CHAPTER – XIII

EVALUATION OF BIOLOGICAL DIETS FOR POSTLARVAE USING RNA/DNA RATIO AS NUTRITONAL STATUS INDICATOR

The commercial importance of shrimp has attracted considerable attention of researchers on its feeding biology (Dall *et al.*, 1990) and nutritional requirements (Akiyama, 1992). The demand for vital nutrients in adults and early life stages of various shrimp species has been widely studied (Boonyaratpalin, 1998: Teshima, 1998; Kurmaly *et al.*, 1989a,b; Kanazawa, 1990; Chandge and Paulraj, 1998; Jones *et al.*, 1997; Paibulkichakul *et al.*, 1998; Coutteau *et al.*, 2000).

In the shrimp hatcheries, production of high quality postlarvae need rearing the same under constant, close to optimal conditions with required quantity of nutritionally-sufficient food. One of the major factors affecting the postlarval quality in the hatcheries is the nutritional stress, other being the thermal and osmotic stress. The poor nutritional condition of the postlarvae can directly lead to high mortality by starvation or indirectly through prolonged stage duration and predation.

Among the shrimp larval stages, zoeal stages are herbivorous, mysIs and postlarval stages are more carnivorous (Jones *et al.*, 1997) and so the latter are fed with live feed *Artemia*. But, unreliable supply, nutritional quality (demanding enrichment thus raising cost) and exorbitant price of *Artemia* cysts have made hatchery technicians to stop

feeding *Artemia* mostly after PL_{10} stage and go for higher dosage of artificial diet. However, this practice in the hatcheries has resulted in limited success and has been reported due to the requirement for exogenous enzymes supplied by live feeds, supporting digestion and growth (Kurmaly *et al.*, 1990; Jones *et al.*, 1993). So, a cheap alternative diet which can supply above nutrients and boost growth is the need of the hour for the late nursery stage postlarvae.

The soft tissues of food organisms like squid, molluscs and fish have been recommended as suitable protein sources for shrimp (Deshimaru *et al.*, 1985: Penaflorida, 1989). These feeds are found to be rich in lipids, cholesterol and other sterols, phospholipids and essential amino acids (Deshimaru, 1982). They are also characterized as containing betaine and nucleotides which have been documented as having attractant qualities (Jones *et al.*, 1997). The possible growth promoting effect of such biological feeds in nurseries, especially as a supplementary diet after the PL_{10} stage when they are weaned to compound diet totally, needs to be studied.

In a rearing system, suboptimal conditions such as food limitation and nutritionally–deficient diet may exert bioenergetic effects which, in general, can be measured as a decline in the rates of development and growth or in changing proportions of chemical constituents of larval biomass. Biochemical analysis, which determines the quantities of such components that serve as energy substrates, could be one of the indicative measures to show changes of nutritional conditions. Among the biochemical indices, the ratio of RNA to DNA has been proven to be a reliable indicator of nutritional condition (Buckley, 1980, 1984; Clemmesen, 1987; Raae *et al.*, 1988) and growth of larval and juvenile fish (Bulow, 1970; Robinson and Ware, 1988, Imsland *et al.*, 2001) as well as number of crustaceans (Wang and Stickle, 1986; Juinio and Cobb, 1994). Moss (1994 a.b) used this

ratio to discriminate successfully between growth rates in juvenile *Litopenaeus vannamei* exposed to different feeds. Nunez *et al.* (2002) has used the same technique to study the effect of various algal feeds on shrimp larvae.

The use of RNA/DNA ratio is based on the assumption that RNA content per cell varies with growth because, RNA serves as both a template and an organizer for protein synthesis (Leslie, 1955). The DNA content per cell is assumed to be constant in normal somatic cells within a given species and this amount is not altered by starvation or other stressors (Munro and Fleck, 1969; Bulow, 1987). Several studies have shown that there is a linear relationship between the rate of protein synthesis and the RNA/DNA ratio (Buckley, 1980, 1984; Goolish *et al.*, 1984).

