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Aquaculture
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Aquaculture 239 (2004) 351–373
www.elsevier.com/locate/aqua-online

Nutritional and bacterial profiles of juvenile *Artemia* fed different enrichments and during starvation

Arthur J. Ritar[a,*], Graeme A. Dunstan[b], Matthew M. Nelson[c], Malcolm R. Brown[b], Peter D. Nichols[b], Craig W. Thomas[a], Edward G. Smith[a], Bradley J. Crear[a], Sagiv Kolkovski[d]

[a] *Marine Research Laboratories, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Nubeena Crescent, Taroona, Tasmania 7053, Australia*
[b] *CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7001, Australia*
[c] *School of Zoology, University of Tasmania, GPO Box 252-05, Hobart, Tasmania 7001, Australia*
[d] *Research Division, Department of Fisheries, P.O. Box 20 North Beach, Western Australia 6920, Australia*

Received 8 April 2004; received in revised form 16 June 2004; accepted 16 June 2004

Abstract

Biochemical and bacterial profiles of juvenile *Artemia* (1.5 mm, 5 days old) were examined in two experiments designed to manipulate their nutritional composition. In Experiment 1, *Artemia* were enriched for up to 36 h with the diatom *Chaetoceros muelleri*, the chromist *Schizochytrium* (Algamac 3050®) or a squid oil emulsion. In Experiment 2, *Artemia* were enriched with the same diets for 6 h, and then starved for 24 h at 4, 18 or 28 °C. *Artemia* did not survive beyond 24 h enrichment on Algamac and survival was low on oil emulsion, contrasting with the rapid growth and high survival on *C. muelleri*. Fatty acid (FA) content of *Artemia* increased marginally after enrichment with *C. muelleri*, with a marked increase over 36 h enrichment in the percent long-chain polyunsaturated FA (LC-PUFA). FA content increased by 60% and 140% after enrichment with Algamac and squid oil emulsion, respectively, and %LC-PUFA also increased. FA content and %LC-PUFA in starved *Artemia* declined by up to 57% at 28 °C whereas the proportional loss was < 30% at 4 °C. Ascorbic acid (AsA) content in *Artemia* increased four-fold over 36 h enrichment with *C. muelleri* and declined or did not change with the other enrichments. AsA was retained during starvation of 6 h-enriched *Artemia* with all treatments. α-Tocopherol (α-T) concentration increased three-fold to a peak after 6 h on oil emulsion and increased two-fold after 24 h on *C. muelleri* while decreasing by 50% during 12 h on Algamac. α-T declined during starvation by up to 50% (depending on temperature) following enrichment with oil emulsion although there was no change with *C. muelleri* or Algamac. For *Artemia*

* Corresponding author. Tel.: +61-362277294; fax: +61-362278035.
E-mail address: Arthur.Ritar@utas.edu.au (A.J. Ritar).

doi:10.1016/j.aquaculture.2004.06.016

enriched 12 h on *C. muelleri* or 6 h on oil emulsion, bacterial abundance was not elevated but further enrichment resulted in increases by two- to three-fold. Bacterial abundance in *Artemia* enriched for 6 h on Algamac increased four-fold and by 24 h, most *Artemia* had perished. Total numbers of heterotrophic bacteria and *Vibrio* spp. were similar after enrichment for 6 h with *C. muelleri* or oil emulsion and after 24 h starvation at 18 or 28 °C. Starvation at 18 or 28 °C after Algamac enrichment increased bacterial numbers up to four-fold. For *Artemia* starved at 4 °C for 24 h, there was a marked reduction in bacterial numbers for all enrichments, indicating that cool temperatures may be bactericidal. Enrichment of juvenile *Artemia* for 6 h achieved substantial biochemical improvement while minimising bacterial contamination, and is regarded as a suitable protocol before feeding to target species such as spiny lobster phyllosoma larvae.

Keywords: Juvenile *Artemia*; Fatty acids; Nutrition; Starvation; Bacteria; *Vibrio*

1. Introduction

Artemia are used widely for the feeding of larvae of many crustaceans and finfish. The larvae of some aquaculture species, such as spiny lobster phyllosoma of *Jasus edwardsii*, prefer larger *Artemia* juveniles (≥ 1.5 mm long, 5 day old) as prey to meet their food intake requirements (Ritar et al., 2003a). Although *Artemia* are not the natural prey of these animals, they are simple to prepare, are attractive in texture and behaviour and have been used effectively as the major or sole component to culture phyllosoma (Kittaka, 1994; Tong et al., 1997; Moss et al., 2000; Ritar et al., 2002, 2003a), presently making them a diet of choice.

The biochemical composition of *Artemia* is regarded as important in optimizing larval nutrition for survival and growth of aquaculture species (McEvoy and Sargent, 1998; Narciso et al., 1999). Most enrichment procedures and products have been developed to improve the nutritional quality of *Artemia* metanauplii (Rees et al., 1994; McEvoy and Sargent, 1998; Sorgeloos et al., 2003) but these may not be suitable for juvenile *Artemia*. The behaviour of juvenile *Artemia* appears to be significantly different to metanauplii in that they ingest and metabolise enrichment diets more rapidly (Dhont and Lavens, 1996). However, the systematic investigation of the most appropriate protocol for enrichment of juveniles and nutrient losses during starvation has yet to be undertaken.

Research on enriching *Artemia* metanauplii has concentrated on increasing the lipid content and especially the amounts of the long-chain polyunsaturated fatty acids (LC-PUFA), docosahexanoic, eicosapentanoic and arachidonic acids (DHA, EPA and ARA, respectively) (Barclay and Zeller, 1996; Sargent et al., 1999a,b; Sorgeloos et al., 2001), with a recent focus on the ratio of DHA/EPA (Evjemo et al., 1997; McEvoy et al., 1996; Narciso et al., 1999). These LC-PUFA, which are usually low in abundance in *Artemia*, are regarded as essential for decapod crustaceans (Castell, 1982; Kanazawa, 1982; Cahu and Fauvel, 1986; Sargent, 1995) and must be supplied in the diet (Kanazawa et al., 1979; Deering et al., 1997). Understanding the accumulation and loss of essential FA (EFA) in *Artemia* metanauplii during enrichment and starvation has helped the development of feeding strategies for finfish larvae (Estévez et al., 1998; Evjemo et al., 2001) and this is also needed for juvenile *Artemia*.

The lipid composition of unenriched juvenile *Artemia* is considerably different to phyllosoma at hatch (Phleger et al., 2001; Smith et al., 2002) and during the development of later stages of wild larvae (Phleger et al., 2001). This suggests that *Artemia* should be enriched to improve their lipid profile for feeding to phyllosoma. Indeed, it is possible to alter the composition of developing larvae in culture by feeding them enriched *Artemia* (Nelson et al., 2002; Smith et al., 2002). Other nutritional components, such as vitamins C (ascorbic acid) and E (tocopherols), may also need to be boosted to further improve phyllosoma performance (Smith et al., 2004a). However, spiny lobster larval cultures are often plagued by sudden mortalities, which have been attributed to colonisations by bacterial pathogens. Therefore, enrichment procedures that also minimise the transfer of bacteria via *Artemia* to larval cultures will be as vital as improvements in their biochemical composition.

Various enrichments that have been used to improve the composition of *Artemia* fall into three groups: live microalgae, spray-dried algae and marine oil emulsions. This study used one representative from each of these groups: a live microalgal diatom *Chaetoceros muelleri*, a commercial spray-dried alga Algamac 3050 and a custom-made squid oil emulsion, with both of the later enrichments high in DHA. Nutritional and bacterial profiles of juvenile *Artemia* were examined during enrichment for up to 36 h and following starvation for 24 h at 4, 18 or 28 °C, mimicking the changes during chilled storage or when fed to phyllosoma from temperate or tropical environments, respectively.

