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Effects of *Artemia* enrichment with microalgae on the survival and growth of *Panulirus homarus* phyllosoma larvae

*J. T. Mary Leema, M. Vijayakumaran, R. Kirubagaran and K. Jayaraj

National Institute of Ocean Technology, Pallikaranai, Chennai-600 100, Tamil Nadu, India.
**E-mail: leemaroy2002@yahoo.com*

Abstract

Growth of early phyllosoma stages of the spiny lobster, *Panulirus homarus* fed with *Artemia* enriched with different species of microalgae was studied. Freshly-hatched phyllosoma were grown at a density of 10 L^{-1} in 60 L upwelling tanks supplied with flow-through sea water at 27±0.5 °C. *Artemia* nauplii were enriched with one of three microalgae strains, *Chlorella vulgaris* (Chlorophyceae), *Chaetoceros gracilis* (Bacillariophyceae) and *Spirulina platensis* (Cyanophyceae). The nauplii were provided with microalgae at a concentration of 1.5 $\times 10^6$ cell ml^{-1} for 6 hours for enrichment. A fourth group was maintained as a control and was fed unenriched *Artemia* nauplii. Survival rate of newly-hatched larvae cultured to Stage II with *Artemia* nauplii enriched with *C. vulgaris, C. gracilis, S. platensis* and unenriched *Artemia* were 58.89±1.11, 57.78 ±2.22, 66.11±0.56 and 40.0 ± 1.67% respectively. The survival rate from Stage I to Stage II larvae was significantly ($p < 0.05$) higher in all the groups fed with enriched *Artemia* nauplii. The highest survival rate (29.20 ±1.36%) from Stage II to Stage III instar 2/3 was obtained in the group fed with *Artemia* nauplii enriched with *C. vulgaris*. Similarly the shortest moult interval from Stage I to III instar 2 (19.5 ±1.44 days) was also observed in this feeding regime. The phyllosoma larvae fed with *C. vulgaris* enriched nauplii, attained Stage III instar 3 in 22-25 days whereas in the other groups the larvae were in Stage III instar 1/2 at the end of 30 days. The lowest range of total aerobic heterotrophic bacterial load (THB) (0.9 $\times 10^3$ ±0.14 to 11.0$\times 10^3$±1.15) and *Vibrio* spp. load (0.81 $\times 10^3$ ±0.02 to 5.73 $\times 10^3$ ±0.2 CFU, ml^{-1}) from day 0 to day 30 in culture water was observed in the group fed with nauplii enriched *C. vulgaris*. Significant ($p < 0.05$) size differences were observed in different groups. Thus larval survival, size, moult interval and bacterial load of culture water were significantly influenced by the use of *Artemia* enriched with microalgae.

Keywords: *Panulirus homarus*, phyllosoma, *Artemia*, microalgae, larval rearing

Introduction

Completion of the larval cycle of spiny lobsters under controlled conditions is a challenge to researchers around the world with success being achieved in at least 8 spiny/rock lobster species (Phillips and Smith 2006). Recent success in production of puerulus and juveniles of the tropical spiny lobster, *Panulirus ornatus* by the Lobster Harvest, Australia; the Australian Institute of Marine Science and the Queensland Department of Primary Industry and Fisheries (Cairns) suggests that the development of commercial hatchery technologies for this and other tropical species is a real prospect Peter Rogers (2010). Large scale production of spiny lobster seeds in hatchery is possible only by satisfying the nutritional and the environmental requirements of phyllosoma larvae (Moss *et al*., 1999; Tong *et al*., 2000; Bermuda *et al*., 2002). Design of experimental culture systems for phyllosoma larvae has been addressed (Illingworth *et al*., 1999; Ritar *et al*., 2002) but to date little is known on their nutritional or health requirements. The first major breakthrough in *P. japonicus* phyllosoma culture was achieved by Inoue (1978). His success was partly due to the use of brine shrimp *Artemia* nauplii as food for the early stages. However, the *Artemia* nauplii are an incomplete food source, because of the low levels of eicosapentaenoic acid (EPA, 20:5n-3) and

