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Modulation of Estrone Exposure Effects Mediated Through Environmental Factors in Male Fathead Minnows, *Pimephales promelas*

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MODULATION OF ESTRONE EXPOSURE EFFECTS MEDIATED THROUGH ENVIRONMENTAL FACTORS IN MALE FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

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Laboratory exposures indicate that estrogens and estrogen mimics can cause endocrine disruption in male fathead minnows (*Pimephales promelas*). In the wild, conditions are not static as is often the case in the laboratory. Changes in environmental parameters can trigger physiological and anatomical changes in fish that have the potential to alter the uptake and observed effects of estrogenic chemicals. To explore the role of environmental variables on the expression of biomarkers of estrogenic exposure, adult male *P. promelas* were exposed to estrone under various environmental conditions (differing temperatures, diets, salinities and dissolved oxygen concentrations) in the laboratory for 21 days in a flow-through system. Plasma vitellogenin, morphological characteristics, hematological parameters, and histopathology were assessed to determine the severity of estrogenic effect. Plasma vitellogenin was most drastically elevated in fish exposed to estrone at a low temperature (18°C) and fed a restricted diet, and was not significantly elevated over the control when fish were exposed to the same estrone concentration (78 ng/L at a high temperature (26°C) and fed a restricted diet. This may have implications in field studies taken during seasons in which these conditions are present, and vitellogenin is used as an indicator of the health of an aquatic system. Salinity at 10 ppm and 50 ppm added NaCl had no significant effect on biomarkers of estrogenic exposure, however estrone concentrations in excess of 85 ng/L corresponded with significantly reduced body condition factor compared to control. Fish exposed to estrone (13 and 51 ng/L) at low dissolved oxygen (hypoxic) conditions showed significantly greater increase in plasma vitellogenin concentrations in comparison to those exposed at near-saturated dissolved oxygen. This effect was not observed in fish exposed to much higher estrone concentrations (292 and 390 ng/L). Significant reductions in hematocrit and gonadosomatic index compared to control were also noted at high (282 ng/L) estrone concentrations. These data indicate that environmental conditions modulate the effects of estrogenic exposure in male *P. promelas.* We anticipate that accounting for a

spectrum of environmental conditions may be necessary for laboratory exposures designed to assess the impact of exogenous estrogenic chemicals.

ACKNOWLEDGEMENTS

For my dad.

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Chapter I

LITERATURE REVIEW

Biology of the Fathead Minnow, *Pimephales promelas*

The fathead minnow, *Pimephales promelas*, is a teleost fish belonging to the family Cyprinidae (Geiger et al., 1988). Males are generally larger than females, and may exceed 9 cm in length (Scott, 1964). Both sexes are deep bodied, with a single, soft-rayed dorsal fin, slightly forked caudal fin, a blunt head with a cranial hump (more prominent in males) and a small, subterminal mouth (Nelson and Paetz, 1992). During breeding season, *Pimephales promelas* exhibit strong sexual dimorphism. Males develop broad, black vertical bands, three rows of keratinized nuptial tubercles on the snout, and a thick dorsal pad anterior to the dorsal fin (Smith, 1979; Fig. 1.2). The species is distributed widely throughout North America (Fig. 1.1), and has been introduced beyond its native range as a result of its popularity as a baitfish among anglers (Pflieger, 1975). *Pimephales promelas* are omnivorous feeders and can tolerate a range of pH and salinities (McCarraher and Thomas, 1968), making them a highly successful cyprinid species that plays an important role in various food webs (Nelson and Paetz, 1992; Jackson and Mandrack, 2002). They also possess an interesting, and highly debated evolutionary adaptation: the ability to secrete a

pheromone alarm substance, called Schreckstoff, which elicits an escape response when epidermal cells sustain mechanical damage (Smith, 1986; Mathis and Smith, 1992). Due to ease of culturing, wide geographical range, rapid development (sexual maturity by four months post-hatch), ecological relevance, fractional spawning and sensitivity to environmental pollutants (Denny, 1987; Geiger et al., 1988; Jensen et al., 2001) the fathead minnow is a popular model for toxicity testing.

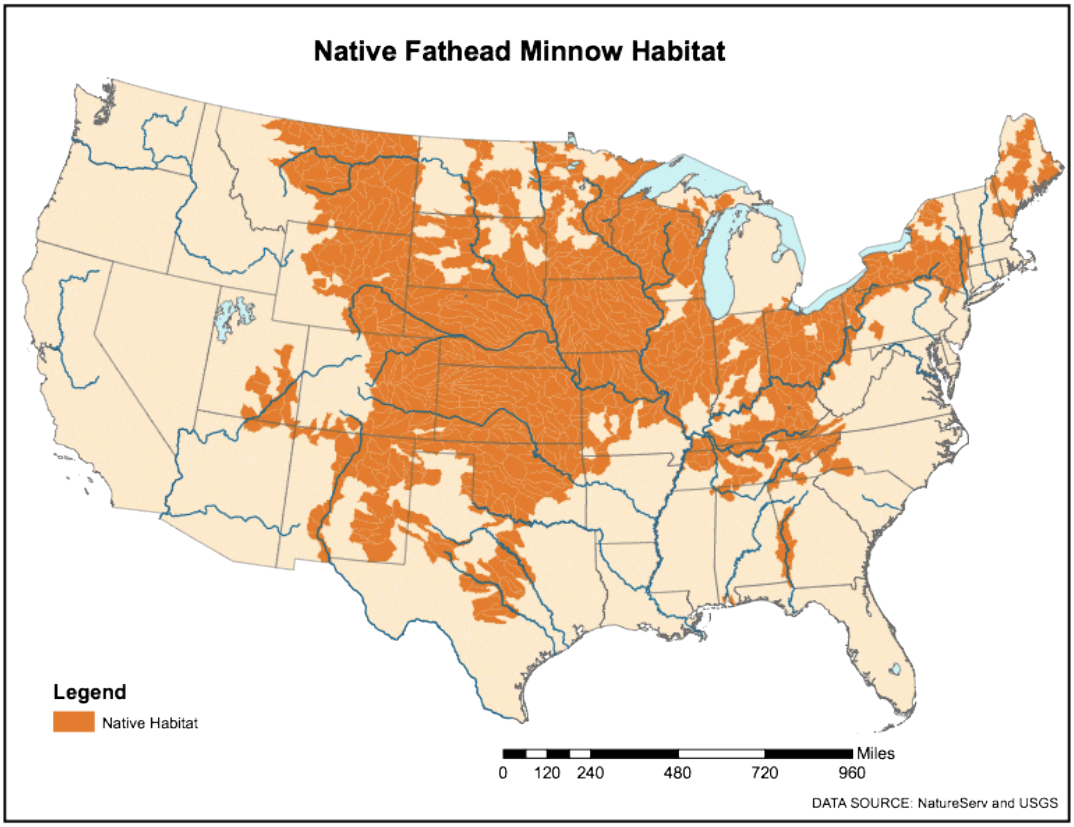

Figure 1.1. Native range of *Pimephales promelas* (Credit: William Heikkila).

As a fractional spawner, *Pimephales promelas* can spawn continuously under the right conditions in the laboratory (Gale and Buynak, 1982; Jensen et al., 2001). In

nature, spawning begins when temperatures reach approximately 15.6°C and photoperiod approaches 16 hours light/8 hours dark, and continues until the fall, until temperatures drop below 15.6°C to 18.4°C (Prather, 1957; Duda, 1989; Danylchuk and Tonn, 2001). Male *Pimephales promelas* exhibit distinct, territorial behavior during the breeding season (McMillan and Smith, 1974). He will find a small overhanging rock or fallen debris and use his tubercles to clean the overhead surface, before attempting to attract females whilst driving away other males. If he is successful, the female deposits the eggs in a single layer on the overhead surface. The male then guards the eggs aggressively, using his tubercles to drive away intruders. The spongy dorsal pad is used to clean and agitate the eggs to prevent stagnation until they hatch in approximately 4-5 days (Geiger et al., 1988; Nelson and Paetz, 1992; Ankley and Villanueve, 2006).

Figure 1.2. Male *Pimephales promelas* with a prominent dorsal pad.

The hypothalamic-pituitary-gonadal axis (Fig. 1.3) is largely conserved across vertebrates (Ankley and Johnson, 2004; Norris, 2007; Silverthorn, 2013). In normal female *Pimephales promelas*, stimulation of follicular development originates in the hypothalamus. Here, gonadotropin-releasing hormone is secreted via direct innervation to the pituitary gland. This action signals the release of two gonadotropic hormones: luteinizing hormone and follicle-stimulating hormone. Luteinizing hormone stimulates the ovarian thecal cells to release androgens, which diffuse to the granulosa cells of the ovary. Here, follicle-stimulating hormone facilitates aromatase production, converting the androgens into estrogen (Hadley and Levine, 2006). The estrogen produced is responsible for oocyte development, secondary sex characteristics, negative feedback to the hypothalamus and pituitary, and hepatic secretion of vitellogenin, an egg-yolk lipoprotein expressed in oviparous species, into the bloodstream (Hoar and Randall, 1988; Hadley and Levine, 2006). Production of vitellogenin is triggered via the binding of estrogens to hepatic estrogen receptors. This binding triggers the dissociation of heat-shock proteins, causing the receptors to become activated and dimerize. Upon recruiting co-activator proteins, they bind to palindromic estrogen response element sequences on DNA, facilitating transcription of vitellogenin mRNA (Hadley and Levine, 2006; Hoar and Randall, 1988; Sumpter and Jobling, 1995). Exogenous estrogens (or estrogen mimics) can bind hepatic estrogen receptors in the same fashion as endogenous estrogens (Ankley and Johnson, 2004).

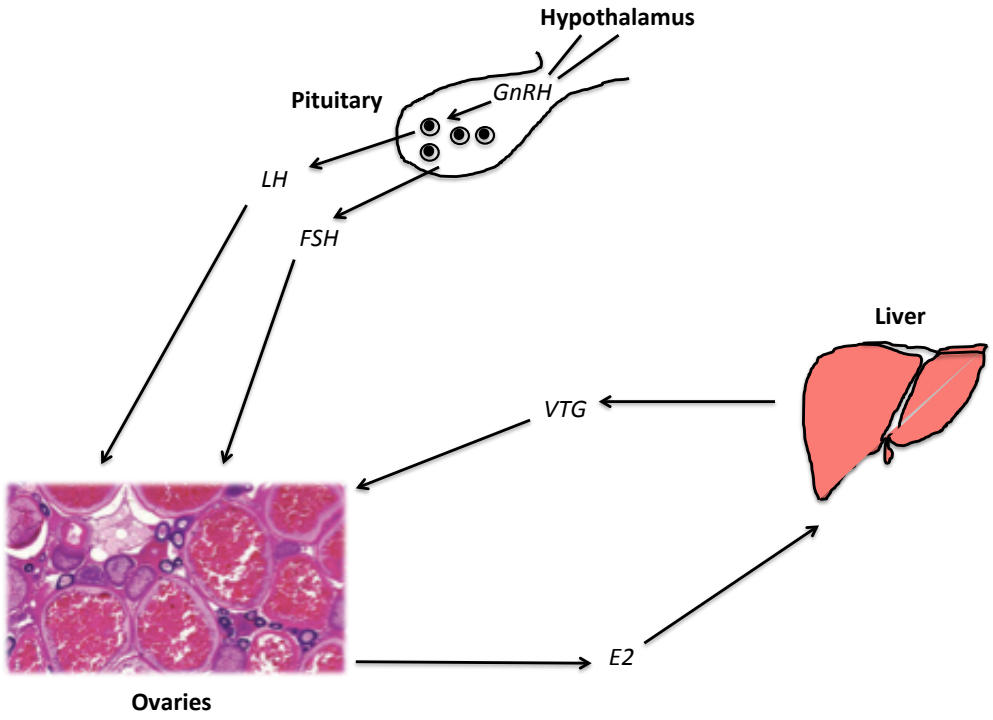

Figure 1.3. A depiction of the *Pimephales promelas* hypothalamic-pituitary-gonadal axis.

Endocrine Disruption

Endocrine disrupting chemicals can interfere with reproduction and development in both humans and wildlife (Colborn et al., 1993). Estrogens, and estrogen mimics, are a potent class of endocrine disrupting chemicals that interfere directly with the hypothalamic-pituitary-gonadal axis. Male hepatocytes contain the machinery necessary for vitellogenin synthesis. Thus, the levels of vitellogenin circulating in blood plasma can be used to assess estrogen exposure in male fish (Sumpter and Jobling, 1995; Panter et al., 1998; Thorpe et al., 2007; Matozzo et al., 2008; Shappell et al., 2010). Estrogen-induced vitellogenesis in male summer flounder (*Paralichthys dentatus*) injected with a high dose of estradiol (10 mg/kg of body

weight) resulted in toxic accumulation of the protein in the liver, kidneys and testes of treated fish. This accumulation was associated with obstruction of renal glomeruli and hepatocytic hypertrophy, and ultimately resulted in the death of the fish (Folmar et al., 2001). Since males lack a normal repository for vitellogenin - its intended target is the ovary in females - it is suggested that it becomes concentrated in kidney and liver, whereby organ failure can occur (Thorpe et al., 2007). The inability for male teleosts to effectively eliminate vitellogenin is further exemplified in Thorpe et al. (2007). The authors found that fish exposed to 29 and 60 ng estradiol/L exhibited elevated plasma vitellogenin concentrations over control for 70 days *after* cessation of exposure.

Vitellogenin induction is not the extent of the biological effect of estrogen exposure in male fish. While it remains challenging to link organismal effects with population decline (Mills and Chichester, 2005; Palace et al., 2009), there is evidence to suggest that elevated plasma vitellogenin levels are indicative of poor reproductive success. Kidd et al. (2007) observed a population collapse and concurrent elevations in plasma vitellogenin concentrations in *Pimephales promelas* following exposure to ethynylestradiol, a synthetic estrogen found in birth control pharmaceuticals, in a whole-lake study. Ethynylestradiol is approximately six times as potent as estrone (Schultz et al., 2013) and tends to persist for a greater duration in water (Ying et al., 2002). Morphological and behavioral perturbations have also been observed in association with estrogenic exposure. Histological analysis of river roach (*Rutilus rutilus*) revealed intersex incidence in up to 100% of fish sampled downstream from wastewater effluent in several rivers throughout the British Isles (Jobling et al., 1998).

Control sites averaged from 4% to 18.1% intersex incidence. Intersex, the presence of oocytes in male gonadal tissue, has been found to be indicative of poor reproductive success in the rainbow darter (*Etheostoma caeruleum*) (Fuzzen et al., 2015). Microscopic analysis of estrogen-exposed male tissues can also reveal reduced masculinity (percentage of mature spermatozoa) in gonad histology (Vajda et al., 2011), and hepatocyte hypertrophy due to vitellogenic activity (Wester et al., 2003; Wolf et al., 2005). Reduction in the prominence of male secondary sex characteristics (tubercles, dorsal pad, and banding) has been observed in association with estrogen receptor agonists in male *Pimephales promelas* (Miles-Richardson, 1999; Harries et al., 2000; Vajda et al., 2011), and estrone has been shown to reduce the escape response of exposed *Pimephales promelas*, involved in predator avoidance (McGee et al., 2009).

Occurrence of Estrogenic Chemicals in the Environment

Environmental estrogens often originate from agricultural runoff (Soto et al., 2004; Matthiessen et al., 2006; Chen et al., 2010) and developed urban areas (Barber et al., 2011; Lee et al., 2011) (Table 1.1). Estradiol, the hormone synthesized by follicle cells surrounding the developing oocyte (Hoar and Randall, 1988), is perhaps the most commonly studied natural estrogen with regards to endocrine disruption in fish (Panter et al., 1998; Miles-Richardson et al., 1999; Folmar et al., 2001; Jensen et al., 2001; Leino et al., 2005; Hyndman et al., 2010). Research suggests, however, that *estrone* may be of greater relevance. It has historically been suggested that estrone is less potent than estradiol, however relative potency estimates vary from 5% to 80%,

and seem to depend on the endpoint being measured (Hoar and Randall, 1988; Thorpe et al., 2003; Van den Belt et al., 2004; Schultz et al., 2013). For example, recent work has shown that potency is quite similar between the two when vitellogenin induction is considered as an endpoint (Dammann et al., 2011). A review of several assessments of occurrence (Table 1.1) suggests that estrone is found at environmental concentrations of about seven times that of estradiol (Kolpin et al., 2002; Soto et al., 2004; Mattheissen et al., 2006; Sarmah et al., 2006; Ferrey et al., 2008; Sellin et al., 2009; Chen et al., 2010; Writer et al., 2010; Barber et al., 2012; Alvarez et al., 2013). Estrone is a natural metabolite of estradiol, and is excreted by animals and humans (Ying et al., 2002). In addition, estradiol can undergo oxidative conversion to estrone in the environment (Fig. 1.4) (Writer, et al., 2012; Mashtare et al., 2013). Writer et al. (2012) found that despite attenuation of estradiol after being released into a stream from a point source, biotransformation to estrone can prolong the downstream impacts of the chemical. Recent work has also discovered that urine associated with livestock may increase the leaching ability of estrogens in soil and thereby increase their propensity for migration into freshwaters (Lucas and Jones, 2009). Reviews of environmental surveys by Panter et al. (1998) and Dammann et al. (2011) determined that environmental concentrations ranged from about 10 ng estrone/L to 100 ng estrone/L (Shore et al., 1993; Environment Agency, 1996; Kolpin et al., 2002; Wang et al., 2008; Thorpe et al., 2008; Writer et al., 2010). Environmental concentrations vary both spatially and temporally (Martinovic-Weigelt et al., 2013) and are affected by seasons and flow rates (Kolpin et al., 2004). For this reason it is important to

consider the possibility of ecological traps: in this case, point source releases that attract organisms (Kristan, 2003). Effluent sources, where pollutant concentration is highest, may attract fishes due to heat and oxygen gradients (Spigarelli et al., 1982).

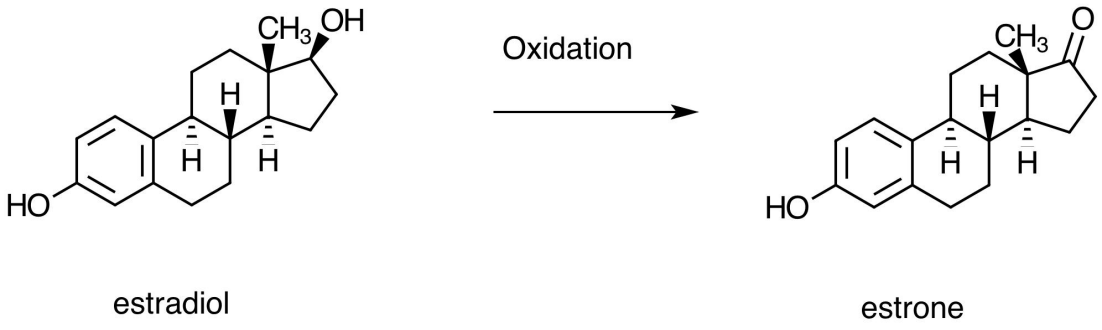

Figure 1.4. Oxidation of estradiol to estrone.

Table 1.1. Occurrence of estradiol and estrone as reported by seven recent studies. The values indicated in the table are the highest environmental concentrations observed within each study in which both estrone and estradiol were detected.

Study	Source	Estradiol (ng/L)	Estrone (ng/L)	Ratio (E1/E2)
Barber et al., 2012	Forested Lake	0.1	1.5	15.0
Writer et al., 2010	Forested Lake	0.4	1.1	2.8
MPCA, 2008	Residential Lake	0.4	1.1	2.8
Kolpin et al., 2002	Susceptible Streams	51.0	112.0	2.2
Sellin et al., 2009	Wastewater Effluent	14.5	22.9	1.6
Soto et al., 2004	Beef Cattle Effluent	3.2	8.3	2.6
Matthiessen et al., 2006	Dairy Cattle Effluent	0.5	9.3	18.6
Sarmah et al., 2006	Dairy Cattle Effluent	331.0	3057.0	9.2
Chen et al., 2010	Swine/Cattle/Chicken Effluent	46.5	398.0	8.6
Alvarez et al., 2013	Swine Effluent	59.2	298.0	5.0
	Average	**50.7**	**390.9**	**6.8**

The Toxicology of Climate Change

Global warming is occurring at an alarming rate, and estimations predict that this trajectory will persist (Murdoch et al., 2000; Adrian et al., 2009). Based on a

report by Oreskes (2004), all queried scientists agreed that climate change is happening, with 97% attributing the effects to anthropogenic influence (AAAS, 2014). Since the end of the last ice age (the Pleistocene Epoch), about 11,700 years ago (Andersen and Borns, 1994), and the beginning of our current epoch (the Holocene), the average global temperature has increased by about 5°C. In the past millennium, however, average global temperature has increased by approximately 0.8°C (AAAS, 2014). This is an 18-fold increase in warming rate. Climate change-induced physical, chemical and biological alterations threaten the integrity of aquatic ecosystems (Tonn, 1990; DeStasio et al., 1996; Justic et al., 1996; Murdoch et al., 2000; Jackson and Mandrack, 2002; Poff et al., 2002; Ficke et al., 2007; Williamson et al., 2008; Adrian et al., 2009; Whitehead et al., 2009; Jeppesen et al., 2010; Taner et al., 2011; Trolle et al., 2011). Changes in surface water temperature (Williamson et al., 2008; Adrian et al., 2009), salinity (Schlenk and Lavado, 2011; Cañedo-Argüelles, 2013), pH (Brander, 2007; Jackson et al., 2001), nutrient profile (Schindler, 2006; Jeppesen et al., 2010), thermal stratification in lakes (Murdoch et al., 2000; Taner et al., 2011; Trolle et al., 2011), and dissolved gases (Justic et al., 1996; Noyes et al., 2009) threaten to alter food web interactions and physiological function of individuals in aquatic ecosystems, especially those species occupying what is already the edge of their physical and chemical tolerances (Johnston and Dunn, 1987; Jackson and Mandrack, 2002; Winder and Schindler, 2004; Noyes et al., 2009). These alterations associated with climate change may serve as a modulator of the toxicological fate of anthropogenic pollutants (Schiedek et al., 2007; Noyes et al., 2009; Holmstrup et al.,

2010). Increases in severe rain events and changes in physiochemical water parameters may alter the concentrations and bioavailability of organic pollutants, such as endocrine-disrupting estrogens (Schwarzenbach et al., 2003; Matthiessen et al., 2006; Noyes et al., 2009).

