



**RMP**  
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## Estrogen receptor in vitro assay linkage studies

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## **Estrogen receptor *in vitro* assay linkage studies**

### **Study Objectives and Applicable RMP Management Question**

Our Phase 1 study indicated that it would be possible to link *in vitro* ER assays to *in vivo* endpoints (Mehinto et al. 2018). These conclusions were based on several experiments that were performed at the University of Florida and SCCWRP. Our conclusion was that in order to see higher order effects in *Menidia*, the dose of estrogens in the *in vivo* assay needed to be above the EC<sub>50</sub> point for the *in vitro* assays. However, the dose response curve for the *in vivo* endpoints was broad starting at 20 ng E2/L and progressing to 20, 200 and 500 ng/L. Our Phase 1 experiments did not include the resolution to reduce uncertainty in estimated quantitative *in vitro* to *in vivo* linkage factors. While the data allowed us to extrapolate those values, it was decided that the experiment should be repeated another time with more intervening estradiol concentrations, to get a better determination.

A second goal of the study was to measure estrogen equivalencies at 6 locations in San Francisco Bay, testing both the water and sediments, to determine the usefulness of the approach. Results from this study will begin to enable managers to determine whether or not additional cleanup is necessary for treated effluents that are disposed into sensitive estuarine environments.

This study addressed the following RMP management question (MQ):

MQ1. Are chemical concentrations in the Estuary at levels of potential concern and are associated impacts likely?

### **Study Objectives**

- (1) To repeat the *in vivo* portion of the linkage study with additional concentrations around the likely EC<sub>50</sub> for *in vivo* responses.
- (2) To test water and sediments from 6 locations in San Francisco Bay for estrogenic equivalencies as a pilot test for this approach.

### **Summary of Study**

#### **A. Repeat of *in vivo* linkage study with additional concentrations of E2.**

For this study, we exposed juvenile *Menidia* (30 days post hatch) to seven nominal concentrations of E2 including 2, 10, 20, 40, 80, 200 and 500 ng/L for 28 days. The exposures were carried out in eight different tanks per condition. At 28 days, fish in half of the tanks were sacrificed to examine them for changes in gene expression and to examine them for sex ratio. Fish in the remaining four tanks per condition were placed in clean water and cultured for an additional 60 days, at which time they were sacrificed to examine them for sex ratio. We used an LC MS/MS method to quantify the actual E2 concentrations in each of the exposure tanks. These measurements indicated that the actual concentrations were below our desired concentrations. We had unusual problems with this experiment and will repeat the entire experiment again.

For gene expression studies, we isolated livers of individual fish after 28 days of exposure. The idea was to examine expression of vitellogenin (Vtg) and choriogenin, two genes that are responsive to estradiol. Unfortunately, we had major problems this year with RNA integrity during isolation and this reduced the number of samples we could examine. We have since determined the source of contamination in our laboratory and have rectified the problem. We were able to identify gene expression for Vtg in a subset of the samples.

In our Phase 1 studies we saw a trend towards feminization at 200 ng E2/L and 100% feminization at 500 ng E2/L. In the present study, sex ratio showed a trend towards feminization at the higher concentrations of E2 but the sex ratio did not reach 100% females. From measured actual concentrations, we know that we did not reach 500 ng/L in the present experiment. Taking the actual concentrations into consideration, data we obtained is similar to data from Phase 1.

#### **B. Test of water and sediment samples collected from San Francisco Bay.**

We extracted estrogenic chemicals from water and sediment samples following established procedures. We employed the GeneBlazer estrogen receptor alpha transactivation assay to measure estrogen equivalencies in these samples.

**Water samples.** There were three water grab samples from each of six locations. There was no detectable E2 equivalence at site LSB02 or SOSL16. The other four sites appeared to have very low levels of E2 equivalence that were below the level of quantitation, and thus could not be reported except in a semi-quantitative fashion. Not all of the grab samples at each site presented with the same equivalency, and the differences may be related to variations in extraction efficiency and ER $\alpha$  transactivation assay, as each of the water samples were treated as separate samples from extraction through assay. The sites that were positive but not quantifiable for E2 equivalency were LSB01, SOSL15, SOSL40, and SB056.

**Sediment samples.** There appeared to be measurable levels of E2 equivalency in sediment samples from some of the sites. There were no detectable levels in sediments from SOSL40, SOSL16 and SB056. Two subsamples, one from LSB02 and one from SOSL15 appeared quite high in their responses. However, the other two subsamples from the same locations had no detectable levels of estrogen equivalency. Since the samples were from the same homogenized composite samples, this suggests that there was inadvertent contamination at the University of Florida of the two highly estrogenic samples and these will need to be re-run in the assay for verification. An alternative explanation suggests that the extraction method was not optimum for polar compounds from sediments, suggesting that a better method should be developed.

The move forward plan is to repeat the exposure experiments this year, at no cost, to get a better range of exposures for the determination of the linkage between *in vitro* and *in vivo* responses. We will repeat the seven concentrations of E2 to get good linkage information between 20 and 200 ng E2/L.

#### **Introduction**

There are many reviewed studies that have documented the occurrence of pharmaceuticals and personal care products in surface waters in our environment at concentrations that have biological activities in aquatic organisms (reviewed in (Cooke et al. 2013). Alterations among higher order endpoints have been observed including gonadal sex differentiation, which causes a disproportionate female sex ratio; changes in secondary sex characteristics; reduction in reproduction and growth; and alterations of courting behavior, among others (Matthiessen and Sumpter 1998; Rodgers-Gray et al. 2001; Adedjei et al. 2012; Baumann et al. 2014). The most studied are chemicals that either mimic the function of 17 $\beta$ -estradiol (E2) or interfere with the biosynthesis or metabolism of the endogenous hormone. As might be anticipated, it is now recognized that the activities of multiple estrogen mimics when present together as mixtures are additive (Brian et al. 2007), making it problematic to monitor effluents using the “one chemical at a time” approach.

