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Optimum level of fish liver oil as enrichment for *Artemia* fed to the tiger tail seahorse *Hippocampus comes* for reproduction and juvenile survival

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Abstract. Reproductive performance of broodstocks and juvenile stress resistance of seahorse *Hippocampus comes* fed *Artemia* enriched with varying fish liver oil (FLO) were evaluated. *Artemia* was enriched in seven media with 0%, 1%, 2%, 3%, 4%, 5% and 10% FLO supplemented with egg yolk, baker's yeast and vitamins 12 h before feeding to seahorse broodstocks. In general, response parameters increased at lower concentration of FLO, peaked at 5% and decreased at 10%; thus, for parabolic relationships, the quadratic regression was appropriate to estimate quantitative optimum level. *Artemia* enrichment with FLO increased considerably its saturated FA content in the form of myristic acid and the total HUFA content, particularly DHA and arachidonic acid. Feeding seahorse broodstock with FLO enriched *Artemia* resulted in: (1) significant increase in brood size both during the first and second brooding, (2) significant shortening of interbrooding period, and (3) significant extension of the time to total mortality during the salinity shock test of juveniles. Quantitative estimates of mean optimum FLO level for the enrichment of *Artemia* fed to *H. comes* was 6.3% (range 5.9–7.0%) using each response parameter fitted in a quadratic model.
Key Words: enrichment, *Artemia*, *Hippocampus comes*, fish liver oil, HUFA.

Introduction. The tiger tail seahorse (*Hippocampus comes* Cantor, 1850) is a high economic value fish species with more than 169 countries engaged in its culture and trade. In the wild, seahorses live in marine habitats, including sea grass beds, coral reefs, mangroves, and estuaries (Lourie et al 1999). In recent years, the quality of the environment for wild seahorses has degraded resulting in the decline of their population. Another cause for the observed decline is overfishing; the global trade in seahorses was estimated to consume at least 20 million animals annually (Vincent 1996). Seahorses are also exploited for use in traditional medicine and curios.

Seahorse fishing occurs in tropical areas such as Vietnam, Thailand, and the Philippines which are the main countries exporting seahorses in the international market together with India and China. The supply of seahorses to the international marine aquarium trade has been affected by the listing of all seahorse species on the Convention on the International Trade in Endangered Species (CITES Appendix II, http://www.cites.org, AC19 Doc. 16.1, accessed 22/06/12). At present, the species on this list are not threatened that might lead to extinction but might become so unless trade is strictly controlled and monitored.

In recent years, many countries have undertaken projects to protect and conserve seahorses. Seahorse aquaculture has a great potential in integrating both conservation and sustainable development goals as an alternative livelihood for fishers (Job et al 2002). However, there is a dearth of information regarding the suitable culture and nutrition of most tropical Indo–Pacific species (Truong 1998). Information on techniques

of reproduction, nutrition and culture are essential to the development of seahorse aquaculture that is aimed to reduce pressure on the wild population.

H. comes is a seahorse species found in the Indo-Pacific that is of high economic value as medicinal products. Like all seahorse species, the female seahorse *H. comes* deposits her eggs into the male's brood pouch, where they are fertilized (Perante et al 2002). The male then protects the young in the pouch, provides oxygen through a capillary network, osmoregulates the developing embryos and transfers nutrients to his offspring.

To maintain good quality broodstock for hatchery operations, appropriate nutritional requirements of the cultured species should be supplied. In the wild, seahorses feed on zooplankton such as amphipods, rotifers, copepods and other small creatures such as fish and shrimp larvae (Perante et al 2002). In captive conditions, seahorses are mostly fed with copepods, *Artemia, Acetes* and *Mysid* but limited studies have been conducted regarding their relative contribution to seahorse nutrition and reproductive performance. These live food organisms may not necessarily contain all the essential nutrients required by seahorses but may serve as organisms for bioencapsulation. This is the process of incorporating nutrients or medicines into living organism that can then be fed to the target larval fish.

Fish liver oil contains the most important fatty acids for marine fish (Immanuel et al 2004). Information is scarce on the nutrition of *H. comes* broodstock and to our knowledge, no study has been previously conducted on bioencapsulating fish liver oil in *Artemia* fed to *H. comes* to improve its reproductive performance. The study aimed to determine the effect of fish liver oil to enrich *Artemia* at different concentrations on the reproductive performance and salinity stress tolerance of the newborns of *H. comes*.

