Dietary astaxanthin levels affect growth, carotenoid digestibility and the deposition of specific carotenoid esters in the Giant Tiger Prawn, *Penaeus monodon*.

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Prawn Carotenoid Digestibility and Function

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Abstract

The predominant carotenoid in prawn tissues is astaxanthin (Axn) and its role in pigmentation has been well studied. However, the effects of dietary Axn on other prawn physiological performance measures are uncertain and dietary carotenoid uptake and tissue deposition are poorly understood. This study fed juvenile prawns (*Penaeus monodon*) diets that contained 0, 25, 50 or 100 mg kg^{-1} Axn for 6 weeks. Animals fed carotenoid free diets had significantly reduced growth than those fed carotenoids, but survival was unaffected. Carotenoid uptake (digestibility) improved as dietary carotenoid levels increased, and was 98.5% in animals fed 100 mg kg^{-1} Axn. After 6 weeks, whole body carotenoid levels were significantly depleted in 0 or 25 mg kg^{-1} fed animals, compared with those fed 50 or 100 mg kg^{-1}. Specific fatty acid esters of Axn accumulated in epithelial tissue, with mono-esters enriched saturated fatty acids, while di-esters were enriched with monounsaturated and polyunsaturated fatty acids. These data suggest a minimum dietary requirement of 25 mg kg^{-1} Axn in clear water systems to maintain growth performance, and 50 mg kg^{-1} or more to avoid depletion of body carotenoid levels and improve efficiency of utilisation. These results also implicate specific fatty acids in the function of carotenoid esters within prawn tissues.

Introduction

Carotenoids as an essential nutrient for prawns are poorly understood. Crustaceans cannot synthesise carotenoids and must obtain them from their diets (Goodwin, 1952), although they are able to inter-convert different carotenoids (Schiedt *et al.*, 1993, Castillo *et al.*, 1982). All wild and cultured crustacean species report the presence of free and esterified forms of various carotenoids, predominantly astaxanthin (Axn) (Castillo *et al.*, 1982, Lenel *et al.*, 1978, Tanaka *et al.*, 1976, Sachindra *et al.*, 2005). The distribution of these forms of carotenoids also varies with species, life history stages, developmental stage, moult stage and the organ or tissue of the animals (Ribeiro *et al.*, 2001, Lenel *et al.*, 1978, Sachindra *et al.*, 2005, Okada *et al.*, 1994, Pan and Chien, 2000, Dall, 1995, Petit *et al.*, 1998, Pan *et al.*, 1999, Valin *et al.*, 1987, Katayama *et al.*, 1971, Petit *et al.*, 1997).

Much of the work on carotenoid nutrition in prawns has focussed on optimising pigmentation levels, where dietary levels of Axn have been recommended at between 80-100 mg kg^{-1} for *Penaeus monodon* (*P. monodon*) (Menasveta *et al.*, 1993, Niu *et al.*, 2012, Okada *et al.*, 1994, Yamada *et al.*, 1990, Wade *et al.*, 2015). Reports of the effect of dietary carotenoid supplementation on growth and survival in *P. monodon* have been mixed, where some studies recorded no effects (Pan *et al.*, 2001, Boonyaratpalin *et al.*, 2001), while others recorded improvements recorded in growth, survival or both (Darachai *et al.*, 1998, Niu *et al.*, 2012, Niu *et al.*, 2014). Post-larvae of *P. monodon* greater survival when fed algal Axn (*Haematococcus pluvialis*) supplemented diets (Darachai *et al.*, 1998). The two studies performed with *P. monodon* juveniles recorded higher growth and survival when fed dietary carotenoids (Niu *et al.*, 2012, Niu *et al.*, 2014), but used no less than 100 mg kg^{-1} Axn. Carotenoid digestibility was also shown to be very high, greater than 90% in *Penaeus monodon* for diets exceeding 100 mg kg^{-1} Axn (Niu *et al.*, 2012, Niu *et al.*, 2014), but has not been assessed below this level. The detection of performance differences in shrimp fed dietary carotenoids between early work and more recent studies is potentially reflective of improvements in trial maintenance, animal health, quality of feed ingredients, system design and animal husbandry. However, the requirement for carotenoids

in prawn diets is not widely accepted, required levels are poorly characterised and carotenoid digestibility has not been investigated below 100 mg kg^{-1}.

Other than pigmentation, the function of carotenoids in prawn tissues have been mainly inferred from the innate oxygen free radical scavenging or photoprotective properties of Axn (Britton, 2008, Miki, 1991). Carotenoids and carotenoid esters appear in early juvenile stages (Berticat *et al.*, 2000, Mantiri *et al.*, 1995, Petit *et al.*, 1991), and are predominantly thought to play a role in the storage of carotenoids in various tissues and organs. Other carotenoid functions include conversion to retinoids that play a prominent role in prawn development and differentiation (Linan-Cabello *et al.*, 2002). Increased levels of dietary carotenoid resulted in the accumulation of carotenoid esters in crustaceans (Yamada *et al.*, 1990, Supamattaya *et al.*, 2005, Boonyaratpalin *et al.*, 2001, Barclay *et al.*, 2006, Kumar *et al.*, 2009, Wade *et al.*, 2008). The esterification of carotenoids has been implicated in the regulation of pigmentation in several species (Wade *et al.*, 2005, Wade *et al.*, 2012, Tume *et al.*, 2009).

