



***Dunaliella salina* in aquatic organisms**

Fish

Carotenoids contribute to the health and reproduction of fish, as well as their pigmentation.

Pigmentation

In wild and cultured masu salmon (*Onchorhynchus macrostomus*), and in wild manchurian trout (*Brachymystax lenok*) the major carotenoids are zeaxanthin, betacarotene and isocryptoxanthin; lutein, canthaxanthin and astaxanthin are among the minor carotenoids (beta-carotene, zeaxanthin, and lutein are components of Nutra-Red™ and Algro™) (Baek and Ha, 1998).

In cultured flounder, *Paralichthys olivaceus*, colour was improved by feeding diets supplemented with beta-carotene or with lutein esters. The main carotenoids in the flesh of the flounder were lutein and zeaxanthin (Ha et al. 1993).

Marine fish larvae

In general, most marine fish larvae are cultured in tanks using 'green water' technique. Different algae such as *Nannochloropsis oculata*, *Tetraselmis* sp., *Isochrysis* sp. and others are added to the rearing tanks at different densities. It is unclear what is the role of the algae and what is the mechanism by which it contributes to the larvae (in most cases, the algae cell is too hard for the larvae to digest and often it will evacuate the larvae gut intact. However, it is clear that these algae are essential for larvae culture. Algae culture in hatchery is considered to be labour intensive as well as require light and in cold climate energy to heat the rearing containers (tanks, beg etc.). Recent results from preliminary studies demonstrated that barramundi (*Lates calcarifer*) larvae preforme similarly (both grow and survive) when live algae (*Nannochloropsis oculata*), green algae paste (*Chlorella* or *Nannochloropsis*) or *NutraPlus* was added to the rearing tanks in standard 'green water' technique.

Fish reproduction and health

The carotenoids beta-carotene, lutein, canthaxanthin and astaxanthin are found in the eggs, ovaries, testicles, and milt (sperm) of fish including salmon, trout and sole. Pigmented eggs have a higher rate of fertilisation than non-pigmented eggs. It is thought that the carotenoids may have a positive effect on sperm motility, may have a role in the respiration of the eggs, and may protect the eggs against UV light damage (Hamdorf, 1960; Hartmann, et al. 1947; Jitariu, et al. 1975)

When Japanese parrotfish (*Oplegnathus fasciatus*) and spotted parrotfish (*Oplegnathus punctatus*) were fed with rotifers which had been supplemented with beta-carotene, survival rates of larvae were higher, and production of lymphocytes in response to challenge was greater, indicating greater resistance to infections (Tachibana, et al. 1997).

In yellowtail, (*Seriola quinqueradiata*) the carotenoid content of eggs was greatly affected by the carotenoid in the broodstock diets. Egg quality was highest in eggs with a strong yellow colour and a high content of lutein and zeaxanthin (Verakunpiriya, et al. 1996).



Ornamental fish

Lutein: The colour of the native Korean bitterling (*Rhodeus uyekii*), is enhanced by addition of carotenoids to the diets; the best enhancement was with lutein supplementation (lutein is a component of *NutraPlus* and *Algro*TM) (Kim et al. 1999).

Carotenoid accumulation from diet is important in sexual colouration and subsequent breeding of guppies (*Poecilia reticulata*); the females prefer males with brighter orange carotenoid-containing spots (Grether, et al. 1999).

The skin of sailfin mollies, *Poecilia latipinna* contains the carotenoid, beta-carotene. Unlike some other poecillid fishes, sailfin mollies rely primarily on carotenoids for their pigmentation (Blanchard, et al. 1991)

Prawns, and other crustacea

Crustacea are capable converting carotenoids such as beta-carotene, alpha carotene, lutein to the red pigment astaxanthin. (Katayama, et al (1973) Astaxanthin forms a carotenoprotein, from which the astaxanthin is released when crustacea are cooked. In the wild, crustacea ingest a mixture of carotenoids from algae and zooplankton.

ALGROTM IS A NATURAL REPLACEMENT FOR THAT WILD FOOD SOURCE OF CAROTENOIDS.

