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Integrative Assessments of Niche Dimensions and Multiple Stressor Effects on Endangered Delta Smelt (*Hypomesus transpacificus*)

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Glossary

11-Beta-HSD-1	11-Beta-Hydroxysteroid-Dehydrogenase-Type1
11-Beta-HSD-2	11-Beta-Hydroxysteroid-Dehydrogenase-Type2
ACTH	Adrenocorticotropic Hormone
ANOVA	Analysis of Variance
β-actin	Beta-Actin
cDNA	Complementary DNA
DDT	Dichlorodiphenyltrichloroethane
Delta	Sacramento-San Joaquin River Delta
DNA	Desoxyribo Nucleic Acid
DO	Dissolved Oxygen
Dph	days post hatch
EC	Electric Conductivity
EDC	Endocrine Disrupting Chemicals
EF-α	Elongation-factor alpha
EST	Expressed Sequence Tag
ETM	Estuarine Turbidity Maximum
FCCL	University of California, Davis Fish Conservation & Culture Laboratory
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GLM	General Linear Model
GLUT2	Glucose Transporter 2
GO	Gene Ontology Database
GR2	Glucocorticoid Receptor 2
GST	Glutathione-S-Transferase
HIF1-α	Hypoxia Inducible Factor
Hood	California Department of Water Resources Water Quality Monitoring Station at Hood
HPI	Hypothalamus-Pituitary-Interrenal
HSP70	Heat Shock Protein 70-kDa
IACUC	UC Davis Institutional Animal Care and Use Committee
IGF	Insulin like Growth Factor

KCl	Potassium Chloride
KEGG	Kyoto Encyclopedia of Genes and genomes
LSZ	Low-Salinity Zone (LSZ),
MC2R	Melanocortin Receptor 2
MR1	Mineralocorticoid Receptor 1
N/A	indicates values are not applicable.
Na/K-ATPase	Sodium/Potassium ATPase
NFkB	Nuclear Factor k-Beta
NH4+ Trans	Ammonium Transporter
NTU	Nephelometric Turbidity Unit
PBS	Phosphate-Buffered Saline
PC	Principal Component
PCA	Principal Component Analysis
PCB	Polychlorinated Biphenyl
POMC	Pro-opiomelanocortin
Ppt	Parts Per Thousand
PSU	Practical Salinity Unit
qPCR	Quantitative Polymerase Chain Reaction
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
Sal	Salinity
SC	Specific Conductance
SD	Standard Deviation
SE	Standard Error
SFÄ	San Francisco Ästuar
SFBD	San Francisco Bay Delta
SFE	San Francisco Estuary
SGK3	Serum/Glucocorticoid regulated Kinase
SL	Standard Length
SRGB	Sacramento River at Garcia Bend
SRWTP	Sacramento Regional Wastewater Treatment Plant

SSJFD	Sacramento - San Joaquin Flussdelta
T	Temperature
T0	Time point at test start,
T24	Time point at test termination.
TAN	Total Ammonia-N
TIE	Toxicity Identification and Evaluation (TIE)
Tur	Turbidity
X2	Position of the 2-ppt-salinity isohaline

Preface

The intention of this work is to contribute to the understanding of the effects of crucial abiotic parameters and multiple stressors on fish species. This is demonstrated on the endangered Delta Smelt (*Hypomesus transpacificus*).

Essential background information about niche concepts, environmental stress, stress response of an organism, estuary ecosystems, species biology of the Delta Smelt, and goals and objectives are described in the introduction. Subsequently four chapters examining important aspects crucial for the endangered Delta Smelt are presented. First, experimental conditions were clearly defined using fish stocking density as a confounding factor. Next, niche assessments for crucial abiotic parameters such as turbidity, salinity, as single stressors and as multiple stressors in combination are presented in chapter two and three. In the last chapter the effects of complex mixture of stressors in the form of a contaminant mixture on the Delta Smelt are investigated. Each chapter was published or is currently under review or preparation as an independent research publication in a peer reviewed journal in a slightly modified form, meeting the respective journal requirements. The chapters are followed by a general discussion that encompasses the relevance and significance of confounding factors, niche assessments, and multiple stressor scenarios for fishes.

Summary

Ecosystems worldwide are exposed to physicochemical stressors, evoked by natural environmental alterations and anthropogenic influences that can pose threats to biodiversity. Environmental and recent human impacts can cause significant levels of stress to organisms, determining their survival or extinction. Stressors can be biotic or abiotic factors, and seldom occur singly in the natural environment but rather act in a complex interplay creating a multiple-stressor scenario. Estuary ecosystems are highly dynamic, and are paragons for multiple stressor scenarios as they represent the interface between coastal marine and freshwater habitats, and are usually heavily anthropogenically influenced. The San Francisco Estuary (SFE) and Sacramento-San Joaquin River Delta (Delta) in California, USA, are among the most anthropogenically modified, managed, and threatened ecosystems in the world, and a severe decline in biodiversity has been observed over the past decades. One species of major concern is the native Delta Smelt (*Hypomesus transpacificus*), which is endemic to the SFE and Delta. The Delta Smelt is listed as threatened and endangered under both the US Federal and California State endangered species acts, as their population has significantly declined in recent decades. Anthropogenic threats and abiotic factors, such as contaminants, low turbidity associated with limited primary production, and salinity associations with habitat limitations, have been postulated to contribute to its decline.

This dissertation investigated the effects of several different biotic stressors (stocking density), abiotic stressors (turbidity, salinity) and anthropogenic stressors (contaminants) on the physiological response of an endangered fish species. Special focus was put on fundamental niche assessments using turbidity as an exemplary abiotic factor. In addition multiple stressor scenarios testing turbidity and salinity in combination, and a complex mixture of contaminants were assessed. A multiple endpoint approach with endpoints from different levels of biological organization was utilized to assess effects. The overall goal of this work was to contribute to the understanding of the reasons leading to population declines. In several tests the effects of fish stocking density, turbidity, salinity and contaminants were evaluated on early life stages of this species.

First, experimental protocols were optimized using fish stocking density as a representative example for a potential confounding factor, as it is known to impact overall stress of fish/Delta Smelt. The aim of this chapter was to determine optimal density ranges within experimental vessels for this species, towards planned multiple stressor studies. Late-larval Delta Smelt (60 days post hatch: dph) were maintained under culture conditions for a period of 24 h, at five different densities: 7, 14, 28, 42, and 56 fish in circular fish tanks containing 8L water. Stress related endpoints of two different levels of biological organization; cortisol concentrations along with stress-related molecular biomarkers pertaining to cortisol production via the Hypothalamus-Pituitary-Interrenal (HPI) axis were

measured. Stress levels were significantly higher at densities of 7, 14, and 56 individuals, suggesting an optimal stocking density in the range of 28 to 42 fish per vessel. This test allowed for the determination of maximum fish numbers per vessel, and further supports empirical observations that late larval Delta Smelt should be exposed, acclimated, and cultured in groups rather than as individuals or in low numbers. This approach represented a powerful experimental design to address fish stocking densities and to evaluate the use of molecular and biochemical endpoints on fish stress, that are easily applicable to a broad range of fish species.

Secondly, the effects of turbidity as a single stressor on late-larval Delta Smelt were tested. In particular for larval and early juvenile stages, turbidity has been postulated as an essential requirement for visualization of prey, thus being a crucial parameter for survival. The aim of this study was to determine optimal turbidity requirements for late larval Delta Smelt, assessed through multiple determinants of different levels of biological organization such as survival, feeding behavior, cortisol, and expression of genes associated with cortisol production and general stress. Late-larval Delta Smelt (60 dph) were exposed for 24 h to turbidities of 5, 12, 25, 35, 50, 80, 120, and 250 NTU, at a low light intensity of 48.07 Lux (SD±=4.8). Survival was reduced at turbidities of 5, 120, and 250 NTU, was greatest between 25 and 80 NTU, and was proportional to feeding performance. Cortisol levels also corresponded with mortality and feeding performance. Principal Component Analysis (PCA) of genomic responses revealed clustering of treatments 25, 35, 50, and 80 NTU, differentiating from responses at the lower and higher turbidities. This multiple endpoint approach proved to be a powerful tool to determine optimal habitat requirements and performance in response to environmental variables. Knowledge on habitat preferences concerning environmental variables may help identify priority areas for Delta Smelt and aids resource managers maintaining suitable turbidity levels beneficial for Delta Smelt conservation.

In the fourth chapter the combination of different turbidities and salinities which are both known to vary within estuaries were tested on juvenile Delta Smelt representing a multiple-stressor scenario. Both factors are influenced by water flows through the SFE and Delta, and are known to be of critical importance to the completion of the Delta Smelt's life cycle. The effects of turbidity and salinity on feeding performance and physiological stress response of Delta Smelt were investigated using food intake, transcriptomic assessments as endpoints. Juvenile Delta Smelt were exposed for 2h to a matrix of turbidities and salinities ranging from 5 to 250 Nephelometric Turbidity Unit (NTU) and 0.2 to 15 parts per thousand (ppt), respectively. Salinity did not significantly impact feeding performance; however, increasing turbidities resulted in reduced feeding rates, especially at 250 NTU. Sodium potassium ATPase - (*Na/K-ATPase*) – an indicator of osmoregulatory stress, and hypothalamic proopiomelanocortin (*POMC*) – a precursor protein to Adrenocorticotropic hormone

(*ACTH*; expressed in response to biological stress) were significantly affected by salinity, with greater induction at salinities > 12 ppt, and at < 2 ppt. Glutathione-S-transferase (GST), a phase II detoxification enzyme that protects cells against reactive oxygen species (ROS), was significantly affected by both salinity and turbidity. Overall these findings suggested that turbidity is an important determinant of feeding behavior, whereas salinity is an important factor influencing the cellular stress response. No clear relationship between turbidity and salinity was found in this study on juvenile Delta Smelt.

Lastly, the sublethal effects of contaminant mixtures on larval Delta Smelt were studied. Anthropogenic activities such as wastewater effluents discharge, along with agricultural and urban runoff, are among the primary sources of contaminants. Transcriptional responses were evaluated in larval Delta Smelt following exposure to water samples collected at the Department of Water Resources Field Station at Hood, a site of concern situated upstream of known Delta Smelt spawning habitats, and downstream of the Sacramento Regional Wastewater Treatment Plant (SRWTP). Microarray assessments indicated effects on the energy metabolism, the lipid metabolism, the amino acid metabolism, genetic information processing (transcription, translation), the nervous system, the immune system, development and upon muscle activity. Further, transcriptional responses of fish exposed to water samples from Hood were compared with exposures to 9% effluent samples from SRWTP, water from the Sacramento River at Garcia Bend (SRGB); upstream of the effluent discharge, and SRGB water spiked with 2 mg/L total ammonium; 9% effluent equivalent. Results indicated that transcriptomic profiles (microarrays and qPCR) from Hood were similar to SRWTP effluent and ammonium spiked SRGB water, but significantly different from SRGB. However, SRGB samples were significantly different to laboratory controls, suggesting that SRWTP effluent is not solely responsible for the responses determined at Hood, that ammonium exposure likely enhances the effect of multiple-contaminant exposures, and the observed mortality at Hood was caused by the combination of effluent discharge and contaminants arising from upstream of the tested sites. This study demonstrated that transcriptomic responses in fishes can be valuable endpoints for the identification of contaminant sources that occur at sublethal concentrations in surface waters.

In conclusion, for the conservation of the Delta Smelt, it could be demonstrated that all stressors, individually as well as in combination, play a crucial role for the survival of this sensitive species. The studies presented herein highlighted the importance of measuring multiple endpoints across different biological organizations in ecological stressor assessments of species of conservation concern. Effective species conservation is only possible with a detailed knowledge of the effects of

each abiotic stressor individually, and in combination, allowing resource managers and conservation biologists to make informed decisions as they relate to each species.

Zusammenfassung

Weltweit werden Ökosysteme von physisch chemischen Stressfaktoren beeinflusst, die von natürlichen Umweltveränderungen und anthropogenen Einflüssen hervorgerufen werden und somit potentiell die Biodiversität gefährden können. Umwelt- und anthropogene Einflüsse können erheblichen Stress auf Organismen ausüben und damit über deren Überleben oder Aussterben bestimmen. Stressfaktoren können sowohl biotischer also auch abiotischer Natur sein. Sie kommen in der Natur selten einzeln vor, sondern häufig als komplexe Kombinationen multipler Stressfaktoren, welche simultan auf Organismen wirken.

Ästuare Ökosysteme repräsentieren Paradebeispiele für Lebensräume, in denen multiple Stressfaktoren vorherrschen, weil sie die Schnittstelle zwischen Marin- und Süßwasserlebensräumen darstellen. Ästuare sind als hochdynamische Lebensräume bekannt und sind im Allgemeinen stark vom Menschen beeinflusst. Das San Francisco Ästuar (SFÄ) und Sacramento - San Joaquin Flussdelta (SSJFD) in Kalifornien, Vereinigte Staaten von Amerika, gehören zu den mit am stärksten vom Menschen veränderten, regulierten, und bedrohten Ökosystemen der Welt. Seit mehreren Jahrzehnten wurde dort ein starker Rückgang der Biodiversität beobachtet. Eine besondere Bedeutung hat dabei die einheimische Fischart Delta Smelt (*Hypomesus transpacificus*), welche endemisch im SFÄ und im SSJFD ist. Aufgrund seines starken Rückgangs in den vergangenen Jahrzenten, ist der Delta Smelt sowohl auf der kalifornischen als auch auf der USA-weit gültigen Roten Liste der vom Aussterben bedrohten Arten als bedroht und gefährdet eingestuft. Es wird angenommen, dass sowohl abiotische Stressfaktoren, wie z.B. Trübung und Salzgehalt als auch anthropogene Einflüsse, wie z.B. Verschmutzungen, zum Rückgang der Art beigetragen haben.

Diese Dissertation beschäftigt sich mit der Untersuchung von Effekten diverser biotischer (Besatzdichte) und abiotischer Faktoren (Trübung, Salzgehalt) sowie anthropogener Stressfaktoren (Schadstoffe) auf eine bedrohte Fischart. Der besondere Fokus der Arbeit liegt dabei auf der Bewertung des Nischenkonzepts, welches beispielhaft anhand des abiotischen Faktors Trübung untersucht wurde. Ausserdem wurden die Effekte von mehreren Stressfaktoren in Kombination (Trübung, Salzgehalt) und eine komplexe Mischung von Schadstoffen untersucht. Um die Effekte auf den Delta Smelt zu bestimmen, wurden Versuchsansätze mit verschiedenen Endpunkten unterschiedlicher biologischer Organisationsebenen verwendet. Das übergeordnete Ziel der Arbeit ist, am Beispiel des Delta Smelts die Rolle der genannten Faktoren im Zusammenhang mit dem Rückgang von Fischpopulationen in Ästuaren zu betrachten. In verschiedenen Experimenten wurden die Effekte von Besatzdichte, Trübung, Salzgehalt und Schadstoffen auf die frühen Lebensstadien dieser sehr sensitiven Fischart untersucht.

In einem ersten Schritt wurden die Versuchsmethoden optimiert. Dies geschah beispielhaft anhand des Faktors Besatzdichte. Dieser Faktor kann die Gesamtstressreaktion in Fischen und damit auch im Delta Smelt beeinflussen. Das Ziel dieser Studie war, die optimale Besatzdichte für die Versuchsbecken, die in den darauf folgenden Hauptversuchen verwendet werden sollten, für den Delta Smelt zu bestimmen. Sechzig Tage alte Delta Smelts wurden zu den in der Fischzucht üblichen Wasserbedingungen in fünf verschiedenen Besatzdichten für 24 Stunden in runden Fischbecken mit einem Volumen von 8 l Wasser exponiert. Als Endpunkte der Stressantwort wurden sowohl die Cortisolkonzentration als auch molekulare Biomarker, die die Cortisolproduktion durch den Hypothalamus-Hypophysen-Interrenal-Regelkreis kontrollieren, bestimmt. Stresslevel in Besatzdichten von 7, 14, und 56 Fischen pro Becken waren deutlich höher als in Besatzdichten von 28 und 42 Fischen pro Becken. Dies zeigt, dass eine Besatzdichte im Bereich von 28 bis 42 Fischen pro Becken für diese Fischart als optimal angesehen werden kann. Durch diesen Test war es nicht nur möglich, die geeignete Fischanzahl pro Becken zu bestimmen, sondern auch empirisch gewonnenes Wissen wissenschaftlich zu verifizieren, nämlich dass spät-larvale Delta Smelts eher in Gruppen als allein oder in geringer Anzahl exponiert, akklimatisiert und gezüchtet werden sollten. Dieser Versuchsansatz stellt ein geeignetes experimentelles Design dar, um Besatzdichten von Fischen mit Hilfe molekularer und biochemischer Endpunkte zu analysieren. Zudem ist dieser Versuchsansatz sehr gut auf andere Fisch- und Beckenarten übertragbar.
In einem zweiten Schritt wurde der Effekt von Trübung auf das spätlarvale Stadium des Delta Smelts analysiert. Es wird angenommen, dass Delta Smelts im larvalen als auch im juvenilen Stadium ein bestimmtes Maß an Trübung benötigen, um ihre Beutetiere visuell erkennen zu können. Somit ist der Parameter Trübung unabdinglich für ihr Überleben. Daher war es das Ziel dieser Studie, den optimalen Toleranzbereich des spätlarvalen Delta Smelts für den Parameter Trübung unter Verwendung mehrerer unterschiedlicher Endpunkte wie Mortalität, Fressverhalten, Cortisolspiegel und Expression von Genen, die für die Cortisolproduktion und den Gesamtstress verantwortlich sind, zu bestimmen. Sechzig Tage alte Delta Smelts wurden für 24 Stunden den Trübungsstufen 5, 12, 25, 35, 50, 80, 120 und 250 NTU bei einer niedrigen Lichtintensität von 48,07 Lux (SD±=4,8) ausgesetzt. Die Überlebensrate war in sehr geringer Trübung von 5 NTU und in sehr hoher Trübung von 120 und 250 NTU stark reduziert. Im Bereich von 25 bis 80 NTU war die Überlebensrate am höchsten. Zudem war die Überlebensrate proportional zum Fressverhalten. Gemessene Cortisolspiegel stimmten mit den Ergebnissen von Überlebensrate und Fressverhalten überein. Die Analyse der Genexpression mit Hilfe der Principal Component Analysis (PCA) ergab ein Gruppierung der Trübungslevels 25, 35, 50 und 80 NTU und eine klare Separation der niedrigen und höheren Trübungslevel. Dieser Versuchsansatz, bei dem verschiedene Endpunkte auf unterschiedlichen Ebenen biologischer

Organisation gemessen wurden, hat sich als bewährte Methode herausgestellt, um optimale Habitatanforderungen und biologische Fitness in Hinblick auf Umweltvariablen zu bestimmen. Detailierte Informationen und umfangreiches Wissen hinsichtlich der Lebensraumansprüche bezüglich wichtiger Umweltfaktoren ist unabdingbar, um geeignete Lebensräume für den Delta Smelt zu identifizieren. Ausserdem hilft dies örtlichen Resourcenmanagern, die Trübungslevels auf einen angemessenen Bereich einzustellen, der dem Delta Smelt und seiner Arterhaltung dienlich ist.

In einem dritten Schritt wurde eine Kombination der abiotischen Stressfaktoren Trübung und Salzgehalt am Delta Smelt getestet, um eine Multistresssituation exemplarisch zu untersuchen. Beide Faktoren werden durch die hydrologischen Fließbedingungen im SFÄ und SSJFD beeinflusst und sind von enormer Wichtigkeit für den Lebenszyklus des Delta Smelts. Die Effekte von Trübung und Salzgehalt auf das Fressverhalten und die physiologische Stressreaktion des Delta Smelts wurden mittels Fütterungsversuchen und Analyse der Genexpression untersucht. Juvenile Delta Smelts wurden für 2 Stunden in einem Matrixsystem Kombinationen unterschiedlicher Trübungen und Salzgehalten exponiert. Die getesteten Trübungsstufen lagen im Bereich von 5 bis 250 NTU und der Salzgehalt lag in einem Bereich von 0,2 bis 15 part per thousand (ppt). Salinität hatte keinen signifikanten Effekt auf das Fressverhalten, wohingegen in höheren Trübungen, insbesondere in 250 NTU, die Futteraufnahme reduziert war. Das Gen Natrium-Kalium ATPase, welches ein Indikator für osmoregulatorischen Stress ist, sowie das Gen hypothalamisches Pro-Opiomelanocortin, ein Vorläuferprotein des adrenocorticotropischen Hormones, waren signifikant durch Salzgehalt beeinflusst, mit stärkeren Effekten in Salzgehalten > 12 ppt und < 2 ppt. Ein weiteres Gen, Glutathion-S-Transferase, welches ein gleichnamiges Enzym kodiert, das in Entgiftungsprozessen der Phase II agiert und Zellen vor reaktiven Sauerstoffradikalen schützt, wurde sowohl von Salzgehalt als auch Trübung signifikant beeinflusst. Insgesamt weisen diese Ergebnisse darauf hin, dass Trübung das Fressverhalten beeinflusst, während sich Salinität auf die zelluläre Stressreaktion auswirkt. Es konnte keine eindeutige Korrelation zwischen den beiden Faktoren festgestellt werden.
Abschliessend wurden die subletalen Effekte einer Schadstoffmischung auf den Delta Smelt getestet. Anthropogene Einträge wie z.B. Kläranlagenabwasser, landwirtschaftliche und städtische Einträge gehören zu den primären Quellen von Schadstoffen. Wasserproben wurden von zwei unterschiedlichen Probennahmestellen entnommen und larvale Delta Smelts für 7 Tage darin exponiert. Eine Probennahmestelle befindet sich in der Nähe des Ortes Hood und der dort gelegenen Freilandstation des Amts für Wasserresourcen, Bundesstaat Kalifornien liegt. Diese Station liegt flussaufwärts von potentiellen Delta Smelt-Laichgründen und unterhalb der städtischen Kläranlage von Sacramento (SRWTP). Veränderungen der Genexpression auf transkriptionaler Ebene wurden mit Hilfe der Microarray-Technologie gemessen. Daraus ergab sich, dass der Energie-, Fett-

und Aminosäurenstoffwechsel, die Verarbeitung genetischer Informationen (Transkription, Translation), das Nerven- und Immunsystem sowie die Entwicklung und Muskelaktivität der Fische signifikant beeinträchtigt waren. Diese Ergebnisse wurden mit drei weiteren Expositionsversuchen verglichen. Es wurden Fische in Sacramento Flusswasser, welches in der Nähe von Garcia Bend, oberhalb der SRWTP genommen wurde, exponiert. In weiteren Versuchen wurde das Flusswasser von Garcia Bend mit 9% Kläranlagenabwasser von der SRWTP verdünnt oder mit 2mg/L Ammonium versetzt und die Fische darin exponiert. Die gemessenen Genexpressionsmuster von Hood waren mit denen vom SRWTP Abwasser und dem Flusswasser versetzt mit Ammonium vergleichbar, jedoch signifikant unterschiedlich vom Flusswasser, welches oberhalb der SRWTP genommen wurde. Ausserdem waren die Genexpressionsmuster von oberhalb der SRWTP signifikant unterschiedlich von den Kontrollen, was darauf hinweist, dass die SRWTP nicht den einzigen Faktor darstellt, der zu den Genexpressionmustern in Hood beiträgt. Somit ist es höchstwahrscheinlich, dass Ammonium den Effekt der Schadstoffmischung verstärkt und dadurch die erhöhte Sterberate am Ort Hood durch eine Kombination von Kläranlagenabwasser und Schadstoffen, die an Orten weiter flussaufwärts eingetragen werden, resultiert. Mit dieser Studie konnte gezeigt werden, dass das Messen von Genexpressionsmustern auf transkriptionaler Ebene in Fischen ein sehr nützlicher Endpunkt sein kann, der die Identifizierung von Schadstoffquellen, sogar in subletalen Konzentrationen, in Gewässern ermöglicht.

Für die Arterhaltung des Delta Smelts lässt sich schlussfolgernd sagen, dass durch diese Studien gezeigt werden konnte, dass alle Stressfaktoren einzeln sowie in Kombination eine wichtige Rolle für das Überleben dieser sensiblen Art spielen und in ein Monitoringprogramm aufgenommen werden sollten. Die hier präsentierten Ergebnisse sind wissenschaftlich fundierte Fallbeispiele für die Bestimmung diverser Endpunkte auf unterschiedlichen Ebenen biologischer Organisation. Dieser Ansatz ermöglicht die Bewertung von Effekten ökologischer Umweltfaktoren auf bedrohte Tierarten. Effektive Maßnahmen zur Arterhaltung gefährdeter Tierarten sind nur dann möglich, wenn umfangreiches Wissen über die Effekte von jedem einzelnen abiotischen Stressfaktor und deren Kombinationen bekannt ist. Dieses erlaubt Ökologen, Umweltmanagern und Biologen, bestmögliche und der Arterhaltung dienliche Entscheidungen zu treffen.

1. Introduction

1.1 Niche Concepts and Niche Dimensions

The concept of the niche was coined almost a century ago, and was mostly influenced by the studies of Grinnell (Grinnell 1917), Elton (Elton 1927) and Hutchinson (Hutchinson 1957). It remains a fundamental concept within the fields of ecology, physiology, and population biology (Chase and Leibold 2003; Pörtner et al. 2010), and combines two crucial qualities: 1. that environmental factors influence the organism's performance; thus control the distribution in the habitat and 2. that the genetic adaptation of an organism restricts it to a specific range of environmental conditions (Hooper et al. 2008). One of the fundamental and crucial goals of ecology and physiology is to understand the abundance and distribution of a species. The terms 'fundamental' and 'realized' niche are specific to niche theory and are interconnected, and often used to describe the distribution of species. The fundamental niche was described by Hutchinson (Hutchinson 1957) as a 'n-dimensional hypervolume', which represents the full range (e.g., of an abiotic factor) in which a species could potentially exist, whereas the term 'realized' niche also takes biotic factors (e.g., predation, species competition) into account (Chase and Leibold 2003). The interspecific and intraspecific relations of a species cause the limits of the realized niche to be narrower than the limits of the fundamental niche. Ecologists and physiologists utilize the concepts in different contexts; ecologists being predominantly interested in studying the biotic aspects of the niche, while physiologists mostly focus on the organism's performance relative to abiotic factors (Pörtner et al. 2010). Measuring the organismal performance using integrative methods, across multiple levels of biological organization from molecular levels, to whole organism responses, allows for determination of niche breadth and dimensions (Pörtner et al. 2010).

1.2 Environmental Stress

Ecosystems worldwide are constantly changing as they are subjected to physicochemical stressors. Change is evoked by natural environmental alterations and by anthropogenic influences, with the latter posing threats to biodiversity. Five major threats to aquatic biodiversity have been identified, namely habitat destruction and degradation, overexploitation, flow modification, invasive species and environmental pollution (Dudgeon et al. 2006; Geist 2011; Kennish 2002). Evolutionary effects and recent human impacts can cause significant levels of stress to organisms, determining the survival or extinction of populations, and ultimately species. Environmental stress is difficult to define and scientists use terms such as 'stressor', 'stress' and 'stress response', interchangeably and

without proper definitions (Schulte 2014). However, a stressor is generally defined as an environmental factor that elicits a detrimental physiological response from an organism. Stressors can be biotic factors, for example food limitation, predation threat, diseases, intraspecific interactions, or abiotic factors, such as temperature, salinity, oxygen, and contaminants. In this work the term 'stressor' is referred to as environmental variables and anthropogenic influences. The term stress response is used to describe the physiological response on the organism. Stressors, whether abiotic or biotic, seldom occur singly in an environmental setting but rather act in a complex interplay creating a multiple-stressor scenario (Breitburg et al. 1998; Schindler 2001). Highly dynamic ecosystems such as estuaries are a paragon for these conditions.

1.3 Stress Response

Threatened or disturbed homeostasis, caused by either internal or external stressors, can be restored by a complex repertoire of adaptations comprising physiological and behavioral responses (Chrousos 1998; Chrousos and Gold 1992; Schulte 2014). Stress responses in fish can be categorized as primary, secondary, and tertiary (Barton 2002; Iwama 1998; Wendelaar Bonga 2011), representing multiple levels of biological organization, and are used as measurements of organismal performance, e.g., for niche assessments (Pörtner et al. 2010).

The primary stress response is characterized by immediate endocrine changes in stress hormones such as catecholamines, adrenocorticotropic hormone (ACTH), and cortisol (Barton 2002; Iwama 1998; Wendelaar Bonga 2011). A frequently evaluated primary stress pathway is the Hypothalamic-Pituitary-Interrenal- axis (HPI-axis), which is responsible for cortisol production, and is comprised of a series of genes that encode for hormones with specific functions. When a stressor leads to the production of corticotropin-releasing factor by the hypothalamus, it triggers the release of ACTH by corticotropic cells. ACTH is derived from Pro-opiomelanocortin (POMC), and binds to specific receptors (melanocortin receptor type 2; MC2R) in the interrenal cells in the head kidney. Through activation of cAMP signaling pathways, MC2R stimulates the cortisol production and its release into the blood. Cortisol has the ability to enter cells and binds to specific receptors such as mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Alsop and Aluru 2011).

The secondary response is described by the physiological effects of the primary stress hormones, which among other, causes the activation of metabolic pathways, hydromineral balance, immune and respiratory functions (Barton 2002; Iwama 1998; Wendelaar Bonga 2011).

The tertiary response encompasses changes in behavior and physiology that are observed at the whole animal level, such as growth, development, reproduction, disease resistance, and survival (Barton 2002; Iwama 1998; Wendelaar Bonga 2011).

It is important to emphasize that not all stressors and environmental changes will cause a similar stress response, for instance hypoxia as a stressor in fish does not necessarily evoke high levels of cortisol, even when it negatively impacts performance (O'Connor et al. 2011). Furthermore, it is important to determine whether environmental changes pose a stressful or non-stressful situation, and to what extent the intensity and time interval of the stressor influences the respective stress response (Schulte 2014). Under some circumstances fishes are able to employ adaptation and phenotypic plasticity, which is defined as the ability of an organism to exhibit several phenotypes depending on the environment (Agrawal 2001), and consequently improve tolerance levels and performance (McBryan et al. 2013; Schulte et al. 2011). Performance will decline, or increase, dependent on stressor intensity and duration, which in turn will be affected by acclimation, performance capacity, and specialization. Measuring performance of organisms as a response to a stressor allows the assessment of niche dimensions (Pörtner et al. 2010). The niche dimensions for a certain factor that controls the distribution of a species, can be described by the law of tolerance proposed by Shelford (1931) using performance curves (Figure 1). The law emphasizes on the one hand, that the success of an organism is contingent on the presence of optimal abiotic and biotic conditions, and on the other, that quantitative or qualitative surplus, or lack of any one or multiple factors that approximate the limits of tolerance for an organism, govern its absence or failure (Allaby 2010).

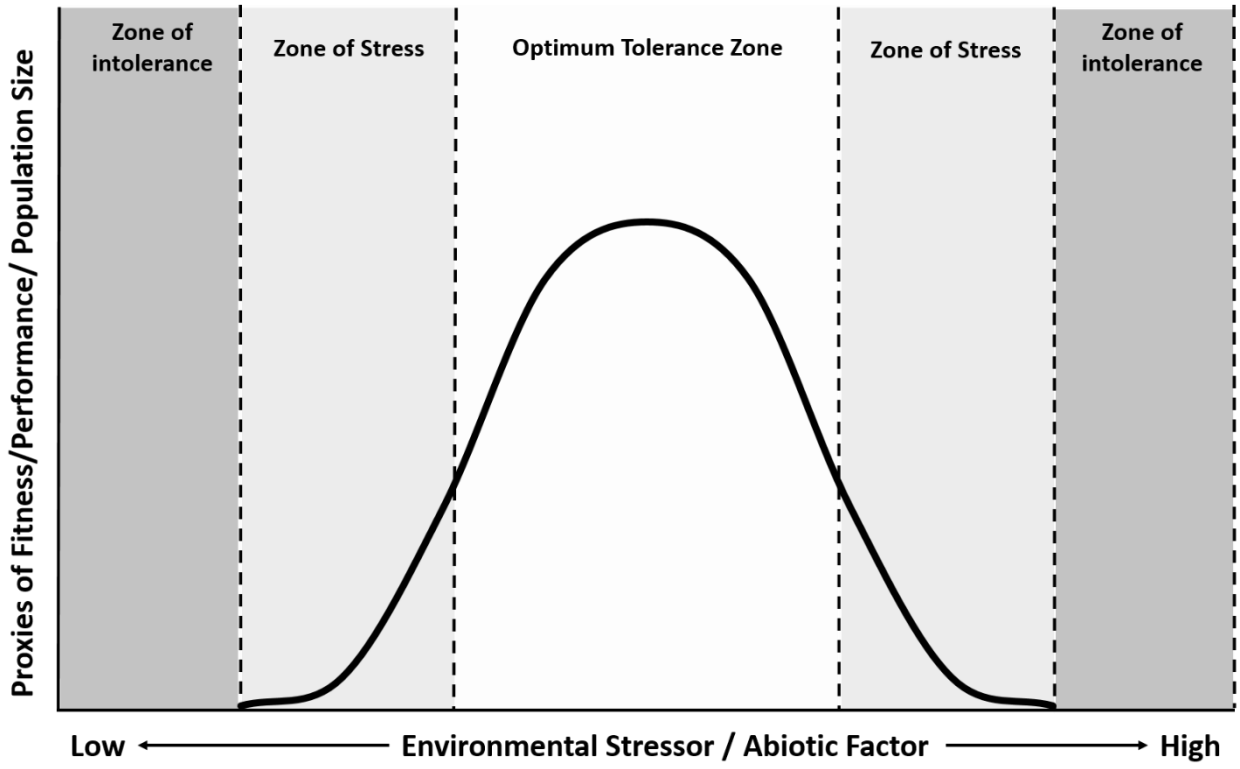

Figure 1. Conceptual model of a the law of tolerance, adapted and modified from Shelford (1931). Proxies of fitness, performance, or population size, are presented as a function of an environmental stressor such as salinity, temperature, or turbidity, results in a bell-shaped curve, which allows for the identification of different zones of tolerance.

1.4 Estuary Ecosystems and Important Stressors

Estuaries are a unique type of ecosystem denoted by the interface between coastal marine and freshwater habitats (Wołowicz et al. 2007), and provide recreational, cultural, and aesthetic services (Ghermandi et al. 2011), are of high economic value to fisheries (Houde and Rutherford 1993), and are characterized by some of the highest biodiversity and production in the world (Bianchi 2006). Several definitions of the term 'estuary' have been proposed over the past century (Elliott and McLusky 2002; Hobbie 2000; Pritchard 1967), however, recently the term has been defined as 'a coastal indentation that has a restricted connection to the ocean and remains open at least intermittently.' (Snedden et al. 2012). An estuary is organized in three regions: '1.) A *tidal river zone* or fluvial zone characterized by lack of ocean salinity but subject to tidal rise and fall of sea level. 2.) A *mixing zone* (the estuary proper) characterized by water mass mixing and the existence of strong gradients of physical, chemical, and biotic quantities reaching from the tidal river zone to the seaward location of a river mouth bar or ebb-tidal delta. 3.) A *nearshore turbid zone* in the open

ocean between the mixing zone and the seaward edge of the tidal plume at full ebb tide.' (Snedden et al. 2012) (Figure 2).

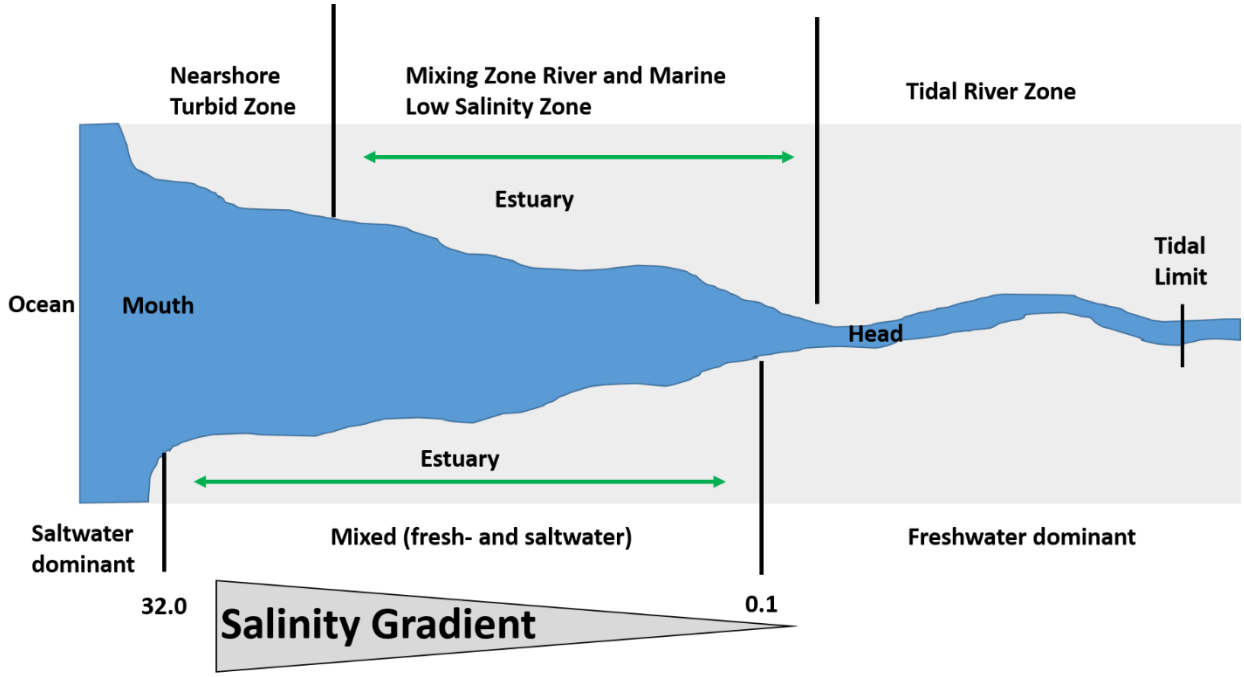

Figure 2. Conceptual model of the generalized process and salinity gradient of an estuary (Jackson 2013). Modified from Pritchard (1967) and Dalrymple et al (1992), depicting the different regions of an estuary.

It needs to be emphasized that boundaries between these zones are highly variable and positions of all zones change continuously depending on tidal cycles as well as annual cycles and long-term geological timescales (Snedden et al. 2012). Due to zonation, estuaries should be viewed as natural continua from fresh water streams to sea without clear separation, instead of isolated biological units (Wołowicz et al. 2007). Due to complex hydrodynamics consisting of freshwater outflow, saltwater intrusion and tidal influence, these ecosystems are extremely variable over space and time, and abiotic interacting factors such as salinity, suspended materials (turbidity), temperature and oxygen can, and do change rapidly (Bianchi 2006; Wołowicz et al. 2007). Because of the highly variable ambient living conditions, estuaries are considered as extreme environments with increased ecological stress on species communities, which make them highly interesting research sites for studies on comparative behavior, ecophysiology, and ecotoxicology (Wołowicz et al. 2007).

Estuaries are among the most biologically productive environments in the coastal zone and provide spawning grounds for numerous fish species (Jackson 2013). The wetlands function as traps of sediments, nutrients, and runoff, acting as natural filters to maintain water quality (Jackson 2013). Over the past century, estuaries worldwide faced immeasurable human impacts, causing additional anthropogenic stressors to evolve and persist, which lead to severe decline in biodiversity (Kennish

2002; Lotze et al. 2006). Marine pollution, nutrient enrichment and organic carbon loading (eutrophication), chemical contaminants, fisheries exploitation, invasive and introduced species, fresh water diversions, shoreline development and dredging, habitat alteration, habitat degradation and loss have been identified in the past, and are predicted in the future, to pose main threats to estuaries (Kennish 2002), together with sea level rise, land subsidence, and anthropogenic climate change (Harley et al. 2006; Scavia et al. 2002). Abiotic factors, combined with anthropogenic influences, create challenging conditions of interacting stressors determining an organism`s survival in these habitats.

Taking all these aspects into account it becomes clear that in estuaries turbidity, salinity, and anthropogenic stressors in form of contaminants deserve specific explanation, definition, and description. An overview about the nature of each factor and its impact on the ecosystem is given below.

1.4.1 Turbidity

Turbidity, often explained as the cloudiness of water, is defined as an expression of the optical property causing light scattering and light absorption, rather than direct light transmission through a water sample (Rice et al. 1994). To measure turbidity in water samples, multiple measurement techniques such as Jackson candle method (Jackson turbidity unit), Nephelometer (nephelometric turbidity unit), transparency tube, and secchi disk/depth have been used historically and recently (Myre and Shaw 2006). Turbidity depends on a number of interacting abiotic and biotic factors which jointly determine the properties, and resulting impact on the habitat and associated organisms. Important factors contributing to the effects of turbidity are thus light intensity, suspended material, and water depth (Lee and Rast 1997), which can vary locally depending on habitat structure. The suspended material can consist of inorganic material e.g., sediments such as clay, silt, and sand (USEPA 1999) and of organic material such as zooplankton, phytoplankton, algae, and detritus. Rivers transport suspended material to the ocean in a periodic manner which depends on seasonal (Tamura et al. 2010) and long-term (Inman and Jenkins 1999) changes. Transport of sediment particles from the head waters of rivers to the estuary is dependent on basin geology (determined by erodibility of sediments, size, and relief), water flow, anthropogenic flow alterations to the drainage basin (land use and land cover) or the river channel (damming, diking, diversions, concretization) (Inman and Jenkins 1999). In an estuary, the deposition, transport, and entrainment of sediments are determined by river morphology, ebb-tide scenarios, and wave process, all operating over different time scales (Nichols and Biggs 1985). Zooplankton often has a high biomass

in estuaries, and species composition varies within each estuary based on geography, temperature, and salinity regime (Benfield 2012). Phytoplankton and algae are important for the primary production and are crucial in the carbon and nutrient cycle, along with production of oxygen (Paerl and Justić 2012). Furthermore, in most coastal ecosystems approximately 50 % of the primary production can be attributed to phytoplankton (Harding Jr et al. 2002). Fast growth rates allow phytoplankton communities to spread very far in a short amount of time, and to potentially form dense 'blooms' which can affect water quality and eventually discolor affected waters (Paerl 1988). Estuarine turbidity can be caused by chlorophyll, algal photo pigments and other colored organic material (Gallegos et al. 1990).

