Fish & Shellfish Immunology
August 2015, Volume 45, Issue 2, Pages 933-939
http://dx.doi.org/10.1016/j.fsi.2015.05.041
http://archimer.ifremer.fr/doc/00270/38116/

Rearing effect of biofloc on antioxidant and antimicrobial transcriptional response in *Litopenaeus stylirostris* shrimp facing an experimental sub-lethal hydrogen peroxide stress

Cardona Emilie [1, 2, *], Saulnier Denis [1], Lorgeoux Bénédicte [1], Chim Liet [2], Gueguen Yannick [1, 3]

[1] Ifremer, Centre Océanologique du Pacifique, Unité de recherche Resources Marines, B.P 7004, 98719 Taravao, French Polynesia
[2] Ifremer, Unité de recherche Lagons, Ecosystèmes et Aquaculture Durable en Nouvelle Calédonie B.P. 2059, 98846 Nouméa, New Caledonia
[3] Ifremer, UMR 5244 IHPE, UPVD, CNRS, Université de Montpellier, F-34095 Montpellier, France

* Corresponding author : Emilie Cardona, email address : emiliecardona2@gmail.com

Abstract :

This study compares the antioxidant and antimicrobial transcriptional expression of blue shrimps reared according to two different systems, BioFloc Technology (BFT) and Clear sea Water (CW) and their differential responses when facing an experimental sublethal hydrogen peroxide stress. After 30 days of rearing, juvenile shrimps were exposed to H2O2 stress at a concentration of 30 ppm during 6 hours. The oxidative stress caused by H2O2 was examined in the digestive glands of the shrimp, in which antioxidant enzyme (AOE) and antimicrobial peptide (AMP) gene expression were analysed by quantitative real-time PCR. Results showed that rearing conditions did not affect the expression of genes encoding AOEs or AMPs. However, H2O2 stress induced a differential response in expression between shrimps from the two rearing treatments (BFT and CW). Comparative analysis of the expression profiles indicates that catalase transcripts were significantly upregulated by H2O2 stress for BFT shrimps while no change was observed for CW shrimps. In contrast, H2O2 caused down-regulation of superoxide dismutase and glutathione transferase transcripts and of the three AMP transcripts studied (penaeidin 2 and 3, and crustin) for CW shrimps, while no effect was observed on BFT shrimp transcript levels. These results suggested that BFT shrimps maintained antioxidant and AMP responses after stress and therefore can effectively protect their cells against oxidative stress, while CW shrimp immune competence seems to decrease after stress.

Highlights

► Contrasted rearing conditions, biofloc and clear water, did not affect the expression of genes encoding antioxidant enzymes and antimicrobial peptides. ► Biofloc shrimps maintained antioxidant and antimicrobial peptide responses after H_2O_2 stress while the immune competence of clear water shrimp seemed to decrease after stress. ►Biofloc seems to improve the immune resistance of animals against stress.

Keywords : biofloc, shrimps, antimicrobial peptides, antioxidant enzymes

1. Introduction

In shrimp aquaculture, intense interest has focused on biofloc technology (BFT). BFT is a rearing system with zero or minimal water exchange. Conglomerates of microbes, algae, protozoa and other organisms, together with detritus and dead organic particles, develop in the water column (Avnimelech, 2009). The intensive microbial community present in this system can be used as a water quality treatment system for the pond and microbial protein can serve as a feed additive. At the present time, when shrimp production faces many losses due to disease outbreaks, the use of BFT can restrain the development of shrimp diseases. One explanation is that, with zero or minimal water exchange, BFT improves biosecurity because the exclusion of pathogens is enhanced by limiting contact with water from external aquatic ecosystems (Taw, 2013). Another explanation is that the microbial proteins assimilated by shrimp are believed to confer beneficial effects on well-being and shrimp immune status (Avnimelech, 2009). Nevertheless, the influence of biofloc on shrimp immune status has been poorly documented (Xu and Pan, 2013, 2014; Kim *et al.*, 2013; Ekasari *et al.*, 2014).

Because of their lack of acquired immunity, marine invertebrates' defence against invading pathogens relies solely on innate immune mechanisms (Mitta *et al.*, 2000). In shrimp these include: 1) pattern recognition receptors (PRR) (Wang and Wang, 2013); 2) humeral

responses characterized by the expression of antimicrobial peptides (AMPs), (Destoumieux *et al.*, 1997) but also coagulation and melanisation by so-called clotting (Omori *et al.*, 1989; Yeh *et al.*, 1998; Chen *et al.*, 2005) and prophenoloxidase activating systems (Sritunyalucksana and Söderhäll, 2000; Lee *et al.*, 2002; Charoensapsri *et al.*, 2011); 3) cellular responses, mainly performed by haemocytes, such as phagocytosis (Song *et al.*, 1994), chemotaxis with haemocyte migration into inflammatory foci (Munoz *et al.*, 2002), release of humeral defence components (agglutinins, coagulation and phenoloxidase enzymes, antimicrobial peptides), encapsulation and nodule formation (Holmblad and Söderhäll, 1999).

Animal stress induced by sub-lethal H_2O_2 concentration is a method rarely used to induce stress and therefore original. It seemed interesting to study the response of animals to H_2O_2 stress because, although oxygen radical stress and antioxidant protection are coming increasingly into focus in physiological research on marine invertebrates, only a few studies have considered the effects of elevated concentrations of reactive oxygen species (ROS) on invertebrate physiology (Abele-Oeschger *et al.*, 1997). Among commonly used biomarkers of immunity, antioxidant enzymes (AOEs) are directly involved in scavenging ROS and play pivotal roles in preventing damage generated by oxidative stress. ROS have been identified as major initiators of tissue damage and can upregulate enzyme activity, signal transcription, and gene expression (Massafra *et al.*, 2000). The antioxidant defence and immune systems are closely linked to responses to pathogens and other stress-related issues that might lead to respiratory burst (Holmblad and Söderhäll, 1999). Measuring AOE expression after environmental stressors such as pH (Wang *et al.*, 2009), temperature, salinity, hypoxia (De Zoysa *et al.*, 2009) and H_2O_2 stress (De Zoysa *et al.*, 2008) has proven to be a very good tool to study the responses of aquatic invertebrates. In contrast, very few studies have used antimicrobial peptide (AMP) analysis to investigate the impact of environmental stress on invertebrate immunology (Mitta *et al.*, 2000, Cellura *et al.*, 2007; Li *et al.*, 2009). The major studies on AMPs have focused on their identification, characterization, and regulation following pathogen infection (Bachère *et al.*, 2004). However, investigating AMPs can provide a unique opportunity to greatly advance current understanding in the field of ecological immunology and especially the impact of environmental stressors (Ellis *et al.*, 2011).

