A Review of Carotenoid Utilisation and Function in Crustacean Aquaculture

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Abstract

Studies over a number of years have consistently shown that dietary carotenoid supplementation is beneficial for crustacean aquaculture across a range of commercially relevant parameters. Most obvious is the effect on pigmentation, where carotenoid inclusion levels in feeds and duration of feeding diets with carotenoids have been extensively optimised across many species to improve product colour, and subsequently quality and price. However, beneficial effects of carotenoid inclusion have increasingly been demonstrated on other parameters including survival, growth, reproductive capacity, disease resistance and stress resistance. A number of natural and synthetic carotenoid sources have been utilised in crustacean aquaculture. This review focuses on the type, metabolic conversion and function of carotenoids used in crustacean nutrition, and explores the physiological benefits this class of molecules brings to these animals.

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1 Introduction

Carotenoids form the basis of the pigmentation of a wide variety of aquatic organisms (Matsuno, 2001, Britton and Goodwin, 1982, Maoka, 2011), and marine animals extensively utilise a variety of properties that carotenoids possess. Due to their diversity and broad distribution, carotenoid types, structure, metabolism and function have been extensively studied across a wide range of organisms (Britton *et al.*, 2008). Among those organisms studied, crustaceans utilise a range of different carotenoids that vary across species, within individual crustacean tissues or are dependent on various physiological, geographic or ecological parameters (Castillo *et al.*, 1982).

Very little attention has been paid to the specific effects of carotenoid supplementation in crustacean aquaculture, aside from the affect on pigmentation (Bjerkeng, 2008). Up until recently, the physiological effects beyond pigmentation have been inferred from other studies, mostly from fish. The present review summarises the recent progress in the use of carotenoids as a dietary nutrient in crustacean aquaculture, and outlines the effects of this dietary carotenoid supplementation on various aspects specific to crustacean physiology.

2 Carotenoids in Crustaceans

2.1 Tissue Distribution and Carotenoid Types

The majority of crustaceans and crustacean tissues attribute their colouration to the presence of various carotenoids. This topic has largely been covered extensively in the past (Castillo *et al.*, 1982, Lenel *et al.*, 1978) and is not the focus of this review. 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.*, 1976a). 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). 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). The accumulation of certain carotenoids in the tissues of different crustaceans not only indicates that these animals are able to interconvert one carotenoid to another, but also implies that there is a specific function for particular carotenoid in certain tissues.

2.2 Carotenoid Interconversion and Metabolism

Like most animals, crustaceans cannot synthesise carotenoids and must obtain them from their diets (Goodwin, 1952). However, for some time there has been strong evidence that various Decapod crustaceans can convert different dietary carotenoids (including canthaxanthin, lutein or zeaxanthin) into the predominant carotenoid Axn (Castillo and Lenel, 1978, Castillo *et al.*, 1980, Chien and Jeng, 1992, Kour and Subramoniam, 1992, Petit *et al.*, 1991, Yamada *et al.*, 1990, Castillo and Negre-Sadargues, 1995, Negre-Sadargues *et al.*, 1993, Mantiri *et al.*, 1995, Vernon-Carter *et al.*, 1996, D' Abrahmo *et al.*, 1983, Tanaka *et al.*, 1976b, Tanaka *et al.*, 1976a). Many years ago, the carotenoid metabolic transformative capacity of crustaceans was summarised (Negre-Sadargues, 1978, Schiedt *et al.*, 1993, Castillo *et al.*, 1982). The major pathway by which β-carotene is converted to Axn is summarised in Figure 1, although it would appear that crustaceans are able to perform a variety of other carotenoid transformations (Castillo *et al.*, 1982). Crustaceans fall into two broad classes based on their metabolic conversion capacity: those that can convert β-carotene to Axn in their internal organs, such as Penaeid shrimp; or those that can convert β-carotene to Axn in their internal organs but also convert metabolic intermediates in other tissues of their body, such as lobsters and crabs(Katayama *et al.*, 1973). Dietary paprika has been used as a source of carotenoids in *P. monodon* broodstock diets, suggesting that the carotenoids α-carotene, α-cryptoxanthin and capxanthin present in paprika were converted into Axn (Wyban *et al.*, 1997). Similarly, *M. japonicus* has been shown to produce Axn from α-carotene, canthaxanthin, echinenone or zeaxanthin (Chien and Jeng, 1992, Tanaka *et al.*, 1976b, Yamada *et al.*, 1990). Carotenoid metabolic capacity is active throughout crustacean early larval and post-larval development (Mantiri

et al., 1995, Mantiri *et al.*, 1996, Petit *et al.*, 1991, Berticat *et al.*, 2000), where the carotenoids may be metabolised as a source of retinoids (Dall, 1995). Yet despite the increase in genomic knowledge of crustaceans, including the sequencing of the complete genome of Daphnia, there has been very little progress in defining the biochemical pathways responsible carotenoid metabolism in this Class of animals. The variation in different carotenoid types across different developmental, physiological and ecological parameters strongly suggests that crustaceans utilise specific carotenoids for different functions during developmental processes or in response to environmental circumstances.

2.3 Carotenoid Sources in Crustacean Aquaculture

Sources of carotenoids that have been used in crustacean diets include synthetic carotenoids (Castillo and Negre-Sadargues, 1995, Chien and Jeng, 1992, Negre-Sadargues *et al.*, 1993), Antarctic krill (Maoka *et al.*, 1985), brine shrimp (Pan and Chien, 2003), shrimp by-products (Mandeville *et al.*, 1991, Chakrabarti, 2002, Meyers and Bligh, 1981), microalgae (Sommer *et al.*, 1991, Supamattaya *et al.*, 2005, Armenta-Lopez *et al.*, 2002, Chien and Jeng, 1992), blue green algae (Liao *et al.*, 1993, Okada *et al.*, 1991), and plant extracts (Vernon-Carter *et al.*, 1996, D' Abrahmo *et al.*, 1983, Arredondo-Figueroa *et al.*, 2003). More recently, other potential sources of carotenoids for crustacean aquaculture have been investigated, including genetic engineering of higher plants to accumulate high levels of ketocarotenoids such as Axn (Han *et al.*, 2013). Studies assessing the effect of different sources of carotenoids on pigmentation in crustaceans are summarised in Table 1.

3 Carotenoid Function in Crustaceans

Carotenoids are known to be involved in a large number of physiological functions in plants and animals, and these functions are largely based on the structure of the carotenoid (Britton, 2008, Goodwin, 1986). As the major carotenoid in crustacean tissues, Axn provides functions that include pigmentation, photoprotection, antioxidant and a source of provitamin A (Britton, 2008). Benefits to the animal include the enhancement of growth, higher survival, increased stress resistance and improved reproductive potential

(Kumar *et al.*, 2009, Supamattaya *et al.*, 2005, Niu *et al.*, 2014, Paibulkichakul *et al.*, 2008, Linan-Cabello *et al.*, 2002a). An example of these benefits was observed in crayfish exposed to pollution, which had lower levels of vitamins and carotenoids in the hepatopancreas, suggesting these may play a role in tolerating polluted environments (Barim and Karatepe, 2010). The conversion of carotenoids into other biologically active molecules, such as Provitamin A and retinoids has also been implicated (Linan-Cabello *et al.*, 2002a). Since the initial proposals of carotenoid function in crustaceans, there has been substantial progress in gathering scientific evidence to support the range of proposed functions of Axn and its effects on crustacean physiology, which will be discussed in further detail in the following sections.

