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Windows of Susceptibility and Consequences of Early Life Exposures to 17β−estradiol on Medaka (*Oryzias latipes*) Reproductive Success

Crystal S.D. Lee Pow,[*,†] Kedamawit Tilahun,[†] Kari Creech,[†] J. Mac Law,[‡] W. Gregory Cope,[§] Thomas J. Kwak,[§,∥] James A. Rice,[§] D. Derek Aday,[§] and Seth W. Kullman[†]

[†]Department of Biological Sciences, Environmental and Molecular Toxicology Program, North Carolina State University, 850 Main Campus Drive, Raleigh, North Carolina 27606, United States

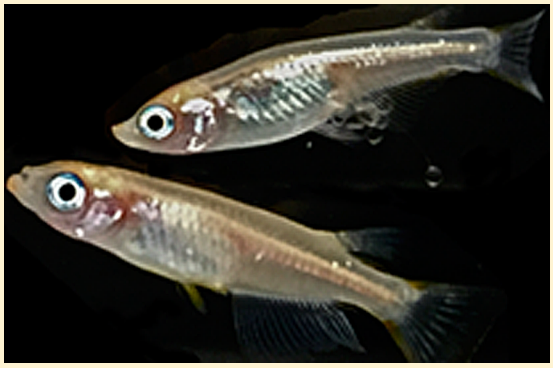
[‡]Department of Population Health and Pathobiology, North Carolina State University, 1060 William Moore Drive, Raleigh, North Carolina 27607, United States

[§]Department of Applied Ecology, North Carolina State University, 127 David Clark Laboratories, Raleigh, North Carolina 27695, United States

[∥]United States Geological Survey, North Carolina Cooperative Fish and Wildlife Research Unit, Raleigh, North Carolina 27695, United States

Supporting Information

ABSTRACT: Estrogens and estrogen mimics are commonly found in surface waters and are associated with deleterious effects in fish populations. Impaired fertility and fecundity in fish following chronic exposures to estrogens and estrogen mimics during critical windows in development are well documented. However, information regarding differential reproductive effects of exposure within defined developmental stages remains sparse. In this study, reproductive capacity was assessed in Japanese medaka (*Oryzias latipes*) after exposure to two concentrations of 17β−estradiol (E2β; 2 ng/L and 50 ng/L) during four distinct stages of development: gonad development, gonad differentiation, development of secondary sex characteristics (SSC) and gametogenesis. Exposure to E2β did not adversely impact survival, hatch success, growth, or genotypic ratios. In contrast, exposure to 50 ng/L E2β during SSC development altered phenotypic ratios and SSC. Exposure to both E2β treatments reduced reproductive capacity (fertility, fecundity) by 7.3−57.4% in adult medaka breeding pairs, with hindrance of SSC development resulting in the largest disruption in breeding capacity (51.6−57.4% decrease) in the high concentration. This study documents differential effects among four critical stages of development and provides insight into factors (window of exposure, exposure concentration and duration of exposure period) contributing to reproductive disruption in fish.



INTRODUCTION

Many classes of chemicals bind estrogen receptors and disrupt endogenous estrogen signaling in humans and wildlife.[1−5] These estrogenic endocrine active compounds (EACs) are exogenous compounds or mixtures that alter functions of the endocrine system with the potential to cause adverse effects in individuals, their progeny or their subsequent progeny.[6] Many estrogenic EACs have been detected in surface waters globally[2,7−11] and have been linked to a number of adverse effects in fish. For example, estrogen contaminants can suppress primary and secondary sex characteristics (SSC) in male fish[1,4,12,13] and alter testicular development and function via a marked increase in apoptosis of spermatogonia cells (primordial germ cells), reduced spermatogenesis and feminization of the seminiferous tubules.[4,14−17] The culmination of these cellular events often results in a defined reduction of male reproductive output.[4,14] Equally important at the population level are effects of estrogenic contaminants on female teleost reproduction. Exposure to estrogen contaminated waters can disrupt hormonal feedback signals, modulate endogenous circulating hormone levels, and alter ovarian development.[18−21] Exposure has also been found to delay oocyte development and cause the formation of atretic oocytes.[15,16,18,22] The effects in male and female fish can be linked to reduced breeding capacity and declines in fish populations.[20,23−27]

Disruption of reproductive success can occur during several periods of development, resulting in organizational and activational effects. Exposures during early windows of development (organization phase) may impact primordial cell differentiation, growth, and gonad development and can lead to detrimental organizational effects, which are permanent.[28,29] In contrast, exposures within the activational phase of develop-

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ment typically influence reproductive maturation, including spermatogenesis and oogenesis.[28,29] These exposures occur at later stages in life and can lead to activational effects, which can be mitigated after termination of exposure and are typically associated with a biological compensatory response that facilitates a reduction in exposure or effect.[30,31] In a study from the United Kingdom, roach (*Rutilus rutilus*) were exposed to wastewater treatment plant effluent during early life stage development (hatch to 300 days post hatch).[32] Their results suggest that exposure led to different magnitudes of feminization. Similarly, Hirai et al. document differential disruption of fecundity and fertility in Japanese medaka (*Oryzias latipes*) following E2β exposure at two periods, fertilization to 31 days post fertilization and fertilization to 81 days post fertilization.[33] While numerous studies have documented impaired fertility and fecundity in fish following chronic exposures to estrogens and estrogen mimics within both the organizational and activational phases,[4,21,22,34,35] there remains a paucity of information regarding differential reproductive effects of exposure within defined developmental stages. This study examined reproductive dysfunction following exposure during four critical stages of development (susceptibility) in medaka: gonad development, gonad differentiation, development of SSC, and gonad maturation.

