Linkage of *In Vitro* Assay Results With *In Vivo* End Points
Final Report – Phase 1
June 2, 2014

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The goal of this project is to establish quantitative linkages between the *in vitro* receptor-based assays and traditional endpoints of adversity in an estuarine fish model, the common silverside (*Menidia beryllina*), which is an established EPA model for estuarine toxicity. To work out the method for this type of linkage analysis, we decided to concentrate on chemicals that are found in wastewaters that behave as weak estrogens. We are in the midst of our analyses, which we should complete in the next 3-4 months for work promised for year 1. So far we have had substantial success with our approach and a few problems that we are in the process of solving. This report is organized around the milestones set up in our proposal.

Proposed Deliverables and Time Line

Deliverable	**Completion Date**
Task 1 Convene focus group and develop actionable plan	**CSD + 1 month**
Task 2 Develop molecular biomarkers for *Menidia*	**CSD + 4 months**
Task 3 Laboratory tests: Early life stage exposures and *in vitro* bioassays	**CSD + 9 months**
Task 4 Field-collected sample exposures	**CSD + 18 months**
Task 5 Chemical analysis of CECs	**CSD + 21 months**
Task 6 Reporting	**Mid-term (Year 1): CSD + 12 months** **Final: CSD + 24 months**

Task 1 Convene focus group and develop actionable plan

Researchers from the Denslow Lab at the University of Florida and from SCCWRP met at the start of the project to plan how the project would be approached. In addition we have had several conference calls to coordinate experimental approaches and we have emailed each other with specific protocols to get input from all sides. We decided to use *Menidia beryllina* as the test species as this fish is reported to be sensitive to contaminants, inhabits estuarine locations in CA and the San Francisco Bay area and is used by EPA as a test organism **(Figure 1)** (Chapman et al. 1995). Drs. Connon and Susanne Brander are also using this fish as a model for the San Francisco Bay area and we agreed to collaborate with them on aspects of this project. They have agreed to make available to us gene sequences they have obtained from a transcriptomics project. This task was completed.

Figure 1. *Menidia beryllina* as a test organism

Task 2. Develop molecular biomarkers for *Menidia*

For this task we agreed to develop quantitative PCR (Q-PCR) assays to evaluate at least 10 different genes for their expression *in vivo*. Five of the genes were for evaluation in early life stage (ELS) and five for evaluation of critical genes in juvenile fish. These gene expression measurements are important to set up the linkage of the *in vitro* assays to responses *in vivo*. Detailed descriptions of the methods used are in Appendix A.

While we promised only ten assays for genes by Q-PCR, we have actually prepared 13 assays. We validated 7 assays that had been previously developed by Susanne Brander for *Menidia*, as part of the Ph.D. dissertation (Brander 2011). These assays were for Vitellogenin (Vtg), estrogen receptor alpha (ERα), estrogen receptor beta a (ERβa), androgen receptor (AR), Choriogenin L (Chg), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cytochrome P450 A1 (Cyp1A) **(Appendix B, Supplementary Figure 1)**.

We also developed and validated assays for an additional 6 genes: insulin growth factor 1 (IGF-1); steroidogenic acute regulatory protein (StAR); growth hormone receptor (GHR); brain aromatase (cyp19b); anti-Mullerian hormone (amh); and doublesex and mab-3 related transcription factor 1 (DMRT1), and two more housekeeping genes ribosomal protein L8 (rpL8) and 18S ribosomal RNA (18S rRNA) **(Appendix B, Supplementary Figure 1)**. As expected, Vtg and ERα were expressed predominantly in the liver of females. We were hopeful that DMRT1 would be related to sex and be expressed exclusively in males and serve as a male biomarker, but we found that it was expressed in the gonads of both males and females. Expression levels were higher in males than in females, but it would be difficult to use this gene as a biomarker of genetic sex since it is expressed in both sexes. DMRT1 serves as a biomarker of sex determination in medaka, but not in many other fish species (Guo et al. 2005; Johnsen and Andersen 2012; Hattori et al. 2013).

We optimized the QPCR assays for each of the genes (Appendix B, Supplementary Figure 2). The amplicons were specific for the genes of interest, only one product was seen in melting experiments and the efficiency of amplification was between 95-105%. All RNA samples passed quality control standards with high A260/A280 ratios and good RNA integrity numbers. All total RNA samples were treated with DNase to remove traces of contaminating DNA. The assays were deemed of good quality to assess relative changes in gene expression with exposures.

Dr. Richard Connon (UC Davis) shared sequences for *Menidia beryllina* that he obtained from a transcriptome project funded by another source. We will complete RNA-Seq experiments in collaboration with Drs. Connon and Susanne Brander in phase 2 of this project. The scope of this collaboration has been focused to include exposures of early life stages to 17β-estradiol (E2), nonylphenol (NP), bifenthrin (BF) and vehicle control.

The original deliverables for this task have been completed.

Task 3. Laboratory tests: Early life stage and juvenile exposures and *in vitro* bioassays

There were three parts for this task; (1) development of the *in vitro* assays to determine EC50's for each of the estrogens; and performance with (2) *in vivo* assays with early life stage fish; and (3) *in vivo* assays with juveniles undergoing gonadal tissue differentiation. All of these deliverables have been completed.

A. *In vitro* Bioassays (UF)

We used InVitrogen GeneBlazer assays to derive estrogen equivalence relationships among the test substances: E2, E1, 4-NP, and BPA. We also tested bifenthrin and galaxolide. All chemicals were purchased from Sigma Chemical Co, with the exception of galaxolide, which was custom synthesized by Dr. John Rimoldi (University of Mississippi), a colleague of Dr. Dan Schlenk. Consequently all work with galaxolide will be in collaboration with Drs. Rimoldi and Schlenk.

The InVitrogen assays are cell-based estrogen receptor (ER) transactivation assays. They depend on a human cell line that normally does not express ERs. To make this cell line, the ligand-binding domain of human ER alpha was attached to the GAL4 DNA binding domain of a yeast factor and this construct was stably transfected into the human cell line. In addition, a reporter gene that codes for the beta lactamase protein under the control of 5 estrogen response elements was also stably transfected into the same cell line. When estrogen or an estrogen mimic come into the cells, they bind to the ligand-binding domain of the ER, alter the conformation of the receptor allowing it to bind to the promoter region (control region) of the reporter gene. This causes the beta lactamase mRNA to be transcribed, and then translated into protein. To confirm that this protein has been expressed and is active, the detection system uses a substrate that the beta lactamase can specifically cleave, thereby causing a signal to be emitted. This is a very sensitive assay for estrogen activation of its receptor.

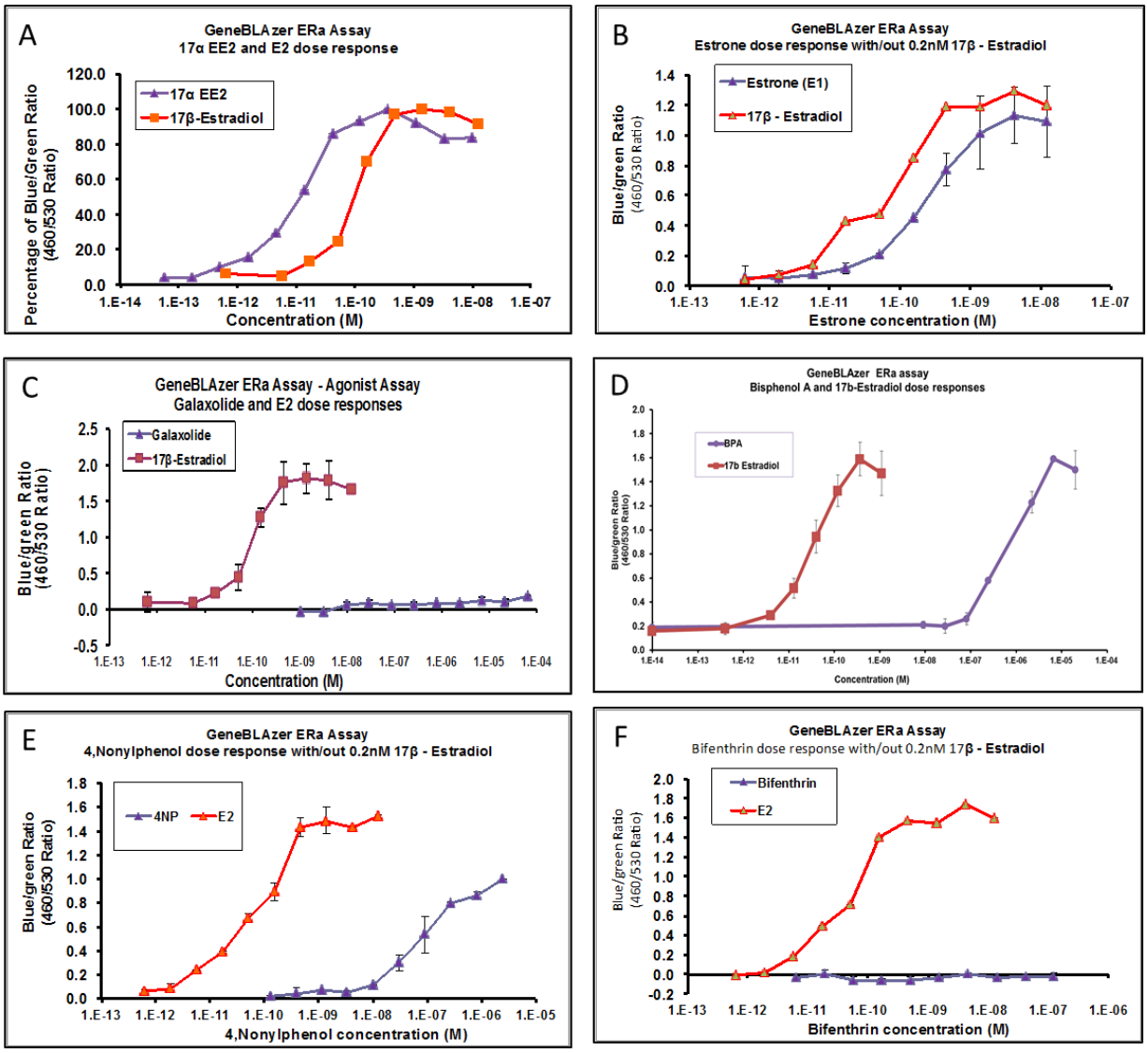


Figure 2: Dose response of InVitrogen ERα GripTite Division Arrested cells to strong and weak ER agonists. Cells were plated in triplicate in 96-well clear bottom plates and dosed with strong and weak ER agonists for 18 h in the presence of 0.5% DMSO, loaded with LiveBLAzer™-FRET B/G substrate (2 h), and fluorescence emission was recorded at 460 and 530 nm using a BioTek Synergy H1 Hybrid Reader.

All InVitrogen assays were performed in agonist and antagonist modes for all the chemicals. For the agonist mode we used at least 9 different concentrations of the test chemical, at half log intervals and a negative control. A positive control (E2) was performed, as well, in order to compare its response with the weaker estrogens. We saw positive signals for 17α–ethinylestradiol (EE2), E2, estrone (E1), 4-nonylhenol (4NP) and bisphenol A (BPA). There was no signal in agonist mode for bifenthrin (BF) and an extremely weak signal for galaxolide (GAL) (**Figure 2).** All specific methods for this assay are described in detail in **Appendix A.** We calculated EC50's for EE2, E2, E1, 4NP and BPA **(Table 1).**

Table 1. EC50 values for tested chemical

Chemical	EC50 (M)
17a-ethinyl estradiol (EE2)	1.11E-11
17b-estradiol (E2)	3.96E-11
Estrone (E1)	2.52E-10
4-Nonylphenol (4NP)	8.57E-8
Bisphenol A (BPA)	4.7E-7

We also performed the assay in antagonist mode in the presence of 0.2 nM E2, a concentration that should produce about 80% of the maximum signal (**Figure 3**). When we added the test chemicals to these assays, we saw a small amount of antagonism for E1 and NP at the lower concentrations, a phenomenon that has been described before (Kim et al. 2002). These chemicals bind to the ligand-binding domain of the receptor but at very low concentrations they do not transactivate the receptor. But, because the ligands are present, E2 is less efficient at binding and thus there is a little bit of competition.

In the case of galaxolide and bifenthrin, the antagonism is very pronounced at the lower concentrations. Bifenthrin appears to be an antagonist also at the higher concentrations. The molecular mechanisms by which bifenthrin acts on fish is still debated in the literature (Brander et al. 2012; Riar et al. 2013). It is possible that bifenthrin is metabolized to a more active metabolite such as to 4-hydroxy bifenthrin and that this activates estrogen receptors. In our hands this metabolite does not activate the human ERα in the Invitrogen Assays, but apparently this metabolite is quite potent on fish ERβ's (Brander, personal communication). Another possibility is that bifenthrin or a metabolite may act at a different point on the HPG axis, resulting in overall estrogenic activity *in vivo* (Riar et al. 2013).

