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Proteomic response of *Macrobrachium rosenbergii* hepatopancreas exposed to chlordecone: Identification of endocrine disruption biomarkers?

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ABSTRACT

The present work is the first study investigating the impacts of chlordecone, an organochlorine insecticide, on the proteome of the decapod crustacean Macrobrachium rosenbergii, by gel-free proteomic analysis. The hepatopancreas protein expression variations were analysed in organisms exposed to three environmental relevant concentrations of chlordecone (i.e. 0.2, 2 and 20 µg/L). Results revealed that 62 proteins were significantly up- or down-regulated in exposed prawns compared to controls. Most of these proteins are involved in important physiological processes such as ion transport, defense mechanisms and immune system, cytoskeleton dynamics, or protein synthesis and degradation. Moreover, it appears that 6% of the deregulated protein are involved in the endocrine system and in the hormonal control of reproduction or development processes of *M. rosenbergii* (e.g. vitellogenin, farnesoic acid o-methyltransferase). These results indicate that chlordecone is potentially an endocrine disruptor compound for decapods, as already observed in vertebrates. These protein modifications could lead to disruptions of M. rosenbergii growth and reproduction, and therefore of the fitness population on the long-term. Besides, these disrupted proteins could be suggested as biomarkers of exposure for endocrine disruptions in invertebrates. However, further investigations are needed to complete understanding of action mechanisms of chlordecone on proteome and endocrine system of crustaceans.

1. Introduction

During the last decades, the proteomic approach has been developed progressively in the field of ecotoxicology in order to increase the understanding of adverse impacts of chemicals in exposed organisms (Rodríguez-Ortega et al., 2003; Sanchez et al., 2011; Wright et al., 2012). Proteomic analyses aim to obtain a quantitative description of protein expressions in order to identify changes following exposure to environmental stress conditions such as temperature fluctuation, parasitism, and exposure to environmental pollutants (Cao et al., 2009; Giusti et al., 2013). Moreover, the analysis of pollutant effects on the proteome of an exposed species could allow to investigate potential new biomarkers (Ankley et al., 2009; Rodríguez-Ortega et al., 2003; Sanchez et al., 2011), widely employed to investigate the presence of xenobiotics and evaluate their consequences on biota (Hiramatsu et al., 2005; Ringwood et al., 2008; Rodríguez-Ortega et al., 2002).

Until recently, most ecotoxicological studies involving the proteomic approaches were carried out using the two-dimensional gel electrophoresis (2D) method, which is a two-dimensional gel-based analysis (i.e. first according to the isoelectric point of proteins and secondly based on the molecular weight of proteins), followed by protein identifications by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) (Gomiero et al., 2006; Görg et al., 2004). However, although 2D is commonly used, this approach is labor

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intensive and has some limitations, such as: under-selection of some protein categories, limited dynamic range, co-migration of proteins, and the necessity to run many replicates (Görg et al., 2004; Zhou et al., 2012). To overcome some of these limitations, a new proteomic approach called “shotgun” proteomic or gel-free proteomic has been developed over the years (Baggerman et al., 2005). In this approach, proteins are extracted from tissues and immediately cleaved into peptides using proteolytic enzymes. Next, peptides are separated in liquid chromatography coupled with a tandem mass spectrometric analysis (LC-MS/MS), and the peptide identifications allow to determine the protein content of the initial sample (Williams, 1999). Moreover, this approach allows to study a multiplicity of proteins simultaneously and therefore, analyze all proteins present in samples (Cao et al., 2009; Williams, 1999). Until now, among invertebrates, most proteomic studies have focused on bivalve species (Sanchez et al., 2011). Few ecotoxicoproteomic studies have been carried out using crustaceans (Boulangé-Lecomte et al., 2016; Gismondi et al., 2015; Trapp et al., 2014), however no study on protein expression changes in response to a chlordecone exposure has been reported.

Chlordecone (CLD) is an insecticide commonly used in the French West Indies (FWI) in particular in Guadeloupe to control the banana weevil *Cosmopolites sordidus* from 1972 to 1993. A few years after the introduction and use of CLD, widespread pollution of soils, rivers, wild animals and aquatic organisms was reported (Cavelier, 1980; Snegaroff, 1977). Indeed, CLD is persistent and accumulates in food webs (Cabidoche and Lesueur-Jannoyer, 2012; Clostre et al., 2013). Since Hammond et al. (1979) demonstrated that CLD can bind to estrogen receptors in rats, many studies investigated the endocrine effects of CLD in various vertebrate species (Curtis and Beyers, 1978; Donohoe and Curtis, 1996; Eroschenko, 1981; Guzelian, 1982), and some invertebrate species (Oberdörster and Cheek, 2001; Schimmel et al., 1979; Zou and Bonvillain, 2004). However, in aquatic ecosystems, endocrine effects of CLD were mainly studied in vertebrates and information about its effects on invertebrates is still limited, since previous studies mainly concerned the observation of morphological characteristics impacted by CLD. Nevertheless, all previous studies carried out on invertebrates led to the hypothesis that CLD could be an EDC in exposed invertebrates. For example, Giusti et al. (2014) showed reduction of the oviposition and the fecundity of the gastropod *Lymnaea stagnalis*. Recently, Legrand et al. (2016) highlighted alterations in the expression of genes involved in reproduction, development and growth of the crustacean *Eurytemora affinis* exposed to CLD. In the same way, our previous studies highlighted that CLD exposure affected the 20-hydroxyecdysone concentration (i.e. molting hormone), the chitobiase activity (i.e. molting enzyme), as well as the vitellogenin and vitellogenin receptor gene expression in the decapod *Macrobrachium rosenbergii* (Lafontaine et al., 2016a, b).

