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Effects of Estrone and Temperature on the Predator-Prey Relationship Between Bluegill Sunfish and Fathead Minnows

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Effects of Estrone and Temperature on the Predator-Prey Relationship Between Bluegill Sunfish and Fathead Minnows

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Abstract

Contaminants of emerging concern (CECs), for example estrone (E1), and their effects to aquatic organisms have been researched consistently, however little is known about how temperature can influence exposure effects of CECs. Chemical exposures and temperature have been shown to independently affect fish and their populations, but little is known about their impact on predator-prey relationships. Previous studies indicate that changes arise through behavioral and physiological changes in either predator or prey. To test the effects of E1 and temperature on predator-prey relationships, adult bluegills and larval fathead minnows were exposed to E1 (125, 625 ng/L) or an ethanol control for 30 days at four temperatures (15°C, 18°C, 21°C, 24°C) to reflect natural variation between seasons. Behavioral experiments were conducted to assess the effects of temperature and estrone exposure on minnows in the larval life stage (i.e., foraging and predator evasion). Significant differences due to water temperature were observed in body length, escape angle, and total escape response of predator evasion responses. On day 30 of exposure, predation trials were performed using one adult sunfish and a group of five control and five exposed (125 or 625ng/L) larvae. Exposed larvae (125ng/L: 49.2%; 625ng/L: 52.9%) displayed a concentration-dependent reduction in survival in comparison to the control minnows (74.2%) following predation from the sunfish predator. Additionally, the prey catching abilities of the sunfish may have been affected, potentially mitigating the predation effects on the minnows. In addition, a series of anatomical and physiological endpoints were explored to assess the independent and interactive effects of estrone exposure and temperature on plasma vitellogenin induction, blood glucose, histology, and morphometric indices on sunfish in the adult life stage. This study provides evidence that minnow populations may suffer due to impaired predator evasion performance and provides information for environmental agencies evaluating ecological effects of exogenous estrogens and climate change.

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Chapter 1: Literature Review

1.1 Introduction

Anthropogenic stress on the environment has become a large concern among scientists in recent years. Pollution release into the environment is one source of environmental stress that receives much attention and research focus. Specifically, concerns have emerged regarding how pharmaceutical, agricultural, industrial, and natural compounds may impact the environment and the organisms exposed as a result of environmental contamination. Many of the compounds that fall into the aforementioned groups have been categorized as contaminants of emerging concern (CECs). CECs are an extensive group of chemicals and compounds that have been recently encountered in the environment and/or for which little information has been gathered. These compounds have been documented to have the potential to cause adverse health effects and ecological impairment (Colborn, 1998; U.S. EPA, 2015).

Unfortunately, assessing the effects CECs have on an individual, population, or community in the environment is complicated due to the effects temperature can have on the rates at which CECs degrade and the rates at which aquatic organisms take up these compounds. Due to this relationship with temperature, the effects CECs may have on aquatic organisms is likely varied throughout seasons. Regrettably, very few toxicological experimental protocols consider this temperature dependency (Jin et al., 2009). Therefore, there is a lack of understanding in how ambient temperature can modify the overall effects of CECs on the behavior and physiology of fish. Such exposures could potentially threaten the overall population of many aquatic organisms by affecting metabolism and/or reproduction (Smith, 1978; Brown et al., 1987; Miller et al., 2007; Elliot et al., 2014). To address the lack of information in this area, the objective of this research is to examine how ambient water temperature modifies the effects of estrone (E1), an environmentally relative estrogenic CEC, on the predator-prey relationship between bluegill sunfish (*Lepomis macrochirus*) and fathead minnows (*Pimephales promelas*).

1.2 Endocrine Disruption in Aquatic Environments

Endocrine active compounds (EACs) are a group of distinct CECs that are capable of endocrine disruption by way of two major pathways: 1. EACs act as antagonists to endogenous hormones by binding to receptors, thus preventing normal binding activity and the following responses; 2. EACs act as agonists to endogenous hormones by promoting activity through mimicry (Jobling et al., 1998; Guillette and Gunderson, 2001; Pait and Nelson, 2002; Sumpter and Johnson, 2005). EACs can be found in most chemical classes including pesticides, steroid hormones, compounds used to produce plastics and other consumer products, industrial by-products and pollutants, and organic pollutants (Metzler & Pfeiffer, 2001; Pait and Nelson, 2002). This diversity of compounds enters aquatic environments through a variety of point and nonpoint sources.

Treated effluent from wastewater treatment plants typically contains an assortment of chemicals that have the potential to disturb normal endocrine functions in many aquatic organisms. Many of these chemicals are EACs that are considered estrogenic in nature (Routledge et al., 1998; Kolpin et al., 2002). In fact, the most frequently studied EACs are estrogenic compounds (Pinto et al., 2014). Municipal wastewater treatment facilities display mixtures that contain both natural estrogens, such as estrone (E1), 17-β estradiol (E2), estriol (E3), and synthetic estrogens such as ethynylestradiol (EE2). E1, E2, E3, and the synthetic EE2 have all been connected to vitellogenin biosynthesis in male fish (Sumpter and Jobling, 1995; Routledge et al., 1998; Larsson et al., 1999). While E1 and E2 are naturally excreted through human wastes, oral contraceptives and other various pharmaceutical products are responsible for most of the EE2 found within wastewater treatment plant effluent (Wise et al., 2011). These estrogenic compounds are in fact so common they have been found in many streams and rivers across the U.S and many other countries (Ternes et al., 1999; Kolpin et al., 2002; Murk et al., 2002; Servos et al., 2005; Lishman et al., 2006). Although the half-lives of these compounds are relatively short (E1: 19 hours, E2: 8-36 hours, E3: 5 hours, EE2: 10-36 hours) (Wentz, 1988), meaning they are rapidly degraded in the

environment, these compounds are continuously released into the aquatic environment through wastewater effluent. This continuous release brings forth a scenario in which the half-lives of these compounds are surpassed by their introduction rates into the environment (Daughton, 2002). While EACs typically aren't acutely toxic at the concentrations present in the environment, exposure at these concentrations has demonstrated sub-lethal effects through impairment of normal functions and growth in exposed organisms.

Exposure to these estrogenic compounds during larval development or sexual maturity has been shown to have adverse effects in fish at molecular, physiological and behavioral levels. Common effects related to estrogen exposure in juveniles includes promoting abnormal tissue development (Gimeno et al., 1996), changes in behavior (McGee et al., 2009), and sexual differentiation (Panter et al., 2002; Nash et al., 2004). Regarding adult fish, common effects include development of ovo-testis or intersex fish (Wanga et al., 2008), vitellogenin (vtg) production in males (Panter et al., 1998; Dammann et al., 2011), reduced quality of semen (Bjerselius et al., 2001), and repressed somatic and gonadal growth (Bjerselius et al., 2001; Dammann et al., 2011). These changes, among others, in reproduction, growth, and behavior could have lasting impacts on the survival of the population, ultimately leading to a decline (Brown et al., 1987; Miller et al., 2007; Elliot et al., 2014).

During a study in an experimental lake, the population of fathead minnows was chronically exposed to low levels of EE2, which is hypothesized to be responsible for the resulting crash of the population (Kidd et al., 2007). This outcome proposes that the individual-level effects of chronic exposure to compounds that are estrogenic in nature can cause drastic shifts in population levels, which has the potential to affect whole aquatic ecosystems. Another study that used red shiners, also known as red-horse minnows, found exposure to E2 lead to various negative reproductive side effects, ultimately ending with reduced reproductive success with no viable progeny produced (McGree et al., 2010). Given

effects like this on reproductive success, complete population collapses of small prey species could occur, leading to negative effects on the organisms that rely on their presence to survive.

1.3 Estrogens and Their Mechanisms of Action

Estrogens, a class of steroid hormones originally derived from cholesterol, can be naturally secreted by the body or synthetically produced. Female vertebrates are capable of producing E1, E2, and E3, which regulate many of the processes involved with female reproduction as well as sexual maturation (Norris, 2007). These estrogens, which function similarly in fish, regulate sexual differentiation and reproductive functions. When the development of the follicle occurs in fish, the hypothalamus secretes gonadotropin-releasing hormone via direct innervation with the pituitary gland. This action stimulates the secretion of luteinizing hormone and follicular-stimulating hormone from the pituitary gland (Hadley and Levine, 2006). Luteinizing hormone stimulates the release of androgens from ovarian thecal cells (Hadley and Levine, 2006). These androgens disperse to granulosa cells located within the ovarian tissue (Hadley and Levine, 2006). Follicle-stimulating hormone promotes the production of aromatase in the ovary, which acts to convert androgens into estrogens (Hadley and Levine, 2006). These estrogens target two subtypes of nuclear estrogen receptors; estrogen receptor α and estrogen receptor β. The liver and gonads, as well as other various tissues, contain estrogen receptors α, while estrogen receptors β are more limited to specific tissues such as liver, brain, and bladder (Socorro et al., 2000). The estrogen 17β-estradiol acts upon estrogen receptors located in the liver by binding to the receptor, thus causing the activation and recruitment of co-activator proteins. These co-activator proteins bind to specific palindromic DNA sequences, thus enabling the transcription of vtg mRNA (Hoar and Randall, 1988; Sumpter and Jobling, 1995; Hadley and Levine, 2006). Externally derived estrogens can elicit a similar response through binding receptors found in the liver, thereby mimicking natural estrogens (Ankley and Johnson, 2004).

1.4 Temperature and the Effects of Estrogen Exposure on Aquatic Biota

Due to nearly constant environmental changes and the fact that some environmental factors can interact with chemical pollutants, it can be very difficult to predict exposure responses within organisms (Brian et al., 2008). Temperature is a key factor in regard to environmental influence on many aquatic species, including teleost fish (Jin et al., 2009). During a 3-year experiment, researchers found that temperature influences the spawning patterns and egg quality in Atlantic halibut (Brown et al., 2006). In addition to direct effects on embryonic duration and egg survival, temperature also typically has effects on size at hatching, developmental rate, and survival, indicating that temperature influences reproductive physiology and development in fish (Pankhurst and Munday, 2011). Fish at the larval stage are usually more sensitive than adults to environmental fluctuations, and therefore could potentially be especially vulnerable to climate change. While examining the influence of temperature on the growth and reproductive status of fathead minnows, it was revealed that females raised at the highest experimental temperature had ovarian tissue lacking germ cells and containing considerable amounts of undetermined tissue (Brian et al., 2011). Furthermore, both males and females exhibited variation in expression of secondary sexual characteristics (Brian et al., 2011). All fish, including fathead minnows and bluegill sunfish, are ectothermic. Therefore, ambient water temperature has the potential to impact fish on many biological levels, from molecular responses, such as the rate of vtg expression (Brain et. al., 2008; Körner et al., 2008), to ecological responses, such as the timing of reproduction (Gillet and Quétin, 2006).

Both temperature and exposure to environmental estrogens have been shown to influence the physiology and reproduction in fish, and other aquatic organisms, individually, but the interaction of these two factors and the resulting effects have hardly been researched (Körner et al., 2008; Jin et al., 2009). Past studies have examined the effects due to temperature stressors on vtg expression. One study measured a cortisol response in salmonids exposed to EE2 and found a positive correlation between temperature and vtg production (Körner et al., 2008). These findings indicate that temperature modifies

estrogen-induced vtg induction. Another study, that exposed fathead minnows to a mixture of estrogenic chemicals, found there was no effect of temperature on the magnitude of the vtg response after 2 weeks of exposure to the estrogenic mixture. They did, however, reveal that after being exposed for 24 hours and also for 7 days, that the response was induced more rapidly at a higher temperature (Brian et al., 2008).

1.5 The Bluegill Sunfish: A Model Predator Species

Bluegill sunfish (*Lepomis macrochirus*), from the family Centrarchidae, can grow up to 30.5 cm when mature and live in a range of aquatic habitats in North America. Bluegills, a teleost fish, can live in either deep water or very shallow water and typically move back and forth between the two depending on the time of day and the season. Bluegills can adapt to almost any type of water, excluding cold bodies of water. Typically, bluegills seek warm shallows that are still and calm, where they commonly hide among water plants and tree stumps (Casterlin and Reynolds, 1978). As omnivores, bluegills typically eat aquatic insects, invertebrates, mollusks, and small fish, such as the fathead minnow. It is known that bluegill sunfish typically select their prey based on size. When the prey species is scarce, they are eaten as they are encountered regardless of size. However, when there is an abundance of the prey species, bluegill sunfish typically tend to eat the individual that appears to be the largest (Werner and Hall, 1974; O'Brien et al., 1976). As the temperature of the water becomes colder, bluegill sunfish tend to eat less frequently and become more dormant in general. Laboratory tests have shown that bluegills don't typically feed when water temperature falls outside a range of 10 °C to 30 °C (Sternberg, 1996).

1.6 The Fathead Minnow: A Model Prey Species

Fathead minnows (*Pimephales promelas*) are a teleost fish that is commonly used for research in aquatic toxicology due to its accelerated sexual maturation, widespread availability, and ability to endure harsh and variable conditions, (Ankley and Villeneuve, 2006; Jensen et al., 2001). Belonging to the family Cyprinidae (Geiger et al., 1988), fathead minnows are small, ray-finned fish typically reaching lengths of about 2.5-7.5 cm long once mature (Nelson and Paetz, 1992). Fathead minnows can potentially

live up to 3-4 years; however, few live past 2 years in their natural habitat due to predatory pressure and additional environmental influences (Kidd et al., 2007). As habitat generalists, fathead minnows can be found in a variety of freshwater habitats across North America, where they characteristically consume algae, protozoa, and aquatic invertebrates (Zimmer et al., 2002). Although fathead minnows typically favor a gentle stream or pond, the species has a high tolerance for variable environmental conditions such as high temperatures, murky water, inconsistent pH, high salinity, and/or oxygen deficient waters (Sommer, 2011). As previously mentioned, fathead minnows are a common prey species for many other larger fish including bluegill sunfish (*Lepomis macrochirus*).

