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Biological, physiological, immunological and nutritional assessment of farm-reared *Litopenaeus stylirostris* shrimp affected or unaffected by vibriosis

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Abstract:

Shrimp aquaculture in New Caledonia is subject to seasonal mortalities during grow-out due to highly virulent *Vibrio nigripulchritudo* (Vn). To understand the mechanisms affecting shrimp resistance and leading to significant mortality, a shrimp ecophysiology and immunology survey was conducted on two farms, the first considered as a "control" farm (HC), the second affected by the disease (DF). Mortality observed during the survey at DF started 50 days after stocking and was typical of this disease. The main observations regarding shrimp were: (a) growth was not affected by the disease and was faster in the DF than in the HC pond; (b) disease did not affect one sex more than the other, or a specific part of the population in terms of weight; (c) the physical condition of shrimp did not specifically allow us to foresee disease outbreak; (d) shrimp at late premolt stage D_2 and early postmolt stage A appeared to be at some points of the mortalities – but not continuously – the most sensitive to disease; (e) physiological, immunological and nutritional parameters of uninfected shrimp in the DF pond were altered, suggesting that environmental stress occurred just before the first mortalities; (f) data suggest that Vn-infected shrimp are more stressed than the presumed healthy shrimp. Combined with pathological and environmental knowledge gained in parallel during this survey, a conceptual model is proposed. Results suggest that an unstable environment induced conditions (i) stressful for the shrimp, increasing their susceptibility to bacterial infections and (ii) favoring the proliferation of the pathogen in the pond. The combination of these two processes could lead to significant mortality.

Highlights

► Shrimp health was surveyed on pond affected or unaffected by a vibriosis. ► Shrimp in molt stages A and D2 appeared to be the most sensitive. ► Even the health of uninfected shrimp was altered just before the first mortalities. ► Infected shrimp are more stressed than the presumed healthy shrimp. ► From our multidisciplinary approach, a conceptual model is proposed.

Keywords: Vibriosis ; Penaeid shrimp ; Field survey ; Physiology ; Immunology ; Nutritional status

1. Introduction

Aquaculture of the blue shrimp *Litopenaeus stylirostris* in New Caledonia is a developing industry with an almost virus-free status. The domesticated species is tolerant of IHHNV, the only significant virus present. However, the industry is affected by seasonal mortalities, either during inter- and cold seasons ("Syndrome 93") (Mermoud et al., 1998, Goarant et al., 2004a), or during the warm season ("Summer Syndrome") (Goarant et al., 2006a, Lemonnier et al., 2006). These two pathologies reduce the profitability of the industry and are therefore its main concerns. In both cases, mortalities are related to septicemic vibriosis due to *Vibrio penaeicida* and *Vibrio nigripulchritudo* during grow-out (Goarant et al., 2006b). The origin of these diseases cannot be explained by a single factor such as the presence of *Vibrio*. Indeed, *V. penaeicida* can be found in the hemolymph of apparently healthy shrimp (Goarant et al., 2004b) without mortality outbreaks, and *V. nigripulchritudo* can be detected in disease-free ponds and shrimps (Goarant et al., 2006a). It is rather the combination of several factors that account for disease, in particular, it is suspected, the interaction between the pond ecosystem, the shrimp and the pathogen (Sniezsko, 1974; Lightner and Redman, 1998).

To understand the mechanisms affecting shrimp resistance and leading to significant mortality, a multidisciplinary field survey linking pathology study (Goarant et al., 2006a), pond ecosystem study (Lemonnier et al., 2010) andshrimp ecophysiology and immunology (this study) was conducted on rearing ponds at two farms, one affected by Summer Syndrome (designated farm DF for “diseased farm”), the other a healthy “control” farm (farm HC). The aims of the pathology and the pond ecosystem studies were to follow and describe the pathogen dynamics in shrimp and the ecosystem in both farms and to compare the environmental conditions between the healthy and the diseased ponds. During this survey, no mortalities linked to Summer Syndrome were recorded in HC pond. In DF pond, the first mortalities were typically observed around d50, when the mean weight of shrimp was 5.9 g.

The results from the pathology study indicate that the shrimp larvae used were free of *V. nigripulchritudo* at the time of stocking. *Vibrio* was detected for the first time in a shrimp sampled 40 days after stocking in DF group. After that, the prevalence increased and fluctuated between 16.7% and 93.3% until the end of the survey. Moribund/dead shrimp were septicemic with *V. nigripulchritudo*. This *Vibrio* was also found in the sediment and the water at some points of the survey. This bacteria cell was also observed in the HC pond, but without mortality outbreak (Goarant et al., 2006a). The environmental study showed a higher variability of phytoplankton in farm DF compared to farm HC. The beginning of the mortality outbreak at DF followed an ecosystem shift characterized by a change in the phytoplankton community (Lemonnier et al., 2010). As part of this study, this paper presents results on the biological, physiological and immunological parameter survey of shrimp.

Various relevant shrimp characteristics were studied throughout rearing, which was implemented simultaneously on diseased and healthy farms. Animals were examined for physical/health condition, including weight, growth, molt stages, hemolymph clotting time, exoskeleton examination for necrotic lesions on the external body surface, gut emptiness and gross signs on body and appendages such as opaqueness of abdominal muscle, deformities, and colored gills and appendage segments. Most of those indicators are proposed by Fegan and Clifford (2001) for routine examinations for monitoring shrimp health on shrimp farms, and may be observed in shrimp affected by vibriosis (Lightner, 1988). Plasmatic physiological indicators of stress response – osmotic pressure (OP), total proteins (TP), oxyhaemocyanin (OH), magnesium ions (Mg ions), glycemia, lactate – were selected in relation to results of previous studies carried out on *L. stylirostris* either under experimental conditions (Mugnier and Justou, 2004) or in field experiments (Lemonnier et al., 2004; Mugnier et al., 2006). Variation of OP was studied as a non-specific indicator commonly used for detecting physiological stresses, including *L. stylirostris* (Lignot et al., 2000). TP can

serve as a significant source of metabolic energy for crustaceans (Claybrook, 1983). OH is the main protein in the hemolymph and is applied in several functions such as oxygen transport, enzymatic activities, osmoregulation and buffering (Paul and Pirow, 1997/98). The Mg ions play an important role as a co-factor in enzyme systems and as a modulator of hemocyanin in crustaceans (Morritt and Spicer, 1993). Variations of blood glucose levels have been observed under several different environmental and physiological conditions (Hall and VanHam, 1998). Lastly, lactate formation can be expected if higher energy production is induced by environmental or physiological changes and anaerobic metabolism occurs. As well as these physiological indicators, the total hemocyte count (THC) was measured as a basic indicator of the immunological status of shrimp, since hemocytes are involved in most of the immune mechanisms in crustacean (Johansson et al., 2000). Because the hepatopancreas plays a key role in digestive processes, various indicators were also examined in the hepatopancreas, such as the hepatosomatic index (HSI), glucose and TP concentrations, and enzymatic activities such as trypsine. The aim was to gain insights into the dynamics of hepatic energy reserves utilization in relation to mortality outbreak. The effect of the presence of *V. nigripulchritudo* in shrimp hemolymph on physiological indicators was analysed.

