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Effect of crude oil petroleum hydrocarbons on protein expression of the prawn *Macrobrachium borellii*☆,☆☆

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ABSTRACT

Hydrocarbon pollution is a major environmental threat to ecosystems in marine and freshwater environments, but its toxicological effect on aquatic organisms remains little studied. A proteomic approach was used to analyze the effect of a freshwater oil spill on the prawn *Macrobrachium borellii*. To this aim, proteins were extracted from midgut gland (hepatopancreas) of male and female prawns exposed 7 days to a sublethal concentration (0.6 ppm) of water-soluble fraction of crude oil (WSF). Exposure to WSF induced responses at the protein expression level. Two-dimensional gel electrophoresis (2-DE) revealed 10 protein spots that were differentially expressed by WSF exposure. Seven proteins were identified using MS/MS and *de novo* sequencing. Nm23 oncoprotein, arginine methyltransferase, fatty aldehyde dehydrogenase and glutathione S-transferase were down-regulated, whereas two glyceraldehyde-3-phosphate dehydrogenase isoforms and a lipocalin-like crustacyanin (CTC) were up-regulated after WSF exposure. CTC mRNA levels were further analyzed by quantitative real-time PCR showing an increased expression after WSF exposure. The proteins identified are involved in carbohydrate and amino acid metabolism, detoxification, transport of hydrophobic molecules and cellular homeostasis among others. These results provide evidence for better understanding the toxic mechanisms of hydrocarbons. Moreover, some of these differentially expressed proteins would be employed as potential novel biomarkers.

1. Introduction

Oil spills are one of the most tragic environmental disasters causing long-term damage to the ecosystem and human health (Goldstein et al., 2011). In addition to acute oil spills, aquatic pollution by petroleum is also caused by chronic discharges which, though smaller can also have serious effect, are particularly critical in rivers and coastal waters.

Although the most striking feature of an oil spill is the surface slick or mousse layer of crude oil, the water soluble fraction (WSF) is the responsible for the toxic effects. The WSF is quite different from the parent oils and it is mostly a mixture of low-boiling aromatic hydrocarbons, closely resembling the fuel diesel oil (Heras et al., 1992). Remarkably, regardless of the utmost importance of petroleum in aquatic pollution there is little information about its effect on organisms and our understanding of the defense mechanisms that develop in cells to overcome the toxic effects of hydrocarbons is far from being complete. Even in ecologically and economically important groups such as crustaceans, there are only a few reports in which the metabolic alteration by freshwater hydrocarbon pollution has been studied. La Plata River estuary is an area exposed to several xenobiotics and the most contaminated region of the country in terms of hydrocarbons (Sur, 1994). Thus, it is important to evaluate the ecotoxicological effects of hydrocarbons on the species of this community. One such species, representative of the benthic community of the Plata River area, is the autochthonous freshwater prawn *Macrobrachium borellii* selected for the present study. Previous studies on the metabolic alteration by freshwater hydrocarbon pollution in crustaceans have mostly been performed using this prawn as a non-model organism, and have shown that sublethal WSF exposure affects lipid anabolism and catabolism, biological membranes fluidity, increase on vitellogenins and induce *M. borellii* antioxidant defense system (Lavarías et al., 2005, 2006, 2007, 2011; Garcia and Heras, 2012).

Abbreviations: 2-DE, Two dimensional gel electrophoresis; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, Dithiothreitol; ESI, Electrospray ionization; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GST, Glutathione S-transferase; MS/MS, Tandem mass spectrometry; MW, Molecular weight; pI, Isoelectric point; WSF, Water soluble fraction of crude oil; TOF, Time of flight.

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The need to detect and assess the impact of pollutants, particularly at sublethal concentrations has led to the development of a range of biological markers or biomarkers measured in a number of different species. The use of biomarkers has been proposed as a sensitive "early warning" tool (Cajaraville et al., 2000). In particular, proteomic and other "omic" studies provide an overview of the biochemical alterations caused by pollutants offering the potential to discover novel biomarkers of exposure and effects. However, their use is still incipient in the ecotoxicological field, though it is rapidly changing. Limitations also exist when employing these technologies. An important disadvantage to aquatic pollution studies is that transcriptomes of very few aquatic invertebrate species have been sequenced to date, thus making protein identification difficult (Ralston-Hooper et al., 2011). Moreover, the only aquatic invertebrate whose genome has been sequenced is the planktonic crustacean *Daphnia pulex* (Colbourne et al., 2011).