3.1 Carotenoids and Crustacean Colouration

The best-established function of carotenoids in crustaceans is pigmentation. Colour plays a major role in consumer acceptability, perceived quality and price paid for commercial crustacean species (Parisenti *et al.*, 2011b, Shahidi *et al.*, 1998, Chien and Jeng, 1992, Erickson *et al.*, 2007). Many species of crustacean lose or do not develop pigmentation if not supplied a diet with sufficient carotenoids. Among these included hermit crabs (Castillo and Negre-Sadargues, 1995), red king crabs (Daly *et al.*, 2013), crayfish (Sommer *et al.*, 1991), clawed lobsters (Tlusty and Hyland, 2005), spiny lobsters (D' Abrahmo *et al.*, 1983, Barclay *et al.*, 2006), and shrimp (Dall, 1995). In shrimp, poor pigmentation was initially described as a disease status (Howell and Matthews, 1991), although this was subsequently shown to be ameliorated by dietary carotenoid supplementation (Menasveta *et al.*, 1993). Recently, pigmentation in banana shrimp has been shown to be heritable (Nguyen *et al.*, 2014), potentially through improvements in pigment retention. Crustacean colour variations have also been observed that are unrelated to dietary carotenoids. Indeed, much of the colour variation between species is thought to be attributable to differences in the sequence and expression pattern of pigment gene crustacyanin (Wade *et al.*, 2009), which will be discussed in more detail in later sections. Rare genetic colour mutations have been observed in clawed lobsters, predominantly Homarid species (Haggin, 2012), but also in prawns and crabs. The spiny lobster

Panulirus cygnus undergoes a colour change from deep red to pale pink during a migratory period (Phillips, 1983). This colour change has been attributed to a developmental ontogenic change that provides protective camouflage during migration, as it was not prevented by dietary carotenoid supplementation or triggered by background substrate colour (Wade *et al.*, 2008). In another example of colour variation, seasonal appearance of pink crab disease was shown to be caused by a parasitic infection (Stentiford *et al.*, 2002). Similarly, colour transitions have been observed between juvenile and adult stages of crabs (Krause-Nehring *et al.*, 2010).

3.1.1 Carotenoid Type, Inclusion Levels and Feed Duration

The majority of the focus of dietary carotenoid inclusion has been on the effects on crustacean pigmentation, having been studied over many years across a range of crustacean species. These studies have been summarised in Table 1. In general, pigment development is largely dependent on the amount of carotenoid in the feed and the duration for which it is fed. Dietary Axn concentrations between 50-100 mg/kg fed for one month were sufficient to produce optimal pigmentation in a range of shrimp species (Niu *et al.*, 2012, Niu *et al.*, 2014, Yamada *et al.*, 1990, Petit *et al.*, 1997). However, 80-100 mg/kg dietary Axn supplementation produced a darker external colour more rapidly, although similar pigmentation levels were achieved over a longer duration of feeding at 50 mg/kg (Chien and Jeng, 1992, Tlusty and Hyland, 2005, Barclay *et al.*, 2006). Pigmentation of red king crabs was also significantly improved over a 56 day period when diets were supplemented with 380 mg/kg Axn (Daly *et al.*, 2013), but no lower inclusion levels or shorter feeding periods were tested. There is clear evidence that as dietary carotenoid levels increase, so does the Axn content of the animal, particularly the Axn esters (Yamada *et al.*, 1990, Supamattaya *et al.*, 2005, Boonyaratpalin *et al.*, 2001, Barclay *et al.*, 2006, Kumar *et al.*, 2009, Wade *et al.*, 2008, Wade *et al.*, 2015b). In order to maintain initial carotenoid levels, spiny lobsters required 90 or 120 mg/kg dietary Axn (Barclay *et al.*, 2006). In some cases, the body concentration of carotenoids (mg/kg dry weight) decreased as shrimp grew (Pan *et al.*, 2001, Pan *et al.*, 1999), while in others the carotenoid concentration was maintained as the animals grew (Yamada *et al.*,

1990, Wade *et al.*, 2015b). Accordingly, some studies report that the whole body tissue Axn concentration is an appropriate indicator of body color of shrimp (Menasveta *et al.*, 1993, Negre-Sadargues *et al.*, 2000), while others suggest Axn concentration isn't necessarily reflective of body colour (Tume *et al.*, 2009). Clearly, further work is required to provide some clarity to the objectivity of this method of assessment.

The type of dietary carotenoid also affects the rate at which pigmentation is developed. Shrimp (*P. monodon*) fed dietary Axn at 100 mg/kg showed the highest levels of tissue Axn (16.5 mg/kg body weight) which was 23% and 43% higher than animals fed 100 mg/kg canthaxanthin or β-carotene, respectively (Yamada *et al.*, 1990). Pigmentation of juvenile Kuruma shrimp, *Marsupenaeus japonicus*, was better when animals were fed 100 mg/kg Axn for one month, compared with animals fed 50 mg/kg Axn or 20 – 200 mg/kg β-carotene (Chien and Jeng, 1992). A similar improved carotenoid tissue deposition was also observed in shrimp fed 100 mg/kg Axn, compared with either canthaxanthin or an Axn-canthaxanthin mixture (Negre-Sadargues *et al.*, 1993). For *P. monodon* to achieve a similar colour to that achieved using 50 mg/kg dietary Axn over 4 weeks, β-carotene was required at 125 mg/kg over 7-8 weeks, which was reduced to 5-6 weeks by using 175 mg/kg (Boonyaratpalin *et al.*, 2001). Shrimp fed a diet supplemented with *Artemia* nauplii (which were enriched with 80% canthaxanthin) for 4 weeks had improved deposition of free and esterified Axn compared with those fed a diet supplemented with mauxia shrimp (55% β-carotene) (Pan and Chien, 2003). Dietary supplementation of 200-300 mg/kg of the β-carotene enriched microalgal pigment from *Dunaliella* was required for optimal pigmentation in *Penaeus monodon* (Supamattaya *et al.*, 2005). These observations support that the efficiency with which carotenoid intermediates are converted to Axn depends on their position in the relevant metabolic conversion pathways. Dietary Axn levels greater than 200 mg/kg did not lead to improvements in pigmentation or tissue carotenoid accumulation (Yamada *et al.*, 1990, Merchie *et al.*, 1998), but other potential benefits of these high dietary carotenoid levels were not examined in these studies. Later sections of this review will explore further research in this area.

3.1.2 Chromatophores and Pigmentory Effectors

The colour of crustaceans is present in either the exoskeleton, or in pigment structures within the underlying hypodermal layer known as chromatophores (Rao, 1985). These structures are able to expand and contract, which strongly contributes to the degree of individual colouration, particularly for species with thin opaque shells like shrimp (Fingerman, 1965, Fingerman, 1966). Such physiological colour changes can be rapid, are reversible and often rhythmic in some species of crustaceans. This expansion and contraction is controlled by hormones secreted from glands in the eyestalks of crustaceans: pigment dispersing hormone (PDH) and red pigment concentrating hormone (RPCH), as a response to various physiological cues (Bagnara and Hadley, 1973, Rao, 2001). These cues can span aspects such as background colour, light source and photoperiod (Latscha, 1990, Rao, 1985).