Material and Method

Fish liver oil bioencapsulation in Artemia. The emulsion for bioencapsulation were prepared following the method of Immanuel et al (2007). Six different emulsions were prepared containing 0%, 1%, 2%, 3%, 4%, 5% and 10% fish liver oil supplemented with hard-boiled egg yolk (1.0 g), baker's yeast (0.2 g), water-soluble (10 g) and fat-soluble vitamins (2 g) in 100 ml seawater. Baker's yeast was added to prevent starvation of *Artemia* in the control (0%) which is known to reduce their lipid content (Immanuel et al 2004; Immanuel et al 2007). The mix was homogenized for 5 min, the stability of the emulsion checked and stored at 4^{o}C until use.

Into each prepared emulsion was added the adult *Artemia* (15-20 days old, mean size 8.1 mm) at a density of 200 *Artemia* L^{-1}. Aeration was provided to the containers to keep the oxygen level at 5 ppm. The period of bioencapsulation was 12 h (i.e. a previously determined period in which *Artemia*'s gut was observed microscopically as being already full) under ambient temperature of 28±1^{o}C. After 12 h, *Artemia* were harvested and fed to seahorse broodstock.

Fatty acid analysis. Random samples of 5 mg *Artemia* from each treatment were used for the fatty acid analysis. The data were expressed as fatty acid methyl esters (FAME).

The preparation of FAME was carried out using an acetylchloride/methanol mixture (1:20, v/v) as the esterification reagent (Lepage & Roy 1984). The fatty acid compositions were determined using a Chrompack CP9001 gas chromatograph (Macclessfield, UK) equipped with a temperature-programmable on-column injector. Injections were performed into a polar 50 m capillary column, BPX70 [SGE Analytical Science (Ringwood, Vic., Australia), 50 m x 0.32 mm ID, 0.25 µm film thickness], with a hydrogen flame ionization detector and helium as a carrier. Individual FAME was identified in comparison with authentic standard reference mixtures (Nu-Chek-Prep.). Integration and calculations were performed on a computer with a software program MAESTRO (Chrompack).

Reproductive performance. A total of 42 seahorse (*H. comes*) broodstocks (21 males and 21 females, 1.5 years old and 12 cm standard length) were divided into eighteen 16

L tanks at one pair per replicate tank. Seawater used was sand-filtered and passed through biofiltration as well as ultraviolet radiation. Water parameters such as temperature, salinity, dissolved oxygen, pH and ammonia were monitored in all the tanks.

During the experiment, seahorse broodstocks were fed enriched adult *Artemia* twice a day (08:00 h and 16:00 h) and the daily number of *Artemia* fed was maintained at 100 individuals per seahorse. Aeration was temporarily suspended during feeding to facilitate prey capture by the broodstock. Faeces and uneaten prey organisms (prey mortality due to unsuccessful prey attacks) were siphoned out once a day at 07:30 and 80% of the water was replaced.

After the seahorse gave birth, the seahorse parents were transferred to another tank. The reproductive performance of seahorse such as interbrooding period (i.e. number of days between the first and second brooding), brood size (i.e. the number of newborn seahorses) and length and body weight of the newborns were assessed. Standard length (SL) was determined as follows (Lourie et al 1999):

SL = head length (HL) + trunk length (TrL) + tail length (TaL)

Salinity shock as test for resistance of newborn seahorses. The quality of the newborn seahorse in terms of stress resistance against abrupt salinity change was assessed by subjecting them to salinity shock. Twenty progenies from each treatment were taken at random and abruptly immersed into two liters of 0 ppt salinity water. The time until total mortality was recorded for 3 replicates of each treatment.

Estimation of requirements and statistical analysis. Data were analyzed by fitting quadratic regression equation used in fish to estimate protein and amino acids (Chiu et al 1988; Zeitoun et al 1976). This model was deemed appropriate for the treatment of almost a hyperbolic data in which the response parameters reached a peak and declined at the highest level of the independent variable (i.e. concentration of FLO). In this method, a quadratic equation is used to fit the response data obtained from feeding a dietary series:

$$R = a + bI + cI^2$$

where R is the measured response; I is the dietary nutrient concentration; and a, b, and c are constants that are calculated to provide the best fit of the data. The value of I that produces the maximum response I_{max} is calculated as follows:

$$I_{max} = -0.5\ (b/c)$$

Standard error of the mean (SEM) were calculated for all mean values. Data were subjected to analysis of variance (ANOVA) and Tukey-Kramer Range Test to determine differences in means ($p<0.05$). All statistical analyses were done with the Statistical Package for the Social Sciences (SPSS) Version 17 software (Chicago, Illinois, USA).

