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Linkage of *In Vitro* Assay Results With *In Vivo* Endpoints

Prepared by

Nancy Denslow, University of Florida

Kevin Kroll, University of Florida

Sumith Jayasinghe, University of Florida

Olanike Adeyemo, University of Florida

Candice Lavelle, University of Florida

Erchao Li, University of Florida

Alvine C. Mehinto, Southern California Coastal Water Research Project

Steve Bay, Southern California Coastal Water Research Project

Keith Maruya, Southern California Coastal Water Research Project

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Final Report – Phase 1 & Phase 2
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University of Florida: Nancy Denslow, Kevin Kroll, Sumith Jayasinghe, Olanike Adeyemo, Candice Lavelle and Erchao Li

SCCWRP: Alvine C. Mehinto, Steve Bay and Keith Maruya

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Executive summary
The goal of this project was to determine quantitative linkages between *in vitro* bioanalytical (IVB) assays and higher-order-endpoints that may relate to population level effects in estuarine fish. The fish model chosen for the *in vivo* endpoints was the inland silverside (*Menidia beryllina*), which is an established fish model for estuarine toxicity and for which established USEPA protocols exist. This project focused on estrogenic chemicals commonly found in wastewater effluent including 17β estradiol (E2), estrone (E1) and 4-nonylphenol (NP), which range in potency for the estrogen receptor. We used commercially available *in vitro* assays for human estrogen receptor alpha transactivation to determine the 50% effect concentration (EC_{50}) for the different chemicals. *In vivo* endpoints included alterations in molecular gene expression and higher-order-endpoints normally used in risk assessment, such as gonadal tissue differentiation, growth and survival. Molecular gene expression endpoints were for genes related to brain development (brain aromatase in larvae) and sex differentiation and reproduction (larvae and juveniles). The higher endpoints were survival, growth of larvae, gonadal tissue differentiation, and growth of juveniles. Larvae were exposed for 7 days and juveniles for 28 days. A subset of juveniles that were exposed for 28 days to estrogenic chemicals were then transferred to clean water and cultured for an additional 60 days to determine whether the effects observed in the constant exposure for 28 days were reversible. In addition to the individual chemicals, we also exposed larvae and juvenile fish to diluted effluent (5 to 50% dilutions) from a wastewater treatment plant that discharges into San Francisco Bay.

EC_{50} values obtained from the *in vitro* assays for the three estrogenic chemicals were 22 ± 2.1 ng E2/L, 81 ± 14 ng E1/L and 20.5 ± 1.6 μg NP/L. In the larval assay, we were able to measure changes in gene expression for three genes: brain aromatase b (Cyp19b), vitellogenin (Vtg) and choriogenin L (Chg), at concentrations that exceeded the EC_{50} points for the *in vitro* assays. All three of these genes are known to have estrogen response elements in their promoters in other fish (Teo et al. 1998; Callard et al. 2001; Chang et al. 2005; Yu et al. 2006; Le Page et al. 2008) and are likely to have them also in *Menidia*. There were no effects of the chemical on larval survival, except at 200 μg NP/L, which led to 100% mortality. There was a trend to decreased growth with increased concentrations for both E2 and NP but due to the shortness of the exposure (7 days) and variability in growth rate among replicate fish, this was not significant.

Similar gene expression and growth results were obtained for juvenile fish and in addition, we were able to measure effects on gonadal differentiation. The two genes selected for gene expression studies, Vtg and Chg, showed dose response increases in expression starting at the EC_{50} concentrations for the *in vitro* assays. We saw estrogen-dependent changes in gonadal tissue differentiation and for some of the exposures altered growth after 28 days of exposure. After two months of culturing in clean water, some of the organismal effects appeared to be reversed, but others persisted, especially for the higher concentrations of estrogens. For E2, there was significant delay for male gonadal tissue differentiation at doses greater than the EC_{50} even after 2 months in clean water. In addition, females had extensive atresia of their ovaries. In the case of E1, feminization effects persisted only at 300 ng E1/L. The effects observed at 100 ng E1/L in the 28-day exposure seemed to be reversed. Nonylphenol showed no effects on gonadal tissue differentiation after 28 days but had increased numbers of undifferentiated fish after the two month grow out in the groups that had been originally treated with the higher nonylphenol concentrations, suggesting that male differentiation had been delayed by the 28-day exposure. Effects on growth were less dramatic, with only some of the conditions showing

statistically significant effects, and these appeared not to be dose-dependent. It would be prudent to repeat the grow-out phase of the experiment one more time to determine if the observed effects are repeatable.

Our results clearly show that it is possible to establish linkages between the screening level in *in vitro* assays and higher order endpoints in fish that are influenced by estrogen, such as growth and gonadal sex differentiation (Table 1). As expected, the *in vitro* responses are the most sensitive endpoints tested and using them as a monitoring tool can provide a margin of safety for aquatic organisms that may be more sensitive to estrogenic chemicals than *Menidia*. Moreover, the margin of safety provided, represented by the ratio of *in vivo* to *in vitro* EC_{50}s ranged from 0.9 to 9 for the potent estrogen E2 to 1.2 for E1 and 2.4 to 3.4 for NP (Table 1). There were no significant effects on growth, but merely trends, by any of the chemicals tested, suggesting that a higher "n" may be required to see appropriate effects on this endpoint. Using more concentrations for *in vivo* tests can refine these ratios.

Of the endpoints we chose to examine, the most sensitive endpoints were at the molecular level for genes known to contain strong estrogen response elements in their control regions, including Vtg, Chg, and Cyp19b. Expression of the mRNAs for Vtg and Chg is specific to females as shown in previous studies with other fish species. Vtg and Chg are synthesized in the liver and are transported through the blood to developing oocytes in female ovaries. Cyp19b expressed mainly in the preoptic area, mediobasal hypothalamus and telencephalon regions of the brain has been linked to brain sexualization and plasticity during development.

Table 1. Quantitative linkages between higher order endpoints and *in vitro* assays for *Menidia*

Chemical	EC_{50} IVB assay (ng/L)	Larval Stage		Juvenile Life Stage					
		EC[a] Gene expression (ng/L)	Ratio[b]	EC[a] Gene expression (ng/L)	Ratio[b]	EC[a] gonadal differentiation (ng/L)	Ratio[b]	EC[a] growth (ng/L)	Ratio[b]
E2	22 ± 2	200	9	20	0.9	200	9	NE[c]	
E1	81±14			100	1.2	100	1.2	NE[c]	
NP	20,500 ±1,640	70,000	3.4	50,000	2.4	NE[c]		NE[c]	

[a]EC, lowest effect concentration at which endpoint was minimally affected
[b]Ratio of higher order endpoint/in vitro assay
[c]NE, no effect

The juvenile fish test was appropriate to assess higher order endpoints such as gonadal tissue differentiation. Larval stages were the least sensitive for growth, probably due to the short duration of the 7-day assay. To capture female tissue differentiation, a sensitive endpoint with good extrapolation possibilities to population level effects (White et al. 2017), the juveniles required 28 days of exposure. In addition, it was necessary to feed the fish twice a day during the exposure paradigm. While the long exposure periods needed to observe the apical effects might preclude their routine implementation, we have identified opportunities to further streamline the *Menidia* life stage exposure protocols.

Exposure to diluted effluent from a secondary WWTP resulted in little to no observed effects on either life stage. As a first example of how the *in vitro* bioanalytical assay would act in screening

mode, the estrogen receptor (ER) transactivation assay response revealed very low equivalent concentrations of estrogenic chemicals (< 5 ng/L) in the diluted effluent used for exposure, confirming its concordance with the molecular and *in vivo* responses observed in either *Menidia* life stage.

This study is among the first to address the linkage between *in vitro* bioassays and higher order effects in estuarine fish in a quantitative way. It also, in a grander scheme, shows the utility of *in vitro* bioassays as monitoring tools for estuarine receiving waters such as those of San Francisco Bay. The project calibrated the *in vitro* bioassays to higher order endpoints in fish showing that the *in vitro* assays were more sensitive than *in vivo* endpoints used for regulatory purposes. EC_{50} values for the *in vitro* bioassays are likely protective of the environment including more sensitive fish species. As an added benefit, *in vitro* bioassays employed as a screening element in a comprehensive, tiered monitoring program, are certainly more cost effective compared to performing *in vivo* assays. While estrogens represent a small fraction of the endocrine active chemicals found in receiving waters, this pilot study has shown that it is possible to make linkages with *in vivo* endpoints for specific contaminants. Additional studies with other potentially bioactive contaminants and different *Menidia* life stages and hormonal systems would add to this developing monitoring tool. This project was made possible by funding from SFEI and by leveraging the work with other projects and collaborators.

Introduction

A growing number of contaminants of emerging concern (CECs) are found routinely in permitted discharges and their receiving waters. Analytical methods currently exist only for a few CECs, which are routinely performed by commercial service laboratories. Analytical methods for most other CECs are still largely in development in academic labs and are not routinely performed. As the development and manufacture of chemicals presents an ever-changing landscape, CECs that are produced in high volumes and/or that are discharged via treated municipal wastewater, industrial effluent or stormwater runoff represent a moving target for environmental quality managers tasked with assessing and/or mitigating their potential for impact.

The CECs of most concern are those that may be potent at trace concentrations (parts per trillion range) and work as endocrine disruptors. Their presence in water bodies may be harmful to aquatic biota inhabiting these locations. Such endocrine disrupting chemicals can interact directly with soluble hormone receptors or can interfere with the natural synthesis or metabolism of endogenous hormones and thereby impede normal function of these processes in exposed organisms. Most attention has been focused on chemicals, which act as estrogens or androgens or their antagonists. Estrogens are important in reproduction, brain development and programming of tissue differentiation during early development (Feist and Schreck 1996; Mandiki et al. 2005; Vetillard et al. 2006; Lassiter and Linney 2007; Remage-Healey and Bass 2007; Zhang et al. 2008; Tomy et al. 2009).

In our own previous work, exposure of fathead minnows (*Pimephales promelas)* to concentrations of ethinylestradiol (EE2) at 2 ng/L induced pericardial/yolk sac edema (Johns et al. 2009). The estrogenic mycotoxin zearalenone (exposure range of 2-50 ng/L) also resulted in myocardial edema (Johns et al. 2009). In addition, we analyzed a limited set of gene expression changes including Vtg, steroidogenic acute regulatory (StAR) protein, insulin-like growth factor 1 (IGF-1) and growth hormone (GH), among others, which were altered by exposures to EE2 and zearalenone. Since gene expression occurs fundamentally earlier than tissue alterations, changes in gene expression can be used to indicate bioavailability and bioactivity of the chemicals *in vivo*. This is the premise for the adverse outcome pathway concept that has been

introduced into ecotoxicology by Ankley and his coworkers (Ankley et al. 2010) as a means to integrate molecular endpoints into risk assessment.

Concurrently, novel *in vitro* methods based on receptor binding or transactivation have been developed that are extremely sensitive to detect target chemicals or multiple chemicals with the same mode of action. Work is being performed to adapt these *in vitro* bioassays for water quality assessment and monitoring purposes (Escher et al. 2014; Mehinto et al. 2015). Few studies, however, link results from such *in vitro* assays with higher order *in vivo* effects that result in adversity for survival, growth, reproduction, or susceptibility to disease. While the *in vitro* assays are likely to be more sensitive than *in vivo* endpoints, they serve to provide a conservative screening level for endocrine linked contaminants. In the current study, we focused only on estrogen receptor (ER) mediated effects since there was substantial information in the literature for estrogens that could help us establish linkages from *in vitro* to *in vivo* effects. However, this is not to say that other modes of action should be dismissed. It is now clear that glucocorticoids (GR), progestins (PR), and peroxisome proliferator-activator receptor (PPAR) agonists may be even more important than estrogens in the environment (Kugathas and Sumpter 2011; Kugathas et al. 2013; Den Broeder et al. 2015; Fent 2015; Frankel et al. 2016; Margiotta-Casaluci et al. 2016; Nakayama et al. 2016). These other modes of action should be investigated in future projects.

The goal of this project was to establish quantitative linkages between the *in vitro* receptor-based assays and traditional endpoints of adversity in a sensitive estuarine fish model, the inland silverside (*Menidia beryllina*), which is an established USEPA model for estuarine toxicity.

The specific tasks of the project were to: (1) use commercially available *in vitro* assays to test both estrogenic and anti-estrogenic properties of individual estrogenic chemicals. (2) Develop sensitive molecular assays based on quantitative polymerase chain reaction (Q-PCR) to evaluate changes in gene expression related to higher order changes in phenotype. (3) Test larvae for growth and survival, as these represent higher order endpoints. (4) Test juveniles for growth and gonadal tissue differentiation. 5) Test a complex effluent for estrogen equivalencies (EEQs) and effects on larvae and juvenile selected endpoints.

This report is comprehensive on the work that was accomplished. The detailed report covering work from year one of the study is in Appendix B. The current report reflects detailed discussion of work accomplished in year 2. Specified methods for year 2 are covered in Appendix A.

Methods

1. *In vitro* assays

We used InVitrogen GeneBlazer human ERα GripTite Division Arrested cells to derive EEQ relationships among several test substances: E2, E1, NP, and bisphenol A (BPA). All of these transactivate the estrogen receptor. We also tested bifenthrin (BIF), a pyrethroid insecticide, and galaxolide (GAL), a musk. BIF was from a leveraged collaboration with Dr. Susanne Brander and Dr. Richard Connon. Both GAL and BIF are estrogenic in fish. All chemicals were purchased from Sigma Chemical Co, with the exception of GAL, which was custom synthesized by Dr. John Rimoldi (University of Mississippi), a colleague of Dr. Dan Schlenk. The transactivation assays were completed in year 1 of the project and details about their use for agonist and antagonist evaluation can be found in Appendix B. Using the assays, we were able to identify EC_{50}s for chemicals of interest to this study (Table 2).

Table 2. EC_{50} values for tested chemical

Chemical	EC_{50} (M)	Concentrations in ng/L
17α-ethinylestradiol (EE2)	1.11E-11	3.3
17β-estradiol (E2)	7E-11	22 ± 2.1
Estrone (E1)	2.52E-10	81 ± 14
4-Nonylphenol (NP)	8.57E-8	20,500 ±1,640

2. Choice of organism

For this work, we chose the estuarine species, *Menidia beryllina* (Figure 1), also known as silversides, as the test organism. This fish inhabits estuarine locations across the US and in particular it is found in San Francisco Bay and thus it is a good model for endangered species in the Bay (Chapman et al. 1995). The USEPA accepts *Menidia* as a test species for population level effects and protocols for measuring growth are available. During the course of this project, we learned a great deal about its biology, growth, and tissue differentiation. We have developed an excellent toolbox, which will be useful for future work. Critical for the success of this project was determining the period of gonadal differentiation in females, which starts at 35 days post hatch (35 dph) and determining the appropriate diet for juvenile *Menidia* between 30 and 58 dph. Details about these experiments are in Appendix A.

Figure 1. *Menidia beryllina* as a test organism.

The *in vivo* endpoints investigated were focused on survival, growth and gonadal differentiation. We chose two life-stages for this work: larvae between 10 to 17 dph and juveniles 30 to 58 dph. Larvae were selected for growth and survival and juveniles were selected for growth and gonadal tissue differentiation in addition to gene expression changes. Larvae studies were performed at SCCWRP and juvenile studies were performed at the University of Florida, following best practices.

Larval growth and survival assays were conducted for 7 days using 10-day-old *Menidia beryllina* larvae based on the USEPA protocol guidelines (Chapman et al. 1995). Triethylene glycol (TEG) was used as a vehicle in order to completely mix the estrogenic chemicals in the water column. The concentration of TEG was 50 ul/L final solutions. Concentrations of chemicals were chosen to bracket the EC_{50}s determined by the *in vitro* assays. Details of the experimental methods employed are in Appendix A.

Juvenile *Menidia* were exposed for 28 days to the test estrogenic chemicals. At the end of the exposure period, a subset of juveniles was evaluated for growth, gene expression and sex ratio by histology. A second group of fish was then transferred to clean water and cultured for an additional 60 days to determine whether the effects observed in the constant exposure for 28 days were reversible. Details of the experimental methods are in Appendix A.

3. Gene expression studies

Since environmental estrogens activate estrogen receptors (ERα and ERβ) causing them to bind to estrogen response elements in susceptible genes, the bioavailability and activities of

estrogenic contaminants can be measured *in vivo* by examining the increase of expression of mRNAs for genes regulated by the receptors. Genes that are responsive to estrogens and linked to higher order endpoints (Filby et al. 2007; Brander 2011; Brander et al. 2012; Bugel et al. 2014) were selected for this work (Table 3). Results of these changes are shown along with the higher order endpoints below. Details for the experimental conditions employed are described in Appendix A.

Table 3. Molecular endpoints evaluated via Q-PCR

mRNAs evaluated	**Larval exposures**	**Juvenile exposures**	**Comments**
GAPDH[a]	X	X	Housekeeping gene used for normalization
Vitellogenin	X	X	Known to increase in response to estrogens. Associated with reproduction
Choriogenin L	X	X	Known to increase in response to estrogens. Associated with reproduction
Cyp19b	X		Brain aromatase, sensitive to estrogens

[a]GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

Results

Appendix B contains the results from Phase 1, which were indispensable for setting up the experiments that were pursued in Phase 2. Here we limit our discourse to results accomplished in Phase 2.

1. Larval assays

This set of experiments encompassed gene expression studies in the whole organism after 7 days of exposure to the chemicals in addition to growth and survival. Nominal concentrations for E2 were 20, 200 and 2000 ng/L and for NP, 20, 70 and 200 µg/L. Actual concentrations of E2 and NP were measured using ELISA and they were close to the nominal concentrations (Table 4).

Table 4. Actual concentrations of E2 and NP used for exposures of larvae.

17β estradiol (ng/L)		**4-Nonylphenol (µg/L)**	
Nominal concentration	Measured concentration (± SEM)	Nominal concentration	Measured concentration (± SEM)
0	< dl*	0	<dl**
20	14 (± 2)	20	28 (±4)
200	151 (± 7)	70	56 (±9)
2000	1330 (± 38)	200	176 (±22)

* E2 ELISA detection limit is < 3ng/L

**NP ELISA detection limit is < 5 µg/L

(a) Gene expression studies for *Menidia* larvae exposed to specific chemicals.
Total RNA was extracted from pooled larvae (10 larvae per beaker) and processed for gene expression analyses by Q-PCR. Gene expression changes were quantified for Vtg, Chg, and Cyp19b. 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). Gene expression increases were observed for all three genes at test concentrations above the calculated EC_{50}s for E2 (22 ng/L) and NP (20.5 µg/L) (Fig. 2), clearly establishing the quantitative linkage between *in vitro* assay responses and gene expression changes in larvae.

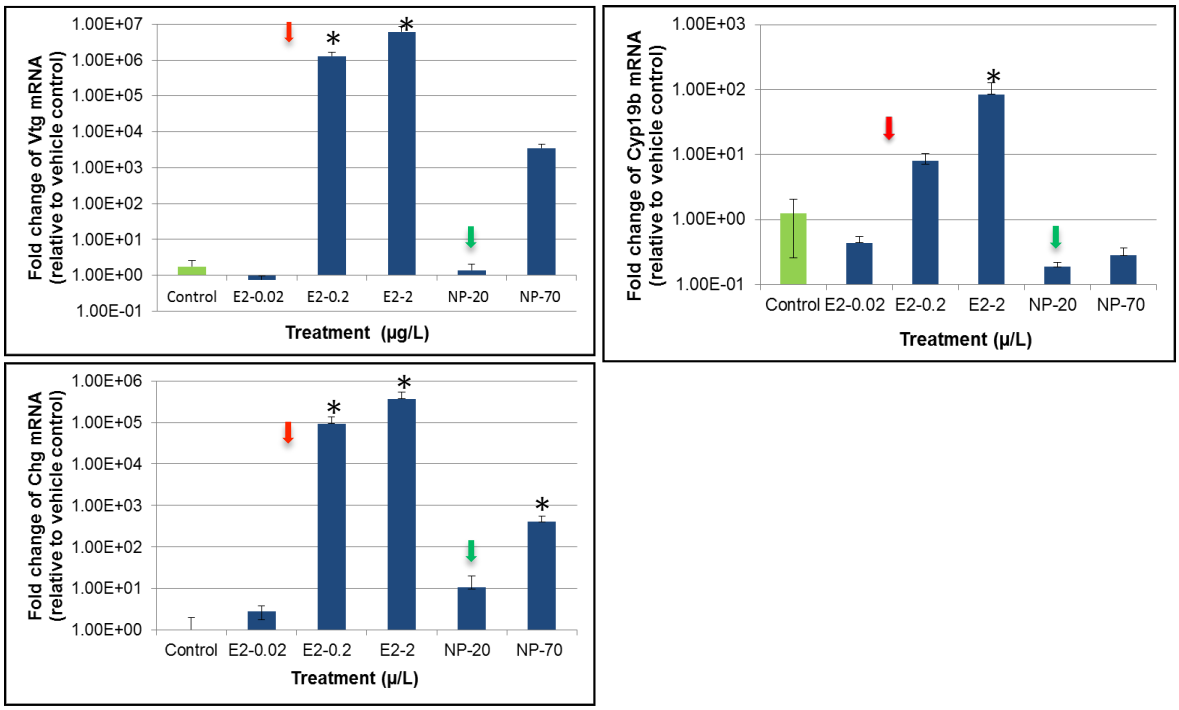


Figure 2. Gene expression changes for Vtg, Cyp19b, and Chg in larvae. Larvae (10 dph) were exposed for 7 days to three concentrations of E2 and NP, as described in **Appendix A**. All larvae from the highest NP exposure (200 µg/L) died. The red and green arrows in the figures denote the EC_{50} points for E2 and NP determined by *in vitro* bioassays. Statistical significance, ** p value < 0.001

(b) Effects of specific estrogenic chemicals on survival of larvae.

There were no survival effects for most of the individual chemical concentrations, except for the highest nonylphenol concentration tested, where all the larvae died (Table 5 and Fig. 3). This was expected as estrogens are not generally overtly toxic and they are thought instead to mainly cause non-lethal effects in fish, for example in growth and reproduction. However, the highest concentration of NP used for the larval study is above the LC_{50} of 70 µg/L for *Menidia* larvae (USEPA 2005).

