Liposoluble vitamins in Crustacean feed: Metabolic and Histological responses

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Vitamins are vital for normal growth and survival of living organisms and they are distributed in feedstuffs in small quantities. This review is focused on the liposoluble vitamins (A, D, E and K) in the diets and metabolic responses of the Argentine penaeoid shrimps *Pleoticus muelleri* and *Artemesia longinaris*, distributed along the South American coast line. Growth, survival and histological analyses serve as indicators of the nutritional value derived from vitamin deficiency. Liposoluble vitamins are also related to stress, antioxidant defense and immune response of shrimps. Effective diet for shrimp culture that provide not only macronutrients including protein and lipid but also micronutrients such as vitamins for optimal growth is an ever improving subject. This review may help formulating suitable feeds for shrimps.

Keywords: Aquaculture, *Artemesia longinaris*, Calciferol, Decapoda, Immunity responses, Menadione, Nutrition, Oxidative stress, Philloquinone, *Pleoticus muelleri*, Retinol, Shrimps, Tocopherols, Tocotrienols

Introduction

The sustainable growth of the aquaculture industry depends up on the availability of nutritious feeds a challenge that can only be met with updated information on the nutritional requirements of shrimp. Knowledge on digestive physiology and metabolism is a prerequisite for understanding and utilizing the nutritional data in feed formulation. Optimized feeds and supplements not only improve the growth, health and survival of different ontogenetic stages of shrimp, but also help to reduce pollution in the aquatic environment[1].

Farmed shrimp have a specific requirement for at least 40 essential nutrients, hence, improvement in feed formulation techniques as well as rations on the basis of individual digestible nutrient levels rather than on crude gross nutrient levels call attention[2]. Feed formulations for shrimp use vitamin levels based on information derived from studies on related species. Widely divergent quantities of vitamins, generally much higher levels, have been added into shrimp feeds using vitamin premixes[3]. On other hand, changes in metabolic requirements can be induced by manipulating food composition; for example, increasing the lipid level in diet, increases the demand for vitamins probably as a physiological response[4].

In this review, we report the development of various feed formulations for crustaceans, particularly, the Argentine penaeoid species, Argentine shrimp *Pleoticus muelleri* (Bate) and shrimp *Artemesia longinaris* (Bate) focusing liposoluble vitamins (A, D, E and K) in the diets and metabolic and physiological responses and histological analysis as indicator of vitamin deficiency. Further, we discussed liposoluble vitamins in relation with stress, antioxidant defense and immune response and also compared with those reported for other crustaceans.

Penaeoid Argentine shrimp

There are two commercial penaeoid species in the Argentine waters, *P. muelleri* and *A. longinaris*. They live in waters with temperatures between 6 and 23°C and salinities from 31.5 to 33.5 ppt. The Argentine Red Shrimp is an open thelycum species, distributed along the South American coastal waters from Rio de Janeiro, Brazil (23°S) to Santa Cruz, Argentina (49°45′S). Males reach 50 g and females 90 g total weight, and their spawning capacity can be over 360000 eggs per female[5]. This species also has a high commercial value with >80% of the catches exported to Spain and Italy. The shrimp *A. longinaris* is a closed thelycum species that shows a similar distribution in the northern area but reaches only the latitude of 43°S. It is abundant in the southern part of Buenos Aires (Mar del Plata, Bahia Blanca) and the northern part of Patagonia (Rawson). Males reaching

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12 g and females 15 g, this species is traded mainly in the internal market[6].

Both species have seasonal and annual catches fluctuations, for what it is important to establish the feasibility of culturing them on commercial basis to provide continuous supply to the market. Previous studies have been done, mainly with *P. muelleri*, on different aspects on the biology, nutrition, maturation, large-scale larval culture and pond culture. There are previous studies about liposoluble vitamins in Argentine shrimp with respect to nutritional requirements[7-10].

Liposoluble vitamins in formulated diets and deficiency responses

Vitamins are complex organic substances of small molecular size. They are distributed in feedstuffs in small quantities and form a distinct entity from other major and minor food components. Its importance as essential constituents in the diets came to light in the early part of this century; however, nutrition research on aquatic organisms was partly due to the inherent problems posed by the aquatic medium (vitamin leaching). As vitamin requirements remain unknown, formulated feeds may still be deficient in certain vitamins even after supplementation[11]. Mc Laren *et al*.[12] developed a vitamin diet test for fish containing crystalline vitamins, casein, dextrin and oils with crab meal or dried liver as the source for the antianaemic factor.

Subsequently, the vitamin free casein purified diet, which is widely used as the standard diet for determining the qualitative and quantitative requirements of a number of aquatic species, has been developed[13]. The reference diet with vitamin free casein as proposed by Conklin[14] and Castell *et al*.'s[15] formulated diet containing a crab protein concentrate as the principal protein source yielded satisfactory growth and survival of marine and freshwater crustaceans.

Dietary vitamin requirements in cultured crustaceans have been originally demonstrated with dietary trials of growth and survival. However, it is also possible that vitamin deficiencies and over fortifications may be manifested through changes in the midgut gland cells[4,9,16,17]. The midgut gland is the main digestive organ of crustaceans and pathological changes of these cells would result in a general impairment of digestive functions. This condition may be responsible for slowing signs of growth and increased mortality associated with nutrient deficiencies[18].

