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Ovarian maturation and haemolymphatic vitellogenin concentration of Pacific white shrimp *Litopenaeus vannamei* (Boone) fed increasing levels of total dietary lipids and HUFA

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Abstract

Two experiments were carried out to determine the quantitative lipid requirements for ovarian maturation of *Litopenaeus vannamei* (Boone) broodstock. The first experiment tested the effect of total dietary lipid (TDL) levels of 8.1%, 8.8%, 9.8% and 11.2% on the gonadosomatic index (GSI), hepatosomatic index (HSI), survival and body weight. The second experiment tested the effect of total dietary levels of highly unsaturated fatty acids (TDH) 0.6%, 1.3%, 2.0% and 2.7% on the same parameters mentioned above and on the haemolymphatic vitellogenin (vg) concentration. The GSI was higher in females fed 8.1% TDL as compared with those fed 8.8% TDL ($P \leqslant 0.05$), and higher in females fed 8.1% and 8.8% TDL than in those fed 9.8% and 11.2% TDL. Increasing TDH levels led to increasing levels of highly unsaturated fatty acids (HUFA) in the ovaries ($r^2 = 0.999$). However, the GSI was not affected by dietary treatments ($P > 0.05$). The vg concentration was higher in animals receiving 2.0% TDH as compared with those receiving 2.7% TDH. In conclusion, high TDL levels affected ovarian maturation in a negative way, why TDH levels had no pronounced effect on ovarian maturation within the range tested in this study.

Keywords: shrimp, maturation, vitellogenin, lipids, HUFA

Introduction

Published studies on the quantitative dietary requirements of penaeid shrimp broodstock are very limited. Most broodstock nutrition studies are based on trial and error experiences or experimental research using fresh food items (Harrison 1990). Purified or semi-purified artificial broodstock diets which guarantee good performance are yet to be developed. Carefully formulated practical feeds, on the other hand, may have a performance close to that of fresh food (Wouters, Nieto & Sorgeloos, 2000), but do not allow a precise control of the levels of a certain nutrient (Benzie 1997). Lipids may be the exception to the rule, as practical ingredients can be defatted and different oils with distinct lipid compositions can be used for the formulation of experimental diets.

In a previous study (Wouters, Molina, Lavens & Calderón, in press) with the Pacific white shrimp *Litopenaeus vannamei* (Boone), the changes that occur in the lipid composition of the body tissues during ovarian maturation were described, showing a high increase in total lipid levels in the ovaries of maturing females and high levels of highly unsaturated fatty acids (HUFA) in mature ovaries. Various studies on other shrimp species revealed similar trends: *L. setiferus* (Middleditch, Missler, Hines, McVey, Brown, Ward & Lawrence 1979), *Marsupenaeus japonicus* (Bate) (Teshima &

Kanazawa 1983), *M. kerathurus* (Forskal) (Mourente & Rodriguez 1991) and *Pleoticus muelleri* (Bate) (Jeckel, Aizpun de Moreno & Moreno 1989). The authors are aware of only one study that addresses the requirements of shrimp broodstock for total dietary lipids (TDL). Bray, Lawrence & Lester (1990) demonstrated an effect of TDL levels on the reproductive performance and offspring quality of the marine shrimp *L. stylirostris* (Stimpson). Direct evidence of the effect of dietary lipids on ovarian maturation was, however, not provided.

The importance of dietary HUFA for crustacean maturation and reproduction has been recognized (Harrison 1990). Middleditch *et al.* (1979) and Lytle, Lytle & Ogle (1990) attribute the success of fresh food maturation diets (squid, bivalves, bloodworms) to their high HUFA content. In agreement, dietary n-3 HUFA have been shown to improve reproductive performance and/or offspring quality in *L. vannamei* (Cahu, Guillaume, Stéphan & Chim 1994; Wouters, Gómez, Lavens & Calderón 1999) and other shrimp species (Xu, Ji, Castell & O'Dor 1994; Cahu, Guillaume, Stéphan & Chim 1995). Alava, Kanazawa, Teshima & Koshio (1993) obtained retarded ovarian maturation in *M. japonicus* fed a HUFA-free diet. However, Cahu *et al.* (1994, 1995) and Wouters *et al.* (1999) could not find evidence to support the hypothesis that high dietary HUFA levels promote ovarian maturation.