This experiment was carried out to evaluate the *in vivo* use of biological feeds for postlarvae in a commercial shrimp hatchery. In addition to growth estimation, difference in RNA/DNA ratio of shrimp exposed to different feeding regimes was also carried out to assess the feeds.

MATERIALS AND METHODS

These experiments were carried out in the commercial shrimp hatchery's outdoor tanks provided with moveable transparent roof top to control sunlight. The *Fenneropenaeus indicus* postlarvae at stage 8 (PL_8) from a single larval rearing tank were transferred from the hatchery's larval rearing section to the outdoor 5 ton tanks and stocked at a density of 20/litre (i.e. 100.000/tank). The postlarvae were starved for 48 hours before the commencement of the experiment.

Four treatments were tested and consisted of PL fed with (1) mixture of microalgae (control named M), (2) commercial postlarval feed (C), (3)commercial feed and biological feeds combination (CB), (4)combination of microalgae, commercial feed, and fresh biological feeds (MCB). It is worth mentioning here that in hatcheries, microalgae are cultivated together with the postlarvae in the nursery tanks. Each treatment had three replicates. Before the start up of the experiment, initial wet weight of forty postlarvae from each replicate was weighed on a digital balance (0.1 mg accuracy).

The water exchange given was 100% per day using UV-irradiated seawater filtered down to 5 μ level. The postlarvae were fed with a commercial feed, Higashi feed no.2 (up to PL_{14}) and 3 (from PL_{15} to PL_{20}) (Higashimaru feeds India Ltd, Cochin, India), quantity as advised by the manufacturer.(Table 45). The feed had a minimum crude protein content of 48% and crude fat of 8%. The fresh feeds used included locally procured fresh squid, mussels and oil sardine. The fresh mussels, squid and sardine were cleaned and processed before use. All the feeds were cut into small pieces and equal quantities of all the feeds were squeezed through 500 to 600 micron mesh in a bucket with water for feeding.

The microalgae were supplied from the indoor culture facility of the hatchery. The fresh biological feeds were fed only during the day time (4 feedings) and were fed separately one hour before feeding the commercial diet. The biological feeds were supplemented at a rate equivalent to 20% of the commercial diet fed per day (on dry weight basis). The microalgae mix included *Chaetoceros muelleri* (CS-176) and *Tetraselmis suecica* (CS-187) and the total algal density was maintained at 40,000 cells/ml at a concentration ratio of 3:1 respectively.

Table 44. Standardized nursery feeding schedule for 100,000 postlarvae, with a commercial postlarval feed

Postlarval stage	Quantity of feed/ feeding (g)	Feedings/day
PL_{10}	10	6
PL_{11}	11	6
PL_{12}	12	6
PL_{13}	13	6
PL_{14}	14	6
PL_{15}	15	6
PL_{16}	17	6
PL_{17}	19	6
PL_{18}	20	6
PL_{19}	22	6
PL_{20}	25	6

The experiment was carried out for a period of 10 days (up to postlarva 20 stage). At the end of the experiment, forty postlarvae from each replicate were weighed. Abdominal muscle tissue was excised and stored immediately at -80° C until nucleic acid analysis. Temperature and dissolved oxygen (DO) concentrations were measured using handheld oxygen meter (YSI model 550 A) and salinity was determined using refractometer. The water temperature was maintained between 28.3 to 29.2 °C, DO concentration was kept between 6.4 and 7.2 mg/l and salinity was maintained at 32 ppt.

Total RNA and DNA were extracted using the procedure of Schmidt and Thannhauser (1945) as modified by Munro and Fleck (1969) and quantified using the dual wavelength method (Munro and Fleck, 1969; Ashford and Pain, 1986). RNA and DNA concentrations were expressed as µg nucleic acid per 100 mg wet weight.

During the experiment, change in weight (mg) per week was calculated. The data were subjected to one way analysis of variance (ANOVA) to compare the treatments

means and differences in mean were evaluated using Tukey's studentized range test. Simple linear regression equation was fitted using least-squares method.

RESULTS

The results of the growth experiments are given in table 45.

