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Impact of bisphenol-A and synthetic estradiol on brain, behavior, gonads and sex hormones in a sexually labile coral reef fish

Jose A. Gonzalez [a], Abigail R. Histed [a], Ewelina Nowak [a], Dominica Lange [a], Sarah E. Craig [a], Coltan G. Parker [a,b], Achint Kaur [a], Supriya Bhuvanagiri [a], Kevin J. Kroll [c], Christopher J. Martyniuk [c], Nancy D. Denslow [c], Cheryl S. Rosenfeld [d], Justin S. Rhodes [a,b,e,*]

[a] The Beckman Institute for Advanced Science and Technology, University of Illinois, d0e N. Mathews Ave, Urbana, IL 61801, United States of America
[b] The Neuroscience Program, University of Illinois, 405 N. Mathews Ave, Urbana, IL 61801, United States of America
[c] Department of Physiological Sciences and Center for Environmental and Human Toxicology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610, United States of America
[d] Department of Biomedical Sciences, Bond Life Sciences Center, Thompson Center for Autism and Neurobehavioral Disorders, MU Institute for Data Science and Informatics, and Genetics Area Program, University of Missouri, Columbia, MO 65211, United States of America
[e] Department of Psychology, University of Illinois, 405 N. Mathews Ave, Urbana, IL 61801, United States of America

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ABSTRACT

Endocrine disrupting chemicals, such as bisphenol A (BPA) and ethinylestradiol (EE2), are detected in the marine environment from plastic waste and wastewater effluent. However, their impact on reproduction in sexually labile coral reef fish is unknown. The objective of this study was to determine impacts of environmentally relevant concentrations of BPA and EE2 on behavior, brain gene expression, gonadal histology, sex hormone profile, and plasma vitellogenin (Vtg) levels in the anemonefish, *Amphiprion ocellaris*. *A. ocellaris* display post-maturational sex change from male to female in nature. Sexually immature, male fish were paired together and fed twice daily with normal food (control), food containing BPA (100 μg/kg), or EE2 (0.02 μg/kg) ($n = 9$ pairs/group). Aggression toward an intruder male was measured at 1, 3, and 6 months. Blood was collected at 3 and 6 months to measure estradiol (E2), 11-ketotestosterone (11-KT), and Vtg. At the end of the study, fish were euthanized to assess gonad morphology and to measure expression of known sexually dimorphic genes in the brain. Relative to control, BPA decreased aggression, altered brain transcript levels, increased non-vitellogenic and vitellogenic eggs in the gonad, reduced 11-KT, and increased plasma Vtg. In two BPA-treated pairs, both individuals had vitellogenic eggs, which does not naturally occur. EE2 reduced 11-KT in subordinate individuals and altered expression of one transcript in the brain toward the female profile. Results suggest BPA, and to a lesser extent EE2, pollution in coral reef ecosystems could interfere with normal reproductive physiology and behavior of the iconic sexually labile anemonefish.

1. Introduction

As ocean pollution becomes more widespread, chemicals leaching into the water continue to be a pressing concern (Atkinson et al., 2003; Goksøyr, 2006; Oberdörster and Cheek, 2001). Among these chemicals are several endocrine disrupting chemicals (EDCs), which can act as estrogen receptor agonists and interfere with normal reproduction in animals (Bhandari et al., 2015; Oehlmann et al., 2009; Oehlmann et al., 2000; Scholz and Klüver, 2009; Söffker and Tyler, 2012; Teuten et al., 2009). Two common EDCs in the aquatic environment are bisphenol A (BPA) and 17α-Ethinylestradiol (EE2) (Arditsoglou and Voutsa, 2012; Aris et al., 2014; Calderón-Moreno et al., 2019; Ozhan and Kocaman, 2019). BPA can leach into the water from plastics, where it is used as a plasticizer, stabilizer, and antioxidant (Koelmans et al., 2014; Oehlmann et al., 2009; Sajiki and Yonekubo, 2003; Teuten et al., 2009; Yamamoto and Yasuhara, 1999). Conversely, EE2 is a synthetic estrogen commonly found in birth control pills and can reach the ocean from wastewater effluent of manufacturing plants, hospitals, and human waste (Bhandari et al., 2015; Monteiro and Boxall, 2010; Rzymski et al., 2017).

Coral reef fish that demonstrate sexual plasticity during their lifetime

* Corresponding author at: Beckman Institute, 405 N. Mathews Ave., United States of America.
E-mail address: jrhodes@illinois.edu (J.S. Rhodes).

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are potentially at higher risk for adverse effects from EDCs. This is because their sex determination is not genetically programmed, but instead is plastic and strictly dependent upon environmental cues (Francis, 1992). Among these species is *Amphiprion ocellaris*, the False Percula clownfish or common anemonefish. Anemonefish live in small groups with one alpha female, one beta male, and sometimes a few lower ranking non-reproductive males (Mitchell, 2005). A male transforms into a female if the female is removed from the group (Casas et al., 2016; Fricke and Fricke, 1977; Godwin, 1994; Godwin and Thomas, 1993; Madhu and Madhu, 2006) or if two males are paired together (Fricke, 1983; Dodd et al., 2019). Females are typically larger and the sexes exhibit a number of sexually dimorphic behaviors (Buston, 2003; DeAngelis and Rhodes, 2016). Males are the primary caretakers of the eggs. Females are more aggressive than males, and are the primary defenders of the territory against intruders (Fricke, 1979). As a male transforms into a female, multiple whole organismal changes take place. The fish becomes larger. Morphological changes occur in the brain that result in alterations in behavior and regulation of the gonads (Dodd et al., 2019). In addition, female anemonefish have higher transcript levels of brain aromatase (*cyp19a1b*) and lower transcript levels of isotocin receptor (*itr*) relative to males (DeAngelis et al., 2018). A recent transcriptomics study of the brains of *A. bicinctus* at various stages during transition from male to female found increased expression of coagulation factor XIII A chain (*f13a1*), and decreased expression of popeye domain containing 3 (*popdc3*) (Casas et al., 2016). Their gonads transform from containing a mixture of testicular tissue and non-vitellogenic eggs to containing mostly vitellogenic eggs (yolked eggs) and no testicular tissue (Dodd et al., 2019; Fricke and Fricke, 1977; Godwin, 1994; Madhu et al., 2010). In addition, females have high levels of circulating 17β-estradiol (E2) in their blood and low 11-ketotestosterone (11-KT), the main bioactive androgen in fish, whereas males have the opposite profile (DeAngelis and Rhodes, 2016). Once transformation to a mature female is complete, the fish is unable to transform back to male. This irreversibility back to male is potentially a problem for the species because if all fish in a group differentiate into females due to EDCs, it would result in a paucity of males to fertilize and tend to the eggs.

The goal of this study was thus to determine how aggression toward intruders, brain gene expression, gonad differentiation, and sex hormone profiles are potentially altered by exposure to environmentally relevant doses of BPA and EE2. Our hypothesis was that fish in the BPA and EE2 treatment groups would show characteristics suggestive of brain and gonad feminization, as has been observed in freshwater fish and terrestrial animals (Bhandari et al., 2015; Oehlmann et al., 2009; Oehlmann et al., 2000; Teuten et al., 2009). Specifically, we expected BPA and EE2 treatments to increase aromatase (*cyp19a1b*) and *f13a1* gene expression in the brain, and to decrease isotocin receptor (*itr*) and *popdc3* gene expression. We further expected these EDCs to increase territorial defense against intruders, reduce testicular tissue, increase vitellogenesis, decrease 11-KT and increase circulating concentrations of E2 and Vtg.

2. Methods

2.1. Animals and husbandry

All fish used in this study were *Amphiprion ocellaris* offspring bred in-house from brood stock obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL). Offspring were raised in groups of 50–80 in 20-gallon long (30″ × 12″ × 12″) aquaria connected to a system approximately 200 gal in total. At the time when the fish were approximately 1-year old, they were removed from group housing and randomly paired together in isolated 10-gallon aquaria. This pairing was done to simulate the normal conditions by which reproductive pairs of anemonefish develop and differentiate (Dodd et al., 2019; Fricke, 1983). Typically, the larger fish in the pair will grow over a period of 1–2 years before eventually transforming into a female, characterized by approximately 100% vitellogenic oocytes in the gonad. During this time, the subordinate remains close to the original size and differentiates as a reproductive male, characterized by approximately 70% of the gonad consisting of testicular tissue and the remaining gonad consisting of non-vitellogenic oocytes (Dodd et al., 2019). Importantly, before the gonadal transformation occurs in both fish and reproduction commences, both fish in the pair maintain small gonads with a small percentage of testicular tissue and mostly non-vitellogenic oocytes (Dodd et al., 2019).

