Combined effect of external ammonia and molt stage on the blue shrimp Litopenaeus stylirostris physiological response

Chantal Mugnier* and Carole Justou

Laboratoire d'Aquaculture Calédonien, IFREMER, BP2059, 98846 Nouméa Cedex, New Caledonia

*: Corresponding author : cmugnier@ifremer.fr, Tel.: +687-28-51-71; fax: +687-28-78-57

Abstract:

The effect of ambient ammonia and molt stage on the physiological response of the blue shrimp Litopenaeus stylirostris was studied. Shrimps were submitted to 54.6 mg l^{-1} ammonia-N (1.76 mg l^{-1} NH_3-N) for 24 hours. Only shrimps in stages C, D_0, D_1 and D_2 were used for the analysis. Haemolymph was assayed for osmoregulatory capacity (OC), magnesium ion (Mg ion), total proteins, oxyhaemocyanin, lactate and glucose. Molt stage had an effect on OC, Mg ion and total proteins in control shrimps, and on OC, Mg ion and lactate in treated animals. Ammonia treatment decreased OC and lactate, and increase Mg ion concentration in haemolymph, for all molt stages. It decreased significantly total proteins and oxyhaemocyanin for stages D_1 and D_2, and increased glucose concentration for stages C and D_0. There was a combined effect of treatment and molt stage only on total proteins concentration. The effects of an external factor (ammonia), an internal one (molt stage) and the combination of both, and the usefulness of using physiological parameters measured in this study as tools to detect stress, are discussed.

1. Introduction

Farming of the blue shrimp <u>Litopenaeus stylirostris</u> in earthen ponds in New Caledonia is a developing industry. However, it is facing episodes of mortality due to a member of the Vibrionaceae <u>V. penaeicida</u> (Costa et al., 1998), which can also be found in the haemolymph of apparently healthy shrimp (C. Goarant, pers. com.). Variations in environmental factors seem to have an impact on the mortalities observed (Mermoud et al., 1998). Environmental factors may induce a stress response in shrimps and, as a consequence, a decrease in immune defence and an increased susceptibility to pathogens (Le Moullac and Haffner, 2000; Horowitz and Horowitz, 2001). Ammonia-N concentration in ponds is one of the main environmental factors that can induce a stress in shrimp. In intensive culture system, accumulation of ammonia-N, which is the principal end-product of nitrogenous compounds, has a deleterious effect on fish and crustaceans (Colt and Armstrong, 1981). In water the equilibrium between unionised ammonia (NH_3) and ammonium ion (NH_4^+) is pH-, temperature- and salinity- dependent. Of the ammonia species, NH_3 is the most toxic to aquatic life. The effects of ammonia-N on shrimp or other decapods physiological response are relatively well documented (Wajsbrot et al., 1990; Young-Lai et al., 1991; Chen and Cheng, 1993a; Schmitt and Uglow, 1997; Racotta and Hernandez-Herrera, 2000; Harris and al., 2001).

Despite moult cycle has an effect on numerous physiological functions in Crustacea, molt stage of the animal except intermolt has received little attention. However, it has been shown that shrimps at pre- and postmolt stages are more sensitive to stress than intermolt animals (Wajsbrot et al., 1990). Premolt animals are also less resistant

to experimental infection with pathogenic bacteria than intermolt animals (Le Moullac et al., 1997; Cheng et al., 2003).

