รายงานวิจัยฉบับสมบูรณ์

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การเพิ่มสารอาหารแอสทาแซนธินและ highly unsaturated fatty acid เพื่อปรับปรุงการเจริญพันธุ์ในกุ้งกุลาดำ (*Penaeus monodon* Fabricius) จากบ่อเลี้ยง

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สัญญาเลขที่ BGJ/18/2543

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2. นายซลี ไพบูลย์กิจกุล	ภาควิชาวิทยาศาสตร์ทางทะเล คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

สารบัญ

Nutritional enrichment of astaxanthin and highly unsaturated fatty acids for maturation improvement of pond-reared broodstock black tiger shrimp (*Penaeus monodon* Fabricius)

Introduction

In Thailand, high quality shrimp larvae have been mostly produced by wild-caught broodstocks. Although the wild-caught broodstocks produce high quality shrimp larvae, they may be carriers of viral diseases (Flegel and Alday-Sanz, 1998; Otta et al., 1999) and cause mass mortality of shrimp after 1 or 2 months of rearing in the earthen pond. The amounts of matured wild female rapidly decrease on time to time. Then, the substitution of wild-caught broodstocks with pond-reared broodstocks will be sustainable developed. However, pond-reared broodstocks are not favorite use for broodstock because they have lower maturation performance than wild-caught broodstocks. One of the main causes of low maturation performance is due to less nutritional information. Therefore, the study of pond-reared broodstocks nutrition to improve maturation and larvae production is necessary.

Astaxanthin and lipid are important nutrient to promote reproductive performance in shrimp (Middleditch et al., 1979; Millamena, 1989; Bray et al., 1990; Menasveta et al., 1994a; Pangantihon-Kuhlmann et al., 1998). The proposed functions for astaxanthin in aquaculture have been those of provitamin A activity, antioxidant properties, positive effect on embryonic and larval development, cellular protection from photodynamic damage, enhancement of growth, maturation and formation of in-chain epoxides that act as oxygen reserves under anoxic condition (Torrissen, 1990). Astaxanthin has been reported as the most frequent end product of carotenoid metabolism in crustaceans (Katayama et al., 1972a) and identified as the main

pigment in the adult prawn, *Penaeus japonicus* (Katayama et al., 1971; 1972b). A variety of factors affects amounts and distribution of carotenoids in crustaceans, including embryogenesis, reproductive cycle, molting, background colors, and hormonal control (Goodwin, 1960).

Lipid plays major roles in reproductive processes of crustaceans. Levels and composition of dietary lipids profoundly affect ovarian maturation and reproductive success (Harrison, 1990). Middleditch et al. (1979; 1980) investigated that penaeid shrimp needs polyunsaturated fatty acid (PUFA) in order to develop shrimp gonad tissue. The purpose of this study is to improve reproductive performance of pond-reared black tiger shrimp broodstock *Penaeus monodon* by dietary fish oil and astaxanthin.

Materials and methods

Experimental design

The experiment was operated 120 days. The experimental design was 2×2×2 factorials in completely randomized design. Two levels of fish oil 3 and 8%, 2 concentrations of astaxanthin 100 and 500 mg kg^{-1} and 2 sexes (male and female) were used. The composition of the tested diet was described in **Table 1**. Eight replication was done in each treatment.

Diet and dietary preparation

The experimental diets contained 48% crude protein. The four experimental diets were: LFLA, supplemented with 3% fish oil and 100 mg kg^{-1} astaxanthin; LFHA, supplemented with 3% fish oil and 500 mg kg^{-1} astaxanthin; HFLA, supplemented with 8% fish oil and 100 mg kg^{-1} astaxanthin; and HFHA, supplemented with 8% fish oil and 500 mg kg^{-1} astaxanthin. Dietary ingredients were grounded into powder and mixed by a twin blade rolling mixer for 30 min. Astaxanthin were added to contain levels according to formula. Astaxanthin source was a synthetic one and bought from Hoffman-La Roche, Switzerland. Vitamin A, C and E were added in experimental diets at levels of 20 000 IU kg^{-1}, 200 and 100 mg kg^{-1}, respectively. The homogenized mixture of each diet's mesh was pelletized by a pelleting machine (CPM, California Pelleting Machine), streamed for 5 min and then dried by hot air oven at 60 °C for 2 hr. The pelleted dietary size was 3 mm of diameter and 5 mm of length. The pelleted diets were kept in dark container and flushed with nitrogen gas before storage at -20 °C.

Table 1. Feed ingredients of broodstock shrimp diets.

Ingredients	Dry weight (g 100 g^{-1} of diet)			
	LFLA	LFHA	HFLA	HFHA
Fish meal	56	56	56	56
Shrimp head meal	10	10	10	10
Wheat flour	16	16	16	16
Refined tuna fish oil	3	3	8	8
Chlorophyll pink[a]	0.125	0.625	0.125	0.625
Cellulose	9.758	9.258	4.758	4.258
Mineral mixture[b]	1	1	1	1
Vitamin mixture[c]	1	1	1	1
Cholesterol[d]	1	1	1	1
Lecithin[e]	1	1	1	1
Binder[f]	1	1	1	1
Vitamin A[g]	0.04	0.04	0.04	0.04
Vitamin C[h]	0.057	0.057	0.057	0.057
Vitamin E[i]	0.02	0.02	0.02	0.02
Total	100	100	100	100

[a]Chlorophyll pink contains 8% active form of astaxanthin, Roche.

[b]Mineral mixture 100 g contains: K_2HPO_4 2.0 g, $Ca_3(PO_4)_2$ 2.720 g, $MgSO_4$ $7H_2O$ 3.041 g, NaH_2PO_4 $2H_2O$ 0.790 g.

[c]Vitamin mixture 10 g contains: ρ-aminobenzoic acid 10.0 mg; biotin 0.40 mg, inositol 400.0 mg; nicotinic acid, 40.0 mg; Ca-pantothenate, 60.0 mg; pyridoxine-HCl, 12.0 mg; riboflavin, 8.0 mg; thiamin-HCl, 4.0 mg; menadione, 4.0 mg; cyanocobalamine, 0.08 mg; calciferol, 1.20 mg; folic acid, 0.80 mg; choline chloride, 120.0 mg.

[d]Ninety five percent cholesterol, laboratory grade, Sigma.

[e]Soy lecithin, feed grade.

[f]Aquabind, Du Pont.

[g]Five hundred thousand IU g^{-1}, feed grade, Roche.

[h]Stay C 35%, Roche.

[i]Fifty percent, feed grade, Roche.

Experimental animals

Shrimp used in the experiment were subadult *P. monodon* at 48-50 g body weight in female and 35-38 g body weight in male. All of the shrimp were collected from a commercial earthen pond at the age of 6 months. The broodstocks were acclimated under experimental condition (temperature 28 ± 1 °C and salinity 35 ppt) for at least 15 days before beginning of the experiment. Shrimp were doubly tagged, a plastic numbering glued at the carapace and a rubber tube with the same number around the eyestalk for biological data monitoring on an individual basis such as molting, and/or ovary maturation. At initial, all broodstock shrimp were randomly selected into each experiment unit.

Experimental pond and reared condition

The rearing system in this experiment was an indoor closed recirculating water system covered with shading to reduce light intensity of about 100%. The system was a circular shape, consisted a rearing tank with 30 tons of water and a biological treatment unit of 8 tons of water at the center. Water depth was maintained at 1.0 m. The detail of the experimental pond has been described by Menasveta (1982). Thirty two rectangular net cages with 0.36 m^2 of rearing area each were established in the rearing unit. At initial, 2 broodstock shrimp, 1 female and 1 male were stocked in each cage.

Feeding strategy

The experimental feeding regime, pelleted diet and fresh diet were fed 3 times a day at 0600, 1200 and 1800. The fresh diet was chopped squid (*Loligo sp.*) given 10% of shrimp body weight at 0600. The pelleted diets fed at 1200 and 1800 for about

2% of shrimp weight a day. Uneaten diets, fecal matters and particular detritus were removed before the first feed.

Data collection

The newly molted female broodstocks were induced to spawn by unilateral eyestalk ablation. In female shrimp, ovarian development was observed by flashing a light through dorsal part of the abdomen every 2 days (Motoh, 1981).

The gravid broodstock in ripe stage was sacrificed for detecting number of eggs and tissues sampling of muscle, hepatopancreas, ovary and shell for analyzing astaxanthin contents and fatty acids concentration. In male, spermatophores were obtained by electrical stimulation at 2-4 volts, and 0.3-0.5 amperes using a method similar to that described by Sandifer et al. (1984). Amount of spermatozoa was determined at the end of experiment in term of total sperm count followed by Leung-Trujillo and Lawrence (1987). The broodstocks growth rates were measured by weighting every 30 day. Survival of shrimp was not determined because dead shrimp were replaced with the new same sex shrimp in the first two months of experiment. At the end of experiment, the remainder shrimp were sacrificed and then muscle, hepatopancreas, ovary and shell were collected for astaxanthin and fatty acid analysis using high performance liquid chromatography and gas chromatography as method described by Weber (1988) and Christie (1989), respectively.

Proximate analysis of the experimental diets

The experimental diets were analyzed for crude protein, lipid, ash, fiber and moisture with methods as described by AOAC (1995).

Water quality

Ammonia, nitrite and nitrate (NH_3-N, NO_2-N and NO_3-N) in water were determined weekly by using test kits (Sera, Germany). Temperature, dissolved oxygen, alkalinity, and pH were monitored every day during the experiment period.

Statistical analysis

Effects of astaxanthin, fish oil and sex on weight gain, amount of eggs and spermatozoa, astaxanthin contents and fatty acid concentration were analyzed using Analysis of Variance and Duncan's New Multiple Range Test (Cody and Smith, 1997).

Results

Water quality at the experiment was maintains as described in **Table 2**. All water quality parameters were in the range that was suitable for aquatic animals. The proximate analysis of experimental diets and astaxanthin content are show in **Table 3**. Average protein content in LFLA, LFHA, HFLA and HFHA diets was 48.64 ± 1.71, 48.96 ± 0.28, 47.52 ± 0.20 and 48.83 ± 0.83%, respectively. Astaxanthin concentrations in LFLA, LFHA, HFLA and HFHA diets were 45.58 ± 1.71, 296.41 ± 2.86, 46.16 ± 1.30 and 264.55 ± 6.00 mg kg^{-1}, respectively. The fatty acid contents in diets and fresh diet, squid was showed in **Table 4**.

Table 2. Water quality of the broodstock shrimp experiment.

Parameters	Range
Salinity (ppt)	35 ± 1
Temperature (°C)	28 ± 1
Dissolved oxygen (mg l^{-1})	6.5-7.7
Alkalinity (mg l^{-1})	148-220
pH	7.5-8.5
Ammonia (mg l^{-1})	0-0.5
Nitrite (mg l^{-1})	0-0.3
Nitrate (mg l^{-1})	0-20

The effect of dietary fish oil, astaxanthin and sex on average weight gain of shrimp is shown in **Figure 1**. There was no significant ($P>0.05$) interaction between fish oil and astaxanthin, and fish oil and sex on average weight gain but astaxanthin and sex had significant ($P<0.05$) interaction. The effect of fish oil on average weight gain was discussed separately, while the effect of astaxanthin and sex on average

weight gain was reported together. Shrimp fed diet containing fish oil 3 and 8% had not significantly different (P>0.05) on average weight gain after 4 months of experiment. In shrimp fed diet supplemented with astaxanthin 100 mg kg^{-1}, female shrimp had significantly higher average weight gain (P<0.05) than those of male. In shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1}, no significant difference (P>0.05) of sex was found on average weight gain. In female, there was not significant difference (P>0.05) among levels of astaxanthin on average weight gain, while male shrimp fed supplemented with astaxanthin 500 mg kg^{-1} had significantly greater average weight gain (P<0.05) than those of obtained astaxanthin 100 mg kg^{-1}.

Table 3. Proximate analysis of experimental broodstock diets as fed basis (means ± s.d.).

Proximate	Diets			
	LFLA	LFHA	HFLA	HFHA
Protein (%)	48.64 ± 1.71	48.96 ± 0.28	47.52 ± 0.20	48.83 ± 0.83
Lipid (%)	7.70 ± 0.03	7.78 ± 0.16	11.51 ± 0.13	12.04 ± 0.30
Moisture (%)	13.56 ± 0.11	12.18 ± 0.08	12.95 ± 0.16	12.87 ± 0.23
Ash (%)	12.51 ± 0.10	12.52 ± 0.37	12.20 ± 0.51	12.40 ± 0.06
Fiber (%)	2.83 ± 0.04	2.92 ± 0.31	2.96 ± 0.17	2.61 ± 0.11
NFE[a] (%)	14.76 ± 1.85	15.64 ± 0.16	12.86 ± 0.85	11.25 ± 0.82
Astaxanthin(mg kg^{-1})	45.58 ± 1.71	296.41 ± 2.86	46.16 ± 1.30	264.55 ± 6.00

[a]NFE = Nitrogen free extract.

Table 4. Fatty acid composition of the experimental diet and squid (means ± s.d., % of total fatty acids).