Results

Water quality. Physico-chemical parameters of the seawater in the culture tanks such as water temperature (29.1±0.1°C), pH (8.2±0.0), salinity (30.4±0.0 ppt), dissolved oxygen (5.9±0.1 ppm) and ammonia (0.08±0.01 ppm) were all optimal for the rearing of *H. comes* and did not depart considerably from the means.

Fatty acid analysis. The fatty acid composition of the enriched *Artemia* with various levels of fish liver oil (FLO) is shown in Table 2. In general, fatty acids increased in content relative to the negative control enrichment (0% FLO) with the exception of the linoleic acid (C18:2n-6) and eicosapentaenoic acid (C20:5n-3) which showed a slight decrease. The highest increase of 258% was in arachidonic acid (20:4n-6) followed by 56 and 45% increase in total HUFA and myristic acid, respectively. Only arachidonic acid

(ARA) and total HUFA content of *Artemia* showed a remarkable relationship with the level of FLO i.e. increased with increasing proportion of FLO in its enrichment (R^2 = 0.86 and 0.96, respectively, Table 1). Thus, optimum levels of FLO for enrichment were estimated using the quadratic model for these two response parameters (Figures 1 and 2). The estimated amount of 6.0% (R^2 = 0.96, p = 0.002) and 7.0% FLO (R^2 = 0.86, p = 0.019) resulted in the maximum estimated arachidonic acid and total HUFA responses, respectively. Quadratic regressions were also done for both total HUFA and ARA contents of *Artemia* with the other response parameters (Table 2). Most of the response parameters exhibited significantly high R^2 values (range of 0.79 to 0.94) except that between total HUFA and interbrooding period with a R^2 value of 0.58 and was not significant (p = 0.170) (Table 2). This indicated that non-linear relationships between response parameters and ARA or total HUFA were strong and significant.

Table 1

Fatty acid composition (%DM) of enriched *Artemia* with various concentrations (0–15%) of fish liver oil (FLO), R^2 of the linear correlation analysis and average percent increase over the unenriched *Artemia* (0% FLO)

Fatty Acid	*Symbol*	*Fish liver oil (%)*								*R^2*	*Ave.% inc.**
		FLO	*0*	*1*	*2*	*3*	*4*	*5*	*10*		
Myristic	C14:0	3.02	2.18	3.29	3.64	2.29	3.52	3.12	3.14	0.20	45.3
Palmitic	C16:0	13.38	14.63	15.45	15.80	14.58	14.93	15.59	14.93	0.08	4.0
Palmitoleic	C16:1n7	7.51	6.12	7.07	6.40	6.20	5.89	5.52	5.89	0.41	0.7
Stearic	C18:0	7.42	7.00	6.64	7.12	7.33	7.31	7.07	7.81	0.7	3.1
Oleic	C18:1n9	24.73	25.22	26.85	26.33	27.05	25.72	23.22	25.72	0.11	2.4
Vaccenic	C18:1n7	7.95	8.43	9.06	9.14	9.42	8.10	8.33	8.10	0.30	3.1
Linoleic	C18:2n6	18.21	17.60	17.15	17.28	17.13	16.26	14.25	16.26	0.57	-6.9
Linolenic	C18:3n6	5.12	4.21	5.73	5.27	5.55	4.56	4.45	5.17	0.0	21.7
Linolenic	C18:3n3	0.61	1.63	1.82	1.72	1.86	1.83	1.48	1.60	0.18	5.4
Arachidonic	C20:4n6	2.26	0.86	2.34	2.62	3.48	3.52	3.81	2.68	0.96	257.6
EPA	C20:5n3	5.85	4.56	4.59	4.29	5.11	4.14	4.03	5.12	0.35	-0.3
DPA	C22:5n3	1.72	Nd	Nd	Nd	Nd	0.22	1.05	0.43	-	-
DHA	C22:6n3	2.41	Nd	Nd	Nd	Nd	0.51	1.88	0.84	-	-
ΣHUFA		12.24	5.42	6.93	6.91	8.59	8.39	10.77	9.07	0.86	55.8
ΣPUFA		36.18	28.86	31.63	31.18	33.13	31.04	30.95	32.1	0.32	9.7
DHA:EPA		0.41	-	-	-	-	0.12	0.47	0.16		

Nd: Not detected; Fatty acid content is expressed as percent fatty acid methy esters (FAME) and % DM;
*Ave. % inc. = [(value at 0% FLO – ave. increase at 1 to 10% FLO)/value at 0% FLO].