Another characteristic that affects the turbidity in an estuary is the region of Estuarine Turbidity Maximum (ETM), which is defined as the area where the concentration of the suspended material is 10-100 times higher, than in adjacent riverine or coastal regions of the estuary (Schubel 1968), and roughly tracks the salinity boundary between fresh water and sea water (Bianchi 2006). It has been suggested that the particle density in ETMs is caused by a complex suite of mechanisms such as gravitationally induced residual currents, tidal asymmetry, and tidal straining of particles on the ebb tide (reviewed in Bianchi 2006). ETM zones are very important for larval life stages of fishes (Dauvin and Dodson 1990; Dodson et al. 1989; North and Houde 2001; Shoji et al. 2005; Sirois and Dodson 2000a; Sirois and Dodson 2000b) as they provide a high density of phytoplankton and zooplankton biomass, and reduced predation risk due to turbid conditions (Chesney 1989).

Turbidity may affect aquatic organisms in multiple ways; for example, by decreasing light intensity leading to decreased primary production and less food availability, reduced visibility of prey, reduced food availability in the benthos due to smothering, or by clogging gill filaments and gill rakers leading to respiratory impairments, and can also reduce avian, piscivory and mammal predation (Bruton 1985). In some cases turbidity, suspended solids, and siltation have been classified as a pollutant (Kennish 1997). Thus, turbidity is a significant parameter in ecosystems impacting aquatic habitats at multiple levels of biological organization.

1.4.2 Salinity

Sea water contains a distinctive composition of dissolved solids and gases, and a varying quantity of suspended organic and inorganic matter (Bianchi 2006; Nicol 1960). The two most important substances are Sodium (Na^+) and Chloride (Cl^-), which represent about 85 % of the total composition of sea salt (Nicol 1960). Salinity was first defined by Forch et al. (1902) in the early 20th century as

'the weight in grams of the dissolved inorganic matter in one kilogram of seawater after all bromide and iodide have been replaced by the equivalent amount of chloride and all carbonate converted to oxide'. Later assessments measured salinity in relationship to chlorinity, and many studies present salinity values this way (Bianchi 2006). In 1978 salinity was newly defined by taking the conductivity ratio between the water sample and a standardized potassium chloride (KCl) solution into account, and was referred to as *practical salinity scale* (Bianchi 2006; Lewis 1980). According to the new salinity scale, the standard seawater sample with a salinity (*S*) of 35 (without ‰ units) has a conductivity ratio of 1 at 15°C and 1 atmosphere, using a potassium chloride (KCl) solution of 32.4356 g in a 1 kg mass of solution (Bianchi 2006; Lewis 1980).

Salinity is crucial to estuarine circulation and biological productivity (Jackson 2013), and estuaries have been classified based on prevailing salinity structures. Four different types can be distinguished: a) *well mixed estuaries* (minimal vertical stratification in salinity), b) *partially mixed estuaries* (to some degree limited vertical mixing), c) *highly stratified estuaries* (lower fresh water discharge than sea water), and d) *salt wedge estuaries* (e.g., fjords) (Bianchi 2006; Jackson 2013). Furthermore, salinity represents an abiotic factor of critical importance to aquatic organisms (Nicol 1960), affecting osmoregulation. Osmoregulation is defined as 'the maintenance of consistent cellular or organismal fluid composition and volume' and represents a crucial and fundamental process (Evans 2011). If a fish is in freshwater, it is susceptible to gain water and lose ions by the passive transport along the concentration gradient, whereas if the fish is in salt water, it is susceptible to lose water and gain ions (Schulte 2011). Both scenarios require different compensation mechanisms. In freshwater the fish takes up ions actively through the gills and the intestine, whereas the fish in saltwater actively excretes ions and drinks water to compensate for the imbalance of homeostasis (Schulte 2011). Most of the fish species possess adaptive mechanisms allowing them to cope with a fresh water or sea water environment on a very limited tolerance range to salinity, a quality referred to as 'stenohaline' (Evans 2011; McCormick et al. 2013; Schulte 2011). Conversely, a few species possess the ability to respond to a wide range of environmental salinities, using adaptive mechanisms; a characteristic called 'euryhaline' (Evans 2011; McCormick et al. 2013; Schulte 2011), making those species suitable for inhabiting estuaries and intertidal habitats (Schulte 2011), or allowing them to perform e.g., anadromous and catadromous migrations between fresh water and salt water (Cooke et al. 2011; Righton and Metcalfe 2011). If fish species are exposed to wide environmental changes in salinity, it requires tolerance or functional modes of regulation towards osmotic stress. This is an energetically costly challenging because salinity levels outside the tolerance range can affect the exchange of water across the cell membrane (Schulte 2011).

1.4.3 Pollution

Contaminants are one of the main threats to aquatic ecosystems and biodiversity (Dudgeon et al. 2006; Geist 2011) with numerous chemicals from different anthropogenic sources entering aquatic ecosystems worldwide (Scholz et al. 2012). Aquatic ecosystems can be polluted by non-point sources as well as point sources. Non-point sources of pollution are agricultural run-off, urban run-off, construction run-off, mining run-off, septic systems, landfills/spills, and silvicultural run-off, whereas point sources originate, for example, from municipal sewage treatment plants, industrial facilities, and combined sewer overflows (Kennish 2002; USEPA 2009). Because human population is highly concentrated at coastal areas (Vitousek et al. 1997) coastal and marine ecosystems such as estuaries are confronted with an increased load of anthropogenic contaminants. A major source of contaminants in estuaries is the effluent of wastewater treatment plants (Kennish 2002) which usually consists of a complex mixture of numerous pesticides, nutrients (Jassby 2008; Parker et al. 2012a), pharmaceuticals (Carballa et al. 2004; Corcoran et al. 2010; Daughton and Ternes 1999; Nakada et al. 2006; Snyder et al. 2003; Sumpter 2005), hormones (Huang and Sedlak 2001; Kolpin et al. 2002), pathogens (Levantesi et al. 2010; Toze 2006; Ye and Zhang 2011), and ammonia (Jassby 2008; Parker et al. 2012a). Wastewater effluent has been a research topic for decades and more recent studies have primarily focused on effects of endocrine disrupting chemicals (EDC) on aquatic organisms (e.g., Ankley et al. 2009; Brander 2013; Söffker and Tyler 2012). Due to the complex mixture of compounds with varying, mostly unknown and undetectable concentrations and effects, the effluent of a wastewater treatment plant can be considered a multiple stressor in the aquatic ecosystems. Additionally, wastewater effluent is one of the largest sources of ammonia in estuaries (Kennish 2002; Parker et al. 2012a). Ammonia has been demonstrated to have detrimental effects on fish that includes impaired cell membrane permeability/transport leading to elevated energy consumption (Martinelle and Haggstrom 1993), histological alterations such as gill lamellae fusions, glomerular nephritis, and hydropic degenerations, resulting in negative effects on the immune system making the fish more susceptible to pathogens and parasites (Benli et al. 2008; Evans et al. 2006). Given its presence in water bodies worldwide due to the discharge of wastewater effluent and its effects on fish, ammonia is an important factor to be considered in contaminant effect assessments in fish.

This dissertation investigates the effects of two abiotic factors turbidity, salinity and their combination as multiple stressors and of an anthropogenic stressor of growing concern pollution, on an estuarine fish species of conservation concern, the Delta Smelt (*Hypomesus transpacificus*).

1.5 Delta Smelt, *Hypomesus transpacificus*: Species Biology, Habitat, Status, and Threats

1.5.1 Species Biology and Habitat

The Delta Smelt (*Hypomesus transpacificus*) belongs to the family of the *Osmeridae* and is a translucent fish with a cucumber-like smell. This pelagic fish species is endemic to the San Francisco Estuary (SFE hereon) and Sacramento-San Joaquin Delta (Delta hereon), has predominantly an annual life cycle and grows up to a length of 60-70 mm Standard Length (SL), sometimes reaching up to 120 mm SL, when reaching the second year (Bennett 2005). As a euryhaline fish species, adult Delta Smelt are mostly found in waters having a salinity of 2-6 ppt but recent findings indicate that a portion of the population remains freshwater year around (Sommer et al. 2011). Their preferred rearing grounds are in shallow (< 3 m), open waters of the estuary where fresh water and brackish water mix. Delta Smelt are relatively poor swimmers, usually found in low flow velocity zones, swimming in short strokes followed by rest periods (Swanson and Young 1998). Delta Smelt are planktivorous feeders and primarily prey on copepods, cladocerans, and amphipods. Beginning in September and October, the adult fish migrate from the Western part of the estuary at a speed of about 1.8-6.3 km per day (Sommer et al. 2011) to the fresh water region which can take several months (Moyle 2002). Spawning usually takes place from March to June (Bennett 2005) and occurs at night (Baskerville-Bridges et al. 2005; Lindberg et al. 1997; Mager et al. 2004). A simplified conceptual model of the Delta Smelt`s life cycle is shown in Figure 3.

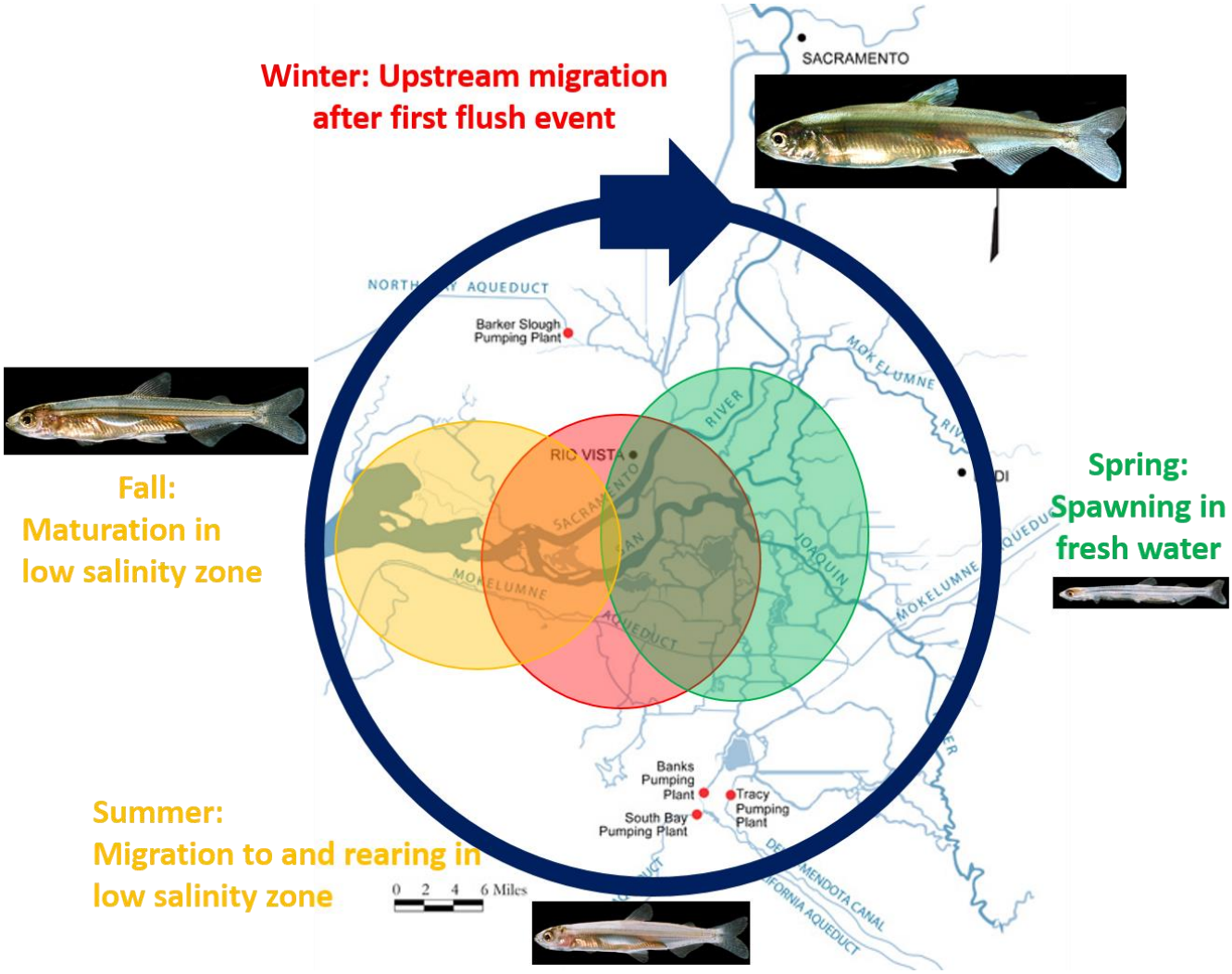

Figure 3. Delta Smelt life cycle relative to seasonal and geographic distribution; simplified and modified from Bennett (2005). Colors correspond with life stage and season, and changing position of the low salinity zone. Delta Smelt photos source: Rene Reyes US Department of the Interior, Bureau of Reclamation.

Delta Smelt eggs are demersal and adhesive, sticking by means of a tiny stalk to hard substrates, and are usually broadcast to bottom substrate, during the spawning event. Depending on water temperature, the eggs hatch after 9-13 days at 14.8-16.5 °C (Mager et al. 2004) or 8-10 days at 17 °C (Baskerville-Bridges et al. 2005) with feeding starting 4-5 days later. Freshly hatched larvae possess a lipid globule allowing them to stay off the bottom (semibuoyant) and allowing them to feed on microscopic prey such as rotifers. With the development of fins and the swim bladder (20-25 days post hatch; dph) they become functionally buoyant; the activity level and mobility of the larvae increases, hence fish develop the ability to move higher in the water column off the substrate (Mager et al. 2004). It has been demonstrated that the larval stage requires a certain turbidity to elicit a feeding response (Baskerville-Bridges et al. 2004; Baskerville-Bridges et al. 2005; Lindberg et al. 2013). More buoyant larvae (age 40-60 dph), with a total length of 16-18 mm, are carried by

currents downstream into the mixing zone where they manage to stay buoyant, likely benefitting from the ETM and the high productivity of phytoplankton and zooplankton (Moyle 2002; Moyle et al. 1992). Since this zone is characterized by a salinity of 0.5-6 ppt, the ETM, and is highly dynamic due to tidal and spatial cycles the Delta Smelt needs to cope with a variety of environmental stressors. Due to its annual life cycle, growth in Delta Smelt is accelerated with the major growth phase after fish reach 30 mm fork length and the variety of food prey increases (Grimaldo et al. 1998). Juvenile fish have a fork length of 40-50 mm by the beginning of August (Erkkila 1950). After that, growth slows down over the next months as energy resources are allocated in the development of gonads (Moyle 2002).

1.5.2 Status and Threats

The Delta Smelt was once abundant in the SFE and Delta (Erkkila 1950; Stevens and Miller 1983), but has been subject to decline over past decades (Moyle 2002; Moyle et al. 1992) with a steep step decline since the early 2000s (Feyrer et al. 2007; Sommer et al. 2007). It is listed as endangered and threatened under California State and Federal Endangered Species Acts, respectively (CDFW 2014; USFWS 1993). Because of its listing, and the continual decline in numbers, the Delta Smelt is considered an indicator species for the health of the SFE and Delta ecosystem. The SFE and Delta are among the most anthropogenically influenced and modified surface water systems in the world (Nichols et al. 1986). Compared to other estuaries, the SFE has experienced the largest increase in human population growth and exhibits the highest human population density living in regions in and adjacent to the ecosystem (Lotze et al. 2006). Habitat degradation, flow modification, pollution, introduced species, and overexploitation have been well documented (Nichols et al. 1986). For the Delta Smelt, several factors have been postulated to cause the observed decline.

Entrainment losses due to water diversions is one factor, which is caused on the one hand by many small water diversions from farmers in the estuary as well as by the construction of two water projects, Central Valley Project (in 1937) and State Water Project (in 1960) (Hanak et al. 2011) diverting water to Southern California. Changing outflow conditions due to water diversions, severe weather conditions (drought, heavy rain fall) have been discussed as another factor that affects the Delta Smelt population (Moyle 2002). Another cause is habitat alterations such as land reclamation (dikes, ditches) and hydraulic mining debris (increased sediment transport) which caused severe degradation of the ecosystem (Dyke and Wasson 2005; Nichols et al. 1986), further reducing suitable habitat for the Delta Smelt.

Introduced species pose another threat. Overall 234 species have been introduced to the SFE and Delta with another 125 species being cryptogenic (Cohen and Carlton 1998). One of the most important invasions for the ecosystem was the introduction of the overbite clam (*Potamocorbula amurensis*) (Carlton et al. 1990; Nichols et al. 1990), which may indirectly impact the Delta Smelt`s decline by significantly decreasing the phytoplankton biomass (Jassby 2008), which in turn affected the zooplankton community (Winder and Jassby 2011), and thus altering the food availability and composition for this fish. There is some evidence that competition with, and predation by, introduced species also play significant roles; e.g., the inland silverside (*Menidia audens*) has been found to prey on Delta Smelt (Baerwald et al. 2012). Another factor is the loss of genetic integrity due to hybridization with introduced Smelt species such as the Wakasagi Smelt (*Hypomesus nipponensis*) (Fisch et al. 2014; May 1996; Trenham et al. 1998). With declining fish numbers, the Delta Smelt faces a bottleneck; resulting in loss in genetic diversity (Fisch et al. 2011).

Turbidity has been shown to be crucial to the Delta Smelt, and a potential factor determining distribution in its habitat. Two hypotheses have been postulated, potentially explaining how turbidity is important for the Delta Smelt. First Delta Smelt are thought to use the turbidity to hide from predators and second that turbidity is needed as visual contrast allowing for easy prey detection. It has been documented that the Delta Smelt is associated with turbid water (Bennett and Burau 2014; Feyrer et al. 2007; Grimaldo et al. 2009) and are usually found in turbidities > 12 NTU (Sommer and Mejia 2013). In addition studies on the feeding response of early life stages of Delta Smelt have found a positive relationship between turbidity and feeding behavior (Baskerville-Bridges et al. 2004; Baskerville-Bridges et al. 2005). It has been postulated that increased pulse turbidity from first flush events (first storms in the rainy season; autumn) is a cue for the annual spawning migration to the Northern Delta (Grimaldo et al. 2009; Sommer et al. 2011). To this date the relationship between the factor turbidity and its physiological impacts on the Delta Smelt are poorly understood and preferences and tolerance ranges for the fish are only loosely determined. There is a great lack of studies, investigating the relationship between turbidity and this fish species, how turbidity is important, and what the effects on the physiological stress response are. Turbidity in the SFE and Delta is determined by the amount of suspended sediments (Cloern 1987; Ganju et al. 2007; Schoellhamer et al. 2012) as well as organic matter (McGann et al. 2013) such as phytoplankton (Jassby 2008) and zooplankton (Winder and Jassby 2011). Turbidity has decreased over the past decades (Feyrer et al. 2007; Jassby 2008; Jassby et al. 2002; Thomson et al. 2010) and several causes have been discussed. Introduction of invasive species such as clams (Carlton et al. 1990; Nichols et al. 1990) and waterweed (Ferrari et al. 2014; Yarrow et al. 2009) as well as dam construction and sediment trapping in reservoirs, reduced major flood events (Baxter et al. 2010; Jassby 2008; Jassby

et al. 2002; Wright and Schoellhamer 2004), nutrient forms and ratios (Glibert et al. 2011; Parker et al. 2012b), and herbicides (Blaser et al. 2011; Orlando et al. 2013) have been postulated as factors contributing to decreases in turbidity in the delta. This reduced turbidity is associated with low primary productivity, resulting in a food-limited estuary (Kimmerer et al. 2005), which in turn affects the abundance of planktivorous species in the SFE and Delta. The establishment of a culture facility, and culture techniques, has been an important step towards the conservation of this vulnerable fish species (Baskerville-Bridges et al. 2005; Lindberg et al. 1997; Lindberg et al. 2013; Mager et al. 2004). Two goals are pursued with launching the captive breeding program for Delta Smelt, 1) creation of a demographically and genetically robust population functioning as a genetic bank for the event of the species` extinction, and theoretically constituting a source of fish for restocking actions 2) supplying researchers with experimental fish without having to exploit valuable specimens from the wild (Fisch et al. 2013; Fisch et al. 2010; Fisch et al. 2009; Lindberg et al. 1997; Lindberg et al. 2013). There is a lack of studies that address important factors contributing to the decline of the Delta Smelt, and the mechanisms behind those factors are poorly understood. There is a need for fundamental research towards the identification of key stressors and threats, as well as their interactions. It is of utmost important that factors driving their population decline are understood, and detailed knowledge is required in order to sustainably manage and conserve this important and sensitive fish species, along with the entire estuary ecosystem.

Another important anthropogenic stressor are contaminants, of which numerous have been detected throughout the Delta and SFE (Parker et al. 2012a; Scholz et al. 2012; Weston and Lydy 2010), and are a potential contributor to fish population declines. The variety of chemical substances identified include mercury, organochlorides such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyl (PCB) (Thompson et al. 2000), metals (Hornberger et al. 1999; Luoma et al. 1990), pesticides from agricultural and urban sources (Amweg et al. 2005; Bergamaschi et al. 2001; Brander et al. 2013; Kuivila and Hladik 2008; Kuivila and Foe 1995b; Weston et al. 2009; Weston and Lydy 2010), pharmaceuticals from wastewater effluent discharge (Jeffries et al. 2015a), as well as nutrients and ammonium in form of effluent from municipal wastewater treatment plants (Nichols et al. 1986; Parker et al. 2012a). Some of these contaminants have been linked directly with detrimental effects to Delta Smelt (Connon et al. 2011a; Connon et al. 2011b; Kuivila and Moon 2004b; Scholz et al. 2012). Due to its brief life span and limited range in the SFE and Delta, the Delta Smelt is potentially continually exposed to numerous classes of contaminants throughout its entire life cycle (National Research Council 2012).

1.6 Goals and Objectives

Comprehensive knowledge and integration of information on autecology (habitat, distribution, life cycle, physiological response) and synecology (structure, development and distribution) of a species of conservation concern are fundamental to implement effective management and conservation efforts (Geist 2011). The overall goal of this study was to enhance the knowledge of the understanding of single and multiple stressors on an endangered fish species in heavily anthropogenically impacted ecosystems, the SFE and Delta. The study aims were two-fold: On one hand the study aimed to gain autecological information on abiotic factors turbidity and salinity towards the determination of the fundamental niche for Delta Smelt, and on the other to augment the knowledge on the understanding of effects of contaminants as potential anthropogenic stressors on the endangered Delta Smelt.

Turbidity and salinity requirements were assessed as a single and in combination, evaluating the effects on physiological stress at multiple levels of biological organization. With the acquired knowledge, this study aimed to define requirement and tolerance ranges for turbidity and salinity, and evaluate their biological significance for Delta Smelt in order to aid resource managers and conservationists to make informed decisions on management actions and conservation efforts.

In a multiple stressor scenario the effects of a contaminant mixture (wastewater effluent), with a special focus on ammonia, were determined, with the goal of identifying and understanding the physiological mechanisms affected by exposure to a mixture of environmentally relevant contaminants.

1.6.1 Confounding Factors

Before testing biologically relevant hypotheses on Delta Smelt, clearly defining the experimental conditions and fully developing all methodology was crucial. The goal of this chapter was to define suitable experimental conditions with minimal confounding stress, using stocking density as an example. A wide range of stocking densities for the experimental design were assessed using biochemical (cortisol) and molecular biomarkers of physiological stress as endpoints. A second goal of this study was to optimize cortisol measurement methods for whole body homogenates, as well as molecular biomarkers performance for physiological stress assessments.

Hypothesis 2.1: Because Delta Smelt are described as an aggregating pelagic species (Bennett 2005; Moyle 2002), the stress response will be elevated at low, as well as at high stocking densities.

Hypothesis 2.2: Stress biomarkers at multiple different levels of biological organization molecular and biochemical will deliver consistent results in physiological stress assessments.

1.6.2 Niche Assessments Turbidity

As highlighted above, turbidity is of crucial importance to the Delta Smelt. Thus the aim of this study was to test the effects of a range of environmentally relevant turbidities on the physiological stress responses of late-larval Delta Smelt, in order to evaluate turbidity requirements. A combination of mechanistic investigations (gene expression, plasma hormones and metabolites) were contrasted with ecological performance measures (feeding performance, and survival). Using a multiple level approach, the goal was to define the fundamental niche, and niche dimensions, for turbidity for late-larval Delta Smelt. Furthermore, the study aimed to demonstrate how the measurement of multiple endpoints at multiple levels of biological organization allow for integrative assessments of the factors effects on an organism, in order to make informed decisions in support of the species' conservation.

Hypothesis 3.1: Different levels of turbidity have an effect on the physiological stress response of Delta Smelt, which in turn affects feeding performance and survival.

Hypothesis 3.2: Several endpoints evaluated at multiple levels of biological organization (primary, secondary, tertiary stress response) deliver similar results, allowing for determination of tolerance ranges and niche dimensions.

1.6.3 Multiple Stressors: Turbidity and Salinity

One important source of turbidity in estuaries is the resuspension of sediments in the mixing zone of salt water and freshwater. The goal of this study was to test how turbidity and salinity, as single parameters, and in combination, affect the physiological response of Delta Smelt at multiple levels of biological organization. In an experimental matrix system, mechanistic investigations (i.e., measuring the stress response using gene transcription and cortisol), were combined with organismal and ecological measures of performance (i.e., feeding performance) under environmentally relevant combinations.

Hypothesis 4.1: The response of Delta Smelt to physicochemical parameters can be determined by measuring endpoints of different levels of biological organization.

Hypothesis 4.2: The existing interaction between turbidity and salinity affects the physiological response of the Delta Smelt differently than turbidity and salinity as a single stressor.

1.6.4 Multiple Stressors Contaminants

In this chapter the effects of contaminant mixtures on Delta Smelt were assessed. In a laboratory experiments Delta Smelt were exposed to river water samples from a site downstream of a wastewater treatment plant (Hood), and responses were compared to results from exposures to water samples from upstream of the wastewater treatment plant, water samples from upstream of the wastewater treatment plant spiked with ammonium chloride, water samples from upstream of the wastewater treatment plant diluted with wastewater effluent as well as to laboratory controls. Molecular biomarkers were used to identify significant effects of the physiological response, potentially explaining the mechanisms behind the response. One goal was to assess the water quality at Hood from a contaminant perspective. Additionally the study aimed for the identification of important metabolic pathways affected by the contaminant mixture.

Hypothesis 5.1: Ammonia in combination with an unknown mixture of contaminants from a waste water treatment plant exerts greater toxicity than as a single substance.

Hypothesis 5.2: Assessment of different mixtures of contaminants, which will result in distinct gene transcription patterns allow the identification of specific sources of contaminants.

2. Confounding Factors

A similar version of this chapter was submitted to Aquaculture.

Hasenbein, M., Fangue, N.A., Geist, J.P., Komoroske, L.M., Connon, R.E. (2015**),** *Physiological Stress Biomarkers reveal Stocking Density Effects in late larval Delta Smelt (Hypomesus transpacificus)*, *Aquaculture*: Accepted

Abstract

Suboptimal fish stocking densities in experimental systems may elicit stress responses that can affect experimental results. Fish species, age and size, water chemistry and flow, and physical characteristics of the experimental system (e.g., tank, cage) are among the parameters to be considered when determining stocking densities. However, systematic studies to define fish densities minimizing stress in experimental systems are rarely performed. This is particularly true when working with species of low aquaculture value or a non-model test species such as the Delta Smelt (*Hypomesus transpacificus*). The aim of this study was to use physiological stress biomarkers to determine suitable fish densities for specific experimental vessels routinely used for this species. We maintained late larval Delta Smelt (60 days post hatch; dph) over a period of 24h, at five different densities: 7, 14, 28, 42, and 56 fish per 8 L circular fish tank. We assessed whole body cortisol and transcriptomic biomarkers that lead to cortisol production to quantify stress levels. Both marker types delivered similar results. Cortisol levels were lowest at densities of 28 and 42 fish per tank, whereas lowest fish densities (7 and 14 fish per tank) evoked the highest stress levels. Genes such as Mineralocorticoid Receptor 1 and Glucocorticoid Receptor 2, as well as 11-Beta-Hydroxysteroid-Dehydrogenase-2 depicted lowest expression levels at stocking densities 28 and 42, and elevated expression levels for stocking densities 7 and 14. Our data support observations that late larval Delta Smelt should be exposed, acclimated, and cultured in groups rather than as individuals or in low numbers. This study indicates the importance of adequately defining experimental conditions that minimize stress, specifically when stress is measured as an endpoint. In addition to classical cortisol measurements, responses of the transcriptome also appear suitable in assessing stress responses in fish, and in determining optimal holding conditions, particularly if short-term responses are the study focus.

Keywords: Stocking Density, Molecular Biomarkers, Stress Response, Cortisol, Delta Smelt

Introduction

Fish stocking density is known to affect physiological and behavioral responses in fishes, and previous studies have utilized a variety of endpoints to measure these responses (Adams et al. 2007; Ellis et al. 2002; Martins et al. 2012; Turnbull et al. 2005). Commonly applied methods used in density studies include assessments of parameters involved in aquaculture production and nutritional status (mortality, body condition factor, food intake, food conversion rate, growth, and size variation), health condition (blood parameters, fin damage, gill condition, and spleen condition), as well as stress indicators (cortisol and glucose concentration, and oxygen consumption) (Ellis et al. 2002; North et al. 2006). Some endpoints, such as those involved in aquaculture production, nutritional status, health condition, and behavior can take longer to manifest and are thus predominantly applied to experiments lasting several weeks to months (Adams et al. 2007; Aketch et al. 2014; Alanärä and Brännäs 1996; Boujard et al. 2002; Brown et al. 1992; de Oliveira et al. 2012). In contrast, other parameters involved in the immediate stress response, e.g., cortisol levels and gene expression, change rapidly and can be used to determine short term stress and stress recovery at time points ranging from several hours to a few days (Caipang et al. 2008a; Caipang et al. 2008b; Fast et al. 2008). The use of molecular biomarkers as stress indicators in aquatic organisms has been successfully demonstrated in various studies addressing stress resulting from turbidity and salinity (Hasenbein et al. 2013), contaminants (Connon et al. 2011a; Garcia-Reyero et al. 2008; Geist et al. 2007), density effects (Caipang et al. 2008a; Caipang et al. 2008b; Gornati et al. 2004a; Gornati et al. 2004b; Salas-Leiton et al. 2010), as well as recovery from stress (Wiseman et al. 2007). Besides commonly assessed plasma cortisol levels (e.g., Pickering and Pottinger 1989), whole-body cortisol levels have also been used successfully to determine stress levels in fish (Cachat et al. 2010; Ramsay et al. 2006; Ramsay et al. 2009), but few studies have associated them with responses on the transcriptome.

Since stocking density impacts physiological and behavioral responses in fishes, defining suitable stocking densities is important in aquaculture, as well as in experimental investigations testing biologically-relevant hypotheses. In addition, studies on several different fish species using the same experimental protocol revealed species-specific differences in the intensity and direction of the stress response after density manipulation (Pottinger 2010). Stress and aggression can increase at low densities when social hierarchies instead of schooling behaviors occur. On the other hand, high stocking densities can also result in an increase in stress levels due to competition. Studies on different species and different experimental settings have indicated that both high and low stocking

densities can cause severe stress in fish (Aketch et al. 2014; Alanärä and Brännäs 1996; Brown et al. 1992; Costas et al. 2008; Jørgensen et al. 1993).

Generalized stress responses in fishes can be activated by diverse and sometimes subtle stressors (reviewed in Barton 2002; Wendelaar Bonga 1997), and any additional stress, such as one resulting from unfavorable stocking density, can act as a confounding factor impacting experimental results, which in turn can potentially lead to misinterpretation of data. Thus assessing density stress is particularly crucial when evaluating stress responsive parameters (e.g., cortisol, glucose) as experimental endpoints. Moreover, any experimental protocol should also try to minimize stress of the organisms for animal welfare reasons. Experimental stocking densities, however, are often exclusively based on numbers of individuals needed for specific assessments; e.g., pooling organisms to achieve a certain yield (Alsop and Vijayan 2008), accepted sample sizes (n-values) (Jensen 1972), or empirical observations (e.g., replication, independence of samples to increase statistical power). For instance, in eco-toxicological testing, the number of organisms used per treatment tends to follow specific guidelines such as standard protocols implemented by the U.S. Environment Protection Agency (USEPA 2002), the Organization for Economic Co-operation and Development (OECD 2006) or the International Organization of Standardization (ISO 2014). Furthermore, concerning the use of animals in experimentation, there is a requirement that researchers seek reasonable alternatives, in the absence of which, this obligation extends to minimizing numbers used (Schechtman 2002).

Experimental protocols do not always account for the physiological impacts of stocking density. For example, Oikari (2006) reviewed the use of caging techniques in field-based eco-toxicological studies, and highlighted that stress susceptibility in fish is rarely incorporated into toxicological evaluations. The review further indicates that test acceptance standards are usually based on survival, regardless of density, thus somewhat biased results are likely accepted. Oikari (2006) further recommends the development of suitable and stress-free stocking densities, in particular when assessing physiological responses associated with organismal stress as an endpoint to answer a specific research question.

A wealth of information on stocking density effects is available for aquaculture-relevant species, from which experimental stocking densities that minimize stress can be derived. Examples of such species include salmon (*Salmo salar*) (Adams et al. 2007; Turnbull et al. 2005; Turnbull et al. 1998), rainbow trout (*Oncorhynchus mykiss*) (Ellis et al. 2002), artic charr (*Salvelinus alpinus*) (Brown et al.

1992; Jørgensen et al. 1993) or model species such as zebrafish (*Danio rerio*) (Gronquist and Berges 2013; Pavlidis et al. 2013; Spence and Smith 2005), fathead minnow (*Pimephales promelas*) (Ankley and Villeneuve 2006; Smith et al. 1978), and Japanese medaka (*Oryzias latipes*) (Davis et al. 2002; USEPA 1991). When working experimentally with non-model and/or endangered fish species, data on optimal stocking densities may be limited, and thus important to determine. Here, we use the Delta Smelt (*Hypomesus transpacificus*) as an example, non-model fish to demonstrate the importance of stocking density determination in experimental biology.

The Delta Smelt is a fish species endemic to the Sacramento-San Joaquin River Delta, California, USA, and was listed as threatened in 1993 under both State and the Federal Endangered Species Act (CDFW 2014; USFWS 1993) and as endangered under California State Endangered Species Acts in 2010 (CDFW 2014). In response to the specie's decline, the University of California, Davis Fish Conservation & Culture Laboratory (FCCL) was founded with the mission to develop intensive fish culture techniques to create a genetically and demographically robust captive population and provide a supply of fish for research purposes (Fisch et al. 2013; Lindberg et al. 2013). Information on appropriate stocking densities for this species beyond those used for facility production (Baskerville-Bridges et al. 2005; Lindberg et al. 2013) is still limited, yet there is an increasing number of studies that use this species in eco-toxicological and eco-physiological testing (Connon et al. 2011a; Connon et al. 2011b; Connon et al. 2009; Hasenbein et al. 2013; Komoroske et al. 2014; Swanson et al. 2000; Swanson and Young 1998). We have used this species towards establishing links between density-dependent physiological stress responses, with the aim of estimating the stocking densities that minimized stress in late larval Delta Smelt for a commonly used experimental design. Because Delta Smelt are described as an aggregating pelagic species (Bennett 2005; Moyle 2002), we hypothesize that the stress response will be elevated at low densities, as well as at high stocking densities. Therefore, this study aims to determine suitable densities to be used for experimental designs.

We measured whole body cortisol levels and compared those with quantification of stress-related molecular biomarkers (gene transcription levels) involved in cortisol production; a key function of the Hypothalamic-Pituitary-Interrenal (HPI)-axis, in order to determine lowest density-dependent stress as indicative of optimal test conditions. This study demonstrates that adequately defined experimental conditions, to the end that stress is reduced to a minimum, are crucial to experimental biology.

Material & Methods

Fish exposures

Fish and experimental space were provided by the University of California, Davis Fish Conservation and Cultural Laboratory (FCCL; Byron, CA, USA). Late larval Delta Smelt (60 dph) were exposed in triplicate, in aerated facility water at densities of 7, 14, 28, 42, or 56 fish per black circular fish tank (8L) (2 Gallon Black Plastic Pail; Item # 3539; United States Plastic Corporation®, USA) for a period of 24h at a light dark cycle of 16h : 8h. Fish were fed prior to the experiment, but were kept unfed throughout the test duration in order to avoid stress variability related to feeding. All handling, and experimental procedures were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC Protocol # 16591). Dissolved oxygen, salinity, specific conductance, pH, turbidity, ammonia (total ammonia-N), and light intensity were monitored at test initiation and at test termination (Table 1). Temperature was recorded throughout the experiment using iBCod submersible temperature loggers (Alpha Mach Inc., Ste-Julie, QC, Canada). Tank control fish were sampled using a 1 L beaker directly from the facility rearing tank previous to test initiation to determine baseline stress, following the same process used to transfer fish to the experimental tanks. At experiment termination, fish were immediately euthanized with an overdose of 50 mg × L^{-1} tricaine methanesulfonate (MS-222; Finquel, Argent Laboratories, Redmond, WA, USA), at neutral pH, buffered with sodium bicarbonate. Whole fish were transferred into 1.5 ml microcentrifuge tubes and snap frozen in liquid nitrogen. Samples were stored at -80°C for subsequent biochemical and molecular analysis.

Cortisol assessments

Whole body cortisol was assessed in a total of nine fish from each treatment (three per replicate), using methods established for zebrafish (Alsop and Vijayan 2008; Cachat et al. 2010); volumes of solutions used were optimized for use in Delta Smelt (Hasenbein et al. 2013). In brief, samples were defrosted on ice and homogenized for 2 min in 1 ml ice-cold 1 × PBS buffer (Phosphate buffered Saline, Bio Ultra, Sigma Aldrich®, Lot # BCBD7118) using a TissueLyzer LT (Qiagen©, Venlo, Limburg, Netherlands) at a frequency of 50 Hz. The resulting homogenate was divided in equal amounts of 500 µl, and used for cortisol and total protein determination. Samples were processed at 4°C throughout the complete extraction procedure. Cortisol was extracted from the homogenate by adding 2.5 ml diethyl ether (VWR International LLC, Radnor, PA, USA) and subsequent vortexing for 1 minute. A total of three washing steps with diethyl ether were performed in order to achieve maximum yield. After each washing step, samples were centrifuged immediately for 7 minutes at 3200 g at 4°C using a refrigerated centrifuge (Sorvall Biofuge Primo R, Kendro Laboratory Products,

Hanau, Germany). The supernatant was transferred to a 15 ml Pyrex glass tube for diethyl ether evaporation, which was performed using an air pump with a 0.22 µm Millipore Express filter (EMD Millipore Corporation, Billerica, MA, USA). Dried samples were resuspended in 200 µl ice-cold 1 × PBS buffer and incubated overnight at 4°C for resuspension. Cortisol assays (Salivary Cortisol, Enzyme Immunoassay Kit, Salimetrics, Inc., State College, PA, USA) were performed according to manufacturer`s instructions, and cortisol levels (mg × dL^{-1}) were calculated with a four-parameter sigmoid standard curve (minus curve fit). Cortisol levels were normalized to total protein and denoted as cortisol concentration (pg cortisol × µg $protein^{-1}$). The second half of the homogenate was used to determine protein content. Following centrifugation at 16500 g for 30 min at 4°C (Beckman Allegra 21R Centrifuge, Beckman Coulter Inc., Indianapolis, IN, USA) the supernatant of each sample was collected and used for total protein content determination following manufactures' protocol (BCA Protein Assay Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Quantitative PCR

Total RNA was extracted from whole-body homogenates. RNA extractions were performed according to manufacturer's protocols using the RNeasy Mini Qiacube Kit (Qiagen©, Venlo, Limburg, Netherlands) utilizing a Qiacube (Qiagen©, Venlo, Limburg, Netherlands). Qualitative and quantitative RNA determination was conducted using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA); 260/280 and 260/230 ratios ranged from 1.98-2.20 and 1.45-2.35, respectively. Integrity of total RNA was assessed by electrophoresis on a 1% (w/v) agarose gel stained with SYBR Dye (SYBR Safe DNA Gel Stain, Invitrogen™, Life Technologies™, Carlsbad, CA, USA). Eight to nine fish per treatment (2-3 from each replicate dependent on survival) were assessed by quantitative polymerase chain reaction (qPCR). Complementary DNA (cDNA) synthesis was performed with 1 µg total RNA per sample, using Reverse Transcriptase III (SuperScript® III Reverse Transcriptase, Invitrogen™, Life technologies™, Carlsbad, CA, USA). Primer and probes for qPCR analyses were designed using Roche Universal Library Assay Design Center (https://www.roche-applied-science.com). Designed primers were purchased through Eurofins MWG Operon (http://www.eurofinsdna.com), and fluorescent probes were obtained from Applied Biosystems (Applied Biosystems®, Life technologies™, Carlsbad, CA, USA). Quantitation of transcription was performed using SDS 2.4 software (Applied Biosystems®, Life technologies™, Carlsbad, CA, USA). Responding genes were normalized using a normalization factor calculated based on the geometric mean of two control genes; *Glyceraldehyde-3-phosphate Dehydrogenase* (GAPDH), *Elongation-factor alpha* (EF-α). Normalization was performed according to the "geNorm" algorithm version 3.5 as described in Vandesompele et al. (2002). Genes were selected based on

their involvement in the HPI-axis (Table 1). *Pro-Opiomelanocortin* (POMC) is the precursor of Adrenocorticotropic Hormone (ACTH) that binds to Melanocortin Receptor 2 (MC2R) in interrenal cells, which in turn stimulates cortisol production through adenylate cyclase and cAMP-dependent signaling pathways (Alsop and Aluru 2011). Cortisol signaling is further mediated by two important receptors, *Glucocorticoid Receptor 2* (GR2), and *Mineralocorticoid Receptor 1* (MR1), which are both ligand-activated transcription factors (Alsop and Vijayan 2008; Prunet et al. 2006). Mineralocorticoids and glucocorticoids bind to MR1 and GR2 inducing the stimulation or repression in transcription of target genes (Tomlinson et al. 2004). *11-Beta-Hydroxysteroid-Dehydrogenase-Type1* (11-Beta-HSD-1) and *11-Beta-Hydroxysteroid-Dehydrogenase-Type2* (11-Beta-HSD-2) are the genes coding for two enzymes which are directly involved in the activation and inactivation of glucocorticoids; e.g., cortisol (Krozowski et al. 1999). In particular, 11-Beta-HSD-1 is a bidirectional enzyme that functions first and foremost as a reductase by converting cortisone into cortisol and also can function as a dehydrogenase by converting cortisol into cortisone (Krozowski et al. 1999; Tomlinson and Stewart 2001). In contrast, 11-Beta-HSD-2 is a unidirectional enzyme that converts cortisol into cortisone (Krozowski et al. 1999; Tomlinson and Stewart 2001), and protects MR1 from occupation by cortisol (Stewart and Mason 1995; Tomlinson and Stewart 2001). In addition *Glutathione-S-Transferase* (GST) a key enzyme for cellular detoxification (Choi et al. 2008) was measured. GST defends cells against Reactive Oxygen Species (ROS) and responds to osmotic stress in fishes (Choi et al. 2008).