While the gold standard is to measure higher order alterations directly *in vivo*, the experiments are costly in both time and money and require the use of hundreds to thousands of fish and furthermore are impractical to run routinely. Based on the recommendations of the National Academy of Sciences (NRC 2007), toxicologists are turning their attention to high throughput *in vitro* assays that are specific for mechanism of action and which are much more cost effective than *in vivo* assays (Dix et al. 2007; Judson et al. 2009; Martin et al. 2009; Conley et al. 2016). However, before these assays can be used in a regulatory framework, it is important to establish linkages from the *in vitro* assays to *in vivo* end points. While some studies have done this with fresh water fish, studies with estuarine fish are lacking. For the fresh water fish, it is now clear that affinity of a chemical for the ligand-binding domain of the estrogen receptor is a good predictor of higher order effects (Miyagawa et al. 2014). A strong linkage between the two for estuarine fish would enable managers to monitor routinely for estrogens in San Francisco Bay with a bioanalytical test. The gap in being able to predict *in vivo* endpoints from *in vitro* assays currently precludes this approach from being widely used.

## **Methods:**

### ***In vivo* responses of Menidia to E2**

Lab reared *Menidia* (25 day post hatch) were purchased from a bioassay supplier (Aquatic Biosystems, Ft. Collins, CO), and acclimated at the University of Florida for 5 days before exposures. Upon arrival and during the experiments, the fish were fed live brine shrimp nauplii. Feeding rates were maintained for each aquarium by pipetting an equal volume of the live feed to each tank. Feeding rates were increased and verified every few days. Water quality (dissolved oxygen, pH, ammonia) was verified weekly or as needed.

### **Exposure of Menidia to E2**

Juvenile *Menidia* (starting at 30 dph) were treated for 28 days with either no chemical or with seven different concentrations of 17 $\beta$  estradiol (0, 2, 10, 20, 40, 80, 200, 500 ng/L). To make the final concentrations, first a stock solution of 17 $\beta$  estradiol was made in ethanol and then triethylene glycol (TEG) was added as a carrier. The stock solution was used to spike artificial sea water (Instant Ocean 15ppt salt) to the final concentrations stated above. TEG final concentration in all dilutions and vehicle control was at 50  $\mu$ l/ liter of test water. This method was a modification of the standardized test guidelines for early life testing (US-EPA 1995; Denslow et al. 2016). The test solutions were added to 50-gal (200 L) fiberglass source tanks (one for each of the test solutions) and these were used to supply eight 2.5-gal (10 liter) glass aquariums. A pump and ChemFluor<sup>TM</sup> tubing were used to deliver the water to each tank. Each aquarium was aerated with a glass pipette and the intensity of aeration was adjusted as the fish grew. Each aquarium comprised fifteen 30-day post hatch and tanks were maintained at 23  $\pm$ 1 $^{\circ}$ C and constant photoperiod (18L:6D). Water quality was intermittently checked to ensure lack of ammonia or nitrite. Water was refreshed at 65% (4.0 L) of the solution daily. Fresh solutions were made every 9 days. A 1-liter water sample was taken from the source tank 24 h after it was made for actual concentration verification and was stored at 4  $^{\circ}$ C with azide until measured.

At the end of the exposure period, fish in 4 of the 8 tanks per condition were sacrificed and evaluated for gene expression and sex ratio by histology. The second group of tanks for each condition was then transferred to clean water (again made up with Instant Ocean, 15 ppt) and fish were cultured for an additional 60 days to determine whether the effects observed in the constant exposure for 28 days were reversible.

### **Water Chemistry**

One liter of test water from each exposure solution including nominal concentrations of 2, 10, 20, 40, 80, 200 and 500 ng/L from three preparation dates were removed from the source tanks 24 h after they were made and azide was added and the water was stored at 4 °C until tested. Deuterated E2 (d5-E2) standard was added to each volume of water and the test water was concentrated on a C-18 solid phase extraction column (Waters) and eluted with MEOH. The eluted samples looked dirty so the sample was then extracted with tert-butyl methyl ether (MTBE), following the usual method of extracting hormones from aqueous media (Matyash et al., 2008). The samples were dried down and then reconstituted in MEOH. To quantify E2 in the solutions, calibration solutions were prepared using various E2 concentrations (from 0.5 to 600 pg/μL). The samples were then injected into a C18 column (Zorbax Eclipse-C18, 2.1 x 100 mm, 3.5 μm (Phenomenex)) and analyzed by mass spectrometry on a QTRAP 6500, using binary pumps with mobile phase A: 0.2 mM NH<sub>4</sub>F in water and mobile phase B: MEOH. Estradiol was measured in negative phase using MRM and monitoring two ion transitions, 271.0 → 145 for quantitation, and 271.0 → 143 for qualification. Data were collected and processed using Analyst and Multiquant software, respectively. The concentrations of E2 in each exposure solution were interpolated from calibration curves. The calibration graph was described by the equation  $y=ax+b$  with  $R^2 \geq 0.99$ .

### **Histology and sex determination**

Each carcass collected at time of experiment takedown was individually processed to verify sexual differentiation. After removing the liver for RNA, the remainder of the carcass was preserved in 10% buffered formalin for histological verification of sexual differentiation. The tail, post genital pore was removed from the preserved fish using a scalpel blade. The anterior portion of each fish was removed posterior to the pectoral fins (chest level). The resulting “stumps” were processed in formalin and put into paraffin so that the tail end pointed upward. When sectioning the stumps, the gonad was visible in slices anterior to the genital pore. Sex was verified and checked by two persons for each individual.

### **Gene Expression**

After 28 days of exposure, four fish from each aquarium were euthanized with anesthetic (MS-222, 150 ppm), individually weighed, and photographed. The livers were removed, flash frozen in liquid nitrogen, and stored at -80 °C for RNA quantification. Liver RNA was purified using STAT-60 (Tel-test) and DNase treated using a PerfeCTa kit (Quanta). cDNA from 300 ng total RNA was synthesized using Script TM kit (Quanta). Quantity and quality of RNA was measured using Nanodrop. cDNA was diluted 1:20 and mixed with SYBR Green (SsoAdvance™, Biorad) containing the forward and reverse primers (10 μM) for Vtg. Gene expression quantification was made using the CFX Connect Real-Time System (Biorad).