Results indicated that the postlarvae could assimilate the microalgae and grow for a short period, but the growth and survival were significantly poorer ($P< 0.01$) than the other treatments. The commercial diet (C) compared to algae (M) fed tanks registered significant improvement in growth and survival ($P<0.01$). The addition of fresh biological feeds in C treatments (i.e. CB treatment) resulted in better weight gain ($P<0.05$) and survival ($P>0.05$). Best results for both growth and survival were given by the MCB treatment. Even though, the addition of microalgae to CB regime improved survival and growth (in MCB regime), the addition did not make any significant difference ($P>0.05$),

Table 45. Growth characteristics of *F. indicus* postlarvae from tanks fed microalgae mix (M), commercial feed (C), commercial feed and biological feed (CB) and all above three (MCB) for 10 days.

Treatment	Initial wet weight (mg)	Final wet weight (mg)	Weight gain/week	Survival (%)
M	5.18 ± 0.04	46.11 ± 0.40	28.65 ± 0.28^{a}	71.93 ± 2.20^{a}
C	5.25 ± 0.03	55.80 ± 0.31	35.42 ± 0.31^{b}	86.07 ± 1.11^{b}
CB	5.23 ± 0.03	65.20 ± 0.35	42.00 ± 0.26^{c}	91.77 ± 1.44^{bc}
MCB	5.20 ± 0.03	67.70 ± 0.29	43.75 ± 0.25^{c}	95.20 ± 1.54^{c}

Values are average of 40 postlarvae per treatment (± S.E.). Values in same column with different superscript differ significantly ($P<0.05$).

Mean RNA concentration of shrimp ranged from 345.80 μg/100 mg wet weight with shrimp fed algal food to 396.7 μg/100 mg with shrimp fed all three diets (Fig. 34). RNA content of shrimp fed algae alone differed significantly from all other treatments. The addition of biological feeds into commercial diet regime (C) did not significantly improve the RNA content ($P>0.05$). However, addition of both microalgae and biological feed to C treatment (i.e. MCB treatment) made significant improvement in RNA concentration ($P<0.01$). Regression analysis of the relationship between RNA content and growth rate gave the equation $Y=-68.77+0.29X$ (fig.38) with r^2 value of 0.87 ($P<0.001$).

Mean DNA concentration was lowest with shrimps from MCB treatment (58.3 μg/100 mg wet weight) (Fig. 35). The diatom treatment recorded the maximum DNA value of 63.4 μg/100 mg wet weight and differed significantly from all other treatments ($P<0.005$). The values of DNA also differed significantly between C and CB treatment as well as between CB treatment and MCB treatment.

Mean RNA/DNA ratio differed significantly ($P<0.05$) between all treatments except between the CB and MCB treatments (Fig.36). The ratio ranged from 5.46 (M treatment) to 6.82 (MCB treatment). The C and CB treatments recorded ratio of 5.95 and 6.51 respectively.

There was a highly significant ($P<0.001$) positive relationship between shrimp growth rate and RNA/DNA ratio for all treatments at the end of experiment (Fig. 37). Regression analysis proved that above 90% of the variation in growth rate could be explained by changes in RNA/DNA ratio.

Fig. 34. RNA concentrations (µg/100 mg wet weight) of postlarvae from treatments fed microalgae (M), compounded diet (C), comp. diet and biological feed (CB) and all three (MCB). Values are means ± S.D.

