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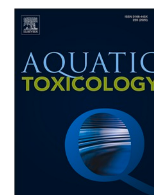

Widespread alterations upon exposure to the estrogenic endocrine disruptor ethinyl estradiol in the liver proteome of the marine male fish *Cyprinodon variegatus*

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ABSTRACT

Quantitative proteomic changes in the liver of adult males of Sheepshead minnow (*Cyprinodon variegatus*) upon exposure to ethinyl estradiol (EE2) were assessed to provide an advanced understanding of the metabolic pathways affected by estrogenic endocrine disruption in marine fish, and to identify potential novel molecular biomarkers for the environmental exposure to estrogens. From a total of 3188 identified protein groups (hereafter proteins), 463 showed a statistically significant difference in their abundance between EE2 treatment and solvent control samples. The most affected biological processes upon EE2 exposure were related to ribosomal biogenesis, protein synthesis and transport of nascent proteins to endoplasmic reticulum, and nuclear mRNA catabolism. Within the group of upregulated proteins, a subset of 14 proteins, involved in egg production (Vitellogenin, Zona Pellucida), peptidase activity (Cathepsine E, peptidase S1, Serine/threonine-protein kinase PRP4 homolog, Isoaspartyl peptidase and Whey acidic protein), and nucleic acid binding (Poly [ADP-ribose] polymerase 14) were significantly upregulated with fold-change values higher than 3. In contrast, Collagen alpha-2, involved in the process of response to steroid hormones, among others, was significantly downregulated (fold change = 0.2). This pattern of alterations in the liver proteome of adult males of *C. variegatus* can be used to identify promising novel biomarkers for the characterization of exposure of marine fish to estrogens. The Whey acidic protein-like showed the highest upregulation in EE2-exposed individuals (21-fold over controls), suggesting the utility of abundance levels of this protein in male liver as a novel biomarker of xenoestrogen exposure.

1. Introduction

The list of endocrine disrupting compounds (EDCs) grows as new materials appear, resulting from advances in industry, pharmaceuticals, or agriculture, among others. Despite these compounds exhibiting features that are useful for our society, they also entail an environmental problem derived from their inappropriate disposal in the environment, with aquatic ecosystems being the major final sink of these chemicals (Kime, 1998). According to the World Health Organization (WHO), an EDC is an exogenous chemical or a mixture of chemicals that alters the function of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny, or subpopulations (WHO World Healt Organization, 2002). As a proof of the potentially dangerous effects caused by these chemicals, the European Commission applies to them the category of very high regulatory concern (REACH regulation (EC) No 1907/2006).

Xenoestrogens are synthetic chemicals that show affinity to estrogen receptors (ER), and thus trigger the biochemical pathways activated by the natural female hormone estradiol (E2) (Beiras, 2018). Estrogenic EDCs may act through genomic pathways that involve interaction with nuclear ER, or rapid non-genomic pathways mediated by membrane-bound ER (Kiyama and Wada-Kiyama, 2015). The US Environmental Protection Agency (US-EPA) has tested about 1800 substances so far for ER bioactivity (EDSP, 2020). However, the list of candidates is continuously growing as new potential EDCs are identified by new and more sensitive techniques, such as transcriptomic or

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proteomic analysis (Benninghoff, 2007; Wetmore and Merrick, 2004).

Ecotoxicological studies have used diverse molecular methodologies to study effects of EDCs. Transcriptomics and proteomics are increasingly used in ecotoxicology to achieve precise and sensitive measurements of the mechanisms of action of toxicants and to characterize changes in the cellular metabolism caused by different experimental conditions (Calzolai et al., 2007). However proteomic methodologies have not drawn the same attention as other omics approaches such as transcriptomics (Liang et al., 2020) despite the synergies between gene transcription and protein synthesis. For example, while the effects at the transcript level provide information regarding initial responses that occur rapidly after exposure to a stressor, the effects observed at the protein level give a more physiologically relevant functional knowledge of the regulation of gene expression (Silvestre et al., 2012). Moreover, often there is a lack of correlation between mRNA and protein levels (Diz and Calvete, 2016; Vogel and Marcotte, 2012) and a number of studies have shown that a considerable percentage of dysregulated proteins were not detected at the transcript level (Mack et al., 2006; Maziarz et al., 2005). This observation emphasizes the need to interpret transcriptomic and proteomic results in a complementary way. Several methods have been used for proteomic analysis, and high-throughput quantitative shotgun proteomics with isobaric labeling (e.g., iTRAQ or TMT) are currently considered one of the most promising methodologies in the field, since it allows to identify and quantify several multiplexed protein samples in a single analysis (Diz and Sánchez-Marín, 2021; Pappireddi et al., 2019). Whereas precise quantification of a limited number of well-known proteins is possible thanks to immunoassays, such as ELISA or Western blot, high-throughput (shotgun) proteomic analysis allows to quantify theoretically the whole set of proteins expressed in one cell, tissue, or organism at one specific time through a direct detection of the proteins. The liver, an organ of crucial relevance in the hypothalamus-pituitary-gonadal axis and main organ for detoxification in all vertebrates (Kime, 1998), is particularly useful for the evaluation of the pollutant effects through this technique.

There is evidence that exposure to xenoestrogens induces the expression of egg protein precursors such as vitellogenin (VTG) and zona pellucida (ZP) in the liver of fish, which is the reason why they have been used as biomarkers for estrogen exposure (Knoebl et al., 2004; Martyniuk et al., 2020; Wit et al., 2010). A general accepted mechanism involves the binding of estrogens, including xenoestrogens, to the ER to form a complex that migrates into the nucleus and binds to genome regions called estrogen responsive elements (EREs), promoting the downstream transcription of several genes, including Vtg and Zp (Klinge, 2001). However, many other molecular mechanisms and functional pathways are also involved in the molecular response to estrogenic EDCs. Unlike the nuclear ER, the G protein-coupled estrogen receptor (GPER) is located in the oocyte membrane. When coupled to E2, the GPER was reported to affect liver weight (Chaturantabut et al., 2019) and present an inhibitory action on oocyte maturation (Pang and Thomas, 2010). GPER is also involved in the Vtg gene expression (Chen et al., 2019; Garcia-Reyero et al., 2018). A working hypothesis of the present study, based on results reported from previous work in different species, was that proteins involved in the signaling pathways mediated by GPER could also be affected by xenoestrogens.