The average global surface water temperature is expected to rise by 1.5°C to 5.8°C by the year 2100 (Houghton et al., 2001). Furthermore, this projected increase is expected to be more dramatic in the United States (Wigley, 1999; Poff et al., 2002). This change bears the risk of damaging sensitive ecosystems, especially those with species occupying the extremes of their thermal tolerance (Tonn, 1990; Walther et al., 2002; Rijnsdorp et al., 2009). If species are lost, complex food web interactions may be disrupted, resulting in a domino effect of perturbations that may ultimately result in a loss of organismal diversity and shifts in species niche occupation (Winder and Schindler, 2004; Mooij et al., 2005; Taner et al., 2011). Depending on the severity of the temperature change, metabolic changes in ectothermic organisms, such as macroinvertebrates (Schiedek et al., 2007) and fish (Roberts, 2012), and temperature-modulated changes in reproductive function (Smith, 1978) could conceivably pose a threat to population stability. Increased temperature has the effect of increasing metabolic rate and enzyme activity in fish (Evans and Claiborne, 2006). Such effects may increase overall pathological biosynthesis of estrogen-induced vitellogenin in male fish (Hoar and Randall). An increase in surface water temperature is also accompanied by a reduction in oxygen solubility, and therefore a reduction in dissolved oxygen necessary for fish respiration (Hughes, 1965; Roberts, 2012). This

reduction in dissolved oxygen necessitates physiological (Blewett et al., 2012) and anatomical changes (Evans, 1987) to increase gill respiration efficiency. An increase in respiratory rate and surface area of the lipophilic gill tissues, the proposed site of uptake for a host of xenobiotic chemicals (Evans, 1987; Blewett et al., 2012), is likely to increase the uptake of estrogens and thereby enhance their biological consequences. Alternatively, increases in water temperature may increase the degradation rate of estrogens (Raman et al., 2001; Chen et al., 2010), reducing aqueous concentrations and potentially reducing the bioavailability of estrone to the fish.

Food web alterations may have additional implications in contaminant toxicity as well. Increased nutrient intake has the potential to enhance reproductive activity in *Pimephales promelas* (Smith, 1978). For decades, the aquaculture industry has recognized the importance of adequate diet to facilitate reproduction in fish (Luquet and Watanabe, 1986; Izquierdo et al., 2001). Vitellogenesis involves the synthesis of egg-yolk lipoprotein and is therefore an energy-intensive process (Hoar and Randall, 1988; Babin et al., 2007). By increasing nutrient intake and subsequently providing greater availability of the necessary biological macromolecules for vitellogenesis, it is feasible that pathogenic vitellogenin plasma concentrations could be enhanced. Conversely, it is possible that climate change disturbances in food webs may lead to diet reduction (Jackson and Mandrak, 2002; Brander, 2007) and stress induction (Andersen et al., 1991). The stress hormone, cortisol, is known to enhance the rate of vitellogenesis in female Asian stinging catfish (*Heteropneustes fossilis*) (Sundararaj et al., 1982).

Hydrological alterations associated with climate change are predicted to alter the salinity of aquatic systems (Moore et al., 1997). An increase in the number and intensity of severe rain events (Poff et al., 2002; Noyes et al., 2009; Jeppesen et al., 2010; Hooper et al., 2013; AAAS, 2014), rising sea levels that result in saltwater intrusion into freshwater systems (Murdoch et al., 2000), and increasing periods of drought (Williamson et al., 2008; Whitehead et al., 2009; Taner, 2011) are predicted to occur with climate change, and will contribute to freshwater hypersalinization due to increased evaporation (Justic et al., 1996; Jeppesen et al., 2010; Hooper et al., 2013). Urban areas of the United States have long reported incidence of high chloride concentrations relating to road salt usage (Oliver et al., 1974; Benbow and Merritt, 2005; Jackson and Jobbagy, 2005; Kaushal et al., 2005; Siegel, 2007; Kelly et al., 2010; Cañedo-Argüelles, 2013; Corsi et al., 2015). Data trends show that concentrations are increasing over time, and regularly violate the United States Environmental Protection Agency chronic water quality criteria concentration of 230 ppm chloride (Oliver et al., 1974; Corsi et al., 2015). Chloride concentrations in excess of 800 ppm have been reported in New Hampshire during times of low water flow in the summer and fall, while natural background concentrations fluctuate between 1 and 10 ppm in unaffected waterways (New Hampshire Department of Environmental Sciences, 2013). Chloride concentrations in metropolitan areas of the northeastern United States are some of the highest during the winter months, when road salt application is at its peak. In this region, concentrations of up to 4,629 ppm chloride have been reported (Kaushal et al., 2005). Increases in chloride

concentrations can cause hypoxic conditions through reduction in oxygen solubility (Roberts, 2012) and incomplete vertical mixing of surface waters (meromixis), which can result in benthic oxygen depletion (Canada Council of Ministers of the Environment, 2011).

The toxic effect of hypersalinization on freshwater organisms has been extensively documented (Adelman et al., 1976; Evans, 1987; Peterson and Meador, 1994; Benbow and Merritt, 2005; Canada Council of Ministers of the Environment, 2011; Elphick et al., 2011; Cañedo-Argüelles et al., 2013). The chronic chloride toxicity threshold for *Pimephales promelas* (252 ppm) is relatively low when compared with other freshwater organisms (Siegel, 2007). Unnaturally high chloride concentrations can interfere with the sodium pump in fish gills, and ultimately cause mortality due to massive osmoregulatory failure (Adelman et al., 1976; Evans et al., 1999). In addition to direct mortality, increases in chloride concentrations have the potential to increase the potency of concurrent xenobiotic chemicals by increasing receptor sensitivity (Schlenk and Lavado, 2011). Also, by reducing oxygen solubility, and necessitating respiratory changes, they may increase uptake of estrogens at the gills (Blewett et al., 2012). Lastly, bioavailability may be increased by chloride reducing solubility of estrone (30 mg/L at 25°C) in water. This is known as the "salting out" effect (Schwarzenbach et al., 2003), whereby water molecules bind strongly to salts, making them unavailable to dissolve organic compounds, and possibly increasing the degradation half-life of the compound (Song and Brown, 1998; Noyes et al., 2009).

Historical data (Stahl et al., 2013) and models of freshwater systems (Moore et al., 1997; Jeppesen et al., 2010) frequently indicate increasing prevalence of hypoxic conditions as a result of climate change, due to increased surface water temperature (Murdoch et al., 2000), prolonged thermal stratification (Taner et al., 2011) and increased nutrient runoff (Adrian et al., 2009) in densely populated and agricultural areas. Incidentally, these are the areas most commonly associated with the discharge of contaminants (Soto et al., 2004; Matthiessen et al. 2006; Chen et al. 2010; Barber et al., 2011; Lee et al., 2011; Martinovic-Weigelt et al. 2013). Hypoxic conditions have been shown to suppress reproductive function in *Cyprinus carpio* (Wu et al., 2003), *Micropogonias undulatus* (Thomas et al., 2006), and *Fundulus grandis* (Landry et al., 2007), and increase estrogen receptor transcriptional activity synergistically in combination with estradiol (Yi et al., 2009). As with hypersalinization, hypoxia has been implicated in an increase of uptake (Blewett et al., 2012) and toxicity of certain chemicals in fish (Ficke et al., 2007). The affinity for organismal congregation near effluent discharge (Spigarelli et al., 1982) may place organisms at additional risk (see "ecological traps" later in this section). Behavioral changes in an oxygen gradient are, of course, not limited to spatial migration. Respiratory movements are increased in low oxygen conditions in order to maintain homeostatic balance in fishes (Gee et al., 1978; Wares and Igram, 1979). The lipophilic gill tissues of teleost fish are thought to be a primary site for uptake of steroidal compounds (Blewett et al., 2012). As has been suggested previously, the increased rate of opercular movement, resulting from hypoxic conditions (Gee et al., 1978; Wares and Igram, 1979), may result in an

increase in volume of chemical exposure, and a subsequent increase in uptake of steroidal compounds such as estrogens, thereby exacerbating their effects. In a study of the effect of hypoxia on responses to a mixture of estrogenic chemicals, however, no effect was found (Brian et al., 2009). The interaction between dissolved oxygen and individual estrogenic compounds has, to our knowledge, not been measured.

The Need for a Dynamic Approach to Toxicity Testing

Toxicity testing is often over-simplified. Culture guidelines set forth by regulatory agencies have generally recommended that water quality parameters be kept constant (Table 1.2; Denny, 1987; U.S. Environmental Protection Agency, 1998). There are benefits to consistent methodology as outlined in these protocols. They allow information from various studies to be more readily compared, and provide a framework for aquatic toxicologists to perform exposures that contribute to the vast ecotoxicological database of knowledge. There are, however, serious limitations to this type of testing. Fish, in a very Darwinian sense, are not genetically identical. Variations in individual biology between organisms within treatments will inevitably lead to variation in dose-response. Other factors, including seasonal fluctuations (Denton and Yousef, 1975; Smith, 1978) and down regulation of estrogen receptors via differing concentrations previously circulating androgens (Poulin et al., 1989), further emphasize the limitations of standardized exposures with the hope of comparing them to each other, or, perish the thought, using the results to create environmental standards that will be applied to a broad geographical or climatic range. At the heart of this type of toxicity testing is the desire to identify sources of negative

effects so that the information can be used to prevent damage to sensitive ecosystems. To generate environmentally relevant results from toxicity tests, the researcher must identify what *environmentally relevant* actually means, and the answer is complicated. To an organism that inhabits a broad ecological niche, such as *Pimephales promelas* environmentally relevant means a range of temperatures, pH, salinities, and so on. If toxicity testing is performed under a variety of such parameters, the results can then be applied to localized situations.

The following work is not an attempt to provide a complete summary of the toxicity of estrone by testing its biological effects under all possible conditions. While such an undertaking would certainly be noble, it is beyond the scope of this thesis. The purpose of the following research can be summarized with a single question: Does environmental variation impact estrone's capacity to disrupt endocrine function in male *Pimephales promelas*? If so, the results should be used as a starting point to justify a more complex series of toxicity tests. These tests would involve exposing organisms under a range of various environmental conditions, to generate results that could be used in creating more effective regulatory standards for a variety of microhabitats.

Table 1.2. The recommended water quality parameters for *Pimephales promelas* reproductive tests (U.S. Environmental Protection Agency, 1998).

Water Characteristic	Recommended Range
Temperature (°C)	24.0 - 26.0
Dissolved Oxygen (mg/L)	>4.9
pH	6.5 - 9.0
Alkalinity (mg/L as calcium carbonate)	>20
Total Organic Carbon (mg/L)	≤5
Un-ionized Ammonia (µg/L)	<35

Chapter II

MODULATION OF ESTRONE EXPOSURE EFFECTS MEDIATED THROUGH DIETARY AND TEMPERATURE REGIMENS IN MALE FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

INTRODUCTION

In the past 100 years, the rate of average global temperature rise has increased by approximately 20-fold (AAAS, 2014), and is likely to continue following this trend (Murdoch et al., 2000; Adrian et al., 2009). This warming is predicted to co-occur with environmental consequences, including increased severe rainfall events (Poff et al., 2002; Noyes et al., 2009; Jeppesen et al., 2010; Hooper et al., 2013; AAAS, 2014), oceanic acidification (Noyes et al., 2009), hypersalinization of freshwater systems (Justic et al., 1996; Jeppesen et al., 2010; Hooper et al., 2013), and surface water temperature increases (Williamson et al., 2008; Adrian et al., 2009), all of which have an effect on aquatic ecosystems (Johnston and Dunn, 1987; Noyes et al., 2009; Whitehead et al., 2009; Stahl et al., 2013). The average global surface water temperature is expected to rise by 1.5°C to 5.8°C by 2100 (Houghton et al., 2001) and the increase may be more dramatic in the United States (Wigley, 1999; Poff et al., 2002). This distortion of the environment can ultimately lead to the loss of

populations, especially in those species occupying the thermal extremes of their select niche (Tonn, 1990; Walther et al., 2002; Rijnsdorp et al., 2009). As a result, food web disruption can occur, resulting in a further loss of organismal diversity and shifts in species occupation (Winder and Schindler et al., 2004; Mooij et al., 2005; Taner et al., 2011).

Aquatic ecosystems also face the burden of anthropogenic pollution. In addition to traditional sources, such as eutrophication due to human activity (cultural eutrophication) (Correll, 1998; Schindler, 2006; Smith et al., 2006; Jeppesen et al., 2010) and metal pollution (Kock et al., 1996; Khan et al., 2008), another threat faces aquatic organisms. Industrialization, agriculture and urbanization have lead to an increase in environmental contaminants of emerging concern (Colborn et al., 1993; Barber et al., 2011). These include pesticides, personal care products, and hormones that eventually end up in waterways through wastewater effluent and non-point runoff (Kolpin et al., 2002; Soto et al., 2004; Matthiessen et al. 2006; Chen et al. 2010; Barber et al., 2011; Lee et al., 2011; Martinovic-Weigelt et al., 2013). A subset of these chemicals, known as endocrine disruptors, have sublethal effects on the organism that can cause physiological abnormalities in the endocrine system, such as reproductive dysfunction (Colborn et al., 1993; Jobling et al., 1998; Schoenfuss et al., 2002; Vajda et al., 2008, Schultz et al., 2013). Estrogens are a widespread class of endocrine disruptors that act upon the reproductive system of male fish (Schultz et al., 2013). Introduction of 17α-ethinyl estradiol, a synthetic estrogen found in female birth control and often found in the environment (Kolpin et al., 2002), resulted in

Pimephales promelas population collapse in a lake in western Ontario, Canada (Kidd et al., 2007). Environmental factors such as heavy and sporadic rainfall events associated with climate change may increase the rate of estrogenic introduction into aquatic systems (Noyes et al., 2009), while hypersalinization increases their bioavailability due to the "salting out" effect whereby the solubility of organic compounds is decreased by increased aqueous salinity (Schwarzenbach et al., 2003). In combination with the aforementioned effects, increased metabolism and temperature-modulated changes in reproductive function (Roberts, 2012; Smith, 1978) could perceivably pose a serious threat to fish populations.

The goal of this experiment was to test whether or not changes in temperature and nutritional regimen would influence the observed biological effects of estrone, a common estrogen found in the environment, on *Pimephales promelas*, a common North American baitfish, and an integral part of the freshwater food web. It was predicted that (1) higher temperatures would enhance the biological effects of estrone, (2) an unrestricted diet would enhance the biological effects of estrone, and that (3) the combination of (1) and (2) would create a "worst case scenario" in which the measured pathological effects of estrone are additively maximized. Spawning of *Pimephales promelas* begins when temperatures reach approximately 15.6°C and continues until the fall, until temperatures drop below 15.6°C to 18.4°C (Prather, 1957; Duda, 1989; Danylchuk and Tonn, 2001). Increased temperature has the effect of increasing metabolic rate and enzyme activity (Evans and Claiborne, 2006). It was predicted that, for this reason, overall biosynthesis of estrone-induced vitellogenin, an

egg-yolk precursor protein produced in male fish exposed to estrogenic chemicals (Hoar and Randall, 1988). While estrone is normally only produced in female oviparous fish to accommodate oogenesis, it is pathologically produced in male fish exposed to exogenous estrogenic chemicals (Sumpter and Jobling, 1995; Ankley et al., 2001; Matozzo et al., 2008; Bartell and Schoenfuss, 2012). Temperature increase is also accompanied by a reduction in oxygen solubility, and therefore a reduction in dissolved oxygen (Roberts, 2012). A reduction in dissolved oxygen necessitates physiological (Blewett et al., 2012) and anatomical changes (Evans, 1987) related to gill respiration in fish. An increase in respiratory rate and surface area of the lipophilic gill tissues (Blewett et al., 2012), the proposed site of uptake for a host of xenobiotic chemicals, is likely to increase uptake of estrone and thereby enhance its biological effects. Alternatively, increases in water temperature may increase the degradation rate of estrone (Raman et al., 2001), reducing aqueous concentrations and exposure. This, in turn, may reduce the overall effect on the organism. Increased nutrient intake is projected to facilitate the energy intensive biosynthesis associated with vitellogenesis (Hoar and Randall, 1988; Babin et al., 2007). Furthermore, if excess food is present, it may serve as a "sink" for which estrogens can bind and be removed from the aqueous medium occupied by fish. This may reduce exposure to the chemical. If a subsequent reduction in food availability occurs, necessitating consumption of the previously extraneous nutriment, exposure may be increased. Restricted nutrition may also increase plasma concentrations of the stress hormone, cortisol (Andersen et al., 1991), which is known to enhance the rate of vitellogenesis

in fish (Sundararaj et al., 1982). It is worth noting that increased temperature may also increase plasma cortisol concentrations in fish (Ryan, 1995).

Ideally, the results from this experiment will aid in the interpretation of biological effects observed in the laboratory and in the environment. This may, in turn, facilitate a deeper understanding of these effects in the context of non-static physical water parameters associated with a dynamic climate.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the St. Cloud State University Institutional Animal Care and Use Committee.

Experimental Design

Replicate 21-day exposures were conducted in the Aquatic Toxicology Laboratory at St. Cloud State University (St. Cloud, MN). The first exposure was conducted from October 5th, 2012 to October 26th, 2012 and the second exposure was conducted from November 27th, 2012 to December 18th, 2012. Previously published flow-through exposure protocols (Schoenfuss et al., 2008) were modified to accommodate a 2x3x2 (temperature, estrone, and feeding regime) experimental design. Fish were kept on a 16 h light/8 h dark cycle. Well water was diverted into one of two head tanks, one of which was maintained at 18°C, the other at 26°C. The temperature-controlled head tanks were fed into mixing tanks, where they were combined with a concentrated stock to create either an ethanol control, a low estrone treatment (15 ng/L nominal) or

a high estrone treatment (100 ng/L). Estrone treatments were established in a treatment-specific common mixing tank, prior to being split into the corresponding aquaria, ensuring equal concentrations in each aquarium. Furthermore, each of the resulting groups was fed either a restricted (0.75% body weight/day) or an *ad libitum* (3% body weight/day) diet. The resulting treatments totaled twelve, with mature male fathead minnows (6 months in age) divided into two tanks of ten individuals per treatment. On the 22nd day, all fish were sacrificed in accordance with St Cloud State University Institutional Animal Care and Use Committee (IACUC) approved protocols.

Exposure Chemicals

Estrone (Sigma-Aldrich, St. Louis, MO) exposure solutions were prepared daily from aliquots of an estrone solution in 100% ethanol. Control treatments received 2 mL of 100% ethanol to rule out any effect from the ethanol carrier. Aliquots were stored at 4°C until use.

Each day, beginning on the first day of exposure, one treatment-specific aliquot was mixed with 10 L of ground water from a dedicated well in an amber glass bottle. Stir bars and plates were assigned to each amber glass bottle containing treatment solution. This method insured continuous agitation to avoid settling of the chemicals. Stainless steel tubes were used to draw the solution into a stainless steel mixing chamber via a Cole-Palmer Masterflex 7523-40 peristaltic pump at a nominal rate of 7 mL/min. Ground water from the same dedicated well was gravity-fed into the mixing chamber at a rate of 200 mL/min to achieve the final treatment concentration. Mixture

was achieved by allowing the combined solution to fall over two barrier-walls within the mixing tank. The final solution was delivered directly to four aquaria via tubing extending from the bottom of the mixing tank. Water exchange rate was approximately 7 exchanges/aquarium/day.

Exposure Organisms

Adult male fathead minnows were obtained exclusively from a laboratory supplier (US EPA Cincinnati). Mortality was checked on a daily basis. Fish were kept at one of two constant water temperatures (18°C and 26°C). Frozen brine shrimp (*Artemia* spp.) and frozen blood worms (*Glycera* spp.) were obtained separately (Brine Shrimp Direct, Ogden, UT) and mixed. Food portions of 0.75% fish body weight were calculated assuming the average weight of a mature male fathead minnow to be approximately 3.3 g. Restricted fish were fed one 0.75% body weight aliquot per day and *ad libitum* fish were fed four aliquots (3% body weight). All fish maintenance was carried out in accordance with St. Cloud State University's IACUC policies.

Water Quality Analysis

Water temperature was recorded every 10 minutes using a HOBO Data Logger (Onset Computer Corporation, Bourne, MA). Separately, additional daily water temperatures were taken as a failsafe, along with pH (General PH-501, General Tools & Instruments, New York, NY) and conductivity measurement (General CO-502, General Tools & Instruments, New York, NY). Total water hardness, free chlorine, total chlorine and alkalinity were assessed periodically (~every 3 days) using

AquaChek 5-in-1 Water Quality Test Strips (Hach Company, Loveland, CO).

Water Chemistry Analysis

At exposure days 5, 10, 15, 20, and on the day of dissection, two 120 mL water samples were taken from each treatment. Water samples were stored at -20°C for 24 hours, then transferred to -80°C for storage until analysis as described in Shappell et al. 2010.

Plasma Vitellogenin Analysis

Blood was obtained from the severed caudal vasculature using a heparinized capillary tube after fish were anesthetized in 0.1% MS-222 (Argent Laboratories, Redmond, WA). Plasma was obtained following blood centrifugation at 8050 x *g* for 8 min at 4°C and stored at -80°C prior to analysis. Plasma vitellogenin concentrations (µg/L) were quantified via competitive antibody-capture ELISA using and 8 point serial dilution standard (4.8 µg/mL to 0.0375 µg/mL). Detailed methods of the assay are described in Shappell et al. (2010).