The aim of the present work was to study the physiological response of sub-adult L. stylirostris to an environmental stress in relation with molt stage. So far this species has received little attention compared to Penaeus vannamei and P. monodon, which are the main cultivated species in the world. However, L. stylirostris is cultivated in America and the shrimp aquaculture in New Caledonia is devoted to this species. The aim of this work was also to test physiological indicators that can be assayed from a haemolymph sample in order to monitor and analyse shrimp response under environmental variations. Thus physiological responses in terms of osmotic regulation, and metabolic responses, to a sub-lethal level of ammonia-N corresponding to 2 mg l^{-1} NH_3-N were studied. Some physiological parameters that can be involved in stress response or be affected by stress were selected for this study. Variation of osmoregulatory capacity (OC), which is the difference in osmotic concentration between haemolymph and surrounding water, was studied as a non-specific indicator commonly used for detecting physiological stresses, including in L. stylirostris (Lignot et al., 2000). Other selected parameters measurable in haemolymph are: total proteins, oxyhaemocyanin, magnesium (Mg) ion, glucose and lactate. Total proteins can serve as a significant source of metabolic energy for crustaceans (Claybrook 1983). It was shown that protein concentration decreases under ammonia stress (Chen et al., 1993, Chen and Cheng 1993a). Oxyhaemocyanin is the main protein in the haemolymph and is implied in several functions like oxygen transport, enzymatic activities, osmoregulation or buffering (Paul and Pirow, 1997/98). The Mg ion plays an important role as a co-factor in enzyme systems and as a modulator of the hemocyanin of crustacean arthropods (Morrit and Spicer,

1993), and variations of its concentration in shrimp haemolymph have been observed under different stress conditions (Boglio, 1995). Variations of blood glucose levels have been observed under several different environmental and physiological conditions (Hagerman et al., 1990; Hall and Van Ham, 1998). Lastly, lactate formation could be expected if a higher energy production is induced by environmental or physiological changes and anaerobic metabolism occures.

2. Material and methods

2.1. <u>Experimental animals</u>

The experiment took place in the Caledonian Aquaculture Laboratory of IFREMER in New Caledonia, on sub-adults (average weight 21.4 ± 2.0 g) of <u>L. stylirostris</u> reared in an earthen growout pond. The shrimps were transported to five 200 l indoor tanks (20 shrimps per tank) with static aerated sea water at 25°C and a salinity of 32‰. They were acclimated for 2 days before the experiment started (Soyez, 1997) and were fed commercial pellets. They were not fed 12h before and during the experiment. Faeces were removed during partial water exchange the days preceding the experiment.

2.2. <u>Moult stage determination</u>

Six moult stages were defined according to the retraction of the epithelium within setae of the antennal scale (Drach, 1939; Chan et al., 1988). Shrimps were classified as A and B for respectively the early and late post molt stages, C for intermolt and D_0, D_1, D_2 for premolt stages. D_2 was the late premolt stage prior to ecdysis, when

epidermis is at maximal retraction and it is possible to distinguish the developing seta.

2.3. Experimental procedure

Shrimps were exposed for 24h to a sub-lethal concentration of 2 mg l^{-1} NH_3-N. Ammonia test solutions were prepared by dissolving the required amount of ammonium chloride in seawater. The amount of ammonium chloride to be added to obtain a NH_3-N concentration of 2 mg l^{-1} was calculated according to the equation of Bower and Bidwell (1978) based on a salinity, pH and temperature of respectively 32‰, 7.81 and 25°C. These parameters were measured again at the end of the experiment. Three tanks out of 5 received ammonium chloride.

After 24h exposure to ammonia, haemolymph samples were collected via the ventral sinus using disposable syringes and needles and were immediately distributed as follows: A 10-µl subsample of haemolymph was used for measurement of osmotic pressure with a vapour pressure Wescor osmometer. A 60-µl subsample was diluted in 1140 µl of distilled water and oxyhaemocyanin absorbance was measured at 335 nm (characteristic of oxyhaemocyanin) using a Spectronic Genesys^{TM} 5 spectrophotometer within a maximum of 50 min after sampling. The oxyhaemocyanin concentration was calculated using an extinction coefficient (E^{mM}_{1cm}) of 17.26 (Chen and Cheng, 1993b). A 135-µl subsample of haemolymph was mixed with 15µl of 10% sodium citrate and centrifuged 5 min at 800g, 5°C. The supernatant (plasma) was stored at –80°C for further biochemical analysis.