Fatty acid	Pelleted diets				Squid
	LFLA	LFHA	HFLA	HFHA	
14:0	4.1 ± 0.2	4.8 ± 0.1	3.0 ± 0.3	4.3 ± 0.2	2.2 ± 0.4
16:0	22.8 ± 1.0	23.9 ± 3	17.3 ± 2.1	22.0 ± 0.1	26.3 ± 0.6
16:1(n-7)	4.3 ± 0.3	5.2 ± 0.1	3.2 ± 0.3	4.7 ± 0.3	0.6 ± 0.1
18:0	6.0 ± 0.0	5.5 ± 1.0	6.8 ± 0.5	6.3 ± 0.2	7.7 ± 0.5
18:1(n-9)	15.7 ± 0.4	12.9 ± 1.0	14.1 ± 1.4	13.6 ± 0.1	2.8 ± 0.1
18:1(n-7)	2.7 ± 0.1	2.6 ± 0.2	3.7 ± 0.6	2.7 ± 0.1	1.3 ± 0.1
18:2(n-6)	11.9 ± 0.2	9.0 ± 0.7	9.4 ± 1.2	7.6 ± 0.1	0.2 ± 0.1
19:0	0.3 ± 0.0	0.4 ± 0.1	0.9 ± 0.2	0.4 ± 0.0	0.2 ± 0.0
18:3(n-3)	1.1 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	0.0 ± 0.1
18:4(n-3)	1.3 ± 0.0	1.2 ± 0.2	1.5 ± 0.1	1.4 ± 0.0	0.3 ± 0.1
20:0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	nd[a]
20:1(n-9)	0.6 ± 0.1	0.7 ± 0.0	0.9 ± 0.0	0.8 ± 0.1	1.5 ± 0.6
20:3(n-6)	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	nd
20:4(n-6)	1.4 ± 0.1	1.4 ± 0.2	1.8 ± 0.1	1.7 ± 0.3	5.3 ± 0.5
20:4(n-3)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.0 ± 0.0
20:5(n-3)	6.7 ± 0.1	6.6 ± 0.4	7.7 ± 0.2	7.1 ± 0.1	8.7 ± 0.3
21:5(n-3)	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.2 ± 0.0	0.1 ± 0.1
22:5(n-6)	0.9 ± 0.0	1.1 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	2.2 ± 0.2
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0 .0 ± 0.0	nd
22:5(n-3)	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	0.7 ± 0.2
22:6(n-3)	14.9 ± 0.3	16.6 ± 1.5	20.6 ± 0.3	19.2 ± 0.4	36.2 ± 0.6
Σ Saturated[b]	35.5 ± 1.3	37.8 ± 2.4	30 ± 2.1	35.4 ± 0.3	38.5 ± 0.7
Σ Monoenes	24.3 ± 0.4	23.1 ± 0.7	23.8 ± 1.3	23.0 ± 0.2	7.0 ± 0.4
Σ n-6 PUFA	14.5 ± 0.4	12.0 ± 0.5	13.4 ± 1.1	11.0 ± 0.1	8.3 ± 0.4
Σ n-3 PUFA	25.7 ± 0.5	27.1 ± 2.3	32.8 ± 0.3	30.5 ± 0.6	46.2 ± 0.7
Σ n-3 HUFA[c]	23.2 ± 0.5	25.0 ± 2.0	30.2 ± 0.3	28.1 ± 0.6	45.9 ± 0.6

[a]nd: not detected.

[b]Sums include minor fatty acids not shown in table.

[c] 20:3(n-3).

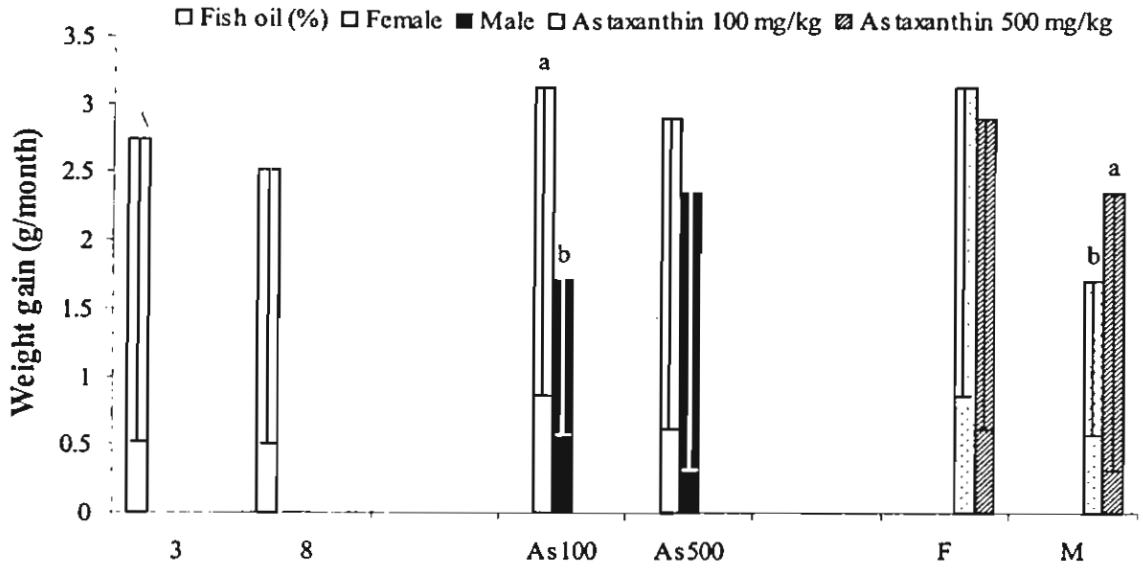

Figure 1. Effect of fish oil and sex on weight gain of broodstock *P. monodon* (As100, Astaxanthin 100 mg kg^{-1}; As500, Astaxanthin 500 mg kg^{-1}; F, Female; M, Male). The different superscripts within each group at the top of each bar are significantly different ($P<0.05$).

Effect of fish oil and astaxanthin on amount of egg in female shrimp is shown in **Figure 2**. The interaction between fish oil and astaxanthin on amount of egg had not significant difference ($P>0.05$). Therefore, the effects of fish oil and astaxanthin on amount of egg were discussed independently. Female shrimp fed diet containing 8% fish oil had significantly greater amount of eggs ($P<0.05$) than those fed diet containing 3% fish oil. Female shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1} had higher amount of eggs ($P<0.05$) than those fed diet supplemented with astaxanthin 100 mg kg^{-1}.

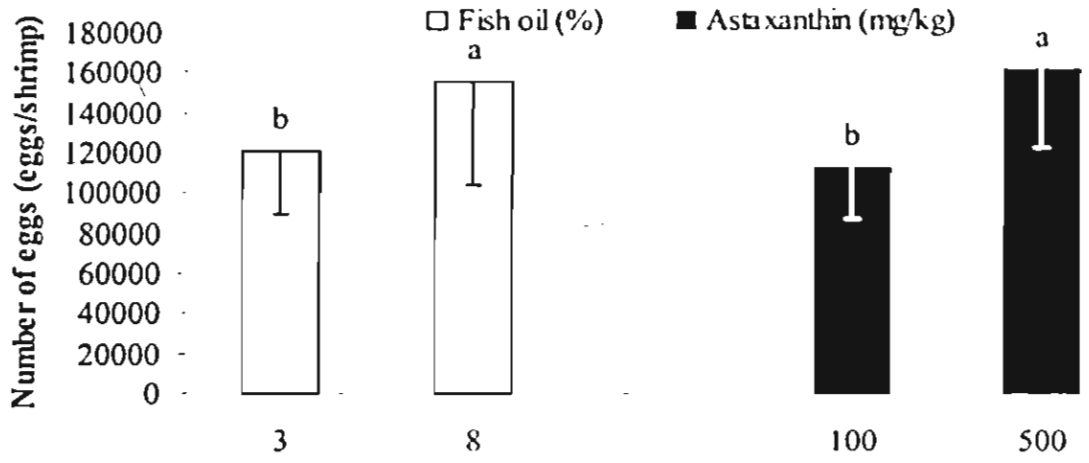

Figure 2. Effect of fish oil and astaxanthin on amount of egg of female broodstock *P. monodon*. The different superscripts within each group at the top of each bar are significantly different ($P<0.05$).

The effect of dietary fish oil and astaxanthin on amount of spermatozoa is shown in **Figure 3**. The effects of fish oil and astaxanthin on amount of spermatozoa were discussed independently because there was no significant ($P>0.05$) interaction between dietary fish oil and astaxanthin. Male shrimp fed diet containing 8% fish oil had significantly greater amount of spermatozoa ($P<0.05$) than those fed diet containing 3% fish oil. Male shrimp fed diet supplement with astaxanthin 500 mg kg^{-1} had higher amount of spermatozoa ($P<0.05$) than those fed diet supplemented with astaxanthin 100 mg kg^{-1}.

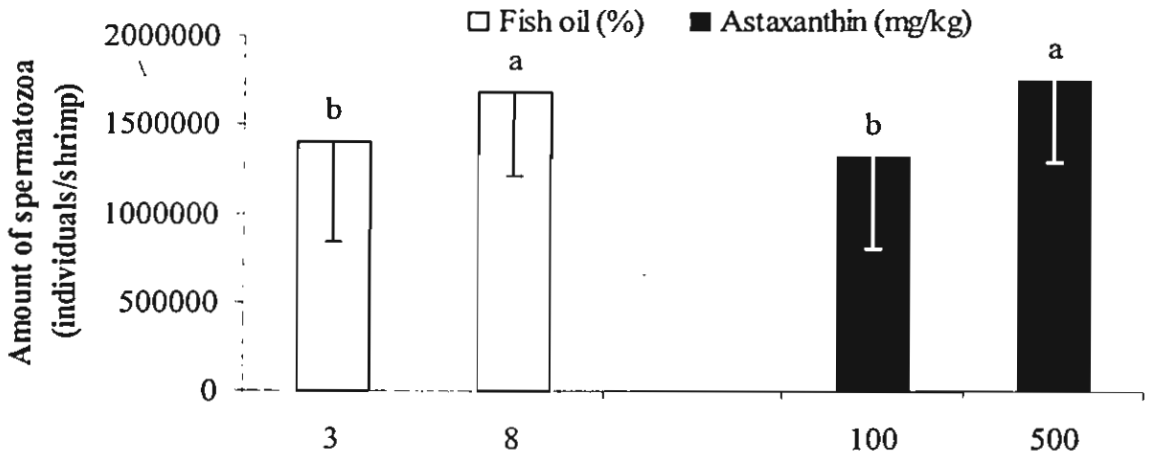

Figure 3. Effect of fish oil and astaxanthin on amount of spermatozoa of male broodstock *P. monodon*. The different superscripts within each group at the top of each bar are significantly different ($P<0.05$).

The effect of dietary fish oil, astaxanthin and sex on astaxanthin accumulation in muscle is presented in Figure **4a**. There was no significant ($P>0.05$) interaction between fish oil and astaxanthin, fish oil and sex, and astaxanthin and sex on astaxanthin accumulation in muscle. Therefore, the effects of fish oil, astaxanthin and sex on astaxanthin accumulation in muscle were discussed independently. No significant differences ($P>0.05$) among levels of astaxanthin and sex were found on astaxanthin content in muscle. Shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1} had significantly higher astaxanthin content in muscle ($P<0.05$) than those fed diet supplemented with astaxanthin 100 mg kg^{-1}.

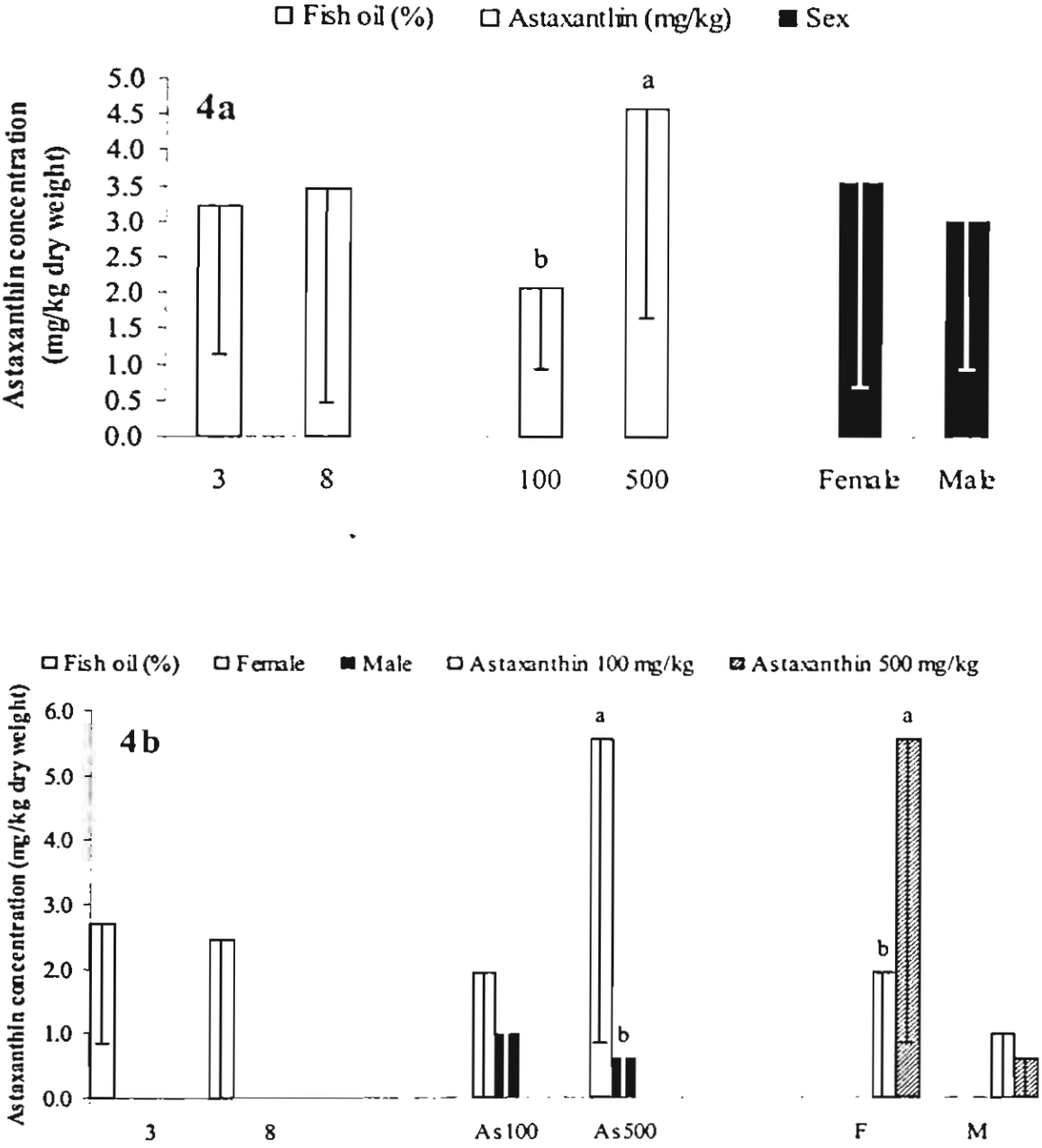

Figure 4. Effects of fish oil, astaxanthin and sex on astaxanthin content in muscle (4a), hepatopancreas (4b), ovary (4c) and shell (4d) of broodstock *P. monodon* (As100, Astaxanthin 100 mg kg^{-1}; As500, Astaxanthin 500 mg kg^{-1}; F, Female; M, Male). The different superscripts within each group at the top of each bar are significantly different (P<0.05).

