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AN HYPOTHESIS ON CRUSTACEAN PIGMENTATION METABOLISM: L-CARNITINE AND NUCLEAR HORMONE RECEPTORS AS LIMITING FACTORS

BY

ABDALLAH GHONIMY[1,2,3]), LAURA S. LÓPEZ GRECO[4]), JIAN LI[1,2,6]) and NICHOLAS M. WADE[5,6])

[1]) National Key Laboratory of Mariculture Biobreeding and Sustainable Goods, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

[2]) Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266071, China

[3]) Fish Farming and Technology Institute, Suez Canal University, Ismailia 41522, Egypt

[4]) Universidad de Buenos Aires, CONICET, Instituto de Biodiversidad y Biología Experimental y Aplicada (IBBEA), Facultad de Ciencias Exactas y Naturales, Departamento de Biodiversidad y Biología Experimental, Laboratorio de Biología de la Reproducción y el Crecimiento de Crustáceos Decápodos, Buenos Aires, 1428EGA, Argentina

[5]) The Roslin Institute and Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Edinburgh EH25 9RG, Scotland, U.K.

ORCID iDs: Ghonimy: 0000-0001-6865-1123; López Greco: 0000-0003-2921-497X; Li: 0009-0008-9969-9523; Wade: 0000-0001-7235-6274

ABSTRACT

Astaxanthin (Axn) is the primary pigment molecule in crustaceans associated with quality, health and growth traits, leading to increased marketing value. Axn can be contained within the protein complex crustacyanin (CRCN) to produce an array of different shell colours, or esterified with fatty acids (FA) for storage but also contributing additional red colouration. L-Carnitine (LC) has a major role in FA oxidation and mitochondrial function optimization, which could influence the proportion of Axn complexed with FA or CRCN. Peroxisome proliferator activated receptors (PPARs) have important roles in FA and Axn uptake, and stored lipid oxidation affecting Axn homeostasis and storage in lipid bodies. Whether Axn could increase PPAR signalling and carnitine palmitoyl transferase activity, leading to induction of lipid metabolism, is not known in crustaceans. Several FA have been shown to preferentially form FA Axn-esters, including saturated fatty acids (SFA) such as C16:0 and C18:0, mono-unsaturated fatty acids (MUFA) such as C16:1 and C18:1, and poly-unsaturated fatty acids (PUFA) such as C20:4, C20:5, and C20:6. We hypothesize that manipulating the dietary ratios and inclusion of LC, Axn, and specific FA may be able to further improve pigment utilization, lipid metabolism, health, and growth in crustaceans.

[6]) Corresponding authors; e-mails: lijian@ysfri.ac.cn; nwade@ed.ac.uk

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Key words. — Astaxanthin, astaxanthin ester, carnitine, crustacyanin, long chain fatty acid, PPAR

RÉSUMÉ

L'astaxanthine (Axn) est la principale molécule de pigment chez les crustacés, associée à des caractéristiques de qualité, de santé et de croissance, ce qui se traduit pars une valeur commerciale accrue. L'Axn peut être contenue dans le complexe protéique crustacyanine (CRCN) pour produire une gamme de couleurs diverses de la cuticule ou être estérifiée avec des acides gras (FA) pour le stockage mais aussi contribuer à une coloration rouge additionnelle. La L-carnitine (LC) a un rôle important dans l'oxydation des FA et l'optimisation de la fonction mitochondriale, ce qui pourrait influencer la proportion des complexes de Axn avec FA ou CRCN. Les récepteurs activés par les proliférateurs de peroxysomes (PPARs) jouent un rôle important dans l'absorption des FA et de Axn, et l'oxydation des lipides stockés ce qui affecte l'homéostasie de Axn et le stockage des lipides dans les tissus. On ne sait pas si, chez les crustacés, l'Axn pourrait augmenter la signalisation PPAR et l'activité de la carnitine palmitoyltransférase, provoquant une induction du métabolisme lipidique. Plusieurs acides gras ont montré former préférentiellement des Axn-esters d'AF, incluant des acides gras saturés (SFA) comme C16:0 et C18:0, des acides gras mono-insaturés (MUFA) comme C16:1 et C18:1, et des acides gras poly-insaturés (PUFA) comme C20:4, C20:5 et C20:6. Nous émettons l'hypothèse que la manipulation des ratios diététiques de LC, Axn et de FA spécifiques, pourrait permettre d'améliorer l'utilisation des pigments, le métabolisme des lipides, la santé et la croissance chez les crustacés.