2. Materials and methods

2.1. Artemia production

Decapsulated *Artemia* cysts (E.G. grade, Artemia Systems, INVE, Belgium) were hatched in 50 l conical tanks at 28 °C in 1 μm-filtered seawater (SW) with vigorous aeration and a 150-W incandescent light suspended 0.5 m above the water surface. Hatched nauplii were rinsed for 5 min and stocked in 670 l tanks at 5 *Artemia* ml^{-1}, followed by the addition of *C. muelleri* on the first day. *Artemia* were then fed twice-daily on a commercial brine shrimp diet (Eyre Peninsula Aquafeeds, South Australia) consisting of rice pollard, soya flour and wheat flour. The diet was prepared by homogenising ingredients in 500 ml seawater in a blender for 15 min (Sunbeam, Australia) and screening to a particle size of 40 μm. *Artemia* were cultured for 5 days to reach approximately 1.5 mm.

2.2. Experimental design

2.2.1. Experiment 1: time-course enrichment

Three enrichments were used to examine the time-course uptake of FA, ascorbic acid (AsA; vitamin C) and tocopherols (T; vitamin E) in juvenile *Artemia*:

1. *C. muelleri*—pre-heated to 28 °C and completely exchanged every 6 h after rinsing *Artemia*. The concentration was $2.7 \pm 0.1 \times 10^6$ cells ml^{-1}. Algae were cultured according to the method of Coutteau (1996) and harvested on day 5 during logarithmic phase.

2. AlgaMac 3050 (spray-dried cells of *Schizochytrium* sp. algae, Aquafauna Bio-Marine, USA)—blended for 3 min in 500 ml SW pre-heated to 28 °C and completely exchanged every 12 h at a rate of 0.3 g l^{-1} after rinsing *Artemia*.
3. Emulsion containing 54% squid oil (prepared at Fisheries Western Australia)—blended for 3 min in 500 ml SW pre-heated to 28 °C and completely exchanged every 12 h at a rate of 0.3 g l^{-1} after rinsing *Artemia*. The oil emulsion included AsA as mixed sodium and calcium salts of ascorbyl phosphate (Rovimix, Stay-C 35, Roche) at 40 mg g^{-1} with <0.2 mg g^{-1} of free AsA, and mixed tocopherols (Cognis Australia) at 40 mg g^{-1}.

2.2.2. Experiment 2: enrichment and starvation

Six enrichments were used to examine the depletion during starvation of FA, AsA and tocopherols in juvenile *Artemia*:

1. *C. muelleri* logarithmic ($2.1 \pm 0.1 \times 10^6$ cells ml^{-1})—produced and exchanged as for Treatment 1, Experiment 1 and harvested on day 5 during the logarithmic phase.
2. *C. muelleri* stationary ($2.6 \pm 0.1 \times 10^6$ cells ml^{-1})—produced and exchanged as for Treatment 1, Experiment 1 and harvested on day 8 during the stationary phase.
3. Algamac 0.15 g l^{-1}—prepared and exchanged as for Treatment 2, Experiment 1 at 0.15 g l^{-1}.
4. Algamac 0.3 g l^{-1}—prepared and exchanged as for Treatment 2, Experiment 1 at 0.3 g l^{-1}.
5. Oil emulsion 0.3 g l^{-1}—prepared and exchanged as for Treatment 3, Experiment 1 at 0.3 g l^{-1}.
6. Oil emulsion 0.6 g l^{-1}—prepared and exchanged as for Treatment 3, Experiment 1 at 0.6 g l^{-1}.

For both experiments, *Artemia* were enriched in triplicate 25 l buckets. Buckets received 200,000 × 1.5 mm *Artemia* and the volume was made up to 20 l with pre-heated (28 °C) 1 μm-filtered SW after addition of enrichment and maintained at 28 ± 1 °C. In all treatments, *Artemia* were aerated vigorously (large bubbles), with *Artemia* in Algamac or oil emulsion receiving additional oxygenation through fine air stones to maintain >90% oxygen saturation. *Artemia* were harvested by pouring through a 125 μm screen and rinsing with 1 μm-filtered SW for approximately 5 min before the initial enrichment and then at each further exchange of enrichment. In the first experiment, *Artemia* were continuously enriched up to 36 h with *C. muelleri* or oil emulsion, and sampled at 0, 3, 6, 12, 24 and 36 h for biochemical analyses. *Artemia* were enriched with Algamac only up to 24 h, although high mortality precluded sampling beyond 12 h. For Experiment 2, *Artemia* were enriched for only 6 h. Triplicate samples of enrichments were collected during the experiments. Each *Artemia* sample comprised 3 l of the contents from each bucket (containing approximately 30,000 *Artemia* or 180 mg dry matter, DM) screened through a 63 μm mesh, rinsed and resuspended in 1 l of 1 μm-filtered SW. Samples were screened, rinsed with 0.5 M ammonium formate and stored in liquid nitrogen before freeze-drying prior to analysis.

2.3. Density and size of Artemia

In Experiment 1, *Artemia* densities, lengths and dry matter were evaluated at each sampling from each bucket. To calculate densities, three 10 ml samples were taken from each replicate, poured through a 250 μm screen and *Artemia* were counted under a dissecting microscope. Mortalities were evident at the 12 h sampling, so at 24 and 36 h, six samples containing 15–30 *Artemia* were taken from each replicate to calculate survival. These were viewed by eye and actively swimming *Artemia* were assessed to be survivors; all others were counted as mortalities. The lengths of *Artemia* ($n = 10$) were measured using a dissecting microscope, digital camera and Scion Image Beta 4.0.2 software (Scion, Frederick, MD, USA). In Experiment 2, only initial measurements were taken before enrichment and there were negligible mortalities.

2.4. Bacteriology

In Experiment 1, a total of 40 *Artemia* (20 per replicate) were sampled from each of the three treatments to enumerate heterotrophic bacteria per *Artemia* at 0, 6, 12 and 36 h during enrichment. In Experiment 2, *Artemia* were sampled similarly at 0 h and after 6 h at the highest density of each enrichment, i.e. *C. muelleri* at stationary phase, Algamac at 0.3 g l^{-1} and oil emulsion at 0.6 g l^{-1}, and after starvation for 6 and 24 h. These times coincided with samples collected for lipid analysis and were taken immediately prior to water exchanges and re-feeding. *Artemia* were homogenised, serially diluted (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) and plated onto ZoBells Marine agar medium or thiosulphate citrate bile salts (TCBS) agar medium (Amyl Media AM243 or AM187, respectively, Australia) for the measurement of total marine heterotrophic bacteria and number of *Vibrio* spp., respectively. After the culture media had been incubated for 24 h at 25 °C, plates containing between 50 and 300 colony forming units were selected and enumerated.

2.5. Biochemical analyses

The fatty acid content of diets, *Artemia* during enrichment and starved *Artemia* were analysed in duplicate. Fatty acid methyl esters (FAME) were formed directly by treating samples in a solution of methanol/chloroform/hydrochloric acid (10:1:1) and analysed with an HP5890A gas chromatograph (Agilent Technologies, USA) and Finnigan Thermoquest GCQ GC-mass spectrometer (Austin, TX, USA) as detailed elsewhere (Lewis et al., 2000).

AsA was extracted from dried *Artemia* (10 mg) using metaphosphoric acid (3%) and acetic acid (8%), derivatised and analysed by HPLC (Brown et al., 1998).

For the analyses of tocopherols (α-, δ- and γ-T), dried *Artemia* (10 mg) were transferred to 10 ml plastic centrifuge tubes, together with 2 ml methanol containing 2 mg butylated hydroxy-toluene. Samples were sonicated (Labsonic 1510 sonic probe; 30 s at 100 W), vortexed (10 s) and left at room temperature for 1 h. Samples were centrifuged (1000 × g, 10 min), the supernatant filtered through a 0.45 ìm syringe filter (Alltech) and analysed by HPLC (Huo et al., 1999).

2.6. Statistical analyses

Statistical analyses were performed using JMP version 5.0 (SAS Institute) for one-way ANOVA and Tukey–Kramer HSD tests for post-hoc multiple comparisons (Sokal and Rohlf, 1995). After arcsin $\sqrt{}$ transformations were performed, survival data were normal and homogeneous. The level of significance for all analyses was $P<0.05$. Data are presented as mean ± standard error unless stated otherwise.

