From ‘Omics to Otoliths: Establishing *Menidia* Species as Bioindicators of Estrogenic and Androgenic Endocrine Disruption.

By

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DISSERTATION

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DAVIS

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Acknowledgments

In 2005, frustrated with the lack of creativity involved in my job and desiring to do "real science," I decided to apply to a Ph.D. program. My decision to join the Pharmacology and Toxicology Graduate Group at UC Davis five and a half years ago has turned out to be one of the best decisions I have ever made. The few semesters I spent on campus were short, but laid the foundation for my research. Through the support of great people like Alan Buckpitt, Inge Werner, Marion Miller, Ron Tjeerdema, Judy Erwin and Linda Deavonic (Aquatic Toxicology Lab) I was able to take full advantage of the resources available.

I met my eventual advisor, Gary Cherr, in 2005 during a visit to the Bodega Marine Lab. I was intrigued by his research and wasn't discouraged by the lack of immediate funding. I will be forever grateful for his help over the next year reading through what seemed like the countless applications for funding that I drafted, which ultimately resulted in a 3-year fellowship that funded the majority of my research. I am also thankful for both the financial and emotional support that he gave me when the state budget crisis temporarily suspended this fellowship. Because of this I barely had a hiccup in my progress. His unwavering enthusiasm for science and seemingly innate ability to be pleasant in the face of almost any situation are traits that I do my best to emulate in both my career and personal life.

My committee has been extremely helpful in guiding me through the journey from proposal to completion. The research experience I gained in Inge Werner's lab prior to beginning work on my dissertation helped me to develop suitable questions and her feedback allowed me to refine my ideas. Mike Denison always had good suggestions for alternative approaches and his thorough edits have made me a better scientific writer.

Although my name will be first on the publications that result from this dissertation, it was completely and truly a collaborative effort. To begin with, none of this research would have been possible without the support of Bodega Marine Lab faculty and staff. I greatly appreciated the sage advice and reminders to have a sense of humor from Jim Clegg and the help I received from the Chang lab. I have Kitty Brown to thank for the space I obtained to house my fish and the Aquatic Resources Group for teaching me how to keep them alive. Finally, without the support and assistance of Carol Vines, Suzy Jackson and others in the Cherr Lab I would never have produced the photogenic Western blots that appear in Chapter 1, among many other results.

While in graduate school, one of my primary goals was to obtain as many research tools as possible. I was fortunate to be at UC Davis, where it was possible to find experts in many toxicological and ecological sub-fields both on and off campus. Through seeking out new ways of answering research questions I formed a number of collaborations along the way. I am incredibly thankful for the opportunity to work with Richard Connon, Bryan Cole, Jim Hobbs, Swee Teh, Kelly Smalling and individuals in the Denison, Moyle, and Young Labs, which enabled me to broaden my skill set and to form working relationships that will last well beyond my pre-doctoral research days.

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I must sincerely thank my parents and siblings for being eternally supportive of me, even as I went back for yet another round of schooling. My in-laws have also had nothing but praise for my endeavors. I look forward to seeing them in the audience as I walk across the stage this coming June to receive my diploma. Finally, my thanks goes beyond what words can convey for the constant support and love I receive from Will White, my best friend, husband and collaborator. He and our beautiful baby girl Vera inspire me to do my best work every day of the year.

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Curriculum Vitae

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Education

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2004 Wildlife Technician, USGS Patuxent Wildlife Research Center, Laurel, MD

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Publications

in prep Brander SM, He G, Smalling KL, Denison MS, Cherr GN. The *in vivo* estrogenic and *in vitro* anti-estrogenic activity of permethrin and bifenthrin.

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Pharmacology and Toxicology

From ‘omics to otoliths: Establishing *Menidia* species as bioindicators of estrogenic and androgenic endocrine disruption

Abstract

A large body of work has established a link between endocrine disrupting compounds (EDCs) and physiological abnormalities in fishes. EDCs, which include pesticides such as pyrethroids, can mimic, synergize or antagonize the effects of endogenous hormones. Both theory and empirical data confirm that EDCs can also cause fish population decline. However, to date few studies have attempted to link realistic environmental EDC mixtures with responses at multiple tiers of the biological hierarchy, including population level impacts. Additionally, although EDCs currently are known to impact a wide array of fishes, most studies continue to use a few standard laboratory denizens. A need exists for an estuarine model fish, particularly considering that estuaries are utilized by many fish species for part of their lives and are subject to many EDC inputs. Silversides (*Menidia spp*.) are well appointed for this role. Furthermore, the San Francisco Bay (SFB) estuary, home to introduced silversides, represents an ecosystem in need of a surrogate to evaluate EDC impacts. As such, an assessment of SFB’s Suisun Marsh, an area with documented pyrethroid presence and EDC inputs, using silversides was undertaken. The first objective was to establish *Menidia* species as an EDC bioindicator. The second objective was to measure environmental impacts at multiple biological scales and use correlations between scales to discern potential mechanisms of disruption. The third objective was to develop and utilize techniques in the laboratory to further understanding of EDC environmental impacts. Clear links were observed between the biological scales examined for signs of endocrine disruption in *Menidia* at two sites within Suisun Marsh of the SFB estuary,

which included alterations in sex ratio, gonadal morphology and expression of estrogen responsive genes. Additionally, my findings suggest that the pyrethroids bifenthrin and permethrin are contributing to the incidence of endocrine disruption in SFB fishes. Finally, I developed a whole body homogenate ELISA that allows the detection of *Menidia* choriogenin, an estrogen responsive protein, in these small fish from which plasma cannot be obtained. Ultimately this work establishes *Menidia* spp. as a sensitive EDC bioindicator and expands the universe of responses that may be evaluated in both the field and the lab.

Dissertation Introduction

Over the past two decades, a large body of work has established a link between endocrine disrupting compounds (EDCs) and a number of physiological and behavioral abnormalities in fishes. EDCs are chemicals that can disrupt endocrine function by mimicking, synergizing or antagonizing the effects of endogenous hormones, such as estrogen or testosterone (Rempel & Schlenk 2008). Estrogenic or androgenic EDCs originate from a variety of sources, including urban or agricultural runoff (e.g., pesticides) and components of treated wastewater effluent (Pait & Nelson 2002). EDCs are widespread in the aquatic environment, occurring even at supposedly pristine sites (Anderson et al. 2006). Examples of hormonal disruption in fishes produced by EDCs include altered secondary sexual characteristics, males producing egg proteins (vitellogenin, choriogenin), and reduced sperm quality (Bogers et al., 2006, Metcalfe et al., 2001, Singh & Singh, 2008). Both theory and empirical data confirm that EDCs can also cause declines in fish populations (Gurney, 2006, Kidd et al., 2007), and links between biochemical endpoints and population persistence have been made (Ankley et al., 2008). However, to date few studies have attempted to link complex environmental EDC mixtures with responses at multiple tiers of the biological hierarchy, including population level impacts.

Furthermore, the universe of potential EDCs is expanding as new pesticides and pharmaceuticals enter the marketplace. For example, the use of a relatively new class of pesticides, pyrethroids, has increased considerably in usage as pesticides considered to be more toxic are phased out (Werner & Moran 2008). Pyrethroids are highly toxic to fish and aquatic invertebrates at ppb or pptr (parts per billion, parts per trillion) levels via prolonging the opening of voltage-depended ion channels (Burr & Ray, 2004, DeLorenzo et al., 2006). In addition to effects perpetrated by the intended mechanism,

recent results from *in vitro* assays reveal that some pyrethroids can act as estrogens, anti-estrogens and/or anti-androgens (Chen et al., 2002, Sun et al., 2007). Permethrin and bifenthrin, which have confirmed *in vitro* and *in vivo* endocrine activity, are two of the most frequently detected pyrethroid pesticides in aquatic ecosystems (Amweg et al., 2006). While existing studies have evaluated the endocrine activity of permethrin and bifenthrin, none have assessed the endocrine concentration-response in fish at the ppb or pptr concentrations found in aquatic ecosystems.

Although EDCs currently impact a wide array of fish species, most EDC studies continue to use a few standard laboratory denizens to assess their impact (e.g., zebrafish, medaka, fathead minnow) (Scholz and Mayer, 2008, Segner, 2009, Ankley et al., 2010). As such, environmental risk assessment and assumptions about sensitivity to EDCs are primarily based on these few species. Furthermore, two of these species are not native to North America, and the fathead minnow, which is native, only represents freshwater fishes. Hence, a need exists for an EDC model species for North American estuarine fishes, particularly considering that estuaries are utilized by many species of fish for at least part of their lives and are subject to an extensive range of inputs likely to contain EDCs (Oberdorster & Cheek, 2000, Ridgway & Shimmield, 2002).

Menidia beryllina, an atherinid fish commonly known as the inland silverside, is well appointed as an alternative estuarine model species. To begin with, the sex ratio of *Menidia* species is susceptible to alteration by estrogen exposure during the larval period (Duffy et al., 2009). They are part of the U.S. Environmental Protection Agency's Whole Effluent Toxicity Testing Program (EPA 2002) and atherinids as a phylogenetic group have been shown to be more sensitive to toxicants in comparison to some other species (Clark et al., 1985). They are widely distributed and important in estuarine food webs (Clark et al., 1985) and are found in estuarine and brackish habitats throughout coastal North America (Middaugh & Hemmer, 1982). The San Francisco Bay (SFB) estuary,

home to introduced silversides (*Menidia audens*, Mississippi silverside), is representative of an ecosystem in need of a surrogate to evaluate potential EDC impacts. It is the largest Pacific estuary in North or South America, is ecologically important (Nichols et al., 1986), subject to a diverse array of anthropogenic inputs including EDCs (Lavado et al., 2009, Ostrach et al., 2008, Johnson et al., 1998), and is home to a number of declining fish species (Feyrer et al., 2007). Currently a model species for assessing EDC impacts has not been established in this region.

To resolve the above-mentioned gaps in knowledge, the work described in this dissertation was designed to assess EDC impacts in the SFB estuary's Suisun Marsh, an area with documented pyrethroid presence and varied EDC inputs, using the silverside as a surrogate. The objectives of were threefold. The first objective was to establish *Menidia* species (*M. beryllina, M. audens*) as a bioindicator for EDCs. The second objective was to measure EDC impacts in the wild at multiple biological scales and use correlations between scales to discern potential mechanisms of disruption. The third objective was to develop and utilize techniques in the laboratory that would further our understanding of EDC impacts in the environment, focusing on pyrethroids.

Chapter 1

An approach to detecting estrogenic endocrine disruption via choriogenin expression in an estuarine model fish species

Abstract

A large body of work has established a link between endocrine disrupting compounds (EDCs) and a number of abnormalities in fishes. However, most EDC studies use several standard laboratory denizens to assess their impact, so assumptions about sensitivity are primarily based on these few species. Additionally, existing methods rely on obtaining sufficient plasma to measure EDC biomarkers. Our objectives were a) to establish a new model species for estuarine fishes, b) to evaluate endocrine impacts with a highly sensitive and specific biomarker, and c) to develop a method for the analysis of this biomarker in small fish that do not possess sufficient blood plasma for protein measurement. As such, we created a polyclonal antibody (Ab) to the estrogen-responsive proteins chorion and choriogenin (Chg) in *Menidia beryllina*, found throughout coastal North America and already utilized in EPA Whole Effluent Testing. We then validated the Ab by using it to measure the response to aqueous ethinylestradiol (EE2) through the development an ELISA using *Menidia* whole body homogenate (WBH). Sensitivity of the Ab to *Menidia* WBH is greater than that of the commercially available option. ELISA sensitivity, with a detection limit of 5 ng/mL and a working range of 22.6 – 1370.9 ng/mL, was comparable to ELISAs developed to measure plasma Chg. To our knowledge this is the first ELISA method developed for the detection of Chg using WBH. Including additional model species and methods allowing the evaluation of alternative sample matrices will contribute to an enhanced understanding of inter-species differences in EDC response.

1. Introduction

Over the past two decades, a large body of work has established a link between endocrine disrupting compounds (EDCs) and a number of physiological abnormalities in fishes. EDCs are chemicals that can disrupt endocrine function by mimicking, synergizing or antagonizing the effects of endogenous hormones, such as estrogen (Rempel and Schlenk, 2008). Estrogenic EDCs originate from a variety of sources, including agricultural runoff (e.g., pesticides) and components of treated wastewater effluent (Pait and Nelson, 2002). Males exposed to estrogenic EDCs during sensitive life stages may exhibit secondary sexual characteristics of the opposite gender or change sex entirely (Bogers et al., 2006). More subtly, exposed adult male fish often produce female reproductive proteins (Cheek et al., 2001). Recently it was reported that these subtle molecular level changes could precipitate population decline (Kidd et al., 2007).

Although EDCs currently impact a wide array of fish species, most EDC studies continue to use a few standard laboratory denizens to assess their impact (e.g., zebrafish, medaka, fathead minnow) (Scholz and Mayer, 2008, Segner 2009, Ankley et al., 2010). As such, environmental risk assessment and assumptions about sensitivity to EDCs are primarily based on these few species. Furthermore, two of these species are not native to North America, and the fathead minnow, which is native, only represents freshwater fishes. Hence, a need exists for an EDC model species for North American estuarine fishes, particularly considering that estuaries are utilized by many species of fish for at least part of their lives and are subject to an extensive range of inputs likely to contain EDCs (Oberdorster and Cheek, 2000, Ridgway and Shimmield, 2002).

Menidia beryllina, an atherinid fish commonly known as the inland silverside, is well-appointed as an alternative estuarine model species. The sex ratio of *Menidia* species is susceptible to alteration by estrogen exposure during the larval period (Duffy

et al., 2009). They are part of the U.S. Environmental Protection Agency's Whole Effluent Toxicity Testing Program (EPA 2002) and atherinids as a phylogenetic group have been shown to be more sensitive to toxicants in comparison to some other species (Clark et al., 1985). They are widely distributed and important in estuarine food webs (Clark et al., 1985) and are found in estuarine and brackish habitats throughout coastal North America (Middaugh and Hemmer, 1982). Additionally, ease of rearing and commercial availability allow for naïve fish to be assessed readily in the laboratory.

One of the most effective ways to evaluate whether a fish has been exposed to an estrogenic EDC is to utilize an indicative molecular biomarker. The majority of studies designed to detect estrogenic endocrine disruption at the molecular level have used the estrogen-responsive female reproductive protein vitellogenin (Vtg) as a biomarker (Rempel and Schlenk, 2008). Although Vtg is a good option for the detection of exposure to estrogenic compounds, an alternative indicator of such exposure is choriogenin (Chg). Chgs are the precursors to the chorion, or egg coat of the oocyte, and they have been shown previously to be more sensitive than Vtg to certain xenoestrogens (Arukwe et al. 2000). Chgs have also been demonstrated to be a sensitive biomarker in Japanese medaka (Lee et al., 2002), a species closely related to silversides.

Previous studies have used commercially available antibodies for cross-species detection of protein expression in response to endocrine disruptors (Palumbo et al., 2009, Tyler et al., 1999). Although these assays are usually robust, ideally such studies should use an assay that is optimized for the species of interest, especially considering the inter-species variation in reproductive proteins such as chorion and its precursor Chg, even within the same phylogenetic group (Schmehl and Graham, 1987). Due to such differences, cross-reactivity of antibodies may not be sufficient, resulting in under-detection of changes in protein expression. Alternatively, non-target proteins may be misidentified, leading to over-detection.

Additionally, the higher sensitivity of species-specific probes allows flexibility of sample type evaluated. The majority of assays developed to detect the expression of female reproductive proteins in response to EDC exposure use blood plasma. While plasma is easily obtained from many fishes, this is not the case for juveniles or for small-bodied species. An alternative to using plasma is to instead use whole body homogenate. This allows assessment of additional fish species and enables the inclusion of juveniles, which are more sensitive to EDCs than adults (Peters et al., 2009). Although whole body homogenate is a complex matrix, it can be successfully used in conjunction with a highly specific antibody (Holbech et al., 2001).

In light of the issues highlighted above, the objectives of our study were a) to establish a new surrogate for North American estuarine fishes, b) to evaluate endocrine impacts with a highly sensitive and specific biomarker, and c) to develop a method for the analysis of this biomarker in small juvenile fish that do not possess sufficient plasma for protein measurement. To address the first objective, we chose the silverside (*Menidia beryllina*) as an alternative to typically utilized species. In lieu of using the only commercially available antibody (anti-salmonid Chg, Biosense, Bergen, Norway), we isolated the chorion (egg coat) of the silverside, for which Chg is the precursor, and used it to generate a specific polyclonal antibody directed against chorion. Since chorion and Chg differ from one another only by a cleaved C-terminal partial sequence (Sugiyama et al. 1999), the antibody created in our second objective also identifies Chg. Finally, an indirect ELISA method was developed with this antibody for the measurement of Chg proteins in whole body homogenate in order to meet the third objective. Application of this method confirmed the expression of Chgs in silversides exposed to a known estrogen. To the best of our knowledge, this is the first ELISA method developed for the detection of Chg using whole body homogenate.

2. Methods

2.1 Solubilization and Isolation of Chorion

Procedures below were based on methods from Oppen-Berntsen et al. (1990). First, gonads were dissected from eleven gravid females collected from Denverton Slough, Solano County, CA (+38° 11' 56.76", -121° 54' 39.31") on April 21, 24 and May 5, 2009. Mature oocytes from ovaries that weighed between 99 – 333 mg each were then homogenized using a glass mortar and pestle to break open oocytes and separate cytoplasm from the insoluble chorion. Ice cold homogenization buffer (200 mg/ml) containing 100 m*M* EDTA and 500 m*M* NaCl, at a pH of 8.3, was used to thoroughly homogenize the combined oocytes. Following homogenization, chorions were rinsed three times with 5 ml and then twice with 2.5 ml of homogenization buffer, and three times with 5 ml of distilled and deionized water. Prior to each rinse the homogenate was resuspended and then centrifuged using a hand-powered centrifuge. Supernatants were removed and discarded after each rinse.

The final chorion pellet (670 mg wet weight) was separated into ten roughly equivalent aliquots of 51 – 79 mg each. A chorion solubilization buffer (Oppen-Bernsten et al. 1990) containing 100 m*M* Tris-HCl, 8 *M* urea, 300 *M* beta-mercaptoethanol (BME), 100 m*M* EGTA, and 1% SDS was added at a ratio of 50 mg/mL to each aliquot. Following the addition of buffer, each aliquot was heated for 20 minutes at 70° C with periodic vortexing during heating to quicken solubilization. Aliquots were then sonicated using a Microson Ultrasonic Cell Disruptor (Heat Systems Ultrasonics, Inc.) for 30 seconds each and subsequently reheated for 5 minutes. Solubilized chorion glycoproteins were then dialyzed overnight against 600 mL of a 50 m*M* Tris-HCl, 25% glycerol solution at pH 8.8. Chorion aliquots of 0.5 mL each were then dialyzed a second time against 1.5 l of phosphate buffered saline (PBS) containing 2 *M* urea, overnight followed by 3 changes of solution every 2 hours, to allow for protein measurement via a

BCA protein assay (Pierce, Rockford, IL, USA) for which interference by this concentration of urea does not occur.

2.2 Antibody Development

Solubilized chorion (660 µg) glycoprotein was loaded into a Bio-Rad preparatory SDS gel which was then electrophoresed at 160 volts / 35 amps for approximately 60 minutes. The gel was then stained with Coomassie Blue and chorion bands at 69, 55, 44 kDa were visualized and excised. Tris buffered saline was added to excised bands to a volume of 2 mL and gels were thoroughly homogenized using a glass mortar and pestle. Homogenate was frozen at -80^{o} C and transported on liquid nitrogen to the UC Davis School of Veterinary Medicine's Comparative Pathology Laboratory for antibody development. Homogenate (1 mL) was injected into two New Zealand white rabbits biweekly for twelve weeks, for a total of 6 injections per rabbit. Test bleeds were taken prior to the start of antigen injections and one week after each antigen injection was given.

Immunoglobulin G (IgG) from rabbit serum was purified by precipitation with 3 additions of 0.8 g/mL ammonium sulfate (Page and Thorpe, 2007), for a final concentration of 24 g/mL. Following each addition of ammonium sulfate, serum was shaken at 4^{o}C for two hours. Afterwards serum was centrifuged at 10,000g for ten minutes, resuspended in 24 g/mL ammonium sulfate and centrifuged again. The pellet was then resuspended in PBS to the original serum volume and purified via overnight dialysis against PBS using a 3.5 kDa sized slide-a-lyzer cassette (Pierce ,Rockford, IL, USA). To verify that the rabbits were mobilizing an immune response specifically to chorion proteins after the first injection, western blots were run using IgG precipitated from test bleeds as an antibody against chorion. The serum with the strongest immune response to chorion proteins was selected as the source for the final antibody.

2.3 Whole Body Homogenates

Juvenile silversides (*Menidia beryllina* - 60 days old) were purchased from Aquatic Biosystems (Ft. Collins, CO). After one week of acclimatization, 10 fish per replicate were exposed for 14 days to a methanol (MeOH) control, 1 ng/L, 10 ng/L, and 50 ng/L ethinylestradiol (EE2, Sigma Aldrich,St. Louis, MO). EE2 was spiked into laboratory control water with a salinity of 5 ppt. Fish were maintained in 3L glass jars containing 1L test water in each and the experiment was maintained at a 14 hr /10 hr light/ dark cycle and temperature maintained at 21 $\pm$ 2 ^{o}C. Each jar was aerated and dissolved oxygen, ammonia and pH were measured daily prior to water changes. Fish were fed live or frozen *Artemi*a nauplii each day at least one hour prior to water change. At the end of the exposure fish were anesthetized on ice and immediately snap-frozen on liquid nitrogen and stored at -80 ^{o}C. Based on methods from Holbech et al.(2001), we homogenized whole fish from each replicate together in approximately 5 mL liquid nitrogen with a ceramic mortar and pestle. Once ground to a powder, two volumes of 50 mM tris-HCl homogenization buffer with protease inhibitor (Roche COmplete Mini, Roche Chemical, Basel, Switzerland) was added to each sample. The sample was further homogenized in a Fisher Scientific Tissuemiser (ThermoFisher Scientific, Waltham, MA, USA) at approximately for 60 seconds. Homogenate was centrifuged at 4^{o}C for one hour at 20,800 ×g and the resulting supernatant was removed and centrifuged again at 14,857 ×g rpm for 15 minutes to ensure removal of all solids. The final supernatant was stored immediately at -80 ^{o}C and a BCA protein assay (Pierce, Rockford, IL, USA) was used to quantify the protein content of each sample.

2.4 Whole Body Homogenate Western Blots

A 10% Tris SDS PAGE gel (Lonza, Basel, Switzerland) was loaded with 10 mL each of whole body homogenate (WBH) from the methanol control treatment and from

the 50 ng/L ethinylestradiol (EE2) treatment. The gel was run on a BioRad model 1000/500 power supply / power pack 200 at 160 volts / 35 amps and transferred to a nitrocellulose membrane using a Trans-Blot SD semi-dry transfer cell (BioRad, Hercules, CA, USA) for one hour at 250 mVolts / 10 amps. The membrane was blocked for 6 hours at 4°C in PBS containing 5% nonfat dry milk, incubated overnight at 4°C in IgG from final serum diluted at 1:10,000 in PBST containing 5% milk, extensively washed to remove unbound antibody and then incubated for one hour in Sigma goat anti-rabbit horse radish peroxidase conjugated secondary antibody. Four washes of 5 minutes each were performed using PBST after blocking, and eight washes of 5 minutes each were done after primary Ab incubation and secondary Ab incubation, respectively. The final blot was imaged for five minutes using West Pico Chemoluminescence (Pierce, Rockford, IL, USA) in a UVP BioImaging Systems (Upland, CA, USA), EPI Chemi II Benchtop Darkroom.

