Pyruvate carboxylase as a sensitive protein biomarker for exogenous steroid chemicals

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ABSTRACT

Assessing protein responses to endocrine disrupting chemicals is critical for understanding the mechanisms of chemical action and for the assessment of hazards. In this study, the response of the liver proteome of male rare minnows (*Gobiocypris rarus*) treated with 17 β -estradiol (E2) and females treated with 17 α -methyltestosterone (MT) were analyzed. A total of 23 and 24 proteins were identified with differential expression in response to E2 and MT, respectively. Pyruvate carboxylase (PC) was the only common differentially expressed protein in both males and females after E2- and MT-treatments. The mRNA as well as the protein levels of PC were significantly down-regulated compared with that of the controls ($p < 0.05$). Our results suggest that endocrine disruptors interfere with genes and proteins of the TCA cycle and PC may be a sensitive biomarker of exposure to exogenous steroid chemicals in the liver of fish.

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1. Introduction

For decades, attention has been given to endocrine disrupting chemicals (EDCs) found in the environment due to their long term adverse effects on development and reproduction in aquatic organisms at biologically relevant concentrations (Pickering and Sumpter, 2003; Segner et al., 2003; Sumpter and Jobling, 2013). Estrogenic chemicals, such as estrone (E1), 17 β -estradiol (E2), estriol (E3) and 17 α -ethinylestradiol (EE2) and androgenic chemicals, such as methyltestosterone, testosterone, and androstenedione have been detected in a number of water systems at concentration levels between 1 and 100 ng/L (Backe et al., 2011; Chang et al., 2011; Jiang et al., 2012; Zhou et al., 2012). Even at low ng/L, these exogenous steroid chemicals have the potential to produce detrimental biological effects. For example, exposure to both androgens and estrogens can cause alterations in growth and development, and altered sex differentiation in fish during early life stages of development (Leet et al., 2011). In addition to endocrine disruption, androgenic and estrogenic exposure can induce genotoxic damage,

modulated immune function, and alter metabolism (Filby et al., 2007).

To assess risk of exposure to these EDCs, a variety of physiological and molecular biomarkers have been developed (Ankley and Johnson, 2004; Rotchell and Ostrander, 2003). Physiological biomarkers include secondary sexual characteristics, gonadosomatic indices, plasma steroids, and gonadal histology, and these endpoints have contributed significantly for guiding interspecies assessments of EDCs. However, the aforementioned physiological endpoints provide little insight into the molecular mechanisms underlying biological responses and are susceptible to life stage and individual/species differences (Hutchinson et al., 2006). Molecular biomarkers include the transcript levels and protein abundance of vitellogenin (VTG) and other genes central in the estrogen response pathway, for example estrogen receptors (*er α* , *er β 1*, and *er β 2*) and various enzymes involved with sex hormone biosynthesis (e.g. cytochrome P450 aromatase). Molecular endpoints have been measured to better define pathways and mechanisms of both estrogenic and androgenic disruption (Celander, 2011; Martyniuk and Denslow, 2012). VTG is one of the most well documented molecular biomarker for estrogenic disruption, and this molecule can be induced in both juvenile and adult fish in response to estrogens or estrogen mimics (Denslow et al., 1999; Heppell et al., 1995; Matozzo et al., 2008). The *vtg* transcript can also be induced rapidly in the

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order of hours to days, thus it can be useful in short-term *in vivo* screens (Hutchinson et al., 2006; Marlatt et al., 2008). The expression of *vtg* is regulated by E2, which is synthesized by CYP19A1, the enzyme responsible for the conversion of testosterone (T) into E2 (Cheshenko et al., 2008; Rotchell and Ostrander, 2003). VTG can also be induced by androgens, but the response is much more variable compared with estrogens (Bogers et al., 2006). For androgenic substances, only male morphological characteristics and the androgen receptor are suggested to be reliable biomarkers (Hutchinson et al., 2006). For instance, histology of the gonads can be used to evaluate the effects of androgenic exposure. However, gonad morphology can also vary depending on the degree of androgen aromatization, making the assessment of responses to androgens potentially more challenging than estrogens (Leet et al., 2011). Therefore, it is of great importance to develop reliable biomarkers for androgenic disruptors.

Early discoveries of biomarkers for steroid hormones in ecotoxicology were based primarily on empirical observations, such as the alteration of sex ratio and breeding success (Hill Jr. and Janz, 2003). However, while more traditional endpoints used in ecotoxicology to assess growth, development and reproductive output are informative for risk assessment and can indicate exposure, they are limited in their ability to uncover the association between molecular responses and adverse outcomes in the whole organism (Van Aggelen et al., 2010). The development of molecular biomarkers such as VTG provides the potential to identify mechanisms of estrogenic disruptors. However, traditional, single endpoint bioassays for xenoestrogens provide little insight about the physiological consequences of exposure and generate limited information for characterizing the complex stress and toxin-metabolism pathways (Celander, 2011). Recent technological advances in high-throughput toxicogenomics and toxicoproteomics provide powerful tools for screening and validating molecular biomarkers for endocrine disruption (Benninghoff, 2007; Martyniuk et al., 2012a). A paradigm shift has been realized, whereby single-biomarker analysis is being replaced by multi-parametric analysis of genes or proteins (Kulasingam and Diamandis, 2008). Proteomic analysis, a powerful tool for global evaluation of protein expression, has been widely used in aquatic toxicology. Several previous studies have focused on the application of quantitative proteomics for identifying and quantifying differentially expressed proteins in response to EDCs (Biales et al., 2011; De Wit et al., 2010; Fang et al., 2010; Martyniuk et al., 2009, 2010, 2012b; Ralston-Hooper et al., 2012; Sanchez et al., 2011). Hundreds of proteins were identified in these studies; however few of them have been systematically analyzed for their correlation with endocrine disruption. Thus, except for a few examples, reliable biomarkers for exogenous steroid chemicals have not been completely characterized using quantitative proteomics.