Vitamin A

Vitamin A is the generic descriptor for compounds with the qualitative biological activity of all-*trans*-retinol, and can be found in animals as retinol, retinal and retinyl esters[19]. Retinoid, or their precursors, is essential in the diet of vertebrates[20], and their function as a component of retinal pigment in the eye has long been known. In addition, they have a fundamental role in gene transcription and are essential in cell division, differentiation and reproduction[21]. In crustaceans, vitamin A is involved in mucopoly-saccharide metabolism, visual pigments and maintenance of differentiated epithelia in various physiological systems, calcium transport across some membranes, in reproduction, embryonic development and in cellular and sub-cellular membrane integrity[22]. Vitamin A has also been related to the normal ovarian development of kuruma shrimp *Marsupenaeus japonicus* (Bate)[23], whiteleg shrimp *Litopenaeus vannamei* (Boone)[24], and fleshy prawn *Fennero-penaeus chinensis* (Osbeck)[25].

Liñán-Cabello *et al*.[26] highlighted the importance of retinoids in crustaceans as the retinoic acid involved in the activation of nuclear receptors of retinoids and in body development and function.

On other hand, Fisher *et al*.[27] found large quantities of retinoids in the eyes of euphausiid species but only small amounts in the rest of the body. In various other crustaceans, retinoid was either absent or constituted 1 mg g^{-1} wet mass. In Penaeidea, some pelagic or deep-sea species had no retinoids in the eyes and only trace amounts or none in the body. Shallow water species such as the northern brown shrimp *Farfantepenaeus aztecus* (Ives) had none in the body, but 4 mg g^{-1} in the eyes[28], a similar situation was found in the rusty crayfish *Orconectes rusticus* (Girard)[29].

Carotenoid function in crustaceans has normally been ascribed to pigmentation, as source of provitamin A and as antioxidants. The requirements for vitamin A depend largely on dietary sources of the provitamins or carotenoid. Crustaceans cannot synthesize carotenoids *de novo* but alter dietary carotenoids by oxidation and deposit them in their tissues[26]. It was also suggested that vitamin A supplementation is unnecessary when fish oils and carotenoids are added to diets for green tiger shrimp *Penaeus semisulcatus* (De Haan)[30].

Carotenoids, as a dietary supplement, play an important role in human health by acting as biological antioxidants that protect cells and tissues from the damaging effects of free radicals. In crustaceans, carotenoids stimulate the immune system, increase stress tolerance, serve as a source of vitamin A, and enhance embryonic development[26]. Díaz *et al.*[31] compared the growth and survival of juvenile shrimp *P. muelleri* fed diets containing different levels of carotenoids (50 and 100 mg astaxanthin kg^{-1} diet; 50 and 100 mg (3-carotene kg^{-1} diet). These authors have not founded evidence that support a possible influence of (3-carotene or astaxanthin on growth or survival. Analogous results have been reported for other penaeoid species fed carotenoid supplemented diets[32].

Many studies have evaluated the dietary essentiality of vitamin A for penaeids[3,7,17,33-36]. He *et al.*[34] demonstrated that at least 130 mg vitamin A in feed of *L. vannamei* prevent deleterious effects on growth, on other hand Liang and Ji[37] recommended about 40 to 60 mg vitamin A kg^{-1} to achieve good survival of *F. chinensis* larvae. Otherwise, it was demonstrated in *P. muelleri* and *A. longinaris*, that there is no relation between the weight gain percent- survival of shrimp and the level of vitamin A in feeds[7,35].

Shiau and Chen[38] reported that juveniles of giant tiger prawn *Penaeus monodon* (Fabricius) showed increased content of total lipids in the midgut gland with increasing levels of dietary vitamin A. There was a similar tendency in the content of total lipid in the whole body of juvenile kuruma prawn *M. japonicus*[36]. These findings differ with those reported for other aquatic species, as high contents of dietary vitamin A decreased the total lipid content in whole body of fish, such as guppy *Poecilia reticulate* (Peters)[39], greasy grouper *Epinephelus tauvina* (Forskål)[40] and olive flounder *Paralichthys olivaceus* (Temminck and Shlegel)[41].

Vitamin D

Commonly known as sunshine vitamin, vitamin D exists in two forms: D2 (ergocalciferol) as found in some food, fish in particular, and D3 (cholecalciferol also called 7-dehydrochelosterol) as formed in most animal tissues by the rupture of one of the ring bonds of 7-dehydrocholesterol by ultraviolet radiation[11]. It is a steroid hormone and is well-known in vertebrates for maintaining calcium and phosphorus homeostasis and promoting bone mineralization. It modulates

reproductive processes, and may have a beneficial effect on insulin action by stimulating the expression of insulin receptors and thereby enhancing insulin responsiveness for glucose transport and has a modulating effect on the immune system. This vitamin does not function as a cofactor in any enzyme system[42,43].

