A differential proteomic approach to assess the effects of chemotherapeutics and production management strategy on giant tiger shrimp *P. monodon*

Frédéric Silvestre a,⁎, Huynh Thi Tu a,c, Amandine Bernard a, Jennifer Dorts a, Marc Dieu b, Martine Raes b, Nguyen Thanh Phuong c, Patrick Kestemont a

a Unité de recherche en Biologie des Organismes (URBO), Facultés Universitaires Notre-Dame de la Paix, rue de Bruxelles 61, B-5000, Namur, Belgium
b Unité de Recherche en Biologie Cellulaire, Facultés Universitaires Notre-Dame de la Paix, rue de Bruxelles 61, B-5000, Namur, Belgium
c College of Aquaculture and Fisheries, The University of Can Tho 3/2 Str., Campus II, Can Tho City, Vietnam

A R T I C L E  I N F O

Article history:
Received 27 January 2010
Received in revised form 28 June 2010
Accepted 28 June 2010
Available online 6 July 2010

Keywords:
Antibiotics
Biomarker
Black tiger shrimp
Intensive aquaculture
Proteomics
Sarcoplasmic calcium-binding protein

A B S T R A C T

The intensification of shrimp farming has been related to the increasing use of chemotherapeutics and potentially suboptimal rearing conditions. For the purpose of assessing the stress level of cultured giant tiger shrimp *P. monodon*, a proteomic analysis (2D-DIGE) was performed on hemolymph. On the one hand, shrimp were exposed for 7 days to the antibiotics enrofloxacin or furazolidone via feed (4 g kg⁻¹) under laboratory conditions. On the other hand, shrimp were submitted to enrofloxacin directly in field conditions in Vietnam, for which two different culture systems were distinguished (intensive and improved extensive). No significant different protein abundance pattern was induced by antibiotics under laboratory conditions, while only one protein spot displayed a 1.53-fold reduction in intensity after exposure to enrofloxacin in improved extensive ponds. When we compared the proteome of shrimp bred either in intensive or in improved extensive system, we observed 9 protein spots displaying significant difference in abundance. Among them, 3 spots of hemocyanin were under-expressed in shrimp from improved extensive ponds. At the opposite 2 spots corresponding to Sarcoplasmic Calcium-binding Protein (SCP) were less abundant in hemolymph of shrimp from intensive ponds. These results demonstrate that the very subtle effects of tested antibiotics on patterns of hemolymph protein expression are overwhelmed by the effects of conditions encountered in different production management systems, such as different oxygen and nitric concentrations.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

Black tiger shrimp (*P. monodon*) production in the Mekong River Delta (MRD), in Vietnam, has grown rapidly during the last decade, reaching 259,476 t in 2005 and moving from low to high intensity (MOF, 2005). In 2003, shrimp farming systems in Vietnam comprised 3% of semi-intensive and intensive, 22% of improved extensive and 75% of extensive culture farms. Intensive systems accounted for 10% while improved extensive culture accounted for 60% of the total shrimp production. The intensive culture system is characterised by small and relatively deep ponds (2000 to 5000 m², depth of 1.2–1.5 m), water exchange regulated by pumps and aeration systems, high stocking densities (20–40 shrimp/m²) and the use of commercial shrimp feed. In contrast, the improved extensive culture system features larger, shallower ponds (up to 10 ha and depth of 0.7–1 m), water exchange by tide, low stocking densities (2–7 shrimp/m²), and the use of natural or supplementary home-made feed (Le and Munekage, 2004).

More than 80% of total Vietnamese shrimp production comes from the MRD, increasing environmental pressure in this region. Dierberg and Kiatissimkul (1996) grouped environmental impacts of the shrimp industry into 7 major categories: mangrove/wetland destruction, saltwater intrusion, land subsidence, water quality impairments, sediment disposal, abandoned shrimp ponds, and displaced traditional livelihoods. Moreover, the culture intensification process demands more and more chemicals and drugs to prevent and treat disease problems. Frequently used products include pesticides, disinfectants, antibiotics and fertilizers (Graslund et al., 2003). These chemicals can reach the surrounding environment and consequently impair water quality, leading to detrimental impacts on wildlife. Moreover, there is an increasing awareness that exposure to those products could slow down growth, favour diverse pathologies, and increase cultured shrimp mortality via a higher stress level and a depletion of the immune response (Le Moullac and Haffner, 2000).

