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Embryonic Exposure to the Synthetic-Progesterone Levonorgestrel (LNG) Results in Hyperactive Behavior in Zebrafish (*Danio rerio*)

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EMBRYONIC EXPOSURE TO THE SYNTHETIC PROGESTERONE LEVONORGESTREL (LNG) RESULTS IN HYPERACTIVE BEHAVIOR IN ZEBRAFISH (*DANIO RERIO*)

A Thesis

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ABSTRACT

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I assessed the effects of embryonic exposure to LNG on locomotor activity in zebrafish (*Danio rerio*). I performed an *in-silico* assessment of LNG using Quantitative Structure-Activity Relationships (QSAR) software to determine the predicted bioavailability, physiological half-life, and potential efficacy of LNG. There were no observable differences in hatching rate or morphology between treated and control groups. On 5 dpf (days post-fertilization), all treated, and control fish were assayed for changes in locomotor activity and thigmotaxis using computational locomotor activity software. Fish exposed to a 5-ng dose of LNG showed significantly increased hyperactive behavior and thigmotaxis compared to control fish ($p<0.05$). In a separate experiment, embryos were assessed at 2 dpf for an effect of LNG on heart rate. Fish exposed to LNG had significantly increased heart rates compared to controls. Collectively, these data indicate that exposure to low, environmentally relevant levels of LNG cause anxiety-like behavior in zebrafish.

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CHAPTER I

INTRODUCTION

Developed nations have witnessed increases in behavioral, social, and psychiatric disorders in adolescents and young adults over the last 50 years (Gray, 2011; Kelleher et al., 2000). These behavioral pathologies range in severity from attention-deficit disorder, to anxiety and depression, to suicidal tendencies (Fombonne, 1995; Prosser and Mcardle, 1996; West and Sweeting, 2003). The etiologies of these disorders are difficult to trace, and studies indicate that numerous psychosociological factors likely contribute to the observed rise in incidence, including social media, sociological pressures, and low self-esteem. (Susman, 1991; Smoller, 2003; McEwen, 2003; Inoff-Germain, 1988). Another factor thought to contribute to the onset and development of these disorders is the environment; specifically, exposure to anthropogenic molecules such as endocrine disrupting chemicals (EDCs). Endocrine disrupting chemicals are a heterogenous group of chemicals that disrupt (or have the potential to disrupt) endocrine processes in organisms. However, identification of a potential environmental EDC threat to behavior is complicated by difficulties in determining the relevant concentration, mode of action, and critical window of developmental exposure. Previous studies have shown EDCs that target the brain and alter neuroendocrine processes have the potential to induce behavioral disorders (Barrett et al., 2003; Kajta and Wójtowicz, 2013; Parihar et al., 2013). The fetal brain is particularly vulnerability to exogenous influences especially areas like the hypothalamus, which is associated with the neuronal pathways involved in anxiety/hyperactivity. Consequently,

accurate identification and knowledge of EDCs that disrupt neurodevelopment is critical to improving our understanding of how the environment influences behavior.

Levonorgestrel (LNG) is a synthetic progestin used in contraceptive pills that has been found at high concentrations in surface water worldwide (Chang et al., 2009; King et al., 2016; Nasuhoglu et al., 2012). The ubiquitous distribution of LNG in the environment, combined with its high bioavailability and steroidal properties, makes LNG a potentially disruptive environmental EDC. Studies conducted in humans, rodents, and fish suggest that steroidogenic pathways could hold the key to unlocking the relationship between an EDC, fetal neurogenesis, and endocrine-linked heath issues later in life. Alterations in fetal neurodevelopment correlate with anxiety and depression (Barret and Swan 2015; Kallak et al., 2017). Steroid receptors for androgens (androgen receptor; AR) and estrogens (estrogen receptor; ER) play a facilitative role in the establishment of neuronal pathways that control behavior (Chen et al., 2014). A recent *in vitro* study using an ER-transfected human glial cell line showed that LNG can disrupt brain development via the ER (Hinfray et al. 2016). Furthermore, LNG has an affinity for progesterone receptors (PR) and ARs (Frank Stanczyk 2007). Sprague-Dawley female rats chronically exposed to daily doses of 20-µg LNG via subcutaneous injection from the first day of pregnancy until pup delivery had offspring that expressed anxiety-like and autism-like behavior as adults (Follesa et al., 2002; Zou et al., 2017). These studies suggest that exposure to LNG during a critical window of neurodevelopment could lead to later manifestation of anxiety-like behavior. Therefore, the goal of this study was to determine if exposure to an environmentally relevant dose of LNG could cause anxiety-like behavior in zebrafish (*Danio rerio*).

My ***hypothesis*** was that embryonic exposure to LNG results in anxiety-like behavior. To test this hypothesis, I exposed zebrafish embryos to various concentrations of LNG for 5 days

and conducted a series of behavioral assays to assess the effect of LNG on locomotive activity, thigmotaxis, and heart rate. The following experiments were conducted:

Experiment 1: Identify if environmentally relevant doses of LNG increases locomotion in zebrafish. Quantitative-structure activity-relationship (QSAR) computer software was used to establish a dose curve for LNG. The doses of 2.5, 5, 50, 100 ng/L were chosen since they are within the range of doses found in the environment (Runnalls et al., 2010; Vulliet et al., 2011; Al-Odaini et al. 2013). While, 500 and 1000 ng/L were chosen as supra-environmental doses.

Next, identify an estrogen dose that alters larval behavior for use as a positive standard. Estrogen is a known stimulator of neurodevelopment. Previous studies have shown that embryonic exposure to estrogen increases hyperactivity. Embryos were exposed to different concentrations of estrogen to identify the lowest effective dose that causes hyperactivity in zebrafish.

Finally, the doses 2.5-1000 ng/L of LNG which were tested for toxicity effects by monitoring hatching rate. This allows us to measure morphological changes as a result of various doses of LNG.

Experiment 2: Determine if low-dose LNG affects thigmotaxis, and heart rate. The results from Experiment 1 were used to inform the choice of LNG dose used in thigmotaxis assays and to assess heart rate. The automated video-tracking software EthoVision XT (Noldus Information Technology, Netherlands) was used to record and analyze the effects of LNG exposure on locomotion and thigmotaxis at 5 days post-fertilization (dpf). Heart rate was assessed by counting heart beats under an inverted microscope for 15 seconds.

CHAPTER II

BACKGROUND SECTION

Behavior and Mental Health

The World Health Organization states that mental health includes a perceived well-being, ability to function and understand, and self-awareness of one's capabilities with a lack of mental illness (Murth et al., 2001). The National Institute of Mental Health (NIMH) defines generalized anxiety disorder (GAD) as a constant feeling of uneasiness, restlessness, and physiological automatic behavior such as trembling and twitching in non-stressful conditions (NIMH, 2020). However, characterization of anxiety-related behaviors is difficult due to the co-expression of other mental health disorders (Merikangas et al., 2010). Anxiety is a thus a broad term that refers to a spectrum of behavioral disorders that cause distress and psychological dysfunction in day-to-day life. For example, locomotive behaviors such as hyperactivity and impulsivity are defined as anxiety-like behaviors (Jensen et al., 1993). Increased heart rate can also be a characteristic of anxiety (Mezzacappa et al. 1997). Although the anxiety phenotype may manifest though numerous different behaviors, all are believed to stem from changes in neurosecretory pathways.

Mammalian studies strongly indicate that anxiety-like behaviors result from changes in neurotransmitter secretion (Charney and Redmond, 1983; Scantamburlo et al., 2007). The anterior cingulate cortex (ACC) in the prefrontal cortex (PFC) of the brain, which regulates impulse control, emotion, and error-related negativity, has been shown to have greater activity in study participants with self-reported anxiety than those without (Weinberg et al., 2010).

Sylvester et al. (2012) utilized magnetic resonance imaging to link increased excitation in error-monitoring brain regions such as the anterior insula, and decreasing function in the regions such as the dorsolateral prefrontal cortex that are responsible for executive control over amygdala activity, leading to anxiety-like maladaptive behavioral responses. Both genetics and the environment can alter neuronal transmission. 'Environment' is a broad term that includes both biotic (living) and abiotic (non-living) factors that can alter an organism's behaviors. Natural and anthropogenic abiotic molecules are of particular interest because of their ability and alter brain function when exposed at early developmental periods.

Environmental Endocrine-Disrupting Chemicals

Endocrine-disrupting chemicals are abiotic environmental factors that can or have the potential to disrupt endocrine function in organisms, including neuroendocrine regulation in the brain (Diamanti-Kandarakis et al., 2009). However, it can be challenging to identify and characterize EDCs. The chemical structure of the molecule can be informative regarding its potential mode of action, half-life, and bioavailability. However, elucidating the true efficacy and mode of action requires investigation because all EDCs have the potential to target and disrupt multiple organ systems. Moreover, the dose-dependent effects of an EDC on a cell are often non-monotonic. In some cases, low doses of an EDC can have more adverse effects than higher ones. Organisms may also be temporally sensitive to EDCs, with the severity of effects dependent on the developmental time of exposure (e.g., gestational vs adult). Thus, numerous factors must be considered when studying EDCs.