In the present study, differential in-gel electrophoresis (DIGE) was performed to compare the liver proteome response in rare minnow after E2 and 17 α -methyltestosterone (MT) treatments. The rare minnow is distributed mostly in the upstream region of the Yangtze River and in the Sichuan Province of China. This cypriniformes species has been used extensively in recent years for aquatic toxicity testing due to its small size (30–80 mm total length), ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates (Zha et al., 2007; Zhou et al., 1995). The fish is considered to be an appropriate and useful species for the assessment of EDCs (Fang et al., 2010; Li et al., 2009; Wei et al., 2008; Yang et al., 2010). The candidate proteins were further assessed as possible protein biomarkers for exogenous steroid chemicals using real-time PCR. We also measured the response of select proteins in rare minnow liver using Western blot. In addition to identifying hepatic proteins responsive to E2 and MT,

this study provides proof of principle that proteomics is a useful approach for future ecotoxicological research using rare minnow.

2. Materials and methods

2.1. Experimental design and sample collection

Experimental design and exposures are similar to those previously published (Zha et al., 2007). Please refer to the [Supporting Information](#) for specific details. Briefly, juvenile rare minnows (~3 months old) were randomly divided into eight groups of thirty fish and exposed to one of three concentrations (control, 1, 10, and 100 ng/L; nominal concentrations) of E2 or MT for 120 days. We point out that the exposure period in this study commenced when fish were >90 dph, following sexual differentiation in this species. After 120 d of exposure, fish were sexually mature and were anesthetized on ice and tissues were collected.

2.2. Protein extraction and separation

Protein extraction and DIGE were performed according to Tan et al. (2012). Please refer to the [Supporting Information](#) for specific details.

2.3. Image acquisition and analysis

The gels were scanned with Typhoon Trio variable mode imager (GE Healthcare, NJ) at wavelengths specific for the CyDyes (488 nm for Cy2, 532 nm for Cy3, and 633 nm for Cy5). The images were analyzed using Image Master 2D Platinum 7.0 (GE Healthcare) software (Guipaud et al., 2007). Gels from male and female fish ($n = 6$ gels/group) were analyzed separately ([Supporting Information](#)). Spots were detected with the estimated number of 2000 and then matched simultaneously from 6 gels. The abundance measurements are made directly to the internal standard without interference from gel-to-gel variation. Protein spots with at least a 2-fold change and a p -value <0.05 were assigned as significant differentially expressed protein spots. In addition, some spots with a ratio of 1.5–2 were also selected as proteins of interest and identified.

2.4. Protein identification and network analysis

Following the image acquisition, gels were stained with silver nitrate and used for in-gel digestion. The resulting peptide mixtures were subjected to matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometer (MALDI-TOF/TOF-MS) analysis according to Meng et al. (2009) using a Bruker UltraFlex MALDI-TOF/TOF instrument. The mass signals generated from the MS mode and the MS/MS mode were combined for protein identification in an NCBI *Danio rerio* database (38841 sequences, released February 4, 2013) using the Mascot search engine (www.matrixscience.com). Peptide and protein identifications were accepted if C.I. % (Confidence Interval %) values were greater than 95%. The identified proteins were then matched to specific processes or functions by searching Gene Ontology (<http://www.geneontology.org/>) and KEGG (<http://www.genome.jp/kegg/>) database. Network analysis was performed according to previous studies in teleost fishes exposed to estrogens and androgens (Martyniuk et al., 2010, 2012b). Please refer to the [Supporting Information](#) for more specific details of in-gel digestion, mass spectrometry, database search conditions and network analysis.

2.5. Validation of differentially expressed proteins

Real-time PCR and Western blot analysis were employed to validate the differentially expressed proteins. The transcripts of alcohol dehydrogenase 8a (*adh8a*), eukaryotic translation elongation factor 2b (*ef2b*), vitellogenin 1 (*vtg1*) and pyruvate carboxylase (*pc*) were examined by quantitative real-time PCR. Expression of the housekeeping β -actin gene was unaffected under experimental conditions (data not shown) and thus it was used as an internal control. Gene names, accession numbers, forward and reverse primer sequences, and amplicon sizes are listed in [Supplemental Table S1](#). Real-time PCR was performed according to Li et al. (2009). We also explored in more detail the response of PC in males and females exposed to E2 and MT. Unlike the DIGE analysis, which measured the liver proteome in only males exposed to E2 and females exposed to MT, western blotting was performed in both sexes for both E2 and MT treatments. Please refer to the [Supporting Information](#) for more specific details of real-time PCR and Western blot analysis.

