Research Article

Metabolic and immune status of Pacific white shrimp *Penaeus vannamei* concerning farming conditions

Juan Pablo Apún-Molina[1] , Arlett Robles-Romo[2]
Takeo Matsumoto[3] & Ilie S. Racotta[2]
[1]Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional
Unidad Sinaloa, Instituto Politécnico Nacional, Guasave, Sinaloa, México
[2]Centro de Investigaciones Biológicas del Noroeste
Instituto Politécnico Nacional, La Paz, B.C.S., México
[3]Servicios Acuícolas Profesionales S.A. de C.V., Culiacán, Sinaloa, Mexico
Corresponding author: Ilie S. Racotta (iracotta@cibnor.mx)

ABSTRACT. Biomarkers for monitoring shrimp health have been proposed but scarcely evaluated at the farm level. We analyzed several indicators of energy status in shrimp under farming conditions concerning stocking densities ($100\,m^{-2}$ with biofloc, 15 and $7\,m^{-2}$). The influence of the year's season (temperature) was also analyzed, and, finally, an unfortunate event of White spot syndrome virus (WSSV) infection occurred on the $7\,m^{-2}$ farms, adding another condition. At shrimp sampling from ponds, the effect of acute handling stress was also analyzed for indicators typically affected by such procedure with a 3- and 10-fold increase in glucose and lactate levels in hemolymph, respectively, regardless of density. This response was partially blunted at lower temperatures and WSSV incidence. Increased levels of protein in the hepatopancreas, adenylic energy charge (AEC) in both hepatopancreas and muscle and phosphagens in muscle were observed in shrimp from the $100\ m^{-2}$ farms, suggesting a better nutritional and energetic status in shrimp cultured at high density with biofloc technology. Shrimp with WSSV presented lower hemocyanin levels in hemolymph, most likely associated with its role in the immune response. In WSSV-infected shrimp, the stress response regarding glucose increase was blunted, whereas a stress-induced increase in triglycerides (TG) levels in hemolymph was observed only with WSSV. Increased TG levels in those shrimp hepatopancreas could indicate a switch from carbohydrate to lipid-based metabolism associated with the preferential use of carbohydrates (Warburg effect) for virus replication in the early infection state.

Keywords: *Penaeus vannamei*; metabolism biomarkers; adenylic energy charge; stocking density; seasonal temperature; white spot syndrome virus; Sinaloa, Mexico

INTRODUCTION

Pacific white shrimp (*Penaeus vannamei*) is the dominant species cultured worldwide. However, shrimp farming has generally encountered pathogen threats, particularly from viruses like White spot syndrome virus (WSSV) and, more recently, bacterial diseases like Acute hepatopancreatic necrosis disease

(AHPND) (Martínez-Córdova et al. 2016, Cornejo-Granados et al. 2017). Shrimp culture at high densities represents a clear advantage in terms of yield. However, the first studies indicated that high stocking densities result in lower growth survival, poor water quality, and the emergence of pathogen outbreaks (Allan & Maguire 1992). Several negative outcomes of shrimp super-intensive culture have been overridden by

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new technologies such as bioflocs and recirculation systems (RAS) (for review, see Emerenciano et al. 2022). However, little is known about the stress implied at high stocking density and its influence on shrimp's immune and metabolic status and their resistance to pathogens (Aguilar et al. 2012, Apún-Molina et al. 2017, Guemez-Sorhouet et al. 2019, Baladrat et al. 2022).

It is well known that environmental stress, characterized mainly by changes in physicochemical variables, decreases immune response capacity (for reviews, see Millard et al. 2021, Mengal et al. 2023). However, physical stress induced by handling does not affect such capacity in a chronic situation (Mercier et al. 2006). In contrast, on a short-term basis, handling enhances this capacity regarding total hemocyte count and superoxide anion production (Mercier et al. 2009). Stress situations are typically detected in penaeid shrimp and other animals from different metabolic indicators that conform to the physiological stress response. For example, glucose and lactate increased in response to handling procedures such as repeated sampling (Racotta & Palacios 1998) or persecution, leading to an escape response (Yu et al. 2009, Robles-Romo et al. 2016). Similarly, several situations of chemical or environmental stress are also characterized by an increase in hemolymph glucose and lactate, as are the cases of formalin exposure (Van Ham & Hall 1998), ammonia exposure (Racotta & Hernández-Herrera 2000, Mugnier & Justou 2004), immersion or hypoxia (Van Ham & Hall 1998, Racotta et al. 2002).

In the context of energy available to perform the biological functions of an organism, the stress response represents an energy reallocation from processes such as growth and reproduction to maintain energy homeostasis (Sokolova et al. 2012). At the cellular level, a bioenergetic steady state implies a balance between synthesis and degradation of ATP (Atkinson 1968). Thus, the concept of adenylic energetic charge (AEC) is a useful tool that indicates the ratio between "charged" power (ATP) and total adenylic nucleotides. While the AEC should be regulated at the cellular level, their levels vary in response to external and internal shocks; thus, it has been considered as an indicator of the "welfare" of an organism, as well as a diagnostic tool on the capacity body's response to environmental changes (Ivanovici 1980). Therefore, AEC determination, together with levels of phosphagens such as arginine phosphate (ArgP), as an immediate source of energy for the ATP, have been used for multiple applications in aquatic invertebrates, such as ecotoxicology, aquaculture, and fisheries.

This paper aims to obtain information on energy flows in shrimp directly in farming conditions, including two putative conditions of stress: high density and temperature (season of the year). Unfortunately, an unexpected incidence of WSSV occurred on one farm. However, we decided to keep the sampling and obtain further data from this farm as it adds another (biological) stressor: disease resulting from a common pathogen. Finally, the effect of acute handling/ sampling stress was also analyzed for some indicators typically affected by stress to assess if the different farming conditions could affect the characteristics of the short-term response. Therefore, sampling was intended to establish baseline levels of physiological indicators of health status and the magnitude of the stress response induced by shrimp capture in different grow-out conditions.

MATERIALS AND METHODS

Sampling design

Three farms were selected for the present study to consider different cropping systems in commercial shrimp production, mainly based on stocking density differences and biofloc technology (Table 1). Unfortunately, a WSSV outbreak occurred in the extensive farm, as observed by external signs and confirmed by PCR (Apún-Molina et al. 2017). Nevertheless, we decided to keep this farm with the corresponding sampling and analysis, as WSSV will probably affect shrimp responses, adding another component to the comparison.

Shrimp were sampled directly from ponds for all sampling dates (Table 1), considering two groups of organisms for each sampling date-farm combination.

The baseline group sought to minimize stress sampling: shrimp were captured from the feed tray within less than 1 min to avoid any stress response that usually takes several minutes (Aparicio-Simon et al. 2010, Robles-Romo et al. 2014).

In the stress group, shrimp were caught with a cast net and kept in a 60 L plastic container with water without aeration for 20 min. Samples were taken from each shrimp, simulating routine biometry. In addition to handling and crowding, shrimp were exposed to hypoxic stress, attaining levels of 0.5 mg L^{-1}.