Table 1. Primer and probe sequences of genes used as molecular biomarkers to determine stress levels in late larval Delta Smelt (Hypomesus transpacificus).

Gene name	Gene code	Primer 5'→3'	Primer 3'→5'	Probe #	% Efficiency
Glutathione-S-Transferase	GST	aatctccctggcagacattgtt	ggccggctctcaaacacat	127	108
Mineralocorticoid Receptor 1	MR1	tttctacactttccgcgagtca	tgatgatctccaccagcatctc	39	99
Glucocorticoid Receptor 2	GR2	catcgtgaagcgtgaggagaa	tgcatggagtccagtagtttgg	129	98
Pro-opiomelanocortin	POMC	tgttcacctgtgcaggtctga	gagaagctctcttccgtggaca	127	102
11-Beta-Hydroxyteroid-Dehydrogenase Type 1	11-Beta-HSD-1	cgtgtcgtctctgctggcta	ggcgaacttggtggaggag	55	109
11-Beta-Hydroxyteroid-Dehydrogenase Type 2	11-Beta-HSD-2	tcctgccatcctcctacaagac	tctggaccaggtgtttgaactg	14	106
Beta Actin	β-actin	tgccacaggactccatacc	catcggcaacgagaggtt	11	107
Glyceraldehyde 3-phosphate dehydrogenase[b]	GAPDH	tccacgagaaagacccaact	cacgccagtagactcaacca	159	95

Statistical Analysis

Data analysis was performed using R version 3.0.2 for statistical computing (R-CoreTeam 2014) and associated packages. Cortisol data and gene expression data were tested for normal distribution using the Shapiro Wilk normality test and homogeneity of variances was determined using the Fligner-Killeen Test. Cortisol data were log-transformed to meet assumptions of normality. Effects of density on cortisol levels and transcription of individual genes were evaluated with nested analysis of variance (ANOVA) tests using lme4 (Bates et al. 2014) as described in Ruohonen (1998). Density was defined as a fixed categorical main effect, and experimental replicate (bucket) was treated as a random effect nested within each density treatment. Statistical significance was determined using a significance level of α=0.05 for all tests. Post Hoc contrasts were tested using lmerTest (Kuznetsova et al. 2014) to determine pairwise significant differences where appropriate. Principal Component Analysis (PCA) was performed on the normalized qPCR dataset in order to analyze transcription patterns of all genes measured. PCA scores were calculated using the covariance matrix. Principal component 1 and 2 were determined to explain the majority of the variation in the data, using a Scree Test (Scree plot) as described in (D'Agostino and Russell 2005).

Results

Physicochemical Parameters

Dissolved oxygen, specific conductance, salinity, pH-value, and turbidity remained stable across all densities and replicates over the 24h test period (Table 2). Light intensity was 40.3 Lux (SD± 7.9). Final ammonium concentrations across densities 7 to 56 ranged from 0.15 to 0.26 mg × L^{-1}.

Table 2. Physicochemical water parameters. Means with standard deviations across all densities and replicates over the 24h exposure time.

Parameter / Time point	Mean / SD±	Facility water	Density 7	Density 14	Density 28	Density 42	Density 56
DO mg × L^{-1} / T0	Mean	9.8	9.5	9.4	9.4	9.2	9.1
	SD±	0.3	0.0	0.0	0.0	0.1	0.1
DO mg × L^{-1} / T24	Mean	N/A	10.1	10.0	10.0	9.9	9.9
	SD±	N/A	0.1	0.1	0.1	0.1	0.1
SC µS × L^{-1} / T0	Mean	497	494	499	496	493	496
	SD±	4	1	1	4	2	2
SC µS × L^{-1} / T24	Mean	N/A	500	501	499	498	502
	SD±	N/A	3	3	3	2	2
pH / T0	Mean	7.9	8.0	8.0	8.0	8.0	8.0
	SD±	0.2	0.0	0.0	0.0	0.1	0.3
pH / T24	Mean	N/A	7.7	7.7	7.8	7.8	8.0
	SD±	N/A	0.0	0.0	0.1	0.1	0.2
Sal PSU / T0	Mean	0.2	0.2	0.2	0.2	0.2	0.2
	SD±	0.0	0.0	0.0	0.0	0.0	0.0
Sal PSU / T24	Mean	N/A	0.2	0.2	0.2	0.2	0.2
	SD±	N/A	0.0	0.0	0.0	0.0	0.0
Tur NTU / T0	Mean	13.1	13.7	14.0	13.6	13.5	14.0
	SD±	0.7	0.1	0.4	0.9	0.4	0.5
Tur NTU / T24	Mean	N/A	12.5	13.5	12.9	13.0	13.4
	SD±	N/A	0.5	0.2	0.6	0.3	0.6
TAN mg × L^{-1} / T0	Mean	0.1	0.1	0.1	0.1	0.1	0.1
	SD±	0.0	0.0	0.0	0.0	0.0	0.0
TAN mg × L^{-1} / T24	Mean	N/A	0.2	0.2	0.2	0.2	0.3
	SD±	N/A					
T °C	Mean	16.6	15.8	15.8	15.8	15.8	15.8
	SD±	0.2	0.7	0.7	0.7	0.7	0.7

Abbreviations: DO (Dissolved Oxygen, mg/L), SC (Specific Conductance µS/cm adjusted to 25°C), pH-value, Sal (Salinity, Practical Salinity Unit; PSU), Tur (Turbidity, Nephelometric Turbidity Units; NTU), TAN (Total Ammonia-N), T (Temperature °C), SD± (Standard Deviation). N/A indicates values are not applicable. T0 = Time point at test start, T24 = Time point at test termination.

Cortisol

Cortisol levels were highest (up to 0.700 pg cortisol × µg protein^{-1}) at lowest fish densities (7 and 14 fish per vessel) and decreased with increasing fish densities, with only 0.193 pg cortisol × µg protein^{-1} measured at a density of 42 specimens per tank (Figure 4). At a further increased fish density to 56 specimens per tank, cortisol levels again increased to a similar level as with 28 fish per tank. Normalized cortisol levels (mean ± SD) are listed in table 3. Detailed statistical information is listed in the appendix table 14 (Table 15).

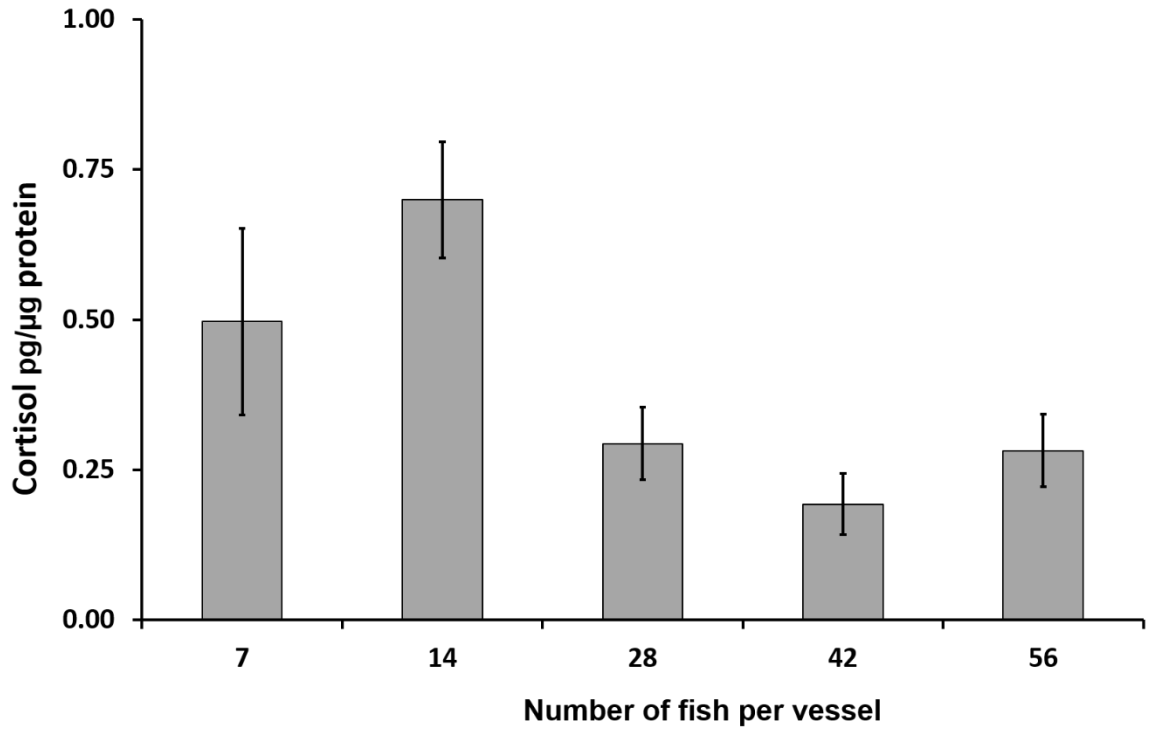

Figure 4. Whole body cortisol levels for different Delta Smelt densities in pg cortisol × µg protein-1. Arithmetic mean across replicates (N=3) with bars indicating standard errors.

Quantitative PCR

Significant differences were detected for GR2 between densities of 7 and 42 fish (pairwise P=0.002) and densities 14 and 42 (pairwise P=0.021) (Figure 5) with density 42 having the lowest expression levels and density 7 having the highest expression levels. Although not statistically significant, visual comparison of cortisol levels with mRNA expression levels of 11-Beta-HSD-2 revealed a similar pattern for densities 7 to 42 (Figure 4 and Figure 5). Furthermore, mRNA expression patterns of genes involved in cortisol production and regulation such as MR1, GR2, and 11-Beta-HSD-2 exhibited lowest expression levels in densities 28 and 42, but were not significant, and POMC a gene involved in initiating and stimulating the cortisol synthesis, had lowest expression levels at density 28. Relative transcript levels of genes (mean ± SD) are listed in Table 3. Detailed statistical information is listed in the appendix table 15 (Table 15).

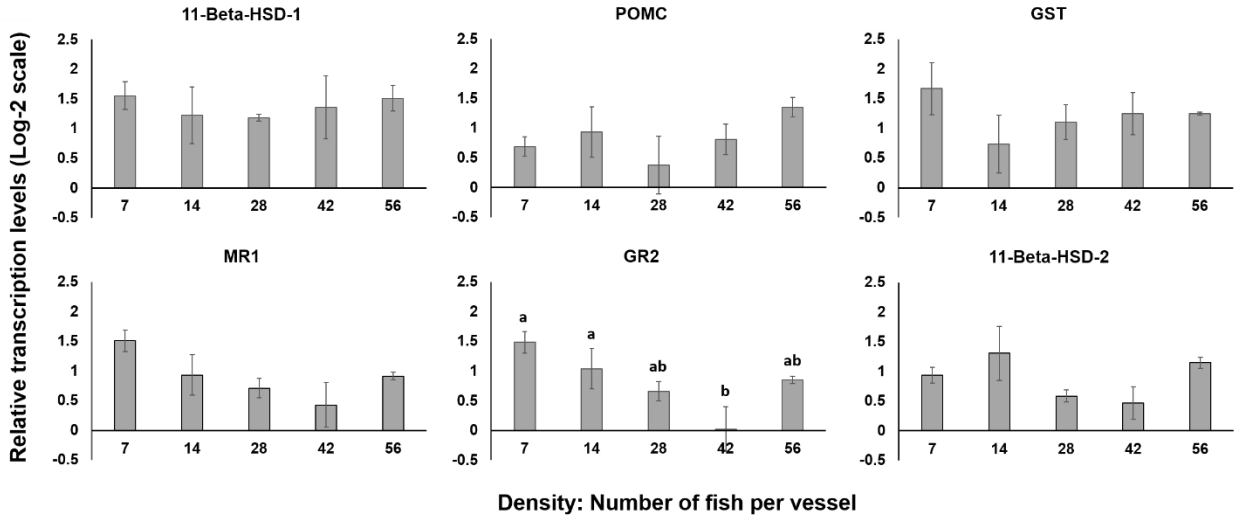

Figure 5. Arithmetic mean Log-2 fold change (N=3) in gene transcription per density level for all genes, relative to normalization factor calculated for two control genes. Bars indicate standard errors. Letters indicate significance differences (Nested ANOVA, Pairwise Post Hoc, significance level α=0.05) between density levels. Abbreviations: POMC (*Pro-opiomelanocortin*), MR1 (*Mineralocorticoid Receptor 1*), GR2 (*Glucocorticoid receptor 2*), 11-Beta-HSD-1 (*11-Beta-Hydroxysteroid-dehydrogenase Type 1*), 11-Beta-HSD-2 (*11-Beta-Hydroxysteroid-dehydrogenase Type 2*), GST (*Glutathione-S-Transferase*).

Table 3. Relative transcript levels (Log-2 fold change) of genes and normalized whole body cortisol levels (pg cortisol × µg protein-1) used as biomarkers to determine stress levels in late larval Delta Smelt (Hypomesus transpacificus).

Density	Biomarker	11-Beta-HSD-1	POMC	GR2	MR1	11-Beta-HSD-2	GST	Cortisol
7	Mean	1.51	0.65	1.48	1.49	0.93	1.66	0.48
	SD±	0.5	0.4	0.6	0.6	0.7	0.8	0.1
14	Mean	1.23	0.94	1.04	0.93	1.3	0.74	0.70
	SD±	0.9	0.9	1.0	0.9	1.0	1.0	0.1
28	Mean	1.18	0.38	0.66	0.71	0.59	1.1	0.29
	SD±	1.1	1.2	0.8	0.8	0.5	1.3	0.1
42	Mean	1.36	0.81	0.02	0.43	0.47	1.24	0.18
	SD±	1.2	1.1	1.1	1.0	1.1	1.0	0.0
56	Mean	1.51	1.35	0.85	0.91	1.15	1.25	0.28
	SD±	0.8	0.9	0.7	0.6	0.6	1.2	0.0

Abbreviations: GST: Glutathione-S-Transferase, POMC: Pro-Opiomelanocortin, MR1: Mineralocorticoid receptor 1, GR2: Glucocorticoid receptor 2, 11-Beta-HSD-1: 11-β-Hydroxysteroid-Dehydrogenase-Type 1, 11-Beta-HSD-2: 11-β-Hydroxysteroid-Dehydrogenase-Type 2, SD: Standard Deviation, SE: Standard Error.

Principle Component Analysis (PCA)

Principal component 1 explained 73.2 % and principal component 2 15.3 % of the variation among stress responsive genes, jointly describing 88.5 % of the variation (Figure 6). The respective biplot, plotted as an insert in Figure 3, elucidates that genes MR1, POMC, and 11-Beta-HSD-1 loaded heavily on principal component 1, whereas 11-Beta-HSD-2, GR2, and GST loaded more on principal component 2. Densities of 28 and 42 fish clustered together, whereas the lowest assessed densities of 7 and 14 clustered more closely with 56.

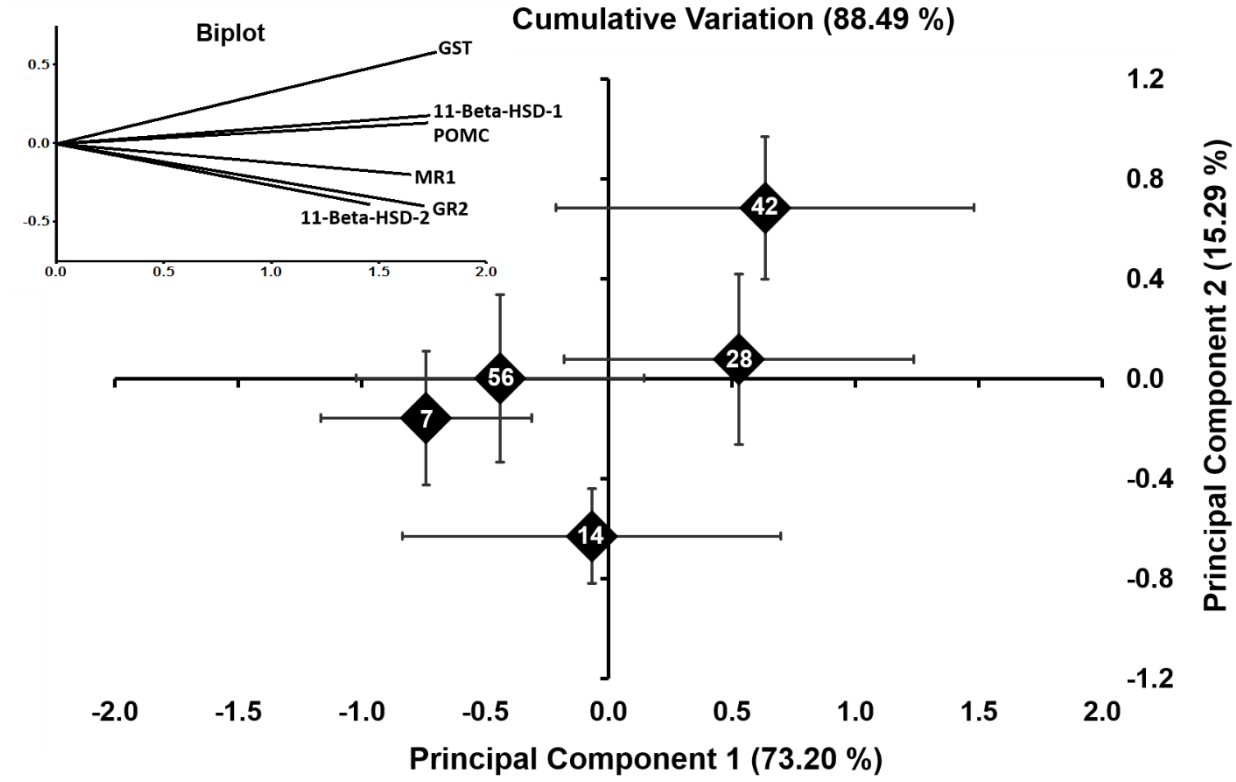

Figure 6. Graphical depiction of the scores of principal components 1 and 2 for centroid graph for each treatment. Percentages give the amount of variation explained by the respective principal component. Numbers indicate density levels (# of fish per vessel). Insert represents respective biplot indicating genes driving the clustering. Legends of X-axis and Y-axis of the centroid graph are also applicable to the biplot. Abbreviations: POMC (*Pro-opiomelanocortin*), MR1 (*Mineralocorticoid Receptor 1*), GR2 (*Glucocorticoid receptor 2*), 11-Beta-HSD-1 (*11-Beta-Hydroxysteroid-dehydrogenase Type 1*), 11-Beta-HSD-2 (*11-Beta-Hydroxysteroid-dehydrogenase Type 2*), GST (*Glutathione-S-Transferase*).

Discussion

This study emphasizes that fish stocking density has a significant effect on fish physiological responses, and the importance of evaluating density stress, towards determining optimal conditions

when planning an experimental design. Both biochemical (cortisol) and molecular (gene transcription) endpoints delivered consistent results as indicators of stress in stocking density experiments. The assessment of stress determined by using two mechanistic approaches can thus be used to establish a more comprehensive evaluation of density stress at multiple levels of biological organization, providing a higher level of confidence in subsequent experimental data.

Response pattern of genes utilized in this study, such as MR1, GR2, and 11-Beta-HSD-2 supported the findings of whole body cortisol levels having lower stress levels at densities 28 and 42, whereas others (POMC, GST, and 11-Beta-HSD-1) did not. This is not surprising, as similar results between genes and their later product (proteins, hormones or metabolites) are not necessarily expected. Studies investigating the relationship between mRNA levels and their later products focus on proteins (Martyniuk et al. 2009; Martyniuk et al. 2012) rather than hormones/metabolites. Nonetheless, certain aspects of the mRNA-protein relationship also apply to mRNA-hormone relations, and several factors influence this relationship. Martyniuk et al (2012) highlights that different kinetics and turnover rates, experimental lag times and sampling time points, as well as biological regulatory factors involved in transcription and translation contribute to the gene-protein relationship. One explanation for the low correspondence between genes POMC, 11-Beta-HSD-1, GST with cortisol patterns is a potential negative feedback loop mechanism which is common between protein and the respective gene, and also known in hormones (Martyniuk et al. 2009). While a single endpoint such as cortisol could potentially be sufficient to establish differences between treatments, our study highlights the benefits of having multiple endpoints, thus providing a greater overview of the mechanisms behind physiological stress responses.

Both the low and high fish stocking densities tested here affected physiological stress responses of late larval Delta Smelt. Results support our hypothesis for this pelagic species; with elevated stress levels observed at low stocking densities, as well as at elevated stocking densities. Both cortisol levels and gene transcription response indicated that lowest stress levels were found at stocking densities in the range of 28 to 42 fish per 8L vessel, suggesting this range as favorable within this test's conditions. These results are consistent with fish density values reported for optimal Delta Smelt culturing methods, which when converted to the same water volume used here, represent a stocking density range of four to eight fish × L^{-1} for fish of similar age (41-80 dph) (Lindberg et al. 2013). Although Delta Smelt are not described as a schooling fish species, aggregations have been observed in the field (Bennett 2005; Moyle 2002). Aggregation likely provides the Delta Smelt with safety in numbers, and protection from potential predators. This could explain lower stress levels in

treatments 28 and 42 fish per 8L vessel compared to low and high stocking densities. Low stocking density might not allow for aggregation of sufficient fish to form a desirable sized group resulting in elevated stress.

Stress levels did not increase significantly in highest stocking density treatments of 56 fish per 8L vessel. This could stem from the fact that the highest tested density treatment of 56 fish was still within the stocking density range (30-60 fish per 8L vessel) of the optimal culturing (Lindberg et al. 2013), and that water quality parameters remained in an acceptable range. Studies on stocking density effects in rainbow trout indicated that water quality affected certain physiological responses more than stocking density (Person-Le Ruyet et al. 2008).
Aside from water chemistry, additional factors such as water flow and physical characteristics of the experimental system (e.g., vessel shape and depth) play an important role for fish stocking density (Pickering 1992). Often the applied density is only valid for the particular system used, in a particular hatchery/facility, and stocking densities must be modified before other systems are being utilized (Ellis et al. 2001). Numbers given for optimal cultural methods for Delta Smelt are determined for a recirculating holding system with freshwater supply, and bio media filter, whereas the approach described herein used a static system.

Other factors such as fish species, age and size need to be considered for determining fish stocking density (Pickering 1992). Differences in responses to stocking density levels have been observed in a number of species. Arctic charr, for example, were observed in several studies to have higher growth rates, greater length and weight, lower aggressive interactions (Brown et al. 1992; Jørgensen et al. 1993; Wallace et al. 1988) and the highest feeding activity (Alanärä and Brännäs 1996) at high densities. Further, this species was observed to have lowest cortisol levels in higher densities compared to low densities (Vijayan and Leatherland 1988). Pigfish (*Orthopristis chrysoptera*), a marine baitfish, also had higher survival rates in higher densities compared to low densities (DiMaggio et al. 2014). Other species have shown a different response to increased densities. For instance, in Senegalese sole (*Solea senegalensis*; Kaup 1858), highest stress levels were found at high density treatments (Costas et al. 2008). These differences can primarily be explained by the distinct biology of each fish species; for example, fishes could be exhibiting specific territorial or schooling characteristics, significantly influencing density driven responses.

Although fish stocking densities have been studied intensively, mostly for aquaculture purposes, this study is one of few to specifically investigate the stocking density for an experimental protocol, and

the first to conduct such evaluations using Delta Smelt. Fish stocking density has been discussed as an important factor in experimental biology, in the context of field experiments using cages as exposure vessels (Oikari 2006). Improper fish stocking density can lead to enhanced stress levels and thus to a false or elevated stress response to the biological stressor tested, which in turn leads to misleading results and potentially misinterpretation of data. Therefore, it is of utmost importance to adequately define the fish stocking density for the experimental protocol, with the aim of providing holding conditions with minimal stress, especially when measures of stress are being used as endpoints.

Conclusion

In this study we showed that fish stocking density has an effect on the physiological stress response of larval Delta Smelt. Both endpoints used in this approach delivered consistent results and are suitable for stress detection in fish stocking density experiments. The results highlight that taking stocking density in consideration as a potential confounding factor might benefit the quality of the experiment and quality of the stress response measured in the experiment.

3. Fundamental Niche Turbidity

The content of this chapter is currently in preparation for submission to a peer reviewed journal.

Hasenbein, M., Fangue, N.A., Geist, J.P., Connon, R.E. *Multiple endpoints of different levels of biological organization allow integrative assessments on environmental stressors in an endangered fish species.*
(In Preparation)

Abstract

Estuary ecosystem are highly managed and modified by anthropogenic activities and belong to some of the most threatened ecosystem types in the world. Turbidity is an important and highly variable parameter in estuary ecosystems known to affect several fish species such as the endangered Delta Smelt (*Hypomesus transpacificus*). This pelagic fish species in endemic to the San Francisco Estuary and Sacramento-San Joaquin River Delta, which is known to depend on turbidity for the completion of its life cycle. We exposed late larval Delta Smelt (60 days post hatch dph) to environmentally relevant algae induced turbidity levels ranging from 5 to 250 NTU for 24h. We used a combination of mechanistic investigations (molecular biomarkers and cortisol) and behavioral and whole organism endpoints (feeding and survival) with the goal to comprehensively assess and determine the tolerance range for turbidity. All endpoints at multiple levels of biological organization delivered consistent results and identified the range between 25 and 80 NTU as optimal under tested conditions. Survival was highest in treatments 12 to 80 and reduced in low (5 NTU) and very high turbidities (120, 250 NTU). Feeding was elevated in turbidities 25 to 80 NTU and decreased in low (5, 12 NTU) and very high turbidities (120, 250 NTU). Cortisol was lowest in 35 to 80 NTU and elevated in very low turbidities 5, 12, 25 NTU. Principal component analysis performed on the set of molecular biomarkers revealed separate clustering of distinct groups of low, medium, and high turbidities. Measuring several endpoints across multiple levels of biological organization allows for comprehensive assessments and provide a high level of confidence in the data. Measuring mechanistic endpoints such as cortisol and molecular biomarkers enables the identification of adverse outcomes and provide explanations for results observed in behavioral and whole organism endpoints such as feeding and survival. Turbidity has been demonstrated to affect fish on a population-level and needs to be considered in habitat and water management.

Introduction

Estuaries are among the most threatened and highly anthropogenically modified and managed ecosystems worldwide (Kennish 2002) and the San Francisco Estuary and Sacramento-San Joaquin River Delta in California, USA (after this referred to as 'Delta') is a paragon (Nichols et al. 1986) representing the hub of the Californian fresh water supply (Lund et al. 2010; Service 2007). Estuaries are a unique type of ecosystem that are denoted by the interface between coastal marine and riverine freshwater habitats (Wołowicz et al. 2007). Characterized by mixing zones of freshwater outflow, saltwater intrusion and a distinctive recurring ebb tide - flood scenario, they provide a unique but very dynamic habitat for highly specialized and adapted species. The mixing zone often to referred to as 'low salinity zone' or 'zone of turbidity maxima' is centered around a salinity of 2 PSU (Jassby et al. 1995; Kimmerer et al. 2013) with at variation in salinity of 0.5-6 PSU (Brown et al. 2014), is characterized by estuarine turbidity maxima (ETM) (Peterson et al. 1975; Schoellhamer 2001). The ETM often is associated with the limit of salt intrusion and is characterized by an increase of suspended particles, phytoplankton, and zooplankton (Peterson et al. 1975; Roman et al. 2001; Sanford et al. 2001), thus representing a zone with higher turbidity which is important for fish larvae (Dodson et al. 1989; North and Houde 2001). Along with other water physicochemical parameters, turbidity plays an important role in estuarine ecosystems. It is known, for example, to influence several trophic levels by altering species composition (Lunt and Smee 2014), and can potentially affect the feeding strategy of fish (Hecht and Van der Lingen 1992) and respective predator-prey interactions.

Turbidity, often explained as the cloudiness or murkiness of water, is defined as an expression of the optical property causing light scattering and light absorption rather than direct light transmission through a water sample (Rice et al. 1994). Important factors contributing to the effect of turbidity are light intensity, suspended material, and water depth (Lee and Rast 1997). These parameters can interact with each other confounding the effect on the fish. Much research has been conducted on evaluating the effects of turbidity on the vision of fish, and predator-prey interactions (Gardner 1981; Gregory and Northcote 1993; Horppila et al. 2004; Miner and Stein 1996; reviewed in Utne-Palm 2002). Turbidity has been demonstrated to affect fish in different ways depending on their specific foraging type and associated behavior. Large predatory fish which detect the prey visually from a greater distance are negatively affected by turbidity, as an increased number of suspended particles can influence the light scattering and thus interfere with prey detection (Chesney 1989; Fiksen et al. 2002; Giske et al. 1994; reviewed in Utne-Palm 2002). Small planktivorous fish, or larval fish, may benefit from a certain degree of turbidity with an increase of reactive distance (distance between predator and prey at time of detection) (Miner and Stein 1996; Utne-Palm 2002), and

elevated feeding in mid-range turbidity levels (Boehlert and Morgan 1985). While turbidity can potentially enhance the contrast between the prey and its background (Hinshaw 1985), it can also lower the risk of predation providing a necessary level of security (Gregory and Northcote 1993), therefore influences feeding and stress levels in fish and represents an important environmental stressor.

Turbidity in the San Francisco Estuary and Delta has decreased over the past decades (Thomson et al. 2010) and several causes have been discussed to cause these trends. Introduction of invasive species such as clams (Carlton et al. 1990; Nichols et al. 1990) and waterweed (Ferrari et al. 2014; Yarrow et al. 2009) as well as dam construction and sediment trapping in reservoirs, reduced major flood events (Baxter et al. 2010; Jassby 2008; Jassby et al. 2002; Wright and Schoellhamer 2004), nutrient forms and ratios (Glibert et al. 2011; Parker et al. 2012b), and herbicides (Blaser et al. 2011; Orlando et al. 2013) have been postulated as factors contributing to decreases in turbidity in the Estuary and Delta. Reduced turbidity is associated with low primary productivity, resulting in a food limited estuary (Kimmerer et al. 2005). In the San Francisco Estuary and the Delta, turbidity levels vary tremendously between location and range from 1.4- 219.7 NTU have been reported (Werner et al. 2010b). Other studies indicated the maximum around 350 NTU (Bennett and Burau 2014). In this study we focus on the range of 5-250 NTU.

The interaction between an organism and changes in its environment can be evaluated by assessing physiological stress responses. Physiological stress responses in fish can be categorized as primary, secondary, and tertiary stress response (Barton 2002; Iwama 1998; Wendelaar Bonga 2011). The primary stress response is characterized by immediate endocrine changes in stress hormones such as catecholamines, adrenocorticotropic hormone (ACTH), and cortisol (Barton 2002; Iwama 1998; Wendelaar Bonga 2011). The Hypothalamic-Pituitary-Interrenal- axis (HPI-axis), is responsible for cortisol production, and is comprised of a series of genes that encode for hormones with specific functions. A received stressor leads to the production of corticotropin-releasing factor by the hypothalamus, which triggers the release of ACTH by corticotropic cells (Alsop and Aluru 2011). ACTH is derived from a gene referred to as Pro-opiomelanocortin (POMC), and binds to specific receptors (melanocortin receptor type 2, MC2R) in the interrenal cells in the head kidney (Alsop and Aluru 2011). Through activation of cAMP signaling pathways MC2R stimulates the cortisol production and its release into the blood (Alsop and Aluru 2011). Cortisol has the ability to enter cells and binds to specific receptors mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Alsop and Aluru 2011). *Glucocorticoid Receptor 2* (GR2), and *Mineralocorticoid Receptor 1* (MR1), which are both ligand-activated transcription factors (Alsop and Vijayan 2008; Prunet et al. 2006) are mediators for cortisol signaling. Mineralocorticoids and glucocorticoids bind to MR1 and

GR2 inducing the stimulation or repression in transcription of target genes (Tomlinson et al. 2004). *11-Beta-Hydroxysteroid-Dehydrogenase-Type1* (11-Beta-HSD-1) and *11-Beta-Hydroxysteroid-Dehydrogenase-Type2* (11-Beta-HSD-2) are the genes coding for two enzymes which are directly involved in the activation and inactivation of glucocorticoids; e.g., cortisol (Krozowski et al. 1999). In particular, 11-Beta-HSD-1 is a bidirectional enzyme that functions first and foremost as a reductase by converting cortisone into cortisol and also can function as a dehydrogenase by converting cortisol into cortisone (Krozowski et al. 1999; Tomlinson and Stewart 2001). In contrast, 11-Beta-HSD-2 is a unidirectional enzyme that converts cortisol into cortisone (Krozowski et al. 1999; Tomlinson and Stewart 2001), and protects MR1 from occupation by cortisol (Stewart and Mason 1995; Tomlinson and Stewart 2001). Cortisol has been demonstrated to control itself suppression by a negative feedback loop on the HPI-axis (Bradford et al. 1992; Fryer and Peter 1977). The secondary response is described by the physiological effects of the primary stress hormones, which among other things the activation of metabolic pathways, hydromineral balance, immune and respiratory functions (Barton 2002; Iwama 1998; Wendelaar Bonga 2011). The tertiary response encompasses changes in behavior and physiology observed at the whole animal level, such as growth, development, reproduction, disease resistance, survival (Barton 2002; Iwama 1998; Wendelaar Bonga 2011).

In the San Francisco Estuary and Delta a severe decline of biodiversity, referred to as the pelagic organism decline (POD) has been documented over the past decades (Baxter et al. 2008; Baxter et al. 2010; Brooks et al. 2012; Sommer et al. 2007; Thomson et al. 2010). Most affected are two introduced species striped bass (*Morone saxatilis*) and threadfin shad (*Dorosoma pentenense*) and two native species longfin smelt (*Spirinchus thaleichthys*) and Delta Smelt (*Hypomesus transpacificus*) (Sommer et al. 2007; Thomson et al. 2010).

The Delta Smelt is a fish species endemic to the San Francisco Estuary and Delta, and is listed as endangered and threatened under California State and Federal Endangered Species Acts, respectively (CDFW 2014; USFWS 1993). Because of its listing, and the continual decline in numbers, the Delta Smelt is considered an indicator species for the health of the Delta ecosystem. In the field Delta Smelt abundance has been linked to the low salinity zone (LSZ) (Moyle et al. 1992) and it has been documented that the Delta Smelt are associated with turbid water (Bennett and Burau 2014; Feyrer et al. 2007; Grimaldo et al. 2009). Studies in a laboratory setting on the feeding response of early life stages of Delta Smelt have found a positive relationship between turbidity and feeding behavior (Baskerville-Bridges et al. 2004; Baskerville-Bridges et al. 2005). Further it has been discussed that increased pulse turbidity from first flush events might be a cue for the annual spawning migration of Delta Smelt to the northern delta (Grimaldo et al. 2009; Sommer et al. 2011). Results of previous studies investigating turbidity effects on juvenile life stages showed enhanced

feeding at low turbidities and constant feeding up to 120 Nephelometric Turbidity Units (NTU) with a strong decline in feeding at 250 NTU (Hasenbein et al. 2013). Stress-related biomarkers indicated a flat lining expression up to 120 NTU and a very high expression at 250 NTU (Hasenbein et al. 2013). Differences between life stages have been observed in environmental parameters such as temperature and salinity (Komoroske et al. 2014) and it is expected that this is true for turbidity, too.

The aim of this study was to test the effects of environmentally relevant turbidities on the physiological stress responses of late-larval Delta Smelt. We hypothesized that different turbidity levels would affect overall physiological stress, which would in turn affect survival, but that maximum prey capture ability (feeding) would occur at optimal turbidity requirements. Thus, in order to determine the preferred range of turbidities for late-larval Delta Smelt, we conducted a combination of mechanistic investigations (gene expression, plasma hormones and metabolites) and contrasted these with ecological performance measures (feeding ability, and survival).

Material & Methods

Study Animals

Late-larval Delta Smelt (*Hypomesus transpacificus*) (60 days post hatch, dph) were provided by the Fish Conservation and Culture Laboratory (FCCL) UC Davis in Byron, CA, USA, where feeding ability and physiological response tests were conducted. Fish were cultured according to cultural protocols described in Baskerville-Bridges et al. (2005) and Lindberg et al. (2013), and fish were held at water temperatures of 17.47 °C (± 0.05 SE) and turbidities of 9.94 NTU (± 0.25 SE) for a period of 8 weeks prior to test. Light intensity for larval and late larval life stages are 4-5 and 1-2 µmol/m^2/s respectively (Lindberg et al. 2013), which equals 40-50 Lux and 10-20 Lux, respectively.

Fish Exposures

Late larval Delta Smelt (60 dph) were exposed at a density of 30 fish per bucket to different turbidities 5, 12, 25, 35, 50, 80, 120, and 250 NTU (nominal values) in aerated facility water in black circular fish tanks (static water system), 2.5 gal (8L volume) (2 Gallon Black Plastic Pail; Item # 3539; United States Plastic Corporation®, USA), for a period of 24h at a light dark cycle of 16h:8h. *Nannochloropsis* algae (Nanno 3600 – High yield grow out feed, Reed Mariculture Inc., USA) were spiked into exposure vessels to achieve desired turbidities. Light intensity was kept constant at 48 Lux (SE = 1.13). The interaction between light intensity and turbidity, observed in previous studies (Baskerville-Bridges et al. 2004), as well as limited water depth within the exposure vessel were considered during planning of the experiment. The exposure vessel and light intensity were chosen according to cultural protocols. Fish stocking density were determined in preliminary tests and was chosen to be in the optimal range, but slightly modified to meet requirements of limited fish numbers. Salinity was kept constant at 2 PSU for the feeding test, whereas the physiology test had a salinity of 0.2 PSU. Fish were fed in the facility tanks prior to test setup, but were kept unfed throughout test duration. For both tests, physicochemical water parameters such as dissolved oxygen, salinity, specific conductance, pH-value, turbidity, and ammonia (total ammonia-N) were monitored at test initiation and at termination. Ammonia was measured in control treatments only, as color of *Nannochloropsis* algae interfered with the measurement method. Temperature remained stable in the physiological tolerance range and was measured in exposure vessels every minute using temperature loggers. All parameters are displayed in Table 5 and Table 6.

a) Feeding Test

After 24h fish were fed with live newly hatched *Artemia fransciscana* in solution (100 ml per bucket, density of 462 *Artemia* per ml (SE=21), total food density in the vessel was 5-6 *Artemia* per ml of water) for a period of 7.5 min (a time determined in preliminary tests to show 50 % gut fullness). At test termination, fish were immediately euthanized, with an overdose of tricaine methanesulfonate (MS-222; Finquel). Specimens were transferred into 15 ml tubes filled with 70% Ethanol and preserved until further processing. Fish were measured for weight and length and dissected for gut content (# of *Artemia* ingested) under a microscope (40x resolution). Survival was recorded at test termination and percentage of survival was calculated based on fish numbers at test setup and test termination. Treatment 120 NTU had only three replicates due to the failure of air stones in one of the replicates.

b) Physiology Test

At test termination fish were immediately euthanized, within the exposure vessel, with an overdose of tricaine methanesulfonate (MS-222; Finquel). Specimens were transferred into 1.5 ml tubes (Eppendorf) and subsequently snap frozen in liquid nitrogen. Samples were stored at -80°C until biochemical and molecular analysis.