A trial carried out by the Thai Department of Fisheries (Appendix 1) has shown that addition of *Algro*TM to pelleted diets successfully pigmented *Penaeus monodon* (black tiger prawns). The trials showed that pigmentation of raw and cooked prawns is equivalent to or better than that achieved by addition of astaxanthin to the feed, and that *Algro*TM is a more cost effective and natural source of carotenoids. As a result of the trial, it is recommended that *Algro*TM is added to feed 6 weeks prior to harvest.

Penaeus japonicus also uses beta-carotene as a source of pigment, converting it into astaxanthin. When three diets containing beta-carotene, astaxanthin and canthaxanthin were fed to *P.japonicus*, the pigmentation and free astaxanthin content of body tissues were the same, after 8 weeks, for all of the dietary groups. Nutritional deficiency with respect to carotenoids is the cause of blue disease in farmed *P.monodon*. "Blue" *P.monodon* had only 4.3-7ppm total carotenoid in their exoskeletons, compared with 26.3ppm and 25.3ppm in the exoskeletons of wild and normally pigmented farmed prawns. Yamada et al (1990). It has been shown that carotenoids are essential growth factors in penaeid prawns, replacing any requirement for vitamin A (retinoids) in the diet. Howell, and Matthews (1991), Dall, W. (1995)

Marine filter feeders

Corals, both soft and hard can grow and survive when fed *Dunaliella* paste (as Nutra-RedTM). Moreover, public aquariums and coral farms / holding facilities trialed the red paste during the past two years with great success. The Aquarium of Western Australia (AQWA) is currently using *NutraPlus* as the sole feed for both soft and hard corals as well as for sponges.

Sea urchins

Beta-carotene is a common major carotenoid found in sea-urchins, in the gonads, tests and



Spines (Tsushima, and Matsuno, 1990). When *Algro*TM was added to prepared feeds, it improved the colour (both red and yellow colour value) of sea urchins. Addition of 250mg carotenoids as *Algro*TM/kg feed (that is, 12.5g *Algro*TM per kg feed) was optimum, and yielded a roe product of high quality, with respect to colour, taste and size (Robinson and Castell, 2000).

Brine shrimp and rotifers

Dunaliella salina is a major component of the diet of wild harvested brine shrimp, *Artemia spp.* Addition of *Algro*TM or *NutraPlus* to the feeding regime of cultured brine shrimp enhances their natural colour and their nutritional properties (Tachibana, et al. 1997, Kolkovski, pers. comm., 2004). *Artemia* can be grown on *NutraPlus* as the sole feed or the paste can be mix with a commonly used feeds such as rice bran. The carotenoids accumulate in the *Artemia*, when feeding or enriching it for at least 24 to 48 hours prior to serving it to fish or other organisms. The result is bright red *Artemia*. The carotenoids are then pass to the target organism. Brine shrimp as well as other crustaceans (prawns *Penaeus sp.*) are capable converting carotenoids such as beta-carotene, alpha carotene and lutein to the red pigment astaxanthin (Katayama, et al. 1973). In the wild, crustacea ingest a mixture of carotenoids from algae and zooplankton. *NutraPlus* and *Algro*TM are natural replacements for that wild food source of carotenoids.

Abalone and molluscs

When added to prepared diets for farmed abalone, *Algro*TM may act as a feed attractant, and improved the colour of the shell (personal communication from Australian customer). Currently, *Algro*TM is added to commercial abalone feeds in Australia.

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Appendix 1.

Trial of Algo™ in pigmentation of shrimp, *P.monodon*

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Introduction

The rapid expansion of aquaculture for the commercial production of fish and crustaceans has led to an ever-increasing demand for improved and cost-effective feeds. One important aspect of this is pigmentation. Especially in the case of salmonid fish and crustaceans, the consumer is attracted by bright and appropriate coloration which is associated with freshness and quality of the product. The desired coloration must be preserved through storage, processing and cooking. In salmonids and crustaceans, this colour is provided by carotenoids, the usual main pigment being astaxanthin (3,3'-dihydroxy- β , β -carotene-4,4'-dione) in the free or esterified form.

In the live crustaceans the orange-red colour of the astaxanthin may be modified to brown, purple, green or blue through the formation of carotenoprotein complexes; the red colour is revealed on cooking.

