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Investigation of an *Artemia*-based Diet for Larvae of the Mud Crab *Scylla serrata*

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Abstract

Techniques for the mass propagation of the mud crab *Scylla* spp. in hatcheries are in demand to supply the requirements of an expanding growout industry. Culture of the crab larvae currently utilizes an *Artemia*-based diet through most of the hatchery cycle. A series of experiments was designed to test the performance of the nauplii of different commercially available *Artemia* cyst products, sourced from various geographic regions, and nauplii enriched with a commercial lipid emulsion. The influence of microalgae addition to the larval culture medium to allow continuous enrichment of *Artemia* was also tested. Each of the test diets was analyzed biochemically and the resulting profile, combined with larval performance data was used to determine the relationships between larval growth and mortality and nutritional components.

Nauplii of eight *Artemia* cyst products were tested, but only two promoted growth and survival through the first metamorphosis from the zoea to the megalopa stage. Crab larval growth and survival were similar among the *Artemia* types fed up to the onset of metamorphosis, at around days 12 to 16. From this point onwards, the 'good' and the 'poor' *Artemia* types diverged. Larvae fed with nauplii of the 'poor' *Artemia* types failed to complete the metamorphic molt. To obtain maximum larval performance when using an *Artemia* nauplii diet, mud crab hatcheries need to be selective of cyst source.

Enriching *Artemia* nauplii elevated total lipid levels and those of a number of fatty acids, including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). However, it did not influence the ability of larvae to complete development. The nutritional component that best correlated with larval survival was phospholipid. It is suggested that further work is required to elucidate the role of phospholipid in the diet of mud crab larvae.

Introduction

Aquaculture production of mud crabs *Scylla* spp. contributes a large proportion to the world production of the genus (FAO 1999). Pond growout is currently dependent on the capture of wild juvenile crabs and poses a major constraint to mud crab industry development (Liong 1992; Rattanachote and Dangwatanakul 1992; Shelley and Field 1999). Consequently there has been an increasing interest in the development of hatchery production techniques. Larviculture of mud crabs is currently based on a diet of *Artemia* for the later larval instars as other live food sources,

particularly rotifers, are less suitable (Zeng and Li 1999; Simon 1975; Brick 1974). Declining survival near the end of the larval cycle and a high incidence of failure to complete the first metamorphosis indicate a possible nutritional deficiency.

Commercially exploited sources of the brine shrimp *Artemia* occur throughout the world and it is well documented that the nutritional content of newly hatched nauplii varies considerably among the types sourced from different geographic locations (Leger et al 1987). Selection of *Artemia* type for feeding cultured marine larvae is therefore critical to meet their nutritional requirements. Moreover, *Artemia* cyst production has recently been declining resulting in cyst price increases, with some cyst types becoming unavailable.

A series of experiments were conducted to investigate nutritional factors associated with the use of *Artemia* nauplii to feed mud crab *S. serrata* larvae. The experiments were designed to test the performance of five different commercially available *Artemia* cyst products sourced from various geographic regions. Furthermore, the potential benefits of nutritionally enhancing three *Artemia* types were investigated through the use of a lipid enrichment emulsion and the algae, *Nannochloropsis oculata* and *Isochrysis galbana*.

Materials and Methods

The production of mud crab larvae followed the standard protocol of BIARC (Mann et al 1999). Larvae were cultured in 3 l culture units maintained under controlled laboratory conditions with a photoperiod of 14 l : 10 D and light intensity of 1800 lux.

Three-liter aquarium bowls containing 2 l of sea water and provided with gentle aeration were used. A wide bore pipette was used to transfer the larvae and the culture containers were stocked with 50 larvae at the start of each experiment. For each culture, media were changed daily by transferring the larvae to replacement cultures containing fresh seawater and food. Larvae were counted and instar stage was recorded during the transfer. The culture experiments lasted for 18 to 21 days.

Rotifers *Brachionus plicatilis* were supplied at $10 \cdot ml^{-1}$ as food for the first zoeal instar and *Artemia* nauplii at a density of $5 \cdot ml^{-1}$ for the subsequent stages. In all treatments, newly hatched nauplii were used except when nutritional enrichment of the nauplii was carried out in trial 1.

Ranges of water parameters during the experimental procedures were: temperature, 27.5 to 30.0ºC; salinity, 33 to 36 ppt; pH, 7.9 to 8.2; dissolved oxygen, near saturation.