1.1. Culture systems

Abbreviations: 2D-DIGE, 2 dimension differential in gel electrophoresis; DO, dissolved oxygen; MRD, Mekong River Delta; MRL, Maximum residue limit; PAP, Protein abundance profile; SCP, Sarcoplasmic calcium-binding protein.

⁎ Corresponding author. Tel.: +32 81 72 42 85; fax: +32 81 72 43 62.
E-mail addresses: frederic.silvestre@fundp.ac.be (F. Silvestre), httu@ctu.edu.vn (H.T. Tu), jennifer.dorts@fundp.ac.be (J. Dorts), marc.dieu@fundp.ac.be (M. Dieu), martine.raes@fundp.ac.be (M. Raes), ntphuong@ctu.edu.vn (N.T. Phuong), patrick.kestemont@fundp.ac.be (P. Kestemont).

© 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbd.2010.06.003
Among the most often used chemotherapeutics, enrofloxacin, an antibiotic belonging to the quinolone family, has gained growing attention. As the excess amount of enrofloxacin can be harmful to people, the European Union fixed the maximum residue limit (MRL) for importation at 100 μg/kg in the edible tissues of fish and other aquatic animals (European Council Regulation, 2002). Furazolidone belongs to the group of nitrofurans with antibacterial agents and has been widely used in aquaculture. It has also been used as a food additive for the treatment of gastrointestinal infections in cattle, pigs and poultry (Alexander et al., 2001; Balizs and Hewitt, 2003; Hoogenboom et al., 2002). In long-term studies with experimental animals, furazolidone and its metabolite (3-aminoo-2-oxazolidinone; AOZ) have shown carcinogenic and mutagenic characteristics (Auro et al., 2004; Timperio et al., 2003). This has led to a prohibition of furazolidone for the treatment of animals used for food production in Europe in 1995 (European Council regulation, 1995).

In addition to the potential hazard for cultured shrimp of using chemotherapeutics and chemicals, animals reared under intensive conditions are likely to encounter suboptimal and stressful environmental conditions, such as elevated water temperature, low dissolved oxygen, lower salinity, and be exposed to toxic compounds such as hydrogen sulphide, methane, ammonia and nitrite (Lee and Wickins, 1992). Tu et al. (2008) has shown that black tiger shrimp reared in intensive farms presented a higher level of lipid peroxidation in hepatopancreas, suggesting that intensive conditions could trigger an oxidative stress.

Proteomics has undergone tremendous advances over the past few years, and technologies have noticeably matured. Molecular biomarkers that will be developed using this approach have the potential of providing early detection of environmental stress, inferred mechanism of action, and relatively efficient monitoring of the environment (Calzolai et al., 2007; Lemos et al., 2010). Despite these developments, biomarker discovery remains a challenging task due to the complexity of the samples (e.g. serum, other bodily fluids, or tissues) and the wide dynamic range of protein concentrations (Domon and Aebersold, 2006). Moreover, the benefits of “–omics” technologies have primarily been realised for well-characterised and sequenced model species. Unfortunately, these species may be inappropriate from an ecotoxicological perspective and for aquaculture production (Forné et al., 2010). Therefore, there is a need to explore new ways to exploit proteomics for commercially important species such as the giant tiger shrimp. Using differential in gel electrophoresis (DIGE) technique, we analysed the effects of enrofloxacin and furazolidone, supplied via feed at doses close to that used by farmers, on the hemolymph of black tiger shrimp (10 ± 1.3 g) were obtained from extensive shrimp farms in Ca Mau province. Upon arrival, shrimp were acclimated to laboratory conditions in composite tanks (capacity 2 m³) filled with natural aerated water of 15 g/L salinity at 30 °C–32 °C for 2 weeks. During the acclimation period, shrimp were fed with commercial shrimp feed (35% crude protein, 5% lipid, 3% calcium, 1% phosphorous, 2% premix vitamin, Charoen Pokphand aquafeed, Thailand). Enrofloxacin or furazolidone powder (98% purity) (Sigma-Aldrich, Germany) was mixed with commercial shrimp feed at a dose of 4 g/kg. Medicated-feed was coated with 3% water and 3% squid oil, and kept frozen at −20 °C until use. Shrimp were divided into 9 composite tanks at a stocking density of 70 shrimp per tank at the beginning of the experiment. Three tanks received feed with enrofloxacin, and 3 other tanks received feed with furazolidone for 7 days. Shrimp from the 3 remaining tanks were fed with non medicated-feed as controls. Shrimp were fed 4 times daily at 8:00, 11:00, 14:00 and 17:00. Feeding rate was 3–5% of body weight. No mortality was observed during the experiment.