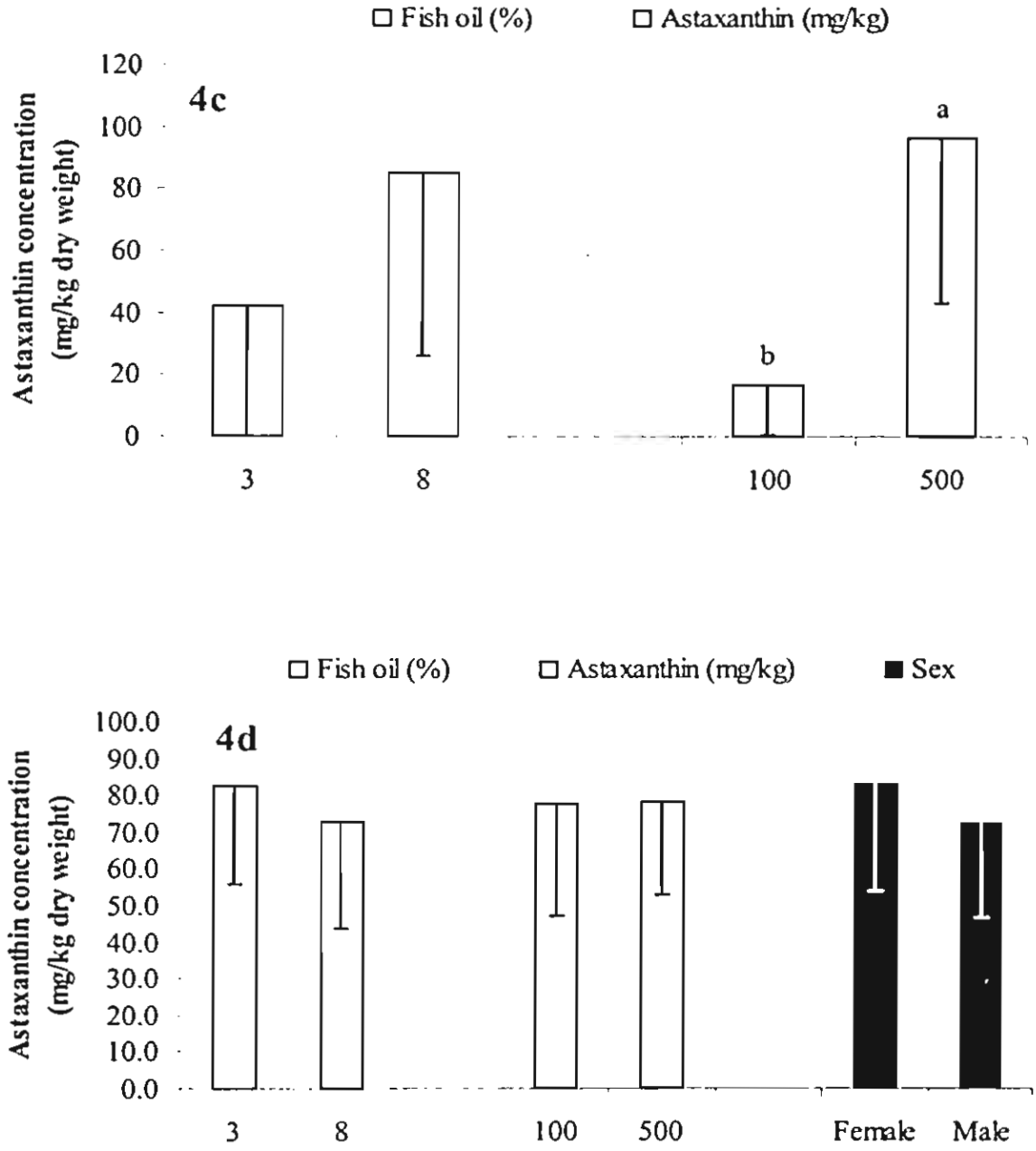

Figure 4. (Continued)

The effect of dietary fish oil, astaxanthin and sex on astaxanthin accumulation in hepatopancreas of shrimp is shown in **Figure 4b**. There was no significant ($P>$ 0.05) interaction between fish oil and astaxanthin, and fish oil and sex on astaxanthin accumulation in hepatopancreas but interaction between astaxanthin and sex was

found significant difference (P<0.05). Thus, the effect of fish oil on astaxanthin accumulation in hepatopancreas was discussed individually, while the effect of astaxanthin and sex on astaxanthin accumulation in hepatopancreas was discussed together. There was not significantly different (P>0.05) between 2 levels of fish oil on astaxanthin content in hepatopancreas during experimental period. In shrimp fed diet supplemented with astaxanthin 100 mg kg^{-1}, no significant difference (P>0.05) of sex was found on astaxanthin accumulation in hepatopancreas. In shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1}, female shrimp had significantly higher astaxanthin accumulation in hepatopancreas (P<0.05) than those of male. Female shrimp fed supplemented with astaxanthin 500 mg kg^{-1} had significantly greater astaxanthin accumulation in hepatopancreas (P<0.05) than those of obtained astaxanthin 100 mg kg^{-1}, while in male shrimp, there was not significant difference (P>0.05) among levels of astaxanthin on astaxanthin accumulation in hepatopancreas.

The effect of dietary fish oil and astaxanthin on astaxanthin accumulation in ovary is shown in **Figure 4c**. The effects of fish oil and astaxanthin on astaxanthin accumulation in ovary were discussed independently because there was no significant (P>0.05) interaction between them. No significant difference (P>0.05) of fish oil was found on different astaxanthin content in ovary, while female shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1} had significant higher astaxanthin content in ovary (P<0.05) than those fed diet supplemented with 100 mg kg^{-1}.

The effect of dietary fish oil, astaxanthin and sex on astaxanthin accumulation in shell is presented in **Figure 4d**. There was no significant (P>0.05) interaction between fish oil, astaxanthin and sex on astaxanthin accumulation in shell. Therefore, the effects of fish oil, astaxanthin and sex on astaxanthin accumulation in the shell

were discussed independently. There was no significantly different (P>0.05) in levels of fish oil, astaxanthin, and sex on astaxanthin content in shell.

Fatty acid contents in muscle, hepatopancreas and ovary of female and male broodstock are shown in **Table 5**. The effects of fish oil, astaxanthin and sex on on fatty acid content in muscle, hepatopancreas and ovary were discussed independently because there were no significant (P>0.05) interaction between fish oil and astaxanthin, fish oil and sex, and astaxanthin and sex.

The effects of fish oil is shown in **Table 6**. No significant difference (P>0.05) of dietary fish oil was found on 18:2(n-6), 18:3(n-3), and total saturated contents in muscle, hepatopancreas and ovary. Shrimp fed diet containing 8% fish oil had significant higher 20:4(n-6), 20:5(n-3), total monoenes and total n-6 PUFA contents in hepatopancreas (P<0.05) than those fed diet containing 3% fish oil. Shrimp fed diet containing 8% fish oil had significant higher 22:6(n-3), total n-3 PUFA and total n-3 HUFA contents in muscle, hepatopancreas and ovary (P<0.05) than those fed diet containing 3% fish oil.

The effect astaxanthin is shown in **Table 7**. There was no significant (P>0.05) of 18:2(n-6), 18:3(n-3), 20:4(n-6), 20:5(n-3), total saturated, total monoenes and total n-6 PUFA contents in muscle, hepatopancreas and ovary on shrimp fed diet containing differed levels of astaxanthin. While, shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1} had significant greater 22:6(n-3), total n-3 PUFA and total n-3 HUFA contents in muscle and ovary than those fed diet supplemented with astaxanthin 100 mg kg^{-1}.

Table 5. Fatty acid composition (means ± s.d., % of total fatty acids) in muscle, hepatopancreas and ovary of female, and muscle and hepatopancreas of male broodstock *P. monodon*.

Fatty acids	Female			Male	
	Muscle	Hepatopancreas	Ovary	Muscle	Hepatopancreas
14:0	2.6 ± 2.3	1.1 ± 1.0	1.3 ± 1.6	3.6 ± 3.4	1.9 ± 2.1
16:0	36.0 ± 21.8	14.4 ± 13.9	15.1 ± 14.8	39.1 ± 23.1	24.4 ± 20.4
16:1(n-7)	4.4 ± 3.4	1.7 ± 1.8	1.6 ± 1.7	4.8 ± 3.6	3.4 ± 3.1
18:0	9.2 ± 5.1	10.5 ± 4.7	9.6 ± 3.2	8.0 ± 5.1	7.1 ± 1.7
18:1(n-9)	8.6 ± 4.8	19.0 ± 7.2	19.7 ± 5.9	7.0 ± 4.6	15 ± 7.8
18:1(n-7)	1.4 ± 0.8	4.2 ± 1.4	3.4 ± 1.1	1.1 ± 0.8	3.2 ± 1.5
18:2(n-6)	5.0 ± 3.1	7.2 ± 4.2	3.7 ± 2	4.6 ± 3.1	7.0 ± 4.3
19:0	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
18:3(n-3)	0.3 ± 0.2	0.5 ± 0.3	0.2 ± 0.1	0.2 ± 0.2	0.6 ± 0.3
18:4(n-3)	0.5 ± 0.4	0.8 ± 0.3	0.7 ± 0.2	0.5 ± 0.3	0.8 ± 0.4
20:0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:1(n-9)	0.4 ± 0.2	2.2 ± 1.1	1.2 ± 0.4	0.3 ± 0.2	1.3 ± 0.4
20:3(n-6)	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
20:4(n-6)	5.5 ± 3.0	4.9 ± 2.5	6.5 ± 2.3	4.9 ± 3.4	3.1 ± 0.8
20:4(n-3)	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	0.3 ± 0.2
20:5(n-3)	9.8 ± 5.4	8.1 ± 2.5	11.3 ± 4.2	8.5 ± 5.5	5.7 ± 2
21:5(n-3)	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.1
22:5(n-6)	0.5 ± 0.3	1.4 ± 0.6	1.1 ± 0.3	0.5 ± 0.4	1.2 ± 0.6
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
22:5(n-3)	0.2 ± 0.2	0.7 ± 0.6	0.5 ± 0.4	0.2 ± 0.2	1.1 ± 0.6
22:6(n-3)	10.6 ± 6.3	17.3 ± 10.1	18.2 ± 10.9	11.1 ± 7.4	18.1 ± 8.3
Σ Saturated[a]	52.1 ± 19.9	28.4 ± 14.7	28.8 ± 16	55.5 ± 22	36.6 ± 22.6
Σ Monoenes	15.5 ± 3.2	29.4 ± 8.4	28.2 ± 5.2	14.1 ± 2.6	24.7 ± 6.5
Σ n-6 PUFA	11.0 ± 6.0	14.1 ± 4.4	11.7 ± 4.0	10.0 ± 6.6	11.8 ± 5.2
Σ n-3 PUFA	21.5 ± 11.5	28.1 ± 9.7	31.4 ± 11.9	20.4 ± 13.2	26.8 ± 11.7
Σ n-3 HUFA[b]	20.7 ± 11.1	26.7 ± 9.8	30.5 ± 11.9	19.7 ± 12.8	25.4 ± 11.1

[a]Sums include minor fatty acids not shown in table.

[b] 20:3(n-3).

Table 6. Effect of fish oil on fatty acid content (means ± s.d., mg g^{-1} dry weight) in muscle, hepatopancreas and ovary. Mean with the different superscripts within each row indicate a significant difference ($P<0.05$).

Fatty acid	Tissue	Fish oil (%)	
		3	8
18:2(n-6)	Muscle	1.42 ± 0.28	1.33 ± 0.50
	Hepatopancreas	8.93 ± 6.49	11.42 ± 8.82
	Ovary	5.10 ± 0.8	3.10 ± 2.45
18:3(n-3)	Muscle	0.06 ± 0.05	0.07 ± 0.05
	Hepatopancreas	0.64 ± 0.52	0.97 ± 0.65
	Ovary	0.20 ± 0.08	0.26 ± 0.13
20:4(n-6)	Muscle	1.53 ± 0.32	1.55 ± 0.25
	Hepatopancreas	3.91^{b} ± 1.72	7.82^{a} ± 5.89
	Ovary	6.65 ± 1.01	6.84 ± 1.26
20:5(n-3)	Muscle	2.68 ± 0.19	2.69 ± 0.36
	Hepatopancreas	7.40^{b} ± 3.89	12.85^{a} ± 6.74
	Ovary	11.23 ± 1.45	12.34 ± 1.27
22:6(n-3)	Muscle	2.62^{b} ± 0.99	3.65^{a} ± 0.45
	Hepatopancreas	13.11^{b} ± 9.59	37.76^{a} ± 15.24
	Ovary	11.08^{b} ± 11.72	29.68^{a} ± 7.19
Σ Saturated	Muscle	27.10 ± 30.37	23.18 ± 22.48
	Hepatopancreas	39.69 ± 32.33	51.62 ± 30.94
	Ovary	43.10 ± 35.2	66.28 ± 56.24
Σ Monoenes	Muscle	5.59 ± 3.88	5.30 ± 2.39
	Hepatopancreas	30.57^{b} ± 20.65	49.43^{a} ± 19.81
	Ovary	27.98 ± 4.71	36.54 ± 12.02
Σ n-6 PUFA	Muscle	3.08 ± 0.27	3.03 ± 0.56
	Hepatopancreas	14.78^{b} ± 8.86	22.90^{a} ± 11.94
	Ovary	13.25 ± 1.75	11.7 ± 2.58
Σ n-3 PUFA	Muscle	5.58^{b} ± 0.95	6.65^{a} ± 0.64
	Hepatopancreas	23.49^{b} ± 12.62	55.79^{a} ± 21.3
	Ovary	23.98^{b} ± 12.57	44.24^{a} ± 8.57
Σ n-3 HUFA	Muscle	5.37^{b} ± 0.96	6.43^{a} ± 0.64
	Hepatopancreas	22.00^{b} ± 11.86	53.37^{a} ± 20.58
	Ovary	23.08^{b} ± 12.52	43.26^{a} ± 8.47

Table 7. Effect of astaxanthin on fatty acid content (means ± s.d., mg g^{-1} dry weight) in muscle, hepatopancreas and ovary. Mean with the different superscripts within each row indicate a significant difference ($P<0.05$).