2. Material and methods

2.1. Field survey procedure

The survey was implemented from October 2002 (d32 after stocking) to January 2003 (d80) on Diseased and Healthy Farms. Two 3-ha earthen ponds, one in each farm, were stocked the same day with postlarvae (PL) originating from the same hatchery batch, at a density of 28 PL m^{-2} in farm DF and 35 PL m^{-2} in farm HC. Shrimp at both farms were fed the same commercial pellets throughout the survey. On each farm and thoughout rearing, daily pond

mortalities were evaluated by counting dead and moribund shrimps at the pond edges. The ponds were managed by the technical staff of each farm using to their standard techniques.

Shrimp were sampled alternately from rearing day 32, on even dates at farm DF and uneven dates at farm HC. Sampling was carried out before the shrimp were fed in order to avoid any variation in physiological parameters due to food intake (Lignot et al., 1999). One hundred shrimp were caught quickly with a castnet in two different locations in the pond (50 in each location, designed as representative of the pond by the farmers) and placed in aerated seawater. Shrimp were individually weighed, their sex and molt stage determined, and the body and exoskeleton examined for physical/health condition: necrotic or melanized lesions (black spots) on the external body surface, colored gills, reddish appendage segments as a result of chromatophore expansion, gut emptiness, deformity of exosqueleton or rostrum and opaqueness of abdominal muscle were recorded. Moribund/dead shrimp were also sampled at DF, in order to find out if shrimp affected by mortalities corresponded to a specific part of the population in terms of weight, sex and molt stage.

Two molt stages were selected for the study of physiological and immunological states of shrimp: the intermolt stage C, which most studies on shrimps are concerned with, and the late premolt stage D2. This second molt stage was selected because shrimps at premolt stage are more sensitive to stress and less resistant to bacterial infection than intermolt animals (Le Moullac et al., 1997; Mugnier and Justou, 2004). Of the 100 shrimp caught, the first 15 at stage C and 15 at stage D_2 were sampled for hemolymph and hepatopancreas analysis. Hemolymph samples were collected rapidly from the ventral sinus using disposable syringes and needles and were immediately (within 10-15 sec) distributed as follows. A 10-µl subsample of hemolymph was used for the measurement of OP with a Wescor osmometer. A 10-µl subsample of hemolymph was diluted in 390 µl of distilled water and OH absorbance was measured at 335 nm (characteristic of OH). The OH concentration was calculated using

an extinction coefficient (E^{mM}_{1cm}) of 17.26 (Chen and Cheng, 1993). A subsample of hemolymph was mixed with 10% sodium citrate as anti-coagulant (9 volumes hemolymph for 1 volume citrate) and centrifuged 5 min at 800g, 5°C. The supernatant (plasma) was stored at -80°C for further biochemical analysis. A 10-µl subsample was diluted in 30 µl of Alsever with 10% formalin for counting the THC from day 50 only, when mortalities started in DF pond. The number of hemolymph samples which coagulated within 10 seconds was recorded. Sampled shrimps were dissected and the hepatopancreas was carefully removed and weighed. HSI was calculated individually as the ratio between wet hepatopancreas weight and total wet body weight. The hepatopancreases were immediately frozen in liquid nitrogen and back in the laboratory were stored at -80°C until analysis. Dead and weak shrimp were collected twice daily along the pond edges, either by farm staff or scientists Weight, physical condition, sex and molt stage were also recorded on fresh dead shrimp and weak shrimp. The analyses of *V. nigripulchritudo* presence in the hemolymph of apparently healthy shrimp allowed us to differentiate the presumed healthy (Vn- shrimp) from the infected (Vn+ shrimp) (Goarant et al., 2006a).

2.2. Molt stage determination

Six molt 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 the early and late postmolt stages respectively, C for intermolt and D_0, D_1, D_2 for premolt stages. D_0 was the very early premolt stage, when the epidermis starts to retract. D_2 was the late premolt stage prior to ecdysis, when the epidermis is at maximal retraction and it is possible to distinguish the developing seta.

2.3. Plasma constituent analysis

A 25-µl subsample of plasma was mixed with 50 µl ice-cold 6% perchloric acid and centrifuged for 15 min at 13000g for deproteinisation. pH of supernatant was neutralized with 14µl of KOH 3M (Paterson, pers. com.). Commercial kits formerly adapted to a microplate were used to determine glucose (Sigma) (Glycemia), lactate and Mg ions (Biomérieux). Absorbance was read on a microplate reader (Digiscan Asys Hitech 340) and concentrations were calculated from a standard curve of known substrates. Glucose and lactate were assayed on deproteneized plasma. Plasma level of TP was measured by the method described by Lowry et al. (1951) adapted to microplate technique, using bovine serum albumin (Sigma, molecular weight: 66,000 daltons) as a standard.

2.4. Total hemocyte count (THC)

Numeration of hemocytes was carried out within 72h following sampling, using an inverted phase contrast microscope (Leica).

2.5. Hepatopancreas analysis

Glucose, TP and trypsine activity were assayed on hepatopancreas extracts. Hepatopancreas was weighed with a microbalance and cut into small pieces, which were homogenized either in chilled (1:10 w:v) distilled water for glucose and total proteins assay or in chilled Na-phosphate buffer 10 mM at a final concentration of 100 mg/ml, then centrifuged at 20000 x g for 20 min at 4°C for trypsine assay. Samples were analysed individually. For TP assay, proteins were extracted with NaOH 2N. TP was measured by the Lowry and al. (1951) technique adapted to the microplate method, using bovine serum albumin (Sigma, molecular weight: 66,000 daltons) as a standard. Glucose was assayed with the Dubois method. For trypsine activity, supernatant was dissolved at 1:5 v/v in chilled TRIS buffer pH 8. Trypsine activity was evaluated by the rate of hydrolysis of N-Benzoyl-l-Arginine-*p*-nitroanilide

(BAPNA) (SIGMA) as a synthetic substrate. BAPNA (1 mM) was dissolved in 1 ml of dimethylsulfoxide (DMSO) and made up to 100 ml with Tris buffer, pH 8 containing 20 mM $CaCl_2$. Assay was adapted from Erlanger et al. (1961) to a microplate. Hepatopancreas extracts (25 µl) were added to 225 µl of substrate solution at 25°C and changes of absorbance at 405 nm (microplate reader, Digiscan Asys Hitech 340) were recorded over a 10 min period (50 measures per min). Trypsin activity units were expressed as the change in absorbance min^{-1} mg^{-1} of protein of the enzyme used in the assay (ΔAbs min^{-1} mg^{-1} $prot^{-1}$).