The present study aimed to investigate the variations of protein expressions in the hepatopancreas tissue of the invertebrate decapod *M. rosenbergii* exposed to three environmental concentrations of CLD, using a “gel-free” proteomic approach. *M. rosenbergii* is one of the biggest freshwater prawns located in all tropical and subtropical area, and widely cultivated for food, as for example in Guadeloupe where it is one of the most important economic resources (New, 2002). Therefore, *M. rosenbergii* can be considered as a good model for investigations on endocrine disruptions in decapods, and for wild *Macrobrachium* spp. living in freshwater ecosystems of these regions (*M. faustinum, M. carcinus*, *M. acanthurus,…*). Results could improve the understanding of the toxic action of chlordecone in invertebrates, and allow the identification of proteins useful in the development of endocrine disruption biomarkers.

2. Materials and methods

2.1. Tested organisms

Post-larvae of *M. rosenbergii* (approximately 2 g, 1.4 cm cephalothorax length, sexually immature) were provided by an aquaculture farm (OCEAN-SA) located at Pointe-Noire (Guadeloupe, FWI) in a geographic area free of CLD contamination. Before the exposure experiment, pretests were carried out to evaluate the CLD concentration in tissues of prawns from Pointe-Noire and results have shown no contamination (concentrations below the limit of detection) (data not shown). Prawns used for the proteomic experiment were transferred to the laboratory (Marine Laboratory of University of the French West Indies, Guadeloupe), and acclimated for one week in glass aquaria filled with 28 L of tap water prefiltered through activated carbon. Aquaria were under constant aeration with a 12 h light/dark photoperiod.

During acclimation, prawns were fed once daily with one artificial shrimp pellet per individual (complete food for rearing, Le Gouessant, France). A constant water temperature of 27.6 ± 0.2 °C was maintained, and pH remained at 7.57 ± 0.03 throughout the experiment. These values are in accordance with optimal water temperature and pH commonly used in prawn farms (New, 2002).

2.2. Experimental design

Post-larvae of *M. rosenbergii* were exposed in the laboratory for 30 days at three CLD concentrations, i.e. 0.2 µg/L, 2 µg/L and 20 µg/L, which were chosen for their environmental relevance in surface water in Guadeloupe. Indeed, in 2003, it was measured CLD concentrations ranging from 0.17 µg/L to 4.4 µg/L. Besides, some contamination peaks reaching 7–9 µg/L were measured (GREPP, 2004; InVS-Inserm, 2009). The water contamination was performed by spiking 28 L of tap water prefiltered through activated carbon in each experimental aquarium with 56 µL of acetonic solution of CLD (0.1 µg/µL, 1 µg/µL and 10 µg/µL), in order to obtain final CLD concentrations of 0.2 µg/L, 2 µg/L and 20 µg/L respectively. In parallel, a “solvent control” consisting of tap water prefiltered through activated carbon spiked with 56 µL of acetone was run. During the 30 days of exposure, *M. rosenbergii* were fed daily with one artificial shrimp pellet per individual (complete food for rearing, Le Gouessant, France). Exposure media were renewed every 96 h in order to maintain constant each CLD concentration. At the end of the 30-day exposure, 4 prawns per condition (i.e. 4 replicates) were sampled, immediately frozen in liquid nitrogen and stored at −80 °C until proteomic analysis.

2.3. Proteomic analysis

2.3.1. Extraction of protein fractions

Each prawn was dissected and the hepatopancreas tissue was collected and solubilized in Tris HCl 10 mM, pH 7.4, SDS 4% containing Protease Inhibitor Cocktail EDTA-free and DNAse. The samples were sonicated twice for 30 s, and homogenized by vortex for 30 min at room temperature, before storage overnight at 4 °C. The protein concentration of samples was quantified using a *RC DC*™ Protein Assay Kit (Biorad, USA).

The samples were reduced and alkylated before applying the 2D Clean-Up kit (GE Healthcare Life Sciences, USA) according to the manufacturer's recommendations, in order to eliminate impurities not compatible with mass spectrometry analysis. After the washing steps, protein pellets were solubilized in 50 mM bicarbonate ammonium. Each sample was digested for 16 h at 37 °C in trypsin solution (ratio trypsin/total proteins (w: w) 1/50) and then, after a 5 times dilution with acetonitrile (80% v-v), 3 h at 37 °C with fresh add of trypsin (ratio trypsin/total proteins (w: w) 1/100). After the digestion step, samples were resuspended in 0.1% formic acid. For each sample, an aliquot corresponding to 3.5 µg of digested proteins was purified using a Zip-

Table 1
Proteins with at least 1.5-fold changes, in at least one exposure condition, in hepatopancreas of *M. rosenbergii* exposed for 30 days at 0.2, 2 and 20 µg/L of CLD, and significantly identified ($p \leq 0.05$ for downregulated proteins, $p \geq 0.95$ for upregulated proteins) with the Uniprot Crustacea database. PLGS Score = Score of protein identification calculated by PLGS (high score means high confidence of identification).

