ABSTRACT

PROTEOMIC CHARACTERIZATION OF TESTICULAR PROTEIN EXPRESSION IN MALE HORNYHEAD TURBOT EXHIBITING HIGH PLASMA ESTROGEN LEVELS

By

Cody David Larsen

December 2014

In selected locations offshore of urban southern California, male hornyhead turbot (*Pleuronichthys verticalis*) exhibit elevated plasma concentrations of the female sex steroid, 17β-estradiol (E2). Males sampled from Santa Monica Bay have consistently elevated E2 concentration (>1000 pg/ml), as much as 10 times higher than males from locations offshore of Orange County. Since estrogens, including E2, are at undetectable levels in the ocean discharge of regional wastewater treatment plants, it was of interest to determine whether testicular expression of steroidogenic enzymes and other proteins impacting estrogen production may be altered and linked to endogenous E2 production. In this study, proteomics was used to screen for changes in testicular protein expression. In addition, proteomes of fish experimentally treated with E2 were compared to a control. These experiments resulted in the molecular characterization of 30 unique proteins, and provided some insight into potential underlying molecular mechanisms associated with this aberrant E2 phenotype.

PROTEOMIC CHARACTERIZATION OF TESTICULAR PROTEIN EXPRESSION IN MALE HORNYHEAD TURBOT EXHIBITING HIGH PLASMA ESTROGEN LEVELS

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LIST OF SYMBOLS AND ABBREVIATIONS

3α-HSD	3-alpha Hydroxysteroid Dehydrogenase
3β-HSD	3-beta Hydroxysteroid Dehydrogenase
5α-DHT	5-alpha Dihydrotestosterone
11-KT	11-Ketotestosterone
11β- HSD-1	11-beta Hydroxysteroid Dehydrogenase-type-1
11β- HSD-4	11-beta Hydroxysteroid Dehydrogenase-type-4
11β- HSD-10	11-beta Hydroxysteroid Dehydrogenase-type-10
2D-GE	Two-Dimensional Gel Electrophoresis
ANOVA	Analysis of Variance
AR	Androgen Receptor
ATP	Adenosine Triphosphate
BRG1	Brahma-Related Gene 1
BW	Body Weight
cc	Cubic Centimeter
CCAR1	Cell Cycle and Apoptosis Regulator 1
CDC5L	Cell Division Cycle 5-Like
CLAEMD	City of Los Angeles Environmental Monitoring Division
cm	Centimeter
EDCs	Endocrine Disrupting Compounds
E1	Estrone

E2	17-beta Estradiol
E3	Estriol
EE2	17-alpha Ethinylestradiol
EEQ	Estrogen Equivalents
ER	Estrogen Receptor
ERE	Estrogen Response Element
ERα	Estrogen Receptor-alpha
ERβ	Estrogen Receptor-beta
ERγ	Estrogen Receptor-gamma
FSH	Follicle Stimulating Hormone
g	Gram
GABA	γ-Aminobutyric Acid
GnRH	Gonadotropin-Releasing Hormone
GTF3C5	General Transcription Factor IIIC5
HPG axis	Hypothalamo-Pituitary-Gonadal axis
HTP	Hyperion Treatment Plant
hr	Hour
LH	Luteinizing Hormone
m	Meters
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
mg	Milligram
min	Minute
ml	Milliliter

mm	Millimeter
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry/Mass Spectrum
MW	Molecular Weight
n	Sample Size
NCBI	National Center for Biotechnology Information
ng	Nanogram
NR	Nuclear Receptor
OC	Orange County
OCSD	Orange County Sanitation District
P450 2N	Cytochrome P450 family 2 subfamily N
P450 IV B1	Cytochrome P450 IV B1
P450-11β	Cytochrome P450 11-beta hydroxylase
P450-17α	Cytochrome P450 17-alpha hydroxylase
P450arom	Cytochrome P450 Aromatase
P450scc	Cytochrome P450 Side Chain Cleavage
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
pI	Isoelectric Focusing Point
pg	Picogram
PPAR	Peroxisome Proliferator-Activated Receptor
R	Correlation Coefficient

RA	Retinoic Acid
RAR	Retinoid Acid Receptor
RIA	Radioimmunoassay
RXRβ	Retinoid X Receptor-beta
SEM	Standard Error of the Mean
SMB	Santa Monica Bay
StAR	Steroidogenic Acute Regulatory Protein
SW	Salt Water
T	Testosterone
TR	Thyroid Hormone Receptor
WWTP	Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

The urban oceans and waterways around the world receive continual effluence of runoff and wastewater derived from human activities. In the Southern California Bight, more than one billion gallons of treated wastewater are released from wastewater treatment plants (WWTPs) into the coastal ocean environment every day (Kennish, 1997). The wastewater carries human-derived contaminants from the homes, industries and streets of this densely populated metropolitan area (Kennish, 1997, Braga et al., 2005, Kim et al., 2007, Liu et al., 2009). Such contaminants may include organochlorine pesticides, pharmaceutical estrogen mimics (e.g., 17α-ethinylestradiol) and other pharmaceuticals, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), surfactants (e.g., alkylphenol, nonylphenol), plasticizers (bisphenol A), endogenous steroid hormones (e.g., 17β-estradiol, estrone), and many others (Braga et al., 2005, Cai et al., 2007, Kim et al., 2007, Liu et al., 2010). Several of the compounds from these different classes of contaminants are also known as "endocrine disrupting compounds" (EDCs), since they significantly alter endocrine systems in wildlife, which can impact physiological homeostasis (Hashimoto et al., 2000, Brown et al., 2007, Brown et al., 2008, Colman et al., 2009, Desforges et al., 2010). The complete removal of environmentally relevant concentrations of EDCs from WWTPs has proven to be difficult, resulting in contaminants passing through to the ocean environment (Snyder et al., 2003, Auriol et al., 2006, Kim et al., 2007, Kasprzyk-Hordern et al., 2009, Liu et al.,

2010). Although endogenous hormones and pharmaceuticals present in the WWTP effluents are derived from or intended for humans, respectively, these compounds can have physiological effects on other organisms–particularly other vertebrates.

Endocrine systems are highly conserved among mammals and other vertebrates. For example, the endocrine system of all vertebrates relies on the neuroendocrine control of the hypothalamo-pituitary-gonadal (HPG) axis for reproduction. In addition, the key steroidogenic enzymes present in humans, such as steroidogenesis acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450-side chain cleavage (P450scc), 17α-hydroxylase, and aromatase (P450arom), all have sequence identities that are greater than 45% between piscine and mammalian species (Bauer et al., 2000). The primary form of active ovarian estrogen in humans, 17β-estradiol (E2), is the same as found in fish and other vertebrates (Lintelmann et al., 2003). After humans synthesize and use E2 in their body, much of the hormone is subsequently converted into less active forms, particularly estrone (E1), to be excreted from the body. In fact, humans excrete urine and feces that contain as many as 15 different endogenous metabolized estrogens, which then can enter into the wastewater systems and end up in the ocean environment (Xu et al., 2005). It has been estimated that E1, E2, and estriol (E3) from human urine make up 66-82% of the total amount of estrogen equivalents (EEQ; a measure of estrogenic activity) found in WWTP influents, while the other 18-34% is likely due to the other various forms of less active estrogenic compounds (Liu et al., 2009). In addition to endogenous estrogens, pharmaceutical compounds such as 17α-ethinylestradiol (EE2) are excreted in human urine, once again ending up in the environment (Brown et al., 2007, Brown et al., 2008, Colman et al., 2009).

The Impact of EDCs on Wildlife

Many EDCs have been demonstrated to have adverse effects on wildlife physiology, including reproduction. EDCs can interfere with endocrine systems in several ways: mimicking or preventing the effects of endogenous hormones, altering hormone receptor function or expression, or altering the synthesis, metabolism, and excretion of endogenous hormones (Ropero et al., 2006). The endogenous hormones affected include sex steroids, such as androgens and estrogens, as well many other types of hormones from steroids to thyroid hormones to peptide/protein hormones.

An example of a well-studied estrogenic EDC is EE2, which is a highly stable synthetic estrogen intended for human-use (birth control) and which is more potent than natural estrogens. EE2 bio-accumulates due to its lipophilic properties and resistance to metabolic transformation processes. A study measuring EE2 in shorthead redhorse suckers (*Moxostoma macrolepidotum*) in a Canadian river detected the compound in 50% of fish samples collected near a WWTP outfall, while no EE2 was detected in fish samples from a reference site 26 km away (Al-Ansari et al., 2010). Paternal exposure to EE2 in rainbow trout (*Oncorhynchus mykiss*) has been found to reduce embryonic survival of offspring (Brown et al., 2007). In a follow-up study, *O. mykiss* exposed to EE2 were found to suffer from altered gamete production, forming aneuploid sperm (Brown et al., 2008). In addition, late gastrulation, early organogenesis, and a disruption in blastula formation occurs in zebrafish exposed to environmentally relevant levels of EE2 (0.5, 1.0, 2.0 ng/L) (Soares et al., 2009).

Exposure to environmental estrogens can induce other reproductive phenotypic changes, such as male production of female-associated reproductive proteins (like

vitellogenin or other egg-associated components) and the development of intersex (expressing characteristics of both sexes, including "ovo-testis"). Vitellogenin is an egg yolk precursor protein that is expressed in the livers of reproductively active female fish, and it is widely used as a biomarker of estrogen exposure and effects in males, which normally do not produce vitellogenin. In one recent study, male teleost fish from 43 rivers worldwide had elevated plasma vitellogenin levels that were correlated with upstream human population centers (Desforges et al., 2010). Similarly, wild male flounder (*Pleuronectes yokohamae*) from urban Tokyo Bay, Japan, had elevated plasma vitellogenin levels, as well as gonadal abnormalities, when compared with fish from a non-urban reference site (Hashimoto et al., 2000). An evaluation of the occurrence of intersex in 16 species of black basses (*Micropterus* spp.) from nine rivers in the United States found the presence of testicular oocytes in 3% of the fish sampled. Of the fish sampled, largemouth bass (*M. salmoides*) and smallmouth bass (*M. dolomieu*) had the highest occurrence of intersex in males at 18% and 33%, respectively (Hinck et al., 2009). Each sampling site in the study was found to have estrogenic EDCs present in the surface waters and sediments, including pesticides and PCBs. In freshwater bream (*Abramis brama*), ovo-testis occurred in 4-16% of phenotypic males residing in three different Dutch rivers, with highest incidence (16%) in a river receiving the WWTP effluent from urban residential areas and farmland (Reinen et al., 2010). Adipose tissue analysis of the fish sampled in this study detected the presence of multiple EDCs (bisphenols, aklylphenols, pesticides, flame-retardants like PBDE), including steroid hormones. Therefore, disruption of male reproductive phenotypes, due to estrogenic EDCs, has been detected in several aquatic environments worldwide.

In addition to reproductive endocrine disruption, contaminant exposures may also cause other kinds of endocrine disruption. A study by Brar et al. (2010) investigated thyroid disruption in two fish species (*Cymatogaster aggregata* and *Leptocottus armatus*) from several areas of the San Francisco Bay. Plasma concentrations of thyroxine were significantly lower in fish sampled from highly impacted sites as compared with fish from locations with reduced urban contaminants. The study also observed significant correlations between hepatic concentrations of PCBs and thyroid hormones, including reduced concentrations of triiodothyronine, suggesting an alteration of the deiodinase activity in the thyroid endocrine system (Brar et al., 2010). In a recent study from our laboratory, PCB levels were correlated to disruption of the stress endocrine system that generates the steroid hormone, cortisol, in English sole residing at Southern California WWTP impacted locations (C. Waggoner, personal communication).

There are many factors that may determine a fish species' susceptibility to environmental contaminants, including migratory behavior (or lack thereof), dietary preferences, and size of home range. A large number of EDCs tend to be lipophilic compounds that, when released into the ocean, bind to particles that settle and accumulate in the bottom sediments. The lipophilic properties of these compounds allow them to pass through plasma membranes, resulting in uptake and accumulation in the cells and tissues of benthic infauna, such as polychaetes and mollusks (Kennish, 1997, Riva et al., 2010). Bioaccumulation of EDCs derived from the sediments is often observed to move through an ecosystem's food chain. Fish species with greater susceptibility to EDC accumulation and exposure are those that have a dietary preference of polychaetes, mollusks, and other benthic infauna.

A High E2 Phenotype in Male Hornyhead Turbot

The hornyhead turbot (*Pleuronichthys verticalis*) is a bottom-dwelling flatfish that feeds primarily on benthic infauna, particularly tube-dwelling polychaetes and the siphons of clams (Kramer, 1991, Cooper, 1995). Hornyhead turbot are believed to be non-migratory (Allen, 1982), and they have a slow and linear growth rate, living to a maximum age of ~25 years (Cooper, 1995). They are commonly found at depths ranging from 10-200 m (Allen, 1982), and exhibit relatively high densities near the WWTP outfalls, which are located at a depth of 60 m in Southern California. These aspects of the hornyhead turbot have attracted its use as a model species for studying and monitoring bioaccumulation of environmental contaminants (*http://www.ocsd.com/opengov/annual-reports/-folder-388*).

Hornyhead turbot of Southern California exhibit extraordinarily high levels of E2 in blood plasma, which may be due to environmental endocrine disruption. Prior studies in our laboratory have shown that male hornyhead turbot exhibit plasma E2 concentrations as high as or higher than that of reproductive females (300-1400 pg/mL; Reyes, 2006; Petschauer, 2008; Reyes et al., 2012). Two of the locations in Southern California that have been emphasized in these studies are Santa Monica Bay (SMB) and the San Pedro Shelf offshore of Orange County (OC). Hornyhead turbot males from SMB consistently exhibit >3-fold higher E2 concentrations when compared with males sampled from the San Pedro Shelf (Reyes, 2006; Hagstrom, 2008; Reyes et al., 2012). This difference has been hypothesized to be due to influences of the WWTP outfalls of the City of Los Angeles' Hyperion Treatment Plant (HTP), which is centrally located within SMB, and the Los Angeles County Sanitation District, located immediately southward on the Palos

Verdes Peninsula (Reyes, 2006; Petschauer, 2008). The San Pedro Shelf, in contrast, represents a more open environment in which oceanic currents more efficiently disperse effluents originating from the Orange County Sanitation District (OCSD); additionally, the OCSD outfall is located four miles offshore on the outer edge of the shelf.