There is no indication that crustaceans regulate serum calcium levels in an analogous fashion as that of vertebrates. Although the mineralized cuticle serves admirably as both a skeleton and a protective integument, its rigid structure presents problems to crustacean's growth. Crustaceans solved this difficulty with the molting. Some argue that the resorptive processes in the molting is primarily for conservation of organic constituents, not calcium. However, the existence in many crustaceans of several mineral storage mechanisms, such as gastroliths, indicate a kind of regulation, and thus a possible regulatory role of vitamin D. Gastroliths are paired mineralized structures formed between the epidermal and cuticular lining of the stomach and have a typical cuticular structure. Several problems arise in attempt to assign gastroliths and mineral regulation to a vitamin D system; gastroliths are not found in all crustaceans and their presence is not restricted to species inhabiting a specific environment related to calcium availability. They appear superfluous, particularly in marine species, which presumably can fulfill their calcium requirements from the surrounding medium. Most evidences indicate the molt and the formation of the gastroliths are both regulated by an ecdysteroid hormone, also gastroliths stores contain only a small portion both of the calcium that is resorbed from the integument before molting and of the calcium required following the molt[14].

Deficient dietary vitamin D causes poor growth, high mortality, reduced appetite and darkening of midgut gland in *M. japonicus* and *L. vannamei*[33,34]. Shiau and Hwang[44] estimated a requirement of 0.2 mg vitamin D3 kg^{-1} diet for *P. monodon* and Reddy *et al.*[3] observed that individuals of the same species fed vitamin D deficient feed grew poorly.

To determine the effects of vitamin D in growth and survival of *A. longinaris*, two trials were conducted with different levels of this nutrient in semi purified diets (0; 0.200; 0.375; 0.750 and 1mg kg^{-1}). The results of this study indicated that the dietary vitamin D requirements of this species, was around 0.375 and 0.750 mg kg^{-1} diet[10], these levels are

significantly higher than those found in *P. monodon*[44]. It is difficult to compare the requirements of different penaeoid shrimp species due to the different experimental conditions; for example, Shiau and Hwang[44] carried out their investigations employing juvenile stages of *P. monodon*, meanwhile adults were used by Pereira[10].

Vitamin E

Vitamin E is the terminology for a group of lipid-soluble compounds, α, β, γ and δ-tocopherols and α, β, γ and δ-tocotrienols. The vitamin E compound with highest biological activity is α-tocopherol, its main function is to protect unsaturated lipids in living tissues against free-radical-mediated oxidation. As a fat soluble vitamin, it is the most effective chain-breaking, lipid soluble antioxidant in biological membranes, where it contributes to membrane stability and protecting critical structures against damage from oxygen free radicals and reactive products of lipid peroxidation[45,46].

Several studies showed that dietary vitamin E was required by the water flea *Daphnia magna* (Straus)[47] and *Moina macrocopa* (Straus)[48]. Kanazawa[33] found that addition of vitamin E to feed results in improved survival of larval *M. japonicus* and He *et al.*[34] reported that *L. vannamei* showed significantly lower survival and weight gain when fed vitamin E free diet for 8 wk. Reddy *et al.*[3] found significantly depressed growth and poor appetite in shrimp *P. monodon* fed a vitamin E deficient diet.

Two trials to evaluate the growth and survival of shrimp *P. muelleri* fed semipurified diets containing different levels of vitamin E have been carried out[8]. In the first experiment the author did not find any relation between weight gain and survival to the dietary level of vitamin E (0; 100; 600 and 1500 mg kg^{-1} diet). However, the second trial showed a positive correlation between the amount of vitamin E in diets and growth (1250; 1500 and 1750 mg kg^{-1} diet) and the lowest weight gain was observed in shrimp fed with 2000 mg kg^{-1} diet. The best results for *P. muelleri* were obtained when supplemented with 1750 mg kg^{-1} feed. A similar requirement (1500 mg kg^{-1}) was obtained for shrimp *A. longinaris* previously[49].These requirements are significantly higher than the dosages described for other Penaeoidea. An optimum requirement of 99 mg vitamin E kg^{-1} diet was determined for *L. vannamei*[34,50] and for maximal growth of *P. monodon*[45] the level of vitamin E required was

85-95 mg vitamin E kg^{-1}. Aquatic animals have high levels of unsaturated fatty acids to maintain cell membrane fluidity especially at low temperatures. This may explain why *P. muelleri* and *A. longinaris*, as species that live in cold waters of South West Atlantic, need an important amount of Vitamin E in the diet, for these, it is assumed that this compound plays an important role in both Argentine penaeoid species.

In the Indian prawn *Fenneropenaeus indicus* (Milne Edwards) Ouraji *et al.*[51] determined the effect of 100 and 300 mg vitamin E kg^{-1} diet along with two levels of lipid (9 and 14%) and their interaction on growth performance and consequently evaluated the fatty acid composition and lipid stability on its muscle tissue during frozen storage. Results showed that an increasing concentration of vitamin E from 100 to 300 mg kg^{-1} in diet reduced the rate of lipid oxidation in the muscle tissue during frozen storage and caused higher highly unsaturated fatty acids (HUFA) retention in muscle of shrimp fed high lipid level diets. However, there was no significant interaction between dietary vitamin E and lipid levels on growth performance. They also described malonaldehyde as a secondary oxidation product of polyunsaturated fatty acids and its analysis is one of the most popular and commonly used methods for tissue peroxidation determination and an increasing dietary vitamin E level significantly lowered the malonaldehyde values in shrimp muscle by lowering the rate of lipid peroxidation during frozen storage.