1.7 Vitellogenin: Estrogenic Exposure Biomarker

Vitellogenin (vtg), a phospholipoglycoprotein, is a precursor protein of egg yolk that is ordinarily found solely in the blood or hemolymph of females (Maitre et al., 1985). Vtg is commonly used as a biomarker of exposure to environmental estrogens, which stimulate elevated levels in males as well as females (Arukwe et al., 1999; Jones et al., 2000). Vtg is released into the bloodstream from the liver, where it is then transported to developing eggs and typically circulates at concentrations of about 10-100 mg/ml in maturing female fish (Giesy and Snyder, 1998). Production of vitellogenin is regulated by E2, which is primarily produced in the developing ovary. E2 produced in response to a surge of gonadotropin in the ovary leads to an increase in vtg synthesis in the liver with transport to the ovary following. Signals transmitted through the hypothalamic-pituitary-gonadal (HPG) axis cause fluctuations in circulating vtg concentrations, due to season, sex (Wallaert and Babin, 1994), age (Wallaert and Babin, 1994), temperature (Mackay and Lazier, 1993; Wallaert and Babin, 1994), contaminants (Ruby et al., 1986), and endogenous hormones (Lazier et al., 1996).

Many previous studies, using vtg as a biomarker, have demonstrated elevated vtg levels in response to various environmental estrogens such as E1, E2, E3, and EE2. One such study reported estrogen induced vtg synthesis in the liver of juvenile Atlantic salmon (*Salmo salar*) using

immunohistochemical analysis (Arukwe et al., 1999). Another study, which involved experimentally exposing male red shiners (*Cyprinella lutrensis*) to E2 for 83 days, observed that the exposure resulted in elevated concentrations of plasma vitellogenin, spermatogenesis changes, altered mating behavior, and overall reduced reproductive success resulting in a lack of viable progeny (McGree et al., 2010).

1.8 Biological Endpoints

Due to the nature of EACs, mortality is not an applicable endpoint as it is with many other environmental pollutants. Fish, as well other aquatic organisms, are exposed to EACs during their entire lifecycle. Similar to mammals, the hypothalamic-pituitary-gonadal (HPG) axis interacting with CECs could link analogous endpoints between taxa. Furthermore, small fish bioassays highlight reproductive and developmental significances through 3-6 month maturation and relatively short lifespan (Ankley et al., 2008).

Typically, a variety of endpoints are used for better interpretation of the response to exposure, largely due to biological variation among individuals possibly confounding findings when solitary endpoints are used. Therefore, combining elements of histology and ethology offers a well-rounded approach to best quantify exposure response.

Internally, gonadal somatic index (GSI) [(testes mass, g/fish mass, g) x 100] and hepatic somatic index (HSI) [(liver mass, g/ fish mass, g) x 100] can serve as important biomarkers (Shapell et al., 2010). GSI, as an important biomarker, can be used to estimate the reproductive condition of the test subject (Zeyl et al., 2014). On the other hand, HSI can be used to detect the hazardous effects of various environmental stressors (Sadekarpawar and Parikh, 2013). Livers of fathead minnows that were exposed to the effluent of a bleached sulfite mill were roughly two to three times larger than the livers of their control counterparts. Therefore, when the comparing the HSI between the two, individuals that were exposed exhibited higher values (Parrott et al., 2003). Yet another important index, the body condition factor (BCF) [(total mass, g)/ (total length, mm) x 100,000] can be used to gauge the relative metabolic

health of fish (Elliot et al., 2014). Additionally, a multitude of histological techniques have been employed as an effective measure for distinguishing indicators of adverse effects to cells and tissues. During examination of gonadal tissues, individuals considered as intersex have been recognized via oocyte development in biologically male fish (Blazer et al., 2007). Analysis of the liver using histological techniques can serve as an indicator of exposure by means of the development of liver vacuoles and other various liver histopathologies and abnormalities (Palace et al., 2009; Viganó et al., 2010). As a result of estrogen exposure, the livers of exposed fish have been reported to be enlarged (Herman and Kincaid, 1988). Additionally, the livers of fish that have been exposed to estrogen have been reported to have 'hole-like' lesions, accumulate amorphous eosinophilic material, have increased vacuolization (Wester and Canton, 1986), and/or have less basophilic hepatocytes (Weber et al., 2003). Simply put, male livers have been shown to resemble the liver of a vitellogenic female in which there is increased basophilia and enlarged nuclei. The accumulated amorphous eosinophilic material has been identified using electrophoresis and immunohistochemistry as masses of vitellogenin and a chorion glycoprotein, which are both produced in response to estrogenic exposure (Hamazaki et al., 1987; Van Den Belt et al., 2002).

Behavioral assays have been successfully used to assess impairment due to exposure to contaminants. Exposure to specific chemicals can induce changes in normal activities and aggression. An increase in movement and foraging activity in response to exposure to EACs can result in an increase in somatic growth (Bell, 2004). Of course, there is a tradeoff between the time spent foraging and the risk of predation (Werner and Anholt, 1993). This so-called risky behavior has the potential to lead to entire populations suffering through reduced survival to reproductive age (Scott and Sloman, 2004). Even when risky foraging behavior isn't exhibited, estrogens have been shown to have adverse effects on predator evasion responses. Generally, a delayed response to a stimulus is seen in larval fishes that have been exposed to estrogenic compounds (McGee et al., 2009). These behavioral changes have the potential to contribute to a population decline through predatory pressure.

Genetic diversity within a population is directly linked to selection pressures and their effect on reproductive success. Varying responses to environmental heterogeneity, disease, and other numerous selection pressures determine adaptive traits via survival and subsequent reproductive success. A plethora of factors impact an individual's development before reaching reproductive maturity. Factors that influence an individual's reproductive potential include genetic variation, life history, sexual selection, and various environmental conditions. In addition, biased sex ratios, embryo viability, and survival into adulthood are all potential reproductive modes to quantify impacts on populations as a whole (Larsson and Forlin, 2002; Hill and Janz, 2003; Lange et al., 2001; Segner et al., 2003). Ultimately, relating effects on an individual level to entire populations requires examination of broad endpoints.

Chapter 2: Exposure to Estrone Alters Predator-Prey Dynamics in Freshwater Fish

2.1 Introduction

A variety of contaminants are known to impair the ability of aquatic organisms to perceive, recognize, and/or appropriately respond to biotic stimuli (Fabian et al. 2007; Cripps et al. 2011). For example, male fishes exposed to environmental estrogens show reduced courtship effort and levels of aggression (Colman et al., 2009; Saaristo et al., 2010) and female fishes show corresponding changes in mate choice (Partridge et al., 2010). Similar contaminant-induces behavioral alteration can reduce the ability of exposed individuals to react to predators, either by impairing sensory systems that are important for the detection of potential threats (Scholz et al. 2000; Faucher et al. 2006) or induce changes in locomotor responses; for example, by increasing reaction time or decreasing the speed of escape (McGee et al. 2009; Painter et al. 2009; Ward et al. 2017). Exposure may also promote risky behaviors, such as a reduction in schooling or increased activity (Nakayama et al. 2005; Bell 2004; Brodin et al., 2013).

Comparatively, less is known about the effects of contaminants on ecological function or community dynamics, despite the fact that these compounds have significant potential to disrupt ecological processes (Clotfelter et al., 2004). Kidd et al. (2007, 2014) showed the collapse of a fathead minnow (*Pimephales promelas*) population in an experimental lake following chronic exposure to low concentrations of a synthetic estrogen, 17α-ethinylestradiol, was associated with both increased abundance of zooplankton and invertebrate species and a decline in the abundance of predatory lake trout (*Salvelinus namaycush*). This study highlights the importance of studying whole ecosystem effects because different species can be impacted by chemical exposures and temperature fluctuations to differing degrees. Additionally, this study highlights the importance of examining how natural, abiotic factors can influence contaminant-induced changes to not only individuals, but to species and population dynamics.

Subtle, contaminant-induced changes in the behavior of individuals can have measurable effects at population and community levels. In the context of predator-prey interactions, exposure to a variety of contaminants reduces the ability of prey to recognize predators (Scholz et al., 2000; Mandrillon and Saglio, 2007), impairs escape responses or other anti-predator behaviors (Scott and Sloman, 2004; McGee et al., 2009; Rearick, 2013), and increases risky behavior (Brodin et al., 2013); these behavioral alterations can ultimately increase vulnerability to predation (Mesa et al., 1994; Scholz et al., 2000; Scott and Sloman, 2004; Mandrillon and Saglio, 2007; McGee et al., 2009; Brodin et al., 2013; Rearick, 2013). Notably however, exposure to contaminants can also have adverse effects on predator success, potentially mitigating increased vulnerability of prey (Weis and Khan, 1990; Grippo and Heath, 2003). For example, Gregg et al. (1997) showed that darter goby exposed to diesel fuel had reduced rates of prey capture. Other studies have reported that exposure reduces motivation to forage (Brown et al., 1987; Weis and Khan, 1991; Smith et al., 1995). Collectively, these findings indicate that the effects of exposure on the outcomes of predator-prey interactions are complex; accurate predictions of the outcomes of exposure on predator-prey interactions therefore require that the independent and interactive effects of exposure on both the predator and prey be known (Burger, 1997).

Here, we investigated the effects of a common environmental estrogen, estrone (E1) on the outcomes of predator-prey interactions between a common forage fish, larval *P. promelas*, and piscivorous sunfish (*Lepomis macrochirus*) across a range of temperatures reflective of natural spring and summer variation. Few studies have explored how variation in the ambient environment might impact the effects of exposure on complex species interactions (Hayden et al., 2015) despite the fact that temperature is a key factor regulating developmental, physiological, and metabolic processes in fish (Beitinger at al., 2000; Pankhurst and Munday, 2011). Natural seasonal fluctuations in ambient temperature therefore have potential to modulate contaminant-induced changes in predator-prey interactions. Moreover, predators and prey may differ in their responses to such fluctuations (Stenseth et al., 2002; Freitas et al., 2007)

because thermal sensitivities can vary between species (Johnston and Temple, 2002; Guderley, 2004). We exposed mature bluegill sunfish (*Lepomis macrochirus*) and larval fathead minnows (*Pimephales promelas*) to environmentally relevant concentrations of E1 (i.e., E1LOW or E1HIGH) or to a solvent control at one of four temperatures (15, 18, 21, 24°C) and conducted a factorial, competitive, predation experiment to test the hypothesis that the biological effects of estrogen exposure on the predator-prey relationship between bluegill sunfish and fathead minnows are modulated by ambient temperature. Our data showed that exposure to E1 reduced overall prey-capture success by predatory sunfish at the majority of temperatures tested. However, within trials exposed larvae were disproportionately consumed compared with control fish.

2.2 Methods and Materials

2.2.1 Experimental Design

To test the hypothesis that estrogenic exposure and temperature affects the survival of fathead minnows following predation, we separately but simultaneously exposed adult bluegill sunfish and larval fathead minnows to either a low or high concentration of estrone (i.e., E1LOW or E1HIGH), or to an equivalent volumetric percentage of solvent at one of four temperatures (15, 18, 21, or 24°C) for 30 days (total of 12 treatments). The temperature range used in the experiment approximated the range of natural spring and summer variation in northern waterways and was well within the thermal tolerance limits for *L. macrochirus* (Stuber et al., 1982) and *P. promelas* (Pyron and Beitinger, 1993). Water quality parameters, including pH, temperature (°C), and dissolved oxygen (mg/L) were monitored on a daily basis using a handheld multi-parameter sampling instrument (model 556 MPS, YSI Instruments, OH, USA). We assessed the effects of temperature and estrogenic exposure on the escape performance of larval minnows in response to a simulated predator on day 21 of exposure. On day 30, we conducted predation trials to examine the effects of temperature and estrogenic exposure on larval predation risk and the prey-capture effectiveness of predatory sunfish. All subjects were sacrificed at the conclusion of the

experiment via an overdose of NaCO2-buffered MS-222 (Western Chemical, WA, USA). The Institutional Animal Care and Use Committee at St. Cloud State University approved all procedures and maintenance protocols used in the experiments (protocol number 8-73).

2.2.2 Exposure chemicals

Powdered estrone (≥ 99% purity, Sigma-Aldrich, St. Louis, MO) was dissolved in 100% ethanol (EtOH) to create stock solutions and stored in 1 mL aliquots at -20°C for the duration of the experiment. Aqueous exposure solutions with nominal concentrations of 125 and 625 ng/L for the E1low and E1high treatments, respectively, were prepared every 3 days in darkened glass carboys by adding an appropriate amount of the stock to 10 L of conditioned well water. An aqueous control treatment was also prepared that contained an equivalent volumetric percentage of solvent (EtOH). Treatment concentrations used in the study were selected for consistency with previous work (Ward et al., 2017; Cox et al. 2017) and because they fall within the environmental range reported by Kolpin et al. (2002). Aqueous exposure solutions were thoroughly mixed by agitating the carboys for 10 s before tightly covering the necks of the carboy with aluminum foil.