3. Results

3.1. Time-course enrichment (Experiment 1)

3.1.1. Artemia size, survival and dry matter

Initial *Artemia* length was 1.46 ± 0.03 mm and increased significantly during enrichment with *C. muelleri* to 1.77 ± 0.04 mm at 36 h and with Algamac to 1.60 ± 0.04 mm at 24 h, while there was no change in *Artemia* fed oil emulsion over the 36 h (Fig. 1a). The population density of *Artemia* before enrichment was 14.2 ± 1.0 *Artemia* ml^{-1} (Fig. 1b). Density declined to 10.3–12.6 *Artemia* ml^{-1} ($P<0.05$) for all treatments during enrichment until 12 h and then remained constant in *C. muelleri*. Density continued to decline to 3.1 *Artemia* ml^{-1} in Algamac at 24 h and to 4.9 *Artemia* ml^{-1} in oil emulsion at 36 h. The proportion of live *Artemia*, which is an instantaneous assessment of survival, was high at 24 and 36 h when enriched in *C. muelleri* (97 ± 1% and 92 ± 1%, respectively), and was lower ($P<0.05$) when enriched in oil emulsion (65 ± 2% and 44 ± 1%, respectively). The proportion of live animals was only 14 ± 2% for *Artemia* in Algamac at 24 h after which this treatment was discontinued. The changes in *Artemia* length, density and survival were reflected in biomass per litre (DM l^{-1}) (Fig. 1c). For *Artemia* before enrichment, biomass was 0.075 g l^{-1} and there was little change during the first 12 h for any enrichment. However, after 36 h biomass was reduced by 63% for the oil emulsion and increased by 137% in *C. muelleri*.

3.1.2. Microbiology

The total number of heterotrophic bacteria per *Artemia* immediately before enrichment was 15.5 ± 4.7 × 10^3 (Fig. 2). Bacterial counts remained at this level in *Artemia* enriched with *C. muelleri* at 6 and 12 h but had increased ($P<0.05$) at 36 h by 190%. For the oil emulsion, counts also remained low after 6 h enrichment, then increased progressively at 12 and 36 h (190% and 415%, respectively). By contrast, counts in Algamac enrichment increased markedly by as early as 6 h and remained elevated at 12 h (442% and 345%, respectively).

3.1.3. Fatty acid composition

The FA content of logarithmic phase *C. muelleri* was 150 mg g^{-1} and % abundance of individual FA, in decreasing order, was: 16:3n-4 26%, EPA 21%, 16:1n-7 20%, 14:0 8%, palmitic acid (16:0) 7% and ARA 2% (Table 1). FA content in Algamac was 587 mg g^{-1} and proportional abundance of individual FA, in decreasing order, was: DHA 36%, 16:0

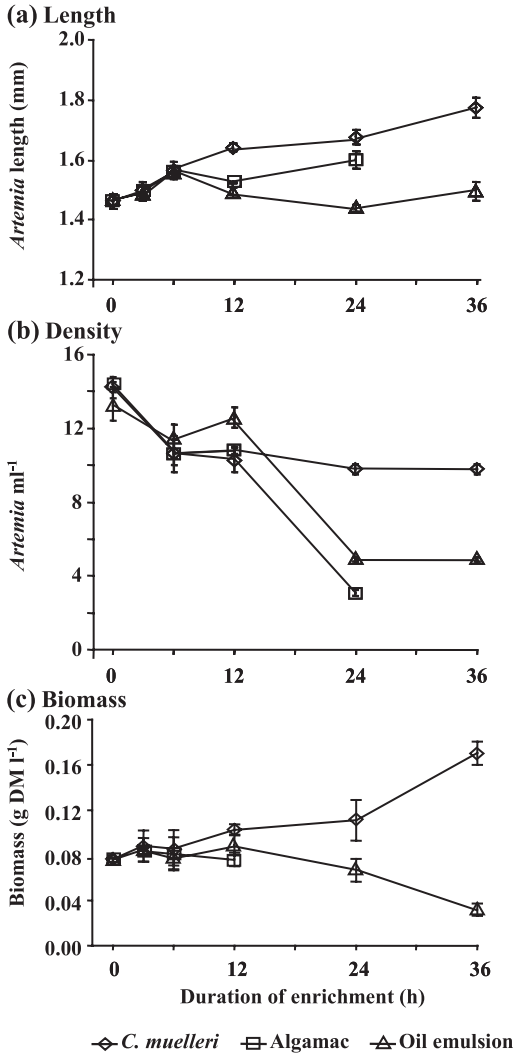

Fig. 1. The effect of enrichment of juvenile *Artemia* with *C. muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}) on (a) length (mm), (b) density (*Artemia* ml^{-1}) and (c) biomass (g DM l^{-1}). Data are mean ± S.E.M. (Experiment 1).

28%, DPA(6) (22:5n-6) 15%, 14:0 11%, EPA 2% and ARA 2%. FA content in the oil emulsion was 655 mg g^{-1} and the proportional abundance of individual FA, in decreasing order, was: 16% each for DHA, 16:0 and oleic acid (18:1n-9c), and EPA 11%.

Pre-enriched *Artemia* contained 123 mg g^{-1} FA and were low in ARA, EPA and DHA (Table 1, Fig. 3) and high in 18:1n-9 (39%) and 18:2n-6 (31%). FA content increased with all enrichments with the degree of increase and changes in profiles reflecting the composition of the diets. Maximum FA content of *Artemia* in *C. muelleri* was achieved

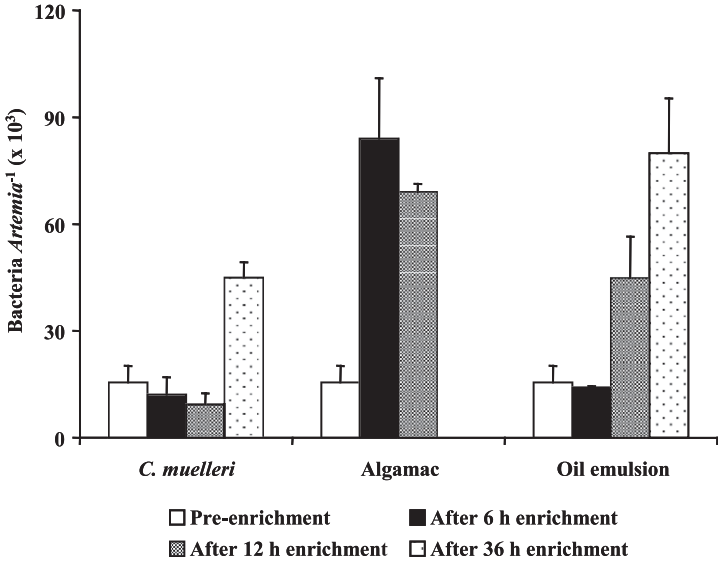

Fig. 2. Total number of heterotrophic bacteria (mean ± S.E.M.) on juvenile *Artemia* during enrichment with *C. muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}; Experiment 1).

after only 6 h enrichment (144 mg g^{-1}) and the composition continued to change over the following 30 h in which EPA doubled (from 8% to 17%) and C_{18} FA reduced by 50%. FA content of *Artemia* fed Algamac rapidly peaked by 6 h (198 mg g^{-1}) with increases in ARA (2%), EPA (4%), DPA(6) (7%) and DHA (17%) and C_{18} FA reduced by 50%.

The highest FA content was achieved in *Artemia* enriched with oil emulsion for 36 h (293 mg g^{-1}). EFA were incorporated into *Artemia* at high rates somewhat reflecting the enrichment, although the relative increase in DHA was considerably less than for ARA and EPA. The percentage sum PUFA was elevated after enrichment on all diets and increased over the enrichment period, reaching its highest after 12 h in Algamac (to 52%), which was at the expense of MUFA.