It is difficult to compare the requirements of the different penaeoid shrimps due to the different experimental conditions. Kanazawa[33] and He *et al.*[34] carried out their investigations employing larval states of *F. indicus* and *L. vannamei*, respectively, meanwhile adults of *P. muelleri* and *A. longinaris* were used by Fernández-Gimenez[7]. Sometimes, it is useful to compare the symptoms associated to the absence of vitamin E in the diet.

When working with vitamin E, it is also important to consider the lipid concentration in feed because this compound's level increases when polyunsaturated fatty acid's level increases in feed. Considering previous investigations, it is possible that the differences in the estimated requirement may vary upon feed composition. The incorporation of good quality oils to feeds protects the lipids during the manufacture and storing and apparently reduces the vitamin E requirement in the trout[52] and the channel catfish[53]. The level and state of oxidation of

polyunsaturated lipids in food, as well as the presence of the other antioxidants and selenium, affect the dietary level of vitamin E required by fish[54].

He and Lawrence[50] fed shrimp *L. vannamei* with feed supplemented with fatty acids and corn oil without vitamin E, but with vitamin C, a great antioxidant. Fernández-Gimenez *et al.*[8] used refined fish oil completely free of vitamins and antioxidants on formulated feed for shrimp *P. muelleri*, besides in these foods the only protection against lipid peroxidation was supplied by vitamin E supplementation.

Vitamin K

Vitamin K is associated with normal blood coagulation and calcium deposition in vertebrates. It is required for carboxylation of glutamate residues to Gamma-carboxyglutamate residues in many proteins, which are necessary for normal blood coagulation. Philloquinone (vitamin K1) is synthesized by plants and algae, whereas the menaquinone family (MK-*n)* (vitamin K2) includes products of bacterial biosynthesis. Vitamin K2 is a cofactor for a number of bacterial steroidal-*P*-dehydrogenases and it is involved in the blood clotting process in animals and the production of some of the intermediates of metabolic pathways. Water soluble salt of the synthetic menadione is used in animal diets: fish feeds are commonly supplemented with menadione sodium bisulfite[55].

Deficiency of vitamin K results in anemia and prolonged coagulation time in fish, and it is highly effective in preventing the moltinate-induced anemia in common carp[56]. In crustaceans, vitamin K is required for larval kuruma shrimp, *M. japonicus*[33], *P. monodon*[57], *F. chinensis*[58], *P. muelleri* and *A. longinaris*[9].

Vitamins D and K are involved in calcium and phosphorus transport in vertebrates[59], although vitamin K has been poorly studied in crustaceans, it has been proposed to play the same role as in vertebrates. Moreover, Shiau and Liu[57] found in the midgut gland of *P. monodon* evidence of a carboxylase and a protein precursor dependant on menadione and they proposed that calcium deposition in juvenile tiger prawn positively correlates with the levels of vitamin K and D in diet. They noticed higher weight gain in grass shrimp *P. monodon* fed diets containing 35 mg menadione kg^{-1} diet.

Shiau and Liu[58] recorded high weight gain of individuals of *F. chinensis*, given diets supplemented with vitamin K. They obtained good weight increase for juvenile shrimp fed diets between 160 and 320 mg menadione kg^{-1}, determining an optimal value of 185 mg kg^{-1}. In contrast to these, no effect in weight gain and survival for *L. vannamei* fed a full vitamin supplemented diet and a feed without vitamin content was observed[34]. Sarasa[9] found no statistical differences in weight gain and survival for shrimp *P. muelleri* and *A. longinaris* fed a fully supplemented diet and 0, 160, 240 and 320 mg vitamin K3 diet[-1].

Histological studies as evidence of vitamin deficiencies

Analyses of the midgut gland are supported by several nutritional researches although information on vitamin deficiencies using histological examinations is scarce in shrimp. The midgut gland is the largest organ in decapod crustaceans and has many biological functions, including synthesis and secretion of digestive enzymes, absorption of digested products, maintenance of mineral reserves and organic substances, lipid and carbohydrate metabolism, distribution of stored reserves during the molt cycle and catabolism of some organic compounds[59]. It is a conspicuous and bilobulated gland composed of simple, blind-ended tubules, and leads to the pyloric stomach. Each tubule has three defined zones: distal, middle and proximal and is surrounded by connective tissue. There can be identified four cell types as well: E cells (embryonic), F (fibrillar), R (resorptive) and B (secretory) form the epithelium[59]. The digestion process is cyclical, and the specific role of the different cellular types has been determined through morphological, ultrastructural, histochemical, and immunohistochemical studies[59].

It has been noted that the midgut gland undergoes histological and histochemical modifications in response to physiological demands, such as molt[60] and reproduction[61], pollution[62], and that there is a correlation between the midgut gland structure and the shrimp's physiological condition and nutrition[9,10,34]. Rodriguez Souza *et al.*[63] suggest that histological criteria constitute a practical mean for preliminary assessment of the acceptability and nutritional value of diets for the crustaceans.