A successful model species for laboratory-based reproductive toxicity testing, medaka is an extensively studied small fish. Similar to the function of the SRY gene in humans, Medaka undergo genetic sex determination via the Y-specific DM-domain gene (*dmy*),[36,37] located on the Y chromosome. During medaka embryogenesis gonadal tissue develops and bilateral lobed gonads are present but are undifferentiated at hatch.[37,38] From hatch to 30 dph gonads undergo rapid differentiation.[37,38] Differentiation of medaka testes is driven by the presence of *dmy*, which can be detected shortly before hatch.[37,39] In the absence of *dmy*, gonads differentiate into ovaries.[39] Hormones produced by the gonads regulate SSC development, which occurs from approximately 30−70 dph.[12,40] Medaka's SSC are sexually dimorphic and critical during reproduction. During courtship, medaka males use their anal and dorsal fins to hold the female and keep their cloaca in close proximity.[41] Medaka also have a rapid generation time (2−4 months) and produce an abundance of eggs when reproductively mature (10−30 eggs per female per day)[42] making it a useful model species in a study of this magnitude. As such the OECD and U.S. EPA Endocrine Disruptor Screening program (EDSP) have established the Medaka Extended One-Generation Reproduction Test (MEOGRT) as a tier II screening for endocrine disrupting compounds.[43]

Here we investigate differential impacts of E2β on reproductive parameters within defined susceptible stages of development. To compare critical windows of exposure, laboratory medaka populations (male and female) were exposed to two environmentally relevant concentrations of 17β−estradiol (E2β; 2 ng/L and 50 ng/L). The low-concentration treatment (2 ng/L) is within the range reported by several studies that have analyzed E2β concentrations in surface water across the globe.[11,17,44] Concentrations as high as 50 ng/L and higher have also been detected.[7] In addition, the 50 ng/L treatment was selected as a positive control based on Seki et al.,[45] who illustrated reproductive disruption following chronic exposure to 27.9 ng/L E2β. Exposure occurred during periods of both organizational (gonad development and gonad differentiation) and activational development (SSC development and gametogenesis). At reproductive maturity breeding capacity (fertility and fecundity) was assessed.

EXPERIMENTAL METHODS

Exposure Stocks. A stock of 17β−estradiol (E2β) was purchased from Steraloids (Newport, RI) and dissolved in ethanol (EtOH) to generate two stocks of 500 μg/L and 20 μg/L, which were stored at −20 °C to preserve chemical integrity. The same stocks were used throughout the experiment to make 50 and 2 ng/L treatments. Rearing media (5.1 mM NaCl, 0.12 mM KCl, 0.198 mM $MgSO_4 \cdot 7H_20$, and 0.081 mM $CaCl_2 \cdot 2H_20$ in deionized and filtered Picopure water; 1000 mL) was spiked with 100 μL of stock solutions to achieve an EtOH concentration of 0.01%. An EtOH (0.01%) treatment was used as a solvent control. Throughout the experiment, ∼500−1000 mL of treatment solutions were subsampled, pre and post exposure, for solid phase extraction. Subsamples were loaded onto 500 mg Oasis HLB Vac Cartridges (Waters). Cartridges were eluted using 12 mL of methanol. Samples were solvent exchanged into EtOH to a final volume of ∼0.5−1 mL. Analytical analysis was conducting using UPLC/MS/MS. Details on filtration, solid phase extraction and UPLC/MS/MS analysis can be found in Supporting Information (SI). Chemical analyses of E2β stocks were within 97.5% (standard error: ±7.5%) of nominal (target) concentrations (SI Table SI-1). Analyses of 2 ng/L and 50 ng/L E2β treatment solutions pre and post exposure were 84.2% (standard error: ±10.0%) of nominal concentrations. In EtOH stock and treatment solutions, E2β was not detected.

Medaka Rearing. All medaka care and maintenance protocols used in this study were approved by the North Carolina State University (NCSU) Institutional Animal Care and Use Committee. Orange−red outbred medaka were obtained from a breeding colony at the NCSU Aquatic Research Facility. During all four developmental stages of susceptibility, herein referred to as windows of exposure (SI, Figure SI-1), fish were maintained in rearing media. Treatments were conducted in glass beakers by static renewal, with 100% renewal of toxicant and rearing media every 3 days. During exposure windows, water was maintained at 24.2 °C (±0.9 °C), pH 7.1 (±0.3) and 0.0 mg/L ammonia (±0.1 mg/L). Following exposure, fish were maintained in rearing media under standard recirculating conditions within an Aquatic Habitat Culturing Unit (AHAB; Aquatic Habitats, Apopka, FL). Temperature, conductivity and pH of water in the AHAB unit were monitored and maintained at 24.9 °C (±1.2 °C), 1.7 μS/cm (±0.3 μS/cm), and pH 7.3 (±0.3). Fish were kept under a light:dark cycle of 16:8 h and fed daily ad libitum with dry food (Otohime B1, Reed Mariculture, Campbell, CA).

Gonad Development (4 h Post Fertilization [hpf] to Hatch, Exposure Window 1). Embryos were collected from the breeding colony and assessed for fertilization under a dissecting microscope.[40] Approximately 322 embryos were divided into groups of 20−25 embryos and randomly assigned to treatments (EtOH control, 2 ng/L E2β, 50 ng/L E2β), with five replicate groups per treatment. Fertilized eggs were cleaned and maintained in 20 mL glass beakers containing 10 mL of treated rearing media. Mortality and hatch success were monitored daily. Total body length was measured (see length and SSC subsection) at time of hatch (∼10 days). Larvae were transferred to clean media and maintained in 1.5 L tanks with static renewal of untreated media every 3 days. At 30 dph, larvae were transferred into an AHAB unit for a grow-out

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period and breeding assessment at adulthood. At approximately 100 dph, fish were anesthetized to measure length, assess SSC, and genotype (see fin clip genotype subsection). A subset of genotypic males (n = 4−6) and females (n = 4−6) from each treatment was euthanized for gonadal histological analysis. Genotypic males and females were maintained separately until pairing into breeding groups. Twelve breeding pairs from each treatment were formed at 112 dph for breeding assessment (see below). Genotypic males and females with unaltered SSC were selected for breeding assessment.

