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Effect of diets containing different levels of highly unsaturated fatty acids on physiological and immune responses in Pacific whiteleg shrimp *Litopenaeus vannamei* (Boone) exposed to handling stress

Laurence Mercier[a], Ilie S. Racotta[a, d], Gloria Yepiz-Plascencia[b], Adriana Muhlia-Almazán[b], Roberto Civera[a], Marcos F. Quiñones-Arreola[a], Mathieu Wille[c], Patrick Sorgeloos[c] and Elena Palacios[a, e, *]

[a] Centro de Investigaciones Biológicas del Noroeste, Mar Bermejo 195, Playa Palo de Santa Rita, La Paz, Baja California Sur 23090, Mexico
[b] Centro de Investigación en Alimentación y Desarrollo, Carretera a La Victoria, Km. 0.6 A.P. 1735, Hermosillo, Sonora 83000, Mexico
[c] Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Rozier 44, B-9000 Gent, Belgium
[d] Ifremer, UMR M100, Laboratoire de Physiologie des Invertébrés, BP 70, Centre de Brest, 29280 Plouzané - France (on sabbatical leave)
[e] UMR/CNRS Université de Bretagne Occidentale; Institut Universitaire Européen de la Mer LEMAR-Laboratoire des sciences de l'environnement marin (UMR 6539) Technopole Brest Iroise, 29280 Plouzané - France (on sabbatical leave)

*: Corresponding author : E. Palacios, email address : epalacio@cibnor.mx

Abstract:

Juveniles fed a diet containing a low or a high level of highly unsaturated fatty acids (HUFA) for 38 days were exposed to handling stress. In a first experiment, stress was applied daily for 30 days, after which the physiological and immunological variables were measured, whereas in a second experiment, stress was applied once and samples were obtained 1 and 24 h after the stressor event. Shrimp that were stressed for 30 days showed significantly lower survival, final weight and feed consumption compared with unstressed shrimp. The concentration of the high-density lipoprotein β-glucan-binding protein was significantly higher in shrimp fed the high-HUFA diet. The glucose concentration in the haemolymph was significantly higher in long-term stressed shrimp compared to controls. The lactate level in the haemolymph was significantly lower in shrimp fed the high-HUFA diet. Lactate and glucose in the haemolymph increased in the 1-h stressed shrimp, but returned to normal levels in 24-h stressed shrimp. A negative effect of repeated-handling stress applied for 30 days was mainly observed on biological performance, whereas the single-stressor event had a more pronounced effect on shrimp physiological and immune responses measured 1 and 24 h after the stressor. A beneficial role of enrichment with HUFA on tolerance to handling stress was observed on immune response capacity.

Keywords: anion superoxide; clotting time; glucose; haemocyte; HDL–BGBP; HUFA

1. Introduction

Penaeid shrimp, like other crustaceans, have a limited ability to synthesize highly unsaturated fatty acids (HUFA) from the polyunsaturated linoleic (18:2n-6) and linolenic (18:3n-3) acids (Castell 1983; D'Abramo 1997). Consequently, HUFA, such as docosahexaenoic (22:6n-3, DHA), eicosapentaenoic (20:5n-3, EPA), and arachidonic (20:4n-6, ARA) acids are considered essential and dependent on dietary supply. HUFA enrichment in diets improved growth and/or survival in larvae (Léger & Sorgeloos 1992), postlarvae (Millamena, Bombeo, Jumalon & Simpson 1988; Rees, Curé, Piyatiratitivorakul, Sorgeloos & Menasveta 1994), and juveniles (González-Félix, Gatlin, Lawrence & Perez-Velazquez 2003) of penaeid shrimp.

A greater resistance to environmental short-term stressors, such as a decrease in salinity and temperature, was also observed after having fed shrimp with diets containing high levels of HUFA (Tackaert, Abelin, Dhert & Sorgeloos 1989; Rees *et al*. 1994; Kontara, Coutteau & Sorgeloos 1997; Chim, Lemaire, Delaporte, Le Moullac, Galois & Martin 2001; Palacios, Bonilla, Pérez, Racotta & Civera 2004). In fish (Kiron, Fukuda, Takeuchi & Watanabe 1995; Bell, Ashton, Secombes, Weitzel, Dick & Sargent 1996; Montero, Socorro, Tort, Caballero, Robaina & Vergara 2004) and molluscs (Delaporte, Soudant, Moal, Lambert, Quéré, Miner, Choquet, Paillard & Samain 2003), dietary HUFA were shown to influence the immune system. This effect in penaeid shrimp is still poorly studied and to date, only the work of Chim *et al*. (2001) has shown an increase of two immune parameters, the respiratory burst and agglutination titres of plasma after having fed juvenile *Litopenaeus stylirostris* (Stimpson) with a diet enriched with HUFA for 28 days.

During grow-out, shrimp are usually exposed to short-term (hours to a few days) and/or long-term (one to several weeks) stressful conditions produced by environmental variations, biological conditions, or handling procedures. Stress plays a very important role in shrimp susceptibility to disease because it reduces their immune defence capacity (Le Moullac & Haffner 2000). Determination of immunological variables, such as total haemocyte count (THC), capacity of reactive oxygen intermediate production, and plasma protein concentration have been suggested as parameters to monitor immune status (Rodríguez & Le Moullac 2000). Haemolymph clotting time that is also a component of the immune system, was proposed as an indicator of stress in crustaceans (Jussila, McBride, Jago & Evans 2001). A high density lipoprotein present in haemolymph acts as a beta glucan binding protein (HDL-BGBP) and is involved in the recognition of extraneous bodies (Vargas-Albores & Yepiz-Plascencia 2000). It has been suggested that HDL-BGBG could be implicated in the beneficial effect of HUFA on the capacity of immune response (Chim *et al*. 2001) and it has been observed that HDL-BGBP mRNA levels are influenced by diet (Muhlia-Almazán, Sánchez-Paz, García-Carreño, Peregrino-Uriarte & Yepiz-Plascencia 2005) .

Measurement of physiological variables, such as glucose (Hall & van Ham 1998; Racotta & Palacios 1998; Mugnier & Justou 2004), lactate (Racotta & Palacios 1998), and osmoregulatory capacity (Charmantier & Soyez 1994; Lignot, Trilles & Charmantier 1997) have also been proposed as indicators of stress. Other physiological variables, such as haemocyanin, total proteins, total lipids, triacylglycerides, and cholesterol were shown to be useful for monitoring shrimp nutritional status and health (Sánchez, Pascual, Sánchez, Vargas-Albores, Le Moullac & Rosas 2001; Pascual, Gaxiola & Rosas 2003; Rosas, Cooper, Pascual, Brito, Gelabert, Moreno, Miranda & Sánchez 2004; Carrillo, Zaldívar & Rosas 2006).

In a previous study, we exposed *Litopenaeus vannamei* (Boone) to a repeated handling stress for 4 weeks and observed decreased levels of protein, total lipids and triglycerides levels in haemolymph of stressed shrimp at the end of the experiment (Mercier, Palacios, Campa-Córdova, Tovar-Ramírez, Hernández-Herrera & Racotta 2006). However, immune parameters such as THC and superoxide anion production measured in that study were not affected by the stressful conditions. To test if this lack of effect on immune parameters were a result of an adaptation to a long-term stressor, in the present work we compare the influence of long vs. short term handling stress on some physiological and immune responses in *L. vannamei.* The possible beneficial role of HUFA counteracting adverse effects of long or short-term stress was also evaluated.