Several studies have been carried out to characterize different physiological stages of the shrimp *P. muelleri* and *A. longinaris* under environmental and nutritional stress, evaluating the functional morphology of the midgut gland. Fernández-Gimenez[7] studied the effects of vitamin A to *P. muelleri´s* feed. Shrimp fed a vitamin free diet, 40 and 100 mg vitamin A kg^{-1},

manifested severe changes including shrinkage of cells and desquamation of apical borders, cellular death determinants, tissue disorganization and cellular lysis (Fig. 1A). Individuals fed 140 mg vitamin A kg^{-1} showed less cellular damage. Some disorganized zones could be seen with hypertrophy and hyperplasia; however abundant secretion was identified in the lumen. The midgut gland of shrimps fed with 180 mg vitamin A kg^{-1} was structurally normal and every cellular type could be identified in the tubules with their characteristic brush border (Fig. 1B). Over 180 mg kg^{-1} shrimp showed glands with an evident tissue disorganization, cellular retraction, hypertrophy and cellular lysis. The best well conserved structure of the gland was described for individuals given 160 mg vitamin A, in these cases some hypertrophic cells and retraction in a less percentage were found. Fernández-Gimenez[7] also studied the effects of different levels of

dietary vitamin A on the cytology of *A. longinaris'* midgut gland. Shrimps given between 0 and 100 mg vitamin A kg^{-1} diet, showed several histological changes, including hypertrophy and hyperplasia of tubular epithelium, folding of basal membrane and piknotic nucleus. In addition, individuals given 100-200 mg vitamin A kg^{-1} diet presented hyper-vacuolization, desquamation of apical border's cells, tissue disorganization, cellular lysis and blurred intercellular spaces. Haemocytic infiltration and some nodules were also identified (Fig. 2A). Individuals fed diets containing 300 mg vitamin A kg^{-1} showed slight damage of the midgut gland.

Pereira[10] evaluated the responses of *A. longinaris* to dietary vitamin D3. Shrimps fed 0.375 and 0.75 mg vitamin D kg^{-1} in feed seemed to conserve the gland as well as the ones given a complete diet (fishmeal, soybean meal and squid protein). Shrimps fed 0, 0.2 and 1 mg vitamin D kg^{-1} diet, showed several cytological damages as well as increased tubular lumen, basal membrane folding, loss of brush border and cellular necrosis (Fig. 2B). The ones given 1 mg vitamin D kg^{-1} diet presented foamy cells.

Fernández-Gimenez[7] evaluated the effect of different levels of vitamin E and the synthetic antioxidant butylated hydroxytoluene (BHT) in semipurified diets (0, 100, 600, 1250, 1500, 1750 and 2000 mg vitamin E kg^{-1} and 16 mg BHT kg^{-1} diet) in the midgut gland of shrimp *P. muelleri*. The gland of individuals fed 1750 mg vitamin E kg^{-1}, showed a well preserved cytology, and every cellular type could be recognized as well as the brush border and secretion inside the tubules indicating cellular activity. Treatments without vitamin E and the ones with 100 and 600 mg, presented cellular retraction and a picnotic
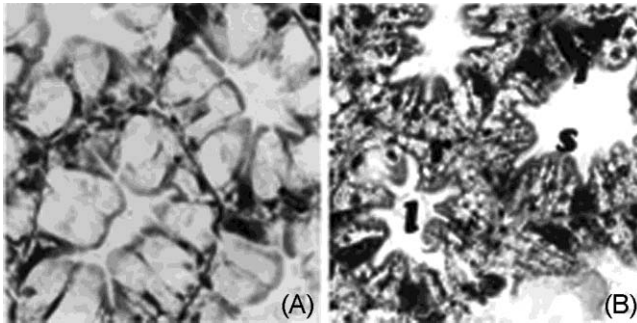

Fig. 1—Midgut gland of *Pleoticus muelleri* fed a diet (A) without vitamin A. (0 mg vitamin A kg^{-1}) [Transverse section through tubules revealed severe cytological alterations; complete loss or destruction of epithelial cells and scarce intertubular space (450X)]; and (B) with 180 mg vitamin A kg^{-1}. [Transverse section through tubules, showing all cellular types and brush border (similar to wild shrimps). f, F cell; l, lumen; r, R cell; s, brush border (450X)]
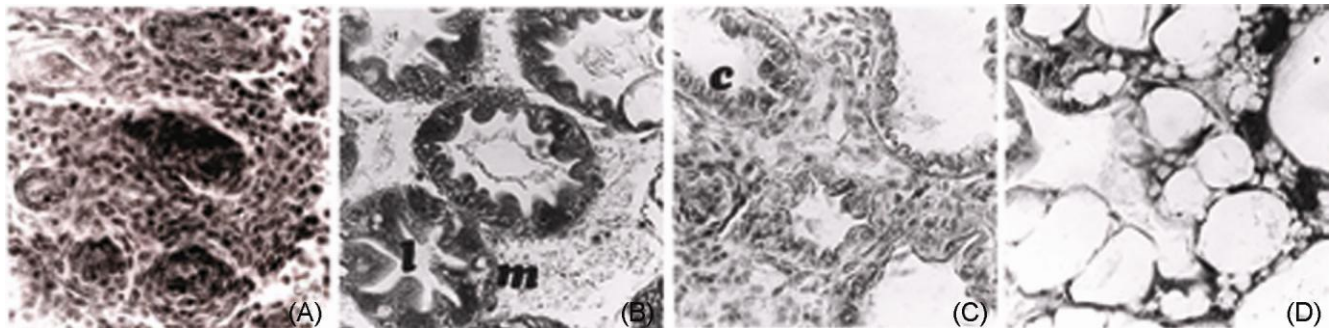