2.5 ELISA

To perform the ELISA with WBH, we first diluted samples in 1:10 in coating buffer (30 mM Na_2CO_3, 70 mM $NaHCO_3$, ph 9.6, Roche COmplete Mini) and loaded 50 mL sample per well, 3 replicates per sample. Standards of known concentrations of chorion in 1:10 WBH were used as an internal control. After coating for 3 hours at 4°C, wells were washed twice with 200 μL each PBS and 200 μL PBS + 5% powdered milk was added to each well. Sample wells were blocked for 1.5 hours, followed by a second 2× PBS wash. *Menidia* chorion primary polyclonal antibody diluted at 1:1000 in PBS with protease inhibitor (Roche COmplete Mini) was then added at 50 μL per well and incubated overnight at 4°C. Following incubation with primary, wells were washed 2× with 200 μL PBS. Then 50 μL per well of a 1:2500 dilution of secondary antibody (goat anti-rabbit HRP conjugate) in PBS containing 5% milk was added and incubated for 3-4

hours at 4°C. Following incubation wells were washed 4× with 200 μL PBS and 50 μL per well 1 step ultra TMB **(**tetramethylbenzidine) (ThermoFisher Scientific, Waltham, MA) was added. Plates were incubated at 25 °C in the dark for 20-30 minutes until sufficient blue color was produced to differentiate the dilutions of chorion-spiked standard. Then 50 mL sulfuric acid (2N) was added to each well to stop the reaction. Wells were read in a GENios plate reader (Tecan, Mannedorf, Switzerland) at an absorbance of 450 nm.

2.6 Data Analysis

Standard curves were calculated by fitting a four parameter logistic model (Rodbard 1981) to log-transformed absorbance and chorion values using nonlinear least squares in R 2.11 (R Core Development Team 2010). The working range was calculated by estimating 20 – 80% binding. Inter- and intra-assay precision was determined by back-calculating chorion standard values from the observed absorbance values using the equation of the line of the standard curve. A nondimensional coefficient of variation of the back-calculated chorion values was calculated by dividing the standard deviation by the grand mean of chorion standards for each data set. A coefficient of variation <15% was considered acceptable (Table 1).

Potential differences between ELISA treatments were analyzed with an ANOVA, followed by a Tukey test. All absorbance values were normalized by protein content prior to statistical analysis.

3. Results

3.1 Test and Final Bleeds

Following the first injection of chorion proteins isolated from *Menidia* oocytes (Figure 1.1), both rabbits mobilized an immune response. A western blot using IgG

isolated from serum sampled after the first test bleed at dilutions of 1:1,000, 1:2,000 and 1:5,000 showed recognition of bands at 69, 55, and 44 kDa, matching the molecular weights of those originally excised (Figure 1.2). The lowest band in Figure 1.2 represents products of protein breakdown. The initial antigen response was strongest towards the 55 kDa subunit. Serum sampled from both rabbits pre-injection did not react with chorion proteins (Figure 1.2). The IgG isolated from the final bleed reacted with chorion proteins at the same molecular weights at a more dilute concentration (1:10,000 than the IgG from the first test bleed (Figure 1.3). For final bleed IgG, the band at 44 kDa was slightly darker than those at 69 and 55 kDa.

3.2 Ethinylestradiol Exposure

Following optimization of the chorion antibody discussed above, the response of juvenile silversides to an estrogenic compound was evaluated and confirmed. Response to a known synthetic estrogen, ethinylestradiol (EE2), was confirmed in WBH using IgG purified from final serum. The antibody recognized choriogenin (Chg), the precursor to chorion, in WBH from naïve fish exposed to 50 ng/L EE2 for 14 days. Chg was not detected in control naïve fish (Figure 1.4). Chg bands resolved at approximately 49 and 55 kDa.

3.3 Assay Precision and Sensitivity

An evaluation of inter- and intra-assay precision showed that back-calculated values nearly matched chorion standards, attesting to the accuracy of the method (Table 1). Inter-assay back-calculated ranges (n=2) were 38.9 – 39.0, 7.79 – 7.80, 1.56, and 0.312. Intra-assay back-calculated ranges (n=3) were 29.9 – 30.0, 5.99 – 6.00, 1.19 and 0.239-0.240. Variance was extremely low, ranging from 1.6 e-07 to 6.3 e-07 for inter-assay and from 4.7 e-06 to 3.5 e-05 for intra-assay. The minimal detection limit was 5

ng/mL, and the working range was 22.6 – 1,370.9, representing 20 – 80 percent binding. These values are slightly higher but comparable to those from assays developed for the measurement of Chg in the plasma of other fish species (Table 1.1).

To determine whether our antibody was more sensitive than the commercially available option, anti-salmonid Chg (Biosense, Bergen, Norway), the two were directly compared in a concurrently-run ELISA. Chorion standards spiked in a 1:10 dilution of whole body homogenate and coating buffer assayed with both the anti-salmonid Chg Ab and anti-*Menidia* chorion Ab were better detected with the latter at all concentrations (Figure 1.5). The minimal detection limit of anti-*Menidia* Chg Ab (5 ng/mL) was two orders of magnitude lower than that of the anti- salmonid Ab (120 ng/mL).

3.4 ELISA

To demonstrate that the *Menidia* anti-chorion Ab is capable of detecting Chg induced by an estrogenic compound commonly found in the aquatic environment in a dose-dependent manner, juvenile *Menidia beryllina* were exposed to three increasing concentrations of ethinylestradiol (EE2) for 14 days and then WBH was assessed via ELISA. Juvenile fish exposed to 1, 10 and 50 ng/L EE2 all expressed Chg at levels significantly higher than the methanol control (MeOH). Mean concentrations in ng Chg / μg protein for MeOH, 1, 10, and 50 ng/L were ($0.002 + 2.1 \times 10^{-4}$ / - 8.9×10^{-3}, 1.866 + 5.1×10^{-1} / - 5.9, 10.739 + 3.7 / - 2.8 e01, and $50.938 + 2.2 \times 10^{1}$ / $-1.2 \times 10^{2)}$, respectively. Upper and lower confidence intervals are not equal and are listed separately because data were not normally distributed (Figure 1.6).

4. Discussion

The egg coat of most teleost fishes is composed of two layers: the outer and inner vitelline envelope, with the inner layer composed of three protein subunits comprising

the majority of the chorion. The origin of the proteins that comprise the chorion is extra-ovarian, in that chorion precursors (Chgs) are synthesized in the liver under the influence of estrogen (Murata et al., 1995). Chorions and Chgs differ from one another only in that Chgs undergo a post-translational modification which involves cleavage of a short C-terminal partial sequence before the Chg is incorporated into the inner vitelline envelope of the egg (Sugiyama et al., 1999). For this reason it is possible to use anti-chorion as an antibody for Chg.

The molecular weights of the three *Menidia* chorion components identified, 69, 55, and 44 kDa, and the Chgs identified via western blot ranging from 55-48 kDa, were similar to those of other fish species. Japanese medaka have three major chorion subunits at 76, 74, and 49 kDa (Murata et al., 1991). Both herring and eelpout have at least three chorion-related polypeptides, which resolve in the range of 44-55 kDa (Griffin et al., 1996, Larsson et al., 2002). The Chgs of rainbow trout migrate to 60, 55, and 50 kDa (Oppen-Berntsen et al., 1992), those of the red lipped mullet are at 51 and 44 kDa (Hong et al., 2009). Although the molecular weights of chorion proteins and Chgs from these four species are similar to one another, they likely differ enough between species that the use of a commercially available Chg antibody made to another species may result in poor detection and an under estimation of these proteins. This was demonstrated in our comparison of the *Menidia* anti-chorion antibody with the commercially available anti-salmonid Chg antibody.

The ELISA using antibody made specifically to *Menidia* chorion is two orders of magnitude more sensitive (minimal detection limit 5 ng/mL) than that conducted using the commercially available anti-salmonid Chg polyclonal antibody from BioSense (minimal detection limit 120 ng/mL). The minimal detection limit of our assay is comparable to detection limits of other Chg ELISAs that used plasma as the sample matrix (1.56 ng/mL, 1-2 ng/mL) (Fujita et al., 2005; Prakash et al., 2007). This is

notable since whole body homogenate is a much more complex matrix than plasma, although we were not able to match the sensitivity of a WBH ELISA developed to detect Vtg in zebrafish (0.2 ng/mL) (Holbech et al., 2001), possibly due to differences in the way the antibody was prepared and samples were purified. Our inter- and intra-assay variation was within acceptable limits and is comparable to the plasma assays mentioned above and to the zebrafish WBH Vtg ELISA (Holbech et al., 2001). Our working range is higher in comparison to previously developed ELISAs for detection of choriogenin in plasma, likely due to the complexity of the WBH matrix. However, choriogenin expression appears to be higher in our WBH samples than was reported in studies with plasma, and we were able to measure choriogenin induced by ethinylestradiol concentrations as low as 1 ng/L with very low variance.

Like vitellogenin (Vtg), Chg is normally only induced by endogenous estrogen in mature females (Oppen-Berntsen et al., 1990, 1992). In comparison to Vtg, Chg expression has been shown to be a more sensitive marker of response to estradiol and to the xenoestrogens DDT, nonylphenol and bisphenol-A (BPA) in salmonids (Celius and Walther, 1998, Arukwe et al., 2000, Ackermann et al., 2002, Fujita et al., 2004). However, it is unclear if this translates to other species. Expression of Chg genes, the precursors to chorion, can begin as soon as four hours after exposure to estradiol in the liver parenchymal cells of fish (Murata et al., 1997). The measurement of protein response rather than mRNA allows for detection of estrogen exposure that may have occurred weeks prior to sampling. This has been shown with male silversides captured from sites contaminated with EDCs, where it can take up to 12 weeks of depuration in lab control water for the level of Chg expression to return to control (unexposed) levels (Brander unpublished data).

We developed our antibody to purified native *Menidia* chorion proteins, rather than using a synthetic peptide. Synthetic peptides can be problematic if they are made to

the portion of the protein that lacks the ideal immune-responsive epitope, which may lead to poor immunogenicity or exhibit cross-reactivity to non-target proteins. The immune response to purified chorion by New Zealand white rabbits was rapid and robust, and was specific to the 3 chorion protein subunits originally excised. Prior to ELISA development, the specificity of the response to the proper proteins was confirmed via western blot in both whole body homogenate and plasma. Our antibody recognized all *Menidia* chorion polypeptides in juveniles exposed to the synthetic estrogen ethinylestradiol via assessment by both Western blot and ELISA.

Concentrations of Chg in plasma have been reported in two species of salmonids. In Masu salmon (*Onchorynchus masou*) injected with 5 mg/kg estradiol, a peak of 5 μg/mL was reached twenty four hours post-injection (Fujita et al., 2004). Levels in Sakhalin Taimen salmon (*Hucho perryi*) were much higher eight days after injection with 2 mg/kg estradiol, at 41.8 μg/mL (Chg H) and 110.5 μg/mL (Chg L) (Shimizu et al., 2000). In Masu salmon, Chg concentrations in females just prior to ovulation were 610 and 980 μg/mL. In our study, concentrations of total Chg reached a mean of 50.9 ng Chg/μg protein in fish exposed to 50 ng/L ethinylestradiol for 14 days. While not directly comparable with the aforementioned plasma ELISAs since both the exposure route and sample matrix differed, proportional Chg expression in WBH was greater than that previously detected in plasma in other species. This could be due to the longer exposure period, the higher potency of ethinylestradiol in comparison to estradiol, and because the exposed juvenile fish were likely expressing Chg in multiple tissues (plasma, liver and mucus) (Meucci et al., 2005).

4.1 Conclusion

The use of whole body homogenate allows the detection of Chg in small fish and in juveniles from which plasma is either difficult or impossible to obtain. This is

particularly useful in light of the heightened sensitivity of juvenile fish to EDCs (Jin et al. 2010; Peters et al. 2009). Using silversides as a surrogate for examining the impact of endocrine disruption in North American estuaries is supported by of the high sensitivity of juveniles to ethinylestradiol (EE2) in this study. This is highly relevant to environmental exposures since EE2 is ubiquitous in the aquatic environment due to its presence in treated wastewater at concentrations at or near what were used in our exposures. Future assays could include samples from wild fish caught at sites with suspected exposure to EDCs. Additionally, since silversides are found in estuaries throughout North America, they have the potential to be used as surrogates for fish species that are endangered and cannot be sampled in large numbers. The responses of silversides could potentially be extrapolated to species of interest (Wenger 2008). In fact, two studies following up on the present study, using wild silversides, are underway in the San Francisco Bay estuary, a region experiencing declines in numerous fish species (Feyrer et al., 2007).

The development of high throughput assays for alternative fish species that have the potential to be used as surrogates for endangered species is of great importance, as relying on a limited number of fish species to represent responses across a range of taxa may lead to an underestimation of toxicity (Banks et al., 2010). Additionally, including more species representative of estuaries will inform efforts to improve water conditions in these highly impacted regions that many fish utilize for at least part of their lives. Risk assessment decisions will be better informed and it will contribute to an enhanced understanding of inter-species differences in response to EDCs. The pairing of well-chosen high throughput assays with environmentally relevant surrogate species is key to discerning the ecological impact of endocrine disruption.

Table 1.1 **ELISA precision and sensitivity**. The precision of the assay is demonstrated by comparing measured chorion values to those back-calculated from the standard curve using the 4 parameter logistic model. The minimal detection limit and working range are compared to assays developed for the measurement of Chg in the plasma of other fish species.

Analysis	Chg μg/mL	Back-Calculated Range	Coefficient of Variation[a]	Detection Limit	Working Range[b]
Inter Assay	39	38.9 – 39.0	5.5 e-07	5 ng/mL	22.6 – 1370.9 ng/mL
	7.8	7.79 – 7.80	6.3 e-07		
	1.56	1.56	2.4 e-07		
	0.312	0.312	1.6 e-07		
				other assays	other assays
Intra Assay	30	29.9 – 30.0	4.7 e-06	1.56 ng/mL[c]	6-200 ng/mL[c]
	6.0	5.99 – 6.00	3.5 e-05	1 - 2 ng/mL[d]	1/2–128/256 ng/mL[d]
	1.2	1.19	1.4 e-05		
	0.240	0.239 – 0.240	1.7 e-05		

a.) Coefficient of Variation = standard deviation / grand mean

b.) Working range is defined as the EC20 – EC80 (20% - 80% binding)

c.) Matrix = plasma (Prakash et al., 2007)

d.) Matrix = plasma, Chg H and L measured with separate antibodies (Fujita et al., 2005)

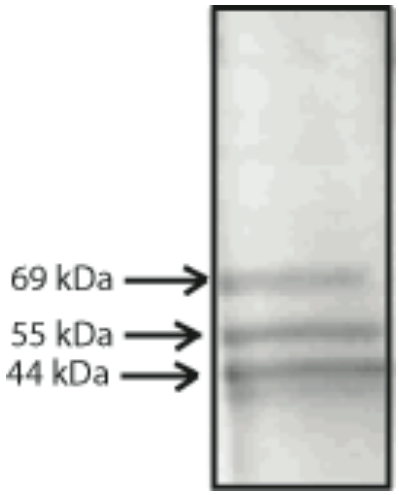


Figure 1.1 **Coomassie-stained SDS-PAGE of chorion proteins excised for use as antigen in rabbit.** Chorion proteins extracted from Menidia oocytes were run on SDS-PAGE and stained with Coomassie blue. Bands were excised and combined with Tris-buffered saline and Freund's adjuvant and injected into New Zealand white rabbits biweekly for 12 weeks.

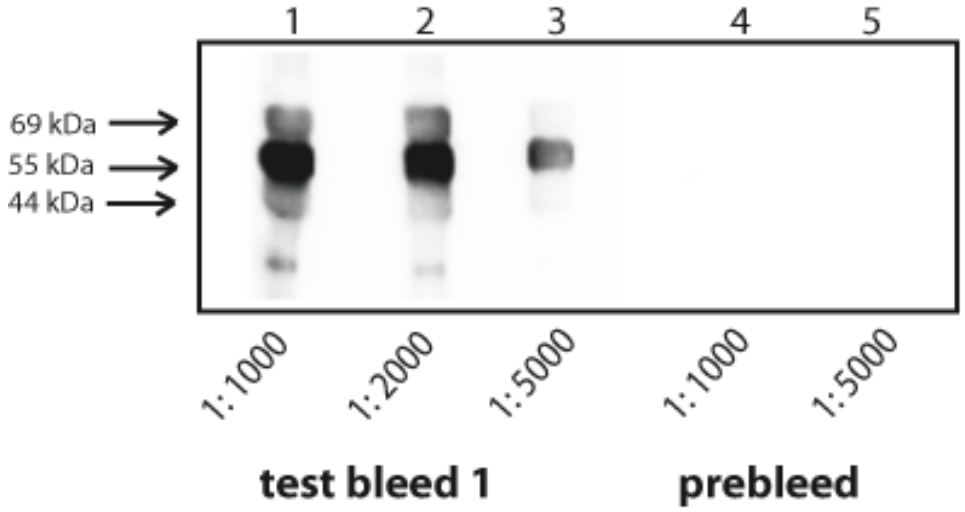


Figure 1.2 **Western blot of *Menidia* chorion proteins incubated with first test bleed IgG.** Chorion was run on SDS-PAGE and then incubated with IgG isolated from the first test bleed from rabbits injected with the chorion antigen. Lanes 1, 2, and 3 represent serum from one rabbit sampled 2 weeks after the first injection of *Menidia* chorion proteins. Decreasing concentrations of purified IgG show decreasing signal. Serum sampled pre-injection, shown in lanes 4 & 5, does not react with the antigen.

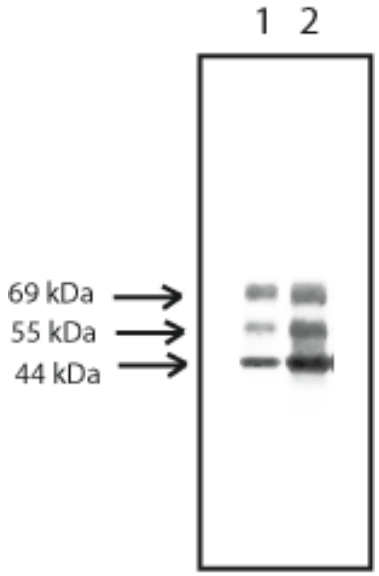


Figure 1.3 **Western blot of *Menidia* chorion proteins incubated with final test bleed IgG.** On SDS-PAGE, 1:10,000 final chorion IgG was incubated with serially diluted amounts of chorion. As expected, bands migrated to 69, 55 and 44 kDA. Protein amounts are 0.076 µg chorion in lane 1 and 0.305 µg chorion in lane 2.

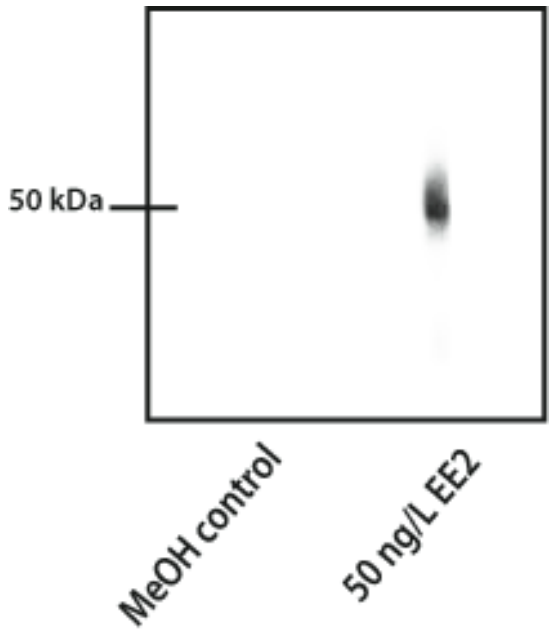


Figure 1.4 **Western blot of whole body homogenate from juvenile *Menidia* incubated with final test bleed IgG**. On SDS-PAGE, IgG from final serum at a concentration of 1:10,000 was incubated with WBH . Lane 1 is WBH from naïve fish exposed to methanol (MeOH) control, and lane 2 is WBH from naïve fish exposed to 50 ng/L ethinylestradiol (EE2) for 14 days.

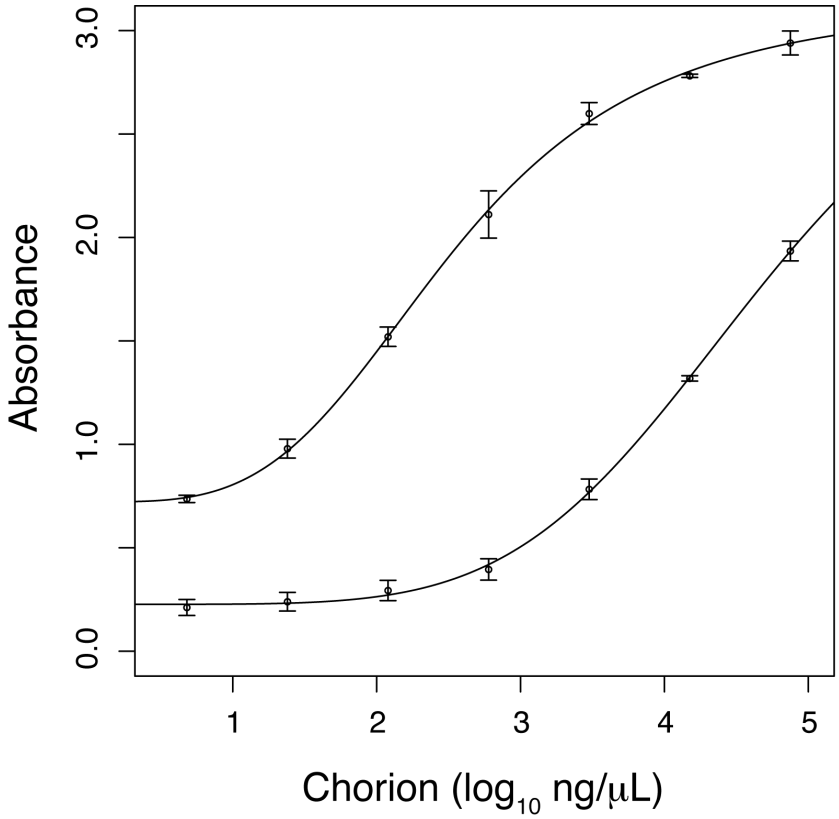


Figure 1.5 **Comparison of anti-*Menidia* chorion Ab to anti-salmonid Chg Ab.** Known amounts of chorion spiked in 1:10 dilution of whole body homogenate were incubated with 1:1000 BioSense anti-salmonid Chg antibody and 1:1000 anti-*Menidia* chorion antibody. ELISA absorbance was read at 490 nM and standard curves were fit using a 4 parameter logistic model.