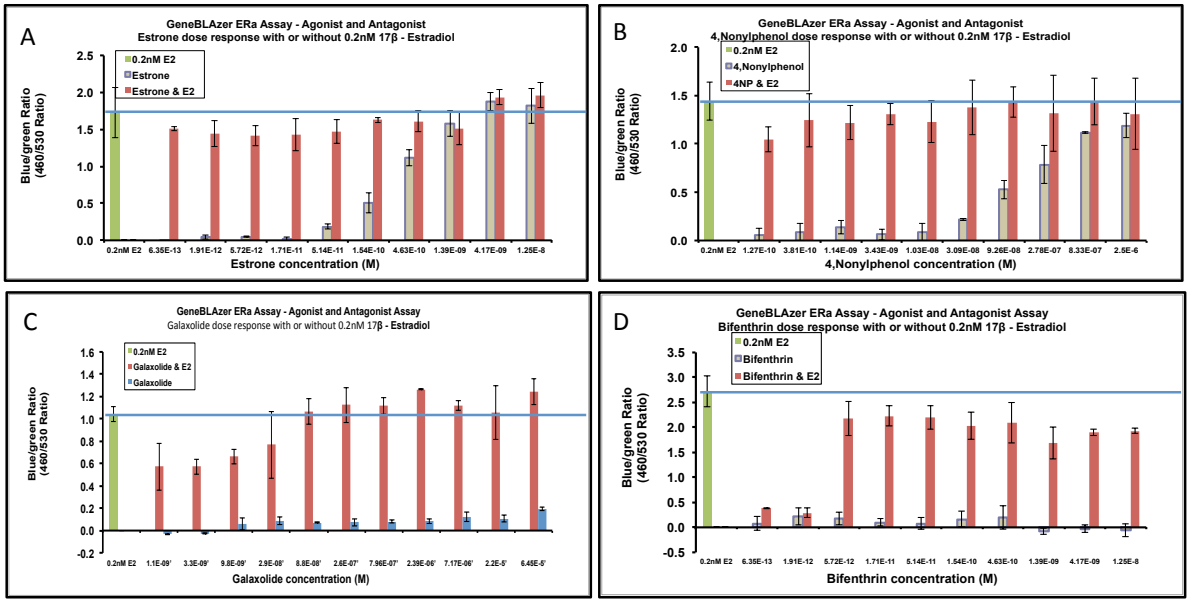


Figure 3. Antagonist mode for the InVitrogen ERα assay. Cells were plated in triplicate in 96-well clear bottom plates and dosed a mixture of E2 (0.2 nM E2 final concentration in wells) with respective concentrations of those chemicals for 18 h in the presence of 0.5% DMSO, loaded with LiveBLAzer™ FRET B/G substrate (2 h), and fluorescence emission was recorded at 460

and 530 nm using a BioTek Synergy H1 Hybrid Reader. The Blue/Green ratio of 0.2 nM E2 alone is given for the comparison.

B. *In vivo* early life stage assays (SCCWRP)

Early life stage (ELS) assays were conducted using 10-day-old *Menidia beryllina* larvae following the EPA protocol. The laboratory set up is shown below for the exposures in beakers (**Figure 4**). The specific methods that were employed for the assay are found in Appendix A. Table 2 contains the nominal concentrations of chemicals that were used.

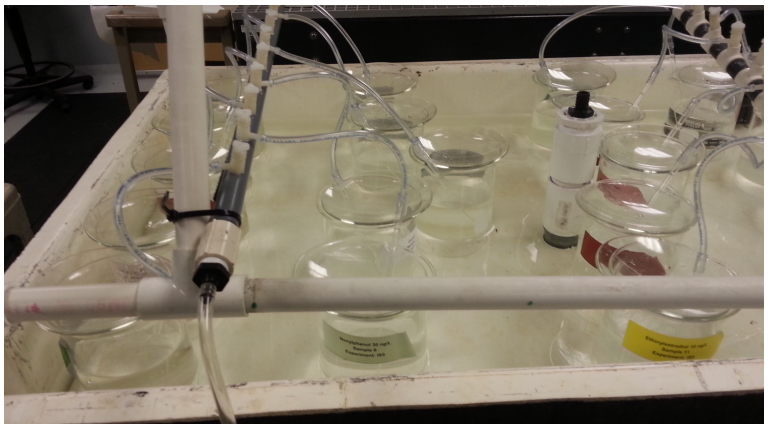


Figure 4. Experimental set up for testing early life stages of *Menidia beryllina* at SCCWRP.

The first experiment was an exposure of 10 day old *Menidia* larvae to E2 following the EPA protocol. A 7-day exposure was conducted with seawater (control), 0.02% methanol (solvent control), 10, 30, 100 and 300 ng E2/L and 10 ng EE2/L as positive control. Exposure concentrations for E2 were selected based on observations from exposure of juveniles conducted at UF. The endpoints of the ELS assay were growth (measured as dry weight) and survival. On day 0, a subsample of fish was used to calculate the average dry weight per larvae. On day 7, the surviving larvae were preserved in liquid nitrogen for subsequent Q-PCR analyses. Fish subsamples were used to estimate the mean dry weight per larvae for each treatment.

Experimental results: Exposure to E2 had no significant effects on survival or growth (**Table 2** and **Figure 5**). Similar exposure experiments were performed with E1, 4NP, BPA and GAL using the concentrations described in **Table 3**.

Table 2: Summary data for 7-day exposure of *Menidia* larvae to various concentrations of E2

Treatment	Seawater control	Methanol control	10 ng/L 17β-estradiol	30 ng/L 17β-estradiol	100 ng/L 17β-estradiol	300 ng/L 17β-estradiol	10 ng/L ethinylestradiol
Survival (%)	87.8	92.7	89.7	89.8	90.1	87.0	93.6
Sig diff from control (one-way ANOVA)	No	No	No	No	No	No	No
Mean dry wt/larvae (mg) ±SD	0.64 ± 0.17	0.68 ± 0.17	0.62 ± 0.21	0.65 ± 0.18	0.67 ± 0.10	0.80 ± 0.07	0.77 ± 0.06
Sig diff from control (one-way ANOVA)	No	No	No	No	No	No	No
Mean temp. (°C)	25.1	25.1	25.0	25.1	25.1	25.1	25.0
Mean salinity (ppt)	15.2	15.1	15.2	15.1	15.1	15.1	15.1
Mean DO (mg/L)	7.19	7.00	6.91	7.22	6.90	7.10	6.93
Average pH	8.29	8.18	8.11	8.19	8.20	8.20	8.17

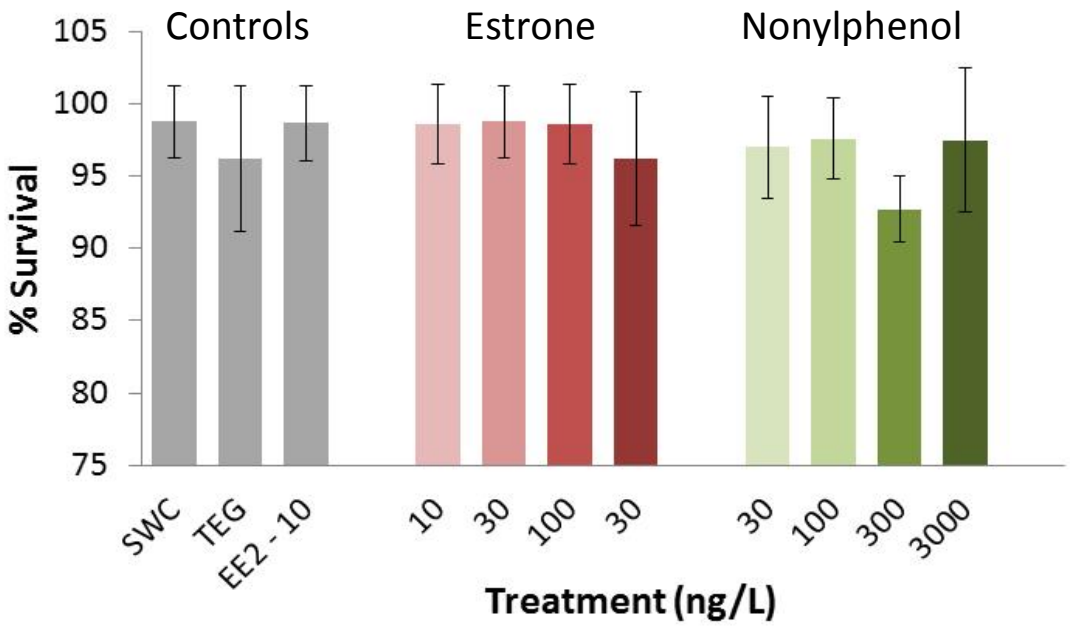


Figure 5. Effect of estrone and nonylphenol exposures on survival of *Menidia* larvae exposed for seven days.

Table 3: *Menidia beryllina* were exposed to the following treatments for seven days.

Treatment	**Nominal concentration**
Seawater control (artificial seawater)	
Vehicle control (TEG)	50 µL/L
EE2 (positive control)	10 ng/L
E1	10, 30, 100, 300 ng/L
4NP	30, 100, 300, 3,000 ng/L
BPA	300, 1,000, 3,000, 30,000 ng/L
Galaxolide	300, 1,000, 3,000, 30,000 ng/L

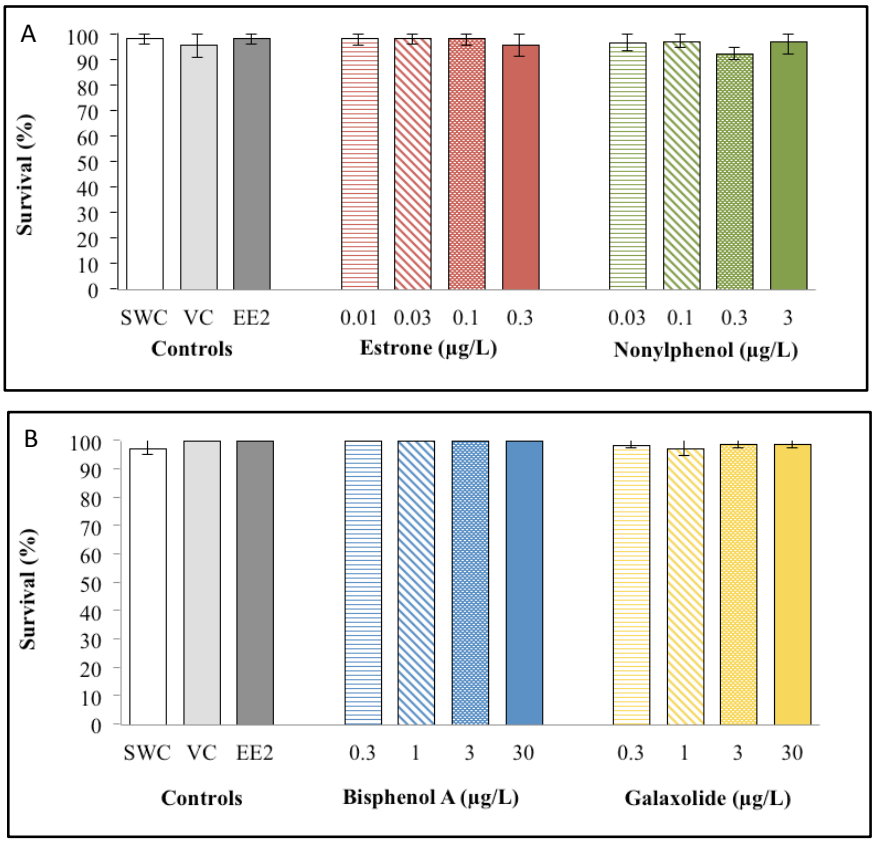


Figure 6: Mean survival (%) for *Menidia beryllina* larvae 7-day exposure to test chemicals. Error bars represent standard deviation (20 fish/replicate, 4 replicates/treatment) and (*) denotes a significant difference compared to the seawater control (SWC). A) Experiment 1- *Menidia* larvae were exposed to seawater only (SWC), a vehicle control (0.005% TEG; VC), a positive control (EE2), and four concentrations of E1 and 4NP. B) Experiment 2- *Menidia* larvae were exposed to SWC, VC, EE2 and four concentrations of BPA and GAL.

Exposure of *Menidia* larvae to test concentrations of E1, 4NP, BPA or galaxolide had no significant effect on survival. In both sets of experiments, the mean survival was greater than 95% for all treatments (**Figure 6)**. It was observed that the growth rate was highly variable among larvae. No significant differences were found in the mean dry weight of exposed larvae compared to larvae in the seawater and/or vehicle controls (**Figure 7**).