Another *a priori* focus of interest of the present study were proteins involved in the biotransformation of xenobiotics, due to the presence of other nuclear receptors that can play a role in their sensing and elimination, such as the pregnane X receptor (PXR) and the constitutive androstane/activated receptor (CAR) (Timsit and Negishi, 2007; Tolson and Wang, 2010). UDP-glucuronosyl-transferase (UGT), and cytochrome P450 (CYP) family proteins have been observed linked to the activation of PXR and CAR (Tolson and Wang, 2010) and alterations in their transcript levels have been reported in fish after exposure to estrogenic EDCs (Hultman et al., 2015).

Whereas indication of xenoestrogen exposure was traditionally tracked by using individual biomarkers such as VTG or ZP induction in male or juvenile fish (Arukwe and Goksøyr, 2003; Purdom et al., 1994), recent technological advances that enable the massive parallel quantification of hundreds or thousands of proteins make it possible to perform a holistic description of the effects of xenoestrogens on the whole hepatic proteome. This approach, using the synthetic hormone 17α-ethinylestradiol (EE2) as a potent model estrogen, has been previously used in freshwater fish, such as the fathead minnow (*Pimephales promelas*) or the zebrafish (*Danio rerio*) (Alcaraz et al., 2021; Rose et al., 2002; Wit et al., 2010), and a few studies with marine fish, such as the mangrove rivulus (*Kryptolebias marmoratus*) (Voisin et al., 2019).

Taking into account a generalized lack of validation of reported effects at the protein level and the variety of molecular mechanisms and metabolic pathways that have been suggested to be involved in estrogenic endocrine disruption, a non-target quantitative proteomic analysis can be particularly useful to validate previously reported biomarkers but also to decipher the full range of xenoestrogen effects along molecular pathways. Non-target proteomics can help to identify novel biomarkers to be added to the current toolbox to assess the exposure of fish to estrogens.

Fish with a short life cycle can be used for toxicity studies because of their easy handling and the possibility to extrapolate observed effects to other vertebrates (Blanton and Specker, 2007). Aquatic ecotoxicology studies have focused mainly on freshwater model species, such as zebrafish or fathead minnow (Ankley and Villeneuve, 2006; Bambino and Chu, 2017), with marine species receiving less attention, probably due to the more demanding conditions of marine cultures. However, some marine fish species have been proposed as ecotoxicological models, such as the marine medaka (*Oryzias melastigma*) (Dong et al., 2014; Kim et al., 2016; Kong et al., 2008), or the Sheepshead minnow (*Cyprinodon variegatus*) (Schnitzler et al., 2017). With this work, we extend the use of *C. variegatus* as a marine model species to ecotoxicology studies based on proteomic approaches, thanks to the high number of nucleotide and protein sequences available in different public repositories.

With the aim to explore the value of *C. variegatus* as marine fish model for testing estrogenic endocrine disruption, we evaluated the effects of an exposure to EE2 on the liver proteome. We hypothesized that the proteomic profile of the liver in adult males should present identifiable differences between EE2-exposed and non-exposed individuals helpful to identify a pattern of alteration characteristic of estrogenic EDCs. Identification of potential novel biomarkers for estrogenic endocrine disruption in this species was also an objective of this study, through the exploration of the results from a non-target proteomics analysis.

2. Material and methods

2.1. Exposure to 17α-ethinylestradiol

C. variegatus adult males (335 days old, 4.40 ± 0.32 cm and 1.67 ± 0.40 g) were provided by ECIMAT (CIM, University of Vigo). All experimental procedures described below were approved by the Ethical Committee for Animal Testing of the University of Vigo (code ES360570181401). Exposures were conducted in glass vessels filled with 1.8 L of 1 μm-filtered natural sea water diluted to reach a salinity of 25±0.2 ppt. Culture conditions, monitored daily, were as follows: temperature 18.8 ± 0.2 °C, pH 7.94±0.03, dissolved oxygen saturation 99.3% ± 1.1 and 16:8 h of light:dark photoperiod. Ammonium and phosphate levels were measured in four random vessels and never exceeded 0.60 mg/L or 0.2 mg/L, respectively. Males were set individually in the vessels under continuous aeration, daily food supply based on Gemma Diamond 1.0 pellets (Skretting, France) *at libitum* and daily 90% water renewal. After 1 week of acclimation, 15 fish were exposed for 7 days to 100 ng/L of EE2, (98% purity, CAS Number: 57–63–6, Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO), hereafter referred as EE2 treatment. After daily water renewal, a pulse of

appropriate volume of EE2 stock dilution was carefully and homogeneously added in each of fifteen vessels to keep a constant EE2 concentration over the course of the experiment. The same number of fish were treated with an equal amount of DMSO for 7 days, hereafter referred to solvent control (SC). DMSO levels were kept at 0.001% v/v. In order to assess actual EE2 concentrations in the exposure water, samples were taken from three SC vessels and three EE2 vessels immediately after the EE2 pulse (t0). The samples were kept at 4ºC and later sent to the Institut Quimic de Sarrià (IQS) where they were analyzed by an Acquity H—Class UHPLC coupled to a triple quadrupole MS/MS. The results of the analysis showed an EE2 concentration below the limit of detection for the SC, and 86.3 ± 8.2 ng/L of EE2 in the t0. In order to assess stability of EE2 in the water, additional samples were taken from three EE2 vessels immediately after the pulse and 24 h later (t24). Results showed a stability of 98.8% for 10 ng/L of EE2 and of 99.3% for 1000 ng/L of EE2 after 24 h.

Throughout the duration of the experiment no fish showed signs of disease after daily visual inspection, such as modification of the skin color, ventilation rate, swimming behavior, loss of appetite and external injuries. After exposure, all fish were euthanized with phenoxiethanol (Merck KGaA, Germany), their size and body weight were measured, livers were dissected, weighed and immediately frozen in liquid nitrogen before long-term preservation at −80 °C. The mean size and body weight (see above) of the fish did not present significant differences between treatments according to the Student's *t*-test ($p = 0.40$ and $p = 0.52$, respectively), nor did the mean weight (0.026 ± 0.010 g) of the liver ($p = 0.83$).