2.1.2. Field study
Three intensive shrimp ponds in Soc Trang province (area of 2000 m²; stocking density of 20 shrimp/m²) and 3 improved extensive shrimp ponds in Ca Mau province (area of 2000–6000 m²; stocking density of 4–5 shrimp/m²) were selected for the field experiment. After two months of stocking, shrimp (mass from 8.8 to 10 g) were fed with medicated-feed containing 4 g enrofloxacin/kg (prepared as described above) for 7 days. Feeding was done 4 times daily.

2.1.3. Sampling and biological material collection
In the laboratory study, shrimp were sampled after 7 days of medication. In the field study, shrimp were sampled at day 0 and after 7 days of medication. At each sampling time, 6 shrimp were sampled in each tank or pond. Hemolymph was withdrawn from the ventral vessel with a syringe containing 0.3 mL of sodium citrate (1%) to avoid clotting, for a total hemolymph volume of about 1 mL per shrimp. Immediately after sampling, hemolymph samples from 6 shrimp were pooled and kept at −80 °C until analysis. In ponds, temperature, salinity, pH and dissolved oxygen (DO) were recorded directly at day 0 and at day 7 using a water quality meter (YSI Environmental 556, USA). Water in each pond was also sampled for nitrite, nitrate and hydrogen sulphide analysis.

2.2. Protein extraction and CyDyes labelling
Proteins were extracted in 1:10 w:v lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris), and sonicated twice 10 s on ice. The soluble protein fractions were harvested by centrifugation at 12,000 g for 15 min at 4 °C and the pellet discarded. The pH of the soluble protein extract was adjusted to 8.5 by addition of appropriate 50 mM NaOH volume and protein concentration was analysed using the Biolog protein assay as described by the manufacturer (BioRad, UK). For DIGE minimal labelling (Unlu et al., 1997), 25 μg of protein sample were labelled with 200 pmol of fluorescent amine reactive Cyanine dyes freshly dissolved in anhydrous dimethyl formamide following the manufacturer’s recommended protocols (GE Healthcare). Labeling was performed on ice for 30 min in the dark and quenched with 1 mM lysine for 10 min on ice. Cy3 and Cy5 were used to label samples, while a mix sample composed of equal amounts of proteins from each replicate was minimally labelled with Cy2 and was used as the internal standard. The 3 labelled mixtures were combined and the total proteins (75 μg) were added v:v to reduction buffer (7 M urea, 2 M thiourea, 2% DTT, 2% CHAPS, 2% IPG 4–7 buffer) for 15 min at room temperature.

2.3. Protein separation by 2-dimensional electrophoresis
Prior to the first dimension separation of proteins, IPG strips (24 cm, pH 4–7; GE Healthcare) were rehydrated overnight with 450 μL of a rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG 4–7 buffer, 2% DTT). The sample sets containing the labelled mixtures were then cup-loaded onto the IPG strips, and isoelectric focusing was performed on an Ettan™ IPGphor 3 isoelectric focusing unit (GE Healthcare). The electrophoresis condition was set at 20 °C for a total of 68 300 Vh at 50 μA of current per strip. Prior to SDS-PAGE, focused IPG strips were equilibrated in buffer (50 mM Tris, 6 M urea,
30% glycerol, 2% SDS, pH 8.8) containing 1% DTT and then 2.5% iodoacetic acid for 2×15 min. Strips were then loaded onto a 14% 24 cm, 1 mm thick, acrylamide gel. The strips were overlaid with 1% agarose in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and run in an Ettan™ DALTsix electrophoresis unit (GE Healthcare) at constant 3 W/gel at 15 °C until the blue dye front had run off the bottom of the gels.