Each 10-gallon aquarium housing a pair of fish contained a 6-inch diameter terra cotta pot to serve as the shelter and territory, a small heater, and a sponge filter. Conditions were set to mimic the natural environment with a pH between 8.0 and 8.4, temperature range of 79–82 °F, photoperiod of 12:12 (lights on at 0700 h and off at 1900), and specific gravity of 1.026. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee (protocol # 18071).

2.2. Experimental design

A total of 27 pairs of fish were formed as described above ($n = 54$ fish). The experiment was temporally separated into three cohorts (12 pairs, 24 fish in cohort 1; 12 pairs, 24 fish in cohort 2; 3 pairs, 6 fish in cohort 3; treatments were equally represented within cohorts). Body lengths and weights ranged from 5.4 to 7.5 cm and 2.9 to 10.3 g at the time when fish were paired. Pairs were randomly assigned to one of three groups (control, BPA, or EE2) with $n = 9$ pairs per group. The control group received plain food (0.8–1 mm size Golden Pearls from Brine Shrimp Direct, Ogden, Utah). The BPA and EE2 groups received the same food except with either 100 μg BPA per kg food or 0.2 μg EE2 per kg food incorporated into the food (see Food preparation section below). The doses were chosen based on analysis of concentrations of these compounds in wastewater and levels found in estuarine systems (Arditsoglou and Voutsa, 2012; Aris et al., 2014; Calderón-Moreno et al., 2019; Hemming et al., 2001; Koelmans et al., 2014; Ozhan and Kocaman, 2019) and were designed to mimic those that this species may be exposed to in the wild. We chose to administer the compounds through the food instead of the water mainly for logistical reasons: 1) our system is not equipped for changing water on a continuous basis, and 2) it is cost prohibitive to replace the large amount of chemicals and salt water required to maintain this oceanic species. This method of delivering the compounds through the fish food has been used previously (Colli-Dula et al., 2014; Martyniuk et al., 2020; Martyniuk et al., 2013; Martyniuk et al., 2011), and models the passage of chemicals through the food chain (Savoca et al., 2021), in addition to through the water from the chemicals leaching from the uneaten food.

Fish were fed twice a day, in the morning and afternoon, for 6 months. Unfortunately, it was not possible to measure the amounts of the food that each fish in the pair ate, and sometimes, food was observed left uneaten. Fish were measured for behavioral response to an intruder (see below) at the 1-month, 3-month and 6-month time-points. At each of these sampling points, the day before behavioral testing, all animals were measured for body weight and body length. One day after the behavioral testing, at the 3-month and 6-month time-points, blood was taken from the caudal vein to measure concentration of 11-ketotestosterone (11-KT), estradiol (E2), and vitellogenin (Vtg) in the plasma following established procedures (DeAngelis and Rhodes, 2016; Denslow et al., 1999; Dodd et al., 2019). Smaller fish were more difficult to extract blood from and therefore blood sample size varied by month. At the 6-month time-point, immediately after the blood draw, fish were euthanized by cervical transection. The whole brain (excluding the spinal cord and olfactory bulbs) of each fish was dissected out within 3.5 min of removal from their home tank. Brains were immediately placed in centrifuge tubes on dry ice, and then stored at −80 °C until RNA extraction for reverse transcription (rt)PCR analysis (see below). The

bodies were placed in vials containing 4% paraformaldehyde chilled at 4 °C and then stored in the refrigerator. The following morning, the bodies were moved to 30% sucrose solution for cryopreservation and returned to the refrigerator for 48 h. Bodies were then placed in Tissue Mounting Media (Tissue-Tek O.C.T. Compound, Sakura, Finetek) and frozen in the −80 °C freezer.

In addition to processing the experimental fish as described above, we also euthanized 2 established reproductive males and 2 established reproductive females from the colony to serve as a reference for interpreting the outcomes, whether indicative of feminization, masculinization or neither. These data are included in the figures for reference but were not used in the statistical analyses. Additional data from previously published studies are also included in the figures to supplement these numbers and are cited accordingly in the figure legends (DeAngelis et al., 2018; DeAngelis and Rhodes, 2016; Dodd et al., 2019).

2.3. Food preparation

For the BPA diet, 5 mg BPA (Sigma Aldrich Cat# 239658) was crushed into powder form and dissolved into Menhaden Oil (Aquatic Nutrition Inc., FL) to a final concentration of 5 μg BPA/mL of Menhaden Oil. For the EE2 diet, the EE2 was dissolved into 100% ethanol to a concentration of 5 mg EE2/mL of ethanol. This solution was then diluted in Menhaden Oil to a final concentration of 10 ng EE2/mL of Menhaden Oil (Colli-Dula et al., 2014). The Menhaden oil solutions were mixed into the Golden Pearl pellets by using a KitchenAid Mixer by slowly adding 2 mL of oil into 100 g of food and mixing for 25–40 min until all clumps were gone and oil was mixed evenly in the food.

2.4. Behavioral response to an intruder

All fish were tested at 1 month, 3 months, and 6 months of exposure. Fish were examined in their home aquariums, the same environment where they were exposed to the endocrine disruptors. Between the hours of 12:00 PM and 4:00 PM, a video camera was set up in front of the aquarium and fish were allowed to acclimate to its presence for 10 min. After acclimation, an intruder group-housed fish was introduced into the aquarium. This intruder was chosen specifically to be a length in between the length of the two fish in the pair. Video recordings proceeded for 10 min and were analyzed in their entirety to record total number of lunges, chases, bites, and aggressive face-to-face, side-to-side, and quivering displays made by each fish toward the intruder. Videos were analyzed by investigators blind to the experimental treatments. A single investigator analyzed all the videos for a given behavior for consistency. Following previously described methods, all aggressive events were summed together to produce one index of aggression per fish (DeAngelis et al., 2020; DeAngelis et al., 2017; Phillips et al., 2020; Yaeger et al., 2014). Lunging toward the intruder was the most common behavioral act of aggression in the present dataset. Approximately 84.6% of the total aggressive acts we recorded were in this category, followed by face-to-face displays (6.3%), biting (2.9%), aggressive quivering (2.8%), chasing (2.0%), then side-to-side displays (1.4%). Lunging was observed in all except one of the videos, but the other behaviors were frequently not observed. Rather than discount them, we opted to make the composite aggression index, which we reasoned would better represent the range of aggression observed by the fish.

In addition to conducting the behavioral test on the experimental fish as described above, we also conducted the intruder test exactly the same way for 12 established reproductive pairs in our colony to serve as a reference.

2.5. Brain gene expression rtPCR analysis

2.5.1. RNA extractions and cDNA synthesis

Following previously established procedures (DeAngelis et al., 2018), whole brain RNA extractions were performed using the RNeasy Lipid Tissue Mini Kit (Qiagen, Cat. No. 74804) following manufacturer instructions. RNA concentrations were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). RNA quality number (RQN) and 28S/18S ratios were determined by using a Fragment Analyzer Automated CE System (Advanced Analytical AATI) at a concentration of 100 ng/μL (see Supplementary Table 1 for results). Average RIN score was 8.8 ± 0.52 SD. With remaining aliquots, RNA concentrations were normalized to 500 ng/μL prior to reverse transcription. RNA was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Cat. No. 1708890) following manufacturer's instructions, in a total volume of 20 μL per reaction. To control for potential genomic DNA contamination, samples were treated with RNase free DNase I (New England BioLabs, Cat. No. M0303S) and negative control reactions were run without the iScript transcriptase.

2.5.2. Primer validation

Previously validated primers for *cyp19a1b*, *itr*, and *actb* in *A. ocellaris* were used (DeAngelis et al., 2018) (Table 1). Beta actin (*actb*) was chosen as a reference gene due to its consistent Ct values across groups, and because it has previously been validated and used as a reference gene for *A. ocellaris* whole brain analysis (DeAngelis et al., 2018; Iwata et al., 2012). Primers for *popdc3* (GenBank # 111588979) and *f13a1* (GenBank # 111580199) were designed using Primer-BLAST software (NCBI) and PrimerQuest (IDT Technologies) against sequences specific to *A. ocellaris* (Tan et al., 2018). Primers were analyzed for hairpins, and primer dimers using OligoAnlyzer (IDT Technologies) prior to serial dilution validations. Only primers showing hairpin analysis with ΔG (kcal·mole^{-1}) values < 2, and primer-dimer values of ΔG < 6 were tested in serial dilutions. All primers were commercially purchased from IDT technologies (Coralville, IA). Following cDNA synthesis, samples were diluted in a 4-fold series and checked for optimal efficiencies and a single melt curve. All primers displayed an efficiency between 94 and 108% and an R^2 value, (which refers to the standard curve relating a serial dilution of a pooled sample to the Ct values) of over 0.98 (see Supplementary Table 2).