2.2. Protein extraction and quantification

Liver samples were processed following general guidelines and protocol described in Diz & Sánchez-Marín (2021) with minor modifications. Specifically, proteins were extracted in lysis buffer (7 M urea; 2 M thiourea; 4% CHAPS) by sonication, running 6 pulses at 20% amplitude of 5 s each with pauses of 30 s on ice to avoid overheating. Samples were then centrifuged at 21,000 × g, 60 min at 4 °C and the supernatant was collected to a new microtube. Protein quantification was performed by using a modified version of the Bradford method compatible with lysis buffer components (Ramagli and Rodriguez, 1985) and adapted for microplate volumes. Five pooled samples (biological replicates) were prepared for each treatment (SC and EE2), each pool made by mixing an equal amount of proteins (35 μg) extracted from three randomly selected fish from each treatment. Pooling samples for proteomic analysis is a valid and convenient approach to increase statistical power for detection of expression differences while keeping experiment costs affordable (Diz et al., 2009). Hence each pooled sample contained a total of 105 μg of proteins, and final concentration was adjusted to 0.5 μg/μL using ultra-pure water. There were no significant differences in the average weight of fish livers among pooled samples (1-way ANOVA; $F_{1,9} = 0.8814$, $p = 0.557$).

2.3. Isobaric labeling and fractionation of protein samples

Proteins were acetone precipitated and trypsin digested following TMT10-plex manual guidelines (Thermo Scientific; Pub No. MAN0016969) with minor modifications described in Diz & Sánchez-Marín (2021). Briefly, 600 μL of pre-chilled HPLC acetone were added to 100 μL (50 μg) of protein from each pooled sample, kept frozen at −20 °C for 2 h, centrifuged twice (8000 × g, 15 min at 4 °C) and supernatant removed. Protein pellet was resuspended in 100 μL of TEAB 100 mM, cysteine residues reduced in 5 μL of TCEP 200 mM at 55 °C for 1 h and alkylated in 5μL of iodoacetamine 375 mM for 30 min, protected from light. Proteins were digested by adding 1 μg of sequencing grade modified trypsin to each sample (Promega, Madison, WI) dissolved in 10 μL of 100 mM TEAB. The reaction took place overnight at 37ºC. Resulting peptides from each pooled sample were labelled with TMT10plex amine reactive reagents (Thermo Scientific). Five tags were used for the five pooled samples (biological replicates) from the EE2 treatment, while the other five available tags were used for tagging peptides of the five pooled samples from the SC treatment.

Labeled peptides from all pooled samples were mixed, and approximately 30 μg of peptides from this mix were separated into eight fractions using a high pH reversed-phase peptide fractionation kit following the manufacturer's instructions (Thermo scientific; Pub. No. MAN0015701). Briefly, the reversed-phase column was compacted by centrifugation, preconditioned with acetonitrile and centrifuged, washed with TFA 0.1% and centrifuged. Then, the sample mix was added to the column and washed with 5% acetonitrile and eight consecutives elutions were done by adding sequentially an increasing amount of acetonitrile (from 10 to 50% v/v), elution products (fractions) collected, dried out in a speed vacuum-concentrator for 4 h and preserved at −80C until mass spectrometry (MS) analysis.

2.4. Mass spectrometry analysis (ESI-MS/MS)

Peptides from each fraction were resuspended in 0.5% formic acid and analyzed by ESI-MS/MS in an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) with a Proxeon EASY-nLC 100 UHPLC system (Thermo Fisher Scientific). Peptide separation was performed using a precolumn Acclaim PepMap 100 (75 μm x 2 cm, REF:164,946) followed by a reversed-phase PepMap C18 column (50 cm x 75 μm ID, 2 μm particles, 100 Å pore side, REF:ES903). A flow rate of 300 nL/min of acetonitrile in linear increasing gradient concentration (from 5% to 25%) was set for 240 min. HCD was used as ion collision method. Other parameters for MS/MS analysis were set as described in Romero et al. (2019). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD027547 (accession) and 10.6019/PXD027547 (DOI).

2.5. Protein identification and quantification

PEAKS Studio v.8.0 software (Bioinformatics Solutions Inc., Waterloo, Canada) was used for matching MS/MS spectra against a *C. variegatus* proteome database downloaded from Uniprot (proteome accession: UP000265020, 31,881 protein sequences; September 6th, 2019) supplemented with protein sequences from common mass spectrometry contaminants (cRAP protein sequences downloaded from https://www.thegpm.org; November 19th, 2019). Identification analysis setup was as follows: False Discovery Rate (FDR), calculated from numbers of decoy and target sequence hits, was set at 1% at PSM level, 8.5% at protein level; 10 ppm and 0.02 Da were set for precursor and fragment ion tolerance respectively; up to one missed trypsin cleavage site allowed. Methionine oxidation was set as variable modification, while carbamidomethylation of cysteine and TMT 10plex (N-terminal, K) were set as fixed modifications. For quantification, peptide intensities for each sample were normalized based on the total sum of intensities within channel (tandem mass tag, TMT) and in relation to the reference channel. Only identifications of unique peptides with ion intensities higher than 1×10^2 were used for protein quantification.

2.6. Statistical analysis of protein abundance data

A Student's *t*-test was performed for each protein using the normalized abundance values transformed to logarithmic scale in order to assess whether the observed differences in the average expression values between samples from EE2 and CS can be explained just by chance (*p-value* < 0.05). Multiple hypothesis testing problem was corrected following the strategy discussed in Diz et al. (2011) using SGoF software to carry out the correction by using different methods (Carvajal-Rodríguez et al., 2009). Results after applying the SGoF correction are finally discussed in the present work.

2.7. Protein annotation

Protein nomenclature, by default in this study, was set according to Uniprot database (Uniprot 28/01/21). For cases where protein name was labelled as "Uncharacterized protein" in Uniprot, a manual reannotation was done by using the BLASTp search against non-redundant protein sequences of *C. variegatus* of NCBI (33,467 sequences, June 2021).

In the particular case where different isoforms of VTG were found, to avoid uncertainties due to different alternative names provided in the database from automatic annotation and their specific relevance as potential EDC biomarkers, the specific VTG nomenclature followed in the literature for fish was used. To achieve this, manual annotated sequences belonging to *Actinopterygii* species found in the literature (Finn and Kristoffersen, 2007) and VTG sequences found in this study were compared through phylogenetic analysis. A phylogenetic tree based on protein sequences was built with MEGA-X software version 10.1.8 (Kumar et al., 2018). Default settings were used for MUSCLE algorithm, ignoring gaps. The evolutionary relationship among sequences was inferred using p-distance and Neighbor-Joining methods (Saitou and Nei, 1987) with all parameters set as default.

