Temporal Patterns of Induction and Recovery of Biomarker Transcriptional Responses to 4-Nonylphenol and 17β-Estradiol in the Estuarine Arrow Goby, *Clevelandia Ios*

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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-disrupting 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 in estuarine mud flats on the west coast of North America. Male gobies were exposed to waterborne 4-NP at 10 μg/L or 100 μg/L for 20 days followed by a 20 day depuration period. Additional males were treated with 17β-estradiol (E2; 50 ng/L). 4-NP at 100 μg/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 the pattern by E2, while *chgHm* and *esr1* mRNA induction by 4-NP lagged 2–11 days behind the responses of these transcripts to E2. These findings establish 4-NP concentration- and time-dependent induction patterns of choriogenin and vitellogenin transcription following exposure to environmentally relevant 4-NP concentrations, while concurrently demonstrating tissue-specific induction patterns for *esr1* by estrogenic compounds. Environ Toxicol 32: 1513–1529, 2017.

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

1. INTRODUCTION

Biologically relevant end points including molecular, cellular, and physiological responses are commonly used 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 be 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 responses generally recover more quickly after the cessation of chemical exposure than do population- or community-level indicators and therefore enable better early detection of changes in environmental

Additional Supporting Information may be found in the online version of this article.

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stressors (Stegeman et al., 2012; Wu et al., 2005). For that reason, molecular responses such as changes in patterns of gene expression have been suggested to be particularly effective biomarkers (Flouriot et al., 1996). However, in order to use 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 responses caused by exposure to chemical contaminants, but also the temporal scale of biomarker recovery after cessation of chemical exposure (Wu et al., 2005).

Relatively few studies have examined the induction and recovery dynamics of biomarker responses in nonmodel taxa even though knowledge of those dynamics is crucial for effective biomarker use for monitoring habitats where variation in contaminant input can be pulsatile. Estuary ecosystems, for instance, can receive intermittent inputs of synthetic and naturally occurring chemicals from residential, industrial, and agricultural activities on land in accordance with periodic rainfall events (e.g., Kennish, 1997; DeLorenzo, 2015). Chemical contamination of estuaries has been documented in most coastal areas of the world (Islam and Tanaka, 2004; Weis, 2014), and while sewage waste remains the most common source of coastal marine pollution, contaminants rountinely 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 the chemicals accumulate within the tissues of estuarine organisms (McCain et al., 1988; Weis, 2014).

Many of the chemical contaminants that have been documented in estuaries exhibit endocrine-active properties and are classified as endocrine-disrupting chemicals (EDCs) given their potential to interfere with endogenous hormone signaling pathways (Tyler et al., 1998; Oberdörster and Cheek, 2001; Porte et al., 2006; 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 by disrupting pathways underlying the synthesis, secretion, transport, metabolism, or excretion of hormones (Crisp et al., 1998; Khetan, 2014). Endocrine signaling pathways regulate physiological homeostasis, growth, reproductive function, and behavior, and there is extensive evidence linking exposure to EDCs to a range of developmental, metabolic, reproductive, and behavioral impacts in marine organisms (Matthiessen, 2003; Porte et al., 2006; Vandenberg et al., 2012; Weis, 2014).

The alkylphenol 4-nonylphenol (4-NP), for instance, is a well-established endocrine-disrupting chemicals (EDC) that has recently been detected at elevated concentrations in coastal estuaries along the Pacific Ocean coastline of California, USA (Diehl et al., 2012; Maruya et al., 2015). Marine invertebrates including mussels (*Mytilus californianus*), oysters (*Crassostrea gigas*), and ghost shrimp (*Neotrypaea californiensis*)—as well as demersal fishes including arrow gobies (*Clevelandia ios*) and staghorn sculpins (*Leptocottus armatus*)—from several of California's estuaries exhibited some of the highest tissue burdens of 4-NP documented worldwide (Diehl et al., 2012). Maruya and coworkers (2015) likewise detected 4-NP in the range of 300–700 ng/g wet mass in *Mytilus* mussels and from 700 to 900 ng/g dry mass in sediments from several coastal habitats in California. Although the sources of this coastal 4-NP pollution are not known, Diehl and coworkers (2012) found high 4-NP concentrations in sludge from septic systems in a nearby coastal community, as well as in river water (1.0 ± 0.3 μg L^{-1}; mean ± 1SE) and sediment (1.0 ± 0.3 mg kg^{-1} dry weight; mean ± 1SE) located 100 m downstream of a wastewater treatment facility near Morro Bay, California, USA. Those data suggest that effluent from local treatment facilities or septic tank discharge may be a source of 4-NP for coastal marine habitats. A separate 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). Additionally, 4-NP has been detected in application water and runoff from agricultural fields in California irrigated with reclaimed wastewater (Xu et al., 2009). Farming land use may therefore also contribute to 4-NP contamination in California's coastal waters and estuaries.