2.5.3. Relative gene expression quantification

Gene expression was quantified using the SSo Advanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Cat. No. 1725271) and CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Cat. No.1855201). All reactions were run in triplicate. The cycle parameters were 95 °C for 120 s, 95 °C for 5 s, 60 °C for 30 s, then plate read and repeated for 40 cycles. Following the 40 cycles, a melt curve was performed with an increase in temperature from 65 °C to 95 °C at 0.5 °C increments, followed by a final plate read.

The cycle threshold (Ct) was calculated automatically in the CFX Connect™ software. Only triplicate reactions with a Ct standard error (SE) under 0.85 were used in statistical analysis. Relative gene expression was computed using the following established formula, which compares the Ct of the gene of interest to the Ct of the reference gene (*actb*): $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.6. Gonadal histology

Bodies were serially sectioned at 40 μm in the sagittal plane using a cryostat (Thermo Scientific, Microm HM 550) and placed directly on subbed slides. Slides were stored in the −80 °C freezer until staining. The day before staining, slides were left out over night to defrost then stained using a Harris Hematoxylin solution as previously described (Dodd et al., 2019).

Sections containing the gonads were photographed using an Axiocam (MRc 5) camera mounted to a Zeiss light microscope running Axio Imager software. To measure total volume of the gonad, we selected 50% of the sections with gonad at random and measured the area of the gonad in the sections. Photographs were taken at 25× total magnification. The photos were outlined using Adobe Photoshop CS6 software.

Table 1
Base-pair sequences for the primers used to measure expression of each gene are given below. Melt temperatures, and efficiency measures specific to each primer pair are also indicated.

Target			Melt		
Sequence	Forward primer	Reverse primer	Temperature	Efficiency	R^2
actb	GTTGGTGATGAAGCCCAGAG	ATCTTCTCCATGTCATCCCAGT	55.7 °C	100.6	0.997
cyp19a1b	CAGCGAGCAACTACTACAACAA	ACATGGTACACCGCAGAC	55.0 °C	108.1	0.981
popdc3	ACCCTGTGTGCTGATAACCC	GATCATAGCGGTGCCCAGAT	57.1 °C	101.6	0.996
itr	GGAGCATCACACAGACTTCC	GGCCACATGTATGTATCTGAAGG	54.5 °C	104.2	0.992
f13a1	TCAACACCTTCCTACGCTGTC	CCTGGTACGGTTTCGGTCAA	57.0 °C	94.7	0.990

We then multiplied the average area, by the total number of sections through the gonad, by the thickness of the sections (40 μm) to obtain total volume of gonad per individual. To estimate the proportion of the gonad containing testicular tissue, non-vitellogenic oocytes, or vitellogenic oocytes, for each individual, 20 sections through the gonads were chosen at random for analysis from the series. Photographs were taken at 100× total magnification. First the gonad was outlined, and then the Quick Selection tool was used to differentiate testicular tissue from ovarian tissue semi-automatically with manual correction until the tissue types were differentiated sufficiently following established procedures (Dodd et al., 2019). The area fraction of the gonad covered by each of these tissue types (% testicular tissue, % ovarian-non-vitellogenic, % ovarian-vitellogenic) was evaluated for each of the sampled sections. The estimated fractions were averaged over the 20 sections to produce one value per individual for each of the tissue types for statistical analysis. To obtain total volume of testicular tissue, non-vitellogenic oocytes, and vitellogenic oocytes, total volume of the gonad was multiplied by the percentages of each tissue type for each individual separately.

2.7. Plasma sex steroid hormone measurements

Plasma was assayed using previously validated commercially available enzyme immunoassay kits for E2 (Calbiotech, Lot NO. ESG4324, range of 3–300 pg/mL) and 11-KT (Cayman Chemical, Item No. 582751, range 0.78–100 pg/mL) following previously established procedures (DeAngelis and Rhodes, 2016). Each plasma sample was diluted 1:30 in the assay buffer prior to analysis following kit instructions. All samples were run in duplicate in the same assay to avoid inter-assay variation. Subsequent absorbance was read using the Epoch Microplate Spectrophotometer (BioTek Instruments) following the manufacturer's instructions.

2.8. Plasma vitellogenin measurements

Relative plasma vitellogenin (Vtg) concentrations were determined using an indirect assay developed against largemouth bass Vtg that we determined also recognized clownfish Vtg. Since we did not have purified clownfish Vtg for the standard curve, we used largemouth bass vitellogenin for this purpose to allow relative comparisons between groups. LMB Vtg standard was purified by anion exchange chromatography as described previously (Denslow et al., 1999). Monoclonal antibody, Mab 3G2 (HL1393) was used as the primary antibody in an indirect ELISA assay. Clownfish plasma samples diluted 1:200, 1:10,000 and 1:100,000 were loaded in the wells of a microtiter plate (NUNC) and allowed to bind for 24 h at 4 °C in a humidified Tupperware container. In separate wells, largemouth bass purified vitellogenin standards (0, 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μg/mL) were used to generate a standard curve. To account for matrix suppression of signal in the ELISA, male largemouth bass plasma (devoid of Vtg) was added to each well of the standard curve to match the dilutions tested.

In brief, the plate was washed 3× with tris buffered saline with tween-20 (TBST, 30 mM Tris, 150 mM NaCl, 2% Tween 20), blocked for 2 h with 1% BSA in TBST, and rewashed before adding the primary antibody (1 μg/mL) to the wells and incubated overnight (4 °C). The plate was then washed 3× with TBST. This was followed by addition of an amplifying secondary antibody (anti-mouse IgG-biotin) diluted 1:1000 with TBST and incubated 2 h at room temperature. The plate was washed, and a final reagent (streptavidin alkaline phosphatase conjugate) diluted 1:1000 in TBST was added for 2 h (R/T).

The plate was washed and a substrate (PNPP, 1 step Thermo-Scientific) was added to each well. The resulting color reaction was measured at 405 nm on an ELISA plate reader (SpectraMax Plus384, Applied Biosystems) and Vtg concentrations were determined using the SoftMax Pro analysis program. The limit of detection for the LMB Vtg ELISA was 0.001 mg/mL. All assays were performed in triplicate and reported as the mean of the three measurements. The coefficient of variation was <10% for all samples analyzed. Inter- and intra-assay variability was measured by analyzing positive controls on several plates and different assays, and is typically <10%, and <5%, respectively.

2.9. Statistical methods

SAS (9.3) Proc Mixed was used for all statistical analyses. $P < 0.05$ was considered statistically significant. Standardized residual distributions from the statistical models were evaluated for meeting assumptions of normality by ensuring skewness was between −1 and 1 and kurtosis between −2 and 2 following accepted practices (Kim, 2013). If the data did not meet these assumptions, the variable was raised to a power for which the residual distribution met these criteria. The following variables were raised to the power of 0.5: total aggression toward intruder, concentration of 11-KT in the plasma, brain aromatase (*cyp19a1b*) and *itr* gene expression. Percent testicular tissue and concentration of vitellogenin in the blood plasma was raised to the power of 0.2, and the log base 10 was used for *f13a1* gene expression. The other variables were not transformed because the raw values met the assumptions of the statistical tests.

Body length and weight were analyzed using a mixed effects linear model with time-point (1, 3, or 6 months), dominance status (larger versus smaller fish in the pair), and treatment group (control, BPA, or EE2) entered as factors in the model, with tank entered as a random variable to account for the repeated measures structure in the data. The behavioral data (total number of aggressive acts toward the intruder in the 10 min test) were analyzed the same way. Concentrations of E2 and 11-KT in the blood plasma were analyzed a similar way except for there were only 2 time-points (3 or 6 months). Total volume of the gonad, and proportion comprised of the different tissue types (e.g., testicular tissue, non-vitellogenic oocytes), and brain gene expression measurements were analyzed similarly except without the time-point variable since these measures were taken only once, at the end of the study. The gonad volume data were also analyzed including length and weight as covariates to account for allometry.

Effect sizes (η^2) for a given factor were calculated using the formula (Bakeman, 2005):

$$\eta^2 = 1 - \frac{residual\ variance\ with\ factor\ in\ the\ model}{residual\ variance\ with\ factor\ removed}$$

Tukey post hoc tests were used to evaluate pairwise differences between means. A Fisher's exact test was used to compare the proportion of fish that displayed vitellogenic oocytes between groups.

Note, for all outcome variables reported in the figures, we also included data from reproductively active males and females to use as a reference for interpretation. However, these data were not used in the statistical analysis of comparisons between the groups (control, BPA, and EE2). The majority of these data were from previously published sources, and statistical significance for sex differences had already been established. However, the behavioral response of a reproductive pair to an intruder had not previously been published. These data were analyzed using a simple *t*-test between males versus females.