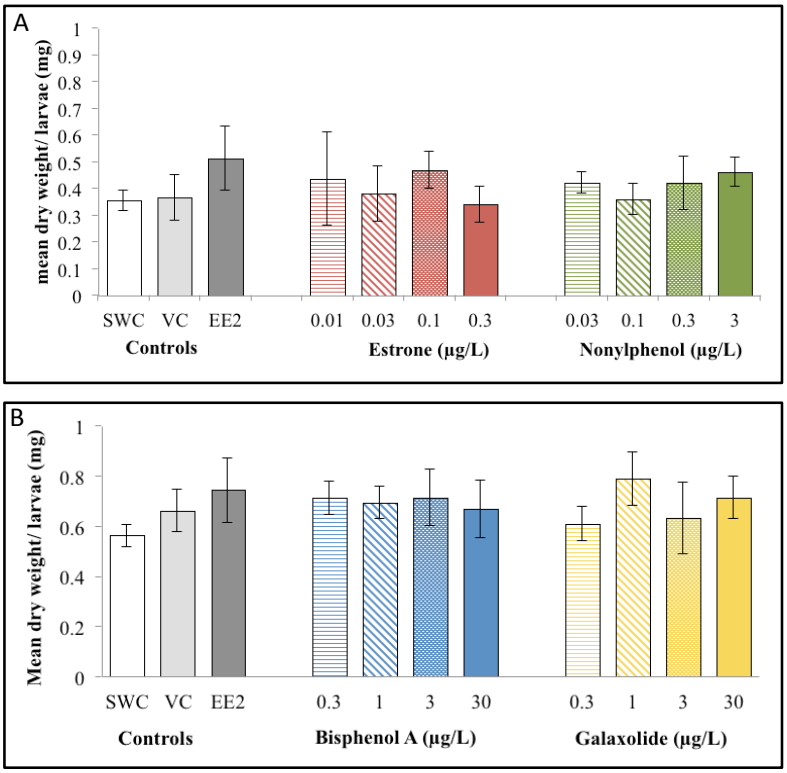


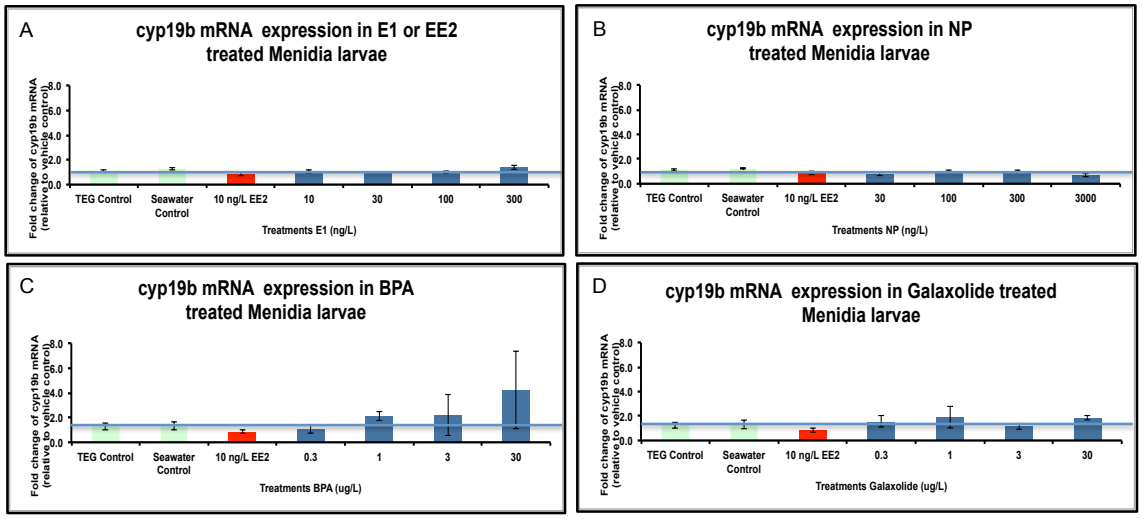
Figure 7: Effects of A) E1, 4NP, B) BPA and galaxolide on the mean dry weight of *Menidia* larvae after 7 days of exposure. There were no differences in growth among the chemical exposed larvae and those in seawater (SWC), vehicle control (VC) and EE2. Error bars represent standard deviation (5 larvae/replicate, 4 replicates/treatment) and (*) denotes a significant difference compared to SWC.

Gene expression studies for *Menidia* larvae

We performed Q-PCR for 5 genes that were expected to relate to effects from estrogen exposure and to higher order apical endpoints. Two of the genes were associated with expected responses to E2, cyp19b (brain aromatase) and StAR (steroidogenic acute regulatory protein) **(Figure 8**). Cyp19b has been shown to have estrogen response elements in its promoter in several teleost species (Callard et al. 2001; Chang et al. 2005; Le Page et al. 2008). StAR is a protein that controls the rate-limiting step for the initiation of steroidogenesis as it shuttles cholesterol into mitochondria for transformation into sex steroids (Chen et al. 2014).

BPA was the only test chemical to show a dose-dependent increase in Cyp19b and an increase in expression of StAR mRNA in larvae. This appeared to be a non-monotic effect with a larger increase at 1 ug/L than at higher concentrations. BPA effects on StAR are known from mammalian systems and fish (Zhou et al. 2008; Liu et al. 2012). GAL showed a trend toward increases in StAR in a dose-responsive manner. It is clear from the literature that GAL can affect steroidogenesis by altering expression of several of the genes in the pathway, but not StAR (Li et al. 2013). However, this study was performed with H295R cells and they may not reflect the *in vivo* actions of GAL for early life stage fish.

Cyp 19 B



StAR

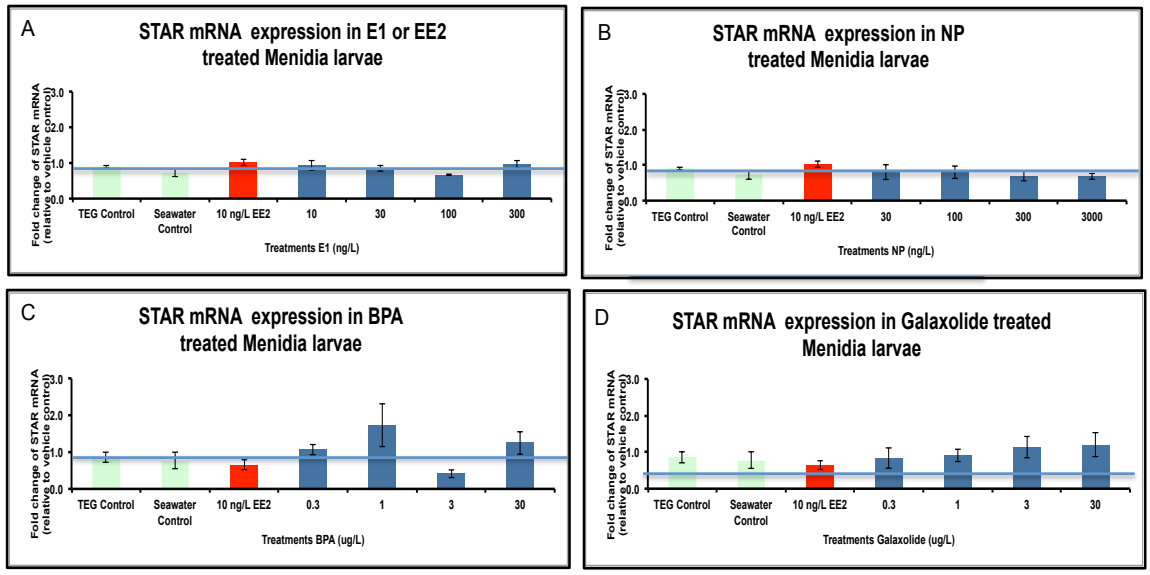
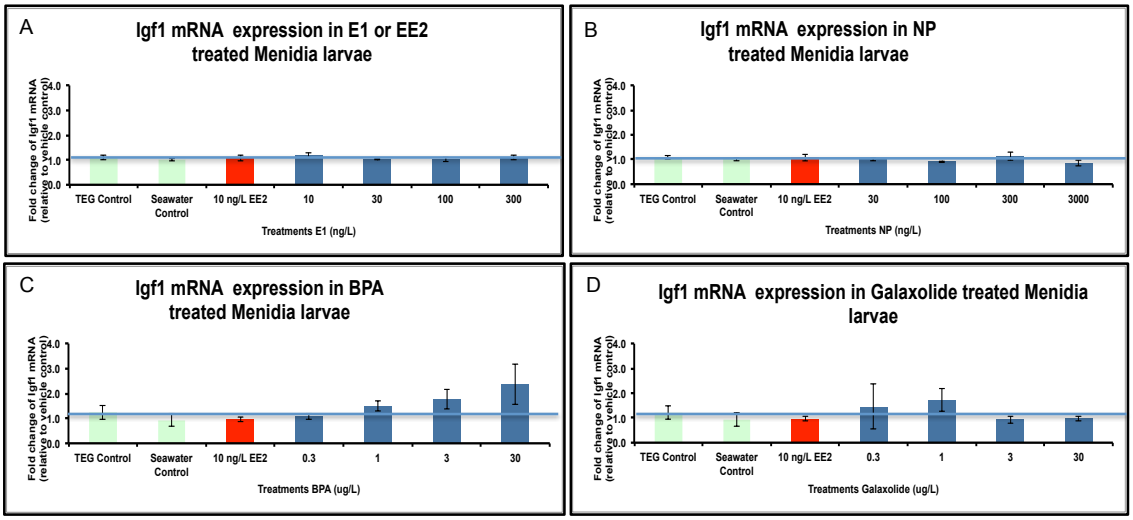


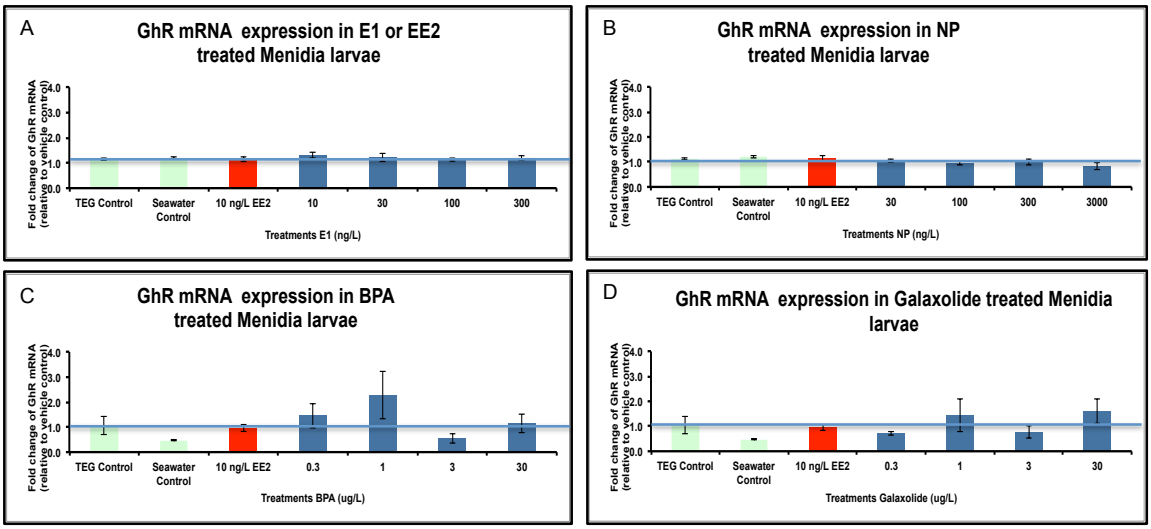
Figure 8. QPCR analysis of Cyp19b and StAR in *Menidia* larvae.

