available that have examined the exposure effects of various EDC classes on the FHM.

The Role of Hormones in the Regulation of Fish Behavior During Gametogenesis

In the aquatic environment, pheromones play a pivotal role in the regulation of various behaviors such as feeding, predator avoidance, and reproduction. The definition of a pheromone has evolved over time to accommodate new findings as a result of these advances, with the most recent being molecules that are released from one individual and elicit a specific reaction in one or more other organisms (Sorensen and Wisenden 2015). Over the past few decades, an increased understanding of the pheromonal role of excreted hormones such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and its conjugates, prostaglandins (PGs) and androgens (androstenedione), play in the control of courtship and spawning. Much of this is a result of advances in technology that has allowed for increased detection, identification, and quantification of such compounds.

Much of the research involving the reproductive functions of pheromones has focused on the goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) due to their hardiness, relatively well-understood endocrine system, and displays of identifiable reproductive behaviors. As such, they are the focal species of this discussion involving the role of the various steroid hormones in the context of oogenesis and their association with pheromonal communication.

In reproductively mature female fish, favorable environmental cues induce increases in GnRH synthesis, leading to the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH travels to follicular thecal cells, where it stimulates the conversion of cholesterol to testosterone (T). Testosterone then diffuses into surrounding granulosa cells where it is converted $_{to}$ an estrogen (17β - estradiol, E_2). Estradiol then travels to the liver where it stimulates the production of vitellogenin, a protein that is sequestered as a nutrition source for embryonic and early fry development. In goldfish, females release high concentrations of E_2 during this period of development via urine (Yamazaki 1990), and that males are attracted to E_2 and respond with behavioral changes (Kobayashi et al. 2002), suggesting that E_2 may serve as a recrudescence pheromone. However, based on changes in electrical potentials recorded via electro-olfactography, goldfish are unable to detect E_2 or its common metabolites (Sorensen et al. 1987), suggesting the releaser hormone is either an unknown metabolite or is a secondary product produced as the result of E_2 exposure. Once vitellogenesis is completed, FSH production decreases and LH production increases, leading to decreased E_2 production and a switch to the production of the androgen androstenedione (AD). Female goldfish excrete AD via

their gills during this time period, causing decreased LH levels in exposed males (Stacey 1991) and increased agonistic behavior (Poling et al. 2001). In response to photo-thermal cues, increasing LH surges cause AD production to decrease, and the production of a maturation inducing steroid (MIS) to increase. In goldfish, this MIS has been identified as the progestogen DHP. Together with the prostaglandin $PGF_{2\alpha}$, DHP endogenously causes migration and breakdown of the germinal vesicle in follicles, leading to ovulation. Release of DHP and its sulfonated/glucuronidated conjugates (DHP S/G) have been shown to increase male following and inspection behaviors in carp (Sorensen and Wisenden 2015), although the release of each is regulated differentially (DHP released through the gills and DHP S/G released via urine pulses). Ovulation occurs fairly rapidly after the induction of DHP synthesis, and causes the eggs to begin the production of a prostaglandin ($PGF_{2\alpha}$). In females, circulating $PGF_{2\alpha}$ stimulates female oviposition behavior within minutes (Sorensen et al. 1995). During this short time period, $PGF_{2\alpha}$ is released via the gills as well as conjugates via urine, causing a releaser effect on male behavior, LH concentration, and milt volume (Sorensen and Wisenden 2015).

The Evolution of Pheromones

The evolution of chemical communication has been a topic of debate for some time, particularly in regard to the forces driving their origin as either actively excreted compounds used as cues by the sender (allomone) and the recipient (kairomone), or whether recipients have evolved to detect and respond to passively released hormones and metabolites ("chemical spying") (Sorensen and Stacey 2004). Because the pheromonally active hormones and their conjugates identified thus far are

unspecialized and utilized endogenously for gametogenesis, it has been proposed that pheromonal communication developed initially as a form of chemical spying (Sorensen, 2015). Males able to detect hormones such as DHP, $PGF_{2\alpha}$, and their conjugates released by females as a passive consequence of oogenesis may have obtained a higher level of reproductive fitness compared to those unable to detect and utilize these signals (Sorensen and Wisenden 2015). This would have allowed for chemical spying to develop into chemical communication by creating a selection pressure on females, resulting in the development and refinement of these signaling substances. The alternative conclusion, that chemical communication developed first, requires females to produce novel compounds that are excreted into the environment without the guarantee that males would be able to detect and interpret them into a biological response. Both of these assumptions are non-adaptive and least parsimonious in terms of evolution, requiring the active development of both an excretion system by females and a method of detection by males.

Support has been garnered for the hypothesis that pheromonal PGFs originated in an ancestor common to all cypriniforms, and that detection of steroids evolved after cyprinids diverged. Several species of teleosts have shown that the ability to detect PGs, whereas the ability to detect free and conjugated steroids is reserved within cyprinids (Sorensen and Wisenden 2015). In terms of reproductive modes, it appears that egg scattering species such as salmon (Olsén et al. 2001), trout (Essington and Sorensen 1996), and chars (Sveinsson and Hara 2000) rely more upon PGs as a method of pheromonal communication. In contrast, FHMs and other species requiring the performance of time intensive behaviors such as the selection and

guarding of nest sites and the display of intricate reproductive courtship and spawning behaviors may be more reliant on steroid hormones such as progestogens for communication, which are released hours ahead of ovulation. As steroid hormones are typically released over long periods of time as a by-product of endogenous mechanisms resulting from oogenesis and maturation, they would logically be more appropriate than PGs which are released over a short time period.

Fathead Minnow Reproductive Behavior

The reproductive behavior of wild fathead minnows was first described over 40 years ago by McMillan and Smith (1974), indicating that males and females display clear and distinct territorial, courtship, and spawning behaviors that can be easily classified and quantified. Once males become sexually mature, they select a nest area typified by an overhanging object that provides a surface area underneath. Males will then display several types of territorial behavior, including hovering (male remains stationary underneath nest structure with slight undulation of the caudal fin to maintain position in the water column), rubbing (male vigorously rubs the top and sides of the structure with his dorsal pad), and circling (male swims in a circular path beneath the breeding structure). When approached by another male, males will display agonistic behaviors in an attempt to defend their territory. Charging (male rapidly swims towards its opponent), butting (where the male moves towards its opponent and makes contact with the end of its snout) and biting are commonly exhibited by competing individuals, culminating in the retreat of one individual away from the breeding structure.

When a territorial male is presented with a reproductively receptive female, he will attempt to lead her into the breeding structure by displaying approach behaviors. During this time, the male will leave the breeding structure and rapidly approach the female, stopping 1 - 2 cm away before turning around and re-entering the structure. At this point, the female will either follow the male back into the structure, or will ignore the advance. As such, these approaches can be classified as either successful or failed. The number of successful approaches by males is typically low compared to the number of failed approaches. Once the male has been able to successfully lead a female into the breeding structure, they will typically perform a series of lateral quivers where the male and female parallel each other and undulate their whole bodies rapidly in concert. Occasionally, the male will initiate a spawning arch by pushing the female up towards the surface of the breeding structure, flipping her upside down. At this time, one or two adhesive eggs are deposited on the breeding tile, which are then immediately fertilized by the male. The duration of interplay between lateral quivers and spawning arches occurs from approximately 30 sec to 2 min at a time, after which the female will exit the breeding structure.

In aquatic toxicology, the analysis of courtship and reproductive behaviors has been identified as an important endpoint (Ankley and Villeneuve 2006, Orlando and Ellestad 2014) and in a limited number of studies has been successfully utilized to describe the effects of several endocrine disruptors including multiple estrogens (Länge et al. 2001, Martinović et al. 2007) and the anti-androgen vinclozolin (Makynen et al. 2000). However, no previous research has been performed that has examined the effects of gestagens on reproductive behavior in fish.

Based on what is currently known about the presence of gestagens in the environment, their modes of action, and their potential to disrupt multiple reproductive endpoints in aquatic species, the research contained in this dissertation investigates two key questions, including:

1) To determine the exposure effects of several gestagens on the behavior, reproduction, and development of secondary sex characteristics in the fathead minnow
2) To determine the exposure effects of several gestagens on sperm motility both *in vitro* and *in vivo* in the fathead minnow

Previous research has shown that exposure to the 19-nortestosterone derived progestins elicits the masculinization of female FHMs after 21+ day exposure, and exposure to a range of gestagens quickly shuts down reproduction (Runnalls et al. 2013). Thus, it was expected that exposure to both GES and LNG for 8 days would cause the development of male SSCs in females, and exposure to GES, LNG, and P_4 would cause a decrease in egg deposition. As behavior have been described as an extremely sensitive endpoint in terms of toxicological studies (Orlando and Ellestad 2014a), it was expected that FHMs exposed to all chemicals would show rapid alterations of key reproductive behaviors related to nest acquisition and defense, courtship, and spawning. As no previous studies have examined the impacts of progestins on sperm motility, it was hypothesized that *in vivo* exposure to progestins would cause the alteration of sperm motility through changes in steroidogenic pathways and gametogenesis, and *in vitro* exposure would cause effects due to the binding of receptors on the sperm membrane.

Chapter 2: Effects of Ethanol, a Common Carrier Solvent Used In Aqueous Exposure Experiments, on the Fecundity and Sexual Development of the Fathead Minnow (*Pimephales promelas*)

Introduction

Understanding the impact of various endocrine disrupting chemicals (EDCs) on aquatic organisms is an ongoing and important research area. The United States Environmental Protection Agency (USEPA) and other agencies, such as the United Nations Organization for Economic Cooperation and Development, have developed guidelines for assays to determine the potential exposure effects on reproductive fitness, morphology, endocrinology, and other relevant endpoints of the fathead minnow (*Pimephales promelas*, FHM). Due to the wide range of EDCs that are tested, the ability to dissolve a specific compound in water varies considerably due to differences in physiochemical properties. While the USEPA suggests that test chemicals be used without carrier solvents and those with low solubility be prepared using a saturator column or similar device, such technologies are difficult to use reliably and require frequent chemical analyses to assure accurate dosing over the course of a study. As such, many studies utilize carrier solvents (e.g. ethanol (EtOH), methanol, and dimethyl sulfoxide) to solubilize more lipophilic test chemicals. Some of these same solvents are known to cause acute toxicity. For example, methanol and dimethyl sulfoxide exposure studies report LC_{50} values in red drum (*Sciaenops ocellatus*) (Robertson et al. 1988), medaka (*Oryzias latipes*), pejerry (*Odontesthes bonariensis*), rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) (Suzuki et al. 1995), and in adult salmon (*Salmo salar*) (Benville et al. 1968), trout (*Oncorhynchus mykiss*) (Benville et al. 1968, Poirier et al. 1986), tilapia (*Oreochromis mossambicus*) (Kaviraj et al. 2004), bluegill sunfish (*Lepomis macrochirus*) (Poirier et al. 1986), and FHM (Poirier et al. 1986).

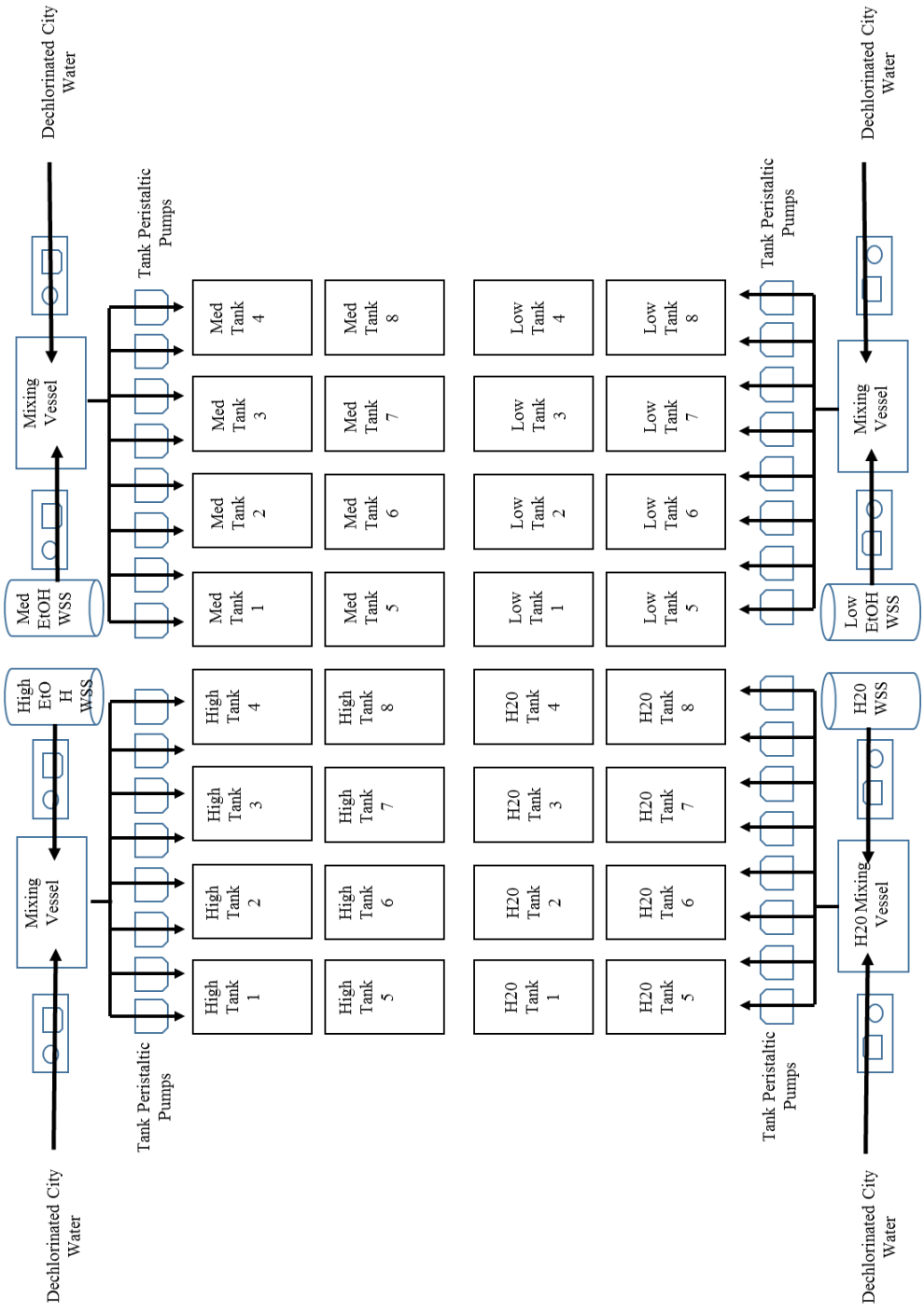
Compared to the studies cited above, the effects of sublethal concentrations on more sensitive endpoints such as behavior, development, and physiology are fewer in number. For example, EtOH increases larval zebrafish startle response behavior, neuronal cell death, and skeletal dysmorphogenesis of the craniofacial and neurocranial skeleton at concentrations as low as ~ 0.02% (Carvan et al. 2004), as well as increased latency of startle responses and increases nearest-neighbor distance in adult zebrafish at concentrations as low as 0.25% (Dlugos and Rabin 2003, Gerlai et al. 2006). However, no such studies have been performed to examine the effects of EtOH alone on the fecundity and sexual development of the FHM. For aquatic toxicology studies that have utilized EtOH as a solvent, concentrations vary considerably for different model organisms, ranging for zebrafish from 0.01% (Örn et al. 2003) to 2.9% (Bilotta et al. 2004), for Japanese medaka from 0.01% to 0.04% (Wu et al. 2008), and for FHMs from 0.00003% (Paulos et al. 2010, Runnalls et al. 2013) to 0.00085% (Painter et al. 2009). Additionally, EtOH has also been identified as an environmental pollutant, constituting up to 90% of organic content in winery wastewater effluent (Colin et al. 2005). While vineyard and winery effluent has been the focus of previous studies examining the effects of certain pesticides such as diuron, azoxystrobin (Bony et al. 2008) and vinclozolin (Makynen et al. 2000, Martinović et al. 2008) on the viability, morphology, physiology, and reproduction of aquatic organisms, no published studies are available which have examined the potential impacts of EtOH as a reproductive toxicant and/or EDC in fish. As such, this study tested if exposure to EtOH would affect fecundity and development of secondary sex characteristics in adult FHMs.

Materials and Methods

Exposure System Design

The design of the laboratory exposure system used in this study followed the approach and recommendations of the USEPA EDSP test guidelines (OPPTS/USEPA 2009). Thirty-two 19 L glass tanks containing a single PVC breeding tile (~ 3" x 4") were used. Groups of eight tanks were utilized for each treatment, and partitions were placed between groups to prevent cross-contamination. Opaque dividers were placed between each tank to limit visual interaction between individuals in adjacent tanks, and blackout curtains were hung around the perimeter of the system to prevent interference from external stimuli. Full spectrum fluorescent lighting was used to provide a consistent photoperiod of 16 h light: 8 h dark. For each treatment, dechlorinated freshwater was delivered to a glass-mixing vessel using a peristaltic pump (Cole Parmer Masterflex 7553-50 w/ MasterFlex Easyload II Head 77200-62) at a rate of 500 mL/ min. During the 7 d exposure period, a separate peristaltic pump (Gilson Minipuls 3) added EtOH from the working stock solution at 1 mL/min to achieve the final EtOH concentrations of 0.0% (water control), 0.00001%, 0.0001%, and 0.001%. For each of the 32 exposure tanks, an individual peristaltic pump (APT Instruments SP200VOHD) delivered the EtOH treatments and water control into each tank at a rate of 55 mL/min, equating to 6 volume changes per 24 h (Figure 2.1).

Figure 2.1



Graphical representation of the flow through exposure system utilized for this study. Low = 0.00001% EtOH, Med = 0.0001% EtOH, and High = 0.001% EtOH.

Experimental Organisms

Approximately 4 mo old male and female FHMs were obtained from Aquatic BioSystems (Fort Collins, CO) and quarantined in our breeding colony for one month under optimal photothermal and water quality conditions (water temperature: 25°C ± 1; dissolved oxygen, DO: ≥ 4.9 mg/L, pH: 7.4 - 7.6, photoperiod: 16 h light: 8 h dark). All experiments were conducted under an approved University of Maryland IACUC protocol (R-13-60). Fish were fed twice daily on a diet of Tetramin® flake food and freshly hatched brine shrimp. Individuals that showed developmental deformities or signs of illness were removed and not utilized for the experiment. After the quarantine period, fish were transferred to the exposure system and separated into breeding triads (1 male: 2 females) and acclimated for 2 wks in dechlorinated tap water.

To ensure that all triads were reproductively active, all breeding tiles were checked for the presence of eggs daily at 1500 h during the acclimation period. Any breeding tiles that had been used for spawning were removed from the tank and a fresh tile was supplied. Triads that had not produced eggs by day 5 were replaced with new fish from the breeding colony. Using this method, we ensured that all triads used for this study (n = 8 for, 0.0001%, and 0.001%, and water control; n = 7 for 0.00001% due to a single damaged tank) had laid eggs at least once by the conclusion of the acclimation period. Following the acclimation period, triads were exposed using the flow-through system to one of the EtOH treatments or water-only control for 7 d.

Fecundity

To test for effects on fecundity, all breeding tiles and surfaces of the tank were examined daily at 1500 h for the presence of embryos. These tiles were then exchanged with fresh tiles, and the number of eggs deposited by each triad quantified.

Secondary Sex Characteristics

After the 7 d exposure period, all fish were euthanized using buffered MS-222 (300 mg/L, pH 7.4), and examined for the presence of male secondary sexual characteristics (SSC), including the presence of nuptial tubercles, dorsal fin spot, and fatpad. Fish standard length and mass were obtained, after which gonads were extracted, weighed for determination of gonadosomatic index, and processed for histological analysis.

Gonad Histology

All histology chemicals were purchased either through Fisher Scientific (Waltham, MA, USA) or VWR (Radnor, PA, USA). Gonads from one-half of randomly chosen fish were preserved in Davidson's fixative for 24 h and transferred to 10% neutral buffered formalin for further histological processing and analysis following USEPA protocol (OPPTS/USEPA 2009). Fixed tissues were dehydrated in a graded series of EtOH, cleared in xylene, and embedded in paraffin wax. Wax blocks were faced and sectioned at 5 μm using a rotary microtome and mounted on glass slides. Samples were then stained with Harris' hematoxylin and eosin-Y alcoholic and cover-slipped using Permount ®. Slides were examined independently by two observers using a Zeiss Axioplan microscope to (1) confirm the sex of each

fish and (2) check for the presence of spermatozoa, spermatids, and spermatocytes in testes and vitellogenic, cortical alveolar, and pre-follicular oocytes in ovaries.

Statistical Analyses

All statistical analyses were performed using SAS Studio (version 3.3, Cary, NC). All data sets were first checked for normality and homoscedasticity. To examine for differences in fecundity and SSCs, treatments groups were compared using ANOVA followed by post-hoc analysis using Tukey HSD with significance set at $p < 0.05$. Unless otherwise noted, all data are presented as the mean ± SEM.

Results

Fecundity

For each treatment, the number of eggs laid per day is presented in Table 2.1. As documented in previous studies, egg production by each FHM triad varied greatly between days. No differences were observed between the control and 0.00001% treatments ($p = 0.3123$). However, triads treated with both 0.0001% and 0.001% showed substantial decreases in average egg production, with a complete shutdown of egg deposition after day 3 (Table 2.1).

Table 2.1

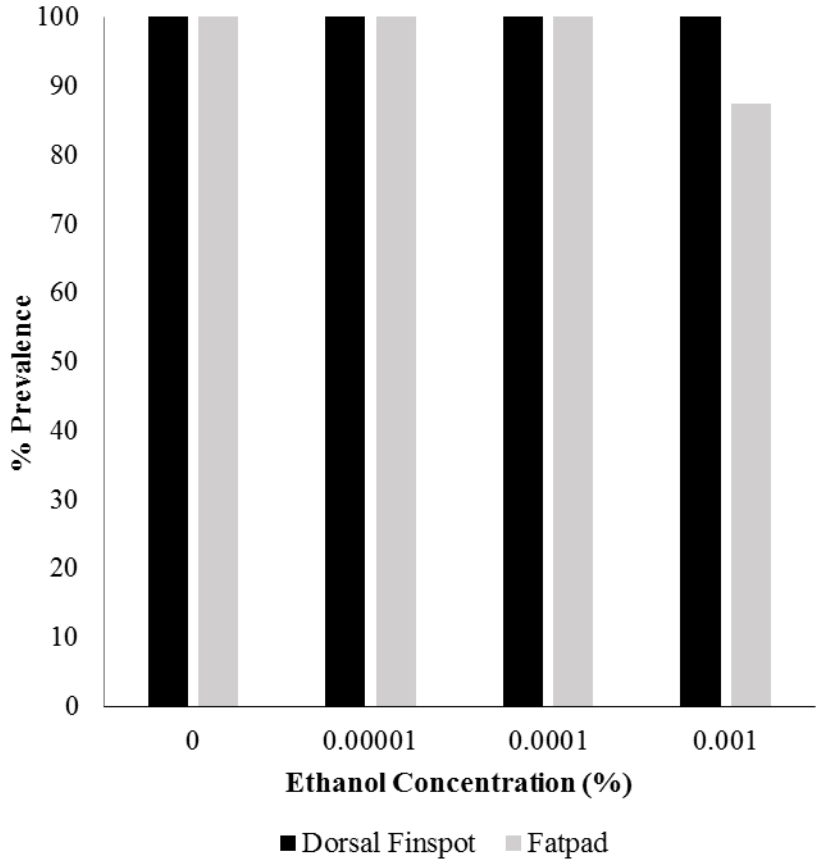
	Day							
Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Total**
Water Only Control	99	308	79	0	0	4	66	556^{A}
0.00001% EtOH	49	56	146	56	0	131	21	459^{A}
0.0001% EtOH	0	45	54	0	0	0	0	99^{B}
0.001% EtOH	0	0	74	0	0	0	0	74^{B}

Daily and overall total egg deposition rates produced by triads (n = 8) from each treatment. Superscript letters indicate significant ($p < 0.05$) differences.