Plasma Cortisol Analysis

In addition to being evaluated for vitellogenin concentrations, plasma samples were also tested for cortisol concentration (µg/L) using a cortisol enzyme immunoassay (EIA) (Cortisol Express EIA Kit # 500370, Cayman Chemical Company, Ann Arbor, MI). An eight-point standard for the assay was produced via serial dilution of a 50 ng/mL bulk standard. EIA buffer was used to dilute the standard by 50%, seven times, generating a range of concentrations from 5 ng/mL to 39.1

pg/mL. The plate wells were pre-coated with goat anti-mouse immunoglobulin G antibody. Samples of 5 µL of fish plasma were diluted to 1:40 and 1:80, then allowed to incubate for 2 hours at room temperature in the coated wells with a competing cortisol-acetylcholinesterase (AChE) conjugate tracer and mouse monoclonal antibody. Following this incubation, the plates were washed using a Stat Fax 2600 Microplate Washer (Awareness Technology, Palm City, FL). Ellman's Reagent, containing the substrate for AChE, was then added at a rate of 200 µL/well and incubated in the dark for 60 minutes (<https://www.caymanchem.com/pdfs/500370.pdf>). Absorbance values were measured on a Multiskan EX spectrophotometer (Thermo Scientific, Waltham, MA) and interpreted using the included Ascent Software.

Blood Glucose Analysis

Using 1 µL of blood, a TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT) was used to quantify blood glucose concentration (mg/dL).

Hematocrit Analysis

Blood was taken via the caudal artery using 75mm heparinized capillary tubes (ClearCRIT Plastic Capillary Tubes, Separation Technology Inc., Sanford, FL). Capillary tubes were sealed (Critoseal, Oxford Labware, St. Louis, MO) and placed in a microhematocrit centrifuge (HERMLE Z200A, Labnet International Inc., Woodbridge, NJ) for 3 minutes at 968 x *g*. Hematocrit was measured using a Spiracrit Micro-Hematocrit Tube Reader (Clay-Adams Inc., New York, NY).

Organosomatic Indices

Prior to dissection, individual fish were weighed (0.01 g precision, Taylor Stainless Steel Food Scale, Taylor Precision Products, Oak Brook, IL) and measured for length to calculate body condition factor (BCF = body weight/total length^3) (Fulton, 1904). To calculate hepatosomatic index (HSI = liver weight/whole body weight x 100) and gonadosomatic index (GSI = gonad weight/whole body weight x 100), liver and gonads were excised and immediately weighed (0.001 g precision, Mettler Toledo AG245, Columbus, OH).

Secondary Sex Characteristics

Prior to dissection, an assessment of the male sexual characteristics of individual fish was performed. Tubercles, dorsal pad and banded coloration were given a separate rating of 1, 2 or 3 based on prominence (1 being the least prominent, 3 being the most prominent) (Smith, 1978). The three values were added together for as a method for comparing morphological sexual maturity between treatments.

Histopathology

Liver and gonads were excised from fish and placed in histological cassettes (Tissue-Loc Histoscreen Cassettes, Thermo Scientific, Kalamazoo, MI) in 10% buffered formalin for at least 24 hours. Dehydration and tissue preparation was achieved following previously prescribed procedures (Carson, 1997). Tissues were embedded in paraffin. Small samples (~1 mm diameter) of liver and gonad were sectioned at approximately 5 μm (Olympus Cut 4055 Microtome, Olympus America

Inc., Center Valley, PA). Tissues were stained using a standard haematoxylin and eosin counter stain (Gabe, 1976; Carson, 1997).

Liver tissues were graded on a scale of 1 to 4 based on cellular vacuolization. A grade of 1 indicates that no vacuoles are visible under a 40x magnification, a grade of 2 indicates that less than 25% of the visible tissue is occupied by vacuoles, a grade of 3 indicates that 25-50% of the visible tissue is occupied by vacuoles, and a grade of 4 indicates that the majority of the visible tissue consists of vacuoles.

Statistical Analysis

A multiple analysis of variance was used to test for interactions between variables. Tukey's post-tests were utilized to determine differences between means.

RESULTS

Chemical Data and Survival Rates

Estrone concentrations were higher in experiment 2 (low treatment: 14±2.1 ng/L, high treatment: 135±20.7 ng/L) than in experiment 1 (low treatment: 9±1.6 ng/L, high treatment: 78±22.9 ng/L) (Table 2.1). In both experiments, survival was higher in the 18°C treatments (100% overall survival in experiment 1 and 99% overall survival in experiment 2) than in the 26°C treatments (87% overall survival in experiment 1 and 83% overall survival in experiment 2) (Table 2.2).

Table 2.1. Average aqueous estrone concentrations in flow-through exposures; chemical analysis methods are consistent with those described in Shappell et al. 2010; samples were taken on days 5, 10, 15 and 20 of the exposure.

	Estrone Treatment (Nominal)	Average Measured Estrone Concentration
Experiment 1	Control	0 ng/L
	15 ng/L	9±1.6 ng/L
	100 ng/L	78±22.9 ng/L
Experiment 2	Control	0 ng/L
	15 ng/L	14±2.1 ng/L
	100 ng/L	135±20.7 ng/L

Table 2.2: Percent survival and number of survivors (survived full exposure/total fish at beginning of experiment) within individual treatments in experiment 1 and experiment 2.

		18°C, Low Feed	18°C, High Feed	26°C, Low Feed	26°C, High Feed
Experiment 1	**EtOH**	100% (20/20)	100% (21/21)	76% (16/21)	95% (19/20)
	9 ng estrone/L	100% (18/18)*	100% (25/25)	74% (17/23)	100% (19/19)
	78 ng estrone/L	100% (20/20)	100% (22/22)	90% (18/20)	90% (19/21)
* accidental female in tank found dead on day 5 of exposure - not counted towards mortality					
		18°C, Low Feed	**18°C, High Feed**	**26°C, Low Feed**	**26°C, High Feed**
Experiment 2	**EtOH**	100% (20/20)	100% (23/23)	95% (21/22)	60% (12/20)
	14 ng estrone/L	100% (19/19)	95% (19/20)	89% (17/19)	88% (15/17)
	135 ng estrone/L	100% (20/20)	100% (20/20)	80% (12/15)	84% (16/19)

Physiological Endpoints

Estrone had the most significant effect (p<0.0001) on mean plasma vitellogenin concentration in both experiments (Table 2.3, 2.4). In both experiments, low estrone-treated fish (9 ng estrone/L in experiment 1, 14 ng estrone/L in

experiment 2) did not exhibit plasma vitellogenin concentrations that were significantly (p<0.05) elevated over the control (Fig. 2.1). In contrast to experiment 2, temperature had a significant effect on mean plasma vitellogenin concentrations in experiment 1. Additionally, there was significant interaction between variables in experiment 1 (Table 2.3). Fish exposed to a high estrone (78 ng estrone/L) concentration at 18°C, and fed a restricted diet, had a mean plasma vitellogenin concentration that was significantly higher than all other treatments, while fish exposed to the same estrone concentration at 26°C, and fed a restricted diet, had a mean plasma vitellogenin concentration that was not significantly elevated over control (Fig. 2.1).

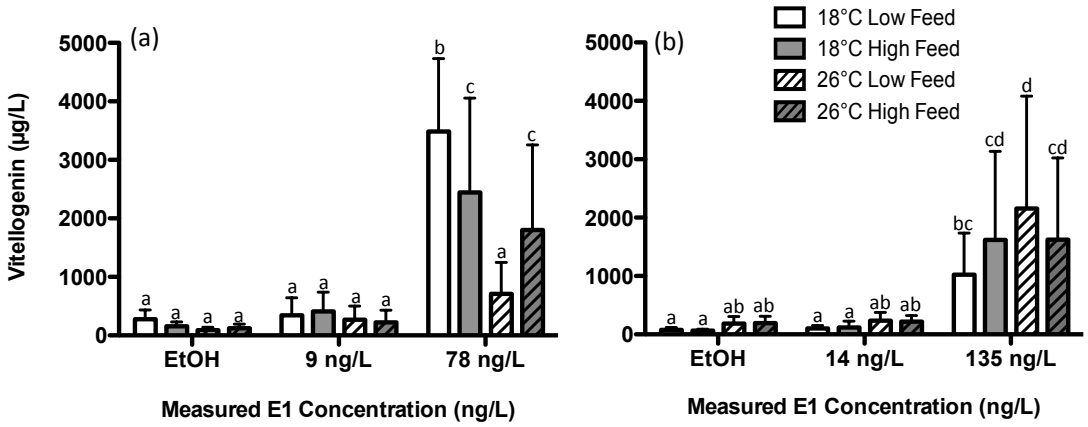

Figure 2.1. Plasma vitellogenin concentrations of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; letters above standard deviation bars indicate significantly different means at p<0.05; Multiple Analysis of Variance; Tukey post-test to compare means.

Hematocrit was not affected by estrone concentration in either experiment, however temperature significantly impacted mean hematocrit in experiment 1 (Fig.

2.2). In experiment 1, hematocrit is elevated in the 18°C estrone-treated (both 9 ng/L and 78 ng/L) fish when compared with 26°C estrone-treated fish (Fig. 2.2a). In both experiments, fish exposed to high estrone at 18°C, fed *ad libitum*, had the highest mean hematocrit. Mean blood glucose concentrations were not significantly different in experiment 1 (Fig. 2.3a), however temperature significantly affected the mean in experiment 2 (Table 2.4). In experiment 2, mean blood glucose was always higher in fish being fed an *ad libitum* diet, when compared with their restricted diet counterpart treatment. This was seen to a higher degree in the 18°C treatments (Fig. 2.3b).

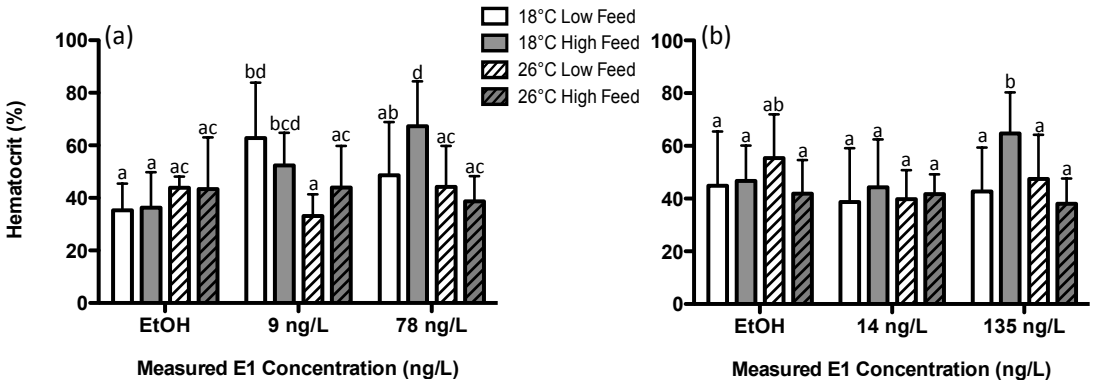

Figure 2.2. Hematocrit of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; letters above standard deviation bars indicate significantly different means at $p<0.05$; Multiple Analysis of Variance; Tukey post-test to compare means.

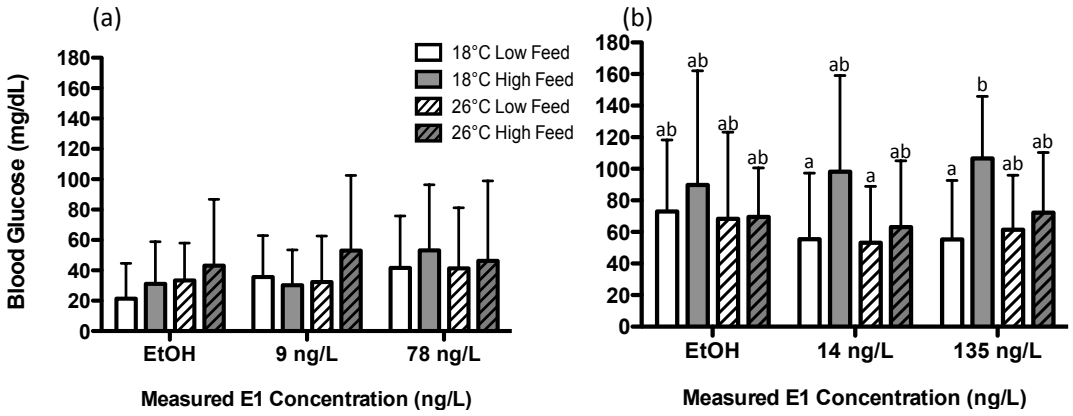

Figure 2.3. Blood glucose concentrations of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; no significant differences between means in experiment 1 (b); letters above standard deviation bars indicate significantly different means at $p<0.05$ (b); Multiple Analysis of Variance; Tukey post-test to compare means.

Morphological Endpoints

Mean body condition factor was not significantly variable between any treatments in experiment 1 (Table 2.3), however effects from temperature were present in experiment 2 (Table 2.4). Fish exposed at 18°C had higher mean body condition factor than fish exposed at 26°C in experiment 2, regardless of feeding regime (Fig. 2.4b).

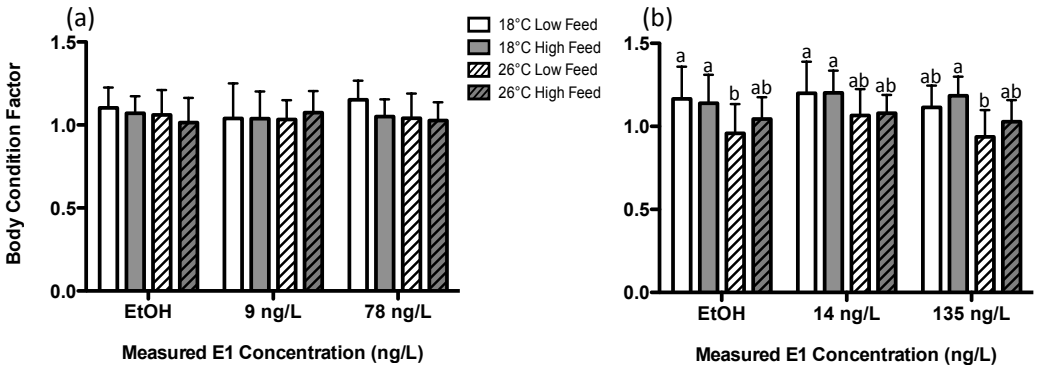

Figure 2.4. Body condition factor of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; no significant differences between means in experiment 1 (b); letters above standard deviation bars indicate significantly different means at $p<0.05$ (b); Multiple Analysis of Variance; Tukey post-test to compare means.

Gonadosomatic index was significantly altered by temperature in both experiments (Table 2.3, 2.4). Males exposed at 18°C had greater mean gonadosomatic index in both experiments, but more dramatically so in those exposed to the highest estrone concentration (135 ng estrone/L) in experiment 2 (Fig. 2.5). Hepatosomatic index followed a similar, although less significant trend in the first experiment (Table 2.3, Fig. 2.6a), although temperature had no impact in the second experiment (Table 2.4, Fig. 2.6b). Hepatosomatic index appeared instead to be diet-dependent in experiment 2 (Table 2.4). Fish fed an *ad libitum* diet (3% body weight/day) consistently had higher mean hepatosomatic index in comparison with fish fed a restricted diet (0.75% body weight/day) in all 26°C treatments (Fig. 2.6b).

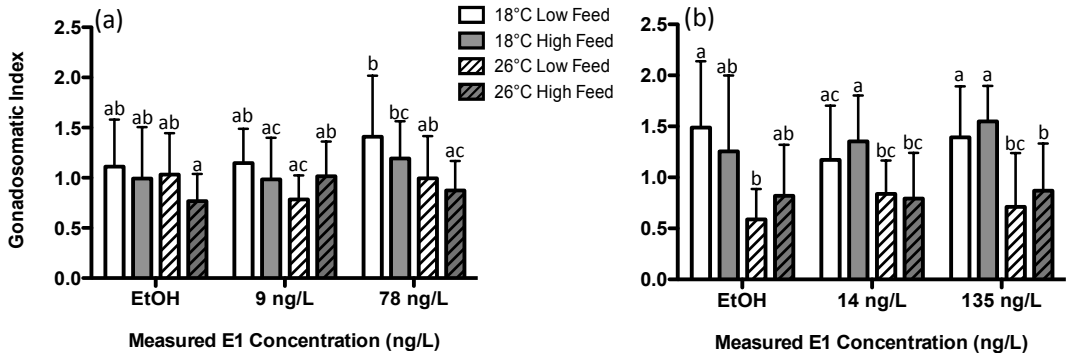

Figure 2.5. Gonadosomatic index of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; letters above standard deviation bars indicate significantly different means at $p<0.05$; Multiple Analysis of Variance; Tukey post-test to compare means.

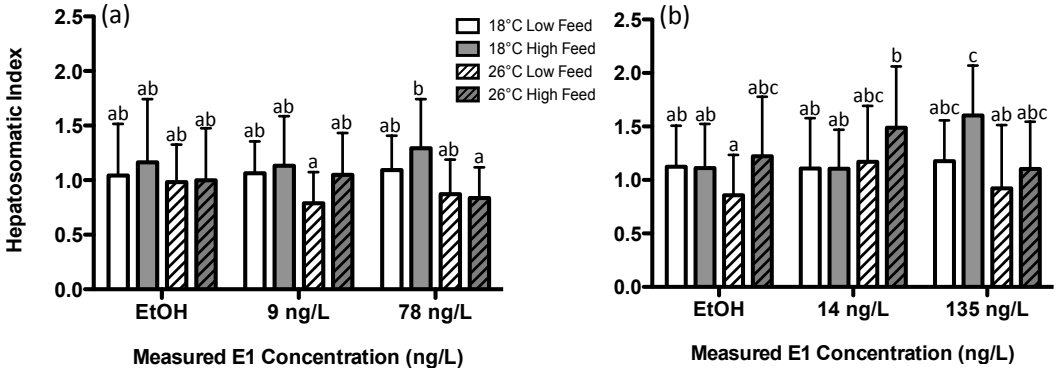

Figure 2.6. Hepatosomatic index of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; letters above standard deviation bars indicate significantly different means at $p<0.05$; Multiple Analysis of Variance; Tukey post-test to compare means.

Secondary sex characteristics did not vary significantly between treatments in experiment 1 (Table 2.3, Fig. 2.7a). In experiment 2, secondary sex characteristics were greater in magnitude in fish exposed at 18°C (Fig. 2.7b, 2.8b).

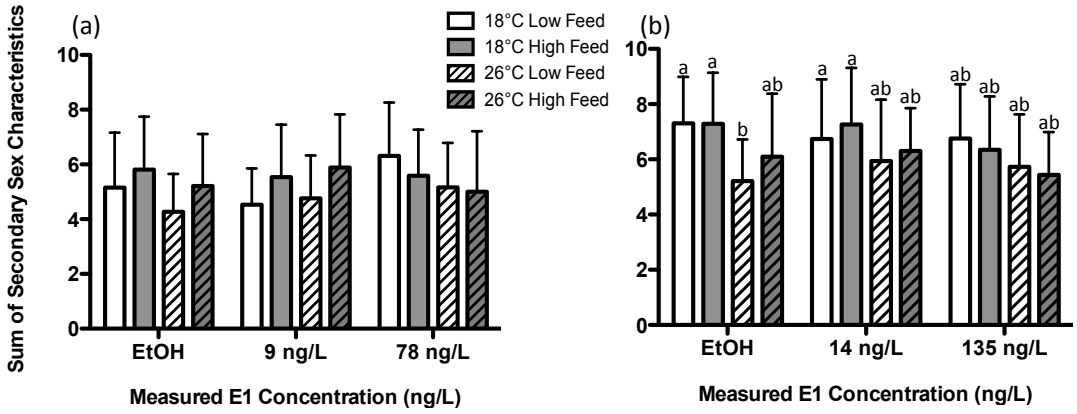

Figure 2.7. Sum of secondary sex characteristics of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; no significant differences between means in experiment 1 (a); letters above standard deviation bars indicate significantly different means at $p<0.05$ (b); Multiple Analysis of Variance; Tukey post-test to compare means.

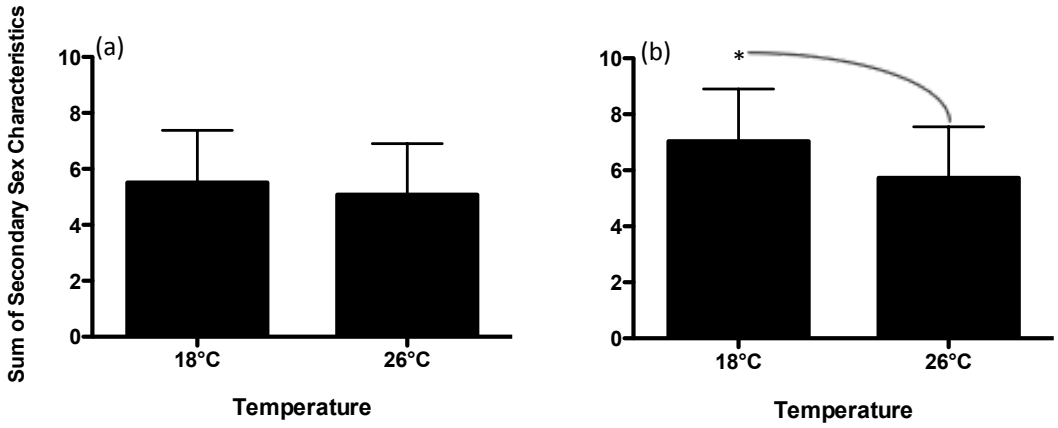

Figure 2.8. Sum of secondary sex characteristics of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); no significant differences between means in experiment 1 (a); * indicates significance at $p<0.05$ (b); bars indicate standard deviation; t-test with temperature treatments compared independent of estrone and feeding regime.