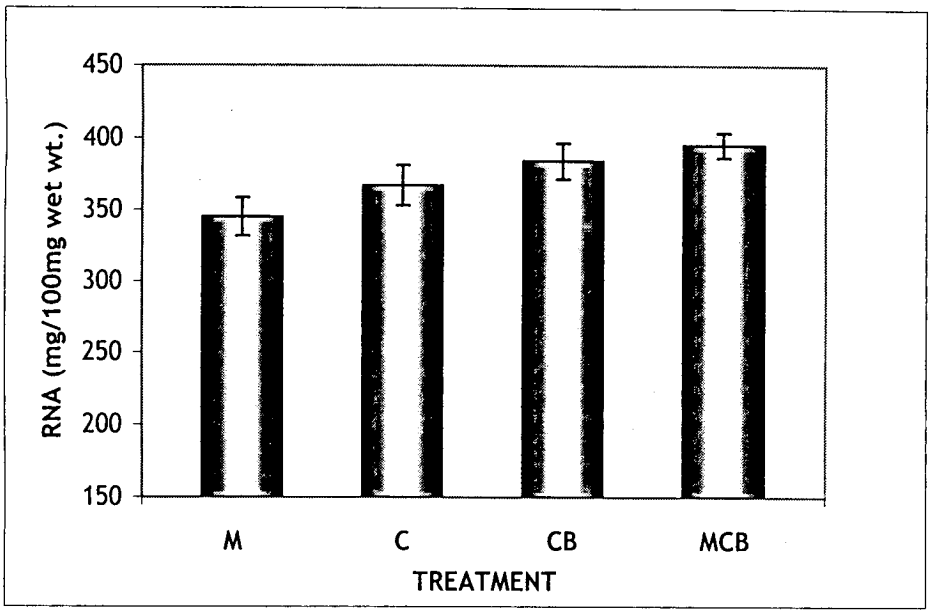


Fig. 35. DNA concentrations (µg/100 mg wet weight) of postlarvae from treatments fed microalgae (M), compounded diet (C), comp. diet and biological feed (CB) and all three (MCB). Values are means ± S.D.

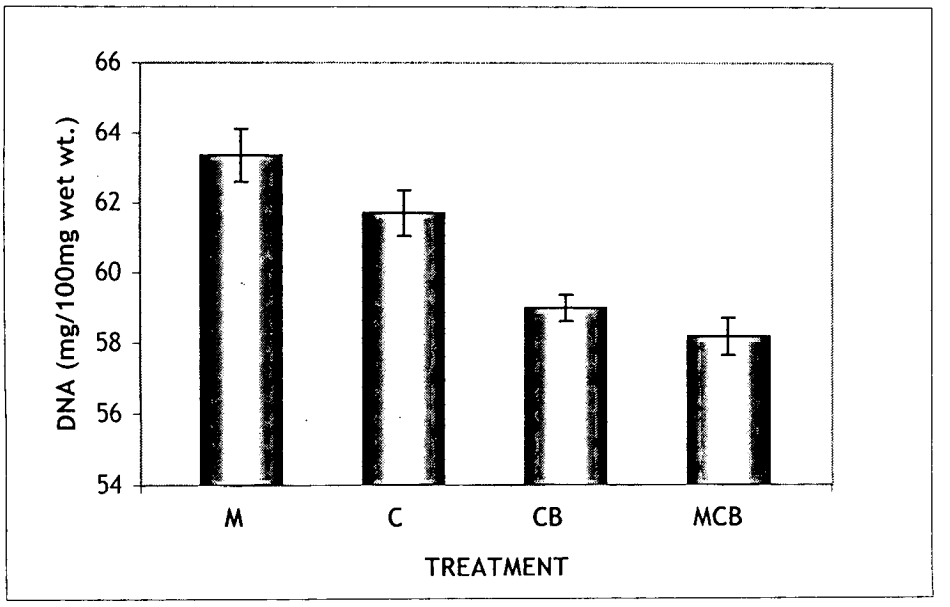


Fig. 36. RNA/DNA ratio of postlarvae from treatments fed microalgae (M), compounded diet (C), comp. diet and biological feed (CB) and all three (MCB). Values are means ± S.D.