Fig. 2—Midgut gland of *Artemesia longinaris* fed a diet (A) deficient in vitamin A, showing haemocytic infiltration and nodules (450X); and (B) without vitamin D. [Details of tubules showing several cytological damages as well as increased tubular lumen, basal membrane folding, loss of brush border and cellular retraction. l, lumen; m, basal membrane folding (450X)]; (C) with 500 mg vitamin E kg^{-1}. [Transverse section showing deterioration of gland with abundant haemocytic infiltration and severe cell retraction. c, cell retraction (450X)]; and (D) deficient in vitamin K. [Transverse section through tubules revealed severe hypertrophy and cellular lysis (450 X)]

nuclei or loss of nucleus. However, in all the cases a small part of the organ seemed to conserve its normal structure. Shrimps fed with 1250 mg vitamin E kg^{-1} diet showed desquamation of the apical zone and degradation of the basal membrane and some cases of retraction and hypertrophy. Further, addition of 1500 and 2000 mg vitamin E kg^{-1} diet caused malnourishment, cellular and nuclear retraction, desquamation and hypertrophy. Supplementation of BHT resulted in cellular hypertrophy and tubular disorganization.

The study of *A. longinaris'* midgut gland fed with diets containing <1000 mg vitamin E kg^{-1} showed a severe deterioration of the gland with connective tissue and haemocyte infiltration, some cases of encapsulation, cell retraction, hypertrophy and hyperplasia, lysis and necrosis (Fig. 2C). Individuals given >1500 mg vitamin E, presented reduced inter-tubular spaces, brush border and abundant secretion of the lumen indicating that both structure and functionality of the gland are well preserved, scarce signs of cellular retraction and lysis. The supplementation of BHT affected highly the cytology of *A. longinaris*. However, necrotic focuses with haemocytic infiltration were not found for this treatment[7].

Sarasa[9] observed histological alterations which indicated that supplementation of menadione in the diet was necessary to maintain the normal structure of midgut gland of *P. muelleri*. Diets without vitamin K or with 160 and 320 mg vitamin K kg^{-1} led to severe changes if the cytology of the gland, including cellular retraction, absence of brush border, hypertrophy, desquamation and necrosis. Shrimps fed with 240 mg vitamin K kg^{-1} diet in addition to complete diet showed a normal functional morphology of midgut gland.

Studying the same diets on *A. longinaris*, Sarasa[9], found out that inadequate levels of menadione (160 and 320 mg vitamin K kg^{-1}) and the absence of this compound, generated cellular hyperplasia and hypertrophy, disorganization of the midgut gland tissue and shrinkage of cells (Fig. 2D). The most preserved organs were described for individuals given complete diets or 240 mg vitamin K kg^{-1} supplemented ones.

The most notable changes reported for *P. muelleri* and *A. longinaris* fed with different levels of liposoluble vitamins, included cellular and nuclear retraction, desquamation or detachment of cells and damage of the basal lamina, and have been reported for other penaeid species, such as *P. monodon* and *F. indicus*[3,17].

Interaction between vitamins and alkaline phosphatase activity

Certain physiological processes in organisms are regulated via formation (phosphorylation) or rupture (dephosphorylation) of phosphate esters. Alkaline phosphatase (EC 3.1.3.1) is an ubiquitous metallo-enzyme occurs widely in nature, from bacteria to man, located in the cell membrane and is involved in several essential functions in mammals. This enzyme is a homodimer and each catalytic site contains three metal ions, i.e. two Zn and one Mg, necessary for enzymatic activity. Found in kidney, liver, intestine and bones in vertebrates, its activity determination is often used in clinical and ecotoxicological studies, abnormal levels in serum point out the existence of degenerative bone disease or liver damage[64,65].

Alkaline phosphatase has been studied and characterized in several invertebrates, and the activity was established in haemocytes of mud crab *Scylla serrata* (Forskål)[66], while Shiau and Hwang[44] and Lovett *et al.*[67] observed enzyme activity in midgut gland of *P. monodon* and in gills of the blue crab *Callinectes sapidus* (Rathbun), respectively. Pereira *et al.*[68] showed the phosphatase activity in two tissues of shrimp *A. longinaris*, describing higher activity values on haemocytes than midgut gland.

Vijayavel and Balasubramanian[69] suggested that phosphatases play major roles in the molting physiology of many crustaceans. In the green crab *S. serrata*, this enzyme is important in absorption of phosphate and calcium from seawater and for the integument formation[70].

Pinoni *et al.*[71] demonstrated the presence of two forms of alkaline phosphatase (AP) in muscle chelates of crab *Neohelice granulate* (Dana), and evaluated its activity in the presence and absence of inhibitor levamisole. They found that AP insensitive to levamisole is influenced by the salinity of the medium, suggesting a possible role for itself in physiological processes such as cell growth and differentiation, mobilization of energy sources and osmoionorregulation.

