CALFED BAY-DELTA PROGRAM

CALFED SCIENCE FELLOWS PROGRAM

Sea Grant California

In cooperation with the

California Sea Grant College Program

FELLOWSHIP APPLICATION COVER PAGE

APPLICANT TYPE ☐ Postdoctoral Researcher ☒ Ph.D. Graduate Student

PROJECT NUMBER not assigned

PROJECT TITLE Endocrine Disruption in the Delta: Confirming Sites of Known Estrogenicity with Outplants, Histology, and Choriogenin Level Measurements.

FINANCIAL SUMMARY

First Year CALFED Funds Requested:	$43,104
Total CALFED Funds Requested:	$129,124
Duration:	3 years
Proposed Start/Completion Dates:	Summer 2007 - Summer 2010

APPROVAL SIGNATURES

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Will animal subjects be used? ☒ Yes ☐ No

APPROVAL DATE: PROTOCOL #: PENDING: X

Does this application involve any recombinant DNA technology or research? ☐ Yes ☒ No

2) Proposed Research

Introduction:

Endocrine disrupting compounds (EDCs) are chemicals (most often anthropogenic) that can disrupt endocrine function by mimicking or enhancing the effects of endogenous hormones (estrogen, progesterone, testosterone), altering hormonal mechanisms and metabolism, and modifying hormone receptor levels (Oberdorster and Cheek 2000). EDCs originate from a variety of sources, such as agricultural runoff (e.g., pesticides), oil refineries (e.g., polyaromatic hydrocarbons), municipal sewage treatment outfalls (e.g., ethinylestradiol), and contaminants associated with urban and residential activities (e.g., phthalates) (Atkinson 2003; Pait and Nelson 2002).

These chemicals are potentially devastating to the reproductive systems of fishes. Following EDC exposure, male fish often produce female reproductive proteins, such as vitellogenin (yolk protein) and choriogenin (egg envelope protein), and may also develop malformed or tumorous testes (Anderson et al. 2006). In females, EDC exposure can depress egg production and cause gonadal lesions and tumors(Oberdorster and Cheek 2000; Pait and Nelson 2002). Effects may extend beyond individual fish to the entire population; for example, altered hormone levels can modify mating behavior and skew population sex ratios (Brian et al. 2006). EDCs are widespread in the environment (Pait and Nelson 2002), and are even found in sites considered to be relatively pristine, such as Carpinteria Salt Marsh (Anderson et al. 2006), a California Natural Reserve System site near Santa Barbara. As the sublethal effects of endocrine disruption may be subtle and can occur at the cellular receptor level (Thomas and Doughty 2004; Thomas et al. 2006), their influence can easily be overlooked.

Such compounds may be responsible for the relatively recent but significant decline in aquatic species in the upper San Francisco estuary (Sacramento San-Joaquin Delta), known collectively as the “Pelagic Organism Decline” (POD). Maintaining the ecological health of areas in the Sacramento-San Joaquin Delta that are key to the stability of endangered and threatened endemic species, such as salmonids and the Delta smelt, is imperative. Only through monitoring for such effects via the detection of changes in or abnormal expression of female-specific gene products, for example, can impacts in reproductive capabilities be detected, followed by specific management practices to reduce endocrine disrupting contaminants from entering waterways.

In salmonids, deleterious effects of endocrine disrupting compounds (17β-estradiol and nonylphenol) on the parr-smolt transformation have been observed (McCormick et al. 2005). In addition, Delta smelt, for example, may be particularly susceptible to the sublethal effects of endocrine disrupting compounds due to their life history characteristics (Bennett 2005; Moyle et al. 1992). Being a species of uniquely low fecundity (Moyle et al. 1992), a further reduction in reproductive capability due to the effect of estrogenic compounds on males could potentially devastate this fragile population. By monitoring for EDCs in areas used for spawning by species such as the

Delta smelt and various threatened salmonid species (such as the Chinook Salmon), the capacity for EDCs to influence population decline can be elucidated.