Table 5. Survival endpoint for Menidia larval exposures

Treatment	N[b]	Mean survival (%)	SEM
0.005% TEG[a] control	4	96.9	3.12
0.02 µg E2/L	4	94.6	2.27
0.2 µg E2/L	4	94.4	3.23
2.0 µg E2/L	4	95.8	2.67
20 µg NP/L	4	98.8	1.25
70 µg NP/L	4	97.4	1.48
200 µg NP/L	4	0	0

[a]TEG, triethylene glycol vehicle at 50 µl/L final concentration
[b]N, is the number of beakers used, each with 20 larvae

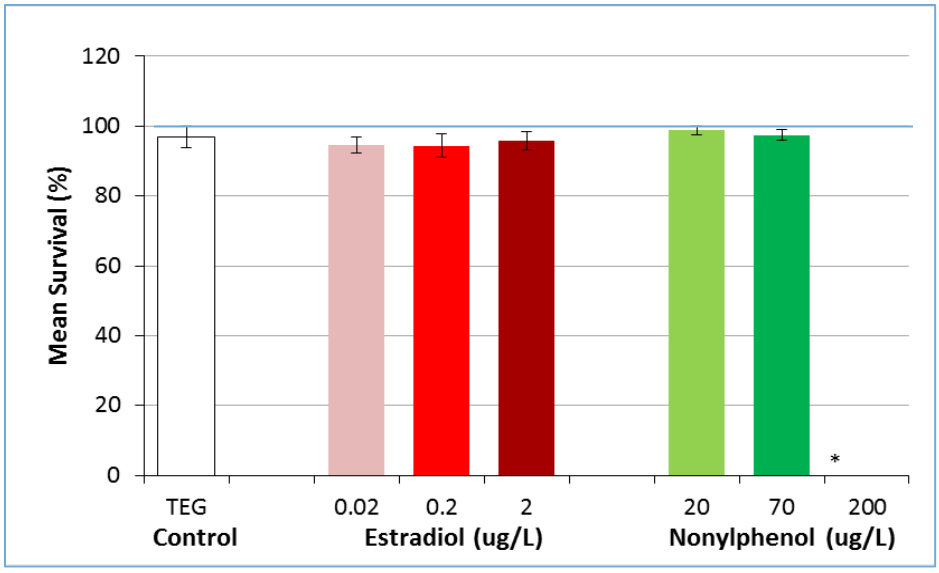


Figure 3. Mean survival (%) for *Menidia beryllina* larvae 7-day exposure to test chemicals. Error bars represent standard deviation (20 fish/replicate, 4 replicates/treatment) and (*) denotes a significant difference compared to the TEG control.

(c) Effects of specific estrogenic chemicals on growth of larvae.
All fish (except those exposed to the highest NP dose) grew significantly compared to day 0 controls ($p<0.01$) (Table 6; Figure 4). There were no statistical differences between either the E2- or the NP-exposed fish and TEG controls. But, there was a trend in growth reduction for both chemical exposures with increasing dose (Figure 4). The NP-exposed fish had 20 - 30% less biomass than the TEG controls. It is likely that 7 days grow out is insufficient time to clearly observe effects on growth, and in the future, it is our recommendation to at least double this

period of time. It could also be that this endpoint is not particularly adequate for estrogens in larvae.

Table 6. Effects of estrogens on growth of *Menidia* larvae

Treatment	N	Mean dry weight per fish (mg)	SEM
Day 0- control fish	5	0.172	0.010
0.005% TEG control	4	0.457	0.039
0.02 µg E2/L	4	0.586	0.042
0.2 µg E2/L	4	0.516	0.032
2.0 µg E2/L	4	0.431	0.029
20 µg NP/L	4	0.361	0.019
70 µg NP/L	4	0.315	0.068

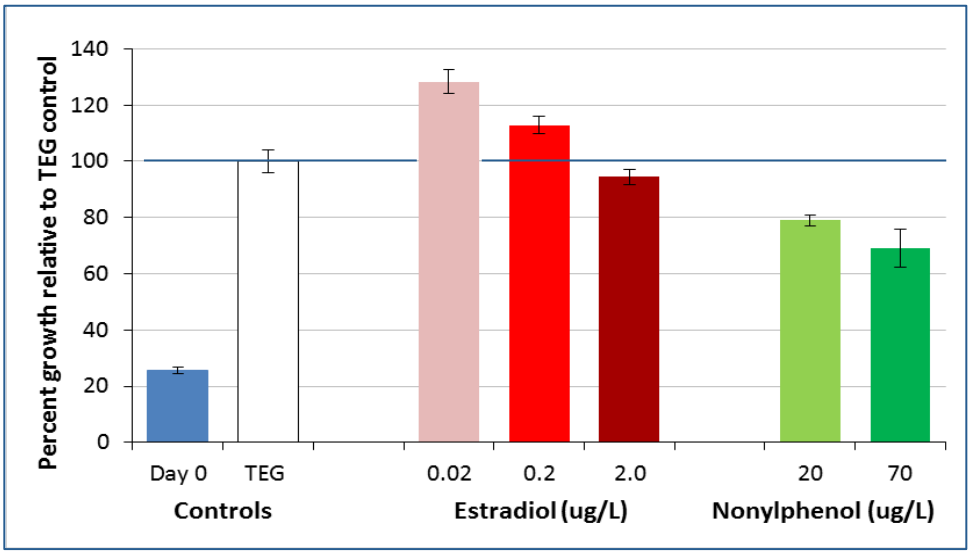


Figure 4. Effects of E2 and NP on mean dry weight of *Menidia* larvae after 7 days of exposure. There were no statistical differences in growth among the chemical exposed larvae and those in TEG, vehicle control. Error bars represent standard error of the mean (5 larvae/replicate, 4 replicates/treatment).

(d) Effects of WWTP effluents on larval endpoints

Before conducting the larval and juvenile effluent exposures, we screened four treated wastewater effluents and identified the plants with the highest estrogenic activity (Table 7). Three batches of 24-hour composite effluents obtained from an unnamed wastewater treatment plant (CA #3) were used for the larval exposures. Upon arrival, the samples were diluted in

natural seawater to produce 3 dilutions: 5%, 10% and 25% effluent samples. Diluted effluent samples were then aerated for 24 hours to remove un-ionized ammonia. The larval toxicity test was conducted for 7 days based on US-EPA guidelines, and details of the exposure are in **Appendix B.** Due to the elevated levels of total ammonia in the effluent exposures (up to 10 mg/L), an ammonia control treatment (~ 8 mg/L) was added. For each batch of effluent used in the exposure, EEQs were measured in the 3 dilutions of effluent using the bioanalytical Invitrogen assays for estrogen receptor (ER) and E2 for the standard curve (Table 8).

Table 7. Estrogen bioanalytical equivalent values (EEQ) for 4 treated wastewater effluents derived from the ER Invitrogen assay

Sample ID	EEQ (ng E2/L)
CA #1	6.4
CA #2	13
CA #3	17
CA #4	2.3

Table 8. Estrogen equivalencies in diluted effluent samples used for testing.

	Mean EEQ (ng E2/L)		
	Expected*	Measured	SEM
SW	0	<BDL	-
NH3	0	< BDL	-
5% effluent	0.85	0.66	0.06
10% effluent	1.7	1.40	0.19
25% effluent	4.25	4.32	0.27

*Expected concentrations were based on CA #3, which showed an EEQ of 17 ng E2/L

Effects of WWTP effluents on gene expression in larvae

None of the treatments resulted in significant change in gene expression (Fig. 5). There were a couple of spurious points that appear to increase expression for an effluent category, but these were not significant and were very low increases compared to the logarithmic increase seen with estrogenic chemical exposures. We conclude that the effluent did not cause changes in gene expression that were relevant to estrogen receptor activity. The highest dose employed for the larvae was 25% effluent, and at this dilution the total estrogenic equivalency was approximately 4 ng/L, a concentration that is much lower than the $EC_{50.}$ Therefore, the lack of response fits data obtained with estrogens in laboratory studies.

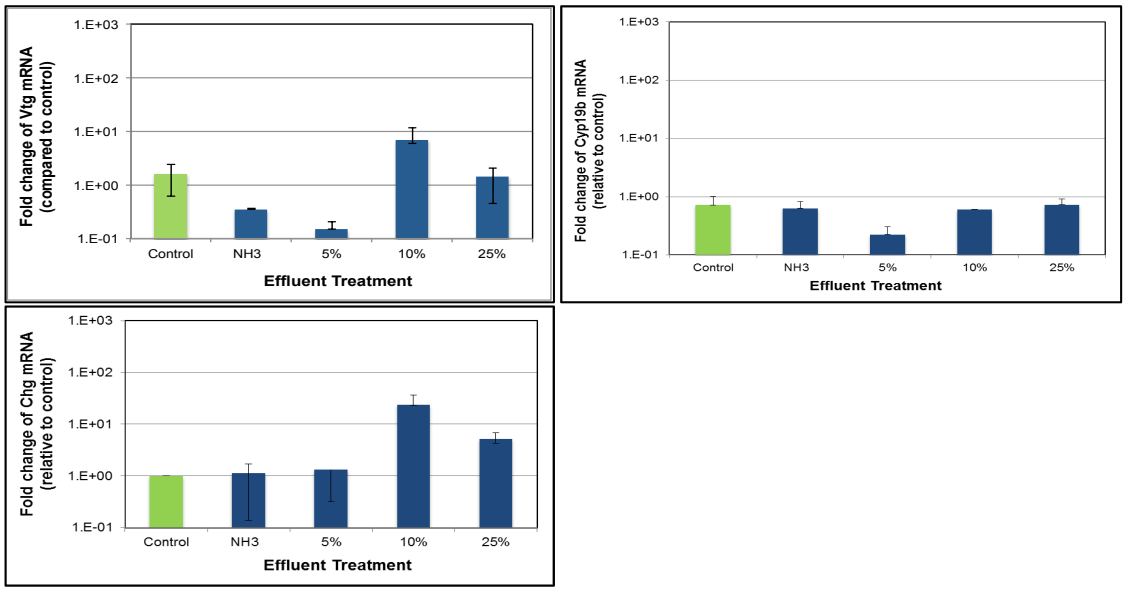


Figure 5. Gene expression changes in larvae with exposure to effluent. Larvae (10 dph) were exposed for 7 days to three dilutions of effluent as described in **Appendix A**. Q-PCR determination of relative levels of expression for Vtg, Chg, and the brain aromatase, Cyp19b. There were no significant differences among the effluent dilutions.

Survival and growth

There were no effects on survival by any of the treatments (Fig. 6). In addition, there were no effects on growth of larvae (Fig. 7).

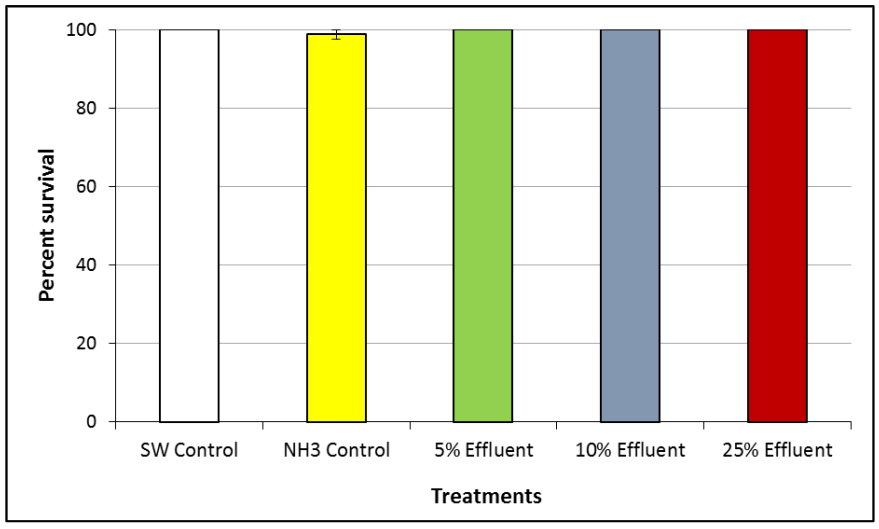


Figure 6. Effect of WWTP effluents diluted to 5%, 10% or 25% on survivability of larvae using the USEPA test.

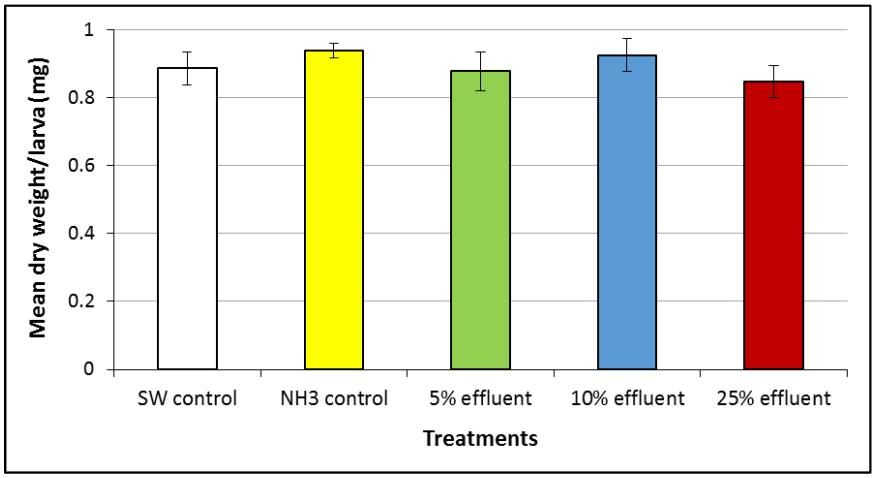


Figure 7. Effect of effluent treatment on growth of fish. Effluent treatments varied between 5% and 25% effluent. An NH3 control at 8 mg/L was included, although NH3 was as high as 10 mg/L in the highest effluent condition.

2. Juvenile assays for gene expression, gonadal differentiation and growth

Juvenile fish 30 dph were exposed to estrogenic chemicals (E2, E1 and NP) or diluted wastewater effluent for 28 days to capture the period of female gonadal differentiation. In addition, fish were evaluated for growth. There was significant development required for this assay to pinpoint the gonadal development period and to overcome initial issues with nutrition. A full description of the methods, including analysis of actual concentrations used for the exposures, is found in **Appendix A**.

(a) Measured concentrations of chemicals.
Measured concentrations of chemical at the end of each 10-day period are found in Table 9 and represent the lowest concentrations to which the juveniles were exposed during that period. Treatment solutions were prepared each 10 days in large containers and delivered to the tanks on a daily basis, as described in Appendix A.

(b) Gene expression studies for juvenile *Menidia* exposed to specific chemicals.
At the end of the 28-day exposure, livers were extracted from *Menidia* for evaluation of gene expression for Vtg and Chg, two genes that are expressed in liver and are associated with reproduction. Exposure of *Menidia* to individual estrogenic chemicals showed a clear effect of dose on gene expression. At the end of the exposure, *Menidia* were 58 dph. Females had differentiated ovaries but there were no males, instead in the control conditions there were several fish with undifferentiated gonads. We checked to see if Vtg expression was different in females compared to the undifferentiated fish (Fig. 8). At the lower concentrations of E2 (2 and 20 ng/L) there was a trend for an increase in Vtg expression over controls for females but not for undifferentiated fish. At the higher concentration (200 ng/L) there is no substantive difference between the response of females and undifferentiated fish and at the highest concentration only females were produced. The differences seen at the lower concentrations can be explained by the “memory” effect (Tan et al. 1996). Once estrogen starts to be synthesized in an organism, the endogenous E2 up-regulates the number of ERs available and thus fish containing the larger number of ERs will transactivate genes more quickly and will be more sensitive to estradiol. However, this effect appears to be abolished by 28 days of exposure to higher E2 concentrations. For the rest of the exposures, we did not separate out females from

undifferentiated fish since there were no statistical differences among them and to get a better "n" for the higher concentrations.

Table 9. Measured concentrations of E2, E1 and NP for the juvenile assays

17β-Estradiol ng/L				
nominal	day-10	day-20	day 28	mean
0	0	na	0	bdl
2	0.8	na	1.7	1.3
20	8.9	na	13	11.0
200	209	na	138	173.5
500	528	na	570	549.0

Estrone ng/L				
nominal	day-10	day-20	day 28	mean
0	0	0	0	bdl
10	3.4	2.2	3.8	3.1
30	40	19	12.8	23.9
100	34.6	29	79	47.5
300	148	160	124.3	144.1

Nonylphenol ng/L				
nominal	day-10	day-20	day 28	mean
0	0	0	0	bdl
10,000	5,000	17,100	17,600	13,233
20,000	30,000	25,200	15,600	23,600
50,000	47,500	45,800	56,000	49,767
70,000	89,200	54,400	70,400	71,333

The results showed a clear correlation between the *in vitro* assays and gene expression (Figs 9-11). In every case, the concentration of estrogenic chemical needed to be greater than the EC_{50}s determined by the *in vitro* assays to result in a significant difference. In the case of NP treated fish, there was great variability in the response, suggesting that incomplete occupancy of ERs by this weak estrogenic chemical may account for this effect.

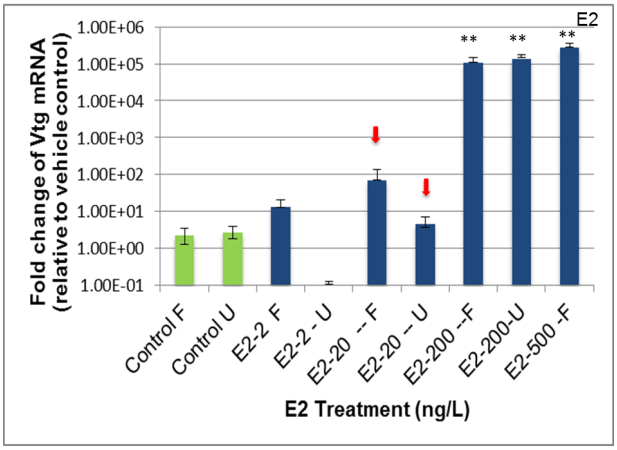


Figure 8. Effect of E2 dose on vitellogenin expression in female (F) and undifferentiated (U) Menidia after 28 days of exposure. Error bars are standard error of the mean. The red arrow in the figure denotes the EC_{50} point for E2 derived from *in vitro* assays. * p value < 0.001.

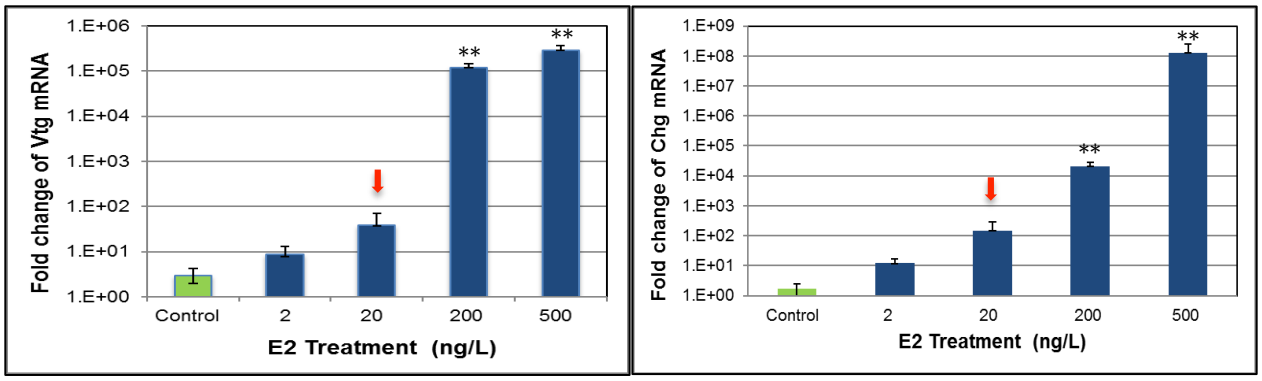


Figure 9. Q-PCR to determine differential expression of Vtg and Chg in juvenile *Menidia* exposed to increasing concentrations of E2 over 28 days. Error bars reflect standard error of the mean. The red arrows in the figures denote the EC_{50} point for E2 derived from the *in vitro* assay. ** = p value < 0.01.

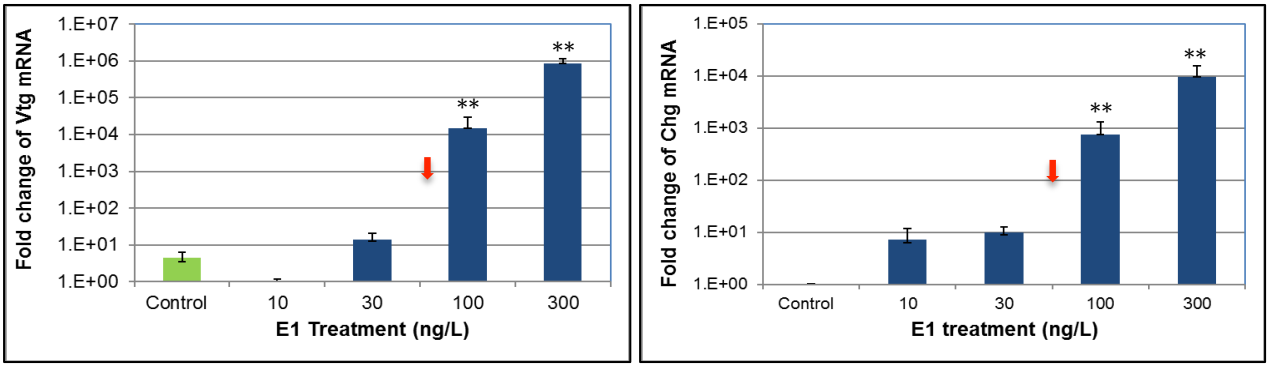


Figure 10. Q-PCR to determine differential expression of Vtg and Chg in juvenile *Menidia* exposed to increasing concentrations of E1 over 28 days. Error bars reflect standard error of the mean. The red arrows in the figures denote the EC_{50} point for E1 derived from *in vitro* assays. ** = p value < 0.01.

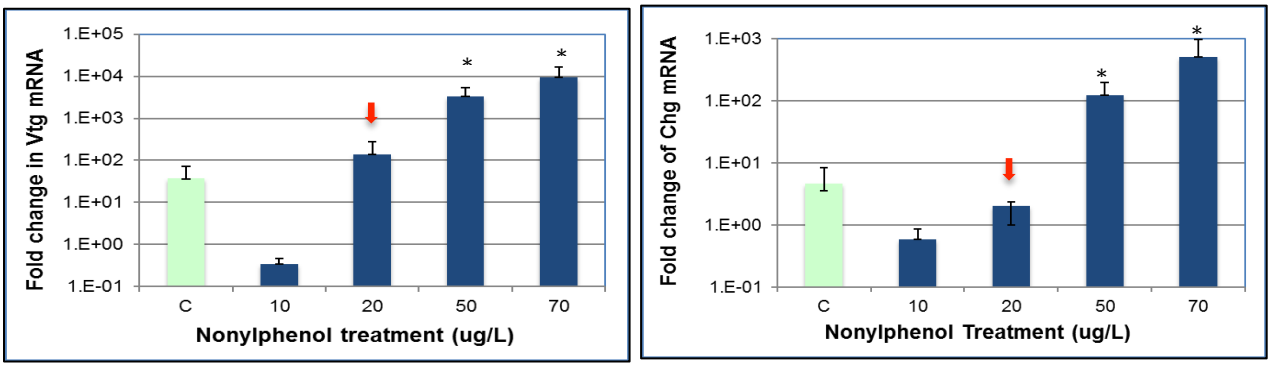


Figure 11. Q-PCR to determine differential expression of Vtg and Chg in juvenile *Menidia* exposed to increasing concentrations of NP over 28 days. Error bars reflect standard error of the mean. The red arrows in the figures denote the EC_{50} point for NP derived from the *in vitro* assay. * = p value < 0.05; ** p value < 0.01.