No significant trends in liver vacuolization were seen in experiment 1 (Fig. 2.9a, 2.11a). In fact, none of the individual means differed from one another in either

experiment (Fig. 2.10), however, when estrone treatments were examined independent of temperature and feeding regime, the 14 ng estrone/L treatment (experiment 2) is significantly higher than both the control (EtOH) and high estrone treatment (135 ng estrone/L) (Fig. 2.10a). Percent of mature spermatozoa was temperature-dependent in both experiments (Table 2.3, 2.4), however this is the extent of the similarity. When we examined the percentage of spermatozoa in 18°C and 26°C treatments independent of estrone concentration and feeding regimen, we found opposite effects between the two experiments. In the first experiment, the 26°C treatment exhibited a higher percentage of mature spermatozoa, whereas in the second experiment, the 18°C treatment exhibited a higher percentage of spermatozoa.

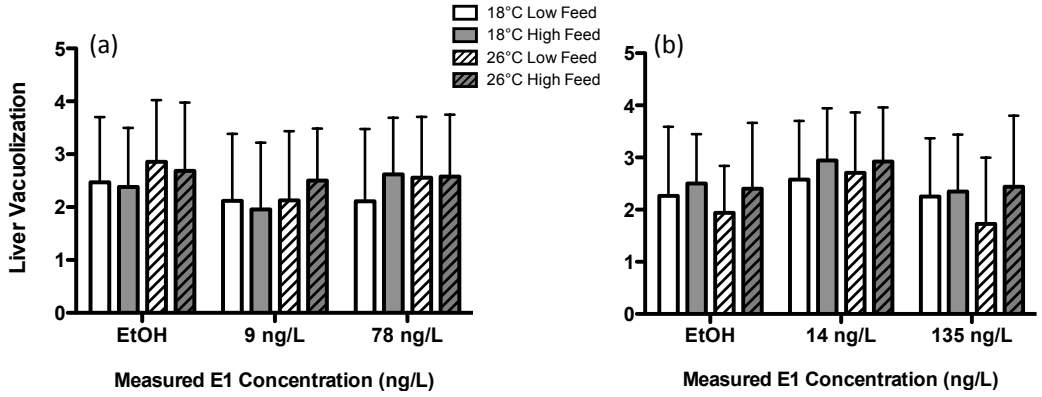

Figure 2.9. Liver vacuolization of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); no significant differences between means; bars indicate standard deviation; Multiple Analysis of Variance; Tukey post-test to compare means.

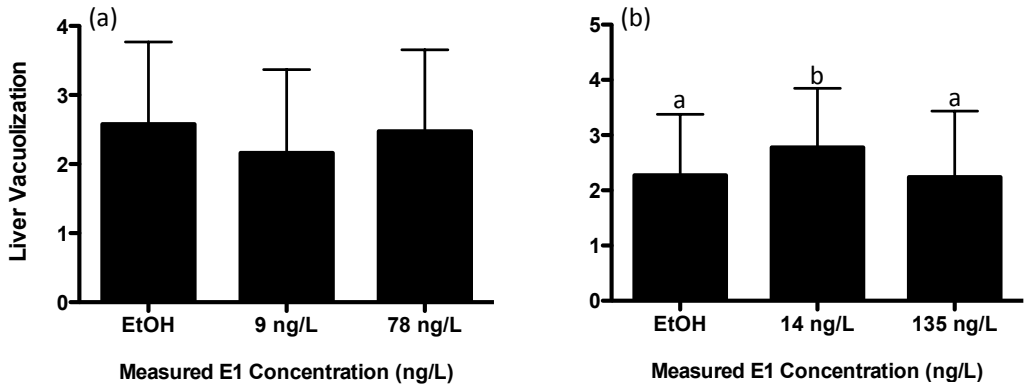

Figure 2.10: Liver vacuolization of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); no significant differences between means in experiment 1 (a); letters above standard deviation bars indicate significantly different means at $p<0.05$ (b); One-Way ANOVA with estrone treatments compared independent of temperature and feeding regime, followed by Tukey post-test.

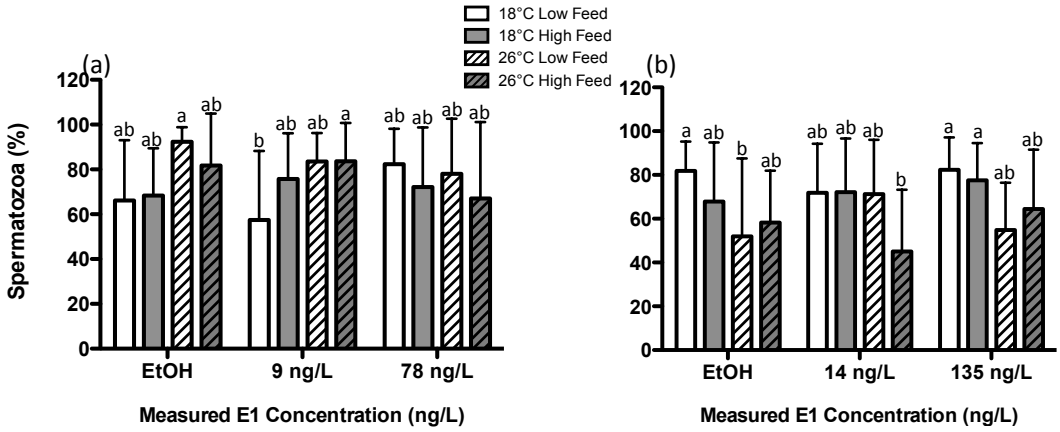

Figure 2.11. Spermatozoa percentage of male *P. promelas* after 21 days of exposure at 18°C and 26°C, and fed either a restricted (0.75% body weight) or an *ad libitum* (3% body weight) diet; letters above standard deviation bars indicate significantly different means at $p<0.05$; Multiple Analysis of Variance; Tukey post-test to compare means.

Table 2.3. A depiction of p-values associated with dependent and all combinations of independent variables in experiment 1. Significant values (p<0.05) are in bold.

Experiment 1 – p values							
	E1	Temp	Feed	E1*Temp	E1*Feed	Temp * Feed	E1*Temp *Feed
Length, mm	0.11	0.38	0.29	0.98	0.85	0.73	0.85
Weight, g	0.21	0.83	0.53	0.71	0.63	0.41	0.73
Testis, g	**0.0032**	**0.0037**	0.32	0.19	0.10	0.12	0.20
Liver, g	0.76	0.08	0.27	0.69	0.74	0.80	0.91
Condition Factor	0.87	0.20	0.45	0.48	0.44	0.53	0.76
Gonadosomatic	0.057	**<.0001**	**0.05**	0.19	0.18	0.30	0.13
Hepatosomatic	0.93	**0.02**	0.24	0.58	0.86	0.78	0.60
Second. Sex Sum	0.58	0.20	0.13	0.34	0.15	0.67	0.95
Cortisol	0.42	0.77	0.80	0.80	0.99	0.54	0.30
Vitellogenin	**<.0001**	**0.0005**	0.98	**0.0005**	0.98	**0.023**	**0.010**
Hematocrit	0.08	**0.0087**	0.30	**0.004**	0.64	0.88	**0.026**
Blood Sugar	0.16	0.31	0.16	0.57	0.99	0.55	0.50
Liver Vacuolization	0.45	0.34	0.73	0.98	0.84	0.97	0.74
% Spermatogonia	0.39	0.78	**0.02**	**0.013**	0.11	**0.02**	0.31
% Spermatocytes	0.30	**<.0001**	0.45	**0.049**	0.20	0.99	0.41
% Spermatids	0.87	**0.009**	0.97	0.31	0.50	0.46	0.67
% Spermatozoa	0.59	**0.004**	0.23	**0.006**	0.06	0.09	0.28

Table 2.4. A depiction of p-values associated with dependent and all combinations of independent variables in experiment 2. Significant values (p<0.05) are in bold.

Experiment 2 – p values							
	E1	Temp	Feed	E1*Temp	E1*Feed	Temp * Feed	E1*Temp *Feed
Length, mm	0.73	**0.0016**	0.12	0.93	0.23	0.99	0.30
Weight, g	0.28	**<.0001**	0.053	0.87	0.25	0.96	0.22
Testis, g	0.84	**<.0001**	0.51	0.66	0.93	0.69	0.45
Liver, g	0.30	**<.0001**	**0.0012**	**0.018**	0.44	0.68	**0.047**
Condition Factor	0.07	**<.0001**	0.23	0.78	0.47	0.49	0.87
Gonadosomatic	0.51	**0.0002**	0.87	0.53	0.66	0.92	0.71
Hepatosomatic	0.16	0.19	**0.024**	**0.013**	0.55	0.51	0.26
Second. Sex Sum	0.55	**0.0026**	0.90	0.37	0.71	0.85	0.98
Cortisol	0.52	0.36	0.90	0.97	0.94	0.86	0.31
Vitellogenin	**<.0001**	0.18	0.91	0.49	0.98	0.33	0.36
Hematocrit	0.25	0.26	0.63	0.25	0.20	**0.013**	0.27
Blood Sugar	0.69	0.09	**0.015**	0.94	0.55	0.09	0.80
Liver Vacuolization	**0.041**	0.35	0.23	0.67	0.88	0.77	0.69
% Spermatogonia	0.67	**0.0018**	0.53	0.32	0.86	0.71	0.57
% Spermatocytes	**0.052**	**0.0016**	**0.0027**	0.66	0.13	0.84	**0.0033**
% Spermatids	**0.018**	0.29	0.06	0.63	0.98	0.34	**0.021**
% Spermatozoa	0.17	**0.0003**	0.06	0.49	0.53	0.99	0.06

DISCUSSION

The goal of this experiment was to determine whether temperature and diet play a role in modulating the observable biological responses to exogenous estrone in adult male fathead minnows (*Pimephales promelas*). It was predicted that, due to changes in gill ventilation and physiology (Evans, 1987; Blewett et al., 2012; Roberts, 2012), metabolic rate (Evans and Claiborne, 2006), and increased estrogen receptor sensitivity (Blair et al., 2000), fish exposed to estrone at 26°C would exhibit an increase in severity of estrogenic effects in comparison to fish exposed at 18°C. Additionally, it was predicted that fish fed an *ad libitum* (3% body weight/day) diet would exhibit biological markers of estrogenic exposure more dramatically than fish fed a restricted diet (0.75% body weight/day). Diet adequacy would increase the chance of metabolic availability of biological macromolecules necessary to respond to estrogenic exposure, particularly with regards to egg-yolk producing vitellogenesis, a well established biomarker of estrogenic exposure in male fish (Sumpter and Jobling, 1995; Ankley et al., 2001; Matozzo et al., 2008; Bartell and Schoenfuss, 2012) with a high energy requirement (Hoar and Randall, 1988; Babin et al., 2007). The data do not corroborate these predictions. Nonetheless, the metabolic and reproductive implications of temperature and diet are demonstrated to a high degree in this study, and prove to be as important as actual xenobiotic chemical concentrations when considering biological endpoints in exposure studies.

Temperature had the most significant biological effect on *Pimephales promelas* in both experiments in terms of the number of dependent variables

significantly impacted (Table 2.3, 2.4). Mortality is perhaps the most pressing endpoint affected by temperature. By facilitating more prolific microbial growth that may be harmful to fish (Roberts, 2012) and increasing metabolic demand (Johnston and Dunn, 1987; Gillooly et al., 2001), it seems a temperature increase created a systemic burden on the fish. In experiment 1, 16 fish died in the 26°C treatments throughout the duration of the exposure (21 days), while all fish survived the exposure in the 18°C treatments. In experiment 2, 19 fish died in the 26°C treatments and only one died in the 18°C treatments. This has profound implications in the context of climate change-induced increases in surface water temperature (Pörtner and Knust, 2007; Noyes et al., 2009).

Estrone had no significant effect in elevating plasma vitellogenin concentrations in fish exposed to aqueous concentrations of 9 ng estrone/L and 14 ng estrone/L (Fig. 2.1) at any temperature or feeding regimen. Concentrations of 78 ng estrone/L and 135 ng estrone/L did, however elicit a response that resulted in significant elevation in plasma vitellogenin concentrations over the control (Fig. 2.1). Interestingly, plasma vitellogenin concentrations were significantly lower in the high estrone treatment in the second experiment (135 ng estrone/L) than in the first experiment (78 ng estrone/L). This may be due to tapering dose response curves to estrogenic chemicals have been reported previously (Wester et al., 2003; Brian et al., 2008). Another factor could be hepatic and renal toxicity similar to that which has been observed in male summer flounder (*Paralichthys dentatus*) injected with estradiol (Folmar et al., 2001). The high estrone treatment in experiment 2 had the

greatest mortality rate of any of the estrone treatments in either exposure (15%). Individuals with a reduced susceptibility to estrone's vitellogenin-inducing effects may have been inadvertently selected for in the sample, resulting in lower mean vitellogenin. Organismal variability also exists between fish, but perhaps more importantly, seasonal variability plays a role in reproductive function in fathead minnows (McMillan and Smith, 1974; Denton 1975; Smith 1978). The difference of two months (September to November) may have played a role in the rate of vitellogenesis, as the timeframe is moving farther away from peak spawning season for fathead minnows (Fig. 2.13), and the mean vitellogenin concentration was indeed lower in the second experiment (November).

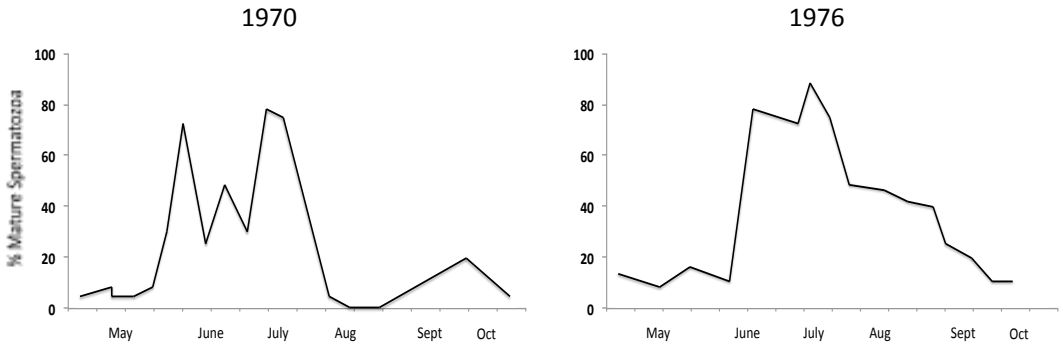

Figure 2.13: Values on the x-axis represent the percentage of mature spermatocytes, which peaks in late spring and early summer, then tapers off through the fall. Adapted from *Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, Pimephales promelas* by R.J. Smith (1978).

Vitellogenin concentrations in the high estrone (135 ng estrone/L) treatments in experiment 2 (Fig. 2.1b) did not exhibit statistical differences due to temperature or diet (Table 2.4). It is likely that any modulating effect that the temperature or diet may

have on vitellogenesis is shrouded by the overwhelming effect of estrone in this case, and we see an effective maximization of dose-response. Additional treatments of doses in excess of 135 ng estrone/L would be necessary to confirm this. At 78 ng estrone/L, however, we see significant interactions between estrone, temperature, and feeding regimen (experiment 1, Fig. 2.1a). In complete contrast to the predicted trends, the low temperature (18°C), restricted diet (0.75% body weight/day) treatment had a significantly higher mean vitellogenin concentration than any other treatment. We propose that this elevation in vitellogenin is a response to two factors: enhancement of estrogen-induced vitellogenesis due to the stress hormone cortisol and optimal breeding temperatures near 18°C that trigger reproductive potency. It has been demonstrated that cortisol (10 – 20 µg/100 mg body weight) has an enhancing effect on induction of vitellogenesis via 17β-estradiol in Asian stinging catfish (*Heteropneustes fossilis*) through purported effects on peptide hormones and enzyme induction (Sundararaj et al., 1982). Additionally, fathead minnow spawning tends to begin when water temperatures reach about 14°C to 18°C (McCarraher and Thomas, 1968). Evidence from Smith 1978 suggests that reproductive efficacy peaks early in the spawning season, then tapers off as water temperatures warm (Fig. 2.13). Also worth mentioning with regards to the 78 ng estrone/L treatment (experiment 1) is the effect of high temperature (26°C) and restricted diet (0.75% body weight/day). This was the only treatment with a mean plasma vitellogenin concentration that was not significantly greater than that of the control. We suggest that this is due to the issue of a temperature-induced metabolism increase along with simultaneous food deprivation

– a situation that has potential environmental relevance in the face of climate change (Brander, 2007; Pörtner and Knust, 2007; Adrian et al., 2009; Noyes et al., 2009). In order to maintain homeostasis at a high metabolic rate, the organism will likely forgo reproductive functions (particularly energy-intensive functions such as vitellogenesis), in order to survive (Luquet and Watanabe, 1986; Izquierdo et al., 2001). Additionally, fish exposed at higher temperatures and fed a more liberal diet may consume more estrone-bound food due to increased metabolic demand. This may explain the increase magnitude of vitellogenin response that we see in the 26°C, *ad libitum* diet treatment over fish exposed at the same temperature, but fed a restricted diet. Interestingly, we do not see the same affect in the 135 ng estrone/L (high temperature, restricted diet) treatment in experiment 2. This is perhaps due to the increase in estrone concentration (~2x) over the first experiment, which may be sufficient to override metabolic safeguards for survival. This group (135 ng estrone/L, 26°C, restricted diet) exhibited lower survival (80%) than fish exposed to 78 ng estrone/L (90% survival) under the same environmental conditions in experiment 1 (Table 2.2).

Hematological endpoints in this study provided insight into temperature-modulated metabolic function in fish. In high estrone treatments for both experiments, we see significant elevations in hematocrit in fish exposed at 18°C and fed *ad libitum* over other treatments (Fig. 2.2). This is unexpected, as elevated hematocrit is often associated with hypoxia (Gallaugher et al., 1995), which is more likely in the 26°C treatment. In a study of physiological responses to temperature acclimation and confinement stress in striped bass (*Morone saxatilis*), a reduction in hematocrit was

noted (Davis and Parker, 1990). Interestingly, this response was slow or non-existent at lower temperatures. Perhaps lower temperatures buffer the stressful effects of estrone in this case (Folmar et al., 2001). The hematocrit in the 18°C, restricted diet treatment that would otherwise be buffered is lower due to nutritional deficiency, potentially resulting in a reduction in erythrocytosis.

A similar trend is present for blood glucose concentrations in experiment 2, where we see an elevation in blood glucose in all 18°C, *ad libitum* treatments (Fig. 2.3b). This may be a result of high carbohydrate bioavailability, but decreased cellular uptake. Larsen et al. (2001) hypothesized that temperature-related insulin fluctuations may be related to changes in insulin receptor internalization and turnover in liver tissues. Additionally, insulin receptor binding affinity is thought to be positively correlated with temperature increase (Freychet et al., 1971).

Anatomical endpoints were also highly dependent on temperature, but were largely unaffected by estrone concentration (Table 2.3, 2.4). Variability in mean gonadosomatic index (Fig. 2.5) and percentage of mature spermatozoa (Fig. 2.11) is strongly attributed to temperature in both experiments (Table 2.3, 2.4). As discussed previously, *Pimephales promelas* begin spawning at temperatures of 14°C to 18 °C (McCarraher and Thomas, 1968), spermatogenesis peaks shortly thereafter (Fig. 2.13), and the process is largely temperature-controlled (Smith, 1978). This explains the greater mean gonadosomatic index in the 18°C-exposed fish from both experiments (Fig. 2.5). The same trend is present in mean sum of secondary sex characteristics in experiment 2 (Fig. 2.7b) and is most likely related to seasonal changes in reproductive

morphology as well (Smith 1978). While the gonad maturity (percent of mature spermatozoa) follows the expected trend of elevated mean in the 18°C-exposed fish in experiment 2 (Fig. 2.12b), the opposite is true for experiment 1 (Fig. 2.12a). It is worth noting, however, that the difference between the mean percentages of mature spermatozoa between temperatures (9.8±3.2) in experiment 1 (Fig. 2.12a) is not as great as the difference between the mean temperatures (17.38±3.5) in experiment 2 (Fig. 2.12b), nor was the difference in mean gonadosomatic index as great in experiment 1 (Fig. 2.5). This may explain why body condition factor was significantly greater in the 18°C-exposed fish in experiment 2 (Fig. 2.4b), but not in experiment 1 (Fig. 2.4a). Mean hepatosomatic index in experiment 1 (Fig. 2.6a) followed the same temperature trends observed in gonadosomatic index. This is potentially due to pathological hepatocytic hypertrophy and hyperplasia associated with increased estrogen receptor activity (Dietrich and Krieger, 2009) as reflected by a corresponding trend in plasma vitellogenin concentrations (Fig. 2.1a) at low temperatures. Vacuolization of the liver was insignificant in experiment 1, but is significantly greater in the 18°C-exposed fish in experiment 2, perhaps due to the metabolic burden associated with increased reproductive function (Roberts, 2012; Smith, 1978; Fig., 2.13) resulting in increased nutrient storage (Hoar and Randall, 1988; Dietrich and Krieger, 2009).