2. Materials and methods

Diet preparation

A HUFA-enriched diet was formulated at the Centro de Investigaciones Biológicas del Noroeste (CIBNOR) using the software MIXIT-WIN® (Agricultural Software Consultants, San Diego, CA, USA) and it was made as follows: Dry ingredients were pulverized, passed through a 0.25-mm mesh sieve, and mixed in a baker mixer. Soybean lecithin and 50% HUFA emulsion (ICES Standard Reference Emulsion 50/0.6/C, Ghent University, Ghent, Belgium; ICES 1997) were added to dry ingredients and homogenized. Water was blended into the mixture to attain a consistency appropriate for pelleting. The resulting mixture was passed through a meat grinder and a 3-mm die. The diet was dried in a forced-air oven at 40°C until reaching 9-10% moisture content, and was then stored in plastic bags at –20°C. A control diet containing low-HUFA content was also prepared, as described above, substituting the 50% HUFA emulsion by a 0% HUFA emulsion (ICES Standard Reference Emulsion 0/-/C, Ghent University, Ghent, Belgium). Both diets were formulated to be isolipidic and iso-nitrogenous, according to *L. vannamei* nutritional requirements (Akiyama & Dominy 1989; Camba, Pedrazzoli,Yaguachi & Akiyama 1993). Diet formulations and proximate compositions are presented in Table 1. The profile of fatty acids was also determined and the results are shown in Table 2. For convenience, experimental diets were termed low- and high-HUFA diets (0% or 50% HUFA emulsion enrichment, respectively).

Animals and experimental designs

Juvenile Pacific whiteleg shrimp *L. vannamei* were captured from culture ponds at CIBNOR and only animals believed to be healthy of 11.1 ± 0.7 g (mean ± standard deviation, SD) were used for the experiments. Selected shrimp were transferred to 18 outdoor concrete tanks (1.38 × 1.11, side by side) at density of ~ 20 organisms m^{-2} (30 shrimp/tank, i.e. 270 shrimp in 9 tanks for each dietary treatment, see also specific experimental designs for each experiment). Tanks were continuously supplied with filtered seawater (15-µm filter) at a rate of 1.2 L min^{-1}, corresponding to a 200% daily water exchange, and each tank was also equipped with one air stone. Temperature was maintained at 26.6 ± 1.3°C with salinity at 36 ± 1 g L^{-1} Photoperiod and illumination was natural. Tanks were shaded by a metal roof.

Shrimp were first acclimated for five days to the tanks, while they were fed twice daily a commercial diet (35% protein, PIASA, La Paz, B.C.S., Mexico). Low-HUFA and

high-HUFA diets were then provided. Total daily feed was initially set at 4% of the biomass in each tank, distributed manually in two rations (50% at 09:00 am and 50% at 06:00 pm). Rations were adjusted daily, based on apparent consumption, where the added feed closely matched the maximum feed ingested, with little or no feed remaining. Two experiments were conducted: in Experiment 1 (12 tanks), the stressor was applied daily for 30 days; in Experiment 2 (6 tanks), the stressor was applied once at the end of the experimental period. In this way, the effects of long vs. short-term stress could be compared.

Experiment 1: Each dietary treatment was randomly assigned to six tanks. Then for each treatment, three were submitted to a repeated handling stressor and three were kept as a control. The stressor was applied each morning. Stress procedure consisted of capturing shrimp and transferring them to confinement conditions (30 shrimp in 6 L seawater) for 5 min. Shrimp were then exposed to air for 10 s and finally returned to their tanks. To avoid adaptation of the shrimp to the long-term stressor, as suspected in previous work (Mercier *et al*. 2006), shrimp air exposure was increased by 5 s each week. Shrimp in the control tanks were not subjected to stress and special care was taken to make sure that shrimp were minimally disturbed. In each tank, a semi-quantitative evaluation of feed consumption was made every morning before the first feeding. Uneaten pellets were removed with a siphon and the weight of the recovered feed was determined using pre-defined measures. Exuviates were also removed daily and counted to determine the number of total moults by treatments. Dead animals were replaced by tagged shrimp to maintain the same density. At day 38 after initiating the experiment (30 d on stress schedule), shrimp were sacrificed to measure the biochemical composition and immunological variables in haemolymph. Tagged animals were not sampled. Body weight was recorded for all sampled shrimp at the end of the experiment.

Experiment 2: Each dietary treatment was randomly assigned to three tanks. After 38 d, a single handling stressor was applied, using the same procedure than for experiment 1. Samples for measurements of biochemical composition and immunological variables were obtained at 1-h and 24-h after the stressor event. Control shrimp were sampled directly from the tanks. As in Experiment 1, dead shrimp were replaced by tagged shrimp, which were not sampled.

Sampling and analyses of biochemical composition, immunological variables, and osmotic pressure

Shrimp were not fed 12 h before and during sample collection. Haemolymph (200 µl) was obtained from the ventral sinus at the base of the first abdominal segment using a 3-ml syringe rinsed with a 5% sodium oxalate in isotonic saline cooled anticoagulant solution (Mercier *et al*. 2006): 60 shrimp in Experiment 1 (n = 15 for each “diet-stress” combination) and 36 shrimp in Experiment 2 (n = 6 for each “diet-time after stress” combination). Lactate, glucose, total protein, haemocyanin, triacylglycerides, cholesterol, and total lipid concentrations were determined in haemolymph. Techniques used for haemolymph collection and analyses of metabolic variables were previously described in Mercier *et al*. (2006).

For osmotic pressure and clotting time, haemolymph was obtained from different shrimp without using anticoagulant: 36 in Experiment 1 (n = 9 for each “diet-stress” combination) and 36 in Experiment 2 (n = 6 for each “diet-time after stress” combination). Haemolymph samples were withdrawn without anticoagulant from the pericardial cavity through the intersegmental membrane between the cephalothorax and the abdominal segment. A first sample (10 µL) was obtained using a pipette with a tip cooled to 4 °C for the determination of osmotic pressure, using a vapour pressure

osmometer (Model 5520, Wescor, Logan, UT, USA). A second sample of haemolymph was collected from the pericardial cavity to determine clotting time, as described by Jussila *et al*. (2001), with minor modifications. Briefly, a capillary test tube (inner diameter: 1.1–1.2 mm; length: 75 mm; Corning, Garner, NC, USA) was filled with haemolymph to 30% capacity. The tube was then turned slowly several times to allow haemolymph flowing through the tube until haemolymph set. Time required for haemolymph to clot in the tube was recorded as clotting time. When haemolymph did not clot within 60 s, the result was not considered.

Haemolymph from the ventral sinus was collected in another set of shrimp, but using EDTA as the anticoagulant (Vargas-Albores, Guzmán & Ochoa 1993) with haemolymph: EDTA dilution of 1:2.5. A total of 36 shrimp were used in Experiment 1 (n = 9 for each "diet-stress" combination) and 36 shrimp in Experiment 2 (n = 6 for each "diet-time after stress" combination). Total haemocyte count and superoxide anion production were analysed from haemolymph samples, as previously described (Mercier *et al*. 2006).