Short-term exposure to black substrates has been shown to improve prawn pigmentation through expansion of hypodermal chromatophores (Parisenti *et al.*, 2011a, Tume *et al.*, 2009, Wade *et al.*, 2015a). An example of the effect that background exposure has on the chromatophores in shrimp epithelial tissue is shown in Figure 2. In addition to expanding and contracting, the chromatophores completely change their pigment content in response to different substrates. In response to dark backgrounds, animals with expanded chromatophores contained high levels of free Axn, while white adapted animals with contracted chromatophores contained high levels of Axn mono-esters (Tume *et al.*, 2009, Wade *et al.*, 2015b). This expansion was also shown to be linked with the accumulation of the colour protein crustacyanin in the hypodermal tissues (Wade *et al.*, 2012), presumably bound to free Axn to create the darker colouration. Tank colour was also shown to affect larval colour, survival and development in crabs (Rabbani and Zeng, 2005). When exposed to constant light, the body color of shrimp (*P. aztecus*) faded and chromatophores lost their diurnal rhythm (Lakshmi *et al.*, 1976). Similarly, the body color of *P. monodon* also became faint when cultured indoors under low light intensity less than 1000 lx (Tseng *et al.*, 1998). However, shrimp (*P. monodon*) subjected to constant light maintained higher carotenoid levels as they grew (Pan *et al.*, 2001). Without addition of Axn in diet, metal halide illumination at 2500 lux resulted in the

significant accumulation of Axn in whole body of *L. vannamei* to over 4 mg/kg, compared with animals held in complete darkness at just over 2 mg/kg (You *et al.*, 2006).

Lastly, the colour of *P. monodon* has been observed to become redder when subjected to thermal and hypoxic stress, but this pigment effect was reversible when the stress was removed and hypoosmotic stress had no effect on colour (de la Vega *et al.*, 2007). Hypoxia was shown to increase the levels of CRCN-C1 abundance in the hepatopancreas of *Litopenaeus vannamei* (Jiang *et al.*, 2009), although why this may be occurring is not understood. Other reports of the effect of stress on pigmentation are largely anecdotal, and there is presently very little understanding of why this might be occurring.

3.1.3 Carotenoproteins and Crustacyanin

Carotenoids and associated carotenoprotein complexes have been found in many invertebrate species with tissue distribution ranging from the skin and gonads to the blood, eggs and shell (Zagalsky, 1985, Lakshman and Okoh, 1993, Cheesman *et al.*, 1967, Bhosale and Bernstein, 2007). Carotenoprotein complexes can be divided into two types: lipovitellins and true carotenoproteins. Lipovitellins possess a less stable and non-specific association of the carotenoid with the lipid portion of a lipoprotein and are responsible colouration of such tissues as the blood, epithelium, eggs and ovaries (Zagalsky, 1985, Cheesman *et al.*, 1967). True carotenoproteins display a highly specific and stoichiometric relationship between the carotenoid and a carotenoid binding protein (CBP), and appear to be particularly widespread among the animals in class *Crustacea* as the mechanism of shell colour production (Zagalsky, 1985, Lakshman and Okoh, 1993, Cheesman *et al.*, 1967).

Pigmentation in crustaceans is produced by a combination of the abundance and degree of expansion of different coloured chromatophores, yellow, blue and red (Rao, 1985), although visibility of chromatophores can be influenced by the thickness of the exoskeleton in some species. As noted earlier, dietary Axn supplementation increases the abundance of epithelial Axn, particularly Axn esters (Yamada *et al.*, 1990, Supamattaya *et al.*, 2005, Boonyaratpalin *et al.*, 2001, Barclay *et al.*, 2006, Kumar *et al.*, 2009, Wade *et al.*, 2015b). Similarly, background colour modifies pigment proportions in epithelial tissues, with

296 contracted chromatophores containing high levels of carotenoid esters, and
297 expanded chromatophores containing high levels of free Axn (Tume *et al.*, 2009,
298 Wade *et al.*, 2015b, Wade *et al.*, 2015a) Within the exoskeleton and hypodermal
299 tissue of crustaceans, free Axn is often bound within a multimeric protein
300 complex called crustacyanin (CRCN) (Wald *et al.*, 1948). CRCN is a member of the
301 lipocalin protein family, a functionally diverse group of proteins that bind small
302 hydrophobic molecules such as steroid hormones, carotenoids, odourants and
303 pheromones (Flower, 1996, Flower *et al.*, 2000). The interaction of CRCN and
304 Axn modifies the naturally red carotenoid to blue or any other colour in the
305 visible spectrum, producing the diverse array of colours seen in the exoskeleton
306 of crustaceans (Cianci *et al.*, 2002). During cooking, this interaction is disrupted,
307 releasing the distinct red colouration of cooked seafood. The dimeric β-
308 crustacyanin (β-CRCN) is formed by two types of CRCN subunits (A and C, also
309 called H_1 and H_2) in association with two Axn molecules (Cianci *et al.*, 2002).
310 Eight of these dimers form a larger molecular weight complex known as α-
311 crustacyanin (α-CRCN), which has been extensively studied using
312 crystallographic techniques (reviewed in, (Chayen *et al.*, 2003, Zagalsky, 2003)).
313 At present, two genes that encode CRCN-A and CRCN-C have been identified
314 across a range of crustaceans (Wade *et al.*, 2009, Ertl *et al.*, 2013, Wang *et al.*,
315 2007). Their expression is restricted to the outer layer of the hypodermis (Wade
316 *et al.*, 2009, Wang *et al.*, 2007), and the spatial regulation of the *CRCN* genes is
317 thought to define the species-specific shell colors and patterns that different
318 crustaceans display (Wade *et al.*, 2009). In further support of this theory,
319 reconstitution of recombinant CRCN monomers (either A or C) formed
320 complexes with distinct absorption spectra, and the presence of CRCN in various
321 species correlated with the ability to produce certain shell colours (Ferrari *et al.*,
322 2012).
323 The development of colour over time in pigment deficient clawed lobsters
324 (*H. americanus*) was dependent on dietary carotenoid concentration, and
325 progressed over three months through either a predominantly red or a
326 predominantly blue phase before achieving a colour considered equivalent to
327 those from the wild (Tlusty and Hyland, 2005). In freshwater shrimp
328 (*M. rosenbergii*), external colour was removed by specific knockdown of a CRCN