Preliminary data from the CalFed Science Program project "Identifying Causes of Feminization of Chinook Salmon in the Sacramento and San Joaquin River System" (Project SCI-05-C111; D. Sedlack, P.I.) indicates that significant estrogenic activity can be detected in water samples from a number of locations in the SSJ watershed (Figure 1). Preliminary sampling of Threadfin Shad, Splittail, Steelhead, and Delta Smelt plasma as part of this project indicates that choriogenin (egg envelope or zona radiata protein) antibodies recognize proteins in males of these species (Spies and Cherr, preliminary data). Furthermore, some of these fish were not reproductive, suggesting exposure to chemicals that prematurely induced choriogenin expression. The proposed study represents an extension of the above CalFed-funded project (currently in progress). I will coordinate my research with their sampling and use sites where Sedlack et al. have confirmed the presence of EDCs. My work will utilize POD species as well as a ubiquitous (Bennett, 2005) resident indicator fish species (*Menidia beryllina*), which will be used as an indicator of exposure to reproductive contaminants. *Menidia beryllina*, more commonly known as the inland silverside, has a well-known reproductive life history (Middaugh and Hemmer 1981; Middaugh and Lempesis 1976), can be easily collected and tolerates a wide range of salinities (Bennett 2005). Because *M. beryllina* has a relatively small homerange (Hoff 1972), EDCs detected in wild fish can be linked to exposure at the site from which they are collected. Unlike the rainbow trout (*Onchorynchus mykiss*) used to confirm estrogenicity in Sedlack and colleagues' CalFed study, inland silversides exist in all areas of the Delta and are therefore representative of the response of both freshwater and saltwater species. By comparing the responses of threatened fishes such as salmonid species and the Delta smelt with a ubiquitous and well-studied sentinel species, we can most effectively identify the specific chemicals that are potentially at fault for their precipitous decline throughout the watershed.

Questions/Objectives:

1. Is a specific physiological response ("biomarker") indicative of exposure to endocrine disrupting compounds (EDCs) found at sites with confirmed estrogenicity in the Sacramento-San Joaquin Delta (refer to Figure 1)?
2. Do chemicals collected in the field with passive samplers produce effects in laboratory animals that match those seen in the field? Do isolated EDCs collected via passive samplers from Dr. Sedlack's study (SPMDs) and our passive samplers (POCIS) produce the same or similar results? What EDCs in particular are responsible for these effects?
3. What are the effects (i.e. choriogenin induction or gonadal malformations) of environmentally relevant potencies and potential interactive effects of EDC combinations in laboratory testing?
4. Do sites with confirmed occurrences of endocrine disruption in *M. beryllina* have a wild fish with similar manifestations? How do our results compare with those of Sedlack and colleagues?

Hypotheses:

1. EDCs will be detected at the majority of sites with confirmed estrogenicity (detected levels of estrogen - confirmed by Sedlack et al.), and they will elicit choriogenin production, gonadal lesions/tumors, and/or ovo-testes (Figure 2) in male and juvenile inland silversides.
2. Laboratory results using EDC extracts will agree with field results and extracts isolated from sites found to cause endocrine disruption in vitro in rainbow trout (*Onchorynchus mykiss*) and hepatocyte cultures in the study led by Dr. Sedlack and Dr. Schlenk, will cause a similar effect when injected into *M. beryllina*.
3. Environmentally relevant EDC concentrations will elicit an effect. Synergistic, antagonistic and additive interactive effects will also be observed.
4. Wild male and juvenile fishes (Delta smelt, salmonids, and other endemic Delta species) will also exhibit choriogenin induction and/or gonadal malformations at sites where endocrine disruption is observed with *M. beryllina*.

Approach/Plan of Work:

I plan to evaluate the response of the fish *Menidia beryllina* (the inland silverside), at sites important to threatened and endemic species in the Sacramento San-Joaquin Delta. First I will determine the spatial distribution of EDC effects within silverside populations at each site. Second, I will use a transplantation experiment to compare EDC effects between sites and to laboratory controls. A number of sites with confirmed estrogenic activity from the study mentioned above that range from the Upper Sacramento river southward to the Merced River (Figure 1.) will be used. The proposed study sites include areas exposed to a variety of potential EDC sources, including pesticides and urban runoff.