Other genes chosen to evaluate embryos were related to growth and sex. These included IgF1 (insulin like growth hormone 1); ghr (growth hormone receptor) (Filby et al. 2006; Beckman 2011; Fuentes et al. 2013) and antimullerian hormone (amh) (Schulz et al. 2007; Hattori et al. 2013) (**Figure 9).**

Igf1


A
Igf1 mRNA expression in E1 or EE2 treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
10
30
100
300
Treatments E1 (ng/L)
B
Igf1 mRNA expression in NP treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
30
100
300
3000
Treatments NP (ng/L)
C
Igf1 mRNA expression in BPA treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments BPA (ug/L)
D
Igf1 mRNA expression in Galaxolide treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments Galaxolide (ug/L)

GhR


A
GhR mRNA expression in E1 or EE2 treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
10
30
100
300
Treatments E1 (ng/L)
B
GhR mRNA expression in NP treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
30
100
300
3000
Treatments NP (ng/L)
C
GhR mRNA expression in BPA treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments BPA (ug/L)
D
GhR mRNA expression in Galaxolide treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments Galaxolide (ug/L)

Amh

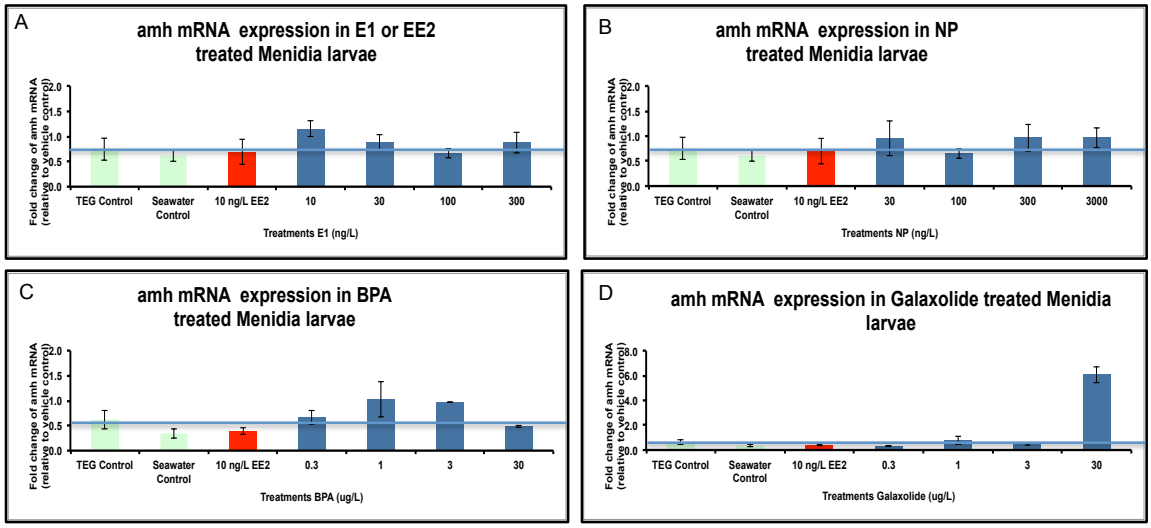


Figure 9. QPCR analyses for Igf1, GHR and Amh for *Menidia* larvae. The Y axis for amh in response to GAL is different than for the other contaminants.

E1 and NP showed effects only on amh, with a higher increase in mRNA steady state levels at the lower concentration of 10 ng/L E1 and 30 ng/L NP. But, these effects were not large and not significant. BPA on the other hand showed a dose-dependent response on IgF1 and non-monotonic effects on ghr and amh, with 1 ug/L showing maximal response. GAL showed a non-monotic dose response for igf1 (maximal response at 0.3 and 1 ug/L (concentrations that were antiestrogenic in the Invitrogen assay). The response for GAL was variable for ghr but showed a significant increase in amh at 30 ug/L.

We also tested DMRT1, hoping that it would be able to distinguish genetic males from genetic females. While initial tests looked promising, we found that DMRT1 was expressed more in adult male gonads than in female gonads, as reported for other fish by others (Guo et al. 2005). However, because it was expressed in both sexes, it did not work as a good biomarker of genetic sex. Nevertheless we tested to see if its expression could be altered by estrogens in *Menidia* embryos (**Figure 10**). The main effect we saw was a reduction in expression by the strongest estrogen EE2 at a concentration of 10 ng/L.

DMRT1

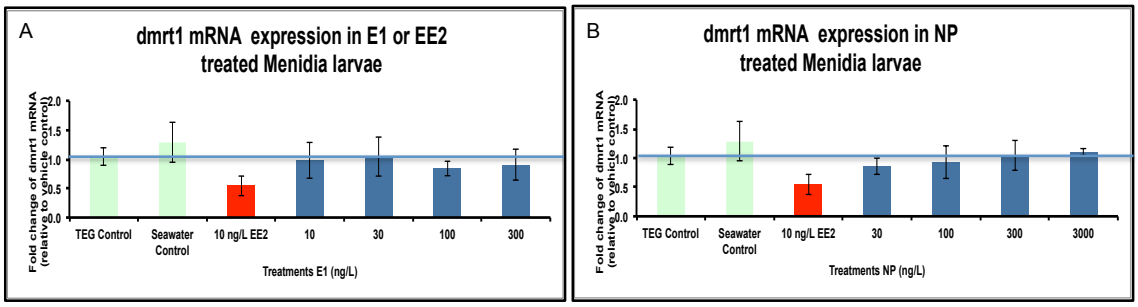


Figure 10. QPCR analysis of dmrt1 in *Menidia* embryos in response to EE2, E1 and NP.

C. Juvenile assays (UF) – Exposure procedures for juvenile fish were developed at the University of Florida. A full description of this assay is found in the **Appendix A**. The initial plan was to expose juvenile fish for 10 days over the period of gonadal differentiation, which we had expected to occur between day 50 and 60 in *Menidia* but the livers were too small to dissect out. In addition, sex determination is temperature dependent (absent exogenous contaminants) and occurs after a fish has reached 20-35 mm in length (Conover and Fleisher 1986). Our first pilot test was with E2 at 4 concentrations half log apart (3, 10, 30 and 100 ng/L) (**Figure 11**). We observed a high degree of variability in fish size at day 10, preventing us from separating livers from all fish. Thus, we used whole fish for Q-PCR analysis.

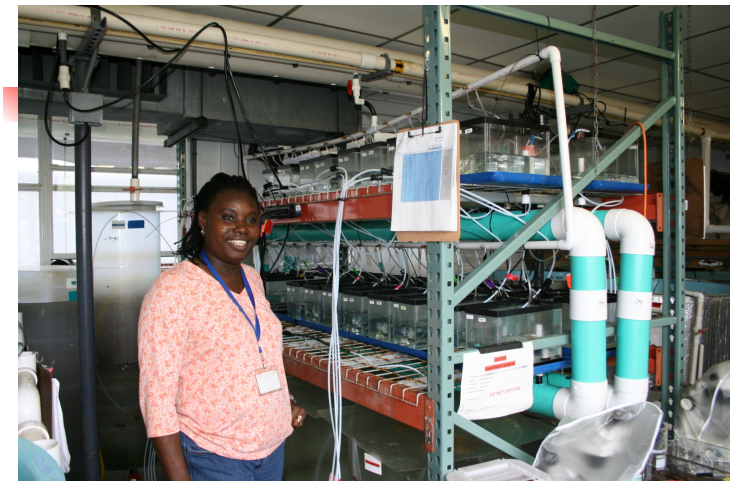


Figure. 11. Experimental set up for juvenile *Menidia beryllina* at the University of Florida.

In the initial experiment, we were able to see Vtg increase in whole fish but only at the 100 ng/L concentration (**Figure 12)**. Interestingly, when we conducted Q-PCR for Chg we observed elevated Chg levels in whole fish at much lower concentrations of E2, starting at 3, 30 and 100 ng/L compared to vehicle control. This was reported previously by Brander (Brander 2011), suggesting that chg is more sensitive than Vtg.

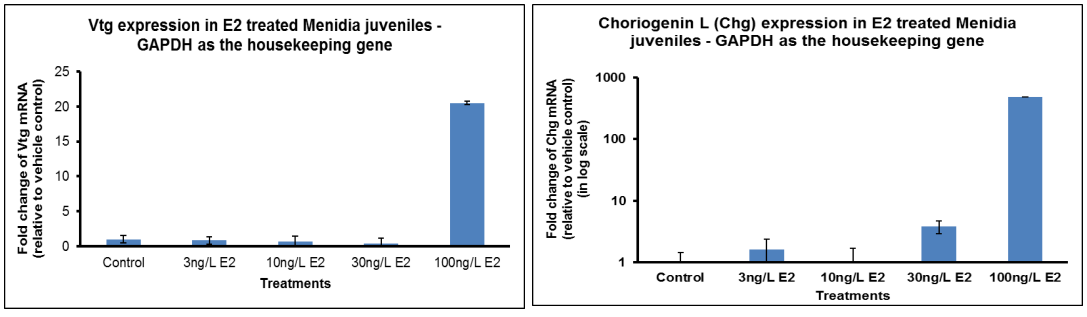


Figure 12. Figure 12. Treatment of *Menidia* juveniles with different levels of E2 resulted in elevated levels of vitellogenin (vtg) and Choriogenin L (Chg). *Menidia* juveniles (~ 50 days old) were exposed to E2 (3, 10, 30 and 100ng/L) for 10 days (50% daily static renewal) for 10 days. Total RNA was extracted from whole-body homogenates and, following reverse transcription, Vtg and Chg were PCR-amplified from cDNA template using Q-PCR. GAPDH was

used as an internal control. Fold change data are mean ± standard deviation relative to vehicle control.

The experiment was repeated, this time allowing fish to be exposed for 21 days to 71 days post hatch (dph). By the end of these longer exposures, fish size was indeed larger, allowing for excision of livers from all fish as well as identification of differentiated gonads. We used this experimental paradigm for the remaining test chemicals (E1, NP, BPA and GAL, Table 3). We used 10 ng/L EE2 as a positive control. Endpoints measured were length and condition factor, histopathology of the gonad and gene expression changes for 5 genes: ERα, ERβ, AR, Chg and Vtg.

Table 3. Nominal and actual concentrations for juvenile *Menidia* 21- to 71-day exposures to estrogenic test chemicals. Actual concentrations were determined by ELISAs specific to each chemical, as described in Appendix A.

Exp I

17β-estradiol ng/L	
nominal	actual
0	0
10	11
30	29
100	95
300	206

Exp II

Nonylphenol ng/L	
nominal	actual
0	tbd
30	tbd
100	tbd
300	tbd
3000	tbd
10,000	tbd

Ethinyl estradiol ng/L	
nominal	actual
10	2.1

Exp III

Estrone ng/L	
nominal	actual
0	0
10	7
30	40
100	131
300	303

Exp IV

Bisphenol A ng/L	
nominal	actual
0	0
300	383
1000	2077
3000	4531
30000	31674

[a]The ELISA assay for NP was back ordered, so we have not been able to confirm the actual concentrations used.

Length and condition factor:
We saw no effects on growth or condition factor; again this is probably due to the great variation in size of the fry at the beginning of the experiment. Data for this endpoint is found in Appendix A, Supplemental Figure 3.

Sex differentiation of the gonads determined by histology:
Fish were fixed in formalin and then trimmed under a dissecting microscope to generate mid-sections of gonadal tissue. This was done by removing the tail about 1 mm post cloaca and the upper part of the body posterior to the heart. The fist mid-section was then embedded in paraffin to the tail pointed up and sliced sagittally at several levels to ensure capture of gonadal tissue. Details of these methods are in the appendix. The sex of each fish was verified by visual inspection using a compound microscope at 20 and 40X. Figure 13 shows typical histological sections at 40X and 100X.

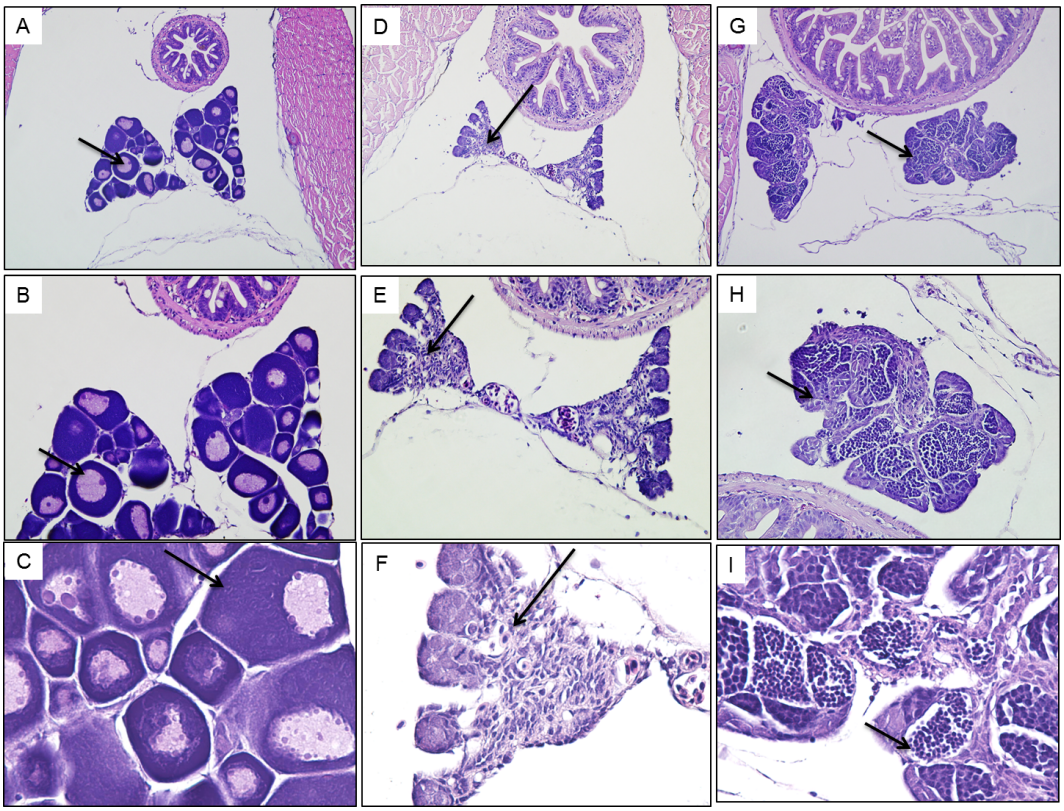
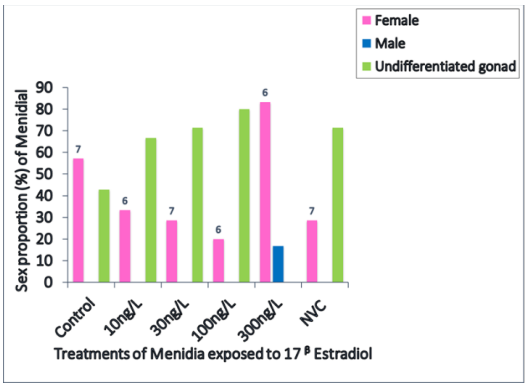
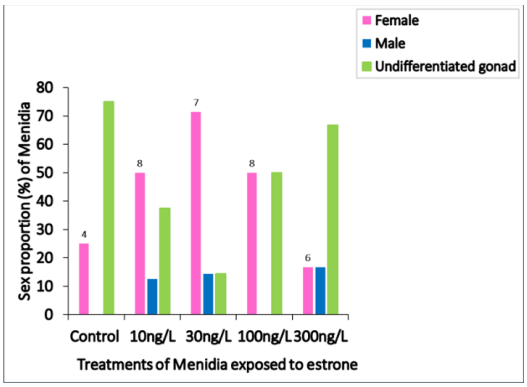