Three studies in other crustacean species have analysed the specific fatty acid attachments of carotenoid esters. In the red crab langostilla *Pleuroncodes planipes*, C16:0 and C18:1n-9 saturated fatty acids accumulated in astaxanthin diesters and polyunsaturated C20:5n-3 and C22:6n-3 accumulated in astaxanthin mono-esters (Coral-Hinostroza and Bjerkeng, 2002). Of the major astaxanthin esters identified in the shrimp *Pandalus borealis*, both the astaxanthin mono-esters and diesters were found to contain both C12:0 and C18:1 fatty acids, with di-ester forms predominating (Breithaupt, 2004). The carapace of the spiny lobster *Panulirus japonicus* was found to contain 42% and 12% astaxanthin di-esters and monoesters, respectively, with both monoesters and diesters composed of predominantly C16:0 or C18:0 fatty acids (>40%), and a further 30% of monoesters containing C16:1 and C18:1 fatty acids (Maoka and Akimoto, 2008). Other than a role in storage, functional roles that fatty acid esterification may provide to the carotenoid molecule are poorly understood.

This study sought to define the effects of a range of dietary Axn inclusion levels on growth and carotenoid digestibility in *P. monodon*. Animals were fed for 6 weeks on one of four diets that contained 0, 25, 50 or 100 mg kg^{-1} Axn. We also quantified whole body carotenoid levels, and identified specific fatty acids that form esters of Axn in the epithelial tissue of *P. monodon*.

Materials and Methods

Animal Handling

Live shrimp (*P. monodon)* were obtained from commercial farms and maintained at CSIRO laboratories at Bribie Island. For all trials, filtered seawater was heated then pumped through the tanks at 1.2 L min^{-1} maintaining water temperatures at 28˚C and salinity at 35 g L^{-1}. Animals were held in a total of 60 red tanks that held 80 L seawater in each. The experiment was conducted indoors under low artificial light conditions and a 12-12 light dark photoperiod. The experimental treatments were composed of a total of twelve replicate tanks for each of the four diets, each of which contained six individually eyetagged *P. monodon*. Animals across the experiment were within the size range 7.25 ± 1.34 g.

Growth Trial

Nine tank replicates were used to assess growth over time, with three further replicate tanks used to assess nutrient digestibility in week 7. Animals were fed once per day for 6 weeks on one of four formulated diets that differed only in the amount of Axn (Table 1) in the form of Carophyll Pink (DSM Nutritional Products). Amount of feed consumed was calculated as the amount of feed offered less the uneaten feed that was recorded from the number of pellets remaining after 24 hours. Animal weights and feed intake were collected at day 0, 14, 28 and 42. An initial sample of three groups of six shrimp was taken at random from the pool of individuals used to stock the experiment and stored at -20˚C until processing. A single animal was sampled at random from each of the 9 tank replicates at day 42 and pooled into three groups for whole animal carotenoid analysis and similarly stored at -20˚C until processed.

Digestibility Trial

Three additional tank replicates were used to assess nutrient digestibility after 6 weeks of feeding one of the four formulated diets that differed only in the amount of Axn in the form of Carophyll Pink. Aeration was kept to a small stream to provide gentle mixing and aeration without suspending any faecal material. Lighting intensity was kept as low as possible on a 12 h light:dark cycle, and only red hand held spotlights used to aid in siphoning and minimise disturbance of

the animals. The animals were fed a restricted ration (approx 1 g) once per day and allowed 60 minutes to consume the ration, before all uneaten food was collected, dried and weighed. Two hours after feed was first offered, and at each hour for the next 3 hours, all faeces were siphoned into a labelled bucket unique to each tank and allowed to settle briefly before the faeces are transferred to a 10 mL centrifuge tube. The seawater was then decanted and replaced with deionised water and the volume made up to 10 mL before centrifugation at 2000 rpm for 30 sec. All fluid was then decanted and the tube capped and frozen. The frozen faecal pellet was then transferred to a sample vial for pooling (same tank, but pooled over time) until sufficient dry matter of faeces (~1 g) had been collected (approximately 7 days). At the end of this period, animals were similarly stored at -20˚C until processing. For this experiment, the amount of feed consumed during the uptake trials was calculated as the amount of feed offered less the uneaten feed that was collected, dried and weighed separately from each tank. Differences in the ratios of the parameters of dry matter, lipid or carotenoid to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility (AD_{diet}) for each of the nutritional parameters examined in each diet based on the following formula:

$$AD_{diet} = \left(1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right) \right) \times 100$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (in this case total carotenoid) content of the diet and faeces respectively. All calculations were performed at the tank replicate level. Carotenoid retention (*y*) was estimated using the following formula:

$$y_{Axn} = (intake_{Axn} / gain_{Axn}) \times 100$$

Chemical analysis

Diet, shrimp and faecal samples were analysed for dry matter, ash, lipid and carotenoid content. Diet and faecal samples were in addition analysed for yttrium content. Shrimp samples were minced whilst frozen, then refrozen and freeze-dried to completion. A sub-sample of the original frozen mince was