2.6. Data and statistical analysis

Biological parameters

Weight data from the field survey were log transformed and the slopes of the linear regression obtained for each farm were compared using Student's t-test. Comparisons of weight of apparently healthy shrimp between farms, and within farm DF between apparently healthy shrimp and dead/moribund shrimp, were carried out with ANOVA at 5% significance threshold (Statview Computer Software). Differences in mortality between shrimp at different molt stages, and comparisons between apparently healthy shrimp and moribund/dead shrimp for sex and molt stage, were analysed by Chi-square tests (<chi>2) at 5% significance threshold.

Physiological and immunological aspects

Samplings were grouped in 4-day periods so as to have similar periods comparable between farms. Comparison for each rearing period was then carried out with ANOVA at the 5% significance threshold (Statview Computer Software). Data were expressed and plotted as mean ± standard error. Comparison between farms was carried out on Vn- shrimp by ANCOVA (statgraph 5.1 software) with farm and molt stages as factors and shrimp weight as

cofactor. In DF, comparison was carried out between Vn+ and Vn- shrimp with *Vibrio* and molt stage as factors and shrimp weight as cofactor. Correlations between hemolymph and hepatopancreas parameters were analysed (Statview Computer Software).

3. Results

3.1. Mortality and final survival

No mortality related to *V. nigripulchritudo* was observed at HC. However, some mortality occurred between d45 and d49 (Fig. 1A). Hydromedusae *Clytia sp.* (Le Borgne pers. com.) at high concentration (between 15 and 20 individuals L^{-1}) in pond water were noticed for the first time at d41 at this farm. After d49, 0 to 3 hydromedusae L^{-1} were counted. Mortality in the pond stopped when the concentration decreased to fewer than 5 individuals L^{-1}. Despite this mortality, the final survival was 62%, as generally observed on this farm. In DF pond, the first dead shrimp were observed around d50 (Fig. 1B), when shrimp sampled in the pond had an average weight of 5.9 ± 0.1 g. Two main periods of mortality were observed: one between d54 and d64 and the other peaking at d77. An intermediate period was observed between d65 and d71. Final survival at harvest was 27%.

3.2. Weight

Weight equations of the power regression were (weight) = 0.017(day)^1.498 for HC (r^2 = 0.97) and (weight) = 0.004(day)^1.887 for DF (r^2 = 0.99). The curves crossed at d54 for an average weight of 6.9 g (Fig. 2). The slope obtained with the linear regression was significantly higher at DF than in the HC group ($p < 0.001$). Before d54, daily average weight of shrimp from HC was higher than in the DF group, and conversely after d54. Weight of moribund and dead shrimp was not significantly different from the weight of apparently

healthy shrimp throughout the survey, except on days 56, 66, 70, and 76, when dead shrimp weighed less than apparently healthy shrimp (ANOVA, $p < 0.05$).

3.3 Sex

There were 47.2% males in the HC group and 49.7% in the DF group, with no difference in sex distribution in the population (<chi>2, $p = 0.106$) at either farm. No difference in weight gain could be observed between males and females at either farm. Summer syndrome mortality affected both females (n = 344) and males (n = 307) (<chi>2, $p > 0.05$) in the DF group.

3.4 Molt stages

The mean percentages of each molt stage observed during the survey are reported in Table 1. There were no significant differences between farms for each stage (ANOVA, $p > 0.20$). Nor were there any differences found when looking separately at the period before mortality (d32-49) and the period during mortality (d50-80). The molt stage could be identified in 89.3% of the dead/moribund shrimp (658 animals) at DF. The percentage of shrimp at stages A and D_2 was significantly higher in moribund/dead than apparently healthy shrimp (<chi>2, $p < 0.05$) (Table 2). Conversely, there were fewer moribund/dead shrimp at stage D_1 than in the sampled population, and no difference for stages B, C and D_0 (Table 2). Moribund/dead shrimp at stage A were observed between d54 (30%) and d60, and on d70 (13%), d74 (4.4%) and d76 (7.9%), roughly corresponding to the beginning of peak mortalities, while at the same time a maximum of 2% of the population was at stage A (Fig. 1B). The difference between moribund/dead and healthy shrimp at stage D_2 was particularly noticeable at the beginning of mortality at days 54, 56 and 58 with respectively 30%, 58% and 36% of moribund/dead shrimp at late premolt stage, as against 12%, 25% and 24% in the apparently healthy shrimp (<chi>2, $p < 0.05$). This significant difference was also observed at days 68

and 70 with 50% and 51% of the dead/moribund shrimp at stage D_2 as against 24% and 11% in the sampled population.

3.5. Physical/health conditions

Shrimp with deformities represented less than 0.5% of the sampled population in both farms (Table 3). The number of shrimp with an empty gut was significantly higher in HC than in DF group (Table 3). Peaks of animals with empty gut were observed around d43-47, 55, 61-65 and d75 in the HC group. This number remained low throughout the survey in the DF group, but tended to increase from d70 onwards. All moribund/dead shrimp had an empty gut. The percentage of shrimp with necrotic lesions on the external body surface was less than 1.7% of the sampled shrimp in both farms (Table 3). Animals with orange-colored gills were three times more frequent in the DF group compared to HC (Table 3). Necrotic lesions were observed only at the end of the survey, after d70 for both farms. HC had twice the percentage of shrimp with red-colored appendage segments compared to the DF group (Table 3). Most of the shrimp with colored appendage segments in HC group were observed during the medusa bloom, and especially after d65. In general, shrimp at stage D_2 were more likely to have red-colored appendage segments (<chi>2, $p < 0.05$), except during the period d47-49 of rearing, when all molt stages were equally affected. The overall number of shrimp with opaqueness of abdominal muscle was twice as high in HC than in the DF group (Table 3). They were observed at several periods in the HC group, but the highest peak was between d53 and d55, with more than 60% of the sampled animals being affected. A smaller peak was also observed in the DF group between d46 and d48. Only 4.8% of the moribund shrimp had an opaque abdominal muscle in the DF group, a percentage significantly lower than that observed in apparently healthy shrimp (<chi>2, $p = 0.0002$). All molt stages could similarly be affected by opaqueness of abdominal muscle.

3.6. Physiological and immunological parameters

The mean percentage of animals with hemolymph that coagulated within 10 seconds was significantly higher in DF than in the HC group (11.1% against 4.6%, <chi>2, $p < 0.0001$). At HC, the highest percentages were observed between d39 and d51, with a peak (20% of the animals sampled) at d45 (during the medusa bloom). At DF, mean peaks were observed at d52-54, d64 and d74-76 with 48%, 38% and 30% respectively of the sampled animals affected.