Male FHM Secondary Sex Characteristics

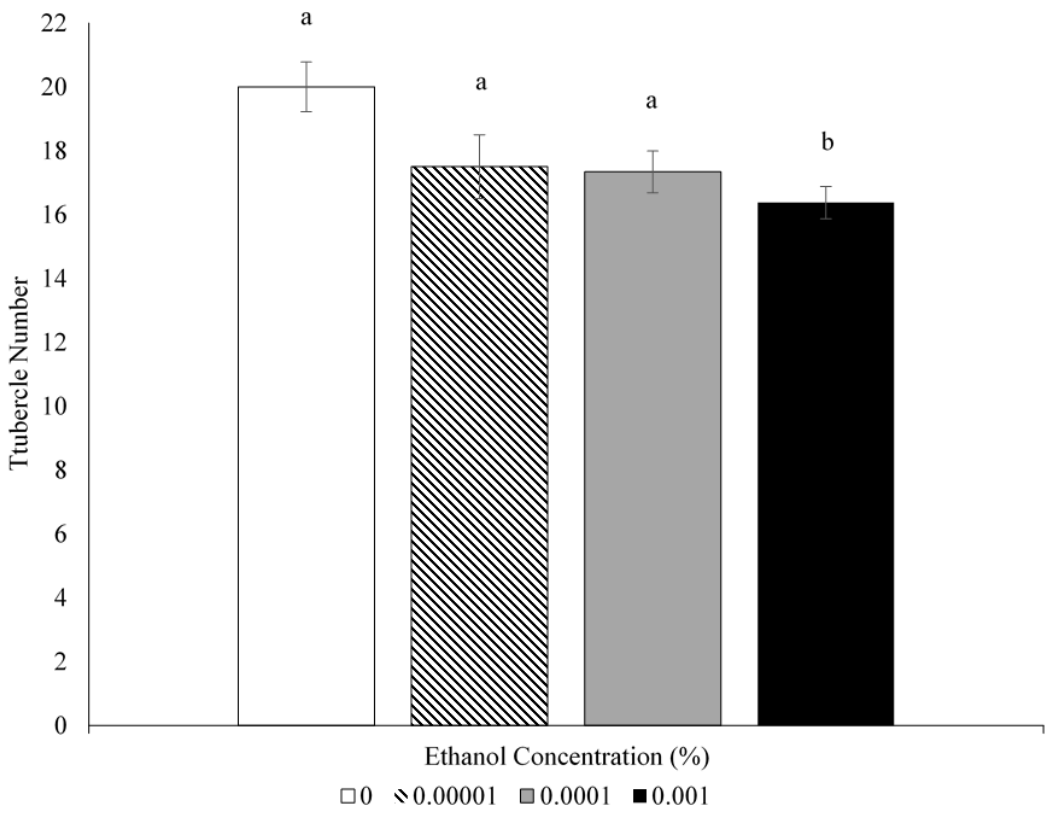
Dorsal fin spots, fatpad formation, and nuptial tubercles were absent on females for all treatments. No statistical differences in male fatpad formation (p = 0.8201) and dorsal fin spot (p = 0.0932) presence were found in any of the treatments (Figure 2.2). A significant decrease (p = 0.0301), however, was observed in the average number of nuptial tubercles present on males in the highest ethanol, 0.001% treatment (Figure 2.3). No significant differences were observed between the other three treatments (Figure 2.3).

Figure 2.2



Presence of dorsal fin spots and fatpad presence on male FHMs (n = 7 for 0.00001% EtOH treatment, n = 8 for all other treatments) from each of the treatments.

Figure 2.3

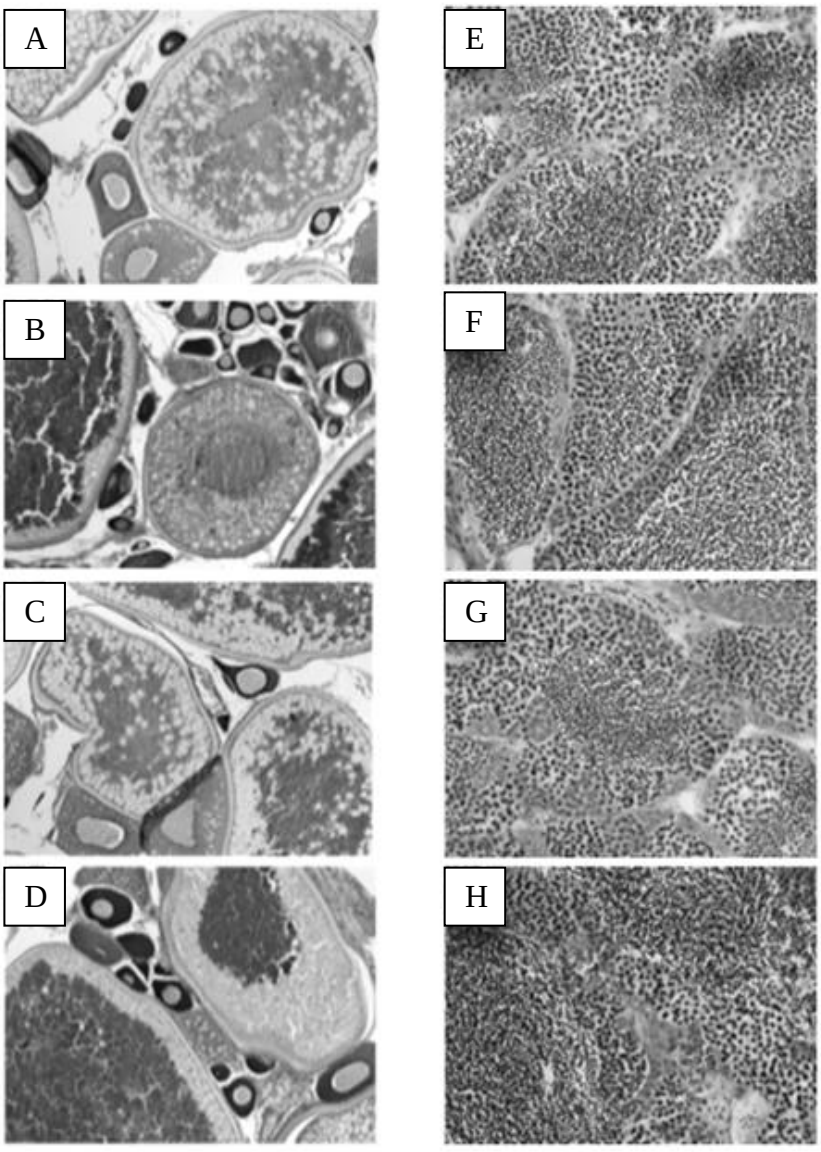


Average nuptial tubercle counts displayed by male FHMs (n = 7 for 0.00001% EtOH treatment, n = 8 for all other treatments) from each treatment. Letters indicate significant ($p < 0.05$) differences.

Gonad Histology

There were no discernable differences present for either males or females across treatments in either gonad mass normalized to body mass, gonadosomatic index (GSI), or stages of gametogenesis. No significant differences in GSI were determined among treatments. Histological examination of the testes of males from each of the treatments revealed that all stages of spermatogenesis (spermatozoa, spermatids, spermatocytes, and spermatogonia) were present (Figure 2.4). All ovaries collected from females exposed to each treatment contained vitellogenic, cortical alveolar, pre-follicular oocytes, and oogonia (Figure 2.4).

Figure 2.4



Micrographs of ovarian tissue of female FHMs treated with (A) H_2O only control, (B) 0.001%, (C) 0.0001%, and (D) 0.00001% ethanol treatments (all at 100X total magnification). Micrographs of testicular tissue of male FHMs treated with (E) H_2O only control, (F) 0.001%, (G) 0.0001%, and (H) 0.00001% ethanol treatments (all at 400X total magnification).

Discussion

While EtOH has been widely utilized as a carrier solvent for short-term reproductive testing using the FHM, final exposure concentrations vary widely across studies. Previous exposure studies were often performed using only a water-only control, hindering the ability to discern the effects of EtOH alone on reproduction through methods via meta-analyses. While multiple studies have examined the effects of EtOH on zebrafish development (Bilotta et al. 2004), visual function (Bilotta et al. 2002), and behavior (Gerlai et al. 2006), no known studies have been published that directly examine the exposure effects of EtOH on FHM reproduction.

At the two highest EtOH concentrations, exposure to 0.0001 and 0.001% caused a significant decrease in average egg production, with no production during days 4-7. Although the mechanism causing this decrease and cessation is unclear, possible factors include alterations in reproductive behavior, steroidogenesis, or HPG axis regulation of reproduction.

In zebrafish, EtOH exposure has been found to interact strongly with the brain's reward reinforcement circuitry, causing lasting adaptations that ultimately result in behavioral changes (Kily et al. 2008), and has also been found to cause increases in acetylcholinesterase (AChE) activity in the brain (Rico et al. 2007). Decreases in AChE and acetylcholine have been associated with alterations of searching behavior (Norton and Bally-Cuif 2010) and conditioned place preference behaviors (Ninkovic et al. 2006) in zebrafish. This supports the possibility that EtOH exposure may be causing the disruption of key reproductive behaviors and, ultimately, the deposition of eggs by FHMs. Thus EtOH exposure in our study may

be altering brain concentrations of acetylcholine and/or its metabolizing enzyme, AChE.

While little research exists examining the effects of EtOH on these specific endpoints in fish, a number of studies have been performed with mammals that, together with EDC exposure studies with FHMs, can be used to support a tentative mechanism(s) that led to the severe and rapid decline in fecundity documented in this study. EtOH is known to exert negative effects on male rat reproduction by altering steroidogenic enzyme transcription, specifically via decreases of *P*-450 17α-hydroxylase/C17-20 lyase mRNA levels (Shi et al. 1998). Oral administration of EtOH to adult male Wistar rats decreases serum testosterone levels, increases serum estradiol concentrations, and decreases 3β-hydroxysteroid dehydrogenase and 17-ketosteroid reductase enzyme activity (Rengarajan et al. 2003). While it is difficult to correlate results between mammalian and teleost models, the observed changes of egg deposition in our study may also be attributed to changes in male reproductive behaviors due to alterations of the steroidogenic pathway.

While no studies have examined the impacts of EtOH on gonadotropins in fish, changes in luteinizing hormone (LH) and follicular stimulating hormone (FSH) may serve a role in the disruption of reproduction as a result of exposure, and may also affect additional changes in the steroidogenic pathway described previously. In male Wistar rats, oral administration of EtOH was shown to decrease LH receptors in Leydig cells (Rengarajan et al. 2003), and to decrease serum LH and pituitary LHβ subunit mRNA levels *in vivo* in castrated male rats after intraperitoneal injection (Emanuele et al. 1991). Decreases in LH releasing hormone- stimulated LH secretion

were also noted as a result of *in vitro* EtOH exposure of female rats (Alfonso et al. 1993). As LH in fish has been shown to mediate the final maturation of oocytes and spermatozoa and the secretion of testosterone from Leydig cells in males (Evans et al. 2013), it is also possible that the exposure to EtOH results in the disruption of final oocyte maturation in females and spermatogenesis in males. While no histological differences were detected during our study, further research using longer exposure periods may reveal increases in apoptotic follicles or decreases in spermatozoa or their sperm motility.

The decrease in numbers of male nuptial tubercles (which have been shown to be induced by exogenous androgens in female FHM) in the highest EtOH treatment provides further support for alterations of the steroidogenic pathway, although further research examining changes in plasma steroid concentrations is required to verify this hypothesis. Because concurrent changes in androgen dependent SSC expression in the 0.0001% treatment were not observed, it is reasonable to conclude that these changes were, at least initially, due to shifts in factors such as reproductive behavior, rather than changes in steroidogenesis or circulating hormone concentrations. As males that were exposed to the highest EtOH concentration exhibited decreases in nuptial tubercle count in addition to significant decreases in fecundity of the triad, it appears that EtOH may produce effects via a number of mechanisms that are dosage dependent. Further research utilizing both shorter (e.g. 24 h, 48 h, etc.) and longer exposure periods (e.g. 2 wks, 3 wks, etc.) are required to confirm and elucidate these differences.

In conclusion, the observation of negative effects on reproduction and alternations in sexual development in FHMs exposed to previously reported EtOH concentrations highlights the importance of this study. Based on the results of this research, it is recommend that future studies use final ethanol concentration no greater than 0.00001% and that potential effects on other species should be examined as sensitivities to ethanol may vary. Future studies examining the effects of EtOH on reproductive behavior, steroidogenesis, and neurotransmitter functionality would elucidate the underlying mechanisms controlling the disruption of reproduction as a result of exposure. As EtOH has been measured entering the environment from specific sources (i.e. vineyard wastewater effluent), this study also provides support for research to ascertain the prevalence of EtOH pollution in the environment, as well as to determine the impacts of such EtOH pollution on aquatic ecosystems receiving such effluent.

Chapter 3: Exposure to the Contraceptive Progestin, Gestodene, Alters Reproductive Behavior, Arrests Egg Deposition, and Masculinizes Development in the Fathead Minnow (*Pimephales promelas*)

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Introduction

Currently, there is great concern regarding the effects of endocrine disrupting chemicals (EDC) that enter aquatic ecosystems from wastewater treatment plants, agricultural runoff, and paper mill plants on the reproductive health of aquatic organisms (Aris et al. 2014, Söffker and Tyler 2012). One of the most studied EDCs is 17α-ethinylestradiol (EE_2), an estrogen that is combined with a progestin in human contraceptives and some hormone replacement therapeutics. Recently, there has been increased interest regarding the effects of gestagens on the reproduction of aquatic organisms. Gestagens include endogenous progestogens (e.g. progesterone and 17α,20β-dihydroxy-4-pregnen-3-one) and synthetic progestins (e.g. GES and LNG), and are classified as compounds that mediate physiological processes and behavior through progesterone receptors. In vertebrates, progestogens play critical roles in the control of reproduction, including regulation of gamete maturation, gestation, and species-specific behaviors (Boni et al. 2007, Tubbs et al. 2010, Wasserman et al. 1982, Zhu et al., 2003). Progestins are not only used as human pharmaceuticals, but are also commonly used as veterinary pharmaceuticals for growth promotion and to synchronize reproduction. Thus, they have been frequently detected in water impacted by human and/or livestock waste (Bartelt-Hunt et al. 2012, Feng et al. 2008, Kraeling and Webel 2015). Moreover, paper mill effluent has been documented to contain phytosterols that are microbially converted to steroid hormones including progesterone (Jenkins et al. 2003). As a result, gestagens can enter the aquatic environment via a myriad of sources such as wastewater treatment plant effluent, runoff from agricultural fields and livestock farms, and paper mill effluent. While

relatively few in number, studies examining environmental concentrations of gestagens have found progestin concentrations up to 188 ng/L in wastewater treatment plant effluent, 34 ng/L in surface waters, and progestogen concentrations in excess of 10,000 ng/L in animal agricultural lagoons (Fent 2015, Orlando and Ellestad 2014).

Progestins have been specifically designed as ligands for human nuclear progesterone receptors (PR) and have been categorized into generations (I – IV). Progestins in each generation share a common parent molecule, with progestins in each successive generation exhibiting progressively greater specificity for the human PR (Africander et al. 2011, Sitruk-Ware et al. 2012). Several generations have cross-reactivity with the human androgen and other steroid hormone receptors, resulting in unwanted side effects in women including hirsutism and unwanted weight gain. Previous research using *in vitro* receptor transactivation luciferase assays has shown that some commonly used progestins (e.g. GES, LNG, and norethindrone) express high affinity for the FHM (*Pimephales promelas*) nuclear androgen receptor (nAR) and little to no affinity for the FHM nPR, indicating that some progestins likely induce biological effects through mostly AR mediated pathways (Ellestad et al. 2014, Runnalls et al. 2013). In addition, corroborating results have recently been published on a distantly related fish, the Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) (Bain et al. 2015).

Thus far, research on the exposure effects of gestagens on aquatic wildlife have mainly focused on the reproduction of aquatic vertebrates (Fent 2015, Kumar et al. 2015, Orlando and Ellestad 2014). While exposure to gestagens has been shown to

decrease egg deposition, increase ovarian apoptosis, and alter development of secondary sexual characteristics in fishes (Paulos et al. 2010, Pinter and Thomas 1997, Runnalls et al. 2013, Svensson et al. 2013, Zeilinger et al. 2009, Zucchi et al. 2012), our understanding of the cause of these changes remains uncertain. Although behavior has been recognized as a sensitive endpoint that is critical for successful reproduction, comparatively few studies have examined EDC exposure effects on behavior in fish (León-Olea et al. 2014, Söffker and Tyler 2012).

This study investigated the exposure effects of gestodene (GES) on the number of eggs deposited, secondary sex characteristics, and reproductive behaviors of FHMs. A relatively short, 8 d exposure period was selected due to an interest in examining the potential short-term effects of GES exposure on both behavior and egg deposition. The FHM is an established model for aquatic toxicology and is part of Tier 1 assays that comprise the USEPA Endocrine Disrupter Screen Program, EDSP (OPPTS/USEPA 2009). In addition to the attributes that make FHMs suitable for the EDSP, they also exhibit relatively sophisticated and easily identifiable reproductive behaviors related to territorial defense, nest tending, courtship, and mating (Cole and Smith 1987) making them well suited for examining exposure effects on behavior. GES was selected for the exposure because of its documented effects on FHM reproduction (Runnalls et al. 2013), as well as its strong binding affinity for the FHM AR (Ellestad et al. 2014, Runnalls et al. 2013). It was hypothesized that following GES exposure female FHM morphology would be masculinized, egg deposition decreased, and that the reproductive behavior of both sexes would be altered.

Materials and Methods

Chemical Stock Preparation

Superstock solutions were prepared by dissolving GES (Steraloids Inc., CAS 60282-87-3) in 95% EtOH (Fisher Scientific), aliquoted, and stored at -20 °C. Each day, working stock solutions were made by a 1:2000 dilution of the superstock solution with 95% EtOH in an aluminum foil covered glass container. Fish were exposed using a flow-through system to four treatments consisting of water control, EtOH control, 10 ng/L GES, and 100 ng/L GES. EtOH concentrations for all treatments did not exceed 0.0000095%.

Fathead Minnow Exposure

Four-month old adult FHMs were obtained from Aquatic Biosystems (CO, USA) and quarantined for one-month in the lab. After quarantine, one male and two female FHMs (breeding triad per tank) were transferred from the breeding colony to the exposure system and acclimated for two weeks in dechlorinated tap water. Fish were fed twice with Tetramin © flake food and once daily with frozen adult brine shrimp. Water quality, including water temperature, pH, and dissolved oxygen, was monitored daily, and photoperiod (16 L : 8 D) was maintained using an automatic timer with lights on at 0800 h. To ensure that all triads were reproductively active, all breeding tiles were checked for the presence of eggs daily at 1500 h during the acclimation period. Any breeding tiles that had been used for spawning were removed from the tank and replaced with a fresh tile. Triads that had not deposited eggs by day 5 were replaced with fish from the breeding colony. All triads used for

this study (n = 8 for H_2O control, 10 ng/L GES, and 100 ng/L GES, with n = 7 for EtOH control due to tank damage) had laid eggs at least once by the conclusion of the acclimation period. All experiments were conducted under an approved University of Maryland IACUC protocol (R-13-60).

After the acclimation period, triads were continuously exposed to one of four treatments (water control, EtOH control, 10 ng/L GES, and 100 ng/L GES) using a flow through system that allowed for one turnover every 4 hrs (55 mL / min). The exposure consisted of a 24 h transition period, when dosing of GES was initiated, and a subsequent 8 d exposure period. For each treatment, water samples were collected daily in amber glass bottles at 1300 h dipped into a randomly selected tank and immediately frozen at -20 °C. Water samples were frozen upon collection and shipped overnight on ice to the USGS Organic Geochemistry Research Laboratory, Lawrence KA.

To determine potential effects on egg deposition, all breeding tiles and surfaces of the tank were examined daily at 1500 h, and the number of eggs deposited by each triad quantified. To examine reproductive behaviors, a 1 h video was recorded from each tank starting at 0800 h on days 1, 2, and 8. To prevent observer bias, video files were automatically labelled with random alphanumeric titles by the recording software before being analyzed. Post-analysis, results were matched to appropriate tanks and treatments using a key generated by the recording software. To analyze the videos, an independent observer examined one 30 s segment every 5 min, and specific reproductive behaviors displayed by both males and females were quantified in terms of frequency or time as they occurred inside the breeding tile (as

described in Cole and Smith (1987). These behaviors were further classified into categories involving courtship, mating, aggression, and nest tending (Table 3.1).

Table 3.1

Ethogram of courtship and spawning behaviors of importance for determining the effects of endocrine disruption

Classification	Behavior	Description (modified from Cole and Smith (1987)
Courtship	**Approach**	Male swims rapidly towards female, ceasing forward motion approximately 3-5 cm away
Courtship	**Lead**	Male positions himself close to female and performs a rapid straight line or zig-zag swimming pattern towards the breeding tile. Leads can be either successful (where female follows male and remains inside breeding tile) or unsuccessful (female does not follow male, or does not remain inside breeding tile for more than 1-2 seconds
Mating	**Lateral Quiver**	Male assumes a position parallel to female, and both individuals simultaneously perform a series of rapid lateral undulations lasting approximately 2-5 seconds
Mating	**Spawning Arch**	During a lateral quiver, the male will flip female upside down using his pectoral fins, during which the female will deposit 1-2 adhesive eggs onto the breeding tile which are immediately fertilized by male
Aggression	**Nip**	Typically directed towards the body or caudal fin, individual attempts a rapid bite
Nest Tending	**Ceiling Rub**	Individual approaches ceiling of tile and moves a portion of dorsal pad or tubercles across the ceiling in a rapid rubbing motion, typically lasting around 2-10 seconds in duration

After the 8 d exposure period, all fish were euthanized using excess MS-222 (300 mg/L, pH 7.4) and examined for the presence of secondary sexual characteristics, including the presence of nuptial tubercles, dorsal fin spot, and fatpad. One gonad lobe was processed for histology and the other snap-frozen in liquid nitrogen for future studies. All chemicals were purchased either through Fisher Scientific (Waltham, MA, USA) or VWR (Radnor, PA, USA), unless otherwise noted. For each sex, gonads from one-half of randomly chosen fish (8 females and 4 males) were preserved in Davidson's fixative for 24 h and stored in neutral buffered formalin for further histological processing and analysis following standard USEPA protocols (OPPTS/USEPA 2009). Gonads from females that exhibited masculinized behaviors and were not randomly selected initially were also preserved. Fixed tissues were embedded in paraffin wax, sectioned on a rotary microtome (5-6 μm), and alternating ribbons from half the gonad thickness were mounted on glass slides, stained with hematoxylin and eosin, and mounted with glass coverslips. Slides were examined by two researchers independently using a Zeiss Axioplan microscope to confirm the sex of each fish and check for the presence of three stages of gametogenesis: spermatozoa, spermatids, and spermatocytes in testes and vitellogenic, cortical alveolar, and pre-follicular oocytes in ovaries.

Water Chemistry Analysis

Upon receiving the water samples at the USGS Organic Geochemistry Research Laboratory, water samples were logged in, thawed, and aliquots pipetted into 2 mL glass chromatography vials to which 5 μL of a 5% tetrasodium EDTA solution was added. The bulk sample and the vialed samples were then refrozen until

analysis. Samples were analyzed using a Waters Corp. (Milford, MA) Acquity H-Class Bio ultrapressure liquid chromatograph (UPLC) and AB/Sciex (Ontario, Canada) API 5500 tandem mass spectrometer (MS/MS). Samples were analyzed for GES and progesterone-d10 as an internal standard. To compensate for potential matrix effects, each sample was analyzed unspiked and then spiked using stacked direct aqueous injection of 95 µL of sample and 5 µL of a solution (A mix) containing 1.9 ng/mL of internal standard followed by 5 µL of a solution (SA mix) containing 1.9 ng/mL of the internal standard and of GES, respectively. Addition of 5 µL of the 1.9 ng/µL A and SA solutions provided the equivalent mass of the injection of 95 µL of a sample with an analyte concentration of 100 ng/L. The compounds were separated using a 20 min gradient separation. The samples were analyzed by MS/MS using scheduled multiple reaction monitoring (SMRM) in positive-ion mode. The SMRM progestin method was set with a 1 min acquisition window centered on the retention time for each analyte. The declustering potential (DP), entrance potential (EP), collision energy (CE), and collision exit potential (CXP) was optimized for each MRM transition pair. Specific UPLC and MS/MS conditions are presented in Table 3.2.