(c) Sex differentiation of the gonads determined by histology

Sexual differentiation was determined by examining sagittal sections of formalin-fixed fish for a region extending from the cloaca to the heart. The sex of each fish was verified by visual inspection using a compound microscope at 20, 40X and 60X. Figure 12 shows typical histological sections at 40X and 60X.

Exposures resulted in severe feminization of fish at the highest doses of E2 and E1, as expected. However, there were no changes in any of the NP-treated fish (Fig 13), but we were not able to achieve the same ratios with respect to the EC_{50} values for NP. In the E2 and E1 cases, the concentrations required to show a significant difference in sex ratio were above the EC_{50}s obtained with the *in vitro* assays, again showing the linkage between the *in vitro* assay and this higher order endpoint. The EC_{50} for E2 was 22 ng/L, but effects on sex ratio were not seen until 200 ng/L, suggesting that the effective dose is somewhere between 20 ng/L and 200 ng/L. For E1, the EC_{50} is 81 ng/L and we observed sex ratio effects by 100 ng/L. The lack of effects for nonylphenol is harder to explain. In the *in vitro* assay, the activity never reached the same plateau as for the stronger estrogens. Perhaps NP just does not occupy enough ERs to have a decisive effect on tissue differentiation even at the high dose of 70 µg/L. We were not able to expose them to 200 µg/L, as that was a lethal dose for the larval test.

(d) Effect of exposure to estrogens on growth over 28 days.

Growth was measured after 17 days of exposure by taking a photo of the juvenile fish in each aquarium and calculating their area and length from the pictures using image J (Schneider et al. 2012) and then again at 28 days of exposure by measuring the weight of the fish that were sacrificed. There were few effects seen at 17 days (Fig 14), mainly due to the high variability of sizes in each of the treatment conditions.

At the end of the 28-day exposure period, we sacrificed half of the fish for growth and sex ratio determination. The weights of the fish were measured by balance (Fig. 15). There are trends towards decreased body weight in each of the exposures. The high point seen in 20 µg NP/L is due to the presence of 4 significantly larger fish in that group.

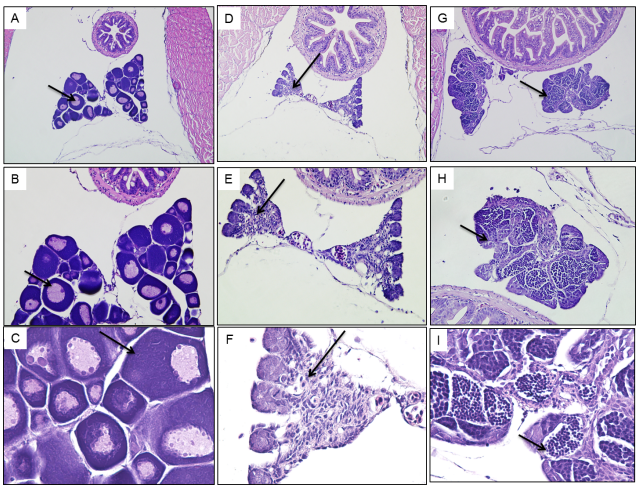


Figure 12. Histological sections of *Menidia* stained with Hematoxylin and Eosin stain (H&E stain). Typical sections showing (A-C) oogonia, typical in females; (D-F) undifferentiated gonadal tissue (gonia) and (G-H) spermatogonia, typical in males. Photomicrograph of sex differentiation top row is 20X, middle row is 40X and bottom row is 60X.

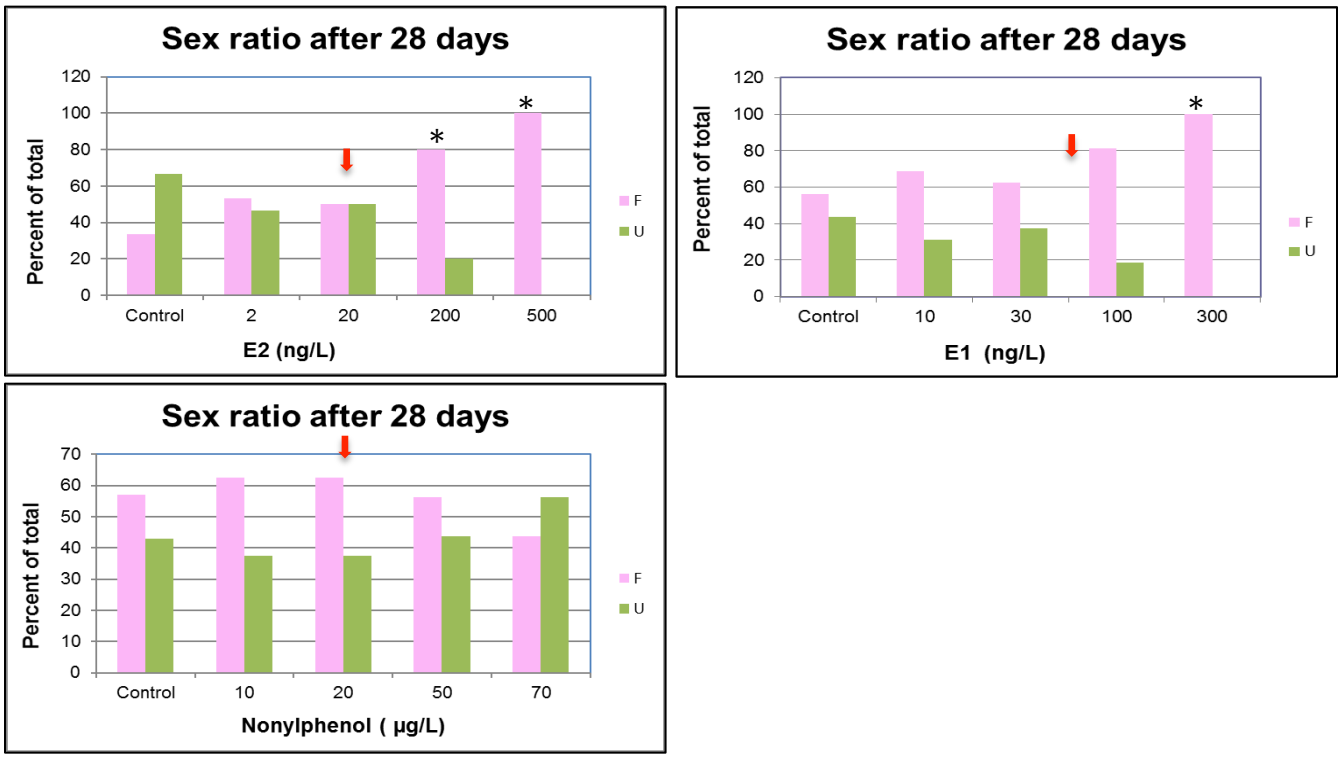


Figure 13. Sex differentiation of *Menidia* exposed to increasing estrogenicity over 28 days, starting with 30 dph fish. The red arrows in the figures denote the EC_{50} points for E2, E1 and NP derived from *in vitro* assays. *indicates statistical significance at $p<0.05$ and ** at $p<0.001$. F= female; U = undifferentiated.

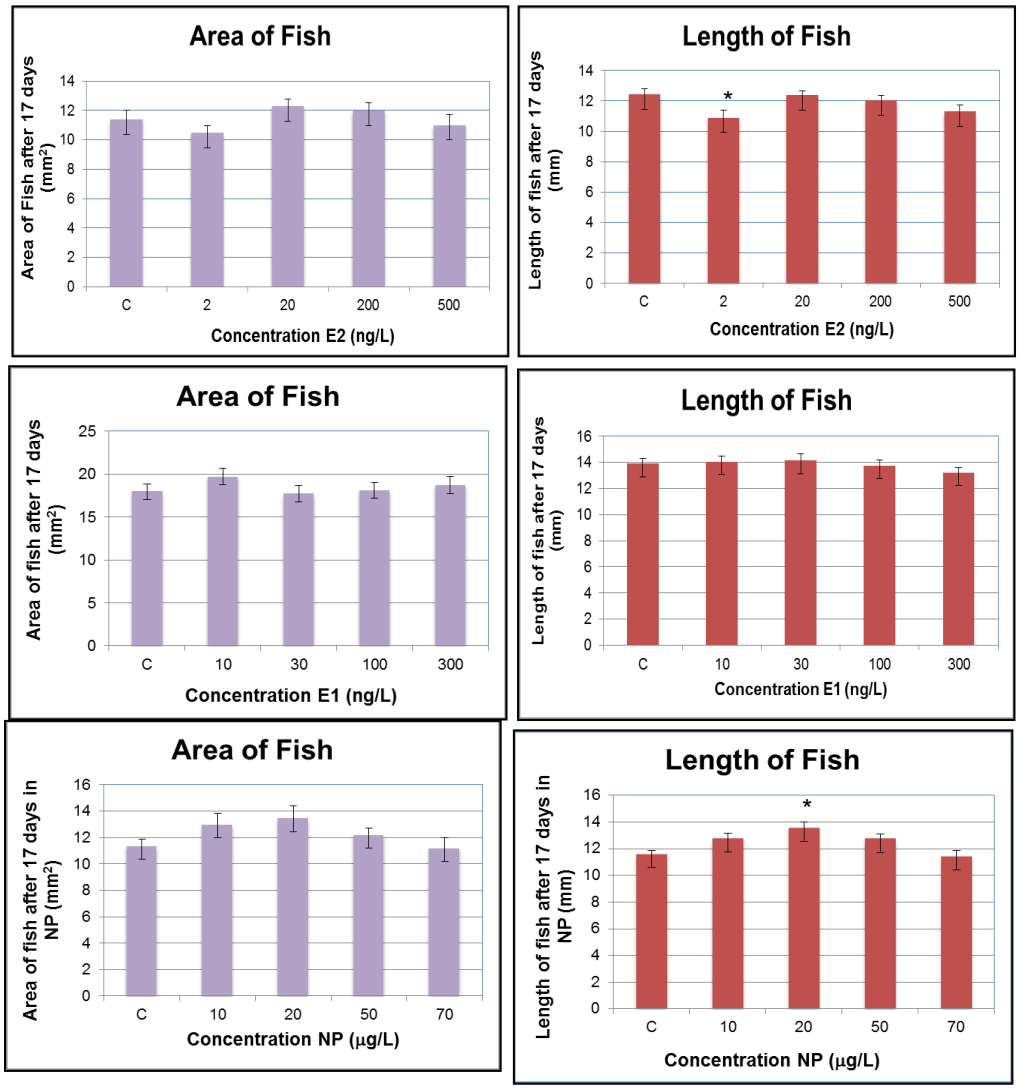


Figure 14. Area and length of fish measured after 17 days of exposure to E2, E1 or NP. Pictures were taken and analyzed using Image J. Asterisks above columns indicate comparisons that are statistically significantly different from control ($p<0.05$).

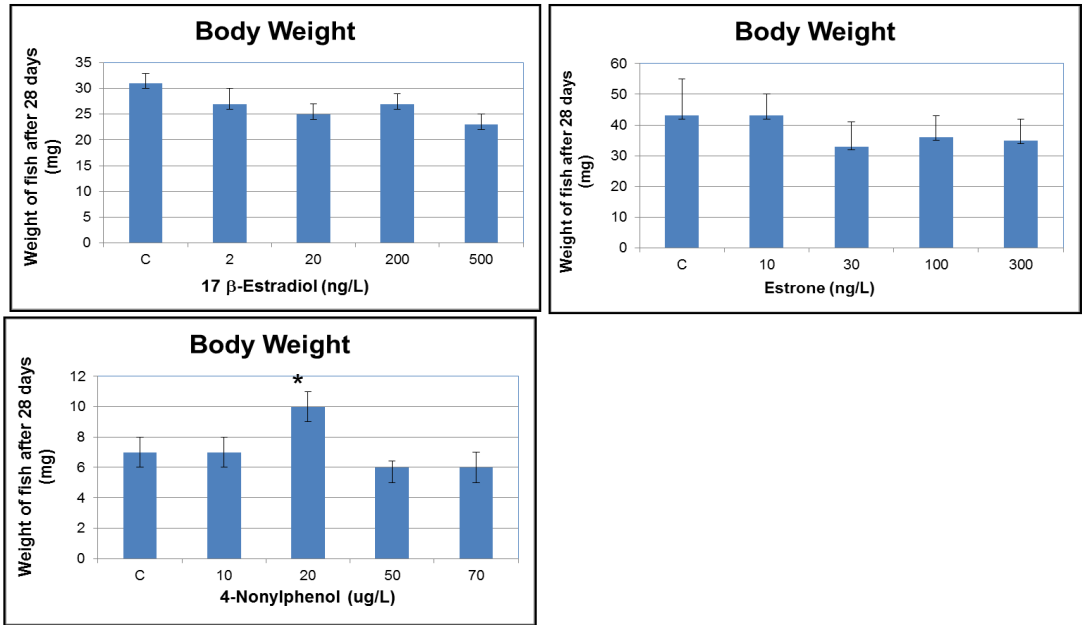


Figure 15. Body weight of fish measured after 28 days of exposure to E2, E1 or NP. Asterisks above columns indicate comparisons that are statistically significantly different from control ($p<0.05$).

(e). Exposure of juvenile *Menidia* to effluent for 28 days

The University of Florida received three shipments of effluent (60 gal each) from the water treatment plant in California for evaluation of estrogenic effects in juvenile fish. It took 4-6 days of transport across country by FEDEX in PTFE bag-lined barrels. When the effluent arrived, we noted that ammonia levels were greater than 200 mg/L and nitrite levels were in the toxic range (10-20 mg/L). The water samples were treated to remove ammonia and nitrite before using for fish exposures. Water samples were then adjusted to 15 ppt salt for exposures of juvenile fish. Details about the treatment are in **Appendix A**.

Samples of water were collected for analysis of estrogenic equivalencies immediately upon receipt of sample, after treatment to remove ammonia and nitrite and at the end of the exposures of diluted samples (Table 10). We used 5%, 20%, and 50% effluent exposures and de-chlorinated carbon-filtered laboratory water as a control. Water was distributed to quadruplicate tanks (8 L/tank) and was changed by 75% on a daily basis. Each shipment of water was used over 10 days and a liter was collected on the last day for estrogen equivalency measurements.

From data shown in Table 10, it is clear that the grab samples of effluent used for this experiment were variable in estrogenic equivalencies. It is likely that the extra 4 days of travel experienced for the second shipment caused further degradation of estrogenic equivalencies, as these were the lowest of the three shipments. Furthermore, it is clear that the treatment to remove total ammonia nitrogen and nitrites further degraded estrogenic compounds. Thus, the waters that were used for the exposures had very low levels of estrogen, below the detection limit of the assay.

Molecular endpoints were extremely variable among fish and did not show any trends. The effluent exposures did not appear to influence Vtg or Chg gene expression, two of the most sensitive biomarkers for estrogenic exposure.

Higher order endpoints were also evaluated. There was no overt mortality in any of the test exposures, thus the test solutions were not toxic to fish. We measured length and area after 17 days of exposure (Fig. 16) and overall body weight at 28 days of exposure (Fig. 17).

Table 10. GeneBLAzer ERa DA cell assay for effluents used in juvenile exposures

UF Sample ID	treatment	sampled	SCCWRP ID	Holding time (d)	GeneBLAzer ERa EEQ (ng E2/L)
Sew- GR I	pre-trt[a]	2.25.16	2016-0118	69	5
	post -trt[b]	3.15.16	2016-0119	69	< 0.48
	control	3.29.16	2016-0124		< 0.48
	5%	3.29.16	2016-0125	69	< 0.48
	20%	3.29.16	2016-0126	69	< 0.48
	50%	3.29.16	2016-0127	69	< 0.48
Sew- GR II	pre-trt (lost)[c]	3.8.16	2016-120	40	2.1
	post -trt	4.6.16	2016-121	40	< 0.48
	control	4.14.16	2016-128		< 0.48
	5%	4.14.16	2016-129	40	< 0.60
	20%	4.14.16	2016-130	40	< 0.60
	50%	4.14.16	2016-131	40	< 0.60
Sew- GR III	pre-trt	4.12.16	2016-122	21	7.1
	post -trt	4.28.16*	2016-123		1.2
	control	4.19.16	2016-132		< 0.60
	5%	4.19.16	2016-133	21	< 0.60
	20%	4.19.16	2016-134	21	< 0.60
	50%	4.19.16	2016-135	21	0.84

[a]pre-trt, effluent water sampled before treatment to reduce total ammonia to non-toxic levels
[b]post-trt, effluent water sampled post treatment
[c]pre-trt (lost), effluent barrels were lost on transport by FEDEX to UF, barrels arrived 4 days later than expected
*post-trt (4/28/16) – forgot to sample after treatment

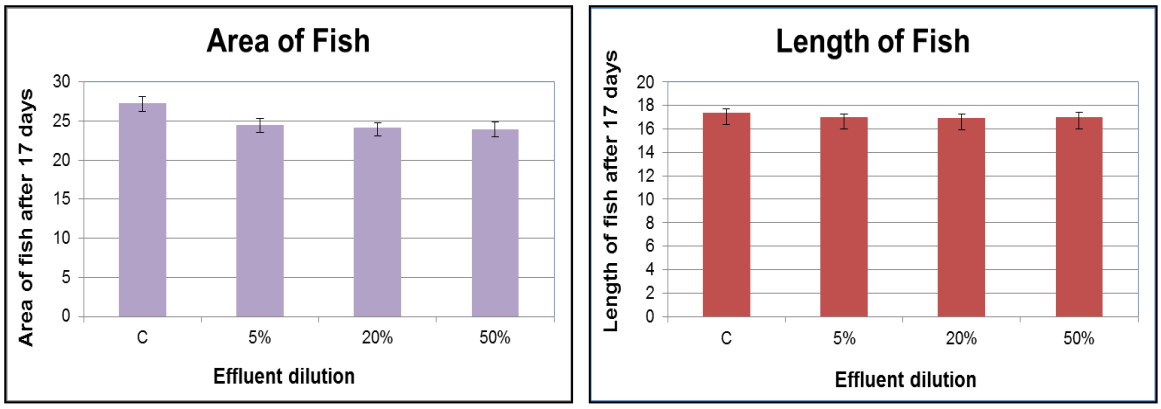


Figure 16. Area and length of fish after 17 days of exposure were measured from a picture taken at the same magnification using Image J.

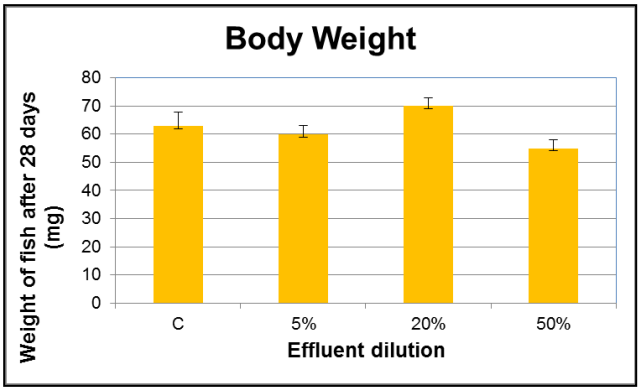


Figure 17. Body weight measured on a balance of fish exposed for 28 days to three dilutions of effluent. Letters over columns indicate comparisons that are significantly different ($p<0.05$).

In summary, the wastewater effluent that was tested had low estrogenic equivalency as determined by the *in vitro* assay (Table 10). At these levels of estrogenic equivalency one would not expect bioactivity through ERs. After 28 days, fish exposed to 20% effluent appeared larger than controls while those exposed to 50% effluent appeared smaller than controls, but neither of these measurements were significant. This effect was likely not through ERs, since biomarkers that are sensitive for estrogen signaling were not activated by the exposures up to 50% effluent. This also may be due to the high variability that exists in fish for this endpoint.

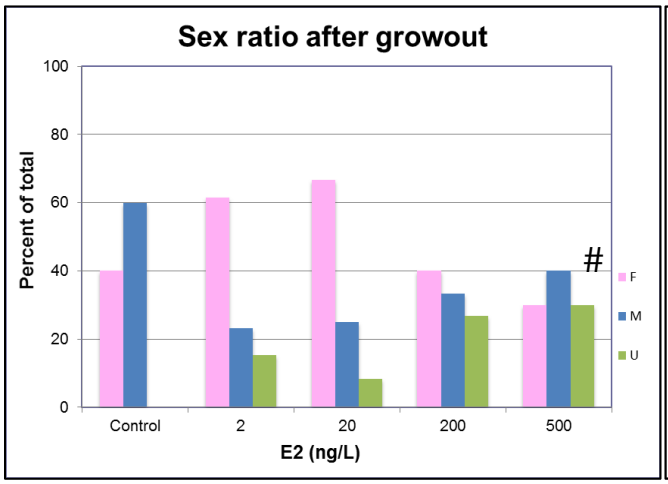
3. Grow out of *Menidia* in clean water for two months after exposures

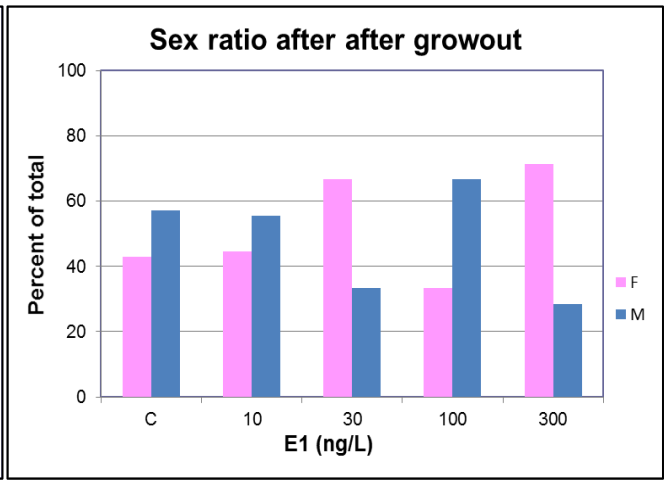
A subset of *Menidia* was transferred to clean water for an additional 60 days after the 28-day exposure to determine whether the effects seen immediately after the constant exposure regimen persisted. In addition, it was important to capture the sexual differentiation of testis in males. As for the 58-dph fish, we analyzed gonadal differentiation in the grow-out fish by histology (Fig. 18-19) and growth by weight of fish as well as by analysis of pictures using ImageJ for length and area (Figs. 19-21).