In our study, we explore the transcriptional responses of genes coding either for AOEs or AMPs in shrimp during contrasted rearing conditions, biofloc *versus* clear seawater, and experimentally induced stress produced by a sub-lethal dose of hydrogen peroxide.

Materials and method

Shrimps

The 12 day-old shrimps post-larvae *Litopenaeus stylirostris* used in this experiment were supplied by the hatchery of the Aquaculture Technical Centre of Tahiti (French Polynesia). Shrimps acclimated during 15 days in a 25 m^3 clear seawater tank (300% water renewal per day) and were fed three times per day with commercial feed at 20% of the shrimp biomass (SICA grower 40).

Biofloc production

The biofloc culture was established before the experiment in 4 tanks (250 L) with sub-adult shrimps (mean weight: 20 g, biomass: 500 $g.m^{-2}$) and fed twice per day with commercial shrimp feed (SICA grower 40) during 30 days. The shrimps were removed before the beginning of the experiment. Aeration was delivered continuously via an air stone in each tank. No water exchange was performed. Tanks were covered with a shade net to control the sunlight (70% inhibition of light).

Experimental design

Shrimps were caught in clear water tanks using a cast net and randomly distributed into 8 tanks (250 L). One hundred individuals (0.07±0.02g) were put into each tank (400 shrimps. m^{-2}). Each tank was continuously aerated with an air stone. No water was exchanged during the experimental period in the biofloc rearing system and a water renewal rate of 300% per day was applied in the clear seawater rearing system.

Two treatments with four replicate tanks for a period of 30 days were tested: clear seawater (CW) and biofloc (BFT). Shrimps were fed *ad libitum* 3 times per day (07:00 am, 01:00 pm and 05:00 pm) with commercial shrimp feed (SICA® grower 40). The pellet given to shrimp was the only source of carbon. It is equivalent to a C/N ratio of 8/1.

H_2O_2 stress, sampling and conservation

To achieve H_2O_2 stress, water renewal was operated in the biofloc tank during 12 hours to place the shrimp in sea clear water. During stress, all shrimps were maintained under the same conditions (clear seawater, temperature = 26.3°C, salinity = 34.5‰, pH = 8.20).

Then, all shrimps remaining in the tanks were stressed by immersion for 6 hours with a sub-lethal concentration of H_2O_2 (30 ppm) added directly to the rearing tank.

Only shrimps in the inter-molt phase were sampled for molecular analysis. Molting stages were determined by microscopic examination of antennal scales according to the method of Drach and Tchernigovtzeff (1967). This was to minimize variations, because changes in physiological parameters are generally observed during the molting cycle in crustaceans.

The sampling was performed before and after 6 hours of stress; ten shrimps per tank were caught and put directly in iced seawater (0°C). Because of their small size, only digestive gland tissues were sampled.

The digestive glands of 10 intermolt shrimps per tank (before and after H_2O_2 stress) were removed. The tissues were immediately pooled in RNA Later (Sigma®), refrigerated at 4°C for 12 hours and kept at − 80 °C until analysis.

Antioxidant and AMP gene expression analysis by relative quantitative real-time PCR (q-PCR)

Total RNA from the digestive glands was extracted using the Trizol method (Invitrogen, USA) according to the manufacturer's instructions. The quantity and quality of each RNA were assessed by measuring their absorbance at 260 and 280 nm using a Nanodrop 1000 Spectrophotometer (Thermo Scientific) associated with ND-1000 V3 7.0 software. A DNAse treatment to remove residual DNA was carried out using the Ambion DNAse free kit, following the manufacturer's instructions.

First-strand cDNA was synthesized with 500 ng of total RNA in each reaction system using the Roche© transcriptor first strand cDNA synthesis system according to the manufacturer's protocol. All cDNAs were diluted 1/100 with nuclease-free water and stored at -20 °C until used as templates in real-time quantitative PCR (qRT-PCR).

Specific primers for Catalase (CAT), Glutathione peroxidase (GPX), Super oxide dismutase (SOD), and glutathione transferase (GSHT) were obtained by alignment of the most conserved regions from those sequences registered in Genbank. Primers for AMP genes, lysozyme (Lyso), Peneaidin 2 and 3 (Pen 2, Pen 3) and Crustin (Cru) were obtained from De Lorgeril *et al.* (2008). The primer sequences are shown in table 1.

Real-time qRT-PCR was carried out in a Stratagene Mx3000P machine (Agilent Technologies) using Brilliant® II SYBR® Green QPCR Master Mix following the manufacturer's recommendations. The reactions were mixed in a volume of 25 µL containing 12.5µL SYBR Premix, 10 µL cDNA (diluted 1/100), and 1.25 µL each of the 4 µM forward and reverse primers. After initial denaturation at 95°C for 10 min, 40 cycles of amplification were carried out starting at 95 °C for 30 s, followed by 45 s at 57 °C and 45 s at 72 °C, with a final extension at 95°C for 1 min, 30 sec at 55°C and at 95°C for 30 sec followed by a final cycle for differentiation curve analysis.

To determine the RT-PCR efficiencies of each primer pair used, standard curves were generated using five serial dilutions (one log of dilution) of a pool of one hundred cDNA samples from the hepatopancreas. The primers' ability was validated when the amplification efficiency varied between 90 and 110%.

Then, all samples collected during the experiment were run in duplicate. Relative gene expression levels were normalized against two specific house-keeping genes, Elongation factor (EF) and glyceraldehyde-3-phosphate-deshydrogenase (GADPH), and each value was calculated in reference to CW shrimps before stress (relative expression = 1) according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis of the data was carried out using XLSTAT software 2012. Percent data (survival rate) were normalized using an arcsine transformation before analysis. The normality of the data distribution and homogeneity of variance were tested for all zootechnical and molecular analysis data using the Shapiro-Wilk test and F-test, respectively. The zootechnical data were normally distributed and variances were homogenous. Hence, the effects of the rearing treatments were tested using a one-way analysis of variance. The molecular results were not normally distributed; the effects of the rearing treatments were tested using the Kruskal-Wallis test.

Results

Survival and growth

After 30 days of rearing, BFT shrimp presented a significantly higher survival rate compared to CW shrimp (99.30 ± 1.30 % *vs.* 42.17 ± 15.35 %, $p<0.01$). The mean body weight of the

184 BFT shrimp at the end of rearing (0.88 ± 0.19 g) was significantly higher than in the CW
185 treatment (0.21 ± 0.04 g) (p=0.001).