329 homolog using RNAi (Yang *et al.*, 2011). In this study, the blue pigment attributed
330 to the Axn-CRCN interaction was removed by decreasing CRCN gene expression,
331 and hence protein abundance, which modified the shrimp colour to red.
332 Although not directly measured, the red colour that remained was likely the
333 underlying red chromatophores containing predominantly Axn esters. This
334 suggests that colour could be preferentially deposited in different
335 chromatophores, although how this might be regulated is not understood.
336 Exposure to white substrates significantly decreased the amount of CRCN
337 protein in shrimp hypodermal tissue, along with decreased free Axn levels and
338 increased Axn ester levels (Wade *et al.*, 2012). Exposure to black substrates
339 significantly increased the abundance of epithelial CRCN protein (Wade *et al.*,
340 2012), indicating the presence of this protein was critical to redistributing
341 hypodermal pigments and achieving optimal cooked colour (Wade *et al.*, 2012).
342 However, CRCN gene expression did not vary across the moult cycle or in
343 response to substrate colour (Wade *et al.*, 2012). Albino colour morphs of shrimp
344 (*F. merguiensis*) displayed significantly reduced expression of the CRCN-A and C
345 genes compared with other shrimp, as well as a range of other genes potentially
346 involved in the regulation of crustacean colour (Ertl *et al.*, 2013). However,
347 expression levels of CRCN were not significantly different between light and dark
348 coloured shrimp, and there was no correlation between levels of CRCN gene
349 expression and Axn content (Ertl *et al.*, 2013). Despite extensive knowledge of
350 the mechanism by which CRCN binds Axn to produce crustacean colour, there is
351 very little known about how CRCN gene expression is regulated or how the CRCN
352 protein complexes form or are modified in the crustacean exoskeleton.
353
354 *3.2 Carotenoids and Growth and Survival*
355 Reports of the effect of dietary carotenoid supplementation on growth and
356 survival in crustaceans have been mixed, with virtually all research having been
357 conducted on shrimp. Some studies reported no significant difference in growth
358 in shrimp that had received dietary carotenoid supplementation (Pan *et al.*,
359 2001, Negre-Sadargues *et al.*, 1993, Boonyaratpalin *et al.*, 2001). However, an
360 increasing number of studies have shown that either growth or survival, or both,
361 are significantly improved when shrimp are fed a diet that contains carotenoids

362 compared with diets that do not (Niu *et al.*, 2012, Niu *et al.*, 2014, Supamattaya *et*
363 *al.*, 2005, Yamada *et al.*, 1990, Kumar *et al.*, 2009, Chien and Shiau, 2005, Petit *et*
364 *al.*, 1997, Darachai *et al.*, 1998, Chien and Jeng, 1992, Flores *et al.*, 2007, Zhang *et*
365 *al.*, 2013).
366 Early reports describing the beneficial effects of Axn on shrimp growth were
367 assessed on postlarvae (Darachai *et al.*, 1998, Chien, 1996) with evidence that
368 Axn supplementation shortened the moult frequency (Petit *et al.*, 1997). Larval
369 stages and postlarvae of *P. monodon* showed greater survival and were longer
370 when fed algal Axn (*Haematococcus pluvialis*) supplemented diets (Darachai *et*
371 *al.*, 1998). Studies on *M. japonicus* juveniles demonstrated that growth
372 performance was similar in shrimp over 8-weeks whether or not 100 mg/kg
373 carotenoid was included (Yamada *et al.*, 1990). However, by the end of 8 weeks
374 animals without dietary carotenoid contained significantly less total carotenoid
375 than those fed 100 mg/kg, and their survival had dropped from 91.3% to 57.1%
376 (Yamada *et al.*, 1990). In a separate experiment by the same authors but using
377 smaller animals, animals that had received 100 mg/kg Axn for 8 weeks had
378 grown significantly better than those that had not been fed Axn, while survival
379 was unaffected (Yamada *et al.*, 1990). Between these two experiments, there was
380 a marked difference in the total carotenoid content prawns at the beginning of
381 the experiment, with poor survival over 8 weeks recorded when initial
382 carotenoid content was low (15.6±0.8 mg/kg). Significant correlations have been
383 observed between tissue carotenoid concentration and survival (Chien and Jeng,
384 1992) or specific growth rate (You *et al.*, 2006).
385 Since this initial work, the vast majority of studies have focussed on the giant
386 tiger shrimp, *Penaeus monodon*. Shrimp fed 125-300 mg/kg of algal extract for 8-
387 weeks showed higher weight gain and survival compared with controls
388 (Supamattaya *et al.*, 2005). When fed with 100mg/kg Axn combined with 1%
389 cholesterol for 74 days, shrimp showed higher weight gain and survival
390 compared with those fed diets without carotenoids (Niu *et al.*, 2012), with
391 apparent Axn digestibility of approximately 98%. In a similar study, shrimp fed
392 100 mg/kg Axn combined with 1% cholesterol also showed significantly higher
393 weight gain and survival (Niu *et al.*, 2014), and showed similarly high (>90%)
394 Axn digestibility. Although less studied, other species have shown a similar

response. Post-larval shrimp (*L. vannamei*) fed 80 mg/kg Axn for 6 weeks showed an increased daily growth coefficient and a reduced moult frequency compared with those animals that had not been fed dietary Axn, but survival was unaffected (Flores *et al.*, 2007). Shrimp (*L. vannamei*) fed either 100, 200 or 400 mg/kg Axn for 30 days showed improved weight gain and survival compared with those without dietary carotenoids (Niu *et al.*, 2009). After 56 days, shrimp (*L. vannamei*) fed 125 or 150 mg/kg Axn had higher weight gain than those fed 25, 50, 75 or 100 mg/kg Axn (Zhang *et al.*, 2013), but survival was unaffected. In freshwater *Macrobrachium*, inclusion of 50, 100 or 200 mg/kg Axn improved growth over the reference (Kumar *et al.*, 2009). Shrimp (*M. japonicus*) had improved survival from 37% to over 50% when fed diets containing carotenoids over 9 weeks, (Chien and Shiau, 2005), with a complementary increase in body Axn levels, but no effect on growth. Improved survival, but not growth, was also recorded in red king crab juveniles fed 380 mg/kg Axn for 56 days (Daly *et al.*, 2013).

Combined, these data suggest that survival is not affected when carotenoids are maintained at a certain level, perhaps between 10-15 mg/kg body weight for *P. monodon*, but survival is compromised below that level without carotenoid supplementation. Where tissue carotenoid levels are initially high, perhaps above 20 mg/kg, further carotenoid supplementation allows improved growth. Variability in animal performance in growth trials may be explained by a range of factors, including animal health, quality of feed ingredients, system design and animal husbandry. Detection of growth differences in shrimp fed dietary carotenoids in more recent studies may reflect improvements in trial maintenance and animal husbandry. The study by (Pan *et al.*, 2001) had shown there was no significant increase in survival in animals fed carotenoids compared with those that were not, although overall survival was less than <30% across the experiment, and this low level of survival casts aspersions on the validity of this work. Despite this, it was demonstrated that higher tissue carotenoid levels were correlated with higher survival (Pan *et al.*, 2001). Carotenoid levels in shrimp at the beginning of the study will also be critical, as carotenoid stores in animal tissues may compensate for the lack of dietary carotenoids at least through the initial stages of an experimental growth trial.