The present study aimed at determining quantitative lipid requirements for ovarian maturation of wild *L. vannamei* females. A first experiment was performed to establish an optimal TDL level for ovarian maturation. A second experiment investigated the effect of total dietary HUFA (TDH) on ovarian maturation and haemolymphatic vitellogenin (vg) concentrations.

Materials and methods

Animals

Wild female *L. vannamei* broodstock was bought from fishermen in Ayangue and San Pedro (Guayas Province, Ecuador), treated with formalin upon arrival and acclimated to the experimental conditions for a period of 2–3 weeks. Then, individual molt staging was carried out by microscopical examination of the setal base of uropod clips as described by Robertson, Bray, Trujillo & Lawrence (1989), and ovarian maturity staging was carried out by visual examination. Immature animals in intermolt stage were selected and ablated at the end of the acclimation phase.

Table 1 Lipid level (% of dry matter, DM), daily ingestion (% DM of biomass), resulting total dietary lipid levels (TDL in percentage DM) and daily lipid uptake (mg DM) per g biomass for the different artificial broodstock diets used in experiment 1

Lipid level (%)	Ingestion rate (% day^{-1})	TDL (%)	Lipid uptake (mg)
8.1	2.11 ± 0.29^a	8.1	2.529
9.0	2.36 ± 0.22^a	8.8	2.944
10.3	2.21 ± 0.14^a	9.8	3.096
12.2	2.11 ± 0.11^a	11.2	3.394

Within a column, values with a common superscript are not statistically different ($P > 0.05$).

Diet

In a preliminary trial, the effect of fresh food replacement with experimental broodstock diet was tested at levels 0%, 50%, 75% and 100% (dry matter, DM). The 100% replacement level gave comparatively poor results in terms of survival (55% vs. 80–90%) and gonadosomatic index (0.9% vs. 1.4–1.9%). As such, a fresh food replacement level of 75% was selected for the present study. Frozen fresh food items were squid and mussel in a 2:1 ratio. During acclimation, a total feeding rate of 2.1% d^{-1} DM of the total shrimp biomass was applied. After ablation, the feeding rate was increased to 3–4% day^{-1}.

Two experiments were conducted. In experiment 1, all experimental diets were formulated to provide an isonitrogenous 52% protein level but with different levels of total lipids (Table 1). In experiment 2, the diets were formulated to be isonitrogenous (52% protein) and isolipidic (8.1% lipids) but with different HUFA levels (see Table 3). To exert better control on lipid and HUFA levels, animal meals were partially defatted with chloroform and methanol according to a modified version of the method described by Folch, Lees & Stanley (1957). Then, the defatted meals where placed in a fume hood to remove residual solvents. In experiment 1, total lipid levels were altered by increasing portions of cod liver oil. In experiment 2, HUFA levels were altered by varying the relative portions of HUFA-rich fish oil and HUFA-free coconut oil. Neutral lipids that supplied fatty acids (FA) as triacylglycerides (TAG) were selected according to the recommendations of Glencross & Smith (1997). The HUFA-rich oil was a combination of EPAX 1040 (53.6% n-3 HUFA; Pronova Biocare, Norway) and ARASCO (40.3% n-6 HUFA; Martek Biosciences, USA).

All ingredients were mixed with 45% (w/w) heated water and the resulting dough was pelleted with a meat grinder, dried in a ventilated oven at 60 °C for 2 h and kept in sealed nitrogen-flushed plastic bags at –20 °C until use. During the first week of the acclimation phase of experiment 2, animals were fed on an artificial diet with low HUFA content (0.2% DM). During the second and third week, they were fed the different experimental diets.