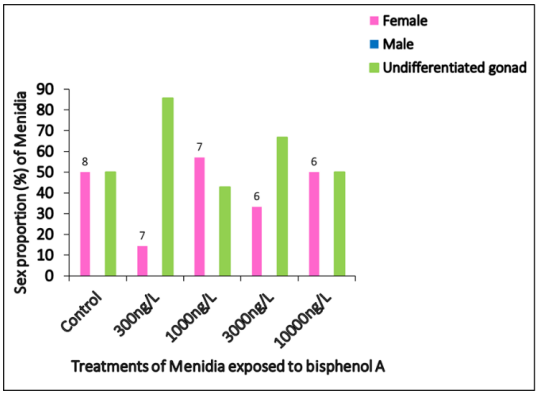
Figure 13. Histological sections of 71-day old *Menidia* stained with Hematoxylin and Eosin stain (H&E stain). Typical sections showing (A-C) oogonia; typical in females; (D-F) undifferentiated gonadal tissue (gonia) and (G-H)spermatogonia, typical in males. Photomicrograph of sex differentiation top row is 20X, middle row is 40X and bottom row is 60X.

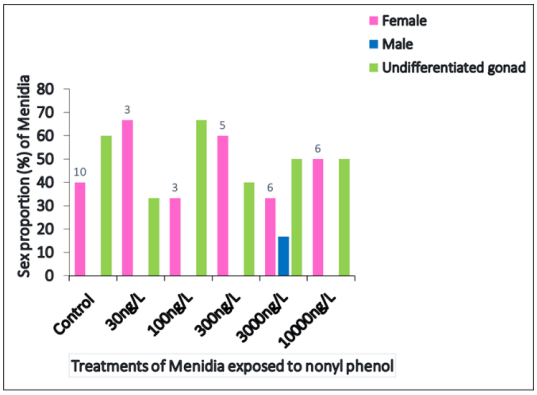
Sex differentiation in *Menidia* is controlled by temperature and length of fish (Conover and Fleisher 1986). Our results suggest that full gonadal differentiation may require a longer window, as many of the gonads were undifferentiated. As noted above, there was substantial size difference among the fry, and this may have contributed to the variance seen in sexual differentiation of gonadal tissue, but we did not set out to test the idea that size and sexual differentiation were correlated and thus we lack data to confirm that hypothesis. We also did not perform Q-PCR for DMRT1 in these fish as a possible measure of genetic sex, but as indicated above, this marker is not fool proof for *Menidia*.

We had expected that gonadal tissue differentiation would have been completed by 71 dph (Conover and Fleisher 1986). Our data suggests that female ovarian tissue differentiates within this time frame but male gonadal tissue differentiation may take longer. For groups with at least 8 fish with detectable gonads, we saw mostly either females or undifferentiated tissue. We cannot comment on whether gonads observed would subsequently differentiate into male tissue.









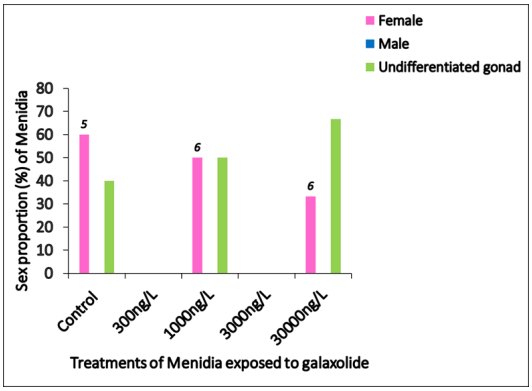


Figure 14. Proportion of females (pink), males (blue) and undifferentiated tissue (green) in *Menidia* after 21 days of treatment and at 71 days of age. The number above each pink columns is the number of fish per group that were analyzed, which was dependent on our ability to identify gonadal tissue in a given specimen. Very few males were identified.

Other than for E2 at 300 ng/L, the proportion of females based on gonadal tissue observations did not seem to differ from controls. For E1, there seemed to be an increased proportion of females with increasing concentration up to 100 ng/L, with, in contrast, a drop at the highest concentration (300 ng/L) where there seemed to be a higher proportion of undifferentiated gonads. This suggested a delay in gonadal maturation due to the high concentration of chemical; however, the power of the experiment was low and this should be repeated. There was no apparent or obvious effect on the proportion of females due to BPA, 4NP or GAL.

Size influence on sexual differentiation: As mentioned above, we had a wide variety of sizes of fish in the experiment, and it was possible that gonadal differentiation occurs at a specific fish size. Generally as seen below, fish with male phenotypes were bigger than the females and the undifferentiated ones. Statistical significance could not be established because in all the cases (signified by the lack of standard deviation) the number of males corresponded to a single fish. Undifferentiated fish were about the same size as females.

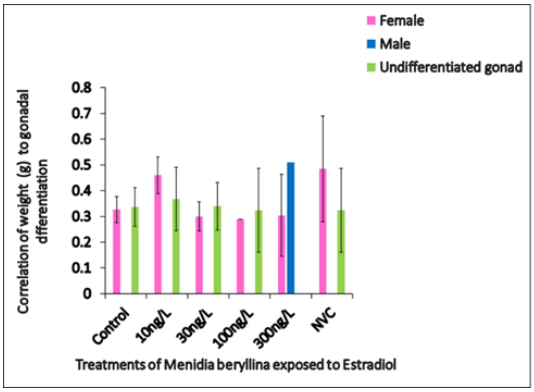
The variation in length was not dose dependent. No male was identified in the control in all treatments despite the fact that they had comparable weight and length. Additionally in control for all exposures, females were identified at varying lengths from a low of 16 mm to a high of 28 mm. In fish exposed to contaminants, females were also identified from a low length of 16 mm (E1 and GAL) to a high of 28 mm (BPA) and 29 mm (E2). Males were 25 mm (E1, E2), 20 mm and 28 mm (4NP); while undifferentiated fish also ranged widely, from the lowest at 16 mm (E1, GAL) to the highest at 28 mm (E2) and 29 mm (BPA). Sex was determinable with a compound microscope at 20X magnification for differentiated males and females, while undifferentiated ones could only be confirmed at 40x magnification.

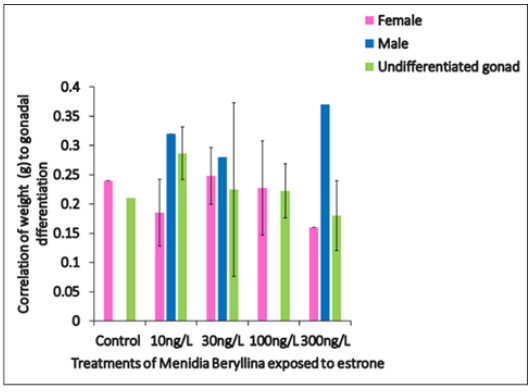
Table 4. Weight and length of fish by sex determination.

Treatment	**Female fish[a] weight (g) & Length(mm)**	**Male fish[a] Weight (g) & Length(mm)**	**Undifferentiated fish[a] Weight (g) & Length(mm)**
17β estradiol, 300 ng/L	0.3±0.15; 21±0.09	0.51; 25	none
Estrone, 10 ng/L	0.19±0.05; 19±0.2	0.32; 22	0.29±0.04; 22±0.06
Estrone, 30 ng/L	0.24±0.05; 22±0.1	0.28; 22	0.23±0.1; 20±02
Estrone, 3,000 ng/L	0.16; 18	0.37; 25	0.18±0.06; 18.5±0.2
NP, 3,000 ng/L	0.5; 25	0.48; 28	0.41; 25

[a]Entries without standard deviations are examples of a single fish.

Correlation of weight to sex proportion: We also examined if overall weight of the fish had an influence on sexual differentiation of the gonad (**Figure 15**). Although not dose dependent, generally in most of the treatments the mean weight of differentiated fish were higher than the undifferentiated. The general exception was with the controls, where the undifferentiated fish had higher mean weight than differentiated fish, but this varied with the set examined. This is probably due to the high variance in fish size at the beginning of the experiment of the 50 dph fish.





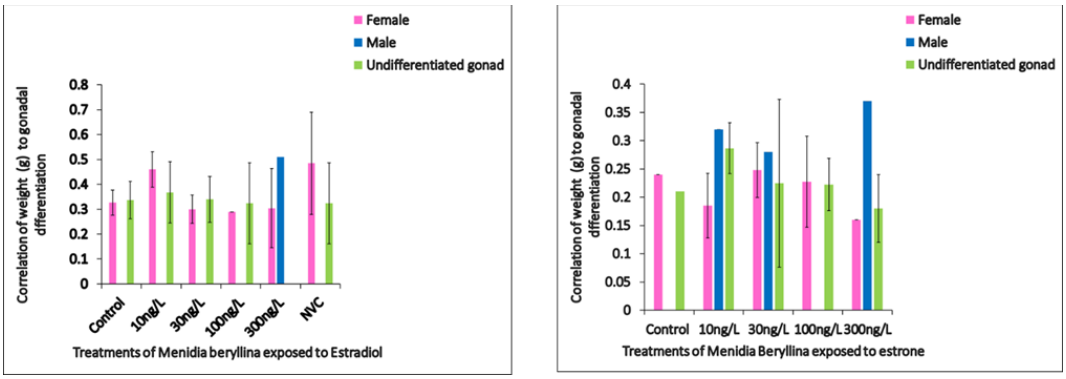


Figure 15. Correlation of weight of fish to sex identification.

Temperature

(Strussmann et al. 2010) reported that family Atherinopsidae to which *Menidia beryllina* belong show temperature-dependent sex determination (TSD) which might also make them prone to dysfunctions such as highly skewed sex ratios. In the present study, mean exposure temperatures during the 21 day period were 22.8 ± 1.5°C (E2), 22.6 ± 1.1°C (E1), 21.2 ± 1.9 °C (BPA), 22.8 ± 0.98°C (4NP) and 22.7 ± 1.0°C (GAL). The maximum mean temperature did not exceed 22.8 ± 1.5°C and the minimum temperature range did not fall below 19°C during the period of exposure. According to Duffy et al, (Duffy et al. 2010) these temperatures fall within an intermediate sex ratio-producing temperature (21°C) as opposed to temperatures that feminize (15°C) and masculinize (28°C) reported for Atlantic silversides, *Menidia menidia*.