Mots clés. — Astaxanthine, ester d'astaxanthine, carnitine, crustacyanine, acides gras à longue chaîne, PPAR

INTRODUCTION

Carotenoids have a range of beneficial effects in crustaceans, including enhanced pigmentation, reproductive output, disease resistance, and environmental stress resistance, all likely attributable to enhanced antioxidant activity (Liñán-Cabello et al., 2002; Wade et al., 2017a). Specifically, the terminal ring moiety structure is the functional group in the Axn molecule with the antioxidant activity (Goto et al., 2001; Kamath et al., 2008; Venditti et al., 2011). Pigmentation level associates with marketing value (Shahidi et al., 1998; Erickson et al., 2007; Parisenti et al., 2011) and growth traits in shrimp (Hasan et al., 2022). In fact, cooked colour is correlated with live shrimp colour (Hasan et al., 2022), where the cooking temperature dissociates the Axn-protein complex resulting in the characteristic red colouration of seafood (Tume et al., 2009; Hasan et al., 2022).

Crustaceans can not synthesize Axn and it must be sourced from their diets (Goodwin, 1952). In shrimp, carotenoids can be acquired from different sources with different supplementation regimes that optimize cooked colour (Wade et al., 2017a), but the colouration is also influenced by genetics (Hasan et al., 2022), light intensity (You et al., 2006), background colour (Tume et al., 2009), or combinations of these factors (Wade et al., 2015). Notably, background colour and

light intensity do not interfere with the accumulated quantity of Axn (Wade et al., 2017b; Díaz-Jiménez et al., 2018).

Pigmentation related feed additives substantially increase the feed cost (Stachowiak & Szulc, 2021), in addition carotenoid requirements differ among species to obtain optimal colour appearance, since *Penaeus monodon* Fabricius, 1798 is darker than *Penaeus vannamei* Boone, 1931 (formerly also referred to as *Litopenaeus vannamei* (Boone, 1931)) at the same body concentration of Axn (Wade et al., 2017b). Pigment occurs in different forms with different colours, including free-Axn, esterified-Axn, or protein-Axn (Tlusty & Hyland, 2005; Tejera et al., 2007; Wade et al., 2009). The proportion of esterified-Axn and protein-Axn forms modulates the final external colour appearance and might also affect Axn function in crustaceans. The metabolic function, formation and health benefits for these molecules are well established in humans (Cao et al., 2023), with evidence accumulating in aquatic animals such as crustaceans (Wade et al., 2017a).

L-Carnitine (LC) has a major role in the oxidation of long chain FA and stored lipids in fish and crustaceans (Lavarías et al., 2009; Safari et al., 2015; Ghonimy et al., 2018). Given the important role of Axn-esters and their storage in lipid bodies, we propose an additional role for LC in Axn metabolism.

The Peroxisome proliferator activated receptor (PPAR) is a master transcriptional regulatory factor, where PPAR-α manipulates the transport activity of FA (CD36) and LC (organic cation transporter novel 2, OCTN2) (Leonardsson et al., 2004; Roberts et al., 2011). Meanwhile, PPAR-γ manipulates FA synthesis and storage in peripheral tissues (Tontonoz & Spiegelman, 2008; Lee et al., 2017). In addition, CD36 is a FA and Axn transporter manipulated by PPAR-α in humans (During et al., 2005; Mashurabad et al., 2016). Therefore, we hypothesise that PPAR-γ and PPAR-α might have a role in Axn uptake and storage in crustaceans and the regulation of Axn metabolism by nuclear receptor expression and signalling should be investigated.

We therefore propose that manipulating the dietary supply of LC and Axn could enhance Axn uptake and Axn liberation from its esterified forms, leading to improved pigmentation and the potential to decrease Axn quantity in crustacean diets. In this research note, we have reviewed the mutual interaction between Axn and LC metabolism in terms of LC, Axn, and PPAR activities.

