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Journal

Archives of Environmental Contamination and Toxicology, 71(2)

ISSN

0090-4341

Authors

Cole, Bryan J
Brander, Susanne M
Jeffries, Ken M
et al.

Publication Date

2016-08-01

DOI

10.1007/s00244-016-0282-8

Peer reviewed

Published in final edited form as:

Arch Environ Contam Toxicol. 2016 August ; 71(2): 210–223. doi:10.1007/s00244-016-0282-8.

Changes in *Menidia beryllina* gene expression and *in vitro* hormone receptor activation following exposure to estuarine waters near treated wastewater outfalls

Bryan J. Cole[*], **Susanne M. Brander**[#], **Ken M. Jeffries**[*], **Simone Hasenbein**[*], **Guochun He**[°], **Michael S. Denison**[°], **Nann A. Fangue**[‡], **Richard E. Connon**[*]

[*]Department of Anatomy, Physiology & Cell Biology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, California, 95616.

[#]Department of Biology & Marine Biology, University of North Carolina, Wilmington, 601 South College Road, Wilmington, North Carolina, 28403.

[‡]Wildlife, Fish & Conservation Biology, University of California, Davis, One Shields Avenue, Davis, California, 95616.

[°]Department of Environmental Toxicology, University of California, Davis, One Shields Avenue, Davis, California, 95616.

Abstract

Fishes in estuarine waters are frequently exposed to treated wastewater effluent, among numerous other sources of contaminants, yet the impacts of these anthropogenic chemicals are not well understood in these dynamic and important waterways. Inland silversides (*Menidia beryllina*) at an early stage of development (12 days post hatch) were exposed to waters from two estuarine wastewater treatment outfall locations in a tidal estuary, the Sacramento/San Joaquin Delta, CA, USA, that had varied hydrology and input volumes. The genomic response caused by endocrine disrupting compounds in these waters was determined using quantitative PCR on a suite of hormonally-regulated genes. Relative androgenic and estrogenic activities of the waters were measured using CALUX reporter bioassays. The presence of bifenthrin, a pyrethroid pesticide and known endocrine disrupting compound, as well as caffeine and the anti-inflammatory pharmaceutical ibuprofen, which were used as markers of wastewater effluent input, were determined using instrumental analysis. Detectable levels of bifenthrin (2.89 ng L^{-1}) were found on one of the sampling dates, and caffeine on all sampling dates, in water from the Boynton Slough. Neither compound was detected at the Carquinez Strait site, which has a much smaller effluent discharge input volume relative to the receiving water body size compared to Boynton Slough. Water samples from both sites incubated in the CALUX cell line induced estrogenic and androgenic activity in almost all instances, though the estrogenicity was relatively higher than the androgenicity. Changes in the expression of endocrine-responsive genes and indicators of general chemical stress were observed following a 96-hour exposure to waters from both locations. The relative levels of endocrine response, changes in gene expression and contaminant concentrations were greater in water from the Boynton Slough site, despite those effluents undergoing a more advanced treatment process. The availability of a widely geographically distributed estuarine model species (*M. beryllina*) now allows for improved assessment of treated effluent impacts across brackish, estuarine, and marine environments.

Introduction

Fishes living in estuarine environments are subjected to particularly high levels of anthropogenic pollution and other types of deleterious environmental impacts, largely due to the tendency for highly urbanized areas to center around estuaries (Diehl et al. 2012; Ridgway and Shimmield 2002). Pollutants can originate from activities associated with urban runoff, shipping and numerous other maritime activities, as well as from treated wastewater effluent discharge originating from urban, industrial, and agricultural sources (Metcalfe et al. 2001; Weston et al. 2009; Weston and Lydy 2010). Of particular concern in estuarine waters are endocrine disrupting compounds (EDCs), which can interfere with, or alter, the process of hormone signaling in fish (Brander 2013; Metcalfe et al. 2001), resulting in a wide range of genetic and physiological abnormalities (Doyle et al. 2013; Jeffries et al. 2008; Le Page et al. 2011; Scholz and Mayer 2008; Tabb and Blumberg 2006; Tetreault et al. 2011). Such exposures can cause changes in the expression of genes that control, or are controlled by hormone signaling, and might have negative repercussions for fish development, reproduction and ultimately impact populations, communities, and ecosystems (Brander 2013; Harris et al. 2011; Hazlerigg et al. 2014).

Much of the research examining the impacts of EDCs has been performed on fish under controlled laboratory conditions, where fish are exposed to concentrations of individual or, much more rarely, to combinations of chemicals (Thorpe et al. 2003). Few field experiments have tested effluent from outfalls located in estuaries, and as such, the complexities introduced by tidal fluctuations and changes in salinity, among other natural environmental variations, are poorly understood. Controlled experiments targeting individual chemicals are essential to understanding the direct impacts of EDCs on gene expression, but cannot assess the effects on wild fishes exposed to variable chemical combinations, which is essential information that directly pertains to monitoring and conservation efforts.

The Sacramento/San Joaquin (SSJ) Delta in California, USA, the largest estuary on the West coast of North America, is an excellent example of an estuary where environmental stressors, largely arising from heavy usage by humans, occur in an area of critical ecological functions (Nichols et al. 1986). Of particular interest in this estuary is the decline of pelagic fish species such as the delta smelt (*Hypomesus transpacificus*), the longfin smelt (*Spirinchus thaleichthys*), as well as numerous salmonids (Sommer et al. 2007). Habitat degradation, habitat loss, competition with introduced species and decreased food availability have all been the subject of much study, but critical understanding of the sublethal effects of contaminants on biota in the SSJ Delta have been largely overlooked (Brooks et al. 2012). Two sites receiving discharge of treated wastewater effluent from Publically Owned Treatment Works (POTW); Boynton Slough and Carquinez Strait (Figure 1), are of particular interest, and were chosen because they represent the varied sources and ramifications of exposure to treated wastewater in the SSJ Delta and other estuaries worldwide. Boynton Slough, which consists of narrow marshland streams, receives approximately 64.4 million liters per day (MLPD) of advanced secondary treated effluent wastewater from domestic, commercial and industrial sources via the Fairfield Suisun Sewer District, which serves Fairfield, Suisun City and Traverse Air Force Base (Board 2013). The

Carquinez Strait site lies in a much more urbanized area than Boynton Slough. The area near the Carquinez Strait water collection site receives deep-water effluent discharge of approximately 85.2 MLPD of industrial coolant waters from a sugar refinery and 3.5 MLPD of secondary treated municipal sewage wastewater from Crocket Community Services District (Board 2012). Carquinez Strait is a much larger body of water, thus the percentage of effluent water compared to total volume is much lower than that of Boynton Slough. However, in addition to the indicated discharge waters, there are several other nearby POTW effluent discharge sites, as well as a large diversity of recreational and commercial maritime activities at Carquinez. Lastly, Boynton Slough is characterized by lower salinity (1–5 ppt) compared to Carquinez Strait (12–16 ppt), though both are tidally influenced.