It is well known that the animals are unable to biosynthesize carotenoids de novo. The carotenoids required for pigmentation thus have to be provided in the feed. Thus the desired coloration of salmonids and of crustaceans can be achieved by including in the feed astaxanthin, which is the carotenoid responsible for the natural colour. The astaxanthin is absorbed and deposited in the tissues intact. The cost of synthetic astaxanthin is high, however, so it is commercially desirable to identify alternative,

more cost-effective means of achieving the desired coloration. There are several reports that crustaceans have the metabolic activity to modify structurally carotenoids that they obtain from their diet. In particular, the ability to introduce oxygen functions into dietary β -carotene to form canthaxanthin (β , β -carotene-4,4'-dione) or astaxanthin has been noted and various alternative pathways or reaction sequences have been proposed (see Goodwin/Castillo for reviews). The phenomenon has been studied most extensively with *Artemia* and *Daphnia* species. In these examples, the normal main carotenoid and end product of metabolism is canthaxanthin. In the case of other decapod crustaceans, e.g. *Panulirus japonicus*, the main carotenoid is generally astaxanthin, and the conversion of β -carotene into astaxanthin by introduction of hydroxy groups at C(3) and C(3') and of keto groups at C(4) and C(4') has been reported. This raises the possibility that β -carotene or products rich in β -carotene could provide an alternative and cheaper means of achieving the desired coloration. Indeed, there is a report that feeding preparations of *Spirulina*, a cyanobacterium that has β -carotene as its main carotenoid, did lead to effective carapace pigmentation in *Penaeus monodon*. We now report the results of controlled feeding trails with the tiger shrimp *Penaeus monodon*. Coloration, astaxanthin content, growth, health and immune response parameters have been compared in shrimps fed diets supplemented with astaxanthin (50 ppm) or with an algal preparation (*Dunaliella salina*) containing β -carotene as its only major carotenoid (fed at 125 ppm or 175 ppm).

Materials and Methods

Experimental diets. The basic diet consisted of the following ingredients (g/kg): fish meal (280), shrimp head meal (100), squid meal (50), wheat gluten (60), soybean meal (100), wheat flour (200), rice flour (91), fish oil (20), lecithin (20), cholesterol (5), vitamin mix (3.3), choline (3), vitamin E



(1.5), vitamin C (1), minerals (40), zeolite (15) and antioxidant (BHT) (0.2). Carotenoid supplements were added as follows.

Diet I (control): Cellulose (10g/kg); no added carotenoid

Diet II: Algal preparation (6.25g/kg), cellulose (3.75g/kg); 125 ppm ®- carotene

Diet III: Algal preparation (8.75g/kg), cellulose (1.25g/kg); 175 ppm ®- carotene

Diet IV: Astaxanthin formulation (0.62g/kg), cellulose (9.38g/kg); 50 ppm Astaxanthin

The algal preparation consisted of dried cells of *Dunaliella salina*, produced in Western Australia. The astaxanthin (synthetic racemate) was obtained as Carophyll pink (Roche), astaxanthin content 8%. The dried ingredients were mixed in a Hobart mixer for 10 minutes, then water (300 ml/kg) was added and the mixing was repeated for a further 10 min. The mixture was then extruded through a 1.5 mm diameter die in a meat grinder. The spaghetti-like feed was cut into pellets (length 0.2-1.0 cm), which were dried in an oven at 60°C for 4-6 hours or until the moisture content was less than 10%. The dried feed pellets were separated into three sizes by sieving and packed in plastic bags.

Experimental Animals

Post-larvae of *Penaeus monodon* (10,000) were obtained from the hatchery and stocked in a 5-ton indoor concrete tank and fed with a commercial diet for 1-2 months or until they attained 1.2g weight.

Growth and Pigmentation Trials

Shrimps of the same size and colour were selected and stocked at 20 animals per aquarium in twenty four 200 litre aquaria, equipped with aeration. Conditions of low salinity (13 ppt) were used and the water exchange rate was 20% every 2 days. The four experimental diets were assigned randomly to the 24 aquaria (i.e. six replicates of each). The animals were fed five times per day and feed was provided at 3-7% of body weight per day, depending on the size of the animals (Table 1). The colour and health of the shrimps were observed every day during feeding and the number and total weight were noted every two weeks. At the end of the feeding trial, coloration of the animals before and after cooking was assessed visually by means of a 'Salmo Fan' and recorded by photography. Shrimps from each treatment were freeze-dried and crushed and the powdered material was sealed under N₂ and transported to Liverpool for carotenoid analysis. Transport time was approximately 48 hours.

