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developmental, neurological, and muscular function can therefore be linked to behavioral performance. Our methodology and approach has been carefully designed to link genomic responses with those observed at higher levels of biological organization, thus allowing for adverse outcome pathways to be synchronously investigated.

### **3. Approach and Scope of Work (Tasks and Objectives)**

Of critical importance to our understanding of the vulnerability of organisms to environmental stress is a comprehensive understanding of the physiological "weak links" that underlie organismal tolerance. Environmental stressors elicit complex physiological responses and it is often unclear which molecular pathway(s) are the organism's critical point(s) of failure. With continuous concerns surrounding pelagic organism declines, anthropogenic impacts to the Bay-Delta, and a forecast of continual climate change over the coming decades, there is an urgency to address these questions in the most efficient manner. In the post-genomic era, we have access to a number of sensitive genomics-enabled techniques that provide us with the capability of investigating the molecular response of many cellular pathways simultaneously. Gene regulation, the process of altering mRNA transcripts for the required synthesis of proteins, is one of the most rapid and versatile ways in which an organism can respond to an environmental stressor. Since the ability of an organism to adjust to a changing environment will be driven by complex changes in gene regulatory networks and subtle changes in numerous cellular pathways, the use of genomics tools will be particularly useful and efficient in elucidating the cellular-level responses to temperature and salinity challenges facing delta smelt. Suites of differentially regulated genes can provide a physiological signature of organismal condition as well as uncover the molecular mechanisms underlying physiological sensitivity, thus gene expression studies stand to be an excellent approach for understanding the broad and integrated responses of aquatic organisms facing environmental stressors (Hofmann and Todgham, 2010; O'Donnell et al., 2010; Todgham et al., 2007; Todgham and Hofmann, 2009). In turn, these types of data can be used to identify physiological thresholds of present day populations and their potential to tolerate future climate change scenarios.

Tasks: To achieve our objectives, the proposal has been divided into 4 tasks.

*Task 1* relates to the project administration and management, including training and supervision. It will integrate communication of progress. Bi-weekly meetings will be conducted with immediate students and personnel, quarterly meetings will be scheduled with co-investigators to discuss progress in each of the tasks. The preparation of progress reports, presentations at Bay-Delta Science Conferences and other relevant events, along scientific papers for publication, will be coordinated at these meetings. As such this task spans the duration of the proposed research; years 1-3. Drs. Connon and Fangue will contribute 18% and 10% respectively and be responsible for the project oversight. Dr Lindberg will assist 5% time to assist with experimental design and physiological performance tests, as well as preparation of manuscripts for publication.

*Task 2* involves the development of an oligonucleotide microarray for delta smelt, further enhancing existing tools developed by Dr. Connon and co-workers. Dr. Connon will be the principal person responsible for overseeing the microarray development, with validation tests and protocol establishment jointly conducted with Dr. Fangue and the graduate student. Development will take place during the first year of the project and is expected to take between 8

and 12 months, including time for training, and aforementioned validation and protocol establishments.

*Task 3* entails conducting temperature and salinity tolerance tests on larval, juvenile and adult delta smelt. We will first acclimate fish to selected physicochemical ranges based on previous research and then conduct a series of tolerance, preference and behavioral tests. These tests will be conducted spanning the three year proposal. Specific timing of each test will depend on availability of delta smelt life-stages. Due to the number of required tests, and the temporal constraints in delta smelt availability, this task is scheduled to span the duration of the project; years 1-3. All staff and students will be involved in designing and/or conducting exposure tests.

*Task 4* involves the use of the microarray developed in task 2, along with standard quantitative PCR verification, to assess patterns of gene expression in delta smelt exposed to the tested physicochemical variables in Task 3. Genomic assessments are planned for project years two and three. The graduate student will primarily be responsible for conducting the genomic research as part of the PhD thesis, will be supervised and coordinated by Drs. Connon and Fangue, and will be assisted by an undergraduate student in conducting RNA extractions and cDNA syntheses.

**Objectives:** Our overall approach and scope of work interlinks these tasks into specific objectives, in order to address our research questions. Firstly, we will use 454-sequencing technology to generate genome-wide fully annotated sequence data required to construct an oligonucleotide microarray for the delta smelt (*Objective 1*), building on and enhancing research conducted by Dr. Connon and co-workers at the UC Davis Aquatic Toxicology Laboratory. We will then quantify the physiological tolerance limits and behavioral responses of delta smelt to salinity and thermal challenges (*Objective 2*). Upon establishing physiological tolerances we will use the next-generation delta smelt microarray to assess the impacts of temperature and salinity variations on larval, juvenile and adult delta smelt (*Objective 3*.) Finally we will integrate and mechanistically contrast genomic data with adverse outcomes at higher levels of biological organization, establishing sensitivity thresholds for delta smelt with regards to temperature and salinity variations in their habitat (*Objective 4*).

