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Thermal modulation of anthropogenic estrogen exposure on a freshwater fish at two life stages

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ABSTRACT

Human-mediated environmental change can induce changes in the expression of complex behaviors within individuals and alter the outcomes of interactions between individuals. Although the independent effects of numerous 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, we exposed reproductively mature and larval fathead minnows (*Pimephales promelas*) to three environmentally relevant concentrations (14, 22, and 65 ng/L) of a common environmental estrogen, estrone (E1), at four water temperatures (15, 18, 21, and 24 °C) reflecting natural spring and summer variation. We then conducted a series of behavioral experiments to assess the independent and interactive effects of temperature and estrogen exposure on intra- and interspecific interactions in three contexts with important fitness consequences; reproduction, foraging, and predator evasion. Our data demonstrated significant independent effects of temperature and/or estrogen exposure on the physiology, survival, and behavior of larval and adult fish. We also found evidence suggesting that thermal regime can modulate the effects of exposure on larval survival and predator-prey interactions, even within a relatively narrow range of seasonally fluctuating 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.

1. 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 (Global Biodiversity Outlook II, 2006; 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 such as dissolved oxygen, pH, salinity, UV radiation or temperature (Crain et al., 2008; Häder and Gao, 2015; Heugens et al., 2001; Holmstrup et al., 2010; Laskowski et al., 2010) have the 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 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). Although the outcomes of these multi-factor interactions are often cumulative or synergistic, they can also be unpredictable (Christensen et al., 2006; Muthukrishnan and Fong, 2014; O'Gorman et al., 2012; Shears and Ross, 2010; see also Crain et al., 2008; Darling and Cote, 2008), or vary across space or time (Molinos and Donohue, 2010; Newman and Clements, 2008), including life stage (Przeslawski et al., 2015; Salice et al., 2011). Thus, concerted efforts to understand 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 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 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

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exposed individuals (Kuiper et al., 1998). Because rates of introduction typically exceed chemical half-lives (Daughton, 2002), endocrine disrupting chemicals (EDCs) are common 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 (Bhandari et al., 2015; McGee et al., 2009; Niemuth and Klaper, 2015; Saaristo et al., 2010; van Aerle et al., 2002; Ward and Blum, 2012). Furthermore, empirical work and population modeling have convincingly demonstrated that these individual-level effects can dramatically impair the viability and sustainability of aquatic populations (Brown et al., 2015; Kidd et al., 2007; Palace et al., 2009).

Efforts to assess the impacts of EDCs on natural populations, however, are complicated by the fact that rates of chemical degradation in the environment (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; Heugens 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 (Brown et al., 2015; Hallare et al., 2005; Heugens et al., 2001, 2003; Khan et al., 2006). 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 (Körner et al., 2008; Korsgaard et al., 1986; Mackay and Lazier, 1993), 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 to influence mortality and physiological impairment (Gordon, 2003; Heugens et al., 2001).

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 represents 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, such as predator-prey relationships, competition for resources, or reproduction, have 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 laboratory 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), 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, specifically predator evasion, foraging efficiency, and 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 exposure at a single life stage (but see Oliveira et al., 2009; Parrott and Blunt, 2005; 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.

2. Material and methods

2.1. 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 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 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 a lethal concentration of $NaCO_2$-buffered MS-222 (Western Chemical, WA, USA). The subjects were dissected (adults) or stored in RNA*later*® (Thermo-Fisher Scientific, MA, USA) (larvae) for use in a separate study. All procedures, and care and maintenance protocols, were approved by the Institutional Animal Care and Use Committee (protocol number 8-73) at St. Cloud State University.

2.2. Subjects, housing and maintenance

Six-month-old, reproductively mature *P. promelas* were purchased from a laboratory 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 this species 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). During this time holding temperatures were increased or decreased as necessary to reach the experiment-specific ambient water temperature. 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.) 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 daily for mortality.

2.3. Exposure chemicals

Estrone is a common natural estrogen discharged in wastewater effluent, and is representative of a broad class of steroidal hormones and other chemicals with estrogenic activity. In a U.S. national survey, Kolpin et al. (2002) reported concentrations of E1 in rivers ranging from <5 ng/L to 112 ng/L.