In addition, although several proteomic studies have evaluated the effect of aquatic pollutants using invertebrate species - mostly bivalve mollusks (Sanchez et al., 2011) - very few proteomic studies have employed crustaceans, and they are restricted to the marine environment (Gomiero et al., 2006; Silvestre et al., 2006). Recently the first toxicology study with a proteomic approach in freshwater environment was applied to evaluate the effect of the herbicide atrazine in two amphipod species (*Diporeia sp* and *Hyalella azteca*) (Ralston-Hooper et al., 2011). However, there are no studies analyzing the effect of freshwater hydrocarbon pollution on protein expression.

The aim of the present work was to evaluate the effect of the WSF on the protein expression profile of the freshwater prawn *M. borellii*, with a view to identify potential pollution biomarkers and begin to understand WSF mechanism of action.

2. Materials and methods

2.1. Sample collection

The work has been carried out in accordance with the EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

Adults of *M. borellii* (Crustacea: Decapoda: Palaemonidae) (n = 100, weighing 0.65–3.00 g (40–66 mm) were collected in March from a watercourse of Plata River estuary, Argentina (34°57′40″ S, 57°46′40″ W). Prawns were taken to the laboratory, separated by sex when appropriate and kept in dechlorinated running water at 22 ± 2 °C under a 14:10 h L:D photoperiod. During conditioning (10 days), a pelleted artificial diet, formulated according to Collins and Petriella (1999), was fed *ad libitum* on a daily basis. The animals were starved for 48 h before experiments, a standard practice in our laboratory.

2.2. Preparation of the WSF

The water-soluble fraction was prepared mixing light crude oil (obtained from Santa Cruz, Argentina) and gas oil (YPF) in tap water at a ratio of 1:1:100 (v/v/v). These were stirred at low speed for 24 h and allowed to settle for additional 48 h. Stirring was made in a cold room at 4 °C in a 10 L stainless steel mixing vessel equipped with a mechanical stirrer and a bottom drain. Fresh WSF batches were prepared every 2 days and collected daily using the bottom drain. The resulting WSF is quite different from the parent oils. It is rich in methyl- and alkyl-substituted monoaromatic and lower molecular weight polyaromatic hydrocarbons and has very low concentrations of C12–C24 n-alkanes, as we have previously reported (Heras et al., 1992).

2.3. Exposure experiments

Exposure assays were performed using sublethal concentrations of WSF previously determined for this prawn (Lavarías et al., 2004). Adults were exposed to 0.6 ppm WSF at 22 ± 2 °C and 14:10 h L:D photoperiod for 7 days without feeding. Control groups were kept in clean water. Prawns were held individually in 0.3 L flasks with the lid sealed. Medium was changed every day. Males and females were exposed separately. For each sex, and treatment, two pools of animals were processed as described in 2. 4. Animals were not under hypoxia and showed no signs of breathing alterations under this experimental setting.

2.4. Protein sample preparation

After exposure, midgut glands (hepatopancreas) from 19 to 25 individuals of the same sex were dissected for proteomic analysis. The number of animals was chosen to provide an adequate amount of protein. Prawns molting during the bioassay were discarded. Briefly, hepatopancreas were homogenized in 10 mM Tris–HCl pH 7.4 buffer and subjected to sequential centrifugations to obtain the cytosolic fraction as described elsewhere (Pasquevich et al., 2011). All centrifugations were done at 4 °C. Cytosol was then transferred to a clean tube and incubated 2 h at 37 °C with nucleases. Proteins were precipitated in a solution of 10 mM trichloroacetic acid, 20 mM DTT in acetone at −20 °C overnight. Ratio of sample: buffer was kept 1:4 (v/v). Precipitated samples were centrifuged and the supernatant removed. Protein pellets were washed twice using 20 mM DTT in 100% cold acetone and then centrifuged for 5 min at 5300 g. The remaining acetone was evaporated by leaving the pellet at room temperature. Dried pellets were then diluted in 500 μL rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.8% ampholytes). Protein concentration was determined using a RC DC Protein Assay kit (Kit #500-0122, Bio Rad) following manufacturer's instructions.