Steroids are lipophilic, can cross the blood-brain barrier, and play a strong regulatory role in neuronal development and neurotransmitter secretion. Thus, EDCs that mimic or manipulate steroids can be particularly disruptive of normal organismal functioning. For example, bisphenol A (BPA) is a xenoestrogen primarily known for its actions on reproductive function via ER binding and activation (Maffini et al., 2006). Prenatal exposure to BPA, measured in urine samples from pregnant mothers, has been shown to be associated with anxiety-like behavior in males and hyperactivity in female adolescents later in life (Harley et al. 2013; Braun et al., 2011; Perera et al,. 2012). Zebrafish have also been shown to exhibit hyperactivity following embryonic exposure to BPA due to AR-mediated, accelerated neuronal development of the hypothalamus (Kinch et al., 2015). The same study revealed bisphenol S(BPS) exposure, an analog used in place of BPA in commercial products, during the same embryonic period resulted in hyperactive behavioral phenotype (Kinch et al., 2015).

Similarly, ethinyl estradiol (EE), a synthetic estrogen used in combination with a progestin as a contraceptive, has been shown to have an observable effect on adult rodent behavior when exposed during early development in various rodent models. Ryan and Vandenbergh administered EE (5 μg/kg/day) daily via oral gavage to pregnant mice from the third day of conception until 21-days postnatal development. Subsequently, the offspring were observed to have anxiety-like behavior when compared to controls in both the elevated-plus maze and light/dark preference chamber assays (Ryan and Vandenbergh, 2006). Likewise, Arabo et al. observed with the free exploration and elevated plus-maze test anxiety-like behavior in the offspring of adult rats whose mothers were injected with EE (15 μg/kg/day) daily on the 9-14th day of pregnancy (Arabo et al., 2005). The same regiment of prenatal dosing with EE resulted in anxiety-like behavior in the offspring as adult rats when tested with the elevated plus maze,

photophobia, and burying behavior assays (Dugard et al, 2001). On the same subject, Zaccaroni et al. exposed fetal Sprague Dawley rats to lower doses EE of 4 and 400ng/kg/day, but with a longer exposure timeframe from gestational day 5 until postnatal day 32. Nevertheless, both of the dose EE2 treatment group displayed increased levels of anxiety in the novel place preference test at 40-45 post-natal date (Zaccaroni et al., 2016). Interestingly, the novel tank test, similar to the novel place preference test, revealed the progeny of zebrafish exposed to 1.2ng/L EE showed increased anxiety in comparison to control offspring (Volkova et al., 2015). These studies highlight the feature of developmental periods of vulnerability to exposure of exogeneous chemicals having observable behavioral changes later in life.

Methoxychlor (MXC), an organochlorine insecticide EDC, has displayed anxiety-inducing behavior later in life after exposure during early developmental periods. Similar to the other EDC's mentioned, MXC is classified as a xenoestrogen, as well as, AR antagonist due to its metabolites (Gaido et al., 2000). Martini et al. administered MXC to pregnant CD-1 mice (20 µg/kg) via ingestion from the 11^{th} day of gestation until 8 days after birth. In adulthood in the offspring, anxiety-like behavior was observed with the elevated plus-maze and open field test (Martini et al., 2014).

This link between early endocrine disrupting chemicals exposure and behavioral changes later in life is thought to be due to changes in the mechanism of ER mediated serotonin release which has been seen in different organismal models. In zebrafish, exposure of a high dose of E_2(0.005 µM) from embryo fertilization to larvae resulted in anxiety-like behavior in a thigmotaxis assay in 6dpf larvae (Ulhaq and Kishida, 2018). The same study revealed with immunohistochemistry doses of 0.001, 0.005, and 1 µM of E_2 were able to alter serotonin positive neurons in the hypothalamus (specifically the raphe and pretectal and thalamic complex)

as early as 48hpf(hours post-fertilization). Also, at 96hpf, the tryptophan hydroxylase isoforms (tph1a, tph1b, tph2), the enzymes responsible for synthesis of the neurotransmitter serotonin, were measured by semi-quantitative PCR and resulted in altered serotonin mRNA levels. The anxiety-like behavior characteristic was attenuated partially by adding ICI, an estrogen receptor blocker.

The plasticity of differentiating glial cells and neurons in the developing brain makes it vulnerable to exogenous influences. Several studies in mammals have demonstrated that EDCs can reprogram neuronal secretion and communication as late as the onset of puberty (Kawai et al., 2007; Castro et al., 2015; Adewale et al., 2011; Mahoney and Padmanabhan, 2010;Rasier et al., 2007 and 2008). When neuronal development is impaired, behavioral, or cognitive disorders can arise (Rice and Barone, 2000; de Graaf-Peters, 2006). Moreover, physiological processes controlled by the neuroendocrine system, such as physical changes during puberty, have been hinted by clinical studies to be delayed or initiated early by EDCs (Yum et al., 2013; Leijs et al., 2008). For example, the temporally sensitive development of secondary characteristics in both adolescent males and females had been shown to be vulnerable to the EDC polychlorinated aromatic hydrocarbon in a human study (Den Hond et al., 2002). At molecular level, the morphology and differentiation capacity of the cells responsible for the initiation of puberty have been shown to be influenced by steroidal hormones. *In vivo* studies on developing rodent hypothalamic glial, astroglia, and neuronal cell cultures clarified E_2 and ER being integral to cell differentiation by modulating trophic effects (Dueñas et al., 1996). In adult and juvenile female rats, chronic exposure to BPA, a xenoestrogen, from early gestation were shown to have lower hypothalamic ER expression (Rebuli et al., 2014). For these reasons, exogeneous substances with

the ability to mimic steroids are of substantial concern, especially for their effects on neuroendocrine-regulated development processes.

Estrogen, the Brain, and Behavior

The neuronal circuitry of the limbic system regulates developmental behavioral responses to external cues, modulated by steroidal hormones (Sokolowski and Corbin, 2012). Circulating and brain levels of the sex hormones estrogen (E_2) and testosterone primarily regulate neuronal development of the hypothalamus and amygdala as seen in rodent models (Romeo et al., 2000; Kim et al., 2011). In turn, the hypothalamus and amygdala are associated with the development of anxiety-like behaviors at an early age (Martin et al., 2009). However, the development of specific limbic system neural circuitry and how steroidal hormones influence them needs to be better understood.

The limbic system consists of the hypothalamus, basal ganglia, cingulate gyrus, thalamus, hippocampus, and amygdala. All of these areas are positioned between the cerebral cortex and brain stem and each component of the limbic system has neural circuitry connecting to the hypothalamus (Isaacson, 2001). The neural circuitry of the limbic system extends from the cortical to the subcortical area, to the diencephalon. The almond shaped amygdala is below the temporal lobe and is connected to the medial preoptic area and ventromedial portions of the hypothalamus. The nuclei of the amygdala that are known to be affiliated with anxiety are the basolateral amygdala, central amygdala, and the medial nucleus (Linsambarth et al., 2017). The basolateral amygdala interacts with the lateral hypothalamus influence the hypothalamus in motivated behavior (Reppucci and Petrovich, 2016). Interestingly all of these same regions work

with the hypothalamus to influence different behaviors. The hypothalamus has 11 nuclei, but the parvocellular and anterior periventricular nuclei are of particular interest for this study due to their roles in the manifestation of anxiety-like behavior and in stress responses (Scantamburlo et al., 2007). In a study of 60 psychiatric patients, anxiety behavior was believed to be a result of serotonin abnormalities (Apter et al., 1990). Additionally, the anterior hypothalamus is associated with both anxiety and aggressive behavior that are regulated by serotonin (Delville et al., 2000; Ricci et al. 2012).

Changes in neurodevelopmental processes involving the limbic system have been associated with a range of adolescent anxiety-like behaviors. For example, magnetic resonance imaging (MRI) neuroimaging in adolescent children has shown increased amygdala volume and metabolic rate to be associated with GAD (De Bellis et al., 2000). Additionally, Milham et al. (2005) found a reduction in left amygdala gray matter volume in pediatric patients with anxiety disorders when compared to patients without anxiety. In another part of the limbic system, the hypothalamus insults have had association with anxiety disorders. Weissenberger et al. (2001), by parent report, found higher rates of anxiety disorders in 12 patients with hypothalamic hamartoma when compared to their control siblings.

In rodents, the embryonic rostral/ventral diencephalon gives rise to the hypothalamus at about mid-gestation (Shimamura et al., 1995). The primordium is induced during neural plate formation (Xie and Dorksky, 2017). In mice, dividing radial glial cells (RGC) of the hypothalamus derive from the ventricular zone and produce glial and neuronal precursors that extend into the parenchyma (Robins et al., 2013). Transcription factors, like a basic helix-loop-helix (bHLH), aid in the differentiation of the precursors into a neuron. Importantly, *in vivo* embryonic mice studies have suggested the differentiation process is dependent on steroidal

hormone signaling (Varshney et al., 2017). In mammals, neurons of the limbic system are produced from early embryonic neurogenesis until late gestation, when the majority of the neurons have migrated (Sokolowski and Corbin; 2012).In embryonic zebrafish, in the absence of exogeneous signaling, hypothalamic progenitors have been observed to be self-regenerating neural progenitors (Duncan et al., 2016). Transcription factors such as insulin-like growth factor-1 (IGF-1) that regulate the development of hypothalamic neurons have been shown to be sensitive to steroidal hormones in *in vivo* rat fetuses (Duenas et al., 1996).