Powdered estrone (≥99% purity) was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in 100% ethanol (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 (5×) and high (25×) 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.

2.4. Exposure apparatus and regime

The fish were maintained throughout the exposure period in 12 L plexi-glass aquaria (30.5 × 30.5 × 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 airstone, 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 (0.0002% v/v), 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 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 including 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 (Axys Analytical Services, Sydney, BC, Canada; Method MLA-075, 2014) (total n = 21 samples, 6-8 samples per concentration).

2.5. Reproduction, survival and growth

During the exposure 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 it into a breeding basket located in the parental aquarium. From these 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 for 21 days, beginning from the day that the eggs were laid, and then used in the behavioral assays.

2.6. 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 conditioned well water (aerated for 24 h prior to use). Larval assays were conducted on day 21 of larval exposure in a 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 assay.

2.7. 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 (see Supplementary video 1). Importantly, variation in predator avoidance performance correlates 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 locomotive mechanics (McGee et al., 2009). Briefly, at the start of each trial, a random subject was placed into a clear-bottomed, 5-cm-diameter testing arena containing 10 mL of conditioned well water. The arena was centered 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 × 1 mm grid to allow for quantification of the response, and illuminated via a Kessil A150 fiber optic light source (Richmond, CA) angled 20 cm above the arena. Subjects were permitted to acclimate to the arena for 1 min, after which the stimulus (~0.5 s in duration) was delivered. Subject responses were recorded using a Redlake MotionScope (Tucson, AZ) high-speed camera (1000 frames s^{-1}) positioned ~25 cm vertically above the test arena.

One observer, blinded 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 (National Institutes of Health, Bethesda, MD). 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 standard body length (BL). 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 per 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 simultaneously takes into account 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 <6 ms were considered false starts and were discarded prior to statistical analysis.

2.8. 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 treatments. The night before each trial, we placed two random 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 using a Canon NTSC Optura 20 digital Hi-8 video camera positioned 40 cm above the test arena, and illuminated by a Kessil A150 fiber optic light source.

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 ± SD, 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 Na_2CO_3-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-field microscope (Center Valley, PA). We subtracted the number of remaining prey from the initial quantity to obtain the number of prey items eaten in each trial. Similar to the procedure described for experiment 1, we used ImageJ to measure the BL of each fish in each trial from video images; we examined the relationship between size and consumption in preliminary analyses (see 'Statistics').

2.9. 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 an 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-μm mesh bottom that permitted the passage of water. The arena was located in the center of a plastic chamber (40 × 30 × 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 BL. 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.

2.10. 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. 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 × 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 and McLennan, 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 to 3 h rest period after ~20 to 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 towards 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 (McMillan and Smith, 1974; Pyron and Beitinger, 1989), were recorded in real time. A butt was defined as a slow approach towards 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 towards the intruding male, accompanied by propulsive tail beats 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.

2.11. 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. We similarly used a chi-square test to compare the survival of adult fish among treatments. The effects of exposure concentration and temperature on clutch size, fertilization success, hatching latency, and larval survival were examined via ANOVAs; larval growth (BL 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 BL 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 among-treatment responses of larvae 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, temperature, and the interaction between these two terms. In preliminary models for experiment 2 we also included the average BL 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 size 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 BL 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 size 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 the 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. We removed the size term prior to final analyses. Unless otherwise indicated, data were analyzed using SPSS (v 21) (IBM, New York, USA). For ANOVAs, we estimated the influence of each fixed effect on subject responses, relative to the other factors in the model, via the partial variance statistic (η^2). For all analyses, we examined the effect size for significant pairwise posthoc comparisons via Cohen's d.

3. Results

3.1. Exposure conditions

Measured E1 concentrations (mean ± SD) were 13.82 ± 7.15, 22.27 ± 9.13 and 65.39 ± 27.70 ng/L for the $E1_{LOW}$ (n = 6 samples), $E1_{MED}$ and $E1_{HIGH}$ (n = 7 and 8 samples for the $E1_{MED}$ and $E1_{HIGH}$ treatments, respectively) exposure treatments; all three values were within the environmental range reported by Kolpin et al. (2002). 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 comparable throughout the experiment, and were well within tolerance limits for the study species.