186 **Expression profiling of antioxidant enzyme genes**

187 For all genes studied, the rearing period did not induce a difference of expression between the
188 two conditions. However, H_2O_2 stress did cause different patterns of expression between the
189 two conditions (Figure 1). As a reminder, relative gene expression levels were normalized
190 with two specific housekeeping genes, Elongation factor (EF) and glyceraldehyde-3-
191 phosphate-dehydrogenase (GADPH), and each value was calculated in reference to CW
192 shrimps before stress. SOD and GSHT for CW shrimps showed a significantly decreased
193 relative transcript abundance 6 hours after stress, 0.39 ± 0.03 and 0.31 ± 0.12, respectively
194 (p=0.03 for both genes), while no significant change was observed for BFT shrimps (0.84 ±
195 0.26 and 0.97 ± 0.65, p>0.05). The difference between BFT and CW shrimps after stress was
196 significantly different for SOD and GSHT (p< 0.01 and p=0.04, respectively). Nevertheless,
197 no effect of stress was observed on GPX expression for animals under either condition. CAT
198 showed a distinct pattern compared to other antioxidant enzymes. BFT shrimps displayed a
199 significant increase in relative RNA abundance after stress (p=0.04) compared to CW
200 shrimps, in which the relative abundance of CAT RNA did not change (2.39 ± 1.49 *vs.* 0.76 ±
201 0.28, p=0.02).

202 **Expression profiling of antimicrobial peptide genes**

203 As for antioxidant enzyme genes, for all AMPs genes studied, the rearing period did not
204 induce differences in expression between the two conditions. However, H_2O_2 stress caused
205 different patterns of expression under both conditions (Figure 2). For the penaeidin family, the
206 relative transcript abundance of Pen 2 and Pen 3 decreased significantly after stress in CW
207 shrimps (p=0.03 for both genes). On the contrary, in the BFT shrimps, no change in Pen 2
208 and Pen 3 transcript abundance was detected after stress compared to before stress (p>0.05).
209 However, significant differences were observed between rearing conditions after stress;
210 respectively, for CW and BFT, 0.15 ±0.08 *vs.* 0.94 ± 0.40 (p=0.03) for Pen 2 and 0.14 ± 0.06
211 *vs.* 0.88 ± 0.53 (p=0.02) for Pen 3. Similar to the penaeidin family, another AMP (Cru)
212 transcript showed a significant decrease in RNA abundance 6 hours after stress in CW
213 shrimps, (0.12 ± 0.04, p=0.03), but not in BFT shrimps (0.41 ± 0.23, p> 0.05). The difference

between BFT and CW shrimps was significant (p=0.03). No effect of induced stress was observed on lysozyme expression in animals from the two conditions.

Discussion

At the present time, when shrimp production faces many losses due to disease outbreaks, the use of BFT can restrain the development of shrimp diseases. It has been hypothesized that the biofloc allowed the shrimp to better withstand environmental stress or infection by pathogenic bacteria or viruses by stimulating non-specific immunity. Recently, several authors determined that BFT stimulated the non-specific immunity of shrimp (Kim *et al.*, 2013; Xu and Pan, 2013, 2014; Ekasari *et al.*, 2014) and one recent work showed that BFT improved shrimp resistance to infectious myonecrosis virus infection (Ekasari *et al.*, 2014). In this research, the antioxidant and antimicrobial transcriptional responses before and after experimental sub-lethal hydrogen peroxide stress in blue shrimps reared according to two different systems, BFT and CW, were compared in order to improve our understanding of BFT's action on antioxidant defences and the humeral immune system of shrimps at the transcriptional level. We undertook, for the first time, to monitor AOE and AMP gene expression in shrimps under different rearing conditions, BFT and CW, and after induced stress.

Biofloc rearing conditions improves shrimp growth and survival

Our results showed that the BFT system promotes better growth (x 4.2) and survival (x 0.4) of *Litopenaeus stylirostris* juvenile shrimps compared to the CW system. Such results have already been described (Moss and Pruder, 1995; Cohen *et al.*, 2005; Azim and Little, 2008; Mishra *et al.*, 2008). According to these authors, the improved performances can be related to the consumption of biofloc by the shrimp, as a source of bacteria, microalgae and zooplankton, which could enhance shrimp nutrition and immunity. In the biofloc system, its natural productivity plays an important and complementary nutritional role for the shrimp in addition to the artificial pellets (Moss and Pruder 1995; Epp *et al.*, 2002; Tacon *et al.*, 2002; Burford *et al.*, 2004). Biofloc is known to be an important source of proteins (Wasielesky *et al.*, 2006) and also of lipids (Crab *et al.*, 2010). Moreover, biofloc can contain microbially bioactive components such as carotenoids, vitamins (Ju *et al.*, 2008) and glutathione (Cardona *et al.*, in prep.). Major nutrients like proteins, lipids, antioxidant and vitamins, carotenoids and

minerals are known to participate, in different ways, in nutritional modulation of immune responses (Trichet, 2010).

Shrimp AOE and AMP gene expression are not modified by rearing conditions

However, in the absence of any particular stressful conditions, we showed that the transcriptional responses of AOE and AMP genes of shrimp were not different between the different rearing conditions. With the significantly different growth and survival results obtained, we could expect a differential response between animals from the two conditions, as shown by others authors. Indeed, Kim *et al.* (2013) showed that biofloc rearing improved immune-related gene expression in *L.vannamei* post-larvae. Nevertheless, these authors used different biomarkers of shrimp immunity than those used in this study. Indeed, the genes targeted were involved in prophenoloxidase (ProPo) cascade activation (6 studied genes: prophenoloxidase 1, prophenoloxidase 2, prophenoloxidase activating enzyme, serine protease, and masquerade-like serine protease). The ProPo cascade can be activated by components of the cell wall like β-1,3-glucan, lipopolysaccharide, and peptidoglycan, elicitors found abundantly on biofloc particles (Johansson & Söderhäll, 1985; Van de Braak *et al.*, 2002; Amparyup *et al.*, 2013). Indeed, bacteria were one of the principal constituents identified in biofloc particles, with a high concentration ranging from 10^6 to 10^9 cell.mL^{-1} (Otoshi *et al.*, 2006; Burford *et al.*, 2003; Avnimelech, 2009; Kim *et al.*, 2013).

Moreover, in addition to activating the ProPo cascade, earlier studies have revealed that biofloc led to an increase in both total haemocyte count and the phagocytic response in shrimp hemolymph, whereas respiratory burst, antibacterial and bacteriolytic activities were not affected (Xu and Pan, 2013; Ekasari *et al.*, 2014). The presence and digestion of the biofloc ingested by the shrimp may release substances in the gastrointestinal tract that could potentially stimulate cellular defences (phagocytosis and the proPo cascade) and the release of more haemocytes into the circulation without a noticeable effect on humeral defence factor (such as AMPs and lysozyme) production (Xu and Pan, 2013). Our results are in accordance with these assertions and confirm that at the gene expression level there is no effect of the BFT condition by comparison to CW.