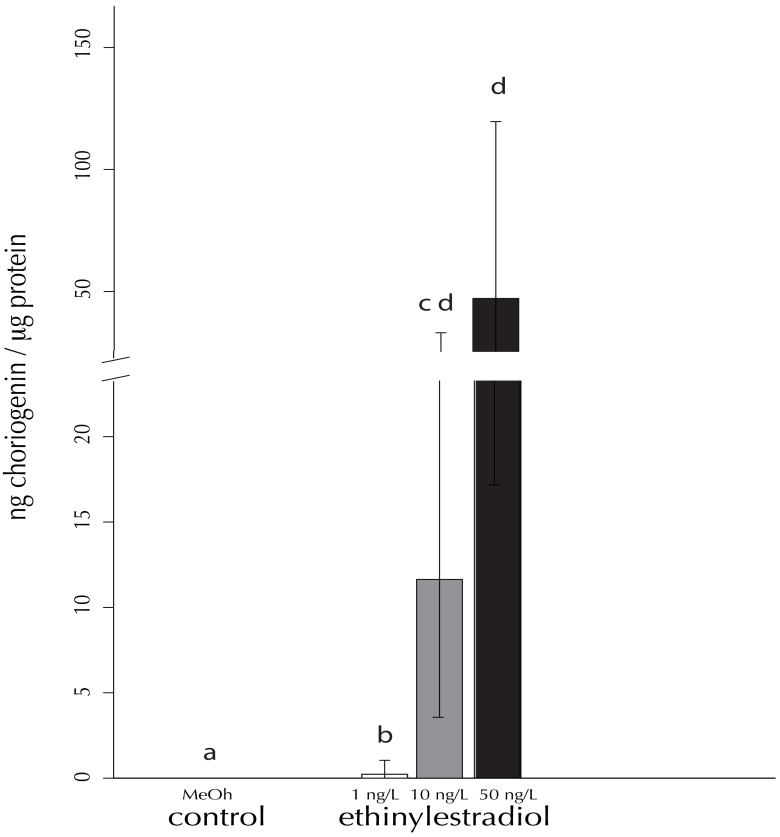


Figure 1.6 **Choriogenin expression in juvenile *Menidia beryllina* exposed to ethinylestradiol.** Whole body homogenate (1:10 homogenate:coating buffer) from juvenile Menidia beryllina exposed for 14 days to 1, 10, and 50 ng/L ethinylestradiol was incubated with 1:1000 anti-Menidia chorion antibody. ELISA absorbance was read at 490 nM. Bars sharing the same letter are not significantly different at the $\alpha < 0.05$ level.

Chapter 2

The *in vivo* estrogenic and *in vitro* anti-estrogenic activity of permethrin and bifenthrin

Abstract

Pyrethroids, a class of pesticides increasingly used for urban and agricultural applications, are highly toxic to fish and aquatic invertebrates at ppb or pptr levels. In addition to effects produced via their action on sodium channels, recent results from *in vitro* and *in vivo* assays reveal that some pyrethroids can act as estrogens, anti-estrogens and/or anti-androgens. Several pyrethroid metabolites have been shown to have greater endocrine activity than their parent compounds. Permethrin and bifenthrin, which have confirmed endocrine activity, are two of the most frequently detected pyrethroid pesticides in aquatic ecosystems. We evaluated the *in vivo* concentration-dependent ability of bifenthrin and permethrin to induce choriogenin (a biomarker of estrogenic activity) in *Menidia beryllina*, a fish species known to reside in pyrethroid contaminated aquatic habitats. We then compared the *in vivo* fish response to that of an *in vitro* assay by employing the CALUX (Chemical Activated Luciferase Gene Expression) assay, examining the potential for agonism, antagonism, or synergism of pyrethroid pesticides with endogenous hormones. Juvenile *Menidia beryllina* exposed to three concentrations of bifenthrin (1, 10, 100 ng/L), permethrin (0.1, 1, 10 μg/L), and EE2 (1, 10, 50 ng/L) had significantly higher relative ng/mL choriogenin (Chg) expressed in whole body homogenate than controls. The positive control ethinylestradiol exhibited a traditional sigmoidal concentration response, but the concentration response curves for induction of Chg by bifenthrin and permethrin were unimodal (inverted U-shaped response), suggesting that response decreased as concentration of both pyrethroids increased. While the Chg response indicated that bifenthrin and permethrin or its metabolites act as estrogen agonists, the CALUX assay detected antagonism of the estrogen response.

The combined results from the *in vivo* and *in vitro* aspects of our study suggest that the metabolites of bifenthrin and permethrin may act as estrogen receptor agonists while the parent compounds act as estrogen receptor antagonists.

1. Introduction

Pyrethroid pesticide use has undergone a considerable increase as organophosphate pesticides are phased out due to concerns regarding mammalian toxicity (Epstein et al., 2000, Sudakin & Power, 2007, Werner & Moran, 2008). Pyrethroids, which are not acutely toxic to mammals at concentrations applied or found in the environment, are highly toxic to fish and aquatic invertebrates at ppb or pptr levels (Burr & Ray, 2004, DeLorenzo et al., 2006). It has been argued that pyrethroid toxicity is lower in aquatic environments due to the lipophilic nature of these compounds, which tend to bind to sediments instead of remaining dissolved, reducing their bioavailability (Leahey 1985). However, these compounds may remain in the water column for days to weeks after introduction (Bondarenko 2006) and are soluble enough to render biological harm, considering that concentrations as low as ng/L may impact sensitive organisms (Werner & Moran, 2008, Brander et al., 2009).

Pyrethroids disrupt the nervous system via prolongation of the opening of voltage-dependent ion channels, the consequences of which are convulsions, paralysis and eventual mortality (Werner and Moran, 2008, Burr and Ray, 2004). Sublethal neurotoxic effects include impaired swimming ability in both fish and invertebrates (Christiansen et al., 2005, Beggel et al., 2010, Brander et al., in prep) and reduced ability to avoid predators (Floyd et al., 2008,). In addition to effects produced by the intended mechanism, recent results from *in vitro* assays reveal that some pyrethroids can act as estrogens, anti-estrogens and/or anti-androgens (Chen et al., 2002, Sun et al., 2007). Pyrethroid metabolites are reported to have even greater endocrine activity than their

parent structures (Tyler et al., 2000, McCarthy et al. 2006). Pyrethroids also have considerable endocrine activity *in vivo,* particularly bifenthrin, permethrin and permethrin metabolites. These can induce vitellogenin in male fish, an egg yolk protein normally found only in females (Nillos et al., 2010).

Permethrin and bifenthrin, which have confirmed *in vitro* and *in vivo* endocrine activity, are two of the most frequently detected pyrethroid pesticides in aquatic ecosystems (Amweg et al., 2006). Both are used in agriculture, but the more toxic bifenthrin is also increasingly used for landscaping and structural pest control under the trade name Talstar™ (Weston et al., 2005, 2009, Werner & Moran, 2008, Domagalski et al., 2010). Due to their increasing presence in aquatic ecosystems and their potential to cause endocrine disruption, here we describe studies examining the biochemical mechanisms and endocrine disrupting effects of environmentally relevant pyrethroid concentrations utilizing a combination of *in vitro* and *in vivo* approaches.

2. Materials and Methods

2.1 Bioassay

We conducted a 14d static aqueous exposure (with daily water renewal) using 65-70d old *Menidia beryllina* (Aquatic Biosystems, Fort Collins, CO). Stock solutions were made in methanol. For these studies, fish were exposed to three concentrations of bifenthrin (1, 10, 100 ng/L) or permethrin (0.1, 1, 10 μg/L) spiked into lab control water consisting of distilled water and filtered sea water (5 uM) at a salinity of 5 ± 1 ppt. Different ranges of concentrations were used because bifenthrin and permethrin differ substantially in toxicity, the former being more toxic. Additionally, 10 μg/L permethrin has already been shown to induce estrogen-responsive proteins, so we wanted to evaluate concentrations close to but lower than this. Ethinylestradiol was included in

these studies as a positive control (1, 10, 50 ng/L) and methanol (0.02%) spiked into lab control water as the negative control. Permethrin (purity 99%) and ethinylestradiol (purity 99%) were obtained from Sigma Aldrich (St. Louis, MO), and bifenthrin (purity 99%) from the USGS Analytical Chemistry Laboratory (Sacramento, CA).

Fish were maintained in 3L glass jars with 1L test water in each. The experiment was maintained at a 14 hr /10 hr light/ dark cycle and temperature was controlled at 21 $\pm$ 2 ^{o}C. Each jar was aerated and dissolved oxygen, ammonia and pH were measured daily prior to water changes. Fish were fed live or frozen *Artemia* nauplii each day at least one hour prior to water change. For the bioassays, there were 4 replicates per treatment (except the positive control which had 2 replicates), with 10 fish per replicate.

At test termination, fish were anesthetized on ice, immediately snap-frozen with liquid nitrogen and stored at -80 ^{o}C. Whole fish from each replicate were pooled and pulverized in ~5 mL of liquid nitrogen with a ceramic mortar and pestle. Once ground to a powder, a 50 mM tris-HCl homogenization buffer with protease inhibitor (Roche COmplete Mini) was added at a ratio of 1 ml buffer:2g tissue and further homogenized with a Fisher Scientific Tissuemiser (ThermoFisher Scientific, Waltham, MA, USA) for 60 seconds. The homogenate was centrifuged for one hour at 20,800 ×g and the resulting supernatant was removed and recentrifuged at 14,850 ×g for 15 minutes to ensure removal of all particulates. The final supernatant was stored immediately at -80 ^{o}C and the protein concentration of each sample quantitated using the BCA protein assay (Pierce, Rockford, IL, USA).

2.2 ELISA

A *Menidia* polyclonal antibody, produced and optimized from *Menidia* chorion as previously described (chapter 1) was used to measure the relative amount of Chg expressed in whole body homogenate (WBH). This indirect ELISA method was based

on methodology developed by Palumbo et al. (2009). This methodology allows accurate quantitation of the amount of Chg in each sample relative to *Menidia* chorion levels expressed in the absence or presence of ethinylestradiol.

2.3 CALUX assay

Estrogen and androgen receptor-based CALUX mammalian cell bioassays (Rogers and Denison, 2000, Ahn et al., 2008) were used to determine the concentration-dependent agonist and antagonist effects of pyrethroids used in these studies. The CALUX bioassay experiments were carried out as described in detail in Ahn et al., 2008. The sources of 17-α testosterone and 17-α estradiol used in all CALUX bioassays were Alltech (State College, PA, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively.

2.4 Analytical Chemistry

Pyrethroid spiked water samples, both new and 24 hours old, were sent to the USGS Analytical Laboratory (Sacramento, CA) for confirmatory chemistry. Methods were performed as described in Hladik et al. 2008. All concentrations of bifenthrin and permethrin had acceptable recoveries (80%–110%).

2.5 Data Analysis

ELISA data were first quantitated by comparison of absorbance values to a chorion standard curve to obtain Chg equivalents. Equivalents were then divided by the protein concentration per well to normalize the data. Because the analysis included pseudoreplicates (each plate contained multiple wells with samples from the same treatment; such wells are not statistically independent replicates; Hurlbert 1984), it was important to account for the potential underestimation of variability introduced by

pseudoreplication, using a no-intercept model with a random effect of replicate within plate (Pinheiro & Bates, 2000). However, because the variance components of the random effects were estimated to be equal to zero, a traditional linear model (followed by Tukey test) was ultimately used to test for differences in Chg expression among experimental treatments.

In order to choose the concentration-response curve that was best supported by our data, a model selection approach using maximum likelihood estimation was used to find the parameter values for each concentration-response (and their variances) that most parsimoniously fit the observed data. We then choose the most parsimonious model (balancing model fit against model complexity). While a sigmoidal concentration-response curve would be expected for increasing concentrations of a toxic compound, bifenthrin and permethrin data appeared to fit a unimodal response model. Therefore both a sigmoidal curve (3 parameter logistic model) and a unimodal curve (a modified 3 parameter logistic model; Brain & Cousens, 1989) were fit to the data. The most parsimonious model was determined using Akaike's Information Criterion corrected for small sample sizes (AIC_c)(Burnham & Anderson, 2002), a model selection method previously used in toxicological studies (Brander et al., 2009). All analyses were performed with the statistical software R 2.11.1 (R Development Core Team, 2010).

For the CALUX bioassays, an individual *t*-test was used to determine whether treatments were significantly different from the relevant positive control (estrogen or testosterone), expressed as 100% cellular response. Resulting *p*-values were Bonferroni corrected to account for multiple comparisons. Differences among treatments in estrogen or testosterone equivalents were tested using an ANOVA followed by a Tukey test.

3. Results

3.1 Differences between Treatments

Juvenile *Menidia beryllina* exposed to all three concentrations of bifenthrin (1, 10, 100 ng/L), permethrin (0.1, 1, 10 mg/L), and EE2 (1, 10, 50 ng/L) have significantly higher relative ng/mL chorion expressed in whole body homogenate than the methanol control (Figure 2.1). None of the pyrethroid treatments were significantly different from one another when compared using ANOVA ($p > 0.05$), however a trend towards an inverse correlation between response and increasing concentration with both permethrin and bifenthrin is apparent (Fig. 2.1). The results pertaining to this trend will be further explained in section 3.2.

Permethrin and bifenthrin treatments were not significantly different from positive controls of 1 and 10 ng/L EE2 over a 100-fold concentration range, with the exception of 1 ng/L bifenthrin, which had a significantly higher relative Chg level than that produced by 1 ng/L EE2 ($p = 0.022$). However, all permethrin and bifenthrin concentrations trend towards having higher relative expression of Chg than the 1 ng/L EE2 treatment, and lower relative expression than the 10 ng/L treatment. A concentration-dependent increase in Chg levels was observed with EE2 exposure, with the 1 ng/L EE2 treatment is significantly different from both 10 ng/L and 50 ng/L EE2. While the 10 and 50 ng/L EE2 treatments were not significantly different, the trend (mean values) was progressively higher with increasing concentration.

3.2 Concentration Response

Concentration response curves for ethinylestradiol (Figure 2.2), permethrin (Figure 2.3), and bifenthrin (Figure 2.4) were generated using maximum likelihood estimates (MLE) and curve fit was evaluated using AICc (Burnham and Anderson, 2002; AIC adjusted for small sample sizes). As expected, ethinylestradiol has a traditional sigmoidal concentration response (Figure 2.2), with a delta $AIC_c = 0$ for the sigmoidal

model fit (Table A.1, Appendix A). Due to limited data, we are not able to display the entire curve.

In contrast to the results for EE2, the concentration response curve for induction of chorion by bifenthrin and permethrin is not sigmoidal, but a unimodal, or biphasic curve (Figures 2.3, 2.4). A unimodal model, in which the response peaks and then begins to decrease as concentration increases, has a more parsimonious fit to the data (AICc = 0) than a linear or sigmoidal model despite having more parameters (delta AICc = 0.3 and 5.0, respectively). All curves were fit to log transformed data $\pm$ 0.01 to subvert the problem of taking the log of concentration 0 (MeOH control).

3.3 CALUX assay

The estrogen and androgen receptor CALUX assays did not detect any estrogenic or androgenic activity, or anti-androgenic activity that was significantly different from controls for any of the permethrin or bifenthrin concentrations tested. Although it appears that AR synergism is occurring, this is most likely due to a problem with low activity in the positive control in that assay. For reasons unknown the testosterone control in the AR antagonism assay had only half of the androgenic activity that the same testosterone control concentration did in the AR agonism assay. Additionally, since there is no concentration-dependence of the effect, it is highly unlikely that either pyrethroid is acting as an testosterone synergist. In the ER antagonism assay, however, an initial bifenthrin concentration-dependent decrease in the ability of EE2 to induced estrogen receptor-dependent reporter gene activity (1-100 ng/L bifenthrin) was observed with a recovery of activity with higher bifenthrin concentrations. In contrast, although some permethrin-dependent reduction in estrogenic activity was observed (~30-40% of maximal estradiol activity), there was no concentration-dependence of this inhibitory

effect. Thus, this inhibition is unlikely to result from an antagonist effect of permethrin on the estrogen receptor or estrogen receptor-signaling pathway.

3.4 Chemistry

As was expected, there was a decrease from the original spiked concentrations of bifenthrin and permethrin over a 24-hour period. While initial actual concentrations were within 90 – 111 % of nominal concentrations, actual concentrations measured just prior to daily bioassay water changes had decreased to 64 -73 % of nominal. These decreases in concentration likely result from adsorption of bifenthrin and permethrin to walls of the glass jars used, food particles, and absorption of the compounds by fish.

4. Discussion

The first objective of our study was to evaluate the concentration-response to bifenthrin and permethrin *in vivo* at environmentally relevant concentrations, in a fish species known to reside in pyrethroid contaminated aquatic habitats. To achieve this goal we evaluated the effects of a range of bifenthrin and permethrin concentrations near those found recently in storm drain run-off and streams in Northern California (Weston et al., 2009, Domagalski et al., 2010) on *Menidia beryllina* (inland silverside). Our second objective was to utilize a biomarker of estrogenic endocrine disruption more sensitive than vitellogenin, the egg yolk protein typically measured following exposure to suspected EDCs (Rempel & Schlenk, 2008) using a species-specific antibody to Chg, an egg coat protein. The third objective was to compare the fish response to that of an *in vitro* assay by employing the CALUX (Chemical Activated Luciferase Gene Expression) assay. With the CALUX assay it was possible to examine the potential for additivity, antagonism, or synergism of pyrethroid pesticides with endogenous hormones.

Most interestingly, it appears that the lowest concentrations of both bifenthrin

and permethrin induced the greatest response. The best-fit curve for both pyrethroids indicates a biphasic or unimodal response. In contrast, EE2 displayed the more typical sigmoidal response. Although the fit as evaluated by AICc is not as strong for bifenthrin as it is for permethrin, the graphical trend is the same and because these two compounds are similar and likely share the same mechanisms of endocrine disruption, the unimodal curve is undoubtedly a better fit for both than the sigmoidal curve.

Other studies have noted that some estrogenic compounds (i.e. BPA) may act in this manner, inducing an inverted "U" shaped or unimodal dose response due to low dose stimulation and higher dose inhibition (Alworth et al., 2002, Welshons et al 2003, Calabrese 2001, 2008). For example, in another study with fish, the estrogenic activity of the pyrethroid metabolite 3-PBOH begins to decrease once reaching a concentration of 25 mM (Nillos et al., 2010). Potential reasons for higher response at a lower concentration are numerous. It is known, for example, that exposure to increasing concentrations of hormones or hormone-mimicking compounds does not necessarily result in a linear response, particularly with receptor-mediated responses that can saturate or vary depending on the concentration. For this reason inverted U-shaped dose or concentration response curves are relatively common in endocrine studies (Welshons et al., 2003). Also, because fish have a low metabolic capacity (Glickman et al., 1982) for compounds such as pyrethroids, decreased conversion of the parent compound to more active metabolites at higher concentrations may result in decreased estrogenicity, which would result in lower expression of Chg at higher exposure levels. Additionally, stimulation of crosstalk or competing cellular responses at higher concentrations (Silva et al., 2010) could lower the overall response of the biomarker being measured. Computer simulations indicate that a unimodal dose-response can occur when receptor homodimerization is disrupted. In particular, a mixed-ligand homodimer can be formed with an endogenous ligand and an exogenous ligand such as

an EDC (Li et al., 2007). This then can change how the dimer binds to the DNA hormone response element and may impact gene transcription and ultimately protein expression. This may occur whether the EDC is acting an agonist or an antagonist (Li et al., 2007).

Evidence for a difference in estrogenicity between the parent pyrethroid compounds and their metabolites is demonstrated by the results of the CALUX assay, which natively expresses the human estrogen (in the BG-1 cells) or androgen receptor (in the T-47D cells) but does not account for hepatic metabolism as our *in vivo* exposures did. Using a cell line that expresses hER and hAR is comparable to evaluating the fish ER and AR response *in vitro* since human and fish steroid receptors are highly conserved (Shyu et al., 2011, Pakdel et al., 1989). As has been demonstrated in other studies with permethrin (Kim et al., 2004, Chen et al., 2002), the CALUX assay results suggest that both bifenthrin and permethrin act as estrogen antagonists (Figure 2.5). The antagonism of bifenthrin clearly increases with increasing concentration to a maximum of 100 ng/L, while permethrin's antagonistic properties do not correlate with concentration tested. This could indicate that bifenthrin has higher affinity for or efficacy at the estrogen receptor than permethrin does, or that toxicity is occurring via other mechanisms with permethrin affecting the cells' ability to respond. The antagonism exhibited by both pyrethroids may account for the lower Chg response seen in the juvenile silversides at higher concentrations of both pesticides. As more of the parent compound was introduced to the silverside's system, a smaller fraction of it may have been converted into estrogenic metabolite(s) and more of the antagonistic parent was left to potentially compete for binding sites. Although graphically it appears that both compounds are also acting as androgen synergists, the lack of a concentration-response and known issues with the testosterone control in androgen antagonism assay make synergism of testosterone by pyrethroids highly unlikely.

Upon an examination of other research findings along with ours, it is possible that the lack of a consistent linear relationship between antagonism and concentration may be caused by bifenthrin and permethrin acting via different mechanisms at different concentrations or conditions. In a prior study, permethrin inhibited 17 β-estradiol induced proliferation of the MCF-7 human breast carcinoma cell line and blocked binding of estradiol to ER in uterine cytosol (Chen et al., 2002). However in a later study performed in rat uteri cells, permethrin was found not to competitively inhibit binding of estradiol to ER (Kim et al., 2004). At a 10^{-7} M, a higher concentration than used in our study, both cis-bifenthrin and permethrin have been shown to activate expression of the estrogen-linked transcription factor pS2 (Zhao et al., 2010, Chen et al., 2002), but in others permethrin (10^{-4} M, 10^{-7} M) wasn't found to induce pS2 expression (Goh et al., 1999, Kim et al., 2004). At high enough concentrations (mg/L) permethrin has also been shown to act as a weak androgen agonist and antagonist (Tyler et al., 2000, Sun et al., 2007), but the environmental relevance of this is highly questionable. Permethrin's agonistic or antagonistic ER and AR actions are clearly dependent on the combination of cell line, concentration used and endpoint examined, and considering the results of our study bifenthrin appears to exhibit similar contradictory behavior. However, particularly in the case of permethrin, it may be that antagonism is occurring due to overall cellular toxicity and not being mediated through an estrogen responsive pathway, since no significant change in antagonism was seen with increasing concentration spanning several orders of magnitude.

Neither compound acted as an estrogen agonist in the CALUX assay at any concentration, a finding that is somewhat surprising considering results of previous research that indicate bifenthrin and permethrin operate as ER agonists via the classical nuclear receptor pathway (Taylor et al.. 2000, Wang et al., 2007). The difference seen may be accounted for by use of the CALUX assay, rather than the YES assay, which is the

cell line type most frequently used for testing pyrethroid endocrine activity to date. Notably, unless modified to do so, the YES assay does not discriminate well between agonists and antagonists (McCarthy et al., 2006). This indicates that the transfected ER in yeast cells may not be functionally normal and may lack key regulatory factors. Additionally, nearly all previous cell line studies conducted with pyrethroids used concentrations that were higher than those used in our study, by as much as an order of magnitude (Tyler et al., 2000). The importance of using an *in vivo* system to compare *in vitro* responses to (Taxvig et al., 2011), the choice of cell line, and of testing environmentally relevant concentrations is abundantly clear considering our findings.

To our knowledge this is first study to test the endocrine concentration-response of bifenthrin and permethrin at environmentally relevant concentrations. Additionally, this is the first study to use cell lines with natively expressed ER or AR to evaluate pyrethroid endocrine activity. Together the fish bioassay and cell line assays evaluate the endocrine activity in a range of concentrations of each pyrethroid both with and without hepatic metabolism.