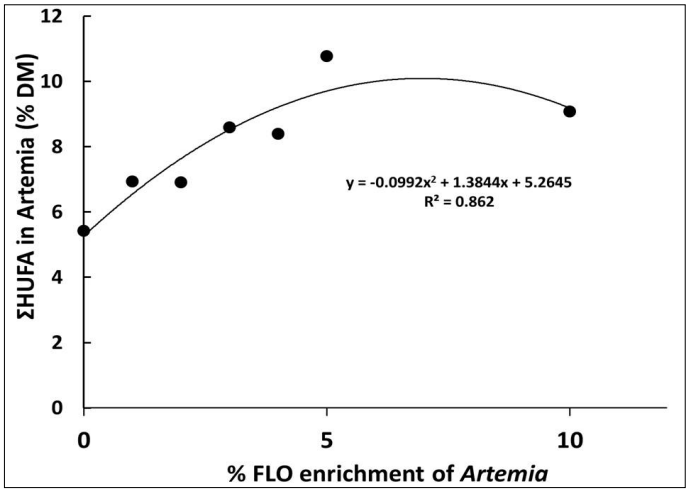

Figure 1. Total HUFA in FLO-enriched Artemia fitted in a quadratic model.

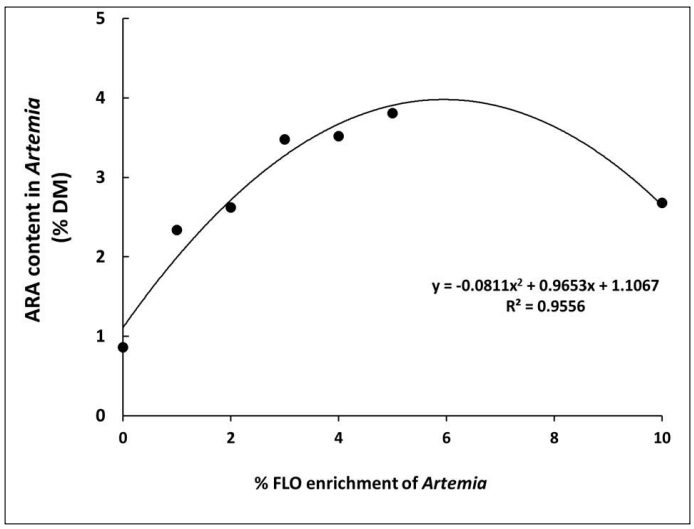

Figure 2. Arachidonic acid levels in *Artemia* enriched at different FLO fitted in a quadratic model.

Table 2

Quadratic or linear regression of the various response parameters with total HUFA or arachidonic acid as independent variable and the corresponding R^2 values of the equation

I	*R*	Regression equation ($R = a + bI + cI^2$)	R^2	*P*
Total HUFA	Time until total death	$R = 21.42 + 0.094I + 0.14I^2$	0.91	0.008
	Interbrooding period	$R = 27.43 - 1.53I + 0.04I^2$	0.59	0.170
	Brood size (1st)	$R = 87.36 - 5.07I + 1.17I^2$	0.88	0.015
	Brood size (2nd)	$R = -0.28 + 16.36I + 0.48I^2$	0.84	0.026
ARA	Time until total death	$R = 27.26 - 2.55I + 1.36I^2$	0.90	0.011
	Interbrooding period	$R = 19.22 + 1.67I - 0.69I^2$	0.79	0.044
	Brood size (1st)	$R = 111.34 - 27.60I + 10.49I^2$	0.94	0.004
	Brood size (2nd)	$R = 129.57 - 37.24I + 16.00I^2$	0.84	0.024

I = dietary nutrient conc.; R = measured response; a, b and c are constants that are calculated to provide the best fit of the data.