Water stability of the pellets was tested in duplicate by placing 2 g in a 100-mL bottle and shaking automatically at 70 r.p.m. during 6 h. Then, the content was recovered on a 150-μm-eyed metal screen, dried and weighed.

Diet ingestion was estimated over a 3-day period in each tank. Before a new feed ratio was given (four times daily), the remaining feed was recovered and weighed after drying at 60 °C for 24 h. This allowed calculation of the amount of feed (DM) that was ingested per unit shrimp biomass on a daily basis.

Total dietary levels of lipids and HUFA were estimated to account for the input by the partial feeding of fresh ingredients (squid and mussel). The reported total dietary levels represent as such the complete dietary regime. The daily uptake of lipid and HUFA was estimated based on dietary levels, diet ingestion and shrimp biomass.

Culture conditions and monitoring

The following culture conditions were used in both experiments: 2-m^2 fibreglass tanks, five female *L. vannamei* per tank, 1 air stone diffuser per tank, 1 ton sand-filtered and UV-treated sea water per tank, 250% daily water exchange, 33 g L^{-1} average salinity, 6.3 mg L^{-1} average dissolved oxygen, 8.1 average pH. During acclimation, the water temperature was 24–25 °C. After ablation, temperature was kept at 28.5–29.5 °C by heating. Four transparent roof sheets allowed a natural photoperiod (12 h light: 12 h dark) with maximum light intensities at the water surface of 3000 Lux at mid-day.

Ablation was applied to the selected animals by cutting one eyestalk and cauterizing the wound. At the moment of ablation, the intact eyestalk was marked with a coloured ring to allow individual monitoring.

Both experiments ended when the majority of the females had some degree of ovarian maturation and just before the first mature female was ready for spawning. This was 21 and 17 days post-ablation for experiment 1 and 2 respectively. Then, all animals were weighed and examined visually for ovarian maturation. Haemolymph samples were taken from all animals in experiment 2. Finally, animals were dissected, while keeping them on ice, for determination of the gonadosomatic index (GSI = 100% × gonad weight/total body weight) and hepatosomatic index (HSI = 100% × hepatopancreas weight/total body weight). Gonads of females with maturity stage 1 (early maturing, GSI ~2%) were sampled for FA analysis. Stage 1 was chosen to make certain that enough sample would be available for each treatment. Only ovaries with similar stages of maturation were analysed to allow easier interpretation of the comparative FA results.

Vitellogenin determination

Haemolymph samples were taken from the ventral region of the first abdominal segment with disposable sterile insulin syringes containing 0.1 M Na_2CO_3. The samples were kept in Eppendorf tubes at –20 °C for further analysis.

Polyclonal antibodies against *L. vannamei* vitellogenin (vg) where prepared at CENAIM. Therefore, vitellogenin–vitellin (vg–vt) was purified from *L. vannamei* ovaries by means of the ultracentrifugation method described by Lee & Puppione (1988). Protein concentrations were determined according to Lowry, Rosebourgh, Farr & Randall (1951), and rabbits were immunized with an antigen: Freund adjuvant mix of 121 μL: 1000 μL by subcutaneous injection and two repetitive doses of an antigen: adjuvant mix of 61 μL: 500 μL. Then, blood samples were taken and the serum containing the antibodies was obtained. The specificity of this antibody was tested with Western blot and immunoblot assays against the antigen, ovary samples and haemolymph of male and female *L. vannamei*. The vg concentrations were determined with an indirect enzyme-linked immunosorbent assay (ELISA) in microwell plates as described in Okumura, Han, Suzuki, Aika & Hanyu (1992). A standard curve was constructed with the purified vg–vt and the ELISA was calibrated using different dilutions.

Lipid analysis

Experimental diets (experiments 1 and 2) and gonad samples (experiment 2) were kept in dark glass vials, nitrogen-flushed and sealed with Teflon® screw caps. They were then transported by air in a Styrofoam box with dry ice to the Ghent University (Belgium) for lipid analyses. Moisture was determined on triplicate samples by drying in an oven at 60 °C during 24 h. Triplicate biochemical analyses where carried out according to the following standard procedures: total lipids (TL) extracted according to the method described by Folch *et al.* (1957) as modified by Ways & Hanahan (1964); FA composition was analytically verified by flame ionization detection (FID) after injecting the sample into a Chrompack CP9001 gas chromatograph according to the procedure described by Coutteau & Sorgeloos (1995).