### **Evaluation of San Francisco Bay water and sediment samples**

Grab samples of water and sediment were collected at various locations in the Bay by SFEI personnel. Samples sent to the University of Florida for evaluation are listed in Table 1.

Table 1. Collection sites for water and sediments in San Francisco Bay

SiteID	Region	Target Latitude	Target Longitude
SB056	South Bay	37.56052	-122.131
LSB01	Lower South Bay	37.49878	-122.082
LSB02	Lower South Bay	37.46282	-122.105
SOSL15	Extreme Lower South Bay	37.45178	-122.062
SOSL16	Extreme Lower South Bay	37.45758	-122.04
SOSL40	Extreme Lower South Bay	37.46212	-122.022

### Water Extraction

One liter of water was collected from each site (in triplicate) by SFEI personnel and shipped to the University of Florida on ice. Upon receiving the samples, the bactericide, sodium azide (1 g/L) was added to each container and water samples were stored at 4° C until processed. Water was collected from 6 different sites in triplicate (n= 6 sites x 3 reps = 18 total).

Debris and algae were removed from each container by vacuum filtration using a glass 1.6 µm fiber filter (Whatman, GF/C). One liter of each filtered sample was concentrated using solid phase extraction on a mixed resin cartridge (HLB, 500mg, Oasis) which captures both hydrophobic and hydrophilic contaminants. Each column was preconditioned with 10 ml of acetone/hexane (1:1), methanol and water. Binding compounds were eluted using methanol and then acetone/hexane (10 ml each). The eluant was dried down using nitrogen in a warm bath (30° C) and brought up to 1.0 ml final volume with DMSO.

### Sediments

Sediment samples were collected by SFEI personnel and shipped to the University of Florida on ice. Samples were collected from 6 sites and were homogenized composites and separated into triplicate replicates (6 sites x 3 reps = 18 samples). Each sample was treated as an individual sample. The vials containing grey clay-like sediment and seawater were mixed with a stainless-steel spatula into a thick slurry. The slurry was quickly poured into aluminum drying dishes and allowed to dry at room temperature (2-3 days). Some samples contained sand which was heavy and this was avoided during decanting. Three grams of the dried "clay" sediment was weighed into glass vials, mixed with 10 ml of acetone/hexane (1:1), and incubated in a 32 °C bath for 1 hour. After cooling, the sample was sonicated for 4 min (8 sec-on, 2 sec-off, at 50% µ) in a container of wet ice to prevent heating, using a method that we developed and validated for organochlorine substances (Dang et al., 2016). The mixture was centrifuged for 5 min at 150 x g and the organic supernatant transferred to a new glass vial using a glass Pasteur pipet. A second extraction and sonication treatment were repeated on each sample and the extracts were pooled. The organic extract was evaporated in a warm water bath (30 °C) using nitrogen gas. The extracts were reconstituted with 1.0 ml methanol, vortexed, and split into 2 glass HPLC vials. One vial was evaporated and then reconstituted with 0.5 ml DMSO for receptor assay analysis. We plan to repeat the extraction process by adding deuterated E2 (d5-E2) standard to measure the recovery of estrogenic chemicals by LC MS/MS.

### GeneBlazer ERa transactivation assay

#### Preparation of samples and standards

The method we used was similar to our previously published method (Escher et al. 2014, Mehinto et al. 2015, Mehinto et al. 2018).

Water and sediment extracts in DMSO were prepared for ER Receptor Transactivation Assay the day of running the analysis. Sample extracts were diluted 1:200, 1:400, 1:800, and 1:1600 in

DMEM buffer (phenol free-DMEM, carbon stripped FBS, Pyruvate, non-essential amino acids, penicillin/streptomycin, 5% DMSO) in a cell culture hood. A standard curve of 17 $\beta$ -estradiol was generated in DMEM buffer at the following final concentrations:  $1 \times 10^{-8}$ ,  $3.3 \times 10^{-9}$ ,  $1.1 \times 10^{-9}$ ,  $3.7 \times 10^{-10}$ ,  $1.2 \times 10^{-10}$ ,  $1.4 \times 10^{-11}$ ,  $1.4 \times 10^{-12}$ ,  $4.5 \times 10^{-12}$ ,  $1.5 \times 10^{-12}$  M. All samples and standards contained the same amount of DMSO (0.5%).

### **ER Receptor Assay**

A vial of division arrested cells (GeneBlazer DA ER $\alpha$ , Invitrogen) were removed from a liquid nitrogen Dewar, and quickly thawed in a 37 °C bath for 3-4 min. The outside of the vial was sterilized with 70% ethanol and then transferred to a cell culture hood. All of the following steps were performed aseptically in a cell culture hood.

The cells were washed in 10 ml of DMEM buffer (No DMSO) and centrifuged at 200 x g for 5 min (25 °C). The wash buffer was removed with a transfer pipette leaving a cell pellet and 0.5 ml buffer behind. Ten ml of fresh DMEM buffer was added, vortexed, and a subsample removed for cell density determination using a hemocytometer and Trypan Blue stain (0.4%). The cells were further diluted with DMEM buffer to a final concentration of 550,000 cells/ml (50,000 cells/well), as directed by the manufacturer.

Ninety microliters of the cell/DMEM mix was added to a black wall/clear bottom 96 well tissue culture plate (Costar 3603) to all the wells except the “No cell” controls (containing DMEM buffer without cells) using an electronic multichannel pipette. The solution was mixed 3X before adding to each well to ensure homogeneity of cell density. Ten microliters of diluted samples and 17 $\beta$ -Estradiol standards were added to designated wells in triplicate. In addition, two other controls, 1). No DMSO and 2). A standard curve Blank were prepared by adding 10  $\mu$ l of DMEM buffer (no DMSO) and 10  $\mu$ l of DMEM buffer with 0.5% DMSO (no 17 $\beta$ -estradiol), respectively. Each plate was covered and put into a sterile 37 °C incubator for 24 hrs. All samples except the No-DMSO control contain 0.5% DMSO in DMEM buffer.