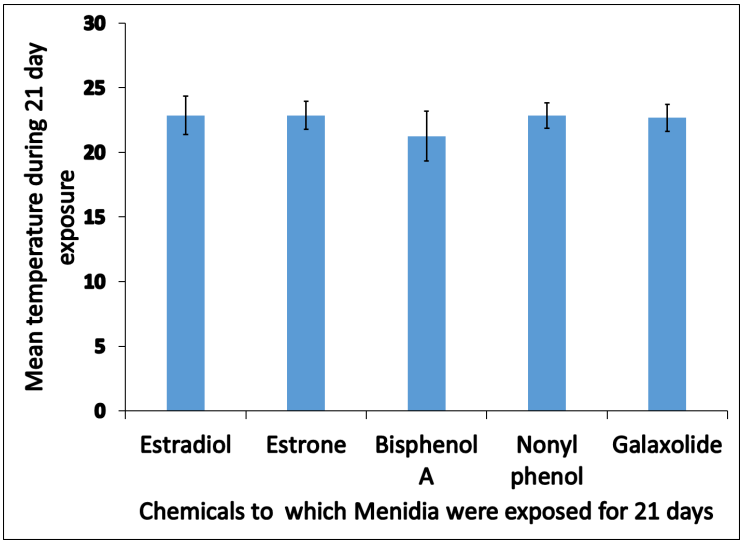


Figure 16. Mean temperature during the 21 day exposures

Influence of contaminants on growth

It was difficult to get a clear understanding of the effects of the different contaminants on growth of the juveniles. The 45-day old fish that were received were different sizes when they arrived and we distributed them randomly to the test tanks. We did not separate them out by size. We did notice that placing them in contaminant tanks increased the variability tremendously of the sizes of the fish and this was not dependent on sexual differentiation. In **Figure 17**, we plotted the overall weight of the fish for those that we checked for sexual differentiation as a function of their contaminant concentration for two of the contaminants, a relatively strong estrogenic contaminant, E1, and a weaker estrogen, NP. As can be seen from these graphs, the controls appear to have less variance in their size than the contaminant treated fish. We get a similar plot for fish length, but with a less pronounced effect. For other exposures, there was no difference in the variance of control and exposed fish. More work will need to be done to determine if this is a real phenotypic change, or just a random selection of fish, since our n is small.

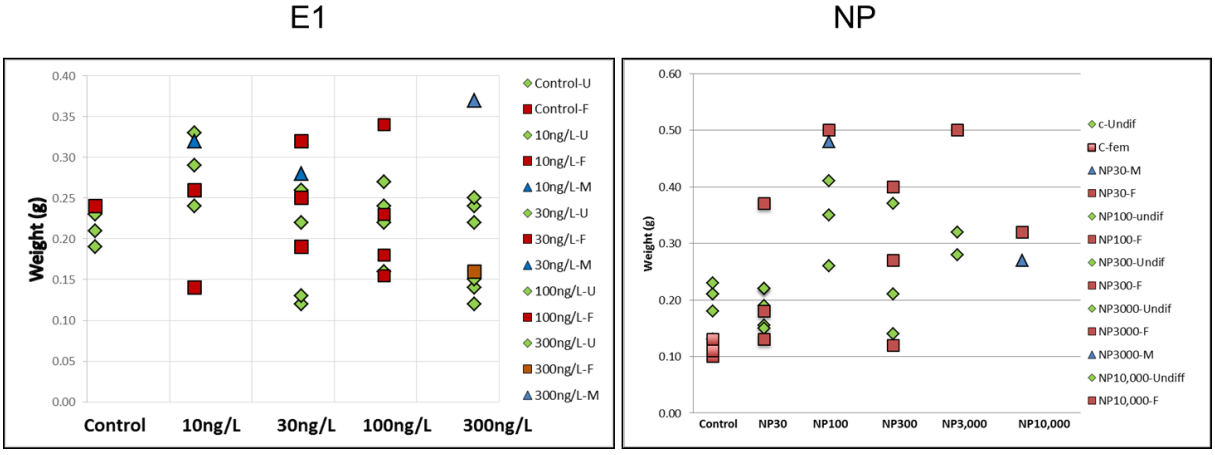
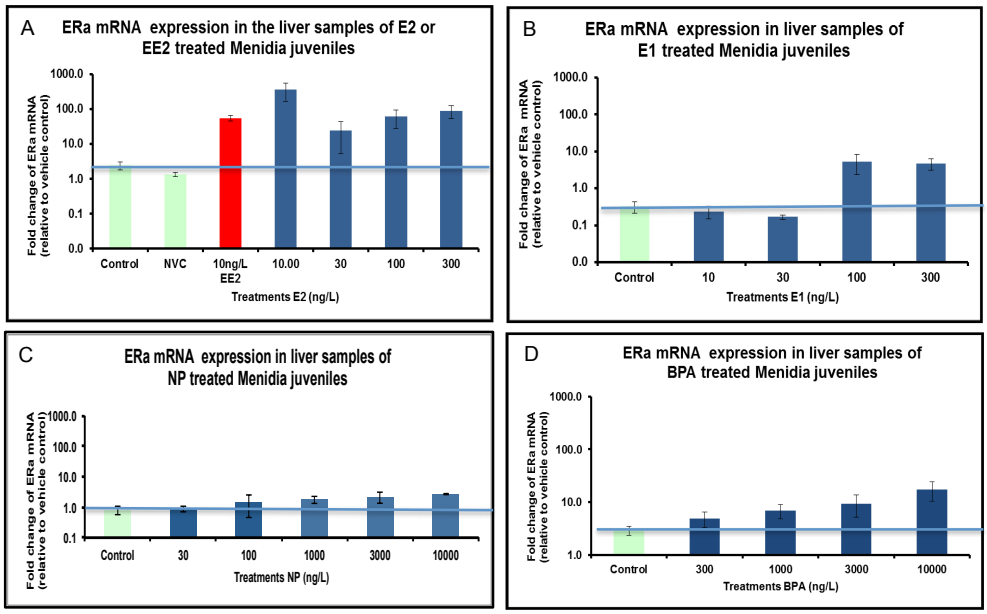


Figure 17. The effect of estrogenic contaminants on weight of fish. This represents only those fish that were used for sex determination by histology. Red squares, females; blue triangles, males; and green diamonds, undifferentiated gonadal tissues.

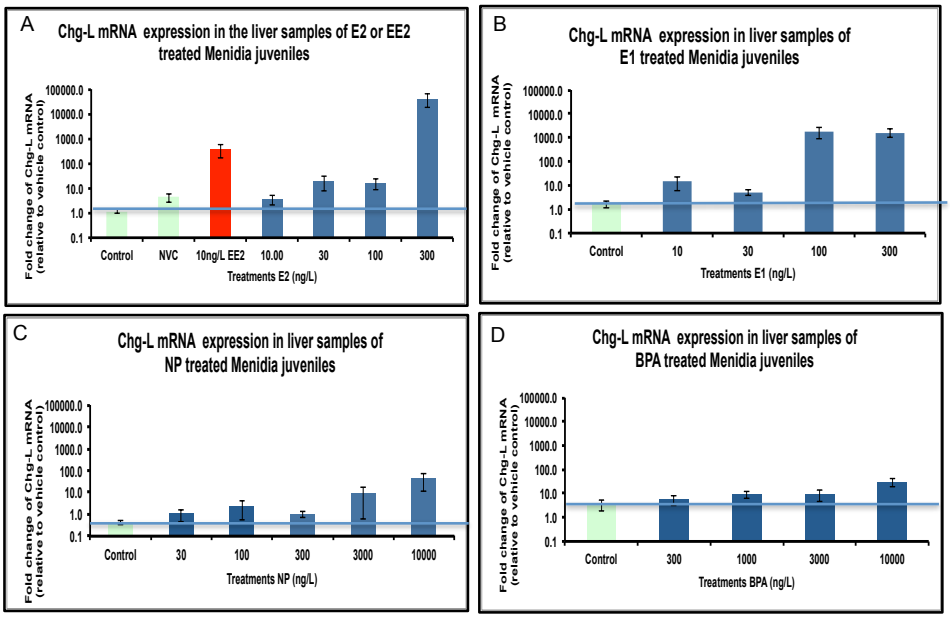
Molecular biomarkers for juvenile *Menidia* exposed to contaminants.

We tested the livers from exposed juvenile *Menidia* for differential expression of 5 genes that could be related to endocrine disruption: estrogen receptor alpha (ERa), Vitellogenin (vtg), choriogenin (chg), androgen receptor (AR), and estrogen receptor beta (ERb). The different treatments resulted in dose-dependent increases in ERa, vtg and chg, in consonance with other studies in fish (Sabo-Attwood et al. 2004; Yu et al. 2006; Chen et al. 2008) (**Fig 18**). The effects on AR and ERβ differed by treatment. The two relatively potent estrogens, E2 and E1, appeared to have a dampening effect on the expression of the two genes by almost two fold. We observed a dampening of ERβ by relatively strong estrogens previously in other fish species (Sabo-Attwood et al. 2004). On the other hand, 4NP and BPA seemed to have a dose-dependent increase of expression (**Figue 19**). This has also been seen for ERβ in other fish (Chandrasekar et al. 2010; Palermo et al. 2012). It is known that these two chemicals have other endocrine activities besides activating the soluble ER. They both can function as an anti-estrogen at low concentrations, as demonstrated by the *in vitro* assays and both also function as anti-androgens. BPA also affects the thyroid hormone axis. Thus, their effects on these two other genes may be due to other activities.

ERa


A
ERa mRNA expression in the liver samples of E2 or EE2 treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
0.1
0.0
Control
NVC
10ng/L EE2
10.00
30
100
300
Treatments E2 (ng/L)
B
ERa mRNA expression in liver samples of E1 treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
0.1
0.0
Control
10
30
100
300
Treatments E1 (ng/L)
C
ERa mRNA expression in liver samples of NP treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
0.1
Control
30
100
1000
3000
10000
Treatments NP (ng/L)
D
ERa mRNA expression in liver samples of BPA treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
Control
300
1000
3000
10000
Treatments BPA (ng/L)

Chg


A
Chg-L mRNA expression in the liver samples of E2 or EE2 treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
NVC
10ng/L EE2
10.00
30
100
300
Treatments E2 (ng/L)
B
Chg-L mRNA expression in liver samples of E1 treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
10
30
100
300
Treatments E1 (ng/L)
C
Chg-L mRNA expression in liver samples of NP treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
30
100
300
3000
10000
Treatments NP (ng/L)
D
Chg-L mRNA expression in liver samples of BPA treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
300
1000
3000
10000
Treatments BPA (ng/L)

Vtg

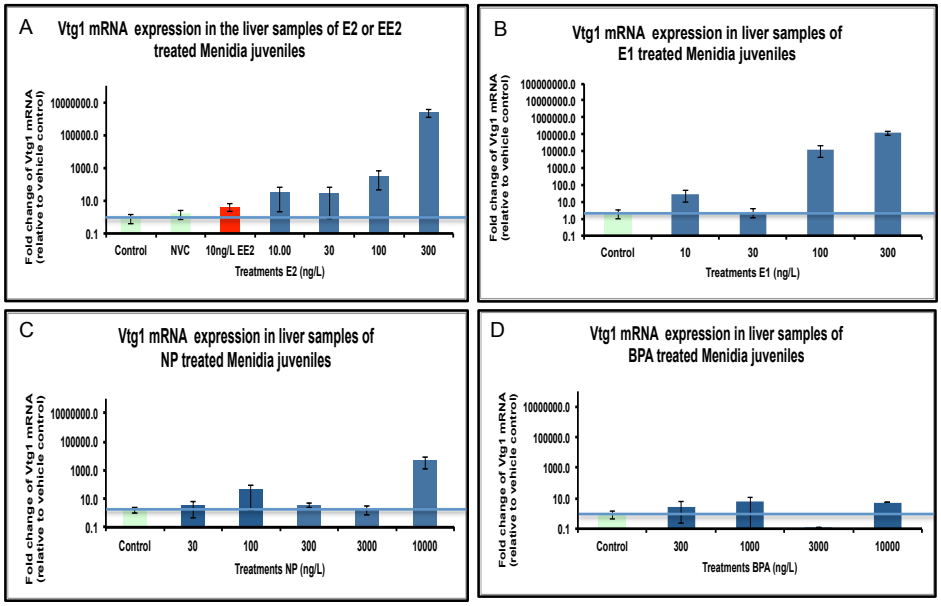
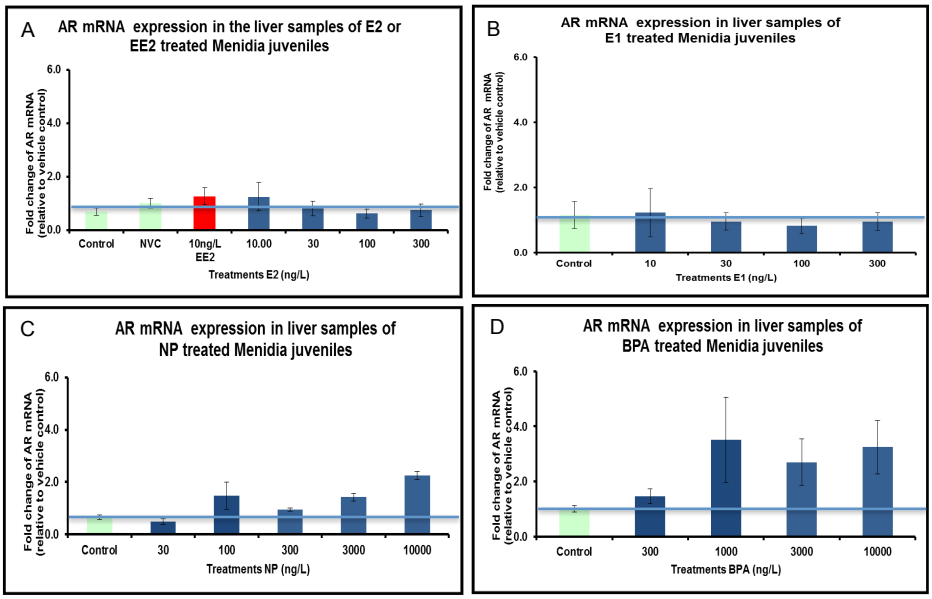


Figure 18. Q-PCR results for ERa, Chg and Vtg on juvenile *Menidia* exposed to E1, E2, 4NP and BPA for 21 days. GAPDH was used as an internal control. Fold change data are mean ± standard error relative to vehicle control. The horizontal line indicates the level of the control.