The biochemical composition of the hemolymph, muscle, and hepatopancreas was determined in both groups (baseline and stress) to assess whether there was a differential stress response depending on the culture condition, as a clear stress response was previously

Table 1. Shrimp sampling in farms. Culture on the intensive farm (Servicios Acuícolas Profesionales S.A. de CV) was done with biofloc technology. WSSV: white spot syndrome virus.

Farm system	Density (shrimp m^{-2})	Stocking date	Sample 1	Weight (g)	TºC	Sample 2	Weight (g)	TºC
Intensive (I)	100	Apr/2013	Jul/2013	9.0 ± 1.52	31.5	Nov/2013	15.0 ± 2.84	21.2
Semi-intensive (S)	15	Apr/2013	Jul/2013	15.0 ± 3.86	31.3	Sep/2013	35.01 ± 4.93	30.8
Extensive (E-WSSV)	7	May/2013	Sep/2013	25.2 ± 0.73	30.9			

characterized for some of the variables measured, namely glucose and lactate levels in hemolymph (Racotta & Palacios 1998, Mercier et al. 2006, Aparicio-Simón et al. 2010). However, immune responses and bioenergetic status were analyzed only in the baseline group.

Two samples were taken along the production cycle for intensive and semi-intensive farms. In contrast, one sampling was possible for the extensive farm due to a WSSV outbreak that caused mass mortalities afterward.

Therefore, three kinds of responses were analyzed for each farm-sampling date combination: i) biochemical composition of hemolymph, hepatopancreas, and muscle, ii) immunological variables in hemolymph, and iii) energy charge in muscle.

Sample collection and biochemical analyses

The two groups of shrimps (baseline and stress) were considered only for such analyses (n = 15 shrimps for each group-farm-sampling date combination). The hemolymph (approximately 400 μL) was collected from the ventral sinus at the base of the first abdominal segment using a 1-mL syringe rinsed with 5% potassium oxalate in isotonic saline-cooled anticoagulant solution (Racotta & Hernández-Herrera 2000). After hemolymph sampling, shrimp were weighed, frozen in liquid nitrogen, and stored at -70°C for further tissue analysis. Hemolymph was centrifuged at 1350 g for 10 min at 4°C; plasma was separated from precipitated cells for biochemical analyses.

For hemocyanin determination, hemolymph samples were diluted 1:20 with isotonic saline, absorbance was recorded at 335 nm, and the concentration was calculated using an extinction coefficient of $E_{1\%}$ = 2.83 for shrimp hemocyanin (Hagerman 1983). Plasma was diluted 1:100 with isotonic saline for protein determination, according to Bradford (1976), using a commercial chromogen reagent (Bio-Rad) and bovine serum albumin (Sigma) as standard.

Commercial kits were used to determine glucose (GOD-PAP, Randox), lactate (PAP, Randox), and triglycerides (GPO-PAP, Randox). Methods were adapted to a microplate, using 20 μL plasma and 200 μL enzyme chromogen reagent (Palacios et al. 2000). The absorbance was recorded at 490 nm for triglycerides and glucose and at 540 nm for lactate on a microplate reader, and concentrations were calculated from a standard substrate solution.

The hepatopancreas and the first abdominal segment muscle were dissected from the frozen shrimp; one sample (~100 mg) of each tissue was homogenized in 5 mL cold 10% trichloroacetic acid (TCA). Homogenates were centrifuged at 3000 g at 5°C for 15 min, and the resulting deproteinized supernatant was used to determine glycogen by the anthrone colorimetric reaction (Van Handel 1965) and lactate by the same kit as for plasma. Another sample (~100 mg) was lyophilized, rehydrated, and homogenized in saline solution to determine total proteins according to Bradford (1976) and triglyceride by the same kit as for plasma. Total triglycerides were not determined in muscle because of the lack of sensitivity of this techniques in this tissue.

Immunological analyses

For these analyses, only unstressed shrimp (baseline group) were considered (10 per farm for each farm-sampling date). One hundred microliters of hemolymph from the ventral sinus were collected using oxalate as an anticoagulant for hemocyte count. In contrast, a second hemolymph sample without anticoagulant was collected from the pericardial cavity to determine clotting time. For this purpose, a drop of hemolymph was immediately placed on a clean glass slide and slightly tilted in various directions on the slide until clotting, recording the time elapsed.

From the first sample, 50 μL of hemolymph was diluted (1:5, v/v) in a solution of 4% formaldehyde in isotonic saline, and total hemocyte count (THC) was determined with a hematocytometer in two replicates for each sample (Mercier et al. 2006).

Energy charge analyses

The shrimps used for these analyses (10 per farm-sampling date, only unstressed shrimp) were

immediately immersed in liquid nitrogen and then stored at -80°C for further analysis of adenylic nucleotides and arginine phosphate (ArgP) as previously described (Robles-Romo et al. 2014, Apún-Molina et al. 2017). The muscle from the first and second abdominal segment and the entire hepatopancreas were pulverized under cryogenic conditions (liquid nitrogen) in a ball mill mixer (MM400, Retsch, Haan, Germany) for 2 min at a frequency of 25 s^{-1}. The frozen powder (~150 mg) was homogenized in 1.5 mL cold TCA with a rotor/stator mechanical homogenizer (model Tempest IQ2, the VirsTis Company) under cold-ice conditions. The crude extract obtained was centrifuged at 3000 *g* for 10 min at 4°C, and the resulting supernatant was neutralized with dichloromethane at a 1:2.6 ratio. A two-phase system was obtained in this way, in which the superior aqueous phase contained nucleotides at a neutral pH.

Nucleotides were separated by ion pairing reverse-phase high-performance liquid chromatography system (model 1100, Agilent Technologies, Santa Clara, CA) with an octadecylsilane (ODS) C18 column (Hyper Clone 150 mm long, 4.6 mm width, 3 µm particle size diameter, Phenomenex, Torrance, CA), with a security guard cartridge C18 (40 mm long, 3.0 mm width, Phenomenex). Separation of nucleotides was done under isocratic conditions, using a mobile phase of 0.15 M NaH$_2$PO4 buffer, 3 mM tetrabutylammonium as the ion-pairing agent, 8% methanol, adjusted at pH 6.0 with 0.1 N NaOH. The chromatograph was operated at a 0.8 mL min^{-1} flow rate, and, under these conditions, the separation of nucleotides was complete in less than 20 min. The nucleotides were detected at 254 nm (Agilent detector coupled to HPLC system). Identification and concentration calculations of nucleotides were performed with standards of ATP, ADP, and AMP (all from Sigma, St. Louis, MO).