Functional annotation for all the identified proteins and a gene ontology (GO) enrichment analysis based on Fisher exact test (FDR = 0.05) were performed using Omicsbox software (version 1.4.12). To identify the enriched terms resulting from the exposure to EE2, the set of differential expressed proteins were used as test set whereas all quantified proteins in the experiment were considered as the reference set.

3. Results

3.1. Differential expressed proteins

Mass spectrometry data analysis reported 37,364 identified and quantified peptides that correspond to 3188 protein groups (hereafter proteins). A total of 647 proteins showed statistically significant differences ($p<0.05$) in their abundance between EE2 treatment and solvent control samples. After applying the SGoF correction, 463 proteins remained statistically significant. A low positive false discovery rate (q-value = 0.11) obtained for these 463 altered proteins reinforces the choice of them for further analysis (see file S1). The comparison of protein abundance between treatment and control samples was expressed as effect size (fold change, FC) and statistical significance (p-value) (Fig. 1). Of these dysregulated proteins, fourteen significantly upregulated proteins with the largest effect sizes (FC > 3) stood out; Whey acidic protein-like (WAP), Poly [ADP-ribose] polymerase 14 (PARP14), Zona pellucida proteins 1, 3 and 4 (ZP1, ZP3, ZP4), Pre-mRNA processing factor 4Bb (PRP4), Vitellogenin domain-containing protein (VTG domain), Vitellogenin proteins Ab and C (VTGAb, VTGC), Isoaspartyl peptidase/L-asparaginase (ASRGL1), two sequences of VWFD domain-containing protein (VWFD domain), Cathepsin E (CTSE) and Peptidase S1 domain-containing protein (PAS1), whereas one significant downregulated protein stood out, Collagen type V alpha 2b (COL5A2).

3.2. Functional enrichment analysis

The biological processes significantly affected by EE2 exposure, according to the enrichment analysis, were related to the synthesis of proteins in the cytoplasm (cytoplasmic translation (GO:0002181) and nuclear-transcribed mRNA catabolic process (GO:0000956)), the transport of nascent proteins to the endoplasmic reticulum (SRP-dependent cotranslational protein targeting to membrane GO:0006614) and processes of ribosomal biogenesis, such as the biogenesis of the large subunit (GO:0042273), small subunit assembly (GO:0000028) and rRNA export from nucleus (GO:0006407), among others (Fig. 2a).

Concerning to the effects at the molecular function level (Fig. 2b),

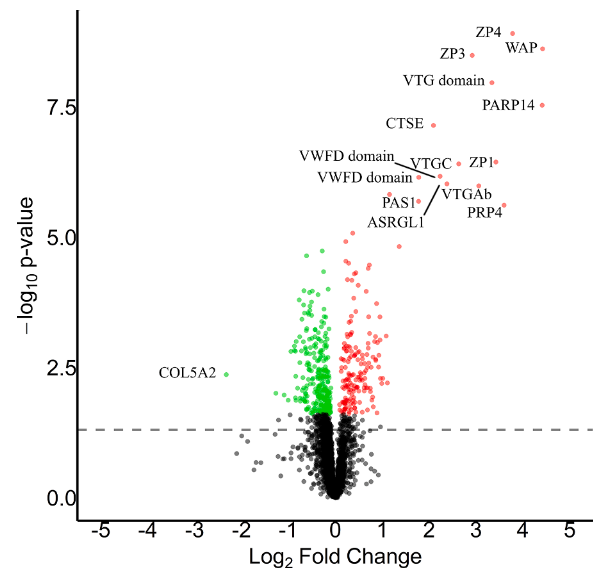

Fig. 1. Volcano plot for identified and quantified proteins in the present study. Red and green dots indicate proteins that were differentially expressed, up- or down-regulation respectively, in EE2 treatment compared to control after SGoF correction. Horizontal line represents a p-value threshold of 0.05, at logarithmic scale ($-\log_{10}$p).

this analysis found terms related to mRNA and rRNA binding (GO:0003729, GO:0019843) and the structural constituent of ribosomes (GO:0003735) significantly enriched in the set of affected proteins. A careful inspection of identified proteins related to the ribosome structure provides strong evidence of a generalized upregulation of this functional component in response to EE2, that is, 57 ribosomal proteins (FC between 1.12 and 1.90). Therefore, our results strongly suggest that the cellular machinery related to protein synthesis was broadly affected in response to EE2 exposure.

Endopeptidase inhibitor activity (GO:0004866) was another molecular function significantly altered by the EE2 exposure. In fact, the most affected protein in this study, which showed the highest upregulation level found (FC = 21.4, p < 0.001) was the WAP-like (accession A0A3Q2D0I0), that presents a peptidase inhibitor activity according to the functional annotation. On the other hand, the molecular function of endopeptidase regulation activity (GO:0061135) was also found altered by EE2. More specifically, twelve proteins involved in the regulation of the peptidase activity were affected by EE2 (Table 1) belonging to different protein families. Among the three identifications found belonging to the Peptidase A1 family, the cathepsin E was significantly upregulated (p < 0.001, FC = 4.27). This protein was among proteins with the highest fold-change in the experiment (Fig. 1). From the seventeen proteins with serine-type endopeptidase activity there were ten that showed significant differences of abundance, one upregulated, the peptidase S1 domain-containing protein (PAS1), and the remaining proteins downregulated. PAS1, with a FC of 3.43, was found among the proteins with greater fold-change compared to the majority of other proteins. Finally, the isoaspartyl peptidase/L-asparaginase was the only identification belonging to the Ntn-hydrolase family that showed statistically significant differences. This protein was also one of the proteins with greater effect size (FC = 5.21) highlighted in Fig. 1.

