Aquaculture Research, 2010, **41**, e393–e403 doi: 10.1111/j.1365-2109.2009.02469.x

Fatty acid profiles of spiny lobster (*Panulirus homarus*) phyllosoma fed enriched *Artemia*

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Abstract

Three different life stages of spiny lobster larvae (phyllosoma) of *Panulirus homarus* were fed A1-Selco-enriched *Artemia* in two culture treatments, one with the microalgae *Nannochloropsis salina* (green water) and the other without the microalgae (clear water) to assess the ability to manipulate their fatty acid composition. Phyllosoma fed with 3-h A1-Selco-enriched *Artemia salina* attained Stage VIII (5.3 mm) and Stage V (3.4 mm) in 42 days in the green and clear water treatments respectively. The higher content of the essential fatty acids in *N. salina* (eicosapentaenoic acid, 25.8%; arachidonic acid, 9.5%; and docosahexaenoic acid, 4.2%) in the green water system increased the fatty acid content of the live food *Artemia*, and ultimately the phyllosoma. In spite of phyllosoma being fed with enriched *Artemia* in the clear water system, the total polyunsaturated fatty acid content of the early (Stages I–III) and mid stage (Stages IV–V) phyllosoma were significantly smaller (18.8% and 14.6% respectively) ($P < 0.05$) than in the green water system (25.3% and 21.2% respectively). These results indicate the positive role of the microalgae in boosting the essential fatty acid content of lobster larvae.

Keywords: phyllosoma, fatty acids, *Nannochloropsis salina*, *Panulirus homarus* L.

Introduction

The spiny lobster, *Panulirus homarus* (Decapoda, Palinuridae) is one of the major lobster species distributed along the southern Indian coastline. It is a potential species for aquaculture, with wild fisheries accounting for over US$1.2 billion in export earnings. Increasing global demand, high product value and recent concern over the sustainability of wild stocks have prompted research in the commercial hatchery production of larvae for both replenishment of wild populations and commercial ongrowing. However, because of several possibilities, the hatchery larval rearing of the spiny lobster has been unsuccessful to date. A major problem in the successful culture of spiny lobster phyllosoma (larvae) has been the lack of fundamental information on diet and feeding habits (Mikami, Greenwood & Takashima 1994). Because the natural prey items of phyllosoma are largely unknown, the nutritional requirements of these animals are unlikely to be fully met in the aquaculture environment. *Artemia* are reported to be a suitable feed for lobster phyllosoma (Vijayakumaran & Radhakrishnan 1986; Radhakrishnan & Vijayakumaran 1993; Kanazawa 1994) as they are able to provide baseline nutrition (Nelson, Mooney, Nichols, Phleger, Smith & Ritar 2002) and they are a soft-bodied prey item that facilitates capture. Research on enriching *Artemia* with polyunsaturated fatty acids (PUFAs) before their use as live prey for lobster larvae has received considerable attention to increase larval survival rates (Nelson *et al.* 2002). The n3 PUFAs such as eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3), and n6 PUFA viz., arachidonic acid (AA, 20:4n6) and docosapentaenoic acid (DPA, 22:5n6), are of specific importance for the nutrition of finfish and crustacean larvae. These PUFAs, which are usually present in low concentrations in unenriched *Artemia*, are regarded as essential for crustaceans

(Lavens & Sorgeloos 2000). This suggests that *Artemia* should be enriched to improve the fatty acid profile for feeding to phyllosoma, with the ultimate aim to provide the correct balance of fatty acids in the diet of lobster larvae.

Several commercial enrichment diets such as DHA-Selco, A1-Selco and Protein Selco are used to supplement the PUFA profiles of *Artemia* and other live feeds used in aquaculture (Coutteau, Castell, Ackman & Sorgeloos 1996; Cho, Hur & Jo 2001). The fatty acid compositions of numerous marine microalgae have been determined for use in aquaculture, particularly as they are the base component of many marine food webs (Volkman, Jeffrey, Nichols, Rogers & Garland 1989; Vazhappilly & Chen 1998). The longer n3 and n6 fatty acids ($\geq$ 18 carbon atoms) dominate the composition of marine microalgae species and are able to be transferred to higher organisms via live feeds (Renaud, Parry & Thinh 1994), such as *Artemia*. It was reported that microalgae species belonging to the Prymnesiophytes (e.g. *Pavlova* spp. and *Isochrysis* spp.) and Cryptomonads (e.g. *Pyrenomonas* spp. and *Guillardia* spp.) have a high proportion of DHA, whereas eustigmatophytes (*Nannochloropsis* spp.) and diatoms have higher proportions of EPA and AA (Nichols, Holdsworth, Volkman, Daintith & Allanson 1989; Martínez-Fernández, Acosta-Salmón & Southgate 2006). Interest in the culture of these microalgal species that are tolerant to tropical culture conditions has increased over recent years, and is essentially important to cater the need of tropical mariculture (Nelson *et al.* 2002). The provision of PUFAs, in particular DHA, EPA and AA, in the diet of cultured larvae is of critical importance as many marine animals appear to have a limited ability to synthesize them *de novo* (Wantanabe 1993; Bell & Sargent 1996; Sargent, McEvoy & Bell 1997). Inadequate inclusion of DHA in cultured marine larvae frequently leads to problems such as visual impairment due to impaired rod function, decreased efficiency in capturing prey at low light intensities and changes to physiological and behavioural processes dependent on neuroendocrines (Wantanabe 1993). Arachidonic acid has been demonstrated to be essential for the production of eicosanoids and provide the basis for cyclooxygenase action to produce prostaglandins (Chakraborty, Chakraborty & Radhakrishnan 2007). An increment in AA during enrichment might reflect the increasing ability for AA-rich immune cells to fight surface-associated bacteria. The metabolic role of AA as the main PUFA in phosphoinositol, a key molecule in transduction signalling, was established (Bell & Sargent 2003). Detailed studies examining the appropriate ratios of DHA, EPA and AA have revealed that given a sufficient amount of DHA, an excess of EPA is not deleterious, whereas the inclusion of AA should be moderated because of generalized biochemically induced stress through excess eicosanoid production (Vazhappilly & Chen 1998).

In the present study, the effect of enrichment duration of *Artemia* using a commercial enrichment product (A1-Selco) on their fatty acid composition was examined. Three different life stages of *Artemia* (instar II, metanauplii and juvenile) were enriched with A1-Selco for different time periods and an optimum time period for enrichment (3 h) was selected as the standard enrichment duration that was later used for all the three life stages of *Artemia*. There are a number of methods used to culture phyllosoma larvae: the green culture water system and the clear water system that does not include algae (Radhakrishnan, Chakraborty, Thangaraja & Unnikrishnan 2009). During this study, the phyllosoma culture treatments were maintained in the two different systems, the green water system using the microalgae, *N. salina*. We report the changes in the fatty acid composition of enriched *Artemia vis-à-vis* different stage phyllosoma in the two water systems.