Environmental relevance is afforded by the use of *Menidia* as our study species. Silversides are ubiquitous in and easily caught from estuarine, brackish and freshwater habitats (Middaugh & Hemmer, 1982), they are exposed to run-off containing pyrethroids throughout North America and are commercially available for laboratory studies. Furthermore, the inland silverside has a higher published permethrin LC50 than many other fish species (Werner & Moran, 2008). Although this may contribute to increased survival, it potentially renders silversides more susceptible to pyrethroid-induced endocrine disruption.

Early life exposure to these low concentrations of pyrethroids may be particularly damaging to silverside populations, since the sex ratio of *Menidia* spp. has been shown to be susceptible to estrogen exposure during the larval period (Duffy et al. 2009).

Notably, our study is the first to look at protein-level endocrine disrupting effects of pyrethroids on juvenile fishes, which are more sensitive to such environmental EDC perturbations (Jin et al., 2010) and more likely to experience long-term developmental changes such as reduced fecundity and/or intersex when exposed to EDCs (Peters et al., 2009).

The ability of pyrethroids to induce egg protein expression in fish, indirectly indicating ER binding and/or activation of the estrogen response element, has been demonstrated in several other studies. For example, Nillos et al. found that adult male medaka exposed to 10 μg/L permethrin expressed significantly higher amounts of vitellogenin (Vtg, yolk protein) in plasma than controls (Nillos et al., 2010) and a study with male zebrafish showed that 100 ng/L permethrin induced expression of two different Vtg genes (Jin et al., 2008). In studies with exposure to aqueous bifenthrin, male medaka (*Oryzias latipes*) expressed Vtg in response to a 10 ng/ml (Wang et al., 2007) and larval fathead minnows (*Pimephelas promelas*) exhibited a concentration-dependent increase in Vtg expression when exposed to ppb concentrations (Beggel et al., 2011). Although vitellogenin is a reliable marker of estrogenic endocrine disruption, it may not be the most sensitive protein based indicator of estrogenic activity. We instead measured expression of Chg, an egg coat protein demonstrated to be more sensitive than vitellogenin in previous studies (Celius & Walther, 1998, Arukwe et al., 2000), using an antibody we derived from *Menidia beryllina* oocytes (see chapter 1). Like vitellogenin, this protein is normally only induced by endogenous estrogen in mature females (Oppen-Berntsen et al., 1990, 1992). In comparison to Vtg, Chg expression has been shown to be a more sensitive marker of response to the xenoestrogens DDT and bisphenol-A (BPA) (Celius & Walther, 1998, Arukwe et al., 2000). Although the mechanism behind Chg's higher sensitivity is not yet known, it may be because the coat of the oocyte is laid down prior to deposition of the yolk in a normal mature female

responding to endogenous estrogen (Wourms 1976) or that this gene contains more estrogen response elements than the vitellogenin gene.

Consistent with previous results, our study found that all concentrations (0.1, 1, 10 μg/L permethrin and 1, 10, 100 ng/L bifenthrin) significantly induced the expression of the Chg egg coat protein in juvenile *Menidia beryllina* in comparison to MeOH controls. Several of these concentrations are an order of magnitude lower than those shown in previous studies to induce estrogen-dependent mRNA or protein production. Notably, the concentrations used in our study decreased substantially over the 24-hour time course between water changes (Table 2.2), indicating that fish were responding much lower concentrations than nominal during a time period of unknown length each day..

The combined results from the *in vivo* and *in vitro* aspects of our study, along with the findings of others, make a compelling case for the metabolites of bifenthrin and permethrin being more estrogenic than their parent compounds. For example, one recent study found that the pyrethroid metabolites 3,4- hydroxyphenoxy-benzyl alcohol (3,4-PBOH) and 3-phenoxy-benzyl alcohol (3-PBOH), which are produced following hepatic metabolism via cleavage and hydroxylation (P450s), induced higher expression of Vtg in rainbow trout hepatocytes than the parent compound (Nillos et al., 2010). Previous studies performed with permethrin found that 3-PBOH had 100-fold the estrogenicity of permethrin and confirmed estrogenic activity in several other metabolites (Tyler et al., 2000, McCarthy et al., 2006). Our findings that permethrin and bifenthrin appear to be estrogenic in the presence of hepatic metabolism but anti-estrogenic in its absence support the results of other studies, although further research is needed to determine this conclusively.

4.1 Conclusions

The coalescence of our findings with those of others leads us to conclude that permethrin and bifenthrin possess endocrine activity in both their metabolized and unmetabolized form. Due to inconsistencies between studies in terms of concentrations and assays used, it can be difficult to pinpoint the exact mechanism(s) by which endocrine disruption occurs. The evidence suggests that the parent compounds of permethrin and bifenthrin act as weak estrogen agonists or antagonists depending on the concentration, and as stronger estrogen agonists when metabolized via hydrolysis or photolysis in the environment or via organismal P450 enzymatic breakdown.

Although the lack of a clear linear response makes it difficult to assess the risk of permethrin and bifenthrin in the wild, the endocrine disruption caused by these compounds clearly exacerbates their toxicity in aquatic ecosystems. Furthermore, the ability of these compounds to exert effects on the endocrine system of fishes at concentrations regularly detected in watersheds is cause for great concern, especially when considering recent research that suggests changes in reproductive protein expression in fishes may precipitate population decline (Kidd et al., 2007) and that the pyrethroid mixtures that occur in aquatic habitats likely have additive endocrine toxicity. Also, although fish may have a low capacity to metabolize pyrethroids to more estrogenic metabolites, microbial enzymes can hydrolyze the parent compounds once they are mobilized in the environment (McCarthy et al., 2006). The ubiquitous nature and persistence of pyrethroids further enhance the threat they pose to fish and invertebrate populations.

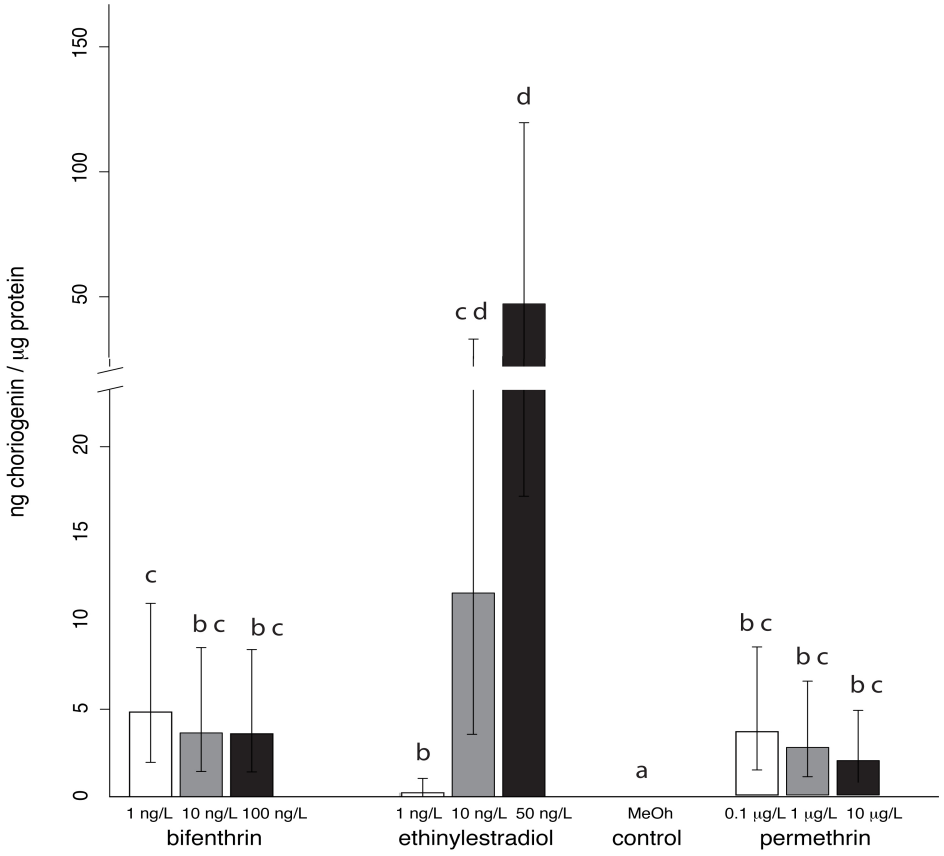


Figure 2.1 **Effect of bifenthrin, permethrin and ethinylestradiol on choriogenin expression in juvenile *Menidia beryllina***. Fish were exposed to the indicated concentration of each compound in water (with daily water changes) for 14 days followed by determination of choriogenin levels. Values represent the mean ± 95% confidence limits of 4 pooled replicates of 6-10 fish each (bifenthrin, permethrin) and 2 pooled replicates of 8-10 fish each (ethinylestradiol) as determined by ANOVA. Significant differences between treatments were determined via a Tukey test. Treatments that were not significantly different $p > 0.05$) are indicated by the same letter and treatments that are significantly different from each other ($p < 0.05$) are indicated by different letters.

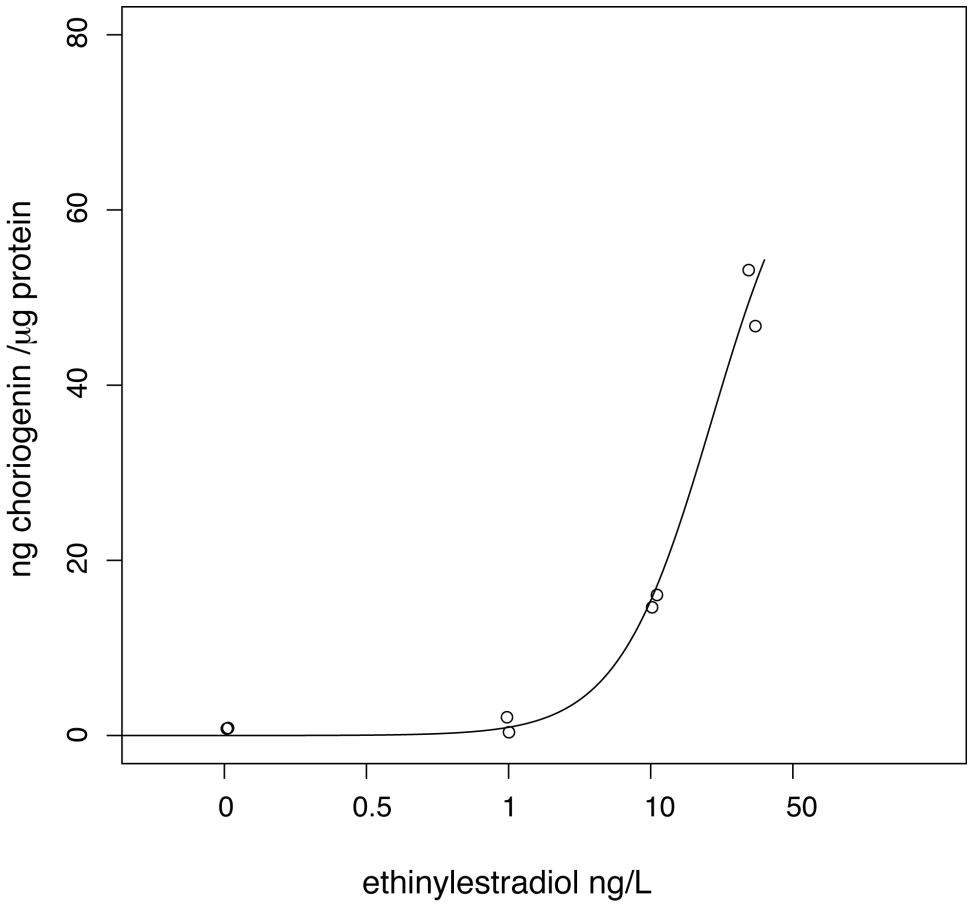


Figure 2.2 **Choriogenin concentration response of juvenile *Menidia beryllina* to ethinylestradiol.** Most parsimonious curve fit to ethinylestradiol concentration-response data (sigmoidal). Each point is represented by the combined homogenate of 10 juvenile *Menidia beryllina* exposed for 14 days to the corresponding concentration.

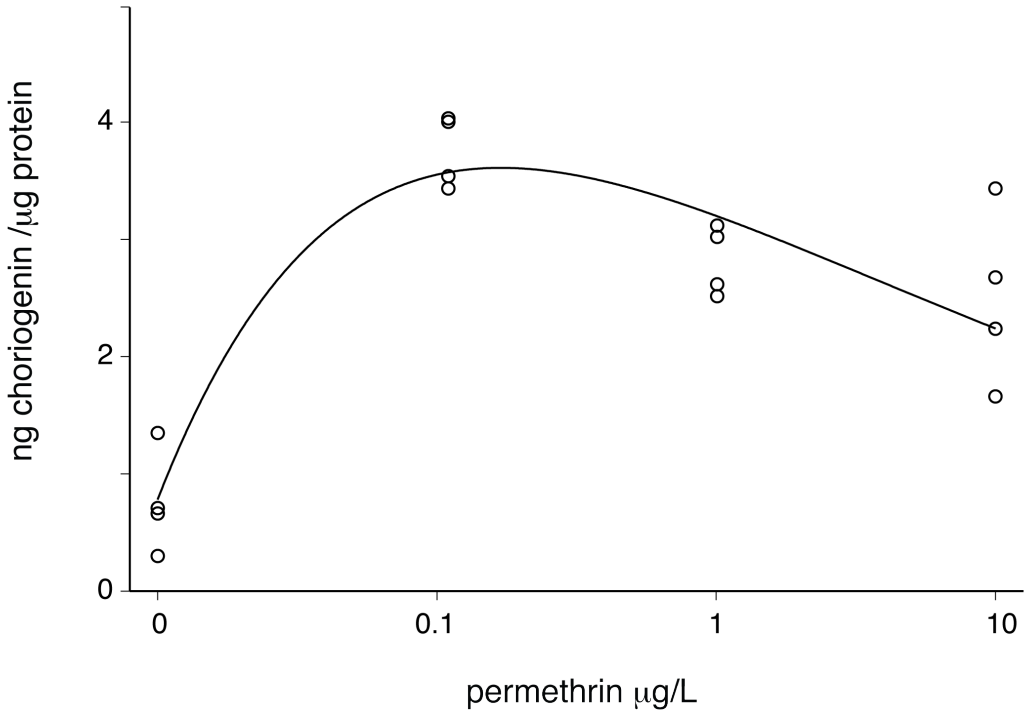


Figure 2.3 **Choriogenin concentration-response of juvenile *Menidia beryllina* to permethrin.** Most parsimonious unimodal curve fit to permethrin concentration-response data. Each point is represented by the combined homogenate of 10 juvenile *Menidia beryllina* exposed for 14 days to the corresponding concentration.

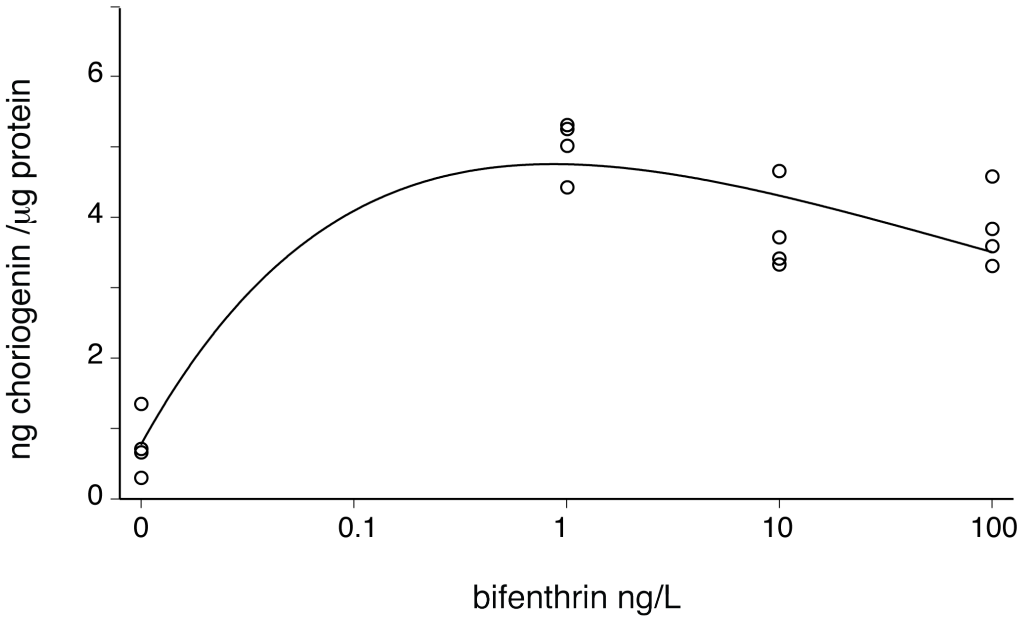


Figure 2.4 **Choriogenin concentration-response of juvenile *Menidia beryllina* to bifenthrin.** Most parsimonious unimodal curve fit to bifenthrin concentration-response data. Each point is represented by the combined homogenate of 10 juvenile *Menidia beryllina* exposed for 14 days to the corresponding concentration.

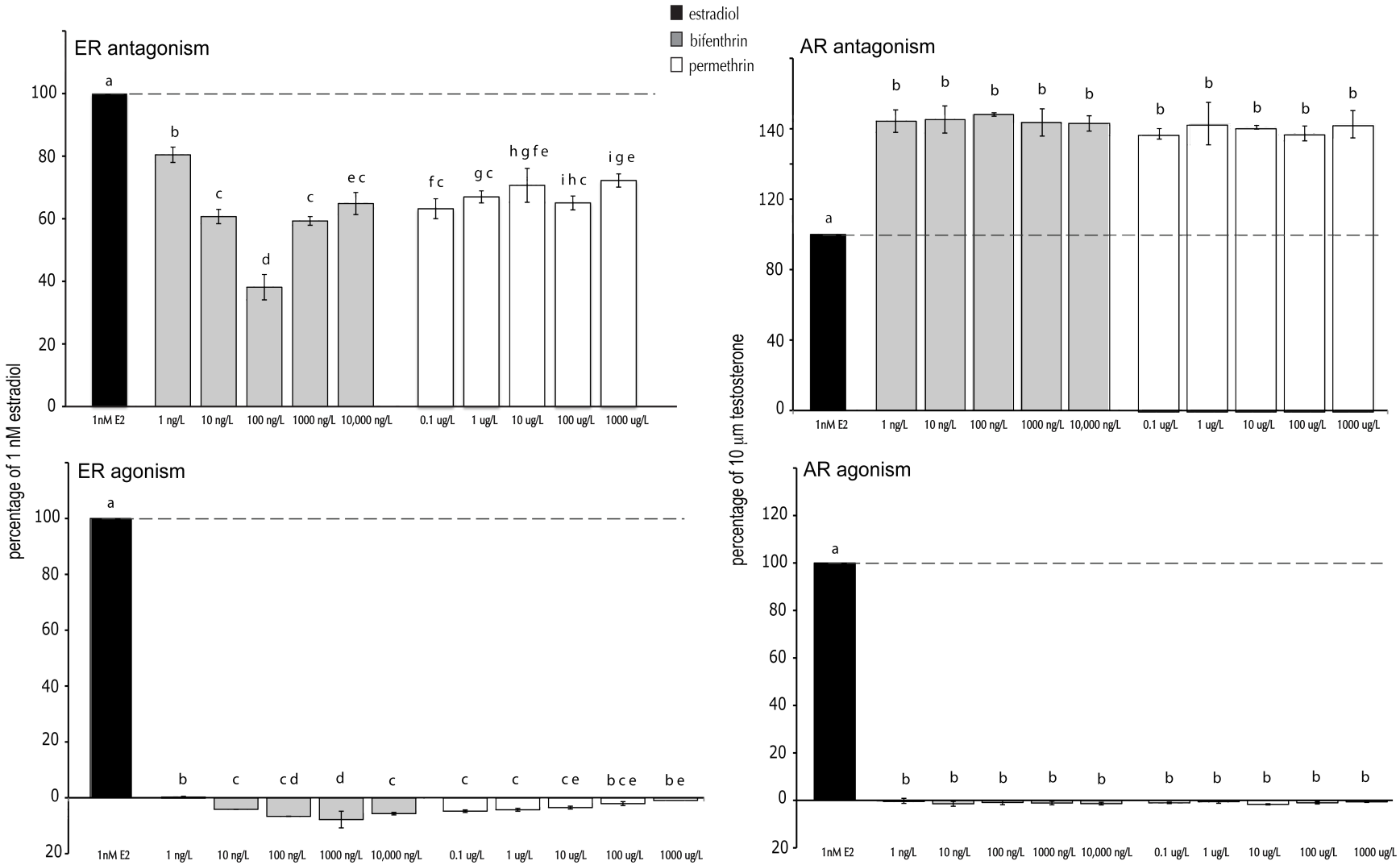

estradiol
bifenthrin
permethrin
ER antagonism
AR antagonism
ER agonism
AR agonism
percentage of 1 nM estradiol
percentage of 10 µm testosterone
1nM E2
1 ng/L
10 ng/L
100 ng/L
1000 ng/L
10,000 ng/L
0.1 ug/L
1 ug/L
10 ug/L
100 ug/L
1000 ug/L

Figure 2.5 **Activation and inhibition of the ER and AR CALUX by bifenthrin and permethrin.** CALUX ER and AR agonist and antagonist activity are presented as a percentage of the estradiol (1 nM) or testosterone (10 μM) positive control (100%). Error bars represent 95% confidence intervals. With ER and AR antagonism, values below 100% indicate the occurrence of antagonism, while values significantly above 100% indicate that synergism occurred. With ER and AR agonist activity, values below 20% of the estradiol or testosterone control indicate that bifenthrin and permethrin are not ER or AR agonists. Significant difference of all treatments from E2 or T controls in all 4 assays was determined via Bonferroni corrected individual t-tests ($p < 0.05$) and is indicated with the letter "a." Tukey test results are represented by letter codes "b – i" above each bar, treatments with different letters are significantly different from each other ($p < 0.05$), treatments sharing letter codes are not significantly different ($p > 0.05$).

Table 2.1 **Chemical analysis of aqueous permethrin and bifenthrin concentrations.** Concentrations of bifenthrin and permethrin were measured via GC/MS in newly spiked laboratory control water and 24 hours later in water that had been used for the *Menidia beryllina* 14 day bioassay.

compound	**nominal**	**actual (new)**	**actual (24 hr)**
bifenthrin	1 ng/L	0.898 ng/L	0.733 ng/L
	10 ng/L	9.514 ng/L	6.890 ng/L
	100 ng/L	111 ng/L	71 ng/L
permethrin	0.1 μg/L	0.092 μg/L	0.068 μg/L
	1 μg/L	1.05 μg/L	0.641 μg/L
	10 μg/L	9.12 μg/L	6.52 μg/L

Chapter 3

From 'omics to otoliths: Correlated responses of an estuarine fish to endocrine disrupting compounds across biological scales.