The results of this study indicate that temperature has profound effects on various physiological and morphological aspects of *Pimephales promelas*, especially those related to metabolism. There is some evidence that low temperature (18°C), in

combination with a restricted diet (0.75% body weight/day), may increase the rate of estrone-induced (78 ng estrone/L) vitellogenesis in male fish (Fig. 2.1a). In contrast, it seems that diet restriction at higher temperatures (26°C) may offset vitellogenin induction (Fig. 2.1a). Analyses of vitellogenin concentrations, gonadosomatic index, secondary sex characteristics, hepatosomatic index and liver vacuolization suggest seasonal and temperature related trends (Denton and Yousef, 1975; Smith 1978), play a strong role in fish physiology and measured biological endpoints that are often used to determine sublethal toxicity in fishes (Colborn et al., 1993; Jobling et al., 1998; Schoenfuss et al., 2002; Vajda et al., 2008, Schultz et al., 2013). This subject requires further exploration, as the seasonality of exposure may play a large role in the perceived effects of pollutants tested on whole animals in a laboratory setting or samples taken *in situ*. Admittedly, increased temperature seemed to have the most dramatic consequence in comparison with other variables tested in this study: higher mortality. This is not to say that estrogens should be disregarded, as their effects on fish populations have been verified with a degree of confidence (Kidd et al., 2007). Ideally, laboratory testing on *Pimephales promelas*, and possibly other organisms, should be done at series of relevant temperatures in order to best assess the toxicity of pollutants in the laboratory, with the intention of extrapolating the findings to generate environmental standard

CHAPTER III

MODULATION OF ESTRONE EXPOSURE EFFECTS MEDIATED THROUGH SALINITY IN MALE FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

INTRODUCTION

The toxic effect of hypersalinization on freshwater organisms has been extensively documented (Adelman et al., 1976; Evans, 1987; Peterson and Meador, 1994; Benbow and Merritt, 2005; Canada Council of Ministers of the Environment, 2011; Elphick et al., 2011; Cañedo-Argüelles et al., 2013). Data indicate that environmental chloride concentrations are increasing over time, especially in urban areas, as road salt application increases (Oliver et al., 1974; Corsi et al., 2015). During times of low water flow in the summer and fall, chloride levels may exceed 800 mg/L, while normal levels fluctuate between 1-10 ppm (New Hampshire Department of Environmental Sciences, 2013). Watersheds in metropolitan areas of the northeastern United States can become particularly saline in the winter months, with concentrations of up to 4,629 ppm chloride recorded (Kaushal et al., 2005). Additionally, it is predicted that biogeochemical alterations associated with climate change will alter the salinity of aquatic systems (Moore et al., 1997). As the planet warms, we will see an increase in severe rain events (Poff et al., 2002; Noyes et al., 2009; Jeppesen et al.,

2010; Hooper et al., 2013; AAAS, 2014), rising sea levels that coincide with saltwater intrusion into freshwater habitat (Murdoch et al., 2000), and drought (Williamson et al., 2008; Whitehead et al., 2009; Taner, 2011), all of which have the potential to alter the salinity of freshwater systems (Justic et al., 1996; Jeppesen et al., 2010; Hooper et al., 2013). More insidious that direct toxicity, perhaps, is the interplay between increasing chloride concentrations and a host of xenobiotic chemicals (Heugens et al., 2001; Schiedek et al., 2007; Fortin et al., 2007).

High salinity has been shown to increase the toxicity of xenobiotic chemicals, such as aldicarb, fenthion, and L-selenomethionine phorate, through activation to more toxic intermediates following saltwater acclimation in anadromous fish (those which migrate into freshwater to spawn) (Schlenk and Lavado, 2011). There is also evidence that high salinity may increase the uptake of estrogen into fish tissues (Blewett et al., 2012). In contrast, metal toxicity is reported to increase with decreasing salinity (Hall and Anderson, 1995). Estrogens can act alone as a potent environmental endocrine disruptor (Colborn et al., 1993; Jobling et al., 1998; Schoenfuss et al., 2002; Vajda et al., 2008, Schultz et al., 2013), and are known for their relative ubiquity (Kolpin et al., 2002; Lee et al., 2011) especially in areas associated with anthropogenic influence, such as agricultural operations (Matthiessen et al. 2006; Chen et al. 2010) and densely populated urban areas (Barber et al., 2011; Lee et al., 2011; Martinovic-Weigelt et al. 2013). Interestingly, the influence of salinity on estrogenic effects in fish has, to our knowledge, only been narrowly explored (Schlenk and Lavado, 2011; Blewett et al., 2012).

This study seeks to determine whether salinity has the effect of modulating the observed biological effects of estrone, a common environmental estrogenic contaminant (Kolpin et al., 2002; Lee et al., 2011), on adult male fathead minnows (*Pimephales promelas*). Fathead minnows have a low chloride toxicity threshold (252 ppm, chronic exposure) when compared with other freshwater organisms (Siegel, 2007). We predicted that, in the presence of elevated aqueous chloride concentrations, the observed biological effects of estrone would be exacerbated. As salinity is increased, the solubility of oxygen in water is reduced (Roberts, 2012). It is suggested that the lipid-rich gill tissue is the primary site of entry for lipophilic estrogens into the bloodstream of teleosts (Blewett et al., 2012). Since increasing salinity decreases oxygen solubility in the water, changes in gill ventilation must accommodate this change to keep oxygen uptake constant (Roberts, 2012). A threefold increase in the uptake of estrogen was observed in 50% seawater over freshwater in the euryhaline fish (Blewett et al. 2012). An increase in salinity can also result in stress induction (Siegel, 2007; Roberts, 2012). A common stress hormone, cortisol, has been shown to produce an enhancing effect on estrogen-induced vitellogenesis in female Asian stinging catfish (10 – 20 µg cortisol/100 mg body weight) (Sundararaj et al., 1982; Hoar and Randall, 1988). Additionally, the bioavailability of estrone may be increased by the reduction in solubility of the chemical in saline water. This is known as the "salting out" effect (Schwarzenbach et al., 2003), whereby water molecules bind strongly to salts, making them unavailable to dissolve organic compounds, and

possibly increasing the degradation half-life of the compound (Song and Brown, 1998; Noyes et al., 2009).

MATERIALS AND METHODS

Ethics Statement

This study was approved by the St. Cloud State University Institutional Animal Care and Use Committee.

Experimental Design

Replicate flow-through exposures of mature male fathead minnows to a well water control, low estrone (15 ng/L nominal) or high estrone (100 ng/L nominal) treatment were maintained at the St. Cloud State University Aquatic Toxicology Laboratory in St. Cloud, MN. Chemical treatments were delivered to the aquaria using modifications to published flow-through protocol (Schoenfuss et al., 2008). In the first experiment, low estrone and high estrone treatments were exposed in well water, at low salinity (10 ppm added NaCl) or at high salinity (50 ppm added NaCl). In the second exposure, the low salinity treatment was eliminated due to minimal differences between well water control (93 ppm Cl) and 10 ppm added NaCl (96 ppm Cl) treatments. Each treatment was replicated in two aquaria, with 10 fish per tank (n=20). In the first experiment, a subset of fish was dissected on day 10, in order to obtain some data in the case that fish would not survive the standard 21-day exposure (Denny, 1987). The remainder of the fish dissected after 20 days of exposure. In the second experiment, fish were exposed for only 10 days. Exposures were maintained

on a 16 h light/8 h dark cycle. The first exposure began on October 29th, 2012, with dissections taking place on November 9th, 2012 (day 10) and November 19th, 2012 (day 20). In the second experiment, exposure began on November 15th, 2012, and all fish were dissected on November 25th, 2012. Fish exposures were performed in accordance with the St. Cloud State University Institutional Animal Care and Use Committee (IACUC) policy.

Exposure Chemicals

Estrone (Sigma-Aldrich, St. Louis, MO) exposure solutions were prepared daily from aliquots of a concentrated estrone solution in 100% ethanol. Controls received 100% ethanol equivalent to that in estrone treatments. Aliquots were stored at 4°C until use.

Each day of the exposure, one treatment-specific aliquot was mixed with 10 L of ground water from a dedicated well. The solutions were kept in amber glass bottles during the exposure. Stir bars and plates were assigned to each amber glass bottle containing treatment solution. This method insured continuous agitation to avoid settling of the chemicals. Stainless steel tubes were used to draw the solution into a stainless steel mixing chamber via a Cole-Palmer Masterflex 7523-40 peristaltic pump at a nominal rate of 7 mL/min. Well water or saline water was also fed into the mixing chamber at a rate of 200 mL/min to achieve the final treatment concentration. Mixture was achieved by allowing the combined solution to fall over two barrier-walls within the mixing tank. The final solution was delivered directly to four aquaria via tubing extending from the bottom of the tank. Water exchange rate was approximately seven

exchanges/aquarium/day.

Exposure Organisms

Adult male fathead minnows (6 months old) were obtained from a laboratory fish rearing facility (US EPA, Cincinnati, OH), and were acclimated in untreated well water for approximately 3 weeks prior to exposure. Survival was assessed on a daily basis. All fish were fed an ad libitum diet of frozen brine shrimp (*Artemia* spp.) and frozen blood worms (*Glycera* spp.) and kept at a constant temperature of approximately 23°C. All fish maintenance was carried out in accordance with St. Cloud State University's IACUC guidelines.

Water Quality Analysis

Physical parameters (temperature, pH, and conductivity) of each treatment were recorded once daily (YSI Model 556 MPS, YSI Environmental, Yellow Springs, OH). Total water hardness, free chlorine, total chlorine and alkalinity were measured periodically using AquaChek 5-in-1 Water Quality Test Strips (Hach Company, Loveland, CO).

Water Chemistry Analysis

At exposure days 5, 10, 15, 20, and on the day of dissection, two 120 mL water samples were taken from each treatment. Estrone concentrations were determined using previously published methods from Dammann et al., 2011. Ion concentrations were analyzed using EPA method 200.7 (Martin et al., 1992) and 300.0 (Pfaff, 1993).

Plasma Vitellogenin Analysis

Blood was obtained from the severed caudal vasculature using a heparinized capillary tube after fish were anesthetized in 0.1% MS-222 (Argent Laboratories, Redmond, WA). Plasma was obtained following blood centrifugation at 8050 x *g* for 8 min at 4°C and stored at -80°C prior to analysis. Plasma vitellogenin concentrations (µg/mL) were quantified via competitive antibody-capture ELISA using and 8 point serial dilution standard (4.8 µg/mL to 0.0375 µg/mL). Detailed methods of the assay are described in Shappell et al. (2010).

Blood Glucose Analysis

Using 1 µL of blood, a TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT) was used to quantify blood glucose concentration (mg/dL). Blood glucose was only measured in the second experiment.

Hematocrit Analysis

Blood was taken via the caudal artery using 75mm heparinized capillary tubes (ClearCRIT Plastic Capillary Tubes, Separation Technology Inc., Sanford, FL). Capillary tubes were sealed (Critoseal, Oxford Labware, St. Louis, MO) and placed in a microhematocrit centrifuge (HERMLE Z200A, Labnet International Inc., Woodbridge, NJ) for 3 minutes at 968 x *g*. Hematocrit was measured using a Spiracrit Micro-Hematocrit Tube Reader (Clay-Adams Inc., New York, NY).

Organosomatic Indices

Prior to dissection, individual fish were weighed (0.01 g precision, Taylor

Stainless Steel Food Scale, Taylor Precision Products, Oak Brook, IL) and measured for length to calculate body condition factor (BCF = body weight/total length^3) (Fulton, 1904). To calculate hepatosomatic index (HSI = liver weight/whole body weight x 100) and gonadosomatic index (GSI = gonad weight/whole body weight x 100), liver and gonads were excised and immediately weighed (0.001 g precision, Mettler Toledo AG245, Columbus, OH).

Secondary Sex Characteristics

Prior to dissection, an assessment of the male sexual characteristics of individual fish was performed. Tubercles, dorsal pad and banded coloration were given a separate rating of 1, 2 or 3 based on prominence (1 being the least prominent, 3 being the most prominent) (Smith, 1978). The three values were added together as a method for comparing morphological sexual maturity between treatments.

Histopathology

Liver and gonads were excised from fish and placed in histological cassettes (Tissue-Loc Histoscreen Cassettes, Thermo Scientific, Kalamazoo, MI) in 10% buffered formalin for at least 24 hours. Dehydration and tissue preparation was achieved following previously prescribed procedures (Carson, 1997). Tissues were embedded in paraffin. Small samples (~1 mm diameter) of liver and gonad were sectioned at approximately 5 µm (Olympus Cut 4055 Microtome, Olympus America Inc., Center Valley, PA). Tissues were stained using a standard haematoxylin and eosin counter stain (Gabe, 1976; Carson, 1997).

Liver tissues were graded on a scale of 1 to 4 based on cellular vacuolization. A grade of 1 indicates that no vacuoles are visible under a 40x magnification, a grade of 2 indicates that less than 25% of the visible tissue is occupied by vacuoles, a grade of 3 indicates that 25-50% of the visible tissue is occupied by vacuoles, and a grade of 4 indicates that the majority of the visible tissue consists of vacuoles.

Testes were assessed based on the proportion of sperm cells (spermatogonia, spermatocytes, spermatids, and spermatozoa) present in the visible tissue. An experienced grader assigned cell percentage values (totaling 100%) to the samples. The overall maturity of the sample was calculated as:

$$\text{Gonad maturity rating} = ((\%_{\text{spermatogonia}}) + (\%_{\text{spermatocytes}} \times 2) + (\%_{\text{spermatids}} \times 3) + (\%_{\text{spermatozoa}} \times 4)) / 100$$

Statistical Analysis

All data was assessed for normality using the Kolmogorov-Smirnov using Graphpad Prism software (Prism 5.0 statistical package, GraphPad Software, Inc., Oxnard, CA). Data were then analyzed using a two-way ANOVA followed by a Bonferroni post-test to determine treatment effects (NaCl, estrone, or interaction). Additionally, individual means were compared using a one-way ANOVA followed by a Tukey post-test.

RESULTS

Chemical Data and Survival Rates

Estrone concentrations were lower in experiment 2 (low treatment: 7±1.8 ng/L, high treatment: 85±32.3 ng/L) than in experiment 1 (low treatment: 16±5.8 ng/L, high treatment: 138±97.3 ng/L) (Table 3.1). Chloride concentrations between the well water control and the 10 ppm added NaCl treatment differed by only about 2%, and thus this treatment was dropped from the second experiment (Table 3.2). Survival was high in both 10-day exposures (>88% in all treatments) (Table 3.3). Immediately following the 10-day dissection of experiment 1, there was a massive die off (~50%). This is believed to have been linked to routine cleaning, which may have introduced bleach into the system.

Table 3.1. Average aqueous estrone concentrations in flow-through exposures; chemical analysis methods are consistent with those described in Shappell et al. 2010; samples were taken on days 5, 10, 15 and 20 of the exposure.

	Estrone Treatment (Nominal)	Average Measured Estrone Concentration
Experiment 1	Control	0 ng/L
	15 ng/L	16±5.8 ng/L
	100 ng/L	138±97.3 ng/L
Experiment 2	Control	0 ng/L
	15 ng/L	7±1.8 ng/L
	100 ng/L	85±32.3 ng/L

Table 3.2. Average aqueous chloride concentrations in flow-through exposures; samples were taken on days 5, 10, 15 and 20 of the exposure

	NaCl Added	Measured Total Sodium Concentration	Measured Total Chloride Concentration
Experiment 1	None (Well Water)	27±0.3 ppm	93±0.1 ppm
	10 ppm	30±0.4 ppm	96±0.5 ppm
	50 ppm	59±4.1 ppm	141±4.7 ppm
Experiment 2	None (Well Water)	29±0.1 ppm	93±0.1 ppm
	50 ppm	48±5.4 ppm	126±0.7 ppm

Table 3.3. Survival for each treatment across both experiments; four fish were taken from each tank (12 per treatment) for the day 10 dissection, these fish were not counted towards the total for day 21 survival calculation; mass casualties in experiment 2 due to experimental error with bleach (see discussion).

		Well Water	10 ppm Added NaCl	50 ppm Added NaCl
Experiment 1, Day 10	**Control**	88% (35/40)	-	-
	16 ng/L estrone	95% (38/40)	98% (39/40	95% (38/40)
	138 ng/L estrone	100% (40/40)	95% (38/40)	98% (39/40)
Experiment 1, Day 21	**Control**	78% (28/36)	-	-
	16 ng/L estrone	67% (24/36)	94% (34/36)	92% (33/36)
	138 ng/L estrone	39% (14/36)	43% (15/36)	94% (34/36)
Experiment 2	**Control**	89% (16/18)	-	-
	7 ng/L estrone	94% (16/17)	-	94% (16/17)
	85 ng/L estrone	94% (17/18)	-	89% (16/18)

Physiological Endpoints

Exposure to 85 ng/L (experiment 2) and 138 ng/L (experiment 1) estrone resulted in significant increase in plasma vitellogenin concentrations over control fish. Neither aqueous estrone concentrations of 16 ng/L (experiment 1) and nor 7 ng/L (experiment 2) resulted in significant vitellogenin increase over control. Furthermore,

the NaCl treatments used in this study did not significantly modulate vitellogenin levels in estrone-exposed male fathead minnows (Fig. 3.1).

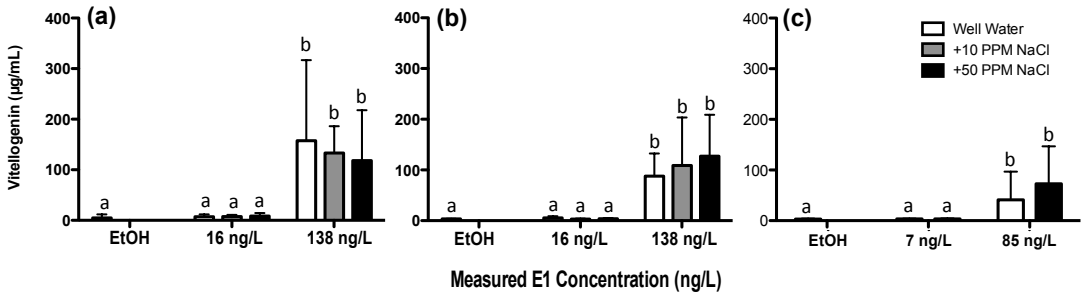

Figure 3.1. Plasma vitellogenin concentrations of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); letters above standard deviation bars indicate significantly different means at $p<0.05$; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

Estrone concentration accounted for all variance in hematocrit, while added NaCl showed no significant effect. Hematocrit did not differ significantly from control in the treatments of either of the 10-day exposures (Fig. 3.2a, c). In the 21 day exposure (experiment 1), hematocrit was significantly elevated over control ($p<0.05$) in fish exposed to 138 ng/L estrone at 50 ppm added NaCl (Fig. 3.2b). Blood glucose (measured only in experiment 2) was not significantly effected by aqueous estrone concentration or added NaCl (Fig. 3.3).

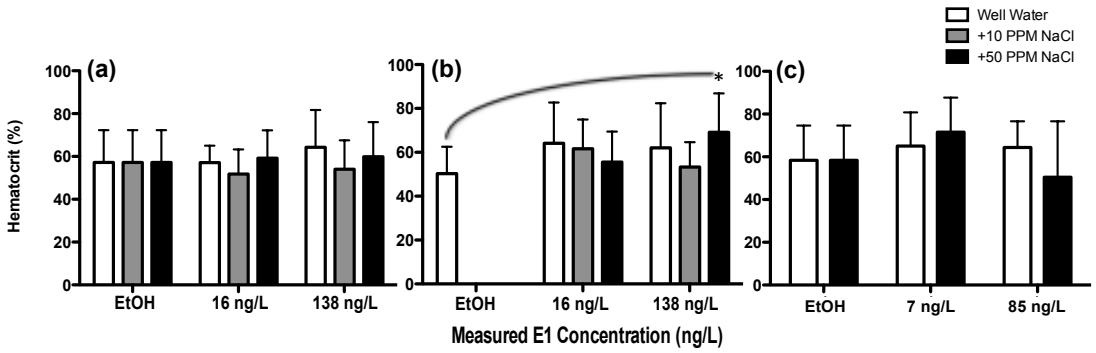

Figure 3.2: Hematocrit of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); * indicates significance at $p<0.05$; bars indicate standard deviation; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

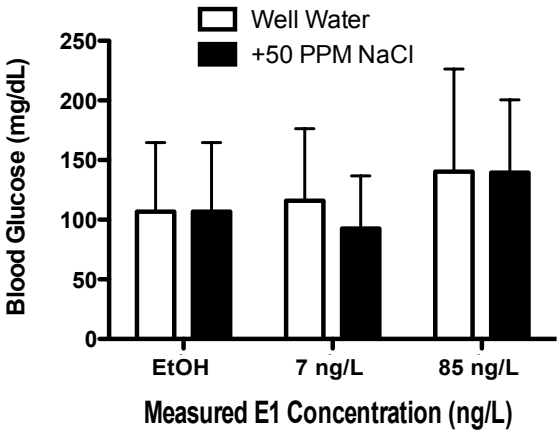

Figure 3.3. Blood glucose concentrations of male *P. promelas* after 10 days of exposure to estrone at 0 and 50 ppm added NaCl (experiment 2); blood glucose was not recorded for experiment 1; no significant differences between means; bars indicate standard deviation; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

Morphological Endpoints

While added NaCl concentration did not significantly alter the observed magnitude of secondary sex characteristics (tubercles, dorsal pad, and color of male fish), estrone was responsible for some variance in fish exposed for 21 days (experiment 1) and in experiment 2 (Fig. 3.4). When examined independent of NaCl treatment, a decreasing trend in secondary sex characteristics is observed with increasing aqueous estrone concentration in all three data sets (Fig. 3.5), however, the majority of the means were not significantly different (p<0.05).