Materials and methods

Chemicals and reagents

All solvents used for sample preparation were of analytical grade and the solvents used for mass spectrophotometer analyses were of LC grade (E-Merck, Darmstadt, Germany). Analytical-grade solvents were redistilled in an all-glass system. Unless otherwise stated, starting material, reactants and solvents were used as such, or purified and dried by standard means.

Production of green water and *Artemia*

Green water culture

Nannochloropsis salina stock was cultured in Walne's/Conway's medium following an established procedure (Brown, Skabo & Wilkinson 1998). Two sets of cylindro-conical non-transparent indoor rearing FRP tanks (180 cm high) with a white inner surface and a water holding capacity of 300 L were provided with mild aeration under a light intensity of 5000–6000 lx (LT Lutron, LX, 101 Luxmeter, Lutron Electronics, Coopersburg, PA, USA). In one set of the (three) tanks, the inoculation culture of *N. salina*

(7 L, 30–40 million cells mL^{-1}) was added 4 days before the introduction of phyllosoma, while the other set of (three) tanks was kept as a control without adding microalgae, hereafter termed as the green water and clear water respectively. The green water system was regularly replenished with *N. salina* to maintain the cell concentration and log phase of growth. Partial exchange ($\sim$ 30%) of water with algae was conducted on a daily basis. Nutritional management of the larval rearing tanks and different environmental parameters were as indicated in an earlier report (Radhakrishnan *et al.* 2009).

Artemia culture

About 0.5–1.0 g of encapsulated *Artemia salina* cysts (Brine shrimp eggs, pro 80^{TM}, Ocean Star International, Snowville, UT, USA) were hydrated and disinfected in 0.4% aqueous sodium hypochlorite solution (w/v) for 2 h following the established procedure of Lavens and Sorgeloos (2000), and hatched following the procedure by Chakraborty *et al.* (2007). Freshly hatched nauplii were harvested after 18 h, separated from the hatching debris and unhatched cysts and thoroughly rinsed with seawater on a 120 μm screen. The *Artemia* nauplii instar-II (0.45–0.48 mm), *Artemia* metanauplii (1.1–1.3 mm, 5 days old) and juveniles (1.5–1.6 mm, 9–10 days old) were grown in microalgal water (*N. salina*) until they were enriched with A1-Selco (a PUFA concentrate, INVE, SA, Belgium). Before the feeding experiment, enrichment was carried out on the three different life stages of *Artemia* for various durations (1–48 h, depending on the stage) to standardize the optimum enrichment period with respect to PUFA content in the live food and an enrichment time of 3 h turned to be suitable.

Feeding of phyllosoma with *Artemia*

Details of collection and broodstock spawning of *P. homarus* have been reported previously (Radhakrishnan & Vijayakumaran 1993; Radhakrishnan *et al.* 2009). In brief, after hatching the berried female *P. homarus* was removed and newly hatched phyllosoma larvae were transferred to larval-rearing tanks (200 L of either green or clear seawater). The green water tanks are the same inoculated with *N. salina* as described previously. The larvae were stocked at a stocking density of 10 larvae L^{-1}. As the result of *Artemia* enrichment studies, a 3 h enrichment protocol for using A1-Selco was selected. All *Artemia* used in phyllosoma feeding were reared according to this protocol. Phyllosoma were fed newly hatched enriched *Artemia* nauplii from hatch – Stage III (10 days) at a density of 1.0 nauplii mL^{-1}. Stages IV–V (15 days) phyllosoma were fed enriched metanauplii daily at a density of 3–5 mL^{-1}. From Stage VI onwards, the larvae were fed with enriched juveniles at a rate of 3–5 mL^{-1}. Phyllosoma feeds were given following the daily water exchange, and flushing away of the uneaten *Artemia*.

Fatty acid analyses

Samples were taken for fatty acid analyses from different life stages of *Artemia* enriched with A1-Selco (instar-II, metanauplii and juveniles), *N. salina* and three different stages of spiny lobster phyllosoma (Stages I–III, IV–V and VI–VIII). Biomass of microalgae (50 mg), phyllosoma (50 mg) and *Artemia* (0.5 g) was harvested by centrifugation at 3000 *g* for 5 min, and the lipids and fatty acid methyl esters (FAME) were prepared following an established method (Bligh & Dyer 1959; Morrison & Smith 1964). Gas chromatographic (GC) analyses were performed using a Perkin Elmer AutoSystem XL, Gas Chromatograph (Perkin Elmer, Waltham, MA, USA) equipped with an Elite-5 (crossbond 5% diphenyl–95% dimethyl polsiloxane) capillary column (30 m $\times$ 0.53 mm i.d., 0.5 μm film thickness, Supelco, Bellfonte, PA) using a flame ionization detector. The temperature programme and instrument parameters were as described earlier (Chakraborty & Paulraj 2007, 2008). Fatty acid methyl esters were identified by a comparison of retention times with the known standards (37-component FAME Mix, PUFA-3, PUFA-1, BAME; Supelco). Fatty acid composition was expressed as the percentage of the total fatty acids (% TFA) of each fraction. *N*-acyl pyrrolidides for use in GC-mass spectroscopic analyses were synthesized by a reaction between pyrrolidine (1 mL) and FAME (1 mg) in acetic acid (0.1 mL) under reflux (100 °C, 2 h) as described earlier (Chakraborty & Paulraj 2009). The distinct fragmentation peaks of FAMEs and pyrrolidides are described in Table 1.

Bacteriological quality of culture water in green water and clear tanks

The culture water was collected weekly from both green and clear water tanks, and serially diluted with sterile seawater. Water samples were collected for the analysis of bacteriological quality before water exchange. The samples thus prepared were spread on Petri dishes (90 mm diameter) preset with ZoBell's

Table 1 Gas chromatographic mass spectroscopic (GC-MS) fragmentation pattern of fatty acid methyl esters (FAMEs) and *N*-acyl pyrrolidides