3.3. Proteins related to estrogen responsive elements

Based on the hypothesis that exposure to EE2 could induce a rapid response through a genomic pathway and with the aim to study the effects of this genomic response at the protein level, the set of *C. variegatus* proteins induced when the EREs are activated was inspected (Table 2). To identify proteins regulated by ERE in

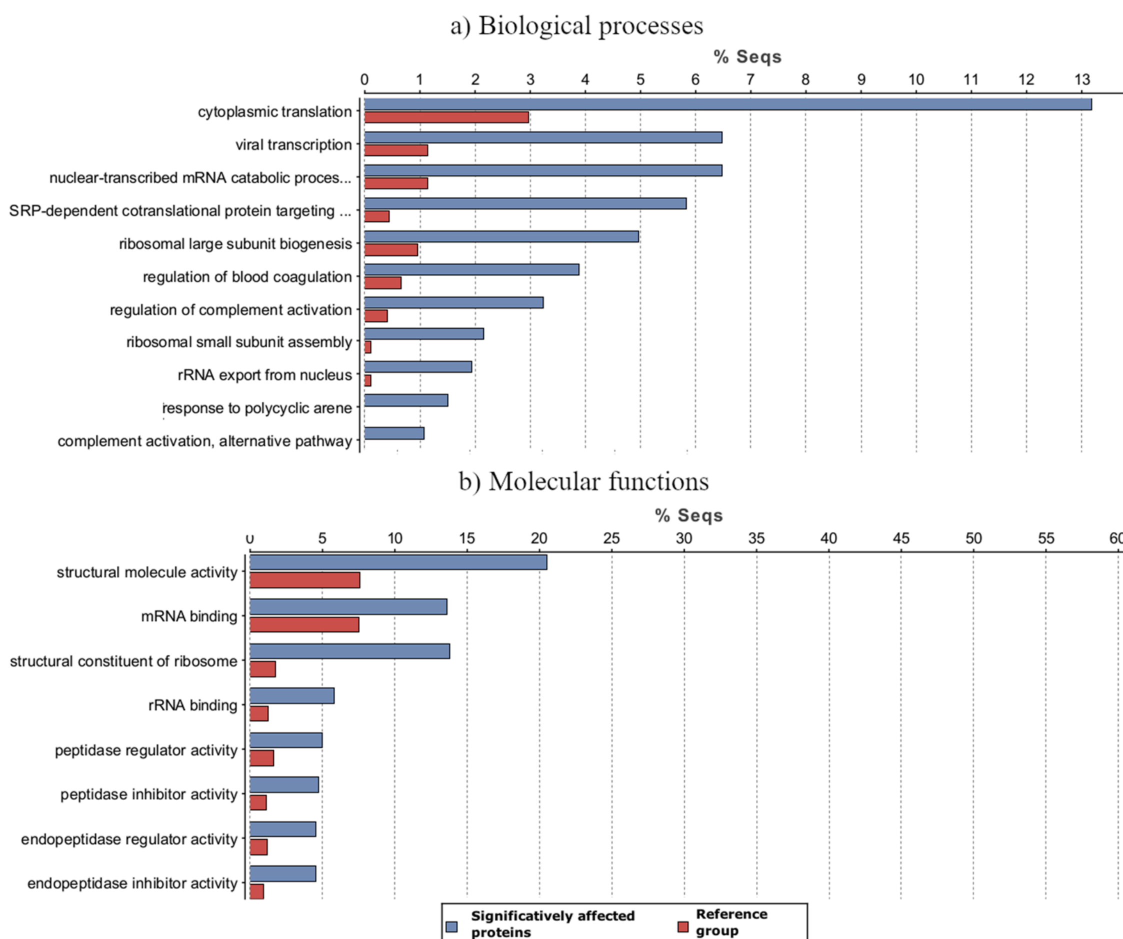

Fig. 2. Enriched bar chart representing the enriched Biological Processes (a) and Molecular Functions (b) according to proteins included in each functional category for the test (significantly affected by EE2) and reference set (all the quantified proteins of the study). For the Biological Processes only the most specific GO terms are shown (with the lowest level in the GO directed acyclic graph, not shown).

C. variegatus, a search for orthologs was performed using ERE-modulated proteins from different species found in the literature (Klinge, 2001). Among them, several proteins directly involved in oogenesis were found. A variety of VTG isoforms were upregulated by the EE2 treatment in this study, particularly VTGAb and VTGC (FC = 8.35 and 6.21 respectively). Both protein names were set following the nomenclature proposed by Finn and Kristoffersen (2007), reannotated according to the clustering results shown in the phylogenetic tree (Fig. 3). In addition of the two VTG isoforms, three protein sequences that contain a vitellogenin-domain were also detected upregulated, one corresponding to the N-terminal region (FC = 10.13), and two more corresponding to different forms of the VWDF domain (FC = 4.71 and 3.44). Another protein related to oogenesis is the ZP protein (also termed choriogenin), of which there were three different isoforms identified in the present study: ZP1, ZP3 and ZP4 (according to NCBI predicted names), showing a significant upregulation when fish were exposed to EE2 (FC between 7.6 and 14.8). Cathepsin E, another protein regulated by EREs with endopeptidase activity (see Section 3.2), was found significantly upregulated (FC= 4.27, $p < 0.001$). In contrast, two proteins, complement C3 and serotransferrin, both proteins related to an ERE, were found downregulated by the EE2 treatment (FC = 0.65 and 0.66, respectively).

3.4. Proteins related to membrane estrogen receptor and biotransformation of xenobiotics

Only two out of sixteen identified proteins in the present study involved in the signaling pathways mediated by GPER were affected. Both are isoforms of the Guanine nucleotide-binding protein (4b and 1a) and were found significantly downregulated (FC=0.84 and 0.71, respectively) (see Table S2). Therefore, results from present work suggest that EE2 does not cause remarkable and widespread effects on GPER-related protein abundances contrarily to the initial hypothesis.

In relation to the biotransformation of xenobiotics, thirty-three proteins belonging to the CYP family, and fifteen belonging to UGT family were identified in this study. Seven of the CYPs and one UGT showed statistically significant differences in abundance in this study (Table S3). The direction of expression differences was dependent on protein family, with CYP1 and CYP4 proteins slightly downregulated (FC between 0.69 and 0.88), whereas CYP2 proteins were consistently upregulated (FC between 1.9 and 2.2).

4. Discussion

In the present study, focused on the effects of exposure of male *C. variegatus* to 100 ng/L EE2 for 7 days, 3188 proteins were identified and quantified, 463 of which were found significantly affected by EE2 exposure. Previously, a two-dimensional in-gel-electrophoresis (2D-DIGE) approach was used in the freshwater fish zebrafish by Wit et al. (2010), and which detected 1500 protein spots in liver samples, 127 of which were significantly differentiated after exposing males to 30 ng/L EE2 for 4 days. In contrast, a shotgun proteomics approach detected 3767 proteins in embryos-larvae of the freshwater species Fathead minnow, 244 of which were affected by exposure to 100 ng/L EE2 after 4 days (Alcaraz et al., 2021). From the comparison of the results obtained

Table 1
Proteins found in *C. variegatus* male liver proteome related with peptidase activity. Proteins are classified by families. "Accession" column points to the corresponding accession number from Uniprot; Protein names are set from different sources: Uniprot-TrEMBL or NCBI (*); FC is the fold-change when EE2 exposure (100 ng/L) and solvent control abundance are compared. The p-value and q-value were obtained from the results of *t*-test comparing protein abundances between treatment (EE2) and solvent control samples. For those non-significant proteins after SGoF correction, q-values are not provided.