Estuaries are vital ecosystems that serve as nursery habitat or breeding grounds to many commercially important species (Kennish, 2002). The presence of 4-NP contamination in estuaries and nearby coastal areas is therefore of high concern given evidence for 4-NP's estrogenic activity and acute toxicity (Soto et al., 1991; White et al., 1994; Servos, 1999; Cravedi and Zalko, 2005). 4-NP has been shown to bind nuclear estrogen receptors (ERs) (White et al., 1994; Routledge and Sumpter, 1996; Tabira et al., 1999; Laws et al., 2000; Preuss et al., 2006), although the affinity of 4-NP for nuclear ERα (*esr1*) is orders of magnitude less than the affinity of 17β-estradiol (E2), the principle estrogen in blood circulation in most fishes (Kwack et al., 2002; Bonefeld-Jorgensen et al., 2007). 4-NP has also been demonstrated to trigger nongenomic estrogenic effects via its binding to membrane ERs (mERs) (Loomis and Thomas, 2000; Thomas and Dong, 2006; Kochukov et al., 2009). Previous studies show that 4-NP sequesters in the liver, brain, gills, gonads, and tissues of fish (Ahel et al., 1994; Lewis and Lech, 1996; Coldham et al., 1998) and can cause negative consequences for reproduction and development (Colborn et al., 1993; Jobling et al., 1996; Christiansen et al., 1998;

Tabata et al., 2001; Chandrasekar et al., 2011). Male fish exposed to 4-NP, for instance, have been found to exhibit a suite of defects including altered testicular structure, decreased sperm counts, intersex gonads, disrupted reproductive cycles, liver damage, and reduced growth (e.g., Christiansen et al., 1998; Servos, 1999; Tanaka and Grizzle, 2002; Kaptaner and Ünal, 2011; El-Sayed Ali et al., 2014; Traversi et al., 2014). And, even though 4-NP is approximately 1500 times less potent than E2 (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., Soto et al., 1991; White et al., 1994; Arukwe et al., 2001, 2002; Ackermann et al., 2002; Genovese et al., 2011; Brander et al., 2012).

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*). The arrow goby is a small teleost fish that is a common benthic inhabitant of Pacific coast bays and estuaries in North America from British Columbia, Canada to Baja, Mexico (Eschmeyer et al., 1983). Arrow gobies occupy intertidal mud flat habitats and forage over the benthos during high tide. At low tide, these fish 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* (Hoffman, 1981). The arrow goby is thus regularly in physical contact with estuarine sediments rich in organic material where 4-NP can accumulate and persist for decades (Garrison and Hill, 1972; Blackburn and Waldock, 1995; Ying et al., 2002; Lavado et al., 2009; Sharma et al., 2009). Arrow gobies collected from Morro Bay, California, by Diehl and coworkers (2012) were found to have high 4-NP tissue concentrations (mean: 237 ng g^{-1} wet mass) above those of the surrounding sediment in this estuary (mean: 53 ng g^{-1} dry mass). However, it is still unclear whether arrow gobies in these habitats are experiencing the 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 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 concentrations of 10 μg/L (low dose) and 100 μg/L (high dose) for 20 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 20 days of exposure to 4-NP or E2, gobies were transferred to clean water for a 20 day depuration period to characterize the time course of transcriptional recovery. At several time points throughout the exposure and depuration periods, we quantified changes in the relative transcript abundance of genes encoding the choriogenins *chgL* and *chgHm*, vitellogenins *vtgA* and *vtgC*, nuclear estrogen receptors (ERs) *esr1* and *esr2a*, and the steriodogenic enzyme *cyp19a1b*. These choriogenin and vitellogenin genes are not normally expressed at high levels in the liver of juvenile or male fish, but display inducible expression by estrogens and a variety of xenoestrogens (Jobling and Sumpter, 1993; Hylland and Haux, 1997; Jones et al., 2000; Denslow et al., 2001; Hemmer et al., 2001; Lee et al., 2002; Lange et al., 2012; Yamaguchi et al., 2015; Hara et al., 2016). Since the expression of all three *vtg* genes is strongly upregulated by estrogens (Flouriot et al., 1997), changes in liver *vtg* transcript abundance and Vtg protein concentration in blood circulation are consider to be among the most reliable and tractable biomarkers for xenoestrogen exposure identified to date (Arukwe and Goksøyr, 2003; García-Reyero et al., 2004; Hutchinson et al., 2006). The estrogen receptor genes *esr1* and *esr2a* encode two of three nuclear ERs present in Actinopterygiian fishes (Hawkins et al., 2000). Gene transcription for these ERs can be autoinduced by estrogen exposure (Yadetie et al., 1999; Arukwe et al., 2001). Cytochrome P450 aromatase (Cyp19a1) enzymes convert testosterone to E2, and gene transcription for the brain isoform (*cyp19a1b*) can be upregulated by exposure to compounds with estrogenic properties (Kishida and Callard, 2001; Menuet et al., 2004). Quantification of these gene transcripts under an experimental design of exposure-depuration was selected to impart a detailed picture of the temporal dynamics for induction and recovery of hepatic and brain mRNA biomarkers of 4-NP or E2 exposure.