docosahexaenoic acid (DHA, 22:6ù-3) generally thought to be required for successful development of crustacean larvae (Sorgeloos, 2001). There are a number of standard enrichment techniques used to improve the nutritive value of *A. franciscana*, especially with respect to the essential n-3 fatty acids and ascorbic acid (AA) (Lèger *et al*., 1987; Gapasin *et al*., 1998). Short term (6-24 hours) enrichment of *Artemia* nauplii with high concentration of HUFA rich microalgae is one of the techniques that has been utilized to improve the nutritional quality (Reitan *et al*., 1993). Microalgae have also been shown to affect the microbial community of the water, the live feed (Nicolas *et al*., 1989) and the micro flora of the larval intestine (Skjermo and Vadstein, 1993). The aim of the present study is to examine the effect of feeding microalgae enriched *Artemia* nauplii to phyllosoma of the tropical spiny lobster, *P. homarus*. The efficacy of enrichment was measured in terms of the survival rate and disease resistance by the larvae.

Material and Methods

***Culture System*:** Sea water was drawn through subsurface filters from the intertidal zone and passed through a rapid sand filter and chlorinated before entering the storage tanks. From the storage tank it was dechlorinated by passing through a charcoal filter and sent through a series of 10, 5 and 1µm cartridge filters with final disinfection by passage through ultraviolet radiation before letting into the 80L upwelling Fibre Reinforced Plastic (FRP) tanks (Fig.1). To create upwelling movement in the tank, water was jetted through fine holes present in the

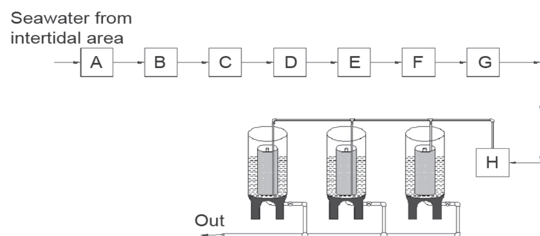

Fig. 1. Schematic diagram of the flow through system used for phyllosoma rearing. A- Rapid sand filter; B-Electrical chlorinator; C-Storage tank; D-Charcoal filter; E-10µ filter; F-5µ filter; G-1µ filter; H-UV radiation

bottom of the inlet pipe. The water level of the tank maintained at 60L by height positioning of a centre overflowing outlet pipe (Fig. 2). The inlet and outlet pipes were encircled by a larger diameter centre stand pipe with holes and covered with nylon screens (250 µm) to retain the phyllosoma and *Artemia* nauplii. Moderate aeration was provided at the centre of the tank with air stones. A ball valve controlled the rate of flow into each tank which was adjusted to (150 ml min^{-1}) to maintain a water turnover of 3-4 times day^{-1}.

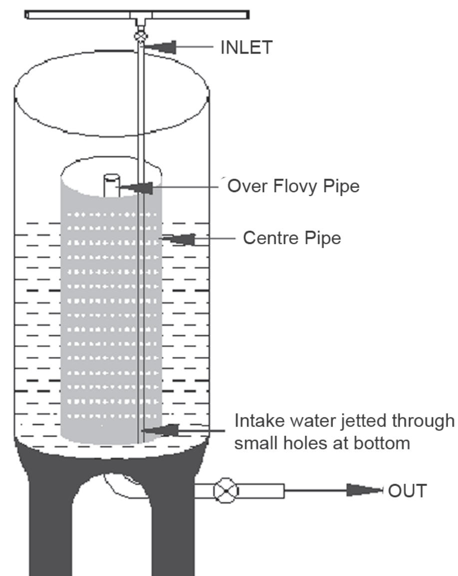

Fig. 2. Upwelling tank used for *Panulirus homarus* phyllosoma culture

***Larval maintenance*:** Freshly hatched phyllosoma larvae from captive *P. homarus* broodstock were grown at a density of 10 larvae L^{-1} in upwelling tanks containing 60 L sea water in the flow-through system described above. The hydrographic parameters recorded during the experiment were: temperature 27 ± 0.5 °C, salinity 33 ± 2, DO > 4mg l^{-1}, ammonia < 1ppm and pH 7.8 ± 0.5. The unconsumed feed and debris from the tanks were removed daily in two steps as described by Ritar *et al*. (2002). Initially, the 250 µm screen covering the drain pipe was removed and replaced with a 500 µm screen for the passive escape of the *Artemia* and the suspended debris. This was followed by bottom siphoning to remove the debris and

remaining food prior to the replacement of 250 µm screen on the drain pipe. Once in five days the number of phyllosoma were counted and transferred to clean upwelling FRP tanks.