AR



ERβ

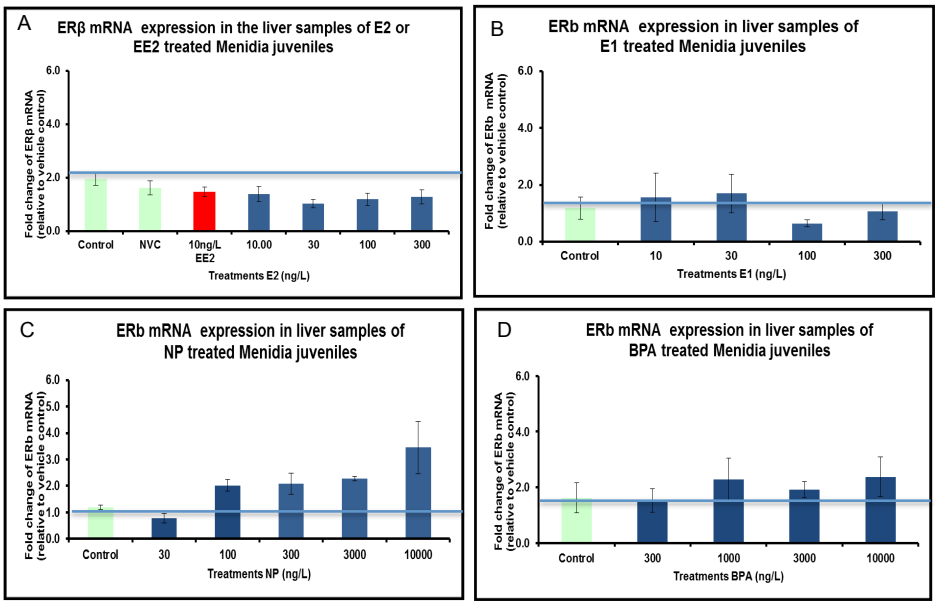


Figure 19. Q-PCR results for AR and ERβ on juvenile *Menidia* exposed to E1, E2, 4NP and BPA for 21 days. GAPDH was used as an internal control. Fold change data are mean ± standard error relative to vehicle control. The horizontal line indicates the level of the control.

Conclusions:

1. Several molecular biomarkers for gene-specific expression were developed for *Menidia beryllina* using Q-PCR.

2. The *in vitro* response of a commercially available estrogen receptor transactivation assay was characterized for E1, E2, 4NP, BPA, GAL and bifenthrin, referenced to the strong agonist EE2. The potency of our test estrogens was as follows:
   E2 > E1 > 4NP > BPA >> GAL, bifenthrin

3. Survival and growth of *Menidia* larvae were not affected by nominal exposure concentrations as high as 300 ng/L of E1; 3000 ng/L of E2; 3 ug/L of 4NP and 30 ug/L for BPA and GAL. Actual exposure concentrations for this series of experiments needed to more completely interpret these observations will be determined in Year 2.

4. Gene expression studies for *Menidia* (larvae) indicated different activities of the estrogenic compounds. The exposures for the larvae were only for seven days possibly insufficient time for a robust transcriptional effect. We have not yet measured the actual concentrations for the exposures.

   a. We had expected to see increases in Cyp19b with all estrogenic chemicals and not GAL because promoters for Cyp19b in fish are known to have estrogen response elements (EREs). To our surprise, only BPA showed a positive dose-dependent response. It is possible that we misidentified the gene sequence, something we will work on more in the next period.

b. *Menidia* larvae-- StAR gene. Only GAL showed a linear dose response, but BPA showed what appeared to be an inverted U shape curve for this gene. This is the main regulator of stroidogenesis.
c. *Menidia* larvae – IgF1 gene is associated with growth. Only BPA produced a linear dose responsive association, despite not being able to observe actual growth in the larvae.
d. *Menidia* larvae – GhR is also associated with growth. Only BPA showed a response, but this was inverted U shaped curve with a maximum effect at 1 ug/L
e. *Menidia* larvae – Amh is associated with being male. BPA showed an inverted dose response curve and GAL showed a high induction but only at the highest concentration of 30 ug/L.
f. *Menidia* larvae – DMRT1 is associated in some fish with maleness. In other fish it is expressed both in males and females, but at much higher levels in males. The only notable effect was seen with ethinylestradiol at 10 ng/L where we saw a distinct depression of expression of this gene.

5. Gene expression studies in juveniles. Strong and weak estrogens behaved as anticipated with biomarkers known to chart estrogenic effects, including Era, Chg and Vtg. Effects on AR and ERb by some of the weak estrogens are probably more related to their other activities, for example it is known that both NP and BPA can act as antiandrogens and that BPA also can suppress transcription of the thyroid hormone receptor (Rostkowski et al. 2011; Sheng et al. 2012).
    a. *Menidia* juveniles – ERa strong dose response for all of the chemicals tested. E2 reached a plateau at low concentrations as seen in o0ther studies. NP was the weakest of the responses.
    b. *Menidia* juveniles – Chg -- Nice dose responses for all the chemicals tested. BPA was weaker than NP.
    c. *Menidia* juveniles – Vtg – Nice dose responses for all the chemicals tested. BPA was weaker than NP
    d. *Menidia* juveniles – AR – We expected no response from pure estrogens and that was the case for E2 and E1, but very strong response for NP and BPA
    e. *Menidia* juveniles – ERb – in other studies, pure estrogens tend to downregulate this gene. We saw that effect with E2 and E1, but NP and BPA upregulated this gene.

6. Gonadal tissue developed during 21 to 71 day *Menidia* exposures was disproportionately female and/or undifferentiated. To put the role of chemical exposure in perspective, the development of males (based on gonadal tissue development) needs to be further investigated.

7. *Menidia* size is critical to allow for excision of gonadal and liver tissue for determination of sex and biomarkers of sexual reproductive status (Chg, Vtg). Our initial experiments suggested that at 21 days, ovarian tissue has differentiated but not testicular tissue, suggesting that to capture this tissue we would need to treat the fish for a longer period of time at our temperature and water conditions. We will perform an additional experiment to verify the time frame for testicular differentiation.

8. Initial observations indicate that we should get a better handle on effects on growth by separating out fish by size at both the larvae and juvenile stages and that we should better understand the time frame for testicular differentiation.

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Appendix A: Methods

A. *In vitro* bioassays for ERa and EEQ calculation (UF)

Exposure solution extracts were made up in DMSO and were stored at -80^{o}C until bioanalysis. ERa- GripTite DA cells plated with ~50,000 cells per well in a 96-well clear bottom plate. Cells were stimulated with different concentrations of the reference chemical (E2) or estrogen mimic in the presence of 0.5% DMSO overnight. The following day, cells were loaded with LiveBLAzer™-FRET B/G Substrate and incubated in the dark for 2 hrs. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader (BioTek Synergy H1 Hybrid Reader) and the calculated Blue/ Green Ratios plotted against the indicated concentrations of the chemical (EE2, E2, E1, BPA or NP).

To calculate EEQs in the exposure extracts a previously described (Escher et al. 2014) was followed. Samples were analyzed on the same plate as a standard curve of E2 for the ERα assay and then used to calculate bioanalytical equivalent concentrations (BEQs). To calculate the EEQs of any exposure solution extract, EC_{10} or $ECIR_{1.5}$ values of the exposure solution extract and the reference chemical (E2) were calculated first. Then, the EC_{10} or $ECIR_{1.5}$ value of the reference chemical (E2) was divided by the respective value of the exposure solution extract.

B. Fish larval exposures (SCCWRP)

Inland silverside (*Menidia beryllina*) were purchased from Aquatic BioSystems. Nine-day old larvae were acclimated in 1 L glass beakers containing 800 mL of artificial seawater (Instant Ocean) at 15 parts per thousand (ppt) for 24 h. The following day, the animals were inspected and replaced when necessary to ensure that each beaker contained 20 larvae at the beginning of the exposures. Larvae were fed newly hatched brine shrimp throughout the exposure until 1 day before the end of the exposures. For this study, larvae were exposed for 7 days to four concentrations of the following endocrine disrupting compounds (EDCs): estrone and nonylphenol (experiment 1), and bisphenol A and galaxolide (experiment 2). Each experiment also included a seawater control, a vehicle control (0.005% triethylene glycol; TEG), and a positive control (17α-ethinylestradiol). Table 1 describes the different treatments and concentrations used in this study. Each treatment consisted of four replicate beakers.

The exposures were conducted using a static system. Test solutions were prepared daily and used to change 75% of the water in each beaker. Water quality parameters were routinely measured and maintained throughout the exposures within the following range: temperature of 24 ± 1 ^{o}C, salinity of 15 ± 1 ppt, dissolved oxygen > 6.5 mg/L, pH 7.95 ± 0.20 and ammonia <0.2 mg/L. The nominal concentrations are found in Table 2 of the main report.

Water chemistry

At day 0, 1, 3, 5, and 7, composite water samples (from all 4 replicates per treatment) were collected for chemical analyses. Samples were preserved with 5 mL of methanol, pH adjusted to 7 using 1M hydrochloric acid and solid phase extracted using Oasis HLB 6cc cartridges.

Apical and molecular endpoints

The number of dead larvae was recorded daily and used to calculate the percent survival for each treatment. Effects of EDCs on growth were examined by measuring the biomass. Five fish per replicate beaker were placed in small pre-weighed aluminum pans and dried for 24 h at 60^{o}C. The following day, the pans were weighed and the average weight per fish was estimated.

The rest of the larvae (12-15) were flash frozen in liquid nitrogen and preserved at -80^{o}C. The samples were sent to University of Florida for RNA extraction using RNA Stat-60 and cDNA synthesis.

Statistical analyses
The effects of EDCs on percent survival and mean dry weight per larvae (mg) were determined by one-way analysis of variance (ANOVA) using the statistical software package R. Level of significance was set at $p < 0.05$.

C. Juvenile fish exposures – UF
Lab reared *menidia* (45 day post hatch) were purchased from a bioassay supplier, Aquatic Biosystems (Ft Collins CO), and acclimated for 5 days before exposure. Upon arrival and during the experiments, the fish were fed live brine shrimp nauplii (BSN) (2-3 days post hatch) daily. Feeding rates were maintained for each aquarium by washing (15 ppt seawater) and concentrating live brine shrimp using a 150um filter, and 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.

We attempted to use artificial diets, but were not successful. In a pilot study, we realized that Menidia appear to only ingest feeds in the water column. If food is uneaten, it goes to the bottom of the tank where it quickly compromised the water quality and was difficult to remove. BSN remain alive and swimming for several days in the test water. However, un-hatched brine shrimp eggs appear also to be ingested by the fish, accumulate in the gut, and can cause mortality in 1-2 weeks. It is difficult to remove all the unhatched cysts from the live brine shrimp due to their size and buoyance. In the future, we will use chemically de-chorionated brine shrimp eggs which can be digested and minimize mortalities due to feeding.

Chemicals
All chemicals were initially dissolved in 95% ethanol with the exception of Galaxolide, which was an ethanol/DMSO (1:1) combination in a sealed GC container to prevent volatilization. Dilutions of the dissolved chemical stock solutions (10 mg/ml) were further diluted in triethylene glycol (TEG) to create individual spiking solutions for each dose. The final concentration of TEG (containing the test chemical) was maintained at 50µl/ liter of test water. The nominal and actual concentrations of the test solutions are in Table 3 of the full report.

Exposure Solutions
City water used for these experiments was carbon filtered to remove chlorine and potential hydrophobic contaminants. Salt water (15 ppt) was prepared using Instant Ocean in a 400 gallon fiberglass tank with heavy aeration. Prepared saltwater was pumped thru a 25 micron filter to remove any fine debris.

Exposure solutions were stored in a 50 gallon fiberglass tank that was continually mixed by mild aeration. The water in each tank was changed daily (50%) by partially draining each aquarium. Fresh solutions were then pumped into each aquarium using Chemfluor tubing. This tubing has been used and validated by the EPA to be low or non-binding for chemicals. Fifteen 50-day post hatch *Menidia* were exposed to the test solutions for 21 days in 2.5 gallon glass aquaria, containing 4 liters of test water, and aerated with a glass pipette. All exposures were run in quadruplicate. One liter water samples from each of the bulk water holding tanks was collected for chemical analysis.