2.6. Statistical analysis

One-way ANOVA was used for statistical analysis of proteomic data. Protein spots with at least a 2-fold change and a p -value < 0.05 were assigned as significant differentially expressed protein spots. For Western blots and qPCR, statistical analysis of variance was performed in SPSS (version 13.0; USA), and a Levene's test of homogeneity of variance and LSD t -test were used. All quantitative data are expressed as means $\pm$ S.E. A probability of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Proteomic analysis

After E2 treatment, 47 significantly altered proteins (ratio ≥ 2 , $p < 0.05$) were detected in male rare minnow across all concentrations. Twenty-three protein spots showing sufficient intensity, clear separation from surrounding spots, and consistent spot shape and size were excised, subsequently in-gel digested by trypsin, and then successfully analyzed by MALDI-TOF/TOF (Fig. 1A). Among these, 14 proteins were up-regulated and 9 proteins were down-regulated after E2 treatments compared to that of controls (Table 1). These proteins were primarily involved in protein catabolic process, carbohydrate metabolic process, signal transduction, oxidation–reduction process in addition to other functions. Specifically, proteins associated with tricarboxylic acid (TCA) cycle were significantly affected; pyruvate carboxylase (PC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were increased and cytoplasmic aconitate hydratase (ACO1) was decreased (Table 1).

Following MT treatment, 44 significantly altered proteins (ratio ≥ 2 , $p < 0.05$) were detected and 24 protein spots were selected and identified by MALDI-TOF/TOF (Fig. 1B). Among these, 9 proteins were up-regulated and 15 proteins were down-regulated compared to that of controls (Table 2). These proteins were primarily associated with chemical response, organ development, protein catabolic process, carbohydrate metabolic process, signal transduction, oxidation–reduction process as well as other functions. Specifically, vitellogenin 1 precursor (VTG1), eukaryotic translation elongation factor 2, like (EEF2), alcohol dehydrogenase (ADH), and pyruvate carboxylase (PC) were significantly affected in the liver after MT treatments (Table 2). Of these, VTG1, EEF2 and ADH are associated with chemical stimulus. Interestingly, PC was also identified as a protein differentially expressed after E2 treatments in males and showed the opposite response to MT (i.e.

increasing with E2) (Table 2). Therefore, VTG1, EEF2, ADH, and PC were selected to be further validated by real-time PCR.

3.2. Relationship between mRNA and proteomic data of PC, VTG1, EEF2 and ADH

The relative levels of *vtg1*, *eef2b* and *adh8a* in males and females after exposure of E2 and MT were determined (Supplemental Fig. S1) and the expression patterns generated after MT exposures were compared to corresponding protein levels. The protein abundance levels of PC, VTG1, EEF2, and ADH were down-regulated in all MT-treated groups compared with control groups (Table 2). VTG1 exhibited a 2-fold decrease in protein abundance following 1 ng/L MT treatment while EEF2 and ADH showed a 2-fold decrease in fish exposed to 10 ng/L MT. The mRNA level of *vtg1* significantly decreased in response to 100 ng/L MT but increased in individuals exposed to 1 and 10 ng/L MT ($p < 0.05$, Fig. 2A). The protein abundance of EEF2 was significantly decreased by MT in 10 ng/L concentration and the mRNA level of *eef2b* was significantly decreased by 100 ng/L MT (Fig. 2A). ADH displayed a decrease in protein abundance but an increase in *adh8a* mRNA levels (Fig. 2A). These results showed that the mRNA levels of VTG1, EEF2 and ADH showed, in general, opposite response patterns compared to protein data collected with DIGE.

DIGE showed that PC was significantly down-regulated following MT treatment (−2.0 fold in 1 ng/L group and −2.33 fold in 10 ng/L group, upper graph in Fig. 2B), corresponding to the expressions of *pc* mRNA which was also significantly decreased with 10 ng/L MT ($p < 0.05$, upper graph in Fig. 2B). Thus, the response of *pc* mRNA and PC protein were consistent in the livers of female rare minnows exposed to MT. Conversely, PC was significantly up-regulated in livers of male rare minnows exposed to E2 (2.61 fold in 1 ng/L group, lower graph in Fig. 2B), in contrast to *pc* mRNA levels which were significantly down-regulated following 1 and 10 ng/L E2 treatment ($p < 0.05$, lower graph in Fig. 2B).

Fig. 1. Representative two-dimensional gel image of the Cy2-labeled proteins that comprise the internal standard. The 47 identified protein spots are marked with master numbers and displayed. (A) There were 23 identified protein spots significantly altered in livers of male rare minnows exposed to E2. (B) There were 24 identified protein spots significantly altered in livers of female rare minnows exposed to MT. The information for the proteins corresponding to the spot numbers is presented in Tables 1 and 2.

Table 1

A detailed list of protein spots identified by MALDI-TOF/TOF MS from the liver of male rare minnow following E2 exposure.