***Mortality and moults determination*:** Moult determination was done daily by removing the exuvia floating in the water column or attached to the walls of the tank. Dead larvae were counted daily and reconfirmed by counting the survivors once in every five days. Once in 15 days a random sample (n=10) of larvae from each culture tank was utilized for measuring the total length (from the anterior tip of the cephalic shield between the eyestalks to the posterior tip of the abdomen and cephalic width (left and right extremes of the cephalic shield)) on a Nikon (model SMZ-1500) spectrozoom microscope (Japan). Phyllosoma were later returned to the culture tank. The larval stages were recognized following to Berry (1971) and Radhakrishnan and Vijayakumaran (1995).

Artemia nauplii enrichment: *Artemia franciscana* cysts from the Great Salt Lake Utah (OSI, USA) were used for feeding phyllosoma. The corion of the cysts was removed by decapsulation according to the method of Sorgeloos *et al.* (1986). Hatching of the decapsulated cysts were done daily in 200 L conical FRP tanks in filtered (2µ) aerated sea water at 26-28 ^{o}C. Twenty four hours after hatching, the nauplii were transferred to the enrichment system. The enrichment of nauplii was done by feeding with the respective axenic microalgae (*C. vulgaris*, *C. gracilis* sp. or *S. platensis*) in the logarithmic phase at a concentration of 1.5×10^{6} cell.ml^{-1} for 6 hours. The enriched *Artemia* were harvested using on 80 µm nylon net, washed thoroughly to remove the debris before feeding to phyllosoma larvae.

Experimental design: Phyllosoma larvae were divided into four groups with each group receiving one of the treatments given here. Group 1: Fed *Artemia* nauplii enriched with *C. vulgaris* (Chlorophyceae)- marine strain;Group 2: Fed *Artemia* nauplii enriched with *C. gracilis* (Bacillariophyceae); Group 3: Fed *Artemia* nauplii enriched with *S. platensis* (Cyanophyceae); and Group 4: control fed unenriched *Artemia* nauplii.

Bacterial load of culture water: Total aerobic heterotrophic bacterial (THB) load of culture water was estimated by the dilution plate method in which 0.1 ml aliquots of undiluted to 10^{-3} dilution of culture water samples were spread plated in triplicate on Zobell Marine Agar (ZMA-2216 Himedia Ltd, India) and incubated at 37 ^{o}C for 24hours prior to enumeration. The diluent blanks were prepared with 50% aged sterile (autoclaved at 121 ^{o}C, 15psi, 20 minutes) sea water.

For the estimation of total *Vibrio* spp. serial diluted (undiluted to 10^{-3}) 0.1 ml aliquots of culture water samples were spread plated in triplicate on Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS-M189 (Himedia, Bombay, India) and incubated at 37Ú C for 24 hours prior to enumeration.

Statistical analysis: Data presented are the mean ± SEM. Statistical analyses were done using one way analysis of variance (ANOVA) followed by Tukey – Kramer HSD tests for post–hoc comparison. Statistics were executed using the statistical program SPSS ver.17. Significant levels for all analyses were set to $p < 0.05$.

Results

Effect of Artemia enrichment on phyllosoma survival rate: There was significant effect of *Artemia* enrichment with different microalgae on the larval survival rate (Fig. 3). The survival rate from Stage I to Stage II larvae were significantly ($p < 0.05$) higher in all the groups fed with *Artemia* sp. enriched