Initial work by Reyes (2006) found that plasma E2 concentrations in hornyhead turbot males are significantly higher than in males of a number of other fish species measured in the area (Dover sole, English sole, Pacific sanddab, California halibut). This suggests that there is some behavioral and/or physiological feature of the hornyhead turbot that makes them more susceptible to impacts of EDCs than other similar flatfish. Alternatively, it is possible that male hornyhead turbot naturally exhibit high E2 levels. In recent experiments performed by our laboratory, relocating wild male hornyhead turbot with high estrogen phenotypes from the SMB environment to clean seawater tanks reduced their plasma E2 concentrations. After a period of four weeks, plasma E2 concentrations reached ≤70 pg/ml, similar to those seen in males of other local fish species (Iwanski, 2011). This suggests that the abnormally high estrogen phenotype in male hornyhead turbot may be induced by environmental influences.

The high estrogen phenotype is hypothetically due to environmental disruption of steroidogenesis. The mechanisms of this disruption, however, are not well understood. The synthesis of steroid hormones (steroidogenesis) is a process in which the precursor molecule, cholesterol, undergoes progressive modifications to generate the different steroids (Stocco and Clark, 1996). Steroidogenic acute regulatory protein (StAR) is involved in the transport of cholesterol into the mitochondria, which is subsequently modified into steroid hormones. This activity of StAR is a rate-limiting step in

steroidogenesis, and it is a suspected target of EDCs, whereby altered StAR expression will have significant effects on the rate of steroidogenesis (Stocco and Clark, 1996; Bauer et al., 2000). Estrogens (e.g., E1, E2) as a class of steroid hormones are formed from precursor androgens (e.g., androstenedione, testosterone), converted by the P450arom enzyme. P450arom is also a potential target of EDCs, as it can have significant effects on the production of estrogens (Thibaut and Porte, 2004; Whitehead and Rice, 2006). Another pathway to E2 production can involve the enzyme 17β-hydroxysteroid dehydrogenase type-1 (17β-HSD-1), which converts E1 into E2. The 17β-HSD-1 enzyme has also been found to be a target of EDCs (Whitehead and Rice, 2006).

Alteration of the steroidogenesis pathways does occur in SMB hornyhead turbot males. The testicular transcript levels of StAR and P450arom mRNA were significantly increased in fish exhibiting increased plasma estradiol concentrations (Hagstrom, 2008). The degree to which these mRNA transcript levels reflect differences in the active proteins was not included in that study, and remained unknown. Furthermore, additional differences in the molecular phenotype are likely beyond these two genes, which could include changes in other steroidogenic enzymes (e.g., alterations in androgen production pathways), pituitary hormone receptors (e.g., for FSH or LH), relevant signaling and transcription factors, or alterations in other receptors known to interact with estrogen. The hypothesis of this Master's Thesis is that high circulating concentrations of E2 in male hornyhead turbot will be significantly correlated to an altered testicular proteome, potentially impacting steroidogenesis and circulating E2 levels. By identifying the proteins that are altered in association with different levels of E2, mechanisms underlying increased E2 biosynthesis may be elucidated.

While a major objective of the present study is to identify the potential molecular mechanisms by which the testis may aberrantly produce E2, it is also important to remember that altered protein levels in the testis may also reflect the local actions of elevated E2 itself. Identified proteins exhibiting altered levels may reflect transcription and/or translation being secondarily regulated by estrogen, rather than an initial environmental impact. This Master's Thesis presents comparative protein profiling by 2D-GE of total protein extracts from male testes of hornyhead turbots, of both wild caught populations and fish treated with E2. Reported data include the identification of numerous proteins that demonstrated differential protein levels between sample groups, providing new insight into the potential mechanisms of the observed aberrant male E2 phenotype.

CHAPTER 2

MATERIALS AND METHODS

Two experiments were performed to test the hypothesis of this Master's Thesis. The first experiment screened the testicular proteomes of wild-caught male hornyhead turbot, looking for altered protein abundance between a low E2 group (<300 pg/ml) and a high E2 group (>1000 pg/ml). For the second experiment, wild-caught male hornyhead turbot were relocated into a laboratory environment for a minimum of four weeks and subsequently injected with either a high dose of E2 or vehicle for a control. Testicular proteomes of injected fish were then screened for altered protein abundance between the E2-treated and control. Proteins with altered abundance were then identified using MALDI-TOF/TOF mass spectrometry.

Field Study: Sites and Fish Sampling

Sampling in SMB was done in collaboration with the City of Los Angeles Environmental Monitoring Division (CLAEMD). On February 2, 2012, hornyhead turbot were collected in the vicinity of the HTP outfall (located 5 miles off El Segundo, CA; Figure 1) by standard otter trawl along the 60 m bathymetric line, using CLAEMD's monitoring vessel, *La Mer*. The two sampling sites in SMB were Z2 (33˚ 54.412' N, 118˚ 31.606' W) and D1T (33˚ 54.166' N, 118˚ 32.221' W). For each site, bottom trawl time ranged from 14-17 min, traveling a distance of 450 m at a speed of 2-2.5 knots. Net retrieval from the bottom environment took approximately 10 min, followed by 30+ min of handling, sorting, and captivity in buckets. After 55-60 min from net-on-bottom,

standard weight (g) and length (mm) of all fish were measured, followed by blood and tissue collection (testis and liver). Blood was collected from the caudal vein using a heparinized 22g, 3.5 cm needle and a 1 cc syringe. Blood samples were placed into 1.5 ml plastic epitubes and centrifuged for 5 min at 5000 rpm to isolate the plasma (Centrifuge 5415C, Eppendorf, Westbury, NY), which was then pipetted into fresh epitubes; in the field, plasma was initially placed on dry ice, and then later stored at -80°C in the laboratory until needed for analysis. Thirty individuals were targeted between the two sites, with an expected sex ratio of 50:50. Sampling yielded 10 fish from D1T (5 male, 5 female), and 13 fish from Z2 (11 male, 2 female), with standard lengths ranging from 125 mm to 155 mm, and body weights ranging from 55 g to 115 g. For males, both testis were removed, placed into 1.5 ml plastic epitubes, frozen and stored as described above. For females, ovarian tissue was removed and stored, as above, for potential use in future studies. Whole livers were also removed, placed in pre-weighed foil (to later calculate HSI), and frozen and stored as described above. Hepatic tissues were taken for potential contaminant analysis and proteomic analysis for potential future studies.

Sampling from the San Pedro Shelf in OC was done in collaboration with the OCSD. On March 5, 2012, hornyhead turbot were collected by otter trawl along the 60 m bathymetric line at two trawl stations used by OCSD's Ocean Monitoring Program, T11 (33˚ 36.055' N, 118˚ 05.199' W) and T1 (33° 34.641' N, 118° 00.567' W). T1 is located in the vicinity of the OCSD outfall, while T11 is located north of the outfall area (Figure 1). Trawling procedures were identical to those used in SMB. Thirty fish were targeted between the two OCSD sites, with an expected sex ratio of 50:50. Sampling yielded 16

fish from T11 (12 male, 4 female), and 17 fish from T1 (11 males, 6 females), with standard lengths ranging from 157 mm to 176 mm, and body weights ranging from 92 g to 150 g. Blood and tissue collection procedure were performed as described above.

Plasma Estradiol Measurement

Concentrations of E2 in blood plasma samples were measured by specific radioimmunoassay (RIA) using commercially available reagents from Diagnostic Systems Laboratory (Beckman-Coulter, Inc., Webster, TX), Santa Cruz Biotech (Santa Cruz, CA), and Sigma Chemical Co. (St. Louis, MO). The RIA protocol has been routinely used and validated for this species in our laboratory (Environmental Endocrinology Laboratory, CSULB, Reyes, 2006, Rempel et al., 2006, Petschauer, 2008, Reyes et al., 2012). Hormone concentrations were determined from a standard curve of %B/Bo versus concentration of unlabeled ligand added at 0.01-1000 ng/ml, using Sigma Plot v.11.2 software (SPSS Inc., Chicago, IL).

Testicular Proteome Evaluation

Fish exhibiting low (<300 pg/ml) and high (>1000 pg/ml) plasma E2 concentrations were selected from the field samples for proteomic analysis of their testicular tissue. Proteomes of sampled fish were compared with each other using two-dimensional gel electrophoresis (2D-GE). Stored testicular tissues were removed from the -80°C freezer, then immediately homogenized, kept at 0°C at all times, and separated into aliquots for electrophoresis and protein assays. Protein concentration for each sample was determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) following manufacturer's instructions. Samples were then normalized by protein content (to 500 µg each) and prepared for 2D-GE using the ReadyPrep 2-D Clean Up procedure

(Bio-Rad Laboratories, Hercules, CA). Gels using 300 µg, 400 µg, and 500 µg protein content were initially compared to identify maximum protein content range for optimal gel quality (e.g., minimal streaking); 500 µg was the upper limit of the ReadyPrep 2-D Clean Up procedure and produced optimal 2D gel results. After being dissolved in rehydration buffer (ReadyPrep procedure), the proteins were subjected to isoelectric focusing in one dimension, using 11cm IEF strips with a pH range of 3-10 on a Bio-Rad Protean IEF cell. The second dimension of separation was by mass/charge ratio on 12% Bis-Tris polyacrylamide gels (Protean Plus, BioRad) in a Laemmli buffer system. Gels were then exposed to a fixing solution (65% nanopure water, 30% methanol, 5% acetic acid) for 1.5 hours, and stained using Coomassie Blue (BioRad) to visualize the testicular proteome of each sample. Gel images were captured using an Investigator Pro-Pic II Scanner (Genomic Solutions, Ann Arbor, MI). The proteomes of fish from the low E2 group and high E2 group were scanned and compared using Progenesis™ Same Spots software (Nonlinear Dynamics, Durham, NC). Proteins that exhibited significant differences (either between sampling regions or in correlation with plasma E2 concentrations) were then targeted for subsequent mass spectrometry analysis and molecular identification.

Target proteins were excised from the gel and gel plugs were rinsed with nanopure water, and then de-stained using 1:1 (v/v) 50% acetonitrile/25 mM ammonium bicarbonate. Gel plugs were then de-salted by repeating a nanopure water wash several times, and dehydrated using 100% acetonitrile with vacuum drying for 15 min in a Speed-Vac centrifuge. Protein samples from gel plugs were digested using trypsin cocktail (ProMega, Fisher Biotech, Tustin, CA; dissolved in 50 mM ammonium

bicarbonate) overnight at 37°C with gentle shaking. Tryptic peptides were extracted from the gel plugs using a 1% trifluoroacetic acid/50% acetonitrile solution and then vacuum dried. Samples were further de-salted using C18 Zip-Tips (Millipore), following the manufacturer's protocol. Each sample was then dissolved in 0.1% trifluoroacetic acid and spotted on a MALDI plate in duplicate. Matrix (α-cyano-4-hydroxy-cinnamic acid; CHCA) was then added on top of each spot on the plate. Plated samples were then analyzed on a MALDI TOF/TOF 4800 mass spectrometer (Applied Biosystems, Foster City, CA) in the IIRMES Proteomics Center (www.IIRMES.org). Proteins were identified using the Mascot search engine (Matrix Sciences, Boston, MA) and NCBI and Swiss-Prot MS spectra databases for all Vertebrata (broad range) and Actinopterygii (narrow range). Protein matches were primarily determined by protein scores, ion scores, and their associated confidence indices, although high scores and confidence indices did not necessarily confirm a positive identification and required evaluation of duplicate sample analysis, repeated matches, and cross-validating the experimental and theoretical values of MW and pI. Every protein identity reported in this study underwent this entire validation process. It was expected that potential molecular targets of endocrine disruption in the testicular tissue were to be identified using this approach.

Laboratory Study: Source of the Experimental Fish

Live hornyhead turbot were collected in the field and subjected to acclimation in a laboratory environment for a minimum of four weeks. A previous study from our laboratory showed that after a period of four weeks in a clean laboratory environment, elevated estrogens (including E2) in male hornyhead turbot return to normal male levels

(Iwanski, 2011). This study aimed to replicate this acclimation, and then subject the fish to E2 treatment.

Initial collections occurred with CLAEMD during their scheduled winter trawls between January 23-29, 2013 at the Z2 and D1T trawling sites (as described above). Fish trawling procedures were identical to those described above, after which fish were kept in aerated coolers of SW and transported back to CSULB. Water changes were done every hour to maintain a suitable temperature. The fish were transferred to the CSULB Marine Laboratory, and the aerated coolers were placed in the walk-in cold room (16°C) overnight to allow temperatures and conditions to stabilize. The following day, the fish underwent a parasite removal procedure, beginning with a 1 hr formalin dip (1 ml formalin/1 gallon SW) in a heavily aerated bucket. Each fish was then individually inspected for external parasites, which were removed with forceps. The deparasitised fish were then quarantined in fresh SW overnight in the same cold room. On the following day, the fish were measured for weight (g) and standard length (mm), tagged just below the dorsal fin, and randomly distributed amongst three 500 gallon tanks filled with re-circulated, filtered SW (obtained from Catalina Water Company, Long Beach, CA) and a sandy substrate.

The target number of fish for this experiment was at least 30, providing a potential sample size of 15 males (assumed sex ratio of 50:50); this species does not exhibit obvious external sexual dimorphism, and therefore sex must be confirmed during dissection. The CLAEMD winter trawls only provided 19 fish total, so additional collections were made with OCSD during their scheduled winter trawl on March 13, 2013. Fish were trawled and collected from OCSD sites T1, T11, and T17 (as described

above), which provided enough fish to reach the target number for this experiment. These fish underwent the same acclimation, deparasitation, and tagging procedures as those collected with CLAEMD. During the acclimation period of this experiment, fish were fed diced pieces of market squid (*Doryteuthis opalescens*) three times a week.