ArgP was also analyzed in samples from the same extract used for nucleotides analysis by HPLC, according to Viant et al. (2001), using reverse-phase Sphere Clone NH$_2$ column (250 mm length, 4.6 mm width, 5 µm particle size diameter, Phenomenex) fitted with an NH$_2$ security cartridge (40 mm length, 3 mm width, Phenomenex). Identification and concentration calculations of ArgP were performed with a standard of purified ArgP (Santa Cruz Biotechnology, Santa Cruz, CA).

All solvents used for HPLC analysis were prepared using HPLC-grade commercial reagents and de-ionized water and then filtered using a 0.45 µm nylon membrane. Data are presented as µmol g^{-1} of tissue (wet weight).

Statistical analysis

The variables were checked for normality and homogeneity using Shapiro-Wilk and Levene's tests. When one of these conditions was not fulfilled, data were logarithm transformed and tested again. Two-way ANOVA was used for biochemical composition to test the main and interactive effects of the farming system and handling stress. When the interaction between both factors was significant, a Newman-Keuls test for mean comparisons was used to compare individual means; otherwise, when only one or both main effects were significant, global means within each factor are mentioned in the text to indicate the particular influence of density and WSSV exposure. For immunological and bioenergetics variables, one-way ANOVA was used to test the farming system's effects on unstressed shrimp's baseline values. All analyses were performed using Statistica 8.0 (StatSoft, Tulsa, OK). Data are reported as mean ± standard deviation, and differences were reported as significant if $P < 0.05$.

RESULTS

Metabolic variables analyzed in hemolymph

A significant effect of farm conditions was observed for total protein in hemolymph. Significantly higher levels ($P < 0.05$) were obtained in shrimp sampled in July in the semi-intensive farm (global mean of both baseline and stress groups: 132.6 mg mL^{-1}), whereas the lowest values were observed in September in the same semi-intensive farm and the second sampling of the intensive farm in November (93.4 and 91.7 mg mL^{-1}, respectively). No significant effect ($P < 0.05$) of stress conditions for handling and overcrowding in any of the farms was observed (Fig. 1a). Similarly, the concentration of hemocyanin in the hemolymph was also significantly higher ($P < 0.05$) in shrimp from the semi-intensive farm in July (global mean 90.1 mg mL^{-1}). In this case, the lowest values were observed for an extensive farm with the incidence of WSSV (38.5 mg mL^{-1}) (Fig. 1b).

The glucose level increased significantly in shrimp following handling stress. Nevertheless, as shown by the significant interaction, this increase was significant only in the case of intensive farm in July (27.7 to 82.6 mg dL^{-1}) and semi-intensive farm in September (35.9 to 92.3 mg dL^{-1}). In contrast, the stress-induced hyperglycemia was completely blunted in the extensive farm with an incidence of WSSV, and the second sampling done in November on the intensive farm. (Fig. 2a). A similar effect was observed for the lactate concentration, with an up to 10-fold increase in shrimp
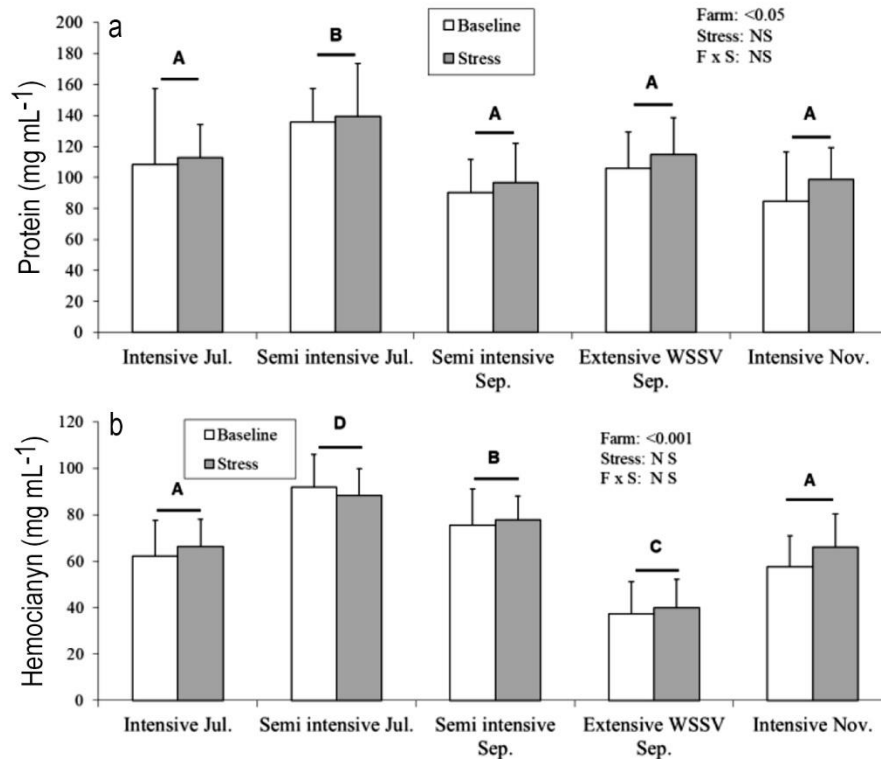
Figure 1. a) Total proteins and b) hemocyanin levels in hemolymph of white shrimp *Penaeus vannamei* from different farms according to the culture density and incidence of White spot syndrome virus (WSSV). Samplings were done at different crop cycle times, under basal conditions or following handling and crowding stress. The data are presented as mean ± standard deviation. Two-way ANOVA significances are inserted in figure (NS: not significant). By the Newman-Keuls test, capital letters indicate significant differences between global means obtained for each farm/sampling date, regardless of stress condition that was not significant.

collected in the intensive farm in July and the semi-intensive farm in both sampling times. A lower, around three-fold, non-significant ($P < 0.05$) increase was observed in shrimp from the extensive farm with the incidence of WSSV and in the second sampling November of the intensive farm (Fig. 2b). For triglyceride levels in hemolymph, a significant main effect of the farm was observed with higher levels in November *vs*. July samplings for the intensive farm. A significant effect ($P < 0.05$) of stress was also observed. However, the individual mean comparison showed that the increase was significant only for the extensive farm with the incidence of WSSV (61.3 *vs*. 80.2 mg dL^{-1}) (Fig. 2c).

Metabolic variables analyzed in tissues

Protein levels in hepatopancreas were significantly affected ($P < 0.05$) by farm, handling stress, and interaction between both factors (Table 2). Protein levels were significantly lower in shrimp from the semi-intensive farm in July than in other farms/

sampling dates. The effect of stress was observed as a decrease in protein levels only for this farm in September. Glycogen concentration in hepatopancreas showed a significant variation between farms, with the lowest levels for semi-intensive farm during July (global mean regardless of stress condition: 8.1 mg g^{-1}) that were significantly different from the same farm in September (11.4 mg g^{-1}) (Table 2). The highest values were obtained on the farm infected with the virus (global mean 14.6 mg g^{-1}), while in the intensive farm, relatively low values were obtained in both sampling periods (9.1 to 10.2 mg g^{-1}).