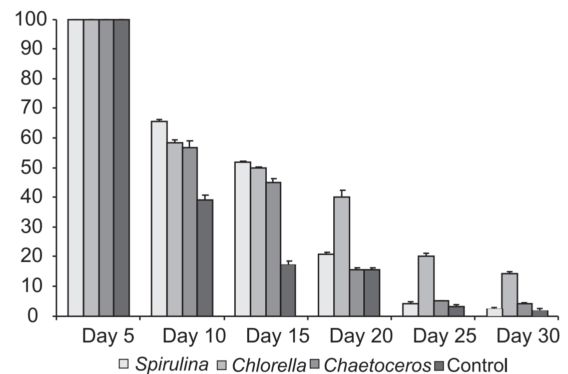

Fig 3. Survival rate of *Panulirus homarus* phyllosoma larvae fed with *Artemia* enriched microalgae; values are mean ± SEM

with microalgae than the control group fed with unenriched *Artemia*. The group fed with *Artemia* sp. enriched with *C. vulgaris* had the highest survival rate (29.20 ± 1.36%) from Stage II to Stage III instars 1/2 and the larvae attained Stage III instar 3 only in this group. Lowest survival rate (4.52 ± 0.29) from Stage II to Stage III instars 2/3 was obtained in the control group fed with unenriched *Artemia* sp.

***Effect of Artemia enrichment on phyllosoma size*:** The increases in length and width of newly hatched phyllosoma to Stage II fed on unenriched *Artemi*a were significantly lower than the groups fed on *Artemi*a enriched with microalgae. The increase in size of phyllosoma larvae fed on *Artemi*a enriched with *C. vulgaris* was significantly ($p < 0.05$) larger than all other groups (Table 1). Moult interval from Stage I to II did not differ significantly ($p > 0.05$) between the groups. However, the lowest moult interval from Stage II to III instar 3 was observed in the phyllosoma group fed on *Artemi*a enriched with *C. vulgaris* (Table 2).

Effect of Artemia enrichment on microbial load of phyllosoma culture water: Highest total aerobic bacterial load (THB) was observed in the culture water of phyllosoma fed with *Artemia* without any enrichment (Fig. 4). THB load of the culture water of phyllosoma fed with *Artemia* enriched with

Table 2. Moult interval (days) of *Panulirus homarus* larvae from Stage I to Stage III when fed on *Artemia* enriched with different microalgae. Values are mean ± SEM. Different alphabets indicate significance at $p < 0.05$

Stage	*Chlorella*	*Chaetoceros*	*Spirulina*	None
I to II	6-8	7-8	7-9	8-10
II to III instar 2	11-14	11-18	12-19	12-20

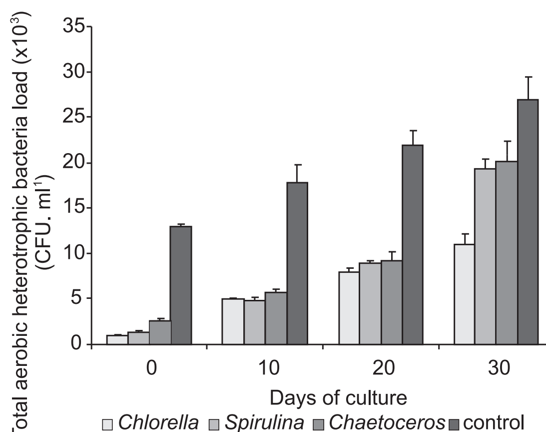

Fig. 4. Total aerobic heterotrophic bacterial load (mean ± SEM) of culture water of *Panulirus homarus* phyllosoma fed with *Artemia* enriched with different microalgae and control (unenriched *Artemia*)

Table 1. Survival and growth of *Panulirus homarus* larvae from Stage I to Stage III when fed on *Artemia* enriched with different microalgae. Values are mean ± SEM

