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Full length article

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



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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 h. 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 AOE 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.

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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 [5]. 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 [67]. Another explanation is that the microbial proteins assimilated by shrimp are believed to confer beneficial effects on well-being and shrimp immune status [5]. Nevertheless, the influence of biofloc on shrimp immune status has been poorly documented [27,36,73,74].

Because of their lack of acquired immunity, marine invertebrates' defence against invading pathogens relies solely on innate immune mechanisms [48]. In shrimp these include: 1) pattern recognition receptors (PRR) [71]; 2) humeral responses characterized by the expression of antimicrobial peptides (AMPs) [22], but also coagulation and melanisation by so-called clotting [16,52,75] and prophenoloxidase activating systems [15,37,62]; 3) cellular responses, mainly performed by haemocytes, such as phagocytosis [59], chemotaxis with haemocyte migration into inflammatory foci [51], release of humeral defence components

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(agglutinins, coagulation and phenoloxidase enzymes, antimicrobial peptides), encapsulation and nodule formation [32].

Animal stress induced by sub-lethal H₂O₂ concentration is a method rarely used to induce stress and therefore original. It seemed interesting to study the response of animals to H₂O₂ 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 [1]. 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 [45]. The antioxidant defence and immune systems are closely linked to responses to pathogens and other stress-related issues that might lead to respiratory burst [32]. Measuring AOE expression after environmental stressors such as pH [69], temperature, salinity, hypoxia [24] and H₂O₂ stress [23] 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 [13,40,48]. The major studies on AMPs have focused on their identification, characterization, and regulation following pathogen infection [7]. 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 [26].

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.

2. Materials and method

2.1. 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 m³ 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).

2.2. 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).

2.3. 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 ± 0.02 g) 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.

2.4. H₂O₂ stress, sampling and conservation

To achieve H₂O₂ stress, water renewal was operated in the biofloc tank during 12 h 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 h with a sub-lethal concentration of H₂O₂ (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 [25]. 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 h 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 H₂O₂ stress) were removed. The tissues were immediately pooled in RNA Later (Sigma[®]), refrigerated at 4 °C for 12 h and kept at – 80 °C until analysis.

2.5. 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 Ref. [21]. 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 12.5 µL 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 s at 55 °C and at 95 °C for 30 s

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.

| Name | Gene name | Sequence 5'–3' | Size | Primer size | Tm | GeneBank |
|-----------|--|--------------------------|--------|-------------|----|----------|
| Pen 2 – F | Peneaidin 2 | GTCTGCCTGGTCTTCTGG | 178 pb | 19 | 60 | AY351655 |
| Pen 2 – R | Peneaidin 2 | CGAACCTGCTGCAGCAATTG | | 20 | 62 | |
| Pen 3 – F | Peneaidin 3 | CCATGCGCTCGTGGTCTG | 211 pb | 19 | 64 | AY351656 |
| Pen 3 – R | Peneaidin 3 | GAACGCGCTTGAAGGTGGTAA | | 22 | 64 | |
| Lyso – F | Lysozyme | GGCTTGGACACAGGGTTACC | | 20 | 59 | CV699332 |
| Lyso – R | Lysozyme | CGTCTGCACGTCAGCTGTG | | 20 | 59 | |
| Cru – R | Crustin | GTGATTCTGTGGCGCCTCTT | 395 pb | 30 | 63 | |
| Cru – F | Crustin | TCTTGACCAATACCTGCAG | | 30 | 60 | |
| GPX – F | Glutathione peroxidase | TCAACAGCTGATCCCGCT | 157 pb | 19 | 59 | |
| GPX – R | Glutathione peroxidase | CCTTGCCGATGAGGAATTT | | 19 | 60 | |
| SOD – F | Super oxide dismutase | GCAATGAATGCCCTTCTACC | 247 pb | 20 | 60 | |
| SOD – R | Super oxide dismutase | CAGAGCCTTCACTCCAACG | | 21 | 60 | |
| GSHT – F | Glutathione transferase | CTGGAGAAGCTGCACGAAG | 198 pb | 19 | 60 | |
| GSHT – R | Glutathione transferase | GTCACGTTCTGTGCTTGC | | 19 | 60 | |
| CAT – F | Catalase | TACTGCAAGTTCCATTACAAGACC | 285 pb | 24 | 61 | |
| CAT – R | Catalase | GTAATTCCTTGGATTGCGGTCA | | 22 | 61 | |
| EF – F | Elongation factor 1 α | CGTTCGCGTATCATGTTCTTGATG | 382 pb | 35 | 60 | AY117542 |
| EF – R | Elongation factor 1 α | GGTGCTGGACAAGCTGAAGGC | | 31 | 60 | |
| GADPH – F | Glyceraldehyde-3-phosphate-dehydrogenase | CGTTGACACCACTTCA | 146 pb | 18 | 59 | AI770197 |
| GADPH – R | Glyceraldehyde-3-phosphate-dehydrogenase | GTGTGCGGTCTCAACATGGA | | 30 | 55 | |

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-dehydrogenase (GADPH), and each value was calculated in reference to CW shrimps before stress (relative expression = 1) according to the $2^{-\Delta\Delta Ct}$ method [42].