2.2.3 Water Quality

To establish the performance of the exposure system, we collected four 1L water samples from each treatment head tank in HDPE bottles. Water samples were immediately frozen and remained frozen until estrogen analysis was performed. Solid phase extraction was performed following the methods outlined by Arditsoglou and Voutsa (2008). To perform the analysis, water samples (500 mL) were spiked with 1.80 ppb of estrone-2,3,4-$^{13}C_3$ internal standard. Suspended solids were removed by filtration through GH Polypro 47mm 0.45μm hydrophilic polypropylene membrane filters. Samples were extracted with 300mg Oasis-HLB solid phase extraction cartridges. The solid phase was first preconditioned with 3 mL methanol followed by 3 mL of water. After the sample had been applied, the

solid phase was dried using air pulled by vacuum, and the analytes were eluted with 5 mL of acetone. HPLC-MS/MS was performed using the methods outlined by Gentili and colleagues (2002). Estrone concentration was measured using an Agilent 1200 HPLC / 6410 triple quadrupole mass spectrometer. Extract (20 μL) was injected onto Agilent Poroshell EC-C18 column using mobile phase was a binary mixture of methanol and 0.1% v/v NH_3 in water with a linear gradient starting from 20% holding for 2 min and increasing to 100% methanol over 4 minutes. The flow rate was 0.250 mL/min at a column temperature of 60°C. Negative ion mode was used for analysis with a -5000V ionization voltage and collision voltage of +135V. Detection was done in MRM mode using the $M\text{-}H^-$ ions: estrone, 269.2 $\rightarrow$145.1 *m*/*z*; estrone-$^{13}C_3$, 272.2 $\rightarrow$148.1 *m*/*z*.

2.2.4 Exposure Regime and Apparatus

2.2.4.1 Sunfish

Adult bluegill sunfish were obtained from 10,000 Lakes Aquaculture (Osakis, MN) and treated with Fungal Cure (API Fishcare). The sunfish were exposed to E1low, E1high, or the EtOH control treatment for 30 days at 15, 18, 21, or 24°C under flow-through conditions (Zhao et al., 2017). Subjects were maintained for the duration of the experiment in eight 52-L aquaria (15 fish per aquarium) under a 16:8 h light:dark cycle; two aquaria for each concentration of E1 and four aquaria for control subjects. Subjects were fed using a mixture of blood worms and brine shrimp ad libitum twice daily. In addition, sunfish were periodically offered live minnows to condition them to the novel food source.

2.2.4.2 Minnows

Minnow larvae (1-day post-hatch [dph]; Environmental Consulting and Testing, Superior, WI) were randomly assigned to 1-L glass jars containing either E1low, E1high or the solvent control (~30 minnows per jar) and maintained for 30 days under a 50% daily static renewal protocol and a 16:8 h light: dark cycle. Each day, half of the water in each jar was removed and replaced with fresh E1-treated water

or control water taken directly from the flow-through exposure lines to the sunfish tanks. As appropriate to the treatment, water temperature was maintained at 15, 18, 21, or 24°C throughout the exposure period using water baths or heating pads. Minnows were fed freshly hatched brine shrimp ad libitum twice daily, beginning 2 dph. Larvae were tested on day 21 of exposure in predator escape assays. After testing, the subjects were returned to their exposure jars until day 30, when predation trials were conducted.

2.2.5 Predation Trials

We conducted a factorial predation assay that paired control and exposed larvae in a competitive setting, thereby permitting direct estimates of increased predation mortality due to exposure (see 'Supplementary material' for a description of the factorial experimental design). Trials were conducted in opaque-walled PVC arenas with a 104-cm diameter and a water depth of 25 cm (total volume: 212 L). Twenty evenly spaced artificial plants were added to each arena to provide refuge for the larvae. Trials were conducted at 15, 18, 21, or 24°C (±1°C), as appropriate to the exposure treatment. The arena was drained and scrubbed between trials to remove any residual chemical cues.

Focal sunfish were fasted for 72 h before being used in the experiment to maximize motivation to forage. At the start of a trial, one sunfish (control, E1low or E1high) was placed in the arena and given approximately 1.5 hours to acclimate. At the end of the acclimation period, one group of control larvae and one group of exposed larvae were simultaneously introduced to the arena. Trials conducted at 18, 21, and 24°C paired 5 exposed and 5 control larvae (10 total larvae); trials conducted at 15°C paired 4 exposed and control minnows, respectively, due to availability of individuals. In each trial, the focal sunfish was permitted to forage for larvae for 1 hour, after which the test was stopped, and the sunfish removed via a hand net. Surviving larvae were immediately captured and transferred to a glass beaker for identification of group assignment (see 'Fluorescent staining', below). We assessed predation upon exposed vs control larval subjects by comparing the relative proportion of exposed vs control minnows in each trial that survived.

2.2.6 Florescent Staining

To facilitate the identification of surviving larvae, subjects from either the control or the exposed group were marked 1 day before the trial using a fluorescent SE-MARK calcein dye (Western Chemical, Ferndale, Washington, USA) according to approved US Food and Drug Association Investigational New Animal Drug protocols (FDA INAD 10-987). The group selected to undergo staining in each trial was randomly determined to prevent mark-associated bias. Larvae from the control, E1low and E1high treatments were maintained for 6 hours in separate stain baths created by adding a 1.0% calcein stock solution to conditioned well water until a concentration of 250 mg/L was reached. Preliminary trials confirmed that florescence persisted until the end of the experiment and no abnormal behaviors were observed during staining. The larvae recovered from each trial were identified using a SE-MARK detector (Western Chemical, Femdale, Washington, USA) to illuminate fluorescently marked fish.

2.2.7 Predator Escape Performance

Predator escape performance was assessed following previously established protocols (McGee et al., 2009; Painter et al., 2009). In each trial, a single larva was placed in a clear-bottomed testing dish containing 10 mL of aerated well water. The dish was positioned on a 1 mm x 1 mm grid with a vibrational chip positioned beneath it that delivered a non-point source stimulus to the larva. and illuminated via a fiber optic light source angled 20 cm above the arena. Subjects were permitted to acclimate to the arena for 1 min, after which the stimulus (~0.5 s in duration) was delivered. Subject responses were recorded using a Redlake MotionScope (Tucson, AZ) high-speed camera (1000 frames s-1) positioned ~25 cm vertically above the test arena.

Latency to the induction of the escape response, escape velocity, turning angle, and total escape response were quantified from the videos using the software program ImageJ (National Institutes of Health, Bethesda, MD). Standard body length (BL) was calculated by digitizing the anterior tip of the snout and the posterior tip of the tail on a still frame from each. Two additional landmarks were digitized

on the grid at a 1 mm distance to account for scale. Latency was recorded as the length of time to induction of movement (in ms). Velocity was calculated during the first 40 ms after the initiation of movement and adjusted to BL per ms. The total escape response of each subject was calculated as BL/(latency in ms + 40 ms). Responses were classified as false starts if the individual initiated the response prior to 5 ms or after 300 ms and were discarded prior to statistical analysis. Each larva was only tested once.

2.2.8 Statistics

We compared the survivals of sunfish and larval minnows, respectively, during the exposure period using chi-square tests. Differences in larval growth (BL on day 21) across treatments were tested via ANOVA.

We compared the among-treatment escape performance of larvae to a simulated predator using ANOVAs. To improve normality, we log-transformed larval escape velocity and latency to first response prior to analyses. We estimated the influence of each variable of interest on subject responses, relative to the other factors in the model, via the partial variance statistic ($\eta 2$). For all analyses, we examined the effect size of for all significant pairwise posthoc comparisons via Cohen's d.

Initial analyses indicated that neither overall larval survival in the predation trials, nor overall prey capture success by sunfish, varied across the 9°C temperature range tested (Ps >0.05). We therefore compared the percent survival of exposed and control larvae within each temperature treatment using Wilcoxon sign rank tests. To assess the effects of exposure on the prey-capture success of predators, we calculated the average proportion of total larvae consumed (exposed + control/2) and compared the proportion of prey items captured across exposure treatments using Kruskal-Wallis tests. Because preliminary analysis indicated that control-treatment predators in larval E1low and E1high trials consumed non-exposed larvae, and E1low and E1high larvae with similar frequencies (all Ps >0.05), we pooled control predators in final analyses.

2.3 Results

2.3.1 Water Quality

Measured E1 concentrations (mean ± SD) were 90 ± 17.6 and 414 ± 146 ng/L for the E1LOW (n = 16 samples) and E1HIGH (n = 15 samples) treatments, respectively. Estrone was not detected in control samples. Water temperatures throughout the exposure period were 16.2 ± 1.08, 18.3 ± 0.62, 21.8 ± 0.40, and 24.1 ± 0.60°C, for the 15, 18, 21, and 24°C treatments, respectively. Water quality in the exposure tanks also remained stable throughout the experiment (dissolved oxygen = 5.50 ± 0.94 mg/L; pH = 8.34 ± 0.23).

2.3.2 Larval Survival and Growth

Neither survival during the exposure period nor growth were affected by E1 concentration or temperature for minnow larvae and sunfish. Survival at day 30 was high and consistent across treatments, ranging from (mean ± SD) 85.5% ± 13.2% to 89.5% ± 8.7% for larvae and 88.25% ± 3.7% to 93.5% ± 11.1% for sunfish. Larval BL on day 21 was also similar among the 12 treatments. Mean body length (± SD) ranged from 7.69 ± 1.48 to 8.89 ± 1.23: Figure 2.1); an ANOVA revealed no significant effect of temperature or concentration level, or an interaction between the two factors on growth (overall model: $F_{11,170} = 0.880$, $P = 0.561$).

2.3.3 Predation Trials

A total of 219 sunfish and 2,096 minnow larvae were used in the predation trials (n = 6-19 trials per exposure scenario; Table 2.1). Across all temperatures and predator exposure treatments, the mean (±SD) survival of control, E1low, and E1high larvae were 74% ± 23%, 49% ± 24%, and 53% ± 23%. In all 16 exposure scenarios, exposed larvae were more likely to suffer predation compared with controls; this difference was statistically significant in 14 of 16 test combinations (Figure 2.2; Table 2.1). Exceptions to this finding occurred only at 24°C (predator: E1high, Prey: E1high) and 21°C (predator:

control, prey: E1low). In these tests, there was no difference in the survival of paired control vs exposed fish.

Across all temperatures and exposure treatments, the mean (±SD) prey-capture success of sunfish in the control, E1low, and E1high treatments were 45% ± 18%, 29% ± 17%, and 31% ± 18%. Exposure had a significant effect on prey-capture success at all temperatures tested (Table 2.2). At three of the four temperatures tested (15°C, 18°C, 24°C), pairwise post hoc analyses indicated that predator success was negatively correlated with exposure level (Figure 2.3); nonexposed sunfish successfully captured and consumed more larvae than sunfish exposed to E1low ($<0.001 < P < 0.05$) or E1high ($<0.001 < P < 0.018$). However, at 21°C sunfish exposed to E1low and E1high consumed more larvae than control subjects (control vs E1low: $P < 0.001$; control vs E1high: $P < 0.001$; Figure 2.3). There was no difference in the proportion of available prey consumed by sunfish exposed to E1low or E1high at any temperature (all $P > 0.05$).

Table 2.1: Wilcoxon sign rank tests comparing the percent survival of larval fathead minnows exposed to either a low (PreyLOW) or high (PreyHIGH) dose of E1 for 30 days with that of nonexposed larvae (control) in predation trials. Significant effects are given in bold.

	Treatment			Wilcoxon Test	
	Predator	**Prey**	**n**	***Z***	***P***
15°C	Control	Low	11	-2.52	**0.012**
	Control	High	12	-2.18	**0.029**
	Low	Low	11	-2.71	**0.007**
	High	High	12	-2.06	**0.039**
18°C	Control	Low	17	-3.46	**0.001**
	Control	High	14	-3.45	**0.001**
	Low	Low	19	-2.69	**0.007**
	High	High	17	-2.29	**0.022**
21°C	Control	Low	9	-1.37	0.169
	Control	High	11	-2.59	**0.009**
	Low	Low	6	-2.02	**0.043**
	High	High	12	-2.48	**0.013**
24°C	Control	Low	15	-3.37	**0.001**
	Control	High	12	-3.12	**0.002**
	Low	Low	17	-3.16	**0.002**
	High	High	13	-1.51	0.131

Table 2.2: Kruskal-Wallis tests comparing predation success (% total larvae consumed) of control sunfish, or sunfish exposed to either a low ($Pred_{LOW}$) or high ($Pred_{HIGH}$) dose of E1 for 30 days. Significant results are given in bold.