2.5. Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis was the method of choice as 2D gel methodologies are well-established, and continue to be a stalwart of proteomics research worldwide (Nesatyy and Suter, 2008). It was carried out with immobilized pH gradient (IPG)-isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension (Görg et al., 1988). Pools were analyzed by triplicate. The IEF was performed using an Ettan IPGphor III (GE Healthcare) and 7 cm linear pH 4–7 Immobiline dry strips (GE Healthcare). This pH range was chosen as it is the pI of most hepatopancreas proteins. Rehydration of the strip and loading of the samples were carried out overnight at room temperature in a dilution buffer (0.002% w/v bromophenol blue, 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% v/v, IPG Buffer 4–7 linear (GE Healthcare)) containing 120 μg protein. After IEF, the Immobiline dry strips were equilibrated at room temperature for 20 min in a buffer containing: 75 mM Tris–HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue and 1% w/v DTT, and then alkylated for 20 min in the same buffer, but with 4.5% w/v iodacetamide in place of DTT. For SDS-PAGE second dimension, the IPG strips were sealed on the top of 1.5 mm thick 12% polyacrylamide gels, with MW standards (GE Healthcare) run in parallel. Vertical electrophoresis was carried out at 30 mA per gel. Gels were stained with Sypro Ruby Protein Gel Stain (Molecular Probes), which has many advantages such as a linear quantitation range of over three orders of magnitude, a lower detection limit (less than 1 ng), it stains most classes of proteins and it is compatible with subsequent analysis of proteins by mass spectrometry (Dunn, 2002). Gels were then scanned using a laser blue light (450 nm) in a Storm 840 instrument (Amersham Bioscience). Images were analyzed with Image master 2D platinum software (Amersham Bioscience). For each matched set, the optical densities of spots were averaged.

Spots identified in treatment groups as differing from controls (t test, $\alpha = 0.05$) were manually excised and send for identification to the Proteomic Service of the National Centre of Biotechnology,

CSIC, Madrid, Spain ("Centro Nacional de Biotecnología", a member of ProteoRed network) as described below.

2.6. Mass spectrometry analysis

In-gel tryptic digestion was performed in reducing conditions, deposited in 96-well plates and processed automatically in a Proteineer DP (Bruker Daltonics, Bremen, Germany). The digestion protocol used was based on Schevchenko et al. (1996). Peptides were extracted and dried by speed-vacuum centrifugation and resuspended in 4 μL of MALDI solution (30% acetonitrile + 15% isopropanol + 0.1 % TFA). 20% of each peptide mixture was deposited onto a 386-well OptiTOF™ Plate (Applied Biosystems, Framingham, MA, USA) and allowed to dry at room temperature. A 0.8 μL aliquot of matrix solution (3 mg/mL CHCA in MALDI solution) was then added onto dried digest and allowed to dry at room temperature.

For MALDI-TOF/TOF analysis, samples were automatically acquired in an ABi 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems) in positive ion reflector mode. PMF and MSMS fragment ion spectra were smoothed and corrected to zero baseline using routines embedded in ABI 4000 Series Explorer Software v3.6. Internal and external calibration allowed to reach a typical mass measurement accuracy of <25 ppm. To submit the combined PMF and MS/MS data to MASCOT software v.2.1 (Matrix Science, London, UK), GPS Explorer v4.9 was used, searching in the non-redundant NCBI protein database. Some of the samples were also analyzed by NanoLC ESI-MSMS using an Ultimate 3000 nanoHPLC (Dionex, Sunnyvale, CA, USA) coupled to an HCT Ultra ion-trap mass spectrometer (Bruker Daltonics). The LC system was coupled via a nanospray source (Bruker Daltonics) to a 3D ion trap mass spectrometer operating in positive ion mode with the capillary voltage set at 1400 V. Automatic data-dependent acquisition allowed to obtain sequentially both full scan (m/z 350–1500) MS spectra followed by tandem MS CID spectra of the four most abundant ions. Dynamic exclusion was applied to prevent the same m/z from being isolated for 1 min after its fragmentation.