The objective of this study was to determine the extent to which fish in environmentally and hydrologically different estuarine areas are exposed to EDCs, and what the resultant impacts on gene expression are. Inland silversides (*Menidia beryllina*), a non-native naturalized fish in the SSJ Delta now developed as a model species for assessing the impacts of EDCs to estuarine organisms (Brander et al. 2012a; Brander et al. 2013), were chosen to assess the effects of these chemicals during the critical period of sexual differentiation.

All collected water samples were tested for the presence of the pesticide bifenthrin, which is frequently detected in the SSJ Delta and in treated wastewater effluent (Weston et al. 2013) and known to act as an estrogen mimic (Chen et al. 2002; Tyler et al. 2000). In addition, samples were tested for contaminants representative of those found in treated wastewater effluent: the anti-inflammatory pharmaceutical ibuprofen and the stimulant caffeine (Thomas and Hilton 2004). Following exposure, we evaluated changes in expression of a suite of endocrine-related genes, many of which have been shown in microarray analysis of silversides to be responsive to bifenthrin treatment at environmentally-relevant concentrations (Brander et al. *In review*). Finally, because these waters contain numerous additional chemicals that were not analyzed for, many of which may be estrogenic or androgenic, *in vitro* cell-based CALUX reporter gene bioassays (Rogers and Denison 1999) for estrogenic, androgenic, and anti-estrogen/androgenic chemicals were performed on all collected water samples to assess the different competing factors that can impact the *in vivo* responses to these water samples. We hypothesized that water sampled from each site would induce responses both *in vivo* and *in vitro*, but that Boynton Slough would cause a stronger response in both, considering the lower dilution volume for effluent discharged into that water body.

Materials and methods

Water collection and fish exposures

Menidia beryllina were purchased from Applied Biosystems (Fort Collins, CO) at 8 days post hatch (dph), arriving at a salinity of 18 ppt. They were gradually acclimated to the desired salinities (less than 4 ppt decrease per day), matching water collections from each investigated site prior to exposures. Site water exposures were performed on fish for 96 hours at 20°C, beginning at 12 dph and ending at 16 dph (average fork length 19 mm). This research was approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC #16845). Throughout acclimation and exposure tests, the fish were

fed *ad libitum*, 3 times per day, with *Artemia franciscana* nauplii (Argent Chemical Laboratories, WA, USA).

Site water was collected by boat from within a 50 meters radius of POTW effluent discharge locations (Figure 1) in Boynton Slough (38° 12’ 30.60” N, 122° 3’ 25.26” W) and Carquinez Strait (38° 04’ 16.81” N, 122° 15’ 12.88” W). Sample waters used for 96 hour *in vivo* exposures to 12 dph *Menidia berylina*, as well as for chemical analyses and *in vitro* CALUX assays were collected on April 25, 2014. Eight days prior to this, to assess endocrine disruptive potential and to get a better perspective on the chemicals present, on April 17, and 21, water samples were collected solely for chemical analyses and for use in CALUX assays (Table 1). Water samples used for exposures were collected in 18.9 L amber LDPE (low density polyethylene) Cubitainers® and stored at 4°C for no more than 96 hours following EPA guidelines. Cubitainers® were further rinsed with ambient water prior to filling. Salinity-matched controls were conducted for each site alongside the experimental exposures, with Carquinez Strait controls at a salinity of 12.5 ppt, and Boynton Slough controls at 1.0 ppt.

Exposures to the site water began on April 26, 2014 with daily water changes and feeding with *Artemia nauplii*. The following physicochemical parameters were monitored daily: temperature, pH, dissolved oxygen, temperature-adjusted electric conductivity (at 20°C), hardness, alkalinity, ammonia concentration as nitrogen and unionized ammonia. Exposures were performed in sterilized 500 mL glass beakers, with 4 replicates of 10 fish each per treatment. Following the exposure, fish were sacrificed with a lethal dose of tricaine methanesulfonate (MS-222/Finquel; Argent Labs, Redmond, WA) at a dosage of 50 mg/L, at neutral pH, buffered with sodium bicarbonate ($NaHCO_3$), flash frozen in liquid nitrogen, and stored at −80°C for later processing.

One-liter grab samples were collected for chemical analyses and CALUX assays, in pre-labeled and kilned amber type III glass bottles (950 mL Wide Mouth Glass Packers with PTFE-lined white PP cap, Thermo Fisher Scientific, Waltham, MA). Samples were collected just below the water surface at the specified locations in the Carquinez Strait and Boynton Slough. Bottles were rinsed with sample water once before filling, and filled leaving as little head-space as possible. Samples were transported on ice to the laboratory and stored in the dark at 4°C until extraction for chemical analysis. Samples used for CALUX assays were treated with 10% methanol then stored at 4°C for less than 14 days before use.

Homogenization and collection of RNA

Total RNA was extracted from whole 16 dph fish using RNeasy Kits (Qiagen, Valencia, CA, USA) following the manufacturer protocol. Total RNA concentration and purity (260/280, 260/230 ratios) were determined using a Nanodrop ND1000 (Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was visually verified via observation of 28S and 18S ribosomal RNA bands, by running on a 1% w/v agarose gel with SYBR Safe (Thermo Fisher Scientific, Carlsbad, CA) and visualized on a UV light box. Extracted total RNA was stored at −80°C for later analysis via qPCR.

Quantitative real-time polymerase chain reaction

To evaluate gene expression responses to exposure to SSJ Delta waters, 10 genes of interest and 1 reference gene were chosen for quantitative real-time polymerase chain reaction (qPCR) analysis (Table 2). Genes were chosen to represent general stress responses, involvement in toxicant defense, or response to endocrine-active compounds. QPCR primers and probes were designed using the Universal ProbeLibrary (UPL) Design Center (Roche, Basel, Switzerland). Quantitative PCR was performed on 24 fish in total for each salinity control and site exposure, sampled equally from each of the 4 replicate beakers (n=6). No-template controls were performed for all qPCR assays, and were all negative. No-primer RT controls were performed during efficiency tests.

Two μg of total RNA was used for cDNA synthesis. The total volume of each sample was brought up with nuclease free water to 24 μL total, then 4 μL of genomic DNA (gDNA) wipeout buffer (Qiagen) was added and incubated at 42°C for 2 min. CDNA synthesis reactions consisted of 8 μL of 5X Quantiscript RT Buffer, 2 μL RT Primer Mix, 2 μL Quantiscript RT (Qiagen) along with 24 μL of nuclease free water containing 2 μg of sample RNA. Samples were incubated at 42°C for 30 min, followed by 95°C for 3 min to stop the reaction, and then held at 4°C until they were moved to −20°C for storage until use in the qPCR reactions.

The qPCR reactions used a 1:6 dilution of template cDNA. Reactions were performed using an ABI HT 7900 A FAST Sequence Detection System (Applied Biosystems, Grand Island, NY, USA) in 384-well plates. Reactions consisted of 6 μL of PCR Master Mix (Qiagen), 0.6 μL of UPL fluorescent probe and forward/reverse primers, 2.4 μL nuclease-free water, and 3 μL of 1:6 diluted cDNA. Cycling conditions were 2 min at 50°C, 10 min at 95°C, 40x cycles of 15 s at 95°C and 60 s at 60°C. Cycle thresholds (Ct) were obtained using SDS 2.4 (Applied Biosystems), and data were normalized to RPL7, a reference gene that was analyzed and confirmed for stability using GeNorm (Vandesompele et al., 2002).