2.4. Plasma constituent analysis

A 50-µl subsample of plasma was mixed with 100 µl ice-cold 6% v/v perchloric acid and centrifuged for 15 min at 13000g for deproteinisation. The supernatant was neutralized with 14µl of KOH 3M (Paterson, pers. com.). Commercial kits were used for total dissolved ammonia-N ($NH_4^+ + NH_3$) (Sigma Chemical), glucose (Sigma), lactate (Biomérieux) and magnesium (Biomérieux) determinations. Except for total dissolved ammonia, kits were adapted to a microplate. Absorbance was read on a spectrophotometer (Spectronic Genesys™ 5) for ammonia. It was read on a microplate reader (Digiscan Asys Hitech 340) for glucose, lactate and magnesium, and concentrations were calculated from a standard curve of substrate. Glucose and lactate were assayed on deproteneized plasma. Plasma level of total proteins was measured by the Lowry et al. (1951) technique adapted to microplate method, using bovine serum albumin (Sigma, molecular weight: 66,000 daltons) as a standard. Ammonia was measured as a control for ammonia-N contamination in shrimps in molt stages C and D_2.

2.5. Water analysis

Osmotic pressure of the water was measured as for the haemolymph, with the Wescor osmometer. Total dissolved ammonia-N ($NH_4^+ + NH_3$) concentrations in the water were measured by the Koroleff method (1976) adapted to seawater. Absorbance was read at 630 nm on the spectrophotometer. Final NH_3-N concentrations were calculated according to the equation of Bower and Bidwell (1978) based on the final salinity, pH and temperature of the water in tanks.

2.6. <u>Data and statistical analysis</u>

The OC was calculated as the difference in mOsm l^{-1} of osmotic pressure between seawater and shrimp haemolymph.

The ratio of oxyhaemocyanin to protein was calculated by dividing the concentration of oxyhaemocyanin (mmol l^{-1}) by that of protein (mmol l^{-1}), which was converted from mg ml^{-1} to mmol l^{-1} by dividing by 66 (Chen and Cheng, 1993b).

There were not enough animals in stages A and B in the experiment for statistical analysis, thus data analysis was conducted on animals in stages C, D_0, D_1 and D_2. Data are plotted as mean ± standard error.

Principal Component Analysis (PCA) was applied to the data designed as observations. All the physiological parameters were defined as variables except the oxyhemocyanin/proteins ratio which is a combination of both parameters. The PCA gives a n-m dimensional plane with the possibility to have, in the same factorial space, observation and variable projections. It was used as a descriptive technique (Statlab computer software).

Data were analysed with two-way and one-way ANOVA followed by the PLSD Fisher test at the significant threshold of 5% (Statview computer software).

3. Results

Figure 1 presents the row projections in the space of the two first correspondence factors (CF) which correspond to axes 1 and 2 on the figure, after submitting data to PCA. This projection plane summarizes 68.8% of the total variance: 50.6% for the CF1 and 18.2% for the CF2. Thirty eight percent of the animals analysed explain 80% of the first axis (CF1). Amongst the 38%, positive data (56%) come from control animals except for one value (treated shrimp in stage C), and mainly from

intermolt stages (77% of C + D_0 molt stages, 15% of D_1, 8% of D_2), and negative

ones (44%) come all from treated animals mainly in premolt stages (73% of D_2, 18%

of D_1, 9% of D_0 and none of C). Eighty % of the second axis is explained by 23% of

the animals (87% of treated and 13% of control shrimps).

Data of control shrimps, whatever the stage, are explained mostly by OC, lactate,