2.6. 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.

3. Results

3.1. Survival and growth

After 30 days of rearing, BFT shrimp presented a significantly higher survival rate compared to CW shrimp ($99.30 \pm 1.30\%$ vs. $42.17 \pm 15.35\%$, $p < 0.01$). The mean body weight of the BFT shrimp at the end of rearing (0.88 ± 0.19 g) was significantly higher than in the CW treatment (0.21 ± 0.04 g) ($p = 0.001$).

3.2. Expression profiling of antioxidant enzyme genes

For all genes studied, the rearing period did not induce a difference of expression between the two conditions. However, H₂O₂ stress did cause different patterns of expression between the two

conditions (Fig. 1). As a reminder, relative gene expression levels were normalized with two specific housekeeping genes, Elongation factor (EF) and glyceraldehyde-3-phosphate-dehydrogenase (GADPH), and each value was calculated in reference to CW shrimps before stress. SOD and GSHT for CW shrimps showed a significantly decreased relative transcript abundance 6 h after stress, 0.39 ± 0.03 and 0.31 ± 0.12 , respectively ($p = 0.03$ for both genes), while no significant change was observed for BFT shrimps (0.84 ± 0.26 and 0.97 ± 0.65 , $p > 0.05$). The difference between BFT and CW shrimps after stress was significantly different for SOD and GSHT ($p < 0.01$ and $p = 0.04$, respectively). Nevertheless, no effect of stress was observed on GPX expression for animals under either condition. CAT showed a distinct pattern compared to other antioxidant enzymes. BFT shrimps displayed a significant increase in relative RNA abundance after stress ($p = 0.04$) compared to CW shrimps, in which the relative abundance of CAT RNA did not change (2.39 ± 1.49 vs. 0.76 ± 0.28 , $p = 0.02$).

3.3. Expression profiling of antimicrobial peptide genes

As for antioxidant enzyme genes, for all AMPs genes studied, the rearing period did not induce differences in expression between the two conditions. However, H₂O₂ stress caused different patterns of expression under both conditions (Fig. 2). For the penaeidin family, the relative transcript abundance of Pen 2 and Pen 3 decreased significantly after stress in CW shrimps ($p = 0.03$ for both genes). On the contrary, in the BFT shrimps, no change in Pen 2 and Pen 3 transcript abundance was detected after stress compared to before stress ($p > 0.05$). However, significant differences were observed between rearing conditions after stress; 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 vs. 0.88 ± 0.53 ($p = 0.02$) for Pen 3. Similar to the penaeidin family, another AMP (Cru) transcript showed a significant decrease in RNA abundance 6 h after stress in CW 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.

4. Discussion

At the present time, when shrimp production faces many losses

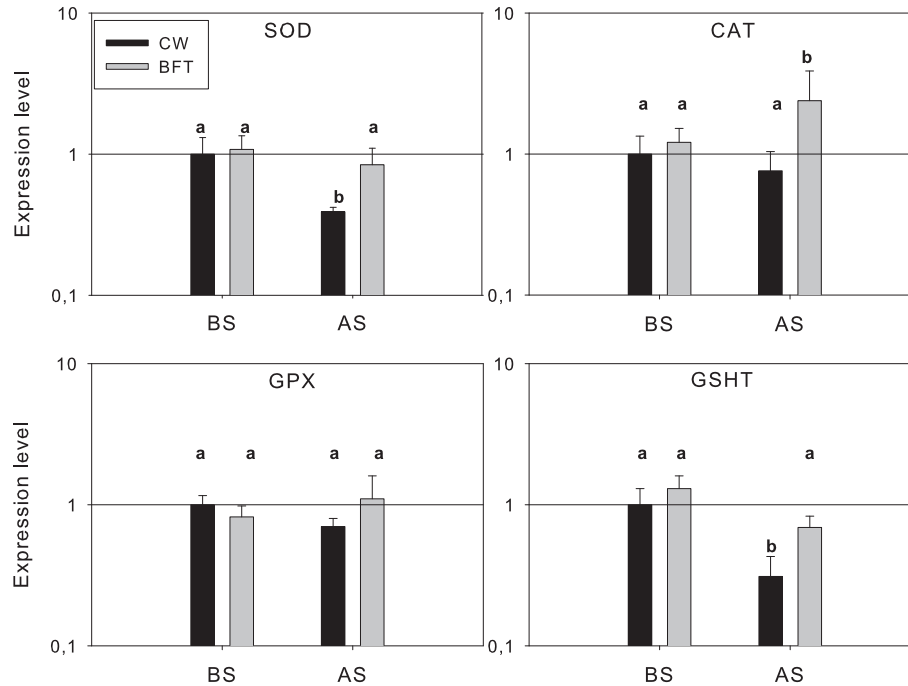


Fig. 1. Expression profiles of genes coding for the antioxidant enzymes Super oxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX) and Glutathione transferase (GSHT) in animals under both conditions both before (BS) and after stress (AS).

