EVALUATION OF THE ESTROGENIC AND OSMOREGULATORY IMPACTS OF
EXPOSURE TO 4-NONYLPHENOL POLLUTION IN THE ESTUARINE ARROW

GOBY, *CLEVELANDIA IOS*

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Kaitlin Marie Johnson

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COMMITTEE MEMBERSHIP

TITLE: Evaluation of the Estrogenic and Osmoregulatory Impacts
of Exposure to 4-Nonylphenol Pollution in the Estuarine
Arrow Goby, *Clevelandia ios*

AUTHOR: Kaitlin Marie Johnson

DATE SUBMITTED: May 2016

COMMITTEE CHAIR: Sean Lema, Ph.D.
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Lars Tomanek, Ph.D.
Associate Professor of Biological Sciences

COMMITTEE MEMBER: Jason Blank, Ph.D.
Associate Professor of Biological Sciences

ABSTRACT

Evaluation of the Estrogenic and Osmoregulatory Impacts of Exposure to 4-Nonylphenol Pollution in the Estuarine Arrow Goby, *Clevelandia ios*

Kaitlin Marie Johnson

Recent evidence indicates that some of California's coastal estuaries are contaminated with the chemical 4-nonylphenol (4-NP). Tissue burdens of 4-NP detected in the intertidal arrow goby (*Clevelandia ios*) in California are among the highest recorded worldwide, however, it remains unknown whether these fish are impacted by this 4-NP contamination. 4-NP is an established endocrine disrupting compound with estrogenic properties that can alter reproductive function. Furthermore, evidence that estrogens can modulate iono- and osmo-regulatory function in fishes implies that estuarine fishes exposed to 4-NP may also experience an impaired ability to maintain hydromineral balance. In Chapter 1 of this research, the time course of detectable xenoestrogen biomarker responses including gene transcripts encoding vitellogenins (*vtgA* and *vtgC*), choriogenins (*chgL* and *chgHm*), and estrogen receptors *esr1* and *esr2a* were examined using quantitative real-time reverse transcription PCR (qRT-PCR) in adult male arrow gobies exposed to either 4-NP or E2. Specifically, adult gobies were treated with 4-NP at 10 µg/L (low 4-NP dose), or 4-NP at 100 µg/L (high 4-NP dose), ethanol vehicle (negative control), or 17β-estradiol (E2) at 50 ng/L (positive control) for 21 days. This 21 day exposure period was followed by a 21 day depuration period to

assess the time pattern of biomarker recovery. Results from these experiments indicated that 4-NP can induce increases in relative mRNA levels encoding vitellogenins, choriogenins, and estrogen receptor *esr1* – but not *esr2a* – in the liver within 72 hrs, and that these transcriptional changes return to pre-exposure levels within 12 days of the termination of 4-NP or E2 exposure. In sum, these findings validate the use of mRNA levels for several estrogen-responsive genes as accurate biomarkers for short-term 4-NP exposure in the arrow goby. In Chapter 2, I evaluated the effects of 4-NP and E2 exposures on the osmoregulatory ability of *C. ios*. I exposed adult arrow gobies to 4-NP (10 µg/L or 100 µg/L) or E2 (50 ng/L) for 14 days, and then transferred the fish from a 33 ppt salinity (control) environment to either 20 ppt, or 5 ppt conditions. Whole body water content was then measured, and the relative mRNA levels for the ion channels $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -cotransporter1 (*nkcc1*) and Na^+/H^+ exchanger-3 (*nhe3*), and the aquaporin water channel aquaporin-3 (*aqp3*) were quantified in the gill epithelium by qRT-PCR. Results showed that fish treated with 4-NP exhibited higher whole body water content, suggesting that 4-NP exposure results in excessive water uptake during hypoosmotic challenge. 4-NP treated gobies also exhibited elevated *nkcc1* and reduced *nhe3* and *aqp3* mRNAs in the gill even prior to transfer of fish from the 33 ppt acclimation salinity. At 6 hrs after salinity transfer, transcripts encoding *nkcc1* remained elevated in the gill epithelium of 4-NP treated gobies transferred to 20 ppt or maintained at 33 ppt (salinity control), while *nhe3* and *aqp3* mRNAs were still less abundant in gills of these fish. These findings point to impaired maintenance of water balance in gobies exposed to 4-NP, with those changes in fluid homeostasis possibly mediated in part by changes in gill ionic regulation. Taken as a entirety, the findings provided by this research reinforces

accumulating data showing the potential for 4-NP to disrupt reproductive physiology in vertebrates, and points to the possibility that 4-NP may impair the ability of fish to regulate ion and water balance under changing salinity conditions.

Keywords: pollution, estuary, endocrine disruption, osmoregulation, nonylphenol

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INTRODUCTION

Estuaries and coastal waters receive inputs of both synthetic and naturally occurring chemicals from residential, industrial, and agricultural activities on land (Kennish, 1997; Sun et al., 2012; DeLorenzo, 2015). Chemical contamination of estuarine and coastal environments has been documented in most coastal areas of the world (Islam and Tanaka, 2004), and while sewage waste remains the most common source of coastal marine pollution, the variety of contaminants commonly identified within coastal and estuarine habitats include fertilizers, pesticides, agrochemicals, heavy metals, polyaromatic hydrocarbons (PAHs), and persistent organic pollutants (POPs) such as polychlorinated biphenols (PCBs), polybrominated diphenyl ethers (PBDEs) and dichlorodiphenyltrichloroethane (DDT) (Islam and Tanaka, 2004; Sun et al., 2012). Depending on the physical structure and properties of the chemical, such contaminants may be found in a dissolved phase within the water column or may become concentrated in sediments and in many cases accumulate within the tissues of marine organisms (McCain et al., 1988; Weis, 2014).

Some of these contaminants exhibit endocrine-active properties and are classified as endocrine disrupting chemicals (EDCs) given their potential to interfere with the endogenous hormone signaling pathways of organisms (Oberdörster and Cheek, 2001; Porte et al., 2006; Tyler et al., 1998; Vandenberg et al., 2012). Such interference can occur when chemical pollutants interact directly with hormone receptors either as agonists that mimic hormone ligand activation or as antagonists that block endogenous hormone binding, or when they disrupt pathways underlying the synthesis, secretion, transport, metabolism or excretion of hormones (Crisp et al., 1998; Khetan, 2014).

Endocrine signaling pathways regulate a suite of physiological processes including metabolic homeostasis, growth, reproductive function, and behavior, and there is extensive evidence linking exposure to EDCs to a range of developmental, metabolic, osmoregulatory, reproductive, and behavioral impacts in marine organisms (Matthiessen, 2003; Porte et al., 2006; Weis, 2014, McCormick et al., 2005), the severity of which depends on several interacting factors including the exposure duration, chemical concentration, and the taxon or population experiencing the exposure (Vandenberg et al., 2012).

The alkylphenol, 4-nonylphenol (4-NP), is one well-established EDC that has recently been detected at elevated concentrations in select coastal areas along the Pacific Ocean coastline of California, USA (Diehl et al., 2012; Maruya et al., 2014). 4-NP is a breakdown product of the nonionic surfactants alkylphenol ethoxylates (APEOs) (Ahel et al., 1994a,b; Giger et al., 1994; Ying et al., 2002). APEOs are synthesized as either nonylphenol ethoxylates, which constitute ~80% of worldwide APEO production, or octylphenol ethoxylates that comprise the remaining ~20% of production (Brook et al., 2005). Nonylphenol is generated when APEOs within products such as plastics, detergents, agriculture sprays, or toilet paper degrade, and nonylphenol enters the marine environment largely via sewage effluent and landfill leaching (Ahel et al., 1994b; Jobling, 1993; Gehring et al., 2004; Soares et al., 2008) or via run-off from agricultural land use. Many of these nonylphenols have been shown to be more toxic than their precursor APEOs (Sonnenschein and Soto, 1998; Sharma et al., 2009) due to the fact that nonylphenols are less soluble in water because they lack hydrophilic moieties, and easily adhere to deposits rich in organic material (Giger et al., 1994; John et al., 2000).

Anaerobic environments such as sediments are particularly prone to the accumulation of 4-NP, and studies have shown that 4-NP can persist for decades in the sediments of rivers and estuaries (Blackburn and Waldock, 1994; Garrison and Hill, 1972; Ying et al., 2002; Sharma et al., 2009).

Nonylphenol ethoxylates are highly lipophilic ($\log K_m = 4.8$ to 5.3) and will accumulate in the tissues of organisms, and can bioaccumulate moderately (for 4-NP: $\log K_{OW} = 3.8$ to 4.8) (Cravedi and Zalko, 2005; Mayer et al., 2007; USEPA, 2005). Marine invertebrates including mussels (*Mytilus californianus*), oysters (*Crassostrea gigas*), and ghost shrimp (*Neotrypaea californiensis*), and fishes including arrow gobies (*Clevelandia ios*) and staghorn sculpins (*Leptocottus armatus*) from several of California's estuaries have exhibited some of the highest recorded tissue burdens of 4-NP documented worldwide (Diehl et al., 2012). The arrow goby is an ideal candidate for examining the negative effects of 4-nonylphenol on endocrine, reproductive, and osmoregulatory function because it is a common benthic inhabitant of Pacific coast bays and estuaries where it regularly comes in to physical contact with sediments rich in organic material where 4-NP can accumulate (Lavado, 2009; Blackburn and Waldock, 1995; Garrison and Hill, 1972; Ying et al., 2002; Sharma et al., 2009). Arrow gobies range in North America from British Colombia, Canada to Baja, Mexico (Eschmeyer and Herald, 1983) and occupy intertidal mud flat habitats where they forage over the benthos during high tide but take refuge in the burrows of mud-dwelling invertebrates such as the ghost shrimp *N. californiensis*, mud shrimp *Upogebia pugettensis*, and innkeeper worm *Urechis caupo* at low tide (Hoffman, 1981). In contrast to many other goby species, where both males and females invest effort in parental care, *C. ios* shows no sign of egg protection during the

period of incubation (Eschmeyer et al. 1983) but rather directs more reproductive effort into laying multiple batches of eggs (fecundity: -3×10^2 - 1.2×10^3 eggs per spawning event) over a larger geographical range (Dawson et al. 2002), making them very plentiful along the coast. While some phylogenetically distant gobiid relatives of *C. ios* such as the bluebanded goby (*Lythrypnus dalli*) and the blackeye goby (*Rhinogobiops nicholsii*) exhibit sequential hermaphroditism (Munday 2006), there is no evidence that the arrow goby or its better studied sister taxon, the tidewater goby (*Eucyclogobius newberryi*), are able to change sex (Swenson, 1995).

In this research, I examined the impacts of 4-NP exposure on the reproductive physiology and osmoregulatory abilities of adult arrow gobies. Specifically, I validated the following xenoestrogen biomarkers; vitellogenin A, vitellogenin C, choriogenin L, choriogenin H minor, estrogen receptor alpha, estrogen receptor gamma, cytochrome P450 aromatase B, and determined the time course of their detectable responses to 4-NP. I then tested whether 4-NP interrupts the ability of the arrow goby to osmoregulate by exposing gobies to varied levels of salinity and 4-NP and evaluating the response of Na⁺/K⁺ ATPase enzyme activity in the gills and quantifying relative gene expression levels for several ion and water transport proteins (e.g., sodium-hydrogen exchanger-3, *nhe3*; sodium-potassium-chloride co-transporter 1, *nkcc1*; aquaporin-3, *aqp3*) which are critical for maintaining osmotic balance during transition from seawater to fresh water salinity.

CHAPTER 1

Temporal patterns of induction and recovery of biomarker transcriptional responses to 4-nonylphenol and 17 β -estradiol in the estuarine arrow goby, *Clevelandia ios*

ABSTRACT

Several estuaries along the Pacific Ocean coast of North America were identified recently as having elevated 4-nonylphenol (4-NP) in sediments and biota, raising concerns about reproductive impacts for wildlife given 4-NP's established estrogenic activity as an endocrine disruption compound. Here we characterize 4-NP mediated induction and recovery of estrogen-sensitive gene transcripts in the arrow goby (*Clevelandia ios*), an intertidal fish abundant on estuarine mud flats on the west coast of N. America. Male gobies were exposed to waterborne 4-NP at 10 μ g/L or 100 μ g/L for 21 days followed by a 21-day depuration period. Additional males were treated with 17 β -estradiol (E2; 50 ng/L). 4-NP at 100 μ g/L elevated hepatic mRNAs encoding vitellogenins A (*vtgA*) and C (*vtgC*) and choriogenin L (*chgL*) within 72 h, and choriogenin H minor (*chgHm*) within 12 days. Hepatic mRNAs encoding estrogen receptor alpha (*esr1*) were also elevated after 12 days of 4-NP exposure, but returned to pre-exposure levels at 20 days even under continuing 4-NP treatment. 4-NP did not alter mRNA levels of estrogen receptor gamma (*esr2a*) in the liver, or of *esr1*, *esr2a*, and cytochrome P450 aromatase B (*cyp19a1b*) in the brain. The temporal pattern of initial induction for hepatic *vtgA*, *vtgC* and *chgL* transcripts by 4-NP mirrored that of E2, while *chgHm* and *esr1* mRNA induction by 4-NP lagged 2 to 11 days behind the responses of these transcripts from E2. These findings establish 4-NP concentration- and time-

dependent induction patterns of choriogenin and vitellogenin transcription following exposure to environmental relevant 4-NP concentrations, while concurrently demonstrating tissue-specific induction patterns for *esr1* by estrogenic compounds.

Keywords: biomarker, choriogenin, endocrine disruption, estradiol, estrogen receptor, estuary, fish, nonylphenol, vitellogenin, xenoestrogen

INTRODUCTION

Estuaries are vital ecosystems that serve as nursery habitat or breeding grounds to many commercially important species (Kennish, 2002), and the presence of 4-NP contamination in estuaries and nearby coastal areas of California is of high concern given evidence for 4-NP's estrogenic activity and acute toxicity (Cravedi and Zalko, 2005; Servos, 1999; Soto et al., 1991; White et al., 1994). 4-NP has been shown to bind nuclear estrogen receptors (ERs) (Laws et al., 2000; Preuss et al., 2006; Routledge and Sumpter, 1996; White et al., 1994; Tabira et al., 1999), even though the affinity of 4-NP for nuclear ER α (esr1) is orders of magnitude less than the affinity of 17 β -estradiol (E2), the principle estrogen in blood circulation in most fishes (Bonefeld-Jorgensen et al., 2007; Kwack et al., 2002). 4-NP has also been demonstrated to trigger nongenomic estrogenic effects via its binding to membrane ERs (mERs) (Kochukov et al., 2009; Loomis and Thomas, 2000; Thomas and Dong, 2006). Previous studies show that 4-NP is sequestered in the liver, brain, gills, gonads and tissues of fish (Ahel et al., 1994; Coldham et al., 1998, Lewis and Lech, 1996) and can cause negative physiological changes that alter reproduction and development (Chandrasekar et al., 2011; Christiansen et al., 1998; Colborn et al., 1993; Jobling et al., 1996; Tabata et al., 2001). Male fish exposed to 4-NP, for instance, have been found to exhibit a suite of varying effects including altered testicular structure, decreased sperm counts, intersex gonads, disrupted reproductive cycles, liver damage, and reduced growth (e.g., Christiansen et al., 1998; El-Sayed Ali et al., 2014; Kaptaner and Unal, 2011; Servos, 1999; Tanaka and Grizzle, 2002; Traversi et al., 2014). Even though 4-NP is approximately 1500 times less potent than E2 in estrogenic activity (Butwell et al., 2002), exposure to 4-NP either at concentrations in the

range found commonly in sewage effluents or for only a brief duration of exposure is capable of activating intracellular ERs and inducing the expression of estrogen-regulated genes in juvenile and male fish (e.g., Ackermann et al., 2002; Arukwe et al., 2001, 2002; Brander et al., 2012; Genovese et al., 2011; Soto et al., 1991; White et al., 1994).