Chemical analysis

Selection of chemicals for analysis: Both a pesticide (bifenthrin, an EDC), and pharmaceutical (ibuprofen), as well as caffeine, all known to occur in association with wastewater treatment effluents, were analyzed for. Prior studies evaluating the effects of bifenthrin and ibuprofen on *M. beryllina* have been conducted (Brander et al. 2012b; DeGroot and Brander 2014; Jeffries et al. 2015), thus they were considered important contaminants to evaluate in this context.

Bifenthrin: Within 24h, water samples were filtered (baked 1μm Grade GF/F microfiber filter, Whatman), spiked with trans-permethrin (dimethyl D6, EQ Laboratories, Atlanta, GA, USA) as a recovery surrogate, extracted using preconditioned solid phase extraction cartridges (Supelclean ENVI™ - C18, 500 mg, Sigma-Aldrich, St. Louis, MO, USA), and evaporated to 0.4 mL at 40°C under a gentle stream of nitrogen using a Turbovap (Biotage, Charlotte, NC, USA). Filter papers were extracted with 2 × 75 mL dichloromethane:acetone (50:50, v/v), evaporated, solvent exchanged into hexane, recombined with the corresponding whole water extract and concentrated to 0.4 mL. The internal standard 4,4’ dibromo-

octafluorobiphenyl (Chem Service, West Chester, PA, USA) was added to all concentrated extracts in order to correct quantitative differences in extract volume as well as to monitor instrument conditions. Extracts were analyzed using an HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an HP-5973N quadrupole mass spectrometer detector operated in electron capture negative ionization mode (GC-ECNI-MS) with methane as the reagent gas. The gas chromatograph was equipped with a split-splitless injector (280°C, splitless, 1.5-minute purge time) and a Supelco DB-5MS column (30 m × 0.25 mm with a 0.25 μm film thickness) with Helium as the carrier gas. Instrumental calibration was performed using nine sets of calibration standard solutions (purchased as 100 μg/ml solution in acetonitrile, Chem Service, West Chester, PA), the surrogate trans-permethrin D6 and the internal standard; 4,4' dibromo-octafluorobiphenyl in hexane. Quality-assurance/quality-control was conducted by analyzing a method blank of deionized water (Milli-Q), to ensure that no contamination occurred during sampling extraction and analysis. Instrumental limit of detection for bifenthrin was 1 ng L^{-1}. No bifenthrin was detected in the method blank. Surrogate recovery was on average 111.21% with a range between 102.01 – 116.59% confirming high extraction efficiency. Reported values were not corrected for surrogate recovery.

Ibuprofen and caffeine: Samples were submitted to the California Department of Fish and Wildlife Water Pollution Control Laboratory (Rancho Cordova, CA) for quantification of ibuprofen and caffeine. These samples were analyzed by gas chromatography (Agilent 6890 plus; Agilent Technologies Inc., Santa Clara, CA) with dual columns (DB5 and DB7). The surrogates Gemfibrozil D6 and Sulfamethazine were used with average recoveries of 106.3%, with a range of 83.0% and 123.0% and 84.1% with a range of 71.8% and 94.0%, respectively. The limit of detection for ibuprofen was 32 ng L^{-1} and 20 ng L^{-1} for caffeine.

CALUX assay to determine estrogen or androgen receptor activities

The CALUX mammalian cell bioassay utilizes recombinant human ovarian carcinoma (BG-1) cells (BG1Luc4E2) or breast cancer (T47-D) cells (T47LucARE), which have been stably transfected with an estrogen-responsive or androgen-responsive luciferase reporter plasmid, respectively. These CALUX cell lines respond to estrogenic (BG1Luc4E2) or androgenic (T47LucARE) chemicals with the induction of expression of firefly luciferase that is directly proportional to the degree of activation of the estrogen or androgen receptor (ER or AR) signaling pathways, respectively (Brander et al. 2012b; Rogers and Denison 1999). Extract preparations from water grab samples to be incubated with the BG1Luc4E2 and T47LucARE cells were performed as previously described (Brander et al. 2013; Giudice and Young 2011). In brief, a bottle control was performed with distilled water held in an identical sampling bottle for the same period of time as the grab samples were stored and extracted in the same manner. Method controls of methanol or methanol combined with the appropriate hormone were also performed (not shown). Receptor agonism is determined by an increase in luciferase activity above the bottle control. Antagonism is determined by a decrease in luciferase activity from the bottle control when the samples were co-exposed to either 1 nM estradiol or 10 μM testosterone. All grab samples were concentrated 2500× and exchanged into dimethylsulfoxide (DMSO). Three technical replicates were performed for each assay.

Statistical analysis

Quantitative PCR data were adjusted using the relative quantification (−ΔΔCt) method (Livak and Schmittgen 2001). Fold-change data are reported as the −ΔΔCt gene transcription relative to RPL7 and normalized to the mean transcription of each gene corresponding to the experimental controls from each site. Differences in gene expression (i.e. mRNA levels) between sites were assessed using a linear mixed effects model (R, 2.15.3, www.r-project.org) that included plate as a factor, to account for any variability introduced by running multiple qPCR plates. Results were considered to be significant at an alpha of ≤ 0.05. CALUX response data were analyzed using JMP (SAS Institute, Cary, North Carolina, USA). Results were analyzed by ANOVA followed by a Dunnet's post-hoc test to determine statistically meaningful differences from controls at a $p<0.05$. Error bars in all figures indicate one standard deviation from the mean.

Results

96-hour water exposures

Water physicochemical parameters remained stable throughout each four-day sample collection batch (Table 3), but varied among the three sample time points, with salinity, and associated electrical conductivity ranging from 11.1 to 16.1 ppt, and 16.83 to 25.80 mS cm^{-1} in Carquinez Strait exposures, and from 1.0 to 1.2 ppt, and 1.70 to 2.19 mS cm^{-1} in Boynton Slough exposures, respectively (Tables S1 and S2). Control water adequately matched water samples from the POTW effluent discharge points in terms of physicochemical parameters. There was no mortality associated with any of the ambient waters, nor controls.

Chemical analysis for contaminants in site water

All collected waters from April 17–25, 2014 were analyzed for the presence of bifenthrin, ibuprofen, and caffeine (Table 4) to determine how concentrations and rate of appearance of each chemical varied for the different collection sites beyond solely during the period of *in vivo* fish exposures. Bifenthrin was only detected in the April 21 collection from Boynton Slough, and ibuprofen was not detected in any samples. Caffeine was found at low concentrations in all samples from Boynton Slough, but not in any of the Carquinez Strait samples.