	Peaks (*m/z*)	
	FAMEs	***N*-acyl pyrrolidides[a]**
Saturated fatty acid (SFA)		
Molecular ion $(M)^+$	Prominent	Prominent
Monounsaturated fatty acid (MUFA)		
Molecular ion $(M)^+$	Not prominent	Prominent
Polyunsaturated fatty acid (PUFA)		
Molecular ion $(M)^+$	Not prominent	Very prominent
Base peak (100%)	1-methoxyethenol (*m/z* 74)	McLafferty rearrangement ion, 1-(pyrrolidin-1-yl)-ethenol (*m/z* 113)
Other prominent peaks	1-methoxyprop-2-en-1-ol (*m/z* 88)	1-(pyrrolidin-1-yl)-prop-2-en-1-ol (*m/z* 127)
Tropylium ion (*m/z* 91)	Prominent	Not prominent
Fingerprinting peaks	The *m/z* fragment ions at a difference of *m/z* 14 (not prominent)	Uniform distribution of fragment peaks at *m/z* 14 U except in the vicinity of the double bond, where the interval is *m/z* 12 U (prominent peaks)
McLafferty rearrangement ion	McLafferty ion (*m/z* 222)	Not apparent

**N*-acyl pyrrolidides were synthesized by reaction between pyrrolidine (1 mL) and FAME (1 mg) in acetic acid (0.1 mL) under reflux (100 °C, 2 h). GC-MS analyses were performed using EI mode in a Varian GC (CP-3800, Varian, Walnut Creek, CA, USA) interfaced with a Varian instrument 1200 L single quadruple mass spectrometer. The GC was equipped with WCOT-fused silica capillary column of high polarity (DB-5; 30 m × 0.25 mm i.d., 0.39 mm o.d., and 0.25 μm film thickness).

Marine (ZMA, Difco Laboratories, Detroit, MI) and thiosulphate citrate bile sucrose (TCBS) agar media. ZoBell's Marine and TCBS media were used to identify heterotrophic marine bacteria and *Vibrio* sp. respectively. The plates were incubated overnight at 37 °C, and the total bacterial counts were recorded (Igarashi, Kittaka & Kawahara 1990) as colony-forming units ($CFU\,mL^{-1}$).

Statistical analyses

Percentage composition of FAME in phyllosoma, diets and *Artemia* sampled at different enrichment times were subjected to a one-way analysis of variance. Correlations between *Artemia* chemical components and A1-Selco components were investigated using SPSS (ver. 10.0) software. A significance level of 95% ($P \leq 0.05$) was used throughout. *Arc sin* transformation was used before the statistical analyses of FAME data expressed in percentages. All measurements were performed in triplicate ($n = 3$), and the values were averaged.

Results

Fatty acid composition of the enrichment product and *Artemia* enriched for up to 48 h

Enrichment product (A1-Selco)

As evident from Table 2, the important PUFAs dominant in A1-Selco in decreasing order of proportional abundance of TFA were EPA (10.8%) > DHA (8.8%) > AA (5.4%). Among n3 PUFAs, EPA was found to be the most abundant in A1-Selco followed by DHA. The fatty acid 18:2n6 was found to be most dominant followed by 20:4n6 among n6 PUFAs (Table 2).

Enrichment of Artemia

Artemia nauplii (instar-II) possessed a fatty acid pattern with significantly high 18:2n6 (8.5%), 18:3n3 (4.1%), EPA (3.2%) and negligible DHA (0.1%). The fatty acid composition of enriched *Artemia* varied, and was a function of the enrichment time. Total PUFA in A1-Selco-enriched *Artemia* nauplii remained the same during the first 3 h of enrichment (22.8–23.5%), and decreased afterwards (4.7% after 48 h) (Table 2). However, EPA and DHA were present in significantly ($P < 0.05$) higher proportions after 3 h of enrichment (7.5% and 1.8% respectively), reducing with further enrichment. The proportion of AA remained unchanged up to 3 h of enrichment decreasing significantly with longer enrichment durations (Table 2). Similar results were observed for the enrichment studies for *Artemia* metanauplii and juveniles (Table 3). For example, EPA and DHA were found to be at their peaks (5.2% and 1.5% respectively) in *Artemia* metanauplii enriched for 3 h, and significantly ($P < 0.05$) reduced thereafter with further enrichment (Table 3). Similarly, EPA and

Table 2 Percentage of total fatty acid composition (% TFA) of *Artemia* nauplii (instar-II) and of *Artemia* enriched with A1-Selco for up to 48 h

	Fatty acids (% TFA)							
			Instar-II *Artemia* A1-Selco enrichment durations (h)					
Fatty acids	*Artemia* nauplii (instar-II)	A1-Selco	1	2	3	8	24	48
Saturated fatty acids								
14:0	0.32c ± 0.05	7.60b ± 0.53	0.41c ± 0.04	1.52c ± 0.13	3.15b ± 0.16	5.82b ± 0.48	24.05a ± 1.50	37.71a ± 2.84
15:0	0.21c ± 0.03	0.54c ± 0.08	0.28c ± 0.03	0.32c ± 0.05	0.37c ± 0.03	1.50b ± 0.10	3.64b ± 0.27	7.43a ± 0.59
16:0	8.07b ± 0.29	12.25a ± 0.61	8.15b ± 0.26	7.60c ± 0.36	8.90b ± 0.47	10.35b ± 0.89	14.50a ± 1.14	12.80a ± 0.96
18:0	4.91a ± 0.13	3.14b ± 0.24	4.72a ± 0.15	4.96a ± 0.28	4.95a ± 0.18	3.02b ± 0.23	2.46c ± 0.15	2.11c ± 0.08
ΣSFA	13.51c	23.53c	13.56c	14.4c	17.38c	18.04c	44.65b	60.05a
Monounsaturated fatty acids								
16:1n7	9.62b ± 0.26	17.12a ± 0.96	9.91b ± 0.63	10.16b ± 0.73	10.62b ± 0.53	10.18b ± 0.65	7.44b ± 0.52	3.25c ± 0.14
16:1n9	16.30a ± 0.85	0.12c ± 0.02	16.48a ± 1.29	15.31a ± 0.96	9.25b ± 0.38	8.07b ± 0.18	7.59b ± 0.38	6.82b ± 0.55
18:1n7	2.21c ± 0.15	ND	2.23c ± 0.15	5.25b ± 0.29	9.10a ± 0.82	7.75a ± 0.67	1.52c ± 0.11	1.18c ± 0.05
18:1n9	24.26a ± 1.59	23.30b ± 1.06	24.38b ± 1.57	25.10b ± 1.43	25.18b ± 1.93	29.24a ± 1.83	25.67b ± 1.18	19.16c ± 0.73
20:1n9	ND	ND	0.14c ± 0.02	0.58a ± 0.04	ND	ND	0.25b ± 0.02	0.11c ± 0.02
ΣMUFA	52.39a	40.54b	53.14a	56.4a	54.15a	55.24a	42.47b	30.52c
Polyunsaturated fatty acids								
16:3n4	0.41a ± 0.03	0.43a ± 0.07	0.43a ± 0.08	0.45a ± 0.06	0.45a ± 0.04	0.38b ± 0.03	0.20c ± 0.03	ND
16:3n6	8.08a ± 0.27	0.62c ± 0.11	6.15a ± 0.36	4.37a ± 0.33	1.23b ± 0.07	1.40b ± 0.06	1.45b ± 0.12	0.86c ± 0.05
18:2n6	8.50a ± 0.79	5.40b ± 0.35	5.40b ± 0.21	5.59b ± 0.28	4.59b ± 0.32	3.11c ± 0.29	ND	ND
18:3n3	4.10a ± 0.16	ND	4.51a ± 0.17	4.73a ± 0.45	5.52a ± 0.40	4.43a ± 0.18	2.10b ± 0.19	1.49b ± 0.10
20:4n6	2.30a ± 0.18	1.50b ± 0.14	2.39a ± 0.23	2.39a ± 0.14	2.42a ± 0.17	1.96b ± 0.05	0.18c ± 0.02	ND
20:5n3	3.18c ± 0.11	10.81a ± 0.60	3.35c ± 0.09	4.13b ± 0.21	7.47b ± 0.61	5.82b ± 0.49	3.28c ± 0.28	2.14c ± 0.16
22:6n3	0.10c ± 0.02	8.75a ± 0.24	0.79c ± 0.05	1.10b ± 0.05	1.78b ± 0.13	0.94b ± 0.07	0.25c ± 0.01	0.18c ± 0.02
ΣPUFA	26.67a	27.51a	23.02b	22.76b	23.46b	18.04b	7.46c	4.67c
n3/n6	0.39c	2.60a	0.62c	0.80c	1.79b	1.72b	3.45a	4.43a