Another group of 36 shrimp in Experiment 1 (n = 9 for each "diet-stress" combination) was used to determine fatty acids in haemocytes and the quantification of the HDL-BGBP in plasma, using EDTA as the anticoagulant, as described above. Haemolymph was then transferred to a sterile glass tube and centrifuged at 800 *g* for 5 min (5°C). Precipitated haemocytes were carefully transferred to a glass vial, using a sterile Pasteur pipette and plasma was transferred to microtubes that were immediately frozen at –70°C. For the determination of fatty acids, pools of haemocytes from three organisms were made for each treatment (n = 3 for each "diet-stress" combination). For the quantification of HDL-BGBP, plasma samples were individually analysed.

Fatty acid and HDL-BGBP analyses

Fatty acid composition in haemocytes was determined, as described by Palacios, Racotta, Aparicio, Arjona and Martínez-Palacios (2007). Briefly, total lipids were extracted with chloroform/methanol. Fatty acids were separated into neutral and polar fractions. Each fraction was trans-esterified with boron-trifluoride methanol and methyl esters were analysed using gas chromatography coupled to a flame ionization detector.

Concentration of HDL-BGBP was determined by an indirect enzyme-linked immunosorbent assay (ELISA), following the protocol described by Kemeny and Challacombe (1988) and using as a primary antibody a polyclonal antibody raised against L. vannamei HDL-BGBP in rabbits. As we previously shown by Western blotting, the antibody is highly specific for HDL-BGBP and has no cross-reactivity to other shrimp hemolymph proteins (Yepiz-Plascencia, Vargas-Albores, Jimenez-Vega, Ruiz-Verdugo & Romo-Figueroa 1998). Plasma samples (containing the HDL-BGBP) from the different treatments were diluted (1:2400) and then 100 L were added in the first row and serially diluted down the 96 well ELISA plates to be coated. The plates were covered with aluminum foil and incubated overnight at 4°C. After incubation, the plates were washed once with TBS-T buffer (0.10 M Tris-HCl, 0.05% Tween 20, pH 7.4, washing buffer), and the non-specific binding sites were blocked with washing buffer plus 3% skim milk for 1 h at room temperature. After 4 washes of 3 min each, 100 µL of the anti-HDL-BGBP polyclonal antibody diluted 1:2000 were added. The plates were incubated for 2 h at room temperature, then the wells were washed, as described above, and goat anti-rabbit IgG antibody, conjugated with alkaline phosphatase (Bio-Rad 170-6518, Hercules, CA, USA; dilution 1:3000), was added. The plates were incubated 1 h at room temperature, washed as described above, and 100 µL of substrate solution (alkaline phosphatase substrate kit, Bio-Rad 172-1063, Hercules, CA, USA) was added to each well. After color development, the reaction was stopped by adding 100 µL (0.4 N) NaOH.

Absorbance was recorded at 405 nm with a microplate reader. In each plate, two negative controls were included, one omitting the primary and the other the secondary antibody solutions. A standard curve prepared with purified HDL-BGBP as reported before (Yepiz-Plascencia et al. 1998) was also included in each plate and used to calculate the concentration of HDL-BGBP in the samples. All determinations were done in duplicate.

Statistical analysis

Variables were checked for normality and homogeneity (Sokal & Rohlf 1995) and when one of these conditions was not fulfilled, data were transformed and tested again. Two-way analyses of variance (ANOVA) were applied to all metabolic and immunological variables, using low-HUFA and high-HUFA diets as the first independent variable and stress treatments (for Experiment 1: control and stress groups; for Experiment 2: control, 1-h, 24-h) as the second independent variable. Two-way ANOVA were also used to analyse initial and final weight, survival, total moults, and feed consumption measured in shrimp from Experiment 1. Differences between means for each group (individual means) were determined by a post-hoc Tukey test only when the interaction between both independent variables was significant. Otherwise, significant differences between global means (pooled means of either dietary treatments or stress condition) are indicated in the text of the result section. Analyses were performed using STATISTICA^{TM} version 5.5 and differences were reported as significant if $P \leq 0.05$. Data are reported as mean ± standard error (SE).

3. Results

Experimental diets showed a similar proximate composition (Table 1), but a different fatty acid composition (Table 2). The high-HUFA diet was characterized by a HUFA content of 2.1%, while the low-HUFA diet contained only 0.07% HUFA. Consequently, HUFA was considered the most determining factor of the nutritive quality of the dietary treatments.

Experiment 1

Biological performance

No difference in weight was observed at the beginning of the experiment. In contrast, shrimp exposed to handling stress for 30 d in both HUFA treatments showed a significant lower weight (global mean: 11.6 ± 0.1 g) than control shrimp (global mean: 12.8 ± 0.2 g) (Table 3). Survival was relatively high by the end of experiment, although the significant interaction indicated that handling stress decreased survival only for shrimp fed the low-HUFA diet. Number of total moults was significantly higher for shrimp exposed to the long-term stressor (global means of both dietary HUFA treatments: 35 ± 2.8 for control vs. 49 ± 3.0 for stressed shrimp) and no difference in response to diet was observed. Although experimental diets were well accepted by the shrimp, consumption of the high-HUFA diet was lower compared to the low-HUFA diet (global means: 11.1 ± 0.1 for the low-HUFA diet vs. 10.6 ± 0.1g for the high-HUFA diet), and the long-term stressor also significantly affected feed consumption (global means: 11.3 ± 0.1 for control vs. 10.5 ± 0.1 g for stressed shrimp).

Haemolymph biochemical composition and osmotic pressure

A significantly lower concentration of haemocyanin and total proteins was observed in shrimp exposed to the handling stress, compared to control shrimp (haemocyanin global means: 82.9 ± 3.5 for control vs. 73.6 ± 3.0 mg mL^{-1} for stressed shrimp; total protein global means: 112 ± 2 for control vs. 106.5 ± 2 mg mL^{-1} for stressed shrimp) (Table 4). A significantly higher concentration of glucose was also observed in stressed shrimp (14.2 ± 1.1 for control vs. 23.3 ± 2.0 mg dL^{-1} for stressed shrimp). Dietary HUFA content did not influence these metabolic variables. In contrast, shrimp fed the high-HUFA diet were found to have a significantly lower lactate concentration, regardless of stress conditions (5.7 ± 0.3 for the low-HUFA diet vs. 4.8 ± 0.2 mg dL^{-1} for the high-HUFA diet). No effects of the long-term stressor and dietary HUFA were observed for triacylglycerides, cholesterol, total lipid concentrations, and osmotic pressure.

Fatty acid composition of haemocytes

Shrimp fed the high-HUFA diet showed a significantly higher proportion of HUFA in the neutral lipids of their haemocytes compared to shrimp fed the low-HUFA diet (15.4 ± 2.2 for the low-HUFA diet vs. 23.4 ± 1.5% of total fatty acids for the high-HUFA diet, data not shown). This effect was even more evident in the polar lipids of the haemocytes (18.9 ± 3.1 for the low-HUFA diet vs. 33.1 ± 1.1% of total fatty acids for the high-HUFA diet, data not shown). Proportions of ARA and EPA in control shrimp fed the high-HUFA diet were about threefold higher than those fed the low-HUFA diet, and an approximate fourfold proportion was found for DHA (Fig. 1). In addition to the dietary effect, the proportion of EPA and DHA in the polar fraction was affected by the long-term stressful conditions. Shrimp fed the low-HUFA diet and exposed to the long-term stressor showed a significantly higher proportion of these two fatty acids in the polar lipids of their haemocytes, compared to control shrimp fed the same diet. Although not significant, the same trend was observed in the neutral lipids.