Additionally, the effect of the stressor was an overall decrease in glycogen levels in this condition (global mean regardless of farm/sampling date: 11.7 *vs*. 9.7 for control *vs*. stressed shrimp, respectively). However, although no significant interaction was obtained when the influence of stress was analyzed for each farm, the effect was statistically significant ($P < 0.05$) only in the farm with WSSV. For triglycerides, significantly higher levels ($P < 0.05$) were observed in
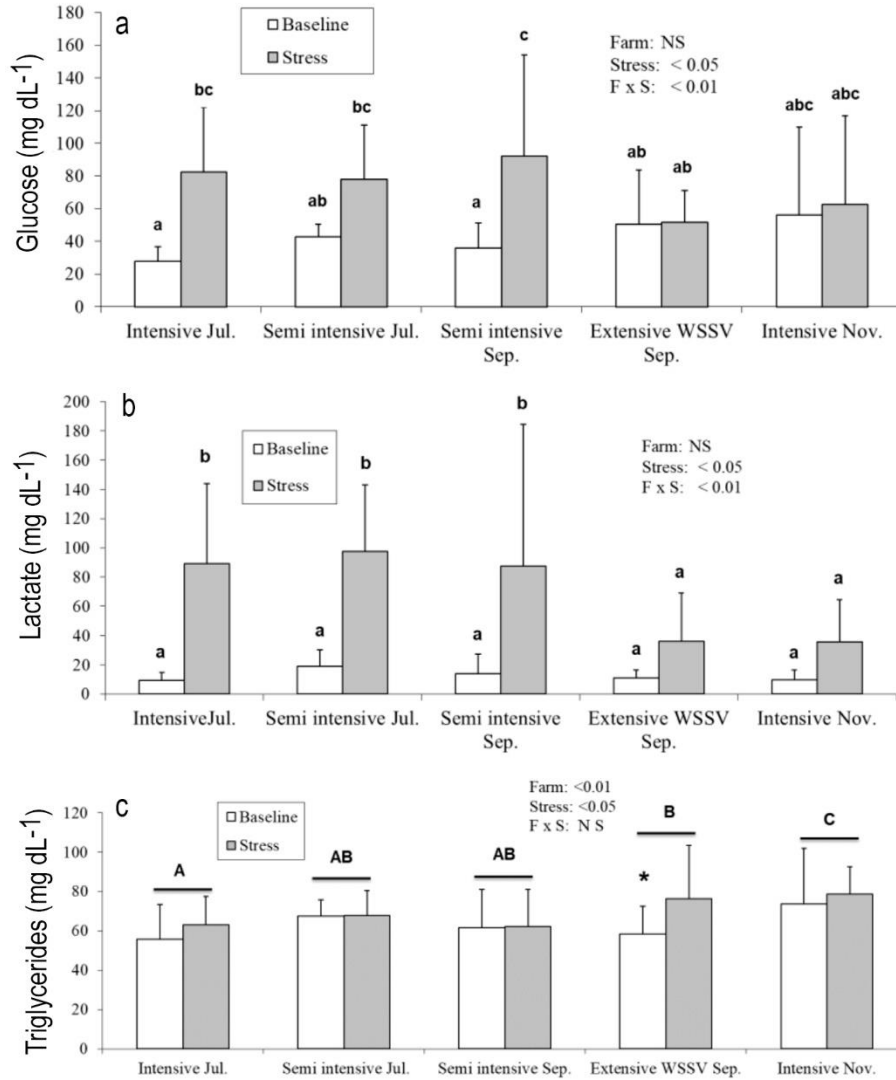
Figure 2. a) Glucose, b) lactate, and c) triglycerides levels in hemolymph of white shrimp *Penaeus vannamei* from different farms according to the culture density and incidence of white spot syndrome virus (WSSV). Samplings were done at different crop cycle times, under basal conditions or following handling and crowding stress. The data are presented as mean ± standard deviation. Two-way ANOVA significances are inserted in figure (NS: not significant). For glucose and lactate levels with significant interaction, individual means with different small letters are significantly different by the Newman-Keuls test. For triglycerides, capital letters indicate significant differences between global means obtained for each farm/sampling date, regardless of stress condition; *indicates the difference between basal and stress groups.

organisms from the farm with the incidence of WSSV (89 mg g^{-1}) compared to other shrimp farms (from 59 to 66 mg g^{-1}), regardless of stress condition (Table 2). Lactate levels were also significantly higher in shrimp farms with WSSV infection (2.15 mg g^{-1}) compared to other shrimp farms that generally had values between 0.55 and 1.1 mg g^{-1}. No effect of handling stress was observed on lactate levels (Table 2).

The influence of stress on protein levels in muscle depended on a particular farm/sampling date, as indi-

cated by a significant interaction (Table 2). Although stress tends to generally decrease protein levels from 536.3 to 485.7 mg g^{-1} (global means for all farms), this effect was significant only for the intensive farm in November, with a decrease from 594.2 mg g^{-1} for the basal group to 351 mg g^{-1} for stress group. The same trend, although not significant, was also observed for the semi-intensive farm in July and the extensive farm with WSSV. Glycogen levels in muscle were not significantly affected ($P < 0.05$) by farm or stress and

Table 2. Metabolic variables (mean ± standard deviation) in white shrimp *Penaeus vannamei* tissues from different farms according to the culture density and incidence of white spot syndrome virus (WSSV). Samplings were done at different crop cycle times, under basal conditions or following handling and crowding stress. Two-way ANOVA results are indicated in the last two columns, indicating the main effects of the farm (F) and stress (S), as well as the interaction between both factors (F×S); * and **$P < 0.05$ and 0.01, respectively, NS: not significant. Individual means with different low letters were significantly different. Otherwise, when a significant main effect of the farm was obtained, capital letters within the columns indicate significant differences between the overall means of each farm, independent of stress condition. The significance between the baseline and stress groups is indicated only in the ANOVA columns for a significant main effect of stress since this factor has only two levels.