	Kruskal-Wallis test		
Temperature	**n**	***Z***	***P***
15°C	46	10.39	**0.006**
18°C	67	34.63	**<0.001**
21°C	38	9.33	**0.009**
18°C	57	19.04	**<0.001**

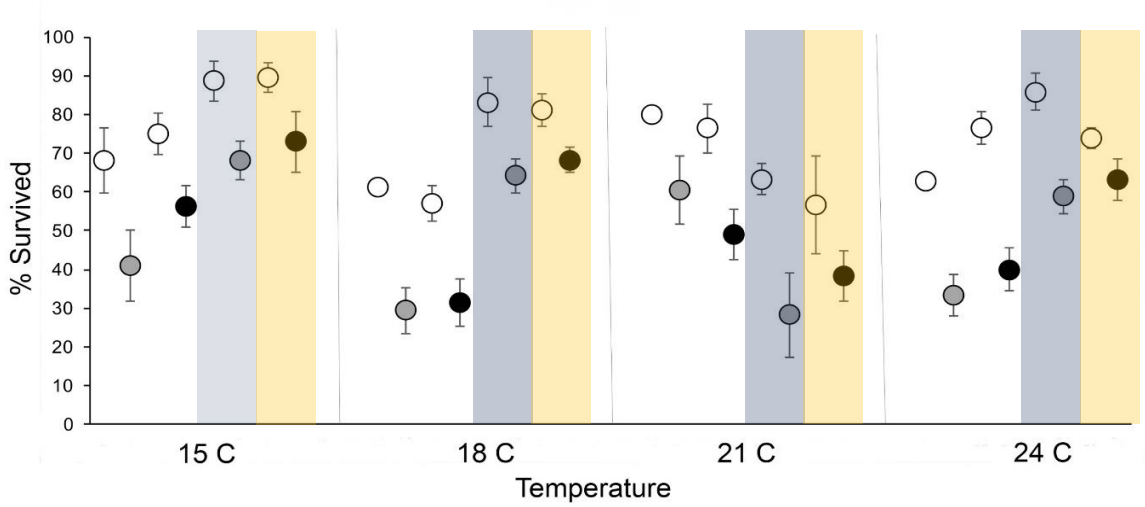

Figure 2.1: Percent survival of larval fathead minnows in competitive predation trials. Larvae were exposed to either a low (E1LOW; gray symbols) or high (E1HIGH; black symbols) dose of E1 for 30 days or to an equivalent volumetric percentage of solvent (control; white symbols). Larvae in the presence of control sunfish are indicated by the white bar, larvae in the presence of E1LOW exposed sunfish are indicated by the gray bars, and larvae in the presence of E1HIGH exposed sunfish are indicated by the tan bar Points and whiskers are mean ± SEM.

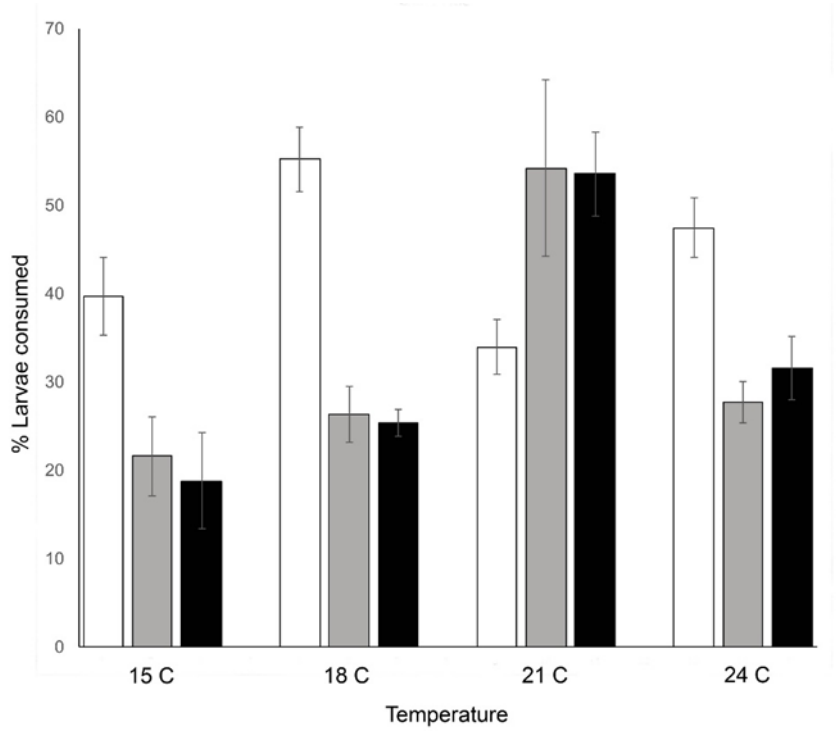

Figure 2.2: Predation success of predatory sunfish (% of total larvae in the trial consumed). Sunfish were exposed to either a low ($E1_{LOW}$; gray symbols) or high ($E1_{HIGH}$; black symbols) dose of E1 for 30 days or to an equivalent volumetric percentage of solvent (control; white symbols). Bars and whiskers represent the mean ± SEM.

2.3.4 Predator Escape Performance

A total of 182 minnow larvae were used in escape performance trials (n=9-28 across treatments). We found significant main effects of temperature, but not E1 concentration or an interaction between the two factors, on aspects of the innate, evasive predator escape response. An ANOVA indicated that total escape performance was reduced at colder temperatures (Table 2.3). Pairwise post-hoc tests revealed that the escape performance of larvae reared at 15°C (0.002 ± 0.002 BL/ms) was significantly reduced compared with that of larvae reared at either 18°C (0.003 ± 0.004 BL/ms; P = 0.012; Cohen's d = 0.32) or 21°C (0.003 ± 0.004 BL/ms; P = 0.013; Cohen's d = 0.32; Figure 2.4A). No other significant pairwise differences were observed (all Ps >0.05).

The observed temperature-related reduction in escape performance was primarily driven by a significant effect of temperature on larval escape velocity (Table 2.3). Pairwise post-hoc tests indicated that the velocity of larvae reared at 15°C (0.012 ± 0.009 BL/ms) was significantly reduced compared with that of larvae reared at either 18°C (0.018 ± 0.012 BL/ms; P = 0.003; Cohen's d = 0.57) or 21°C (0.018 ± 0.012 BL/ms; P = 0.007; Cohen's d = 0.57; Figure 2.4B). By contrast, we did not observe significant effects of temperature or E1 exposure on latency to first response (Table 2.3). Mean (± SD) latencies ranged from 81.18 ± 53.16 ms to 147.77 ± 110.51 ms across treatments (Figure 2.4C)

We also found a significant main effect of temperature on turning angle (Table 2.3). Consistent with the temperature-dependent variation in other aspects of the escape response that we observed, turning angle differed in larvae reared at 15°C (127.61 ± 69.10 degrees) compared with that of larvae reared at either 18°C (134.37 ± 97.83 degree; P = 0.018; Cohen's d = 0.08) or 21°C (121.33 ± 76.32; P = 0.001; Cohen's d = 0.09; Figure 2.4D).

Table 2.3: Results of ANOVAs examining the effects of temperature (15, 18, 21 and 24°C) and E1 concentration (control, $E1_{LOW}$ or $E1_{HIGH}$) on the predator escape performance of larval minnows. Significant effects are given in bold.

Parameter	Effect	F	*df*	*P*	η^2
Velocity	Concentration	0.65	3, 170	0.526	0.008
	Temperature	4.51	2, 170	**0.005**	0.074
	Concentration *x* Temperature	0.70	6, 170	0.650	0.024
Latency	Concentration	0.37	3, 170	0.690	0.004
	Temperature	0.28	2, 170	0.841	0.005
	Concentration *x* Temperature	0.70	6, 170	0.650	0.024
Total escape response	Concentration	0.32	3, 170	0.726	0.004
	Temperature	3.14	2, 170	**0.027**	0.052
	Concentration *x* Temperature	0.96	6, 170	0.451	0.033
Angle of escape	Concentration	0.50	3, 170	0.951	0.001
	Temperature	4.63	2, 170	**0.004**	0.076
	Concentration *x* Temperature	0.66	6, 170	0.682	0.023

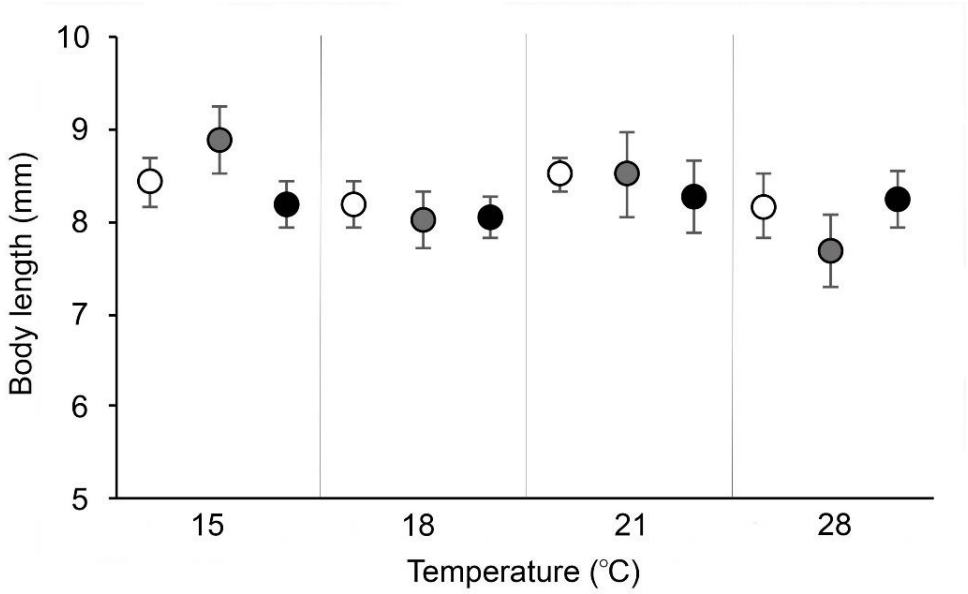

Figure 2.3: Body length (mm) of larval fathead minnows exposed to either a low (E1LOW; gray symbols) or high (E1HIGH; black symbols) dose of E1 or to an equivalent volumetric percentage of solvent (control; white symbols) and reared at one of four temperatures (15, 18, 21 and 24°C). Points and whiskers are mean ± SEM.

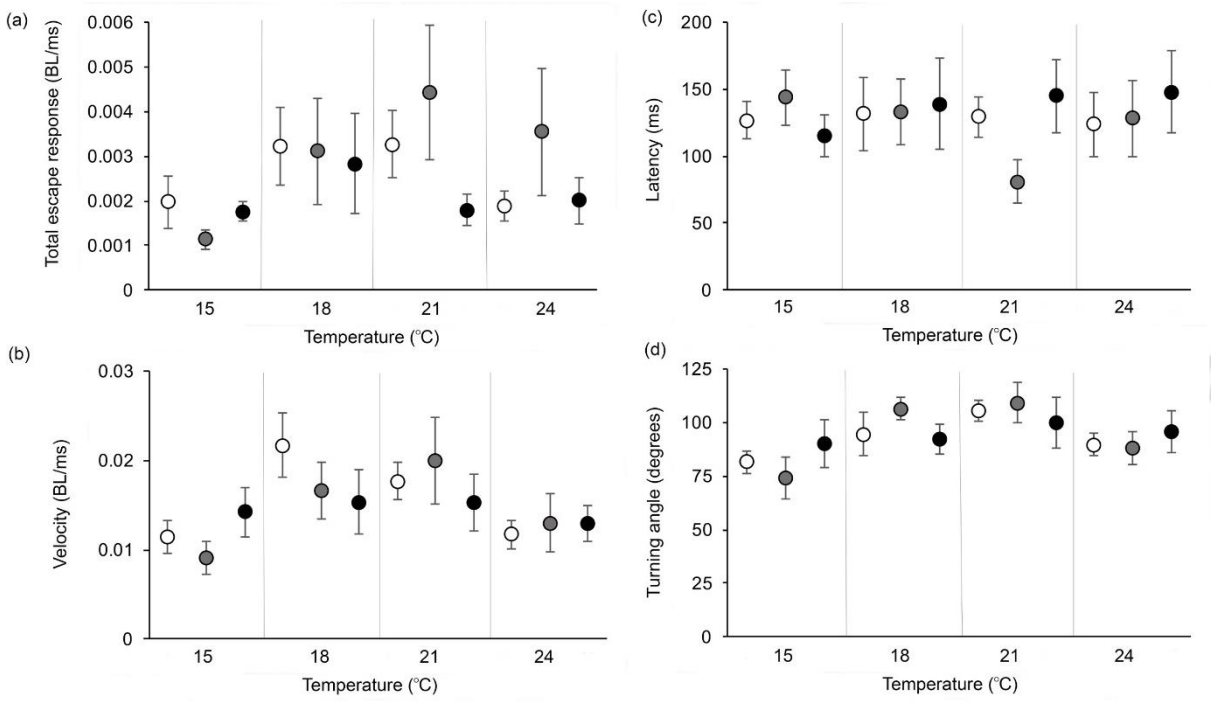

Figure 2.4: Responses of larvae exposed to E1 or an EtOH solvent control (control), at four ambient temperatures. (a) Total escape response (BL/ms). (b) Escape velocity (BL/ms) over the first 40 ms of the response. (c) Latency (in ms) to the induction of the response. (d) Turning angle, relative to the initial position of the head (in degrees). White circles represent the EtOH solvent control exposure group. Gray and black circles represent the E1LOW and E1HIGH exposure treatments, respectively. Points and error bars depict mean ± SEM.

2.4 Discussion

In this study, we examined the extent to which varied ambient temperature and estrogenic exposure modifies the behavior of two fish species. We hypothesized that, similar to the findings of previous studies (McGee et al., 2009, Rearick, 2013) a concentration dependent impairment to the predator evasion response would ultimately lead to decreased survival following exposure to a predator. Our results showed two main findings; first, we established that E1 and temperature independently influence the survival and behavior of both larval fathead minnows and mature bluegill sunfish; Second, taken together our data suggest that individuals in the larval stage are more susceptible than adults to the effects of natural abiotic variation on aquatic contaminants.