The following day, 30  $\mu$ l of a mix of fluorescent substrate, Live Blazer FRET and cell viability reagent (PrestoBlue, Invitrogen) were added to all the wells. Each plate was covered with an aluminum foil seal and incubated for 2 hrs at room temperature in the dark. Using a fluorescent plate reader (Biotek Synergy H1) and analysis program (Gen5 2.0), each plate was scanned at 3 different excitation/emission wavelengths: 1) Blue (excitation 409 nm, emission 460 nm), 2) Green (excitation 409 nm, emission 530 nm), and 3) Cytotoxicity (excitation 560 nm, emission 590 nm). The output data was analyzed by subtracting the average from the “no cell” control data from all of the wells for both the blue and green output data. Then a ratio of the blue/green data was determined. The average of the “blank” or DMSO control well (Buffer, cells, & DMSO) was subtracted from the blue/green ratio to give the final normalized data. Standard curve data was plotted, and samples displayed as bar graphs at each dilution.

## **Results**

### **A. *In vivo* responses of Menidia to E2**

#### **1. Actual concentrations of E2 in test tanks**

The actual concentrations of E2 in each of the test conditions was measured by LC MS/MS using deuterated E2 as an internal spike. The measured concentrations were much lower than the nominal values. Control waters were devoid of E2 as expected.



Table 2. Measured concentrations of E2 in the tanks

Nominal Concentrations	Average Measured Amount
C	BDL
2 ng/L	1.9 ng/L
10 ng/L	4.5 ng/L
20 ng/L	12 ng/L
40 ng/L	20 ng/L
80 ng/L	25 ng/L
200 ng/L	130 ng/L
500 ng/L	268 ng/L

## 2. Gene expression studies

Menidia (30 days post hatch, dph) were treated for 28 days with varying concentrations of E2 from 2 to 250 ng/L. After 28 days of exposure, Menidia were sacrificed and livers were extracted for total RNA. Vitellogenin (Vtg) mRNA expression was measured as a function of E2 treatment (Fig. 1). There is a gradual increase in Vtg with increasing concentrations, but actual E2 concentrations had to be higher than the EC<sub>50</sub> of the *in vitro* assay to produce significant levels of Vtg mRNA (marked by the red arrow in Fig. 1). This experiment fits well with the experiments previously performed.

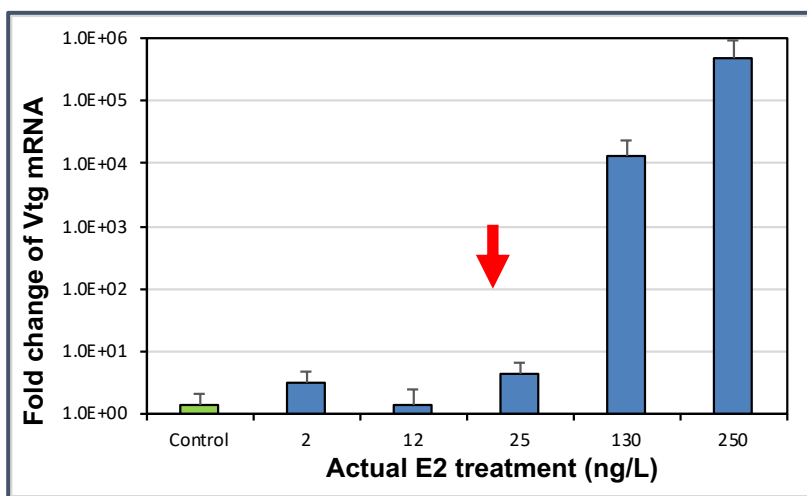


Figure 1. Vtg expression in Menidia after 28 days of treatment with increasing concentrations of E2. The red arrow points to the EC<sub>50</sub> concentrations obtained with *in vitro* estrogen receptor transactivation assays.

## 3. Sex Ratio

We processed 3 to 4 fish per tank for histology to determine sex. Fig. 2 shows the sex ratio obtained using nominal concentrations of E2. The sample number per tank was low so the total fish per condition were pooled and sex ratio was determined as a percent of total. As before, higher estrogen concentrations appear to bias the sex ratio towards female. However, even at the highest concentration tested, only 67% of the total were female. This was an unexpected result as we expected the highest concentration to be 500 ng E2/L. Our actual concentrations were much lower than we anticipated. An examination of the temperature logs showed that water temperatures were between 22 °C and 23 °C

throughout the experiment, which is a neutral temperature giving equal numbers of males and females. *Menidia* have temperature-dependent sex determination.