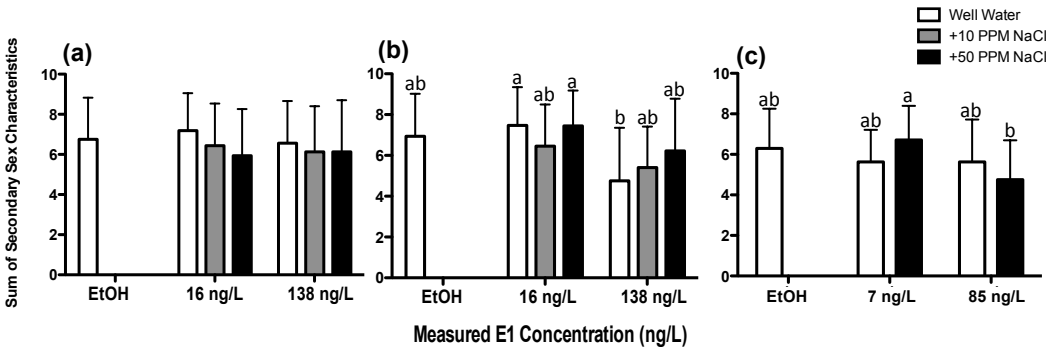

Figure 3.4. Sum of secondary sex characteristics of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); letters above standard deviation bars indicate significantly different means at p<0.05; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

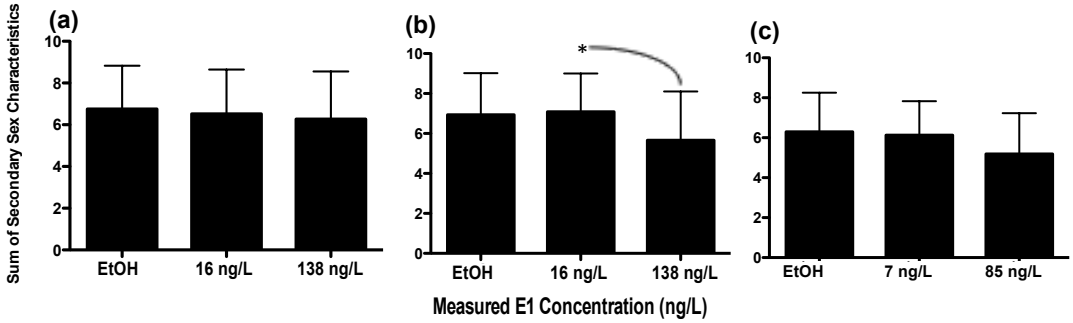

Figure 3.5. Sum of secondary sex characteristics of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1, and 10 days of estrone exposure in experiment 2; * indicates significance at $p<0.05$; bars indicate standard error; One-Way ANOVA with estrone treatments compared independent of added NaCl, followed by Tukey post-test.

Added NaCl did not significantly alter body condition factor, however, aqueous estrone concentration had a significant effect on variance in mean body condition factor (Fig. 3.6, 3.7). A significant decrease in body condition factor in both 10-day exposures can be seen in the high (138 ng/L and 85 ng/L) estrone treatments (Fig. 3.7a, c). The effect in the 21-day exposure (experiment 1) differs in that the 138 ng/L aqueous estrone, 50 ppm added NaCl treatment is elevated over control (Fig. 3.6b). Added NaCl did not significantly alter organosomatic indices in either of the experiments, nor did estrone (Fig. 3.8, 3.9).

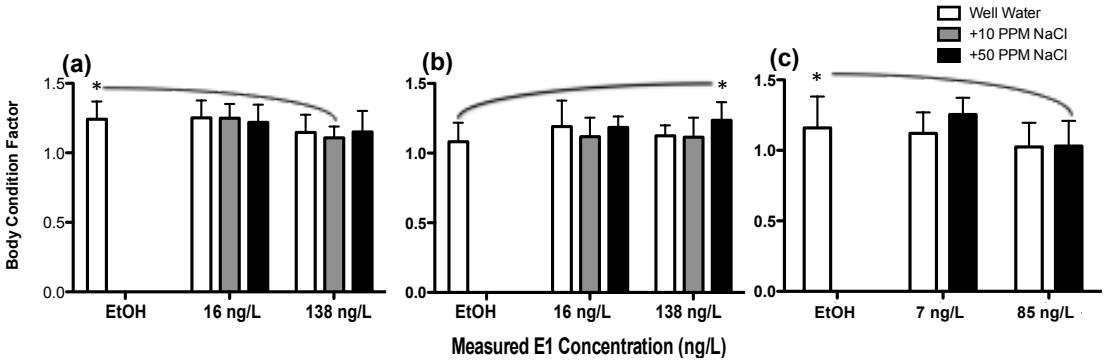

Figure 3.6. Body condition factor of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); * indicates significance at $p<0.05$; bars indicate standard deviation; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

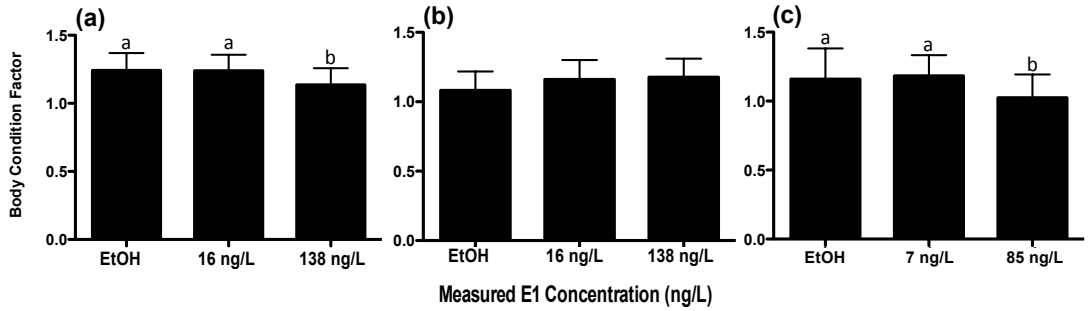

Figure 3.7. Body condition factor of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1, and 10 days of estrone exposure in experiment 2; letters above standard deviation bars indicate significantly different means at $p<0.05$; One-Way ANOVA with estrone treatments compared independent of added NaCl, followed by Tukey post-test.

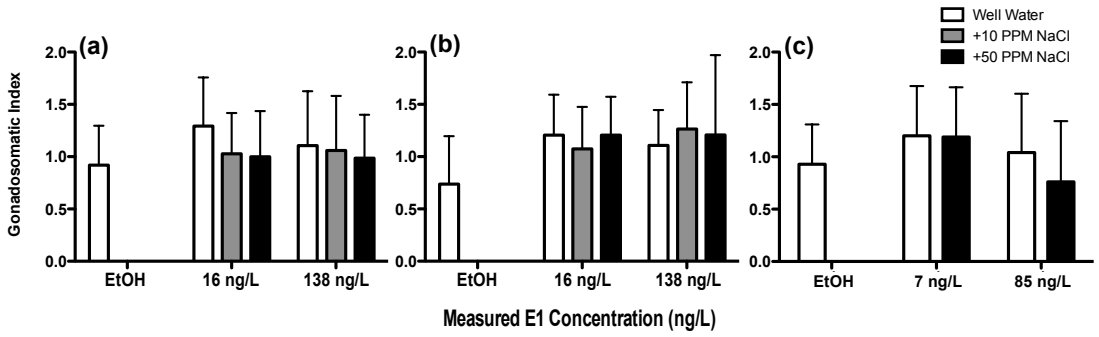

Figure 3.8. Gonadosomatic index of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); no significant differences between groups; bars indicate standard deviation; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

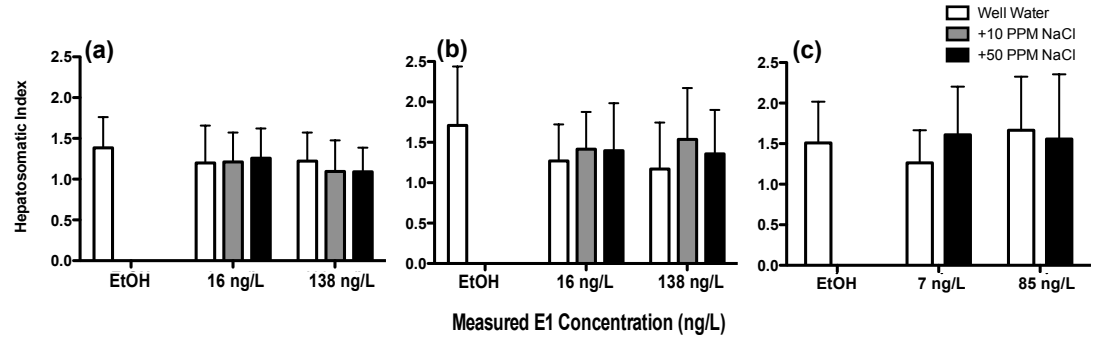

Figure 3.9. Hepatosomatic index of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); no significant differences between groups; bars indicate standard deviation; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

Liver vacuolization showed no trends in the 21-day exposure, however increased vacuolization was observed at increasing aqueous estrone concentrations in the 10-day exposed fish from experiment 1 (Fig. 3.10, 3.11). In experiment 2, we see

significantly increased vacuolization in the low estrone treatment (7 ng/L) when compared with the high treatment (85 ng/L) when means are observed independent of added NaCl concentration (Fig. 3.11). Calculated gonad maturity GPA was not significantly variable between treatments in either of the experiments (Fig. 3.12).

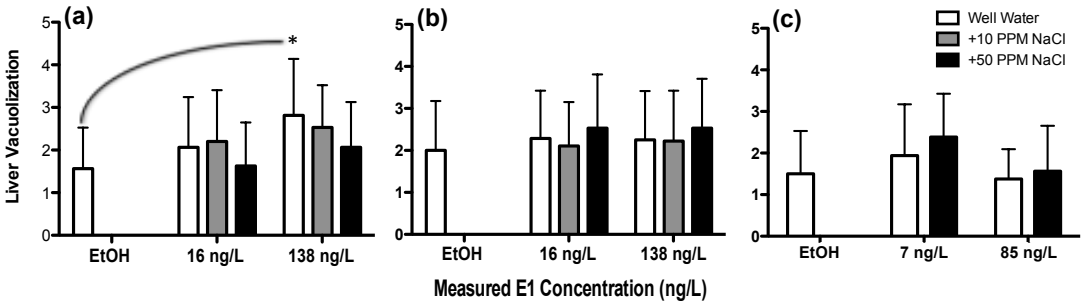

Figure 3.10. Liver vacuolization of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); * indicates significance at $p<0.05$; bars indicate standard deviation; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

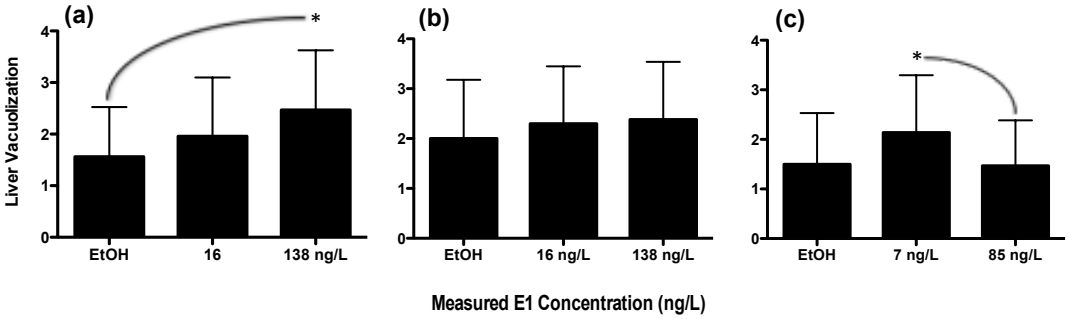

Figure 3.11. Liver vacuolization of male *P. promelas* after 10 days of exposure to estrone during experiment 1; * indicates significance at $p<0.05$; bars indicate standard deviation; One-Way ANOVA with estrone treatments compared independent of added NaCl, followed by Tukey post-test.

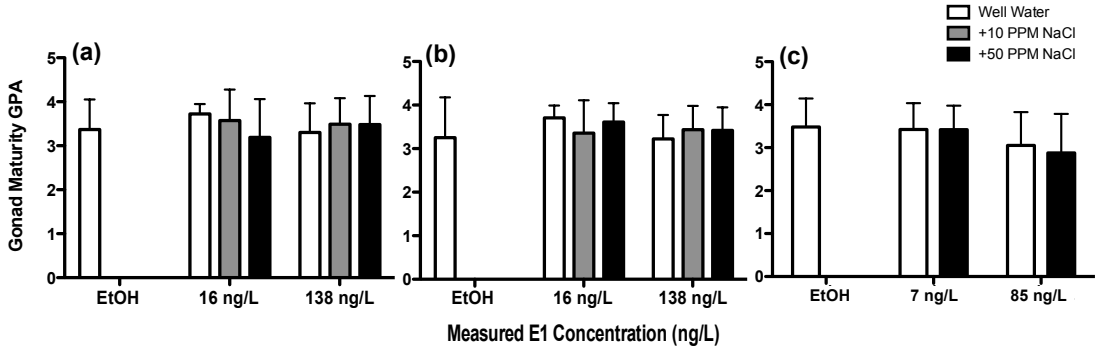

Figure 3.12. Gonad maturity "GPA" of male *P. promelas* after 10 days of exposure (a) and 21 days of exposure (b) to estrone during experiment 1 at 0, 10 and 50 ppm added NaCl, and 10 days of estrone exposure in experiment 2 at 0 and 50 ppm added NaCl (c); no significant differences between groups; bars indicate standard deviation; Two-Way ANOVA followed by Bonferroni post-test; One-Way ANOVA followed by Tukey post-test.

DISCUSSION

The current study examined the impact of salinization on the biological effects (Colborn et al., 1993; Jobling et al., 1998; Schoenfuss et al., 2002; Vajda et al., 2008; Martinovic-Weigelt et al., 2013) of a common aquatic pollutant, estrone (Kolpin et al., 2002; Lee et al., 2011; Schultz et al., 2013). It was hypothesized that the addition of 10 and 50 ppm NaCl would increase receptor sensitivity (Schlenk and Lavado, 2011) and steroid uptake (Blewett et al. 2012), thereby exacerbating the observed biological effects associated with estrogen exposure in male fathead minnows. Based on the results, we reject this hypothesis. None of the variability in any of the endpoints measured can be significantly ($p \geq 0.05$) attributed to salinity flux.

Elevated plasma vitellogenin concentrations in male fish are a hallmark of estrogenic exposure (Sumpter and Jobling, 1995, Matozzo et al., 2008). It was

expected that salinity would play a direct role in modulating this response, however, the only significant variability in the ANOVA was due to estrone. Vitellogenin concentrations in male fish exposed to aqueous concentrations of 138 ng/L (experiment 1) and 85 ng/L (experiment 2) estrone were significantly elevated over control, while concentrations of 16 ng estrone/L (experiment 1) and 7 ng estrone/L were not sufficient to cause significant vitellogenin induction (Fig. 3.1). These data suggest that in systems where estrone equivalents are ≥16 ng/L, plasma vitellogenin measurement may not serve as an effective endpoint to quantify the effects of exogenous compounds. We can also extrapolate from these low estrone treatments that, although there was not a "saline control" in this experiment, added NaCl concentrations of 50 ppm alone are not sufficient to elucidate a vitellogenic response.

Interestingly, hematocrit was only elevated in the 21-day fish exposed to a high concentration of estrone (138 ng/L). Whether this is a result of increased exposure duration or an artifact of potential exposure to a chlorine disinfectant is cause for further examination. This may be of particular relevance, as wastewater treatment often results in a discharge of chlorine (Government of Canada, 1993), which may potentially coincide with hormone discharge (Ankley et al. 2007) and formation of additional toxic organic chlorinated compounds (Emmanuel et al., 2004). Toxicity of chlorine in fish is attributed to the oxidation of hemoglobin to methemoglobin and subsequent anoxia (Grothe and Eaton, 1975), which may in turn trigger an increase in erythrocyte production. The majority of the chlorine-related mortality occurred in the high estrone treatment. Whether or not this was due to the combined effects of the two

chemicals is impossible to determine, but may be, based on knowledge of hepatic, renal, and osmoregulatory toxicity mechanisms for both chemicals (Adelman et al., 1976; Evans, 1987; Peterson and Meador, 1994; Folmar et al, 2001; Benbow and Merritt, 2005; Canada Council of Ministers of the Environment, 2011; Elphick et al., 2011; Cañedo-Argüelles et al., 2013). It is also possible that elevated hematocrit in surviving fish is just an adaptive response to prolonged low dissolved oxygen associated with higher salinity. Perhaps 10 days was not a sufficient time-frame for this response, and is therefore not seen in either 10-day exposure. Gill deformations were noted in some fish in the NaCl treatments, and may also have played a role in reducing the efficiency of gas exchange, thereby necessitating an increase in erythrocyte production.

In both experiments, we see a decreasing trend in secondary sex characteristic intensity as the concentration of estrone is increased. While the effect was only significant in the 21-day exposure, this observation is substantiated by the literature, which suggests that fathead minnow male secondary sex characteristics are accentuated at high androgen levels (Smith, 1974; Ankley et al., 2001) and reduced by estrogen receptor agonists (Harries et al., 2000). This notion is further corroborated by longstanding knowledge of feedback loops associated with the vertebrate hypothalamic-pituitary-gonadal axis. At moderate concentrations, estrogens elicit negative feedback suppression of gonadotropin-releasing hormone (Dorling et al., 2003), however at high concentrations, for periods of time in excess of 36 hours,

feedback becomes positive and gonadotropin release (particularly luteinizing hormone) is stimulated (Silverthorn, 2013).

Body condition factor of fish exposed to high estrone concentrations in both of the 10-day exposures was significantly lower than both control and low estrone-treated fish. Hepatic and renal dysfunction associated with high vitellogenin values has been observed in male summer flounder (*Paralichthys dentatus*) injected with 17β-estradiol (0.1 – 10.0 mg 17β-estradiol/kg body weight) (Folmar et al., 2001). Reduction in hepatic-metabolic function could be responsible for the weight loss in these fathead minnows. Increased hepatic vacuolization in the day-10 data from experiment 1 (Fig. 3.10) is consistent with these findings. Interestingly, trends in mean hepatosomatic index do not show that liver weights are proportionally higher, though, in these fish. This likely indicates a lack of true hypertrophy, but rather a simple increase in stress-induced energy storage, which may be detrimental to liver function.

While this study determined that added NaCl concentrations of 10 and 50 ppm do not significantly alter the observed biological effects of estrone, it fails to fully answer the question as to whether salinity plays a role in the severity of estrogenic effects in male fathead minnows. This is due to several factors. Most importantly, this study lacked a low-salinity control. The chloride concentration of the well water used in this exposure was approximately 95 ppm (Table 3.2). This makes the addition of 50 ppm NaCl (30.3 ppm chloride) less dramatic, and introduces the possibility of a fish population that is already acclimating to what may be relatively high salinity in comparison with their previous culture parameters, considering that normal chloride

concentrations in freshwater systems range from 1-10 ppm (New Hampshire Department of Environmental Sciences, 2013). Behavioral observations of a subset of fish may serve as an indication of acclimation from a different water source. For example, it would be useful to observe the rate of opercular movements. A reduction in opercular movements over time may indicate changes in respiratory physiology and morphology associated with acclimation to lower dissolved oxygen associated with higher salinity (Blewett et al., 2012). All treatments were low in comparison with the United States Environmental Protection Agency (USEPA) chronic water quality criteria concentration of 230 ppm chloride (U.S. Environmental Protection Agency, 1988) and the chronic toxicity threshold for the fathead minnow of 252 ppm chloride (Siegel, 2007). Some metropolitan watersheds may see levels exceeding 2,000 ppm (Kaushal et al., 2005). Finally, this experiment, if replicated, should contain a true saline control (no estrone) to absolutely rule out any biological effects that might arise solely due to salinity.

The observations from this study provide some valuable insight into the biological effects of estrone, particularly with regards to vitellogenin induction thresholds (there is a lack of significant induction at concentrations ≤16ng/L). It also serves as a lesson in laboratory maintenance, especially when using chemicals, like sodium hypochlorite, that are known to be acutely toxic to aquatic life. As is often the case, new ideas can potentiate from perceived mistakes. The introduction of a chlorinated substance into the system, and the observed effects certainly raise questions and open the door for further exploration. We should also further explore the

issue of interaction between salinity and estrogenic effects. Based on the threat of climate change-associated salinity fluctuations in freshwater systems (Justic et al., 1996; Moore et al., 1997; Jeppesen et al., 2010; Hooper et al., 2013) and the chloride-associated physiological mechanisms that have the potential to modulate estrogenic effects (Hoar and Randall, 1988; Song and Brown, 1998; Schwarzenbach et al., 2003; Noyes et al., 2009; Schlenk and Lavado, 2011; Blewett et al., 2012; Roberts, 2012) it would be warranted to revisit this issue. Ideally this would be achieved using total chloride concentrations consistent with environmental fluctuations as described in Corsi et al. 2015. Environmental concentrations are consistently higher than those tested in this study, and may be sufficient to corroborate the original hypothesis.

CHAPTER IV

MODULATION OF ESTRONE EXPOSURE EFFECTS MEDIATED THROUGH DISSOLVED OXYGEN IN MALE FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

INTRODUCTION

Anthropogenic influences, such as agricultural operations (Soto et al., 2004; Matthiessen et al. 2006; Chen et al. 2010) and urban runoff (Barber et al., 2011; Lee et al., 2011; Martinovic-Weigelt et al. 2013) place a chemical burden on aquatic systems. Estrogenic compounds, such as estrone (E1), 17ß-estradiol (E2), and the synthetic 17α-ethynylestradiol (EE2), exhibit widespread occurrence (Kolpin et al., 2002; Lee et al., 2011) and interfere with reproductive function in fish (Colborn et al., 1993; Jobling et al., 1998; Schoenfuss et al., 2002; Vajda et al., 2008, Schultz et al., 2013). While examination of dose-dependent effects of individual aquatic contaminants is relatively straightforward due to standardized culture techniques (Denny, 1987), there is still much to be known about the effects of chemical mixtures and the interaction between physical environmental parameters. The need for this type of research is becoming increasingly urgent as the ecological reach of human impact broadens and as the introduction and fate of biologically active chemicals is altered in the face of a

changing climate (Oreskes, 2004).