total proteins and haemocyanin, when treated animals refer more to Mg ion and
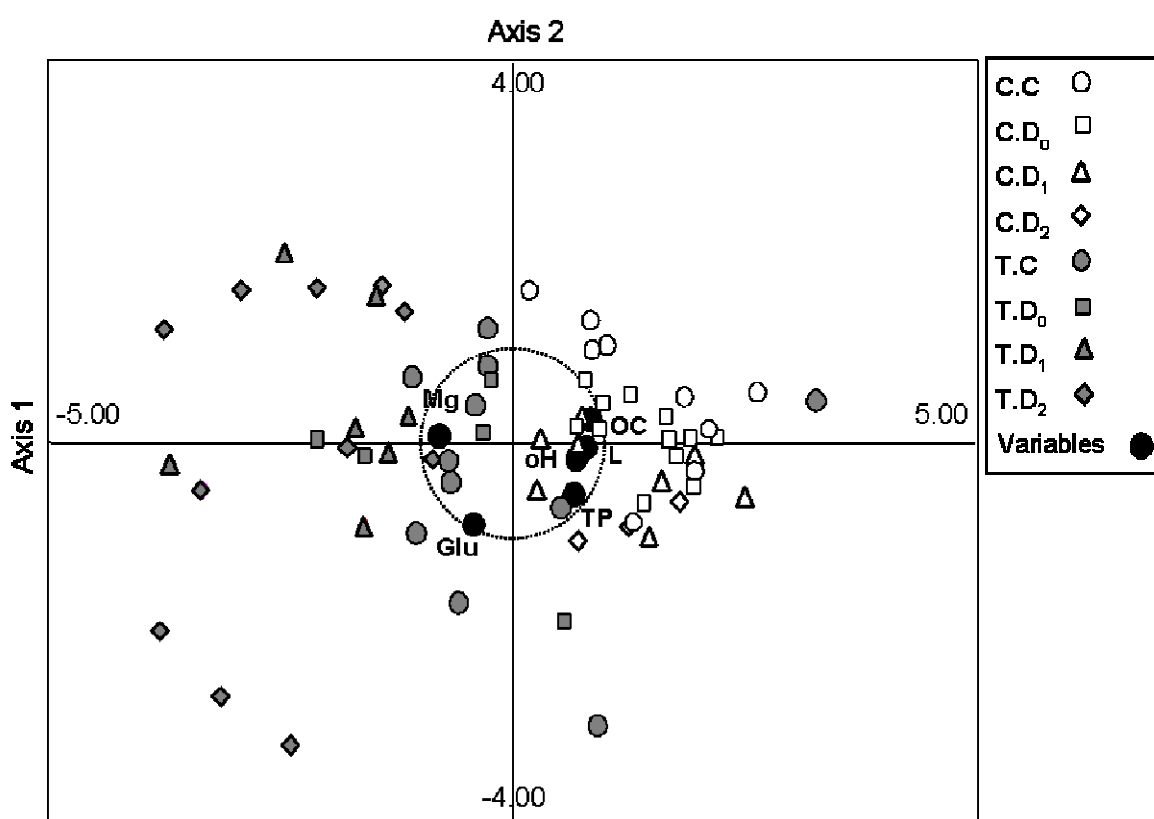
glucose concentration.

Fig. 1. Row projections in the two first factor space (axes 1 and 2) of the Principal Component Analysis conducted on 6 variables (physiological parameters: osmoregulatory capacity (OC), lactate (L), oxyhaemocyanin (oH), total proteins (TP), glucose (Glu) and Mg ion (Mg)) and 92 individual observations taking into account the treatment (control C., treated T.) and the different molt stages.

3.1. Effect of short-term exposure to ammonia-N on haemolymph constituents

Final ambient total ammonia-N concentrations are presented in Table 1. They corresponded to 0.08 mg l^{-1} NH_3-N for control tanks and 1.79 mg l^{-1} NH_3-N for treated tanks.

Table 1

Mean total ammonia-N concentrations (mg l^{-1}) in the medium and in the haemolymph of the shrimps in stages C and D_2 (Mean ± standard error)

	Medium	Haemolymph	
		Stage C	Stage D_2
Control	2.57 ± 0.08	2.31 ± 0.51	4.24 ± 1.05
		(n=18)	(n=8)
24h ammonia treatment	54.60 ± 2.50	45.99 ± 2.59	50.96 ± 3.32
		(n=10)	(n=10)