Differential AOE and AMP responses induced by H_2O_2 stress between shrimps from BFT and CW

After a H_2O_2 stress treatment, a strong effect of the rearing condition was noticed in AOE and AMP gene expression levels. The antioxidant defence system is inducible at a moderately high concentration of H_2O_2, which is hypothesized to also act as a messenger of signal transduction by regulating the mRNA level through activation of signal pathways (Ji, 1995; Sen and Packer, 1996). In this study, the expression of CAT was significantly increased in the digestive gland after 6 hours under H_2O_2 exposure for animals reared in BFT, while no change in RNA transcript abundance was recorded for CW shrimps. This result suggested that H_2O_2 stimulated the expression of CAT in BFT animals. AOEs' function is to catalyse the conversion of H_2O_2 into water and molecular O_2 to protect the organism from peroxidation (Zhang *et al.*, 2008). This result seems to show that excess H_2O_2 that penetrates cells and tissues would be more easily detoxified and neutralized in the cytosol by catalase in BFT shrimp than in CW shrimp. The increase in CAT RNA expression in BFT shrimp could correspond to an adaptive antioxidant stress response (Bigot *et al.*, 2010). In contrast, the absence of a response in the CW shrimps could reflect an inappropriate response of the organism to neutralize peroxides and their potential involvement in oxidative cellular damage (Meng *et al.*, 2014). GPX also catalyses the conversion of H_2O_2 into H_2O and O_2. The absence of significant differential expression before and after 6 hours under stress could be explained by it being too short a time for the animals to react or to the paucity of biological replicates (n=4) to draw out significant differences. Indeed, Fu *et al.* (2012) showed that GPX mRNA transcription increased significantly between 8 h and 12 h after H_2O_2 injection. Stress induced significant down-regulation of SOD and GSHT in CW animals only. Similar to our findings on CAT genes, this could be explained by a diminished capacity for the CW organism to protect itself against oxidative stress (Bigot *et al.*, 2010) and the degradation of cells by H_2O_2. All together, the difference in response between animals from the two rearing conditions suggests that CW shrimp did not seem able to abolish cytotoxic effects due to H_2O_2-induced oxidative stress, while BFT shrimp seemed to present an adaptive antioxidant stress response. For information, we had measured SOD and CAT enzymatic activities. However, the observed standard deviations of the results were very important. Thus, it was very difficult to define correlations with the molecular data. These observations could be explained by the fact that it is not so easy to measure and interpret enzymatic activities on crude extract. Many activity inhibitors could be present in the extract. Therefore, we have decided not to show these biochemical results.

In addition to AOE production, haemocytes are able to synthesize soluble antimicrobial peptides (AMPs). Measuring humeral immunity offers a further insight into the impact of stressors on haemocyte functionality, associated with an organism's immunocompetence (Ellis *et al.*, 2011). This study shows differences in AMP gene expression levels according to the rearing conditions of *L. stylirostris* facing a H_2O_2 stress. The transcript levels of three out of four AMP genes studied (Pen 2 and 3, Crustin) appeared to be significantly modified after stress in CW shrimps only. The reported modulations in mRNA quantities were distributed in circulating haemocytes present in the digestive gland, since in invertebrates AMPs are produced mostly by haemocytes (Bachère *et al.*, 2004). In our experiment, no cell counts were performed before RNA extraction. Total RNA quantities were adjusted before reverse transcription and cDNA concentrations were adjusted another time in order to use identical template quantities in qPCR. Meanwhile, we have no data on the quantitative and qualitative composition of the various haemocytes sampled. The decreases in the AMP transcripts of CW shrimps could be explained by two phenomena: (i) a decrease in the relative number of haemocytes expressing the considered mRNA (Cellura *et al.*, 2007) induced by H_2O_2 cytotoxicity and (ii) a migration of haemocytes from the digestive gland towards exposed tissue to H_2O_2 as is often observed after infection by a pathogen or by injury (Bachère *et al.*, 2004). However, the observed differences in AMP transcript level could be compared to the data observed for shrimp survival under the BFT and CW conditions. We can suppose that the differential AMP transcriptional response after stress could be related to an improved immune ability of BFT animals facing a H_2O_2 stress. Nevertheless, further study is needed to confirm this assumption.

Conclusion

The rearing condition, BFT or CW, did not affect gene expression encoding antioxidant enzymes and antimicrobial peptides, although BFT significantly improved the growth and survival of animals. However, H_2O_2 stress induced a differential response in AOEs and AMPs between shrimp from BFT and CW. Biofloc shrimps seem to maintain their antioxidant and antimicrobial peptide responses after H_2O_2 stress, while clear water shrimp immune competence seemed to decrease after stress.

Acknowledgements

This study was sponsored by the Government and Southern, Northern and Island Provinces of New-Caledonia and the management of marine resources and mining of Tahiti. We would like to thank the Technical Centre for Aquaculture of Tahiti for their help during the running of the experiment, Julie Fievet for help during the sampling and Julien De Lorgeril for the discussions on shrimp antimicrobial peptides.

References

Abele-Oeschger, D., Tüg, H., Roettgers, R., 1997. Dynamics of UV-driven hydrogen peroxide formation on an intertidal sandflat. Limnology and oceanography, 42(6), 1406-1415.

Abele, D., Burlando, B., Viarengo, A., Pörtner, H.O., 1998. Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 120(2), 425-435.

Abele, D., Puntarulo, S., 2004. Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 138(4), 405-415.

Amparyup, P., Charoensapsri, W., & Tassanakajon, A., 2013. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish & shellfish immunology, 34(4), 990-1001.

Avnimelech, Y., 2009. Biofloc Technology - A practical guide Book. Baton Rouge, Louisiana, United States, The World Aquaculture Society.

Azim M.E., Little D.C., Bron J.E., 2008. Microbial protein production in activated suspension tanks manipulating C:N ratio in feed and the implications for fish culture. Bioresource Technology, 99 (2008), pp 3590–3599.

Bachère, E., Gueguen, Y., Gonzalez, M., De Lorgeril, J., Garnier, J., Romestand, B., 2004. Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. Immunological reviews, 198(1), 149-168.

Bartlett, T.C., Cuthbertson, B.J., Shepard, E.F., Chapman, R.W., Gross, P.S., Warr, G.W., 2002. Crustins, homologues of an 11.5-kDa antibacterial peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*. Journal of Marine Biotechnology (NY) 4 (3): 278-293.

Becerra-Dorame, J.M., Martinez-Cordova, L. R., Martínez-Porchas, M., Hernández-López, J., López–Elías, J. A., & Mendoza–Cano, F., 2012. Effect of using autotrophic and

heterotrophic microbial-based-systems for the pre-grown of *Litopenaeus vannamei*, on the production performance and selected haemolymph parameters. Aquaculture Research.