The interpretation of MS/MS spectra was done either by *de novo* sequencing or by database search using the Mascot algorithm (Matrix Science Ltd.). *De novo* analysis of MSMS spectra that did not match against peptide sequences already present in databases was performed using the software PEAKS (Bioinformatics solutions). PEAKS analysis offers a variable number of candidates (usually 5) representing the best solutions for the experimental MSMS spectra.

A fold mean change was calculated for significant proteins (t test, $p \leq 0.05$) by dividing expression levels of treated animals over those observed in the controls.

2.7. Crustacyanin gene expression analysis

To evaluate if the over-expression of crustacyanin (CTC) was also exerted at the transcriptional level, the CTC gene expression was chosen for further analysis by quantitative real time PCR (qPCR). β-Actin was used as reference gene. Bioassays were performed as mentioned above and then 3 groups of 3 prawns each were sampled from control and WSF-exposed prawn. This design was replicated for each sex. Hepatopancreas samples were preserved in RNAlater (Qiagen).

Total RNA was isolated using an RNeasyMini kit (Qiagen), with on-column DNA digestion. RNA was quantified by Quant-iT RNA assay kit (Invitrogen) followed by electrophoresis for integrity assessment.

Degenerate primers were designed from related species and data obtained by the proteomic analysis of *M. borellii* CTC (Forward 5´-AA GGCTTCGACGTCAAGACTGCC-3´ and reverse 5´-TATGCTGTCGAAGGCT GCTCTGC-3´) to render a product of 290 bp. β-Actin gene was 700pb and oligonucleotides were designed from *Penaeaus* (*Litopenaeaus*) *vannamei* (Forward 5´-CATCACACTTTCTACAACGA-3´ and reverse 5´-AGTGATTTCCTTCTGCATC-3´).

Both products were cloned and sent for sequencing to the Molecular Biology laboratory of the "Centro de Investigación en Alimentación y Desarrollo A.C., Unidad Mazatlán en Acuicultura y Manejo Ambiental", Sinaloa, México, where a Licor IR2 DNA Sequencer was used. BLASTN analysis (Altschul et al., 1997) confirmed that the nucleotide sequences obtained were of the desired genes. Definitive primers for qPCR were designed using Primer3 software (Rozen and Skaletsky, 2000) (see Table 1 for sequences).

Two-step RT PCR was carried out with an iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio Rad). Amplification was performed in a Stratagene Mx3000P QPCR System (Stratagene) with 1 μg reverse transcribed total RNA for each sample (control and WSF treatment). PCR conditions were: one cycle 95 °C for 3 min and 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. A dissociation protocol was carried out to ensure a unique amplicon. The calibration curve method was used for the analysis of data obtained from the RT PCR system. Standard curves were constructed to confirm the efficiency of primers.

Reactions containing primer pairs without template were included as blank controls. The assay was performed in triplicate for each of the three independent biological replicates performed for each sex analyzed. Gene expression was presented using the method described by Pfaffl (2001).

2.8. Statistical analysis

GraphPad Prism 4 software (GraphPad, San Diego, CA, USA) was used for Statistical analysis and plotting. Student t-test was used to analyze the differences between controls and treated expression proteins. Normal distribution of the data was checked using the modified Shapiro–Wilks normality test. A P value <0.05 was taken as the level of significance.

3. Results

3.1. Macrobrachium borellii cytosolic proteome

The use of two-dimensional polyacrylamide gel electrophoresis (2-DE) coupled with protein identification by mass spectrometry allowed the first overview of the proteome of a southern South American crustacean and the analysis of some alterations of its expression caused by hydrocarbon exposure. The cytosolic proteome of *M. borellii* hepatopancreas is shown in Fig. 1A.

Table 1
Primers designed for real-time RT-PCR analysis of crustacyanin.