On March 21, 2013, there was an anomalous spike in the ammonia level of the water system at the Marine Laboratory, and in the following days several of the fish were lost before and after the ammonia levels subsided. As a result, additional fish were collected during OCSD trawls on July 9, 2013 (at trawling sites T1, T12, T22, and T23) and during CLAEMD trawls on August 21-30, 2013 (at trawling sites Z2, 9292, 9326, and C1). Ten healthy fish were ultimately available for the experiment; these fish completed a minimum of four weeks of captivity with periods of captivity of 4, 5, 12, 26, or 40 weeks.

Laboratory Study: Treatments and Sampling

Individual hornyhead turbot were randomly selected to be in the E2 treatment group and the control group. The treatment group was subjected to two E2 injections (3 μg/g body weight), 48 hr apart (similar to Madsen and Korsgaard, 1991). The control group was injected with only the vehicle (vegetable oil) under the same regimen. Blood and tissue samples were taken 24 hr after the second injection. There were nine males and one female, providing sample sizes of four for the control group and five for the treatment group. Standard lengths ranged from 118 mm to 159 mm, and body weights ranged from 40 g to 88.7 g.

Testicular tissue samples from the experimental fish were subjected to proteomic analysis, as described above, comparing the E2 treatment and control groups. Fish

injected with E2 were assumed to have relatively high E2 values, whereas those injected with vehicle were assumed to have relatively lower E2 values.

Data Analysis

Data sets of hormone concentrations, protein levels (normalized spot volumes), and protein identifications were generated. For the field study, 39 fish were collected from SMB (sites Z2 and D1T) and OC (sites T1 and T11) during the winter of 2012; mean E2 concentrations were compared for differences between the four field sites using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post-hoc test. For proteomic analyses, fish were divided into a low E2 group (n=4) and a high E2 group (n=6). In the laboratory experiment, groups consisted of an E2-injected treatment group (n=5) and vehicle-injected control group (n=4). Student's t-tests were used to determine the mean differences in protein levels (normalized spot volume) between the low E2 and high E2 groups, and between the E2-treated and control groups in the laboratory study. In addition, Pearson product-moment correlation analyses were carried out between hormone concentrations and protein level changes in order to determine significant relationships among parameters. SigmaPlot v.11.2 software was used to conduct all statistical tests; mean differences or correlation coefficients (R) were considered significant when $p<0.05$.

CHAPTER 3

RESULTS

Plasma Estradiol Concentrations in Field-Caught Male Hornyhead Turbot

Hornyhead turbot were sampled at the T1 and T11 locations in OC, and the Z2 and D1T locations in SMB, to evaluate whether there were differences in plasma E2 concentrations. Fish sampled from the D1T and Z2 sites exhibited mean E2 concentrations of 1494.3 ± 178.5 pg/ml and 601.6 ± 77.3 pg/ml, respectively (Figure 2). In contrast, the mean E2 concentrations of fish sampled from the T1 and T11 sites were 214.9 ± 67.2 pg/ml and 444.4 ± 51.9 pg/ml, respectively. The mean E2 concentration of fish sampled at D1T was significantly higher than that in all other groups ($p<0.001$). The E2 concentration of fish sampled at Z2 was significantly higher than that in the T1 group ($p<0.013$), but not different than the T11 group. In addition, no differences in plasma E2 concentration were noted between fish sampled at T1 and T11.

Field Study: Identification of Proteins from the Testicular Proteome

Hornyhead turbot samples were selected from the above field sites and divided into two groups, one exhibiting relatively lower E2 concentrations (<300 pg/ml), and one exhibiting high E2 concentrations (>1000 pg/ml). All individuals in the low E2 group were from OC sites, and all individuals in the high E2 group were from SMB sites. The individual testicular proteomes of fish from the two groups were then compared using 2D-GE. Proteins from the testicular tissue samples were separated by their isoelectric focusing point in the first dimension and by molecular weight in the second. The highest

quality gels produced were then digitally scanned and analyzed using Progenesis SameSpots^{TM} software, as described in the Methods. Using the software, a calibrator gel (synthesized *in silico*) was generated by aligning all gel samples (n=10), which was subsequently used to compare the protein spot volumes between each individual gel. This method produced normalized spot volumes for each individual protein on each gel, for a total of 867 proteins captured in the images. The normalized spot volumes of these proteins were then analyzed for differential levels between the low E2 (n=4) and high E2 (n=6) groups. This analysis found 51 proteins that exhibited differential levels between the two groups (Table 1; $p<0.05$). MALDI-TOF/TOF mass spectrometry was then used to analyze the peptide mass fingerprints for each protein, elucidating the molecular identities of 27 of these proteins (listed in Table 2). The locations of the identified proteins are shown in a representative stained 2D gel in Figure 3.

Differences in Testicular Protein Levels

Table 1 lists all 51 proteins that exhibited significantly different levels between the low E2 and high E2 groups; protein levels ranged from -1.7 fold reduced to +2.0 fold increased in the high E2 group as compared with the low E2 group. Of the 27 proteins whose molecular identity was elucidated, two were enzymes known to be involved in steroidogenic pathways or steroid metabolism. Among the other identified proteins, there were several that may have effects on the testicular cell physiology, including endogenous metabolism, xenobiotic metabolism, and a few that may have indirect or direct impacts on estrogen production and signaling pathways.

The first steroidogenic enzyme was identified as L-3-hydroxyacyl-coenzyme-A dehydrogenase, but is also known in the literature as 17β-hydroxysteroid dehydrogenase

10 (17β-HSD-10; Yang et al., 2005). This is a mitochondrial enzyme that is involved in the metabolism of branched-chain fatty acids and steroids, and was significantly up-regulated in the high E2 group (1.4-fold; p=0.0416; Figure 4; Table 1). Two isoforms of the steroidogenic enzyme cytochrome P450 11-beta, also known as 11-beta-hydroxylase (P450-11β), showed a significant decrease in the high E2 group. Isoform 680 decreased by 1.2-fold (p=0.0088; Table 1) and isoform 706 decreased by 1.4-fold (p=0.0236; Table 1). An identification of a partial fragment of P450-11β was also made, and this protein also exhibited a significant decrease in the high E2 group (1.4-fold; p=0.0347; Figure 4; Table 1).

Several of the identified proteins involve nuclear and gene regulatory functions. Retinoid X receptor beta (RXRβ), a nuclear receptor that binds with hormone response elements, exhibited reduced levels in the high E2 group (1.2-fold, p=0.0240; Table 1). Transcription activator BRG1-like (partial; BRG1), a transcriptional coactivator involved with nuclear hormone receptors, was significantly increased in the high E2 group (1.2 fold; p=0.0131; Table 1). CCAR 1 protein, a cell cycle and apoptosis regulator that recruits to endogenous nuclear receptor target genes in response to hormones, exhibited a 1.2-fold increase in the high E2 group (p=0.0381; Table 1). Chromatin modification-related protein eaf6 (Eaf6), which is involved in histone acetylation and transcription regulation, was reduced in the high E2 group (1.2-fold; p=0.0124; Table 1). Cell division cycle 5-like protein (CDC5L), a DNA-binding protein involved in cell cycle progression, exhibited a 1.6-fold increase in the high E2 group (p=0.0047; Figure 4; Table 1). Protein SDA 1 homolog, which is involved in protein transport and ribosome biogenesis, was significantly increased in the high E2 group (1.2-fold; p=0.0053; Table 1).

In addition, there were several proteins involved in cellular metabolism and energetics. Cytochrome P450 IV B1 (P450 IV B1), an enzyme known to be involved in both endogenous and xenobiotic metabolism, exhibited a 1.2-fold increase in the high E2 group (p=0.0065; Table 1). A cytochrome P450 2N, a subfamily of enzymes involved in the metabolism of arachidonic acid and xenobiotics, exhibited a 1.5-fold decrease in the high E2 group (p=0.0275; Table 1). Aldo-keto reductase family 1 member A1a (aldehyde reductase) exhibited a 1.2-fold increase in the high E2 group (p=0.0495; Table 1). Mitochondrial aconitase 2, an enzyme that catalyzes the conversion of citrate to isocitrate in the citric acid cycle, was significantly up-regulated in the high E2 group (1.2-fold; p=0.0371; Table 1).

Several other protein spots that exhibited significant site-related differences had identifications that matched proteins present in the searched databases; however, they were either unnamed protein products or uncharacterized proteins: unnamed protein product (1.3-fold increase; p=0.0092; Table 1); uncharacterized protein LOC678611 (1.4-fold increase; p=0.0238; Table 1); unnamed protein product (1.3-fold increase; p=0.0416; Table 1); unnamed protein product (2.0-fold increase; p=0.0428; Table 1 & Figure 4); unnamed protein product (1.4-fold increase; p=0.0488; Table 1).

Relationships Between Plasma Estradiol and Testicular Protein Levels

Pearson product-moment correlation analyses were used to determine the relationships between plasma E2 concentration (pg/ml) and protein spot volume (arbitrary units) among the 10 samples of field caught males in which testicular proteome was analyzed. Many of the proteins that exhibited significant site-related differences (described above) also demonstrated significant correlations with plasma E2

concentration, further supporting their candidacy as proteins associated with the molecular mechanisms causing this high estrogen phenotype, or in the response to it. In addition, several proteins exhibited levels that significantly correlated with E2 concentration, but did not exhibit significant site-related differences. This introduced additional testicular protein candidates altered in association with the high estrogen phenotype.

There were 40 proteins that were significantly correlated with plasma E2 concentrations ($p<0.05$; Table 3), and six proteins that had a trending relationship with plasma E2 ($p>0.05$; Table 3). The levels of one steroidogenic enzyme, P450-11β, demonstrated a strong positive correlation with plasma E2 concentration (R=-0.796, $p=0.00592$; Figure 5). The levels of several proteins involved in gene regulation also exhibited strong correlations with plasma E2 concentration, including RXRβ (R=-0.833, $p=0.0028$; Figure 5), BRG1 (R=0.834, $p=0.0027$; Figure 5), CCAR1 (R=0.682, $p=0.0297$), Eaf6 (R=-0.692, $p=0.0267$) and SDA1 homolog (R=0.878, $p=0.00084$). One metabolic enzyme, P450 IV B1, was also found to be strongly correlated with plasma E2 concentration (R=0.773, $p=0.0088$; Figure 5). Another metabolic enzyme, P450 2N, was not significantly correlated with E2, but demonstrated a trending relationship (R=-0.622, $p=0.0548$).

The molecular identity of a number of proteins that exhibited strong correlations with plasma E2 (R>0.60) were unable to be elucidated in the present study (Figure 7). These proteins will be targets for future identification by peptide mass fingerprint analysis using MALDI-TOF/TOF mass spectrometry.

Laboratory Study: E2-Responsive Proteins in the Testicular Proteome

Hornyhead turbot were collected from the wild and transported back to the CSULB Marine Laboratory, where they were transferred into tanks and maintained in aquaria with clean sediment and seawater (see Methods). A previous study by Iwanski (2011) demonstrated that after a period of four weeks in captivity, elevated E2 in field-caught male hornyhead turbot subsided to levels expected in normal males (<50 pg/ml). In the present study, captivity in the aquarium environment for at least four weeks was followed by injection of the fish with either 3 μg E2/g body weight (treatment) or vehicle (control), and their gonadal tissues were sampled after 48 hours. The testicular proteome of the treatment and control groups were then compared using 2D-GE. Using the same methods as before, 2-D gels were aligned and used to compare the protein spot volumes between each individual sample. All 867 spot numbers and positions from the earlier field study gels were maintained and transferred to this new set of gels to allow for direct comparisons. The normalized spot volumes of these proteins were then analyzed for differential levels between E2-treated (n=5) and control (n=4) groups. This analysis found 24 proteins that exhibited significant differential levels between the two groups (Table 4; $p<0.05$), with two additional proteins showing non-significant trends (Table 4; $0.06>p>0.05$). MALDI-TOF/TOF mass spectrometry was then used to analyze the peptide mass fingerprints for each of these proteins, resulting in the elucidation of the molecular identity of 14 of the proteins (proteins listed in Table 4 have their molecular identification data provided in Table 2). The mapped locations of these proteins are shown in the representative stained 2D gel in Figure 3.

Of the 14 identified proteins, one was the steroidogenic enzyme 17β-HSD-10, which demonstrated a 1.6-fold increase in the E2 treatment group (p=0.0005; Table 4; Figure 6), similar to the 1.4-fold increase in the wild-caught high E2 group. Several other proteins are known to be involved in gene regulation. Prohibitin, a protein that mediates transcriptional repression of nuclear hormone receptors, exhibited a 1.3-fold increase in the E2 treatment group (p=0.0025; Table 4; Figure 6). Transcription factor IIIC63 (GTF3C5) exhibited a 1.5-fold decrease in the E2 treatment group (p=0.0140; Table 4; Figure 6). Two isoforms of the CCAR1 protein showed decreases in the E2 treatment group; isoform 927 showed a 1.3-fold decrease (p=0.0210; Table 4), while CCAR1 isoform showed a 1.4-fold decrease (p=0.0218; Table 4; Figure 6). This pattern differs from the wild-caught high E2 group, where CCAR1 exhibited a 1.2-fold increase.

Unidentified Proteins Exhibiting Significant Changes and Correlations

There were several proteins in both the field study and the laboratory study that demonstrated differential protein levels between groups, and/or that demonstrated significant correlations with E2, yet their identities were unable to be elucidated. For example, proteins #984 (R=0.776, p=0.00838), #1906 (R=0.813, p=0.00426), and #2430 (R=0.785, p=0.00719) all showed strong positive correlations with E2 (Figure 7), but were unable to be identified in the present study. These have strong potential as candidate proteins of the underlying molecular mechanism of this high estrogen phenotype, and will be the target of future molecular characterization studies.