3.1.4. Ascorbic acid (AsA) and tocopherol (T) concentrations

Concentrations of AsA in *C. muelleri* and Algamac were 0.60 and 0.04 mg g^{-1}, respectively. The assay did not detect free AsA in the oil emulsion as the ascorbate was present as a stable phosphate ester. *Artemia* initially contained 0.14 mg AsA g^{-1} and there was no significant increase after enrichment with Algamac or oil emulsion (Fig. 4a). In contrast, the concentration of AsA in *Artemia* fed *C. muelleri* increased more than four-fold over the enrichment period to 0.63 mg g^{-1}.

C. muelleri and Algamac contained 0.30 and 0.001 mg α-T g^{-1}, respectively. Low concentrations of γ-tocopherol (γ-T; 0.02 mg g^{-1}) were also detected in *C. muelleri*. The oil emulsion contained high concentrations of mixed tocopherols; 9.9 mg α-T g^{-1}, 19.6 mg γ-T g^{-1}, and 5.2 mg δ-T g^{-1}. Concentrations of tocopherols in enriched *Artemia* reflected that of their diets. After 6 h enrichment with the oil emulsion, there was a three-fold increase in α-T from an initial concentration of 0.10 mg g^{-1}, although further

Table 1
Percentage composition of major (>3%) fatty acids in pre-enriched *Artemia* and in *Artemia* enriched with *C. muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}) for up to 36 h (Experiment 1)

	Newly hatched phyllosoma[a]	Enrichments			Pre-enriched *Artemia*	*Artemia* enriched with									
		C. muelleri	Algamac	Oil emulsion		*C. muelleri* for				Algamac for		Oil emulsion for			
					0 h	6 h	12 h	24 h	36 h	6 h	12 h	6 h	12 h	24 h	36 h
14:0	1.5	8.4	10.5	4.2	0.5	2.3	2.8	3.6	4.1	2.8	2.1	1.0	1.0	1.0	1.3
16:2n-4	–	3.3	0.1	–	–	0.9	1.1	1.5	0.4	0.1	0.1	–	–	–	–
16:3n-4	–	26.0	0.1	0.1	0.5	5.9	8.0	10.3	11.3	0.3	0.4	0.4	0.3	0.3	0.4
16:1n-7	3.9	20.4	0.3	4.7	1.8	7.0	9.3	12.0	14.9	1.1	1.2	2.7	2.8	3.1	3.7
16:0	8.3	6.6	28.3	15.9	9.1	8.7	8.6	8.3	8.5	14.8	13.2	10.3	10.1	9.3	10.3
18:2n-6	1.5	0.8	0.0	4.1	31.2	22.9	19.2	14.8	11.7	17.4	17.0	21.1	18.3	16.0	13.8
18:1n-9c/ 18:3n-3	15.7	3.1	0.1	15.7	38.7	28.3	22.8	17.4	13.3	22.4	21.9	31.1	29.5	28.8	27.6
18:1n-7	4.6	0.7	0.1	3.7	4.4	3.7	3.7	4.0	4.4	2.7	2.8	4.5	4.7	4.7	5.0
18:0	4.2	0.7	0.6	3.0	4.9	4.2	4.4	4.5	5.0	3.3	3.4	4.1	3.8	3.2	3.2
20:4n-6 ARA	7.1	2.0	2.1	1.4	0.6	1.2	1.4	1.7	1.9	2.2	2.8	1.1	1.2	1.5	1.5
20:5n-3 EPA	17.8	20.9	2.3	11.3	1.7	8.4	11.6	15.1	17.0	4.0	5.6	7.0	8.8	10.9	11.3
22:5n-6 DPA(6)	–	–	15.2	0.4	–	0.2	0.1	0.1	0.1	6.5	6.7	0.2	0.3	0.3	0.5
22:6n-3 DHA	14.8	1.0	36.1	16.2	0.1	0.8	0.5	0.4	0.6	16.6	16.5	6.1	7.9	8.9	8.9
Other[b]	14.8	6.0	4.3	19.4	6.4	5.7	6.3	6.3	6.8	5.9	6.3	10.4	11.1	12.0	12.6
Sum branched	–	–	0.2	1.1	1.7	0.6	1.2	1.0	0.9	0.8	0.9	1.3	1.1	1.0	1.2
Sum SFA	16.1	17.0	40.1	24.4	16.0	16.7	17.1	17.8	19.1	21.9	19.8	16.8	16.2	14.6	15.8
Sum MUFA	31.9	26.4	0.9	33.4	47.3	41.2	38.0	35.5	34.9	27.7	27.5	42.7	41.7	41.8	42.2
Sum PUFA	46.5	56.6	58.8	41.1	35.0	41.4	43.6	45.7	45.1	49.6	51.8	39.2	41.0	42.5	40.8
Total FA mg g^{-1} DM	70	150	587	655	123	144	136	140	141	198	195	178	191	222	293

Data are mean ± S.D. values; $n=2$; (–), below detection.

ARA, arachidonic acid; EPA, eicosapentanoic acid; DPA(6), docosapentanoic acid; DHA, docosahexanoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[a] Newly hatched phyllosoma profile (target predator species) is included for comparison (Smith et al., 2003b).

[b] Other minor fatty acids included: 12:0, i14:0, C_{14} PUFA, 14:1, br15:0, i15:0, a15:0, 15:0, C_{16} PUFA, i16:0, 16:1n-7t/16:2n-7, 16:1n-9c, 16:1n-5c, 16:1n-13t, 16:0 fatty aldehyde (Falde), 16:1 Falde, br17:0, i17:0, a17:0, 17:1, 17:2, 17:0, C_{18} PUFA, 18:3n-6, i18:0, 18:4n-3, 18:1n-7t, 18:1n-5c, 18:0 Falde, 18:1 Falde, i19:0, 19:1, 20:3n-6, 20:4n-3, 20:2n-6, 20:1n-7c, 20:2, 20:1n-5, 20:1n-11c, 20:1n-9c, 20:0, C_{21} PUFA, 21:0, 22:4n-6, 22:5n-3, C_{22} PUFA, 22:1n-9, 22:1n-7, 22:1n-11, 22:0, C_{24} PUFA, 24:1, 24:0.

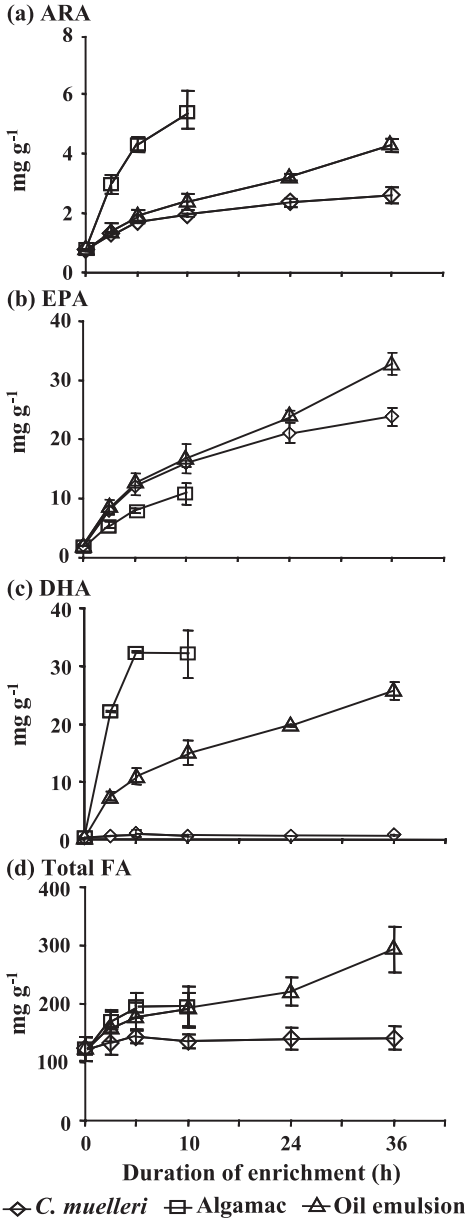

Fig. 3. Content (mean ± S.D., mg g^{-1} DM) in juvenile *Artemia* of (a) arachidonic acid (ARA), (b) eicosapentanoic acid (EPA), (c) docosahexanoic acid (DHA) and (d) total fatty acids during enrichment with *C. muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}; Experiment 1).