Immune variables

Immune variables analysed in haemolymph were not affected by either handling stress or dietary HUFA, except for the concentration of HDL-BGBP, which was significantly higher in shrimp fed the high-HUFA diet, regardless of stress condition (52.5 ± 3.1 for the low-HUFA diet vs. 66.0 ± 4.3 µg mg^{-1} total proteins for the high-HUFA diet) (Fig.2).

Experiment 2

Haemolymph biochemical composition and osmotic pressure

A significant drop of haemocyanin concentration was obtained 24-h after applying the handling stressor (96.2 ± 4.2 for control, 98.5 ± 4.1 at 1-h, 82.3 ± 3.6 mg mL^{-1} at 24-h) (Table 5). In contrast, the concentration of total proteins significantly increased 1-h

after stressing the shrimp (106 ± 4.5 for control, 135.3 ± 6.2 mg mL^{-1} at 1-h). Technical problems prevented the measurement of total proteins at 24-h. Concentration of total proteins was also influenced by dietary HUFA and a significantly higher content was observed in shrimp fed the high-HUFA diet (113.4 ± 4.4 for the low-HUFA diet vs. 128 ± 8.3 mg mL^{-1} for the high-HUFA diet). Lactate and glucose concentrations in haemolymph were significantly higher 1-h after having stressed shrimp, but both returned to a level similar to control shrimp at 24-h (lactate global means: 5.7 ± 0.4 for control, 7.2 ± 0.8 at 1-h, 5.4 ± 0.4 mg dL^{-1} at 24-h; glucose global means: 16.7 ± 1.5 for control, 56.2 ± 6.5 at 1-h, 22.4 ± 2.8 mg dL^{-1} at 24-h). Shrimp fed the high-HUFA diet had a significantly lower osmotic pressure compared to shrimp fed the low-HUFA diet (828.9 ± 12.4 for the low-HUFA diet vs. 786.9 ± 6.1 mOsm kg^{-1} for the high-HUFA diet). Triacylglycerides, cholesterol, and total lipid concentrations were not significantly affected.

Immune variables

A significant main effect of stress was observed for the THC, with a significant increase 1-h after the application of the stressor (12.9 ± 2.3^{b} for control, 20.4 ± 1.5^{a} at 1-h, 13.9 ± 1.6^{b} ×10^{6} haemocytes ml^{-1} haemolymph at 24-h) (Fig. 3). This effect was more pronounced in shrimp fed the low-HUFA diet. As shown by a significant interaction, an increase in the production of superoxide anion was also observed 1-h after stressing the shrimp, but only for those fed the high-HUFA diet. Clotting time was significantly higher at 1-h than at 24-h after the stressor was applied, regardless of dietary treatment (12.8 ± 0.8^{ab} for control, 13.7 ± 0.6^{b} at 1-h, 11.6 ± 0.4^{a} s at 24-h).

4. Discussion

Biological performance

The long-term stressor affected the final survival, weight, feed consumption, and moulting frequency of shrimp. The lower final weight observed in stressed shrimp reflects a reduced growth rate. In fish, decreased growth is considered a good indicator of long-term stress (Pickering 1993). Interestingly, stressed shrimp presented a higher total number of moults. These results suggest that moulting is not automatically followed by increased weight and could be triggered by a long-term handling stressor. The higher moulting frequency observed in stressed shrimp is in disagreement with several studies: Clark (1986) reported an inhibition of moulting in *Penaeus semisulcatus* De Haan submitted to hypoxia for 17 days. Hewitt and Duncan (2001) showed a lower moulting rate in *Marsupenaeus japonicus* (Bate) maintained at high temperatures (36°C) for 12 days. Staples and Heales (1991) observed a longer intermolt period at extreme salinities (5 and 55‰) in *Fenneropenaeus merguiensis* (De Man). However, an increased moulting frequency was observed in other situations, such as high ammonia levels in *M. japonicus* (Chen & Kou 1992) and low salinity (3‰) in crabs at instars C5 and C6 (Spivak 1999). This suggests that depending on the nature of the stressor, different results can be obtained. A higher incidence of physical injury produced by the long-term stressor could explain the lower feed consumption observed in stressed shrimp. The higher moulting frequency also may have affected feed consumption as shrimp do no

eat just before and after ecdysis (Sánchez-Paz García-Carreño, Hernández-López, Muhlia-Almazán & Yepiz-Plascencia 2007).

In this study, dietary levels of HUFA were within the range of those used in other studies conducted with juvenile penaeid shrimp (Chim *et al*. 2001; González-Félix, Gatlin, Lawrence & Perez-Velazquez 2002). The supplementation of HUFA in the diet partially counteracted the negative effect of handling stress on survival, indicating a beneficial effect of their inclusion in the diet although no improvement of growth was observed. A positive effect of dietary DHA on tolerance to various stressful conditions, such as extreme temperature and salinity, exposure to air, and low dissolved oxygen was observed by Kanazawa (1997) in larvae of the Red Sea bream. HUFA supplementation also increased survival in a salinity stress test in shrimp postlarvae (for review, Palacios & Racotta 2007). Shrimp fed the high-HUFA diet had a lower osmotic pressure, i.e. higher osmoregulatory capacity, than those fed the low-HUFA diet, but only in the acute stress and not in the long-term stress. This was mainly a result of high values of osmotic pressure 24-h after applying the stressor in the low-HUFA diet. It could be suggested that short-term stress produces a loss of hypo-osmoregulatory capacity that is partially counteracted by high HUFA levels in the diet. Similar results were obtained for hyper-osmoregulatory capacity in shrimp exposed to low salinity stress during 4 days (Chim *et al*. 2001).

Effects of stressor on immune response

Long-term handling stress did not alter the shrimp immune response. A similar result was obtained by Mercier *et al*. (2006) and it was then suggested that a shrimp adaptation to stressful conditions was occurring. To overcome long-term stress adaptation, in the present study, we intensified the magnitude of the handling stressor and increased progressively the duration of emersion, but even then we failed to observe any effect on immune variables. In contrast, short-term stress affected THC and haemolymph clotting time, regardless of HUFA supplementation. The higher THC 1-h after having applied the handling stressor in shrimp fed the low-HUFA diet is in disagreement with several studies that report a decrease of THC in crustaceans exposed to stressful conditions (Sánchez *et al*. 2001; Le Moullac, Soyez, Saulnier, Ansquer, Avarre & Levy 1998; Cheng, Liu, Hsu, Chen 2002; Perazzolo, Gargioni, Ogliari & Barraco 2002). However, Jussila *et al*. (2001) also observed an increase in THC 1-h after induced swimming activity for 2 min in the western rock lobster *Panulirus cygnus*. Clotting time increased 1-h after applying the stressor and decreased at 24-h. Hyalinocytes participate in the clotting process by releasing a calcium ion-dependent transglutaminase that catalyses the polymerization of clotting proteins present in the plasma (Lee & Söderhäll 2002). Although we did not do a differential count of hemocytes, a change in the proportion of hyalinocytes in haemolymph 1-h after the short-term stressor may explain the increase in clotting time.