Individual fatty acids are expressed as percentage of total identifiable fatty acids.ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids. Data are presented as mean values of three samples (mean ± standard deviation). Row values with different alphabet subscripts (a–c) are significantly different ($P < 0.05$), ND, fatty acid identified as trace, but not integrated.

DHA were present in significantly higher proportions after 3 h of enrichment in juvenile *Artemia* (4.7% and 1.3% respectively), reducing significantly ($P < 0.05$) with further enrichment. The content of AA in *Artemia* metanauplii and juveniles were found to reach their peaks after 3 h of enrichment (2.1% and 1.7% respectively) (Table 3).

Fatty acid profiles of green water and phyllosoma fed enriched *Artemia* in green and clear water systems

The percentage of total PUFA was high (42.3%) in this microalgae and was dominated by EPA (25.8%), AA (9.5%) and DHA (4.2%) with an n3/n6 ratio of 2.55 (Table 4). Early-stage phyllosoma (Stages I–III) reared in green water and fed with A1-Selco-enriched *Artemia* for 3 h exhibited significantly higher total PUFA, DHA and EPA concentration (25.3%, 2.4% and 10.3% respectively) than those reared in clear water (18.8%, 1.0% and 6.2% respectively), thus probably reflecting a dietary input from the microalgae of the green water system (Table 4). A contrary result was noted in AA with a significantly lower value in the green water system (3.0%) than in the clear water system (4.2%). Mid-stage phyllosoma (Stages IV–V) fed A1-Selco-enriched *Artemia* nauplii and cultured in green water were found to have significantly higher contents of total PUFA (21.2%), DHA (2.1%), AA (3.9%) and a significantly higher n3/n6 ratio (1.5), compared with those cultured in clear water (14.6%, 1.0%, 2.0% and 1.2 respectively). Advanced stages of phyllosoma (Stage VI onwards) were cultured only in the green water system as the clear water system crashed due to high mortality. The patterns of total PUFA and individual PUFAs (DHA, EPA and AA) were comparable with ($P < 0.05$) those for Stages IV–V phyllosoma cultured in green water. However, the n3/n6 ratio was significantly lower

Table 3 Percentage of total fatty acid composition (% TFA) of *Artemia* metanauplii (1.10–1.30 mm, 5 days old) and juveniles (1.55–1.58 mm, 9–10 days old) enriched with A1-Selco for 2–8 h

	***Artemia* A1-Selco enrichment durations**					
	***Artemia* metanauplii**			**Juvenile *Artemia***		
Fatty acids	**2 h**	**3 h**	**8 h**	**2 h**	**3 h**	**8 h**
Saturated fatty acids						
14:0	3.15c ± 0.25	3.29c ± 0.14	6.39a ± 0.40	3.82b ± 0.18	4.18b ± 0.33	8.47a ± 0.61
15:0	0.39c ± 0.03	0.93b ± 0.06	2.03a ± 0.18	0.93b ± 0.03	1.25b ± 0.08	1.50b ± 0.13
16:0	12.05c ± 0.95	12.27c ± 1.27	14.11b ± 0.73	14.06b ± 0.96	14.39b ± 0.81	19.38a ± 1.87
18:0	6.27b ± 0.53	6.33b ± 0.26	7.22a ± 0.51	6.37b ± 0.53	6.42b ± 0.36	7.69a ± 0.25
ΣSFA	21.86c	22.82b	29.75a	25.18b	26.24b	37.04a
Monounsaturated fatty acids						
16:1n7	10.68b ± 0.87	11.38a ± 0.69	10.83b ± 0.58	10.74b ± 1.35	10.85b ± 0.97	10.72b ± 0.59
16:1n9	14.57a ± 1.16	9.26b ± 0.43	8.64b ± 0.71	13.81a ± 0.84	9.49b ± 0.53	7.26c ± 0.47
18:1n7	3.62b ± 0.18	4.14a ± 0.31	3.56b ± 0.13	3.69b ± 0.22	4.30a ± 0.18	3.59b ± 0.33
18:1n9	28.11b ± 1.73	29.62a ± 1.52	29.75a ± 1.80	28.15b ± 1.69	29.57a ± 1.46	28.33b ± 2.52
20:1n9	ND	ND	ND	ND	ND	ND
ΣMUFA	56.98a	54.4b	52.78c	56.39a	54.21b	49.90c
Polyunsaturated fatty acids						
16:3n4	ND	ND	0.12b ± 0.02	0.12b ± 0.03	0.29a ± 0.03	0.19b ± 0.02
16:3n6	3.29a ± 0.21	2.43b ± 0.14	2.49b ± 0.17	2.87b ± 0.26	2.65b ± 0.14	2.27b ± 0.19
18:2n6	4.51a ± 0.35	3.17b ± 0.17	3.04b ± 0.23	3.63b ± 0.28	2.08c ± 0.07	1.83c ± 0.14
18:3n3	3.17b ± 0.28	4.23a ± 0.36	3.78b ± 0.19	2.03c ± 0.02	2.15c ± 0.11	1.94c ± 0.07
20:4n6	1.86b ± 0.09	2.07a ± 0.11	1.61b ± 0.05	1.38c ± 0.11	1.68b ± 0.15	1.26c ± 0.12
20:5n3	3.82b ± 0.17	5.15a ± 0.43	3.27b ± 0.28	3.26b ± 0.21	4.67a ± 0.70	2.89b ± 0.23
22:6n3	0.95b ± 0.05	1.54a ± 0.12	0.73b ± 0.04	0.71b ± 0.05	1.28a ± 0.09	0.62b ± 0.05
ΣPUFA	17.6a	18.59a	15.04b	14.00b	14.8b	11.00c

Individual fatty acids are expressed as percentage of total fatty acid. Fatty acid data are presented as mean ± SD. Other notations are as indicated under Table 2.