Maruya and coworkers (2014) detected elevated 4-NP burdens in the range of 300-700 ng/g wet mass in *Mytilus* mussels and from 700-900 ng/g dry mass in sediments from several coastal habitats in California. Although the main source of this coastal 4-NP pollution is not fully known, Diehl and coworkers (2012) found high concentrations of 4-NP in sludge from septic systems in a coastal community and in river sediment adjacent to a wastewater treatment facility near Morro Bay, California, USA, suggesting that effluent from local treatment facilities or septic tank discharge may be a source of 4-NP to coastal marine habitats. An analysis of geographic variation in 4-NP tissue burdens in mussels in California revealed that contamination was highest in locations that receive storm water discharge (Dodder et al., 2014), therefore, 4-NP contamination in California's coastal waters could also originate in lesser part from farming land use since 4-NP has also been detected in both application water and runoff from agricultural fields in California irrigated with reclaimed wastewater (Xu et al., 2009).

The aim of this study was to characterize the time course of induction and recovery of gene transcriptional responses to 4-NP exposure in the liver and brain of the arrow goby (*C. ios*). Because the arrow goby is a common benthic inhabitant of Pacific coast bays and estuaries where it resides in the burrows of mud-dwelling invertebrates, it is regularly in physical contact with estuarine sediments rich in organic material where 4-NP can accumulate and persist for decades (Lavado, 2009; Blackburn and Waldock,

1995; Garrison and Hill, 1972; Ying et al., 2002; Sharma et al., 2009). Arrow gobies collected from Morro Bay, California, by Diehl and coworkers (2012) were found to have 4-NP tissue concentrations a full magnitude (10x) higher than that found in the surrounding sediment. However, it is still unclear whether arrow gobies in these habitats are experiencing detrimental physiological or fitness consequences under conditions of such NP pollution, and it is critical to understand how potential gene transcript biomarkers change temporally under 4-NP exposure in order to apply these biomarkers in environmental monitoring efforts using this species.

Here, we exposed male arrow gobies in the laboratory to dissolved phase 4-NP at environmentally-relevant concentrations of 10 µg/L (low dose) and 100 µg/L (high dose) for 21 days. We also exposed another group of gobies to E2 (50 ng/L) for the same duration to provide a positive control comparison for estrogen-induced effects. Following 21 days of exposure to 4-NP or E2, gobies were transferred to clean water for a 21 day depuration period to permit characterization of the time course of transcriptional recovery. At several time points throughout the exposure and depuration periods, we quantified changes in the relative transcription abundance of genes encoding the choriogenins *chgL* and *chgHm*, vitellogenins *vtgA* and *vtgC*, nuclear estrogen receptors (ERs) *esr1* and *esr2a*, and the steroidogenic enzyme *cyp19a1b*. These choriogenin and vitellogenin genes are not normally expressed at high levels in the liver of juvenile or male fish, but expression can be induced by estrogens and a variety of xenoestrogens (Flouriot et al., 1997; Hylland and Haux, 1997; Jones et al., 2000; Lange et al., 2012; Lee et al., 2002; Yamaguchi et al., 2015, Hara et al., 2016), making changes in the transcript abundances of *cgh* and *vtg* mRNAs and their corresponding proteins among the most

reliable biomarkers for xenoestrogen exposure identified to date (Garcia-Reyero, 2004; Arukwe and Goksøyr, 2003). The estrogen receptor genes *esr1* and *esr2a* encode two of three nuclear ERs present in Actinopterygian fishes (Hawkins et al, 2000). Gene transcription for these ERs can be autoinduced by estrogen exposure (Yadette et al., 1999; Arukwe et al., 2001). Cytochrome P450 aromatase (*Cyp19a1*) enzymes convert testosterone to E2, and transcription of the brain isoform (*cyp19a1b*) can be up-regulated by exposure to compounds with estrogenic properties (Kishida and Callard, 2001, Menuet et al., 2004). This experimental design was selected to provide a detailed picture of the temporal dynamics of induction and recovery of hepatic and brain mRNA biomarker regulation with 4-NP or E2 exposure.

MATERIALS AND METHODS

Sequencing of partial cDNAs encoding estrogen-responsive genes

RNA extraction

A female arrow goby (body mass: 0.52 g, standard length: 38.90 mm) was collected from its burrow within the intertidal mud flats of the Morro Bay estuary (35.345173°N, -120.843945°W) near Morro Bay, CA, USA, using slurp guns on 3 February 2012. The fish was euthanized using tricaine methanesulfonate (MS222, Argent Laboratories, Redman, WA, USA), and the brain and liver tissues were dissected, frozen in liquid N₂, and stored at -80°C. Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) with bromochloropropane for phase separation. The resulting total RNA was quantified (P300 NanoPhotometer, Implen, Westlake Village, CA, USA) and DNase I treated using the Turbo DNasefree Kit (Ambion, Grand Island, NY, USA).

Degenerate primer PCR & cDNA sequencing

Total RNA was reverse transcribed and PCR was performed using degenerate primers designed to consensus regions of cDNA or gene sequences for choriogenin L major (*chgL*) and choriogenin H-minor (*chgHm*), vitellogenin A (*vtgA*) and C (*vtgC*), estrogen receptors alpha (*esr1*) and gamma (*esr2a*), and the brain isoform of the steroidogenic cytochrome P450 aromatase enzyme (*cyp19a1b*). All degenerate primers are provided in Supplemental Materials, Table 1. Degenerate primers for *chgL* were made using cDNA sequences from *Cyprinodon variegatus* ([AY598616](#)), *Fundulus heteroclitus* ([AB533330](#)), and *Kryptolebias marmoratus* ([EU867503](#)), and for *chgHm* using available cDNAs from *C. variegatus* ([AY598615](#)), *F. heteroclitus* ([AB533329](#)), *K. marmoratus* ([EU867502](#)), and *Cichlasoma dimerus* ([EU081905](#)). Degenerate primers to *vtgA* were designed from *Thunnus thynnus* ([FJ743688](#)), *Pargus major* ([AB181838](#)), and *Labrus mixtus* ([FJ456934](#)), and to *vtgC* from *T. thynnus* ([GU217573](#)), *L. mixtus* ([FJ456936](#)), and *Morone Americana* ([DQ020122](#)). Primers to *esr1* were made to consensus regions of sequences from the yellowfin goby *Acanthogobius flavimanus* ([AB290321](#)), *Sebastes schlegelii* ([FJ594994](#)), *Chrysophrys major* ([AB007453](#)), and *Acanthopagrus schlegelii* ([AY074780](#)), and to *esr2* using sequences from *A. flavimanus* ([AB290322](#)), *Paralichthys olivaceus* ([AB070630](#)) and *Perca flavescens* ([DQ984125](#)). Degenerate primers to the *cyp19a1b* were designed to cDNA sequences available from *F. heteroclitus* ([AY494837](#)), *Poecilia reticulata* ([AT395692](#)) and *Jenynsia multidentata* ([EU851873](#)). A partial cDNA encoding 60S ribosomal protein L8 (*rpl8*) was amplified and sequenced for use as a housekeeper control gene using degenerate primers designed to consensus regions of

cDNA sequences from *Pimephales promelas* ([AY919670](#)), *F. heteroclitus* ([AY725217](#)), *Danio rerio* ([BC065432](#)), *Lates calcarifer* ([GQ507429](#)), and *S. schlegelii* ([AB491052](#)).

First-strand cDNA was generated from total RNA extracted from both liver and brain in 20 µL reactions containing 4.2 µg of RNA (4.0 µL), 1.0 µL of random primers (random hexadeoxynucleotides; Promega Corp., Madison, WI, USA), 1.0 µL of deoxynucleotide triphosphates (dNTPs, 100mM, Promega Corp.), 0.25 µL of recombinant RNasin® ribonuclease inhibitor (20U·µL⁻¹, Promega Corp.), 5.5 µL of nuclease-free H₂O, and 4.0 µL of 5x buffer, 3.0 µL of MgCl₂ (25 mM), and 0.5 µL of GoScript™ reverse transcriptase enzyme (Promega Corp.). Reverse transcription reactions were run under a thermal profile of 25°C for 5 min, 42°C for 1 h, and the 70°C for 15 min, according to the protocol of the GoScript™ Reverse Transcription System (Promega Corp.).

First-strand cDNA was then amplified in reactions comprised of 25 µL of GoTaq® Colorless Master Mix (Promega Corp.), 21 µL of nuclease-free H₂O, 1 µL each of forward and reverse degenerate primer (50 µM), and 2 µL of cDNA. Each PCR was run using thermal conditions of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 49-54°C for 30 s, and 72°C for 1-1.5 min, and finally 72°C for 2 min. The resulting PCR products were then amplified by a second round of PCR using nested forward and reverse primers and similar thermal cycling conditions. Finally, PCR products were examined by gel electrophoresis using 1.2% ethidium bromide agarose gels (E-Gel® agarose gels; Life Technologies, Grand Island, NY, USA), and any resulting products of expected size were purified (QIAquick PCR purification kit; Qiagen, Valencia, CA, USA) and Sanger sequenced (MCLab, South San Francisco, CA, USA). The resulting nucleotide sequences

were then aligned using Sequencher v.4 software (GeneCodes Corp., Ann Arbor, MI, USA) and BLAST searched against existing entries in the NCBI database (www.ncbi.nlm.nih.gov) to confirm partial cDNA identity. The resulting sequence assemblies generated partial length cDNAs for choriogenins *chgL* (549 bp nucleotides in length; GenBank accession no. [KU886161](#)) and *chgHm* (397 bp, [KU886160](#)), vitellogenins *vtgAa* (1,100 bp, [KU886159](#)) and *vtgC* (962 bp, [KU886158](#)), estrogen receptors *esr1* (696 bp, [KU886157](#)) and *esr2a* (756 bp, [KU886156](#)), the brain isoform of aromatase *cyp19a1b* (797 bp, [KU886155](#)) from the arrow goby. We also obtained a partial, 328 bp nucleotide cDNA encoding *rpl8* ([KU886162](#)) for use as an internal control gene for quantitative real-time PCR.

NP exposure and recovery experiment

Animals

Adult arrow gobies (*Clevelandia ios*) were collected at low tide on 21 August 2013 and 25 August 2013 from the burrows of ghost shrimp (*Neotrypaea californiensis*) or polychaete worms within the mud flats of Morro Bay estuary, CA, USA, using slurp guns. Gobies were transported back to California Polytechnic State University's Center for Coastal Marine Sciences facility in Avila Beach, CA. The fish were housed in 3 L acrylic tanks with flow-through filter seawater (33 ppt salinity) under ambient photoperiod, and fed an *ad libitum* diet of tropical fish flake (International Pet Supplies and Distribution, Inc., San Diego, CA, USA) for at least 21 days prior to commencing experimental treatments. All experimental procedures were approved by the Animal Care and Use Committee of California Polytechnic State University, San Luis Obispo (Protocol #1301).

Experimental design

Arrow gobies (standard length: 37.93 ± 0.27 mm; body mass: 0.54 ± 0.01 g; mean $\pm$ SEM) were divided arbitrarily into mixed-sex groups of 25 fish that were each assigned to closed-system, 19 L glass aquaria assigned to one of the following treatments: high dose 4-NP (100 μ g/L 4-NP in 0.0001% ethanol), low dose 4-NP (10 μ g/L 4-NP in 0.0001% ethanol) or control (0.0001% ethanol vehicle only). An additional set of fish were exposed to 17 β -estradiol (E2; 50 ng/L in 0.0001% ethanol) as a positive control comparison. Four replicate aquaria were used for each treatment. Experimental 4-NP (mixture of C₁₅H₂₄O isomers, purity >98.5%, ACROS Organics) and E2 (Sigma-Aldrich, St. Louis, MO, USA) treatments were created by first dissolving each compound in absolute ethanol and then adding the dissolved phase chemical to an experimental tank. Complete seawater and chemical exposure renewal of each treatment tank occurred every 2-3 days. All exposure tanks were oxygenated with air and positioned within a larger flow-through water bath (approx. 1135 L) to maintain all treatment tanks at ambient ocean temperatures ($15.1 \pm 1.6^{\circ}\text{C}$; mean $\pm$ SD) (HOBO Pendant® Temperature/Light Data Logger, Onset Computer Corp., Bourne, MA, USA) throughout the experiment.

Each 19 L treatment tank initially contained clean, filtered seawater at the time that gobies were introduced to the tanks, and fish were maintained in the treatment tanks under these clean seawater conditions for 48 hours prior to the addition of 4-NP (high or low dose), ethanol vehicle, or E2 positive control. Pre-exposure collections ('baseline' samples, Day 0) of gobies ($n = 6-10$ per treatment) were conducted on the morning immediately prior to commencing chemical exposure treatments. For this baseline

collection and each sampling time thereafter, gobies were captured haphazardly from the 19 L treatment tanks using dip nets, euthanized in MS222, and then measured and weighed. Liver and brain tissues were flash frozen in liquid N₂ and subsequently stored at -80°C. The gonad tissues were also dissected for visual confirmation of gonadal sex. The remaining carcass tissues of each fish were stored in EPA-certified glass vials and stored at -80°C for subsequent quantification of 4-NP body contaminant loads.

Immediately after the collection of the ‘baseline’ (Day 0) fish samples, 4-NP, 17β-estradiol (positive control), or ethanol vehicle (negative control) was added to each respective treatment tank (**Fig. 1**). Gobies ($n = 5-10$) were then sampled again from each treatment tank at time points of 24 hrs (Day 1), 72 hrs (Day 3), 12 days (Day 12), and 20 days (Day 20) after commencing 4-NP or E2 exposures, followed by a 20 day depuration period where gobies were maintained in clean seawater without additions of 4-NP, E2 or ethanol vehicle. Gobies were sampled during this depuration period at time points of 72 hrs (Recovery Day 3), 12 days (Recovery Day 12) and 20 days (Recovery Day 20) after the change to untreated seawater to evaluate the time course of transcript biomarker recovery from dissolved phase 4-NP exposure.

Quantification of estrogen-responsive mRNA levels using qPCR

Total RNA was extracted from the liver and brain tissues using Tri-Reagent as described above. The resulting RNA was quantified (P300 Nanophotometer), DNase I treated (Turbo DNasefree Kit; Ambion), and then quantified again. Total RNA was reverse-transcribed in 18 µL reactions containing 3.6 µL 5× buffer (Promega, Madison, WI, USA), 2.7 µL MgCl₂, 0.9 µL deoxyribonucleotide triphosphates (dNTPs; 10 mM),

and 0.9 μ L random hexamer primer (Promega, Madison, WI), 0.225 μ L recombinant RNasin RNase inhibitor (Promega), 0.675 μ L GoScript reverse transcriptase enzyme (Promega) and 9.0 μ L of total RNA template (25 ng/ μ L) under a thermal profile of 25°C for 5 min, 42°C for 1 h and 70°C for 15 min.