Health Trials

Animals: Health brown-black specimens of *P. monodon*, in the size range 12-15g, were purchased from a shrimp farm where no disease outbreak had been reported. They were kept in a 3-ton concrete tank equipped with aeration and were acclimatized to the test conditions for 1 week before the experiments were performed. The animals were fed high-quality shrimp feed four times daily at a total of 3% body weight per day. They were examined for parasitic infestation and bacterial infection by routine diagnosis and PCR analysis was used to test for white spot virus.

Test Conditions

Two sets of experiments were performed in, respectively, four 3-ton concrete tanks with a stocking density of 80 shrimps per tank and four 200-litre glass aquaria with a stocking density of 10 shrimps per aquarium. In each set, four groups of animals were compared. These were fed, respectively, experimental diets I-IV four times daily at 3% of body weight per day. The salinity of the water was kept constant at 20 ppt and the water temperature was maintained at 29-30° by means of a 1 kW heater



in each concrete tank throughout the experiment. Analysis of immune function. The immune functions of the test shrimps were evaluated in the following ways.

- a) Total haemocyte count. Blood from each shrimp was withdrawn from the base of a walking leg by means of a 1 ml syringe with 23G needle. The blood was immediately diluted with 0.15% trypan blue and was counted under a light microscope. The results were reported as number of haemocytes per mm³.
- b) Phenoloxidase activity. Phenoloxidase activity in the haemocytes was determined by the method of Smith and Soderhall (1983). Blood was collected as described in (a), with the use of 3% L-cysteine as an anticoagulant. After centrifugation the pellet was washed three times with lobster haemolymph medium (LHM) at pH 7.4 before resuspension in cacodylate buffer (pH 7.4). The haemocytes were disrupted by sonication for 3 seconds at 40, before centrifugation at 10000 rpm at 4°C for 5 minutes. The supernatant (haemocyte lysate, HLS) was collected for phenoloxidase analysis. For this, HLS (200 μ l) was incubated with trypsin solution (200 μ l) [?]. After 2 minutes, L-DOPA (200 μ l, 3 mg/ml) was added. The reaction was monitored by determination of light absorbance at 490 nm every 2 minutes. The protein content of the HLS was determined by the Lowry method (Lowry et al, 1958).
- c) Clearance ability. Each shrimp was injected with a known number of bacterial (8.75 x 10⁶ efu). Three hours after the injection, blood was withdrawn and the number of bacteria in the blood was counted by the plate count technique. Estimation of disease resistance. Disease resistance was tested by challenging the shrimps with pathogenic luminescent bacteria (*Vibrio harveyi*) or white-spot disease virus. A 23 hour culture of *V. harveyi* was used and the bacterial suspension was adjusted to a cell density of 1 x 10⁷ efu/ml. Each shrimp was injected with 0.1 ml of the suspension. Mortality was recorded for 10 days post injection. Stock white-spot disease virus was thawed from -70°C. The original concentration of virus was 1:2 and it was diluted to 1:2500 with LHM. Each shrimp was injected with the diluted virus (0.1 ml). Mortality was recorded over 5 days.

Carotenoid analysis

Extraction and determination of total carotenoid content.

The samples were provided as dried, crushed material. They were used for analysis exactly as received and were analysed only for carotenoid. Other factors, e.g. residual water content, were not checked. The dried material was not uniform. There was wide variation in the appearance of individual particles, which ranged in colour from white to deep-red, as is to be expected considering the non-uniform distribution of pigment in the animal. Each powdered specimen was mixed thoroughly before sampling for analysis. Five replicate samples from each of the four test diets were analysed and three replicate extractions and analyses were performed for each. Dried shrimp powder, as received, (0.5g) was weighted accurately, then moistened with water (1 ml) and allowed to swell for a few minutes. Tetrahydrofuran (THF, 4 ml) was added and the suspension was mixed first on a whirlimixer, then by sonication for 15 seconds. The solid material was allowed to settle and the clear liquid was pipetted into a centrifuge tube. The extraction was repeated three or four times, until no more colour was extracted. The combined extracts were then centrifuged at 4500 rpm for 5 minutes. The clear supernatant was made up to a known volume with THF. The UV/Vis absorption spectrum was recorded against a blank of wet THF. The carotenoid concentration was determined as astaxanthin equivalents, from the absorbance at a λ_{max} (ca. 480 nm) and a A_{1%} value of 2100 [Reference]. The samples were then evaporated to dryness under N₂ and stored under N₂ at 20°C to await HPLC or other analysis.