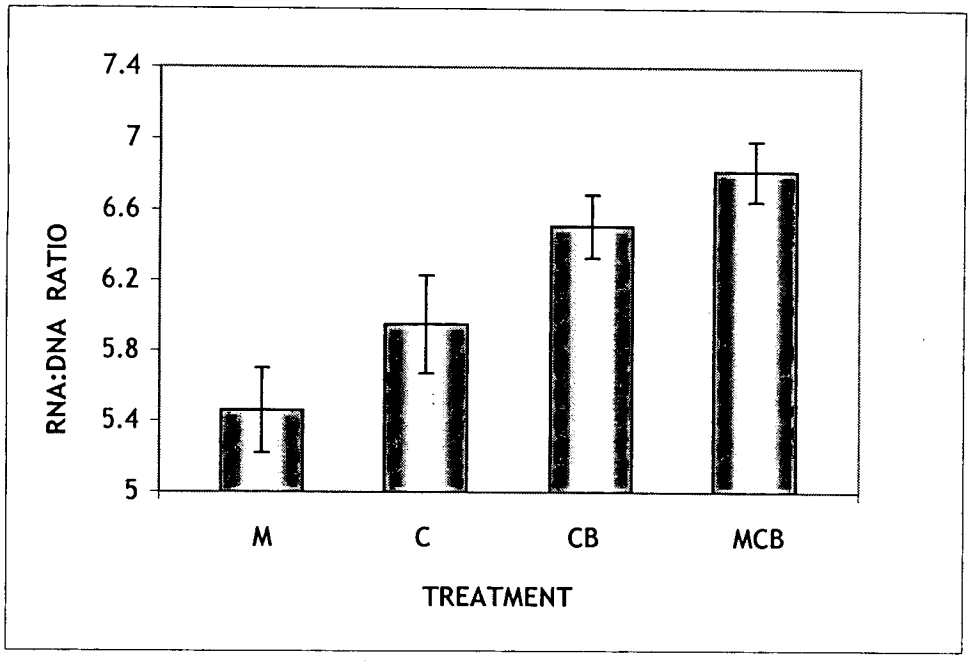


Fig. 37. Regression line for RNA/DNA ratio on shrimp growth rate (mg/week) for all replicates of all treatments at the end of ten day experiment.

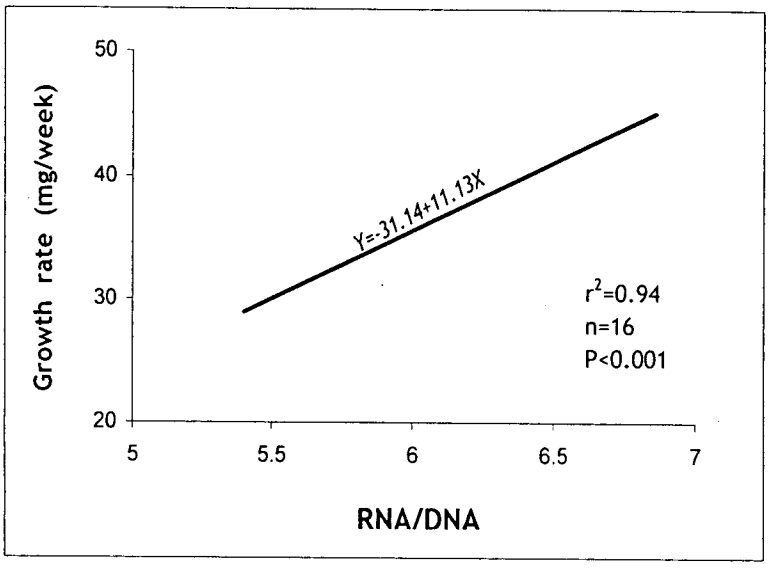
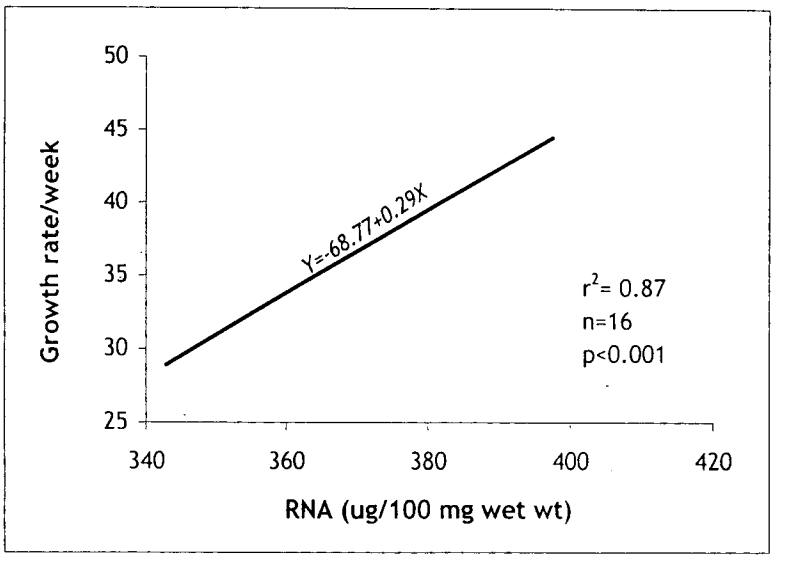