Gene-specific oligo primers for SYBR green real-time quantitative reverse transcription polymerase chain reaction assays (qRT-PCR) were designed for choriogenins *chgL* and *chgHm*, vitellogenins *vtgAa* and *vtgC*, estrogen receptors *esr1* and *esr2a*, brain aromatase *cyp19a1b*, and ribosomal protein *rpl8*. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA), and primer nucleotide sequences are provided in Table 2 of the Supplemental On-Line Materials. Quantitative RT-PCR reactions (16 μ L) contained 8.0 μ L SYBR Green Master Mix (Life Technologies, Grand Island, NY, USA), 1.0 μ L each of forward and reverse primer (10 μ M), 4.5 μ L nuclease-free water, and 1.5 μ L of reverse-transcribed cDNA template. The PCR thermal profile for each reaction was 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A dissociation stage consisting of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds was also included for each reaction to confirm amplification of a single product and the absence of primer-dimers. For each gene a standard curve was made from pooled RNA samples representing all treatments. These standards were serially diluted and each standard was assayed in triplicate. For each gene, correlation coefficients (r^2) for the standard curve were always greater than $r^2 = 0.90$. Controls for DNA contamination were run via the inclusion of RNA samples that were not reverse transcribed. For each gene, resulting mRNA levels were calculated based on the standard curve, normalized to *rpl8* mRNA

abundance from that same tissue of the individual fish, and then expressed as a value relative to baseline mRNA abundance of the ‘baseline’ (Day 0) measurement of the vehicle control group.

Statistical analysis

Relative mRNA abundance data did not conform to the assumptions of normality and were therefore $\log_{10}(x + 1)$ transformed. The transformed mRNA expression data for the two 4-NP dose treatment and control vehicle groups were then analyzed using two-factor ANOVA models with ‘treatment’ and ‘sampling day’ as factors. When significant main effects or interactions were identified in the two-factor ANOVA models, a Dunnett’s test was then run to identify which treatment groups differed significantly within each sampling day. Given that the aim of exposing fish to E2 (positive control) was to provide a temporal comparison of the induction of gene transcription, these E2-exposed fish were analyzed separately using a one-factor ANOVA model with ‘sampling day’ as the main effect factor. Pairwise *post hoc* comparisons were then conducted using Tukey HSD multiple comparison tests to identify significant differences in relative mRNA levels among sampling days. All statistical analyses were two-tailed and performed using the statistical software package JMP v10 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Liver choriogenin and vitellogenin mRNA levels are altered by 4-NP

The relative abundance of gene transcripts encoding choriogenin *chgL* in the liver

increased ~21-fold in fish exposed to the high dose of 4-NP after 72 h of treatment (treatment*sampling date, $F_{14,158} = 2.995$, $p = 0.0004$) (**Fig. 2A**). Hepatic *chgL* mRNA levels in these high dose 4-NP exposed males were elevated throughout the entire 4-NP exposure period and still remained elevated at 72 h following the transfer of fish to clean water (depuration). The hepatic abundance of transcripts encoding *chgHm* was likewise observed to be approximately 13-fold higher in males exposed to the high dose 4-NP treatment compared to the control group beginning 12 d after the start of 4-NP exposure, and approximately 16-fold higher after 20 d of exposure (treatment*sampling date, $F_{14,157} = 4.056$, $p < 0.0001$) (**Fig. 2B**). Similar to *chgL*, transcript abundance for *chgHm* remained significantly elevated through 72 h of depuration. Transcripts encoding *chgHm* suggested a more rapid induction in gene expression in some males beginning 72 h after commencing 4-NP exposure, but the high level of individual variation in that gene transcription response precluded statistical significance in pairwise comparisons. As expected, E2 elevated both *chgL* (**Fig. 2A**; $F_{7,55} = 11.593$, $p < 0.0001$) and *chgHm* (**Fig. 2B**; $F_{7,55} = 17.521$, $p < 0.0001$) mRNA levels in the liver within 24 h of hormone treatment. Transcript abundances for both choriogenin genes remained elevated from pre-treatment baseline levels through the entire E2 exposure period but returned to baseline after 72 h of depuration for *chgL* and after 12 days for *chgHm*.

Gene transcripts encoding the *vtgAa* mRNAs were also significantly up-regulated in the liver by the high dose 4-NP exposure (treatment*sampling date, $F_{14,153} = 3.5245$, $p < 0.0001$) (**Fig. 2C**). Transcript abundance for *vtgAa* were increased ~60-fold by 4-NP within 72 h, and remained elevated throughout the entire exposure period. Hepatic *vtgAa* mRNA abundance remained significantly elevated though 72 h of the depuration period,

but had returned to baseline levels by 12 days after transfer to clean water. 4-NP exposure likewise induced a ~23-fold increase in *vtgC* mRNA levels within 72 h after dosing (**Fig. 2D**) (treatment*sampling date, $F_{14,146} = 2.5039$, $p = 0.0032$), and *vtgC* mRNA levels in fish exposed to the high dose of 4-NP remained significantly elevated until 12 days after depuration. These patterns of 4-NP induction of *vtgAa* and *vtgC* mirrored the temporal induction of these transcripts by E2, with E2 up-regulating hepatic mRNA levels for both *vtgAa* ($F_{7,55} = 9.671$, $p < 0.0001$) and *vtgC* ($F_{7,55} = 3.5017$, $p = 0.0036$) within 72 h. However, 4-NP induction of *vtgAa* peaked at only ~40% of the maximum transcript abundance elevation induced by E2 (**Fig. 2C**), and *vtgC* peaked at ~10% the maximum mRNA level caused by exogenous E2 (**Fig. 2D**).

Hepatic estrogen receptor mRNA levels

Relative gene transcript abundance for *esr1* became elevated in the liver of fish treated with the high dose of 4-NP (**Fig. 4A**) (treatment*sampling effect, $F_{14,146} = 1.7944$, $p = 0.0444$). This 4-NP induced increase in hepatic *esr1* mRNA levels was not statistically significant until day 12 of exposure, even though an increasing trend appeared as soon as 24 h after commencing exposure. Treatment of fish with exogenous E2 likewise elevated liver *esr1* mRNA abundance. This effect of E2 was rapidly detectable at 24 h and peaked as a more than 75-fold elevation at 72 h, but then declined to a ~15-fold elevation over pre-exposure expression levels until the end of E2 exposure at 21 days (**Fig. 4A**). Hepatic transcript abundance for *esr2a* was not affected by 4-NP at either the high or low dose exposures (**Fig. 4B**) (treatment*sampling date, $F_{14,154} = 0.6100$, $p = 0.8539$), and was likewise not affected by E2 treatment ($F_{7,53} = 1.0552$, $p =$

0.4050) suggesting the absence of estrogen-response transcriptional regulation for *esr2a* in the liver.

Brain aromatase and estrogen receptor mRNA levels

In the brain, the relative mRNA levels of *cyp19a1b* were observed to be elevated 3-fold at 12 days and ~2.5-fold at 20 days after commencing E2 treatment (**Fig. 5A**) ($F_{7,54} = 5.1060$, $p = 0.0002$). Transcript abundance for *cyp19a1b* declined back to baseline levels by 20 days after depuration. In contrast to E2, neither dose of 4-NP had an effect on brain *cyp19a1b* transcript abundance (**Fig. 5A**) (treatment effect: $F_{2,153} = 0.8234$, $p = 0.4408$; treatment*sampling day interaction: $F_{14,153} = 0.3851$, $p = 0.9775$).

4-NP exposure likewise did not alter the relative abundance of *esr1* (treatment effect: $F_{2,153} = 0.2667$, $p = 0.7663$; treatment*sampling day interaction: $F_{14,153} = 0.5800$, $p = 0.8777$) or *esr2a* (treatment effect: $F_{2,153} = 0.2019$, $p = 0.8174$; treatment*sampling day interaction: $F_{14,153} = 0.8982$, $p = 0.5621$) gene transcripts in the brain. E2 likewise had no effect on transcript abundance of either estrogen receptor *esr1* ($F_{7,54} = 0.6503$, $p = 0.7124$) or *esr2a* ($F_{7,54} = 1.6777$, $p = 0.1351$) in the brain.

DISCUSSION

Biologically-relevant end points including molecular, cellular, and physiological responses are commonly used to indicate when organisms have been exposed to chemical contaminants and to identify habitats where wildlife may be experiencing the toxic effects of chemical pollution. In order to be useful for environmental monitoring, however, such biological end points or ‘biomarkers’ must be quantitatively measurable,

predictable and reproducible across a variety of chemical exposure conditions, and indicative of changes in exposure over time (Hutchinson et al., 2006; Lam, 2009). There is evidence that biomarkers at lower levels of biological organization such as molecular or cellular biomarkers generally recover more quickly after the cessation of chemical exposure than do population- or community-level indicators and therefore enable better early detection of environmental stressors (Stegemen et al., 1992; Wu et al., 2005). For that reason, molecular responses such as changes to patterns of gene expression have been suggested to be particularly effective biomarkers (Flouriot et al., 1996). However, in order to employ gene expression patterns as reliable biological indicators for environmental monitoring, it is necessary to understand not only the timing of initial and maximal induction of gene expression response after chemical exposure, but also the time scale of biomarker recovery – and in some cases, adaptation – after cessation of chemical exposure (Wu et al., 2005). And yet, few studies have examined these induction and recovery dynamics, even though recognition of such dynamics is crucial for the effective use of biomarkers, especially for monitoring habitats such as estuaries where variation in contaminant concentrations can be pulsatile with periodic rainfall events (e.g., DeLorenzo, 2015).

In this study, we characterized the timing of induction and recovery for estrogen-responsive gene expression to 4-NP to establish and validate mRNA abundance changes as reliable biomarkers for environmental monitoring using the estuarine arrow goby. Our data indicated that the relative mRNA expression of both choriogenins (*chgL* and *chgHm*) and vitellogenins (*vtgAa* and *vtgC*) rose significantly in the liver in response to both 4-NP and E2. While significant elevations in hepatic *chgL* and *chgHm* mRNAs were detected

within 24 h of E2 treatment, increases in *vtgAa* and *vtgC* were first observed after 72 h of E2 exposure, suggesting a slower up-regulatory transcriptional response of these Vtg genes compared to the Chg mRNAs. Likewise, we observed rapid transcriptional induction of estrogen receptor *esr1* mRNAs in the liver within 24 h of E2 exposure. This E2-induced elevation in *esr1* mRNAs peaked at 72 h and then declined by 12 days even during continuing E2 dosing. Hepatic transcripts encoding *esr1* were similarly elevated by the 100 ug/L (high dose) 4-NP exposure, with significant elevations observed after 12 days of treatment, but mRNA levels returning to baseline, pre-exposure abundance at 20 days, even under continuing 4-NP exposure. These distinct patterns for the initial induction, maximum induction, and recovery for Chg, Vtg, and ER mRNAs after E2 or 4-NP treatment point to variation in the utility of these genes as biomarkers for the biomonitoring of 4-NP and xenoestrogen exposure in the arrow goby and possibly other fish (Wu et al., 2005), and are likely related to the distinct functional roles of these genes in the reproductive physiology of teleost fishes.

Time-course of choriogenin and vitellogenin biomarker induction and recovery

The inner layer of the fish egg envelope (chorion), termed the zona radiata, is comprised of three glycoprotein subunits ZI-1, ZI-2 and ZI-3 derived from precursor proteins synthesized in the liver from the genes *chgH*, *chgHm*, and *chgL*, respectively (Murata et al., 1994,1997; Sugiyama et al., 1999). The resulting choriogenin proteins are then transported in blood circulation to the ovary for incorporation into developing oocytes (Murata et al., 1997; Yilmaz et al., 2015, Hara et al., 2016). It is well established that the expression of *chg* genes in fish is regulated by estrogens and xenoestrogens

(Arukwe and Goksøyr, 2003; Lee et al., 2002; Rhee et al., 2009; Yamaguchi et al., 2015). In adult male medaka (*Oryzias latipes*), for example, the hepatic abundance of mRNAs encoding both *chgH* and *chgL* rises within 4-8 hours of E2 treatment (Murata et al., 1997; Yamaguchi et al., 2015), and male sheepshead minnow (*Cyprinodon variegatus*) treated with dissolved phase E2 at doses of 182 ng/L or greater exhibited significant elevations in *chg* gene transcript abundance within 2 days of exposure (Knoebl et al., 2004). While Knoebl and coworkers (2004) also observed similar E2 dose thresholds for induction of Chg genes, several studies provide evidence that changes in mRNA levels of *chgL* are a more sensitive biomarker for estrogenic effects than *chgHm* both for their higher and more rapid inducible response. For instance, male medaka treated with waterborne 4-NP at 50 ug/L exhibited detectable *chgL* mRNA levels after 6 days as assessed via semi-quantitative RT-PCR, while *chgHm* transcripts required 4-NP at 100 ug/L to express bands at this same 6 day exposure duration (Lee et al., 2002). A similar lower dose threshold for *chgL* transcription induction was observed in response to bisphenol A, suggesting a general pattern of more sensitive estrogenic induction for *chgL* than for *chgHm* (Lee et al., 2002). Given our data showing a similar timing of induction of *chgL* and *chgHm* following 4-NP and E2 exposures, we interpret previously observed differences in xenoestrogen induction of *chgL* and *chgHm* as likely resulting from the dissimilarities in magnitude of relative transcript abundance responses for these two Chg genes rather than being indicative of differences in the timing of E2 or xenoestrogen induction. We observed that E2 induced a greater than 350-fold increase in hepatic *chgL* mRNA levels, but only a ~180-fold increase in *chgHm* mRNAs within 72 h of commencing treatment of male gobies with E2. It is important to note, however, that 4-

NP exposure at 100 ng/L significantly increased *chgL* mRNA levels by ~30-fold, but *chgHm* mRNAs by ~40-60-fold within 72 h to 12 days of treatment, suggesting that the relative magnitude of transcript up-regulation of these two Chg genes may vary depending on the estrogen or xenoestrogen compound.

Similar to the Chg envelope proteins, results from our current study with male arrow gobies showed that relative mRNA expression of the two examined vitellogenins, *vtgAa* and *vtgC*, rose significantly in the liver following exposure to either E2 or the 100 µg/L dose of 4-NP. Most teleost fishes have evolved at least three forms of Vtg proteins with each protein encoded by one or more genes: *vtgAa* and *vtgAb* encode full length Vtg proteins, while the *vtgC* gene encodes a truncated Vtg lacking three yolk protein domains (Finn and Kristoffersen, 2007; Reading et al. 2009; Reading and Sullivan 2011; Wang et al., 2005; Williams et al., 2014; Yilmaz et al., 2015). The relative expression levels of these genes and, subsequently, proportional composition of yolk proteins derived from the different forms of vtgs varies among fish taxa (Williams et al., 2014). The yolk of the marine goldsinny wrasse (*Ctenolabrus rupestris*) is almost completely comprised of VtgAa protein (Kolarevic et al., 2008), while the ratio of VtgAa:VtgAb:VtgC proteins is 9:15:1 in the yolk of oocytes from barfin flounder, *Verasper moseri* (Sawaguchi et al., 2008). Genes encoding these Vtg yolk precursor proteins are typically only expressed in liver of female fish, but can be induced hepatically in males by chemicals with estrogenic activity (Denslow et al., 2001; Garcia-Reyero, 2003; Hemmer et al., 2001; Hylland and Haux, 1997; Jobling and Sumpter, 1993). Since the expression of all three *vtg* genes is strongly up-regulated by estrogens (Flouriot et al., 1997), changes in *vtg* transcript abundance and Vtg protein concentration in blood circulation are considered to be among

the most reliable and tractable biomarkers for xenoestrogen exposure identified to date (Arukwe and Goksøyr, 2003; Garcia-Reyero, 2004; Hutchinson et al., 2006).