In addition, aromatase, an enzyme localized in RGCs, has been shown to contribute to neurogenesis (Forlano et al., 2001; Radakovits et al., 2009). Aromatase was shown shown to be altered in E_2 exposed zebrafish, resulting in an anxiety-like behavior (Ulhaq and Kishida, 2018). However, numerous E_2 signaling pathways affiliated with E_2 production can be altered by xenoestrogens resulting in anxiety-like behavior.

The neurotransmitters in the limbic system most strongly associated with anxiety are serotonin, dopamine, norepinephrine, and GABA (Nuss, 2015; Linsambarth et al., 2017; Zweifel et al., 2011). Each of these neurotransmitters has been shown to be influenced by steroidal hormones that modulate anxious behavior, especially E_2 (Imwalle et al., 2005; Tian et al., 2013; Pandaranandaka et al., 2006). Pandaranandaka et al. 2006 showed that treatment with E_2 reduced anxiety in ovariectomized rats, along with concentrations of serotonin, dopamine, and GABA in regions of the limbic system (Pandarankandaka et al., 2006). This result could be an effect of E_2 stimulating progenitor cell division, as observed in the ventricular zone (Martínez-Cerdeño et al., 2006). Notably, in rodents much of hypothalamic paraventricular nucleus is affected by serotonin, which increases neuronal excitability (Ho et al., 2007). In mammals, E_2 can potentially increase the capacity of serotonin synthesis and modulate anxiety-like behavior (Hiroi et al.,

2006). This capacity magnifies the effect that even exogeneous steroidal hormones potentially have on neural development and the function of behavioral regions of the brain.

Levonorgestrel (LNG)

Levonorgestrel is an oral contraceptive hormone previously identified as a potential EDC. Through feces and urine, LNG and its metabolites are able to enter the environment (Besse and Garric et al., 2009; López de Alda and Barceló, 2000).Furthermore, LNG enters aquatic environments through wastewater treatment plant effluent and agricultural runoff (Chang et al., 2009; Mansell et al., 2011). It has been found at concentrations ranging from 0.02 – 213 ng/L in environmental surface waters and sediments and is believed to be only partly metabolized by wastewater treatment (Runnalls et al., 2010; Vulliet et al., 2011; Al-Odaini et al. 2013).

Levonorgestrel is a synthetic progesterone that has been shown in rodent models to exert its effects via progesterone- and androgen-mediated pathways in the hypothalamus and pituitary (Lemus et al., 1992). Levonorgestrel has been suggested to not have a binding affinity for ER but does have a high affinity for PRs and ARs in those same regions as shown by an *in vitro* displacement analysis test in various rat endocrine tissues (Lemus et al., 1992). However, Rabe et al. (2000) observed *in vitro* LNG inducing ER-beta activity with ER-beta transfected COS-7 cells, fibroblast-like cells from monkey kidney tissue, in a dose dependent manor. Furthermore, metabolites of LNG have been shown to have ER binding affinity in HeLa cells, an immortal cell line derived from cervical cancer of an African-American woman, transfected with ER, which could further convolute results (García-Becerra, 2002). The ability of LNG to potentially modulate multiple steroidal processes makes it a viable candidate to regulate neurogenesis.

In vivo studies conducted at different developmental periods indicate that LNG may induce behavioral alterations. In rodents, prenatal exposure to LNG is associated with an increased risk of autism-like behavior (Zou et al., 2017). In addition, rodents chronically exposed to LNG as adults were found to express anxiety-like behavior in the elevated plus-maze (Follesa et al., 2002; Porcu et al. 2012). In rabbits, behavioral alterations have been suggested to be a result of a shift in steroidal hormones concentration effecting neuroendocrine activity (Kawakami and Sawyer; 1959). In an adult human clinical study of oral contraceptives, LNG, in combination with other contraceptives, was able to affect the hypothalamic-pituitary-gonadal axis, leading to lower E_2 and FSH concentrations (Vandever et al., 2008).

Zebrafish as an *In Vivo* Model for EDC Screening

Zebrafish are a viable *in vivo* model for EDC screening because their brain developmental dynamics are analogous to higher vertebrae (Segner, 2009; Blader and Strahle, 2000; Lohr and Hammerschemidt, 2011). The enzymes associated with hormone regulation, and hormone signaling pathways and receptors, are conserved between humans and zebrafish and serve similar functions (Machluf et al., 2011). Moreover, the embryonic period of zebrafish development is comparable to neurogenesis during human gestational development during the 2^{nd} trimester (Kinch et al., 2015). In reference to the neuroendocrine system, most of the anatomical and functional features of the hypothalamus are conserved in mammals and teleost fish (Xie and Dorsky, 2017; Löhr and Hammerschmidt, 2011). The lacto-, somato-, corticio-,

thyro-, and gonado- trope hypothalamic cell types are conserved, as are hypothalamic regulators (Löhr and Hammerschmidt, 2011).

Nevertheless, there are differences found between zebrafish and humans/mammals. Zebrafish behavior is not fully translational to humans since not all neuronal behavioral circuitry has been studied, and thus the data generated are limited in their applicability (Kastenhuber et al., 2010). Another general limitation associated with the use of a zebrafish model is that behavioral endpoints can vary across studies, with similar parameters quantified differently (e.g., velocity versus relative locomotive activity), and differences in acclimation times used (Kinch et al., 2015; Chen et al., 2011; Ulhaq and Kishida, 2018; Kalueff et al., 2013). However, this methodological limitation can be minimized by using velocity as a measurement, which is a more objective parameter to measure locomotive activity. Additionally, utilizing the same acclimation period utilized in pervious behavioral assays can help mitigate variability. In reference to the anatomy, the major hypothalamic differences between mammals and zebrafish primarily lie in morphology and include a reduced periventricular nucleus in mammals and a median eminence that is almost completely absent in zebrafish. But hypothalamic neurogenesis by proliferation and differentiation has been shown to comparable in zebrafish and humans (Xie and Dorsky, 2017).

Taken together, the characteristics of zebrafish embryonic development allow for rapid, high-throughput screening of EDCs, and a better understanding of the mechanisms underlying altered neuroendocrine-related development. Moreover, the high fecundity of zebrafish allows for the completion of data-rich experiments, and the *ex utero* developmental system allows for the manipulation of developmental periods comparable to human gestational periods, without maternal influence. Because zebrafish progress through relatively brief developmental periods,

including early neuronal development, data may be generated quickly; indeed, behavioral assays may be performed as early as 5 dpf. Most importantly, however, the HPG axis and behavioral responses are conserved in mammals and fish, allowing the zebrafish to act as a viable animal model of anxiety (Bencan et al., 2009).

CHAPTER III

MATERIALS AND METHODS

General Animal Housing

All protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Rio Grande Valley. A cross-strain of adult wild-type AB and TL was received from Texas A&M University and used for the experiment. On weekdays, adult zebrafish were fed dry flake food (Wardley Advanced Nutrition Tropical Fish) two times a day, at 10:30 AM and 4:30 PM, and were fed a supplementary meal of brine shrimp at 2:00 PM. On weekends, the zebrafish were fed dry flake food once a day at 12:00 PM. The amount of food given was limited to that which could be consumed within 5 minutes. Zebrafish were maintained under a 14 hours light: 10 hours dark light-dark cycle. Water quality was monitored daily to ensure that ammonia, nitrate, and nitrite levels were appropriate (API Freshwater Master test kit). Salt levels were also monitored daily via a conductivity meter, and salt was added if the value was found to be below optimum and water was added if found to be above (>300 μS/cm, <1000 μS/cm). Tank pH (>6.8,<7.6) and temperature (25.0-29°C) were also measured. The tank heater would be adjusted to be kept within the appropriate temperature. The tank pH would be adjusted by adding water conditioner. Ten-percent water changes were performed as needed to maintain water parameter values within an acceptable range, in addition to changing tanks. A 200-micron filter pad collected waste such as zebrafish feces and was changed daily. A 50-micron cannister collected smaller particulates and was changed at weekly intervals.

QSAR Predictive Modeling

Quantitative structure-activity relationship (QSAR) models were used to screen LNG for qualities characteristic of EDCs by utilizing the Organization for Economic Co-operation and Development QSAR toolbox as *in-silico* software. To understand this, levonorgestrel's prevalence in a water treatment plant and environmental mediums were analyzed. Furthermore, the physiochemical properties of LNG (molar mass, water solubility, vapor pressure, and log kow, melting point) were utilized in the sewage fugacity model for predicted fate in a wastewater treatment facility. As well, level III fugacity model in QSAR utilized the same physiochemical properties to estimate half-life of LNG in air, water, soil, and bottom sediment, which consists of varying pockets of air, water, minerals, and organic substances. Temperature in the simulation was set to 25°C. Next, LNG's chemical structure was analyzed for ER-binding potential against the ER binding database(ERBA OASIS).