BIOCHEMICAL ASPECTS

Dietary influence

Like all animals, crustaceans cannot synthesize Axn and it must be obtained from their diets (Wade et al., 2017a). Crustaceans also have the capacity to

convert different dietary carotenoids (such as β-carotene, or lutein) to Axn via various metabolic pathways (Wade et al., 2017a; Yu & Liu, 2020). In general, a dietary carotenoid concentration of between 50 and 100 mg/kg feed is sufficient to generate an optimal pigmentation level after one month in the shrimp's body. However, levels exceeding 100 mg/kg feed can generate a darker pigmentation level more rapidly, while levels below 50 mg/kg feed required longer to achieve the same level of pigmentation (Wade et al., 2017a). As the dietary Axn level increases, the Axn deposition in animal tissues increases, especially the Axn esters (Wade et al., 2017a,b). Beyond the role of Axn in pigmentation, there may be a threshold for total amounts of Axn-esters in order to observe improved performance in stressful environments (Wade et al., 2017b), an effect that can only be maintained through adequate dietary carotenoid inclusion or improvements in dietary carotenoid utilization.

Genetic influence

The manner in which Axn interacts with the protein crustacyanin (CRCN) has been fascinating to scientists for more than 70 years (Wald et al., 1948; Zagalsky, 2003). This mechanism was subsequently shown to be derived from the formation of a multimeric protein called α-crustacyanin built from two smaller 20 kDa proteins CRCN-A and CRCN-C (Chayen et al., 2003). These CRCN genes and the mechanism of colour formation has been shown to be unique to crustaceans (Wade et al., 2009). Budd et. al. (2017) expanded our understanding of these in penaeid shrimp, where 12 species were shown to have an excess of 35 genes encoding for CRCN proteins, including at least 6 in some species that each have functional roles in pigmentation (Budd et al., 2017). Most recently, several hundred predicted CRCN genes were annotated as CRCN from the genomes of *P. vannamei* (cf. Zhang et al., 2019) and *P. monodon* (cf. Uengwetwanit et al., 2021; Huerlimann et al., 2022), although in many cases these genes may not be functional or have been incorrectly annotated as CRCN.

Astaxanthin homeostasis is further influenced by genes regulating absorption, transportation and deposition of Axn, which is quite well understood in many fish species, including salmon and rainbow trout (Houston et al., 2009; Baranski et al., 2010; Lehnert et al., 2019), but less knowledge exists in crustaceans. Although not yet having identified major genes impacting pigmentation in shrimp, heritability estimates for both colour and growth traits ranged from moderate to high (Nguyen et al., 2014; Giang et al., 2019; Hasan et al., 2022).

The spiny lobster, *Panulirus cygnus* George, 1962 undergoes an ontogenic colour change from a deep red to a pale pink during migration, potentially as a mechanism for protective camouflage, a transition that was not prevented

by dietary carotenoid supplementation or provoked by the background substrate colour (Wade et al., 2008). In the freshwater shrimp *Macrobrachium rosenbergii* (De Man, 1879), the external colour was removed by the knockdown of a CRCN homologue (Yang et al., 2011). This functional downregulation of pigmentation was similarly performed for several CRCN isoforms in *P. monodon* (cf. Budd et al., 2017). Little more is known about the genetic mechanisms controlling CRCN expression in crustaceans, or the genetic mechanisms influencing carotenoid homeostasis through absorption, tissue accumulation, transfer, or metabolic transformations.

Pigment structures

In shrimp, the opaque shells are thin, which is allowing the underlying chromatophores to expand and contract, thus impacting the degree of colouration (Wade et al., 2017a). In lobsters, the phenotypic colour of the lobster is a combination of pigments embedded in different layers with various forms of Axn (Tlusty & Hyland, 2005). The free Axn accumulates in the epidermal layer (below the cuticle layer), while α-CRCN and β-CRCN accumulate in the cuticle layer, in addition to the crustochrin accumulation of another pigment protein in the epicuticular layer (Tlusty & Hyland, 2005).

In crustaceans, pigment dispersing hormones (PDH) control circadian rhythms and other environmental cues of pigment diffusion in the chromatophores (Strauss & Dircksen, 2010). In this way, CRCN abundance and chromatophore dispersal would be independent of Axn-esters within the epithelial tissue. Shrimp adapted to black substrates increased levels of the Axn-CRCN complex without modification of CRCN gene expression (Wade et al., 2012; Wade et al., 2015). In this context, studies on Axn-CRCN complex formation should consider the hormonal measures as they are main regulators for pigmentation distribution.