Our data provide further evidence for the validity of elevated relative mRNA levels of these *vtg* genes as reliable biomarkers of exposure to 4-NP in teleost fishes (Arukwe and Goksøyr, 2003; Bowman et al., 2003; Hemmer et al., 2002). 4-NP elicited significant increases in *vtgAa* and *vtgC* relative mRNA levels within 72 h of exposure. The magnitude of 4-NP induction for *vtgAa* was nearly 2 times greater than that observed for *vtgC*. A similar magnitude difference in transcriptional induction between *vtgAa* and *vtgC* was also observed in response to E2 treatment. This observation corresponds with the findings of Meng and coworkers (2010), who observed that relative levels of full length *vtg* transcripts (*vtg*-1, *vtg*-2, *vtg*-4, and *vtg*-5) in zebrafish (*Danio rerio*) showed higher magnitude peak induction following exposure to 17 α -ethinyloestradiol (EE2), than did mRNA levels for *Danio vtg*-3, which encodes a truncated Vtg protein homologous to *vtgC* in other fishes. Fathead minnows (*Pimephales promelase*) treated with ethinyl estradiol (EE2) likewise showed differential maximum transcriptional induction responses depending on the *vtg* gene, with levels of *vtgC* mRNA peaking four orders of magnitude lower than the maximum levels for *vtgA* mRNAs (Miracle et al., 2006). Taking into account this evidence from multiple independent studies, *vtgAa* mRNA levels indeed appear to exhibit a greater response to estrogenic compound exposures than *vtgC* transcripts.

In terms of biological response recovery, our results showed that these Chg transcriptional changes induced by 4-NP or E2 exposure recovered to pre-exposure expression levels within 12 days of depuration. Recovery of *vtg* mRNA levels from their

4-NP induced elevation varied depending on the *vtg* gene; transcripts encoding *vtgAa* remained elevated at 72 h after depuration, while *vtgC* mRNA abundance was still elevated significantly even at 12 days after depuration, albeit at a low, 1.7-fold level compared to the peak induction levels of more than 23-fold. Transcripts encoding *vtgC* also showed limited evidence of declining abundance levels between 72 h and both 12 days and 20 days of 4-NP treatment, suggesting the possibility of a modest compensatory response under continuous 4-NP exposure (e.g., Calabrese and Baldwin, 2001; Genovese et. al. 2012). Hepatic levels of both *vtg* genes had returned to pre-exposure baseline abundance before 20 days of clean water exposure, demonstrating that neither 4-NP nor E2 caused long-term changes in the relative expression levels of these genes.

This rapid recovery of *vtg* mRNA levels following termination of E2 or 4-NP exposure in the arrow goby mirrors the recovery timing observed previously for Vtg pathways in other fishes. For example, sheepshead minnow treated with dissolved phase E2 (0.089 or 0.71 ng/L) or para-NP (5.6 or 59.6 mg/L) for 15 days still exhibited elevated hepatic *vtg* mRNA and plasma Vtg protein levels 2 days after cessation of chemical exposures, but had declined by 4 days and were at pre-exposure levels within 8 days of depuration (Hemmer et al., 2002). In a study of the South American cichlid *Cichlasoma dimerus* exposed to octophenol (150 µg/L) for 28 days, Genovese and coworkers (2012) observed that hepatic mRNAs for *chgL* and *chgH* returned to control, unexposed levels within 1 to 3 days of depuration, while *vtgAb* mRNAs remained elevated 7 days after transfer to clean water, but had returned to control levels prior to the next sampling time at 14 days.

Based on the time course of induction and recovery observed in these and other

studies, hepatic choriogenins *chgL* and *chgHm* and vitellogenins *vtgAa* and *vtgC* exhibit characteristics of fast-induction and fast-recovery corresponding to a type 5 biomarker according to the temporal induction-recovery classification proposed by Wu and coworkers (2005). Such type 5 biomarkers are highly sensitive to fluctuations in environment levels of chemical contaminants and respond rapidly to a pulse increase or decline in pollution (Wu et al., 2005). Increases in *chg* mRNAs do not appear to show significant adaptation even under long-term estrogen or xenoestrogen exposure (e.g., Ackerman et al., 2002; Giesy et al., 2000) and the maximum induction levels of these genes is correlated with the concentration, and in some cases short-term duration, of exposure (e.g., Knoebel et al., 2004). Those characteristics indicate that changes in the relative abundance levels of these genes should serve as tractable, dose-response biomarkers for evaluating variation in 4-NP contamination in coastal estuaries, where rainfall often generates episodic increases in pollution levels (Kennish, 1997; Sun et al., 2012; DeLorenzo, 2015).

Estrogen receptor induction varies with receptor type and tissue

The genomic actions of estrogens such as E2 occur via hormone binding to nuclear estrogen receptors (ERs), which then bind to estrogen responsive elements (EREs) within the promoter regions of genes to modify their transcription (Beato, 1991). Xenoestrogens such as 4-NP can likewise bind these ERs and block endogenous estrogen access (McLachlan, 1993), ultimately inducing the synthesis of estrogen responsive genes (Yadete et al., 1999). Three nuclear ERs have been identified in teleost fish, *esr1*, *esr2a*, and *esr2b* (Hawkins et al., 2000). Although all three receptors are expressed in several

organs and tissues, the highest expression levels of these nuclear ER genes typically occur in the liver and gonad, with lower levels in the brain, pituitary, intestine, and skeletal muscle (Hawkins et al., 2000; Tchoudakova et al., 1999; Socorro et al., 2000; Menuet et al., 2002). Tissue distribution patterns for expression of ERs, however, can vary between fish taxa. For instance, transcripts encoding *esr1* and *esr2b* are principally expressed in the liver of fathead minnows (Filby and Tyler, 2005), while *esr2a* is expressed principally in the intestine and ovaries of this Cyprinid species (Filby and Tyler, 2005, Wu et al., 2001, Bouma and Nagler, 2001). However, in studies of nuclear ERs from two other cyprinid fishes, transcripts encoding *esr1* in African catfish (*Clarias gariepinus*) and the *esr2a* in goldfish (*Carassius auratus*) were each most highly expressed in pituitary and brain, with considerably lower levels of expression in the gonad and liver (Ma et al., 2000; Choi and Habibi, 2003; Teves et al., 2003). Such species variation in *esr* gene expression is likely attributable to several factors including differences in circulating E2 concentrations or estrogen function between the sexes or developmental stages of fish examined and taxonomic variation in reproductive life history including breeding seasonality (e.g., Hernández et al., 1992; Marlatt et al., 2010).

Our results here with the arrow goby point to another factor that might help explain observed species differences in ER mRNA expression: differences in estrogen-induced transcriptional sensitivity between *esr* genes. Specifically, we observed that hepatic transcript abundance for *esr1* in adult male arrow gobies was up-regulated over 40-fold within 24 h and over 75-fold by 72 h following the start of E2 exposure. 4-NP exposure likewise up-regulated liver *esr1* transcript abundance, with *esr1* mRNAs increased 21-fold after 12 days of treatment with 100 µg/L 4-NP. Relative mRNAs for *esr1* remained

elevated only briefly during both the 4-NP and E2 exposures and returned to pre-exposure baseline abundance prior to cessation of 4-NP or E2 exposure, implying adaptation of the *esr1* transcriptional response under prolonged 4-NP or E2 exposure. Neither 4-NP nor E2, however, had any effect on hepatic *esr2a* mRNA levels, suggesting that *esr2a* transcriptional regulation in the liver of adult male arrow gobies may be insensitive to estrogenic compounds.

Several prior studies support this finding that teleost nuclear ER genes can differ in their sensitivity to estrogen and xenoestrogen transcriptional induction (Boyce-Derricott et al., 2010; Huang et al., 2010; Nelson and Habibi, 2013, Tohyama et al. 2015). In male fathead minnow, for instance, E2 (100 ng/L) was observed to up-regulate hepatic *esr1* relative mRNA levels but had no effect on transcript abundance for either *esr2a* or *esr2b* (Filby and Tyler, 2005). Similarly, exposure of male goldfish to exogenous E2 via silastic implants of 100 µg/g body mass strongly up-regulated hepatic *esr1* (ER α) mRNA levels, had no effects on *esr2a* (ER β 2) mRNAs, and down-regulated transcript abundance for *esr2b* (ER β 1) (Marlatt et al., 2008). Rainbow trout (*Oncorhynchus mykiss*) treated with aqueous phase E2 (473 ng/L) for seven days showed elevations in mRNAs for two *esr1* paralogs encoding α -type receptors ER α 1 and ER α 2, but no change in abundance for transcripts encoding the β receptors ER β 1 and ER β 2 (Osachoff et al., 2013). Meucci and Arukwe (2006) found that exposure of juvenile Atlantic salmon, *Salmo salar*, to waterborne 4-NP at doses of 5 to 50 µg/L up-regulate hepatic *esr1* mRNA levels after 3 and 7 days of exposure, but also observed that an *esr2* transcript was temporarily reduced in relative abundance after 3 days of 4-NP exposure, only to recover to pre-exposure levels by 7 days of 4-NP treatment. The picture that has emerged from these and other

studies that have examined ER regulation in the liver confirms E2 autoinduction of hepatic *esr1* transcription across oviparous fishes examined to date, but suggests that E2 effects on hepatic *esr2a* and *esr2b* transcription vary depending on the species and life history stage of individuals being examined (Nelson and Habibi, 2013).

The differing sensitivities of the teleost fish *esr* genes to estrogen-induced transcription are likely linked to divergent functional roles for these nuclear ER receptors, which itself can vary among tissues. While it is sometimes suggested that the strong correlation in up-regulation between *esr1* and *vtg* mRNAs in the liver indicates *esr1*/ER α mediation of *vtg* gene regulation, results from experimental studies indicate that it is the *esr2*/ER β receptor that mediates Vtg regulation by estrogens (Nelson and Habibi, 2013). In rainbow trout hepatocytes, Vtg production was induced by the mammalian ER β receptor agonist diarylpropionitrile, but not by the mammalian ER α agonist propylpyrazole-tiol (Leanos-Castaneda and Van Der Kraak, 2007). In this same study, the ER α antagonist methyl-piperidino-pyrazole failed to inhibit E2-induced Vtg production. Similarly, Yamaguchi and coworkers (2014) aimed to clarify the differing roles of ER subtypes in the mediation of E2-induced hepatic choriogenin production in medaka (*Oryzias latipes*) and observed that the induction of hepatic *chgH* transcription was strongly dependent on the dose of an ER α selective agonist (Orthoester-2k), implying *esr1*/ER α mediation of liver *chgH* induction. In contrast, hepatic *chgL* mRNA abundance was observed to be more responsive to the ER β -selective ligand 2-(4-hydroxyphenyl)-5-hydroxy-1,3-benzoxazole, suggesting that an *esr2*/ER β may be involved in E2-mediated *chgL* transcriptional induction (Yamaguchi et al., 2014). While these and similar studies are helping to elucidate the distinct functional roles of ERs in E2-regulated *vtg* and *chg*

transcription, it is important to note that the specificity of these mammalian ER agonists and antagonists likely varies depending on evolved variation in ER structure among teleost fishes, and that many ligands may interact with multiple ERs – or other receptor pathways – to directly or indirectly influence *vtg* or *cgh* regulation (Chakraborty et al., 2011; Huang et al., 2010). For that reason, it might be expected that alternative experimental approaches could provide conflicting information about ER subtype function in *Vtg* and *Chg* regulation. While such data are limited at present, one recent study using *esr* receptor-specific morpholino (MO) oligonucleotides to disrupt *esr* gene expression in zebrafish embryos found that *esr1* and *esr2b* MOs both prevented E2 induction of *vtg* and *esr1* mRNAs (Griffin et al., 2013), suggesting that the findings from agonist/antagonist studies have not yet revealed the complete picture of ER subtype function in regulating *Vtg* and *Chg* biomarker expression.

Brain aromatase regulation

In the brain, one of the best established gene targets of E2 action is the brain isoform of cytochrome P450 aromatase B (Kishida and Callard, 2001), an enzyme involved in the conversion of testosterone to estrogen. Actinopterygian fishes possess two forms of steroidogenic aromatase enzyme genes, *cyp19a1a* and *cyp19a1b*, which evolved as a result of a gene duplication event (Diotel et al., 2010). These genes show distinct tissue-specific expression patterns with the *cyp19a1a* gene (ovarian aromatase, or aromatase A) expressed primarily in ovary and the *cyp19a1b* (brain aromatase, or aromatase B) expressed in radial glial cells in the brain (Tchoudakova and Callard, 1998; Forlano et al., 2001; Menuet et al., 2002; Zhang et al., 2004). Brain aromatase

transcription in fish has previously been shown to be induced by estrogens (e.g., Kishida and Callard, 2001; Menuet et al., 2004), and our data here revealed that male arrow gobies treated with waterborne E2 (50 ng/L) exhibited nearly 2- to 3-fold greater *cyp19a1b* mRNA levels in the brain after 12 days and 20 days of exposure. This E2-induced increase in brain *cyp19a1b* mRNAs was short-term, however, and *cyp19a1b* transcript abundance declined to baseline, control levels by 72 h after termination of the exposure. We did not, however, detect any changes in brain *cyp19a1b* transcript abundance caused by 4-NP at either exposure dose.

E2 regulation of *cyp19a1b* occurs in part via a nuclear ER pathway, and two EREs have been identified in the 5' flanking region of the *cyp19a1b* genes from goldfish and zebrafish (Callard et al., 2001). While it remains unclear which ER subtype(s) mediate this *cyp19a1b* up-regulation by E2, morpholino inhibition of *esr2b* was found to impair E2-induced *cyp19a1b* transcription in zebrafish embryos (Griffin et al., 2013). In the present study, we tested for E2 and 4-NP effects on the relative abundance of *esr1* and *esr2a* transcripts in the whole brain, but found no evidence for regulation of either receptor gene by either of these chemicals. While our results with brain *esr* regulation differ from some previous findings in teleosts (e.g., Depiereux et al., 2014; Marlatt et al., 2008), they are consistent with other studies. For instance, waterborne 4-NP and E2 exposures for up to six days had no effect on brain *esr1* mRNA abundance in rainbow trout fry (Vetillard and Bailhache, 2006). In one recent study by Xing and coworkers (2016) using cultured radial glial cells from goldfish, E2 was observed to increase the abundance of transcripts encoding both *cyp19a1b* and *esr2b*, but not *esr1* or *esr2a*. In the same study, this E2 effect on *cyp19a1b* mRNAs was also linked to E2-induced

recruitment of the dopamine D1 receptor and the level of phosphorylated cyclic AMP response element binding protein (p-CREB). Xing and colleagues (2016) suggested, however, that this synergistic interaction between E2 and dopamine pathways in up-regulating *cyp19a1b* is not solely mediated by *esr2b*, but also requires involvement of endogenous *esr1* and *esr2a* expression. While future studies are needed to explore these interactions between aromatase, E2, and dopamine pathways in the brain more fully, the inconsistent and variable responses of ER transcriptional expression to E2 in teleost fishes indicate that *esr* genes have limited utility as biomarkers for xenoestrogen exposure.

CONCLUSIONS

Our data provides evidence for concentration- and time-dependent induction of liver *vtg* and *chg* gene expression in male arrow goby after exposure to environmentally relevant concentrations of 4-NP. Maximum hepatic induction of these genes occurred within 72 h to 12 days of exposure – depending on the gene – and recovery of mRNA levels took between 3 and 12 days following depuration. Our data also demonstrate that the ER gene *esr1* exhibits tissue-specific transcriptional regulation by E2. Taken together, these findings validate the utility of relative mRNA levels of *vtg* and *chg* genes as accurate hepatic biomarkers for xenoestrogen exposure in the arrow goby and provide a foundation for assessing whether male gobies in estuaries along the Pacific Coast of N. America are being impacted by 4-NP pollution in these habitats (Diehl et al., 2012; Maruya et al., 2015).