Gonad Differentiation (Hatch to 30 dph, Exposure Window 2). Larvae (n = 300) were collected from the breeding colony on the day of hatch and divided into 15 groups of 20 larvae each. Groups were randomly assigned to a treatment (EtOH control, 2 ng/L E2β, 50 ng/L E2β), with five replicate groups per treatment. Larvae were maintained in 1 L beakers containing 0.5 L of treated rearing media for 30 days and mortality was monitored daily. At 30 dph, larvae were removed from treatments and imaged (see below) to determine length. Fish were then transferred into the AHAB unit for analysis, grow-out and breeding assessment, as described in the gonad development subsection. Genotypic males and females with unaltered SSC were selected for breeding assessment.

Development of SSC (30 to 80 dph, Exposure Window 3). Juvenile fish at 30 dph (n = 300) were collected from the breeding colony and divided into 15 groups of 20 fish each. Groups were randomly assigned to a treatment (EtOH control, 2 ng/L E2β, 50 ng/L E2β), with five replicate groups per treatment. Fish were maintained in 2 L beakers containing 1.5 L of treated rearing media and mortality was monitored daily. At 80 dph, fish were removed from treatments and total body length was measured. Fish were then transferred into the AHAB unit for analysis, grow-out and breeding assessment, as described in the gonad development subsection.

Gametogenesis (Post 112 dph, Exposure Window 4). Sixty adult fish were collected from the breeding colony at ∼100 dph. These fish were anesthetized to measure length, assess SSC and genotype. Genotypic males and females were maintained separately until pairing into breeding groups. At 112 dph, 30 males and 30 females were divided into pairs with one male and one female each. Breeding pairs were kept in 1 L beakers with 0.5 L rearing media and breeding was monitored for 7 days to determine baseline levels of reproductive output. At 119 dph, males and females were separated for a 7 day depuration period by placement into individually labeled containers with 0.5 L of media. Males and females were subsequently returned to identical breeding pairs and randomly assigned treatments (EtOH control, 2 ng/L E2β, 50 ng/L E2β) at 126 dph, with 10 replicates per treatment. Breeding pairs were continuously exposed to each treatment for 14 days. Breeding was assessed daily for the 14 day exposure period. Following exposure (at 140 dph), five to six pairs of fish from each treatment were euthanized for histological assessment. In addition, a subset of breeding pairs from each treatment (n = 4−6) was maintained in untreated media for an additional 14 days to assess potential latent effects on reproductive parameters.

Length and SSC. Impacts of treatments on phenotypic sex determination, growth, length and SSC were characterized throughout this experiment. Following exposure during gonad development and differentiation, total length (mm) was measured by placing live fish in a beaker and imaging them when still. Images were taken with a Nikon SMZ1500 camera (Nikon Instruments, Inc. Melville, NY) coupled to NIS Elements F 3.2 software (Nikon Instrument, Inc.). Total length was calculated using the measurement tool in Adobe Photoshop CS5 (Adobe Systems, Inc. San Jose, CA). At reproductive maturity for all exposure groups (∼100 dph), fish were anesthetized in cold water (∼4 °C) for 30 s and imaged to assess SSC and total length. Gross assessments of SSC included determining presence or absence of urogenital papillae (SI Figure SI-2A), papillary processes (SI Figure SI-2B) and dorsal notch. A 5 mm dorsal fin clip was also taken for genotyping before returning animals to rearing media.

Fin Clip Genotype. Genotyping for *dmy* was conducted using fin clips. Dorsal fin clips (5 mm) were cut and dissolved in Proteinase K in NTES buffer (50 mM Tris−HCl, 50 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 8.0) overnight. Genomic DNA was extracted using saturated NaCl and purified with EtOH washes. Forward (5′−CATGAGCAAGGAGAAGCA−3′) and reverse primers (5′−GGCCGGGTCCCCGGGTG−3′) were used in polymerase chain reactions (PCR) to co-amplify *dmrt1ay* and *dmy*. The forward primer was designed using Primer3[46,47] to a conserved region of both *dmrt1ay* (NM001278904), an autosomal paralog, and *dmy* (NM001104680). The reverse primer was designed according to Matsuda et al. (PG17.5).[36] Amplicons from PCR reactions were used to identify genetic male (*dmrt1ay*$^+$, *dmy*$^+$) and female (*dmrt1ay*$^+$, *dmy*$^-$) genotypes (SI Figure SI-2C). All mating pairs were established based on genotypic sex determination.

Histology. Fish were euthanized using an overdose (300 mg/L) of tricaine methanesulfonate (M-222, Sigma Aldrich, St. Louis, MO) and then fixed in Modified Davidson Fixative for 24 h (35.15% distilled water, 31.35% of 100% ethanol, 22% of 37% formaldehyde, and 11.5% glacial acetic acid). Following the 24 h fixation, fish were placed into 70% EtOH and samples were held at room temperature until processing. Tissue samples were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (H&E) by the NCSU College of Veterinary Medicine Histopathology Laboratory (Raleigh, NC). All sections were examined to assess gonad development and gonad phenotype with light microscopy by a board certified (ACVP) veterinary pathologist.

Breeding Assessment. At 112 dph a single genotypic male and a single genotypic female from each treatment window were combined into breeding pairs. For exposure groups at gonad development, gonad differentiation and SSC development, breeding pairs were monitored for a total of 14 days in the AHAB system. AHAB tanks contained mesh filters, baffles, or netting (SI Figure SI-3) to ensure capture of deposited eggs from each pair. Breeding pairs from the reproductive maturity exposure group were maintained in 1 L beakers containing 0.5 L of rearing media for the entirety of the experiment (i.e., baseline, exposure, and postexposure periods) as described in Miller et al.[14] Eggs were collected daily from each breeding pair for 14 days. Individual eggs were assessed for fertilization under a dissecting microscope.[40]

Statistical Analysis. Statistical analyses were conducted with Prism 5.0 (GraphPad, La Jolla, CA). A Sharpiro−Wilk test and Bartlett's test were used to test data for normality and equal variances. A one-way analysis of variance (ANOVA) was used to evaluate the effect of treatment on time to hatch and length from all exposure windows, coupled with a Tukey's multiple comparison test if significant ANOVA differences were detected. Data that did not meet assumptions of normality or equal variance were analyzed with nonparametric methods. The

