12-2016

Modulation of Estrogenic Effects Via Temperature on Two Life Stages of Fathead Minnow

Megan K. Cox
St. Cloud State University, mkcox@stcloudstate.edu

Modulation of Estrogenic Effects Via Temperature on Two Life Stages of Fathead Minnow

by

Megan K. Cox

B.S., St. Cloud State University, 2014

A Thesis

Submitted to the Graduate Faculty of

St. Cloud State University

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science in

Cell and Molecular Biology

December 2016

Thesis Committee:

Dr. Heiko Schoenfuss, Chairperson
Dr. Jessica Ward
Dr. Mark Mechelke
Dr. Paige Novak

Abstract

Human-mediated environmental impacts can induce changes in the expression of complex behaviors within individuals, and alter the outcomes of interactions between individuals. Although the independent effects of a number of important stressors on aquatic biota are well documented (e.g., exposure to environmental contaminants), fewer studies have examined how natural variation in the ambient environment modulates these effects. In this study, factorial experiments were conducted to assess the influences of temperature and estrogen concentration on two life stages of fathead minnow (*Pimephales promelas*). Larval and adult minnows were exposed for 21 or 30 days, respectively, to 3 concentrations of estrone (nominally at 25, 125, and 625 ng/L) or to an ethanol carrier control (0 ng/L), at four water temperatures (15, 18, 21, and 24 °C) reflecting natural seasonal variation. A series of behavioral experiments were conducted to assess the independent and interactive effects of temperature and estrogen exposure on intra- and interspecific interactions in three contexts with important fitness consequences (i.e., reproductive behavior, foraging, and predator evasion). In addition, a series of anatomical and physiological endpoints were explored to assess the independent and interactive effects of chemical exposure on plasma vitellogenin induction, blood glucose, hematocrit, histology, and morphometric indices. Evidence was obtained suggesting that thermal regime can modulate the effects of exposure on larval survival, larval predator-prey interactions, and adult physiological and anatomical endpoints, even within a relatively narrow range of ambient temperatures. These findings improve our understanding of the outcomes of interactions between anthropogenic stressors and natural abiotic environmental factors, and suggest that such interactions can have ecological and evolutionary implications for freshwater populations and communities.

Acknowledgements

For my Grandmom,

Who inspired me through her actions,

And taught me I could be both strong and kind.

For my parents,

I hope I make you proud.

And for my advisors and greatest mentors,

Dr. Jessica Ward and Dr. Heiko Schoenfuss,

Whom without I may never have come this far,

Thank you for your encouragement and for your guidance.

Table of Contents

List of Tables

Table

List of Figures

Figure

CHAPTER 1: LITERATURE REVIEW

Introduction

Anthropogenic stress on the environment is of growing global concern. One form of stress of particular relevance to the environment is pollutant discharge. In recent years, the production, use, and disposal of a variety of agricultural, industrial, pharmaceutical, and naturally excreted compounds have led to significant adverse outcomes with respect to ecological and human health (Kolpin 2002). Many of these compounds have been classified as contaminants of emerging concern (CECs), broadly defined as recently detected chemicals in the environment or chemicals for which little is known, that have potential to cause health risk and ecological harm (US EPA 2014).

However, assessing the effects of CECs on individuals is complicated by the fact that rates of CEC degradation in the environment, and rates of uptake by aquatic organisms, are temperature dependent, and thus likely to be seasonally variable. Most established experimental protocols for toxicological experiments fail to take abiotic environmental factors, such as temperature, into account (Jin et al. 2009). Thus, little is known about how variability in ambient temperature modulates the effects of CECs on the behavior of fish and other aquatic organisms despite the fact that temperature-modulated effects of exposure (Smith 1978), such as an increased metabolic rate or reproductive dysfunction, could feasibly pose a threat to the stability of aquatic populations. To address this knowledge deficit, the aim of this research is to examine how temperature modulates the effects of a common estrogenic CEC, estrone (E1) on a freshwater forage fish, *Pimephales promelas* at two life stages (i.e., 21 days post-hatch and at reproductive maturity). Regulatory guidelines often focus the effects of exposures to xenobiotic compounds based on only a single (adult) life stage; however, the developmental processes of most fishes are heavily regulated by the endocrine system (Janz and Weber 2000) and individuals in exposed populations are therefore particularly susceptible to perturbation by CECs during the early ontogenetic

stages of life (Guillette et al. 1996; Koger et al. 2000; Metcalfe et al. 2000; Liney et al. 2005; Blazer et al. 2012). Thus, a robust understanding of the importance of the effects of CEC exposure on individuals and populations requires knowledge at multiple life stages (Staples et al. 2011).

Endocrine Disruption in Aquatic Environments

Endocrine disrupting compounds (EDCs) are a special class of CECs that are commonly found in aquatic environments and mimic or antagonize natural endocrine function through either modulation or disruption (Jobling et al. 1998). Examples of contaminants with endocrine active potential include steroid hormones, pesticides, plasticizers, and organic pollutants. These EDCs enter aquatic ecosystems through a variety of point and non-point sources, and are often pervasive and/or widely distributed throughout the environment.

Wastewater treatment plants discharge effluents that contain complex mixtures of chemicals with endocrine active properties, many of which are estrogenic in nature (Routledge et al. 1998; Kolpin et al. 2002; Thorpe et al. 2003). Numerous U.S. streams have been shown to contain measurable quantities of estrogenic organic wastewater contaminants (Kolpin et al. 2002). Although these compounds can be rapidly degraded in the environment, they are frequently present during critical periods of development and reproduction. Moreover, the continuous release of these estrogenic compounds through wastewater effluent generates a “pseudo-persistent” scenario wherein chemical half-lives are exceeded by effluent introduction rates (Daughton 2002). Exposure to estrogenic EDCs at critical life stages, such as during larval development or at sexual maturity, have been shown to result in adverse molecular, behavioral, and physiological effects in fish, including changes in the growth, behavior, and sexual differentiation of juveniles (Panter et al. 2002; van Aerle et al. 2002, McGee et al. 2009) and the development of ovo-testis and induced vitellogenin (vtg) production in males, reduced semen quality, inhibited somatic and gonadal growth, altered female fecundity, and changes in male and female aggressive and sexual behavior and/or secondary sexual characteristics in adults (Panter et al. 1998; Shioda and Wakabayashi 2000; Bjerselius et

al. 2001; Saaristo et al. 2010; Dammann et al. 2011). Evidence suggests that these individual-level changes can lead to population declines by reducing juvenile recruitment below threshold levels required for population persistence (Brown et al. 1987; Miller et al. 2007). Such a reduction in recruitment is hypothesized to have been responsible for the dramatic collapse of the fathead minnow (*P. promelas*) population in an experimental lake observed by Kidd et al. (2007), following chronic exposure to low levels of 17α-ethynylestradiol. This outcome suggests that the individual-level effects of persistent exposure to estrogenic compounds at environmentally observed concentrations can translate into impaired sustainability for aquatic populations.

Estrogens and Their Mechanisms of Action

Estrogens are steroid hormones that can either be produced naturally or synthetically. Female vertebrates naturally produce the estrogen hormones, E1, 17β-estradiol, and estriol (Norris 2007), which regulate a variety of biological functions. For example, female sexual development in humans is regulated by naturally occurring 17β-estradiol. 17α-ethynylestradiol is a commonly used synthetic hormonal contraceptive. In fish, estrogens also regulate sexual differentiation and reproductive function. Follicular development in fish originates in the hypothalamus, where gonadotropin-releasing hormone secretion occurs via direct innervations to the pituitary gland. The action stimulates the release of luteinizing hormone and follicular-stimulating hormone from the pituitary gland. Luteinizing hormone acts upon ovarian thecal cells to liberate androgens, which diffuse to the granulosa cells found in ovarian tissue. Follicle-stimulating hormone facilitates aromatase production in the ovary, converting androgens into estrogens (Hadley and Levine 2006). The gonadal steroid hormones, androgens and estrogens, are initially derived from cholesterol and are necessary for sexual maturation of both sexes. The first biosynthetic step converts cholesterol into androgens. Aromatization of androgens converts the compounds into estrogens. Testosterone is converted to17β-estradiol and androstenedione is converted to E1, with further conversion into17β-estradiol (Nelson and Habibi 2013).

The estrogens produced are targeted for two subtypes of nuclear estrogen receptors, estrogen receptor α and estrogen receptor β. Estrogen receptors α can be found in various tissue types including the liver and gonads, whereas estrogen receptors β are more exclusive to particular tissue types such as liver, brain, and bladder (Socorro et al. 2000). The estrogen 17β-estradiol is responsible for acting on hepatic estrogen receptors by binding the receptor and causing the activation and recruitment of co-activator proteins to bind to palindromic DNA sequences facilitating the transcription of vtg mRNA (Hoar and Randall 1988; Sumpter and Jobling 1995; Hadley and Levine 2006). Exogenous estrogens can facilitate a similar response through binding hepatic receptors, thereby mimicking natural estrogens (Ankley and Johnson 2004).

The Fathead Minnow: A Model Species in Ecotoxicology

Fish models are commonly used to evaluate organismal responses to endocrine disruption because the hypothalamic-pituitary-gonadal (HPG) axis of fish shares a high degree of conservation with that of other vertebrates. Thus, the responses of fish to EDCs can be extrapolated to infer potential modes of action and mechanisms to other vertebrates, including humans (Ankley and Johnson 2004). One model species commonly used in ecotoxicological studies is the fathead minnow, *P. promelas. Pimephales promelas* is a teleost fish that belongs to the family Cyprinidae (Geiger et al. 1988). It is a fractional spawner, meaning that fish can spawn continually for several months in the laboratory (Brungs 1971; Jensen et al. 2001). Because of this trait, and other advantages such as established culturing techniques, rapid sexual maturation, easy accessibility, and the capacity to withstand harsh conditions, *P. promelas* is a popular model aquatic organism for ecotoxicological research (US EPA 1987; Geiger et al. 1988; Jensen et al. 2001).

Fathead minnows are small-bodied, ray-finned fish, ranging in size from 2.5-7.5 cm long at maturity (Nelson and Paetz 1992). They are relatively short-lived with an average life span of 3-4 years; however, few live past 2 years of age in native habitats due to predation and other environmental factors (Kidd et al.

2007). *Pimephales promelas* is a popular bait fish for anglers (USGS 2015) and is ubiquitous throughout much of North America, partly due to anthropogenic introductions. They are habitat generalists that can be found in a variety of freshwater habitats across the geographic range, where they feed on algae, protozoa, and aquatic invertebrates (Zimmer et al. 2002). Although *P. promelas* prefers a slow-moving stream or pond habitat, the species has a high tolerance for variable or degraded environments consisting of elevated temperatures, turbid conditions, high salinities, variable pHs, and/or poorly oxygenated waters (Sommer 2011). Therefore, introduced *P. promelas* have been known to outcompete and consequently displace vulnerable native populations of congeners (USGS 2015).

The breeding season for reproductively mature *P. promelas* typically begins in May and extends through August. A single female may produce from 6800 to more than 10,000 eggs during a given year, and may participate in 16-26 spawning events (Ross et al. 2001). Spawning typically begins in the spring, once water temperatures reach approximately 15°C and under a 16:8 hour light:dark photoperiod. Spawning continues into early fall or until the ambient conditions are no longer favorable (Prather 1957; Duda 1989; Danylchuk and Tonn 2001). During the reproductive season fathead minnows exhibit strong sexual dimorphism; males develop a large, mucous-secreting fatty dorsal pad, nuptial keratinized tubercles, and a distinctive change in coloration (Smith 1974; Smith 1978). Reproductive success is dependent upon the ability of the male to acquire a mate and defend the nest site against other competitors (Sommer 2011; Martinovic-Weigelt et al. 2012; Fig. 1.1). Males utilize their tubercles by bumping intruders to guard their nest sites, and exhibit distinct territorial behavior during the breeding season (McMillian and Smith 1974). The mucous-secreting dorsal pad has fungicidal properties that the male rubs over the nest to disinfect the eggs (Kottelat and Freyhof 2007). It has also been postulated that the mucous may improve egg attachment and provide a chemical indicator marking the nesting site, providing a potential reproductive cue as to the status of the individual (Smith and Murphy 1974).

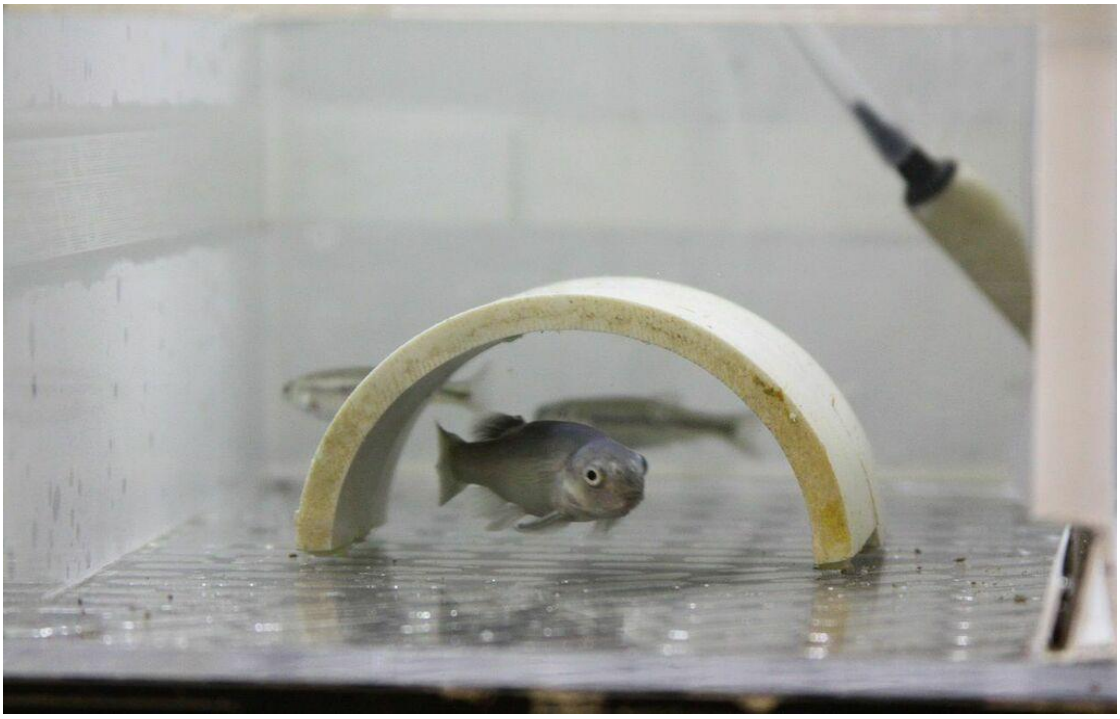

Fig. 1.1. Male *P. promelas* exhibiting nesting behavior while attempting to attract resident females**.** (Credit: Michelle Matsuura)

Temperature and the Effects of Estrogen Exposure on Aquatic Biota

Organisms live in an ever-changing environment, and some environmental factors can interact with chemical challenges to produce unpredictable organismal responses (Brian et al. 2008). Temperature is considered to be a primary factor of environmental influence on aquatic species, including teleost fish (Jin et al. 2009). For example, a 3-year experiment by Brown et al. (2006) showed that spawning patterns and egg quality in Atlantic halibut are influenced by temperature, indicating that temperature plays a vital role in the reproductive physiology and development of fish. Fathead minnows exposed to 17α-ethinylestradiol (0-32 ng/L) during the embryonic stage exhibited decreased growth (length/weight) at high concentrations (Parrott and Wood 2002). Brian et al. (2011) investigated the influence of temperature on the growth and reproductive status of *P. promelas*; while the authors reported no significant effect of temperature on sexual differentiation, they found that females reared at the highest

temperature had ovarian tissue that lacked germ cells and contained substantial amounts of undetermined tissue. In addition, both sexes of fish displayed variation in expression of secondary sexual characteristics. Estrogen exposure has also been found to decrease egg production and fertilization success (Dammann et al. 2011), thus potentially limiting egg availability and viability. Most fishes, including *P. promelas*, are ectothermic; therefore, water temperature has the potential to exert effects at multiple levels of organization, from molecular responses (i.e., the rate of vtg expression; Brain et. al. 2008; Körner et al. 2008) to ecological responses (i.e., the timing of reproduction; Gillet and Quétin 2006).

Whereas temperature and environmental estrogens have been individually shown to influence the physiology and reproductive functioning of fish, the outcome from the interaction between these factors have rarely been investigated (Körner et al. 2008; Jin et al. 2009). It is feasible that temperature-modulated changes in function (Smith 1978), such as an elevated metabolic rate or reproductive dysfunction, could pose a threat to population stability; but little is known with respect to long-term implications, or whether the effects of exposure at some particular life stages are more dependent on temperature than at others. Körner et al. (2008) examined the effects of temperature stress – measured as a cortisol response – on vtg expression in salmonids upon waterborne exposure of 17α-ethinylestradiol. The study found a positive temperature relationship with vtg synthesis, indicating that temperature modulates estrogen-induced vtg induction. A study investigating the relative potency and effectiveness of E1, 17β-estradiol, and estriol in sex reversal in the ectothermic red-eared slider (*Trachemys scritpa)*, which demonstrates temperature-dependent sex determination, found that varying temperature during incubation with administration of exogenous hormones produced a synergistic effect, skewing sex in a temperature and exposure consistent manner (Crews et al. 1996).

Temperature and Foraging Ability in Exposed Biota

The bioenergetic demands of consumption and metabolism are costly to any organism, but are especially costly to organisms under environmental stress. Feeding behavior is an extremely sensitive

indicator of environmental stress (Beitinger 1990). Smith and Weis (1997) reported that killifish from mercury-contaminated and reference sites differed in the frequency of prey capture attempts by 50%. A 28-day sub-lethal dioxin exposure also yielded a reduced feeding rate in juvenile trout (Mehrle et al. 1988). Brown et al. (1987) found that larval large-mouth bass exposed to 67 or 88 μg/L pentachlorophenol for 8 weeks post-hatch exhibited significantly fewer predacious attempts towards brine shrimp than unexposed larval fish. A study looking at the interactive effects of temperature and carbon dioxide on foraging behavior of juvenile coral reef fish found that foraging activity and food consumption decreased at the highest temperature parameter (31.5 ºC) (Nowicki et al. 2012). Thus, sustaining foraging consumption may diminish energy efficiency if the thermal optimum for food, assimilation, and growth has been exceeded, resulting in significant challenges for growth and survival.

Multiple pollutants have been shown to elicit decreases in food consumption, which could have severe repercussions for the survival, growth, and reproduction of exposed individuals (Beitinger 1990; Atchison et al. 1996). Preliminary studies investigating heavy metal exposure on foraging behavior have focused on the mechanistic parameters of feeding behavior such as consumption rate, feeding attempts, and capture efficiency; ultimately, these studies have generally found a significant decrease in food consumption (Beitinger 1990; Weber et al. 1991; Atchison et al. 1996). Fathead minnows exposed for 4 weeks to 0, 0.5 or 1.0 mg/L lead as lead acetate, a neurotoxin, were fed 20 *Daphnia magna*, and the total time spent foraging, the number of failed feeding attempts, and the foraging distance were observed (Weber et al. 1991). Compared to unexposed fish, the amount of time spent foraging, and the number of failed attempts to catch prey was significantly greater in exposed *P. promelas.* Combined with research demonstrating that the toxicant potency of estrogens increases with temperature (Korsgaard et al. 1986), it is reasonable to hypothesize that exposure to E1 will reduce feeding rates in *P. promelas* in a manner similar to other pollutants, and that these effects will be more pronounced at higher temperatures.

Temperature as a Modulator of Behavioral Interactions at Multiple Life Stages

Predator escape behavior exhibited by larval teleost fishes is induced by an external stimulus (Nissanov and Eaton 1989). The success of a predator escape maneuver varies greatly with motor performance, and with behavioral responsiveness (Webb and Zhang 1994) and the controlled directionality of the prey's response (Eaton et al. 2001). Previous studies have demonstrated that larval escape performance is impaired by even short-term exposure to environmental concentrations of EDCs, especially estrogenic compounds (McGee et al. 2009; Painter et al. 2009). McGee et al. (2009) established that juvenile *P. promelas* exposed to E1 for 12 days in a static renewal system exhibited an overall reduced predator avoidance response, delayed C-start latency period, and a decrease in total escape response performance. Both motor performance and responsiveness are susceptible to changes in ambient temperature (Webb and Zhang 1994), and thus elevated temperatures may exacerbate the effects of EDC exposure. Alternatively, in aquatic organisms, a decline in aerobic scope characterizes the commencement of thermal limitation (Pörtner and Knust 2007). The decline in aerobic scope is induced by the limited capacity of circulatory and ventilatory systems to match oxygen demand. A restriction such as this affects all levels of higher function, including muscular activity and behavior (Pörtner and Knust 2007). Exposure to E1 may also impair the detection and central sensory processing of the external stimulus (Eaton et al. 2001). Temperature sensitivity of the sensory pathway may be attributable to cellular and synaptic influences that establish M-cell excitability, and ultimately behavioral responsiveness (Fay and Ream 1992). On the other hand, Preuss and Faber (2003) found that acute changes of temperature had an opposite effect on C-start kinematics and behavior. Specifically, cooling slowed motor performance, and increased response latency and the likelihood of an inappropriate response towards the stimulus, suggesting that higher ambient temperatures result in a more balanced neuromuscular response during an attempted predation event.