Gene expression responses to SSJ Delta water exposure

Water from both sites caused a significant change in the level of mRNA of genes important in controlling hormone signaling and development in the 12 dph fish (Figure 2 and 3). Both forms of the aromatase gene were significantly differentially expressed ($p<0.05$); with increased expression of CYP19a, and decreased expression of CYP19b, in the fish exposed to Boynton Slough POTW discharge water compared to salinity matched controls (Figure 2). Additionally, two of the three estrogen receptors, ESR1 and ESR2, also showed altered expression levels compared to controls. A major chemical detoxification gene CYP1a, showed increased expression ($p<0.05$), consistent with exposure to a wide variety of chemicals (Figure 2). Fish exposed to water from Carquinez Strait POTW discharge showed

significantly decreased expression ($p<0.05$) of three major hormone receptors (Figure 3): the androgen receptor (ARx) and two estrogen receptors (ESR2 and ESR3).

CALUX assays for estrogen and androgen receptor agonists and antagonists

To understand baseline levels of endocrine disruption, water samples, including the final one used for fish exposures, were tested in the CALUX bioassays for their effect on estrogen or androgen receptor-dependent gene expression. All 2500x concentrated water samples from the Boynton Slough and Carquinez Strait induced luciferase activity in the BG1Luc4E2 cell line, indicating the presence of chemicals that mimic 17β-estradiol (i.e., they act as estrogen receptor agonists) are present at both sites, on all three of the dates sampled (Figure 4A). In contrast, none of the samples from either site inhibited estradiol-dependent induction of luciferase gene expression (i.e. none acted as estrogen receptor antagonists). All of the samples from both sites also induced luciferase activity in the T47LucARE cell line, indicating the presence of testosterone-like chemicals (i.e., androgen receptor agonists) at these sites with significant increases in receptor-binding activity at both sites from all days (Figure 4B). Similar to results with the estrogen receptor, none of the samples inhibited testosterone-induced reporter gene induction (i.e. the samples did not contain androgen receptor antagonists).

Discussion

Understanding the impacts of anthropogenic contaminants in dynamic estuarine habitats is crucial to managing these susceptible areas, but studies are difficult for many reasons. While research has been conducted on the effects of complex mixtures on fish in the SSJ Delta, either by assessing responses in the resident fish *M. beryllina* (Brander et al. 2013) or by reconstituting Delta-specific mixtures for laboratory exposures (Schlenk et al. 2012), little light has been shed on the cumulative effect of complex endocrine-active mixtures on estuarine fish species. Although a number of studies have exposed various fish species to wastewater (i.e. (Barber et al. 2011; Liney et al. 2006; Tetreault et al. 2011), the vast majority of these types studies have been done in freshwater. Hence there is little information available for marine or estuarine species, even though treated wastewater effluent is frequently pumped into estuarine ecosystems.

In this study, we sought to further expand use of *M. berylina* as a model species for examination of estuarine endocrine disruption by specifically evaluating molecular-level responses to wastewater exposure. These responses were linked to overall estrogenic or androgenic activity of site water samples via CALUX assays, rather than conducting extensive analytical chemistry to develop a likely incomplete list of contaminants. CALUX assays have been previously used to relate the overall endocrine activity in concentrated water samples to fish response (Brander 2013; Brander et al. 2012b). Here, they are used as a relative comparison between the sites for the sampled water's capacity to activate or inhibit estrogen and androgen receptors. Although the CALUX assay uses human cell lines, due to the similarity in sequence of two estrogen receptors (ER alpha and ER beta, of which CALUX has both) and the androgen receptor between fish and humans (Pakdel et al. 1989; Shyu et al. 2011), the CALUX is a useful tool to use in this context for the measurement of

overall endocrine activity. These results were utilized as an indicator of total endocrine disruption potential of the two sites. Although there are limitations to this assay in that it does not contain fish hormone receptors, it allows for the determination of potential EDC impacts from complex environmental mixtures in an efficient and cost-effective manner.

Water collected near two POTW estuarine effluent discharge sites caused significant changes in gene expression of 12 dph silversides in an acute (96-hour) assessment, compared to salinity-matched controls. While the two sites elicited qualitatively different responses, they cannot be quantitatively compared due to large differences in salinity necessitating controls specific to each site. The site-specific changes in gene expression were likely caused by differences in the chemicals present in samples from each site, which also resulted in differential activities detected in the CALUX reporter assays for estrogenic and androgenic chemicals.

Comparing the gene expression responses of 12 dph fish between the two sites, there were both induction and repression responses caused by Boynton Slough POTW outfall waters, compared to the more consistently decreased expression of hormone receptors for estrogens and androgens caused by Carquinez Strait waters. A greater number of genes were significantly affected by exposure to water from Boynton Slough site as compared to that from Carquinez Strait. In the Boynton Slough samples, expression of both CYP19b and CYP19a aromatase genes (Kishida and Callard 2001; Trant et al. 2001) were altered, which could have a functionally significant influence on development as these genes are responsible for converting testosterone into estradiol (Simpson et al. 1994) and are known to be important in sex determination, particularly in fish with temperature sensitive sex determination such as *Menidia* spp. (Duffy et al. 2010). Interestingly, the CYP19a gene showed a larger relative change in expression than CYP19b when compared to controls. Fishes are unique amongst vertebrates in that they have two isoforms of the CYP19a gene. The increased expression of CYP19a seen in Boynton-exposed larvae likely represents increased activity of the CYP19a1b isoform (brain aromatase), as gonads are not differentiated in 12 dph larvae. However this could not be specifically verified because it was not possible to differentiate the two isoforms in the *Menidia* transcriptome sequence, thus a general CYP19a probe was used here, which could detect both isoforms. Where it has been described, the CYP19a1b isoform is primarily found in the neuroendocrine portion of the brain (Blázquez and Somoza 2010), where it plays a role in the establishment of neuroendocrine circuitry during the period of sexual differentiation (Gennotte et al. 2014). Furthermore, this isoform may play a role in temperature-sensitive sex determination (Blázquez and Somoza 2010), and is highly responsive to estrogenic compounds due to the presence of an estrogen-responsive DNA element in the promoter region of this gene. Modulation of CYP19b expression suggests alteration of neurogenesis in these larvae (Piferrer and Blázquez 2005). On the whole, changes in the expression of both CYP19 isoforms indicate that these larval fish could be predisposed to gonadal abnormalities or reduced fecundity in adulthood, due to early alterations to their hypothalamic-pituitary-gonadal axis, as has been shown in another Atherinid species (*Oryzias melastigma*) exposed to EDCs during the larval and juvenile stages (Ye et al. 2014). Furthermore, a recent study conducted by Mills and colleagues (Mills et al. 2014) demonstrated an association between altered brain and gonadal aromatase in adult teleosts and reduced gonadal somatic index.