Table 3.2

SPECIFIC TANDEM MASS SPECTROMETRY CONDITIONS FOR MEASURING GES	
ULTRA-PRESSURE LIQUID CHROMATOGRAPHY (UPLC) CONDITIONS	
ANALYTICAL COLUMN	Waters BEH 50 x 2mm with 1.7 μL packing with matching BEH guard column
MOBILE PHASE	A, 0.1% formic acid; B, methanol
SAMPLE TEMPERATURE	5 °C
COLUMN TEMPERATURE	40 °C
FLOW RATE	0.4 mL/min
TANDEM MASS SPECTROMETRY (MS/MS) CONDITIONS	
CURTAIN GAS	20 L/h
GAS 1	40 L/h
IONSPRAY VOLTAGE	2500 V
COLLISION GAS	8
SOURCE TEMPERATURE	650 °C
TARGET SMRM CYCLE	0.25 s
RESOLUTION	Unit

An 8-point standard curve of solutions ranging from 0.5 to 1000 ng/L was analyzed with the samples using stacked injections to draw up 95 μL of standard solution and 5 μL of A mix. The experimental samples then were analyzed as A-SA pairs as previously stated. A laboratory water blank sample was analyzed after the standard curve and after the analysis of each A-SA paired sample. Two MRM (protonated molecule-daughter ion) transitions were monitored for each analyte. Analytes were identified based on their analyte retention time, defined as the average ratio of the two daughter ions of the protonated analyte calculated from the standard curve ± 25% and their retention time to each other (± 1 s). Sample runs were processed using AB/Sciex Multiquant 3.0 and samples were initially quantitated using the 8-point standard curve. The resulting processed file was exported to Excel (Microsoft, 15.0) and quantitation was performed using the method of standard addition.

Statistical Analyses

Statistical analyses were performed using SAS Studio 3.3 (Cary, NC). All data sets were checked to assure normality and homoscedasticity. For the total number of eggs deposited, secondary sexual characteristic development (number of tubercle number only, as fin spot and fatpad were documented as present or not present), and male reproductive behaviors, treatments groups were compared with using a one-way ANOVA followed by a pairwise post-hoc analysis using Tukey's HSD test with significance set at $p < 0.05$. To test for main (treatment, time) and interaction effects on daily egg production, a two-way ANOVA was also performed followed by a pairwise post-hoc analysis using Tukey's HSD test with significance

set at $p < 0.05$. All displays of male behaviors by females were considered to be significant, as male behavior in female fish was only present in GES treated females and completely absent in control female fish. Unless otherwise noted, all data are shown as mean ± SEM.

Results

Water Chemistry

Daily measured concentrations of GES found in each treatment are shown in Table 3.3. No GES was detected in either the water or EtOH control samples. Inexplicably, no GES was detected in the 10 ng/L tank on day 3. Following the recommendations in the USEPA test guidelines (OPPTS/USEPA 2009), calculations including ½ of the limit of quantification (1 ng/L), thus 0.5 ng/L, and calculations excluding day 3 from the 10 ng/L treatment are both shown in Table 3.3. With day 3 excluded, concentrations for the 10 ng/L treatment ranged from 8 - 12 ng/L, with an overall average of 9.7 ± 0.5 ng/L. Concentrations for the 100 ng/L ranged from 40 - 140 ng/L, with an average of 91.2 ± 11.9 ng/L.

Table 3.3

GES Water Chemistry Results

Treatment	Day	ng/L	Treatment	Day	ng/L	Treatment	Day	Ng/L	Treatment	Day	Ng/L
W	1	nd	**E**	1	nd	**10 ng/L**	1	8	**100 ng/L**	1	100
W	2	nd	**E**	2	nd	**10 ng/L**	2	10	**100 ng/L**	2	120
W	3	nd	**E**	3	nd	**10 ng/L**	3	nd	**100 ng/L**	3	100
W	4	nd	**E**	4	nd	**10 ng/L**	4	12	**100 ng/L**	4	50
W	5	nd	**E**	5	nd	**10 ng/L**	5	11	**100 ng/L**	5	140
W	6	nd	**E**	6	nd	**10 ng/L**	6	10	**100 ng/L**	6	80
W	7	nd	**E**	7	nd	**10 ng/L**	7	9	**100 ng/L**	7	40
W	8	nd	**E**	8	nd	**10 ng/L**	8	8	**100 ng/L**	8	100
Mean ± SEM		nd			nd			8.5 ± 1.3 ng/L			91.2 ± 11.9 ng/L
Mean ± SEM (w/o day 3)								9.7 ± 0.5 ng/L			

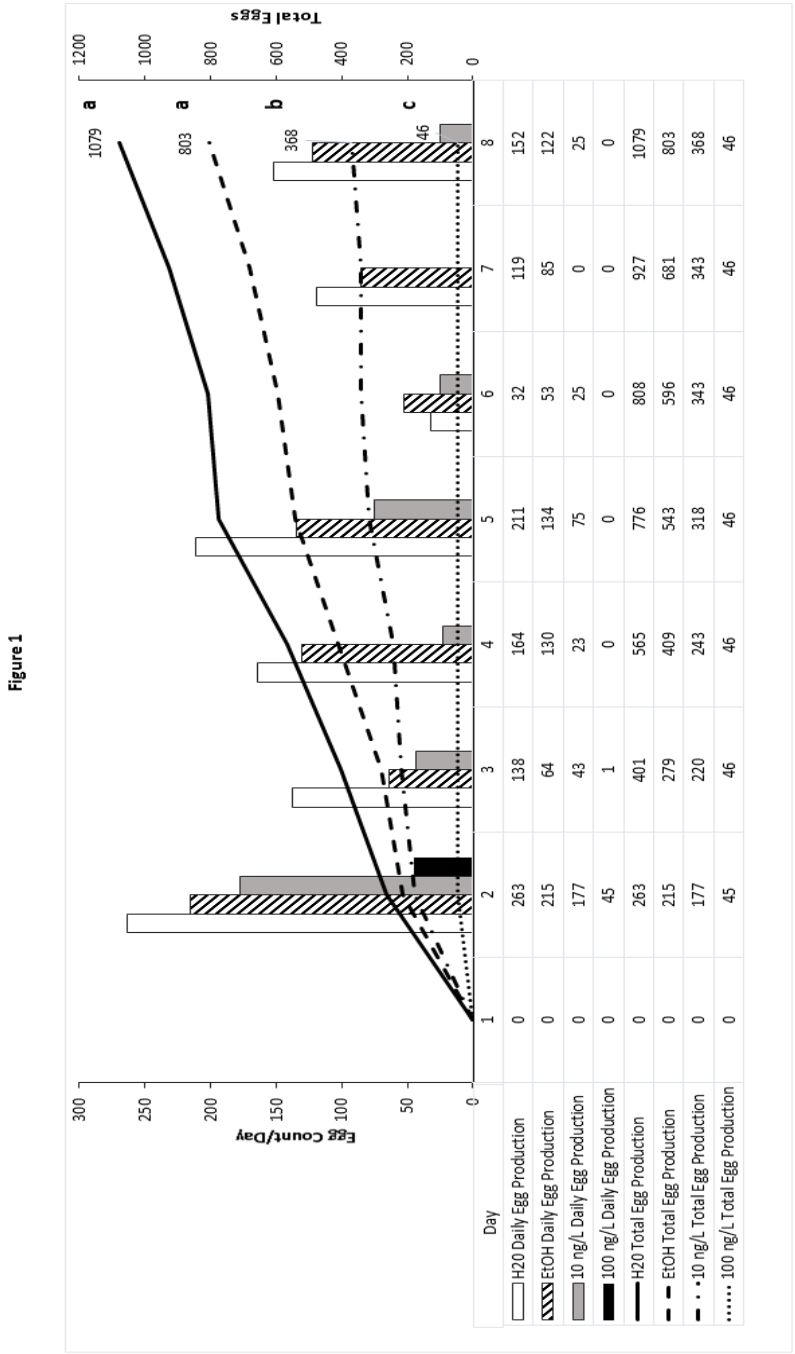
Gonadal Morphology

No treatment effects were observed on the gonadosomatic index, i.e., gonad mass as percentage of the total body mass ($p = 0.787$). Histological examination of the gonad confirmed that the initial determination of sex at dissection was accurate, and that no intersex or gross pathological differences were detected. Slides were also examined for differences in the presence of three stages of gametogenesis (spermatozoa, spermatids, spermatocytes in testes; vitellogenic, cortical alveolar, and pre-follicular oocytes in ovaries). All stages of gametogenesis were observed in all gonads and no treatment-associated differences were observed.

Egg Deposition

For each treatment level, the average daily number of eggs and the total number of eggs deposited were calculated (Figure 3.1). We found that both treatment ($p = 0.0001$) and time ($p = 0.0072$) affected egg deposition and there was no interaction between the two variables ($p = 0.9904$). There was a strong decrease in daily egg deposition in the 10 ng/L treatment and a complete cessation of deposition by day 3 for the 100 ng/L treatment. No differences were found in the total number of eggs deposited between the two controls, and significant decreases were seen for both the 10 ng/L and 100 ng/L treatments (Figure 3.1). However, no differences in daily egg or total eggs deposited by the fish between the 10 and 100 ng/L treatments were found (Figure 3.1). When the number of eggs deposited each day was compared, significantly lower numbers of eggs were deposited on days 1 and 6, and significantly higher numbers of eggs deposited on day 2 and there was no difference in egg deposition in days 3-5 and 7-8.

Figure 3.1

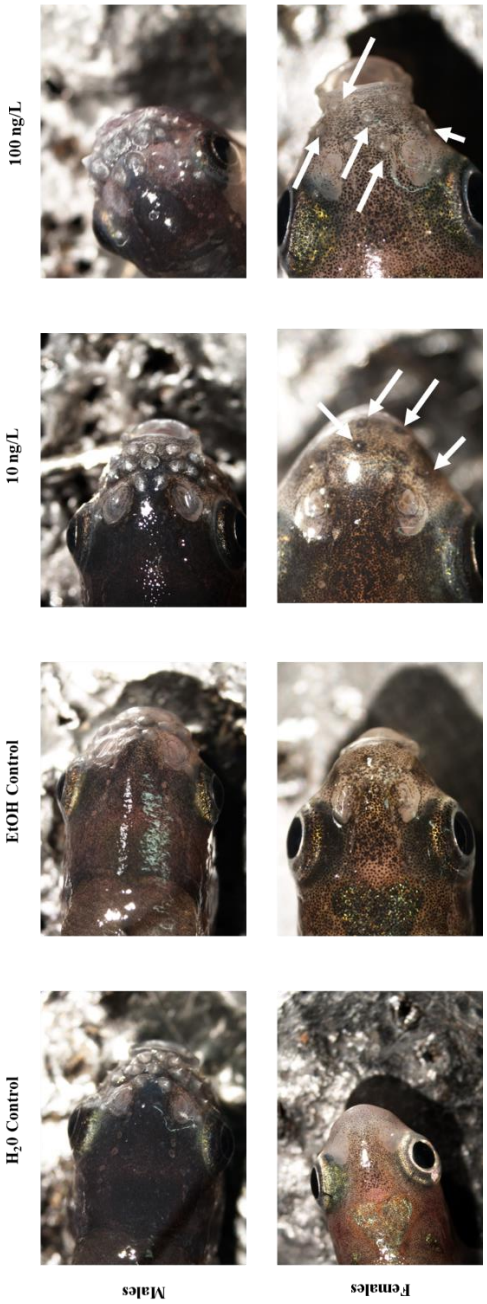


Mean (± SEM) egg production and total egg production for the H_2O control, ethanol (EtOH) control, 10 ng/L gestodene (GES), and 100 ng/L GES treatments. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).

Secondary Sex Characteristics

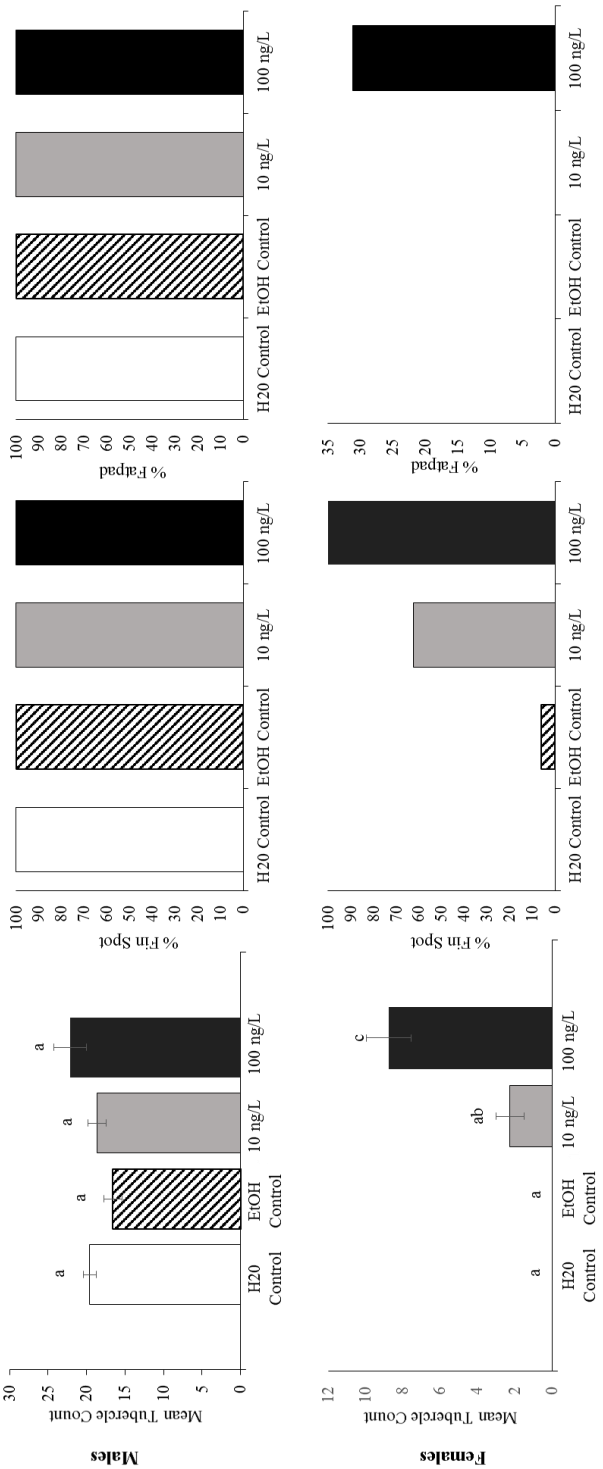
Males from all treatments exhibited nuptial tubercles, fatpad, and dorsal fin spot with no visual effects from exposure to GES (Figures 3.2 and 3.3). Females from both water and EtOH controls did not display tubercles or fatpads. With the exception of a single EtOH female, dorsal fin spots were absent from control females. Conversely, females showed apparent dose dependent masculinization in both the low and high GES treatments, with 40% of the 10 ng/L treated females and 90% of the 100 ng/L treated females having nuptial tubercles (Figures 3.2 and 3.3). Females exposed to 100 ng/L of GES developed greater numbers of tubercles than both the controls and 10 ng/L treatment ($p = 0.045$). Regarding the other secondary sex characteristics examined, 60% of females in the 10 ng/L and 100% of females in the 100 ng/L treatment developed dorsal fin spots (Figures 3.2 and 3.3). Fatpads were only present in females in the 100 ng/L treatment, and at relatively lower frequencies (0.30) compared to the other male-typical secondary sex characteristics (Figures 3.2 and 3.3).

Figure 3.2



Photographs of nuptial tubercles exhibited by males (top) and females (bottom) for the H_2O control, ethanol (EtOH) control, 10 ng/L gestodene (GES), and 100 ng/L GES treatments. Arrows highlight individual tubercles present on females. Sample size of 8 for all groups except EtOH control, which has 7.

Figure 3.3

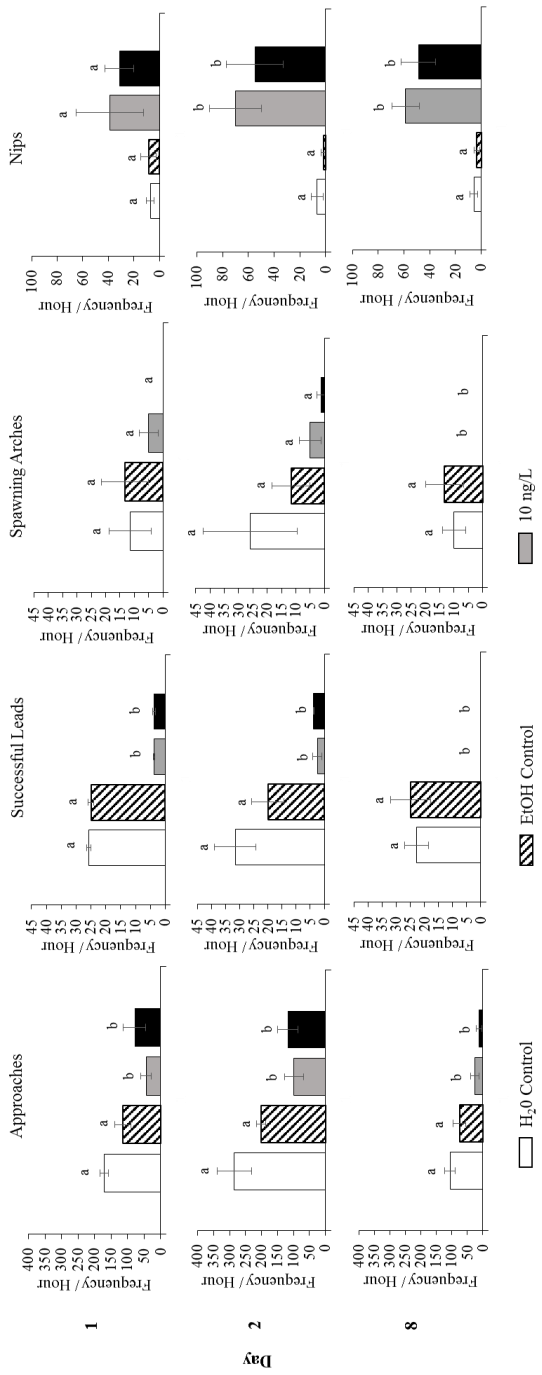


Mean tubercle count (± SEM), total % fin spot presence, and total % fatpad presence exhibited by males and females from H_2O control, ethanol (EtOH) control, 10 ng/L gestodene (GES), and 100 ng/L GES treatments after 8 days of exposure. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).

Male Behavior

GES altered various male FHM behaviors, beginning as early as the first day of exposure. Males from both the 10 and 100 ng/L treatments had fewer approaches to females on days 1, 2 and 8 ($p < 0.0001$), with no differences found between the 10 ng/L and 100 ng/L treatments ($p = 0.621$, Figure 3.4). There were fewer successful lead attempts by GES treated males compared to control males on days 1, 2, and 8, with no differences between the 10 and 100 ng/L treatments ($p = 0.421$, Figure 3.4). Spawning arch events remained consistent among treatments for days 1 and 2, but spawning arches in males were completely absent on day 8 within both the 10 and 100 ng/L treatments. While not significant ($p = 0.4213$), GES exposed males trended toward a greater number of nipping behaviors on day 1. Both 10 ng/L and 100 ng/L treated males displayed a significant increase in the frequency of nipping behaviors on days 2 ($p = 0.0088$) and 8 ($p = 0.0003$). No differences were found among all treatments on days 1, 2, and 8 for the number of observed ceiling rubs, lateral quivers, or total time spent in the breeding tile displayed by males ($p \geq 0.05$).

Figure 3.4

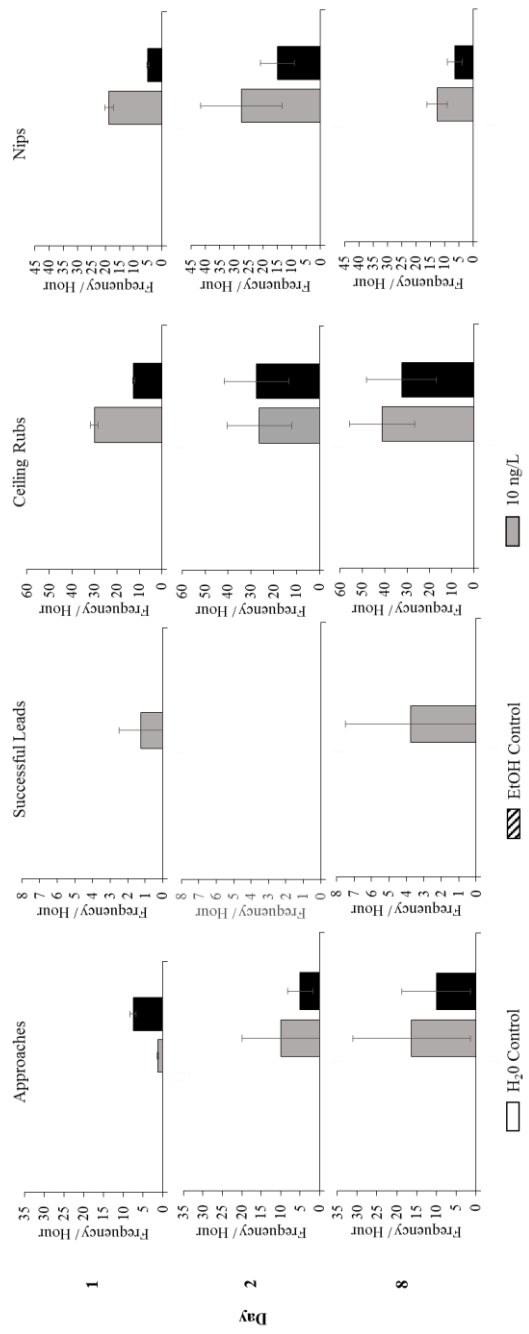


Mean frequencies (± SEM) of approach, lead, spawning arch, and nipping behavior exhibited by males from H_2O control, ethanol (EtOH) control, 10 ng/L gestodene (GES), and 100 ng/L GES treatments after 1, 2, and 8 days of exposure. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).

Female Behavior

In addition to the alteration of male behaviors, we also observed the general masculinization of behaviors in female fish in the 10 and 100 ng/L treatments. These females showed increased aggression towards males in the form of nipping behavior, and were observed guarding and cleaning breeding tiles after just one day of exposure (Figure 3.5). Interestingly, in 25% of GES treatment tanks (2 of 8 tanks for both 10 and 100 ng/L treatments), females exhibited male-typical behaviors such as approaching other females and successfully leading them back to the spawning tile where an attempt was made to perform lateral quivers. Females in both the water and EtOH controls did not exhibit any male typical behaviors.

Figure 3.5



Mean frequencies (± SEM) of approach, lead, spawning arch, and nipping behavior exhibited by females from H_2O control, ethanol (EtOH) control, 10 ng/L gestodene (GES), and 100 ng/L GES treatments after 1, 2, and 8 days of exposure. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).

Discussion

While progestins have been widely utilized by humans as a component of birth control pharmaceuticals since the 1960s, few studies have examined the exposure effects of such chemicals on the reproduction of fish and other aquatic wildlife. Those that have been performed have tested their effects over relatively long 21 - 28 d periods of exposure and have measured effects on egg deposition, hormone levels, and secondary sex characteristics, but did not include a behavioral component. Behavior is known to be a sensitive endpoint for fish exposed to endocrine disrupting chemicals and other toxicants (Aris et al. 2014, Söffker and Tyler 2012). Given the specific interest in potential effects on behavior, a shorter exposure period of 8 d was chosen to enable the documentation of reproductive behaviors at days 1, 2, and 8, yet was sufficiently long to measure effects on egg deposition as shown by other researchers using progestins similar to GES. Development of secondary sex characteristics, gonadal morphometrics (wet weight and GSI), and histopathology were also examined at the end of the study.

We chose GES for the exposure component because it was the most potent activator of the FHM nAR *in vitro* (Ellestad et al. 2014) and because of the exposure effects *in vivo* (Runnalls et al. 2013). Although there are no known environmental measurements for GES as of yet, concentrations of all other progestins have been reported in the single to low hundreds of ng/L (Fent 2015); thus, the 0, 10, and 100 ng/L concentrations were selected for our study. Using liquid chromatography tandem mass spectrometry, the concentrations of exposure system samples taken daily were measured, with results in reasonable agreement to the nominal GES

concentrations. Water quality measurements, including dissolved O_2, pH, and temperature were also measured daily, and they remained within the recommended range (OPPTS/USEPA 2009).