2.4. Image analysis and statistics

Labelled proteins were visualized using a Typhoon 9400 imager (GE Healthcare) at 3 specific wavelengths (488 nm for Cy2, 532 nm for Cy3, 633 nm for Cy5). Resolution was of 100 μm. Image analysis was performed using DeCyder BVA 5.0 software (GE Healthcare). Briefly, the Differential In-Gel Analysis (DIA) facility co-detected (the estimated number of spots for each codetection was set to 2500) and differentially quantified the protein spots in each image, using the internal standard sample as a reference to normalize the data. At a second step, the Biological Variation Analysis (BVA) was used to calculate ratios between samples and internal standard abundances as XML file for use with ProteinScape 2.0 (Bruker) with Mascot 2.2 as search engine (Matrix Science). Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at one. Carbamidomethylation was allowed as fixed modification and oxidation of methionine as variable modification. Mass tolerance for monoisotopic peptide window was 10 ppm and MS/MS tolerance window was set to 0.05 Da. The peak lists were searched against the full NCBInr database (9694989 sequences downloaded on September 15th 2009). Scaffold (version Scaffold-2_06_01, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.2) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.). Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nevzhitinskii et al., 2003).

3. Results

In the present work we used protein extracts from *P. monodon* hemolymph to compare protein abundance profiles (PAPs) among animals exposed via feed to 2 antibiotics, enrofloxacin or furazolidone, in laboratory and in field conditions. A total number of spots between 2139 and 2394 were detected on the 2D gels with the DeCyder software. With our criteria for significance (p<0.01) and experimental design (3 replicates of 6 independent individuals), we could only identify a limited impact of the tested antibiotics on PAPs. Dige analysis revealed that only one protein spot (spot number 1280) was significantly affected by enrofloxacin treatment (Fig. 1). The decrease of abundance for this spot, unidentified, was 1.53-fold in shrimp exposed for 7 days to the fluoroquinolone in improved extensive ponds. No significant antibiotic effect was highlighted under laboratory conditions and in shrimp reared in intensive farms.

Water physicochemical parameters were significantly different among different culture systems. In Table 1 we can observe that temperature was higher of 2.2 °C and that dissolved oxygen was

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physicochemical parameters of water from intensive (Soc Trang province) and improved extensive (Ca Mau province) ponds. Mean values ± standard deviations (N = 3).</td>
</tr>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td>Temperature (°C)</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Salinity (g/L)</td>
</tr>
<tr>
<td>NO₂ (mg/L)</td>
</tr>
<tr>
<td>NO₃ (mg/L)</td>
</tr>
<tr>
<td>NH₃ (mg/L)</td>
</tr>
<tr>
<td>H₂S (mg/L)</td>
</tr>
</tbody>
</table>