Spot ID ^a	GI accession no.	Protein name	MW/PI	Score	Matched peptides	Fold change ^b		
						1 ng/L Vs ctl	10 ng/L Vs ctl	100 ng/L Vs ctl
Protein catabolic process								
187	161612168	Dmgdh protein	97.9/6.54	58	2	−2.13	−1.93* ^c	1.14
582	41282194	Glutamate dehydrogenase 1b	60.3/8.45	108	2	−1.76*	−1.80*	−0.98
740	40548302	Carboxypeptidase A precursor	47.8/6.36	37	1	1.42	1.46	2.88
761	82187435	4-Hydroxyphenylpyruvate dioxygenase	45.4/6.33	91	2	−1.84*	−0.99	1.11
1060	61806624	Six-cysteine containing astacin protease 3 precursor	29.5/6.16	137	3	0.97	0.98	2.66
1079	13811441	Proteasome subunit alpha type 6-B	28.9/7.99	36	1	1.51*	1.45	2.11
1088	62531021	Try protein	27.2/7.46	34	1	−0.90	−0.96	2.37
1112	62531021	Try protein	27.2/7.46	48	1	2.17	2.02	3.10
Carbohydrate metabolic process								
174	77993336	Cytoplasmic aconitate hydratase	99.5/6.45	146	3	−2.00	−2.74	−1.10
268	18858695	Pyruvate carboxylase, mitochondrial	130.7/6.44	34	1	2.61	1.63*	1.08
425	31872040	Transketolase	68.7/6.81	54	1	−1.62*	−1.78*	−1.04
922+	50540366	ATP-citrate synthase	120.6/6.83	58	11	−1.59*	−1.57*	−0.96
990	169403947	Glyceraldehyde-3-phosphate dehydrogenase	36.0/8.2	36	2	3.98	3.10	1.16
1005+	169403947	Glyceraldehyde-3-phosphate dehydrogenase	36.0/8.2	68	7	2.56	3.73	1.29
Signal transduction								
137+	29179488	Mvp protein	95.1/5.48	72	7	−1.54*	−2.10	−0.90
1039+	326664643	PREDICTED: laminin subunit alpha-3	115.7/5.77	58	20	3.09	2.50	1.50*
1077+	62955541	Ras homolog gene family, member Ub	27.1/9.04	69	8	1.54*	1.51*	2.10
Oxidation—reduction process								
509	9622234	Catalase	59.8/8.12	83	2	1.23	−1.62*	1.27
1090	41056185	Uncharacterized protein LOC393297	29.3/5.20	36	2	−1.40	1.10	2.20
Other function								
701	41054972	40S ribosomal protein SA	34.2/4.75	33	1	2.69	2.13	1.22
705+	326665514	PREDICTED: NACHT, LRR and PYD domains-containing protein 3-like	161.3/1.57	61	13	−1.86*	−1.43	−1.24
724+	63101292	Zgc: 112061 protein	104.6/6.58	60	9	1.01	1.34	2.48
1076+	41056139	tRNA-dihydrouridine synthase 3-like	76.3/8.50	59	13	0.93	1.07	3.21

^a Proteins identified by PMF noted by “+” following spot ID.^b The average fold changes as compared to the controls. Bold character indicates significant fold change values of significantly altered protein spots (ratio ≥ 2 , $p < 0.05$). The down-regulations are noted by “−”.^c Asterisk (*) indicates the protein of interest with a ratio of 1.5–2.

3.3. Evaluation of PC by Western blot and real-time PCR

The protein levels of PC in the livers of rare minnows in response to E2 and MT were further evaluated by Western blot to verify DIGE results (Fig. 3). In general, there was a down-regulation of PC protein in male and female fish following both E2 and MT treatments and this was observed in one or two concentrations (Fig. 3). Following E2 exposure, PC protein showed approximately a 2-fold change in the livers of males at 1 and 10 ng/L (Fig. 3A). In females, there was no significant effect of E2 on PC protein levels (Fig. 3B). After MT treatment, the protein levels of PC were significantly down-regulated at 100 ng/L in the livers of males (Fig. 3C). In females, PC was significantly down-regulated at 10 ng/L MT (Fig. 3D). Thus, in both males and females, Western blot analysis suggested that the abundance of PC protein decreased with E2 and MT treatments. The Western blot results of females exposed to MT were consistent with our DIGE observation. However, the results of males exposed to E2 were inconsistent between the two techniques; we hypothesize that this may be due to posttranslational modifications of PC or other regulatory mechanisms.

The *pc* mRNA levels in liver from males and females were also measured in order to compare the transcript to the protein (Fig. 4). The levels of *pc* mRNA were down-regulated in all liver samples in response to E2 and MT. The mRNA levels of *pc* were significantly decreased in the livers of both male and female rare minnows in response to 1 and 10 ng/L E2 ($p < 0.05$, Fig. 4A and B). With MT treatment, the mRNA levels of PC were significantly decreased in all treatment groups in the livers of males (Fig. 4C) and down-regulated in the 10 ng/L group of females ($p < 0.05$) (Fig. 4D). These results demonstrate that the levels of *pc* mRNA corresponded to that of the protein levels measured using Western blot (Fig. S2).