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Table 1. Percent Survival and Mean Total Length (mm) of Medaka from Four Windows of Exposure

window	treatment	N	survival (%)[a]	time to hatch (d)	total length (mm)	
					post exposure	reproductive maturity
1	EtOH	105	84.89 (24.15)	11.31 (0.40)	5.53 (0.11)	26.60 (1.76)
	2 ng/L E2β	105	86.12 (16.34)	11.77 (0.46)	5.55 (0.10)	26.64 (1.40)
	50 ng/L E2β	112	84.89 (24.15)	11.13 (0.69)	5.65 (0.13)	24.76 (1.84)
2	EtOH	100	81.00 (14.75)	NA	9.21 (0.41)	25.62 (1.20)
	2 ng/L E2β	100	85.00 (7.91)	NA	9.52 (0.86)	25.65 (1.08)
	50 ng/L E2β	100	70.00 (39.21)	NA	8.67 (0.30)	25.71 (0.23)
3[b]	EtOH	190	42.18 (22.46)	NA	19.17 (2.28)	24.37 (1.71)
	2 ng/L E2β	203	35.12 (25.00)	NA	18.77 (2.77)	23.76 (0.58)
	50 ng/L E2β	224	34.25 (35.76)	NA	19.52 (2.82)	23.99 (1.93)
4	EtOH	20	100 (0.00)	NA	NA	26.50 (2.56)
	2 ng/L E2β	20	100 (0.00)	NA	NA	26.23 (2.89)
	50 ng/L E2β	20	100 (0.00)	NA	NA	25.36 (2.72)

[a]Survival was determined post exposure. [b]Analysis of SSC (3) was done on a composite of 3 replicate experiments. Standard deviation in parentheses. d: days. mm: millimeters. NA: not applicable. No significant differences (F-test or Kruskal–Wallis, $p > 0.05$) were detected among the treatments within windows 1–4; gonad development (1), gonad differentiation (2), development of SSC (3), and gametogenesis (4).

effect of treatment on hatch success, time to hatch and survival were analyzed using the Kruskal–Wallis test, coupled with a Wilcoxon multiple comparison test. Chi-square (χ^2) analysis was conducted to determine the effect of treatments on genotypic sex ratio, phenotypic sex ratio and SSC. To assess the effect of treatment on fertility and fecundity, cumulative egg production and cumulative fertilized eggs were analyzed within each breeding group. To analyze accrual of eggs, cumulative egg density at day 14 (Cum_{MAX}) was normalized to the control treatment, where the maximum and minimum values of the EtOH solvent control were used to calculate percent of control. An ANOVA was used to analyze differences in Cum_{MAX} and cumulative egg production or fertilization rate (slope) among treatments.

RESULTS

Exposure Window 1: Gonad Development (4 hpf to Hatch). Exposure to E2β during gonad development did not alter hatching success, time to hatch or growth (Table 1). Hatching success (survival) ranged from 84.7% to 87.6% within this exposure window and there was no difference (Kruskal–Wallis, $p > 0.05$) among treatment groups. No difference was detected (F-test, $p > 0.05$) in time to hatch or total body length at hatch among treatments. At reproductive maturity (~100 dph), there was also no difference (F-test, $p > 0.05$) in total body length among the treatment groups. In contrast, exposure to E2β led to discrepancies between genotype (presence/absence of *dmy*), phenotype (expression of SSC) and gonad phenotype. Analysis of genotype and phenotype (Figure 1) revealed three XY females (i.e., genetic male with urogenital papillae present and dorsal cleft and papillary processes absent) and two XX males (i.e., genetic female with urogenital papillae absent and dorsal cleft and papillary processes present). However, there were no statistical differences (χ^2, $p > 0.05$) in both genetic and phenotypic sex ratios among the three treatments. Papillary processes were visibly absent in three XY males from E2β treatments: one fish from the 2 ng/L E2β treatment and two fish from the 50 ng/L E2β treatment. Conversely, urogenital papillae were present in a single XY male from the 50 ng/L E2β treatment. There was no overall significant difference (χ^2, $p > 0.05$) in the SSC expression among any treatment groups. Histological assessment (SI Table SI-2) at the time of reproductive maturity revealed that the 50 ng/L E2β treatment altered gonad development; the gonads from one XY male in the 50 ng/L E2β treatment exhibiting the presence of ovaries.

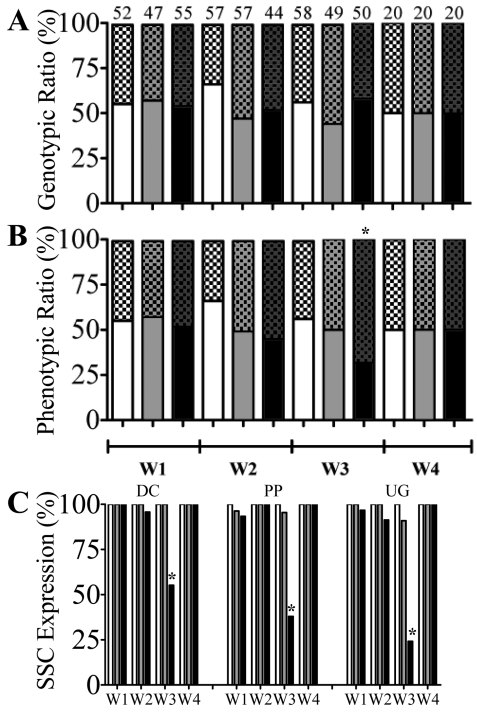

Figure 1. Genotypic and phenotypic sex ratios of male and female medaka ~100 dph for windows 1–4; gonad development (1), gonad differentiation (2), development of SSC (3), and gametogenesis (4). (A) Percent XY males (expressing *dmy*; solid bars) and percent XX females (lacking *dmy*; checkered bars) were calculated for EtOH control (white), 2 ng/L E2β (gray) and 50 ng/L E2β (black). (B) Percent XY males expressing male SSC (solid bars) and percent XX females (expressing female SSC; checkered bars). (C) Percent of XY males expressing dorsal cleft (DC) and papillary processes (PP) and lacking urogenital papillae (UG). Asterisks denote significant difference (χ^2, $p < 0.05$) from EtOH treatment within each window. Analysis of SSC was done on a composite of three replicate experiments.