Effect of HUFA on fatty acid composition of haemocytes and immune response

To our knowledge, this is the first time that fatty acid composition of haemocytes is analysed in penaeid shrimp. We found increased levels of HUFA in the neutral and polar lipids of haemocytes with increased level of all three HUFA (ARA, EPA and DHA) in the diet. In *L. vannamei*, incorporation of HUFA in relation to their dietary level has been reported for other tissues, such as the hepatopancreas (González-Félix *et al*. 2002;

2003; Palacios *et al*. 2004; Hurtado, Racotta, Arjona, Hernández-Rodríguez, Goytortúa, Civera & Palacios 2006), muscle (González-Félix *et al*. 2002; 2003), and gills (Palacios *et al*. 2004; Hurtado *et al*. 2006). The present work shows that HUFA are not only accumulated in haemocytes in relation to diet, but also when they are required. The higher proportion of EPA and DHA in the neutral and polar lipids in stressed shrimp fed the low-HUFA diet showed a capacity of shrimp to retain these two HUFA, despite their low level in the diet. This probably indicates a biological function of these two fatty acids in the haemocytes during stressful conditions.

Our main hypothesis was that the increased level of HUFA in haemocytes could improve the immune defence potential of shrimp, especially under stress. In mammals, there is good evidence that HUFA modulate immune response by influencing the physical properties of immune cell membranes and membrane-associated enzymes and receptor sites (Peck 1994). Additionally, HUFA can influence the immune system through the production of eicosanoids, alteration of gene expression, lipid peroxidation, or apoptosis (de Pablo, Puertollano & Álvarez de Cienfuegos 2002). In fish, HUFA have been shown to affect immune response influencing the cytotoxic, chemotactic (Ashton, Clements, Barrow, Secombes & Rowley 1994), phagocytic activity, and the production of antibodies (Kiron *et al*. 1995). Chim *et al*. (2001) also demonstrated an increase in the agglutination titre of plasma and the haemocyte oxidative activity in the blue shrimp *L. stylirostris* fed a diet enriched with HUFA. In our study, except for the diet-related increase in anion superoxide production in the short-term stress experiment, we did not find any effect of the dietary enrichment of HUFA on the immune variables directly related with haemocytes such as THC, anion superoxide production, and haemolymph clotting time. However, the concentration of HDL-BGBP was significantly affected by the supplementation with HUFA. HDL-BGBP is a lipoprotein involved in the shrimp defence system because it recognizes invading microorganisms and amplifies the activation of the prophenoloxidase activating system (Vargas-Albores & Yepiz-Plascencia 2000). Thus, increased concentration of HDL-BGBP in shrimp fed the high-HUFA diet could enhance their immune response when exposed to pathogens. In *L. vannamei***,** HDL-BGBP is synthesised in the hepatopancreas (Yepiz-Plascencia, Gollas Galván, Vargas-Albores & Garcia-Bañuelos 2000) and it contains approximately 57% of lipids (Ruiz-Verdugo, García-Bañuelos, Vargas-Albores, Higuera-Ciapara & Yepiz-Plascencia 1997). Supplementation with dietary HUFA might have increased the concentration of HUFA in the hepatopancreas, influencing the formation of HDL-BGBP.

Additionally, shrimp fed the high-HUFA diet presented an increased anion superoxide production 1-h after being subjected to the stressor, whereas this response was not observed in those fed the low-HUFA diet. The possible involvement of increased HUFA levels in the haemocytes is suggested through different possible mechanisms. ARA is a precursor of the leukotriene LTB4 which was showed to enhance the generation of reactive oxygen species in mammals (Calder 2001). DHA and EPA could also play an important role in the increase of superoxide anion production. In clams, Delaporte *et al*. (2003) reported that high dietary EPA and ARA levels have positive effects on the oxidative activity of haemocytes, as well as on the concentration of haemocytes and phagocytosis. EPA can provide energy to haemocytes and thus, greater capacity to respond to stimulation (Delaporte *et al*. 2003).

Biochemical composition of haemolymph

A rapid increase of glucose occurred in haemolymph after applying the short-term handling stressor. Hyperglycaemia is a common response observed after exposing

penaeid shrimp to various kinds of short-term stressors, such as repeated sampling (Racotta & Palacios 1998), exposure to formalin (van Ham & Hall 1998), hypoxia or emersion (Hall & van Ham 1998), and exposure to high ammonia concentrations (Racotta & Hernández-Herrera 2000; Mugnier & Justou 2004). A higher glucose concentration also occurred in shrimp submitted to the long-term stressor. However, short-term stress produces a large glucose increase (Experiment II); a long-term stress that increases progressively in intensity can produce a response but not of the same magnitude (Experiment I), and a long-term stress that is similar in magnitude throughout the experiment (Mercier *et al*. 2006) does not produce a statistically different glucose response. In fish, Pickering & Pottinger (1989) also reported that the increase in cortisol levels during long-term stress is not as pronounced as during long-term stress.

A slight increase in lactate concentration also occurred only in haemolymph of shrimp exposed to the short-term stressor. These results parallel those in muscle (results not shown), where lactate concentration was significantly higher in shrimp sampled 1-h after the stressor was applied, but at 24-h, dropped to levels significantly lower than control shrimp (7 ± 0.4 for control, 8.4 ± 0.5 at 1-h, 5.5 ± 0.4 mg g^{-1} at 24-h). However, the relative magnitudes of glucose and lactate increases confirm the previous observations made by Racotta & Palacios (1998), as well as by Mugnier & Justou (2004) that lactate is a less sensitive indicator of stress than glucose, at least under some conditions.

It has to be pointed out that shrimp fed the high-HUFA diet and submitted to the long-term stressor had a lower lactate concentration in the haemolymph. Shrimp sampling could induce a rapid stress response, possibly characterized by an increased lactate concentration within minutes, as recently observed (Racotta, unpublished observations). Such stress response could be partially blunted by high HUFA levels in the diet. This counteracting effect of HUFA was also partly observed in the short-stress experiment since the magnitude of the lactate increase at 1-h was higher with the low-HUFA diet than with the high-HUFA diet, although the interaction was not significant. Changes in fuel metabolism in shrimp submitted to long-term stress were also evident with their use of proteins to overcome the stress threat. This effect could contribute to explain the lower growth during long-term handling stress, as previously discussed. In contrast, total lipids and particularly triacylglycerides did not seem to have been used as a significant source of metabolic energy. Cultivated shrimp are well adapted to use proteins for supplying energy, growth, osmoregulation, and producing glycogen and glucose through the gluconeogenic pathway (Rosas, Cuzon, Gaxiola, Pascual, Taboada, Arena & Van Wornhoudt, 2002). Furthermore, the decrease in haemolymph total proteins observed under long-term stress may reveal a response to stress of the immune system because it relies strongly on circulating proteins. The decrease of haemocyanin might be interpreted as a physiological and immune response to stress because in crustaceans, this protein is implied in several functions, such as oxygen transport, protein storage (Paul & Pirow 1998), osmotic regulation (Paul & Pirow 1998), anti-fungal defence (Destoumieux-Garzón, Saulnier, Garnier, Jouffrey, Bulet & Bachère 2001), and it has a phenoloxidase-like activity (Adachi,Wakamatsu, Ito, Miyamoto, Kokubo, Nishioka & Hirata 2005).