*p<0.05; ** p<0.01 between intensive and improved extensive ponds.
2 mg/L higher in improved extensive ponds (p<0.0011). On the other hand, nitrite (p<0.0008), ammonia (p<0.0184) and hydrogen sulphide (p<0.0012) were all lower in improved extensive ponds (up to 3.8-fold lower for nitrite) than in intensive ones. The type of culture management system seems to induce a strong variation in the PAPs. When patterns are compared between shrimp reared in intensive and improved extensive ponds at day 0 (before the use of antibiotics), a significant variation in abundance was observed for 9 protein spots at p<0.01. Among the protein spots showing significant variation in intensity between both culture systems, five decreased in improved extensive reared animals, with abundance factors comprised between −1.47 and −2.18 (Figs. 1, 2). Three of these spots have been identified as hemocyanin in *Penaeus monodon* (spots 926, 1166, 1302) with the help of as many as 7 different peptides. Hemocyanins are copper-containing oxygen transport proteins found in the hemolymph of many invertebrates. In arthropodan hemocyanins exist as hexamers comprising 3 heterogeneous subunits and possess 1 oxygen-binding centre per subunit. Four other protein spots showed an increase in abundance in shrimp from improved extensive ponds, ranging from 3.29 to 4.39-fold. Two of them were unambiguously identified as the Sarcoplasmic Calcium-binding Protein (SCP) by the means of 2 peptide sequences (Table 2; Fig. 3). This protein exists in shrimp as polymorphic dimers (alpha–alpha, alpha–beta, beta–beta), the alpha chain having been entirely sequenced in 1984 (Takagi and Konishi, 1984). The alpha chains are formed of about 190 amino acids and have three functional Ca\(^{2+}\)-binding sites, which are common to EF-hand type Ca\(^{2+}\)-binding protein, and which have high affinity for Ca\(^{2+}\). This protein is related to the parvalbumins of vertebrates and is abundant in fast-contracting muscle. However, its expression is also observed in other crustacean tissues such as gill, hepatopancreas, intestine or antennal gland (Gao et al., 2006).
Integrated aquaculture management has been proposed to achieve industry sustainability. Components of a sound management plan should include the promotion of a research program for determining impacts from the shrimp industry and require an environmental impact assessment tool (Dierberg and Kiattisimkul, 1996). The issue of animal welfare in aquaculture is of growing interest, and there is an increasing consumer demand for documentation of safe and ethically defendable food production. Consequently, sustainable development of shrimp culture necessitates that animals should be in good health condition, unstressed, and that as low amounts of chemicals as possible should be used. For this purpose the use of biomarkers in cultured shrimp can be of great interest. Comprehension of the mechanisms related to the sub-lethal effects caused by environmental stressors upon shrimp metabolism would help to develop sensitive and precise diagnostic tools with a predictive capability in assessing the toxic effects, thus metabolism would help to develop sensitive and precise diagnostic tools with a predictive capability in assessing the toxic effects, thus permitting early intervention to improve aquatic animal health and welfare.

4. Discussion

Integrated aquaculture management has been proposed to achieve industry sustainability. Components of a sound management plan should include the promotion of a research program for determining impacts from the shrimp industry and require an environmental impact assessment tool (Dierberg and Kiattisimkul, 1996). The issue of animal welfare in aquaculture is of growing interest, and there is an increasing consumer demand for documentation of safe and ethically defendable food production. Consequently, sustainable development of shrimp culture necessitates that animals should be in good health condition, unstressed, and that as low amounts of chemicals as possible should be used. For this purpose the use of biomarkers in cultured shrimp can be of great interest. Comprehension of the mechanisms related to the sub-lethal effects caused by environmental stressors upon shrimp metabolism would help to develop sensitive and precise diagnostic tools with a predictive capability in assessing the toxic effects, thus permitting early intervention to improve aquatic animal health and welfare.

Table 2

Putative identification of spots from 2D maps by LC–MS/MS. Hemolymph proteome of shrimp from Ca Mau (improved extensive) or Soc Trang Province (intensive) ponds.