Behavior is the product of intricate physiological and developmental processes. In males, the sex hormone testosterone mediates the expression of male agonistic behavior (Lephart 1996). Male fish rely upon intraspecific aggression to secure nesting sites, compete for mates, and defend the nest; the successful defense of a male's nesting site is crucial to his reproductive success (Martinovic-Weigelt et al. 2012). Numerous studies have shown that exposure to xenoestrogens decreases the expressions of male nesting (Brian et al. 2006) and aggressive behaviors (Bell 2001; Majewski et al. 2002; Coleman et al. 2009). Saaristo et al. (2010) observed the aggressive behavior of unexposed male sand gobies (*Pomatoschistus minutus*), and males exposed to 17α-ethinylestradiol, in response to unexposed intruding males during periods of courtship and nest defense. They found that exposed males were less aggressive towards rival males than control males; however, interestingly, this decrease in aggression did not result in an increase in nest takeover rates. It is reasonable to expect that male *P. promelas* exposed to E1 may show similar decreases in aggression performance compared to control males, which may be further exacerbated at elevated temperatures.

Dynamic Ecotoxicology

Ecotoxicology recognizes the need for a dynamic approach when linking cellular and behavioral responses to ecosystems; this thesis research connects physiological and behavioral changes of organisms exposed to aquatic contaminants to a broader ecological relevance, to account for natural abiotic factors, for example temperature. Bioactive compounds, such as estrogenic EDCs, have the capacity to cause harm to wildlife populations and individuals at multiple life stages via various modes of action. This research investigates how temperature modulates the effects of E1 on different life stages of *P. promelas* by measuring a suite of physiological, reproductive, and behavioral endpoints.

Pimephales promelas is a keystone prey species in aquatic environments, and population declines could have long-term impacts at several trophic levels (Kidd et al. 2007; Palace et al. 2009). Understanding how environmental parameters interact with anthropogenic stressors, such as EDC

exposure, to influence key metrics such as growth, development, reproduction, physiology, and behavior can provide more robust data for predicting population dynamics under real world scenarios. The results of these experiments could provide a baseline for regulatory agencies interested in assessing the ecological effects of environmental estrogens and climate change, and could be broadly extended to infer the effects of other contaminants, and at other aquatic trophic levels. The findings could also be used to improve population models by incorporating both reproductive and non-reproductive endpoints.

CHAPTER 2: THERMAL MODULATION OF ANTHROPOGENIC ESTROGEN EXPOSURE ON A FRESHWATER FISH, *PIMEPHALES PROMELAS*, AT TWO LIFE STAGES

Introduction

Human-mediated environmental changes to aquatic ecosystems are occurring at an unprecedented rate, with potentially severe repercussions for resident wildlife. Habitat alteration or loss (e.g., nutrient loading, increased sedimentation, or physical changes due to land-use), invasive species introductions, over-harvesting, and influxes of aquatic contaminants have globally recognized, clear, and adverse effects on the health and viability of aquatic biota (Keister et al. 2010). Such stressors are typically studied in isolation; however, interactions among multiple anthropogenic stressors, or between stressors and natural abiotic environmental factors (e.g., dissolved oxygen, pH, salinity, UV radiation or temperature) (e.g., Heugens et al. 2001; Crain et al. 2008; Holmstrup et al. 2010; Laskowski et al. 2010; Häder and Gao 2015) have significant potential to modulate or exacerbate the impacts of human-mediated environmental change at both individual and population levels. For example, interactions between inputs of inorganic nutrients (i.e., fertilizer) and organic matter have been shown to alter the dynamics of food webs in marine intertidal ecosystems (O'Gorman et al. 2012). Changes in the toxicities of aquatic contaminants in response to variation in UV-B exposure or salinity are also well documented (Hall and Anderson 1995; Pelletier et al. 2006). While the outcomes of these multi-factor interactions are often cumulative or synergistic, they can also be unpredictable (e.g., Christenson et al. 2006; Shears and Ross 2010; O'Gorman et al. 2012; see also Crain et al. 2008; Darling and Cote 2008), or vary across space or time (Newman and Clements 2008; Molinos and Donohue 2010), including life stage (Salice et al. 2011; Przeslawski et al.; 2015). Thus, concerted efforts aimed at understanding of the impacts of anthropogenic change under more complex, real-world scenarios are of key importance for predicting and mitigating adverse effects on aquatic ecosystems.

Freshwater fish populations are often geographically restricted, and are thus likely to be especially vulnerable to declines in abundance or extirpation due to anthropogenic stress (Dudgeon et al. 2006; Heino et al. 2009). Among the most pressing primary threats to freshwater fish is chemical pollution; urban, industrial and agricultural runoffs, and wastewater treatment plants, continually discharge contaminants into rivers and streams (Kolpin et al. 2002), many of which bind to organismal hormone receptors and disrupt the normal endocrine functioning of exposed individuals (Kuiper et al. 1998). Because rates of introduction typically exceed chemical half-lives (Daughton 2002), EDCs are frequently present in the environment during critical life stages, such as during early development or at reproductive maturity. Exposure to EDCs has been shown to induce a variety of adverse molecular, behavioral, and physiological effects in both juvenile and adult fish (e.g., van Aerle et al. 2002; McGee et al. 2009; Saaristo et al. 2010; Ward et al. 2012; Bhandari et al. 2015; Niemuth and Klaper 2015). Furthermore, empirical work and population modeling have convincingly demonstrated that these individual-level effects can dramatically impair the viability and sustainability of aquatic populations (Kidd et al. 2007; Palace et al 2009; Brown et al. 2015).

Efforts to assess the impacts of EDCs on natural populations, however, are complicated by the fact that rates of chemical degradation in the environment (e.g., Starner et al. 1999), and uptake and elimination by organisms (Gordon 2003), are dependent on the ambient temperature of the environment (Cairns et al. 1975; Heugans et al. 2001). In fish and other ectothermic aquatic species, temperature governs a wide array of fundamental physiological processes, including sexual determination, rates of early development, cellular signaling, biochemical reactions, and basal metabolic activity (Crockett and Londraville 2006; Ospina-Alvarez and Piferrer 2008), with potential to modulate the responses of organisms to toxicants in various ways (Heugens et al. 2001, 2003; Hallare et al. 2005; Khan et al. 2006; Brown et al. 2015). For example, increases in temperature have been shown to exacerbate EDC-induced production of vitellogenin (vtg; an egg yolk protein precursor normally only found in females) in juvenile

salmonids (Korsgaard et al. 1986; Mackay and Lazier, 1993; Korner et al. 2008), and to influence EDC-induced skewed sex ratios in zebrafish (Brown et al. 2015). At higher temperatures, EDC exposure also synergistically increases mortality and impairs embryogenesis (Osterauer and Kohler 2008). Cumulatively, the data collected to date suggest that chemical toxicants can interact with the thermal conditions in complex ways to influence mortality and physiological impairment (Heugans et al. 2001; Gordon 2003).

By comparison, little is known regarding the interactive effects of temperature and EDC exposure on the behavior of fish and other aquatic organisms (Manciocco et al. 2014). This deficit is significant, because an individual's behavior is the product of a complex set of integrated physiological and developmental responses to the environment (Clotfelter et al. 2004), and altered inter- and intraspecific trait-mediated behavioral interactions that impact individual fitness (e.g., predator-prey relationships, competition for resources, reproduction) have significant potential to reduce population abundances and alter the structure and function of aquatic communities (Clotfelter et al. 2004; Kidd et al. 2014).

In this study, we conducted a factorial experiment in the lab to determine the extent to which temperature modulates the survival, development, reproductive physiology and interspecific (foraging ability, predator evasion) and intraspecific (male-male competition) behavioral interactions of a freshwater fish, the fathead minnow (*Pimephales promelas*), exposed to a common environmental estrogen, estrone (E1) at two life stages (i.e., during larval development and at sexual maturity). Our aims were threefold; first, we tested the general hypothesis that temperature modulates the dose-dependent effects of estrogen exposure at both larval and adult life stages. Second, we assessed the extent to which independent and interactive effects of E1 exposure and temperature differ across fitness contexts (i.e., predator evasion, foraging efficiency, territorial defense). Third, we compared the general susceptibility of fish to behavioral impairment during early development, and at sexual maturity. To date, most single studies have focused on the effects of exposures to endocrine disrupting compounds at a single life stage

(but see Parrott and Blunt 2005; Oliveira et al 2009; Schultz et al. 2012 for examples to the contrary); but growth and survival during the early stages of life, and successful reproduction at maturity, all directly impact individual fitness. Thus, knowledge regarding the effects of contaminant exposure at multiple life stages is a prerequisite to accurately assessing and predicting impacts under complex, real-world scenarios.

Methods and Materials

Experimental Design

To test the hypothesis that the biological effects of estrogen exposure are modulated by ambient temperature, we exposed breeding groups of fathead minnows (two mature females, one male) to a low, medium or high concentration of E1 (i.e., $E1_{LOW}$, $E1_{MED}$, or $E1_{HIGH}$) dissolved in ethanol (EtOH), or to EtOH alone (Control), at one of four temperatures (15, 18, 21, 24°C) for 30 days (16 total treatments; 10–14 breeding groups per treatment). These temperatures reflect natural spring and summer seasonal variation in northern temperate streams, rivers and lakes and are well within the thermal tolerance limits for *P. promelas* (Pyron and Beitinger 1993). Throughout the exposure period we monitored the fecundity and fertility of females and males. Beginning on day 10 and lasting through day 17, we collected one clutch of eggs from each breeding pair and placed it in a breeding basket in the parental aquarium. On days 29 and 30, we tested the parental subjects in two behavioral assays designed to assess the independent and interacting effects of temperature and estrogen exposure on the foraging ability of males and females, and the territorial aggression of resident male fish towards a conspecific male intruder. We conducted two additional assays to assess the predator escape performance and foraging ability of exposed and control 21-day-old larval fish reared at different temperatures. All subjects were sacrificed immediately following the completion of testing via an overdose of MS-222 (Sigma-Adrich, MO, USA).

The subjects were dissected (adults) or stored in RNA*later*® (Thermo-Fisher Scientific, MA, USA) (larvae) for use in a separate study.

Subjects, Housing, and Maintenance

Six-month old, reproductively mature *P. promelas* were purchased from a certified disease-free culturing facility (Environmental Consulting and Testing, WI, USA) and shipped to St Cloud State University at bi-monthly intervals between March and July 2015. We chose *P. promelas* to test the hypothesis that the biological effects of estrogen exposure are modulated by ambient temperature because it is widespread in North America, and considered to be a model species for ecotoxicology research (Ankley and Villeneuve 2006). Upon arrival (day 0), the fish were introduced directly into the exposure apparatus and permitted to acclimate to their surroundings for 24 h before the experiment was started (day 1). The fish were maintained under a 16 h light: 8 h dark photoperiod, and fed an *ad libitum* diet of frozen brine shrimp (*Artemia franciscana*, San Francisco Bay Brand Inc., CA, USA) and bloodworms (*Glycera* spp., Brine Shrimp Direct, UT, USA twice daily for the duration of the experiment. F1 generation larvae were fed newly hatched brine shrimp (Brine Shrimp Direct, UT, USA) twice daily, beginning two days after hatching. The aquaria were cleaned of debris and monitored for mortality on a daily basis.

Exposure Chemicals

Estrone is a common natural estrogen discharged in wastewater effluent, and can be considered to be representative of a broad class of steroidal hormones and other chemicals with estrogenic activity (Kolpin et al. 2002). Powdered E1 (≥ 99%) was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in 100% EtOH (1687.5 μg/mL). In accordance with EPA guidelines for short-term exposure studies, this solution was then serially diluted with EtOH to produce low, medium (5x) and high (25x) treatment stock solutions with nominal concentrations of 67.5, and 337.5, and 1687.5 μg/mL, verified for accuracy before the start of the experiment using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The three E1 solutions, and an EtOH solvent control, were stored in amber glass bottles at 4°C

for the duration of the experiment. For all treatments, aqueous exposure solutions were prepared every three days in darkened glass carboys via the addition of an appropriate quantity of stock solution to 10 L of conditioned, non-chlorinated well water (two carboys per treatment). Each solution was thoroughly mixed by agitating the bottles for 10 s and the neck of each bottle was covered tightly with aluminum foil.

Exposure Apparatus and Regime

The fish were maintained throughout the exposure period in 12 L plexi-glass aquaria (30.5 x 30.5 x 30.5 cm) divided in half by the addition of a stainless steel mesh partition to accommodate two breeding groups, each of which was composed of one male and two females (total of 56 breeding groups; 10-14 aquaria per treatment). Each aquarium was covered on the sides and back with neutral-colored contact paper, and equipped with an air stone, a semi-circular polypropylene spawning tile, and a mesh basket to prevent egg predation. An LED strip light placed ~30 cm above each aquarium provided illumination.

Water amended with E1, or an equivalent volumetric percentage of EtOH, was continuously gravity-fed to the aquaria from eight stainless steel mixing chambers (two chambers per treatment). Each mixing chamber served eight aquaria. A Cole-Palmer Masterflex 7523-40 peristaltic pump (Vernon Hills, IL) was used to draw the exposure solutions from the carboys into the mixing chambers via stainless steel tubes. A continuous flow of ground water from a dedicated well was added to the exposure solution in each mixing tank at a flow rate of approximately 900 mL/min for estimated final E1 aquarium concentrations of 5, 25, and 125 ng/L (i.e., low, medium and high treatments). Throughout the 30-day exposure period, the incoming ground water was maintained at a constant temperature (i.e., 15, 18, 21 or 24 °C) via a thermostat-controlled head tank. Due to space and equipment restrictions, separate 30-day exposures were conducted for each temperature. The temperature order, and the spatial locations of the E1 mixing tanks relative to one another were randomized at the start of the study.

Water quality parameters (i.e., dissolved oxygen, total dissolved solids, pH, salinity, and temperature) were measured daily using a handheld multi-parameter sampling instrument (model 556

MPS, YSI Instruments, OH, USA). The presence of chlorine was monitored twice weekly using water quality test strips (Hach, CO, USA). In addition, water samples were collected in 1 L high-density polyethylene (HDPE) containers from the outflow of the stainless steel mixing tanks at three-day intervals throughout the exposure period and frozen at -20°C for chemical analysis of E1. The E1 concentrations of two randomly selected samples of each of the 12 treatments were measured at the conclusion of the experiment via LC-MS/MS (total n = 24 samples, 8 samples per concentration).

Reproduction, Survival, and Growth

During the exposure period reproductive groups were assessed daily for spawning activity. Fecundity (number of eggs laid in each clutch), and fertilization success (the proportion of fertilized eggs, identified by the presence of eyespots) were recorded for each clutch. Once eyespots appeared (~3 days) we removed the spawning tile and replaced it with a fresh one. However, between exposure days 10 and 17 we collected one clutch from each breeding group and placed into a breeding basket located in the parental aquarium. From these clutches we recorded latency to first hatch (in days, from the time that the eggs were laid) and the number of eggs that successfully hatched to produce free-swimming larvae; these larvae were reared in the exposure tanks to day 21 and then used in the behavioral assays.

Behavioral Assays

We performed four behavioral experiments designed to assess the independent and interactive effects of temperature and estrogen exposure on intra- and interspecific interactions in three contexts (reproduction, foraging, and predator evasion). All trials were performed in clean water. Larval assays were conducted on day 21 in a larval testing chamber at room temperature between 0800 h and 1300 h, under differential lighting. Depending on the experiment and the number of surviving larvae we tested the responses of one to six subjects from each clutch. Because male territory defense tests were conducted *in situ* (i.e., in each focal male's home tank), all adult assays were conducted on day 29 or 30 between 0800

h and 2200 h at the ambient temperature maintained during exposure. Each larval or adult subject was only used once in a given experiment.

Experiment 1: Effects of E1 and Temperature on Larval Escape Performance

The 'C-start' is a broadly conserved evasive locomotor fixed-action pattern response exhibited by fishes and other aquatic organisms (Domenici and Blake 1997; Hale et al. 2002). The response is initiated by the perception of a stimulus, and is manifested by bending the body into a C-shape followed by a bout of burst swimming away from the stimulus at a 90° angle. Importantly, variations in aspects of the response correlate with the probability of surviving a predatory attack (Walker et al., 2005).

We assessed larval escape performance using an established methodology for the quantification of fast-start (C-start) locomotive mechanics (McGee et al. 2009). Briefly, at the start of each trial, a randomly selected subject was placed into a clear-bottomed, 5-cm-diameter testing arena containing 10 mL of conditioned well water. The arena was positioned on a pad containing a vibrational chip used to deliver a non-point source stimulus to the subject. The pad was covered with a 1 mm x 1 mm grid to allow for quantification of the response, and illuminated via a Kessil A150 fiber optic light source (CA, USA) 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 (M1, Tucson, AZ) high-speed camera (1000 frames s^{-1}) positioned ~25 cm directly above the test arena.

One observer, who was blind to the treatment identity of the fish, quantified the latency to the induction of the escape response, escape velocity, turning angle, and total escape response from the videos using the software program ImageJ (NIH, MD, USA). Two anatomical landmarks, the anterior tip of the snout and the posterior tip of the tail, were digitized on each video and used to calculate total body length (TL). 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 body lengths (BL) per ms according to Blob et al. (2007). The total escape response of each subject was calculated as BL/(latency in ms + 40 ms); we included this value because it takes into account potential changes in both velocity and latency (McGee et al. 2009). We measured the turning angle as the angle of rotational movement, relative to the initial head position at the onset of the stimulus. Trials with latency responses less than 10 ms were considered to be false starts and were discarded prior to statistical analysis.

Experiment 2: Effects of E1 and Temperature on Larval Foraging Ability

We conducted a larval foraging assay to assess differences in foraging ability among the 16 treatments. The night before each trial, we placed two randomly selected test subjects from the same clutch in a 3.8-cm-diameter feeding arena containing 10 mL of conditioned well water. Test subjects were deprived of food for 18 h prior to testing to ensure complete evacuation of their digestive system, confirmed in preliminary tests by viewing the transparent larvae under a microscope. Trials were recorded for posterity using a Canon NTSC Optura 20 digital Hi-8 video camera positioned 40 cm directly above the test arena, and illuminated by the same fiber optic light source used in experiment 1.

Subjects were permitted to acclimate to the arena for 1 min at the start of each trial. Following the acclimation period, we administered a prey aliquot consisting of a known quantity of freshly hatched *Artemia* nauplii (mean 31 ±4, range 22-37) to the center of the arena via a glass pipette. The subjects were permitted to forage freely for 60 s, and then immediately euthanized via the lethal addition of 2 mL $NaCO_2$-buffered MS-222 administered directly to the test arena with a dedicated glass pipette. The test subjects were removed from the arena, and 2 mL of formalin was added to euthanize the surviving *Artemia*. One observer, who was blind to the treatment identity of the test subjects, counted the number of surviving *Artemia* under an Olympus dark-scope microscope (PA, USA). We subtracted the number of remaining prey from the initial quantity to obtain the average number of prey items eaten in each trial.

Similar to the procedure described for experiment 1, we used ImageJ to measure the TL of each fish in each trial from video images; we examined the relationship between size and consumption in preliminary analyses (see 'Statistics').

Experiment 3: Effects of E1 and Temperature on Adult Foraging Ability

For consistency with experiment 2, we determined the ability of individual adult fish to capture and consume prey using a similar, but appropriately modified procedure. Test subjects were deprived of food for 24 h prior to testing. At the start of each trial, one male or female fish was placed in a 20-cm-diameter stainless steel arena equipped with a 240-micron mesh bottom that permitted the passage of water. The arena was located in the center of a plastic chamber (40 x 30 x 23 cm) containing water at the appropriate exposure temperature (i.e., 15, 18, 21, or 24°C). One end of a piece of flexible PVC tubing was positioned at the center of the arena just under the water surface, to allow for food delivery. The other end of the tube was attached to a syringe mounted on a retort stand outside of the apparatus that was not visible to the test subject.