There are few studies in invertebrates which link the alkaline phosphatase activity and levels of vitamins in the diet. Fu *et al.*[72] investigated the interaction between vitamins A and D and alkaline phosphatase activity in Pacific abalone *Haliotis discus hannai* (Ino). The authors observed that dietary vitamin A generally increased the enzyme activity in viscera except the excessive supplement which

decreased phosphatase activity and dietary vitamin D significantly increased enzyme activity. On other hand, in crustaceans, Shiau and Hwang[44] suggested that alkaline phosphatase may be involved in the metabolism of calcium and phosphorus in shrimp, they reported that absence and levels above 0.1 mg vitamin D kg^{-1} in *P. monodon* diet, correlates with a reduced activity of this enzyme.

Alkaline phosphatase activity was evidenced by Pereira *et al.*[68] in haemocytes and midgut gland of shrimp *A. longinaris*. The purpose of this study was to evaluate the enzyme activity in relationship with different doses of vitamin D3 (0, 0.2, 0.375, 0.75 and 1 mg vitamin D3 kg^{-1}) in feed and to estimate its potential use as biomarker for nutritional stress. The results demonstrated higher enzymatic activity values in haemocytes than in midgut glands. The highest enzyme activity in haemocytes was observed in shrimps fed without vitamin D (1.23 abs min^{-1} mg protein^{-1}). However, in midgut gland, the activity varied from 0.141 to 0.297 abs min^{-1} mg protein^{-1}, with the highest values on 0 and 1 mg vitamin D kg^{-1} feed. Histological analysis of the midgut gland confirmed a good health of the shrimp fed 0.375 and 0.750 mg vitamin D3 kg^{-1} feed and were used as optimal values for determining enzymatic activity. The results indicated that alkaline phosphatase activity in *A. longinaris* was influenced by dietary vitamin D and may be used as a biomonitor of nutritional stress.

Sarasa[9] observed an inverse relationship between alkaline phosphatase activity in midgut gland of *P. muelleri* and levels of vitamin K3 in diet. In larvae of white shrimp *Litopenaeus setiferus* (Perez and Kensley), it was demonstrated that alkaline phosphatase activity is situated along the midgut gland, suggesting that absorption is widespread. However in juveniles, the activity is restricted to the midgut gland and midgut region. Alkaline phosphatase activity in the digestive gland of decapods has been associated with metabolites transmembrane transport; tissues in which this enzyme activity is present are generally thought to function in active transport absorption[73]. Using histochemical methods, Monin and Rangneker[74] observed that midgut gland cells of crab *S. serrata*, showed a positive reaction for alkaline phosphatase only at the brush border. Physiological processes, as molting, also affect AP activity. Meyran and Craf[75] observed periodical changes in the enzyme activity in the posterior caeca of the amphipod

Orchestia cavimana (Heller), using cytochemical techniques during the molting cycle; suggesting that this enzyme may be involved in calcium transport. Other vitamins had also a similar effect on alkaline phosphatase activity of midgut gland. Sarasa[9] observed that shrimp *P. muelleri* responded to different levels of vitamin K in feed; in this case the highest phosphatase alkaline activity was linked to several histological changes in the midgut gland.

Dietary vitamin modulation of antioxidant defense and immune response

Oxygen is an essential compound of living organisms and the generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, commonly referred as prooxidants, is inevitable in aerobic metabolism of the body. ROS cause lipid oxidation, protein oxidation, DNA strand break and base modification, and modulation of gene expression which finally lead to several pathological states including apoptosis[76].

Therefore, in living systems an equilibrium exists between the rate of production and the rate of breakdown of ROS. Under normal physiological states, harmful effects of ROS are effectively neutralized by antioxidant defense system of organisms, which generally comprises of both enzymes and small antioxidant molecules. However, an alteration of the prooxidant and antioxidant equilibrium in favor of the former, results in oxidative stress[77].

Aquatic pollution is a major contributor of oxidative stress in fish and crustaceans. These pollutants are taken up by aquatic organisms from sediments, suspended particles matter with toxic properties and food sources[78]. A significant role of antioxidant defenses is recognized in protecting the aquatic animals from the oxidative stress which resulted due to their exposure to various agents such as xenobiotics, nutritional imbalance and hypoxia. Among the various antioxidants, vitamin E is unique and deserves special attention for its important role in maintaining the quality of aquaculture products. It also provides additional health protection through its immunostimulant property[77].

In crustaceans, the midgut gland, rich in lipids and fat soluble vitamins, regulates the body metabolism. It is recommended as an environmental indicator of water pollution[79] and as biomarker of oxidative

stress[80]. Barim and Karatepe[78], determined that vitamins A, E, C and β-carotene in midgut gland and muscle tissues of danube crayfish *Astacus leptodactylus* (Eschscholtz) were significantly higher in unpolluted water, than polluted, providing the evidence that vitamins A, E, C and β-carotene are sensitive indicators of aquatic pollution.

Dandapat *et al.*[77] who studied the effect of vitamin E in diets (200, 400 and 600 mg kg^{-1}) on lipid peroxidation in gills and midgut gland of the giant river prawn *Macrobrachium rosembergii* (De Man) suggest that vitamin E is capable of reducing lipid peroxidation and can modulate the antioxidant defense system in gills and midgut gland, nevertheless, the response is highly specific. It is further observed that highest dose of vitamin E (600 mg kg^{-1}) could not render much additional protection in both the tissues.