Accession	Protein name	*p*-value	FC	q-value
Ntn-hydrolase family				
A0A3Q2DF27	Isoaspartyl peptidase/L-asparaginase (ASRGL1)*	9.54E-07	5.2	1.75E-04
A0A3Q2CE59	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase isoform X1*	0.30	0.9	
A0A3Q2D7G8	Asparaginase-like protein 1	0.52	0.9	
Peptidase A1 family				
A0A3Q2DE82	Cathepsin E-like* (CTSE)	7.19E-08	4.3	1.75E-04
A0A3Q2FFT0	Cathepsin D	0.032	0.8	
A0A3Q2FFH7	Cathepsin D	0.97	1.0	
Serine-type endopeptidase activity (GO:0,004,252)				
A0A3Q2CK81	Peptidase S1 domain-containing protein (PAS1)	2.06E-06	3.4	2.97E-04
A0A3Q2DW28	Hepatocyte growth factor-like protein*	0.0001	0.7	6.54E-03
A0A3Q2D697	Plasminogen	0.0002	0.6	9.88E-03
A0A3Q2EB49	Hyaluronan-binding protein 2*	0.0006	0.7	1.88E-02
A0A3Q2CA44	Complement factor B-like*	0.0006	0.7	1.94E-02
A0A3Q2FYQ9	Complement C2-like*	0.002	0.7	3.02E-02
A0A3Q2CDE2	Coagulation factor VIIi	0.007	0.8	5.88E-02
A0A3Q2G8B8	Complement factor I*	0.009	0.8	6.34E-02
A0A3Q2CDX6	Prothrombin	0.013	0.9	7.90E-02
A0A3Q2CDN9	Coagulation factor X	0.022	0.8	1.02E-01
A0A3Q2E057	Peptidase S1 domain-containing protein	0.063	0.8	
A0A3Q2DT34	Peptidase S1 domain-containing protein	0.12	0.9	
A0A3Q2FTP4	Peptidase S1 domain-containing protein	0.13	1.1	
A0A3Q2C965	Peptidase S1 domain-containing protein	0.13	0.7	
A0A3Q2D2T2	Peptidase S1 domain-containing protein	0.14	0.8	
A0A3Q2DFD0	Vitamin K-dependent protein C*	0.15	0.9	
A0A3Q2E4F6	Peptidase S1 domain-containing protein	0.34	0.7	

in studies that use traditional gel-based approaches versus those that use non-target LC-based proteomics, a superiority of non-target proteomics can be seen in the number of detections and the precision of the quantification. A screenshot of the early response to an exposure of estrogens is shown in our study that encompasses changes that may be temporal, for balancing the dysregulations occurred, or may be of a more permanent nature, as demonstrated Voisin et al. (2019) who found long term effects over the proteome of the marine fish mangrove rivulus (*Kryptolebias marmoratus*) 140 days after chronic exposures to 4 ng/L EE2, with 49 differentially expressed proteins in the liver related to transcription, lipid/steroid metabolic process and amino acid/protein metabolic process, among others.

Several biological processes and cellular functions were overrepresented in the set of proteins affected by EE2, according to the enrichment analysis, most of them related to different aspects of protein synthesis. Proteins involved in the mRNA translation biological process were affected by the treatment. The detection of upregulated proteins with the molecular function of mRNA binding reinforces this result. A nuclear-transcribed mRNA catabolic process was also overrepresented in our results. The translation of mRNA into proteins is a ribosome-mediated process and in line with this observation, the function of structural constituents of ribosomes and rRNA binding were also enriched in the set of EE2-affected proteins, as well as the process of biogenesis of the large ribosomal subunit, the small ribosomal subunit assembly, and the rRNA export from nucleus. The process of SRP-dependent cotranslational protein targeting to membrane was another overrepresented function in the set of differentially expressed proteins due to EE2 exposure. Interestingly, this process is directly connected to protein synthesis due to its involvement in the transportation of newly synthesized proteins to the endoplasmic reticulum (Keenan et al., 2001). These effects suggest a scenario with a high synthesis and transport of proteins involved in egg production (for instance, VTG, ZP), which is a key response to the exposure to EE2 in males. These results are in line with those reported by Wit et al. (2010), who found alterations on biosynthetic and protein processes as well as RNA metabolism, among other altered functional pathways. Voisin et al. (2019) also found long term effects on 7 ribosomal proteins, 140 days after a 28 days EE2 exposure.

The increased detected synthesis of proteins involved in egg production as VTG or ZP and related mechanisms, is consistent with the well-known genomic pathway of estrogenic signaling through a nuclear estrogen receptor, which consists of modulating the expression of several genes regulated by EREs (Klinge, 2001). In this study, eleven proteins regulated by EREs and affected by the EE2 exposure were detected (Table 2), nine of them upregulated with FC values between 3.4 and 13.8. Although this pathway was not yet specifically demonstrated in *C. variegatus,* ERE-modulated proteins were described in other species, and the interpretation is justified by the fact that the segment of the ER sequence of amino acids with affinity for the ERE (the C-terminal region) is highly conserved among vertebrates (Nelson and Habibi, 2013).

Results from our study provide evidence through a direct measure at the protein level that VTG is a valid biomarker for estrogenic endocrine disruption for *C. variegatus,* which is in agreement with previous results from immunoassays (Folmar et al., 2000). It is important to note that this evidence of the EE2 effects is provided at isoform level, VTGAb (FC= 8.35) and VTGC (FC= 6.21). Upregulation of protein expression at different VTG isoforms in fish liver upon exposure to EE2 has been only reported in a proteomic work in zebrafish (Wit et al., 2010). Three isoforms of VTG have been described in teleosts and their functionality could explain the presence of VTGAb and VTGC and the absence of VTGAa in the results of this study. VTGC is in charge of nutrients supply for larvae, and VTGAa and VTGAb contribute also to hydration of the embryo (Finn et al., 2002), bring an extra stability during the lipid cargo in the liver and allow a better solubility in plasma, due to the PV region (Finn, 2007). VTGAa is also responsible for the buoyancy of the pelagic eggs (Finn et al., 2002), a function not needed in *C. variegatus* due its benthic eggs. The fact that VTGAa was not found in the protein database together with this observation, suggests that VTGAa is not present in *C. variegatus*.