The inland silverside, an atherinid introduced to California marshes approximately 35 years ago {Bennett, 2005 #214}, is commonly used in the laboratory and field as a study species for physiological and toxicological studies (Fuller et al. 2004; Middaugh et al. 1996; Ward et al. 2006). Its habitat and diet engender contact with sediment-bound and soluble contaminants: although it dwells in the water column, it preys upon benthic organisms in addition to pelagic copepods (Bennett 2005). Male silversides can be identified externally (Huber and Bengtson 1999), and the reproductive life history of silversides is well-known (Middaugh and Hemmer 1981). Although some EDCs can be measured using water and sediment chemistry alone, the integration of contaminants in water, sediment, and food sources and the effect of their metabolites can only be assessed using an aquatic organism such as the inland silverside. Inland silversides have been maintained successfully in field caging experiments at or near sites proposed for use in this study (Bennett 2005).

Experimental Design

Stage 1: Initial assessment of EDC levels. Wild fish (males and immature juveniles only) will be collected from 2-3 areas at each site (Figure 1) in early spring and blood samples

will be taken for choriogenin analysis. Adult male and immature silversides will be collected from all sites (fish can be sexed visually (Chizinkski et al. 2007; Huber and Bengtson 1999). Sites in which fish exhibit elevated choriogenin will be used for outplanting in Stage 3. Because silversides are generally site-attached (remain in a roughly 100x100 m region (Hoff 1972), elevated choriogenin measurements can be linked to the site of capture. May want to give a little collection detail: beaches? Net type? Blood collection in lab?, etc.

Stage 2: Choriogenin analysis. Plasma collected either via heart puncture or caudal vein (50-100 μL) will be centrifuged for analysis (Anderson et al. 2006). A Bio-Rad protein assay (Bradford method) will be performed to determine the plasma protein concentration, and choriogenin levels will be analyzed via Western blotting or ELISA. For the Western blots, samples (10 μg protein/lane) will be subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using 10% gels (160 volts for one hour), followed by semi-dry transfer to nitrocellulose membranes (10 volts for one hour). After blocking the membranes with 3% non-fat milk in PBS, they will be probed with primary antibody (anti-ZRP - zona radiata protein IgG). The membranes will then be probed with secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase and visualized using epi-chemiluminescence). Scanning and quantification will be performed on a UVP Epi Chemi Darkroom. An ELISA in 96 well microtiter plates will be performed with plasma samples (10 μg total protein) incubated at 4 ^{o}C for 15 hours. The plates will be washed twice with PBS, and then blocked with PBS-T (0.05% Tween in PBS) containing 1% BSA for 1 hour at room temperature. The plates will then be incubated with primary antibody for 2 hours at room temperature, washed 4 times in PBS-T, and then incubated with secondary antibody for one hour at room temperature. The plates will be washed again (six times) in PBS-T and an enzyme reaction will be developed using *o*-phenylenediamine dihydrochloride and 0.012% hydrogen peroxide in 0.05 phosphate-citrate buffer. The reaction will be stopped by adding 2M HCl, and the absorbance read at 490 nM on a microplate reader. All samples will be quantified by comparison to the plasma of a known female silverside as the standard control. This is a well-established assay in the Cherr laboratory (Figure 3).

Stage 3: Outplants and passive contaminant collection. In the late spring, male young-of-the-year silversides will be collected from a population in an unimpacted area of the Delta (in collaboration with Dr. David Sedlack and Dr. Bill Bennett) and relocated to the Bodega Marine Laboratory (BML), where they will be kept in aquaria for several weeks while baseline (i.e., normal) plasma choriogenin levels are analyzed and depuration/disinfection (i.e., removal of parasites, etc.) occurs. Fish will then be transported to each site and placed in Vexar™ mesh cages, which prior experience has shown to have a negligible chance of escape (A. Brooks, pers. comm.). Caged *Menidia beryllina* will only be placed at sites in which the species has already been introduced and established. The number of fish per cage will be determined by conducting a power analysis prior to beginning this experiment. Choriogenin samples will be taken from caged fish every two weeks for 3 months and measured as detailed above in Stage 2. Following the field experiment, fish will be returned to BML for histological analyses of gonads (formalin fixation and paraffin embedding for sectioning via microtome

(Anderson et al. 2006)). To confirm that abnormalities observed in caged fish are a product of EDC exposure, control fish will be held in aquaria at BML during the field experiment. Control fish will be subject to the same regime of choriogenin and gonad sampling / histology as caged fish. During the outplanting experiment passive samplers (Polar Organic Chemical Integrative Samplers - POCIS) will be deployed at the site of each cage to collect contaminants (Stuer-Lauridsen 2005).