Subjects were permitted to acclimate to the arena for 10 min at the start of each trial. Following the acclimation period, we gravity-fed a prey aliquot of 30 mature *Daphnia pulex* in 10 mL conditioned well water to the center of the arena via the feeding tube. The subject was permitted to forage freely for 5 min before the test was stopped, and the subject removed from the arena via a hand net and measured for SL. We then removed the arena from the water chamber, drained the water, and counted the number of *Daphnia* that remained on the mesh. The arena was thoroughly washed between trials to remove any potential odor cues. For the same reason, we also replaced the water in the chamber between trials.

Experiment 4: Effects of E1 and Temperature on Male Territorial Aggression

All tests examining the independent and interactive effects of E1 exposure and temperature on the agonistic behavior of male minnows were conducted by a single observer; daily observations made during the exposure period indicated that all males readily established territories under the provided spawning tile (Ward, unpubl. data). In each test we presented the focal subject (i.e., the resident male) with a randomly selected, non-exposed, conspecific male intruder within a 9-cm-diameter x 12-cm-tall cylindrical glass jar capped with fine mesh. The size of the jar permitted limited movement of the stimulus male, thereby minimizing variation in intruder behavior across trials (Ward et al. 2006). In total, we used 26 stimulus males. Each male was used between 3 and 16 times and males were placed in an 8 L tank and given a 1-3 hour rest period after ~ 20-30 min of testing. After testing, we recorded the size (BL) of each stimulus male using digital calipers. The BL of the resident male was similarly obtained at the conclusion of the experiment. The resident (mean ± SD, 53.09 ± 4.98; range, 43.00-79.00) and stimulus males (52.01 ± 4.12; range, 44.81-61.61) tested were comparable in size across all treatments. However, we calculated the size ratio of the two males in each test and included this value as a covariate in preliminary statistical analyses (see 'Statistics').

To begin each trial, the jar was placed in the center of the tank, at a distance of 15 cm from the spawning tile. The behavior of each focal subject toward the intruding male was directly observed for 5 min from behind a blind, and the frequencies of two well-described aggressive behaviors, butting and strikes (MacMillan and Smith 1974, Pyron and Beitlinger 1989), were recorded in real time. A butt was defined as a slow approach toward the intruding male that culminated in closed-mouth contact between the snout of the resident male and the jar. A strike was defined as a fast approach toward the intruding male, accompanied by propulsive tailbeats that culminated in either a closed-mouth bump or open-mouth snap. In addition, we recorded the latency (in s) to the first agonistic response.

Statistics

We compared the level of spawning activity among treatments using a chi-square test based on the total number of clutches produced in each treatment. The effects of exposure concentration and temperature on clutch size, fertilization success, hatching latency, and larval survival were examined via ANOVAs; larval growth (TL on day 21) was tested via Generalized Estimating Equations (GEE) (Hardin and Hilbe, 2012), to account for possible genetic correlations among individuals from the same clutch. As appropriate, we arcsine or log transformed the proportions of fertilized eggs, surviving larvae, and larval TL to meet parametric assumptions. We used pairwise post-hoc tests (Least Significant Difference; LSD) to compare dependent variables across levels for significant effects.

We directly compared the responses of larvae from the 12 treatments to the simulated predator (experiment 1), and their foraging abilities (experiment 2), using GEE models. For experiment 1 we fit marginal models to each of our three continuous response variables (latency to first response, escape velocity and total escape response) using an identity link function. For experiment 2, we fit the model to the number of prey eaten specifying a Poisson distribution with a log link function. For each model we selected and validated the appropriate correlation structure using the Quasi Likelihood Under Independence Model Criterion (QIC) (Hardin and Hilbe, 2012; Pan, 2001). All models tested the effects of E1 concentration (Control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$), temperature (15, 18, 21, or 24°C) and the interaction between these two terms. In preliminary models for experiment 2 we also included the average TL of the two larvae in each trial as a covariate, to account for differences in prey consumption due to size. We did not find a significant main effect of size on the number of prey eaten in each trial, nor evidence of significant interactive effects with temperature or concentration. Therefore, we removed the TL term prior to final analyses.

We examined whether adult fish from the 16 treatments differed in the number of prey consumed (experiment 3) using a Generalized Linear Model (GLM) with a negative binomial distribution and log

link function, which corrects for the presence of zeros in the dataset (O'Hara and Kotze 2010). We specified temperature, E1 concentration, and sex (male or female) as fixed factors in the model and included the interaction terms. In preliminary models we examined the influence of size on prey consumption by including SL as a covariate. We did not find a significant main effect of size on the number of prey eaten in each trial, nor evidence of significant interactive effects with temperature or concentration. Therefore, we removed the SL term prior to final analyses.

We examined variation in male agonistic behavior towards conspecific males (experiment 4) using one-way ANOVAs with either the frequency of agonistic displays or latency to response specified as the dependent variable, and E1 concentration, temperature, and the interaction between these terms specified as fixed factors. Preliminary analysis indicated that numbers of butts and strikes were significantly positively correlated (Pearson correlation: $r = 0.54$, $P < 0.001$). Therefore, we additively combined these values for each male prior to statistical analysis. We also included the size ratio of the two males in each test as a covariate in preliminary statistical analyses but did not find significant main or interactive effects of the size difference between the resident and stimulus males on the number of, or delay in, agonistic responses performed by the resident male. Therefore, we removed the SL term prior to final analyses.

Results

Exposure Conditions

Collapsing over all temperatures, actual E1 concentrations (mean ± SD) were 14.17 ± 3.09, 25.43 ± 9.96 and 65.39 ± 27.70 for the low (n = 7 samples), medium and high (n = 8 samples per treatment) exposure treatments. Water temperatures remained stable through the exposure period; over all concentrations the mean (± SD) daily temperatures recorded for the 15, 18, 21, and 24°C treatments were 15.82 ± 0.82, 18.36 ± 0.74, 20.94 ± 0.64, and 23.45 ± 0.87°C, respectively. Water quality measurements (dissolved oxygen = 8.30 ± 1.74 mg/L; pH = 7.76 ± 0.30; conductivity = 0.91 ± 0.06 mS/cm; salinity

(0.45 ± 0.01 g/L; and chlorine: undetectable) were also relatively uniform throughout the experiment, and were within tolerance limits for the study species.

Reproduction, Survival and Growth

A total of 642 adult *P. promelas* was used in this study (428 female, 214 male). Adult survival on Day 30 was high (range: 92 - 99% across treatments) and similar across the 16 treatments ($\chi^2 = 0.999$, df = 14, P =0.95. The number of clutches produced during the exposure period was variable, ranging from 12 (in the $E1_{HIGH}$ treatment at 21°C) to 51 (in the control and $E1_{MED}$ treatments, both at 24°C) (Fig. 2.1A); however, the amount of spawning activity did not differ statistically among treatments ($\chi^2 = 1.120$, df = 9, P = 0.99).

We observed a significant effect of E1 exposure on the mean number of eggs laid in a single clutch (Table 2.1). Post-hoc tests indicated that females exposed to $E1_{HIGH}$ laid significantly fewer eggs than those in the $E1_{LOW}$ treatment (P = 0.006; Fig. 2.1B). We also found a significant effect of water temperature on clutch size (Table 2.1). In general, females maintained at lower temperatures laid more eggs than those maintained at higher temperatures (Fig. 2.1B). Post hoc tests revealed that clutches laid at 21°C had significantly fewer eggs than those laid at 15°C (P < 0.001) or 18°C (P < 0.001). Clutch sizes were also smaller at 24°C compared with 15°C (P = 0.001). By contrast, we did not observe a significant interaction between temperature and concentration on clutch size, or significant independent or interactive effects of concentration and temperature on fertilization success (Table 2.1; Fig. 2.1C); the proportion of fertilized eggs ranged from 0.60 ± 0.32 for fish maintained under control conditions at 15°C to 0.81 ± 0.21 for fish exposed to $E1_{LOW}$ at 15°C. Water temperature, but not E1 concentration or the temperature *x* concentration interaction, had a significant effect on hatching latency (Table 2.1). Collapsing across all concentrations, hatching latency was negatively related to temperature, with significant step-wise reductions in the duration of embryonic development observed with each temperature increase (all Ps < 0.001) (Figure 2.1D).

Larval survival on Day 21 varied across treatments from 4% ± 5% (in the $E1_{LOW}$ treatment, at 24°C) to 26% ± 16% (in the control treatment, at 15°C) (Fig. 2.1E). The number of larvae maintained at 21°C that survived to day 21 was insufficient for behavioral or statistical analysis; therefore, we excluded this temperature treatment in subsequent analyses. Although we did not observe significant main effects of either temperature or E1 concentration on larval survival, we did observe a significant temperature *x* concentration interaction (Table 2.1); mean (± SD) survival at 15°C was significantly greater for control subjects than for subjects exposed to $E1_{LOW}$ (9% ± 4%, P = 0.002), $E1_{MED}$ (9% ± 7%, P = 0.004) or $E1_{HIGH}$ (5% ± 3%, P < 0.001). Survival did not differ among exposure levels at 18°C or 24°C (all Ps > 0.05).

Rearing temperature, but not concentration or the associated interaction, had a significant effect on larval growth (Fig. 2.1F). Larval size (body length) on day 21 was positively linearly related to the ambient temperature (Table 2.1). Post-hoc tests indicated that larvae reared at 24°C were significantly larger than those reared at 15°C or 18°C (both Ps < 0.001). Larvae reared at 18°C were also larger than those raised at 15°C (P = 0.004).

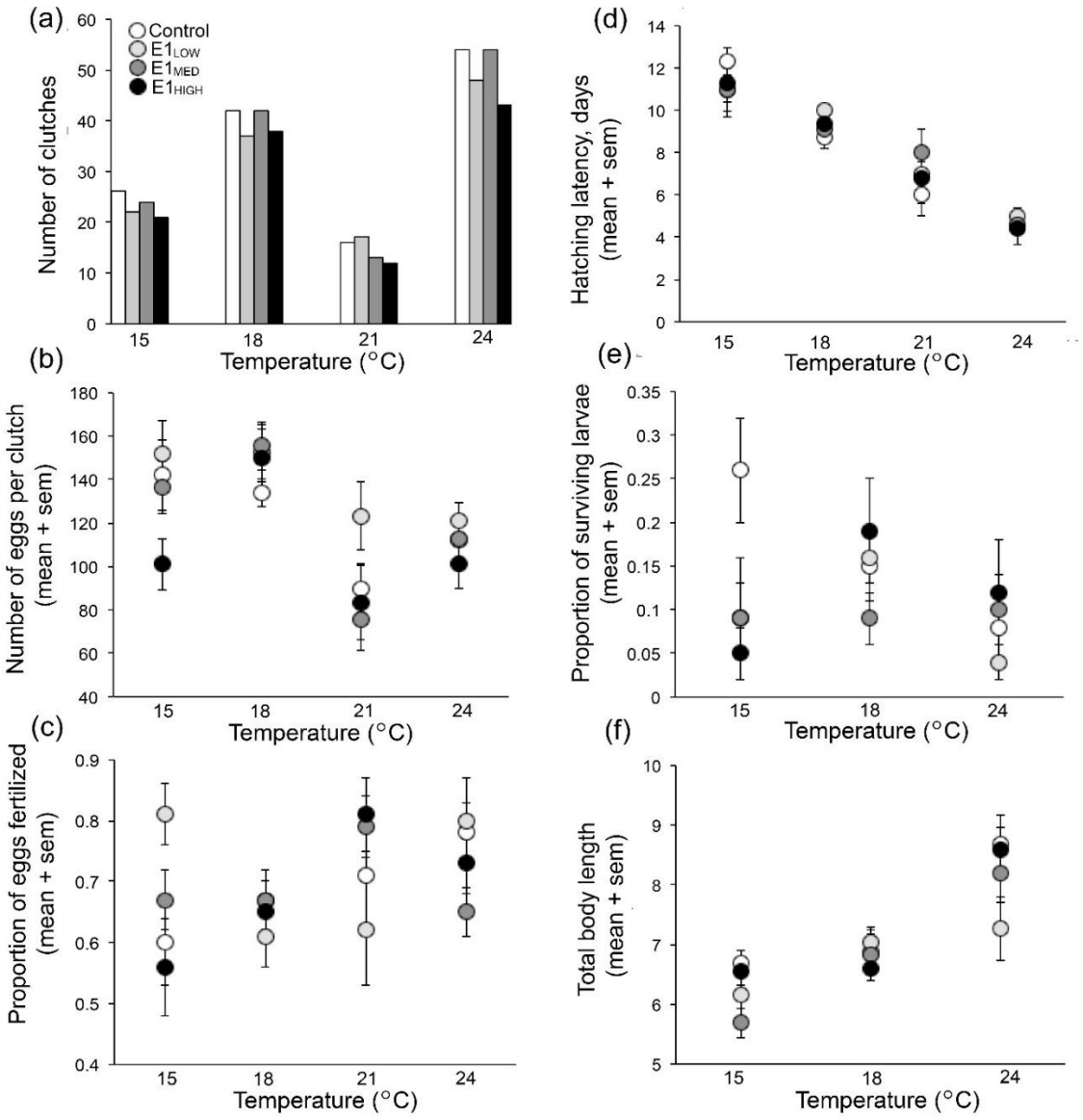

Fig. 2.1. Reproduction, growth, and survival of fathead minnows (*Pimephales promelas*) over a 30-day exposure period at four ambient temperatures. (A) Level of spawning activity observed in each factorial temperature and E1 exposure concentration combination (n = 9 – 51); (B) Female fecundity, indicated by the number of eggs (n = 9 – 51); (C) Male fertilization success, measured as the proportion of eggs laid that showed evidence of eyespots (n = 9 – 51); (D) Hatching latency, measured as the length of time (in

days) to the first day of hatching (n = 3 – 12); (E) Larval survival, measured as the proportion of successfully hatched eggs per clutch that survived to day 21 (n = 4 – 9); (F) Body size (total length) of larvae on exposure day 21 (n = 3 – 19). For all panels, white bars or symbols represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars or symbols represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (25ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± SEM.

Table 2.1: Results of ANOVAs or GEE examining the effects of temperature (15, 18, 21 and 24°C and E1 concentration (Control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on the survival, reproduction and growth of fathead minnows, *Pimephales promelas*, at two life stages. Significant effects are given in bold ($\alpha < 0.050$).

Parameter	**Effect**	**F**	***df***	***P***
Fecundity	Concentration	2.67	3,492	**0.047**
	Temperature	13.78	3,492	**<0.001**
	Concentration *x* Temperature	1.19	9,492	0.297
Fertility	Concentration	0.59	3,390	0.623
	Temperature	1.34	3,390	0.260
	Concentration *x* Temperature	1.72	9,390	0.083
Hatching latency	Concentration	0.24	3,103	0.871
	Temperature	59.20	3,103	**<0.001**
	Concentration *x* Temperature	0.70	9,103	0.700
Larval survival	Concentration	1.90	3,85	0.136
	Temperature	2.39	2,85	0.098
	Concentration *x* Temperature	2.49	6,85	**0.029**
Larval growth	Concentration	4.56	3	0.297
	Temperature	48.30	2	**<0.001**
	Concentration *x* Temperature	11.35	6	0.078

Experiment 1: Effects of Temperature and Concentration on Predator Evasion Performance

In total, we examined the innate, evasive locomotor responses of 152 larvae (n = 3 – 19 across treatments), depending on the number of surviving individuals available for testing. We found significant main effects of concentration and temperature on escape velocity, as well as a significant interaction between the two factors (Table 2.2). Overall, velocity was negatively related to temperature (Fig. 2.2A); average (± SD) escape velocities of larvae reared at 15, 18 and 24°C were 0.030 ± 0.003, 0.021 ± 0.001, and 0.019 ± 0.003 BL/ms, respectively. Pairwise post-hoc tests indicated that larvae reared at 15°C exhibited faster escape velocities than those maintained at either 18°C (P = 0.026) or 24°C (P = 0.002), but no other significant pairwise differences were observed (all Ps > 0.05). By contrast, escape velocities showed an inverted 'U' shaped distribution with respect to exposure concentration (Fig. 2.2A); average (± SD) escape velocities of larvae exposed to control, $E1_{LOW}$, $E1_{MED}$ and $E1_{HIGH}$ were 0.022 ± 0.002, 0.026 ± 0.003, 0.026 ± 0.002, and 0.022 ± 0.003 BL/ms, respectively; larvae exposed to $E1_{MED}$ were significantly faster than subjects in either the control (P = 0.006) or $E1_{HIGH}$ (P = 0.003) treatments. Pairwise comparisons indicated that escape velocities were statistically similar among all other treatments (Ps > 0.05). We also observed a significant interaction between E1 concentration and temperature that influenced escape velocity (Table 2.2), indicating that temperature modulates the effects of E1 exposure on escape performance; at 15°C subjects exposed to $E1_{MED}$ exhibited a significantly enhanced escape speed compared to control subjects (P = 0.005). Escape velocities were similar among control and exposed subjects at higher temperatures (all pairwise *P*s > 0.05 at 18°C and 24°C).

Temperature, but not exposure concentration, also had a significant effect on latency (Table 2.2); collapsing over all temperatures, the mean (± SD) latencies in the 15, 18, and 24°C treatments were 87.97 ± 96.95, 130 ± 134.95, and 171.91 ± 195.40 ms, respectively, suggesting that latency is negatively related to thermal regime (Fig. 2.2B). Post-hoc tests indicated that the latencies of subjects reared at 24°C were

significantly longer than those raised at 15°C (P = 0.013); no other significant pairwise differences were found (Ps > 0.05).

We did not detect significant effects of exposure concentration or temperature, nor a significant temperature *x* concentration interaction in GEE models examining the total escape response or the turning angle ($0.06 \leq P \leq 0.89$; Table 2.2; Fig. 2.2C, 2.2D). However, the total escape response exhibited a linear trend consistent with the interpretation that performance decreases with increasing temperature (Fig. 2.2C); over all concentrations tested, the mean (± SD) total escape responses for subjects reared at 15, 18, and 24°C were 0.008 ± 0.007, 0.006 ± 0.007, and 0.005 ± 0.007, respectively.

Table 2.2: Results of GEE models examining the effects of ambient temperature (15, 18, and 24°C and E1 concentration (Control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on larval predator evasion performance. Significant effects are given in bold ($\alpha < 0.050$).