Chemical Treatment

Stock solutions of LNG (Sigma-Aldrich) and E_2 (Sigma-Aldrich) were obtained in powdered form and were dissolved in ethanol (EtOH) and diluted with Zebrafish embryo media (E3) to the final concentrations of 2.5,10,50,100,500 nM for E_2 and 2.5, 5, 50, 100, 1000 ng/L for LNG as indicated in the experiments. The range of E_2 doses were chosen on previous literature for studying ER binding in zebrafish (Menuet et al., 2002). The LNG doses of 2.5, 5, 50, 100 ng/L were chosen since they are within the range of doses found in the environment (Runnalls et al., 2010; Vulliet et al., 2011; Al-Odaini et al. 2013). While, 500 and 1000 ng/L were chosen as supra-environmental doses. To generate the treatments, 0.1 g of LNG was

dissolved in 1 L of 100% EtOH and diluted with E3, by serial dilution from the 1000 ng/L, to generate solutions with concentrations of 100, 50, 25, 5 and 2.5 ng/L LNG. A 0.04% solution of tricaine (Sigma-Aldrich) was made by dissolving tricaine in E3.The vehicle control in each experiment was the dose of EtOH (0.001%) used for the highest dose of either E_2 or LNG in each experiment.

Experimental Design

Two groups of approximately 15 randomized pairs of adult male and female zebrafish were bred once a week at a ratio of 1:1 or 3:4 (m:f). Zebrafish were introduced to breeding tanks on the main zebrafish habitat system(temperature-controlled recirculating aquatic housing unit) after the 4:30 PM feeding on Tuesdays and Wednesdays to maintain fish at the optimum temperature. Males and females were separated by a divider. At the onset of light of the following day, the breeding tank divider was lifted and breeding was permitted for 45 minutes. After this period, the embryos were collected, washed with E3 according to Brand et al. (2002), and transferred to a 96 cell culture plate with E3 (Brand et al., 2002). The embryos were transferred into wells with a cut pipette tip containing approximately 100 μL of E3. The E3 was subsequently drawn out of the well with a pipette tip and replaced with either 200 μL of E3, or a LNG treatment mixed with E3. The embryos were kept in a dark incubator at 28°C until used in experiments.

The experimental design of the E_2 dose-curve consisted of replicate 96-well plates that included a control(E3 only), negative control of tricaine, and different doses of E_2 ranging from 2.5-500 nM. Tricaine was administered at 5dpf before testing. The top row (A) was assigned as the control treatment. Row B was the negative control of tricaine treatment. Row H was also assigned to the control to increase the sample size for the group for more accurate results. Rows

C, D, E, F, and G were assigned different doses of estrogen, but the row order of the doses was randomized across replicate plates to control for plate locality effects. No embryos were placed in wells in the A and B columns to avoid potential edge effects (Lundholt et al., 2003).

Each 96-well plate for the LNG exposures had a control, vehicle, positive control, and negative control treatment, as well as doses of LNG of 2.5, 5, 25, 100, 500, 1000 ng/L. A different treatment was assigned to each row. The control, vehicle, positive control, and negative control were assigned to same top four rows of the plate. Row A was the control, row B was the vehicle, row C was the negative control of tricaine, and row D was the E_2 positive control. The remaining E, F, G, and H rows contained the LNG treatments. Again, the row order of the dose was randomized across replicate plates.

For the thigmotaxis assay, the experimental design on the 6-well plates included the control, vehicle, tricaine, and the 5 ng/L LNG dose. To maintain equal representation on each plate, row A consisted of the control, E_2 as the positive control, and tricaine as the negative control. All of the wells of row B were assigned to the LNG dose.

Hatching

To evaluate the effect of LNG on larval morphology and development, embryos at each concentration of LNG were assessed for hatching from 24 hpf to 120 hpf. Hatching occurs when the zebrafish larva breaks the egg membrane by uncurling into a linear orientation. Delayed hatching is considered a sublethal indicator of impaired development (Martinez et al., 2019). Embryos were assessed under a microscope fitted with a Leica ICC50 HD at 3, 24, 48, 52, 55, 72, 76, 79, 96, 100, 103, and 120 hpf for evidence of hatching and morphological abnormalities,

including a bent spine or coagulation (Martinez et al., 2019). Fertilization was verified at the 24 hpf timepoint by visualization of heartbeat and pigmentation.

Behavioral Assays

A large number of behavioral assays have been used to study pathological behaviors in zebrafish. Most characterization of pathological behaviors has been undertaken via the interpretation of quantitative endpoints derived from automated video-tracking software. These assays can be conducted from as early as 5 dpf or on fish at the adult stage (Kalueff et al., 2013). However, adults typically have a greater range of quantifiable behavioral endpoints. The large amount of data that can be collected automatically allows for the construction of robust behavioral profiles in response to novel and tried compounds that can be confidently compared with control subjects (Rihel and Schier, 2012).

The behavioral analysis set-up was modeled after previous zebrafish studies (Cario et al., 2011; Zhou et al., 2014). Each 96-well plate was positioned on an x-ray viewing box on top of a fluorescent light source. Trials were recorded with a consumer-grade camcorder (Sony ILCE-6000). The video camera and 96-well plate was surrounded by a black curtain during each trial to prevent outside disturbance. At the start of each trial, the fish were allowed a 5-minute acclimation period. After this time, the movement of each fish was recorded for 5 minutes.

Locomotor Activity

In humans, a hyperactive behavioral phenotype is an anxiety-related behavior (Jensen et al., 1993). Quantifying locomotive behavioral phenotypes in zebrafish have translational value because similar behavioral phenotypes have been seen in adolescent and larval zebrafish (Ahmad

et al., 2012). Therefore, analyzing locomotive behavioral activity can further our understanding of how exposure to LNG during embryonic development can affect zebrafish at later life stages (Colwill and Creton, 2011). A locomotor behavioral assay was conducted on zebrafish larvae 5 dpf between 10 AM and 2 PM. Zebrafish were maintained from 0-5 dpf in their designated treatment, as well as throughout the recording. After 5 mins of acclimation, locomotor activity was recorded for 5 minutes.

Thigmotaxis

Thigmotaxis is an indicator of anxiety-like behavior in both rodents and zebrafish that is characterized by staying close to the wall of an arena. The introduction of a zebrafish into a novel environment typically results in the expression of anxiety-like behaviors such as thigmotaxis (Blaser and Gerlai, 2006), which can be quantified by measuring the total amount of trial time that the subject spends near the wall. These data can be supplemented by other quantifiable behavioral endpoints, such as temporal-spatial frequency, locomotive activity, and the number and length of locomotive freezes (Kalueff et al., 2013).

A thigmotaxis assay was performed as described in Ulhaq and Kishida (2018) and Schnoor et al. (2012). The thigmotaxis assay was conducted using 5-dpf zebrafish larvae, individually transferred into a 6-well plate before being placed in the trial set-up. The larvae were permitted to acclimate to their surroundings for 6 minutes before the trial started. Trials were recorded for 5 minutes. After recording was complete, the larvae were euthanized by being subjected to a low temperature (0-2°C). EthoVision XT was set up to include both an outer and inner zone. The outer zone was approximately 4 mm in length, which is the width of the free

space between the wall and the inner zone (approximate length of zebrafish larvae). The inner zone consisted of the rest of the area within the well (Fig. 1).

Measurement of Heart Rate

Heart rate is an autonomic physiological parameter that has been shown in humans to be associated with anxiety; in zebrafish, exposure to estrogen at a concentration of 5 μM has been shown to increase heart rate (Mezzacappa et al., 1997; Ulhaq and Kishida, 2018). Therefore, I included this parameter in the study because it bridges an autonomic physiological function known to be sensitive to estrogenic activity with anxiety-related behavior. At 2 dpf, embryos from a 96-well plate immersed in 200 μL of their respective treatment medium were placed under an inverted microscope and acclimatized for 15 minutes to ensure that the heart beat was stabilized. Afterwards, heart beats were counted manually for 15 seconds with a clicker.

Video Analysis

The behavior of each fish was automatically quantified using Noldus EthoVision XT15 software. Each video was converted from an MTS file into an MP4 file before analysis for compatibility with the software. Measurements taken from the video were calibrated by vertical and horizontal lines along the well plates (12.7 cm x 8.5 cm) and pixels were converted into cm. Using the multi-arena module, each well was delineated as a separate arena using the shape tool and labeled. The detection settings were adjusted for each well by adjusting the gray scale value to ensure that each larva was being reliably tracked. For thigmotaxis, the time was quantified, in seconds, when located in the outer zone of the arena. The locomotive activity of the zebrafish larvae would be quantified as velocity as cm per second. Once values were obtained and

statistical analyses had been completed, values were converted to percentage values relative to the control for easier under stability and comparability between groups.

Statistical Analysis

Data were analyzed using GraphPad Instat 3 and are shown as mean ± SEM unless stated otherwise. The hatching data was analyzed with one-way ANOVA with Dunnet's post-hoc-test. Preliminary screening indicated that the data for the E_2 and LNG locomotive activity assays were non-normal. Therefore, dose curves were analyzed with non-parametric Dunn's multiple comparison tests followed by Kruskal-Wallis post-hoc tests. Subject responses from the thigmotaxis assay were evaluated by one-way ANOVA followed by Tukey's Honestly Significantly Different (HSD) post-hoc tests. Differences between groups were considered to be significant when $p < 0.05$.