3.2. 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 ($\chi^2 = 0.99$, 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 54 (in the control and $E1_{MED}$ treatments, both at 24 °C) (Fig. 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 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. 1b; Cohen's d = 0.26). We also found a significant effect of water temperature on clutch size (Table 1). In general, females maintained at lower temperatures laid more eggs than those maintained at higher temperatures (Fig. 1b). Post-hoc tests revealed that clutches laid at 21 °C had significantly fewer eggs than those laid at 15 °C ($P < 0.001$; Cohen's d = 0.60) or 18 °C ($P < 0.001$; Cohen's d = 0.81). Clutch sizes were also smaller at 24 °C compared with 15 °C ($P = 0.001$; Cohen's d = 0.32). 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 1; Fig. 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 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; Cohen's d = 1.02–1.38) (Fig. 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. 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 1); mean (±SD) survival at 15 °C (25% ± 16%) was significantly greater for control subjects than for subjects exposed to $E1_{LOW}$ (9% ± 12%, $P = 0.002$; Cohen's d = 1.09), $E1_{MED}$ (9% ± 17%, $P = 0.004$; Cohen's d = 0.93) or $E1_{HIGH}$ (5% ± 8%, $P < 0.001$; Cohen's d = 1.53). 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. 1f). Larval size (body length) on day 21 was positively linearly related to the ambient temperature (Table 1). Posthoc tests indicated that larvae reared at 24 °C were significantly larger than those reared at 15 °C or 18 °C (both Ps < 0.001; Cohen's d = 1.33 and 0.94, respectively). Larvae reared at 18 °C were also larger than those raised at 15 °C ($P = 0.004$; Cohen's d = 0.30).

3.3. Experiment 1: effects of temperature and E1 concentration on predator evasion performance

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). Overall, velocity was negatively related to temperature (Fig. 2a); average (±SD) escape velocities of larvae reared at 15, 18 and 24 °C were 0.030 ± 0.020, 0.024 ± 0.016, and 0.019 ± 0.017 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 ($P = 0.026$; Cohen's d = 0.33) or 24 °C ($P = 0.002$; Cohen's d = 0.59), 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. 2a); average (±SD) escape velocities of larvae exposed to control, $E1_{LOW}$, $E1_{MED}$ and $E1_{HIGH}$ were 0.022 ± 0.016, 0.026 ± 0.017, 0.026 ± 0.017 and 0.022 ± 0.017 BL/ms, respectively; larvae exposed to $E1_{MED}$ were significantly faster than subjects in either the control ($P = 0.006$; Cohen's d = 0.18) or $E1_{HIGH}$ ($P = 0.003$; Cohen's d = 0.23) 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), 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 ($P = 0.005$; Cohen's d = 0.23). Escape velocities were similar among control and exposed subjects at higher temperatures (all pairwise Ps > 0.05 at 18 °C and 24 °C).

Temperature, but not exposure concentration, also had a significant effect on latency (Table 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. 2b). Posthoc tests indicated that the latencies of subjects reared at 24 °C were significantly longer than those raised at 15 °C ($P = 0.013$; Cohen's d = 0.54); no other significant pairwise differences were found (Ps > 0.05).