Historical data (Stahl et al., 2013) and models of freshwater systems (Moore et al., 1997; Jeppesen et al., 2010) frequently indicate increasing prevalence of hypoxic conditions as a result of climate change, due to increased surface water temperature (Murdoch et al., 2000), prolonged thermal stratification (Taner et al., 2011) and increased nutrient runoff (Adrian et al., 2009) in densely populated and agricultural areas. Incidentally, these are the areas most commonly associated with the discharge of contaminants (Soto et al., 2004; Matthiessen et al. 2006; Chen et al. 2010; Barber et al., 2011; Lee et al., 2011; Martinovic et al. 2013). Hypoxic conditions suppress reproductive function in *Cyprinus carpio* (Wu et al., 2003), *Micropogonias undulatus* (Thomas et al., 2006), and *Fundulus grandis* (Landry et al., 2007), and increase estrogen receptor transcriptional activity synergistically in combination with estradiol (Yi et al., 2009). Hypoxia has also been implicated in an increase of uptake (Blewett et al., 2012) and toxicity of certain chemicals in fish (Ficke et al., 2007). The affinity for organismal congregation near effluent discharge (Spigarelli et al., 1982) may place organisms at additional risk. Behavioral changes in an oxygen gradient are, of course, not limited to spatial migration. Respiratory movements are increased in low oxygen conditions in order to maintain homeostatic balance in fishes (Gee et al., 1978; Wares and Igram, 1979). The lipophilic gill tissues of teleost fish are thought to be a primary site for uptake of steroidal compounds (Blewett et al., 2012). For this reason, we suggest that a higher rate of opercular movement, resulting from hypoxic conditions (Gee et al., 1978; Wares and Igram, 1979), may result in an increase in volume of

chemical exposure, and a subsequent increase in uptake of steroidal compounds such as estrogens. By measuring plasma vitellogenin concentrations in male fathead minnows (*Pimephales promelas*) we can quantify the impact of estrone on individual fish (Sumpter and Jobling, 1995; Matozzo et al., 2008). In a study of the effect of hypoxia on responses to a mixture of estrogenic chemicals, no effect was found (Brian et al., 2009). The interaction between dissolved oxygen and individual estrogenic compounds has, to our knowledge, not been measured.

In this study, we examine the effect of dissolved oxygen concentration on observed biological endpoints commonly associated with estrogenic exposure. It was hypothesized that hypoxia would intensify the observed effects of exposure to a single estrogenic compound. However, if this was rejected, the data would indicate that biomarkers of estrogen-induced endocrine disruption are expressed similarly regardless of dissolved oxygen conditions, as observed with mixtures in Brian et al. 2009. Estrone (E1) was used as a representative estrogenic compound due to its environmental relevance, as it is often found at concentrations higher than other estrogenic chemicals (Kolpin et al., 2002,; Dammann et al., 2011) due to oxidative breakdown of estradiol after it is introduced into the environment (Ying et al., 2002). If dissolved oxygen is found to significantly modulate induction of estrogenic exposure biomarkers, it will serve to indicate that water quality parameters are a pertinent variable to be considered when measuring the effects of estrone, and possibly other steroidal chemical pollutants.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the St. Cloud State University Institutional Animal Care and Use Committee.

Experimental Design

Replicate flow-through exposures of mature male fathead minnows (6 months old, Environmental Consulting & Testing, Superior, WI) to either an ethanol carrier control, low estrone (51 ng/L in Experiment 1; 13 ng/L in Experiment 2) or high estrone (390 ng/L in Experiment 1; 282 ng/L in Experiment 2) treatment was maintained for 21 days at the St. Cloud State University Aquatic Toxicology Laboratory in St. Cloud, MN. Fish were kept on a 16 h light/8 h dark cycle. Exposure began on August 23rd and ended on September 14th for experiment 1 and began on October 31st, 2014 and ended on November 21st, 2014 for experiment 2. Chemical treatments were delivered to the aquaria using modifications to published flow-through protocol (Schoenfuss et al., 2008). Some modification of the configuration was necessary in order to minimize dissolved oxygen in the non-aerated treatments by decreasing surface agitation in the stainless steel mixing tanks. Ethanol control, low estrone and high estrone treatments were exposed at either a low (no aeration) or a high (aerated) dissolved oxygen (DO) concentration. Each treatment was replicated in two aquaria, with 10 fish per tank (N=20). On day 22, all fish were dissected in

accordance with St. Cloud State University Institutional Animal Care and Use Committee (IACUC) policy.

Exposure Chemicals

Estrone (Sigma-Aldrich, St. Louis, MO) exposure solutions were prepared daily from aliquots of an estrone solution in 100% ethanol. Control treatments received 2 mL of 100% ethanol to rule out any effect from the ethanol carrier. Aliquots were stored at 4°C until use.

Each day, beginning on the first day of exposure, one treatment-specific aliquot was mixed with 10 L of ground water from a dedicated well. The solutions were kept in amber glass bottles during the exposure. Stir bars and plates were assigned to each amber glass bottle containing treatment solution. This method insured continuous agitation to avoid settling of the chemicals. Stainless steel tubes were used to draw the solution into a stainless steel mixing chamber via a Cole-Palmer Masterflex 7523-40 peristaltic pump at a nominal rate of 7 mL/min. Ground water from the same dedicated well was gravity-fed into the mixing chamber at a rate of 200 mL/min to achieve the final treatment concentration. Mixture was achieved by allowing the combined solution to fall over two barrier-walls within the mixing tank. The final solution was delivered directly to four aquaria via tubing extending from the bottom of the tank. Water exchange rate was approximately seven exchanges/aquarium/day.

Exposure Organisms

Adult male fathead minnows were obtained exclusively from a laboratory supplier

(Environmental Consulting & Testing, Superior, WI). Survival was assessed on a daily basis. All fish were fed an ad libitum diet of frozen brine shrimp (*Artemia* spp.) and frozen blood worms (*Glycera* spp.) and kept at a constant 25°C. Fish were placed in tanks 7 days prior to estrone exposure to acclimate to their respective dissolved oxygen concentration. All fish maintenance was carried out in accordance with St. Cloud State University's IACUC guidelines.

Water Quality Analysis

Physical parameters (temperature, conductivity, total dissolved solids, salinity, dissolved oxygen, pH, and oxidation reduction potential) of each aquarium were recorded each morning using a (YSI Model 556 MPS, YSI Environmental, Yellow Springs, OH). In addition, dissolved oxygen and temperature were recorded during the afternoon and evening in addition to the aforementioned reading. Total water hardness, free chlorine, total chlorine and alkalinity were measured periodically using AquaChek 5-in-1 Water Quality Test Strips (Hach Company, Loveland, CO).

Water Chemistry Analysis

Water samples were collected from the mixing tank outflow on days 5, 10, 15, and 20 of exposure. On the day of dissection, two 120 mL water samples were taken from each treatment. Water samples were stored at -20°C. Aqueous estrone concentrations from experiment 1 were determined using LCMS as described in Shappell et al., 2010. A DetectX Estrone Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI) was used to measure estrone water concentrations from experiment 2.

Samples and standards were added to clear, 96-well microtiter plates coated with an antibody to capture rabbit antibodies. The addition of a polyclonal estrone antibody initiates binding of an estrone-peroxidase conjugate. After an incubation the plate is washed and a substrate is added, and reacts with the bound estrone-peroxidase conjugate. This induces color generation that was measured using a Multiskan EX spectrophotometer (Thermo Scientific, Waltham, MA) at 450 nm. Samples and standards (7-point curve) were run in duplicate to ensure consistency.

Plasma Vitellogenin Analysis

Blood was obtained from the severed caudal vasculature using a heparinized capillary tube after fish were anesthetized in 0.1% MS-222 (Argent Laboratories, Redmond, WA). Plasma was obtained following blood centrifugation at 8050 x *g* for 8 min at 4°C and stored at -80°C prior to analysis. Plasma vitellogenin concentrations (µg/mL) were quantified via competitive antibody-capture ELISA using and 8 point serial dilution standard (4.8 µg/mL to 0.0375 µg/mL). Detailed methods of the assay are described in Shappell et al. (2010).

Blood Glucose Analysis

Using 1 µL of blood, a TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT) was used to quantify blood glucose concentration (mg/dL).

Hematocrit Analysis

Blood was taken via the caudal artery using 75mm heparinized capillary tubes (ClearCRIT Plastic Capillary Tubes, Separation Technology Inc., Sanford, FL).

Capillary tubes were sealed (Critoseal, Oxford Labware, St. Louis, MO) and placed in a microhematocrit centrifuge (HERMLE Z200A, Labnet International Inc., Woodbridge, NJ) for 3 minutes at 968 x *g*. Hematocrit was measured using a Spiracrit Micro-Hematocrit Tube Reader (Clay-Adams Inc., New York, NY).

Organosomatic Indices

Prior to dissection, individual fish were weighed (0.01 g precision, Taylor Stainless Steel Food Scale, Taylor Precision Products, Oak Brook, IL) and measured for length to calculate body condition factor (BCF = body weight/total length^3) (Fulton, 1904). To calculate hepatosomatic index (HSI = liver weight/whole body weight x 100) and gonadosomatic index (GSI = gonad weight/whole body weight x 100), liver and gonads were excised and immediately weighed (0.001 g precision, Mettler Toledo AG245, Columbus, OH).

Secondary Sex Characteristics

Prior to dissection, an assessment of the male sexual characteristics of individual fish was performed. Tubercles, dorsal pad and banded coloration were given a separate rating of 1, 2 or 3 based on prominence (1 being the least prominent, 3 being the most prominent) (Smith, 1978). The three values were added together as a method for comparing morphological sexual maturity between treatments.

Histopathology

Liver and gonads were excised from fish and placed in histological cassettes (Tissue-Loc Histoscreen Cassettes, Thermo Scientific, Kalamazoo, MI) in 10%

buffered formalin for at least 24 hours. Dehydration and tissue preparation was achieved following previously prescribed procedures (Carson, 1997). Tissues were embedded in paraffin. Small samples (~1 mm diameter) of liver and gonad were sectioned at approximately 5 µm (Olympus Cut 4055 Microtome, Olympus America Inc., Center Valley, PA). Tissues were stained using a standard haematoxylin and eosin counter stain (Gabe, 1976; Carson, 1997).

Liver tissues were graded on a scale of 1 to 4 based on cellular vacuolization. A grade of 1 indicates that no vacuoles are visible under a 40x magnification, a grade of 2 indicates that less than 25% of the visible tissue is occupied by vacuoles, a grade of 3 indicates that 25-50% of the visible tissue is occupied by vacuoles, and a grade of 4 indicates that the majority of the visible tissue consists of vacuoles.

Testes were assessed based on the proportion of sperm cells (spermatogonia, spermatocytes, spermatids, and spermatozoa) present in the visible tissue. An experienced grader assigned cell percentage values (totaling 100%) to the samples. Furthermore, to represent the overall maturity of the sample, we used:

$$\text{Gonad maturity rating} = ((\%_{\text{spermatogonia}}) + (\%_{\text{spermatocytes}} \times 2) + (\%_{\text{spermatids}} \times 3) + (\%_{\text{spermatozoa}} \times 4)) / 100$$

Statistical Analysis

All data was assessed for normality using the Kolmogorov-Smirnov using Graphpad Prism software (Prism 5.0 statistical package, GraphPad Software, Inc., Oxnard, CA). Data were then analyzed using a two-way ANOVA followed by a Bonferroni post-test. Additionally, some data were re-interpreted using a one-way

ANOVA followed by a Tukey post-test.

RESULTS

Chemical Data and Survival Rates

The average measured aqueous estrone concentrations were 51 ng/L (low estrone) and 390 ng/L (high estrone) in experiment 1, and 13 ng/L (low estrone) and 282 ng/L (high estrone) in experiment 2. Dissolved oxygen averaged 1.5±0.34 mg/L (low dissolved oxygen) and 9.4±0.41 mg/L (high dissolved oxygen) in experiment 1 (Fig. 4.1a) and 1.8±0.38 mg/L (low dissolved oxygen) and 8.9±0.55 mg/L (high dissolved oxygen) in experiment 2 (Fig. 4.1b). Dissolved oxygen was not recorded from days 17-20 in experiment 1 due to equipment malfunction. Survival was lowest in the high estrone, low dissolved oxygen treatment in both experiments (75% in experiment 1, 80% in experiment 2) (Table 4.1).

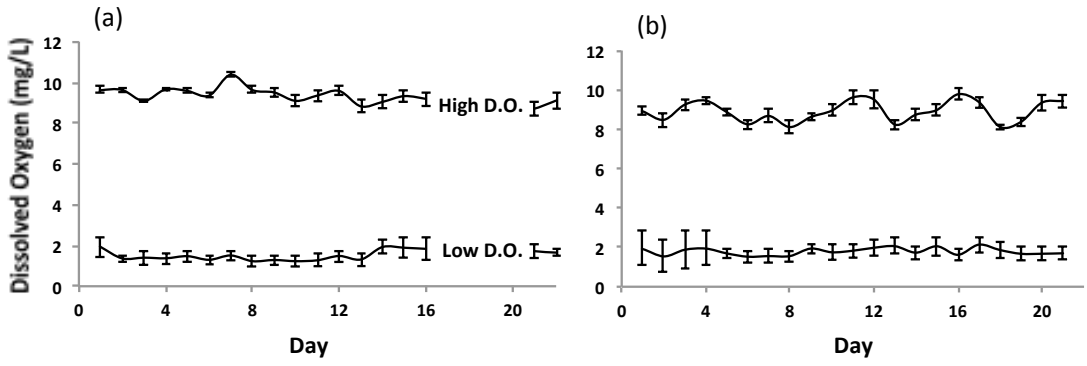

Figure 4.1. Measured dissolved oxygen values for experiment 1 (a) and experiment 2 (b); bars represent standard deviation.

Table 4.1. Percent survival and number of survivors (survived full exposure/total fish at beginning of experiment) within individual treatments in experiment 1 and experiment 2.

		Low D.O.	High D.O.
Experiment 1	**EtOH**	80% (16/20)	90% (18/20)
	51 ng/L estrone	95% (19/20)	100% (20/20)
	390 ng/L estrone	75% (15/20)	95% (19/20)
Experiment 2	**EtOH**	90% (18/20)	100% (20/20)
	13 ng/L estrone	85% (17/20)	100% (20/20)
	282 ng/L estrone	80% (16/20)	90% (18/20)

Physiological Endpoints

In both experiments, plasma vitellogenin concentrations were significantly elevated over control in all estrone-treated groups. Plasma vitellogenin concentrations did not differ significantly between high and low estrone treatments in either experiment. In both experiments, the low estrone, low dissolved oxygen treatment had significantly higher plasma vitellogenin concentrations than low estrone, high dissolved oxygen treatment (Fig. 4.2, Two-Way ANOVA followed by Bonferroni Post-Test, $p<0.05$).

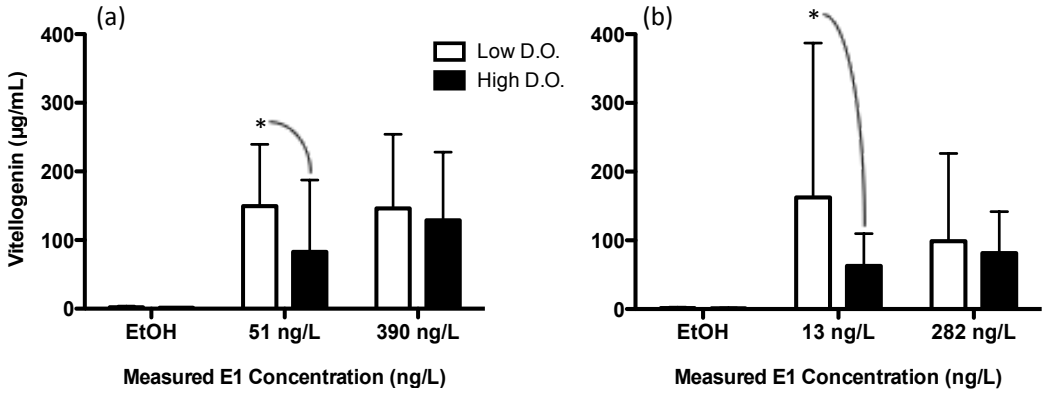

Figure 4.2. Plasma vitellogenin concentrations of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); bars indicate standard deviation; * indicates significance at $p<0.05$; Two-Way ANOVA followed by Bonferroni post-test.

Blood glucose concentration was not significantly impacted by dissolved oxygen in either experiment (Fig. 4.3). In experiment 1, blood glucose concentrations of low estrone-treated *P. promelas* were significantly lower than the control (One-Way ANOVA with estrone treatments compared independent of dissolved oxygen, followed by Tukey post-test, $p<0.05$; Fig. 4.4), however, these results were not replicated in experiment 2.

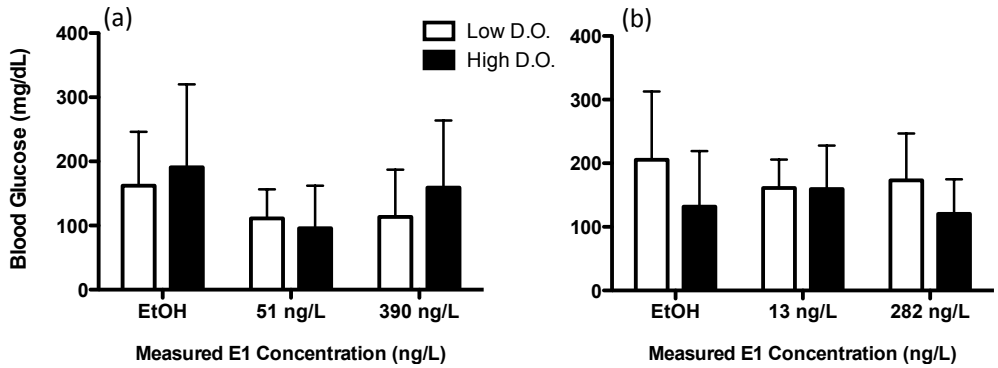

Figure 4.3. Blood glucose concentrations of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); bars indicate standard deviation.

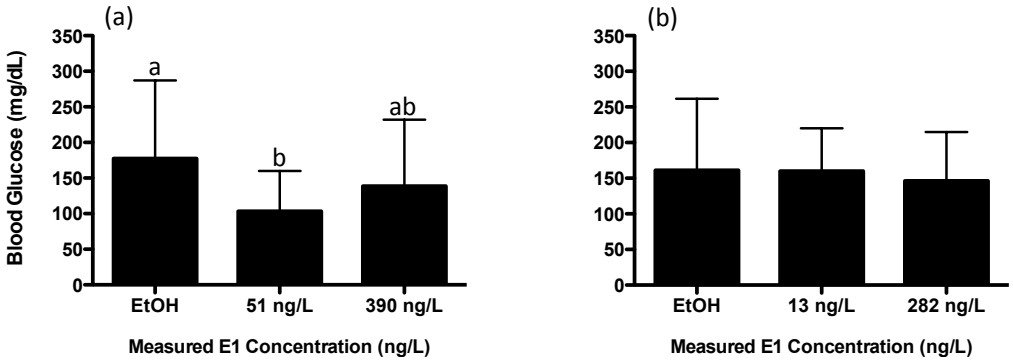

Figure 4.4. Blood glucose concentrations of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); letters above standard deviation bars indicate significant differences at $p<0.05$; One-Way ANOVA with estrone treatments compared independent of dissolved oxygen, followed by Tukey post-test.

Hematocrit percentage was not significantly affected by dissolved oxygen in either experiment (Fig. 4.5). Estrone was responsible for all observed variation in hematocrit percentage. In both experiments, high estrone-treated fish showed the lowest hematocrit percentage (Fig. 4.6). In experiment one, the hematocrit percentage

of high estrone-treated (390 ng/L) fish were only significantly lower than the low estrone-treated fish. In experiment two, however, high estrone-treated (282 ng/L) fish had significantly lower hematocrit than both control and low estrone-treated (13 ng/L) fish.

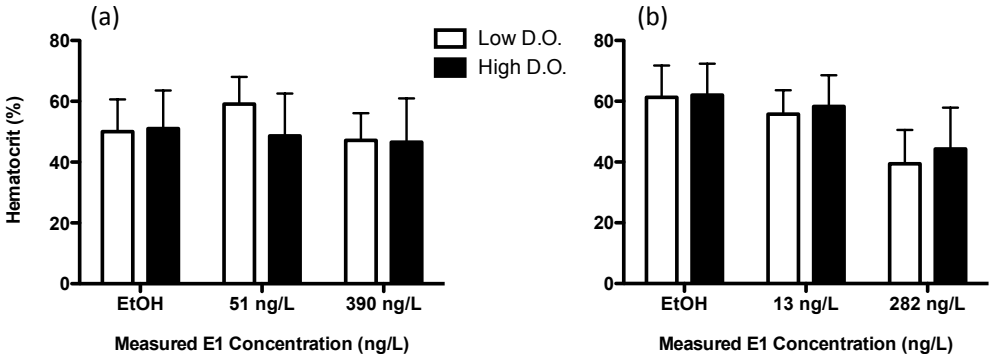

Figure 4.5. Hematocrit of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); bars indicate standard deviation.

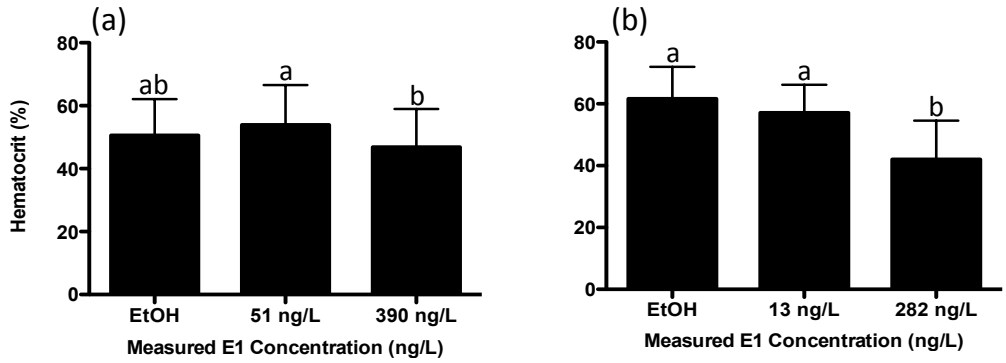

Figure 4.6. Hematocrit of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); letters above standard deviation bars indicate significant differences at $p<0.05$; One-Way ANOVA with estrone treatments compared independent of dissolved oxygen, followed by Tukey post-test.