A total of eight *Artemia* cyst products or types were tested. Three were sourced from the Great Salt Lakes (GSL A, GSL B, GSL C), one from San Francisco Bay (SFB), one from China (CHIN) and three were reported to be selected strains from varied sources unspecified by the supplier (SEL A, SEL B, SEL C). The selected strains were promoted as high nutrition products.

Three separate trials were conducted. The first trial investigated the effect of nutritionally manipulating three *Artemia* types SEL A, GSL A, GSL B. A commercial lipid emulsion (Super Selco, INVE Aquaculture) was used to enrich *Artemia* metanauplii according to the manufacturer's instructions. Continuous enrichment of *Artemia* nauplii was done through direct addition of two species of microalgae, both singly and in combination, to the larval culture tanks. The algal species were *Nannochloropsis oculata* and Tahitian *Isochrysis galbana* at 5 x 10^5 and 1 x 10^5 cells·ml^{-1} respectively. This trial was designed as a balanced 4-way factorial combination of treatments.

Algae or lipid emulsion enrichment in trial 1 did not influence the performance of the larvae, and were omitted in trials 2 and 3. These two trials followed a randomized complete block design of five *Artemia* types with three replicates each. One of the *Artemia* treatments in trial 3 failed leaving only four *Artemia* types for analysis.

Biochemical analyses were conducted on newly hatched *Artemia* nauplii of six different types as well as the Super Selco enriched metanauplii of the three types used in the first trial. The diets were analyzed for proximate content (protein, lipid, ash, gross energy) as well as for phospholipid, fatty acid profile, amino acid profile and vitamins A, E and C.

Statistical Methods

Analysis of individual trials

Day 0 was designated as the day that the *Artemia* nauplii were introduced, and all survival and growth measurements were calculated for each culture unit. This corresponds to day 5 of actual larval development taken from the time of hatching. The daily growth data were analyzed as a growth index corresponding to the average instar number of larvae remaining in the culture per day, starting with zoea-1 as the first instar.

The repeated measures of data, percent survival and growth index, were subjected to analysis of variance (split for time) following the AREPMEASURES procedure of Genstat 5 (Payne et al. 1993). Larval survival was analyzed using data converted from percent mortality per day, and growth index from growth increments per day. For survival data, the treatment by time interactions were examined using the protected least significant difference (LSD) test at $P = 0.05$.

Cross trials meta-analysis

Each *Artemia* type in the three combined trials separately contributed an estimate of daily mortality and growth increment from day 0 to day 6, from day 6 to trial end, and also during the entire trial period. The eight newly hatched and three chemically enriched *Artemia* diets have 13 independent observations for correlation and regression analyses. The fatty

acids that had all observations below the reporting limit of 0.1 $mg \cdot g^{-1}$ dry weight of sample were excluded from the analyses.

Profile analysis

Hierarchical cluster analysis, following the HCLUSTER procedure of Genstat (Payne et al. 1993) was used to investigate biochemical groupings among the *Artemia* types, while follow-up univariate analysis of variance determined mean profiles.

Results

Feeding the mud crab larvae with *Artemia* nauplii enriched with the commercial lipid booster did not affect ($P > 0.05$) larval survival or growth (Fig. 1). In this trial enriched *Artemia* were on the average almost 200 mm longer than newly hatched nauplii (658 mm and 463 mm respectively). The addition of *N. oculata* or *I. galbana* alone or in combination, to the culture medium also had no effect ($P > 0.05$) on larval survival or growth (Fig. 2).

In all three trials, *Artemia* type significantly influenced ($P < 0.05$) larval survival (Fig. 3). Larval growth, was also significantly affected ($P < 0.05$) by *Artemia* type in trials 1 and 3 (Fig. 3). In all trials, similar larval performance was observed in the treatments initially (Fig. 3), with survival percentages and growth indices diverging in the latter phase of the culture period. This pattern was statistically investigated using the sequential LSD testing that determined the day on which significant ($P < 0.05$) differences between treatments first occurred. Percent survival was found on days 13, 14 and 17 for trials 1, 2 and 3 respectively. For growth data, the treatments first diverged on days 12, 12 and 14 for trials 1, 2 and 3 respectively.