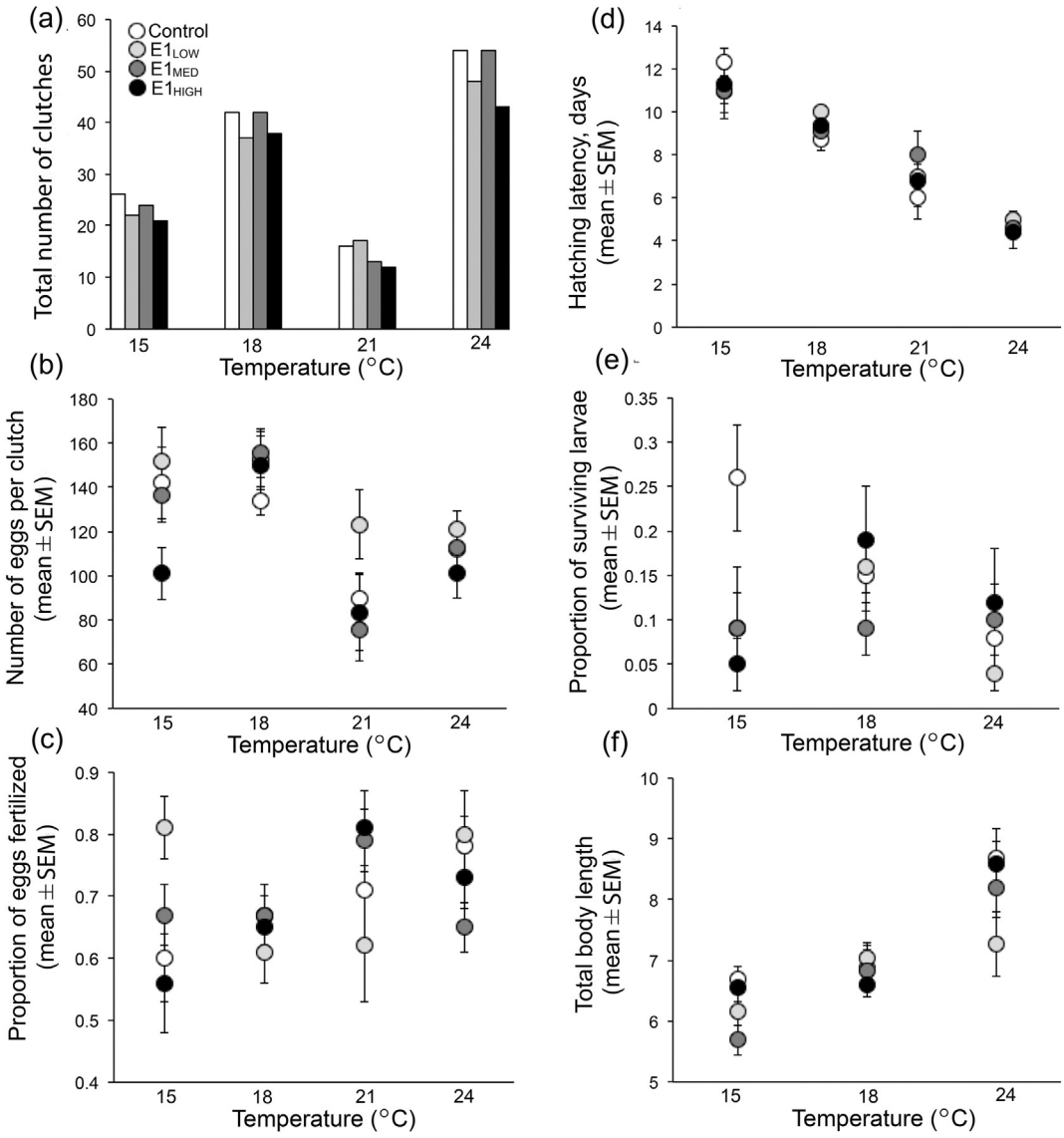

Fig. 1. Reproduction, growth, and survival of fathead minnows (*Pimephales promelas*) over a 30-day exposure period at four ambient temperatures. (a) Total level of spawning activity observed in each factorial temperature and E1 exposure concentration combination (n = 12–54); (b) female fecundity, indicated by the number of eggs (n = 12–54); (c) male fertilization success, measured as the proportion of eggs laid that showed evidence of eyespots (n = 10–51); (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 = 3–12); (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), $E1_{MED}$ (22 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± SEM.

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 ($0.06 \le P \le 0.89$; Table 2; Fig. 2c, d). However, the total escape response exhibited a linear trend consistent with the interpretation that performance decreases with increasing temperature (Fig. 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.