2. MATERIALS AND METHODS

2.1. Sequencing of Partial cDNAs Encoding Estrogen-Responsive Genes

2.1.1. 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 methansulfonate (MS222, Argent Laboratories, Redman, WA, USA), and the brain and liver tissues were dissected, frozen in liquid N_2, 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 DNAfree Kit (Ambion, Grand Island, NY, USA).

2.1.2. Degenerate Primer PCR and cDNA Sequencing

Total RNA was reverse transcribed, and PCR was then 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 supporting information 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 for *vtgA* were designed from *Thunnus thynnus* (FJ743688), *Pargus major* (AB181838), and *Labrus mixtus* (FJ456934), and for *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 *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 using total RNA extracted from both liver and brain in 20 μL reactions containing 4.75 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, 100 mM, Promega Corp.), 0.25 μL of recombinant RNasin® ribonuclease inhibitor (20 U μL^{-1}, Promega Corp.), 5.5 μL of nuclease-free H_2O, and 4.0 μL of 5× buffer, 3.0 μL of $MgCl_2$ (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 H_2O, 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 (Molecular Cloning Laboratories, 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 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* (1100 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.

2.2. NP Exposure and Recovery Experiment

2.2.1. Animals

Adult arrow gobies (*C. ios*) were collected at low tide on 21 August 2013 and 25 August 2013 from the burrows of ghost shrimp (*N. californiensis*) or polychaete worms within the mud flats of Morro Bay estuary, CA, USA, using slurp guns. Fish were transported back to California Polytechnic State University's Center for Coastal Marine Sciences facility in Avila Beach, CA. The gobies 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).

2.2.2. Experimental Design

Arrow gobies (standard length: 37.93 ± 0.27 mm; body mass: 0.54 ± 0.01 g; mean ± SEM) were divided haphazardly into mixed-sex groups of 25 fish that were each transferred to closed-system, 19 L glass aquaria assigned to one of the following treatments: 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) or control (0.0001% ethanol vehicle only). An additional set of fish were exposed to 17β-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 $C_{15}H_{24}O$ isomers, purity

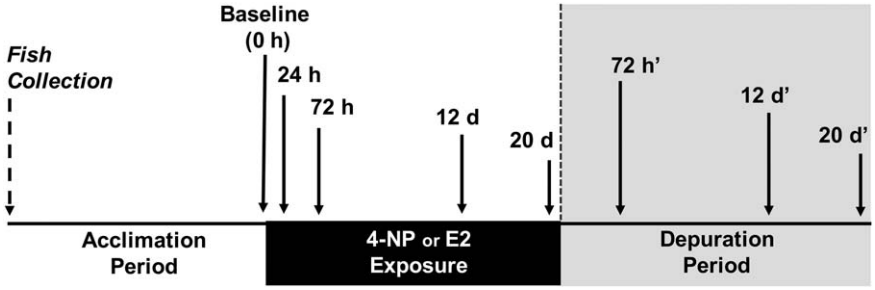


Fig. 1. Experimental design illustrating the 3 week pre-exposure acclimation period, 4-NP or E2 exposure period with sampling time points at 0 h (baseline), 24 h, 72 h, 12 d, and 20 d, and depuration period with sampling at 72 h, 12 d, and 20 d.