Stage 4: Chemical extract verification. Extracts collected from POCIS samplers (EST Laboratories) used in our study (for polar organic contaminants) and C18 filter extracts from Dr. Sedlack's study will be injected into naïve fish (*M. beryllina*) in the laboratory and an endocrine disruption response (choriogenins and ovotestes in males) will be verified and compared to the response of the rainbow trout (*Onchorynchus mykiss*) used in Dr. Sedlack's study. POCIS samplers provide time-integrated average concentrations of organic chemicals, and can be deployed for a period of several months (http://www.cerc.usgs.gov/pubs/center/pdfDocs/POCIS.pdf).

Selected chemical analyses of the active extracts will be conducted in collaboration with Dr. Sedlack at UC Berkeley, an experienced chemist and the lead P.I. on the CalFed funded project "Identifying Causes of Feminization of Chinook Salmon in the Sacramento and San Joaquin River System". The Supercritical Fluid Extraction (SFE) method (using CO_2) will be used to produce a fractionated, high purity, low volume extract in the desired solvent for the subsequent bioassays and in vivo confirmation of potency and effect. The solvent strength of supercritical CO_2 can be adjusted by making small changes to pressure, temperature, and/or the amount or type of organic modifier added. The sensitivity of SFE allows the extraction of compounds with a wide range of chemical properties, including estrogenic compounds such as nonylphenol (Lee et al. 1997) and ethinyl estradiol (Lee et al. 2004), that are free of natural organic matter and other interference. Fractions for use in bioassays can be produced by collecting the supercritical fluid extract offline on a solid phase trap (with activated carbon or C18) and then separating the analytes via chromatography during elution with a non-toxic solvent such as ethanol or water (Eskilsson et al. 2001; van Bavel et al. 1996; Wolfe et al. 1995). An automatic fraction collector will be used. Extracts will then also be analyzed via LC-MS or GC-MS, in order to determine the presence of any unknown compounds. Depending on chemical analyses, specific EDCs present in the extracts will be isolated in order to determine individual potency. Depending on the outcome of this work, I will also perform a laboratory investigation of the additive or interactive effects of EDCs that occur together at the same site.

Stage 5: As suggested in a recent review, it is important that the effects of environmentally relevant potencies and potential interactive effects of EDC combinations not be overlooked (Eggen et al. 2004). I will conduct laboratory tests using environmentally relevant concentrations and exposures (exposure through water rather than injection) to confirm the toxicity observed in the tests above. I will also look at the effect of EDC mixtures, particularly combination(s) of estrogenic and anti-estrogenic or androgenic compounds isolated from passive sampler extracts. By injecting fish with two EDCs simultaneously one can gauge how chemicals with varied

properties become either synergistic, additive, or antagonistic when acting in concert with one another in vivo. Injections will be performed as described in Stage 4, and the same endpoints (ovo-testes and choriogenin expression) will be measured. The extent of this portion of the study will depend on the identity of the chemicals extracted. Include standard response to E2 and plan to express choriogenin response to extracts as E2 equivalents.

Stage 6: In cooperation with Bob Spies of Applied Marine Systems (a colleague of Dr. Cherr's), Dr. Sedlack's group, the California Dept. of Fish and Game, and NOAA, wild fish will be collected from sites shown in Figure 1 and from the screens at the Tracy and Byron pumping facilities (Figure 4), as well as from IEP and CDFG collecting trips. As outlined in the previous stages, these fish will be examined for choriogenin expression and gonadal malformation. Expectations are that it will be possible to collect a diversity of fishes, many of which will represent threatened species (i.e. Delta smelt, salmonids, threadfin shad).

Timeline

Year 1

a) Field experiments
   - initial assessment (choriogenin measurements from wild fish): SUMMER 2007
   - caging experiment: SUMMER/FALL 2007
b) Choriogenin measurement & Histology: FALL 2007
c) Chemical extract measurement and validation (bioassays): WINTER 2007
d) Data analysis: WINTER / SPRING 2008

Year 2

a) Repeat caging experiment: SPRING/SUMMER 2008
b) Choriogenin measurement & Histology: FALL 2008
c) Chemical extract measurement and validation: WINTER 2008
d) Data analysis: WINTER / SPRING 2009

Year 3

a) Laboratory experiments using EDC mixtures: SPRING 2009
b) Assays of wild fish from pumping stations and other sites: SUMMER 2009
c) Choriogenin & Histology on wild fish: FALL 2009
d) Data analysis: WINTER 2009 / Spring 2010