Bigot, A., Vasseur, P., Rodius, F., 2010. SOD and CAT cDNA cloning, and expression pattern of detoxification genes in the freshwater bivalve *Unio tumidus* transplanted into the Moselle river. Ecotoxicology, 19(2), 369-376.

Burford M.A., Thompson P.J., Bauman H., Pearson D.C., 2003. Microbial communities affect water quality, shrimp performance at Belize Aquaculture. Global Aquaculture Advocate 6, 64–65.

Burford, M.A., Thompson, P.J., McIntosh, P.R., Bauman, R.H., Pearson, D.C., 2004. The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero exchange system. Aquaculture 232, 525–537.

Cellura, C., Toubiana, M., Parrinello, N., Roch, P., 2007. Specific expression of antimicrobial peptide and *HSP70* genes in response to heat-shock and several bacterial challenges in mussels. Fish & shellfish immunology, 22(4), 340-350.

Chandra, J., Samali, A., Orrenius, S., 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radical Biology and Medicine, 29(3), 323-333.

Charoensapsri, W., Amparyup, P., Hirono, I., Aoki, T., and Tassanakajon, A., 2011. Pm PPAE2, a new class of crustacean prophenoloxidase (proPO)-activating enzyme and its role in PO activation. Developmental & Comparative Immunology, Vol. 35, No. 1, pp. 115-124.

Chen, M.Y., Hu, K.Y., Huang, C.C., Song, Y.L., 2005. More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation. Developmental & Comparative Immunology, Vol. 29, No. 12, pp. 1003-1016.

Cohen, J.M., Samocha, T.M., Fox, J.M., Gandy, R.L., Lawrence, A.L., 2005. Characterization of water quality factors during intensive raceway production of juvenile *Litopenaeus vannamei* using limited discharge and biosecure management tools. Aquacultural engineering, 32(3), 425-442.

Crab, R., Chielens, B., Wille, M., Bossier, P., Verstraete, W., 2010. The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae. Aquaculture Research, 41(4), 559-567.

De la Vega, E., O'Leary, N. A., Shockey, J. E., Robalino, J., Payne, C., Browdy, C. L., ... & Gross, P. S., 2008. Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (*Lv*ALF):

A broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Molecular immunology, 45(7), 1916-1925.

De Lorgeril, J., Saulnier, D., Janech, M. G., Gueguen, Y., & Bachere, E. (2005). Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with Vibrio penaeicida. Physiological genomics, 21(2), 174-183.

De Lorgeril, J., Gueguen, Y., Goarant, C., Goyard, E., Mugnier, C., Fievet, J., ... & Bachère, E., 2008. A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a *Vibrio* infection. Molecular immunology, 45(12), 3438-3445.

Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J., & Bachère, E., 1997. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). Journal of Biological Chemistry, 272(45), 28398-28406.

De Zoysa, M., Pushpamali, W.A., Oh, C., Whang, I., Kim, S. J., Lee, J., 2008. Transcriptional up-regulation of disk abalone selenium dependent glutathione peroxidase by H_2O_2 oxidative stress and *Vibrio alginolyticus* bacterial infection. Fish & shellfish immunology, 25(4), 446-457.

De Zoysa, M., Whang, I., Lee, Y., Lee, S., Lee, J. S., Lee, J., 2009. Transcriptional analysis of antioxidant and immune defense genes in disk abalone (*Haliotis discus discus*) during thermal, low-salinity and hypoxic stress. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 154(4), 387-395.

Drach, P., Tchernifovtzeff, C., 1967. Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. Vie Milieu 18, 596-609.

Ellis, R.P., Parry, H., Spicer, J.I., Hutchinson, T.H., Pipe, R.K., Widdicombe, S., 2011. Immunological function in marine invertebrates: responses to environmental perturbation. Fish & shellfish immunology, 30(6), 1209-1222.

Ekasari, J., Azhar, M. H., Surawidjaja, E. H., Nuryati, S., De Schryver, P., & Bossier, P., 2014. Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources. Fish & shellfish immunology.

Epp, M.A., Ziemann, D.A., Schell, D.M., 2002. Carbon and nitrogen dynamics in zero-water exchange shrimp culture as indicated by stable isotope tracers. Aquaculture Research, 33(11), 839-846.

436 Fu, M., Zou, Z., Liu, S., Lin, P., Wang, Y., Zhang, Z., 2012. Selenium-dependent glutathione
437 peroxidase gene expression during gonad development and its response to LPS and
438 H_2O_2 challenge in *Scylla paramamosain*. Fish & shellfish immunology, 33(3), 532-
439 542.
440 Galloway, T.S., Depledge, M.H., 2001. Immunotoxicity in invertebrates: measurement and
441 ecotoxicological relevance. Ecotoxicology, 10(1), 5-23.
442 Hikima, S., Hikima, J. I., Rojtinnakorn, J., Hirono, I., Aoki, T., 2003. Characterization and
443 function of kuruma shrimp lysozyme possessing lytic activity against *Vibrio* species.
444 Gene, 316, 187-195.
445 Holmblad, T., Söderhäll, K., 1999. Cell adhesion molecules and antioxidative enzymes in a
446 crustacean, possible role in immunity. Aquaculture, 172(1), 111-123.
447 Ji, L.L., 1995. Exercise and free radical generation. Role of cellular antioxidant systems.
448 Holoszy J. Exercise and Sports Science Review, Williams and Wilkins Co, Baltimore,
449 Maryland, pp. 135–166.
450 Johansson, M. W., Söderhäll, K., 1985. Exocytosis of the prophenoloxidase activating system
451 from crayfish haemocytes. Journal of Comparative Physiology B, 156(2), 175-181.
452 Ju Z., Forster I., Conquest L., Dominy W. 2008. Enhanced growth effects on shrimp
453 (*Litopenaeus vannamei*) from inclusion of whole shrimp floc or floc fractions to a
454 formulated diet. Aquaculture Nutrition 14.
455 Kim, S. K., Pang, Z., Seo, H. C., Cho, Y. R., Samocha, T., Jang, I. K., 2013. Effect of bioflocs
456 on growth and immune activity of Pacific white shrimp, *Litopenaeus vannamei*
457 postlarvae. Aquaculture Research, 45(2), 362-371.
458 Lee, K. Y., Zhang, R., Kim, M. S., Park, J. W., Park, H. Y., Kawabata, S.-i. and Lee, B. L.,
459 2002. A zymogen form of masquerade-like serine proteinase homologue is cleaved
460 during pro-phenoloxidase activation by Ca2+ in coleopteran and *Tenebrio molitor*
461 larvae. European Journal of Biochemistry, 269: 4375–4383. doi: 10.1046/j.1432-
462 1033.2002.03155.x
463 Le Moullac, G., Haffner, P., 2000. Environmental factors affecting immune responses in
464 Crustacea. Aquaculture, 191(1), 121-131.
465 Li, H., Parisi, M.G., Toubiana, M., Cammarata, M., Roch, P., 2008. Lysozyme gene
466 expression and hemocyte behaviour in the Mediterranean mussel, *Mytilus*
467 *galloprovincialis*, after injection of various bacteria or temperature stresses. Fish &
468 shellfish immunology, 25(1), 143-152.