Fig. 38. Regression line for RNA concentration on shrimp growth rate (mg/week) for all replicates of all treatments at the end of ten day experiment.



DISCUSSION

It is generally believed that postlarval shrimps require a higher dietary protein level than older shrimp (Chen *et al.*, 1985; Goddard, 1996). The quality of diet required to achieve maximal growth also is size dependent (Chen *et al.*, 1985), small shrimp being more dependant than larger ones on animal protein. Studies carried out to know the nutritional requirements of shrimp postlarvae have emphasized the requirement of protein, lipid, carbohydrate, vitamins and minerals (Jones *et al.*, 1997). According to Gopal and Paulraj (1993), the optimum dietary protein level for juvenile *F. indicus* was from 35 to 37.5%. It is important that quality of protein (essential aminoacid profile and digestibility) also influence the quantity required. Higher net protein utilization and biological value have been reported with clam meal and fish meal by Ali (1992). Gopal and Paulraj (1993) also reported that clam meal and fish meal significantly improved survival and growth in *F. indicus.*

Squid meal and fish meal have been suggested as good protein sources for *Penaeus monodon* juveniles with an essential aminoacid index of 0.96 each (Penaflorida, 1989). Deshimaru *et al.* (1985) found that *P. monodon* fed with soft parts of clam *Venerupis philippinarum* attained good growth and feed efficiency. They showed the soft parts are rich in polar lipids and sterols and in essential amino acids such as methionine and arginine. A mixture of taurine and aminoacids designed to mimic clam extract showed good performance in terms of growth and feed efficiency in *P. monodon* (Hartati and Briggs, 1993).

High nutritional value of squid has been reported and is attributed to its aminoacid composition which is similar to that of shrimp (Bray *et al.*, 1990a) and because it contains high sterol levels (Wouters *et al.*, 2001a). Cruz-Richque *et al.* (1989) studied effects of squid extracts in the diet of *M. japonicus* and found that the same enhanced growth. The nutritional composition of squid, sardine and mussel has been reported to have good amount of essential fatty acids namely 20:5 n-3 (EPA), 22:6 n-3 (DHA) and 20:4 n-6 (Merican and Shim, 1994; Cahu *et al.*, 1995). Read (1981) reported that *F. indicus* has limited capacity to elongate and desaturate 18 n-3 and 18 n-6 fatty acid into 20C and 22C of n-3 and n-6 fatty acids. The same also indicated that high HUFA diet gave superior weight gain, survival and feed conversion. Influence of HUFAs on growth has been reported in other shrimp species also (Kanazawa *et al.*, 1979c; Leger *et al.*, 1985; Rees *et al.*, 1994; Kontara *et al.*, 1997; Teshima, 1998; Gonzalez-Felix *et al.*, 2002). The fresh biological feeds are also characterized as containing betaine and nucleotides which have been documented as having attractant qualities (Jones *et al.*, 1997).

In this experiment also, it could very well be suggested that the improvement in survival and growth by the addition of biological feeds was due to extra nutrient contribution. It could also be that addition of fresh feeds increased the shrimp moulting rate, as it has been reported with using clam, *Villorita cyprinoides* for *F. indicus* juveniles (Regunathan, 1992).