(1.3) in Stages VI–VII than in Stages IV–V (1.5) phyllosoma (Table 4).

Size, survival rate and time frame for the developmental stages of *P. homarus* phyllosoma

The total length of phyllosoma reared in the green water system was found to be 2.6 mm at Stage III (Fig. 1). However, lobster larvae cultured in the clear water system were found to be smaller (2.1 mm at Stage III) than those reared in green water. At the end of the 42-day culture period, phyllosoma in the green water system had advanced to Stage VIII (5.3 mm) with a survival rate of about 33%, while those in the clear water system only reached Stage V (3.4 mm) and had very low survival (Fig. 1). The intermoult interval of phyllosoma reared in the clear water was found to be longer than that recorded in the green water system. The lobster larvae were recorded to moult six times with a total intermoult period of 5–9 days to attain Stage V in the clear water system, but nine times with a total intermoult period of 4–6 days to advance to stage VIII in the green water system.

Microbial quality of green and clear water systems used to culture phyllosoma

Heterotrophic marine bacteria and *Vibrio* counts were generally much lower (20×10^3 and 0.3×10^3 CFU mL^{-1} respectively) in the green than in the clear water systems during the 6-week experimental period (Fig. 2). The mid phase of the culture period exhibited high bacterial load in clear water (total heterotrophic marine bacterial count, 64×10^3 CFU mL^{-1} and *Vibrio* count, 2.2×10^3 CFU mL^{-1}), while in the green water system a sharp decrease in total heterotrophic marine count to 1×10^3 CFU mL^{-1} and total *Vibrio* count to 0.2×10^2 CFU mL^{-1} after stage IV were apparent (Fig. 2).

Table 4 Percentage of total fatty acid composition (% TFA) of newly hatched *Panulirus homarus* phyllosoma larvae and of three subsequent life stages (I–III, IV–V and VI–VII) fed with A1-Selco enriched (for 3 h) *Artemia* in green and clear water rearing systems

	Fatty acids (% TFA)						
	Phyllosoma						
Fatty acids	Newly hatched phyllosoma	*Nannochloropsis salina*	Stages: I–III		Stages: IV–V		Stages: VI–VII
			Green water	Clear water	Green water	Clear water	Green water
Saturated fatty acids							
14:0	2.49a ± 0.15	0.39b ± 0.03	2.85a ± 0.15	2.96a ± 0.16	4.15a ± 0.23	5.48a ± 0.43	4.28a ± 0.29
15:0	1.80c ± 0.09	3.10b ± 0.24	2.72b ± 0.06	3.59a ± 0.21	2.97b ± 0.17	3.53b ± 0.21	3.56a ± 0.12
16:0	12.22c ± 0.83	21.0a ± 1.05	13.94c ± 0.60	16.62b ± 1.39	14.69b ± 0.84	17.24b ± 0.76	15.28b ± 1.36
18:0	7.35b ± 0.60	0.94c ± 0.08	8.00b ± 0.38	7.19b ± 0.53	10.84a ± 1.32	12.91a ± 1.05	12.87a ± 1.13
ΣSFA	23.86b	25.43b	27.51b	18.81c	32.65a	39.16a	35.99a
Monounsaturated fatty acids							
16:1n7	4.36b ± 0.21	20.1a ± 0.68	5.87b ± 0.42	5.26b ± 0.30	4.24b ± 0.11	3.29b ± 0.16	3.11b ± 0.08
16:1n9	1.53b ± 0.06	ND	ND	ND	ND	1.57b ± 0.05	2.08a ± 0.16
18:1n7	4.31b ± 0.17	0.50c ± 0.02	6.20a ± 0.31	8.48a ± 0.76	6.92a ± 0.53	7.11a ± 0.69	8.23a ± 0.53
18:1n9	24.60a ± 1.72	9.40b ± 0.75	28.18a ± 1.85	29.83a ± 2.57	25.81a ± 1.79	28.06a ± 1.61	24.35a ± 2.07
20:1n9	2.34a ± 0.16	ND	0.95b ± 0.07	1.69a ± 0.13	1.30b ± 0.08	ND	1.27b ± 0.08
ΣMUFA	37.14c	30.0b	41.2a	45.26a	38.27c	40.03b	39.04b
Polyunsaturated fatty acids							
16:3n4	0.42a ± 0.03	0.05b ± 0.00	0.20a ± 0.02	0.15a ± 0.03	ND	ND	ND
16:3n6	1.28b ± 0.10	0.31c ± 0.02	2.52a ± 0.10	1.82b ± 0.07	1.93a ± 0.13	1.25b ± 0.14	2.18a ± 0.19
18:2n6	3.15a ± 0.16	2.10b ± 0.11	3.59a ± 0.26	2.53b ± 0.18	2.65b ± 0.20	3.28a ± 0.18	2.53b ± 0.06
18:3n3	0.30b ± 0.02	0.40b ± 0.03	3.27a ± 0.13	2.89a ± 0.05	3.78a ± 0.42	3.16a ± 0.25	3.93a ± 0.25
20:4n6	4.96b ± 0.38	9.50a ± 0.37	3.01c ± 0.46	4.22b ± 0.31	3.85b ± 0.27	2.03c ± 0.07	4.18b ± 0.33
20:5n3	5.34c ± 0.42	25.80a ± 2.35	10.34b ± 0.71	6.16c ± 0.49	6.88c ± 0.51	3.80c ± 0.15	5.93c ± 0.40
22:6n3	1.98b ± 0.09	4.18a ± 0.19	2.36b ± 0.15	1.04c ± 0.08	2.10b ± 0.09	1.04c ± 0.02	1.97b ± 0.14
ΣPUFA	17.43c	42.34a	25.29b	18.81c	21.19b	14.56c	20.72b
n3/n6	0.81c	2.55a	1.77b	1.75b	1.51b	1.22c	1.33c

No survival of phyllosoma in the clear water system after Stage V was apparent; Stages I–III phyllosoma fed on *Artemia* nauplii; Stages IV–V phyllosoma fed on *Artemia* metanauplii; Stages VI–VII phyllosoma fed on juvenile *Artemia*. Individual fatty acid is expressed as percentage of total identifiable fatty acids (mean ± SD). Other notations are as indicated under Table 2.