Li, H., Toubiana, M., Monfort, P., Roch, P., 2009. Influence of temperature, salinity and *E. coli* tissue content on immune gene expression in mussel: Results from a 2005–2008 survey. Developmental & Comparative Immunology, 33(9), 974-979.

Liu, K. F., Chiu, C. H., Shiu, Y. L., Cheng, W., Liu, C. H., 2010. Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish & shellfish immunology, 28(5), 837-844.

Livak, K.J., Schmittgen, T. D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2ΔΔCTMethod. Methods, 25(4), 402-408.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656-666.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology, 101(1), 13-30.

Massafra, C., Gioia, D., De Felice, C., Picciolini, E., De Leo, V., Bonifazi, M., Bernabei, A., 2000. Effects of estrogens and androgens on erythrocyte antioxidant superoxide dismutase, catalase and glutathione peroxidase activities during the menstrual cycle. Journal of endocrinology, 167(3), 447-452.

Meng, X.L., Liu, P., Li, J., Gao, B.Q., Chen, P., 2014. Physiological responses of swimming crab *Portunus trituberculatus* under cold acclimation: antioxidant defense and heat shock proteins. Aquaculture.

Mishra J.K., Samocha, T.M., Patnaik, S., Speed, M., Gandy, R.L., Ali, A., 2008. Performance of an intensive nursery system for the Pacific white shrimp, *Litopenaeus vannamei*, under limited discharge condition. Aquacultural Engineering38, 2–15.

Mitta, G., Hubert, F., Dyrynda, E.A., Boudry, P., Roch, P., 2000. Mytilin B and MGD2, two antimicrobial peptides of marine mussels: gene structure and expression analysis. Developmental & Comparative Immunology, 24(4), 381-393.

Moss, S.M., Pruder, G.D., 1995. Characterization of organic particles associated with rapid growth in juvenile white shrimp, *Penaeus vannamei* Boone, reared under intensive culture conditions. Journal of Experimental Marine Biology and Ecology, 187(2), 175-191.

Muñoz, M., Cedeño, R., Rodríguez, J., Van Der Knaap, W. P., Mialhe, E., Bachère, E., 2000. Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. Aquaculture, 191(1), 89-107.0

Muñoz, M., Vandenbulcke, F., Saulnier, D., Bachère, E., 2002. Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. European Journal of Biochemistry, *269*(11), 2678-2689.

Omori, S.A., Martin, G.G., Hose, J.E., 1989. Morphology of hemocyte lysis and clotting in the ridgeback prawn, *Sicyonia ingentis*. Cell and Tissue Research, Vol. 255, No. 1, pp. 117-123.

Otoshi, C. A., Holl, C. M., Moss, D. R., Arce, S. M., Moss, S. M., 2006. Super-intensive RAS trial yields encouraging shrimp harvest at Oceanic Institute. Global Aquaculture Advocate, 9(4), 64-65.

Patil, P.K., Bhuvaneswari, T., Ezhil Praveena, P., Otta, S.K., 2014. Probiotics and immunostimulants in shrimp aquaculture. Pins Aquaculture- .training Manual on, 81.

Pallavi, P. N., Babu, K. N., Reddy, D. C., Kalarani, V., 2012. Antioxidant defenses and oxidative stress parameters in tissues of Penaeus monodon acclimated to different salinities. World, 4(5), 539-549.

Qiu, J., Wang, W.N., Wang, L.J., Liu Y.F., Wang A.L., 2011. Oxidative stress, DNA damage and osmolality in the Pacific white shrimp, Litopenaeus vannamei exposed to acute low temperature stress, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, Volume 154, Issue 1, June 2011, Pages 36-41, ISSN 1532-0456, http://dx.doi.org/10.1016/j.cbpc.2011.02.007.

Rauen, U., Petrat, F., Li, T., De Groot, H., 2000. Hypothermia injury/cold-induced apoptosis—evidence of an increase in chelatable iron causing oxidative injury in spite of low O2−/H2O2 formation. The FASEB Journal, 14(13), 1953-1964.

Sen, C. K., Packer, L., 1996. Antioxidant and redox regulation of gene transcription. The FASEB journal, 10(7), 709-720.

Song, Y.L., Hsieh, Y.T., 1994. Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: Analysis of reactive oxygen species. Developmental & Comparative Immunology, 18(3), 201-209.

Song, Y.L., Li, C.Y., 2014. Shrimp immune system-special focus on penaeidin. Journal of Marine Science and Technology, 22(1), 1-8.

Sotelo-Mundo, R. R., Islas-Osuna, M. A., de-la-Re-Vega, E., Hernández-López, J., Vargas-Albores, F., Yepiz-Plascencia, G., 2003. cDNA cloning of the lysozyme of the white shrimp *Penaeus vannamei*. Fish & shellfish immunology, 15(4), 325-331.

Sritunyalucksana, K., Söderhäll, K., 2000. The proPO and clotting system in crustaceans. Aquaculture, 191(1), 53-69.

Trichet, V.V., 2010. Nutrition and immunity: an update. Aquaculture Research, 41(3), 356-372.

Van de Braak, C. B. T., Botterblom, M. H. A., Taverne, N., Van Muiswinkel, W. B., Rombout, J. H. W. M., Van der Knaap, W. P. W., 2002. The roles of haemocytes and the lymphoid organ in the clearance of injected *Vibrio* bacteria in *Penaeus monodon* shrimp. Fish & shellfish immunology, 13(4), 293-309.

Victor, B., Narayanan, M., Jones Nelson, D., 1990. Gill pathology and hemocyte response in mercury exposed *Macrobrachium idea* (Heller). Journal of Environmental Biology, 11(1), 61-65.

Tacon, A.G.J., Cody, J., Conquest, L., Divakaran, S., Forster, I.P., Decamp, O., 2002. Effect of culture system on the nutrition and growth performance of Pacific white shrimp *Litopenaeus vannamei* (Boone) fed different diets. Aquaculture Nutrition 8, 121–137.

Taw, 2013. Shrimp farming : biofloc as biosecurity?. Book of abstract of Biofloc technology and shrimp disease workshop. World Aquaculture society HoChiMinh.