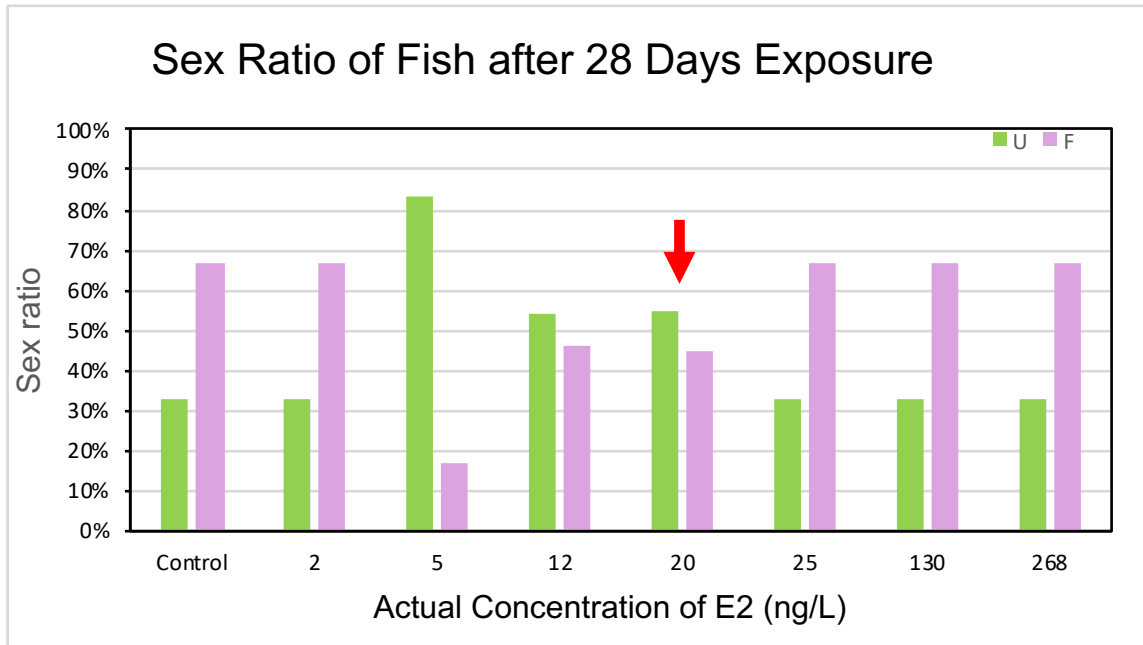


Figure 2. Sex ratio of juvenile *Menidia* cultured in different E2 concentrations for 28 days. Green bars represent sexually undifferentiated fish and pink bars represent females. The red arrow indicates the EC<sub>50</sub> for E2 in the *in vitro* assay. N = 11-13 fish per group with most groups containing 12 fish.

We also processed the 3-month-old fish to look for sex ratio after an additional 2 months of culture in clean water. Mature male testis contained clusters of mature sperm and these were clearly distinguishable from female ovaries with many oocytes at different stages of maturation (Fig. 3).

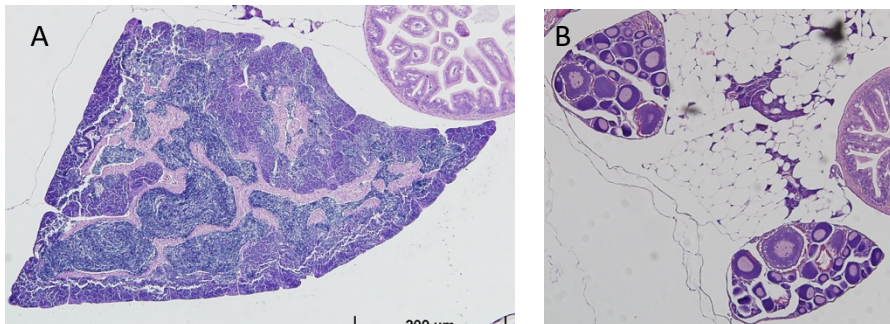


Figure 3. Histology of control fish that were 3 months old after culturing for two months in clean water. A) Males and B) Females. Gonads were excised from fish and fixed for histopathology examination.

Gonads of females that were previously treated with the higher levels of E2 showed a high degree of atresia, suggesting that there were some long lasting effects in the ovaries. Fig. 4 illustrates some examples.

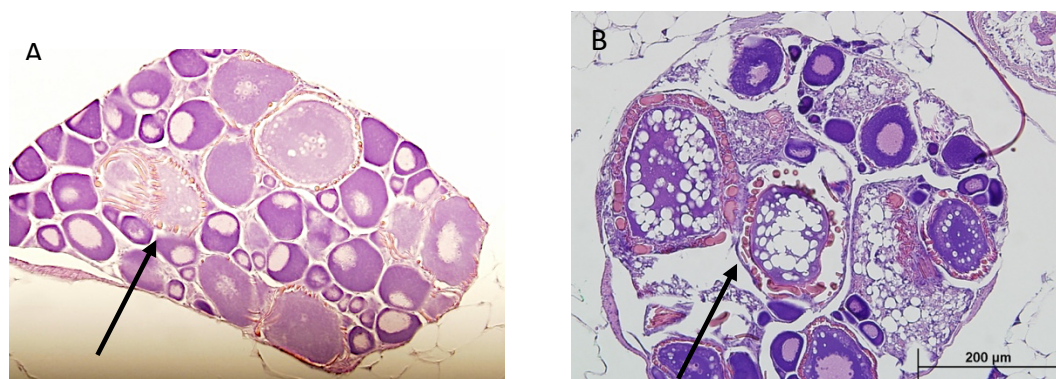


Figure 4. Examples of atresia seen in ovaries of fish treated with higher levels of E2 for one month before transfer to clean water for 2 months. Black arrows point to atretic oocytes.

There were no significant differences in sex ratio after two months of further culturing in clean water (Fig. 5). Only one fish in the control group still had undifferentiated gonads. The trends towards a skewed sex ratio observed after 28 days in the higher concentrations of E2 were totally gone after two months in clean water. It is possible that *Menidia* are still very plastic in sex determination at these early stages in life.

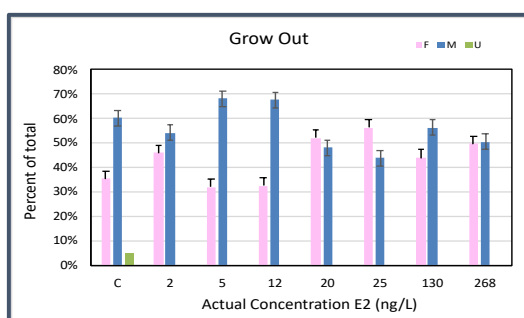


Figure 5. Sex ratio of *Menidia* cultured for 28 days in different E2 concentrations and then placed in clean water for an additional 60 days. Green bar in the control represents sexually undifferentiated fish, blue bars are mature males and pink bars represent females. N = 20-27 fish per group. Error bars represent standard errors, as each tank in the group of 4 were evaluated separately for sex differentiation.

## B. Evaluation of San Francisco Bay water and sediment samples

### 1. Water grab samples

We tested the extracts of the water samples for estrogen equivalency using the Gene Blazer ER alpha transactivation assay. The standard curve for this assay met all of the criteria for the assay (Mehinto et al., 2015) and Figure 6.