***Objective 1:*** Enhance and advance delta smelt-specific molecular tools developed over the past three years, by providing a more extensive genomic coverage (*Task 2*).

Dr Connon and co-workers developed a delta smelt microarray using a traditional cDNA library, containing 8,448 gene fragments (Connon et al., 2009), representing approximately 2000 unique genes. This technology has been successfully applied to investigate the effects of contaminants of concern in Delta waters on larval delta smelt; described above. Data obtained using these cDNA microarrays, were suitable for these studies, but limited in terms of genome response coverage. One of the goals of this proposal is to further develop the genomic tools available in delta smelt to allow for a more thorough assessment of the physiological capacity of these fish. We will sequence the transcriptome, or the suite of expressed mRNA transcripts, of delta smelt exposed to a number of stressors and environmental conditions. The gene sequences generated will then be annotated (i.e. protein name and function assigned) using computer software and available sequence for other species (Center for Genomics and Bioinformatics, Indiana University). From this well-annotated transcriptome sequence, a custom-designed oligonucleotide microarray will be built as a tool for research investigating the physiological mechanisms underlying the sensitivity of delta smelt to a variety of environmental challenges. It

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will immediately result in a publication in a genomics or bioinformatics journal but perhaps more notably will provide a state of the art platform for environmental genomics-based research in delta smelt.

**Method:** In order to capture a diversity of mRNA transcripts, we will utilize archived RNA from previous delta smelt studies conducted in our lab (Connon et al., 2009; Markiewicz et al., 2010; Reece et al., 2009; Werner et al., 2008) that have been exposed to a variety of environmental stressors, including ammonia, copper, select pesticides and variations in salinity and temperature. This approach will increase our ability to capture sequence information for the expression of rare transcripts. Total RNA will be pooled into a single sample and shipped on dry ice to the Center for Genomics and Bioinformatics (CGB) facility at Indiana University, where it will undergo quality assessment, cDNA library construction, sequencing using 454 technology, and sequence assembly and annotation (see budget justification, task 2, for detailed information on 454 project costs provided by the CGB facility).

To develop the microarray platform, annotated gene sequence will be uploaded into a java-based oligonucleotide probe design software program called YODA (Yet-another Oligonucleotide Design Application) (Nordberg, 2005). This program will generate three unique, 60 base pair probes for each gene in regions of high binding efficiency. The probe set from all the genes will then be uploaded into eArray, a web-based microarray design program offered by Agilent Technologies, for creation of a microarray platform with the appropriate internal controls and triplicate probe replication. This process will result in a well-designed microarray that will be commercially manufactured by Agilent Technologies and made openly available to the research community interested in conducting genomics studies on delta smelt.

**Objective 2: Investigate the effects of temperature and salinity on the physiological tolerance limits, behavioral responses and swimming performance in Delta smelt (Task 3).**

These experiments will examine the physiological effects of temperature and salinity on 3 key life stages of the delta smelt. Acclimation and test ranges are based on studies carried out within the Department of Wildlife and Fish Biology, UC Davis (Swanson et al., 2000), and the delta smelt Fish Conservation and Culture Laboratory, UC Davis (Baskerville-Bridges et al., 2004a). We have designed a 3x3x3 experimental matrix with a 14-day minimum gradual acclimation period to 3 temperatures (10, 15, and 20°C), 3 salinities (2, 8, and 14ppt) and 3 smelt life stages (larvae, juvenile, and adult). Each temperature and salinity combination will be replicated 3 times in individual tanks housing between 20 and 30 fish, depending on particular age classes. These acclimation treatments were chosen to include ranges of temperature and salinities likely encountered by smelt in the Bay-Delta, as well as high and low tolerances reported in the cited literature for larvae, juveniles and adults. Once acclimation is complete, thermal and salinity tolerance and preference measures will be conducted on 8 fish from each experimental treatment replicate for a total of n=24 for each condition and age class.