Díaz *et al.*[81] evaluated the free radical scavenging properties of *P. muelleri*´s midgut gland extracts by electron paramagnetic spin resonance spectrometry methods (EPR) against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. This study was carried out to characterize different physiological stages of the shrimps under environmental and nutritional stress. Feeding trials were carried out on juveniles and feeds with different concentrations of vitamins A and E were formulated. The control groups were fed with fresh squid mantle and with a vitamin free diet. For all treatments, the extracts exhibited strong DPPH radical scavenging activity, suggesting that the tissue is a powerful natural antioxidant. Individuals fed with different concentrations of vitamin E showed the strongest effect of the DPPH radicals, reducing the DPPH radicals to 50%, after an incubation period of 3 min. In contrast, the extracts of control shrimp, fed with squid mantle, had the weakest antioxidant activity. Further, they suggest that loss of individual antioxidants and/or generation of oxidation products from the shrimps can be measured as an index of oxidative stress and the presence of vitamins A or E in feed can provide immediate protection against free radicals.

On the other hand, the immune system in crustaceans is less developed than in vertebrates. Shrimps have a wide array of non-specific factors, both cellular as well as humor, which are involved in defense against pathogens. It may be possible to stimulate the non-specific defense mechanisms system of shrimps and enhance the disease resistance[82].

The immune system can be influenced by a wide range of factors including diseases, pollutants, hormones and diet (nutrition). Vitamin E has been shown to influence various parameters of the immune response and disease resistance in *P. monodon*. Lee and Shiau[46] evaluated the effect of dietary vitamin E on juvenile grass shrimp and demonstrated that the non-specific immune responses of shrimp improved as the level of vitamin E increased in the diet, reaching a peak after the requirement (85-89 mg kg^{-1}).

Kanagu *et al.*[82] clearly demonstrated the importance of vitamins as immunomodulators in shrimps. Vitamins C and E are considered as potential immunomodulators in shrimp disease management. An immunomodulator is a chemical drug, stressor or action that elevates the non-specific defense mechanism. The authors observed that vitamin C exhibited more immunoestimulant effect rather vitamin E in *P. monodon*. Likewise, Fernández-Gimenez *et al.*[83] evaluated different doses of vitamin D in the diet of *A. longinaris* and observed that plasma phenoloxidase activity was higher in control treatments (0.375 and 0.75 mg vitamin D kg^{-1} diet) than the ones with 1 mg/kg vitamin D in diet. However, total phenoloxidase activity in lysate haemocytes was similar in all treatments; suggesting there is no relationship between plasma phenoloxidase activity and immune response in haemocytes.

Salinity is an important abiotic factor affecting the immune system of penaeid shrimps. Although juvenile and adult penaeids are competent hyper-/hypo-osmoregulators, acute salinity changes may cause stress to the shrimp, and thus facilitate infections. Liu *et al.*[84] examined the effect of vitamin E and salinity on antioxidant enzyme activities in *L. vannamei* and demonstrated that vitamin E might be useful as an effective antioxidant by regulating osmotic balance and resistance to salinity changes in shrimp.

Conclusion

Penaeoid shrimps, *A. longinaris* and *P. muelleri* are two important marine resources in the Argentine Sea, with commercial importance. Both species have seasonal and annual fluctuations in catches. It is, therefore, important to establish the feasibility of culturing them on commercial basis to provide continuous supplies of these species to the market. Successful culturing of aquatic species should have tools that are able to measure the interaction

Table 1—Dietary liposoluble vitamin dosages recommended for some Decapod crustaceans

Species	Vitamin	Suitable/optimum dosages (mg kg^{-1} diet)	References
Artemesia longinaris	A	300	Fernández-Gimenez[7]
	D	0.375-0.750	Pereira[10]
	E	1500	Fernández-Gimenez[7]
	K	240	Sarasa[9]
Pleoticus muelleri	A	180	Fernández-Gimenez[7]
	E	1750	-do-
	K	240	Sarasa[9]
Fenneropenaeus chinensis	A	40-60	Liang & Ji[37]
	K	185	Shiau & Liu[58]
Fenneropenaeus indicus	E	100-300	Ouraji *et al.*[51]
Litopenaeus vannamei	A	130	He *et al.*[34]
	E	99	He *et al.*[34]; He & Lawrence[50]
Macrobrachium rosembergii	E	600	Dandapat *et al.*[77]
Penaeus monodon	D	0.2	Shiau & Hwang[44]
	E	85-95	Hsu & Shiau[45]
	K	35	Shiau & Liu[57]

between food, environment and physiology of the organisms.

Dietary vitamin requirements in crustaceans (Table 1) have been demonstrated with dietary trials for growth and survival; however, vitamin deficiencies and over-fortifications may be manifested through changes in the midgut gland cells. As the midgut gland is the primary digestive organ of crustaceans, pathological changes of these cells result in a general impairment of the digestive function. Biochemical studies have demonstrated the relationship between levels of vitamin in diet and enzyme activity, such as, alkaline phosphatases and digestive proteinases, and the important role of vitamins as antioxidants and immunomodulators. This review provides a better understanding of relation between liposoluble vitamins in diet and metabolic, physiological and histological responses, and may help in formulating suitable feeds for shrimps.

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