	Up-regulated protein name	Accession N°	Taxonomy	PLGS score	Biological function
1	Farnesoic acid O-methyltransferase	A0PGI8	*L. vannamei*	3174	Endocrine system
2	Vitellogenin	Q8ISB2	P. semisulcatus	165	Endocrine system
3	70 kDa heat shock protein	D6BP38	*P. varians*	3903	Immunity and defenses
4	Beta-1,3-glucan-binding protein	P81182	*L. vannamei*	167	Immunity and defenses
5	Bip	J7K1E9	*L. vannamei*	3456	Immunity and defenses
6	Calmodulin	B6DYD6	*P. clarkii*	32493	Immunity and defenses
7	Catalase	H8XYP6	*M. rosenbergii*	3373	Immunity and defenses
8	Cathepsin L	D7F2M6	*P. varians*	23285	Immunity and defenses
9	Chitinase 3	H8YI19	*P. japonica*	28637	Immunity and defenses
10	Cytochrome P450	H6UXP1	*M. nipponense*	9886	Immunity and defenses
11	Ferritin	I1VWN8	*M. rosenbergii*	11888	Immunity and defenses
12	Glucose-regulated protein 78	B8LF10	*F. chinensis*	5292	Immunity and defenses
13	Heat shock protein 70	Q194W6	*C. sapidus*	5394	Immunity and defenses
14	Lectin 1	I6W7T5	*M. rosenbergii*	1569	Immunity and defenses
15	Lectin 2	I6V2P8	*M. rosenbergii*	38288	Immunity and defenses
16	Lectin 3	I6W5B6	*M. rosenbergii*	7194	Immunity and defenses
17	Lipopolysaccharide and beta-1,3-glucan binding protein	C7DZ96	*M. rosenbergii*	29040	Immunity and defenses
18	Prophenoloxidase	Q58HZ8	*M. rosenbergii*	258	Immunity and defenses
19	Protein disulfide-isomerase	C0JBY4	*L. vannamei*	7676	Immunity and defenses
20	Superoxide dismutase [Cu-Zn]	Q45Q33	*M. rosenbergii*	39780	Immunity and defenses
21	Transglutaminase	F1JZV5	*M. rosenbergii*	345	Immunity and defenses
22	Calreticulin	E2DRF0	*P. monodon*	7418	Protein synthesis and degradation
23	Elongation factor 2	I6VB26	*S. paramamosain*	9432	Protein synthesis and degradation
24	Proteasome subunit alpha type	E9GIX9	*D. pulex*	1174	Protein synthesis and degradation
25	Ribosomal protein	D7F2L2	*P. varians*	4553	Protein synthesis and degradation
26	Alpha-spectrin	D0UN94	*L. emarginata*	1985	Cytoskeleton
27	Enolase	I6P4W6	*M. rosenbergii*	35490	Glucose metabolism
28	Glyceraldehyde 3-phosphate dehydrogenase	G3C6U6	*H. adactyla*	13148	Glucose metabolism
29	Phosphoenolpyruvate-carboxykinase	Q86R97	*N. granulata*	1187	Glucose metabolism
30	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	P35316	*A. franciscana*	2542	Ion transport
31	Calsequestrin	F8QXM4	*S. paramamosain*	2415	Ion transport
32	Sarcoplasmic calcium-binding protein 1	P05946	*A. leptodactylus*	21632	Ion transport
33	Sarcoplasmic/endoplasmic reticulum calcium ATPase	P86911	*C. opilio*	3092	Ion transport
34	V-type proton ATPase	D3PHZ2	*L. salmonis*	352	Ion transport
35	Arginine kinase	E2JE77	*M. rosenbergii*	25441	ATP metabolism
36	ATP synthase	D2CNK5	*L. vannamei*	6645	ATP metabolism
37	14-3-3 protein epsilon	H9CWV0	*S. paramamosain*	25152	Signal transduction
38	ADP-ribosylation factor 1	C1BTJ2	*S. louse*	1910	Signal transduction
	Down-regulated protein name	**Accession N°**	**Taxonomy**	**PLGS score**	**Biological function**
39	Male reproductive tract specific Kazal type proteinase inhibitor	Q1EF71	*M. rosenbergii*	49666	Endocrine system
40	Sperm gelatinase	I6R3T3	*M. nipponense*	5911	Endocrine system
41	Crustin	B8LG64	*M. rosenbergii*	2423	Immunity and defenses
42	Pen a 1 allergen	Q3Y8M6	*F. aztecus*	10021	Immunity and defenses
43	Enhancer of split mbeta protein	*C1BU45*	*L. salmonis*	1200	Protein synthesis and degradation
44	Glutamate dehydrogenase	Q0KHB4	*L. vannamei*	5701	Protein synthesis and degradation
45	Histone H2A	D2DSH4	*S. paramamosain*	10552	Protein synthesis and degradation
46	Histone H3	I6P4G7	*M. rosenbergii*	14244	Protein synthesis and degradation
47	Ubiquitin	D7RF65	*E. sinensis*	12638	Protein synthesis and degradation
48	Actin 1	O96657	*P. monodon*	49838	Cytoskeleton
49	Gelsolin cytoplasmic	Q27319	*H. americanus*	899	Cytoskeleton
50	Myosin heavy chain isoform 1	E9FZS9	*D. pulex*	30703	Cytoskeleton
51	Tropomyosin	D3XNR9	*M. rosenbergii*	16125	Cytoskeleton
52	Troponin	P05547	*A. leptodactylus*	22629	Cytoskeleton
53	Alpha-1,4 glucan phosphorylase	E9G2G6	*D. pulex*	4181	Glucose metabolism
54	Pyruvate kinase	B1N690	*L. vannamei*	3444	Glucose metabolism
55	Triosephosphate isomerase	K0E682	*L. vannamei*	2895	Glucose metabolism
56	Alpha-amylase	E9GXM0	*D. pulex*	930	Carbon metabolism
57	Endo-1,4-beta-glucanase	Q1A366	*M. lar*	6409	Carbon metabolism
58	Clathrin	D0UQ16	*N. oerstedii*	1524	Ion transport
59	Sodium/potassium-transporting ATPase subunit alpha	Q95PC2	*P. marmoratus*	8141	Ion transport
60	Hemocyanin	F5CEX2	*M. nipponense*	17775	Oxygen transport
61	Na + /Ca2 + -exchanger	Q8WPE2	*P. scaber*	1109	Signal transduction
62	Crustacyanin-like lipocalin	A4Z4V4	*M. rosenbergii*	5561	Pigmentation