**Thermal tolerance methodology:** Critical thermal maxima (CTMax) will be determined as an index of thermal tolerance. A fish's CTMax is defined as the temperature at which fish lose the ability to escape conditions that will ultimately lead to death. During the CTM trial, water temperatures are constantly increasing at a rate of 0.3°C/min, which is slow enough to quickly equilibrate with the fish's body temperature, but fast enough to prevent thermal acclimation during the experimental trial. Individual fish will be held in plexiglass vessels, and trials will continue until a repeatable, ecologically and ethically defensible endpoint is reached. A CTMax

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represents a lethal endpoint in nature, but will be non-lethal in the laboratory as the fish must recover when returned to acclimation temperatures. Loss of equilibrium (LOE) is the most common endpoint in fish thermal tolerance studies and will be used in these experiments.

Salinity tolerance methodology: Salinity tolerance will be conducted in much the same way as CTMax trials. Briefly, individual fish will be held at their acclimation temperatures and experimental salinities will be increased at a rate of 3ppt per hour until LOE is reached as the experimental endpoint. From these data, we will be able to quantify tolerance limits, determine if they differ between life-stages or with varying acclimation conditions.

Thermal and salinity preference trials: A fish's preferred temperature is a behavioral index of physiological optima. In many fish species, there is general agreement between performance optima of physiological correlates such as feeding, growth, metabolic rates, swimming performance, and reproduction with preferred temperatures (reviewed in Fanguie et al. 2009). We will utilize a minimum of 10 fish from each of our previously described acclimation groups, individually subjected to thermal and salinity preference trials. Preferred temperatures in delta smelt will be quantified in our laboratory using a thermal gradient device (Myrick et al., 2004). Fish will be exposed to the thermal gradient and their occupied temperatures recorded. Temperature selection data is bounded by upper and lower avoidance temperatures, and the preferred temperature will be calculated as a statistical measure of central tendency (mode) of the temperature distribution reflecting the temperature most often occupied by the fish during the trial. Similarly, a fish's preferred salinity often reflects the salinity at which osmoregulation costs are low. Simple modification of the annular thermal preference device will allow for salinity preference determination from salinities ranging from 0-16ppt.

For each temperature and salinity preference trial, image analysis will be performed using Ethovision Behavior Software XT 6 (Noldus Information Technology Inc. Leesburg, VA), that can track an organisms' position at a speed of 29 data-points per second. These positions can be used to measure quantitative behavioral performance parameters such as distance moved, velocity, levels of mobility, rotation, angular velocity, meander, and turn angle. During the preference trials fish will be individually video monitored for up to 5 minutes using a CCD digital camera recording through a WinTV-HVR-850 hybrid TV stick connected to a portable laptop. Specific protocols are in place at the UC Davis Aquatic Toxicology Laboratory for conducting video-imaging assessments on delta smelt.

**Objective 3: Investigate the effect of temperature and salinity to understand the mechanisms behind physiological tolerances (Task 4).**

We will use the results of our thermal and salinity tolerance tests (Objective 2) to inform our experimental design for this objective. Based on these data, we will conduct a series of sub-lethal thermal, salinity, and these stressors combined to assess molecular mechanisms underlying tolerance thresholds. The proposed molecular techniques are commonly used in our laboratory.

Thermal challenge experiments: Each age class acclimated to 10, 15, and 20°C and 2ppt will be subjected to a 1-hour, acute thermal challenge as follows: 10°C acclimated fish will be challenged with 15, 20, and 25°C; the latter being a critical maximum, 15°C acclimated fish to 20 and 25°C, and 20°C acclimated fish to a 25°C exposure. Published (Swanson et al., 2000) and unpublished results suggest that delta smelt can tolerate brief exposures to 25°C, but we will confirm our selection of sub-lethal temperatures in our thermal tolerance assessments. We chose to focus on increasing temperature as this is the most likely scenario for Delta smelt in the

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estuary where reproductive timing is such that environmental temperatures are increasing through development as the summer proceeds. Following the 1-hour heat stress, fish will be euthanized with a lethal dose of MS-222 and frozen at -80°C until RNA extraction for quantitative PCR and microarray analyses of gene expression. Including the appropriate controls and handling controls, this experimental design will generate 81 samples. For each of the 3 age classes, RNA samples will be analyzed in a design where the heat stressed samples will be compared to the corresponding controls. In combination, if we analyze the following: (1) three age classes and (2) four heat stress temperatures, the total number of microarrays required will be 108 (printed on an Agilent 4 plex, totaling 27 slides). The justification for the purchase of these microarrays is outlined in the Budget Justification.