Abstract

Endocrine disrupting chemicals (EDCs) agonize, antagonize or synergize the effects of endogenous hormones and cause physiological abnormalities in fishes. Both theory and empirical data confirm that EDCs can also cause declines in fish populations. However, few studies have attempted to link complex environmental EDC mixtures with responses at multiple tiers of the biological hierarchy, including population-level effects. Additionally, the use of environmentally relevant fishes as model species from regions with demonstrated fish population decline is lacking. To this end, we undertook a four-tiered investigation into estrogenic and androgenic EDC effects in *Menidia audens* (Mississippi silverside), an atherinid distributed throughout the impacted San Francisco Bay (SFB) estuary. Our main objective was to integrate observations at each biological scale with the goal of determining whether the reproductive health of *Menidia* populations was being negatively impacted by sites receiving either urban run-off and treated wastewater effluent or ranch run-off, and if so what the mechanism(s) of endocrine disruption may be. Clear links were observed between the four biological scales. At the ranch site, where primarily estrogenic compounds were present, males had significantly higher and females trended towards higher expression of estrogen-responsive genes. At the urban/wastewater outfall site, which is more polluted and contaminated by both estrogens and comparatively higher concentrations of androgens, both males and females had relatively low expression of estrogen-responsive genes, males had lower GSI and higher incidence of severe testicular necrosis, and the proportion of females caught throughout the spawning season was significantly lower

than the ranch site and in comparison to observations of *Menidia* sex ratios in other populations. *Menidia* appear to be highly sensitive to EDCs and my results suggest that the population at the urban/wastewater site is being negatively impacted by these contaminants at several biological scales.

1.Introduction

Endocrine disrupting chemicals (EDCs) agonize, antagonize or synergize the effects of endogenous hormones and are known to cause a number of physiological and behavioral abnormalities in fishes (Rempel & Schlenk, 2008). EDCs originate from a variety of sources and are widespread in the aquatic environment (Pait & Nelson, 2002, Anderson et al., 2006). Examples of hormonal disruptions in fishes produced by EDCs include altered secondary sexual characteristics, males producing egg proteins (vitellogenin, choriogenin), and reduced sperm quality (Bogers et al., 2006, Metcalfe et al., 2001, Singh & Singh, 2008). Both theory and empirical data confirm that EDCs can also cause declines in fish populations (Gurney, 2006, Kidd et al., 2007). Recent studies have utilized the results from single EDC laboratory exposures to produce predictive population models (Miller et al., 2007, Ankley et al., 2008), have assessed multiple genomic and organismal level endpoints in response to known environmental mixtures (Filby et al., 2007), and have correlated EDC-perturbations in gonad or gene expression changes with reduced reproductive performance or varying degrees of urbanization (Harris et al., 2011, Crago et al., 2011). However, to date few studies have attempted to link complex environmental EDC mixtures with responses at multiple tiers of the biological hierarchy, including population level effects.

Additionally, the use of environmentally relevant fishes as model species in many studies is lacking. Most EDC studies continue to use several common laboratory denizens to assess impacts (e.g., zebrafish, medaka, fathead minnow) (Scholz and Mayer,

2008, Segner, 2009, Ankley et al., 2010). As a result, assumptions about sensitivity to EDCs are primarily based on these few species. Relying on a limited number of fish species to represent responses across a range of taxa may lead to an underestimation of toxicity, since surrogate species are often less sensitive than the species of interest (Banks et al., 2010). This is of particular concern when considering threatened or endangered species. Assessing the status of threatened species is a challenge, however, since these species often cannot be sampled. In this situation the use of a resident fish as a surrogate is a better alternative to evaluating the response of typically utilized lab species. The San Francisco Bay (SFB) estuary, the largest Pacific estuary in North or South America, is ecologically important (Nichols et al., 1986), subject to a diverse array of anthropogenic inputs including EDCs (Lavado et al., 2009, Ostrach et al., 2008, Johnson et al., 1998), and is home to a number of declining fish species (Feyrer et al., 2007). It is representative of an ecosystem in need of a surrogate to evaluate potential EDC impacts.

The use of markers from several different levels of biological organization using a resident model fish allows for inferences to be made about the overall impact on the reproductive health and potential population consequences for that species. To this end, we undertook a four-tiered investigation into estrogenic and androgenic EDC effects on *Menidia audens*, a euryhaline atherinid distributed throughout the San Francisco Bay (SFB) estuary (Moyle 2002) that is sensitive to EDCs (Duffy et al., 2009). Our main objective was to integrate observations at each biological scale with the goal of determining whether the reproductive health of *Menidia* was being negatively impacted by sites receiving either urban run-off and treated wastewater effluent or ranch run-off, and if so what the mechanism(s) of endocrine disruption may be. Our hypothesis was that the site receiving urban run-off and effluent would be more disrupted than the site receiving only ranch run-off. As such, we measured overall estrogenic and androgenic

activity at each site using recombinant cell lines containing an estrogen- or androgen-sensitive reporter gene and determined via chemical analysis whether particular hormones, alkylphenols, and pesticides were present. Organism level endpoints were an examination of relative changes in endocrine-related gene expression, growth, and gonad health in *Menidia audens*. At the population level, sex ratio over two spawning seasons was measured. Ultimately our goal is to develop an approach for other estuarine systems in North America, the majority of which contain *Menidia* species (Strussman et al., 2010) that could similarly be utilized as a surrogate in EDC studies.

2. Methods

2.1 Fish Collection and Processing

Fish were seined monthly from the urban beach (+38° 13' 5.47", -122° 1' 48.50") and the ranch beach (+38° 11' 56.76", -121° 54' 39.31") (Figure 3.1) in Solano County, California, USA. A 30' x 3' seine with $^1/_4$" mesh and a 3'x3'x3' with $^1/_8$" mesh inset pocket was used (Middaugh & Hemmer, 1992). Captured fish were kept in a cooler with aeration and transported back to the UC Davis Bodega Marine Lab, Sonoma County, California for processing. During the 2009 sampling season approximately 20 fish from each site were kept alive and held in aquaria at 5-10 ppt salinity for 4-5 months to serve as depurated controls for gene expression analyses. The standard length of remaining fish was measured, then they were sacrificed and livers were immediately removed and snap-frozen on liquid nitrogen for mRNA extraction. Gonads were removed and weighed and then preserved for 24 hours in Davidson's solution (Johnson and Braunbeck, 2009) followed by storage in phosphate buffered 10% formalin. Fish mass was taken after gonad removal and gonad weight was added to fish mass to generate a total weight for GSI (gonadal somatic index = gonad mass / total mass) calculation. After weighing fish, heads were removed for otolith extraction. Sagittal otoliths were

dissected, mounted on slides, photographed, and growth increments were counted and measured based on methods from Hobbs et al. (2007).

2.2 Water Collection

Water was collected from 1-2 feet below surface in I-Chem 200 series 32 ounce amber glass bottles from 5 sites: urban slough (+38° 13' 16.08", -122° 2' 52.86"), urban/wastewater (wwtp) beach (+38° 13' 5.47", -122° 1' 48.50"), ranch beach (+38° 11' 56.76", -121° 54' 39.31"), wastewater treatment outfall (+38° 12' 30.60", -122° 3' 25.26"), and downstream of outfall (+38° 12' 30.66", -122° 3' 12.54"). Bottles were rinsed with sample water once before filling, leaving as little head-space as possible. Samples were then kept on ice in coolers until extraction began the following day.

2.3 Sample Preparation for CALUX

All glassware was washed with soap and acetone and baked in a muffle furnace at 232° Cprior to use. Prior to extraction samples were shaken thoroughly to evenly distribute any suspended solids and poured into a filter holder set-up 250 mL at a time (Fisher Scientific, Waltham, MA, USA), creating two replicates for each grab sample. Samples were extracted under 15-20 mm/Hg vacuum pressure through a 934-AH Whatman filter. Following extraction, samples were acidified to pH 2 with 10N HCl to improve the binding of EDCs with hydroxyl functional groups to the C-18 cartridge. A 24-cartridge Supelco solid phase extraction manifold (Supelco, Bellefonte, PA, USA) attached to a vacuum flask was used to extract filtered samples through OASIS HLB 6cc cartridges (Waters, Milford, MA, USA). Cartridges were pre-conditioned using 5 mL each of EtAc:AOc (75:25), 5 mLs MeOH and 5 mLs acidified MilliQ water. Samples were extracted over an hour-long period at 5 mm/Hg. Prior to elution, cartridges were washed twice with 5 mL 5% MeOH. Cartridges were then eluted twice with 4 mL

EtAc:AOc (75:25) into glass tubes and dried under a nitrogen evaporator at 60-70 °C. Samples collected on 17 October 2009 were concentrated 2500× with DMSO, while samples collected on 28 March 2010 were concentrated 4500×. Samples suspended in DMSO were capped and frozen at -20 °C until being evaluated for estrogenic, androgenic, anti-estrogenic and anti-androgenic activity using recombinant cell bioassays as described by Ahn et al. (2008).

2.4 mRNA extraction

Snap-frozen livers dissected from wild silversides collected from June through September of 2009 were individually homogenized in microcentrifuge tubes using a polypropylene pestle in ice-cold buffer containing 1 mL Trizol reagent. Homogenized samples were then incubated for 5-10 minutes at room temperature, after which 200 mL chloroform was added to each. After shaking each tube vigorously by hand for 15-30 seconds, the homogenized Trizol chloroform mixture was centrifuged at 13,400 rpm for 15 minutes at 4° C. Following centrifugation, the aqueous phase was transferred to a new tube and an equal volume of isopropanol was added and mixed thoroughly by inversion. Samples were then centrifuged again at 10,000 rpm for 15 minutes at 4° C. Supernatant was discarded following centrifugation and the remaining pellet washed three times with 1 mL 75% molecular grade ethanol by centrifuging for 5 minutes at 10,000 rpm at 4° C. The pellet was then dried in a vacuum centrifuge for 5 minutes at low temperature and resuspended in 100 mL nuclease-free water. This was heated to 60° C for 1 minute and quantified on a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Samples with an mRNA content of $\geq$100 ng were kept for cDNA synthesis and stored at -80° C.

2.5 cDNA synthesis

A master mix (10 mL) of 100 mM dNTP (Invitrogen, Carlsbad, CA, USA), nuclease-free water (GeneMate, BioExpress, Kaysville, UT, USA), RNaseOUT

(Invitrogen), and SuperScript III (Invitrogen), 5X Frist Strand Buffer, 0.1 M DTT and 300 ng/mL random primers was added to 10 mL of extracted RNA. Tubes were then centrifuged for 30 seconds and placed in a Mastercycler (Eppendorf International, Hamburg, Germany) running an hour-long cDNA synthesis program (50 minutes at 50 ^{o}C, 5 minutes at 95 ^{o}C). Samples were then frozen at -80^{o} C.

2.6 Primer Design / BLAST

Genes of interest from at least three to five species of fish and one mammal were identified using BLAST (Basic Local Alignment Search Tool, NIH National Center for Biotechnology Information). Areas of high convergence were identified and assessed for adequate GC content ($\geq$50%) and length. The primer for thyroid receptor a was obtained from Galay-Burgos et al. (2008).

2.7 PCR

PCR products were produced using the forward and reverse primers designed as described above for each gene of interest, Taqman Universal PCR Master Mix (Roche, Indianapolis, IN, USA) containing 10X Buffer, 100 mM dNTP, MgCl and Taq, and nuclease-free water (GeneMate). The Taqman / primer mix was loaded into a 96-well plate and run in a Mastercycler (Eppendorf International) on a three hour-long PCR program. Samples were run on an agarose gel infused with ethidium bromide; bands were visualized under UV light. Those that matched or were close to the expected amplicon length were cut out of the gel with a clean razor blade and sent for sequencing.

2.8 Cloning

A TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) was used to clone PCR products that were not successfully sequenced after the initial PCR. The reaction was

transformed into chemically competent *E. coli* cells. PCR product, salt solution, DNase free water, and a pCR 4-TOPO Vector were mixed gently and incubated for thirty minutes at room temperature. Following incubation, a vial of One Shot *E. coli* cells was heat-shocked for 30 s at 42° C and 250 mL of autoclaved S.O.C. or L.B. broth medium containing ampicillin was added using aseptic technique. Tubes were then shaken at 37° C for one hour and then contents of the transformation reaction were spread onto prewarmed plates containing S.O.C. or L.B. medium and 15 g/L agar and incubated overnight at 37° C. Approximately 4-6 colonies were chosen for analysis and cultured overnight at 37° C. Plasmid DNA was then isolated via centrifugation and sequenced to confirm presence and correct orientation of the insert.

2.9 Sequence Identification

Sequences generated via PCR and or cloning were analyzed using ORF (open reading frame) Finder (http://www. ncbi.nlm.nih.gov/projects/gorf/). The two longest predicted ORFs were entered into BLAST to determine protein identify. In all cases with the exception of the androgen receptor, a perfect match was generated. Because it is unknown if the androgen receptor identified is the isoform alpha or beta, we have named it “androgen receptor x.”

2.10 qPCR

Genes for q-PCR assessments were selected according to level of expression significance, knowledge base from literature, and functional classification. Primer and probes for q-PCR analyses were designed using Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com). Designed primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com), and TaqMan probes were supplied by Roche. Sequences for all genes assessed by q-PCR analyses have

been submitted to GenBank (http://www.ncbi.nlm.nih.gov). Respective primers and probe systems for investigated genes are detailed in Table 1, including a reference gene encoding for GAPDH. Control fish and those caught from both the urban and ranch influenced sites were assessed using q-PCR. Complementary cDNA was synthesized using 1.0 ng total RNA, with random primers and SuperScript® III reverse transcriptase (Invitrogen, Carlsbad, CA), and diluted to a total of 120 mL with nuclease free water to generate sufficient template for q-PCR analysis. TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA) was used in q- PCR amplifications. SDS 2.2.1 software (Applied Biosystems, Foster City, CA) was used to quantify transcription.

2.11 Histology

Gonad tissue samples fixed in 10% PBS buffered formalin were dehydrated in a graded ethanol series and embedded in paraffin. Tissue blocks were sectioned (4μm thick) and stained with hematoxylin and eosin (H & E). Tissue sections were examined under a BH-2 Olympus microscope for common and/or significant lesions. Lesions in testes were qualitatively scored on a scale of 0 = not present, 1 = mild, 2 = moderate, and 3 = severe. Although ovaries were also sectioned, most were not of a high enough quality to be scored.

2.12 Chemical Analysis

2.12.1 Steroids and Alklyphenols in Surface Water

Methods for the extraction of water grab samples for steroid and alkylphenol measurement were performed as described in Mansell et al. (2011).

2.12.2 Pesticides in Surface Water

Surface water samples (1L) were filtered using 0.7 μm glass fiber filters (GF/F) (Whatman, Florham Park, New Jersey), extracted onto Oasis HLB solid-phase extraction (SPE) cartridges (6cc, 500 mg, 60 μm, Waters Corportation, Milford, Massachusetts), dried, eluted with ethyl acetate, reduced to 200 μL and analyzed for a suite of 56 pesticides by gas chromatography –mass spectrometry operating in electron ionization mode (GC-EIMS). Prior to extraction, samples were spiked with $^{13}C_3$-atrazine, and diazinon diethyl-d_{10} (Cambridge Isotopes, Andover Massachusetts) as recovery surrogates (Hladik et al., 2008).

2.12.3 Pesticides in polyethylene devices

Low density polyethylene (PE) membranes (Brentwood Plastics, Brentwood, MO; 70 $\pm$ 1 μm) was pre-cleaned by soaking in dichlormethane (DCM) for 48 hrs followed by methanol (MeOH) for 24 hrs and finally deionized water for 24 hrs. PE was stored in glass jars in deionized water prior to use to minimize the effects of airborne laboratory contaminants. Polyethylene devices (PEDs), which were deployed approximately 2 feet below the water's surface at the wastewater outfall and the ranch beach in polypropylene holders, were left at either the ranch beach or wastewater outfall for a period of 14 – 19 days, at which point they were removed and placed on ice until extraction. Field deployed polyethylene devices (PEDs) were extracted based on methods modified from published sources (Adams et al., 2007, Anderson et al., 2008 and Lohmann et al., 2004). Prior to extraction PEDs were rinsed with deionized water and wiped with a damp Kim-wipe to remove any debris and biofouling. PEDs were spiked with 100 μL of a 2 ng/μL solution of ring-$^{13}C_{12}$-*p,p'* DDE and phenoxy-$^{13}C_6$-*cis*-permethrin used as recovery surrogates and extracted twice with 60 mL of DCM using a sonicator bath for 30 minutes each. The sample extracts were combined, dried over sodium sulfate (Na_2SO_4), reduced

to 0.5 mL using a Turbovap II evaporation system (Biotage LLC, Charlotte, NC) and analyzed for 56 pesticides using GC-EIMS.

2.12.4 Instrumental analysis

All sample extracts (1 µL injection volume) were analyzed on an Agilent 5975 gas chromatograph (GC)/electron ionization mass spectrometer (EI-MS) (Folsom, CA, USA). Analyte separation on the GC was achieved using a 30 m x 0.25 mm i.d., 0.25 µm DB-5ms fused silica column (Agilent Technologies, Folsom, California) with helium as the carrier gas. The temperature of the splitless injector was held constant at 275° C. The temperature program was 80° C (hold 0.5 min), increase to 120° C at 10° C/min, increase to 200° C at 3° C/min (hold 5 min), followed by a third increase to 219° C at 3° C/min, and a final increase to 300° C at 10° C/min (hold 10 min). The transfer line, quadrupole and source temperatures were 280° C, 150° C and 230° C, respectively. Data for all pesticides was collected in selective ion monitoring mode (SIM) with each compound having one quantifier and 1-2 qualifier ions (Table B.1, Appendix B).

Instrument calibration was achieved using calibration concentration standards that spanned the linear range of instrument response. Calibration curves were considered acceptable if the R^2 for each individual compound was greater than 0.995. The responses of the instrument was monitored every 6-8 samples with mid-level check standards. The instruments were considered to be stable if the recovery of the check standards fell within the range of 80-115 % of the nominal standard concentration. If environmental sample concentrations fell outside the linear range of the instrument, the samples were diluted appropriately and re-analyzed.

2.12.5 Detection Limits

Surface-water method detection limits (MDLs) were validated for the majority of the pesticides previously (Hladik et al., 2008) using the EPA procedure described in 40 CFR Part 136 (U.S. Environmental Protection Agency, 1992). Water samples used to determine MDLs were collected in 2005 from the Sacramento River at Miller Park. MDLs for all compounds in water ranged from 0.9 to 10.1 ng/L and instrumental limits of detection (LOD) ranged from 0.5 to 1.0 ng/L (Table B.1, Appendix B). Analytes detected at concentrations greater than the instrumental LOD but less than the MDL were reported as estimates. Instrumental LODs for pesticides measured in PEDs ranged from 5 to 10 ng/PED (Table B.2, Appendix B).

2.12.6 Quality Assurance

Pesticide concentrations in water and PEDs were validated against a comprehensive set of performance based quality assurance/quality control (QA/QC) criteria including laboratory blanks, matrix spikes, and surrogate recovery. One laboratory water blank was processed to test the cleanliness of the laboratory procedures. No pesticides were detected in any of the blank samples. Ring-$^{13}C_3$-atrazine and diethyl-d_{10} diazinon were used as recovery surrogates to assess the efficiency of sample extraction. Percent recovery of surrogates for all samples analyzed (including QC samples) ranged from 77% to 101% with a mean ($\pm$ standard deviation) of ring-$^{13}C_3$-atrazine and diethyl-d_{10} diazinon of 90 $\pm$ 6 % and 90 $\pm$ 7 %, respectively. Two water samples were spiked in the laboratory with pesticides and the percent recovery ranged from 82 to 110% with a median of 92%.

No pesticides were detected in the two blanks processed with the PED samples. Percent recoveries of the surrogates, ring-$^{13}C_{12}$-*p,p'* DDE and phenoxy-$^{13}C_6$-*cis*-permethrin ranged from 72 to 101 %. The mean ($\pm$ standard deviation) of ring-$^{13}C_{12}$-*p,p'*

DDE and phenoxy-$^{13}C_6$-*cis*-permethrin of 88 ± 8% and 87 ± 9 %, respectively. Two samples were spiked with pesticides and processes with PED samples and the percent recovery of matrix spiked samples ranged from 70 to 99 % with a median of 85 %.

2.13 Data Analysis

Unless otherwise indicated, all calculations were performed in R version 2.11 (R Development Core Team 2010).

2.13.1 CALUX

Dose-response curves for CALUX exposures to 17β-estradiol (concentrations of 1×10^{-15} to 1×10^{-6} M) and testosterone (concentrations of 1×10^{-12} to $\times10^{-5}$ M) were fit using logistic regression with binomial error and logit link. These curves were then used to estimate the relative equivalent concentration of estrogen or testosterone in each environmental sample analyzed with CALUX. Confidence intervals (95%) on the equivalent concentrations were estimated using a Monte Carlo approach: we used the means and covariances of the logistic model coefficients to simulate a distribution of 1000 different values of those coefficients; we then used that distribution to simulate a distribution of the equivalent hormone concentration for each CALUX sample.

Differences among sites were tested using ANOVA followed by Tukey test. Data were log-transformed in order to ensure homogeneity of variances.

2.13.2 Gene Expression

Prior to analysis, all gene expression data were normalized to GAPDH (reference gene) and to expression of the same gene from depurated control fish from each site. Differences in gene expression between sites were assessed using *t*-tests on normalized data. We report results as fold-change in expression, which we calculated from

normalized data using the $\log_2(-DC_t)$ method (Livak and Schmittgen, 2001). A heat map representing relative changes in transcript levels was produced using Genesis 1.0 software (Institute for Biomedical Engineering, Graz University of Technology).

2.13.3 Histology

Ordinal necrosis ratings were converted into a binomial metric for analysis using logistic regression. The necrosis rating for each sample was classified as ≥ 1, ≥ 2, or ≥ 3; three separate logistic regressions were then used to determine whether sites differed in the proportion of observations in each of those categories. Because the data exhibited quasi-separation, models were fit using Firth's bias-reduced logistic regression (Heinze and Ploner, 2003; package logistf in R 2.11).

2.13.4 Length, Sex Ratio, GSI

Fish length, sex ratio, and GSI were expected to vary over the sampling period, so we tested for difference among sites in those variables while including year and Julian data as covariates in a linear model (length) or logistic regression (sex ratio and GSI).

2.13.5 Otolith growth rate analysis

Silversides lay down daily rings on their otoliths, which are calciferous structures in the inner ear that are used as gravity, balance, movement, and directional indicators. These structures have been used for decades to measure the growth rates of *Menidia* species and other fishes (Barkman and Bengtson, 1987). The width of daily otolith increments is proportional to daily somatic growth in *Menidia menidia* (Barkman and Bengtson, 1987). We examined daily otolith increments from fish captured from the ranch and urban/wwtp beaches from March-September 2009. Only growth during the first growing season was examined because growth slows in winter, changing the

relationship between otolith size and somatic size (e.g., Hobbs et al., 2007, Panella 1971). The onset of winter growth is indicated by a dark band; we only examined growth rings preceding that band.

Examination of growth trajectories for each fish (plots of otolith radius at each increment versus age) indicated that all fish had approximately linear growth trajectories, so we used linear regression to model radius as a function of age (attempts to fit nonlinear, saturating growth functions failed to converge). There was no evidence for seasonal effects on growth, so we pooled fish across collection dates to test for the effects of site and sex on growth rate. There was also no evidence for a decrease in growth rate with age over the first growing season, so differences in the final age of sampled fish did not bias the estimates of growth rate.

We fit linear regression models with a random effect for fish (thus controlling for the non-independence of increment widths within each fish; Laird and Ware 1982). We fit models with fixed effects for age, site, sex, and all of their interactions, then removed non-significant interaction terms ($p > 0.1$) in a stepwise manner. Mixed-effects models were fit using function lme in the nlme package version 3.1 (Pinheiro and Bates, 2000) for the statistical program R version 2.11.1 (R Development Core Team 2010); note this approach is equivalent to repeated-measures ANOVA for longitudinal data (Laird and Ware, 1982).