Genes name	Primer	Sequence (5´–3´)	Annealing temperature of primer (°C)	Product length (bp)
CTC	F	CAGAAGGGAACCAGCTCAAG	60	195
	R	CGAAGAAGAAGGCGAAGTCT		
β-actin	F	TCGTGCTCGACTCTGGTGATGG	60	173
	R	GATTTCTCGCTCAGCCGTGGTG		

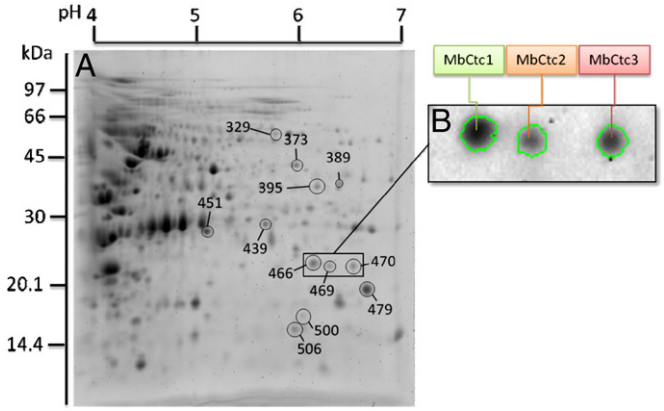

Fig. 1. Proteome of *Macrobrachium borellii* hepatopancreas. A. Cytosolic proteins (120 μg) were analyzed by 2-DE (IEF pH 4–7, second dimension in 12 % SDS-PAGE). Proteins were stained with Sypro Ruby and visualized using a blue light source. B. Magnification of protein spots 466, 469 and 470 identified by tandem MS as crustacyanin isoforms.

3.2. Differential protein expression

Search, detection, and identification of differentially expressed protein markers was performed using a comparative proteomic approach of hepatopancreas' proteins, obtained after exposing the organisms to sublethal concentrations of the WSF. The number of recognizable spots on the gels averaged 99.3 ± 8.9 in females and 138.8 ± 13.9 in males using Sypro Ruby staining and Image Master 2-DE Platinum software.

Differential expression analysis was performed for males and females under the same conditions. Females showed significant differences in several expressed proteins (Table 1, Fig. 2). Bioinformatic analysis of peptide mass spectra of these proteins allowed the identification of 7 of the 10 differentially expressed proteins, usually revealing similarities with proteins belonging to families or orders taxonomically close to this non-model organism (Table 2). Among them, one of the three CTC isoforms named MbCtc1, MbCtc2, MbCtc3 (Fig. 2B) was selected for subsequent analysis of its mRNA abundance, as described below. Remarkably, males showed lower altered expression of the same proteins as females, which was not statistically significant (Fig. 2).

3.3. Analysis of CTC mRNA by real time PCR

CTC was selected to study the transcription level of its mRNA on WSF-exposed prawn. Its mRNA quantification showed an over expression in WSF-exposed prawns in both females and males, which was several times higher in the former (Fig. 3).

4. Discussion

No proteomic information for crustaceans of the Plata basin was available. This study, although not comprehensive, provides information on differentially expressed proteins of part of *M. borellii* cytosolic proteome that could be employed in pollution studies.

Two-dimensional electrophoresis analysis of part of the hepatopancreas cytosolic proteome revealed 10 differentially expressed proteins and, in spite of limitations generated by the lack of genome sequencing of aquatic invertebrates, 7 were identified by mass spectrometry. The number of differentially expressed proteins obtained is in the same range of several other pollution studies using 2-DE (Silvestre et al., 2006; Leung et al., 2011; Zhu et al., 2012; Schmidt et al., 2013). Four of them, namely, Nm23 oncoprotein, arginine methyltransferase, fatty aldehyde dehydrogenase and glutathione S-transferase (GST; EC 2.5.1.18) were down-regulated after WSF exposure, whereas two glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) isoforms and a lipocalin-like CTC were up-regulated. These proteins displayed functions related with carbohydrate and amino acid metabolism, detoxification, small hydrophobic-binding proteins and cellular homeostasis, among others, giving, to our knowledge, the first insight into the WSF-induced responses of a decapod crustacean at the protein expression level. These results evidenced that the use of 2-DE and *de novo* sequencing allows investigating the cellular responses to pollutants, even in organisms with few genomic sequences available in databases.