Breeding assessment at reproductive maturity revealed that exposure to E2β during gonad development led to altered

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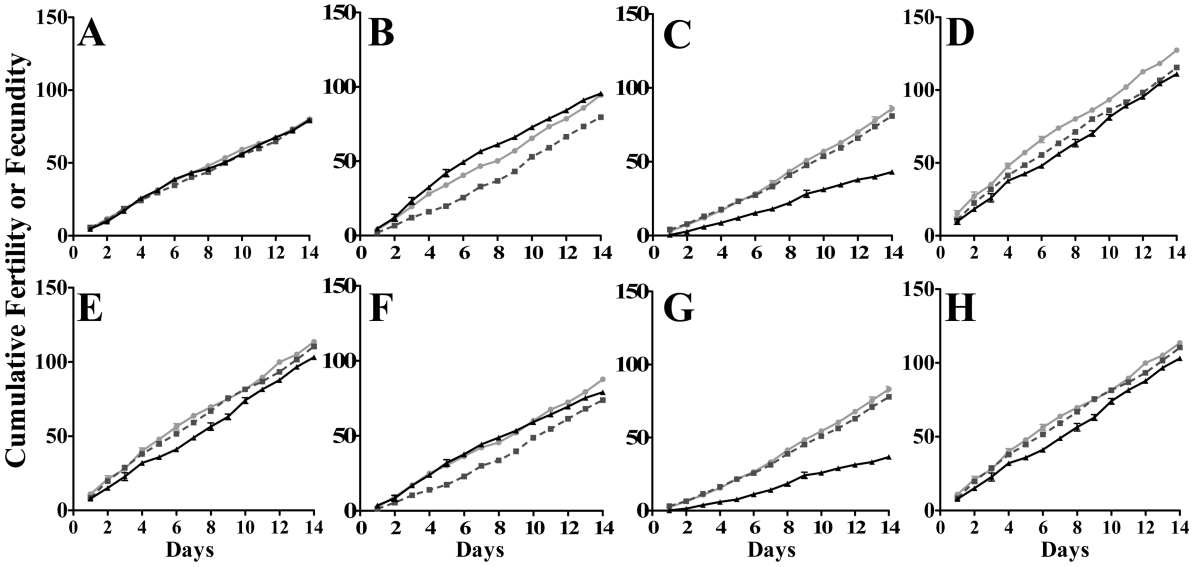

Figure 2. Cumulative fecundity (A–D) and fertility (E–H) of reproductively mature breeding pairs from windows 1–4. Breeding pairs of EtOH control (light gray), 2 ng/L E2β (dashed gray) and 50 ng/L E2β (black) breeding groups that were exposed during gonad development (A and E), gonad differentiation (B and F), development of SSC (C and G), and gametogenesis (D and H). Analysis of SSC was done on a composite of three replicate experiments.

Table 2. Mean Maximum Cumulative (Cum_{MAX}) Number of Eggs and Slope of Fertility and Fecundity of Breeding Pairs from Windows 1–4

			fecundity		fertility	
window	treatment	N	cum_{MAX}[a]	slope	cum_{MAX}[a]	slope
1	EtOH	12	100.00 (1.59)a	5.63 (0.11)a	100.00 (1.68)a	5.37 (0.11)a
	2 ng/L E2β	12	98.56 (2.23)a	5.45 (0.08)a	92.67 (2.34)b	4.83 (0.10)b
	50 ng/L E2β	10	99.23 (1.79a	5.57 (0.14)a	91.65 (1.86)b	4.93 (0.11)b
2	EtOH	12	100.00 (1.51)a	6.72 (0.10)a	100.00 (1.71)a	6.31 (0.10)a
	2 ng/L E2β	12	83.55 (1.22)b	6.06 (0.18)b	83.68 (1.44)b	5.68 (0.17)b
	50 ng/L E2β	12	101.01 (1.54)a	6.88 (0.24)a	89.83 (1.60)c	5.85 (0.14)c
3[b]	EtOH	12	100 (2.55)a	6.47 (0.14)a	100.00 (2.46)a	6.32 (0.14)a
	2 ng/L E2β	11	94.07 (1.90)a	5.96 (0.14)b	93.49 (1.95)a	5.80 (0.13)b
	50 ng/L E2β	10	48.36 (1.88)b	3.45 (0.07)c	42.60 (2.13)b	3.01 (0.10)c
4	EtOH	9	100.00 (1.53)a	8.32 (0.19)a	100.00 (1.71)a	7.66 (0.16)a
	2 ng/L E2β	9	89.41 (1.09)b	7.72 (0.15)b	96.97 (1.19)b	7.51 (0.14)a
	50 ng/L E2β	7	85.45 (1.37)b	7.73 (0.10)b	89.86 (1.45)b	7.32 (0.12)a

[a]Maximum cumulative eggs normalized to ETOH treatment. [b]Analysis of SSC (3) was done on a composite of 3 replicate experiments Standard deviation in parentheses. Letters denote significant difference (ANOVA, $p < 0.05$) between treatments within windows 1–4; gonad development (1), gonad differentiation (2), development of SSC (3), and gametogenesis (4).

reproductive capacity. During this exposure window there was no significant difference (F-test, $p > 0.05$) in cumulative egg production (Cum_{MAX} and rate) among treatment groups (Figure 2A, Table 2). There was, however, a significant effect (F-test with Tukey's test, Cum_{MAX}: $p = 0.011$, slope: $p = 0.002$) on fertility in both E2β treatments. The Cum_{MAX} values from the 2 ng/L and 50 ng/L E2β treatments were 7.3–8.3% less than Cum_{MAX} from the EtOH solvent control treatment. Egg fertilization rate (slope) in the EtOH treatment was 0.4–0.5 more eggs per day than in both E2β treatments (Figure 2E).