Morphological Endpoints

Body condition factor, gonadosomatic index, and hepatosomatic index were not affected by dissolved oxygen (Fig. 4.7, 4.8, 4.10). In experiment 1, control fish had significantly elevated body condition factor over low estrone-treated fish, however no such relationship was observed in experiment 2. Gonadosomatic index was not significantly variable between any treatments except for in the case of the high estrone treatment (282 ng/L) in experiment 2, which was significantly lower than both control and low estrone-treated (13 ng/L) fish (Fig. 4.9). In experiment one, hepatosomatic index in high estrone-treated fish was significantly lower than both control and low estrone-treated fish. Hepatosomatic index did not differ from control in either of the estrone treatments in experiment 2.

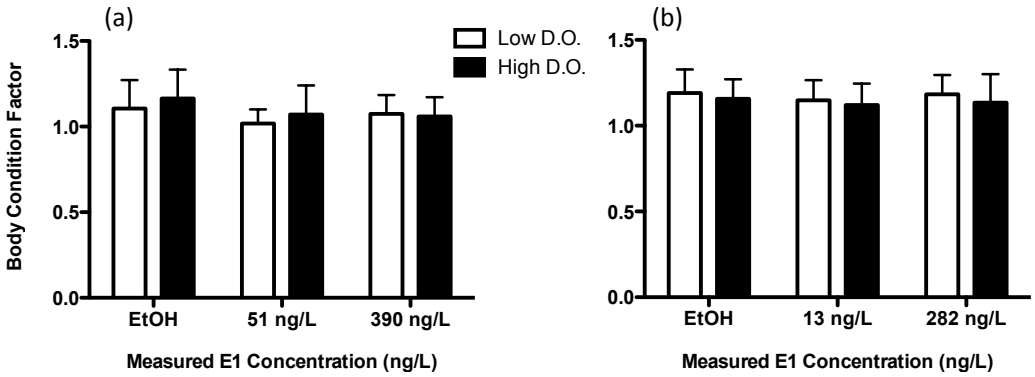

Figure 4.7. Body condition factor of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b).

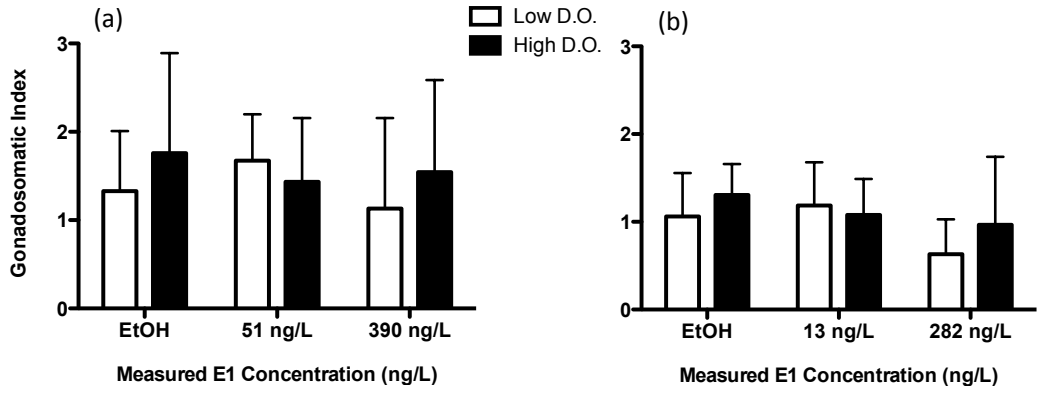

Figure 4.8. Gonadosomatic index of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); bars indicate standard deviation.

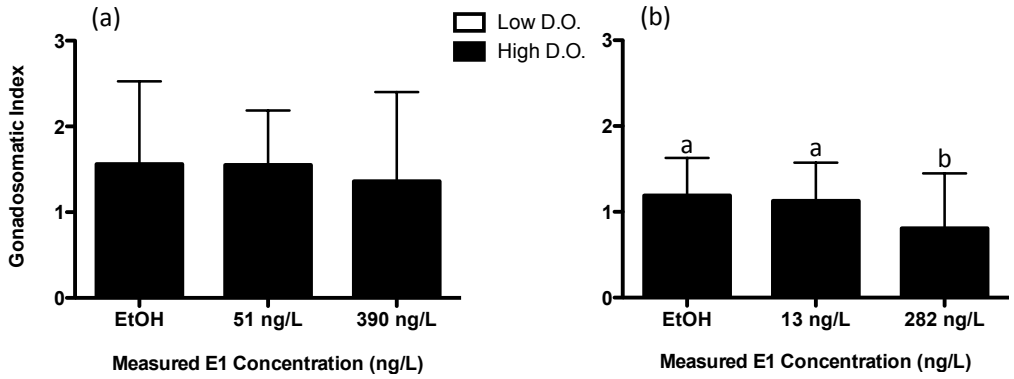

Figure 4.9. Hematocrit of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); letters above standard deviation bars indicate significant differences at $p<0.05$; One-Way ANOVA with estrone treatments compared independent of dissolved oxygen, followed by Tukey post-test.

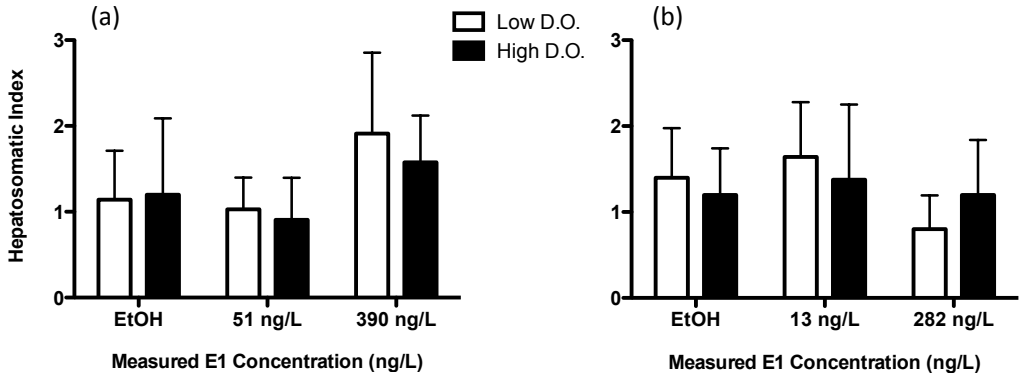

Figure 4.10. Hepatosomatic index of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); bars indicate standard deviation.

In experiment 2, no significant effects on secondary sex characteristics were observed as a result of either dissolved oxygen or estrone. In experiment 1, the sum of secondary sex characteristics was dissolved oxygen-dependent in fish exposed to 51 ng/L. Low estrone-treated fish (51 ng/L) exposed at low dissolved oxygen had significantly lower secondary sex characteristics sums than low estrone-treated fish exposed at high dissolved oxygen (Fig. 4.11).

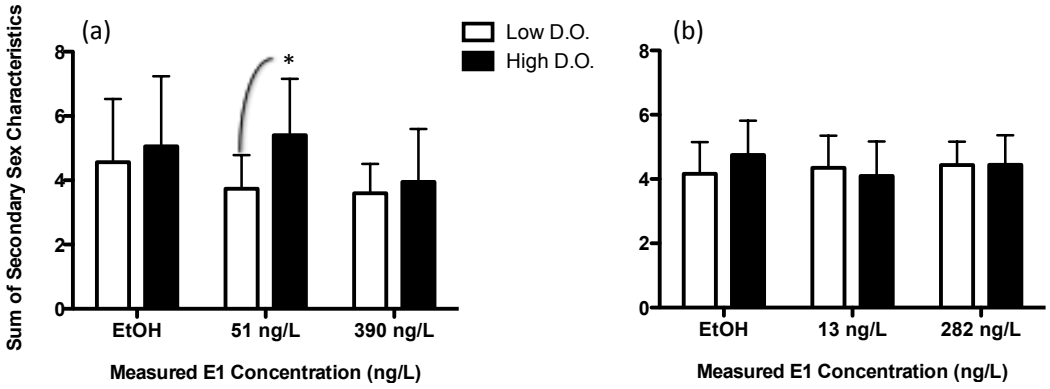

Figure 4.11. Sum of secondary sex characteristics of male *P. promelas* after 21 days of exposure to estrone during experiment 1 (a) and experiment 2 (b); bars indicate standard deviation; * indicates significance at $p<0.05$; Two-Way ANOVA followed by Bonferroni post-test.

DISCUSSION

This study was a series of two experiments designed to determine whether dissolved oxygen would alter physiological and morphological endpoints measured to quantify the effects of estrone, a known endocrine-disrupting chemical in fish (Panter et al., 1998; Van den Belt et al., 2004; Dammann et al., 2011). Estrone exposure caused vitellogenin induction in all treatments. There was no statistical difference between high and low treatments. This is surprising due to the disparity between measured concentrations (approximately a four-fold difference in experiment 1 and a 22-fold difference in experiment 2). Previous, unpublished data (Chapter 2, 3) has indicated that estrone concentrations as high as 16 ng/L had no significant effect in inducing vitellogenin concentrations over control fish. Therefore, we estimate that the ELISA chemical data from the second exposure is inaccurate and requires further

examination using methods that were utilized in experiment one (LCMS) as described in Shappell et al., 2010.

Of particular interest is the disparity in vitellogenin concentrations between the high and low dissolved oxygen treatments in low estrone-treated fish from both experiments. Changes in gill physiology and ventilation occur to facilitate oxygen uptake (Blewett et al., 2012). Increases in gill surface area have been documented in Cyprinidae exposed to hypoxic conditions (Evans, 1987). In a low oxygen environment, opercular movements may increase to keep oxygen uptake constant (Roberts, 2012). An increase in flow rate of estrone-laced water past the gills is the likely cause of increased uptake. Observation during the exposure indicated that fish in low dissolved oxygen treatments were respiring more rapidly, however this could not be quantified. In addition to modified respiration, it was noted that fish in low dissolved oxygen treatments tend to congregate near the inflow, as opposed to high dissolved oxygen treatments in which fish were much more mobile. This behavior may have additional implications, such as the possibility of ecological traps: in this case, point source releases that attract organisms (Kristan, 2003). Effluent sources, where pollutant concentration is highest, may attract fishes due to heat and oxygen gradients (Spigarelli et al., 1982). In a low oxygen environment, fish may congregate near surface-agitated water to increase oxygen consumption. If the source of the surface agitation is a wastewater discharge, for example, where pollutants enter the system in the least diluted state.

The difference in vitellogenin induction between low and high dissolved oxygen that was present in the low estrone treatments of both experiments was not mirrored by the high estrone treatments of either experiment. It is likely that at these concentrations (390 ng/L in experiment 1 and 282 ng/L in experiment 2) the dose response is maximized regardless of dissolved oxygen's effect on uptake of estrone.

While blood glucose concentrations did not appear to have any meaningful significant trends in this data set, it is a worthy endpoint for future studies. The simplicity of blood glucose testing alone makes it worthwhile, though it does have pitfalls. As a short-term analysis of metabolic stress, blood glucose may be skewed by the stress of moving the fish prior to dissection in laboratory exposures such as this one (David and Parker, 1990).

The average percentage of blood cell volume in high estrone-treated (282 ng/L) fish was significantly lower than both control and low estrone-treated (13 ng/L) fish in experiment 2. At first glance, one may attribute this difference to nutritional deficit due to a high rate of vitellogenesis in estrone treated fish. The vitellogenin data, however, does not fully corroborate this assumption. While not significantly different ($p \geq 0.05$), the average plasma vitellogenin concentration in low estrone-treated fish was in fact greater (113 µg/L) than that of the average high estrone-treated fish (90 µg/L) in experiment 2. It is also worth noting that this phenomenon (decreased hematocrit) does not occur in experiment one, however, the estrone concentrations in experiment 2 are likely higher than what was indicated by the ELISA (the ELISA indicates that the low estrone treatment averaged 13 ng/L, a concentration that is not

likely to induce vitellogenesis per data from chapter 2 and 3). This data, therefore, suggests that at high doses, however, estrone may have physiological ramifications other than vitellogenin induction, which place the organism under further metabolic stress. Renal toxicity due to very high doses of estradiol has been observed in male summer flounder (*Paralichthys dentatus*) (Folmar et al., 2001). It is possible that kidney damage occurred in the 282 ng estrone/L treatment (experiment 2), and resulted in reduced production of erythropoietin production in the kidneys, in turn reducing red blood cell production (American Society of Hematology, 2015).

While dissolved oxygen had no effect on the morphological endpoints measured in this study, the variation caused by estrone is worth examination. Gonadosomatic index in the highest concentration of estrone tested (282 ng/L) was significantly lower than both the control and low estrone treatment in the same experiment (2). Vitellogenin concentrations, interestingly, were the lowest, on average, in the treatment group with the highest estrone concentration (Fig. 4.4), further reinforcing the thought that vitellogenin may not provide a comprehensive quantification of estrone's effects at high doses.

Variation in the sum of secondary sex characteristics in low estrone-treated fish in experiment 1 raises questions. Low estrone-treated fish exposed at low dissolved oxygen had significantly lower secondary sex characteristics sums than low estrone-treated fish exposed at high dissolved oxygen (Fig. 4.11). This data is consistent with significant variations in vitellogenin concentrations in the same treatments in experiment 1, with low estrone-treated, low dissolved oxygen fish

displaying higher plasma vitellogenin concentrations than low estrone-treated, high dissolved oxygen fish. This may suggest a correlation between morphologically dominant males and reduced vitellogenin expression. This relationship is not mirrored in the second experiment, however, where the difference in vitellogenin induction between low and high dissolved oxygen is the greatest (Fig. 4.2b). Still this potential relationship is cause for further examination of the correlation between secondary sex characteristics and vitellogenin induction.

This study provides some indication that laboratory chemical toxicity tests should be performed under various dissolved oxygen concentrations to account for potential changes in uptake due to gill ventilation changes (Blewett et al., 2012). With a warming climate, other parameters are sure to change, and the "norm" of today may no longer be so tomorrow. Further testing is necessary to determine how the interaction between aquatic contaminants and water parameters such as temperature, salinity and pH affect the endpoints we measure to determine toxicity. Additionally, effort should be made to examine interactions between different pollutants, since they rarely, if ever are found individually (Kolpin et al., 2002). Studies performed under a range of chemical concentrations could be used to create a system for setting water quality standards based on the properties of individuals systems. This would reduce or potentially eliminate the possibility of further damaging sensitive ecosystems due to laboratory toxicity testing that is unrepresentative of the environment it is intended to represent.

CHAPTER V

CONCLUSION

Key Findings

The preceding research sought to determine whether environmental variables would alter the observed biological effects of estrone in adult male fathead minnows (*Pimephales promelas*). A series of laboratory exposures were conducted, in replicate, to determine if temperature and diet (TEFE), salinity (SAL), and dissolved oxygen (DO) would significantly alter physiological and morphological effects. The cumulative findings suggest that all of the parameters, aside from salinity, do significantly alter biological measurements. These data emphasize the importance of considering physical and chemical environmental parameters when assessing the effects of exogenous estrone on male *Pimephales promelas*.

The effects of estrone on vitellogenin induction in male *Pimephales promelas* are apparent in every exposure. In the TEFE and SAL studies, aqueous concentrations of 16 ng estrone/L and lower had no significant effect in elevating plasma vitellogenin concentrations. Different detection methods were used to quantify aqueous estrone concentrations in the DO study, and elevations in plasma vitellogenin concentrations were observed at concentrations below 13 ng estrone/L. It is possible that a sampling

or assay protocol error lead to a misreading of aqueous concentrations. If this was indeed the case, then the data suggest that at environmental estrone concentrations below 16 ng/L (SAL study), plasma vitellogenin quantification may not be a sufficiently sensitive endpoint to assess the biological effect of estrogenic chemicals. Significant interactions between estrone, temperature, and feeding regimen (TEFE study) at aqueous an concentration of 78 ng estrone/L suggest that low temperatures (18°C), in combination with diet restriction (0.75% body weight/day), may exacerbate the induction of vitellogenesis in male *Pimephales promelas* due to increased cortisol production and optimal breeding temperatures occurring near 18°C (Prather, 1957; Danylchuk and Tonn, 2001; Smith, 1978). Furthermore, evidence from the same study suggest that high temperatures (26°C), in combination with diet restriction may suppress vitellogenesis, probably as a result of forgone reproductive function in order to sustain survival at a high metabolic rate under diet restrictions (Luquet and Watanabe, 1986; Izquierdo et al., 2001). Differences in chloride concentrations in the SAL study were not sufficient to modulate estrogenic response. The chloride concentrations tested, however, fall well below the United States Environmental Protection agency's chronic water quality criteria threshold of 230 ppm (U.S. Environmental Protection Agency, 1988). This subject should be revisited, especially in light of recent evidence that chloride often persists at aqueous concentrations much higher that those tested in this study (Corsi et al., 2015), and the determination that base chloride levels were already relatively high in the control water source (93 ppm). If the SAL study were run again, it would be advisable to bring the total chloride

concentration up to at least 230 ppm and perhaps approach the chronic toxicity threshold for *Pimephales promelas* (252 ppm) in order to see how the organism responds to estrone when chloride-induced osmoregulatory stress is induced to a higher degree (Adelman et al., 1976; Evans et al., 1999; Siegel, 2007). Increased vitellogenin induction was observed at low dissolved oxygen concentrations and low estrone concentrations in the DO study. It is predicted that this modulation in response is due to physiological and morphological respiratory changes that occur as a result of hypoxia (Evans, 1987; Blewett et al., 2012). It is also important to note that the modulation of vitellogenin induction due to temperature changes and feeding regimen changes were only observed in the first experiment, at 78 ng estrone/L, and were not observed in the second experiment, when the concentration was much higher (135 ng estrone/L). A similar effect was evident in the DO study, in which modulation of vitellogenin induction via a difference in dissolved oxygen was only observed at low estrone concentrations. These data suggest that, at high enough estrone concentrations, the modulation of estrogenic effects, through environmental variation, is overwhelmed by the effect of estrone.

Hematological parameters provided some insight into metabolic function and possibly indirect effects of estrone exposure. Blood glucose was found to be elevated in all fish exposed at 18°C and fed an *ad libitum* diet in experiment 2 of the TEFE study. The same trend is not observed in the first TEFE experiment, however this effect can be explained by a high carbohydrate bioavailability, but reduced cellular uptake due to changes in insulin function at low temperatures (Freychet et al., 1971;

Larsen et al., 2001). While hematocrit did not show consistent trends throughout all three studies, in the DO study, there is some evidence to suggest that renal toxicity (Folmar et al., 2001) may lead to reduction in hormonal stimulation of red blood cell production (American Society of Hematology, 2015).

The effects of temperature were again evident upon examination of sex-based morphological endpoints. In the TEFE study, gonadosomatic index was significantly elevated in the 18°C in both experiments. Data from the second experiment indicate that sum of secondary sex characteristics and gonad maturity to follow the same trend – an increase in sexual maturity at lower temperatures. A review of the literature (Denton and Yousef, 1975; Smith, 1978) suggests that gonad histology and reproductive function peak early in the spawning season, when temperatures warm above 15.6°C. As temperatures increase, the percent of mature sperm tapers off until fall (Smith, 1978). Vitellogenin data also indicate a possible deceasing trend that may be seasonal in nature. Within studies, we see a decrease in average vitellogenin in the high estrone treatment from experiment 1 to experiment 2. This is evident in all three studies, but the means only differ significantly within the TEFE study and SAL study (t-test, $p<0.05$).

Future Directions

When conducting whole-organism exposures, there is an innate level of uncertainty that one must contend with. Disease, genetics, or poor handling of the organisms can influence the perceived outcome of the study in ways that cannot be predicted. What's more, there is much ado about conducting "clean" experiments. In

truth, there can be no such thing as a clean, whole-organism experiment, at least when using model species without an identical genetic profile. It is for this reason that we propose that the utmost level of transparency be undertaken when reporting results of toxicology tests. To do so, we should embrace the inevitable variability in out experiments and record all possible parameters. In this way, we can more effectively interpret results in the context of specific exposure scenarios. If taken a step further, and by intentionally varying the parameters, one can generate results that apply to multiple scenarios. This is arguably a much more useful approach to toxicity testing. Ideally, tests conducted at intervals of various combinations of parameters would be used for regulatory determination. By doing so, it is possible to generate a database of toxicity values for a given toxin based on environmental parameters. To determine the chronic toxicity threshold for a given microhabitat, one could simply refer to the conditions that correspond to monitoring data from that location and look up the value. These values could then be used to set pollution limits to be enforced in specific areas.

Results from the preceding studies also indicate a need for baseline data on the model organism being used. For example, the potential for seasonal variability in the reproductive capacity of *Pimephales promelas* in the laboratory is largely unknown. Smith (1978) discusses seasonal changes in gonad histology as a function of temperature, however, our results suggest that *Pimephales promelas* may retain some of this seasonal variation in the laboratory regardless of temperature.

Additionally, taking some baseline data on the organisms prior to exposure may prove to be useful when trying to interpret certain endpoints. While some

measurements could be tedious and possibly stressful to the fish, analysis of secondary sex characteristics prior to exposure, for example, would be easily and quickly accomplished with very little handling of the specimens. A change in the average sum of secondary sex characteristics could then be assessed. As fish may already be morphologically dominant or subordinate prior to being placed in the exposure, a single reading at the end may be deceptive. It may also be useful to examine the relationship between individual variation and pathological responses to chemicals. For example, it should be assessed whether males with more masculine features (high gonadosomatic index, prominent secondary sex characteristics, circulating androgen levels, etc.) respond differently to estrogenic chemicals when compared with less masculine individuals. If a differing intensity of response was established quantitatively, it could be used to standardize the interpretation of observed biological effects and create a more accurate overall pathological assessment.

The results of the three studies undertaken for this thesis project are sufficient to justify further examination of the complex interaction between contaminants, such as estrone, and environmental variables. This examination should consist of observations of a pollutant's biological effects across a wide range of chemical and physical parameters that represent the distribution of the organism. Using this information, we can generate a regulatory framework that better protects specific microhabitats from the toxic effects of endocrine disrupting chemicals and other environmental pollutants.

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