analysed for its moisture content by gravimetric analysis following oven drying at 105˚C for 24 h. Dry matter of other samples was calculated by gravimetric analysis of a milled sample of freeze dried material following oven drying at 105˚C for 24 h. Total yttrium concentrations were determined for diet and faecal samples after mixed acid digestion using inductively coupled plasma mass spectrophotometry (ICP-MS). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Total lipid content of the diets, animals and faeces was determined gravimetrically following extraction of the lipids using chloroform:methanol (2 : 1) under low light conditions. Whole animal, diet or faecal total lipid extracts were dried under nitrogen and resuspended in 1 mL of hexane for carotenoid analysis by HPLC. HPLC separation followed previous methods (Wade *et al.*, 2012). Peak areas were grouped according to elution times into Free Axn (6 to 12 min), Axn mono-esters (12 to 32 min) and Axn di-esters (32 to 60 min) and quantified using Empower Pro software (Waters Corporation). Free Axn concentration was calculated using peak areas against Axn standards of known concentration, and then normalised against the dry weight of the original tissue extracted.

Carotenoid thin layer chromatography

Pigmented epithelial tissue was dissected from the abdominal segments of individual *P. monodon* from initial stock animals. Whole tissue was homogenised in 2 ml water containing the Complete protease inhibitor cocktail (Roche) using a Precellys 24 tissue homogenizer (Bertin Technologies). Insoluble material was separated by centrifugation at 13 000 x g for 5 min at 4˚C. The blue soluble liquid fraction was transferred to a new tube and freeze dried, while the red insoluble pellet was retained separately. Carotenoids were extracted from whole dissected epithelial tissue as a crude extract (CE), the soluble protein fraction (Sol) and the insoluble epithelial tissue that remained after the removal of soluble proteins (Insol). The carotenoids were extracted from this pooled material using acetone until no further pigment remained. This extract was dried under nitrogen and resuspended in hexane, and was termed the crude extract (CE) for further analysis. The carotenoids extracted from the CE, Insol and Sol fractions were run

on HPLC as outlined above. They were also separated by thin layer chromatography (TLC) on pre-coated aluminium plates (20x20cm^2, 0.25-mm thickness, DC-Alufolien, Kieselgel 60, product number 7731, Merck), using a mobile phase of acetone:hexane (30:70 v:v). A standard of free Axn was also included. The retardation factor (R_f) was calculated as the ratio of the distance moved by each band relative to the solvent front. Large preparative TLC plates were prepared using multiple loadings of the Insol extract. Resolved TLC bands were scraped from the plate under low light conditions and pooled, followed by elution from the silica matrix with hexane. Extracted bands were re-confirmed as mono or di-ester peaks by HPLC as described earlier.

Identification of lipid classes

The six purified bands from TLC (F, M1, M2, D1, D2, D3) along with a sample of the whole Insol extract (W) was subject to fatty acid methyl esterification (FAME) and gas chromatography (GC) according to established methods (Coutteau and Sorgeloos, 1995). C21 was incorporated at a known concentration across samples as an internal reference, and individual fatty acids were identified based on retention times against a standard of known fatty acids. Due to the method of extraction and band purification, absolute quantity of fatty acid peaks could not be reliably determined.

Statistical Analysis

The statistical significance of diet, time, or an interaction of these two variables was assessed by 2-way analysis of variance (ANOVA) of growth data, followed by Fischer's test allowing 5% error. Where comparison between individual measurements was required, statistical significance was assessed by single factor ANOVA, followed by Fischer's test allowing 5% error. All statistical analyses were performed using StatPlus:Mac 2009 (AnalystSoft Inc).

Results

Shrimp Performance

Shrimp weight over time was significantly restricted in animals that were not fed carotenoids. Statistical analysis showed a significant effect of diet ($F = 10.42$, $p < 0.001$) and time ($F = 64.95$, $p < 0.001$) on growth, as well as a significant interaction of diet and time ($F = 3.59$, $p = 0.002$). Shrimp weights had significantly increased over the 6-week trial in all animals fed carotenoids, but had not significantly increased in animals fed a carotenoid free diet. Weights of animals fed diets without carotenoids were significantly lower ($p < 0.001$) than all other treatments at day 28 and 42 (Figure 1). Survival across the experiment was 91.7%, but was highest in the no carotenoid treatment (94.4%) which also showed the worst growth performance (Table 2). After 6-weeks, weight gain ($F = 20.26$, $p < 0.001$), feed intake ($F = 25.33$, $p < 0.001$) and FCR ($F = 38.91$, $p < 0.001$) were each significantly poorer in animals fed a carotenoid free diet, while the weight gain and FCR of those fed 25, 50 or 100 mg kg^{-1} Axn were the same (Table 2). Prawn weight gain without dietary carotenoids was restricted to around 1 g (Table 2), and most of this growth occurred within the first 2 weeks. Although animals fed a carotenoid free diet ate significantly less, their FCR was also much worse than those animals that had carotenoid supplementation. Overall, animals fed a carotenoid free diet performed particularly poorly, and this was not entirely related to reduced feed intake.