While GES exposure did not appear to affect development of secondary sex characteristics in male FHMs, a strong masculinizing effect was documented in females. The masculinization of female secondary sex characteristics was similar in scope, but differed in apparent sensitivity of secondary sex characteristics to GES exposure to that noted previously. Based on these results, the development of dorsal fin spots in GES exposed females appears to be the most sensitive of the secondary sex characteristics examined, followed by nuptial tubercle formation and fatpad formation (the latter of which was only seen in the 100 ng/L treatment). These results differ somewhat from a previous study which reported that dorsal fin spot and fatpad formation were the most sensitive secondary sex characteristics, and that nuptial tubercles were present, but only in the highest, 100 ng/L, exposure concentration. Other studies using other 19-nortestosterone derived progestins, norethindrone or LNG, have reported masculinized skin pigmentation or fin spot, as well as nuptial tubercles, but neither measured the presence of fatpad formation (Paulos et al. 2010, Zeilinger et al. 2009). Furthermore, female FHMs also developed secondary sex characteristics similar to reproductive males when exposed to the environmental androgen, 17β–trenbolone, including darkened skin pigmentation, nuptial tubercles, and fatpad (Ankley et al. 2003). These studies together with *in vitro* receptor transactivation research (Bain et al. 2015, Ellestad et al. 2014) indicate that GES and

other early generation progestins are producing their effects in fish at least in part through AR mediated pathways.

GES exposure negatively affected the number of eggs deposited by FHM triads on breeding tiles, compared to egg deposition in water (1,079 eggs) or EtOH (803 eggs) control triads. Importantly, the EtOH control numbers are based on 7 tanks, whereas the water control (and GES treatment) levels have 8 tanks. If EtOH control eggs are adjusted to account for the tank that was damaged (918 eggs), and the water and adjusted EtOH control number of egg deposited averaged (998 eggs), there is a 63% decline in the number for eggs in the 10 ng/L treatment group (369 eggs). Using a similar approach, the number of eggs deposited by the 100 ng/L treatment group declined by more than 95% from 998 to 46 total eggs deposited in 8 d. The finding that GES exposure precipitously decreases egg deposition by FHM triads agrees with previous research; which found similar decreases in egg deposition, but in a 21 d exposure experiment using paired FHMs. In combination, these results confirm that GES has rapid, strong, and adverse effects on reproduction.

The deleterious effect of GES on FHM egg deposition is certainly important, but how does GES exposure lead to such decreased egg deposition? Three hypotheses for how GES exposure dramatically decreased egg deposition through exposure are proposed: (1) dysfunction of the brain-pituitary-gonadal axis regulation of reproduction; (2) gonadal toxicity resulting from histopathological alterations; and (3) altered reproductive behavior of males, females, or both sexes. These hypotheses are not necessarily all-inclusive, and more than one may be involved simultaneously in the strong decline and arrest of egg deposition in this study and others.

Dysfunction of the Brain-Pituitary-Gonadal Axis

Previous studies where progestin exposure causes decreases in egg deposition are all 21 - 28 d exposures, which is far longer than the present study at 8 d. Some of these studies examined the histology of the gonad and report effects on gametogenesis in both sexes and hypothesized that normal brain-pituitary-gonadal regulation of gametogenesis had been altered (Fent 2015, Kumar et al. 2015). Studies have measured circulating concentration of sex steroid hormones and expression of gonadotropin genes. In female FHMs exposed to GES or LNG, concentration of steroid hormones 17β-estradiol and testosterone were decreased and, in males, testosterone and 11-ketotestosterone were increased (Runnalls et al. 2013a). Female FHMs exposed to norethindrone had similarly decreased circulating concentrations of 17β-estradiol, but testosterone was decreased in males exposed to the highest concentration of this progestin.

In similar studies, the frog (*Xenopus laevis*) was exposed to LNG for approximately 28 d from Nieuwkoop Faber (NF) stage 46 – 55 (period of sexual differentiation), transferred to water only, and followed by sampling at NF 58 and NF 66, when the expression of luteinizing hormone (LHβ) and follicle stimulating hormone (FSHβ) genes were quantified (Lorenz et al. 2011). The expression of LHβ and FSHβ in the brain and pituitary (combined) in both males and females decreased at 10^{-9} and 10^{-8} M at NF 58 and at just the 10^{-8} M concentration of LNG at the older stage, NF 66. While not significant, another study reported a trend in lower mean value of LHβ in adult female FHMs exposed to another progestin, norethindrone (Petersen et al. 2014). Presently, there are no published studies examining the

exposure effects on brain neuropeptides (gonadotropin releasing hormone, kisspeptin, gonadotropin-inhibitory hormone etc.) that regulate pituitary gonadotropins. As such, further research is needed to test this hypothesis.

Gonadal Toxicity Resulting in Histopathology

In addition to dysregulation of the brain-pituitary-gonad axis, GES and other progestins could be acting directly upon the gonad and affect gametogenesis directly or affect steroid hormone synthesis. GES is a potent agonist of the FHM AR and, as such, may produce similar effects as have been reported in previous research where fish have been exposed to 17β-trenbolone, a known environmental androgen. There was no difference in gonadosomatic index (percentage gonad mass relative to body mass), but there were histopathologies as documented in FHM males and females exposed to trenbolone for 28 d (Ankley et al. 2003). As characterized by the authors, histopathologies of the testis included hyperproduction of sperm (greatly expanded seminiferous tubule lumens filled with spermatozoa). In the ovaries of trenbolone treated females, there was no evidence of recent ovulation, and there was increased number of pre-ovulatory atretic follicles (Ankley et al. 2003).

In the present study, there were no observable differences in ovaries or testes across treatments. There was no difference in gonadosomatic index, which is a measure of the reproductive state of the fish (Christians 1999). The gonadosomatic index was supplemented with histological analysis of gonadal sex and three stages of gametogenesis, which confirmed that the initial determination of gonadal sex from gross examination during dissection of the fish and extraction of the gonad was accurate and agreed with the histology. In addition, all ovaries contained

vitellogenic, cortical alveolar, and prefollicular oocytes, and all testes contained spermatozoa, spermatids, and spermatocytes. While not quantitative, these histological results support the conclusion that any effect of GES on the sexual differentiation of the gonad or stages of gametogenesis was not observed. The only other known study with GES did not report histology (Runnalls et al. 2013). Importantly, this study differs from other progestin exposure studies in that it did not find treatment effects on the gonads of either sex. These differences indicate that GES may affect egg deposition via different mechanism(s) compared to LNG and other progestins. It is also possible that effects on the gonad require longer exposure periods to develop and are not observable from an 8 d exposure to GES as was conducted here.

Altered Reproductive Behavior

Reproductive behaviors were examined on day 8 to both ascertain the consistency of any exposure effects on behavior observed during days 1 and 2 and to determine if effects were altered by the duration of exposure. The recently published study on the exposure effects of another progestin, LNG, on the eastern mosquitofish is the first to expressly address effects on reproductive behavior (Frankel et al. 2016b). Males paired with females exposed to LNG for 8 d exhibited reduced interest in courtship and mating. One study by another lab provides anecdotal evidence that male FHMs exposed to the progestins LNG and drospirenone for 28 d displayed increased aggression and a lack of interest in the breeding tiles (Zeilinger et al. 2009).

Most fish use excreted amino acids, prostaglandins, and steroid hormones as pheromones (Sorensen 2015). Among other behaviors, pheromones enable chemical communication of the state of reproductive readiness and help coordinate reproductive behaviors in fishes. Of the reproductive pheromones used by cyprinid fishes, such as goldfish and carp, females release androgens (androstenedione), progestogens (free and conjugated 4-pregnen-17,20 –diol-3-one, DHP and DHP-S), and prostaglandins ($PGF_{2\alpha}$), which males use to identify individuals nearing ovulation. Receptors for some of these pheromones have been reported at the mRNA transcript level in the olfactory epithelium of other cyprinid species (i.e. goldfish and zebrafish (Kolmakov et al. 2008). Environmental measurement of steroid hormones downstream of animal agriculture facilities and human waste water treatment plants have been suggested by others to be potential pheromone signaling disrupters (Kolodziej et al. 2003, Kolodziej et al. 2004).

How pheromones work through olfactory receptors to contribute to the brain-pituitary-gonadal regulation of gametogenesis is poorly understood. However, given the presence of membrane progesterone receptors in the olfactory epithelium (Hamdani and Døving 2007) and that androgenic and progestogenic pheromones are known to orchestrate final maturation of gametes and spawning behavior (Sorensen and Stacey 2004), it is reasonable to hypothesize that GES and other environmental gestagens could alter normal reproductive behavior in exposed fishes.

In the present study, aqueous exposures to GES caused rapid and profound effects on the normal reproductive behavior of both male and female FHMs, and these effects on behavior were maintained through the end of the experiment. In

contrast to the effects of GES on secondary sex characteristics, both male and female FHMs appear to exhibit altered behaviors after just one day of GES exposure. Males showed less interest in courtship and mating (as evidenced by changes in approach and lead attempts), and showed increased aggression towards females. Increased male aggressive behavior and nest acquisition have been noted in other studies examining exposure effects to androgens such as 17α-methyltestosterone and 11-ketotestosterone (Munakata and Kobayashi 2010, Pankhurst et al. 1999). The results presented here provide further evidence that GES action is mediated through the AR, and that GES and likely other 19-nortestosterone derived progestins function as environmental androgens.

Females also became more aggressive during GES exposure, accompanied by decreases in their willingness to be led by males and related courtship and spawning behaviors. While low in frequency, females from two of eight tanks in both the 10 ng/L and 100 ng/L treatments were observed competing with males for territory acquisition and performing male-typical reproductive behaviors such as nest tending, approaches towards other females, and lateral quiver attempts with other females.

This study documents that relatively short-term exposure of adult FHMs to the human contraceptive progestin, GES, at environmentally reasonable concentrations induces rapid and profound negative effects on reproductive behavior, egg deposition, and sexual development. Because of the rapidity of the changes in behavior and the lack of deleterious effects on gonads of GES-exposed fish, it may be that the effect on egg deposition in this study is primarily the result of altered reproductive behavior. Further, it is hypothesized that the altered reproductive behavior is due to disrupted

pheromonal signaling and or direct effects on the neuroendocrine regulation of reproduction. The alteration of FHM reproductive behaviors may serve as either the initial or main driving force of the observed decline in egg deposition, providing an explanation for the disparity between the rapid shutdown of egg production observed in this and other studies, along with potential subsequent changes in the gonad during longer exposures. Regardless of mechanism, rapid and strong responses from GES exposure suggest that exposed populations of wild fish would be similarly affected.

Taken together, results presented here combined with previous research strongly suggest that certain progestins working through fish androgen receptors may be affecting population recruitment and, by extension, aquatic ecosystem health.

Chapter 4: The Effects of Progesterone and Levonorgestrel on the Reproductive Behavior, Fecundity, and Morphology of the Fathead Minnow (*Pimephales promelas*)

Introduction

In Chapter 3, short-term exposure to the progestin gestodene (GES) was found to elicit strong effects in adult FHMs, including masculinization of secondary sexual characteristics of females after 8 days, shutdown of egg deposition after 4 days, and alteration of normal reproductive behaviors in just one day. While the results strongly suggest that 1) 19- nortestosterone derived progestins as a class of compounds can cause reproductive disruption over a short period of time and 2) reproductive behavior is an extremely sensitive endpoint that can be used to determine the presence of progestin contamination in aquatic environments, further research is required to validate and expand upon these findings. While GES has been shown to be the most potent progestin in terms of transactivation of the FHM androgen receptor (Ellestad et al. 2014), it is currently not approved for human use by the U.S. Food and Drug Administration but is used extensively in Europe. Although the presence or absence of this compound in the environment has yet to be widely defined due to limited sampling studies, it is likely that GES contamination is limited in the United States. Many of the other progestins that activate fish androgen receptors and affect reproduction are utilized in the United States as part of contraceptive and hormone replacement therapies. The progestin levonorgestrel (LNG) is commonly utilized in the United States, and has been shown to activate androgen receptors in the FHM (Ellestad et al. 2014) and Murray-Darling rainbowfish (Bain et al. 2015), albeit at higher concentrations than observed with GES. Exposure to LNG for $\geq$ 21 days has been shown to cause numerous detrimental effects on several aquatic species (Bain et al. 2015, Orlando and Ellestad 2014). Some of the

more salient findings include decreased egg production in FHMs, decreased mRNA expression of AR and increased expression of nPR in zebrafish, decreased mRNA expression of Hsd3β, Hsd20β, Cyp17, AR, ERα, and FSHβ in FHM larvae, and masculinization of female FHMs (Runnalls et al. 2013). In the United States, LNG has been detected in surface waters at concentrations up to 38 ng/L (Kumar et al. 2015), and has also been found as a component of wastewater treatment plant effluent in various other countries (Kumar et al. 2015, Orlando and Ellestad 2014).

In addition to progestins, scientists studying the effects of progestogens on the reproduction and viability of aquatic organisms have shown that progesterone (P_4) activates FHM nPR with no affinity for FHM nAR. P_4 disrupts the reproduction and development of aquatic organisms, including decreases in male FHM fecundity (DeQuattro et al. 2012) and changes in FHM mRNA expression (Garcia-Reyero et al. 2013, Zucchi et al. 2013). P_4 has been measured in the environment at concentrations up to 375 ng/L both in the United States and in other countries (Orlando and Ellestad 2014), and has been shown to enter the environment as both a component of wastewater treatment plant effluent, kraft mill effluent and runoff from agricultural farms (Orlando and Ellestad 2014). Compared to those found for progestins, measured environmental P_4 levels have been considerably higher. While several studies have been conducted to examine the effects of long term ($\geq$ 21 day) exposure of FHMs to P_4 on viability, fecundity, and morphology, effects on reproductive behaviors have yet to be examined.

Thus, because it is important to determine the potential effects of LNG and P_4 on the reproduction, morphology, and behavior of the FHM, a study was designed

using the same methods from the GES experiment to examine impacts after 8 days of exposure. This allowed for 1) further investigation on the effects of different progestin compounds on reproduction, 2) a comparison of the effects between a progestin and a progestogen, and 3) validation of reproductive behavioral analyses as a method to determine the presence of both progestins and progestogens. To remain environmentally relevant, nominal concentrations of 10 and 100 ng/L for LNG and 30 and 300 ng/L for P_4 were selected.

Materials and Methods

Chemical Stock Preparation

Superstock solutions were prepared by dissolving lyophilized LNG (Steraloids Inc., CAS 60282-87-3) or P_4 (Steraloids Inc., CAS 57-83-0) in 95% EtOH (Fisher Scientific), aliquoted, and stored at -20 °C. Separate 8 day exposures were conducted for each chemical. Each day, working stock solutions were made by a 1:2000 dilution of the superstock solution with 95% EtOH in an aluminum foil covered glass container. Using this method, fish were exposed using a flow-through system to four treatments. For LNG, treatments consisted of a water control, EtOH control, 10 ng/L LNG, and 100 ng/L LNG. For P_4, treatments consisted of a water control, EtOH control, 30 ng/L P_4 and 300 ng/L P_4. EtOH concentrations for all treatments did not exceed 0.0000095%.

Fathead Minnow Exposure

For each chemical exposure, sexually mature adult FHMs (~ 150 dph) were obtained from Aquatic Biosystems, CO, USA and quarantined for one-month in the

lab. After quarantine, groups of one male and two females (breeding triad per tank) were transferred from the breeding colony to the exposure system and acclimated for two weeks in dechlorinated tap water. Fish were fed twice daily with Tetramin ® flake food and once daily with frozen adult brine shrimp. Water quality, including water temperature, pH, and dissolved oxygen, was monitored daily, and photoperiod (16H : 8 D) was maintained using an automatic timer with lights on at 0800 h and off at 1600 h. To ensure that all triads were reproductively active, the breeding tile in each tank was checked for the presence of eggs daily at 1500 h during the acclimation period. Any breeding tiles that had been used for spawning were removed from the tank and replaced with a fresh tile. Triads that had not deposited eggs by day 5 were replaced with fish from the breeding colony and their reproductive status examined before the start of the experiment. All triads used for this study (n = 8 for H_2O control, 10 ng/L LNG, 100 ng/L LNG, 30 ng/L P_4, and 300 ng/L P_4 and n = 7 for EtOH control due to tank damage) had laid eggs at least once by the conclusion of the acclimation period. All experiments were conducted under an approved University of Maryland IACUC protocol (R-13-60).

After the acclimation period, triads were continuously exposed to one of the four treatments using a flow through system that allowed for one turnover every 4 hrs (55 mL / min). The exposure consisted of a 24 h transition period, when dosing was initiated, and a subsequent 8 d exposure period.

To determine potential effects on egg deposition, all breeding tiles and surfaces of the tank were examined daily at 1500 h, and the number of eggs deposited by each triad quantified. To examine reproductive behaviors, a 1 h video was

recorded from each tank starting at 0800 h on days 1, 2, and 8. To prevent observer bias, video files were automatically labeled with random alphanumeric titles by the recording software before being analyzed. Post-analysis, results were matched to appropriate tanks and treatments using a key generated by the recording software. Behavioral analyses were conducted using JWatcher (V 0.9). An independent observer examined videos, and the frequency of ceiling rubs, approaches, leads, lateral quivers, and spawning arches were quantified using timepoint sampling (one 30 s segment every 5 min). For the purpose of this study, these behaviors were further classified into categories involving courtship, mating, aggression, and nest tending.

After the 8 d exposure period, all fish were euthanized using excess MS-222 (300 mg/L, pH 7.4) and examined for the presence of secondary sexual characteristics, including the presence of nuptial tubercles, dorsal fin spot, and fatpad. One gonad lobe was processed for histology and the other snap-frozen in liquid nitrogen for future studies. All chemicals were purchased either through Fisher Scientific (Waltham, MA, USA) or VWR (Radnor, PA, USA), unless otherwise noted. For each sex, gonads from one-half of randomly chosen fish (8 females and 4 males) were preserved in Davidson's fixative for 24 h and stored in neutral buffered formalin for further histological processing and analysis following standard USEPA protocols (OPPTS/USEPA 2009). Fixed tissues were embedded in paraffin wax, sectioned on a rotary microtome (5-6 μm), and alternating ribbons from half the gonad thickness were mounted on glass slides, stained with hematoxylin and eosin, and mounted with glass coverslips. Slides were examined by two researchers

independently using a Zeiss Axioplan microscope to confirm the sex of each fish and check for the presence of three stages of gametogenesis: spermatozoa, spermatids, and spermatocytes in testes and vitellogenic, cortical alveolar, and pre-follicular oocytes in ovaries.

Statistical Analyses

Statistical analyses were performed using SAS Studio 3.3 (Cary, NC). All data sets were checked to assure normality and homoscedasticity. For the total number of eggs deposited, secondary sexual characteristic development (number of tubercle number only, as fin spot and fatpad were documented as present or not present), and male reproductive behaviors, treatments groups were compared using a one-way ANOVA followed by a pairwise post-hoc analysis using Tukey's HSD test with significance set at $p < 0.05$. To test for main (treatment, time) and interaction effects on daily egg production, a two-way ANOVA was also performed followed by a pairwise post-hoc analysis using Tukey's HSD test with significance set at $p < 0.05$. Unless otherwise noted, all data are shown as mean ± SEM.

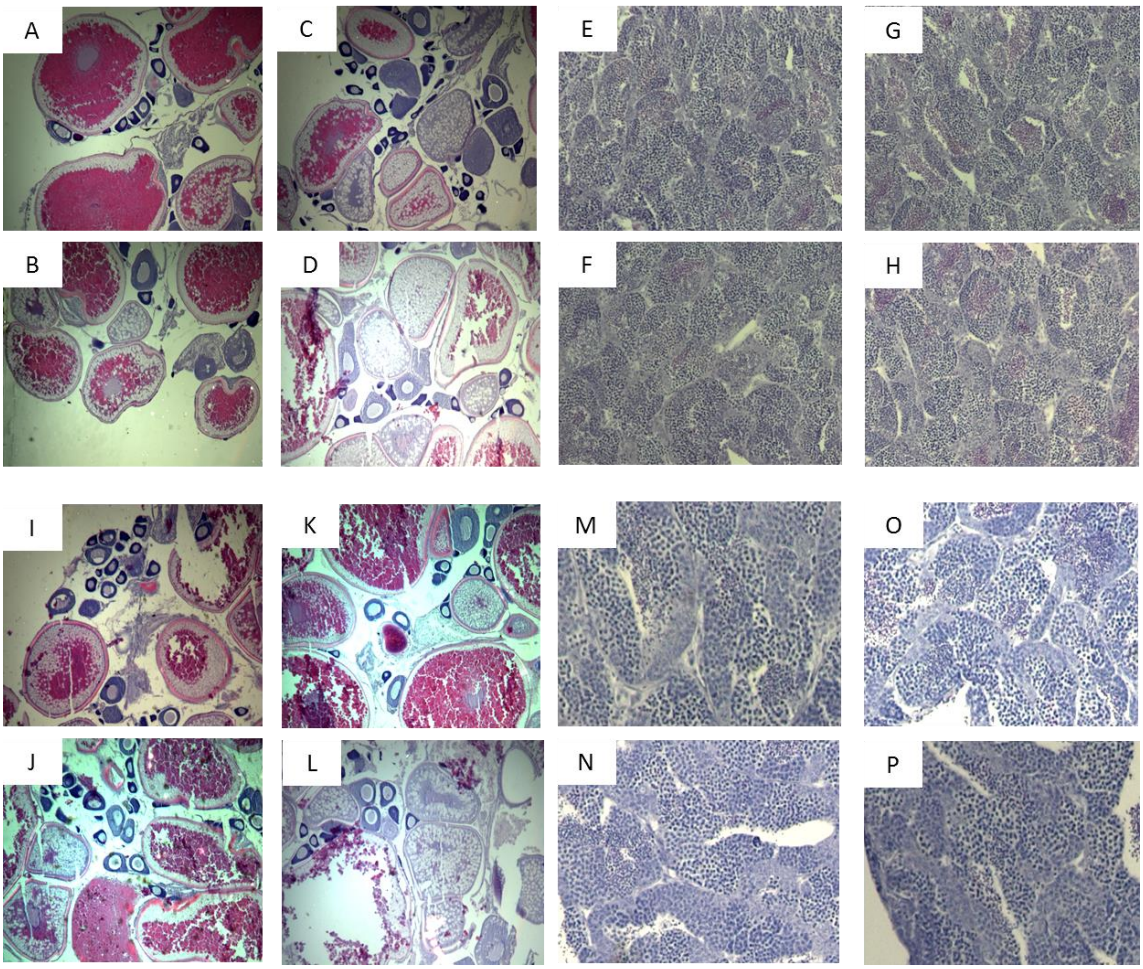
Results

Gonadal Morphology

No treatment effects were observed for either LNG ($p = 0.533$) or P_4 ($p = 0.640$) on the gonadosomatic index. Histological examination of the gonad confirmed that the initial determination of sex at the time of establishing triads was accurate, and that no intersex or gross pathological differences were present. All stages of

gametogenesis were observed in all gonads and no treatment-associated differences were observed (Figure 4.1).

Figure 4.1



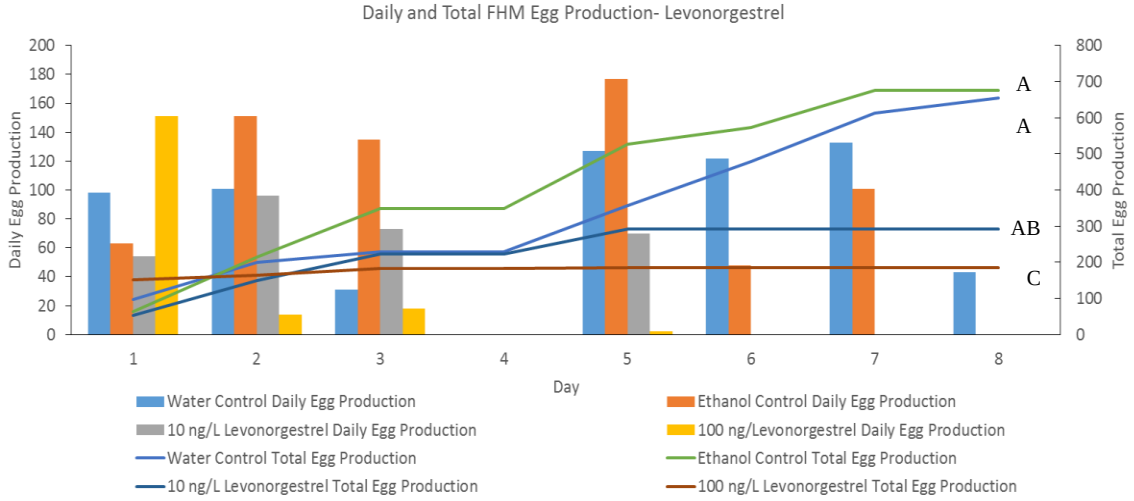
Micrographs of ovarian tissue of female FHMs treated with (A + I) H_2O control, (B + J) EtOH Control, (C) 10 ng/L LNG, (D) 100 ng/L LNG, (K) 30 ng/L P_4, and (L) 300 ng/L P_4. Micrographs of testicular tissue of male FHMs treated with (E + M) H_2O control, (F + N) EtOH Control, (G) 10 ng/L LNG, (H) 100 ng/L LNG, (O) 30 ng/L P_4, and (P) 300 ng/L P_4.