Pigmentation proteins

In the case of crustaceans, Axn occurs in different forms including free (red), esterified (pink), or protein-bound (any colour from red to blue). The protein crustacyanin (CRCN) belongs to the lipocalin superfamily as a functional protein, that is capable of binding hydrophobic molecules such as Axn (Flower, 2000). The CRCN protein is a unique evolutionary adaptation protein that allowed crustaceans to produce a diversity of colours and patterns (Wade et al., 2009). The Axn-CRCN complex has at least three forms including α-CRCN (blue) (fig. 1), β-CRCN (purple), and crustochrin (yellow) (Tlusty & Hyland, 2005; Tejera et al., 2007; Wade et al., 2009). There has been considerable interest in the structural chemistry that underlies the coloration mechanism in lobsters (Chayen et al., 2003).

Fig. 1. Blue body syndrome in the shrimp *Penaeus vannamei* Boone, 1931. The left individual demonstrates the potential effect of reduced Axn-ester storage and increased protein bound Axn to generate the blue colour appearance, while the right shrimp represents the normal colour.

Although in general darker colour contained the greater total carotenoid levels, there is additional importance of carotenoid forms and their distribution that has effects on the external colour (fig. 2). When each carotenoid form was quantified from differently coloured shrimp, surprisingly the most amount of carotenoid was found in the least pigmented animals, likely through the constriction and perhaps intentional conservation of pigment molecules within the epithelial tissue. Meanwhile, brown/red and blue shrimps showed a similar amount of free Axn and di-esterified Axn, but varied in the amount of mono-esterified Axn, with increased free Axn in protein-bound form present in the darkest individuals.

Esterified astaxanthin

Free-Axn and Axn-ester distribution differs among species, regarding the animal's physiological status and organ or tissue type, and such diversity can significantly increase the complexity and interaction with other molecules or membranes (Britton, 2008; Wade et al., 2017a). In crustaceans, Axn has been consistently shown to combine with FA of different lengths whether they are

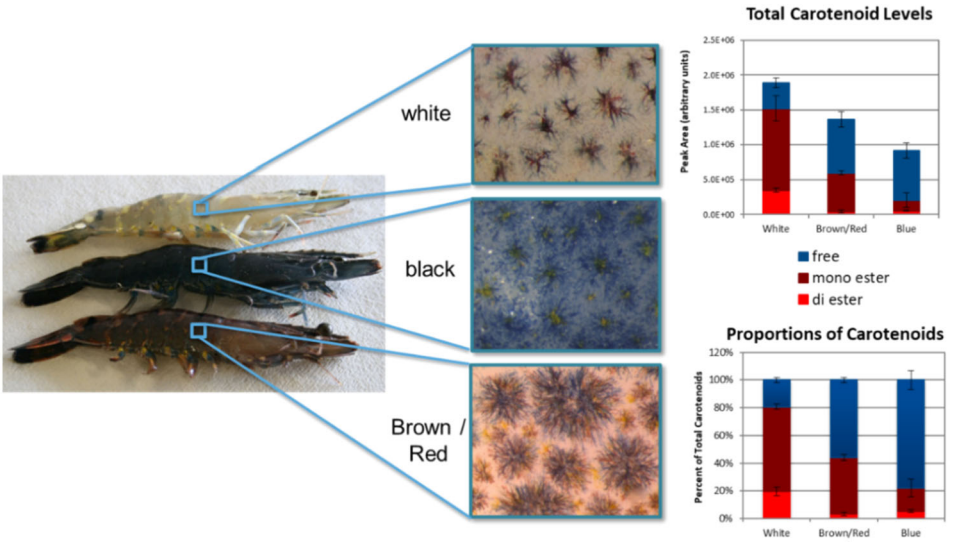

Fig. 2. Astaxanthin quantity and esterification among natural colour variation in the shrimp *Penaeus monodon* Fabricius, 1798. Examples of three individual shrimp within a population that display different colours and distribution of epithelial pigments, which also significantly impacts total carotenoid levels and the proportion of mono- and di-esters of Axn within their exoskeletons.

monoesters (C12:0, C14:0, C16:0 and C18:0) or diesters (C16:1, C18:1, C20:4, C20:5 and C22:6) (Coral-Hinostroza & Bjerkeng, 2002; Breithaupt, 2004; Moaka & Akimoto, 2008; Wade et al., 2017b), and the high Axn-ester/total Axn ratio could increase purple colour other than greenblack colour in individuals of the Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards, 1853 (Li et al., 2020).