3.4. Protein networks reveal that differentially expressed proteins are primarily involved in metabolism and stress

Protein interaction networks were constructed to obtain a more holistic view of differentially expressed proteins in the female and male liver. Consistent with steroid action in the liver, cellular functions of proteins that were affected by both E2 and MT included those related to energy metabolism and general stress (Fig. 5). The protein network in males revealed that proteins differentially affected by E2 were involved in processes such as the pentose-phosphate shunt and general stress (e.g. catalase activity and reactive oxygen species) (Fig. 5A). The protein network in female revealed that proteins differentially affected by MT were involved in processes such as glycogen phosphorylation and degradation, glucose metabolism, translation, and purine metabolism (Fig. 5B). There were three entities in the pathways that were affected by both E2 and MT. These included glucose metabolism, tricarboxylic acid cycle, and PC. MT treatment in females decreased all three entities while E2 treatment in males increased both glucose metabolism and PC, while the tricarboxylic acid cycle was down-regulated. All abbreviations for the protein networks are found in the Figure caption.

4. Discussion

Exogenous steroid chemicals are widely detected in a number of water systems, ranging from several to hundreds ng/L (Backe et al., 2011; Chang et al., 2011; Jiang et al., 2012; Zhou et al., 2012). These compounds produce detrimental biological effects even at low ng/L levels (Filby et al., 2007; Leet et al., 2011). To identify putative biomarkers of exogenous steroid chemicals, the hepatic proteome of

Table 2

A detailed list of protein spots identified by MALDI-TOF/TOF MS from the liver of female rare minnow following MT exposure.

Spot ID ^a	GI accession no.	Protein name	Biological process/Molecular function	MW/PI	Score	Matched peptides	Fold change ^b		
							1 ng/L Vs ctl	10 ng/L Vs ctl	100 ng/L Vs ctl
Response to chemical stimulus									
46	166795887	Vitellogenin 1 precursor	Response to estradiol stimulus; response to xenobiotic stimulus	150.3/8.68	132	2	−2.10	−1.49	−1.34
175	28278942	Eukaryotic translation elongation factor 2, like	GTP catabolic process; response to chemical stimulus	96.4/6.28	54	2	−1.74 ^{*c}	−2.30	−1.35
850	15428578	Alcohol dehydrogenase	Ethanol metabolic process; response to chemical stimulus	41.3/8.3	50	1	−1.19	−2.00	−0.97
Organ development									
753	6636384	Alpha-cardiac actin	Atrioventricular canal development; endocardial cushion formation	42.3/5.29	303	5	0.97	−2.01	−1.66*
754+	113951763	Olfactomedin-like protein 3B precursor	Multicellular organismal development	45.2/5.6	59	6	−1.77*	−2.68	−1.18
874+	50539690	Intraflagellar transport protein 172 homolog	Pronephros development; regulation of TOR signaling cascade	199.0/5.6	58	14	1.49	1.21	2.08
Carbohydrate metabolic process									
105	18858695	Pyruvate carboxylase, mitochondrial	Gluconeogenesis	130.7/6.44	68	1	−2.00	−2.33	−1.53*
213	56606106	Liver glycogen phosphorylase	Carbohydrate metabolic process	111.8/5.50	275	4	−1.88*	−2.49	−1.34
1086+	47085697	Hypoxanthine–guanine phosphoribosyltransferase	Hypoxanthine metabolic process; purine ribonucleoside salvage	24.9/6.21	73	13	−1.85*	−1.55*	−1.20
1120	47271422	Triosephosphate isomerase B	Gluconeogenesis; glycolysis	27.1/6.9	62	2	1.67*	1.85*	1.57*
1295	37748736	ATPase, H+ transporting, lysosomal, V1 subunit B, member a	ATP hydrolysis coupled proton transport; ATP metabolic process	56.3/5.39	87	4	2.00	0.92	0.78
Protein catabolic process									
127	47937910	Aars protein, partial	Alanyl-tRNA aminoacylation	84.7/5.39	82	4	−2.39	−3.06	−2.18
287	66910298	Si:dkey-276i5.1 protein	Glycyl-tRNA aminoacylation	87.1/6.8	62	4	1.80*	2.17	1.56*
729	33416881	LOC402826 protein	Histidine catabolic process	49.0/6.17	151	3	−1.85*	−2.13	−1.51*
Oxidation–reduction process									
553	61806472	Uncharacterized protein LOC541322	Oxidation–reduction process	61.1/5.62	45	2	0.86	−1.33	−1.69*
1065	148226847	Peroxioredoxin-4 precursor	Antioxidant activity; peroxiredoxin activity	29.4/6.30	34	1	1.46	1.56*	1.56*
Signal transduction									
468+	326667642	PREDICTED: cyclin-G-associated kinase	AMP biosynthetic process; purine ribonucleoside salvage	62.3/8.46	62	8	4.43	2.39	1.44
755	27882111	Adka protein		37.9/5.43	45	1	1.69*	1.96*	1.69*
845+	326673035	PREDICTED: tyrosine-protein phosphatase non-receptor type 13		185.6/6.06	64	17	−1.78*	−1.39	−1.02
1035+	326674793	PREDICTED: mdm2-binding protein-like		96.0/8.83	58	6	1.68*	2.00	1.49
Other function									
93	292619409	PREDICTED: hypothetical protein LOC794259	Gene silencing by RNA; nucleic acid phosphodiester bond hydrolysis	291.6/6.76	42	1	−1.50*	−2.29	−1.36
138	30351169	p100 co-activator variant 1		100.7/6.93	70	2	−1.48	−2.91	−1.53*
200	90093334	Transitional endoplasmic reticulum ATPase-like	ATP binding	90.2/5.44	52	1	−1.66*	−2.00	−1.50*
1050+	326680510	PREDICTED: zinc finger protein 41		50.3/9.30	71	11	2.72	2.17	2.48