Exposure Window 2: Gonad Differentiation (Hatch to 30 dph). Exposure to E2β during gonad differentiation did not alter survival or growth in medaka (Table 1). Fish exhibited no difference (Kruskal–Wallis, $p > 0.05$) in mortality (70–85%) among the treatment groups (Table 1). There was also no difference (F-test, $p > 0.05$) in length following exposure or at reproductive maturity (~100 dph). Conversely, exposure to E2β during gonad differentiation led to discrepancies in genotype, phenotype and gonad phenotype. Analysis of genotype and phenotype identified one XX male from the 2 ng/L E2β treatment and three XY females from the 50 ng/L E2β treatment (Figure 1). However, there were no significant differences (χ^2, $p > 0.05$) in phenotypic or genotypic sex ratios among the three treatments. Papillary processes were visibly absent in one XY male from the 50 ng/L E2β treatment and urogenitial papillae were present in two XY males from the 50 ng/L E2β treatment. There was no overall difference (χ^2, $p > 0.05$) in the SSC expression among treatment groups.

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Histological assessment (SI Table SI-2) at the time of reproductive maturity revealed that both E2β treatments altered gonad development. One XX female from the 2 ng/L treatment exhibited testes and three XY males from the 50 ng/L treatment exhibited ovaries.

Exposure to E2β during gonad differentiation altered reproduction, with the 2 ng/L treatment having a larger impact than the 50 ng/L treatment. At reproductive maturity, fecundity from the 2 ng/L E2β treatment was attenuated and Cum_{MAX} was 16.5−17.5% lower (F-test with Tukey's test, $p < 0.0001$) than in the EtOH and 50 ng/L E2β treatments (Figure 2B, Table 2), which were not different from one another (F-test with Tukey's test, $p > 0.05$). Similarly, the 2 ng/L E2β treatment produced 0.7−0.8 fewer eggs per day (F-test with Tukey's test, $p = 0.010$) compared to both EtOH and 50 ng/L E2β treatments, which were also not different (F-test with Tukey's test, $p > 0.05$). Breeding pairs in the 2 ng/L and 50 ng/L E2β treatments fertilized 16.3% and 10.2% fewer eggs (F-test with Tukey's test, $p < 0.0001$) than those in the EtOH treatment (Figure 2F). This relationship was also observed in eggs fertilized per day, where 0.5−0.6 more eggs (F-test with Tukey's test, $p < 0.0001$) were fertilized per day in the EtOH treatment compared to the 2 ng/L and 50 ng/L E2β treatments.

Exposure Window 3: Development of SSC (30−80 dph). Mortality was high during this exposure window (7.0−57.5% survival; SI Table SI-3), as a result the entire experiment was repeated two additional times with similar outcomes to achieve an adequate number of fish and breeding pairs for further analysis. Data were pooled from the three experimental replicates for exposure during development of SSC. Mortality for the experimental replicates can be found in the SI (Table SI-3). During SSC development, exposure to E2β did not alter composite survival or growth (Table 1). Kruskal−Wallis analysis found no difference ($p > 0.05$) among the three treatment groups (Table 1). There was also no difference (F-test, $p > 0.05$) in length post-exposure or at reproductive maturity (~100 dph). Both E2β treatments altered genotype, phenotype and gonad phenotype among the laboratory fish. Analysis of genotype and phenotype revealed 1 XX male from the 2 ng/L E2β treatment and 13 XY females from the 50 ng/L E2β treatment (Figure 1). While there was no treatment effect (χ^2, $p > 0.05$) on genotypic sex outcomes, phenotypic sex ratio was significantly (χ^2, $p = 0.035$) shifted toward a female-dominant phenotype population in the 50 ng/L E2β treatment relative to the EtOH solvent control treatment. Analysis of SSC revealed that papillary processes were visibly absent in 1 and 18 XY males from the 2 ng/L and 50 ng/L E2β treatments, respectively. In addition, urogenital papillae were present in 2 XY males from the 2 ng/L E2β treatment and 22 from the 50 ng/L E2β treatment. In 13 XY males from the 50 ng/L E2β treatment, the dorsal cleft was not present. Attenuation of male SSC and expression of female SSC by fish in 50 ng/L E2β treatments were significant (χ^2, $p \leq 0.0004$) relative to the EtOH and 2 ng/L treatments during SSC development. There was no significant difference (χ^2, $p > 0.05$) between the EtOH and 2 ng/L treatments. Histological assessment (SI Table SI-2) at the time of reproductive maturity revealed that both E2β treatments produced intersex in the gonad. One XX female from the 2 ng/L treatment developed spermatocytes within the ovaries and two XY males from the 50 ng/L treatment developed oocytes within the testes.

Exposure to E2β during SSC development also altered reproductive capacity at the time of reproductive maturity. There was no difference (F-test, $p > 0.05$) in the Cum_{MAX} of egg production or fertilization between the 2 ng/L E2β treatment and the EtOH controls (Figure 2C and G, Table 2). The 50 ng/L E2β treatment attenuated fecundity and fertilization by 45.7−57.4%, relative to fecundity and fertilization in the EtOH control and 2 ng/L E2β treatments. There were significant (F-test, $p < 0.0001$) concentration-dependent decreases in rates of egg production and fertilization. The 2 ng/L E2β treatment reduced production and fertilization rates by 0.5 eggs per day, relative to rates in the EtOH control. Egg production and fertilization rates in the 50 ng/L E2β treatment were reduced by 3.0−3.3 eggs per day, relative to rates in the EtOH treatment.

Exposure Window 4: Gametogenesis (Post 112 dph). Prior to conducting the gametogenesis exposure there were no differences in length (F-test, $p > 0.05$; Table 1) or expression of SSC (χ^2, $p > 0.05$; Figure 1) among the three treatments. There was no mortality throughout gametogenesis exposure (Table 1). Following the experiment, reproductive capacity was assessed and there were several breeding groups that produced fewer than 30 eggs over the 7 day baseline assessment. These groups were excluded from analyses. As a result, one breeding group was excluded from the ETOH control treatment and the 2 ng/L E2β treatment, and three breeding groups were excluded from the 50 ng/L E2β treatment. There were no differences (F-test, $p > 0.05$) in fertility and fecundity during the baseline period among the treatments (SI Figure SI-4, Table SI-4).