Fatty acid	Tissue	Astaxanthin (mg kg^{-1})	
		100	500
18:2(n-6)	Muscle	1.43 ± 0.24	1.32 ± 0.52
	Hepatopancreas	9.63 ± 4.87	10.8 ± 9.82
	Ovary	4.68 ± 1.95	3.44 ± 2.24
18:3(n-3)	Muscle	0.08 ± 0.04	0.05 ± 0.05
	Hepatopancreas	0.77 ± 0.51	0.85 ± 0.70
	Ovary	0.25 ± 0.13	0.22 ± 0.11
20:4(n-6)	Muscle	1.59 ± 0.32	1.49 ± 0.24
	Hepatopancreas	6.99 ± 6.00	5.06 ± 3.32
	Ovary	6.52 ± 1.07	6.94 ± 1.19
20:5(n-3)	Muscle	2.65 ± 0.23	2.72 ± 0.34
	Hepatopancreas	11.01 ± 7.00	9.62 ± 5.39
	Ovary	11.52 ± 0.81	12.1 ± 1.79
22:6(n-3)	Muscle	2.86^{b} ± 1.09	3.43^{a} ± 0.62
	Hepatopancreas	22.53 ± 18.39	29.34 ± 17.12
	Ovary	13.67^{b} ± 16.05	27.60^{a} ± 6.79
Σ Saturated	Muscle	27.8 ± 28.25	22.55 ± 24.7
	Hepatopancreas	47.84 ± 34.56	44.33 ± 29.82
	Ovary	39.78 ± 32.73	68.94 ± 55.81
Σ Monoenes	Muscle	5.89 ± 3.13	5.03 ± 3.17
	Hepatopancreas	39.83 ± 18.71	41.15 ± 25.24
	Ovary	27.45 ± 5.26	36.96 ± 11.44
Σ n-6 PUFA	Muscle	3.17 ± 0.33	2.95 ± 0.51
	Hepatopancreas	19.31 ± 8.65	18.85 ± 13.37
	Ovary	12.85 ± 2.71	12.02 ± 2.1
Σ n-3 PUFA	Muscle	5.86^{b} ± 1.02	6.39^{a} ± 0.85
	Hepatopancreas	37.67 ± 24.16	43.11 ± 24.11
	Ovary	26.92^{b} ± 17.66	41.88^{a} ± 7.88
Σ n-3 HUFA	Muscle	5.61^{b} ± 1.01	6.22^{a} ± 0.83
	Hepatopancreas	35.71 ± 23.69	41.10 ± 22.93
	Ovary	26.00^{b} ± 17.51	40.92^{a} ± 7.86

The effect of sex on fatty acid content in muscle, hepatopancreas and ovary is shown in **Table 8**. There was no any significant ($P>0.05$) of all fatty acid contents in muscle, hepatopancreas and ovary between male and female shrimp.

Table 8. Effect of sex on fatty acid content (means ± s.d., mg g^{-1} dry weight) in muscle, hepatopancreas and ovary. Mean with the different superscripts within each row indicate a significant difference (P<0.05).

Fatty acid	Tissue	Sex	
		Female	Male
18:2(n-6)	Muscle	1.34 ± 0.46	1.43 ± 0.31
	Hepatopancreas	10.65 ± 8.92	9.54 ± 5.53
	Ovary	3.99 ± 2.09	
18:3(n-3)	Muscle	0.07 ± 0.05	0.07 ± 0.05
	Hepatopancreas	0.79 ± 0.65	0.86 ± 0.55
	Ovary	0.23 ± 0.11	
20:4(n-6)	Muscle	1.55 ± 0.26	1.52 ± 0.32
	Hepatopancreas	6.90 ± 5.74	4.33 ± 1.56
	Ovary	6.76 ± 1.09	
20:5(n-3)	Muscle	2.72 ± 0.24	2.62 ± 0.36
	Hepatopancreas	11.48 ± 6.86	8.16 ± 4.05
	Ovary	11.84 ± 1.39	
22:6(n-3)	Muscle	3.03 ± 1.05	3.40 ± 0.51
	Hepatopancreas	25.57 ± 17.92	27.09 ± 18.3
	Ovary	21.41 ± 13.17	
Σ Saturated	Muscle	28.49 ± 30.95	18.91 ± 13.42
	Hepatopancreas	46.80 ± 30.28	44.55 ± 35.38
	Ovary	55.98 ± 46.85	
Σ Monoenes	Muscle	5.72 ± 3.69	4.94 ± 1.83
	Hepatopancreas	44.05 ± 23.67	34.30 ± 18.18
	Ovary	32.73 ± 10.05	
Σ n-6 PUFA	Muscle	3.03 ± 0.48	3.08 ± 0.39
	Hepatopancreas	20.53 ± 12.56	16.47 ± 8.18
	Ovary	12.39 ± 2.27	
Σ n-3 PUFA	Muscle	6.04 ± 1.07	6.32 ± 0.74
	Hepatopancreas	41.04 ± 23.88	39.66 ± 25.02
	Ovary	35.23 ± 14.49	
Σ n-3 HUFA	Muscle	5.83 ± 1.07	6.10 ± 0.74
	Hepatopancreas	39.02 ± 23.12	37.74 ± 24.04
	Ovary	34.29 ± 14.41	

Discussion

Many reports studied potential of pond-reared shrimp broodstock for larvae production by comparing with wild-caught broodstock (Menasveta et al., 1993, 1994b; Sangpradub et al., 1994; Ramos et al., 1995; Cavalli et al., 1997; Palacios et al., 1999). Menasveta et al. (1994b) reported total eggs produced by large females was significantly greater than for small shrimp; nevertheless, shrimp source (pond-reared and wild-caught) and size had no significant influence on amount of eggs spawned per spawning event. Palacios et al. (1999) reported that wild-caught shrimp had higher mating and spawning frequencies compared to pond-reared broodstock. However, the number of nauplii per spawn was higher for wild shrimp, but fertilization and hatching rates were higher for pond-reared spawners.

The result of this study illustrated that fish oil could enhance reproductive performance in pond-reared broodstock shrimp. Shrimp fed diet containing high level of fish oil can produce higher amount of eggs and spermatozoa than those fed diet containing low level (**Figure 2** and **3**) and they accumulated high contents of 20:4(n-6), 20:5(n-3) and total n-6 PUFA in hepatopancreas (**Table 6**). Many reports noted that n-6 PUFA, especially 20:4(n-6), is precursor in the synthesis of prostaglandins in vertebrates and insects which serve many functions in reproduction, spanwing and larval production (Middleditch et al., 1979; Johnson et al., 1983; Spaziani et al., 1991; Alava et al., 1993; Harrison, 1997).

Our study found that 22:6(n-3) had the most effect to improve reproductive performance in pond-reared broodstock shrimp. The positive relationship between 22:6(n-3), total n-3 PUFA and n-3 HUFA contents in muscle, hepatopancreas and ovary, and amount of egg and spermatozoa in pond-reared broodstock shrimp was

found on this study. Shrimp fed diet supplemented with high level of fish oil had significantly higher amount of egg and spermatozoa, and 22:6(n-3), total n-3 PUFA and n-3 HUFA contents in muscle, hepatopancreas and ovary than those fed diet supplement with low level of fish oil. This was also reported by Kanazawa et al. (1979) and Xu et al. (1994) as 22:6(n-3) had more effective as an essential fatty acid than other essential fatty acids. Cerolini et al. (1997) and Conner et al. (1997) noted that n-3 and n-6 PUFA may have important roles for sperm production. The results of this study indicate that fish oil contained high contents of total n-3 PUFA and n-3 HUFA that could improve reproductive performance of pond-reared shrimp.

The current study indicated that astaxanthin could improve reproductive performance of pond-reared shrimp. Shrimp fed diet supplemented with high level of astaxanthin had significantly higher amount of egg and spermatozoa than those fed diet supplemented with low level of astaxanthin. Moreover, high astaxanthin accumulation in muscle and ovary were detected in shrimp fed diet supplemented with high level of astaxanthin (**Figure 4a** and **4c**). A similar several previous studies showed the important effects of astaxanthin on ovarian maturation (Menasveta et al., 1994a; Sagi et al., 1996; Pangantihon-Kuhlmann et al., 1998; Ribeiro et al., 2001). During ovarian maturation, crustaceans accumulate and mobilize astaxanthin from hepatopancreas to ovary via the haemolymph with lipovitellin in the oocytes (Nelson et al., 1988; Quinito et al., 1989; 1990; Harrison, 1990).

Astaxanthin may be promoting amount of spermatozoa in male broodstock via the provitamin A activity. Ratinoid act on the testicular development, especially on germ cell, via retinoic acid receptor and/or retinoid X receptors (Boulogne et al., 1999). Akmal et al.(1997) reported that mutational studies of spermatid have

identified retinoic acid receptor alpha as having an essential role in spermatogenesis. Characterization of retinoic acid receptor alpha expression revealed the time and location of the vitamin A requirement during spermatogenesis.

In this study, the amounts of astaxanthin in diet supplemented with astaxanthin 100 and 500 mg kg^{-1} were 45.87 ± 1.51 and 280.48 ± 4.43 mg kg^{-1}, repectively. The analytical concentration of astaxanthin in diet supplemented with astaxanthin 500 mg kg^{-1} was less than the expected concentration due to loss of astaxanthin during feed pelleting process and feed storage.

This study found that shell was the main tissue for astaxanthin deposit and muscle and hepatopancreas were the subsequent tissues for astaxanthin deposit. Similar with reported by Negre-Sandargues et al. (1993) as dietary astaxanthin was found to be stored in the integument (carapace and epidermis) and hepatopancreas. Moreover, the all-trans-astaxanthin was found as the main form of astaxanthin in this study. The 9-cis and 13-cis astaxanthin was found minor content. This was also reported by Muriana et al. (1993) as study of astaxanthin identify using HPLC suggested all-trans-astaxanthin to be the main component, which was accompanied by an epimer and its cis-isomer.

This study was not detected the interaction between dietary fish oil and astaxanthin, and fish oil and sex, while dietary astaxanthin had interaction with sex on astaxanthin content in hepatopancreas and growth of shrimp (**Figure 1** and **4b**). Female shrimp fed diet containing high level of astaxanthin had significantly higher accumulate astaxanthin content in hepatopancreas than those fed diet containing low level of astaxanthin but male shrimp had not significantly different astaxanthin content in hepatopancreas among levels of astaxanthin supplemented in shrimp diet.

Astaxanthin had high accumulated in muscle, hepatopancreas and ovary of female shrimp fed diet containing high astaxanthin supplementation, while it had only high accumulated in muscle of male shrimp. This indicated that both sexes of shrimp had different rates of astaxanthin accumulation and transfer among tissues during reproductive maturation. Dall et al. (1995) reported that the maturing ovary of *P. esculentus* contained high levels of carotenoids.

The interaction between astaxanthin and sex on weight gain indicated that both sexes of shrimp required astaxanthin for growth at different levels. Effect of astaxanthin on weight gain had not found in female broodstock shrimp but detected only in male broodstock shrimp. Male shrimp fed diet containing high level of astaxanthin had significantly greater weight gain than those fed diet containing low level of astaxanthin. It may be that female shrimp accumulated high content of astaxanthin during ovarian maturation. The astaxanthin content had higher than the requirement of astaxanthin for growth. So, the study found no effect of astaxanthin on growth of female shrimp. However, Negre-Sandargues et al. (1993) reported that shrimp fed diet supplemented with astaxanthin were not significantly different on growth rate.

The result of this study found that fish oil had not effect on growth of pond-reared broodstock shrimp after 4 month of experiment. The average total lipids of experimental diet supplemented with 3 and 8% fish oil were 7.74 ± 0.10 and 11.78 ± 0.22%, respectively. The total lipids in diets of this study found in range of lipid requirement levels for juvenile to broodstock shrimp. In crustacean diet, the best survival and growth responses are achieved when the dietary level of one oil or a

mixture of oils is between 5 to 8% (D'Abramo, 1997). Bray et al. (1990) reported that the broodstock diet supplemented with 11.1% lipid has high nauplii production.

The result of present study found that shrimp fed diet containing high level of astaxanthin had higher astaxanthin content in muscle and ovary than those fed diet containing low level of astaxanthin ovary (**Figure 4a** and **4c**). The accumulated astaxanthin in muscle and ovary had effect on accumulation of 22:6(n-3), total n-3 PUFA and total n-3 HUFA contents in muscle and ovary (**Table 7**). It may be that the accumulated astaxanthin esterified to long chain fatty acids, especially 22:6(n-3). So, the increasing of fatty acid content in muscle and ovary was found, when shrimp had increased astaxanthin content in their tissues. Meyers and Latscha (1997) reported that astaxanthin is present in nature either in the free form, esterified to long chain fatty acids, or associated with proteins forming carotenoproteins. All three forms are found in crustaceans.