Data Management

I will compare endpoints (choriogenin levels, ovo-testes, etc.) at field sites and laboratory controls using one-way ANOVAs, with site status as a fixed effect and location as a random effect. This analysis will determine whether EDC effects are present at protected and restored sites (relative to controls) and whether these effects

differ between the two types of site. In the lab-based studies, I will generate dose-response relationship curves for each endpoint using extracts (SPMD, POCIS) from each site. I will then compare these curves using General Linear Models (GLMs) to determine whether extract potency differs amongst sites, different EDC types and / or different species (Stage 6).

Output and Anticipated Products / Benefits:

While endpoints such as the LC50 (lethal concentration for half of the population) may give ecologists a starting point in their understanding of toxic effects, they do little to explain the collective effect of sub-lethal, yet persistent, concentrations on an ecosystem. Nonetheless, the majority of water-quality-monitoring work is concerned only with the mortality of individual organisms, which neglects to address the effect of contaminants in an environmentally relevant and realistic setting. The research I am proposing will determine whether it is necessary to regularly consider sublethal endpoints related to reproductive health in water-quality assessments, beginning with an assessment of ecosystems vital to the survival of threatened and endemic Delta species, such as the Delta smelt. Only by quantifying the health of these organisms will CalFed have the ability to properly implement and plan future restoration projects, select areas to be protected, and effectively maintain those that already are.

This project will directly benefit one of the key components of CalFed's mission, that being to improve ecosystem quality. A thorough assessment of endocrine disruption in areas of the Delta with declining populations of key species will contribute to improving and increasing aquatic and terrestrial habitats and improving ecological functions in the Bay-Delta system to support sustainable populations of diverse and valuable plant and animal species (http://science.calwater.ca.gov/pdf/psp/PSP_2006_intro_063006.pdf). These ecosystems cannot be improved upon, restored, or expanded with a successful outcome until existing problems are elucidated and eliminated. Also, by identifying the chemicals that are contributing to population decline, we are adding emphasis to the importance of the improvement of existing water treatment techniques and the development of new technology in the future.

This project will benefit CalFed and my community mentor, Bruce MacFarlane of NOAA, by extending and confirming the results of work already being done through the CalFed funded project : "Identifying Causes of Feminization of Chinook Salmon in the Sacramento and San Joaquin River System", and ultimately contributing to elucidating the cause of pelagic organism decline. The scientific significance of the results from this study will be greatly strengthened by confirming the effects observed with another species, which I will accomplish through the use of *M. beryllina*. The research of Dr. MacFarlane, an expert on salmonids with a background in toxicology, will benefit immensely from the information gathered during this research project, as NOAA is also greatly concerned with the decline of fish in the Delta. Furthermore, Dr. MacFarlane is also peripherally involved in the CalFed project already underway, which will allow for greater coordination between Dr. Sedlack's project and this one. Under Dr. MacFarlane's mentorship, I will be able to further the goals of NOAA, and potentially

assist with developing new testing strategies that will allow NOAA and its sister agencies (EPA, CDF&G, etc.) to better understand population decline with new tools. Also, as *Menidia beryllina* is already an approved EPA test species, instituting a new model for the monitoring of endocrine disrupting compounds in the future through government agencies such as NOAA and the EPA, for example, may be less complex. Finally, as he is a an adjunct professor at the University of Santa Cruz, I will benefit by having access to the many resources in Marine Ecology and Biology available there.