The kinetics of physiological parameters of Vn-free shrimp at stages C or D_2 is presented on Fig. 3. Results from ANCOVA (Table 4) showed that all hemolymph parameters studied were significantly different between farms, except OP ($p = 0.06$): Mean glycemia, Mg ions, OH, TP and THC were higher in shrimp from farm HC than from DF, while lactate was lower. At the beginning of the survey (d32-40) and comparatively to DF, HC was characterized by shrimp with a concentration of Mg ions 2.7 times higher (Fig. 3C) and a glycemia 1.9 times lower (Fig. 3E). A positive peak in OP (Fig. 3A) and negative peaks in TP (Fig. 3B) and OH (Fig. 3F) were also observed during the period d37-40. Between d41 and d48 parameters developed in a similar way for both farms and no significant differences were found whatever the parameter studied. During the following period (d49-d56), characterized by the beginning of the mortality at DF, glycemia and TP were lower in shrimp from farm DF than from farm HC, while OP tended to be higher in HC than in the DF group. Glycemia remained significantly lower in the DF group until d76, except during the period d61-d68. The lack of significance for this period may be explained by the low number of Vn-free shrimp in the sample jeopardizing the statistical power of our analysis (n = 5), as 80% of shrimp were carrying Vn (Vn+) (Goarant et al., 2006a). During the last period of the survey

(d71-80), a significant increase was observed in the HC goup, but also in the DF group, while between d69-72 the concentration was close to the basal level (d32-40) observed during the first part of the survey at DF.

In the period d61-68, farm DF presented a lower OH than farm HC (Fig. 3F), as opposed to higher lactate concentrations (Fig. 3D). Lack of data between d65-68 due to insufficient intermolt Vn- shrimp prevented us from properly comparing THC (Fig. 3G), but it was significantly higher at HC than in the DF group from d69 to d72.

3.7. Hepatopancreas analysis

Table 4 shows statistical results from ANCOVA, with a significant farm effect on HSI, TP and trypsine activity. At the beginning of the survey, HSI was lower in the HC group comparatively to DF (Fig. 4A). From d53 onwards, this index was similar at both farms. From d45 to d48, TP was particularly high at DF (168.3 ± 14.8 $mg.ml^{-1}$). From d49 it dropped sharply and remained up to 25% lower than the concentration observed in the HC group until the end of the survey (Fig. 4B). Glucose concentrations were not significantly different between ponds (Table 4, Fig. 4C). Trypsine activity was significantly lower at HC compared to the DF group (Table 4), though from d41 on, the patterns were similar in both ponds (Fig. 4D).

3.8. Presence of *V. nigripulchritudo* in shrimp hemolymph

At DF, Vn+ shrimp were observed from d40 onwards in the population sampled (Goarant et al., 2006a). Within the Vn+ shrimp population, the percentage of premolt shrimp was significantly higher than that of intermolt shrimp (80.4% against 49.4%) (<chi>2, $p = 0.0005$) when mortality appeared (d51-60). Subsequently the difference was no longer significant. Analysis of indicators of physical condition did not show any significant difference between

Vn+ and Vn-. The percentage of shrimp with reduced clotting time was significantly lower in Vn+ shrimp compared to Vn- shrimp (respectively 10.2% as against 16.7%; <chi>2, $p < 0.05$). Glycemia, OH, TP and THC were significantly lower, and OP and Mg ions were significantly higher in Vn+ shrimp than Vn- shrimp (Table 5). The differences for the parameters analysed in the hepatopancreas between Vn+ and Vn- shrimp were not significant (Table 5).

4. Discussion

Physical/health conditions were not characteristics of shrimp sampled in the DF pond before and during mortality. As for many types of disease (e.g. yellowhead virus), these overall signs are not sufficiently specific to detect an outbreak of Summer Syndrome and to help us understand the mechanisms affecting shrimp resistance, especially since some of them, such as empty gut and opaqueness of abdominal muscle, were mostly observed at HC without any link with mortality.

Peaks of shrimp with a reduced hemolymph clotting time were observed during mortalities linked to vibriosis or when hydromedusae were present. This reduced clotting time could be the result of an immunostimulatory effect rather than a disease symptom, as clotting time is usually delayed in diseased shrimp (Lightner, 1988; Fegan and Clifford, 2001, Song et al., 2003). It could also be a short-term response to external stressful conditions, as observed for some crustaceans (Jussila et al., 2001).

Results showed that growth was not affected by the disease and was faster at DF than in the HC group. In a previous study on Summer Syndrome, a possible negative effect of fast growth on the resistance of shrimp to infection and/or environmental stress was suspected (Lemonnier et al., 2006). This effect has already been demonstrated for the cladoceran *Daphnia magna* (Barber et al., 1994; Smolders et al., 2005). A higher growth rate implies an

increase in the molting frequency (Gauquelin et al., 2007), and high molting frequency might not only increase energy expenditure for exuviations, but also alter the animal's entire energy allocation strategy (Cockcroft and Wooldridge, 1985). Extra energy allocated to growth and molting may be derived from other functions involved in reactions and/or adaptation to environmental variations, including response to pathogens. However, as the growth rate was also relatively high in the HC group, it cannot be assumed that it is a causative factor on its own, but could be one promoting factor among others.

In our study, moribund and dead shrimp were observed during periods corresponding to peak molting, as previously reported by Fegan and Clifford (2001) in ponds infected with WSSV. Stages D_2 and A were the molt stages the most affected by mortality at the beginning of peak mortalities, while premolt stage D_1 was the least affected. Interestingly, shrimp at postmolt stage B were not particularly sensitive, as the percentage observed in moribund/dead shrimp was exactly the same as that observed in the sample population. Le Moullac et al. (1997) have shown that *L. stylirostris* challenged at premolt stage D are more sensitive to a *Vibrio* infection than animals at intermolt stage C. Experimental infection of the white shrimp *L. vannamei* with a pathogenic strain of *Vibrio alginolyticus* showed that shrimp at postmolt stage (A + B) were more sensitive to infection than shrimp at premolt stage (D_0 to D_3) (Liu et al., 2004). Similarly, preliminary experimental work in *L. stylirsotris* infected with *V. nigripulchritudo* gave the same result (De Decker and Goarant, pers. com.). On the other hand, late premolt and postmolt shrimp *L. stylirostris* are more sensitive to stressful conditions such as severe hypoxia than intermolt animals (Mugnier and Soyez, 2005) and the combination of two stressful conditions such as ammonia and hypoxia can lead to mortality affecting more than 50% of the shrimp at late premolt stage and none of the other stages (Mugnier et al., 2008). It is too early to conclude that there is a predominant effect of

environmental conditions rather than *Vibrio* infection on shrimp mortality affecting one molt stage rather than another, but this hypothesis needs to be tested.