As such, the attachment of FA has been implicated with the regulation of pigmentation, improved resistance to environmental stress, and potentially other performance enhancements in several crustacean species. We are just beginning to understand how these key FA Axn esters might play a role in regulating and modulating Axn function in vivo.

Metabolic mechanism of astaxanthin uptake

In crustaceans, several aspects of carotenoid uptake and metabolism are poorly characterized due to a variety of biological constraints, including the lack of tissue culture models. In human cell culture, β-carotene absorption can be manipulated via passive diffusion (Hollander & Ruble, 1978), scavenger-receptor class B-type I (SR-B1) (During et al., 2005), and cluster of differentiation 36 (CD36) (Lee et al., 2017). CD36, a FA carrier and also a member of the B class scavenger receptors, transfers β-carotene from apical to intracellular space (Lee et al., 2017).

In mammals, peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes. PPAR-α has an important role in the regulation of the β-oxidation in the liver, while PPAR-β has an important role in regulating β-oxidation in skeletal muscle (Leonardsson et al., 2004; Roberts et al., 2011). Whereas PPAR-γ is predominantly expressed in the peripheral tissues where the FA are synthetized and stored (Tontonoz & Spiegelman, 2008; Lee et al., 2017). In fact, PPAR-α regulation includes LC transport, FA uptake and activation in both mitochondria and peroxisome cell components (Rakhshandehroo et al., 2007).

Both CD36 and SR-B1 are FA and Axn transporters that are manipulated by PPAR-α in humans (During et al., 2005; Mashurabad et al., 2016; Lee et al., 2017). In addition, the PPAR-signalling pathway mediates Axn metabolism as part of homeostatic regulation (Bohn, et al., 2019). On the other hand, Axn dietary supplementation induces PPAR activity leading to activation of lipid metabolism (Choi, 2019), therefore a feedback mechanism could exist between Axn and PPAR molecules (fig. 3). PPAR-α increases lipoprotein lipase gene expression, and down-regulates hepatic apolipoprotein C-III in humans (Gervois et al., 2000). In fact, dietary Axn increases polar MUFA or PUFA in the tissues of the kuruma shrimp, *Penaeus japonicus* Spence Bate, 1888 (earlier also referred to as *Marsupenaeus japonicus* (Spence Bate, 1888)) (Wang et al., 2019), and those

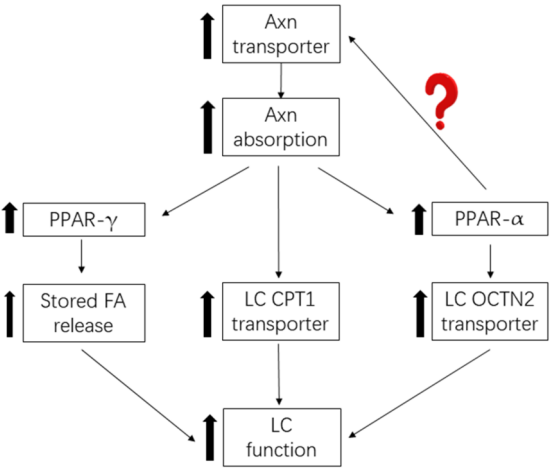

Fig. 3. The potential mutual interaction among L-carnitine (LC), astaxanthin (Axn), and peroxisomal proliferator activator receptor (PPAR). An increased level of Axn absorption activates the function of PPAR-γ and PPAR-α. PPAR-γ activates the release of stored FA, and PPAR-α activates the function of organic cation transporter novel 2 (OCTN2), which increases the LC uptake and transportation among tissues. Axn preserves carnitine palmitoyl transferase activity (CPT1a), the rate limiting step of FA translocatation from the cytoplasm to the mitochondrial interspace, which improves LC function. The question mark indicates the poorly investigated aspect in crustaceans.

molecules are all located on the lipid drops' lipoprotein surface (Niu et al., 2014). This suggests a possible effect of PPAR-α on astaxanthin liberation from the Axn-lipoprotein complex.

Combined, we propose that PPAR-γ and PPAR-α might have important central roles in the regulation and modulation of Axn uptake and storage in crustacean tissues and therefore provide a link between dietary carotenoids and carotenoid utilization.