3. Results

3.1 CALUX

Estrogenic activity was detected above solvent control (DMSO) levels in all samples collected from Suisun Marsh. Estrogen equivalents in samples collected from the urban/wwtp beach, urban slough, ranch beach, wastewater outfall and 300 meters downstream of the outfall on 17 October 2009 and 28 March 2010 were 7.15e-12 & 1.48e-

12M, 1.41e-11 & 2.38e-12M, 9.77e-12 & 2.38e-12M, 1.59e-11 & 3.25e-12M, and 2.04e-11 & 4.09e-12M, respectively (Figure 3.2). Although samples from 28 March 2010 were more highly concentrated (4500x) than those collected on 17 October 2009 (2500x), estrogen equivalents were lower at all sites sampled on 28 March 2010. On both sampling dates, the sample taken 300 meters downstream of the outfall was highest in estrogen equivalents, although not significantly different from the outfall on 28 March 2010 and not significantly different from any of the other sites on 17 October 2009.

Androgenic activity was also detected above solvent control (DMSO) levels at all sites sampled. Testosterone equivalents in samples collected from the urban beach, urban slough, ranch beach, wastewater outfall and 300 meters downstream of the outfall on 10/17/2009 and 3/28/2010 were 1.58e-08 & 3.33e-08M, 2.30e-08 & 3.28e-08M, 7.08e-09 & 1.20e-08M, 2.49e-08 & 5.15 e-07M, and 2.13e-08 & 1.12e-07M, respectively (Figure 3.2). In contrast to estrogen equivalents, testosterone equivalents at all sites sampled were higher on 28 March 2010 (4500x) than on 17 October 2009 (2500x). While androgen equivalents were significantly higher at the outfall on 28 March 10, all urban sites were similar in androgenic activity on 17 October 2009. Overall, all urban sites had significantly higher activity than the ranch site on both dates. No anti-estrogenic or anti-androgenic activity was detected (data not shown).

3.2 Gene Expression

Vitellogenin (Vtg) and choriogenin L (ChgL), genes induced by estrogen, had significantly higher expression in males at the site exposed to ranch run-off than males exposed to urban run-off and treated wastewater effluent (Figure 3.3). Expression of estrogen receptor alpha (ESR1) was also higher in ranch males, although not significant. Expression of three estrogen-related genes in females, Vtg, ChgL, and ESR1, were also higher on average in ranch females compared to urban females, although not significant

due to high variation (Figure 3.3). No significant differences were found between the ranch and urban sites in either males or females in expression of thyroid receptor alpha (TRa), insulin-like growth factor 2 (IGF-2), androgen receptor X (ARx), or estrogen receptor g (ESR3). However, expression of all transcripts measured (Vtg, ChgL, ESR1, ESR3, ARx, IGF-2, TRa) generally clustered together by site overall (Figure 3.4), red signifying upregulation and green down-regulation. The reference gene, GAPDH, was expressed at similar levels in control, ranch and urban fishes.

3.3 Histology

The proportion of observations of mild and moderate germ cell necrosis (rating = 1, 2) in testes from males caught at the ranch and urban beaches was not significantly different, although testes from urban males trended higher in both (Figure 3.5). The proportion of observations of severe necrosis (rating = 3) was significantly higher in males caught at the urban beach. No cases of intersex were observed. A micrograph of a normal testes and a severely necrotic testes are shown in Figure 3.6.

3.4 Gonadal Somatic Index

The gonadal somatic index (GSI) was significantly higher in males caught at the ranch beach in both 2009 and 2010 ($p=0.0148$) (Figure 3.7). Male GSI was significantly lower in 2010, and a negative relationship between increasing julian date and GSI was found ($p = 6.08\times10^{-8}$). No significant difference was observed between urban and ranch females ($p=0.2760$), and although no significant relationship existed between GSI and julian date, a negative trend is apparent. Because no females were seined from the urban beach after July in either 2009 or 2010, analysis was ended at that time point.

3.5 Growth

Otolith radius increased with fish age (as expected) but there were significant effects of both site and sex on that growth rate ($p < 0.0001$). Males grew slower than females (as expected), but fish from the urban / wastewater site grew more slowly than those from the ranch site (Fig. 3.8).

3.6 Standard Length

Analysis of each sex separately found that males caught at the urban beach were significantly longer than males at the ranch beach (p = 8.36e-07). There was also a significant negative relationship between increasing Julian date and standard length (p = 0.0004), and males caught in 2010 were significantly smaller than those caught in 2009 (p = 0.01) (Figure 3.9). A significant difference was not detected between females captured at the two sites (p > 0.05), and although there was a trend towards decreasing size with increasing Julian date (p = 0.055) and smaller size in 2010 (p = 0.059), these relationships were not significant (Figure 3.9).

When sexes were analyzed together, as expected females were found to be significantly larger than males at the ranch beach (p = 0.0128). But unexpectedly, males from the urban beach were significantly larger than females caught there in both years (p = 0.0132) (Table 3.2).

3.7 Sex Ratio

The proportion of female fish caught from the urban beach was significantly lower than the proportion caught at the ranch beach in both 2009 and 2010 (p = 0.00128). Additionally, a lower proportion of female fish was caught at both beaches in 2010 compared to 2009 (p = 0.02228) (Figure 3.10).

3.8 Chemistry

Water sampled from the urban and ranch beaches, urban slough and the outfall contained detectable levels of 17β estradiol (E2), estrone (E1), nonylphenol (NP) and octylphenol (OP) (Figure 3.11). Levels of E2 and E1 were surprisingly higher in the urban slough than at the outfall. Nonylphenol levels trended highest at the outfall, but octylphenol was found at roughly equivalent concentrations at all sites sampled. Only one sample was available for analysis from the urban slough, therefore standard error could not be calculated for measurements from this site.

The majority of the pesticides, herbicides and fungicides for which analysis was conducted in both water grab samples (taken on the first and last date of PED deployment) and PEDs were not detected. However, the following contaminants were detected in PEDs left at the wastewater outfall from 4 August – 17 August 2009: bifenthrin, dieldrin, fipronil, permethrin, p p – DDD, pp – DDE, pp – DDT, PCA (pentachloroanisole), PCNB (pentachloronitrobenzene), and trifluralin (Table 3.3). These contaminants were also detected in PEDs left at the ranch site from 11 June or 16 June – 1 July 2009, with the exception of PCA, PCNB, and permethrin. With the exception of the persistent legacy contaminant dieldrin, all contaminants detected in PEDs were found at higher amounts at the wastewater outfall / urban site than at the ranch site. Hydrophilic chemicals that tend not to adsorb to substrates such as plastic or sediments were found in water grab samples. Water samples from the wastewater oufall / urban site contained: DCA, atrazine, carbaryl, diazinon, fipronil, hexazinone, metolachlor, simazine, and trifluralin. At the ranch site all of these contaminants were also detected, with the exception of DCA, diazinon, hexazinone, metolachlor, and trifluralin. Although a slightly higher concentration of atrazine was detected at one ranch sampling location in comparison to the wastewater outfall, all other contaminants

were at higher concentrations in water sampled from the vicinity of the outfall (Table 3.3).

4. Discussion

The need for EDC studies that examine and link impacts at multiple biological scales, including the population level, has been suggested by several recent reviews in the field (Clements 2000, Denslow & Sepulveda 2007, Rempel & Schlenk 2008) and such studies are becoming increasingly common. For example, lab studies with the fathead minnow and other species have been used to predict population trajectories (Ankley et al., 2008, Miller et al., 2007, Gutjahr-Gobell et al., 2005) and changes in reproductive biomarkers have been linked to land use (urbanization) and to exposure to complex environmental mixtures in the lab (Crago et al., 2011, Filby et al., 2007). To date, the most direct link between lower level molecular endpoints (Vtg expression) and population persistence has been demonstrated in a study undertaken by Kidd et al. (2007), who observed a population crash in fathead minnows exposed for multiple years to ppb levels of ethinylestradiol in an experimental lake. Our study expands upon these efforts that primarily utilized exposures to known chemicals or conducted exposures in the lab with standard test species by evaluating the impact of environmental mixtures with measured levels of endocrine activity in the field on several tiers of the biological hierarchy in wild fish. It encompasses endpoints with both high ecological significance (sex ratio, growth) and high mechanistic significance (gene expression, histopathology) (Denslow & Sepulveda 2007).

To date, a study on both estrogenic and androgenic endocrine disruption has yet to be conducted on fishes in the SFB estuary. Recently awareness of EDC prevalence has increased, with estrogenic activity documented in the watershed's rivers (Lavado et al., 2009) and agricultural drain water (Johnson et al., 1998). Discerning impacts on SFB

fishes, however, is challenging because the regions' many highly impacted native species cannot be collected in large enough numbers due to population decline (Feyrer et al., 2007). Selection of the introduced *Menidia audens* (Mississippi silverside) as a surrogate for EDC studies is appropriate as it is distributed through the entire estuary and shares life history traits such as habitat use, diet and lifespan with some endangered fishes (Moyle 2002).

At this study's initiation, it was hypothesized that endocrine disruption would primarily be seen at the urban/ wastewater treatment outfall site, and that despite some ranching activity present at the ranch site, minimal evidence of endocrine disruption would be observed here. In effect, the ranch beach was meant to be a reference for the beach contaminated by urban run-off and treated wastewater effluent, for lack of other comparable seining beaches that are not exposed to EDCs within the SFB estuary. While now it is known that EDCs are clearly present at both sites, responses at the ranch site were limited to the molecular level while putative impacts at all levels of biological organization were observed at the urban site.

4.1 Site Activity and Chemistry

Although many studies have utilized the CALUX assay in the laboratory and in the field to measure endocrine activity in samples (Giudice & Young, 2010, Van der Linden et al., 2008, Houtman et al., 2004, Murk et al., 1996), few have attempted to link responses or contaminants in fish with the measured levels of endocrine activity detected in complex environmental samples (Houtman et al., 2007). The value of this effect-based approach is that it allows assessment of complex environmental mixtures and incorporates the activity of unknown EDCs unlikely to be identified by even an extensive chemical analysis. The CALUX assay cells natively express either the estrogen receptor (ER) or androgen receptor (AR). It can detect compounds that exert effects either by

binding directly to a nuclear steroid receptor or indirectly (Rogers & Denison, 2000), by binding to non-genomic steroid receptors (Thomas 2003), or by altering transcription factors or via crosstalk with other receptors (Tilghman et al., 2010, Palumbo et al., 2009). Although the ER and AR CALUX used in this study were of human origin, it is known that both the ER and AR are highly conserved from lower to higher vertebrates (Le Drean et al., 1995).

Significantly higher levels of androgenic activity were detected at all sites sampled on the urban and treated wastewater effluent influenced side of the marsh, with the highest levels observed at the wastewater outfall at both sampling dates. Estradiol equivalents, however, were several orders of magnitude lower than the testosterone equivalent concentrations measured and although they trended higher downstream of the outfall and at the outfall, estradiol equivalents at the ranch beach were roughly equivalent to those at the urban slough and urban beach on both sampling dates. Although samples were more highly concentrated on the 28 March 2010 sampling date in comparison to 17 October 2009, estradiol equivalents were lower in the spring, likely due to dilution from winter rains. In contrast, testosterone equivalents were lower in the fall in comparison to the spring, which could indicate that androgenic contaminants come from different sources than estrogenic contaminants do.

These findings run counter to a number of studies that have reported high levels of estrogens or estrogenic effects in treated wastewater effluent compared to other sites (Vajda et al., 2008, Ma et al., 2007, Tilton et al., 2002). Very low levels of natural estrogens were detected at both the urban/wastewater outfall site and the ranch site, and although levels of estrogenic contaminants such as DDT, DDE, DDD, permethrin, and bifenthrin (Kojima et al., 2004, Brander et al., Chapter 2) were also detected and were present at higher levels at the urban site than the ranch site, overall estrogenic activity did not largely differ.

According to other studies, androgens and androgenic compounds comprise a sizeable proportion of the EDCs present in treated wastewater effluent (Chang et al., 2011, Kolodziej et al., 2003) and a diverse array of compounds, from fungicides to PAHs, can act as androgen agonists depending on the concentration present (Tamura et al., 2006). For example, both atrazine and simazine, which were detected in PED extracts (simazine at much higher levels at the urban site), have demonstrated *in vitro* androgenic activity (Orton et al., 2009). The AR CALUX assay may have also been sensitive to natural and synthetic glucocorticoids and progestins present as pharmaceuticals in treated wastewater effluent, as it has been shown in previous studies that it sometimes does not discriminate well between these three types of compounds (Van der Linden et al., 2008). Considering that the natural androgens testosterone and androstenedione were not detected, the contribution of xenoandrogens to measured AR CALUX activity at the urban site may be significant.

4.2 Gene expression

Evaluating the expression of endocrine sensitive genes is an effective and increasingly common approach used to discern mechanisms of action of EDCs or EDC mixtures. As seen in other studies, males caught from the ranch site that had been exposed to water with measured estrogenic activity were expressing both Vtg and ChgL (Filby et al., 2007). However, males from the urban site had comparably low levels of Vtg and ChgL expression, even though both sites had roughly equivalent levels of estrogenic activity. Additional observations, albeit statistically insignificant, follow this trend in that males at the ranch site also had comparatively higher expression of ESR1 and females at the ranch site had higher expression of Vtg, ChgL, and ESR1 than urban females.

The observed difference in expression of these genes in males and females between the ranch and urban sites could be attributed to the presence of androgenic EDCs, as detected by the AR CALUX, at the urban site. For example, a study in which female fish were exposed to androgenic EDCs showed that their Vtg expression decreases relative to controls (Seki et al., 2006). Studies targeting fish gonads have found that additions of androgens to ovarian tissue cause a dose-dependent decrease in estradiol production (Braun & Thomas 2003), which would lead to a decrease in or absence of Vtg production. The trend towards lowered expression of estrogen-responsive genes in urban females during the reproductive season could indicate masculinization due to androgenic EDC exposure, and potentially lowered fecundity. However, it should be noted that it is difficult to make a direct comparison between activity in grab samples measured via CALUX and gene expression in fish exposed over a prolonged period.

4.3 Gonad Health

Gonad health in silversides was evaluated using two endpoints, gonadal somatic index (GSI) and histology. The appearance of histological sections, coupled with analysis of Vtg expression, is considered one of the most sensitive endpoints for determining whether endocrine disruption has occurred (Dang et al., 2011). Exposure to both estrogenic and androgenic compounds has been shown to reduce GSI and increase the incidence of germ cell necrosis, which may represent an interruption of spermatogenesis (Velasco-Santamarla et al., 2010, Leon et al., 2007, Blazer 2002).

Intersex has been observed downstream of other wastewater outfalls (Tetreault et al., 2011) but was not detected here. However, moderate levels of necrosis were higher (marginally significant) and severe cases of necrosis were significantly higher at the urban / wastewater outfall site. Additionally, GSI was significantly lower in urban males.

Both lowered GSI and necrosis could result in lowered sperm count and hence lower fecundity of urban males.

4.4 Growth

Otoliths are rarely utilized in toxicological studies, yet they have been shown in one study to be a more sensitive measure of growth than merely focusing on somatic changes (Rose et al., 2005). To our knowledge, otoliths have not been used to compare the growth rates of fish exposed to different types of EDCs, which may modify growth via interactions with the insulin-like growth factor system (Reinecke 2010).

Pottinger et al. (2010) found that the length, body mass and growth rate of sticklebacks influenced by wastewater effluent were greater than that of reference populations. While our study did find that males caught from the urban site were significantly longer than males caught from the ranch site, examination of otoliths revealed that the growth rate of urban males was significantly slower than ranch males. Urban males were also significantly larger than urban females, which is surprising considering that in *Menidia* populations female fecundity is strongly correlated with increasing SL and weight therefore females are typically larger than males (Middaugh and Hemmer, 1992). This is another indication, along with reduced GSI and testicular necrosis, that overall fecundity is lower at the urban / wastewater outfall site.

The reason for differences is unclear and the difference in overall growth rate could be due to differences in food availability between the two sites. However, this does not explain males being larger than females at the urban site. It is possible that because silversides have temperature sensitive sex determination, that some fish born in the early spring that were genotypically female (more females produced at colder temperatures) actually became phenotypically male due to early life exposure to androgenic EDCs. As a result, these early season urban males had a longer period to grow than ranch males

born later in the year, so regardless of the slower growth rate urban males were significantly longer overall.

4.5 Population level

Because silversides have temperature sensitive sex determination (TSD), they follow a seasonal pattern of spring female-biased sex ratios and summer to fall male-biased ratios. In natural populations, females are produced in excess earlier in the season so they have time to grow larger and hence have the ability to carry more eggs (Conover & Kynard, 1981, Hubbs et al., 1976). While this expected pattern was observed at both the urban and ranch sites, the urban site had a significantly lower proportion of females throughout the entire breeding season in both 2009 and 2010. Additionally, although females are produced in excess earlier in the year, the population should even out to approximately a 50:50 ratio when the entire year is taken into account (Conover & Van Voorhees, 1990). Hence, it appears that the population exposed to urban run-off and treated wastewater effluent, which has been demonstrated via the CALUX assay to contain significantly higher amounts of androgens or xenoandrogens, may be undergoing masculinization.

Results are also not always clear-cut, particularly with complex environmental mixtures. For example, studies have shown simultaneous expression of Vtg in male fish and male biased sex ratios in the same population downstream of wastewater or pharmaceutical discharges (Rempel et al., 2006, Sanchez et al., 2011). Even compounds that are considered to be estrogenic, such as nonylphenol, can exert unexpected effects at the population level. For example, in a study performed with Japanese medaka, which are in the same phylogenetic group (Atherinomorpha) as silversides, the two highest concentrations of nonylphenol had a decreased proportion of females in comparison to controls (Nimrod & Benson, 1998). Furthermore, compounds found in wastewater

effluent such as the synthetic progestins levonorgestrol and norethindrone and glucocorticoids have been demonstrated to masculinize and to reduce the fecundity of various fish species (Zeilinger et al., 2009, Paulos et al., 2010, Hattori et al., 2009, Yamaguchi et al., 2010, Knapp et al., 2010). Still another potential factor involved in masculinization is exposure to hypoxic events, which have been shown to masculinize Atlantic croaker (Thomas & Rahman 2010), and these are known to occur in Suisun Marsh. The masculinization of *Menidia audens* may have occurred due to the interplay of several of these dynamics.

4.6 Conclusions

Clear links were observed between the biological scales examined for signs of endocrine disruption in *Menidia audens*. At the ranch site, where primarily estrogenic compounds were present, males had significantly higher and females trended towards higher expression of estrogen-responsive genes. At the urban/wastewater outfall site, which is contaminated by both estrogens and comparatively higher concentrations of androgens, both males and females had relatively low expression of estrogen-responsive genes, males had lower GSI and higher incidence of severe testicular necrosis, and the proportion of females caught throughout the spawning season was low compared to the ranch site and in comparison to observations of *Menidia* sex ratios in other populations.

Additionally, it was found that males at the urban site were significantly larger (in SL) than females. This finding runs counter to the reproductive strategy of most atherinid fishes, in which females are larger than males in order to maximize their capacity to carry oocytes. Males were larger despite having a slower growth rate than urban females, which may be due to genetically female larval silversides being masculinized early in the season, when colder temperatures should result in a female biased population. Future research will seek to confirm whether this is occurs

experimentally, both in the laboratory and in the field, using genetic markers of sex determination.

The development of a multi-tiered approach for alternative fish species that have the potential to be used as surrogates for endangered species is of great importance. Mathematical modeling approaches are currently being developed to predict the population trajectory of silversides exposed to EDCs (Brander and White, unpublished), the goal being to extrapolate these predictions to endangered fish species with similar life histories. Finally, since *Menidia* species are found on the East, West, and Gulf coasts of the United States (Middaugh & Hemmer, 1982), their utility could potentially be expanded to estuaries nationwide.

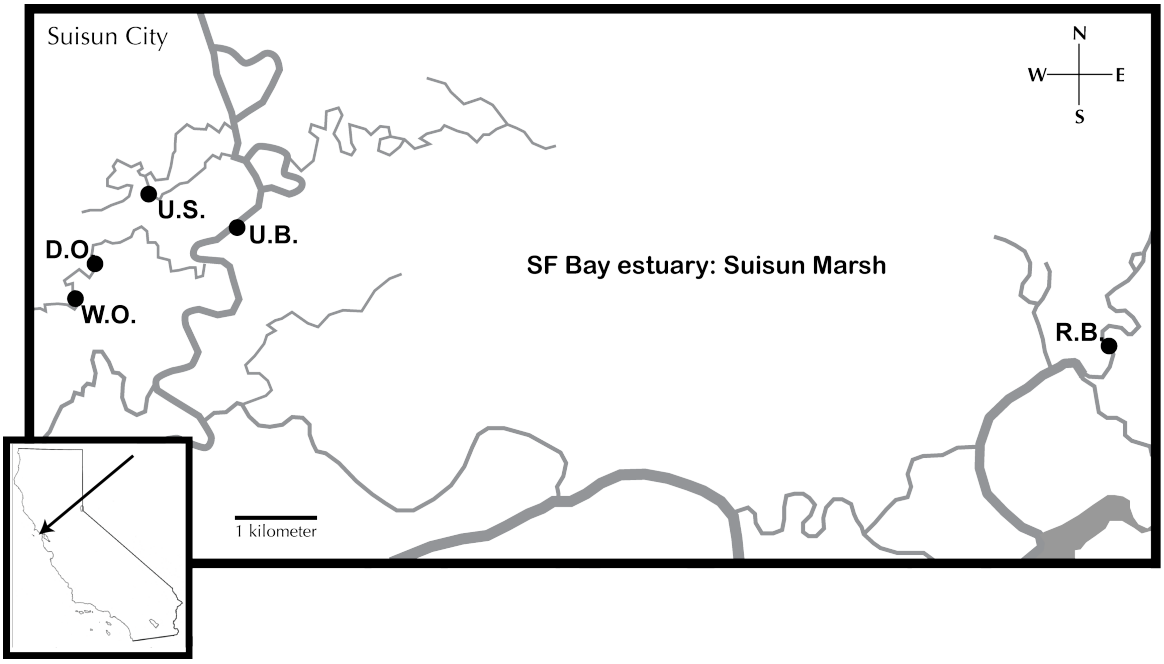


Figure 3.1 **Map of study sites, Suisun Marsh, San Francisco Bay**. Suisun Marsh is located approximately 96 kilometers NE of San Francisco (SF) Bay. Water samples were collected for reporter gene assay and hormone measurement from D.O. (downstream of outfall), W.O. (wastewater outfall), U.S. (urban slough), U.B. (urban beach), and R.B. (ranch beach). PEDs (polyethylene devices - passive samplers) were deployed at W.O. and R.B. Fish were collected via beach seine from U.B. and R.B.

Table 3.1 **Primers for qPCR analysis**. Primers were selected based on expression significance, literature and functional classification and designed using Roche's Universal Probe Library Assay Design Center.