3.4. Experiment 2: effects of temperature and E1 concentration on prey capture success of larval minnows

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. 3a. The GEE revealed a significant effect of temperature on capture success (Table 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 11.06 ± 5.64, 14.98 ± 5.85, and 17.15 ± 5.50, respectively. Post-hoc tests revealed that prey capture was significantly enhanced at each step-wise increase in temperature ($0.012 \le P < 0.001$; Cohen's d = 0.38–1.09). We did not detect a significant main effect of E1 concentration on prey capture success (Table 3). However, we did find a significant temperature *x* concentration interaction (Table 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 ($P = 0.013$; Cohen's d = 0.89) or $E1_{LOW}$ treatments ($P = 0.001$; Cohen's d = 1.71). 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 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	η^2
Fecundity	Concentration	2.67	3492	**0.047**	0.016
	Temperature	13.78	3492	<**0.001**	0.077
	Concentration × temperature	1.19	9492	0.297	0.021
Fertility	Concentration	0.59	3390	0.623	0.004
	Temperature	1.34	3390	0.260	0.009
	Concentration × temperature	1.72	9390	0.083	0.038
Hatching latency	Concentration	0.24	3103	0.871	0.007
	Temperature	59.20	3103	<**0.001**	0.633
	Concentration × temperature	0.70	9103	0.700	0.058
Larval survival	Concentration	1.90	3,85	0.136	0.051
	Temperature	2.39	2,85	0.098	0.043
	Concentration × temperature	2.49	6,85	**0.029**	0.134
Larval size	Concentration	4.56	3	0.297	
	Temperature	48.30	2	<**0.001**	
	Concentration × temperature	11.35	6	0.078	

3.5. Experiment 3: effects of temperature and E1 concentration on prey capture success of adult minnows

The average number of prey successfully identified, localized and captured by either male or female subjects in each of the 16 treatments are shown in Fig. 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 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; Cohen's d = 0.39–0.86). Subjects also consumed more prey at 21 °C than 18 °C (P = 0.011; Cohen's d = 0.49). Only one exception to this general trend was observed; subjects consumed more prey at 15 °C than at 18 °C (P = 0.005; Cohen's d = 0.50).

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 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.

3.6. Experiment 4: effects of temperature and E1 concentration on territorial aggression

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 $E1_{HIGH}$. Accordingly, there was 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 5). Males exposed to $E1_{HIGH}$ performed fewer aggressive acts than males exposed to $E1_{MED}$ (P = 0.005; Cohen's d = 0.66) or control subjects (P = 0.005; Cohen's d = 0.62) (Fig. 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 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 ≤ Ps ≤ 0.001; Cohen's d = 0.36–0.71) (Fig. 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 5).

By contrast, neither E1 concentration nor temperature had independent or interactive effects on the latency to first response by resident males (Table 5), although there was a trend for latency to decrease with increasing temperature (Fig. 4d); 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 s, respectively.

Table 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 × temperature	14.37	9	**0.026**
Latency	Concentration	1.76	3	0.625
	Temperature	6.87	2	**0.032**
	Concentration × temperature	10.32	6	0.112
Total escape response	Concentration	3.57	3	0.312
	Temperature	4.88	2	0.087
	Concentration × temperature	12.02	6	0.061
Angle of escape	Concentration	2.80	3	0.423
	Temperature	1.30	2	0.521
	Concentration × temperature	2.29	6	0.891

4. Discussion

In this study, we examined the extent to which variation in the ambient temperature modulates the behavior and physiology 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.

4.1. Independent and interacting effects of temperature and EDC exposure on behavior

4.1.1. 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., 2001). The response is initiated when one of the two M-cells is activated and the signal is propagated along the axon, causing the contralateral trunk muscles to contract into the characteristic "C" shape (Eaton et al., 2001). 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 motoneuron transmission is also affected by a variety of chemicals (Carlson et al., 1998), resulting in impaired 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 response compared with fish exposed at 18 °C (Xia et al., 2015). With the exception that larvae