Tip (Billerica, USA) C18 High Capacity according to the manufacturer's recommendations.

Then, samples were evaporated to dryness in a speed vacuum. Peptides were conditioned at 3.0 µg in ammonium formiate 100 mM, with 150 fmoles in Yeast Alcohol Dehydrogenase (ADH, accession number P00330) per volume injected for the MassPREP protein digestion standard mixtures (MPDS, Waters corporation, USA). The internal standards spiked with MPDS mix 1 or MPDS mix 2, allow

technical verification of the whole 2D-UPLC separation, MS^E data acquisition with additional ion mobility separation and PLGS identification process. ADH being present at ratio MPDS mix1/MPDS mix 2 = 1.

2.3.2. Samples analyses on a nanoUPLC-SynaptTM HDMSTM G2 system

Protein samples were analysed using a *nano*Acquity UPLC© (Waters corporation, USA) separation system. This system uses a unique combination of two C18 chromatographic separation performed at different pH (i.e. pH = 10 and then pH = 3). Protein samples were then analysed with the Synapt™ HDMS™ G2 mass spectrometer (Waters corporation, UK) which uses an electrospray ionisation source (ESI) and allows sensitive detection (lower limit around 0.1–1 fmol of protein) with high resolution and high accuracy (within 10 ppm) for the analysed peptides. All the peptides are fragmented. Then, each sequence and identity can be obtained from database searches and correlation to the accurate mass measured for each parent peptide fragmented.

2.3.3. Identification of proteins and PLGS analysis

As the transcriptome of the crustacean decapod *M. rosenbergii* has not been sequenced yet, protein identification was performed by homology using ProteinLynx Global Server Software v2.5 (PLGS, Waters, USA) and the Crustacea database extracted from UNIPROT (www.uniprot.org). This database search also involves the search on a randomized database recomputed from the original database to evaluate the risk of false positive protein identification. For identification, the minimum to consider is at least two different peptides per protein identified and a control of the false-positive rate, which must be as low as possible (the false-positive rate will be of maximum 1% because of the setting used in PLGS database search). Biological functions were obtained by using the UniProtKB section of the UniProt website (www.uniprot.org) and the AmiGO section of GeneOntology website (http://amigo1.geneontology.org).

2.3.4. Analysis of PLGS results

After sample analyses in LC-ESI-MS/MS^E, PLGS Software has been used to compare proteins identified in each condition two by two. Then, the expression levels of proteins have been normalized using the expression level of the standard protein alcohol dehydrogenase (ADH – see Section 2.3.1). Results obtained were sorted according to three parameters. First, only proteins identified without ambiguity were selected. Then, only the proteins having a *p*-value lower than 0.05, and higher than 0.95 were retained. Indeed, a *p*-value is calculated by PLGS Expression E (Richardson et al., 2012), and values between 0 and 0.05 represent a 95% likelihood of under-expression, while a value between 0.95 and 1 indicates a 95% likelihood of over-expression. Finally, a 1.5-fold expression change was used as significant level (ratio of the protein amount less than 0.67 and greater than 1.5).

3. Results

3.1. Protein identification

This study was carried out to investigate the difference in protein expression of *M. rosenbergii* between control conditions (i.e. without pollutant stress) and CLD exposure. “Shotgun” proteomic analysis of hepatopancreas of *M. rosenbergii* allowed to identify 120 proteins without ambiguity (i.e. false-positive rate less than 1%) in all experimental conditions. Among these proteins, multiple comparisons of protein expressions revealed that the expression of 62 proteins were significantly different in exposed prawns compared to control, according to the 1.5-fold change criterion (Table 1). Results revealed that 38 proteins were up-regulated and 24 proteins were down-regulated.