Ammonia-N in the haemolymph of control shrimps (Table 1) were not significantly different between stages C and D_2 (p>0.05). No difference was also observed in the ammonia treated group (p>0.1). No mortality was recorded during the experiment. Ammonia exposure treatment increased variability between individuals as shown on Figure 1 (PCA). It had an overall (all molt stages combined) significant effect on all parameters (p ≤ 0.0018), except for the oxyhaemocyanin/total protein ratio. Haemolymph glucose and Mg ion levels were significantly higher after 24h treatment, while a significant decrease was observed for OC, lactate, total proteins and oxyhaemocyanin levels (Fig. 2). OC (p ≤ 0.0055), lactate (p ≤ 0.0134) and Mg

ion (p ≤ 0.0020) concentrations were affected at all stages. Ammonia-N treatment decreased OC for 30% (stage C) to 56% (stage D_2) (Fig. 2A), lactate level for 37% (stage C), 42% (stages D_0 and D_2) and 53% (stage D_1) (Fig. 2F). An increase of 70% (stage C) to 120% (stage D_2) was observed for Mg ions concentration (Fig. 2B). Plasma glucose level did not change for stages D_1 and D_2, but ammonia treatment increased glucose levels by 30% in stage C and 25% in stage D_0 (Fig. 2G). No differences between control and treated animals in stages C and D_0 were observed for total proteins level (figure C) and oxyhaemocyanin concentration (Fig. 2D), when total proteins and oxyhaemocyanin concentration in stages D_1 and D_2 decreased similarly between 17 to 20%.

3.2. Effect of molt stage and sex on haemolymph constituents

A significant difference ($p<0.02$) between stages was observed in control shrimps for OC (Fig. 2A), Mg ion (Fig. 2B), and total proteins (Fig. 2C). The highest OC (163 ± 3 mOsm l^{-1}) was observed in stage C, and the lowest (131 ± 13 mOsm l^{-1}) in stage D_2 (Fig. 2A). Mg ion concentration was lower in stage C (7.3 ± 0.4 mmol l^{-1}) compared to stages D_1 (10.2 ± 0.4 mmol l^{-1}) and D_0 (9.4 ± 0.2 mmol l^{-1}), but not stage D_2 (8.4 ± 0.6 mmol l^{-1}) (Fig. 2B). Total proteins level was significantly higher in stages D_0 and D_1 compared to stage C (Fig. 2C). No differences were observed for oxyhaemocyanin concentration, oxyhaemocyanin/total proteins ratio, lactate and glucose levels. Differences between molt stages disappeared after 24h ammonia-N treatment for total proteins ($p>0.05$). However, levels of Mg ion were significantly higher ($p<0.05$) in stages D_1 and D_2 compared to stage C. Differences between stages appeared for lactate level after 24h ammonia-N treatment ($p<0.01$). Mean lactate

levels in stages D_1 (12.3 ± 1.5 mg dl^{-1}) and D_2 (11.3 ± 0.7 mg dl^{-1}) were lower than that in stage C (17.3 ± 1.2 mg dl^{-1}) (Fig. 2F).