Estrogenic exposures have been shown to impact various physiological (Purdom et al., 1994; Ward et al., 2017) and behavioral (Coe et al., 2008; Colman et al., 2009; McGee et al., 2009) aspects in many species of freshwater fish. Additionally, these exposure effects have provided evidence indicating predator-prey relationships and aquatic food webs may be altered both directly and indirectly (Hallgren et al., 2014; Kidd et al., 2014). Organisms sharing the same habitat are both exposed to aquatic pollutants, however one species may be more susceptible than the other (Burger, 1997). Our results show that larvae exposed to E1 for 30 days had a reduced survival (E1LOW: 49.2%; E1HIGH: 52.9%) in the presence of a predator compared to their control counterparts (74.2%), regardless of ambient water temperature. These findings are consistent with a previous study in which, following similar predation trials after exposure to 38 or 103 ng/L of E2, the survival of exposed larvae was about 45% in comparison to the control larvae at 55% (Rearick, 2013). Subsequent multi-generation population modeling showed a steady projected decline in the fathead minnow population (Rearick, 2013). Additionally, the E1 exposure effects on the larval minnows were somewhat mitigated because the predatory sunfish displayed a reduced success in prey capture. On average, sunfish exposed to E1 successfully captured 32.4% (E1LOW) and 32.0% (E1HIGH) in comparison to their control counterparts with a success rate of 44.7%. Additionally, sunfish reared at 15°C were less successful in prey capture, potentially due to the resulting temperature-dependent decrease in metabolism.

Fluctuations in ambient water temperatures can potentially have negative impacts on ectothermic organisms, such as fish, as their body temperatures fluctuate with water temperature. Additionally, the rates of most biological processes and biochemical reactions in the body increase roughly exponentially with temperature (Zuo et al., 2011). The rates of growth and development are not exempt. Typically, larval growth is slower when reared in colder water temperatures, largely due to a slowed metabolism (Gillooly et al., 2001; Zuo et al., 2011). Generally, food consumption increases, then peaks at an optimal temperature, and decreases once temperatures rise past the optimum (Brett and Groves, 1979). Our data

show that larval fathead minnows were similar in size at 21 days of exposure regardless of ambient water temperature. These similarities in size can potentially be attributed to density dependent growth and exploitation competition (Arthur and Dixon, 1994; Vandenbos et al., 2006). The minnows raised at 15°C had the lowest survival of the four temperatures, suggesting that a lower density of larvae allowed for increased individual growth.

The predator evasion response, or C-start response, is regulated almost wholly by the Mauthner cells (M-cells), two large cells located in the hindbrain (Eaton et al., 2001). When one of these cells is activated, the predator evasion response is triggered and the signal travels along the axon. This transmission ultimately causes the trunk muscles on the opposite side of the body from the activated M-cell to contract, forming the characteristic "C" shape (Korn and Faber, 2005). Chemical exposure and temperature change have been shown to independently influence larval escape performance via effects on the activity of the M-cells. Changes in temperature can potentially alter the balance between excitatory and inhibitory neurotransmission onto the M-cells, resulting in behavioral changes (Preuss and Faber, 2003; Szabo et al., 2008). Effects on the M-cells and resulting behaviors have been shown to be intensified at higher ambient temperatures (Xia et al., 2015). Transmission of the signal is also affected by a plethora of chemicals (Carlson et al., 1998), resulting in impairment to predator escape performance (McGee et al., 2009; Painter et al., 2009). For example, juvenile *P. promelas* exposed as embryos to 50 ng/L of E1 displayed an increased latency in the initiation of the C-start response and a reduction in the total escape response in comparison to their control counterparts (McGee et al., 2009). We did not find that E1 exposure significantly impaired the predator evasion response, leading us to believe there is some other underlying mechanism responsible for the reduction in survival following exposure to a predator. However, we did find that temperature significantly impacted the turning angle, the escape velocity and the total escape response. Our data shows that, on average, larvae reared at 15°C and 24°C suffered significant reductions to escape velocity and the total escape response.

Chapter 3: Temperature Modulates the Effects of Estrogenic Exposure in a Piscivore Freshwater Fish

3.1 Introduction

The impacts of the near ubiquitous presence of endocrine active compounds (EACs) on aquatic ecosystems have become a growing concern to environmental biologists and natural resource managers. Previous studies have provided ample evidence that EACs, especially estrogenic EACs, can affect anatomy, physiology and behaviors of exposed organisms, ultimately impacting predator-prey relationships. However, few studies have assessed how these changes are modulated by abiotic factors such as environmental temperature. The current study, therefore, evaluates how temperature modulates the effects of a commonly occurring estrogenic EAC in a piscivore fish whose predation performance was shown to be impacted by EACs in prior studies (McGee et al., 2009; Rearick, 2013; Ward et al., 2017).

EACs may disrupt the endocrine system by acting as hormone agonists, promoting activity by mimicking endogenous hormones (Jobling et al., 1998; Guilette and Gunderson, 2001; Pait and Nelson, 2002; Sumpter and Johnson, 2005). Indeed, many receptor agonists in the aquatic environment exhibit estrogenic activity (Routledge et al. 1998; Kolpin et al. 2002; Pinto et al., 2014) and may enter the aquatic environment with treated wastewater effluent (Purdom et al., 1994; Folmar et al., 1996; Harries et al., 1997; Desbrow et al., 1998; Routledge et al., 1998; Baronti et al., 2000). Estrone (E1), 17-β estradiol (E2), estriol (E3), and the synthetic ethynylestradiol (EE2) have all been documented in aquatic ecosystems and may induce vitellogenin (vtg) biosynthesis in male fish (Sumpter and Jobling, 1995; Routledge et al., 1998; Larsson et al., 1999). E1 and E2 are naturally excreted through human and animal wastes, oral contraceptives and other varying pharmaceuticals are responsible for most of the EE2 found within wastewater treatment plant effluent (Wise et al., 2011). These estrogenic compounds are commonly found in streams and rivers across the U.S and many other countries (Ternes et al. 1999; Kolpin et al. 2002; Murk et al., 2002; Servos et al., 2005; Lishman et al., 2006). Although the half-lives of

these compounds are relatively short (E1: 19 hours, E2: 8-36 hours, E3: 5 hours, EE2: 10-36 hours) (Wentz, 1988), the almost constant release of these estrogens with treated wastewater effluent brings forth a scenario in which the half-lives of these compounds are surpassed by their introduction rates into the environment (Daughton, 2002). E1, a breakdown product of E2 (Writer et al., 2012; Ankley et al., 2017), is frequently used in toxicology studies because aquatic concentrations of E1 are typically higher than E2 (Alvarez et al., 2013) and the biological effects of E1 are less understood (Dammann et al., 2011; Feifarek et al., 2018; Shappell et al., 2018).

Temperature is a key environmental factor regulating metabolism and activity among aquatic ectothermic species, including teleost fishes (Jin et al., 2009). As ectotherms, fish can be impacted by ambient water temperature across biological levels, from molecular responses, such as the rate of vtg expression (Brain et. al., 2008; Körner et al., 2008; Cox et al., 2018), to ecological responses, such as the timing of reproduction (Gillet and Quétin, 2006). During a 3-year experiment, researchers found that higher water temperature resulted in lower egg production, reduced fecundity, and a lowered fertilization rate in Atlantic halibut (Brown et al. 2006). In addition to direct effects on embryonic duration and egg survival, temperature also typically effects larval size at hatching, developmental rate, and survival, demonstrating that temperature influences reproductive physiology and development in fish (Pankhurst and Munday, 2011). While examining the influence of temperature on the growth and reproductive status of fathead minnows, Brian and colleagues (2011) found that female fish reared at the highest experimental temperature (32°C) displayed ovarian tissue that was lacking germ cells and contained considerable amounts of undetermined tissue (Brian et al., 2011). Furthermore, females reared at the highest experimental temperature displayed reduced ovipositor length, while males displayed fewer tubercles and less prominent fat pads (Brian et al., 2011). While effects of temperature have been demonstrated in many single species tests, the effects of temperature on complex inter-species relationships are poorly understood. In one study, researchers demonstrated that elevated temperature

resulted in an increase to the predator (Pseudochromis fuscus) attack speed while simultaneously impairing the prey's (Pomacentrus wardi) ability to escape, resulting in increased predation rates (Allan et al., 2015).

Assessing the effects of EACs on fish populations in the environment is complicated by the temperature dependency of EACs degradation. Previous studies have provided ample evidence supporting the hypothesis that at higher temperatures, the rates of degradation are more rapid for E1 (Guo et al., 2012; Cox et al., 2018) and E2 (Li et al., 2005; Guo et al., 2012). Furthermore, uptake of estrogenic EACs by fish may also be affected by water temperature with higher uptakes as respiration increases with temperature (Beamish and Mookherjii, 1964). As a consequence, effects of EACs on aquatic organisms are likely variable across seasons (Jin et al., 2009).

Much research effort has been put towards evaluating the effects of EACS to aquatic organism, where researchers have consistently demonstrated that EACs have adverse effects using single species tests, however less is known about how exposure to EACs can disrupt the balance of the relationship that exists between a predator and prey. One study has demonstrated that, regardless of ambient water temperature, fathead minnows (prey) exposed to two concentrations of E1 survived at a 20% lower rate following predation in comparison to their control counterparts (Korn et al., in prep). Additionally, predatory bluegill sunfish exposed to the same E1 treatments as the fathead minnows, displayed a 10% reduction in their ability to capture prey compared to control sunfish regardless of ambient water temperature (Korn et al., in prep). Effects to complex species interactions are important to consider because species rarely exist in isolation. Increased research efforts in this area can make toxicological studies more relevant to real world scenarios and aid agencies interested in environmental preservation.

Behavioral changes in freshwater fish can have lasting impacts on the population. For instance, reduced foraging and efficiency can affect growth rate (El-Sayed, 2002). Additionally, reduced ability to avoid predators can increase mortality rates. Prey exposed to pollutants may fail to recognize predators

(Kolmakov et al., 2009), exhibit risky behavior or hyperactivity (Mesa et al., 1994; Zhou and Weis, 1998), display an impairment to predator avoidance performance (McGee et al., 2009; Painter et al., 2009), reduced stamina (Mesa et al., 1994), and display inability to properly school (Mesa et al., 1994). These exposure effects can ultimately lead to an increased vulnerability to predation (Scott and Sloman, 2004). If individuals to higher concentrations of contaminants are easier for predators to capture, this can also facilitate the transfer of the contaminants to higher trophic levels. Altered predator-prey interactions involve organisms from two trophic levels and can cause changes in populations of predators, prey, or both and thus affect the community. In contaminated environments, both predator and prey are exposed, and one may be more susceptible than the other. Previous studies have shown that estrogenic compounds can alter predator-prey relationships after demonstrating reduced survival in fish (*Pimephales promelas*) exposed to an estrogenic compound following exposure to a predator (Rearick, 2013; Korn et al., in prep). The predator species (*Lepomis macrochirus*) was also affected, showing an impairment in the ability to capture prey following exposure to E1 that may potentially mitigate effects seen in the prey species (Korn et al., in prep).

In the current study, bluegill sunfish (*Lepomis macrochirus*), from the family Centrarchidae, were chosen as predators as they are native to a range of aquatic habitats in North America and are proficient at catching prey fish including juvenile fathead minnows (Ehlinger and Wilson, 1988). When foraging, bluegill sunfish typically tend to eat the individual that appears to be the largest (Werner and Hall, 1974; O'Brien et al., 1976). As ambient water temperature becomes colder, bluegills tend to eat less frequently and become more dormant. Laboratory tests have demonstrated that bluegills don't typically feed or consistently grow when water temperature falls outside a range of 10 °C to 30 °C (Stuber et al., 1982; Sternberg, 1996) with peak spawning occurring at 24 °C to 27 °C (Stuber et al., 1982).

The goals of the current study were to (i) assess the biological response in fish exposed to E1 at several water temperatures. (ii) link organismal effects seen as a result of estrogenic exposure to

previously demonstrated decreased survival in prey and decreased capture success in predators (Korn et al., in prep). Specifically, we tested the hypotheses that (i) estrogenic exposure induces biosynthesis of vtg and decreased biological indices in male fish; that (ii) estrogenic exposure doesn't negatively impact female fish; and that (iii) sunfish exposed to E1 at higher temperatures exhibit decreased biological indices.

3.2 Methods and Materials

3.2.1 Experimental Design

3.2.1.1 Adult Exposures

Four 30-day exposures were conducted in the St. Cloud State University Aquatic Toxicology Laboratory in St. Cloud, Minnesota. Adult bluegill sunfish (10,000 Lakes Aquaculture, Osakis, MN) were exposed using a previously published protocol for flow-through exposure systems (Zhao et al., 2017). Analytically confirmed E1 concentrations of 90 ± 17.6 ng/L and 414 ± 147 ng/L and an ethanol carrier control were used. Four exposures were run at 15, 18, 21, and 24 °C, for a total of 12 treatments (for logistical reasons, each temperature was run independently). For a given temperature, eight large aquaria (52 Liters) were used to rear sunfish with two of these aquaria for each of the two concentrations of E1 and four aquaria for the ethanol control. The start of exposures was staggered by aquarium and day to provide for sufficient time to conduct the lengthy predation trials following the exposure. Each aquarium contained 15 randomly assigned adult bluegill sunfish (not divided by sex) raised under a 16:8 h light:dark cycle. Fish were fed using a mixture of blood worms and brine shrimp *ad libitum* twice daily. Additionally, sunfish were periodically fed larval fathead minnows to familiarize them with the prey species. Water quality parameters, including temperature, pH, and dissolved oxygen were recorded every three days using a handheld multi-parameter sampling instrument (model 556 MPS, YSI Instruments, OH, USA). On Day 30 of the exposure period, predation trials occurred, and the fish were sacrificed via an overdose of MS222, following methods approved by the St Cloud State University Animal Care and

Use Committee (IACUC protocol SCSU 8-73). Standard ecotoxicology biomarkers including blood plasma vtg (using ELISA methodology; Schultz et al., 2013; Thomas et al., 2017) and blood glucose (Bevelhimer et al., 2014) were measured. The liver and gonads from each bluegill sunfish were harvested, weighed, and analyzed for histopathological changes. Organosomatic indices and condition factor were also calculated using organ weight, wet fish weight, and total length of the fish. Aqauria were monitored daily for mortality.