Cross-trials analysis

The proximate and vitamin composition as well as the levels of phospholipid and the fatty acids EPA and DHA are listed in table 1. Initially, simple correlations among the full set of biochemical variables were tabulated. These showed a high degree of correlation among the independent X-variables. This means that for each Y-variable, there was a number of models that gave approximately equal fits.

Multiple linear models were investigated using the fast screening method of Furnival (1971); and models with three-parameters were selected as the most appropriate for both whole-trial mortality and growth estimates. The best three models based on R^2 significant at $P < 0.05$ are listed in table 2. Interpretation of the 3-parameter models was complicated by the high level of correlation among the independent variables and the small sample size, particularly of the successful *Artemia* types.

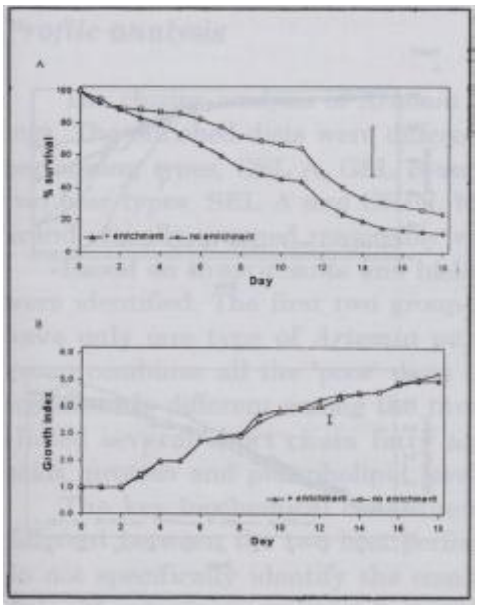

Fig. 1. Survival (A) and growth (B) of mud crab larvae fed newly hatched *Artemia* nauplii and *Artemia* nauplii enriched with a commercial lipid emulsion. Points are means of pooled data among different *Artemia* types. Survival is expressed as percent of original number of larvae and growth as growth index per day (= mean instar stage). Vertical bar represents LSD (P = 0.05).

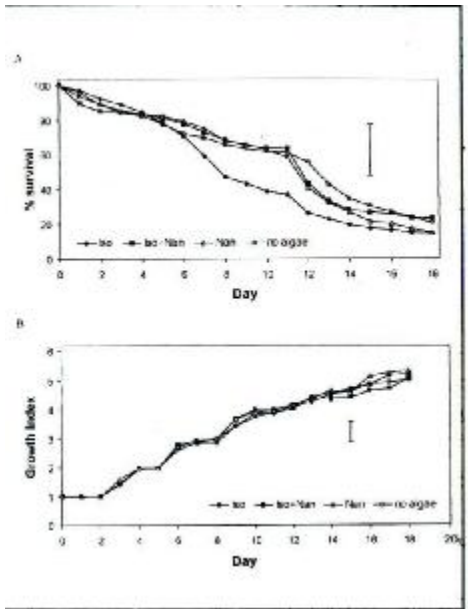

Fig. 2. Survival (A) and growth (B) of mud crab larvae cultured continuously in the presence of two species of microalgae, both alone and in combination. Survival is expressed as percent of original number of larvae and growth as growth index (= mean instar stage). Vertical bar represents LSD (P = 0.05). (Iso = *Isochrysis galbana*; Nan = *Nannochloropsis oculata*)

Table 1. Proximate composition and levels of vitamins A, C and E, phospholipid and the acids EPA and DHA in newly hatched nauplii and enriched metanauplii of tested *Artemia* types

	Sel A	Sel B	Sel C	GSL A	GSL B	GSL C	SFB	CHIN	Sel A+e	GSL A+e	GS B+e
% Protein	55.0	58.9	60.5	58.0	59.6	58.6	59.7	60.1	50.4	56.1	55.8
% Ash	7.1	6.3	6.5	6.9	7.2	7.1	6.7	7.5	9.2	8.1	8.2
% Lipid	19.4	19.3	15.1	18.9	15.7	19.5	17.1	17.8	33.2	25.7	25.7
G E M J/kg	22.2	23.3	23.4	22.8	22.9	22.2	23.8	22.0	25.0	23.6	24.4
Vitamin C $mg \cdot g^{-1}$ dry wt	495	444	610	636	669	644	790	548	392	426	545
Vitamin A $mg \cdot g^{-1}$ dry wt	34.5	37.2	2.9	43.5	41.0	30.1	nd (<0.5)	12.0	59.3	187.0	61.1
Vitamin E $mg \cdot g^{-1}$ dry wt	6.0	5.4	16.5	6.7	5.1	11.2	56.1	12.0	145.9	81.5	68.2
Phospholipid $mg \cdot g^{-1}$ dry wt	60.0	54.1	52.9	54.1	54.0	57.2	57.5	59.2	61.9	59.0	52.6
EPA $mg \cdot g^{-1}$ dry wt	15.49	23.05	3.58	5.72	2.61	4.96	2.72	15.32	62.14	39.23	41.44
DHA $mg \cdot g^{-1}$ dry wt	nd (<0.1)	0.31	nd (<0.1)	nd (<0.1)	nd (<0.1)	nd (<0.1)	nd (<0.1)	nd (<0.1)	21.72	15.78	18.25