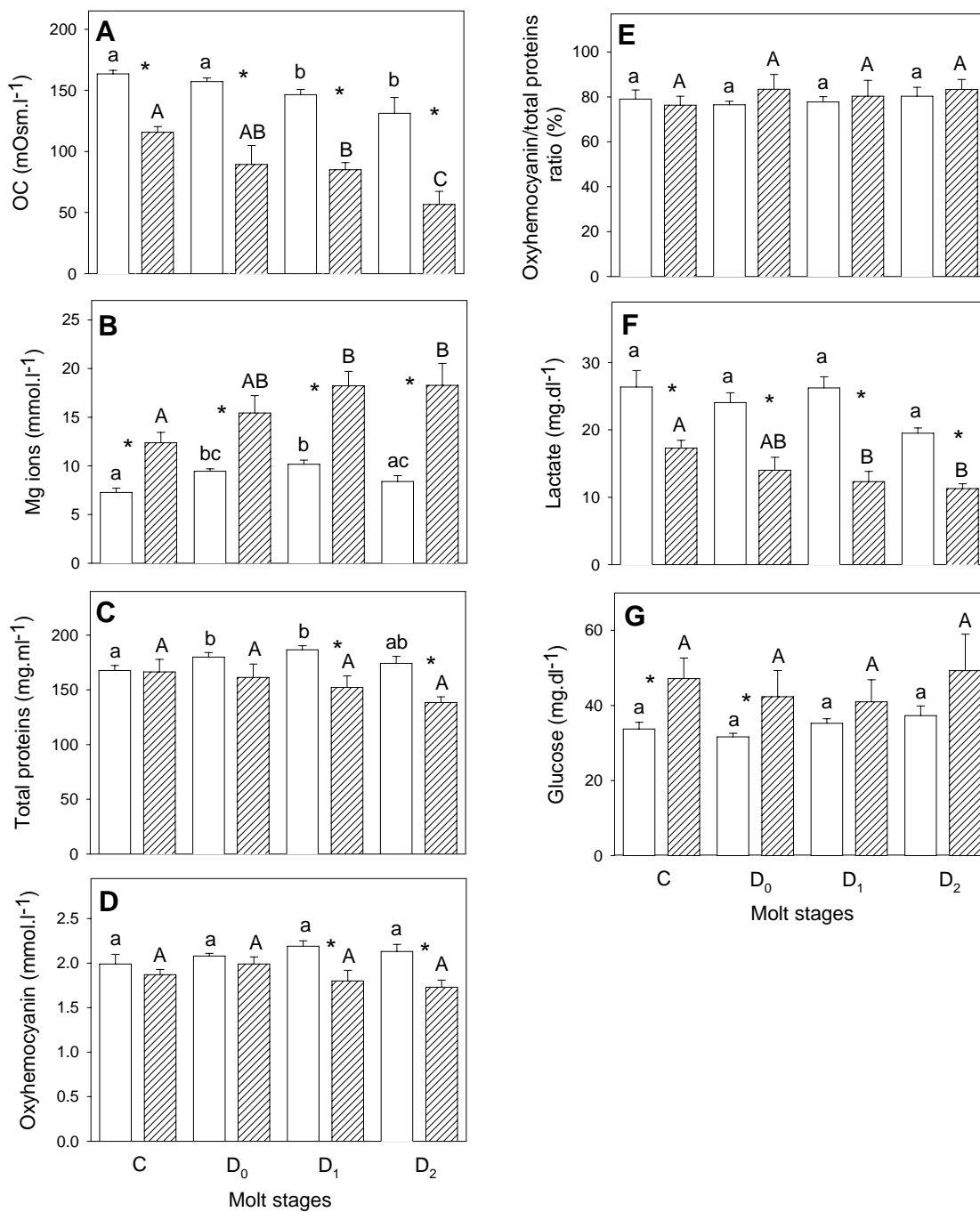No differences were observed between male and female (P>0.20 for all parameters).

Fig. 2. Mean (± S.E.) osmoregulatory capacity (OC) (A), Mg ion (B), total proteins (C), oxyhaemocyanin (D), oxyhaemocyanin/total proteins ratio (E), lactate (F) and glucose (G) concentrations in the haemolymph of L. stylirostris at different molt stages. Comparisons of controls (open bars) with shrimps exposed to ammonia (bars with pattern). Mean values from 16-18 determinations for control stages C, Do, D1; 3-8 determinations for control stage D2, and 5-11 determinations for treated animals. Bars within control or treatment groups with different letters are significantly different (p<0.05). Significant difference between control and treated animals are shown by *.

4. Discussion

4.1. Ammonia levels

Ambient ammonia in control tanks was below the safe level defined for P. monodon adolescent (4.26 mg l^{-1} ammonia-N), and the NH_3-N concentration was equivalent to the safe level (0.08 mg l^{-1}) (Chen et al., 1990), or below the safe level defined for P. vannamei (0.16 mg l^{-1} NH_3–N (Lin and Chen, 2001).

Mean haemolymph ammonia-N level in control shrimp in stage C was close to the value observed in control P. chinensis (27g weight) (2.71± 0.04 mg l^{-1}) by Chen et al. (1993), and lower than that observed in P. japonicus (4.45 ± 0.44 mg l^{-1} (stage C, 14g) (Chen and Kou, 1991). Haemolymph ammonia concentration of treated shrimps in stage C reached 84% of the ambient ammonia-N concentration, which is similar to what was obtained by Chen et al. (1993) in stage C adults of P. chinensis (80%) after 24h treatment with 20 mg l^{-1} ammonia-N. An increase of ammonia in the plasma with increasing ambient ammonia-N concentration was also observed by Chen and Kou (1991) in P. japonicus (stage C). Chen et al. (1994) observed in P. monodon that