In the DF group, decreasing glycemia observed in Vn-free shrimp appeared just before the first dead shrimp were observed. Hypoglycemia is a long-term stress indicator, as observed in *Nephrops norvegicus* submitted to hypoxia for 3 weeks (Hagerman et al., 1990). The data suggest an increase of carbohydrate catabolism and turnover, as indicated by hypoglycemia (Fig. 3E), and concomitantly the production of lactate (Fig. 3D) as the end-product of glycolysis. Lactate is produced by muscular work (functional anaerobiosis) and excess of lactate is transferred from the muscle to the hemolymph. However, Racotta and Hernandez-herrara (2000) suggested a reduced use of carbohydrate through anaerobic metabolism when animals are exposed to stress. Furthermore, decrease of both TP (Fig. 3B) and OH (Fig. 3F) is probably the result of proteolysis to satisfy increased metabolic demand caused by a stressor situation involving reduced availability of energy. Under experimental conditions, it was also demonstrated for several shrimp species including *L. stylirostris* that TP and OH decrease under environmental stress (e.g. Chen and Cheng, 1995; Mugnier and Justou, 2004). The increase in oxygen demand along with the increase in metabolic demand represents a conflicting situation for the animals, because more oxygen is needed at the tissue level. It would be reasonable to suggest that energy obtained from anaerobic metabolism could partially compensate for this problem. Stress therefore has a considerable and rapid effect on respiratory function. Wabete et al. (2008) demonstrated that *L. stylirostris* is characterized by a low arterial hemolymph pressure and at 28°C by a very high O_2-demand to ensure high cellular O_2 requirements and O_2 consumption. During stressful events, a mismatch between O_2-demand and O_2-supply could contribute to the increase in the anaerobic metabolism of the shrimp observed in our survey through impairment of their hemolymph O_2-carrying capacity.

The difference in studied physiological parameters between Vn+ and Vn- shrimp (hypoglycemia, lower OH, TP and THC and higher PO and Mg ions in Vn+ shrimp) suggest that Vn+ shrimp are more stressed than Vn- shrimp. The effect of pathogen on crustacean physiology has already been observed. Increase in Mg ions is a stress indicator as observed in *P. monodon* and *L. stylirostris* (Boglio , 1995; Boglio and Goarant, 1996), but also an indicator of infection as observed in *M. japonicus* infected with a virus (Hennig et al., 1998). Moreover, experimental infections with white spot syndrome virus (WSSV) in *P. indicus* showed pathogen effects on variations in physiological and immunological parameters, such as hyperglycemia, decrease in OH and THC, and increase in PT (Yoganandhan et al., 2003). Similarly in *L. vannamei* infected with Taura virus, decreases of PT, OH and THC were observed (Song et al., 2003). Hyperglycemia and increase in lactate were also observed in *L. vannamei* challenged with *V. alginolyticus* (Hsieh et al., 2008). A sublethal infection with *Vibrio* led to a negative effect on THC in *L. stylirostris* (Goarant and Boglio, 2000) as well as a decrease in antioxidant defences (Castex et al., 2009). Several factors can cause a reduction of hemocyte numbers, such as hemocyte infiltration in infected tissues, low cell replacement by hematopoietic organs and hemocyte death through apoptosis. Immune assessment conducted in farm-reared *L. vannamei* infected by IMNV virus showed that the immune system of shrimp responds only at a late stage of the disease (Costa et al., 2009).

Environmental factors may also have an effect on immunological response (Le Moullac and Haffner, 2000), including on THC and on susceptibility to Vibrio (Le Moullac et al., 1998; Liu et al., 2004; Cheng et al., 2007). THC values during d49-60 (between $0.84\ 10^7 \pm 0.13\ 10^7$ and $1.35\ 10^7 \pm 0.12\ 10^7$ hemocytes/ml hemolymph) is low compared to mean values observed in *L. stylirostris* in New Caledonia ($3.2\ 10^7$ hemocytes/ml hemolymph, Goarant,

pers. Com.). As data before d49 are lacking, it is not possible to say whether this was a transitory value. THC in Vn- shrimp located in ponds affected by the disease remains lower than usual till the end of the survey, suggesting a potential effect of the environment. Attention needs to be paid in future to observing the immunological responses (Bachère, 2000). Different cell types (hyaline, semi-granular and granular) and antimicrobial peptides could be monitored to analyze more precisely the immunological responses of shrimp to vibriosis in pond aquaculture (Le Moullac and Haffner, 2000; Rolland et al., 2010).

The hepatopancreas is considered to be the main storage organ in shrimp and HSI is used to follow the general nutritional status of crustaceans. Data from our survey were in the range of values found by Castex et al. (2008) for 9-10 g *L. stylirostris* (0.045 - 0.048), but higher than those reported for *L. vannamei* by Sánchez-Paz et al. (2007). At the beginning of the survey, HSI was about 20% higher in the DF group compared to HC, suggesting better digestion and adsorption of food, which in turn contribute to improving hepatic storage in the digestive gland. Moreover the number of shrimp with an empty gut in the morning before feeding was significantly higher in HC than in the DF group. On the basis of these results, it cannot be assumed that shrimp feeding is a disease causative factor. However, from a few days before the beginning of mortalities linked to Summer Syndrome up until the end of the survey, total protein concentrations in hepatopancreas were lower in the DF group than in HC (25% lower at the end of the survey), indicating a decrease in protein storage. It is generally accepted that protein is the main energy source in crustaceans. No difference between Vn+ and Vn- shrimp could be shown, suggesting that the presence of the pathogen in hemolymph cannot by itself account for this result.

Trypsin is considered to be the most important enzyme in digestive dietary protein, and together with chymotrypsin is the most abundant proteolityc enzyme in the digestive gland of

crustaceans. Trypsin activity (TA) is modulated by several internal and external factors such as genetic factors, frequency of feeding, origin and quantity of dietary protein, molting and stress (see the review by Sainz Hernández and Córdova Murueta, 2009). Results on TA in the digestive gland showed that enzyme activity was significantly higher in the DF group compared to HC. Apart from a stress factor, the others factors involved in the TA were similar. One hypothesis is that the difference between ponds could be due to a stress effect as observed by Córdova Murueta et al. (2004) in experimental conditions.

Shrimp have a limited but effective lipid and CHO metabolism that is used according to specific energetic and/or physiological and/or structural demands (Charmantier et al., 1994). For example, under short-term starvation, a rapid decrease of plasma and hepatopancreas glucose is detected (Sánchez-Paz et al., 2007). Results from our survey show no difference between the two ponds, suggesting that animals were able to regulate the glucose concentration in the digestive gland whatever the pathological or/and environmental conditions.

The differential evolution of physiological and immunological parameters of pathogen-free shrimp in DF group from d49 tends to indicate that shrimp were affected by the environmental conditions. During mortality outbreaks, pond DF water column parameters did not generally present values independently considered to be stressful as defined in experimental conditions, but rather high variability and unstable values, such as for dissolved oxygen (Lemonnier et al., 2006; Lemonnier et al., 2010). Variations were fast, setting in a few days (or even in a few hours) and it is difficult to describe precisely the dynamic especially as many phenomena were occurring at the same time.