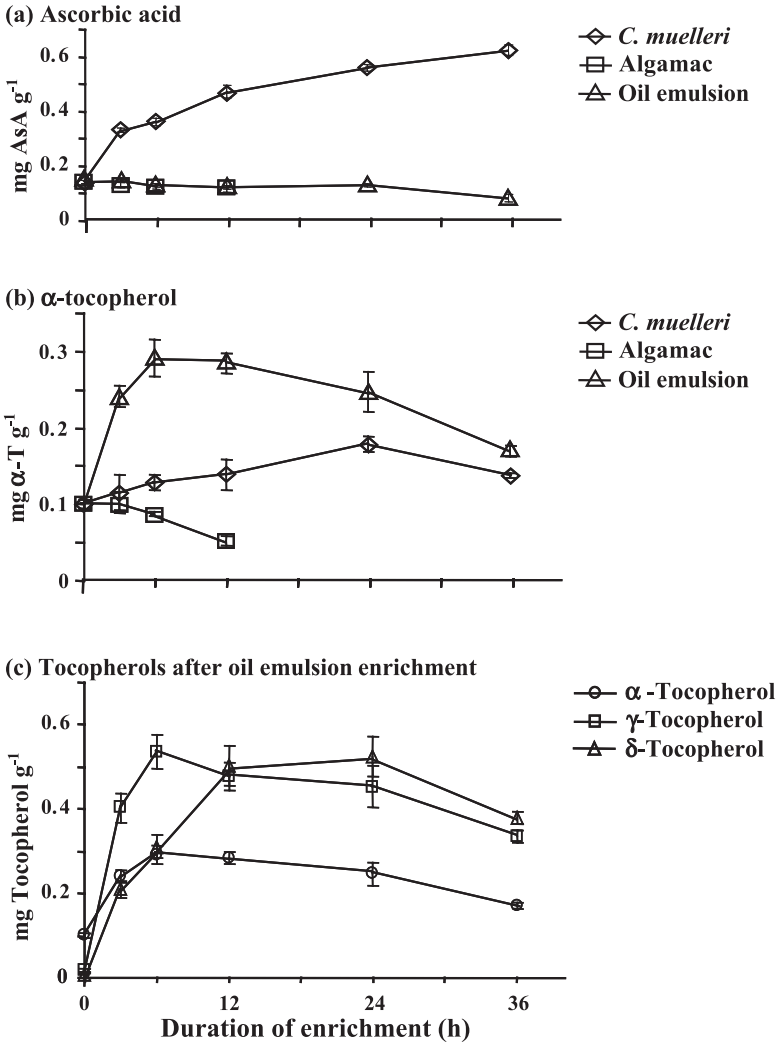

Fig. 4. Content (mean ± S.D., mg g^{-1} DM) in juvenile *Artemia* of (a) ascorbic acid, or (b) α-tocopherol (α-T) during enrichment with *C. muelleri* (logarithmic phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.3 g l^{-1}), and of (c) α-, γ- and δ-T during enrichment with oil emulsion (0.3 g l^{-1}; Experiment 1).

enrichment caused a reduction in α-T (Fig. 4b). Enrichment with *C. muelleri* produced an 80% increase in α-T after 24 h, whereas enrichment with Algamac produced a 50% reduction in α-T by 12 h.

Apart from their content of α-T, *Artemia* initially contained trace amounts of γ-T (0.02 mg g^{-1}) and δ-T (0.01 mg g^{-1}). Concentrations of these tocopherols did not change in *Artemia* enriched with *C. muelleri* and Algamac. However, both their concentrations increased significantly in *Artemia* fed oil emulsion to over 0.50 mg g^{-1} at 6 h for γ-T and 12 h for δ-T (Fig. 4c).

3.2. Enrichment and starvation (Experiment 2)

3.2.1. Artemia size and microbiology

Initial *Artemia* length was 1.52 ± 0.04 mm. The total number of heterotrophic bacteria per *Artemia* immediately before enrichment was $10.5 \pm 0.8 \times 10^3$ (Fig. 5a). For *Artemia* enriched for 6 h at 28 °C, bacterial counts declined by 81% in *C. muelleri*, increased by 29% after enrichment with Algamac or did not change after enrichment with oil emulsion. After 24 h starvation at 4 °C, counts mostly declined by 90–99% for *Artemia* in all enrichments and were lowest in *C. muelleri*-enriched *Artemia*. After 24 h starvation at 18 °C, counts were 31% and 65% less in *Artemia* enriched with *C. muelleri* and oil emulsion, respectively, but increased by 182% with Algamac. After 24 h starvation at 28 °C, bacterial numbers were elevated for *Artemia* in Algamac (170%) and oil emulsion (20%) but were still 70% lower for *Artemia* enriched with *C. muelleri* than pre-enriched *Artemia*. *Vibrio*. spp. counts were generally 30–70% lower and followed a similar pattern to total counts, except for Algamac-enriched *Artemia* held at 18 °C in which counts remained low (Fig. 5b).

3.2.2. Fatty acid composition

FA contents of logarithmic and stationary phases of *C. muelleri* were similar (92 and 108 mg g^{-1}, respectively) but compared to Experiment 1, were 39% lower. However, the proportional abundances of individual FA were similar to Experiment 1. FA contents of Algamac and oil emulsion were 529 and 789 mg g^{-1}, respectively, and the FA profiles were similar to Experiment 1.

FA content and profile of *Artemia* after 6 h enrichment reflected the three diets and were similar to Experiment 1. FA content increased by 20% after enrichment with *C. muelleri* (to 125–133 mg g^{-1}) regardless of whether logarithmic or stationary phase cultures were used (Fig. 6d), and the FA profiles were also similar. FA content increased by 60% and 100% for *Artemia* enriched with Algamac (to 169–173 mg g^{-1}) and oil emulsion (to 192–210 mg g^{-1}), respectively, and the FA profiles between enrichment rates were similar. There was no difference in the improvement in DHA, EPA or ARA content after enrichment of *Artemia* within diet type, i.e. logarithmic or stationary phase of *C. muelleri*, or either dose rate of Algamac or oil emulsion. ARA content was about 40% higher after enrichment with Algamac than with oil emulsion or *C. muelleri* (5.3, 3.2 and 3.1 mg g^{-1}, respectively; Fig. 6a). In contrast, EPA content of enriched *Artemia* was highest with oil emulsion, and similar with Algamac and *C. muelleri* (21, 9 and 8 mg g^{-1}, respectively; Fig. 6b). DHA was highest using Algamac (19% of total fatty acids), followed by oil emulsion (10%) and *C. muelleri* (0.2%), which equated to 34, 21 and 0.3 mg g^{-1} *Artemia*, respectively (Fig. 6c).