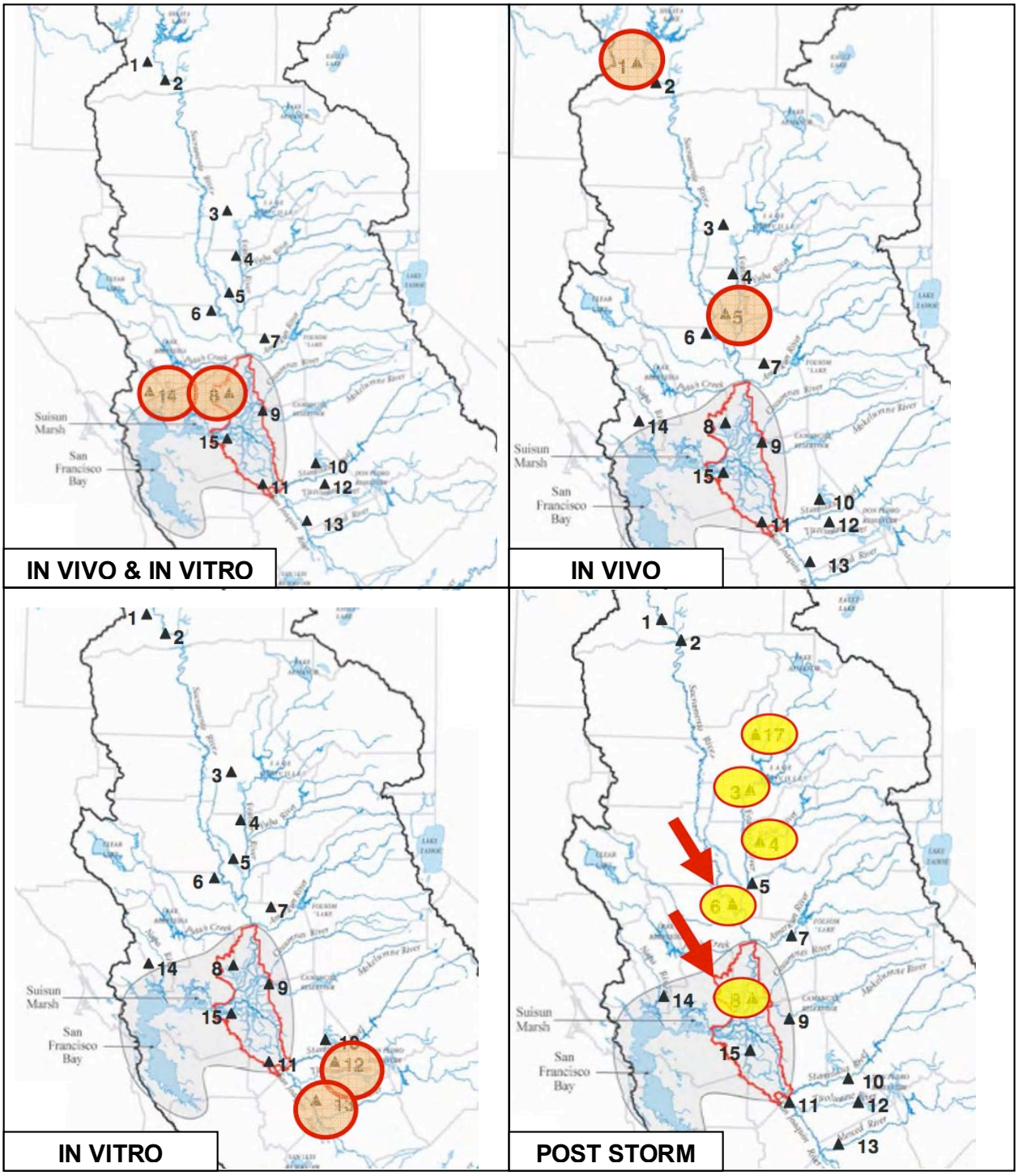
My major professor and research mentor, Dr. Gary Cherr, also the chair of the designated emphasis in reproductive biology, will benefit by having the opportunity to commit to a project that plays to his strengths in both reproductive biology and marine toxicology. His role in the PEEIR program (Pacific Estuarine Ecosystem Indicator Research Consortium) over the past several years has established himself as a leader in EDC-related research (www-bml.ucdavis.edu/**peeir**/index.htm). He will benefit by being able to use the skills and techniques developed under PEEIR on a larger scale through collaborating with Dr. Sedlack at UC Berkeley and Dr. MacFarlane at NOAA during this project.

The benefits I as a fellow would gain during this project are numerous. Amongst my community mentor, major professor, and fellow collaborators on the current CalFed project discussed above, I will have concurrent access to an experienced aquatic ecologist at NOAA with strong connections to other government agencies and to UC Santa Cruz, will work daily with one of the leading reproductive biologists and environmental toxicology professors at UC Davis, and will be able to consult with Ph.D.'s who have expertise in chemistry, environmental engineering, and environmental science at UC Berkeley (Dr. Sedlack) and UC Riverside (Dr. Schlenk). Most importantly, this fellowship would fully support the majority of my dissertation research.