| | Sampling dates in farm | | | | | | | | | | Two-way ANOVA | | |
| | July (intensive) | | July (semi-intensive) | | September (semi-intensive) | | September WSSV (extensive) | | November (intensive) | | | | |
	Basal	Stress	Basal	Stress	Basal	Stress	Basal	Stress	Basal	Stress			
Hepatopancreas											F	S	F×S
Total proteins (mg g⁻¹)	156.3 ± 53.8[a]	144.2 ± 29.4[a]	68.7 ± 205[b]	71.4 ± 15.0[b]	153.2 ± 59.6[a]	82.0 ± 31.7[b]	141.0 ± 61.5[a]	131.1 ± 36.0[a]	127.6 ± 25.9[a]	124.7 ± 31.7[a]	**	*	*
Glycogen (mg g⁻¹)	10.24 ± 2.78 AB	9.79 ± 3.13	9.53 ± 5.69 A	6.83 ± 1.97	12.06 ± 4.83 B	10.7 4 ± 4.30	17.61 ± 7.93 C	11.73 ± 4.64	9.12 ± 2.05 AB	9.42 ± 3.30	**	*	NS
Triacylglycerides (mg g⁻¹)	70.2 ± 14.3 A	61.2 ± 11.55	59.9 ± 20.5 A	66.6 ± 17.1	67.5 ± 18.0 A	64.9 ± 11.7	83.8 ± 63.3 B	94.3 ± 38.6	57.2 ± 12.6 A	60.5 ± 13.7	**	NS	NS
Lactate (mg g⁻¹)	1.09 ± 1.32 A	0.93 ± 0.69	1.01 ± 0.85 A	0.89 ± 0.17	0.82 ± 0.66 A	1.01 ± 1.36	2.10 ± 1.86 B	2.20 ± 2.55	0.61 ± 0.54 A	0.55 ± 0.46	**	NS	NS
Muscle													
Total proteins (mg g⁻¹)	439.2 ± 173.9[ab]	536.4 ± 81.30[bcd]	548.70 ± 112.6[bcd]	469.9 ± 106.8[abc]	475.9 ± 78.2[abcd]	553.5 ± 66.6[cd]	619.2 ± 118.8[d]	517.9 ± 148.6[bcd]	594.2 ± 84.0[cd]	351.0 ± 243.8[a]	NS	*	**
Glycogen (mg g⁻¹)	5.23 ± 1.51	5.67 ± 2.36	4.84 ± 2.48	4.65 ± 0.93	5.04 ± 2.63	4.15 ± 1.51	3.99 ± 2.01	4.40 ± 2.17	5.30 ± 2.55	4.90 ± 1.59	NS	NS	NS
Lactate (mg g⁻¹)	8.09 ± 3.06[d]	7.53 ± 2.52[cd]	7.61 ± 4.95[cd]	2.88 ± 1.05[a]	3.66 ± 1.08[a]	3.43 ± 1.12[a]	4.82 ± 1.97[ab]	7.11 ± 1.29[cd]	8.59 ± 0.68[d]	5.83 ± 0.39[bc]	**	*	*

showed values between 4 and 5.7 mg g^{-1} (Table 2). Significant effects of farm, stress, and interaction were observed in lactate levels in muscle. The highest lactate levels were observed in shrimp from the intensive farm sampling in both July (7.8 mg g^{-1}) and November (7.2 mg g^{-1}) (Table 2).

Additionally, lactate levels decreased by the stress condition of 6.54 to 5.35 mg g^{-1}, considering the mean values of all farms (global average). However, as indicated by the interaction, the effect of stress was significant only for the semi-intensive farm in July (from 7.6 to 2.9 mg g^{-1}) and for the intensive farm in November (from 8.6 to 5.8 mg g^{-1}). In contrast, for the extensive farm with WSSV, lactate significantly increased from 4.8 to 7.1 mg g^{-1}.

Immunological variables in hemolymph

The highest THC was observed in the semi-intensive farm in July with a value higher than 6 million cells, followed by the intensive farm also in the July sampling, while the lowest values were found in the samplings between September and November of the three farms (between 2.7 and 4 million cells, see Fig. 3a for significant differences). Hemolymph clotting time was significantly longer ($P < 0.05$) in shrimp of the extensive farm exposed to the WSSV in September (33.1 s), compared with values obtained in July for both the intensive and semi-intensive farms (20 to 21 s). Intermediate values were observed in the semi-intensive farm in September (29.2 s) and the intensive farm in November (26 s) (Fig. 3b).

Energy charge and phosphagens in tissues

In muscle and hepatopancreas, significantly lower values ($P < 0.05$) of AMP and ADP were observed, as well as significantly higher values of ATP in the intensive farm shrimp compared to the semi-intensive farm in the July sample (Table 3), which in turn resulted in a significant difference for the energy charge (0.76 vs. 0.63) in hepatopancreas and (0.92 vs. 0.75) in muscle (Figs. 4a-b).

In the September sampling, shrimp from the farm with white spot incidence had lower values of ATP and AEC (0.54) in hepatopancreas compared to their homologs of the semi-intensive white spot-free farm that were sampled in the same month (0.80) (Table 3, Fig. 4b). However, the opposite effect was observed in muscle with significantly higher levels ($P < 0.05$) of ATP in shrimp from the WSSV farm, although the difference was not significant ($P > 0.05$) for AEC (Table 2, Fig. 4a). ArgP levels in muscle were significantly higher in intensive farm shrimp in both

samplings (4.9 µmol g^{-1}) compared to shrimp from other farms (2.8 µmol g^{-1}) (Fig. 4c).

DISCUSSION

Analyzing biochemical and immunological biomarkers of health status directly at farm conditions is valuable in terms of a direct application to their value to evaluate the physiological condition of shrimp during commercial grow out that implies inevitably different stressful situations (Tu et al. 2010). However, at that level, in contrast to an almost fully controlled experimental level, it is almost impossible to dissociate between different factors that could affect such indicators. Nevertheless, with the sampling schedule used in the present study, it seems reasonable to dissociate among three different identified factors or stressful conditions by specific comparisons between farms:

i) Stocking density by comparing the intensive and semi-intensive farms in July sampling, as the second sampling is not comparable between both farms due to different dates (September vs. November).

ii) Temperature and other environmental factors associated with the sampling date are mainly compared by samplings of July and November at the intensive farm and, to a lesser degree, July and September at the semi-intensive farm because of minimal temperature differences between these two sampling months. However, shrimp size/weight could also have influenced differences between sampling dates on both farms.

iii) The incidence of pathogens, i.e. WSSV, was measured by comparing the semi-intensive and the extensive farms with the incidence of WSSV, both sampled in September. Although there was a difference between stocking densities (7 vs. 15 shrimp m^{-2}), it is considered minimal, and the influence of WSSV should be prevalent.