Discussion

Artemia are well known for their value as one of the major live foods for the larvae of penaeids and lobsters (Sorgeloos, Coutteau, Dhert, Merchie & Lavens 1998). However, *Artemia* is deficient in essential PUFAs, particularly EPA and DHA (Brown 1994; Kanazawa 1994). Optimizing the biochemical composition of *Artemia* is important to improve survival and growth of lobster phyllosoma (Nelson *et al.* 2002). The commercial product A1-Selco was used to enhance the PUFA content of *Artemia* nauplii, metanauplii and juveniles. The fatty acid composition of enriched *Artemia* of each life stages varied according to the enrichment time. A 3-h enrichment resulted in the highest EPA and DHA contents. Increasing the enrichment time resulted in a lower essential PUFA content. Thus, an enrichment time of 3 h appeared to be adequate.

Enrichment beyond 3 h reduced the PUFA content in *Artemia*, possibly due to the fact that bioaccumulation of PUFAs in the active functional metabolic pool occurs at an insufficient rate (Mourente & Tocher 1994; Tocher & Ghioni 1999). This can be explained due to the limited capacity of *Artemia* for the desaturation and elongation of shorter chain length fatty acids to provide an adequate amount of essential fatty acids like EPA and DHA (Kanazawa, Teshima & Ono 1979; Castell 1982). It is apparent that *Artemia* nauplii supplemented with A1-Selco preferentially catabolize these essential PUFAs to SFAs when enriched for >3 h (Chakraborty *et al.* 2007).

In an earlier study of Radhakrishnan and Vijayakumaran (1993), poor survival and longer moulting interval of *P. homarus* phyllosoma under laboratory culture conditions were attributed to poor water quality and nutritional deficiencies. The phyllosoma

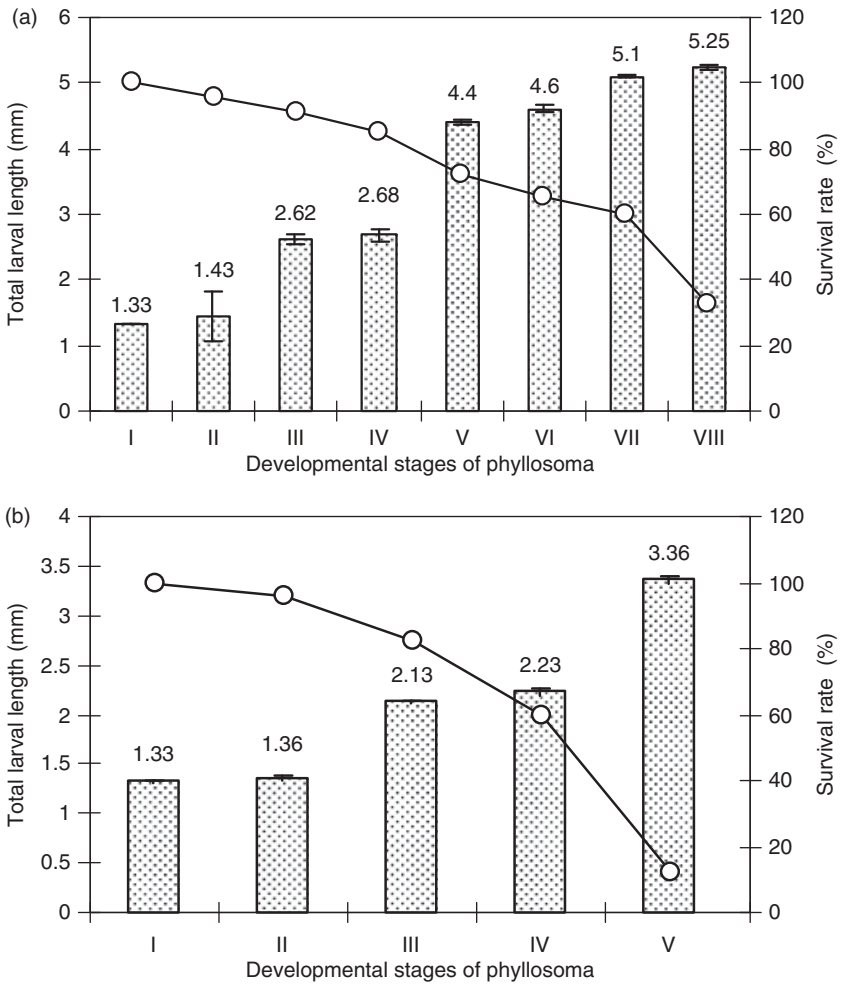

Figure 1 Differential size pattern (total length) and survival rate (%) of *Panulirus homarus* phyllosoma in (a) green water and (b) clear water rearing systems. No survival of phyllosoma was apparent after Stage V in the clear water system. Phyllosoma were able to attain Stage VIII with a size of 5.25 ± 0.04 mm and a survival rate of 33% in the green water system.

reared in the green water system reached Stage VIII after 42 days, whereas those in the clear water system only attained Stage V (3.4 mm) during the same culture period. The faster development of phyllosoma in the green water rearing system was possible due to the combined effect of the two factors *viz.*, live feed *Artemia* enriched with PUFA and microalgae *N. salina*, and perhaps the effect of the latter only.

Total PUFA in early-stage phyllosoma (Stages I–III) fed with enriched *A. salina* was found to be 25% in the green water rearing system, compared with 19% in the clear water rearing system. The former system yielded early-stage phyllosoma (Stages I–III) with 10.3% EPA, 3.0% AA and 2.4% DHA, compared with 6.2%, 4.2% and 1.0%, respectively, in clear water ($P < 0.05$). The microalgae contained a range of PUFAs such as EPA, AA and DHA in appreciable quantities (25.8%, 9.5% and 4.2% respectively), and apparently contributed to the higher content of these fatty acids in *P. homarus* phyllosoma reared in green water. Because of the direct correlation between n3 PUFA with larval growth and the known limited ability of many marine larvae to synthesize n3 essential fatty acids, they must be supplied externally (Mourente & Tocher 1994). These observations further indicate that when phyllosoma were cultured in green water, they were able to upgrade their own fatty acid content to a satisfactory level by utilizing excessive amounts of *N. salina*. However, these long-chain n3 PUFAs exhibited a reduction in their composition in advanced-stage phyllosoma (Stage IV onwards). The total PUFA content of Stages IV–V phyllosoma was