"+e" indicates lipid emulsion enriched metanauplii.

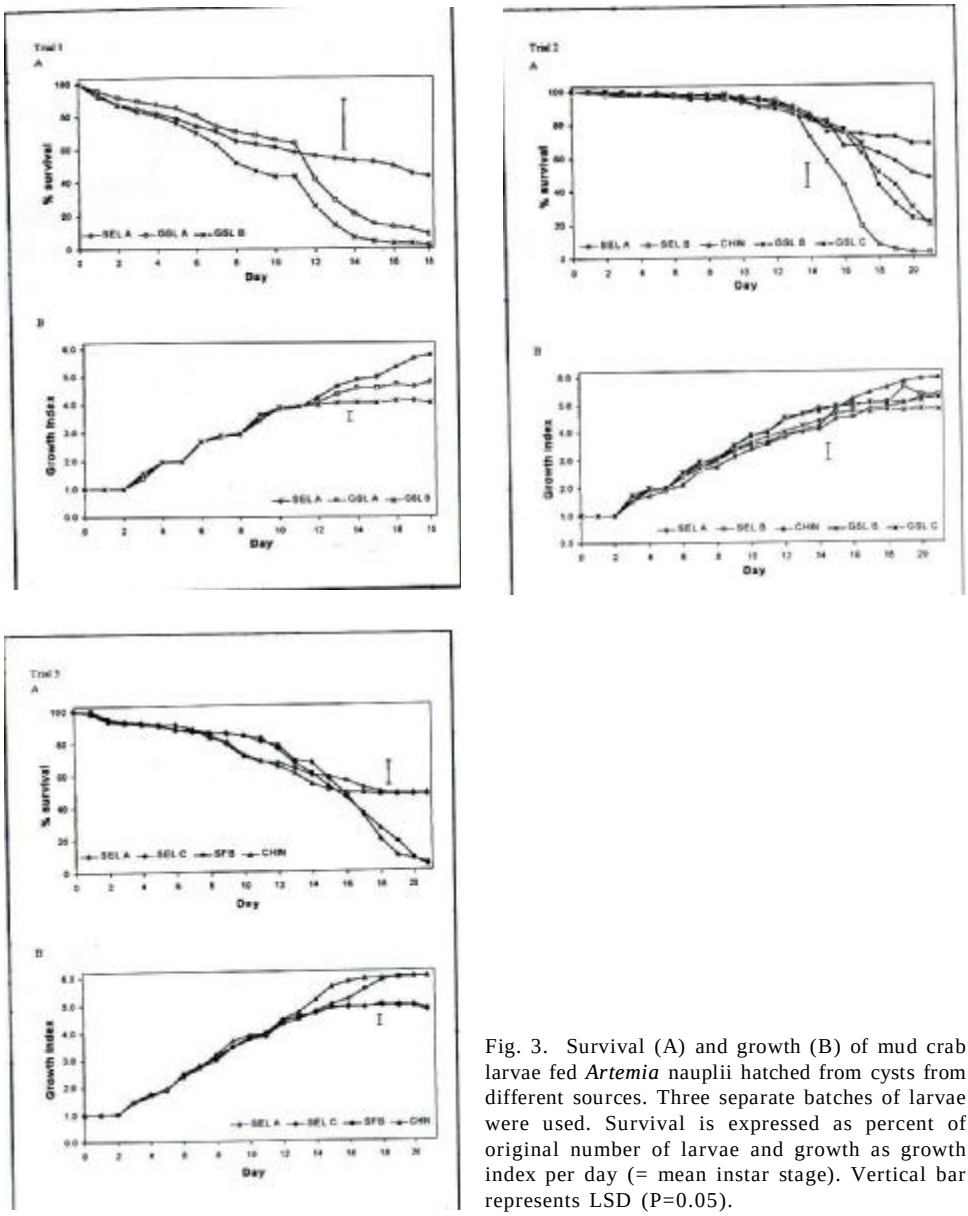

Fig. 3. Survival (A) and growth (B) of mud crab larvae fed *Artemia* nauplii hatched from cysts from different sources. Three separate batches of larvae were used. Survival is expressed as percent of original number of larvae and growth as growth index per day (= mean instar stage). Vertical bar represents LSD (P=0.05).

Table 2. Best three-parameter models for mortality (M %/day) and growth (G /day) over the whole-trial

	Adjusted R^2
Mortality	
M = 24.4 - 0.413´Phospholipid + 0.0209´Vitamin A + 0.128´FA 18:1n-9	0.899
M = 47.9 - 0.544´Phospholipid + 0.0227´Vitamin A - 0.441´Leucine	0.880
M = 32.0 - 0.530´Phospholipid + 0.0204´Vitamin A + 0.111´Lipid %	0.876
Growth	
G = 0.352 - 0.00788´Proline + 0.00782´FA 18:2n-6 - 0.00147´FA 18:3n-3	0.556
G = 0.338 - 0.01000´Proline + 0.00813´FA 18:2n-6 + 0.00275´FA 16:1n-7	0.544
G = 0.289 - 0.00905´Proline + 0.00651´FA 18:2n-6 + 0.00575´FA 18:1n-7	0.536

Profile analysis

The cluster analysis of *Artemia* biochemistry identified four main groupings. The enriched diets were different from the rest, and three of the poorer performing types, GSL A, GSL B and GSL C, formed a group. However, the two best types, SEL A and CHIN, had quite different profiles. The Chinese brand actually grouped reasonably well with the worst treatment, SEL B.

Based on these results and biological reasons, three basic profile groups were identified. The first two groups are representatives of 'good' diets, and have only one type of *Artemia* per group, CHIN and SEL A. The third group combines all the 'poor' diets together. Key indicator variables, those significantly different among the three groups, identified by this analysis included several short chain fatty acids, fatty acid 20:4n-6, several amino acids, protein and phospholipid level.

The key biochemical constituents identified in the profile analysis as different between the two best performing *Artemia* types against the others, do not specifically identify the combination of nutrients expected in 'good' diets. This is due mainly to the two 'good' types being quite different in biochemical profile.

Discussion