CHAPTER 2

Disruption of Osmoregulatory Ability of the Estuarine Arrow Goby (*Clevelandia ios*) by 4-Nonylphenol and 17 β -Estradiol Exposure

ABSTRACT

Recent evidence indicates that some of California's coastal estuaries are contaminated with the chemical 4-nonylphenol (4-NP). The compound 4-NP is a well-established endocrine-disrupting chemical with estrogenic properties, and exposure to 4-NP has been found to alter estrogen hormone signaling in many marine organisms. 4-NP has been shown to regulate osmoregulatory function and we hypothesize that estuarine fishes exposed to 4-NP in California's estuaries might suffer deleterious impacts due to impaired osmoregulatory abilities in the rapidly changing salinity conditions of coastal estuaries. The aim of our study was thus to determine if 4-NP interrupts the ability of the estuarine arrow goby (*Clevelandia ios*) – a benthic fish abundant on the mud flats of California's estuaries – to osmoregulate under changing salinity conditions. Adult mixed-sex groups of arrow gobies were exposed in seawater (33 ppt) to either high dose 4-NP (100 ug/L), low dose 4-NP (10 ug/L), 17 β -estradiol (50 ng/L; positive control), or ethanol vehicle only (negative control) for 12 days. Fish were then collected from each treatment tank at times representing either a baseline (0 hrs; all fish at 33 ppt salinity) sample, or at 6 hr or 24 hr time points following transfer to 20 ppt or 5 ppt salinities. Additional fish were maintained at the acclimation salinity (33 ppt) for the duration of the experiment as a salinity control. Whole body water content was then measured in all fish at 24 hr after salinity transfer. In addition, the relative abundance of gene transcripts encoding the

relative mRNA levels for the ion channels $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -cotransporter1 (*nkcc1*) and Na^+/H^+ exchanger-3 (*nhe3*), and the aquaporin water channel aquaporin-3 (*aqp3*) was measured in the gill epithelium using real-time quantitative reverse transcription PCR. Results indicated that treatment groups at both 33 ppt (salinity control) and 20 ppt that were dosed with either E2 or high 4-NP showed significant reductions in relative *nhe3* and *aqp3* mRNA levels in the gill epithelium compared to unexposed, control fish. Furthermore, at 24 hr after salinity transfer, gobies from these same treatment groups also showed significantly higher total body water content compared to their respective control groups. These findings suggest that 4-NP exposure can induce significant changes in osmoregulatory balance in arrow gobies in patterns that lead to an increased influx or reduced elimination of water upon experiencing lower salinity environments.

Keywords: ionregulation, endocrine disruption, osmoregulation, nonylphenol

INTRODUCTION

In light of accumulating evidence that the Earth's global climate system is changing as a result of anthropogenic activities (IPCC, 2014), there is an increased need to understand how shifting environmental conditions associated with a changing global climate will impact the transport, accumulation, metabolism and toxicity of chemical pollutants (Dalla Valle et al., 2007; Hooper et al., 2013; Moe et al., 2013; Noyes, 2009; Noyes and Lema, 2015a). Environmental parameters predicted to change under shifting climatic conditions include temperature, precipitation, and both pH and dissolved O₂ in aquatic systems, and many of these parameters have been demonstrated to impact the toxicity of chemical pollutants (Holmstrup et al., 2010; Noyes and Lema, 2015; Schiedek et al., 2007). And yet, to date, relatively few studies have evaluated how the developmental, physiological, and behavioral impacts of chemical contaminant exposure are influenced by variation in environmental conditions (e.g., temperature, salinity, pH) in an ecologically relevant context (Noyes and Lema, 2015), and there remains a lack of information on whether the detrimental impacts of chemical pollutants for both organism and ecological health will be exacerbated by global climate change (Hooper et al., 2013; Noyes, 2009).

Aquatic ectotherms such as fish may be particularly vulnerable to increased toxicity from chemical contaminants under a changing climate since several of the environmental parameters predicted to change in freshwater and marine aquatic systems have been linked to key aspects of physiological performance in these taxa. For instance, temperature is well established to influence multiple aspects of physiology in fish including metabolic oxygen demand (Clarke and Johnston, 1999; Myrick and Cech,

2000), food conversion efficiency (Myrick and Cech, 2000; Wurtsbaugh and Cech, 1983), and rates of growth and development (Carveth et al., 2007). Temperature can also influence the rate of chemical uptake from water by fish (Blewett et al., 2013a,b), and changes in toxicity for fish under elevated thermal conditions are likely to emerge from the interactions of temperature-associated variation in chemical uptake and shifts in metabolic processes (e.g., Lemly, 1993; MacLeod and Pessah, 1973; Reid et al., 1997). What is more, adaptive variation in the physiological responses of fish to environmental variation has been documented extensively across taxa (e.g., Eddy and Handy, 2012), and it is probable that the susceptibility of fish to enhanced chemical toxicity will be distributed unevenly across species and populations depending on factors that include the severity of climate change in a geographic region, the composition and concentration of chemical exposure, and the evolutionary history of the taxon and prior experience in habitats with stable or variable environmental conditions.

Aquatic species inhabiting coastal estuaries may be particularly vulnerable to impacts from the combined influences of chemical toxicity and climate change given that estuaries can receive a mixture of fertilizers, pesticides, agrochemicals, heavy metals, polyaromatic hydrocarbons (PAHs), and persistent organic pollutants (POPs) from residential, industrial, and agricultural activities on land (Islam and Tanaka, 2004; Kennish, 2001; Sun et al., 2012; DeLorenzo, 2015). Estuaries have also been identified as one of the marine habitats most vulnerable to climate change (Kennish, 2002; Robinson et al., 2013). Climate change is predicted to result in increasing habitat loss in estuaries from the combined effects of coastal subsidence, erosion and rising sea levels (Kennish, 2002; Robins et al., 2016; Robinson et al., 2013). In addition, estuaries are

expected to receive increasing nutrient and sewage inputs leading to eutrophication, which combined with elevated temperature conditions should lead to more frequent hypoxia events (Kennish, 2002; Robins et al., 2016; Sheahan et al., 2013). As atmospheric warming changes hydrological cycles, changes in local precipitation patterns are predicted to lead to altered river flows. While specific predictions on changing river flow rates vary geographically, in several regions shifting trends in winter and summer precipitation are expected to generate reduced freshwater flows in summer and increased flows in winter (e.g, Bromirski et al., 2003; Robins et al., 2016) as well as an increase in the frequency and severity of extreme rainfall events (Diffenbaugh et al., 2005). Shifting rainfall and river flow rates will affect estuarine salinities, turbidity, and the dynamics of chemical contaminant and nutrient inputs, making the species that inhabit these coastal marine ecosystems particularly vulnerable to detrimental impacts from the combined influences of climate change and chemical pollution (DeLorenzo, 2015; Schlenk and Lavado, 2011).

As transition zones between freshwater ecosystems and the ocean, estuaries are among the most productive ecosystems on Earth and commonly serve as important nursery grounds to many species of fishes and invertebrates, while also providing water filtration, nutrient cycling, and protection to coastal communities during storms (Barbier, 2011; Beck et al., 2001). A unique feature of most organisms that reside in estuaries is their ability to maintain internal hydromineral balance under rapidly changing environmental salinities. In euryhaline estuarine fishes, for instance, hydromineral balance is mediated, in part, by the movement of ions and water across the gill (Hwang et al., 2011). Under increased salinity conditions such as during a rising tide, euryhaline

fishes may ingest seawater in an attempt to balance out the osmotic loss of water to their hypersaline environment, and then excrete the excess ions (e.g., Na^+ , Cl^-) via ion channels in the gill epithelium (reviewed by Hirose et al., 2003; Hwang and Lee, 2007; Hwang et al., 2011). This process is accomplished by Na^+ , K^+ , and Cl^- entering mitochondrion-rich cells (MRCs) in the epithelium from the blood via basolateral cotransport of Na^+ , K^+ , and Cl^- ions by the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter-1 protein (NKCC1). Na^+ is actively pumped out of the cell via the $\text{Na}^+/\text{K}^+/\text{ATPase}$ (NKA) (Silva et al., 1977), which facilitates the buildup of a K^+ concentration gradient and voltage gradient as K^+ extrudes back into the plasma through K^+ channels (Degnan, 1985). Cl^- is extruded across the apical membrane by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Marshall and Singer, 2002). In addition, recent evidence suggests that water movement across the gill epithelium is regulated via changes in aquaporin-3 (AQP3) expression, which assists with the maintenance of basolateral cell hydraulic conductivity when fish are in fresh water conditions (Marshall, 2013).

Gill iono- and osmoregulation is mediated by a variety of transcriptional regulators and hormones including cortisol, prolactin and estrogens such as 17β -estradiol (E2) (Carrera et al., 2006; McCormick et al., 2013; McCormick, Lerner et al, 2005; Lerner et al., 2012). In the euryhaline fishes *Fundulus heteroclitus* and *Oreochromis mossambicus*, E2 reduces gill NKA activity during transitions from lower salinity to greater salinity (Mancera et al., 2004; Vijayan et al., 2001). Similar reductions in gill NKA activity following E2 exposure have been observed in juvenile Atlantic salmon *Salmo salar* (Lerner et al., 2007). Such hormonal effects on gill osmoregulatory function can, however, vary depending on environmental context, species, and even the life history

stage or reproductive state of the individual (e.g., McCormick et al., 1991; McLeese et al., 1994; Schreiber, 2001). For instance, in the euryhaline fish *Sparus auratus*, E2 did not alter gill NKA activity following transfer from seawater to fresh water, but did diminish the reduction in plasma osmolality and Na⁺ concentration resulting from this salinity change (Guzman et al., 2004). Accordingly, exposure to chemical contaminants with estrogenic activity may also have the potential to disrupt ionoregulation across the fish gill (Carrera et al., 2006; Lerner et al., 2012), although to date very few studies have tested for such effects.

Recently, several estuaries along the Pacific Ocean coast of North America were identified as having elevated NP contamination in sediments and biota (Diehl et al., 2012; Maruya et al., 2014). Mussels (*Mytilus* spp.), shrimp, and fishes including the arrow goby (*Clevelandia ios*) and staghorn sculpin (*Leptocottus armatus*) from these estuaries were found to have among the highest tissue burdens of NP documented worldwide (Diehl et al., 2012). NP, a breakdown product from a subgroup of nonionic surfactants, alkyphenol ethoxylates, is released during the degradation of products such as plastics, detergents, agriculture sprays, and toilet paper (Fairey et al., 1997; Sharma et al., 2009; Soares et al., 2008), and can also be introduced into the environment via waste water discharge or direct run-off in agriculture intensive areas (Gehring et al., 2004; Zgola-Grzeskowiak et al., 2009). NP is an established estrogen-active substance and has been shown in laboratory studies to cause negative effects on reproductive function in fishes (Ackerman et al., 2002; Sakamoto and McCormick, 2005; Lerner et al., 2012). Given NP's estrogenic activity, it is also possible that NP exposure could impact the osmoregulatory ability of fishes in contaminated estuaries or of fishes migrating through estuarine

environments (Lerner et al, 2005; Lerner et al., 2012; Hanson et al., 2010; McCormick et al., 2005). In Atlantic salmon (*Salmo salar*), for instance, acute exposure to NP (100 $\mu\text{g}\times\text{L}^{-1}$) produced decreased plasma Na^+ concentration in juvenile fish maintained in freshwater, and also decreased plasma Cl^- concentration in salmon transferred to a salinity of 30 ppt (Lerner et al., 2007a). Atlantic salmon exposed to NP in the yolk-sac larval life stage had lower gill NKA activity and elevated plasma Cl^- upon transfer to seawater conditions 1 year later during smoltification, the developmental period when salmon are migrating downstream and transitioning from freshwater streams and rivers to the ocean environment (Lerner et al., 2007b). These NP-exposed Atlantic salmon smolts also showed reduced seawater preference in a pattern similar to the reduced preference of E2-exposed smolts (Lerner et al., 2007b), suggesting that NP's effects on ion regulation may have been mediated by the compound's estrogenic effects.

The aim of this study is to determine if exposure to 4-NP interrupts the ability of the estuarine arrow goby (*Clevelandia ios*) to regulate hydromineral balance under changing salinity conditions representative of the salinities experienced by arrow gobies in their natural habitat during a coastal rainstorm event. The arrow goby is a small, benthic gobiid fish that is highly abundant along the coast of California, USA, and can be found as far north as British Columbia, Canada and as far south as Baja California, Mexico. This goby occupies the burrows of polychaete worms and shrimp at low tide, where it is in contact with low oxygen sediments rich in organic material where NP can accumulate and persist for decades (Lavado et al., 2009). Diehl and coworkers (2012) documented mean NP tissue burdens up to 40,100 $\text{ng}\times\text{g}^{-1}$ lipid weight in arrow gobies in estuaries of California, USA. Such elevated tissue burdens point to the possibility that

the iono- and osmo-regulatory ability of these gobies could be impacted detrimentally. To test this idea, we examined whether exposure to dissolved phase NP ($100 \mu\text{g}\times\text{L}^{-1}$ or $10 \mu\text{g}\times\text{L}^{-1}$) or E2 ($\mu\text{g}\times\text{L}^{-1}$) influenced the ability of adult male arrow gobies to acclimate to acute changes in environmental salinity from seawater condition (33 ppt) to reduced salinities of either 20 ppt or 5 ppt for up to 24 h. We measured body water content of these fish 24 h after salinity transfer as an overall gauge of the effects of NP or E2 exposure on osmoregulation. We also quantified the relative abundance of gene transcripts encoding the bumetanide-sensitive $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter 1 (*nkcc1*) and Na^+-H^+ exchanger isoform 3 (*nhe3*) ion transporters, as well as mRNAs for the aquaporin 3 (*aqp3*) channel, in the gill epithelium to provide insights into the mode of impacts for NP's effects on ionoregulation by the gills.

MATERIALS AND METHODS

Isolation and sequencing of partial cDNAs from the arrow goby

RNA extraction and reverse transcription

A male arrow goby (standard length: 42.00 mm; body mass: 0.80 g) was collected by slurp gun from its burrow in the intertidal mud flats from the Morro Bay estuary near Morro Bay, California, USA (35.345173°N , 120.843945°W) on 3 February 2012. The goby was euthanized (tricaine methanesulfonate, MS222; Argent Laboratories, Redman, WA, USA), and the gills were dissected, flash frozen in liquid N_2 , and then stored at -80°C . Total RNA was extracted from the gills using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) with bromochloropropane as the phase separation reagent. The resulting total RNA was subsequently quantified (P300

NanoPhotometer, Implen, Westlake Village, CA, USA) and DNase I treated using the Turbo DNafree Kit (Ambion, Grand Island, NY, USA).

Total RNA was reverse transcribed in 20 µl reactions containing 4.0 µl GoScript™ reverse transcriptase (Promega Corp., Madison, WI, USA), 4.0 µL of 5x buffer, 3.0 µL of MgCl₂ (25 mM), 1.0 µL of random primers (random hexadeoxynucleotides; Promega Corp.), 1.0 µL of deoxynucleotide triphosphates (dNTPs, 100mM, Promega Corp.), 0.25 µL of recombinant RNasin® ribonuclease inhibitor (20U·µL⁻¹, Promega Corp.), 5.5 µL of nuclease-free H₂O, and 4.0 µL of total RNA (322 ng·µL⁻¹). Reverse transcription followed a thermal profile of 25°C for 5 min, 42°C for 1 h, and the 70°C for 15 min, according to the protocol of the GoScript™ Reverse Transcription System (Promega Corp.).