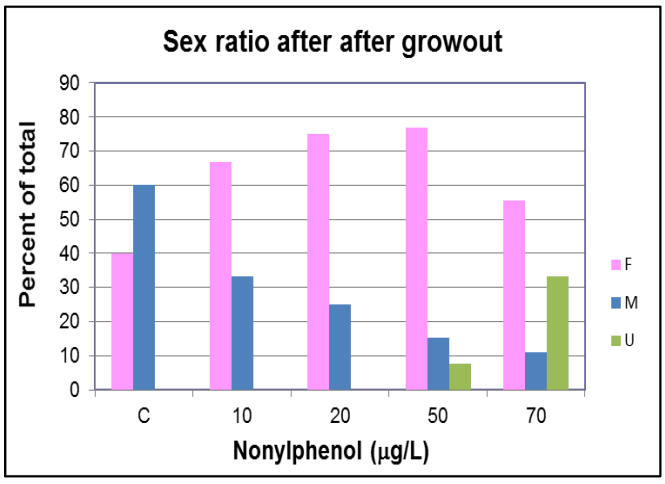
The results suggest that permanent changes may continue to persist for two months after the initial exposure to strong estrogens. For weaker estrogens, the tissue effects may be reversible.

(a) Sex differentiation

As mentioned above, females differentiate their gonads between days 35 and 45 but males require up to 120 days to differentiate testis. At the 58 dph time point (28 days of constant exposure to control or estrogenic substances) we observed females and undifferentiated tissues, as expected. The ratio of female to undifferentiated tissue was skewed to females if E2 or E1 concentrations were used above the EC_{50} for the respective *in vitro* assays. The ratio of females to undifferentiated fish increased in a dose-responsive manner with 100% of the fish presenting as female at 500 ng E2/L and 300 ng E1/L. Persistent exposure to strong estrogens is known to feedback to the hypothalamus to repress normal signaling for reproduction (Dickey and Swanson 1998; Colli-Dula et al. 2014). This would affect steroidogenesis that gives rise to both testosterone and estradiol. Both are needed for tissue differentiation to occur.







Numbers of fish per group in grow-out experiment					
E2 (ng/L)	C	2	20	200	500
# fish/ group	15	13	12	15	10
E1 (ng/L)	C	10	30	100	300
# fish/ group	7	9	12	6	7
4 NP (ug/L)	C	10	20	50	70
# fish/ group	5	6	4	13	9

Figure 18. Sex ratio determined by analysis of gonad histology for fish that were exposed for 28 days and then allowed to grow for two months in clean water. Fish were treated as controls or with increasing concentrations of E2, E1 or NP for 28 days and then placed in clean water for 60 days. Pink refers to confirmed females, blue to confirmed males and green to undifferentiated tissues. # (p=0.052)

After 2 additional months of culture in clean water, some of the effects from the early exposure were still present. At the highest concentrations of estrogens, we now saw some males, which were not evident at the 58 dph time point, suggesting that some of the fish were able to reverse from the female phenotype. Since we do not have a marker for genetic sex, it is not possible to determine if the ones that reverted were genetically determined to be male. For the strongest estrogen tested, E2, it seems that female tissues may have gone through apoptosis in order to form first undifferentiated tissue that was then converted in some fish to male tissue (Fig. 18). Reversal of gonadal tissue under the influence of hormones has been described for other fish such as zebrafish (*Danio rerio*) (Uchida et al. 2004; Sun et al. 2013), medaka (*Oryzias latipes*) and tilapia (*Oreochromis niloticus*) (Paul-Prasanth et al. 2013).

There are no published studies showing that *Menidia* can also reverse their gonads and more work is probably necessary to establish this. It was interesting to see that undifferentiated gonads were more noted in fish that had been treated with higher estrogen equivalencies. It also seems that the treatments delayed final differentiation, as by two months no undifferentiated fish should be present, based on our initial experiments on sexual differentiation. New studies suggest that there may be a biomarker for genetic sex in *Menidia* but further work is necessary to validate this biomarker.

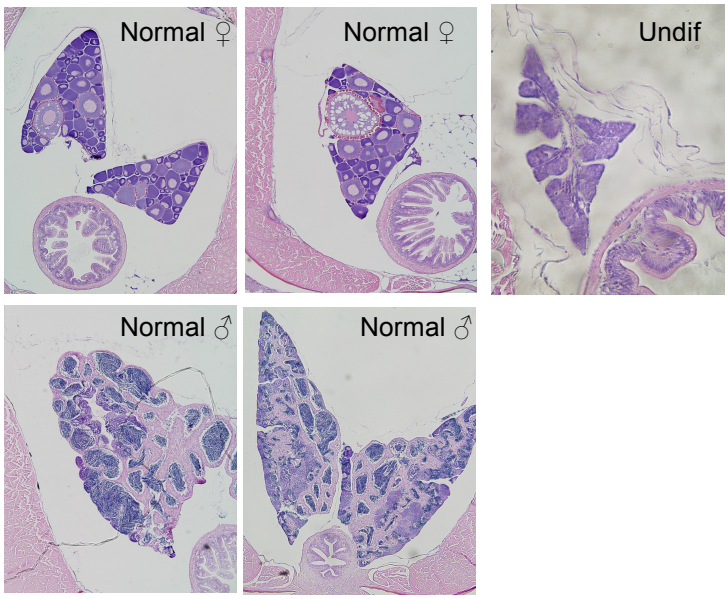


Fig. 19. Representative histology used to distinguish females from males and undifferentiated tissues after 2 months in clean water.

Examples of histology slides that were used to distinguish females from males and undifferentiated tissues are shown in Fig. 19. At the high concentrations of E2, only one female in the treated group was fully vitellogenic. The other females showed gonadal atresia (Fig. 20). Effects for sex differentiation from exposure to NP seemed to follow the same pattern as seen for E2, with the higher concentrations of NP resulting in several fish with undifferentiated gonadal tissues. In this case, it is possible that exposure to NP simply delayed gonadal differentiation for males. The lack of statistical significance is due to the small sample size. Future work should increase “n”. In the case of E1, the effects observed in 58 dph fish seemed

to reverse for males, producing more males than females in the 100 ng E1/L condition but still maintaining a higher proportion of females in the 300 ng E1/L condition.

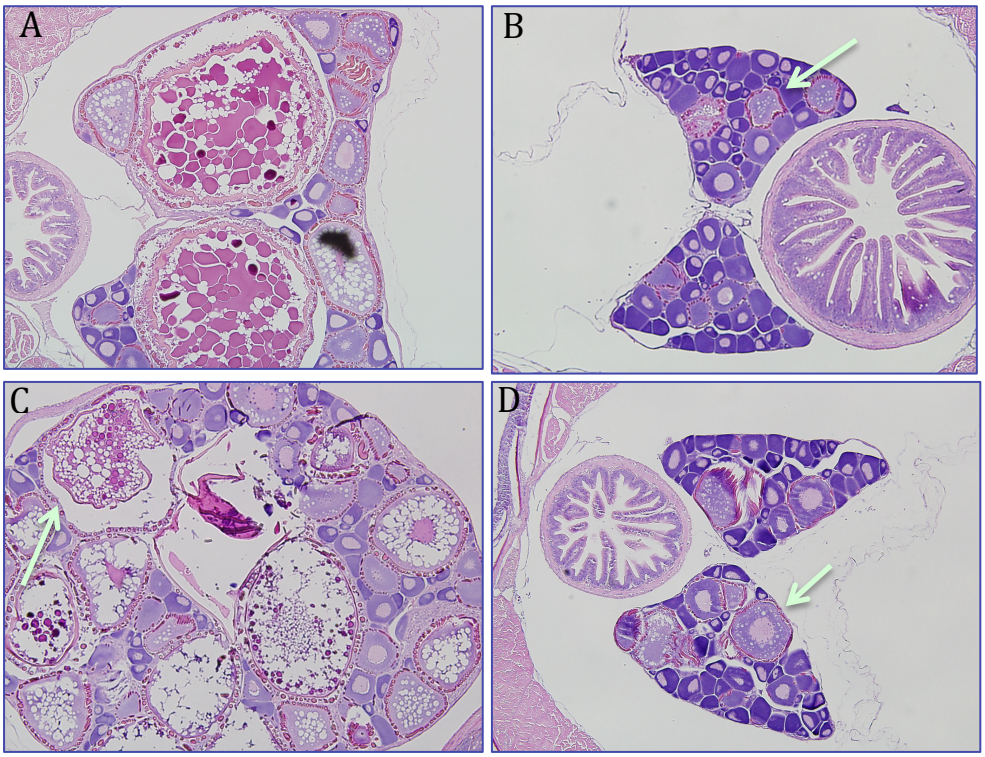


Figure 20. Female gonadal histology in fish 188 dph. Histology of (A) a control mature oocyte with Vtg (pink material) in the ovary of a control fish and atretic oocytes in *Menidia* exposed to (B-C) 20 ng E2/L and (D) 500 ng E2/L for 28 days and then cultured in clean water for two months. Oocytes should be filled with Vtg at this stage.

(b) Growth

We also measured growth after the two months in clean water either by weighing the fish or by taking the total length of the fish and area from a picture and using ImageJ to evaluate growth among groups (Fig 21). Effects on body weight appeared to differ by chemical. In the case of E2, fish treated with 20 ng/L seemed to be larger than other fish, perhaps reflecting the stimulation of growth *in vivo* by low concentrations of E2 (Ng et al. 2001). For E1, the opposite effect was seen, where there was a dip in body weight just below the EC_{50}, and thereafter there was a steady increase in body weight. In the case of nonylphenol, fish decreased in body weight at the EC_{50} and showed a progressive dose response decrease in weight. Some of this unevenness may be due to inherent variability of this endpoint with outbred fish.

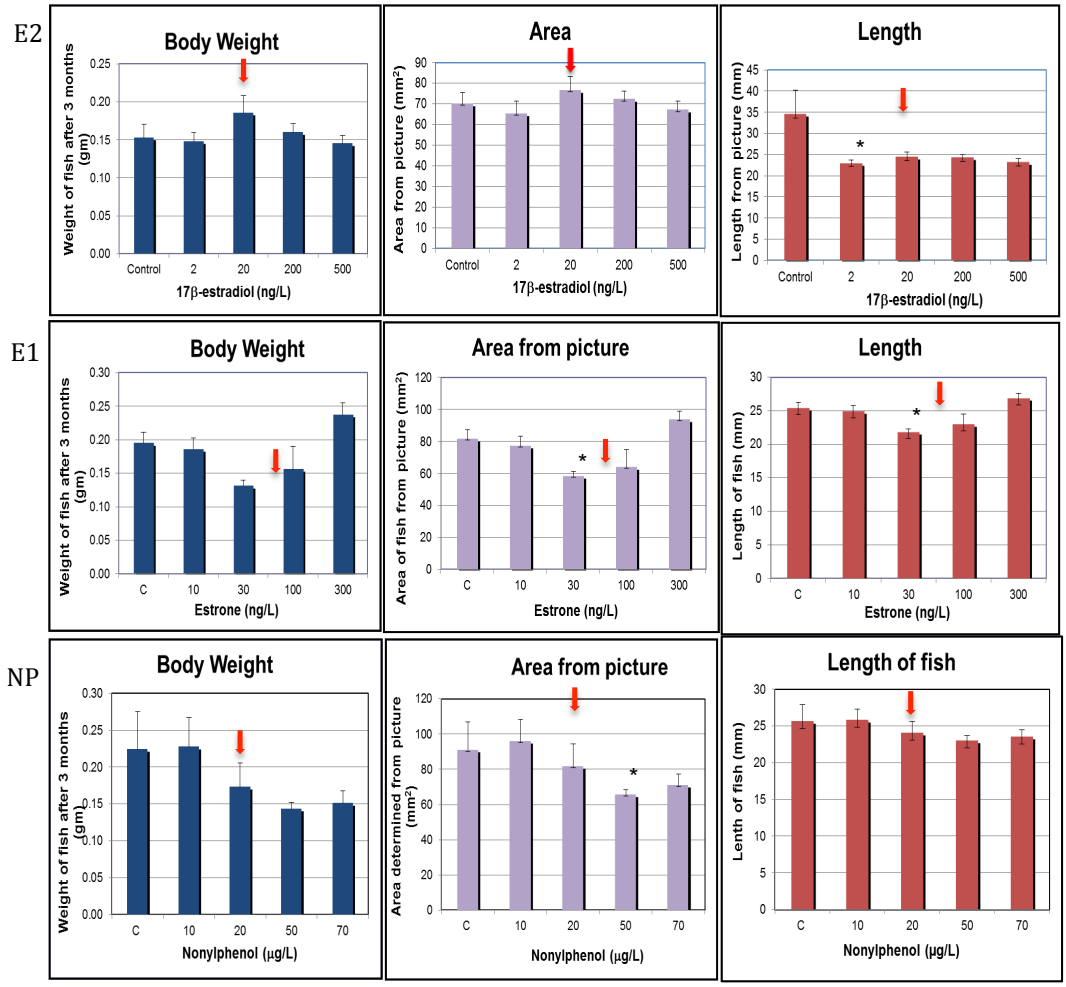


Figure 21. Body weight (measured by balance) and measurements of area and length from pictures by ImageJ. Unexposed fish and fish exposed to increasing concentrations of E2, E1 or NP for 28 days were then placed for two months in clean water. Error bars represent standard error of the mean. * indicates $p<0.05$.

(c) Exposures to effluent

There was no effect on sex ratio for fish exposed to effluent dilutions for 28 days and then placed into clean water for 2 months (Fig 22). This was expected, as there were no effects immediately during the 28-day exposures to effluent dilutions, in which estradiol equivalents were lower than the EC_{50} for the *in vitro* tests.

Exposure of fish to 5% and 20% effluent had no effect on body weight, an observation that was similar to the findings at 28 days of exposure (Fig. 23). Fish exposed to 50% effluent were significantly smaller than controls. This followed the same trend that was observed at 28 days of exposure in the diluted effluent, suggesting that the additional two months in clean water did not reverse the effects on growth seen from the initial exposures. Measurements from pictures of the fish via Image J, showed to statistical significance among the groups.

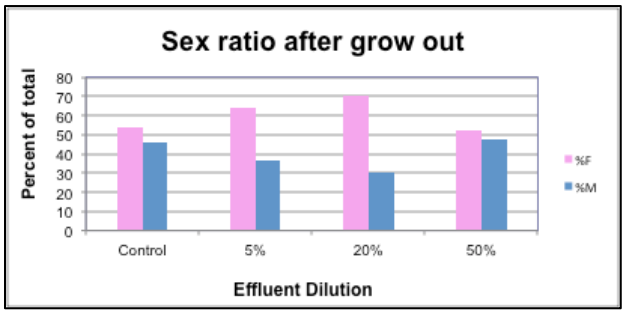


Figure 22. Sex ratio of *Menidia* exposed to diluted effluent for 28 days and then placed into clean water for 2 months. There were no statistically significant alterations in sex ratio in the tested effluent dilutions.

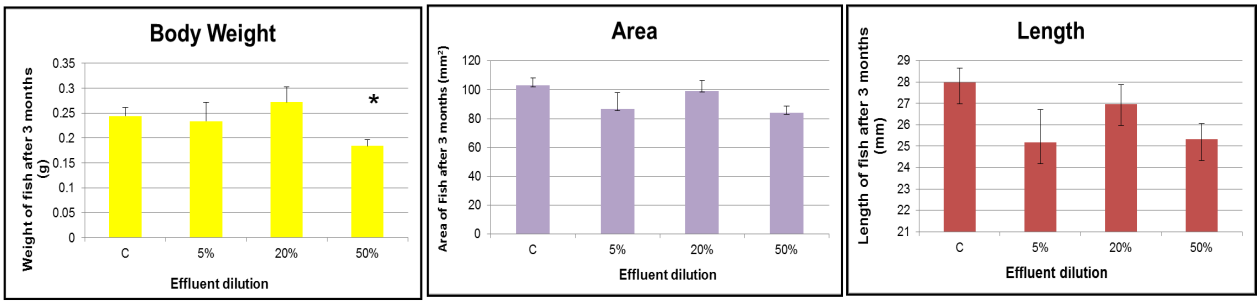


Figure 23. Measured body weight (measured by balance), area, and length obtained from a picture of *Menidia* that were originally exposed to diluted effluent for 28 days and then placed in clean water for two months. Error bars are standard error of the mean. Asterisk above the column indicates significantly lower body weight compared to controls ($p<0.05$).

Discussion

The goal of the experiments was to determine whether it would be possible to link activity in ER-transactivation *in vitro* assays with apical changes *in vivo* in *Menidia* with increasing concentrations of estrogenic chemicals. Our original hypothesis was that estrogens effect their changes in apical endpoints by binding and transactivating ERs, which in turn alter gene expression as the basis for the phenotypic changes observed. If the critical point were the transactivation of the ER, then only concentrations above this level would be needed to have effects on higher organismal levels. This idea is consonant with the adverse outcome framework (Fig. 24) that has as its premise that contaminants initiate their adverse outcomes through a series of steps that begin with a molecular initiating event (Ankley et al. 2010).

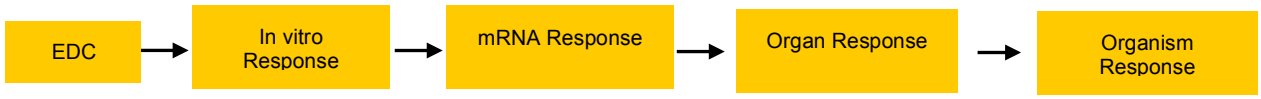


Figure 24. Representation of an adverse outcome pathway (AOP)

We were able to confirm with our study that this paradigm works for strong estrogens that can be found in wastewater such as estradiol (E2) and estrone (E1) and to a lesser extent for weak estrogens such as 4-nonylphenol (NP). In larval studies (10-17 dph), the individual estrogens

had no significant effect on growth and survival, except for a trend in lower weight observed for NP and mortality at the highest concentration. These endpoints were also not affected by exposures of up to 25% effluent. However, there was a clear effect by E2 and NP on gene expression with increases in Vtg, Chg, and Cyp19b at concentrations higher than the EC_{50}s measured by the *in vitro* assay.

For juvenile fish (30-58 dph), exposure to the chemicals for 28 days clearly showed effects on tissue differentiation (tilting the ratio towards females) but only for the strong estrogens E2 and E1 and only at concentrations that were higher than the EC_{50}s measured in the *in vitro* assays. Further culture of *Menidia* for an additional two months in clean water reversed some of the severe ratio tilting towards females. Interestingly, it appeared that some of the fish were able to reverse the estrogenic effects on tissue differentiation, with many fish appearing as either undifferentiated or male. We had calculated that by the end of the experiment all fish would be terminally differentiated, but it appeared that the effects of estrogen were reversed and fish were readjusting to their genetic sex. Reversal of estrogenic effects has been observed in other fish (Paul-Prasanth et al. 2013) and this point needs to be further investigated for *Menidia*.

Superposition of higher order effects from larvae and juvenile fish on the *in vitro* dose response curve (Fig 25), suggests that the *in vitro* assays are more sensitive than *in vivo* but with not as pronounced a difference as we hypothesized in the beginning. We only have a few concentrations for the *in vivo* assays, and better precision of measurements would indicate a better fit.

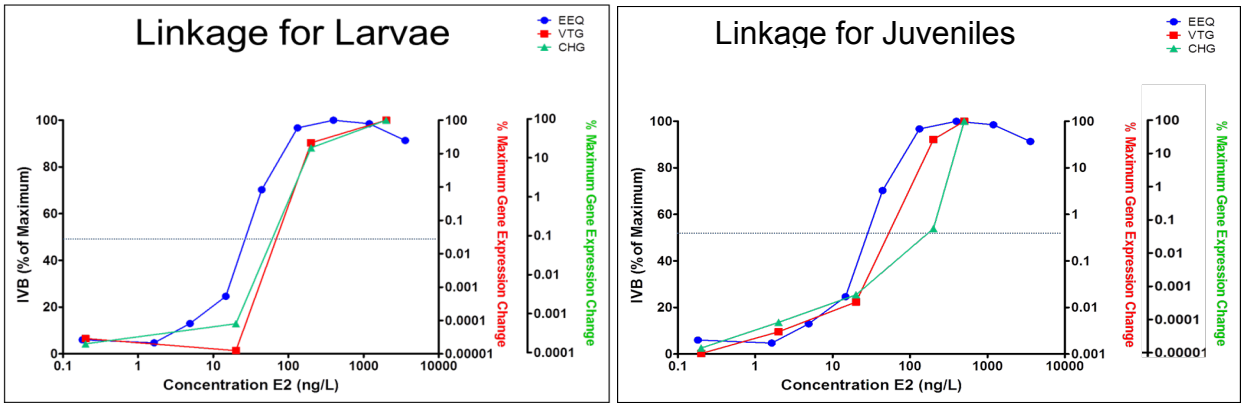


Figure 25. Superposition of gene expression endpoints onto the curve for the in vitro GeneBlazer receptor assay for estrogenic equivalents (EEQ). For each data set, numerical values were converted to percent of maximum response. In the case of gene expression, for larvae the maximum increase was 6 million- and 372,000-fold for Vtg and Chg, respectively at 2000 ng/L. In the case of juveniles, maximum increases were 290,000- and 125 million-fold for Vtg and Chg, respectively at 500 ng/L.

In effluent exposures, the measured estrogen equivalencies in the dilutions used were all less than the EC_{50}'s for E2. Therefore, we were not surprised to see an absence of effects on growth and survival for the early stage fish or on endpoints of concern in juvenile fish.

Conclusions:

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

   E2 > E1 > NP >> BPA >> GAL

2. These chemicals did not statistically affect survival and growth for either the larval or juvenile stage fish. There was a trend towards decreased growth in larvae, but this was not statistically significant.

3. Gene expression studies for *Menidia* larvae indicated that concentrations above the EC_{50} for the *in vitro* assay were necessary to see changes in genes known to have estrogen response elements (EREs) in their promoters, including Vtg, Chg, and Cyp19b.

4. Juvenile fish gonadal differentiation and growth endpoints were sensitive to estrogens, but only at doses that were greater than the EC_{50}s measured in the *in vitro* assays.