3.2.1.2 Exposure Solutions

Powdered estrone (≥ 99% purity, Sigma-Aldrich, St. Louis, MO) was dissolved in 100% ethanol to form exposure solution spikes. The E1 solution spikes and an ethanol solvent control, were stored in the 1 mL aliquots at -20°C for the duration of the experiment. Every three days, for each treatment, exposure solutions were prepared in darkened glass carboys by adding the appropriate 1 mL aliquot to 10 L of conditioned, non-chlorinated well water to make the exposure solutions, ethanol carrier control (EtOH control), a low dose treatment (E1LOW), and a high dose treatment (E1HIGH). Each solution was thoroughly mixed by agitating the carboys for 10 s. Immediately following agitation, the neck of each carboy was tightly covered with aluminum foil to prevent chemical degradation.

3.2.2 Water Chemistry

To establish the performance of the exposure system, we collected four 1L water samples from each treatment head tank in HDPE bottles. Water samples were immediately frozen and remained frozen until estrogen analysis was performed. Solid phase extraction was performed following the methods outlined by Arditsoglou and Voutsa (2008). To perform the analysis, water samples (500 mL) were spiked with 1.80 ppb of estrone-2,3,4-$^{13}C_3$ internal standard. Suspended solids were removed by filtration through GH Polypro 47mm 0.45μm hydrophilic polypropylene membrane filters. Samples were extracted with 300mg Oasis-HLB solid phase extraction cartridges. The solid phase was first

preconditioned with 3 mL methanol followed by 3 mL of water. After the sample had been applied, the solid phase was dried using air pulled by vacuum, and the analytes were eluted with 5 mL of acetone. HPLC-MS/MS was performed using the methods outlined by Gentili and colleagues (2002). Estrone concentration was measured using an Agilent 1200 HPLC / 6410 triple quadrupole mass spectrometer. Extract (20 μL) was injected onto Agilent Poroshell EC-C18 column using mobile phase was a binary mixture of methanol and 0.1% v/v NH_3 in water with a linear gradient starting from 20% holding for 2 min and increasing to 100% methanol over 4 minutes. The flow rate was 0.250 mL/min at a column temperature of 60°C. Negative ion mode was used for analysis with a -5000V ionization voltage and collision voltage of +135V. Detection was done in MRM mode using the $M\text{-}H^-$ ions: estrone, 269.2 $\rightarrow$145.1 *m*/*z*; estrone-$^{13}C_3$, 272.2 $\rightarrow$148.1 *m*/*z*.

3.2.3 Biological Endpoints

3.2.3.1 Organosomatic Indices

Sunfish were anesthetized using 250 mg/L tricaine methane sulfonate (MS-222) buffered to pH 7.2 using equal weight sodium bicarbonate (Argent Laboratories, Redmond, WA) and wet body weights and total body lengths were obtained for each sunfish (0.01 g precision, Acculab Vicon, Edgewood, NY). Additionally, gonads and livers from each fish were removed and weighed (0.001 g precision, Mettler Toledo AG245, Columbus, OH). Wet body weight and total body length were utilized for calculation of the condition factor (CF) [(total mass, g/(total length, mm)^3) x 100,000] (Fulton, 1904). Liver and wet body weights were utilized for calculation of the hepatosomatic index (HSI) (liver weight/whole body weight × 100). Gonad and wet body weights were utilized for calculation of the gonadosomatic index (GSI) (gonad weight/whole body weight × 100).

3.2.3.2 Blood Glucose

Following anesthetization, the tail was severed and a ~1 µL sample of whole blood was obtained from the caudal vasculature of each fish. Blood glucose was then measured for each sunfish using a digital glucose monitor (TRUEbalance Blood Glucose Monitor, Moore Medical, Farmington, CT).

3.2.3.3 Plasma Vtg

Following anesthetization, blood was collected from the caudal vasculature via tail severance and centrifuged at (8000xg for 12 minutes at 4°C). Plasma was collected and stored at -80°C for later analysis. Plasma vtg was measured using a competitive antibody-capture ELISA (Thomas et al., 2017) using a species-validated anti-vitellogenin antibody and purified vtg as standard. The procedure used species-validated polyclonal anti-sunfish vtg antibody and purified sunfish vtg. Microtiter plate wells were coated with 600 ng species-validated vtg in a carbonate coating buffer (pH 9.6). A pre-competition step was achieved with the antibody (1:20,000 final dilution) and either standard vtg, control plasma, or sample plasma in 1% BSA/PBS (pH 7.5). Following incubation, this mixture was loaded into the wells and subsequently incubated for 1 hour at room temperature, followed addition of the secondary antibody (anti-rabbit IgG-HRP, Sigma-Aldrich, St. Louis, MO) at a concentration of 1:10,000. The substrate, tetramethylbenzidine (TMB) was added and incubated for 16 minutes at room temperature and color development was measured at 620 nm on a Multiskan EX plate reader (Thermo Fisher, Waltham, MA) and sample values were calculated using the associated Multiskan Ascent software. Eight-point standard curves with a range of 5.0 to 0.039 µg/mL were prepared using two-fold serial dilutions.

3.2.3.4 Histopathology

Previously described histological protocols were followed (Thomas et al., 2017). Briefly, tissues were fixed in formalin for at least 1 week. To prepare for analysis, tissues were dehydrated in a series of ethanol and xylene baths using a Leica automated tissue processor TP 1050 (Leica, Wetzlar, Germany) and subsequently embedded in paraffin using a Thermo Scientific Microm EC 350-1 embedding station

(Waltham, MA). Embedded tissues were sectioned at 5 μm thickness in two separate locations in the tissue (~100 μm apart) using a Reichert-Jung cassette microtome (Leica, Wetzlar, Germany). Slides containing the sectioned tissues were stained using standard hematoxylin and eosin counter-staining techniques previously described in Carson (1997) in a Leica ST5010 Autostainer XL. FHistological sections were ranked on semi-quantitative scales (1–4) for vacuolization of the liver (1, <25% of total area; 2, 25-50% of area; 3, 50-75% of area; 4, >75% of area vacuolated) and the presence/absence of proteinaceous fluid was also noted. The developmental stage of the gonad (testis or ovary) was also histologically evaluated based on the percentage of cell types observable in the field of view (female: perinuclear oocyte, cortical alveolar, early vitellogenic, late vitellogenic; male: spermatogonia, spermatocyte, spermatid, spermatozoa). All assessments of histological samples were performed blinded to eliminate observational bias by the assessor. Random slides (~10%) were evaluated a second time to determine between analysis variance. The values obtained from the two analyses differed by <3%.

3.2.4 Statistical Analysis

Males and Females were separately analyzed using multivariate analysis of variance (MANOVA) with temperature, concentration specified as fixed factors and HSI, GSI, CF, glucose, and plasma vtg concentration as dependent variables. Histological data was analyzed using two-way ANOVA followed by Tukey's multiple comparison post hoc test. Dependent variables were log transformed where necessary to improve normality. Where appropriate, pair-wise post hoc tests (Least Significant Difference; LSD) were used for comparison of dependent variables across levels. All analyses were conducted using SPSS (v21).

3.3 Results

3.3.1 Water Quality and Exposure Conditions

Water quality remained stable throughout the experiment (dissolved oxygen = 5.50 ± 0.94 mg/L; pH = 8.34 ± 0.23; and chlorine: undetectable) and were well within species tolerance limits (Holland et

al., 1974). Measured E1 concentrations (mean ± SD) were 90 ± 17.6 and 414 ± 146 ng/L for the $E1_{LOW}$ (n = 16 samples) and $E1_{HIGH}$ (n = 15 samples) treatments, respectively. Estrone was not detected in control water samples. Water temperatures throughout the exposure period were 16.1 ± 1.05, 18.5 ± 0.60, 21.9 ± 0.39, and 24.2 ± 0.65°C, for the 15, 18, 21, and 24°C treatments, respectively. Water quality and exposure conditions are separated by temperature and treatment (Table 3.1).

Table 3.1: Descriptive water chemistry of measured E1 concentrations collapsed across all temperatures and the exposure conditions, temperature, and dissolved oxygen from four subsequent 30-day exposure experiments with mature adult bluegill sunfish. Average ± SD calculated for all measured parameters with sample size in parenthesis.

Treatment Group	Measured E1 concentration (ng/L)	Nominal Temperature (°C)	Measured Temperature (°C)	Measured Dissolved Oxygen (mg/L)
Ethanol control	No E1 detected (16)	15	15.5 ± 0.68 (32)	6.11 ± 0.89 (10)
		18	18.3 ± 0.53 (32)	5.46 ± 0.55 (10)
		21	21.5 ± 0.46 (29)	6.10 ± 0.62 (9)
		24	23.8 ± 0.59 (28)	5.77 ± 0.64 (9)
E1 Low	90 ± 17.6 ng/L (16)	15	15.7 ± 0.71 (32)	6.57 ± 0.63 (10)
		18	18.1 ± 0.53 (32)	6.18 ± 0.44 (10)
		21	21.5 ± 0.44 (29)	6.04 ± 0.62 (9)
		24	23.8 ± 0.59 (28)	5.61 ± 0.81 (9)
E1 High	414 ± 146 ng/L (15)	15	15.5 ± 0.73 (32)	6.61 ± 0.66 (10)
		18	18.2 ± 0.52 (32)	5.58 ± 0.34 (10)
		21	21.5 ± 0.39 (29)	5.86 ± 0.60 (9)
		24	23.8 ± 0.45 (28)	5.77 ± 0.41 (9)

3.3.2 Fish Survival

Survival during the exposure period was not affected by E1 exposure or water temperature for either minnow larvae or sunfish. Survival at day 30 was high and consistent across treatments, ranging from (mean ± SD) 88.25% ± 3.7% to 93.5% ± 11.1% for sunfish. Survival for each temperature and treatment can be found in table 3.2.

Table 3.2: Survival of adult bluegill sunfish for each treatment (EtOH control, E1LOW, and E1HIGH) and temperature (15, 18, 21, 24°C).

Treatment Group	Nominal Temperature (°C)	Survival of Sunfish (%)
Ethanol control	15	93
	18	92
	21	92
	24	85
E1 Low	15	100
	18	97
	21	83
	24	73
E1 High	15	77
	18	100
	21	97
	24	100

3.3.3 Biological Responses

A total of 289 sunfish (males: n = 187, females: n = 102) were used to evaluate biological responses to estrogenic exposure at different temperatures. We found significant effects due to temperature and E1 treatment independently in the different measured endpoints. Most notably, males were significantly impacted by E1, while only vtg in females was impacted by E1.

3.3.3.1 Effects of E1 Exposure on Male Organismal Endpoints

There were significant effects of E1 exposure on the GSI in male bluegill sunfish (P=0.000; Figure 3.3A) with control males displaying higher values for GSI than those treated with E1LOW (P=0.0259) and E1HIGH (P=0.0288). Additionally, significant effects of E1 concentration on glucose (P=0.005; Figure 3.2C) were also observed. Blood glucose levels were higher in males exposed to E1LOW than in control males (P=0.0028) and those exposed to E1HIGH (P=0.0059). Significant effects of E1 concentration of plasma vtg (P=0.000; Figure 3.2E) were also observed. Plasma vtg concentrations were higher in both males exposed to E1LOW (P=0.0117) and E1HIGH (P<0.0001) in comparison to their control counterparts with males exposed to E1HIGH reaching the max level of detection.

Additionally, gonad maturity was significantly impacted by E1 exposure (Figure 3.3C; stages impacted: spermatogonia (P=0.002), spermatocytes (P=0.023), spermatozoa (P=0.000); stages not impacted: spermatids) Finally, there were no effects of E1 exposure in liver vacuolization in the adult male sunfish.

3.3.3.2 Effects of Temperature on Male Organismal Endpoints

Temperature had a significant effect on the condition factor in males (P=0.021; Figure 3.1) with CF being highest at 18°C (vs 15°C: P=0.0052; 21°C: P=0.0089). Temperature also significantly impacted HSI in males (P=0.000; Figure 3.2A), subjects reared at 15°C had significantly higher HSI values than those reared at higher temperatures (vs 18°C: P=0.0170; 21°C: P=0.0346; 24°C: P=<0.0001). Temperature also had significant effects to plasma vtg concentrations in males (P=0.000; Figure 3.2E) with males reared at 24°C displayed reduced plasma vtg concentrations than fish reared at lower temperatures (vs 15°C: P=0.0048; 18°C: P=0.0111; 21°C: P=0.0311). Additionally, temperature significantly impacted gonad maturity in male sunfish (Figure 3.3C; stages impacted: spermatogonia (P=0.028), spermatocytes (P=0.000), spermatids (P=0.000), spermatozoa (P=0.020)). Finally, there were no effects of E1 exposure in liver vacuolization in the adult male sunfish.