Although they live in water, shrimps are also in contact with the pond bottom and sediment. pH values in farm DF sediment were close to stressful values: 80% of values were below 6.6

(Lemonnier et al., 2010), the upper limit below which osmoregulation in *L. stylirostris* is affected (Lemonnier et al., 2004). However, similar values of pH were observed in sediment at farm HC from d50 without any physiological response being detected. But experimental studies where one parameter such as ammonia, hypoxia or nitrites is studied in controlled conditions do not reflect the complexity of the pond environment and the fact that the impact of stress increases in a synergic rather than a cumulative fashion. For instance, it has been shown that low dissolved-oxygen levels increase the toxicity of ammonia to the shrimp *L. stylirostris* (Mugnier et al., 2008). Unfortunately, few studies have been conducted so far on the effects of combined environmental conditions. A cumulative effect of these environmental conditions may have decreased the resistance of shrimps to the disease. Another hypothesis is that an unknown environmental factor or factors not examined in this study are involved.

A conceptual model

Figure 5 presents a model of the disease conceptualized from the literature (Sniezsko, 1974; Lightner and Redman, 1998) and all the results from this survey (Goarant et al., 2006; Lemonnier et al., 2010; this study). In this model, we postulate that mortalities occur in an unstable environment, characterized by sudden phytoplanktonic changes and abiotic parameters (pH, ammonia, etc.) close to stressful values. Such conditions, which may occur in combination with an other unknown triggering factor, weaken the host physiological status, as suggested by the results of our comparative study between the two farms and the evolution of physiological parameters, increasing shrimp susceptibility to bacterial infections (Fig. 5, arrow 1). A potentially negative effect of excessively fast growth on shrimp resistance to infection and/or environmental stress is suspected and will be addressed by further investigations. The molt stage could also play a role in disease outbreak (Fig. 5, arrow

2). While the occurrence of pathogenic *Vibrio* isolates in the pond does not necessarily lead to mortality outbreaks, it has been previously reported that the colonization of the pond ecosystem by the pathogen occurs at the onset of the disease (Goarant et al., 2007). The presence of the pathogen in pond sediment before mortalities suggests that sediment could be a potential infecting reservoir (Walling et al., 2010). It has also been shown that *V. nigripulchritudo* is able to survive in sediment throughout an 18-week drying period and therefore from one rearing cycle to the next (Labreuche, pers. com.). However, further studies are needed to determine the environmental factors controlling pathogen proliferation and virulence in ponds (Fig. 5, arrow 3). Our results suggest that Vn-infected shrimp are more stressed than presumed healthy shrimp. However, the data do not allow us to definitely conclude whether the physiological and immunological responses observed in Vn-infected shrimp result either from the presence of the pathogen in shrimp hemolymph or from a different shrimp susceptibility (Fig. 5, arrows 4). Our results suggest that an unstable environment could be the key factor explaining the presence of stressed shrimp and the proliferation of the highly virulent pathogen in ponds. The combination of these two conditions may induce mortality outbreaks.

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Table 1: Mean percentage of each molt stage observed during the period d32-80 in the sampled populations of control farm (farm HC) and farm affected by the "summer syndrome" (farm DF).

	Molt stages					
	A	B	C	D_0	D_1	D_2
Farm HC	0.4 ± 0.2	18.4 ± 2.4	25.9 ± 2.4	10.8 ± 0.9	21.2 ± 2.3	23.7 ± 2.2
Farm DF	0.7 ± 0.3	19.3 ± 1.8	27.1 ± 1.9	12.8 ± 1.6	19.7 ± 2.4	20.3 ± 2.1

Table 2: Percentages of each molt stage in dead/moribund and apparently healthy shrimp (sampled population) in diseased farm DF affected by the "summer syndrome" during the period of mortality (d50-80).

	Molt stages					
	A	B	C	D_0	D_1	D_2
Sampled population	0.7	19.7	25.9	16.3	17.1	20.3
Dead/moribund	5.3	19.9	29.2	12.3	4.8	28.5
	*P<0.01	P=0.94	P=0.20	P=0.06	*P<0.01	* P<0.01

*Significant differences between population and dead/moribund shrimp for each molt stage (ANOVA)

Table 3: Physical/health conditions: Percentages of shrimp which presented rostrum or exoskeleton deformity, an empty gut, necrotic lesions on the external body surface, orange-coloured gills, red-coloured appendage segments and opaqueness of abdominal muscle in the sampled population of control farm (farm HC) and farm affected by the summer syndrome (farm DF).

	Deformity	Empty gut	Necrotic lesions	Coloured gills	Red segments	Opaqueness of abdominal muscle
Farm HC	0.5	13.7	1.7	1.1	5.4	19.8
Farm DF	0.3	9.1	0.4	3.1	2.5	9.0
	$p = 0.28$	$*p < 0.01$	$*p < 0.01$	$p = 0.07$	$*p < 0.01$	$*p < 0.01$

*Significant differences between farms (Chi^2)

Table 4: Statistical results (p values) of ANCOVA with farm and molt stage as factors and weight as cofactor in shrimp free of pathogen, farm DF.

	Parameter	Farm	Molt stage	Weight
Hemolymph	Glucose	< 0.01	< 0.01	< 0.01
	Oxyhaemocyanin	0.03	< 0.01	< 0.01
	Mg ions	< 0.01	< 0.01	0.13
	Total proteins	0.04	< 0.01	< 0.01
	Osmotic pressure	0.06	< 0.01	< 0.01
	Lactate	< 0.01	< 0.01	< 0.01
	THC	< 0.01	< 0.01	< 0.01
Hepatopancreas	Hepatosomatic index	< 0.01	0.70	< 0.01
	Total proteins	< 0.01	0.66	< 0.01
	Glucose	0.16	0.81	< 0.01
	Trypsine activity	< 0.01	0.22	0.79

Table 5: Comparison between VN+ and Vn- shrimp in DF. Statistical results (p values) of ANCOVA with *Vibrio nigripulchritudo* and molt stage as factors and weight as cofactor.

	Parameter	V nigri	Molt stage	Weight
Hemolymph	Glucose	< 0.01	< 0.01	< 0.01
	Oxyhaemocyanin	< 0.01	< 0.01	< 0.01
	Mg ions	< 0.01	< 0.01	< 0.01
	Total proteins	< 0.01	< 0.01	< 0.01
	Osmotic pressure	< 0.01	< 0.01	0.33
	Lactate	< 0.01	< 0.01	< 0.01
	THC	0.01	0.03	0.46
Hepatopancreas	Hepatosomatic index	0.87	0.46	< 0.01
	Total proteins	0.10	0.86	< 0.01
	Glucose	0.97	0.37	0.28
	Trypsine activity	0.87	0.10	< 0.01