PCR amplification and sanger sequencing

PCR was performed on the gill first-strand cDNA library using degenerate primers designed to consensus regions of the bumetanide-sensitive Na⁺/K⁺/2Cl⁻ cotransporter 1 (*nkcc1*), Na⁺-H⁺ exchanger isoform 3 (*nhe3*), and aquaporin 3 (*aqp3*) from other teleost fishes. Degenerate primers for *nkcc1* were designed to consensus regions of the coding region of this gene from mummichog *Fundulus heteroclitus* ([AY533706](#), [DR442079](#) and [GT098098](#)), Indian ricefish *Oryzias dancena* ([GQ862972](#)), and the European seabass *Dicentrarchus labrax* ([AY954108](#)). Primers for *nhe3* were designed using available cDNAs from the sheepshead minnow *Cyprinodon variegatus* ([HM142345](#)), *F. heteroclitus* ([AY818825](#) and [DR046872](#)), the tilapia *O. mossambicus* ([AB326212](#)) and longhorn sculpin *Myoxocephalus octodecemspinosus* ([EU909191](#)), and for *aqp3* using

nucleotide consensus regions of cDNAs from *F. heteroclitus* ([EU780154](#)), *D. labrax* ([DQ647191](#)), and *O. mossambicus* ([AB126941](#)). All degenerate primers are provided in Table 3. A partial cDNA encoding ribosomal protein L8 (*rpl8*) was isolated and sequenced previously from the arrow goby (KU886162; Johnson and Lema, submitted), and was used as a possible internal control gene. A partial cDNA sequence for elongation factor 1 (*ef1 α*) was also amplified and sequenced using degenerate primers described elsewhere (Lema, 2010) as a second control gene.

Complementary DNA was amplified in 50 μ l reactions containing 25 μ l of GoTaq® Colorless Master Mix (Promega), 1 μ l each of forward and reverse degenerate primer (50 μ M), and 21 μ l of nuclease-free H₂O under a profile of 95°C for 2 min, followed by 30-35 cycles 95°C for 30 s, 51-54°C for 30 s and 72°C for up to 1.5 min, and then 72°C for up to 3 min. Following electrophoresis on 1.2% ethidium bromide gels, any PCR products with bands of expected size were cleaned (QIAquick PCR Purification Kit, Qiagen), quantified (NanoPhotometer P300, Implen, Westlake Village, CA, USA), and Sanger sequenced (Molecular Cloning Laboratories, South San Francisco, CA, USA). The resulting nucleotide sequences were aligned using Sequencher v5.1 software (Gene Codes Corp., Madison, WI, USA) and the identity of each assembled cDNA sequence was confirmed via BLAST comparison against previously published sequences in teleost fishes (<http://blast.ncbi.nlm.nih.gov/>). These methods identified partial cDNAs from *C. ios* encoding a 893 bp partial cDNA for *nkcc1* ([KJ957838](#)), a 614 bp nucleotide cDNA for *nhe3* ([KJ957837](#)), and a 598 bp partial cDNA encoding *aqp3* ([KJ957836](#)), as well as a 818 bp nucleotide partial cDNA encoding *ef1a* (KX098508).

Salinity challenge following NP or E2 exposure

Animals

Adult arrow gobies were collected at low tide on 18 April 2014 from the burrows of ghost shrimp (*Neotrypaea californiensis*) or polychaete worms within the mud flats of Morro Bay estuary near Morro Bay, California, USA, using slurp guns. The fish were transported back to California Polytechnic State University's Center for Coastal Marine Sciences facility in Avila Beach, CA. The fish were housed in 3 L acrylic tanks with flow-through filter seawater (33 ppt salinity) and fed an *ad libitum* diet of tropical fish flake (International Pet Supplies and Distribution, Inc., San Diego, CA, USA) for three weeks prior to commencing experimental treatments. A prior study evaluating estrogen-sensitive biomarkers indicated that a three week captive holding period was sufficient for residual 4-NP clearance from arrow gobies collected from the Morro Bay estuary (Johnson and Lema, submitted). The Animal Care and Use Committee of California Polytechnic State University approved all experimental procedures (Protocol 1301).

Experimental design

Adult mixed-sex arrow gobies (standard length: 29.58 ± 0.24 SEM) divided arbitrarily into groups of ~ 30 fish were exposed in 19 L glass closed-system seawater tanks (33 ppt) to one of the following treatments for 2 weeks: high dose 4-NP (100 µg/L 4-NP in 0.0001% ethanol), low dose 4-NP (10 µg/L 4-NP in 0.0001% ethanol), 17β-estradiol (E2; 50 ng/L in 0.0001% ethanol), or control (0.0001% ethanol vehicle only). Experimental 4-NP (mixture of C₁₅H₂₄O isomers, purity >98.5%, ACROS Organics) and

E2 (Sigma-Aldrich, St. Louis, MO, USA) treatments were created by first dissolving each compound in absolute ethanol and then adding the dissolved phase chemical to an experimental tank. Complete seawater and chemical exposure renewal of each treatment tank occurred every 2 days. All exposure tanks were oxygenated with air and positioned within a larger flow-through water bath (approx. 1135 L) to maintain all treatment tanks at ambient ocean temperatures ($19.3 \pm 4.7^{\circ}\text{C}$; mean $\pm$ SD) (HOBO Pendant® Temperature/Light Data Logger, Onset Computer Corp., Bourne, MA, USA).

After 14 days, gobies ($n = 10$ per treatment) were collected from each treatment tank for baseline (time = 0 hrs) samples at 33 ppt salinity. For this baseline collection and each sampling time thereafter, gobies were captured arbitrarily from their treatment tanks using dip nets, euthanized in MS222, and then measured and weighed. Gill tissue was dissected from each fish and divided into portions which were either flash frozen in liquid N_2 for later RNA extraction or stored in a sucrose- Na_2EDTA -imidazole (SEI) buffer and frozen for later evaluation of NKA enzyme activity. Immediately after the collection of the ‘baseline’ (time= 0 hrs) fish, the remaining fish from each treatment group were transferred to tanks containing water of 33 ppt, 20 ppt, or 5 ppt salinity without added 4-NP, E2, or ethanol control. Fish were then sampled again at time points of 6 hrs and 24 hrs ($n = 10$ fish per treatment and sampling time) after salinity transfer.

Quantification of gill ion channel and aquaporin mRNA levels using qPCR

Total RNA was extracted from the gills of the arrow gobies using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) with bromochloropropane. The resulting RNA was quantified (P300 Nanophotometer, Implen) and DNase I treated

(Turbo DNase-free Kit, Ambion). The resulting DNase-treated RNA was then quantified again and all experimental RNA samples from a given tissue were diluted to the same concentration. Total RNA was reverse-transcribed in 12 μ L reactions containing 2.4 μ L iScript Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 3.6 μ L nuclease-free water and 6.0 μ L of total RNA template (23.5 ng/ μ L) under a thermal profile of 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min.

Gene-specific oligo primers for SYBR green real-time quantitative reverse transcription polymerase chain reaction assays (qRT-PCR) were designed for *nkcc1*, *nhe3*, and *aqp3*, as well as both *rpl8* and *efla* for use as internal control genes. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA), and primer nucleotide sequences are provided in Table 4. Quantitative PCR reactions were run on the 7300 Real-Time PCR System (Applied Biosystems) in 16 μ L volumes containing 8.0 μ L SYBR Green Master Mix (Life Technologies, Grand Island, NY, USA), 0.8 μ L each of forward and reverse primer (10 μ M), 4.9 μ L nuclease-free water, and 1.5 μ L of reverse-transcribed cDNA template. The PCR thermal profile for each reaction was 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A dissociation stage consisting of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds was also included for each reaction to confirm amplification of a single product and the absence of primer-dimers. For each gene a standard curve was made from pooled RNA samples representing all treatments. These standards were serially diluted and each standard assayed in triplicate. For each gene, correlation coefficients (r^2) for the standard curve were always greater than $r^2 = 0.93$. RNA samples that were not reverse transcribed were included as controls for DNA contamination. For each gene,

resulting mRNA levels were calculated based on the standard curve and then expressed as a value relative to the control 33 ppt at either baseline, 6 hrs, or 24 hrs mRNA abundance.

Quantification of total body water content

The small body size of the arrow goby prohibits the collection of blood in sufficient volumes for plasma osmolality quantification. For that reason, we instead quantified total body water content to further evaluate osmoregulatory impacts of 4-NP and E2 exposure. Fish from the 24 hrs time point only were weighed to obtain a wet body mass, dried for 7 days at 55 °C, and then weighed again to provide a dry body mass. Total body water content was then calculated using the following equation: $(M_b - M_{b(d)})/M_b$, where M_b represents wet body mass and $M_{b(d)}$ represents dry body mass, as described previously (Plaut, 1998).

Statistical analyses

The relative mRNA expression data for the two 4-NP dose treatments, E2, and control vehicle group at both 6 hours and 24 hours were analyzed using two-factor ANOVA models with ‘salinity’ and ‘treatment’ as factors. When significant main effects or interactions were identified in the two-factor ANOVA models, a Dunnett’s test was then run to identify which treatment groups differed significantly within each sampling time point. The baseline transformed mRNA expression data was analyzed using a oneway ANOVA model with ‘treatment’ as the only factor and a Dunnett’s test was run to determine which treatment groups differed significantly at baseline 33 ppt. The total

body water content data was also analyzed using two-factor ANOVA models with ‘salinity’ and ‘treatment’ as factors. When significant main effects or interactions were identified in the two-factor ANOVA models, a Dunnett’s test was then run to identify which treatment groups differed significantly within each sampling time point. All statistical analyses were two-tailed and performed using the statistical software package JMP v10 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Effects of 4-NP on whole body water content

Examination of whole body water content (% mass) in adult gobies sampled 24 hr after salinity transfer revealed variation in body water content linked to both salinity challenge and 4-NP exposure (treatment*salinity, $F_{6, 117} = 9.2525$, $p < 0.0001$) (**Fig. 6**). Specifically, whole body water % increased in control fish from 78.9 ± 0.35 % (mean $\pm$ SEM) in gobies maintained at 33 ppt, to 79.8 ± 0.34 % in gobies transferred to 20 ppt, and to 80.5 ± 0.39 % in gobies transferred to 5 ppt for a 24 h period ($F_{2,29} = 4.157$, $P = 0.0259$). Gobies treated with low dose 4-NP and maintained at 33 ppt (salinity control) exhibited elevated whole body water of $82.7 \pm 0.8\%$, compared to mean values ranging from 78.3 to 79.4% for the other treatment groups maintained at this 33 ppt acclimation salinity, suggesting that 4-NP induced changes in osmoregulatory balance in fish maintained under stable seawater conditions. Low dose 4-NP-treated gobies likewise exhibited elevated body water % after transfer to 20 ppt conditions compared to unexposed, control fish. The pattern of this 4-NP effect differed, however, in gobies transferred from 33 ppt to 5 ppt, where whole body water % was observed to be elevated

at $83.0 \pm 0.8\%$ in fish treated with the high dose of 4-NP, compared to unexposed, control fish ($80.5 \pm 0.5\%$) transferred to 5 ppt. Exposure to E2 did not alter whole body water content significantly compared to control fish.

4-NP alters the transcriptional responses of gill ion channel and aquaporin-3 during salinity challenge

Gobies exposed to 4-NP or E2 had altered levels of mRNAs encoding *nkcc1* (**Fig. 7A**), *nhe3* (**Fig. 7B**), and *aqp3* (**Fig. 7C**) in the gill at the baseline (0 hr) sampling period prior to transfer from 33 ppt to reduced environmental salinities, suggesting that both 4-NP and E2 can impact the dynamics of ion and water movement in the gill epithelium independent of any change in osmotic conditions. At this baseline (0 hr) sampling point, the relative abundance of *nkcc1* mRNAs was significantly elevated in both the low and high 4-NP treatment groups relative to the control group (treatment, $F_{3,86} = 6.9859$, $p = 0.0003$) (**Fig. 7A**). Gill *nhe3* transcript abundance was reduced ~67% in the high 4-NP treatment group compared to *nhe3* mRNA levels in control fish ($F_{3,86} = 3.6666$, $p = 0.0154$) (**Fig. 7B**), while low dose and high dose 4-NP treated gobies exhibited gill *aqp3* mRNA levels that were reduced approximately 70% and 80%, respectively, compared to the relative levels of *aqp3* transcript observed in control fish prior to salinity transfer (**Fig. 7C**) ($F_{3,86} = 4.086$, $p = 0.0092$). The relative abundance of transcripts encoding *aqp3* were also altered by exogenous E2 supplementation, with gill *aqp3* mRNAs ~1.8-fold higher in abundance in E2-treated gobies compared to control fish.

Transfer of gobies from the 33 ppt acclimation salinity to either 20 ppt or 5 ppt revealed significant impacts of 4-NP or E2 treatment on the transcriptional responses of

nkcc1, *nhe3*, and *aqp3* in the gill to reduced environmental salinities. At 6 hr after salinity transfer, *nkcc1* mRNAs were elevated significantly in gobies treated with E2 or the high dose of 4-NP and either maintained at the 33 ppt control salinity or transferred to 20 ppt (treatment*salinity, $F_{6,119} = 2.7779$, $P = 0.0145$) (**Fig. 8A**). Gobies exposed to E2 or 4-NP and then transferred to 5 ppt, however, exhibited a similar relative abundance of gill *nkcc1* mRNAs as that observed in control fish transferred to this same 5 ppt salinity. At the 24 hr sampling time after salinity transfer, gill *nkcc1* mRNAs were only observed to be elevated in E2 exposed fish (treatment*salinity, $F_{6,119} = 7.8109$, $P < 0.0001$) (**Fig. 9A**). No effects of 4-NP treatment on gill *nkcc1* expression were observed at 24 hr.

The transcriptional response of *nhe3* in the gill to decreased environmental salinity was altered by both E2 and 4-NP exposure. At the 6 hr sampling time, gobies exposed to E2 and the high dose 4-NP treatment both exhibited highly depressed gill *nhe3* mRNA levels compared to control and low dose 4-NP fish (**Fig. 8B**) (treatment*salinity, $F_{6,111} = 7.8474$, $P < 0.0001$). Gill *nhe3* mRNA levels were similarly observed to be reduced at 6 hr in gobies exposed to E2 or high dose 4-NP and transferred to 20 ppt, but not in gobies transferred to 5 ppt. Gill *nhe3* mRNA abundance generally declined with transfer to reduced salinities, as observed in both control and low dose 4-NP fish. The absence of significant E2 or 4-NP effects on gill *nhe3* following transfer to 5 ppt therefore may result more from this pattern of decreasing gill *nhe3* mRNA abundance upon exposure to lower salinity conditions, rather than from any recovery from E2 or high dose 4-NP effects. The overall effects of osmotic conditions on gill *nhe3* mRNA levels was diminished by 24 hr after salinity transfer, as indicated by the absence of any salinity-associated variation in *nhe3* transcript abundance in unexposed, control fish (**Fig. 9B**).

Even so, gobies treated with the low dose of 4-NP had significantly lower *nhe3* mRNA levels in the gill than control fish at the 24 hr sampling time under both 33 ppt and 20 ppt conditions (treatment, $F_{3, 115} = 5.6300$, $p = 0.0012$; salinity, $F_{2, 115} = 3.4306$, $p = 0.0357$).

Acute challenge with reduced environmental salinity resulted in a decreased relative abundance of *aqp3* transcripts in the gill in unexposed, control fish sampled 6 hr after salinity transfer (**Fig. 8C**). This salinity effect, however, was only observed in control fish, as both E2 and 4-NP exposed gobies sampled at 6 hr already exhibited significantly reduced gill *aqp3* mRNA levels in the 33 ppt environment. The abundance of *nhe3* mRNAs remained low in fish that were transferred to either 20 ppt or 5 ppt (treatment*salinity, $F_{6, 112} = 3.8287$, $p = 0.0016$). These E2 and 4-NP associated reductions in gill *aqp3* mRNAs were less apparent by 24 hr after salinity transfer (**Fig. 9C**), although significant reductions in gill *aqp3* mRNA abundance were still observed in gobies exposed to 4-NP and transferred to either 20 ppt (low dose 4-NP treatment only) or 5 ppt (high dose 4-NP treatment only).