net ammonia-N uptake occurred at ambient ammonia-N greater than 5 mg l^{-1} after 24h exposure. Final NH_3–N ambient concentration in treated tanks (1.79 mg l^{-1}) was less than the 2 mg l^{-1} expected. However, it was above the safety level (Lin and Chen, 2001) and below the lethal level, as no mortality was recorded during the 24h experiment, whatever the molt stage. Only a few shrimps seemed to be weak after 24h exposure. In comparison, no mortalities were recorded in adolescent P. monodon (5g) exposed for 24h to 90 mg l^{-1} ammonia-N (1.62 mg.l^{-1} NH_3), or for 72h to 50 mg l^{-1} (0.90 mg l^{-1} NH_3) (Chen et al., 1990). Moreover, the duration of treatment was shorter than the LT_{50} (the time required to kill half of the population) defined by Chen et al. (1990), which were 114h for shrimps exposed to 50 mg l^{-1} ammonia-N and 31.2h for shrimps exposed to 100 mg l^{-1} (1.80 mg l^{-1} NH_3).

4.2. Internal effect due to molt cycle (control shrimp)

The blue shrimp L. stylirostris is a hypo-osmoregulator in 32 ‰ seawater. However, the difference of osmolarity between the medium and the haemolymph is reduced during molting. Dependence on external factors gradually declines in older molt stages suggesting a reduction in integument permeability as the exoskeleton hardens (Hunter and Uglow, 1993). We observed in our study that OC decreased with the late premolt stages. A correlation with molt stage was also observed on total proteins and Mg ion with a slight but significant increase of concentration during the early premolt stages and then a decrease, which could be attributed to haemolymph dilution before ecdysis. In Crangon crangon, reasonably constant total proteins concentrations are maintained during early to mid premolt stages, but drop between late premoult and immediate premoult (Hunter and Uglow, 1993). In other species like P. monodon (Ferraris et al., 1986) or L. vannamei (Cheng et al., 2002), total

protein concentrations remained stable throughout the molting cycle. No effect due to molt cycle was observed on glucose, oxyhaemocyanin and the hemocyanin/total proteins ratio.

4.3. External effect due to ammonia treatment

Ammonia treatment affected the shrimps as shown by the variation of most of the physiological parameters studied. Effect of ammonia on osmoregulation has been demonstrated in P. japonicus and the American lobster Homarus americanus (Young-Lai et al., 1991). Lin et al., (1993) showed in P. japonicus that the effect of ammonia on osmoregulation was dose- and exposure-time dependant, and the effect reversible. They suggested that the decrease in OC was the result of an impaired Na^+ and Cl^- regulation. Decrease in haemolymph total proteins with increased ambient ammonia-N may be due to an increase in catabolism of proteins to adjust osmoregulation (Ferraris et al., 1986). Decrease of both total proteins and oxyhaemocyanin when animals are exposed to ambient ammonia is probably a result of proteolysis as evidenced by an increase in free amino acids in P. monodon (Chen et al., 1994). It may also result of an increased permeability to water under ammonia exposure. Similarly, increase in Mg ion concentration under stress (capture, air exposure) was reported by Boglio and Goarant (1996) for L. stylirostris, which may be due to impaired osmoregulation and flow into the haemolymph by diffusion.

Racotta and Hernandez-Herrera (2000) observed that oxygen consumption increases with increasing ambient ammonia in P. vannamei, but there was no hyperglycemia, nor significant decrease of lactate. These authors suggested a reduced use of carbohydrate through anaerobic metabolism in shrimp exposed during 24h to 30.2 mg.l^{-1} ammonia-N. In our study we do observe a decrease in lactate level, and an

increase in glucose concentration, at least in stages C and D_0. It is possible that the ambient concentration of ammonia, which was 1.8 times higher in our studies (54.6 mg l^{-1}) lead to an increased effect on shrimp physiology. To our knowledge, there are no other works published on the effect of ambient ammonia on shrimp glucose and lactate levels in haemolymph.