>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 (approximately 1135 L) to maintain all treatment tanks at ambient ocean temperatures (15.1 ± 1.6°C; mean ± 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 h 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 exposures. 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 N_2 and subsequently stored at −80°C. The gonad tissues were also dissected for visual confirmation of gonadal sex.

Immediately after the collection of the "baseline" (Day 0) fish samples was complete, 4-NP, E2 (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 h (Day 1), 72 h (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 h (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.

2.2.3. Quantification of Estrogen-Responsive mRNAs 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 DNAfree 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 $MgCl_2$, 0.9 μL deoxyribonucleotide triphosphates (dNTPs; 10 mM), and 0.9 μL random hexamer primer (Promega, Madison, WI), 0.225 μL recombinant RNasin RNase inhibitor (Promega), 0.675 μL GoScript reverse transcriptase enzyme (Promega), and 9.0 μL of total RNA template (25 ng/μL) 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 supporting information. Quantitative RT-PCRs (16 μL) contained 8.0 μL SYBR Green Master Mix (Life Technologies, Grand Island, NY, USA), 1.0 μL each of forward and reverse primer (10 μM), 4.5 μL nuclease-free water, and 1.5 μL of reverse-transcribed cDNA template. The PCR thermal profile for each reaction was 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. A dissociation stage consisting of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s 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 with each standard assayed in triplicate. Correlation coefficients (r^2) of the standard curve for each gene 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