Statistical analysis

Each tank was considered as an experimental unit for replication. In both experiments, four treatments were tested with four replicates each. Five animals were stocked in each tank at the beginning of the experimental phase. At the end, all tanks had at least four surviving animals. Weights, GSI, HSI and haemolymph concentrations of the animals were averaged per tank to represent a tank mean. Normal probability plots and the Levene test for homogeneity of variances were used to verify the assumptions for further analysis. There was no need to transform data. One-way ANOVA and subsequent Duncan's multiple-range test for detection of treatment differences (Mead, Curnow & Hasted 1993) were used for statistical analyses (STATISTICA, Statsoft). Vg concentrations and GSI were strongly correlated during early maturation (see Fig. 2). Therefore, analyses of vg concentrations were carried out with adjusted means (Steel & Torrie 1980), accounting for the diet-dependent covariate GSI by dividing vg concentration with the corresponding value y from the polynomial regression equation $y = -0.0962(\mathrm{GSI})^2 + 0.8287(\mathrm{GSI}) - 0.0983$ (see Fig. 2). Unadjusted means are given in this publication. According to the recommendations of Mendoza, Revol, Fauvel, Patrois & Guillaume (1997), vg concentrations of females with a final GSI < 1.75% were also compared in order to differentiate immature (stage 0) females.

Results

Experiment 1

Moisture and water stability of the diets were 7.5 ± 0.3% (mean ± SD) and 76.6 ± 8.0% respectively. The daily ingestion rates were relatively high for all diets and did not differ ($P > 0.05$) between them (Table 1). Total lipid levels of the artificial broodstock diets ranged from 8.1% to 12.2%. The squid/mussel mix that comprised 25% of the total dietary feeding regime had a total lipid level of 8.2% DM. Corresponding TDL levels and daily uptake of lipid per g biomass were estimated and are presented in Table 1.

No differences ($P > 0.05$) in survival, weight, or HSI were detected among dietary treatments (Table 2). However, the GSI was significantly higher ($P \leqslant 0.05$) with 8.1% TDL and 8.8% TDL than with the remaining levels. The GSI was also significantly higher ($P \leqslant 0.05$) with 8.1% TDL than with 8.8% TDL.

Experiment 2

Moisture content and water stability of the diets were 7.2 ± 0.9% and 78.4 ± 6.3% respectively. No differences were detected ($P > 0.05$) among the ingestion rates of the different broodstock diets (Table 3). HUFA levels of the experimental broodstock diets of experiment 2 varied between 0.2% and 3.1%. The squid/mussel mix had a HUFA level of 1.7% DM. Corresponding TDH levels and daily uptake of HUFA per g biomass were also estimated (Table 3).

The lipid level and FA composition of the experimental artificial diets and the fresh food mix are given in Table 4. Total lipid levels ranged from 8.2% to 8.6%. The FA composition of the diets were those that were aimed at during formulation.

The FA composition of the ovaries dissected from early maturing females (GSI ~2) at the end of the experiment are presented in Table 5. The ovarian FA composition reflected the FA composition of the diet fed to the animals. High positive correlations were found between the HUFA level in the diet and the HUFA level in the gonads, and between the

Table 2 Survival, body weight, hepatosomatic index (HSI) and gonadosomatic index (GSI) of *Litopenaeus vannamei* (Bonne) broodstock fed increasing total dietary lipid levels (TDL): mean values (± SE) at the end of experiment 1