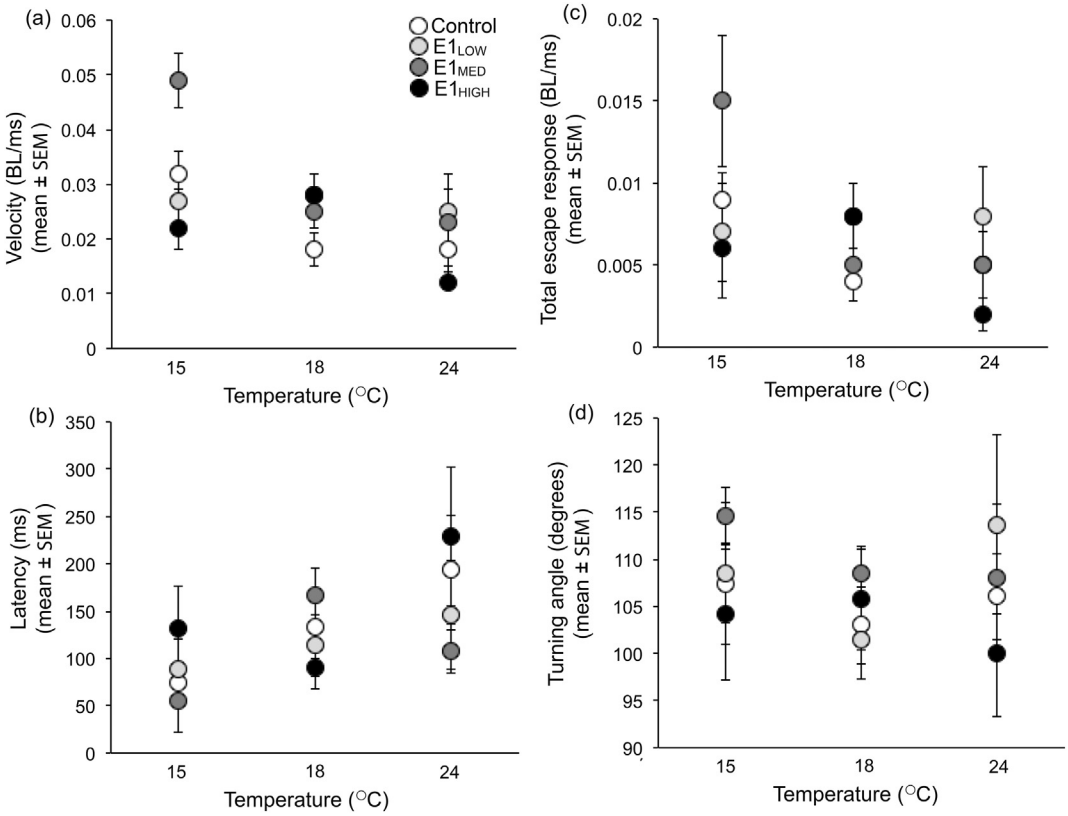

Fig. 2. Responses of larvae exposed to E1 or an EtOH solvent control (control), at three ambient temperatures. Sample sizes ranged from 3 to 19 across treatments. (a) Escape velocity (BL/ms) over the first 40 ms of the response; (b) latency (in ms) to the induction of the response; (c) total escape response (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), $E1_{MED}$ (22 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± SEM.

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 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 the $E1_{HIGH}$ 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., Batty and Blaxter, 1992; Webb, 1978; reviewed in Domenici 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 at 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 et al., 1996; Krupczynski and Schuster, 2013; Preuss and Faber, 2003). 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).

4.1.2. Male-male aggression

Estrogen-induced reductions in the nesting behavior (Brian et al., 2006) and aggression (Bell, 2001; Colman et al., 2009; Saaristo et al., 2010) of male fish are well described. 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 demonstrating that estrogenic EDCs decrease levels of androgens (Coe et al., 2008; Salierno and Kane, 2009) that regulate 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 higher temperatures (i.e., 21 °C and 24 °C, which corresponds to the optimal thermal breeding regime; Brian et al., 2011; Smith, 1978); 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*) (Kochhann 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). 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