Anticipated Per Year Outcomes

After year 1, the sites that elicit responses characteristic of endocrine disruption will be identified, and confirmatory chemistry and laboratory bioassays will be underway. I anticipate that by the end of this first year we will be able to hone in on a suite of compound(s) responsible for eliciting reproductive toxicity in *M. beryllina*. Repeating these experiments in the second year will confirm the initial results and simultaneously strengthen the findings of Dr. Sedlack's CalFed project. The third year will result in elucidation of the effects of EDC mixtures and also confirm reproductive toxicity in wild fish. Ultimately this project will allow future EDC research in the Delta to be more narrowly focused, as the issues that are of highest importance will be brought to light.

List of Figures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POST STORM
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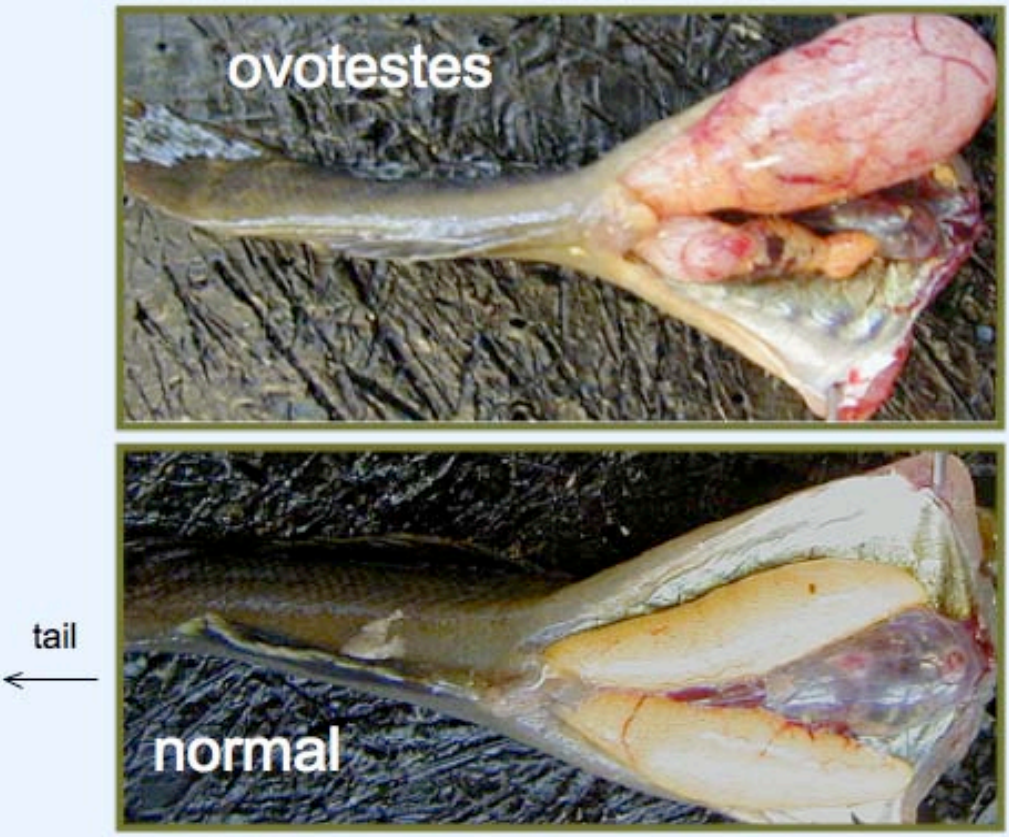