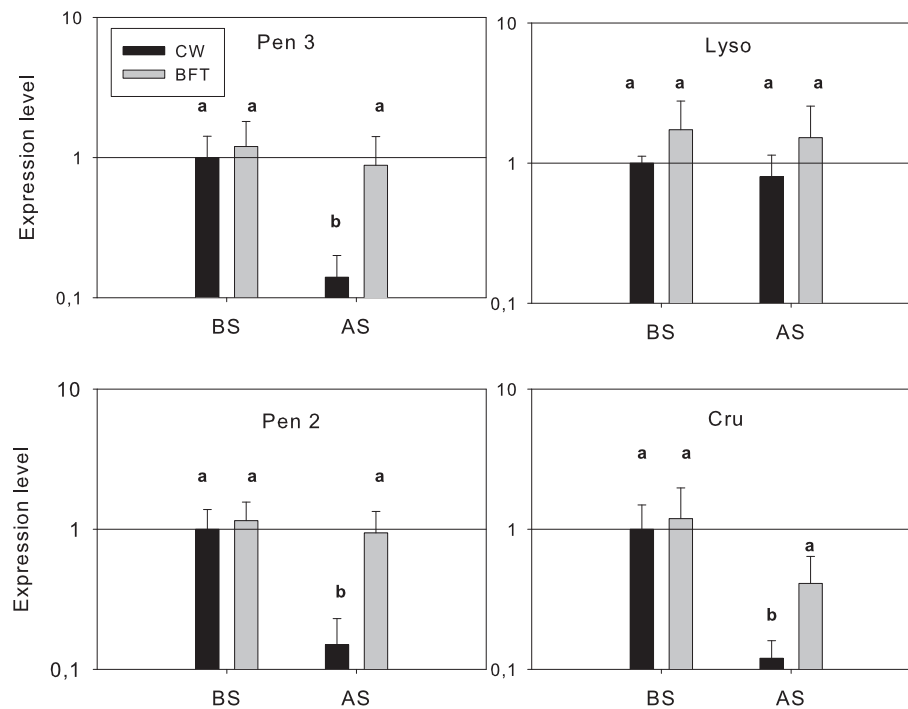


Fig. 2. Expression profiles of genes coding for AMPs, Lysozyme (Lyso), Peneaidin 2 and 3 (Pen 2 and Pen 3) and Crustin (Cru) in animals from both conditions both before (BS) and after stress (AS).

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 [27,36,73,74] and

one recent work showed that BFT improved shrimp resistance to infectious myonecrosis virus infection [27]. 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.

4.1. 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 *L. stylirostris* juvenile shrimps compared to the CW system. Such results have already been described [6,17,47,49]. 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 [12,28,49,66]. Biofloc is known to be an important source of proteins [72] and also of lipids [18]. Moreover, biofloc can contain microbially bioactive components such as carotenoids, vitamins [35] 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 [63].

4.2. 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 [36]; 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 [4,34,64]. 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 [5,11,36,53].

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 [27,73]. 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 [73]. 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.

4.3. Differential AOE and AMP responses induced by H₂O₂ stress between shrimps from BFT and CW

After a H₂O₂ 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 H₂O₂, which is hypothesized to also act as a messenger of signal transduction by regulating the mRNA level through activation of signal pathways [33,58]. In this study, the expression of CAT was significantly increased in the digestive gland after 6 h under H₂O₂ exposure for animals reared in BFT, while no change in RNA transcript abundance was recorded for CW shrimps. This result suggested that H₂O₂ stimulated the expression of CAT in BFT animals. AOE's function is to catalyse the conversion of H₂O₂ into water and molecular O₂ to protect the organism from peroxidation [76]. This result seems to show that excess H₂O₂ 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 [10]. 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 [46]. GPX also catalyses the conversion of H₂O₂ into H₂O and O₂. The absence of significant differential expression before and after 6 h 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 [29]; showed that GPX mRNA transcription increased significantly between 8 h and 12 h after H₂O₂ 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 [10] and the degradation of cells by H₂O₂. 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 H₂O₂-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 [26]. This study shows differences in AMP gene expression levels according to the rearing conditions of *L. stylirostris* facing a H₂O₂ 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 [7]. 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 [13] induced by H₂O₂ cytotoxicity and (ii) a migration of haemocytes from the digestive gland towards exposed tissue to H₂O₂ as is often observed after infection by a pathogen or by injury [7]. 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 H₂O₂ stress. Nevertheless, further study is needed to confirm this assumption.

5. 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, H₂O₂ stress induced a differential response in AOE and AMPs between shrimp from BFT and CW. Biofloc shrimps seem to maintain their antioxidant and antimicrobial peptide responses after H₂O₂ stress, while clear water shrimp immune competence seemed to decrease after stress.

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