In addition, the effect of acute sampling stress (handling, crowding, and hypoxia) was also analyzed on biochemical variables in hemolymph and tissues that are typically representative of stress response (e.g. glucose and lactate levels in hemolymph). The purpose was to assess if a chronic stressful condition inherent to farming conditions of this study (temperature, stocking density, or WSSV infection) will affect subsequent acute stress, as commonly observed for other stress situations. Regarding the response to such acute stressors, glucose and lactate increased, whereas glycogen levels in hepatopancreas decreased, confirming
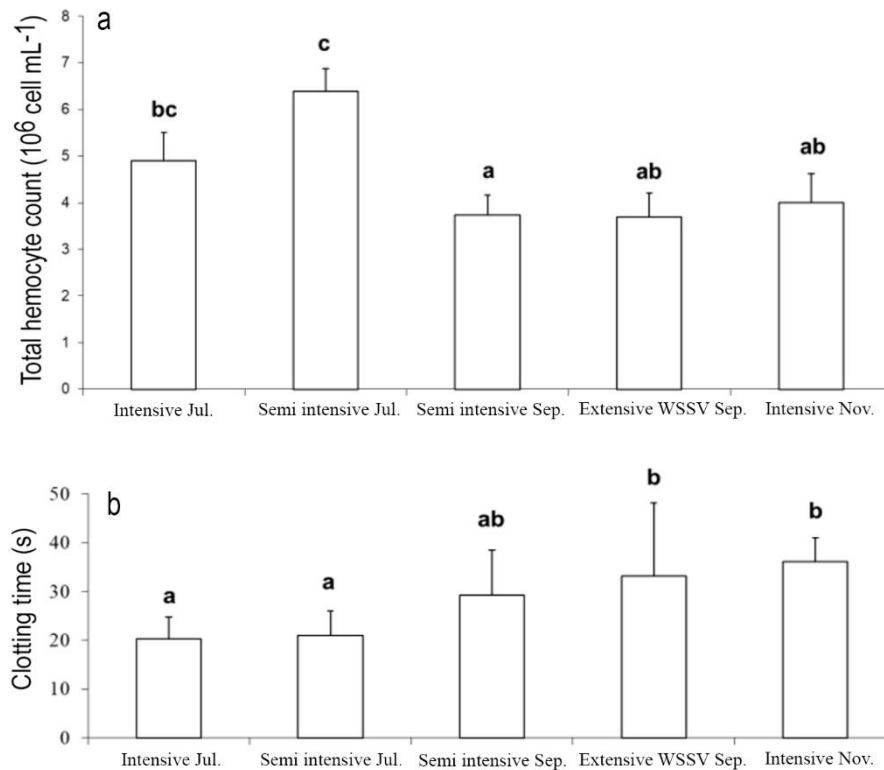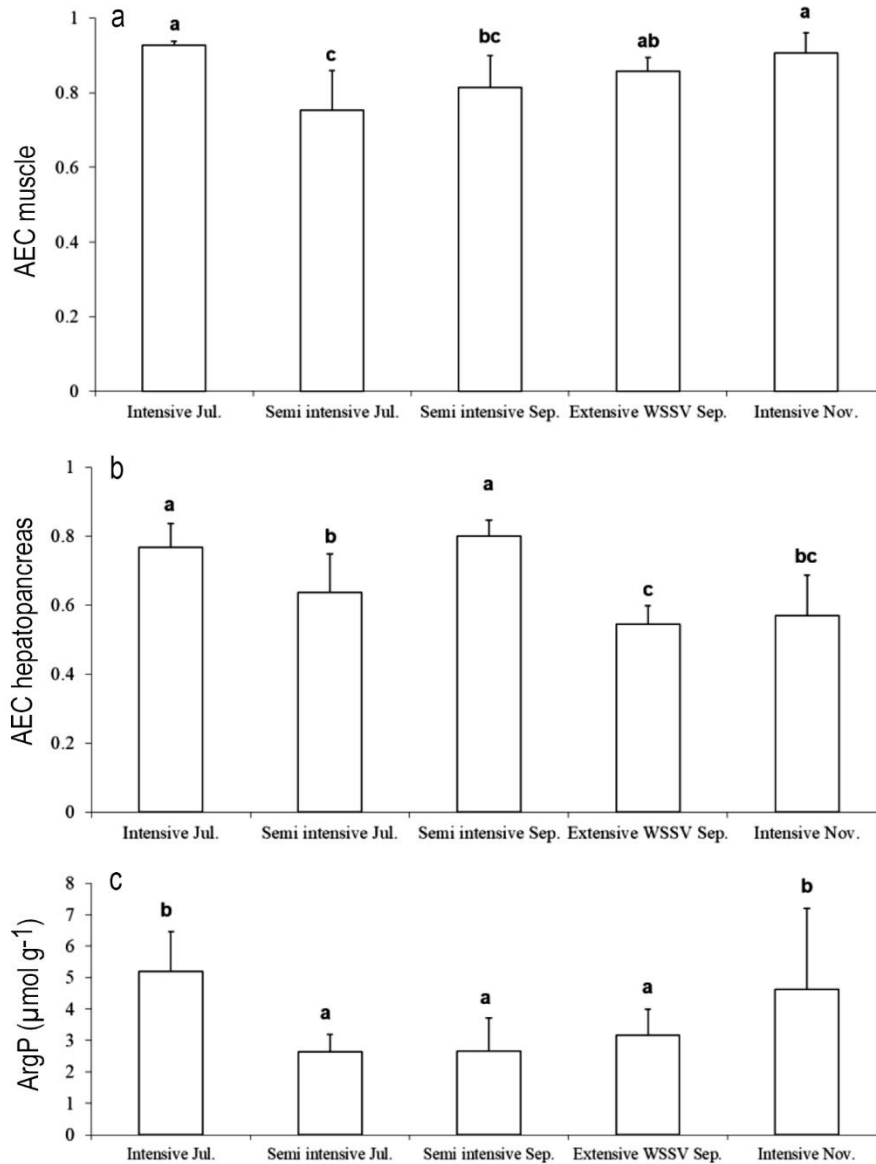
Figure 3. a) Hemolymph total hemocyte count and b) clotting time of white shrimp *Penaeus vannamei* from different farms according to the culture density and incidence of White spot syndrome virus (WSSV). As indicated, samples were done under basal conditions without stress at different crop cycle times. The data are presented as mean ± standard deviation. Following unifactorial ANOVA and Newman-Keuls test means with different letters are significantly different.

Table 3. Levels of individual nucleotides (AMP, ADP, ATP) and total adenylic nucleotides in hepatopancreas and muscle (mean ± standard deviation, µmoles g^{-1} wet weight) of white shrimp *Penaeus vannamei* in three different farms according to the culture density and the incidence of White spot syndrome virus (WSSV). Samplings were made at different times of the crop cycle as indicated, only under basal conditions without stress. Following one-way ANOVA and the Newman-Keuls test means with different letters are significantly different.

Tissue	Intensive July	Semi-intensive July	Semi-intensive September	Extensive WSSV September	Intensive November
Hepatopancreas					
ATP	0.72 ± 0.22[c]	0.48 ± 0.19[ab]	0.61 ± 0.09[bc]	0.42 ± 0.13[ab]	0.30 ± 0.28[a]
ADP	0.35 ± 0.09[a]	0.33 ± 0.13[a]	0.22 ± 0.06[a]	0.49 ± 0.13[b]	0.31 ± 0.22[a]
AMP	0.09 ± 0.03[a]	0.16 ± 0.06[b]	0.07 ± 0.03[a]	0.30 ± 0.06[c]	0.15 ± 0.06[b]
Total adenylic nucleotides	1.16 ± 0.28[a]	0.97 ± 0.32[ab]	0.90 ± 0.13[ab]	1.20 ± 0.25[a]	0.76 ± 0.50[b]
Muscle					
ATP	6.92 ± 1.23[a]	4.75 ± 1.26[b]	5.31 ± 1.29[b]	7.05 ± 0.76[a]	6.55 ± 1.42[a]
ADP	1.01 ± 0.19[b]	2.53 ± 0.85[a]	2,02 ± 0.51[a]	2.18 ± 0.47[a]	1.33 ± 0.88[b]
AMP	0.07 ± 0.03[a]	0.69 ± 0.41[c]	0.38 ± 0.32[b]	0.27 ± 0.13[ab]	0.13 ± 0.16[ab]
Total adenylic nucleotides	8.00 ± 1.36[a]	7.97 ± 0.88[a]	7.71 ± 0.82[a]	9.49 ± 0.76[b]	8.01 ± 1-93[a]

previous studies analyzing the effect of handling (Aparicio-Simón et al. 2010, Robles-Romo et al. 2016) or hypoxia (Martínez-Antonio et al. 2019). However, the magnitude and statistical significance of the responses depended on the conditions of each farm and

season of the year. Following the increase in glucose levels and metabolism associated with stress, a decrease in glycogen levels in the hepatopancreas was observed in stressed shrimp. However, this response was significant only on the extensive farm and will be