Egg Deposition

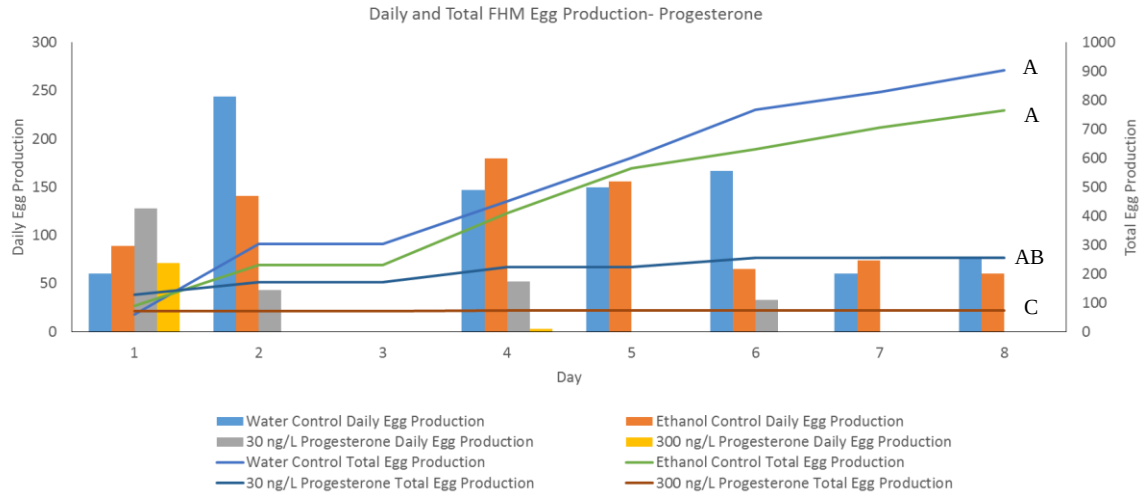
For each treatment level, the average daily number of eggs and the total number of eggs deposited were calculated (Figure 4.2). For LNG, there was a decrease in daily egg deposition in both the 10 ng/L and 100 ng/L treatments, leading to complete cessation of deposition by day 5. In terms of average egg deposition rates, no differences were found in the total number of eggs deposited between the two controls, as well as between the EtOH control and 10 ng/L treatment. A significant decrease in egg deposition was found between the 100 ng/L LNG treatment and all other treatments (Figure 4.2A). For P_4, decreases in egg deposition were apparent from day 2, with complete cessation observed starting on day 4 for both 30 ng/L and 300 ng/L treatments (Figure 4.2B). No differences in average egg deposition were found between the two controls, as well as between the EtOH and 30 ng/L P_4 treatment. A significant decrease in average egg deposition was observed between the 300 ng/L P_4 treatment and all others.

Figure 4.2

A



B

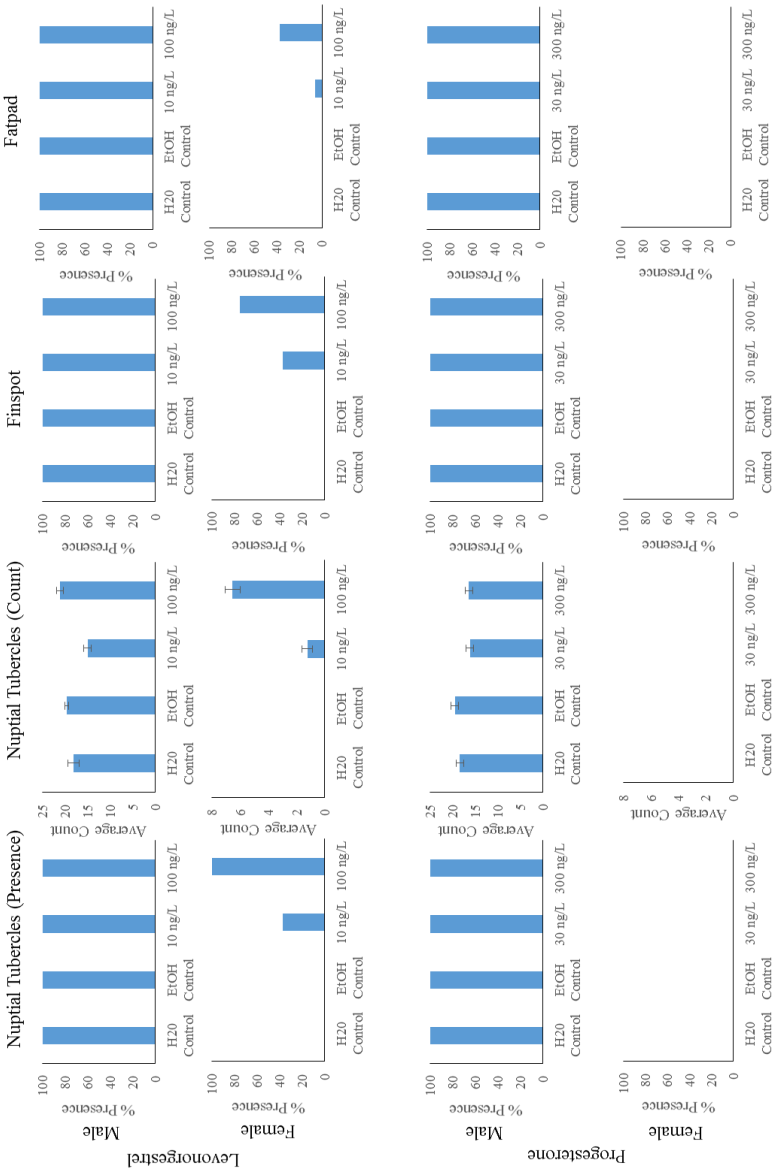


4.1 A- Mean (± SEM) egg production and total egg production for the H_2O control, ethanol (EtOH) control, 10 ng/L LNG, and 100 ng/L LNG treatments. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).
4.1 B- Mean (± SEM) egg production and total egg production for the H_2O control, ethanol (EtOH) control, 30 ng/L P_4, and 300 ng/L P_4 treatments. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).

Secondary Sex Characteristics

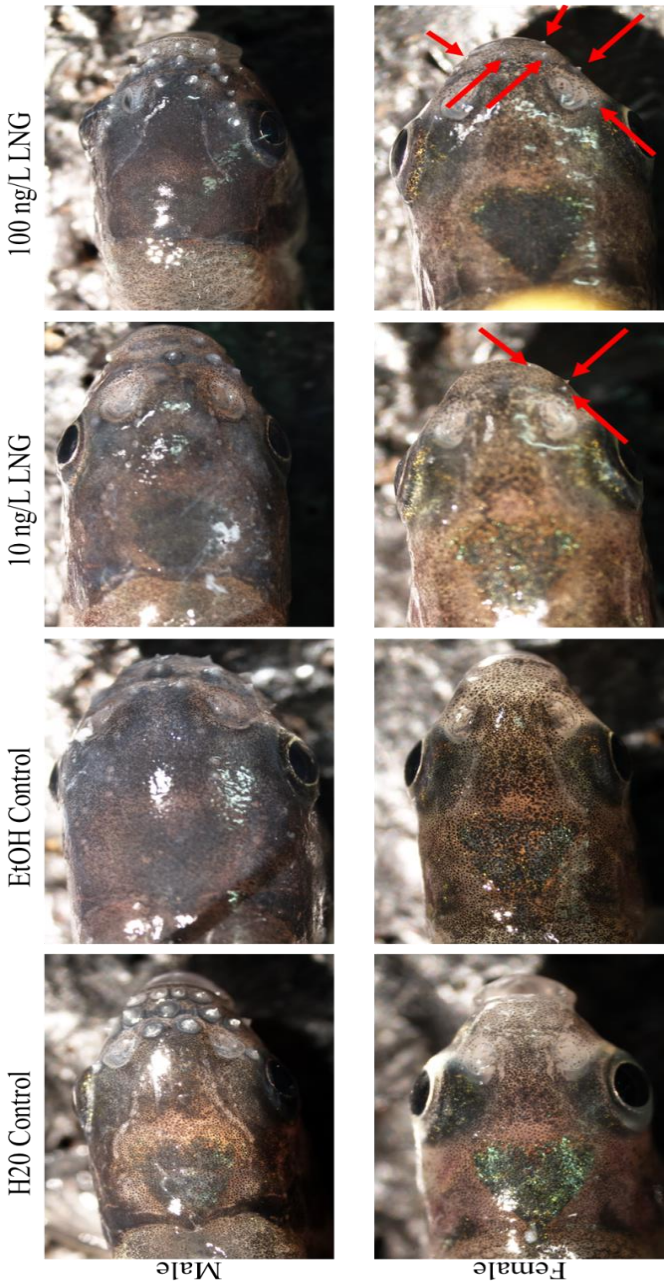
Males from both the LNG and P_4 treatments all exhibited nuptial tubercle, fatpad, and dorsal fin spot development (Figure 4.3). Females exposed the 10 ng/L LNG and 100 ng/L LNG treatments exhibited masculinization, including the development of nuptial tubercles (40% and 100%, respectively) (Figure 4.4), dorsal finspots (40% and 75%, respectively) and fatpads (10% and 31.25%, respectively). Females from all treatments in the P_4 study did not show any signs of masculinization.

Figure 4.3



Percent presence and average count of nuptial tubercles, percent presence of fin spot, and percent presence of fatpads on both male and female FHMS for each LNG and P_4 treatment. Sample size of 8 for all groups except EtOH control, which has 7.

Figure 4.4



Photographs of nuptial tubercles exhibited by males (top) and females (bottom) for the H_2O control, ethanol (EtOH) control, 10 ng/L LNG, and 100 ng/L LNG treatments. Arrows highlight individual tubercles present on females. Sample size of 8 for all groups except EtOH control, which has 7.

Behavioral Results

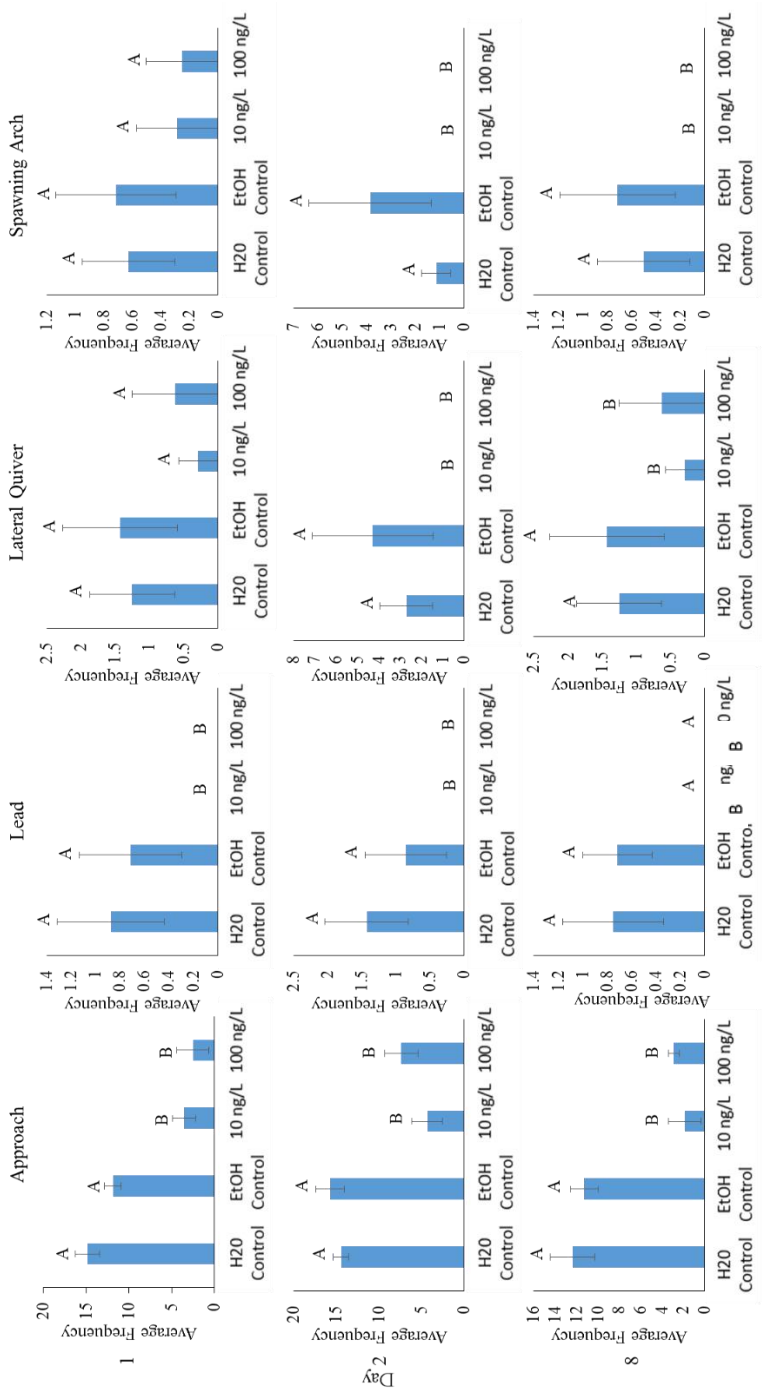
Levonorgestrel (LNG)

Exposure to LNG altered reproductive behaviors as early as the first day of the exposure period. After one day of exposure, triads in both the 10 ng/L and 100 ng/L treatments exhibited a decrease in approach behavior ($p = 0.0266$) which continued on days 2 and 8 (Figure 4.5). Leading behavior ceased on day 1, while lateral quiver, and spawning arch behaviors were all absent on days 2 and 8 (Figure 4.5). No differences in nest tending behavior or time spent in the breeding tile were observed. Additionally, no aggressive nipping or butting behaviors by either female or male fish were observed during the study. Interestingly, females from one triad in the 10 ng/L LNG treatment and two triads in the 100 ng/L treatment were observed exhibiting male nest tending behaviors on day 8.

Progesterone (P_4)

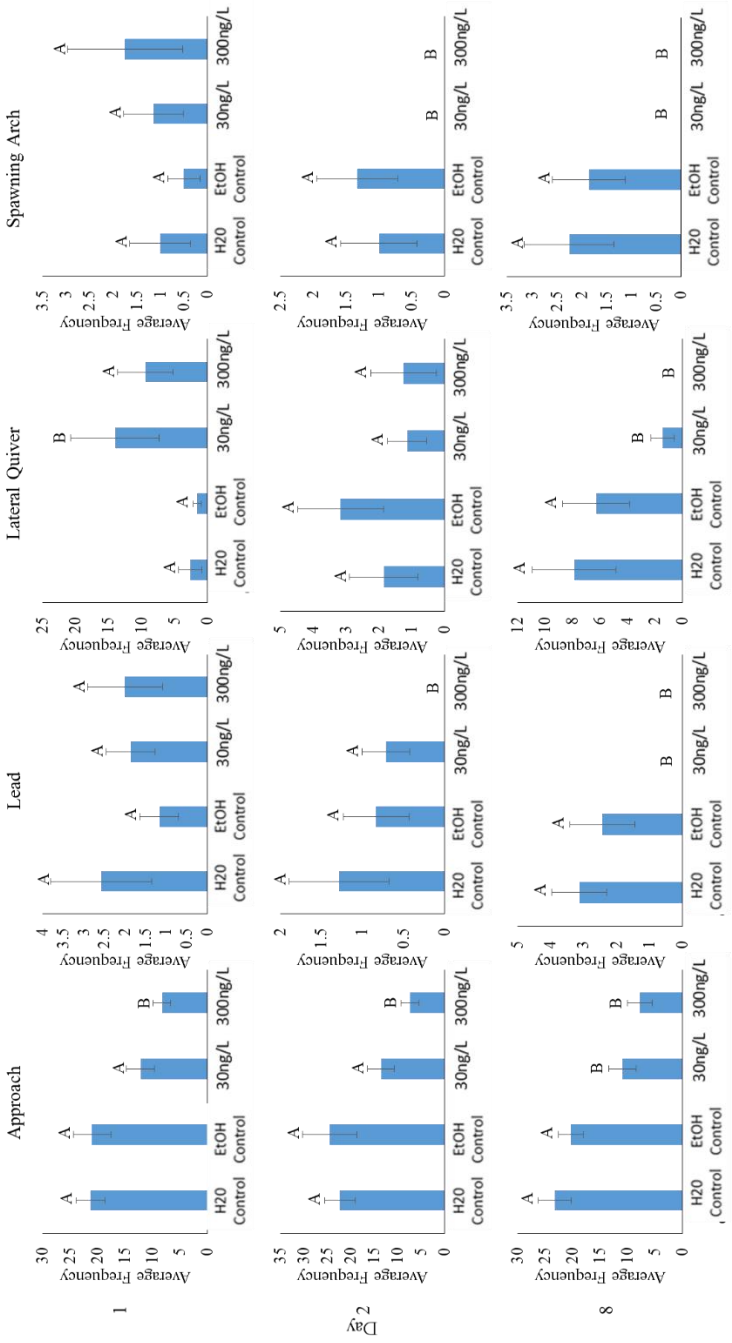
When compared to controls, triads exposed to P_4 at both 30 ng/L and 300 ng/L also exhibited altered normal reproductive behaviors (Figure 4.6). Approach behavior was significantly reduced on day 8 for the 30 ng/L treatment, and was also decreased on days 1, 2, and 8 for the 300 ng/L treatment. Lead behavior was completely absent on day 8 for the 30 ng/L treatment as well as on days 2 and 8 for the 300 ng/L treatment. Surprisingly, the frequency of lateral quivers increased in the 30 ng/L treatment on day 1, showed no difference compared to controls on day 2, and was significantly decreased on day 8. Triads exposed to both the 30 ng/L and 300 ng/L treatments exhibited decreased lateral quivers and cessation of spawning arches on days 2 and 8.

Figure 4.5



Mean frequencies (± SEM) of approach, lead, lateral quiver, and spawning arch behavior exhibited by males from H_2O control, ethanol (EtOH) control, 10 ng/L LNG, and 100 ng/L LNG treatments after 1, 2, and 8 days of exposure. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).

Figure 4.6



Mean frequencies (± SEM) of approach, lead, lateral quiver, and spawning arch behavior exhibited by males from H_2O control, ethanol (EtOH) control, 30 ng/L P_4, and 300 ng/L P_4 treatments after 1, 2, and 8 days of exposure. Sample size of 8 for all groups except EtOH control, which has 7. Letters indicate significant differences ($p < 0.05$).

Discussion

LNG

Previous studies suggested that exposure to LNG would disrupt several key aspects of reproduction in the fathead minnow at low ng/L concentrations. Exposure of adult FHMs to LNG caused the development of male secondary sexual characteristics in females and the cessation of egg deposition after 21 days of exposure (Runnalls et al. 2013), and in females decreases pregnenolone, DHP, testosterone, and 11-KT plasma concentrations after 7 days of exposure (Overturf et al. 2014). Exposure to LNG masculinizes females and disrupt courtship and display behavior in eastern mosquitofish after 8 days (Frankel et al. 2016b). While this was the first study to examine the effects of LNG exposure on FHM reproductive behavior, it was hypothesized that exposure would decrease both courtship and spawning behavior similar to those found as a results of GES exposure in Chapter 3 based on its ability to activate FHM AR.

LNG affected reproductive behaviors, resulting in decreased approach and leading behaviors on day 1, and disruption of all other reproductive behaviors on day 2. While males and females exposed to LNG did not exhibit increased aggression, the observation of some females exhibiting nest tending behaviors suggests that LNG also masculinizes female behavior, but with less severity compared to the results found during the GES study (Chapter 3). The possibility that the differences in observed effects are due to the relatively lower receptor activation of LNG for fish AR compared to GES (Ellestad et al. 2014) is supported by results showing that egg deposition was shut down on day 3 for the 100 ng/L GES treatment (Frankel et al.

2016a), but required 5 days to affect the same result for the 100 ng/L LNG treatment. While the physical masculinization of females was observed in response to both LNG and GES exposure, a higher proportion of females exhibited dorsal finspots and fatpads in the GES study (Frankel et al. 2016a) than those exposed to LNG for both the 10 ng/L and 100 ng/L treatments. When FHMs were exposed to other strong AR agonists such as 17β-trenbolone(Ankley et al. 2003) and 17α-methyltestosterone(Pawlowski et al. 2004) similar effects regarding female masculinization and disruption of egg production were observed, providing further evidence that the decreased effects could be due to differences in AR activation. These results provide additional evidence supporting the detrimental effects of progestin exposure on the fecundity, morphology, and behavior of a model aquatic species. As effects were observed after just 8 days of exposure, it appears that $\geq$ 21 d exposures are insufficient to fully elucidate the rapid effects of LNG on FHM reproduction. As such, future studies using shorter exposure periods (minutes, hours, days, etc.) and more sensitive endpoints (reproductive behavior, gene expression, etc.) are required to further assess its potential for environmental impact.

While the mechanism(s) controlling these changes remain unclear, several possible mechanisms may be operating either independently or synergistically. In fish, several endogenous hormones have been shown to serve as reproductive pheromones, including the androgen androstenedione and its conjugates (Sorensen 2015). Androstenedione has been shown to bind the nAR of goldfish (Wells and Van Der Kraak 2000), and when excreted by non-ovulating females serves as a rapid repressor of male mating behaviors (Sorensen and Wisenden 2015). If

androstenedione is able to exert changes in reproductive via the activation of nAR, it is possible that LNG may mimic the pheromonal effects of androstenedione, resulting in the rapid suppression of courtship and mating behaviors. In goldfish, it has been found that exposure of olfactory epithelium to DHP, DHP-S, androstenedione, and PGs cause the stimulation of neurons that lead to the lateral part of the medial olfactory tract, which in turn have been found to be responsible for the mediation of reproductive behaviors (Hamdani and Døving 2007). While the response of the lateral medial olfactory tract to LNG was not examined in this study, it is possible that LNG causes changes in reproductive behaviors through this mechanism. As such, future research examining the behavioral response of FHMs to androstenedione and LNG in conjunction with its ability to affect action potentials in the olfactory system may help to test this hypothesis.

The observed impacts of LNG on reproduction may also be attributed to changes in the mRNA transcript profiles of several genes involved with steroidogenesis. When juvenile FHMs were exposed to a low concentration (16.3 ng/L) of LNG for 28 d, the down-regulation of 3β-HSD, 20β-HSD, CYP17, AR, ERα, and FSH was observed (Overturf et al. 2014). Additionally, when adult female FHMs were exposed to 100 ng/L of LNG *ex-vivo* for 7 d, they exhibited decreases in DHP, pregnenolone, testosterone, and 11-ketotestosterone production (Overturf et al. 2014). While the effects of LNG on GnRH and gonadotropins such as LH and FSH are currently unknown, it is likely that LNG may also disrupt normal GnRH signaling and subsequent pituitary gonadotropin release. Taken together, these findings suggest that the masculinization of females, suppression or cessation of egg deposition, and

shutdown of select reproductive behaviors may have also been due to shifts in hormone levels.

P_4

It was expected that P_4 would cause the disruption of egg deposition, decrease the presence of male secondary sexual characteristics, and cause the disruption of both male and female reproductive behaviors. During this study, the cessation of egg deposition after 4 d of exposure to both concentrations of P_4 was noted, as well as behavioral alterations after just 1 d of exposure. P_4 did not masculinize females, and did not have any significant effect on the expression of SSCs or GSI in males. The observed decrease in fecundity supports previous research which noted reduced egg deposition after 21 d of exposure (DeQuattro et al. 2012). While DeQuattro et al. (2012) observed an increase in male GSI, it was not observed here. This was perhaps due to the shorter exposure period compared to the other study (8 d vs 21 d). As P_4 has been shown to weakly transactivate FHM nPR receptors (Ellestad et al. 2014), increase testosterone production in FHM testes *in vitro* (Chishti 2013), and decrease prostaglandin E synthase 3b expression (Chishti 2013), it was surprising that no effects on the expression of androgen induced SSCs in males was observed. It is possible that males are able to utilize a compensatory mechanism involving the conversion of P_4 into T via changes in steroidogenesis. While decreases of mPRβ expression as a result of progesterone exposure have only been noted in female FHMs (Petersen et al., 2015), it is possible that males are also able to alter nPR expression as a way of mitigating the effects of P_4 exposure. Such a mechanism would also explain

why these changes were only observed as a result of longer exposure periods or through *in vitro* methods, which result in the decoupling of the HPG axis.