Parameter	**Effect**	χ^2	***df***	***P***
Velocity	Concentration	10.71	3	**0.013**
	Temperature	10.00	2	**<0.007**
	Concentration *x* Temperature	14.37	9	**0.026**
Latency	Concentration	1.76	3	0.625
	Temperature	6.87	2	**0.032**
	Concentration *x* Temperature	10.32	6	0.112
Total escape response	Concentration	3.57	3	0.312
	Temperature	4.88	2	0.087
	Concentration *x* Temperature	12.02	6	0.061
Angle of escape	Concentration	2.80	3	0.423
	Temperature	1.30	2	0.521
	Concentration *x* Temperature	2.29	6	0.891

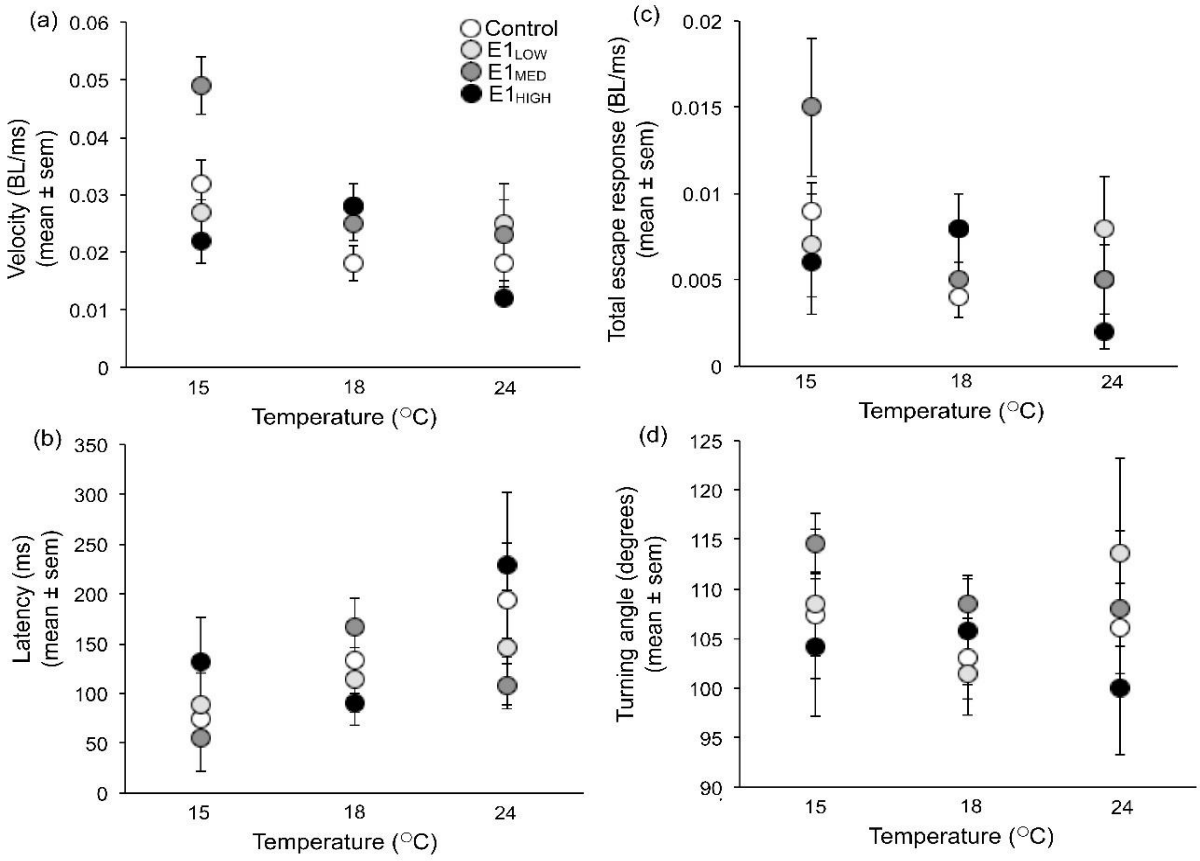

Fig. 2.2. Predator evasion responses of larvae exposed to E1 or an EtOH solvent control (Control), at alternative ambient temperatures. Sample sizes ranged from 3 to 19 across treatments. (A) Escape velocity (BL/ms) over the first 40 ms of the response; (B) Latency (in ms) to the induction of the response; (C) Total escape response (BL/ms); (D) Turning angle, relative to the initial position of the head (in degrees). White circles represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black circles represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (25 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± sem.

Experiment 2: Effects of Temperature and Concentration on Prey Capture Success of Larval Minnows

In total we conducted a total of 144 foraging trials (n = 3 – 23 across treatments, depending on the number of surviving larvae available for testing). The average proportion of prey successfully identified, localized and captured by larvae in each of the 12 treatments is shown in Fig. 2.3A. The GEE revealed a significant effect of temperature on capture success (Table 2.3). Overall, prey capture increased linearly with rearing temperature; collapsing over all concentration levels, the mean (± SD) numbers of prey consumed at 15, 18, and 24°C were 10.5 ± 0.82, 14.97 ± 0.68, and 17.52 ± 0.85, respectively. Post-hoc tests revealed that prey capture was significantly enhanced at each step-wise increase in temperature ($0.012 \leq P < 0.001$). We did not detect a significant main effect of E1 concentration on prey capture success (Table 2.3). However, we did find a significant temperature *x* concentration interaction (Table 2.3), suggesting that temperature modulates the effects of estrogen exposure on larval foraging ability. Post-hoc comparisons indicated that at the lowest temperature tested (15°C), subjects exposed to $E1_{MED}$ consumed significantly fewer prey than those in the Control ($P = 0.013$) or $E1_{LOW}$ treatments. Capture success did not differ among fish reared at different exposure concentrations at higher temperatures (18 or 24°C; all pairwise Ps > 0.05).

Table 2.3: Results of GEE examining the effects of ambient temperature (15, 18, and 24°C) and E1 concentration (Control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on the consumption of live prey by larval *Pimephales promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	**F**	***df***	***P***
Concentration	5.80	3	0.122
Temperature	30.66	2	**<0.001**
Concentration *x* Temperature	22.14	6	**<0.001**

Experiment 3: Effects of Temperature and Concentration on Prey Capture Success of Adult Minnows

The average proportions of prey successfully identified, localized and captured by either male or female subjects in each of the 16 treatments are shown in Fig. 2.3B. A total of 364 subjects (183 male and 181 female) were used in foraging trials (n = 12 – 28 across treatments). The GLM revealed a significant main effect of temperature (15, 18, 21, or 24 °C) on the number of prey captured (Table 2.4). Similar to the findings for experiment 2, capture rates were greater at higher temperatures. Pairwise post-hoc tests indicated that subjects consumed more prey at 24°C than at lower temperatures (21, 18, or 15°C; all Ps ≤ 0.006). Subjects also consumed more prey at 21°C than 18°C (P = 0.011). Only one exception to this general trend was observed; subjects consumed more prey at 15°C than at 18°C (P = 0.005).

By contrast, we did not observe significant main effects of either sex or E1 concentration on subject responses, nor significant interaction terms involving these factors (Table 2.4). These results indicate that male and female subjects captured and consumed prey at similar rates, and that capture success was unaffected by E1 exposure.

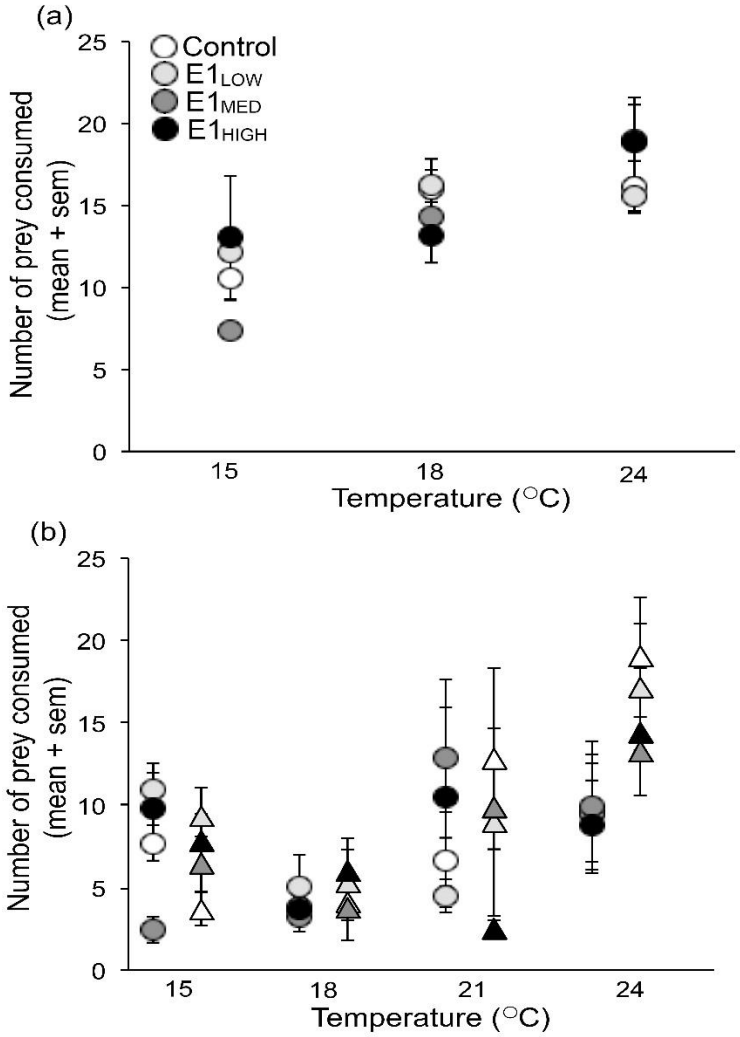

Fig. 2.3. Prey consumption by (A) larvae or (B) adult subjects exposed to varying concentrations of E1 or solvent (Control) at different ambient temperatures. Sample sizes across treatments in (A) ranged from 3 to 23. Sample sizes in (B) ranged from 12 – 28. White symbols represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black symbols represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (25 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments, respectively. Points and error bars depict means ± sem. Circles and triangles in (B) represent the number of prey consumed by males and females, respectively.

Table 2.4: Results of GLM examining the effects of temperature (15, 18, 21, and 24°C), E1 concentration (Control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) and sex on the consumption of live prey by adult *Pimephales promelas*. Significant effects are given in bold ($\alpha < 0.050$).

Effect	**F**	***df***	***P***
Concentration	1.32	3	0.725
Temperature	49.69	3	**<0.001**
Sex	1.23	1	0.268
Concentration *x* Temperature	15.37	9	0.081
Concentration *x* Sex	2.23	3	0.526
Temperature *x* Sex	4.60	3	0.203
Concentration *x* Temperature *x* Sex	14.99	9	0.091

Experiment 4: Effects of Temperature and Concentration on Territorial Aggression

In total, we examined the responses of 207 males towards a conspecific intruder (n = 8 – 16 across treatments). Resident males consistently responded to the presence of an intruder by approaching the restrained male and performing aggressive displays (bumps and strikes); 127 of the 207 subjects tested (61%) exhibited at least one act of territorial aggression towards the intruder. Of the 80 subjects who failed to exhibit an aggressive response, 32 (40%) were exposed to the highest concentration of E1 ($E1_{HIGH}$). Accordingly, there was a significant main effect of E1 exposure concentration on the number of aggressive acts (i.e., the combined number of bumps and strikes) performed by resident males (Table 2.5). Males exposed to $E1_{HIGH}$ performed fewer aggressive acts than males exposed to $E1_{MED}$ (P = 0.005) or

control subjects (P = 0.005) (Fig. 2.4A). No other significant pairwise differences among exposure treatments in the number of aggressive were observed (all Ps > 0.05).

Temperature also had a significant effect on male intraspecific interactions (Table 2.5). Males maintained at 21°C were significantly more aggressive than those maintained at any other tested temperatures (i.e., 15, 18, or 24°C) ($0.048 \leq P\text{s} \leq 0.001$) (Fig. 2.4A). Subjects maintained at other temperatures did not differ in the number of territorial aggressive acts performed (all *Ps* > 0.05). However, we did not find evidence that temperature significantly modulates the effects of E1 exposure (Table 2.5)

By contrast, neither E1 concentration nor temperature had independent or interactive effects on the latency to first response by resident males (Table 2.5), although there was a trend for latency to decrease with increasing temperature (Fig. 2.4B); over all concentrations the average latencies (± SD) of subjects maintained at 15, 18, 21, and 24°C were 92.70 ± 85.58, 97.03 ± 80.55, 77.62 ± 84.72, and 56.94 ± 71.24, respectively**.**

Table 2.5: Results of ANOVA examining the effects of temperature (15, 18, 21, and 24°C) and E1 concentration (Control, $E1_{LOW}$, $E1_{MED}$, $E1_{HIGH}$) on aggressive displays performed by a territorial male *Pimephales promelas* towards a conspecific intruder, and the latency to first aggressive response. Significant effects are given in bold ($\alpha < 0.050$).

Parameter	**Effect**	**F**	***df***	***P***
Aggressive displays	Concentration	3.80	3,191	**0.011**
	Temperature	5.90	3,191	**0.001**
	Concentration *x* Temperature	0.98	9,191	0.462
Latency	Concentration	1.50	3,111	0.219
	Temperature	1.55	3,111	0.205
	Concentration *x* Temperature	0.73	9,111	0.685

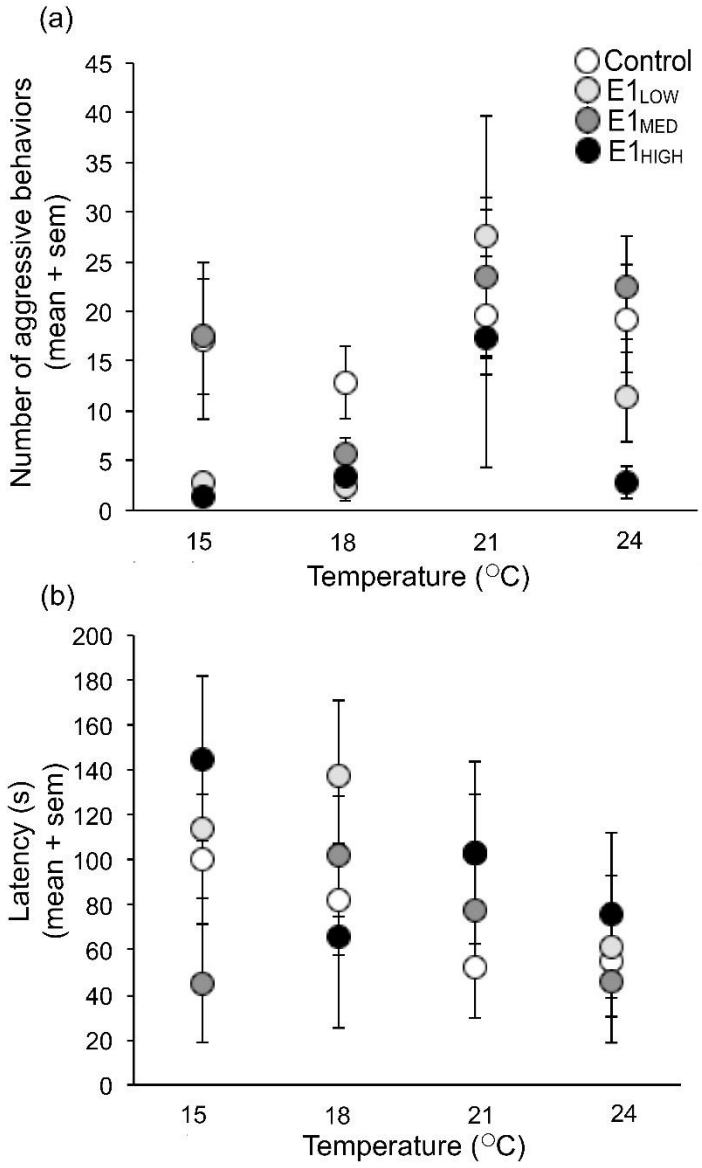

Figure 2.4. Aggressive responses of resident males exposed to varying concentrations of E1 at different ambient temperatures (n = 8 – 16). (A) Combined number of butts and strikes; B) Latency to first aggressive act (s). White circles represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black circles represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (25 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± sem.

Discussion

In this study, we examined the extent to which variation in the ambient temperature modulates the behavior and physiology effects of fish exposed to an estrogenic contaminant, at two life stages. Our results yielded several main findings; first, we confirmed that E1 and temperature independently influence aspects of the survival, reproduction and behavior of both larval and mature fish. Second, we found some evidence indicating that the effects of contaminants can vary with thermal regime. Third, taken together our data suggest that individuals in earlier life stages are more susceptible to the modulating effects of natural abiotic variation on anthropogenic stressors.

Independent and Interacting Effects of Temperature and EDC Exposure on Behavior

Predator Evasion

The C-start startle response is almost entirely regulated by a pair of large, easily identifiable Mauthner cells (M-cells) found in the hindbrain (Eaton et al. 1981). The response is initiated when one of the two M-cells is activated and the signal is then propagated along the axon, which descends caudally into the spinal cord, crosses the midline and synapses on moto-neurons on the opposite side of the body causing the contralateral trunk muscles to contract into the characteristic “C” shape (Eaton et al. 1981). Both changes in temperature and exposure to contaminants have the potential to influence escape performance through their effects on the activity of neural M-cells. Temperature alterations shift the balance between excitatory and inhibitory transmission onto the M-cells with corresponding changes in behavior (Preuss and Faber 2003; Szabo et al. 2008). M-cell to moto-neuron transmission is also affected by a variety of chemicals (Carlson et al 1998), resulting in impaired larval escape performance (McGee et al. 2009; Painter et al. 2009). Moreover, these effects are exacerbated at higher temperatures (Xia et al. 2015). For example, McGee et al. (2009) showed that juvenile *P. promelas* exposed as embryos to 50

ng/L of E1 exhibited a delay in the initiation of the C-start response compared to control fish, and a reduction in total escape performance. Juvenile qingbo, *Spinibarbus sinensis*, exposed to perfluorooctane sulfonate for four weeks at 28°C also exhibited riskier behavior and impairment of the fast-start escape response compared with fish exposed at 18°C (Xia et al. 2015). With the exception that larvae reared at 15°C and exposed to an intermediate concentration of E1 (i.e., $E1_{MED}$) had a *faster* escape velocity compared with subjects exposed to higher or lower levels of estrogen, we did not find that exposure to E1 significantly impaired the larval fast-start startle response. However, consistent with previous work suggesting that the effects of exposure on aquatic organisms are magnified at higher temperatures (Noyes et al. 2009), compared to control subjects, fish exposed to $E1_{HIGH}$ under warmer conditions (24°C) showed response latencies that were on average 18% longer and swimming speeds that were 50% slower; combined, these factors contributed to a 2.6-fold reduction in the total escape performance of larvae exposed to $E1_{HIGH}$ at 24°C.

Studies investigating the influence of temperature on the startle response generally report that C-start kinematics and behavioral responsiveness are either unaffected by, or improve in parallel with, temperature over a limited thermal window (e.g., Webb 1978; Batty and Blaxter 1992; reviewed in Dominici and Blake 1997). Consistent with these findings, total escape responses and turning angles exhibited by the larval *P. promelas* in our study were relatively stable across the 9°C temperature range tested here. Interestingly however, larvae reared 24°C had slower swimming speeds and longer response latencies than those reared at 15°C. One potential explanation for these results is that the trials were conducted at room temperature (~21°C), which represented an acute increase in temperature for the fish acclimated at 15°C or 18°C and a decrease in temperature for fish acclimated at 24°C. Fish acclimated to colder temperatures frequently show an improved swimming performance at warmer temperatures, and vice versa (Johnson and Bennett 1995; Preuss and Faber 2003; Krupczynski and Schuster 2013). For

example, short-horned sculpins, *Myoxocephalus scorpius*, acclimated to 5°C demonstrated higher swim velocities when tested at 15°C compared to when tested at 5°C (Beddow et al. 1995).

Male-Male Aggression

Estrogen-induced reductions in the nesting behavior (Brian et al. 2006) and aggression (Bell 2001; Coleman et al. 2009; Saaristo et al. 2010;) of male fish are well described. For example, Saaristo et al. (2010) compared the aggressive responses of unexposed male sand gobies (*Pomatoschistus minutus*), and males exposed to 17α-ethinylestradiol, toward unexposed intruding males during courtship and nest defense and found that exposed males were less aggressive towards rivals. Consistent with these previous studies, the resident male minnows in our study exposed to the highest concentration of E1 exhibited more than a 2.5-fold reduction (across all temperatures) in aggression towards conspecific intruders compared to control subjects. These data are consistent with evidence indicating that exposure to estrogenic EDCs is associated with decreased levels of androgens (Coe et al. 2008; Salierno and Kane 2009) responsible for regulating the expression of reproductive behavior in fish (Liley and Stacey 1983; Mayer et al. 2004).

The reproductive males in our study were also generally more aggressive at temperatures on the higher end of the thermal range (i.e., 21°C and 24°C, which corresponds to the optimal thermal breeding regime; Smith 1978; Brian et al. 2011); at 21°C and 24°C males performed approximately 2-fold and 1.5-fold more aggressive displays than at 15°C, respectively. At these same, higher temperatures, males performed approximately 2.5-fold and 2-fold more aggressive displays than at 18°C. Higher levels of aggression at warmer temperatures have also been reported in other species, including dwarf cichlids (*Apistogramma agassizii*) (Kochhan et al. 2015), damselfish (*Pomacentrus bankanensis*) (Biro et al. 2010) and mosquitofish (*Gambusia holbrooki*) (Carmona-Catot et al. 2013) and likely reflect the regulatory influence of exogenous proximate cues on endogenous reproductive physiology (Munro et al. 1990; Rocha et al. 2008). In seasonally reproducing fish such as *P. promelas*, reproductive behavior (i.e.,

courtship, male-male aggression, and parental care) typically begins in the spring once ambient temperatures reach 18°C (Smith, 1978), with maximal growth and survival of offspring occurring between 20°C and 24°C (Brian et al. 2011). Significantly, heightened aggression at the optimal breeding temperature eroded the aggressive disparity between males exposed to high concentrations of E1 and those in other treatments; at this temperature all males were highly aggressive. One potential explanation for these results is that higher levels of circulating androgens under peak spawning conditions compensate for the effects of estrogen exposure. In fish, 11-ketotestosterone (11-KT) is the main androgen associated with aggression and dominance (Taves et al. 2009) and endogenous levels of 11-KT and testosterone are highest during the pre-spawning and spawning periods (Borg 1994; van Breukelen et al. 2015). Dominant males (e.g., territory-holding males such as those in the present study) also have higher levels of circulating androgens than subordinate males (e.g., non-territorial males) (Parikh et al. 2006).