CHAPTER 4

DISCUSSION

Male hornyhead turbot sampled in the vicinity of the HTP in SMB have elevated estrogen concentrations in their blood plasma (Reyes, 2006; Hagstrom, 2008; Petschauer, 2008; Reyes et al., 2012), and this previous finding was confirmed in the present study. It is hypothesized that the high E2 concentration is an aberrant phenotype caused by environmental conditions, given regional differences between SMB (high E2) and other locations (e.g., low E2 in fish sampled from Orange County coastline). It is possible that these males naturally exhibit high E2 levels, although studies performed by our laboratory suggest that this is not the case. For example, wild-caught male hornyhead turbot exhibiting high E2 were relocated and kept in clean seawater tanks, and after four weeks their plasma E2 concentrations subsided to ≤70 pg/ml, similar to levels in males of other local fish species (Reyes, 2006; Iwanski, 2011). This suggests that the abnormally high E2 phenotype may be induced by environmental factors that cause endocrine disruption, and possibly an altered gonadal proteome as evaluated in the present study.

In order to provide insights into the gonadal mechanisms that may be involved in the observed high estrogen phenotype in hornyhead turbot males, comparative protein profiling of the testis of both wild-caught fish and E2-treated fish was carried out using 2D-GE. In comparing the proteome profiles of wild-caught fish, the testicular proteomes of individuals exhibiting low E2 plasma concentrations (<300 pg/ml) were used as a reference for the proteomes of individuals exhibiting high E2 plasma concentrations

(>1000 pg/ml). The results from this analysis identified changes in a broad functional spectrum of protein groups, including some involved in steroidogenesis, and others involved in nuclear hormone binding, gene regulation, and intracellular transport. While changes of these proteins may be related to the molecular mechanisms underlying the aberrant production of E2 due to (unknown) environmental factors, some of these changes may also reflect local actions of elevated E2 on testicular function.

In order to elucidate the difference between proteins potentially impacted by the environment and those regulated by elevated E2 levels, wild-caught fish were also relocated from the environment to aquaria for a minimum of four weeks and then experimentally treated with E2. The testicular proteomes of these animals were compared with control animals in order to screen for proteins associated with the experimentally elevated E2. The results from this analysis identified changes in proteins from functionally similar groups as compared with those described above in the low E2 and high E2 wild-caught fish. Three of the proteins exhibiting differences were common between the wild-caught high E2 group and the experimental E2-treated group. This suggests that the observed differences in these three proteins, in the wild-caught high E2 group, are likely due to the secondary effects of elevated E2.

Altered Steroidogenic Enzymes Potentially Impact Testicular E2 Production

Alterations in steroidogenic enzyme levels can have significant effects on the production and regulation of gonadal steroid hormone levels (Stocco and Clark, 1996; Bauer et al., 2000; Whitehead and Rice, 2006). Male reproductive function, such as spermatogenesis, is controlled by the release of hypothalamic gonadotropin-releasing hormone (GnRH), pituitary gonadotropins, and gonadal androgens as well as estrogens.

These hormones are regulated by complex feedback loops along the HPG axis. GnRH and gonadotropin secretion in males is not only modulated by androgens, but also by E2, as reviewed by Tilbrook and Clarke (2001). Furthermore, in females, this negative feedback is broken during follicle and oocyte maturation, when there is a surge in circulating estrogen, as reviewed by Naftolin et al. (2007). Synaptic plasticity in the arcuate nucleus is induced by E2, causing a disinhibition of the GnRH neurons and a subsequent estrogen-induced gonadotropin surge (Pau and Spies, 1997). In the present study, male hornyhead turbot exhibit plasma E2 concentrations as high, and sometimes higher, than that of reproductive females (Reyes, 2006; Petschauer, 2008; Reyes et al., 2012). These reproductive female concentrations of E2 may conceivably cause loss of negative feedback regulation by E2 at the pituitary level, potentially elevating the release of luteinizing hormone and resulting in subsequently increased testicular steroid production. Testosterone (T) is an immediate precursor to E2 and is converted by the steroidogenic enzyme P450arom. In the present study's screening effort, P450arom protein was not detected; however, in previous studies, males exhibiting high E2 phenotypes had increased mRNA transcript levels of P450arom when compared to low E2 males (Hagstrom, 2008; Iwanski, 2011). A study by Horvath et al. (1997), however, demonstrated that male rats lack estrogen-induced plasticity in the arcuate nucleus 24 hr after being injected with 100 μg E2. In the present study, however, male hornyhead turbot are suspected of having long-term E2 exposure, as fish of varying sizes (age) exhibit high plasma E2.

In previous studies, testicular mRNA transcript levels of other enzymes involved in estrogen production, such as StAR, were upregulated in hornyhead turbot males

exhibiting a high E2 phenotype (Hagstrom, 2008). These enzymes were not identified in the proteomes evaluated in the present study; however, other enzymes involved in steroidogenic pathways demonstrated significant differences in protein levels between the different sample groups. L-3-hydroxyacyl-coenzyme-A dehydrogenase (SCHAD) showed an increase in the both the wild-caught high E2 group and the E2-treated group. This is a multifunctional enzyme that has several alternative names throughout its history in the literature, including SC-HMAD (1995), HADH II (1996), ERAB (1997), SCHAD (1998), ABAD (1999), and 17β-HSD-10 (2001) (Yang et al., 2005). The latter, 17β-HSD-10, is the internationally agreed upon classification for this mitochondrial hydroxysteroid dehydrogenase. It is a single-domain, multifunctional enzyme, and its catalytic efficiency in the oxidation of E2 is comparable with 17β-HSD-4 (He et al., 1999; Nordling et al., 2001). It can inactivate E2 by converting it to E1, and it also has strong 3α-HSD activity, converting 5α-androstanediol and allopregnanolone into 5α-dihydrotestosterone (5α-DHT) and 5α-dihydroprogresterone (He and Yang, 2006). This suggests a potential decrease in allopregnanolone, a modulator of $GABA_A$ receptors, and 5α-androstanediol, a weak estrogen that binds to estrogen receptor beta (ERβ). This may also cause an increase in 5α-DHT, a potent androgen, and an increase in 5α-dihydroprogesterone. This potential increase in 5α-DHT production from the 5α-androstanediol precursor may reduce the demand from testosterone, another 5α-DHT precursor. This decreased demand would increase the available testosterone, allowing for it to be used as a precursor for E2. In addition, Jazbutyte et al. (2009) reports that estrogen receptor alpha (ERα) interacts with 17β-HSD-10 in the mitochondria, and it is implicated in the regulation of intracellular estrogen levels. When E2 concentration is

low, ERα forms a complex with 17β-HSD-10; when E2 increases, its higher binding affinity for ERα displaces 17β-HSD-10, subsequently causing the β-oxidation of E2 into E1. In a previous study, hornyhead turbot with high E2 also demonstrated high E1 levels (Iwanski 2011). The observed up-regulation of this enzyme in both the wild-caught high E2 group and E2-treated group suggests that this is a likely a response to elevated E2, rather than a molecular mechanism contributing to the high E2 phenotype.

Another steroidogenic enzyme, P450-11β, exhibited a decrease in two isoforms in the wild-caught high E2 group, and was negatively correlated with E2. P450-11β is an important steroidogenic enzyme that is involved in the biosynthesis of 11-ketotestosterone (11-KT) in the testis of fish, and the biosynthesis of glucocorticoids (e.g., cortisol) in the interrenal tissues (fish equivalent of adrenal cortex). In a study by Jiang et al. (1996), hCG induced P450-11β transcription in eel testis, increasing 11-KT production 1-3 days after injection. After 6-9 days, there was a subsequent decrease of P450-11β transcript levels, likely due to negative feedback from elevated 11-KT serum concentration. The reduction observed in P450-11β in the high E2 group may be due to elevated 11-KT serum concentration; however, 11-KT was not measured in the present study. P450-11β transcript levels are highly expressed in the testis during stage I and II of spermatogenesis, followed by a decrease during late spermatogenesis (Liu et al., 2000). Another study treated male rainbow trout with 20 μg E2/g BW for 10 days during testis differentiation, and also post-differentiation, and looked at steroidogenic enzyme mRNA levels in the testis. In the differentiating group treated with E2, P450-11β transcripts were absent; in the post-differentiation group, there was a rapid decrease in P450-11β transcripts in response to E2 treatment (Govoroun et al., 2001). This evidence

suggests that reduced levels of P450-11β is in response to the high E2 concentration in the hornyhead turbot, and may be having a detrimental effect on their reproductive capacity and a reduction in 11-KT production. A previous study, however, suggests that 11-KT levels are not impaired in high E2 males, as demonstrated by a seasonal increase in 11-KT associated with spawning (Reyes et al., 2012).

Altered Testicular Response to Estrogen

E2 is an important regulator of growth, differentiation, and cellular functions in a wide array of target tissues. Its actions take place in not only the male and female reproductive tissues, but in skeletal, cardiovascular, and neural tissues as well. The effects of E2 are can be mediated through a wide variety of estrogen receptors (ERs). ERα and ERβ are the most well-known ERs, however, a third estrogen receptor, ERγ, was identified in Atlantic croaker testis (Hawkins et al., 2000). Another ER includes G protein-coupled estrogen receptor (GPER), which is a membrane surface receptor that activates a protein kinase C (Thomas et al., 2010). The molecular mechanisms of E2 signaling via estrogen receptors (ERs) have many signaling pathways, but their actions are primarily either ligand-dependent transcription, or cell-surface signaling, as reviewed by Hall et al. (2001). The ligand-dependent mechanism of ER action involves the binding of ligand (i.e. E2) to a nuclear receptor (NR). This forms a complex which has a DNA-binding domain that binds to an estrogen response element (ERE) on DNA, promoting transcription of estrogen responsive genes. Interestingly, ERs are not the only NRs that have binding affinity to EREs. An element of the ER DNA-binding domain, the P-box, defines the DNA-binding specificity for NRs. The P-box of ER is similar to the P-boxes of retinoic acid receptor (RAR), retinoid X receptor (RXR), estrogen-related

receptor (ERR), and thyroid hormone receptor (TR), meaning that each NR has the capability of binding to EREs (Pettersson and Gustafsson, 2001). In addition, the binding of NRs to DNA often involves the dimerization of receptors, cross-talk among NRs, and coactivation and corepression factors (Pettersson and Gustafsson, 2001; Bolt et al., 2013).

In the present study, RXRβ was decreased in the wild-caught high E2 group and demonstrated a negative correlation with E2. RXRβ interacts with multiple hormone response elements, including the ERE. In the human breast cancer cell line MCF-7, RXRβ regulates ERE-driven reporter activity in a ligand-dependent pathway, causing inhibition in the absence of the RXR ligand 9-*cis*-retinoic acid (RA). RXRβ also inhibits the action of ER with a mechanism more complex than simple competition of ERE binding (Segars et al., 1993). In rat hippocampal slice cultures, RXRβ stimulates steroidogenesis in response to RA treatments by increasing the levels of steroidogenic enzymes P450-17α and P450arom (Munetsuna et al., 2009). This stimulated E2 and T synthesis 2-fold, consistent with the 1.7-fold increase of P450-17α, which produces precursors for E2 and T. Interestingly, the increase P450arom was not essential for enhancing estradiol synthesis. A study by Nunez et al. (1996) observed the presence of both RXR and peroxisome proliferator-activated receptor (PPAR) in reproductive tissue. They formed a heterodimer that was capable of activating estrogen responsive genes through the direct binding to EREs. RXRβ can also form a heterodimer with androgen receptor (AR), and can suppress AR target genes in the presence of RA, or act as a coactivator of AR target genes in the absence of a ligand (Chuang et al., 2005). These studies suggest that RXRβ possesses a multifaceted role in the regulation in ER- and AR-

target genes, potentially influencing the response to E2 and possibly steroid hormone synthesis. For future studies, measurements of RA concentration may help to elucidate the regulatory role of RXRβ in the transcription of mentioned genes.

A transcription factor, BRG1, increased in the wild-caught high E2 group, and had a strong positive correlation with E2. BRG1 is the central catalytic ATPase subunit in the chromatin-modifying SWI/SNF complex, which disrupts the chromatin architecture of target promoters (Xu et al., 2004; Trotter and Archer, 2007). BRG1 is an essential component for nuclear receptor-mediated transcription, and the availability of ATP as a substrate for BRG1 is necessary for its chromatin-remodeling activity (Xu et al., 2004). In some cases, BRG1 can also act as a corepressor by forming complexes that remodel target promoters, silencing the genes (Underhill et al., 2000; Gaston and Jayaraman, 2003). Furthermore, there is evidence that BRG1 is recruited to ER in response to estrogen, and the subsequent transcriptional activation of estrogen-responsive genes requires functional BRG1 (DiRenzo et al., 2000). The mechanism of action for this coactivation is regulated by simultaneous histone acetylation by other factors. In the presence of estrogen antagonists, however, BRG1 is required for transcriptional repression of estrogen-responsive genes (Zhang et al., 2007). The increase in BRG1 in the wild-caught high E2 group in the current study, and its correlation with E2, may indicate that this is a candidate molecular mechanism involved in the alteration of estrogen-responsive gene function. Its role in histone acetylation and estrogen-responsive gene activation may contribute to an imbalance of estrogen-responsive transcript levels, potentially resulting in a cascade of effects on hormone feedback, and the activation or repression of other genes.

Another transcription factor, prohibitin (PHB), works with BRG1 as a corepressor of ER-driven transcription suppression in the presence of an estrogen antagonist (Zhang et al., 2007). The PHB-mediated repression is dependent on the presence of BRG1, and conversely, PHB is required for BRG1 recruitment. Another role PHB has been reported to play is in the repression of estrogen signaling via ERα. In the absence of E2, PHB associates with the estrogen-regulated pS2 promoter of ERα target genes, but in the presence of E2, it dissociates from the promoter (He et al., 2008). In the present study, there was no detected change in PHB in the high E2 group; however, there was an increase in PHB in response to E2-treatment. This suggests that there may be increased PHB–mediated repression of estrogen-induced ERE activity in the E2-treated fish. When estrogen levels are high, the repression of estrogen-responsive genes can be important in regulating negative feedback (Tee et al., 2004; Docquier et al., 2013). The undetected change of PHB in wild-caught high E2, in conjunction with elevated BRG1, suggests in imbalance of coactivation and corepression of estrogen-responsive genes and subsequent protein translation in a physiological system subjected to high circulating E2 plasma concentration. This imbalance may, in turn, affect the complex network of factors involved in the regulation of hormone levels.