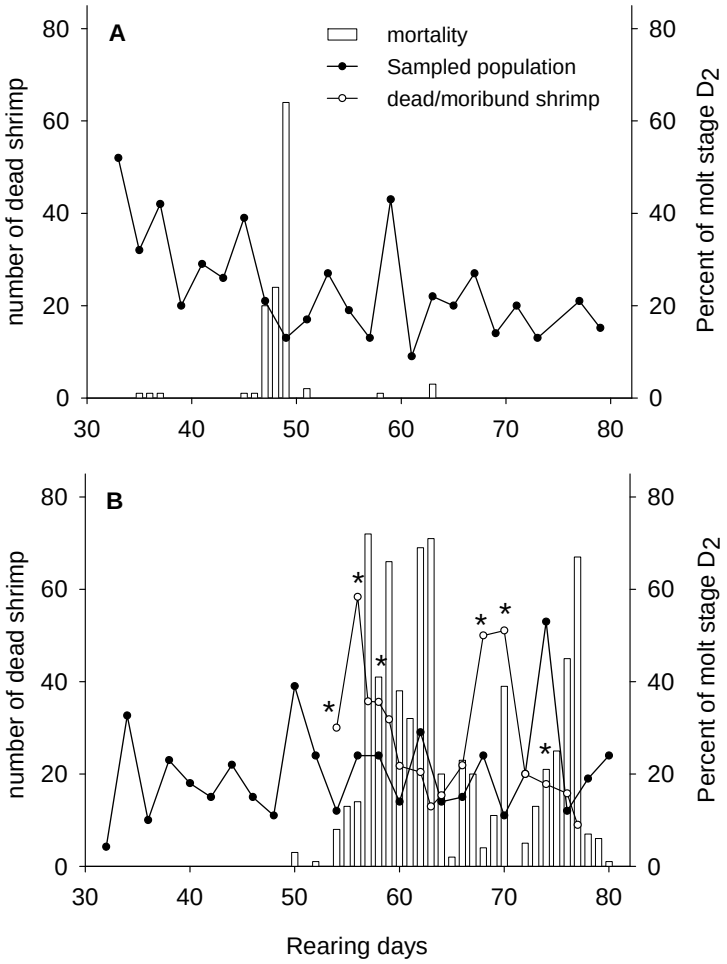


Figure 1: Daily number of dead shrimp observed and percentages of apparently healthy and dead/moribund shrimp in late premolt stage D_2 in (A) farm HC and (B) farm DF.

*indicates significant difference between apparently healthy and dead/moribund shrimp in stage D_2 (<chi>2, $p < 0.05$)

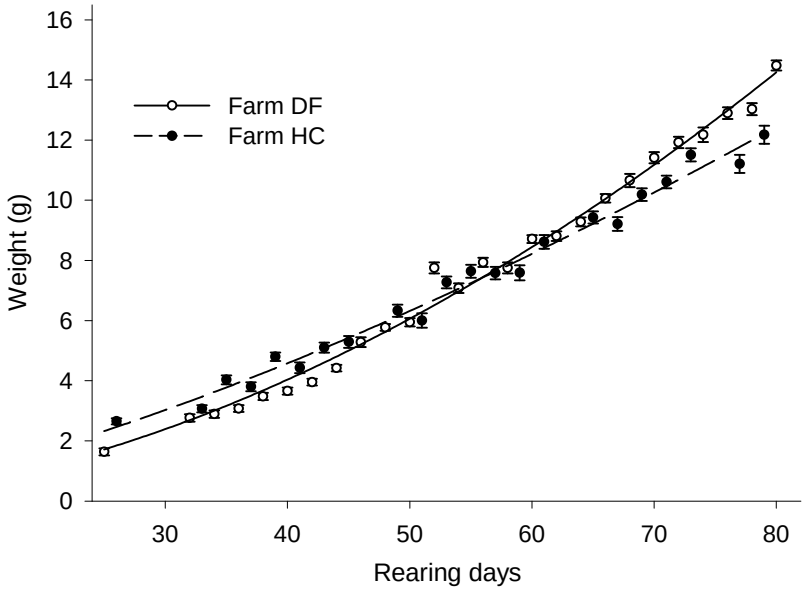
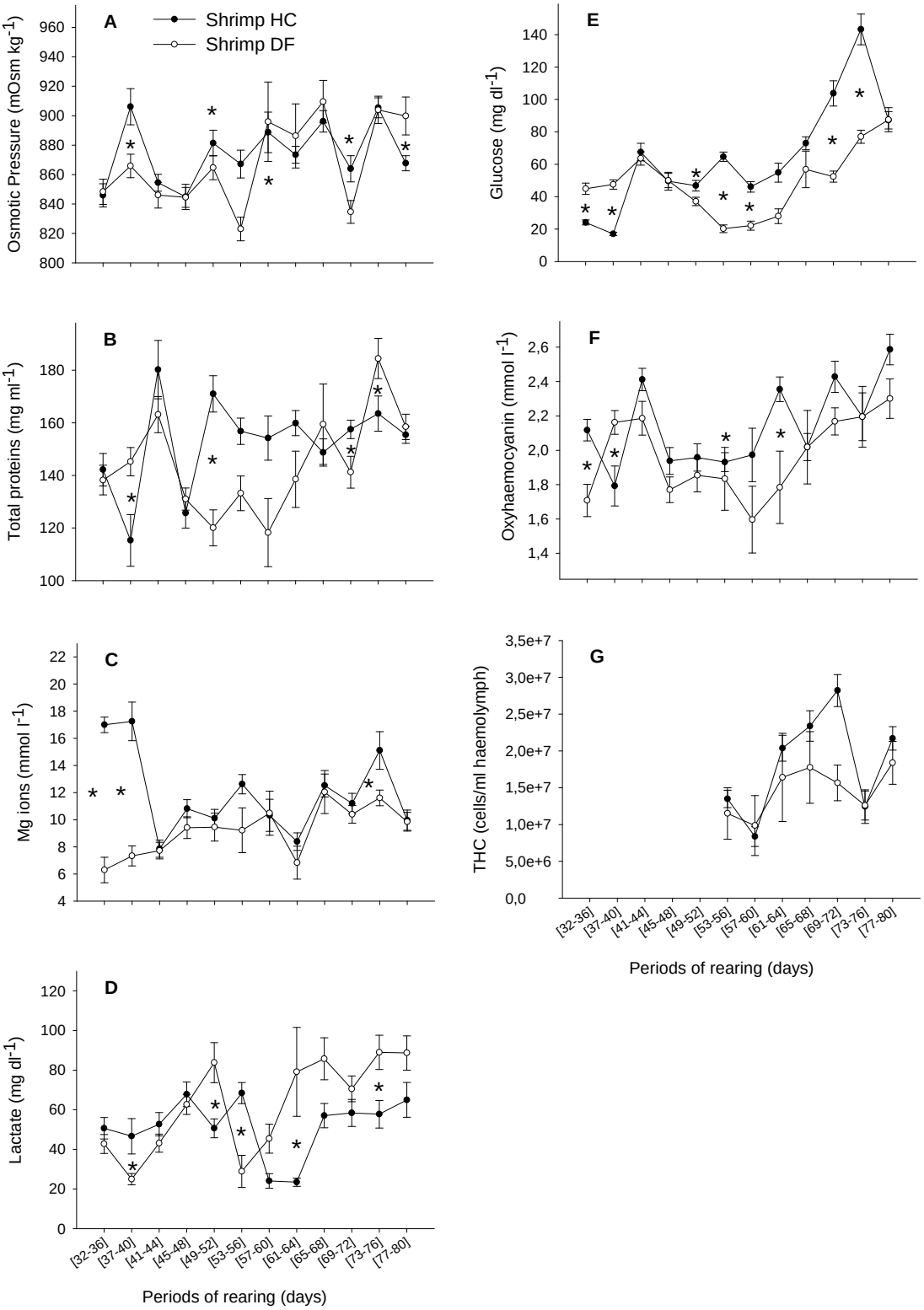


Figure 2: Daily average (± S.E.) weight for farm HC (black circles) and farm affected by Summer Syndrome (farm DF, open circles).