Transcript Name	**Primer Sequence**	**Probe Number**
Menidia ESR1 forward	ACGCTTCCGCATGCTCA	Roche #15
Menidia ESR1 reverse	CTCCATTGTGCCAGTGCAGA	
Menidia ESR3 forward	CATTATGCCCTCCACGCACT	Roche #52
Menidia ESR3 reverse	GACCATCCTGGGAAACTGATCTT	
Menidia ARx forward	ATCCGCATGCAGTGCTCATA	Roche #31
Menidia ARx reverse	CCCCAGACCTCGTATTCAACG	
Menidia ChgL forward	CATCCAGTCATCAGTCATGAGTTTC	Roche #82
Menidia ChgL reverse	GGTCCCGTTTTCTGCAGTTAAG	
Menidia TRa forward	TGTCGGACGCCATATTCGAT	Roche #51
Menidia TRa reverse	CCTCGGTGTCATCCAAGTTGA	
Menidia GAPDH forward	GGTGGTGAACACACCAGTGG	Roche #159
Menidia GAPDH reverse	CACGAGAGGGACCCAACTAACA	
Menidia Vtg forward	GTAGAGTTCATGAAGCCCATGCT	Roche #108
Menidia Vtg reverse	AAATCAATGTAAGCGGCAAAGG	
Menidia IGF2 forward	GGCTGCCTTCCTATTCCACAC	Roche #38
Menidia IGF2 reverse	GCAGGTCATACCCGTGATGC	

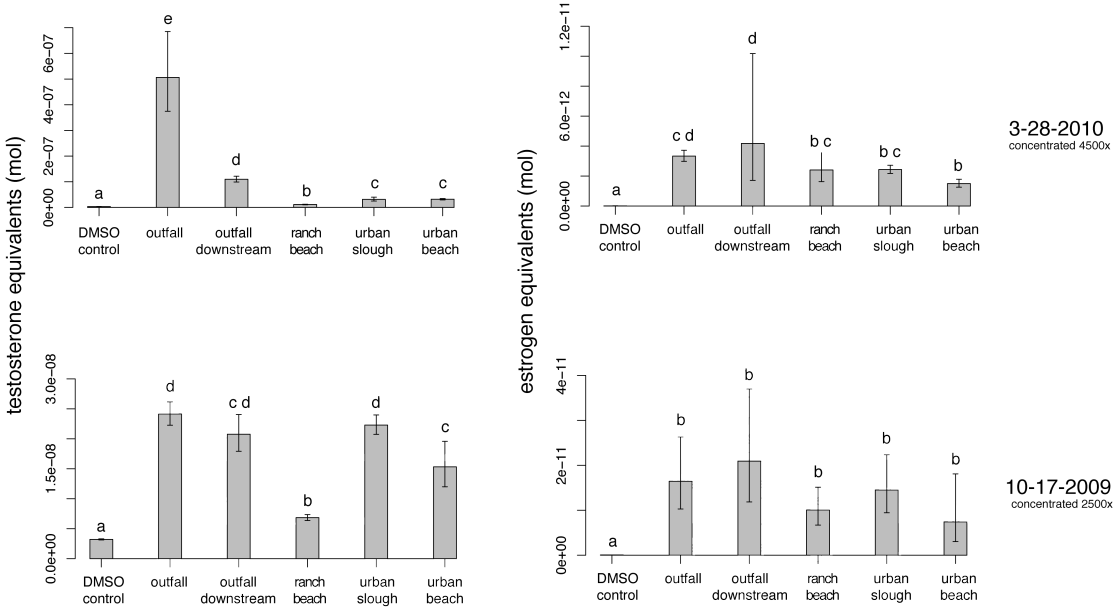


Figure 3.2 **Estrogen and testosterone equivalents measured by CALUX.** Estrogen and testosterone equivalents were measured in water grab samples collected from the urban beach, urban slough, ranch beach, wastewater outfall and downstream of outfall on 17 Oct 2009 and 28 Mar 2010. Samples collected on 17 Oct 2009 were concentrated 2500x and those collected on 28 Mar 2010 were concentrated 4500x, and both resuspended in the control solvent DMSO. An ANOVA was run for results of each hormone and sampling date combination and error bars represent 95% confidence intervals. Significant differences between treatments were determined via Tukey test. Treatments that are not significantly different ($p > 0.05$) share the same letter and treatments that are significantly different from each other ($p < 0.05$) do not share a letter.

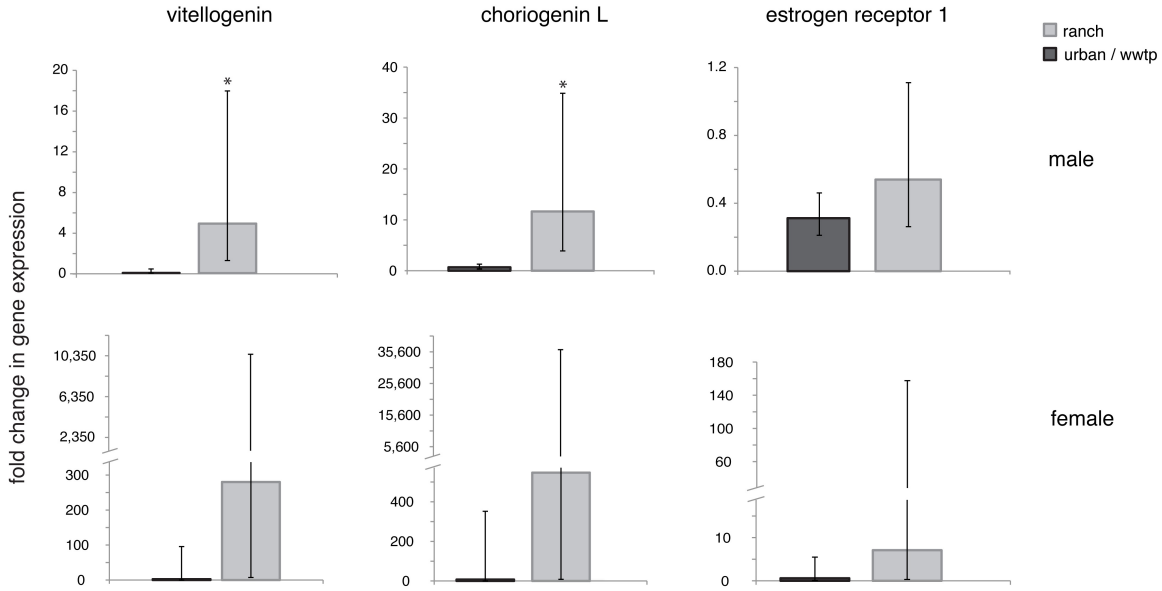


Figure 3.3 **Differential expression of estrogen sensitive transcripts by site and sex.** Expression of vitellogenin and choriogenin L in ranch males (n=11) was significantly higher ($p < 0.05$) than in urban/wwtp males (n=11), and expression of ESR1 trended higher in ranch males. Expression of vitellogenin, choriogenin and ESR1 were not significantly different but all trended higher in ranch females (n=6) compared to urban/wwtp females (n=6). All fish used for qPCR were caught between June and September of 2009. Error bars represent 95% confidence intervals. Mean expression for each transcript was transformed to fold change in expression using the log 2(−Delta Ct) method.

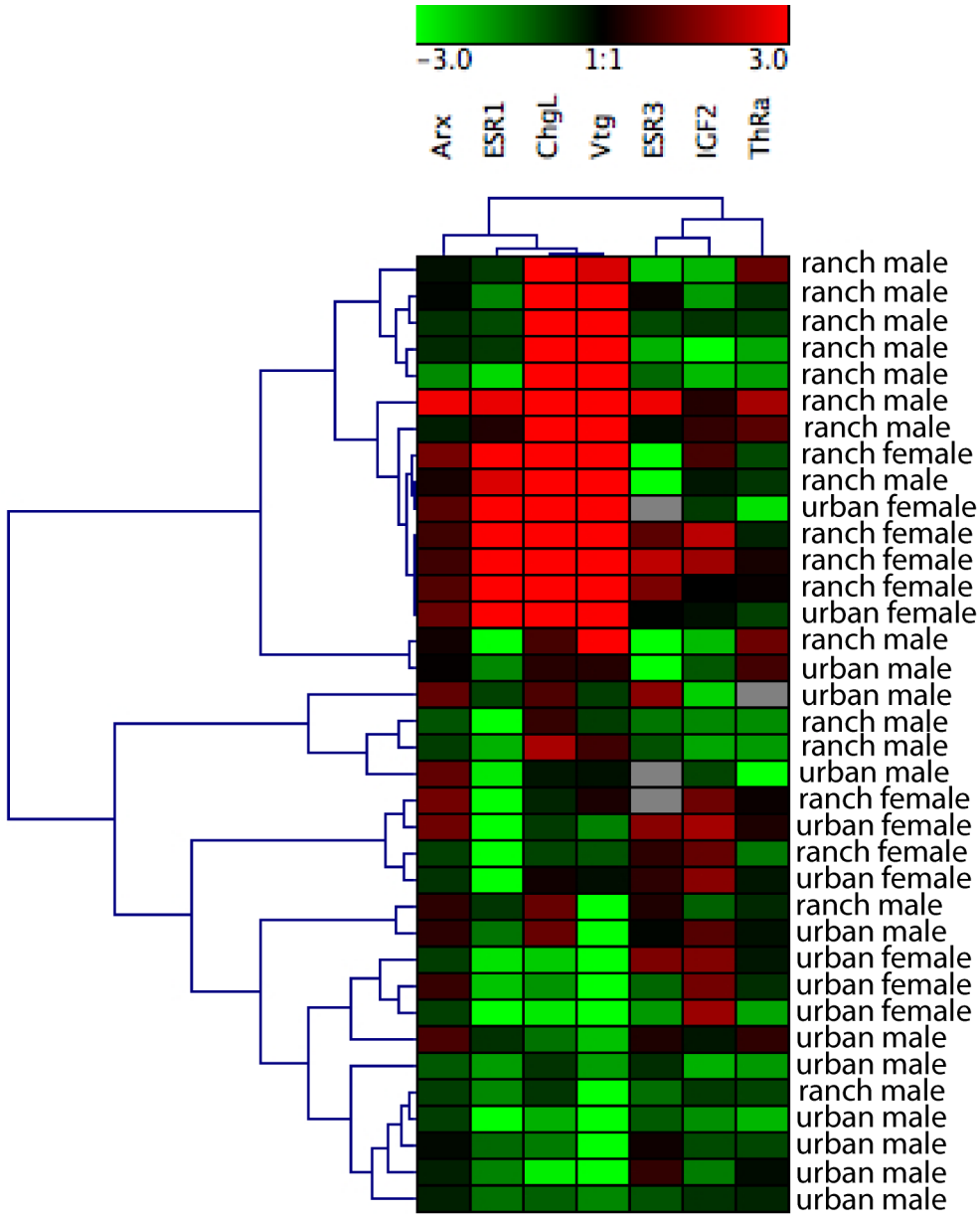


Figure 3.4 **Heat map of transcript expression by site and sex.** Although vitellogenin and choriogenin fold changes in expression were the only transcripts found to be significantly different between sites, expression of transcripts generally clustered according to site and sex, particularly in males. Cluster analysis was performed using Genesis 1.0 software. Red signifies an upregulation in fold expression, while green signifies down-regulation.

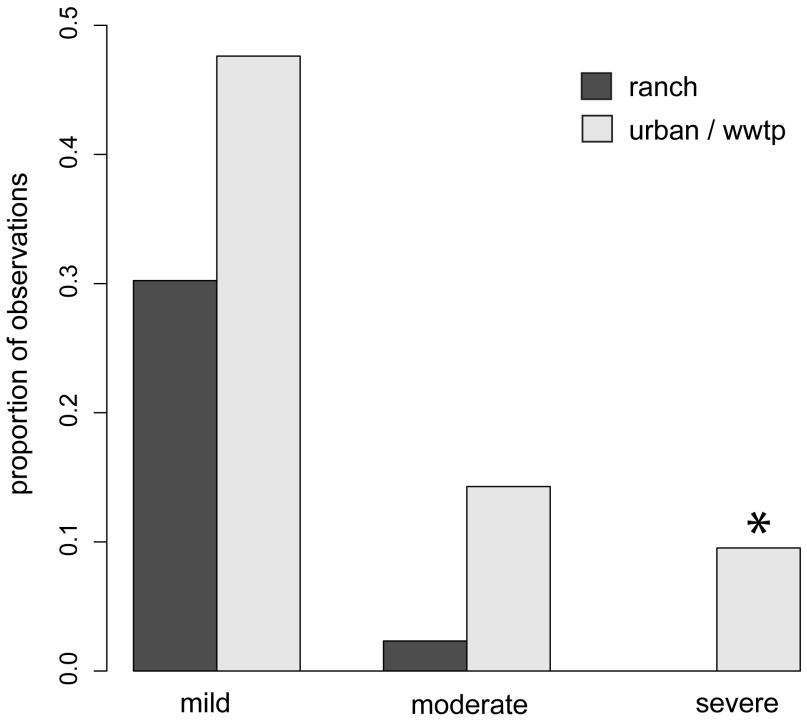
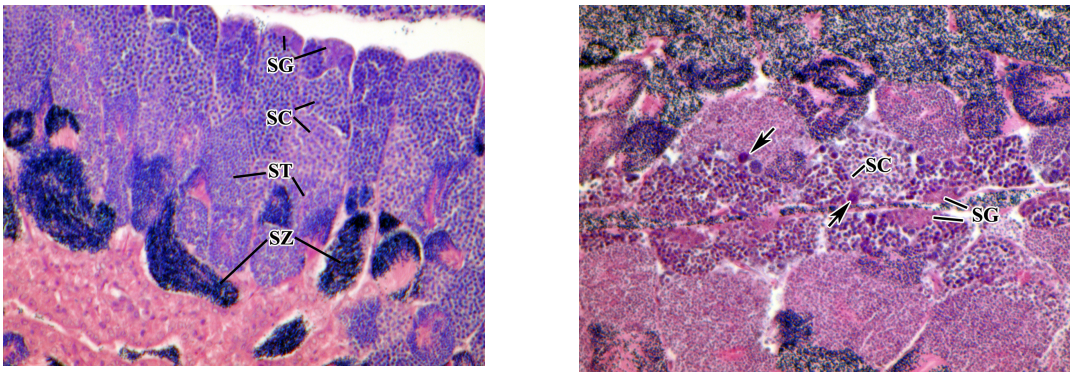


Figure 3.5 **Histological evaluation of testes by site.** Testes sectioned from ranch (n = 43) and urban/wwtp males (n = 42) caught from March through August in both 2009 and 2010 were evaluated for necrosis and qualitatively scored on a scale of 1 = mild, 2 = moderate, and 3 = severe. Data were analyzed using bias-reduced logistic regression. Significance at $p < 0.05$ is indicated by *.



a) b)

Figure 3.6 **Comparative testicular morphology**. a) Normal testicular morphology of ranch male. SG = spermatogonia;SC = primary and secondary spermatocytes; ST= spermaids; and SZ = spermatozoa in an H&E paraffin section. b) Abnormal testicular morphology of urban/wwtp male showing severe germ cell necrosis (arrows) at the spermatocyte (SC) stage. Sg = spermatogonia in an H&E paraffin section.

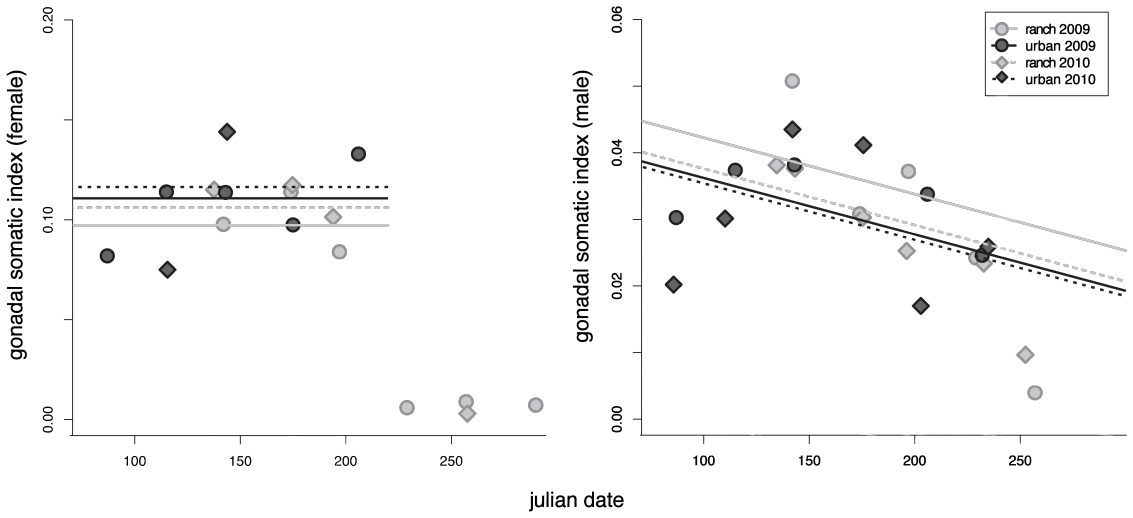


Figure 3.7 **Gonadal somatic index by site, sex and year**. GSI in ranch males (n = 61) was significantly higher than that in urban males (n = 76). There was also a significant negative relationship between male GSI and increasing Julian date. There was no significant difference between ranch females (n = 27) and urban females (n = 15), nor was there a significant decrease in GSI over time; however a trend is apparent. No females were caught from the urban beach after Julian date 225 in 2009 or 2010, so the regression was truncated at day 225.

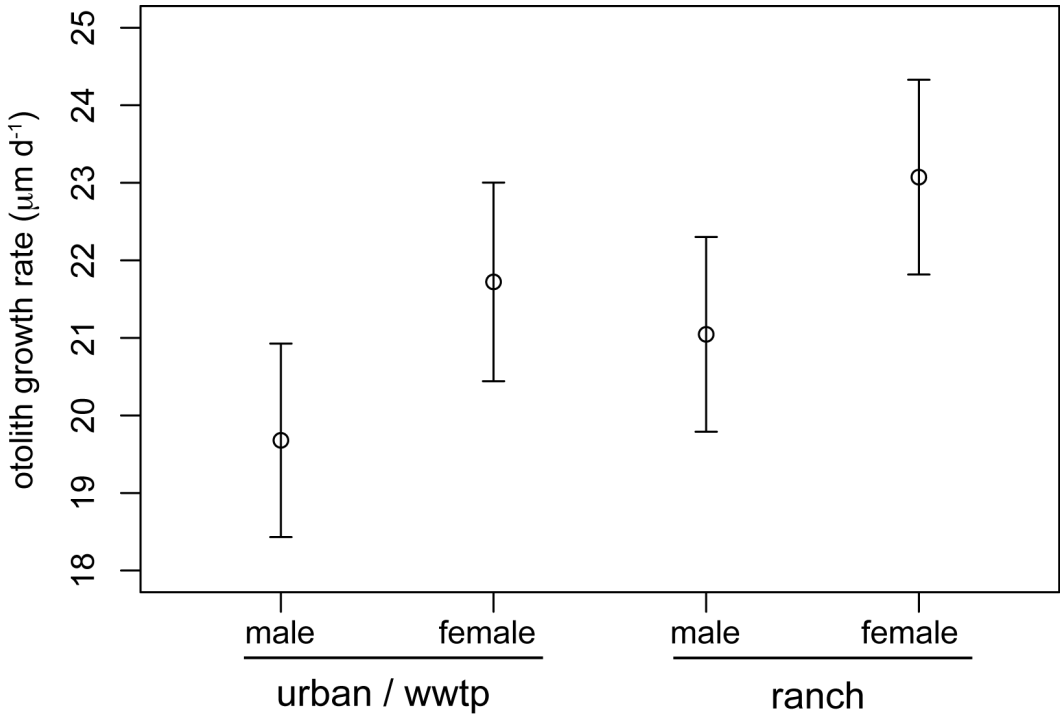


Figure 3.8 **Otolith growth rates by site and sex**. Growth rate as estimated by incremental otolith diameter increase was significantly higher at the ranch site than at the urban site. While females grew significantly faster than males overall, females (n = 10) at the urban site grew more slowly than those at the ranch site (n = 19), likewise for urban males (n = 47) versus ranch males (n = 26).

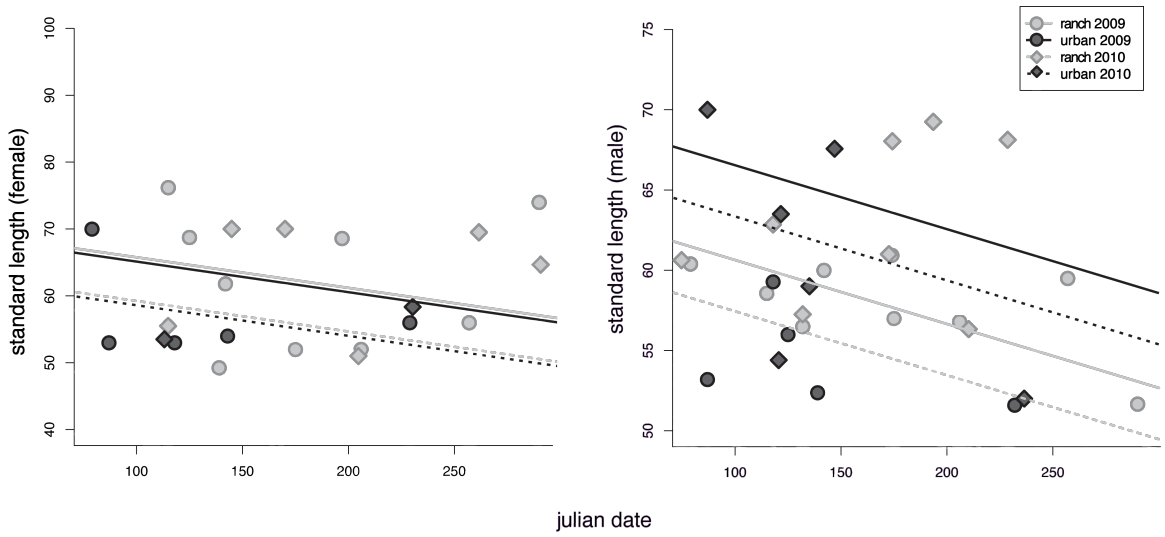


Figure 3.9 **Mean standard length by site, sex and year**. Standard length (SL) was used as a measure of comparative growth between sites and sexes. In both years, SL was significantly larger in males at the urban site than in males at the ranch site. There was no significant difference in SL between females from the two sites.