CHAPTER IV

RESULTS

Experiment 1: Investigate the Effects of LNG on Development and Morphology

QSAR Analysis

Table 1 shows the QSAR predicted percentage of the LNG mass distribution during wastewater treatment generated from the BioWin simulation. Specifically, 6.69% of the predicted mass will be in the primary sludge which has undergone sedimentation and chemical precipitation and only 5.72% would be predicted to amass in the waste sludge. The volatilization process removes volatile organic compounds by vaporizing dissolved substances which is typically volatile organic compounds (high vapor pressure and low water solubility). Levonorgestrel possess neither of these chemical properties thus is predicted to not vaporize or have mass accumulate in the settling volatilization. Aeration of the water is utilized for removing dissolved gas, but LNG would not be predicted to be affected by the process with 0% mass being lost. Only 0.18% of the LNG mass would be predicted to be affected by biodegradation with 0.03%, 0.01%, and 0.14% being lost to primary, settling, and aeration biodegradation activity, respectively. The end result is the final water effluent would be predicted to be have an 87.42% mass distribution of LNG. This is indicative of LNG not being biodegradable and able to persist throughout a wastewater treatment plant recycling into the environment.

Levonorgestrel's half-life, in hours, in different common environmental mediums was estimated by using BIOWIN (*Ultimate*)/AOP programs in QSAR (Table 2). Levonorgestrel's physicochemical properties of molar mass, water solubility, vapor pressure, melting point, and log Know were used to calculate the half-life. In sediment medium, LNG was projected to have the highest half-life at 13,000 hours which is nearly a year and a half. On the same note, LNG's half-life was seen to be only 2880 hour long in soil and the half-life of 1440 hours seen in the water medium. While having a very low (relative to the others) predicted half-life at 1.41 hours in air.

QSAR was also utilized to analyze LNG's chemical structure (Fig. 2A) for ER binding potential. First, LNG's molecular weight was compared against the ER-binding database (ERBA OASIS). Initially, QSAR's results projected, LNG was labeled as having "strong" to "very strong binding" potential to ER due to having a molecular weight of 312 daltons (Fig 2B) and possessing a hydroxyl group (-OH) (Fig. 2A). However, LNG's chemical structure was analyzed for accessible -OH and amino groups. Levonorgestrel's -OH is deemed impaired due to being attached to a ring with a substituent (Fig 2A). In comparison, the chemical structure of representative ligands with non-impaired OH groups ligands were provided in fig. 2C. These examples possess a 5-6 member carbon ring without substitutions at one of the position next to these groups: (C{H2}C{H}O{H}, c{H}cO{H}, etc.). In fig. 2D an illustration of ligand binding sites on ER predicts that 5-6 member carbon ring chemicals without an unhindered OH functional group would bind to site A. However, LNG has a hindered OH-functional group (Fig 2A), thus QSAR predicted that the ER-binding potential of LNG is low. Based on these predictions, the relevant doses of LNG found in surface waters (2.5, 5.0, 50,100 ng/L), as well

as, amounts higher reported in the environment (500,1000 ng/L) were used for the rest of the study.

As a quality control for the LNG experiments E2 was tested a various does to determine which dose increased velocity as an indicator of locomotor activity in 5dpf zebrafish

Embryos were exposed to E_2 beginning at 3 hpf, and swimming activity was measured at 5 dpf and compared to non-treated and negative controls. Figure 3 is a bar graph that shows the mean (SEM) velocity of 5 dpf zebrafish in the various treatment groups. Exposure to 100 nM of E_2 significantly ($P<0.01$) increased the velocity of the zebrafish relative to the control (Fig. 3), which is validated measurement for locomotor activity. However, no significantly increased activity was seen in response to any of the other E_2 doses (2.5 nM,10 nM,50 nM, 500 nM). Fish exposed to tricaine (a negative control) also exhibited behavior that was significantly lower from all of the E_2-treated groups, as well as the control. Tricaine was found to be significantly different from E_2 doses ranging from 2.5-100 nM ($P<0.001$). However, fish in the 500 nM E_2 group and control had the same significant difference from the negative control ($P<0.01$).

Assessing the Effects of LNG on Locomotion

Embryos were exposed to various doses of LNG from 3 hpf to 5 dpf at which time they were recorded for the locomotion assay, similar to the assay that was undertaken for E_2 (Fig. 4) A vehicle control group was included that was treated with the highest concentration (0.001%) of EtOH used dilute LNG and E_2 in the experiments. E_2 at a concentration of 100 nM was included as a positive control, and was found to be significantly higher from the non-treated control ($P<0.001$), vehicle-treated control ($P<0.001$), and negative control tricaine groups ($P<0.001$). Zebrafish in the 5 ng/L LNG group exhibited significantly increcased swimming velocity

compared to fish in the non-treated control($P<0.001$), vehicle treated control ($P<0.001$), and negative control tricaine ($P<0.001$) groups. Fish in the treated with other LNG doses (2.5, 25,100, 500, 1000 ng/L) did not exhibit significantly different responses from those observed in the control, vehicle, or E_2 groups. Fish in the tricaine group also had responses that were significantly lower from all of the LNG and E_2 dose groups, as well as control and vehicle. The results of this assay identified the 5ng/L dose of LNG was capable on increasing locomotor activity.

Hatching

A one-way ANOVA with Dunnett's post-hoc test indicated that there was no effect of LNG on the development (hatching time) of zebrafish after exposure to LNG at concentrations ranging from 5-1000 ng/L (Fig. 5). The zebrafish were observed at 3, 24, 48, 52, 55, 72, 76, 79, 96, 100,103, and 103 hpf under a microscope. The non-treated control, vehicle treated, and the LNG treated groups were shown to be hatching typically at the 52 hpf timepoint. When the zebrafish larvae emerged from the embryo into a linear body position, they were considered hatched. When comparing the control and vehicle groups to the LNG treatment groups, the mean hatching time were not significantly different with very little variation. In this experiment, there was no evidence indicating that any LNG dose had an effect on early developmental morphology. Therefore, those same doses would be utilized in the behavioral assay experiments.

Experiment 2: Assessing the Effects of LNG on Thigmotaxis and Heart Rate.

In the next experiment, I used the 5 ng does of LNG to determine its effects on thigmotaxis in 5dpf zebrafish. First, the velocity of the zebrafish thigmotaxis assay was analyzed to ensure that the effect of LNG on locomotor activity was consistent with results from the

previous experiments. As expected, Fig. 6 shows that both larvae in the 100 nM E_2 and 5 ng/L LNG groups showed a significantly ($P<0.001$) increased mean velocity compared with to controls. Fish exposed to the negative control, tricaine, showed a significantly slower velocity compared to fish in the E_2 ($P<0.001$) and LNG ($P<0.001$) groups, but not the control ($P>0.05$). Visualization of locomotive activity tracks from a representative 6-well plate shows that the fish in the LNG 5 ng/L group appear to move more compared to those in the control and tricaine groups, as well as the 100 nM E_2 (Fig. 7). Next, these same fish we assayed for thigmotaxis.

Fig 8 shows that fish exposed to 100 nM of E_2 and 5 ng/L LNG spent a significantly greater proportion of time in the outer zone compared to the control and tricaine groups (Fig. 7). The tricaine group was also significantly negatively different from the control.

Finally, a heart rate assay was performed at to determine if the 5 ng LNG dose altered heart rate in zebrafish at 2 dpf (Fig. 9). The heart rate was significantly increased in both the positive control E_2 ($P<0.001$) and 5 ng/L treated LNG ($P<0.05$) groups compared to the vehicle ($P>0.05$), tricaine ($P>0.05$), and control groups ($P>0.05$). An E_2 dose of 5 nM was used because previous studies have reported that this dose is sufficient to significantly increase heart rate (Ulhaq and Kishida, 2018). The average heart rate of fish in the tricaine group was significantly decreased compared to the E_2 ($P<0.001$) and LNG ($P<0.001$) groups, but was similar to both the vehicle ($P>0.05$) and control ($P>0.05$).

Discussion

Although a majority of studies have shown that the fetal and adolescent periods of development are particularly vulnerable to exogeneous chemicals (Spear, 2000, 1991; Spyker, 1975; Rice and Barone, 2000), fetal exposure to LNG has not been shown to cause noticeable

birth defects (De Santis et al., 2005). The primary goal of this study was to evaluate the effect of exposure to environmentally relevant doses of LNG during embryonic development on locomotive activity, thigmotaxis, and heart rate as indicators of anxiety. This endeavor is important because anxiety disorders affect approximately 31.9% of American adolescents (Merikangas et al., 2010). Moreover, studies of mammals suggest that exposure to prenatal contraceptives may be a causal factor (Li et al., 2018). The results of this study are consistent with the hypothesis that low levels of LNG induce anxiety-related behavior. In this study, I showed that the period corresponding to neuroendocrine development is sensitive to the exogeneous steroids LNG and E_2. Interestedly, lower doses of LNG induced anxiety-like behavior, however higher doses did not. The zebrafish exposed to LNG showed increased velocity in response to a 5 ng/L dose but did not show increased velocity until reaching close to the 1000 ng/L dose, but not significantly higher. This resulted in an inverted U-shape, which is similar to the non-monotonic dose response curve observed in response to other EDCs, such as BPA (Hill et al., 2018). A non-monotonic dose response curve suggests that LNG may act in a non-linear fashion, with lower doses resulting in a higher likelihood of developing an anxious behavioral phenotype. This is important since lower doses of LNG have been found in environmental water sources with.