The Venn diagram shows the number of proteins which abundance was significantly altered in each CLD exposure condition, compared to controls (Fig. 1 - see Supplementary data for more details). We observed

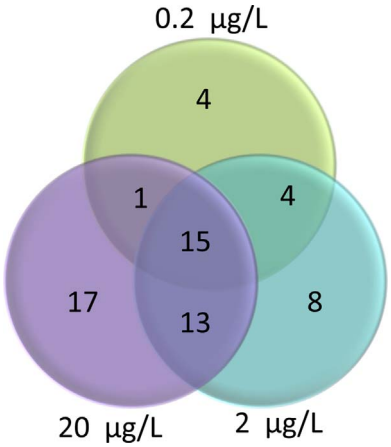

Fig. 1. Venn diagram of total proteins with at least 1.5-fold expression change and significantly identified in hepatopancreas of *M. rosenbergii* exposed for 30 days to the three chlordecone concentrations.

that the higher the CLD exposure concentration, the higher the number of proteins impacted, which is in agreement with a dose-response relationship. Indeed, most proteins were impacted in prawns exposed to 2 µg/L or 20 µg/L of CLD. Approximately 50% of the proteins altered following a CLD exposure were only disrupted by one concentration of CLD (i.e. 29 proteins), compared to controls, while 15 proteins were altered, whatever the CLD concentration of exposure.

The identified proteins were functionally categorized based on the UniProtKB annotation of biological processes. The proteins differently expressed in exposed prawns compared to controls were principally involved in 9 biological functions; for example, ion transport, immunity and defenses, or ATP metabolism (Table 1, Fig. 2). Results highlighted five biochemical processes representing almost 80% of the total of identified proteins impacted. Moreover, we observed that the disrupted proteins are involved in the defense mechanisms and immunity (i.e. 32% of total altered proteins), protein synthesis and degradation (i.e. 15%), cytoskeleton and muscle movements (i.e. 11%), and glucose metabolism or ion transport (i.e. 10% each). Finally, proteomic analysis revealed that 6% of the proteins deregulated by CLD exposure were involved in the endocrine system.

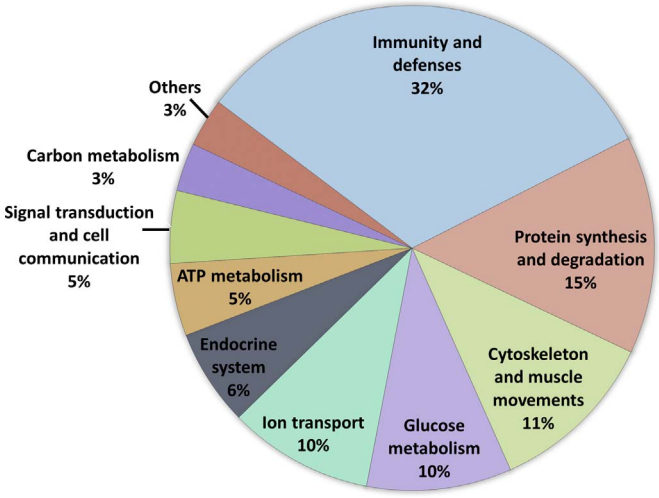

Fig. 2. Graphical view representing the percentage of proteins within each functional category as a function of the total protein number with at least 1.5-fold expression change and significantly identified in hepatopancreas of *M. rosenbergii* exposed to chlordecone.

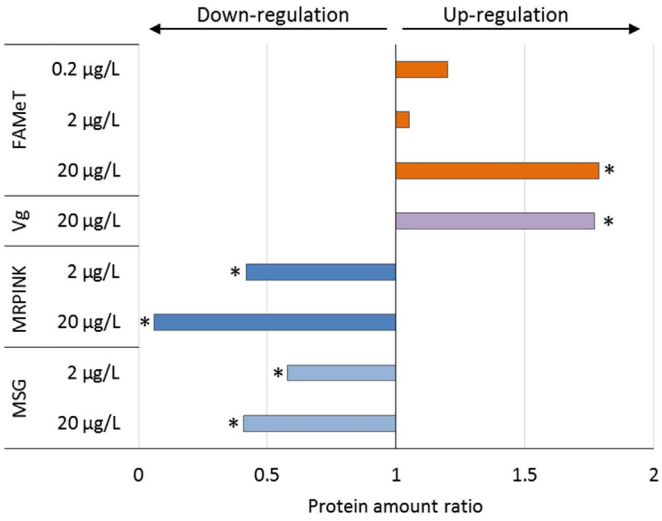

Fig. 3. The four proteins involved in hormonal process and whose abundance was modified in *M. rosenbergii* exposed to CLD as compared to controls. The three conditions of exposure were represented by the CLD concentrations (i.e. 0.2 µg/L, 2 µg/L and 20 µg/L). Vg = Vitellogenin; MRPINK = Male reproductive tract specific kazal type proteinase inhibitor; MSG = Sperm gelatinase; FAMeT = Farnesoic acid o-methyltransferase. Asterisks represent a statistically significant difference compared to controls.