3.3 Carotenoids and Tolerance to Disease and Stress

This section will focus on studies where dietary carotenoids have been supplied, then the capacity to tolerate an induced stress has been directly tested under controlled conditions, and the effects on survival or other biochemical parameters assessed. The improved survival described in the previous section was reported after a period of 8-9 weeks of a growth feeding trial in experimental systems using different carotenoids (Axn, β-carotene or canthaxanthin). However, more recent studies have been designed to specifically assess whether responses to acute and chronic stresses, such as hypoxia, salinity or viral infection, are improved after long periods of dietary carotenoid supplementation. Analysis on shrimp (*F. chinensis*) showed that hypoxia alone triggered significant up-regulation of proteins involved in immunity (chymotrypsin and carboxypeptidase), and down regulation of proteins involved in energy production (citrate synthase, ATP synthase), metabolism (transketolase and esterases) and antioxidant capacity (glutathione peroxidase and cMnSOD) (Jiang *et al.*, 2009). Dietary levels of 125 and 150 mg/kg Axn fed to shrimp (*L. vannamei*) for 56 days lowered total antioxidant status, superoxide dismutase (SOD), and catalase activities than those animals fed 25, 50, 65 or 100 mg/kg (Zhang *et al.*, 2013). Carotenoids were found to be less abundant in the digestive gland and ovary of farmed *L. vannamei* compared with wild animals, and levels were concluded to be insufficient to neutralise oxidative stress during ovarian development (Linan-Cabello *et al.*, 2003). Crayfish exposed to pollution had lower levels of vitamins and carotenoids in the hepatopancreas, suggesting these may play a role in tolerating polluted environments (Barim and Karatepe, 2010).

Similar to growth and survival, the majority of work on tolerance to stress has been performed on shrimp. Early studies showed that larval stages of *P. monodon* supplemented with algal carotenoids were more resistant to low salinity stress than those with synthetic Axn or controls (Darachai *et al.*, 1998). Similarly, survival of *P. monodon* postlarvae during a low salinity stress test exposure to 4 hours of low dissolved oxygen (< 1.0 mg/L) was improved in

461 shrimp (*P. monodon*) fed 360 mg/kg Axn for one week (Chien *et al.*, 1999). In a
462 separate test, these shrimp were also shown to be more tolerant of lower oxygen
463 levels in a lethal oxygen test (Chien *et al.*, 1999). Dietary Axn supplementation at
464 80 mg/kg enhanced antioxidant capacity in tiger shrimp (*P. monodon*)
465 postlarvae, which resulted in a significant improvement in recovery to both
466 thermal and osmotic stress (Chien *et al.*, 2003). In this study, higher body Axn
467 levels were recorded, total antioxidant status (TAS) was reduced and superoxide
468 dismutase (SOD) levels were reduced. The authors also speculated that
469 hepatopancreas function was improved due to lower levels of aspartate
470 aminotransferase (AST), a blood marker of liver integrity in mammalian systems,
471 being identified in the circulating hemolymph. However, both AST and alanine
472 aminotransferase (ALT) levels were reduced by thermal and osmotic stress,
473 which was opposite to the expected effect of stress. The inclusion of 80 mg/kg in
474 diets for 8-weeks improved shrimp (*P. monodon*) resistance to ammonia stress,
475 and animals showed higher total antioxidant status and lower SOD levels (Pan *et*
476 *al.*, 2003). AST and ALT levels were lowered by Axn supplementation, and were
477 negatively correlated with TAS. However, aminotransferase levels were not
478 correlated with survival, and may indicate that shrimp mortality was unrelated
479 to hepatopancreas damage. When fed 300 mg/kg of algal carotenoids for 8
480 weeks, *P. monodon* showed improved tolerance to a nine day period of daily
481 hypoxic stress (<1.0 mg/L) and also higher resistance to WSSV infection
482 (Supamattaya *et al.*, 2005).
483 Studies in other shrimp also showed similar effects. In *M. japonicus*, inclusion of
484 at least 50 mg/kg dietary Axn, from either synthetic or algal sources, resulted in
485 improved survival to low oxygen stress (Chien and Shiau, 2005). Significantly
486 greater levels of Axn had accumulated during the 9-week feeding trial, along with
487 a reduced oxygen consumption rate, suggesting that Axn may be acting as an
488 intracellular oxygen reserve or as a potent cellular antioxidant. Total carotenoid
489 levels were highest in animals that showed the highest survival, yet total
490 hemocyte count was lower and hemolymph phenoloxidase activity was
491 unchanged. Post-larval shrimp (*L. vannamei*) fed 80 mg/kg Axn for 6-weeks
492 showed significantly higher osmoregulatory capacity than those without dietary
493 Axn after salinity was reduced from 35 to 3 gL^{-1} (Flores *et al.*, 2007). This was

494 coupled with significantly increased levels of hemocytes, hemocyanin and
495 glucose in the hemolymph, and reduced levels of hemolymph lactate (Flores *et*
496 *al.*, 2007). In a hypoxia stress test, postlarval shrimp (*L. vannamei*) fed either 200
497 or 400 mg/kg Axn recorded significantly higher survival, but no other
498 physiological parameters were measured (Niu *et al.*, 2009). More recently,
499 freshwater prawns showed a significant increase in phenoloxidase activity and
500 total hemocyte count after 28 days of consuming carotenoid fortified diets
501 (Kumar *et al.*, 2009), although no direct stress test was performed on the animals
502 in this study. Systemic injection of Axn into the same species caused an increase
503 in the total hemocyte count and an increased resistance to bacterial infection,
504 although there was no complementary increase in antioxidant indicators
505 (Angeles *et al.*, 2009). After low dissolved oxygen challenge, shrimp
506 (*L. vannamei*) fed 75-150 mg/kg Axn for 56 days had higher survival than those
507 animals fed 25 or 50 mg/kg Axn, and this was potentially linked with higher
508 expression of hypoxia inducible factor 1 alpha (HIF-1α), cytosolic manganese
509 superoxide dismutase (cMnSOD) and catalase in Axn fed animals (Zhang *et al.*,
510 2013). After 74 days feeding 100 mg/kg Axn or 250 mg/kg β-carotene, improved
511 growth performance and survival in juvenile *P. monodon* was coupled with lower
512 malondialdehyde levels (an indicator of lipid peroxidation) after a simulated live
513 transport test (Niu *et al.*, 2014). In addition, expression levels of heat shock
514 protein 70 (Hsp-70) were significantly elevated under hypoxia compared with
515 normoxia, and further up-regulated under hypoxic conditions without dietary
516 carotenoids (Niu *et al.*, 2014). Although counter-intuitive, the expression of
517 hypoxia inducible factor 1 alpha (HIF-1α) was decreased under hypoxic
518 condition, but were higher in animals fed β-carotene suggesting that the
519 response to hypoxia had been alleviated (Zhang *et al.*, 2013, Niu *et al.*, 2014).
520 In summary, data consistently demonstrate that dietary carotenoids increase the
521 total antioxidant capacity in the haemolymph of crustaceans, coupled with
522 decreased activity of other antioxidant enzymes. This may occur through
523 increased Axn levels in the haemolymph and tissues, improved oxygen carrying
524 capacity, decreased oxidation of polyunsaturated fatty acids or cellular proteins
525 or decreased activation of stress response systems. Combined, these data suggest
526 that the stress response is reduced in animals receiving dietary carotenoids