These data shed insight on the importance of steroidal hormones in the development of behavioral disorders, possibly through neurogenic-mediated mechanisms. In addition, these data provide further clarification on the periods of development at which exposure to exogeneous hormones may result in a permanent change in brain morphology (Nugent et al., 2011).

The underlying mechanism leading to the observed behavioral differences in the thigmotaxis and locomotive behavioral assays could potentially be related to altered

neurogenesis in the hypothalamus, as suggested by previous studies (Kinch et al., 2015). In adult zebrafish, ERs and aromatase B are coexpressed in hypothalamic radial glia and have been suggested to control migrational and differentiation as consequence of E_2 exposure (Diotel et al., 2013). One potential mechanism that could be affected by LNG, E_2 biosynthesis can be catalyzed by cytochrome P450 aromatase, which is carried out by the product of the *cyp19a1* gene (Cui et al., 2001; Simpson et al., 2001). Developmental studies of zebrafish have shown that expression of brain aromatase is increased in embryos 12 hpf and is regulated by a positive feedback loop with estrogen acting on the estrogen response element of cyp19a1b (Sherilyn et al., 2006). LNG has a demonstrated ability to induce brain aromatase in zebrafish-specific bioassays, offering a potential mechanism for the modulation of E_2 and associated anxiety-like behavior (Hinfray et al., 2016). Studies have shown that metabolites of LNG have estrogen-binding affinity (García-Becerra, 2002). Similarly, the QSAR results were indicative of LNG having ER binding potential due to having a low molecular weight "strong-very strong binding" affinity (Fig. 2B), but OH-hinderance does not allow for this. Thus, LNG has low binding affinity for ER, so not likely binding directly to ER and could be either increasing aromatase or aromatase is converting to ER. Alternatively, this process could be facilitated through the AR, as suggested by Kinch et al. (2015) who reported that aromatase is an AR-mediated neurogenesis process during early hypothalamic development (Kinch et al., 2015). But the effect of LNG on AR could depend on the developmental stage. Liang et al.'s (2015) study on juvenile zebrafish suggested exposure of the progestin norgestrel did not affect AR gene transcription but was able to alter through an androgen receptor-mediated pathway.

Additional research is now required to reveal the molecular actions of LNG that result in a heightened anxious phenotype. Ulhaq and Kishida (2018) demonstrated that 1 μM of E_2 was

sufficient to induce anxiety-like behavior, as assessed by a thigmotaxis assay, but 0.005 μM E_2 was not (Ulhaq and Kishida, 2018). Saili et al. (2012) found that exposure to 0.1 μM E_2 led to a hyperactive behavior phenotype in zebrafish larvae. Similar findings have been reported for rodents, but with E_2-associated with reductions in anxiety-like behavior at different estrogen doses (Walf et al., 2009). Taken together, these findings demonstrate that E_2 exerts biphasic dose-dependent effects on behavior, which could be similar to what is occurring with LNG.

Conclusion

The study showed that developmental exposure to an environmentally relevant, low dose of LNG resulted in the expression of anxiety-like behavior. Both LNG and E_2 showed concentration-specific effects in their ability to induce behavioral alterations. These data indicate that embryonic zebrafish are susceptible to the effects of E_2 and LNG during neuronal development. Considering the similarity of results obtained in response to E_2 and LNG, it is possible that LNG acts in an estrogenic manner to facilitate the development of anxious behavior through modification of neurogenesis.

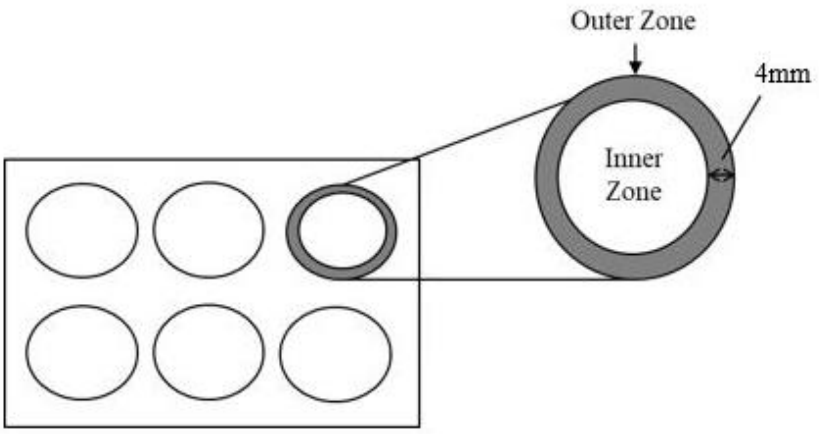


Figure 1: Thigmotaxis arena design

The inner zone included the central area of the well, with a diameter of 31mm. The outer zone consisted of a ring with a width of 4 that encircled the inner zone and included the wall of the arena.

Location in Treatment Plant	Predicted % Mass Distribution
Primary Sludge	6.69%
Waste Sludge	5.72%
Primary Volatilization	0.00%
Settling Volatilization	0.00%
Aeration Off Gas	0.00%
Primary Biodegradation	0.03%
Settling Biodegradation	0.01%
Aeration Biodegradation	0.14%
Final Water Effluent	87.42%

Table 1: Quantitative structure-activity relationship analysis of LNG's water treatment prediction

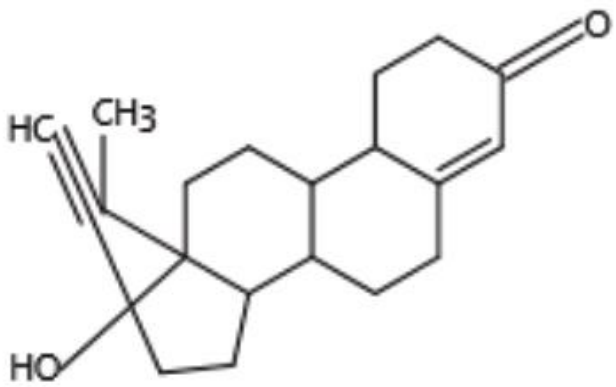
Predicted fate of LNG after wastewater treatment processing. % was equal to the total amount.

Medium	Half-life (Hours)
Sediment	13,000
Soil	2880
Water	1440
Air	1.41

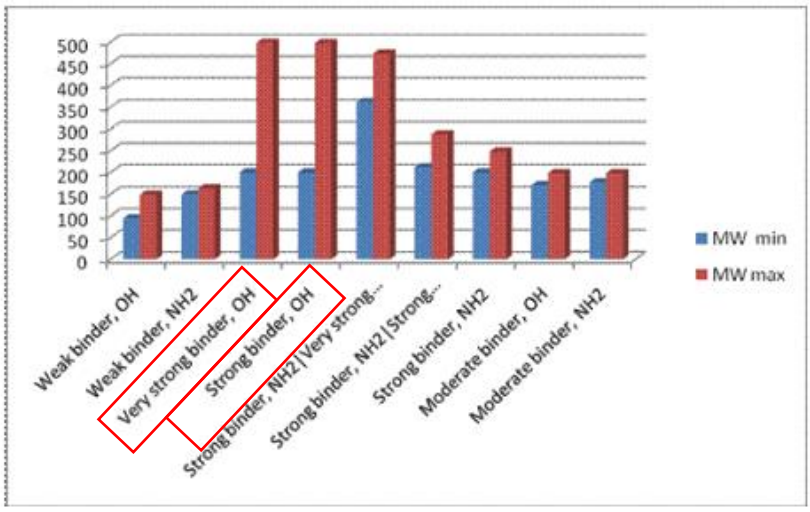
Table 2: Quantitative structure-activity relationship analysis of LNG's bioavailability

The predicted half-life of LNG in different, common environmental media.

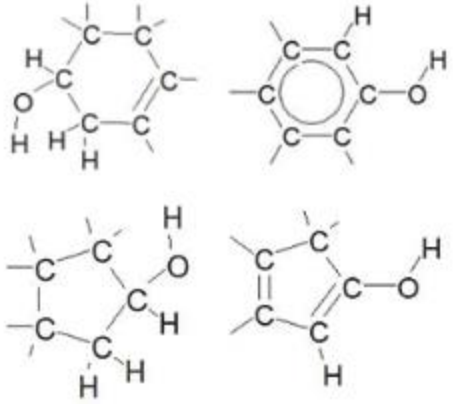
A)


O
HC
CH3
HO

B)


500
450
400
350
300
250
200
150
100
50
0
Weak binder, OH
Weak binder, NH2
Very strong binder, OH
Strong binder, OH
Strong binder, NH2 | Very strong...
Strong binder, NH2 | Strong...
Strong binder, NH2
Moderate binder, OH
Moderate binder, NH2
MW min
MW max

C)



D)

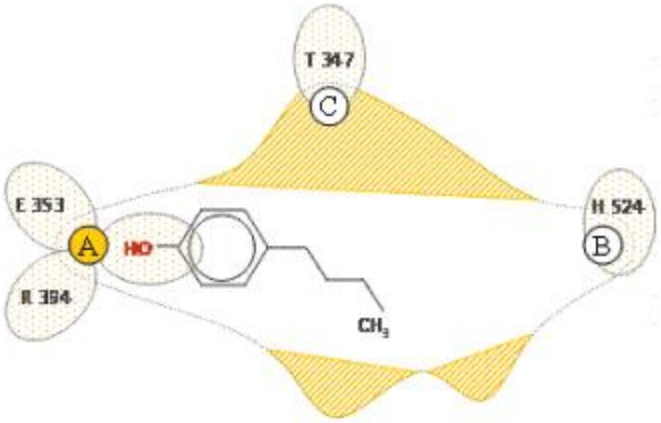


Figure 2: Estrogen receptor binding prediction report of LNG from QSAR program
Estrogen receptor binding prediction report of LNG from QSAR program. A) Chemical structure of LNG with a hydroxyl group on the lower left portion attached. B) A graph comparison of ER-binding potential as shown by ER-binding database (Estrogen Receptor Binding Affinity OASIS) showing that chemicals possessing a hydroxyl (-OH) group with molecular weight (MW) between 150 Daltons and 500 have binding potential to ER. LNG possesses a MW of 312 Daltons categorizing it as a strong binder or very strong binder (marked by red rectangles). C) Four hydroxyl ligand chemical structures representing non-impaired -OH groups to compare to LNG. In comparison to the non-impaired -OH groups, LNG has an impaired -OH group. D) A schematic representation of a hydroxylated ligand interacting at site A of the ER binding pocket is shown. Chemicals with a single 5-or 6-member carbon ring structure with an unhindered -OH (one in the para- or meta-position on the ring) are ER binders. LNG was shown to hindered OH thus would be predicted to not have this same type of binding.