	Dietary treatment			
	8.1% TDL	8.8% TDL	9.8% TDL	11.2% TDL
Survival (%)	90 ± 20 ^{a}	90 ± 12^{a}	86 ± 10^{a}	96 ± 10^{a}
Body weight (g)	45.4 ± 4.23^{a}	43.5 ± 4.26^{a}	46.2 ± 5.8^{a}	43.9 ± 0.8^{a}
HSI (%)	2.55 ± 0.12^{a}	3.00 ± 0.18^{a}	2.85 ± 0.10^{a}	2.80 ± 0.25^{a}
GSI (%)	3.01 ± 0.25^{c}	2.07 ± 0.60^{b}	1.07 ± 0.27^{a}	1.22 ± 0.41^{a}

Within a row, values with a common superscript are not statistically different ($P > 0.05$).

n-3:n-6 HUFA ratio of the diet and the n-3:n-6 HUFA ratio of the gonads (Fig. 1).

Table 6 presents survival rates, body weights, organ indices and haemolymphatic vg concentrations. Survival, body weight, HSI and GSI did not differ among dietary treatments ($P > 0.05$).

The haemolymphatic vg concentration of animals fed 2.0% TDH was significantly higher ($P < 0.05$) than that of animals fed 2.7% TDH. The remaining treatments had intermediate vg concentrations, not different from each other ($P > 0.05$). The same differences were detected when only vg concentrations of immature females (GSI < 1.75%) were compared. A polynomial regression equation gave the best-fitted line between GSI and vg concentration (Fig. 2), showing a rapid increase in vg concentrations of the haemolymph during early maturation, a plateau in mid-maturing females and a decrease in late-maturing females. The correlation between GSI and vg concentrations was particularly strong during early maturation.

Table 3 HUFA level (% of dry matter, DM), daily ingestion (% DM of biomass), resulting total dietary HUFA levels (TDH in percentage DM) and daily HUFA uptake (mg DM) per g biomass for the different artificial broodstock diets used in experiment 2

HUFA level (%)	Ingestion rate (% day^{-1})	TDL (%)	HUFA uptake (mg)
0.2	2.34 ± 0.34^{a}	0.6	0.217
1.1	2.56 ± 0.50^{a}	1.3	0.452
2.1	2.73 ± 0.47^{a}	2.0	0.743
3.1	2.73 ± 0.23^{a}	2.7	1.061

Within a column, values with a common superscript are not statistically different ($P > 0.05$).

Discussion

The results of experimentt 1 clearly demonstrate that the ovarian maturation (GSI) is influenced by TDL levels. This is in line with the observations made by Wouters *et al.* (in press), who demonstrated a 2.5-fold increase in ovarian total lipids in the early stage of maturation of *L. vannamei* and concluded that a substantial part of the accumulated lipids must come from diet intake. Under these circumstances it is normal to expect that lipid requirements of maturing shrimp are higher than those of juveniles or non-reproductive adults. This is also why commercial broodstock diets contain around 10% total lipids, i.e. 3% to 5% higher than shrimp grow-out diets (D'Abramo 1989; Bray *et al.* 1990). Nevertheless, our results indicate that TDL levels of 9% or higher affect ovarian maturation in a negative way. A TDL level of 8.1% gave the highest GSI. Lower TDL levels were not tested. This seems to agree with the findings of Bray *et al.* (1990), who obtained a decrease of the percentage of the females mating per night when increasing TDL levels from 11.1% to 13.9% with menhaden oil. Two possible reasons for this are given by D'Abramo (1989): (1) exceeding high levels are not effectively processed by the animal, and (2) excess lipid levels (~ energy levels) may inhibit appetite. Equal ingestion rates of all the diets in experiments 1 and 2 rule out the second possibility. The optimum TDL level determined by Bray *et al.* (1990) was higher than the one obtained in the present study. This could be due to differences between species (*L. vannamei* vs. *L. stylirostris*), lipid sources (cod liver oil vs. menhaden oil) or availability of other energy sources (e.g. 52% vs. 62% protein).