CCAR1 is a transcription factor that acts as a coactivator of AR by promoting and stabilizing the binding of the AR/GATA2 complex to AR-responsive genes, increasing transcriptional activity (Seo et al., 2013). The depletion of CCAR1, however, reduced androgen-dependent expression of AR-target genes (Seo et al., 2013). Reduction of CCAR1 also inhibits estrogen-induced expression of ER-responsive genes, and is a key regulator of nuclear receptor transcription complex recruitment (Kim et al., 2008; Yu et

al., 2011). In the present study, CCAR1 increased in the wild-caught high E2 group and had a positive correlation with E2. In response to E2-treatment, however, two isoforms were identified, both of which decreased. These findings suggest that CCAR1 may be increasing the transcriptional activity of ER- and AR-responsive genes and/or protein translation in the wild-caught high E2 group. In the E2-treated group, the decreased CCAR1 suggests an inhibition of AR- and ER-responsive genes, and that the increase observed in the wild-caught high E2 group may be due to environmental factors.

Several other proteins involved in transcriptional regulation were identified in this study. GTF3C5 decreased in the E2-treated group. GTF3C5 directly interacts with steroid receptor RNA activator protein (SRAP), a positive regulator of AR, ERα, ERβ, glucocorticoid receptor (GR), and PPARγ (Chooniedass-Kothari et al., 2010). Eaf6 decreased in the wild-caught high E2 group, and also negatively correlated with E2. Eaf6 is a subunit of the NuA4 histone acetyltransferase complex, which is involved in chromatin-remodeling (Doyon and Cote, 2004). The function of NuA4, however, is not affected by the absence of Eaf6 (Mitchell et al., 2008). This suggests that decreased Eaf6 has a negligible effect on the high E2 phenotype. CDC5L, a cell cycle regulator and pre-mRNA splicing factor, increased in the high E2 group. Pre-mRNA splicing by spliceosomes is a necessary step in the formation of translatable mRNA, performing intron excision, as reviewed by Jurica and Moore (2003). CDC5L is an essential component in the second catalytic step of pre-mRNA splicing, and its function is key in the regulation of mitotic progression (Ajuh et al., 2001; Mu et al., 2014). This increase in CDC5L may indicate that there are more pre-mRNA transcripts available to be spliced into translatable mRNA.

Transcriptional regulation can alter the available mRNA transcript levels of genes, but these transcripts must then be translated by ribosomes into proteins to become physiological effectors. SDA1 is a protein required for exporting 60S pre-ribosomal subunits into the cytoplasm (Saracino et al., 2004). Nuclear 60S pre-ribosomal subunits begin to mature in the nucleoplasm and are transported into the cytoplasm where they become the 60S ribosome. This transportation of pre-ribosomal RNAs is fundamental for gene expression, as reviewed by Kohler and Hurt (2007). An SDA1 homolog increased in the wild-caught high E2 group and had a strong positive correlation with E2, but had no changes detected in the E2-treated group. This increase in the SDA1 homolog suggests an increased availability of the 60S ribosome in the cytoplasm, increasing the potential for mRNA translation. This further suggests that there is potentially increased protein and enzyme production in the wild-caught high E2 group, and it is not a secondary effect of increased E2 plasma concentration. Additionally, the effects of altered transcript levels in the high E2 group may potentially be amplified by this increased translational availability. This supports the idea that the increase of mRNA transcript levels of steroidogenic enzymes (StAR, P450arom) detected in hornyhead turbot testis (Hagstrom, 2008; Iwanski, 2011) are being translated into functional enzymes.

Altered Cellular Metabolism in Response to Environmental Factors

Having balanced cellular energetics is crucial for the proper function and growth of an organism. Physiological processes such as fuel intake, storage, and expenditure are controlled by the metabolism of endogenous and exogenous compounds, which rely on the coordinated action of a suite of transcription factors and coregulators. Hormonal

control of these processes has been well documented in the literature, as reviewed by Giguere (2008), demonstrating the transcriptional roles that nuclear hormone receptors play in homeostasis, including GR, TR, PPAR, ERRα, and ERRβ. Another study reported that ERRγ is a transcriptional regulator of phosphatidic acid phosphatase, LIPIN1 mRNA expression (a gene involved in the regulation of intracellular lipid levels), and also inhibits hepatic insulin signaling (Kim et al., 2011). Furthermore, LIPIN1 regulates PPARγ transcriptional activity (Kim et al., 2013), demonstrating the complex regulatory interactions between NHRs, cellular energetics, and metabolic function. In the present study, numerous proteins and enzymes involved in cellular metabolism were found to differ among sample groups, many of which were correlated with E2.

The cytochrome P450 enzymes are a superfamily of mono-oxygenases that, in general, function to make compounds more hydrophilic, improving their excretability. They are found in all kingdoms of life, and are important in the detoxification of xenobiotics, and the synthesis and breakdown of endogenous compounds, such as fatty acids and hormones. In the present study, there were two cytochrome P450 enzymes that were altered in the wild-caught high E2 group, but no changes were noted in response to E2 treatment. The P450 IV B1 enzyme increased in the high E2 group and was positively correlated with E2, while a member of the cytochrome P450 2N family decreased in the high E2 group and was not correlated with E2.

The P450 IVB1 enzyme bioactivates and metabolizes a wide variety of endogenous substrates (e.g., sterols, thromboxanes, vitamins, arachidonic acid), and xenobiotics, such as valproic acid (pharmaceutical anti-depressant), *p*-xylene (manufacturing chemical used to make polyester), and numerous aromatic amines (Baer and Rettie, 2006). The

increases observed in this enzyme may indicate an increased demand for endobiotic and/or xenobiotic metabolism. Furthermore, evidence shows potential regulation of the CYP4B1 gene by 5α-DHT (Isern and Meseguer, 2003). Interestingly, this coincides with the increase of 17β-HSD-10 in the high E2 group, a previously described enzyme that directly contributes to the production of 5α-DHT. Additionally, CYP4B1 gene expression has been shown to be mediated by RXR binding (Waxman, 1999), a NR that was also altered in the wild-caught high E2 group, as described above.

The P450 2N enzymes are known for their ability to metabolize arachidonic acid, converting it into epoxyeicosatrienoic acids (Oleksiak et al., 2000). Arachidonic acid exhibits non-competitive inhibition of the specific binding of estrogen to the ER in peripheral tissues such as the prostate, while having a potentiating effect in brain ERs (Kato, 1989). Decreases of this enzyme in the wild-caught high E2 group may be causing an increase in free arachidonic acid, promoting the inhibition of estrogen signaling. Furthermore, there was no change in the E2 treatment group, indicating that this may be a contributing factor to the high E2 phenotype found in wild-caught male hornyhead turbot.

A major mechanism for the metabolism of endogenous and exogenous compounds is oxidation-reduction, a reaction catalyzed by enzymes called oxidoreductases. One such enzyme, aldehyde reductase, was identified in the present study, and increased in the wild-caught high E2 group. This enzyme catalyzes the reduction of aldehydes and ketosteroids, while also catalyzing the oxidation of hydroxysteroids and *trans*-dihydrodiols of PAHs (Hyndman et al., 2003). It also reduces 16-ketoestrone into 16-keto-α-estradiol, which is an immediate precursor of the weak estrogen E3 (Stimmel et

al., 1950; O'connor et al., 1999). The increase of this enzyme suggests testicular cells are being exposed to environmental factors, such as oxidative stressors, and increasing this enzyme may be a response to mitigate the damaging effects of free radicals.

Many of these metabolic reactions serve to regulate the energetics of a cell. Aconitase 2, for example, is an enzyme that catalyzes the interconversion of citrate to isocitrate in the citric acid cycle—a process that generates energy in the form of ATP, through the oxidation of acetyl-CoA. In the wild-caught high E2 group, there was an increase in this enzyme, suggesting altered fuel metabolism. This may be a cellular response to increase fuels to support increased energy requirements due to cell physiological stress. Interestingly, several key enzymes of the citric acid cycle, including aconitase 2, are regulated by E2, DHT, and EDCs, as reviewed by Chen et al. (2009). A study by Pastorelli et al. (2005) found an increased expression of mitochondrial aconitase 2 in ovarectomized female mice treated with E2, providing evidence for aconitase 2 expression being responsive to E2. In the present study, however, there was no detected change in aconitase 2 in response to E2 treatment, although comparing these studies may be ineffectual due to the sex and species differences. This may indicate an environmental impact on this enzyme in the wild-caught high E2 group, perhaps in response to 5α-DHT or the presence of EDCs.

Closing Comments

In the present study, a high concentration of E2 in the blood plasma of male hornyhead turbot from SMB was confirmed, and the phenotype of the impacted testicular tissue was characterized at the molecular (protein) level. A key finding of this study is the quantified differences of proteins between sample groups, many of which are

involved in transcriptional regulation. Coactivators and corepressors of NRs counter each other to form a critical balance of transcriptional regulation (as outlined in the Discussion section), serving to maintain appropriate responses to hormones, such as negative feedback.

The abundance profile of these proteins suggests that there may be a suppressed testicular response to estrogen in the high E2 group. Reduced RXRβ, increased BRG1, and an unchanged level of PHB could hypothetically be working together to reduce the E2 responses via inhibition of ER signaling. This proteomics study did not identify ERs, but changes in ER abundance profiles could also lead to a reduced testicular response to high E2. These factors may be contributing to an altered hormone response, impeding normal hormone level regulation.

The present data also show that there was an overall increase in enzymes involved in cellular metabolism and oxidative stress in the wild-caught high E2 group, but these enzymes had no apparent changes between the experimental control and E2-treated groups, with the exception of creatine kinase. This may suggest that these fish are experiencing oxidative stress, potentially due to their environment. Hepatic tissue samples were collected in tangent with the plasma and testis samples used in this study, allowing for future contaminant analyses. Such analyses may provide further insights into the impact that the environment may be having on the physiological status of these fish in the wild.

The use of proteomics in this study has allowed for a novel perspective on the complex physiological status of these impacted fish. The research led to several interesting new hypotheses about the potential causes and effects related to this aberrant

phenotype. Future studies should test these hypotheses, as well as continue to characterize the unidentified proteins that exhibited obvious changes in this study. A more targeted proteomics approach (i.e. isolation of endoplasmic reticula to enrich for metabolic/steroidogenic enzyme proteins) may also provide greater resolution, and allow for the detection of changes unseen in this study.

APPENDIX

TABLES AND FIGURES

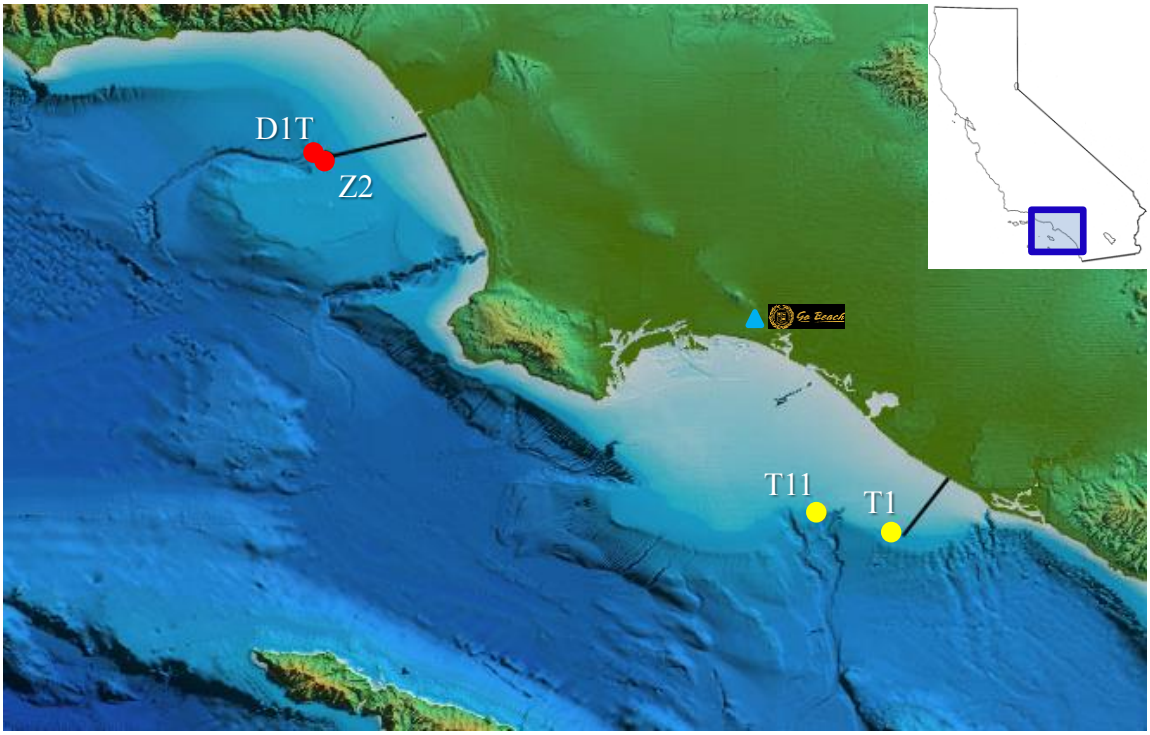

FIGURE 1. Sampling sites used to collect hornyhead turbot for this study, located within the ocean monitoring jurisdictions of the Orange County Sanitation District (OCSD) and the City of Los Angeles Environmental Monitoring Division (CLAEMD). OCSD site T1 is located 6.7 km offshore, at 60 m depth, and is proximal to the treated wastewater discharge ("outfall") environment. OCSD site T11, also at 60 m depth, is located 7.7 km north of T1. CLAEMD sites Z2 and D1T are located 8.0 km offshore, at 60 m depth, within proximity of the HTP outfall.