Figure 4. Adenylic energy charge (AEC) a) in muscle and b) hepatopancreas, and c) concentrations of arginine phosphate (ArgP) in muscle of white shrimp *Penaeus vannamei* from different farms according to the culture density and incidence of White spot syndrome virus (WSSV). As indicated, samples were done under basal conditions without stress at different crop cycle times. The data are presented as mean ± standard error. Following unifactorial ANOVA and Newman-Keuls test means with different letters are significantly different.

discussed below. Finally, a surprising result was a lower protein content in the muscle of stressed shrimp. On the contrary, exposure to several days of chronic hypoxia (2.5 mg L^{-1}) increased protein levels in muscle (Seidman & Lawrence 1985, Racotta et al. 2002). We could speculate that the stressor applied in this study (handling, crowding, and 0.5 mg L^{-1} hypoxia) is considerably different in exposure time (20 min *vs.* two weeks) and intensity and then could induce proteolysis to free amino acids to fuel energy demand caused by

stress or for specific protein synthesis (e.g. hemocyanin, hypoxia-inducible factor, heat shock proteins) in the hepatopancreas.

Stocking densities

Protein and hemocyanin levels in hemolymph were higher in the semi-intensive farm compared to the intensive one for the July sampling. Variable effects were observed for high-density stress in laboratory conditions with no effect (Aguilar et al. 2012) or a

decrease only for hemocyanin (Apún-Molina et al. 2017), in contrast to the present results obtained at pond level. Several studies have indicated that the level of protein in the hemolymph represents a good indicator of the nutritional status of shrimp (Rosas et al. 2002, Pascual et al. 2003), which could explain why farms with lower density with a probable greater availability of natural food could present a higher level of protein, given that natural biota contributes to the nutrition of *P. vannamei* (Leber & Pruder 1988, Burford et al. 2004). However, the results of hemolymph contrasted with other indicators of nutritional status, such as protein and glycogen in hepatopancreas, with lower levels in shrimp reared at lower density (semi-intensive farm). Moreover, energetic status inferred from AEC and ArgP levels was also lower in those shrimps. Therefore, additional factors such as biofloc rearing in the intensive farm seem likely to be involved. Indeed, the microbial community represents an additional food web for shrimp and could explain a higher physiological condition (Emerenciano et al. 2022). In turn, biofloc culture implied a highly efficient aeration compared to traditional aeration in the semi-intensive in which some degree of hypoxia was probably occurring, resulting in increased hemolymph levels of protein and hemocyanin as previously observed in shrimp under chronic hypoxic conditions (Racotta et al. 2002).

Several studies have analyzed the influence of high density on the immune response capacity, obtaining different results. In the short term (3 to 12 h), they get a high-density depressed immune system, inferred from various immune effectors, including THC (Lin et al. 2015). On a long-term basis, other studies did not obtain any influence of high density on THC (Apún-Molina et al. 2017, Baladrat et al. 2022). Similarly, in the present work, THC and clotting time were not affected by stocking density when comparing the intensive farm (100 shrimp m^{-2}) with the semi-intensive one (15 shrimp m^{-2}).

Stress response regarding glucose and lactate increase was similar in magnitude in both farms sampled in July and September for the semi-intensive farm, indicating that a previous putative chronic stress condition (high density) does not affect the response to an acute stressor during sampling. In contrast, Martínez-Antonio et al. (2019) observed that shrimp under chronic stress conditions imposed by low salinity and reared at high densities presented a lower ability to respond to subsequent acute stressors such as hypoxia or escape response. Such a topic deserves further research in shrimp, as multiple stress conditions exist in culture conditions, and overall performance needs to understand what kind of stressors could affect capacities to overcome subsequent stressors. On the other hand, glycogen in hepatopancreas was differently affected by sampling stress with a decrease only for the semi-intensive farm, contrary to an expected more accentuated acute sampling stress response following putative chronic stress due to high density occurring in the intensive farm. However, as previously discussed, the biofloc could produce higher nutritional conditions and overcome the apparent dual stress condition.

Based on the results obtained in the present work, it could be speculated that 100 m^{-2} is not a stressful density at the pond level, and the overall nutritional and physiological condition of shrimp seems even better than at lower density, most likely due to biofloc. Similarly, the use of biofloc allowed doubling density (postlarvae, PL) from 300 to 600 PL m^{-3} without affecting the overall performance and physiological condition in terms of glycogen levels in hepatopancreas and AEC in muscle, which were otherwise affected at 600 PL m^{-3} in the absence of biofloc (Guemez-Sorhouet et al. 2019). These results align with the trend toward super-intensive shrimp farming, especially when combined with biofloc technology (Emerenciano et al. 2022).

Sampling date (temperature)

The magnitude of the sampling stress response is the most striking result. Indeed, the hyperglycemic stress response was no longer present in November for two reasons: higher baseline levels and lower stress levels compared to July, although not significantly. On the other hand, the magnitude of the stress-induced lactate increase is considerably lower and no longer significant in the November sampling compared to July. Our research team previously observed such differences when sampling shrimp from ponds at different times of the year with a cast net, implying a stronger stress response in autumn and spring samplings compared to winter (Arcos et al. 2009). A lower stress response can be attributed to lower temperatures related to the season of the year. Indeed, hypothermia is used in shrimp as a sedative procedure to reduce metabolic activity before hemolymph sampling (Pascual et al. 2003).

Similarly, when shrimp were captured from ponds with a cast net, the resulting stress response characterized by increased glucose and lactate levels was partially blunted by placing shrimp in water at 5°C below pond temperature (Zamora 2012). The decrease in muscle protein related to sampling stress discussed previously was observed only in November in contrast

to July, when protein levels tend to increase, although not significantly. Again, this result could be surprising, especially if a low temperature attenuated the stress response. Other factors such as shrimp age or size influence such response; for example, bigger shrimp could elicit stronger muscle contraction, affecting the protein contractile machinery.