Whole Body Carotenoid Abundance

Carotenoids were extracted from whole animals that had been homogenised and freeze dried. When quantified on a µg carotenoid per g dry weight basis (Figure 2A), the total Axn content had significantly decreased in all treatments compared with initial levels ($F = 6.76$, $p = 0.007$). However, there was no significant difference in total Axn levels between the different dietary treatment groups (Figure 2A). Although paler in colour (data not shown), animals fed without dietary carotenoids did not turn blue or have total Axn levels as low as those with "Blue Colour Syndrome" (Menasveta *et al.*, 1993). The proportions of free Axn and Axn esters were then separated quantified from whole carotenoid extracts using HPLC. There was a significant accumulation of carotenoid mono-esters in animals

fed 50 mg kg^{-1} (t = 5.19, p < 0.001) or 100 mg kg^{-1} (t = 8.25, p < 0.001) dietary carotenoid compared with 0 mg kg^{-1} fed animals (Figure 2B). However, no significant elevation in Axn mono-esters was achieved using dietary levels of 25 mg kg^{-1}, and even at 100 mg kg^{-1} Axn mono-esters could not be maintained at initial levels. Dry weight carotenoid levels were then adjusted relative to the total weight of the prawns from each group (Figure 2B). Data showed that total carotenoid levels incrementally increased in the animals as a whole, such that total Axn levels had significantly increased when fed 50 mg kg^{-1} (t = 3.34, p = 0.006) or 100 mg kg^{-1} (t = 6.72, p < 0.001) dietary Axn compared with those fed without dietary Axn. Only animals fed without dietary carotenoids showed significantly lower total astaxanthin levels than initial animals (t = 4.38, p = 0.001), and were particularly depleted in Axn mono-esters. After 6 weeks, only animals fed 100 mg kg^{-1} Axn contained levels higher than those in initial animals, although this increase was not significant (t = 2.34, p = 0.037). These data demonstrated that carotenoids become depleted in *P. monodon* without dietary supplementation of 50 mg kg^{-1}.

Carotenoid Digestibility

Faecal samples were collected from three replicate tanks for each treatment, and digestibility analysed for a range of parameters (Table 3). Based on the formulations, very little difference was expected between the apparent digestibility of DM, lipid or protein, and this was true for the diets containing 25, 50 and 100 mg kg^{-1} Axn (Table 3). However, lipid digestibility was significantly lower in the carotenoid free diet, from approximately 80% in carotenoid diets down to 65% in carotenoid free diets. These differences in lipid digestibility may further explain the poor growth performance and FCR observed in this treatment, but whether they are due to the absence of dietary carotenoid alone requires further confirmation. Carotenoid digestibility showed an incremental increase in apparent digestibility of carotenoid as the level of dietary carotenoid increased (Table 5), although this was not significant (F = 4.418, p = 0.066). However, the increased digestibility from 65.3% in the 25 mg kg^{-1} treatment to 98.5% in the 100 mg kg^{-1} treatment was significant (t = 2.97, p < 0.018).

Identification of Carotenoid Fatty Acid Esters

The potential importance of esterification in the regulation and storage of dietary carotenoids led us to separate different potential forms contained within prawn epithelial tissues. HPLC revealed that soluble and insoluble epithelial fractions contained distinct proportions of carotenoid esters (Figure 3A). The blue aqueous soluble fraction contained 91.9% free Axn and 8.1% mono-esters, while the insoluble fraction contained 27.7% free Axn, 60.2% mono-esters and 12.1% di-esters. These three epithelial fractions (crude extract, soluble and insoluble) were separated by thin layer chromatography alongside free Axn, and revealed several distinct carotenoid ester forms (Figure 3B). In line with the HPLC result, the soluble epithelial fraction contained predominantly free Axn, while the Insol fraction contained the majority of carotenoid esters. Three fractions of Axn di-esters (D1, D2, D3) two Axn monoester fractions (M1 and M2) and free Axn (F) were most abundant in the Insol material. Each band was purified separately on preparative TLC plates, and the fatty acids contained within each fraction analysed (Table 4). The M1 and M2 mono-ester bands were highly enriched with saturated fatty acids (SFAs), particularly 16:0 and 18:0 fatty acids. Conversely, the D1, D2 and D3 di-ester bands were highly enriched with monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs), particularly 22:1n-9, 20:3n-3, 20:5n-3 and 22:2 fatty acids.

Discussion

The present study demonstrated a clear effect of dietary Axn supplementation on growth performance in *P. monodon*, and suggests that at least 25 mg kg^{-1} is an essential dietary Axn requirement in this species. After only four weeks, animals without dietary carotenoid supplementation recorded significantly poorer growth when fed diets containing 25 mg kg^{-1} Axn (Figure 1), but showed no effect on survival (Table 2). This effect was similar to the only studies in *P. monodon* that observed reduced growth in animals fed carotenoid free diets compared those fed 100 mg kg^{-1} (Niu *et al.*, 2012, Niu *et al.*, 2014). When body weight was taken into account, total body carotenoids (μg) were depleted over time in animals fed 0 or 25 mg kg^{-1} Axn, but were maintained at approximately 90% of the initial total carotenoid levels when fed diets containing 50 mg kg^{-1} (Figure 2B). Only animals that received 100 mg kg^{-1} dietary Axn showed increased total body carotenoid levels, approximately 20% higher than the initial animals (Figure 2B). Although feeding 25 mg kg^{-1} Axn for 6 weeks showed no growth effects, significant carotenoid depletion also occurred at this inclusion level, and such negative growth effects may appear if fed over a longer duration. This suggests that, under these conditions in this clear water system, there is actually a requirement of between 50 to 100 mg kg^{-1} dietary Axn to maintain total body Axn levels over time.