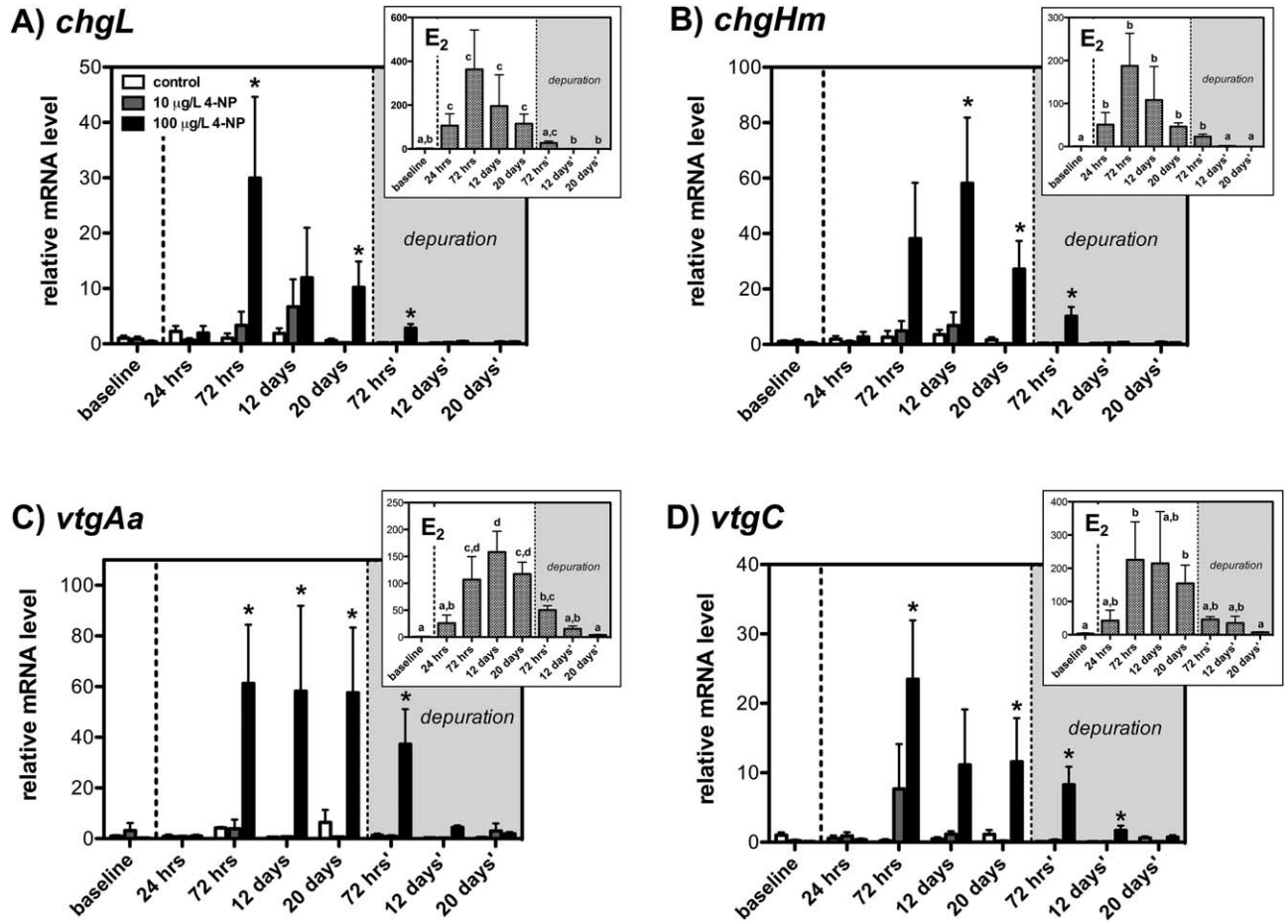


Fig. 2. Relative mRNA expression of choriogenin mRNAs encoding *chgL* and *chgHm* and vitellogenins *vtgAa* and *vtgC* in the liver of male arrow gobies exposed to either ethanol vehicle control, 4-NP at 10 μg/L, or 4-NP at 100 μg/L for a 20 day period followed by a 20 day depuration. Statistical significance was evaluated first with two-way ANOVA models and then with *post hoc* Dunnett's tests within each sampling day (* denotes $p < 0.05$) *Inset graphs*: Relative mRNA expression of *chgL*, *chgHm*, *vtgAa*, and *vtgC* in the liver of male arrow gobies exposed to 17β-estradiol [E2] at 50 ng/L (positive control). Statistical significance was evaluated with one-way ANOVA models and then with *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).

curve, normalized to *rpl8* mRNA abundance from that same tissue of the individual fish, and then expressed as a value relative to mRNA abundance of the "baseline" (Day 0) measurement of the vehicle control group.

2.3. Statistical Analyses

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 treatments and the control vehicle group were 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).

3. RESULTS

3.1. 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 remained elevated 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 days after commencing 4-NP exposure, and approximately 16-fold higher after 20 days 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* showed evidence of a more rapid induction in gene expression in some males beginning 72 h after commencing 4-NP exposure, but the large variation among individual fish 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 pretreatment 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 *vtgAa* were also significantly upregulated 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* 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 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 upregulating 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).

3.2. 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. 3A) (treatment * sampling effect, $F_{14,146} = 1.7944$, $p = 0.0444$). The increase in hepatic *esr1* mRNA levels caused by 4-NP was not statistically significant until day 12 of exposure, even though an increasing trend appeared apparent as soon as 24 h after commencing exposure. Treatment of fish with exogenous E2 likewise elevated liver *esr1* mRNA abundance. This effect of 4-NP was rapidly detectable at 24 h and peaked as a 75-fold elevation at 72 h, but