Table 3.2 **Mean standard length compared by site, sex, and year**. Standard length (SL) was compared between sexes.

coefficient	**estimate**	**standard error**	**p value**
intercept	65.08	1.95	< 2e-16
sex	4.08	1.63	0.01
field site	5.85	1.31	1.15e-05
julian date	-0.04	0.01	9.81e-05
year 2010	-3.89	1.24	0.00
sex * field site	-6.53	2.62	0.01

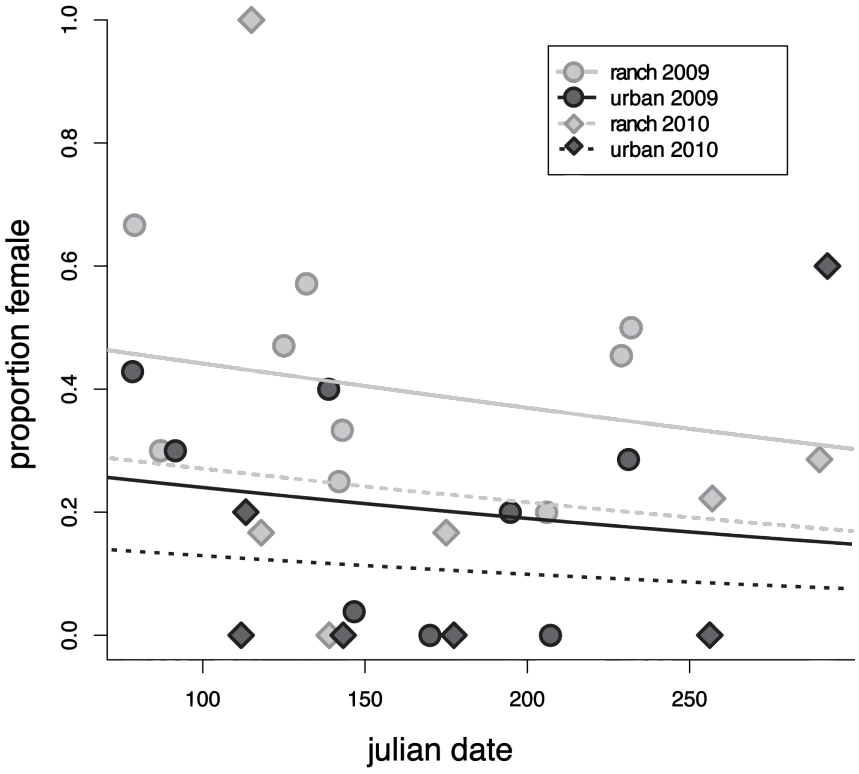


Figure 3.10 **Sex ratio by site and year**. A regression model was fit to the sex ratio of fish caught at each sampling event found a significantly higher proportion of female fish at the ranch site in comparison to the urban site in both years. There was an overall significant negative relationship between proportion female and increasing julian date, and a lower proportion female overall in 2010 when compared with 2009.

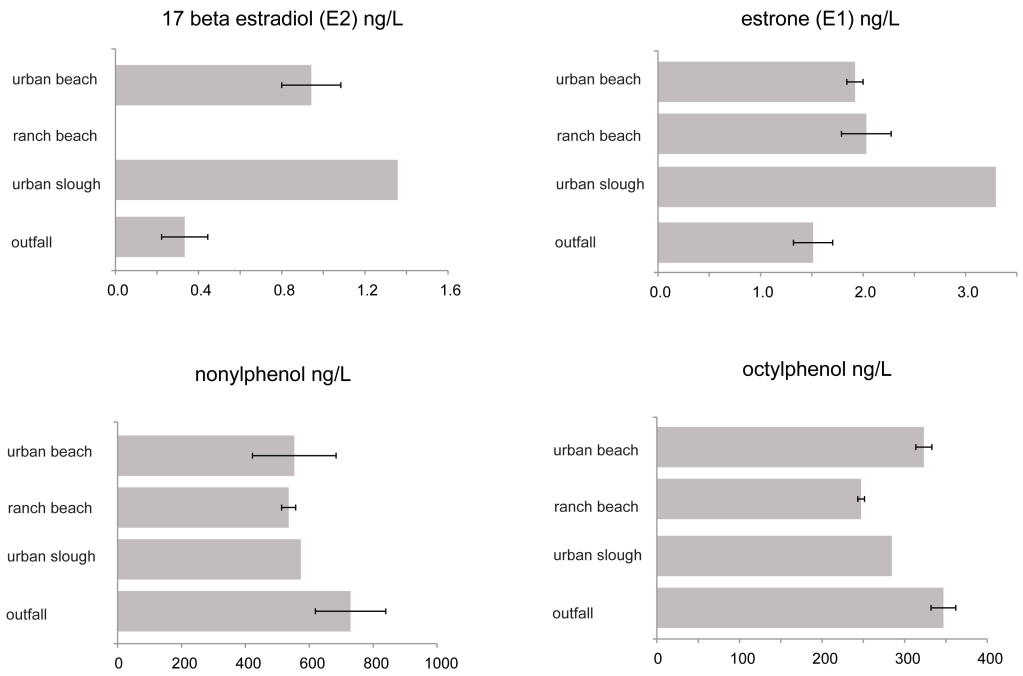


Figure 3.11 **Hormone and alkylphenol water chemistry**. Levels of 17 alpha and beta estradiol, estrone, testosterone, androstenedione, progesterone, estriol and the alkylphenols nonylphenol and octylphenol at ng/L were measured in grab samples from the wastewater outfall (n=3), ranch beach (n=2), urban slough (n=1), and urban beach (n=2). Error bars represent standard error. Testosterone, androstenedione, progesterone, estriol, and 17α-estradiol concentrations were measured but were below detection or quantitation limits. Nonylphenol and octylphenol concentrations are estimated, levels measured were outside of the quantitative range.

Table 3.3 **Pesticide, herbicide, and fungicide passive sampler and water chemistry**. Levels of frequently used contaminants and legacy pollutants were measured both in polyethylene devices (PEDs – passive sampler) and grab samples from the wastewater outfall, and two different locations at the ranch beach. The first position in each cell is the amount detected in the PED in ng/PED (n=4), and the second position in each cell is the amount detected in grab samples taken at the beginning and end of each PED deployment period in ng/L (n=2).

Site	Dates	3,4-DCA	3,5-DCA	Atrazine	Bifenthrin	Butylate	Carbaryl	Carbofuran	Chlorpyrifos	Clomazome
Ranch South	6/11/09 – 7/01/09	nd / nd	nd / nd	nd / 2.0 [a]	15.7±1.9 / nd	x / nd	nd / 11.0±3.2	nd / nd	nd / nd	nd / nd
Ranch North	6/16/09 – 7/1/09	nd / nd	nd / nd	nd / 3.4 [a]	6.0±0.76 / nd	x / nd	nd / nd	nd / nd	nd / nd	nd / nd
Wastewater Outfall	8/04/09 – 8/17/09	nd /7.95±0.55	nd / nd	nd / 2.9 [a]	32.2±4.7 / nd	x / nd	nd /35.0±14.9	nd / nd	nd / nd	nd / nd
		Cycloate	Cyfluthrin	Cyhalothrin	Cypermethrin	DCPA	Deltamethrin	Diazinon	Dieldrin	EPTC
Ranch South	6/11/09 – 7/01/09	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	43.0±3.7 / x	nd / nd
Ranch North	6/16/09 – 7/1/09	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	49.4±7.4 / x	nd / nd
Wastewater Outfall	8/04/09 – 8/17/09	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / 9.3±4.8	30.9±3.9 / x	nd / nd
		Esfenvalerate	Ethalfluralin	Etofenprox	Fipronil	Hexazinone	Malathion	Methidathion	Methoprene	Methylparathion
Ranch South	6/11/09 – 7/01/09	nd / nd	nd / nd	nd / nd	5.9±1.04 / nd	x / nd	nd / nd	nd / nd	nd / nd	nd / nd
Ranch North	6/16/09 – 7/1/09	nd / nd	nd / nd	nd / nd	nd / nd	x / nd	nd / nd	nd / nd	nd / nd	nd / nd
Wastewater Outfall	8/04/09 – 8/17/09	nd / nd	nd / nd	nd / nd	7.9±3.7/9.0±1.4	x / 12.1 [a]	nd / nd	nd / nd	nd / nd	nd / nd
		Metolachlor	Molinate	Napropamide	Oxyfluorfen	*p p*' -DDD	*p p*' -DDE	*p p*' -DDT	PCA	PCNB
Ranch South	6/11/09 – 7/01/09	nd / nd	nd / nd	nd / nd	nd / nd	3.2 ±0.50 /nd	8.9±0.59/ nd	3.7±0.65 / nd	nd / nd	nd / nd
Ranch North	6/16/09 – 7/1/09	nd / nd	nd / nd	nd / nd	nd / nd	3.3±0.65 / nd	12.0±0.80/ nd	4.8±0.59 / nd	nd / nd	nd / nd
Wastewater Outfall	8/04/09 – 8/17/09	nd / 6.3±3.9	nd / nd	nd / nd	nd / nd	6.4 ±0.35/ nd	24.3±2.2/ nd	11.0±0.68 / nd	23.2±2.7 / nd	7.1±0.46 / nd
		Pebulate	Pendimethalin	Permethrin	Phenothrin	Phosmet	PBO	Prometryn	Propanil	Propyzamide
Ranch South	6/11/09 – 7/01/09	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd
Ranch North	6/16/09 – 7/1/09	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd
Wastewater Outfall	8/04/09 – 8/17/09	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd	nd / nd
		Remethrin	Simazine	Tau-fluvalinate	Tefluthrin	Tetramethrin	Thiobencarb	Trifluralin		
Ranch South	6/11/09 – 7/01/09	nd / nd	nd / 16.4±4.0	nd / nd	nd / nd	nd / nd	nd / nd	4.0±1.2 / nd		
Ranch North	6/16/09 – 7/1/09	nd / nd	nd / 14.4±4.2	nd / nd	nd / nd	nd / nd	nd / nd	2.5±0.42 / nd		
Wastewater Outfall	8/04/09 – 8/17/09	nd / nd	nd / 43.0±1.5	nd / nd	nd / nd	nd / nd	nd / nd	5.7±0.23 / 2.1±0.70		

a) Cannot calculate standard deviation, chemical was only detected in one replicate

Final Discussion

The objectives of this dissertation were threefold. The first objective was to establish *Menidia* species as a bioindicator for EDCs. The second objective was to measure EDC impacts in the wild at multiple biological scales and use correlations between scales to discern potential mechanisms of disruption. The third objective was to develop and utilize techniques in the laboratory that would further our understanding of EDC impacts in the environment. This work expands upon previous EDC research in several ways. To begin with, the field of aquatic endocrine disruption was in want of an estuarine study species that could be used as a surrogate for threatened fishes. *Menidia* species are highly sensitive (Clark et al., 1985), available commercially, and are ubiquitous throughout coastal North America (Middaugh & Hemmer, 1982). Additionally, the high-throughput assay developed for *Menidia* species allows for the measurement of endocrine responses in whole body homogenate, making the assessment of small juvenile fish possible. Such as assessment was carried out using juvenile *Menidia beryllina* exposed to environmentally relevant concentrations of a newer-use pyrethroid pesticides. Finally, this research expands upon previous efforts that primarily utilized exposures to known chemicals or conducted exposures in the lab with standard test species by evaluating the impact of environmental mixtures with measured levels of both estrogenic and androgenic endocrine activity in the field on several tiers of the biological hierarchy in a wild fish (*Menidia audens*). This was the first such study to be performed in the San Francisco Bay (SFB) estuary, the largest estuarine region on the west coast of North America.

Recently awareness of EDC prevalence in the SFB estuary has increased, with estrogenic activity documented in the watershed's rivers (Lavado et al., 2009) and agricultural drain water (Johnson et al., 1998). Discerning impacts on SFB fishes,

however, is challenging because the region's many highly impacted native species cannot be collected due to population decline (Feyrer et al., 2007). Selection of the introduced *Menidia audens* (Mississippi silverside) as a surrogate for EDC studies is appropriate as it is distributed through the entire estuary and shares life history traits such as habitat use, diet and lifespan with some endangered fishes (Moyle 2002). Silversides are well appointed as surrogates because they are easily collected in the field and have a small home range that allows toxicological responses to be linked to a specific site (Hoff 1972), and have established sensitivity to toxicants. For example, the sex ratio of *Menidia* spp. is susceptible to alteration by estrogen exposure during the larval period (Duffy et al., 2009). They are part of the Environmental Protection Agency's Toxicity Testing Program (US EPA 2002), and Atherinids as a phylogenetic group are sensitive in comparison to other species (Clark et al., 1985). Their potential as an EDC surrogate species extends to other regions, as they are ubiquitous in and easily caught from estuarine, brackish and freshwater habitats throughout coastal North America (Middaugh & Hemmer, 1982).

Clear links were observed between the biological scales examined for signs of endocrine disruption in *Menidia audens* in Suisun Marsh of the San Francisco Bay estuary. At the ranch site, where primarily estrogenic compounds were present, males had significantly higher and females trended towards higher expression of estrogen-responsive genes. At the site exposed to urban run-off and treated wastewater effluent, which is more polluted and contaminated by both estrogens and comparatively higher concentrations of androgens, both males and females had relatively low expression of estrogen-responsive genes. Furthermore, males had lower gonadal somatic index (GSI) and higher incidence of severe testicular necrosis, and the proportion of females caught throughout the spawning season was low compared to the ranch site and in comparison to observations of *Menidia* sex ratios in other populations. Additionally, it was found

that males at the urban site were significantly larger (in SL) than females. This finding runs counter to the reproductive strategy of most atherinid fishes, in which females are larger than males in order to maximize their capacity to carry oocytes. Males were larger despite having a slower growth rate than urban females, which may be due to genetically female larval silversides being masculinized early in the season, when colder temperatures should result in a female biased population. Future research will seek to confirm whether this is occurs experimentally, both in the laboratory and in the field, using genetic markers of sex determination.

Compounds that could be acting at EDCs as low concentrations leading to the above-described impacts include pyrethroid pesticides, particularly bifenthrin and permethrin. Pyrethroids are known to be present throughout the SFB estuary (Weston & Lydy, 2010). To our knowledge the dose-response of the endocrine effects of bifenthrin and permethrin at environmentally relevant concentrations had yet to be evaluated prior to our study. Additionally, this was the first study to use cell lines with natively expressed ER or AR to evaluate pyrethroid endocrine activity. The coalescence of our findings with those of others leads us to suggest that the parent compounds of permethrin and bifenthrin act as estrogen antagonists, and as estrogen agonists when metabolized (see Chapter 2). Although the lack of a clear linear concentration-response makes it difficult to assess the risk of permethrin and bifenthrin in the wild, the endocrine disruption caused by these compounds clearly exacerbates their toxicity in aquatic ecosystems. Furthermore, the ability of these compounds to exert effects on the endocrine system of fishes at concentrations regularly detected in watersheds is cause for great concern, especially when considering recent research that suggests changes in reproductive protein expression in fishes may precipitate population decline (Kidd et al., 2007) and that the pyrethroid mixtures that occur in aquatic habitats likely have additive endocrine toxicity.

The integration of approaches in the field and laboratory is challenging but necessary for accurate evaluation of EDC impacts. Our development of an ELISA that uses whole body homogenate allows the detection of Chg in small fish and in juveniles from which plasma is either difficult or impossible to obtain. This is particularly useful in light of the heightened sensitivity of juvenile fish to EDCs (Jin et al., 2010, Peters et al., 2009) and expands the universe of responses that may be evaluated in both the field and lab. Additionally, evaluating the expression of endocrine sensitive genes is an effective and increasingly common approach used to discern mechanisms of action of EDCs or EDC mixtures. Our development of qPCR primers and probes for a selection of endocrine-related genes *Menidia* species has led to successful funding for development of a *Menidia* microarray. The availability of high throughput assays for both protein and gene expression in *Menidia* will lead to better-informed risk assessment decisions and it will contribute to an enhanced understanding of inter-species differences in response to EDCs. The pairing of well-chosen high throughput assays with environmentally relevant surrogate species is key to discerning the ecological impact of endocrine disruption.

The development of a multi-tiered approach and high throughput assays for an alternative fish species that has the potential to be used as a surrogate for endangered species is of great importance, and the results of this study have lead to the funding of two additional multi-year projects focusing on endocrine disruption in *Menidia audens* in the SFB estuary. Mathematical modeling approaches are also being developed to predict the population trajectory of silversides exposed to EDCs, with an overall goal to extrapolate these predictions to endangered fish species (Brander and White, unpublished). Finally, since *Menidia* species are found on the East, West, and Gulf coasts of the United States (Middaugh & Hemmer, 1982), their utility could potentially be expanded to estuaries nationwide. Including more species representative of estuaries

will inform efforts to improve water conditions in these highly impacted regions that many fish utilize for at least a portion of their lives.

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Appendix A

Table A.1 **Maximum likelihood estimates for ethinylestradiol, bifenthrin, and permethrin best fit models for concentration-response**. Parameters are defined as follows: g is the maximum, a is the exponential intercept term, b is the exponential rate term (rate of decrease after peak), and f is the rate of increase. Coefficients, estimates, and SE are shown for the most parsimonious model for each compound as indicated by ΔAICc = 0.

treatment	coefficient	estimate	standard error	ΔAICc
ethinylestradiol	g	81.2884	84.9811	sigmoidal 0.0 unimodal 3.64
	a	4.1983	0.7636	
	b	-2.7442	1.9826	
bifenthrin	f	0.4692	0.0509	unimodal 0.0 sigmoidal 2.02
	g	8.33e03	2.18e07	
	a	7.4687	2.61e03	
	b	0.4834	0.4514	
permethrin	f	0.4724	0.0276	unimodal 0.0 sigmoidal 4.25
	g	1.11e04	1.94e07	
	a	8.1498	1.75e03	
	b	0.744	0.4028	

Appendix B

Table B.1 Pesticide type, CAS number, physical -chemical properties and GC-EIMS quantifier and qualifier ions for the pesticides measured.

	CASRN	Type	Solubility (mg/L)	log K_{ow}	Quantifier ion	Qualifier ion (s)
3,4-DCA	95-76-1	D	580	2.7	161	163
3,5-DCA	626-43-7	D	784	2.9	161	163
Alachlor	15972-60-8	H	130	2.9	188	160, 238
Allethrin	589-74-2	I	0.0001	5.0	123	136, 124
Atrazine	1912-24-9	H	28	2.8	200	215, 173
Bifenthrin	82657-04-3	I	0.1	7.3	181	166
Butylate	2008-41-5	H	40	4.2	146	174, 156
Carbaryl	63-25-2	I	32	2.4	144	115, 116
Carbofuran	1563-66-2	I	291	1.6	164	149
Chlorpyrifos	2921-88-2	I	0.3	5.1	314	197, 258
Clomazome	81777-89-1	H	1100	2.5	204	125, 240
Cycloate	1134-23-2	H	75	4.1	154	155
Cyfluthrin	68359-37-5	I	0.0012	5.6	163	127, 199
Cyhalothrin	68085-85-8	I	0.005	6.8	181	197, 161
Cypermethrin	52315-07-8	I	0.004	6.6	163	127, 181
DCPA	1861-32-1	H	0.5	3.9	301	299, 332
Deltamethrin	52918-63-5	I	0.2	4.6	253	172, 181
Diazinon	333-41-5	I	38	3.8	179	199, 304
EPTC	759-94-4	H	344	3.2	128	132
Esfenvalerate	66230-04-4	I	0.002	6.2	225	125, 167
Ethalfluralin	55283-68-6	H	0.2	5.1	276	316, 292
Etofenprox	80844-07-1	I	0.0225	6.9	163	164
Fenpropathrin	39515-41-8	I	14.1	6.2	181	265, 125
Fipronil	120068-37-3	I	1.9	4.0	367	369, 351
Fipronil desulfinyl	205650-65-3	D	NA	NA	388	333, 390
Fipronil sulfide	120067-83-6	D	0.54	NA	351	353, 255
Fipronil sulfone	120068-36-2	D	NA	NA	383	385, 255
Hexazinone	51235-04-2	H	33,000	3.8	171	128
Malathion	121-75-5	I	130	3.5	123	173, 158
Methidathion	950-37-8	I	220	2.2	145	125
Methoprene	40596-69-8	I	1.4	5.2	111	107, 191
Methylparathion	298-00-0	I	55	3.0	263	109, 246
Metolachlor	51218-45-2	H	530	3.1	162	238, 240
Molinate	2212-67-1	H	800	3.2	126	98
Napropamide	15299-99-7	H	73	3.4	100	115, 128
Oxyfluorfen	42874-03-3	H	0.1	3.8	252	300, 317
p,p'-DDD	72-54-8	D	0.005	5.1	235	237, 165
p,p'-DDE	72-55-9	D	0.0014	5.7	318	246, 316
p,p'-DDT	50-29-3	I	0.001	6.9	235	237, 165
Pebulate	1114-71-2	H	60	3.8	128	132
Pendimethalin	40487-42-1	H	0.275	4.6	252	191, 162
Pentachloroanisole (PCA)	1825-21-4	D	<1	5.5	265	280, 267
Pentachloronitrobenzene (PCNB)	82-68-8	F	0.44	4.64	295	293, 265
Permethrin	52645-53-1	I	0.006	6.1	183	165, 127
Phenothrin	26002-80-2	I	0.0097	6.1	123	183
Phosmet	732-11-6	I	25	2.8	160	133

Piperonyl butoxide	51-03-6	S	14.3	4.8	176	177
Prometryn	7287-19-6	H	33	3.5	241	184, 226
Propanil	709-98-8	H	229	2.3	161	163, 317
Remethrin	10453-86-8	I	37.9	5.4	123	128, 171
Simazine	122-34-9	H	2.0	2.2	201	186, 188
Tefluthrin	79538-32-2	I	0.016	6.4	177	197, 161
Tetramethrin	7696-12-0	I	1.83	4.6	164	123, 165
t-Fluvalinate	102851-06-9	I	0.001	4.6	250	252
Thiobencarb	28249-77-6	H	28	3.4	100	125, 257
Trifluralin	1582-09-8	H	0.3	5.3	306	264
D, degradate; H, herbicide; I, insecticide						

Table B.2 Method detection limits and instrument limits of detection for pesticides in surface water and polyethylene devices.

	Surface Water		**PEDs**
	MDL (ng/L)	**LOD (ng/L)**	**LOD (ng/PED)**
3,4-DCA	8.3	1.0	10
3,5-DCA	7.6	1.0	10
Alachlor	1.7	1.0	5
Allethrin	6.0	1.0	5
Atrazine	2.3	0.5	5
Bifenthrin	4.7	0.5	5
Butylate	1.8	1.0	5
Carbaryl	6.5	1.0	5
Carbofuran	3.1	1.0	5
Chlorpyrifos	2.1	0.5	5
Clomazome	2.5	1.0	5
Cycloate	1.1	1.0	5
Cyfluthrin	5.2	1.0	5
Cyhalothrin	2.0	0.5	5
Cypermethrin	5.6	1.0	5
DCPA	2.0	0.5	5
Deltamethrin	3.5	1.0	5
Diazinon	0.9	1.0	5
EPTC	1.5	0.5	5
Esfenvalerate	3.9	0.5	5
Ethalfluralin	3.0	0.5	5
Etofenprox	2.2	1.0	5
Fenpropathrin	4.1	1.0	5
Fipronil	2.9	0.5	5
Fipronil desulfinyl	1.6	0.5	5
Fipronil sulfide	1.8	0.5	5
Fipronil sulfone	3.5	0.5	5
Hexazinone	8.4	1.0	10
Malathion	3.7	1.0	5
Methidathion	7.2	1.0	5
Methoprene	6.4	1.0	5
Methylparathion	3.4	1.0	5
Metolachlor	1.5	1.0	5

Molinate	3.2	1.0	5
Napropamide	8.2	1.0	5
Oxyfluorfen	3.1	1.0	5
p,p'-DDD	4.1	0.5	5
p,p'-DDE	3.6	0.5	5
p,p'-DDT	4.0	0.5	5
Pebulate	2.3	0.5	5
Pendimethalin	2.3	0.5	5
Pentachloroanisole (PCA)	4.7	0.5	5
Pentachloronitrobenzene (PCNB)	3.1	0.5	5
Permethrin	3.4	0.5	5
Phenothrin	5.1	1.0	5
Phosmet	4.4	1.0	5
Piperonyl butoxide	2.3	1.0	5
Prometryn	1.8	1.0	5
Propanil	10.1	1.0	10
Remethrin	5.7	1.0	5
Simazine	5.0	1.0	5
Tefluthrin	4.2	1.0	5
Tetramethrin	2.9	1.0	5
t-Fluvalinate	5.3	1.0	5
Thiobencarb	1.9	0.5	5
Trifluralin	2.1	0.5	5