Cortisol Assessments

Whole body cortisol was assessed in a total of 4-7 fish from each treatment depending on survival, using methods established for zebrafish (Alsop and Vijayan 2008; Cachat et al. 2010). Volumes of solutions were optimized for use in juvenile Delta Smelt (Hasenbein et al. 2013) and further adjusted for larval fish. In brief, samples were defrosted on ice and homogenized in 1 × PBS buffer using a TissueLyzer LT. The resulting homogenate was divided in equal amounts of 500 µl, and used for cortisol and total protein determination. Cortisol was washed out of the homogenate using diethyl ether. Ether was evaporated and cortisol in dried samples was resuspended in 1 x PBS buffer. Cortisol assays (Salivary Cortisol, Enzyme Immunoassay Kit, Salimetrics, Inc., State College, PA, USA) were performed according to manufacturer`s instructions, and cortisol levels (mg × dL^{-1}) were calculated with a four-parameter sigmoid standard curve (minus curve fit). Cortisol levels were normalized to total protein and denoted as cortisol concentration (pg cortisol × µg $protein^{-1}$). The second half of the homogenate was used to determine protein content. Following centrifugation at 16500 g for 30 min at 4°C the supernatant of each sample was collected and used for total protein content determination following manufactures' protocol (BCA Protein Assay Kit, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Quantitative PCR

Total RNA was extracted from whole-body homogenates. RNA extractions were performed according to manufacturer's protocols using the RNeasy Mini Qiacube Kit (Qiagen©, Venlo, Limburg, Netherlands). Qualitative and quantitative RNA determination was conducted using a NanoDrop ND1000 Spectrophotometer; 260/280 and 260/230 ratios ranged from 2.04-2.18 and 1.94-2.35, respectively. Integrity of total RNA was assessed by electrophoresis on a 1% (w/v) agarose gel. Depending on survival, the stress response and physiology gene transcription of five to eight fish per treatment were assessed by quantitative polymerase chain reaction (qPCR). Complementary DNA (cDNA) synthesis was performed using Reverse Transcriptase III (SuperScript® III Reverse Transcriptase, Invitrogen™, Life technologies™, Carlsbad, CA, USA) and primer and probes for qPCR analyses were designed using Roche Universal Library Assay Design Center (https://www.roche-

applied-science.com). Quantitation of transcription was performed using SDS 2.4 software (Applied Biosystems®, Life technologies™, Carlsbad, CA, USA). Responding genes were normalized using a normalization factor calculated based on the geometric mean of two control genes; *Glyceraldehyde-3-phosphate Dehydrogenase* (GAPDH) and *Beta-Actin* (*B-actin*). Normalization was performed according to the "geNorm" algorithm version 3.5 as described in Vandesompele et al. (2002). A total of 17 genes were selected based on their involvement in the HPI-axis and in other important functions such as energy metabolism, development and somatic growth, ion homeostasis, oxygen homeostasis, inflammatory response, osmoregulation, and general stress. Genes involved in the HPI-Axis (POMC, 11-Beta-HSD-1, 11-Beta-HSD-2, GR2, MR1, described in the introduction) were evaluated along with other important biomarkers described below. *Glutathione-S-Transferase* (GST) a key enzyme for cellular detoxification (Choi et al. 2008) was measured. GST defends cells against Reactive Oxygen Species (ROS) and responds to osmotic stress in fishes (Choi et al. 2008). Serum/Glucocorticoid regulated kinase (SGK) plays an important role in ion homeostasis and has stimulatory effects on sodium transport via the epithelial sodium channel (Pearce 2001). HSP70 is a gene involved in general stress and heat stress (Fangue et al. 2006; Iwama et al. 1999; Quinn et al. 2011). Glucose transporter (GLUT 2) a gene involved in glucose homeostasis and energy metabolism (Polakof et al. 2012; Wood and Trayhurn 2003). Insulin like growth factor (IGF) is a gene that interacts with growth hormone and is involved in development and somatic growth in fish and highly conserved in all vertebrates (Duan 1997; Moriyama et al. 2000; Reinecke et al. 2005; Wood et al. 2005). Ammonium transporter a gene identified in an earlier study (Connon et al. 2011b). Hypoxia inducible factor 1 (HIF1-α) is a transcription factor that is activated by reduced oxygen concentrations and plays an important role in oxygen homeostasis (Semenza 2002; Semenza 2009) and is regulated by Nuclear Factor-k-Beta (NFkB) (van Uden et al. 2008). NFkB is an important transcription factor playing a crucial role in host defense and in chronic inflammatory diseases (Barnes 1997). Catalase is one of the antioxidant enzymes and protecting the cell from oxidative damage by reactive oxygen species (ROS) (Devasagayam et al. 2004; Hook et al. 2014; Rodríguez-Ariza et al. 1993). NaK ATPase is a key enzyme involved in ion transport during osmoregulation in fish and is a molecular indicator of osmoregulatory stress.

Sequence information, primer efficiency, gene code and gene name are listed in Table 4.

Table 4. Primer and probe sequences of genes used as molecular biomarkers to determine stress levels in late larval Delta Smelt (Hypomesus transpacificus).

Gene name	Gene Code	Primer 5'→3'	Primer 3'→5'	Probe #	% Efficiency
Glutathione-S-Transferase	GST	aatctccctggcagacattgtt	ggccggctctcaaacacat	127	108
Mineralocorticoid Receptor 1	MR1	tttctacactttccgcgagtca	tgatgatctccaccagcatctc	39	99
Glucocorticoid Receptor 2	GR2	catcgtgaagcgtgaggagaa	tgcatggagtccagtagtttgg	129	98
Pro-opiomelanocortin	POMC	tgttcacctgtgcaggtctga	gagaagctctcttccgtggaca	127	102
11-Beta-Hydroxyteroid-Dehydrogenase Type 1	11-Beta-HSD-1	cgtgtcgtctctgctggcta	ggcgaacttggtggaggag	55	109
11-Beta-Hydroxyteroid-Dehydrogenase Type 2	11-Beta-HSD-2	tcctgccatcctcctacaagac	tctggaccaggtgtttgaactg	14	106
Beta Actin	β-actin	tgccacaggactccatacc	catcggcaacgagaggtt	11	107
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	tccacgagaaagacccaact	cacgccagtagactcaacca	159	95
Insulin like growth factor	IGF	gacacgctgcagtttgtatgtg	ccatagcctgccggtttgt	110	100
Serum/Glucocorticoid regulated kinase	SGK3	ttattgagatcaaagcgcatgac	ggtgtgaagggaggtggaatc	85	107
Hypoxia inducible factor	HIF1a	gccatggccaagctcctta	attcagcattggcactaagcac	41	105
Glucose Transporter 2	GLUT2	gccatgtcagttggcctcat	gacatgctgacgtagctcatcc	130	109
Heat shock Protein 70 kD	HSP70	aagattctggagaagtgcaacga	ccttctcagcggtctggttct	20	107
Nuclear factor k-Beta	NFkB	tgcacggatgaacacattgtc	ccaaagtccagaggcttgtca	123	103
Ammonium transporter	NH4 Trans	caggctgtcttatcgcttacgg	cagcgtcatgactaacagctgaa	61	95
Catalase	Catalase	gaccagggcatcaagaacctta	ggatggcgtagtctgggtca	88	97
Sodium Potassium ATPase	Na K ATPase	gtcatcccaatctactgcacca	catgatgtcgccaatcttgc	88	109

Statistical Analysis

Data were analyzed using the '*stats*' package in R-project for statistical computing (version 3.0.2, http://www.r-project.org) (R-CoreTeam 2014). Normal distribution was confirmed for feeding and mortality data using the Shapiro-Wilk Normality Test and homoscedasticity was tested using the Fligner-Killeen Test. One-way ANOVA models were used to test for statistical significant differences followed by a Tukey HSD Post Hoc Test (significance level α=0.05). Cortisol data were Log-2 transformed to meet normality assumption. Homogeneity of variances was confirmed with the Fligner-Killen Test. One-way ANOVA models were used to assess statistical significant differences between treatments. Quantitative PCR data were tested for normality using the Shapiro-Wilk Normality Test and further analyzed for homoscedasticity with the Fligner-Killeen test. Normal distributed genes were analyzed with an One-way ANOVA model followed by a Tukey HSD Post Hoc Test (significance level α=0.05) where appropriate, whereas non-normal distributed genes were analyzed using the Kruskal-Wallis Test (Significance Level α=0.05). Principal Component Analysis (PCA) was carried out on the transcriptomic (qPCR) data set in order to analyze differences in transcription patterns between treatments, as well as determine to which extend these profiles were affected by the treatments. PCA scores were calculated using the covariance matrix and principal components 1, 2, and 3 were determined to explain the majority of the variation in the data, using a Scree Test (Scree plot) as described in D'Agostino and Russell (2005). PCA scores of

Components 1, 2, and 3 were plotted as a centroid graph to interpret, visualize and support clustering of treatments with similar transcriptomic responses. In addition, the respective biplot was plotted to identify the important genes driving the two components and explaining the percentage of variation.
Detailed information about test statistics are listed in the supplementary Table 21 in the appendix.

Results

Physicochemical Parameters

Levels of specific conductance, pH, salinity and temperature remained stable throughout the test duration and were within the acceptable range for these tests. Dissolved oxygen reduced over time in all turbidity levels in the feeding test and all but one turbidity levels of the physiology test. Values ranged over time for the feeding test from 9.79-7.44 mg/L and for the physiology test from 9.53-7.75 mg/L, respectively. Air stones were used to keep algae in suspension in the exposure vessels, but since aeration had to be limited due to sensitivity of the fish (Lindberg et al. 2013), settlement of algae occurred in all turbidity levels of the feeding test and in turbidity levels 25, 35, 50, 80, 120, and 250 NTU of the physiology test, causing the decrease in turbidity measured at 24h. Ammonia concentration increased over time from 0.08 mg/L (± 0.01 SE) at test initiation to 0.15 mg/L (± 0.00 SE) at test termination. Physicochemical water parameters for the feeding test and the physiology test are presented in Table 5 and Table 6, respectively.

Table 5. Physicochemical water parameters of the turbidity feeding test conducted on late larval Delta Smelt 60 dph exposed to different levels of turbidity over 24h. Values are means with standard error across replicates for each turbidity level taken at test initiation T0 and test termination T24.

Treatment Salinity, Turbidity	Average Standard Error (SE)	DO mg/L		Spec. Con. µS/cm		pH		Salinity		Turbidity NTU		Ammonia mg/L NH_3-N		Light intensity (Lux)	Temperature °C
		T0	T24	T0	T24	T0	T24	T0	T24	T0	T24	T0	T24		
2 PSU, 5 NTU	Average	9.79	8.84	3738	3804	7.65	7.22	2.00	2.03	6.18	5.16	Average = 0.08 SE = 0.01	Average = 0.15 SE = 0.004	Average = 48.07 SE = 1.13	Average = 17.52 SE = 0.0037
	SE	0.05	0.05	82	102	0.07	0.04	0.00	0.05	0.22	0.25				
2 PSU, 12 NTU	Average	9.48	8.88	3712	3852	7.82	7.32	2.05	2.05	11.7	10.6				
	SE	0.05	0.08	238	150	0.06	0.02	0.09	0.09	0.2	0.2				
2 PSU, 25 NTU	Average	9.55	8.64	3721	3732	7.89	7.48	1.98	1.98	24.2	19.9				
	SE	0.03	0.15	129	141	0.03	0.04	0.08	0.08	1.0	0.8				
2 PSU, 35 NTU	Average	9.54	8.59	3941	3939	7.88	7.47	2.10	2.10	34.8	27.3				
	SE	0.06	0.11	69	72	0.02	0.02	0.04	0.04	0.8	0.7				
2 PSU, 50 NTU	Average	9.45	8.42	3699	3743	7.87	7.47	1.93	2.00	51.3	43.8				
	SE	0.15	0.11	24	45	0.04	0.01	0.03	0.00	1.0	3.1				
2 PSU, 80 NTU	Average	9.51	8.35	3932	3887	7.93	7.53	2.05	2.05	80.7	58.2				
	SE	0.10	0.24	65	118	0.07	0.03	0.06	0.06	1.4	5.6				
2 PSU, 120 NTU	Average	9.57	8.44	3887	3845	7.90	7.52	2.07	2.07	120	97				
	SE	0.04	0.14	129	174	0.01	0.05	0.07	0.09	2	5				
2 PSU, 250 NTU	Average	9.24	7.44	3760	3909	8.03	7.55	2.05	2.08	251	199				
	SE	0.08	0.21	188	62	0.08	0.03	0.06	0.05	2	4				

Abbreviations: DO: Dissolved Oxygen, Spec. Con.: Specific Conductance, NTU: (Nephelometric Turbidity Unit, SE: Standard Error, T0 = Time point at test start, T24 = Time point at test termination.

Table 6. Physicochemical water parameters of the turbidity physiology test conducted on late larval Delta Smelt 60 dph exposed to different levels of turbidity over 24h. Values for each exposure vessel and respective turbidity level taken at test initiation T0 and test termination T24.

Turbidity (NTU) nominal concentration	DO mg/L		Spec. Con. µS/cm		pH		Salinity PSU		Turbidity NTU		Ammonia mg/L NH_3-N		Light intensity LUX	Temperature °C
	T0	T24	T0	T24	T0	T24	T0	T24	T0	T24	T0	T24		
5	9.47	9.29	764	864	8.17	8.23	0.4	0.4	6.38	6.30				
12	9.53	9.45	692	890	8.63	8.09	0.3	0.4	8.8	11.8				
25	9.43	9.15	768	1083	8.36	8.02	0.4	0.4	26.4	21.9				
35	9.28	9.09	882	858	8.54	7.8	0.5	0.4	32.2	27.3	Average = 0.08 SE = 0.01	Average = 0.15 SE = 0.004	Average = 48.07 SE = 1.13	Average = 17.52 SE = 0.0037
50	8.41	9.19	853	864	8.56	7.74	0.4	0.4	53.2	42.5				
80	9.34	7.75	887	1131	8.49	7.69	0.4	0.5	82.2	62.5				
120	9.32	8.62	1017	1082	8.50	7.76	0.5	0.5	129	123				
250	9.29	8.62	910	1163	8.78	8.41	0.4	0.6	240	202				

Abbreviations: DO: Dissolved Oxygen, Spec. Con.: Specific Conductance, pH-value, PSU: practical salinity unit, NTU: Nephelometric Turbidity Units, T0 = Time point at test start, T24 = Time point at test termination.

Feeding Test

Survival

Survival was on average 81.08 % (± 4.34 SE) across all treatments. Highest survival rates were observed in treatments 12, 25, 35, 50, and 80 NTU with survival rates of 90.00 % (± 4.71 SE), 93.33 % (± 3.60 SE), 87.50 % (± 2.50 SE), 88.33 % (± 5.18 SE), and 87.50 % (± 3.69 SE), respectively (Figure 7). Survival decreased at low turbidities of 5 NTU, averaging 72.50 % (± 3.94 SE), as well as at high turbidities of 120 and 250 NTU, averaging 71.12 % (± 4.01 SE) and 58.34 % (± 6.16 SE), respectively. There were significant differences between treatments 5 and 25 NTU ($P<0.05$), 12 and 250 NTU ($P<0.001$), 25 and 120 NTU ($P<0.05$), 25 and 250 NTU ($P<0.001$), 35 and 250 NTU ($P<0.01$), 50 and 250 NTU ($P<=0.01$), and 80 and 250 NTU ($P<0.01$).

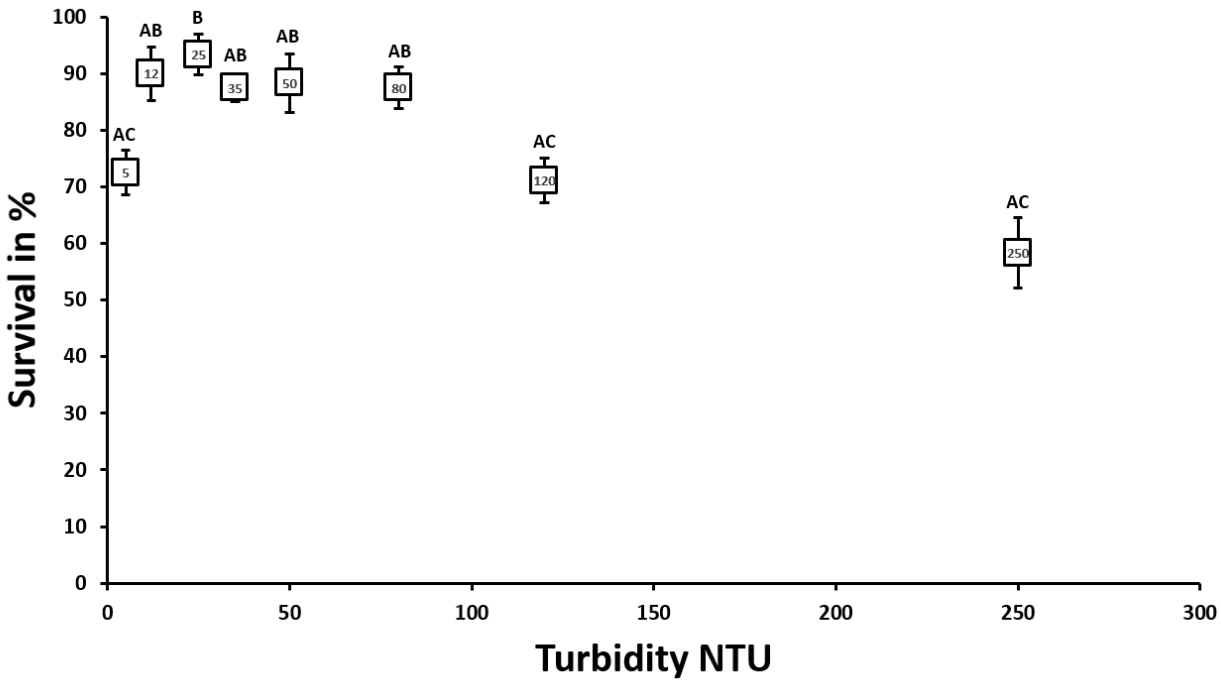

Figure 7. Survival: Percentage survival of 60-dph late larval Delta Smelt after 24h maintenance at turbidity levels between 5 and 250 NTU (N=3-4). Bars depict standard error (SE). Letters indicate significant differences between turbidity levels (Anova, Tukey HSD, α=0.05).

Feeding

Highest feeding rates were recorded at mid-range turbidities of 25 to 80 NTU (Figure 8), where late-larval Delta Smelt ingested a mean of 17 (± 4.40 SE), 25 (± 5.08 SE), 19 (± 4.75 SE), and 22 (± 4.19 SE) *Artemia* per fish within the 7.5 minutes feeding time, respectively. Lower feeding rates were observed at both low and high turbidities with prey ingestions of 8 (± 2.68 SE) and 11 (± 2.17 SE) *Artemia* per fish, at turbidities of 5 and 12 NTU respectively, and 14 (± 4.81 SE) and 5 (± 2.07 SE) *Artemia* per fish, at turbidities of 120 and 250 NTU respectively. Significant differences between feeding rates were detected between 35 and 250 NTU ($P<0.05$). No ingested algae were found.

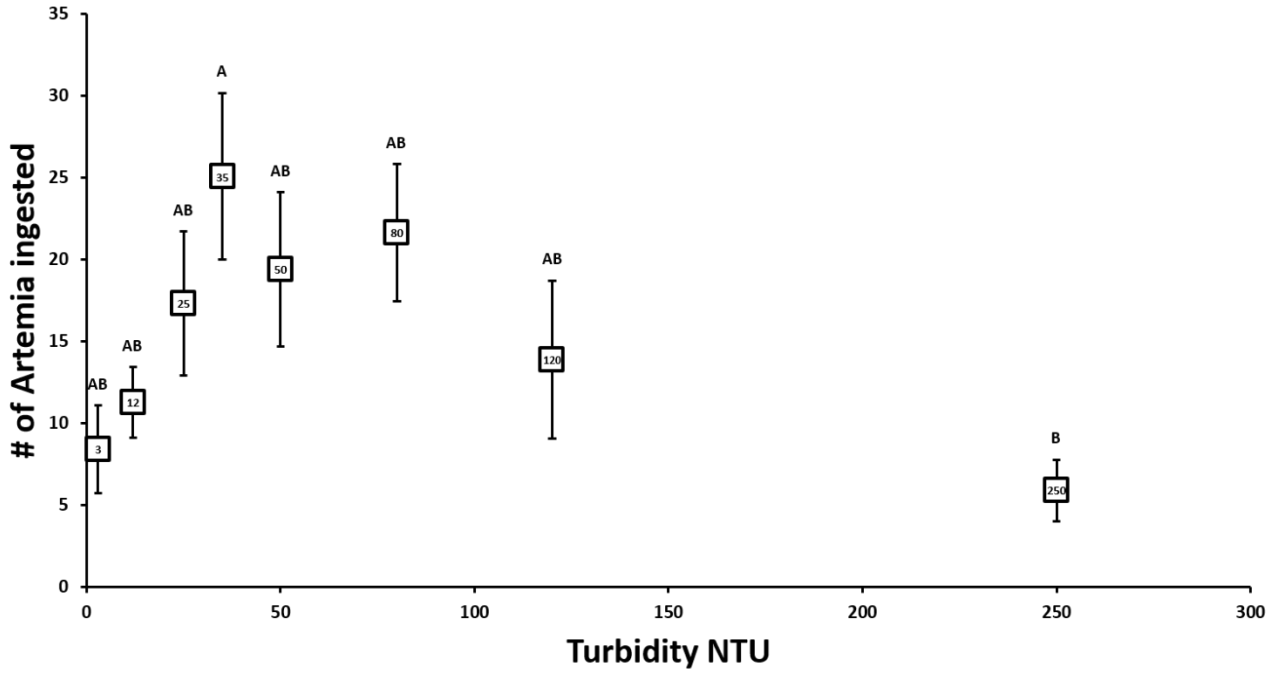

Figure 8. Feeding performance: Mean number of Artemia ingested by 60-dph late-larval Delta Smelt following 24h maintenance at turbidity levels between 5 and 250 NTU (N=3-4). Bars depict standard error (SE). Letters indicate significant differences between turbidity levels (Anova, Tukey HSD, α=0.05).

Physiology Test

Cortisol

No significant differences in whole-body cortisol levels were observed in late-larval Delta Smelt maintained across turbidities of 5 to 250 NTU ($\alpha<0.05$). However, lowest cortisol levels were measured in fish maintained at 50, 80, and 250 NTU (Figure 9), and levels were elevated in fish at treatments 35, and 120 NTU. Fish maintained at lower turbidity levels of 5, 12, and 25 NTU had high levels of cortisol with 25 NTU exhibiting the highest cortisol level, as well as the greatest variability within measurements.

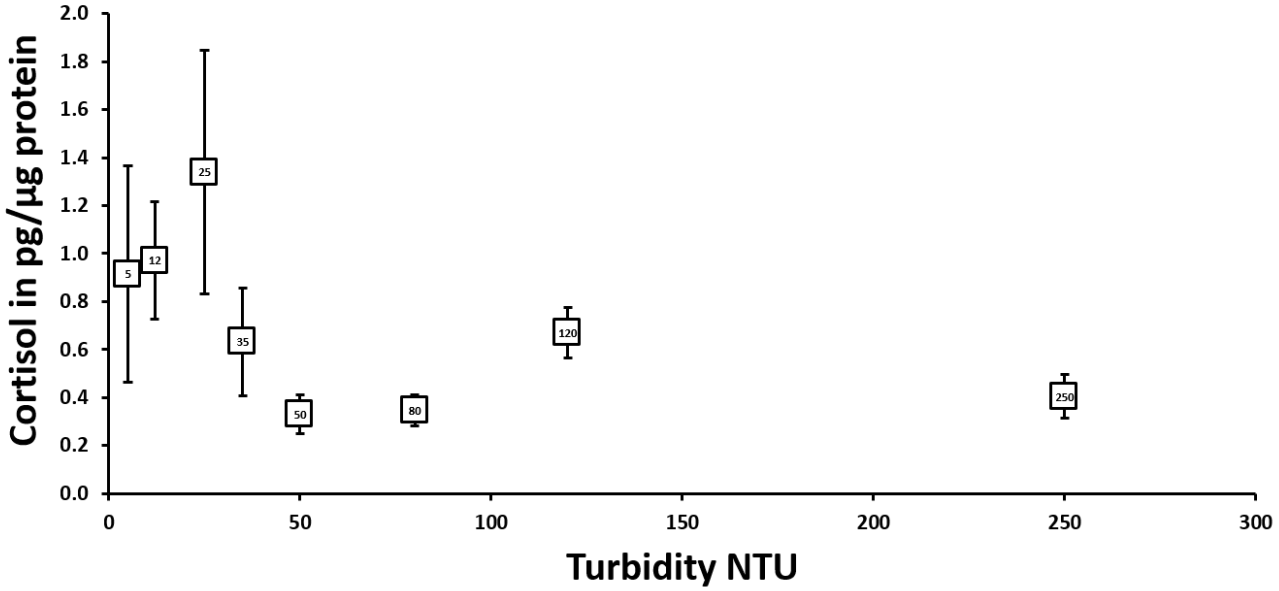

Figure 9. Whole body cortisol levels of 60-dph late-larval Delta Smelt following 24h maintenance at turbidity levels between 5 and 250 NTU. Arithmetic mean across individual fish (N=4-7) Bars depict standard errors (SE).

Quantitative PCR

Four out of 15 genes assessed with qPCR, GST, HSP70, GLUT2 and NH_4^+ Trans, responded significantly in late-larval Delta Smelt (Table 7). GST was significantly differentially expressed at the highest turbidity (250 NTU), relative to 25 ($P<0.05$) and 35 NTU ($P<0.05$), suggesting oxidative stress at higher turbidity. HSP70 indicated highest general stress levels at 120 and 250 NTU, significantly different from the 5 NTU ($P<0.01$), 12 ($P< 0.001$), and 35 NTU ($P<0.05$) groups. No significant differences were observed at 25 NTU, due to high variability in responses. GLUT2 was also highest at high turbidity, significantly differentiating between 250 NTU and 5 NTU ($P<0.01$), 35 NTU ($P<0.05$) and 80 NTU ($P<0.05$). Interestingly, NH_4^+ Trans a gene coding for the ammonia transporter, also responded significantly in fish maintained at 12 NTU relative to 120 ($P<0.001$), and 250 NTU ($P<0.05$).

Table 7. Fold-change in Gene Transcription for each gene measured in late larval Delta Smelt 60 dph maintained for 24h at different turbidity levels. Values above 1.0 indicate upregulated genes and value below 1.0 indicate down-regulated genes. Statistically significant differences are bolded.

Turbidity		11-Beta-HSD-1	POMC	GR2	MR 1	11-Beta-HSD-2	GST	Na/K ATPase	HSP 70	GLUT 2	NH4+ trans	Catalase	SGK3	IGF	NF-KB	HIF 1a
5	Average	2.68	1.12	3.01	2.71	1.33	2.16	1.33	**2.10**	**2.32**	1.28	1.48	0.61	1.83	2.41	0.98
	SE	0.47	0.44	0.85	0.53	0.27	0.31	0.23	0.28	0.24	0.22	0.13	0.10	0.30	0.41	0.19
12	Average	2.34	1.07	2.03	2.38	0.92	2.25	1.08	**1.83**	2.99	**0.71**	1.55	0.49	1.67	2.13	1.11
	SE	0.43	0.31	0.32	0.33	0.17	0.27	0.21	0.25	0.37	0.16	0.32	0.10	0.28	0.23	0.20
25	Average	2.02	0.66	1.69	2.10	1.33	**1.87**	1.04	2.26	2.80	1.60	1.57	0.67	1.62	2.48	1.30
	SE	0.38	0.17	0.21	0.35	0.26	0.36	0.21	0.39	0.55	0.42	0.25	0.16	0.39	0.37	0.32
35	Average	2.46	0.88	2.30	2.34	1.58	**2.00**	1.21	**2.76**	**2.79**	1.85	1.88	1.41	2.46	2.76	1.35
	SE	0.39	0.16	0.35	0.46	0.34	0.21	0.13	0.41	0.38	0.43	0.26	0.76	0.54	0.47	0.28
50	Average	2.60	0.94	2.51	3.23	1.21	3.00	2.20	3.48	3.90	1.90	2.44	0.95	2.71	3.06	1.95
	SE	0.32	0.17	0.28	0.38	0.17	0.39	0.26	0.28	0.54	0.22	0.30	0.16	0.41	0.30	0.33
80	Average	2.42	0.95	2.59	2.60	1.58	2.87	1.60	3.22	**3.43**	1.40	2.13	0.80	2.28	2.97	1.72
	SE	0.61	0.20	0.42	0.35	0.16	0.51	0.09	0.23	0.56	0.22	0.22	0.08	0.33	0.30	0.38
120	Average	3.28	1.87	3.78	3.80	1.56	3.92	1.78	**4.27**	4.82	**3.05**	2.58	0.99	2.49	3.34	1.58
	SE	0.53	0.99	0.80	0.72	0.15	0.71	0.14	0.45	0.60	0.62	0.30	0.17	0.46	0.41	0.28
250	Average	2.84	0.97	2.04	3.15	1.17	**5.36**	1.62	**3.62**	**6.97**	**2.13**	2.37	0.71	2.69	3.61	1.43
	SE	0.37	0.22	0.39	0.57	0.23	1.68	0.29	0.60	1.68	0.59	0.51	0.12	0.45	0.38	0.26

Abbreviations: HSP70: Heat shock Protein 70kD, GLUT 2: Glucose Transporter 2, NH4+ trans: Ammonium transporter, GST: Glutathione-S-Transferase, IGF: Insulin like growth factor, NF-kB, Nuclear factor k-Beta, HIF1a: Hypoxia inducible factor 1 alpha, 11-Beta-HSD-1: 11-β-Hydroxysteroid-Dehydrogenase-Type 1, 11-Beta-HSD-2: 11-β-Hydroxysteroid-Dehydrogenase-Type 2, MR1: Mineralocorticoid receptor 1, GR2: Glucocorticoid receptor 2, POMC: Pro-Opiomelanocortin, SGK3: Serum/Glucocorticoid regulated kinase 3.

Principal Component Analysis (PCA)

Principal Component Analysis was conducted on all transcriptomic data (Table 7, Figure 10). Principal Component 1 (PC1) explained 40.40 % of the variation while Principal Components 2 (PC2), and 3 (PC3) explained 19.60 % and 10.80 % of the variation, respectively, leading to a cumulative variation of 70.80 % (Figure 10a). Visual assessments of the graphical depiction of the Principal Component scores for PC1 and PC2 (Figure 10a) cluster treatments 25, 35, 50 and 80 NTU, more closely with lower turbidities (5 and 12 NTU), with higher turbidity levels separating from the cluster. Respective biplots (Figure 10b) indicate that several different genes such as GR2, MR1, 11-Beta-HSD-1, Glut2, GST, and Catalase loaded heavily on PC 1, while POMC, HIF1a, SGK3, HSP 70, and NH4-trans determined the loading for PC2 (Figure 10b). Differentiation of the highest turbidities (120 and 250 NTU) from all others tested, was more marked when visualizing PC 1 vs PC3 (Figure 10c), whereas PC2 vs PC3 (Figure 10d) highlight differentiation of the lowest turbidities from mid-range, and high treatments. Taken together, this indicates that gene transcription patterns measured across all genes clustered for the low, medium, and high turbidities separately, and are consistent with the responses recorded for survival, feeding, and cortisol levels.

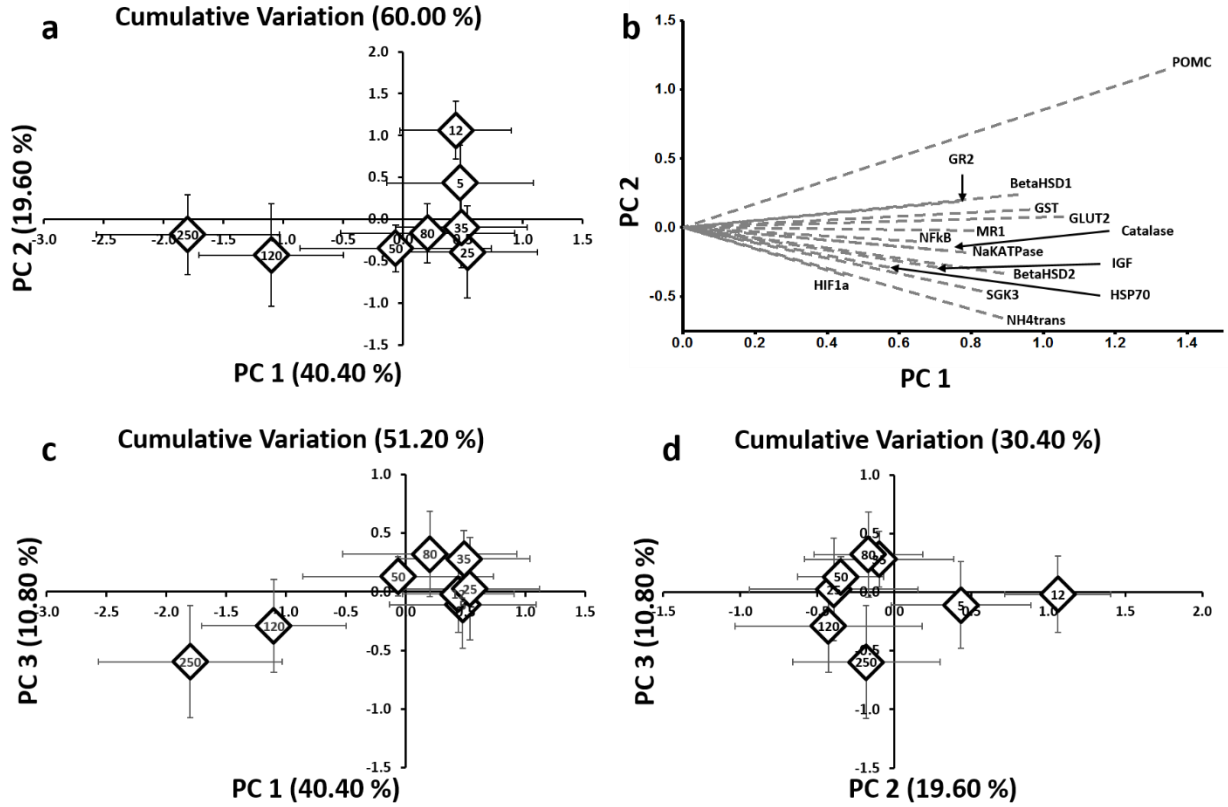

Figure 10. Graphical depiction of the scores of Principal Components 1 and 2 (a); 1 and 3 (c); 2 and 3 (d); centroid graph for each treatment. Percentages indicate the amount of variation explained by the respective principal component. Numbers indicate turbidity levels (NTU). Respective biplot indicates genes driving the clustering of component 1 and 2 is plotted in (b) with gene names. Abbreviations: HSP70: Heat shock Protein 70kD, GLUT 2: Glucose Transporter 2, NH4+ trans: Ammonium transporter, GST: Glutathione-S-Transferase, IGF: Insulin like growth factor, NF-kB, Nuclear factor k-Beta, HIF1a: Hypoxia inducible factor 1 alpha, 11-Beta-HSD-1: 11-β-Hydroxysteroid-Dehydrogenase-Type 1, 11-Beta-HSD-2: 11-β-Hydroxysteroid-Dehydrogenase-Type 2, MR1: Mineralocorticoid receptor 1, GR2: Glucocorticoid receptor 2, POMC: Pro-Opiomelanocortin, SGK3: Serum/Glucocorticoid regulated kinase 3.

Discussion

This study determined and quantified how differences in turbidity affect late-larval Delta Smelt survival, feeding, and mechanistic stress responses over a period of 24h, demonstrating the specific turbidity requirements by this species in a sensitive life stage. Overall, turbidity ranges from 25-80 NTU were determined to be optimal under the tested conditions (specific light intensity and water depth) as evident from the highest survival, feeding and lowest gene transcription changes compared to other treatments. Detrimental effects occurred both at low turbidities of 5 and 12 NTU, as well as at high turbidities of 120 and 250 NTU. Consequently, even short-term (<24 h) exposure to

such conditions are likely to have significant adverse effects on the population level of this endangered species.

The findings of this study are consistent with field observations where Delta Smelt are associated with specific water turbidity, and have been more frequently sampled at turbidities between 10-50 NTU (Bennett and Burau 2014; Grimaldo et al. 2009). Turbidity is influenced by light intensity, water depth, as well as type of suspended material (Lee and Rast 1997); factors that can vary in the field depending on local habitat conditions, but which can be controlled for in laboratory assessments. Standardized turbidity loading using algae in the laboratory is not necessarily the same as in the field since different water components contribute to turbidity. However, since laboratory observations very well correspond with findings where most Delta Smelt are caught in the field, it is likely that they depend on certain turbidity level independent of what material (organic or inorganic) the turbidity consists of. In future studies on different types of suspended materials, and relative mixtures of suspended materials, need to be tested, in combination with different light intensity levels, to further elucidate the interaction between particle types and light intensity, and how fish respond to these variables.

Delta Smelt survival was highest at mid-range turbidities, but reduced at very low and high turbidities. For Delta Smelt culturing procedures, algae induced turbidities and light intensities need to be adjusted for each specific life stage (Lindberg et al. 2013). The light intensities for this test were in the same range as used in the culture facility for late-larval Delta Smelt, but this range of light intensity, at turbidity levels below that used at the facility, would likely lead to increased stress and potential mortality. Another potential cause for the elevated stress and reduced survival observed at low turbidity levels, could stem from the concept that elevated turbidity is beneficial for planktivorous fish, in order to hide from predators. In fact, Delta Smelt are reported to use turbid waters to hide from predators (Moyle 2002), which might explain reduced stress levels in specific and suitable turbidity ranges.

Reduced survival at high turbidities could be caused by gill-clogging, reducing their capacity for respiration. Clogging of fish gillrakers and gill filaments as a result of excess suspended material is not uncommon (Bruton 1985; Sutherland and Meyer 2007; Wong et al. 2013). Although not determined in this study, it is highly likely that algae material restricted respiration by filling the gill cavities. Increased expression of GST at high turbidity is an indication of oxidative stress in gills, which could also result in osmotic imbalance, as well as overall effects on respiration. Highest survival rate across mid-range turbidities (12-80 NTU) indicates the optimal range for late-larval Delta Smelt under test`s conditions. However, increased stress levels and reduced feeding were

determined in treatment 12 NTU survival was still high, indicating sub-lethal stress levels in fish exposed in this treatment; at the specific light intensity and tank depth. Restricted water depth in the exposure vessel prevents the fish from escaping deeper into the water column, in order to avoid elevated light intensities, which is possible in nature. Turbidity and light intensity are therefore likely key factors determining their location in the water column.

Feeding responses expectedly corresponded with survival. A constant feeding rate was observed between 25 NTU and 80 NTU with a high peak at 35 NTU. A similar result was found in studies on European Smelt testing the feeding response to turbidities and light intensities; determining highest feeding rates at 20 NTU and a constant feeding between 20 and 50 NTU (Horppila et al. 2004). The feeding pattern is typical for a planktivorous fish, which benefit from turbid waters by a contrast enhancement that helps them detect the prey items (Boehlert and Morgan 1985; Utne-Palm 2002). Studies on larvae of Pacific Herring (*Clupea harengus pallasi*) assessing the feeding response to different concentrations of suspended sediments found increased feeding rates at intermediate concentrations compared reduced feeding rates in control treatments (no suspended sediments) and a very high concentrations (Boehlert and Morgan 1985). Increased stress has been discussed to lead to reduced food intake and food conversion rate in fish (Wendelaar Bonga 2011), thus the observed low feeding rates at low turbidity levels are likely caused by the increased stress levels, as described above. However, this could also be attributed to backscattering of light. Light intensities above the light saturation level of the fish cause negative effects on prey detection (Utne-Palm 2002), as backscattering of light by the particles can minimize the contrast between the background and the prey item, making it less visible (Cerri 1983; Guthrie 1986; Loew and McFarland 1990). This would reduce the ability of the Delta Smelt to find prey items in the exposure vessel and lead to decreased feeding rates. In high turbidities, reduced feeding is also likely due to elevated stress levels, as detected at the molecular level with fish resulting in oxidative and osmotic stress. Lastly, feeding assessments were conducted using *A. franciscana*, which is used in the culturing procedures and was fed in abundance similar to cultural procedures. It is likely that the feeding results would have been different if other prey species occurring in the Delta Smelt`s habitat would have been used with a different mobility and escape response. Larval fish are constantly swimming and do not perform a coordinated hunt, rather feeding when directly encountering prey items (Bennett 2005). The combination of the swimming behavior and the different prey item might result in a more marked feeding curve pattern due to the interspecific factors between predator and prey. In future studies feeding performance could be further explored by utilizing numerous prey species, in particular, zooplankton species that are commonly consumed in their natural diets.

Turbidity is also known to impact vision, and high turbidities can reduce the reactive distance of a fish (Hecht and Van der Lingen 1992; Miner and Stein 1996; Utne-Palm 2002; Utne 1997) and with that limits the volume of water a fish could search for prey. As with backscattering of light at low turbidity, reduction in feeding at high turbidity may also be caused by an impaired field of vision. Even though larval and juvenile Delta Smelt are planktivorous feeders, it is highly likely that they cope with a wide range of turbidities, however, if turbidities reach threshold levels visibility may be impaired to a degree where prey detection is impossible. We have demonstrated reduced feeding at high turbidities (250 NTU), in prior studies on juvenile Delta Smelt (Hasenbein et al. 2013). The observed feeding in 120 and 250 NTU would therefore be resultant of incidental prey encounters at close proximities.

The realized niche takes into account interactions with interspecific and intraspecific. Being confined to a certain range of turbidity by the threat of predator and within that zone the feeding has to be optimal as well. We have tested the tolerance without predation. With a presence of the predator the activity range of the smelt will be reduced to the turbidity range where vision for the predator might be difficult and Delta Smelt could hide from them. It is likely that they will have more restricted range when predators are present.