5. Gene expression in juveniles also required concentrations above the EC_{50}s for significant effects.

6. Females with differentiated gonads responded to estradiol at lower concentrations than undifferentiated fish, suggesting that they were primed for estrogen activation of ERs.

7. Some of the early effects on tissue differentiation observed at 58 days appeared to be reversed with two additional months of culturing in clean water. However, exposure during tissue differentiation to the higher concentrations of the strong estrogens seemed to have effects that persisted even after two months in clean water.

8. Wastewater effluents had measurable but low concentrations of estrogenic equivalency (17 ng/L for 100% effluent, measured immediately after collection, and 5-7 ng/L, measured after shipment to Florida). Denitrification and pH adjustment to eliminate ammonia seemed to also remove estrogenic equivalencies, potentially through microbial action. Dilutions of tested effluent were below the EC_{50}s measured by the *in vitro* assay. There were no observable effects on higher order endpoints of survival and growth of larvae and or growth in juveniles. There were no observable changes in gene expression for sensitive estrogen biomarkers in either larvae or juveniles.

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Appendix A: Materials and Methods -Year 2

A. Fish larval exposures

Inland silversides (*Menidia beryllina*) purchased from Aquatic BioSystems were acclimated and tested at SCCWRP as described in Appendix B for year 1. Nine-day old *M. beryllina* larvae were purchased from Aquatic BioSystems. Upon their arrival, larvae were inspected to remove the smallest and largest ones and the remaining fish were acclimated in 1 L glass beakers containing 800 mL of diluted natural seawater 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.

Two separate experiments were conducted using a semi-static system with 75% of the test solutions replaced daily. In experiment 1, larvae were exposed for 7 days to 17β-estradiol (0.02, 0.2, 2 µg/L), nonylphenol (20, 70, 200 µg/L), a vehicle control (0.005% triethylene glycol; TEG) and a seawater control. All test solutions were prepared daily. In experiment 2, larvae were exposed to three dilutions of the WWTP effluents (5, 10, 25%), an ammonia control (~8 mg/L), and a seawater control. Three batches of 24-hour effluent composites were used during the 7-day effluent exposure. Upon arrival at SCCWRP, each batch was immediately diluted with natural seawater and aerated for 24 h to reduce the ammonia levels. Each batch of diluted effluents was used to replace the exposure water daily for up to 3 consecutive days before a new batch was used.

All the exposures consisted of four replicate beakers per treatment and the beakers were maintained in a water bath to maintain consistent water temperature. Larvae were fed newly hatched brine shrimp throughout the exposure until 1 day before the end of the exposures. Feeding regime was 0.03 g wet weight per beaker on day 0 - 2, and 0.05 g wet weight per beaker on day 3 – 6. The quantity was adjusted in exposure beakers presenting >20% mortality according to the US-EPA recommendations. Water quality parameters were routinely measured and maintained throughout the exposures within the following range: temperature of 24 ± 1 °C, salinity of 15 ± 1 ppt, dissolved oxygen > 5 mg/L and pH 7.4 ± 0.1 (Appendix A, Table 1). Total ammonia levels were kept below 1 mg/L in all seawater controls and single chemical exposures. However, the levels were higher in the effluent exposures (up to 10 mg/L).

Table 1. Water quality parameters for larval exposures to single chemicals

Water quality parameter	Average	Range among treatments
Temperature (°C)	24.7	24.4 – 24.9
Salinity (‰)	15.3	15.2 – 15.6
Dissolved oxygen (mg/L)	5.85	5.81 – 6.01
pH	7.4	7.3 – 7.4
Ammonia	0.3	0.26 – 0.38

B. Water chemistry for larval exposures

Composite water samples (from all 4 replicates per treatment) were collected for chemical analyses and stored at -20 °C until further analyses at SCCWRP. Samples were preserved with 5 mL methanol, pH adjusted to 7 using 1M hydrochloric acid and solid phase extracted using

Oasis HLB 6cc cartridges. The Abraxis semi quantitative ELISA kits were used for quantification. Data for these analyses are presented in the report.

C. Apical and molecular endpoints for larval exposures

The number of dead larvae was recorded daily and used to calculate the percent survival for each treatment. Effects of estrogenic chemicals 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. Fish for the individual chemical exposures were processed for RNA at SCCWRP using Trizol Plus RNA purification kit. RNA samples were frozen at -80 ^{o}C and shipped on dry ice to the University of Florida for Q-PCR.

Larvae from the effluent exposures were flash frozen in liquid nitrogen and preserved at -80 $^{\circ}$C. The samples were sent to University of Florida for RNA extraction using RNA Stat-60, cDNA synthesis and evaluation by Q-PCR.

D. Bioscreening of effluents

Composite WWTP effluent water samples from the four CA WWTP plants and the diluted effluent samples used in the larval exposure were collected in amber glass bottles containing 50 mg ascorbic acid and 1 g sodium azide for preservation. All samples were processed by solid phase extraction using Oasis HLB cartridges (200 mg, Waters) within 72 hours of collection. Samples were eluted in DMSO and analyzed using the GeneBLAzer ERa assay according to the procedure described previously (Mehinto et al. 2015).

E. Statistical analyses for larval exposures

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 $p < 0.05$.

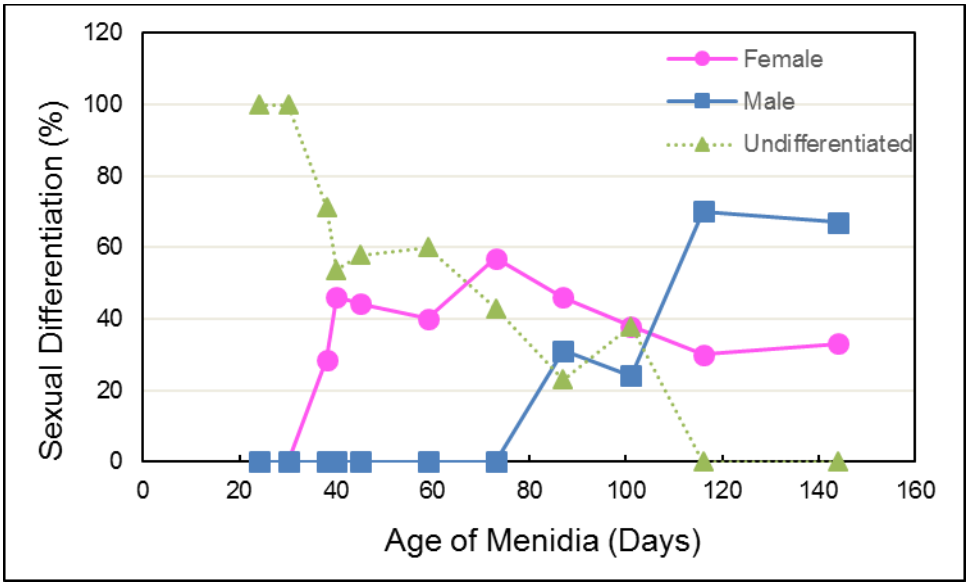
F. Juvenile fish exposures

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.

G. Verification of sexual differentiation in fish.

We found that gonadal differentiation at 23 ±1 ^{o}C occurs between days 30 and 45 for females and between days 87 and 120 for males (Appendix A, Fig. 1). This species also uses temperature for sex determination (Conover and Fleisher 1986; Duffy et al. 2010), so it is important to monitor temperature. By starting our exposures at 30 dph and expanding our exposures to 28 days in year 2, we were able to capture the period of female gonad differentiation, which appears to be sensitive to estrogens.

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.

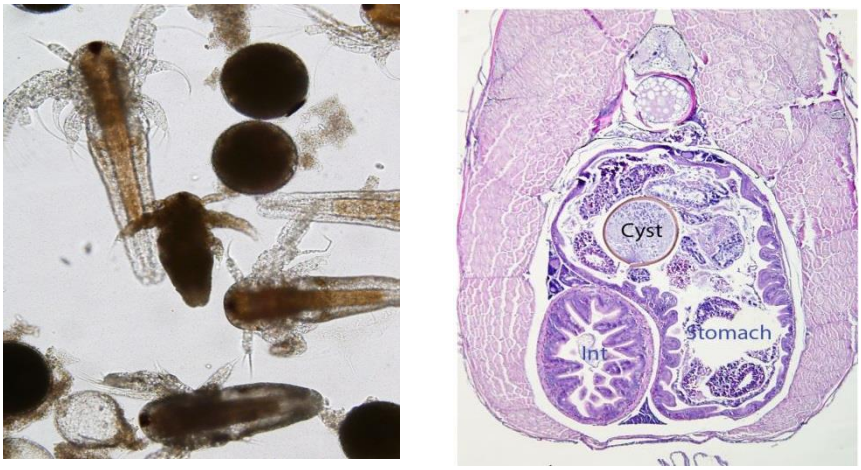


Appendix A, Figure 1. Gonadal tissue differentiation in *Menidia* over time. *Menidia* were cultured at 23 ºC and 15 ppt salinity. Females are depicted by pink circles, males by blue squares and undifferentiated tissue by green triangles. The figure is a compilation of three different experiments. Each time point n=16 fish.

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 would be present at slices anterior to the genital pore. Sex was verified and checked by two persons for each individual.

H. Diet for juvenile *Menidia*

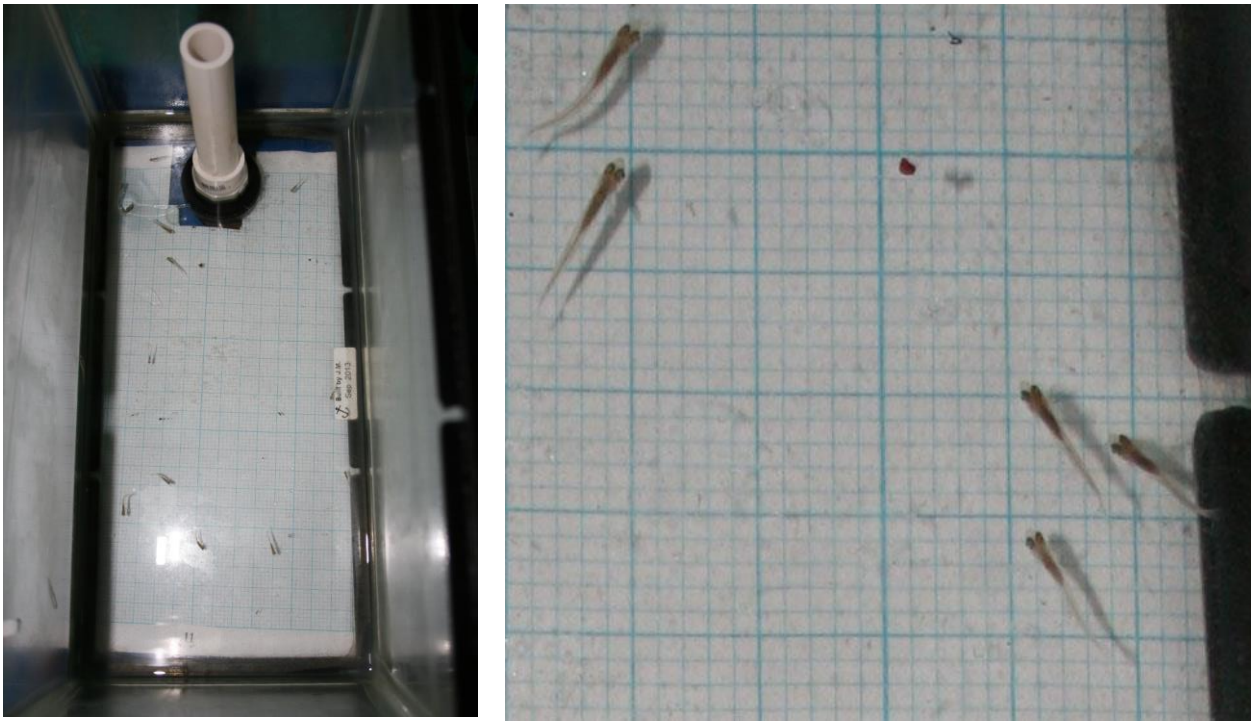
The high mortality of fish across all conditions in year 1 found after the 2nd week of treatment was due to unhatched artemia cysts, provided as a food source. The unhatched eggs are about the same diameter as the gastrointestinal tract of the juvenile *Menidia,* and when these are eaten by the *Menidia*, they get stuck and do not pass through (Appendix A, Fig. 2). We resorted to using a new product, E-Z egg, that are shell-free artemia eggs.



Appendix A, Figure 2. Histology comparing the size of the unhatched artemia cysts and the gastrointestinal tract of *Menidia*. The cysts appeared to be getting stuck in the intestine and caused lack of growth and finally death after about 14 days.

I. Evaluation of growth of juveniles

A better method was developed to evaluate growth in juvenile *Menidia*, based on length and circumference from pictures taken during the month long exposures. Because of initial issues with these measurements in year 1, the experiments were completely repeated in year 2.



Appendix A, Figure 3. Digital images of fish that were used to evaluate length and area of fish. Small squares = 2 mm.

Growth of the fish in each aquarium was measured at three time periods: 0, 17, and 28 days after exposure. At these time points the aquarium was drained to about 0.75 cm and each was

photographed with a digital camera. The low volume ensured each fish was in the same focal length and depth. Each aquarium had grid paper underneath the entire tank with 2.0 mm squares to be used as a standard length reference.

Individual length and area of each fish was measured using the ImageJ program. The 2.0 mm squares beneath the tank were used to calibrate the photo and then make measurements of each live fish within the tank. At the end of the experiment, four fish were randomly removed from each aquarium, weighed, and digitally photographed (with reference length). Data at time of takedown and live time intervals were used to measure and compare growth within an experiment.

J. Exposure system

The same exposure system described in year 1 was used with minor modifications. A 50 gal (200 L) fiberglass source tank containing exposure solution supplied four 2.5 gal (10 liter) glass aquariums with test water. Each system used a pump and ChemFluor™ tubing 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 contained fifteen 30-day post hatch *Menidia* in 15 ppt artificial seawater and tanks were maintained at 23 ±1°C and constant photoperiod (18L:6D). Water quality was intermittently checked to ensure lack of ammonia or nitrite.

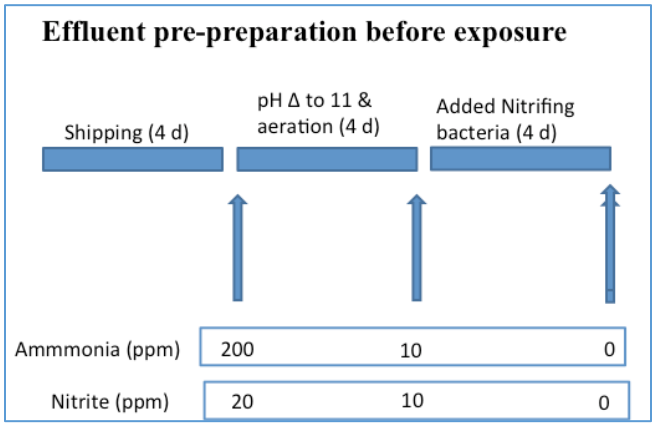
In order to ensure water quality**,** the volume of exposure water of each aquarium was increased 50% to 6.0 liters. Water was refreshed at 65% (4.0 L) of the solution daily. The increased exposure volume and exposure duration required making up 3 different batches of fresh exposure solution during the experiment. Fresh solutions were made every 9 days. A 1 liter water sample was taken from the source tank 9 days after it was made for water chemistry verification and stored at 4 °C.

Exposure Solutions

The fish were exposed to 3 lab made solutions (estrone, 17β-estradiol, and nonylphenol) and final effluent collected from a wastewater treatment facility in CA. The individual chemicals used were dissolved in ethanol and then a carrier, triethylene glycol, to make spiking solutions for each dose. The TEG concentration was maintained at a constant concentration, 50µl/ liter of test water.

Wastewater effluent

Three different 60-gallon grab samples were shipped to the University of Florida for use as an exposure solution. Effluent post treatment was collected into a PTFE bag lining 2 x 30 gallon steel barrels. This was shipped FEDEX truck freight, which took about 4-5 days. The water from each barrel was checked for the presence of chlorine, ammonia, and nitrite. Each shipment contained fish-lethal quantities of ammonia (30-200 ppm), and nitrite (20-30 ppm). In order to remove the ammonia, the pH of the water was adjusted to pH 11.5 using NaOH and heavily aerated. When the ammonia decreased substantially (ie. 200 → 20 ppm), the pH was adjusted to 7.5, and nitrifying bacteria (Turbo Start Fritzyme™) were added to remove the remainder of the ammonia and nitrite. These steps are illustrated in Appendix A, Fig. 4. When the ammonia and nitrite were non-detectable, the salinity of the effluent water was adjusted to 15 ppt using Instant Ocean™ and verified using a refractometer. This water was diluted with 15 ppt artificial seawater made with carbon filtered city water further filtered through a 75 µM filter to remove any debris.



Appendix A, Figure 4. Schematic showing how effluent water was prepared for exposures at the University of Florida.

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 usinq Script TM kit (Quanta). Quantity and quality of RNA was measured using Nanodrop. cDNA was diluted 1:20 and mixed with SYBR Green (SsoAdvance^{TM}, Biorad) containing the forward and reverse primers (10 µM) for each gene of interest. Gene expression quantification was made using the CFX Connect Real-Time System (Biorad).

K. Water Chemistry

Reference chemicals

One liter of test water from exposure solutions was removed from the tanks every 10th day and after 28 days of exposure. This represents the lowest concentrations of reference chemical that the fish experienced. For estrone and 17β-estradiol experiments the test water was concentrated on a C-18 solid phase extraction column (Agilent). Nonylphenol water samples were concentrated on NEXUS columns (Agilent). After elution and dilution into the test range, the concentrations of estrone, 17β-estradiol, and nonylphenol were measured using ELISA kits (Abraxiskits.com).

Wastewater Effluent

One liter of final effluent was collected at 3 different phases of the experiment: 1) Neat after received; 2) Neat post pH change, air bubbling, and addition of nitrifying bacteria; and 3) Diluted test solution (0, 5%, 20%, 50%). Samples collected at each of these phases were concentrated using Oasis HLB columns (Waters). Columns were shipped to SCCWRP for analysis.

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Appendix B

Linkage of *In Vitro* Assay Results With *In Vivo* End Points
Final Report – Phase 1
June 2, 2014
Revised November 21, 2014

University of Florida: Nancy Denslow, Kevin Kroll, Olanike Adeyemo, Candice Lavelle, and Sumith Jayasinghe

SCCWRP: Alvina Mehinto, Steve Bay and Keith Maruya

Linkage of *In Vitro* Assay Results With *In Vivo* End Points
Final Report – Phase 1
November 26, 2014

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 (Fig. 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 Fig. 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 Fig. 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.

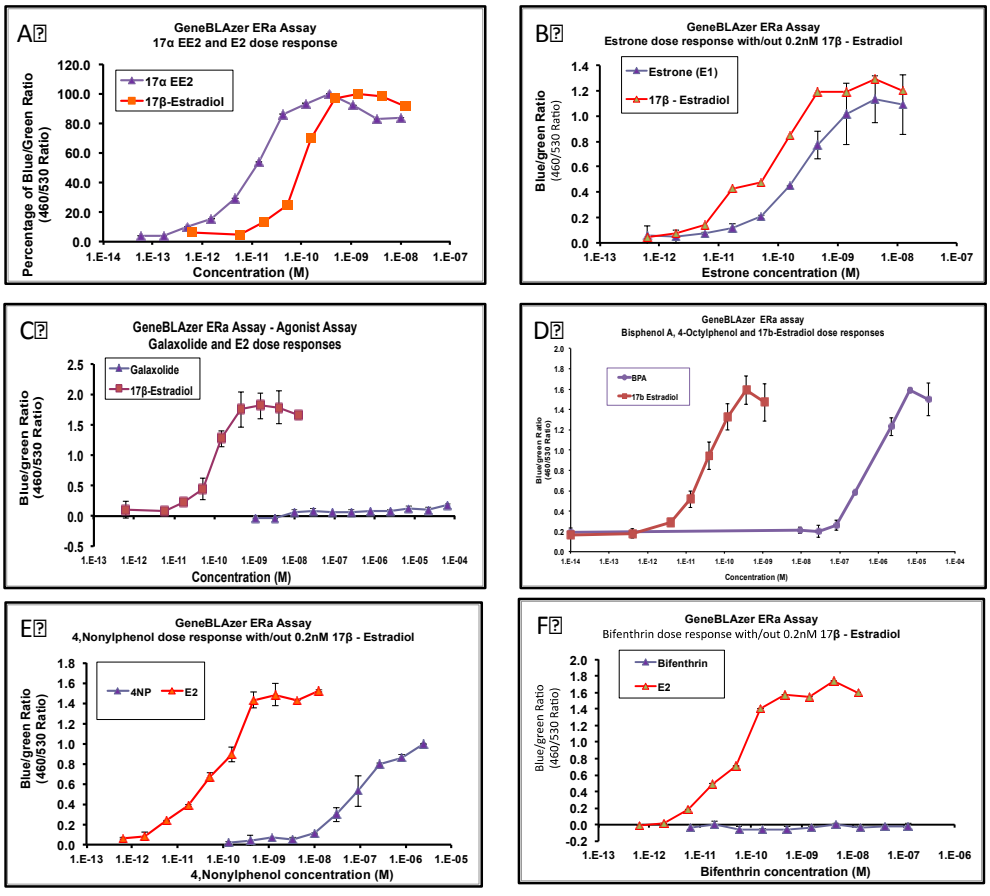


Figure 2: Dose response of InVitrogen ERα GripTite Division Arrested cells to strong and weak ER agonists. Cells were plated in triplicate in 96-well clear bottom plates and dosed with strong and weak ER agonists for 18 h in the presence of 0.5% DMSO, loaded with LiveBLAzer™-FRET B/G substrate (2 h), 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 (17α–ethinylestradiol, EE2) was performed, as well, in order to compare its response with the weaker estrogens. We saw positive signals for EE2, 17β–estradiol (E2), estrone (E1), 4-nonylphenol (NP) and bisphenol A (BPA). There was no signal in agonist mode for bifenthrin (BF) and an extremely weak signal for galaxolide (GAL) (**Fig. 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)	Concentrations in ng/L
17a-ethinyl estradiol (EE2)	1.11E-11	3.3
17b-estradiol (E2)	7E-11	22 ± 2.1
Estrone (E1)	2.52E-10	81 ± 14
4-Nonylphenol (4NP)	8.57E-8	20,500 ±1,640
Bisphenol A (BPA)	4.7E-7	107,000

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 (Fig. 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 capable of activating 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).