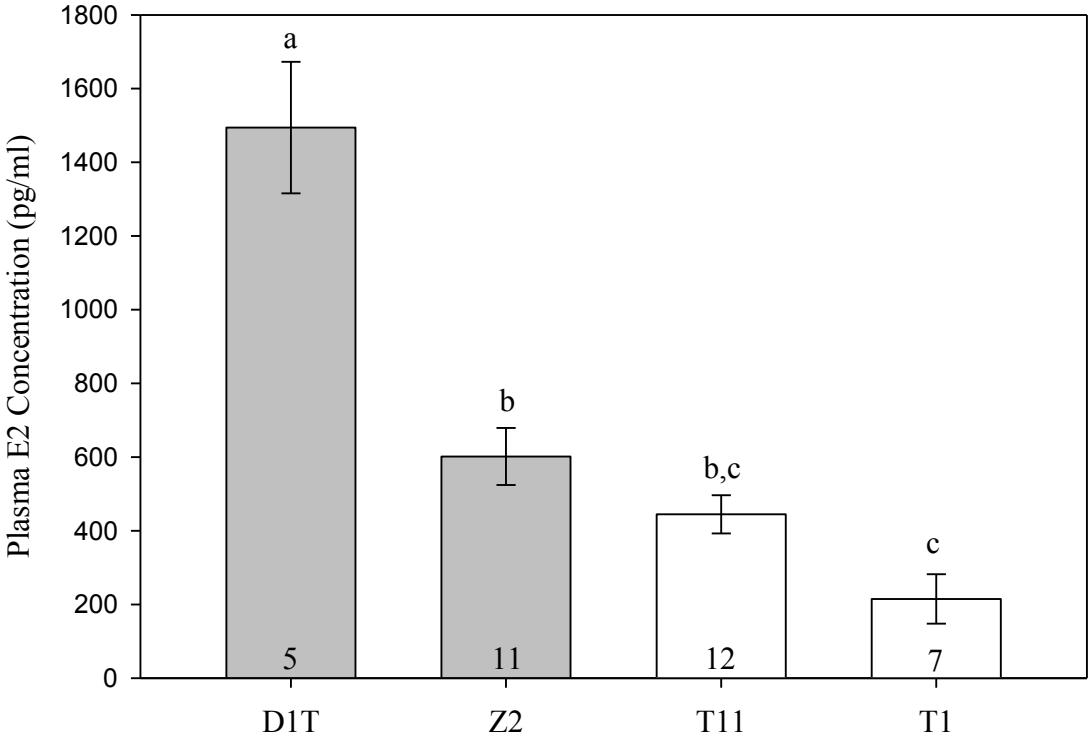

FIGURE 2. Plasma 17β-estradiol (E2) concentrations in hornyhead turbot males sampled from OC sites T11 and T1, and SMB sites Z2 and D1T. E2 was measured by radioimmunoassay, with concentration expressed as mean ± SEM, with *n* at the bottom of each bar. Different letters denote means that are significantly different ($p<0.05$).

TABLE 1. Differences in Testicular Protein Abundance in the High E2 and Low E2 Groups

Study ID	Protein #	Protein ID	Low E2 Group Mean ± SEM (n)	High E2 Group Mean ± SEM (n)	Fold Difference	*p*
1b	1645	L-3-hydroxyacyl-CoA dehydrogenase, partial	640565 ± 57808 (4)	865082 ± 69188 (6)	+1.4	0.0416
2a	459	Cytochrome P450 11-beta, partial	458168 ± 37407 (4)	339272 ± 27866 (6)	-1.4	0.0347
2b	680	Cytochrome P450 11-beta	211675 ± 11750 (4)	170185 ± 5867 (6)	-1.2	0.0088
2c	706	Cytochrome P450 11-beta	136774 ± 12464 (4)	97500 ± 7839 (6)	-1.4	0.0236
3	1930	Retinoid X receptor, beta	164927 ± 8744 (4)	133050 ± 6671 (6)	-1.2	0.0240
4	975	Transcription activator BRG1-like, partial	111257 ± 7907 (4)	137450 ± 4623 (6)	+1.2	0.0131
6a	927	CCAR1 protein, partial	425290 ± 28366 (4)	495988 ± 14844 (6)	+1.2	0.0381
8	1567	Chromatin modification-related protein eaf6	1399203 ± 58136 (4)	1177014 ± 39669 (6)	-1.2	0.0124
9	2219	Cell division cycle 5-like protein-like	159511 ± 12442 (4)	261044 ± 26199 (6)	+1.6	0.0047
10	989	SDA1 homolog	343503 ± 8639 (4)	422477 ± 16775 (6)	+1.2	0.0053
13	2214	Zinc finger protein 618-like	148126 ± 14477 (4)	94390 ± 4262 (6)	-1.6	0.0022
14	326	Putative nuclease HARBI1-like	140279 ± 3816 (4)	165990 ± 6102 (6)	+1.2	0.0139
15	823	Cytochrome p450 IV B1	246246 ± 6287 (4)	292354 ± 9676 (6)	+1.2	0.0065
16	435	Cytochrome P450 family 2 subfamily N protein	85762 ± 5063 (4)	57690 ± 7455 (6)	-1.5	0.0275
17	1641	Aldo-keto reductase family 1, member A1a	538507 ± 35229 (4)	670189 ± 45055 (6)	+1.2	0.0495
18	144	Nucleoside diphosphate kinase B	1319965 ± 51651 (4)	1650512 ± 104392 (6)	+1.3	0.0338
19	2277	Aconitase 2, mitochondrial	562747 ± 19185 (4)	652708 ± 26066 (6)	+1.2	0.0371
20	446	CTP synthase 1	75066 ± 7531 (4)	129794 ± 11691 (6)	+1.7	0.0027
21	1920	Muscle-type creatine kinase CKM1 or Creatine kinase, testis isozyme	2430894 ± 143554 (4)	2022261 ± 66891 (6)	-1.2	0.0195
22	1306	N-alpha-acetyltransferase 15, NatA auxiliary subunit isoform 1	351572 ± 27661 (4)	414137 ± 10803 (6)	+1.2	0.0428
26	2283	Vacuolar sorting protein 54 long isoform	224621 ± 14974 (4)	166022 ± 9954 (6)	-1.4	0.0107
27	438	Apolipoprotein A-IV	217862 ± 15158 (4)	276574 ± 16270 (6)	+1.3	0.0369
31	1795	unnamed protein product	319963 ± 16150 (4)	438957 ± 34833 (6)	+1.4	0.0238
32	1803	uncharacterized protein LOC678611	943665 ± 83627 (4)	736800 ± 39246 (6)	-1.3	0.0364
33	1960	unnamed protein product	229852 ± 78094 (4)	451939 ± 79758 (6)	+2.0	0.0428
34	1990	unnamed protein product	246883 ± 38912 (4)	347721 ± 26111 (6)	+1.4	0.0488
35	2194	unnamed protein product	204884 ± 14059 (4)	276289 ± 22573 (6)	+1.3	0.0416
--	2334		364291 ± 15708 (4)	287552 ± 8049 (6)	-1.3	0.0012

TABLE 1. Continued.

Study ID	Protein #	Protein ID	Low E2 Group Mean ± SEM (n)	High E2 Group Mean ± SEM (n)	Fold Difference	*p*
--	1906		89900 ± 6477 (4)	128694 ± 6517 (6)	+1.4	0.0039
--	1076		223812 ± 11414 (4)	177112 ± 7338 (6)	-1.3	0.0076
--	1774		278927 ± 16807 (4)	355922 ± 15480 (6)	+1.3	0.0092
--	2430		320826 ± 12610 (4)	369539 ± 8786 (6)	+1.2	0.0104
--	2337		241082 ± 5309 (4)	185334 ± 13220 (6)	-1.3	0.0110
--	2357		67361 ± 3227 (4)	104019 ± 10249 (6)	+1.5	0.0121
--	2403		446685 ± 26993 (4)	584571 ± 33710 (6)	+1.3	0.0135
--	2419		144798 ± 13219 (4)	100464 ± 7481 (6)	-1.4	0.0141
--	922		332044 ± 37239 (4)	472467 ± 35196 (6)	+1.4	0.0156
--	874		176132 ± 18682 (4)	124909 ± 8472 (6)	-1.4	0.0201
--	2191		191694 ± 22666 (4)	134818 ± 8906 (6)	-1.4	0.0205
--	2288		436985 ± 8955 (4)	250192 ± 37256 (6)	-1.7	0.0211
--	984		41489 ± 3265 (4)	61708 ± 5795 (6)	+1.5	0.0214
--	2407		104037 ± 9168 (4)	137271 ± 7943 (6)	+1.3	0.0218
--	617		1344570 ± 68695 (4)	1619078 ± 67125 (6)	+1.2	0.0228
--	1523		2655038 ± 250180 (4)	1978369 ± 111239 (6)	-1.3	0.0256
--	1093		150740 ± 20045 (4)	110376 ± 5220 (6)	-1.4	0.0300
--	2098		183680 ± 8909 (4)	267600 ± 30959 (6)	+1.5	0.0324
--	2390		392381 ± 44115 (4)	565808 ± 47835 (6)	+1.4	0.0368
--	593		457094 ± 27014 (4)	362934 ± 24757 (6)	-1.3	0.0375
--	1972		659100 ± 46621 (4)	814454 ± 41368 (6)	+1.2	0.0388
--	1594		382332 ± 29303 (4)	305672 ± 14951 (6)	-1.3	0.0403
--	2207		525087 ± 30940 (4)	427772 ± 25509 (6)	-1.2	0.0491

Note: Normalized protein volumes (arbitrary units) are given as mean ± SEM; *n*=4 for the Low E2 group and *n*=6 for the High E2 group. P-values were generated using Student's t-test.

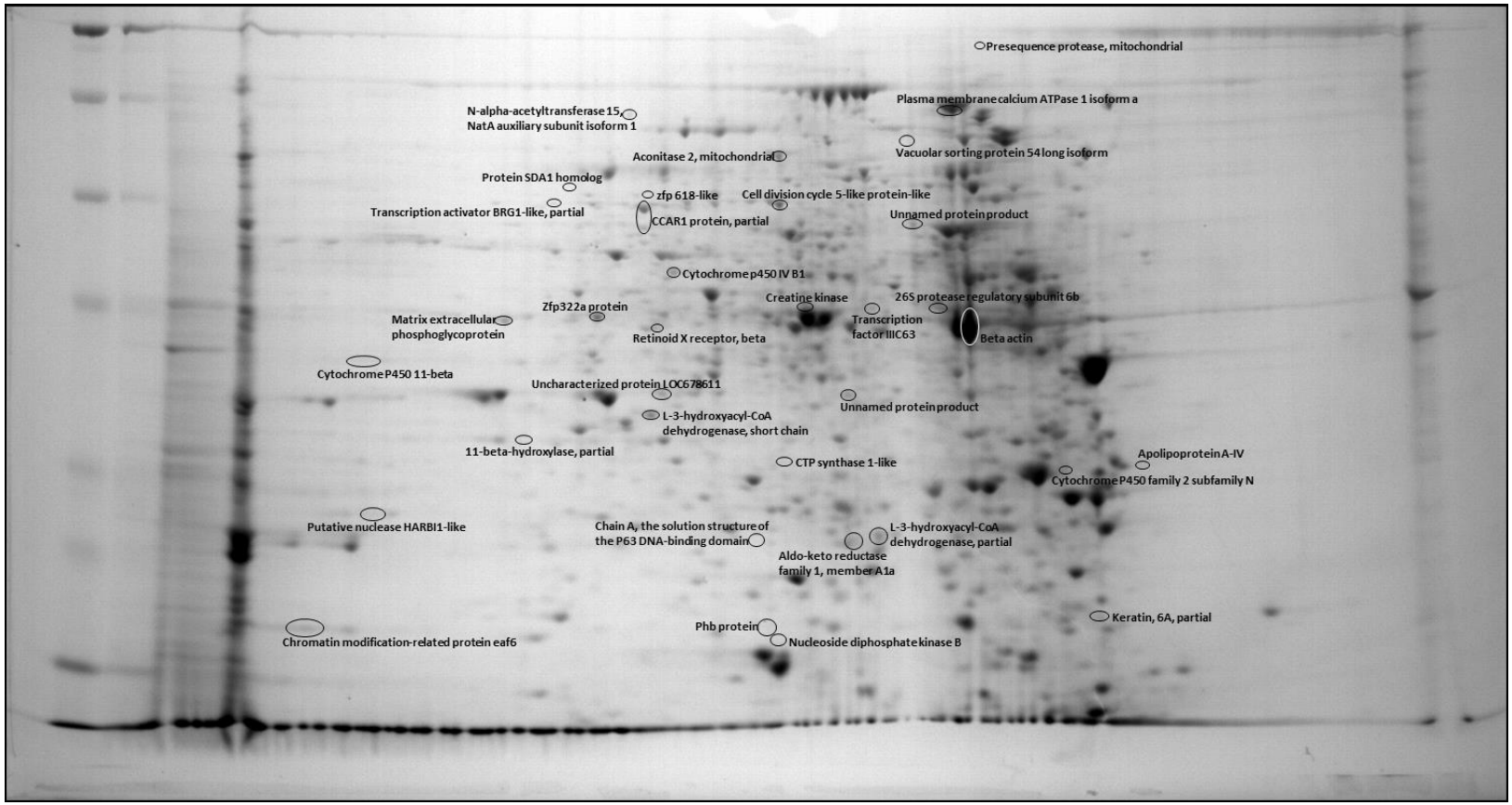

FIGURE 3. Representative 2-D gel stained with Coomassie Blue to visualize the hornyhead turbot testicular proteome. Circled spots indicate proteins whose molecular identities have been determined in this study. Proteins spots were mapped using Nonlinear Dynamics SameSpots™ software. The proteome shown is from an individual used in the E2 injection study (CLAEMD 9326, fish # HT17). Not all proteins identified across all testicular samples can be visualized on this gel image.