The expression of these proteins showed sex-dependent responses as significant changes were observed in WSF-exposed females but only a tendency in males, compared with controls. Females were also more affected than males in crabs exposed to crude oil, crude oil spiked with alkylphenols and 4-nonylphenol (NP) as pollutants (Gomiero et al., 2006), but more work is needed to understand the effect of sex and tissue-specific response to pollutants.

Glyceraldehyde-3-phosphate dehydrogenase participates in the glycolytic pathway catalyzing the synthesis of a high-energy intermediate used in the synthesis of ATP. Induction of GAPDH was also observed in human cells and other crustaceans exposed to other environmental contaminants such as the herbicide atrazine in amphipods (Ralston-Hooper et al., 2011) or under hypoxic conditions in the crustacean *Fenneropenaeus chinensis* (Jiang et al., 2009). The over-expression of GAPDH in hydrocarbon-exposed prawns suggests an increase in energy generating pathways, in agreement with previous reports indicating that WSF causes an increase in energy production by increasing fatty acid consumption and β-oxidation pathway (Lavarías et al., 2006). We can therefore suggest that WSF may increase both carbohydrate and lipid energy producing pathways, though further analysis of alterations in other enzymes of these pathways or biochemical composition of the tissue is needed to validate this hypothesis.

CTC, a carotenoprotein from the lipocalin superfamily responsible for crustacean exoskeleton coloration (Chayen et al., 2003; Wang et al., 2007), is synthesized in hepatopancreas among other organs (Wang et al., 2007). For instance, *Homarus americanus* have several CTC isoforms that are combined in various ways to impart different colors to this lobster (Chayen et al., 2003). Multiple copies of CTC were found in the related species *Macrobrachium rosenbergii* genome (Wang et al., 2007). Likewise, in the present study, three CTC isoforms were identified in *M. borellii* (Fig. 1B), and one of them, MbCtc1, was overexpressed after sublethal WSF exposure. Further, we were able to observe in several prawn exposed to the WSF contaminant a more pigmented carapace, although this possible effect was not quantitatively evaluated. A correlation between exoskeleton coloration changes and CTC expression was observed in *M. rosenbergii* (Yang et al., 2011). Since CTC is such a well-studied protein in crustaceans, it stimulated further study of its expression at the mRNA level which also evidenced an increased CTC expression. The reasons for this increase remain unclear, though one possibility is that the hydrophobic nature of the WSF contaminant may somehow alter CTC regulatory mechanism in *M. borellii* causing its over-expression, but more studies are needed to validate this hypothesis.

After WSF exposure, hepatopancreas exhibits down-regulation of GST. GSTs are known as phase II detoxification enzymes with conjugation activities, though they have other physiological functions such as gene expression regulators or binding proteins (Di Giulio et al., 1995). Given the diverse roles of GST, it is not surprising that GST activity increased in some crustaceans exposed to stress factors (Barata et al., 2005; Anto et al., 2009), while in others expression varied according to which GST isoforms was studied, and appear to react differently, depending on the pollutant (Silvestre et al., 2006). Thus, the diversity of roles and isoforms of GST do not allow a clear interpretation of its function under WSF contamination.

Arginine methyltransferases (EC 2.1.1.125/126) catalyze the methylation of arginine residues within proteins. The role of this