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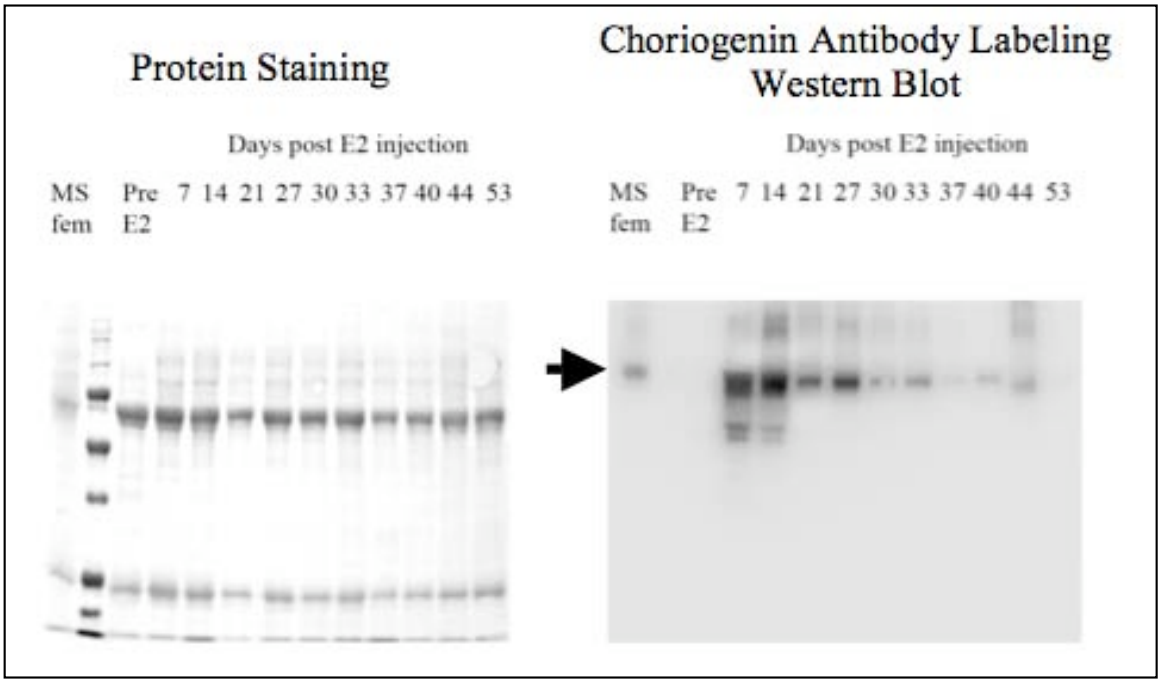
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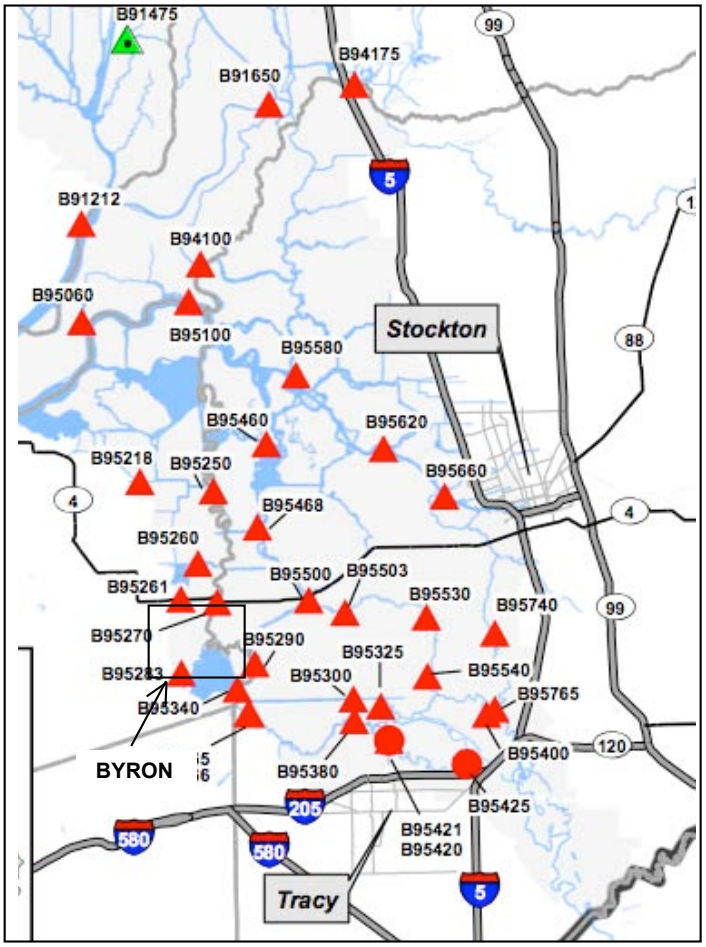

Surface Water Stations Monitored by the California Department of Water Resources

Legend:

- Tide Station (NAVD88)
- Tide Station (NGVD29)
- Stage Station (NAVD88)
- Stage Station (NGVD29)
- Delta Islands
- County Line

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