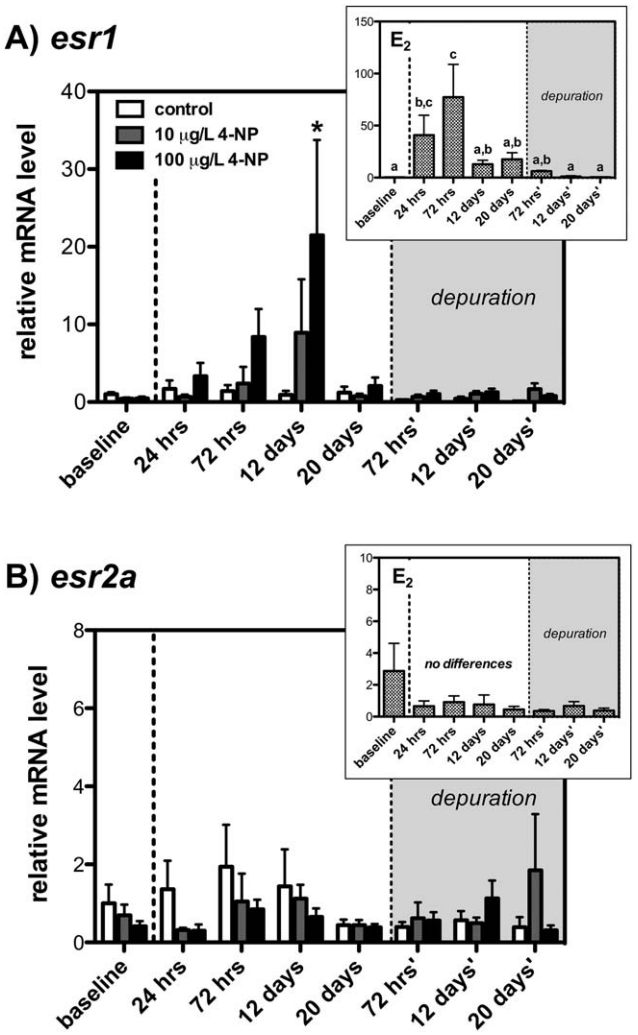
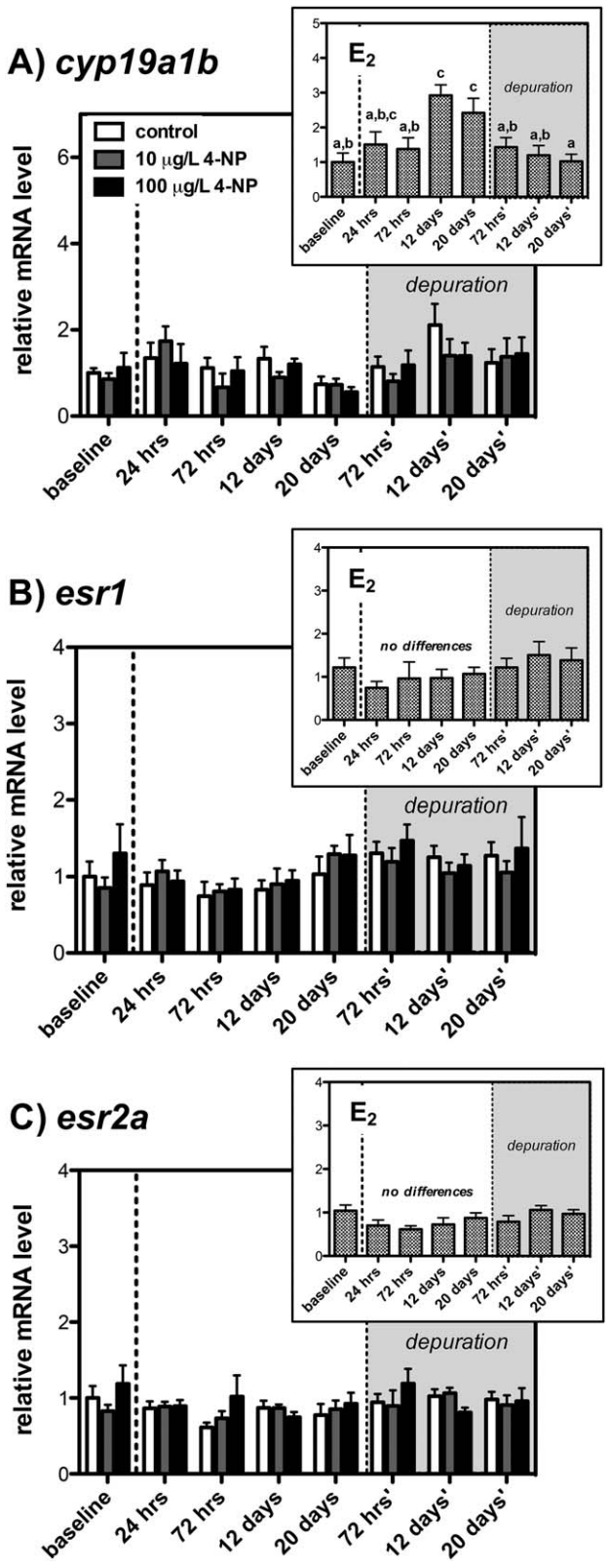


Fig. 3. Relative mRNA expression of estrogen receptors *esr1* and *esr2a* in livers of adult male arrow gobies exposed to either ethanol vehicle control, 4-NP at 10 μg/L (low 4-NP dose), or 4-NP at 100 μg/L (high 4-NP dose) for a 20 day period followed by a 20-day depuration. Statistical significance was 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 was evaluated with one-way ANOVA models 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).

then declined to a ~15-fold elevation over pre-exposure expression levels until terminating E2 exposure after 20 days (Fig. 3A). Hepatic transcript abundance for *esr2a* was not affected by 4-NP at either the high or low doses (Fig. 3B) (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.