The two estrogen receptors for which expression was significantly altered by treatment with Boynton Slough waters oppositely expressed, consistent with a mixed response to the treatment. ESR1 was significantly downregulated, while ESR2 was significantly upregulated. It is established that the three ER isoforms in fishes exhibit different affinities for the endogenous hormone estradiol and for various EDCs. For example, ESR2 has a greater affinity for 17β-estradiol than ESR1 does (Hawkins and Thomas 2004). As such, differences in the direction of response for ESR1 and ESR2 are not unexpected. In the CALUX reporter assay, Boynton Slough waters were particularly estrogenic, so much so that the response in some of the treatments was almost as great as the maximal induction response to estradiol (1nM). It is possible that the mixed response of estrogen receptors observed in the qPCR assays is due to regulatory cascades wherein relatively low levels of hormone or hormone mimics causes an increase in expression of target genes, but as the hormone concentration is elevated further, the response is attenuated through negative feedback loops (Calabrese 2001; Vandenberg et al. 2012; Welshons et al. 2003). Such a response has been observed previously resulting from exposure to potent EDCs such as permethrin and bifenthrin (Brander et al. 2012b). Results indicate that these fish may have been exposed to either very potent estrogenic chemicals, or combined estrogenic and androgenic chemicals with moderate potency, either of which could result in the mixed response of estrogen receptors to site waters.

The CYP1a gene, which showed a large increase in expression in Boynton Slough but not in Carquinez Strait water exposed 12 dph fish, encodes one of the primary proteins involved in detoxifying foreign chemicals, and the gene is known to be highly inducible as a response to exposure to a wide range of pollutants (van der Oost et al. 2003). The induction of CYP1a is mediated by another ligand-dependent receptor, the aryl hydrocarbon (dioxin) receptor (AhR) (Hahn et al. 2006). The Fairfield Suisun Wastewater Treatment Plant, which discharges into Boynton Slough, uses advanced secondary treatment, which is assumed to remove a greater number of EDCs than the plant in Carquinez Strait, which uses secondary treatment. Thus we would expect lower impacts from exposure to Boynton Slough waters because of greater chemical removal. However, the total volume of water present and flow rates through Carquinez Strait are much greater than those in Boynton Slough, resulting in a higher degree of dilution of POTW effluent. Furthermore, the total percentage of treated sewage effluent (95% of the 64.4 MLPD in Boynton Slough is treated effluent VS just 3.5 MLPD in Carquinez Strait) is higher in Boynton Slough as well. Our findings indicate that the advanced sludge treatment used in the Fairfield Suisun Plant may not be completely effective at removing EDCs. These findings highlight the importance of optimally designing wastewater treatment plants with consideration of the hydrology of the system into which treated effluent is discharged, particularly in estuaries where the salinity is variable and flow is not uni-directional, as it would be in a riverine system.

While waters from the Carquinez Strait were less estrogenic than those from Boynton Slough in reporter assays, they still produced significant changes in gene expression in 12 dph fish. Unexpectedly, the response of all gene products that were significantly affected was one of downregulation, despite the activation of both estrogen and androgen receptors as revealed by the reporter gene assays. All three of the responding genes were hormone receptors, including the androgen and two estrogen receptors. Such a decrease in response of

all receptors could have cascading impacts as decreased reception of natural hormones or anthropogenic EDCs leads to diminished gene expression response. Loss of responsiveness to hormones could result in decreased growth rates, thus impacting populations via increased predation or decreased fecundity of smaller fish (Gleason and Bengtson 1996). On the other hand, it is possible that decreased expression of hormone receptors indicates negative feedback may be occurring. The number or activity of hormone receptor proteins, as opposed to the number of mRNA transcripts, has increased to the point that further transcript production is downregulated (Nikinmaa and Rytkönen 2011).

Greater changes in gene expression following exposure to Boynton Slough waters are likely related to the relatively higher concentrations of chemicals in this waterway as compared to Carquinez Strait, however there are other possibilities to explain the effects we observed. Changing salinity can itself affect gene expression and can impact the response following exposure to EDCs, in part due to differences in the uptake of estrogenic chemicals at different salinities. Recently it was shown in another estuarine model species, *Fundulus heteroclitus*, that the uptake of the synthetic estrogen ethyinylestradiol, commonly present in treated effluent, was reduced at higher salinities (Blewett et al. 2013). Additionally, it has been reported that bifenthrin has more potent estrogenicity in steelhead trout (*Oncorhynchus mykiss*) when they were exposed in fresh water close to the salinity of that in Boynton Slough than in higher salinity waters such as Carquinez Straight (Forsgren et al. 2013; Riar et al. 2013). A similar trend indicating higher estrogenicity of bifenthrin at lower salinities was also shown in a recent experiment in silversides (Diaz and Brander, unpublished data). In contrast, other contaminants are more toxic as salinity is increased, especially in hypersaline environments (Lavado et al. 2009; Lavado et al. 2012; Maryoung et al. 2015; Schlenk and Lavado 2011).

Both sites used in this study caused significant changes in gene expression in fish in the early stages of their development and evidence of endocrine disruption in CALUX assays. While the magnitude of the genetic response is relatively small (<1-fold change in gene expression), this could still have repercussions for further development of these fish. Because the majority of significantly responding genes were hormone receptors, which begin signaling cascades that magnify the impact of a hormonal change, or CYP19a and b which are involved in hormone metabolism, there could be large ramifications of even small changes, such as those observed here, as a result of exposure to low concentrations of hormone-mimicking compounds (Vandenberg et al. 2012). Many fish, including silversides, spawn in April-June in the SSJ Delta, so the impacts on expression of hormone receptors and other endocrine genes in developing embryos and juveniles during this time could represent a significant impact on populations of these fish. This is especially true for fish like silversides, which exhibit temperature-dependent sexual differentiation as a mechanism to optimize fecundity (Huber and Bengtson 1999), meaning that environmental parameters can reverse genetic sex determination, hence altering their sex ratios. The window for sex differentiation is not specifically known, but it has been shown to occur in the closely related *Menidia menidia* when larvae are approximately 8–21 mm in length (Conover and Fleisher 1986), likely prior to 20 dph. EDCs, especially at the high levels of potency observed in Boynton Slough samples, could skew male:female sex ratios, thus impacting the growth potential of fish populations (Kidd et al. 2007). In fact, waters approximately 1 km in

distance from Boynton Slough, within the same estuarine system, were shown over a recent two-year study (2009–2010) to have significantly masculinized populations of silversides concomitant with changes in the expression of endocrine-responsive genes in wild fish and both estrogenic and androgenic activity in the same CALUX cell lines (Brander et al. 2013).

Understanding the influence of anthropogenic EDCs on sex determination in silversides and other fishes is crucial to defining the contribution these chemicals play in declining populations of fish in the SSJ Delta and estuaries elsewhere. Furthermore, this study lays the framework for a more in-depth analysis of the numerous wastewater treatment plants that discharge into estuarine ecosystems. A thorough analysis of the effects of varied approaches to wastewater treatment, combined with widely varying salinities, dilution factors, and hydrological dynamics is essential to discerning the impact of anthropogenic activities in estuaries. Such a study would not only be useful for the SSJ Delta, but could inform the design or need for the upgrade of wastewater treatment plants in estuarine systems globally, which historically have been understudied compared to outfalls in freshwater, riverine systems (Desforges et al. 2010; Jobling et al. 2005). The availability of a widely distributed estuarine fish model will now allow for the development of a better understanding of these impacts across brackish, estuarine, and marine environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Linda Deanovic and Marie Stillway University of California Davis Aquatic Toxicology Laboratory for maintaining the silversides and conducting the exposures, and Dennis Cocherell and Matthias Hasenbein for assistance with water sampling. This research was funded by the California Department of Fish and Wildlife (contract #E1183010, to REC and SMB), the State and Federal Contractors Water Agency (contract #06-447-300, to REC), the National Institutes of Environmental Health Sciences [P42-ES004699 (MSD)] and the California Agricultural Experiment Station. Delta Science Postdoctoral Fellowships from the Delta Stewardship Council provided funding for BJC and KMJ.