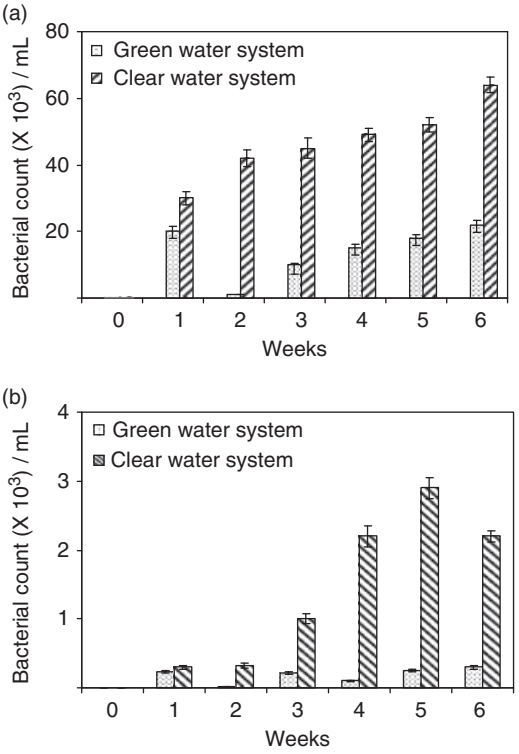

Figure 2 Bacterial load in green water and clear water rearing systems used to culture phyllosoma. Bacterial counts are expressed as colony-forming units (CFU) per mL. (a) Heterotrophic marine bacterial count and (b) bacterial count with respect to *Vibrio* sp. Total counts ranging between 30 and 300 colonies were recorded on the plates. Samples of the culture water from both the experimental tanks were serially diluted in sterile seawater, and grown on ZoBell's 2216 agar (for total heterotrophic marine bacteria) and thiosulphate citrate bile sucrose media (selective media for *Vibrio* sp.).

found to decrease by 16.2% in green and 22.6% in clear water, followed by a significant reduction in Stages VI–VII (20.72% in green water). Eicosapentaenoic acid reduced to 6.9% in Stages IV–V phyllosoma reared in green water and 3.8% in the clear water system. In Stages VI–VII phyllosoma, EPA and DHA were found to be significantly ($P < 0.05$) reduced (5.9% and 2.0% respectively) possibly due to their formation occurring at an insufficient rate by desaturation (by Δ5-fatty acid desaturase) and elongation (by elongase) of 18:3n3 or 18:4n3 (Narciso, Pousao-Ferreira, Passos & Luis 1999). It is apparent that phyllosoma do not store these long-chain PUFAs (EPA and DHA) in similar proportions to other short-chain $C_{16–18}$ PUFAs like 16:3n6, 18:2n6 and 18:3n3. Interestingly, an increase in C_{16-18} PUFAs like 16:3n6 (1.9% in Stages IV–V and 2.2% in Stages VI–VII) and 18:3n3 (3.8% in Stages IV–V and 3.9% in Stages VI–VII) were apparent in phyllosoma reared in green water. It can be concluded that their concentration range was maintained either through input from *N. salina* or through biosynthetic processes. These results indicate the inability of phyllosoma to synthesize EPA and DHA from short-chain precursors (like 18:3n3) at an optimum rate apparently due to the low level of Δ5-desaturase activity.

In addition, the lower bacterial load in the water column during the entire culture period may also benefit the development of phyllosoma in the green water system. The decrease in the bacterial counts in the green water system suggests that bacteria might have been controlled by the antibacterial activities of *Nannochloropsis* (Seraspe, Valenzuela, Maeda & Kamei 2005). Microalgae were reported to have unique antibacterial and/or immunostimulatory properties, thus stimulating the non-specific immune system of phyllosoma and affecting their feeding activity (Igarashi *et al.* 1990). Glycosides and terpenes were isolated from *Nannochloropsis*, and these compounds were reported to have antibacterial properties associated with vibriostatic activity (Seraspe *et al.* 2005). Improved survival of early-stage larvae of *Panulirus japonicus* was achieved with *Nannochloropsis* sp. (Kittaka 1991). Kittaka (1994) stated that the presence of microalgae in rearing tanks not only inhibited the proliferation of microbial contamination but also served as a natural water purifier, therefore enhancing the survival rate, moulting frequency and growth of the larvae.

The content of 20:4n6 was found to 4.2% higher ($P < 0.05$) in clear water at the initial stage (Stages I–III). An increment of 20:4n6 in Stages I–III larvae cultured in clear water might reflect increasing stress levels resulting in a higher production of this fatty acid. It is apparent that some enzymes may not be present in phyllosoma, and their formation may be induced by metabolic stress. However, an advanced-stage phyllosoma (Stages IV–V) reared in clear water may be unable to synthesize the stress enzyme(s) required to produce 20:4n6 in sufficient amounts (2.0%).

In this study, a clear and green water system for feeding larvae of the spiny lobster *P. homarus* with A1-Selco-enriched *Artemia* sp. were compared. The use of *N. salina* as microalgae in the green water system improved the growth and survival of *P. homarus* phyllosoma compared with the clear water culture.

Acknowledgments

The authors are thankful to Prof. (Dr) Mohan Joseph Modayil, Director, CMFRI, Cochin, for providing the necessary facilities to carry out the work. Thanks are also due to Dr Shibu M. Eappen, Scientist B, Sophisticated Test and Instrumentation Centre, Cochin University of Science and Technology, Kerala for recording the mass spectral data.

References

Bell J.G. & Sargent J.R. (1996) Lipid nutrition and fish recruitment. *Marine Ecology Progress Series* **134**, 315–316.

Bell J.G. & Sargent J.R. (2003) Arachidonic acid in aquaculture feeds: current status and future opportunities. *Aquaculture* **218**, 491–499.

Bligh E.G. & Dyer W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry* **37**, 911–917.

Brown M.F. (1994) Modulation of rhodopsin function by properties of the membrane bilayer. *Chemistry and Physics of Lipids* **73**, 159–180.

Brown M.R., Skabo S. & Wilkinson B. (1998) The enrichment and retention of ascorbic acid in rotifers fed microalgal diets. *Aquaculture Nutrition* **4**, 151–156.

Castell J.D. (1982) Fatty acid metabolism in crustaceans. In: *Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition* (ed. by G.D. Pruder, C.J. Langdon & D.E. Conklin), pp. 124–145. Louisiana State University, Baton Rouge, LA.

Chakraborty K. & Paulraj R. (2007) Eicosapentanoic acid enrichment from sardine oil by argentation chromatography. *Journal of Agricultural and Food Chemistry* **55**, 7586–7595.

Chakraborty K. & Paulraj R. (2008) Enrichment of eicosapentaenoic acid from sardine oil with Δ5-olefinic bond specific lipase from *Bacillus licheniformis* MTCC 6824. *Journal of Agricultural and Food Chemistry* **56**, 1428–1433.