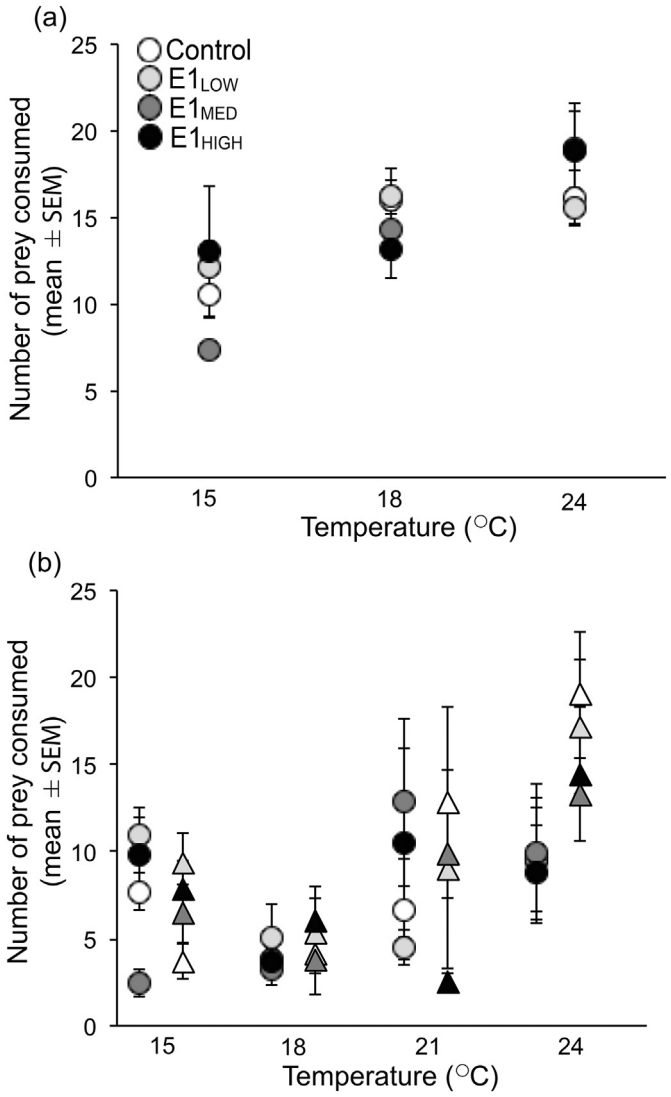

Fig. 3. Prey consumption by (a) larvae or (b) adult subjects exposed to varying concentrations of E1 or solvent (control) at different ambient temperatures. Sample sizes across treatments in (a) ranged from 3 to 23. Sample sizes in (b) ranged from 12 to 28. White symbols represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black symbols represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (22 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.

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).

Table 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 × temperature	22.14	6	<**0.001**

Table 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 × temperature	15.37	9	0.081
Concentration × sex	2.23	3	0.526
Temperature × sex	4.60	3	0.203
Concentration × temperature × sex	14.99	9	0.091

4.1.3. 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, 2013; Sokolova and Lannig, 2008). Exposure to heavy metals, pharmaceuticals, and other contaminants has been shown 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 rutilus*) exposed to 50 ng/L 17α-ethinylestradiol showed reduced foraging success, measured as the number of *D. magna* captured and consumed over a given internal 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, *Perca fluviatilis*; ruffe, *Gymnocephalus cernuus* (Bergman, 1987); and short-horned sculpin, *M. scorpius* (Beddow et al., 1995). 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*;

Table 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	η^2
Aggressive displays	Concentration	3.80	3191	**0.011**	0.056
	Temperature	5.90	3191	**0.001**	0.086
	Concentration × temperature	0.98	9191	0.462	0.044
Latency	Concentration	1.50	3111	0.219	0.039
	Temperature	1.55	3111	0.205	0.040
	Concentration × temperature	0.73	9111	0.685	0.055