The FA profile of starved *Artemia* progressed towards that of pre-enriched *Artemia* (Fig. 6), i.e. lower amounts of LC-PUFA. With all diets, FA content of starved *Artemia* was lower than immediately after enrichment and the reduction was greater during starvation at warmer temperatures. After starvation for 6 h, the % loss of FA was lowest with *C. muelleri*, and for the three enrichments was less at 4 °C (0–15%) than at 18 °C (2–21%) or 28 °C (8–21%). The loss was greater after 24 h starvation, i.e. 20–29% at 4 °C, 19–32% at 18 °C

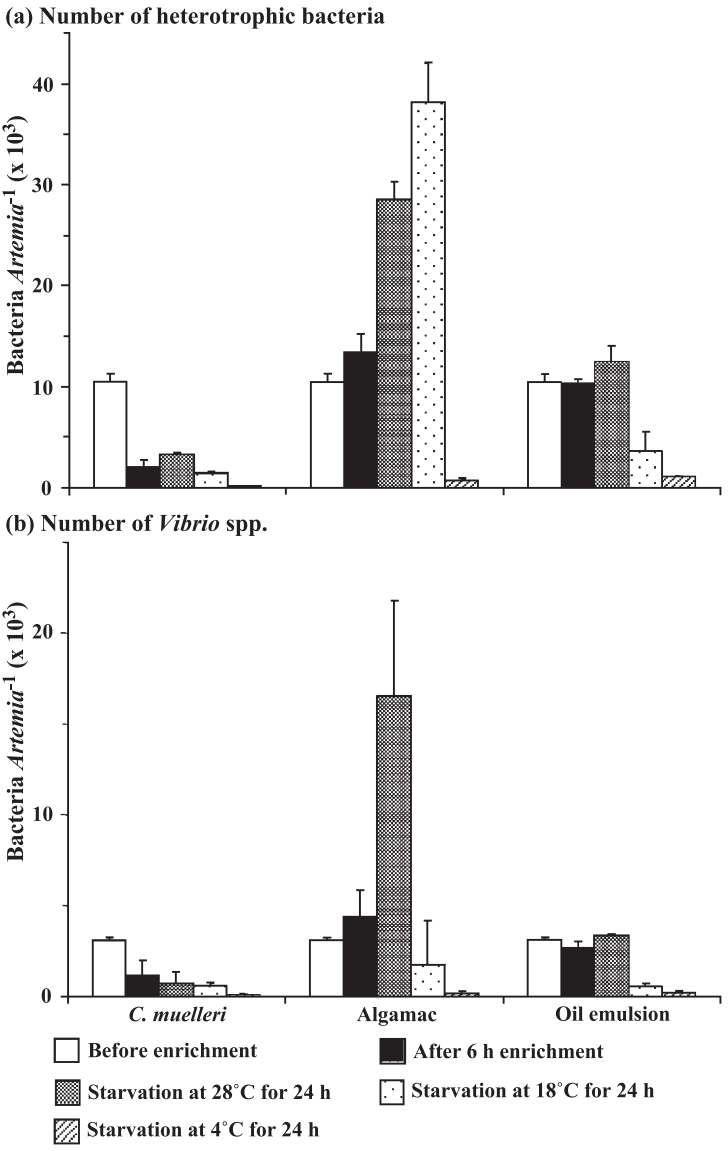

Fig. 5. Total numbers of (a) heterotrophic bacteria and (b) *Vibrio* spp. (mean ± S.E.M.) in juvenile *Artemia* during enrichment with *C. muelleri* (stationary phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.6 g l^{-1}) and after 24 h starvation at 4, 18 or 28 °C (Experiment 2).

and 41–57% at 28 °C, and was similar for all enrichments. The loss of DHA from *Artemia* during starvation at 28 °C was >75% for Algamac and oil emulsion treatments, with reduced losses at cooler temperatures, while there were comparatively minor reductions in EPA and ARA.

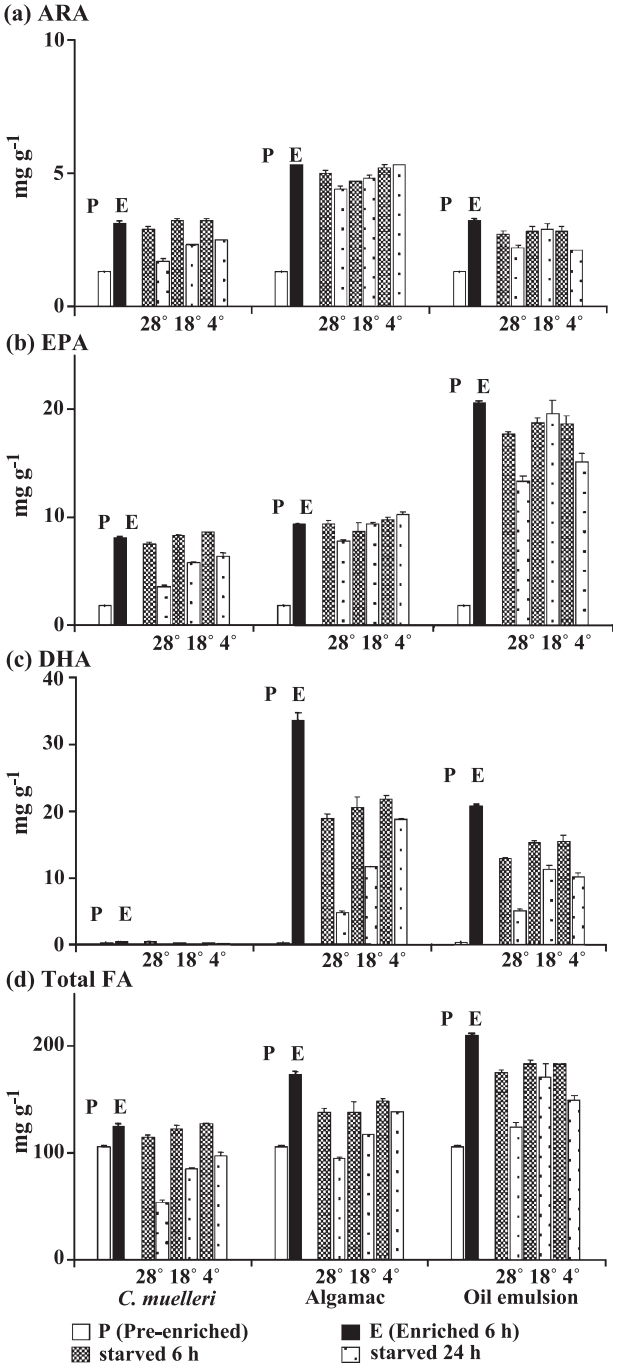

Fig. 6. Content (mean ± S.D., mg g^{-1} DM) in juvenile *Artemia* of (a) arachidonic acid (ARA), (b) eicosapentanoic acid (EPA), (c) docosahexanoic acid (DHA) and (d) total fatty acids during enrichment with *C. muelleri* (stationary phase), Algamac (0.3 g l^{-1}) or oil emulsion (0.6 g l^{-1}; Experiment 2).

3.2.3. Ascorbic acid (AsA) and tocopherol (T) concentrations

AsA concentrations in pre-enriched *Artemia* (0.13 mg g^{-1}) and in *Artemia* after 6 h of the respective enrichments were similar to Experiment 1. AsA did not increase in *Artemia* enriched with oil emulsion or Algamac, whereas *Artemia* enriched with *C. muelleri* (either logarithmic or stationary) increased to 0.50 mg g^{-1}. There were no significant changes in AsA concentrations during starvation at 4, 18 or 28 °C in *Artemia* fed any of the diets.

α-T concentrations in pre-enriched *Artemia* (0.04 mg g^{-1}) were approximately one-third of those in Experiment 1, while after 6 h enrichment, were similar to Experiment 1. *Artemia* concentrations doubled after enrichment with *C. muelleri* (0.07 mg g^{-1}, logarithmic or stationary). Oil emulsion (at either rate) produced 10-fold increases in α-T (0.31–0.39 mg g^{-1}) but there was no change for Algamac. There were also no changes following 6 h starvation of *Artemia* enriched with *C. muelleri* or Algamac. However, in *Artemia* enriched with oil emulsion and starved for 6 h, the α-T decline depended on temperature, i.e. from 0.39 mg g^{-1} initially to 0.29, 0.23 and 0.20 mg g^{-1} at 4, 18 and 28 °C, respectively, while after 24 h starvation, the concentrations decreased by 50% or more.

As in Experiment 1, δ-T and γ-T were only present in *Artemia* after enrichment with oil emulsion with concentrations reaching 1.8 mg γ-T g^{-1} and 0.5 mg δ-T g^{-1}. Depletion during starvation showed similar temperature-dependant trends to α-T, although the rates differed. Hence, concentrations of α-T, δ-T and γ-T reduced by 47%, 33% and 80%, respectively, after *Artemia* were starved for 24 h at 28 °C.