TABLE 2. Proteins Identified by Peptide Mass Fingerprint Analysis using MALDI-TOF/TOF Mass Spectrometry

Category	Study ID	Protein #	Protein ID	Accession No.	Protein MW	Protein PI	Protein Score	Protein Score C.I. %	Total Ion Score	Total Ion Score C.I. %
Steroidogenic	1a	2508	L-3-hydroxyacyl-coenzyme A dehydrogenase	gi|54038609	34474.9	8.56	59	0	49	88.505
	1b	1645	L-3-hydroxyacyl-CoA dehydrogenase, partial	gi|444292668	29709	7.19	54	0	42	48.858
	2a	459	Cytochrome P450 11-beta, partial	gi|388458940	37810.7	9.24	31	0	26	0
	2b	680	Cytochrome P450 11-beta	gi|157311691	60893.9	9.76	42	0	26	0
	2c	706	Cytochrome P450 11-beta	gi|157311691	60893.9	9.76	43	0	26	0
Nuclear	3	1930	Retinoid x receptor, beta	gi|94733756	54140	8.14	67	94.652		
	4	975	Transcription activator BRG1-like, partial	gi|326681197	89855.4	9.25	27	0		
	5	1556	Prohibitin	gi|41351079	29723.8	5.28	72	90.486	48	82.46
	6a	927	CCAR1 protein, partial	gi|34191778	92516.2	8.75	32	0		
	6b	981	CCAR1 protein, partial	gi|37805381	93140.7	9.02	26	0		
	7	747	Transcription factor IIIC63	gi|5281316	60048.3	6.64	29	0	19	0
	8	1567	Chromatin modification-related protein eaf6	gi|225707780	24207.1	9.58	36	0	27	0
	9	2219	Cell division cycle 5-like protein-like	gi|410900814	92713.8	6.73	64	89.329	28	0
	10	989	SDA1 homolog	gi|27545219	79342.2	9.29	26	0		
	11	275	P63 DNA-binding domain	gi|212374861	26272	8.42	36	0		
	12	720	Zinc finger protein 322a	gi|16877820	47525.7	8.92	32	0	25	0
	13	2214	Zinc finger protein 618-like	gi|432887596	98227.1	8.33	70	97.32		
	14	326	Putative nuclease HARBI1-like	gi|348543862	39471.5	9.57	34	0	26	0
Metabolism	15	823	Cytochrome p450 IV B1	gi|180969	59437.3	8.61	30	0	20	0
	16	435	Cytochrome P450 family 2 subfamily N protein	gi|336111742	48264.9	5.88	59	67.775	25	0
	17	1641	Aldo-keto reductase family 1 member A1a	gi|50603905	37024.9	6.86	31	0	18	0
	18	144	Nucleoside diphosphate kinase B	gi|259089317	17303.6	7.68	64	89.81	50	97.317
	19	2277	Aconitase 2, mitochondrial	gi|37046702	85547.8	7.58	91	99.9	83	99.995
	20	446	CTP synthase 1-like	gi|348526077	66632.7	6.05	40	0	40	81.429

TABLE 2. Continued.

Category	Study ID	Protein #	Protein ID	Accession No.	Protein MW	Protein PI	Protein Score	Protein Score C.I. %	Total Ion Score	Total Ion Score C.I. %
Metabolism	21	1920	Muscle-type creatine kinase CKM1	gi|268308331	43204.8	6.58	270	100	242	100
			or Creatine kinase, testis isozyme	UTS1_PLAFE	43261.9	6.5	35	0	35	97.494
	22	1306	N-alpha-acetyltransferase 15, NatA auxiliary subunit isoform 1	gi|348533730	101852	7.71	32	0	24	0
	23	1944	26S protease regulatory subunit 6b	gi|308322395	47363.4	5.07	43	0	26	0
Cell Signaling	24	1478	Metalloproteinase-1	PREP_DANRE	115753.9	6	22	0	22	41.125
	25	1329	Plasma membrane calcium ATPase 1 isoform a	gi|82399351	135357.7	5.58	36	0		
Cellular Transport	26	2283	Vacuolar sorting protein 54 long isoform	gi|42564952	111545.3	6.1	33	0		
	27	438	Apolipoprotein A-IV	gi|206598064	29149	4.7	27	0		
Structural	28	716	Matrix extracellular phosphoglycoprotein	gi|240006679	56050.8	8.62	35	0		
	29	2527	Beta actin	gi|371639041	42241	5.48	626	100	522	100
	30	183	Keratin, 6A, partial	gi|355698804	22394.3	4.97	39	0	26	0
Unknown	31	1795	unnamed protein product	gi|47227272	38278.5	6.58	60	69.226		
	32	1803	uncharacterized protein LOC678611	gi|94536645	51127.9	8.17	58	58.487	28	0
	33	1960	unnamed protein product	gi|47216798	71072.9	8.88	66	92.446	45	81.661
	34	1990	unnamed protein product	gi|47216798	71072.9	8.88	77	99.501	41	44.361
	35	2194	unnamed protein product	gi|47210887	77663	6.07	59	65.471		

Note: Protein spots were assigned a number according to SameSpots™ software (Nonlinear Dynamics, Inc.) mapping. Protein identifications were generated by MASCOT database searches and matching with the NCBI accession number, theoretical molecular weight (MW; kDa), and isoelectric focusing point (pI). The strength of the identification is indicated by the protein score, protein score confidence index, total ion score, and total ion confidence index.

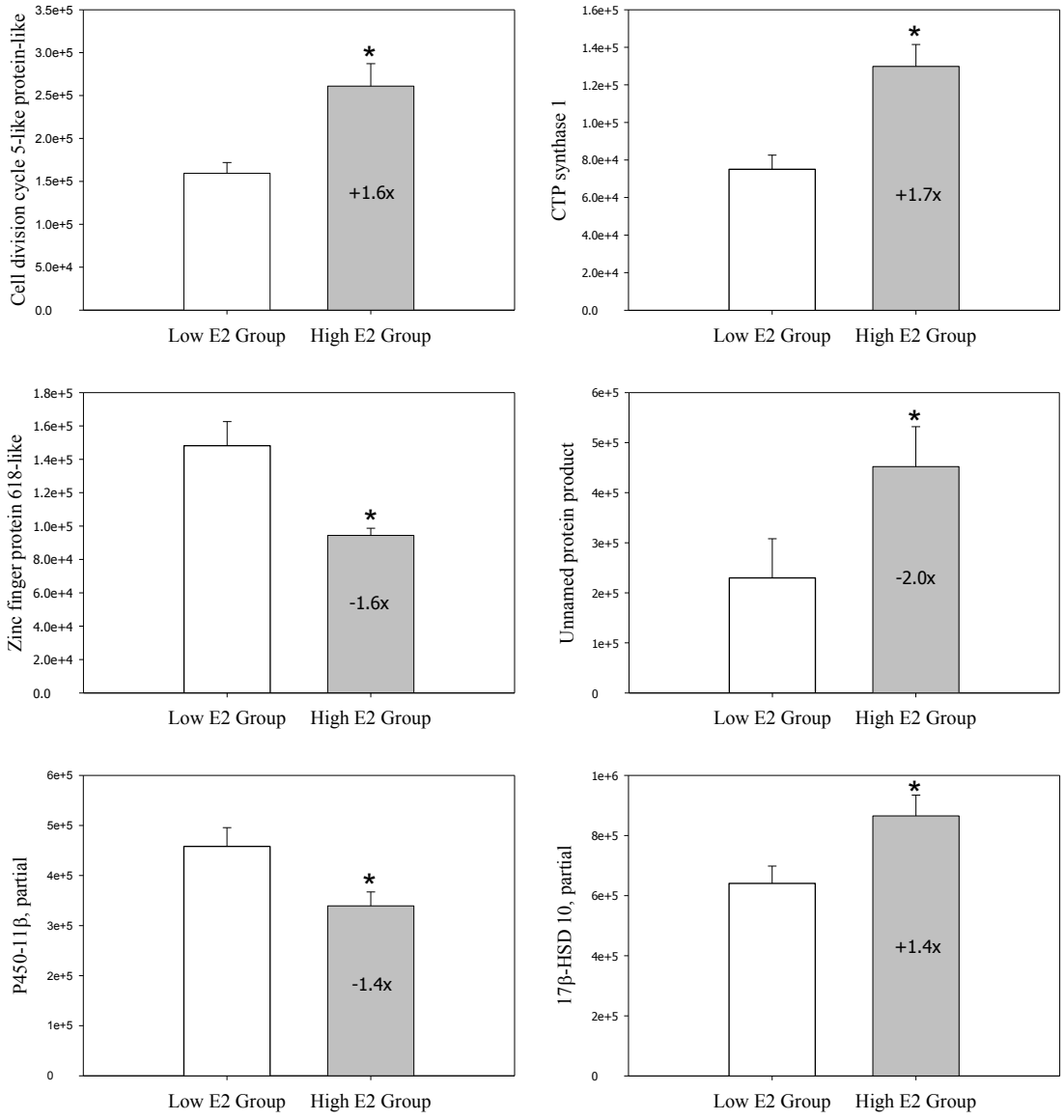

FIGURE 4. E2-related differences in testicular protein abundance of selected proteins. Bars represent mean ± SEM normalized protein volumes (arbitrary units); n=4 for the low E2 group and n=6 for the high E2 group. Proteins represented here show significant E2-related differences in protein abundance (*p<0.05).

TABLE 3. Pearson Product-Moment Correlations Between Plasma 17β-Estradiol Concentration (pg/ml) and Testicular Protein Abundance (normalized spot volume)

Protein #	Protein ID	R	*p*
989	**protein SDA1 homolog**	**0.878**	**0.00084**
975	**transcription activator BRG1-like, partial**	**0.834**	**0.00273**
1930	**retinoid x receptor, beta**	**-0.833**	**0.0028**
2334		**-0.825**	**0.00333**
2282		**-0.824**	**0.0036**
1906		**0.813**	**0.00426**
2301		**-0.802**	**0.0053**
680	**cytochrome P450 11-beta**	**-0.796**	**0.00592**
2430		**0.785**	**0.00719**
984		**0.776**	**0.00838**
823	**cytochrome p450 IV B1**	**0.773**	**0.00879**
2214	**zinc finger protein 618-like**	**-0.762**	**0.0103**
1990	**unnamed protein product**	**0.759**	**0.0109**
2470		**-0.753**	**0.012**
1920	**muscle-type creatine kinase CKM1**	**-0.74**	**0.0144**
2390		**0.74**	**0.0144**
326	**putative nuclease HARBI1-like**	**0.733**	**0.0158**
144	**Nucleoside diphosphate kinase B**	**0.72**	**0.0188**
1648		**0.709**	**0.0216**
2419		**-0.709**	**0.0216**
446	**CTP synthase 1**	**0.704**	**0.0232**
2403		**0.703**	**0.0234**
1567	**chromatin modification-related protein eaf6**	**-0.692**	**0.0267**
927	**CCAR1 protein, partial**	**0.682**	**0.0297**
593		**-0.68**	**0.0305**
1748		**-0.679**	**0.031**
693		**0.678**	**0.0311**
1536		**0.676**	**0.032**
1306	**N-alpha-acetyltransferase 15, NatA auxiliary subunit isoform 1**	**0.672**	**0.0332**
2337		**-0.668**	**0.0348**
799		**-0.666**	**0.0355**
1569		**-0.666**	**0.0356**
1017		**0.665**	**0.0358**
2283	**vacuolar sorting protein 54 long isoform**	**-0.655**	**0.0397**
1076		**-0.648**	**0.0428**
91		**0.648**	**0.0429**
2425		**0.641**	**0.046**
1322		**-0.636**	**0.0479**
390		**0.636**	**0.0482**
617		**0.636**	**0.0482**
1803	uncharacterized protein LOC678611	-0.631	0.0504
1960	unnamed protein product	0.63	0.0508
1777		0.63	0.0509
100		-0.629	0.0512
435	cytochrome P450 family 2 subfamily N protein	-0.622	0.0548
2357		0.621	0.0552

Note: Includes all fish for which testicular proteome was analyzed (n=10). Significant correlations are shown in bold font (p<0.05); relationships approaching significance (0.10<p<0.05) are also presented. For additional information on protein identification, please refer to Table 2.