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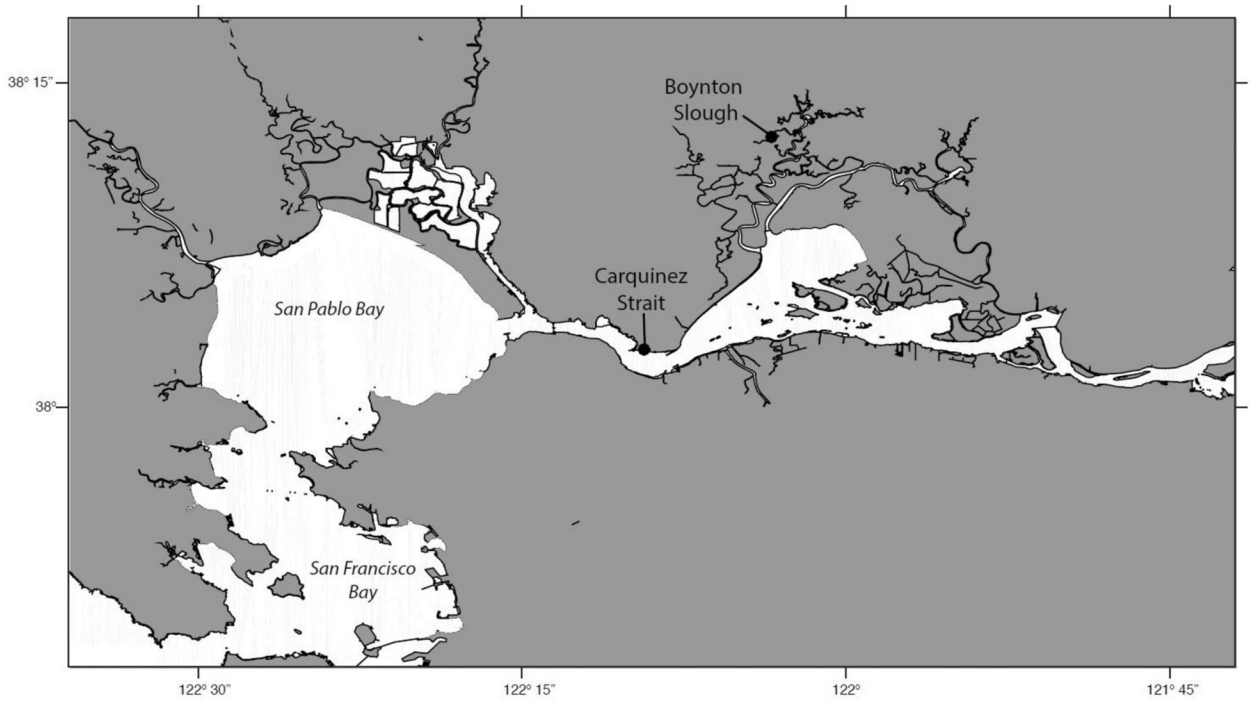

Figure 1:
Location of sampling sites associated with publically owned treatment works (POTW) in the Sacramento-San Joaquin Delta and estuary, California, USA.

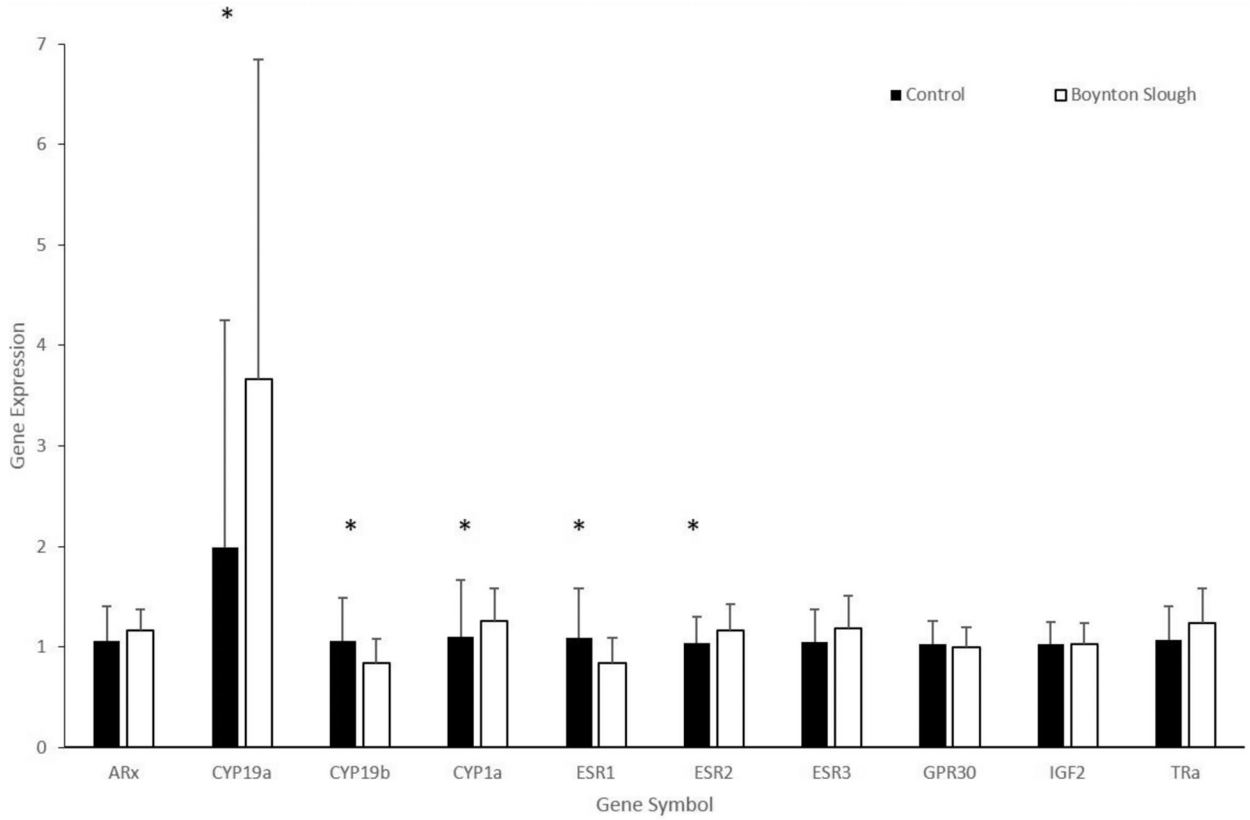

Figure 2:

Gene induction response in 12 days post hatch (dph) inland silversides (*Menidia beryllina*) exposed for 96 hours to water from Boynton Slough collected as a single grab sample on 4/25/14 compared to salinity-matched controls. Fold-change data (labelled Gene expression on the y-axis) are reported as the −ΔΔCt gene transcription relative to RLP7, and normalized to the mean transcription of each gene corresponding to the experimental controls from each site. Genes found to be significantly different from controls ($p<0.05$) are indicated by an asterisk. Values represent the mean ± the standard deviation.