DISCUSSION

Changing climate conditions are predicted to lead to a decreased frequency and increased severity of precipitation events in California (Bromirski et al. 2003; Dettinger, 2005; Harley et al., 2006; Romero-Lankao et al., 2014). For estuaries in California, those changing conditions are expected to result in less freshwater inflow during most months of the year resulting in increased estuarine salinities (Short and Neckles, 1999; Stahle et al., 2001). However, accompanying this overall trend of increasing estuarine salinities with climate change, are less frequent, but more severe winter rainstorms and flood

events which are predicted to generate extreme low salinity events (Cloern et al., 2011; Knowles and Canan, 2002). Changes in runoff volumes associated with shifts in precipitation are also expected to increase chemical contamination to estuaries (Noyes et al., 2009; Wetz and Yoskowitz, 2013). Those combined conditions of increased pollutant input coupled with altered dynamics of salinity variation will represent a multi-stressor effect that could substantially impact aquatic organisms residing in estuarine habitats (DeLorenzo, 2015; Schlenk and Lavado, 2011).

When faced with low salinity conditions, estuarine organisms are constantly at the risk of disruption of hydromineral balance via the combined actions of ion loss and water uptake across permeable surfaces. In order to cope with these challenges, estuarine fishes must be able to switch their ion regulatory mechanisms to actively absorb ions across the gill and gut epithelium and excrete dilute urine (Edwards and Marshall, 2013; Marshall, 2013). However, these abiotic extremes combined with exposure to higher concentrations or more complex mixtures of chemical pollutants may overwhelm the physiological capacity of some estuarine species to respond to changing salinity conditions, ultimately resulting in reduced fitness and population declines (DeLorenzo, 2015; Schlenk and Lavado, 2011).

Here, we examined the interacting effects of acute exposure to waterborne 4-NP or E2 on the osmoregulatory ability of the estuarine arrow goby during a short term (24 hr) salinity challenge. As an estuarine species, arrow gobies experience fluctuating salinity conditions with tidal cycles and rainfall influx into their estuarine environments. While the osmoregulatory ability of the arrow goby has not been studied in detail, other intertidal marine fishes of the gobiid subfamily Gobionellinae (e.g., *Gillichthys mirabilis*)

are euryhaline and tolerant of large changes in environmental salinity (e.g., Evans and Somero, 2008; Larson and Madani 1991; Nagahama et al., 1973).

Our results showed, as expected, that whole body water content increased in arrow gobies that were unexposed to either 4-NP or E2 when transferred from 33 ppt to the reduced salinities of 20 ppt and 5 ppt. When the surrounding salinity drops rapidly, even euryhaline teleost fish will gain some water osmotically due to their blood osmolarity being higher than that of their environment (McCormick et al., 2013). Our data also suggest that 4-NP exposure induced changes in osmoregulatory balance in gobies maintained under stable seawater conditions. After 24 h, fish exposed to low 4-NP in both the 33 ppt and 20 ppt treatment groups experienced significantly higher total body water content than their respective control groups. However, in the 5 ppt treatment group after 24 h only the fish exposed to the high 4-NP treatment group expressed a significant increase in total body water content compared to the control group. The finding that the two different 4-NP groups elicited a significant change in whole body water content while the E2 treated group did not could be due to differential ligand-binding affinities for 4-NP and E2 and the three different estrogen receptors found in teleosts (Hawkins et al., 2000). The *esr2a* receptor (also referred to as the ER gamma) of teleost fishes exhibits divergent binding properties compared to the *esr1* and *esr2b* receptors (Hawkins and Thomas, 2004), and differences in 4-NP and E2 agonist action for ER receptor expression in the gill might contribute to these differential effects on whole body water content. Alternatively, 4-NP exposure has been documented to impact other tissues involved in osmoregulation in fishes such as the kidney (Bhattacharya et al., 2008; Sayed et al., 2011), and it is possible that the differential effects of E2 and 4-NP on whole body

water balance occurred via impacts on renal function or some other toxic effect not related to the estrogenic activity of 4-NP.

In the present study, we also examined how 4-NP or E2 exposure affected the relative abundance of gene transcripts encoding the Na^+/H^+ exchanger-3 (*nhe3*), $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (*nkcc1*), and the aquaporin-3 channel (*aqp3*) in the gill epithelium of arrow gobies as the fish experienced decreasing environmental salinity. Mechanisms of iono- and osmoregulation involving these ion and water transporters in the gill have been well studied in other euryhaline teleost fishes including the estuarine mummichog (*Fundulus heteroclitus*) under scenarios where fish are transitioned from sea water (SW) to freshwater (FW), as well as from FW to SW conditions (Marshall, 2002, 2013; Hirose et al., 2003; Perry et al., 2003; Evans et al., 2005; Hwang and Lee, 2007). In SW adapted fish, salt secretion is mediated by Na^+/K^+ -ATPase activity (NKA), *Nkcc1*, and the cystic fibrosis transmembrane conductance regulator (Cftr) Cl^- channel (Silva et al., 1977; Marshall, 1995; Evans et al., 2005). MR cells in the gills of euryhaline fishes can sense osmolality changes as small as 5 mOsm kg^{-1} , and the slight swelling of ionocytes can stop NaCl secretion (Marshall et al., 2000). This effect is hypothesized to be nearly instantaneous, mediated by increased transcription and protein expression of *Nkcc1* in the basolateral membrane and Cftr in the apical membrane (Marshall et al., 2005b, 2008). *Nkcc1*, is generally found co-localized with NKA and is most likely to be involved in salt secretion, as its mRNA and protein have been reported to be upregulated in the gills of many different teleosts after transfer from FW to SW conditions (Inokuchi et al., 2008; Tipsmark et al., 2002; Hwang and Lee, 2007). Immunohistochemical staining in the gills of sea bass (*Dicentrarchus labrax*) revealed that *Nkcc* protein

localized to the basolateral membrane of secretory MR cells in SW adapted fish, but to the apical regions of MR cells when the fish were in FW and the gills were in an absorptive state (Lorin-Nebel et al., 2006). However, in the pearl spot cichlid, *Etroplus suratensis*, results from a study wherein fish were adapted to FW conditions revealed an almost complete absence of Nkcc protein in the gills (Evans and Somero, 2008). That absence of Nkcc expression supports an alternative model for osmoregulation in freshwater proposed by Evans et al. (1999), who advocated that there is very low abundance of the Nkcc protein in FW acclimated gills.

In our current data with the arrow goby, we did not detect any significant changes in gill *nkcc1* mRNA levels in fish transferred to reduced salinities. While this finding is contradictory to one prior experiment that indicated a decrease in salinity yielded a corresponding decrease in gill *nkcc1* mRNA levels (Lorin-Nebel et al., 2006), it is largely in agreement with several other studies that observed an increase in gill *nkcc1* mRNA levels following transfer of fish to elevated environmental salinities, but no change following transfer to lower salinities (e.g., Cutler and Cramb, 2002; Inokuchi et al., 2008; Scott et al., 2004). A partial explanation for why some studies have observed changes in gill *nkcc1* gene transcript abundance after transfer to reduced salinities but others have not may be related to the time scale of the salinity challenge. Our data revealed that as salinity was reduced relative *nkcc1* mRNA levels remained fairly constant for our control population after both 6 hrs and 24 hrs in their respective salinities. However, this was a short term (24 hr) experiment, and the appearance of FW type chloride cells requires a functional shift of the gills from secretory to absorptive epithelium which can only be achieved after long-term exposure to freshwater (Lorin-Nebel et al., 2006). The 24 hr

period of our salinity challenge was therefore not likely to have resulted in a full shift in ionoregulatory cell types within the gill epithelium, but instead only would detect the initial, short-term transcriptional responses to hyposalinity challenge.

Exposure to low salinities did, however, alter the relative gene transcript abundance for *nhe3* and *aqp3* in the gills of adult arrow gobies. Specifically, we observed that unexposed, control gobies transferred to lower salinity showed reduced gill *nhe3* and *aqp3* mRNA levels at the 6 hr sampling time, but not at the 24 hr sampling time. This result opposes the findings of previous studies which generally have observed an upregulation of *nhe3* mRNA levels in the gill of fish transferred to lower salinity environments (Inokuchi et al., 2008; Watanabe et al., 2008; Choe et al., 2005; Breves et al., 2011). Our results also indicated that the treatment groups at both 33 ppt and 20 ppt that were dosed with either E2 or high 4-NP showed significant reductions in relative *nhe3* mRNA levels in the gill compared to the unexposed, control fish.

In zebrafish and tilapia (*Oreochromis* spp.), Nhe3 located in the apical membrane of gill MR cells has been suggested to be the main transporter for Na^+ uptake and H^+ secretion in low salinity environments (Yan et al., 2007; Watanabe et al., 2008). However, there is a thermodynamic argument that a Na^+/H^+ exchanger could not properly function in a dilute environment with low intracellular Na^+ (Parks et al., 2008), and that a vacuolar-type proton ATPase (V-type ATPase) that is electrically coupled to an apically-located Na^+ channel, allows Na^+ to be exchanged for H^+ secreted by the V-ATPase may also be involved (Reid et al, 2003; Lin et al., 2006; Esaki et al., 2007). And yet, other research has shown that an apically located Na^+/Cl^- cotransporter (NCC) may be the main pathway for ion absorption in FW adapted tilapia (Hiroi et al, 2008; Velan et al., 2011).

In the current study, however, we did not quantify the abundance of mRNAs encoding V-type ATPase or NCC in the gills of arrow goby, although such data might inform mechanisms of Na⁺ uptake in the arrow goby gill. It is also important to point out that while the transfer to lower salinities decreased *nhe3* antiporter mRNA levels in the gill of the arrow goby which appears contrary to findings in other fishes, this opposing result could also be indicative of taxonomic variation in ionoregulatory mechanisms. The cellular mechanisms of ion transport in the gill have been studied in detail in only a few species of euryhaline fishes (e.g., mummichog, tilapia, sheepshead minnow, salmonids), and the arrow goby is phylogenetically distant from those better studied species.

When euryhaline fish are placed in freshwater their gill cells tend to swell because they are gaining water osmotically due to their blood osmolarity being higher than that of the surrounding environment (Avella et al., 2009). Water is able to flow down its concentration gradient across cell membranes through aquaporin water channels located in the plasma membrane of ionocytes (Agre et al., 2002; Zeuthen, 2010). This process of diffusion is slowed by having the aquaporin channels confined to only the basolateral side of the membrane and by having skin and gill epithelia with low osmotic permeability (Kirschner, 1997). Claudins, a family of structural proteins, provide tight intercellular junctions aiding in additional low osmotic and ion permeability via paracellular pathways (Bagherie-Lachidan et al., 2008, 2009).

Following exposure to reduced environmental salinity, the blood plasma will be diluted if fish are not able to quickly regulate the net flux of ion loss and water gain. Some cells have been found to accrue organic osmolytes in an attempt to equalize intra- and extracellular tonicity and prevent the passive movement of water out of the cell

(Yancey, 2005). The expression of genes encoding several osmolyte- producing enzymes were shown to increase during hyperosmotic stress in the longjaw mudsucker, *Gillichthys mirabilis*, suggesting that cell volume regulation through the accumulation of organic osmolytes is central to a hyperosmotic stress response (Evans and Somero, 2008). While the gene expression changes that enable this equalization occur more than 24 hrs after salinity transfer, the expression of core transporters such as Aqp3, Cftr, and subunits of NKA are upregulated more rapidly (McCormick et al., 2013). For instance, in the mummichog, mRNAs encoding *aqp3* were observed to increase in abundance within 6 hrs of beginning a low salinity challenge (Jung et al., 2012).

Our results here with the arrow goby, however, also revealed that even before changing salinities, E2-treated gobies had gill *aqp3* transcript levels that were approximately 1.7 fold higher than that of unexposed, control fish. After 6 hrs of exposure to the different salinity treatments, both the high and low 4-NP as well as the E2 treated groups showed significant reductions in gill *aqp3* mRNA levels at both 33 ppt and 20 ppt. Taken together with the observation that these 4-NP exposed gobies also exhibited altered *nhe3* mRNA levels in the gill, we interpret these data to suggest the possibility that 4-NP may disrupt Na^+ and water transport in the gill.

Supporting that idea, juvenile, freshwater Atlantic salmon treated with 4-NP show reduced plasma Na^+ concentrations, as well as lower plasma Cl^- concentrations following transfer to seawater (Lerner et al., 2007a). Exposure of Atlantic salmon to E2 and 4-NP has also previously been shown to compromise normal physiological changes by reducing growth hormone receptor (GHR) abundance. Smolt development in salmon is influenced by the endocrine axis of growth hormone (GH) and insulin-like growth factor

I (IGF-I) (Young et al., 1989; Sakamoto et al., 1993; McCormick et al., 2000). GH and IGF-I levels should rise during smolting (McCormick, 2001; Lerner et al., 2012) to aid in increased salinity tolerance (Komourdjian et al., 1976; Bolton et al., 1987; Madsen, 1990). However, production of circulating IGF-I is largely due to GH binding to hepatic GHR (Duan, 1998), which E2 and 4-NP are capable of negatively affecting, and therefore ultimately affecting Atlantic salmon smolting capabilities (Lerner et al., 2012). This phenomenon of decreased liver GHR after E2 injection has been observed in Mozambique tilapia (Davis et al., 2008), and has also been linked to a decrease in ionocytes abilities to secrete ions and an increased capacity for ion-uptake (Lerner et al., 2012). However, our results again differ from this theory as they do not indicate that E2 is an endogenous regulator of hyperosmoregulatory abilities but rather indicate a reduced ability to ion regulate. Lerner et al. (2012), also observed an increase in plasma GH for SW acclimated fish after administration of E2 or 4-NP and a reduction in plasma levels of GH in fish acclimated to FW. These differences observed in E2 among the different salinities were attributed to GH's sensitivity to negative feedback by IGF-I being altered by varied environmental salinities (Lerner et al., 2012).

Beyond direct effects of 4-NP and E2 on gill *nhe3* and *aqp3* mRNA levels, it is also important to consider the potential impact of netting stress, and hence cortisol secretion, on the transcript levels of these genes. Some of the differences observed between our baseline (0 hr, 33ppt) and 6 hr (33 ppt) groups may be attributable to E2 and 4-NP modifying how cortisol regulates the transcription of these genes. In teleost fish, cortisol has a dual role as a glucocorticoid regulating metabolism and growth and as a mineralocorticoid regulating hydromineral balance (Eddy, 1981; McCormick et al.,

2008). Cortisol typically increases gill ion uptake in FW acclimated fish (Perry and Bernier, 1999). A normal physiological response to an acute stressor such as netting therefore would be to enhance net ion outflow from the gill when in FW, but increase net ion influx when in SW (Lerner et al., 2007). This response has been attributed to increased catecholamine levels which can affect the vascular resistance of the gills and cause an increase in ion diffusion rates (Eddy, 1981). It is therefore possible that 4-NP and E2 exposure may interact with effects of cortisol on gill ionoregulation caused by netting and tank transfer stress.

CONCLUSIONS

While the fitness effects of 4-NP exposure are challenging to demonstrate for wild fishes, Atlantic salmon exposed to 4-NP have been found to experience a significant inhibition of gill NKA activity and chloride cell development (Madsen et al., 1997), reduced migratory drive (Bangsgaard et al., 2006; Madsen et al., 2004) and in some more extreme cases a major population decline (Fairchild et al., 1999). Our results in the arrow goby provide additional data suggesting that changes in ion and water transport dynamics across the gill may be a contributing factor to fitness impacts caused by 4-NP exposure. With salinity dynamics expected to shift in coastal estuaries under changing global climate conditions, understanding the impacts of environmental chemicals on iono- and osmoregulation will be increasingly important for identifying estuarine species vulnerable to the combined stressors of rapid salinity changes and pollutants (DeLorenzo, 2015; Noyes et al., 2009; Schlenk and Leveno, 2011).