Results from this investigation also confirmed that the postlarvae could assimilate microalgae and attain short-term growth. Gut content analyses of juvenile and subadult *P. monodon* reared in extensive ponds without supplementary feeding (Bombeo-Tuburan *et al.* 1993) or in semi-intensive ponds with supplementary feeding (Focken *et al.* 1998) showed that diatoms and green algae were important food items. This was despite the

dominance of other natural foods whose particle sizes were larger than the algae, such as detritus, plant materials and crustacean parts. Moss (1994b) reported that juvenile *L. vannamei* fed the diatom, *Chaetoceros* sp. exhibited growth not significantly different from a commercial diet fed treatment. However, the lower growth obtained in the present experiment may be due to the length of experimental period. Long-term growth and survival of shrimp fed algae alone was not equivalent to other treatments as algae alone could not satisfy the high dietary protein requirement (Dall, 1990).

Postlarval brownshrimp, *Farfantepenaeus aztecus* could rapidly assimilate the diatom, *Skeletonema costatum*, but shrimp growth was poor when algae was the only food available (Gleason, 1986). Both the algae used here, have been reported as a good source of HUFA. Microalgae grown to late-logarithmic growth phase typically contain 30 to 40% protein, 10 to 20% lipid and 5 to 15% carbohydrate (Brown *et al.*, 1997; Renaud *et al.*, 1999) and is also a good source of minerals (Fabregas and Herrero, 1986). While, *Chaetoceros muelleri* has been reported to contain 20:5 n-3 and 22:6 n-3. *Tetraselmis suecica* has both 20:4 n-3 and 20:5 n-3 (Volkman *et al.*, 1989). The improvement in growth and survival with the MCB treatment could be due to nutritional contribution of algae. Healthy phytoplankton bloom in water is also reported to provide proper turbidity and subsequently stabilize the shrimp (Chien, 1992).

Only limited number of studies have been carried out to use RNA and RNA/DNA ratio as an indicator of nutritional status for shrimp. The RNA value obtained here for the diatom-fed shrimp was comparable to Moss (1994b) who reported a value of 339.99 µg/100 mg wet weight for shrimp fed algae *Chaetoceros* sp. for 5 days. The higher concentration of RNA with shrimp fed commercial diet and biological diet indicate higher nutrient supply and protein synthesis. Higher feeding rate and faster growth in

shrimp and fish resulted in increased RNA concentrations (Bulow, 1970; Moss, 1994 a,b; Chiu and Huang, 1999). Decline in RNA concentration with starvation has been reported widely (Buckley, 1980; Wright and Martin, 1985; Clemmesen, 1987; Mathers *et al.*, 1993, Gwak, 1999).

The relationship between RNA concentration and growth rates in fish larvae also has been experimented (Bulow, 1970; Buckley, 1984). Dagg and Littlepage (1972) found that RNA concentrations of *Artemia salina* were significantly related to growth rate measured by the rate of dry weight increase per individual. Moss (1994b) also reported that variation in growth rate could be explained up to 76% with changes in RNA concentration. In the present experiment, regression analysis affirmed that 87% change in growth could be explained by RNA values (Fig.38).

Mean DNA concentration was lowest with shrimps from MCB treatment (58.3 µg/100 mg wet weight; SE ±0.21). The diatom treatment gave the maximum DNA value of 63.4 µg/100 mg wet weight. The phenomenon of poorly-fed shrimp giving the highest DNA concentration again corroborated with Moss (1994b) who reported highest DNA concentration with starved shrimp juvenile compared to fed ones. Moss (1994a) also noted that the slow growing shrimp had higher DNA concentrations. The DNA content of starved cod (*Gadus morhua)* larvae were generally higher than that of fed larvae (Raae *et al.*, 1988). According to the author, the phenomenon of high DNA in starved larvae could be explained by the collapse of cellular control mechanisms resulting from lack of sufficient nutrition, with residual cellular energy being used for rapid unscheduled DNA synthesis.