Identification and quantitative analysis.



Rapid preliminary analysis by thin-layer chromatography (TLC). Samples of the extracts were subjected to small-scale analysis by TLC on silica with developing solvents of increasing polarity (diethyl ether - light petroleum mixtures). Three main bands were obtained, corresponding to free astaxanthin, astaxanthin monoester and astaxanthin diester (identified by comparison with samples from *Haematococcus pluvialis*, kindly provided by Dr A.J. Young, Liverpool John Moores University). A minor band corresponding to authentic β -carotene was present in the extracts of shrimps fed β -carotene. Saponification. Except under strictly anaerobic conditions, exposure of astaxanthin to alkaline conditions leads to quantitative oxidation to the diosphenol astacene (3,3'-dihydroxy-2,3,2',3'-tetrahydro- β , β -carotene-4,4'-dione). Under the same conditions, astaxanthin acyl esters are hydrolysed and astacene again results. Small portions of the extracts were subjected to alkaline conditions (5% KOH in ethanol, 12 hours) and the carotenoids then extracted into diethyl ether following acidification of the reaction mixture. TLC (silica, diethyl ether) showed astacene to be the major carotenoid present. High-performance liquid chromatography (HPLC). The extracts were analysed quantitatively by HPLC on a silica column acidified with phosphoric acid, according to a standard procedure [Ref] Quantitative carotenoid compositions were determined from peak areas at the monitoring wavelength 480 nm (λ_{\max} of astaxanthin). The pattern of main components present corresponded to the profile of astaxanthin diesters, astaxanthin monoesters and free astaxanthin, as identified in *H. pluvialis* (comparison kindly provided by Dr A.J. Young). All had spectra identical to that of standard astaxanthin. β -carotene was identified by its retention time and absorption spectrum, which were identical to those of an authentic sample.

Results and Discussion

Growth, feed conversion and survival

As shown in Table 2, there were no significant differences between the groups of shrimps fed experimental diets I-IV, in terms of growth (as average final weight), survival rate and feed conversion.

Colour

Visual observations first showed a difference in colour of the fresh shrimps at 4 weeks for those fed diet III (175 ppm β -carotene) and at 5 weeks for those fed diet II (125 ppm β -carotene) or diet IV (50 ppm astaxanthin). The colour changed from bluishlight brown to darker brown and brighter, with time. The colour and colour scores of the experimental shrimps are given in Table 3. The desired colour was attained at 5-6 weeks in the group fed 175 ppm β -carotene and at 7-8 weeks in the groups fed 125 ppm carotene or 50 ppm astaxanthin. There is thus a relationship between the nature and concentration of carotenoid in the feed and the optimal duration of feeding. Feeding the carotenoid for too long may give a colour which is not so attractive to the consumer and is not economical. For prolonged feeding of carotene or astaxanthin, a lower carotenoid concentration is recommended.

Analysis

Pigment extraction.

Efficient extraction of carotenoid from the dried material was not easy to achieve. Several extraction procedures and solvents were tried. The method eventually adopted was efficient and reproducible and gave a clear, coloured extract and an essentially colourless solid residue from which no further pigment could be extracted.