Although intake was significantly lower in animals fed without carotenoids, feed intake alone did not account for the reduced growth performance, and the FCR of animals fed without carotenoids was significantly higher than those fed carotenoids (Table 2). The absolute FCR values were relatively high for each treatment, as expected due to the feed intake being restricted to once per day. Do I need pellet counting method reference here? Past studies have also observed a significant elevation of FCR (Niu *et al.*, 2012, Niu *et al.*, 2014), as well as a reduction in lipid retention when animals were fed carotenoid free diets (Niu *et al.*, 2014). In the present study, that effect has been alleviated by the inclusion of at least 25 mg kg^{-1} dietary Axn. However, our results also showed that at 25 mg kg^{-1} dietary carotenoids were digested at significantly lower efficiency (65.3%) than at 100 mg kg^{-1} (98.5%; Table 3). Past studies that showed >90% carotenoid digestibility at 100 mg kg^{-1} or above (Niu *et al.*, 2012, Niu *et al.*, 2014), but levels

less than 100 mg kg^{-1} were not studied. Digestibility of dietary canthaxanthin (70 mg kg^{-1}) was low, estimated at 50% (Niu *et al.*, 2012), but may be specific to this carotenoid. Feels like I need something here about a similar ingredient that has reduced digestibility at low levels – any examples? I can't find any. Although counter-intuitive, this study showed that Axn digestibility improved as the dietary Axn level increased and was extremely efficient at levels of 100 mg kg^{-1}. When fed at high levels, crustaceans retain dietary carotenoids in esterified form, which become depleted over time when not supplied in the diet and leads to poor animal performance (Menasveta *et al.*, 1993, Niu *et al.*, 2012, Niu *et al.*, 2014). This implies that carotenoids are being continually metabolised and are required for normal prawn growth and health. These have been proposed to cover a range of functions covering pigmentation, antioxidant protection and conversion to retinoids (Linan-Cabello *et al.*, 2002), but most of these functions relate to carotenoids and not carotenoid esters. Poor performance may only become evident below a certain level of total body carotenoids, but this level is poorly defined. In the present study, negative growth effects were observed within 28 days when no dietary carotenoids were supplied, with a significant depletion of total body carotenoids to 30 μg g^{-1} dry weight from initial levels of 50 μg g^{-1} dry weight. Defining an absolute total body carotenoid level has been difficult due to the variation in published literature, which have ranged from 6-86 μg g^{-1} dry weight in studies with cultured *P. monodon* (Latscha, 1990, Sachindra *et al.*, 2005, Tume *et al.*, 2009, Niu *et al.*, 2012, Niu *et al.*, 2014). This variability will be greatly affected by animal size and developmental stage (post-larval, juvenile, adult). Based on the results of the present study, carotenoid levels below 30 μg g^{-1} dry weight or 250 μg total body Axn may be indicative of poor growth in 7-12 g animals. Further refinement of dietary carotenoid levels may be possible, especially for other life stages.

Many studies, including this one, have shown that carotenoids and their esters accumulate in different crustacean species, in specific tissues or at various developmental stages (Ribeiro *et al.*, 2001, Lenel *et al.*, 1978, Sachindra *et al.*, 2005, Okada *et al.*, 1994, Pan and Chien, 2000, Dall, 1995, Petit *et al.*, 1998, Pan *et al.*, 1999, Valin *et al.*, 1987, Katayama *et al.*, 1971, Petit *et al.*, 1997). The proportions of free Axn and Axn esters are also modified in response to certain

environmental cues such as background substrate colour (Wade *et al.*, 2005, Wade *et al.*, 2012, Tume *et al.*, 2009). Our study also demonstrated that unique fatty acids are enriched in mono- and di-esters of astaxanthin in *P. monodon* (Table 4), which are markedly different from other crustaceans (Coral-Hinostroza and Bjerkeng, 2002, Breithaupt, 2004, Maoka and Akimoto, 2008). The esterification of Axn with specific fatty acids and the presence of carotenoid isomers can significantly increase the complexity of the interaction between the carotenoid and other biological molecules or membranes (Britton, 1995, Goodwin, 1986, Liaaen-Jensen, 1997). Feeding extremely high levels (200-300 mg kg^{-1}) of dietary carotenoids has been shown to improve *P. monodon* resistance to a range of physiological stressors, such as improved resistance to salinity (Darachai *et al.*, 1998), dissolved oxygen (Chien *et al.*, 1999, Supamattaya *et al.*, 2005), temperature (Chien *et al.*, 2003) or disease challenge (Supamattaya *et al.*, 2005). Extremely high levels of dietary Axn (500 mg kg^{-1}) also led to an accumulation of Axn along with long chain PUFAs in *P. monodon* ovary tissue, with complementary improvements in reproductive performance (Paibulkichakul *et al.*, 2008). This study has identified specific carotenoid fatty acid esters that accumulate in tissues of *P. monodon* that may underlie these positive effects of dietary carotenoids.