Conclusion

This study documented that biological performance indicators, such as growth, survival, and feed consumption were negatively affected by long-term handling stress, suggesting that such stressful situations could affect shrimp culture. Lactate and glucose

increases in haemolymph are useful indicators of stress only during short-term handling stress. A beneficial role of HUFA enrichment was appreciated on shrimp stress resistance and immune defence potential, as determined by increased concentration of HDL-BGBP, and an increased capacity to produce superoxide anion as a short-term stress response. HUFA supplementation also decreased lactate concentration in the haemolymph of long-term stressed shrimp that can suggest lower stress susceptibility during handling associated to sampling.

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Tables

Table 1. Ingredients and proximate composition of low-HUFA and high-HUFA diets

Ingredients (g 100g^{-1} diet)	Low-HUFA diet	High-HUFA diet
Wheat meal[1]	40.12	40.12
Fish meal[2]	28.03	28.03
Soybean paste[1]	20.00	20.00
Alginic acid[3]	2.00	2.00
Vitamin premix[4]	1.80	1.80
Sodium phosphate dibasic[3]	1.20	1.20
Mineral premix[5]	0.50	0.50
Cholesterol[3]	0.50	0.50
Choline chloride[6]	0.20	0.20
Ascorbic acid[6]	0.09	0.09
Soybean lecithin[7]	1.50	1.50
ICES emulsion, 0% HUFA[8]	4.05	--
ICES emulsion, 50% HUFA[8]	--	4.05
BHT[9]	0.004	0.004
Proximate composition (g 100g^{-1} dry matter, except moisture)[10]		
Moisture	7.82 ± 0.37	7.85 ± 0.38
Crude protein	33.36 ± 1.00	33.44 ± 1.01
Ether extract	6.87 ± 0.60	6.62 ± 0.58
Ash	20.98 ± 1.31	20.60 ± 1.28
Crude fibre	2.98 ± 0.27	2.91 ± 0.26
Free Nitrogen Extract	35.81	36.43

[1]Proteínas Marinas y Agropecuarias (Guadalajara, Jalisco, Mexico).

[2]Promotora Industrial Acuasistemas (La Paz, B.C.S., Mexico).

[3]Sigma (St. Louis, MO, USA).

[4]Vitamin premix (g kg^{-1} premix): vitamin A Acetate‡, 5; vitamin D3‡, 0.001; vitamin E*, 8; vitamin K_3‡, 2; thiamin-HCl‡, 0.5; riboflavin (B_2) ‡, 3; pyridoxine-HCl‡, 1; pantotheic acid‡, 5; nicotinic acid‡, 5; biotin‡, 0.05; inositol‡, 5; vitamin B_{12}‡, 0.002; folic acid‡, 0.18; cellulose*, 865.3. ‡ ICN Biomedicals (Aurora, OH, USA). *Sigma (St. Louis, MO, USA).

[5]Mineral premix (g 100g^{-1} premix): cobalt chloride*, 0.004; cupric sulphate pentahydrate*, 0.25; ferrous sulphate*, 4; magnesium sulphate heptahydrate*, 28.4;

magnesium sulphate monohydrate*, 0.65; potassium iodide*, 0.07; sodium selenite*, 0.01; zinc sulphate heptahydrate*, 13.19; cellulose*, 53.43. * Sigma (St. Louis, MO, USA).

[6]Roche (México, D.F., Mexico).

[7]Distribuidora de Alimentos Naturales y Nutricionales (México, D.F., Mexico).

[8]Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium.

[9]Butylated Hydroxytoluene. ICN Biomedicals (Aurora, OH, USA).

[10]Results are presented as means ± SD, except for the results of the free nitrogen extract that are given as means only.

Table 2. Fatty acid composition (g 100 g^{-1} diet) of the low-HUFA and high-HUFA diets

	Low-HUFA diet (*n* = 2)	High-HUFA diet (*n* = 2)
14:0	1.71	0.31
16:0	2.55	2.06
18:0	0.54	0.52
16:1n-7	0.23	0.27
18:1n-9	1.63	1.48
18:1n-7	0.18	0.22
18:2n-6	3.13	2.81
18:3n-3	0.34	0.34
20:4n-6	0.01	0.09
20:5n-3	0.03	1.21
22:6n-3	0.03	0.79
Σ SAT[1]	8.85	3.17
Σ MUFA[2]	2.15	2.22
Σ PUFA[3]	3.64	5.31
Σ HUFA[4]	0.07	2.10

Results (means) are given in wet weight.

[1]Sum of saturated fatty acids.

[2]Sum of monounsaturated fatty acids.

[3]Sum of polyunsaturated fatty acids.

[4]Sum of highly unsaturated fatty acids (≥four double bonds).

Table 3. Biological performance (means ± SE) of long-term stressed *Litopenaeus vannamei* and controls fed a low-HUFA or high-HUFA diet.

	Low HUFA diet		High HUFA diet		D[1]	S[1]	D×S
	Control	Stress	Control	Stress			
Initial weight (g)[2]	11.1 ± 0.1	11.1 ± 0.1	11.2 ± 0.1	11.1 ± 0.1	NS	NS	NS
Final weight (g)[2]	12.8 ± 0.2	11.7 ± 0.2	12.8 ± 0.2	11.4 ± 0.2	NS	**	NS
Survival (%)[3]	95.0 ± 1.5[a]	90.7 ± 0.3[b]	93.7 ± 0.7[ab]	94.0 ± 0.0[ab]	NS	*	*
Total moults (No. tank^{-1})[3]	38.3 ± 3.3	45.3 ± 5.0	31.7 ± 4.1	52.7 ± 2.6	NS	**	NS
Food consumption (g tank^{-1} day^{-1})[3]	11.5 ± 0.1	10.8 ± 0.1	11.1 ± 0.1	10.1 ± 0.1	**	**	NS

[1]The results of the two-way ANOVA are presented in the last three columns (D = diet, S = stress, NS = not significantly different, * = $P \leq 0.05$, ** = $P \leq 0.01$). When a significant interaction was obtained, a Tukey post-hoc analysis was applied to individual means. Means sharing the same letter are not significantly different ($P > 0.05$). See text for comparison between global means (pooled means of either dietary treatments or stress conditions).

[2]Initial weight was recorded for all shrimp

when stocked in the experimental tanks (n=90 per group), whereas final weight was recorded only for shrimp sampled for the different analyses (n=42 per group).

[3] These variables were recorded per tank (n=3 replicates per group).

Table 4. Haemolymph biochemical composition and osmotic pressure (means ± SE) analysed in long-term stressed *Litopenaeus vannamei* and controls fed a low-HUFA or high-HUFA diet.