Exposure to both E2β treatments during the exposure period altered reproductive capacity in breeding pairs compared to the EtOH control (Figure 2D and H, Table 2). Cumulative egg production was not different (F-test with Tukey's test, $p > 0.05$) in the 2 ng/L E2β and 50 ng/L E2β treatments; both treatments produced 10.6−14.6% fewer eggs than in the EtOH treatment (F-test with Tukey's test, $p < 0.0001$). Similarly, egg production rate was not statistically different (F-test with Tukey's test, $p > 0.05$) between both E2β treatments, and both treatments produced 0.6 fewer eggs per day relative to the EtOH control (F-test with Tukey's test, $p < 0.05$). Cumulative fertilized eggs in the 50 ng/L E2β treatment were 7.1−10.1% lower (F-test with Tukey's test, $p < 0.001$) than Cum_{MAX} in the EtOH and 2 ng/L E2β treatments, which were not significantly different (F-test with Tukey's test, $p > 0.05$) from each other. There was no significant difference ($p > 0.05$) in the fertilization rate among the three treatments. Exposure to E2β during gametogenesis led to the development of intersex in the 50 ng/L treatment. Histological assessment (SI Table SI-2) following exposure revealed that two XY males developed oocytes within their testes.

During the postexposure period breeding capacity from both E2β treatments was also altered. Breeding pairs exhibited an inverse effect compared to exposure period (SI Figure SI-4, Table SI-4). Both fecundity and fertility were greater (F-test with Tukey's test, $p < 0.0001$) in fish from both E2β treatments than in fish from the EtOH control treatment, with one exception: cumulative egg production in the 2 ng/L E2β treatment was not significantly different (F-test with Tukey's test, $p > 0.05$) than Cum_{MAX} in the EtOH treatment (SI Table SI-4). Otherwise, there was a concentration-dependent compensatory response with egg production rate, fertilization rate, and cum_{MAX} of fertilized eggs.

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DISCUSSION

There are growing accounts of the deleterious effects of estrogen contaminant exposure on fish populations.[20,23−27] Previous studies of estrogen (e.g., E2β, 17α-ethynylestradiol (EE2), estriol) and wastewater treatment plant effluent exposure during specific temporal or developmental stages[4,21,26,32,48] or through complete life cycles[22,35,45] have established that exposure can lead to impaired reproductive success. However, from regulatory or management perspectives, it is difficult to prioritize decisions or mitigation efforts without a clear understanding of the most critical stages of susceptibility. In this study, while corroborating previous research documenting direct effects of E2β exposures on breeding capacity, we demonstrated distinct patterns in reproductive sensitivities to exposure during specific stages of development. Assessment of hatching success, time to hatch, survival, and growth in the four windows showed that E2β exposure had no significant nontarget pathologies, consistent with previous studies in medaka and fathead minnows (*Pimephales promelas*).[15,49−52] However, exposure to E2β significantly altered phenotype, gonad phenotype and breeding capacity in medaka.

Our study confirms previous findings[21,48] that E2β exposure has the ability to impair reproductive capacity following time-sensitive estrogen exposures. We also demonstrated that exposure to E2β during all of the critical developmental stages tested altered breeding capacity in medaka, with time-dependent impacts on SSC and gonad development. Development of SSC was the only exposure period with a significant attenuation of male-specific SSC expression. This finding is similar to those of studies with fathead minnows that documented feminization/demasculinization of SSC in males exposed to estrogen contaminated water.[1,13,52]

In addition to a large disruption in reproduction, there was substantial mortality during the SSC development exposure, relative to the other developmental stages. The repeated and consistent mortality of fish during this exposure window was unrelated to experimental conditions (e.g., water temperature, dissolved oxygen, conductivity, pH), which were all similar to the other tests conducted. It is possible that the exposure duration may have contributed to the mortality. The 50 days exposure window during SSC development was longer than the other exposure windows (∼10−30 days). Another possible explanation for the mortality observed during the SSC development stage is the use of 0.01% EtOH. While no solvent toxicity studies have been conducted in medaka during this specific exposure window, González-Doncel et al.[53] observed no developmental effects in medaka embryos exposed to ≤0.25% EtOH. In addition, we did not observe high levels of mortality during the other three exposure windows in this study. Although the elevated mortality remains unexplained, it is a compelling finding that warrants additional study.

Another time sensitive end point that we revealed was the alteration in gonad morphology. E2β induced development of XY fish with ovaries and XX fish with testes (i.e., gonad sex reversal), which only occurred in fish exposed during gonad development and differentiation (i.e., organizational windows). In contrast, intersex (the presence of both male and female gonadal tissue) was only observed in fish exposed after gonads were already differentiated, representing possible activational effects. This finding suggests that E2β induced gonad sex reversal and intersex during very specific stages of development, unlike other studies with medaka[48,54] and rainbow trout (*Oncorhynchus mykiss*),[55] which document intersex in fish from several exposure windows. However, in these previous studies, concentrations were significantly higher (ranging 4−250 μg/L E2β) than in this study. Our test concentrations were 3−4 orders of magnitude lower and more likely to reflect environmental concentrations found in surface waters of the US.[7,11,17,44]

In addition to feminization of XY genetic fish, we documented masculinization of XX genetic fish, albeit in negligible numbers. The detection of testis within a female (XX) fish and spermatocytes within the ovary of a female (XX) fish was unexpected, however, the presence of XX males in wild medaka populations is not uncommon.[56] Several fish species are known to begin their reproductive life as one sex (male or female) and then later in life an environmental cue trigger a switch to another sex.[57] For example, in a study with the cichlid fish (*Cichlasoma dimerus*), the absence of males triggered one or more dominant females to switch to fully functional males.[57,58] Alternatively, sex reversal is known to occur spontaneously (0.1% to >10%) in wild medaka populations.[56,59] Estrogen stress may have triggered the alteration observed in the females in this study. Our findings are similar to findings by Papoulias et al.[15] that found 1 XX male in 20 XX fish after injection of EE2 during embryo development. They observed an XX male in their lowest treatments (0.005 ng EE2/egg) and none in their higher treatments (0.05−5.0 ng EE2/egg). These findings warrant further investigations of the impacts of E2β on female fish.