Combined, evidence suggests that there is a functional role for the accumulation of carotenoids and the formation of specific fatty acid esters, and these may be linked to the metabolism, storage, mobilisation or deposition of astaxanthin within various tissues. The mono-ester form of Axn may be a more mobile or transitory storage form of the carotenoid in *Penaeus monodon*, through attachment of readily available endogenous short chain fatty acids. However, whether other Axn esters may perform specific functions dependent upon their fatty acid attachment requires further study. Presently, there are many aspects of carotenoid esters that are not understood, including whether the carotenoid must move through a mono-ester intermediate before becoming a diester, or whether fatty acid interconversion (elongate or desaturate) can occur while attached to the carotenoid.

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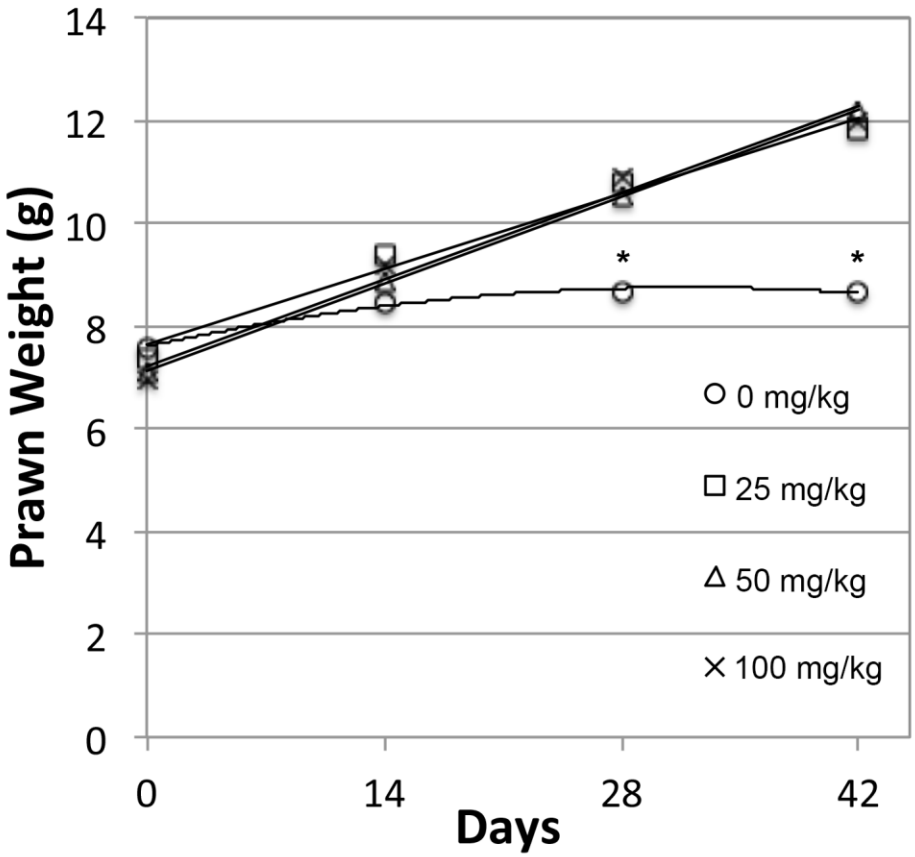

Figure 1. Prawn growth over time at different dietary Axn levels. Prawn weight was measured every two weeks while animals were fed one of four experimental diets containing 0, 25, 50 or 100 mg kg^{-1} Axn. * indicates significant ($p < 0.05$) difference between prawn weight of different diet treatments at a particular time.

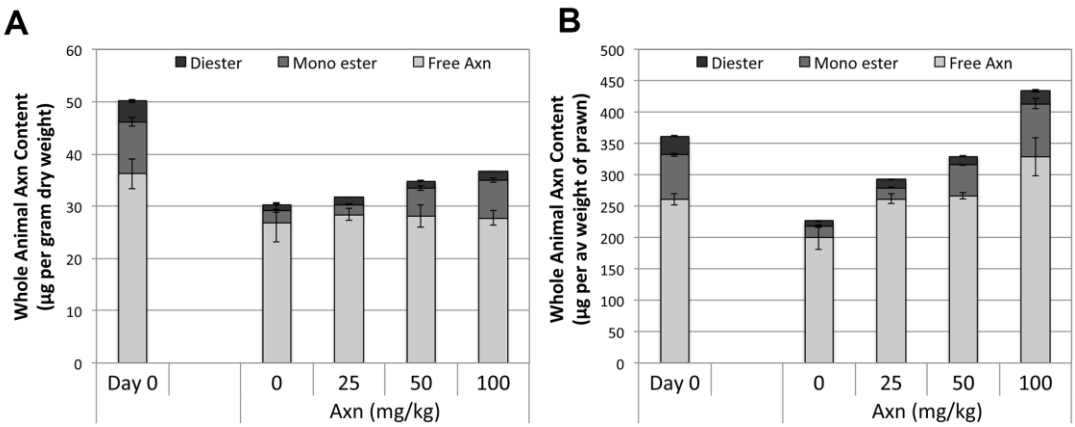

Figure 2. Whole prawn Axn levels after 42 days of feeding different dietary carotenoid levels. Total carotenoids were extracted from freeze dried whole prawns and quantified using HPLC into free, mono-ester or di-ester fractions of Axn. Data is expressed as µg Axn per gram dry weight (A) or converted to total amount of carotenoid contained within the animal (B).