3.3. Brain Aromatase and Estrogen Receptor mRNA Levels

In the brain, relative mRNA levels of *cyp19a1b* were observed to be elevated threefold at 12 days and ~2.5-fold at 20 days after commencing E2 treatment (Fig. 4A) ($F_{7,54} = 5.1060$, $p = 0.0002$). Transcript abundance for *cyp19a1b* declined back to baseline levels after 20 days of depuration. The 4-NP treatments (10 μg/L or 100 μg/L), however, did not have any detectable effects on brain *cyp19a1b* transcript abundance (Fig. 4A) (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 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.

4. DISCUSSION

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*) showed upregulation in the

Fig. 4. 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 10 μg/L, or 4-NP at 100 μg/L for a 20 day period followed by a 20 day depuration. Statistical significance was 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 was evaluated with one-way ANOVA models 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).

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 transcriptional upregulation 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 under continuing E2 dosing. Hepatic mRNAs encoding *esr1* were similarly increased by the 100 μg/L (high dose) 4-NP exposure, with significant elevations observed after 12 days of treatment. Transcript levels of *esr1* returned to baseline, pre-exposure abundance by 20 days, however, 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 reproductive physiology.

4.1. 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, 1997a; 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., 1997b; 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 h of E2 treatment (Murata et al., 1997a; 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 the induction of Chg genes, several other 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 μg/L exhibited detectable *chgL* mRNA levels after 6 days as assessed via semi-quantitative RT-PCR, while *chgHm* transcripts required a 4-NP dose of 100 μg/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 here, where we observed 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 μg/L significantly increased *chgL* mRNA levels by ~30-fold, but *chgHm* mRNAs by ~40- to 60-fold within 72 h to 12 days of treatment, suggesting that the relative magnitude of transcript upregulation for 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*, in the liver was significantly upregulated 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 (Wang et al., 2005; Finn and Kristoffersen, 2007; Reading et al., 2009; Reading and Sullivan 2011; 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*), for example, 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).

Our data provide further evidence supporting the validity of using elevated relative mRNA levels of these *vtg* genes as reliable biomarkers of exposure to 4-NP in male fishes (Hemmer et al., 2002; Arukwe and Goksøyr, 2003; Bowman et al., 2003). 4-NP elicited significant increases in *vtgAa* and *vtgC* relative mRNA levels within 72 h of initiating exposure. The degree of 4-NP induction for *vtgAa* was nearly two magnitudes greater than that observed for *vtgC*. Similar variation 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 found 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 ethinyl estradiol (EE2), than did mRNA levels for *Danio* vtg-3, which encodes a truncated Vtg protein homologous to *vtgC* in other fishes. Fathead minnows (*Pimephales promelas*) treated with EE2 likewise showed varying maximum transcriptional induction responses depending on the *vtg* gene, with levels of *vtgC* mRNAs 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 magnitude 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* mRNAs 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 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 Vtg declined by 4 days and was 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 1–3 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., Giesy et al., 2000; Ackermann et al., 2002) and the maximum induction levels of these genes is correlated with the concentration, and in some cases short-term duration, of exposure (e.g., Knoebl 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).