In summary, dietary fish oil (HUFA) and astaxanthin can promote reproductive performance in pond-reared broodstock *P. monodon* of both sexes. In the current result, 20:4(n-6) and 22:6(n-3) were prominent fatty acids to promote amount of egg and spermatozoa in pond-reared broodstock. The practical pond-reared broodstock shrimp diet supplemented with astaxanthin 300 mg kg^{-1} and/or 12.0% lipid could enhance reproductive performance of broodstock shrimp *P. monodon*.

Recommendation

This study demonstrated the potential dietary fish oil and astaxanthin to improve reproductive performance of pond-reared shrimp *P. monodon* and can be directly applied to aquacultural activity and to further studies. However, to increase the effectiveness, the feed pelleting process could be improved for reduction of astaxanthin loss during preparing pelleted diet.

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Output จากโครงการ

1. Effect of fish oil enrichment on maturation performance of pond-reared black tiger prawn *Penaeus monodon* broodstock. การนำเสนอผลงานแบบโปสเตอร์. การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ครั้งที่ 28, 24-26 ตุลาคม 2545, กรุงเทพฯ.

2. Nutritional enrichment of astaxanthin and highly unsaturated fatty acids for maturation improvement of pond-reared broodstock black tiger shrimp (*Penaeus monodon* Fabricius). อยู่ระหว่างเตรียมต้นฉบับเพื่อส่งตีพิมพ์ในวารสาร Aquaculture

APPENDICES

APPENDIX A

Sumitted paper for Aquaculture

Nutritional enrichment of astaxanthin and highly unsaturated fatty acids for maturation improvement of pond-reared broodstock black tiger shrimp (*Penaeus monodon* Fabricius)

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Abstract

The study of the improvements for reproductive performance of pond-reared black tiger shrimp broodstock *P. monodon* was used diets containing 2 levels of fish oil, 3 and 8%, and 2 levels of astaxanthin 100 and 500 mg kg^{-1}. Female shrimp weighting 48-50 g and male shrimp weighting 35-38 g were randomly separated to fed the experimental diets for 4 months. The result indicated no interaction between fish oil and astaxanthin, and fish oil and sex on average weight gain was found but astaxanthin and sex had significant ($P<0.05$) interaction. Fish oil was no effect on average weight gain. In male, shrimp fed high level of astaxanthin had significantly higher average weight gain ($P<0.05$) than those fed low level of astaxanthin. There was no interaction between fish oil and astaxanthin on amount of egg and

spermatozoa. Shrimp fed diet containing high levels of fish oil and astaxanthin had significantly higher amount of eggs and spermatozoa than those fed diet containing low levels of them. No interaction was found on fish oil and astaxanthin, and fish oil and sex on astaxanthin content in all tissues. Astaxanthin content in all tissues was not significantly different among level of fish oil. On astaxanthin content in muscle, hepatopancreas, ovary and shell, only interaction between astaxanthin and sex was found. In muscle and ovary, shrimp fed diet containing high level of astaxanthin had significantly high astaxanthin content. In hepatopancreas, female shrimp fed diet containing high level of astaxanthin had significantly higher astaxanthin content than male shrimp. In shell, no significant difference among levels of fish oil, astaxanthin and sex was found on astaxanthin content. The interaction between fish oil, astaxanthin and sex was not found on fatty acid content in muscle, hepatopancreas and ovary. Shrimp fed diet containing high level of fish oil and astaxanthin had significantly high 22:6(n-3), total n-3 PUFA and total n-3 HUFA contents in muscle and ovary, whereas 20:4(n-6), 20:5(n-3), 22:6(n-3), total n-6 PUFA, total n-3 PUFA and total n-3 HUFA contents were significantly high accumulated in hepatopancreas of shrimp fed diet containing high level of fish oil. The supplementation of dietary fish oil and/or astaxanthin in practical broodstock shrimp diet could enhance reproductive performance of pond-reared broodstock shrimp.

keyword: HUFA, astaxanthin, broodstock, *Penaeus monodon*, reproductive performance

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1. Introduction

In Thailand, high quality shrimp larvae have been mostly produced by wild-caught broodstocks. Although the wild-caught broodstocks produce high quality shrimp larvae, they may be carriers of viral diseases (Flegel and Alday-Sanz, 1998; Otta et al., 1999) and cause mass mortality of shrimp after 1 or 2 months of rearing in the earthen pond. The amounts of matured wild female rapidly decrease on time to time. Then, the substitution of wild-caught broodstocks with pond-reared broodstocks will be sustainable developed. However, pond-reared broodstocks are not favorite use for broodstock because they have lower maturation performance than wild-caught broodstocks. One of the main causes of low maturation performance is due to less nutritional information. Therefore, the study of pond-reared broodstocks nutrition to improve maturation and larvae production is necessary.

Astaxanthin and lipid are important nutrient to promote reproductive performance in shrimp (Middleditch et al., 1979; Millamena, 1989; Bray et al., 1990; Menasveta et al., 1994a; Pangantihon-Kuhlmann et al., 1998). The proposed functions for astaxanthin in aquaculture have been those of provitamin A activity, antioxidant properties, positive effect on embryonic and larval development, cellular protection from photodynamic damage, enhancement of growth, maturation and formation of in-chain epoxides that act as oxygen reserves under anoxic condition (Torrissen, 1990). Astaxanthin has been reported as the most frequent end product of carotenoid metabolism in crustaceans (Katayama et al., 1972a) and identified as the main pigment in the adult prawn, *Penaeus japonicus* (Katayama et al., 1971; 1972b). A variety of factors affects amounts and distribution of carotenoids in crustaceans,

including embryogenesis, reproductive cycle, molting, background colors, and hormonal control (Goodwin, 1960).

Lipid plays major roles in reproductive processes of crustaceans. Levels and composition of dietary lipids profoundly affect ovarian maturation and reproductive success (Harrison, 1990). Middleditch et al. (1979; 1980) investigated that penaeid shrimp needs polyunsaturated fatty acid (PUFA) in order to develop shrimp gonad tissue. The purpose of this study is to improve reproductive performance of pond-reared black tiger shrimp broodstock *Penaeus monodon* by dietary fish oil and astaxanthin.

2. Materials and methods

The experiment was operated 120 days. The experimental design was 2×2×2 factorials in completely randomized design. Two levels of fish oil 3 and 8%, 2 concentrations of astaxanthin 100 and 500 mg kg^{-1} and 2 sexes (male and female) were used. The composition of the tested diet was described in **Table 1**. Eight replication was done in each treatment.

The experimental diets contained 48% crude protein. The four experimental diets were: LFLA, supplemented with 3% fish oil and 100 mg kg^{-1} astaxanthin; LFHA, supplemented with 3% fish oil and 500 mg kg^{-1} astaxanthin; HFLA, supplemented with 8% fish oil and 100 mg kg^{-1} astaxanthin; and HFHA, supplemented with 8% fish oil and 500 mg kg^{-1} astaxanthin. Dietary ingredients were grounded into powder and mixed by a twin blade rolling mixer for 30 min. Astaxanthin were added to contain levels according to formula. Astaxanthin source was a synthetic one and bought from Hoffman-La Roche, Switzerland. Vitamin A, C and E were added in experimental diets at levels of 20 000 IU kg^{-1}, 200 and 100 mg

kg^{-1}, respectively. The homogenized mixture of each diet's mesh was pelletized by a pelleting machine (CPM, California Pelleting Machine), streamed for 5 min and then dried by hot air oven at 60 °C for 2 hr. The pelleted dietary size was 3 mm of diameter and 5 mm of length. The pelleted diets were kept in dark container and flushed with nitrogen gas before storage at -20 °C.

Shrimp used in the experiment were subadult *P. monodon* at 48-50 g body weight in female and 35-38 g body weight in male. All of the shrimp were collected from a commercial earthen pond at the age of 6 months. The broodstocks were acclimated under experimental condition (temperature 28 ± 1 °C and salinity 35 ppt) for at least 15 days before beginning of the experiment. Shrimp were doubly tagged, a plastic numbering glued at the carapace and a rubber tube with the same number around the eyestalk for biological data monitoring on an individual basis such as molting, and/or ovary maturation. At initial, all broodstock shrimp were randomly selected into each experiment unit.

The rearing system in this experiment was an indoor closed recirculating water system covered with shading to reduce light intensity of about 100%. The system was a circular shape, consisted a rearing tank with 30 tons of water and a biological treatment unit of 8 tons of water at the center. Water depth was maintained at 1.0 m. The detail of the experimental pond has been described by Menasveta (1982). Thirty two rectangular net cages with 0.36 m^2 of rearing area each were established in the rearing unit. At initial, 2 broodstock shrimp, 1 female and 1 male were stocked in each cage.

The experimental feeding regime, pelleted diet and fresh diet were fed 3 times a day at 0600, 1200 and 1800. The fresh diet was chopped squid (*Loligo sp.*) given 10% of shrimp body weight at 0600. The pelleted diets fed at 1200 and 1800 for about

2% of shrimp weight a day. Uneaten diets, fecal matters and particular detritus were removed before the first feed.

The newly molted female broodstocks were induced to spawn by unilateral eyestalk ablation. In female shrimp, ovarian development was observed by flashing a light through dorsal part of the abdomen every 2 days (Motoh, 1981). The gravid broodstock in ripe stage was sacrificed for detecting number of eggs and tissues sampling of muscle, hepatopancreas, ovary and shell for analyzing astaxanthin contents and fatty acids concentration. In male, spermatophores were obtained by electrical stimulation at 2-4 volts, and 0.3-0.5 amperes using a method similar to that described by Sandifer et al. (1984). Amount of spermatozoa was determined at the end of experiment in term of total sperm count followed by Leung-Trujillo and Lawrence (1987). The broodstocks growth rates were measured by weighting every 30 day. Survival of shrimp was not determined because dead shrimp were replaced with the new same sex shrimp in the first two months of experiment. At the end of experiment, the remainder shrimp were sacrificed and then muscle, hepatopancreas, ovary and shell were collected for astaxanthin and fatty acid analysis using high performance liquid chromatography and gas chromatography as method described by Weber (1988) and Christie (1989), respectively.

The experimental diets were analyzed for crude protein, lipid, ash, fiber and moisture with methods as described by AOAC (1995). Ammonia, nitrite and nitrate (NH_3-N, NO_2-N and NO_3-N) in water were determined weekly by using test kits (Sera, Germany). Temperature, dissolved oxygen, alkalinity, and pH were monitored every day during the experiment period. Effects of astaxanthin, fish oil and sex on weight gain, amount of eggs and spermatozoa, astaxanthin contents and fatty acid

concentration were analyzed using Analysis of Variance and Duncan's New Multiple Range Test (Cody and Smith, 1997).

3. Results

Water quality at the experiment was maintains as described in **Table 2**. All water quality parameters were in the range that was suitable for aquatic animals. The proximate analysis of experimental diets and astaxanthin content are show in **Table 3**. Average protein content in LFLA, LFHA, HFLA and HFHA diets was 48.64 ± 1.71, 48.96 ± 0.28, 47.52 ± 0.20 and 48.83 ± 0.83%, respectively. Astaxanthin concentrations in LFLA, LFHA, HFLA and HFHA diets were 45.58 ± 1.71, 296.41 ± 2.86, 46.16 ± 1.30 and 264.55 ± 6.00 mg kg^{-1}, respectively. The fatty acid contents in diets and fresh diet, squid was showed in **Table 4**.

The effect of dietary fish oil, astaxanthin and sex on average weight gain of shrimp is shown in **Fig. 1**. There was no significant (P>0.05) interaction between fish oil and astaxanthin, and fish oil and sex on average weight gain but astaxanthin and sex had significant (P<0.05) interaction. The effect of fish oil on average weight gain was discussed separately, while the effect of astaxanthin and sex on average weight gain was reported together. Shrimp fed diet containing fish oil 3 and 8% had not significantly different (P>0.05) on average weight gain after 4 months of experiment. In shrimp fed diet supplemented with astaxanthin 100 mg kg^{-1}, female shrimp had significantly higher average weight gain (P<0.05) than those of male. In shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1}, no significant difference (P>0.05) of sex was found on average weight gain. In female, there was not significant difference (P>0.05) among levels of astaxanthin on average weight gain, while male shrimp fed

supplemented with astaxanthin 500 mg kg^{-1} had significantly greater average weight gain (P<0.05) than those of obtained astaxanthin 100 mg kg^{-1}.

Effect of fish oil and astaxanthin on amount of egg in female shrimp is shown in **Fig. 2**. The interaction between fish oil and astaxanthin on amount of egg had not significant difference (P>0.05). Therefore, the effects of fish oil and astaxanthin on amount of egg were discussed independently. Female shrimp fed diet containing 8% fish oil had significantly greater amount of eggs (P<0.05) than those fed diet containing 3% fish oil. Female shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1} had higher amount of eggs (P<0.05) than those fed diet supplemented with astaxanthin 100 mg kg^{-1}.

The effect of dietary fish oil and astaxanthin on amount of spermatozoa is shown in **Fig. 3**. The effects of fish oil and astaxanthin on amount of spermatozoa were discussed independently because there was no significant (P>0.05) interaction between dietary fish oil and astaxanthin. Male shrimp fed diet containing 8% fish oil had significantly greater amount of spermatozoa (P<0.05) than those fed diet containing 3% fish oil. Male shrimp fed diet supplement with astaxanthin 500 mg kg^{-1} had higher amount of spermatozoa (P<0.05) than those fed diet supplemented with astaxanthin 100 mg kg^{-1}.

The effect of dietary fish oil, astaxanthin and sex on astaxanthin accumulation in muscle is presented in Fig. **4a**. There was no significant (P>0.05) interaction between fish oil and astaxanthin, fish oil and sex, and astaxanthin and sex on astaxanthin accumulation in muscle. Therefore, the effects of fish oil, astaxanthin and sex on astaxanthin accumulation in muscle were discussed independently. No significant differences (P>0.05) among levels of astaxanthin and sex were found on astaxanthin content in muscle. Shrimp fed diet supplemented with astaxanthin 500 mg

kg^{-1} had significantly higher astaxanthin content in muscle ($P<0.05$) than those fed diet supplemented with astaxanthin 100 mg kg^{-1}.