Analysis of total carotenoid content

The UV/visible light absorption spectra of the total extracts from all the experimental shrimp samples were qualitatively identical in terms of λ_{\max} and lack of fine structure, and were all characteristic of the spectrum of standard astaxanthin in the same solvent. Analysis by thin-layer chromatography and HPLC (see below) confirmed that, in all cases, the bulk of the carotenoid present (approximately 90%) was astaxanthin and its esters. The total carotenoid content could thus be determined, as astaxanthin equivalents, from the quantitative absorption spectra of the total extracts. The results are given in Table 4. The carotenoid content in all three groups fed a diet supplemented with carotenoid was much greater (2-4 fold) than that of shrimps fed the control diet, with no added carotenoid and the carotenoid present was almost all astaxanthin (free and esterified), regardless of whether the carotenoid administered was astaxanthin or β -carotene. This is in agreement with the visual observation of the colour of the shrimps.

Analysis of carotenoid composition

The HPLC profiles of the extracts from all the four groups of experimental shrimps were very similar (Fig. 1). Free astaxanthin was identified by its retention time, absorption spectrum and comparison with an astaxanthin standard. Most other major components also had absorption spectra identical to that of astaxanthin and occurred in patterns characteristic of mixtures of astaxanthin monoesters (t_R 3-5 min) and diesters (t_R 2-3 min), identified by comparison with extracts of *Haematococcus pluvialis*. Astaxanthin and its esters accounted for around 90% of the total carotenoid in all cases. Confirmation of this identification was obtained by alkaline hydrolysis of the extracts, after which astacene (produced under these conditions from free astaxanthin and astaxanthin esters) was in all cases about 90% of the total carotenoid recovered. In specimens that had been fed experimental diets II or III, containing β -carotene, a sharp peak with retention time 1.57 minutes was also seen. This component had retention time and absorption spectrum identical to those of authentic β -carotene. Even in these samples, however, β -carotene was only a minor component of the total carotenoid (no more than 6%). Cooking did not significantly affect the carotenoid content of the shrimps that were fed the carotenoid-supplement diets, but there were quantitative differences in the relative proportions of free and esterified astaxanthin in raw and cooked shrimps (data not shown). Conclusions from the analytical results The carotenoid content in shrimps fed diets containing no carotenoid supplement was, as expected, low. A 2-fold to 4-fold increase in carotenoid content was obtained by feeding the carotenoid-supplemented diets. The desired coloration and astaxanthin content were achieved not only by feeding astaxanthin but also by feeding β -carotene. Feeding β -carotene, at 125 ppm or 175 ppm, resulted in substantially greater carotenoid contents in the shrimps than did feeding astaxanthin at 50 ppm. The carotenoid composition in all cases was similar, with astaxanthin and its esters by far the main components. A relatively small amount of unchanged β -carotene was present in the shrimps that had been fed β -carotene. The shrimp *P. monodon* clearly has the metabolic activity to convert dietary β -carotene into astaxanthin.

Immune response and disease resistance.

The shrimps used in the part of the work were free from bacterial infection and also did not carry the white spot virus (negative PCR test). They showed a low level of infestation with parasitic *Zoothamnium* and were therefore bathed with formalin (35 ppm) for 24 hours before the start of the experiment. The shrimps were then fed the experimental diets I-IV for 4 weeks; at this point those fed



carotenoid-containing diets were considerably darker in colour than those fed the control diet. A number of parameters of health and immune response were compared in the four groups. Two major parameters of immune response in crustaceans are haemocyte count and phenoloxidase activity in the haemocytes (Smith and Soderhall, 1983; Ashida and Soderhall 1984; Lanz et al., 1993). Some substances characteristic of microorganisms, e.g. 1,3-glucan, peptidoglycan, lipopolysaccharide, can induce this enzyme activity (Soderhall, 1981; Smith and Soderhall, 1983; Soderhall and Hall, 1984; Song and Hsieh, 1984).

No significant difference was found in the number of haemocytes in the circulatory system of the shrimps from the four groups, nor in phenoloxidase activity in these haemocytes (data not shown). Clearance ability, i.e. the efficiency with which the shrimp is able to remove foreign particles or microbial cells from the blood circulation, is another parameter that can reflect the immune response of shrimps or other crustaceans (Martin et al, 1993). Again no significant differences were observed between the carotenoid-supplemented and unsupplemented groups in clearance ability, nor in their resistance to pathogenic bacteria or viruses (data not shown).