	Artemia enrichment with			
	Chlorella	*Chaetoceros*	*Spirulina*	None
Survival % from:				
Stage I to II	58.89 ± 1.11[a]	57.78 ± 2.22[a]	66.11 ± 0.56[a]	40.00 ± 1.67[a]
Stage II to III	29.20 ± 1.36[abc]	6.25 ± 0.40[b]	11.77 ± 0.89[c]	4.52 ± 0.29[ac]
Larval total length (mm) at :				
Stage I	0.62 ± 0.01	0.61 ± 0.01	0.62 ± 0.02	0.63 ± 0.02
Stage II	1.84 ± 0.05	1.76 ± 0.06	1.71 ± 0.05	1.70 ± 0.04
Stage III Instar 2	2.83 ± 0.04	2.73 ± 0.03 [c]	2.66 ± 0.04	2.61± 0.05
Larval cephalic shield width (mm) at :				
Stage I	0.62 ± 0.01	0.61 ± 0.01	0.91 ± 0.03	0.63 ± 0.02
Stage II	0.96 ± 0.02	0.91 ± 0.03	0.87 ± 0.01	0.82 ± 0.01
Stage III instar 2	1.56 ± 0.04	1.52 ± 0.04	1.40 ± 0.02	1.28± 0.01

microalgae had a significantly (p< 0.05) lower load than the unenriched control group. There was no significant difference (p>0.05) in THB load of the groups enriched with different microalgae until day 20. However, after day 20 the culture water of phyllosoma fed with *Artemia* enriched with *C. vulgaris* had a significantly lower (p< 0.05) THB and *Vibrio* spp. load. Similarly, the *Vibrio* spp load of the culture water of control group fed with unenriched *Artemia* had a significantly higher load than the other groups (Fig. 5).

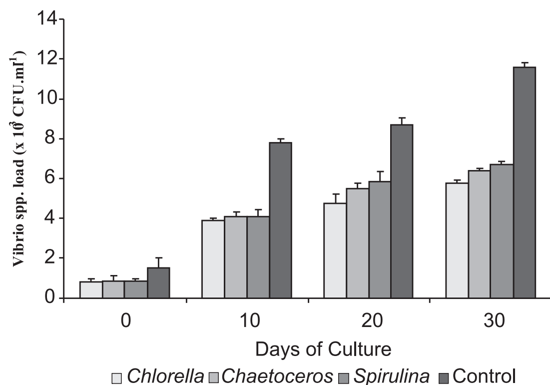

Fig. 5. *Vibrio* spp. load (mean ± SEM) of culture water of *Panulirus homarus* phyllosoma fed with *Artemia* enriched different microalgae and control (unenriched *Artemia*)

Discussion

The essential nutritional requirements of *P. homarus* phyllosoma larvae remain unknown. In the wild the larvae are presumed to consume a wide variety of zooplankton, which differs between seasons and locations (Ritar *et al*., 2003a). Despite the shortcomings in the nutritional value of *Artemia* it is still the most favoured diet in phyllosoma culture due to its ease of availability, preparation and larval attraction (Kittaka, 1994; Moss *et al*., 2000). Enriching the *Artemia* with algae, oil emulsion and other products renders the possibility of altering the biochemical composition of *Artemia* and a concomitant change in the lipid content and fatty acid profile (including EFA and ascorbic acid content) of phyllosoma feeding on it. This in turn, has significant effect on improving growth, resistance to disease and morphological development of phyllosoma larvae (Ritar *et al*., 2003b). Further, Nelson *et al*. (2002) and Smith *et al*. (2002) have reported enhancement of disease resistance and improved survival in spiny lobster phyllosoma fed with enriched *Artemia.* Results of this study confirms these observations as the survival rate from Stage I to Stage II larvae were significantly (p< 0.05) higher in all the groups fed with *Artemia* sp. enriched with microalgae than the control group fed with unenriched *Artemia.* Better growth and shorter moult interval observed in phyllosoma larvae fed on *Artemia* enriched with different microalgae suggests the benefits of microalgae enrichment of *Artemi*a. In an earlier study, *Artemia* nauplii fed on axenic cultures of *Chlorella* sp. recorded rapid growth (Rosowski, 1989) and the phyllosoma group fed on *Artemia* enriched with *C. vulgaris* in this study also showed better survival, growth and shorter moult interval compared to the other groups, suggesting that enrichment with this micro algal species is more effective for early stage *P. homarus* phyllosoma.