Reproductive performance. Final mean length and weight of male broodstock seahorses were 12.3±0.2 cm (range = 12.0-12.6 cm) and 8.5±0.1 g (range = 8.40-8.59 g), respectively. Final mean female length and weight were 10.6±0.2 cm (range = 10.4-10.78 cm) and 6.2±0.1 g (range = 6.1-6.4), respectively. Both final length and weight of either male or female were not affected by the dietary treatments ($p>0.05$, data not shown). Two-tailed Student t-test showed that the male were significantly longer and heavier than the female broodstocks ($p<0.05$).

Brood size (i.e. the number of newborn seahorses) as a function of % FLO enrichment of *Artemia* is shown in Figure 3. Broodstocks gave birth two times in all the treatments within the period of the experiment (from June to September 2009). Brood size increased with increasing levels of FLO during the first and second brooding (R^2 = 0.93 and 0.89, respectively); brood size varied significantly with %FLO in both first and second brooding. Student t-test (not shown) revealed that the number of newborn seahorse released (i.e. brood size) in the second brooding was significantly more than that in the first brooding ($p<0.05$, data not shown)

Interbrooding period (i.e. number of days between the first and second brooding which ranged from 16 to 21 days), in contrast, was negatively correlated with *Artemia* enrichment with FLO (R^2 = 0.94, Figure 4) and varied significantly from each other. The optimum FLO estimated for the shortest interbrooding period was 6.1% using the quadratic model (Table 3).

Mean size of newborn seahorses was uniform at 1.1±0.0 cm and mean weight was 46.5±2.1 mg (range = 45.0–50.0 mg); both length and weight of the newborns were not affected by the dietary enrichment treatment ($p>0.05$, data not shown).

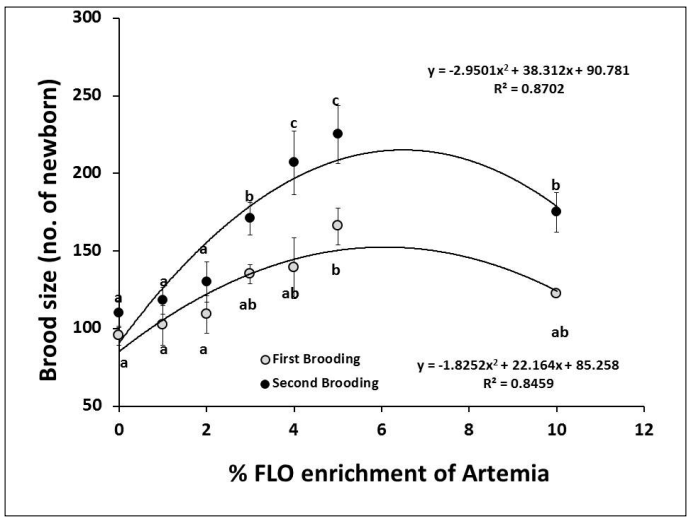

Figure 3. Brood size (i.e. the number of newborn seahorses) during the first and second brooding at various FLO enrichment.

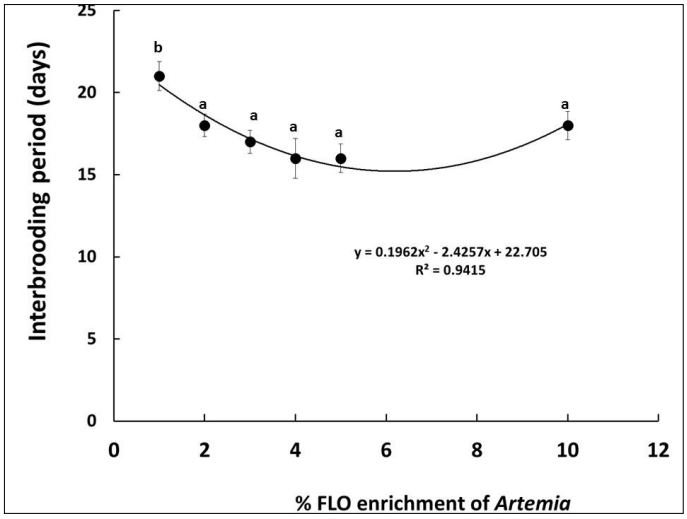

Figure 4. Interbrooding period (i.e. the period between the first and second brooding) of *H. comes* broodstocks at various FLO enrichment.