	Low HUFA diet		High HUFA diet		D	S	D×S
	Control	Stress	Control	Stress			
Haemocyanin (mg ml^{-1})[1]	83.5 ± 4.8	72.1 ± 4.3	82.3 ± 5.2	75.1 ± 4.3	NS	*	NS
Total proteins (mg ml^{-1})[1]	113.4 ± 2.8	103.4 ± 3.1	110.6 ± 2.8	109.6 ± 2.2	NS	*	NS
Triacylglycerides (mg dl^{-1})[1]	50.2 ± 5.7	49.6 ± 7.8	58.6 ± 10.1	94.0 ± 0.0^{ab}	NS	NS	NS
Cholesterol (mg dl^{-1})[1]	33.9 ± 5.0	40.9 ± 8.8	37.7 ± 7.7	25.8 ± 1.8	NS	NS	NS
Total lipids (mg dl^{-1})[1]	211.5 ± 30	214.8 ± 40	238.2 ± 45	126.4 ± 11	NS	NS	NS
Lactate (mg dl^{-1})[1]	5.5 ± 0.3	5.9 ± 0.6	5.3 ± 0.3	4.4 ± 0.2	*	NS	NS
Glucose (mg dl^{-1})[1]	14.1 ± 1.4	20.4 ± 2.2	14.4 ± 1.8	26.2 ± 3.3	NS	**	NS
Osmotic pressure (mOsm kg^{-1})[2]	780.3 ± 11	791.7 ± 6.9	790.5 ± 8.8	779.5 ± 6.9	NS	NS	NS

See Table 3 for statistical analyses.

[1]N = 15 shrimp for each diet-stress combination.

[2]N = 9 shrimp for each diet-stress combination.

Table 5. Haemolymph biochemical composition and osmotic pressure (means ± SE) analysed in short-term stressed *Litopenaeus vannamei* and controls fed a low-HUFA or high-HUFA diet.

	Low HUFA diet			High HUFA diet			D	S	D×S
	Control	1-h	24-h	Control	1-h	24-h			
Haemocyanin (mg ml^{-1})	96.6 ± 6.5	95.1 ± 3.8	77.0 ± 5.0	95.9 ± 5.9	102 ± 7.4	87.7 ± 4.4	NS	*	NS
Total proteins (mg ml^{-1})	104 ± 6	123 ± 3		108 ± 7	148 ± 10		*	**	NS
Triacylglycerides (mg dl^{-1})	61.1 ± 7.9	73.4 ± 17.8	59.4 ± 11.6	60.9 ± 11.3	54.1 ± 6.5	82.8 ± 16.9	NS	NS	NS
Cholesterol (mg dl^{-1})	43.8 ± 6.3	53.5 ± 14.2	45.5 ± 8.5	46.1 ± 9.7	39.9 ± 7.4	60.6 ± 12.6	NS	NS	NS
Total lipids (mg dl^{-1})	291 ± 34	340 ± 72	307 ± 67	284 ± 49	252 ± 46	381 ± 63	NS	NS	NS
Lactate (mg dl^{-1})	6.2 ± 0.7	8.5 ± 0.9	5.3 ± 0.7	5.2 ± 0.5	6.0 ± 1.0	5.5 ± 0.6	NS	*	NS
Glucose (mg dl^{-1})	16.5 ± 2.1	60.1 ± 7.1	18.2 ± 3.3	16.9 ± 2.3	52.3 ± 11.3	26.7 ± 4.0	NS	**	NS
Osmotic pressure (mOsm kg^{-1})	814 ± 18	810 ± 27	860 ± 11	776 ± 6	810 ± 14	775 ± 2	NS	NS	NS

See Table 3 for statistical analyses.

N= 6 shrimp for each diet-time post-stress combination.

Figures

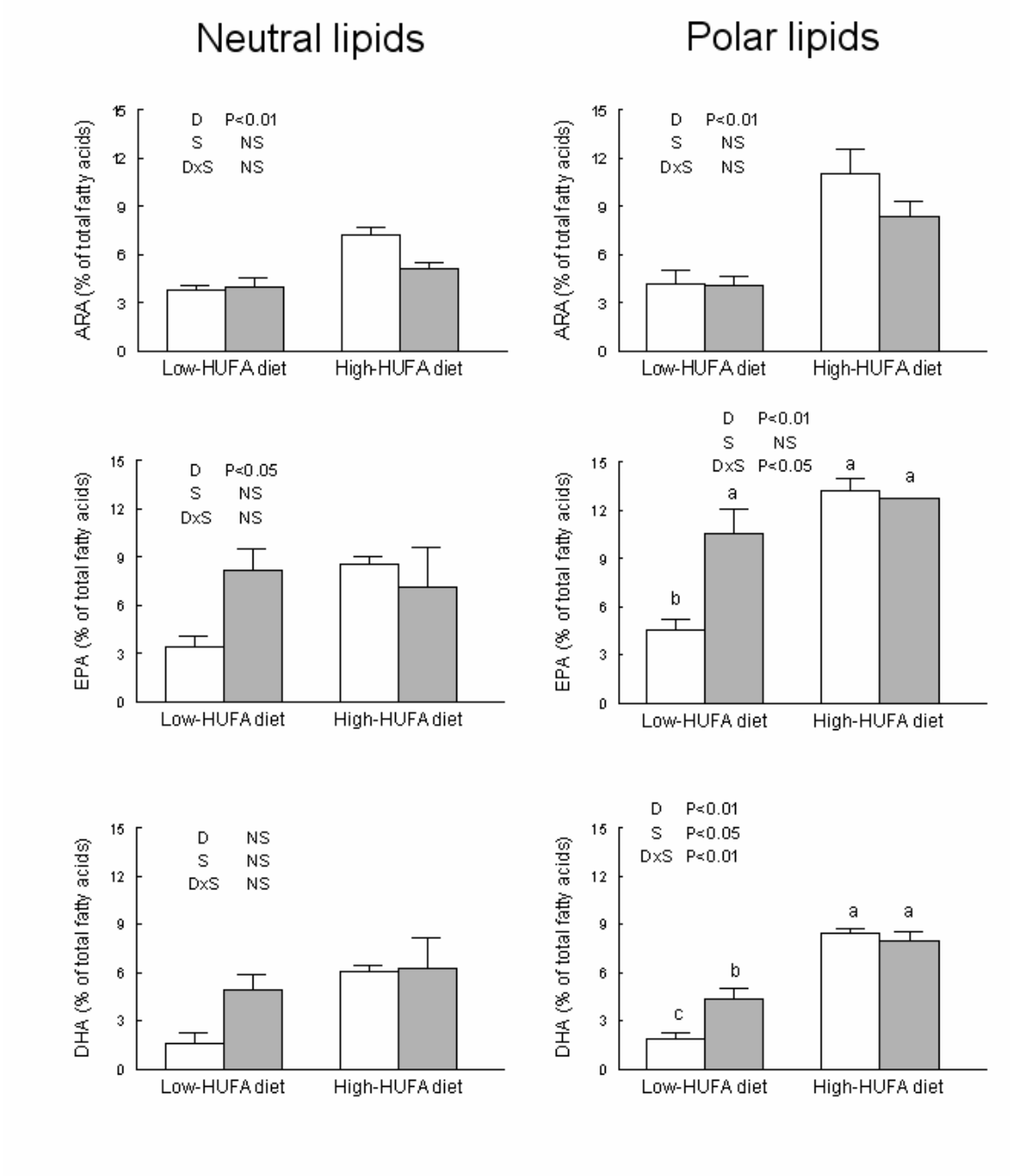

Fig. 1.