Table 4 Total lipid level (% of dry matter, DM) and fatty acid (FA) composition (% of total FA and mg g^{-1} DM) of the experimental artificial broodstock diets and fresh food mix (squid/mussel 2:1) used in experiment 2

	Artificial diets								Fresh Food	
	0.6% TDH		1.3% TDH		2.0% TDH		2.7% TDH			
	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}
Total lipids	8.2		8.5		8.6		8.6		8.2	
14:0	28.3	10.8	16.3	7.5	7.5	4.1	1.5	0.9	4.0	1.8
16:0	23.2	8.8	18.2	8.3	14.5	7.9	12.5	7.9	23.2	10.5
16:1(n-7)	0.9	0.3	1.4	0.6	1.7	0.9	1.9	1.2	2.7	1.2
18:0	20.5	7.8	14.0	6.4	9.2	5.0	5.5	3.5	6.5	2.9
18:1(n-9)	6.0	2.3	7.3	3.3	7.9	4.3	8.6	5.5	3.5	1.6
18:1(n-7)	1.6	0.6	1.7	0.8	1.8	1.0	1.9	1.2	2.3	1.0
18:2(n-6)-c	8.5	3.3	8.0	3.7	7.7	4.2	7.6	4.8	1.1	0.5
18:3(n-6)	0.0	0.0	0.5	0.2	0.8	0.4	1.1	0.7	0.1	0.0
18:3(n-3)	1.8	0.7	1.5	0.7	1.3	0.7	1.2	0.8	0.7	0.3
20:1(n-9)	0.8	0.3	0.8	0.4	0.9	0.5	0.9	0.6	2.2	1.0
20:3(n-6)	0.0	0.0	0.6	0.3	1.0	0.6	1.3	0.9	0.0	0.0
20:4(n-6)	0.4	0.1	6.1	2.8	10.1	5.5	13.0	8.3	3.9	1.8
20:5(n-3)	2.0	0.8	4.6	2.1	6.1	3.3	7.2	4.6	10.3	4.7
22:1(n-9)	0.0	0.0	1.0	0.4	1.4	0.8	1.7	1.1	0.1	0.0
22:5(n-6)	0.0	0.0	0.2	0.1	0.4	0.2	0.5	0.3	1.3	0.6
22:5(n-3)	0.1	0.1	2.1	1.0	3.5	1.9	4.4	2.8	1.0	0.4
24:1(n-9)	0.1	0.0	0.5	0.2	1.7	0.9	2.1	1.3	0.1	0.0
22:6(n-3)	3.0	1.1	10.6	4.9	17.4	9.5	21.6	13.8	20.5	9.3
HUFA	5.7	2.2	24.1	11.0	38.0	20.7	47.4	30.2	38.1	17.3
n-3: n-6 HUFA		12.8		2.7		2.6		2.5		5.4

For clarity only FA yielding > 1% are listed.

Quantitative and qualitative lipid requirements are interrelated. This is why different TDL levels were tested before starting the study on quantitative HUFA requirements. The dietary treatments of experiment 2 had a TDL level between 8.2% and 8.6%, only slightly higher than that formulated. The range chosen for the TDH levels (i.e. 0.6–2.7%) was based on data available in literature for shrimp larvae, post-larvae and juveniles (Xu, Ji, Castell & O'Dor 1993; Rees, Curé, Piyatiratitivorakul, Sorgeloos & Menasveta 1994; Kanazawa 1995; Meridan & Shim 1997). The TDH levels chosen by Cahu *et al.* (1995) for *Fenneropenaeus indicus* broodstock (i.e. 0.56–2.4%) also served as a guideline. However, within this range, no clear influence of dietary HUFA could be detected on survival or ovarian maturation in the present study. At day 17 post-ablation, broodstock of all dietary treatments had a GSI corresponding to the early maturing stage. It appears as such that a TDH level of 0.6% was enough to satisfy HUFA requirements for initial ovarian development. Apart from the finding of Alava *et al.* (1993), who report a reduced GSI in broodstock fed a HUFA-free diet, there is no evidence of an obligatory role of HUFA in the process of sexual maturation. This suggests that HUFA are indeed required, but only in minimum quantities.