Salinity shock as test of resistance of newborn seahorses. All salinity-challenged newborn seahorses died within 26 to 39 min. Newborn seahorses fed enriched *Artemia* with 5% FLO survived significantly the longest at 39 min (Figure 5) but was not significantly different from those fed *Artemia* enriched with 3, 4 and 10% FLO. The estimated level of FLO resulting in the longest time to total mortality was 6.2% - Table 3). We also ran the test on a 30 ppt which is the optimum salinity as a positive control and no newborn seahorse died.

Table 3

Estimation of optimum FLO enrichment of *Artemia* fed to seahorse broodstock using the different response parameters in the quadratic model

Response parameter (I)	*Quadratic equation* $(R = a + bI + cI^2)$*	R^2	*P*	I_{max}
Time until total death	$R = 24.81 + 3.86I - 0.31I^2$	0.89	0.013	6.2
Interbrooding period	$R = 22.98 – 1.70I + 0.14I^2$	0.94	0.028	6.1
Brood size (1st)	$R = 85.26 + 22.16I - 1.83I^2$	0.85	0.024	6.0
Brood size (2nd)	$R = 90.78 + 38.31I - 2.95I^2$	0.87	0.017	6.5

R = measured response; I = dietary nutrient conc.; a, b and c are constants that are calculated to provide the best fit of the data; **I_{max} = -0.5(b/c) = the value of I that produces the maximum response.

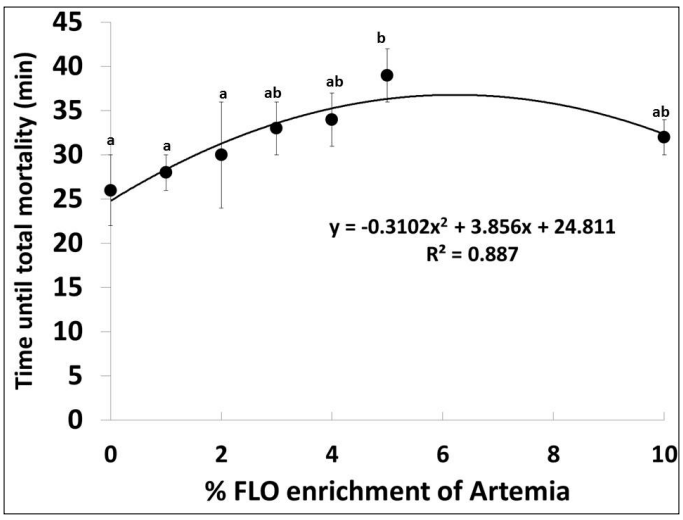

Figure 5. Effect of the level of FLO in the enrichment of *Artemia* on the length of time (min) until total death in newborn seahorses during the salinity shock challenge test.

Discussion. It is common knowledge that in aquatic animals, the nutritional status of broodstocks somehow dictates upon the performance of the newborn juveniles and this generalization could also extend to seahorses. In several genera of the family Syngnathidae (pipefishes, seadragons and seahorses) embryos develop in a brood pouch to protect, aerate, osmotically buffer and nourish the embryos (Azzarello 1991; Quast & Howe 1980; Ripley & Foran 2009). Thus, the nourishment coming from the male parent could impact on the quality of the juvenile to be released as an independent seahorse.

In fish, lipids in broodstock nutrition are one of the most important factors influencing egg quality and fecundity (Rainuzzo et al 1997). The reserves of lipid in the fish egg serve both as substrate for energy metabolism and structural components in membrane (Sargent 1995). Deficiency of HUFAs in the broodstock diets negatively affects fecundity, fertilization rate and hatching rate (Rainuzzo et al 1997). These observations could also be assumed for seahorses. *Artemia* as live food to seahorse broodstock seems to be a part of the practical technique of seahorse culture although as it is, it is deficient nutritionally (Watanabe et al 1983; Webster & Lovell 1990). Indeed, it had no detectable DPA and DHA in its unenriched form as was shown in the present study (Table 1). Similarly shown here, its nutritional value can be manipulated through their diet (Furuita et al 1999; Han et al 2000). It can non-selectively ingest particles that enable them to become bioencapsulaptors (Lim et al 2003; Sorgeloos et al 2001).