Conclusions

For the shrimp *Penaeus monodon* in aquaculture, the normal colour desired by the consumer is only achieved by feeding a diet supplemented with carotenoid. The desired colour was achieved by feeding astaxanthin (50 ppm) for 7-8 weeks before harvest. Similar coloration was achieved by feeding a preparation of algal β -carotene which contains no astaxanthin. β -carotene at 125 ppm also gave the required coloration in 7-8 weeks, whereas the same result was obtained after only 5-6 weeks when β -carotene at 175 ppm was used. The optimal duration of feeding the supplemented diets therefore depends upon which carotenoid is being fed and at what concentration. Although not tested in this study, it is likely that feeding the carotenoid at these concentrations for too long a period may give a colour that is not so attractive to the consumer, as well as not being economically desirable. If pigment-containing feed is supplied for longer periods, a lower concentration of carotene or astaxanthin would be appropriate. Irrespective of whether the shrimps were fed astaxanthin or β -carotene, the main carotenoid accumulated was astaxanthin in free and esterified form, showing that *P. monodon* has the metabolic ability to convert β -carotene into astaxanthin. The supplementation with β -carotene or astaxanthin had no significant effect on growth of the shrimps, average final weight, survival rate, or efficiency of feed conversion. The provision of carotenoids in the feed did not result in any significant difference in the immune response of the shrimps in terms of production of haemocytes, phenoloxidase in the cytoplasmic granules of the haemocytes, or microbial clearance ability, nor in resistance to infectious diseases. Previous studies with *P. japonicus* have shown that dietary astaxanthin, β -carotene or canthaxanthin led to the deposition of mainly astaxanthin esters in the carapace, as in the present study with *P. monodon* (Yamada et al, 1990).

In that work, however, astaxanthin was reported to be more effective for pigmentation than β -carotene or canthaxanthin. The present study did not include a direct comparison of astaxanthin and β -carotene fed at the same concentration, but was designed simply to show that feeding of algal β -carotene was effective and efficient. Also with *P. japonicus*, Chien and Jeng (1993) reported a higher survival rate for animals fed astaxanthin-supplemented diets than for ones fed a supplement of β -carotene or algal meal. This contrasts with the present results obtained with the different species, *P. monodon*. This is a highly significant development for commercial aquaculture, because it shows that a similar result can be achieved by supplementing diets with β -carotene as with astaxanthin. This has clear commercial advantages, since β -carotene is considerably less expensive than astaxanthin.



Table 1. Feeding rate for experimental shrimps

Shrimp Size (g)	Feeding Rate (% body weight/day)
1.0	7.0
1.7	6.0
2.4	5.0
3.3	4.0
4.2	3.5
5.0	3.2
6.0	3.0

Table 2. Growth feed conversion and survival of *P. monodon* fed diets supplemented with various levels of β -carotene and astaxanthin for 8 weeks. Differences were not statistically significant.

Treatment	Average body weight (g)		Feed Conversion Rate	Survival Rate (%)
	Initial	Final		
Control	1.21 \pm 0.01	8.12 \pm 0.53	1.45 \pm 0.11	83.33 \pm 7.53
125 ppm carotene	1.21 \pm 0.01	7.59 \pm 0.59	1.45 \pm 0.11	89.17 \pm 8.61
175 ppm carotene	1.22 \pm 0.01	7.39 \pm 0.75	1.53 \pm 0.16	84.17 \pm 5.85
50 ppm astaxanthin	1.22 \pm 0.01	8.01 \pm 0.54	1.53 \pm 0.11	87.00 \pm 7.58

Table 3. The observed colour and the colour scores (Salmo Fan) for *Penaeus monodon* fed different levels of β -carotene and astaxanthin for 8 weeks.

Treatment	Colour scores boiled shrimp	Colour unboiled shrimp
Control	20-25	blue - light brown
125 ppm carotene	21-33	light - medium brown
175 ppm carotene	30-34	dark brown
50 ppm astaxanthin	20-32	light - medium brown



Table 4. Total carotenoid content of the shrimp *Penaeus monodon* after feeding experimental diets I-IV for 8 weeks. The results were calculated as astaxanthin equivalents.