3. Results

3.1. Body size was unaffected by BPA or EE2 treatment

No effects of the treatments on body size were detected. Collapsed across treatments, the larger fish in the pair (the dominant fish) grew significantly more than the subordinate over the duration of the experiment (Fig. 1). This was indicated by a significant effect of time ($\eta^2 = 0.43$, $F_{3,177} = 48.0$, $p < 0.0001$; $\eta 2 = 0.53$, $F_{3,177} = 63.9$, $p < 0.0001$), status ($\eta^2 = 0.35$, $F_{1,177} = 98.9$, $p < 0.0001$; $\eta^2 = 0.55$, $F_{1,177} = 197.5$, $p < 0.0001$), and status-by-time interaction ($\eta^2 = 0.04$, $F_{3,177} = 5.2$, $p = 0.002$; $\eta^2 = 0.18$, $F_{3,177} = 14.6$, $p < 0.0001$) for body length and weight,

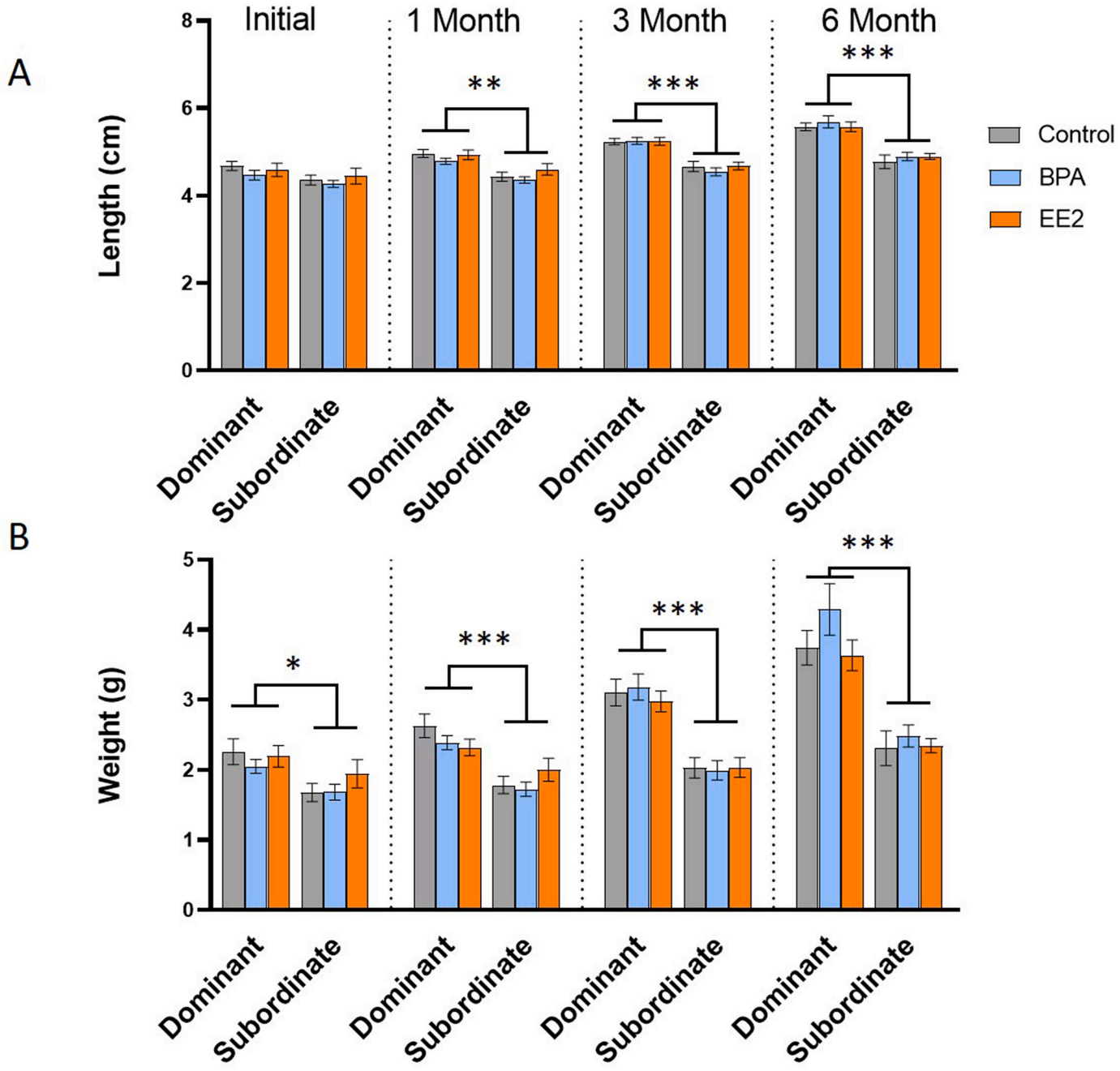

Fig. 1. Body growth across the experiment. A. Mean body length (cm) and B. Mean body mass (g) shown for each group at 1, 3 and 6 months. The dominant fish grew, while the subordinate remained the same size over the course of the experiment. No treatment effects were detected. Standard error bars shown. $n = 9$ per bar, the same individuals each month. $^*p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$.

respectively. No other interactions were significant.

3.2. BPA reduced aggression toward male intruders, EE2 had no significant effect

Total levels of aggression increased as time progressed from 1 to 3 to 6 months ($\eta^2 = 0.04$, $F_{2,139} = 4.4$, $p = 0.01$), collapsed across treatment and dominance status (Fig. 2). The dominant fish in the pair was significantly more aggressive than the subordinate fish collapsed across treatment and time ($\eta^2 = 0.12$, $F_{1,139} = 19.3$, $p < 0.0001$). The main effect of treatment (collapsed across time and dominance status) was also significant ($\eta^2 = 0.07$, $F_{2,139} = 5.6$, $p = 0.005$). Post-hoc tests indicated that BPA treated fish were significantly less aggressive than control fish ($p = 0.003$), displaying an average of 18 (±3.3 SEM) aggressive acts toward intruders in 10 min versus 31 (±3.8) for control. The EE2 group was also slightly less aggressive than the control fish, displaying only 23 (±2.9) aggressive acts, but this did not reach statistical significance ($p = 0.09$). There were no differences between BPA and EE2 ($p = 0.45$). None of the other interactions between treatment, time or dominance status were significant. A reduction in aggression is indicative of a masculinizing or de-feminizing effect as reproductive males are significantly less aggressive than their female partners toward intruders on this test ($\eta^2 = 0.28$, $t_{10} = 2.5$, $p = 0.03$).

3.3. BPA and EE2 altered expression of sexually dimorphic transcripts in the brain

A tissue processing error resulted in some of the samples not being usable for RNA isolation and rtPCR analysis. Even with this limitation, we had sufficient samples to lead to conclusive and robust results. Final sample sizes for rtPCR are shown in Fig. 3. BPA significantly increased brain aromatase (*cyp19a1b*) gene expression by approximately 51% relative to the EE2 group, as indicated by a significant effect of treatment ($\eta^2 = 0.15$, $F_{2,15} = 5.2$, $p = 0.02$; Fig. 3A). No effect of status or interaction between status and treatment were detected. Post hoc tests indicated that BPA was different from EE2 ($p = 0.006$), and there was a trend for BPA to differ from control, but this did not reach statistical significance ($p = 0.11$). EE2 was not different from control. BPA and EE2 significantly reduced *popdc3* gene expression by approximately 66% and 57% relative to the control group, respectively. This was indicated by a significant effect of treatment ($\eta^2 = 0.19$, $F_{2,14} = 4.4$, $p = 0.03$; Fig. 3B). No effect of status or interaction between status and treatment were detected. Post hoc tests indicated control was different from EE2 ($p = 0.03$) and BPA ($p = 0.02$), but EE2 and BPA were not different from each other. No effect of the treatments, dominance status or interactions were detected for *itr* or *f13a1* gene expression (Fig. 3C, D).