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Appendix A: Chapter 1

Table 1. Degenerate primers used for amplification and sequencing of partial cDNAs associated with reproduction from the arrow goby, *Clevelandia ios*.

Transcript	Primer	Nucleotide sequence (5' to 3')
Choriogenin L (<i>chgL</i>)	ChgL_for1d	GCTGTYGARTGYAGAGAGAGYA
	ChgL_rev1d	GTGCGWGCCAKGAACTTTGAG
	ChgL_for2d	CGTGGARGTCAAGAARGACATG
	ChgL_rev2d	GAGCCTGTGATYCTGGCRTC
	ChgL_for3d	CACAATGTGAGCAGYCTTCC
Choriogenin H-minor (<i>chgH-minor</i>)	CgHm_for1d	GGYACTWCTRTTGAGGCTTTGGT
	CgHm_rev1d	GCAHCTKGGYTCACAGYTRT
	CgHm_for2d	TGAGGTTGGTKRSTTCCTCC
	CgHm_rev2d	AAACAAAWCGCCTGTAGTGGGTTGG
	CgHm_for3d	TGCAGCTCCTGGACCTCT
Vitellogenin A (<i>vtgA</i>)	VTGa_for1d	ACCCTCTATGCCATCRCYGAAGA
	VTGa_rev1d	TTCCCTCAKGTCCACRCTGTT
	VTGa_for2d	TTCTGCGTGAGATCRTCCTTCTTG
	VTGa_rev2d	CCATCRAAGGGGGTGAGACCTT
	VTGa_for3d	ACTGCTGCTGGTCTTCCAATGG
	VTGa_rev3d	CAGCCTTTGGTCCAACTWKAACCTC
Vitellogenin C (<i>vtgC</i>)	VTGc_for1d	GCCTGAACCCCHAAGAAARCCTACGA
	VTGc_rev1d	TCCTCAGAGTSTCAAGSAGTTGG
	VTGc_for2d	GCAAGTGTGAGAGYAACTATGCTA
	VTGc_rev2d	TTTBTCACAGCTTTSATTGYCTGG
	VTGc_for3d	CTATGGYTCTCTGGTRTACAAGCACTG
	VTGc_rev3d	CCAAAGCTGGCTCYTCAATGTT
	VTGc_for4d	GGCAGCATTAACCATCATGCGTTC
Estrogen Receptor 1 (<i>esr1</i>)	gobyEsr1-for1d	GGTCACAATGACTATATGTGCCAGC
	gobyEsr1-rev1d	GGTGAGCGTCCAGCATCTC
	gobyEsr1-for2d	GACAGGAATCGGAGGAAGAGCTG
	gobyEsr1-rev2d	TCCATGCCTTTGTTGCTCATGTG
	gobyEsr1-for3d	CGTCTTAGGAAGTGTTACGAAGT
	gobyEsr1-rev3d	TCCATTGTGCCRGTCAGAAAGA
Estrogen Receptor 2a (<i>esr2a</i>)	gobyEsr2a-for1d	CTTCAAGAGGAGCATCCAAGGAC
	gobyEsr2a-rev1d	CAGTGGAGGTGGTCCATGCC
	gobyEsr2a-for2d	GACTACATCTGCCC MGCMACCAATC
	gobyEsr2a-rev2d	ATGTGVGASAGCAGCATGAGCA
	gobyEsr2a-for3d	GACAAGAA YCGCCGTAARAGCTG
	gobyEsr2a-rev3d	CCCACACCAGAGCGTCCGT
Brain Aromatase (<i>cyp19a1b</i>)	bArom-for 1d	GCAGTGTACCACGYTTGAGGAG
	bArom-rev1d	GGGYTGTCAAAGTTTGTCAGGC
	bArom-for 2d	CTACACATCCAGATTTGGSAGCAAA
	bArom-rev2d	TCSGYTTGTGCATGAGTCCAATG
	bArom-for 3d	GATGGAGGGGAGGGGTATCAT
	bArom-rev3d	GAAGAAGAGGCTGATGRAGAGYGT
Ribosomal protein L8 (<i>rpl8</i>)	L8_for1d	ACCGCTTCAAGAAGAGGACMGAG
	L8_rev1d	TGATGGTTACCACCACCGAAGG
	L8_for2d	CAGTTCATCTACTGCGGCAGAA
	L8_rev2d	CCTTCAGGATGGGYTTGTCAATAC

Table 2. Gene-specific primers, for pathways associated with reproductive physiology, for SYBR green quantitative PCR in arrow goby.

Transcript	Primer	Nucleotide Sequence (5' to 3')	Amplicon length (bp)	% efficiency (avg.)
<i>chgL</i>	for	CTCCTATGTCCTGAACTACAATCC	98 bp	98.15%
	rev	GCTCACATTCTGCCTCCTT		
<i>chgH-minor</i>	for	ACGTGGCGTATAACTCCTACTA	112 bp	101.26%
	rev	CAGCATTCTCACCTCCACAA		
<i>vtgA</i>	for	TTGACTACCCACAGCACATC	106 bp	100.24%
	rev	CCTCAGCCATGACAATCTCTAA		
<i>vtgC</i>	for	CCTGGCACAAGCAAATACAC	101 bp	99.11%
	rev	CCATGTCCCTCCACATCTTT		
<i>esr1</i>	for	CATCCTAGACAGAGAGGAAGGA	97 bp	98.74%
	rev	CACCGGAGTTGAGCAAGATAA		
<i>esr2a</i>	for	GCACAGGAGGAAAGCTGTTA	115 bp	101.90%
	rev	CGTCCATGATCCGACCAATC		
<i>cyp19a1b</i>	for	CACGTCGATGCCCTCTATTT	108 bp	103.14%
	rev	ACAGCTTGCCAGGTTTCA		
<i>rpl8</i>	for	TGTACTGCCTGTAGGTGTAATG	141 bp	103.18%
	rev	TGTGGCATAGTTCCTGAAG

Figure 1. Experimental design illustrating the three week pre-exposure acclimation period, 4-NP or E2 exposure period with sampling time points at 0 h (baseline), 24 h, 72 h, 12 d and 20 d, and depuration period with sampling at 72 h, 12 d and 20 d.

LIVER

Figure 2. Relative mRNA expression of choriogenin mRNAs encoding *chgL* and *chgHm* and vitellogenins *vtgA* and *vtgC* in the liver of male arrow gobies exposed to either ethanol vehicle control, 4-NP at 10 µg/L, or 4-NP at 100 µg/L for a 20 day period followed by a 20-day depuration. Statistical significance evaluated first with two-way ANOVA models and then *post hoc* Dunnett's tests within each sampling day (* denotes $p < 0.05$) *Inset graphs:* Relative mRNA expression of *chgL*, *chgHm*, *vtgA* and *vtgC* in the liver of male arrow gobies exposed to 17β-estradiol [E2] at 50 ng/L (positive control). Statistical significance evaluated with one-way ANOVA and then *post hoc* Tukey's tests across sampling days. Prime indicators on x-axis represent time after initial depuration (change to clean water). Error bars represent SEM. ($n = 6-11$ fish per treatment and sampling time).

Figure 3. Phylogenetic analysis of estrogen receptor (*esr*) genes in select fishes and tetrapods. Estrogen receptors group into clades defined as *esr1* or *esr2*, with bony fishes having evolved two *esr2* receptors termed *esr2a* and *esr2b*. Deduced amino acid sequences were aligned using Clustal X, and the phylogeny was constructed using a Neighbor-Joining Method with pairwise deletion of gaps using MEGA (v.5.1) software. Bootstrap values on each node indicate 1000 replicates. The two new *esr* partial cDNA identified from arrow goby are indicated by boxes. GenBank accession numbers for each gene are provided in parentheses.

LIVER

A) *esr1*

B) *esr2a*

Figure 4. Relative mRNA expression of estrogen receptors *esr1* and *esr2a* in livers of adult male arrow gobies exposed to either ethanol vehicle control, 4-NP at 10 µg/L (low 4-NP dose), or 4-NP at 100 µg/L (high 4-NP dose) for a 20 day period followed by a 20-day depuration. Statistical significance evaluated first with two-way ANOVA models and then *post hoc* Dunnett's tests within each sampling day (* $p < 0.05$). *Inset graphs:* Relative mRNA expression of *esr1* and *esr2a* in the liver of male arrow gobies exposed to 17β-estradiol [E2] at 50 ng/L (positive control). Statistical significance evaluated with one-way ANOVA and then *post hoc* Tukey's tests across sampling days. Prime indicators on x-axis represent time after initial depuration (change to clean water). Error bars represent SEM values. ($n=6-10$ fish per treatment and sampling day).

BRAIN

Figure 5. Relative mRNA expression of *cyp19a1b*, *esr1*, and *esr2a* in the brain of adult male arrow gobies exposed to either ethanol vehicle control, 4-NP at 5 µg/L, or 4-NP at 50 µg/L for a 20 day period followed by a 20-day depuration. Statistical significance evaluated first using two-way ANOVA models and then within each sampling day using Dunnett's tests (* $p < 0.05$). *Inset graphs:* Relative mRNA expression of *cyp19a1b*, *esr1* and *esr2a* in the liver of male arrow gobies exposed to 17β-estradiol [E₂] at 50 ng/L (positive control). Statistical significance evaluated with one-way ANOVA and then *post hoc* Tukey's tests across sampling days. Prime indicators on x-axis represent time after initial depuration (change to clean water). Error bars represent SEM values. ($n=6-10$ fish per treatment and sampling day).

Appendix B: Chapter 2

Table 3. Degenerate primers used for amplification and sequencing of partial cDNAs, important to osmoregulatory function, from the arrow goby, *Clevelandia ios*.

Transcript	Primer	Nucleotide sequence (5' to 3')
Na ⁺ /K ⁺ /2Cl ⁻ cotransporter-1 (<i>nkcc1</i>)	NKCC1_for1d	CCA RAT GTG AAC TGG GGY TCW TC
	NKCC1_for2d	CTT CAG RCC SCA GTG TYT GGT G
	NKCC1_for3d	GGT BCA TWC CTT CAC CAA GRA CG
	NKCC1_rev3d	GCT GCC TCC ARC TCC ATG TTG TC
	NKCC1_rev2d	TCC AGC TCR TTG TCW GTG ATY CTC
	NKCC1_rev1d	ATK ACG ATG AGG TTG GCT GTG
Na ⁺ /H ⁺ exchanger isoform 3 (<i>nhe3</i>)	NHE3-for1d	CAT GCC AAA CAA GCT CTT CTT CAS C
	NHE3-for2d	TCA TYG GGA CCT GCT GGA AC
	NHE3-for3d	CTG TCG CTG TGG GGG TGT CA
	NHE3-rev3d	AAC CTT CAT GGC GTA ACG GAC
	NHE3-rev2d	AAG ATG ATG GTT TCC GAG CCG TTG
	NHE3-rev1d	TGA GGA GGA TGA AGC CCG TGT TC
aquaporin 3 (<i>aqp3</i>)	Aqp3-for1d	AAC TGK CCC GCT TCT TYC AGA TCC
	Aqp3-for2d	TT GGC ACB CTC ATC CTT GTR ATG T
	Aqp3-for3d	TCA ACT TTG CCT TYG GCT TYG
	Aqp3-rev3d	CCT TGG GGR ATG GGG TTG TTG
	Aqp3-rev2d	GAC AAT CCA ATS ACC ARA ACC ACA
	Aqp3-rev1d	CGT GGT CCR ADG TCT CTK GC

Table 4. Gene-specific primers, for pathways associated with osmoregulatory function, for SYBR green quantitative PCR in the arrow goby.

Transcript	Primer	Nucleotide Sequence (5' to 3')	Amplicon length (bp)	% efficiency (avg.)
<i>nkcc1</i>	for	GGAGGTCTTACGTTGCTGATT	106 bp	100.97%
	rev	CATGGTCAATGCGGTTGATTT		
<i>nhe3</i>	for	GCTCTGGTCGGTGTGATTT	108 bp	110.18%
	rev	GGAGAGGTATCCAAGGACAAAC		
<i>aqp3</i>	for	GTTGGTGTTGAGTGGTGGTA	128 bp	107.52%
	rev	AGGTCACAGCAGGATTAAGATG		
<i>eflalpha</i>	for	CCAGGAGTGTGTTCCATTAG	86 bp	107.47%
	rev	GTTGGAGACCAGTGAGAAGATG

Figure 6. Whole body water content (% mass) in adult mixed-sex arrow gobies after 24 hrs exposed to: high dose 4-NP (100 ug/L), low dose 4-NP (10 ug/L), 17 β -estradiol (50 ng/L; positive control), or ethanol vehicle only in either 33 ppt, 20 ppt, or 5 ppt seawater. Statistical significance evaluated using two-way ANOVA models and then Dunnett's tests (* p < 0.05).

Figure 7. Relative mRNA gene expression levels for the ion transport proteins, $\text{Na}^+ \text{K}^+ \text{Cl}^-$ cotransporter 1 [*NKCC1*](A), sodium-hydrogen exchanger-3 [*nhe3*](B), and the water transport protein, aquaporin-3 [*aqp3*](C) in the gill of adult mixed-sex arrow gobies exposed in seawater to: high dose 4-NP (100 ug/L), low dose 4-NP (10 ug/L), 17β -estradiol (50 ng/L; positive control), or ethanol vehicle only. Fish were exposed for 12 days, and then fish ($n = 10$ per treatment) were collected from each treatment tank for baseline (0 hrs) samples in 33 ppt salinity. Statistical significance evaluated using two-way ANOVA models and then Dunnett's tests (* $p < 0.05$).

Figure 8. Relative mRNA gene expression levels for the ion transport proteins, $\text{Na}^+ \text{K}^+ \text{Cl}^-$ cotransporter 1 [*NKCC1*](A), sodium-hydrogen exchanger-3 [*nhe3*](B), and the water transport protein, aquaporin-3 [*aqp3*](C) in the gill of adult mixed-sex arrow gobies exposed in seawater to: high dose 4-NP (100 ug/L), low dose 4-NP (10 ug/L), 17β -estradiol (50 ng/L; positive control), or ethanol vehicle only. Fish were exposed for 12 days and then fish from each treatment group were transferred to tanks containing water of 33 ppt, 20 ppt, or 5 ppt salinity, and sampled after 6 hours ($n = 10$ fish per treatment and sampling time). Statistical significance evaluated using two-way ANOVA models and then Dunnett's tests ($*p < 0.05$).

Figure 9. Relative mRNA gene expression levels for the ion transport proteins, $\text{Na}^+ \text{K}^+ \text{Cl}^-$ cotransporter 1 [*NKCC1*](A), sodium-hydrogen exchanger-3 [*nhe3*](B), and the water transport protein, aquaporin-3 [*aqp3*](C) in the gill of adult mixed-sex arrow gobies exposed in seawater to: high dose 4-NP (100 ug/L), low dose 4-NP (10 ug/L), 17 β -estradiol (50 ng/L; positive control), or ethanol vehicle only. Fish were exposed for 12 days and then fish from each treatment group were transferred to tanks containing water of 33 ppt, 20 ppt, or 5 ppt salinity, and sampled after 24 hours ($n = 10$ fish per treatment and sampling time). Statistical significance evaluated using two-way ANOVA models and then Dunnett's tests (* $p < 0.05$).