Concentrations of E1, E2, EE2, NP, BPA, and control solutions were verified using ELISA kits (Abraxis). One liter of each exposure solution was collected at the end of the experiment from the bulk holding tanks and stored at 4°C. E1, E2, and EE2 were concentrated down to 1.0 ml using C18 solid phase extraction cartridges (AccuBOND II ODS-C18, Agilent) and eluted with methanol. NP and BPA SPE concentration utilized a Nexus matrix (BondElut, Agilent). The remaining portion was evaporated with nitrogen and reconstituted in distilled water containing 10% methanol.

Tissue collection

The fish were anesthetized using MS-222 (100 mg/ml). The total weight (to 0.01 g) and lengths (to 0.1 mm) of each fish were recorded. The liver was removed using a dissecting microscope by making a small incision in the chest, and then flash frozen using liquid nitrogen. The remaining carcass for each fish was preserved in 10% buffered formalin for histological verification of sex and reproductive stage. Whole fish were anesthetized, flash frozen, and stored at -80°C as a “back-up” for RNA quantification. A total of 4 livers, and 4 whole fish were collected from each aquarium at the end of the experiment.

Histology

In order to ensure capture of the gonadal tissue during sectioning, the fish were trimmed under a dissecting microscope after formalin fixation. The tail was severed 1mm post the cloaca and then posterior to the heart. The resulting mid-sections were imbedded in paraffin so the tail pointed up and then sliced sagitally at several levels posterior the cloaca to ensure capture of gonad tissue. Histological processing was conducted by Histological Tech Services (Gainesville, FL) and stained by H&E. The sex of each fish was verified by visual inspection using a compound microscope at 20X, 40X and 60X.

Appendix B: Validation of QPCR assays for Menidia beryllina

A) Verification of primer design for QPCR for various genes involved in reproduction. For this set of experiments, liver tissues were obtained from Menidia and then extracted for total RNA. This RNA sample was then evaluated for purity (A260/A280 ratio with the NanoDrop spectrophotomer). Primers were designed for the genes listed below (Table S1). Other primers were from Susanne Brander (Brander 2011). All primers were first verified by regular PCR and migrated into a gel (Fig. S1) and then by Q-PCR to check the linearity of the amplification (Fig. S2).

Table S1. Menidia primers designed and validated for PCR and qPCR

Transcript name	Name of the Primer	Primer sequence
Menidia berulina- insulin-like growth factor i	MB-Igf1-*Fwd*	CGATGTGCTGTATCTCCT
	MB-Igf1-*Rev*	CTCTCTCTCCACAGACAAA
Menidia - STAR	MB-StAR- *Fwd*	GCCAGGACACGATGATTA
	MB-StAR- *Rev*	CTATACAGGTAGGCCCATTC
Menidia - GhR	MB-GhR- *Fwd*	AGCCAGTAGAGACCAAAC
	MB-GhR- *Rev*	GTTGAGGAGCAGACTATGA
Menidia – Brain Aromatase	MB-cyp19b- *Fwd*	GCAGGATGTGATGGAGAA
	MB-cyp19b- *Rev*	CACTGCCTGACGTTATCT
Menidia – anti-mullerian hormone	MB-AMH- *Fwd*	TCCTGATTGGTGGAGAAC
	MB-AMH-*Rev*	CTCAGCTCACACAGGAAC
Menidia- dmrt1	MB-dmrt1-*Fwd*	GACTGTCAATGCCCAAAG
	MB-dmrt1-*Rev*	GCCACAGGACTACAAATC

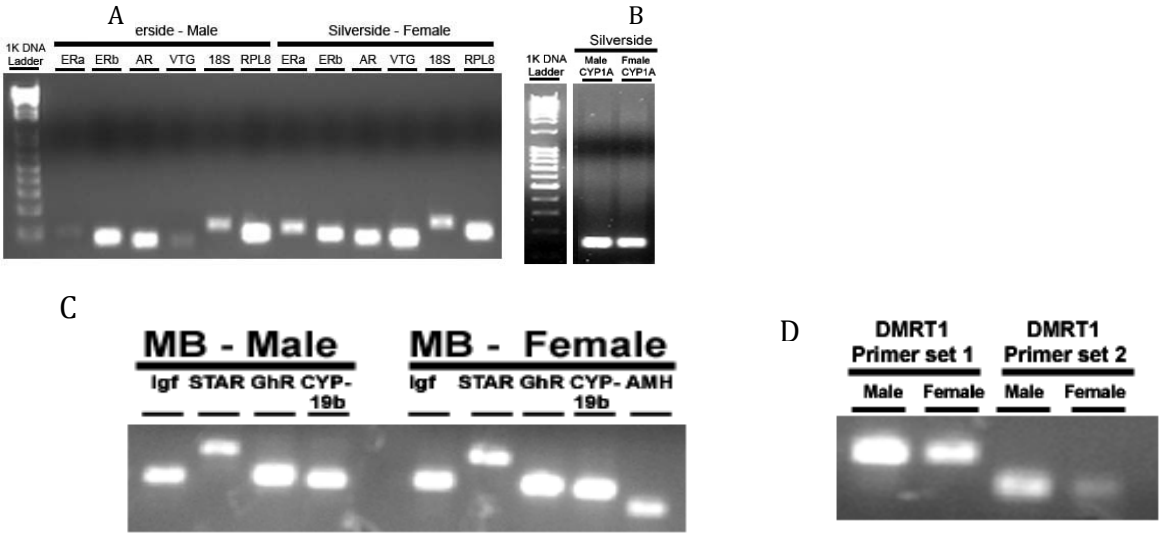
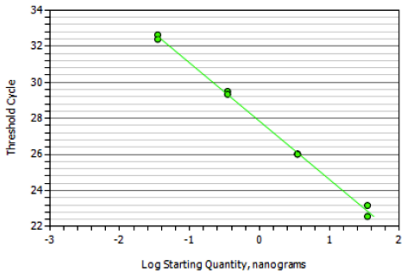


Figure S1: PCR verification of primers for (A) Vtg, ERa, ERb, AR, 18S rRNA and rpl8; (B) CYP1A; (C) Igf, StAR, GhR, Cyp19b, amh and (D) DMRT1 in adult male and female Menidia. Total RNA was extracted from adult Menidia liver tissues and amplified with primers specific for the amplified sequences. Abbreviations: Vtg, Vitellogenin; ERa, estrogen receptor

alpha; ERb, estrogen receptor beta; AR, androgen receptor; 18S rRNA, 18S ribosomal RNA, rpl8, ribosomal protein L8; CYP1A, cytochrome P450 A1; IgF, insulin like growth factor, StAR, steroidogenic acute regulatory protein; GhR, growth hormone receptor; CYP19b, brain aromatase; amh, anti-mullerian hormone; DMRT1, doublesex and mab-3 related transcription factor 1.

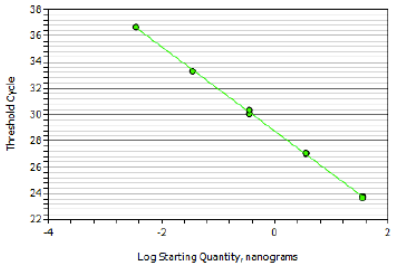
B) Amplification efficiency for each of the primers. Dilution curves were prepared for each of the primers to verify the amplification efficiency. All primer pairs were between 95 and 105 % efficient.

ER alpha



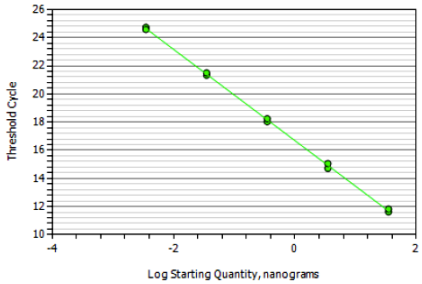
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.9	0.997	-3.233	27.845

ER beta



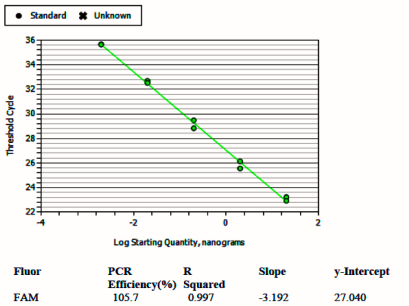
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.8	1.000	-3.211	28.739

VTG1



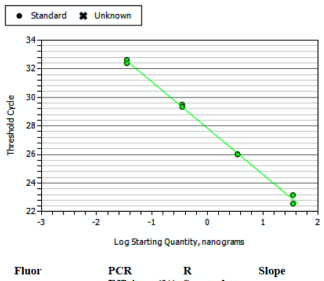
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.5	0.999	-3.240	16.685

Chg-L



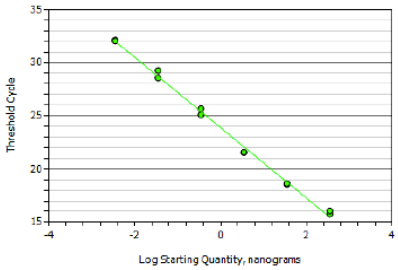
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	105.7	0.997	-3.192	27.040

AR



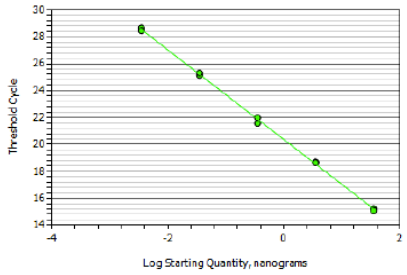
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.9	0.993	-3.223	28.232

GAPDH



Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	100.6	0.996	-3.307	23.889

RLP8

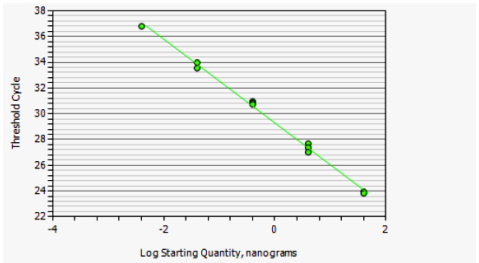


Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	99.5	0.999	-3.333	20.359

Figure S2. Q-PCR assays validation for ERα, ERβ, Chg, AR, Vtg1 and two housekeeping genes, RLP8 and GAPDH for juvenile *Menidia* and of GhR, Cyp19b, IgF1, StAR, amh, & DMRT1 for larval *Menidia*. Efficiency of the reaction should be between 95% and 105% to be useable for measuring changes in gene expression.

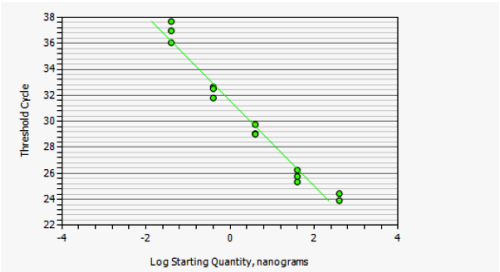
Validation of primers for q-PCR for ELS Menidia.

GhR



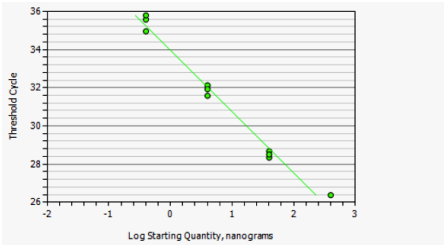
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.1	0.996	-3.250	29.290

Cyp 19b



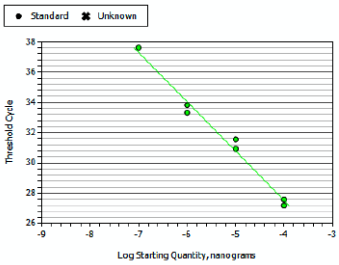
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	102.1	0.968	-3.273	31.584

IgF1



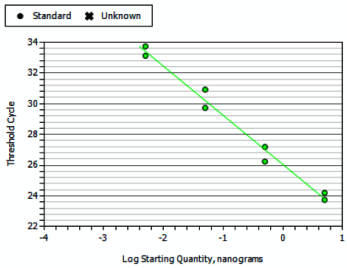
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.9	0.984	-3.211	33.961

StAR



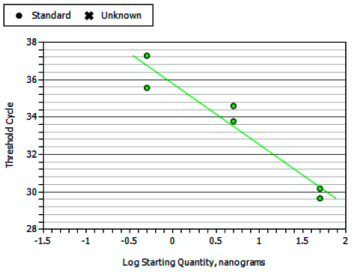
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	102.8	0.983	-3.258	14.491

Amh



Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	105.4	0.984	-3.199	26.046

DMRT1



Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.1	0.927	-3.249	35.781