3.4. BPA significantly feminized the gonads including producing pairs of females; EE2 had no effect at the tested dose

Seven out of the 18 fish in the BPA group displayed zero testicular tissue and the presence of some fraction of vitellogenic eggs in their gonads (ranging from 7% to 77% the total volume of the gonad), indicating that the fish had fully differentiated into a female. Zero fish in the control or EE2 group displayed this gonadal phenotype, i.e., they all had some fraction of testicular tissue present and zero vitellogenic oocytes (Fig. 4). This difference of 7 out of 18 versus 0 out of 36 was significant by Fisher's exact test ($p = 0.0002$). Five of the 7 fish in the BPA group with vitellogenic oocytes were the dominant member of the pair. However, remarkably, in 2 of the 7 cases, the subordinate also displayed vitellogenic oocytes. Hence, in 2 pairs out of the 9 pairs (amounting to an approximate 22% frequency), both the dominant and subordinate fish had vitellogenic oocytes indicating that the pair consisted of two females, a phenomenon which, to our knowledge, has never been observed in the laboratory or the wild. Such a disruption that allowed two females to cohabitate could, in the natural setting, be catastrophic

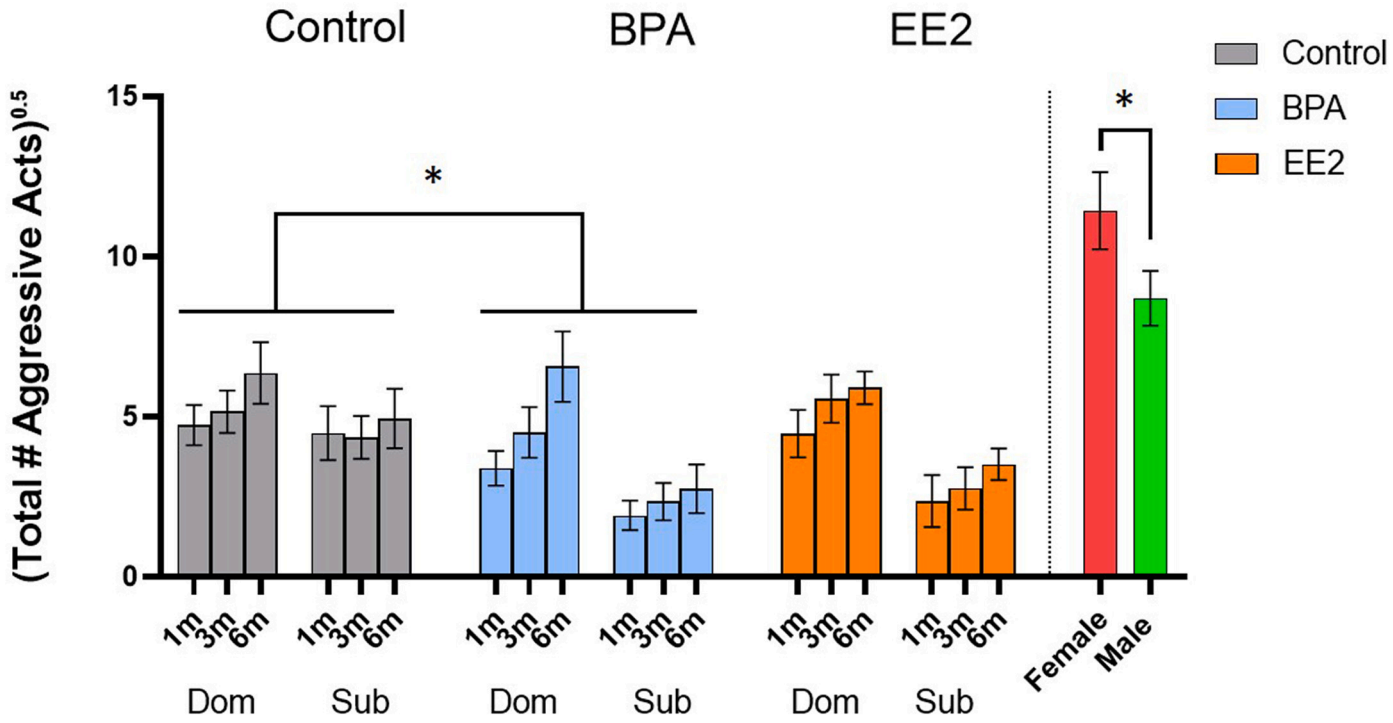

Fig. 2. BPA decreased aggression toward a male intruder, EE2 had no significant effect. Mean total number of aggressive acts (e.g., lunges, bites, chases, and aggressive displays; raised to the power of 0.5 to normalize the otherwise skewed residual distribution) directed toward the male intruder shown for each group at 1, 3, and 6 months. Aggression increased over time, the dominant fish was more aggressive than the subordinate, and BPA decreased aggression relative to the control, collapsed across time and dominance status. No interactions were significant. Standard error bars shown. n = 9 per bar, the same individuals each month. For comparison, aggression levels for the same intruder test are shown for 12 established reproductive male-female pairs. *$p < 0.05$.

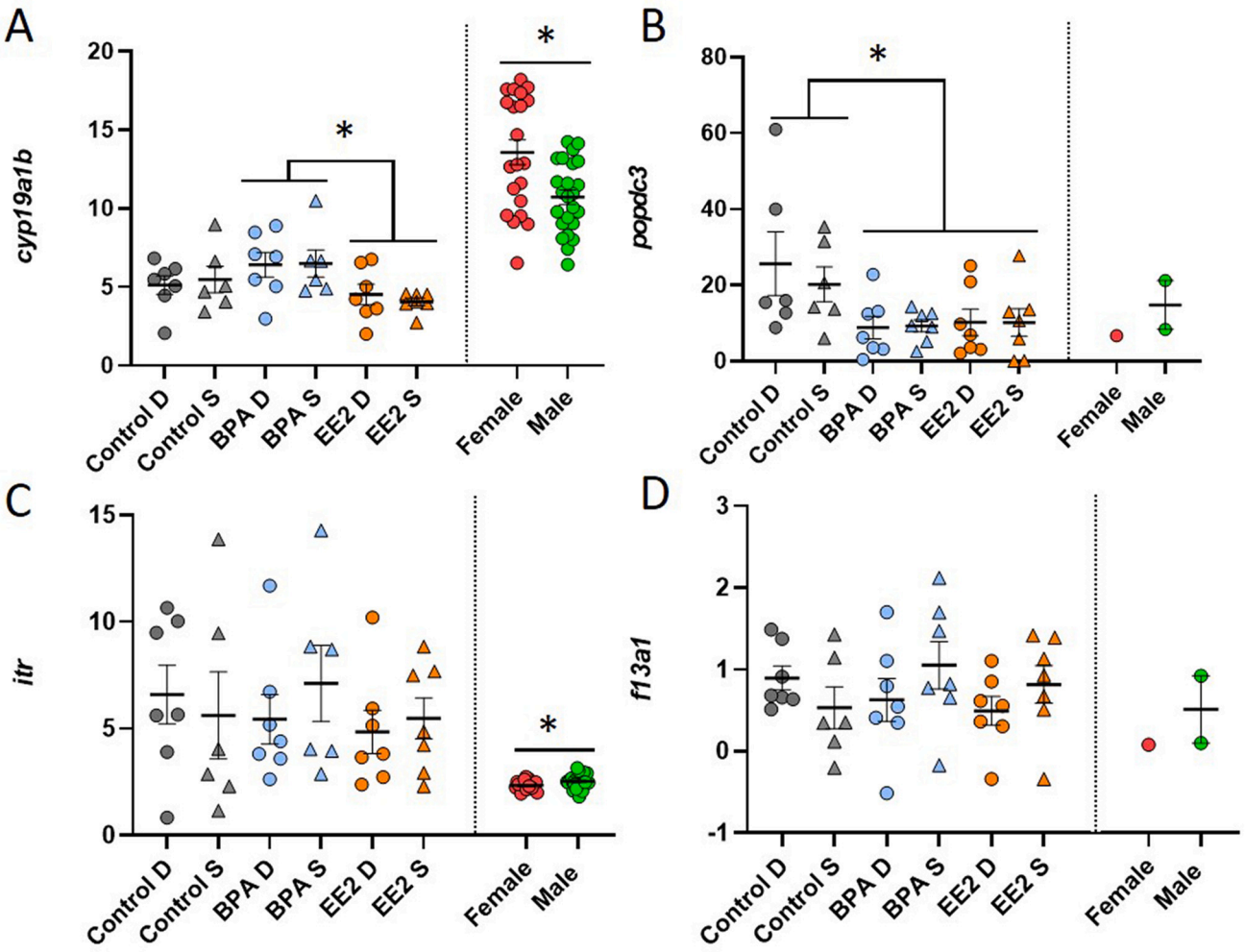

Fig. 3. BPA and EE2 partially feminized brain gene expression. A-D. Relative gene expression levels (arbitrary units) are shown for brain aromatase (*cyp19a1b*), *popdc3*, *itr*, and *f13a1*, respectively. Data are shown for each individual, separately by group. Group means and standard errors are also shown. BPA significantly increased brain aromatase gene expression relative to the EE2 group, and slightly increased brain aromatase gene expression relative to control, though the latter was not statistically significant ($p = 0.11$). EE2 and control groups did not differ from each other. BPA and EE2 displayed similar expression of *popdc3* which was significantly reduced relative to the control group. The treatments did not significantly affect *itr* or *f13a1* expression. For comparison data for established reproductive male-female pairs are also shown. For *cyp19a1b* and *itr* we included data from a previously published manuscript (DeAngelis et al., 2018). *p < 0.05.

for the species.