Prey Capture Success

The bioenergetic demands of prey capture, consumption and metabolism are costly to any organism, but are especially costly to organisms under environmental stress (Heugens et al. 2001; Sokolova and Lannig 2008; Sokolova 2012). Exposure to heavy metals, pharmaceuticals and other contaminants, including estrogens (Hallgren et al. 2014) is known to impair foraging success, and reduce the biomass and growth of adult and juvenile fish both in the lab and in the field (see reviews by Sloman and McNeil 2012; Weis and Candelmo 2012). By contrast, few studies have examined the effects of estrogen exposure on the foraging ability of fish; Cagle (2014) found no effect of 17β-estradiol on the feeding behavior of male *Betta splendens*. Hallgren et al. (2014) reported that juvenile roach (*Rutilus rutilis*) exposed to 50 ng/L 17α-ethinylestradiol showed reduced foraging success, measured as the number of *D. magna* captured and consumed over a given interval of time. With the exception that larvae reared at 15°C and exposed to an intermediate concentration of E1 (i.e., $E1_{MED}$) captured fewer prey than unexposed or $E1_{LOW}$ subjects, neither larval nor adult prey capture rates were significantly affected by

exposure to E1. The differences between our findings and those of Hallgren et al. (2014) could potentially reflect species-specific differences in sensitivity, or the comparatively higher estrogenicity of synthetic estrogen 17α-ethinylestradiol relative to estrone (Van den Belt et al. 2004).

However, prey capture rates were positively related to temperature for both larvae and adult *P. promelas*. Similar associations between prey capture success and elevated temperatures have been reported for a number of fish species, including Australian bass, *Macquaria novemaculeata* (Grigaltchik et al. 2012); dottyback, *Pseudochromis fuscus (*Allan et al. 2015); perch, and ruffe, *Gymnocephalus cernuus (*Bergman 1987*)*. In our study, adult and larval *P. promelas* maintained at 24°C had capture rates that were on average 1.85-fold and 1.66-fold greater than those observed for age-matched individuals maintained at 15°C. Further work is necessary to determine the causal mechanisms underpinning prey-capture success in *P. promelas*; however, temperature-dependent variation in the outcomes of predator-prey interactions could reflect direct differences in attack rate (Persson 1986; Grigaltchik et al. 2012), physiological mechanisms and/or kinetics associated with a predatory strike (Allan et al. 2015), or hunger; although all of the subjects in our study were fasted for the same length of time elevated temperatures may have induced shifts in the metabolic rate with an associated increase in energy requirements (Clarke and Johnston 1999; Noyes et al. 2009).

Survival, Reproduction and Growth of *P. promelas* at two life stages

In natural populations, persistence is dependent not only on the reproductive success or survival of the adult cohort, but also on the survival of the F1 generation to maturity. In our study, larvae exposed to E1 at 15°C were 2.8 times ($E1_{LOW}$) to 5 times ($E1_{HIGH}$) less likely to survive to day 21 than their unexposed counterparts, suggesting that the adverse effects of E1 exposure on early survival are more prevalent at this temperature. In addition, female *P. promelas* in the $E1_{HIGH}$ treatment laid fewer eggs than females exposed to a low concentration of E1; a finding that was also particularly pronounced at 15°C. Reductions in reproductive output and/or survival following estrogen exposure are well-documented in *P.*

promelas (e.g., Parrott and Blunt 2005; Thorpe et al. 2007; Dammann et al. 2011). Moreover, these effects of exposure can be transgenerational (Schwindt 2014), and can reduce recruitment rates below those needed for population persistence (Kidd et al. 2007).

Temperature also had a significant effect on the duration of embryonic development and growth of larval *P. promelas*. Although rates of development can vary widely among species, development within species is largely a function of temperature (Jobling, 1997). Accordingly, eggs laid at 24°C exhibited a significantly shorter hatching latency compared to eggs laid at 15°C. Larvae reared at 24°C were also considerably larger than those reared at 15°C on day 21. By contrast, neither male fertility nor adult survival was affected by exposure to E1 or temperature.

CHAPTER 3: TEMPERATURE MODULATES ESTROGEN EXPOSURE AND THE MEASURABLE BIOLOGICAL EFFECTS ON AQUATIC BIOTA

Introduction

All vertebrates naturally excrete a range of estrogens with urine and feces. Among estrogens, 17β-estradiol (E2) has received much attention due to its prevalence in effluent (Kolpin et al. 2002) and estrogenic potency (Van den Belt et al. 2004). However, during wastewater treatment and in the aquatic environment E2 quickly biodegrades into the more stable estrone (E1) (Vajda et al. 2008; Writer et al. 2012). As a consequence, E1 is the most prevalent estrogen in wastewater effluent (Johnson et al. 2004; Mes et al. 2005) and in the aquatic environment, where E1 concentrations may exceed 100 ng/L (Matthiessen et al. 2006; Chen et al. 2010, Alvarez et al. 2013). Estrone does degrade biologically in wastewater treatment systems and those conditions that support robust nitrification also support E1 degradation (Tan et al. 2013; Shi et al. 2004). Nevertheless, nitrification is very sensitive to temperature, slowing dramatically with decreasing temperature (Wild et al. 1971). Estrogen degradation is also sensitive to temperature, increases in water temperature may increase the degradation rate of estrogens (Raman et al. 2001), and therefore decreasing water temperature could feasibly increase estrogenic concentrations in aquatic ecosystems. Since temperature is not constant in the environment and annual temperature regimes often coincide with critical periods of biological activity (i.e., reproduction during spring warming of aquatic environments), temperature-dependent changes in E1 degradation efficacy during wastewater treatment could have a dramatic impact on receiving waters.

The presence of estrogens in aquatic ecosystems is correlated with disruption in normal physiological and reproductive function. Estrogens present in municipal and industrial effluents have been causally linked to widespread sexual perturbation in exposed wild fish populations (Jobling et al. 1998; Routledge et al. 1998). Exposure of an experimental lake to a synthetic estrogen (17α-ethynylestradiol) caused the collapse of the resident fathead minnow population (Kidd et al. 2007; Palace

et al. 2009). In laboratory studies, plasma vitellogenin (vtg), an egg yolk precursor protein produced by female fish for reproduction, is elevated in males exposed to estrogens (for example: Hemmer et al. 2002; Shappell et al. 2010). Estrogen exposure also decreases reproductive success through reduced fecundity and fertilization success (Panter et al. 1998; Thorpe et al. 2003; Parrott et al. 2005; Dammann et al. 2011).

Organisms live in dynamic environments, and abiotic environmental conditions may interact with chemical challenges to produce complex organismal responses (Brian et al. 2008). Ambient temperature exerts an environmental influence on ectothermic aquatic organisms, including teleost fish (Jin et al. 2009). However, assessing the effects of temperature and estrogenic exposure on individual aquatic organisms is complicated by the temperature dependent rates of E1 degradation in the environment and rates of E1 uptake by aquatic organisms (Starner et al. 1999; Heugans et al. 2001; Gordon 2003). Established experimental protocols for toxicological experiments often fail to take abiotic environmental factors, such as temperature, into account (Jin et al. 2009). Most fishes, including fathead minnows, are ectothermic; therefore, water temperature has the potential to exert effects at multiple levels of organization, from molecular responses (i.e., the rate of vtg expression; Brain et. al. 2008; Körner et al. 2008) to ecological responses (i.e., the timing of reproduction; Gillet and Quétin 2006). Whereas temperature and environmental estrogens have been individually shown to influence the physiology and reproductive functioning of fish, the biological effects resulting from the interaction between these factors has rarely been investigated (Körner et al. 2008; Jin et al. 2009) and is likely multi-faceted.

The goal of the current study was to assess the biological response in fish exposed to E1 under several temperature regimes. Specifically, we tested the following hypothesis: cooler water temperatures will exacerbate the adverse physiological and reproductive effects of E1 exposure on adult *P. promelas*. If the hypothesis is falsified, the data would support the alternative hypotheses that the biological effects of E1 exposure on adult *P. promelas* are exacerbated at higher water temperatures, possibly as a result of the increased organismal metabolism, or that there is no effect of water temperature on exposed organisms

(null hypothesis). Previous studies have demonstrated that chemical toxicants can interact with the thermal conditions in complex ways to influence physiological impairment and survival (Heugans et al. 2001; Gordon 2003); therefore, the alternative hypotheses account for these potential outcomes.

Methods and Materials

Experimental Design

Four successive month-long flow-through exposure experiments were conducted in the Aquatic Toxicology Laboratory at St. Cloud State University (MN) between March and July 2015. A previously published flow-through exposure protocol (Schoenfuss et al. 2008; Hyndman et al. 2010; Shappell et al. 2010) was modified to accommodate a 4 *x* 4 factorial experimental design consisting of graded concentrations of E1: low (E1-L), medium (E1-M), and high (E1-H), at 12.5, 25, and 65 ng E1/L respectively, including an ethanol control (0 ng E1/L; 0.0002% ethanol v/v) at four temperatures (15, 18, 21, and 24°C). E1 (Sigma-Aldrich, St. Louis, MO) was dissolved in 100% ethanol to generate treatment-specific aliquots used to make fresh E1 stock solutions on every third day of the exposure period. Treatment specific aliquots were diluted in 10 L of water and pumped (Cole-Palmer Masterflex 7523-40 peristaltic pump) consistently through stainless steel tubing into a continuous flow of groundwater to achieve the desired exposure concentrations. A mixing tank with two internal chambers was utilized to ensure complete mixing of ground water and E1 stock solution prior to the mixture being distributed equally to all aquaria within a treatment.

Mature (6 month old) fathead minnows (*P. promelas*) were obtained from a dedicated laboratory fish supplier (Environmental Consulting and Testing, Superior, WI). Each exposure temperature was tested singly, at random due to facility size restrictions, for an exposure period of 30 days at all four concentrations of E1. A total of 28 aquaria (volume: 12 L, 30.5 x 30.5 x 30.5 cm) were divided in half by the addition of a stainless steel mesh partition to accommodate two breeding groups, each consisting of one male and two females (56 breeding groups; 10-14 aquaria per treatment). Fish were assessed for

morphological and histopathological endpoints at the end of the 30 day exposure in accordance with approved St. Cloud State University IACUC protocols (# 8-73). Fish were maintained in accordance to U.S. EPA guidelines (Denny, 1987), with the exception of water temperature which was adjusted to meet the experimental objectives. Fish were fed a diet of frozen blood worms (*Glycera* spp.) and brine shrimp (*Artemia* spp.) *ad libitum* twice daily (Brine Shrimp Direct, Ogden, UT) and maintained on a 16:8 h light/dark photoperiod.

Water Quality and Chemistry

Water quality parameters were recorded daily and included temperature, pH, dissolved oxygen, conductivity, salinity, and oxidation reduction potential using a YSI (model 556 MPS; Yellow Springs, OH). Every third day of the exposure total hardness, free chlorine, total chlorine, pH, and alkalinity were screened for using AquaChek 5 in 1 test strips (Hach Company, Loveland, CO). The concentration of the E1 stock solution was verified using LC/MS/MS. Water sample collection occurred the day after an E1 chemical solution renewal. Water samples were collected from the outflow of the mixing tanks in 1-L HDPE bottles and 50 mL conical tubes as a secondary sample, and stored at -20°C until further analysis.

Biological Endpoints

Plasma Vtg Analysis

Heparinized capillary tubes were used for blood collection from the severed caudal vasculature of anesthetized minnows (0.01% neutral buffered MS-222 solution; Argent Laboratories, Redmond, WA). Whole blood was centrifuged at 5,900 x g for 5 minutes at 4°C, before plasma was separated and stored at -80°C for subsequent analysis. Quantification of plasma vtg was achieved through a competitive antibody-capture ELISA (Parks et al., 1999). A two-fold serial dilution was used to prepare an eight-point standard curve ranging from 4.8 µg/L to 0.00375 µg/L. Further elaboration of this method can be found in Parks et al. (1999) and Shappell et al. (2010).

Blood Glucose and Hematocrit

Blood glucose was measured using a digital glucose monitor (TRUEbalance Blood Glucose Monitor, Moore Medical, Farmington, CT), which utilizes a 1 µL sample of whole blood obtained from the caudal vasculature of each fish. To measure hematocrit (i.e., percent packed red blood cells to whole blood), heparinized capillary tubes were used to collect whole blood. Hematocrit tubes were sealed with a clay plug and centrifuged at 3,000 x g for 5 minutes (HERMLE Z200A, Labnet International Inc., Woodbridge, NJ) before hematocrit was determined using a Spiracrit Micro-Hematocrit Tube Reader (Clay Adams Inc., New York, NY).

Organosomatic Indices

Fish were euthanized using a 0.01% neutral buffered MS-222 solution (Argent Laboratories, Redmond, WA) prior to dissection. Each fish was measured for total length (TL) and standard length (SL, in mm) using a metric ruler and for total body weight (in grams, using a digital scale precise to 0.01 g, Acculab Vicon, Edgewood, NY). Body condition factor (BCF), an overall measurement of fish health, was calculated using the formula (BCF = [body weight/total length^3] X 100,000; Fulton 1904). Liver and reproductive organs (i.e., ovaries and testes) were collected and the mass of each organ was recorded using a digital scale precise to 0.001 g (Mettler Toledo AG245, Columbus, OH). A hepatosomatic index value (HSI; liver weight/whole body weight X 100) and a gonadosomatic index value (GSI; gonad weight/whole body weight X 100) were calculated for each fish, respectively.

Secondary Sex Characteristics and Histopathology

Secondary sexual characteristics (SSC) were measured using a scoring system based on prominence of characters using a scale from 1 (least prominent) to 3 (very prominent) to score the fatty dorsal pad, tubercles, and banded coloration intensity, following methods described in Smith (1978). Excised livers and gonads were placed in histological cassettes, and fixed in 10% neutral buffered

formalin. Dehydration protocols described in Carson (1997) were used to process tissue samples in a Leica ASP 300 Automated Tissue Processor (Leica Microsystems Inc., Buffalo Grove, IL). Paraffin embedded tissues were sectioned on a Leica Reichert-Jung 2030 cassette microtome (5 μm sections). Tissues were stained with hematoxylin and eosin counter-stain procedures described in Carson (1997) using a Leica ST5010 Autostainer XL. Liver hepatocyte vacuolization and developmental stage were assessed using bright-field microscopy. Liver vacuolization was based on the prominence of vacuoles in hepatocytes within the field of view (40x magnification), 1 (< 5% vacuolization), 2, (> 25-50% vacuolization), 3, (>50%-75% vacuolization), and 4, (>75% vacuolization). Reproductive maturity was based on the percentage of four main germ cell types during gametogenesis (i.e., spermatogenesis and oogenesis) within the field of view (40x magnification). The presence of eosinic fluid, atritic eggs, and cases of intersex were noted if observed during evaluation. Assessment of histological slides was blinded with the observer unaware of the treatment of each observed section.

Statistics

Males and females were analyzed separately using multivariate analysis of variance (MANOVA) with concentration, temperature, and concentration *x* temperature specified as fixed factors and SL, body weight, gonad weight, liver weight, hematocrit, blood glucose level, plasma vtg concentration, BCF, GSI, and HSI specified as dependent variables. Dependent variables were log transformed where needed for normality. The model for males also included an overall SSC score for each male, based on the degree of development of the tubercles and dorsal pad, and the intensity of banding color. Preliminary analysis revealed that these characters were highly correlated; therefore, each male was assigned a SSC score based on principal components analysis (PCA). The PCA extracted a single component that explained 68% of the variation. All analyses were conducted using SPSS (v21).

Results

Water Quality and Estrogen Concentrations

Environmental conditions were stable throughout the duration of all four temperatures. Collapsing over all concentrations, environmental conditions (mean ± sd) are as follows: 15°C experiment: pH = 7.9±0.3; conductivity = 0.90±0.04; salinity = 0.45±0.02; ORP = 225.3±64.5; 18°C experiment: pH = 7.7±0.2; conductivity = 0.90 ± 0.07; salinity = 0.45 ± 0.01; ORP = 266.9 ± 22.5; 21°C experiment: pH = 8.14 ±0.2; conductivity = 0.63 ± 0.42; salinity = 0.31 ± 0.21; ORP = 167.5 ± 38.0; and 24°C experiment: pH = 7.72 ± 0.3; conductivity = 0.92 ± 0.07; salinity = 0.46±0.01; ORP = 125.3 ± 0.28. E1 concentrations (mean ± SD) were 14.17 ± 3.09, 25.43 ± 9.96, and 65.39 ± 27.70 ng/L for the E1-L (n = 7), E1-M (n = 8) and E1-H (n = 8) treatments respectively (Table 3.1).

Table 3.1: Descriptive water chemistry of measured E1 concentrations collapsed across all temperatures and the environmental conditions, temperature and dissolved oxygen, in four subsequent 30-day exposure experiments with mature fathead minnows (mean ± SD shown for all measured parameters), sample sizes are denoted in parentheses.

Treatment (Abbreviation)	**Measured E1 Concentration (ng/L)**	**Nominal Temperature (°C)**	**Measured Temperature (°C)**	**Measured Dissolved Oxygen (mg/L)**
Ethanol Control (EtOH)	No E1 detected	15	16.8 ± 0.572 (31)	9.73 ± 1.35 (31)
		18	18.9 ± 0.722 (33)	7.97 ± 1.53 (33)
		21	21.2 ± 0.561 (20)	7.19 ± 0.02 (2)
		24	24.5 ± 0.524 (31)	6.95 ± 0.24 (31)
E1 Low (E1-L)	14.26 ± 3.01 (7)	15	17.2 ± 0.761 (31)	9.19 ± 1.29 (31)
		18	18.5 ± 0.432 (34)	8.77 ± 1.14 (34)
		21	21.2 ± 0.492 (20)	6.99 ± 0.00 (1)
		24	24.2 ± 0.590 (31)	7.71 ± 0.21 (31)
E1 Medium (E1-M)	25.43 ± 9.31 (8)	15	16.8 ± 0.745 (31)	9.62 ± 0.95 (31)
		18	18.7 ± 0.532 (34)	7.97 ± 1.31 (34)
		21	21.0 ± 0.561 (20)	6.29 ± 0.00 (1)
		24	24.3 ± 0.591 (31)	7.71± 0.21 (31)
E1 High (E1-H)	65.39 ± 25.20 (8)	15	16.5 ± 0.459 (30)	10.12 ± 1.23 (30)
		18	18.3 ± 0.318 (34)	8.78 ± 1.24 (34)
		21	21.1 ± 0.594 (20)	6.66 ± 0.00 (1)
		24	24.3 ± 0.597 (31)	7.71 ± 0.28 (31)

Fish Survival

In total, 642 fish (428 females and 214 males) were analyzed in the current study. Fish survival across all thermal regimes was excellent (range: 92 – 99%) and similar when compared across all 16 treatments ($\chi^2 = 0.99$, df = 14, P =0.95). Collapsing across all concentrations, percent survival was observed to be the lowest in the 24°C temperature exposure at 92%. Collapsing across all temperatures, the E1-M concentration had the lowest percent survival at 95%.

Physiological and Anatomical Endpoints

For both male and female subjects, the MANOVA models revealed significant overall effects of E1 exposure, temperature, and concentration *x* temperature on organismal endpoints. All significant independent and interactive effects of water temperature and E1 exposure are summarized in (Table 3.2).

Table 3.2. Summary of significant results for the independent and interactive effects of temperature and E1 exposure on exposed male and female physiology and morphology. The direction of arrows indicates worsening effects as temperature or concentration increases.

	MALES			**FEMALES**		
	Temp.	Conc.	Temp. *x* Conc.	Temp.	Conc.	Temp. *x* Conc.
Physiological Endpoint						
Plasma vtg	↓	↑	↓			
Blood glucose	↑	↓	↓			↓
Hematocrit		↑			↓	
Morphological Endpoint						
TL						
SL				↓		↓
Total weight				↓	↓	↓
Liver weight	↓			↓	↓	↓
Gonad weight	↓			↓		
BCF	↓			↓	↓	
HSI				↓		↓
GSI	↓			↓		
Liver histology						
Gonad histology						
SSC	↑			---		---

Male Results

Effects of E1 Exposure on Male Organismal Endpoints

There were significant effects of E1 exposure on physiological endpoints such as plasma vtg concentrations in male fathead minnows (vtg: $F_{3,169} = 15.85$; $P < 0.001$). Post-hoc tests indicated that plasma vtg concentrations were significantly lower in control males than in exposed males (E1-L, E1-M, and E1-H; all Ps < 0.001). Significant effects of E1 concentration on blood glucose ($F_{3,169} = 5.10$; $P = 0.002$) and hematocrit ($F_{3,169} = 4.88$; $P = 0.003$) were also observed. Blood glucose levels were higher in males exposed to E1-L than in males exposed to either E1-M or E1-H ($P < 0.001$). Hematocrit was significantly lower in E1-H exposed males compared with control ($P = 0.037$) and E1-L ($P < 0.001$) exposed males, and lower in males exposed to E1-M than E1-L ($P = 0.013$). No statistically independent effects of E1 exposure were found on male morphological endpoints such as TL, SL, total weight, liver weight, gonad weight, BCF, HSI, GSI, SSC, including gonad and liver histology.