In the case of bisphenol A we see no effects at the lower concentrations but we see antiestrogenic activity at concentrations higher than 6E-7 M. In agonist mode, this concentration is on the plateau. This suggests that bisphenol A binds to the ligand binding site and blocks estradiol from effectively entering for transactivation.

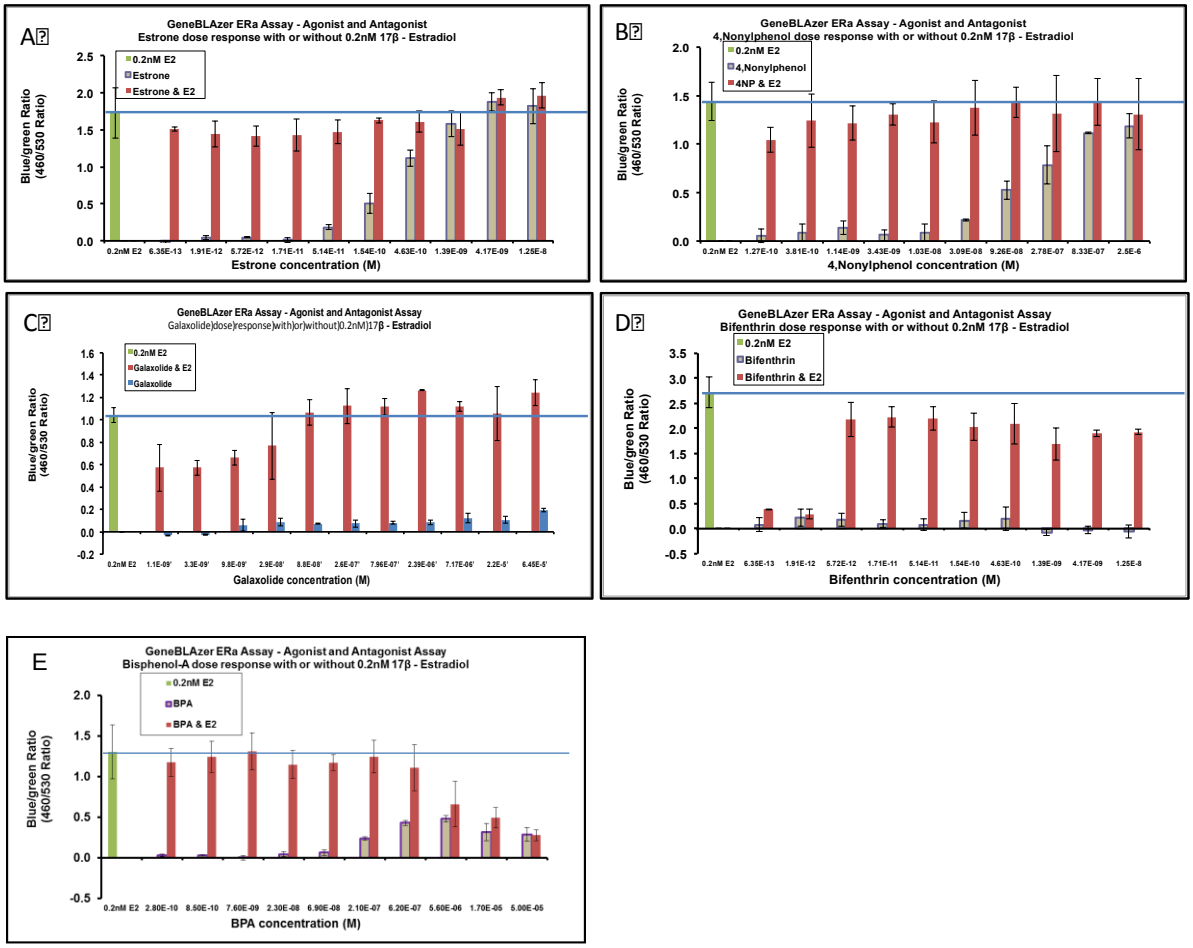


Figure 3. Antagonism of the ERα assay. Cells were plated in triplicate in 96-well clear bottom plates and dosed a mixture of E2 (0.2 nM E2 final concentration in wells) with respective concentrations of those chemicals for 18 h in the presence of 0.5% DMSO, loaded with LiveBLAzer™ FRET B/G substrate (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. (A) Estrone, (B) Nonylphenol, (C) Galaxolide, (D) Bifenthrin and (E) Bisphenol A.

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 (Fig. 4). The specific methods that were employed for the assay are found in Appendix A. Table 3 contains the nominal concentrations of chemicals that were used.

Fig. 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, NP, BPA and GAL using the nominal and actual concentrations described in Table 3. As can be seen, the actual concentrations measured for BPA and NP were below what was expected, suggesting that the chemicals are binding to surfaces. We will repeat the exposures for NP and confirm the exposure concentrations for E1 and E2 in Year 2.

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

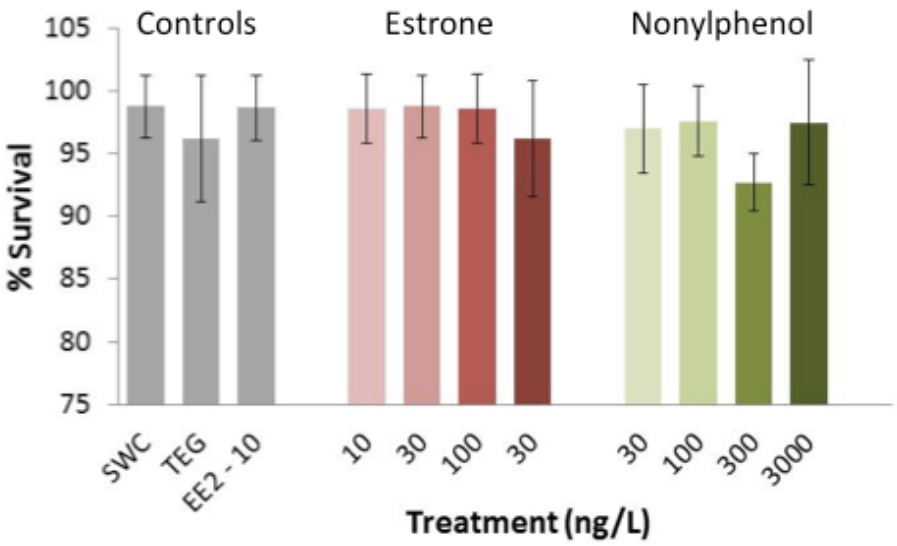


Figure 5. Effect of estrone and nonylphenol exposures on survival of Menidia larvae exposed for seven days.

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

Treatment		**Chemical concentrations (ng/L)**			
Seawater control (artificial seawater)	Nominal	0			
Vehicle control (TEG)	Nominal	50 µL/L			
EE2 (positive control)	Nominal	10			
	Measured	TBD			
Estrone (E1)	Nominal	10	30	100	300
	Measured	TBD	TBD	TBD	TBD
4-Nonylphenol (4NP)	Average recovery (± SD) of spiked surrogate: 73.8% (± 24.6)				
	Nominal	30	100	300	3,000
	Measured	19.2	18*	14*	92.9
Bisphenol A (BPA)	Average recovery (± SD) of spiked surrogate: 65.2% (± 14.2)				
	Nominal	300	1,000	3,000	30,000
	Measured	7.3	33.2	58.9	14,087
Galaxolide	Average recovery (± SD) of spiked surrogate: 75.9% (± 5.4)				
	Nominal	300	1,000	3,000	30,000
	Measured	283.2	788	2,867	18,928.5

TBD – To be determined

*Additional water samples will be analyzed to verify the exposure concentrations

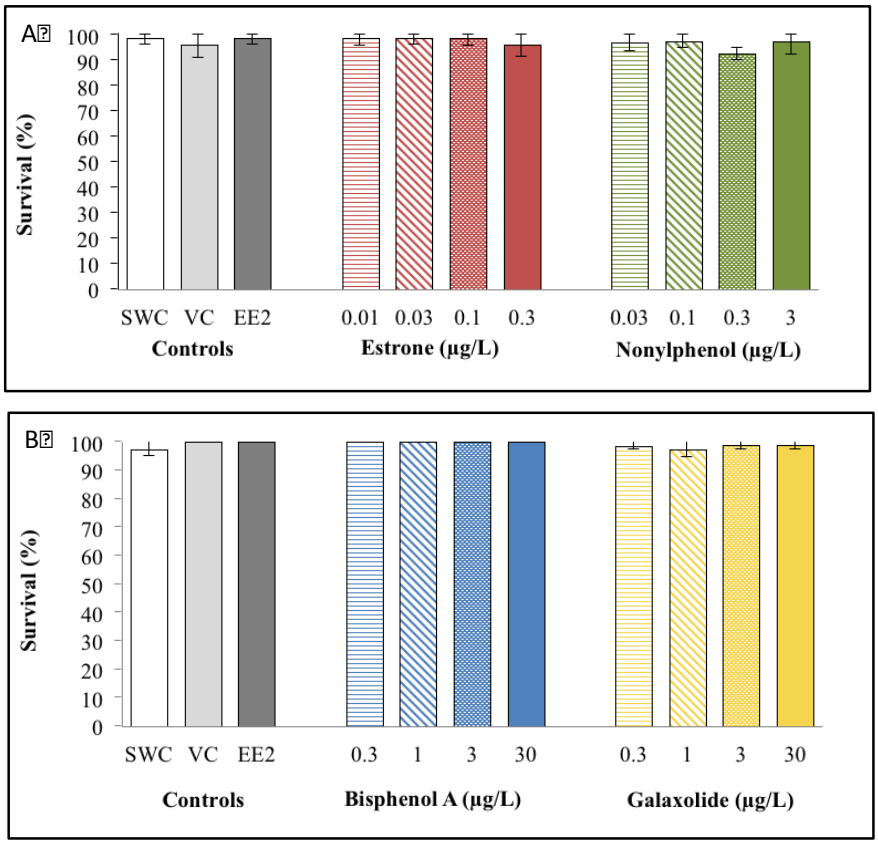


Figure 6: Mean survival (%) for *Menidia beryllina* larvae 7-day exposure to test chemicals. Error bars represent standard deviation (20 fish/replicate, 4 replicates/treatment) and (*) denotes a significant difference compared to the seawater control (SWC). A) Experiment 1- Menidia larvae were exposed 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**).

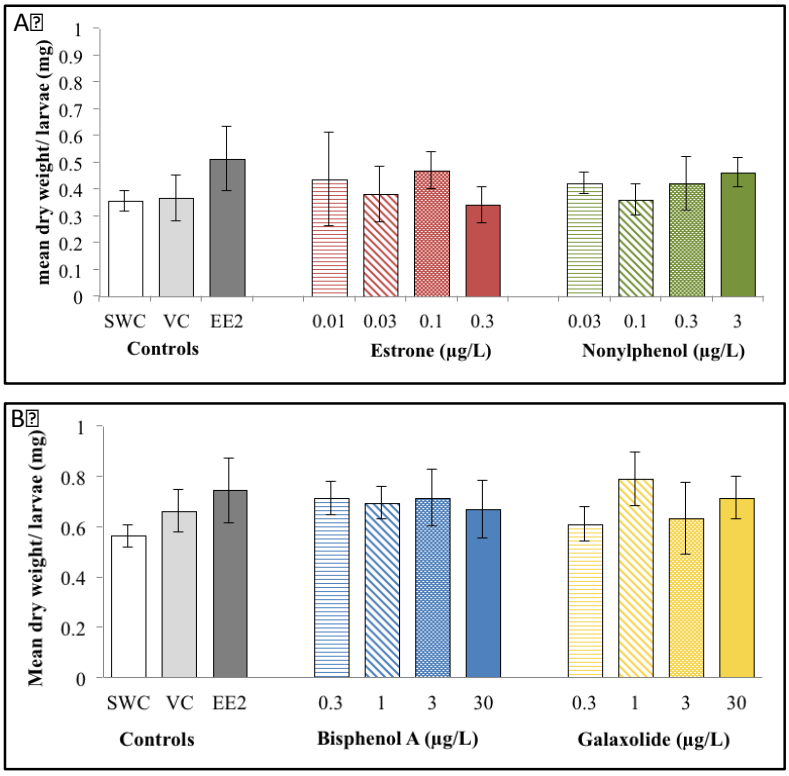


Figure 7: Effects of A) E1, 4NP, B) BPA and galaxolide on the mean dry weight of Menidia larvae after 7 days of exposure. There were no differences in growth among the chemical exposed larvae and those in seawater (SWC), vehicle control (VC) and EE2. Error bars represent standard deviation (5 larvae/replicate,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

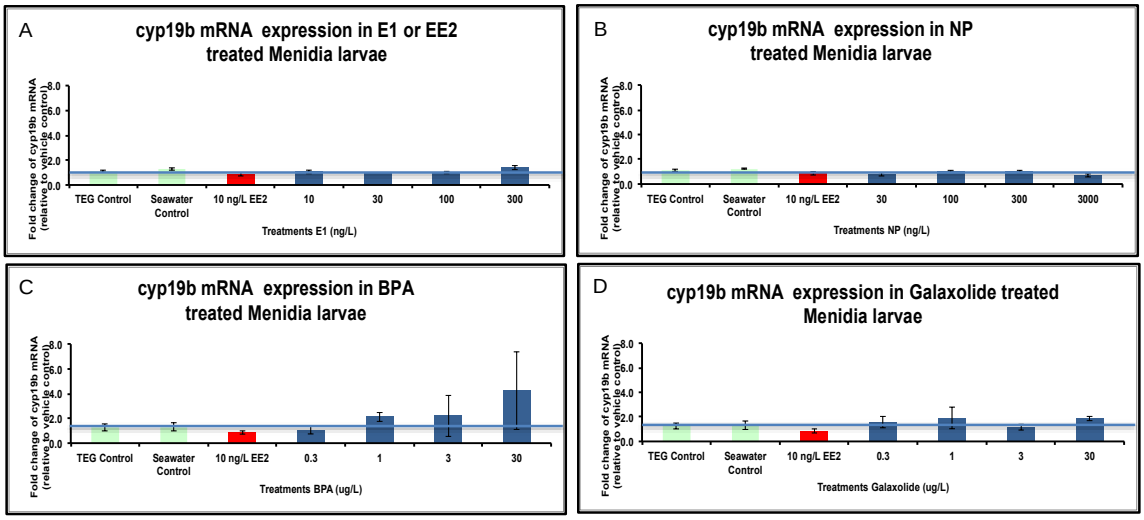
Gene expression studies for Menidia larvae

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) **(Fig. 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.

Because of the lack of results, we repeated the Q-PCR analysis for Cyp19b and also tested for changes in Vtg and Chg expression as a function of chemical concentrations for E1 and NP (Fig. 11). These results suggested that the actual concentrations for BPA and NP were much lower than predicted, supporting the validity of the measured concentrations (Table 3).

Cyp19B



StAR

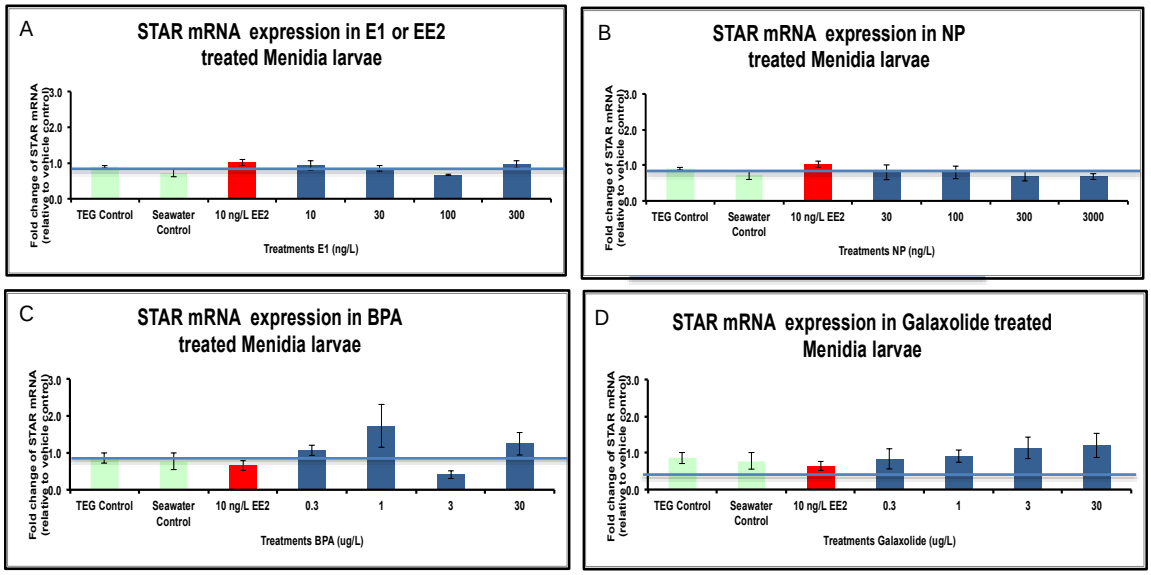
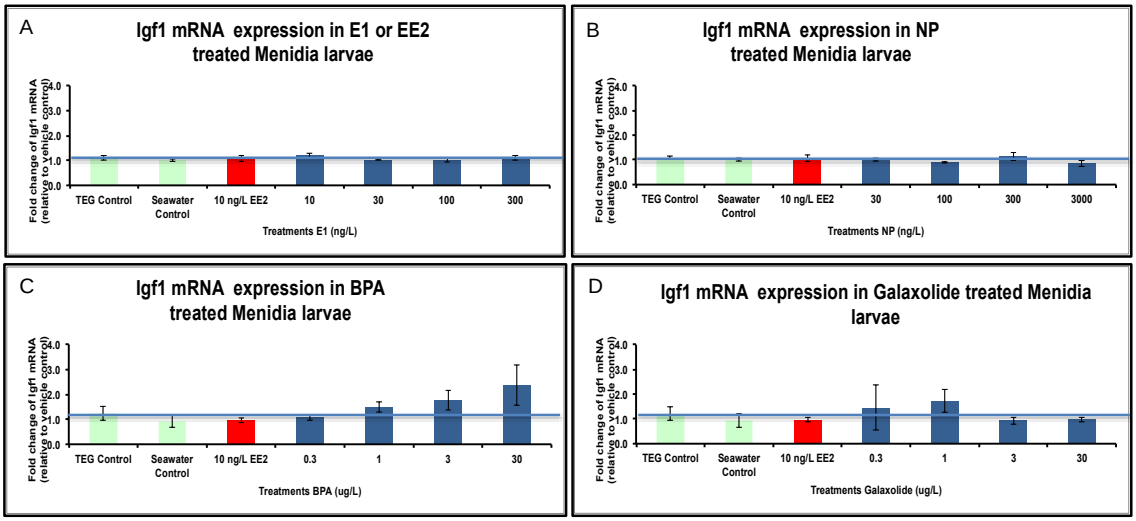


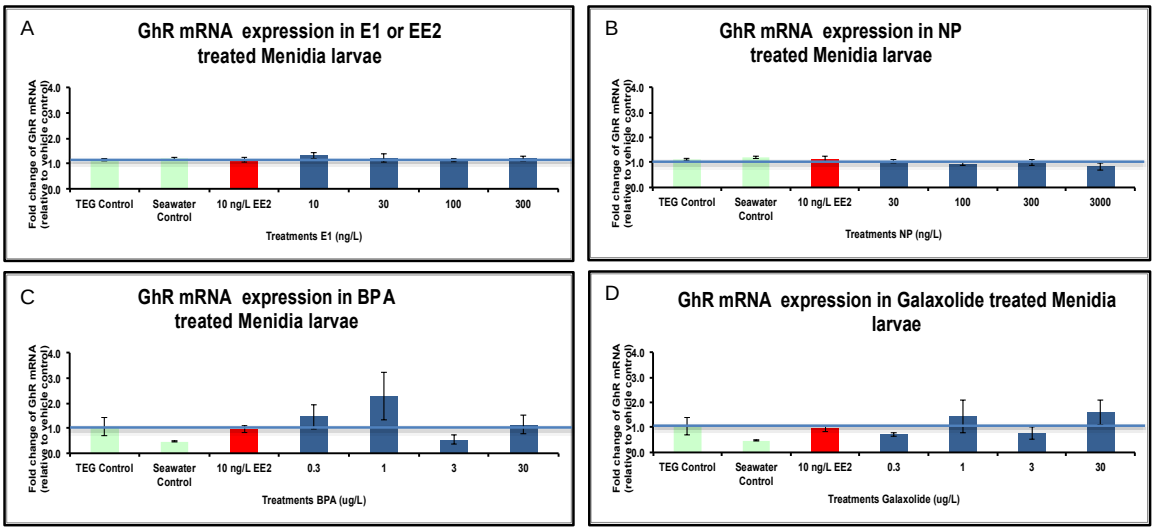
Figure 8. QPCR analysis of Cyp19b and StAR in *Menidia* larvae.

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) (Fig. 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

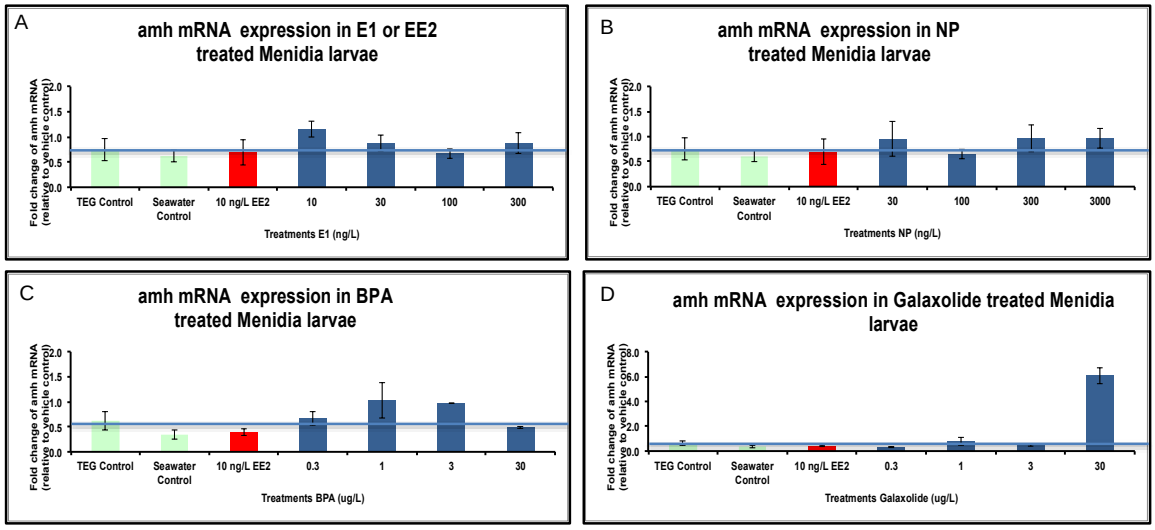


Figure 9. QPCR analyses for Igf1, GHR and AMH for *Menidia* larvae. The Y axis for AMH in response to GAL is different than for the other contaminants.