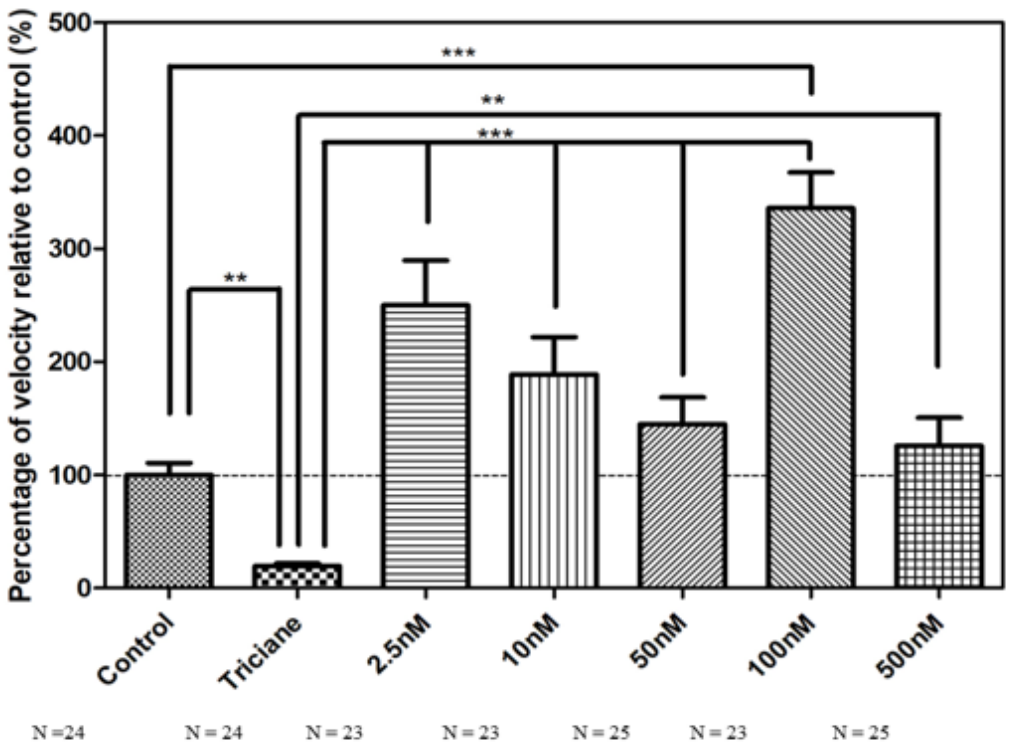


Figure 3: Dose curve of estrogen reveals 100 nM estrogen exposure during neuronal development induces hyperactivity at 5 dpf

Embryos were exposed at 3 hpf and velocity was measured at 5 dpf. Tricaine was utilized at a negative control. Data are shown as mean ± SEM; *P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001 Kruskal-Wallis test, Dunn's post-hoc test.

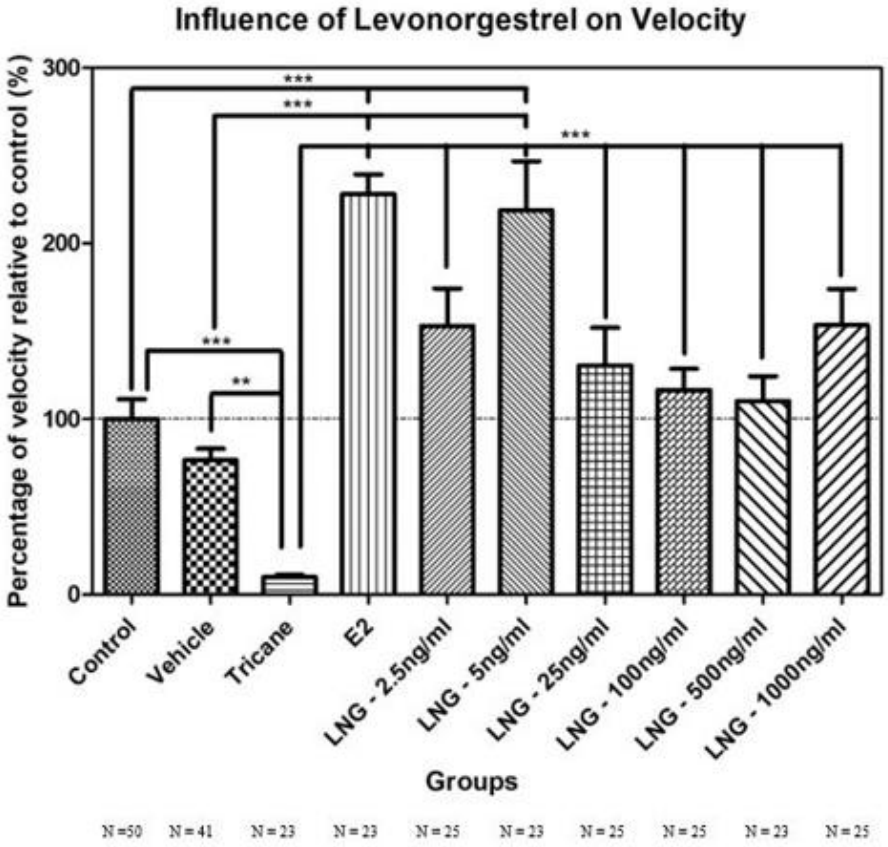


Figure 4. Developmental exposure of embryos to 5 ng/L of LNG induces hyperactivity at 5 dpf

Embryos were exposed at 3 hpf and velocity was measured at 5 dpf. Vehicle was the highest dose of EtOH used for LNG. Estrogen at 100 nM was used as a positive control. Tricaine was utilized as a negative control. Data are shown as mean ± SEM;*P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001 Kruskal-Wallis test, Dunn's Post Test.

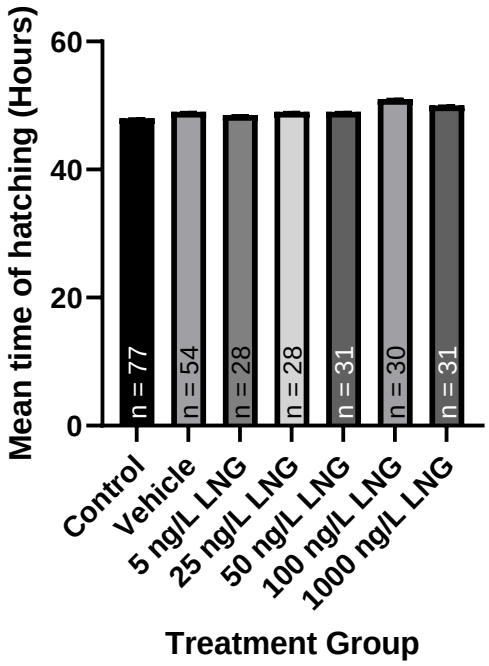


Figure 5: Hatching time

The hour of hatching was recorded for zebrafish embryos exposed to 5, 25, 50, 100, 1000 ng/L of LNG, vehicle(EtOH), and an unexposed control group.