Astaxanthin and lipid interaction

As it is highly hydrophobic, Axn readily associates within lipid micelles (Chimsung et al., 2014), where the lipids act as Axn carrier molecules (Rajasingh et al., 2006). In salmonids, increased dietary levels of MUFA or SFA were correlated with enhanced Axn uptake and retention (Olsen et al., 2005) and negatively correlated with dietary omega 3 PUFA (Bjerkeng et al., 1999; Olsen et al., 2005). But in other studies in fish, higher dietary lipid content including PUFA and cholesterol increased Axn absorption (Nickell, 1998; Bjerkeng & Berge, 2000; Rørå et al., 2005; Ytrestøyl et al., 2005; Chimsung et al., 2013). Combining supply of dietary algal carotenoids with different oil sources, particularly C18:1n-9 FA sourced from canola oil, optimized the carotenoid digestibility in Atlantic salmon (Courtot et al., 2022). The interaction between FA source and carotenoid uptake is still poorly understood in fish, with little to no equivalent knowledge in crustaceans.

Cholesterol homeostasis is another important component of lipid and carotenoid uptake and transport, with several links to regulation by nuclear receptors (Shahoei et al., 2019). Cholesterol positively impacted dietary utilization of Axn, but not β-carotene, and increased Axn transport through the blood stream by inducing lipoprotein formation in the black tiger shrimp, *P. monodon* (cf. Niu et al., 2014). In addition, dietary cholesterol interacted with Axn to improve pigmentation and promoted Axn deposition in the tissues of the kuruma shrimp by inducing lipase secretion (Wang et al., 2019).

Fatty acid and LC interaction

LC facilitates long chain FA translocation to the mitochondrial matrix where the β-oxidation takes place, which is the rate-limiting step in β-oxidation controlled by the activity of the mitochondrial enzyme carnitine palmitoyltransferase 1 (CPT1a). Noteworthy, activated SCFA and MCFA are freely crossing the mitochondrial membranes (Guzman & Geelen, 1993). In our previous work, we have reviewed and investigated the potential conflicting factors with LC function in monogastrics such as dietary components of methionine, lysine, fibres, and minerals (Ghonimy

et al., 2018; Tjale et al., 2022). However, LC supplementation increased lipid oxidation and growth in the prawn, *Macrobrachium borellii* (Nobili, 1896) and the clawed crayfish, *Pontastacus leptodactylus* (Eschscholtz, 1823) (earlier also referred to as *Astacus leptodactylus* Eschscholtz, 1823) (Lavarías et al., 2009; Safari et al., 2015).

LC plays a vital role in optimizing mitochondrial function including proton accumulation in the form of ATP in crustaceans (Li et al., 2019). LC increases mitochondrial biogenesis and gene expression of various mitochondrial components via mediating the accumulation of reactive free radicals. Since the electron transfer chain (ETC) reaction generates reactive oxygen species in the mitochondria, LC modulates oxidative stress directly by free radical scavenging and Fe^{2+} ions' chelation activities, and indirectly by decreasing ROS and reactive nitrogen species producing enzymes (Modanloo & Shokrzadeh, 2019). Optimized ETC reactions lead to a decreased flow of protons out of mitochondria by involving them in ATP production (Modanloo & Shokrzadeh, 2019; Lee et al., 2022). In fact, it was suggested that Axn-protein complex formation was associated with acid-base change in the carotenoid conformation through proton relocation on the end ring of Axn (Begum et al., 2015).

The link between astaxanthin, fatty acid and LC

We propose that combined regulatory mechanisms of Axn, FA and LC metabolism, possibly through a series of unidentified nuclear receptors, can further enhance the utilization efficiency of dietary pigments. The effect of dietary LC to induce lipid metabolism could sequester the free FA pool and release stored lipids, whereas the LC-induced optimization effect on mitochondrial function could reduce mitochondrial proton leakage and prevent Axn oxidation. Free FA sequestration may decrease FA availability to form new Axn-esters, while the reduction free FA from circulating lipid bodies could liberate FA from Axn-esters, allowing more free Axn to be available for CRCN binding in pigment complexes. In addition, the optimized mitochondrial proton production could increase ATP production, and thereby increase the proton availability for the Axn protein binding required during pigment formation. Combined, these postulates highlight the importance and metabolic interaction of FA esters and LC, and the regulation of carotenoid esterification as a key factor in crustacean pigmentation. Activated FAs represent an ester in the form of acyl-CoA (Grevengoed et al., 2014). In this esterification reaction, diacylglycerol O-acyltransferases are candidate enzymes for the esterification reaction of Axn-ester formation (Chen et al., 2015), whereas the palmitoyltransferase is a required enzyme for the esterification reaction of LC-ester formation (Di Lisa et al., 1995). Elevated triglyceride and FA levels