Effects of Temperature on Male Organismal Endpoints

Temperature had a significant effect on plasma vtg concentration in males ($F_{3,169} = 5.17$, $P = 0.002$), plasma vtg concentrations were higher at 15°C than at 18°C ($P = 0.018$) or 21°C ($P < 0.001$). Temperature also had a significant effect on blood glucose levels ($F_{3,169} = 5.\ 10$, $P = 0.002$), subjects maintained at 24°C had significantly lower blood glucose levels compared to subjects maintained at cooler temperatures (15°C: $P = 0.046$; 18°C: $P = 0.017$; 21°C: $P = 0.001$).

Temperature also produced significant independent effects on morphological indices. Significant effects of temperature were observed for male gonad weight ($F_{3,170} = 5.12$; $P = 0.002$) and liver weight ($F_{3,169} = 13.96$; $P < 0.001$). Gonad weight at 15°C was significantly greater than at 21°C ($P = 0.001$) or 24°C ($P = 0.008$). Liver weights were higher at the two lowest temperatures (15°C and 18°C) than they were at either of the two higher temperatures (21°C and 24°C) (all Ps < 0.003). Significant effects of

temperature on BCF ($P = 0.002$) and GSI ($P < 0.001$) were also observed. Post-hoc analysis showed that the body condition of males maintained at the highest temperature (24°C) was significantly reduced compared to that of males maintained at lower temperatures (15°C: $P = 0.011$; 18°C: $P = 0.027$; 21°C: $P = 0.009$). The average GSI was greater at 15°C than at any of the higher temperatures ($0.032 \leq Ps < 0.001$). There was a significant effect of temperature on the degree of development of male SSC ($F_{3,169} = 4.59$, $P = 0.004$). Post-hoc tests indicated that male SSC scores were higher at 24 °C than at 15 °C ($P = 0.005$). We did not observe a significant effect of temperature on TL, SL, total weight, HSI, or either histological endpoint.

Interactions Between E1 Exposure and Temperature on Male Organismal Endpoints

A significant interaction between water temperature and E1 exposure concentration was observed for plasma vtg concentration ($F_{9,170} = 2.99$, $P = 0.002$); in general, the differences in vtg concentration between control and exposed males were more pronounced at cooler temperatures. Post-hoc analysis showed that plasma vtg levels were significantly reduced in control males compared to exposed males at temperatures 15°C, 18°C, and 21°C ($0.034 \leq Ps < 0.001$; Fig. 3.1A). However, there were no differences in the amount of plasma vtg produced by control males and exposed males at 24°C. A significant temperature *x* concentration interaction was also observed for blood glucose ($F_{9,169} = 3.74$, $P < 0.001$; Fig. 3.1B). Control and E1-L males maintained at 18°C had significantly elevated blood glucose compared to E1-M and E1-H exposed males. No other interactive effects of E1 exposure and temperature were observed for any of the other physiological or morphological endpoints.

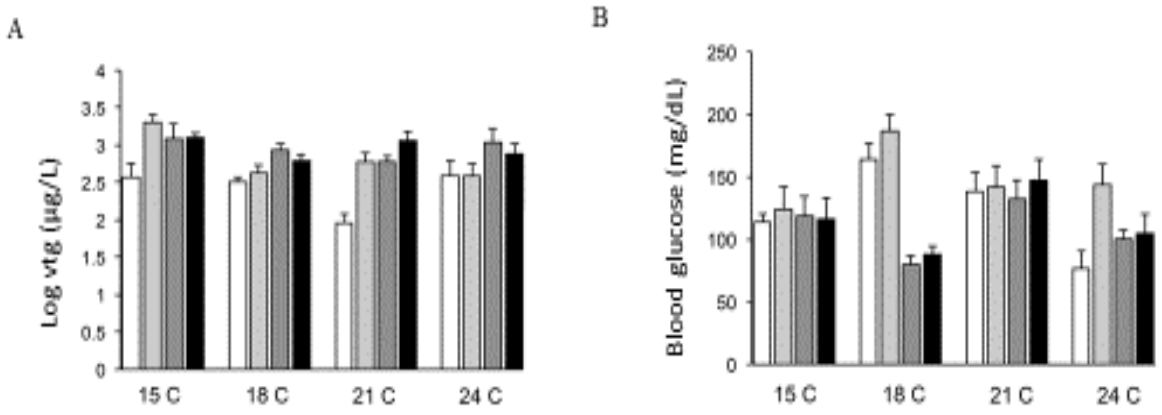

Figure 3.1. Significant interactions of E1 concentration and temperature effects on male physiology (A) log vtg concentrations (µg/L) and (B) blood glucose levels (mg/dL). White bars represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars represent the E1-L, E1-M, and E1-H exposure treatments; bars and error bars depict means ± sem.

Female Results

Effects of E1 Exposure on Female Organismal Endpoints

Female endpoints were not nearly as impacted by exposure as the previously reported males, however a statistically significant effect of E1 exposure on female hematocrit ($F_{3,215} = 2.95$, $P = 0.034$) was observed, and post-hoc tests revealed the hematocrit of control females was greater than E1-H exposed females ($P = 0.015$). As suggested, E1 exposure did not have a significant effect on other physiological endpoints such as plasma vtg concentrations or blood glucose levels.

However, unlike the males, female morphology was affected by E1 exposure. In particular, significant effects of E1 concentration on female fish total weight ($F_{3,215} = 4.64$, $P = 0.004$) and liver weight ($F_{3,215} = 5.71$, $P = 0.001$) were observed. Post-hoc tests indicated that the weights of females

exposed to E1-M had a significantly reduced total weight compared to females exposed to E1-L (P = 0.024). In addition, the liver weights of fish exposed to E1-M were less than the liver weights of control fish (P = 0.030) and fish exposed to E1-L (P = 0.007). Significant effects of E1 concentration were observed for BCF ($F_{3,215}$ = 3.66, P = 0.013) and HSI ($F_{3,215}$ = 3.42, P = 0.018). The BCF of subjects exposed to E1-M was reduced compared with that of fish exposed to E1-L (P = 0.01). We did not observe an effect of E1 exposure on other morphological indices such as TL, SL, gonad weight, HSI, GSI, or gonad and liver histology.

Effects of Temperature on Female Organismal Endpoints

There was a significant effect of temperature for SL ($F_{3,215}$= 15.24, P < 0.001), total weight ($F_{3,215}$= 28.28, P < 0.001), gonad weight ($F_{3,215}$= 6.99, P < 0.001), liver weight ($F_{3,215}$= 25.64, P < 0.001), BCF ($F_{3,215}$= 6.88, P < 0.001), GSI ($F_{3,215}$= 9.09, P < 0.001), HSI ($F_{3,215}$= 12.95, P < 0.001).) observed in female fish. Post hoc tests indicated that females maintained at 15°C and 18°C were longer (SL), heavier (total weight), and had bigger livers (by weight) compared to those maintained at 21°C and 24°C (all Ps < 0.001). Female fish maintained at 15°C and 18°C also had bigger gonads (by weight) compared with those maintained at 21°C (P = 0.03 and P = 0.005, respectively), and at 18°C compared to 24°C (P = 0.008). Significant effects of temperature on BCF (*P* < 0.001), HSI (*P* < 0.001), and GSI (*P* < 0.001) were also observed. Females maintained at the coldest temperature (15°C) had a greater BCF and a higher GSI than those maintained at the highest temperature (24°C; all Ps < 0.001). Females maintained in cooler water also had greater HSI values; HSI was higher at 15°C and 18°C compared to at 21°C (Ps < 0.001) and greater at 18°C than 24°C (P = 0.023). Significant independent effects of temperature were evident throughout all of the anatomical endpoints except for TL and the histological endpoints of interest.

Interactions Between E1 Exposure and Temperature on Female Organismal Endpoints

A significant temperature *x* concentration interaction was detected for female blood glucose ($F_{9,215}$= 2.43, P = 0.012). At 18°C, subjects exposed to E1-L had higher blood glucose levels than those exposed to E1-M (P < 0.001) or E1-H (P < 0.001) (Fig. 3.2).

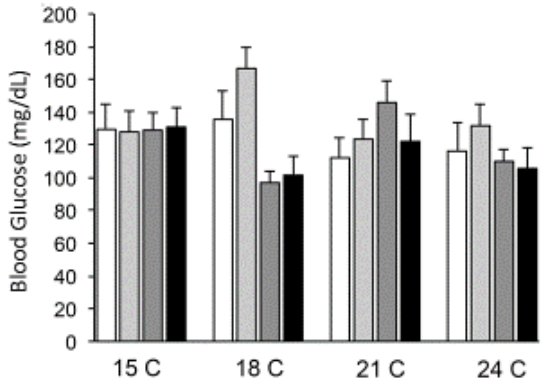

Figure 3.2. Significant interaction of E1 concentration and temperature on female blood glucose levels (mg/dL). White bars represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars represent the E1-L, E1-M, and E1-H exposure treatments; bars and error bars depict means ± sem.

Interactive effects of exposure concentration and temperature were also observed for female morphological indices such as SL ($F_{9,215}$= 3.07, P = 0.002, total weight ($F_{9,215}$= 2.35, P = 0.015), liver weight ($F_{9,215}$= 3.23, P = 0.001), and HSI ($F_{9,215}$= 2.78, P = 0.004). Post-hoc tests indicated that at 15°C females exposed to E1-L were shorter than those in the E1-M (P < 0.001) or E1-H (P = 0.03) treatments (Fig. 3.3A). At 21°C and 24°C subjects exposed to varying concentrations of E1 were similar in size. Differences among the exposure groups in body weight were more pronounced at cooler temperatures. At

15°C, females exposed to E1-L were heavier (had higher body weights) than fish in the control ($P = 0.015$), E1-M ($P < 0.001$), or E1-H ($P = 0.014$) groups. At 18°C, control fish also weighed more than fish exposed to E1-M ($P < 0.001$) or E1-H ($P = 0.023$) (Fig. 3.3B). Differences in the weight of fish from the different E1 exposure groups at warmer temperatures (i.e., 21°C or 24°C) were not observed. Liver weight was negatively linearly correlated to exposure concentration at 18°C; Control subjects had livers that were significantly heavier than those of fish exposed to E1-M ($P < 0.001$) and E1-H ($P < 0.005$), subjects exposed to E1-L had heavier livers than those exposed to E1-M ($P = 0.001$), and subjects exposed to E1-M had heavier livers than those exposed to E1-H ($P = 0.025$) (Fig. 3.3C). Liver weights were similar across exposure groups at all other temperatures. Similarly, control and E1-L subjects at 18°C also had higher HSI values than fish exposed to E1-M or E1-H ($0.037 < P < 0.001$) (Fig. 3.4D). Plasma vtg concentrations for females exposed to alternative concentrations of E1 were not dependent on temperature, suggesting that temperature does not modulate estrogenic activity in exposed females regarding plasma vtg induction, when compared to unexposed females. In addition, significant interactions between E1 exposure concentration and temperature were not observed for the BCF, GSI, or either histological endpoint. Collectively, the results for females suggest that temperature interacts with E1 exposure more intimately concerning morphological endpoints rather than physiological endpoints.

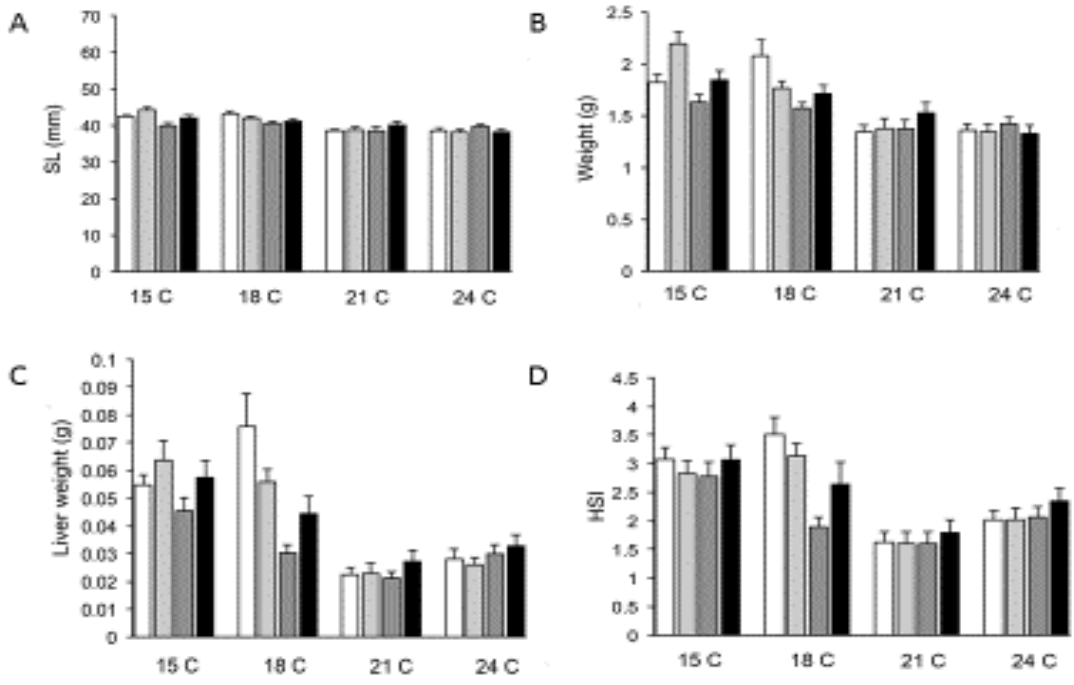

Figure 3.3. Significant interactions of E1 concentration and temperature effects on female morphology (A) SL (mm), (B) total weight (g), (C) liver weight (g), (D) HSI. White bars represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black bars represent the E1-L, E1-M, and E1-H exposure treatments; bars and error bars depict means ± sem.

Discussion

This study examined how water temperature modulates the physiological and reproductive effects of E1 exposure on adult fathead minnows. The results yielded several main independent effects of either E1 exposure concentration or water temperature on many of the biomarkers of interest, confirming that E1 concentration and temperature independently produce effects on fish physiology and morphology in both sexes. Cumulatively, the data suggest that temperature is the main driving force affecting

morphological indices, whereas E1 exposure concentration affects physiological endpoints. We also found evidence supporting the hypothesis that cooler water temperature modulates the estrogenic effects of exposed organisms in both males and females.

Temperature increases the metabolic rate of ectotherms (Johnston and Dunn 1987), subsequently increasing the rate of enzymatic activity (Voet et al. 2013). Enzymes are required for hepatic synthesis of the vtg protein (Hadley and Levine 2006). In males, the presence of vtg in the blood, and related transcription in the liver, are considered to be specific biomarkers for estrogen-mimetic contaminants in the environment (Hoyt et al. 2003). Research on salmonid fishes injected with a natural steroid estrogen suggests that an increase in temperature will be associated with increased estrogenic potency (Korsgaard et al. 1986). However, the results of this study demonstrated the exact opposite. Unexposed males did exhibit lower plasma vtg concentrations than exposed males, however male fish maintained at 15°C were found to have higher concentrations of plasma vtg induction than males maintained at 18°C or 21°C. Increases in water temperature may increase the degradation rate of E1, as demonstrated in Raman et al. (2001) and the present study, thereby reducing aqueous concentrations and bioavailability. The interactive effects of E1 concentration and temperature were evident in exposed males maintained at all temperatures except 24°C, further supporting that increases in temperature may reduce E1 concentrations to a point where male fish are no longer able to illicit a biological effect, or forgo vitellogenesis to maintain homeostasis at a higher metabolic demand (Luquet and Watanabe 1986). Exposed female fish did not display a significant independent or interactive effect of plasma vtg induction compared to control females across treatments. Watanabe et al. (2007) pooled six large exposure studies and systematically evaluated several experimental endpoints to determine “normal” or baseline natural variability in fathead minnows and determined a mean concentration of E2 in unexposed females of 6.07 ± 4.0 ng/mL, whereas the mean E2 concentration in unexposed males was 0.43 ± 0.30 ng/mL. Because females naturally produce estrogens, the concentrations used for this experiment in concurrence with E1 degradation rates

and decreased potency of E1 compared to E2, may not have been great enough to produce an exposure response, whereas male minnows were in a more susceptible range for exposure.

Hematological endpoints can shed insight on fish tolerance to a stressor, such as thermal stress, and the overall physiological state of the organism (Del Río Zaragoza et al. 2008). Temperature increases induce hepatic glycogenesis and glucose concentration in the blood (Radhakrishnaiah and Parvatheswararao 1984). Temperature-induced insulin fluctuations may be correlated to changes in insulin receptor internalization and turnover rate in hepatic tissue (Larsen et al, 2001); in addition, insulin receptor binding affinity is thought to be positively correlated with increased temperatures (Freychet et al. 1971). In this experiment, interactive effects of temperature and E1 concentration on blood glucose concentration were observed for both male and female fish. In males, blood glucose was similar across concentrations with an exception at 24°C (control vs. E1-L) and with males maintained at 21°C (significantly elevated glucose levels in control and E1-L males). Males exposed to higher temperatures and at lower concentrations had elevated glucose levels, demonstrating that increased temperature has an effect on glycogenesis and that being exposed to reduced concentrations lessens the E1 exposure impact. E1-L exposed females maintained at 18°C exhibited a similar pattern, higher blood glucose concentrations compared to E1-M or E1-H exposed females. Unexposed males and females or males and females exposed to E1-L concentrations displayed higher blood glucose levels, which could indicate a higher energy reserve.

The morphological endpoints in this study were greatly influenced by temperature for males and females. In females all but one anatomical endpoint (i.e. TL) yielded statistically significant effects of temperature. In addition, SL, total weight, liver weight, and HSI in females were significantly affected by the interactions of temperature and E1 concentration. Male anatomy was also influenced by temperature, but to a lesser degree, and did not produce any interactive effects of temperature and E1 concentration on any anatomical endpoint. Overall, unexposed and E1-L exposed females maintained at lower

temperatures were longer, heavier, and had bigger livers than E1-M or E1-H exposed females. Metabolic response to temperature changes is vital to organismal homeostasis under seasonal temperature fluctuations (Bruneaux et al. 2014). Females maintained at lower temperatures have a reduced metabolism, which may lead to a decreasing capacity for detoxifying processes, resulting in the internalization of toxins for longer periods of time.

Collectively, the results of these experiments demonstrate that temperature does modulate estrogenic exposure on adult fathead minnow physiology and morphology. The significant interactions between temperature and E1 exposure observed in this experiment demonstrated that female fish were more susceptible to the temperature-modulating effects of estrogenic exposure on morphometric endpoints. However, males were more susceptible to the interactive effects of temperature and E1 exposure on physiological endpoints. The present study demonstrates that seasonal fluctuation is great enough to induce significant physiological and anatomical changes in fish, and highlights that male and female exposure organisms can act in very opposing ways under identical exposure regimes.

CHAPTER 4: CONCLUSION

Ecotoxicology recognizes the need for a dynamic approach when linking cellular and behavioral responses to ecosystems. This study connects physiological and behavioral changes of organisms exposed to aquatic contaminants to a broader ecological background. Bioactive compounds, such as estrogenic endocrine active compounds, have the capacity to cause harm to wildlife populations and individuals at multiple life stages via various modes of action. *Pimephales promelas* is a keystone prey species in aquatic environments, and population declines could have long-term impacts at several trophic levels (Kidd et al. 2007; Palace et al. 2009). Understanding how environmental parameters interact with anthropogenic stressors, such as E1 exposure, to influence key metrics such as growth, development, reproduction, physiology, and behavior can provide more robust data for predicting population dynamics under real world scenarios.