In addition to producing 7 individuals with vitellogenic oocytes, the remaining individuals in the BPA group displayed significantly increased volume of non-vitellogenic oocytes in the gonad as compared to the other groups. After removing the 7 individuals with vitellogenic oocytes which had large gonads, significant effects of treatment were observed for total volume of the gonad ($\eta^2 = 0.09$, $F_{2,18} = 4.8$, $p = 0.02$; Fig. 4D), and non-vitellogenic volume ($\eta^2 = 0.22$, $F_{2,18} = 5.4$, $p = 0.01$; Fig. 4E), but no change in testicular volume (Fig. 4F). Post-hoc tests indicated that total gonad volume and non-vitellogenic ovarian tissue volume increased in the BPA group relative to EE2 ($P = 0.008$, $P = 0.006$) and control ($P = 0.02$, $P = 0.01$), but no differences between EE2 and control were detected. We also observed a significant effect of status on total volume ($\eta^2 = 0.09$, $F_{1,18} = 10.2$, $p = 0.005$; Fig. 4D), non-vitellogenic volume ($\eta^2 = 0.08$, $F_{1,18} = 9.3$, $p = 0.007$; Fig. 4E), and testicular volume ($\eta^2 = 0.10$, $F_{1,18} = 7.7$, $p = 0.01$; Fig. 4F) with dominant fish displaying larger gonads than subordinate fish. However, this effect went away after including length or weight of the animal as a covariate, since larger fish have larger gonads, and dominant fish are larger than subordinates (Fig. 1). The length and weight covariates were significant in these analyses. For total gonad volume, statistics for length and weight were as follows (length, $\eta^2 = 0.07$, $F_{1,14} = 7.3$, $p = 0.02$; weight, $\eta^2 = 0.08$, $F_{1,18} = 7.7$, $p = 0.01$).

As a result of the larger volume of ovarian tissue, the percentage of testicular tissue was reduced in the BPA group relative to the other groups. This change was reflected by a significant effect of treatment ($\eta^2 = 0.33$, $F_{2,23} = 9.7$, $p = 0.0009$; Fig. 4I). Neither the effect of status nor the interaction between status and treatment were significant. Post-hoc tests indicated BPA was different from EE2 ($p = 0.003$) and the control group ($p = 0.0004$), but EE2 and control were not different from each other (Fig. 4I). Due to large variation, no group differences were detected in the percentage of the gonads composed of non-vitellogenic oocytes (Fig. 4H).

3.5. BPA and EE2 de-masculinized circulating sex steroids

BPA significantly reduced 11-KT to female-typical levels (DeAngelis and Rhodes, 2016). Moreover, this effect occurred in both dominant and subordinate fish (Fig. 5A). In the EE2 group, dominant fish showed higher 11-KT than subordinate. This was indicated by a significant effect of treatment ($\eta^2 = 0.65$, $F_{2,55} = 25.6$, $p < 0.0001$), status ($\eta^2 = 0.13$, $F_{1,55} = 9.0$, $p = 0.004$) and an interaction between treatment and dominance status ($\eta^2 = 0.06$, $F_{2,55} = 3.7$, $p = 0.03$). Post-hoc tests indicated that collapsed across time and dominance status, 11-KT was

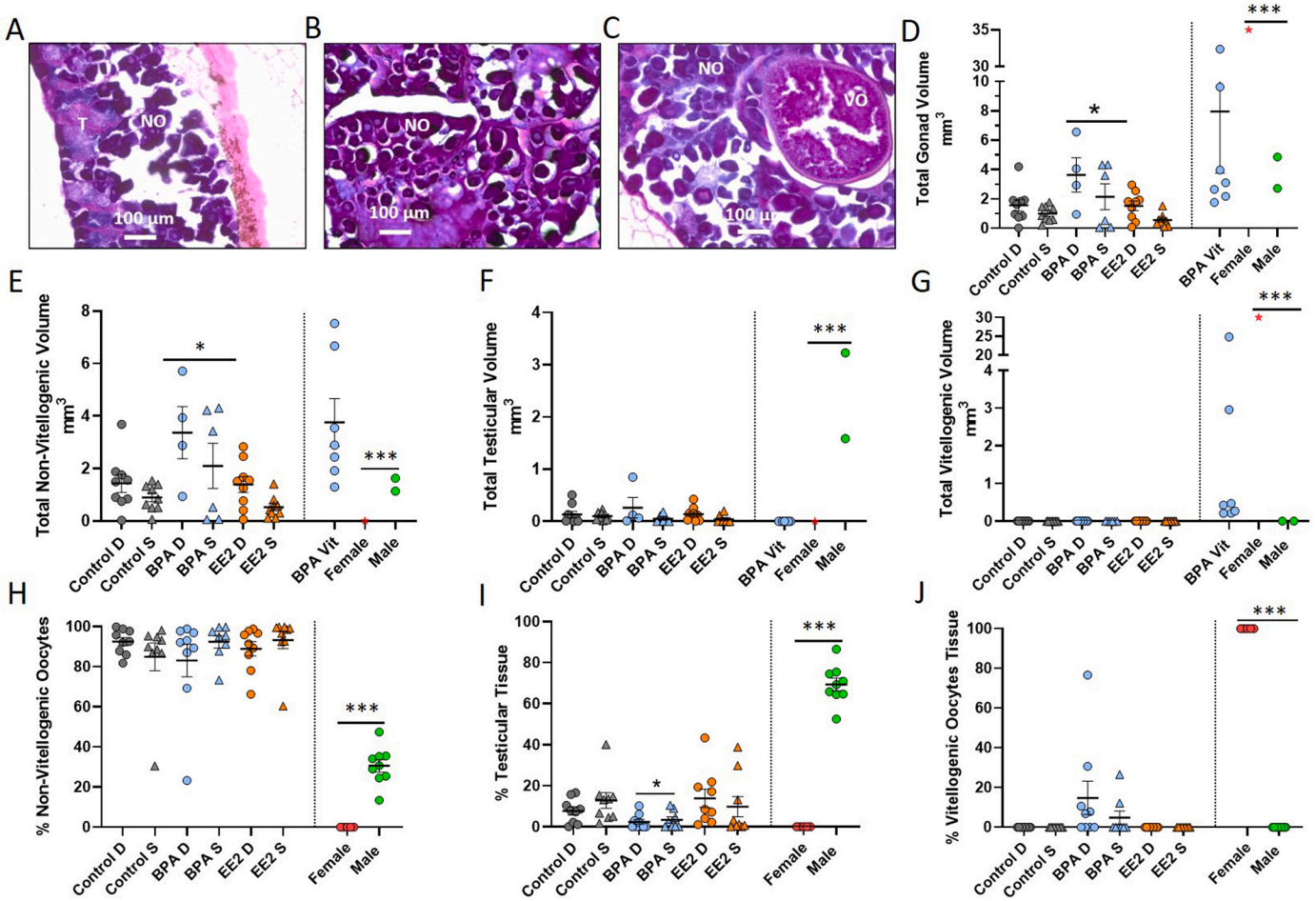

Fig. 4. BPA actively feminized the gonads whereas EE2 had no effect. A. Photograph of a sagittal section through the gonad stained with H&E from a control (untreated) individual. B–C. Representative gonads from two different individuals in the BPA group. "NO" indicates non-vitellogenic oocytes. "VO" indicates vitellogenic oocytes. "T" indicates testicular tissue. D. Total volume of the gonad in cubic mm shown for each group. E. Percent of the gonad composed of testicular tissue. F. Percent of the gonad composed of non-vitellogenic oocytes. G. Percent of the gonad composed of vitellogenic oocytes. H. Total testicular volume in cubic mm. I. Total non-vitellogenic oocyte volume. J. Total vitellogenic oocyte volume. Data are shown for each individual, separately by group. Group means and standard errors are also shown. BPA increased volume of vitellogenic and non-vitellogenic oocytes without changing volume of testicular tissue, resulting in decreased percent of testicular tissue. In panels D and H–J, the 7 individuals from the BPA group that displayed vitellogenic oocytes are shown separately, since the volume occupied by vitellogenic eggs is substantially larger. In addition, we included 2 males, and indicated where females would occur using a red star. We did not measure females for volume of the gonad (or volume of vitellogenic oocytes) since the volume ranges from within the upper range in the figures to orders of magnitude higher when they are carrying well-developed eggs. For panels E–G, data for established reproductive males and females from a previously published study from our lab are shown next to the experimental data (Dodd et al., 2019). *$p < 0.05$, ***$p < 0.0001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significantly lower in the BPA group (average 72 pg/mL ± 8.4 SEM) relative to EE2 ($p < 0.0001$; average 698 pg/mL ± 94.4) and control (p < 0.0001; 676 pg/mL ± 82.9), whereas control and EE2 were not different from each other. Post-hoc tests also indicated that 11-KT was significantly different between the subordinate versus the dominant fish in the EE2 group ($p = 0.0003$; subordinate displayed 397 pg/mL ± 45.0 whereas dominant displayed 867.2 pg/mL ± 127.9) but not in the control or BPA groups. The time when the fish were sampled, 3 months or 6 months did not significantly affect plasma 11-KT levels. No effect of any of the treatments or interactions were detected for plasma E2 levels (Fig. 5B).