Comparison of reproductive dysfunction among the four windows of exposure tested suggests that timing of exposure impacts the magnitude of disruption. Exposure during gonad development and gametogenesis had the least impact on reproductive function. In contrast, exposure during gonad differentiation and SSC development had a greater impact on reproductive function, with exposure during SSC development having the greatest effect. Exposure to 50 ng/L E2β during development of SSC resulted in a significant reduction in breeding success. Other studies have found reduced female preference for and reproductive success of males exhibiting reduced male-specific SSC.[1−3] Our results here support the notion that SSC are critical to reproductive capacity in fish populations. In addition to the large magnitude in reproductive disruption we observed a pronounced shift in phenotypic sex ratio and feminization/demasculinization of SSC following E2β exposure during SSC development. This exposure period was the only window in which there was a significant effect on phenotypic sex ratio in medaka, confirming that impacts to SSC expression may be disruptive to medaka breeding capacity.

Conversely, exposure during gonad development had the smallest impact on reproductive capacity. Similarly, Nakamura and Takahashi[60] showed no effects on testes or ovary development in tilapia (*Tilapia mossambica*) exposed to EE2 prior to gonad differentiation. Results in both medaka and tilapia suggest that during this period, when gonads are developing but are undifferentiated, estrogen exposure may have minimal effects. There may be underlying mechanisms during gonad development in medaka that facilitate adaptation to excess estrogen exposure. Studies have documented significant differences in temporal abundance of estrogen receptors in fish.[61,62] Chakraborty et al.[62] suggest that time-sensitive expression of medaka estrogen receptors may coincide with specific events in gonad development. A gene expression

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analysis of medaka estrogen receptors during our four exposure windows may shed light on possible mechanisms governing the differences in sensitivity that we observed.

Through our investigation of E2β effects on medaka breeding capacity we found possible inherent mechanisms to compensate for reproductive disruption following estrogen exposure. The response of medaka to exposure during gametogenesis was a classic example of "activational disruption" and compensatory mechanisms. Activational effects can be mitigated after exposure cessation,[28,29] and can lead to biological compensatory responses that facilitate a reduction in effect.[30,31] Exposure to E2β during gametogenesis resulted in a 10.2−14.6% reduction in breeding capabilities. Following termination of exposure, E2β treatment groups exhibited a complete renewal of breeding capacity. In fact, during the postexposure evaluation period of gametogenesis exposure, reproduction increased 13.1−76.7% relative to the EtOH control treatment. These results, like those documented with cichlids and fathead minnows,[30,31] suggest an inherent compensatory mechanism following exposure to E2β in some fish species.

Another possible compensatory mechanism was also observed following exposure during gonad differentiation. During the gonad differentiation experiment we observed a nonmonotonic concentration response (i.e., a U-shaped curve), which is typical of endocrine active compounds[63] and points to a possible compensatory mechanism following endocrine disruption.[30,64,65] The 2 ng/L E2β treatment resulted in a larger reduction in reproductive capacity compared to the 50 ng/L E2β treatment. Comparably, other studies have documented nonmonotonic effects of estrogen contaminants on aromatase and gonadotropin-releasing hormone expression in fish.[66,67] Our data suggest a nonmonotonic response of reproductive success after E2β exposure during gonad differentiation. However, further analysis with additional concentrations is needed to be conclusive.

The data presented here suggest that exposure during specific developmental stages leads to differential magnitudes of disruption. It is possible that duration of exposure may also factor into the differences in magnitude. The marginal reproductive dysfunction found in gonad development stage may be attributed to the relatively short exposure time (~11 days) or lengthy recovery period (112 days). In another study with medaka, a long-term exposure (80 days) to E2β reduced fecundity and fertility, but no adverse effects were observed following a short-term exposure (30 days).[33] Similarly, E2β exposure during gametogenesis yielded a lower magnitude of reproductive disruption and its exposure period was also shorter (14 days) relative to gonad and SSC differentiation stages.

Herein we evaluated four important developmental stages critical to reproductive success and demonstrated distinct patterns in disruption among the stages. However, it remains unclear what is governing these differential patterns and factors not addressed in the scope of this study could also account for the results we report. For example, estrogens can affect activational and organization development in the brain, ultimately altering breeding (e.g., dancing, competing, and recruiting) and parental behavior (e.g., nest building and nest guarding) in fish populations.[68−72] In addition, reproductive development is managed by precise gene regulatory networks (e.g., hypothalamus−pituitary−gonadal axis and hypothalamus−pituitary−gonadal−liver axis).[14,52,69] Investigating the effect of estrogen exposure on brain development and gene expression may shed light on behavioral and molecular components driving the differences observed.

While supporting previous studies documenting deleterious reproductive effects following exposure to E2β,[4,21,26,54] our findings also provide further insight into critical stages in development. Our results suggest that not only did timing of exposure and exposure concentration have an effect on the magnitude of reproductive disruption, but possibly duration of exposure as well. We also observed several potentially inherent compensatory mechanisms to estrogen exposure. Overall, this study illustrates the sensitivity of reproductive function in fish populations to EACs. With the human population projected to continue to increase, anthropogenic contaminants like EACs will likely remain pervasive aquatic contaminants across the globe. Understanding the dynamics of reproductive disruption is the first step in mitigating potential adverse outcomes in populations of wild fishes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b01568.

SPE and chemical analysis; analytical chemistry results (Table SI-1); exposure schematic (Figure SI-1); secondary sex characteristics and genotyping (Figure SI-2); AHAB breeding tanks (Figure SI-3); histology results (Table SI-2); survival of SSC experimental replicates (Table SI-3); baseline and postexposure reproduction during gametogenesis (Figure SI-4); and baseline and postexposure reproduction during gametogenesis (Table SI-4)(PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (862) 216-9789; e-mail: cleepow8@gmail.com.

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Notes

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