The effect of dietary fish oil, astaxanthin and sex on astaxanthin accumulation in hepatopancreas of shrimp is shown in **Fig. 4b**. There was no significant ($P>0.05$) interaction between fish oil and astaxanthin, and fish oil and sex on astaxanthin accumulation in hepatopancreas but interaction between astaxanthin and sex was found significant difference ($P<0.05$). Thus, the effect of fish oil on astaxanthin accumulation in hepatopancreas was discussed individually, while the effect of astaxanthin and sex on astaxanthin accumulation in hepatopancreas was discussed together. There was not significantly different ($P>0.05$) between 2 levels of fish oil on astaxanthin content in hepatopancreas during experimental period. In shrimp fed diet supplemented with astaxanthin 100 mg kg^{-1}, no significant difference ($P>0.05$) of sex was found on astaxanthin accumulation in hepatopancreas. In shrimp fed diet supplemented with astaxanthin 500 mg kg^{-1}, female shrimp had significantly higher astaxanthin accumulation in hepatopancreas ($P<0.05$) than those of male. Female shrimp fed supplemented with astaxanthin 500 mg kg^{-1} had significantly greater astaxanthin accumulation in hepatopancreas ($P<0.05$) than those of obtained astaxanthin 100 mg kg^{-1}, while in male shrimp, there was not significant difference ($P>0.05$) among levels of astaxanthin on astaxanthin accumulation in hepatopancreas.

The effect of dietary fish oil and astaxanthin on astaxanthin accumulation in ovary is shown in **Fig. 4c**. The effects of fish oil and astaxanthin on astaxanthin accumulation in ovary were discussed independently because there was no significant ($P>0.05$) interaction between them. No significant difference ($P>0.05$) of fish oil was found on different astaxanthin content in ovary, while female shrimp fed diet

supplemented with astaxanthin 500 mg kg^{-1} had significant higher astaxanthin content in ovary (P<0.05) than those fed diet supplemented with 100 mg kg^{-1}.

The effect of dietary fish oil, astaxanthin and sex on astaxanthin accumulation in shell is presented in **Fig. 4d**. There was no significant (P>0.05) interaction between fish oil, astaxanthin and sex on astaxanthin accumulation in shell. Therefore, the effects of fish oil, astaxanthin and sex on astaxanthin accumulation in the shell were discussed independently. There was no significantly different (P>0.05) in levels of fish oil, astaxanthin, and sex on astaxanthin content in shell.

Fatty acid contents in muscle, hepatopancreas and ovary of female and male broodstock are shown in **Table 5**. The effects of fish oil, astaxanthin and sex on on fatty acid content in muscle, hepatopancreas and ovary were discussed independently because there were no significant (P>0.05) interaction between fish oil and astaxanthin, fish oil and sex, and astaxanthin and sex.

The effects of fish oil is shown in **Table 6**. No significant difference (P>0.05) of dietary fish oil was found on 18:2(n-6), 18:3(n-3), and total saturated contents in muscle, hepatopancreas and ovary. Shrimp fed diet containing 8% fish oil had significant higher 20:4(n-6), 20:5(n-3), total monoenes and total n-6 PUFA contents in hepatopancreas (P<0.05) than those fed diet containing 3% fish oil. Shrimp fed diet containing 8% fish oil had significant higher 22:6(n-3), total n-3 PUFA and total n-3 HUFA contents in muscle, hepatopancreas and ovary (P<0.05) than those fed diet containing 3% fish oil.

The effect astaxanthin is shown in **Table 7**. There was no significant (P>0.05) of 18:2(n-6), 18:3(n-3), 20:4(n-6), 20:5(n-3), total saturated, total monoenes and total n-6 PUFA contents in muscle, hepatopancreas and ovary on shrimp fed diet containing differed levels of astaxanthin. While, shrimp fed diet supplemented with

astaxanthin 500 mg kg^{-1} had significant greater 22:6(n-3), total n-3 PUFA and total n-3 HUFA contents in muscle and ovary than those fed diet supplemented with astaxanthin 100 mg kg^{-1}. The effect of sex on fatty acid content in muscle, hepatopancreas and ovary is shown in **Table 8**. There was no any significant (P>0.05) of all fatty acid contents in muscle, hepatopancreas and ovary between male and female shrimp.

4. Discussion

Many reports studied potential of pond-reared shrimp broodstock for larvae production by comparing with wild-caught broodstock (Menasveta et al., 1993, 1994b; Sangpradub et al., 1994; Ramos et al., 1995; Cavalli et al., 1997; Palacios et al., 1999). Menasveta et al. (1994b) reported total eggs produced by large females was significantly greater than for small shrimp; nevertheless, shrimp source (pond-reared and wild-caught) and size had no significant influence on amount of eggs spawned per spawning event. Palacios et al. (1999) reported that wild-caught shrimp had higher mating and spawning frequencies compared to pond-reared broodstock. However, the number of nauplii per spawn was higher for wild shrimp, but fertilization and hatching rates were higher for pond-reared spawners.

The result of this study illustrated that fish oil could enhance reproductive performance in pond-reared broodstock shrimp. Shrimp fed diet containing high level of fish oil can produce higher amount of eggs and spermatozoa than those fed diet containing low level (**Fig. 2** and **3**) and they accumulated high contents of 20:4(n-6), 20:5(n-3) and total n-6 PUFA in hepatopancreas (**Table 6**). Many reports noted that n-6 PUFA, especially 20:4(n-6), is precursor in the synthesis of prostaglandins in vertebrates and insects which serve many functions in reproduction, spanwing and

larval production (Middleditch et al., 1979; Johnson et al., 1983; Spaziani et al., 1991; Alava et al., 1993; Harrison, 1997).

Our study found that 22:6(n-3) had the most effect to improve reproductive performance in pond-reared broodstock shrimp. The positive relationship between 22:6(n-3), total n-3 PUFA and n-3 HUFA contents in muscle, hepatopancreas and ovary, and amount of egg and spermatozoa in pond-reared broodstock shrimp was found on this study. Shrimp fed diet supplemented with high level of fish oil had significantly higher amount of egg and spermatozoa, and 22:6(n-3), total n-3 PUFA and n-3 HUFA contents in muscle, hepatopancreas and ovary than those fed diet supplement with low level of fish oil. This was also reported by Kanazawa et al. (1979) and Xu et al. (1994) as 22:6(n-3) had more effective as an essential fatty acid than other essential fatty acids. Cerolini et al. (1997) and Conner et al. (1997) noted that n-3 and n-6 PUFA may have important roles for sperm production. The results of this study indicate that fish oil contained high contents of total n-3 PUFA and n-3 HUFA that could improve reproductive performance of pond-reared shrimp.

The current study indicated that astaxanthin could improve reproductive performance of pond-reared shrimp. Shrimp fed diet supplemented with high level of astaxanthin had significantly higher amount of egg and spermatozoa than those fed diet supplemented with low level of astaxanthin. Moreover, high astaxanthin accumulation in muscle and ovary were detected in shrimp fed diet supplemented with high level of astaxanthin (**Fig. 4a** and **4c**). A similar several previous studies showed the important effects of astaxanthin on ovarian maturation (Menasveta et al., 1994a; Sagi et al., 1996; Pangantihon-Kuhlmann et al., 1998; Ribeiro et al., 2001). During ovarian maturation, crustaceans accumulate and mobilize astaxanthin from

hepatopancreas to ovary via the haemolymph with lipovitellin in the oocytes (Nelson et al., 1988; Quinito et al., 1989; 1990; Harrison, 1990).

Astaxanthin may be promoting amount of spermatozoa in male broodstock via the provitamin A activity. Ratinoid act on the testicular development, especially on germ cell, via retinoic acid receptor and/or retinoid X receptors (Boulogne et al., 1999). Akmal et al.(1997) reported that mutational studies of spermatid have identified retinoic acid receptor alpha as having an essential role in spermatogenesis. Characterization of retinoic acid receptor alpha expression revealed the time and location of the vitamin A requirement during spermatogenesis.

In this study, the amounts of astaxanthin in diet supplemented with astaxanthin 100 and 500 mg kg^{-1} were 45.87 ± 1.51 and 280.48 ± 4.43 mg kg^{-1}, repectively. The analytical concentration of astaxanthin in diet supplemented with astaxanthin 500 mg kg^{-1} was less than the expected concentration due to loss of astaxanthin during feed pelleting process and feed storage.

This study found that shell was the main tissue for astaxanthin deposit and muscle and hepatopancreas were the subsequent tissues for astaxanthin deposit. Similar with reported by Negre-Sandargues et al. (1993) as dietary astaxanthin was found to be stored in the integument (carapace and epidermis) and hepatopancreas. Moreover, the all-trans-astaxanthin was found as the main form of astaxanthin in this study. The 9-cis and 13-cis astaxanthin was found minor content. This was also reported by Muriana et al. (1993) as study of astaxanthin identify using HPLC suggested all-trans-astaxanthin to be the main component, which was accompanied by an epimer and its cis-isomer.

This study was not detected the interaction between dietary fish oil and astaxanthin, and fish oil and sex, while dietary astaxanthin had interaction with sex on

astaxanthin content in hepatopancreas and growth of shrimp (**Fig. 1** and **4b**). Female shrimp fed diet containing high level of astaxanthin had significantly higher accumulate astaxanthin content in hepatopancreas than those fed diet containing low level of astaxanthin but male shrimp had not significantly different astaxanthin content in hepatopancreas among levels of astaxanthin supplemented in shrimp diet. Astaxanthin had high accumulated in muscle, hepatopancreas and ovary of female shrimp fed diet containing high astaxanthin supplementation, while it had only high accumulated in muscle of male shrimp. This indicated that both sexes of shrimp had different rates of astaxanthin accumulation and transfer among tissues during reproductive maturation. Dall et al. (1995) reported that the maturing ovary of *P. esculentus* contained high levels of carotenoids.

The interaction between astaxanthin and sex on weight gain indicated that both sexes of shrimp required astaxanthin for growth at different levels. Effect of astaxanthin on weight gain had not found in female broodstock shrimp but detected only in male broodstock shrimp. Male shrimp fed diet containing high level of astaxanthin had significantly greater weight gain than those fed diet containing low level of astaxanthin. It may be that female shrimp accumulated high content of astaxanthin during ovarian maturation. The astaxanthin content had higher than the requirement of astaxanthin for growth. So, the study found no effect of astaxanthin on growth of female shrimp. However, Negre-Sandargues et al. (1993) reported that shrimp fed diet supplemented with astaxanthin were not significantly different on growth rate.

The result of this study found that fish oil had not effect on growth of pond-reared broodstock shrimp after 4 month of experiment. The average total lipids of experimental diet supplemented with 3 and 8% fish oil were 7.74 ± 0.10 and 11.78 ±

0.22%, respectively. The total lipids in diets of this study found in range of lipid requirement levels for juvenile to broodstock shrimp. In crustacean diet, the best survival and growth responses are achieved when the dietary level of one oil or a mixture of oils is between 5 to 8% (D'Abramo, 1997). Bray et al. (1990) reported that the broodstock diet supplemented with 11.1% lipid has high nauplii production.

The result of present study found that shrimp fed diet containing high level of astaxanthin had higher astaxanthin content in muscle and ovary than those fed diet containing low level of astaxanthin ovary (**Fig. 4a** and **4c**). The accumulated astaxanthin in muscle and ovary had effect on accumulation of 22:6(n-3), total n-3 PUFA and total n-3 HUFA contents in muscle and ovary (**Table 7**). It may be that the accumulated astaxanthin esterified to long chain fatty acids, especially 22:6(n-3). So, the increasing of fatty acid content in muscle and ovary was found, when shrimp had increased astaxanthin content in their tissues. Meyers and Latscha (1997) reported that astaxanthin is present in nature either in the free form, esterified to long chain fatty acids, or associated with proteins forming carotenoproteins. All three forms are found in crustaceans.

In summary, dietary fish oil (HUFA) and astaxanthin can promote reproductive performance in pond-reared broodstock *P. monodon* of both sexes. In the current result, 20:4(n-6) and 22:6(n-3) were prominent fatty acids to promote amount of egg and spermatozoa in pond-reared broodstock. The practical pond-reared broodstock shrimp diet supplemented with astaxanthin 300 mg kg^{-1} and/or 12.0% lipid could enhance reproductive performance of broodstock shrimp *P. monodon*.

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Table 1. Feed ingredients of broodstock shrimp diets.

Ingredients	Dry weight (g 100 g^{-1} of diet)			
	LFLA	LFHA	HFLA	HFHA
Fish meal	56	56	56	56
Shrimp head meal	10	10	10	10
Wheat flour	16	16	16	16
Refined tuna fish oil	3	3	8	8
Chlorophyll pink[a]	0.125	0.625	0.125	0.625
Cellulose	9.758	9.258	4.758	4.258
Mineral mixture[b]	1	1	1	1
Vitamin mixture[c]	1	1	1	1
Cholesterol[d]	1	1	1	1
Lecithin[e]	1	1	1	1
Binder[f]	1	1	1	1
Vitamin A[g]	0.04	0.04	0.04	0.04
Vitamin C[h]	0.057	0.057	0.057	0.057
Vitamin E[i]	0.02	0.02	0.02	0.02
Total	100	100	100	100

[a]Chlorophyll pink contains 8% active form of astaxanthin, Roche.

[b]Mineral mixture 100 g contains: K_2HPO_4 2.0 g, $Ca_3(PO_4)_2$ 2.720 g, $MgSO_4$ $7H_2O$ 3.041 g, NaH_2PO_4 $2H_2O$ 0.790 g.