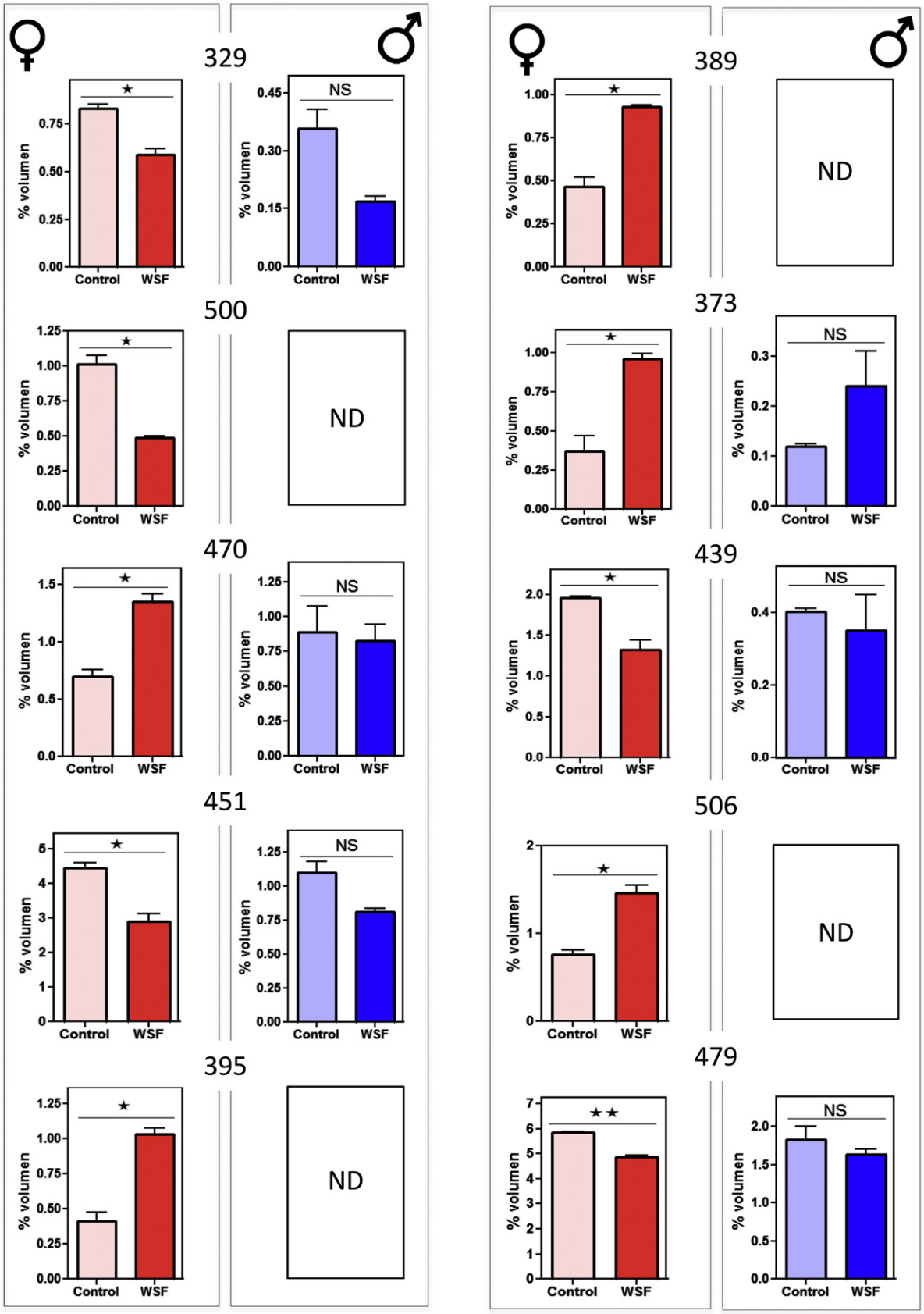

Fig. 2. Differentially expressed proteins in female (red) and male (blue) *Macrobrachium borellii* hepatopancreas after WSF exposure. Bars represent the mean ± 1SE of volume percentage. ND: Not determined, NS: Not significant, * $p < 0.05$, ** $p < 0.01$.

Table 2
Identification of differentially expressed proteins in *Macrobrachium borellii* hepatopancreas after exposure to sublethal concentrations of WSF.

Protein number	pI[a]	MW[a]	Expression[b]		Identification	Species[c]	Taxonomic group	Access number[d]
			Female	Male				
329	5.8	57	0.71	0.47	Fatty Aldehyde Dehydrogenase	*Lepeophtheirus salmonis*	Crustacea-Copepoda	**GenBank ID: ADD38780.1**
389	6.5	40	2.04	[g]	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	*Homarus americanus*	Crustacea-Decapoda	**UniProt ID: P00357.2**
500	6.2	16	0.46	[g]	NA[e]	-	-	-
373	6.0	44	2.48	2.07	NI[f]	-	-	-
470	6.6	23	1.93	1.39	Crustacyanin	*Macrobrachium rosenbergii*	Crustacea-Decapoda	**GenBank ID: ABC88388.1**
439	5.8	28	0.66	0.87	Arginine methyltransferase	*Streptocephalus seali*	Crustacea-Anostraca	**GenBank ID: ACY43145.1**
451	5.2	27	0.65	0.74	Glutathione-S-Transferase	*Eriocheir sinensis*	Crustacea-Decapoda	**GenBank ID: ACU46011.1**
506	6.18	15	1.81	[g]	NI[f]	_	_	_
395	6.3	38	2.16	1	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	*Homarus americanus*	Crustacea-Decapoda	**UniProt ID: P00357.2**
479	6.8	20	0.84	0.89	Nm23 Oncoprotein	*Litopenaeus vannamei*	Crustacea-Decapoda	**GenBank ID: ABI93176.1**