527 which improves survival to that stress, and that Axn is performing a broad
528 protective role against the detrimental effects of oxidative damage in tissues.
529 Similar to growth, many factors can affect survival in experimental systems,
530 which is especially problematic when survival is a key measure of performance
531 against stress. However, clear experimental evidence now exists to show that
532 carotenoid supplementation improves a range of factors to enable crustaceans to
533 tolerate stresses such as disease, hypoxia, temperature and salinity. These effects
534 appear to link the proposed antioxidant function of carotenoids themselves, with
535 physiological improvements in antioxidant capacity in the animals, and
536 improved performance under various stressful conditions. Some inconsistency
537 exists in the physiological responses of animals to dietary carotenoids, which
538 may highlight differences in the way different crustaceans deal with a variety of
539 stressors.
540
541 *3.4 Carotenoids and Reproductive Performance*
542 Nutrition plays a critical role in the reproductive success of crustaceans, and the
543 accumulation of nutrients in the developing ovaries, particularly lipids and
544 carotenoids, has a direct effect on reproductive measures such as egg number,
545 hatching rate and total nauplii produced (Wouters *et al.*, 2001). Very little
546 progress has been made in understanding the basis by which dietary carotenoids
547 improve crustacean reproduction since it was summarised more than ten years
548 ago (Linan-Cabello *et al.*, 2002a). During early maturation, carotenoids
549 accumulate in the hepatopancreas in both free and esterified form, after which
550 they are transported via the haemolymph to the ovaries during secondary
551 vitellogenesis (Harrison, 1990, Vincent *et al.*, 1988). Carotenoid content and type
552 varies greatly during ovarian development (Dall *et al.*, 1995, Linan-Cabello *et al.*,
553 2002b, Linan-Cabello *et al.*, 2003, Vincent *et al.*, 1988, Vincent *et al.*, 1989). The
554 darkening that occurs with this accumulation forms the basis of "staging" female
555 ovaries during ovarian maturation (Wouters *et al.*, 2001). Free and esterified Axn
556 is known to accumulate in the hepatopancreas during ovarian maturation, while
557 levels in the integument remain relatively constant (Dall *et al.*, 1995). Captive
558 shrimp contained less carotenoids, particularly in stage IV ovaries, than their
559 wild caught counterparts (Linan-Cabello *et al.*, 2003), strongly suggesting that

560 broodstock nutrition was deficient. Paprika as a source of dietary carotenoids (α-
561 carotene, α-cryptoxanthin and capxanthin) was shown to improve nauplii quality
562 in *P. monodon* broodstock (Wyban *et al.*, 1997), with the assumption that these
563 carotenoids were able to be converted into Axn. Axn supplemented in
564 broodstock diets for *Penaeus monodon* showed improved spawning and
565 fecundity (Pangantihon-Kuhlmann *et al.*, 1998). In the only recent study, high
566 levels of dietary fish oil and Axn have been linked to improved reproductive
567 performance, as measured by egg and spermatozoa number, in *P. monodon*
568 broodstock (Paibulkichakul *et al.*, 2008). As might be expected, increased dietary
569 fish oil led to accumulation of polyunsaturated fatty acids (PUFAs) in
570 hepatopancreas and ovary tissues, particularly 22:6n-3. However, extremely
571 high levels of dietary Axn (300 mg/kg) also led to an accumulation of Axn along
572 with these long chain PUFAs in ovary tissue (Paibulkichakul *et al.*, 2008).
573 Increased focus may be required on the use of carotenoids in conjunction with
574 other nutrients of reproductive significance, such as long chain PUFAs.
575 The positive effects of Axn can potentially be attributed its extremely high
576 capacity to scavenge oxygen free radicals, and the prevention of peroxidation of
577 PUFAs in tissues and diets (Britton, 2008, Miki, 1991). In various fish species, the
578 accumulation of carotenoids in reproductive tissues through dietary carotenoid
579 supplementation has been shown to improve a number of performance
580 characteristics, such as egg number, egg quality and number of larvae (Bjerkeng,
581 2008). Oxygen free radicals have been shown to attack biomembrane lipids and
582 proteins, leading to deterioration in egg quality (Bromage and Roberts, 1995). In
583 crustaceans, in conjunction with a depletion of carotenoids in the
584 hepatopancreas and ovary, an elevation of superoxide dismutase (SOD) activity
585 was observed in the haemolymph of captive shrimp compared with wild shrimp
586 (Linan-Cabello *et al.*, 2003). This was suggested to reflect the insufficient
587 scavenger activity to neutralize oxidative stress processes during spawning.
588 Normal developmental and physiological processes, such as ovarian
589 development and reproduction, are also potential sources of oxygen free
590 radicals.
591 Although not initially identified as necessary for embryonic development,
592 carotenoids are lost from fish and crustacean embryos prior to the first feeding

593 stages (Bjerkeng, 2008, Dall *et al.*, 1995). This implies the carotenoids present in
594 eggs and pre-feeding embryos are metabolised into other colourless molecules,
595 that in turn potentially perform biological functions. Axn has been proposed to
596 be an important source of Provitamin A and retinoids in eggs and early embryos
597 (Dall *et al.*, 1995, Linan-Cabello *et al.*, 2002a, Miki, 1991). Evidence from a
598 number of different crustaceans suggests that the retinols and other retinoid
599 derivatives play a critical role in developmental processes of crustaceans,
600 including ovarian and larval development (Linan-Cabello *et al.*, 2002a).
601 Crustaceans possess a number of retinoids and retinoic acid receptors in
602 crustaceans and the enhancement of the ovarian development in shrimp
603 suggests an important role of these metabolites in shrimp physiology for their
604 successful aquaculture. Carotenoids are the sole source of retinoids in
605 crustaceans, and their role as bioactive molecules may have been largely
606 overlooked (Linan-Cabello *et al.*, 2002a).
607
608

4 Conclusion

Carotenoids are considered a semi-essential nutrient that promotes optimal survival and growth at low dietary inclusion levels, approximately 25 mg/kg dietary Axn. Studies demonstrate that some form of dietary carotenoid intake is required in order to maintain carotenoid levels over time as animals grow, whether that intake is from natural pond biota or formulated into feeds. This amount is estimated at 50 mg/kg dietary Axn to maintain between 20-25 mg/kg body weight Axn for juvenile *P. monodon*. Increasingly, evidence suggests that specific carotenoids accumulate in different crustacean tissues over various life history stages. At present this minimum body Axn level is poorly defined, but whole body Axn levels may improve survival and growth across various stages of commercial production.

Optimal shrimp pigmentation can be achieved within several weeks by including Axn in the diet at levels of between 50-100 mg/kg, which can be reduced by using higher dietary inclusion levels. At these and even higher inclusion levels, utilisation efficiency of dietary carotenoids is extremely high and often exceeds 90%. In Penaeid shrimp, the amount of carotenoid required to be deposited in the tissues to achieve optimal colour is around 30-50 mg/kg body weight. However, this amount does not result in the same overall colour of different species, i.e. *P. monodon* is darker than *L. vannamei* at the same body Axn level. In other crustaceans, this body Axn level may need to be significantly higher. Background colour and light intensity are highly effective at redistributing carotenoid pigments, both to make shrimp darker or lighter in colour. Optimal pigmentation can lead to substantially higher sale prices, but there can be a preference for either darker or lighter shrimp depending on the target market.