3.2. Comparative analysis of proteins identified and involved in hormonal process

As CLD is suspected of being an endocrine disruptor in invertebrates, and especially in *M. rosenbergii* according to our previous results (Lafontaine et al., 2016a, b), results analysis focused on the 6% altered proteins involved in the endocrine system, which was represented by 4 proteins (Fig. 3). Among these proteins, 3 are involved in the reproduction process, and 1 in the development process. Indeed, the vitellogenin (Vg) protein was significantly up-regulated in prawns exposed to 20 µg/L of CLD; while the male reproductive tract specific kazal type proteinase inhibitor (MRPINK) and the sperm gelatinase (MSG) were significantly down-regulated in prawns exposed to 2 and 20 µg/L of CLD. Besides, the farnesoic acid o-methyltransferase (FAMeT) enzyme was up-regulated in all the exposed prawns, but only significantly in prawns exposed to 20 µg/L of CLD.

4. Discussion

Like aquatic vertebrates, aquatic invertebrates are exposed to several EDCs (Kloas et al., 2009; Meyer-Reil and Köster, 2000; Sanchez et al., 2011). However, few studies have been conducted on these species to evaluate the impacts of EDCs (Sanchez et al., 2011) by using a proteomic analysis. This study was devoted to characterizing global effects of CLD on the hepatopancreas proteome of *M. rosenbergii*, and especially on the endocrine system processes since CLD is suspected of being an EDC for invertebrates.

4.1. Global overview

The Venn diagram indicated that the number of modified proteins increased with CLD concentration of exposure. Moreover, results also showed that at least 9 biological processes were impacted by the three CLD exposures among which: stress response, cytoskeleton, protein synthesis and degradation or endocrine system. Most altered proteins were involved in immunity and defense processes (Table 1), including detoxification process, biotransformation process (cytochrome P450), stress (oxidative) response (Hsp70, Bip, calmodulin, transglutaminase), antioxidant mechanism (superoxide dismutase, catalase, ferritin) and immune system (lectins, chitinase, crustin, prophenoloxidase, protein disulfide-isomerase). These proteins were principally up-regulated in CLD-exposed prawns.

Xenobiotics are known to induce biotransformation process in exposed organisms (James and Boyle, 1998). Biotransformation is a complex mechanism that involves phase I and phase II enzymes which transform endogenous compounds or xenobiotics into more soluble compounds in order to facilitate their excretion (Koenig et al., 2012; Snyder, 1998). In crustaceans, this mechanism mainly occurs in the detoxifying organ, the hepatopancreas (James and Boyle, 1998; Snyder and Mulder, 2001). The increase of cytochrome P450 monooxygenases (CYP450) enzyme regulation observed in this study (n°10, Table 1), could be an induced response due to the CLD exposure. Indeed, CYP450 are the main detoxification enzymes involved in the phase I of the biotransformation process, and one of the more widely used biomarkers for biochemical response pathways (Snyder and Mulder, 2001). Moreover, this result could be correlated to those of Gaume et al. (2014) who measured an increase of the CYP450 gene expression in *M. rosenbergii* exposed to CLD for 8 days.

As exposure to xenobiotics and CYP450 activity may also lead to increased oxidative stress (e.g. production of reactive oxygen species - ROS), an up-regulation of antioxidant enzymes often occurs (Leung et al., 2011). Among antioxidant enzymes, superoxide dismutase (SOD) and catalase play a key role in cell protection from ROS (Mahaffey et al., 1982; Rodríguez-Ortega et al., 2002). Therefore, the up-regulation of SOD and catalase (n°20 and 7, Table 1), observed in the present work, could allow the elimination of ROS produced during a CLD exposure. This hypothesis is supported by the increase of catalase gene expression observed by Gaume et al. (2014) in *M. rosenbergii* exposed to CLD. Moreover, the increase of antioxidant enzymes in presence of CLD reflects the activation of defense mechanisms in exposed organisms to thwart ROS toxicity, as already observed in several organisms exposed to toxic organic compounds (Fercha et al., 2013; Horion et al., 2015; Snyder and Mulder, 2001).

Besides, the expression of cathepsin L was also up-regulated in exposed prawns, as compared to controls (n°8, Table 1). Cathepsin L is a proteolytic enzyme involved in protein degradation during immunity and digestion, but may also play a role in the uptake of vitellogenin during ovarian maturation and ecdysis in crustaceans, since this enzyme appears to be under the ecdysteroid control (Matsumoto et al., 1997; Qian et al., 2014; Zhao et al., 2013). Therefore, its deregulation, due to CLD exposure, could impact the molting and the reproduction processes of *M. rosenbergii* (see Section 4.2).

CLD is an organochlorine insecticide that acts by altering sodium channels, essential for the transmission of nerve impulses in organisms (Guzelian, 1982; Newhouse et al., 2009). Indeed, CLD was reported to produce neurotoxicity by inhibiting the Na^+/K^+-ATPase activity in fish (Desaiah and Koch, 1975) and rat (Bansal and Desaiah, 1985; Guzelian, 1982; Mishra et al., 1980). Our results showed the down-regulation of Na^+/K^+-ATPase and Na^+/Ca^{2+}-exchanger (n°59 and 61, Table 1) in *M. rosenbergii* exposed to CLD. Na^+/K^+-ATPase plays a key role in osmoregulation and regulation of membranes functioning. Its inhibition induces the increase of intracellular concentration of Na^+ by diffusion through the plasma membrane, following the concentration gradient (Guzelian, 1982). This Na^+ increase inhibits the Na^+/Ca^{2+}-exchanger and the Ca^{2+} release from the cell, leading to an increase of the intracellular concentration of Ca^{2+}. This increase leads to the activation of contractile proteins causing convulsions, and therefore the death of the target organism. Moreover, the increase of Ca^{2+} concentration in the cell could activate the Ca^{2+} transfer into the lumen of the sarcoplasmic reticulum (Silvestre et al., 2010). This suggestion is supported by our results that shown up-regulation of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (n°30, 32 and 33, Table 1), calreticulin and calsequestrin (n°22 and 31, Table 1), which are proteins that sequester calcium in sarcoplasmic reticulum, rendering it inactive. As Ca^{2+} regulation is crucial for exoskeleton development of crustaceans, its deregulation by CLD exposure could thus induce impairments in the molting process and development of *M. rosenbergii*.