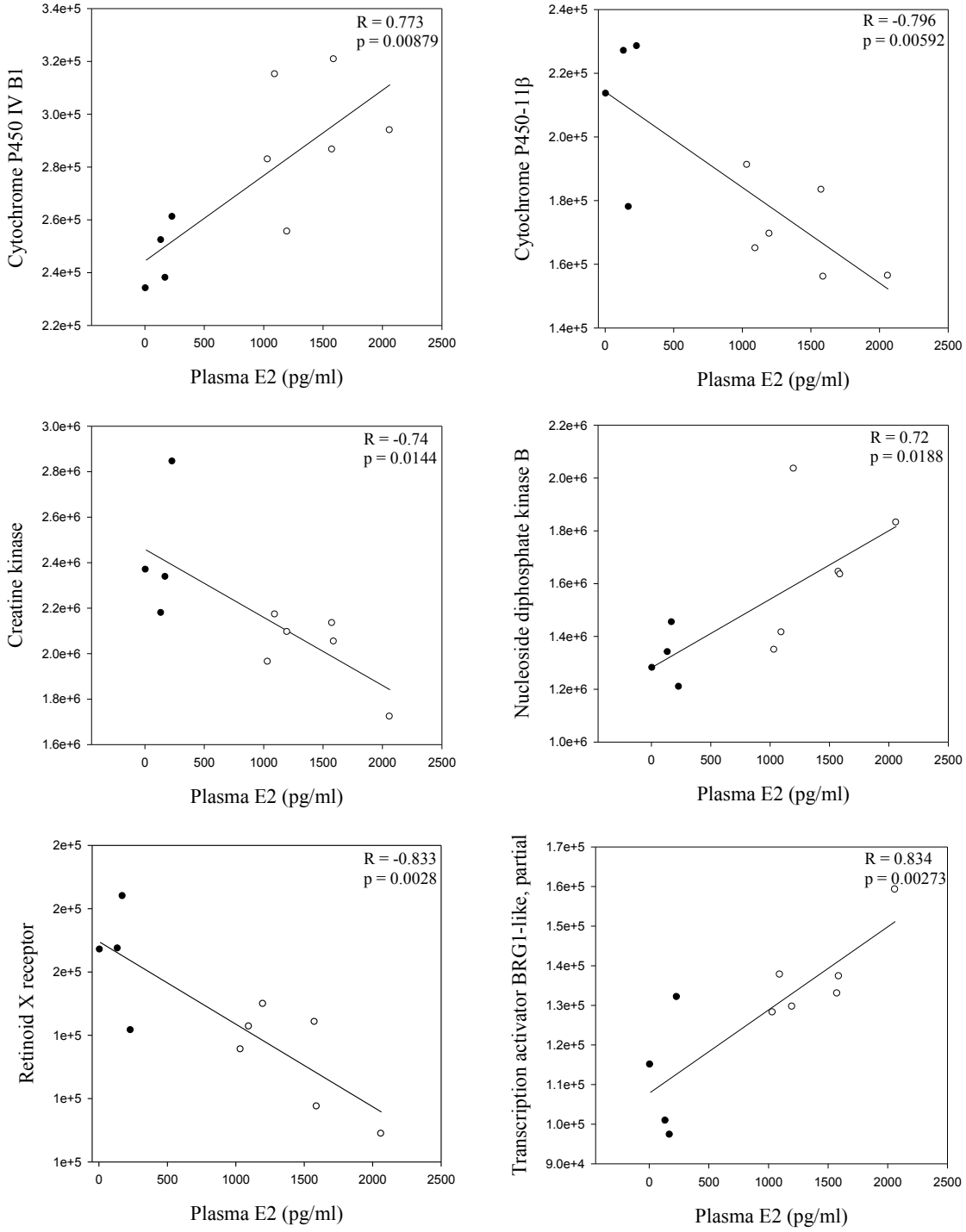

FIGURE 5. Illustration of selected correlations between plasma 17β-estradiol (E2; pg/ml) and normalized protein spot volume (arbitrary units). R and *p*-values are located in the top right of each graph (n=10). Shaded circles represent individuals from the low E2 group; open circles represent individuals from the high E2 group.

TABLE 4. 17β-Estradiol (E2) Treatment-Related Differences in Testicular Protein Abundance

Study ID	Protein #	Protein ID	Control Mean ± SEM (n)	E2 Injected Mean ± SEM (n)	Fold Difference	*p*
1b	1645	L-3-hydroxyacyl-CoA dehydrogenase, partial	439097 ± 24190 (4)	697573 ± 36929 (5)	+1.6	0.0005
5	1556	Prohibitin	137612 ± 3837 (4)	182336 ± 9467 (5)	+1.3	0.0025
6a	927	CCAR1 protein, partial	399006 ± 25129 (4)	312645 ± 17083 (5)	-1.3	0.0210
6b	981	CCAR1 protein, partial	1036769 ± 75364 (4)	737326 ± 73124 (5)	-1.4	0.0218
7	747	Transcription factor IIIC63	151707 ± 10066 (4)	100476 ± 10877 (5)	-1.5	0.0140
11	275	P63 DNA-binding domain	330040 ± 19438 (4)	381205 ± 11778 (5)	+1.2	0.0520
12	720	Zinc finger protein 322a	1018943 ± 60709 (4)	819749 ± 37027 (5)	-1.2	0.0267
21	1920	Muscle-type creatine kinase CKM1 or Creatine kinase, testis isozyme	2186029 ± 117436 (4)	2550134 ± 47803 (5)	+1.2	0.0220
23	1944	26S protease regulatory subunit 6b	488224 ± 55198 (4)	318456 ± 32859 (5)	-1.5	0.0271
24	1478	Metalloproteinase-1	384365 ± 39720 (4)	231045 ± 31481 (5)	-1.7	0.0185
25	1329	Plasma membrane calcium ATPase 1 isoform a	5681463 ± 133660 (4)	4734503 ± 320330 (5)	-1.2	0.0440
26	2040	Vacuolar sorting protein 54 long isoform	141910 ± 8872 (4)	189766 ± 14221 (5)	+1.3	0.0349
28	716	Matrix extracellular phosphoglycoprotein	1336420 ± 103381 (4)	1028706 ± 50616 (5)	-1.3	0.0226
29	2527	Beta actin	5428904 ± 404733 (4)	4346283 ± 269519 (5)	-1.2	0.0552
--	1150		50123 ± 1877 (4)	64363 ± 1656 (5)	+1.3	0.0008
--	2447		142848 ± 14335 (4)	88264 ± 9598 (5)	-1.6	0.0147
--	1975		317935 ± 12517 (4)	223985 ± 2237 (5)	-1.4	0.0160
--	500		144701 ± 19583 (4)	208940 ± 9358 (5)	+1.4	0.0173
--	205		568433 ± 51636 (4)	367771 ± 37996 (5)	-1.5	0.0205
--	724		639081 ± 42765 (4)	500629 ± 32881 (5)	-1.3	0.0314
--	2025		321018 ± 28590 (4)	228735 ± 20214 (5)	-1.4	0.0319
--	461		386737 ± 22674 (4)	454533 ± 15418 (5)	+1.2	0.0335
--	663		237733 ± 11818 (4)	166869 ± 24078 (5)	-1.4	0.0373
--	2283		207449 ± 32201 (4)	136231 ± 7690 (5)	-1.5	0.0404
--	826		191643 ± 8402 (4)	158043 ± 9946 (5)	-1.2	0.0458
--	2293		1554883 ± 210664 (4)	1063342 ± 90517 (5)	-1.5	0.0478

Note: Normalized protein spot volumes (arbitrary units) are given as mean ± SEM; n=4 for the control group (vehicle injected) and n=5 for the treatment group (E2 injected). P-values were generated using a one-way ANOVA.

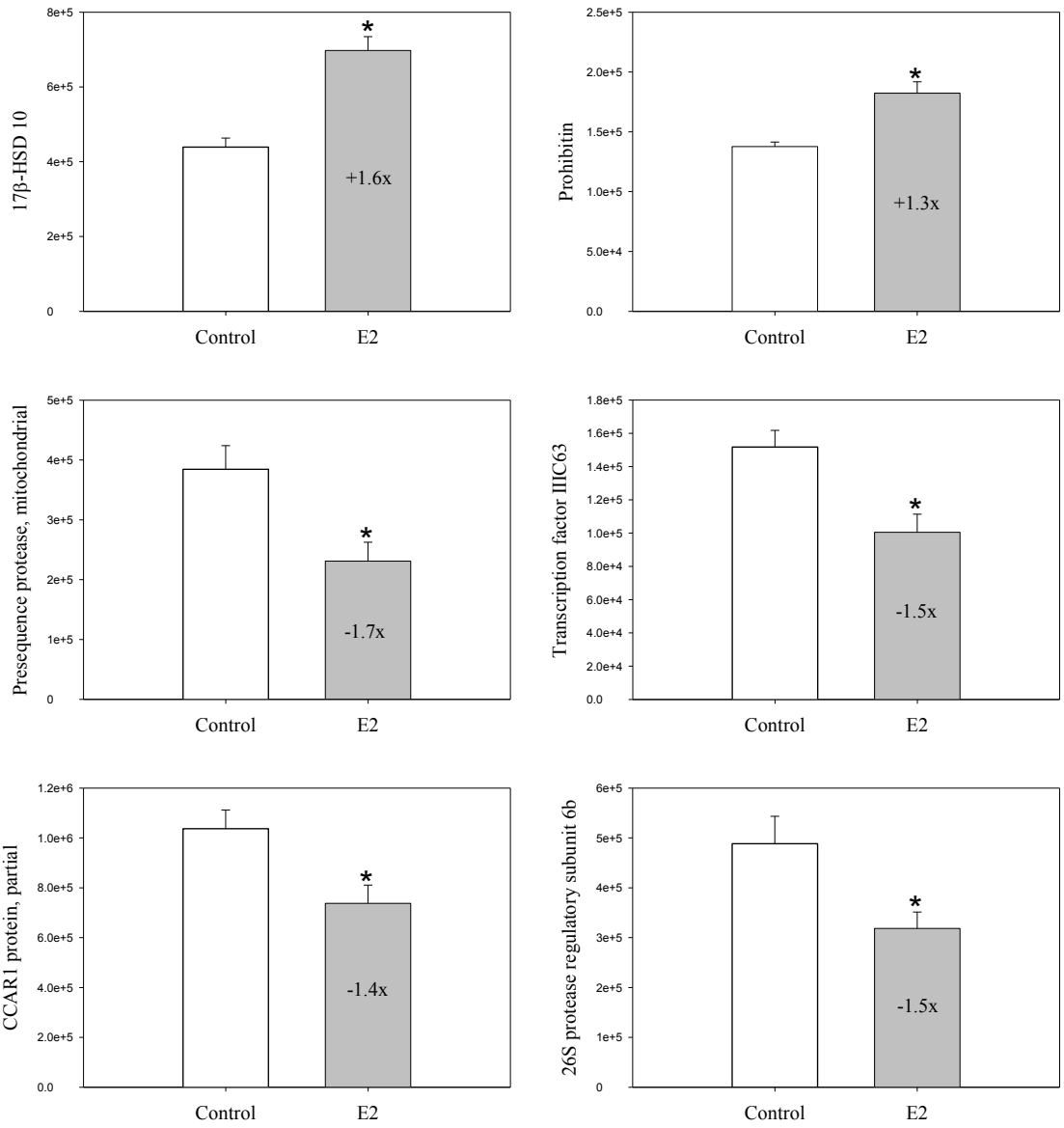

FIGURE 6. 17β-Estradiol (E2) treatment-related differences in testicular protein abundance of selected proteins. Bars represent mean ± SEM normalized protein spot volume (arbitrary units); *n*=4 for the control group (vehicle-injected), and *n*=5 for the treatment group (E2-injected). Fold-differences between groups are provided within the E2 treatment bar. Captive hornyhead turbot were injected with E2 (3 µg /g body weight) or vehicle (control) as described in Methods. Proteins represented here show significant E2 treatment-related differences in protein abundance (*p<0.05).

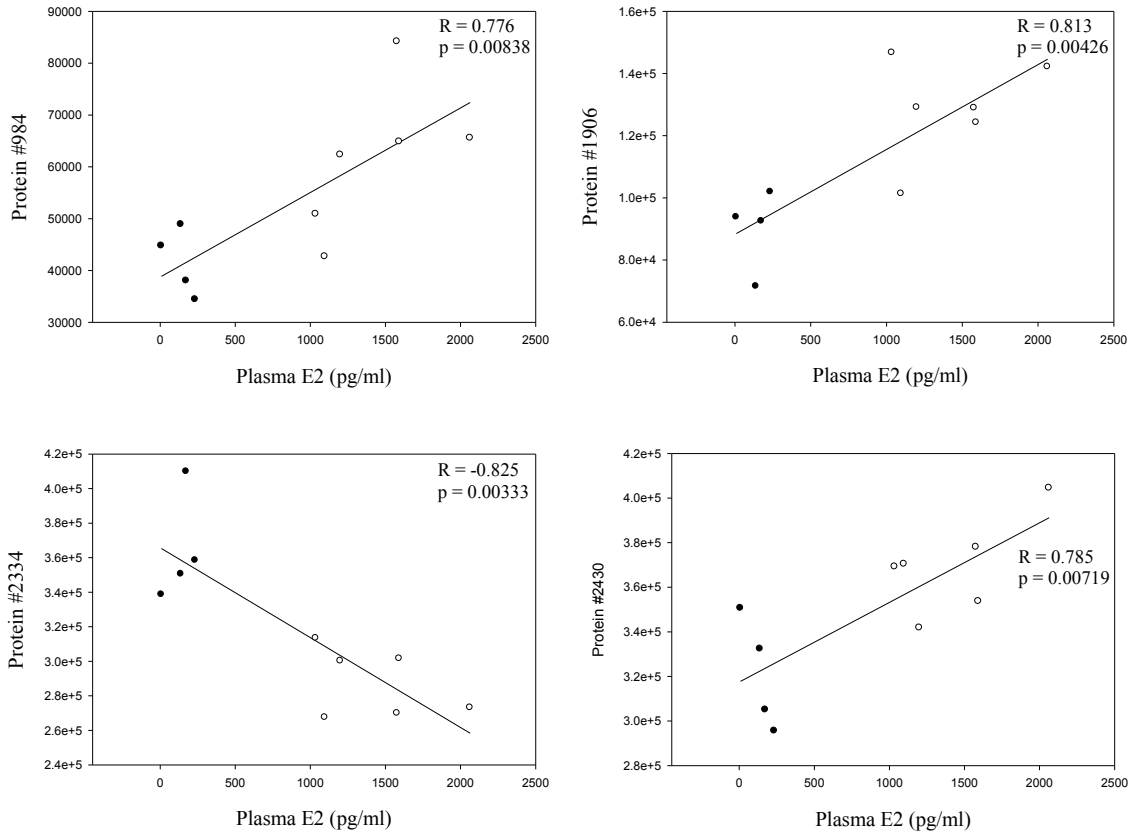

FIGURE 7. Illustration of selected correlations between plasma 17β-estradiol (E2; pg/ml) and normalized protein spot volume (arbitrary units) of unidentified proteins. R and *p*-values are located along the right axis of each graph (n=10). Shaded circles represent the low E2 group; open circles represent the high E2 group. Given the strong correlations, these unidentified proteins are the target of future molecular characterization studies.

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