Salinity challenge experiments: We will follow a similar approach to that above. Each age class acclimated to 2, 8, and 14ppt will be subjected to a 1-hour, acute salinity challenge as follows: 2ppt acclimated fish will be challenged with 8, 14, and 20ppt, 8ppt acclimated fish to 14 and 20ppt, and 14ppt acclimated fish to a 20ppt exposure. Here we focus on increasing salinity as this is the most likely scenario for delta smelt in the estuary resulting from forecast sea level rises. Following the 1-hour salinity stress, fish will be euthanized with a lethal dose of MS-222 and frozen at -80°C until RNA extraction for quantitative PCR and microarray analyses of gene expression. As with the thermal stress tests, this experimental design will generate 81 samples. For each of the 3 age classes, RNA samples will be analyzed in a design where the salinity stressed samples will be compared to the corresponding controls. In combination, if we analyze the following: (1) three age classes and (2) four salinity stress treatments, the total number of microarrays required will be 108 (printed on an Agilent 4 plex, totaling 27 slides). The justification for the purchase of these microarrays is outlined in the Budget Justification.

It is not our intention to perform combined salinity and temperature stress tests within the scope of this proposal, but we will likely use information gained from these studies towards including this in future research.

RNA extraction: Total RNA will be isolated in TRIzol® reagent (Invitrogen) according to manufacturer's instructions. RNA samples will undergo an additional column clean-up step to remove tRNA and any small-size degraded mRNA. Fish of each stage will be sampled immediately prior to thermal/salinity challenge (control), handled, as well as after each exposure to stress. These samples will be used for oligonucleotide microarray analyses (designed in Objective 1), and qPCR to assess the molecular mechanisms behind physiological tolerances of delta smelt to temperature and salinity changes.

Hybridization of delta smelt samples to microarrays: Ten µg of total RNA from each sample will be reverse transcribed (RT) using anchored oligo-dT, random primers and amino-allyl dUTP. Following RT, the RNA template will be removed from the reaction by incubation at 65°C for 15 min in the presence of 0.2M NaOH and 0.1M EDTA. Single stranded cDNA is then labeled by indirect coupling with 1µL of Alexa fluor 555 (reference) or 647 (experimental) succinimidyl ester dyes (Invitrogen) for 1h at room temperature. Unincorporated fluorophors will be removed using a RNA clean-up kit (Qiagen, Valencia, CA, USA). Samples will be quantified spectrophotometrically to ensure high quality cDNA synthesis and dye incorporation before continuing onto the slide hybridization. cDNA will be fragmented prior to being applied to the slide following Agilent's Gene Expression Hybridization Kit (Protocol G4140-90050). Samples will be loaded separately onto each of the four arrays on the slide and hybridized for 17h at 65°C. After hybridization, slides will be washed by immersion in Agilent's Gene Expression



Wash Buffers. Slides will be scanned on an Axon GenePix 4000B scanner (Axon Instruments, Molecular Devices). Note: we have not found it necessary to perform dye swaps using the Agilent microarray, which saves a considerable amount in terms of extra microarrays (Todgham and Hofmann, 2010; O'Donnell et al., 2009).

Analysis of microarray data: Data from the oligonucleotide microarrays used in this project will be gathered using GenePix Pro 4.0 software (Axon Instruments) to measure the ratio of Alexa fluor 555 to 647 fluorescence quantified for each "spot" on the arrays. We will use normalization by design methods to statistically analyze our microarray data. It involves using the experimental design to adjust for array-to-array differences and labeling bias. Here, a gene-by-gene normalization of dye bias is performed. After this analysis, we carry out the empirical Bayes method on the results of each gene-by-gene analysis to moderate variances and, therefore, the test statistics. Additionally, we will calculate adjusted p-values. Thus, most of the methodology, in the end, is very similar to other microarray analysis, and only the normalization differs (Jarrett and Ruggiero, 2008).

Quantitative PCR: qPCR will be used to verify microarray responses. Total RNA will be extracted as described for microarray applications. Complementary cDNA will be synthesized from 1 µg total RNA using 100 units of SuperScript III, 600 ng random hexamer primers, 10 U RnaseOut (Rnase inhibitor), and 1 mM dNTPs (all Invitrogen, Carlsbad, CA, USA) in a final volume of 20 µL. The reverse transcription reaction will be carried out at 50°C for 50 min and terminated by heating for 5 min to 95°C with immediate cooling on ice. Samples will be diluted with nuclease-free water to a total volume of 120 µL and stored at -20 °C for subsequent qPCR assessments. Quantitative PCR measurements will be performed using TaqMan Universal PCR Mastermix (Applied Biosystems) Samples will be prepared in Dr. Connon's lab, and assessments conducted at the Lucy Whittier facility at UC Davis. Samples will be placed in 384-well plates and amplified in an automated fluorometer (ABI PRISM 7900 Sequence Detection System, Applied Biosystems).