Results also showed an up-regulation of the enzymes ATP synthase and arginine kinase (n°36 and 35, Table 1) which could be the consequence of the proton pumps activation, requiring energy. Indeed, the up-regulation of ATP synthase and arginine kinase suggests a significant energy mobilization in hepatopancreas of *M. rosenbergii* exposed to CLD. Previous investigations of the EDC effects on invertebrate proteome have underlined an increase of arginine kinase, e.g. in *Porcellio scaber* exposed to bisphenol A and vinclozolin (Lemos et al., 2010) or in *Gammarus pulex* exposed to polybromodiphenyl ethers (Gismondi et al., 2015). Although the energy metabolism was disturbed after CLD exposure, no consequence could be highlighted because proteins involved in glycolysis and glucogenesis were either up-regulated or down-regulated (e.g. enolase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, triosephosphate isomerase).

4.2. Altered proteins involved in reproduction and development processes

Chlordecone has been identified to have endocrine disruption effects on vertebrates (Donohoe and Curtis, 1996; Flouriot et al., 1996; Hammond et al., 1979), and invertebrates (Lafontaine et al., 2016a, b). Our proteomic analysis underlined some deregulation of proteins hormonally controlled and involved in reproduction or development processes.

4.2.1. Vitellogenin

The expression of vitellogenin (Vg) was significantly up-regulated in *M. rosenbergii* exposed to 20 µg/L of CLD compared to controls (n°2, Table 1). The presence of Vg in sexually immature prawns, as observed here, has already been reported in the oriental river prawn, *Macrobrachium nipponense* and the glass prawn, *Palaemon elegans* (Bai et al., 2016; Sanders et al., 2005). Synthesized during the vitellogenesis in females, Vg is the extraovarian precursor of yolk proteins, called vitellins (Vn) (Jasmani et al., 2004; Matozzo et al., 2008; Sankhon et al., 1999). Vn, which is an intraovarian essential compound for embryonic and larval development, is formed from Vg during its sequestration by the oocytes (Jasmani et al., 2004; Tseng et al., 2001). In vertebrates, Vg synthesis is under the control of endogenous estrogens and estrogen receptor pathways (Sumpter and Jobling, 1995). However, ecdysozoans have lost the steroid receptor family, as suggested by Thornton et al. (2003) and observed by Thomson et al. (2009) in *Daphnia pulex* (i.e. absence of the 3 A group (estrogen) and the 3 C group (androgen, progestogen) receptors). Therefore, the Vg up-regulation observed here could not be explained by an interaction of CLD with estrogen receptors. In crustaceans, the Vg synthesis is regulated by several hormones which could be affected by the CLD exposure, and thus explained the Vg up-regulation, such as: the VIH (vitellogenesis inhibiting hormone) secreted by the X-organ/sinus gland complex, the VSH (vitellogenesis stimulating hormone) secreted by the thoracic ganglion or the VSOH (vitellogenesis stimulating ovarian hormone) secreted by ovaries (Hyne, 2011; Kusk and Wollenberger, 2007; Rodriguez et al., 2007). Moreover, the molting hormone, 20-hydroxyecdyone (20-HE), which is secreted by the Y-organ under negative control of the MIH (molt inhibiting hormone) from the X-organ, is also involved in vitellogenesis (Rodriguez et al., 2007). The increase of Vg in prawns exposed to CLD could be explained by the interaction of CLD with the signaling pathways of these hormones through the X-organ of *M. rosenbergii*, which is one of the main neuroendocrine organs in crustaceans. This hypothesis is in line with our previous study which demonstrated a decrease of the 20-HE concentration in *M. rosenbergii* exposed to CLD (Lafontaine et al., 2016a). Moreover, the increase of Vg observed here is in accordance with the increase of Vg gene expression previously observed in *M. rosenbergii* exposed in-situ, in a pond (i.e. closed aquaculture) contaminated with 0.33 µg/L of CLD (Lafontaine et al., 2016b). In the same way, several investigations of crustaceans exposed to EDCs (e.g. xenoestrogen compounds) shown Vg increase (Billinghurst et al., 2000; Ghekiere et al., 2006; Huang and Chen, 2004; Huang et al., 2006; Oberdörster et al., 2000; Sanders et al., 2005).

The increase of Vg could cause some reproductive impairments such as oocyte production impairments, abnormal structure of oocytes, or decrease of the reproductive capacity (Huang et al., 2006; Jubeaux et al., 2012; Oetken et al., 2004), which could affect the *M. rosenbergii* fitness.