Figure 1. Principal HUFA in the neutral and polar lipids of haemocytes in long-term stressed (grey bars) *Litopenaeus vannamei* and controls (white bars) fed a low-HUFA or high-HUFA diet. Results (means ± SE) are expressed as per cent of total fatty acids and were statistically analysed as described in Table 3. ARA: Arachidonic acid (20:4n-6), EPA: Eicosapentaenoic acid (20:5n-3), DHA: Docosahexaenoic acid (22:6n-3). N = 3

pooled samples (each pool consisted of 3 shrimp) for each diet-stress combination (see Methods).

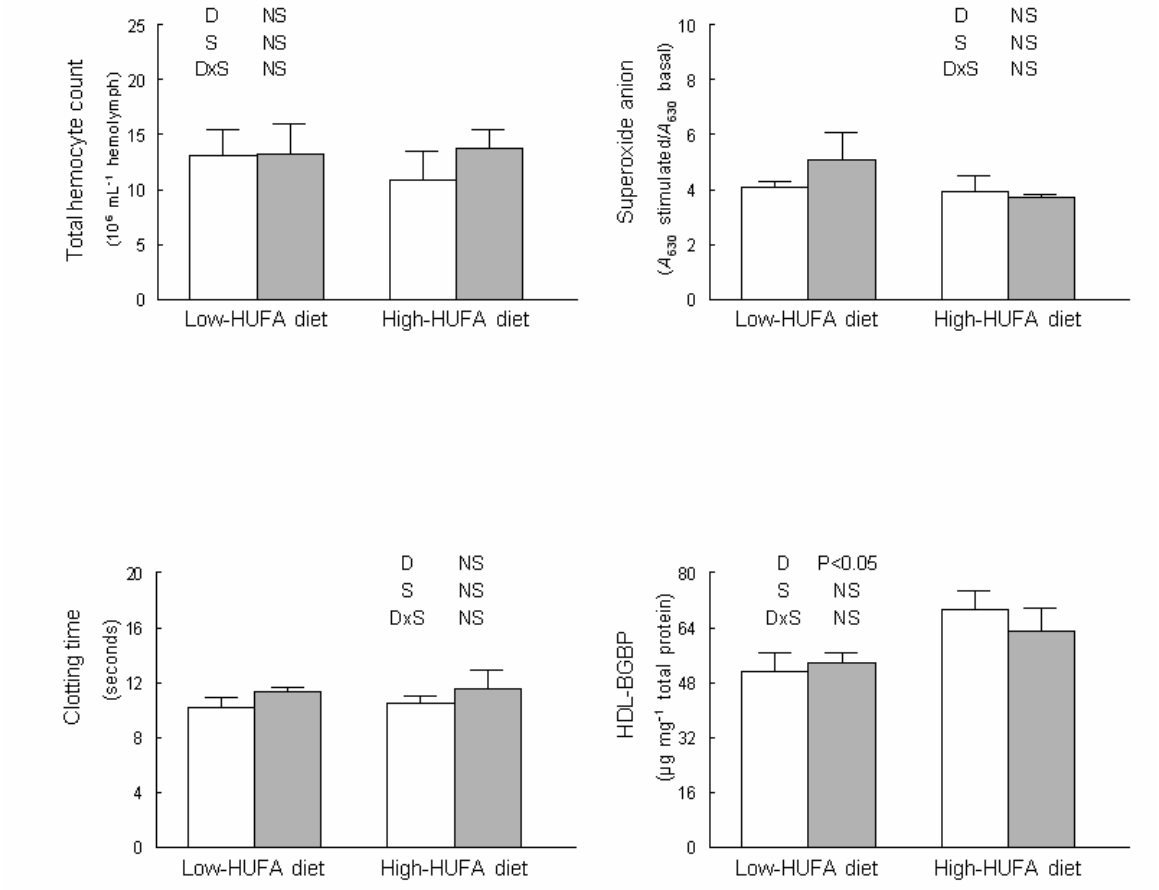

Fig. 2.

Figure 2. Immunological variables analysed in long-term stressed (grey bars) *Litopenaeus vannamei* and controls (white bars) fed a low-HUFA or high-HUFA diet. Results are expressed as means ± SE and were analysed as described in Table 3. N = 9 shrimp for each diet-stress combination.

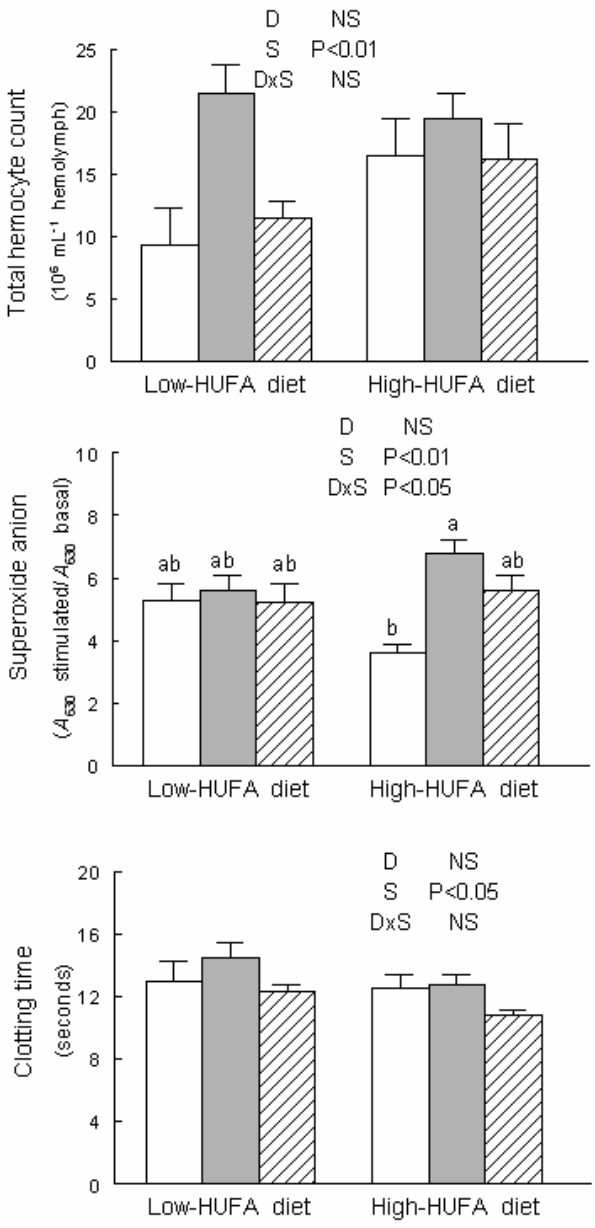

Fig. 3.

Figure 3. Immunological variables analysed in control (white bars), and 1-h (grey bars) and 24-h (striped bars) after applying the short-term stressor in *Litopenaeus vannamei* fed a low-HUFA or high-HUFA diet. Results are expressed as means ± SE and were analysed as described in Table 3. N= 6 shrimp for each diet-time post-stress combination.