4.4. Combined effect of ammonia and molt stage

A two-way ANOVA showed that there was no interaction between treatment and molt stage except for total proteins ($p < 0.05$). Even if the environmental effect seems stronger than internal effect (molt cycle), the PCA representation shows that shrimps in stage D_2 and, in a less extend, D_1 present a higher variability and a stronger physiological response to ammonia than animals in stages C and D_0, indicating that stages D_1 and D_2 seem to be more sensitive to ammonia than C and D_0 animals. The PLSD Fisher test confirms this observation: Only shrimps in stages D_1 and D_2 show significant differences for parameters like total proteins and oxyhaemocyanin. Moreover, for OC and Mg ion, the difference in levels between control and treated shrimps is greater in stage D_2 compared to stage C (respectively 2.3 and 2.2 fold for D_2 against 1.4 and 1.6 for C). Similarly, L stylirostris submitted to experimental hypoxia show a decrease of OC twice more important in stage D_2 than in stage C, and a higher rate of mortality in stage D_2 than in intermolt stage (Mugnier and Soyez, 2001). On the other hand, glucose increase in treated shrimps is significant for animals in stages C and D_0, but not for stages D_1 and D_2. Hardy and al. (1994) observed that the snow crab Chionoecetes opilio submitted to different temperatures showed a greater increase of glucose concentration at a lower temperature in hard-shell crabs than in soft-shell ones. The authors suggested that it could be due to lower

reserves of glycogen in the soft-shell crabs and by the dilution effect of their greater plasma volume. Combined effects of stress and molt stage on physiological response of crustacean are very poorly documented. However it is important to understand these interactions as in aquaculture it is suggested that the shrimps are more sensitive to disease during the molting period when environment is less favourable to the shrimps (Mermoud et al., 1998; Horowitz and Horowitz, 2001). To our knowledge, only the effects of separate factors (i.e. environmental stress or molt stage) on the response to experimental infection have been studied. Thus it has been shown that L stylirostris (Le Moullac et al., 1997) and the giant freshwater prawn Macrobrachium rosenbergii (Cheng et al., 2003), are less resistant to pathogenic bacterial infection in premolt stage than in stage C. On the other hand, a hypoxic stress decreases the resistance of intermolt L. stylirostris to Vibrio infection (Le Moullac et al., 1998). In New Caledonia a large part of the population carry the Vibrio responsible for outbreaks (Goarant, pers. Com.). However, the disease is not systematic, and triggering factors may be implied. Theses factors can as well be external (environment) as internal (molting) or a combination of both.

4.5. Physiological parameters as tools for stress detection

The ability to detect a physiological stress before it causes irreversible damage or death, and in aquaculture to monitor the health of animals, may be obtained by the measure of physiological parameters (Lignot et al., 2000). Coupled with the measure of some environmental parameters (including oxygen, ammonia, temperature), they could be management tools to eliminate or reduce stress factors and improve shrimp health. For this, physiological parameters need to be sensitive and reliable. OC is already known as a good but non-specific stress indicator (Lignot et al., 2000).

However, its use is limited to controlled conditions because it is external salinity dependent. As shown on the PCA, Mg ion and, to a lesser extent, glucose seem to be good candidates. Boglio and Goarant (1996) pointed out that Mg ion could be a good stress indicator, at least for acute stress, for L. stylirostris and for P. monodon. Hyperglycaemia is an indicator of short-term stress (Hall and Van Ham, 1998). A decrease of glucose level can also be observed when perturbation last longer (Hagerman et al., 1990). Results of Racotta and Palacios (1998) on stress of experimental manipulation (repeated sampling) in P. vannamei let suggest that circulating levels of glucose, rather than lactate, appears to be a more sensitive stress indicator. Our recent field surveys tend to confirm interest of both Mg ion and glucose rather than lactate as tools for stress detection (unpublished data). Total proteins and oxyhaemocyanin are also promising candidates. Concentration of ammonia in haemolymph could also be used as an index of ammonia loading for L. stylirostris intensive culture system.

Acknowledgement

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