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein name</th>
<th>Species</th>
<th>Accession no.</th>
<th>MW (kDa)</th>
<th>No. of peptides</th>
<th>% cov.</th>
<th>Sequences</th>
<th>Mascot ion score</th>
<th>X1 Tandem-log(e)</th>
<th>Imp. ext.</th>
</tr>
</thead>
<tbody>
<tr>
<td>926</td>
<td>Hemocyanin</td>
<td>Penaeus monodon</td>
<td>Q95V28</td>
<td>5.1</td>
<td>51.152</td>
<td>8</td>
<td>(R)RDLILYVXERK(I) (R)DAIAHGYVDR(A) (R)AGNHIDIMNER(G) (R)KEDSSAVTPDVPFPATLFEK(T) (R)VFEDELPHNHQQFVR(Q) (K)JIVSNNKGQVEATVR(V) (K)GESAVTPDVPFPATLFEK(T) (K)GQGEVATVR(I)</td>
<td>53.24</td>
<td>1.05</td>
<td>1.94</td>
</tr>
<tr>
<td>1166</td>
<td>Hemocyanin</td>
<td>Penaeus monodon</td>
<td>Q95V28</td>
<td>5.1</td>
<td>51.152</td>
<td>6</td>
<td>(K)VVFENYIQFHD(1) (R)VFEDELPHNHQQFVR(Q) (K)JIVSNNKGQVEATVR(V) (K)GESAVTPDVPFPATLFEK(T) (K)QALGGADSLTDIFSAATGIPN(R) (R)RDILIVER(G) (K)AGNHIDIMNER(G) (K)YGGOPSPRDPMNVNFEDVGVAR(I)</td>
<td>44.44</td>
<td>1.89</td>
<td>2.18</td>
</tr>
<tr>
<td>1302</td>
<td>Hemocyanin</td>
<td>Penaeus monodon</td>
<td>Q95V28</td>
<td>5.1</td>
<td>51.152</td>
<td>3</td>
<td>(K)IDVSNNKGQVEATVR(I) (K)SSESAVTVPDVPSATLFEK(T) (K)EALGGADSLTDIFSAATGIPN(R) (R)RDILIVER(G) (K)AGNHIDIMNER(G) (K)YGGOPSPRDPMNVNFEDVGVAR(I)</td>
<td>65.63</td>
<td>2.07</td>
<td>1.49</td>
</tr>
<tr>
<td>1727</td>
<td>Sarcoplastic</td>
<td>Litopenaeus vannamei</td>
<td>CTA639</td>
<td>4.7</td>
<td>22.078</td>
<td>11</td>
<td>(R)RFIAHGYIIVDR(A) (R)AGNHIDIMNER(G) (K)IDVSNNKGQVEATVR(I) (R)VFEDLPNFGHIQVK(V) (K)VFNHGEYIQHD(-)</td>
<td>72.31</td>
<td>6.51</td>
<td>3.29</td>
</tr>
<tr>
<td>1738</td>
<td>Sarcoplastic</td>
<td>Litopenaeus vannamei</td>
<td>CTA639</td>
<td>4.7</td>
<td>22.078</td>
<td>12</td>
<td>(K)IDVSNNKGQVEATVR(I) (R)VFEDLPNFGHIQVK(V) (K)VFNHGEYIQHD(-)</td>
<td>56.1</td>
<td>1.89</td>
<td>4.02</td>
</tr>
</tbody>
</table>

Legend abbreviations. No., Spot number as given by DeCyder software on the 2-D gel images; Obs., observed; Accession No., Accession number in Swiss-Prot database; pl, theoretical isoelectric point; Mw, theoretical molecular weight; Imp. Ext., fold regulation where a positive value indicates that the protein spot abundance increases in shrimp reared in improved extensive ponds compared to intensive, before any enrofloxacine medication.

Despite the lack of significant result under laboratory conditions and in shrimp reared in intensive ponds, one protein spot (spot number 1280) showed a reduced abundance after exposure to enrofloxacine in improved extensive farms. This fact suggests a modulation of enrofloxacine effect by surrounding environmental conditions. As previously mentioned by Lee and Wickins (1992), we found that, under intensive culture conditions, shrimp can be exposed to suboptimal environmental factors. Compared to improved extensive, water from intensive ponds presented a significant lower temperature as well as a lower dissolved oxygen concentration. In addition, shrimp were stocked at a very high density (20 shrimp/m²) low temperature as well as a lower dissolved oxygen concentration. Further developments, combining 2D-DIGE with immunosubtraction of hemocyanin, will remove a significant fraction of the protein mass, yielding a corresponding enrichment of lower abundant species.

Kiattisimkul (1996) indicated that oxidation of these organic waste compounds can deplete the DO, triggering the formation of toxic metabolites such as hydrogen sulphide, methane, ammonia, and nitrite. In the present study, we actually measured higher levels of hydrogen sulphide, ammonia and nitrite in intensive ponds, suggesting that those shrimp encounter more potentially stressful conditions. Our proteomic results denote an increase in abundance of three spots compared to intensive ponds (Soc Trang). Spots 1727 and 1738 have been identified as sarcoplasmic calcium-binding proteins.

Fig. 3. Two-dimensional views of spots number 1727, 1730 and 1738, differentially up-regulated (p<0.01) in shrimp sampled in improved extensive ponds (Ca Mau) compared to intensive ponds (Soc Trang). Spots 1727 and 1738 have been identified as sarcoplasmic calcium-binding proteins.