Wang, Y. C., Chang, P. S., Chen, H. Y., 2007. Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*. Fish & shellfish immunology, 23(6), 1161-1177.

Wang, W.N., Zhou, J., Wang, P., Tian, T.T., Zheng, Y., Liu, Y., Wei.Jun, M., Wang, A.L., 2009. Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 150(4), 428-435.

Wang, D., Li, F., Chi, Y., Xiang, J., 2012. Potential relationship among three antioxidant enzymes in eliminating hydrogen peroxide in penaeid shrimp. Cell Stress and Chaperones, 17(4), 423-433.

Wang, X. W., & Wang, J. X., 2013. Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections. Fish & shellfish immunology, 34(4), 981-989.

Wasielesky, W., Atwood, H., Stokes, A. Browdy, C.L., 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp *Litopenaeus vannamei*. Aquaculture 258, 396–403.

Xu, W.J., Pan, L.Q., 2013. Enhancement of immune response and antioxidant status of *Litopenaeus vannamei* juvenile in biofloc-based culture tanks manipulating high C/N ratio of feed input. Aquaculture, 412, 117-124.

570 Xu, W.J., Pan, L.Q., 2014. Evaluation of dietary protein level on selected parameters of
571 immune and antioxidant systems, and growth performance of juvenile *Litopenaeus*
572 *vannamei* reared in zero-water exchange biofloc-based culture tanks. Aquaculture, 426,
573 181-188.
574 Yeh, M. S., Chen, Y. L., Tsai, I. H., 1998. The hemolymph clottable proteins of tiger shrimp,
575 *Penaeus monodon*, and related species. Comparative Biochemistry and Physiology Part
576 B: Biochemistry and Molecular Biology, Vol. 121, No. 2, pp. 169-176.
577 Zhang, Q., Li, F., Zhang, X., Dong, B., Zhang, J., Xie, Y., Xiang, J., 2008. cDNA cloning,
578 characterization and expression analysis of the antioxidant enzyme gene, catalase, of
579 Chinese shrimp *Fenneropenaeus chinensis*. Fish & shellfish immunology, 24(5), 584-
580 591.
581
582