Results of enrichment of live feeds like *Artemia* are subject to changes that depend partially on the duration of exposure to the enrichment medium (Tamaru et al 2003). Detectable increases in HUFA content are detectable in *A. franciscana* after exposure from 12 to 24 h (Narciso et al 1999; Tamaru et al 2003). In the present study, exposure of 12 h to the FLO enrichment medium, *Artemia* nutritional value increased considerably in terms of its fatty acid profile. The observation in the present study that the greatest increase in fatty acids was in the total HUFA and the specific HUFA arachidonic acid only partially agreed with the data obtained by Immanuel et al (2007) who have reported that the greatest elevation is in DHA and EPA.

Differences in the number of seahorse juveniles produced may be a function of several factors. One possibility was that the female *H. comes* might have produced fewer eggs to transfer to males and the reason could be partially due to nutrition (Kruger et al 2001; Takasuka et al 2005). Li et al (2005) observe that either deficient or excess dietary n-3 HUFA has a negative effect on egg and subsequent larval quality in the marine teleost *Plectrohyncus cinctus* (Temminck & Schlegel, 1843).

Fecundity is size-dependent in syngnathids (Teixeira & Musick 2001; Vincent & Giles 2003) but male dimensions in seahorses such as the volume of the sealed brood pouch can also be used to predict brood size (Boisseau 1967). The length and weight of males and females were all uniform in the present study, thus, differences in brood size

could not be attributed to these factors, assuming that the volume of pouch could be predicted by the size of the male parent. Diet could be the considerable source of the differences in brood size. Quantitative estimate of brood size peaks at 6.1 and 6.5% FLO in enriched *Artemia* suggesting that the dietary HUFAs could have played an important role in the production of maximum number of seahorse juveniles released. However, it is not understood clearly in the present study the reason why the second brooding produced significantly larger brood size than that in the first brooding.

Brood production by male seahorse was probably limited by the availability of eggs from the female seahorse because of the one-to-one ratio of the pairs per tank in the present study. However, the interclutch intervals (the period in which the female seahorse transfer its egg to the male) are equal to the brooding periods of males in *H. guttatus* (Curtis & Vincent 2006); thus the interbrooding period in the present study could be independent from interclutch intervals of the female seahorse. Among other factors that affect interbrooding period, energetic constraints could be a possibility as is observed in northern anchovy populations (Hunter & Leong 1981). In the present study, the increased level of myristic acid (C14:0) following enrichment might have provided the seahorse broodstock a very good substrate for energy expenditure. The quantitative estimate of 6.1% FLO enrichment of *Artemia* fed to seahorse elicited the shortest interbrooding period in the present study, an estimate very close to other estimates by other response parameters also in the present study.

Seahorses produce well-developed independent juveniles at birth and this could be the reason why they are not prone to high mortality in the wild (Woods 2007). Salinity has been identified as a factor that affects juvenile seahorse survival at least in *Hippocampus kuda* Bleeker, 1852 (Hilomen-Garcia et al 2003). In the present study, we employed salinity shock as a means to swiftly determine the quality of the juveniles (i.e. resistance to sudden salinity changes) that could have stemmed from the dietary treatments. The optimum level of FLO estimated with the quadratic model using the time of total mortality as the response parameter was 6.2% and this value was within the vicinity of estimates using the total HUFA and arachidonic acid (5.9 and 7.0% FLO) as response parameters. This indicated that the total HUFA and arachidonic acid content of the FLO bioencapsulated in *Artemia* could have significantly resulted in the longest protracted time of survival of juveniles whose parents were fed with 5% FLO bioencapsulated in *Artemia*.

Conclusions. In conclusion, reproductive performance of *H. comes* broodstocks and survival of their offspring could be enhanced by bioencapsulating fish liver oil in *Artemia* for 12 h before feeding. *Artemia* enrichment with FLO increased considerably its saturated FA content in the form of myristic acid and its total HUFA content, particularly DHA and arachidonic acid. Feeding seahorse broodstocks with FLO enriched *Artemia* resulted in: (1) significant increase in brood size both during the first and second brooding, (2) significant shortening of interbrooding period, and (3) significant extension of the time to total mortality during the salinity shock test of juveniles. Quantitative estimates of mean optimum FLO level for the enrichment of *Artemia* fed to *H. comes* was 6.3% (range 6.0–7.0%) using each response parameter fitted in a quadratic model.

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