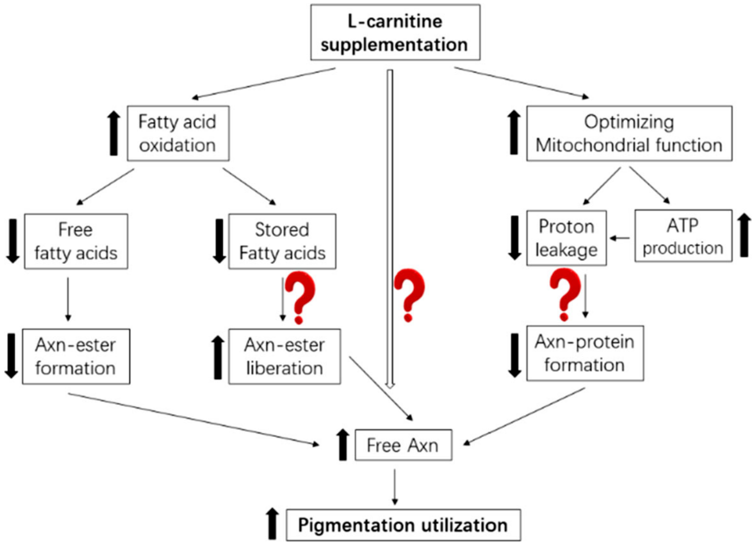

Fig. 4. The effect of L-carnitine (LC) supplementation on astaxanthin utilization in crustaceans. LC has a major role in the oxidation of long chain FA, where dietary supplementation of LC leads to increased FA oxidation and subsequently decreased stored FA. LC optimizes mitochondrial function by decreasing proton leakage out of mitochondria and resulting in an increase in ATP production. The question marks indicate poorly investigated aspects in crustaceans.

increase long chain LC-esters and not the short chain FA in mice intestine, and this accompanied with the increased level of CPT1a (Khatun et al., 2016). Which implied the effect of LC on the availability of long chain FA and subsequently the Axn-ester formation (fig. 4). Axn suppresses the oxidative reaction on CPT1a, which represents a feed-back mechanism between the LC and Axn (Brass, 2000; Aoi et al., 2008).

HYPOTHESIS TESTING

We hypothesize a positive effect of LC on crustacean pigmentation, through a series of potential interactions between LC and FA, and between FA and Axn to impact Axn distribution as CRCN-Axn complexes or Axn esters. To investigate this hypothesis, the esterification of LC and Axn, the complementary antioxidant effect of LC and Axn, CRCN protein complexation, and mitochondrial ATP production are needed to be investigated by varying dietary levels of LC and Axn. However, a set of additional analyses are suggested for each functional molecule. These include quantification of LC, LC-esters, FA, PPAR expression, CPT1a activity and FA transporter activities in different tissues. For Axn, these include quantification of Axn, Axn-esters and Axn-ester transporters in different tissues.

Finally, CRCN protein quantity and gene expression and PDH in different tissues. The CRCN-Axn complex is highly water soluble, meaning it can be extracted and quantified separately from the lipid soluble Axn esters present in the epithelial tissue. Considering there is a strong dietary component, some factors might be important including carotenoid types and sources, FA composition, minerals, and potentially the intestinal microbiota composition. These factors could help define the impact of LC and FA on Axn absorption, haemostasis, and metabolism in crustaceans.

CONCLUSION

LC might liberate Axn from its complex with FA by inducing FA oxidation, and prevent Axn complexation with protein by decreasing proton leakage out of mitochondria. PPAR might increase Axn uptake by inducting FA transporter activity, and liberate Axn-ester from lipid bodies by inducing lipid release for energy production. Several FA were proposed as key molecules in pigmentation regulation based on Axn-ester formation. A dietary mixture of LC, Axn, and specific FA might improve pigment utilization efficiency and reduce dietary inclusion levels and therefore the cost of shrimp diets, while maximizing product quality and value.

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