The experiment clearly demonstrates the variability in performance of mud crab larvae fed *Artemia* sourced from different regions. Only two of the eight *Artemia* types assayed in this experiment supported the development of a significant proportion of larvae to the megalopa stage. One of these came from China, while the other SEL A is marketed as a selected cyst grade from an unspecified source. Using larvae of the crab *Rhithropanopeus harrisii* in a bioassay, Johns et al (1981) also found that Great Salt Lake *Artemia* did not support complete development of the larvae but *Artemia* from Brazil and California promoted high production of postlarvae. The present work and Johns et al (1981) demonstrate larval responses to *Artemia* from different sources that are similar across relatively divergent crab species.

The possibility that the observed performance differences among the different *Artemia* types due to accumulated pesticides in some *Artemia* cysts has been investigated and discounted (Johns et al, 1981; Seidel et al, 1982). It was thought that the most likely source of performance variability lay in the nutritional composition of the *Artemia* nauplii.

Schauer et al (1980) found differences in fatty acid profiles and suggests that growth rate and survival in crustacean larvae were correlated with essential fatty acid content (EFA) in *Artemia*, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The relationship between EFA and larval survival using an *Artemia* diet is not clearly established. Enriching poor performing *Artemia* types with EPA or DHA did not improve larval survival of the crab *Portunus trituberculatus*

(Takeuchi et al 1999), although it was observed that the intermolt period of *P. trituberculatus* shortened with increasing DHA level in the diet. It was observed in this study that EFA did not influence larval performance. Levine and Sulkin (1984), however reported that for *Eurypanopeus depressus crab* larvae, the inclusion of lipids through *Artemia* enrichment was essential for larval survival or growth. Leger et al (1985) and Murthy (1998) also demonstrated that n-3 polyunsaturated fatty acid (PUFA) is critical for crustacean larval survival. This study, however, did not find a link between survival of mud crab larvae and n-3 PUFA level.