Cortisol and molecular biomarkers corresponded with survival and feeding. Elevated cortisol levels at 5, 12, and 25 NTU indicate higher stress in these treatments which may have resulted from a variety of reasons. As discussed above, at the lower turbidity levels (5 and 12 NTU), light sensitivity along with the confined water depth of the exposure vessel may have played important roles. Cortisol levels at 25 NTU, although elevated, were highly variable, with two out of five fish exhibiting extremely high values. Turbidity ranges between 35 and 80 NTU, again resulted in lower levels of cortisol in late-larval Delta Smelt, supporting what we observed in the survival and the feeding response indicating this range as favorable under test conditions. Reduced cortisol levels observed at the highest turbidity (250 NTU) are likely associated with respiration impairment; elevated mortality resulted in these treatments. It appears that environmental stressors above a certain threshold do not lead to the production of cortisol, an expected primary stress response, in fact molecular biomarkers (e.g., GST and HSP70) indicate that secondary stress responses are at play.

Stress biomarkers such as GST involved in oxidative and osmotic stress indicate potential detrimental effects on respiration (as discussed above). However, this study also determined general stress responses; indicated by an elevated transcription of HSP70 at high turbidities (120 and 250 NTU), and more specifically, it highlighted alterations in energy metabolism; as indicated by GLUT 2 which responded significantly at high turbidity (250 NTU).

Increased stress also affects a number of organismal parameters due to reallocation of metabolic energy. Since fish spend more energy on regaining homeostasis (e.g., respiration, locomotion, tissue repair, and hydromineral regulation), less energy can be invested in development and growth. Reduction in growth can in turn, lead to starvation, reduced fitness, and reduced activity, and ultimately death (Wendelaar Bonga 2011). Reduced activity and fitness, has further implications on predator avoidance, as well as to capture prey items. Stress is also known to affect the susceptibility to disease (Pickering and Pottinger 1989; Snieszko 1974), and cortisol has been demonstrated to function as a key mediator modulating the immune response (Tort 2011).
Reduced survival in larval life stages due to elevated or decreased turbidities can have significant impacts on reproduction of the declining Delta Smelt population, long term. Recruitment in numbers of young fish for reproduction at the adult life stage is reduced i.e., less individuals will migrate to the spawning grounds for mating and reproduction. This might further reduce the genetic diversity and might exacerbates the persisting bottleneck (Fisch et al. 2011).

All endpoints (survival, feeding, biochemical (cortisol), and molecular stress parameters) delivered consistent results, providing a high level of confidence in the data. Measuring molecular biomarkers, involved in several different metabolic functions such as stress response (HPI-axis), energy metabolism, general stress, and osmotic stress helps to understand mechanisms behind the physiological adaptations, and to evaluate potential adverse outcomes caused by stressors. Together with cortisol molecular biomarkers provide explanations for behavioral and whole organism endpoints feeding and survival.
In the San Francisco Estuary and Delta the observed decrease in turbidity over the past decades, caused by invasive water weeds, introduced clams, and intensive dam construction might be another contributor to the Delta Smelt`s decline (Carlton et al. 1990; Ferrari et al. 2014; Jassby et al. 2002; Nichols et al. 1990; Yarrow et al. 2009). Our data confirm that turbidity is an important, yet often overlooked environmental variable that contributes to population-level effects in fish; at least for Delta Smelt. Turbidity in the San Francisco Estuary and other estuaries worldwide is often anthropogenically modified, resulting in altered flows, sedimentation, and changes in primary production, thus turbidity itself, and parameters that affect turbidity, must be considered when addressing habitat management.

To our knowledge, this is the first study to assess turbidity effects on the physiological stress response of an endangered fish species using endpoints of multiple different levels of biological organization. Endpoints at multiple levels of biological organization provide tools for a

comprehensive assessment of physiological and behavioral responses, which enable researchers to determine habitat preferences, and can be well transferred to other fish species and stressor types, illustrating wide-use potential in the field of conservation physiology.

4. Multiple Stressors

A similar version of this chapter was published:

Hasenbein, M., L. M. Komoroske, R. E. Connon, J. Geist & N. A. Fangue, 2013. Turbidity and Salinity Affect Feeding Performance and Physiological Stress in the Endangered Delta Smelt. Integrative and Comparative Biology 53(4):620-634 doi:10.1093/icb/ict082.

Abstract

Coastal estuaries are among the most heavily impacted ecosystems worldwide with many keystone fauna critically endangered. The Delta Smelt (*Hypomesus transpacificus*) is an endangered pelagic fish species endemic to the Sacramento–San Joaquin Estuary in northern California, and is considered as an indicator species for ecosystem health. This ecosystem is characterized by tidal and seasonal gradients in water parameters (e.g., salinity, temperature, and turbidity), but is also subject to altered water-flow regimes due to water extraction. In this study, we evaluated the effects of turbidity and salinity on feeding performance and the stress response of Delta Smelt because both of these parameters are influenced by water flows through the San Francisco Bay Delta (SFBD) and are known to be of critical importance to the completion of the Delta Smelt's life cycle. Juvenile Delta Smelt were exposed to a matrix of turbidities and salinities ranging from 5 to 250 nephelometric turbidity units (NTUs) and 0.2 to 15 parts per thousand (ppt), respectively, for 2 h. Best statistical models using Akaike's Information Criterion supported that increasing turbidities resulted in reduced feeding rates, especially at 250 NTU. In contrast, best explanatory models for gene transcription of sodium-potassium-ATPase (*Na/K-ATPase*) - an indicator of osmoregulatory stress, hypothalamic pro-opiomelanocortin - a precursor protein to adrenocorticotropic hormone (expressed in response to biological stress), and whole-body cortisol were affected by salinity alone. Only transcription of glutathione-S-transferase, a phase II detoxification enzyme that protects cells against reactive oxygen species, was affected by both salinity and turbidity. Taken together, these data suggest that turbidity is an important determinant of feeding, whereas salinity is an important abiotic factor influencing the cellular stress response in Delta Smelt. Our data support habitat association studies that have shown greater Delta Smelt abundances in the low-salinity zone (0.5–6.0 ppt) of San Francisco Bay, a zone that is also understood to have optimal turbidities. By determining the responses of juvenile Delta Smelt to key abiotic factors, we hope to aid resource managers in making informed decisions in support of Delta Smelt conservation.

Introduction

Aquatic systems worldwide are changing in profound ways, and as the human population continues to grow, the impacts of anthropogenic stressors are likely to increase (Halpern et al. 2008). Stressors such as habitat loss, over-exploitation, pollution, invasive species, and climatic change have consequences for species' extinctions, loss of biodiversity, and overall ecosystem function (Dudgeon et al. 2006; Geist 2011). Since contemporary aquatic species are being challenged with suites of anthropogenic stressors unlike those which have dominated their evolutionary histories, and because natural systems are almost always simultaneously subject to multiple, and possibly interacting stressors, there is a need to more comprehensively account for this in physiological and ecological studies as well as in conservation planning (Crain et al. 2008). Coastal estuaries are among the most degraded aquatic habitats worldwide, and the management of estuaries and their resources are among our greatest conservation challenges. The San Francisco Bay Delta (SFBD) is one of the most highly anthropogenically-impacted estuaries in the United States (Cloern and Jassby 2012), and its decline is well documented and not in dispute (Healey et al. 2008; Lund et al. 2010). Current water-management practices in the SFBD are challenging because resource managers are charged with achieving co-equal goals: the provision of ecosystem services (largely the provision of a reliable water supply to Californians) and the maintenance of ecosystem health and function. The SFBD is subject to marine influences from the Pacific Ocean mixing with seasonally fluctuating freshwater input from the Sacramento and San Joaquin Rivers (Cloern and Jassby 2012), providing unique gradients in water parameters (e.g., salinity and temperature), and providing the sediment and energy needed to shape the physical habitat. However, diversion of water for anthropogenic demands has vastly reduced the amount of freshwater inflow into the SFBD by approximately 40% in the average year (Lund et al. 2010), and waters of higher salinity now move further upstream during the fall (Cloern and Jassby 2012). Simultaneously, a rise in sea level is expected to force saline waters further inland and upstream (Smithson 2002). The SFBD is substantially different from the historical Delta, and the effects of this change on native species are widespread (Moyle and Bennett 2008; National Research Council 2012). Three pelagic fishes of the upper SFBD estuary (Delta Smelt, *Hypomensus transpacificus*; longfin smelt, *Spirinchus thaleichthys*; and age-0 striped bass, *Morone saxatilis*) have declined sharply since the early 2000s, and their populations have remained low over the past decade. The underlying causes of the pelagic organism decline are vigorously debated, and considerable efforts are underway to evaluate complex multistressor interactions that may be contributing to species' declines such as entrainment (i.e., fish drawn through intakes) at water pumping stations, loss of critical habitat, competition with and predation from non-native species, limited food due to changes in plankton communities, altered abiotic conditions, contaminants, and

poor water quality (Brooks et al. 2012; Brown et al. 2009; Bryant and Souza 2004; Feyrer et al. 2007; Hieb 2005; Sommer et al. 2007; Winder and Jassby 2011). The Delta Smelt is of particular interest as it is an endangered pelagic fish species endemic to the Sacramento–San Joaquin Estuary in northern California; it acts as an indicator of ecosystem health in its habitat range, and has been listed as endangered under both the USA Federal and Californian State Endangered Species Acts. Delta Smelt are an annual, estuarine species able to withstand moderate fluctuations in salinity (Swanson et al. 2000); however, their distribution in the SFBD has been correlated with the low-salinity zone (LSZ), centered at 2 parts per thousand (ppt). The position of the 2-ppt-salinity isohaline (also referred to as ‘‘X2’’) is defined as the distance in kilometers from the Golden Gate Bridge to its location in the estuary (Jassby et al. 1995; Kimmerer 2002). Although adult Delta Smelt have been found to tolerate short-term exposures to salinities up to approximately 19 ppt (Swanson et al. 2000), typically few are observed at salinities >4 ppt, suggesting reduced performance or aversion to environments of higher salinity (Bennett 2005; Kimmerer 2002). If so, then the impact of climatic change pushing the LSZ further upstream could result in the reduction of optimal habitat for this species in the SFBD. Additionally, adult Delta Smelt annually migrate upstream in the late fall–early winter to spawn, where their larvae develop in freshwater habitat until they reach post-larval stages and migrate downstream in the spring toward the LSZ (Bennett 2005). The X2 is seasonally variable and dependent on flow regimes, which can be manipulated; it is closely managed to maintain habitats understood to be optimal for the Delta Smelt. Turbidity in the SFBD can also vary greatly by location and season and is dependent on a range of factors such as tidal flux, wind, rainfall and water flow, algal growth, ambient light levels, and depth. Turbidity levels in excess of 100 NTU occur primarily during winter storms, or during summer when irrigation return-flows contribute substantially to sediment loads (Dahlgren et al. 2004). Turbidity can impact fishes indirectly by influencing primary productivity and thus food-web dynamics, but turbidity levels can also affect some fish species directly. For example, it has been shown that larval Delta Smelt will not feed in clear water (Baskerville-Bridges et al. 2004), and it has been postulated that turbidity provides the visual contrast needed for Delta Smelt to see their prey. However, highly turbid waters may also reduce Delta Smelts’ feeding success, likely by reducing their ability to visually identify the prey; however, this is greatly dependent on light intensity (Baskerville-Bridges et al. 2004). The introduction of non-native species (e.g., the invasion of the suspension-feeding overbite clam, *Corbula amurensis*, believed to be largely responsible for large reductions in plankton populations) has also impacted turbidity in many regions of the SFBD (Carlton et al. 1990; Jassby et al. 2002; Nichols et al. 1986; Nichols et al. 1990). Given the likely sensitivity of Delta Smelt both to salinity and turbidity levels, it is important to understand how they impact Delta Smelts’ performance, survival, and overall

distribution in the SFBD. In this study, we evaluated the effects of turbidity and salinity both on the feeding performance and on the stress response of Delta Smelt because both of these physicochemical parameters are influenced by water flows through the SFBD, and these factors are known to be of critical importance to the completion of the Delta Smelt's life cycle (Bennett 2005). We hypothesize that the response of Delta Smelt to physicochemical parameters can be determined by combining mechanistic investigations (i.e., measuring the stress response using gene transcription and cortisol), with organismal and ecological measures of performance (i.e., feeding ability) under environmentally relevant combinations of environmental stressors. Our aim was to determine whether the habitat associations of Delta Smelt recorded in the field are a consequence of physicochemical parameters, such as turbidity and salinity, rather than other constraints, such as interspecific competition or predator–prey relationships. We highlight how physiological and behavioral studies that consider multiple and potentially interacting stressors are not only mechanistically revealing but also can aid resource managers in making informed decisions in support of fish conservation.

Material & Methods

Study Animals

Juvenile Delta Smelt (120 days post hatch, dph) were raised at the Fish Conservation and Culture Laboratory (FCCL) UC Davis in Byron, CA, following specific aquaculture methods for this delicate species (Baskerville-Bridges et al. 2004; Lindberg et al. 2013). Fish were spawned in April 2012 and reared at 15.4–16.4°C under a natural photoperiod and at low light intensities (1–20 lux; (Lindberg et al. 2013). Fish were fed four times daily to satiation with *Artemia franciscana* (Argent Chemical Laboratories, WA, USA), and acclimation conditions in the holding tanks prior to experimentation were as follows: dissolved oxygen (DO) =8.99 mg O_2 L^{-1} (SE=0.21), pH=7.64 (SE=0.1), salinity=0.2 ppt (SE=0.06), and turbidity=4.56 NTU (SE=0.33). All handling, care, and experimental procedures used were reviewed and approved by the UC Davis Institutional Animal Care and Use Committee (IACUC Protocol 16591).

Fish Exposures

Two experimental tests are presented in this study: one to assess the feeding ability of Delta Smelt in response to exposures to salinity and turbidity, and the second to assess the physiological stress response measured via changes in gene transcription and assays of whole-body cortisol associated with exposures to salinity and turbidity. In both experimental tests, juvenile Delta Smelt (120 dph)

were exposed in 9.5 l black high-density polyethylene buckets (US Plastic Corp.), for a period of 2 h, to an experimental matrix of turbidities and salinities. Preliminary tests in juvenile Delta Smelt held under culture conditions revealed a complete clearance time of 18 h from foregut through the intestine. Fish were therefore starved for 16 h prior to initiation of the test and an additional 2 h during acclimation to the test buckets to ensure their guts were empty. Delta Smelt are commonly caught in the field in turbid waters up to 50 NTU and in the LSZ (0.5 - 6 ppt). Broadly, Delta Smelt habitat extends from the freshwater reaches of the Delta, seaward to ~19 ppt and includes temperatures of 25°C or lower. Distribution of Delta Smelt at a finer scale (i.e., in relation to life stage, tidal, seasonal and diurnal movements, turbidity, salinity, temperature, biotic variables, the environmental conditions conducive to feeding, and how these variables interact) are poorly understood (Bennett 2005; Feyrer et al. 2007; Nobriga et al. 2008), and the mechanisms responsible for complex distribution patterns are largely unknown. In a preliminary, range-finding study, we found elevated mortality after 24 h at turbidities > 120 NTU and salinities of 12 ppt. We therefore chose turbidity and salinity treatments that were physiologically challenging but nonlethal over at least 3 h, and encompass values typical and maximal for the habitats of juvenile Delta Smelt. Turbidity levels were adjusted using Nannochloropsis algae (Nanno 3600-High yield grow out feed, Reed Mariculture Inc., USA), whereas salinity was adjusted using Instant Ocean Sea salt (Spectrum Brand Inc., USA). Handling control samples contained culture water without additional algae or sea salt. Air stones were used in all exposure vessels to maintain oxygen concentrations near saturation. Temperature (°C) was constantly monitored during exposures using iBcod temperature loggers (Alpha Mach Inc., Canada). DO (mg L^{-1}), salinity (ppt), turbidity (NTU), and light intensity (lux) were measured at the beginning of a test using a YSI 55 DO meter (YSI Inc.), a YSI 63 handheld meter (YSI Inc., Xylem Brand, for conductance and salinity), a Hach 2100q portable turbidity meter (Hach company), and a Extech instruments easy view 30 light meter (FLIR commercial Systems). Our experimental design consisted of a 6×5 matrix of turbidities and salinities, respectively. The nominal exposures were 0, 12, 25, 50, 120, and 250 NTU and 0, 2, 6, 12, and 15 ppt. Average measured salinities for each group were 0.2 ppt (SE=0.0), 2.1 ppt (SE=0.1), 6.0 ppt (SE=0.0), 12.0 ppt (SE=0.0), and 15.0 ppt (SE=0.0). Measured average turbidities were 4.13 NTU (SE=0.32), 12.6 NTU (SE=0.3), 25.9 NTU (SE=0.4), 50.9 NTU (SE=1.5), 122 NTU (SE=2.0), and 248 NTU (SE=2.0). Light intensity, pH, and temperature remained constant throughout each test at 1.04 lux (SE=0.34), 8.15 (SE=0.03), and 16.18C (SE=0.1), respectively. DO in all test buckets remained near saturation and ranged from 8.55 to 9.24 mg O2 L^{-1}. Feeding tests were conducted in triplicate, each containing 10 juveniles per bucket (total 30 fish). After the 2-h exposure, fish were fed with freshly hatched live *A. franciscana* in abundance, dispensed in 100 ml solution at a density approximating 600 *Artemia* per ml (593 ±SD

98), based on current culture methodology (Baskerville-Bridges et al. 2004; Baskerville-Bridges et al. 2005) and resulting in a final density of 7 *Artemia* ml^{-1}. Fish were allowed to feed for a period of 25 min (determined through preliminary tests as the optimal time for ingesting *Artemia* to the foregut only and prior to digestion; data not shown) after which time they were immediately euthanized and stored in 75% ethanol for analysis of gut contents. Fish were euthanized with an overdose of MS-222 (Tricaine methanesulfonate, Finquel), at a dosage of 50 mg L^{-1} buffered to a neutral pH with sodium bicarbonate ($NaHCO_3$). Guts were later dissected, and the number of *Artemia* ingested was counted under a dissecting microscope. Termination of the test and sampling of each bucket was accomplished in <20 s. The assessments of physiological stress were conducted similarly using 10 juvenile Delta Smelt per 9.5-L bucket; fish were not fed for the duration of the test. Following the 2-h exposure period, fish were euthanized as described earlier, but whole fish were then immediately snap frozen in liquid nitrogen, and stored at -80°C for assays of gene transcription and measurement of whole-body cortisol.

RNA Isolation and Quantitative Polymerase Chain Reaction Assessments

Total RNA was extracted from whole-body homogenates of individual fish, using TRIzol Reagent, (Ambion RNA, Life Technologies Corporation) according to manufacturer's guidelines. RNA concentrations were determined using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), total RNA 260/280 and 260/230 ratios ranged between 1.86–2.15 and 1.75– 2.05, respectively. Total RNA integrity was verified through electrophoresis on a 1% (wt/vol) agarose gel. Between three and five fish per treatment were assessed by quantitative polymerase chain reaction (qPCR). Complementary DNA (cDNA) was synthesized using 1 mg total RNA per sample. Primers and probes for qPCR analyses (Table 1) were designed using Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com). Primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com), and TaqMan probes were supplied by Roche or Applied Biosystems. SDS 2.2.1 software (Applied Biosystems) was used to quantify transcription, and data were analyzed using the $Log_2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) relative to handling control samples. Differences in transcription were calculated relative to Beta-Actin (b-actin); identified as a suitable reference gene for this assessment using GeNorm (Vandesompele et al. 2002). Genes were selected to determine alterations in osmotic (Na/K-ATPase), oxidative (GST), and general (HSP70) stress, as well as to integrate parameters involved in corticosteroid stress pro-opiomelanocortin (POMC), and regulation (GR2 and MR1), and gluconeogenesis (GLUT2) in Delta Smelt (Table 8).

Table 8. Primer and probe systems used for quantitative PCR analyses.

Gene Name		Primer	Roche Probe No.
Sodium/Potassium ATPase (*Na/K-ATPase*)	Forward: Reverse:	gtcatcccaatctactgcacca catgatgtcgccaatcttgc	88
Heat Shock Protein 70-kDa (*HSP70*)	Forward: Reverse:	aagattctggagaagtgcaacga ccttctcagcggtctggttct	20
Pro-opiomelanocortin (*POMC*)	Forward: Reverse:	tgttcacctgtgcaggtctga gagaagctctcttccgtggaca	127
Glutathioine-*S*-Transferase (*GST*)	Forward: Reverse:	aatctccctggcagacattgtt ggccggctctcaaacacat	127
Glucocorticoid Receptor 2 (*GR2*)	Forward: Reverse:	catcgtgaagcgtgaggagaa tgcatggagtccagtagtttgg	129
Mineralocortin Receptor 1 (*MR1*)	Forward: Reverse:	tttctacactttccgcgagtca tgatgatctccaccagcatctc	39
Glucose Transporter 2 (*GLUT2)*	Forward: Reverse:	gccatgtcagttggcctcat gacatgctgacgtagctcatcc	130
Beta Actin (□-*Actin*)	Forward: Reverse:	tgccacaggactccatacc catcggcaacgagaggtt	12

Measures of Whole-Body Cortisol

Cortisol extraction was performed on five fish per treatment, and the method was modified from studies carried out on zebrafish (Alsop and Vijayan 2009; Cachat et al. 2010; Vijayan and Leatherland 1990). Briefly, samples were thawed and homogenized in 1 ml ice-cold 1 X phosphate-buffered saline (PBS buffer: sodium potassium combo, 0.08 mol L^{-1} disodium hydrogen phosphate, 1.36 mol L^{-1} sodium chloride, mol L^{-1} potassium chloride, 0.017 mol L^{-1} potassium phosphate monobasic) using a hand-held homogenizer. The homogenate was then split in equal amounts (500 µl) into two different tubes, one aliquot was transferred into a pyrex glass tube for the extraction of cortisol and the second aliquot into a 1.5 ml eppendorf tube for determination of protein content. The cortisol extraction was performed three times for maximum yield, using 2.5mL diethyl ether (VWR International LLC, USA) per sample. Glass tubes were capped and vortexed for 1 min. Subsequently, samples were centrifuged for 15 min at 5000 rpm 3214 relative centrifugal forces (RCF) at 4°C. The supernatant was retained and transferred to a new 20 ml glass vial. Samples were left overnight in the fume hood for complete evaporation of diethyl ether before resuspension in 1mL 1 X PBS and stored at 4°C overnight. The cortisol assay (Cortisol Salivary Immunoassay, Salimetrics LLC) was then performed according to the manufacturer's instructions, and cortisol levels (µg d L^{-1}) were calculated with a four-parameter sigmoid standard curve (minus curve fit). Cortisol levels were normalized to the protein content of each sample. Protein concentrations in the homogenate were determined using the bicinchoninic acid method (Pierce, Thermo Fisher Scientific Inc.). Protein samples were

diluted 20-fold with 1 X PBS Buffer to match kit's serum albumin standards, and assay was performed according to manufacturer's instruction. Protein levels (µg m L^{-1}) were calculated using a linear standard curve. Cortisol levels were normalized to total protein and expressed as cortisol concentration (pg cortisol µg $protein^{-1}$).

Statistical Analysis

Statistical analyses were performed using R version 2.15.2 (R-CoreTeam 2014). Specific R packages used are listed with corresponding analyses. For data on feeding, the glmmADMB package (Skaug et al. 2012) was used to construct generalized mixed models to evaluate relationships of salinity and turbidity with number of *Artemia* consumed (Bolker et al. 2009). Models employed a negative binomial distribution with a zero-inflation parameter to meet the needs of the data. Fish length (fork length in millimeters) was included as a covariate to account for variation in feeding response due to fish size. Models were generated for combinations of the hypothesized predictor relationships (9 total) (Table 9) and were compared and selected using Akaike's Information Criterion corrected for small sample sizes (AIC_c; (Anderson and Burnham 2002; Burnham and Anderson 2004). AICc is defined as: -2 log*L* (θ||y) + 2K + (2K [K + 1]/n - K - 1), where log*L* (θ||y) is the maximized log-likelihood of the model parameters given the data, *K* is the number of estimable parameters, and *n* is the sample size (Burnham 2002). The best explanatory model was identified based on AIC_c difference (Δi; i.e., the difference between the AIC_c of model *i* and the lowest AIC_c observed) and Akaike weight (w_i; calculated as the model likelihood normalized by the sum of all model likelihoods). Models with an AIC_c difference (Δi; i.e., the difference between the AIC_c of model *i* and the lowest AIC_c observed) < 2 were considered to be the most favored models, and w_i closer to 1 indicated greater confidence in the selection of the favored explanatory model (Burnham and Anderson 2004). The penalty imposed by AIC_c for each additional parameter in a model is 2K + (2K [K + 1]/n – K - 1), such that the inclusion of an additional parameter may result in a new model that is Δ*i* < 2, even if the parameter has no additional explanatory ability (Arnold 2010; Burnham 2002). Therefore, in the few cases where multiple models had Δ*i* < 2, but differed by the inclusion of an additional parameter (*K*), all models are reported but the more parsimonious model was selected (Arnold 2010). Biochemical data were transformed (cubed root) to meet assumptions of normality and analyzed with the R stats package 2.15.2 (R-CoreTeam 2014). For biochemical and transcriptomic responses, salinity and turbidity were treated as categorical variables in general linear models (GLMs;(Quinn and Keough 2002). Biochemical and transcriptomic responses can reflect a large variety of response curves, such as sublethal thresholds followed by nonmonotonic patterns of upregulation or downregulation as an organism heads toward their tolerance limit for a given

stressor. Since this was the first time biochemical and transcriptional changes have been quantified in Delta Smelt in response to turbidity and salinity, this approach allowed us to focus on a smaller set of models to evaluate the contributions of the estimated parameters (Johnson and Omland 2004), which can then inform future studies characterizing response curves in greater depth (Niehaus et al. 2012). Models were generated, compared, and selected using similar methods as those described above for each gene or biochemical marker. Genes were checked against one another for issues of collinearity (none were found), and GLMs were constructed for each gene response. Model assumptions of normality of residuals were checked graphically, and homogeneity of variances was confirmed with Fligner-Killeen tests (Crawley 2007). Graphics were created in the ggplot2 package version 0.9.3 (Wickham and Chang 2012) and Sciplot 1.1-0 (Morales 2012).

Table 9. Summary of model selection analysis for Delta Smelt Artemia consumption. Best explanatory model selected is indicated by bolding. AICc difference (Δi) is the difference between the AICc of model i and the lowest AICc observed. Akaike weight (w_i) is calculated as the model likelihood, $\exp(-\Delta_i / 2)$, normalized by the sum of all model likelihoods; values close to 1 indicate greater confidence in the selection of a model. All models were adjusted for zero inflation and used a negative bionomial distribution with a log link function.

Model Parameters	Δi	df	w_i
Artemia~NTU+Length	**0**	**6**	**0.443**
Artemia~Salinity*NTU+Length	0.3	8	0.39
Artemia~Salinity+NTU+Length	2	7	0.167
Artemia~Salinity+Length	32.8	6	<0.001
Artemia~1(*null model*)	69.7	3	<0.001
Artemia~NTU	140.2	5	<0.001
Artemia~Salinity*NTU	158	7	<0.001
Artemia~Salinity+NTU	169.9	6	<0.001
Artemia~Salinity	189.5	5	<0.001

Results

Feeding

The full model (Artemia ~ Salinity*NTU + Length) and one including turbidity and the covariate fish length (Artemia ~ NTU + Length) were supported by AIC_c and w_i ($\Delta i < 2$; Table 9). The more parsimonious model (Artemia ~ NTU + Length) was selected as the best explanatory model (Arnold 2010); however, it is possible that salinity and its interactions with turbidity may also influence Delta Smelts' feeding (Figure 13). The best model depicted a negative impact of turbidity on juvenile

smelts' feeding (Figure 13 and supplementary Table 17 Appendix), with the number of *Artemia* ingested per fish particularly reduced at high turbidities (i.e., 250 NTU). The covariate fish size was also present in the supported model; while larger fish consumed more *Artemia*, the slope of this relationship was weak but consistent across all treatments (Figure 12 and supplementary Table 17 Appendix).

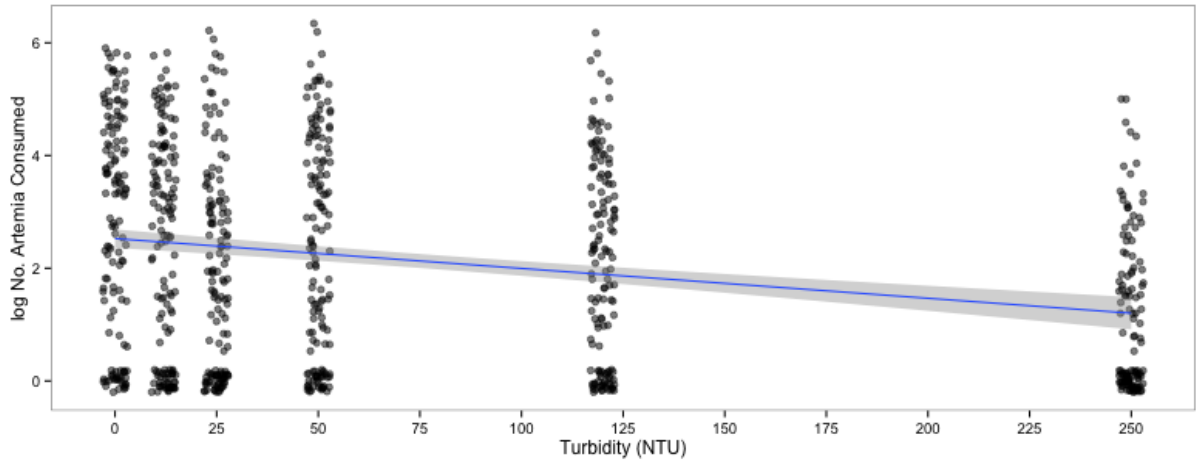

Figure 11. Graphical relationship between turbidity and feeding performance (number of Artemia ingested in 25 min) of juvenile Delta Smelt (see Table 2 for model selection).Line denotes best fit linear regression with shading of 95% confidence intervals. Data points are displayed with random position jitter (x,y= 3,0.2) and opacity to portray density of overlapping individual data points. The y axis is depicted on a log scale to reflect the log link function applied in the negative binomial model.

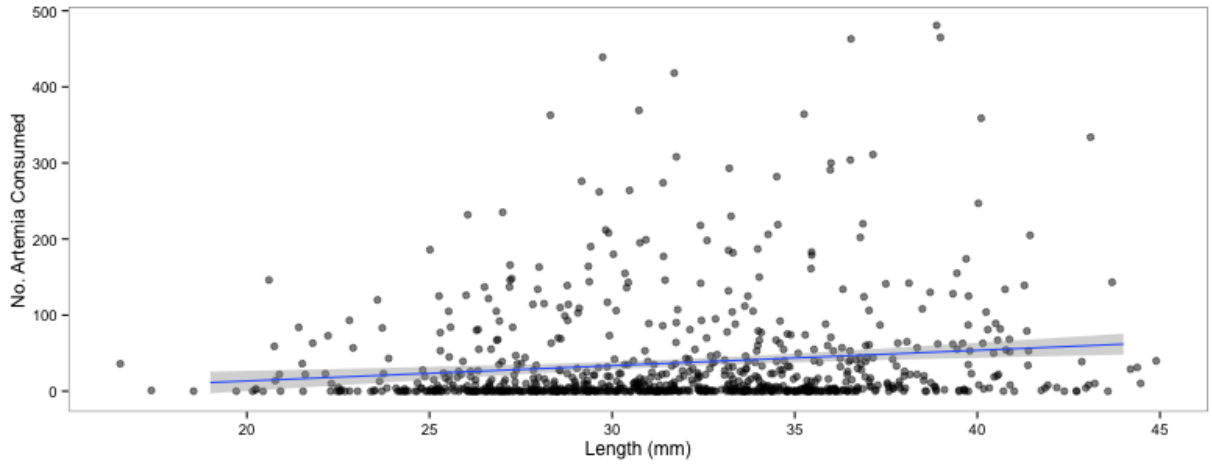

Figure 12. Graphical depiction of the shallow, positive relationship between juvenile Delta Smelt fish length (fork length mm) and number of Artemia ingested during the 25 min. feeding period as covariate in generalized linear mixed model, see Table 2 for specific model output. Line denotes best fit linear regression with shading of 95% confidence intervals. Data points are displayed with random position jitter (x,y= 3,0.2) and opacity to portray density of overlapping individual data points.

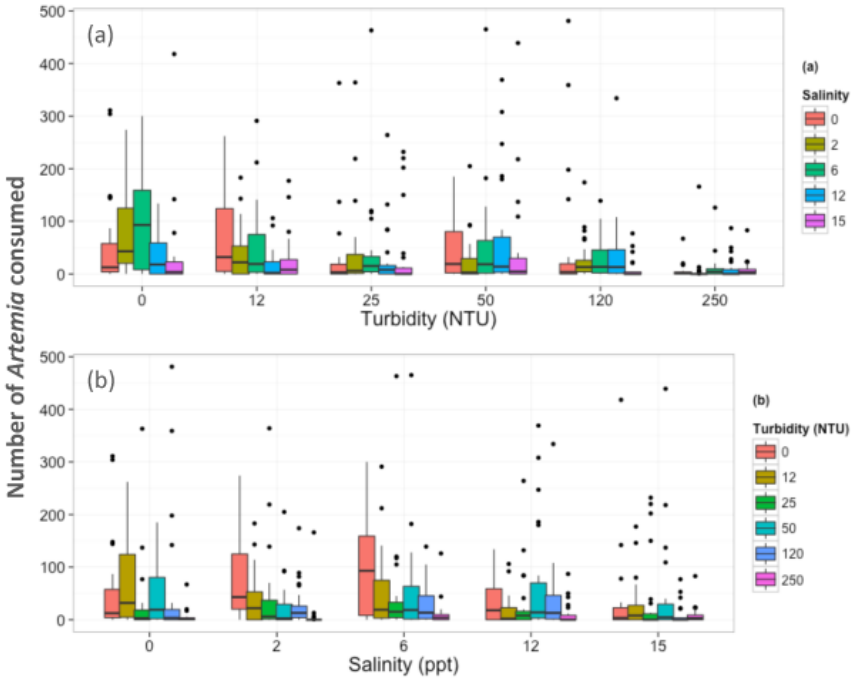

Figure 13. Boxplots displaying the number of Artemia ingested in the 25 min feeding period by 120 dph Delta Smelt dependent on salinity and turbidity acclimations of 2 h. Upper and lower hinges of boxes denote the 75th and 25th percentiles, respectively, and the line within the box demarking the median (50th percentile). Upper and lower whiskers are calculated ± 1.5 * interquantile range; data points which fall outside this range are classified as outliers and represented by individual dots. Panel (a) shows feeding performance in each salinity treatment with respect to turbidity. Panel (b) shows feeding performance in each turbidity treatment with respect to salinity.

QPCR and biochemical assessments

In contrast to our findings that turbidity impacted feeding performance, salinity was the stressor that more consistently related to physiological stress, as measured by qPCR (Table 10 and Figure 14 and Figure 15). The most favored models for *Na/K-ATPase* and *POMC* included only salinity as a predictor, whereas *GST* was best described by an additive model including both salinity and turbidity (GST ~ Salinity + Turbidity). The MR1 ~ Salinity model was also within the Δ*i* = 2 cutoff for model selection (Δ*i* = 1.6); however, the null model was selected because it was more parsimonious and its w_i was substantially above that of MR1 ~ Salinity. Interestingly, the null model was the best-fit model for all other genes, including genes involved in the general response to stress, that is, *HSP70*, and specifically to corticosteroid regulation (*GR2*) and glucose transport (*GLUT2*). GLM tables of the selected models for genes (other than those for which the null model was selected) are detailed in Supplementary Table 18. *Sodium-Potassium ATPase* is a key enzyme involved in ion transport during osmoregulation in fish and is a molecular indicator of osmoregulatory stress. Transcription of *Na/K-ATPase* increased with higher salinities (Supplementary and Figure 14b). *Glutathione-S-transferase* (*GST*) is a detoxification system that defends cells against reactive oxygen species (ROS) and is also known to respond to osmotic stress in fishes (Choi et al. 2008). Increasing salinity and turbidity affected the upregulation of *GST* transcription (Figure 14c). Hypothalamic *POMC* is a precursor protein of many hormonal peptides, including the pituitary adrenocorticotropic hormone (ACTH), which is transcribed in response to biological stress (Palermo et al. 2012). Transcription of *POMC* also increased with salinity (Supplementary Table 18 and Figure 15a). However, as stated earlier, other genes related to glucocorticoid regulation and glucose transport (i.e., *GR2*, *MR1*, and *GLUT2*) did not exhibit the same pattern (Figure 15b-d). The best explanatory model for the response of whole-body homogenate cortisol was $\sqrt[3]{}$ Cortisol ~ Salinity (Table 11and supplementary Table 19 Appendix). Lactate exhibited a similar pattern; however, Δ*i* =1 for the more parsimonious $\sqrt[3]{}$ Lactate ~ 1(null) model, and therefore was selected as more favorable. This was the same for glucose, for which the most parsimonious supported model was $\sqrt[3]{}$ Glucose ~ 1(null). GLM tables of the selected models for cortisol are detailed in Supplementary Table 20 in the appendix.

Table 10. Summary of model selection analysis for Delta Smelt transcriptomic responses. Best explanatory model selected is indicated by bolding. AICc difference (Δ_i) is the difference between the AICc of model i and the lowest AICc observed. Akaike weight (w_i) is calculated as the model likelihood, exp(-Δ_i / 2), normalized by the sum of all model likelihoods; values close to 1 indicate greater confidence in the selection of a model.

Model Parameters	Δi	df	w_i
HSP70~1*(null)*	**0**	**2**	**0.766**
HSP70~Salinity	2.6	6	0.207
HSP70~Turbidity	7.3	7	0.020
HSP70~Salinity+Turbidity	9.4	11	0.007
HSP70~Salinity*Turbidity	34	31	<0.001
Catalase~1*(null)*	**0**	**2**	**0.887**
Catalase~Salinity	4.6	6	0.090
Catalase~Turbidity	7.6	7	0.020
Catalase~Salinity+Turbidity	12.4	11	0.002
Catalase~Salinity*Turbidity	32.4	31	<0.001
Na+/K+ ATPase~Salinity	**0**	**6**	**0.890**
Na+/K+ ATPase~1*(null)*	4.5	2	0.093
Na+/K+ ATPase~Salinity+Turbidity	8.1	11	0.015
Na+/K+ ATPase~Turbidity	12.5	7	0.002
Na+/K+ ATPase~Salinity*Turbidity	19.6	31	<0.001
POMC~Salinity	**0**	**6**	**0.964**
POMC~Salinity+Turbidity	7.3	11	0.025
POMC~1*(null)*	9.1	2	0.010
POMC~Salinity*Turbidity	13	31	0.001
POMC~Turbidity	17.2	7	<0.001
GR~1*(null)*	**0**	**2**	**0.864**
GR~Salinity	5.1	6	0.067
GR~Turbidity	5.2	7	0.065
GR~Salinity+Turbidity	10.6	11	0.004
GR~Salinity*Turbidity	29.3	31	<0.001
MR~1*(null)*	**0**	**2**	**0.681**
MR~Salinity	1.6	6	0.299
MR~Turbidity	7.8	7	0.014
MR~Salinity+Turbidity	9.5	11	0.006
MR~Salinity*Turbidity	29.5	31	<0.001
Glucose Transporter~1*(null)*	**0**	**2**	**0.801**
Glucose Transporter~Salinity	3.2	6	0.161
Glucose Transporter~Turbidity	6.5	7	0.032
Glucose Transporter~Salinity+Turbidity	9.8	11	0.006
Glucose Transporter~Salinity*Turbidity	28.7	31	<0.001
SGK3~1*(null)*	**0**	**2**	**0.866**
SGK3~Salinity	4.1	6	0.112
SGK3~Turbidity	7.6	7	0.020
SGK3~Salinity+Turbidity	11.4	11	0.003

SGK3~Salinity*Turbidity	23.3	31	<0.001
NF KB~1*(null)*	**0**	**2**	**0.724**
NF KB~Salinity	2.3	6	0.229
NF KB~Turbidity	6.1	7	0.034
NF KB~Salinity+Turbidity	8.1	11	0.013
NF KB~Salinity*Turbidity	14.2	31	<0.001
GST~Salinity+Turbidity	**0**	**11**	**0.813**
GST~Salinity	3.8	6	0.123
GST~Salinity*Turbidity	5.3	31	0.058
GST~Turbidity	10.4	7	0.005
GST~1*(null)*	11.6	2	0.002

Table 11. Summary of model selection analysis for Delta Smelt biochemical responses. Best explanatory model selected is indicated by bolding. AICc difference (Δ_i) is the difference between the AICc of model i and the lowest AICc observed. Akaike weight (w_i) is calculated as the model likelihood, exp($-\Delta_i$ / 2), normalized by the sum of all model likelihoods; values close to 1 indicate greater confidence in the selection of a model.