Although presently poorly defined, the carotenoid levels required to elicit the physiological improvements in disease resistance, hypoxia or reproductive performance may be considerably higher than those for pigmentation. These beneficial effects have been demonstrated on various physiological characteristics such as survival, growth and resistance to stress. However, unlike colour, accurate measurement of these effects is often difficult due to a range of external factors. Improvements in research methods and techniques have led to

641 a stronger understanding of the physiological mechanisms underlying
642 carotenoid function in crustaceans. Very little is known about the genetic
643 mechanisms that underlie the absorption, transport, tissue accumulation or
644 metabolic transformations of carotenoids in any animal species. It is reasonable
645 to assume that the accumulation of these carotenoids underpins the
646 physiological changes that lead to improved performance of a variety of
647 commercially relevant traits in aquaculture. More detailed studies are required
648 to define the basis of the benefits of carotenoids in crustacean aquaculture.
649 Although some functions of carotenoids may be preserved, we cannot continue
650 to rely on research from vertebrate systems to draw conclusions on their effect
651 in crustaceans.

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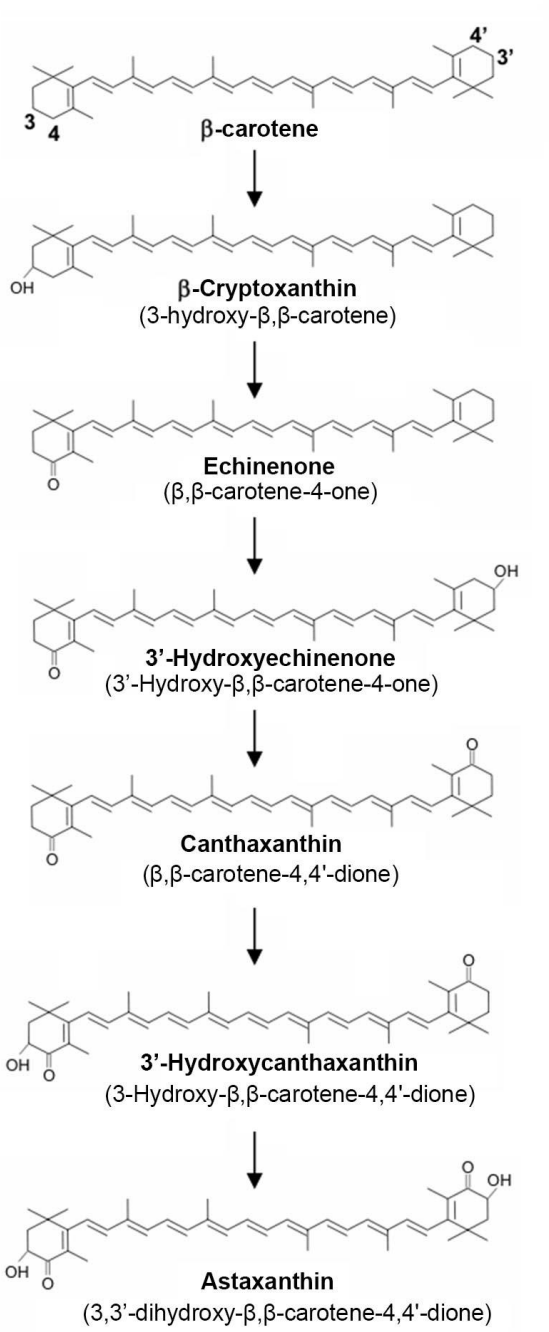
1052 Figure 1. Schematic diagram of the major conversion pathway of β-carotene to
1053 astaxanthin in crustacean tissues.



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1056 Figure 2 The response of crustacean abdominal epithelial chromatophores when
1057 exposed to black (A and C) or white (B and D) coloured substrates.

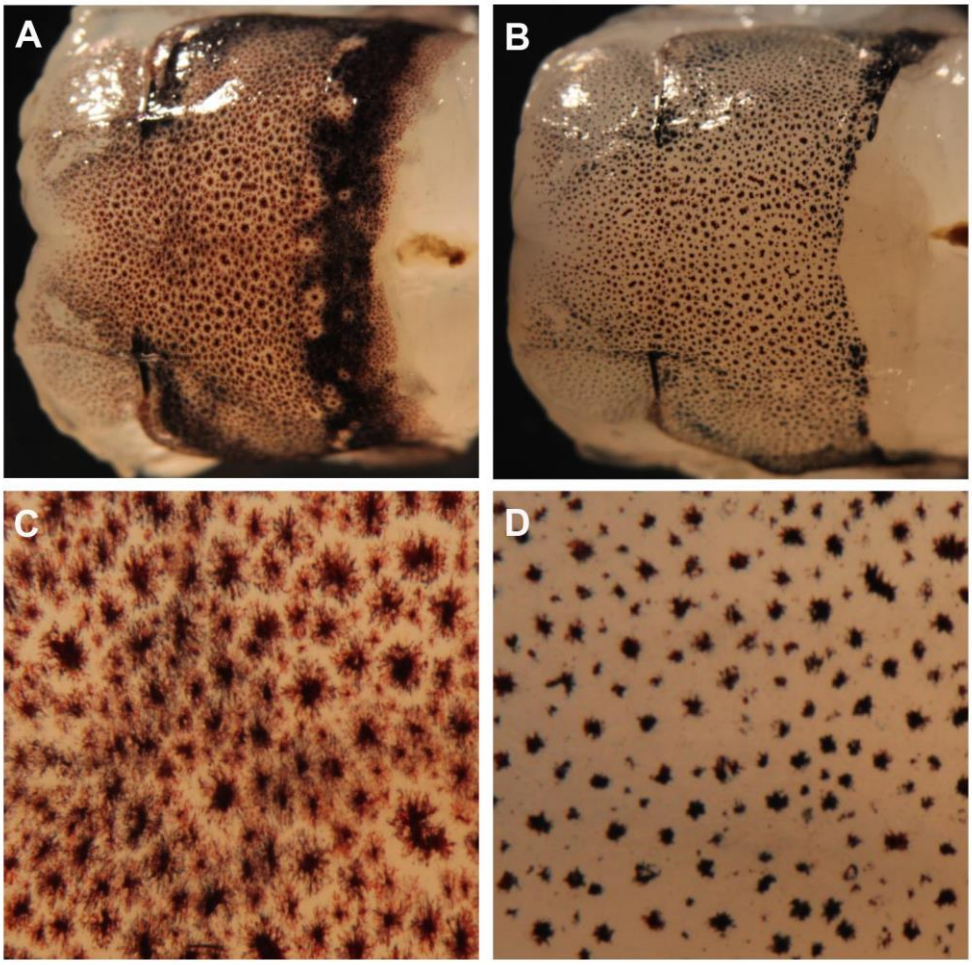

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Table 1. Summary of carotenoid research in crustacean diets that improves pigmentation.