4.2.2. Male reproductive tract specific kazal type protease inhibitor

Results revealed that the male reproductive tract specific kazal type protease inhibitor (MRPINK) and the sperm gelatinase (MSG) were significantly decreased in *M. rosenbergii* exposed to 2 and 20 µg/L of CLD compared to controls. The MRPINK and MSG (n°39 and 40, Table 1) have been identified from adult *M. rosenbergii* (Cao et al., 2007; Li et al., 2008; Qian et al., 2012; Yang et al., 2013), and the present results revealed that they are also present in sexually immature prawns. MRPINK is linked to the male prawn reproduction and plays an important role during the fertilization process, mainly in sperm-oocyte interactions (Cao et al., 2007; Li et al., 2008). Indeed, MRPINK was identified and characterized as having an inhibitory effect on both the gelatinolytic and proteolytic activities of prawn sperm (Li et al., 2009, 2008). These enzymes are involved in the degradation of the vitelline coat by sperm proteases during the fertilization process, and are therefore necessary for sperm penetration of the egg envelope (Li et al., 2008; Vacquier, 1998).

This study revealed a significant decrease of MRPINK in prawns exposed to 2 and 20 µg/L which may result in an increase of gelatinolytic and proteolytic activities of sperm. The disruption of the sperm protease activity during spermatozoid penetration into the egg envelope could have an impact on reproductive capability. Moreover, MRPINK specifically inhibits the activity of *M. rosenbergii* sperm gelatinase (MSG) (Cao et al., 2007; Li et al., 2009, 2008; Qian et al., 2012). MSG is linked to the male reproductive tract and is also involved in the fertilization process, even if its specific role is still unknown. The decrease of MRPINK and MSG in CLD-exposed prawns could prevent the normal progress of the reproduction and could have an impact on the population dynamics on the long-term.

4.2.3. Farnesoic acid o-methyltransferase

Finally, proteomic analysis highlighted an up-regulation of the farnesoic acid o-methyltransferase (FAMeT) in *M. rosenbergii* exposed to CLD, as compared to controls (n°1, Table 1). The FAMeT is a key enzyme involved in the conversion of farnesoic acid (FA) to methyl farnesoate (MF) (Duan et al., 2014; Li et al., 2013). MF, an analog of the juvenile hormone of insects, is a crustacean hormone synthesized by the mandibulary organ, and involved in the regulation of several physiological processes such as growth, reproductive development, ovarian development or metamorphosis (Abdu et al., 1998; Chan et al., 2005; Chang, 1995; Duan et al., 2014; Li et al., 2013; Makkapan et al., 2011; Toyota et al., 2015). Therefore, FAMeT may play key roles in the regulation of the reproduction and growth of crustaceans (Duan et al., 2014; Silva Gunawardene et al., 2002). The increase of the FAMeT protein, observed here, could lead to an increase of MF by higher FA conversion. Moreover, several studies showed that changes in the MF levels appeared to stimulate or inhibit larval development and metamorphosis in exposed crustaceans, resulting in the production of organisms with mixed larval and juvenile physical traits (Abdu et al., 1998; LeBlanc, 2007; Yamamoto et al., 1997). Furthermore, it was shown that high MF levels in ovigerous daphnids resulted in high male/female ratio in offspring (Olmstead and Leblanc, 2002; Rider et al., 2005; Tatarazako et al., 2003). Therefore, these studies allowed to determine the role of MF as the endogenous signal that triggers sexuality in daphnids according to environmental signals and that MF had also regulatory roles in sex differentiation in decapods. Besides, it has been demonstrated that MF may stimulate the synthesis and secretion of ecdysteroids from the Y-organ, and regulate the Vg

production and uptake, which is in accordance with the Vg up-regulation observed here (Chan et al., 2005; Chang et al., 1993; Mak et al., 2005; Nagaraju, 2007).

5. Conclusion

This study revealed that the proteome of *Macrobrachium rosenbergii* was influenced by CLD exposure. Several proteins were significantly up- or down-regulated in exposed prawns compared to controls. Most of these proteins are involved in the ion transport, defense mechanisms and immune system, cytoskeleton, or protein synthesis and degradation. Results provide evidence that CLD does not target one single process in cells but may induce several toxic effects, including immunotoxicity, neurotoxicity, developmental toxicity and reproductive toxicity. Moreover, proteins involved in reproduction and development processes, also showed significant deregulation. As these proteins are closely linked with the hormonal system of crustaceans, the presence study indicates that CLD potentially disrupts the hormonal system of *M. rosenbergii* like endocrine disrupting compounds. Indeed, Vg, MRPINK, MSG and FAMeT play key roles in the regulation of biological processes hormonally controlled by the endocrine system. The modification of their regulation could induce adverse effects on reproduction and/or development and thus, impact the crustacean populations on the long term.

Although this proteomic analysis revealed the impact of CLD on the protein expression of *M. rosenbergii*, and notably on the regulation of proteins involved in endocrine functions of crustaceans, further investigations are needed to complete the understanding. Investigations could especially focus on hormonal pathways, such as the X/Y-organs, their receptors (e.g. ecdysteroid receptor), as well as the production of associated hormones (e.g. MIH, VIH). However, results of the present study raise new focus on proteins that can be used as biomarkers of endocrine disruptor exposure in invertebrates.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2017.03.043.

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