3.3.3.3 Effects of E1 Exposure on Female Organismal Endpoints

There were significant effects of E1 exposure on the plasma vtg concentrations in female bluegill sunfish (P=0.000; Figure 3.2F) with control females displaying lower plasma vtg concentrations than female bluegills exposed to E1HIGH (P=0.0011). Lastly, there were no observed significant effects from E1 exposure on the condition factor, HSI, glucose, liver vacuolization, GSI, or gonad maturity (histology) in adult female sunfish.

3.3.3.4 Effects of Temperature on Female Organismal Endpoints

Temperature had a significant effect on the HSI in females (P=0.000; Figure 3.2B) with fish reared at lower temperatures displaying increased HSI values compared to those reared at higher temperatures (15°C vs 21°C: p<0.0001; 18°C vs 21°C: P=0.0015). Temperature also significantly

impacted glucose levels in females (P=0.007; Figure 3.2D) with females reared at 15°C displaying higher glucose levels than those reared at higher temperatures (vs 21°C: P=0.0001; 24°C: P=0.0095). Temperature also significantly impacted plasma vtg concentrations in females (P=0.039; Figure 3.2F) with females reared at 15°C displaying higher concentrations than fish reared at higher temperatures (vs 21°C: P=0.0400). Additionally, temperature also had effects to the GSI of females (P=0.000; Figure 3.3B) with females at lower temperatures displaying increased GSI values compared to those reared at higher temperatures (15°C vs 21°C: P=0.0009; 18°C vs 21°C: P<0.0001; 18°C vs 24°C: P=0.0293; 21°C vs 24°C: P=0.0377). Lastly, temperature significantly impacted gonad maturity in females (Figure 3.3D; stages impacted: perinuclear oocyte (P=0.006), cortical alveolar (P=0.044), early vitellogenic (P=0.002); stages not impacted: late vitellogenic) Finally, there were no observed significant effects of temperature on the condition factor, liver vacuolization, or plasma vtg concentrations in adult female sunfish.

3.3.3.5 Interactive Effects of E1 Exposure and Temperature

There were no observed significant effects on adult male or female organismal endpoints due to the interaction of ambient water temperature with E1 exposure.

Table 3.3: Results of MANOVAs examining the effects of E1 concentration (control, E1LOW, or E1HIGH) and temperature (15, 18, 21, 24°C) on CF, HSI, glucose, vtg, and GSI. Significant effects are indicated in bold.

Independent Variable	Dependent Variable	Males		Females	
		F	**P**	***F***	**P**
Treatment	CF	1.095	0.337	0.850	0.431
	HSI	1.752	0.177	0.045	0.956
	Glucose	5.397	**0.005**	1.346	0.265
	vtg	109.388	**0.000**	9.325	**0.000**
	GSI	12.218	**0.000**	2.035	0.136
Temperature	CF	3.349	**0.021**	1.920	0.132
	HSI	8.268	**0.000**	6.152	**0.001**
	Glucose	2.473	0.064	4.249	**0.007**
	vtg	11.478	**0.000**	2.908	**0.039**
	GSI	1.137	0.337	7.593	**0.000**

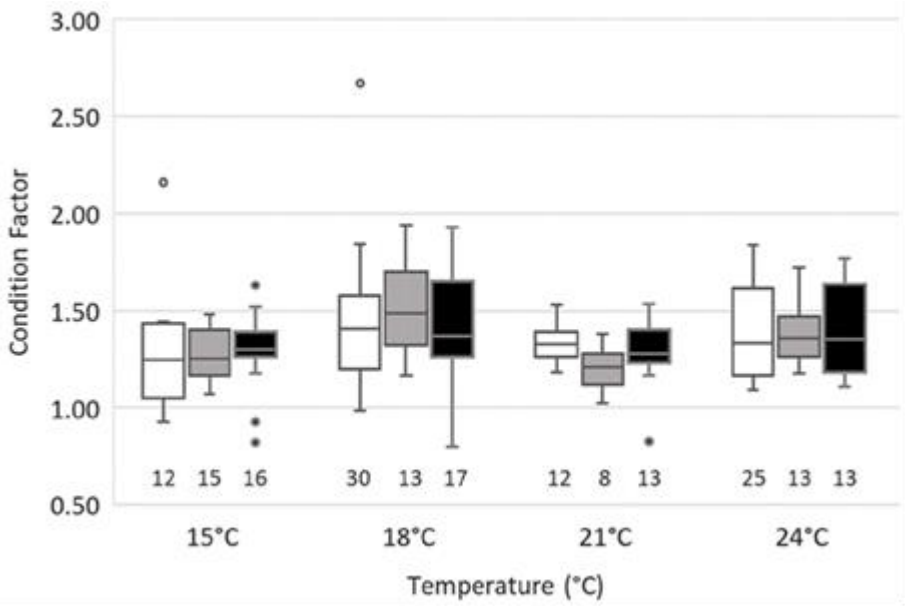

Figure 3.1: Significant effects of temperature on the condition factor in mature male bluegill sunfish. White boxes represent the EtOH control exposure group, gray boxes represent $E1_{Low}$ exposure group, and black boxes represent $E1_{High}$ exposure group. Sample sizes are given beneath each box and whisker.

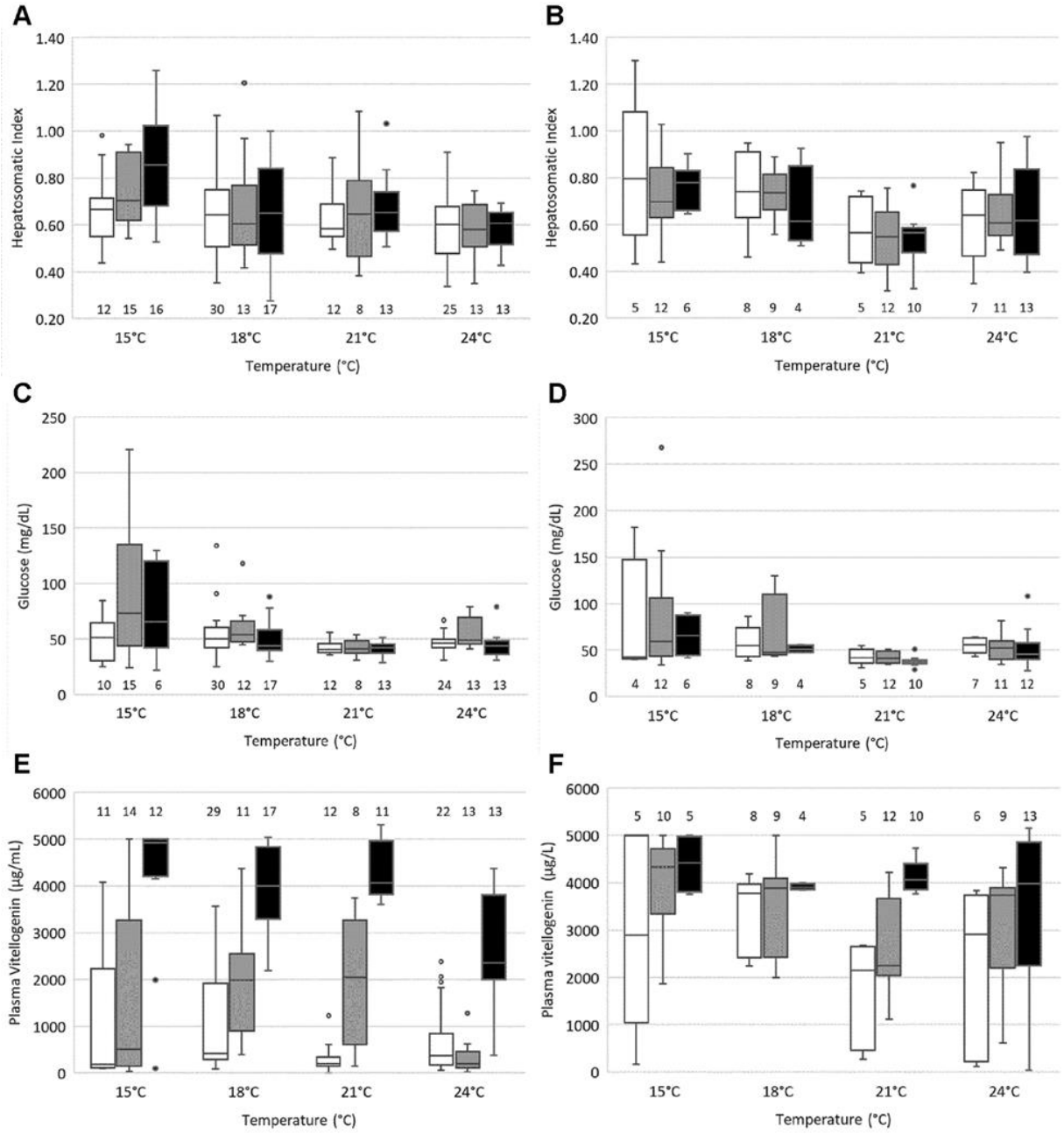

Figure 3.2: Biological responses in adult male and female bluegill sunfish relating to liver functions. (A) HSI in adult male sunfish. (B) HSI in adult female sunfish. (C) Blood glucose (mg/dL) in adult male sunfish. (D) Blood glucose (mg/dL) in adult female sunfish. (E) Plasma vtg concentrations (µg/mL) in adult male sunfish. (F) Plasma vtg concentrations (µg/mL) in adult female sunfish. White boxes represent EtOH control exposure group, gray boxes represent $E1_{Low}$ exposure group, and black boxes represent $E1_{High}$ exposure group. Sample sizes are given beneath or above each box and whisker.

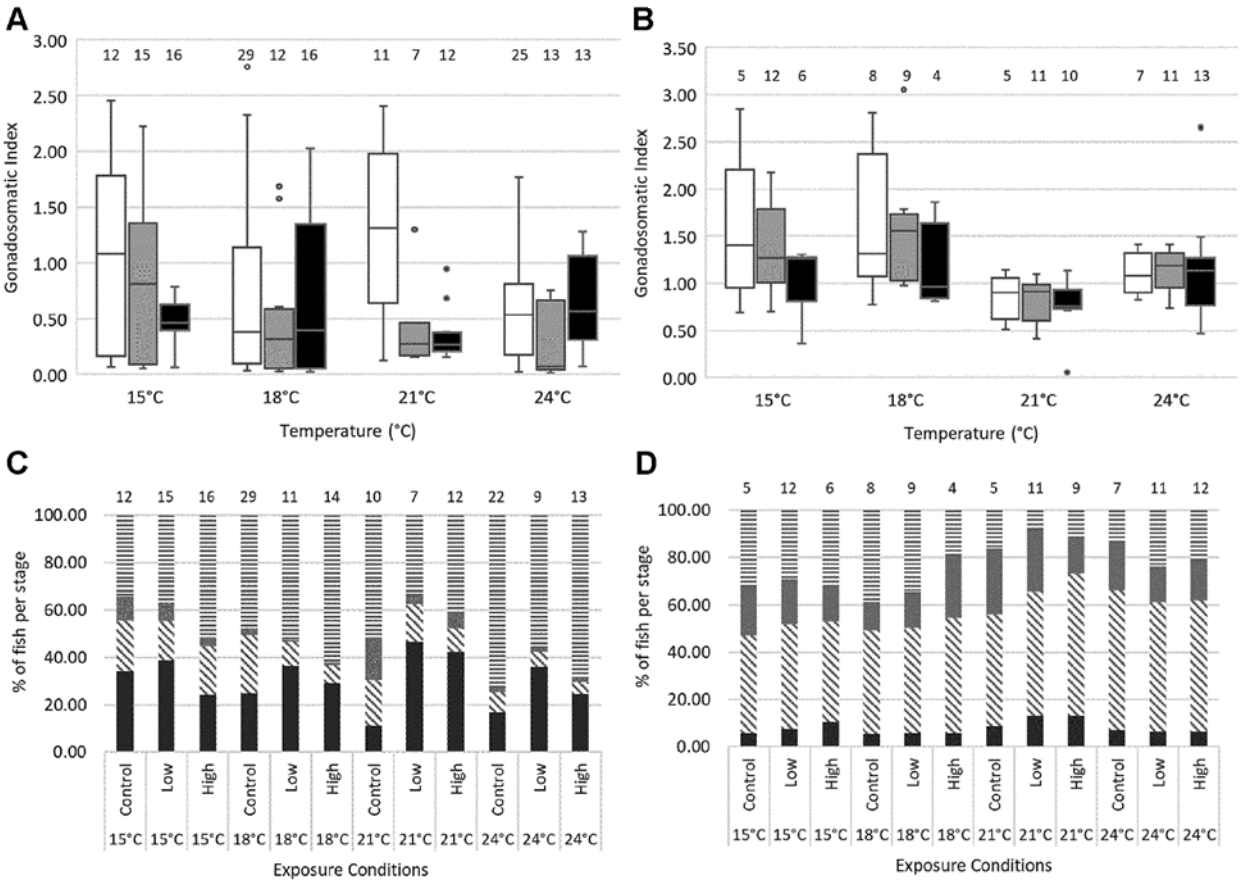

Figure 3.3: Biological responses in adult male and female bluegill sunfish relating to gonad functions. (A) GSI in adult male sunfish. (B) GSI in adult female sunfish. White boxes represent the EtOH control exposure group, gray boxes represent $E1_{Low}$ exposure group, and black boxes represent $E1_{High}$ exposure group. Sample sizes are given above each box and whisker. (C) Gonad maturity in adult male sunfish (from bottom to top: spermatogonia, spermatocytes, spermatids, spermatozoa). (D) Gonad maturity in adult female sunfish (from bottom to top: perinuclear oocyte, cortical alveolar, early vitellogenic, late vitellogenic). Sample sizes are given above each bar.