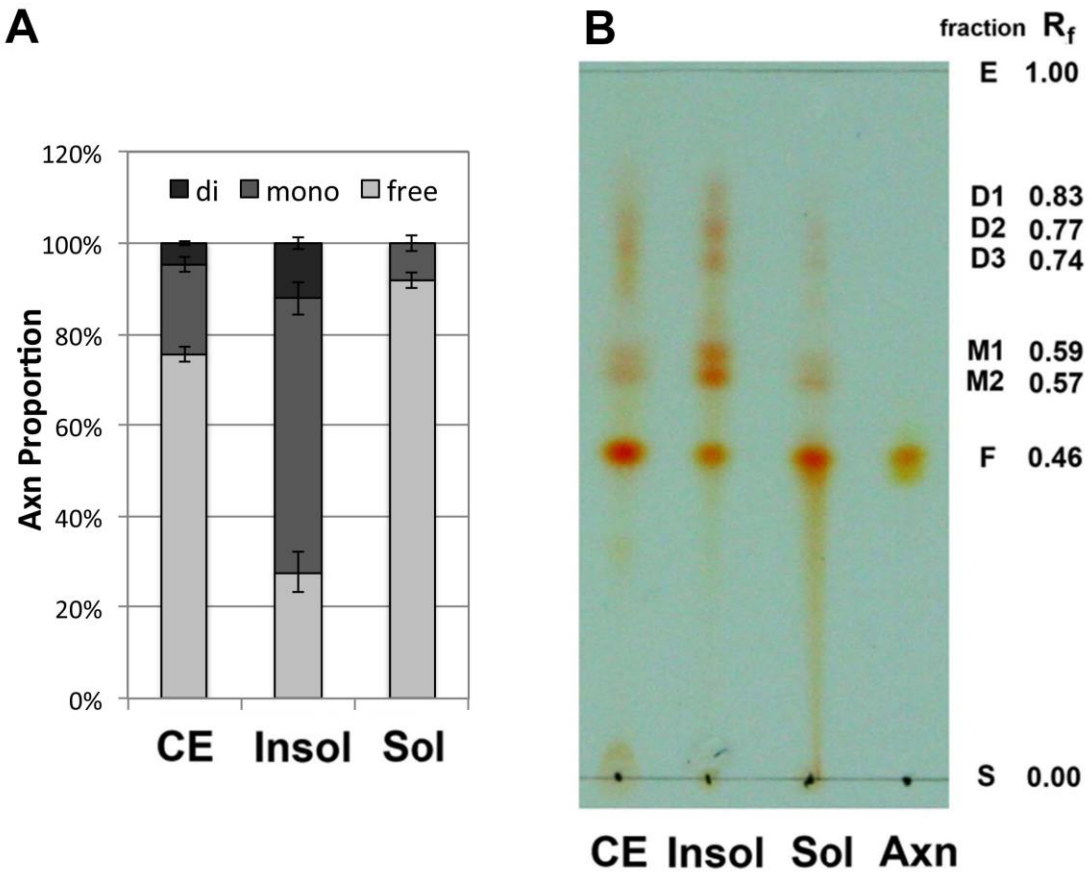

Figure 3. Separation of Axn esters. Whole prawn epithelial tissue carotenoid extract (CE) was separated into soluble (sol) and insoluble (insol) fractions and the proportions of free, mono-ester or di-ester fractions of Axn quantified by HPLC (A). Thin layer chromatography (TLC) was also used to separate these fractions into different free (F), mono-ester (M1, M2) or di-ester (D1, D2, D3) bands (B). The five bands from the insol fraction were used to identify fatty acid classes forming these carotenoid esters.

Table 1. Experimental diet formulations and proximate composition.

Formulation (%)	0 mg kg^{-1}	25 mg kg^{-1}	50 mg kg^{-1}	100 mg kg^{-}
Fish Meal	45.0%	45.0%	45.0%	45.0%
Gluten (wheat)	5.0%	5.0%	5.0%	5.0%
Flour	46.28%	46.25%	46.23%	46.18%
Lecithin	1.0%	1.0%	1.0%	1.0%
Fish Oil	1.5%	1.5%	1.5%	1.5%
Carophyll Pink (10%)	0.000%	0.025%	0.050%	0.100%
Cholesterol	0.10%	0.10%	0.10%	0.10%
Banox E	0.02%	0.02%	0.02%	0.02%
Vit C (Stay C)	0.10%	0.10%	0.10%	0.10%
Vit premix	0.20%	0.20%	0.20%	0.20%
Min premix	0.30%	0.30%	0.30%	0.30%
Yttrium	0.50%	0.50%	0.50%	0.50%
TOTAL	100%	100%	100%	100%
Proximate Composition (dry weight)				
Moisture Content (%)	8.89	9.04	8.67	9.27
Total Protein (%)	43.47	42.68	42.78	44.02
Total Lipid (%)	8.06	8.96	8.49	9.00
Ash (%)	12.57	12.51	12.93	12.66
Carotenoids (mg kg^{-1})	6.14	14.00	38.27	78.21

Table 2. Performance characteristics for animals fed different levels of dietary carotenoids for 6 weeks.