Other differences unrelated to stress response between seasons were higher levels of TG in hemolymph in November for the intensive farm but without differences in hepatopancreas. Therefore, it seems unlikely that the overall TG reserves were affected by season. Still, the more immediate available TG pool for different tissues could be increased as a mechanism for thermal compensation. Therefore, in addition to increased enzymatic activities for lipid metabolism as an overall mechanism to compensate for the decreased temperature-dependent metabolism (Guderley 2004), we suggest an increase in substrate concentration could also contribute to such compensation. An increase in plasma TG at low temperatures, although not significant, was previously observed in *P. vannamei* and was attributed to the rupture of hepatopancreas tubules observed in that study (Wang et al. 2019), although at a considerably lower temperature (13°C) reached in a shorter period (7.5°C d^{-1}), compared to the present study.

No differences in immune or bioenergetic variables were obtained between both seasons for the same intensive farm, except for a lower AEC in hepatopancreas in November. Previous studies have related motor activity to ATP levels and AEC in muscle, although not measured in hepatopancreas (Giesy & Dickson 1981, Robles-Romo et al. 2014). Thus, a lower metabolism in November due to lower temperature could explain such results in hepatopancreas as the most important metabolic organ in crustaceans.

Regarding differences within the same farm regarding sampling date, we could also consider the semi-intensive farm. However, the difference in temperature between July and September was minimal, with average values of 30 and 31°C, respectively. Therefore, additional unknown factors could explain such differences, which consisted first of lower protein levels and hemocyanin in hemolymph in September, which could be related to differential nutritional status or oxygen levels, as previously discussed. The increase in body weight between July and September cannot explain this difference because the concentration of proteins and hemocyanin increases concomitantly with body weight (Rosas et al. 2002). On the other hand, a lower THC and a higher AEC in hepatopancreas were

obtained in September, but no clear explanation can be provided in this case.

WSSV outbreak

In September, the extensive farm suffered a widespread WSSV; therefore, the shrimp sampled showed clear signs of infection. Several differences were observed when comparing the physiological variables obtained in these shrimps with those from other farms, particularly the semi-intensive farm in September, which had the most similar condition. The drastic decrease of hemocyanin as well as of the hemocyanin/protein ratio (0.35 compared to 0.63 to 0.94 for the other farms) implies a particular affectation of this circulating protein, which is most likely related to the role of hemocyanin in the immune response at different levels (Destoumieux et al. 1997, Xu et al. 2008). On the other hand, it is known that the concentration of hemocyanin is affected by hypoxia, molt cycle, and nutritional status (Boone & Schoffenich 1979, Hagerman 1983), which are in general affected by the advanced stage of the disease, as decrease in oxygen uptake and feed intake are well-known alterations in sick shrimp (Yoganandhan et al. 2003)

As for the effect related to the sampling date, the stress response was blunted in terms of plasma glucose and attenuated in terms of plasma lactate by the WSSV outbreak. Viral infection, including WSSV, induced important metabolic changes, especially increased glycolytic flux or Warburg effect (Chen et al. 2011, Apún-Molina et al. 2017). However, some discrepancies about specific effects exist in the literature, probably related to the time-course responses and degree of infection (see Discussion in Apún-Molina et al. 2017). For example, a dual response was observed following WSSV infection in shrimp with a primary increase in glycolytic flux (glucose consumption and lactate production) at 12 h post-infection (hpi) to provide energy for viral replication followed by a second phase in which there is metabolic exhaustion, characterized by an increase in ADP/ATP ratio and a decrease in plasma glucose and lactate from 24 hpi (Chen et al. 2011). In this context, it seems likely that the stress response capacity of shrimp is no longer occurring on extensive farm because of the metabolic incapacity to sustain glycolysis. However, this is not related to a depletion of reserves as glycogen levels in hepatopancreas under baseline conditions, as shrimp from the WSSV farm presented the highest levels of glycogen that decreased to the same level as in the other farms following stress.

Moreover, this farm's lactate levels in hepato-pancreas were the highest, suggesting that the WSSV Warburg effect is still occurring. On the other hand, lactate increased in muscle following stress only in shrimp from this farm in contrast to other farms in which lactate did not change or even decrease (e.g. the semi-intensive farm in July) despite the strong increase in hemolymph for most farms analyzed in the present study. Such difference between muscle and hemolymph lactate levels related to stress was already reported in previous studies, in which it was suggested that the clearance capacity of lactate from muscle to hemolymph is very efficient in shrimp (Aparicio-Simón et al. 2010) but seems to be compromised by WSSV infection. These results indicate that, taken together, the metabolic disruption caused by WSSV alters the subsequent response to acute environmental/ manipulation stressors. However, the precise mecha-nisms and the consequences of survival due to combined infection and stress remain to be established.

Lipid metabolism is also altered during WSSV infection: Hsieh et al. (2008) observed an increase in the concentration of TG at 36 h followed by a decrease from 72 h, whereas Chen et al. (2011) observed a constant decrease in the concentration of TG in plasma in shrimp infected by WSSV and suggests that this reduction was due to the use of TG both for energy production and synthesis of macromolecules used in the replication of the virus. Recently, analysis of lipid droplet staining, lipase activity, and beta oxidation/ lipogenesis inhibition also supported an important role of lipid metabolism in the different stages of virus infection: lipolysis at the viral genome replication stage and lipogenesis at later stages of virion morphogenesis (Ng et al. 2023). Interestingly, in the present work, WSSV-infected shrimp present a TG-based stress response instead of a typical glucose/lactate response, as indicated by the increase in TG in hemolymph following stress. Moreover, the levels of TG in hepatopancreas were also higher in these shrimps, regardless of stress. WSSV induced a sort of switch from carbohydrate to lipid-based metabolism, asso-ciated with the preferential use of carbohydrates (Warburg effect) for virus replication (Chen et al. 2011). Alternatively, shrimps from the extensive farm with WSSV outbreak are already in a sort of metabolic depression in which main reserves of hepatopancreas, such as glycogen and TG, are spared. Such a possibility was suggested in crabs with bacterial infection (Thibodeaux et al. 2009) and shrimp with viral infection (Apún-Molina et al. 2017) based on increased levels of ATP and AEC, which, however, was not the case in the present work as AEC was lower in shrimp from the WSSV farm.

Regarding the two immunological variables measured in the present work, a trend of decreased THC and increased clotting time was observed in shrimp from the WSSV farm compared to other farms. Delayed or even absence of clotting capacity has been previously reported in shrimp with WSSV (Yoganandhan et al. 2003, Apún-Molina et al. 2017) or bacterial infection (Lightner & Lewis 1975). Similarly, decreased THC is a clear sign of infection (Yoganandhan et al. 2003, Vaseeharan et al. 2013) and was explained by hemocyte migration to the site of infection to counteract the pathogen by phagocytosis, production of phenoloxidase and peneaidins, among other immune mechanisms (Song et al. 2003, Joseph & Philip 2007).

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