[c]Vitamin mixture 10 g contains: ρ-aminobenzoic acid 10.0 mg; biotin 0.40 mg, inositol 400.0 mg; nicotinic acid, 40.0 mg; Ca-pantothenate, 60.0 mg; pyridoxine-HCl, 12.0 mg; riboflavin, 8.0 mg; thiamin-HCl, 4.0 mg; menadione, 4.0 mg; cyanocobalamine, 0.08 mg; calciferol, 1.20 mg; folic acid, 0.80 mg; choline chloride, 120.0 mg.

[d]Ninety five percent cholesterol, laboratory grade, Sigma.

[e]Soy lecithin, feed grade.

[f]Aquabind, Du Pont.

[g]Five hundred thousand IU g^{-1}, feed grade, Roche.

[h]Stay C 35%, Roche.

[i]Fifty percent, feed grade, Roche.

Table 2. Water quality of the broodstock shrimp experiment.

Parameters	Range
Salinity (ppt)	35 ± 1
Temperature (°C)	28 ± 1
Dissolved oxygen (mg l^{-1})	6.5-7.7
Alkalinity (mg l^{-1})	148-220
pH	7.5-8.5
Ammonia (mg l^{-1})	0-0.5
Nitrite (mg l^{-1})	0-0.3
Nitrate (mg l^{-1})	0-20

Table 3. Proximate analysis of experimental broodstock diets as fed basis (means ± s.d.).

Proximate	Diets			
	LFLA	LFHA	HFLA	HFHA
Protein (%)	48.64 ± 1.71	48.96 ± 0.28	47.52 ± 0.20	48.83 ± 0.83
Lipid (%)	7.70 ± 0.03	7.78 ± 0.16	11.51 ± 0.13	12.04 ± 0.30
Moisture (%)	13.56 ± 0.11	12.18 ± 0.08	12.95 ± 0.16	12.87 ± 0.23
Ash (%)	12.51 ± 0.10	12.52 ± 0.37	12.20 ± 0.51	12.40 ± 0.06
Fiber (%)	2.83 ± 0.04	2.92 ± 0.31	2.96 ± 0.17	2.61 ± 0.11
NFE[a] (%)	14.76 ± 1.85	15.64 ± 0.16	12.86 ± 0.85	11.25 ± 0.82
Astaxanthin(mg kg^{-1})	45.58 ± 1.71	296.41 ± 2.86	46.16 ± 1.30	264.55 ± 6.00

[a]NFE = Nitrogen free extract.

Table 4. Fatty acid composition of the experimental diet and squid (means ± s.d., % of total fatty acids).

Fatty acid	Pelleted diets				Squid
	LFLA	LFHA	HFLA	HFHA	
14:0	4.1 ± 0.2	4.8 ± 0.1	3.0 ± 0.3	4.3 ± 0.2	2.2 ± 0.4
16:0	22.8 ± 1.0	23.9 ± 3	17.3 ± 2.1	22.0 ± 0.1	26.3 ± 0.6
16:1(n-7)	4.3 ± 0.3	5.2 ± 0.1	3.2 ± 0.3	4.7 ± 0.3	0.6 ± 0.1
18:0	6.0 ± 0.0	5.5 ± 1.0	6.8 ± 0.5	6.3 ± 0.2	7.7 ± 0.5
18:1(n-9)	15.7 ± 0.4	12.9 ± 1.0	14.1 ± 1.4	13.6 ± 0.1	2.8 ± 0.1
18:1(n-7)	2.7 ± 0.1	2.6 ± 0.2	3.7 ± 0.6	2.7 ± 0.1	1.3 ± 0.1
18:2(n-6)	11.9 ± 0.2	9.0 ± 0.7	9.4 ± 1.2	7.6 ± 0.1	0.2 ± 0.1
19:0	0.3 ± 0.0	0.4 ± 0.1	0.9 ± 0.2	0.4 ± 0.0	0.2 ± 0.0
18:3(n-3)	1.1 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	0.0 ± 0.1
18:4(n-3)	1.3 ± 0.0	1.2 ± 0.2	1.5 ± 0.1	1.4 ± 0.0	0.3 ± 0.1
20:0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	nd[a]
20:1(n-9)	0.6 ± 0.1	0.7 ± 0.0	0.9 ± 0.0	0.8 ± 0.1	1.5 ± 0.6
20:3(n-6)	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	nd
20:4(n-6)	1.4 ± 0.1	1.4 ± 0.2	1.8 ± 0.1	1.7 ± 0.3	5.3 ± 0.5
20:4(n-3)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.0 ± 0.0
20:5(n-3)	6.7 ± 0.1	6.6 ± 0.4	7.7 ± 0.2	7.1 ± 0.1	8.7 ± 0.3
21:5(n-3)	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.2 ± 0.0	0.1 ± 0.1
22:5(n-6)	0.9 ± 0.0	1.1 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	2.2 ± 0.2
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0 .0 ± 0.0	nd
22:5(n-3)	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.0	0.7 ± 0.2
22:6(n-3)	14.9 ± 0.3	16.6 ± 1.5	20.6 ± 0.3	19.2 ± 0.4	36.2 ± 0.6
Σ Saturated[b]	35.5 ± 1.3	37.8 ± 2.4	30 ± 2.1	35.4 ± 0.3	38.5 ± 0.7
Σ Monoenes	24.3 ± 0.4	23.1 ± 0.7	23.8 ± 1.3	23.0 ± 0.2	7.0 ± 0.4
Σ n-6 PUFA	14.5 ± 0.4	12.0 ± 0.5	13.4 ± 1.1	11.0 ± 0.1	8.3 ± 0.4
Σ n-3 PUFA	25.7 ± 0.5	27.1 ± 2.3	32.8 ± 0.3	30.5 ± 0.6	46.2 ± 0.7
Σ n-3 HUFA[c]	23.2 ± 0.5	25.0 ± 2.0	30.2 ± 0.3	28.1 ± 0.6	45.9 ± 0.6

[a]nd: not detected.

[b]Sums include minor fatty acids not shown in table.

[c] 20:3(n-3).

Table 5. Fatty acid composition (means ± s.d., % of total fatty acids) in muscle, hepatopancreas and ovary of female, and muscle and hepatopancreas of male broodstock *P. monodon*.

Fatty acids	Female			Male	
	Muscle	Hepatopancreas	Ovary	Muscle	Hepatopancreas
14:0	2.6 ± 2.3	1.1 ± 1.0	1.3 ± 1.6	3.6 ± 3.4	1.9 ± 2.1
16:0	36.0 ± 21.8	14.4 ± 13.9	15.1 ± 14.8	39.1 ± 23.1	24.4 ± 20.4
16:1(n-7)	4.4 ± 3.4	1.7 ± 1.8	1.6 ± 1.7	4.8 ± 3.6	3.4 ± 3.1
18:0	9.2 ± 5.1	10.5 ± 4.7	9.6 ± 3.2	8.0 ± 5.1	7.1 ± 1.7
18:1(n-9)	8.6 ± 4.8	19.0 ± 7.2	19.7 ± 5.9	7.0 ± 4.6	15 ± 7.8
18:1(n-7)	1.4 ± 0.8	4.2 ± 1.4	3.4 ± 1.1	1.1 ± 0.8	3.2 ± 1.5
18:2(n-6)	5.0 ± 3.1	7.2 ± 4.2	3.7 ± 2	4.6 ± 3.1	7.0 ± 4.3
19:0	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
18:3(n-3)	0.3 ± 0.2	0.5 ± 0.3	0.2 ± 0.1	0.2 ± 0.2	0.6 ± 0.3
18:4(n-3)	0.5 ± 0.4	0.8 ± 0.3	0.7 ± 0.2	0.5 ± 0.3	0.8 ± 0.4
20:0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:1(n-9)	0.4 ± 0.2	2.2 ± 1.1	1.2 ± 0.4	0.3 ± 0.2	1.3 ± 0.4
20:3(n-6)	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
20:4(n-6)	5.5 ± 3.0	4.9 ± 2.5	6.5 ± 2.3	4.9 ± 3.4	3.1 ± 0.8
20:4(n-3)	0.0 ± 0.0	0.2 ± 0.2	0.1 ± 0.0	0.0 ± 0.0	0.3 ± 0.2
20:5(n-3)	9.8 ± 5.4	8.1 ± 2.5	11.3 ± 4.2	8.5 ± 5.5	5.7 ± 2
21:5(n-3)	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.1
22:5(n-6)	0.5 ± 0.3	1.4 ± 0.6	1.1 ± 0.3	0.5 ± 0.4	1.2 ± 0.6
24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
22:5(n-3)	0.2 ± 0.2	0.7 ± 0.6	0.5 ± 0.4	0.2 ± 0.2	1.1 ± 0.6
22:6(n-3)	10.6 ± 6.3	17.3 ± 10.1	18.2 ± 10.9	11.1 ± 7.4	18.1 ± 8.3
Σ Saturated[a]	52.1 ± 19.9	28.4 ± 14.7	28.8 ± 16	55.5 ± 22	36.6 ± 22.6
Σ Monoenes	15.5 ± 3.2	29.4 ± 8.4	28.2 ± 5.2	14.1 ± 2.6	24.7 ± 6.5
Σ n-6 PUFA	11.0 ± 6.0	14.1 ± 4.4	11.7 ± 4.0	10.0 ± 6.6	11.8 ± 5.2
Σ n-3 PUFA	21.5 ± 11.5	28.1 ± 9.7	31.4 ± 11.9	20.4 ± 13.2	26.8 ± 11.7
Σ n-3 HUFA[b]	20.7 ± 11.1	26.7 ± 9.8	30.5 ± 11.9	19.7 ± 12.8	25.4 ± 11.1

[a]Sums include minor fatty acids not shown in table.

[b] 20:3(n-3).

Table 6. Effect of fish oil on fatty acid content (means ± s.d., mg g^{-1} dry weight) in muscle, hepatopancreas and ovary. Mean with the different superscripts within each row indicate a significant difference ($P<0.05$).

Fatty acid	Tissue	Fish oil (%)	
		3	8
18:2(n-6)	Muscle	1.42 ± 0.28	1.33 ± 0.50
	Hepatopancreas	8.93 ± 6.49	11.42 ± 8.82
	Ovary	5.10 ± 0.8	3.10 ± 2.45
18:3(n-3)	Muscle	0.06 ± 0.05	0.07 ± 0.05
	Hepatopancreas	0.64 ± 0.52	0.97 ± 0.65
	Ovary	0.20 ± 0.08	0.26 ± 0.13
20:4(n-6)	Muscle	1.53 ± 0.32	1.55 ± 0.25
	Hepatopancreas	3.91^{b} ± 1.72	7.82^{a} ± 5.89
	Ovary	6.65 ± 1.01	6.84 ± 1.26
20:5(n-3)	Muscle	2.68 ± 0.19	2.69 ± 0.36
	Hepatopancreas	7.40^{b} ± 3.89	12.85^{a} ± 6.74
	Ovary	11.23 ± 1.45	12.34 ± 1.27
22:6(n-3)	Muscle	2.62^{b} ± 0.99	3.65^{a} ± 0.45
	Hepatopancreas	13.11^{b} ± 9.59	37.76^{a} ± 15.24
	Ovary	11.08^{b} ± 11.72	29.68^{a} ± 7.19
Σ Saturated	Muscle	27.10 ± 30.37	23.18 ± 22.48
	Hepatopancreas	39.69 ± 32.33	51.62 ± 30.94
	Ovary	43.10 ± 35.2	66.28 ± 56.24
Σ Monoenes	Muscle	5.59 ± 3.88	5.30 ± 2.39
	Hepatopancreas	30.57^{b} ± 20.65	49.43^{a} ± 19.81
	Ovary	27.98 ± 4.71	36.54 ± 12.02
Σ n-6 PUFA	Muscle	3.08 ± 0.27	3.03 ± 0.56
	Hepatopancreas	14.78^{b} ± 8.86	22.90^{a} ± 11.94
	Ovary	13.25 ± 1.75	11.7 ± 2.58
Σ n-3 PUFA	Muscle	5.58^{b} ± 0.95	6.65^{a} ± 0.64
	Hepatopancreas	23.49^{b} ± 12.62	55.79^{a} ± 21.3
	Ovary	23.98^{b} ± 12.57	44.24^{a} ± 8.57
Σ n-3 HUFA	Muscle	5.37^{b} ± 0.96	6.43^{a} ± 0.64
	Hepatopancreas	22.00^{b} ± 11.86	53.37^{a} ± 20.58
	Ovary	23.08^{b} ± 12.52	43.26^{a} ± 8.47

Table 7. Effect of astaxanthin on fatty acid content (means ± s.d., mg g^{-1} dry weight) in muscle, hepatopancreas and ovary. Mean with the different superscripts within each row indicate a significant difference ($P<0.05$).