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 (Fig. 10). The main effect we saw was a reduction in expression by the strongest estrogen EE2 at a concentration of 10 ng/L.

DMRT1

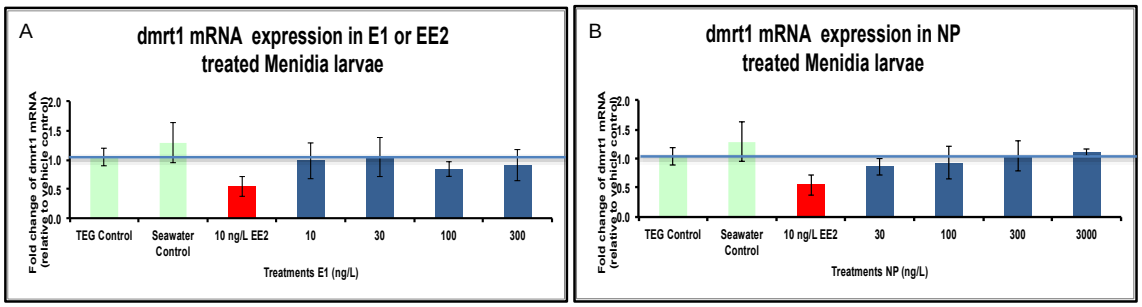


Figure 10. QPCR analysis of dmrt1 in Menidia embryos in response to EE2, E1 and NP.

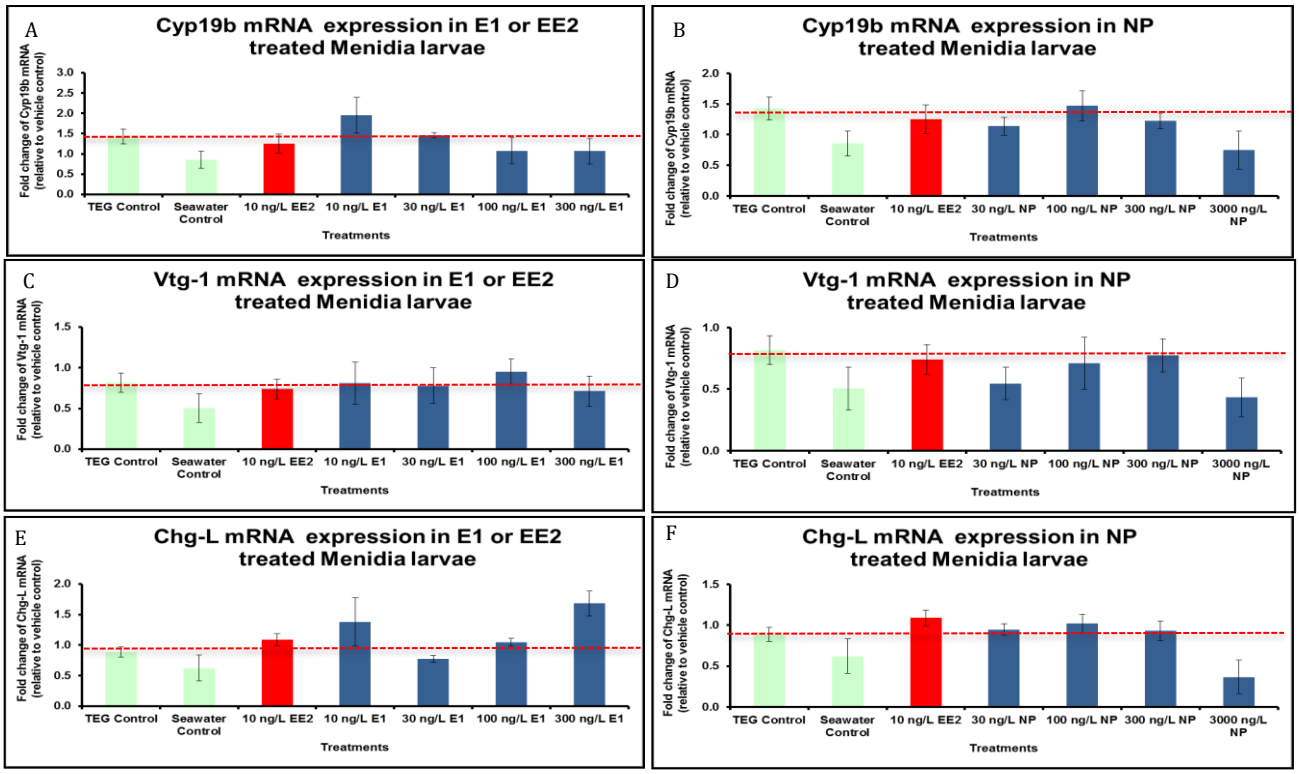


Figure 11. Re-analysis of ELS samples exposed to estrone (A, C, E) and nonylphenol (B, D, F) by QPCR. We measured again the expression levels for brain aromatase(Cyp19b), vitellogenin-1 (Vtg1) and choriogenin-L (Chg-L). Housekeeping gene GAPDH was used as a control and showed reasonable levels of expression.

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) (**Fig. 12**). 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. 12. 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 (**Fig. 13)**. 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.

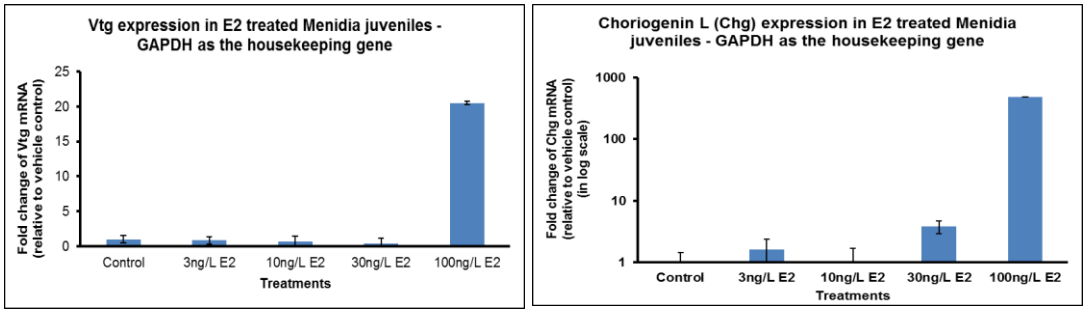


Figure 13. Treatment of Menidia juveniles with different levels of E2 resulted in elevated levels of vitellogenin (vtg) and Choriogenin L (Chg). Menidia juveniles (~ 50 days old) were exposed to E2 (3, 10, 30 and 100ng/L) for 10 days (50% daily static renewal) for 10 days. Total RNA was extracted from 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 4). 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 4. 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 exposure for NP presented with technical problems and thus we plan to repeat this experiment, along with other exposures to get better data.

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.

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 first 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 14 shows typical histological sections at 40X and 100X.

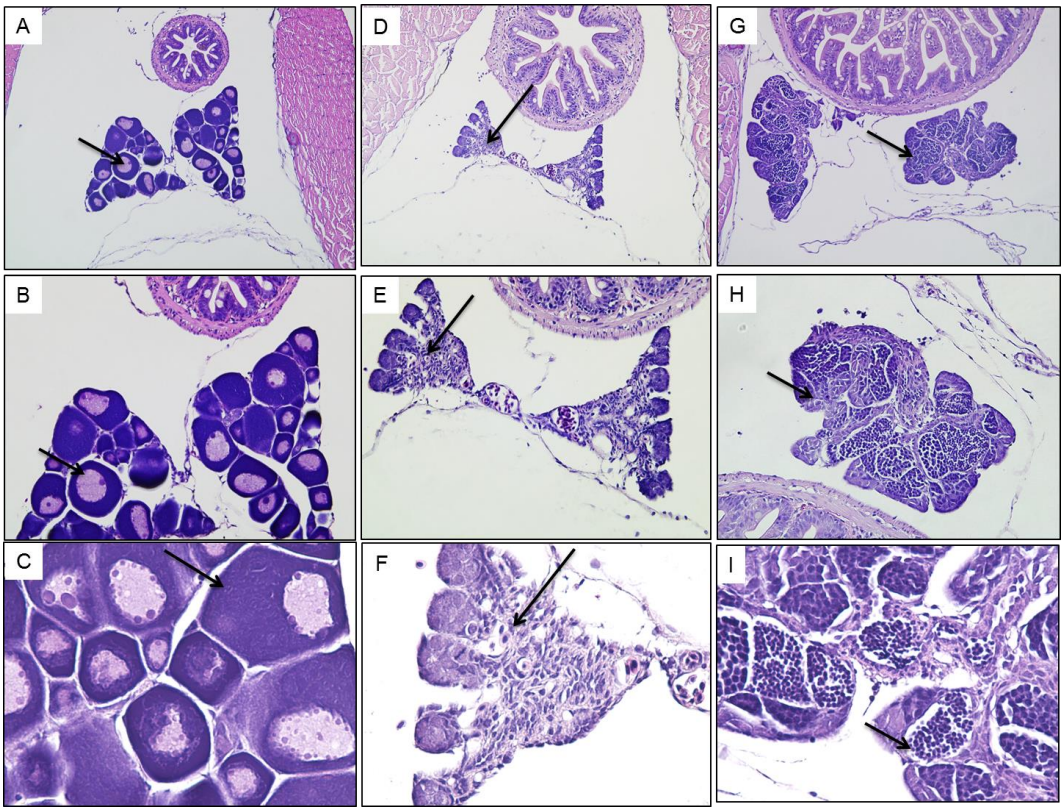


Figure 14. Histological sections of 71-day old Menidia stained with Hematoxylin and Eosin stain (H&E stain). Typical sections showing (A-C) oogonia; typical in females; (D-F) undifferentiated gonadal tissue (gonia) and (G-H) spermatogonia, typical in males. Photomicrograph of sex differentiation top 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.

To get a handle on sexual differentiation we got a new batch of Menidia 45 dph from the vendor and took fish for histology at 45 (immediately when they arrived) and then at two-week intervals for 3 months. We sent fish at monthly intervals (days 73 and 101) for histology and will examine the rest soon (Fig. 15). The fish were fed a formulated diet (Brine Shrimp Flake, Brineshrimpdirect.com) during this trial. To our surprise about 20% of the sampled fish were already differentiated into females at day 45 with the remainder undifferentiated. Males did not appear till day 101. We will examine the intermediate time points soon. This means that we need to start our exposures sooner – possibly at 30 dph. From the graph below it also appears that females reach the 50% point for the population by day 73, suggesting that females are completely differentiated in that time frame, while males just begin to differentiate starting close to the 73 dph. We will test this again, starting at day 30 and proceeding with the exposures till day 71, as previously done. We may also keep a cohort of fish in clean water after this exposure time till day 130, to get full tissue differentiation.

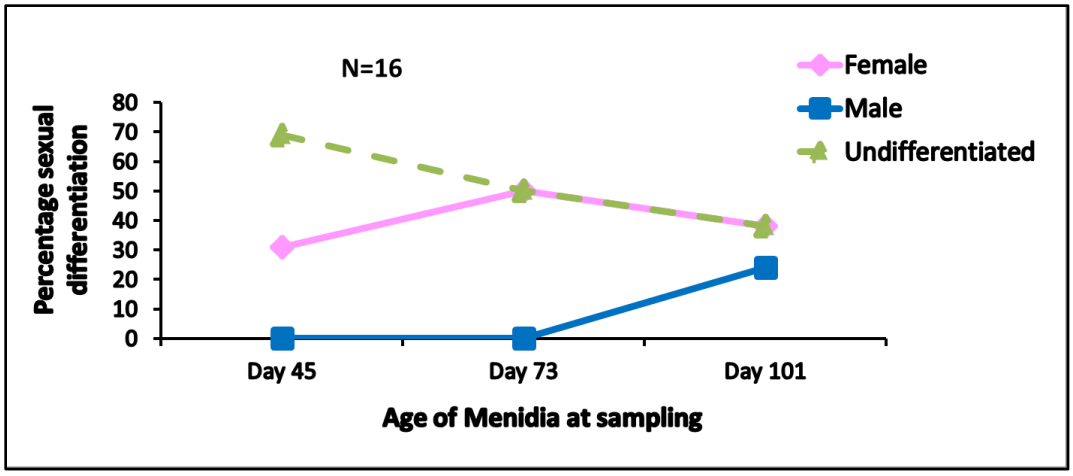


Figure 15. Test of gonadal differentiation for Menidia. Juvenile fish were ordered from the supplier to arrive 45 dph at the University of Florida. 16 fish were sacrificed upon arrival and tested for tissue differentiation by histology. Subsequently 16 fish were collected 73 dph and 101 dph. These were processed for histology as well. We have also collected fish at intermediate time points, which will be processed soon.

Contaminant effects on gonadal differentiation.

In the experiments performed in Phase 1, we took 8 fish per group at 71 dph for gonadal differentiation examination. The results of this effort are plotted below. We have an additional 8 fish per group that we collected as backups and these will be processed soon by histology to see if we can have a stronger dataset for statistical purposes.

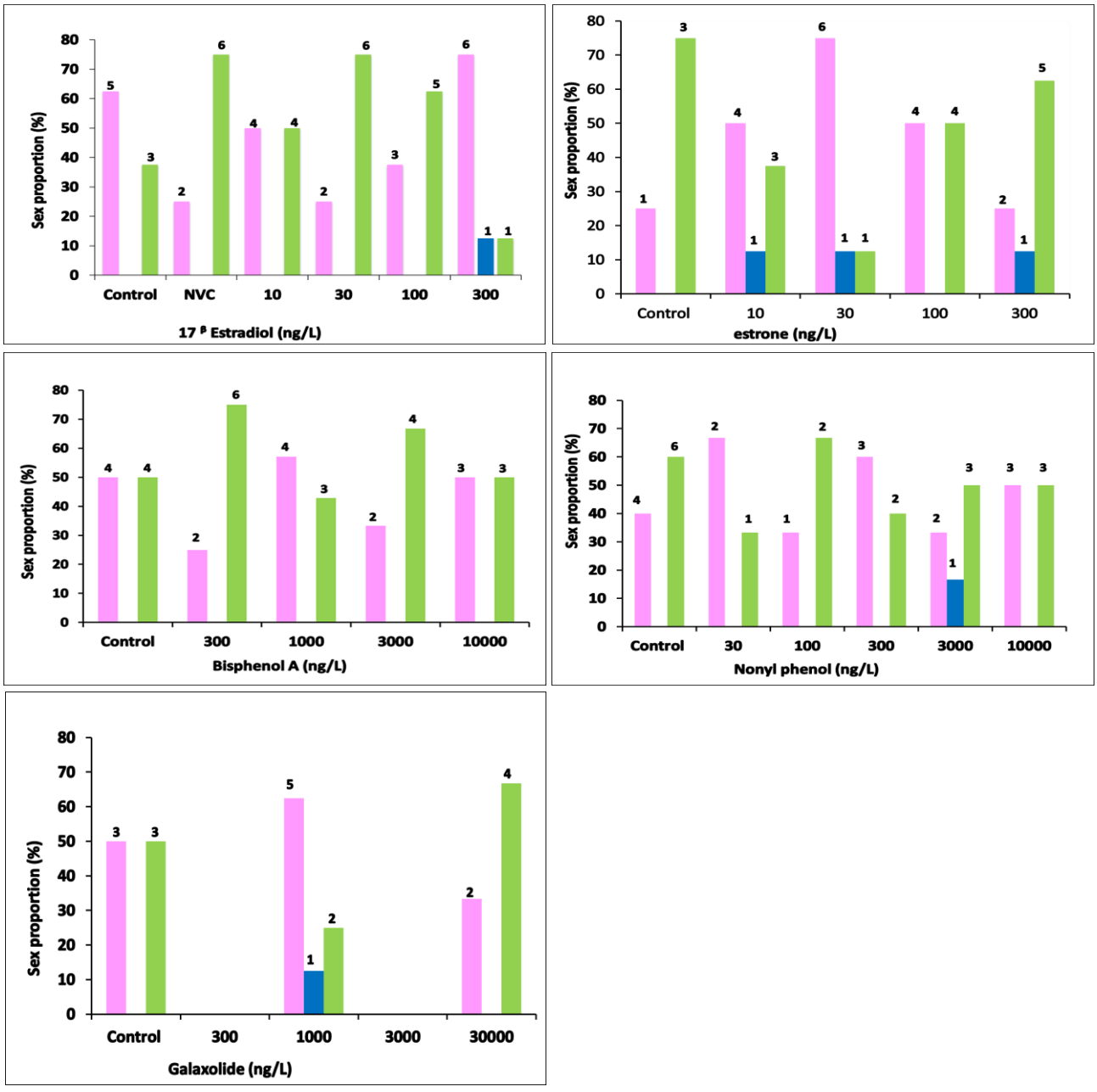


Figure 16. Contaminant induced tissue differentiation. Proportion of females (pink), males (blue) and undifferentiated tissue (green) in Menidia after 21 days of treatment and at 71 days of age. The number above each column is the number of fish per group that were analyzed, which was dependent on our 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, and then 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 (Table 4), 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 1.6mm to a high of 2.8mm. In fish exposed to contaminants, females were also identified from a low length of 1.6mm (E1 and GAL) to a high of 2.8mm (BPA) and 2.9mm (E2). Males were 2.5mm (E1, E2), 2.0mm and 2.8mm (4NP); while undifferentiated fish also ranged widely, from the lowest at 1.6mm (E1, GAL) to the highest at 2.8mm (E2) and 2.9 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; 2.1±0.09	0.51; 2.5	none
Estrone, 10 ng/L	0.19±0.05; 1.9±0.2	0.32; 2.2	0.29±0.04; 2.2±0.06
Estrone, 30 ng/L	0.24±0.05; 2.2±0.1	0.28; 2.2	0.23±0.1; 2.0±0.2
Estrone, 3,000 ng/L	0.16; 1.8	0.37; 2.5	0.18±0.06; 1.85±0.2
NP, 3,000 ng/L	0.5; 2.5	0.48; 2.8	0.41; 2.5

[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 (Fig. 17). 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.

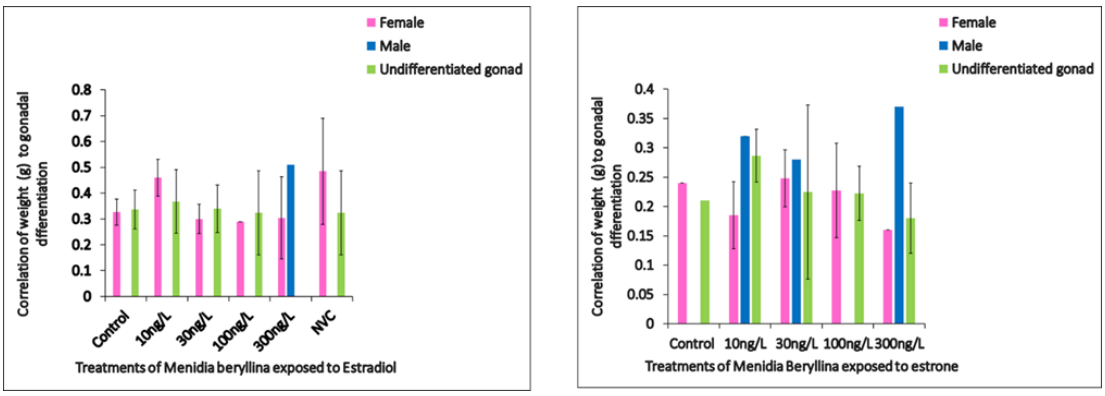


Figure 17. Correlation of weight of fish to sex identification.

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 (E1), 21.2 ± 1.9 (BPA), 22.8 ± 0.98 (4NP) and 22.7 ± 1.0 (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*.

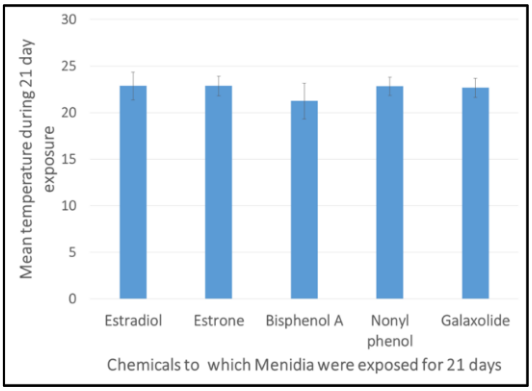


Fig. 18. Mean temperature during the 21 day exposures

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 Fig. 19, 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. 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.

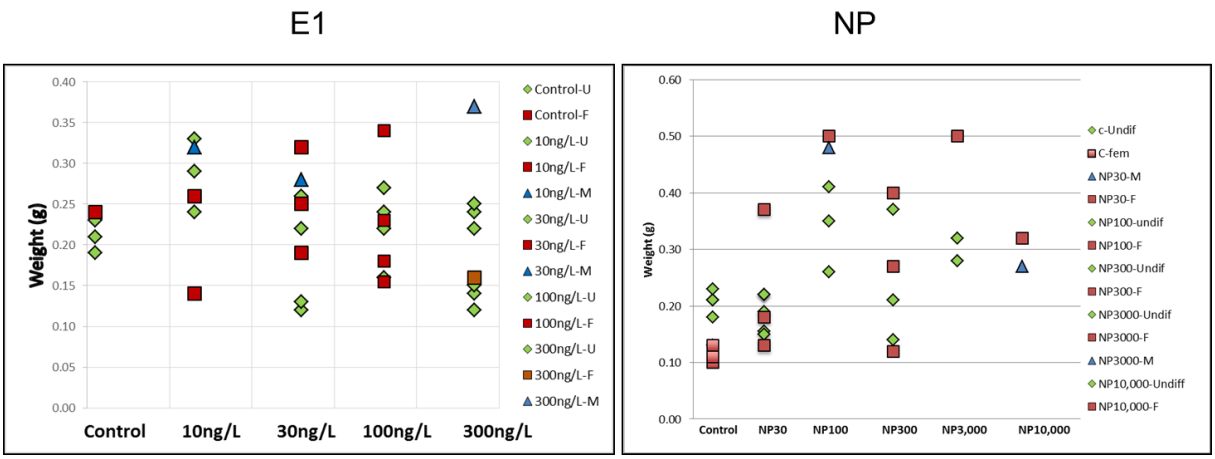
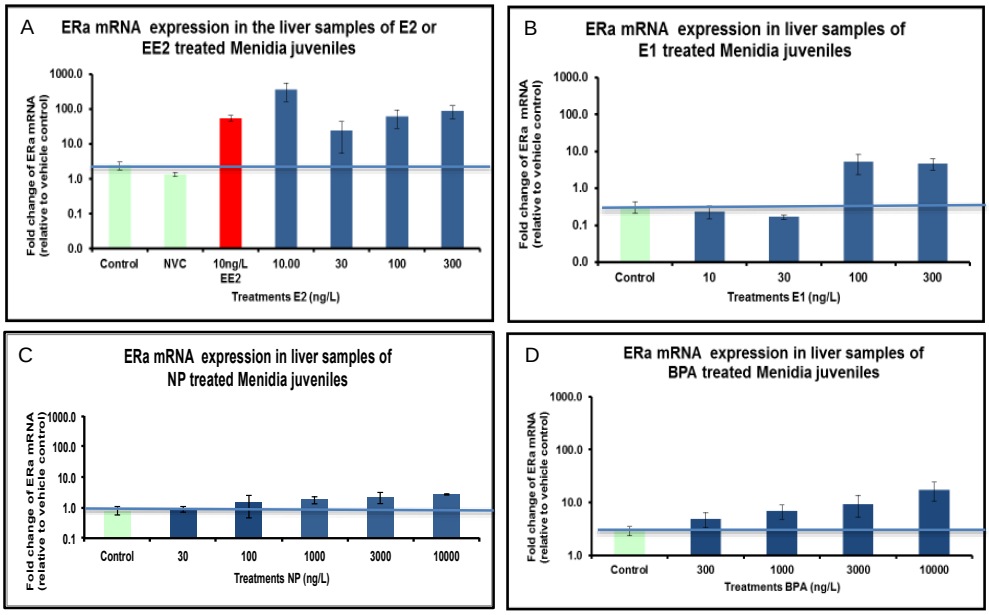


Figure 19. The effect of estrogenic contaminants on weight of fish. This represents only those fish that were used for sex determination by histology. Red squares, females; blue triangles, males; and green diamonds, undifferentiated gonadal tissues.