Taking into account the totality of the experiments performed, the data demonstrate that (i) variation in the sensory responsiveness, kinematics, and outcomes of complex intraspecific and interspecific interactions can occur even within the normal range of seasonally fluctuating temperatures, and that (ii) changes in the thermal regime can interact with chemical stressors in ways that further adversely affect physiology, survival and behavior. The majority of significant interactions between E1 exposure and temperature were observed between exposure treatments at the coldest temperature tested (15°C), potentially reflecting a slower rate of microbial E1 degradation under colder thermal regimes (Cox et al., *in prep*). Support for this hypothesis comes from the finding that concentrations of plasma vtg in male *P. promelas* used in this study were significantly higher at 15°C than at warmer temperatures (Cox et al., *in prep*). Alternatively, metabolic changes associated with elevated temperatures may have resulted in increased rates of degradation and elimination (Noyes et al. 2009).

Many of the significant interactions between temperature and exposure were observed in larvae, which is consistent with previous studies indicating that individuals in exposed populations are

particularly susceptible to perturbation by CECs during early ontogenetic stages (Guillette et al. 1996; van Aerle et al. 2002; Liney et al. 2005; Sloman and McNeil 2012). Greater susceptibilities of fishes at early life stages can be expected, because the developmental processes of most fishes are heavily regulated by the endocrine system (Janz and Weber 2000). However, it is important to note that female morphological indices also displayed significant temperature and E1 exposure interactions, but were relatively unaffected physiologically compared to males. Therefore, this suggests that male and female anatomy and physiology responds differently despite being exposed to identical treatments. Taken together, the data suggest that the effects of EDC exposure may be more pronounced on the offspring of early-spring breeding individuals than on the offspring of individuals breeding later in the season.

Changes in global air and water temperatures due to climate change (O'Reilly et al. 2015) and/or increases in aquatic contaminant loads have potential to directly or indirectly alter the structure and function of populations and communities through changes in survival, reproduction, or altered behavioral interactions (Clotfelter et al. 2004; Tuomainen and Candolin 2011; van Zuiden et al. 2016). While most populations are expected to be impacted by climate change or pollution to some extent, ectothermic species such as fish, amphibians and reptiles, and populations living at the edge of their physiological tolerance range, will likely be particularly vulnerable to interactions between shifting thermal regimes and contaminants (Noyes et al. 2009). Improving our understanding of the effects of interactions among multiple anthropogenic stressors, and between stressors and natural abiotic environmental factors, is therefore of key importance to identifying vulnerable populations, and predicting population dynamics under changing real-world scenarios.

REFERENCES

Allan, B.J.M., Domenici, P., Munday, P.L., McCormick, M.I., 2015. Feeling the heat: the effect of acute temperature changes on predator–prey interactions in coral reef fish. Conserv. Physiol. 3(1): cov011; doi:10.1093/conphys/cov011.

Alvarez, D.A., Shappell, N.W., Billey, L.O., Bermudez, D.S., Wilson, V.S., Kolpin, D.W., Perkins, S.D., Evans, N., Foreman, W.T., Gray, J.L., Shipitalo, M.J., Meyer, M.T., 2013. Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations. Water Res. 47, 3347-3363.

Ankley, G.T., Johnson, R.D., 2004. Small fish models for identifying the effects of endocrine disrupting chemicals. ILAR Journal 45(4), 469-483.

Ankley, G.T., Villeneuve, D.L., 2006. The fathead minnow in aquatic toxicology: Past, present and future. Aquat. Toxicol. 78, 91-102.

Atchison, G.J., Sandheinrich, M.B., Bryan, M.D., 1996. Effects of environmental stressors on interspecific interactions in aquatic animals. In: Newmann, M.C., Hagoe, C.H. (Eds.), Ecotoxicology: A hierarchical treatment. CRC Press, Boca Raton, FL, pp. 319-345.

Axys Analytical Services. 2014. Analysis of pharmaceutical and personal care products and hormones in solid, aqueous, tissue and POCIS samples by LC-MS/MS. AXYS Method MLA-075, Rev 06.01.

Batty, R.S., Blaxter, J.H.S., 1992. The effect of temperature on the burst swimming performance of fish larvae. J. Exp. Biol. 170, 187-201.

Beddow, T.A., Leeuwen, J., Johnston, I.A., 1995. Swimming kinematics of fast starts are altered by temperature acclimation in the marine fish *Myoxocephalus scorpius*. J. Exp. Biol. 198, 203-208.

Beitinger, T.L., 1990. Behavioral reactions for the assessment of stress in fishes. J. Great Lakes Res. 16(4), 495-528.

Bell, A.M., 2001. Effects of an endocrine disrupter on courtship and aggressive behavior of male three-spined stickleback, *Gasterosteus aculeatus*. Anim. Behav. 62, 775–780.

Bergman, E., 1987. Temperature-dependent differences in foraging ability of two percids, *Perca fluviatilis* and *Gymnocephalus cernuus*. Environ. Biol. Fish 19(1), 45-53.

Bhandari, R.K., vom Saal, F.S., Tillitt, D.E., 2015. Transgenerational effects from early developmental exposures to bisphenol A or 17α-ethinylestradiol in medaka, *Oryzias latipes*. Sci. Reports. 5, 9303.

Biro, P.A., Beckmann, C., Stamps, J.A., 2010. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. Proc. Biol. Sci. 277, 71–77.

Bjerselius, R., Lundstedt-Enkel, K., Håkan, O., Mayer, I., Dimberg, K., 2001. Male goldfish reproductive behavior and physiology are severely affected by exogenous exposure to 17β-estradiol. Aquat. Toxicol. 53, 139-152.

Blazer, V.S., Iwanowicz, L.R., Henderson, H., Mazik, P.M., Jenkins, J.A., Alaverez, D.A. et al., 2012. Reproductive endocrine disruption in small-mouth bass (*Micropterus dolomieu*) in the Potomac River basin: Spatial and temporal comparisons of biological effects. Environ. Monit. Assess., 187(7), 4309-4334.

Blob, R.W., Wright, K.M., Becker, M., Maie, T., Iverson, T.J., Julius, M.L., Schoenfuss, H.L., 2007. Ontogenetic change in novel function: waterfall climbing in adult Hawaiian gobiid fishes. J. Zool. (Lond.) 269, 315–324.

Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. 109, 219-245.

Brian, J.V., Augley, J.J., Braithwaite, V.A., 2006. Endocrine disrupting effects on the nesting behavior of male three-spined stickleback *Gasterosteus aculeatus* L. J. Fish Biol. 68, 1883-1890.

Brian, J.V., Beresford, N., Margiotta-Casaluci, L., Sumpter, J.P., 2011. Preliminary data on the influence of rearing temperature on the growth and reproductive status of fathead minnows *Pimephales promelas*. J. Fish Biol. 79(1), 80–88.

Brian, J.V., Harris, C.A., Runnalls, T.J., Fantinati, A., Pojana, G., Marcomini, A., Booy, P., Lamoree, M., Kortenkamp, A., Sumpter, J.P., 2008. Evidence of temperature-dependent effects on the estrogenic response of fish: Implications with regard to climate change. Sci. Total Environ. 397, 72-81.

Brown, A.R., Owen, S.F., Peters, J., Zhang, Y., Soffker, M., Paull, G.C., Hosken, D.J., Wahab, M.A., Tyler, C.R., 2015. Climate change and pollution speed declines in zebrafish populations. Proc. Natl. Acad. Sci. USA 112(11), E1237–E1246.

Brown, J.A., Johansen, P.H., Colgan, P.W., Mathers, R.A., 1987. Changes in the predator-avoidance behavior of juvenile guppies (*Poecilia reticulanta*) exposed to pentachlorophenol. Can. J. Zool. 63, 71-78.

Brown, N.P., Shields, R.J., Bromage, N.R., 2006. The influence of water temperature on spawning patterns and egg quality in the Atlantic halibut (*Hippoglossus L.*). Aquaculture 261, 993-1002.

Bruneaux, M., Nikinmaa, M., Laine, V. N., Lindström, K., Primmer, C. R., Vasemägi, A., 2014. Differences in the metabolic response to temperature acclimation in nine-spined stickleback (*Pungitius pungitius*) populations from contrasting thermal environments. J. Exp. Zool. A., Ecol. Genetics Physiol., 321(10), 550-565.

Brungs, W.A., 1971. Chronic effects of low-dissolved oxygen concentration on the fathead minnow (*Pimephales promelas*). Journal Fisheries Research Board of Canada 28(8), 1119-1123.

Cagle, M., 2014. Sexual dimorphism and potential hormonal modulation of feeding mechanics in Siamese fighting fish, *Betta splendens*. PhD Dissertation.

Cairns, J. Jr., Heath, A.G., Parker, B.C., 1975. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia 47, 135–171.

Carlson, R.W., Bradbury, S.P., Drummond, R.A., Hammermeister, D.E., 1998. Neurological effects of startle response and escape from predation by medaka exposed to organic chemicals. Aquat. Toxicol. 43, 51–68.

Carmona-Catot, G., Magellan, K., Garcıa-Berthou, E., 2013. Temperature-specific competition between Invasive mosquitofish and an endangered cyprinodontid fish. PLoS ONE 8(1), e54734.

Carson, F.L., 1997. Histotechnology. ASCP Press, Chicago, IL.

Chen, T.S., Chen, T.C., Yeh, K.J.C., Chao, H.R., Liaw, E.T., Hsieh, C.Y., Chen, K.C., Hsieh, L.T., Yeh, Y.L., 2010. High estrogen concentrations in receiving river discharge from a concentrated livestock feedlot. Sci. Total Environ. 408, 3223- 3230.

Clarke, A., Johnston, N.M., 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. J. Anim. Ecol. 68, 893–905.

Clotfelter, E., Bell, A., Levering, K.R., 2004. The role of animal behavior in the study of endocrine-disrupting chemicals. Anim. Behav. 68, 665-676.

Coe, T., Hamilton, P., Hodgson, D., Paull, G., Stevens, J., Sumner, K., Tyler, C., 2008. An environmental estrogen alters reproductive hierarchies, disrupting sexual selection in group spawning fish. Environ. Sci. Technol. 42, 5020–5025.

Coleman, J.R., Baldwin, D., Johnson, L.L., Scholz, N.L., 2009. Effects of the synthetic estrogen, 17-ethinylestradiol, on aggression and courtship behavior in male zebrafish (*Danio rerio*). Aquat. Toxicol. 91, 346–354.

Cox, M., Peterson, K., Tan, D., Novak, P., Ward, J., Schoenfuss, H., In prep. Temperature modulates estrogen removal during wastewater treatment and the measurable biological effects on aquatic biota**.**

Crain, C.M., Kroeker, K., Halpern, B.S., 2008. Interactive and cumulative effects of multiple human stressors in marine systems. Ecol. Lett. 11, 1304-1315.

Crews, D., Cantu, A.R., Rhen, T., Vohra, R., 1996. The relative effectiveness of estrone, estradiol-17 beta, and estriol in sex reversal in the red-eared slider (*Trachemys scripta*), as turtle with temperature-dependent sex determination. Gen. Comp. Endocrinol. 102(3), 317-326.

Crockett, E.L., Londraville, R.L., 2006. Temperature, in: Evans, D.H., Claiborne, J.B., editors. The physiology of fish. Boca Raton, London, New York: Taylor and Francis. pp. 231–269.

Dammann, A.A., Shapell, N.W., Bartell, S.E., Schoenfuss, H.L., 2011. Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, *Pimephales promelas*. Aquat. Toxicol. 105, 559-568.

Danylchuk, A.J., Tonn, W.M., 2001. Effects of social structure on reproductive activity in male fathead minnows (*Pimephales promelas*). Behav. Ecol. 12(4), 482-489.

Darling, E.S., Cote, I.M., 2008. Quantifying the evidence for ecological synergies. Ecol. Lett. 11, 1278–1286.

Daughton, C.G., 2002. Environmental stewardship and drugs as pollutants. Lancet 360, 1035-1036.

Del Río Zaragoza, O., Rodríguez, M. H., Bückle Ramirez, L. F., 2008. Thermal stress effect on tilapia *Oreochromis mossambicus* (Pisces: Cichlidae) blood parameters. Mar. Freshw. Behav. Physiol., 41(2), 79-89.

Denny, J.S., 1987. Guidelines for the culture of fathead minnows, *Pimephales promelas*, for use in toxicity tests. EPA/600/3-87/001.

Domenici, P., Blake, R.W., 1997. The kinematics and performance of fish fast-start swimming. J. Exp. Biol. 200, 1165–1178.

Duda, S.W., 1989. Controlled spawning of laboratory reared fathead minnows (*Pimephales promelas*). Environmental Science and Biology Theses. Paper 7.

Dudgeon, D., Arthington, A.H., Gessner, M., Kawabata, O.Z.I., Knowler, D.J., Leveque, C., Naiman, R.J., Prieur-Richard, A.H., Soto, D., Stiassny, M.L.J., Sullivan CA., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biol Rev. 81, 163-182.

Eaton, R.C., Lavender, W., Wieland, C.M., 1981. Identification of Mauthner initiated response patterns in goldfish: evidence from simultaneous cinematography and electrophysiology. J. Comp. Physiol. A 144, 521-531.

Eaton, R.C., Lee, R.K.K., Foreman, M.B., 2001. The Mauthner cell and other identified neurons of the brainstem escape network of fish. Progress Neurobiol. 63, 467-485.

Fay, R.R., Ream, T.J., 1992. The effects of temperature change and transient hypoxia on auditory nerve fiber response in the goldfish (*Carassius auratus*). Hearing Research 58, 9-18.

Ferrey, M., Preimesberger, A., Schoenfuss, H., Kiesling, R., Barber, L., Writer, J., 2008. Statewide endocrine disrupting compound monitoring study. Minnesota Pollution Control Agency, 1-83.

Freychet, P., Roth, J., Neville Jr., D.M., 1971. Insulin receptors in the liver: Specific binding of [125I] insulin to the plasma membrane and its relation to insulin bioactivity. Proc. Nat. Acad. Sci. USA. 68, 1833-1837.

Fulton, T.W., 1904. The rate of growth of fishes. Fisheries Board of Scotland. Annual Report 22 part 3, pp. 141–241.

Geiger, D.L., Call, D.J., Brooke, L.T., 1988. Acute toxicities to organic chemicals to fathead minnows (*Pimephales promelas*). Center for Lake Superior Environmental Studies, Wisconsin.

Gillet C., Quétin P., 2006. Effect of temperature changes on the reproductive cycle of roach in Lake Geneva from 1983 to 2001. J. Fish Biol. 69, 518–534.

Gordon, C.J., 2003. Role of environmental stress in the physiological response to chemical toxicants. Environ. Res. 92, 1-7.

Grigaltchik, V.S., Ward, A.J.W., Seebacher, F., 2012. Thermal acclimation of interactions: differential responses to temperature change alter predator-prey relationship. Proc. Biol. Sci. 279, 4058–4064.

Guillette, L.J., Crain, D.A., Rooney, A.A., Pickford, D.B., 1996. Organization versus activation: the role of endocrine disrupting contaminants (EDCs) during embryonic development in wildlife. Environ. Health Perspec. 103, 157-164.

Häder, D.P., Gao, K., 2015. Interactions of anthropogenic stress factors on marine phytoplankton. Front. Environ. Sci. 3, 1-14.

Hadley, M.E., Levine, J.E., 2006. Endocrinology, Sixth Edition, San Francisco, CA.

Hale, M.E., Long, J.H., McHenry, M.J., Westneat, M.W., 2002. Evolution of behavior and neural control of the fast-start escape response. Evolution. 56, 993-1007.

Hall, L.W., Anderson, R.D., 1995. The influence of salinity on the toxicity of various classes of chemicals to aquatic biota. Crit. Rev. Toxicol. 25, 281–346.

Hallare, A.V., Schirling, M., Luckenbach, T., Köhler, H.R., Triebskorn, R., 2005. Combined effects of temperature and Cd on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. J. Therm. Biol. 30, 7–17.

Hallgren, P., Nicolle, A., Hansson, L.A., Bronmark, C., Nikoleris, L., Hyder, M., Persson, A., 2014. Synthetic estrogen directly affects fish biomass and may indirectly disrupt aquatic food webs. Environ. Toxicol. Chem. 33(4), 930-936.

Hardin, J.W., Hilbe, J.M., 2012. Generalized Estimating Equations, second ed. New York: Chapman and Hall/CRC.

Heino, J., Virkkala, R., Toivonen, H., 2009. Climate change and freshwater biodiversity: detected patterns, future trends and adaptations in northern regions. Biol. Rev. 84, 39–54.

Hemmer, M.J., Bowman, C.J., Hemmer, B.L., Friedman, S.D., Marcovich, D., Kroll, K., Denslow, N., 2002. Vitellogenin mRNA regulation and plasma clearance in male sheepshead minnows, (*Cyprinodon variegatus*) after cessation of exposure to 17β-estradiol and p-nonylphenol. Aquat. Toxicol. 58(1-2), 99-112.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M., Admiraal, W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 2001(31), 247-284.

Hoar, W.S., Randall, D.J., 1988. The physiology of developing fish. Fish physiology 6(A): 348-395.

Holmstrup, M., Bindesbøl, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Sci. Total Environ. 408, 3746–3762.

Hoyt, P.R., Doktyz, M.J., Beattie, K.L., Greenly, M.S., 2003. DNA microarrays detect 4-nonylphenol-induced alterations in gene expression during zebrafish early development. Ecotoxicol. 12, 469-467.

Hyndman, K.M., Biales, A., Bartell, S.E., Schoenfuss, H.L., 2010. Assessing the effects of exposure timing using 17β-estradiol. Aquat. Toxicol. 96, 264-272.

Janz, D.M., Weber, L.P., 2000. Endocrine system, in: Ostrander, G.K., editor, The laboratory fish. Academic Press, London, UK, pp. 415-439.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S., Ankley, G.T., 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas).* Comparative Biochemistry and Physiology 128: 127-141.

Jin, Y., Chen, R., Sun, L., Liu, W., Fu, Z., 2009. Photoperiod and temperature influence endocrine disruptive chemical-mediated effects in male adult zerbrafish. Aquat. Toxicol. 92, 38-43.

Jobling, M., 1997. Temperature and growth: modulation of growth rate via temperature change, in: Wood, C.M., McDonald, D.G., editors, Global warming: implications for freshwater and marine fish. Cambridge Univ. Press, Cambridge, UK, pp. 223–254.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32, 2498–2506.

Johnson, A. C., Williams, R. J., 2004. A Model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. Environ. Sci. Technol. 38, 3649–3658.

Johnson, T.P., Bennett, A.F., 1995. The thermal acclimation of C-start performance in fish: an integrated study of molecular and cellular physiology and organismal performance. J. Exp. Biol. 198, 2165-2175.

Johnston, I.A., Dunn, J., 1987. Temperature acclimation and metabolism in ectotherms with particular reference to teleost fish. Symp. Soc. Exp. Biol. 41, 67-93.

Keister, J.E., Pascual, D.L., Clasen, J.L., Hopfensperger, K.N., Kelly, N., Llopiz, J.K., Moseman, S.M., Petes, L., 2010. Climate and anthropogenic change in aquatic environments: a cross-ecosystem perspective, in: Kemp, P.F., editor, Eco-DAS VIII Symposium Proceedings, ASLO. pp. 1-16.

Khan, M.A.Q., Ahmed, S.A., Catalin, B., Khodadoust, A., Ajayi, O., Vaughn, M., 2006. Effect of temperature on heavy metal toxicity to juvenile crayfish, *Orconectes immunis* (Hagen). Environ. Toxicol. 21, 513–20.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Nat. Acad. Sci. 104, 8897-8901.

Kidd, K.A., Paterson, M.J., Rennie, M.D., Podemski, C.L., Findlay, D.L., Blanchfield, P.J., Liber, K., 2014. Direct and indirect responses of a freshwater food web to a potent synthetic oestrogen. Phil. Trans. R. Soc. B. 369, 20130578.

Koger, C.S., Teh, S.J., Hinton, D.E., 2000. Determining the sensitive development stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17beta-estradiol or testosterone. Marine Environment Research. doi:10.1016/50141-1136(00)00068-4.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. Environ. Sci. Technol. 36, 1202-1211.

Körner, O., Kohno, S., Schönenberger, R., Suter, M.J., Knauer, K., Guillette, L.J. Jr., Burkhardt, Holm, P., 2008. Water temperature and concomitant waterborne ethinylestradiol exposure affects the vitellogenin expression in juvenile brown trout (*Salmo trutta*). Aquat Toxicol. 90(3), 188-96.

Korsgaard, B., Mommsen, T.P., Saunders, R.L., 1986. The effect of temperature on the vitellogenic response in Atlantic salmon post-smolts (*Salmo salar*). Gen. Compar. Endocrinol. 62, 193–201.