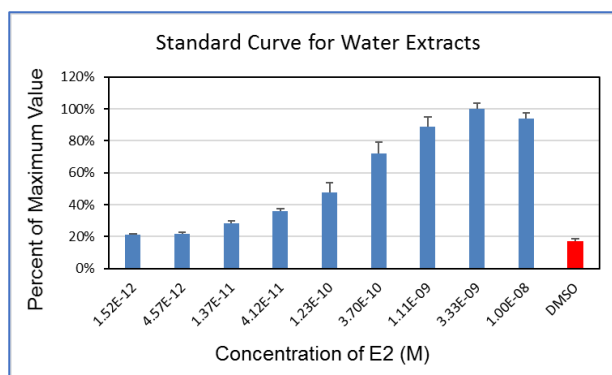


Fig. 6. Standard curve used to evaluate water E2 equivalencies. Blue bars are the individual standards used and the red bar is the DMSO control.

The standard curve was evaluated using Sigma Plot and  $EC_{50}$  values, Max and Min were calculated using a model for sigmoidal dose response curves. Most of the water samples contained EEQs which were quantifiable but below the level of quantitation (Figure 7). We concentrated one liter of water to 1 ml but then performed dilutions in the assay of 1 → 200, 1 → 400, 1 → 800 and 1 → 1,600. Thus, the most concentrated sample represents a concentration of 5X. The recommendation for future water testing would be to concentrate the original sample 4,000-fold and if estrogens are present they would be measurable in the two top dilutions.

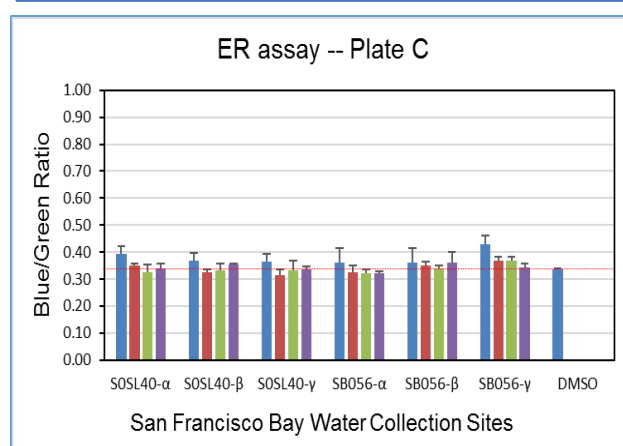
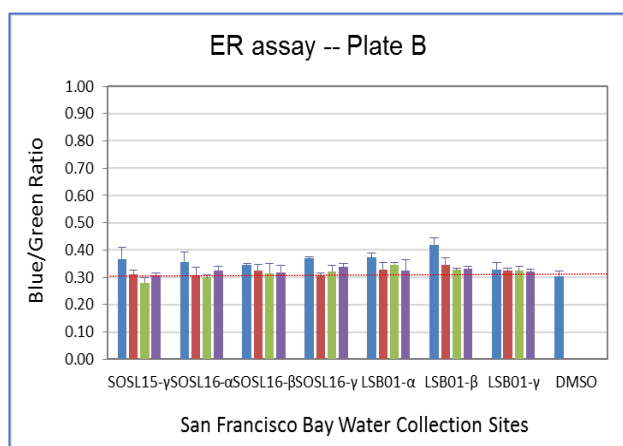
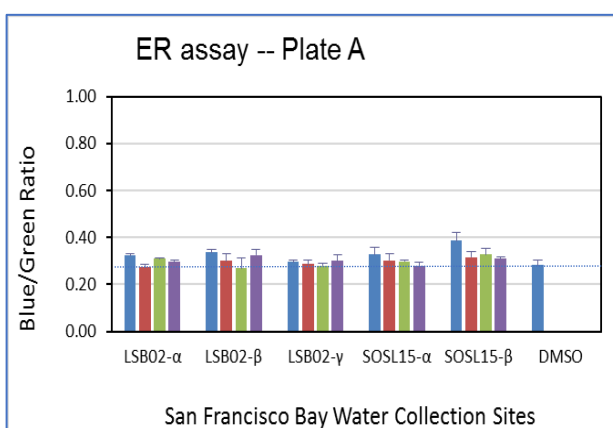
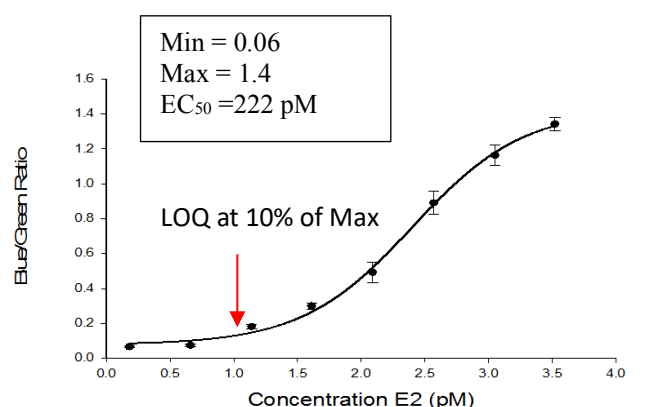


Figure 7. GeneBlazer ERa assay results for water extracts. The dotted line represents the effect in the assay of DMSO. The Y axis in the sample graphs was set to a maximum of 1.0 to match the sediment figures. The 50% effect is about 0.8 on the Blue/Green ratio scale for the standard curve.

## 2. Sediment samples

The standard curve for sediment samples also passed the criteria for acceptance (Mehinto et al, 2015) (Fig. 8).

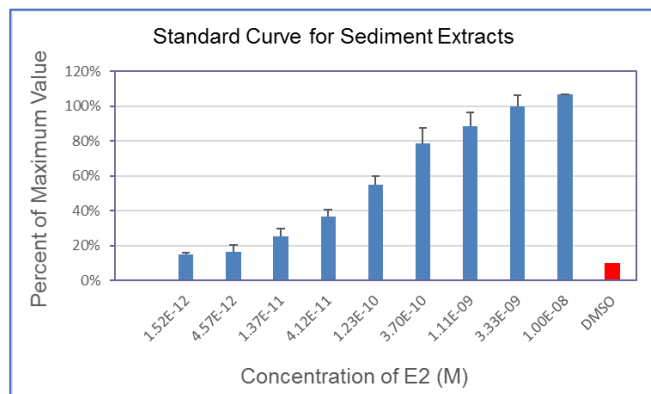


Fig. 8. Standard curve used to evaluate water E2 equivalencies. Blue bars are the individual standards used and the red bar is the DMSO control.