The proteomic approach developed in the present study, based on a simple and non-invasive hemolymph sampling, has the potential to point out sensitive biomarkers distinguishing between different environmental conditions. With this approach, we could identify two forms of the Sarcoplasmic Calcium-binding Protein (SCP) as being less abundant in hemolymph of shrimp sampled in intensive ponds. This protein appears to play an important role in cellular calcium homeostasis. It effectively serves to reduce the availability of Ca$^{2+}$ in the cytoplasm. As Ca$^{2+}$ is of crucial importance during the molting cycle, it is not surprising to observe that SCP expression can be modulated as a function of molting stage (Gao et al., 2006). Besides, SCPs are also found in hemolymph where they were linked to the immune system status in Drosophila melanogaster. In this species, the SCPs increased in hemolymph of immune-challenged flies (Engström et al., 2004). In shrimp, SCPs are down-regulated in gills of animals infected by the yellow-head virus (YHV) (Rattanarajpong et al., 2007). The authors suggested that the decrease of SCPs could play an important role in the shrimp response to viral infection. The resulting impairment of cytosolic Ca$^{2+}$ binding could lead to the activation of enzymes that could damage or destroy the YHV-infected cells. On the other hand, other stressors also seem to be able to interfere with Ca$^{2+}$-binding proteins. For example, cadmium, which has a strong capacity to interact with calcium homeostasis, has been shown to reduce the expression of a Ca$^{2+}$-binding protein in gills of the Chinese crab Eriocheir sinensis (Silvestre et al., 2006). So far, the differential abundance of two forms of SCPs reported in P. monodon between animals reared in intensive and improved extensive systems can’t be directly related to a particular stressor. At our knowledge, no study investigated the effects of environmental parameters on SCP expression. However, our results might suggest that intensive culture conditions could impair the calcium homeostasis of farmed shrimp and, consequently, could also impair their immune system. Consequences could be a higher sensitivity to viral infection and other diseases, requiring the farmers to increase their use of drugs and chemicals. Further developments should use the SCPs in hemolymph as a biomarker of environmental stress in farm shrimp. We therefore recommend further investigation of the links between SCPs in hemolymph and the sensitivity of shrimp to diseases. This should allow monitoring of the stress level of shrimp and should lead to optimization of pond parameters in order to avoid excessive infection, ultimately preventing the use of additional chemicals.
5. Conclusions
The use of a proteomic approach in the field of aquaculture and better pond management in order to point out new biomarkers of environmental stress was lacking up to now. The DIGE approach used in the present work has shown evidence that the proteome in the hemolymph of black tiger shrimp is influenced by the environmental conditions in ponds. In order to achieve sustainability, the aquaculture sector should require an impact assessment tool as an integral component of a sound management plan. Even though enrofloxacin and furazolidone treatments induce only limited effects, the observed modified protein patterns in shrimp reared in different culture systems suggest that the stress level depends upon the system, with a likely higher stress in shrimp reared in intensive ponds, possibly due to lower oxygen and/or higher organic compounds in water. We highlight the possibility to use the abundance of SCPs and hemocyanin in hemolymph as a non-invasive assessment tool to determine the environmental stress level in farmed black tiger shrimp. Further studies should determine whether the decrease of abundance of these proteins could be directly linked to an observable impairment of the productivity and the quality of shrimp.

Acknowledgments
The authors thank Catherine Demazy and Edouard Delaive (URBC), University of Namur (Belgium) for their valuable help during proteomic analysis. We also are grateful to Wes Dowd, University of California Davis (USA), for the proofreading of the present manuscript. This study was jointly supported by Belgian Science Policy Office and the Ministry of Science and Technology in Vietnam (Project BL 12/V07, BL13/V06 and NDT 4/2005). Huynh Thi Tu is holder of CERUNA-FUNDP Ph.D. grant, the University of Namur, Belgium. Frederic Silvestre is a post-doctoral researcher at the FNRS, Belgium. The proteomic and MS facility of the URBC is supported by the FNRS, Belgium.

References