[a] MW and p*I* correspond to experimentally calculated values.
[b] Relative fold change referred to control.
[c] Species in which proteins were identified.
[d] NCBI database accession number.
[e] Not extracted from gels due to technical issues.
[f] Not identified.
[g] Quality of spots precluded analysis.

post-translational protein modification in crustaceans as well as in other well studied members of Arthropoda such as the house fly *Drosophila melanogaster* is still unknown (Boulanger et al., 2004). Fatty aldehyde dehydrogenase (EC 1.2.1.3) protein was also down-regulated. It oxidizes medium- or long-chain aliphatic aldehydes and, to our knowledge, this is the first report of this enzyme in invertebrates. The scarce studies of arginine methyltransferase and fatty aldehyde dehydrogenase in invertebrates preclude any further comparison.

Another down-regulated protein was nm23 (non-metastatic 23). The NM23 family is almost universally conserved across all three domains of life: Eubacteria, Archaea and Eukarya. This protein is a nucleoside diphosphate kinase (NDPK; EC 2.7.4.6) required for endocytosis and to maintain the pool of nucleoside triphosphate needed in biosynthetic reactions. The enzyme is functional as NDPK in shrimps (Quintero-Reyes et al., 2012) and its expression is down-regulated under hypoxic conditions in *Fenneropenaeus chinensis* (Jiang et al., 2009). As an ancient protein, nm23 has acquired many functions, ranging from normal oocyte differentiation or skin homeostasis and repair to control of cell cycle progression, transcription, DNA repair, cell motility and invasion suppression (Bilitou et al., 2009; Jiang et al., 2009). This diversity precludes any propositions regarding the significance of its under expression after WSF exposure.

As a whole, these results indicate that some of the proteins reported here, which had not been evaluated in oil spill pollution studies before, could potentially be employed as early pollution biomarkers once its differential expression has been validated by field studies.

In conclusion, the present study provides evidence that hydrocarbon pollution can affect the protein expression profiles in hepatopancreas of an aquatic organism. To our knowledge, it is the first demonstration of such an effect for water-soluble crude oil hydrocarbons in aquatic biota. Further studies with a similar approach but using other freshwater invertebrates and a range of geographical locations are needed to generalize these findings.

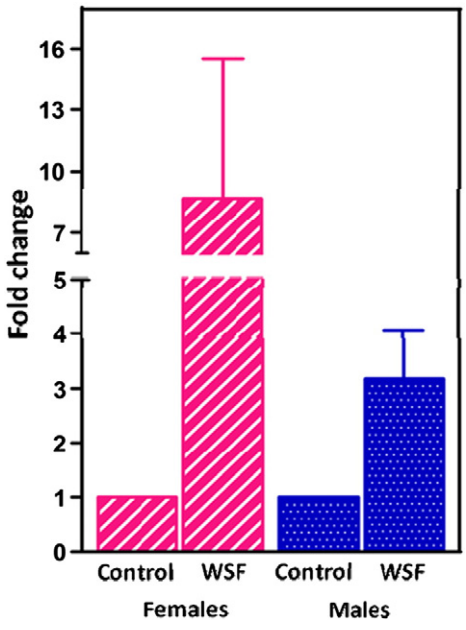

Fig. 3. Crustacyanin mRNA abundance in male and female *Macrobrachium borellii* hepatopancreas after WSF exposure. Bars represent the mean of three determinations ± 1SE.

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M.S.D. is member of CICBA, Argentina. H.H. is member of CONICET, Argentina. M.Y.P. is a CONICET scholarship fellow and a 2009 fellow of SRE, Mexico.

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