Model Parameters	Δi	df	w_i
^{3}VLactate~Salinity	0	6	0.599
^{3}VLactate~1*(null)*	**1**	**2**	**0.355**
^{3}VLactate~Salinity+Turbidity	6.1	11	0.029
^{3}VLactate~Turbidity	7	7	0.018
^{3}VLactate~Salinity*Turbidity	21.7	31	<0.001
^{3}VCortisol~Salinity	**0**	**6**	**0.754**
^{3}VCortisol~Salinity+Turbidity	2.6	11	0.202
^{3}VCortisol~1*(null)*	6.1	2	0.036
^{3}VCortisol~Turbidity	9.4	7	0.007
^{3}VCortisol~Salinity*Turbidity	13.8	31	< 0.001
^{3}VGlucose~1*(null)*	**0**	**2**	**0.638**
^{3}VGlucose~Salinity	1.5	6	0.307
^{3}VGlucose~Turbidity	5.8	7	0.034
^{3}VGlucose~Salinity+Turbidity	7.9	11	0.012
^{3}VGlucose~Salinity*Turbidity	8.4	31	0.010

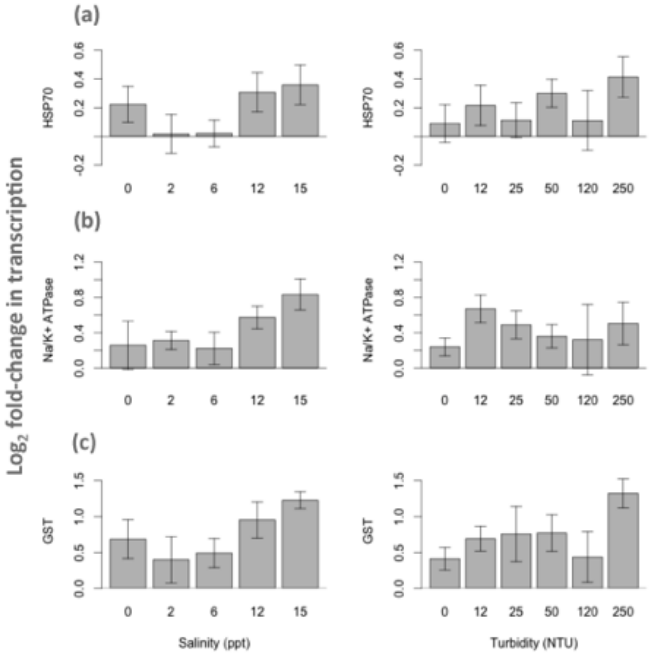

Figure 14. Gene markers for general stress, salinity, and oxidative stress, in 120 dph Delta Smelt dependent on salinity and turbidity variables following a 2 h acclimation period. Log_2 fold-change in gene transcription relative to b-actin, ± standard errors. a) Heat Shock Protein 70 kDa (*HSP70*), b) Sodium-Potassium ATPase (*Na/K-ATPase*), c) Glutathione-*S*-transferase (*GST*).

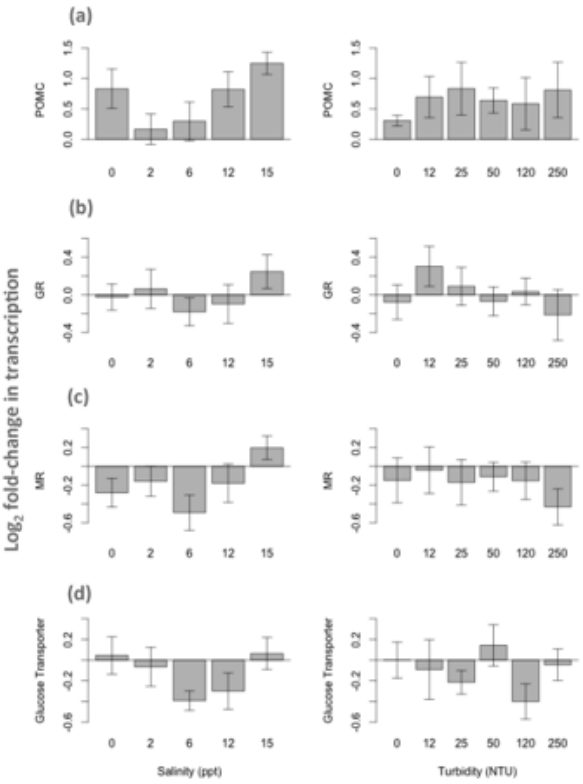

Figure 15. Hypothalamic-pituitary-interrenal (HPI) axis, corticosteroid regulation stress and glucose transporter responses in 120 dph Delta Smelt dependent on salinity and turbidity variables following a 2 h acclimation period. Log_2 fold-change in gene transcription relative to b-actin, ± standard errors. a) Pro-opiomelanocortin (*POMC*), b) Glucocorticoid Receptor 2 (*GR2*), c) Mineralocortin Receptor 1 (*MR1*), and d) Glucose Transporter 2 (*GLUT2*).

Discussion

Turbidity impacted juvenile Delta Smelts' feeding performance over the 2-h exposure period. Overall, there was a negative relationship between turbidity levels and feeding performance, with highest feeding rates at low turbidity (<12 NTU), relatively persistent feeding rates over a broad range of turbidities (12-120 NTU), and a strong decline in feeding rates at levels of 250 NTU. Turbidity, defined as the distance that light penetrates the water column, and caused by individual particles such as suspended sediment, dissolved organic matter, and phytoplankton (Rice et al. 1994), can have both positive and negative effects on fish, depending on the species (Utne-Palm 2002). For instance in bluegill (*Lepomis macrochirus*) and adult cape silverside (*Atherina breviceps*), reduced feeding rates were reported at higher turbidities (Gardner 1981; Hecht and Van der Lingen

1992). This is supported by our findings, in which Delta Smelt exhibited reduced feeding rates at 250 NTU. Studies carried out by Baskerville-Bridges et al. (2004) on larval Delta Smelt found highest feeding rates at 11 NTU and 22.8 lux, and stressed the significant interaction between turbidity and light intensity. This interaction may explain why feeding was highest at low turbidity as the average light intensity utilized in our test was 1.04 lux, matching that of the holding tanks, but lower than the reported tests conducted on larval Delta Smelt. This low light intensity may also have further impeded feeding at the highest turbidity levels of 250 NTU. It is known that increasing turbidity reduces the reactive distance in fish (Utne-Palm 2002), thereby minimizing the volume of water that the fish can potentially search for food (Hecht and Van der Lingen 1992). Delta Smelt are visual predators; thus, feeding is more likely to occur through direct encounters with prey under highly turbid conditions such as the tested 250 NTU. A general observation while conducting this test was that swimming activity increased at low turbidity, and fish were seen more often swimming near the surface in the clear treatments. This higher activity may be due to the fish sensing the handler's presence, thus triggering escape responses and searching behavior, since turbidity is expected to reduce stress levels in fish by providing protection from predators (Gregory and Northcote 1993; Sirois and Dodson 2000b). Our test was designed, however, to function at low light intensity due to the depth restrictions imposed by the exposure vessels. Low light intensity within a shallow container may have allowed Delta Smelt to utilize the available water column; occupying the surface and utilizing darker (simulating depth) zones that would not have been available had higher light intensities been used. It is important to note that this study was conducted using algal suspensions of *Nannochloropsis* to modify turbidity. This is the same algal suspension that is used at the culture facility to rear larval Delta Smelt (Baskerville-Bridges et al. 2005), and there were no signs of ingested algae in the smelts' guts in any of the treatments. Although this approach is not directly representative of the mixed, and highly variable composition of suspended particles, dissolved organic matter, and diverse phytoplankton communities present in the SFBD, our data do provide evidence for relatively consistent feeding performance at turbidities up to 120 NTU at the light intensity assessed. In nature, Delta Smelts' feeding performance may vary due to variation in turbidity as well as to natural light intensity. Delta Smelt are known to be relatively abundant in the LSZ (Hobbs et al. 2006; Moyle et al. 1992), and wild Delta Smelt, collected during monitoring efforts, often are observed in association with turbidities between 10 and 50 NTU (Feyrer et al. 2007). Our data are consistent with these observations and suggest that juvenile Delta Smelt are capable of consistent feeding across the range of turbidities and salinities that are typical of their SFBD habitat. Although turbidity was the factor that significantly impacted juvenile Delta Smelts' feeding performance, the gene transcription and biochemical data suggest that salinity was the dominant

factor affecting osmoregulation and the generalized stress response in juvenile Delta Smelt. During transfer to saltwater, many fish alter gill *NaK-ATPase* enzyme activity, facilitating ion exchange and osmoregulation (Evans 2008). An increase in gill *NaK-ATPase* enzyme activity is often preceded by, or accompanied by, increases in *NaK-ATPase* mRNA expression, and changes in *NaK-ATPase* mRNA expression patterns have been shown in many fish species following transfer to saline conditions; for example, rainbow trout (Richards et al. 2003), killifish (Scott et al. 2004), and Atlantic salmon (Bystriansky and Schulte 2011). In Delta Smelt, the transcription of *NaK-ATPase* as well as *GST* increased in response to increasing salinity. *GST* is a detoxification system that defends cells against ROS, and responds to osmotic stress in fishes (Choi et al. 2008). Taken together, these data suggest that the transcription of *Na/K-ATPase* and *GST* may play a role in juvenile Delta Smelts' responses to osmotic stress.

Cortisol is the main corticosteroid hormone in fish that is expressed in response to stress, and changes in the transcription profiles of osmoregulatory genes in fish gills in response to seawater are thought to be hormonally mediated. Many studies report elevated cortisol following transfer to seawater (McCormick 1995; McCormick 2001), and these increases can be rapid (minutes to hours) and persistent (over days) depending on the severity of the osmoregulatory challenge. Hypothalamic *POMC* is a neuroactive ligand-receptor, and precursor protein to the ACTH, which is expressed in the anterior pituitary and is a major secretagogue of cortisol. That is, *POMC* is a major precursor to the activation of the hypothalamic-pituitary-interrenal (HPI) axis, the system responsible for the secretion of cortisol in teleosts (Alsop and Vijayan 2009; Mommsen et al. 1999; Palermo et al. 2012; Schjolden et al. 2009). Corticosteroid receptors, such as glucocorticoid and mineralocorticoid receptors, function to regulate gene transcriptions that are implicated in responses to stress (Alsop and Vijayan 2009; Savitz et al. 2009). Glucocorticoid receptors are induced by cortisol, while mineralocorticoid receptors mediate effects on electrolyte and fluid balance, and are known to downregulate glucose transport systems (Korgun et al. 2011). It is interesting, therefore, that neither of the measured corticosteroid receptors nor the glucose transporter, responded significantly to the salinity treatments.

In Delta Smelt, hypothalamic *POMC* transcription was induced at high levels of salinity (15 ppt), suggesting greater effects on activation of the HPI axis. However, while the best explanatory models indicated that cortisol from whole-body homogenates also increased at higher salinities, transcription of neither the corticosteroid receptors nor the glucose transporter responded. Vijayan and Leatherland (1990) reported that during chronic stress, plasma cortisol in brook charr are elevated, then fall back to resting levels, and that cortisol clearance levels are dependent on corticoid receptors, binding proteins and tissue uptake of cortisol. It is probable that cortisol

dynamics are complex and highly variable in Delta Smelt, which are notoriously delicate and sensitive to handling stress, with reported mortality rates above 90 % in field-caught adults; this sensitivity has resulted in specific collection and transport procedures published for this species (Swanson et al. 1996). It is known that Delta Smelts' tolerance to stress is limited (Connon et al. 2011a; Connon et al. 2011b; Connon et al. 2009; Swanson et al. 1996) which may account for the variable results related to the cortisol pathways determined in this study. Furthermore, and more specifically, assessments need to be conducted to establish cortisol levels and related genes in Delta Smelt, elicited by differing stressors (e.g., high stocking density, confinement, and toxicant exposure), and at different life stages, to more fully interpret the complexity in the responses measured.

The overall response profiles of gene transcription measured in this study are nonmonotonic. Nonmonotonic dose–response curves are not uncommon, especially in complex mechanistic studies, and inflexions in transcription responses have been postulated to represent thresholds of maximum stress (Connon et al. 2011b; Connon et al. 2012; Conolly and Lutz 2004; Heckmann et al. 2008; Vandenberg et al. 2012). They have been observed following exposure to contaminants at concentrations at which significant mechanistic effects, determined by qPCR, correspond with detrimental effects upon behavior, such as swimming performance (Beggel et al. 2010; Fritsch et al. 2013), and they have been universally acknowledged by endocrinologists (Vandenberg et al. 2012; Zoeller et al. 2012). Transcription of the aforementioned genes (Figure 14 and Figure 15) also profile the same response curve with respect to turbidity. This response curve is mostly inversed from that of the salinity responses, but also follows a nonmonotonic response, increasing with elevated turbidity at either 12 or 25 NTU, and reducing at 50 and 120 NTU, with a secondary and significant upregulation at 250 NTU. This increase and resulting inflexion is likely to represent maximum tolerance levels, beyond which long-term detrimental effects would be observed.

We hypothesized that Delta Smelts' physiological performance in response to physicochemical parameters (i.e., salinity and turbidity) could be determined through measurements of feeding ability and biochemical and molecular parameters that relate to physiological stress. Integrating both assessments (feeding and physiological stress), overall responses following a 2-h acclimation period indicate an optimal performance at low turbidity and at a salinity between above 0 and 6 ppt, with detrimental effects at turbidities above 120 NTU and salinities at or above 12 ppt. These results are supported by field studies that associate Delta Smelts' abundance with 0.5-6 ppt (Hobbs et al. 2006; Kimmerer 2002; Moyle et al. 1992) and turbidities of 10 and 50 NTU (Feyrer et al. 2007). Our study provides insight into optima for juvenile Delta Smelt with respect to the assessed parameters, that will guide future long-term, multi-life-stage evaluations toward assessing the fundamental niche

for this species. Future assessments will address larval, juvenile, and adult life-stages, additional stressors and timescales of exposures, and consider additional light intensities that encompass ecological and seasonal ranges, to more comprehensively assess the habitat requirements of Delta Smelt. Nevertheless, this study has insofar supported past and current field assessments of Delta Smelts' habitat and distribution, and should be informative to SFBD resource managers.

5. Contaminants

The content of this chapter was published:

Hasenbein, M., I. Werner, L. A. Deanovic, J. Geist, E. B. Fritsch, A. Javidmehr, C. Foe, N. A. Fangue & R. E. Connon, 2014. Transcriptomic profiling permits the identification of pollutant sources and effects in ambient water samples. Science of The Total Environment 468–469(0):688-698 doi:http://dx.doi.org/10.1016/j.scitotenv.2013.08.081.

Abstract

Contaminant exposure is one possible contributor to population declines of endangered fish species in the Sacramento-San Joaquin Estuary, California, including the endangered Delta Smelt (Hypomesus transpacificus). Herein we investigated transcriptional responses in larval Delta Smelt resulting from exposure to water samples collected at the Department of Water Resources Field Station at Hood, a site of concern, situated upstream of known Delta Smelt habitat and spawning sites and downstream of the Sacramento Regional Wastewater Treatment Plant (SRWTP). Microarray assessments indicate impacts on energy metabolism, DNA repair mechanisms and RNA processing, the immune system, development and muscle function. Transcription responses of fish exposed to water samples from Hood were compared with exposures to 9% effluent samples from SRWTP, water from the Sacramento River at Garcia Bend (SRGB), upstream of the effluent discharge, and SRGB water spiked with 2 mg/L total ammonium (9% effluent equivalent). Results indicate that transcriptomic profiles from Hood are similar to 9% SRWTP effluent and ammonium spiked SRGB water, but significantly different from SRGB. SRGB samples however were also significantly different from laboratory controls, suggesting that SRWTP effluent is not solely responsible for the responses determined at Hood, that ammonium exposure likely enhances the effect of multiple-contaminant exposures, and that the observed mortality at Hood is due to the combination of both effluent discharge and contaminants arising from upstream of the tested sites.

Introduction

Aquatic ecosystems are among the most diverse ecosystem types worldwide, however, there have been significant declines in biodiversity over the past decades; attributed to habitat destruction and degradation, flow modification, invasive species, overexploitation, and overall water quality (Dudgeon et al. 2006; Geist 2011; Kennish 2002). The Sacramento-San Joaquin Estuary in California is an example of detrimental effects resulting within an aquatic ecosystem with intense anthropogenic impact (Cloern and Jassby 2012; Lund et al. 2010). Endemic to this system is a pelagic fish species

that has exhibited a gradual decline in population since the 1980s (Bennett 2005; Moyle et al. 1992) with a significant step decline recorded in 2000 (Feyrer et al. 2007; Sommer et al. 2007). The Delta Smelt (Hypomesus transpacificus) was classified as threatened under the Federal and State Endangered Species Act (ESA), 1993, and listed as endangered under the Californian Endangered Species Act (CESA) in 2010 (DFG 2011). It is known as a species with an annual life cycle, low fecundity, and a relatively limited habitat range, making it highly susceptible to changes in the Sacramento-San Joaquin Estuary (Moyle et al. 1992). Habitat degradation, habitat loss, competition with introduced species, decreased food availability, along with changes in abiotic water quality parameters like temperature, salinity and turbidity, have all been the subject of critical scrutiny and are considered to play a significant role in declining Delta Smelt numbers (Moyle et al. 1992). Harmful effects on biota in the Sacramento–San Joaquin Estuary are also likely evoked by contaminants entering the delta through anthropogenic activities such as wastewater treatment effluent, and agricultural and urban runoff (Kennish 2002; Kuivila and Foe 1995a; Kuivila and Moon 2004a; Thompson et al. 2000).

The impacts of environmentally relevant concentrations of pollutants on aquatic organisms are often subtle, and thus difficult to determine, however, in the past decade researchers in the ecotoxicogenomics field have successfully evaluated effects of contaminants upon a number of species (Connon et al. 2008; Denslow et al. 2007; Garcia-Reyero et al. 2008; Garcia-Reyero et al. 2009; Garcia-Reyero et al. 2011; Geist et al. 2007; Heckmann et al. 2008; Watanabe and Iguchi 2006). Genomic responses at the individual level, often assessed through microarray technology, have been extrapolated to effects on populations (Connon et al. 2008; Fedorenkova et al. 2010; Heckmann et al. 2008; Miracle and Ankley 2005; Snape et al. 2004; Watanabe and Iguchi 2006) creating a powerful tool for use in risk assessment (Hamadeh et al. 2002; Watanabe and Iguchi 2006). Although genome sequencing for non-model, ecologically relevant species is still in the early stages (Denslow et al. 2007), the use of transcriptome analyses in aquatic toxicology is rapidly growing, and its application has the potential to provide information about mechanisms and modes of action for classes of chemicals, as well as provide specific signatures of toxicity (Connon et al. 2012; Denslow et al. 2007; Hamadeh et al. 2002).

We have previously developed a cDNA microarray for the Delta Smelt (Connon et al. 2009), which was used to assess the effects of single contaminants (i.e. esfenvalerate, copper and ammonia) on larval fish (Connon et al. 2011a; Connon et al. 2011b; Connon et al. 2009). However, the transferability of the methods applied in these studies to complex chemical mixtures commonly encountered in the field has not yet been tested. We utilize microarray and quantitative PCR analyses to assess transcription responses in Delta Smelt exposed to water samples from the

Sacramento River. Samples were collected at the California Department of Water Resources Water Quality Monitoring Station at Hood, a test site of interest and identified as being of poor water quality (Werner et al. 2010b), located downstream of the Sacramento Regional Wastewater Treatment Plant (SRWTP), and at the Sacramento River at Garcia Bend (SRGB), located upstream from the SRWTP effluent outlet. The SRWTP discharges its effluent into the lower Sacramento River, which ultimately leads to Delta Smelt spawning and larval nursery areas. Total ammonium in the Sacramento River, downstream of the SRWTP point of discharge, has been recorded at concentrations up to 1.0 mg/L, whilst concentrations of 0.28 mg/L have been reported directly upstream from known Delta Smelt spawning and nursery areas (Werner et al. 2010a). The effects of ammonia on Delta Smelt have previously been reported (Connon et al. 2011b), however there is a lack of information on the effects of effluent sourced ammonia, within a complex mixture of contaminants, which is integrated into this ambient water toxicity study. The aim of this study was to investigate whether ammonium entering the system as part of a contaminant mixture present in wastewater effluent, or Sacramento River water, would exert greater toxicity than as a single substance. Therefore we compare exposures to wastewater effluent, with upstream river water samples, with and without added ammonium, and contrast responses to down-stream water sample exposures.

Material & Methods

Test Organism

Delta Smelt were obtained from the University of California Davis (UC Davis) Fish Conservation and Culture Laboratory (UCD-FCCL) in Byron, CA, USA and transported to the Aquatic Toxicology Laboratory (presently Aquatic Health Program) UC Davis in black 2.5 gal buckets at a maximum density of 150 fish per bucket. Containers were placed in coolers packed lightly with ice to maintain a temperature of 16 ±2 °C during transport. Hatchery water was also used for laboratory control and low electrical conductivity (EC, adjusted to 20 °C) control treatments. This water was pumped directly from the intake channel of the H.O. Banks Pumping Facility near Byron, CA, and passed through a series of sedimentation beds containing natural vegetation to allow any suspended solids in the water to precipitate. The less turbid water was then exposed to an ozonation system to kill any potentially harmful microbes. Ozonated FCCL water was transported to UCD-ATL, and control waters were prepared for the test one day before fish were collected.

Water Sample Collection

Results of two exposure experiments are presented here: a) Exposure to water sampled from the Sacramento River at Hood, on April 30th, 2009, and b) Upstream and effluent exposures on SRGB and SRWTP water samples, beginning on June 11th, 2009. The latter was conducted to assess the effect and contribution of contaminants, including total ammonium, to sites downstream of the SRWTP outlet, and as such also included SRGB water spiked with ammonium, as detailed below. a) Water from the Sacramento River at California Department of Water Resources Water Quality Monitoring Station at Hood (Coordinates: 38°22'03.6"N 121°31'13.6"W; hereafter referred to as Hood) was collected as a single grab sample. This site is located approximately 8 miles downstream of the SRWTP. The sample was taken from shore and pumped from a depth of approximately 0.5 m using a standard water pump.

b) Sacramento River water was collected at Garcia Bend (SRGB), approximately 2 miles upstream from the SRWTP effluent discharge. This water was tested unaltered (as control), mixed with SRWTP effluent to a final total ammonium concentration of 2 mg L^{-1} (averaging 9% effluent), or spiked with a concentrated stock solution of ammonium chloride (4,000 ppm NH_4CL, Sigma-Aldrich, ACS reagent grade N99%), to match the ammonium concentration of the SRWTP effluent. Final total ammonium concentrations were 1.9 ± 0.23 mg L^{-1} for SRWTP effluent samples and 1.87 ± 0.26 (SD) for spiked SRGB during the 7-d experimental period. SRGB and effluent samples were collected daily, one day prior to being used for testing. SRWTP effluent was collected in form of 24-h composite samples. Twenty liter, clear low-density polyethylene (LPDE) cubitainers (total 700 liters) were used for collection and transport of water samples to the UCD ATL and were kept on ice to maintain sample temperature at 0-6 °C until receipt at UCD ATL, where water samples were stored in an environmental chamber at 4 °C.

Exposures

Fish were maintained for 48 h in test conditions prior to test initiation, and treated with antibiotics (Maracyn and Maracyn-2, Virbac AH Inc., Fort Worth TX), to reduce the likelihood of disease-induced effects, and eliminate possible infections (Connon et al. 2011b). Final antibiotic concentrations were 5.3 mg L^{-1} Maracyn (erythromycin) and 0.26 mg L^{-1} Maracyn-2 (minocycline). Use of test organisms was approved by the UC Davis Institutional Animal Care and Use Committee (Animal Use Protocol for Animal Care and Use #13361). This institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare. The Assurance Number is A3433-01.

Tests were conducted in 10-L aerated aquaria filled with 7-L of water from Hood, SRWTP and SRGB samples, and respective controls. Larval Delta Smelt, 41 days post hatch (dph) and 47-dph, ranging in fork length between 15 and 20 mm, were used for exposures. Experimental control fish for both exposures were maintained in hatchery water from UCD-FCCL. Larval Delta Smelt are known to require low levels of turbidity for feeding (Feyrer et al. 2007; Hasenbein et al. 2013; Hobbs et al. 2006), thus the turbidity of controls was adjusted to match that of the field water samples (Table 13) using Nanno 3600™, a concentrated *Nannochloropsis* algae solution (68 · 109 cells ml^{-1}; Reed Mariculture, Inc. Campbell, CA). No turbidity adjustments were made on field samples from Hood, SRWTP or SRGB. Twelve larval fish were placed into each of four replicate aquaria per experimental treatment. Tests were conducted at 17 ± 1.2 °C, a light: dark cycle of 16 h: 8 h, and animals were fed "ad libitum" three times a day during the acclimation and testing period with live *Artemia franciscana*. At test initiation, water in aquaria was drained to approximately 2 L, replenished with respective experimental water samples, and exposed in a flow-through system at a rate of 5.56 L per day. Fish were exposed for 7 d and EC, dissolved oxygen (DO), pH, temperature, turbidity and total ammonium were measured daily. Dissolved oxygen and EC were measured using YSI 85 meters, and pH was measured with a Beckman 240 pH meter. Turbidity was measured using a Hach 2100P Turbidity meter. Total ammonium was measured using a HACH DR/890 Colorimeter Meter and a HACH AmVerTM Low Range Ammonia Test 'N Tube TM Reagent Set 0-2.5 mgL^{-1} N (HACH Inc.). Unionized ammonia concentrations for all samples were calculated using measured total ammonia-N, temperature, EC and pH. Mortality was recorded on a daily basis, and dead fish were removed. All tests were terminated at the same time of day (noon), when surviving fish were counted, euthanized with buffered (neutral pH, using sodium bicarbonate ($NaHCO_3$)) tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO, USA), rinsed in deionized water, snap-frozen, and stored at −80 °C for subsequent transcriptomic analyses.

Microarray Screening on Field Station Assessments (Hood)

Microarray assessments were carried out on fish exposed to water samples from Hood. Subsequent analyses of SRGB and SRWTP tests were conducted using qPCR on genes identified as responding significantly from the microarray study (see below). We utilized a Delta Smelt cDNA microarray with 8,448 expressed sequence tags (ESTs), the development of which is described in Connon et al. (2009). In brief, purified PCR fragments ranging in size from 1 to 4 kb, were pin-printed in duplicate onto epoxysilane coated glass slides (Schott- Nexterion, USA). PCR fragments were printed without knowledge of sequence annotation, thus only genes that were differentially expressed following exposures were sequenced for identification. Transcriptomic assessments were conducted between

larvae exposed to water from the Sacramento River at Hood and control water (detailed above). Total RNA was extracted from whole, individual fish, using Trizol Reagent (Invitrogen) following manufacturer's guidelines. RNA concentrations were determined using a NanoDrop ND1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), total RNA 260/ 280 and 260/230 ratios ranged between 1.88 and 2.15 and 1.70 and 2.10, respectively. Total RNA integrity was verified through electrophoresis on a 1% agarose gel. Total RNA from 3 fish per replicate, per treatment, was pooled, resulting in four biological replicates and four controls. A total of 500 ng total RNA for each was amplified using a SuperScriptTM Indirect RNA Amplification System (Invitrogen). Resulting amplified RNA (aRNA) was labeled with Alexa fluor dyes ® 555 and 647 (Invitrogen) as per manufacturer's instructions. Two color microarray assessments were carried out using 1 µg of amplified aRNA for each control and exposed sample, including dye swaps for each (total 4 slides), which were hybridized for 16 hours at 42 °C. Slides were scanned using a GenePix 4000B scanner (Axon Instruments). Microarray data, experimental design and hybridization details are available for download through the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov) accession number GSE 40991. Data was analyzed using LIMMA GUI (Linear model for microarray analysis graphical user interface; (Gentleman 2005), written in the R-programming language (available through Bioconductor http://www.Bioconductor.org). Data was normalized using print-tip Lowess and between arrays applying average intensity quantile normalization methods, with background correction. A linear model fit was computed using the duplicates on the arrays and least-squares method, with Benjamini Hochberg false discovery rate adjustment (Benjamini and Hochberg 1995). Only a small proportion of features on the cDNA microarray were previously sequenced (Connon et al. 2011a; Connon et al. 2011b; Connon et al. 2009), thus genes that were differentially expressed following exposure to ambient water from Hood in this study, were sequenced at the CA&ES Genomic Facility, UC Davis. Sequences were annotated according to homologies to protein database searches using translated nucleotide sequences and direct nucleotide queries (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were only annotated if they were found to have a BLASTx match with the expected value smaller than 1×10^{-5} and a score above 30, and each annotation was individually checked for homology by performing a reciprocal blast match. Differentially expressed genes were functionally classified according to the Kyoto Encyclopedia of Genes and genomes (KEGG-http://www.genome.jp/kegg/kegg2.html) into functional groups. Additional information was gathered from literature and the Gene Ontology Database (GO - http://www.uniprot.org/uniprot) to aid classification.

Quantitative Polymerase Chain Reaction (qPCR) Assessments

Specific genes of interest identified through microarray assessments in this study, as well as previous studies conducted on Delta Smelt (Connon et al. 2011a; Connon et al. 2011b; Connon et al. 2009) were selected (Table 12) to conduct comparative quantitative PCR (qPCR) studies amongst Hood, SRWTP effluent, SRGB plus ammonia, SRGB controls and UCD-FCCL controls. A total of 12 fish per treatment; three from each replicate, were assessed by qPCR. All RNA extractions were performed as indicated above and RNA samples were treated with DNase I, (amplification grade, Invitrogen), prior to cDNA synthesis. Complementary DNA (cDNA) was synthesized using 2 µg total RNA, with 50 units of Superscript III (Superscript III Reverse Transcriptase, Invitrogen, Carlsbad, CA, USA), 600 ng random primers, 10 units of RNase Out, and 1 mM dNTPs (Invitrogen) to a final volume of 20 µL. Reactions were incubated for 50 min at 50 °C followed by a 5 min denaturation step at 95 °C. Samples were diluted with the addition of 130 µL nuclease-free water to a total volume of 150 µL for subsequent real-time PCR assessments. Primers and probes for qPCR analyses were designed using Roche Universal Probe Library Assay Design Center (https://www.roche-applied-science.com). Primers were obtained from Eurofins MWG Operon (http://www.eurofinsdna.com), and TaqMan probes were supplied by Roche or Applied Biosystems (Table 12). The assessed efficiency of the primer-probe systems ranged between 90 and 97%. TaqMan Universal PCR Mastermix (Applied Biosystems) was used in qPCR amplifications. SDS 2.2.1 software (Applied Biosystems) was applied to quantify transcription and qPCR data was analyzed using the $Log_2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). Differences in transcription were calculated relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); identified using GeNorm (Vandesompele et al. 2002) as a suitable reference gene for this assessment

Table 12. Primers and probes used for quantitative PCR assessments of gene transcription in Delta Smelt (Hypomesus transpacificus).

Gene Name	Gene Code	Primer 5'→3'	Primer 3'→5'	Roche Probe No. #
Alpha Actin	a-actin	cctgcctcgtcgtactcctg	catcctggcttccctgtcc	*11*
Adenylate Kinase	Adk	ctgtcttctggggacctgttg	ctcctttctgcataattgcctgt	*36*
Amylase	Amy	gatcaccatgttcttgatctgacg	ccatcaatcctgaccaaacctg	*99*
Aspartoacylase	Aspa	cagagccttcacgacagaaa	tgaacctcatagggcaggtc	*22*
Fbxo32 (Atrogin)	Atrogin	ggaagcaccaaagagcgtca	ggcgctgcagaaatccaa	*7*
Calmodulin	Calm2	ttccttattcgacatggatggc	gcagacccagtgactgcatg	*17*
Caspase 3	Casp3	gagaaccggtatgaaccaacg	tccaagcttcccaaacactttc	*159*
Creatine Kinase	CK	cgatcggcgttggagatg	gccaagttcaacgagattctgg	*163*
Collagen XI	ColXI	ccaaaatcgatcaggttccaat	tggttggcatccccaaag	*#*
Estrogen Receptor 1	ESR1	tccaggagctgtctctccat	gagaccgatcatgagcacct	*72*
Keratin 15	Krt15	ccagcaaaaccagttactcctcc	cctgatgagcctccatacctca	*38*
Myosin Light Chain 2	Mlc2	catgggagaccgcttcacc	tgtcgatgggagcttcacg	*10*
Ammonium transporter	NH4 transp	caggctgtcttatcgcttacgg	cagcgtcatgactaacagctgaa	*61*
Pregnane X Receptor	PXR	tgaggcggtggagaagag	gaggcggtggagaagag	*144*
Sarcoendoplasmic Reticulum – Calcium ATPase	SERCa	catgatcattgggggagca	tgctgtgatgacaacgaggac	*148*
Tubulin Cofactor Beta	Tbcb	gactcctgcagctggtatgga	ccagcttctgcaggaacttgtc	*78*
Transforming Growth Factor beta	TGF-b	caacggcatagtgcatgtgg	gaatgtgtgcacgttgttggt	*76*
Thyroid Hormone Receptor alpha	THR-a	gcgtggacaagatcgagaag	tgtgcttgcggtagttgatg	*62*
Transmembrane protein 4 sub-family 4	Tms4sf4	ccctggctctcatctccatc	ccatctttggcatacttcacc	*64*
Tumor Necrosis factor alpha	TNF-a	ctttttccgctgttccatgttc	gttaccagcatacgcagtgtcc	*2*
Tropomyosin	Tpm	tcccttaacagacgcatccag	cagtagccagacgctcctgtg	*101*
Zona Pellucida	Zpa	catgcggctgagtttggataa	tgccattgatagcatcaacttca	*106*
*Glyceraldehyde 3-phosphate dehydrogenase**	GAPDH	tccacgagaaagacccaact	cacgccagtagactcaacca	*159*

 # Custom design (FAM-caacgtcatggtcaatg-BHQ Applied Biosystems), *reference gene.

Statistical Analysis

Quantitative PCR data were assessed separately for Hood and SWRTP tests, relative to respective controls. Statistical significance was tested using Shapiro Wilk normality test on $Log_2^{(-\Delta\Delta Ct)}$ data, followed by Mann Whitney U test to test for significant differences in fold change. The full suite of gene responses, combining data from Hood, and SRWTP and SRGB tests, and respective controls, were then subjected to principal component analysis (PCA) using Genesis software version 1.7.5 36 (Sturn et al. 2002) on delta Ct data relative to GAPDH $Log_2^{(\Delta Ct)}$ to assess genomic profiling similarities between samples.

Results

Water Physicochemistry

Water physicochemical parameters (EC, pH, temperature, DO, and turbidity) remained stable throughout the test and there were no significant differences between treatment groups (Table 13). Nominal total ammonium concentration spiked into SRGB water samples were consistent with measured concentrations. Total ammonium concentrations of Hood, SRGB and laboratory control samples were below the estimated limit of uncertainty of the Hach (Loveland, CO) AmVer Ammonia Test'N Tube Reagent Set (0.067 mg/L).

Table 13. Physicochemical water parameters determined during the tests. Values represent mean and standard deviations (SD) over the 7 d test period.

	Control		Hood		SRGB		SRGB plus 2 mg/L NH_4-N		SRWTP 9% effluent	
Parameter	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Temperature °C	17.0	1.2	16.9	1.2	16.9	0.2	17.0	0.3	16.9	0.4
EC (µS/cm) (20°C adjustment)	179	36	150	30	172	59	221	59	219	60
DO (mg/l)	9.6	0.2	9.8	0.2	9.6	0.2	9.5	0.3	9.6	0.3
pH	7.95	0.14	7.97	0.06	7.89	0.10	7.83	0.08	7.83	0.12
Turbidity (NTU)	7	1	5	4	7	5	6	5	6	5
Measured Ammonia Nitrogen (mg/L)	-		-		-		1.90	0.24	1.96	0.16
Unionized Ammonia (mg/L)	-		-		-		0.038	0.007	0.039	0.010

SRGB: Sacramento River at Garcia Bend, SRWTP: Sacramento Regional Wastewater Treatment Plant, EC: Electric Conductivity, DO: Dissolved Oxygen, NTU: Nephelometric Turbidity Units. "-": Below estimated limit of uncertainty.

Mortality

Delta Smelt mortality after 7-d exposure to water samples from the Sacramento River at Hood was 44.7%, significantly exceeding the mortality in the controls of 14.8% (p b 0.01). The control treatment included a low EC (179 µS cm^{-1}) and low turbidity (5 NTU) adjusted water, these two parameters combined, though predominantly the low EC, may have contributed to the mortality observed, since no mortality was recorded for the fish maintained in non-adjusted culture water from UCD-FCCL (1167 µS cm^{-1} and 11 NTU). There were no significant differences in mortality amongst SRGB (26.5%), SRGB plus 2 mg/L total ammonium (26.1%), and 9% SRWTP effluent (25.0%).

Microarray Assessment (Hood Field Station)

Microarray analysis of Delta Smelt larvae exposed to ambient water collected from Hood identified 103 independent genes responding significantly to the treatment (cut-off p b 0.05). Eighty eight genes were downregulated and only 15 were up-regulated. A total of 94 genes were assigned to a function/pathway, whereas 9 genes remained unknown. The differentially transcribed genes, annotation and functional classifications are presented in Table 14, and supplementary information in the appendix in Table 15 (Table 16). Metabolic pathways responsible for pancreatic secretion, protein digestion and absorption, fatty acid metabolism, pentose phosphate pathways, glycolysis and gluconeogenesis, as well as the starch and sucrose pathway, were affected by exposure. Genes involved in these metabolic pathways included, among others, intestinal fatty acid binding protein 2b (FABP2), carboxypeptidase b (CPB1) and aminopeptidase N (ANPEP), a gene coding for an enzyme that acts as a catalyst in the amino acid cleavage reaction of protein or peptide substrates (Taylor 1993). Energy supply pathways were affected along with metabolism, as indicated by the down regulated transcription of genes such as vacuolar proton pump subunit H (ATPeV54kD), which is involved in the oxidative phosphorylation pathway (Saraste 1999).

Altered transcription of genes assigned to genetic information processing is likely an indication of effects on protein biosynthesis, in particular transcription and translation. The messenger RNA surveillance pathway was potentially affected by exposure, as indicated by the down-regulation of Eukaryotic peptide chain release factor subunit 1 (eRF-1), along with a down-regulation of 60S ribosomal export protein (NMD3). Up-regulation of Nei endonuclease VIII-like 1 (NeiL1) is indicative of base excision repair induction, suggesting oxidative DNA damage resulting from exposure (Bandaru et al. 2002; Das et al. 2007; Dou et al. 2003; Vartanian et al. 2006).

Effects on the immune system were also highlighted through the microarray assessments. Pathways associated with biological defense were affected, in particular with antigen processing and presentation, complement activation and intestinal immune network for immunoglobin A (IgA) production. Genes such as Major Histocompatibility Complex 2 (MHC2), Beta-2-Microglobulin (B2M), Complement factor BF-2 (BF-2), and Complement regulatory protein (CRRY), are associated with the immune system and were significantly down-regulated on exposure (Braciale et al. 1987; Germain and Margulies 1993; Kim and Song 2006; Xie et al. 2003; Zipfel and Skerka 2009).

Neuromuscular system effects were also apparent as indicated by Tubulin Cofactor Beta (TBCB) (Grynberg et al. 2003; Lopez-Fanarraga et al. 2007); implicated in nerve development and cell differentiation, and Taxilin beta-like (TXLNB); a gene promoting motor nerve regeneration (Itoh et al. 2005), both of which were down-regulated. Interestingly, Atrogin-1 (MAFbx32), which is known to be highly expressed during muscle atrophy (Gomes et al. 2001), was significantly up-regulated

following exposure. Furthermore, Transgelin (TAGLN) a gene responsible for muscle development was down-regulated, along with Troponin 1 (TNN1), a muscle filament involved in regulation of striated muscle contraction, through alpha-actin, and tropomyosin binding (Assinder et al. 2009; Lehman et al. 2009). Both Alpha-Actin (ACTA) and Tropomyosin were also affected by exposure, up and downregulated respectively. Parvalbumin Typ 1 (PvalbT1), involved in muscle relaxation after contraction and calcium ion binding, was significantly up-regulated, along with Calmodulin 2 (CAM2) (Celio and Heizmann 1982; Chin and Means 2000; Heizmann 1984). Ictacalcin (ICN) also involved in calcium ion binding and calcium homeostasis was significantly down-regulated (Porta et al. 1996). Genes associated with bone structure and development, were also influenced by exposure, as suggested by the down-regulation of Collagen Type XI (ColXI) and secreted protein, acidic, cysteine-rich (osteonectin) (SPARC), both ofwhich are involved in collagen binding in vertebral development and ossification (Delany and Hankenson 2009; Wargelius et al. 2010).

Table 14. Microarray assessment and functional classification of Delta Smelt (Hypomesus transpacificus) genes responding to Sacramento River water from Hood, as determined by Kyoto Encyclopedia for Genes and Genomes (KEGG) pathway analysis.