4. Discussion

4.1. Fatty acids

The nutritional improvement of *Artemia* with diets rich in FA, and in particular LC-PUFA, has been found to be beneficial when they are fed to marine larvae, especially to finfish, although this is not yet clear for crustaceans (Sorgeloos et al., 2003). The chosen representatives of the three main groups of enrichments had lipid content ranging from >10% for the live microalga to >50% for the spray-dried alga and oil emulsion, and all were rich in one or more of the LC-PUFA. The target predators for nutritional improvement of *Artemia* in our laboratory were newly hatched phyllosoma larvae of the spiny lobster *J. edwardsii*, which typically contain 7–12% ARA, 15–18% EPA and 8–15% DHA as proportions of total FA, and lipid content at hatch may range between 50 and 140 mg g^{-1} DM (Phleger et al., 2001; Smith et al., 2003a,b; Ritar et al., 2003b). Lipid content increases at later stages of development in wild phyllosoma and may reach >300 mg g^{-1} at the final Stage XI before decreasing markedly to <40 mg g^{-1} in the recently settled puerulus (Phleger et al., 2001). This is due to the tremendous energy expenditure of the puerulus during its long oceanic return journey to close inshore (Jeffs et al., 2001) and metabolic maintenance before it starts to feed (Lemmens, 1994). Therefore, a diet that readily allows the accumulation of lipid reserves during larval development in culture would probably assist in the peri-metamorphic transition to puerulus and post-puerulus.

Artemia fed the oil emulsion achieved, after 36 h enrichment, the highest FA and EPA contents, reflecting their diet. In contrast, *Artemia* fed Algamac increased their total FA

content more rapidly and had more ARA, DPA(6) and DHA, as did their diet. Doubling the feeding rate of Algamac (from 0.15 to 0.3 g l^{-1}) or oil emulsion (from 0.3 to 0.6 g l^{-1}) did not appreciably increase the content of ARA, EPA or DHA after 6 h enrichment. Thus, if a short enrichment of 6 h improves efficiency in the hatchery and the high *Artemia* mortalities can be remedied, Algamac may prove to be the most nutritious diet for phyllosoma. Enrichment with *C. muelleri* for 36 h resulted in negligible change in FA content compared to 6 h enrichment. The FA composition of *C. muelleri* is typical of other diatoms, with high levels of 16:0, 16:1n-7, C_{16} PUFA, ARA and EPA, and low levels of C_{18} and C_{22} PUFA including DHA. In this study, there was little difference in the compositions of logarithmic and stationary phase *C. muelleri*. The composition of *C. muelleri* was reflected in gradual changes in these FA in enriched *Artemia* with elevated EPA and low DHA. Decapod crustacean larvae have limited or negligible capacity to convert C_{18} PUFA to LC-PUFA (Suprayudi et al., 2004). With enrichment, C_{18} PUFA were reduced markedly, especially with the *C. muelleri* diet.

The LC-PUFA profile of enriched juvenile *Artemia* generally reflects that of the enrichment, and this offers opportunities for dietary manipulation to meet the needs of the phyllosoma larvae, as found previously by Smith et al. (2002). However, although ARA and EPA accumulation in *Artemia* mirrored the percentage in the enrichment, DHA increased at a lower rate. Estévez et al. (1998) also found previously that *Artemia* metanauplii accumulated less DHA during enrichment and lost it fastest during starvation compared to other LC-PUFA. This is likely to be due to an inability of juveniles to preferentially assimilate DHA during enrichment.

DHA did not undergo significant retroconversion to EPA in juveniles, as is the case with *Artemia* metanauplii (Navarro et al., 1999; Evjemo et al., 2001) because EPA was accumulated at a rate similar to inclusion levels in the enrichments and increased independently of DHA. EPA content of *Artemia* increased after enrichment with the degree dependent on the relative content of the diet. For example, %EPA was highest with the *C. muelleri* diet and increased progressively during 36 h enrichment.

The ARA level in *Artemia* was highest after enrichment with Algamac and was proportionally higher than in the diet, although retroconversion from DPA(6) may be contributing to this increase. Spiny lobster phyllosoma contain 7–8% ARA (Smith et al., 2003b; Phleger et al., 2001; Ritar et al., 2003b), so they probably have a high requirement for this n-6 PUFA during development. Higher ARA content in larvae has been proposed as beneficial for the stress resistance and pigmentation of crustaceans and finfish (Estévez et al., 1997; D'Souza and Loneragan, 1999; Koven et al., 2001). ARA also has a major role as a precursor of eicosanoid hormones required for moulting and stress response (Lytle et al., 1990; Sargent, 1995). It has been suggested that EPA modulates eicosanoid production from ARA, and failure to supply these two FA in the appropriate balance may result in adverse biochemical responses in the predator larvae (Sargent, 1995). The ARA content can be readily incorporated into *Artemia* via supplementation in oil emulsion (Smith et al., 2002).

Smith et al. (2002) found that for juvenile *Artemia* starved at 28 °C for 6 h there were no losses in lipid content although there were significant changes in the relative levels of FA. In contrast, Evjemo et al. (2001) found a greater than 30% loss in lipid content from enriched *Artemia* metanauplii held at 26 °C for 24 h while losses were only 11% at 12 °C.

In this study, the loss in FA during starvation for 24 h was higher, reaching 57% at 28 °C while at 4 °C it was only 29%. However, losses were much higher for DHA than for the other EFA, especially at 28 °C, reaching 86% (in the Algamac treatment). *Artemia* do not store DHA at similar proportions to other EFA (Evjemo et al., 2001). Nevertheless, the decline in DHA appears to occur at a slower rate in enriched juveniles than in metanauplii (Evjemo et al., 2001; Smith et al., 2002) and is lowest at 4 °C, making their refrigerated storage a feasible procedure for delayed feeding to predator larvae.

4.2. Vitamins

Algae are usually high in vitamins, especially in AsA and α-T, while commercial fish oil emulsions, such as the customised squid oil emulsion used here, are often supplemented with vitamins during manufacture. The greatest improvement in AsA concentrations of juvenile *Artemia* occurred after enrichment with *C. muelleri*, increasing four-fold to 0.6 mg g^{-1} DM, which is comparable to the five-fold increase seen previously using *Isochrysis* spp. (Tahitian strain) (Ritar et al., 2003a). These concentrations are similar to unenriched *Artemia* metanauplii, and are probably sufficient to meet normal dietary requirements of most fish and crustacean larvae (Merchie et al., 1997). However, the enrichment we observed with algae was considerably less than the 60-fold increase after direct enrichment with the particulate ascorbyl-2-phosphate (Smith et al., 2004b), which improved the stress resistance of newly hatched phyllosoma (Smith et al., 2004a).

There was no increase in AsA after enrichment with the oil emulsion despite the inclusion of 40 mg ascorbyl phosphate g^{-1} DM. Ascorbyl phosphate is converted to free AsA in other fish and crustacean larvae, presumably by phosphatase enzymes (Gouillou-Coustans et al., 1998; Ruff et al., 2001). In our study, failure to increase AsA in *Artemia* may have been due to an insufficient inclusion in the emulsion, poor absorption or low efficiency of conversion of ascorbyl phosphate to AsA by *Artemia*. Regarding the latter, we incubated *Artemia* with acid phosphatase (Merchie et al., 1997; H. Nelis, personal communication) before AsA assay, but this was inconclusive as we found a poor conversion to AsA by the enzyme.