3.6. BPA feminized circulating Vtg levels, EE2 had no effect at the tested dose

BPA significantly increased plasma Vtg concentration to female-typical levels in both dominant and subordinate fish (Fig. 5C). This was indicated by a significant effect of treatment ($\eta^2 = 0.69$, $F_{2,35} = 22.6$, $p < 0.0001$). Post-hoc tests indicated that collapsed across time and dominance status, plasma Vtg was significantly higher in the BPA group (average 5.4 mg/mL ± 1.40 SEM) relative to the control (p < 0.0001, 0.0 mg/mL ± 0.003 SEM) and EE2 (p < 0.0001, 0.06 ± 0.053 SEM), whereas control and EE2 were not different from each other. The time when the fish were sampled, 3 months or 6 months, and dominance status (whether the fish was the subordinate or dominant member of the pair) did not significantly affect plasma Vtg levels.

4. Discussion

The major finding of the study is that an environmentally relevant dose of BPA partially feminized the gonads and circulating sex steroid hormones of the sexually labile, and iconic coral reef fish, *Amphiprion ocellaris*. Within 3 months of exposure, levels of 11-KT, the major bioactive androgen in fish, plummeted to female-typical levels (Fig. 5A) (DeAngelis and Rhodes, 2016). By 6 months, the gonads of most of these fish retained a small fraction of testicular tissue, but 7 out of 18 fish treated with BPA ended up with zero testicular tissue and the presence of vitellogenic oocytes indicated they had transformed into a female

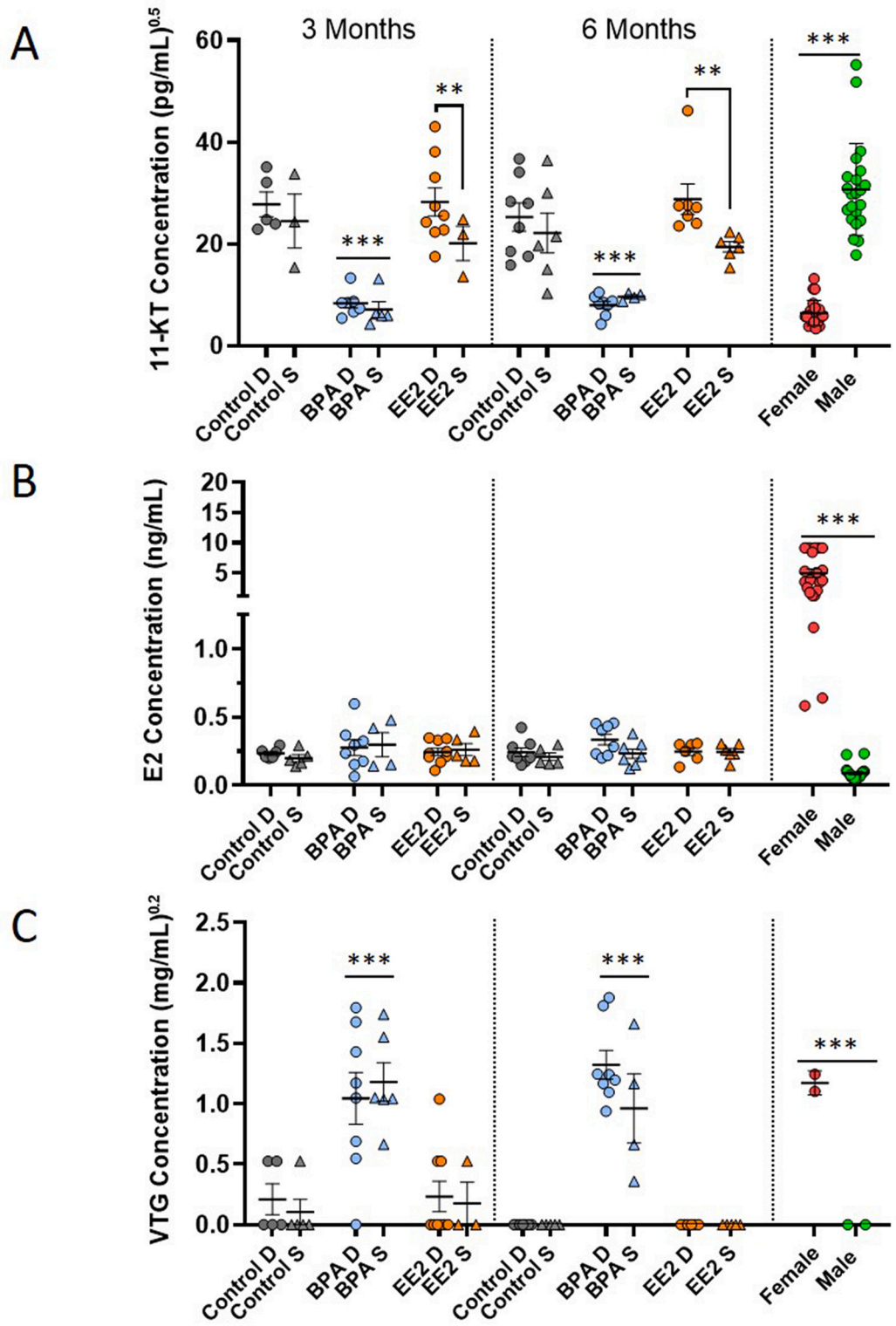

Fig. 5. BPA feminized plasma 11-KT and Vtg; EE2 partially reduced 11-KT. A. Concentration of 11-KT in the plasma in pg/mL (raised to the power of 0.5 to normalize the otherwise skewed residual distribution) B. Concentration of E2 in the plasma in pg/mL. C. Concentration of Vtg in the plasma in mg/mL. Data are shown for each individual, separately by group at 3 and 6 months. Group means and standard errors are also shown. Some of the same individuals were measured in both months. BPA reduced 11-KT and increased Vtg in both dominant and subordinate fish, whereas EE2 reduced 11-KT only in subordinate fish and had no effect on Vtg. No treatment effects were observed for E2. For comparison, data for established reproductive males and females from three of our previously published studies are shown next to the experimental data (DeAngelis et al., 2018; DeAngelis and Rhodes, 2016; Dodd et al., 2019). $^{**}p < 0.001$, $^{***}p < 0.0001$.

(Fig. 4). In 2 out of 9 pairs, both the subordinate and dominant member of the pair had vitellogenic oocytes. This amounts to the prediction that 22% of the pairs will be composed of two females if exposed to the level of BPA used here. If similar findings were to occur in the natural setting, it could be detrimental to the species' survival as anemonefish depend upon a system where the dominant member of the pair differentiates into a female and the subordinate member remains male to facilitate sexual reproduction (Fricke and Fricke, 1977; Fricke, 1979). Whether BPA from plastic waste or other chemicals are contributing to the decline in anemonefish and other coral reef species is an active area of research (Chen et al., 2018; Holzer et al., 2017; Savoca et al., 2021) and it is too early to draw conclusions on wild populations. Freshwater fish exposed to EDCs show dramatic declines in population numbers (Kidd et al., 2007), and the potential exists for population level impacts on coral reef species with exposures, but the risk is yet undetermined.

Although the majority of fish in the BPA group displayed feminized

gonads as indicated by increased presence of non-vitellogenic oocytes and/or presence of vitellogenic oocytes, and subsequently reduced percentage of testicular tissue, many BPA-treated fish displayed normal gonads similar to controls. One possible explanation for this variability is that the individuals consumed different amounts of food, and therefore were exposed to different doses. Unfortunately, we were unable to measure food intake or concentration of the BPA in the tissues. On the other hand, all the individuals exposed to BPA displayed dramatically reduced 11-KT and increased VTG (Fig. 5A, C), diminishing the possibility that dose is responsible for the difference. Moreover, dominant fish which grew faster than subordinate fish (Fig. 1), likely ate more food and therefore received a larger dose, but there was no detectable effect of dominance status on the gonadal composition measures. Perhaps there is a threshold level of BPA needed to affect the gonads, that was not reached in some fish. Another possibility is that the different fish responded in different ways to the endocrine disruptors, given the complexity in the way sex is determined in the species with important contributions from the brain and behavior, as well as the gonads (Dodd et al., 2019).