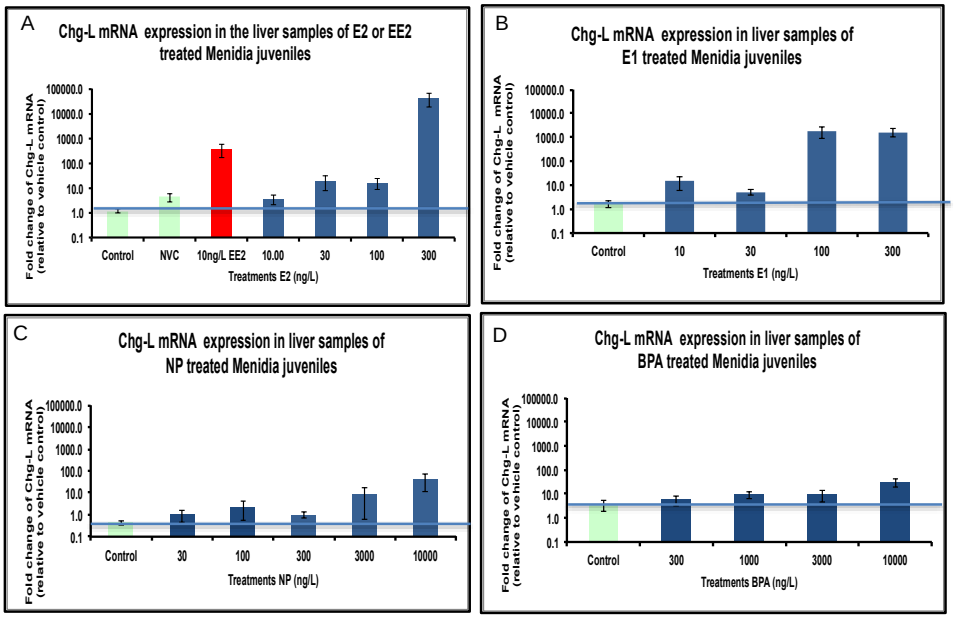
Molecular biomarkers for juvenile Menidia exposed to contaminants.

We tested the livers from exposed juvenile Menidia for differential expression of 5 genes that could be related to endocrine disruption: estrogen receptor alpha (ERα), Vitellogenin (Vtg), choriogenin (Chg), androgen receptor (AR), and estrogen receptor beta (ERβ). The different treatments resulted in dose-dependent increases in ERα, Vtg and Chg, in consonance with other studies in fish (Sabo-Attwood et al. 2004; Yu et al. 2006; Chen et al. 2008) (Fig. 20). 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 (Fig. 21). 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

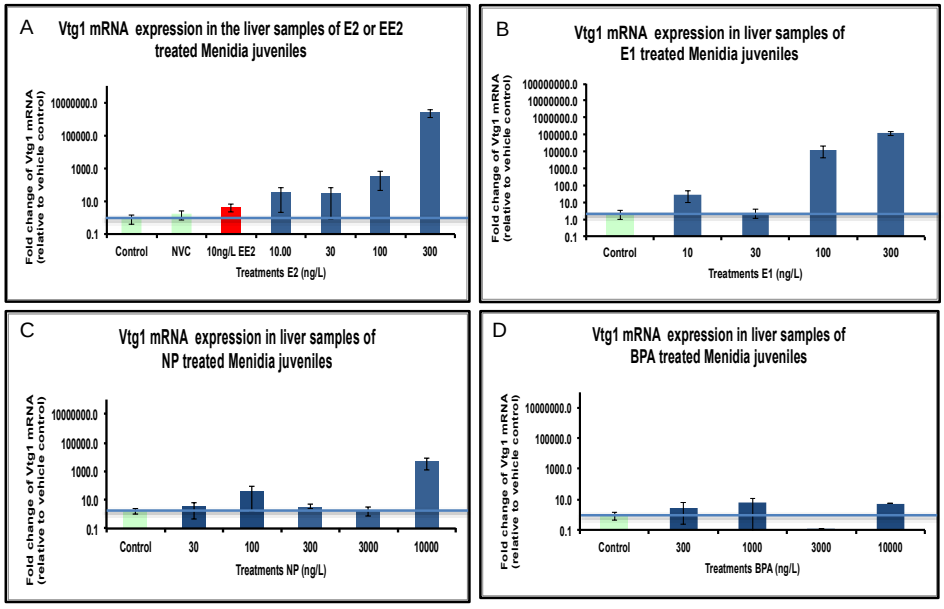
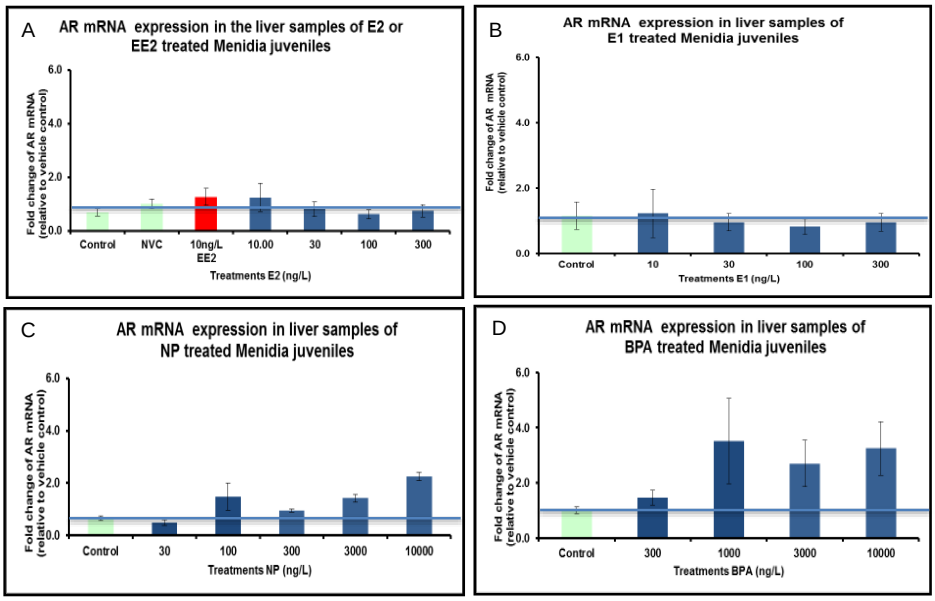


Figure 20. Q-PCR results for ERa, Chg and Vtg on juvenile Menidia exposed to E1, E2, 4NP and BPA for 21 days. GAPDH was used as an internal control. Fold change data are mean ± standard error relative to vehicle control. The horizontal line indicates the level of the control.

AR



ERb

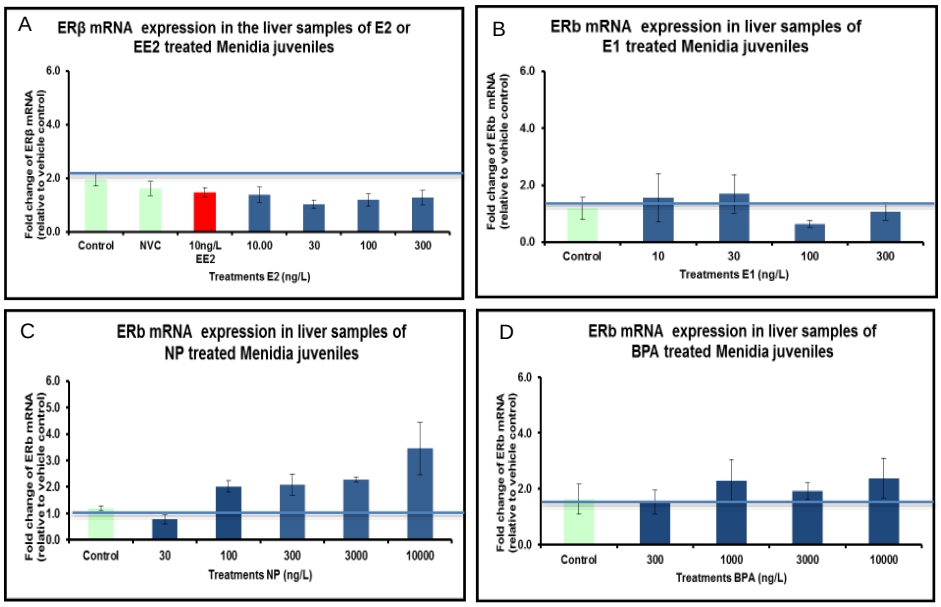


Figure 21. Q-PCR results for AR and ERβ on juvenile Menidia exposed to E1, E2, 4NP and BPA for 21 days. GAPDH was used as an internal control. Fold change data are mean ± standard error relative to vehicle control. The horizontal line indicates the level of the control.

The concentrations for these exposures were picked from the literature. However, only two of the conditions exceeded the EC50 determined from the *in vitro* assays, and this was for E2 and E1. These experiments will be repeated to make sure we have at least two concentrations above the EC50 determined in the *in vitro* assays.

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. We measured actual concentrations of the exposure solutions and found that we were below the desired concentrations for BPA and NP by a large margin. We plan on repeating the exposures for 4NP once we determine how the chemical was lost.

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. However, since the exposure concentrations were below nominal levels for at least two of the chemicals, it is likely that we failed to see gene expression changes for this reason.

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. It is also possible that estuarine fish respond differently than fresh water fish. We will work more on this aspect 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 steroidogenesis.
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 ERα, Chg and Vtg. Effects on AR and ERβ 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 – ERα strong dose response for all of the chemicals tested. E2 reached a plateau at low concentrations as seen in other 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 – ERβ – 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. We have determined that females differentiate within this window but it takes males at least up to 100 days for sexual differentiation to occur. We will continue to refine this experiment in the next period, to make sure we can capture the full window of gonadal tissue differentiation.

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.

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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Methods

A. *In vitro* bioassays for ERα, β and EEQ calculation (UF)

Exposure solution extracts were made up in DMSO and were stored at -80°C until bioanalysis. ERα- 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 °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 3 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°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 feed in the water column. If food was uneaten, it went 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 4 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.

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 (2011) (Table S2). 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 berylina- 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

Table S2. **Primers and probes used for qPCR analysis that came from Susanne Brander (2011)**.

Transcript Name	**Primer Sequence**	**Roche Probe Number**
Fwd: *M.audens* estrogen receptor 1	ACGCTTCCGCATGCTCA	#15
Rev: *M. audens* estrogen receptor 1	CTCCATTGTGCCAGTGCAGA	
Fwd: *M. audens* estrogen receptor 3	CATTATGCCCTCCACGCACT	#52
Rev: *M. audens* estrogen receptor 3	GACCATCCTGGGAAACTGATCTT	
Fwd: *M. audens* androgen receptor x	ATCCGCATGCAGTGCTCATA	#31
Rev: *M. audens* androgen receptor x	CCCCAGACCTCGTATTCAACG	
Fwd: *M. audens* choriogenin L	CATCCAGTCATCAGTCATGAGTTTC	#82
Rev: *M. audens* choriogenin L	GGTCCCGTTTTCTGCAGTTAAG	
Fwd: *M. audens* thyroid receptor alpha	TGTCGGACGCCATATTCGAT	#51
Rev: *M. audens* thyroid receptor alpha	CCTCGGTGTCATCCAAGTTGA	
Fwd: *M. audens* GAPDH	GGTGGTGAACACACCAGTGG	#159
Rev: *M. audens* GAPDH	CACGAGAGGGACCCAACTAACA	
Fwd: *M. audens* Vtg	GTAGAGTTCATGAAGCCCATGCT	#108
Rev: *M. audens* Vtg	AAATCAATGTAAGCGGCAAAGG	
Fwd: *M. audens* insulin-like growth factor 2	GGCTGCCTTCCTATTCCACAC	#38
Rev: *M. audens* insulin-like growth factor 2	GCAGGTCATACCCGTGATGC	

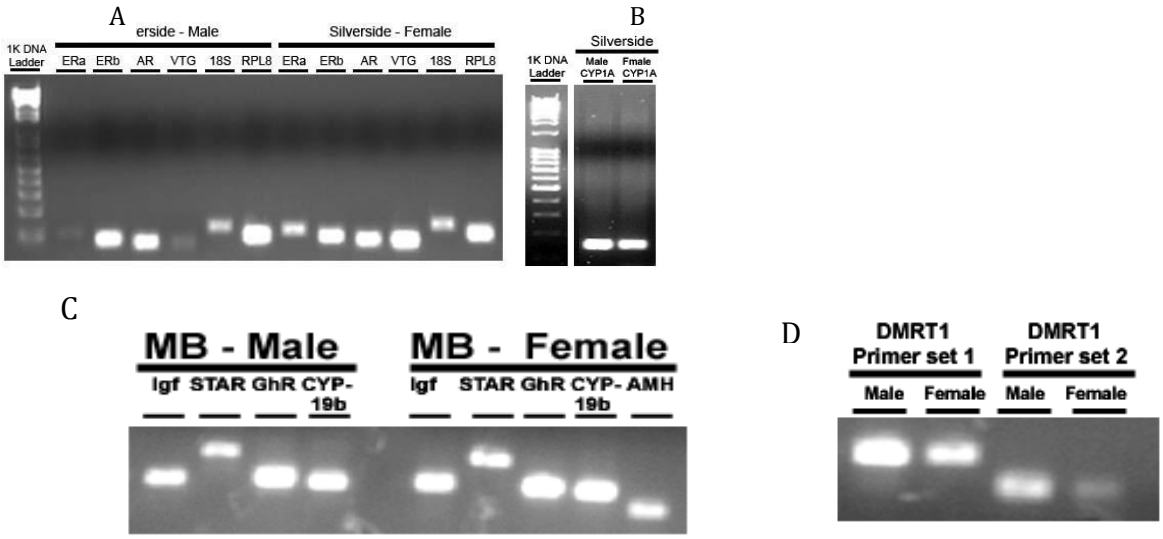
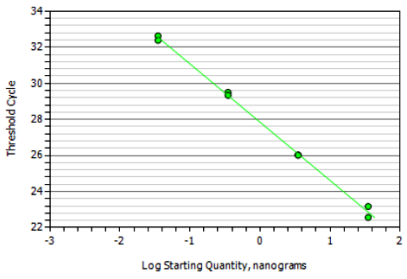


Figure S1: PCR verification of primers for (A) Vtg, ERα, ERβ, AR, 18S rRNA and rpl8; (B) CYP1A; (C) Igf, StAR, GhR, Cyp19b, amh and (D) DMRT1 in adult male and female Menidia. Total RNA was extracted from adult Menidia liver tissues and amplified with primers specific for the amplified sequences. Abbreviations: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

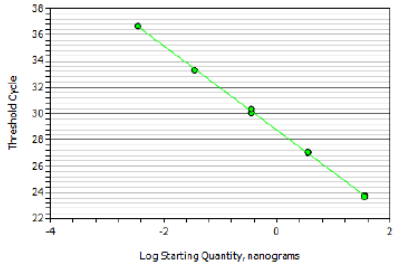
B) Amplification efficiency for each of the primers. Dilution curves were prepared for each of the primers to verify the amplification efficiency. All primer pairs were between 95 and 105 % efficient.

ER alpha



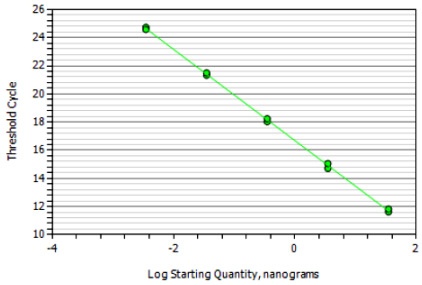
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.9	0.997	-3.233	27.845

ER beta



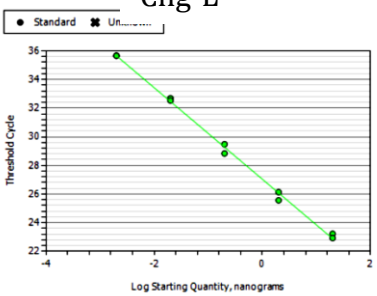
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.8	1.000	-3.211	28.739

VTG1



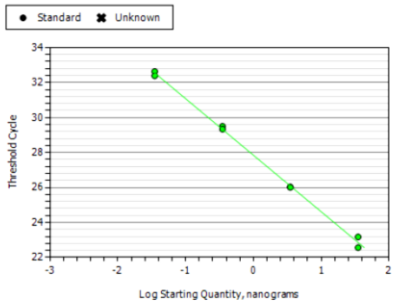
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.5	0.999	-3.240	16.685

Chg-L



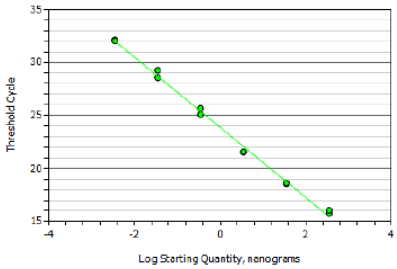
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	105.7	0.997	-3.192	27.040

AR



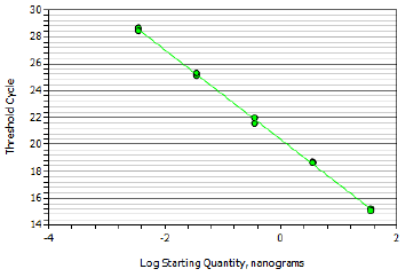
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.9	0.993	-3.223	28.232

GAPDH



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

RLP8

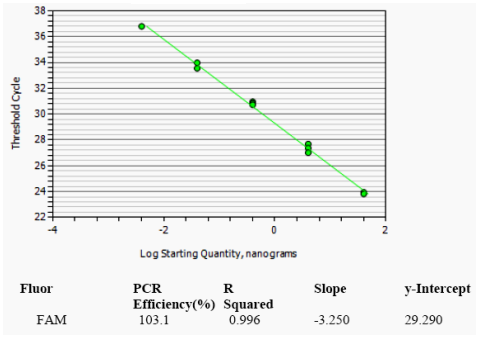


Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	99.5	0.999	-3.333	20.359

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.

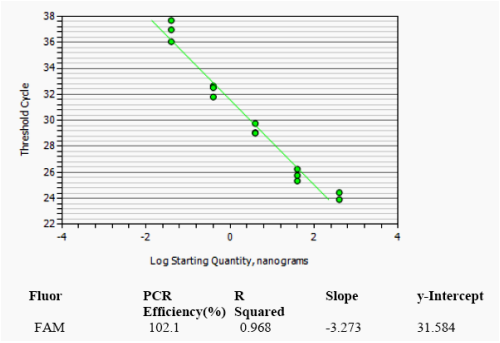
Validation of primers for q-PCR for ELS Menidia.

GhR



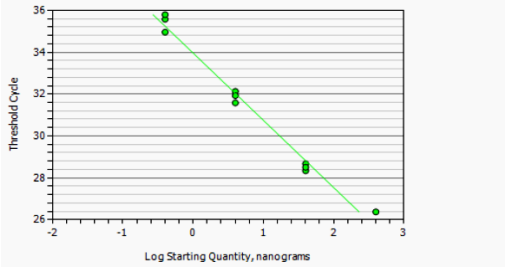
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.1	0.996	-3.250	29.290

Cyp 19b



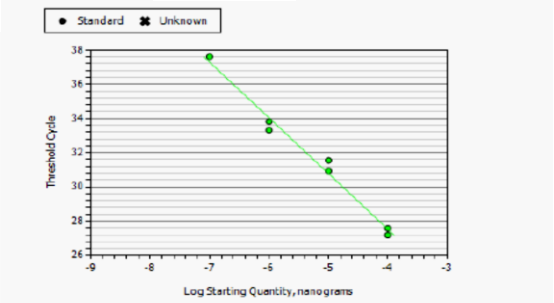
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	102.1	0.968	-3.273	31.584

IgF1



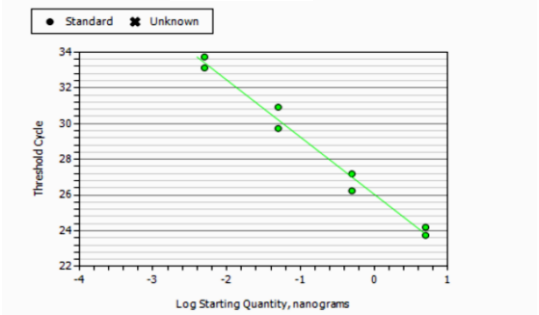
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.9	0.984	-3.211	33.961

StAR



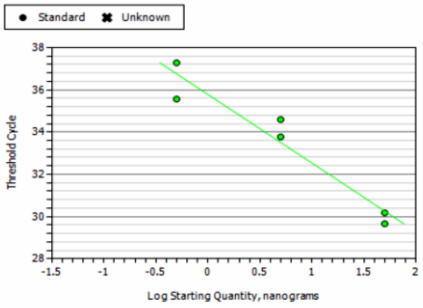
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	102.8	0.983	-3.258	14.491

Amh



Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercep
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