Kottelat, M., Freyhof, J., 2007. Handbook of European freshwater fishes. Retrieved from http://www.fishbase.org/summary/4785 04(21), 8897-8901.

Krupczynski, P., Schuster, S., 2013. Precision of archerfish C-starts is fully temperature compensated. J. Exp. Biol. 216, 3450-3460.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A., 1998. Interaction of oestrogenic chemicals and phytoestrogens with oestrogen receptor b. Endocrinology 139, 4252–4263.

Larsen, D.A., Beckman, B.R., Dickhoff, W.W., 2001. The effect of low temperature and fasting during the winter on metabolic stores and endocrine physiology (insulin, insulin growth-like growth

factor-I, and thyroxine) of Coho salmon, *Oncorynchus* kisutch. Gen. Comp. Endocr. 123, 308-323.

Laskowski, R., Bednarska, A.J., Kramarz, P.E., Loureiro, S., Volker, S., Kudlek, J., Holmstrup, M., 2010. Interactions between toxic chemicals and natural environmental factors—a meta-analysis and case studies. Sci. Total Environ. 408, 3763–3774.

Lephart, E.D., 1996. A review of brain aromatase cytochrome P450. Brain Research Reviews 22, 1-26.

Liley, N.R., Stacey, N.E., 1983. Hormones, pheromones, and reproductive behavior in fish, in: Hoar, W.S., Randall, D.J., Donaldson, E.M.), Fish Physiology, Vol. 9: Reproduction, Part B: Behavior and Fertility Control. Academic Press, New York, pp.1–63.

Liney, K.E., Jobling, S., Shears, J.A., Simpson, P., Tyler, C.R., 2005. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutlius*) exposed to effluents from wastewater treatment works. Environ. Health Perspec. 113, 1299-1307.

Luquet, P., Watanabe, T., 1986. Interaction “nutrition-reproduction” in fish. Fish Physiology and Biochemistry Vol. 2.

Mackay, M.E., Lazier, C.B., 1993. Estrogen responsiveness of vitellogenin gene expression in rainbow trout (*Oncorhynchus mykiss*) kept at different temperatures. Gen. Comp. Endocrinol. 89, 255–266.

Majewski, A.R., Blanchfield, P.J., Palace, V.P., Wautier, K., 2002. Waterbourne 17alpha-ethynylestradiol affects aggressive behavior of male fathead minnows (*Pimephales promelas*) under artificial spawning conditions. Water Quality Research Journal of Canada 37, 697-710.

Manciocco, A., Calamandrei, G., Alleva, E., 2014. Global warming and environmental contaminants in aquatic organisms: the need of the etho-toxicology approach. Chemosphere 100, 1-7.

Martinovic-Weigelt, D., Ekman, D.R., Villeneuve, D.L., James, C.M., Teng, Q., Collette, T.W., Ankley, G.T., 2012. Fishy Aroma of Social Status: Urinary Chemo-Signaling of Territoriality in Male Fathead Minnows (*Pimephales promelas*). PLoS ONE 7(11), 1-9.

Matthiessen, P., Arnold, D., Johnson, A.C., Pepper, T.J., Pottinger, T.G., Pulman, K.G.T., 2006. Contamination of headwater streams in the United Kingdom by oestrogenic hormones from livestock farms. Sci. Total Environ. 367, 616-630.

Mayer, I., Borg, B., Páll, M., 2004. Hormonal control of male reproductive behavior in fishes: a stickleback perspective. Behaviour 141, 1499-1510.

McGee, M.R., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Schoenfuss, H.L., 2009. Predator avoidance performance of larval fathead minnows (*Pimephales promelas*) following short-term exposure to estrogen mixtures. Aquat. Toxicol. 91, 355-361.

Mehrle, P.M., Buckler, D.R., Little, B.B., Smith, L.M., Petty, J.D., Peteman, P.H., Stalling, D.L., De Graeve, G.M., Coyle, J.J., Adams, W.J., 1988. Toxicity and bioconcentration of 2,3,7,8-tetrachlorodibenzo-p-dioxin and of 2,3,7,8-tetrachlorodibenzofuran in rainbow trout. Environ. Toxicol. Chem. 7, 47-62.

Mes, T., de Zeeman, G., Lettinga, G., 2005. Occurrence and fate of estrone, 17β-estradiol and 17α-ethynylestradiol in STPs for domestic wastewater. Rev. Environ. Sci. Biotechnol. 4, 275-311.

Metcalfe, T.L., Metcalfe, C.D., Kiparissis, Y., Niimi, A.J., Foran, C.M., Benson, W.H., 2000. Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to o,p'-DDT in water or through maternal transfer. Environ. Toxicol. Chem. 19, 1893-1900.

Miller, D.H., Jensen K.M., Villeneuve, D.L., Kahl, M.D., Makynen, E.A., Durhan, E.J., Ankley, G.T., 2007. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 26, 521–527.

Molinos, J.G., Donohue, I., 2010. Interactions among temporal patterns determine the effects of multiple stressors. Ecol. Appl. 20, 1794-1800.

Munro, A.D., Scott, A.P., Lam, T.J., 1990. Reproductive seasonality in teleosts: environmental influences. Boca Raton, CRC Press.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15-24.

Nelson, J.S., Paetz, M.J., 1992. The Fishes of Alberta. The University of Alberta Press, Edmonton

Newman, M.C., Clements, W., 2008. Ecotoxicology: a comprehensive treatment. Boca Raton: Taylor and Francis / CRC Press.

Niemuth, N.J., Klaper, R.D., 2015. Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. Chemosphere 135, 38-45.

Nissanov, J., Eaton, R.C., 1989. Reticulospinal control of rapid escape turning maneuvers in fish. Am. Zool. 29, 103-121.

Norris, D.O., 2007. Vertebrate Endocrinology, Fourth Edition. Burlington, MA

Nowicki, J.P., Miller, G.M, Munday, P.L., 2012. Interactive effects of elevated temperature and CO_2 on foraging behavior of juvenile coral reef fish. J. Exp. Marine Biol. Ecol. 412, 46-51.

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environ Int. 35, 971-986.

O'Hara, R.B., Kotze, D.J., 2010. Do not log-transform count data. Methods Ecol. Evol. 1, 118–122.

O'Reilly, C.M., plus 63 others, 2015. Rapid and highly variable warming of lake surface waters around the globe, Geophys. Res. Lett. 42, 10773–10781.

O'Gorman, E.J., Fitch, J.E., Crowe, T.P., 2012. Multiple anthropogenic stressors and the structural properties of food webs. Ecol. 93, 441–448.

Oliveira, R., Domingues, I., Koppe Grisolia, C., Soares, A.M., 2009. Effects of triclosan on zebrafish early-life stages and adults. Environ. Sci. Pollut. Res. Int. 16(6), 679-688.

Ospina-Alvarez, N., Piferrer, F., 2008. Temperature-dependent sex determination in fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of climate change. PLoS ONE. 3(7), e2837.

Osterauer, R., Kohler, H-R., 2008. Temperature-dependent effects of the pesticides thiacloprid and diazinon on the embryonic development of zebrafish (*Danio rerio*). Aquat. Toxicol. 86, 485-494.

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 28, 2677-2684.

Palace, V.P., Evans, R.E., Wautier, K.G., Mills, K.H., Blanchfield, P.J., Park, B.J., Baron, C.L., Kidd, K.A., 2009. Interspecies differences in biochemical, histopathological, and population responses in four wild fish species exposed to ethynylestradiol added to a whole lake. Can. J. Fish. Aquat. Sci. 66, 1920-1935.

Pan, W., 2001. Akaike's information criterion in generalized estimating equations. Biometrics. 57, 120-125.

Panter, G.H., Thompson, R.S., Sumpter, J.P., 1998. Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquat. Toxicol. 42, 243–253.

Panter, G.H., Hutchinson, T.H., Länge, R., Lye, C.M., Sumpter, J.P., Zerulla M., et al. 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-) estrogenic substances. Environmental Toxicology and Chemistry 21: 319-326.

Parikh, V.N., Clement, T.S., Fernald, R.D., 2006. Androgen level and male social status in the African cichlid, *Astatotilapia burtoni*. Behav. Brain Res. 166(2), 291-295.

Parks, L.G., Cheek, A.O., Denslow, N.D., Heppell, S.A., McLachlan, J.A., LeBlanc, G.A., Sullivan, C.V., 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. Comp. Biochem. Physiol. C 123, 113–125.

Parrott, J.L., Wood, C.S., 2002. Fathead minnow lifecycle tests for detection of endocrine-disrupting substances in effluents. Water Qual. Res. J. Canada 37(3), 651-667.

Parrott, J.L., Blunt, B.R., 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20, 131-141.

Pelletier, E., Sargian, P., Payet, J., Demers, S., 2006. Ecotoxicological effects of combined UVB and organic contaminants in coastal waters: a review. Photochem. Photobiol. 2, 981–993.

Persson, L., 1986. Temperature-induced shift in foraging ability in two fish species, roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*): implications for coexistence between poikilotherms. J. Anim. Ecol. 55, 829.

Prather, E.E., 1957. Preliminary experiments on winter feeding small fathead minnows. *Proc. 11th Ann. Conf., Southeast Assn. Game and Fish Comm.*

Preuss, T., Faber, D.S., 2003. Central cellular mechanisms underlying temperature-dependent changes in the goldfish startle-escape behavior. J. Neurosci. 23, 5617-5626.

Przeslawski, R., Byrne, M., Mellin, C., 2015. A review and meta-analysis of the effects of multiple abiotic stressors on marine embryos and larvae. Global Change Biol. 21, 2122–2140.

Pyron, M., Beitinger, T.L., 1993. Temperature tolerance after spawning in female and male fathead minnows, *Pimephales promelas*. Texas J. Sci. 45, 319-333.

Pörtner, H.O., Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. Science 315, 95-97.

Radhakrishnaiah, K., Parvatheswararao, V., 1984. Adaptation to thermal stress in the freshwater eurythermal teleost, *Sarotherodon mossambicus*. Carbohydrate metabolism phosphorylaseactivity. J. Anim. Morphol. Physiol. 31, 195–202.

Raman, D.R., Layton, A.C., Moody, L.B., Easter, J.P., Sayler, G.S., Burns, R.T., Mullen, M.D., 2001. Degradation of estrogens in dairy waste solids: Effects of acidification and temperature. Am. Soc. Agric. Eng. 44, 1881-1888.

Ross, S.T., 2001. The inland fishes of Mississippi. University of Mississippi, Jackson, MS

Routledge, E.J., Sheahan, D., Desbrow C., Brighty G.C., Waldock, M., Sumpter, J.P., 1998. Identification of estrogenic chemicals in STW effluent, 2. In vivo responses in trout and roach. Environ. Sci. Technol. 32, 1559-1565.

Saaristo, M., Craft, J.A., Lehtonen, K.K., Lindström, K., 2010. Exposure to 17-alpha ethinyl estradiol impairs courtship and aggressive behavior of male sand gobies (*Pomatoschistus minutus*). Chemosphere 79, 541-546.

Salice, C.J., Rowe, C.L., Pechmann, J.H.K., Hopkins, W.A., 2011. Multiple stressors and complex life cycles: insights from a population-level assessment of breeding site contamination and terrestrial habitat loss in an amphibian. Environ. Toxicol. Chem. 30(12), 2874-2882.

Salierno, J.D., Kane, A.S., 2009. 17-alpha-ethinyl estradiol alters reproductive behaviors, circulating hormones and sexual morphology in male fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 28, 953-961.

Schoenfuss, H.L., Bartell, S.E., Bistodeau, T.B., Cediel, R.A., Grove, K.J., Zintek, L., Lee, K.E., Barber, L.B., 2008. Impairment of the reproductive potential of male fathead minnows by environmentally relevant exposures to 4-nonylphenol. Aquat. Toxicol. 86, 91–98.

Schultz, M.M., Bartell, S.E., Schoenfuss, H.L., 2012. Effects of triclosan and triclocarban, two ubiquitous environmental contaminants, on anatomy, physiology, and behavior of the fathead minnow (*Pimephales promelas*). Archiv. Environ. Contam. Toxicol. 63(1), 114-124.

Schwindt, A.R., Winkelman, D.L., Keteles, K., Murphy, M., Vajda, A.M., 2014. An environmental oestrogen disrupts fish population dynamics through direct and transgenerational effects on survival and fecundity. J. Appl. Ecol. 51, 582–591.

Shappell, N.W., Hyndman, K.M., Bartell, S.E., Schoenfuss, H.L., 2010. Comparative biological effects and potency of 17-α and 17β-estradiol in fathead minnows. Aquat. Toxicol. 100, 1–8.

Shears, N.T., Ross, P.M., 2010. Toxic cascades: multiple anthropogenic stressors have complex and unanticipated interactive effects on temperate reefs. Ecol. Lett.13, 1149–1159.

Shi, J., Fujisawa, S., Nakai, S., Hosomi, M., 2004. Biodegradation of natural and synthetic estrogen by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*. Water Res. 38(9), 2323-2330.

Shioda, T., Wakabyashi, M., 2000. Evaluation of reproductivity of medaka (*Oryzias latipes*) exposed to chemicals using a 2-week reproduction test. Water Sci.Technol. 42(7-8), 53-60.

Sloman, K.A., McNeil, P.L., 2012. Using physiology and behaviour to understand the responses of fish early life stages to toxicants. J. Fish Biol. 81, 2175–2198.

Smith, G., Weis, J.S., 1997. Predator/prey interactions of the mummichog, *Fundulus heteroclitus*: Effects of living in a polluted environment. J. Exp. Marine Biol. Ecol. 209, 75-87.

Smith, R.J.F., 1974. Effects of 17 α-methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). Can. J. Zool. 52, 1031-1038.

Smith, R.J.F., 1978. Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, *Pimephales promelas*. Can. J. Zool. 56, 2103–2109.

Smith, R.J.F., Murphy, B.D., 1974. Functional Morphology of the Dorsal Pad in Fatheads Minnows (*Pimephales promelas* Rafinesque). Transactions of the American Fisheries Society 103(1), 65-72.

Socorro, S., Power, D.M., Olsson, P.E., Canario, A.V.M., 2000. Two estrogen receptors expressed in teleost fish, *Spasrus aurata*: cDNA cloning, characterization and tissue distribution. J. Endocrinol. 166, 293-306.

Sokolova, I.M., 2013. Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integr. Comp. Biol. 53(4), 597-608.

Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Climate Res. 37(2-3), 181-201.

Sommer, A., 2011. *Pimephales promelas*. Animal diversity Web. Retrieved from http://animaldiversity.ummz.umich.edu/accounts/Pimephales_promelas/

Staples, C.A., Tilghman Hall, A., Friederich, U., Caspers, N., Klecka, G.M., 2011. Early life-stage and multigeneration toxicity study with bisphenol A and fathead minnows (*Pimephales promelas*). Ecotoxicol. Environ. Safety 74(6), 1548-1557.

Starner, K., Kuivila, K.M., Jennings, B., Moon, G.E., 1999. Degradation rates of six pesticides in water from the Sacramento River, California, pp. 89-99. In: U.S. Geological Survey Toxic Substances Hydrology Program Water Resource Investigation Report 99-4018A, Proceedings of the Technical Meeting, Charleston, SC

Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect. 103(7), 173-178.

Szabo, T.M., Brookings, T., Preuss, T., Faber, D.S., 2008. Effects of temperature acclimation on a central neural circuit and its behavioral output. J. Neurophysiol. 100, 2997-3008.

Tan, D. T., Arnold, W. A., Novak, P. J., 2013. Impact of organic carbon on the biodegradation of estrone in mixed culture systems. Environ. Sci. Technol. 47, 12359–12365.

Taves, M.D., Desjardins, J.K., Mishra, S., Balshine, S., 2009. Androgens and dominance: sex-specific patterns in a highly social fish (*Neolamprologus pulcher*). Gen. Comp. Endrocrinol. 161, 202−207.

Thorpe, K.L., Benstead, R., Hutchinson, T.H., Cummings, R.I., Tyler, C.R., 2003. Reproductive effects of exposure to oestrone in the fathead minnow. Fish Physiol. Biochem. 28, 451–452.

Thorpe, K.L., Benstead, R., Hutchinson, T.H., Tyler, C.R., 2007. Associations between altered vitellogenin and adverse health effects in fathead minnows (*Pimephales promelas*). Aquat.Toxicol. 85, 176–183.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 37(6), 1142-1149.

Tuomainen, U., Candolin, U., 2011. Behavioural responses to human-induced environmental change. Biol. Rev. 86, 640–657.

US EPA, 1987. Guidelines for the culture of fathead minnows *Pimephales promelas* for use in toxicity tests. Retrieved from http://nepis.epa.gov/Adobe/PDF/2000HU6Q.PDF

US EPA, 2014. Contaminants of emerging concern. Retrieved from http://water.epa.gov/scitech/cec/

USGS, 2015. Non-indigenous aquatic species. Retrieved from http://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=621

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O., 2008. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. Environ. Sci. Technol. 42, 3407–3414.

van Aerle, R., Pounds, N., Hutchinson, T.H., Maddix, S., Tyler, C.R., 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. Ecotoxicology 11, 423-434.

van Breukelen, N.A., Sneckser, J.L., Itzkowitz, M., 2015. Male convict cichlid 11-ketotestosterone levels throughout the reproductive cycle: an exploratory profile study in laboratory and field populations. PeerJ 3:e949; DOI 10.7717/peerj.949

Van den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R., Witters, H., 2004. Comparative study on the in vitro/in vivo estrogenic potencies of 17β-estradiol, estrone, 17α-ethynylestradiol and nonylphenol. Aquat. Toxicol. 66, 183–195.

Van Zuiden, T.M., Chen, M.M., Stefanoff, S., Lopez, L., Sharma, S., 2016. Projected impacts of climate change on three freshwater fishes and potential novel competitive interactions. Diversity Distrib. 22, 603–614.

Voet, D., Voet, J.G., Pratt, C.W., 2013. Fundamentals of biochemistry, Fourth Edition. Hoboken, NJ.

Walker, J.A., Ghalambor, C.K., Griset, O.L., McKenney, D., Reznick, D.N., 2005. Do faster starts increase the probability of evading predators? Funct. Ecol. 19, 808–815.

Ward, J.L., Blum, M., 2012. Exposure to an environmental estrogen breaks down sexual isolation between native and invasive species. Evol. Appl. doi: 10.1111/j.1752-4571.2012.00283.x.

Ward, J.L., McLennan, D.A., 2006. Changes in agonistic, courtship and parental displays of the male brook stickleback, *Culaea inconstans*, across the breeding cycle. Behaviour 143, 33-56.

Watanabe, K.H., Jensen, K.M., Orlando, E.F., Ankley, G.H., 2007. What is normal? A characterization of the values and variability in reproductive endpoints of the fathead minnow, *Pimephales promelas*. Comp. Biochem. Phys. C. 146, 348-356.

Webb, P.W., 1978. Effects of temperature on fast-start performance of rainbow trout, *Salmo gaidneri*. J. Fish Res. Board Can. 35, 1417-1422.

Webb, P.W., Zhang, H., 1994. The relationship between responsiveness and elusiveness of heat-shocked goldfish (*Carassius auratus*) to attacks by rainbow trout (*Oncorhynchus mykiss*). Can. J. Zool. 72, 423-426.

Weber, D.N., Russo, D.B., Spieler, R.E., 1991. Waterbourne lead affects feeding abilities and neurotransmitter levels of juvenile fathead minnows (*Pimephales promelas).* Aquat. Toxicol. 21, 71-80.

Weis, J.S., Candelmo, A., 2012. Pollutants and fish predator/prey behavior: A review of laboratory and field approaches. Curr. Zool. 58(1), 9-20.

Wild, H. E., Sawyer, C. N., McMahon, T. C., 1971. Factors affecting nitrification kinetics. J. Water Pollut. Control Fed. 43, 1845–1854.

Writer, J.H., Ryan, J.N., Keefe, S.H., Barber, L.B., 2012. Fate of 4-nonylphenol and 17β-estradiol in the Redwood River of Minnesota. Environ. Sci. Technol. 46, 860-868.

Xia, J-G, Ma Y-J, Guo W-M, Huang L, Fu S-J., 2015. Temperature-dependent effects of PFOS on risk recognition and fast-start performance in juvenile *Spinibarbus sinensis*. Aquat. Biol. 24, 101-108.

Zimmer, K.D., Hanson, M.A., Butler, M.G., 2002. Effects of fathead minnows and restoration on prairie wetland ecosystems. Freshwater Biol. 47, 2071-2086.