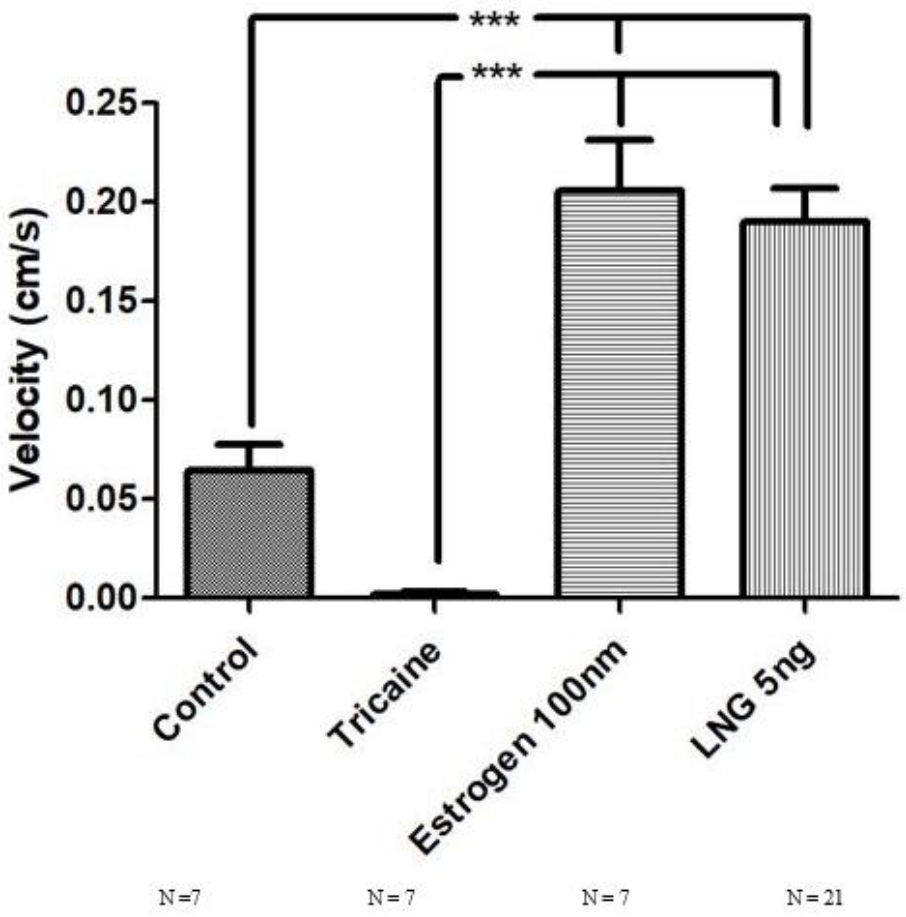


Figure 6: Velocity of thigmotaxis assay larvae

Velocity was measured at 5dpf. Estrogen at 5nM was used as a positive control. Tricaine was utilized as a negative control. Data are shown as ± SEM;*P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001 one-way ANOVA, Tukey's Honestly Significant Difference Test.

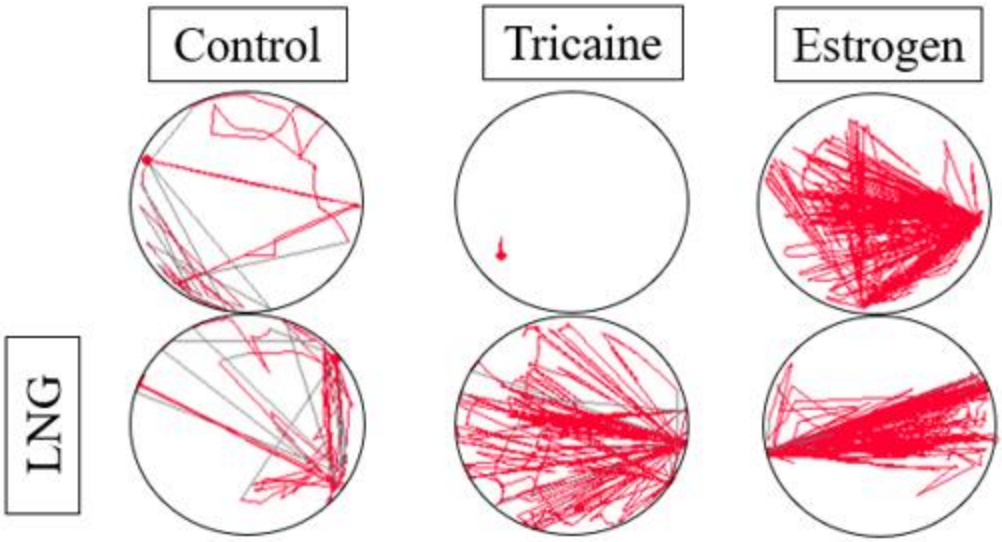


Figure 7: Representative images of movement patterns of fish exposed to E2, LNG, and controls

Representative locomotive activity of one of the 6 well plates of the larvae's movement is shown.

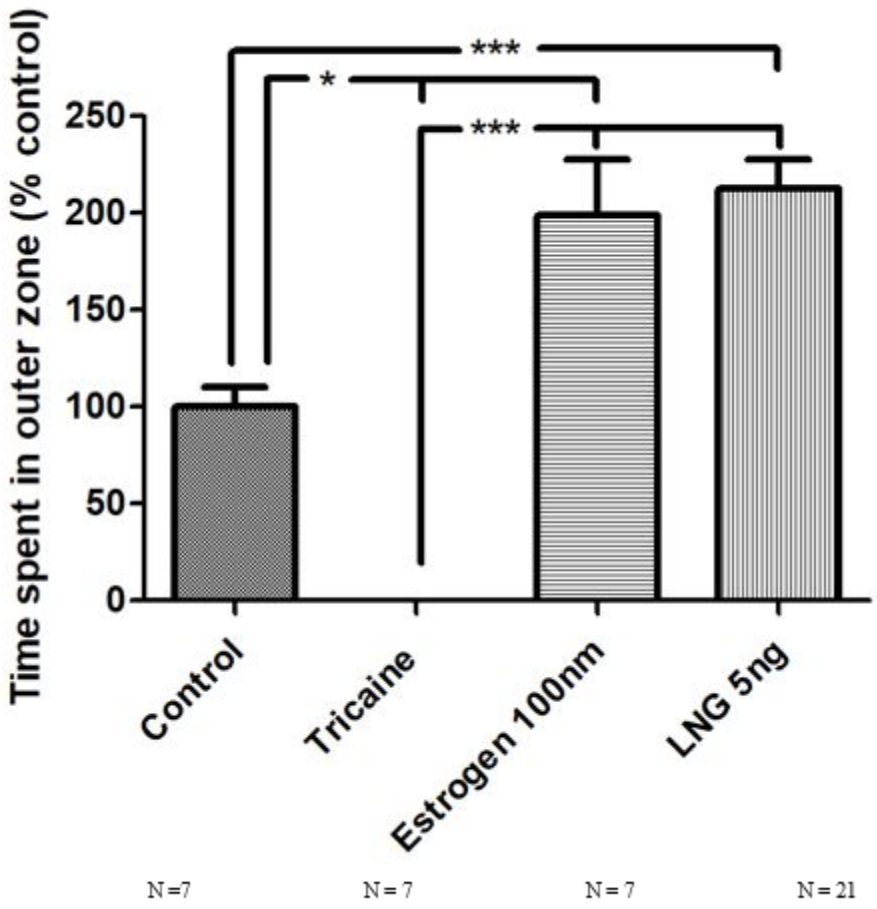


Figure 8: Thigmotaxis assay

Thigmotaxis assay was evaluated at 5 dpf. Estrogen at 100nM was used as positive control. Tricaine was utilized as a negative control. Data are shown as mean ± SEM;*P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001 one-way ANOVA, Tukey's Honestly Significant Difference Test.

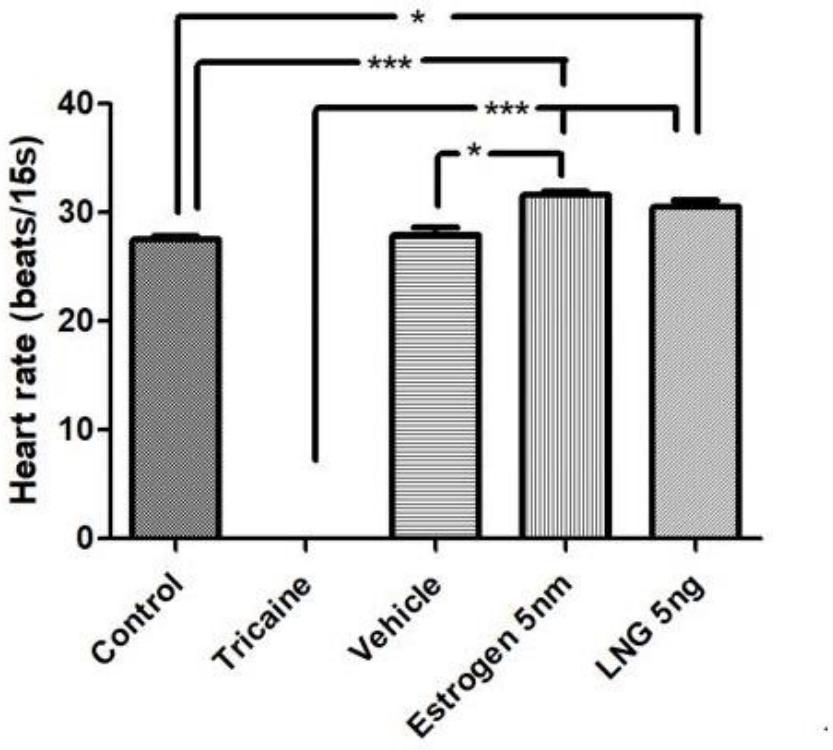


Figure 9: Heart rate measurement in embryos exposed to low doses of E2 and LNG

Heartrate was measured at 2 dpf. Vehicle was the highest dose of ethanol used. Estrogen at 5 nM was used as a positive control. Tricaine was utilized as a negative control. Data are shown as mean± SEM;*P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001 one-way ANOVA, Dunn's Multiple Comparison Test.

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