Reference	Inclusion range	Carotenoid	Source	Optimal Pigmentation
Giant Tiger Prawn (*Penaeus monodon*)				
(Yamada *et al.*, 1990)	0 – 400 mg/kg	Astaxanthin / β-carotene / Canthaxanthin	Synthetic	200 mg/kg Astaxanthin
(Liao *et al.*, 1993)	3%	β-carotene / Zeaxanthin	Spirulina / Krill Oil	3% Spirulina
(Menasveta *et al.*, 1993)	0 – 50 mg/kg	Astaxanthin	Synthetic	50 mg/kg
(Merchie *et al.*, 1998)	230 – 810 mg/kg	Astaxanthin	Synthetic	inconclusive
(Boonyaratpalin *et al.*, 2001)	125 – 175 mg/kg	β-carotene	Algal	125 mg/kg
(Supamattaya *et al.*, 2005)	125 – 300 mg/kg	β-carotene	Algal	200 – 300 mg/kg
(Niu *et al.*, 2012)	70 – 200 mg/kg	Astaxanthin / Canthaxanthin	Synthetic	100 mg/kg Astaxanthin + cholesterol
(Niu *et al.*, 2014)	100 – 250 mg/kg	Astaxanthin / β-carotene	Synthetic	100 mg/kg Astaxanthin + cholesterol
Pacific White Shrimp (*Litopenaeus vannamei*)				
(Vernon-Carter *et al.*, 1996)		Astaxanthin / Lutein	Synthetic / Marigold	Marigold
(Arredondo-Figueroa *et al.*, 2003)	200-250 mg/kg	Capsanthin	*Capsicum annuum*	
(Niu *et al.*, 2009)	0 – 400 mg/kg	Astaxanthin	Synthetic	100 - 200 mg/kg
(Ju *et al.*, 2011)	25 – 150 mg/kg	Astaxanthin	Algal and Synthetic	75 – 100 mg/kg

Kuruma Shrimp (*Marsupenaeus japonicus*)				
(Chien and Jeng, 1992)	50 – 200 mg/kg	Astaxanthin / β-carotene	Synthetic / algal	100 mg/kg Astaxanthin
(Negre-Sadargues *et al.*, 1993)	100 mg/kg	Astaxanthin/ Canthaxanthin	Synthetic	50 mg AX + 50 mg CX
(Petit *et al.*, 1997)	0 – 220 mg/kg	Astaxanthin/ Canthaxanthin	Synthetic / *Artemia*	60 mg/kg Astaxanthin
(Chien and Shiau, 2005)	0 – 100 mg/kg	Astaxanthin	Synthetic / algal	100 mg/kg
Giant Freshwater Prawn *Macrobrachium rosenbergii*)				
(Kumar *et al.*, 2009)	0 – 200 mg/kg	Astaxanthin	Synthetic	200 mg/kg
Hermit Crab (*Clibanarius erythropus*)				
(Castillo and Negre-Sadargues, 1995)	200 mg/kg	Astaxanthin / β-carotene / Canthaxanthin	Synthetic	200 mg/kg Astaxanthin
Red King Crab (*Paralithodes camtschaticus*)				
(Daly *et al.*, 2013)	0 – 380 mg/kg	Astaxanthin	Synthetic / algal	380 mg/kg
American Clawed Lobster (*Homarus americanus*)				
(Tlusty and Hyland, 2005)	0 – 220 mg/kg	Astaxanthin	Synthetic	220 mg/kg
Tropical Spiny Crayfish (*Panulirus ornatus*)				
(Barclay *et al.*, 2006)	30 – 120 mg/kg	Astaxanthin	Synthetic	120 mg/kg

Table 2. Summary of carotenoid research in crustacean diets that improves physiological performance.

Reference	Inclusion level	Species	Response
Growth and Survival			
(Yamada *et al.*, 1990)	100 mg/kg Axn	*M. japonicus*	Improved survival or growth
(Darachai *et al.*, 1998)	various	*P. monodon*	Improved post-larval survival
(Chien and Shiau, 2005)	50-100 mg/kg	*M. japonicus*	Improved survival
(Supamattaya *et al.*, 2005)	300 mg/kg β-carotene	*P. monodon*	Greater weight gain and improved survival
(Flores *et al.*, 2007)	80 mg/kg Axn	*L. vannamei*	Improved growth and moult frequency
(Kumar *et al.*, 2009)	50-200 mg/kg Axn	*M. rosenbergii*	Greater weight gain and improved survival
(Niu *et al.*, 2009)	100-400 mg/kg Axn	*L. vannamei*	Greater weight gain and improved survival
(Niu *et al.*, 2012)	100 mg/kg Axn + cholesterol	*P. monodon*	Greater weight gain and improved survival
(Daly *et al.*, 2013)	380 mg/kg	*Paralithodes camtschaticus*	Improved survival
(Zhang *et al.*, 2013)	125-150 mg/kg Axn	*L. vannamei*	Improved growth
(Niu *et al.*, 2014)	100 mg/kg Axn + cholesterol	*P. monodon*	Greater weight gain and improved survival
Tolerance to Disease and Stress			
(Darachai *et al.*, 1998)	various	*P. monodon*	Improved tolerance to low salinity
(Chien *et al.*, 1999)	360 mg/kg Axn	*P. monodon*	Improved survival to low dissolved oxygen
(Chien *et al.*, 2003)	80 mg/kg Axn	*P. monodon*	Improved recovery from thermal and osmotic stress, enhanced anti-oxidant capacity.

(Pan *et al.*, 2003)	80 mg/kg Axn	*P. monodon*	Improved resistance to ammonia stress, higher anti-oxidant status, lower SOD levels.
(Chien and Shiau, 2005)	50 mg/kg Axn	*M. japonicus*	Improved survival to low oxygen
(Supamattaya *et al.*, 2005)	300 mg/kg Axn	*P. monodon*	Improved survival to daily hypoxia stress, increased resistance to WSSV infection
(Flores *et al.*, 2007)	0-150 mg/kg Axn	*L. vannamei*	Improved tolerance to low salinity
(Niu *et al.*, 2009)	200-400 mg/kg Axn	*L. vannamei*	Improved survival to daily hypoxia stress
(Angeles *et al.*, 2009)	1.34 nmol g^{-1} Axn injected	*M. rosenbergii*	Improved survival to bacterial infection
(Zhang *et al.*, 2013)	75-150 mg/kg Axn	*L. vannamei*	Improved survival to hypoxia stress, increased HIF-1α, cMnSOD and catalase expression.
(Niu *et al.*, 2014)	100 mg/kg Axn 250 mg/kg β-carotene	*L. vannamei*	Improved survival in live transport test, reduced malondialdehyde and HSP-70 levels
Reproductive Performance			
(Wyban *et al.*, 1997)	Various	*L. vannamei*	Improved nauplii quality
(Pangantihon-Kuhlmann *et al.*, 1998)	100 mg/kg Axn	*P. monodon*	Improved spawning and fecundity
(Paibulkichakul *et al.*, 2008)	50-300 mg/kg Axn	*P. monodon*	Increased number of eggs and spermatozoa, accumulation of Axn in ovary tissue