Functional Category	Upregulated Transcripts	Downregulated Transcripts
1 Metabolism		
1.1 Carbohydrate Metabolism		
Pentose Phosphate Pathway [PATH:ko00030]		TALDO1, PRPS, PGI
Glycolysis Glyconeogenesis [PATH:ko00010]		PGI
Starch and sucrose Pathway [PATH:ko00500]		PGI
Amino sugar and nucleotide Metabolism [PATH:ko00520]		CYB5R3, PGI
1.2 Energy Metabolism		SQRDL *
Oxidative Phosphorylation [PATH:ko00190]		ATPeF0E, ATPeV54kD
1.3 Lipid Metabolism		
Arachidonic acid Metabolism [PATH:ko00590]		PTGES
Sphingolipid Metabolism [PATH:ko00600]		ASAH1
Glycerophospholipid metabolism [PATH:ko00564]		PCYT2
1.4 Nucleotide Metabolism		
Purine Metabolism [PATH:ko00230]		PRPS
1.5 Aminoacid Metabolism		
Tryptophan Metabolism [PATH:ko00380]		TPH
Glycine, Serine and Threonin Metabolism [PATH:ko00260]	GATM	Setd8b, GAMT,GNMT
Arginine and Proline Metabolism [PATH:ko00330]	GATM	GAMT
1.6 Metabolism of Other Amino Acids		
Glutathione Metabolism [PATH:ko00480]		ANPEP
Phosphonate and phosphinate metabolism [PATH:ko00440]		PCYT2
1.7 Metabolism of Cofactors & Vitamins		
Porphyrin and chlorophyll metabolism [PATH:ko00860]		FTH1, FTL
1.8 Enzyme Families		
Peptidases		NPEPL1
2 Genetic Information Processing		
2.1 Transcription		
Spliceosome [PATH:ko03040]		HSPA1_8, BUD31
Transcription factors [BR:ko03000]		CEBPD
2.2 Translation		
mRNA surveillance pathway [PATH:ko03015]		eRF-1
RNA transport [PATH:ko03013]		NMD3
Ribosome biogenesis in eukaryotes [PATH:ko03008]		NMD3, NOB1, WDR43, HSR1
Translation factors [BR:ko03012]		eEF-2
2.3 Folding Sorting and Degradation		

Proteasome [PATH:ko003050]	PSMC4	PSMA3, PSMB4, PSME3
Protein Processing in endoplasmic reticulum [PATH:ko04141]		HSPA1_8, SAR1, UBE2G1, TBCB
Ubiquitin mediated proteolysis [PATH:ko04120]		UBE2G1, UBE2L3
SNARE interactions in vesicular transport [PATH:ko04130]		SNAP29
Ubiquitin system [BR:ko04121]	ZFAND2B, FBXO32 MAFbx	KLHL31
Chaperones and folding catalysts [BR:ko03110]		CCT5, CCT8, DNAJC7, TXNIP
2.4 Replication and Repair		
Base excision repair [PATH:ko03410]	NeiL1	
DNA repair and recombination proteins [BR:ko03400]	UBE2V	
Chromosome [BR:ko03036]		H1_5
3 Environmental Information Processing		
3.1 Signal Transduction		EMP55*, LRG1 *
MAPK signaling Pathway [PATH:ko04010]		HSPA1_8, RAP1B
Phosphatidylinositol signaling system [PATH:mcc04070]	CaM2	
Calcium signaling pathway [PATH:mcc04020]	CaM2	
Calcium ion binding *	PvalbT1 *	PvalbT2 *, PvalbT3 *, EPD-1 *, ICN *, SPARC *
Wnt-signaling Pathway [PATH:ko04310]		RHOA
TGF-beta signaling Pathway [PATH:ko04350]		RHOA
3.2 Signal Molecules and Interactions		
Cell adhesion molecules (CAMs) [PATH:ko04514]		MHC2, CNTN1
GTP-binding proteins [BR:ko04031]		RAB27A
Protein binding *		IST1 *, BSDC1 *, FAHD1 *, HIGD1A *, LCN1 *, SDS22 *, ATG101 *, EMP55*
4 Cellular Processes		
4.1 Transport and Catabolism		
Lysosome [PATH:ko04142]	CTSD	ASAH1, ATPeV54kD
Endocytosis [PATH:ko04144]		HSPA1_8, RHOA, VPS28, VPS4
Phagosome [PATH:ko04145]		MHC2, ATPeV54kD
Peroxisome [PATH:ko04146]		PXMP2
Regulation of autophagy [PATH:ko04140]		GABARAP, ATG101*
4.2 Cell Motility		
Regulation of actin cytoskeleton [PATH:ko04810]		RHOA, TNNI1 *
Cytoskeleton proteins [BR:ko04812]		MLC1, Krt4, TNNI1, TNNI2
Muscle Atrophy *	FBXO25_32 MAFbx32 *	
Cell matrix adhesion and Matrix structural constituent *		EPD-1 *, ColXI *
4.3 Cell Growth and Death		
Oocyte meiosis [PATH:mcc04114]	CaM2	
Apoptosis *		MLC1 *, CHAC1, NUPR1 *
cell cycle, cell division *		IST1 *, TXNIP *, WDR82 *

4.3 Cell Communication		
Focal adhesion [PATH:ko04510]		RHOA, RAP1B
Adherens junction [PATH:ko04520]		RHOA
Tight junction [PATH:ko04530]		RHOA
5 Organismal Systems		
5.1 Immune System		BF-2 *
Hematopoietic Cell Lineage [PATH:ko04640]		ANPEP
Antigen processing and presentation [PATH:ko04612]		PSME3, MHC2, HSPA1_8, B2M
T cell receptor signaling pathway [PATH:ko04660]	UBE2V *	RHOA, HSR1 *, RAB27A *, TXNIP *
Chemokine signaling pathway [PATH:ko04062]		RHOA, RAP1B
Leukocyte transendothelial migration [PATH:ko04670]		RHOA, RAP1B
Intestinal immune network for IgA production [PATH:ko04672]		MHC2
Complement and coagulation cascades		CRRY
5.2 Endocrine System		
Renin-Angiotensin System [PATH:ko04614]		ANPEP, THOP1
PPAR signaling pathway [PATH:ko03320]	FABP2	UCP1
Insulin signaling pathway [PATH:mcc04910]	CaM2	
GnRH signaling pathway [PATH:mcc04912]	CaM2	
Melanogenesis [PATH:mcc04916]	CaM2	
5.3 Circulatory System		
Vascular smooth muscle contraction [PATH:ko04270]	CaM2	RHOA
Cardiac muscle contraction [PATH:ko04260]	ActA	TNNI1, Tpm
Relaxation after contraction *	PvalbT1 *	PvalbT2 *, PvalbT3 *
calcium homeostasis *		ICN *
5.4 Digestive System		
Pancreatic secretion [PATH:ko04972]		RHOA, RAP1B, CPB1
Salivary secretion [PATH:mcc04970]	CaM2	
Gastric acid secretion [PATH:mcc04971]	CaM2	
Fat digestion and absorption [PATH:ko04975]	FABP2	
Protein digestion and absorption [PATH:ko04974]		CPB1
Proteolysis *		BF-2 *, LCN1 *
5.5 Nervous System		
Neurotrophin signaling pathway [PATH:ko04722]	CaM2	RHOA, RAP1B
Long-term potentiation [PATH:ko04720]	CaM2	RAP1B
Axon guidance [PATH:ko04360]		RHOA, CNTN1*, St8sia4*
5.6 Sensory System		
Phototransduction [PATH:mcc04744]	CaM2	ARRDC2
Olfactory transduction [PATH:mcc04740]	CaM2	

Sensory perception of taste *		LCN1 *
5.7 Development		
Cell differentiation *		MLC1 *, Tbcb *
Ossification, Collagen binding *		SPARC *
Muscle organ -, Nervous system development *		TAGLN *, Tbcb *, TXLNB*
5.8 Environmental Adaptation		
Stress response, heat response *		HSPB8*, HIGD1A *
Response to oxidative stress	NeiL1	
Facilitate necrotic cell death under different types of stress*		HEBP2 *
6 Not Assigned		
	CG057 , apo14kDa , SLC6A9	FUNDC1 , TNNT3B, apo14kDa, GATLS1, NAT8L, TMEM106B, PWP1

* Denotes genes for which no KEGG pathways were identified, and where functional categories were attributed from KEGG Brite functional hierarchies, gene ontology and related litertature. Abbreviations; Gene names: **ActA**: Alpha Actin; **ANPEP**: Aminopeptidase N; **Apo14kDa**: 14kDa Apolipoprotein; **Arrdc2**: Arrestin domain-containing protein 2; **ASAH1**: N-acylsphingosine amidohydrolase; **ATG101**: Autophagy-related protein 101; **ATPeF0E**: ATP synthase e chain, mitochondrial; **ATPeV54kD**: Vacuolar proton pump subunit H; **B2M**: Beta-2 microglobulin; **BF-2**: Complement factor Bf-2; **BSDC1**: BSD domain containing 1; **BUD31**: BUD31 homolog; **CaM2**: Calmodulin-2; **CCT5**: T-complex protein 1 subunit epsilon; **CCT8**: T-complex protein 1 subunit theta; **CEBPD**: CCAAT/enhancer-binding protein delta; **CG057**: CG057 protein; **CHAC1**: Cation transport regulator-like protein 1; **CNTN1**: Contactin 1a precursor; **ColXI**: Collagen type XI alpha1 short isoform; **CPB1**: Carboxypeptidase B; **CRRY**: Complement regulatory protein **CTSD**: Cathepsin D; **CYB5R3**: NADH-cytochrome b5 reductase; **DNAJC7**: DnaJ homolog subfamily C member 7; **eEF-2**: Elongation factor 2; **EMP55**: 55 kDa Erythrocyte membrane protein; **EPD-1**: Ependymin-1; **eRF-1**: Eukaryotic peptide chain release factor subunit 1; **FABP2**: Intestinal fatty acid binding protein 2b; **FAHD1**: Fumarylacetoacetate hydrolase domain-containing protein 1; **MAFbx32** : F-box only protein 32 Muscle atrophy F-box protein, Atrogin1; **FTH1**: Ferritin, heavy subunit; **FTL**: Ferritin, middle subunit; **FUNDC1**: FUN14 domain-containing protein 1; **GABARAP**: Gamma-aminobutyric acid receptor-associated protein-like 1; **GAMT**: Guanidinoacetate N-methyltransferase; **GATM**: Glycine amidinotransferase, mitochondrial; **GATSL1**: GATS-like protein 1; **GNMT**: Glycine N-methyltransferase; **GPI**: Phosphoglucose isomerase-2; **H1_5**: H1 histone family, member 0; **HEBP2**: Heme-binding protein 2; **HIGD1A**: HIG1 domain family member 1A; **HSPA1_8**: Heat shock cognate 71 kDa protein; **HSPB8**: Heat shock 22kDa protein 8; **HSR1**: GTP-binding protein HSR1; **ICN**: Ictacalcin; **IST1**: KIAA0174-like protein, IST-homolog1; **KLHL31**: Kelch-like protein 31; **KRT4**: Krt4 protein; **LCN1**: Lipocalin precursor; **LRG1**: Leucine-rich alpha-2-glycoprotein; **MCL1**: Myeloid leukemia differentiation protein homologue; **MHC2**: Major histocompatibility complex 2; **NAT8L**: N-acetyltransferase 8-like protein; **NEIL1**: Nei endonuclease VIII-like 1; **NMD3**: 60S Ribosomal export protein NMD3; **NOB1**: RNA-binding protein NOB1; **NPEPL1**: Aminopeptidase-like 1; **NUPR1**: Nuclear protein 1; **PCYT2**: Ethanolamine-phosphate cytidylyltransferase; **PRPS**: Phosphoribosyl pyrophosphate synthetase 1A isoform 1; **PSMA3**: Proteasome subunit alpha type 7; **PSMB4**: Proteasome subunit beta type 4; **PSMC4**: 26S protease regulatory subunit 6B; **PSME3**: Proteasome activator complex subunit 3; **PTGES**: Prostaglandin E synthase 3; **PvalbT1**: Parvalbumin Typ1; **PvalbT2**: Parvalbumin Typ2; **PvalbT3**: Parvalbumin Typ3; **PWP1**: Periodic tryptophan protein 1 homolog; **PXMP2**: Epithelial membrane protein 2; **RAP1B**: Ras-related protein Rap-1b precursor; **RAB27A**: Ras-related protein Rab-27A; **RHOA**: Transforming protein RhoA precursor; **SAR1**: SAR1 gene homolog A; **SDS22**: Protein phosphatase 1 regulatory subunit 22; **SLC6A9**: Novel protein similar to vertebrate solute carrier family 6 (neurotransmitter transporter, glycine); **SNAP29**: Synaptosomal-associated protein 29; **SPARC**: SPARC precursor; **SQRDL**: Sulfide:quinone oxidoreductase; **St8sia4**: Alpha-2,8-polysialyltransferase IV; **TAGLN**: Transgelin; **TALDO1**: Transaldolase; **TBCB**: Tubulin folding cofactor B; **THOP1**: Thimet oligopeptidase; **TMEM106B**: Transmembrane protein 106B; **TNNI1**: Troponin1; **TNNI2**: Troponin, slow skeletal Muscle; **TNNT3B**: Troponin T3b, skeletal, fast isoform 2; **TPH**: Tryptophan hydroxylase; **TPM**: Tropomyosin; **TXLNB**: Taxilin-Beta-like; **TXNIP**: Thioredoxin-interacting protein; **UBE2G1**: Ubiquitin-conjugating enzyme E2 G1; **UBE2L3**: Ubiquitin-conjugating enzyme e2 l3; **UBE2V**: Ubiquitin-conjugating enzyme E2 variant 1; **UCP1**: Mitochondrial uncoupling protein 2; **VPS28**: Vacuolar protein sorting-associated protein 28 homolog; **VPS4**: Vacuolar protein sorting-associating protein 4B; **WDR43**: WD repeat-containing protein 43; **WDR82**: WD repeat domain containing 82 isoform 1; **ZFAND2B**: AN1-type zinc finger protein 2B.

Comparative qPCR (Field Station, Upstream and Effluent Samples)

A set of 22 genes identified as indicated above, were used for comparative qPCR studies amongst Hood, SRWTP effluent, SRGB plus ammonium, and respective controls (Figure 16 A and B). The response profile relative to controls, between the 9% effluent, 2 mg/L ammonium spiked SRGB water, and Hood are comparable. Of significance is downregulation of Collagen XI (ColX1) which, regardless of ammonium concentration, responded similarly to effluent and ammonia spiked SRGB water. The majority of the assessed genes that responded significantly (p b 0.05); 14 of 22, corresponded to microarray data in their up or down regulation. Creatine kinase (CK) and sarcoendoplasmic reticulum calciumATPase (SERCA), genes involved in muscular activity,were significantly down-regulated (p b 0.01) on exposure to Hood while aspartoacylase (ASPA) a gene associated in nerve signaling, was significantly down-regulated ($p < 0.05$) on exposure to Hood, but upregulated on exposure to ammonium spiked SRGB water ($p < 0.05$) and 9% effluent ($p < 0.01$). Correlating transcriptional responses resulting from each treatment, by means of PCA, highlights the similarity of responses between Hood, SRWTP effluent and ammonium spiked SRGB water (Figure 17 A and B), differentiating significantly ($p < 0.05$) to the respective SRGB and laboratory controls. Furthermore, the laboratory control differs significantly ($p < 0.05$) from the SRGB control.

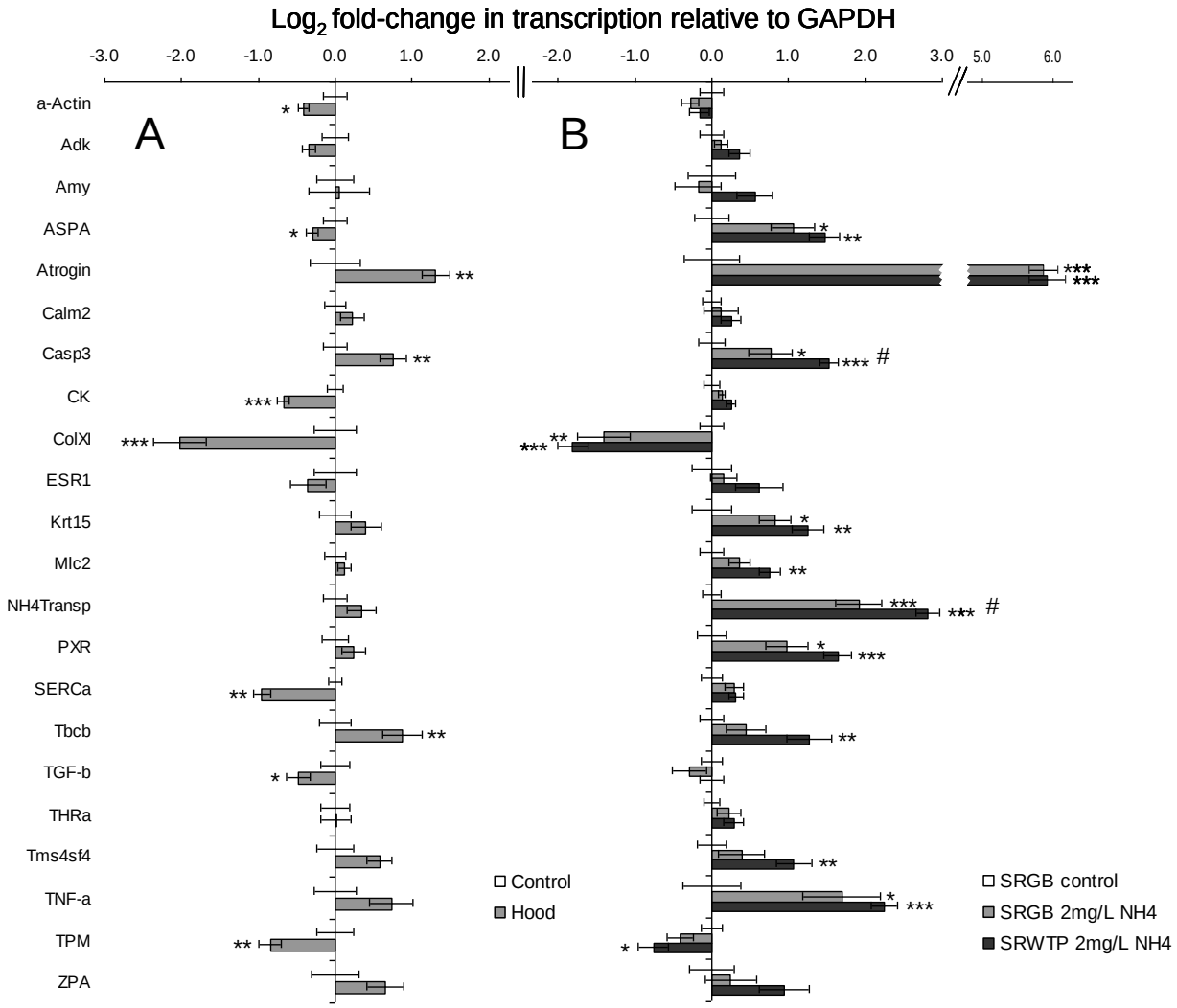

Figure 16. Fold-change in transcription of 22 genes that responded significantly in larval Delta Smelt exposed for 7 d to ambient water samples collected at (A) the lower Sacramento River at the Department of Water Resources Field Station Hood, (B) the Sacramento River at Garcia Bend (SRGB) and 9% effluent from the Sacramento Regional Wastewater Treatment Plant (SRWTP). *, **, and * represent statistical significance at p b 0.05, p b 0.01 and p b 0.001 respectively. # represents statistical significance at p b 0.05 between SRGB spiked with 2 mg/L total ammonium and 9% SRWTP effluent containing 2 mg/L total ammonium. a-actin: Alpha Actin, Adk: Adenylate Kinase, Amy: Amylase, Aspa: Aspartoacylase, Atrogin: Fbxo32 (Atrogin), Calm2: Calmodulin, Casp3: Caspase 3, CK: Creatine Kinase, ColXI: Collagen XI, ESR1: Estrogen Receptor 1, Krt15: Keratin 15, Mlc2: Myosin Light Chain 2, NH4 transp: Ammonium transporter, PXR: Pregnane X Receptor, SERCa: Sarcoendoplasmic Reticulum–Calcium ATPase, Tbcb: Tubulin Cofactor Beta, TGF-b: Transforming Growth Factor beta, THR a: Thyroid Hormone Receptor alpha, Tms4sf4: Transmembrane protein 4 sub-family 4, TNF-a: Tumor Necrosis factor alpha, Tpm: Tropomyosin, Zpa: Zona Pellucida.**

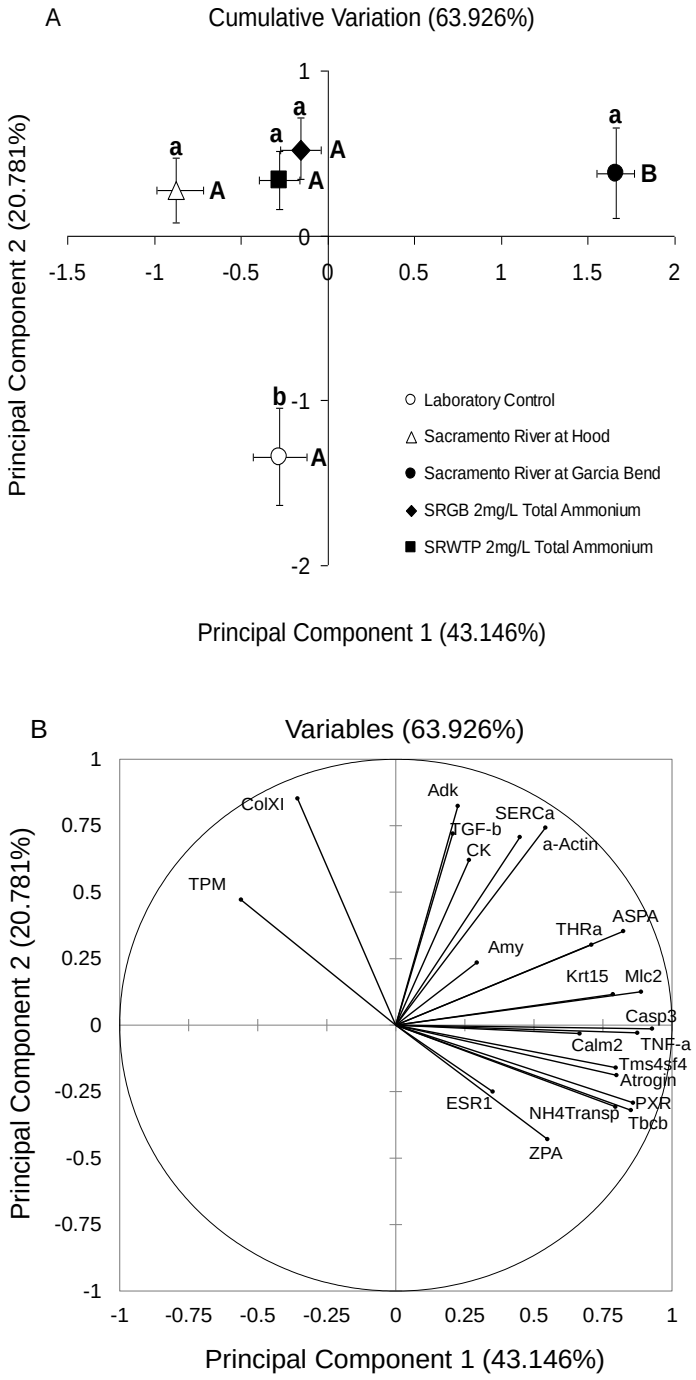

Figure 17. Principal component analysis (A) and respective variables plot (B) of transcriptional responses in larval Delta Smelt exposed for 7 d to ambient water samples collected at the lower Sacramento River at the Department of Water Resources Field Station Hood, the Sacramento River at Garcia Bend (SRGB) and 9% effluent from the Sacramento Regional Wastewater Treatment Plant (SRWTP), and SRGB water spiked with 2 mg/L total ammonium, and UCD-FCCL culture water. Letters a, b, and c upper and lower-case represent significant differences between samples (alpha = 0.05) as determined by PC1 and PC2, respectively.

Discussion

This study utilized a transcriptional profiling approach to assess and monitor the impacts of contaminants in the environment. As demonstrated in the current study, the use of transcription

profiling to assess the impact of complex mixtures will unlikely identify specific contaminants responsible for toxicity, especially without integration of toxicity identification and evaluation (TIE), but it does show promise towards the identification of contaminant sources.

Increased mortality of Delta Smelt following exposure to water from the Sacramento River at Hood could not be attributed to either of the relevant physicochemical parameters (turbidity, conductivity) of this site, when compared to the matching conductivity and turbidity controls, implying that either a combination of these stressors or other types of stressors, potentially contaminants, were responsible for the observed mortality. Such potential contaminant impacts are highlighted in the microarray assessment, identifying effects on important molecular pathways in Delta Smelt exposed to water collected from the Hood field station. However, it must be emphasized, that the necessary addition of algae to adjust the turbidity may have impacted on the overall stress response differences, a factor that was not addressed in this study.

One of the best characterized molecular pathways is that of PPAR metabolic regulation, a pathway of nuclear receptors that function as transcription factors that regulate gene expression, playing an essential role in numerous diverse physiological process including cell differentiation, development, metabolism of carbohydrates, lipids and proteins, and is activated by signals that control energy and nutrient homeostasis (Mandard et al. 2004; Puigserver 2005). The PPARs are master regulators of suites of other genes; thus it is highly likely that changes in their transcriptional activity would affect their numerous target genes. A key factor at the starting point of the PPAR signaling pathway, by which all three known PPA receptors, PPARα, PPARβ/δ, and PPARγ (Michalik et al. 2006) are affected, is the Intestinal FABP2; involved in fat digestion and absorption (Kaikaus et al. 1990). Potential adverse effects on digestion are conditioned by effects on energy supply and may be translated into impaired growth, reduced fitness, significant malnutrition and starvation. Furthermore, dietary protein deficiencies have been reported to affect the immune system and increase susceptibility to contaminants (Banerjee 1999). Furthermore the PPAR signaling pathway is reportedly a target of endocrine disruption, and plays an important role in fatty acid metabolism (Casals-Casas et al. 2008). There are further indications that exposure may have adversely affected energy metabolism, as indicated by the down-regulation of ATPeF0E and ATPeV54kD, which function as proton pumps in the oxidative phosphorylation pathway (Saraste 1999).

Closely linked with the effects on metabolism is the production of reactive oxygen species, which can potentially evoke DNA damage (Devasagayam et al. 2004). In a healthy organism, the generation of pro-oxidants in the form of reactive oxygen species is effectively kept in check by the various levels of antioxidant defense. However, following exposure to adverse physicochemical, environmental or pathological agents, this delicately maintained balance is shifted in favor of pro-oxidants resulting in

'oxidative stress' (Devasagayam et al. 2004), further potentiating the toxicity of contaminants (Banerjee 1999).

NeiL1, which is involved in base excision and repair in DNA bubble formation during replication (Das et al. 2007; Dou et al. 2003; Hu et al. 2005), was up-regulated upon exposure. This increase in transcription potentially indicates that genetic information processing is adversely affected. The base excision repair aspect is not the only indicator of potential effects on genetic information processing. The altered transcription of eRF-1 and NMD3, involved in the mRNA surveillance pathway, and RNA transport (Czaplinski et al. 1998; Kashima et al. 2006), also contributes to this hypothesis. Effects on these pathways may indicate changes in protein biosynthesis, in particular transcription, translation, and RNA degradation and can lead to metabolic impairments.

Effects on development were also highlighted in this study. Down-regulation of collagen XI has been associated with vertebral deformities, specifically in the development of vertebral compact bone (Wargelius et al. 2010). Bone structure might also be negatively affected by the down-regulation of SPARC/Osteonectin BM-40. Studies on bone structure have revealed decreased numbers of osteoblasts and osteoclasts, as well as decreased bone-formation rate and a loss of osteonectin in SPARC-Null mice (Delany and Hankenson 2009), and an increased collagen maturity (Boskey et al. 2003). These findings indicate potential effects on bone formation and bone remodeling, and impaired bone structure accompanying a lack of SPARC/Osteonectin BM-40 in the Delta Smelt. Further a lack of SPARC/Osteonectin BM-40 was shown to have adverse effects on wound healing in mice (Basu et al. 2001), suggesting that similar effects may impact on exposed Delta Smelt.

Effects upon muscle function may be indicative of subsequent effects on swimming performance. Tropomyosin is involved in muscle contraction, interacting with calcium and binding to actin filaments during the contraction cycle. Accumulation of calcium in muscular tissue contributes to muscle degradation, muscular dystrophy and muscle fiber necrosis (Olive et al. 1994). Elevated Ca^{2+}-level in the muscle cells are likely indicated by changes in calmodulin and parvalbumin regulation, since calmodulin is a Ca^{2+}-binding protein (Chin and Means 2000) and parvalbumin is involved in the removal of calcium from myofibrils, and facilitation of muscle relaxation (Rewal et al. 2005), and is localized in fast contracting muscles (Freund 1989). Along with likely muscular atrophy, as suggested by the up-regulation of the ubiquitin mediated Atrogin-1 (MAFbx), it is highly indicative that these effects would impact on both muscle development and function.

Microarray analysis also pointed to potential effects on immune system. The major histocombatibility complex class II (MHCII) and β-microglobulin (B2M) proteins play important roles in antigen processing and presentation pathway. MHCII is directly linked with the T cell receptor signaling pathway (Cresswell 1994; Markmann et al. 1988), which in turn controls the cytokine

production and the activation of other immune cells. B2M is a small protein normally found on the surface of many cells, including lymphocytes and is known to be involved in cell protection (Tanaka et al. 2005). Catabolism of B2M takes place almost exclusively in the kidney and its excretion is an indication of long term nephrotoxicity (Sørensen et al. 1985). Reduced transcription levels of B2M are known to compromise the immune system (Tay et al. 1995). However, the exposure effects upon B2M are not certain, as significant down-regulation was only determined via the microarray assessments, and not through qPCR. An important aspect of the immune defense of an organism is the complement cascade. The complement factor BF-2 and CRRY contribute significantly to the complement cascade and influence the C3 convertase (Milder et al. 2007; Ponnuraj et al. 2004) which is a central step in this system (Sarma and Ward 2011). Complement factor BF-2 is a serine protease, which activates the C3 convertase (Milder et al. 2007; Ponnuraj et al. 2004), while CRRY reduces the C3/C5 convertase activity, via decay accelerating factor (DAF) and membrane cofactor protein (MCP), mediate cell lysis (Nangaku et al. 1997). Furthermore several functions such as cell lysis, chemotaxis, phagocyte recruitment, inflammation and B-cell receptor signaling pathway are associated with BF-2 (Carroll 2004; Walport 2001).

It has previously been demonstrated that contaminants alone can have severe impacts on the immune system (Clifford et al. 2005), and can function as predisposing factors, which accompanied with low levels of pathogen infections, can lead to increased mortalities. The measured immune system responses seen in this study could be resultant of synergistic effects of contaminants with undetermined pathogens. Although fish were treated with antibiotics, which should reduce the risk of bacterial infection, possible infectious agents present in ambient water samples or SRWTP effluent should not be ruled out entirely.

A number of contaminants, including pyrethroids, heavy metals and polycyclic aromatic hydrocarbons, have previously been detected at the Hood site (Werner et al. 2010a). Several sources of agricultural and urban contaminants are located upstream from the site. Effluent discharge from the Sacramento Regional Wastewater Treatment Plant (SRWTP) is one of the largest known sources of ammonium to the lower Sacramento River. Although information on many micro pollutants in the lower Sacramento River is scarce, it is well-known that hormones, pharmaceuticals, personal care products and pesticides are present in wastewater treatment effluent at levels that can cause deleterious effects in fish (Jobling et al. 2009; Jobling et al. 2006; Weston and Lydy 2010). In addition, inflow from the American River, a tributary to the Sacramento River, has been reported to carry a number of pesticides such as the pyrethroid bifenthrin, which is heavily applied for pest control in urban (Weston et al. 2009; Weston and Lydy 2010) as well as agricultural regions upstream. Transcriptional differences were significant between Delta Smelt exposed to water from

Hood, downstream of the wastewater treatment plant, and from the upstream site at SRGB, while exposure to SRWTP effluent and ammonium spiked into water from SRGB resulted in significant similarities to Hood. These responses suggest that ammonium originating from the wastewater treatment had a significant impact on the Delta Smelt. Measured ammonium concentrations in the water from Hood were low, however, and the up-regulation of the ammonium transporter gene on fish exposed to Hood was non-significant, corroborating that ammonium alone is not causing the observed effects, and suggesting that other contaminants acting synergistically with ammonium may be responsible for the observed similarities in transcriptional differences. We have previously reported that ammonium affects cell membrane permeability (Connon et al. 2011b), potentially enhancing the uptake and effects of multiple-contaminant exposure. It is therefore possible that effects of contaminants present upstream (SRGB site) may be enhanced by the addition of ammonium. While SRWTP effluent undoubtedly contributes additional contaminants to the Sacramento River, it is likely not solely responsible for the Delta Smelt mortality observed after exposure to Hood water samples. In fact, PCA supports this hypothesis through the clustering of Hood with SRWTP effluent and ammonium spiked SRGB samples (Figure 17), and the significant differentiation between transcriptional responses in SRGB exposed fish to those from the laboratory control. The findings of this investigation indicate that contaminants originating upstream of Hood are a potential cause for Delta Smelt growth and development abnormalities, leading to the recorded mortality. We have measured transcription indicators of impacts on the energy metabolism, DNA and RNA processing, development of bone and muscle and on the immune system. Previous studies have indicated the presence of numerous contaminants at this site that arise from anthropogenic activities (Werner et al. 2010b) but further investigations upstream from Hood and SRGB are required to determine the source and classes of contaminants in the Sacramento River, and these investigations should include broad-scale chemical analyses. Although SRWTP effluent discharge is reportedly a major source of contaminants, urban and agricultural activities throughout the area, and upstream of the tested sites, are likely to contribute to the complex mixture of compounds adversely affecting the Delta Smelt habitat and population dynamics.

6. General Discussion

This project investigated the effects of different stressors of biotic, abiotic, and anthropogenic nature on an endangered fish species in a highly dynamic, heavily impacted, modified, and managed ecosystem. Laboratory experiments assessing the effects of stocking density (chapter 2), turbidity (chapter 3), turbidity and salinity interactions (chapter 4), and a complex mixture of contaminants (chapter 5) were conducted.

In all studies presented herein, multiple endpoints from different levels of biological organization were assessed; from molecular and biochemical responses, through feeding performance to percentage survival. In chapter 2 molecular stress biomarkers were contrasted with biochemical endpoints such as cortisol overall delivering consistent results and hypothesis 2.2: Stress biomarkers at multiple different levels of biological organization molecular and biochemical will deliver consistent results in physiological stress assessments, could be supported. In chapter 3 molecular biomarkers, whole body cortisol levels, feeding behavior and survival delivered consistent results for turbidity effects on late larval Delta Smelt supporting hypothesis 3.2: Several endpoints evaluated at multiple levels of biological organization (primary, secondary, tertiary stress response) deliver similar results, allowing for determination of tolerance ranges and niche dimensions. The evaluation of responses at multiple levels of biological organization, throughout chapters 2 to 5 confirmed hypothesis 4.1: The response of Delta Smelt to physicochemical parameters can be determined by measuring endpoints of different levels of biological organization. Thus, molecular and biochemical biomarkers have been validated as predictive of responses at higher levels of biological organization. Further studies on contaminant stressors considering the integration of multiple endpoints across different levels of organization have indicated that investigations should never use a single level of biological organization (Clements 2000). Measuring physiological stress using multiple endpoints provides a clear understanding of the mechanistic pathways that impair fitness. In particular, sublethal endpoints such as molecular markers and biochemical endpoints can identify stressful conditions in fish before they are detectable at the behavioral or whole organism level (e.g., feeding performance). In addition consistent results observed across multiple endpoints as indicated in chapter 2 and 3 offers a greater level of confidence of the biological significance of the stressor. To fully comprehend the adverse outcomes of physiological stress, measuring multiple endpoints is of utmost importance, and allows for the evaluation and validation of predictive effect-based biomarkers. This concept and approach of determining endpoints at multiple levels of biological organization, to evaluate potential adverse outcomes, can easily be transferred to other fish species, as well as stressor types, and is a useful approach in conservation physiology (Cooke et al. 2013;

Wikelski and Cooke 2006). The field of conservation physiology uses physiological and mechanistic tools to reveal and understand how environmental stressors and predominantly anthropogenic alterations affect species at different levels of biological organization (Cooke et al. 2013; Wikelski and Cooke 2006). For conservation purposes detailed knowledge about how an organism responds physiologically to an environmental stressor is important and essential for making informed decisions towards conservation efforts (Wikelski and Cooke 2006). This approach can be used for integrative niche assessments as demonstrated in this project (chapters 3 and 4). In fact, integrative niche assessments are most effective when physiological and mechanistic tools are combined and effectively used to understand how environmental stressors affect species at specific levels of biological organization.

When assessing the niche dimensions for a species of interest it is important to clearly distinguish between two niche concepts: 1) the realized niche and 2) the fundamental niche. Ecologists have placed emphasis on the realized niche, which takes abiotic and biotic factors into account. Physiologists, on the other hand, have focused predominantly on the fundamental niche, excluding biotic factors. The fundamental niche for late-larval Delta Smelt, as it relates to turbidity, was assessed using multiple endpoints, and is discussed in chapter 3, which corresponded with each other, indicating an optimum range between 25-80 NTU under the tested conditions. Applying the results to the conceptual model of the law of tolerance from Shelford (1931) (Figure 1, Introduction), it can be determined that ranges < 12 NTU and > 120 NTU are representing zones of intolerance for the late larval stage, whereas turbidity ranges between 12 and 25 NTU and 80 and 120 NTU can be determined as zones of stress, while the range between 25-80 NTU represents the zone of optimal tolerance. A more precise determination of different zones of tolerances is only possible if physiological responses at the sublethal level are combined with whole organism endpoints, with a broader spectrum of turbidities being assessed, thus it is important to highlight that the boundaries of these zones are variable, have some transition zones, and are dependent on other confounding factors such as water depth, and light intensity.

Results presented in chapter 4 revealed that the Delta Smelt juvenile life stage was unaffected at turbidities up to 120 NTU, and preliminary tests on the adult life stage (data not included) did not show any significant response at neither the molecular and biochemical (cortisol, glucose, lactate) levels, and adult feeding performance was highly variable and non-significant. These findings suggest that the fundamental niche for Delta Smelt for turbidity is life-stage specific. Life-stage specificity of tolerances was also identified for other abiotic stressors (temperature and salinity) for the same species (Komoroske et al. 2014). Results for those stressors indicated highest tolerance towards salinity and temperature for the larval stage, contrasting with turbidity as a stressor, which indicates

tightest requirements at larval stages, broader ranges in juveniles, and broadest tolerances in adults. The findings suggests that ontogenetic sensitivities are stressor dependent, and can be inconsistent throughout life stages, emphasizing the need to determine tolerance levels for all life stages and stressors.

In this project special focus was placed on the fundamental niche of Delta Smelt. However to understand the ecological niche concepts in its entirety, investigations on the realized niche encompassing the findings presented herein, are necessary. Specifically this includes biotic factors, interspecific and intraspecific competition, and field assessments. This would need to be combined with several different stressor types of crucial importance, e.g., temperature and multiple stressor scenarios as investigated in chapter 4 and 5. Understanding both niche concepts; fundamental and realized, would broaden our knowledge on ecological niche for this species.

The stressors investigated in this study have different levels of biological complexity, which increases with the number of stressors involved, and the level of ecological relevance. In chapter 2, the stressor stocking density evoked a simple stress response which could be assessed using well established fundamental tools of stress physiology cortisol levels and molecular stress biomarkers. Stocking density exemplifies the importance and need to clearly define all experimental conditions prior to conducting an assessment. Fish stocking density had clear effects on stress as determined using both molecular and biochemical evaluations. Hypotheses 1 in chapter 2; Delta Smelt exhibited higher stress levels at low and high densities, with lower stress responses in intermediate stocking densities; specific to the density range and conditions of the test system; was confirmed by the data. When conducting effect-based assessments, it is essential to minimize additional stress caused by experimental conditions, which could otherwise potentially mask any significance in biological responses (Oikari 2006). This is of even higher importance when endpoints of stress physiology are assessed. Information on parameters such as fish stocking density for a particular species is sometimes unavailable, or may be limited, in particular when the fish species of interest is not of aqua cultural value, not frequently studied, is a non-model species, or is listed as endangered. The aspect of confounding factors is often overlooked, however, the findings presented here highlight the importance of conducting such assessments for non-model species, in particular when attempting to place the stress response of an experiment in context.

In chapter 3, turbidity represented a stressor of higher biological complexity and was demonstrated to have severe impacts on larval Delta Smelt at different levels of biological organization (physiological stress, feeding, survival), indicating the importance, and specific turbidity requirements within their ecosystems. The complexity of interdependence of turbidity with

parameters such as light intensity (light attenuation), water depth, and type of suspended material make turbidity difficult to determine. Turbidity is inconsistent throughout an aquatic system, as its composition varies over space and time. Materials contributing to turbidity originate from river and estuarine sediments organic biomass, phytoplankton and zooplankton, as well as being heavily influenced by weather phenomena such as storms and floods. This makes characterization and demarcation of turbidity difficult, and continuous and accurate monitoring challenging. Because of the interaction with other confounding factors, turbidity can be considered as a factor with a higher level on biological complexity, causing a more complex stress response in the fish.

In chapter 4, turbidity was tested in combination with salinity representing a multiple stressor scenario with an even higher level of biological complexity. Turbidity is known to largely impact on the visual capacity of fishes (Utne-Palm 2002), and was demonstrated herein to influence feeding performance more than the gene expression in juvenile Delta Smelt, whereas salinity affected the gene expression more statistically significantly than feeding behavior. When assessing multiple stressors it is therefore important to consider which response mechanism the respective stressor is predominantly affecting, and whether the different stressors have an effect on the same or on a different response mechanism.

In chapter 5, stressors with the highest level of complexity, namely contaminant mixtures and ammonia in field samples were assessed. The detrimental effects resulting from exposure to a complex mixture of contaminants; specifically how exposure affects the physiological response of the early life stage of the Delta Smelt. Special focus was placed on a field assessment at a site on the Sacramento River at Hood, situated downstream of a municipal wastewater discharge point, that also receives contaminants from multiple other sources, including as agricultural and urban runoff. Survival was contrasted with molecular responses using microarray and qPCR. Molecular biomarkers have been proven to be efficacious in the assessment of the subtle effects resulting from exposure to stressors. Molecular approaches such as quantitative PCR and microarrays have been used successfully in the field of ecotoxicology (e.g., Aggelen et al. 2010; Ankley et al. 2006; Biales et al. 2015; Connon et al. 2009; Connon et al. 2012; Denslow et al. 2007; Fedorenkova et al. 2010; Garcia-Reyero et al. 2008; Garcia-Reyero et al. 2011; Jeffries et al. 2015b; Miracle and Ankley 2005). These tools are highly suitable to assess and detect effects of multiple stressors such as contaminant mixtures. The data presented in this chapter demonstrated that transcriptomic profiling allows the identification of contaminant sources in ambient samples, which confirmed the second hypothesis. Further it was highlighted how transcriptomic assessments can successfully be utilized to investigate the effects of complex contaminant mixtures, within ambient water samples.

All stressors assessed in this project play a crucial role in aquatic ecosystems, and it could be demonstrated that these factors and stressors can have significant effects on Delta Smelt and their survival.