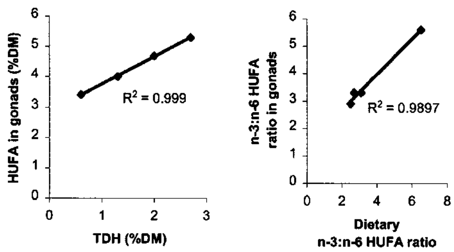

Figure 1 HUFA levels and n-3:n-6 HUFA ratios in the gonads of *Litopenaeus vannamei* (Boone) females as a function of the total dietary HUFA levels (TDH,% of dry matter) and dietary n-3:n-6 HUFA ratios. Best-fitted regression lines are given.

Table 5 Fatty acid (FA) composition (% of total FA and mg g^{-1} DM) of the gonads of early maturing *Litopenaeus vannamei* fed increasing HUFA levels (expt 2). For clarity only FA yielding > 1% are listed

	Dietary treatments							
	0.6% TDH		1.3% TDH		2.0% TDH		2.7% TDH	
	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}	%	mg g^{-1}
14:0	3.8	3.9	3.6	4.1	2.7	3.5	1.8	2.5
16:0	16.7	17.2	18.3	21.0	18.4	23.7	17.6	24.0
16:1(n-7)	6.3	6.4	5.1	5.9	4.1	5.3	3.5	4.8
17:0	0.8	0.8	1.0	1.1	1.0	1.3	0.9	1.3
17:1(n-7)	2.3	2.3	0.0	0.0	0.0	0.0	0.0	0.0
18:0	9.8	10.0	9.2	10.5	8.6	11.1	8.2	11.2
18:1(n-9)	16.2	16.6	14.5	16.7	14.1	18.2	14.6	19.9
18:1(n-7)	3.7	3.8	3.2	3.6	3.3	4.3	3.7	5.0
18:2(n-6)-c	3.8	3.9	3.4	4.0	4.0	5.1	4.6	6.3
20:4(n-6)	4.9	5.0	7.6	8.7	7.6	9.7	9.1	12.4
20:5(n-3)	10.2	10.5	10.4	11.9	9.0	11.6	9.3	12.8
22:5(n-3)	0.9	0.9	0.9	1.1	1.4	1.8	1.7	2.3
22:6(n-3)	16.1	16.5	15.5	17.8	17.3	22.3	17.1	23.4
HUFA	33.1	34.0	35.2	40.4	36.6	47.1	38.5	52.5
n-3:n-6HUFA		5.6		3.3		3.3		2.9

For clarity only FA yielding > 1% are listed.

However, there are at least three reasons why low HUFA levels are not to be recommended for shrimp broodstock diets: (1) increasing TDH levels resulted in increasing ovarian HUFA levels in the present study (see Fig. 1); (2) it is known that HUFA cannot be synthesized de novo by Penaeid shrimp (Kanazawa, Teshima & Tokiwa 1977; Clarke 1982; Chang & O'Connor 1983) or may only be synthesized to a very limited extent (Mourente 1996); and (3) there is proof of the beneficial role of medium to high dietary HUFA levels on other reproductive parameters and on offspring quality (Cahu *et al.* 1994, 1995; Xu *et al.* 1994).

Haemolymphatic vg concentrations are good indicators of the onset of secondary vitellogenesis (extraovarian yolk protein synthesis) during early maturation (Fig. 2), rapidly increasing until reaching a plateau in mid-maturing females. This evolution and the observed vg concentrations are similar to those reported for *F. indicus* (Mendoza & Fauvel 1989). Interestingly, increased vg concentrations could be detected before any increase in ovary weight was appreciated. In our study, however, early maturation was achieved in all dietary treatments, and in consequence haemolymphatic vg concentrations were high in all