Fatty acid \	Tissue	Astaxanthin (mg kg^{-1})	
		100	500
18:2(n-6)	Muscle	1.43 ± 0.24	1.32 ± 0.52
	Hepatopancreas	9.63 ± 4.87	10.8 ± 9.82
	Ovary	4.68 ± 1.95	3.44 ± 2.24
18:3(n-3)	Muscle	0.08 ± 0.04	0.05 ± 0.05
	Hepatopancreas	0.77 ± 0.51	0.85 ± 0.70
	Ovary	0.25 ± 0.13	0.22 ± 0.11
20:4(n-6)	Muscle	1.59 ± 0.32	1.49 ± 0.24
	Hepatopancreas	6.99 ± 6.00	5.06 ± 3.32
	Ovary	6.52 ± 1.07	6.94 ± 1.19
20:5(n-3)	Muscle	2.65 ± 0.23	2.72 ± 0.34
	Hepatopancreas	11.01 ± 7.00	9.62 ± 5.39
	Ovary	11.52 ± 0.81	12.1 ± 1.79
22:6(n-3)	Muscle	2.86^{b} ± 1.09	3.43^{a} ± 0.62
	Hepatopancreas	22.53 ± 18.39	29.34 ± 17.12
	Ovary	13.67^{b} ± 16.05	27.60^{a} ± 6.79
Σ Saturated	Muscle	27.8 ± 28.25	22.55 ± 24.7
	Hepatopancreas	47.84 ± 34.56	44.33 ± 29.82
	Ovary	39.78 ± 32.73	68.94 ± 55.81
Σ Monoenes	Muscle	5.89 ± 3.13	5.03 ± 3.17
	Hepatopancreas	39.83 ± 18.71	41.15 ± 25.24
	Ovary	27.45 ± 5.26	36.96 ± 11.44
Σ n-6 PUFA	Muscle	3.17 ± 0.33	2.95 ± 0.51
	Hepatopancreas	19.31 ± 8.65	18.85 ± 13.37
	Ovary	12.85 ± 2.71	12.02 ± 2.1
Σ n-3 PUFA	Muscle	5.86^{b} ± 1.02	6.39^{a} ± 0.85
	Hepatopancreas	37.67 ± 24.16	43.11 ± 24.11
	Ovary	26.92^{b} ± 17.66	41.88^{a} ± 7.88
Σ n-3 HUFA	Muscle	5.61^{b} ± 1.01	6.22^{a} ± 0.83
	Hepatopancreas	35.71 ± 23.69	41.10 ± 22.93
	Ovary	26.00^{b} ± 17.51	40.92^{a} ± 7.86

Table 8. Effect of sex on fatty acid content (means ± s.d., mg g^{-1} dry weight) in muscle, hepatopancreas and ovary. Mean with the different superscripts within each row indicate a significant difference ($P<0.05$).

Fatty acid	Tissue	Sex	
		Female	Male
18:2(n-6)	Muscle	1.34 ± 0.46	1.43 ± 0.31
	Hepatopancreas	10.65 ± 8.92	9.54 ± 5.53
	Ovary	3.99 ± 2.09	
18:3(n-3)	Muscle	0.07 ± 0.05	0.07 ± 0.05
	Hepatopancreas	0.79 ± 0.65	0.86 ± 0.55
	Ovary	0.23 ± 0.11	
20:4(n-6)	Muscle	1.55 ± 0.26	1.52 ± 0.32
	Hepatopancreas	6.90 ± 5.74	4.33 ± 1.56
	Ovary	6.76 ± 1.09	
20:5(n-3)	Muscle	2.72 ± 0.24	2.62 ± 0.36
	Hepatopancreas	11.48 ± 6.86	8.16 ± 4.05
	Ovary	11.84 ± 1.39	
22:6(n-3)	Muscle	3.03 ± 1.05	3.40 ± 0.51
	Hepatopancreas	25.57 ± 17.92	27.09 ± 18.3
	Ovary	21.41 ± 13.17	
Σ Saturated	Muscle	28.49 ± 30.95	18.91 ± 13.42
	Hepatopancreas	46.80 ± 30.28	44.55 ± 35.38
	Ovary	55.98 ± 46.85	
Σ Monoenes	Muscle	5.72 ± 3.69	4.94 ± 1.83
	Hepatopancreas	44.05 ± 23.67	34.30 ± 18.18
	Ovary	32.73 ± 10.05	
Σ n-6 PUFA	Muscle	3.03 ± 0.48	3.08 ± 0.39
	Hepatopancreas	20.53 ± 12.56	16.47 ± 8.18
	Ovary	12.39 ± 2.27	
Σ n-3 PUFA	Muscle	6.04 ± 1.07	6.32 ± 0.74
	Hepatopancreas	41.04 ± 23.88	39.66 ± 25.02
	Ovary	35.23 ± 14.49	
Σ n-3 HUFA	Muscle	5.83 ± 1.07	6.10 ± 0.74
	Hepatopancreas	39.02 ± 23.12	37.74 ± 24.04
	Ovary	34.29 ± 14.41	

Fig. 1. Effect of fish oil and sex on weight gain of broodstock *P. monodon* (As100, Astaxanthin 100 mg kg^{-1}; As500, Astaxanthin 500 mg kg^{-1}; F, Female; M, Male). The different superscripts within each group at the top of each bar are significantly different ($P<0.05$).

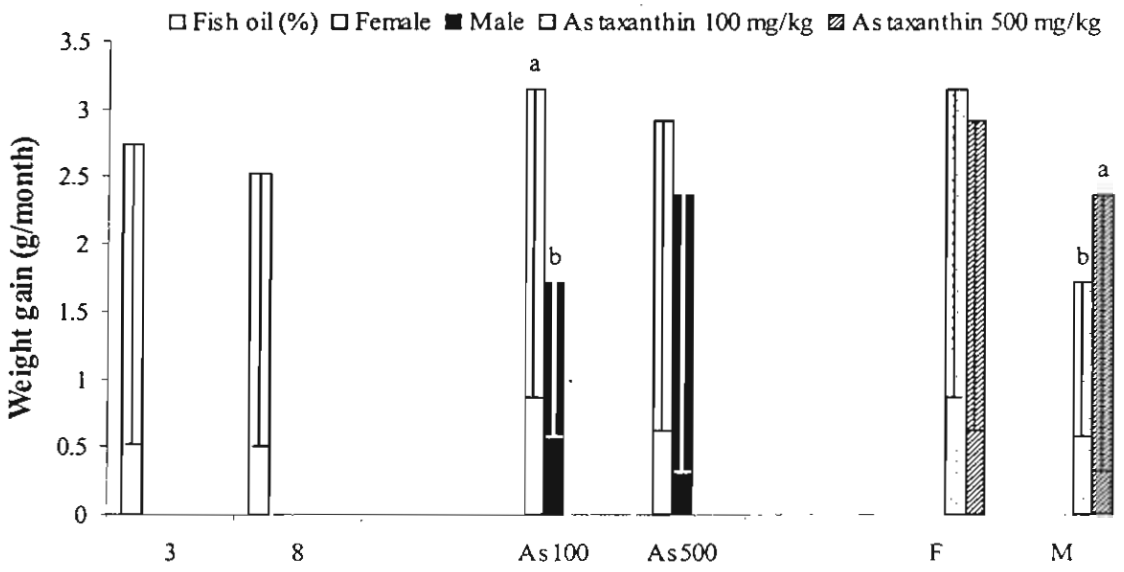

Fig. 2. Effect of fish oil and astaxanthin on amount of egg of female broodstock *P. monodon*. The different superscripts within each group at the top of each bar are significantly different ($P<0.05$).

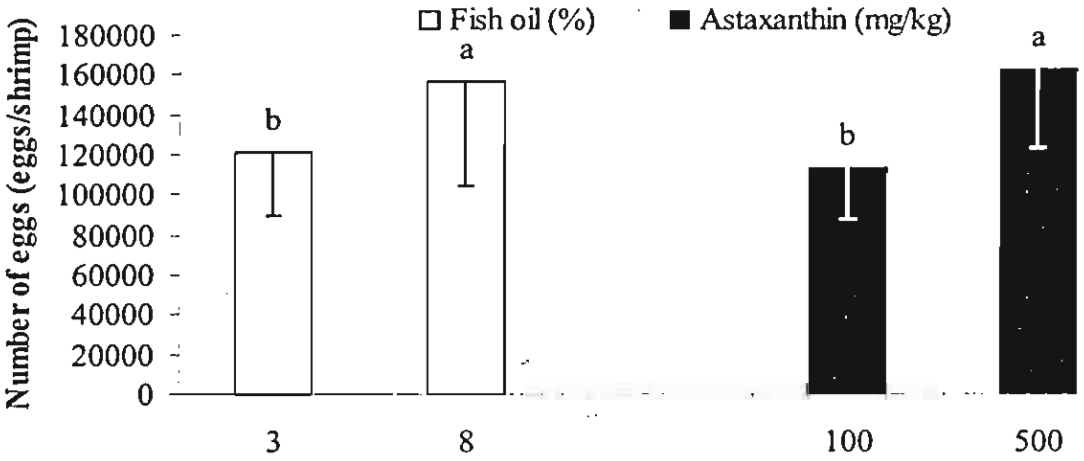

Fig. 3. Effect of fish oil and astaxanthin on amount of spermatozoa of male broodstock *P. monodon*. The different superscripts within each group at the top of each bar are significantly different ($P<0.05$).

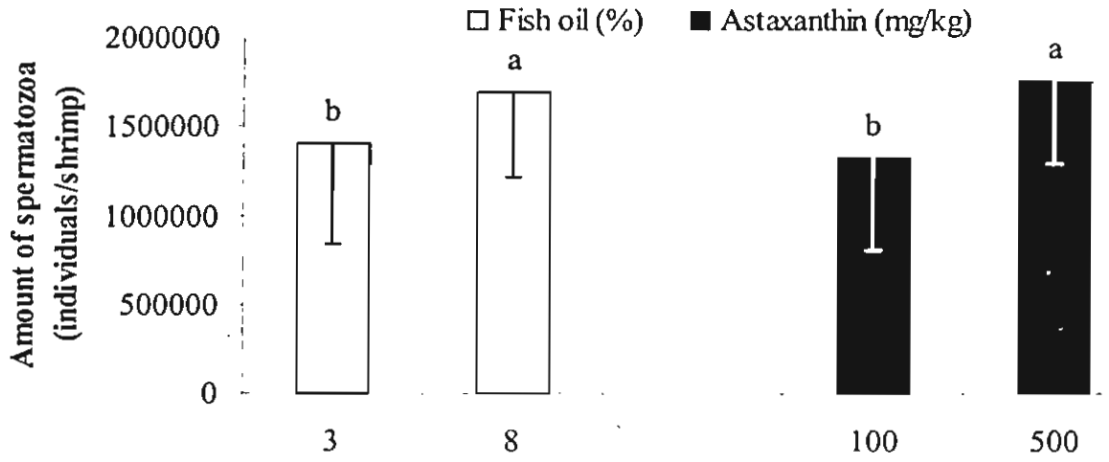

Fig. 4. Effects of fish oil, astaxanthin and sex on astaxanthin content in muscle (4a), hepatopancreas (4b), ovary (4c) and shell (4d) of broodstock *P. monodon* (As100, Astaxanthin 100 mg kg^{-1}; As500, Astaxanthin 500 mg kg^{-1}; F, Female; M, Male). The different superscripts within each group at the top of each bar are significantly different ($P<0.05$).

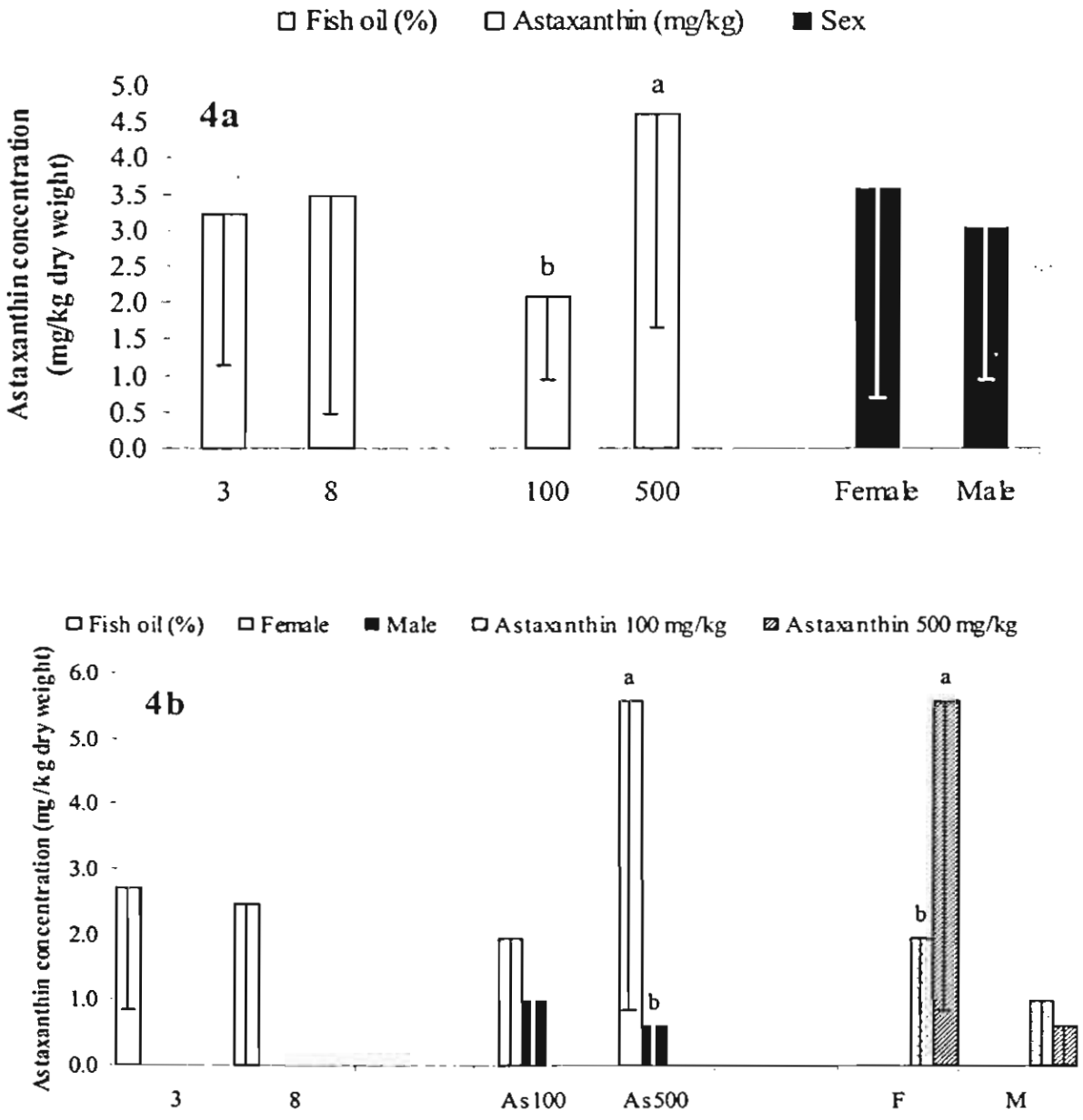

Fig. 4. (Continued)