Several of the sediment extracts showed the presence of estrogenic contaminants. To determine the amount of estrogenic activity in each sample, the samples were compared to the standard curve to determine the amount and the value was then adjusted for dilution and concentration factors and then transformed to ng/g sediment, dry weight. A good diagnostic for positive results in the assay is the decrease in effect (ladder) seen with dilution (Fig. 9).

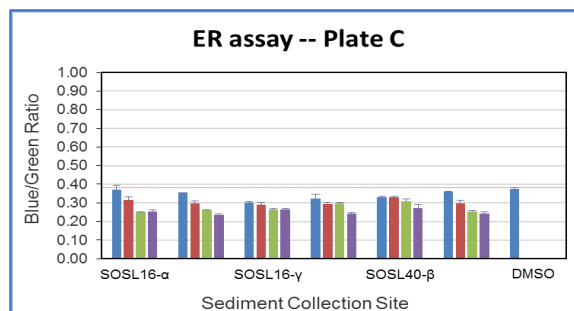
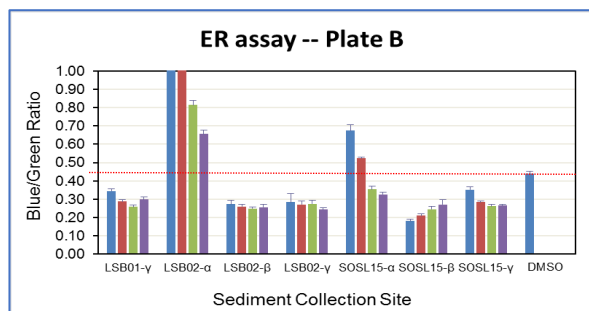
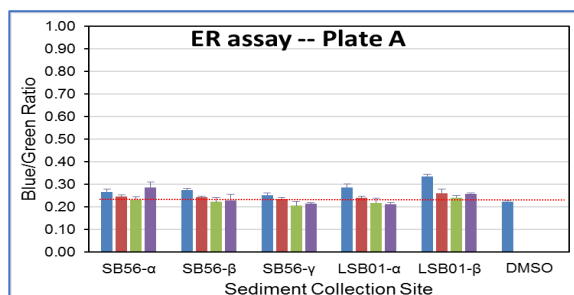
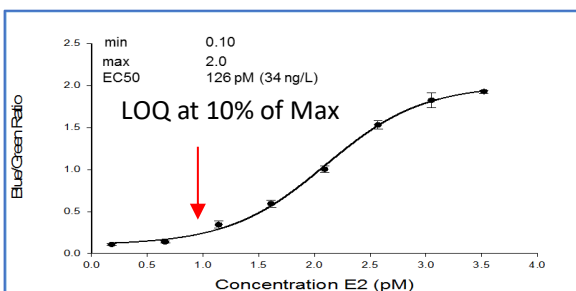


Figure 9. GeneBlazer ERa assay results for sediment extracts. The dotted line represents the effect in the assay of DMSO. Calculations of min, max and EC<sub>50</sub> were calculated from data plotted as percent of maximum response. Note, we think there may have been laboratory contamination of plate B. The Y axis in the sample graphs was set to a maximum of 1.0 which is up to the 50% value on the Blue/Green ratio levels for the standard curve.

After thorough analysis of the data it was determined that the two spikes seen with sediment samples could have been from contamination introduced into the samples during their analysis at the University of Florida, since only one of three replicate samples in each case were positive. However, a second opinion was offered that suggested that the extraction method used for sediments was not optimum for polar estrogenic contaminants and since each of the replicate samples was treated independently, it was possible that in two of the samples, one from each set, fluffy sediment was brought over into the extract which was then further extracted by DMSO when the dried extract was resolubilized.

Table 3 contains the estimated levels of estrogenic equivalence in water and sediments. In this assay the limit of detection (LOD) for water samples was 40 pg E2/L and the LOQ was 150 pg E2/L. For sediments the LOD was 400 pg/g sediment (dry weight) and the LOQ was 3.7 ng/g sediment (dry weight). Values for estrogenicity that are between the LOD and LOQ are listed as below the LOD 0.15 ng/L for water and 3.7 ng/g sediment (dry weight). Sample values that were below the level of detection are listed as BDL.

**Table 3. Estimated EEqs**

San Francisco Bay Water Sites			San Francisco Bay Sediment Sites	
Site Description	Water Collection Site	Estrogen equivalency <b>ng/L</b>	Sediment Collection Site	Estrogen equivalency <b>ng/g</b>
South Bay	SB056-α	BDL	SB056-α	BDL
	SB056-β	BDL	SB056-β	BDL
	SB056-γ	0.89	SB056-γ	BDL
Lower South Bay	LSB01-α	BDL	LSB01-α	≤3.7
	LSB01-β	0.70	LSB01-β	≤3.7
	LSB01-γ	BDL	LSB01-γ	BDL
Lower South Bay	LSB02-α	BDL	LSB02-α	*****
	LSB02-β	BDL	LSB02-β	BDL
	LSB02-γ	BDL	LSB02-γ	BDL
Extreme Lower South Bay	SOSL15-α	BDL	SOSL15-α	*****
	SOSL15-β	0.69	SOSL15-β	BDL
	SOSL15-γ	≤0.15	SOSL15-γ	BDL
Extreme Lower South Bay	SOSL16-α	BDL	SOSL16-α	BDL
	SOSL16-β	BDL	SOSL16-β	BDL
	SOSL16-γ	BDL	SOSL16-γ	BDL
Extreme Lower South Bay	SOSL40-α	≤0.15	SOSL40-α	BDL
	SOSL40-β	BDL	SOSL40-β	BDL
	SOSL40-γ	BDL	SOSL40-γ	BDL

BDL, below limit of detection (LOD). LOQ is 3.7 ng/g sediment. Values listed as below 3.7 ng/g are values that were between the LOD and the LOQ.