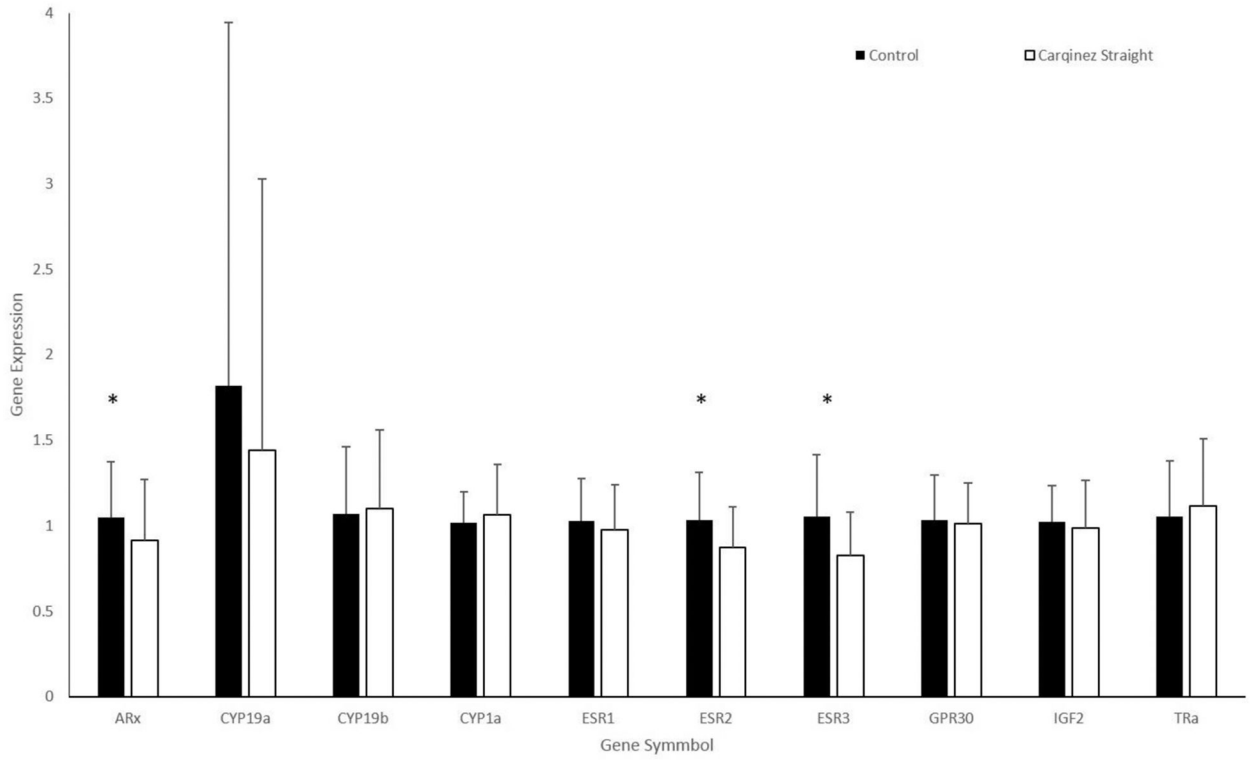

Figure 3:

Gene induction response in 12 days post hatch (dph) inland silversides (*Menidia beryllina*) fish exposed for 96 hours to water from the Carquinez Strait collected as a single grab sample on 4/25/14 compared to salinity-matched controls. Fold-change data (labelled Gene expression on the y-axis) are reported as the −ΔΔCt gene transcription relative to RLP7, and normalized to the mean transcription of each gene corresponding to the experimental controls from each site. Values represent the mean ± the standard deviation, and those samples significantly different from the matched controls ($P<0.05$) are indicated by an asterisk. The three genes showing significantly reduced expression compared to controls were all hormone receptors.