Analysis of quantitative PCR data: We will use SDS 2.2.1 software (Applied Biosystems) to quantify gene amplification in real-time. Gene expression levels will be calculated using the relative quantification  $2^{-\Delta\Delta CT}$  method as described in (Livak and Schmittgen, 2001). Beta-actin, a commonly used house-keeping gene, will be used as reference upon which to normalize expression of the entire suite of genes, though incorporation of other reference genes will be considered favorable. We will perform cluster analysis and gene expression profiling on both microarray and subsequent qPCR verification data to aid mechanistic interpretation of responses. Quantitative PCR data for each individual fish from all tests will be combined into one annotated dataset and subjected to agglomerative hierarchical clustering using Genesis software version 1.7.5 (Sturm et al., 2002). A heatmap profile of gene expression will be generated using cosine correlation, with complete linkage clustering, based on data trends and mean distances, to assess response differences between species to respective treatments. Principal component analysis (PCA) will be conducted in conjunction to correlation heatmaps, to ascertain factors responsible for thermal and salinity stress response variability.

**Objective 4: Link mechanistic tolerance thresholds with adverse outcomes in delta smelt behavior (Tasks 2, 3 and 4)**

Following completion of Objectives 1-3 (Tasks 2-4) we will utilize both genomic and physiological information gained from thermal and salinity tolerance to investigate linkages

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between mechanistic responses with whole individual responses. For example, the level of expression of genes involved in osmoregulation will be assessed in terms of measured loss of equilibrium, or genes involved in neuromuscular activity will be compared to swimming performance responses. Analysis will include correlation between genomic responses and loss of equilibrium and/or swimming performance. Changes in expression of key molecular biomarkers will highlight biochemical pathways responding to contaminant exposure. We will also utilize novel bioinformatic approaches including the Kyoto Encyclopedia for Genes and Genomes (KEGG) to identify and understand key pathways that may indicate thermal and salinity tolerance. For example, neurological and muscular responses measured at genomic level will be directly contrasted with swimming behavior assessed with video-imaging analytical software. Contrasting these responses with higher level effects such as swimming performance will further validate the links between the two-system measurements, utilizing biologically relevant parameters within an adverse outcome pathway assessment.

Achievement of the above objectives will result in an understanding of physicochemical parameter effects on the delta smelt, tools for monitoring changes in environmental stress and recommendations to the Bay-Delta Science program in the form of conceptual models based on response thresholds to temperature and salinity variation on delta smelt.

#### **4. Feasibility**

Feasibility of this project is very high. Drs. Connon, Fangue and Lindberg and other staff are highly qualified and suitably experienced to carry out all aspects of this investigation (see qualifications, below). The proposed molecular, physiological and behavioral methodologies are of common use in our laboratories and all the equipment needed to conduct this project is readily available. Emily Martinez is an incoming graduate student, is funded for three years and has the appropriate skill set to design and conduct experimental manipulations with fishes, as well as analyze behavioral data sets and molecular samples. She will be further trained and supervised by Drs Connon and Fangue

The proposed work will build on and benefit considerably from results of previous and ongoing studies with delta smelt carried out at various laboratories at UC Davis, as well as planned IEP funded studies to assess whether juvenile and adult delta smelt discriminate levels (and types) of turbid and saline environments and make behavioral choices based on this information, and how temperature affects juvenile and adult behavior. These studies are planned to be conducted in September 2010 at the UCD-ATL in collaboration with Dr Lindberg (FCCL). We have carefully designed experiments to answer specific questions of interest to the Delta Science Program, as outlined in our proposal.

Facilities and equipment: The UCD-ATL of the School of Veterinary Medicine (University of California Davis, [www.ucdavis.edu/apc/atl](http://www.ucdavis.edu/apc/atl)) is a State-certified lab with over 20 years of experience in investigating surface water quality and aquatic ecosystem health in watersheds throughout California. ATL activities focus on conducting aquatic organism toxicity tests, as defined by the United States Environmental Protection Agency (US EPA), using standard as well as resident aquatic species. ATL follows protocols and quality assurance criteria established by the US EPA and the State of California's Surface Water Ambient Monitoring Program (SWAMP).

The UCD-ATL is a 3200 ft<sup>2</sup> facility consisting of four individual laboratories and is fully equipped to conduct toxicant exposure and surface water monitoring studies. Available