While both LNG and P_4 ultimately shutdown reproductive behaviors over the course of 8 d, the pattern in which behaviors were altered was markedly different. While a decrease in approach behavior after 1 day of exposure in the 300 ng/L P_4 treatment, some behaviors involving courtship and spawning were either significantly increased (lateral quivers in the 30 ng/L treatment) or showed an increasing trend compared to the controls (lateral quivers and spawning arches in the 300 ng/L treatment). Although the mechanisms controlling these changes are currently unknown, a tentative explanation is possible when placed in the context of pheromonal communication and hormonal control of gamete maturation.

Cyprinids utilize the progestogen DHP as a reproductive pheromone. When expressed by an ovulating female, DHP is detected by the olfactory epithelium in males, causing rapid increases in courtship behaviors (Sorensen and Wisenden 2015). In goldfish and carp, this change in behavior has been shown to be mediated via the prolonged activation of V1r receptors in the bulbular neurons (Pfister and Rodriguez 2005), causing rapid increases in courtship behaviors (Sorensen and Wisenden 2015). As such, other progestogens such as P4 may also cause behavioral changes using the same pathway.

One other possible explanation for the increase in spawning behaviors involves the alteration of gamete maturation in exposed females. In cyprinids, later stage developing follicles begin to produce DHP in response to increased LH stimulation (Evans et al. 2013). DHP then causes the migration and breakdown of the

germinal vesicle, resulting in final oocyte maturation. Once this process is completed, ovulation occurs shortly afterwards and is accompanied by the production and release of $PGF_{2\alpha}$, which has also been shown to cause changes in reproductive behaviors (Harvey and Carolsfeld 1993, Sorensen and Wisenden 2015). Thus, it is possible that the initial uptake of exogenous P_4 by females results in increased rates of follicular maturation, leading to a greater number of ovulating females, an increase in the release of the pheromone $PGF_{2\alpha}$, and ultimately an increase in reproductive behaviors. Studies examining the role of DHP in the final oocyte maturation of FHMs as well as the pheromonal role of DHP, $PGF_{2\alpha}$ and their conjugates in FHM reproduction are required to test these hypotheses.

Conclusions

This study builds upon earlier documentation of the rapid and profound exposure effects of the progestin GES on reproductive behavior, egg deposition, and secondary sexual characteristics at environmental concentrations. Using the same methods, a closer examination of the exposure effects of the progestogen P_4 indicated that concentrations far lower than what has been reported in the environment can also disrupt reproductive behavior and fecundity in the FHM. It is hypothesized that the observed changes for each compound result from pheromonal disruption, changes in steroidogenesis, and/or alterations of neuroendocrine regulation, and that reproductive behavior continues to be the most sensitive endpoint of those tested. As aquatic environments affected by EDCs often contain complex mixtures of androgenic, gestagenic, estrogenic, thyroidogenic, and other neuroactive chemicals, future studies should also be performed using combinations of EDCs from different chemical

classes to determine any additive, synergistic, or antagonistic effects. Given the sensitivity of FHM reproductive behavior to both androgenic and progestogenic substances, the results presented here support the expanded use of behavioral assays as an endpoint for studies examining the effects of other classes of EDCs (e.g. estrogens, thyroid hormones, etc.). In conclusion, this study highlights the need for further examination of the effects of short term gestagen exposure on aquatic organisms using multiple endpoints.

Chapter 5: The *In vivo* and *In vitro* Exposure Effects of Several Gestagens on Fathead Minnow (*Pimephales promelas*) Sperm Motility

Introduction

Compared to our understanding of the effects of gestagens on the embryonic development, fecundity, and reproductive morphology of teleosts, very little is known about their impacts on sperm motility despite that it critically influences fertilization success due to the short duration of activity (Kime et al. 2001). Motility traits such as velocity (Gage et al. 2004) and percentage of motile sperm (Linhart et al. 2000) are both positively correlated with fertilization success in numerous species.

In fish, a combination of endogenous androgens and progestogens mediate the final maturation of gametes (Scott et al. 2010) and cause increased milt volume (Evans et al. 2013). Androgens such as testosterone and 11-ketotestosterone are responsible for the division of spermatogonia into spermatocytes and the liberation of mature spermatids into the testes lumen, respectively (Evans et al. 2013). In males, leydig cells also produce 17α,20β–dihydroxypregnenone which induces DNA replication in Japanese eel (*Anguilla japonica*) spermatogonia (Miura et al. 2006), and capacitate motility via increases in seminal plasma pH in Japanese huchen (*Hucho perryi*) (Higashino et al. 2003). As membrane progesterone receptors have been identified on the sperm of Atlantic croaker (*Micropogonias undulates*) (Tubbs et al. 2010, Tubbs and Thomas 2008), it is possible that progestogens also play a role in sperm motility post-maturation. Currently, it is unclear if fish sperm also contain nuclear androgen receptor (nAR) or membrane androgen receptors (mAR) that can be used to sense and respond to androgenic ligands. However, the presence of at least nAR in fish testes (Evans et al. 2013) and the use of androstenedione by both males (Sorensen et al. 2005) and females (Sorensen and Wisenden 2015) to affect

behavioral changes and decrease milt production (Sloman et al. 2006) suggest that exposure to certain progestins may also have an effect on motility either by direct binding to sperm cells or via activation of neurons in the ventral region of the olfactory bulb (Hamdani and Døving 2007). Gestagens such as gestodene and levonorgestrel strongly activate FHM AR, whereas progesterone only activates FHM nPR (Ellestad et al. 2014).

With the advent of computer-assisted sperm analysis (CASA), it is possible to objectively quantify these endpoints and others such as curvilinear velocity, average path velocity, and percent linearity that are not discernable to the naked eye (Kime et al. 2001). CASA has been successfully used in previous research to examine the effects of EDCs on sperm motility in fish, including progesterone (Murack et al. 2011) and 17β-estradiol (Schoenfuss et al. 2011), 17α-ethinylestradiol (Hashimoto et al. 2009), and clofibric acid (Runnalls et al. 2007), providing an easy and accurate method for identifying the effects of exposure on male gamete quality.

Table 5.1- Parameters Measured Using Computer Assisted Sperm Analysis (CASA)

Parameter	Unit	Description
Curvilinear Velocity (VCL)	μm/sec	Velocity over the actual sperm track
Average Path Velocity (VAP)	μm/sec	Velocity over a calculated, smoothed path
Straight Line Velocity (VSL)	μm/sec	Velocity over the straight-line distance between the beginning and end of the sperm track
Linearity (LIN)	%	Straight-line distance divided by the incremental deviations along the actual path
Beat Cross Frequency (BCF)	Hz	The frequency with which the sperm crosses the smoothed path
Elongation	μm	Ratio of head width to length
Duration	sec	Period of time in which sperm remain actively motile

This study investigated the *in vivo* and *in vitro* effects of gestagen exposure on FHM sperm motility. In experiment 1, male FHMs were exposed to either an EtOH control or one of two concentrations of gestodene (GES), levonorgestrel (LNG), or progesterone (P_4) for 8 days, after which semen samples were collected and analyzed using CASA. Based on the results from Chapters 3 and 4 of this dissertation and studies by other researchers, it was expected that exposure to each of the above chemicals would decrease sperm motility in male FHMs. In experiment 2, semen samples were collected from untreated male FHMs, and aliquots exposed to the same treatments during the initial activation of motility. As this method more closely mimics the exposure route of sperm to pheromones naturally released by males and females, it was hypothesized that motility would decrease in response to AR binding chemicals (resembling androstenedione) and increase in response to PR binding chemicals (resembling DHP).

Materials and Methods

Research Organism Acquisition

Sexually mature male FHMs were selected from a breeding colony maintained at the University of Maryland, College Park. Males were identified as sexually mature through the presence of three secondary sexual characteristics; dorsal fatpads, dorsal finspots, and nuptial tubercles. To facilitate breeding conditions, fish were maintained under optimal conditions (pH= 7.4, dissolved oxygen $\geq$ 95%, water temp = 23.3 - 24.4^{o}C, 16 L: 8 D photoperiod) and fed twice a day to satiation using Tetramin ® flake food and once a day with freshly hatched brine shrimp. All

experiments were conducted under an approved University of Maryland IACUC protocol (R-13-60).

Experiment 1- In vivo Exposure

Exposures for this portion of the study were performed using a static replacement method. Groups of sexually mature males (n = 6) were placed into individual 10 gallon aquariums, containing either an EtOH control, 10 ng/L GES, 100 ng/L GES, 10 ng/L LNG, 100 ng/L LNG, 30 ng/L P_4, or 300 ng/L P_4. The same photothermal and feeding strategies used for the breeding colony were maintained during the exposure period. Ethanol concentrations in each tank did not exceed 0.0000095%. Each day, two 50 % (5 gallon) water changes were performed at 0800 H and 1600 H, and new aliquots of chemical added to maintain nominal concentrations. Superstocks were created by dissolving lyophilized GES (10mg, Steraloids Inc., CAS 60282-87-3), LNG (10 mg, Steraloids Inc., CAS 60282-87-3), or P_4 (30mg, Steraloids Inc., CAS 57-83-0) in 10 mL of 95% EtOH. One mL of each of these was then added to 9 mL of EtOH to create the low treatment superstocks. All superstocks were kept in cold storage (-20^{o}C) when not in use. For each water change, an initial working stock solution was created by diluting 100 uL of each SS into 2,000 mL of dechlorinated city water. From this, 37.85 mL was then added to the appropriate treatment tank. After 8 days, 4 males from each treatment were euthanized using an overdose of MS-222 (300ppm), and both lobes of the testes removed and placed into a 1.5 mL tube containing 1 mL of FHM extender solution (pH 7.58, NaCl 94mM 5.52 g/L, KCl 27mM 2 g/L; Tris–HCl 15mM 2.42 g/L (Tris base adjusted with HCL), glycine 50mM 3.75 g/L) (Runnalls et al. 2007). Testes

were macerated using a pipette tip, and the mixture allowed to rest for 1 min to separate the released spermatozoa from unwanted connective tissue and other debris. Afterwards, 500 uL of the supernatant was removed and placed into a separate 1.5 mL tube and stored on ice. To account for changes in motility as a result of the time required to collect the samples, the order in which fish were processed from each treatment was randomized using a random numbers table.

To activate spermatozoa motility, 10 uL of extended semen from each sample was added to 40 uL of deionized ultrafiltered water (DIUF) in a 1.5 mL tube and the contents gently mixed. A 15 uL aliquot of this solution was then immediately loaded into a Makler chamber (Sefi Instruments, Israel), and the contents analyzed using a Hamilton-Thorne Computer Assisted Sperm Analysis Machine (version 12, Hamilton-Thorne, Beverly, Maine). Parameters measured included total motility, smoothed path velocity (VAP), straight-line velocity (VSL), track velocity (VCL), beat cross frequency (BCF), percent linearity (LIN), elongation (ELONG) and duration. Settings utilized to obtain accurate analyses are included in Table 5.2.

Table 5.2

Setting for Hamilton-Thorne Computer Assisted Sperm Analysis (CASA) Machine

FPS	60Hz
Number of frames	30
Minimum Contrast	45
Minimum Cell Size	2 pix
Cell Intensity	60
STR	VSL/VAP x 100
LIN	VSL/VCL x 100

Experiment 2- In vitro exposure

To determine the effects of gestagen exposure *in vitro*, samples were obtained from sexually mature FHM males (n = 8) using the methods described in experiment 1, resulting in 1 mL of extended semen from each individual. For each fish, a 10 uL aliquot was added to 40 uL of DIUF containing either an ethanol control, 10 ng/L GES, 100 ng/L GES, 10 ng/L LNG, 100 ng/L LNG, 30 ng/L P_4, or 300 ng/L P_4. A 15 uL aliquot of the exposed sample was then immediately placed onto a Makler Chamber (Sefi Instruments, Israel) and the various motion parameters quantified using the Hamilton-Thorne CASA machine. Analyses were obtained at 0 sec (defined as the first observation, < 6 sec after activation), 30 sec, and 60 sec post-activation.

Statistical Analysis

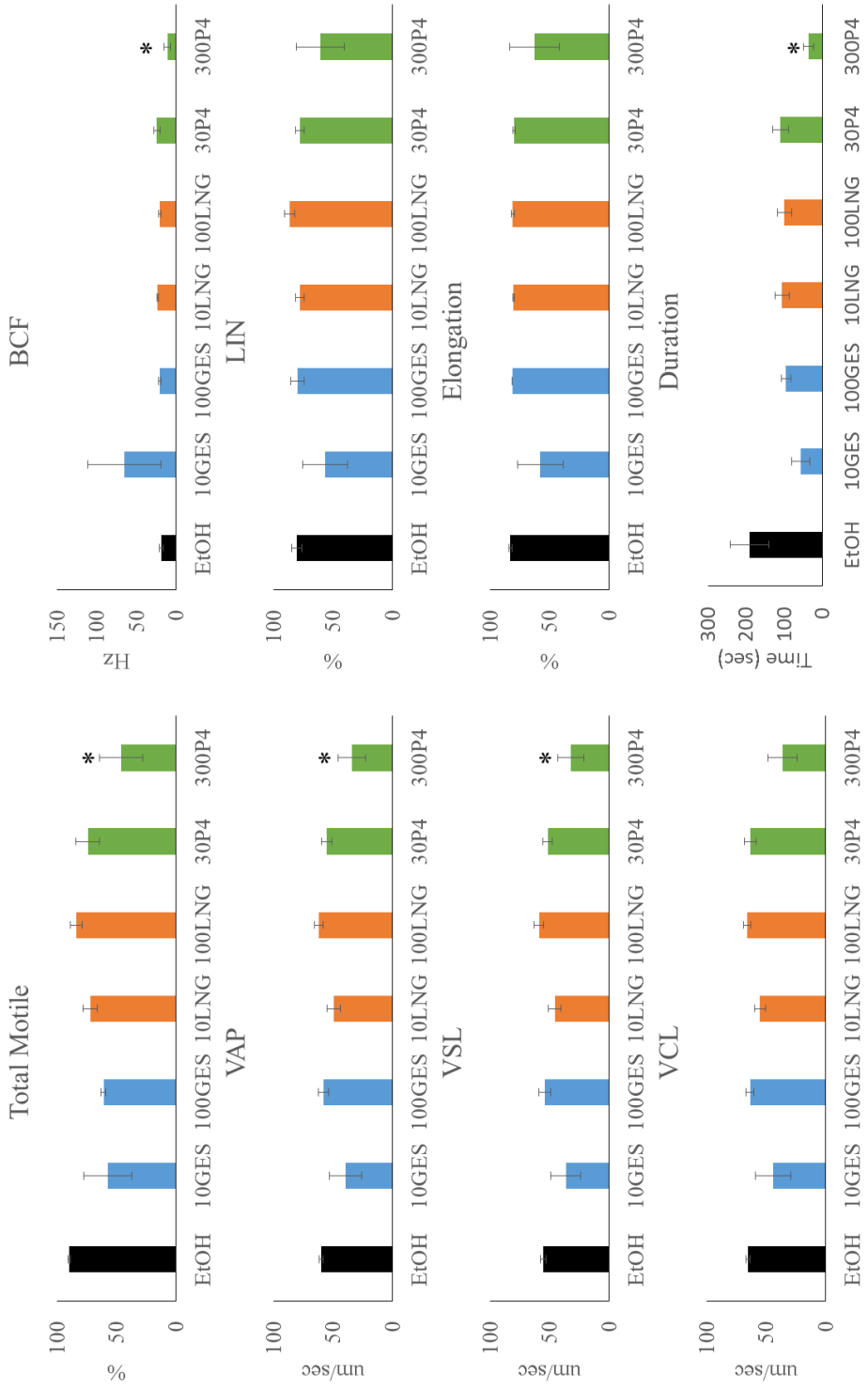
Statistical analyses were performed using SAS Studio 3.3 (Cary, NC). All data sets were checked to assure normality and homoscedasticity. For both the *in vivo* and *in vitro* experiments, treatments were compared to the EtOH control using ANOVA followed by Dunnett's test.

Results

Experiment 1: In vivo Exposure

Spermatozoa isolated from intact male FHMs and exposed *in vivo* for 8 days were not affected by exposure to the progestins LNG and GES at either concentration (Figure 5.1). However, significant decreases in several parameters were observed in the 300 ng/L P_4 treatment. When compared to the control, spermatozoa from the 300 ng/L treatment displayed significant decreases in total motility ($p = 0.0173$), VAP ($p = 0.0311$), VSL ($p = .0371$), VCL ($p = 0.0452$), BCF ($p = 0.0472$), and duration ($p = 0.0243$). No significant differences were found for LIN or elongation. No differences in any of the measured endpoints were observed as a result of the 30 ng/L P_4 exposure.

Figure 5.1

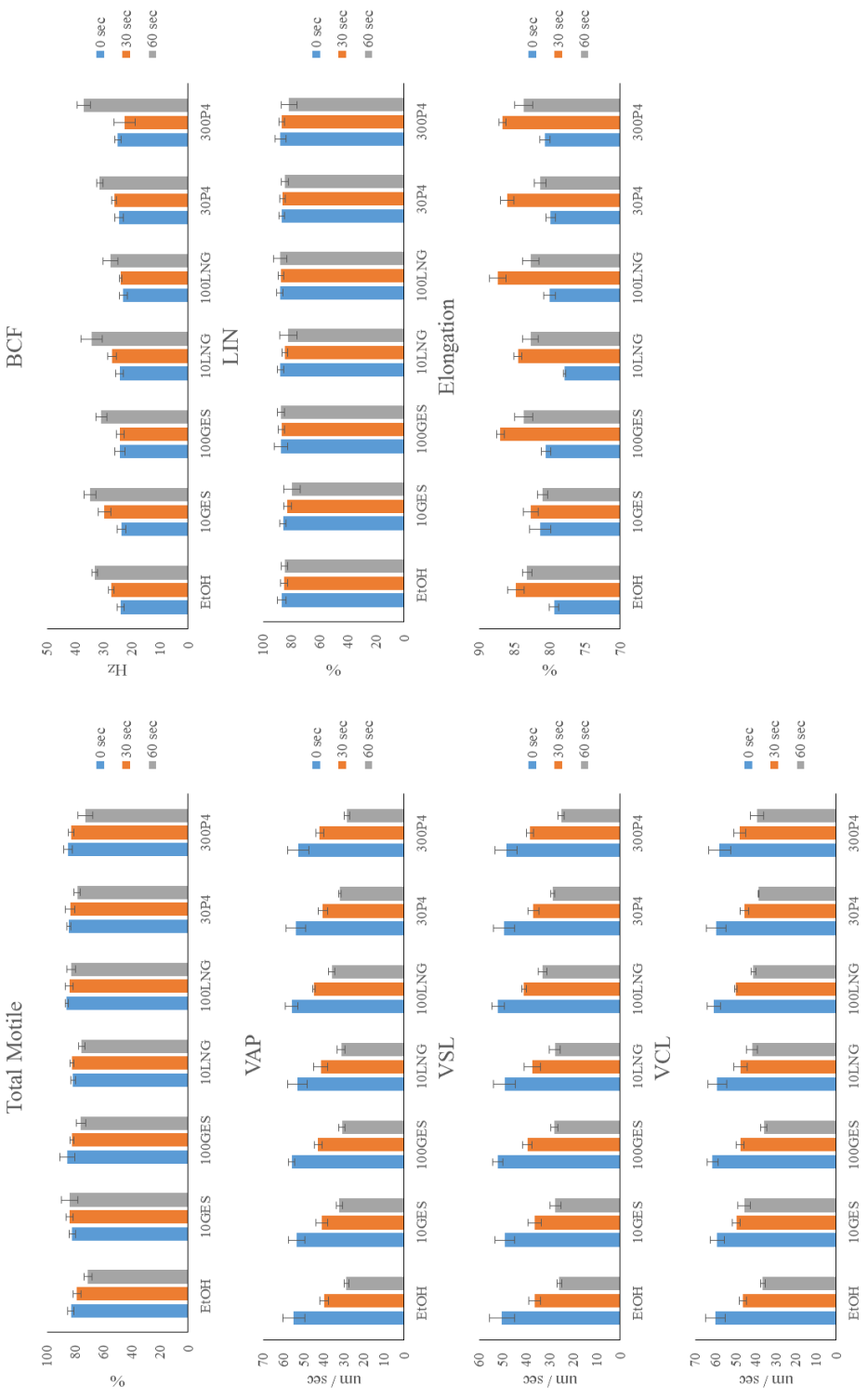


Mean measurements of total motility, VAP, VSL, VCL, BCF, LIN, elongation, and duration of semen obtained from males exposed to each treatment (n = 6) *in-vivo*. Asterisks indicate significant differences ($p < 0.05$) compared to the EtOH control.

Experiment 2: In vitro Exposure

When spermatozoa were exposed to the same treatments *in vitro*, they showed no significant differences in any of the motion parameters tested at 0, 30 and 60 sec (Fig. 5.2). For each of the endpoints, the pattern in which motility changed over time was consistent between treatments (e.g. the relative decrease of VAP over time seen in the EtOH treatment was observed in all other treatments).

Figure 5.2



Mean measurements of total motility, VAP, VSL, VCL, BCF, LIN, and Elongation of FHM semen exposed *in-vitro* to each treatment (n = 8) at 0, 30, and 60 seconds.

Discussion

While CASA has been utilized to examine the motility of fish spermatozoa for the past several decades (Kime et al. 2001), few studies have adapted the technique to examine the effects of endocrine disrupting compounds on male gamete quality. When compared to previous studies examining the motility of FHM spermatozoa, the observed changes in motility of control samples were consistent with results of previous studies (Murack et al. 2011, Schoenfuss et al. 2011), indicating that the males selected for this study were of acceptable quality to allow for a comparison of treatment effects.

Spermatozoa motility duration of freshwater fish species has been shown to be relatively short compared to both saltwater teleost (Browne et al. 2015) and mammalian species. Further, motility has been shown to rapidly decrease shortly after activation (Browne et al. 2015, Cosson et al. 2008) suggesting that results obtained after an extended period post-activation may not accurately represent the impacts of chemical exposure on gamete quality. Compared to the previous study by Murack et al. (2011) which analyzed semen samples 80 sec after activation, the use of a Hamilton-Thorne CASA unit in combination with a Makler chamber to prevent drift allowed for the rapid analysis of semen samples within 6 sec of activation.

As the *in vivo* and *in vitro* experiments were designed to simulate 1) the effects of whole animal short term exposure and 2) the effects of exposure to spermatozoa that are ejaculated into contaminated water, respectively, it appears that the activation processes are unaffected by progestin exposure. While the testes of males from each treatment were not examined histologically in this study, the lack of

differences observed in response to 8 day exposures of GES, LNG, and P_4 in chapters 3 and 4 support the lack of effect on sperm maturation. Multiple androgens, including testosterone and 11-ketotestosterone, have been associated with the division of spermatogonia and the release of spermatids into the lumen of the testes (Evans et al. 2013, Harvey and Carolsfeld 1993). In a closely related species, the release of androstenedione and its conjugates by both non-ovulating females and competing males decreased semen production (Sloman et al. 2006). Additionally, the male olfactory system has been shown to be extremely sensitive to androstenedione (Sorensen et al. 2005). Thus, it was hypothesized in this study that short term exposure of male FHMs to AR-binding progestins would disrupt spermatozoa maturation and alter motility. Previous studies contained in this dissertation examining short term exposure of LNG and GES have reported decreases in egg deposition. Based on the results of the present study, it is unlikely that this decrease is due to alterations of spermatozoa quality, although future studies on the effects of progestin exposure on fertilization success and final oocyte maturation are required to substantiate this claim. It is also unknown whether longer exposure periods would also affect similar results. When male FHMs were exposed *in vivo* to the 300 ng/L P_4 treatment, a decrease in total motility, VAP, VSL, and duration was observed. These results are consistent with those of Murack et al. (2011), who found that 7 d *in vivo* exposure of male FHMs to progesterone at similar concentrations caused a reduction in motility.