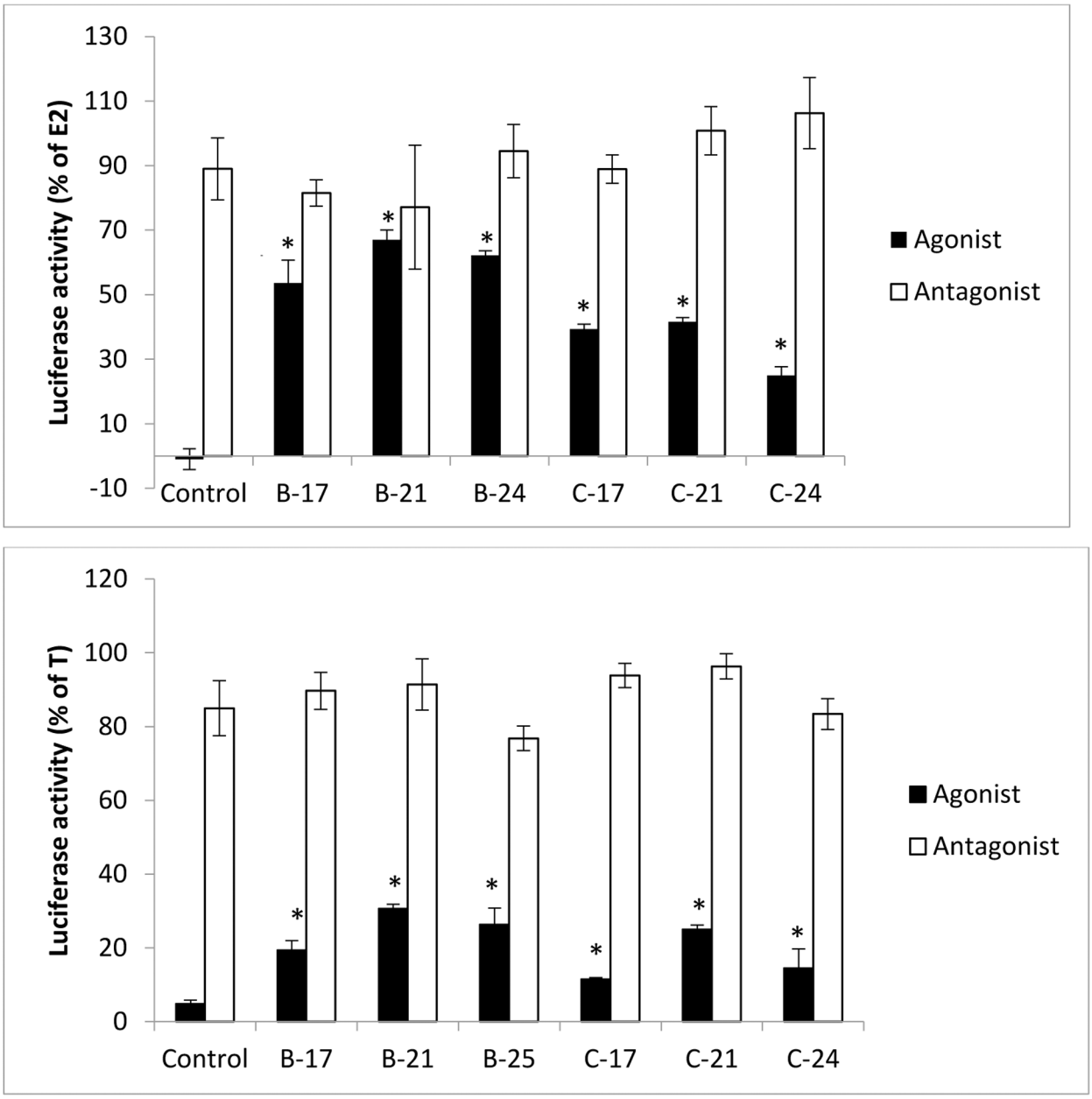

Figure 4:

Agonist and antagonist activity of Boynton Slough and Carquinez Strait water samples in estrogen and androgen receptor responsive CALUX cell lines. Sample names indicate the site and date in April that the grab sample was collected – B-17 is Boynton Slough, collected on 4–17-2014. Values represent the mean ± standard deviation, and significance ($p<0.05$) is indicated by an asterisk. (A) Estrogen-responsive BG1Luc4E2 CALUX cells or (B) testosterone-responsive T47LucARE CALUX cells were incubated with the indicated water sample extract in the absence (for agonist activity (white bars)) or presence of 17β-estradiol (for antagonist activity (black bars)) and luciferase activity determined at 24 hours as described under Materials and Methods.

Table 1:

Water samples were collected on the indicated dates in April of 2014. The indicated assays were performed on the collected water samples from each date.

Collection Date	4/17/2014	4/21/2014	4/25/2014
Water chemistry	Y	Y	Y
CALUX	Y	Y	Y
Fish exposure	N	N	Y

Table 2:

Primers and related information for the genes used to assess the response to exposure to Sacramento/San Joaquin Delta waters in 12 days post hatch Silversides (*Menidia beryllina*).

Function	Symbol	Name	Roche UPL #	Efficiency (%)		Primer Sequence (5' to 3')
Reference	RPL7	60s ribosomal protein 17	31	95.1	F:	aacttcttgtggccgttcaag
					R:	tcgcctccctccacaaagt
Hormone	GPR30	G-protein coupled estrogen	48	104.1	F:	cgtcctctccggcctctac
receptor		receptor 1			R:	tgaggatgttcccaatgaagc
Hormone	ESR3	Estrogen receptor beta b (ESR3)	130	101.4	F:	gattttattcaaccggagcagtg
receptor					R:	catcggctcgtctgatgaact
Hormone	ESR1	Estrogen receptor 1	15	107.8	F:	ctccattgtgccagtgcaga
receptor					R:	acgcttccgcatgctca
Hormone	ESR2	Estrogen receptor 2	52	102.4	F:	gaccatcctgggaaactgatctt
receptor					R:	cattatgccctccacgcact
Hormone	ARx	Androgen receptor	31	99.1	F:	atccgcatgcagtgctcata
receptor					R:	ccccagacctcgtattcaacg
Hormone	TRA	TR alpha	51	101.5	F:	tgtcggacgccatattcgat
receptor					R:	cctcggtgtcatccaagttga
Androgen	CYP19A	Gonadal aromatase	3	104.7	F:	gcctcccacagaccaacaat
metabolism					R:	gccatgctgaggtgttcagtc
Androgen	CYP19b	Brain aromatase CYP19b	25	119.2	F:	cagaacccagatgtggagcag
metabolism					R:	cacagactttcccctgaacacc
Growth	IGF2	Insulin-like growth factor 2	38	101.2	F:	gcaggtcatacccgtgatgc
factor					R:	ggctgccttcctattccacac
Detoxification	CYPla	CytochromeP4501a	17	105.7	F:	ccgacgttacaaccaccatg
					R:	gacgaaatcctccgtcaggtt

Table 3:

Summary of physicochemical parameters from water sampled at Carquinez Strait and Boynton Slough, during a 96-hour, 12 dph *Menidia beryllina* exposure.

		Mean	Range	SD	SE
Carquinez Strait controls	Temperature (°C)	20.4	19.7 – 20.8	0.49	0.25
	pH	8.14	8.12 – 8.17	0.03	0.01
	Dissolved Oxygen (mg L^{-1})	8.35	8.1 – 8.6	0.21	0.1
	Electrical Conductivity (mS cm^{-1}, @ 20°C)	17.75	17.53 – 17.98	0.2	0.1
	Salinity	11.6	11.5 – 11.8	0.13	0.06
Carquinez Strait samples	Temperature (°C)	20.4	19.6 – 21.2	0.66	0.33
	pH	8	7.96 – 8.08	0.05	0.03
	Dissolved Oxygen (mg L^{-1})	8.28	8.0 – 8.6	0.25	0.13
	Electrical Conductivity (mS cm^{-1}, @ 20°C)	17.3	16.83 – 17.74	0.42	0.21
	Salinity	11.3	11.1 – 11.5	0.17	0.09
Boynton Slough controls	Temperature (°C)	20.4	19.4 – 21.2	0.75	0.37
	pH	8.13	8.07 – 8.24	0.08	0.04
	Dissolved Oxygen (mg L^{-1})	8.75	8.4 – 9	0.26	0.13
	Electrical Conductivity (mS cm^{-1}, @ 20°C)	2259.25	2163 – 2388	105.03	52.52
	Salinity	1.25	1.2 – 1.3	0.06	0.03
Boynton Slough samples	Temperature (°C)	20.3	19.6 – 20.9	0.54	0.27
	pH	8.35	8.3 – 8.42	0.06	0.03
	Dissolved Oxygen (mg L^{-1})	8.75	8.4 – 9	0.26	0.13
	Electrical Conductivity (mS cm^{-1}, @ 20°C)	2110.75	2074 – 2153	33.42	16.71
	Salinity	1.2	1.2 – 1.2	0	0

Table 4:

Concentrations of chemicals detected by mass spectrometry for the indicated water samples. All measurements are given in weight by volume amounts of μg L^{-1}.

Chemical	Minimum Detection Limit	Boynton 4/17/2014	Boynton 4/21/2014	Boynton 4/25/2014	Carquinez 4/17/2014	Carquinez 4/21/2014	Carquinez 4/25/2014
Bifenthrin	0.001	ND	0.00289	ND	ND	ND	ND
Ibuprofen	0.02	ND	ND	ND	ND	ND	ND
Caffeine	0.032	0.081	0.052	0.069	ND	ND	ND