4.2. Estrogen Receptor Induction Varies with Receptor Type and Tissue

The genomic actions of estrogens such as E2 occurs via hormone binding to nuclear estrogen receptors (ERs), which then interact with estrogen responsive elements (EREs) within the promoter regions of genes to modify 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 (Yadetie 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 (Tchoudakova et al., 1999; Hawkins et al., 2000; Socorro et al., 2000; Menuet et al., 2002). Tissue distribution patterns for ER expression, however, can also 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 predominantly in the intestine and ovaries of this cyprinid species (Bouma and Nagler, 2001; Wu et al., 2001; Filby and Tyler, 2005). 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, dissimilar estrogen function between the sexes, 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 upregulated over 40-fold within 24 h and over 75-fold by 72 h following the start of E2 exposure. 4-NP exposure likewise upregulated 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 of transcriptional induction to estrogen and xenoestrogens (Boyce-Derricott et al., 2010; Huang et al., 2010a, 2010b; Nelson and Habibi, 2013; Tohyama et al., 2015). In male fathead minnow, for instance, E2 (100 ng/L) was observed to upregulate 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 upregulated hepatic *esr1* (ERα) mRNA levels, had no effects on *esr2a* (ERβ2) mRNAs and down-regulated transcript abundance for *esr2b* (ERβ1) (Marlatt et al., 2008). Rainbow trout (*Oncorhynchus mykiss*) treated with aqueous phase E2 (473 ng/L) for 7 days showed elevations in mRNAs for two *esr1* paralogs encoding α-type receptors ERα1 and ERα2, but no change in abundance for transcripts encoding the β receptors ERβ1 and ERβ2 (Osachoff et al., 2013). Meucci and Arukwe (2006) found that exposure of juvenile Atlantic salmon, *Salmo salar*, to waterborne 4-NP at doses of 5–50 μg/L upregulated hepatic *esr1* mRNA levels after 3 and 7 days of exposure, but also observed that an *esr2* transcript was temporally 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 oviparious fishes examined to date, but also suggests that the effects of E2 on hepatic *esr2a* and *esr2b* transcription vary depending on the species and life history stage being examined (Nelson and Habibi, 2013).

The differing sensitivities of teleost fish *esr* genes to estrogen-induced transcription is likely linked to divergent functional roles for the nuclear ER receptors, with those roles possibly varying themselves among tissues. While it is sometimes suggested that the strong correlation in upregulation between *esr1* and *vtg* mRNAs in the liver infers *esr1*/ERα mediation of *vtg* gene regulation, results from experimental studies indicate that it is the *esr2*/ERβ receptor that mediates Vtg regulation by estrogens (Nelson and Habibi, 2013). In rainbow trout hepatocytes, Vtg production has been shown to be induced by the mammalian ERβ receptor agonist diarylpropionitrile, but not by the mammalian ERα agonist propyl-pyrazole-tiol (Leanos-Castaneda and Van Der Kraak, 2007). In this same study, the ERα antagonist methyl-piperidino-pyrazole failed to inhibit E2-induced Vtg production. Similarly, Yamaguchi and coworkers (2015) 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 ERα selective agonist (Orthoester-2k), implying *esr1*/ERα mediation of liver *chgH* induction. In contrast, hepatic *chgL* mRNA abundance was observed to be more responsive to an ERβ-selective ligand 2-(4-hydroxyphenyl)-5-hydroxy-1,3-benzoxazole, suggesting that an *esr2*/ERβ receptor 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 on *vtg* or *chg* regulation (Chakraborty et al., 2011; Huang et al., 2010a). 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 is 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.

4.3. 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 catalyzing the conversion of testosterone to estrogen. Actinopterygiian fishes possess two forms of steroidogenic aromatase enzyme genes, *cypa19a1a* and *cyp19a1b*, that 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 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* upregulation 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 upregulate the abundance of transcripts encoding both *cyp19a1b* and *esr2b*, but not *esr1* or *esr2a*. In this 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 upregulating *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.

5. CONCLUSION

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).

Looking beyond the relevance of these findings for the arrow goby specifically, this exposure-recovery data will help inform the use of molecular biomarkers as indicators of endocrine disruption and toxicity in demersal fishes more broadly. Marine sediments can be a significant source for the exposure of demersal fishes to 4-NP and other chemicals, and contaminants accumulated in sediment can move into the overlying water via equilibrium partitioning or be taken up via dermal absorption (Salomons et al., 1987). Such uptake mechanisms, in combination with biomagnification, may result in elevated contamination risk for demersal species compared to taxa living in the water column. For example, in coastal southern California, benthic Pleuronectiform fishes including the Dover sole (*Microstomus pacificus*) and hornyhead turbot (*Pleuronichthys verticalis*)—species that live in direct physical contact with the ocean floor—have been documented with muscle burdens of total DDTs 3–4 times higher than those observed in white croaker (*Genyonemus lineatus*), a schooling species that swims above the substrate (Gossett et al., 1983; McDermott-Ehrlich et al., 1978; see also Zeng and Tran, 2002). High burdens of 4-NP have likewise been detected in hornyhead turbot from these same habitats (Maruya et al., 2012), and there is evidence that these fish may be experiencing adverse endocrine effects (Bay et al., 2012). Studies that examine not only the induction—but also the recovery or adaptation—of molecular and physiological responses to chemical exposure will therefore be essential to the use of gene expression patterns in benthic marine fishes as reliable biological indicators for environmental monitoring.

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