	Axn Content (mg kg^{-1})				Pooled
	0	25	50	100	SEM
Survival (%)	94.4	88.9	90.7	92.6	1.9
Start weight (g)	7.58	7.39	7.08	6.95	0.22
End weight (g)	8.67 ^{a}	11.81 ^{b}	12.18 ^{b}	12.00 ^{b}	0.30
Gain (g)	1.09 ^{a}	4.43 ^{b}	5.10 ^{b}	5.05 ^{b}	0.32
Growth (g week^{-1})	0.18 ^{a}	0.74 ^{b}	0.85 ^{b}	0.84 ^{b}	0.05
Feed consumed (g)	148.5 ^{a}	158.2 ^{b}	159.4 ^{b}	159.5 ^{b}	0.91
FCR	23.6 ^{a}	7.3 ^{b}	6.0 ^{b}	5.9 ^{b}	1.17

Superscripts denote significant (P <0.05) differences between measured values across treatments.

Table 3. Apparent Digestibility Coefficients (ADC) for dietary nutrients at different carotenoid inclusion levels.

	Axn Content (mg kg^{-1})				Pooled
	0	25	50	100	SEM
DM (%)	46.6%^{a}	61.7%^{a}	62.1%^{a}	61.5%^{a}	3.24%
Lipid (%)	64.1%^{a}	86.3%^{b}	78.5%^{b}	80.0%^{ab}	2.89%
Protein (%)	61.0%^{a}	58.7%^{a}	64.3%^{a}	67.6%^{a}	2.75%
Carotenoids (%)	nd	65.3%^{ab}	81.5%^{b}	98.5%^{bc}	6.21%

Superscripts denote significant (P <0.05) differences between measured values across treatments. nd indicated that no carotenoid was detected in these samples.

Table 4. Composition of major fatty acids (expressed as % of the total) of astaxanthin fractions from thin layer chromatography of prawn epithelial extracts.

SFA	CE	D1	D2	D3	M1	M2
11:0	0.00	0.00	0.00	0.00	0.25	0.65
12:0	0.00	3.43	2.59	1.37	1.25	2.99
13:0	1.12	0.87	0.00	0.87	0.40	1.94
14:0	0.55	4.20	3.17	4.28	3.19	6.29
15:0	0.45	0.90	0.00	1.47	1.21	1.70
16:0	29.22	18.00	12.44	19.59	43.93	41.37
17:0	1.12	0.39	0.00	1.08	1.03	0.60
18:0	3.28	6.54	6.22	8.04	25.83	8.87
20:0	0.24	0.60	1.18	1.26	0.16	0.25
22:0	0.81	1.19	2.60	1.23	0.37	0.60
23:0	0.09	0.83	1.88	0.98	0.27	0.44
24:0	0.38	0.00	0.00	0.00	0.00	0.00
Sum	37.27	36.94	30.07	40.16	77.90	65.69
MUFA						
14:1n-5	0.30	1.43	0.00	0.00	0.38	1.05
15:1	2.24	2.50	2.43	6.06	0.44	0.60
16:1n-7	0.82	1.78	1.31	1.66	0.88	2.82
17:1	0.22	0.00	0.00	0.00	0.34	0.50
18:1n-9cis	16.56	9.83	4.35	7.50	6.98	6.33
18:1n-9trans	10.47	0.78	0.00	0.39	0.78	1.02
20:1n-11	0.22	1.48	2.30	1.11	0.14	0.16
20:1n-9	0.81	0.00	0.00	0.00	0.35	0.21
22:1n-9	1.23	11.17	14.27	10.60	2.61	4.11
24:1n-9	0.30	0.00	0.00	0.00	0.00	0.00
Sum	33.17	28.97	24.67	27.32	12.89	16.82
PUFA						
16:2or3	2.74	0.88	0.00	0.00	0.00	0.00
18:2n-6 trans	0.23	0.00	0.00	0.00	0.00	0.00
18:2n-6 cis	7.05	1.37	0.52	1.57	0.57	6.01
18:3n-6	0.33	0.82	0.68	0.58	0.32	0.22
18:3n-3	0.21	0.48	0.54	2.18	0.16	0.19
18:4n-3	0.08	0.99	1.58	0.00	0.12	0.58
20:2n-6	0.44	1.32	2.22	0.85	0.33	0.52
20:3n-3	0.08	5.54	7.88	2.98	1.47	1.56
20:5n-3	5.93	5.38	7.52	5.59	1.35	1.86
22:2	0.49	13.63	20.16	14.99	3.44	4.94
22:4n-6	0.65	1.76	0.00	0.00	0.00	0.00
22:5n-3	0.36	0.00	0.00	0.00	0.00	0.00
22:6n-3	9.73	1.92	4.18	3.77	0.96	1.63
Sum	28.31	34.09	45.27	32.52	8.71	17.50
Total	98.7	100.0	100.0	100.0	99.5	100.0
Sum n-3	19.1	15.2	21.7	14.5	4.1	5.8
Sum n-6	9.2	18.9	23.6	18.0	4.7	11.7
(n-3)/(n-6)	2.1	0.8	0.9	0.8	0.9	0.5