Improving the food value of *Artemia* through enrichment prior to feeding fish or crustacean larvae is now a common practice among marine hatcheries due to improved survival, growth and stress resistance (Sorgeloos and Leger 1992). In the current experiment, greatly elevated levels of EPA and DHA were achieved but no improvement in growth and survival of the larvae was observed. This may indicate that the required levels of EPA, DHA and other fatty acid component levels elevated in *Artemia* through enrichment are met in unenriched newly hatched *Artemia* nauplii. Alternatively the balance of nutrients is critical to larval performance and both enriched and newly hatched 'poor' *Artemia* types do not provide this balance. In a review of lipid nutrition of marine fish larvae, Sargent et al (1997) observed that techniques for the manipulation of larval diets continue to develop and greater attention is being directed towards balancing the ratios of important dietary constituents to achieve optimum larval performance.

The insignificant differences in larval survival and growth when fed enriched metanauplii and newly hatched nauplii in trial 1 indicate that *Artemia* size within the range 463 mm to 658 mm does not affect larval performance.

The inflection point of the survival curves is useful for identifying the effect of the inadequate diets on the larvae. Up to the inflection point, occurring on days 12 to 15, all *Artemia* types tested performed similarly but beyond this time there is an obvious decline in survival only of larvae fed the poorer *Artemia*. Days 12 to 15 of the larval development period corresponds to the period when larvae are preparing for or have commenced the metamorphosis to the megalopa stage. Observation of the larvae during this period showed high incidence of molt death syndrome (MDS). MDS refers to larvae that have begun molting but further progress is halted before the exuvium is completely shed. These larvae survive for a short period but can not swim or feed. MDS occurred in all crab larvae fed 'poor' *Artemia* types. Megalops were not produced, indicating the likelihood of a similar nutritional inadequacy in the diets tested.

The two types of algae, *N. oculata* and *I. galbana*, used in trial 1 to enrich *Artemia* were primarily chosen for their complimentary nature of EPA and DHA content (Dunstan et al., 1993). As there was no positive response of the larvae to the addition of these microalgae it was surmised that the algal species used did not improve the larval nutrition. This observation corroborates the finding of Chen and Jeng (1980) that addition of

Chlorella to the culture medium did not influence growth and survival of mud crab larvae. Although Brick (1974) reported the same finding for mud crab larvae up to the last zoea stage, an improved rate of larval metamorphosis to the megalopa occurred. This improvement may have been due to the water conditioning effect of the microalgae rather than nutritional influences. Johns et al (1981) also found that enriching *Artemia* nauplii with *Isochrysis galbana* did not positively influence the survival of *Rithropanopeus harrisii* larvae.

There are two choices to effectively utilize an exclusively *Artemia* based diet in the culture of mud crab larvae. First, to use *Artemia* types that are inherently nutritionally adequate and second, to enrich a poorer *Artemia* type to achieve the desired nutritional profile. The first option may be difficult as high quality *Artemia* may be prohibitively expensive or not commercially available. During the study the two better performing *Artemia* sources (SEL A and CHIN) became unavailable in the market. The second option utilizes the cheaper, commonly available *Artemia* cysts. However, an effective enrichment formulation still needs to be developed for crab larvae. Supplementation of the basic *Artemia* diet with artificial microparticulate diets is another alternative, but this requires a detailed knowledge of the nutritional requirements of the crab larvae.

Artemia phospholipid content was correlated with both crab larval survival and growth and was strongly represented in the models for larval mortality rate. Furthermore, phospholipid was one of the key biochemical constituents identified in the profile analysis which varied between the two best performing *Artemia* brands and the rest. These findings do not however confirm a cause and effect relationship between phospholipid and larval performance, particularly when considering the constraints of the analysis. The present observation corroborates the work of Cotteau et al (1997) which demonstrated that the phospholipid inclusion level, especially phosphatidylcholine and phosphatidylinositol, is more crucial than the total lipid content in the nutritional requirement of marine larval species. In view of the findings presented in this study, further study is recommended to assess the requirement and importance of phospholipid in the diet of mud crab larvae.

To ensure efficiency and long term viability of mud crab hatchery operations it is necessary to identify the critical dietary components and ensure that these can be effectively ingested and assimilated by the larvae.

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