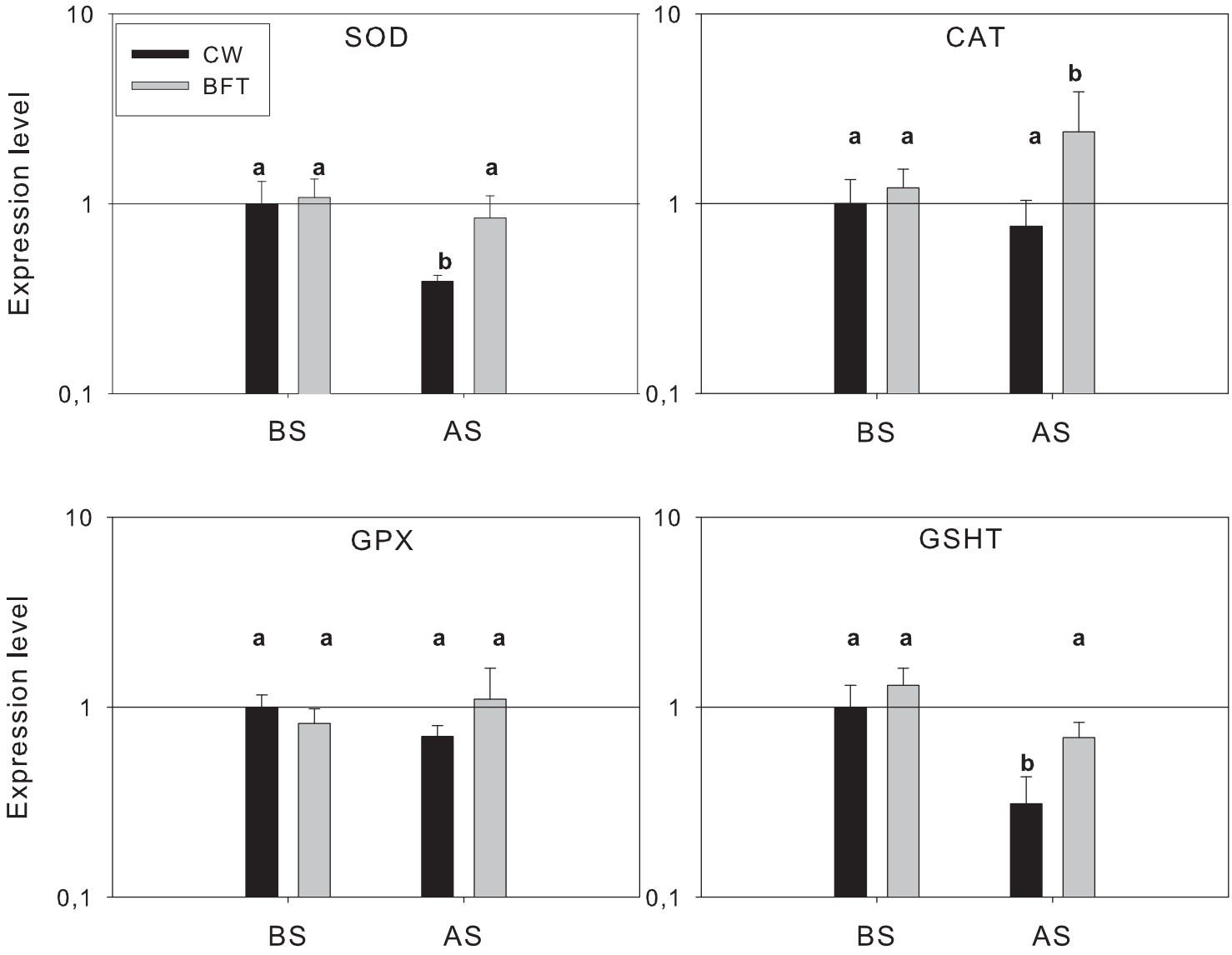
Tables and captions

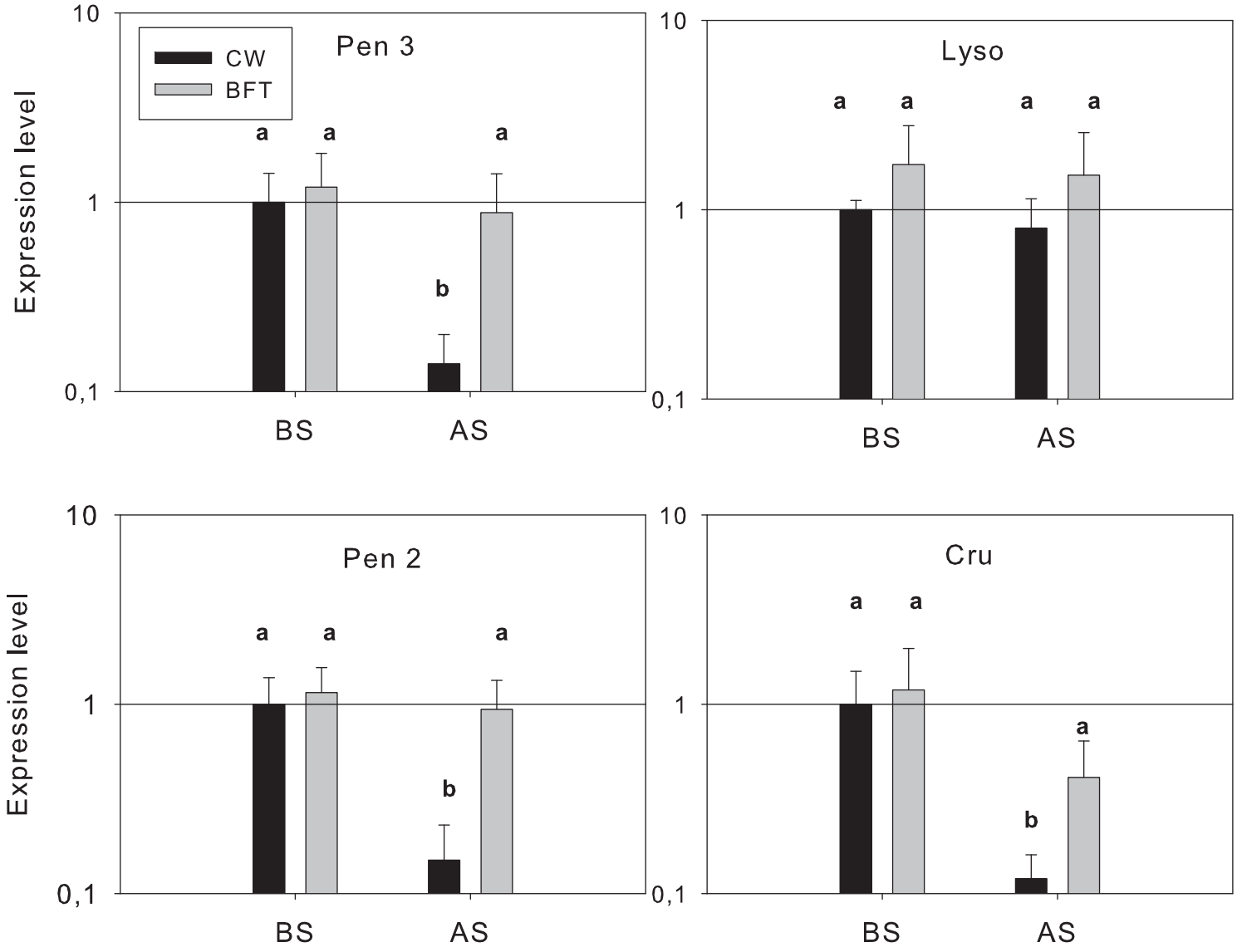
Table 1. PCR primers (F: Forward, R: Reverse) used to amplify antimicrobial peptides (Pen3, Pen2, Lyso, Cru), antioxidant enzymes (GPX, SOD, GSHT, CAT) and house-keeping genes (GADPH, EF) of the shrimp Litopenaeus stylirostris in a real-time PCR procedure.

Figure 1. Expression profiles of genes coding for the antioxidant enzymes Super oxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX) and Glutathione transferase (GSHT) in animals under both conditions both before (BS) and after stress (AS).

Figure 2. Expression profiles of genes coding for AMPs, Lysozyme (Lyso), Peneaidin 2 and 3 (Pen 2 and Pen 3) and Crustin (Cru) in animals from both conditions both before (BS) and after stress (AS).

Name	Gene name	Sequence 5' - 3'	Size	Primer size	Tm	GeneBank
Pen 2 - F	Peneaidin 2	GTCTGCCTGGTCTTCTTGG	178pb	19	60	AY351655
Pen 2 - R	Peneaidin 2	CGAACCTGCTGCAGCAATTG		20	62	
Pen 3 - F	Peneaidin 3	CCATGCGCCTCGTGGTCTG	211pb	19	64	AY351656
Pen 3 - R	Peneaidin 3	GAACGCGCTTGTAAGGTGGTAA		22	64	
Lyso - F	Lysozyme	GGCTTGGCACCAGGGTTACC		20	59	CV699332
Lyso - R	Lysozyme	CGTCTGCACGTCAGCTGTG		20	59	
Cru - R	Crustin	GTGATTCTGTGCGGCCTCTT	395pb	30	63	
Cru - F	Crustin	TCTTGCACCAATACCTGCAG		30	60	
GPX - F	Glutathione peroxidase	TCAACAGCTGATCCCGTCT	157pb	19	59	
GPX -R	Glutathione peroxidase	CCTTGCCGATGAGGAATTT		19	60	
SOD - F	Super oxide dismutase	GCAATGAATGCCCTTCTACC	247pb	20	60	
SOD - R	Super oxide dismutase	CAGAGCCTTTCACTCCAACG		21	60	
GSHT - F	Glutathione transferase	CTGGAGAAGCTGCACGAAG	198pb	19	60	
GSHT - R	Glutathione transferase	GTCACGTTCCTGTGCTTGC		19	60	
CAT - F	Catalase	TACTGCAAGTTCCATTACAAGACG	285pb	24	61	
CAT - R	Catalase	GTAATTCTTTGGATTGCGGTCA		22	61	
EF - F	Elongation factor 1α	CGTTCCGGTGATCATGTTCTTGATG	382pb	35	60	AY117542
EF - R	Elongation factor 1α	GGTGCTGGACAAGCTGAAGGC		31	60	
GADPH - F	Glyceraldehyde-3-phosphate-deshydrogenase	CGTTGGACACCACCTTCA	146pb	18	59	AI770197
GADPH - R	Glyceraldehyde-3-phosphate-deshydrogenase	GTGTGCGGTGTCAACATGGA		30	55	


SOD
CAT
GPX
GSHT
CW
BFT
Expression level
10
1
0,1
a
b
BS
AS


Expression level
10
1
0,1
CW
BFT
Pen 3
a
a
a
b
BS
AS
Lyso
a
a
a
a
Pen 2
Cru

Highlights

- Contrasted rearing conditions, biofloc and clear water, did not affect the expression of genes encoding antioxidant enzymes and antimicrobial peptides.
- Biofloc shrimps maintained antioxidant and antimicrobial peptide responses after H_2O_2 stress while the immune competence of clear water shrimp seemed to decrease after stress.
- Biofloc seems to improve the immune resistance of animals against stress.