Bulow (1970) with golden shiners (*Notemigonus crysoleucus)* suggested that within each time period, DNA concentrations increased with greater weight loss and longer periods of food deprivation. The slight decrease in DNA with increased growth and the slight increase in DNA with increased weight loss were probably due to changes in cytoplasmic volume. With food deprivation, other cellular constituents are metabolized and DNA is preserved (Leslie, 1955). Similar increase in DNA with food deprivation was also reported by Gwak (1999) with larvae and juveniles of Japanese flounder, *Paralichthys olivaceus.*

In the present results, variations in the ratio values were mainly due to the variation in RNA content, with well-fed larvae having higher concentrations of same and registered high ratio. Raae *et al.* (1988) also reported that the ratio of fed cod larvae was always higher than starved one. The RNA/DNA ratio recorded with diatom fed (5.46) and commercial diet fed postlarvae (5.95) were lower than the values reported by Moss (1994b). The author reported a value of 6.15 and 6.01 with *L. vannamei* juveniles fed for 5 days (final weight approximately 1.1 g) with commercial microencapsulated feed and microalgae (*Chaetoceros* sp.) respectively. The wider variation in ratio with diatom-fed shrimp between this experiment and Moss (1994b) could be more due to the shrimp being of different species than nutritive value of microalgae used. It could be that *F. indicus*, could not assimilate algae as much as *L. vannamei.* Postlarval white shrimp *F. setiferus* was reported to be more herbivorous than *F. aztecus* (McTigue and Zimmerman. 1991). Moreover, the better nutritional quality of the algae used in this study was confirmed by comparing with values of Nunez *et al.* (2002). The author reported values of 4.7 and 3.5 with *L. vannamei* larvae fed with *Chaetoceros* sp. G1 and A1 strains respectively. Further, the present study followed a mixed diet regime and not monoalgal diet as used by

Nunez *et al.* (2002). Monoalgal diet regimes could produce shortage of essential nutrients for the development of larvae due to varying biochemical composition (Gopalakrishnan, 1976; Yufera and Lubian, 1990).

The nucleic acid ratio in this study exhibited the same pattern as the growth rate and corroborates with studies of Moss (1994a,b). Nunez *et al.* (2002) noted that shrimp larvae with higher ratio showed better dry and organic biomass and survival. While, the ratio obtained with postlarvae fed for 10 days in the present study could account for 94% variation in growth rate, Moss (1994b) reported that more than 75% of the variation in growth rate could be explained with ratio values. A model that included both RNA/DNA ratios and water temperature accounted for as much as 92% of the variation in growth rates of eight species of larval fish (Buckley, 1984). Though significant linear relationship of RNA concentration with growth rate was established in this study, the accuracy was slightly lesser than RNA/DNA ratio versus growth rate relationship.

These results suggest that regression models used to describe the relationship between growth rate and ratio and/or RNA concentration may be useful in predicting *in situ* growth of juvenile shrimp in the field. Folkvord *et al.* (1996) noted that RNA/DNA ratio of herring larvae (*Clupea harengus*) was significantly correlated to growth rate and larval size. Wright and Martin (1985) illustrated a remarkable relationship between changes in notochord length and RNA/DNA ratio of striped bass (*Morone saxatalis*) larvae.

This study very well demonstrate the greater sensitivity of the RNA/DNA index as an indicator of the physiological condition of shrimp postlarvae as it has been reported with larvae (Nunez *et al.*, 2002) and juveniles (Moss, 1994b). Differences in ratios were

noticed in less than 24 hours after juvenile shrimp were exposed to different food sources (Moss, 1994a). RNA/DNA ratios of juvenile summer flounder (*Paralichthys dentatus*) in the laboratory changed significantly within one day of starvation and refeeding at 16°C (Malloy and Targett, 1994). In addition to the purpose of quickly assessing the quality of diet (present study), it could also be used to study other factors which affect growth, such as environmental conditions (Canino, 1994) and presence of xenobiotic compounds (Barron and Adelman, 1984). Very few studies have analysed effect of different factors on the ratio (Buckley, 1984; Ferguson and Danzmann, 1990; Canino, 1994; Imsland *et al.*, 2001).

In summary, with the analysis of growth rate and RNA/DNA ratio estimation it could be confirmed that biological feeds boosted the growth rate of shrimp. So, in the regular feeding regime of nurseries the fresh feed mix could be used as supplementary feed coupled with required water exchange as left over feed would affect the water quality. Nucleic acid analysis as in the present study could be used to estimate growth rates of juvenile shrimp.