Treatment	Sample	Astaxanthin	Range of 3 replicate extractions	Mean	Range* 1	Range 2
T1. Diet I; Control	T1R1	45	43-46	50	45-54	43-61
	T1R3	49	45-52			
	T1R4	49	46-50			
	T1R5	54	54-54			
	T1R6	54	50-61			
T2. Diet II; supplemented with β -carotene (125 ppm)	T2R1	136	133-142	143	129-159	121-161
	T2R3	159	157-161			
	T2R4	139	129-145			
	T2R5	129	121-140			
	T2R6	150	148-152			
T3. Diet III; supplemented with β -carotene (175 ppm)	T3R1	213	209-216	178	155-213	152-216
	T3R3	161	159-164			
	T3R4	196	193-200			
	T3R5	155	152-157			
	T3R6	164	162-167			
T4. Diet IV; supplemented with astaxanthin astaxanthin (50 ppm)	T4R1	109	106-112	108	104-116	102-116
	T4R3	104	102-105			
	T4R4	106	102-107			
	T4R5	116	116-116			
	T4R6	104	102-105			

*Range 1 Range between highest and lowest samples (mean of 3 replicate extractions)

Range 2 Range between highest and lowest individual values



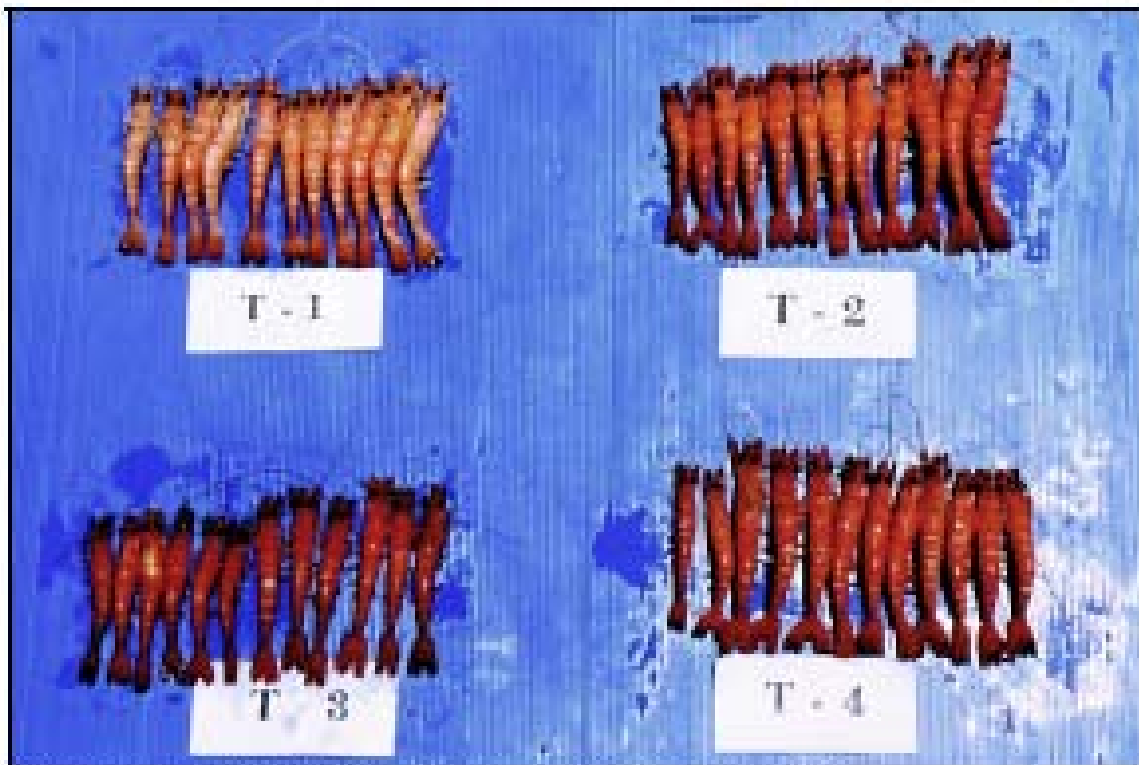
Control



125ppm carotencoids from Algro™



175ppm carotenoids from Algo™



All treatments with 'T-4' being 40ppm carotenoids as astaxanthin

Reference: Unpublished results of Dr Mani Boonjaratpalin and Dr George Britton.

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