Chakraborty K. & Paulraj R. (2009) Selective enrichment of n3 polyunsaturated fatty acids with C_{18}–C_{20} acyl chain length from sardine oil using *Pseudomonas fluorescens* MTCC 2421 lipase. *Food Chemistry* **114**, 142–150.

Chakraborty R.D., Chakraborty K. & Radhakrishnan E.V.R. (2007) Variation in fatty acid composition of *Artemia salina* nauplii enriched with microalgae and baker's yeast for use in larviculture. *Journal of Agricultural and Food Chemistry* **55**, 4043–4051.

Cho S.H., Hur S.B. & Jo J.Y. (2001) Effect of enriched live feeds on survival and growth rates in larval Korean rockfish, *Sebastes schlegeli* Hilgendorf. *Aquaculture Research* **32**, 199–208.

Coutteau P., Castell J.D., Ackman R.G. & Sorgeloos P. (1996) The use of lipid emulsions as carriers for essential fatty acids in bivalves: a test case with juvenile *Placopecten magellanicus*. *Journal of Shellfish Research* **15**, 259–264.

Igarashi M.A., Kittaka J. & Kawahara E. (1990) Phyllosoma culture with inoculation of marine bacteria. *Nippon Suisan Gakkaishi* **56**, 1781–1786.

Kanazawa A. (1994) Nutrition and food. In: *Spiny Lobster Management* (ed. by B.F. Phillips, J.S. Cobb & J. Kittaka), pp. 483–494. Blackwell, Oxford, UK.

Kanazawa A., Teshima S. & Ono K. (1979) Relationship between essential fatty acid requirements of aquatic animals and the capacity for bioconversion of linolenic acid to highly unsaturated fatty acids. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **63**, 295–298.

Kittaka J. (1991) Culture of spiny lobsters from egg to puerulus stage. *Revista de Investigaciones Marinas* **12**, 332–340.

Kittaka J. (1994) Culture of phyllosomas of spiny lobster and its application to studies of larval recruitment and aquaculture. *Crustaceana* **66**, 258–270.

Lavens P. & Sorgeloos P. (2000) The history, present status and prospect of the availability of the *Artemia* cysts for aquaculture. *Aquaculture* **181**, 397–403.

Martínez-Fernández E., Acosta-Salmón H. & Southgate P.C. (2006) The nutritional value of seven species of tropical microalgae for black-lip pearl oyster (*Pinctada margaritifera* L.) larvae. *Aquaculture* **257**, 491–503.

Mikami S., Greenwood J.G. & Takashima F. (1994) Functional morphology and cytology of the phyllosomal digestive system of *Ibacus ciliatus* and *Panulirus japonicus* (Decapoda, Scyllaridae and Palinuridae). *Crustaceana* **67**, 212–225.

Morrison W.R. & Smith L.M. (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *Journal of Lipid Research* **5**, 600–608.

Mourente G. & Tocher D.R. (1994) *In vivo* metabolism of [$^{1-14}$C]-linolenic acid (18:3n-3) and [$^{1-14}$C] eicosapentaenoic acid (20:5n3) in a marine fish: time-course of the desaturation/elongation pathway. *Biochimica Biophysica Acta* **1212**, 109–118.

Narciso L., Pousao-Ferreira P., Passos A. & Luis O. (1999) HUFA content and DHA/EPA improvements of *Artemia* sp. with commercial oils during different enrichments periods. *Aquaculture Research* **30**, 21–24.

Nelson M.M., Mooney B.D., Nichols P.D., Phleger C.F., Smith G.G. & Ritar A.J. (2002) The effect of diet on the biochemical composition of on-grown *Artemia*: potential formulations for rock lobster aquaculture. *Journal of the World Aquaculture Society* **33**, 146–157.

Nichols P.D., Holdsworth D.G., Volkman J.K., Daintith M. & Allanson S. (1989) High incorporation of essential fatty acids by the rotifer *Brachionus plicatilis* fed on the prymnesiophyte alga *Pavlova lutheri*. *Australian Journal of Marine and Freshwater Research* **40**, 645–655.

Radhakrishnan E.V. & Vijayakumaran M. (1993) Early larval development of the spiny lobster *Panulirus homarus*

(Linnaeus, 1758) reared in the laboratory. *Crustaceana* **68**, 151–159.

Radhakrishnan E.V., Chakraborty R.D., Thangaraja R. & Unnikrishnan C. (2009) Effect of *Nannochloropsis salina* on the survival and growth of phyllosoma of the tropical spiny lobster *Panulirus homarus* L. under laboratory conditions. *Journal of Marine Biological Association of India* **51**, 52–60.

Renaud S.M., Parry D.L. & Thinh L.V. (1994) Microalgae for use in tropical aquaculture. 1. Gross chemical and fatty acid composition of twelve species of microalgae from the Northern Territory, Australia. *Journal of Applied Phycology* **6**, 337–345.

Sargent J.R., McEvoy L.A. & Bell J.G. (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* **155**, 127–177.

Seraspe E.B., Valenzuela J.L.B., Maeda H. & Kamei Y. (2005) Bioactivity screening of extracts of *Nannochloropsis* sp. against *Vibrio harveyi* causative agent of luminous vibriosis in penaeid shrimps. *UPV Journal of Natural Science* **10**, 27–36.

Sorgeloos P., Coutteau P., Dhert P., Merchie G. & Lavens P. (1998) Use of brine shrimp, *Artemia* spp. in larval crustacean nutrition: a review. *Reviews in Fisheries Science* **6**, 55–68.

Tocher D.R. & Ghioni C. (1999) Fatty acid metabolism in marine fish: low activity of fatty acyl D5-desaturation in gilthead seabream (*Sparus aurata*) cells. *Lipids* **34**, 433–440.

Vazhappilly R. & Chen F. (1998) Heterotrophic production potential of omega-3 polyunsaturated fatty acids by microalgae and algae-like microorganisms. *Botanica Marina* **41**, 553–558.

Vijayakumaran M. & Radhakrishnan E.V. (1986) Effect of food density on feeding and moulting of phyllosoma larvae of the spiny lobster *Panulirus homarus* (Linnaeus). *Proceedings of the Symposium of Costal Aquaculture* **4**, 1281–1285.

Volkman J.K., Jeffrey S.W., Nichols P.D., Rogers G.I. & Garland C.D. (1989) Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *Journal of Experimental Marine Biology and Ecology* **128**, 219–240.

Wantanabe T. (1993) Importance of docosahexaenoic acid in marine larval fish. *Journal of the World Aquaculture Society* **24**, 152–161.