^a Proteins identified by PMF noted by “+” following spot ID.^b The average fold changes as compared to the controls. Bold character indicates significant fold change values of significantly altered protein spots (ratio ≥ 2 , $p < 0.05$). The down-regulations are noted by “−”.^c Asterisk (*) indicates the protein of interest with a ratio of 1.5–2.

Chinese rare minnows was analyzed after fish were exposed to E2 and MT (1, 10, 100 ng/L) for 120 days. A total of 23 and 24 differentially expressed proteins were identified using the DIGE technique coupled with MALDI-TOF/TOF-MS following E2 and MT exposure, respectively. Most of these proteins were involved in chemical response, organ development, protein catabolic process, carbohydrate metabolic process, signal transduction, oxidation–reduction process and other functions. In a previous study, 2-DE coupled MALDI-TOF/TOF-MS has been applied to investigate the hepatic responses in adult male zebrafish following 1 nM E2 treatment for 14 days (Jin et al., 2010), and 13 significantly altered proteins (ratio > 2) were detected. These proteins were involved in oxidative stress, signal transduction, oxidation–reduction and cellular calcium ion homeostasis. In addition, Martyniuk et al. (2009) used isobaric tags for relative and absolute quantitation (iTRAQ) to study the hepatic proteome response of female fathead minnows exposed to 17 β -trenbolone, a potent pharmaceutical androgen, and flutamide, an anti-androgenic pharmaceutical. The authors identified 45 proteins

that were differently affected ($p < 0.05$) by the treatments of 17 β -trenbolone (5 μ g/L), flutamide (500 μ g/L) or a mixture of both compounds, and these proteins were primarily associated with cellular metabolism and oxidative stress responses. Our findings and that of the previous studies suggest that proteomics methods can characterize putative biomarkers for exogenous steroid chemicals. In addition, biological functions related to the differently expressed proteins in the rare minnow were in general agreement with that of previous reports in fish exposed to sex steroid mimics.

In this study, three down-regulated proteins (VTG1, EEF2, and ADH) detected in the livers of female rare minnows exposed to MT were involved in chemical response. Previous researchers have reported that these proteins are associated with endocrine disruption (De Wit et al., 2010; Lam et al., 2008; Levi et al., 2012; Zheng et al., 2013). Vitellogenin protein was reduced after low MT treatment in the rare minnow. The synthesis of VTG is widely accepted as a biomarker for estrogenic contamination in the aquatic environment (Matozzo et al., 2008). In a study investigating

Fig. 2. Protein and relative mRNA levels of selected proteins in the livers of rare minnows following E2 and MT treatments. The bar charts show the protein levels based upon 2D-DIGE and mRNA levels using real-time PCR. The values represent the average fold changes. Significant changes of mRNA levels as compared to the controls ($p < 0.05$) are indicated by asterisks (*), and pound sign (#) refers to an average increase or decrease higher than 2-fold in protein levels compared to the controls. (A) Differential expression of ADH, EEF2, and VTG1 in the livers of female rare minnows following MT exposure. (B) Differential expression of PC mRNA and protein levels are compared between female and male rare minnows exposed to MT and E2, respectively.

the effects of the aromatase inhibitor fadrozole, a significant decrease in plasma VTG relative to controls was observed and the expression level of *vtg1* was down-regulated in female fathead minnows (Ralston-Hooper et al., 2012). This effect could be the result of increasing levels of non-aromatized T. In our study, the abundance of VTG1 protein was down-regulated in all MT-treated groups and the mRNA level of *vtg1* was significantly decreased at 100 ng/L MT, responses that are consistent with the results of the aforementioned fadrozole exposure. However, at low-concentrations, the mRNA levels of *vtg1* were significantly higher compared to levels in control animals. The results suggest that *vtg1* may also be induced by androgens, a response that has been observed with *in vitro* hepatocyte treatments with androgens (Kim et al., 2003).

The protein EEF2 is involved in protein synthesis. A previous study has demonstrated that EEF2 is up-regulated and subjected to PTMs in adult male zebrafish (*Danio rerio*) exposed to 30 ng/L EE2 for 4 and 28 days (De Wit et al., 2010). In our study, both EEF2 mRNA and protein levels were altered in response to MT. The inconsistent results in directional change between mRNA and protein suggest that EEF2 might be under complex transcriptional/translational control and post-translational regulation. However, as pointed out by others, mRNA and protein correlations can be difficult to capture in short term, single time point experiments (Martyniuk et al., 2012c). Previous studies have demonstrated that treatment with estrogens induces posttranslational modifications in EEF2 (Blake et al., 2005) and this could result in non-congruence between mRNA and protein levels. In addition, the expression

Fig. 3. PC protein quantitation in the livers of rare minnows exposed to E2 and MT by Western blot. Western blots were performed in liver lysates from fish samples as follows; (A) males and (B) females exposed to E2; (C) males and (D) females exposed to MT; and control tissues. Equal amounts of the protein were electrophoresed in each gel lane, and representative immunoblots are shown. β -actin was used as a loading control. The ratios of respective proteins to β -actin were calculated by performing densitometric analysis using VisionWorksLS software, and the data are represented as means $\pm$ S.E. * $p < 0.05$, relative to control.

patterns of *ee2b* mRNA levels after E2 and MT treatments were also compared. The expression of *ee2b* mRNA was significantly altered in fish exposed to E2, but only markedly altered in high exposure concentration of MT. This could be due to the different mechanisms of EE2 regulation in response to steroid treatments.