The three-fold difference in α-T concentration of pre-enriched *Artemia* in Experiment 1 (0.10 mg g^{-1}) compared to Experiment 2 (0.04 mg g^{-1}) was not explored further, but may have reflected compositional variations in batches of the commercial on-growing diet. Enrichment with algae gave moderate (up to double) increases in α-T concentration, i.e. to 0.2 mg g^{-1} after 24 h in Experiment 1 and to 0.07 mg g^{-1} after 6 h in Experiment 2. In another study, where juvenile *Artemia* were cultured wholly on algae, α-T concentrations were 0.04–0.08 mg g^{-1} (Vismara et al., 2003). Optimal concentrations of α-T in live feeds have not been defined for larvae of many species in culture, although zooplankton containing 0.07–0.17 mg g^{-1} supported growth of fish larvae (Atlantic halibut, *Hippoglossus hippoglossus*, Rønnestad et al., 1999; fresh water walleye *Stizostedion vitreum*, Kolkovski et al., 2000). It is yet to be established whether higher doses of this antioxidant are beneficial, but this may be so when larvae are fed high PUFA diets as α-T is degraded to protect PUFA against oxidation (Stéphan et al., 1995). In this context, inclusion of high α-T concentrations (and the less biologically active δ-T and γ-T) in *Artemia* enriched with marine oil emulsions may be warranted because of their corresponding high PUFA.

Following the same reasoning, while Algamac-enriched *Artemia* are also rich in PUFA, their low concentrations of α-T may be suboptimal for larvae. More rigorous experimentation is needed to test these hypotheses.

There was no reduction in AsA concentration during starvation of *Artemia* juveniles for any of the enrichments regardless of temperature, which is in accord with findings for *Artemia* metanauplii enriched with ascorbyl palmitate and later starved (Merchie et al., 1995). In contrast, tocopherols concentrations in *Artemia* enriched with oil emulsion reduced significantly following 24 h starvation, although they remained higher than for pre-enriched *Artemia*.

4.3. Survival, growth and bacterial abundance

Although the primary purpose of enrichment is to improve the nutritional composition of juvenile *Artemia*, there may be other consequences. *Artemia* will use some diets, such as the microalgae *C. muelleri*, as food resulting in a considerable increase in body size and biomass over the 36 h enrichment. In contrast, *Artemia* enriched with Algamac or oil emulsion did not grow under the regime used here. Further, the 30% mortality in *Artemia* stabilised after 6 h enrichment with *C. muelleri*, whereas there were continuing losses in the other enrichments. This was more severe with Algamac, which resulted in almost complete mortality by 24 h despite, maintaining high oxygenation during enrichment, and appeared to be associated with extensive proliferation of bacteria. For all treatments, enrichments were exchanged every 6 h (*C. muelleri*) or 12 h (Algamac and oil emulsion) after rinsing *Artemia*. Thus, it appears that a considerable remnant bacterial burden was carried over into the following enrichment period where there was a further build up. This burden was attached directly to *Artemia* despite rinsing away excess enrichment for at least 5 min in an attempt to reduce the bacterial levels in the culture water (Lavens and Sorgeloos, 1996).

Optimizing the *Artemia* composition with nutrients that are needed for larval development would possibly improve phyllosoma performance in culture, although this is yet to be proven. A recent study in which juvenile *Artemia* were variously enriched failed to demonstrate improvements in larval growth, and more importantly in survival, when fed to phyllosoma (Nelson et al., 2003). It is probable that the nutritional improvement after enrichment of *Artemia* was outweighed by the higher microbial contamination attributable to some of the diets. We found that bacterial numbers increased rapidly to their maximum after 6 h of Algamac enrichment, more than four-fold the levels prior to enrichment. Bacterial proliferation was slower with the oil emulsion, taking at least 12 h to reach comparable bacterial numbers. Elevated numbers of bacteria with the *C. muelleri* enrichment were only apparent at 36 h. *Artemia* typically contain high bacterial loads especially of *Vibrio* spp., including pathogens to marine larvae in culture (Makridis et al., 2000; Olafsen, 2001). Some enrichments may accentuate this problem, leading to complete mortality of larvae in culture when fed contaminated *Artemia* (unpublished data). In contrast, pathogenic *Vibrio* spp. and externally fouling *Leucothrix*-like bacteria are diminished in phyllosoma during starvation because they are not exposed to bacteria-laden *Artemia* (Ritar et al., 2003b) or their organic wastes. Thus, if bacterial numbers are a concern, then a short enrichment, which still allows for significant nutritional improvement

while minimising the microbial burden, should be considered, especially with diets such as Algamac or oil emulsions.

Nutrients that enhance the growth of opportunistic and pathogenic bacteria (Olsen et al., 2000; Olafsen, 2001) are probably higher in the leachate from Algamac particles and in oil emulsion. If *Artemia* are enriched for longer than 6 h with either of these diets, then the high microbial content, which is transferred to culture tanks, would probably reduce larval survival. In fish larvae, these bacteria associated with *Artemia* are consumed and have a seriously adverse impact on gut physiology (Ottesen and Olafsen, 2000) and this is also likely to be the case in lobster phyllosoma.

Bacterial proliferation in *Artemia* fed *C. muelleri* was low, even exhibiting somewhat of a reduction after 6 h enrichment. Some microalgae appear to be naturally bacteriostatic or bactericidal (Kellam and Walker, 1989; Olsen et al., 2000), disinfective properties useful during enrichment of *Artemia* at warm temperature and high nutrient load typical of the intensive, static cultures, which are otherwise conducive to bacterial growth. For example, *C. muelleri* may be used to sanitize the external and internal surfaces of *Artemia* by short-term (0.5–3 h) purging, either alone (Tolomei et al., 2004) or in combination with formaldehyde (unpublished data). These algal treatments markedly reduced the bacterial content (especially *Vibrio* spp.) compared to enriched *Artemia* remaining untreated. It must be cautioned that algal cells may themselves be carriers of bacteria including *Vibrio* spp., occurring in much higher numbers in stationary than logarithmic phase cultures (Salvesen et al., 2000).

The bacterial numbers remained high or even increased during starvation for up to 24 h at 18 or 28 °C, temperatures that mimicked *Artemia* residence in culture for temperate or tropical lobster larvae, respectively. Interestingly, enriched *Artemia* held at 4 °C for up to 24 h displayed a reduction in total and *Vibrio* numbers suggesting this may be a possible means to disinfect *Artemia* of pathogenic *Vibrio* spp. prior to feeding of phyllosoma. However, recent studies showed that *Vibrio* held at low temperatures enter into a state termed "viable but non-culturable" (VBNC) (Jiang and Chai, 1996; Johnson and Brown, 2002). For example, *V. vulnificus* could no longer be detected by direct plating on TCBS agar after 24 h at 4 °C but these previously VBNC cells were able to revert to growth following a temperature upshift before plating (Johnson and Brown, 2002). Furthermore, Oliver (1995) found that VBNC cells have the potential to cause infection following resuscitation. Further work is required to confirm whether storage of *Artemia* at 4 °C results in *Vibrio* spp. becoming VBNC or killed/inactivated before the treatment could be considered a means of disinfection.

5. Conclusions

The type and the duration of enrichment markedly influenced the biochemical composition and bacterial content of juvenile *Artemia*. The greater depletion rate during starvation at higher temperatures indicates that freshly enriched *Artemia* may need to be fed more than once daily in tropical hatcheries to ensure their nutritive value. The minimal loss of enrichment at 4 °C makes refrigerated storage before feeding a viable proposition, with a possible further benefit of *Artemia* disinfection. The use of *C. muelleri* was fortuitous in

exemplifying both its nutritive and antibacterial effects, although some other algae may have equivalent properties. In contrast, Algamac and oil emulsion increased microbial levels probably due to the presence of nutrients conducive to their rapid proliferation. It may be argued that the relative changes in microbial content, particularly of the pathogenic *Vibrio* spp., are of greater importance than changes in the nutritional profile.

On the basis of all parameters examined, enrichment of juvenile *Artemia* for 6 h is most appropriate. In future, other enrichments and feeding strategies, such as mixed diets of bactericidal microalgae in combination with Algamac or vitamin-enhanced oil emulsions, could also be examined. The potential of diets to improve lobster phyllosoma survival and growth needs to be determined in a culture environment where there is no interference from microbial pathogens.

Acknowledgements

The project was supported by the Fisheries Research and Development (Project number 2000/214). We thank Mina Brock for technical assistance with the lipid and vitamin analyses and Danny Holdsworth who managed the GC-MS facility.

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