Spiny lobster phyllosoma culture is often confronted by sudden mass mortalities caused by bacterial pathogens. Hence, enrichment procedures that minimize the bacterial load of *Artemia* fed to larvae are equally crucial as the ones that alter the composition of phyllosoma larvae (Ritar *et al*., 2004). Though the primary purpose of enrichment of *Artemia* with microalgae is to enhance the nutritional quality of *Artemia* there are other interesting consequences like the bacteriostatic activity of many microalgae which renders additional advantage of microbial control to phyllosoma larvae (Ritar *et al*., 2003a). Ritar *et al*. (2004) reported 81% decline in the total number of heterotrophic bacteria per *Artemia* after 6h enrichment with the micro alga *C. muelleri*. In *P. homarus* phyllosoma rearing, the lowest range of total aerobic heterotrophic bacterial load of $0.9 \times 10^3 \pm 0.14$ to $11.0 \times 10^3 \pm 1.15$ CFU.ml^{-1} and *Vibrio* spp. load of $0.81 \times 10^3 \pm 0.02$ to $5.73 \times 10^3 \pm 0.2$ CFU.ml^{-1} from day 0 to day 30 in culture water was observed in the Group fed with *Artemia* sp. enriched with *Chlorella.* Hence it may be inferred that enrichment of *Artemia* with microalgae considerably reduces the bacterial load carried by *Artemia* to the phyllosoma culture water. *Artemia* have been reported to contain high bacterial load especially,

Vibrio spp. pathogenic to crustacean and marine fish larvae (Makridis *et al*., 2000; Olafsen, 2001). Even during the present study, the culture water of phyllosoma fed with unenriched *Artemia* had the highest range of total aerobic heterotrophic bacterial load of 13 $\times 10^3 \pm 0.14$ to $27 \times 10^3 \pm 2.5$ $CFU.ml^{-1}$ and *Vibrio* spp. load of 1.52 $\times 10^3 \pm 0.49$ to 11.59 $\times 10^3 \pm$ 0.2 from day 0 to day 30. It is common to note that *Artemia* enrichment emulsions, such as Algamac, which have improved the biochemical composition of *Artemia* have also accentuated bacterial proliferation and resulted in severe larval mortality (Ritar *et al*., 2003a). This is further exemplified by the fact that phyllosoma larvae exposed to starvation exhibited lesser load of pathogenic *Vibrio* spp. load and external fouling by *Leucthrix* spp. due to minimal exposure to organic load and *Artemia* (Ritar *et al*., 2003b). Some of the microalgae naturally possess bacteriostatic or bactericidal and disinfective (Kellam and Walker, 1989; Olsen *et al*., 2000) properties which are useful during enrichment of *Artemia* at tropical temperatures and high nutrient loads typical of intensive phyllosoma culture systems. Utilizing Denaturing Gradient Gel Electrophoresis (DGGE) for bacterial community profiling Høj *et al*. (2009) isolated antimicrobial compound producing bacteria *Antarcobacteria* from *Artemia* nauplii enriched with microalgae. This further emphasizes the use of microalgae for *Artemia* enrichment as an additional aid to control pathogenic bacteria in phyllosoma culture. Microalgae are reported to contain high concentration of vitamins especially vitamin C, ascorbyl acetate (AsA) vitamin E and alpha tocopherol (α–T) which have been shown to improve disease resistance in phyllosoma larvae (Smith *et al*., 2004). Hence, it is probable that the enrichment of *Artemia* with microalgae may also provide vitamins essential to enhance the disease resistance of phyllosoma larvae. In contrast, commercial *Artemia* enrichment emulsions are supplemented with vitamin additions during manufacture (Ritar *et al*., 2004).

In conclusion, the use of marine microalgae *C. vulgaris*, *C. gracilis* and *S. platensis* for *Artemia* enrichment had a positive impact on the survival rate. The lower bacterial load of culture showed the nutritive and antibacterial effects of microalgae. The relative reduction in the *Vibrio* spp. load in the culture water of phyllosoma larvae fed with *Artemia* enriched with microalgae had greater importance than their nutritive value. On the basis of all the parameters examined, enrichment of *Artemia* with microalgae greatly reduces the magnitude of mortality in the early phyllosoma larvae. Future *Artemia* enrichments should combine bactericidal microalgae with other enrichments like vitamin enhanced oil emulsions for enhancing the survival rate of phyllosoma. Antibacterial activity of the microalgae should be examined in future *Artemia* enrichment experiments.

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