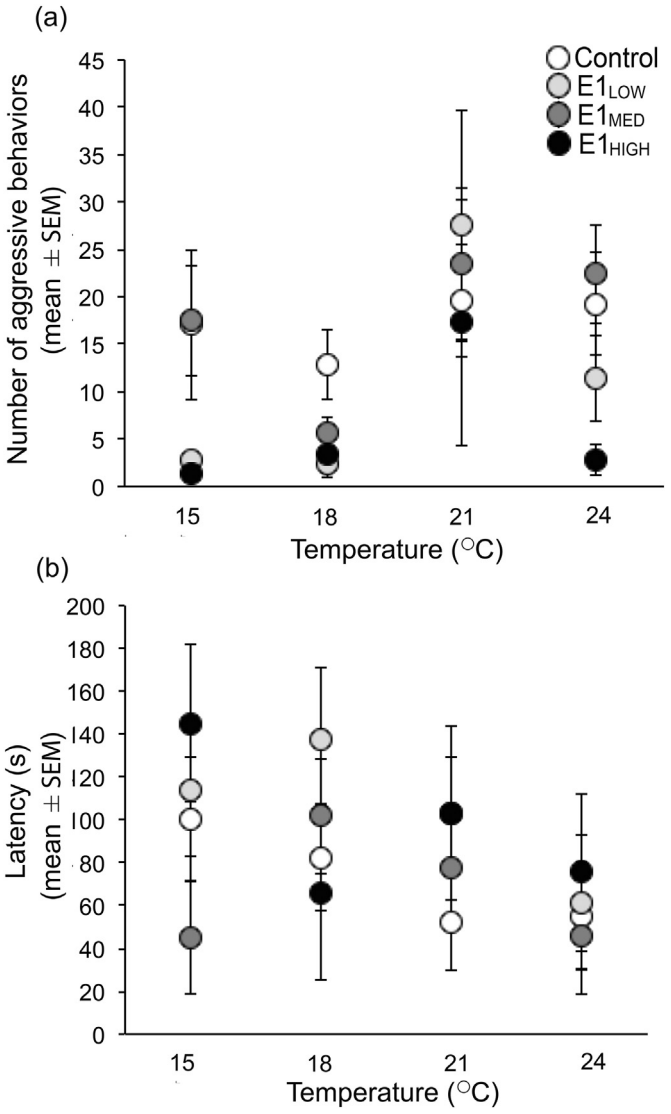

Fig. 4. Aggressive responses of resident males exposed to varying concentrations of E1 at different ambient temperatures (n = 8–16). (a) Combined number of butts and strikes; (b) latency to first aggressive act (s). White circles represent the EtOH solvent control exposure group (0 ng/L E1). Light gray, dark gray, and black circles represent the $E1_{LOW}$ (14 ng/L), $E1_{MED}$ (22 ng/L), and $E1_{HIGH}$ (65 ng/L) exposure treatments. Points and error bars depict means ± SEM.

however, temperature-dependent variation in the outcomes of predator-prey interactions could reflect direct differences in attack rate (Grigaltchik et al., 2012; Persson, 1986), 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).

4.2. 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., Dammann et al., 2011; Parrott and Blunt, 2005; Thorpe et al., 2007). Moreover, these effects of exposure can be transgenerational (Schwindt et al., 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.

5. Conclusion

Our data show that (i) variation in behavioral responsiveness, kinematics, and the outcomes of intraspecific and interspecific interactions can occur within a relatively narrow range of seasonally fluctuating temperatures, and that (ii) changes in the thermal regime can interact with chemical stressors in ways that further influence physiology, survival and behavior. The significant interactions between E1 and temperature that we observed could all be traced to differences 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 review). Support for this hypothesis comes from the finding that concentrations of *vtg* in male *P. promelas* used in this study were significantly higher at 15 °C than at warmer temperatures (Cox et al., in review). Alternatively, metabolic changes associated with elevated temperatures may have resulted in increased rates of degradation and elimination (Noyes et al., 2009). Moreover, all of the significant interactions between temperature and exposure that we observed occurred in larvae, consistent with previous studies indicating that individuals in exposed populations are particularly susceptible to perturbation by EDCs during the early ontogenetic stages of life (Guillette et al., 1996; Liney et al., 2005; Sloman and McNeil, 2012; van Aerle et al., 2002). 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). Taken together, our 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, are likely be particularly vulnerable to interactions between shifting thermal regimes and contaminants (Noyes et al., 2009). Further studies are necessary to improve our understanding of the effects of interactions among multiple anthropogenic stressors, and between stressors and natural abiotic environmental factors. Such studies are likely to be of key importance to identifying vulnerable populations and predicting population dynamics under changing real-world scenarios.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2017.05.015.

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