ADH (or ADH8A, coded by gene *adh8a*) has been classified as a functional zebrafish class I alcohol dehydrogenase (Reimers et al., 2004). Transcriptomics analyses in zebrafish showed that *adh8a* is significantly regulated by estrogenic compounds (Kausch et al., 2008; Lam et al., 2008). In our study, DIGE and real-time PCR showed that ADH was significantly altered in response to E2 and MT. However, the different expression patterns of ADH protein and discrepant trends between males and females in mRNA levels suggest that the regulation of *adh8a* may be influenced by the sex of the organism. Our findings, and those reported by previous studies, suggest that the protein and gene expression patterns of VTG1, EEF2, and ADH may not be reliable molecular biomarkers for the screening of androgenic chemicals.

Pyruvate carboxylase (PC) was the only differentially expressed protein detected in the livers of male rare minnows exposed to E2 and female fish exposed to MT. Similar to VTG1, EEF2, and ADH, the alteration pattern of *pc* mRNA did not typically follow the directional change of that of the protein. In a previous study on the proteomic response of fathead minnow brain to EE2, mRNA levels of *microtubule associated protein tau* (*mapt*) were decreased while

the protein abundance was increased (Martyniuk et al., 2010). These results suggest that the lack of correlation between *pc* mRNA transcript and protein abundance may be due to differential regulatory mechanisms at the level of the transcriptome and proteome. Furthermore, real-time PCR and Western blotting revealed that PC was significantly down-regulated in E2 and MT exposed rare minnows at low concentrations on both transcriptional and translational levels, which indicates that PC is sensitive to the stimulus of these exogenous steroid chemicals. However, the concentration–response effects were not completely linear, which suggests that the dynamics of molecular reactions were complex and the turnover rates between regulation (synthesis and breakdown) of mRNAs and proteins were different. In a previous study of rare minnow, similar results were also observed in many proteins and its mRNA levels, such as TCTP, GST, MSR and others (Wei et al., 2008). Such concentration–response effects were also observed in zebrafish embryo proteome responses to six different phenanthrene concentrations varying from 0.049 to 0.345 mg/L (Gündel et al., 2012). In the present study, there was some discrepancy between the DIGE and western blot results for PC levels in males exposed to E2, which might be the result of posttranslational modifications or other regulatory mechanisms. For example, in other eukaryotes, PC is also expressed as two isozymes (Jitrapakdee et al., 2007) that show differential regulation. This may be the case in the rare minnow and the antibodies may not be specific for one

Fig. 4. Differential expression of PC on mRNA level in the livers of rare minnows exposed to E2 and MT. The relative expression levels of *pc* in the livers of (A) male and (B) female rare minnows after 120 days of exposure to E2 and (C) males and (D) females treated by MT are analyzed by real-time PCR. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. Data expressed as mean $\pm$ S.E of each treatment (males or females, $n = 3$). * $p < 0.05$, relative to control.

Fig. 5. Protein networks for (A) males treated with E2 and (B) females treated with MT. Green indicates that the protein/cell process are down-regulated while red indicates that the protein/cell process is up-regulated. Abbreviations are as follows; AARS, alanyl-tRNA synthetase; ACLY, ATP citrate lyase; ACO1, aconitase 1, soluble; ACTC1, actin, alpha, cardiac muscle 1; ADH1, alcohol dehydrogenase 1A (class I), alpha polypeptide; ADK, adenosine kinase; AMDHD1, amidohydrolase domain containing 1; ASTL, astacin-like metallo-endopeptidase (M12 family); ATP6V1B1, ATPase, H⁺ transporting, lysosomal 56/58 kDa, V1 subunit B1; CAT, catalase; CPA1, carboxypeptidase A1 (pancreatic); CS, citrate synthase; DMGDH, dimethylglycine dehydrogenase; DUS3L, dihydrouridine synthase 3-like (*S. cerevisiae*); EEF1A2, eukaryotic translation elongation factor 1 alpha 2; GAK, cyclin G associated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GARS, glycyl-tRNA synthetase; GLUD1, glutamate dehydrogenase 1; HPD, 4-hydroxyphenylpyruvate dioxygenase; HPRT1, hypoxanthine phosphoribosyltransferase 1; IFT172, intraflagellar transport 172 homolog (*Chlamydomonas*); KIF20A, kinesin family member 20A; LAMA3, laminin, alpha 3; MTBP, Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104 kDa; MVP, major vault protein; OLFML3, olfactomedin-like 3; PC, pyruvate carboxylase; PRDX4, peroxiredoxin 4; PRSS1, protease, serine, 1 (trypsin 1); PSMA6, proteasome (prosome, macropain) subunit, alpha type, 6; PTPN13, protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase); PYGL, phosphorylase, glycogen, liver; RHOU, ras homolog gene family, member U; RPSA, ribosomal protein SA; SND1, staphylococcal nuclease and tudor domain containing 1; TKT, transketolase; TPI1, triosephosphate isomerase 1; VCP, valosin containing protein; ZFP41, zinc finger protein 41 homolog (mouse). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PC isozyme. Despite this, it is clear from these data that PC is sensitive to steroid regulation at the protein level.