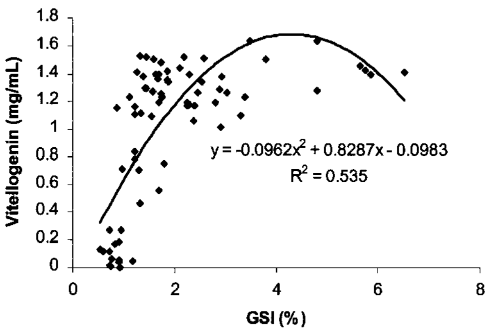

Figure 2 Haemolymphatic vitellogenin concentrations (mg mL^{-1}) as a function of gonadosomatic indices (GSI,%) in all surviving females of experiment 2. The best-fitted line is given.

treatments. Still, it is worthy to note the significant decrease ($P \leq 0.05$) in vg concentration by increasing TDH levels from 2.0% to 2.7%, accompanying a non-significant ($P > 0.05$) decrease in GSI. This might be an indication that 2.7% dietary HUFA is too high for optimal ovarian maturation. In agreement, Rees, Curé, Piyatiratitivorakul, Sorgeloos & Menasveta (1994) stated that an excess of dietary n-3 HUFA may not be beneficial for

Table 6 Survival, body weight, hepatosomatic index (HSI), gonadosomatic index (GSI) and haemolymphatic vitellogenin concentrations (vg) of *L. vannamei* broodstock fed increasing total dietary HUFA levels

	Dietary treatments			
	0.6% TDH	1.3% TDH	2.0% TDH	2.7% TDH
Survival (%)	95 ± 10[a]	95 ± 10[a]	95 ± 10[a]	95 ± 10[a]
Body weight (g)	58.2 ± 6.3[a]	61.1 ± 6.3[a]	54.7 ± 6.0 [a]	56.1 ± 13.3[a]
HSI (%)	2.93 ± 0.38[a]	2.98 ± 0.27[a]	3.09 ± 1.08[a]	2.35 ± 0.78[a]
GSI (%)	2.29 ± 0.78[a]	2.42 ± 0.52[a]	3.20 ± 0.81[a]	2.68 ± 1.16[a]
Vg (mg mL^{-1}) in all females	1.01 ± 0.24[ab]	1.00 ± 0.20[ab]	1.15 ± 0.19[b]	0.84 ± 0.14[a]
Vg (mg mL^{-1}) in immature females	0.78 ± 0.36[ab]	0.81 ± 0.25[ab]	1.20 ± 0.36[b]	0.48 ± 0.49[a]

(TDH): mean values (± SE) at the end of experiment 2.

growth of *Penaeus monodon* (Fabricius) post-larvae, and Dhert, Lavens & Sorgeloos (1992) suggested the same for fish and crustacean larvae in general.

n-3 HUFA are considered to have the highest nutritional value of all essential FA for penaeid shrimp (Kanazawa *et al.* 1977; Xu *et al.* 1993). In consequence, most previous mentioned studies on HUFA requirements only considered n-3 HUFA, in particular eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Still, considerable levels of n-6 HUFA, particularly arachidonic acid (ARA, 20:4n-6), were found in the hepatopancreas, ovaries and nauplii of wild *L. vannamei* (Wouters *et al.*, in press), for which a n-3:n-6 HUFA ratio of 2.7:1 was formulated in the dietary treatments of the current study. This is in agreement with the recommendations of Middleditch *et al.* (1979) and Lytle *et al.* (1990), and takes into consideration that ARA is thought to be a precursor in the synthesis of prostaglandins (Sagi, Silkovsky, Fleisher-Berkovich & Danon 1995; Harrison 1997). The high correlation found between the n-3:n-6 HUFA ratio of the diet and the ovaries in the present study (see Fig. 2), emphasizes the importance of formulating a well-balanced dietary FA composition.

In conclusion, it can be said that the process of ovarian maturation (not necessarily overall reproductive performance and/or offspring quality) was negatively affected by increasing dietary TDL levels from 8.1% to 8.8% or higher. No clear influence of dietary HUFA on ovarian maturation was detected within the range 0.6–2.7% TDH. However, with the combined results of ovarian HUFA levels, haemolymphatic vg concentrations and the findings of previous studies, a TDH level of 2% may be recommendable for normal ovarian development, reproductive performance and offspring quality of penaeid shrimp.

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