In conclusion, an improved CASA protocol was developed to obtain measurements of motility at much earlier time points compared to previous studies,

and was successfully utilized to examine the impacts of both *in vivo* and *in vitro* gestagen exposure on FHM spermatozoa motility. This study supports the relative ease of utilizing spermatozoa motility as an endpoint for endocrine disruption, and provides additional information regarding the mechanisms causing the disruption of other endpoints observed in previous studies. While it appears that some AR binding progestins do not alter sperm motility as a result of short term exposure (seconds or days), it is possible that longer exposure periods may affect decreases in spermatozoa motility. As P_4 at environmentally relevant concentrations decreased spermatozoa motility as a result of *in vivo* exposure, future research should examine the effects of decreased sperm motility on fertilization success.

Chapter 6: Conclusions

Exogenous exposure of several aquatic species to gestagens results in the disruption of several key endpoints related to reproduction, including changes in plasma steroid concentrations, physical masculinization of females, and, most importantly, the decrease or cessation of egg deposition (Kumar et al. 2015, Orlando and Ellestad 2014). While this alteration in fecundity has been noted in studies using fathead minnows, zebrafish, threespine stickleback, and medaka (Kumar et al. 2015), the factors controlling this are unknown. Although behavior, a sensitive apical endpoint for aquatic toxicology studies in fishes and amphibians, is often mentioned as requiring further attention (Orlando and Ellestad 2014, Söffker and Tyler 2012), few studies have focused on examining the impacts of endocrine disrupting chemicals (EDCs) on behavior, and no studies have been performed to examine the effects of gestagens on reproductive behavior and its association with the observed shutdown of egg deposition. Thus, the research presented in this dissertation is the first to explore the effects of exposure to the progestins gestodene (GES), levonorgestrel (LNG), and a progestogen (progesterone, P_4) on reproductive behavior in the fathead minnow (FHM). Novel insights regarding 1) the role of reproductive behavior in the disruption of egg deposition, and 2) the feasibility of behavior as an endpoint for future toxicology studies have been provided.

During the exposure trials, the cessation of egg deposition was observed as early as day 3 for GES and by day 5 for P_4. Notably, the disruption of behaviors associated with successful reproduction was observed after just 24 hrs of exposure to GES and P_4. While the masculinization of females as evidenced by the development of male typical secondary sexual characteristics was observed, these changes were not

present during the first few days of exposure, and did not coincide with the initial decreases in fecundity. No changes in gonadal histology were observed for either the males or females exposed to any of the compounds, suggesting that neither spermatogenesis nor oogenesis was affected by the exposure used herein, and did not factor in the observed decrease in egg deposition. Based on these findings, it is hypothesized that alterations of reproductive behavior plays a pivotal role in the shutdown of egg deposition during the first 8 d of exposure to these gestagens.

It is possible to identify whether a particular triad was exposed to an AR-binding progestin or a progestogen based upon the disrupted set of behaviors. Triads exposed to the AR-binding progestins, LNG and GES, exhibited decreases in courtship and spawning behaviors, but also exhibited increased male aggression and masculinized female behaviors including nest tending, female-female courtship, and female-female spawning activities. When exposed to the progestogen P_4, the same decrease in courtship behaviors was observed, but there were no observations of aggressive behaviors by the males or masculinized behaviors by the females. Although further research examining the effects of estrogenic and thyroidogenic compounds is required, assessment of reproductive behavior appears to be a viable option for rapidly determining the impacts of several classes of endocrine disruptors. It also has the potential to allow researchers to suggest a predicted mode of action (MOA) based on the types of behaviors that are altered.

While exposure to either GES or LNG masculinized females and increase aggression in male behavior, the effect of these alterations on population dynamics has yet to be examined. For example, when competing for nest sites in the wild, male

FHM success has been associated with the increased display of aggressive behaviors (Martinovic-Weigelt et al. 2012). As male FHMs have been shown to become more aggressive as a result of both GES or LNG exposure, it is possible that they would be able to outcompete non-exposed males, creating an unfortunately positive selection pressure for exposed individuals that may also be reproductively compromised.

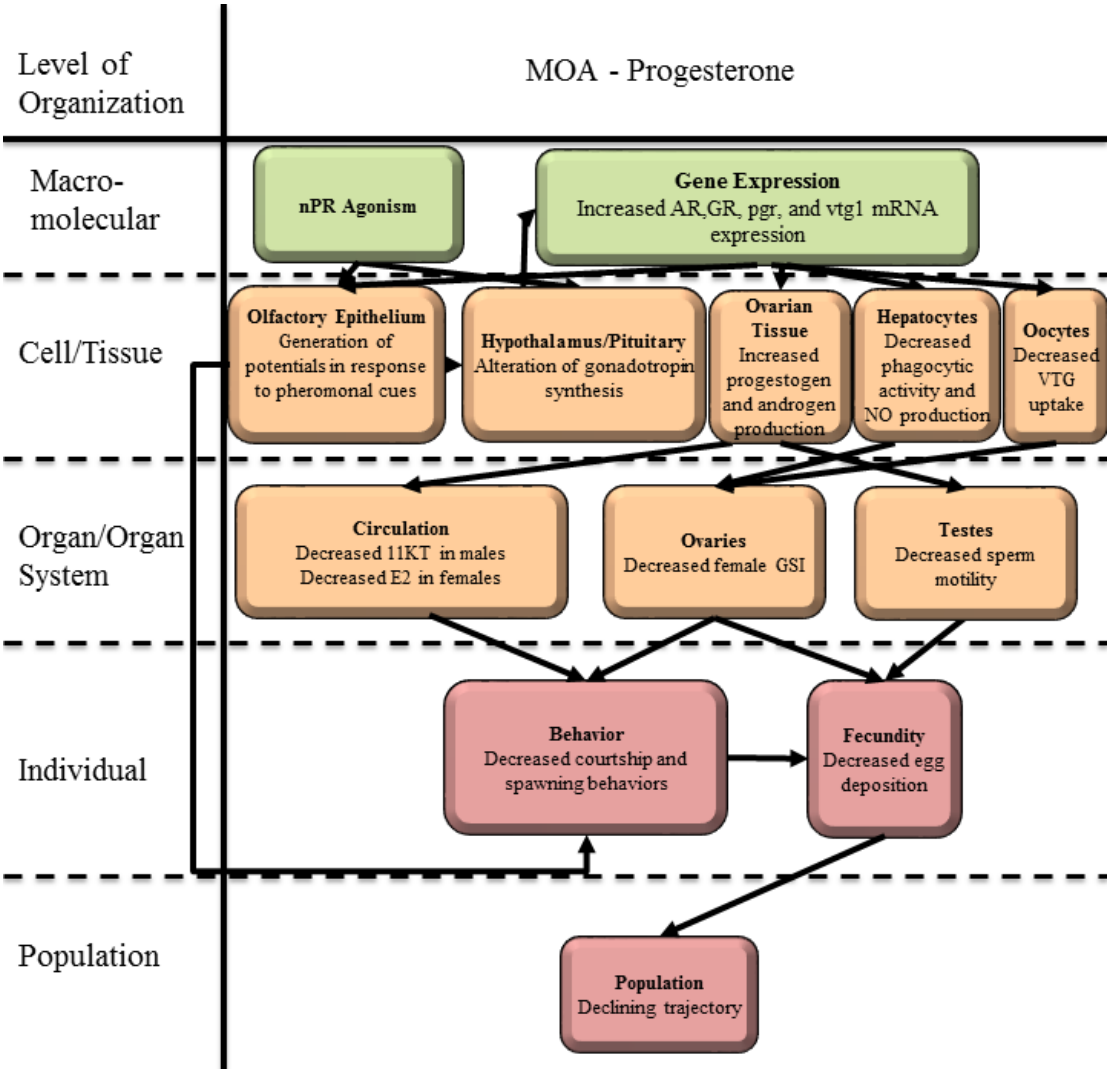
Gamete quality has often been linked to reproductive success in both fresh and saltwater teleost species (Bobe and Labbé 2010, Rurangwa et al. 2004). As this study of *in vitro* or *in vivo* progestin exposure on spermatozoa motility is the first to elucidate whether the observed decrease in fecundity was a result of decreased spermatozoa quality. While there were no observed effects on spermatozoa motility as a result of either GES or LNG exposure, it is possible that longer exposure periods such as those used in the study by Runnalls et al., 2013 would cause an effect in males. Additionally, the lack of effect on spermatozoa motility is consistent with the lack of changes to gonadal histology as a result of GES or LNG exposure, suggesting that the decrease in fecundity is not due to changes in spermatozoa quality. When males were exposed to the progestogen P_4, they exhibited changes in spermatozoa motility similar to those found by Murack et al. (2011). As P_4 continues to be found in the environment as a result of WWTPE and agricultural runoff, further research is required to determine if the observed *in vivo* effects on spermatozoa motility affect fertilization capacity in wild fishes exposed to P_4.

In contrast, the effects of progestins on final oocyte maturation have yet to be examined. As progestin exposure decreases VTG mRNA synthesis (DeQuattro Za 2012) as well as the development of late stage VTG oocytes (Zucchi et al. 2014), it is

possible that changes in follicle development as a result of progestin exposure are responsible for the observed decreases in egg deposition.

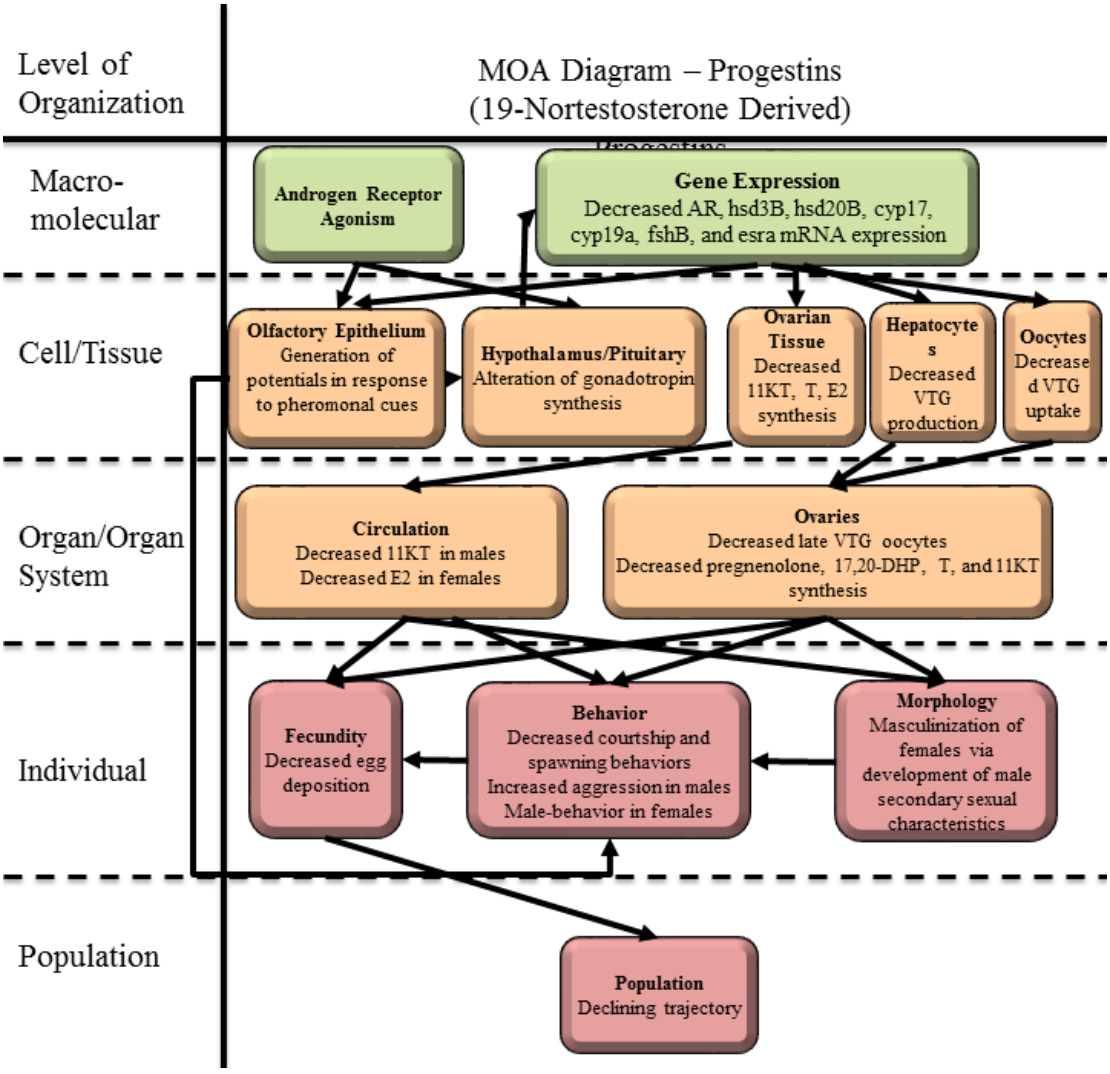
When the behavioral and physiological data gained from these experiments are combined with the results of previous studies, it is possible to propose a mechanism of action (MOA) for the effects of both 19-nortestosterone derived progestins and progestogen exposure on reproduction in the FHM. As the progestins GES and LNG transactivate AR, masculinize females, cause major alterations of reproductive behaviors, and are derived from the same parent compound (19-nortestosterone), the MOAs presented below focus on the dysfunction of reproduction in the FHM as a result of progesterone(Figure 6.1) and 19-nortestosterone derived progestin (Figure 6.2) exposure.

Figure 6.1



Schematic representation of the mechanism of action for progesterone

Figure 6.2



Schematic representation of the mechanism of action for 19-nortestosterone derived progestins

Based on the hypothesized MOAs, it is clear that aqueous exposure to gestagens in a lab setting results in a multitude of negative effects on fishes. However, further research is required to fully elucidate the mechanisms controlling the observed changes in critical endpoints. The ability of gestagens to excite potentials in the olfactory epithelium of both fresh and saltwater teleosts has yet to be examined, as well as their ability to function as pheromonal stimulants or inhibitors. By examining the effects of gestagen exposure on gonadotropin (LH and FSH) expression coupled with changes in plasma steroid concentrations, a greater understanding of the effects of exposure on the hypothalamic – pituitary - gonadal axis could be determined. Studies that may also serve to further increase our understanding of their environmental impact include the examination of 1) the ability of aquatic organisms to recover from various lengths of gestagen exposure if they are later removed from the treatment and placed into a non-treated environment, and 2) the multi-generational effects of gestagen exposure. Because gestagen exposure has been shown to cause immunosuppression and decreased phagocytic activity of head kidney macrophages, interactions with other non-reproductive endpoints such as growth, immunological function, and stress should also be considered when assessing the overall impact of gestagens on health.

Because geographic knowledge of the presence and concentrations of gestagens is limited, increased environmental monitoring will help to gain a better understanding of contamination levels. In doing so, it will be possible to identify areas that are of greater concern due to their ecological functions such as breeding grounds for endangered, economically important, and/or keystone species. Because

the use of contraceptive pharmaceuticals by under-developed countries is increasing, it is possible that geographically associated aquatic environments may also be at increased risk, especially if they lack adequate water sanitation and treatment plant facilities.

While this dissertation has focused on a few compounds, it is important to note that exposures in the environment occur as part of complex mixtures that include multiple classes of bio-active EDCs (e.g. androgens, estrogens, thyroidogens, etc.) and other neuroactive substances. Although many of these chemicals have been shown to act independently, the ability of two or more classes to form additive, synergistic, or antagonistic relationships is poorly understood. Studies examining interactions between an estrogen and antiestrogen (Garcia-Reyero et al. 2009), androgen and antiandrogen (Collette et al. 2010), and progestin and progestogen (Zucchi et al. 2014) have found such effects. Because of the vast number of pharmaceuticals that are released into the aquatic environment, it is probably not feasible to attempt to replicate these whole-effluent mixtures in a lab setting. However, by determining the specific target sites of each compound through molecular methods (e.g. receptor activation assays), it may be possible to perform mixture studies using select chemicals that more accurately approximate environmental conditions. For example, many of the 19-nortestosterone derived progestins have been shown to cause the same kind of effects on reproduction, several of which have been correlated with relative binding assays of each chemical to fish nAR. By first assessing the presence and concentration of each progestin in the

environment, it may then be possible to determine the average binding affinity and their ability to activate receptors.

By determining this value for each set of compounds found in the environmental sample, one could then select a single chemical that, when utilized at the proper concentrations, more accurately depicts the activity of an entire class. While such a model is fairly simplistic, it could provide a working method to examine the interactions and impacts of broad classes of contaminants that have been previously defined by both short-term exposure studies and binding affinity and receptor activation studies to ER, AR, nPR, and their respective membrane receptors.

In conclusion, the results of this research provide an increased understanding of the effects of gestagen exposure on novel endpoints including reproductive behavior and male gamete quality. Furthermore, they serve to both increase our understanding of how these EDCs affect the reproductive fitness of the FHM and providing evidence that further research examining the environmental impact of these chemicals is warranted.

Bibliography

Adlercreutz H, Bannwaert C, Wahala K, Makela T, Brunow G, Hase T, Arosemena PJ, Kellis, JK, Vickery LE. 1993. Inhibition of Human Aromatase by Mammalian Lignans and Isoflavonoid Phytoestrogens. 44 2.

Africander D, Verhoog N, Hapgood JP. 2011. Molecular Mechanisms of Steroid Receptor-Mediated Actions By Synthetic Progestins Used in HRT and Contraception. Steroids 76:636-652.

Alfonso M, Durán R, Marcó J. 1993. *In Vitro* Effect of Ethanol on LH and FSH Secretion by Pituitary Glands of Female Rats. Endocrine Research 19:175-186.

Ankley, G, Jensen, K, Makynen, E, Kahl, M, Korte, J, Hornung, M, Henry, T, Denny, J, Leino, R, Wilson, V, Cardon, M, Hartig, P, Gray, L. 2003. Effects of the Androgenic Growth Promoter 17-Beta-Trenbolone on Fecundity and Reproductive Endocrinology of the Fathead Minnow. Environmental Toxicology and Chemistry, 22(6), 1350-1360.

Ankley GT, Villeneuve DL. 2006. The Fathead Minnow in Aquatic Toxicology: Past, Present and Future. Aquatic Toxicology 78:91-102.

Aris AZ, Shamsuddin AS, Praveena SM. 2014. Occurrence of 17alpha-ethynylestradiol (EE_2) in the Environment and Effect on Exposed Biota: A Review. Environmental International 69:104-119.

Bain PA, Kumar A, Ogino Y, Iguchi T. 2015. Nortestosterone-Derived Synthetic Progestogens do not Activate the Progestogen Receptor of Murray-Darling Rainbowfish (*Melanotaenia fluviatilis*) but are Potent Agonists of Androgen Receptors Alpha and Beta. Aquatic Toxicology 163:97-101.

Bair, GO. 1998. Treatment of Side Effects of Progestins and Progesterone Analogues Used for Birth Control, United States. 5744463http://www.freepatentsonline.com/5744463.html

Bartelt-Hunt SL, Snow DD, Kranz WL, Mader TL, Shapiro CA, Donk SJv, Shelton DP, Tarkalson DD, Zhang TC. 2012. Effect of Growth Promotants on the Occurrence of Endogenous and Synthetic Steroid Hormones on Feedlot Soils and in Runoff from Beef Cattle Feeding Operations. Environmental Science and Technology 46:1352-1360.

Benville PE, Smith CE, Shanks WE. 1968. Some Toxic Effects of Dimethyl Sulfoxide in Salmon and Trout. Toxicology and Applied Pharmacology 12:156-178.

Bilotta J, Barnett JA, Hancock L, Saszik S. 2004. Ethanol Exposure Alters Zebrafish Development: A Novel Model of Fetal Alcohol Syndrome. Neurotoxicology and Teratology 26:737-743.

Bilotta J, Saszik S, Givin CM, Hardesty HR, Sutherland SE. 2002. Effects of Embryonic Exposure to Ethanol on Zebrafish Visual Function. Neurotoxicology and Teratology 24:759-766.

Bobe J, Labbé C. 2010. Egg and Sperm Quality in Fish. General and Comparative Endocrinology 165:535-548.

Boni R, Gualtieri R, Talevi R, Tosti E. 2007. Calcium and Other Ion Dynamics During Gamete Maturation and Fertilization. Theriogenology 68:S156-S164.

Bony S, Gillet C, Bouchez A, Margoum C, Devaux A. 2008. Genotoxic Pressure of Vineyard Pesticides in Fish: Field and Mesocosm Surveys. Aquatic Toxicology 89:197-203.

Braselton WE, Lin TJ, Mills TM, Ellegood JO, Mahesh VB. 1977. Identification and Measurement by Gas Chromatography-Mass Spectrometry of Norethindrone and Metabolites in Human Urine and Blood. Journal of Steroid Biochemistry 8:9-18.

Browne R, Kaurova S, Uteshev V, Shishova N, McGinnity D, Figiel C, Mansour N, Agney D, Wu M, Gakhova E. 2015. Sperm Motility of Externally Fertilizing Fish and Amphibians. Theriogenology 83:1-13.

Carballa M, Omil F, Lema JM, Llompart Ma, García-Jares C, Rodríguez I, Gómez M, Ternes T. 2004. Behavior of Pharmaceuticals, Cosmetics and Hormones in a Sewage Treatment Plant. Water Research 38:2918-2926.

Carson, R. 2002. Silent Spring. Houghton Mifflin Harcourt, 2002.

Carvan MJ, Loucks E, Weber DN, Williams FE. 2004. Ethanol Effects on the Developing Zebrafish: Neurobehavior and Skeletal Morphogenesis. Neurotoxicology and Teratology 26:757-768.

Chang H, Wan Y, Hu HY. 2009. Determination and Source Apportion of Five Classes of Steroid Hormones in Urban Rivers. Environmental Science and Technology 43:7691-7698.

Chishti, YZ, Feswick, A, Munkittrick, KR, Martyniuk, CJ. 2013. Transcriptomic Profiling of Progesterone in the Male Fathead Minnow (*Pimephales promelas*) Testis. General and Comparative Endocrinology, 192, 115-125.

Christians JK. 1999. Controlling for Body Mass Effects: Is Part-Whole Correlation Important? Physiological and Biochemical Zoology 72:250-253.

Colborn T, vom Saal FS, Soto AM. 1993. Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans. Environmental Health Perspectives 101:378.

Colburn T, Dumanoski D, Myers JP. 1996. Our Stolen Future: Are we Threatening our Fertility, Intelligence, and Survival. A Scientific Detective Story:137.

Cole K, Smith RJ. 1987. Male courting behaviour in the fathead minnow, *Pimephales promelas*. Environmental biology of Fishes 18:235-239.

Colin T, Bories A, Sire Y, Perrin R. 2005. Treatment and Valorisation of Winery Wastewater by a New Biophysical Process. Water Science and Technology 51:99-106.

Collette TW, Teng Q, Jensen KM, Kahl MD, Makynen EA, Durhan EJ, Villeneuve DL, Martinović-Weigelt D, Ankley GT, Ekman DR. 2010. Impacts of an Anti-Androgen and an Androgen/Anti-Androgen Mixture on the Metabolite Profile of Male Fathead Minnow Urine. Environmental Science and Technology 44:6881-6886.

Cosson J, Groison AL, Suquet M, Fauvel C, Dreanno C, Billard R. 2008. Studying Sperm Motility in Marine Fish: An Overview on the State of the Art. Journal of Applied Ichthyology 24:460-486.

Davtyan C. 2012. Four Generations of Progestins in Oral Contraceptives. Proceedings of UCLA Healthcare 16.

DeQuattro ZA, Peissig EJ, Antkiewicz DS, Lundgren EJ, Hedman CJ, Hemming JDC, Barry TP. 2012. Effects of Progesterone on Reproduction and Embryonic Development in the Fathead Minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 31:851-856.

Dlugos CA, Rabin RA. 2003. Ethanol Effects on Three Strains of Zebrafish: Model System for Genetic Investigations. Pharmacology Biochemistry and Behavior 74:471-480.

Durhan EJ, Lambright C, Wilson V, Butterworth BC, Kuehl DW, Orlando EF, Guillette LJ, Jr., Gray LE, Jr., Ankley GT. 2002. Evalutation of Androstendione as an Androgenic Component of River Water Downstream of Pulp and Paper Mill Effluent. Environmental Toxicology and Chemistry 21:1973-1976.

Ellestad LE, Cardon M, Chambers IG, Farmer JL, Hartig P, Stevens K, Villeneuve DL, Wilson V, Orlando EF. 2014a. Environmental Gestagens Activate Fathead

Minnow (*Pimephales promelas*) Nuclear Progesterone and Androgen Receptors *in vitro*. Environmental Science and Technology 48:8179-8187.