1 Figure 3: Osmotic pressure (A), Total proteins concentration (B), Mg ions concentration (C),
2 Lactate concentration (D), Glucose concentration (E), oxyhaemocyanin concentration (F) and
3 Total hemocyte count (G) in the hemolymph of pathogen-free (Vn-) shrimps in stage C and
4 D2 from a control farm (Farm HC) and a farm affected by Summer Syndrome (Farm DF)
5 between d32 and d80 of the rearing. Mean ± SE. n= 11-59 for farm HC and 5-58 for farm
6 DF. * indicates significant differences between farms (ANOVA, $p < 0.05$).

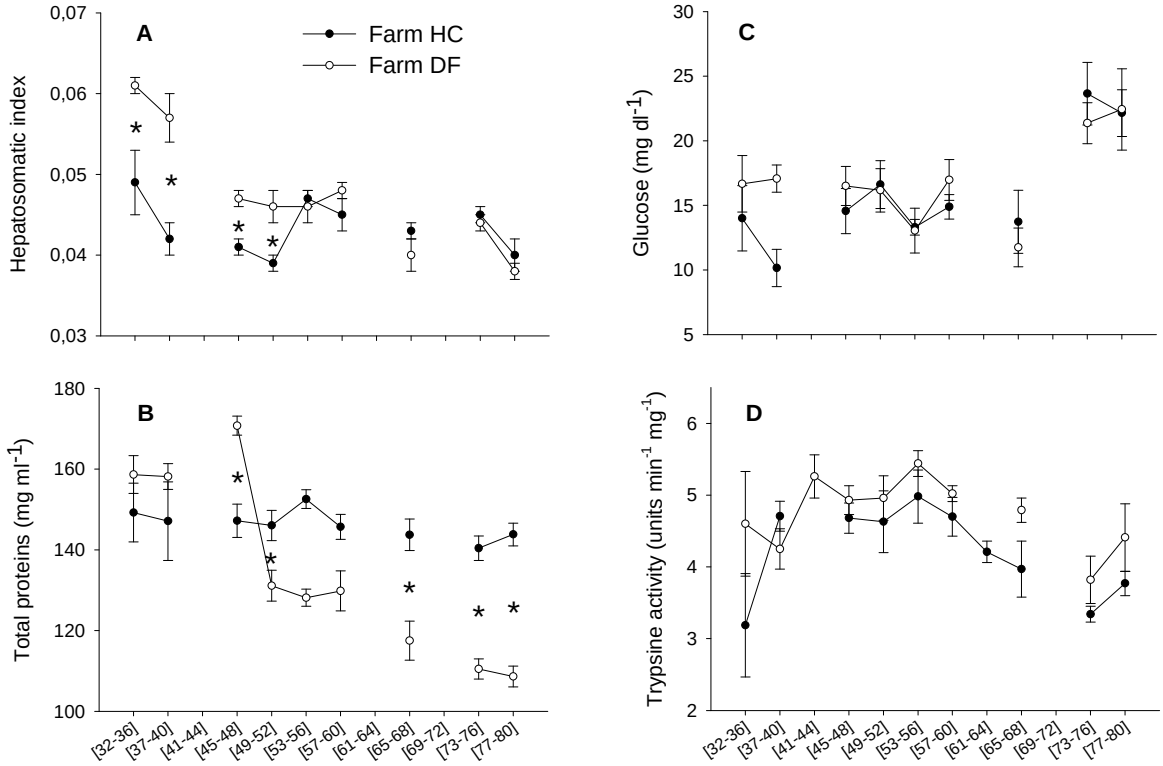
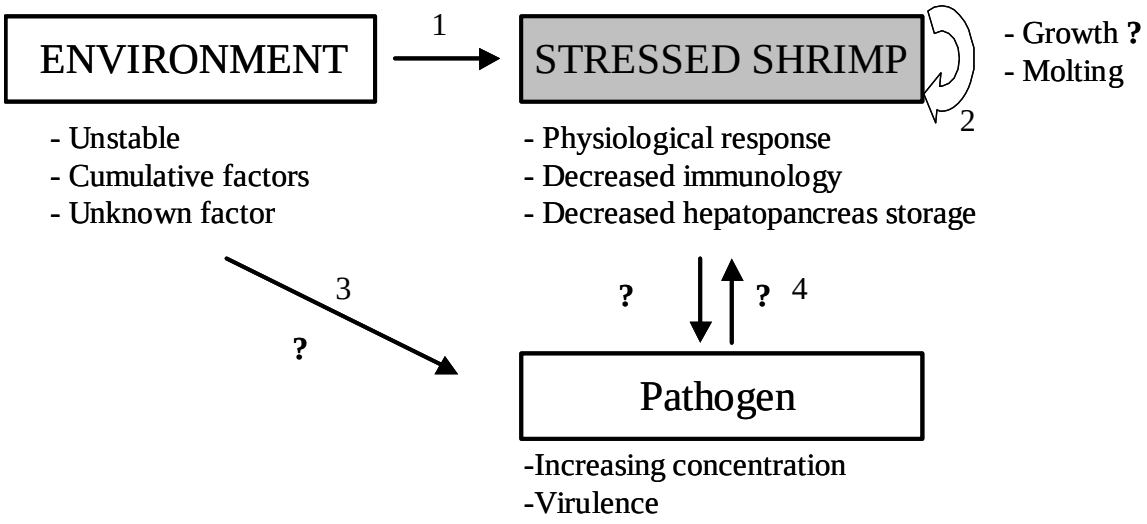


Figure 4: Hepatosomatic index (A), Total protein concentration (B), Glucose concentration (C), trypsine activity in the hepatopancreas of shrimps in stage C from a control farm (Farm HC) and a farm affected by Summer Syndrome (Farm DF) between d32 and d80 of rearing.



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3 Figure 5: Schematic representation of the “disease model” conceptualized from the literature

4 (Sniezsko, 1974; Lightner and Redman, 1998) and from data previously published by

5 Goarant et al. (2006a), Lemonnier et al. (2010) and presented in this manuscript.

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