Due to complex interactions with other factors and highly variable composition as discussed above, turbidity affects the aquatic ecosystems at multiple different levels, through indirect and direct effects (Bruton 1985). In addition it became clear that turbidity is an important, yet often overlooked environmental variable that contributes to population-level effects in fish species; as tested at the example of the Delta Smelt. Asides from the findings presented here, the importance of turbidity has been demonstrated in other estuaries and other species, e.g., the rainbow smelt (*Osmerus mordax*) (Dauvin and Dodson 1990; Dodson et al. 1989; Sirois and Dodson 2000a; Sirois and Dodson 2000b), white perch (*Morone americana*) and striped bass (*Morone saxatilis*) (North and Houde 2001), indicating that it is predominantly larval fish that depend on this abiotic parameter. Generally a continuous monitoring program for turbidity would be beneficial to a lot of fish species especially for the early life stages, as it could aid identify potentially unfavorable turbidity conditions in crucial habitats. In addition monitoring data could help track the fish in their habitat. For Delta Smelt, this approach would need further and detailed assessments, however results from evaluations of Secchi depth and catch per unit effort using modeling techniques, indicated an inverse correlation between the two factors (Latour 2015).

Contaminants in the aquatic environment generally occur as mixtures; rarely as single compounds, and are mostly constituted of undetermined chemicals, of unknown concentrations, and often below the limit of detection of current analytical methods, yet still pose significant threats to aquatic organisms. Many compounds that are commonly detected in surface waters are pesticides (Weston et al. 2013), sometimes with endocrine disrupting properties (EDCs) (Brander 2013), heavy metals (Birch and Taylor 1999; Kennish 2002; Liu et al. 2015), flame retardants (Andresen et al. 2004; Marklund et al. 2005; Sundkvist et al. 2010; Wei et al. 2015), Polycyclic Aromatic Hydrocarbons (PAH) (Oros and Ross 2004; Pereira et al. 1999; Wu et al. 2011), and ammonium (Connon et al. 2011b; Parker et al. 2012a), amongst many others. In order to conserve species and maintain water quality standards a continuous monitoring program for contaminants would be needed, with the aim of identifying contaminant sources as well as conducting comprehensive chemical and toxicological analyses. The implementation of molecular tools into monitoring programs had been suggested (Biales et al. 2015; Connon et al. 2012; Geist 2011) and could facilitate the detection and identification of adverse outcomes in fish, that result from exposure (Ankley et al. 2010). The information gained from a monitoring program could help resource managers take action and make informed decisions in favor of the conservation of fish species and aquatic ecosystems.

Noteworthy, the application of approaches and tools described herein are easily transferrable to a number of fish species, and to the assessment of several different stressor types, highlighting the global relevance of this work.

7. Outlook

The research presented in this study has augmented knowledge on various aspects on experimental methodology, the concept of the fundamental niche, multiple stressors of abiotic factors, and the impact of complex contaminant mixtures as multiples stressors. However, further studies would be beneficial to further elaborate on these findings.

The results of the confounding factors in experimental set ups, demonstrated on the Delta Smelt, have revealed the importance of considering experimental conditions, in particular when assessing stress responses. Such determination of experimental conditions should be applied to all species used in physiological and ecotoxicological testing. In the Delta Smelt stocking density was an important factor, in other species different factors such as vessel shape, flow through systems, different life stages, aeration speeds, feeding versus not feeding during experiments, and exposure times are likely to be similarly important. Assessed endpoints could be implemented in aquaculture methods helping to monitor and detect of unfavorable conditions and to optimize aquaculture techniques.

The work presented here delivered valuable information on the effects of stressors using short-term exposures of up to one week, however, long-term assessments, based on the findings presented here, where fish are exposed to these stressors for several months, would help to further investigate effects on the organisms that may be consequential at the population level. Adaptive mechanisms and phenotypic plasticity, could be evaluated, in conjunction with carefully selected physiological endpoints relative to the long term assessments. For example, food intake could be replaced with growth and development over time, and whole body cortisol (or plasma cortisol) replaced with cortisol measurements in scales as described in Aerts et al. (2015).

This work has comprehensively assessed the fundamental niche of Delta Smelt, as it relates to turbidity, within a laboratory setting, and provided important insight and knowledge about how turbidity affects aquatic organisms, as well as the importance of turbidity in an aquatic ecosystem. However, in order to understand the niche in its entirety, assessments that focus on determining the realized niche; including biotic factors as well as intraspecific and interspecific relationships (e.g., predator prey interaction), are needed along with comprehensive field-based assessments.

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9. Publication List

Peer-reviewed Publications Included in this Thesis

M. Hasenbein*, L. M. Komoroske*, R. E. Connon, J. Geist & N. A. Fangue, 2013. Turbidity and Salinity Affect Feeding Performance and Physiological Stress in the Endangered Delta Smelt. Integrative and Comparative Biology 53(4):620-634 doi:10.1093/icb/ict082. (*equal contribution authors)

M. Hasenbein, I. Werner, L. A. Deanovic, J. Geist, E. B. Fritsch, A. Javidmehr, C. Foe, N. A. Fangue & R. E. Connon, 2014. Transcriptomic profiling permits the identification of pollutant sources and effects in ambient water samples. Science of The Total Environment 468–469(0):688-698 doi:http://dx.doi.org/10.1016/j.scitotenv.2013.08.081.

M. Hasenbein, Fangue, N.A., Geist, J.P., Komoroske, L.M., Connon, R.E. (2015**),** *Physiological Stress Biomarkers reveal Stocking Density Effects in late larval Delta Smelt (Hypomesus transpacificus)*, *Aquaculture*: Accepted

Peer-reviewed Publications not Included in this Thesis

Komoroske, L. M., R. E. Connon, J. Lindberg, B. S. Cheng, G. Castillo, **M. Hasenbein** & N. A. Fangue, 2014. Ontogeny influences sensitivity to climate change stressors in an endangered fish. Conservation Physiology 2(1) doi:10.1093/conphys/cou008.

Jeffries, K. M., L. M. Komoroske, J. Truong, I. Werner, **M. Hasenbein**, S. Hasenbein, N. A. Fangue & R.E. Connon, 2015b. The transcriptome-wide effects of exposure to a pyrethroid pesticide on the critically endangered deltasmelt Hypomesus transpacificus. Endangered Species Research In press doi:10.3354/esr00679.

Selected Oral and Poster Contributions Related to this Thesis

Oral Presentations

Matthias Hasenbein, Nann A, Fangue, Juergen Geist, Lisa M. Komoroske, Richard E. Connon: Physiological stress responses to turbidity in larval Delta Smelt (*Hypomesus transpacificus*). Invited Talk, Interagency Ecological Program Annual Workshop, Folsom, CA, March 2015

Matthias Hasenbein, Lisa M. Komoroske, Bethany DeCourten, Nann A, Fangue, Juergen Geist, Richard E. Connon: Stress responses to turbidity, salinity and temperature in Delta Smelt (*Hypomesus transpacificus*). Invited Talk, Interagency Ecological Program Annual Workshop, Folsom, CA, April 2013

Matthias Hasenbein, Inge Werner, Linda Deanovic, Jürgen Geist and Richard E. Connon: Genomic Assessments in Delta Smelt (*Hypomesus Transpacificus*) Exposed to River Water Downstream of the Sacramento Regional Waste Water Treatment Plant. Nor Cal SETAC Berkeley, CA, May 2012

Poster Presentations

Matthias Hasenbein, Nann A. Fangue, Juergen Geist, Lisa M. Komoroske, Richard E. Connon: Stress Response to turbidity utilizing feeding performance, biochemical, and molecular biomarkers in Delta Smelt (*Hypomesus transpacificus*). Bay Delta Science Conference, Sacramento, CA, Oct 2014

Matthias Hasenbein, Nann A. Fangue, Juergen Geist, Lisa M. Komoroske, Richard E. Connon: Determination of optimal fish density in experimental systems using physiological stress response. Bay Delta Science Conference, Sacramento, CA, Oct 2014

Matthias Hasenbein, Nann A. Fangue, Juergen Geist, Richard E. Connon: Optimal fish density determination using biomarkers of stress, not a rule of thumb. SETAC Europe 24th Annual Meeting, Basel, Switzerland, May 2014

Matthias Hasenbein, Inge Werner, Erika B. Holland Fritsch, Alireza Javidmehr, Linda A. Deanovic, Nann A. Fangue, Juergen Geist, Richard E. Connon: Wastewater treatment associated ammonium impacts on larval Delta Smelt (*Hypomesus transpacificus*). SETAC North America 34th Annual Meeting, Nashville, TN, Oct 2013

Matthias Hasenbein, Lisa M. Komoroske, Nann A. Fangue, Juergen Geist, Richard E. Connon: Effects of Turbidity and Salinity on Feeding Performance and Molecular Stress in Juvenile Delta Smelt (*Hypomesus transpacificus*). 47th Annual Conference of the California-Nevada Chapter of the American Fisheries Society, Davis, CA, May 2013

Matthias Hasenbein, Nann A. Fangue, Lisa Komoroske, Bethany DeCourten, Joan Lindberg, Richard E. Connon: Old standards versus new approaches: Towards Defining the fundamental niche of Delta Smelt (*Hypomesus transpacificus*). Bay-Delta Science Conference, Sacramento, CA, Oct 2012

Matthias Hasenbein, Inge Werner, Linda Deanovic, Juergen Geist, and Richard Connon: Genomics in Environmental Diagnostics: Health Assessments in Delta Smelt (*Hypomesus transpacificus*). Interagency Ecological Program Annual Workshop, Folsom, CA, April 2011

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11. Appendix

Table 15. Supplementary table. Detailed statistical information on individual nested anova models for the main effect density for cortisol and each gene of the qPCR data set, respectively.

Nested Anova Model Parameter	Effect	Sum Sq	DF	Mean Sq	F value	Pr(>F)
Cortisol	Density	0.99	4	0.25	3.28	0.06
GR2	Density	9.87	4	2.47	3.05	0.03
11-Beta-HSD-1	Density	0.68	4	0.17	0.19	0.94
POMC	Density	4.58	4	1.15	1.19	0.37
MR1	Density	5.18	4	1.29	1.82	0.14
11-Beta-HSD-2	Density	4.55	4	1.14	1.54	0.21
GST	Density	3.72	4	0.93	0.73	0.58

Abbreviations: DF: Degrees of Freedom, Sum Sq: Sum of squares, Mean Sq: Mean of squares, GST: Glutathione-S-Transferase, POMC: Pro-Opiomelanocortin, MR1: Mineralocorticoid receptor 1, GR2: Glucocorticoid receptor 2, 11-Beta-HSD-1: 11-β-Hydroxysteroid-Dehydrogenase-Type 1, 11-Beta-HSD-2: 11-β-Hydroxysteroid-Dehydrogenase-Type 2.

Table 16. Supplementary Table S1. Annotation relative fold-change and statistical significance of differentiated genes in Hypomesus transpacificus exposed for 7-d to water samples from the Sacramento River at Hood, downstream of the Sacramento Regional Wastewater Treatment Plant effluent discharge.

ID	Gene most similar to	Gene Symbol	Species match	Score	e-value	P-Value	Fold-Change
DS[96]_01_D_07	F-box protein 32 alpha Atrogin	MAFBX32	*Salmo Salar*	504	9.00E-141	0.000	1.590
DS[96]_02_C_09	ubiquitin-conjugating enzyme E2 G1	UBE2G1, UBC7	*Anoplopoma fimbria*	348	2.00E-94	0.039	0.600
DS[96]_02_D_06	Vacuolar proton pump subunit H	ATPeV54kD	*Salmo Salar*	535	4.00E-150	0.033	0.390
DS[96]_02_D_07	Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAP, ATG8, LC3	*Osmerus mordax*	211	3.00E-53	0.023	0.270
DS[96]_02_D_10	ubiquitin-conjugating enzyme e2 l3	UBE2L3, UBCH7	*Ictalurus furcatus*	317	4.00E-85	0.009	0.370
DS[96]_02_E_03	ubiquitin-conjugating enzyme e2 l3	UBE2L3, UBCH7	*Ictalurus furcatus*	317	4.00E-85	0.014	0.430
DS[96]_02_F_07	Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAP, ATG8, LC3	*Osmerus mordax*	213	4.00E-54	0.036	0.250
DS[96]_03_B_04	N-acylsphingosine amidohydrolase	ASAH1	*Takifugu rubripes*	356	1.00E-96	0.001	0.360

DS[96]_03_B_07	DnaJ homolog subfamily C member 7	DNAJC7	*Dicentrarchus labrax*	342	2.00E-92	0.004	0.590
DS[96]_04_A_01	Ras-related protein Rab-27A	RAB27A	*Osmerus mordax*	299	7.00E-80	0.003	0.530
DS[96]_04_B_03	parvalbumin TYP1	Pvalb, Pva	*Hypomesus transpacificus*	203	5.00E-51	0.010	1.720
DS[96]_04_B_09	leucine-rich alpha-2-glycoprotein	LRG1	*Ctenopharyngodon idella*	93.2	1.00E-17	0.005	0.200
DS[96]_04_D_07	N-acylsphingosine amidohydrolase	ASAH1	*Takifugu rubripes*	345	3.00E-93	0.000	0.360
DS[96]_04_D_10	BUD31 homolog	BUD31, G10	*Osmerus mordax*	299	9.00E-80	0.004	0.480
DS[96]_04_E_01	Transgelin = smooth muscle protein 22-alpha	TAGLN, Sm22, Sm22a	*Osmerus mordax*	293	5.00E-78	0.010	0.270
DS[96]_04_E_11	alpha actin	ACTA1, ACTA	*Salmo trutta*	402	E-110	0.001	2.220
DS[96]_04_H_09	Thimet oligopeptidase	THOP1	*Salmo salar*	306	9.00E-82	0.023	0.270
DS[96]_05_A_02	autophagy-related protein 101	ATG101	*Danio rerio*	186	6.00E-46	0.047	0.160
DS[96]_05_B_10	Proteasome subunit alpha type 7	PSMA3	*Osmerus mordax*	456	8.00E-127	0.036	0.290
DS[96]_05_G_04	Ras-related protein Rap-1b precursor	RAP1B	*Osmerus mordax*	379	1.00E-103	0.044	0.160
DS[96]_06_B_10	Fumarylacetoacetate hydrolase domain-containing protein 1, YisK-like protein	Fahd1,C16orf36, YISKL	*Osmerus mordax*	448	2.00E-124	0.019	0.320
DS[96]_06_D_01	tubulin folding cofactor B	TBCB, CKAP4, CLIMP63, CG22	*Salmo salar*	301	5.00E-80	0.004	0.610
DS[96]_06_E_10	Proteasome activator complex subunit 3	PSME3	*Osmerus mordax*	505	2.00E-141	0.023	0.310
DS[96]_07_B_07	Heme-binding protein 2	HEBP2	*Osmerus mordax*	391	2.00E-107	0.001	0.590
DS[96]_07_C_07	aminopeptidase N, Microsomal aminopeptidase	ANPEP, APN, CD13, PEPN	*Pseudopleuronectes americanus*	199	2.00E-49	0.022	0.600
DS[96]_07_G_03	Ependymin precursor, Ependymin-1	EPD-1	*Osmerus mordax*	411	3.00E-113	0.002	0.430
DS[96]_07_H_05	T-complex protein 1 subunit epsilon	CCT5, TCP-1-epsilon	*Salmo salar*	399	1.00E-109	0.016	0.360
DS[96]_08_D_01	T-complex protein 1 subunit epsilon	CCT5, TCP-1-epsilon	*Salmo salar*	204	3.00E-51	0.004	0.320
DS[96]_08_H_04	small heat shock protein-like = heat shock 22kDa protein 8	HSPB8	*Salmo salar*	137	3.00E-31	0.045	0.400
DS[96]_09_B_05	CCAAT/enhancer binding protein delta2	CEBPD	*Oncorhynchus mykiss*	236	8.00E-61	0.008	0.680
DS[96]_09_B_09	Cation transport regulator-like protein 1	chaC1	*Osmerus mordax*	201	3.00E-50	0.033	0.560
DS[96]_09_D_03	Mitochondrial uncoupling protein 2	UCP1, SLC25A7	*Osmerus mordax*	159	1.00E-37	0.000	0.530
DS[96]_09_D_07	Ferritin, heavy subunit	FTH1	*Osmerus mordax*	203	9.00E-93	0.000	0.640

DS[96]_09_H_08	parvalbumin TYP1	Pvalb, Pva	*Hypomesus transpacificus*	210	6.00E-53	0.000	1.590
DS[96]_10_B_07	Ethanolamine-phosphate cytidylyltransferase	PCYT2	*Salmo Salar*	441	8.00E-122	0.042	0.430
DS[96]_10_E_02	Ferritin, heavy subunit = Ferritin heavy chain	FTH1	*Osmerus mordax*	349	9.00E-95	0.000	0.680
DS[96]_11_B_10	Transgelin = smooth muscle protein 22-alpha	TAGLN, Sm22, Sm22a	*Osmerus mordax*	413	7.00E-114	0.042	0.270
DS[96]_11_E_09	55 kDa erythrocyte membrane protein	MPP1, AAG12, DXS552E, EMP55, MRG1, PEMP	*Salmo salar*	396	1.00E-108	0.039	0.160
DS[96]_11_H_04	Transforming protein RhoA precursor	RHOA	*Esox lucius, Oncorhynchus mykiss*	390	4.00E-107	0.005	0.350
DS[96]_12_D_10	periodic tryptophan protein 1 homolog, Keratinocyte protein IEF SSP 9502	PWP1	*Danio rerio*	329	3.00E-88	0.025	0.220
DS[96]_12_E_09	Lipocalin precursor	LCN1	*Salmo salar*	314	3.00E-84	0.000	0.690
DS[96]_13_A_09	F-box only protein 32 Muscle atrophy F-box protein	MAFBX32	*Salmo salar*	459	2.00E-127	0.000	1.440
DS[96]_13_B_05	invariant chain-like protein 14-1 (MHC class II)	MHC2	*Oncorhynchus mykiss*	198	3.00E-49	0.005	0.390
DS[96]_13_E_10	Tropomyosin	TPM1, TPM2, TPM3, TPM4	*Theragra chalcogramma*	281	2.00E-74	0.040	0.130
DS[96]_13_F_08	HIG1 domain family member 1A	HIGD1A, HIG1, HSPC010	*Anoplopoma fimbria*	159	1.00E-37	0.002	0.670
DS[96]_13_G_05	Epithelial membrane protein 2	PXMP2, PMP22	*Esox lucius*	256	9.00E-67	0.042	0.480
DS[96]_14_A_11	Troponin I, slow skeletal muscle	TNNI1	*Anoplopoma fimbria*	318	2.00E-85	0.016	0.490
DS[96]_14_E_10	alpha-2,8-polysialyltransferase IV	St8sia4	*Oncorhynchus mykiss*	365	2.00E-99	0.008	0.580
DS[96]_14_H_05	N-acetyltransferase 8-like protein, Camello-like protein 3	NAT8L	*Salmo salar*	81.6	3.00E-14	0.011	0.300
DS[96]_15_D_09	complement factor B	CFB, BF-2	*Oncorhynchus mykiss gairdneri*	219	2.00E-55	0.000	0.700
DS[96]_15_H_07	Vacuolar protein sorting-associating protein 4B	VPS4	*Salmo salar*	252	1.00E-65	0.003	0.270
DS[96]_16_B_08	Nucleotide-binding protein-like, GTP-binding protein HSR1	GNL1L, HSR1	*Osmerus mordax*	449	2.00E-124	0.002	0.410
DS[96]_17_G_05	H1 histone family, member 0 / Histone H5A	H1_5	*Danio rerio / Osmerus mordax*	162 /162	9.00E-39 / 1.00E-38	0.037	0.150
DS[96]_18_H_10	Eukaryotic peptide chain release factor subunit 1	eRF-1, erf1	*Osmerus mordax*	332	3.00E-89	0.006	0.320
DS[96]_21_E_09	contactin 1a precursor	CNTN1	*Danio rerio*	109	3.00E-22	0.030	0.240

DS[96]_22_C_04	Beta-taxilin, taxilin beta-like	TXLNB, C6orf198, MDP77	*Salmo salar*	350	9.00E-95	0.047	0.560
DS[96]_24_E_02	T-complex protein 1 subunit theta	CCT8 TCP-1-theta	*Danio rerio*	452	2.00E-125	0.050	0.350
DS[96]_25_D_04	parvalbumin TYP1	Pvalb, Pva	*Hypomesus transpacificus*	203	5.00E-51	0.000	2.220
DS[96]_27_C_10	myeloid leukemia differentiation protein homologue	MCL1	*Salmo salar*	168	6.00E-40	0.005	0.350
DS[96]_28_E_01	Arrestin domain-containing protein 2	Arrdc2, PP2703	*Salmo salar*	462	3.00E-128	0.011	0.230
DS[96]_31_H_02	carboxypeptidase B	CPB1	*Paralichthys olivaceus*	365	2.00E-99	0.003	0.290
DS[96]_32_D_10	CCAAT/enhancer-binding protein delta	CEBPD	*Salmo salar*	297	6.00E-79	0.014	0.330
DS[96]_33_B_04	WD repeat domain containing 82 isoform 1	wdr82	*Salmo salar*	309	7.00E-83	0.041	0.410
DS[96]_35_B_06	Vacuolar protein sorting-associated protein 28 homolog	VPS28	*Osmerus mordax*	458	2.00E-127	0.040	0.170
DS[96]_37_E_07	KIAA0174-like protein = IST1 homolog	IST1, KIAA0174	*Danio rerio*	407	6.00E-112	0.011	0.600
DS[96]_37_G_07	BSD domain containing 1	bsdc1	*Danio rerio*	415	3.00E-114	0.044	0.210
DS[96]_38_C_07	Epithelial membrane protein 2	PXMP2, PMP22	*Esox lucius*	257	4.00E-67	0.002	0.410
DS[96]_38_F_09	Beta-taxilin, taxilin beta-like	TXLNB, C6orf198, MDP77	*Salmo salar*	270	2.00E-70	0.003	0.640
DS[96]_38_H_11	Guanidinoacetate N-methyltransferase	GAMT	*Esox lucius*	409	2.00E-112	0.026	0.550
DS[96]_39_C_08	kelch-like protein 31	KLHL31	*Danio rerio*	519	1.00E-145	0.002	0.510
DS[96]_39_H_02	tryptophan hydroxylase	E1.14.16.4, TPH	*Oreochromis niloticus*	423	1.00E-116	0.003	0.540
DS[96]_41_D_08	GATS-like protein 1	GATSL1	*Danio rerio*	409	1.00E-112	0.017	0.230
DS[96]_41_D_10	sulfide:quinone oxidoreductase, mitochondrial, Cadmium resistance protein 1,Heavy metal tolerance protein 2	SQRDL	*Danio rerio*	318	3.00E-85	0.002	0.500
DS[96]_42_D_06	F-box only protein 32, Atrogin 1	MAFBX32	*Salmo salar*	338	2.00E-91	0.001	1.640
DS[96]_43_D_11	Elongation factor 2	eEF-2, ef2	*Salmo salar*	244	3.00E-63	0.003	0.490
DS[96]_44_A_10	14kDa apolipoprotein	apo14kDa	*Plecoglossus altivelis*	249	1.00E-64	0.009	1.610
DS[96]_44_C_02	parvalbumin beta-1, Parvalbumin TYP3	PvalbB1	*Cyprinus carpio*	182	1.00E-44	0.005	0.600
DS[96]_44_C_08	WD repeat-containing protein 43 UTP5, U3 small	wdr43, KIAA0007, UTP5	*Danio rerio*	250	4.00E-65	0.005	0.300

	nucleolar RNA-associated protein 5						
DS[96]_44_G_12	complement regulatory plasma protein akin to Complement regulatory protein Crry	Crry, Cry, p65	*Paralabrax nebulifer*	179	2.00E-43	0.001	0.380
DS[96]_44_H_06	phosphoglucose isomerase-2	GPI, pgi	*Plecoglossus altivelis altivelis*	508	4.00E-142	0.000	0.700
DS[96]_46_A_12	Transaldolase	TALDO1	*Osmerus mordax*	520	7.00E-146	0.000	0.480
DS[96]_46_B_02	Krt4 protein	KRT4	*Danio rerio*	340	1.00E-91	0.022	0.200
DS[96]_46_D_03	26S protease regulatory subunit 6B	PSMC4, RPT3	*Osmerus mordax*	569	2.00E-160	0.000	1.460
DS[96]_47_A_03	Thioredoxin-interacting protein	TXNIP	*Salmo salar*	381	8.00E-104	0.009	0.340
DS[96]_47_B_10	SAR1 gene homolog A	SAR1	*Danio rerio*	385	2.00E-105	0.016	0.510
DS[96]_47_G_01	Synaptosomal-associated protein 29	SNAP29	*Salmo salar*	270	5.00E-71	0.031	0.280
DS[96]_48_B_12	F-box only protein 32	MAFBX32	*Ictalurus furcatus*	320	5.00E-86	0.000	1.440
DS[96]_50_C_02	60S ribosomal export protein NMD3	NMD3	*Salmo salar*	424	9.00E-117	0.020	0.430
DS[96]_51_B_11	phosphoglucose isomerase-2	GPI, pgi	*Plecoglossus altivelis altivelis*	546	2.00E-153	0.025	0.390
DS[96]_53_B_09	Troponin I	TNNI1	*Epinephelus coioides*	226	1.00E-57	0.022	0.500
DS[96]_55_A_10	Calmodulin-2	CAM2	*Esox lucius*	168	2.00E-40	0.000	4.450
DS[96]_55_F_01	nei endonuclease VIII-like 1	NEIL1	*Danio rerio*	348	3.00E-94	0.000	6.000
DS[96]_56_D_07	Ferritin, heavy subunit = Ferritin heavy chain	FTH1	*Osmerus mordax*	346	5.00E-94	0.001	0.570
DS[96]_57_E_08	parvalbumin beta-1, Parvalbumin TYP3	PvalbB1	*Cyprinus carpio*	180	6.00E-44	0.000	0.670
DS[96]_57_F_12	Aminopeptidase-like 1	NPEPL1	*Danio rerio*	451	5.00E-125	0.012	0.310
DS[96]_58_H_03	T-complex protein 1 subunit theta	CCT8 TCP-1-theta	*Danio rerio*	437	7.00E-121	0.008	0.320
DS[96]_60_C_10	ubiquitin C variant; Ubiquitin-conjugating enzyme E2 variant 1	UBE2V	*Epinephelus coioides*	321	2.00E-86	0.000	1.610
DS[96]_60_D_07	intestinal fatty acid binding protein 2b	FABP2	*Cyprinus carpio*	227	4.00E-58	0.000	1.900
DS[96]_61_D_01	Glycine N-methyltransferase	GNMT	*Esox lucius*	364	3.00E-99	0.003	0.230
DS[96]_61_E_06	Ferritin, heavy subunit = Ferritin heavy chain	FTH1	*Osmerus mordax*	193	5.00E-48	0.021	0.330
DS[96]_61_F_04	Guanidinoacetate N-methyltransferase	GAMT	*Esox lucius*	446	1.00E-123	0.002	0.400
DS[96]_62_H_04	Guanidinoacetate N-methyltransferase	GAMT	*Danio rerio*	339	9.00E-92	0.003	0.340

DS[96]_63_A_08	Ferritin, heavy subunit = Ferritin heavy chain	FTH1	*Osmerus mordax*	367	4.00E-100	0.002	0.400
DS[96]_63_C_08	SPARC precursor	SPARC, ON	*Osmerus mordax*	365	2.00E-99	0.034	0.220
DS[96]_63_E_03	Ictacalcin	ICN	*Salmo salar*	162	1.00E-38	0.006	0.420
DS[96]_63_E_09	Nuclear protein 1, Candidate of metastasis 1, Protein p8	NUPR1, COM1	*Salmo salar*	119	9.00E-26	0.002	0.630
DS[96]_64_F_08	ATP synthase e chain, mitochondrial = ATP5I = ATP5K	ATPeF0E, ATP5I	*Osmerus mordax*	104	5.00E-21	0.037	0.540
DS[96]_65_A_04	novel protein similar to vertebrate solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9)	SLC6A9	*Danio rerio*	367	E-100	0.001	1.960
DS[96]_65_G_02	Synaptosomal-associated protein 29	SNAP29	*Salmo salar*	332	2.00E-89	0.043	0.320
DS[96]_66_H_03	Ferritin, middle subunit	FTL	*Osmerus mordax*	241	3.00E-62	0.026	0.370
DS[96]_67_C_03	parvalbumin TYP1	Pvalb, Pva	*Hypomesus transpacificus*	200	5.00E-50	0.000	2.040
DS[96]_67_C_08	parvalbumin, TYP2	Pvalb, Pva	*Boreogadus saida*	178	2.00E-43	0.001	0.530
DS[96]_67_H_10	N-acylsphingosine amidohydrolase	ASAH1	*Takifugu rubripes*	172	1.00E-41	0.049	0.270
DS[96]_68_F_02	protein phosphatase 1 regulatory subunit 7, Protein phosphatase 1 regulatory subunit 22	PPP1R7, SDS22	*Salmo salar*	426	1.00E-117	0.034	0.210
DS[96]_69_D_10	beta-2 microglobulin	B2M	*Salmo salar*	176	2.00E-42	0.027	0.510
DS[96]_69_G_01	Vacuolar protein sorting-associating protein 4B	VPS4	*Salmo salar*	247	5.00E-64	0.045	0.430
DS[96]_70_A_06	Heme-binding protein 2	HEBP2	*Salmo salar*	333	1.00E-89	0.005	0.250
DS[96]_70_C_05	FUN14 domain-containing protein 1	FUNDC1	*Salmo salar*	283	6.00E-75	0.035	0.180
DS[96]_70_D_05	troponin T3b, skeletal, fast isoform 2, Troponin T	TnTf	*Danio rerio*	234	3.00E-60	0.037	0.390
DS[96]_71_H_11	Mitochondrial uncoupling protein 2	UCP1, SLC25A7	*Osmerus mordax*	329	9.00E-89	0.000	0.630
DS[96]_72_D_12	transmembrane protein 106B	TMEM106B	*Danio rerio*	416	2.00E-114	0.021	0.460
DS[96]_72_E_02	AN1-type zinc finger protein 2B	ZFAND2B, AIRAPL	*Danio rerio*	303	7.00E-81	0.000	2.890
DS[96]_72_F_12	NADH-cytochrome b5 reductase	CYB5R3	*Osmerus mordax*	546	1.00E-153	0.039	0.300
DS[96]_72_G_02	phosphoglucose isomerase-2	GPI, pgi	*Plecoglossus altivelis altivelis*	464	4.00E-129	0.001	0.490
DS[96]_72_H_01	beta-2 microglobulin	B2M	*Hypomesus transpacificus*	245	1.00E-63	0.013	0.180
DS[96]_73_C_07	Prostaglandin E synthase 3	E5.3.99.3, PTGES	*Osmerus mordax*	308	2.00E-82	0.003	0.250

DS[96]_73_C_11	Transforming protein RhoA precursor	RHOA	*Esox lucius*	387	5.00E-106	0.015	0.260
DS[96]_73_F_05	ubiquitin-conjugating enzyme E2 G1	UBE2G1, UBC7	*Esox lucius*	340	2.00E-92	0.043	0.190
DS[96]_74_A_11	CG057 protein; C7orf57 homolog = Gm11992	cg057	*Salmo salar*	186	1.00E-45	0.000	1.900
DS[96]_74_B_05	N-acylsphingosine amidohydrolase	ASAH1	*Takifugu rubripes*	397	6.00E-109	0.016	0.320
DS[96]_74_C_01	14kDa apolipoprotein	apo14kDa	*Plecoglossus altivelis*	253	1.00E-65	0.003	0.580
DS[96]_74_F_08	Ferritin, heavy subunit = Ferritin heavy chain	FTH1	*Osmerus mordax*	367	4.00E-100	0.013	0.390
DS[96]_74_H_05	N-acylsphingosine amidohydrolase	ASAH1	*Takifugu rubripes*	462	3.00E-128	0.001	0.460
DS[96]_74_H_10	heat shock cognate 71 kDa protein	HSPA1_8	*Salmo salar*	392	2.00E-107	0.004	0.210
DS[96]_75_D_04	Gamma-aminobutyric acid receptor-associated protein-like 1	GABARAP, ATG8, LC3	*Osmerus mordax*	238	2.00E-61	0.039	0.250
DS[96]_77_A_09	transmembrane protein 106B	TMEM106B	*Danio rerio*	370	1.00E-100	0.002	0.430
DS[96]_77_E_03	myeloid leukemia differentiation protein homologue	MCL1	*Salmo salar*	210	1.00E-52	0.005	0.440
DS[96]_79_A_01	collagen type XI alpha1 short isoform	COL8A1, COLX1	*Salmo salar*	130	3.00E-28	0.012	0.270
DS[96]_79_A_02	Beta-taxilin-like	TXLNB, C6orf198, MDP77	*Salmo Salar*	370	9.00E-101	0.000	0.570
DS[96]_79_D_08	F-box protein 32 alpha Atrogin	MAFBX32	*Salmo Salar*	505	9.00E-141	0.012	0.510
DS[96]_80_H_08	Wu:fc27e05 protein = RNA-binding protein NOB1	NOB1	*Danio rerio*	268	5.00E-70	0.007	0.330
DS[96]_81_D_07	complement regulatory plasma protein akin to Complement regulatory protein Crry	Crry, Cry, p65	*Paralabrax nebulifer*	188	5.00E-46	0.039	0.290
DS[96]_82_E_02	N-acylsphingosine amidohydrolase	ASAH1	*Takifugu rubripes*	450	9.00E-125	0.000	0.470
DS[96]_82_E_06	parvalbumin, TYP2	Pvalb, Pva	*Boreogadus saida*	177	5.00E-43	0.014	0.490
DS[96]_82_E_07	transmembrane protein 106B	TMEM106B	*Danio rerio*	410	1.00E-112	0.044	0.340
DS[96]_82_F_07	phosphoribosyl pyrophosphate synthetase 1A isoform 1	PRPS, prsA	*Danio rerio*	545	3.00E-153	0.035	0.150
DS[96]_82_G_11	glycine amidinotransferase, mitochondrial	GATM	*Danio rerio*	437	1.00E-120	0.001	2.340
DS[96]_85_A_02	cathepsin D	CTSD, CPSD	*Miichthys miiuy*	439	1.00E-121	0.000	1.510
DS[96]_88_C_07	Proteasome subunit beta type 4	PSMB4, PSMB2, PROS26	*Oncorhynchus mykiss*	503	1.00E-140	0.004	0.520

Table 17. Supplemental Table 1. Mixed model selected for juvenile Delta Smelt Artemia consumption. All models were negative binomial mixed models using the log link function, adjusted for zero-inflation. CI=Confidence interval.

Artemia~NTU+Length

Parameter	Estimate	Std. Error	Lower CI limit (2.5%)	Upper CI limit (97.5%)
(Intercept)	1.86	0.52	0.83	2.89
NTU	-0.01	0.00	-0.01	0.00
Length	0.07	0.02	0.04	0.10

Table 18. Supplemental Table 2. Models selected for gene transcription where the best model was not the null. CI=Confidence interval.

Model	Parameter Level	Estimate	Std. Error	Lower CI limit (2.5%)	Upper CI limit (97.5%)
Na+/K+ ATPase~Salinity	(Intercept)	0.28	0.17	-0.05	0.61
	2ppt	0.04	0.24	-0.43	0.51
	6ppt	-0.09	0.24	-0.56	0.38
	12ppt	0.3	0.24	-0.18	0.77
	15ppt	0.66	0.24	0.19	1.14
POMC~Salinity	(Intercept)	0.78	0.24	0.32	1.25
	2ppt	-0.64	0.34	-1.3	0.01
	6ppt	-0.54	0.34	-1.2	0.12
	12ppt	0.12	0.34	-0.54	0.78
	15ppt	0.58	0.34	-0.08	1.24
GST~Salinity+Turbidity	(Intercept)	0.35	0.26	-0.17	0.87
	2ppt	-0.34	0.26	-0.86	0.17
	6ppt	-0.26	0.26	-0.77	0.25
	12ppt	0.3	0.26	-0.22	0.81
	15ppt	0.61	0.26	0.1	1.13
	12NTU	0.26	0.29	-0.31	0.83
	25NTU	0.37	0.29	-0.19	0.94
	50NTU	0.45	0.29	-0.13	1.02
	120NTU	0	0.29	-0.56	0.57
	250NTU	0.91	0.3	0.33	1.49

Table 19. Whole-body homogenate cortisol levels, expressed as pg.µg total protein-1, for 120 dph Delta Smelt exposed to salinity*turbidity treatments for 2 h.

Salinity (ppt)	Turbidity (NTU)	N	Mean	Median	St. Error	Min	Max
0	0	5	0.85	0.83	0.23	0.27	1.65
0	12	4	1.57	0.78	1.00	0.24	4.49
0	25	5	1.36	0.83	0.64	0.27	3.83
0	50	5	0.46	0.49	0.13	0.08	0.76
0	120	4	0.69	0.47	0.24	0.41	1.41
0	250	5	0.42	0.28	0.22	0.02	1.25
2	0	5	1.24	1.21	0.24	0.65	1.85
2	12	4	1.43	0.74	0.93	0.16	4.10
2	25	5	1.75	0.79	0.93	0.11	5.20
2	50	5	2.33	1.39	1.39	0.11	7.64
2	120	5	4.89	4.03	2.18	1.27	13.30
2	250	5	1.62	0.78	0.57	0.54	3.04
6	0	5	14.20	1.09	11.80	0.09	61.10
6	12	5	3.14	4.23	1.20	0.29	6.47
6	25	5	0.13	0.13	0.03	0.06	0.21
6	50	5	1.02	0.91	0.21	0.53	1.79
6	120	5	3.64	3.00	1.21	1.03	8.15
6	250	5	3.45	2.41	1.49	0.83	9.26
12	0	5	0.29	0.35	0.08	0.06	0.50
12	12	6	0.74	0.38	0.37	0.11	2.47
12	25	4	0.28	0.24	0.08	0.13	0.49
12	50	4	1.56	1.17	0.61	0.66	3.26
12	120	5	1.25	0.54	0.59	0.17	3.26
12	250	4	1.12	1.17	0.48	0.14	1.98
15	0	4	2.63	1.28	1.82	0.08	7.90
15	12	5	0.65	0.20	0.30	0.14	1.42
15	25	5	1.80	1.29	0.68	0.40	4.18
15	50	4	0.88	0.21	0.74	0.02	3.09
15	120	5	1.61	1.14	0.53	0.28	3.33
15	250	5	1.34	0.86	0.89	0.06	4.83

Table 20. Model selected for cortisol (the null model was chosen for other biochemical metrics). CI=Confidence interval.

$^{3}\sqrt{Cortisol}$~Salinity

Parameter Level	Estimate	Std. Error	Lower CI limit (2.5%)	Upper CI limit (97.5%)
(Intercept)	8.58×10^{-03}	9.03×10^{-04}	6.77×10^{-03}	1.04×10^{-02}
2ppt	2.85×10^{-03}	1.27×10^{-03}	3.22×10^{-04}	5.39×10^{-03}
6ppt	3.55×10^{-03}	1.26×10^{-03}	1.03×10^{-03}	6.06×10^{-03}
12ppt	-1.80×10^{-04}	1.28×10^{-03}	-2.73×10^{-03}	2.37×10^{-03}
15ppt	9.78×10^{-04}	1.28×10^{-03}	-1.58×10^{-03}	3.53×10^{-03}

Table 21. Supplementary. Detailed statistical information on individual ANOVA models and Kruskal- Wallis Test for the effect turbidity for endpoints survival, feeding, cortisol and each gene of the qPCR data set, respectively.

Anova Model Parameter	Effect	Sum Sq	DF	Mean Sq	F value	Pr(>F)
Survival	Turbidity	4118	7	588.3	7.862	0.0000689
Feeding	Turbidity	1287	7	183.9	3.12	0.0182
Cortisol	Turbidity	15.34	7	2.191	1.684	0.144
HSP70	Turbidity	5.878	7	0.8398	6.012	0.000037
GLUT 2	Turbidity	10.55	7	1.5079	2.797	0.0151
NH4+ trans	Turbidity	14.86	7	2.123	3.97	0.00152
GST	Turbidity	8.74	7	1.25	2.66	0.02
Catalase	Turbidity	2.612	7	0.3731	1.3	0.269
IGF	Turbidity	2.406	7	0.3437	0.837	0.562
NF-kB	Turbidity	2.654	7	0.3792	1.296	0.271
HIF1a	Turbidity	3.80	7	0.54	0.84	0.56
11-Beta-HSD-2	Turbidity	2.54	7	0.36	0.64	0.72
MR1	Turbidity	3.40	7	0.49	1.31	0.26
GR2	Turbidity	2.29	7	0.33	0.70	0.68
Na/K ATPase	Turbidity	4.49	7	0.64	1.87	0.09
Kruskal Wallis Test	**Effect**	**Chi Squared**	**DF**			**Pr(>F)**
11-Beta-HSD-1	Turbidity	5.43	7			0.61
POMC	Turbidity	1.75	7			0.97
SGK3	Turbidity	5.66	7			0.5795

Abbreviations: DF: Degrees of Freedom, Sum Sq: Sum of squares, Mean Sq: Mean of squares, HSP70: Heat shock Protein 70kD, GLUT 2: Glucose Transporter 2, NH4+ trans: Ammonium transporter, GST: Glutathione-S-Transferase, IGF: Insulin like growth factor, NF-kB, Nuclear factor k-Beta, HIF1a: Hypoxia inducible factor 1 alpha, 11-Beta-HSD-1: 11-β-Hydroxysteroid-Dehydrogenase-Type 1, 11-Beta-HSD-2: 11-β-Hydroxysteroid-Dehydrogenase-Type 2, MR1: Mineralocorticoid receptor 1, GR2: Glucocorticoid receptor 2, POMC: Pro-Opiomelanocortin, SGK3: Serum/Glucocorticoid regulated kinase 3.