Table 3.4: Results of two-way ANOVAs examining the effects of temperature (15, 18, 21, 24°C) and E1 concentration (control, $E1_{Low}$ or $E1_{High}$) on maturity of gonadal cells. Significant effects are indicated in bold.

Sex	Independent Variable	Dependent Variable	*F*	P
Males	Treatment	% spermatogonia	6.523	**0.002**
		% spermatocytes	3.852	**0.023**
		% spermatids	21.108	**0.000**
		% spermatozoa	1.436	0.241
	Temperature	% spermatogonia	3.105	**0.028**
		% spermatocytes	6.318	**0.000**
		% spermatids	30.286	**0.000**
		% spermatozoa	3.363	**0.020**
Females	Treatment	% perinuclear oocyte	0.955	0.389
		% cortical alveolar	0.474	0.624
		% early vitellogenic	0.313	0.732
		% late vitellogenic	0.450	0.639
	Temperature	% perinuclear oocyte	4.460	**0.006**
		% cortical alveolar	2.803	**0.044**
		% early vitellogenic	1.972	0.124
		% late vitellogenic	5.193	**0.002**

3.4 Discussion

The goals of the current study were to (i) assess the biological response in fish exposed to E1 across a range of environmentally realistic water temperatures; and to (ii) link organismal effects seen as a result of estrogenic exposure to previously demonstrated decreased survival in prey and decreased capture success in predators (Korn et al., in prep). The results revealed three key findings. First, E1 concentration and water temperature exhibited significant independent effects on organismal responses in both sexes of adult bluegill sunfish. Second, male sunfish were more severely affected by E1 exposure than female sunfish. Third, the results show evidence that water temperature may modulate the effects of estrogenic exposure on fish, with the effects of exposure typically being more pronounced at cooler ambient water temperatures.

Male and female organisms are commonly affected differently by estrogenic exposures, which has been demonstrated in previous studies (Nash et al., 2004; Kidd et al., 2007; Cox et al., 2018). In the present study, males were more severely impacted by E1 exposure, displaying increased blood glucose concentrations (Figure 3.2C), increased plasma vtg concentrations (Figure 3.2E), GSI (Figure 3.3A), and reduced gonadal maturity (Figure 3.2C), in comparison to their female counterparts, which only displayed increased plasma vtg concentrations (Figure 3.2F). These findings are consistent with the effects seen in *Pimephales promelas* (Cox et al., 2018). While E1 is not the most potent of estrogens, it has been shown that it is a potent enough estrogen to disrupt normal functions in feedback loops and cause adverse effects (Tilton et al., 2001). Likely, because females naturally produce estrogens, experimental concentrations of E1 in the present study may not have been sufficient enough to elicit significant responses. In females, in instances when exogenous estrogen concentrations surpass endogenous concentrations, associated feedback loops can down regulate production in the body to mitigate potential effects of exposure to exogenous estrogens; however, it has been previously demonstrated that exogenous estrogens may be capable of disrupting estrogen feedback loops in aquatic organisms (Tilton et al., 2001). Males, in contrast, do not contain the same feedback loops as females, which likely contributes to why males were affected more severely by estrogenic exposure in the current study. Males that have been chronically exposed to estrogenic compounds may become feminized, in which they begin to lose secondary sex characteristics (Miles-Richardson et al., 1999), display impairments in normal reproductive functions (Woodling et al., 2006), and may even display intersex gonads (Jobling et al., 1998; Van Aerle et al., 2001; Woodling et al., 2006). These observed effects to males have the potential to ultimately influence reproduction, distribution, and abundance of the exposed species, which can lead to indirect effects on other species sharing the same ecosystem.

Many bodily processes, such as metabolic processes, are highly sensitive to ambient water temperature in ectothermic species, such as fish. In general, as water temperature increases, the metabolic

rate in fish also increases (Brett, 1964; Johnston and Dunn, 1987) which in turn accelerates the rates of enzymatic reactions (Voet et al., 2013). This relationship results in roughly a doubling of reaction rates under substrate saturation for every 10°C increase in temperature (Somero, 1978). The biosynthesis of a common biomarker for estrogenic exposure, vtg (Sumpter and Jobling, 1995), requires the use of enzymes (Hadley and Levine, 2006). In previous research, opposing results have been demonstrated. One study, using salmonids injected with estrogen, has suggested that as temperature increases so does the potency of estrogens (Korsgaard et al., 1986). Conversely, another study has demonstrated that E1 exposed male fathead minnows reared at cooler temperatures displayed elevated plasma vtg concentrations (Cox et al., 2018), suggesting that elevated water temperature may not increase estrogen potency. The present study also demonstrated elevated plasma vtg concentrations in E1 exposed male sunfish reared at cooler water temperatures (15, 18 and 21 °C) in comparison to their counterparts reared at 24 °C. Furthermore, plasma vtg concentrations were significantly lower in control males than in those exposed to either concentration of E1. The high-volume turnover rates of the flow through exposure system make it unlikely that reduced bioavailability of E1 occurred at higher water temperatures (Raman et al., 2001). Males reared in higher water temperatures may have exhibited decreased vitellogenesis in order to focus energy towards maintaining homeostasis at a higher metabolic rate (Luquet and Watanabe, 1986). This is supported by the reduced blood glucose concentrations (Figure 3.2C), reduced GSI (Figure 3.3A), reduced HSI (Figure 3.2A), increased condition factor (Figure 3.1), and increase in gonad maturity (Figure 3.3C) exhibited in E1 exposed males at higher temperatures suggesting that energetic reserves necessary for the biosynthesis of vtg were limited. Therefore, the findings of the current study suggest that the effects of estrogenic exposure are more severe at cooler water temperatures than at higher water temperatures. Ultimately, increased research efforts should be put towards studies examining the effects temperature can have on chemical exposures. Such exposures could potentially threaten the overall population of many aquatic organisms, particularly those already on the edge of their thermal tolerance

limits, by affecting metabolism, potentially requiring organisms to shift energy reserves to meet increased energetic demands and/or reproduction by affecting the seasonal timing at which various reproductive events occur (Smith, 1978; Brown et al., 1987; Miller et al., 2007; Elliot et al., 2014). Ultimately, because temperature can have effects on metabolism and likely contaminant induced changes, considering the impacts abiotic stressors, such as temperature, can have on chemical exposures is important.

It has been previously hypothesized that water temperature may influence the potency of various estrogens, with some studies showing evidence to support this hypothesis (Korsgaard et al., 1986; Ward et al., 2017; Cox et al., 2018), while others have shown evidence that doesn't support this hypothesis (Korn et al., in prep). The current study has found no statistically significant effects of interactions between E1 exposure and ambient water temperature; however, trends in the data have shown some evidence indicating potential temperature modulation of E1 exposure. The present study demonstrated evidence to support the hypothesis that temperature modulates estrogenic exposure, with concentration dependent effects being more pronounced at cooler water temperatures. In males, glucose (Figure 3.2C) was highest at 15°C, plasma vtg concentrations (Figure 3.2E) were higher at 15, 18, and 21°C, and condition factor (Figure 3.1) was lowest at 15°C. Additionally, in females, glucose (Figure 3.2D) was highest at 15 and 18°C, plasma vtg concentrations (Figure 3.2F) were highest at 15°C, and the gonads of females (Figure 3.3D) were less mature at 15 and 18°C. These findings are similar to a previously conducted study in which adult fathead minnows were exposed to different concentrations of E1 at four different temperatures (Cox et al., 2018). Cox and colleagues (2018) found significant interactions between temperature and E1 exposure in which exposed males displayed increased plasma vtg concentrations and glucose at cooler water temperatures, and exposed females exhibited increased glucose at cooler water temperatures. Ultimately, the interactions between contaminant induced changes and abiotic factors, such as temperature, are poorly understood and deserve further research efforts.

The effects displayed in single species studies can not only directly affect those exposed but can also indirectly affect other species that may interact with those directly affected, ultimately impacting complex species dynamics and food webs. Previous research has demonstrated both direct and indirect effects of estrogenic exposure, in which the collapse of the fathead minnow population following EE2 exposure resulted in a large population decrease in the trout population, likely due to decreased food sources (Kidd et al., 2007; Kidd et al., 2014). These studies provide evidence that exposure effects can impact multiple species and the complex interactions that exist between them. In addition to demonstrating indirect exposure effects to predator-prey interactions, previous studies have also demonstrated that estrogenic exposure can have direct adverse effects to predator-prey relationships in fish (Rearick, 2013; Korn et al., in prep), with predator species exhibiting a reduced ability to capture prey (Korn et al., in prep) and prey species exhibiting a reduced ability to escape predators (Rearick, 2013; Korn et a., in prep). The physiological effects of E1 exposure in the current study may be correlated to the 10% reduction in ability to capture prey as demonstrated in a related study (Korn et al., in prep). The physiological effects demonstrated in this study may have caused a shift in energy use in the sunfish, causing them to allocate more energy towards the maintenance of vtg biosynthesis, and increased blood glucose during times of stress, rather than focusing energy towards foraging and prey capture. Ultimately, species rarely exist in isolation in natural settings, making the use of multiple species studies more necessary in order to more accurately predict exposure effects in more real-world settings. Collectively, the results of this study provide evidence that temperature, similar to that observed during natural springtime variation, modulates the effects E1 exposure on adult bluegill sunfish.

Chapter 4: Conclusion

The necessity for evaluating interactions between chemicals and abiotic stressors, as well as the resulting effects to complex species interactions is becoming more apparent to researchers in the field of toxicology. The current study connects behavioral and physiological changes of organisms that were exposed to a common aquatic pollutant to broader ecological relevance. Common aquatic pollutants, such as endocrine active compounds that are estrogenic, have the capability to adversely impact native populations and individuals. As a key prey species in many aquatic habitats, adverse exposure effects to *Pimephales promelas* can potentially result in population declines that can have long-term impacts to piscivorous species relying on *P. promelas* as a food source (Kidd et al. 2007; Rearick, 2013; Kidd et al., 2014; Korn et al., in prep). Increasing the amount of research effort put towards understanding how abiotic factors modulate anthropogenic stressors, such as aquatic contaminants, to influence critical aspects of aquatic life such as behavior, reproduction, physiology, and growth and development, can ultimately offer increased insight for predicting effects to complex species interactions and population dynamics under ever-changing, real world scenarios.

Taking into account both of the aforementioned experiments, the data demonstrate that (i) organisms in the larval life stage are more susceptible to adverse effects from exposure to aquatic contaminants (ii) changes in ambient water temperature can influence chemical stressors in ways that negatively impact physiology, behavior, and ultimately survival and complex species relationships. The majority of evidence supporting thermal modulation of E1 exposure was observed between exposure treatments at the coldest tested temperature (15°C) and the highest tested temperature (24°C), with increased adverse effects typically displayed at 15°C, possibly due to a slower rate of E1 degradation at colder water temperatures. This hypothesis was supported by the finding that plasma vtg concentrations in male bluegill sunfish in this study were significantly lower at 24°C than at cooler temperatures.

The present study demonstrated that larval fathead minnows were particularly sensitive to estrogenic exposure, in which E1 exposed larval minnows survived predation 20% less than their control counterparts, which is consistent with previous toxicological research indicating that individuals in exposed populations are especially susceptible to adverse exposure effects during early life stages (Van Aerle et al. 2002; Liney et al. 2005; Sloman and McNeil 2012). Increased susceptibility of fish in early and still developing life stages is anticipated because the endocrine system is largely responsible for regulating many developmental processes (Janz and Weber 2000). Additionally, it is important to note that female biological endpoints displayed significant temperature effects with only plasma vtg concentrations being affected by E1 exposure, while male biological endpoints were affected by temperature similarly but affected by E1 exposure more severely. These differences in biological responses suggest that male and female fish that have been exposed to identical treatments respond differently. Ultimately, taken together, the data suggest that the effects of estrogenic exposure may be more pronounced when the water is cooler in the early spring season, with newly hatched larvae being particularly vulnerable, compared to when the water is warmer later in the season.

Increases in contaminant loads in aquatic environments and/or fluctuations in air and water temperatures as a result of climate change (O'Reilly et al. 2015) can potentially alter complex species interactions and population dynamics directly or indirectly through adverse effects to survival (Kidd et al., 2007; Kidd et al., 2014), reproduction (Kidd et al., 2007), physiology (Kidd et al., 2007), or altered behavior (Clotfelter et al. 2004; Tuomainen and Candolin 2011). Most organisms and habitats are anticipated to be influenced by pollution or climate change to some degree, however ectothermic species including fish, amphibians, and reptiles are likely more vulnerable to the effects of increased pollution and/or climate change (Noyes et al. 2009). Additionally, populations that are already living at the edge of their physiological tolerance limits are expected to be particularly vulnerable pollution and/or climate change. Ultimately, to more accurately identify particularly vulnerable populations and predict

population dynamics in real environmental settings, our understanding of the effects of interactions between anthropogenic stressors and natural abiotic factors, and of the resulting effects to complex species interactions needs to be expanded by allocating further research into this area.

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