\*\*\*\*\* show sediment aliquots that appear to have been contaminated in the laboratory.

## Discussion:

### ***In vitro to in vivo* extrapolation.**

The experiment was performed using the same approach as was used for Phase 1 of this study, except that we doubled the number of exposure tanks from 4 to 8 and at 28 days sacrificed all of the fish in 4 of the tanks. The fish in the remaining 4 tanks were placed in clean water for an additional 2 months, at which time they too were sacrificed for histology. We had significant issues with the experiment that clouded the results. The actual concentrations used were much lower than the nominal. We also suffered severe contamination issues during RNA extraction, which rendered some of the RNA samples unusable for quantification of Vtg and Chg gene expression. Nevertheless, because we used more fish, we had some in each group that we could evaluate for Vtg.

In this experiment, it appears that the high nominal concentrations of estradiol produced a trend towards female-induced sex ratio. However, this trend was much less than was seen in Phase 1 (Denslow et al., 2016). These results are explained due to the much lower actual concentrations than the nominal values. The entire experiment will be repeated this coming year in order to validate the results.

### **Evaluation of San Francisco Bay water and sediment samples**

Grab samples of water and sediments were collected from around the Bay by SFEI staff and mailed to the University of Florida for evaluation.

Water samples contained very low levels of estrogenic equivalency, i.e.  $\leq 0.15$  ng E2/L (the limit of quantitation (LOQ)). Concentrations of estrogens in this range are unlikely to cause biological effects in fish. The calculated EC<sub>50</sub> point for the *in vitro* assay is around 20 ng/L, and in Phase 1 of this project (Denslow et al., 2016), we showed that one needed to be above the EC<sub>50</sub> point to show any subchronic *in vivo* effects in fish. Thus, concentrations in the water are about 100 to 200-fold lower than required to see *in vivo* effects. The maximum BEQ reported for an urbanized river system in Belgium was 0.94 ng E2/L (Vandermarken et al. 2018). PNECs for estrogenicity in aquatic systems have been reported between 0.1 to 1 ng E2/L (Wang et al. 2011; Jarosova et al. 2014; Vandermarken et al. 2018). The large volume of water in the Bay, probably effectively dilutes estrogenic contaminants. In addition, most of the estrogenic chemicals found as contaminants are hydrophobic, making them more likely to adsorb to particulates and end up in the sediments.

Sediment samples in the Bay contained considerably more estrogen equivalence. Two sites showed high values including LSB02 (lower South Bay) and SOSL15 (Extreme Lower South Bay) but only one out of three samples at each location was high. The three sediment samples sent were actually composites that were split into three different aliquots, suggesting the very high concentrations found in the assay were likely due to contamination during preparation of the sample for analysis. Each aliquot was treated as a separate sample, extracted individually and then tested in the assay. However, a second opinion from a chemist was that it was possible that the extraction method for sediments was suboptimal for polar compounds and it was possible that some fluffy sediment material from two of the sediments (the two high samples) actually got carried over into the extract and then was further solubilized by DMSO when the extracts were dissolved. Thus, it is not possible at this time to determine if there is estrogen mimics in the sediments at these two locations and the experiment should be repeated.

Two out of three samples from LSBO1 had quantifiable amounts of estrogen equivalency in the Gene Blazer assay. These are in the range seen at other sites that are thought to be contaminated.

We did not investigate the presence of antiestrogens at these sites, but values in the sediment bioanalytical assay that are below the DMSO control may be due to such substances. In the Phase 1 experiments, we showed that low concentrations of bifenthrin and galaxolide were antiestrogenic in the *in vitro* assay (Denslow et al., 2016), so it is possible that such chemicals are also associated with the sediments.

It is useful to compare these results to other locations in CA. In the Russian River watershed, a freshwater system where only seasonal discharge of treated municipal wastewater effluent is permitted, only one site showed elevated estrogen equivalency (0.09 ng E2 equivalency/g sediment) (Maruya et al., 2018). That value is significantly lower than the sediment concentrations found at LSB02 and SOSL15. In another study, samples of contaminated sediment along the Palos Verdes Shelf and from San Diego were analyzed for estrogen equivalencies (Crago et al., 2016). The Palos Verdes site is known to be contaminated by DDTs and PCBs. The San Diego control site had 0.3 ng E2 equivalency/g sediment, while the Palos Verdes site had 1.3 ng E2 equivalency/g sediment (Crago et al. 2016). Other studies have reported ranges of BEQs of 0.20 to 3.3 ng E2/g dw (Boehler et al. 2017); 0.20 ng E2/g maximum (Vandermarken et al. 2018); and 0.06 to 1.2 ng E2/g for riverine sediments in China (Wang et al. 2014). Thus, the BEQ estimated for LSB02- $\alpha$ , if confirmed, represented an unusually high level that warrants further investigation.

### Conclusions:

1. The results of this study matched the previous results from Phase A that showed that *in vivo* effects in *Menidia* could be expected at aggregate estrogenic concentrations (BEQs) that are higher than the EC<sub>50</sub> for the *in vitro* assay.
2. Because of all the issues that were faced during the experiment, we will repeat the 28-day exposures to better determine the effects of dose on sex differentiation in *Menidia*.
3. There are significant concentrations of estradiol equivalence in sediments at locations in lower South Bay. It would be interesting to determine what chemicals are contributing to this response. Recent publications suggest that sediment may be a sink for endocrine active compounds (Sangster et al. 2014; Zhang et al. 2015).
4. The positive results for estrogens in bay sediments should be followed up to determine the compounds responsible, and the suggestion of antiestrogen activity in some sediments should also be followed up.
5. Water levels of estradiol equivalence are low (below quantitation levels) when they are found. At these concentrations, the levels are not likely to pose a threat to marine fish.
6. One possible factor that could influence the results was the sample collection by SFEI and transport across the country on ice, followed by azide addition to the samples to kill any possible bacterial activity. It might be helpful to add the azide in California before shipping to make sure that bacterial activity that could chew up estrogens is stopped before shipment.



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