While the effect of BPA on gonadal composition was variable, the effect on plasma 11-KT and Vtg levels was highly consistent and robust. BPA dramatically reduced 11-KT in all the individuals (Fig. 5A), and dramatically increased Vtg (Fig. 5C). This result is consistent with the known effects of BPA in terrestrial and freshwater aquatic species (Hemming et al., 2001; Liu et al., 2013; Oehlmann et al., 2009; Oehlmann et al., 2000; Rosenfeld et al., 2017; Teuten et al., 2009; Wang et al., 2019; Zhang et al., 2013). One implication of having low 11-KT could be reduced sperm volume, motility, and velocity (Hatef et al., 2012). We did not extend the experiment out far enough to measure reproductive rates, which would require several years to do. However, future studies should examine the possibility that male fish exposed to BPA that retain testicular tissue, display reduced fecundity.

While BPA had a robust and consistent effect reducing 11-KT and increasing Vtg, BPA had no discernable effect on plasma E2 levels. A signature feature of female *A. ocellaris* is high levels of circulating E2 (DeAngelis and Rhodes, 2016; Dodd et al., 2019). Hence, BPA resulted in partial feminization of the plasma sex steroids, reducing 11-KT but having no effect on E2 (Fig. 3B). Even fish with gonads containing vitellogenic oocytes did not exhibit female-like circulating E2 levels. A possible explanation is that BPA could be acting as an E2 agonist and therefore inhibiting the further production of E2 via negative feedback. This idea is consistent with the literature in which BPA had no significant effect on serum E2 levels or even decreased serum levels (Acconcia et al., 2015; Patel et al., 2017; Wang et al., 2019). An alternative explanation, consistent with no difference in E2 levels, would be that BPA may be inducing feminization through its action as an androgen antagonist instead of an estrogen agonist (Acconcia et al., 2015; Bhandari et al., 2015; Wang et al., 2019). For example, BPA can induce apoptosis of germ cells and Leydig cells in the testis, and hence lead to decreased production of 11-KT (Wang et al., 2019). BPA is also able to impact ovarian maturation without any change in steroidal sex hormones directly through the HPG axis (Wang et al., 2019). The effects of BPA cannot always be predicted by their affinity for estradiol receptor (ER) and can vary based on tissue, species, and life-stage (Acconcia et al., 2015; Bhandari et al., 2015).

In addition to observing no change in plasma E2, we also did not observe any effect of BPA on body size, which is important because an increase in body size is another hallmark sign of feminization in the species. It is unlikely that the lack of changes in E2 and body growth are related, since the body growth occurs before the gonads change and the sex hormones display the female profile under experimental conditions. One explanation is that the mechanisms involved in body growth are separate from those affected by endocrine disruption from BPA. This is consistent with the idea that the body growth occurs months to years before the gonads change and female sex hormones are produced in natural sex change (Dodd et al., 2019).

During natural sex change, body size, brain, and behavior change first, preceding changes in the gonads and sex hormones (Dodd et al., 2019). Clearly this process did not occur in response to BPA since there was no effect on body growth, and the impact on behavior and brain gene expression was subtler and less clearly followed a female trajectory as compared to the gonads and sex hormones. BPA reduced aggression toward intruder males (Fig. 2) which is opposite to our predictions and is more consistent with male behavior. Females are more aggressive than males (Fig. 2) and are the primary defenders of the territory (Fricke, 1979). Also supporting the idea that the brain changes were not consistent with the gonads are the two pairs of fish where both the dominant and subordinate fish had feminized gonads with vitellogenic oocytes. These fish continued to live in pairs in peace. Under natural conditions, two females of this species would not peacefully cohabitate, but rather they would continually fight over the territory, which may turn deadly for one or both of them. The BPA-feminized fish apparently did not recognize one another as females. In future research, the introduction of a novel female intruder to fish under these conditions may help illuminate the extent to which these fish are behaviorally feminized, and the extent to which they are perceived as female by naïve conspecifics.

The idea that feminization of the brain from BPA was incomplete is also supported from the brain gene expression data (Fig. 3). Previously we reported females displayed higher levels of brain aromatase than males (DeAngelis et al., 2018), and here we observed higher expression of brain aromatase in the BPA-treated group, which is consistent with feminization. Aromatase converts testosterone to estradiol in the brain, and displays positive feedback in response to estradiol signaling (Callard et al., 2001; Menuet et al., 2005). Hence, if BPA mimics the action of estradiol, then an increase in aromatase would be predicted. Also consistent with the female profile, BPA-treated fish displayed reduced expression of *popdc3*. This gene, *popdc3*, was discovered in a recent transcriptomics paper to show decreased levels in the brain during sex change from male to female in a different species of anemonefish, *A. bicinctus* (Casas et al., 2016).

While brain aromatase and *popdc3* show evidence for feminization, we observed no changes for *itr* nor *f13a1*. Previously we observed males display higher *itr* gene expression in their brain than females (DeAngelis et al., 2018). A possible explanation is that *itr* expression is differentially regulated when the males begin to care for the eggs (DeAngelis et al., 2020; DeAngelis et al., 2018; DeAngelis et al., 2017), and since none of the fish were reproducing at the end of the study, no differences in *itr* gene expression were induced. In addition, we observed no effect on *f13a1* expression which was identified in the transcriptomics study of *A. bicinctus* as increasing expression in the brains of males transitioning into females (Casas et al., 2016). The explanation for this difference is unclear. The function of *f13a1* in brain feminization is yet unknown. Taken together, the gene expression data suggest partial, incomplete feminization from BPA had occurred, with increases in aromatase and decreases in *popdc3* but no change in *itr* or *f13a1*. Further analysis of a more comprehensive list of genes including, sex steroid receptors, along with neuroanatomical analysis of the sexual dimorphisms in the brain would help resolve the extent to which BPA impacts brain gene expression.

In addition to BPA, EE2 also influenced the outcomes measured here, but the effects were subtler than BPA. This suggests that some of the observed changes on the gonad and behavior seen for BPA may be due to mechanisms other than through classic estrogen receptors. The only effect was reducing 11-KT in subordinate individuals but not dominant individuals (Fig. 3A), and reducing *popdc3* gene expression in the brain (Fig. 5B). This result is consistent with previous studies in freshwater, brackish, and marine fish species where a reduction in 11-KT was observed in the plasma and/or testes (D'Alvise et al., 2020; Flores-Valverde et al., 2010; Meina et al., 2013). However, many previous studies also found EE2 reduces testicular tissue and increases Vtg, which we did not observe (Fenske et al., 2005; Kidd et al., 2007; Länge et al.,

2001; Palace et al., 2002; Schäfers et al., 2007). This difference could be attributed to dose and/or route of administration, as the majority of the previous studies exposed fish to EE2 through the water, and it is not clear how to compare doses administered through the food versus the water. Several studies have found behavioral effects of EE2 (Bhandari et al., 2015; Colman et al., 2009; Reyhanian et al., 2011; Saaristo et al., 2019; Salierno and Kane, 2009) as well as changes in brain gene expression including increased brain aromatase gene expression in the killifish, *Jenynsia multidentata* (Roggio et al., 2014). A study using the same species, *A. ocellaris*, as studied here, found EE2 increased aggression among juveniles rearing in small groups of 3 individuals (Chen and Hsieh, 2017). However, this result is difficult to compare to ours since aggression in our study was directed toward a male intruder, rather than among individuals within a group. Another difference is that Chen and Hsieh (2017) administered 100 μg/kg feed while this study only administered 0.2 μg/kg feed to be more environmentally-relevant. Taken together, further research exploring the effects of different dosages and routes of administration of EE2 is needed before strong conclusions can be made about the impact of this pollutant on anemonefish reproductive behavior and physiology.

5. Conclusion

In conclusion, to the best of our knowledge, this is the first study to explore the impact of the EDCs, BPA and EE2, on the behavior and reproductive physiology of a sexually labile coral reef fish. We observed substantial feminizing effects of BPA on gonadal histology and plasma 11-KT and Vtg levels. Remarkably, in two instances, a pair of anemonefish treated with BPA both developed vitellogenic oocytes indicating they were both females, a result which is potentially catastrophic for a species which depends on social control of sex determination to produce reproductive pairs. Even more concerning is that the behavior and brains of these fish were not consistent with their gonads, suggesting BPA independently dysregulates the brain and periphery. The fitness consequences may be more severe if BPA disrupts the match between reproductive behavior and gonads since synchrony in the brain-gonad axis is required for reproductive success. In contrast, we only observed a modest effect of EE2, slightly decreasing 11-KT levels but only in subordinate members of the pair, and decreasing *popdc3* expression in the brain. We attribute the more modest effects of EE2 to the low dose chosen for this compound. Taken together, results suggest BPA and to a lesser extent EE2 pollution have another potentially disastrous consequence for the health of our ecosystem, threatening the reproductive system of one of the most iconic coral reef species that exists on the planet, along with potentially other vulnerable sexually labile marine fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2021.105043.

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