In this study several ZP isoforms were among the most upregulated proteins: ZP1 (FC= 10.74), ZP3 (FC= 7.57) and ZP4 (FC= 13.76). ZP has been previously used as a biomarker of estrogenic effects in *C. variegatus*

Table 2
Proteins affected by EE2 treatment found in this study whose genes are regulated by an estrogen responsive element. "Accession" column points to the Uniprot accession. Protein names are obtained from different sources; This study [a], Uniprot-TrEMBL [b], or NCBI [c]. Symbols as in table 1. FC is the fold-change when EE2 exposure (100 ng/L) and solvent control abundance are compared. The p-value and q-value were obtained from the results of *t*-test comparing protein abundances between treatment (EE2) and solvent control samples.

Accession	Protein names	*p*-value	FC	q-value
A0A3Q2GA16	Vitellogenin Ab[a] (VTGAb)	1.04E-06	8.3	1.75E-04
A0A3Q2CDP0	Vitellogenin C[a] (VTGC)	3.94E-07	6.2	9.93E-05
A0A3Q2FF28	Vitellogenin domain-containing protein [b]	1.09E-08	10.1	5.50E-06
A0A3Q2DQU0	VWFD domain-containing protein[b]	6.82E-07	4.7	1.44E-04
A0A3Q2DNR5	VWFD domain-containing protein[b]	7.13E-07	3.4	1.44E-04
A0A3Q2E2H1	Zona pellucida sperm-binding protein 4[c] (ZP4)	1.24E-09	13.8	2.19E-06
A0A3Q2FKU6	Zona pellucida sperm-binding protein 1[c] (ZP1)	3.63E-07	10.7	9.93E-05
A0A3Q2E210	Zona pellucida sperm-binding protein 3[c] (ZP3)	3.26E-09	7.6	2.19E-06
A0A3Q2DE82	Cathepsin E-like[c] (CTSE)	7.19E-08	4.3	2.42E-05
A0A3Q2EAS2	Complement C3[c]	0.0017	0.7	0.028
A0A3Q2DRA0	Serotransferrin[b]	0.0071	0.7	0.058

based on reported effects due to exposure to estrogenic compounds at the transcriptomic level (Denslow et al., 2001; Knoebl et al., 2004). Evidence of the induction of ZP proteins by EE2 through a direct measure at protein isoform level is provided for the first time in male fish liver. Interestingly, the ZP2 form was not detected in this study, which is in agreement with the observation reported by Modig et al. (2006), who highlighted that a ZP2 homolog had never been found in fish.

An important link had previously been demonstrated between VTG and ZP overexpression and feminization in zebrafish (Fenske et al., 2009) and the formation of testis-ova in the Japanese medaka (Hirakawa et al., 2012) after chronic exposures to the same and lower concentrations of EE2 to that used in this study. Those severe symptoms related with reproduction caused by exposure to low environmental EE2 concentrations, for which VTG and ZP overexpression can serve as initial biomarkers, have been shown to induce reproductive alterations in fish leading to deleterious effects at the population level (Kidd et al., 2007). This evidence could be strengthened if similar alterations could be also demonstrated in *C. variegatus* after a chronic exposure to EE2, which can be the basis for future studies.

Regarding effects mediated by membrane ER receptors, the interaction of the GPER with xenoestrogens has been recently studied in zebrafish larvae where effects including enhanced liver growth (Chaturantabut et al., 2019) or inhibition of maturation (Pang and Thomas, 2010) as well as vitellogenesis in adult male zebrafish (Chen et al., 2019) have been observed. The proteins related to the activation of GPER were reviewed by Pico and Coort (2019). However, none of these previously reported proteins directly related with the GPER were detected in our study to be significantly affected by EE2, except for the downregulation of A0A3Q2E2V2 and A0A3Q2DW21, two isoforms of the Guanine nucleotide binding protein, 4b and 1a. The molecular effects of the activation of GPER in response to estrogens involve both rapid signaling and transcriptional regulation (Prossnitz and Barton, 2011). The transcriptional regulation, related to the vitellogenesis, acts in conjunction with the nuclear estrogen receptors (Chen et al., 2019), which is in line with the observed alterations in vitellogenesis found in the present study. However the rapid signaling events, that involve the production of cAMP, intracellular calcium mobilization and PI3K activation in mammals, may be undetectable under our experimental conditions if it is assumed that this response is not detectable after 7 days of exposure.

Besides metabolic pathways directly related with ER signaling, effects on other biological processes were also detected. Biotransformation of hydrophobic organic xenobiotics normally takes place in two

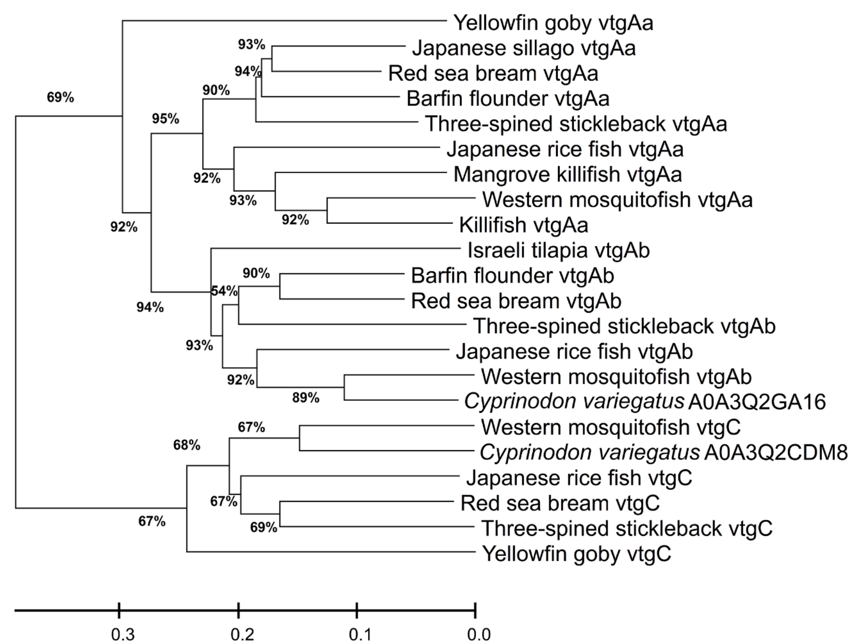

Fig. 3. Phylogenetic tree of Actinopterygii VTG isoforms used by Finn & Kristoffersen (2007) including also the *C. variegatus* VTG forms found in this study, using p-distances and Neighbor-Joining method. Scale refers to the branch length in terms of distance units employed to infer the phylogenetic tree. The percentages of each branch indicate the fraction of iterations that report the same result after a bootstrapping process of 10.000 replicas.