Emanuele MA, Tentler J, Emanuele NV, Kelley MR. 1991. *In vivo* Effects of Acute EtOH on Rat α and β Luteinizing Hormone Gene Expression. Alcohol 8:345-348.

Essington T, Sorensen P. 1996. Overlapping Sensitivities of Brook Trout and Brown Trout to Putative Hormonal Pheromones. Journal of Fish Biology 48:1027-1029.

Evans DH, Claiborne JB, Currie S. 2013. The Physiology of Fishes, Fourth Edition. Taylor & Francis.

Feng J, Lawson MA, Melamed P. 2008. A Proteomic Comparison of Immature and Mature Mouse Gonadotrophs Reveals Novel Differentially Expressed Nuclear Proteins That Regulate Gonadotropin Gene Transcription and RNA Splicing. Biological Reprodution 79:546-561.

Fent K. 2015. Progestins as Endocrine Disrupters in Aquatic Ecosystems: Concentrations, Effects and Risk Assessment. Environmental International 84:115-130.

Frankel TE, Meyer MT, Kolpin DW, Gillis AB, Alvarez DA, Orlando EF. 2016a. Exposure to the Contraceptive Progestin, Gestodene, Alters Reproductive Behavior, Arrests Egg Deposition, and Masculinizes Development in the Fathead Minnow (*Pimephales promelas*). Environmental science & technology 50:5991-5999.

Frankel TE, Meyer MT, Orlando EF. 2016b. Aqueous Exposure to the Progestin, Levonorgestrel, Alters Anal Fin Development and Reproductive Behavior in the Eastern Mosquitofish (*Gambusia holbrooki*). General and Comparative Endocrinology (In Press).

Gage MJ, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal Traits and Sperm Competition in Atlantic Salmon: Relative Sperm Velocity is the Primary Determinant of Fertilization Success. Current Biology 14:44-47.

Garcia-Reyero NM , Martyniuk CJ, Kroll KJ, Escalon BL, Spade DJ, Denslow, ND. 2013. Transcriptional Signature of Progesterone in the Fathead Minnow Ovary (*Pimephales promelas*). General and Comparative Endocrinology, 192, 159-169.

Garcia-Reyero N, Villeneuve DL, Kroll KJ, Liu L, Orlando EF, Watanabe KH, Sepúlveda MaS, Ankley GT, Denslow ND. 2009. Expression Signatures for a Model Androgen and Antiandrogen in the Fathead Minnow (*Pimephales promelas*) Ovary. Environmental Science and Technology 43:2614-2619.

Gerlai R, Lee V, Blaser R. 2006. Effects of Acute and Chronic Ethanol Exposure on the Behavior of Adult Zebrafish (*Danio rerio*). Pharmacology Biochemistry and Behavior 85:752-761.

Hamdani EH, Døving KB. 2007. The Functional Organization of the Fish Olfactory System. Progress in Neurobiology 82:80-86.

Harvey B, Carolsfeld J. 1993. Induced Breeding in Tropical Fish Culture. International Development Research Centre Ottawa.

Hashimoto S, Watanabe E, Ikeda M, Terao Y, Strüssmann CA, Inoue M, Hara A. 2009. Effects of Ethinylestradiol on Medaka (*Oryzias latipes*) as Measured by Sperm Motility and Fertilization Success. Archives of Environmental Contamination and Toxicology 56:253-259.

Higashino T, Miura T, Miura C, Yamauchi K. 2003. Effects of Two Sex Steroid Hormones on Early Oogenesis in Japanese Huchen (*Hucho perryi*). Fish Physiology and Biochemistry 28:343-344.

Jenkins R, Wilson EM, Angus RA, Howell WM, Kirk M. 2003. Androstenedione and Progesterone in the Sediment of a River Receiving Paper Mill Effluent. Toxicological Sciences 73:53-59.

Jensen KM, Korte JJ, Kahl MD, Pasha MS, Ankley GT. 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (Pimephales promelas). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 128:127-141.

Johnson AC, Williams RJ. 2004. A Model To Estimate Influent and Effluent Concentrations of Estradiol, Estrone, and Ethinylestradiol at Sewage Treatment Works. Environmental Science and Technology 38:3649-3658.

Kaviraj A, Bhunia F, Saha NC. 2004. Toxicity of Methanol to Fish, Crustacean, Oligochaete Worm, and Aquatic Ecosystem. International Journal of Toxicology 23:55-63.

Kermen F, Franco LM, Wyatt C, Yaksi E. 2013. Neural Circuits Mediating Olfactory-Driven Behavior in Fish. Frontiers in Neural Circuits 7.

Kily LJ, Cowe YC, Hussain O, Patel S, McElwaine S, Cotter FE, Brennan CH. 2008. Gene Expression Changes in a Zebrafish Model of Drug Dependency Suggest Conservation of Neuro-Adaptation Pathways. Journal of Experimental Biology 211.

Kime DE, Van Look KJW, McAllister BG, Huyskens G, Rurangwa E, Ollevier F. 2001. Computer-Assisted sperm analysis (CASA) as a Tool for Monitoring Sperm Quality in Fish. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 130:425-433.

Kobayashi M, Sorensen PW, Stacey NE. 2002. Hormonal and Pheromonal Control of Spawning Behavior in the Goldfish. Fish Physiology and Biochemistry 26:71-84.

Kolmakov NN, Kube M, Reinhardt R, Canario AVM. 2008. Analysis of the Goldfish *Carassius auratus* Olfactory Epithelium Transcriptome Reveals the Presence of Numerous Non-Olfactory GPCR and Putative Receptors for Progestin Pheromones. BMC Genomics 9:429.

Kolodziej EP, Gray JL, Sedlak DL. 2003. Quantification of Steroid Hormones with Pheromonal Properties in Municipal Wastewater Effluent. Environmental Toxicology and Chemistry 22:2622-2629.

Kolodziej EP, Harter T, Sedlak DL. 2004. Dairy Wastewater, Aquaculture, and Spawning Fish as Sources of Steroid Hormones in the Aquatic Environment. Environmental Science and Technology 38:6377 - 6384.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance. Environmental Science and Technology 36:1202-1211.

Kraeling RR, Webel SK. 2015. Current Strategies for Reproductive Management of Gilts and Sows in North America. Journal of Animal Science and Biotechnology 6:1-14.

Kumar V, Johnson AC, Trubiroha A, Tumova J, Ihara M, Grabic R, Kloas W, Tanaka H, Kroupova HK. 2015. The Challenge Presented by Progestins in Ecotoxicological Research: A Critical Review. Environmental Science and Technology 49:2625-2638.

Länge R, Hutchinson TH, Croudace CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. 2001. Effects of the Synthetic Estrogen 17α-Ethinylestradiol on the Life-Cycle of the Fathead Minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry 20:1216-1227.

Laurell C, Kullander S, Thorell J. 1968. Effect of Administration of a Combined Estrogen-Progestin Contraceptive on the Level of Individual Plasma Proteins. Scandinavian Journal of Clinical & Laboratory Investigation 21:337-343.

León-Olea M, Martyniuk C, Orlando EF, Ottinger MA, Rosenfeld C, Wolstenholme J, Trudeau VL. 2014. Current Concepts in Neuroendocrine Disruption. General and Comparative Endocrinology 203:158-173.

Linhart O, Rodina M, Cosson J. 2000. Cryopreservation of Sperm in Common Carp *Cyprinus carpio*: Sperm Motility and Hatching Success of Embryos. Cryobiology 41:241-250.

Lorenz C, Contardo-Jara V, Trubiroha A, Kruger A, Viehmann V, Wiegand C, Pflugmacher S, Nutzmann G, Lutz I, Kloas W. 2011. The Synthetic Gestagen

Levonorgestrel Disrupts Sexual Development in *Xenopus laevis* by Affecting Gene Expression of Pituitary Gonadotropins and Gonadal Steroidogenic Enzymes. Toxicological Sciences 124:311-319.

Lorenzen A, Hendel JG, Conn KL, Bittman S, Kwabiah AB, Lazarovitz G, Massé D, McAllister TA, Topp E. 2004. Survey of Hormone Activities in Municipal Biosolids and Animal Manures. Environmental Toxicology 19:216-225.

MacLatchy DL, Van Der Kraak GJ. 1995. The Phytoestrogen B-Sitosterol Alters the Reproductive Endocrine Status of Goldfish. Toxicology and Applied Pharmacology 134:305-312.

Makynen E, Kahl M, Jensen K, Tietge J, Wells K, Van Der Kraak G, Ankley G. 2000. Effects of the Mammalian Antiandrogen Vinclozolin on Development and Reproduction of the Fathead Minnow (*Pimephales promelas*). Aquatic Toxicology 48:461-475.

Mansell DS, Bryson RJ, Harter T, Webster JP, Kolodziej EP, Sedlak DL. 2011. Fate of Endogenous Steroid Hormones in Steer Feedlots Under Simulated Rainfall-Induced Runoff. Environmental Science and Technology 45:8811-8818.

Markey CM, Rubin BS, Soto AM, Sonnenschein C. 2002. Endocrine Disruptors: from Wingspread to Environmental Developmental Biology. The Journal of Steroid Biochemistry and Molecular Biology 83:235-244.

Martinovic-Weigelt D, Ekman DR, Villeneuve DL, James CM, Teng Q, Collette TW, Ankley GT. 2012. Fishy Aroma of Social Status: Urinary Chemo-Signalling of Territoriality in Male Fathead Minnows (*Pimephales promelas*). PLoS ONE 7:e46579.

Martinović D, Blake LS, Durhan EJ, Greene KJ, Kahl MD, Jensen KM, Makynen EA, Villeneuve DL, Ankley GT. 2008. Reproductive Toxicity of Vinclozolin in the Fathead Minnow: Confirming an Anti-Androgenic Mode of Action. Environmental Toxicology and Chemistry 27:478-488.

Martinović D, Hogarth WT, Jones RE, Sorensen PW. 2007. Environmental Estrogens Suppress Hormones, Behavior, and Reproductive Fitness in Male Fathead Minnows. Environmental Toxicology and Chemistry 26:271-278.

McCann M, Potter L. 1994. Progestin-Only Oral Contraception: A Comprehensive Review. Contraception 50:S1-195.

McMillan VE, Smith RJF. 1974. Agonistic and Reproductive Behaviour of the Fathead Minnow (*Pimephales promelas Rafinesque*). Zeitschrift für Tierpsychologie 34:25-58.

Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C. 2006. Progestin is An Essential Factor for the Initiation of the Meiosis in Spermatogenetic Cells of the Eel. PNAS 103:7333-7338.

Munakata A, Kobayashi M. 2010. Endocrine Control of Sexual Behavior in Teleost Fish. General and Comparative Endocrinology 165:456-468.

Murack PJ, Parrish J, Barry TP. 2011. Effects of Progesterone on Sperm Motility in Fathead Minnow (*Pimephales promelas*). Aquatic Toxicology 104:121-125.

Nico L, Fuller P, Neilson M. 2014. *Pimphales promelas*. USGS Nonindigenous Aquatic Species Database.

Ninkovic J, Folchert A, Makhankov YV, Neuhauss SC, Sillaber I, Straehle U, Bally-Cuif L. 2006. Genetic Identification of AChE as a Positive Modulator of Addiction to the Psychostimulant D-Amphetamine in Zebrafish. Journal of Neurobiology 66.

Norton W, Bally-Cuif L. 2010. Adult Zebrafish as a Model Organism for Behavioural Genetics. BMC Neuroscience 11:1-11.

Oddens BJ. 1999. Women's Satisfaction with Birth Control: A Population Survey of Physical and Psychological Effects of Oral Contraceptives, Intrauterine Devices, Condoms, Natural Family Planning, and Sterilization Among 1466 women. Contraception 59:277-286.

Olsén KH, Bjerselius R, Mayer I, Kindahl H. 2001. Both Ovarian Fluid and Female Urine Increase Sex Steroid Hormone Levels in Mature Atlantic Salmon (*Salmo salar*) Male Parr. Journal of Chemical Ecology 27:2337-2349.

OPPTS/USEPA. 2009. Fish Short-Term Reproduction Assay. Office of Prevention, Pesticides, and Toxic Substances USEPA. Report no. EPA/740-C-09-007.

Orlando EF, Ellestad LE. 2014. Sources, Concentrations, and Exposure Effects of Environmental Gestagens on Fish and Other Aquatic Wildlife with an Emphasis on Reproduction. General and Comparative Endocrinology 203:241-249.

Örn S, Holbech H, Madsen TH, Norrgren L, Petersen GI. 2003. Gonad Development and Vitellogenin Production in Zebrafish (*Danio rerio*) Exposed to Ethinylestradiol and Methyltestosterone. Aquatic Toxicology 65:397-411.

Overturf MD, Overturf CL, Carty DR, Hala D, Huggett DB. 2014. Levonorgestrel Exposure to Fathead Minnows (*Pimephales promelas*) Alters Survival, Growth,

Steroidogenic Gene Expression and Hormone Production. Aquatic Toxicology 148:152-161.

Painter MM, Buerkley MA, Julius ML, Vajda AM, Norris DO, Barber LB, Furlong ET, Schultz MM, Schoenfuss HL. 2009. Antidepressants at Environmentally Relevant Concentrations Affect Predator Avoidance Behavior of Larval Fathead Minnows (*Pimephales promelas*). Environmental Toxicology and Chemistry 28:2677-2684.

Pankhurst N, Hilder P, Pankhurst P. 1999. Reproductive Condition and Behavior in Relation to Plasma Levels of Gonadal Steroids in the Spiny Damselfish *Acanthochromis polyacanthus*. General and Comparative Endocrinology 115:53-69.

Partnership UCHaP. 2011. Opportunities for Combined Heat and Power at Wastewater Treatment Facilities: Market Analysis and Lessons from the Field.

Patisaul HB, Jefferson W. 2010. The Pros and Cons of Phytoestrogens. Frontiers in Neuroendocrinology 31:400-419.

Paulos P, Runnalls TJ, Nallani G, La Point T, Scott AP, Sumpter JP, Huggett DB. 2010. Reproductive Responses in Fathead Minnow and Japanese Medaka Following Exposure to a Synthetic Progestin, Norethindrone. Aquatic Toxicology 99:256-262.

Pawlowski S, Sauer A, Shears JA, Tyler CR, Braunbeck T. 2004. Androgenic and Estrogenic Effects of the Synthetic Androgen 17alpha-methyltestosterone on Sexual Development and Reproductive Performance in the Fathead Minnow (*Pimephales promelas*) Determined Using the Gonadal Recrudescence Assay. Aquatic Toxicology 68:277-291.

Petersen LH, Hala D, Carty D, Cantu M, Martinovic D, Huggett DB. 2014. Effects of Progesterone and Norethindrone on Female Fathead Minnow (*Pimephales promelas)* Steroidogenesis. Environmental Toxicology and Chemistry 34(2), 379-390.

Petitti DB. 2003. Combination Estrogen–Progestin Oral Contraceptives. New England Journal of Medicine 349:1443-1450.

Pfister P, Rodriguez I. 2005. Olfactory Expression of a Single and Highly Variable V1r Pheromone Receptor-Like Gene in Fish Species. Proceedings of the National Academy of Science U S A 102:5489-5494.

Pinter J, Thomas P. 1997. The Ovarian Progestogen Receptor in the Spotted Seatrout, *Cynoscion nebulosus*, Demonstrates Steroid Specificity Different from Progesterone Receptors in other Vertebrates. Journal of Steroid Biochemistry and Molecular Biology 60:113-119.

Poirier SH, Knuth ML, Anderson-Buchou CD, Brooke LT, Lima AR, Shubat PJ. 1986. Comparative Toxicity of Methanol and N,N-Dimethylformamide to Freshwater Fish and Invertebrates. Bulletin of Environmental Contamination and Toxicology 37:615-621.

Poling KR, Fraser EJ, Sorensen PW. 2001. The Three Steroidal Components of the Goldfish Preovulatory Pheromone Signal Evoke Different Behaviors in Males. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 129:645-651.

Qiao Y, Yang H, Wang B, Song J, Deng A. 2009. Preparation and Characterization of an Immunoaffinity Chromatography Column for the Selective Extraction of Trace Contraceptive Drug Levonorgestrel from Water Samples. Talanta 80:98-103.

Rengarajan S, Malini T, Sivakumar R, Govindarajulu P, Balasubramanian K. 2003. Effects of Ethanol Intoxication on LH Receptors and Glucose Oxidation in Leydig cells of Adult Albino Rats. Reproductive Toxicology 17:641-648.

Rico EP, Rosemberg DB, Dias RD, Bogo MR, Bonan CD. 2007. Ethanol Alters Acetylcholinesterase Activity and Gene Expression in Zebrafish Brain. Toxicology Letters 174:25-30.

Robertson SM, Lawrence AL, Neill WH, Arnold CR, McCarty G. 1988. Toxicity of the Cryoprotectants Glycerol, Dimethyl Sulfoxide, Ethylene Glycol, Methanol,

Sucrose, and Sea Salt Solutions to the Embryos of Red Drum. The Progressive Fish-Culturist 50:148-154.

Runnalls TJ, Beresford N, Losty E, Scott AP, Sumpter JP. 2013. Several Synthetic Progestins with Different Potencies Adversely Affect Reproduction of Fish. Environmental Science and Technology 47:2077-2084.

Runnalls TJ, Hala DN, Sumpter JP. 2007. Preliminary Studies Into the Effects of the Human Pharmaceutical Clofibric Acid on Sperm Parameters in Adult Fathead Minnow. Aquatic Toxicology 84:111-118.

Rurangwa E, Kime D, Ollevier F, Nash J. 2004. The Measurement of Sperm Motility and Factors Affecting Sperm Quality in Cultured Fish. Aquaculture 234:1-28.

Schoenfuss HL, Martinovic D, Sorensen PW. 2011. Effects of Exposure to Low Levels of Water-Borne 17b-Estradiol on Nest Holding Ability and Sperm Quality in Fathead Minnows. Journal of Contemporary Water Research and Education 120:6.

Scott A, Sumpter J, Stacey N. 2010. The Role of the Maturation-Inducing Steroid, 17, 20β-dihydroxypregn-4-en-3-one, in Male Fishes: A Review. Journal of Fish Biology 76:183-224.

Shi Q, Hales DB, Emanuele NV, Emanuele MA. 1998. Interaction of Ethanol and Nitric Oxide in the Hypothalamic-Pituitary-Gonadal Axis in the Male Rat. Alcoholism: Clinical and Experimental Research 22:1754-1762.

Shulman LP. 2011. The State of Hormonal Contraception Today: Benefits and Risks of Hormonal Contraceptives: Combined Estrogen and Progestin Contraceptives. American Journal of Obstetrics and Gynecology 205:S9-S13.

Sitruk-Ware R, Nath A, Mishell DR. 2012. Contraception Technology: Past, Present and Future. Contraception 87(3), 319-330.

Sloman KA, Wilson RW, Balshine S. (200. Behaviour and Physiology of Fish (Vol. 24): Gulf Professional Publishing.

Söffker M, Tyler CR. 2012. Endocrine Disrupting Chemicals and Sexual Behaviors in Fish – A Critical Review on Effects and Possible Consequences. Critical Reviews in Toxicology 42:653-668.

Sorensen P, Brash A, Goetz F, Kellner R, Bowdin L, Vrieze L. 1995. Origins and Functions of F Prostaglandins as Hormones and Pheromones in the Goldfish. In Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish. Austin Texas, FishSymp (Vol. 95, pp. 252-254).

Sorensen PW. 2015. Introduction to Pheromones and Related Cues in Fishes. Pages 1-9 in Sorensen PW, Wisenden BD, eds. Fish Pheromones and Related Cues. Ames, Iowa USA: John Wiley & Sons

Sorensen PW, Hara TJ, Stacey NE. 1987. Extreme Olfactory Sensitivity of Mature and Gonadally-Regressed Goldfish to a Potent Steroidal Pheromone, 17α, 20β-dihydroxy-4-pregnen-3-one. Journal of Comparative Physiology A 160:305-313.

Sorensen PW, Pinillos M, Scott AP. 2005. Sexually Mature Male Goldfish Release Large Quantities of Androstenedione Into the Water Where it Functions as a Pheromone. General and Comparative Endocrinology 140:164-175.

Sorensen PW, Stacey NE. 2004. Brief Review of Fish Pheromones and Discussion of their Possible Uses in the Control of Non-Indigenous Teleost Fishes. New Zealand Journal of Marine and Freshwater Research 38:399-417.

Sorensen PW, Wisenden BD. 2015. Fish pheromones and related cues. John Wiley & Sons.

Stacey, NE. 1991. Hormonal Pheromones In Fish: Status and Prospects. In Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish (Vol. 91, pp. 177-181). FishSymp.

Stacey N, Chojnacki A, Narayanan A, Cole T, Murphy C. 2003. Hormonally Derived Sex Pheromones in Fish: Exogenous Cues and Signals from Gonad to Brain. Canadian Journal of Physiology and Pharmacology 81:329-341.

Suzuki T, Komada H, Takai R, Arii K, Kozima TT. 1995. Relation between Toxicity of Cryoprotectant DMSO and Its Concentration in Several Fish Embryos. Fisheries Science 61:193-197.

Sveinsson T, Hara TJ. 2000. Olfactory Sensitivity and Specificity of Arctic Char, *Salvelinus alpinus*, to a Putative Male Pheromone, Prostaglandin F2α. Physiology & Behavior 69:301-307.

Svensson J, Fick J, Brandt I, Brunstrom B. 2013. The Synthetic Progestin Levonorgestrel Is a Potent Androgen in the Three-Spined Stickleback (*Gasterosteus aculeatus*). Environmental Science and Technology 47:2043-2051.

Tubbs C, Pace M, Thomas P. 2010. Expression and Gonadotropin Regulation of Membrane Progestin Receptor Alpha in Atlantic Croaker (*Micropogonias undulatus*) Gonads: Role in Gamete Maturation. General and Comparative Endocrinology 165:144-154.

Tubbs C, Thomas P. 2008. Functional Characteristics of Membrane Progestin Receptor Alpha (mPRα) Subtypes: A Review with New Data Showing mPRα

Expression in Seatrout Sperm and its Association with Sperm Motility. Steroids 73:935-941.

USEPA. 2008. Clean Watersheds Needs Survey 2008

Vulliet E, Baugros JB, Flament-Waton MM, Grenier-Loustalot MF. 2007. Analytical Methods for the Determination of Selected Steroid Sex Hormones and Corticosteriods in Wastewater. Analytical and Bioanalytical Chemistry 387:2143-2151.

Wasserman W, Richter J, Smith L. 1982. Protein Synthesis During Maturation Promoting Factor and Progesterone Induced Maturation in *Xenopus* Oocytes. Developmental Biology 89:152-158.

Wells K, Van Der Kraak G. 2000. Differential Binding of Endogenous Steroids and Chemicals to Androgen Receptors in Rainbow Trout and Goldfish. Environmental Toxicology and Chemistry 19:2059-2065.

Weston LA, Mathesius U. 2013. Flavonoids: Their Structure, Biosynthesis, and Role in the Rhizosphere, Including Allelopathy. Journal of Chemical Ecology 39:283-297.

Wu M, Chaudhary A, Khan IA, Dasmahapatra AK. 2008. Ethanol Teratogenesis in Japanese Medaka: Effects At the Cellular Level. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 149:191-201.

Yamazaki F. 1990. The Role of Urine in Sex Discrimination in the Goldfish *Carassius auratus*. Bulletin of the Faculty of Fisheries, Hokkaido University 41:155-161.

Zeilinger J, Steger-Hartmann T, Maser E, Goller S, Vonk R, Länge R. 2009. Effects of Synthetic Gestagens on Fish Reproduction. Environmental Toxicology and Chemistry 28:2663-2670.

Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. 2003. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proceedings of the National Academy of Sciences 100:2231-2236.

Zucchi S, Castiglioni S, Fent K. 2012. Progestins and Anti-Progestins Affect Gene Expression in Early Development in Zebrafish (D*anio rerio*) at Environmental Concentrations. Environmental Science and Technology 46(9):5183-92.

Zucchi S, Castiglioni S, Fent K. 2013. Progesterone Alters Global Transcription Profiles at Environmental Concentrations in Brain and Ovary of Female Zebrafish (*Danio rerio*). Environmental Science and Technology 47:12548-12556.

Zucchi S, Mirbahai L, Castiglioni S, Fent K. 2014. Transcriptional and Physiological Responses Induced by Binary Mixtures of Drospirenone and Progesterone in Zebrafish (*Danio rerio*). Environmental Science and Technology 48:3523-3531.