PC is a critical mitochondrial enzyme regulating the citric acid cycle; it also facilitates other biosynthetic reactions by converting pyruvate into oxaloacetate, and is localized within the mitochondrial matrix of the cells of many organs and tissues (Marin-Valencia et al., 2010). In astrocytes, PC is required for gluconeogenesis and glycogen synthesis and to replenish α -ketoglutarate removed from the TCA for the synthesis of glutamine, the main neuronal precursor of γ -aminobutyric acid (GABA). Thus, these inter-related biochemical pathways could impact hypothalamic–pituitary–gonad (HPG) function (Ankley et al., 2009; Marin-Valencia et al., 2010; Murin et al., 2009). Previous studies have observed an increase in glutamate and alanine in the livers of estradiol-treated eelpout (*Zoarces viviparus*), and it was concluded that these increases might indicate E2 influenced energy metabolism in exposed animals (Andreassen et al., 2005). In this study, the decrease of glutamate dehydrogenase and PC were detected in the E2 exposure, which suggests that glutamine synthesis may have been impacted by E2 via down-regulation of PC. PC has been reported to be associated with tumor growth by mediating glucose-dependent anaplerosis, allowing cells to achieve glutamine independence (Cheng et al., 2011). PC is also integrated in other biochemical processes. In a previous study on the toxicity of tributyltin (TBT) on abalone, Zhou et al. (2010) investigated endogenous metabolites and found a decrease in pyruvate. The authors hypothesized that the declining levels of pyruvate indicated that there was a shift in pyruvate metabolism and that this was likely towards the formation of acetyl-coenzyme A. This response could stimulate PC to convert pyruvate into oxaloacetate, leading to further accumulation of TCA cycle intermediates. It is clear that PC is involved in several biochemical pathways and that it exerts its effects via divergent cellular mechanisms. Therefore, disruptions in this protein by environmental contaminants can potentially affect multiple levels of biochemical homeostasis.

There is also a relationship between the expression of PC and human diseases; thus there may be a link between altered PC expression due to environmental contaminants and human health. For example, a deficiency of PC causes multi-organ metabolic imbalance and leads to disease (Marin-Valencia et al., 2010). There is increasing interest in PC recently in endocrinology because of its epidemiological association with type 2 diabetes (Han and Liu, 2010). In a previous study, it was revealed that inhibition of PC reduced plasma glucose and lipid concentrations and that the rate of endogenous glucose production was affected as well, indicating that PC played an important role in gluconeogenesis and energy production (Kumashiro et al., 2013). In the present study, significant alteration of PC and other proteins associated with carbohydrate metabolic processes suggest that gluconeogenesis and energy production are targets of exogenous steroid chemicals. This is supported by our pathway analysis; proteins related to glucose metabolism, tricarboxylic acid cycle, and glycogen degradation were differentially affected by E2 and MT in the liver. Therefore, diseases related to metabolic dysfunction may be exacerbated by EDCs.

PC is responsive to chemicals although physiological and nutritional stressors can also induce changes in PC abundance. Overexpression of *pc* mRNA has been observed to be a response to heat stress and feed restriction and this occurs through the regulation of promoter regions upstream of PC (White et al., 2011, 2012). Previous proteomics studies have shown that PC is significantly down-regulated in the livers after EE2 exposure in male zebrafish (De Wit et al., 2010). In the present study, proteomic observations revealed that PC was significantly down-regulated in livers of rare minnows exposed to both E2 and MT consistent with data in zebrafish. Biales et al. (2011) observed that PC was up-regulated in brains of female zebrafish exposed to prochloraz, a pesticide with

effects on the HPG axis. This is inconsistent with our findings here of a decrease in PC and may be due to chemical or tissue specific regulation. However, these studies and ours suggest that PC is affected by chemicals that perturb the reproductive axis and may be a sensitive biomarker for exogenous steroid chemicals.

In conclusion, we analyzed the hepatic proteome of male and female rare minnow in response to E2 and MT, respectively. The proteomic data presented here complement genomic data obtained from previous studies with estrogens and androgens in the liver of fish. PC was identified to be a sensitive protein that was affected by both E2 and MT, supporting its potential use as a biomarker for EDCs. In addition, this study also demonstrated that DIGE can be a powerful approach for biomarker discovery in ecotoxicology and specifically, in the rare minnow. In the future, additional studies should focus on the regulatory mechanisms of PC by steroid hormones to further validate this protein as a sensitive biomarker for exogenous steroid chemicals.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2014.03.006>.

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