steps, a Phase I reaction frequently consisting of a cytochrome P450 (CYP)-dependent oxidation, and a Phase II conjugation of the oxidized metabolite by transferases such as UGT (Beiras, 2018). CYP genes are grouped according to their sequence homologies in families, such as CYP1, CYP2 or CYP4. According to Villeneuve et al. (2007) and Tolson & Wang (2010), several CYP proteins from different families are expected to be affected if xenobiotic receptors such as CAR or PXR are activated. In this study, of the seven CYPs that showed statistically significant differences in abundance in EE2-exposed fish, distinct patterns of response were obtained for the CYP1 and CYP4 proteins, which were slightly downregulated, compared to the CYP2 proteins, which were consistently upregulated (see Table S3). Unspecific CYP enzymes transform E2 to estriol and 2-hydroxy-estradiol, which are subsequently conjugated with glucuronic acid by UGT (Figure S4), an enzyme that was found to be upregulated in this study (Table S3) and which is also related to CAR and PXP receptors (Tolson and Wang, 2010). The present results support the involvement of the CYP2 family, rather than CYP1A1 or the CYP4 family, in this metabolic pathway, at least as far as biotransformation of EE2 is concerned.

Endopeptidase inhibitor activity was another molecular function affected by EE2 exposure in this study. In particular, the 21.4-fold overexpression of the WAP-like (accession A0A3Q2D0I0) in livers of exposed male fish was the most overexpressed protein in this study. To the best of our knowledge, this protein has never been related to endocrine disruption. However, our results suggest that it could represent a potential candidate that should be explored as a biomarker for estrogen exposure. To the best of our knowledge there is no studies about WAP related to estrogen exposure in fish however, a link between WAP induction and estrogen sensitivity have been reported in human ovary cell lines (Chen et al., 2016). The matching sequence of WAP-like found includes two repeated domains named "four disulfide core domain" or 4-DSC, domain characteristic of the WAP protein but also present in other proteins, since 4-DSC has been also found in other proteins that exhibit peptidase inhibitor activity (Bingle et al., 2002; Ranganathan et al., 1999). Whereas in mammals, WAP act as a nutrient in milk and regulates the development of the mammary glands (Nukumi et al., 2004), the specific function of this protein in fish has not been well stablished. Despite is likely that the protein detected in this study as WAP-like corresponds effectively to a protein with an endopeptidase inhibitor activity, future studies should help to improve the annotation of this (matching) protein sequence clarifying its nomenclature and associated functions. Interestingly, Wit et al. (2010) found proteins related to inhibition and stimulation of protease activities affected in livers of male zebrafish exposed to EE2. Shen et al. (2018) also found the molecular function of endopeptidase inhibitor activity enriched in hepatocarcinoma cells exposed to EE2. In agreement to previous reported results in the literature, stimulation but also inhibition of peptidase activity were found in the enrichment analysis carried out in the present study, these results based on the overexpression found in twelve and five proteins (including WAP-like) respectively. Mechanisms of cell proliferation and apoptotic processes, both regulated by nuclear estrogen receptors (Acconcia et al., 2005), could explain the effects of estrogens over proteins with peptidase and peptidase inhibitor activities, and the found overexpression of the WAP-like protein may work by preventing tissue degradation beyond what is required, keeping the balance between these processes

To the best of our knowledge no scientific literature has been published studying WAP-like proteins in fish (a systematic search in WoS database was carried out using the term "Whey acidic protein", filtered in the categories of "endocrinology metabolism" AND/OR "toxicology", yielding after manual filtering a total of 13 entries, none of them related to fish). Likewise, a review of WAP in lower vertebrates (Smith, 2011) identified no information about the functions of this protein in fish, however, it pointed to the existence of sequences with a WAP domain in teleost fish. Despite this lack of published literature, a high number of related proteins in teleost fish are present in the Uniprot portal: using search terms "whey acidic protein" or "WAP domain-containing protein" in Uniprot (12/08/2021), 372 proteins from 87 species of teleost fish were identified. All entries obtained from this search present some kind of peptidase inhibitory function: a "serine-type endopeptidase inhibitor activity" or the more general "peptidase inhibitor activity". The lack of manual revision of automatic annotations related to the WAP-like might be behind the fact that WAP-like overexpression caused by estrogen exposure has not been reported so far. Future studies on fish estrogenicity by using non-target omics techniques, coupled to an improvement of fish WAP-like annotations, should allow to shed light on the causal factor/s of WAP-like induction and its relation to estrogens.

4.1. Conclusions

In summary, the non-target proteomic analysis allowed to detect a general proteomic response in the male liver of *C. variegatus* exposed to 100 ng/L of EE2, approximately 15% of the quantified proteins were affected. A consistent upregulation of proteins involved in the cellular machinery of protein synthesis was found, involving ribosomal biogenesis and transcriptional processes. Generalized alterations in the abundance of the ribosomal proteins reported in this study should be considered in other similar studies based on gene expression analysis (e.g., by qPCR) because ribosomal proteins have been commonly used as reference genes. The induction of ERE-modulated proteins detected in this study is very likely the main cause of the alterations detected in ribosomal biogenesis and transcriptional processes. These affected proteins are the direct response of the stimulation of the nuclear ER, a key point relevant for ecotoxicology because its promiscuity with several pollutants. Evidence of 463 proteins affected by EE2 was provided, highlighting a widespread alterations of proteins related to egg production, affecting the two isoforms VTGAb and VTGC, and three isoforms of zona pellucida (ZP1, ZP3, ZP4), biotransformation of EE2 (involving some proteins of CYP2 family), peptidase activity (highlighting Isoaspartyl peptidase, cathepsin E and the peptidase S1 domain-containing protein) and endopeptidase inhibitory activity. This latter activity was associated with the most affected protein found in this study, the WAP-like, a surprising result that deserves further research effort to elucidate its role in endocrine signaling and to determine its potential use as a biomarker candidate of estrogenic endocrine disruption.

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CRediT authorship contribution statement

Alexandre M. Schönemann: Methodology, Investigation, Formal analysis, Writing – original draft. **Ricardo Beiras:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision. **Angel P. Diz:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106189.

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